

External Review Draft

**Nanomaterial Case Study:
Nanoscale Silver in Disinfectant Spray**

Disclaimer

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Abbreviations

| | |
|---|---|
| ACGIH | American Conference of Governmental Industrial Hygienists |
| ADME | Absorption, Distribution, Metabolism, and Excretion |
| AFM | Atomic Force Microscopy |
| Ag | Silver |
| Ag(NH₃)₂⁺ | Ionic Diamminesilver |
| Ag⁺, Ag²⁺, and Ag³⁺ | Ionic Silver |
| Ag⁰ | Zero-Valent Silver |
| Ag₂S | Silver Sulfide or Argentite |
| Ag₃AsS₃ | Proustite |
| Ag₃SbS₃ | Pyrrargyrite |
| Ag₅SbS₄ | Stephanite |
| AgCl | Silver Chloride or Cerargyrite |
| AgClO₄ | Silver Perchlorate |
| AgI | Silver Iodide |
| AgNO₃ | Silver Nitrate |
| AgSO₄ | Silver Sulfate |
| AMO | Ammonia Monooxygenase |
| As[V] | Arsenate |
| ATP | Adenosine Triphosphate |
| ATSDR | Agency for Toxic Substances and Disease Registry |
| BAF | Bioaccumulation Factor |
| BASS | Bioaccumulation Aquatic System Simulator |
| BCF | Bioconcentration Factor |
| BET | Brunauer, Emmett, Teller Method |
| BLM | Biotic Ligand Model |
| BSA | Bovine Serum Albumin |
| BSE | Back-Scattered Electron |
| BSI | British Standards Institution |
| C₆₀ | Carbon 60, Buckminster Fullerenes, or Buckyballs |
| Ca²⁺ | Ionic Calcium |
| CaCl₂ | Calcium Chloride |
| CDC | Centers for Disease Control and Prevention |
| CE | Capillary Electrophoresis |

| | |
|-----------------------------------|--|
| CEA | Comprehensive Environmental Assessment |
| CEINT | Center for Environmental Implications of Nanotechnology |
| Cl⁻ | Ionic Chlorine |
| CPB | Cetylpyridine Bromide |
| CPC | Condensation Particle Counter |
| CPF | Chlorpyrifos |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DGT | Diffusive Gradients in Thin Films |
| DLS | Dynamic Light Scattering |
| DOC | Dissolved Organic Carbon |
| EC₅₀ | The Concentration Resulting in a Non-Lethal Toxic Effect in 50% of the Organisms |
| EDS | Energy-Dispersive X-Ray Spectroscopy |
| EDTA | Ethylenediaminetetraacetic Acid |
| E-FAST V2.0 | Exposure And Fate Assessment Screening Tool Version 2.0 |
| EHS | Environmental Health and Safety |
| ELPI | Electrical Low Pressure Impactor |
| EPA | U.S. Environmental Protection Agency |
| EPS | Exopolymeric Substances |
| ESEM | Environmental Scanning Electron Microscopy |
| Fe³⁺ | Ferric Iron |
| FFF | Field Flow Fractionation |
| FIFRA | Federal Insecticide, Fungicide, and Rodenticide Act |
| FMPS | Fast Mobility Particle Sizer |
| GI | Gastrointestinal |
| GSH | Glutathione |
| H₂O₂ | Hydrogen Peroxide |
| HAADF | High-Angle Annular Dark-Field |
| HEPA | High Efficiency Particulate Air |
| HR-TEM | High-Resolution Transmission Electron Microscopy |
| IC₂₀ | Concentration Causing an Inhibitory Response in 20% of the Organisms |
| ICON | International Council on Nanotechnology |
| ICP-MS | Inductively Coupled Plasma-Mass Spectroscopy |
| ICRP | International Commission on Radiological Protection |
| IL-6, IL-8, IL-β | Interleukins |
| INAA | Instrumental Neutron Activation Analysis |
| ISE | Ion-Selective Electrode |

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| ISO | International Organization for Standardization |
| K⁺ | Ionic Potassium |
| K_d | Distribution (or Partition) Coefficient |
| K_{sp} | Solubility Product Constant |
| LC₅₀ | Concentration Resulting in Mortality of 50% of the Organisms |
| LDMA | Long Differential Mobility Analyzer |
| LIBD | Laser-Induced Breakdown Detection |
| LOAEL | Lowest Observed Adverse Effect Level |
| LSPR | Localized Surface Plasmon Resonance |
| MATC | Maximum Acceptable Toxic Concentration |
| MCL | Maximum Contaminant Level |
| Mg²⁺ | Ionic Magnesium |
| MgCl₂ | Magnesium Chloride |
| MIC | Minimum Inhibitory Concentrations |
| MINChar | Minimum Information for Nanomaterial Characterization |
| MIP-2 | Macrophage Inhibitory Protein-2 |
| MTT | 3-(4,5-Dimethylthiazol- 2-Yl)-2,5-Diphenyltetrazolium Bromide |
| N₂ | Nitrogen |
| Na⁺ | Ionic Sodium |
| Na⁺/K⁺ - ATPase | Sodium- and Potassium-Activated Adenosine Triphosphatase |
| NaAgS₂O₂ | Sodium-Silver Thiosulfate |
| NaCl | Sodium Chloride |
| NAG | N-Acetyl-B-D Glucosaminidase |
| nano-Ag | Nanoscale Silver |
| nano-Au | Nanoscale Gold |
| nanomaterials | Nanoscale Materials |
| nano-TiO₂ | Nanoscale Titanium Dioxide |
| NH₂OH.HCl | Hydroxylamine Hydrochloride |
| NIL | Nanoparticle Information Library |
| NIOSH | National Institute for Occupational Safety and Health |
| NOAEL | No Observable Adverse Effect Level |
| NOEC | No Observed Effect Concentration |
| NOM | Natural Organic Materials |
| NOSH | Nanoparticle Occupational Safety and Health Consortium |
| OECD | Organisation for Economic Co-operation and Development |
| OPC | Optical Particle Counter |

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|------------------------------------|--|
| OSHA | Occupational Safety and Health Administration |
| PAA | Polyacrylic Acid |
| PAH | Polycyclic Aromatic Hydrocarbon |
| PBMC | Peripheral Blood Mononuclear Cell |
| PCR | Polymerase Chain Reaction |
| PEC | Predicted Environmental Concentration |
| PEN | Project on Emerging Nanotechnologies |
| PHA | Phytohaemagglutinin |
| PM | Particulate Matter |
| PMFA | Probabilistic Material Flow Analysis |
| PNEC | Predicted No-Effect Concentration |
| PO₄³⁻ | Phosphate |
| PT | Permeability Transition |
| PTP | Proteinaceous Pores |
| REACH | Registration, Evaluation, Authorisation and Restriction of Chemicals |
| RIVM | Netherlands Institute for Public Health and the Environment |
| RO | Reverse Osmosis |
| ROS | Reactive Oxygen Species |
| -S | Inorganic Sulfide |
| S²⁻ | Sulfide |
| SAP | Scientific Advisory Panel |
| SAXS | Small-Angle X-Ray Scattering |
| SCENIHR | Scientific Committee on Emerging and Newly Identified Health Risks |
| SDS | Sodium Dodecyl Sulfate |
| SE | Secondary Electron |
| SEC | Size Exclusion Chromatography |
| SEM | Scanning Electron Microscopy |
| -SH | Thiol |
| SIMPROF | Similarity Profile |
| SMPS | Scanning Mobility Particle Sizer |
| SNOMS | Single-Nanoparticle Optical Microscopy and Spectroscopy |
| SO₄²⁻ | Sulfate |
| SPM | Scanning Probe Microscopy |
| SPR | Surface Plasmon Resonance |
| STEM | Scanning Transmission Electron Microscopy |
| TEM | Transmission Electron Microscopy |

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|------------------------|--|
| TEOM® | Tapered Element Oscillating Microbalance |
| TFF | Tangential-Flow Ultrafiltration |
| TiO₂ | Titanium Dioxide |
| TNF-α | Tumor Necrosis Factor-α |
| WAXS | Wide-Angle X-Ray Scattering |
| XAS | X-Ray Absorption Spectroscopy |
| XRD | X-Ray Diffraction |
| ZnS | Zinc Sulfide |

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Organizational Abbreviations

IOAA: Immediate Office of the Assistant Administrator
NCEA: National Center for Environmental Assessment
NERL: National Exposure Research Laboratory
NHEERL: National Health and Ecological Effects Research Laboratory
NRMRL: National Risk Management Research Laboratory
OA: Office of the Administrator
OAR: Office of Air and Radiation
OCSPP: Office of Chemical Safety and Pollution Prevention
OEI: Office of Environmental Information
OPEI: Office of Policy, Economics, and Innovation
OPP: Office of Pesticide Programs
OPPT: Office of Pollution Prevention and Toxics
ORD: Office of Research and Development
ORISE: Oak Ridge Institute for Science and Education
OSCP: Office of Science Coordination and Policy
OSP: Office of Science Policy
OSRTI: Office of Superfund Remediation and Technology Innovation
OST: Office of Science and Technology
OSWER: Office of Solid Waste and Emergency Response
OW: Office of Water

Preamble

1 This document is part of continuing efforts by the U.S. Environmental Protection Agency (EPA) to
2 understand the science issues and information gaps associated with nanotechnology, consistent with
3 recommendations in the EPA *Nanotechnology White Paper* (U.S. EPA, 2007, [090564](#)) and EPA
4 *Nanomaterial Research Strategy* (U.S.EPA, 2009, [625484](#)). Engineered nanoscale materials
5 (nanomaterials) have been described as having at least one dimension on the order of approximately 1 to
6 100 nanometers (nm). Such materials often have unique or novel properties that arise from their small
7 size.

8 The specific type of nanomaterial considered in this document is nanoscale silver (nano-Ag) as
9 possibly used in disinfectant sprays. This “case study” does not represent a completed or even a
10 preliminary assessment, nor is it intended to serve as a basis for near-term risk management decisions on
11 possible uses of nano-Ag. Rather, the intent is to describe what is known and unknown about nano-Ag in
12 a selected application as part of a process to identify and prioritize scientific and technical information to
13 support long-term assessment efforts. Previous EPA case studies focused on nanoscale titanium dioxide
14 used in drinking water treatment and in topical sunscreen (U.S. EPA, 2009, [225004](#)).

15 This case study is organized around a comprehensive environmental assessment (CEA) framework,
16 which starts with the product life cycle and encompasses fate and transport processes in various
17 environmental media, exposure-dose characterization, and ecological and health effects, as well as other
18 direct and indirect ramifications of both primary and secondary substances or stressors associated with a
19 nanomaterial. The organization of the document reflects the CEA framework: after an overall
20 introduction to this document (Chapter 1) and introductory material on silver and nano-Ag (Chapter 2),
21 Chapter 3 highlights stages of the product life cycle (feedstocks, manufacturing, distribution, storage, use,
22 disposal), followed by Chapter 4 on fate and transport processes, Chapter 5 on exposure-dose
23 characterization, and Chapter 6 on ecological and health effects.

24 Throughout the document are lists of questions that reflect information gaps or possible research
25 issues. Note that some of these questions might be specific to the use of nano-Ag in a disinfectant spray;
26 other research issues might apply more broadly to nano-Ag irrespective of its application; and still other
27 questions might apply even more widely to nanomaterials in general. Readers are encouraged to consider
28 the questions listed throughout the document and offer specific comments on how individual research
29 questions might be more precisely or accurately articulated. If additional questions should be included or
30 if information is already available to address some of the questions posed here, readers are encouraged to
31 provide such comments as well.

1 The CEA approach is both a framework and a process. The latter aspect employs a formal
2 judgment process that will use the information presented in the case study and the questions raised in this
3 document as a starting point to determine priorities among various research topics and directions. After
4 that process has been completed, a final chapter will be added to this document to summarize highlights
5 from preceding chapters and the major research issues that have emerged.

6 Comments should be submitted in accordance with instructions provided in the August 13, 2010,
7 Federal Register notice.

Chapter 1. Introduction to this Document

1.1. Background

1 Nanoscale materials (nanomaterials) have been described as having at least one dimension on the
2 order of approximately 1–100 nanometers (nm) (National Nanotechnology Initiative, 2006, [091186](#)).
3 Engineered nanomaterials are intentionally made, as opposed to being an incidental by-product of
4 combustion or a natural process such as erosion, and often have unique or novel properties that arise from
5 their small size. Like all technological developments, engineered nanomaterials offer the potential for
6 both benefits and risks. The assessment of such benefits and risks relies on information, and, given the
7 nascent state of nanotechnology, much remains to be learned about the characteristics and impacts of
8 nanomaterials to support such assessments. This document is part of an endeavor to identify what is
9 known and, more importantly, what is not yet known that could be of value in assessing the broad
10 implications of certain nanomaterials.

11 The focus of this document is a specific application of a selected nanomaterial: the use of
12 engineered nanoscale silver (nano-Ag) as an agent in disinfectant spray products. The U.S.
13 Environmental Protection Agency (EPA) recently completed similar “case studies” of nanoscale titanium
14 dioxide (nano-TiO₂) used for drinking water treatment and for topical sunscreen (U.S. EPA, 2009,
15 [225004](#)). Such case studies do not represent completed or even preliminary assessments; rather, they are
16 intended as a starting point in a process to identify and prioritize possible research directions to support
17 future assessments of nanomaterials.

18 Part of the rationale for focusing on a series of nanomaterial case studies is that such materials and
19 applications can have highly varied and complex properties that make considering them in the abstract or
20 in generalities quite difficult. Different materials and different applications of a given material could raise
21 unique questions or issues as well as some issues that are common to various applications of a given
22 nanomaterial or even to different nanomaterials. After several individual case studies have been
23 examined, refining a strategy for nanomaterials research to support long-term assessment efforts should
24 be possible.

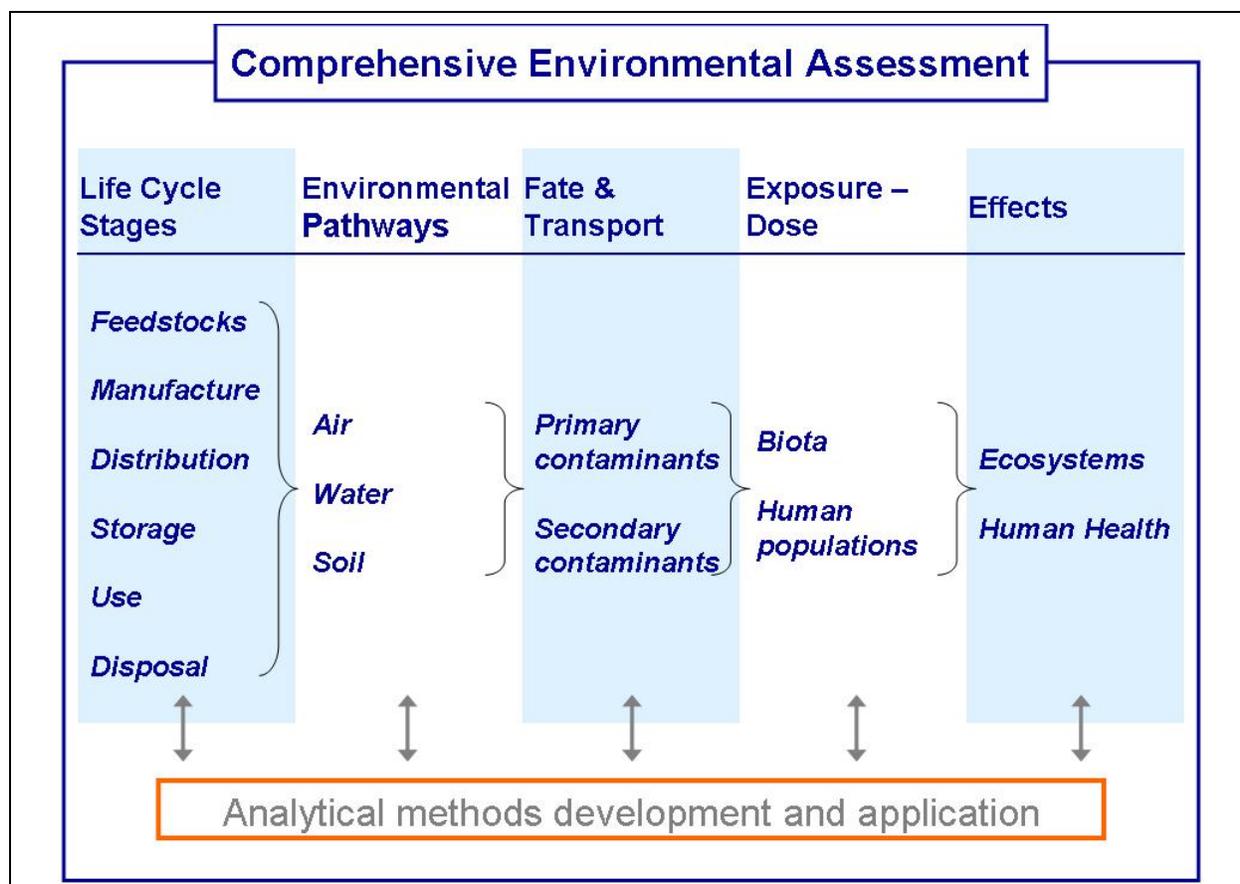
25 The process for selecting nano-Ag in disinfectant spray as a case study involved a workgroup
26 representing several EPA program offices, regional offices, and Office of Research and Development
27 laboratories and centers. The EPA workgroup considered several candidate nanomaterials and voted for
28 their preferences based on, among other things, apparent relevance of the nanomaterial to EPA

1 programmatic interests. The choice of a specific application, disinfectant spray in this case, was
2 determined by a smaller team directly involved in the production of the case study document. Among the
3 factors guiding the selection process at each stage was the potential for exposure of ecological receptors
4 and human populations to the nanomaterial as a function of a particular application. This is not to say,
5 however, that the selection of nano-Ag in disinfectant spray signifies a determination that it presents the
6 greatest potential for exposure of all possible applications, or, for that matter, that any exposure actually
7 occurs to such a product. Rather, the case study on nano-Ag in disinfectant spray and other case studies
8 simply provide a means to focus thinking about the types of information that would be instructive in
9 assessing the potential ecological and health implications of selected nanomaterials.

10 This case study of nano-Ag, like the first case studies of nano-TiO₂ (U.S. EPA, 2009, [225004](#)),
11 incorporates a comprehensive environmental assessment (CEA) framework, which combines a product
12 life-cycle perspective with the risk assessment paradigm (Davis, 2007, [089803](#); Davis and Thomas, 2006,
13 [089638](#)). In essence, risk assessment relates exposure and effects information for a given substance or
14 stressor, and CEA expands on this paradigm by including life-cycle stages and considering both indirect
15 and direct ramifications of the substance or stressor. Figure 1-1 illustrates the principal elements in the
16 CEA approach. The first column of Figure 1-1 lists typical stages of a product life cycle: feedstocks,
17 manufacture, distribution, storage, use, and disposal (which would include reuse or recycling, if
18 applicable). The second column lists environmental pathways or media (air, water, soil) to which
19 nanomaterials or associated materials (e.g., manufacturing by-products) might be released at various
20 stages of the product life cycle. Within these media, nanomaterials or associated materials can be
21 transported and transformed and can interact with other substances in the environment, both natural and
22 anthropogenic; thus, a combination of primary and secondary contaminants can be spatially distributed in
23 the environment (third column, Figure 1-1).

24 The fourth column of Figure 1-1, exposure-dose, goes beyond characterizing the occurrence of
25 contaminants in the environment, as exposure refers to actual contact between a contaminant and
26 organisms (i.e., biota¹ and human populations). Under the CEA approach, exposure characterization can
27 involve aggregate exposure across routes (e.g., inhalation, ingestion, dermal); cumulative exposure to
28 multiple contaminants (both primary and secondary); and various spatiotemporal dimensions (e.g.,
29 activity patterns, diurnal and seasonal changes). Dose is the amount of a substance that actually enters an
30 organism by crossing a biological barrier. Conceptually, dose links exposure with the last column of
31 Figure 1-1, which highlights ecological and human health effects that can result when an effective dose
32 reaches a target cell or organ in a receptor organism or, in an ecological context, when a stressor is at a

¹ The term biota is used throughout this document to refer to organisms other than humans.



Source: Adapted from Davis and Thomas (2006, [089638](#)) and Davis (2007, [089803](#)).

Figure 1-1. Basic structure of CEA as a framework for identifying and prioritizing research efforts.

1 level sufficient to cause an adverse response in a receptor. “Effects” encompasses both qualitative
 2 hazards and quantitative exposure-response relationships.

3 The CEA framework is highly simplified in Figure 1-1. Among the many direct and indirect
 4 impacts that could conceivably be included in a CEA are effects on other materials (e.g., damage to
 5 surfaces of structures, statuary, vehicles), hedonic or aesthetic qualities (e.g., alterations in visibility, taste,
 6 odor), and other possible large scale impacts such as energy consumption, resource depletion, and global
 7 climate change. Although none of these effects are being excluded a priori from consideration here, their
 8 inclusion would depend on having a plausible premise for expecting a discernible impact. If such a
 9 premise can be articulated for additional types of effects, the case study can be expanded to encompass
 10 their consideration within the CEA framework.

11 CEA involves the elaboration and synthesis of information from the elements in all five columns
 12 depicted in Figure 1-1 to systematically evaluate the direct and indirect ramifications of a nanomaterial
 13 and its by-products. Underlying the CEA elements are analytical methods that make detection,

1 measurement, and characterization of nanomaterials in the environment and in organisms possible.
2 Characterization of the substance(s) of interest (e.g., determination of chemical identity, reactivity, purity,
3 and other properties) is a critical aspect of each of the five elements of CEA. Not reflected in Figure 1-1
4 is the process of carrying out CEA, including an essential ingredient in making CEA effective – the
5 inclusion of diverse technical and stakeholder perspectives to ensure that a holistic view is achieved. As
6 either an assessment tool or as a framework for developing a research strategy, CEA is also a *process* that
7 involves an array of participants and contributors in making collective judgments through a formal,
8 structured method.

9 Other efforts have been made to assess the potential risks of nanomaterials by incorporating a life-
10 cycle perspective (Environmental Defense-DuPont Nano Partnership, 2007, [090565](#); Shatkin, 2008,
11 [180065](#); Thomas and Sayre, 2005, [088085](#)) or by using collective expert judgment methods (Kandlikar et
12 al., 2007, [091626](#); Morgan, 2005, [088831](#)), primarily in a risk management context. Although the present
13 document differs somewhat from these other efforts in its purpose – namely, this document is intended to
14 aid in developing a research strategy for the comprehensive environmental assessment of nanomaterial
15 risks – all of these endeavors complement and reinforce one another.

1.2. Purpose of this Document

16 The intent of this document is to highlight what is known and unknown about nano-Ag that could
17 be relevant to conducting a CEA of such nanomaterials. However, the document does not provide an
18 actual assessment or present conclusions regarding possible ecological or health risks related to nano-Ag.
19 Nor does the document purport to present an exhaustive review of the literature. Instead, it attempts to
20 provide a framework for systematically considering current and future information of possible relevance
21 to long-term assessment efforts.

22 It must be emphasized that this case study has been developed without a specific regulatory or
23 policy objective in mind. EPA has the authority to regulate a disinfectant spray product containing
24 nano-Ag under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. §136), and
25 such a product might be of interest in various other policy and regulatory contexts. This document,
26 however, is not intended to serve as a basis for near-term risk management decisions on this specific
27 nanomaterial use.² Rather, the intent is to use this document to identify scientific and technical

² Other EPA efforts are concerned with scientific issues relevant to nanomaterials within a regulatory context. A notable example is a recent review by a FIFRA Scientific Advisory Panel on hazard and exposure issues related to nano-Ag pesticide products (U.S. EPA, 2010, [625619](#)). As noted in the review, the Panel’s purpose is to provide advice and recommendations to EPA on pesticides regarding “the impact of regulatory actions on health and the

1 information that could be pertinent for future assessment efforts. The results of future assessments might,
2 of course, provide input to policy and regulatory decision-making at that time.

3 Focusing on only one possible example of a nano-Ag application obviously does not represent all
4 the ways in which this nanomaterial could be used or all the issues that different applications could raise.
5 By considering this single application of nano-Ag, however, research directions can be identified that
6 might apply not only to this application specifically, but also to nano-Ag in general and perhaps even
7 more broadly to other nanomaterials. The present case study, along with previous case studies of
8 nano-TiO₂ used for drinking water treatment and for topical sunscreen (U.S. EPA, 2009, [225004](#)) and
9 possibly one or more future case studies of other nanomaterials, will be used in the further development
10 and refinement of a comprehensive environmental assessment research strategy for nanomaterials. It is
11 also hoped that this strategy will assist not only the research efforts of EPA but the broader scientific
12 community as well.

13 When implemented, a CEA is meant to be comparative, examining the relative risks and benefits of
14 different formulation options, for example. The focus of a comparative CEA would be guided by risk
15 management objectives. For example, a nano-Ag disinfectant spray might be compared to a disinfectant
16 containing conventional silver, or the comparison might be to a different nanomaterial formulation, such
17 as nano-TiO₂, or to a non-spray product type or some other variable. Given that a number of different
18 options could be of interest to risk managers, considering every potential option in the present case study
19 is not feasible. Therefore, this document focuses solely on nano-Ag as it might be used in a disinfectant
20 spray, which is also consistent with the fact that the case study is not intended to be an assessment at this
21 time, but rather is meant to assist in identifying and prioritizing research related to nano-Ag.

1.3. How to Read this Document

22 This document presents a case study of nano-Ag using the CEA approach as a framework as
23 illustrated in Figure 1-1. For the application of interest (i.e., spray disinfectants containing nano-Ag), the
24 discussion of current knowledge and apparent data gaps is organized by chapter according to the basic
25 structure of a CEA, including life-cycle stages, releases to environmental pathways, fate and transport
26 processes, exposure and dose, and effects in humans and biota. Although this approach facilitates a

environment” (p. ii). Although this purpose fundamentally differs from that of this case study, much of the information included in the Panel’s review is relevant to this case study (reflected by the fact that both publications cite many of the same literature sources). Furthermore, the potential data gaps represented by the questions regarding future research at the end of each chapter in this case study are generally consistent with the research needs the FIFRA Scientific Advisory Panel identified.

1 logical organization and sequential presentation of information, it also results in some
2 compartmentalization into chapters and subsections. The document attempts to bridge such
3 compartmentalization with cross-references between sections and the inclusion of summaries at the end of
4 each chapter.

5 The information presented here was obtained from a variety of published and unpublished sources,
6 including corporate Web sites and personal communications, and some information is based on inferences
7 drawn from other materials or applications. Such information sources are used because of the limited
8 amount of published materials on nano-Ag and its applications in the peer-reviewed literature, coupled
9 with the limited mechanisms for making manufacturer-specific data publicly available. Given that this
10 case study is not an assessment but, rather, a means to identify information gaps and research questions,
11 the use of a range of information sources seems justifiable.

12 Questions posed at the end of Chapters 2–6 reflect information gaps in those respective chapters of
13 the document. Generally, information gaps suggest possible directions for future research. Note that
14 some of these gaps relate specifically to the use of nano-Ag as a disinfectant spray. Other research issues
15 might apply more broadly to nano-Ag irrespective of its application. Still other questions might apply
16 even more widely to nanomaterials in general. Many of these issues overlap with those identified by EPA
17 in the Nanotechnology White Paper (2007, [090564](#)) and the EPA Nanomaterials Research Strategy (2009,
18 [625484](#)). These questions by no means represent a complete or final list of research issues; rather, they
19 are intended to stimulate readers to think of additional questions that could be asked or to consider ways
20 to restate existing questions more precisely or accurately.

21 Readers are encouraged to consider the questions listed throughout the document and offer specific
22 comments on how individual research questions might be articulated more precisely or accurately. If
23 additional questions should be included or if information is already available to address some of the
24 questions posed here, readers are encouraged to provide such comments as well. Note that the
25 fundamental question pervading this document is “What might we need to know to be able to conduct a
26 CEA of nano-Ag in disinfectant spray products?” All other questions should help elucidate answers in
27 relation to this fundamental question.

28 This document is not an end in itself. The information presented in the case study and the
29 questions raised in this document are a foundation for a process to determine priorities among various
30 research topics and directions. After that process has been completed, a final chapter will be added to this
31 document to summarize highlights from preceding chapters and the major research issues that have
32 emerged.

1.4. Terminology

1 A number of terms used in the field of nanotechnology have specialized meanings, and definitions
2 of certain terms could have important legal, regulatory, and policy implications. Not surprisingly,
3 perhaps, defining such words, including the term nanomaterial itself, has often been a matter of
4 considerable interest and debate. For the purposes of this document, however, it is not deemed necessary
5 to have a connotative definition that states the necessary and sufficient conditions that define a
6 nanomaterial. Instead, a denotative approach is used; that is, the term “nanomaterial” in the case study
7 denotes something that most persons would agree is (or at least appears to be) an example of a
8 nanomaterial or a product that incorporates a nanomaterial, regardless of whether a consensus exists
9 regarding what properties or characteristics qualify it as such.

10 Although this case study focuses on “nano-Ag,” readers should note that this term encompasses a
11 variety of materials that might possess a range of physicochemical properties. As a result, not all
12 materials referred to as nano-Ag will necessarily behave in the same manner and exert the same biological
13 effects. Thus, caution in extrapolating from one nano-Ag formulation to another when assessing hazards
14 is appropriate (U.S. EPA, 2010, [625619](#)). Conversely, until more information is available to discern more
15 precisely how various formulations differ in behavior and effects, pooling information from multiple
16 sources can be useful for the purposes of this document, namely to identify potential research directions
17 to pursue.

18 Some other terms used throughout this document are discussed below, primarily to explain how the
19 terms are used here rather than to attempt to provide a formal definition of them.

20 **Nano-Ag.** This document focuses primarily on engineered nanoscale silver (nano-Ag),
21 which usually is in the form of particles in the 1- to 100-nm size range. The term “nano-
22 Ag,” as it is used in this document, refers to a variety of formulations containing silver
23 particles that meet this size-based definition. When reading this document, it is important
24 to understand that the general use of this term encompasses specific formulations that can
25 display a range of characteristics and behaviors depending on the properties of the particle,
26 the experimental or environmental conditions, and other factors.³ Where information is not
27 specific to nano-Ag, the term silver is used without the “nano” prefix.

³ Where sources have provided documentation on size, surface coating, extent of aggregation or agglomeration, and other salient properties or characteristics, this information is included in the case study with sources referenced appropriately.

1 **Conventional silver.** To make an explicit distinction between the nanoscale material and
2 other forms of silver not having the special characteristics of nano-Ag, the term
3 “conventional” is used in this document. Even so, materials described as conventional
4 often contain a range of particle sizes, including some with nanoscale dimensions. In the
5 scientific and technical literature on silver, the terms “**bulk**” and “**ionic**” also are often used
6 to distinguish conventional from nanoscale silver. Additionally, terms such as **ultrafine**,
7 **PM-0.1** (which means particulate matter less than 0.1 micrometer [μm] diameter), and
8 **micronized grade** have been used to denote nanoscale particles, but typically in a
9 particular context or field of specialization such as aerosols and air pollution.

10 **Aggregate and agglomerate.** As discussed in Chapter 4, in many circumstances primary
11 nanoscale particles can aggregate or agglomerate into secondary particles with dimensions
12 greater than 100 nm (a cluster that is sometimes referred to as a colloid, as described
13 below). Specifically, the terms “aggregate” and “agglomerate” are used in the literature on
14 nanomaterials and other fields to indicate the clustering of particles into a single entity of
15 such particles. These two terms can have specific meanings. For example, the British
16 Standards Institution (BSI, 2007, [202162](#)) defines aggregate as a “particle comprising
17 strongly bonded or fused particles where the resulting external surface area may be
18 significantly smaller than the sum of calculated surface areas of the individual components”
19 and notes that “the forces holding an aggregate together are strong forces, for example,
20 covalent bonds, or those resulting from sintering or complex physical entanglement.” The
21 BSI defines agglomerate as a “collection of loosely bound particles or aggregates or
22 mixtures of the two where the resulting external surface area is similar to the sum of the
23 surface areas of the individual components” and notes that “the forces holding an
24 agglomerate together are weak forces, for example van der Waals forces, as well as simple
25 physical entanglement.” However, the meanings of aggregate and agglomerate have
26 sometimes been interchanged, as noted by Nichols et al. (2002, [202114](#)). This difference in
27 meanings across, and sometimes within, the various fields that contribute to nanomaterials
28 research highlights the emerging and multidisciplinary nature of the nanotechnology field.
29 The nanotechnology community is an amalgam of investigators who all study nanoscale
30 materials but whose scientific roots are in various other mature fields spanning toxicology,
31 ecology, colloid science, materials science, and many other disciplines. The customary
32 terminology for aggregates and agglomerates may be well established within one field, but
33 use of these terms may elicit different interpretations within another; as a result, the

1 definitions for these terms are not specific, nor are they consistent. Given this
2 inconsistency in usage and, more importantly, the frequent lack of adequate information to
3 determine which term might be more appropriately applied in a particular study or report,
4 the term “**cluster**” is used in this document to subsume both aggregates and agglomerations
5 of nanoparticles. This term has precedent within multiple disciplines and avoids confusion
6 between potentially inconsistent connotations of the other terms. Note that, in addition to
7 being used as a noun (as explained above), the word “aggregate” is used as an adjective
8 (primarily in Chapter 5) to refer to exposure to a given material from multiple sources,
9 pathways, and routes.

10 **Colloid.** The term “colloid” is used in the literature to refer to a particle or cluster of
11 particles suspended within a given medium and that are smaller than microscale (i.e., less
12 than 10^{-6} m). Wijnhoven et al. (2009, [180201](#)), for example, refer to colloidal silver as
13 comprising silver particles primarily in the range of 250 to 400 nm, thereby distinguishing
14 nano-Ag and colloidal silver. By contrast, Luoma (2008, [157525](#)) describes a colloidal
15 particle as containing multiple atoms of a substance measuring between 1 nm and 1,000
16 nm, and thus a colloid might or might not be a nanoparticle in that context. In this case
17 study, although the term colloid is used at times to refer to a sub-microscale particle
18 (especially if a cited publication uses this terminology), either the more specific term
19 “nanoscale” or a specific size range is used when the particle size is salient to the
20 discussion.

21 The extent to which the properties of a cluster of primary nano-Ag particles that exceeds
22 100 nm are similar to the properties of conventional silver is unclear. Also unclear is the
23 extent to which changes in conditions might initiate the formation, decomposition, or
24 dissolution of a cluster, and there is uncertainty as well regarding what specific factors
25 drive important changes in conditions. As will be discussed in Chapter 4 (Fate and
26 Transport in Environmental Media), disaggregation can occur under some conditions.
27 Given these considerations, this document does not use 100 nm as the essential and
28 exclusive criterion for considering what might be relevant to an evaluation of nano-Ag.
29 This view is consistent with a statement by the European Commission (2008, [196378](#)) that
30 extends the term nanomaterial to encompass “nanostructured materials,” defined by the
31 International Organization for Standardization (ISO) (Technical Specification 27687) as
32 “[a]ggregates and agglomerates, often existing at a micro size, [that] may have some of the
33 behaviour and effects of their smaller sub units, e.g., due to an increased surface area.”

1 **Naturally occurring, incidental, and engineered nanoparticles.** In addition to
2 distinctions based on size of particles, The Project on Emerging Nanotechnologies (2009,
3 [196774](#)) divides nanoscale materials into three classes based on the origin of the particles.
4 Naturally occurring nanosized particles include, for example, particles that originate from
5 volcanic explosions, ocean spray, and soil and sediment weathering and biomineralization
6 processes (which can result in crystals of aluminum and iron oxides with nanometer-scale
7 dimensions). The second class is incidental nanosized particles, which are generated as by-
8 products of processes such as combustion, cooking, or welding. The focus of this report is
9 on the third class of nanoscale materials, *engineered* nanomaterials. This class comprises
10 materials purposely generated for a specific function, such as the carbon nanotubes used in
11 tennis rackets to make them lighter and stronger. In this case study, unless otherwise
12 specified, references to nano-Ag indicate engineered nanoscale materials. Non-engineered
13 types of nanosized silver (from the first or second class) are referred to as nanoscale silver.
14

Chapter 2. Introduction to Silver and Nanoscale Silver

2.1. Conventional Silver

1 Silver (Ag) is rarely found in its pure, free form, but instead is more commonly found as an alloy
2 with gold and other metals. Silver is also associated with minerals, the predominant form of which is
3 argentite (Ag_2S), followed by cerargyrite (AgCl). Other minerals containing silver, in order of
4 prevalence, are proustite (Ag_3AsS_3), pyrargyrite (Ag_3SbS_3), and stephanite (Ag_5SbS_4). Silver can exist in
5 its metallic state (Ag^0) and in three cationic states (Ag^+ , Ag^{2+} , and Ag^{3+}), with Ag^0 and Ag^+ being most
6 common (CRC, 2000, [196090](#)). The most abundant silver compounds in the environment are silver
7 sulfide (Ag_2S), silver nitrate (AgNO_3), and silver chloride (AgCl) (Wiberg et al., 2001, [098733](#)).

8 Silver is naturally released into the environment by wind and water erosion of soils and rocks
9 containing silver. Earth's crust contains approximately 0.1 part per million (ppm) of silver, and soils
10 contain approximately 0.3 ppm (Boyle, 1968, [597512](#)). Ambient water concentrations of 0.2 micrograms
11 per liter ($\mu\text{g/L}$) in fresh water and 0.25 $\mu\text{g/L}$ in sea water have been reported, and background silver has
12 also been found in biota at levels of micrograms per gram ($\mu\text{g/g}$) of tissue, particularly in fish and
13 shellfish.

2.1.1. Historic and Current Uses of Silver and Silver Compounds

14 By 2500 B.C., the Chaldeans in present-day Iraq and Kuwait had mastered a mining technique to
15 extract silver from lead ores. In addition to using silver as a durable metal in the production of jewelry,
16 coins, and utensils, ancient peoples also recognized its potential to keep liquids pure (The Silver Institute,
17 2009, [202655](#)). The Greek historian Herodotus recorded that Cyrus the Great, King of Persia from 559
18 B.C. to 530 B.C., had water drawn from a stream and "... boiled, and very many four-wheeled wagons
19 drawn by mules carry it in silver vessels, following the king wherever he goes at any time ..."
20 (Herodotus, 1920, [594263](#)).

21 The use of silver compounds for therapeutic purposes has a long history. In the 18th century, silver
22 nitrate, often called lunar caustic, was molded into pencil-like forms and used to remove granulation
23 tissue from wounds and to lance abscesses, while powdered silver nitrate was used to kill impurities and
24 to dry open wounds (Klasen, 2000, [202675](#)). In the 19th century, physicians used silver nitrate to promote

1 healing of burns and other wounds, and others began to research the bactericidal effects of silver. As
2 early as 1880, physicians used a silver nitrate eye-drop solution to prevent gonococcal conjunctivitis in
3 newborns. In the early 20th century, surgeons used silver foil, silver sutures, and other silver dressings to
4 prevent infection in surgical wounds. Today, wound dressings containing silver such as Actisorb Silver
5 220 (Johnson and Johnson) and silver-based vascular and urinary catheters are used in healthcare
6 (Chopra, 2007, [157707](#)).

7 In 1839, Louis-Jacques-Mandé introduced a photography technique that used a silver-plated sheet
8 of copper, sensitized with iodide vapors to make the silver react with light, and a salt solution to
9 permanently set the image on the sheet (Department of Photographs, 2004, [202672](#)). Silver is still used in
10 photography today, but with the advent of digital photography, the percentage of its total usage continues
11 to decline, dropping from 39% in 1979 to 13% in 2008 (GFMS, 2009, [201631](#)).

12 Silver has the highest thermal and electrical conductivities of any pure metal across a range of
13 temperatures (CRC, 2000, [196090](#)), and is therefore used in industrial applications such as household
14 switches, switch panels in electrical appliances, batteries, and superconductors (The Silver Institute, 2009,
15 [202685](#)). In 2008, industrial applications of silver accounted for 54% of the total silver used in
16 manufacturing (GFMS, 2009, [201631](#)). Jewelry (19%), silverware (7%), and coins (8%) account for the
17 remaining silver used in manufacturing (GFMS, 2009, [201631](#)). Other industrial uses of silver include as
18 coatings on mirrors and compact discs, in water purification systems, as antibacterial disinfectants, and in
19 rear window defrosters in automobiles (The Silver Institute, 2009, [202685](#)).

20 Silver iodide is used for cloud seeding. This process introduces silver iodide to clouds to induce
21 contact freezing of supercooled liquid water (colder than 0 °C) so that the amount or type of precipitation
22 that falls from clouds is altered (Stroyproject LTD, 2009, [225328](#)). Supercooled liquid water is
23 chemically unstable and thus freezes upon contact with silver iodide, an artificial ice-nucleus. Freezing
24 releases heat to the environment, thereby adding energy to the system and possibly increasing the
25 intensity or duration of precipitation. Silver iodide can be introduced via aircraft or by ground-level
26 dispersion devices (e.g., rockets, anti-aircraft guns, generators). The efficacy of cloud seeding is
27 unknown, but the practice is popular in geographic locations where freshwater supplies are scarce (U.S.,
28 2006, [224918](#)).

2.1.2. Historic Environmental Silver Levels

29 Industrial processes such as smelting and mining, photography, and jewelry manufacture have led
30 to elevated levels of silver being released into the environment (U.S. EPA, 1987, [195458](#)). Typically,
31 areas of elevated silver concentrations occur near sewage outfalls, electroplating plants, mine waste sites,
32 and silver iodide-seeded areas (Eisler, 1996, [196141](#)). Runoff from silver disposal sites can transport

1 silver farther from these locations, and subsequent human activities such as dredging and construction can
 2 further transport the silver (Purcell and Peters, 1998, [195563](#)). As presented in Table 2-1, silver
 3 contamination has been studied in several U.S. locations including the Quinnipiac River (Connecticut)
 4 (Rozan and Hunter, 2001, [202680](#); Rozan et al., 1995, [201632](#)), San Francisco Bay (Flegal et al., 2007,
 5 [195508](#)), and various sites in Michigan (Murray et al., 2004, [082802](#)).

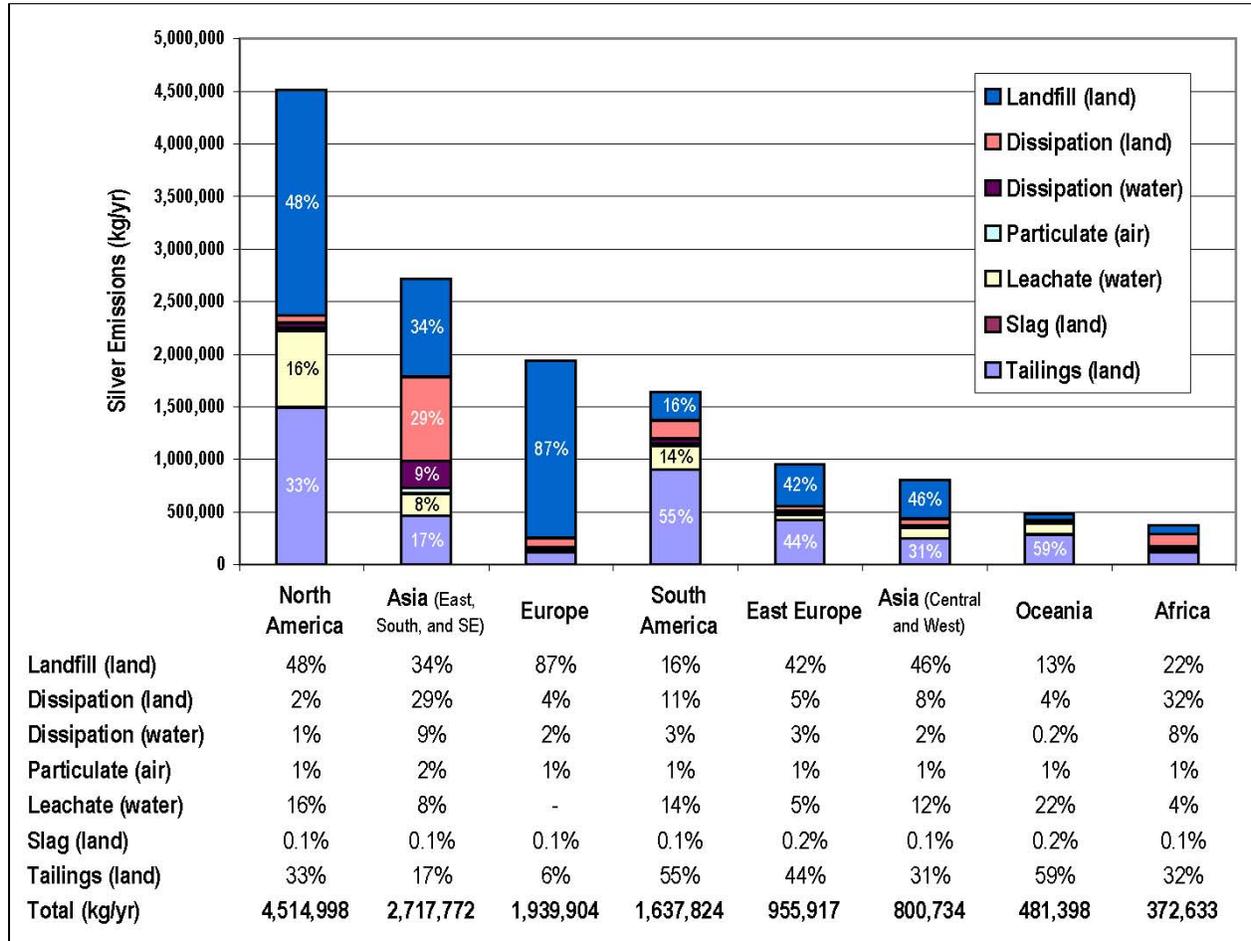
Table 2-1. Selected U.S. studies of silver contamination in the environment.

| Location | Anthropogenic source | Medium | Levels measured | Source |
|-------------------------------|--|--|--|---|
| Quinnipiac River, Connecticut | Manufacturing of silver tableware and decorative items | Water | 5–500 nanograms per liter (ng/L) Max = 800 ng/L | Rozan et al. (1995, 201632) |
| | | Sediment | 15–30 micrograms per gram (µg/g) Max = 250 µg/g | |
| | | Effluent from municipal wastewater treatment plant | 120–180 ng/L | |
| San Francisco Bay | Municipal wastewater treatment plant handling discharge from a photo processing facility | Surface sediments | Lower South Bay 0.052–1.18 µg/g, dry weight Mean = 0.388 µg/g, dry weight | Flegal et al. (2007, 195508) |
| | | Surface water | <0.1–26.3 ng/L (total dissolved silver between 1989 and 2005) | |
| Michigan | None specified | Surface soil | 0.5 ± 0.2 µg/g (commercial) 0.8 ± 0.5 µg/g (residential) 2.3 ± 2.2 µg/g (industrial) | Murray et al. (2004, 082802) |
| | | Sub-surface soil | 0.6 ± 0.4 µg/g (commercial) 0.5 ± 1.1 µg/g (residential) 2.2 ± 4.0 µg/g (industrial) | |

6 In the Quinnipiac River, Rozan et al. (1995, [201632](#)) and Rozan and Hunter (2001, [202680](#)) found
 7 that silver concentrations in the river water peaked at 800 nanograms per liter (ng/L) following rainstorms
 8 due to erosion of the silver-laden soil on the river banks and resuspension of contaminated sediments.
 9 Using cesium-137, Rozan et al. (1995, [201632](#)) dated the highest concentrations of silver in the river bank
 10 to the 1950s, corresponding to the peak production period of the local silver industry.

11 Ecological and toxicological effects have been linked to silver concentrations in the environment in
 12 the ng/L range as demonstrated by several field and dietary toxicity studies summarized by Luoma (2008,
 13 [157525](#)) and described here. Hornberger et al. (2000, [225177](#)) observed biochemical signs of stress, most
 14 notably failure to reproduce, in clam species (*Corbula amurensis*) on a mudflat 2 kilometers (km) from a
 15 domestic-sewage outfall in South San Francisco Bay. Over a 30-year period, the amount of silver in the
 16 waste delivered to this sewage facility decreased, and the facility also improved its treatment process for
 17 wastes containing silver. With these changes, the amount of silver discharged from the sewage facility to
 18 the bay decreased and the reproductive capabilities of the clams recovered (Hornberger et al., 2000,
 19 [225177](#)).

1 In 1978, an estimated 2.47 million kilograms (kg) of silver was emitted to the environment by the
 2 United States alone (Smith and Carson, 1977, [202683](#)). Eighty-two percent of the annual silver loss
 3 originated from anthropogenic activities (42% from the photography industry) (Smith and Carson, 1977,
 4 [202683](#)). In 1978, 3.7% of released silver entered the atmosphere, 28.5% ended up in the aquatic
 5 environment, and 67.8% entered the terrestrial environment (Smith and Carson, 1977, [202683](#)).



Source: Adapted with permission from Eckelman and Graedel (2007, [196138](#))

Figure 2-1. Silver emissions to the environment by geographical region.

The original analysis by Eckelman and Graedel (2007, [196138](#)) examined silver emissions in 64 countries. For this report, the data have been aggregated according to the United Nations' geographical regions (UN Statistics Division, 2008, [597420](#)).

North America: Canada, Mexico, and United States

Asia (East, South, and Southeast): China, Hong Kong, India, Indonesia, Iran, Japan, Malaysia, Philippines, Singapore, South Korea, Taiwan, Thailand

Europe: Austria, Bel-Lux, Denmark, Finland, France, Germany, Greece, Italy, Netherlands, Norway, Portugal, Spain, Sweden, and United Kingdom

South America: Argentina, Bolivia, Brazil, Chile, Colombia, Peru, Venezuela

East Europe: Bulgaria, Poland, Romania, Russia, and Ukraine

Asia (Central and West): Israel, Kazakhstan, Saudi Arabia, Turkey, UAE, Uzbekistan

Oceania: Australia and New Zealand

Africa: Algeria, Cameroon, Egypt, Ethiopia, Ghana, Ivory Coast, Kenya, Morocco, Namibia and South Africa, Nigeria, Sudan, Tanzania, Tunisia, Uganda, Zimbabwe

6 More recently, Eckelman and Graedel (2007, [196138](#)) characterized the emissions of silver based
 7 on data and estimations of environmental releases of silver in 1997 (Figure 2-1). Their assessment began
 8 with the production, fabrication, and import/export data collected and estimated in a material flow

1 analysis by Johnson et al. (2005, [484604](#)) for 64 countries. Johnson et al. concluded that their material
2 flow analysis comprised “well over 90%” of the global silver flow including mining and production,
3 fabrication and manufacture, use, and waste management. Eckelman and Graedel (2007, [196138](#)) further
4 characterized the losses of silver by applying a series of assumptions about the percentage of silver lost in
5 each process. For example, they assumed particulate silver emissions from incineration of municipal
6 solid waste varied directly with the overall income level of the country, ranging from an emission factor
7 of 0.1% for high-income countries to 0.4% for low-income countries. The variance was due to the type of
8 furnace and pollution control technology used; the researchers assumed that high-income countries
9 employ control technologies that are more modern and more efficient than those used in low-income
10 countries. Thus, in high-income countries, more of the silver is captured before it is emitted to the
11 environment resulting in a lower emission rate.

12 Eckelman and Graedel calculated silver emissions for 64 countries, and Figure 2-1 presents their
13 estimates of silver emissions aggregated by geographical regions worldwide. The reported values do not
14 account for transport of the silver from one medium (i.e., air, land, or water) to another after initial
15 emissions. Eckelman and Graedel (2007, [196138](#)) estimated 65% of the 2.84 million kg of silver emitted
16 from the United States enters landfills, 18% is released to the environment via tailings, and 13% is
17 contained in leachate from the mining and productions process.

18 The U.S. Environmental Protection Agency’s (EPA’s) National Secondary Drinking Water
19 Regulations recommend a guideline of less than 0.10 milligram per liter (mg/L) (or ppm) of total silver in
20 drinking water due to the potential cosmetic (not health) effects of silver ingestion, specifically argyria
21 (U.S. EPA, 2009, [225005](#)). States may decide to enforce this standard, but enforcement is not required.
22 Due to lack of evidence, EPA has not prescribed ambient water quality criteria for silver for human health
23 (i.e., to protect from exposure to silver by consumption of contaminated water or organisms).

24 With respect to water quality criteria to protect aquatic life, a few states have set or proposed
25 aquatic life chronic criteria concentrations; for example, the North Carolina Division of Water Quality has
26 proposed a criterion of 0.06 µg/L (NCDENR Division of Water Quality, 2007, [597412](#)). Oregon has
27 established a 0.12-µg/L criterion in its Administrative Rules based on chronic toxicity to rainbow trout
28 and minnows in fresh water and to mysids in salt water (Oregon Department of Environmental Quality,
29 2004, [597421](#)). Texas, New York, and some regions in California have established similar chronic aquatic
30 life criteria concentrations. Several regions in California have established maximum contaminant levels
31 (MCLs) for silver between 5 and 10 µg/L. EPA proposed and then withdrew a chronic toxicity standard
32 in the early 1990s (Ford, 2001, [195509](#); U.S. EPA, 1987, [195458](#)). The Agency has, however, prescribed
33 maximum acute concentrations of 3.2 µg/L in fresh water and 1.9 µg/L in salt water (U.S. EPA, 2009,
34 [225006](#)). These standards are enforced through the issuance of discharge permits at the state level.

2.2. Nanoscale Silver

1 Nanoscale silver is not new. The Lycurgus Cup, a glass and bronze cup on display at the British
2 Museum, was likely created in the 4th Century AD (Evanoff and Chumanov, 2005, [195506](#)). The glass in
3 the cup appears green until light is shone through it, shifting the absorption spectrum so that the glass
4 appears to glow red. Small gold and silver particles were incorporated into the glass used by the original
5 craftsman; in the 1990s, researchers at the British Museum determined the average diameter of the gold
6 and silver particles in the glass to be 70 nanometers (nm). The shift in the apparent color of the cup is due
7 to the gold and silver particles in the glass. The Lycurgus Cup therefore represents what is likely one of
8 the first uses of nanoscale silver. Nanoscale silver also has been found in stained glass. The unique
9 optical properties of nanoscale silver that prompted these early uses are discussed further in Section 2.3.6.

10 The literature suggests that products containing colloidal silver have been available (although not
11 necessarily registered or supported by science) for use by humans as therapeutic agents for more than 100
12 years (Bottomly et al., 1909, [630020](#)), and colloidal silver suspensions containing some particles with at
13 least one dimension in the 1- to 100-nm range were likely employed long before their use in these
14 applications was recorded.

15 More recently, the Woodrow Wilson Center's Project on Emerging Nanotechnologies (PEN) has
16 compiled an inventory of consumer products reported by their manufacturers to contain nanomaterials.
17 Based on the data PEN has collected, nano-Ag appears to be the most common nanomaterial used today
18 in manufactured consumer products (Project on Emerging Nanotechnologies, 2009, [196774](#)). Of the
19 more than 1,015 consumer products included in the August 2009 PEN inventory of nanomaterial-based
20 consumer products, more than 25% are listed as containing nano-Ag. Consumer products based on
21 nano-Ag represent a significant fraction of every product category examined for the PEN inventory.
22 From March to August 2009, the number of products reported to contain nano-Ag rose from 25 to 259, a
23 more than 10-fold increase (Project on Emerging Nanotechnologies, 2009, [196774](#)). When considering
24 this apparent market growth, readers should take into consideration that the products in the PEN inventory
25 were identified through Internet searches for products reported by their manufacturers or distributors to
26 contain silver nanoparticles. Manufacturers' claims that products listed in the PEN inventory contain
27 engineered nanoparticles have not all been independently or scientifically validated.

28 The PEN Silver Nanotechnology Consumer Inventory contains detailed information about current
29 consumer products reportedly containing nano-Ag and indicates that most nano-Ag products claim to
30 eliminate bacteria and their related odors (Fauss, 2008, [196776](#); Project on Emerging Nanotechnologies,
31 2009, [196774](#)). Some products, such as dietary supplements, laundry detergent, body soap, toothpaste,
32 and wall paint, appear to contain colloidal nano-Ag. One manufacturer suggests that dietary supplements
33 containing nano-Ag promote a healthy immune system by inhibiting the growth of and possibly
34 destroying bacteria and viruses in the digestive tract (ConSeal International Inc, 2010, [594257](#)). One

1 manufacturer claims that nano-Ag supplements can defend the body from “colds, flu, and hundreds of
2 diseases (even anthrax)” and that silver has been used throughout history as a cure for more than 650
3 diseases, from AIDS to cancer (Melchizedek, 2010, [098732](#)).

4 According to industry claims, nano-Ag has been fixed to the surfaces of or impregnated in several
5 products, including kitchen utensils, food preservative film, and filters of air and water purification
6 systems (Project on Emerging Nanotechnologies, 2009, [196774](#)). For instance, manufacturers have
7 introduced nano-Ag into cooking utensils to prevent bacterial contamination and to reinforce the strength
8 of the utensils. Nano-Ag can be incorporated into the materials used to produce clothing, socks, fabrics,
9 and shoe soles, purportedly to minimize odors by inhibiting the growth of bacteria, mold, and mildew.
10 Nano-Ag also has been added to medical products, including wound dressings, contraceptives, surgical
11 instruments, bone prostheses, and cardiac catheters (Chen and Schluesener, 2008, [196119](#); Samuel and
12 Guggenbichler, 2004, [202682](#)), some of which might not be included in the PEN consumer product
13 inventory (Wijnhoven et al., 2009, [180201](#)). Other products to which nano-Ag has been reportedly added
14 include home furnishings, cleaning products, food storage containers, kitchen appliances, curling irons,
15 hair dryers, make-up, burn creams, nasal sprays, soaps, and dish detergents
16 (Project on Emerging Nanotechnologies, 2009, [196774](#)).

17 Spray disinfectants represent a category of the nano-Ag cleaning products that could become
18 available for home and garden use in the United States. Products intended to disinfect inanimate objects
19 or otherwise control microorganisms, except on living humans or animals, are considered “pesticides,”
20 and federal law requires that EPA register such products before they may lawfully be sold or distributed in
21 the United States. Spray disinfectants containing nano-Ag particles might be more effective at killing
22 bacteria, for example, than those made with larger, conventional silver particles due to the higher surface
23 area-to-volume ratios of the smaller particles, which could result in increased reactivity. Some
24 manufacturers have claimed that aerosol disinfectant sprays containing nano-Ag kill 99% of bacteria on
25 various surfaces and prevent odor for long periods (ConSeal International Inc, 2010, [594257](#); Shanghai,
26 2009, [594268](#)). Theoretically, nano-Ag sprays could also serve as broad-spectrum fungicides, and the
27 sprays could exhibit antiviral properties (Sun et al., 2005, [202684](#); Wright et al., 1999, [202687](#)).

28 The expanding use of nano-Ag in the consumer market suggests that, depending on the behavior of
29 nano-Ag in the environment, background concentrations of nano-Ag and also of silver in some
30 environmental media could represent additional sources of long-term and incremental exposure to both
31 humans and biota. In the home, nano-Ag spray disinfectants might be applied to walls, tables, beds, and
32 other surfaces to eliminate harmful bacteria, particularly in the kitchen and bathroom. Spray disinfectants
33 with nano-Ag could be used in hospitals, nursing homes, airports, and other public places in efforts to
34 protect people from illness and disease.

2.3. Physical-Chemical Properties of Nanoscale Silver

1 The physicochemical properties of nanoparticles determine both their fate in the environment and
2 their beneficial and harmful effects. Although the size of the nanoparticles can be the most distinguishing
3 property when compared to conventional particles, other unique physical and chemical properties begin to
4 emerge as particles approach the nanoscale range. For this reason, Auffan et al. suggest that “below a
5 critical size, it is not possible to simply scale the properties of bulk materials based on the surface area to
6 predict the properties of nanoparticles” (Auffan et al., 2009, [193255](#)). Other scientists agree that
7 “although some material properties, like chemical composition and crystal structure, are the same on the
8 nanoscale as in the bulk phase, other properties differ... a nanoparticle retains properties of both materials
9 in the bulk phase and molecular precursors” (Sayes and Warheit, 2009, [201592](#)). Therefore, although the
10 physicochemical properties differ, some researchers have found that the effects of nano-silver can be
11 similar to those produced by conventional ionic silver (Pal et al., 2007, [196273](#)), while other studies have
12 shown that this is not the case (Griffitt et al., 2009, [199805](#)). These differing findings led the Federal
13 Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (2010, [625619](#)) to
14 conclude that “comparison[s] of physicochemical properties of the nano [versus] bulk materials are
15 needed.”

16 Exactly which physiochemical properties of engineered nanoparticles, including nano-Ag, can be
17 useful for predicting their behavior and interactions in the environment is unclear. Several organizations
18 and independent researchers have published recommendations on the physicochemical characterization
19 data that should accompany research findings on fate and transport and ecological and human toxicity
20 (MINChar Initiative, 2008, [594265](#); Oberdorster et al., 2005, [090087](#); OECD, 2008, [157512](#); Sayes and
21 Warheit, 2009, [201592](#); Tiede et al., 2008, [196278](#); U.S. EPA, 2010, [625619](#)). These recommendations
22 are based on synthesis of published, peer-reviewed studies on the behavior and effects of nanoparticles,
23 but the recommended properties vary by organization and researcher. Some of the recommendations
24 regarding characterization before, during, and after toxicity studies are further described in Section 6.1.1.

25 In general, the most prescribed physicochemical properties include:

- 26 • Size, including agglomeration and aggregation tendencies;
- 27 • Morphology, including shape and crystal structure;
- 28 • Surface area;
- 29 • Chemical composition;
- 30 • Surface chemistry and reactivity;
- 31 • Solubility; and
- 32 • Conductive, magnetic, and optical properties.

33 Each property, as it relates to nano-Ag and to the other properties identified, is briefly discussed below.
34 For nano-Ag spray disinfectants, Hansen et al. (2007, [093168](#)) suggest that all of the above properties,
35 with the exception of conductive, magnetic, and optical properties, are relevant to conducting a hazard

1 identification. It could also be that these properties, which may affect the efficacy of the final product,
2 offer clues as to which types of nano-Ag may be preferentially commercialized and thus most relevant to
3 study as potential hazards. Additional details on the state of knowledge of physicochemical properties in
4 relevant environmental compartments, exposure routes, and effects are provided in the chapters that
5 follow.

2.3.1. Size

6 In recent years, synthesis methods have been developed to produce nanoparticles, and silver
7 nanoparticles in particular, of various shape and size distributions. See, for example, Bar-Ilan et al. (2009,
8 [191176](#)), Evanoff and Chumanov (2005, [195506](#)), Khaydarov et al. (2009, [194104](#)), and Tolaymat et al.
9 (2010, [625615](#)). Relatively monodisperse particles can be obtained within the size range of 1–100 nm
10 (see Chapter 3 for synthesis methods).

11 The size distribution of nanoparticles, including silver nanoparticles, however, does not necessarily
12 remain constant and depends on the chemical and physical environment surrounding the nanoparticles;
13 silver nanoparticles can agglomerate or aggregate to form larger sized clusters of nanoparticles. How
14 rapidly the particles cluster in an aqueous medium depends on particle collision frequencies (e.g.,
15 Brownian motion and particle concentration), the energy of the particle collisions, the attractive-repulsive
16 properties of the particles involved (e.g., repelling surface charges of two positively charged particles),
17 and the interactions with colloidal materials such as natural organic matter present in the water.
18 Handy et al. (2008, [157562](#)) summarized that “after collision, particles can remain in aqueous phase as
19 single particles or form particle-particle, particle-cluster, and cluster-cluster aggregates.” The dispersion
20 state describes the extent to which particles become clustered by interparticle attractive forces. Surface
21 coatings and stabilizing agents can enhance the stability of the dispersion and maintain the original or
22 intended size distribution.

23 These phenomena can affect the fate and transport of nano-Ag particles in the environment and in
24 humans and biota. Often, coagulation leads to the formation of larger, less mobile particle clusters
25 (AFSSET, 2006, [201630](#); Aitken et al., 2004, [090566](#); Wiesner et al., 2006, [089583](#)). Nano-Ag used in
26 some products can enter the environment as individual nanoparticles or as small clusters. In other cases,
27 the nano-Ag incorporated into consumer products as composites or mixtures could be released into the
28 environment in an encapsulated form (Lowry and Casman, 2009, [196153](#)). The translocation of particles
29 depends in part on their size; hence, clusters of nano-Ag behave quite differently compared to single
30 particles (Ma-Hock et al., 2007, [091320](#)). The size of the nano-Ag (i.e., an individual particle versus a
31 cluster) can determine the likelihood of release of silver ions, sometimes referred to as Ag⁺ ions, from the
32 particle and the particle’s behavior in the environment (O'Brien and Cummins, 2009, [196217](#)). Moreover,

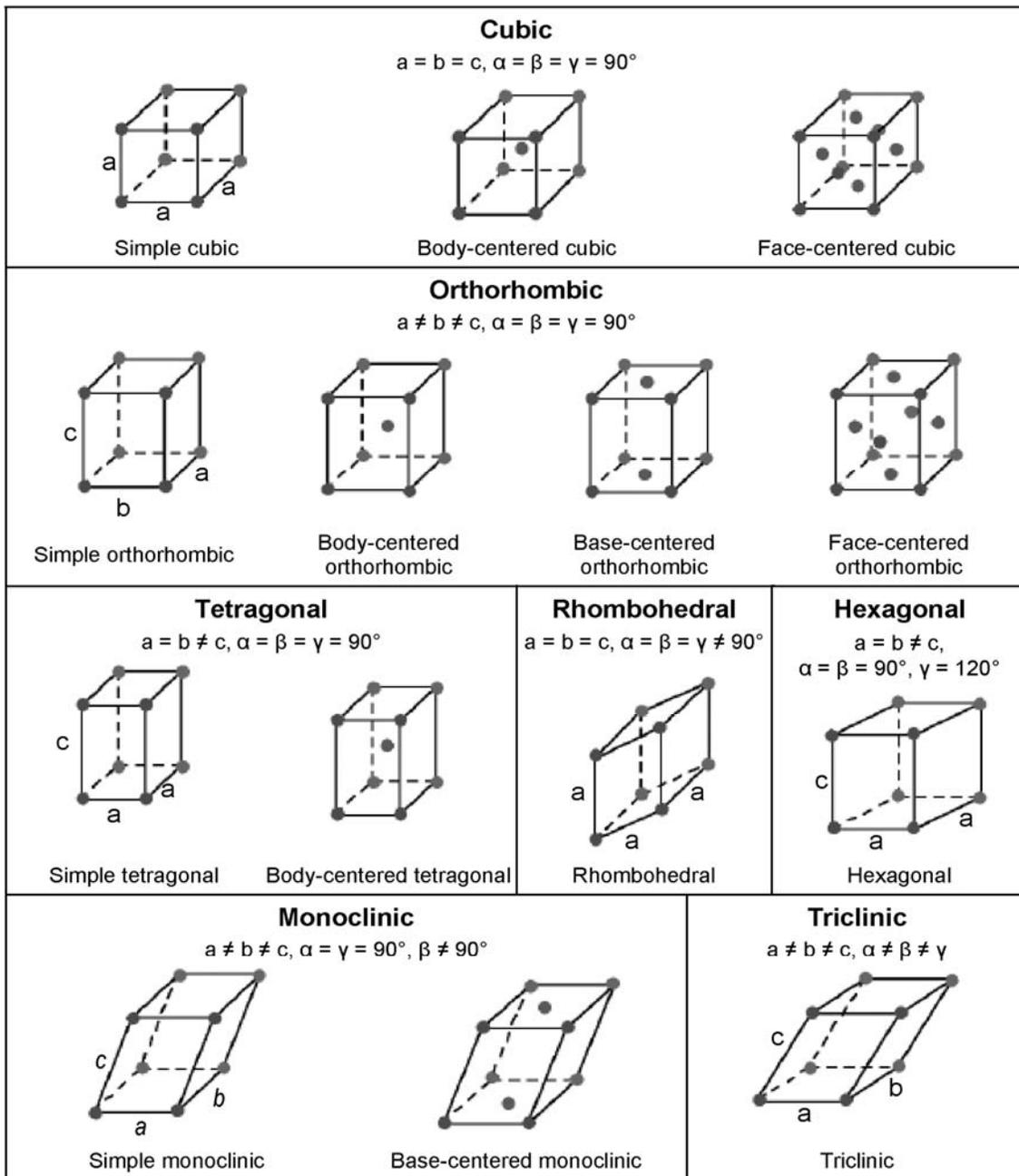
1 size alone might determine particle mobility in the environment and within the body (Chen and
2 Schluesener, 2008, [196119](#)) and enable nano-Ag to enter cells (Bar-Ilan et al., 2009, [191176](#); Morones et
3 al., 2005, [196271](#)). As emphasized by the FIFRA Scientific Advisory Panel (2010, [625619](#)), however, the
4 impact of size on the biological response elicited by nano-Ag particles is less clear. Size-dependent
5 particle mobility is discussed as it relates to potential biotic and human uptake and dose in Chapter 5, and
6 size-dependent ecological and human health effects are discussed in Chapter 6.

2.3.2. Morphology

7 Nano-Ag can be synthesized into various forms, including particles, spheres, rods, cubes, truncated
8 triangles, wires, films, and coatings (Pal et al., 2007, [196273](#); Wijnhoven et al., 2009, [180201](#)). Nano-Ag
9 particles with various crystal structures also can be created. The atoms, molecules, or ions that compose
10 the crystal occur in the same repeated arrangement, or lattice, throughout the crystal (Barron and Smith,
11 2010, [597388](#)). Auguste Bravais, a 19th century mathematician and physicist, determined that in three-
12 dimensional space, 14 lattice configurations are possible such that the arrangements of the points appear
13 identical when viewed from any other point. Figure 2-2 shows seven crystal structures (e.g., cubic,
14 orthorhombic) and the associated 14 Bravais lattices (e.g., simple cubic, body-centered orthorhombic).
15 The crystal structures also are described in terms of the planes that join the points in the lattice. These
16 planes are denoted using Miller Indices, which are the reciprocals of the intercepts of crystal plane with
17 the *x*, *y*, and *z* three-dimensional axes. Figure 2-3 illustrates three common crystal planes.

18 The shape of nano-Ag particles can affect the kinetics of their deposition and transport in the
19 environment. Depending on its surface structure and shape, a nano-Ag particle might exhibit different
20 reactivity (Oberdorster et al., 2005, [090087](#)), as its shape could make it difficult for particles to approach
21 each other. Such shape-related interactions can be controlled in some situations by adding detergents or
22 surface coatings to the particles to change their shape or surface charge (Handy et al., 2008, [157562](#)).

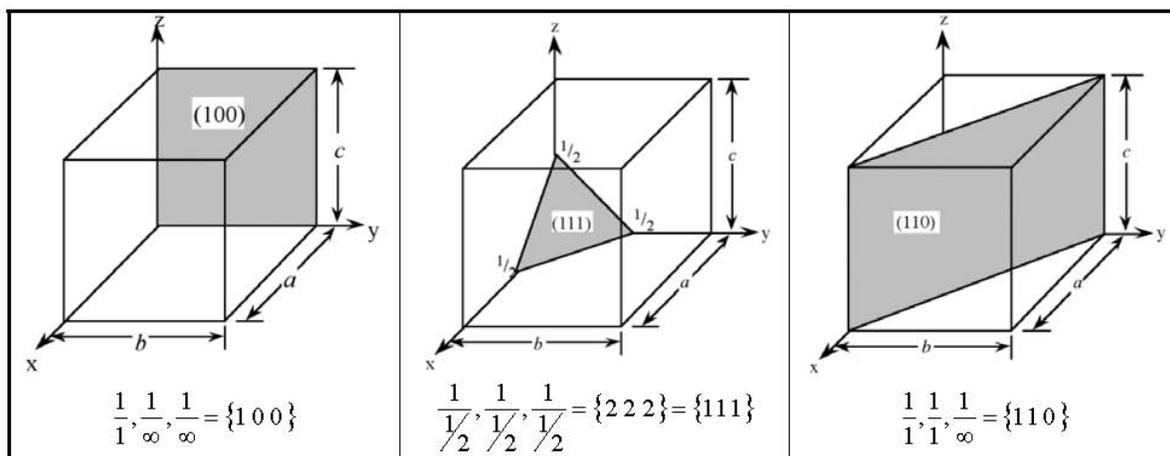
23 Pal et al. (2007, [196273](#)) studied the antibacterial activity (using *Escherichia coli*, or *E. coli*) of
24 silver nanoparticles of various shapes. Results indicated that nano-Ag particles of various shapes could
25 kill *E. coli*, but the inhibition results differed and could be explained based on the percent of active facets
26 in the crystal structure. Specifically, truncated triangular silver nanoplates with a {111} lattice plane as
27 the basal plane displayed the strongest biocidal action, compared to the spherical and rod-shaped nano-Ag
28 particles, indicating that increasing the number of active facets on the surface of a crystalline, or highly
29 ordered, nanoparticle increases its ability to inhibit bacterial growth.



Source: Adapted from Barron and Smith (2010, [597388](#)).

Figure 2-2. Crystal lattice structures.

The axes of a unit crystal are designated a , b , and c , and the angles between the axes are designated with the Greek letter corresponding to the axes not forming the angle. For example, β is the angle between the a and c axes. In each structure, all points are equidistant.



Source: Adapted from Barron and Smith (2010, [597388](#)).

Figure 2-3. Common crystal lattice planes.

The Miller Indices for a given lattice plane describe where the plane intersects the axes of the crystal structure. Depending on the lattice structure, the axes might not intersect at right angles; for example, the a and c axes of a monoclinic crystal do not form a 90° angle. Miller Indices are expressed as the reciprocals of these intersections, and an index of zero indicates that the plane is parallel to the axis in that particular direction.

2.3.3. Surface Area

1 Because of their small size, nano-Ag particles have greater specific surface area when compared to
 2 the same mass of material in larger particles (Luoma, 2008, [157525](#)) and have a greater surface area-to-
 3 volume ratio (Auffan et al., 2009, [193255](#)). Auffan et al. estimate that a 10-nm particle has approximately
 4 35–40% of its atoms on the surface compared to 15–20% of the atoms on a particle larger than 30 nm in
 5 diameter. This large surface area of nanoparticles relative to their mass or volume increases the reactivity
 6 and sorption behavior of the particle (Auffan et al., 2009, [193255](#); Tiede et al., 2008, [196278](#); U.S. EPA,
 7 2010, [625619](#)). Large specific surface area enhances chemical reactivity, which means that smaller silver
 8 nanoparticles have more reaction sites (i.e., sites that can receive electrons) on their surfaces and are more
 9 sensitive to oxygen, a natural electron donor, than larger particles (Auffan et al., 2009, [193255](#)).
 10 Therefore, smaller particles could exhibit greater efficacy as biological agents or stressors in ecosystems or
 11 on human health, as discussed in Section 2.3.4.

12 Surface area also affects the ratio of silver ions on the surface of a silver particle to silver ions that
 13 are “buried” inside the same silver particle. This ratio might also increase as particle size decreases.
 14 Thus, for larger particles with a smaller surface area-to-volume ratio, most of the silver ions might be
 15 unable to interact with the environment or biological surfaces. This idea is further discussed in Section
 16 6.1.

2.3.4. Chemical Composition

1 As mentioned previously, silver can exist in four oxidation states: Ag^0 , Ag^+ , Ag^{2+} , and Ag^{3+} , and
2 the free silver ion under natural conditions is Ag^+ (CRC, 2000, [196090](#)). Of these, Ag^0 and Ag^+ are the
3 most commonly occurring oxidation states in the environment. Based on a review of the existing
4 literature, Wijnhoven et al. (2009, [180201](#)) concluded that environmental and human studies appear to
5 demonstrate that forms of conventional silver that release free silver ions are more toxic than other forms
6 of conventional silver that do not (Wijnhoven et al., 2009, [180201](#)). Speciation would therefore strongly
7 influence how much silver is available to affect living organisms. To achieve stability, positively charged
8 silver ions will associate with negatively charged ligands⁴ (e.g., sulfide in fresh water and chloride in salt
9 water) (Luoma, 2008, [157525](#)); the reader is referred to a detailed discussion of this process in Section
10 4.4. The concentrations of these ligands and the bond strength between the silver ions and the ligands
11 influence the distribution of silver as free silver ions (its more bioavailable form) and the “less available”
12 ligand-bound forms (Luoma, 2008, [157525](#)).

13 Chemical composition also includes the surface coating of the nanoparticle (Sayes and Warheit,
14 2009, [201592](#)). Coatings may be used to stabilize the nanoparticles in solution, to prevent agglomeration,
15 or to add functionality to the nanoparticle depending on its intended use. Surface coatings can influence
16 the reactivity of the nanoparticle in various media including surface water, biological fluids, and
17 laboratory test media (Auffan et al., 2009, [193255](#); Cumberland and Lead, 2009, [199804](#)).

2.3.5. Surface Chemistry and Reactivity

18 The larger surface area and surface charge of nano-Ag particles correlate with the availability of
19 more possible reactive sites and greater chemical reaction potential, adsorption potential, and potential to
20 aggregate or agglomerate. These properties can, in turn, affect the transport properties, behavior,
21 interactions, distribution, bioavailability, and effects of nano-Ag particles in the environment (O'Brien and
22 Cummins, 2009, [196217](#); Wiesner et al., 2009, [194996](#)). Surface charge also influences particle stability
23 in dispersions and overall solubility (Sayes and Warheit, 2009, [201592](#); Tiede et al., 2008, [196278](#)).

24 Coatings on the nanoparticle surface can consist of various chemical compositions, which influence
25 particle behavior and persistence (Handy et al., 2008, [157562](#); Tiede et al., 2008, [196278](#)). Some
26 examples of coatings commonly applied to nanoparticles are provided in Table 2-2. Nano-Ag is often
27 coated with a surfactant, polymer, or polyelectrolyte (Lowry and Casman, 2009, [196153](#)). These coatings
28 can impart charge to the particles (positive or negative) and stabilize them against clustering and

⁴ A ligand is a substance (e.g., atom, molecule, radical, or ion) that forms a complex around a central atom (see Section 4.4).

1 deposition (Nowack and Bucheli, 2007, [092294](#); Wiesner et al., 2006, [089583](#)). For example, some
 2 nano-Ag particles are engineered to remain in water as single particles, making them more water
 3 dispersible by improving their water solubility and suspension characteristics (Luoma, 2008, [157525](#)).
 4 The magnitude of the effect of the coating depends on the type and repulsive forces of the coating. Small-
 5 molecular-weight coatings provide primarily electrostatic stabilization by imparting a surface charge to
 6 the particle. These repulsive forces are fairly weak and are readily blocked by cations in solution. Large-
 7 molecular-weight polymers (uncharged) can provide steric repulsions that stabilize particles against
 8 clustering and enhance transport (Lowry and Casman, 2009, [196153](#)). In a study of nano-Ag disinfectant
 9 spray, Kvitek et al. (2008, [196266](#)) found that nano-Ag particles with different surface coatings varied
 10 with regard to inhibiting bacterial growth, depending on the surface coating and the bacteria tested. Their
 11 findings imply that it is the surface coating used on the particles in a nano-Ag spray disinfectant that
 12 might be the key to product effectiveness and not necessarily the nano-Ag per se.

Table 2-2. Types of common coatings of nano-Ag.

| Type | Coatings |
|----------------------|--|
| Emulsifiers | Bovine serum albumin (BSA) Polyoxyethylene-sorbitan monooleat (Tween 80) Polyvinyl alcohol (PVA) Sodium citrate |
| Surfactants | Cetyltrimethylammonium bromide (CTAB) Daxad 19 Hydrocarbons Polyoxyethylene-sorbitan monooleat (Tween 80) Sodium dodecyl sulfate (SDS) |
| Ligands and Polymers | Polyethylene glycol (PEG) Starches/sugars Polysaccharides Polyvinylpyrrolidone (PVP) |

Note: List of coatings in the table are examples mentioned in the literature summarized in Appendices B and C.

2.3.6. Solubility

13 Solubility influences the fate and behavior of nanoparticles in the environment (Wijnhoven et al.,
 14 2009, [180201](#)) and also the dissolution of the nanomaterial and release of ions (Auffan et al., 2009,
 15 [193255](#)). Metallic conventional silver is insoluble in water while silver salts including silver nitrate and
 16 silver chloride have varying degrees of solubility (i.e., the solubility of silver nitrate in water is 216 grams
 17 per 100 milliliters (g/100 mL) at 20°C while silver chloride is nearly insoluble) (CRC, 2000, [196090](#)).
 18 Refer to Table 4-1 for additional solubility information. Cumberland and Lead (2009, [199804](#)) showed

1 approximately 1% dissolved silver when nano-Ag particles (average size 13–15 nm) were added to a
2 solution containing humic substances and sodium or calcium to mimic environmentally relevant
3 conditions. Griffet et al. (2009, [199805](#)) reported that the total nano-Ag concentration was approximately
4 10 times greater than the soluble nano-Ag concentration at 25 °C in filtered water (0.22-micrometer [μm]
5 filter). In general, a substance's solubility or degradability tends to be inversely related to persistence in
6 environmental media. Persistent materials that are slow to dissolve might be more available to biological
7 systems depending on the fate and transport of the substance in the environment.

8 The rate of dissolution can be considered proportional to particle surface area; therefore,
9 nanoparticles should dissolve faster than larger conventional materials, considering surface area alone
10 (O'Brien and Cummins, 2009, [196217](#)). Particle concentration, surface morphology, surface energy,
11 clustering, and other properties, however, are also relevant when considering dissolution at the nanoscale.
12 For example, clustered nanoparticles can be less soluble than unclustered nanoparticles because the
13 equilibrium solubility of the system is inversely proportional to overall particle size (Borm et al., 2006,
14 [088034](#)). Consequently, increased clustering will tend to result in increased persistence of the
15 nanoparticles in solution. For nano-Ag, solubility also can be modified by surface coatings, as noted in
16 the previous section (Luoma, 2008, [157525](#); SCENIHR, 2006, [194113](#)). The hydrophobic or hydrophilic
17 nature of the coatings and the bonding strength between the coatings and the nano-Ag can affect solubility
18 in different media.

2.3.7. Conductive, Magnetic, and Optical Properties

19 Silver nanoparticles have been studied and characterized by material scientists extensively over the
20 past two decades. In addition to its biocidal effects, nano-Ag, like other noble metals such as copper and
21 gold, interacts strongly with electromagnetic radiation, which causes nano-silver to take on unique
22 conductive, magnetic, and optical properties. These properties facilitate the use of nano-Ag in
23 biomolecular labeling and detection and in other electronic sensor technologies. For example, silver
24 exhibits the highest surface plasmon resonance (SPR) band among all metals, and only silver, gold, and
25 copper display this resonance in the visible spectrum (Evanoff and Chumanov, 2005, [195506](#); Wijnhoven
26 et al., 2009, [180201](#)). (A plasmon is a quantum of a collective oscillation of charges on the surface of a
27 solid induced by a time-varying electric field.) A high SPR means that the electrons on the surface of a
28 particle are highly interactive with electromagnetic fields, and as the surface plasmons resonate, the
29 energy can be detected, quantified, and, in the case of silver, seen in the visible spectrum. Several studies
30 have shown that these properties strongly depend on a particle's size, shape, spatial ordering,
31 composition, and surface properties (Evanoff and Chumanov, 2005, [195506](#); Henglein, 1998, [225173](#); Jin

1 et al., 2001, [225181](#); Kamat, 2002, [224896](#); Murphy and Jana, 2002, [224906](#); Temgire and Joshi, 2004,
2 [224916](#)).

2.4. Analytical Methods for Nano-Silver

3 Accurate analytical methods can help improve understanding of the behavior and properties of
4 nano-Ag particles in various environmental media and can allow for improved characterization of
5 exposure and resulting impacts. The ability to monitor nanoparticles in various media, however, relies on
6 sufficiently sensitive instrumentation. Although measuring only the physicochemical properties that are
7 relevant from a risk assessment or risk management perspective might be desired, the mechanistic
8 understanding of biological effects of nanoparticles is still evolving and this list of physicochemical
9 properties is in flux. Furthermore, because physicochemical properties of nano-Ag are dynamic and
10 depend highly on the surrounding media, instrumentation for characterization in various environmental
11 conditions could be useful (Tiede et al., 2008, [196278](#)). Isolating and recovering nano-Ag particles in
12 sample matrices ranging from animal and plant cells to soil and water could be an important step in the
13 complete characterization of nano-Ag particles.

14 A few recently published review articles summarize current techniques available to characterize
15 engineered nanomaterials (Jiang et al., 2009, [193450](#); Maynard and Aitken, 2007, [090674](#); Powers et al.,
16 2006, [088783](#); Powers et al., 2007, [090679](#); Sayes and Warheit, 2009, [201592](#); Tiede et al., 2008,
17 [196278](#)). This section highlights some of the currently available techniques used specifically in nano-Ag
18 studies; this section, however, is not intended to present a comprehensive literature review of
19 nanomaterial characterization techniques. The reader is referred to Appendix A and the review articles
20 cited above for more information on such methods. Appendix A contains summary tables that present
21 limits of detection for the techniques listed and summarize some of their advantages and disadvantages.
22 Even so, these tables are not offered as definitive summaries of the field and should be viewed as
23 illustrating the complexities in nanomaterial characterization.

2.4.1. Methods for Laboratory Research

24 Several laboratory methods and types of instrumentation are available to characterize nano-Ag (see
25 Appendix A). Although many of these methods are considered accurate techniques for characterization of
26 nano-Ag and incorporate state-of-the-science instrumentation, they are usually resource intensive and
27 require trained specialists. These methods are generally used to develop new synthesis methods, modify
28 surface properties, and study biological interactions at the individual particle level. Despite numerous

1 possible characterization methods, researchers face challenges of maintaining the nanoparticles in
2 suspension during characterization and also determining the dose affecting the tissue or cell.

3 Microscopy techniques such as scanning electron microscopy (SEM) and transmission electron
4 microscopy (TEM) can be applied to study the size, shape, and morphology of individual particles or
5 powder samples. These studies include novel synthesis methods (Siekkinen et al., 2006, [225214](#); Sun and
6 Xia, 1991, [225218](#); Sun et al., 2002, [224912](#)) where nanoparticles are embedded in a matrix. Microscopic
7 techniques may also be used to study the biocidal properties of nanoparticles and to detect the presence
8 and any localization of nanoparticles in biological structures. For example, SEM and TEM studies have
9 shown the presence of nanoparticles within bacterial cells (Baker et al., 2005, [194057](#); Morones et al.,
10 2005, [196271](#); Shrivastava et al., 2007, [196276](#); Sondi and Salopek-Sondi, 2004, [196277](#)); alveolar
11 macrophages (Carlson et al., 2008, [195497](#)); and HIV (Elechiguerra et al., 2005, [225161](#)). Microscopic
12 analyses can be conducted in conjunction with energy-dispersive X-ray spectroscopy (EDS) to determine
13 chemical composition; for convenience, many SEM systems come equipped with EDS.

14 Aerosolized nanoparticulate systems are often studied using ensemble methods. Such methods
15 include laser diffraction (Powers et al., 2006, [088783](#)), dynamic light scattering (DLS) (McMurry, 2000,
16 [081517](#); Murdock et al., 2008, [193563](#)), centrifugal sedimentation, and impaction. Many ensemble
17 techniques are used to characterize particulate or aerosol systems in general and to study ultrafine
18 atmospheric particles, carbon nanotubes, and other nanoengineered materials specifically. Ensemble
19 methods are preferred when obtaining particle-size distribution data.

20 In addition to analytical instrumentation, development of characterization protocols also is relevant
21 so that results are consistent, reproducible, and reliable. Sayes and Warheit (2009, [201592](#)) suggest such
22 protocols emphasize that characterization data for the material should be assessed in the biologically or
23 environmentally relevant media, in the most dispersed state possible, and using more than one method.

2.4.2. Methods and Instrumentation to Assess Environmental Occurrence

24 Detecting nanoparticles in the environment (particularly the natural environment) can be
25 challenging because available analytical methods often are not sufficiently sensitive at environmentally
26 relevant concentrations and cannot distinguish natural materials in the nanoscale size range from
27 manufactured nanomaterials (Domingos et al., 2009, [193347](#); Simonet and Valcárcel, 2009, [193648](#)).
28 Also, many analytical methods require sample treatment and solvent evaporation, which consequently
29 could alter the samples by causing nanoparticle clustering and salt precipitation (Simonet and Valcárcel,
30 2009, [193648](#)). Detecting nanoparticles in water or soil is further complicated by the heterogeneous
31 nature of the sample matrix and the agglomeration and aggregation tendencies of the nanoparticles.

1 Making such measurements in situ would help address physical and other changes in nanoparticles due to
 2 different conditions in the immediate medium. Portable equipment with sufficient sensitivity, however,
 3 has not yet been developed (Simonet and Valcárcel, 2009, [193648](#)). Although traditional methods to
 4 measure metals in samples (e.g., atomic adsorption furnace methods) cannot differentiate between
 5 conventional silver and nano-Ag, these traditional methods can be coupled with other methods to confirm
 6 and quantify the presence of silver in a sample. Methods can also be coupled to enable detection of more
 7 than one parameter simultaneously. For example, field flow fractionation (FFF) can be coupled with
 8 inductively coupled plasma-mass spectrometry (ICP-MS) for both size and chemical analysis.

9 To illustrate the variety of methods available to assess nanomaterials in environmental matrices, a
 10 sample of available methods for analyzing nanomaterials in soil, sediment, and ground water is shown in
 11 Table 2-3.

Table 2-3. Analytical methods for nanomaterials in soil, sediment, and ground water for size fraction and distribution, surface area, and phase and structure.

| Metric | Analytical method | Notes |
|---------------------|--|---|
| Size fractionation | Centrifugation | Analyze aquatic colloids and particles extracted from soil and sediment samples. Nanoparticles must be in solution. |
| | Ultrafiltration – direct-flow ultrafiltration or tangential-flow ultrafiltration (TFF) | |
| | Field flow fractionation (FFF) | |
| | Capillary electrophoresis (CE) | |
| | Size exclusion chromatography (SEC) | |
| Size distribution | Transmission electron microscopy (TEM) | In most cases samples analyzed by electron microscopy will be destroyed and cannot be analyzed by another method (Tiede et al., 2008, 196278). |
| | Scanning electron microscopy (SEM) | |
| | Scanning probe microscopy (SPM) | |
| | Dynamic light scattering (DLS) | |
| | Laser-induced breakdown detection (LIBD) | |
| | Small- and wide-angle X-ray scattering (SAXS/WAXS) | |
| Surface area | BET (Brunauer, Emmett, Teller method of calculating specific surface area) | Only nanoparticles with a regular or pseudo-regular geometry and without significant porosity |
| | Calculation from TEM (length and width) and atomic force microscopy (AFM) (height) measurements, and particle nanocrystalline geometrics | |
| Phase and structure | Electron diffraction | XRD and XAS are non-destructive techniques (Tiede et al., 2008, 196278). |
| | X-ray diffraction (XRD) | |
| | X-ray absorption spectroscopy (XAS) | |
| | Raman spectroscopy | |
| | High-resolution transmission electron microscopy (HR-TEM) | |

Source: Adapted from U.S. EPA (2009, [225004](#)).

12 In a study comparing six analytical methods for determining nanoparticle sizes (TEM, atomic force
 13 microscopy [AFM], DLS, fluorescence correlation spectroscopy, nanoparticle tracking analysis, and field
 14 flow fractionation), Domingos et al. (2009, [193346](#)) concluded that the two most commonly used
 15 techniques reported in the literature (TEM on air-dried samples and DLS) also were the two that appear to

1 be most prone to artifacts. Their recommendation was to use multiple analytical techniques or multiple
2 preparation techniques, or both.

3 Several recent studies have employed ensemble methods to characterize crystal structure, particle
4 size, and morphology of nano-Ag particles in biological matrices. For example, Laban et al. (2009,
5 [199809](#)) coupled TEM with electron diffraction to verify that the particles detected within the embryos of
6 fathead minnows were nano-Ag particles. Similarly Asharani et al. (2008, [194056](#)) combined TEM
7 analysis with energy-dispersive X-ray spectroscopy (EDS) to confirm the presence and location of
8 nano-Ag particles in zebrafish embryos.

2.4.3. Methods and Instrumentation for Assessing Occurrence in the Workplace

9 The potential for workplace exposure to nano-Ag exists at all stages of the manufacturing of
10 nano-Ag and products containing nano-Ag. The monitoring of a specific nanomaterial poses several
11 challenges, due to the presence of background particulate matter generated from other activities that
12 typically occur at industrial sites. Such activities include combustion processes, metal operations where
13 vapors can condense (e.g., soldering, welding, smelting), and mechanical processes (e.g., grinding,
14 blending) (Ono-Ogasawara et al., 2009, [225206](#)). Although standardized protocols exist for monitoring
15 the suspended particulate matter at workplaces, they do not distinguish between ultrafine particles and
16 nanoparticles.

17 Analysis of workplace exposure thus far has focused on measuring nanoparticles in the air.
18 Instruments that can be used for aerosol sampling are available, but most are designed for laboratory use
19 (Nanosafe, 2008, [196066](#)) and lack one or more of the following attributes: portability, ease of use,
20 capacity to distinguish nanoparticles from non-nanoparticles, different size bins in the 1- to 100-nm range,
21 or ability to sample personal breathing zones (Ostraat, 2009, [196077](#)). Engineered nanoparticles can be
22 measured in the workplace using a variety of instrumentation, including: condensation particle counters
23 (CPC), optical particle counters (OPC), fast mobility particle sizers (FMPS), scanning mobility particle
24 sizers (SMPS), electrical low pressure impactors (ELPI), aerosol diffusion chargers, and tapered element
25 oscillating microbalances (TEOM[®]).

26 Several studies have characterized nanoparticles at manufacturing facilities using various analytical
27 methods. Thus far, however, only one study has been identified that characterized nanoparticles at a
28 nano-Ag production facility. Park et al. (2009, [225210](#)) used an SMPS to measure the size of particles in
29 ambient air at a manufacturing facility using liquid-phase processes that produce silver nanoparticles.
30 Also, electrostatic precipitators were used to collect particles on TEM grids to analyze surface
31 morphology.

1 Because analytical instrumentation and techniques for measuring mass and number concentrations
2 of other nanomaterials could be used directly or adapted to characterize silver nanoparticles, a few recent
3 studies characterizing other nanomaterials at manufacturing sites are mentioned here. Fujitani et al.
4 (2008, [225165](#)) characterized fullerenes at a manufacturing facility using an SMPS, optical particle
5 counter, and SEM during non-work and work periods, and during an agitation process. Similarly, Demou
6 et al. (2008, [224884](#)) quantified real-time size, mass, and number concentrations using SMPS and a CPC
7 at a pilot plant producing metal oxide nanostructures.

8 Given the active research in both academic and commercial laboratories to develop new
9 nanomaterial-based technologies, the potential exists for laboratory workers to be exposed. Tsai et al.
10 (2009, [193684](#)) sampled and characterized the ambient air from laboratory hoods using a FMPS and an
11 SEM. These analyses were performed during the handling (i.e., pouring or transferring with a spatula) of
12 nanoalumina and nano-Ag (Tsai et al., 2009, [193684](#)). Inside the fume hood, the researchers observed a
13 shift in the mean particle diameter of the originally spherical nano-Ag particles from around 60 nm to 150
14 nm, indicating clustering of the particles during handling. Based on testing with a 100-gram (g) sample
15 and a 15-g sample of nanoalumina, the researchers concluded that working with smaller quantities of
16 sample decreases the concentration of particles entering the laboratory space from the fume hood by
17 approximately 20%.

18 In recent years, several governmental and environmental organizations have voiced a need for
19 methods and protocols to monitor nanomaterials in the workplace. For example, the National Institute for
20 Occupational Safety and Health (NIOSH) recently published a document entitled *Approach to Safe*
21 *Nanotechnology – Managing the Health and Safety Concerns Associated with Engineered Nanomaterials*
22 (NIOSH, 2009, [196073](#)) in which sampling and monitoring methods and equipment are discussed. The
23 Nanoparticle Occupational Safety and Health (NOSH) Consortium, an industry-led consortium of
24 participants from academia and governmental and non-governmental organizations, is helping to define
25 best practices for working safely with engineered nanoparticles (NOSH, 2008, [196088](#)). The NOSH
26 Consortium has developed portable air monitoring methods suitable for daily monitoring in nanoparticle
27 research and development and in manufacturing settings. In 2008, the NanoSafe2 project, a European
28 Community-sponsored project for safe production and use of nanomaterials, released a report that
29 highlighted findings in measurement methodologies for nanoparticle detection and measurement with
30 various types of online and offline monitoring instruments (Nanosafe, 2008, [196066](#)). The report
31 provides examples of new nano-aerosol measurement equipment that is easy to transport and use. No
32 commercially available equipment, however, is currently available for long-term monitoring. The report
33 also recommends that monitoring at workplaces include not only personal sampling and measurements
34 inside the facility, but also measurements of nanomaterials in drains and in the exhausted air to help
35 ensure protection of the environment. Finally, several companies are developing or have developed air

1 monitoring devices for nanoparticle detection; the parameters that each device measures vary (Bennett,
2 2005, [193820](#); TRS Environmental, 2009, [196057](#); van den Brink, 2008, [196075](#)).

2.4.4. Methods for Quantifying Dose and Dose Metrics

3 To quantify dose, it is necessary to measure physical characteristics of the material in a way that
4 facilitates the analysis of dose-response relationships. Researchers and risk assessors often quantify dose
5 in terms of mass (e.g., in $\mu\text{g}/\text{m}^3$ for inhalation exposures, or in $\text{mg}/\text{kg}\text{-day}$ for ingestion exposures), but,
6 for some substances, mass might not be the physical parameter most closely correlated with the biological
7 response. For example, exposure to asbestos fibers is typically characterized by number concentration
8 (i.e., number of particles in a specific quantity of exposure medium) of fibers of specific shape and
9 composition (Maynard and Aitken, 2007, [090674](#)). For nanoparticles specifically, dose can be measured
10 in terms of number concentration (e.g., the number of particles inhaled per volume of air) and surface area
11 concentration (e.g., the surface area of the particles inhaled per volume of air, m^2/m^3) in addition to mass
12 concentration.

13 In some respects, using mass as the primary metric for characterizing dose is an attractive option
14 for nanoparticles; for example, measuring mass for a pollutant is standard procedure, and instrumentation
15 for conducting such measurements is widely available (Maynard and Aitken, 2007, [090674](#)). Oberdörster
16 et al. (2005, [090087](#)) suggested that measuring mass concentrations for inhalation or intratracheal
17 instillation studies of nanomaterials and conducting gravimetric and chemical analyses of filter samples
18 can provide relatively accurate dose characterization when compared to surface area or particle number
19 metrics. In some cases, it might also be possible to estimate particle surface area or number concentration
20 from measures of mass concentration based on the estimated diameter of the particles; however, when the
21 distribution of particle sizes is wide or the number of very large particles is great, using mass
22 concentration to calculate number concentration could be unreliable. There appear to be other potential
23 drawbacks to using mass as the primary metric for characterizing dose. Nanoparticles have been shown
24 in many cases to be more toxic than heavier particles with the same chemistry; specifically, researchers
25 have shown that the toxicity of insoluble materials increases with decreasing particle size on a mass-for-
26 mass basis (Mark, 2007, [202653](#)). This relationship might occur because the increased specific surface
27 area of nanoparticles provides more area (compared to that of larger particles) for potential biological
28 reactions than their mass alone would predict (Oberdorster et al., 2005, [090087](#)).

29 Evidence suggests that particle number might be highly correlated with health effects and might be
30 a relevant dose metric. Wittmaack (2007, [090452](#)) found for titanium dioxide that particle number is the
31 dose metric that correlates best with pulmonary inflammation response. Devices to count particles are
32 available; however, even the most complex and powerful detection units are limited to the detection of

1 particles with diameter of about 10 nm or greater (Maynard and Aitken, 2007, [090674](#)). These
2 instruments also tend to be very expensive, which could preclude the use of this technology to obtain
3 information about particle size or size distribution. Tsuji et al. (2006, [088088](#)) also questioned the value
4 of measuring only particle number and using this as a dose metric because it does not necessarily
5 correlate with health effects as well as other dose metrics.

6 Surface area might be another appropriate metric for characterizing dose (Oberdörster et al., 2005,
7 [087559](#); Oberdorster et al., 2007, [090923](#); Tsuji et al., 2006, [088088](#)). In general, the increased surface
8 area of nano-sized particles can change their chemical reactivity, bioavailability, and the biological
9 responses they can induce (Luoma, 2008, [157525](#); Mark, 2007, [202653](#)), and thus surface area
10 concentration might be highly correlated with response. Direct, real-time measurement of particle surface
11 area has become possible in recent years. One device, TSI's Nanoparticle Surface Area Monitor (Model
12 3550), filters only particles that deposit in the alveolar or thoracic region of the respiratory system
13 (Maynard and Aitken, 2007, [090674](#)). Such measurement systems, however, are not yet cost-effective.

14 Although identifying a single dose metric that best reflects risk might be desirable, the toxicity and
15 reactivity of nanoparticles appear to be functions of multiple factors, including surface area, number,
16 shape and size, and composition. For this reason, Maynard and Aitken (2007, [090674](#)) suggested that
17 different metrics (e.g., particle number concentration, surface area concentration, mass concentration, or
18 length concentration) might be selected for different aerosol sprays depending on factors such as these.
19 Similarly, Oberdörster et al. (2005, [090087](#)) suggested that mass, surface area, and particle number are
20 essential dose metrics for nanoparticles and that, when possible, dose should be characterized by all three
21 measures.

2.5. Summary of Physicochemical Properties and Analytical Methods

22 The size, morphology, surface area, chemical composition, surface chemistry and reactivity, and
23 solubility of nano-Ag particles are all thought to play a role in determining their use and effectiveness in
24 commercially available spray disinfectant solutions, behavior in the environment, and human and
25 ecological exposure potential and toxicity. These properties are interdependent, however, and can change
26 as nano-Ag particles are dispersed in different solutions, move between environmental compartments, and
27 are transported within living organisms. Adequate characterization of nano-Ag could help when
28 evaluating potential risks associated with its use in disinfectant sprays, and the chapters that follow
29 highlight the physicochemical properties of nano-Ag that might be pertinent at each stage of a
30 comprehensive environmental assessment.

1 Sensitive analytical methods underpin characterizing the presence and physicochemical properties
2 of nano-Ag in the laboratory, natural environment, workplace, and living organisms. Laboratory methods
3 such as spectroscopy, chromatography, electron microscopy, and spectrometry help researchers
4 characterize nano-Ag and its interaction in solution, within organisms, and in cells. These same methods
5 cannot necessarily be used to detect and characterize nano-Ag outside of the laboratory for several
6 reasons. Instruments are not easily portable and are expensive; environmentally relevant nano-Ag
7 concentrations can occur below current method and instrument detection limits; and the methods cannot
8 yet consistently distinguish between naturally occurring nanoparticles and engineered nanoparticles such
9 as those used in nano-Ag spray disinfectants. Additional information on analytical methods is presented
10 in Chapters 4, 5, and 6 regarding specific methods used to study the fate and transport of nano-Ag, the
11 potential exposure of humans and biota to nano-Ag, and the effects of such exposure.

12 Questions reflecting data gaps in the information about physicochemical properties and analytical
13 methods are listed on the following page. They are listed in approximate order of the presentation of
14 information in this chapter. The order is in no way intended to reflect the relative importance of the
15 questions.

Questions about Physicochemical Properties and Analytical Methods

- 2.1. What information could be provided about the nano-Ag contained in spray disinfectants to enable adequate characterization of exposure routes and toxic effects?
- 2.2. How can engineered nano-Ag particles be distinguished from incidental, background, or naturally occurring nano-Ag particles?
- 2.3. Which physicochemical properties of conventional silver can be applied to nano-Ag?
- 2.4. Does the morphology of nano-Ag determine the efficacy of use in spray disinfectants?
- 2.5. How does surface coating affect:
 - 2.5.a. the physicochemical properties of nano-Ag?
 - 2.5.b. toxicity to humans or biota?
- 2.6. What physicochemical properties of nano-Ag can be used to:
 - 2.6.a. predict fate and transport in environmental media?
 - 2.6.b. predict toxicity to humans or biota?
- 2.7. Which physicochemical properties of nano-Ag are most essential to characterize before and during toxicity experiments?
- 2.8. What standardized test methods or characterization protocols are necessary to ensure that research results generated in multiple laboratories are consistent, reproducible, and reliable?
- 2.9. Are there standard nano-Ag reference materials that can be used in exposure and effects testing to aid in comparison of results among investigators?
- 2.10. Do adequate analytical methods exist to detect and characterize nano-Ag in environmental compartments and in biota?
- 2.11. What analytical methods are available to disaggregate nano-Ag particles in preparing environmental samples for analysis?
- 2.12. Do adequate analytical methods exist to detect and characterize exposure to nano-Ag via soil, water, and air?
- 2.13. What new analytical methods would enhance characterization of nano-Ag particles?
- 2.14. For the purpose of assessing potential risk, what metrics are most informative for quantifying dose of nano-Ag?

Chapter 3. Life-Cycle Stages

1 The first step in comprehensive environmental assessment (CEA) is to examine the life cycle
2 stages of the nanoscale product. This chapter provides a description of information available about the
3 life cycle of nano-Ag spray disinfectant products to support the discussions in the chapters that follow
4 about environmental fate and transport, potential exposure pathways for humans and biota, and possible
5 effects resulting from exposure to nano-Ag. As noted in a recent Federal Insecticide, Fungicide, and
6 Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) review of issues related to nano-Ag in
7 pesticides (2010, [625619](#)), any use of nano-Ag as a pesticide (including as a disinfectant) could cause
8 nano-Ag or a related by-product derived from such use to enter the environment. In the environment, the
9 potential exists for human and biotic exposure. For this case study, by-products comprise material waste
10 from feedstock processing and manufacturing, secondary pollutants formed through chemical or other
11 transformations of primary pollutants, and, within organisms, metabolic products derived from primary
12 toxicants (Davis, 2007, [089803](#)).

3.1. Feedstocks

13 Anthropogenic sources of silver emissions into the environment include:

- 14 • mining, smelting, and coal combustion operations;
- 15 • manufacturing, use, and disposal or recycling of products containing silver; and
- 16 • waste discharges from mining operations, industrial processes, and wastewater treatment
17 facilities (Purcell and Peters, 1998, [195563](#)).

18 A report commissioned by The Silver Institute (2009, [202685](#)) estimates that 76.6% of the world's 2008
19 silver supply of 27,631,905 kilograms (kg) came from mining, with North America and Latin America
20 leading the world's mining (GFMS, 2009, [201631](#)). Approximately 28% of this amount was mined in
21 operations where the main revenue source is silver. Of the remaining mined supply, 37% was recovered
22 from lead and zinc mining operations, and the rest from gold (11%), copper (23%), and other metal (2%)
23 mining operations. These ores are typically mined using open-pit or underground methods and are
24 enriched using flotation and smelting processes. The silver metal is extracted electrochemically using the
25 Parkes, Moebium, or Balbach-Thum process. In a silver life-cycle analysis conducted by Johnson et al.
26 (2005, [484604](#)), the authors estimated that 20% of the ore mined for silver enters the environment through
27 mine tailings, although the authors suggest that further research could refine this estimate.

1 The remaining silver in the world's 2008 supply came from other net government sales (3.5% in
2 2008) and the recycling of silver scrap (19.9% in 2008), including silver recovered from jewelry,
3 photographic chemicals, discarded computers, and other manufactured products that originally contained
4 silver components (GFMS, 2009, [201631](#)).

5 There are no reliable estimates of the total worldwide volume of nano-Ag production. The annual
6 survey of The Silver Institute (2009, [202655](#)) states that 95% of annual silver consumption is for
7 industrial, photographic, and jewelry applications. Worldwide nano-Ag production could be as high as
8 5% of total silver production (Mueller and Nowack, 2008, [157519](#)); therefore, Mueller and Nowack
9 (2008, [157519](#)) estimate that a "best guess" for the worldwide production of nano-Ag is 500,000 kg per
10 year. To form their estimate, Mueller and Nowack reviewed data collected by survey and personal
11 communications about the quantity of nano-Ag manufactured in Switzerland and extrapolated from this
12 estimate to apply to the entire world.

13 Elemental silver is not reactive under normal environmental conditions, dissolving only in strong
14 acids to form salts (Wiberg et al., 2001, [098733](#)). Silver nitrate (AgNO_3) is a precursor to many silver
15 compounds, including nano-Ag, and is prepared by dissolving silver metal in nitric acid. Silver halides,
16 which are compounds used in photographic applications, can be produced from AgNO_3 , with halide ions
17 (i.e., fluoride, chloride, bromide, iodide, or astatide) displacing the nitrate (Wiberg et al., 2001, [098733](#)).
18 Reduction of silver salts, such as AgNO_3 or silver cyanide, is one of the principal methods for producing
19 nano-Ag (Evanoff and Chumanov, 2005, [195506](#)).

3.2. Manufacturing

20 Manufacturing procedures for nano-Ag are generally proprietary. For example, the Top Nano
21 Technology company Web site advertises that the company has "kilogram-scale manufacturing"
22 capability for nano-Ag, but information about their manufacturing process is not accessible through the
23 U.S. Patent Office database, the company Web site, or written inquiries to the company (Chou, 2010,
24 [597423](#)). A search of the U.S. Patent Office database did reveal some patented, company-specific
25 manufacturing processes, so those processes, and others described in the peer-reviewed literature, are
26 incorporated into the section that follows. In general, however, limited data were identified on specific
27 points of release or the quantity of nano-Ag released as a result of the manufacturing process. This lack
28 of data is consistent with statements included in the report on nano-Ag from the FIFRA SAP (2010,
29 [625619](#)).

30 The Project on Emerging Nanotechnologies (PEN) (2009, [196774](#)) reports that, as of 2009, the
31 companies that produce spray disinfectant solutions purportedly incorporating nano-Ag include American
32 Biotech Labs, ConSeal International, Inc., Daido Corporation, GNS Nanogist Co., Lion Corporation,

1 Shanghai Huzheng Nanotechnology Co. Ltd., Skybright Natural Health, and Top Nano Technology. This
2 list, however, is not comprehensive, as manufacturing of products potentially containing nano-Ag
3 continues to expand. Furthermore, PEN notes that selection of products for inclusion on the list and the
4 information presented on specific products is based on data that are publicly available on company Web
5 sites, and none of the information has been independently verified (2009, [196774](#)).

3.2.1. Synthesis of Silver Nanoparticles

6 Silver nanoparticles can be synthesized using wet-chemistry methods (chemical reduction), laser
7 ablation, radiolysis, and vacuum evaporation methods. Krutyakov et al. (2008, [594264](#)) and others have
8 published comprehensive reviews discussing the strengths, drawbacks, and challenges of available
9 nanoparticle synthesis methods. Tolaymat et al. (2010, [625615](#)) searched the scientific literature to collect
10 information about how nano-Ag is synthesized. Relying on the synthesis methods described in nearly
11 200 papers, they concluded that the synthesis of nano-Ag particles most often produces spherical particles
12 with a diameter of less than 20 nm.

13 Chemical reduction of silver ions is the primary method for rapidly producing large quantities of
14 silver nanoparticles (Zhang et al., 2007, [224924](#)), and indeed Tolaymat et al. (2010, [625615](#)) reached this
15 same conclusion based on their review of the literature on the synthesis of nano-Ag. The chemical
16 reduction of transition metal salts to generate zero-valent particles (not necessarily nanoparticles) was
17 first described by Faraday in 1857 (Bonnemann and Richards, 2001, [224882](#)). Following this early
18 discovery, Turkevich et al. (1951, [225226](#)) synthesized gold nanoparticles by reducing chloroauric acid
19 with sodium citrate. Subsequently, this method was extended for the synthesis of nano-Ag.

20 With the use of a reducing agent, silver ions (Ag^+) in solution are reduced from a positive valence
21 to a zero-valent state (Ag^0) (Zhang et al., 2007, [224924](#)). Because zero-valent silver tends to
22 agglomerate, a primary challenge is to maintain the nanoparticles in the desired size range. Controlling
23 size range is generally accomplished by using surface coatings such as surfactants, polymers, or
24 stabilizing ligands (Zhang et al., 2007, [224924](#)). The choice of reducing agent and the order and rate of
25 mixing can alter the rates of nucleation and particle growth (Bonnemann and Richards, 2001, [224882](#)).
26 Thus, particle size and dispersion can be controlled by altering the synthesis process. Manipulation of
27 laboratory conditions also enables the shape of the nanoparticles (e.g., rods, wires, disks, spheres) to be
28 controlled (Sun and Xia, 2002, [224914](#); Yu and Yam, 2004, [225238](#)).

29 There are many variations on this basic theme of chemical reduction. The common elements,
30 however, are AgNO_3 as a feedstock; an aqueous solvent or a non-polar solvent; a reducing agent; and a
31 stabilizing agent, most often a surfactant (Goia and Matijevi, 1998, [225169](#)). When AgNO_3 is used as a
32 feedstock, nitrate (NO_3) will likely result from the reaction and may then be a byproduct of concern

1 (Tolaymat et al., 2010, [625615](#)). In their review of nano-Ag synthesis methods reported in the literature,
2 Tolaymat et al.(2010, [625615](#)) found that AgNO₃ is the most commonly reported silver salt precursor used
3 in synthesis. Reduced metal atoms are insoluble and thus tend to cluster, eventually forming solid
4 particles. The driving force behind the reduction is the difference between the reduction and oxidation
5 (redox) potentials of the two half-cell reactions (Goia and Matijevi, 1998, [225169](#)). With a larger redox
6 potential (attained by using a stronger reducing agent such as sodium borohydride, NaBH₄), the system
7 will achieve greater oversaturation. The reaction will be more rapid and more nuclei will form, resulting
8 in smaller particles, as the available silver is distributed among many nuclei. If a weaker reducing agent
9 is used, such as ascorbic acid, the reaction rate can be increased by elevating the temperature. If larger
10 particles are desired, a weaker reducing agent is used because it will cause a slower reaction and lead to
11 the formation of fewer nuclei. The available silver will be consumed by the smaller number of particles,
12 resulting in a larger final particle size. Further growth can occur by continued addition of metal atoms,
13 leading to crystals with a regular shape and few internal grain boundaries. If aggregation takes place, the
14 resulting particles will be spherical and polycrystalline, with internal grain boundaries. Both mechanisms
15 can occur in the same system.

16 Examples of mild reducing agents are sodium citrate (Lee and Meisel, 1982, [225193](#)) and sugars
17 (Kvitek et al., 2005, [225189](#); Panáček et al., 2006, [196274](#)). An advantage of using sodium citrate is that
18 it has low toxicity compared to stronger reducing agents such as sodium borohydride. A disadvantage of
19 sodium citrate is its lower reduction activity, which necessitates higher temperatures and results in longer
20 reaction times (Zhang et al., 2007, [224924](#)). Kvitek et al. (2005, [225189](#)) and Panáček et al. (2006,
21 [196274](#)) used sugar as the reducing agent and ammonia as the complexing agent to form diamminesilver
22 cation [Ag(NH₃)₂⁺]. By controlling ammonia concentration and varying the type of sugar, the researchers
23 could control particle size. The redox potential for Ag(NH₃)₂⁺ is lower than that for silver ions, and the
24 ensuing slower reaction leads to fewer nuclei and larger particles. Kvitek et al. (2005, [225189](#)) obtained
25 particles ranging from 45 to 380 nanometers (nm). Panáček et al. (2006, [196274](#)) obtained particles in the
26 25- to 450-nm range.

27 Leopold and Lendl (2003, [224902](#)) used a stronger reducing agent, hydroxylamine hydrochloride
28 (NH₂OH.HCl), combined with a solution of AgNO₃. According to the researchers, the advantages of this
29 method are a rapid reaction rate, a narrow particle size distribution, reliably reproducible results, and the
30 ability to carry out the process at room temperature. The size distribution of the particles can be
31 controlled by changing the order and rate of mixing of the reactants. Average particle diameter varied
32 from 23 to 67 nm, and the particles were spherical.

33 The choice of stabilizing agent also influences the final product. A method by Sun et al. (2005,
34 [202684](#)) using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (i.e., HEPES buffer) at pH 7.4
35 produced “face-center-cubic phase” particles between 5 and 20 nm, with a mean diameter of 10 nm. In
36 this study, human serum albumin was used to stabilize the particles, rendering them suitable for study of

1 their ability to inhibit HIV growth. Hu et al. (2008, [224894](#)) developed a method to produce very small
2 nano-Ag particles by using polyacrylic acid (PAA) as a surfactant. The authors noted that the carboxylic
3 groups in the PAA bonded well to the silver nanoparticles, effectively limiting their growth. Their method
4 uses polyols as both the solvent and the reductant, and the synthesis is performed at the boiling
5 temperature of the solvent. Through careful selection of the PAA chain length and the specific polyol
6 (ethylene glycol, diethylene glycol, or triethylene glycol), the authors could produce particles <10 nm
7 with good dispersion.

8 Repeatability and reproducibility of nano-Ag synthesis can be augmented by other techniques, such
9 as microwaving or sonication. Yin et al. (2002, [224922](#)), for example, developed a method to synthesize
10 nano-Ag based on the Tollens method for electroless plating for the synthesis of nanoparticles. The
11 researchers mixed a solution of AgNO₃ with an activator (sodium hydroxide) and a reducing agent
12 (formaldehyde and sorbitol). This procedure was carried out in a sonicator (ultrasound bath), which the
13 researchers believed to promote a more uniform concentration profile, leading to a narrow size
14 distribution in the samples.

15 Zhang et al. (2007, [224924](#)) presented a detailed discussion of the micro-emulsion method. This
16 type of synthesis involves either a mixture of water, surfactant, and oil, or a mixture of water, surfactant,
17 co-surfactant, and oil. The system can involve oil micelles in water, or water micelles in oil. The
18 discussion by Zhang et al. (2007, [224924](#)) focused on water-in-oil micro-emulsion, also referred to as
19 “reverse micelles.” The basic principle is that the reduction reaction takes place in water droplets, which
20 are covered by surfactant molecules. As the particles enlarge, the surfactant molecules then become
21 adsorbed to the particle surfaces, halting further growth and preventing them from forming clusters. The
22 diameter and shape of the particles can be controlled by the size and shape of the droplets. This technique
23 can produce relatively stable, small particles (2–5 nm average diameter), with a narrow size distribution.
24 To initiate the reaction, two micro-emulsions are mixed – one carrying the silver salt and the other
25 carrying the reducing agent. The micelles collide, and the reactants mix.

26 In addition to serving as reactors, the reverse micelles act as templates for the shapes of the
27 nanoparticles (Zhang et al., 2007, [224924](#)). A low surfactant concentration gives rise to spherical
28 particles. Higher concentrations can produce rods or columns. In a mixed cationic-anionic surfactant
29 solution, “worm-like” micelles form, producing nanowires. The reducing agent also influences particle
30 morphology, as do the water content, AgNO₃ concentration, and chain length of the alkane used as the
31 solvent. Despite achieving tight control of particle size and flexibility in shape, this method has several
32 disadvantages, including potentially high expense because it requires large amounts of surfactants and
33 solvent. Also challenging is the removal of the surfactants and solvent from the nanoparticles.
34 Furthermore, stable dispersions are obtained only at low concentrations. Because of these disadvantages,
35 the authors noted that the method is not currently suitable for large-scale manufacturing (Zhang et al.,
36 2007, [224924](#)).

1 Wet-chemical methods often leave trace amounts of reducing agents or surfactants on silver
2 nanoparticle surfaces (Amendola et al., 2007, [224880](#)). Some unconventional methods have therefore
3 been developed for applications requiring pure nanoparticles. For example, laser ablation of pure silver
4 immersed in a solvent generated nanoparticles with a logarithmically normal particle size distribution
5 (Amendola et al., 2007, [224880](#)). In an earlier study, laser ablation of bulk silver in aqueous sodium
6 dodecyl sulfate solution generated silver nanoparticles for which the size either increased with the
7 radiation power or decreased with surfactant concentration (Mafune et al., 2000, [224904](#)). Researchers
8 are also investigating the possibility of using plants to synthesize metallic nanoparticles, including
9 nano-Ag (Harris and Bali, 2008, [195522](#)).

10 Several relatively recent patents have been granted for silver nanoparticle synthesis. Although the
11 information obtained from the patents does not generally indicate which, if any, have been adopted for use
12 in large-scale manufacturing, the patents do provide perspective on potential developments. Available
13 descriptions, however, are brief. For example, Oh et al. (2003, [225288](#)) proposed a method to produce
14 silver and silver-alloyed nanoparticles in a surfactant solution. As with other similar methods, the
15 technique involves using a reducing agent to produce silver nanoparticles from a silver salt solution; the
16 use of the surfactant in this method presumably controls nanoparticle size. Holladay et al. (2004,
17 [597403](#)), who are associated with American BioTech, a nano-Ag spray disinfectant manufacturer, hold a
18 patent that describes a method for generating nano-Ag particles between 5 and 20 nm by immersing
19 electrodes (including silver-coated wires) in a 15-gallon plastic container filled with water. The patent
20 claims that the silver particles are dispersed evenly through the water by the rotation of an impeller in the
21 container.

22 Because of the inherent disadvantages of other synthesis methods, it is reasonable to assume that
23 the bulk of nano-Ag is produced using wet-chemistry methods involving liquid-phase materials and
24 processes. A recently published study characterized airborne silver nanoparticles inside a manufacturing
25 facility located in Korea (Park et al., 2009, [225210](#)). This large manufacturing facility, producing
26 3,000 kg of silver nanoparticles per month, uses AgNO₃ as feedstock and employs a chemical reduction
27 method. The manufacturing of nano-Ag occurs in four stages: the chemical reduction step is followed by
28 filtering, drying, and grinding stages. Real-time monitoring and sampling of silver nanoparticles using
29 scanning motility particle sizer (SMPS) and long differential mobility analyzer (LDMA) techniques at the
30 facility indicated that the highest concentration of airborne nanoparticles occurred after the reaction stage,
31 when some aerosolized nano-Ag particles were released into the air. The researchers also noted that
32 particles deposited to the floor and other surfaces following the release of particles to the air at the end of
33 each stage. The study authors did not attempt to quantify emissions data on a mass released-per-mass
34 produced basis. More details on the potential exposure scenarios implied by this study are included in
35 Section 5.3.2.

3.2.2. Manufacturing of Nano-Ag Disinfectant Sprays

1 The production, characterization, and handling of nano-Ag require specialized technical expertise,
2 and the available data suggest that nano-Ag might be produced in-house at companies that manufacture
3 disinfectant sprays (Holladay et al., 2004, [597403](#); Sawafta et al., 2008, [597424](#)). Sawafta et al. (2008,
4 [597424](#)) described a “nanocomposite” of at least two metals, including silver, in solution where the metal
5 nanoparticles are created either by “mechanical/physical size reduction processes” or “co-precipitation
6 processes.” Size reduction is further described with steps including grinding, pulse laser evaporation,
7 sonication, and sorting by centrifugation or magnetic separation. Alternatively, production of nano-Ag
8 might occur at facilities that exclusively produce nano-Ag and other engineered nanomaterials in large
9 volumes, which are then sold to manufacturers of spray disinfectants. When contacted, ConSeal
10 International, Inc. reported that they produce and sell more than 2,500 gallons annually of their nano-Ag
11 disinfectant spray, NanoSil, which they claim contains nano-Ag (Gilmore, 2010, [597422](#)).

12 Based on available patent data and data presented on company Web sites (but unverified), some
13 major operations that manufacture spray bottle products containing nano-Ag involve mixing of various
14 ingredients, mechanical or chemical processes to achieve uniform product consistency, filtration
15 processes to remove impurities, intermediate storage of the prepared bulk spray product in tanks, and
16 finally automated dispensing into bottles (ConSeal International Inc, 2010, [594257](#); Holladay et al., 2004,
17 [597403](#); Park et al., 2009, [225210](#); Sawafta et al., 2008, [597424](#); Shanghai, 2009, [594268](#)). Thermal
18 heating or cooling steps also can be involved, depending on the ingredients, spray formulation, and
19 desired properties. Although bulk spray liquid can be produced in batches in mixing vessels and
20 transferred to intermediate storage, dispensing and packaging can be accomplished using continuous,
21 automated processes. Individual bottles, sealed after completion of quality control, might be packaged
22 into cardboard cartons for distribution and retail sales.

23 A 2008 U.S. patent application (Sawafta et al., 2008, [597424](#)) describes a metallic nanocomposite
24 synthesized for its biocidal properties. As explained in the application, to create a spray disinfectant, the
25 nanocomposite can be combined with hypochlorite or another chlorine-releasing compound;
26 chlorhexidine or another biguanide molecule with the chemical formula $C_2H_7N_5$; quaternary ammonium
27 salts commonly used as germicides, disinfectants, and sanitizers; or other ionic liquids, surfactants, soaps,
28 or detergents.

29 During the manufacture of nano-Ag sprays, there could be releases of nano-Ag, other spray
30 ingredients, or by-products of the spray. Preliminary handling of large quantities of nano-Ag prior to
31 creating the spray disinfectants, such as unpacking, sampling for quality control, measuring, and
32 transporting nanoparticles, could lead to release of nano-Ag to the air or surfaces in the facility.
33 Depending on the quality of packaging and storage conditions at facilities where manufacturers acquire
34 nano-Ag in large volumes and stockpile the raw material for extended periods, nano-Ag and associated

1 substances might be released to ambient air. Similarly, mechanical processes such as mixing, grinding, or
2 agitation of liquids can cause nano-Ag to escape to the ambient air. As Park et al. (2009, [225210](#))
3 demonstrated, wet-chemistry handling processes also can emit nano-Ag to the air. Once bulk spray is
4 produced, other activities such as storage, addition of other ingredients, and dispensing into bottles could
5 result in releases of particles to the environment, resulting in the potential for worker exposure. Available
6 data on exposures are described in Section 5.3.2. In addition to the potential for exposure during routine
7 manufacturing operations, accidental short-term exposure at high doses might occur at spray production
8 facilities. These exposures could occur as a result of incidents ranging from major accidents to medium-
9 scale adverse events, such as a leak or break in process vessels or pipes, to minor events such as small
10 chemical spills.

11 Finally, nano-Ag, other spray ingredients, or by-products from disinfectant spray-manufacturing
12 facilities could enter waste streams including landfill waste and wastewater streams by way of fluids
13 released from flushing and cleaning of processing equipment, improperly treated processing waste, and
14 cleaning of contaminated surfaces.

3.3. Distribution and Storage of Nano-Ag Disinfectant Sprays

15 Disinfectant sprays are most likely distributed in sealed plastic bottles. The principal method of
16 retail distribution likely is through the transport of cardboard cartons, each containing several dozens of
17 spray bottles. Although the boxes with spray bottles might be stored at intermediate distribution facilities,
18 they are apt to be opened only at the retail location where the individual units are ultimately sold to
19 customers. The possible scenarios for releases during transport include damage to the cartons or leakage
20 from the bottles as a result of mishandling of cartons, faulty packaging, or improper stacking of cartons in
21 transport vehicles. If the bottles are sealed properly and not damaged during transport, releases of product
22 prior to use might be limited to breakage of bottles or large-volume spills of the liquid spray at retail
23 locations where silver sprays are sold.

3.4. Use of Nano-Ag Disinfectant Sprays

24 Manufacturers state that their disinfectant sprays can be used on a wide variety of surfaces,
25 including walls, floors, sinks, door knobs, light-switch covers, telephones, appliances, tables, and chairs.
26 Sprays are likely to be used in both residential and institutional settings, such as hospitals, restaurants, and
27 schools. Nano-Ag from the use of sprays likely will be found in the air, on the intended surfaces, and on

1 unintended surfaces contaminated by overspray, including the body and food. Spraying of kitchen
2 surfaces with nano-Ag products could result in the transfer of the particles to food items and to light
3 switches, door knobs, telephones, and other surfaces that are often touched. Additional activities
4 involving the sprayed surfaces could release more nano-Ag or spray by-products. For example,
5 subsequent cleaning of the surface with products containing oxidizing agents, such as hydrogen peroxide,
6 could oxidize the nano-Ag and release ionic silver. Concomitant release of other spray ingredients would
7 also occur and could affect the behavior of nano-Ag. Surfaces sprayed with nano-Ag products could be
8 wiped down with paper towels, disposable dust cloths, or other single-use products; these disposable
9 products then are likely to enter municipal waste collection systems and landfills.

10 Nano-Ag disinfectants sprayed on sinks, bathtubs, and toilets could enter wastewater streams or
11 septic tanks. Similarly, fabric or clothing that is sprayed and then laundered also could release nano-Ag
12 and by-products into wastewater. Benn and Westerhoff (2008, [157595](#)) found that three of six brands of
13 socks containing nano-Ag leached the particles during wash simulations. Their results suggest that
14 nanoparticles that are not incorporated into fabrics but instead are sprayed onto fabrics also might enter
15 wastewater streams.

16 Commercial establishments such as restaurants and hospitals might purchase bulk quantities of
17 spray solutions containing nano-Ag for use with spray-gun applicators. For example, the usage
18 instructions for NanoSil from ConSeal International, Inc. (2010, [594257](#)) suggest using a spray gun or
19 mop for application. Excess product remaining after spraying is likely to be disposed of into municipal
20 wastewater streams, as would the water used to rinse spray guns after use.

21 Nano-Ag disinfectant sprays might also be used outdoors; for example, sprays might be used to
22 disinfect outdoor trash cans, outdoor furniture and children's toys, or boats or other recreational
23 equipment. From these applications, nano-Ag and by-products might directly enter natural waters or soil,
24 rather than being processed at wastewater treatment facilities.

25 Shanghai Huzheng Nanotechnology Co. Ltd. (2009, [594268](#)) reports on their Web site that their
26 products will continue to protect against bacteria for up to 24 hours after application. This claim suggests
27 that particles will continue adhering to the surfaces to which they are applied for up to a day. Research by
28 Brady et al. (2003, [194058](#)) suggests that a silver disinfectant continues to effectively inhibit bacterial
29 growth on a solid glass surface despite repeated rinsing under tap water; other non-silver disinfectants did
30 not show the same effectiveness. A subsequently published letter to the editor of the journal, however,
31 questions the applicability of the results because it could have been the film created by the disinfectant,
32 rather than the silver, that prevented bacterial growth (Schuster et al., 2004, [194077](#)). Additionally, the
33 authors of the letter suggested that incubating the glass tiles under humid conditions between tests rather
34 than at room temperature artificially increased bacteria growth over normal conditions and overstated the
35 effectiveness of the disinfectant spray. They also questioned whether the surface disinfectant would be as
36 effective on porous surfaces such as wood as it purportedly was on glass.

3.5. Disposal of Nano-Ag Disinfectant Sprays

1 The most likely scenario for disposal of spray bottles is through household waste, whether those
2 containers are taken to a landfill or recycled. Regardless of the pathway, any nano-Ag and associated
3 substances remaining in the bottles ultimately would enter municipal solid waste streams. If the waste is
4 incinerated, nano-Ag might be released to the air. If the waste is deposited in landfills, nano-Ag could
5 leach into the soil. Alternatively, if containers are recycled, both workers and consumers could come in
6 contact with nano-Ag in the manufacturing and use of products made from recycled materials that
7 previously contained nano-Ag spray. The disposal of bottles that are unopened or contain unused portions
8 of spray would be an additional source of nano-Ag and other spray ingredients in municipal solid waste
9 streams. It is also possible that disinfectant sprays or materials that have come in contact with them might
10 be disposed of improperly, for example, in wooded areas, rivers, or other illegal dumping grounds. In
11 such cases, nano-Ag could then directly enter the environment.

3.6. Summary of Life-Cycle Stages

12 The life cycle of nano-Ag used in spray disinfectants begins with the extraction or recovery of
13 conventional silver from mining operations. As much as 5% of silver production could be nano-Ag, but
14 substantial uncertainty is associated with this figure (Mueller and Nowack, 2008, [157519](#)). A variety of
15 methods of nano-Ag production are reported in the literature and in patent filings, but how many of these
16 are used on an industrial scale or which are used most frequently in general or in the production of spray
17 disinfectants is unknown. Results of bench-scale syntheses of nano-Ag suggest that using wet chemical
18 processing is more efficient than other production processes; wet chemical processing is likely to result in
19 lower inhalation exposures during the manufacturing stage than solid- or vapor-phase processes. No
20 information specific to releases of nano-Ag and associated substances to the environment during
21 distribution, use, or disposal of spray disinfectants was identified. At any of these life-cycle stages, nano-
22 Ag could be released to the air (especially to indoor air during use) or to surfaces within homes and public
23 spaces. Disposal could result in the release of nano-Ag, other spray ingredients, or nano-Ag by-products
24 to the environment by way of landfills or wastewater streams.

25 Questions reflecting data gaps in the information about life-cycle stages are listed on the following
26 page. They are listed in approximate order of the presentation of information in this chapter. The order is
27 in no way intended to reflect the relative importance of the questions.

Questions about Life-Cycle Stages

- 3.1. What is a reliable estimate of worldwide and domestic nano-Ag production?
- 3.2. What data regarding the physicochemical properties, concentrations, and formulations in nano-Ag spray disinfectants are appropriate for assessing their behaviors in and impacts on the environment?
- 3.3. What are realistic strategies for collecting data on production quantities and product characteristics given that much of this information is proprietary?
- 3.4. What properties of engineered nano-Ag particles that are incorporated in spray disinfectants are different from known properties of colloidal silver?
- 3.5. Which manufacturing methods for nano-Ag and spray disinfectants containing nano-Ag are most common at the industrial scale?
 - 3.5.a. What are the associated feedstocks and by-products; of these feedstocks and by-products, which might be released, in what quantities, and via which pathways?
 - 3.5.b. Does the choice of manufacturing method for nano-Ag or spray disinfectant containing nano-Ag affect the release rate of silver ions?
- 3.6. What changes occur to the physicochemical properties of nano-Ag throughout the material life cycle stages, either as a function of process and product engineering or as a function of incidental encounters with other substances and the environment?
 - 3.6.a. Do the changes that occur as a function of process and product engineering (e.g., the incorporation of nano-Ag into disinfectant sprays) affect the release rate of silver ions such that the rate might differ throughout the life cycle stages?
- 3.7. What are the potential exposure vectors by which nano-Ag or nano-Ag by-products could be released to the environment at the various life-cycle stages?
 - 3.7.a. What information is most relevant (e.g., product handling throughout different life cycle phases, product use patterns, and nanoparticle release rates from products) for determining which of these potential exposure vectors represent the most significant pathway(s) for environmental release?
 - 3.7.b. What are the prevailing release pathways expected to be for nano-Ag and disinfectant sprays containing nano-Ag into the environment?
 - 3.7.c. What are the frequencies and durations of releases of nano-Ag during various life-cycle stages?

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Chapter 4. Fate and Transport in Environmental Media

1 The production, use, and disposal of engineered nano-Ag eventually will lead to its occurrence in
2 air, soil, and water (Wiesner et al., 2006, [089583](#)). Chapter 4 examines what might happen to nano-Ag⁵
3 after its release to the environment at various stages of the product life cycle for spray disinfectants.
4 Nano-Ag released to air, water, or soil could then be transported or transformed through chemical,
5 physical, and biological processes. Although the fate and transport of nano-Ag-associated contaminants,
6 such as waste by-products related to feedstocks and manufacturing, is also of relevance to a
7 comprehensive environmental assessment, the current insufficiency of information on these associated
8 contaminants precludes their coverage in this chapter.

9 Current literature suggests that the fundamental properties governing the environmental fate of
10 engineered nanoparticles in general are not thoroughly understood,⁶ and that studies on fate and transport
11 of nano-Ag, although beginning to emerge, are still relatively few. The lack of data on the fate and
12 transport of nano-Ag by-products and waste produced after disposal precludes a comprehensive
13 discussion in this chapter and represents a potential data gap for a comprehensive environmental
14 assessment of nano-Ag. This chapter does, however, summarize what is known about the environmental
15 behavior and transport and transformation processes of engineered nanoparticles (and specifically nano-
16 Ag, when available), the physical-chemical properties of these particles, and the characteristics of the
17 environmental media that can affect the behavior of these particles.

18 Section 4.1 provides a brief discussion of the chemical and physical characteristics and processes
19 that influence fate and transport of nano-Ag in environmental media. The sections that follow provide the
20 available information regarding nano-Ag behavior in indoor and ambient air (Section 4.2), terrestrial
21 systems (Section 4.3), and aquatic systems (Section 4.4). A discussion of models that might be used for
22 evaluating the fate and transport of nano-Ag, or silver ions released from nano-Ag, in environmental
23 media is provided in Section 4.5.

⁵ Throughout this chapter, the term “nano-Ag” refers to silver nanoparticles that can display a range of properties and behaviors depending on specific characteristics of the particle, environmental conditions, and other factors.

⁶ The recent FIFRA Scientific Advisory Panel review (U.S. EPA, 2010, [625619](#)) came to a similar conclusion.

4.1. Factors Influencing Fate and Transport of Nano-Ag

1 The literature indicates that aerosols of atmospheric nanoscale particles formed by combustion
2 processes (e.g., from cars, incinerators) have been studied at length; relatively little, however, is known
3 about aerosols from solid, *engineered* nanomaterials (Ma-Hock et al., 2007, [091320](#)). Aerosols of
4 engineered nanoparticles are synthesized in the laboratory to have unique physicochemical properties and
5 certain functional properties for use in commercial products (Jiang et al., 2009, [193450](#); Ma-Hock et al.,
6 2007, [091320](#)). These “intentional” nanoparticles are controlled for size and shape and designed for
7 functionality, and might have a surface coating or other surface modifications to help increase the
8 product’s stability and persistence after its release (see Section 4.1.1.1) (Oberdörster et al., 2005, [087559](#)).

9 For decades, health-related aerosol exposures have been represented in terms of mass concentration
10 measurements alone. For assessing exposure to airborne nanoparticles, other factors such as particle
11 number, particle shape and surface area, surface chemistry (including coatings), and the degree to which
12 particles agglomerate or aggregate to form clusters⁷ play a critical role in determining nanoparticle
13 distribution and fate within the environment and in evaluating their potential health impacts (Jiang et al.,
14 2009, [193450](#); Ma-Hock et al., 2007, [091320](#); Maynard and Aitken, 2007, [090674](#)).

15 Once released into the environment, nanoparticles would be expected to behave generally as
16 follows: (1) stay in suspension as individual particles; (2) form clusters with other particles (and
17 potentially deposit or undergo facilitated transport); (3) dissolve in a liquid; or (4) chemically transform
18 based on reactions with natural organic matter or other particles (Luoma, 2008, [157525](#)). As described in
19 the following sections, the distribution and fate of nano-Ag within the environment depends on the
20 physical and chemical processes that occur in the environment (Section 4.1.1) and the characteristics of
21 the environmental system (Section 4.1.2) (Boxall et al., 2007, [157712](#)), as well as characteristics of the
22 particles, as described detail in Section 2.3. The presence of spray ingredients or materials used in the
23 manufacturing process can also potentially affect the environmental behavior of nano-Ag, although no
24 specific information regarding this phenomenon was identified during development of the case study.

25 For the remainder of this chapter, much of the information presented is applicable to engineered
26 nanoparticles in general, as few fate and transport studies specific to nano-Ag were identified.

⁷ As summarized by Nichols et al. (2002, [202114](#)) and discussed in more detail in Chapter 1, the meanings of the terms “aggregate” and “agglomerate” as they refer to the formation of particle “clusters” are sometimes interchanged; thus, the definitions of these terms are neither specific nor consistent. To simplify the discussion for this case study, the term “cluster” is used throughout this document to indicate an aggregate or agglomerate of nanoparticles, regardless of the nature or strength of particle cohesion or the mechanisms by which the particles assemble.

4.1.1. Physical and Chemical Processes in the Environment

1 As discussed above, the particle-specific characteristics of nanomaterials, together with the
2 physical and chemical processes that occur as a result of nanoparticle interactions with co-occurring
3 substances and the surrounding environment, determine their behavior and transport in environmental
4 systems.

4.1.1.1. Persistence

5 Although silver can accumulate in water, sediments, soils, and organisms, silver ions and silver
6 nanoparticles are fundamentally different. Free silver ions can associate with other ions, but the ion itself
7 is intrinsically persistent, although it can be converted to other species (i.e., speciate). In contrast, a nano-
8 Ag particle is not necessarily persistent. Particles can dissolve or disassemble (i.e., physically transform),
9 and they will not necessarily re-form, losing the properties of the primary particle. A study conducted by
10 Liu and Hurt (2010, [625606](#)) demonstrated that nano-Ag particles are not persistent in aquatic systems
11 containing dissolved oxygen. Specifically, in air-saturated water, the amount of dissolved silver released
12 from nano-Ag colloids over one day increased in a linear fashion over time. Increasing pH or increasing
13 concentrations of natural organic matter inhibited the release of dissolved silver in the air-saturated water.
14 In deoxygenated water, dissolved silver release from nano-Ag colloids was completely inhibited.

15 Some types of silver nanoparticles in commercial products are engineered to have charged
16 functional groups or surface coatings so that they remain dispersed. In general, particles that remain
17 dispersed (e.g., through the presence of surface coatings) will tend to exhibit greater persistence in the
18 environment than particles that are not treated to favor dispersal (Luoma, 2008, [157525](#)).

4.1.1.2. Particle Aggregation and Agglomeration

19 The translocation of nanoparticles depends largely on their size; for this, among other, reasons,
20 clusters of engineered nanoparticles will behave quite differently compared to single engineered
21 nanoparticles (Ma-Hock et al., 2007, [091320](#)). Generally, nanoparticle clusters formed through
22 aggregation or agglomeration are less mobile than individual nanoparticles (Nowack and Bucheli, 2007,
23 [092294](#)). The extent of clustering depends on the properties of the particle (e.g., particle shape, size,
24 surface area, surface charge) and the characteristics of the environmental system (Handy et al., 2008,
25 [157562](#); Tiede et al., 2009, [193680](#)).

26 Aggregation and agglomeration affect the potential for release of nano-Ag from products into
27 environmental media (Wijnhoven et al., 2009, [180201](#)). Nano-Ag particles can be specially coated to
28 reduce the formation of clusters so that the high surface reactivity of ionic silver can be exploited
29 (Kandlikar et al., 2007, [091626](#)). Other spray ingredients might also affect nano-Ag clustering.

1 The formation of particle clusters and deposition are closely related phenomena. As described by
2 Navarro et al. (2008, [157517](#)) and Wiesner et al. (2006, [089583](#)), aggregation and agglomeration describe
3 the interaction between two mobile objects (transport). Deposition refers to the settling of a mobile
4 particle onto a land or water surface. Nanoparticles that associate with dissolved materials or particles in
5 the environment likely will be deposited in sediments or soils (Luoma, 2008, [157525](#)), although this
6 outcome can be complicated by processes such as dissociation or resuspension of particles due to changes
7 in aquatic or soil chemistry and physical disturbance and activity of aquatic organisms. The formation of
8 clusters and therefore of larger particles that are trapped or eliminated through deposition or
9 sedimentation affects the concentrations of bioavailable engineered nanoparticles, especially in the water
10 column. Although these nanoparticle clusters become less mobile, uptake by soil- or sediment-dwelling
11 organisms or filter feeders is still possible (Nowack and Bucheli, 2007, [092294](#)).

4.1.1.3. Adsorption

12 Adsorption, conceptually similar to both deposition and the formation of clusters as introduced
13 above, is the binding of molecules or particles to a surface. The potential of the nanoparticle to adsorb
14 onto a surface is influenced by its surface area, surface charge, and degree of clustering, and the presence
15 of surface coatings (O'Brien and Cummins, 2009, [196217](#)). Because of their high surface area-to-volume
16 ratio and complexing capability, nanoparticles can adsorb pollutants or other spray ingredients, which
17 might change the transport, solubility, and bioavailability of both the nanoparticles and the pollutants in
18 the environmental systems and modify their toxic effects (Navarro et al., 2008, [157517](#)).

4.1.1.4. Transport/Mobility Potential

19 Once nanoparticles are released into the environment, their transport is a critical factor in assessing
20 their impact and ultimate fate in the environment. Generally, nanoparticle transport on the molecular or
21 particle scale is dominated by Brownian motion (random motion of small particles suspended in a gas or
22 liquid). Weaker forces such as London or van der Waals are responsible for attachment behaviors that
23 ultimately determine particle mobility (Biswas and Wu, 2005, [088038](#)). Transport or mobility potential of
24 nano-Ag is affected by the characteristics of the nanomaterial (including those of the material matrix and
25 surface coatings), associated substances from manufacturing or product formulation, and the
26 environmental medium (O'Brien and Cummins, 2009, [196217](#)).

27 The greater potential mobility of nanoparticles in the environment relative to the mobility of larger
28 particles implies a greater potential for exposure because they are dispersed over greater distances and
29 their effective persistence in the environment increases. The physical movement of a nanoparticle,
30 however, is restricted by its small size and propensity to adsorb to surfaces (Borm et al., 2006, [089840](#)).

1 Their propensity to adsorb to surfaces or to form clusters can make them less mobile in porous media
2 such as soil (Borm et al., 2006, [089840](#); Wiesner et al., 2006, [089583](#)).

4.1.1.5. Transformation

3 Many nanoparticles will transform physically, chemically, or biologically once released to the
4 environment (e.g., through loss of surface coatings). Transformation can affect size, shape, and surface
5 chemistry of the particles and their coatings, and this process will affect their ultimate distribution,
6 persistence, and toxicity in the environment (Lowry and Casman, 2009, [196153](#)). Transformation can
7 lead to substances that present a very different hazard than does the untransformed material that was
8 originally released (Maynard, 2006, [089448](#)). Particles can dissolve or disassemble, for example, which
9 means they essentially transform, altering the properties of the initial particle (Luoma, 2008, [157525](#)).

4.1.2. Characteristics of the Environmental System

10 In terrestrial and aquatic systems, released nanoparticles (single or matrix-bound) are affected by
11 abiotic factors such as organic matter content, concentrations of ligands, solution ionic strength, pH, and
12 other environmental variables, all of which play a large role in the fate of nanoparticles in the
13 environment. The presence of other spray ingredients or substances used in manufacturing could modify
14 these interactions. In general, these abiotic factors alter the chemistry that determines the extent of
15 aggregation and agglomeration and ultimately influences toxicity (Cumberland and Lead, 2009, [199804](#);
16 Gao et al., 2009, [195514](#); Handy et al., 2008, [157562](#)).

4.1.2.1. Organic Matter Content

17 Silver can be immobilized as a result of sorption or binding to particles (predominantly organic
18 matter). Although silver ions can readily bind to soil and particles, organic matter (containing negatively
19 charged humic and fulvic acids) can also coat the surface of nanoparticles, resulting in particles that
20 disperse rather than form clusters (Handy et al., 2008, [157562](#)). Chen and Elimelech (2007, [597391](#))
21 studied the influence of humic acid on the aggregation kinetics of fullerene (C₆₀) nanoparticles in various
22 monovalent and divalent electrolyte solutions and found that, in the presence of sodium chloride (NaCl),
23 magnesium chloride (MgCl₂), or low-concentration calcium chloride (CaCl₂) electrolyte solutions,
24 adsorbed humic acid on the fullerene nanoparticles led to steric repulsion. Steric repulsion effectively
25 stabilized the nanoparticle suspension and resulted in a remarkable drop in the rate of particle clustering.
26 Conversely, enhanced clustering occurred at higher CaCl₂ concentrations.

4.1.2.2. Concentrations of Ligands

1 Many inorganic (e.g., chloride, sulfides, and sulfates) and organic ligands are commonly present in
2 the environment (Choi et al., 2009, [193317](#)) and also could be present in spray disinfectant product
3 formulations. Ionic silver forms strong complexes with anions (particularly sulfides), resulting in its
4 immobilization in wastewater streams and surface waters (Adams and Kramer, 1999, [202665](#)).

4.1.2.3. pH

5 Environmental conditions such as pH of the surrounding soil or water environment are very
6 important in determining the nature and degree of nanoparticle transport (Boxall et al., 2007, [157712](#)).
7 pH will change the surface charge of particles, thereby affecting the size of the cluster (Dunphy Guzman
8 et al., 2006, [090584](#)). As pH of the system increases, the number of negatively charged sites in the system
9 increases, elevating the potential for adsorption of the nanoparticles to negatively charged species
10 (O'Brien and Cummins, 2009, [196217](#)). The mobility of silver ions increases under conditions of
11 increased acidification (Adams and Kramer, 1999, [202665](#)).

4.1.2.4. Ionic Strength

12 The rate and extent of particle aggregation and agglomeration depend in part on ionic strength and
13 ionic composition. In general, increasing ionic strength (e.g., additions of salt) and the presence of
14 divalent cations such as Ca^{2+} (ionic calcium) and Mg^{2+} (ionic magnesium) increase the rate and extent of
15 clustering and can affect the stable size of the clusters formed (Cumberland and Lead, 2009, [199804](#);
16 Handy et al., 2008, [157562](#); Lowry and Casman, 2009, [196153](#)). According to Lee et al. (2007, [194072](#)),
17 the presence of a sufficiently high concentration of salt (NaCl at 100 millimolar [mM]) appears to reduce
18 the thickness of the electric double-layer on the surface of nanoparticles and decrease the zeta-potential (a
19 measure of stability behavior of a colloid) below its critical point, leading to the formation of nanoparticle
20 clusters.

4.1.2.5. Redox Transformations

21 Reduction-oxidation (redox) reactions (in which the oxidation state of one reactant increases while
22 the other decreases) are important for the degradation of organic compounds. These reactions also are the
23 basis of various precipitation and dissolution processes that influence the sequestration and mobility of
24 inorganic metals. Thus, redox reactions are likely to influence the transformation and fate of
25 nanoparticles. Redox reactions are often mediated by microorganisms, either directly through enzymatic

1 activity or indirectly through the production of biogenic substances (e.g., secretions) that act as oxidants
2 or reductants (Wiesner et al., 2006, [089583](#)).

3 Studies have suggested that in aqueous solution nano-Ag can become oxidized under oxygenated
4 conditions, resulting in silver ions forming on particle surfaces and an increase in dissolved silver
5 concentrations (Gao et al., 2009, [195514](#)). The oxidation or reduction processes can lead to the release of
6 silver ions in solution, but redox reactions can also occur at the surface of nanoparticles leading to change
7 in its crystalline nature (Auffan et al., 2009, [193255](#)). Auffan et al. (2009, [193255](#)) further suggest that
8 the in vitro toxicity of metallic nanoparticles can be predicted based on their redox properties and their
9 ability to be oxidized, reduced, or dissolved within unicellular organisms (see Section 6.1.1).

4.2. Air

10 Nanoparticles can become suspended in air during manufacturing, distribution, or use (Grassian,
11 2009, [196145](#)). As discussed in Section 3.2, nano-Ag, other spray ingredients, or by-products of the spray
12 could be released during the manufacture of nano-Ag disinfectant sprays. Preliminary handling of large
13 quantities of nano-Ag prior to creating the spray disinfectants could lead to release of nano-Ag to the air
14 or surfaces in the facility. Mechanical processes such as mixing, grinding, or agitation of liquids might
15 cause nano-Ag to escape to the ambient air. During the distribution and storage phase, release of nano-Ag
16 in spray products might occur if bottles containing the spray were not sealed properly or if bottles become
17 damaged during transport (see Section 3.3). Whether nano-Ag released to the air during the
18 manufacturing process and product distribution phases would result in localized or more widespread
19 contamination is unclear.

20 The use of spray disinfectants containing nano-Ag will result in direct emissions of nano-Ag and
21 other substances to the indoor microenvironment. By virtue of its use indoors, most of the nano-Ag
22 would be expected to remain in the buildings in which it is used (and be suspended in air or deposited on
23 surfaces, with the potential for resuspension); nano-Ag, however, could be transported from the indoor
24 environment to the outdoor environment, and into the ambient air, water, and soil. In addition, such
25 products might also be used outside (e.g., to disinfect boats, garbage cans, decks, or automobiles or in
26 outdoor stadiums or arenas or in food processing plants that vent to the outside). Although such uses
27 could increase the concentration of nano-Ag in the outside environment, significant dilution in the
28 ambient air could also occur, and the extent to which the resulting concentrations would be discernible in
29 relation to background levels is unknown. Nano-Ag contained in sprays applied to indoor surfaces might
30 subsequently be washed down the drain or wiped with a paper towel or other cloth that is discarded in the
31 trash (thus entering a landfill) or washed in a washing machine (thus entering a wastewater treatment

1 plant). The potential for other spray ingredients to bind to nano-Ag could result in transport of co-
2 pollutants through these pathways or modification of the transport behavior of nano-Ag.

4.2.1. Diffusion

3 Several processes and factors influence the fate of airborne particles in indoor and outdoor
4 environments, including size, chemical characteristics, the nature of interactions with other airborne
5 particles, residence time in the air, and distance traveled prior to deposition (U.S. EPA, 2007, [090564](#)).
6 The fate of airborne particles outdoors could also be influenced by meteorological factors, including
7 wind, temperature, and relative humidity (Navarro et al., 2008, [157517](#)). Nanoparticles might be in the
8 form of single particles or in clusters that are larger than the primary particle (Grassian, 2009, [196145](#))
9 (see Section 2.3). Individual nano-sized particles will likely follow the laws of gaseous diffusion when
10 released to air, with their diffusion rate inversely related to their diameter (i.e., smaller particles will
11 diffuse more quickly) (U.S. EPA, 2007, [090564](#)). Due to their high diffusion coefficients, nanoparticles
12 (based on size alone) should be much more highly mobile than micrometer-sized particles (Aitken et al.,
13 2004, [090566](#)). The dynamics of airborne nano-sized particles suggest that they will generally follow
14 airflows and not be influenced by mechanisms such as settling and inertial deposition (Maynard, 2006,
15 [089448](#)). They are, however, more likely than larger particles to deposit to surfaces via diffusive
16 mechanisms.

17 Once they are emitted to the indoor atmosphere, nanoparticles will diffuse according to a
18 concentration gradient, from high-concentration zones to low-concentration zones. Nanoparticles will
19 mix rapidly through indoor air and quickly disperse, and can be carried by various air movements caused
20 by differences in temperature, ventilation, or the movement of people or objects. For nanoparticle
21 aerosols, concentrations at the emission source can therefore drop fairly quickly. These aerosols can
22 diffuse over greater distances and persist for a relatively long time in the indoor environment (AFSSET,
23 2006, [201630](#); Aitken et al., 2004, [090566](#)), although diffusive deposition will decrease their airborne
24 concentration (see Section 4.2.3).

4.2.2. Particle Aggregation and Agglomeration

25 Nanoparticles in sprays are typically in the form of clusters (Biswas and Wu, 2005, [088038](#)),
26 although some commercial products are engineered to have surface coatings to help stabilize them against
27 aggregation and agglomeration. Particle clusters have a much larger aerodynamic diameter than single
28 nanoparticles and thus disperse and deposit differently. Dispersed nanomaterials have been observed not

1 to yield many single nanoparticles in the atmosphere, even when using relatively high-energy dispersion
2 methods (e.g., through spraying) (Ma-Hock et al., 2007, [091320](#)).

4.2.3. Residence Time

3 Nanoparticles as single particles have short residence times in air because of their rapid diffusion in
4 air, deposition to surfaces, and association with larger particles. They might, however, attach to larger
5 (0.1–1 micrometer [μm]) particles, diffuse over greater distances, and persist in the atmosphere for longer
6 periods of time (AFSSET, 2006, [201630](#); Aitken et al., 2004, [090566](#); Biswas and Wu, 2005, [088038](#)). In
7 general, particles in the 0.1- to 10- μm range have the longest residence time in the atmosphere (Biswas
8 and Wu, 2005, [088038](#)). Longer residence time in the atmosphere allows more time for the particles to be
9 mobilized by wind and other forces; therefore, long-range atmospheric transport of nanoparticles is
10 possible (Wiesner et al., 2006, [089583](#)).

4.2.4. Deposition and Resuspension

11 Particles suspended in the indoor air could be removed from the atmosphere and deposited onto
12 floors, walls, and other surfaces. Single nanoparticles might remain suspended in the air until they are
13 randomly deposited on a surface due to Brownian motion. Due to gravitational settling, larger particles
14 will fall out of the atmosphere more quickly and are likely to be deposited closer to the emission source
15 (AFSSET, 2006, [201630](#); Aitken et al., 2004, [090566](#)).

16 Eventually, all particles in the ambient air are deposited (dry deposition) or washed out (wet
17 deposition) to aquatic or terrestrial systems (e.g., soil and plants) (Mueller and Nowack, 2008, [157519](#)).
18 Some nano-Ag particles that have become deposited could experience secondary transport via wind and
19 become resuspended into ambient air and deposited elsewhere. Once deposited, however, nanoparticles
20 likely would not be resuspended in the air or re-aerosolized (Aitken et al., 2004, [090566](#); Colvin, 2003,
21 [069168](#)). Aerosol particles that contact one another generally stick together because of attractive London
22 or van der Waals forces and form aggregates or agglomerates (Aitken et al., 2004, [090566](#)). London or
23 van der Waals forces also act to keep a particle attached to a surface. Once agglomerated (or attached),
24 small particles would be much more difficult to resuspend.

4.2.5. Additional Factors

25 As discussed in Section 4.1, nanoparticles for use in commercial spray products are synthesized to
26 have unique physicochemical properties and certain functional properties (Jiang et al., 2009, [193450](#); Ma-

1 Hock et al., 2007, [091320](#)). They might be engineered to have a surface coating or other surface
2 modifications to help stabilize them against cluster formation and deposition and to increase the product's
3 persistence after its release (Oberdörster et al., 2005, [087559](#); Wiesner et al., 2006, [089583](#)). Spray
4 ingredients might also affect the transport and persistence of nano-Ag in air. In addition to its physical
5 properties, the transport and ultimate fate of a sprayed product is affected by the environmental or
6 meteorological factors that it encounters (e.g., magnitude of air currents, temperature, relative humidity)
7 (Navarro et al., 2008, [157517](#)).

4.3. Terrestrial Systems

8 Nano-Ag in spray disinfectants can enter terrestrial ecosystems in several ways:

- 9 • During manufacturing, distribution, use, or disposal of nano-Ag spray products, nano-Ag might
10 be transported into ambient air and subsequently deposited or washed out to aquatic or
11 terrestrial systems (Mueller and Nowack, 2008, [157519](#)).
- 12 • Disposal of spray products containing nano-Ag might result in nano-Ag release to soil (Navarro
13 et al., 2008, [157517](#)). Disposal products are either incinerated (whereby nano-Ag could be
14 released into the air and deposited on soil and plants) or deposited in solid waste landfills.
15 Land-filled sewage sludge could result in leaching of the nano-Ag into subsoils and
16 groundwater (Blaser et al., 2008, [193283](#)).
- 17 • Products containing nano-Ag might wash down a sink or bathtub drain or be discharged from a
18 washing machine into a wastewater treatment plant. Sewage sludge (separated during the
19 wastewater treatment process) is sometimes applied as a fertilizer to agricultural soils (Blaser et
20 al., 2008, [193283](#)). Therefore, nano-Ag might be released into soil via sewage sludge. Runoff
21 flows along the ground surface could transfer nanoparticles in the sewage sludge to nearby
22 terrestrial systems or aquatic systems (Blaser et al., 2008, [193283](#); O'Brien and Cummins, 2009,
23 [196217](#)).
- 24 • Some nano-Ag particles that deposit on soil might experience secondary transport via wind and
25 become resuspended into ambient air and re-deposited into aquatic or terrestrial systems.
26 However, as stated previously, once deposited, nanoparticles would be unlikely to resuspend in
27 the air or re-aerosolize given their propensity to agglomerate or attach to surfaces (Aitken et al.,
28 2004, [090566](#); Colvin, 2003, [069168](#)).

29 Overall, information on the fate and transport of nanoparticles in terrestrial systems is limited.
30 Information obtained from the literature provides a general description of the behavior of nanoparticles in
31 soil and plants; information specific to nano-Ag, however, has not been found.

4.3.1. Soil

32 The fate of nanoparticles released to soil is likely to vary depending on the physical and chemical
33 properties of the nanoparticles, the presence of other spray ingredients, and the complex characteristics of

1 the soil environment. Climatic conditions (precipitation and temperature) also can determine how the
2 nanoparticles are transferred (runoff, drainage, leaching) (AFSSET, 2006, [201630](#)).

3 Due to their size, nanoparticles are potentially mobile in soils (AFSSET, 2006, [201630](#)).
4 Nanoparticles are small enough to fit into the spaces between soil particles, and therefore might travel
5 farther than larger particles before becoming trapped in the soil matrix. Alternatively, nanoparticles
6 released to soil can be strongly sorbed to soil due to their high surface areas, and therefore become
7 immobile. The strength of the sorption of any nanoparticle to soil will depend on its size, chemistry,
8 applied particle surface treatment, the presence of other substances, and the conditions under which it is
9 applied (O'Brien and Cummins, 2009, [196217](#); U.S. EPA, 2007, [090564](#)).

10 Properties of the soil environment (e.g., soil type, soil organic matter, pH, ionic strength, presence
11 of other pollutants) also could affect nanoparticle transport. The types and properties of the soil (e.g., clay
12 versus sand) can affect nanoparticle mobility (O'Brien and Cummins, 2009, [196217](#); U.S. EPA, 2007,
13 [090564](#)). Interactions between nanoparticles and soil organic matter alter the degree of nanoparticle
14 aggregation or agglomeration in soils (see Section 4.1.2.1). Soil porewater is generally rich in dissolved
15 organic molecules (e.g., soluble organic matter and humic and fulvic acids) that can enhance colloidal
16 stability of nanomaterials and increase their mobility (Jaisi and Elimelech, 2009, [597406](#)). Changes in pH
17 can affect the size of the cluster, adsorption potential, and mobility in environmental media, as discussed
18 in Section 4.1.2.3. Soils are not static, and changes to their constituents (e.g., the addition of fertilizers or
19 rain) can reduce soil pH and hence can increase the mobility of contaminants (ATSDR, 1990, [196773](#)).
20 As discussed in Section 4.1.2.4, the presence of salt ions in soil can promote the association of
21 nanoparticles, thus reducing their bioavailability or physically restraining nanoparticle-organism
22 interactions. Nanoparticles might adsorb other pollutants, which can change the transport and
23 bioavailability of both the nanoparticles and the other pollutants in the soil (Navarro et al., 2008, [157517](#)).

4.3.2. Plants

24 Plants could be exposed to nano-Ag in air, water, and soil. Airborne nanoparticles could attach to
25 leaves and other aerial parts of plants (Navarro et al., 2008, [157517](#)). To what extent a plant might
26 represent an efficient nano-Ag trap can depend on factors such as leaf area surface, type of plant/tree (e.g.,
27 deciduous versus coniferous), season, and evapotranspiration. Once on the leaf surface, nanoparticles
28 could be translocated to different tissues of the plant. If nano-Ag is present in soils, plant roots could
29 interact with nano-Ag associated with soil material and in soil pore water. The mobility of nanoparticles
30 in pore water is an essential condition for interactions with plant roots or fungal hyphae. In the presence
31 of certain organic compounds, nanoparticles will have improved mobility in soils, and could thus interact
32 more efficiently with plant roots (Navarro et al., 2008, [157517](#)).

1 Some scientists have conducted studies to assess the potential for nanoparticle recovery using
2 plants (i.e., phytoextraction, which can be exploited for phytoremediation). Harris and Bali (2008,
3 [195522](#)) examined the extent of uptake of nano-Ag by two metal-tolerant plants, *Medicago sativa*
4 (alfalfa) and *Brassica juncea* (type of mustard plant). For both species, silver accumulation in plant
5 tissues (i.e., silver uptake) was measured as a function of exposure time and substrate silver
6 concentration. The authors also examined the form and distribution of silver once it was extracted by the
7 plants. In general, for *M. sativa*, silver uptake rate increased as a function of silver concentration in the
8 growth medium and exposure time. The authors' "best" results showed an uptake of approximately
9 13.6 wt.% Ag for the mustard plant. For *B. juncea*, the authors' "best" results showed a large uptake rate
10 (approximately 12.4 wt.% Ag), but silver uptake appeared to be independent of silver concentration in the
11 substrate and exposure time. For both species, the distribution of nano-Ag within the plant (determined
12 using proton induced X-ray emission spectroscopy) was unclear; however, as far as form, silver was
13 stored in the plant cells as discrete nanoparticles. Based on the results of this study, certain plants could
14 be valuable in sequestering nano-Ag for the purposes of phytoremediation.

4.4. Aquatic Systems

15 Nano-Ag in spray disinfectants could be released into aquatic systems in several ways:

- 16 • After its use, a spray containing nano-Ag might be transported into ambient air and
17 subsequently deposited in water bodies.
- 18 • During its use, a product containing nano-Ag could be washed down the sink or bathtub drain,
19 and material could be released into the sewage system, wastewater collection and treatment
20 facilities (U.S. EPA, 2010, [625619](#)), and, eventually, to water bodies.
- 21 • Disposal of spray products containing nano-Ag might result in release of nano-Ag to soil
22 (Navarro et al., 2008, [157517](#)). Disposal of by-products released during the manufacturing
23 process might result in a similar type of discharge to soil. Land-filled sewage sludge could
24 cause the silver to leach into subsoil and groundwater and to migrate to surface water (Blaser et
25 al., 2008, [193283](#); U.S. EPA, 2010, [625619](#)).
- 26 • Products containing nano-Ag might wash down a sink or bathtub drain or be discharged from a
27 washing machine into a wastewater treatment plant. Sewage sludge (separated during the
28 wastewater treatment process) is sometimes applied as a fertilizer to agricultural soils (Blaser et
29 al., 2008, [193283](#)). Therefore, nano-Ag might be released into soil via sewage sludge. Runoff
30 flowing along the ground surface (which causes erosion) could transfer nanoparticles in the
31 sewage sludge to nearby waterways (Blaser et al., 2008, [193283](#); O'Brien and Cummins, 2009,
32 [196217](#)).

33 Overall, few studies are available on the fate and transport of nanoparticles in natural aquatic
34 systems. Information obtained from the literature provides a general description of the behavior of
35 nanoparticles in water and sediments, although information specific to nano-Ag is limited.

4.4.1. Natural Aquatic Systems

1 As discussed in Section 6.2.2, aquatic organisms are highly susceptible to silver ion toxicity in
2 natural waters. Therefore, the behavior of nano-Ag in water will strongly influence whether significant
3 incidences of exposure and toxicity to aquatic organisms can be expected. The key chemical, physical,
4 and environmental factors in natural waters that could affect fate and transport behavior of nano-Ag in
5 aquatic systems are discussed below.

4.4.1.1. Surface Properties

6 As described in Section 2.3, the surface properties of nano-Ag are among the most critical
7 determinants governing its mobility and fate in aquatic systems. Particles in suspension settle at rates that
8 depend on particle size, density, and shape. Waterborne nanoparticles generally settle more slowly than
9 larger particles of the same substance. Due to their high surface area-to-mass ratios, however, nano-sized
10 particles can sorb to sediment particles and become removed from the water column (Oberdörster et al.,
11 2005, [087559](#); U.S. EPA, 2007, [090564](#)). The surface properties of nano-Ag govern its stability and
12 mobility as colloidal suspensions or their clustering into larger particles and deposition in aquatic systems
13 (Navarro et al., 2008, [157517](#)). Nano-Ag particles can be engineered with surface coatings to improve
14 water solubility and suspension characteristics.

4.4.1.2. Ionic Ag and Ag Complexes in Water

15 The mechanisms of action that govern toxicity of nano-Ag particles and ionic silver are the subject
16 of ongoing research, as investigators seek to determine whether nanoscale silver toxicity is due to the
17 particles themselves and their intrinsic properties, due to particles releasing silver ions, or due to some
18 synergistic combination of the two (Lubick, 2008, [195540](#)). For this reason, the behavior of ionic silver is
19 relevant to understanding potential impacts of disinfectant sprays that include nano-Ag. Navarro et al.
20 (2008, [157516](#)) recently showed evidence that both nanoparticles and ions serve as sources of toxicity,
21 with particles sometimes enhancing the toxic effects of ions. A study by Choi et al. (2008, [194060](#)),
22 which examined the inhibitory effects of nano-Ag, silver ions and silver chloride (AgCl) colloids on
23 microbial growth, suggested that nitrifying bacteria are especially susceptible to inhibition by nano-Ag
24 and that the accumulation of nano-Ag could detrimentally affect the microorganisms in wastewater
25 treatment. Results of this study showed that nano-Ag and silver ions inhibited nitrifying bacterial growth
26 by $86 \pm 3\%$ and $42 \pm 7\%$, respectively.

27 The form of silver in the water is governed in part by the nature of the water. In studies over the
28 past few years, a very small proportion of the total dissolved silver in water has been observed to remain
29 as free silver ions, and forms other than silver ion are predominant in the aquatic environment (Blaser et

1 al., 2008, [193283](#); Luoma, 2008, [157525](#)). Table 4-1 lists the various silver complexes that could occur in
 2 solution and provides the solubility product constant (K_{sp}) for each.

Table 4-1. Solubility product constants for various silver complexes.

| Silver complex | Formula | Solubility product constant (K_{sp}) |
|-------------------------------------|--|--|
| Silver(I) sulfide (α -form) | Ag ₂ S | 6.69×10 ⁻⁵⁰ |
| Silver(I) sulfide (β -form) | Ag ₂ S | 1.09×10 ⁻⁴⁹ |
| Silver(I) arsenate | Ag ₃ AsO ₄ | 1.03×10 ⁻²² |
| Silver(I) cyanide | AgCN | 5.97×10 ⁻¹⁷ |
| Silver(I) iodide | AgI | 8.51×10 ⁻¹⁷ |
| Silver(I) phosphate | Ag ₃ PO ₄ | 8.88×10 ⁻¹⁷ |
| Silver(I) sulfite | Ag ₂ SO ₃ | 1.49×10 ⁻¹⁴ |
| Silver(I) bromide | AgBr | 5.35×10 ⁻¹³ |
| Silver(I) thiocyanate | AgSCN | 1.03×10 ⁻¹² |
| Silver(I) chromate | Ag ₂ CrO ₄ | 1.12×10 ⁻¹² |
| Silver(I) oxalate | Ag ₂ C ₂ O ₄ | 5.40×10 ⁻¹² |
| Silver(I) carbonate | Ag ₂ CO ₃ | 8.45×10 ⁻¹² |
| Silver(I) chloride | AgCl | 1.77×10 ⁻¹⁰ |
| Silver(I) iodate | AgIO ₃ | 3.17×10 ⁻⁸ |
| Silver(I) sulfate | Ag ₂ SO ₄ | 1.20×10 ⁻⁵ |
| Silver(I) bromate | AgBrO ₃ | 5.34×10 ⁻⁵ |
| Silver(I) acetate | AgC ₂ H ₃ O ₂ | 1.94×10 ⁻³ |

Increasing Solubility

Source: CRC (2000, [196090](#)).

3 The free silver ion has a strong tendency to associate with negatively charged ions (ligands) in
 4 natural waters to achieve stability. Ligands can occur in solution, on particle surfaces, or on dissolved
 5 organic matter (Luoma, 2008, [157525](#)). Spray ingredients and other substances involved in the
 6 manufacturing process also could act as ligands. The distribution of free silver ions and silver complexes
 7 depends on the concentration of silver, concentrations of the different negatively charged ligands (such as
 8 chloride, sulfide, thiosulfate, and dissolved organic carbon), and the strength of the bond between each
 9 ligand and the silver ion (Blaser et al., 2008, [193283](#); Choi et al., 2009, [193317](#); Luoma, 2008, [157525](#)).
 10 The rest of this subsection focuses on the latter two points; however, quantifying estimated mass releases
 11 of silver from different life cycle phases of spray disinfectants containing nano-Ag, which might change
 12 the silver ion concentration (see Chapter 2), is critical.

13 Ligands that hold silver strongly are abundant in most sediments; therefore, silver ions tend to bind
 14 readily to particulate matter (Blaser et al., 2008, [193283](#); Luoma, 2008, [157525](#)). The availability of
 15 oxygen in sediments tends to dictate the form of silver bound to the particles. Strong complexes with
 16 organic material predominate at the sediment surface, where oxygen is usually present and sulfides

1 typically are not. In deeper sediments, where oxygen is absent, silver forms stable complexes with sulfide
2 (Luoma, 2008, [157525](#)).

3 Silver ions form especially strong complexes with free thiol (-SH) ligands and with the sulfide
4 ligands that are present in natural organic materials dissolved in water. Silver ions might also interact
5 strongly with the chloride anion (Cl^-), although the nature of that reaction differs depending on whether
6 the medium is fresh water or sea water. In general, concentrations of chloride ions are low in fresh water,
7 but silver ions could react with any chloride ions present to produce silver chloride, most of which
8 precipitates out of solution under normal conditions. Dissolved sulfides, organic materials, and chloride
9 ions will likely complex essentially all the free silver ions in fresh waters (making it unavailable for
10 uptake by organisms) and drive the free silver ions to very low levels (Luoma, 2008, [157525](#)).

11 In sea water, chloride occurs in very high concentrations. Multiple chloride ions can react with
12 each silver ion to form dense complexes that keep silver in solution. Although this silver-chloro complex
13 dominates in solution in sea water, sulfide complexes could also be present (Luoma, 2008, [157525](#)).

4.4.1.3. Particle Aggregation and Agglomeration

14 In water, the physical structure of nanoparticles can be modified, and hence the properties of the
15 particles can change (AFSSET, 2006, [201630](#)). The formation of clusters can significantly affect the
16 transport of particles in aquatic systems. Clusters of nanoparticles that settle can be expected to
17 accumulate in sediments (unless disruption of sediments [e.g., through dredging] causes re-mobilization
18 of the sediment particles). Those that do not settle can travel in the water column from the point of
19 release (Lowry and Casman, 2009, [196153](#)). Nanoparticle clusters are less bioavailable than single
20 nanoparticles (Navarro et al., 2008, [157517](#)). The formation of clusters that are trapped through
21 sedimentation affects the concentrations of nanoparticles that are bioavailable to organisms. Although
22 clustered or adsorbed nanoparticles are less mobile, they can still be taken up by sediment-dwelling
23 animals or filter feeders (see Chapter 5) (Nowack and Bucheli, 2007, [092294](#)).

4.4.1.4. Important Environmental Factors

24 As is true for nanoparticles in general, environmental factors that influence the dispersion and
25 deposition behavior of nano-Ag include salinity (ionic strength), the presence of surface coatings (i.e.,
26 engineered surface coating or natural organic matter), pH, and water hardness (the concentration of
27 competing cations, such as Ca^{2+} and Mg^{2+}). Results of a recent study by Gao et al. (2009, [195514](#)) on the
28 behavior of nano-sized silver in complex natural waters have suggested that dissolved organic carbon, pH,
29 and the concentrations of electrolytes (e.g., Ca^{2+} and Mg^{2+}) help control the formation of clusters.

1 Typical aquatic environments, including rivers, lakes, and estuaries, contain monovalent and
2 divalent salts as well as natural organic matter (Saleh et al., 2008, [597414](#)). Particles of all dimensions
3 are more likely to associate as salinity increases (Luoma, 2008, [157525](#)). Thus, nanoparticles will tend to
4 form clusters to a greater degree in salt water (which has a high ionic strength) than in fresh water (Klaine
5 et al., 2008, [193475](#); Lowry and Casman, 2009, [196153](#)). Even small increases in salinity above that of
6 fresh water (~2.5 parts per trillion [ppt]) can cause a rapid loss of colloids through aggregation and
7 precipitation processes (Stolpe and Hasselov, 2007, [524902](#)).

8 As described in Handy et al. (2008, [157562](#)), additions of salt to the medium (increasing ionic
9 strength) could provide charge shielding or compress the charge layer on the surface of the nanoparticles
10 so that particle collisions lead to attachment of particles and therefore clusters. The formation of
11 nanoparticle clusters and precipitation of nanoparticles in sea water could result in the deposition of
12 nanoparticles to sediments, perhaps with subsequent accumulation in the sediments and exposure to
13 benthic organisms (Klaine et al., 2008, [193475](#)). In general, when ionic strength of an aqueous solution
14 increases, the size of particle clusters increases, and the particles become considerably less mobile
15 (AFSSET, 2006, [201630](#)). For conventional silver, Luoma (2008) found that the formation of dense
16 silver-chloro complexes in sea water (as described above in Section 4.4.1.2), however, helps to keep silver
17 in solution. Silver, as part of this tightly bound silver-chloro complex, is more mobile and more reactive
18 than it would be in fresh water (Luoma, 2008, [157525](#)).

19 Another factor affecting the transport and distribution of nanoparticles in the aquatic environment
20 is surface coating, either that which is acquired upon release to the environment (e.g., coating by natural
21 organic matter) or a coating that is engineered onto the nanoparticles (Nowack and Bucheli, 2007,
22 [092294](#)). The interactions between nanoparticles and natural organic matter can influence nanoparticle
23 fate and transport in aquatic systems. The formation of larger nanoparticle clusters by high-molecular-
24 weight natural organic matter compounds might favor deposition of the particles into sediments, likely
25 decreasing their bioavailability. Solubilization by natural surfactants such as lower-molecular-weight
26 natural organic matter compounds, however, might increase their mobility and their bioavailability to
27 organisms (Navarro et al., 2008, [157517](#)).

28 The behavior of conventional silver in aquatic systems, which has been well studied, could also be
29 relevant to understanding the behavior of nano-Ag in these systems. Silver, with a distribution coefficient
30 (K_d) of $10^{4.5}$ – 10^6 , is known to be an extremely particle-reactive metal (Andren and Bober, 2002, [625593](#)).
31 This results in a comparatively short residence time in aquatic systems; silver is quickly scavenged from
32 the water column, ending up in sediments.

33 As a ubiquitous component of aquatic systems, natural organic matter can influence the surface
34 speciation and charge of nanoparticles, thereby affecting their mobility and their propensity to cluster or
35 deposit (Navarro et al., 2008, [157517](#)). Natural organic matter (containing negatively charged humic and
36 fulvic acids) could coat the surface of nanoparticles, resulting in particles that tend to stay dispersed rather

1 than aggregate or agglomerate (Handy et al., 2008, [157562](#)). As mentioned in Section 4.1.2.1, however,
2 in the presence of certain electrolyte solutions containing high CaCl₂, adsorbed humic acid on
3 nanoparticles leads to enhanced particle clustering (Chen and Elimelech, 2007, [597391](#)). Natural organic
4 matter can stabilize particles against forming clusters in water, which can enhance transport in aqueous
5 environments and ground water (Lowry and Casman, 2009, [196153](#)).

6 In addition to natural organic matter, artificially produced organic compounds might be used to
7 stabilize nanoparticle suspensions (Navarro et al., 2008, [157517](#)). Some nano-Ag particles are engineered
8 to disperse and remain as single particles (i.e., not aggregate), increasing the possibility of the persistence
9 and accumulation of non-associated forms in natural waters. Surface coatings can be added to improve
10 water solubility and suspension characteristics (Luoma, 2008, [157525](#)). Metallic nanoparticles are often
11 coated with inorganic or organic compounds to maintain their stability and mobility as colloidal
12 suspensions (Navarro et al., 2008, [157517](#)). The potential effect of spray ingredients co-occurring with
13 nano-Ag on the stability and mobility of suspensions is unclear.

14 The pH of water could influence the rate of nanoparticle clustering, depending on the surface
15 charge of the particles involved (Handy et al., 2008, [157562](#)). In general, the mobility of silver increases
16 under conditions of increased acidification (lowering of pH) (Luoma, 2008, [157525](#)). Water hardness
17 will alter the chemistry that controls particle aggregation and agglomeration (and ultimately ecotoxicity;
18 see Section 6.1) (Handy et al., 2008, [157562](#)). Hard water (in contrast to soft water) has a high mineral
19 content. Nanoparticle surface charge effects could be influenced by the concentrations of competing
20 cations like Ca²⁺ and Mg²⁺ that might screen off a negatively charged surface. Nanoparticle dispersion in
21 aquatic systems will likely be influenced by the free cation concentration (Handy et al., 2008, [157562](#)).

4.4.2. Wastewaters

22 The formation of particle clusters, surface charge, and surface area of nano-Ag, as well as the
23 presence of other spray ingredients and the treatment method in use, will affect removal efficiency and
24 fate of nano-Ag in wastewater (O'Brien and Cummins, 2009, [196217](#)). At a treatment facility, sorption
25 processes and chemical reactions likely would affect nanoparticles. Those nanomaterials that do not sorb
26 during the primary treatment phase could be removed via settling in the secondary clarifier, after which
27 they might become entrapped in larger sludge flocs. Although wastewater treatment plants can remove
28 much of the nano-Ag and associated free silver ions from the wastewater, some silver might survive
29 treatment, remain in the treated water, and ultimately be discharged into water bodies. Additionally,
30 nanomaterials that are removed in the wastewater treatment process could be released into soil via sewage
31 sludge, which is sometimes applied as a fertilizer to agricultural soils (Benn and Westerhoff, 2008,
32 [157595](#); Blaser et al., 2008, [193283](#)). Runoff along the surface of the ground then could transfer

1 nanoparticles in the sewage sludge to nearby terrestrial systems or waterways (Blaser et al., 2008,
2 [193283](#); O'Brien and Cummins, 2009, [196217](#)).

3 In some rural areas of the United States, formal wastewater or solid-waste collection methods
4 might not be available as they are in municipal settings. Some rural households might rely on septic
5 systems that could be compromised or otherwise ineffective or dispose of wastewater through pipes into a
6 pond or the woods. Additionally, rural environments could be exposed to illegal or unmonitored disposal
7 of manufacturing waste products (e.g., into informal landfills, on the side of the road, and into aquatic
8 systems). To the extent that such sources might exist, these exposure pathways should be considered.

4.5. Fate and Transport Models

9 Most current models are not appropriate for use in predicting nano-Ag fate and transport through
10 environmental compartments (U.S. EPA, 2010, [625619](#)). Models of dispersive and convective movement
11 of airborne particles and gases and models of fate and transport of chemicals and particles in surface
12 waters and soils, however, could be linked and adapted for predicting environmental fate and transport of
13 nano-Ag and silver ions released from those particles. The potential influence of other spray ingredients
14 or other substances used in the manufacturing on the fate and transport of nano-Ag could also be
15 incorporated into the model. Such a comprehensive model, however, has yet to be developed for nano-
16 Ag.

17 The U.S. Environmental Protection Agency (EPA) and others have used environmental models
18 widely to simulate diffusive and convective movement of aqueous-phase chemicals through
19 environmental compartments (e.g., soils, sediments, water) and partitioning of the chemicals between
20 media (e.g., between solid and aqueous phases). Examples of such models are included in the Models
21 Knowledge Base compiled by EPA's Council for Regulatory Environmental Modeling.⁸ Transport of
22 nano-Ag clusters or nano-Ag sorbed to organic particles could be simulated with particulate matter (PM)
23 transport models for surface waters. Although adapting models designed to predict fate and transport of
24 suspended solids or organic matter of small sizes (e.g., the Particle Tracking Model, developed by the
25 Army Corps of Engineers) to nanoparticle transport is possible, these models might need to be adapted to
26 include additional forces, including particle clustering, sorption to suspended particles, and possibly
27 colloidal behavior. Evaluation of such models by comparing model outputs with measured values also
28 could be challenging, given that the reliability of analytical methods to detect nanoparticles at
29 environmentally relevant concentrations (i.e., in the nanogram/liter [ng/L] range) is questionable
30 (Demirbilek et al., 2005, [193887](#); Luoma, 2008, [157525](#)). Models that can be used to specifically

⁸ <http://www.epa.gov/crem/knowledgebase/index.htm>

1 estimate the fate and transport of nano-Ag in air and soils have not been developed, but some fate and
2 transport models have been proposed for evaluating the fate and transport of nano-Ag, or silver ions
3 released from nano-Ag, in water and sediments. These models are described below. In addition,
4 Gottschalk et al. (2010, [597400](#)) recently described a probabilistic material flow model used for assessing
5 the environmental exposure to engineered nanoscale titanium dioxide (nano-TiO₂) particles. A brief
6 discussion of this modeling approach is also provided below.

7 Although empirical data on nano-Ag concentrations in the environment are lacking, a recent study
8 by Mueller and Nowack (2008, [157519](#)) used computer modeling to predict nano-Ag concentrations in
9 air, water, and soil in Switzerland based on simple assumptions and a substance flow analysis. The
10 volumes of different environmental compartments for the entire country were calculated as surface area
11 multiplied by depth for soil, surface water, and air:

- 12 • soil volume = agricultural and non-agricultural surface areas multiplied by 0.2- and 0.05-m
13 mixing depths, respectively;
- 14 • surface water volume = surface area multiplied by mixing depth of 3 m; and
- 15 • air volume estimated as volume of air within 1 km of ground level across the country.

16 Homogenous and complete mixing within each medium was assumed. Predicted environmental
17 concentrations (PEC) were calculated for “realistic exposure scenarios” (based on nano-Ag use
18 worldwide, estimated as 500 tons per year) and for “high exposure scenarios” (based on 1,230 tons nano-
19 Ag per year, or 5% of the world-wide extraction of 25,620 tons of silver that is not used in jewelry,
20 photography, or industry). Allocation of world-wide nano-Ag use to Switzerland was based on the
21 country’s share of the total population of industrialized countries (i.e., 0.0068). The investigators
22 estimated that more than 15% of nano-Ag is used in sprays and cleaning agents, and that most (85%) of
23 that nano-Ag is discharged into wastewater from wastewater treatment plants. Of the remaining 15%,
24 approximately 5% is discharged to air, 5% is discharged to soils, and 5% is disposed of in waste
25 incineration plants. For nano-Ag discharged in wastewaters, the investigators further assumed that 97%
26 of nanoparticles are removed in packed-bed filters and that an average 97–99% of suspended particles are
27 removed during treatment. Finally, overflow wastewater discharge during storm events was assumed to
28 be 5–10% of total wastewaters.

29 Blaser et al. (2008, [193283](#)) modeled fate and transport of silver ions, instead of nano-Ag particles,
30 in the Rhine River to assess potential risks from European use and disposal of plastic and textile
31 consumer products containing nano-Ag. They assessed the likely fate of silver ions released to municipal
32 wastewaters from washing and wearing textiles spun with nano-Ag and contact with water of plastics
33 coated or impregnated with nano-Ag. Their model estimated silver ion concentrations in the water
34 column and in the top layer of sediments for three different scenarios (“minimum,” “intermediate,” and
35 “maximum” emission scenarios). Silver ion releases were estimated from silver content in biocidal

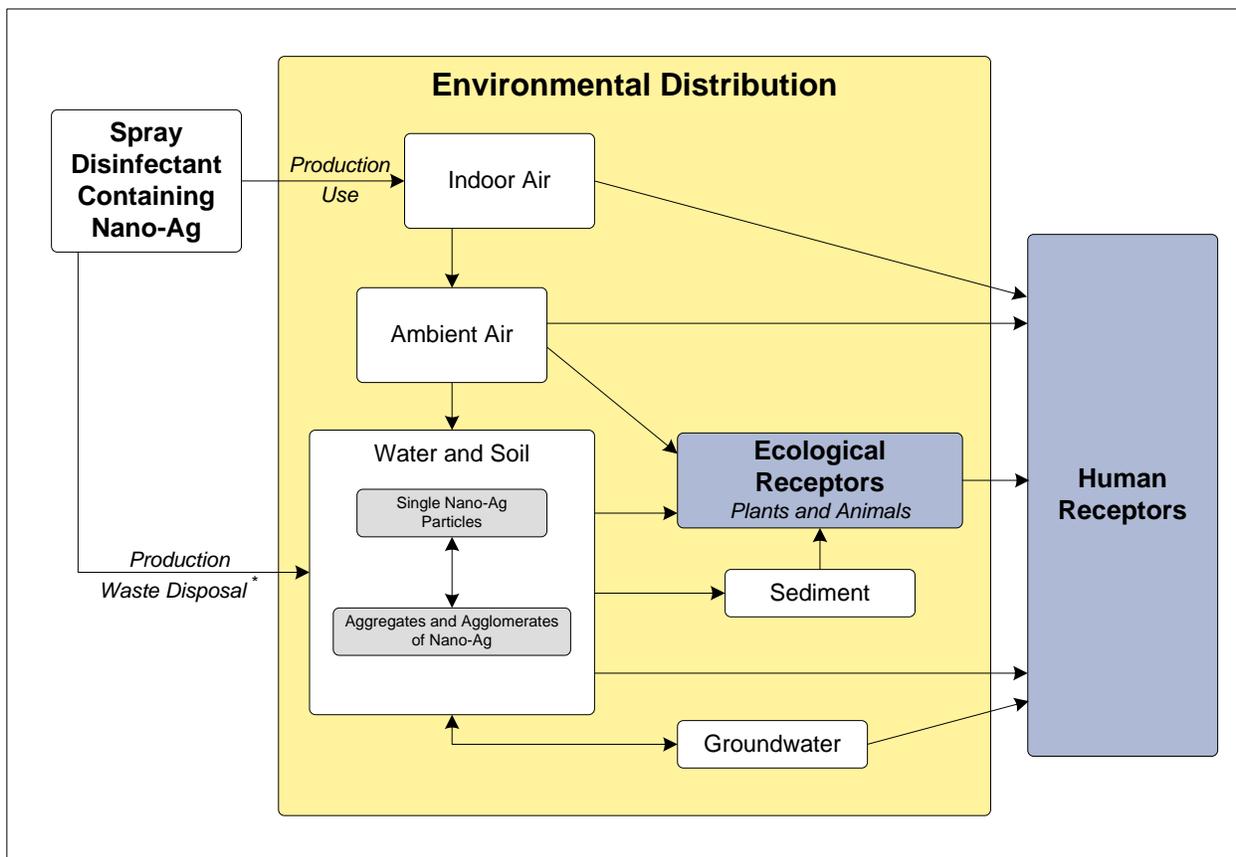
1 plastics and textiles. The fraction of wastewater treated was assumed to be 80–90% and the fraction of
2 silver removed by filtration and treatment was assumed to be 85–99%. Data on release rates of silver ions
3 from different types of plastics embedded with nano-Ag and from products with surface applications of
4 nano-Ag are sparse, and measured rates vary substantially among different formulations.

5 The model, which simulates silver ion fate and transport processes in river waters and sediments,
6 estimated PEC ranges of 4–40, 10–140, and 30–320 ng/L in river water for the “minimum,”
7 “intermediate,” and “maximum” emission scenarios, respectively. Predicted silver ion concentrations
8 increase in both the water column and sediments downstream as the river flows through populated areas
9 with wastewater treatment facilities (Blaser et al., 2008, [193283](#)). In the top layer of the sediment, the
10 PEC ranges for the scenarios were 0.04–2, 0.1–6, and 0.3–14 milligrams per kilogram (mg/kg),
11 respectively. In the interstitial waters of the sediments, calculated PECs for the scenarios were 9, 30, and
12 70 ng/L, respectively. The investigators reported that the PECs calculated for the river water were
13 generally consistent with the range of empirical data available for silver concentrations in river waters
14 (>0.01–148 ng/L). The sediment PECs, however, were generally higher than the range of measured data
15 for river sediments (0.2–2 mg/kg), but they were well below the value of 150 mg/kg reported for
16 measurements taken in heavily affected river beds. The proportion of the silver ions that is likely to be
17 bioavailable, however, depends on the availability of organic and inorganic sulfides and other materials in
18 the river to bind silver ions, as discussed in Section 4.4.1.4 and in Chapter 5.

19 Gottschalk et al. (2010, [597400](#)) developed a probabilistic material flow analysis (PMFA)
20 framework to derive probability distributions of PECs for engineered nano-TiO₂ particles in Switzerland
21 primarily using the data of Mueller and Nowack (2008, [157519](#)). The PMFA calculated, from a whole
22 life-cycle perspective and taking into account uncertainty and variability in model inputs, concentrations
23 of nano-TiO₂ in all “natural” environmental compartments (atmosphere, soil, surface water, sediment, and
24 groundwater) and “technical” environmental compartments (production, manufacturing, use, recycling,
25 and disposal in waste incineration and sewage treatment plants). The same assumptions of a single
26 compartment for all air in Switzerland, all water, and other simplifying assumptions described in the
27 previous paragraph applied. Although the authors believe that this PMFA is applicable to predict
28 concentrations of compounds in the environment when little information is available concerning
29 environmental fate and exposure characteristics, the added value of their approach over that of Mueller
30 and Nowack (2008, [157519](#)) has yet to be evaluated by other researchers. Some of the principal
31 assumptions included in the PMFA (e.g., homogenous mixing of material across all surface waters in
32 Switzerland) were not actually built into the Monte Carlo simulation and sensitivity analyses. Some
33 results (e.g., total mass, mass flux) estimated by the PMFA are in the form of ranges extending more than
34 two orders of magnitude for several environmental compartments and pathways. As also noted by the
35 authors, additional empirical data are still required to generate useful model input distributions.

4.6. Summary of Nano-Ag Fate and Transport in Environmental Media

1 The important potential pathways of nano-Ag and by-products into the environment associated
 2 with the production (inclusive of manufacturing, distribution, and storage), use, and disposal of spray
 3 disinfectants containing nano-Ag are summarized in Figure 4-1. Nano-Ag can be released into air, water,
 4 and soil at various stages of the life cycle. Within these media, nano-Ag can be transported and
 5 transformed and spatially distributed in the environment. Ultimately, ecological or human receptors could
 6 be exposed to nano-Ag and associated contaminants.



Source: Adapted from Nowack and Bucheli (2007, [092294](#)).

Figure 4-1. Potential nano-Ag pathways into the environment associated with production, use, and disposal of spray disinfectants containing nano-Ag.

* Waste disposal includes products containing nano-Ag that might wash down a sink or bathtub drain or be discharged from the washing machine into wastewater treatment plants, land-filled sewage sludge, or sewage sludge used as a fertilizer on agricultural fields. This nano-Ag could then migrate into water or soil media and be distributed throughout various environmental compartments.

1 One of the primary pathways for release of nano-Ag in spray disinfectants could occur through
2 indoor use, where it could be sprayed into the air and onto a surface. Transport of nano-Ag from the
3 indoor environment to the outdoor environment, where it could partition into the ambient air, water, and
4 soil, is then possible. Release of nano-Ag in spray disinfectants might also occur during production or as
5 a result of waste disposal. For example, a product containing nano-Ag that is wiped up with a paper
6 towel and then discarded in the trash could end up in a landfill, with subsequent leaching into subsoil and
7 ground water and possible migration to surface water. A product containing nano-Ag that is washed down
8 a sink or bathtub drain might enter into wastewater treatment plants, and treated water containing nano-
9 Ag subsequently could be released into water bodies. Other spray ingredients and substances involved in
10 the manufacture of nano-Ag sprays could co-occur with nano-Ag in the environment and potentially
11 modify its fate and transport behavior, although information regarding this possibility was not identified
12 during development of this case study.

13 Either a model focused on the movement of airborne particles and gases or one designed to predict
14 the fate and transport of chemicals and particles in surface waters and soils (or a combination of these two
15 model types) could potentially serve as a basis for developing a comprehensive model for predicting
16 environmental fate and transport of nano-Ag and the associated release of silver ions. Such a
17 comprehensive model, however, has yet to be developed for nano-Ag.

18 Questions reflecting data gaps in the information about fate and transport are listed on the
19 following page. They are listed in approximate order of the presentation of information in this chapter.
20 The order in no way, however, is intended to reflect the relative importance of the questions.

Questions about Fate and Transport in Environmental Media

- 4.1. Do the properties of nano-Ag that differ from those of well-characterized colloidal silver, if any, cause them to behave differently in aquatic, terrestrial, and atmospheric environmental compartments?
 - 4.1.a. If they do differ, how do they differ?
 - 4.1.b. Can information about how colloidal silver behaves in these environments be used to understand how nano-Ag behaves?
- 4.2. Does particle size of nano-Ag affect the rate of release of silver ions in environmental compartments?
- 4.3. Does the aggregation state, aggregate size, or aggregate density of nano-Ag affect the rate of release of silver ions in environmental compartments?
- 4.4. Which physicochemical properties of nano-Ag and nano-Ag coatings can best be used to predict its fate and transport in different environmental media?
- 4.5. Is nano-Ag as environmentally persistent as conventional silver?
- 4.6. Does nano-Ag form the same strong complexes with anions as conventional silver, and if so, is it also effectively immobilized in aquatic environments?
- 4.7. How does nano-Ag partition among soil, water, sediment, and air, and what are the key parameters determining this partitioning behavior?
- 4.8. Which environmental factors significantly affect the behavior of nano-Ag in aquatic and terrestrial ecosystems, and by what mechanisms do they impart these effects?
- 4.9. What are the characteristics of nano-Ag surface coatings that affect the transport behavior of nano-Ag within and between environmental compartments, and how is the transport affected?
- 4.10. How effectively is nano-Ag removed from sewage and industrial process water by wastewater treatment technology, and can information on the removal of conventional silver be applied to nano-Ag removal?
- 4.11. To what extent does nano-Ag bind to wastewater sludge and settle out or remain with treated water and enter the downstream aquatic environment?
- 4.12. How could existing models applicable to conventional silver be used to adequately predict the transport and fate of nano-Ag through environmental compartments, or how could they be modified to do so?
- 4.13. What role, if any, does temperature play in the behavior of nanoparticles?

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Chapter 5. Exposure, Uptake, and Dose

1 This chapter examines the potential for biota and humans to be exposed to primary and secondary
2 contaminants associated with nano-Ag in a disinfectant spray product. As described in preceding
3 chapters, nano-Ag and associated materials (e.g., feedstock and manufacturing waste by-products, co-
4 product ingredients) constitute primary contaminants that might be released to different environmental
5 media at one or more stages during the life cycle of the material. Once released, nano-Ag and associated
6 materials could undergo physicochemical and biological transformation processes that might result in
7 exposure of humans and biota to various secondary contaminants. From a comprehensive environmental
8 assessment standpoint, all of these primary and secondary contaminants are of potential relevance. At
9 present, however, attention is directed first at nano-Ag as the primary contaminant of interest.

10 Throughout this chapter, the term nano-Ag is used to refer in general to any type or formulation of
11 engineered silver nanomaterials and might encompass a variety of physical and chemical properties. As
12 noted in Chapter 1, there is no clear demarcation between exposure-dose and effects. Overlap of some
13 information in Chapter 5 relating to exposure and uptake with information in Chapter 6 on effects is
14 unavoidable. To the extent possible, discussion of studies cited in both chapters is limited in Chapter 5 to
15 discussion of exposure-dose and in Chapter 6 to discussion of effects.

16 Exposure to a substance requires contact between the substance and the surface of an organism via
17 one or more environmental media (i.e., water, air, soil). For internal exposure to occur, the substance
18 must penetrate the organism's cell walls, cell membranes, or other barriers between the organism and its
19 environment; that is, the substance must be bioavailable to the organism. Transfer of a substance from
20 any of these environmental media and across exchange boundaries results in an internal dose that is
21 distributed by the circulatory system to organs.

22 Several terms are used throughout this chapter in describing the characterization of exposure-dose
23 (U.S. EPA, 1992, [090324](#); U.S. EPA, 2005, [086237](#)). *Exposure* is contact of an agent with the outer
24 boundary of an organism. *Exposure concentration* is the concentration of a substance in its transport or
25 carrier medium at the point of contact. *Dose* is the amount of a substance available for interaction with
26 metabolic processes or biologically significant receptors after crossing the outer boundary of an organism.
27 *Applied dose* is the amount of a substance presented to an absorption barrier and available for absorption
28 (although not necessarily having yet crossed the outer boundary of the organism). In non-experimental
29 settings, *potential dose*, a more general term, is the amount ingested, inhaled, in contact with the skin,
30 flowing past gills, or in contact with other exchange boundaries (e.g., surface of plant roots). *Absorbed*
31 *dose* is the amount crossing a specific absorption barrier (e.g., gills, digestive tract) through one or more

1 uptake processes. *Internal dose* is a more general term, representing all of the substance that has been
2 absorbed by one or more exchange boundaries. Finally, *delivered dose* is the amount of a substance
3 available for interaction with a particular organ, tissue, or cell. The portal of entry determines the extent
4 to which the substance might be transformed (e.g., by the liver following ingestion) prior to reaching the
5 general circulation. Where the exchange boundary (e.g., gills) is the site of toxic action, potential dose
6 might equal delivered dose for that boundary.

7 Toxic effects depend not only on delivered dose, but also on the timing and patterns of exposure to
8 the substance in environmental media, bioavailability of the substance from a specific medium to
9 particular organisms through their uptake processes, fate of the substance in the organism, and the
10 sensitivity of the organism to the substance. The disposition of the substances within the organism
11 includes its metabolism (possibly to more toxic entities), distribution, storage, and excretion. The
12 behavior of organisms in their environment can play a key role in their exposure profiles. For example,
13 exposure scenarios for children can differ drastically from those for adults because they spend more time
14 in contact with floor surfaces and they mouth a variety of objects that adults would not. Other
15 characteristics of an organism (e.g., age, reproductive status, size, health status, individual exposure to
16 other agents) can modify its sensitivity to a toxic substance, that is, its response to a given exposure, as
17 discussed in Chapter 6.

18 Because limited information is available on releases of nano-Ag during manufacturing, storage,
19 use, and disposal of products such as spray disinfectants, quantitative, data-driven estimates of potential
20 geographic extent of releases and media concentrations of nano-Ag and associated contaminants are not
21 yet possible. Thus, identifying the most likely significant exposure pathways and routes of intake to
22 humans or biota currently relies on conceptual models. Shatkin (2008, [180065](#)) presents the case that a
23 risk assessor can “step through” the life cycle of a specific product, identifying points in the
24 manufacturing, storage, distribution, and application of a new product that might result in releases to the
25 environment and exposures of humans and biota. Thus, a risk assessor can deduce from limited product
26 information, and from general manufacturing and distribution practices for similar products, some likely
27 release points in the life cycle of a new product and the types of environments and media to which the
28 releases might occur.

29 Nano-Ag can be released to the environment at various points in the life cycle of a disinfectant
30 spray, as described in Chapter 3, and many of these release scenarios could be similar to those for nano-
31 Ag contained in other end-use products such as fabrics. Occupational exposures of humans and releases
32 of silver into the environment are possible both during the creation of nano-Ag particles from silver
33 feedstocks and the manufacturing of disinfectant spray products that incorporate nano-Ag. Some of the
34 most likely and significant exposure scenarios can be deduced for humans and biota by using the potential

1 release scenarios described in Chapter 3 and existing knowledge of the characteristics and behavior of
2 nano-Ag and associated substances (e.g., silver ions, transformation products, waste products, by-
3 products) in the environment.

4 This chapter characterizes current knowledge regarding measured exposures to nano-Ag (i.e.,
5 contact between an organism and nano-Ag) and uptake and internal dose (i.e., absorption of nano-Ag by
6 organisms). Exposure and dose data are presented first for biota in Sections 5.1 and 5.2, respectively.
7 Exposure data then are presented for humans in Sections 5.3. General discussions of the potential for
8 aggregate and cumulative exposures involving nano-Ag are presented in Sections 5.4 and 5.5,
9 respectively. Aggregate exposure to nano-Ag from spray products and from other products and sources
10 determines the total potential and internal doses of nano-Ag, as discussed in Section 5.4. Cumulative
11 exposures to multiple substances and other types of nanoparticles along with nano-Ag are examined in
12 Section 5.5. Because limited product formulation data are available, the evidence for co-exposure to
13 other spray constituents is not evaluated in detail, although such an evaluation could be relevant in a
14 comprehensive assessment. Section 5.6 includes a brief discussion of exposure models. Human uptake
15 and dose from exposure to nano-Ag are discussed in Section 5.7.

16 Measurements of existing environmental concentrations of nano-Ag, data on its persistence in the
17 environment, and methods for predicting future concentrations could be useful when assessing current
18 and future exposures of humans and ecosystems from nano-Ag in disinfectant sprays and other consumer
19 products. Also, information on how exposure to nano-Ag particles translates into internal dose of either
20 nano-Ag particles or silver ions, or both, could be relevant to understanding possible modes of action ⁹ of
21 nano-Ag in different groups of organisms. Effects of nano-Ag in the environment (which are discussed in
22 Chapter 6) can occur at exchange barriers between organisms and environmental exposure media – often
23 called portal-of-entry effects – or internal to the organism. Systemic effects depend on the bioavailability
24 of the nano-Ag or release of silver ions from the particles, either or both being absorbed and delivered to
25 target organs or tissues (Lowry and Casman, 2009, [196153](#)). Chronic effects depend, moreover, on the
26 ability of organisms to detoxify or excrete nano-Ag or silver ions derived from the particles. Silver,
27 conventional or nano-sized, that is not excreted can accumulate in an organism (Berthet et al., 1992,
28 [098717](#)), and the silver in organisms can be passed up the food chain (Bianchini and Wood, 2008,
29 [195485](#)). Consequently, the concepts of bioavailability, bioconcentration, and bioaccumulation are
30 fundamental to this discussion of uptake and dose, especially with regard to uptake by biota in ecological
31 food webs. Information on these three concepts, with an emphasis on how they pertain to uptake of

⁹ “Mode of action” is defined and discussed in greater detail in Section 6.2, footnote 8.

1 nano-Ag by biota, is presented at the beginning of Section 5.2.1. This discussion focuses on application
2 of these terms specifically to nanomaterials.

5.1. Biotic Exposure

3 As discussed in Chapter 3 of this document, it is reasonable to infer that, based on likely uses of
4 nano-Ag in spray disinfectant products, two potential types of releases to the environment are down the
5 drain to wastewater treatment plants (with subsequent release of treated water to surface waters) and land
6 application of sewage sludge in agricultural areas, which are similar to releases from nanofunctionalized
7 textiles, as described by Blaser et al. (2008, [193283](#)). Process wastewater containing nano-Ag from
8 manufacturing facilities could also reach wastewater treatment plants and result in nano-Ag entering the
9 environment. Thus, exposure of aquatic organisms via the water column and sediments in riverine
10 through coastal environments is possible. Additional exposure of aquatic animals to silver accumulated in
11 their food could also occur. Luoma (2008, [157525](#)) hypothesizes that concentrations of nano-Ag in
12 aquatic systems will likely be within the same orders of magnitude as conventional silver concentrations
13 during times of historic elevated releases. Luoma expects nano-Ag levels in the South San Francisco Bay,
14 for example, to reach concentrations of 26–189 nanograms per liter (ng/L), as previously observed during
15 the 1980s when discharges of conventional silver to the environment were occurring at a rate of
16 approximately 550 kilograms (kg) per year (Smith and Flegal, 1993, [597415](#)). Furthermore, Luoma
17 argues that nano-Ag discharges at that rate might increase concentrations in the sediment of the San
18 Francisco Bay by more than an order of magnitude from the 2007 baseline, resulting in concentrations of
19 approximately 3 parts per million (ppm). Blaser et al. (2008, [193283](#)) estimated similar concentrations
20 for the Rhine River following anticipated increases in nano-Ag releases. Note, however, that these
21 estimated concentrations represent releases of nano-Ag from all sources, not from spray disinfectants
22 alone. Theoretical projections such as these are the only estimates currently available of potential
23 environmental exposures of nano-Ag because monitoring technology for measuring nanoparticle
24 concentrations in the nanogram per liter (ng/L) range is still under development. It is also not yet possible
25 to estimate the relative contribution of nano-Ag from spray disinfectants to total nano-Ag exposures from
26 all sources.

27 The probable use profile of nano-Ag disinfectant sprays indicates that releases of nano-Ag to the
28 terrestrial environment are not expected to be as great as those to the aquatic environment. As discussed
29 in Chapter 3, however, soil biota and agricultural crops could be exposed where sewage sludge is used to
30 amend soils. Landfilling of sludge or solid waste containing nano-Ag, either from manufacturing
31 processes or from disposal of unused product and cleaning materials, also could result in exposure of
32 terrestrial organisms or leaching of nano-Ag into groundwater. Exposures of reptiles, birds, and

1 mammals could occur through incidental soil ingestion in agricultural areas, but the quantities of ingested
2 nano-Ag are expected to be small and the sensitivity to ingested silver in these organisms is expected to
3 be low (as for humans). Exposures of herbivores to nano-Ag accumulated in plants might occur, as well
4 as exposures of insectivores to nano-Ag accumulated in their prey. Airborne nano-Ag in outdoor
5 environments based on indoor uses is expected to be insignificant; outdoor uses, however, might result in
6 inhalation exposure of animals and exposure of plants through their foliage.

7 It is possible that localized accidental releases or spills can create “hot spots” of nano-Ag
8 contamination. Accidental releases to terrestrial or aquatic environments might occur at bulk material
9 storage facilities or during transport of nano-Ag from one facility to another during the production,
10 manufacture, and distribution of products containing nano-Ag (see Chapter 3). Empirical data or
11 modeling results appear to be lacking regarding accidental releases of nano-Ag throughout the life cycle
12 of nano-Ag sprays and other products.

5.2. Biotic Uptake and Dose

13 Investigations of the absorption, distribution, metabolism, and excretion (ADME), as well as
14 pharmacokinetics, of nanoparticles in general and of nano-Ag in particular have not yet been conducted
15 on species from the major groups of organisms (e.g., algae, macrophytes, higher plants, annelids,
16 echinoderms, arthropods, mollusks, amphibians, reptiles, birds). Some data exist on aquatic species,
17 primarily fish (Handy et al., 2008, [157563](#)). In the context of the current case study, the uptake of
18 nano-Ag in the environment by different groups of organisms would depend on the likelihood of exposure
19 via different pathways leading from the production, storage, use, and disposal of consumer products
20 containing nano-Ag.

21 Few techniques have been developed that can accurately quantify dosimetry for nano-Ag, and
22 therefore differentiating between exposure and uptake in biota is difficult. The primary dose metric used
23 for ecological effects studies is an exposure concentration expressed in terms of mass. Depending on the
24 study, the term “mass” could correspond to the mass of silver in the nanoparticles added, mass of total
25 silver in solution, mass of silver ions, or mass of free silver in solution, although the form is not always
26 specified. Some studies have attempted to normalize the dose across various types of nanoparticles by
27 expressing concentration in terms of moles. Dose-dependent effects have been observed in multiple
28 organisms at various time scales as detailed in Appendix B, but the mechanisms that allow the penetration
29 of nano-Ag into the membranes and interiors of single cells or into the tissues of biota are not yet well
30 understood. The nano-Ag rate of uptake, nano-Ag tissue or whole-body concentrations, and the fate of
31 the nanoparticle at the cell, tissue, and organism level are not currently known (Luoma, 2008, [157525](#)).

1 The discussion of biotic uptake and dose of nano-Ag is divided into four major sections. The first
2 section provides general descriptions of bioavailability, bioconcentration, and bioaccumulation of
3 nano-Ag and conventional silver in biota (Section 5.2.1) as the terms apply to the remainder of Section
4 5.2. The subsequent three sections focus on specific aspects of the uptake of nano-Ag, silver ions, and
5 conventional silver by bacteria and fungi (Section 5.2.2); uptake of nano-Ag, silver ions, and conventional
6 silver by biota in aquatic ecosystems (Section 5.2.3); and uptake of nano-Ag, silver ions, and
7 conventional silver by terrestrial biota in agricultural settings (Section 5.2.4). Throughout, nano-Ag is
8 distinguished from silver ions and from other forms of silver to the extent possible. In addition, dose is
9 addressed, wherever possible, by reporting absorption of nano-Ag or silver ions through the surface of an
10 organism to its interior as distinguished from adsorption of nano-Ag or silver ions onto exterior surfaces
11 (e.g., gills, gastrointestinal [GI] tract).

5.2.1. Bioavailability, Bioconcentration, and Bioaccumulation

12 Chemicals and substances that cannot be biodegraded beyond inorganic compounds can ultimately
13 persist and in some cases accumulate in both environmental media and biota. Bioavailability is defined as
14 the availability of a substance in an environmental medium for absorption by an organism in contact with
15 that medium (e.g., absorption of a substance inhaled with air or ingested with food). Bioconcentration
16 refers to the direct uptake and accumulation of a substance from an external medium (e.g., from water
17 through gills for aquatic organisms), while bioaccumulation occurs from both direct uptake from an
18 external medium *and* ingestion of a substance. Both bioconcentration and bioaccumulation require
19 “bioavailability” of some fraction of the substance in environmental media. Bioconcentration of an agent
20 can occur across cell walls (e.g., plants) or across specialized exchange surfaces (e.g., gills of aquatic
21 invertebrates or fish), whereas bioaccumulation applies to animals only (U.S. EPA, 2003, [201636](#)). In
22 natural environments, the ratio of the chemical concentration in an animal to the chemical concentration
23 in its environment generally is referred to as a bioaccumulation factor. Bioavailability is a prerequisite for
24 toxicity (Luoma, 2008, [157525](#)). Bioavailability, therefore, often is assessed indirectly by evaluating the
25 toxicity of an agent to organisms under specified conditions (Berthet et al., 1992, [098717](#)).

26 Bioavailability of nano-Ag is likely to be a function of its form, characteristics of the
27 environmental exposure medium, and characteristics of the organism for several reasons:

- 28 1. Properties of the nano-Ag such as size, potential to form clusters, or surface properties and
29 coatings can influence the uptake of the nano-Ag or facilitate or preclude its binding to other
30 particles that might or might not be available for uptake.
- 31 2. Characteristics of the environmental medium (e.g., surface water pH, temperature, presence of
32 calcium carbonate, sulfates, other salts, dissolved and particulate natural and other organic
33 materials) can modify the bioavailability of nano-Ag through binding of nano-Ag or silver ions
34 in a nonbioavailable form.

1 3. Characteristics of the organism and its route(s) of intake (e.g., acidic environment of the GI
2 tract, fish gill active transport of silver ions through ionic sodium [Na⁺] uptake channels) can
3 variably affect bioavailability and biocompatibility of nano-Ag.

4 Processes that readily transport nanoparticles from one environmental medium to another reduce
5 bioavailability in the first medium. For example, when nano-Ag is released to aquatic systems, many
6 agents in natural waters can bind to nano-Ag and silver ions and result in precipitation or sedimentation of
7 the silver (see Section 4.4.1). Silver that has precipitated out of solution is no longer directly bioavailable
8 to organisms in the water column; depending on its form, however, it might be more available to
9 organisms in the sediment.

10 Chemicals that sorb to the external surface of organisms, including the epithelial lining of the GI
11 tract in animals, but cannot penetrate the outer layer (e.g., epidermis, cell wall) because of large size,
12 surface charge, or other properties are generally not considered to be bioavailable. Nanoparticles sorbed
13 to the external surfaces of organisms, however, can in some cases damage cell walls or cell membranes or
14 provide a steady release of ions that affect the organisms' performance (e.g., survivorship, growth,
15 reproduction). Different coatings and surface properties of nano-Ag products might enhance or inhibit
16 sorption to gill surfaces or uptake by the GI tract. A nano-Ag particle that associates with and disrupts
17 essential cell processes at the external membrane, or delivers silver ions that do so, therefore, *is*
18 considered bioavailable for purposes of this document. This approach is consistent with the convention
19 used by Luoma (2008, [157525](#)).

20 Attributes of organisms that help to determine bioavailability of silver ions and nano-Ag to them
21 are discussed in Section 5.2.1.1. Environmental factors that can modify bioavailability of silver ions and
22 nano-Ag are discussed in Section 5.2.1.2. Use of bioaccumulation models is discussed briefly in Section
23 5.2.1.3. Potential for bioaccumulation of nano-Ag or silver ions from nano-Ag is examined for aquatic
24 and terrestrial food webs as part of the broader discussion about uptake in aquatic and terrestrial
25 organisms in Sections 5.2.3 and 5.2.4, respectively.

5.2.1.1. Attributes of an Organism that Influence Bioavailability of Silver Ions and Nano-Ag

26 The type of organism, its structure, and its physiology are key determinants of the mechanisms by
27 which it might be able or tend to adsorb silver ions and nano-Ag from the environment. Uptake of silver
28 ions and nano-Ag from the environment can depend on whether the organism is a prokaryote or
29 eukaryote, its cell size, cell wall or membrane construction, type of circulatory system, respiratory
30 physiology, and other major aspects of its body plan and physiology. At this time, however, data
31 concerning the influence of basic phylogenetic attributes on absorption or adsorption of nano-Ag are
32 lacking for most groups (Choi et al., 2009, [193317](#); Handy et al., 2008, [157562](#)). Sections 5.2.2 through
33 5.2.4 examine potential uptake routes for silver ions and nano-Ag for different groups of organisms,

1 describing evidence for the degree of penetration into epithelial and systemic cells and extracellular
2 matrices.

3 The degree to which an organism absorbs silver ions from its environment is likely to depend on
4 many aspects of its condition, possibly including its life stage, reproductive status, existing body burden
5 of silver, osmotic status, and nutritional and overall health condition. These conditions also might affect
6 uptake of nano-Ag. An animal's behavior (e.g., filter feeding) might also influence the amount of
7 nano-Ag to which it is exposed in the environment. Note that if nano-Ag particles adhere to an
8 organism's external surfaces, and if the particles release silver ions, the concentration of silver ions in the
9 immediate vicinity of the organism would be expected to be higher than concentrations of silver ions in
10 the surrounding medium. Measurement of silver ion concentrations in a micro-thin, "unstirred" layer of
11 water or viscous medium (e.g., mucus) immediately surrounding the organism without mixing, and
12 thereby contaminating, that layer with the surrounding medium would be technically challenging.

5.2.1.2. Attributes of the Environment Influencing Bioavailability of Silver Ions and Nano-Ag

13 As described in Section 4.1.2, several characteristics of the environment can affect the fate and
14 transport of nanoparticles in water and in soils. These characteristics are likely to influence the amount of
15 nano-Ag and silver ions that organisms come in contact with in different exposure media. Characteristics
16 of the environment that influence the bioavailability of nanoparticles in general and nano-Ag specifically
17 differ for aquatic and terrestrial systems.

18 For aquatic systems and metallic nanoparticles, distinguishing surface waters by their salinity (e.g.,
19 freshwater, estuarine, marine), hardness, organic matter content, and pH (Section 4.4.1.4) is important.
20 Processes that can remove nano-Ag from suspension and silver ions from solution are noted below (Gao
21 et al., 2009, [195514](#); Luoma, 2008, [157525](#); Wijnhoven et al., 2009, [180201](#)):

- 22 • Aggregation (referred to in this case study as the formation of clusters) of nano-Ag particles
23 with each other into larger particles that settle out of suspension;
- 24 • Complexing of nano-Ag with natural organic matter (NOM), which might create larger particles
25 that settle out of the water column; and
- 26 • Complexing of nano-Ag or silver ions with inorganic materials, forming insoluble precipitates.

27 These processes deposit the silver to the sediments where, depending on the particles and sediment
28 chemistry, the nano-Ag and silver ions might be more or less bioavailable to benthic organisms.

29 To date, essentially no data have been published that indicate the fate of nano-Ag particles released
30 to surface waters (Wijnhoven et al., 2009, [180201](#)). Data are available, however, concerning the
31 bioavailability of silver ions introduced to surface water in the form of the highly soluble silver nitrate
32 (AgNO_3). Therefore, factors that affect the bioavailability of silver ions from AgNO_3 released to surface

1 waters are discussed first. Factors that might influence the bioavailability of nano-Ag particles are
2 considered second.

Environmental Factors that Affect Bioavailability of Silver Ions

3 Many ligands exist for silver ions in natural surface waters (see Section 4.4.1.2). Silver-
4 complexing agents include inorganic ligands (e.g., chloride [Cl⁻], bicarbonate, thiosulfate), simple organic
5 ligands (e.g., amino acids, ethylenediaminetetraacetic acid [EDTA]), and complex polydispersed organic
6 ligands such as humic and fulvic acids (Bianchini and Wood, 2008, [195485](#)).

7 In natural freshwaters, concentrations of free silver ions are likely to be very low (Luoma, 2008,
8 [157525](#)) (see Table 4.1 in Chapter 4, which lists the solubility products of common silver compounds in
9 water). Silver ions bind very strongly to reduced sulfur in natural waters, and sulfide (S₂⁻) concentrations
10 typically exceed silver concentrations in the environment by several orders of magnitude (Blaser et al.,
11 2008, [193283](#)). The general assumption has been that ligands present in the water will reduce
12 bioavailability (and toxicity) of metals to aquatic biota by reducing the free metal ion concentrations
13 (Bianchini and Wood, 2008, [195485](#)). Blaser et al. (2008, [193283](#)) concluded that in freshwater systems,
14 silver is expected to be bound to S₂⁻ either in the form of colloidal Ag₂S clusters or as an Ag₂S surface
15 complex on organic matter. Ag₂S is a relatively stable complex that is insoluble in water (Blaser et al.,
16 2008, [193283](#)).

17 In wastewater treatment plants, silver ions are easily removed because of their strong sorption to
18 suspended particles. Most silver ions that might reach surface waters should rapidly bind to ligands,
19 settle out of the water column, and become incorporated into the sediments, although repeated
20 resuspension back into the water column during scouring by storm events and by bioturbation (animal-
21 sediment interactions) is possible (Blaser et al., 2008, [193283](#)).

22 Toxicity tests with aquatic organisms confirm that bioavailability of silver ions is reduced in the
23 presence of excess sulfides. Reactive S₂⁻, as found in zinc sulfide (ZnS) clusters for example, reduces the
24 acute toxicity of silver ions to both daphnids (Bianchini et al., 2002, [195482](#)) and rainbow trout
25 (*Oncorhynchus mykiss*) (Mann et al., 2004, [202679](#)). With a ratio of sulfide to total silver of 250 to 20,
26 Bianchini et al. (2002, [195482](#)) observed no toxicity in *Daphnia magna* neonates after 48 hours at a silver
27 concentration as high as 2.1 micrograms per liter (µg/L) (or 19 nanomoles per liter [nM] × 108 grams per
28 mole [g/mol] of silver), while in the absence (less than 5 nM) of sulfide, the 48-hour LC₅₀ (the
29 concentration at which 50% of the organisms die) for neonate *D. magna* was estimated to be 0.18 to
30 0.26 µg/L. The daphnids accumulated more total silver in the presence of sulfide than in its absence
31 (Bianchini et al., 2002, [195482](#)) owing to accumulation of unabsorbed sulfide-bound conventional silver
32 on the gills (on thoracic appendages) and in the digestive tract, but the sulfide ligand appeared to shield
33 the organism from toxicity (Bianchini et al., 2005, [195483](#)).

1 In freshwaters, Cl^- can complex with silver ions to form silver chloride (AgCl), reducing the
2 concentration of silver ions, as demonstrated in several studies reported by Bielmyer et al. (2008,
3 [195490](#)). Some investigators have found that “circumneutral” AgCl (i.e., at a pH favoring the neutral
4 complex instead of ionic disassociation) nonetheless can passively enter and accumulate in rainbow trout
5 (Hogstrand and Wood, 1996, [196215](#); Wood et al., 2002, [098735](#)).

Environmental Factors that Affect Bioavailability of Nano-Ag Particles

6 Many environmental factors could affect bioavailability of nano-Ag particles in aquatic ecosystems
7 (see Section 4.4.1.4), including surface water chemistry (e.g., ionic strength, pH, dissolved materials) and
8 suspended solids. Together with physicochemical properties of particles (e.g., size, shape, surface area),
9 environmental factors influence dissolution potential, aggregation potential, resulting particle surface
10 properties, and interactions with dissolved and particulate organic matter in surface water (Boxall et al.,
11 2007, [157712](#)). Also, the capacity of wastewater treatment plants to remove nano-Ag from sewage (and
12 to “dispose” of it in sewage sludge) influences the overall amount of nano-Ag likely to reach surface
13 waters.

14 Uncoated metallic nanoparticles released to surface waters tend to become coated with NOM
15 quickly (Boxall et al., 2007, [157712](#); Gao et al., 2009, [195514](#); Navarro et al., 2008, [157517](#)). In aquatic
16 ecosystems, the organic matter usually originates from one or more of the following sources (Navarro et
17 al., 2008, [157517](#)):

- 18 • Fulvic compounds from humic substances, primarily from decomposition of plant materials
19 from terrestrial sources;
- 20 • Rigid biopolymers including polysaccharides and peptidoglycans produced by phytoplankton or
21 bacteria; and
- 22 • Flexible biopolymer recombination of decomposed organic materials.

23 Sorption to large clusters of organic matter or agglomeration with high-molecular-weight materials tends
24 to remove nanoparticles from solution, depositing them to the sediments (Handy et al., 2008, [157562](#);
25 Klaine et al., 2008, [193475](#)). On the other hand, complexation with low-molecular-weight organic
26 materials might enhance the particles’ ability to stay in suspension (Hyung et al., 2007, [090111](#)). Also
27 relevant to the bioavailability of nano-Ag are any processes that might sequester free metal ions after their
28 release to water from suspended nanoparticles, such as complexation with available sulfides. No studies
29 regarding the specific fate and bioavailability of nano-Ag in the environment were identified for this case
30 study. See Section 6.1 for additional discussion of factors that might affect the bioavailability of nano-Ag
31 as indicated by toxicity tests with specified modifications to either the nano-Ag (e.g., coatings) or the test
32 water (e.g., pH, Cl^- , ionic strength).

1 Toxicity tests have confirmed that sulfides can reduce the bioavailability of nano-Ag or silver ions
2 released from the nanoparticles. Choi et al. (2009, [193317](#)) compared the toxicity, and by inference the
3 bioavailability, of nano-Ag (average size 15 ± 9 nanometers [nm]) to an enriched concentration of
4 nitrifying bacteria in the absence and presence of several possible ligands, including S^{2-} , sulfate (SO_4^{2-}),
5 Cl^- , phosphate (PO_4^{3-}), and EDTA. The source of the bacteria was a local nitrifying activated sludge
6 plant in Missouri (Choi et al., 2008, [194060](#)). The biomass suspensions were aerated with pure oxygen to
7 maintain a dissolved oxygen concentration of approximately 20 milligrams per liter (mg/L) before the
8 nano-Ag or silver ions were added. Sulfide was the most effective of the ligands in reducing toxicity
9 (80% reduction inferred from increased oxygen uptake rates) of the nano-Ag to the bacteria. A back-
10 scattered electron (BSE) detector coupled with a secondary electron (SE) detector was used to locate
11 nano-Ag particles and Ag_2S complexes attached to the surface of the bacteria. Scanning electron
12 microscopy (SEM) in conjunction with energy dispersive x-ray analysis identified elemental composition.
13 These techniques revealed that the nano-Ag particles reacted with the S^{2-} to form new Ag_xS_y complexes
14 and precipitates that did not oxidize during 18 hours of aeration. The toxicity of the test medium was
15 enhanced, however, at S^{2-} concentrations higher than 1 mg/L, presumably from inhibition of bacterial
16 metabolism by the free available S^{2-} (Choi et al., 2009, [193317](#)).

17 In saltwater systems, the high ionic strength of sea water and high concentration of Cl^- favors the
18 formation of nano-Ag clusters and precipitation out of the water column; however, laboratory studies
19 indicate that available natural organic compounds (e.g., humic substances and thiols) can behave as
20 surfactants, binding with nano-Ag and stabilizing some of the particles in suspension (Hyung et al., 2007,
21 [090111](#)). Also in saltwater systems, exopolymeric substances, rich in polysaccharides and anionic
22 colloidal biopolymers, are secreted by phytoplankton and bacteria. Such substances could either protect
23 the organisms from interactions with nanoparticles (e.g., by initiating extracellular aggregation of
24 nanoparticles or by binding metal ions), or enhance interactions (e.g., by adsorbing and holding
25 nanoparticles in the immediate vicinity of cell surfaces) (Miao et al., 2009, [196270](#)).

26 Using sea water, Miao et al. (2009, [196270](#)) demonstrated rapid and complete aggregation of
27 uncoated nano-Ag (60–80 nm), leaving no detectable particles less than 220 nm in size in solution. The
28 addition of natural organic compounds or thiols greatly enhanced the presence of nano-Ag in solution.
29 Miao et al. (2009, [196270](#)) found further that diatoms exposed to nano-Ag in the sea water accumulated
30 silver linearly with the estimated free silver ions in solution at higher nano-Ag concentrations. It was not
31 clear whether the higher accumulation of silver in the diatoms (measured as μg Ag per mg diatom carbon)
32 resulted solely from influx of silver ions into or beyond the cell wall, or included accumulation of neutral
33 $AgCl$, which was a few orders of magnitude more prevalent in the solution (Miao et al., 2009, [196270](#)).

34 Although no studies of nano-Ag removal in wastewater treatment were identified, a study of
35 nanoscale titanium dioxide (nano- TiO_2) removal provides some indication of likely efficacy of nano-Ag
36 removal during treatment. Kiser et al. (2009, [225305](#)) examined the removal of Ti-containing particles at

1 wastewater treatment facilities. At one facility, concentrations of Ti in raw sewage ranged from 100 to
2 almost 3,000 µg/L, with particles greater than 700 nm accounting for the majority. Titanium in the
3 effluent from this (and other) facilities ranged from <5 to 15 µg/L, with almost all particles less than
4 700 nm in size. Removal of Ti from raw waste water ranged between 79% and 95%, depending on time
5 of year and use of filtration. Visualization of the Ti-containing solids remaining in the water after tertiary
6 treatment was conducted using scanning electron microscopy (backscatter mode)/electron dispersive
7 X-ray (SEM/EDX) microanalysis. Most particles comprised Ti and oxygen only, and particle types
8 included both single TiO₂ nanoparticles and aggregates of nano-TiO₂ ranging in size from 50 nm to a few
9 hundred nm. The aggregates appeared to be composed of TiO₂ particles smaller than 50 nm in size.
10 Laboratory tests revealed that TiO₂ particles sorbed onto activated sludge biomass. The investigators
11 concluded that 70 to 85% of TiO₂ particles in wastewater would be removed (assuming biomass in
12 wastewater around 2000–3000 mg/L total suspended solids). The smaller particles are less likely to be
13 removed by sedimentation or filtration if not sorbed to heavier solid particles. Similar removal
14 efficiencies might occur for nano-Ag in sewage input to wastewater treatment facilities.

15 The bioavailability of nano-Ag (and ionic silver released from nano-Ag) in sewage sludge applied
16 to terrestrial environments has not been investigated. Soils contain numerous ligands that can complex
17 nano-Ag and silver ions, and sewage sludge has substantial particulate organic matter to which nano-Ag
18 could bind depending on its size and surface coating (see Sections 4.1.2 and 4.3.1). The degree to which
19 nano-Ag might be available in sewage sludge applied to agricultural fields depends on both properties of
20 the particles and properties of the sludge/soil medium.

5.2.1.3. Bioaccumulation Models

21 Models to estimate bioaccumulation of nano-Ag or silver ions released from nano-Ag in aquatic
22 and possibly terrestrial food webs are relevant because the nano-Ag in spray disinfectants is expected to
23 be released into wastewaters (see Chapter 3). An existing model that could be adapted for aquatic food
24 webs for at least the silver ions released from nano-Ag is the U.S. Environmental Protection Agency's
25 (EPA) Bioaccumulation and Aquatic System Simulator (BASS) model, which is a ligand-binding model
26 for positive ionic inorganic substances that includes toxicokinetic, physiological, and ecological processes
27 affecting chemical uptake directly from water in fish (Barber, 2008, [201628](#)). Models for nano-Ag uptake
28 from food via the GI tract, however, are not yet available.

29 For terrestrial ecosystems, a first step would be to examine the availability of measured
30 conventional silver accumulation factors for terrestrial plants and soil invertebrates. Baes et al. (1984,
31 [073233](#)) cite a plant-soil bioconcentration factor (BCF) value of 0.138 for “above-ground” and 0.10 for
32 “below-ground” terrestrial plant parts consumed by humans, where the plant BCF value is based on total
33 dry weight silver concentrations in the plants and soils. Values less than 1.0 generally indicate a very low

1 concern for bioconcentration (e.g., BCF values <100 are considered of low concern by programmatic EPA
2 offices), which might explain the paucity of data on conventional silver uptake by plants. If the behavior
3 of nano-Ag in the soil is similar to the behavior observed for ionic silver in other plant-soil studies,
4 nano-Ag could accumulate in metal-tolerant plant root systems (Sections 5.2.4.1 and 5.2.4.3). If such
5 accumulation occurs, then small mammals or other biota that consume roots and tubers might be able to
6 hyper-accumulate nano-Ag. No models were identified that investigate this food pathway in terrestrial
7 ecosystems. Reviews of the uptake of inorganic chemicals from soils by earthworms (a commonly
8 studied soil organism) have not included silver (e.g., (Sample et al., 1999, [081977](#))), with the exception of
9 a single BCF value of 1.0 for Ag₂S cited in the review by Ratte (1999, [195564](#)).

5.2.2. Uptake by Bacteria and Fungi

10 Many bacteria and fungi readily take up conventional silver and nano-Ag, as summarized in this
11 section by type of organism.

12 **Bacteria.** Only oxidized nano-Ag particles, that is, particles with chemisorbed silver ions on the
13 surface, exert antimicrobial effects (Lok et al., 2007, [196762](#)). This might be due to the combination of
14 the nano-Ag and the silver ions that are tightly adsorbed (via physical or chemical forces) on the particle
15 surface (Lok et al., 2007, [196762](#)). Reduced nano-Ag appears to be unstable and easily oxidized.

16 Prokaryotes such as bacteria have a cell wall that separates them from their environment. Unlike
17 the more advanced eukaryotes, bacterial cells cannot perform phagocytosis or endocytosis, the two
18 processes by which nanoparticles might be absorbed into eukaryotic cells or organisms. Most bacteria
19 excrete siderophores, which chelate the relatively insoluble Fe³⁺ (ferric iron) ions in the environment,
20 forming a soluble complex that the bacteria absorb by active transport. Although other metal ions (e.g.,
21 aluminum) have been reported to complex with siderophores, no reports of silver ion chelation were
22 found. In the environment, siderophores have the highest affinity for Fe³⁺.

23 The membrane structures of bacteria are classified into two groups: gram-positive and gram-
24 negative. The structural differences occur in the key component of the cell wall, peptidoglycan, located
25 immediately outside the cytoplasmic membrane. The cell wall of gram-positive bacteria (e.g., *Bacillus*,
26 *Clostridium*, *Listeria*, *Staphylococcus*, *Streptococcus*) includes a ~30-nm-thick peptidoglycan layer, while
27 the cell wall of gram-negative bacteria (e.g., *Escherichia*, *Salmonella*, *Pseudomonas*) includes only a thin,
28 ~2- to 3-nm layer of peptidoglycan. The gram-negative cell wall also contains an additional outer
29 membrane composed of phospholipids and lipopolysaccharides facing the external environment. The
30 largest pores in the outer membrane of gram-negative bacteria secrete bacterial proteins out of the cell and
31 can measure almost 10 nm in diameter (Bitter et al., 1998, [597389](#)). These pores are likely to be “open”
32 only as needed for protein transport across the membrane (Filloux, 2004, [597399](#)). The fixed porins,

1 which allow diffusion of smaller molecules in both directions across the outer cell membrane, are smaller,
2 with effective diameters for solutes of 1–2 nm.

3 Studies investigating uptake of nano-Ag by gram-positive bacteria are few (e.g., Panáček et al.,
4 2006, [196274](#)), with most studies focusing on gram-negative bacteria. Sondi and Salopek-Sondi (2004,
5 [196277](#)), Morones et al.(2005, [196271](#)), and Hwang et al. (2008, [194065](#)) have shown that nano-Ag
6 anchors to and penetrates the cell wall of gram-negative bacteria. Wijnhoven et al. (2009, [180201](#)) and
7 others have proposed that the physical penetration changes the structure of the cell membrane
8 (presumably the outer membrane first), possibly increasing its permeability and resulting in uncontrolled
9 transport of materials into and out of the cytoplasm. Others have suggested the antibacterial mechanism
10 of nano-Ag is the formation of free radicals that damage the membrane (Danilczuk et al., 2006, [202670](#);
11 Kim et al., 2007, [202674](#)). Hwang et al. (2008, [194065](#)) proposed a synergistic toxic effect of nano-Ag
12 and silver ions from the nano-Ag in producing reactive oxygen species (ROS) in two oxidative-stress-
13 damaged sensitive strains of bioluminescent bacteria (DS1 and DK1). With nano-Ag sorbed to the
14 bacterial cell wall surface, silver ions can move into the cells and produce ROS inside. Hwang et al.
15 (2008, [194065](#)) hypothesized that the membrane damage caused by the nano-Ag attachment and insertion
16 demonstrated by Morones et al. (2005, [196271](#)) also might disrupt the ion efflux system, thereby
17 preventing expulsion of the silver ions from the bacterium.

18 Pal et al. (2007, [196273](#)) assessed the influence of nano-Ag shape on toxicity to the gram-negative
19 *Escherichia coli* bacterium. They found that truncated, triangular, silver nanoplates exhibited the
20 strongest antibacterial activity and reported the top “basal plane of truncated triangular silver nanoplates
21 [i.e., a {111} facet] is a high-atom-density surface” (Pal et al., 2007, [196273](#)). Images obtained with
22 energy-filtering transmission electron microscopy (TEM) revealed that many of the nano-Ag particles
23 adhered to the cell surfaces were coincident with pits (depressions) in the cell wall. Using high-angle
24 annular dark-field (HAADF) scanning transmission electron microscopy (STEM), Morones et al. (2005,
25 [196271](#)) demonstrated that individual, roughly spherical, nano-Ag with {111} facets attached directly to
26 outer cell membrane. In addition, nano-Ag was found throughout the interior of cells. It has been
27 proposed, however, that physical disruption of cell membrane integrity by nano-Ag might be the primary
28 cause of antibacterial effects, with accumulation of nano-Ag in the cytoplasm occurring as a secondary
29 effect (Neal, 2008, [196069](#)).

30 One difficulty with interpreting literature on the interaction of nano-Ag with gram-negative
31 bacteria is that investigators do not distinguish the cytoplasmic membrane from the cell wall from the
32 external membrane (e.g., Hwang et al., 2008, [194065](#); Morones et al., 2005, [196271](#); Pal et al., 2007,
33 [196273](#)), which together are only 2–3 nm thick. These distinctions are important for gaining a better
34 understanding of the specific site and mechanism of entry for nano-Ag into gram-negative bacteria.

35 **Nitrifying bacteria.** Nitrifying bacteria oxidize inorganic nitrogen compounds for energy
36 (chemoautotrophic), can oxidize ammonium ions to nitrites and nitrates, and are common in municipal

1 wastewaters. Key enzymes for these processes, including ammonia monooxygenase and nitrite
2 oxidoreductase, are organized along internal membrane systems. Choi et al. (2008, [194060](#)) examined the
3 effects of exposing nitrifying bacteria to nano-Ag (average size 14 ± 6 nm), silver ions (from AgNO_3),
4 and AgCl colloids (average size 250 nm). Interactions between microbes and nano-Ag were examined
5 using environmental scanning electron microscopy (ESEM), which allows imaging of hydrous samples.
6 The images revealed that nominally 10-nm nano-Ag from a commercial source, when mixed with
7 nitrifying bacteria in suspension, formed clusters in extracellular polymeric substances (from the
8 bacteria), which resulted in larger particles ranging from 200 nm to a few micrometers. Electron
9 micrographs demonstrated nano-Ag attached to bacterial cells. Choi and Hu (2008, [194061](#)) found that
10 metabolic inhibition of nitrifying bacteria (as inferred from oxygen uptake measurements) corresponded
11 to the fraction of nano-Ag less than 5 nm in diameter, suggesting that only small nano-Ag particles
12 penetrate the cell wall and membrane.

13 **Fungi.** Fungi are eukaryotic organisms with a cell nucleus and distinct organelles. Nano-Ag is
14 fungicidal against many common fungi, including the genera *Aspergillus*, *Candida*, and *Saccharomyces*
15 (Wijnhoven et al., 2009, [180201](#)). Yeast is a unicellular fungus. The cell wall, plasma membrane, and
16 periplasmic space between the wall and membrane together account for approximately 15% of the total
17 cell volume (Feldmann, 2005, [626572](#)). The membrane is selectively permeable, and the cells are capable
18 of both endo- and exocytosis. Both the cell wall and membrane are involved in budding (reproduction).
19 To investigate uptake and mode of action of nano-Ag on microfungi, Kim et al. (2009, [194069](#)) used a
20 budding yeast *Candida albicans* exposed to spherical nano-Ag with an average diameter of 3 nm. TEM
21 revealed that treated fungal cells exhibited pits and holes in their cell walls and transmembrane pores
22 through which cellular constituents could leak. Comparisons, such as exposing yeast to surface-coated
23 nano-Ag or to silver ions from AgNO_3 , were not provided.

24 Nano-Ag can be formed from ionic silver in solution in the presence of some fungi. For example,
25 extracellular nano-Ag particles between 5 and 25 nm in diameter have been produced by exposing the
26 filamentous fungi *Fusarium oxysporum* (Ahmad et al., 2003, [225147](#)) and *Aspergillus fumigatus* (Bhainsa
27 and D'Souza, 2006, [224881](#)) to aqueous silver ions. Nano-Ag 5–15 nm in size is stabilized by proteins
28 secreted by the fungus (Ahmad et al., 2003, [225147](#)). Given the relatively low concentrations of ionic
29 silver in surface waters, this particular mechanism of nano-Ag formation is probably without
30 consequence.

31 Mukherjee et al. (2001, [225202](#)) found “intracellular” nano-Ag of 25 ± 12 nm in *Verticillium*
32 exposed to aqueous silver ions. Electron microscopy revealed that the nano-Ag particles formed adjacent
33 to the cell wall surface but external to the plasma membrane, possibly as a result of reduction by enzymes
34 in the plasma membrane of the ions reaching the periplasmic space. No toxicity to the fungus was
35 observed; the cells continued to multiply after exposure and synthesis of nano-Ag. In this case, the fungi
36 were essentially removing free silver ions from the environment.

5.2.3. Uptake in Aquatic Ecosystems

1 In general, conventional silver contamination of aquatic ecosystems is thought to be of more
2 concern than contamination of terrestrial systems because of the high toxicity of silver ions to many
3 groups of aquatic organisms (Kramer et al., 2009, [196335](#)). Historically, most notable impacts of
4 conventional silver in the environment have been in the immediate vicinity of silver mines (Ratte, 1999,
5 [195564](#)) and in some estuaries receiving wastewaters containing silver from photographic facilities
6 (Flegal et al., 2007, [195508](#)). As a notable example, evaluations in San Francisco Bay suggest that waste
7 silver originating at a photogenic processing plant was discharged from a regional water quality control
8 plant through the late 1970s and subsequently accumulated in estuarine sediments to high levels, leading
9 to its characterization as the “Silver Estuary” (Flegal et al., 2007, [195508](#)). Even after active discharging
10 of silver wastes ceased, silver concentrations remained elevated. For example, in intertidal mudflats of
11 the southern reach of the estuary, or South Bay, concentrations measured during that time were
12 approximately 0.2–0.6 micrograms per gram ($\mu\text{g/g}$) (i.e., two to six times higher than regional
13 background levels of less than 0.1 $\mu\text{g/g}$). Concentrations of silver in the clam *Macoma petalum* dropped
14 from approximately 100 $\mu\text{g/g}$ in the late 1970s to 2–4 $\mu\text{g/g}$ by the late 1990s. Silver concentrations in the
15 Asian clam *Corbula amurensis* dropped from approximately 4 $\mu\text{g/g}$ to 0.5 $\mu\text{g/g}$ over the same time period.
16 In both species, the drop in body tissue silver corresponded with improved maturation of gonadal tissues
17 and readiness to spawn. To date, however, those scenarios appear to be the only real-world situations in
18 which silver contamination has caused high accumulation in biota and adverse effects in aquatic biota (in
19 benthic bivalves).

20 Once nano-Ag reaches an aquatic ecosystem from wastewater treatment facilities, its fate and the
21 likelihood that it will contact aquatic biota depend on many factors. First, properties of the particles (e.g.,
22 size, shape, coatings) and water chemistry (e.g., dissolved organic carbon, ionic strength, pH) will
23 influence the extent to which the particles will remain in suspension, partition to dissolved organic carbon
24 in the water column, form clusters with each other, and adsorb to suspended particles and plankton. As
25 noted in Chapter 4 and Section 5.2.1, the chemistry and content of natural freshwater, estuarine water, and
26 salt water generally favor formation of silver complexes and clusters that settle out of the water column to
27 the surface of the sediment bed. In freshwater systems, the formation of Ag_2S and S^{2-} complexes
28 removes silver ions from solution, while in saltwater systems the formation of AgCl predominates. Silver
29 nanoparticles, on the other hand, might remain in suspension if coated to prevent formation of clusters or
30 complexation with, or adsorption to, other particles. Laboratory tests of the toxicity of nano-Ag to
31 freshwater aquatic organisms generally use one or more methods to ensure suspension of the nano-Ag in
32 the water column. The methods generally do not reflect conditions in natural surface waters. The current
33 consensus is that nano-Ag rarely remains in suspension in natural ecosystems, although opinions differ as

1 to future possibilities as product use grows and technologies for keeping particles in suspension advance
2 (Luoma, 2008, [157525](#)).

3 For animals, uptake of nano-Ag from water or pore water in sediments might occur at the gill
4 surface during respiration, following ingestion with food (e.g., detritus, algae, smaller animals), or dermal
5 absorption, depending on the form and bioavailability of nano-Ag and silver ions in food, water, and
6 sediments. Uptake into an organism versus adsorption to its surface depends on the nanoparticulate
7 chemistry at exterior surfaces, which could cause the particles to form clusters, and the behavior of the
8 particles inside cells and circulatory fluids (e.g., blood plasma) (Handy et al., 2008, [157563](#)). Assuming
9 that nano-Ag particles remain in suspension in the water column, as the particles come in contact with the
10 surfaces of organisms, three scenarios are possible.

- 11 • No interaction; nano-Ag drifts away from organism and continues movement in the water
12 according to Brownian motion. This option seems most likely for nano-Ag with some type of
13 surface coating, either a natural one acquired post-release (e.g., dissolved organic matter) or
14 manufactured (e.g., to keep nano-Ag in suspension until a spray disinfectant is used).
- 15 • Nano-Ag particles adhere (adsorb) to the surface(s) of the organism; for example, to the cell
16 wall surface of phytoplankton and macrophytes, the carapace and appendages of crustacean
17 zooplankton, the epidermis of larval forms of invertebrates, the outer surface of aquatic eggs,
18 and, for larger animals (e.g., mussels, crabs, fish), to the exchange surface of gills, olfactory
19 receptors, and the lining of the GI tract for ingested particles. Uptake of silver ions released
20 from the nano-Ag in the vicinity of organisms is expected to the same degree as uptake of silver
21 ions released from dissolved silver salts, conventional silver powders, or other sources of silver
22 ions.
- 23 • Nano-Ag particles penetrate the surface of an organism. Whether a silver nanoparticle can
24 penetrate the outer cell wall or membrane of an organism depends on properties of the particle
25 and characteristics of the organism. Depending on the organism, various uptake mechanisms
26 are possible for nano-sized particles. Sorption to natural low-molecular-weight organic
27 materials in the surface water or some manufactured surface coatings might facilitate uptake of
28 nano-Ag at the cell wall or cell membrane by increasing the particles' lipophilicity.

29 Where nano-Ag particles do penetrate cell walls, cell membranes, circulatory systems, or
30 interstitial spaces of organisms, they might interfere with cellular structure and function by physical
31 (mechanical) forces, by providing a continuing source of free silver ions, or by both types of action. How
32 the route of uptake of nano-Ag (and silver ions from the particles) is influenced by surface modifications
33 of the particles is currently unknown (Behra and Krug, 2008, [157596](#)). Presumably, smaller particles are
34 more likely to be taken up by endocytosis than larger particles. Routes of uptake for silver ions and nano-
35 Ag are discussed below by type of aquatic organism and environment: (1) freshwater algae, (2)
36 freshwater protozoa, (3) mollusks, (4) aquatic crustacea, (5) fish eggs, (6) freshwater fish, and (7)
37 saltwater fish.

5.2.3.1. Uptake by Algae

1 Freshwater algae appear to take up silver ions rapidly, presumably through a copper ion transporter
2 through the cell membrane (Lee et al., 2005, [224900](#)), resulting in high BCFs (e.g., 10,000 liters per
3 kilogram [L/kg] wet weight) (Ratte, 1999, [195564](#)). To evaluate the toxicity to and uptake of nano-Ag in
4 green algal cells, Navarro et al. (2008, [157516](#)) exposed *Chlamydomonas reinhardtii* for 1 hour to
5 nano-Ag and silver ions with and without cysteine as a ligand to decrease free silver ions in solution. The
6 nano-Ag particles were evaluated for size and zeta (ζ) potential using dynamic light scattering (DLS) and
7 were visually inspected using TEM. Particle size ranged from 10 to 200 nm with a median of 40 nm; the
8 diameter of 98% of particles was within 25 ± 13 nm. As measured by diffusive gradients in thin films
9 (DGT), the maximum concentration of silver ions (most of the labile silver measured) comprised
10 approximately 0.0–1% of the total silver in solution, and the Ag-ion-selective electrode (ISE) method
11 indicated the silver ion concentrations to be 0.7–1.2% of the total silver. With the addition of algae, silver
12 ions dropped to 0.1% after 1 hour. The inhibition of algal photosynthesis in the presence of AgNO_3 was
13 completely eliminated by the addition of equimolar concentrations of cysteine. Several indirect lines of
14 evidence led the authors to conclude that the toxicity of nano-Ag to the algae required interaction with the
15 algae and nanoparticle, but was mediated by silver ions released from the nanoparticles either at the algal
16 interface or near the algal interface where products of algal metabolism, notably hydrogen peroxide
17 (H_2O_2), might be secreted.

18 In saltwater systems, the high ionic strength of sea water and high concentration of Cl^- favors the
19 formation of nano-Ag particle clusters. However, available natural organic compounds (e.g., humic
20 substances and thiols) can behave as surfactants, binding with nano-Ag and stabilizing some of the
21 particles in suspension (Hyung et al., 2007, [090111](#)). Exopolymeric substances secreted by
22 phytoplankton and bacteria (Verdugo et al., 2004, [224920](#)) could either protect the organisms from
23 nanoparticle toxicity or enhance toxicity, as discussed in Section 5.2.1.2 (Wilkinson and Reinhardt, 2005,
24 [224954](#)).

25 Algal uptake of nano-Ag in sea water was examined using the diatom *Thalassiosira weissflogii*
26 (Miao et al., 2009, [196270](#)). Diatoms are eukaryotic single-celled algae encased in a silica-based rigid
27 “frustule” (essentially two glass half shells, or valves, fused together). Nutrients were limited to enhance
28 excretion of carbohydrates by the cells. Initial solutions of 60- to 80-nm nano-Ag in deionized water
29 were well dispersed, but when added to sea water, nano-Ag rapidly formed clusters, with no particles
30 detectable in a less than 200-nm filtrate. The addition of natural organic compounds (fulvic acid from the
31 Suwannee River) stabilized some of the nano-Ag in suspension, and the addition of thiols increased nano-
32 Ag particle concentrations in the less than 220-nm filtrate by a factor of 100. Because toxicity was not
33 enhanced by the addition of thiols, Miao et al. (2009, [196270](#)) concluded that one or more protective
34 mechanisms occurred: the carbohydrate coating of the silica shell repelled the particles, the nano-Ag

1 particles were too large to penetrate pores in the shells, or the coating of nano-Ag with organic material
2 prevented interaction with the diatoms. The exopolymeric coatings excreted by the cells contain
3 covalently bound proteins that might bind silver ions. They concluded further that the observed toxicity
4 of the nano-Ag was due to the release of free silver ions in the vicinity of the cell; nano-Ag did not
5 penetrate the cells.

6 Whether inside the algal cell or sorbed to the surface, nano-Ag can act as a continuing, slow-
7 release source of silver ions (Lubick, 2008, [195540](#)). To date, the data are consistent with nano-Ag
8 adsorption to the outer cell wall and release of silver ions in the immediate vicinity of the cell wall.

5.2.3.2. Uptake by Protozoa

9 Using the single-celled flagellate protozoan *Paramecium caudatum*, Kvittek et al. (2009, [195535](#))
10 demonstrated that nano-Ag particles approximately 30 nm in diameter were not toxic at concentrations as
11 high as 25 mg/L (paramecia survived for 7 days; the 1-hour LC₅₀ was 39 mg/L), whereas silver ions
12 caused immediate death of all of the paramecia at concentrations as low as 0.4 mg/L (see Section 6.2.2).
13 The size and ζ potential of the nano-Ag particles were measured by DLS, and size was confirmed with
14 TEM. Modifying the nano-Ag with Tween 80 (1% w/w), a nonionic surfactant, increased the
15 bioavailability (as reflected in measures of toxicity) of the nano-Ag to the paramecia somewhat, with a
16 measured 1-hour LC₅₀ of 16 mg Ag/L.

5.2.3.3. Uptake by Bivalve Mollusks

17 Investigators have noted that surface sediment-dwelling and filter-feeding mollusks are prime
18 candidates for uptake of engineered nanoparticles released to the environment, particularly if the
19 nanoparticles associate with natural particles (Moore, 2006, [089839](#)). Mollusks accumulate conventional
20 pollutants sorbed to suspended particles and sediment (Galloway et al., 2002, [224888](#); Livingstone, 2001,
21 [225197](#)). Lamellibranch bivalves (e.g., mussels, scallops, cockles, most clams) filter food from the water
22 passing over their gills, with cilia moving the food particles to the mouth. Nanoparticles might be trapped
23 and ingested with the phytoplankton and suspended detritus that comprise the food (Wijnhoven et al.,
24 2009, [180201](#)). Moore (2006, [089839](#)) postulated that benthic marine bivalves such as the blue mussel
25 (*Mytilus edulis*) might absorb ingested nanoparticles by endocytosis.

26 Moore et al. (1997, [202119](#)) and Owen (1970, [224908](#)) demonstrated uptake of nano-sized particles
27 and their deposition in the digestive glands of mussels and cockles (*Cardium edule*), respectively. In the
28 case of mussels, the nanoparticles were composed of sucrose polyester oil (particle diameters not
29 reported). The in vitro experiments demonstrated that isolated hepatopancreatic digestive cells from the
30 mussel took up the particles by endocytosis and subsequent attachment into the lysosomal degradative

1 compartment (Moore et al., 1997, [202119](#)). In the case of cockles, animals were fed nanoparticles
2 comprising colloidal graphite and iron oxide in vivo, and animals were sacrificed and tissues removed and
3 fixed at several time intervals. The electron micrographs of digestive cells showed that some phagosomes
4 in the cells engulfed single particles (phagocytosis) of 50 to 300 nm in diameter. Smaller nanoparticles
5 appear to have been ingested by pinocytosis (i.e., pinocytic vesicles with a characteristic granular outer
6 coat). Both types of vesicles transferred their contents to primary phagosomes in subapical regions of the
7 digestive cells, which ultimately connected to the lysosomal degradative compartment (Owen, 1970,
8 [224908](#)).

9 Although uptake of nano-Ag by bivalve mollusks has not yet been evaluated, the rate of uptake
10 likely would depend on the rate at which the animal moves water over its gills and the proportion of
11 nano-Ag particles large enough to be trapped by the gill lamellae, but small enough not to be ejected from
12 the food ingestion stream. Water filtration rates would depend on temperature and the size of the mollusk,
13 as well as reproductive status and other factors.

5.2.3.4. Uptake by Aquatic Crustacea

14 Uptake of silver ions from water has been examined for several species of crustacea, with BCFs as
15 high as 1,100 and >2,200 for *Gammarus pulex* (Terhaar et al., 1977, [202115](#)) and *D. magna*, respectively
16 (Garnier-Laplace et al., 1992, [098719](#)). Uptake of silver ions is associated with branchial sodium- and
17 potassium-activated adenosine triphosphatase (Na⁺/K⁺-ATPase) in both crayfish (Grosell et al., 2002,
18 [195518](#)), which are tolerant of conventional silver, and in daphnids (Bianchini and Wood, 2003, [195486](#)),
19 which are sensitive to conventional silver. Uptake of nano-Ag or silver ions from the nanoparticles could
20 occur in crustacea through the adhesion of nano-Ag or nano-Ag clusters to the external carapace (e.g.,
21 planktonic crustacean) or gills (macrocrustacea). In addition, transport of ingested nano-Ag across the gut
22 epithelium by endocytosis might be possible.

23 Zooplankton feed by filtering large volumes of water through setae (i.e., structures similar to tiny
24 combs) on their appendages. The setae collect larger bacteria, algal cells, and possibly nanoparticle
25 clusters (Baun et al., 2008, [157598](#)). Ingestion of nano-TiO₂ clusters has been verified by their presence
26 in the gut of *D. magna* (Figure 2 in Baun et al., 2008, [157598](#)). Adhesion of aggregations of nano-TiO₂ to
27 the exoskeleton and antennae of *D. magna* and carbon-60 (C₆₀) to antennae of the marine copepod *Acartia*
28 *tonsa* also have been demonstrated (Figure 3 in Baun et al., 2008, [157598](#)). Uncoated nano-Ag particles
29 also could sorb to the exoskeleton and antennae of planktonic invertebrates.

30 Although studies specific to the uptake of nano-Ag by crustaceans are lacking, multiple studies
31 have demonstrated the toxicity of nano-Ag to these organisms (see Section 6.2.2.2 and Appendix B).
32 Those studies indicate that uptake, or at least sorption to gill and possibly GI epithelia, with subsequent
33 release of silver ions, does occur.

5.2.3.5. Uptake by Vertebrate Eggs

1 Several university laboratories are assessing uptake of chemicals and nanoparticles by eggs and
2 embryos of the freshwater zebrafish *Danio rerio*. Three have published recently on uptake of nano-Ag in
3 aqueous suspension (Asharani et al., 2008, [194056](#); Bar-Ilan et al., 2009, [191176](#); Nallathamby et al.,
4 2008, [191175](#)) and all have reported that nano-Ag can be incorporated in the body of developing
5 embryos. Asharani et al. (2008, [194056](#)) prepared nano-Ag “capped” with either soluble potato starch or
6 bovine serum albumin (BSA) to prevent cluster formation (both agents are considered nontoxic). TEM
7 and surface plasmon resonance (SPR) analyses of stock solutions indicated an average size range of 5–20
8 nm, with a broader distribution of sizes for the BSA-capped particles. Embryos of an unspecified age
9 (likely less than 6 hours post-fertilization) were exposed for 72 hours to one of five concentrations of
10 capped nano-Ag or only to the capping agent or to control water. Concentration-dependent toxicity was
11 observed as described in Section 6.2.2.3 and Appendix B. Tissue analysis revealed significantly higher
12 concentrations of residual total silver in test organisms than in the controls, although no concentration
13 data were provided in the study report (Asharani et al., 2008, [194056](#)). TEM examination of older
14 zebrafish embryos showed nano-Ag deposits throughout the body, including the trunk, tail, skin, heart,
15 and brain, and clusters of nano-Ag throughout the epidermis. Closer examination of the trunk and tail
16 showed most nano-Ag particles deposited inside cell nuclei, with fewer in the cytoplasm. In the nuclei,
17 nano-Ag particles were found as distinct clusters. The investigators noted that nano-Ag appeared as
18 clusters in most organs except the brain, where they remained dispersed.

19 Laban et al. (2009, [199809](#)) also observed “clumps” of nano-Ag distributed throughout fathead
20 minnow (*Pimephales promelas*) embryos after nano-Ag had attached in large quantities to the chorion
21 surface. Asharani et al. (2008, [194056](#)) reported that nanoparticles that enter early embryonic cells (e.g.,
22 4-cell stage) have a high chance of being distributed throughout the embryo, although they did not state
23 whether any of their embryo exposures started at that stage (e.g., less than 1.5 hours after fertilization). It
24 is also possible that nano-Ag particles could be transported across the epidermis of later stage embryos.

25 Smaller nano-Ag particles can be absorbed more readily than larger nano-Ag particles due to their
26 size. While investigating the toxic mode of action of nano-Ag, Bar-Ilan et al. (2009, [191176](#)) exposed 4-
27 to 6-hour-old (post fertilization) sphere-stage embryos to four different size groups (3, 10, 50, and
28 100 nm) and concentrations of either nano-Ag or nanoscale gold (nano-Au), which is relatively inert.
29 Exposure water was renewed daily for up to 10 days. The smaller size groups (3 and 10 nm) of nano-Ag
30 produced a higher incidence of sublethal effects than the larger sizes (50 and 100 nm), although mortality
31 was similar across sizes. Exposure to nano-Au in the same size categories and series of concentrations
32 produced no measureable toxicity. Instrumental neutron activation analysis (INAA) demonstrated that
33 both nano-Ag and nano-Au adsorbed to or were taken up by the embryos. The investigators provided no

1 information on whether the size of the nano-Au influenced the quantity of gold associated with the
2 embryo, although they cited other studies indicating better “translocation” of smaller sizes.

3 The method by which nano-Ag enters the outer egg chorion was elucidated using real-time
4 visualization of nano-Ag particles traversing the zebrafish chorion (Lee et al., 2007, [194072](#); Nallathamby
5 et al., 2008, [191175](#)). Nano-Ag has the highest quantum yield of Rayleigh scattering of the “noble” metal
6 nanoparticles, and scattering intensity is proportional to the volume of the particles. The bright particles
7 can be observed directly using dark-field single-nanoparticle optical microscopy and spectroscopy
8 (SNOMS). Also, the localized surface plasmon resonance (LSPR) spectra (wavelength/color) show size
9 dependence, which allows a size category to be estimated for nanoparticles of different visible colors.
10 Using these techniques, Lee et al. (2007, [194072](#)) and Nallathamby et al. (2008, [191175](#)) estimated the
11 proportion of nano-Ag used in their initial experiments to be ~75% particles of 5–15 nm diameter, ~23%
12 particles of 16–30 nm, and only ~1% particles as large as 31–46 nm. They prepared the particles by
13 reducing silver perchlorate (AgClO_4) and then washing and centrifuging the resulting nano-Ag to obtain
14 highly purified particles without surface coating. By adjusting the concentration of NaCl in the medium
15 to maintain a low ionic strength solution, they could keep the ζ potential of the particles high, and the
16 particles were stable in solution (i.e., did not form clusters) for months (Nallathamby et al., 2008,
17 [191175](#)).

18 Lee et al. (2007, [194072](#)) also demonstrated that the single nanoparticles in the test water did not
19 form clusters and could pass through zebrafish chorionic pores (500–700 nm in diameter) by diffusion
20 (Brownian motion). Some single particles remained in the pores, but most passed through into the
21 chorionic fluid where they continued to move by Brownian motion, as demonstrated by real-time video.
22 Some particles also diffused back out of the egg through the chorionic pores. Few clusters of nano-Ag
23 were observed in the fluid. Estimated diffusion coefficients for the nano-Ag in the chorionic fluid
24 revealed a viscosity gradient across the embryo, yolk, and the chorion. The nano-Ag that stayed (possibly
25 adsorbed) in the chorionic pores appeared to serve as nucleation sites for nano-Ag cluster formation,
26 physically clogging some chorionic pores, which would limit oxygen and waste exchange between the
27 embryo in the egg and its environment. This laboratory also demonstrated a counterclockwise movement
28 of fluid within the chorion with a range of viscosity gradients, with slower movement of nano-Ag in the
29 more viscous portions of the chorionic fluid (Nallathamby et al., 2008, [191175](#)).

30 Most zebrafish embryos treated with nano-Ag concentrations less than 0.08 nM at or before the
31 cleavage stage (8-cell) completed development at 120 hours post-fertilization (Lee et al., 2007, [194072](#)).
32 Examining these fish using SNOMS, researchers identified nano-Ag embedded in multiple organs,
33 notably in the retina, brain, heart, gill arches, and tail. Thus, some of the nano-Ag particles that entered
34 the egg by diffusion reached the embryo the same way and were taken up into the body of the developing
35 embryo, possibly by endocytosis. Higher exposure concentrations resulted in higher incidence of
36 deformed and dead embryos, as discussed in Section 6.2.2.3.

5.2.3.6. Uptake by Freshwater Fish

1 Fish are exposed to chemicals in solution or in suspension in water at both their gills and GI
2 epithelia. Between the aquatic milieu and the external surface of the fish is an “unstirred layer,” usually
3 with polyanionic mucus secretions (Handy et al., 2008, [157563](#)). The unstirred layer tends to be more
4 viscous and move more slowly than bulk water, thereby holding nanoparticles at the external surface of
5 the organism (Handy et al., 2008, [157562](#)). The various ligands present on the cell surface also are
6 predominantly anionic. Nanoparticles should generally diffuse across the mucous layer more slowly than
7 single molecules such as electrolytes and metal ions, and cationic nanoparticles might bind to strands of
8 mucoproteins hindering their uptake (Handy and Shaw, 2007, [093122](#); Handy et al., 2008, [157563](#)). Cell
9 surfaces also might present ligands for nano-Ag (e.g., gill epithelium is predominantly anionic) (Handy
10 and Eddy, 2004, [594262](#)).

11 At the gills, metal cations can move through the epithelial membrane using specialized cation
12 transporter channels. Most nano-Ag particles, however, should be too large to traverse the cation
13 channels, which have a pore diameter less than 1 nm. In the gut, where endocytosis is one method by
14 which the epithelial cells absorb nutrients, nanoparticle uptake through vesicular transport is possible
15 (Handy et al., 2008, [157563](#)).

16 Nano-Ag, however, need not cross epithelial membranes to affect fish; adsorption to the gill
17 membranes is sufficient for nano-Ag to deliver silver ions, which are toxic to fish. Investigators have
18 hypothesized and provided evidence that exposure to silver ions blocks the Na^+/K^+ -ATPase active
19 transport of Na^+ from fresh water across the gills into fish (Bury et al., 1999, [195494](#); Morgan et al., 1997,
20 [597411](#)). Recent studies suggest that the mechanism of action of silver ions on the gill might be more
21 complex and that silver ions are absorbed by the basolateral gill membrane into the bloodstream, where
22 they then travel to and concentrate in the kidneys. Bury (2005, [195495](#)) found increased sodium ion
23 efflux rather than decreased sodium ion uptake in juvenile rainbow trout (*O. mykiss*) exposed to sublethal
24 concentrations of silver ions (added as AgNO_3). The fishes' Na^+ ionic balance was restored by the 21st
25 day of exposure, while kidney K^+ -dependent *p*-nitrophenol phosphatase activity was reduced and the total
26 silver concentration in both gills and kidneys was elevated 20-fold. Indirect in vitro evidence suggested
27 that the gill basolateral membrane could sequester silver in membrane vesicles. The fish could then
28 absorb the silver or expel it back into the surrounding water (Bury, 2005, [195495](#)).

29 Many studies measure total silver tissue burden following exposure to nano-Ag, although some
30 traditional methods used for tissue analysis (such as mass spectrometry) cannot distinguish silver
31 nanoparticles from silver ion concentrations (Griffitt et al., 2009, [199805](#)). A chronic study (10–38 days)
32 investigating the effect of nano-Ag on caudal fin regeneration in *D. rerio* reported that nano-Ag had
33 penetrated fish organelles, including mitochondria, nuclei, and blood vessels (Yeo and Pak, 2008,

1 [191177](#)). The investigators measured the residual silver concentrations in fish muscle, intestine, and
2 testes following exposure to nano-Ag, but did not report the specific particle size or the age of the test
3 organism. They did report, however, that the total silver concentration was highest in the muscle 2 hours
4 post exposure, but that total silver concentrations in the muscle and testes decreased to nearly zero by
5 about 100 hours post exposure. Conversely, total silver concentrations in intestinal tissues of zebrafish,
6 both with and without amputated fins, continued to increase through 140 hours post exposure (Yeo and
7 Pak, 2008, [191177](#)).

5.2.3.7. Uptake by Saltwater Fish

8 Many investigators believe the uptake and effects of nano-Ag in saltwater fish occur by different
9 mechanisms than in freshwater fish. Freshwater and anadromous fish must be able to maintain body
10 fluids that are hyperosmotic compared with surrounding fresh waters, and they do so with active ionic
11 transport. Silver ions released by nano-Ag near or sorbed to the gill membranes are actively absorbed by
12 the Na^+/K^+ -ATPase ion channels in the gills. Saltwater fish, on the other hand, are somewhat hypo-
13 osmotic with respect to surrounding sea water, and free silver ions in the water column readily bond with
14 abundantly available Cl^- to form AgCl , which precipitates from solution. For example, investigations of
15 the gulf toadfish (*Opsanus beta*), which can survive in a wide range of salinities if acclimated, have
16 indicated uptake of silver. Above the isosmotic point of approximately 32% sea water, toadfish drink the
17 water and absorb Na^+ , Cl^- , and water across the GI tract and actively excrete Na^+ and Cl^- across the gills
18 and secrete ionic magnesium (Mg^{2+}) into urine (Wood et al., 2004, [195587](#)). At lower salinities, the
19 toadfish actively takes up Na^+ and Cl^- across the gills and retains the ions in the kidneys. Wood et al.
20 (2004, [195587](#)) acclimated toadfish to salinities ranging from 2.5 to 100% sea water, followed by 24-hour
21 exposure to $2.18 \mu\text{g Ag/L}$ as $^{110\text{m}}\text{Ag}$ -labelled AgNO_3 . Speciation of silver varied with salinity: the silver
22 chloride anion AgCl_3^{2-} dominated at 100% salinity and declined with decreasing salinity, while another
23 silver chloride anion, AgCl_2^- , dominated at intermediate salinities (10–60% sea water). Neutral dissolved
24 AgCl^0 was negligible at higher salinities but gradually increased with decreasing salinity to approximately
25 equal to AgCl_2^- at the lowest salinity (2.5%). At all salinities, total silver ion concentrations in solution
26 decreased over the 24-hour exposure period due to adsorption and precipitation. Only 5% of the total
27 silver initially present in the test solutions was accounted for by the amount found in the toadfish.
28 Maximum total silver accumulation in the toadfish occurred at the lowest salinity tested, 2.5% sea water;
29 minimum uptake occurred at 40% sea water.

30 Wood et al. (2004, [195587](#)) also found that silver concentrations in bile were higher at lower sea-
31 water concentrations. Of the toadfish tissues, the liver showed the highest internal accumulation of silver,
32 while muscle concentrations were lowest. The authors concluded that silver ions entering the gills were
33 efficiently absorbed by the blood and distributed to other organs. They attributed the variation in patterns

1 of total silver accumulation in different tissues and at different salinities to at least two factors: (1)
2 salinity-dependent changes in the silver speciation and (2) salinity-dependent changes in the
3 ionoregulatory physiology of the fish (Wood et al., 2004, [195587](#)). The relative importance of ingestion
4 and GI tract absorption increased with increasing salinity after the isosmotic point.

5 Nichols et al. (2006, [195550](#)) examined total silver accumulation in gills and plasma of toadfish
6 exposed for the longer time duration of 6 days. The investigators found the same pattern of decreasing
7 silver accumulation with increasing salinity, which was expected given the lower bioavailability of AgCl
8 complexes formed at higher salinities. The group also compared total silver uptake in water with and
9 without NOM obtained from the Suwannee River. The addition of NOM appeared to reduce gill
10 accumulation of silver in toadfish only at salinities less than 40% sea water. At higher salinities, the
11 organic matter did not appear to influence silver accumulation in gills. In contrast, the addition of organic
12 matter appeared to increase silver concentrations in blood plasma at salinities less than 40% sea water.
13 The investigators hypothesized that the organic matter helped to keep more silver in solution, facilitating
14 gill uptake of silver (Nichols et al., 2006, [195550](#)).

15 Information presented in this section regarding the uptake of conventional silver might be relevant
16 for this case study; however, no data specific to the uptake of nano-Ag by saltwater fish were identified.
17 Additional information would be useful to fully assess the potential for exposures for saltwater fish.

5.2.3.8. Bioaccumulation in Aquatic Food Webs

18 As discussed previously, nano-Ag that sorbs to or is absorbed by organisms is defined as
19 bioavailable for purposes of this document. A consumer organism (e.g., herbivore, carnivore) that feeds
20 on smaller organisms (e.g., phytoplankton, zooplankton, eggs, small fish) will ingest the nano-Ag and
21 other conventional silver compounds that are on or in their food. The question then becomes to what
22 extent the nano-Ag on or in the food is bioavailable to the consumer, either via interaction with the gut
23 epithelial cells or absorption by those cells followed by passing the particles through to the circulatory
24 system. This question is examined for water-column and sediment communities. What remains unknown
25 for all groups is how the route of uptake of nano-Ag (and silver ions from the particles) is influenced by
26 surface modification of the particles (Behra and Krug, 2008, [157596](#)).

Water-Column Organisms

27 Algae, the primary producers in the water column, show high bioconcentration of silver inside cells
28 when exposed to free silver ions, with BCF values as high as 10,000 to 100,000 in some studies (Ratte,
29 1999, [195564](#)). However, studies of algae exposed to nano-Ag focus on toxicity endpoints (e.g., growth
30 inhibition) and contribution of silver ions to toxicity, not on calculating BCF values (e.g., Navarro et al.,
31 2008, [157517](#)). Concentrations of total silver versus nano-Ag on or in the cell walls or in the cell

1 cytoplasm have not been explored. At a minimum, however, algal cells might concentrate nano-Ag
2 particles relative to particulate concentrations in the water column by adhesion of nano-Ag to the external
3 cell wall, as has been demonstrated for bacteria (Morones et al., 2005, [196271](#)).

4 Bioconcentration or bioaccumulation of free silver ions in filter-feeding zooplankton, the next step
5 up pelagic food webs, is on the order of a factor of 1,000–5,000, somewhat lower than for algae (Ratte,
6 1999, [195564](#)). Zooplankton might sorb nano-Ag to setae, cilia, antennae, other appendages, gills, and
7 the GI tract epithelium (Baun et al., 2008, [157598](#)). This sorption would not be considered
8 bioaccumulation for those organisms, but could lead to bioaccumulation in their consumers.

9 Bioaccumulation of nano-Ag would require uptake of the particles after ingestion, possibly by
10 endocytosis along the consumer's GI tract. Bioconcentration of silver ions could occur if nano-Ag
11 particles sorbed to gills, and then the water's chemistry promoted the release of silver ions from the
12 particles. On the other hand, crustacean zooplankton, which shed their carapace at regular intervals,
13 might facilitate sedimentation of nano-Ag particles sorbed to their exoskeletons (Ratte, 1999, [195564](#)).

14 Some forms of complexed silver are bioaccumulated in aquatic crustaceans without evidence of
15 toxicity or internal absorption. Several studies have demonstrated bioaccumulation of silver atoms bound
16 with inorganic (-S) or thiol (-SH) sulfides without evidence of toxicity, presumably owing to a lack of free
17 silver ions (Bianchini et al., 2002, [195482](#); Kramer et al., 2009, [196335](#)). In the presence of S²⁻,
18 complexed silver can be ingested and accumulated in the digestive tract of *D. magna*, enhancing the
19 apparent "whole-body" silver burden even though none is absorbed into the body of the animal (Bianchini
20 et al., 2005, [195483](#)). Although not associated with internal accumulation or toxicity of silver ions to the
21 *D. magna* themselves, the silver is passed along the food chain to consumers of *D. magna* (Bianchini and
22 Wood, 2008, [195485](#)). For the consumers, the ingested silver might or might not be adsorbed or absorbed
23 by the GI tract.

24 Fish exhibit low BCF values for silver ions relative to BCFs reported for algae and zooplankton,
25 and the potential for bioconcentration or bioaccumulation of nano-Ag, either in solution or in food, has
26 not been examined. BCF values for free silver ions of approximately 1 to 350 have been measured for the
27 body and viscera of fish on a wet-weight basis compared with surrounding water (i.e., for *Cyprinus*
28 *carpio*, *P. promelas*, and *O. mykiss*) (Ratte, 1999, [195564](#)). Measures of bioaccumulation of total silver
29 from the diet were not found. For ingested nano-Ag that is adsorbed to or in food, the low pH in the gut
30 might favor formation of the relatively insoluble AgCl from silver ions released from silver nanoparticles
31 (Panyala et al., 2008, [195554](#)), thereby favoring dissolution of the nano-Ag in the gut. However, larger
32 nano-Ag particles with smaller surface-to-volume ratios might take longer to dissolve, and they could be
33 excreted in feces if not absorbed or adsorbed by the gut. For mammals, there is evidence that small nano-
34 Ag particles can be absorbed into the blood stream, accumulated in the liver, and excreted back into the
35 gut lumen in bile through exocytosis (Sadauskas et al., 2007, [091407](#)). The silver then might be
36 reabsorbed by the gut or excreted in feces (Sadauskas et al., 2007, [091407](#)).

1 The evidence cited above indicates that in general, bioaccumulation of silver atoms or compounds
2 appears to decrease with increasing trophic level in water-column food webs. The highest BCF values are
3 reported for algae (i.e., 10,000 to 100,000), with lower values reported for zooplankton (e.g., 5,000) and
4 fish (i.e., 1 to 350). Fish show limited absorption of silver from their diet or from water in the first place,
5 and hence limited accumulation (Ratte, 1999, [195564](#)). No data are available on potential
6 bioaccumulation of nano-Ag specifically.

7 The extent to which water column biota might alter nano-Ag after coming in contact with
8 nanoparticles in the environment is another consideration for food chain accumulation (Behra and Krug,
9 2008, [157596](#)); no studies were identified, however, that examined the bioavailability of nano-Ag in
10 various water column biota to consumer organisms up the food chain.

Sediment Organisms

11 Sediment is likely to be an important sink for nano-Ag clusters, nano-Ag, silver ions sorbed to
12 particles, and silver ions precipitated in insoluble compounds. Detritus (i.e., decaying plant and animal
13 materials) and microorganisms form the base of food webs originating in the benthos. Benthic
14 detritivores and filter-feeders contact and ingest relatively large quantities of detritus, associated
15 microorganisms, and plankton near the sediment surface. Thus, silver in sediments could enter aquatic
16 food webs through benthic organisms.

17 In general, BCF values for small crustaceans exposed to soluble silver salts added to
18 sediment/water systems are close to or less than 1 (Ratte, 1999, [195564](#)). BCF values measured for the
19 same organisms (e.g., *G. pulex*, *Chironomus luridus*) in water only, however, can be three orders of
20 magnitude higher (e.g., 1,100) (Garnier-Laplace et al., 1992, [098719](#); Ratte, 1999, [195564](#)). These
21 findings suggest that much of the silver in sediments is not bioavailable, presumably due to the high
22 availability of ligands for silver ions in sediments.

23 One category of benthic organism that can bioaccumulate total silver by a factor of 1,000 or more
24 is bivalve mollusks (Ratte, 1999, [195564](#)). As filter feeders, bivalves can accumulate silver ions directly
25 from water and from any silver on or in their food near the sediment/water interface. Terhaar et al.
26 (Terhaar et al., 1977, [202115](#)) determined wet-weight BCF values of up to 1,400 for the freshwater
27 bivalve *Ligumia* spp. In San Francisco Bay, the *Macoma petalum* clam accumulated total silver at
28 concentrations five to seven times higher than in the phytoplankton (Reinfelder et al., 1998, [156047](#)) and
29 acquired between 40 and 95% of the silver through their diet (Griscom et al., 2002, [088266](#)).

30 The extent to which silver transfer from the benthos into aquatic food chains might occur depends
31 on many factors, including bioavailability and the consumer organisms. No studies of bioaccumulation of
32 nano-Ag in benthic invertebrates or their predators were identified.

5.2.4. Terrestrial Ecosystems

1 The uptake of dissolved silver ions and complexes from soils by terrestrial plants and soil micro-
2 and macrofauna has been investigated in a few laboratory and field experiments. Uptake of nano-Ag by
3 soil invertebrates has been investigated in the laboratory.

5.2.4.1. Uptake by Terrestrial Plants

4 Available evidence indicates that terrestrial plants take up silver ions from highly contaminated
5 soils. Agricultural plants grown on soils amended with sewage sludge spiked with Ag₂S showed only
6 below-ground bioconcentration of silver. Total silver concentrations in the roots of corn, lettuce, oats,
7 turnips, and soybeans ranged from 2.0 to 33.8 milligrams per kilogram (mg/kg), whereas silver
8 concentrations in the aboveground parts of the same plants were below detectable levels (<1 mg/kg)
9 (Hirsch, 1998, [224892](#)). Because Ag₂S is less soluble than other forms of silver, however, it is not
10 conclusive that silver accumulation in plant tissues is the result of ionic silver uptake through plant roots.
11 Other studies indicated that other crops and grasses also accumulated more silver in the root systems than
12 in other parts of the plants. Mushrooms accumulated silver to concentrations of 120–150 mg/kg dry
13 weight when grown on compost amended with sewage sludge containing conventional silver (Falandysz
14 et al., 1994, [224886](#)). Concentrations of silver in trees grown in areas subject to silver iodide (AgI) cloud
15 seeding exhibited total silver concentrations between 1 and 13 mg/kg in aboveground leaves, twigs, bark,
16 and wood (Klein, 1978, [224898](#)). In this case, exposure through deposition on leaves would have been
17 possible in addition to root exposures.

18 Harris and Bali (2008, [195522](#)) demonstrated “hyper” accumulation of silver ions by *Brassica*
19 *juncea* (a mustard plant) and *Medicago sativa* (alfalfa), two species known to be metal tolerant. The
20 plants were grown hydroponically from seeds for 4 weeks in demineralized water, and then moved to
21 Petri dishes containing aqueous solutions of AgNO₃ at concentrations up to 1% silver by weight. The
22 ratios of silver in the plant tissues (entire plant assays) to the silver concentration in the aqueous growth
23 medium ranged from 6 to 67 for alfalfa and from 10 to 124 for mustard. TEM with energy dispersive
24 x-ray spectroscopy revealed clusters of large numbers of spherical-shaped silver nanoparticles with a size
25 distribution reportedly centered around 50 nm. The authors did not report which plant tissues were
26 examined (e.g., root, stem, leaves). The sequestration of nano-Ag led the authors to recommend using
27 these plant species to synthesize large quantities of nano-Ag particles.

28 Nowack and Bucheli (2007, [092294](#)) hypothesize that nanoparticles in general could interact with
29 plant roots through several mechanisms, including adsorption onto the surface of roots, assimilation into
30 root cell walls, and uptake into root cells. The authors also suggest that nanoparticles might diffuse into
31 the apoplast (i.e., intercellular space); from this location, they could possibly be taken up by apoplastic

1 membranes or could enter the xylem at sites of damage. Based on observations of the behavior of other
2 metal complexes in plants, it might be possible for nanoparticles to be transported to plant shoots once
3 they are present in the xylem. Silver nanoparticles have not been observed inside plant tissues other than
4 roots, but total silver concentrations in zucchini shoots exposed by roots to nano-Ag in hydroponic
5 solution were almost five times higher than in zucchini shoots exposed to equivalent concentrations of
6 conventional (powdered) silver (Stampoulis et al., 2009, [199839](#)). Those results suggest that nano-Ag
7 formulations facilitate silver uptake by plants compared with conventional silver formulations, perhaps
8 owing to higher silver ion release rates from nano-Ag, with absorption of silver ions (and possibly nano-
9 Ag) by the roots and transport of at least silver ions to shoots.

5.2.4.2. Uptake by Soil Macrofauna

10 The limited available studies suggest that invertebrates may take up silver ions from soil, but this
11 has not been conclusively determined. Nematodes, multicellular, usually microscopic, organisms in soil
12 communities that feed on bacteria and detritus, have been shown to take up nano-Ag. Roh et al. (2009,
13 [195565](#)) demonstrated uptake of nano-Ag particles in the nematode *Caenorhabditis elegans* using
14 genomic, proteomic, and cellular-level endpoints. Three-day-old nematodes cultured on agar growth
15 media with *E. coli* for food were exposed to nano-Ag in water for 24 or 72 hours. The nano-Ag particles
16 (less than 100 nm in size) were dispersed in deionized water by sonication for 13 hours, stirring for
17 7 days, and filtering through a cellulose membrane with pore size of 100 nm to remove nano-Ag clusters
18 in solution. Measures of light scattering using dark-field microscopy indicated uptake of nano-Ag into
19 the body of the nematodes and aggregation of nano-Ag predominantly around the uterine area. The
20 investigators used physical rather than chemical means to disperse the nano-Ag in aqueous solution to
21 provide relevance to environmental exposures, and they demonstrated that nano-Ag was absorbed into the
22 body of the nematode by some unidentified mechanism(s). In a soil environment with organic matter
23 capable of sorbing nano-Ag, however, nano-Ag and silver ions might be largely immobilized and not
24 available for uptake by nematodes.

5.2.4.3. Terrestrial Food Webs

25 As discussed in Section 4.3, the major pathway by which nano-Ag from indoor uses of spray
26 disinfectants could reach terrestrial ecosystems is expected to be application of sewage sludge to soils
27 (e.g., for agriculture). It is also possible that terrestrial organisms might be exposed to nano-Ag in
28 contaminated water from flooding or crop irrigation, or possibly through deposition of nano-Ag
29 suspended in air following outdoor use of nano-Ag products. With the possible exception of areas near
30 silver mining operations (Kramer et al., 1994, [098723](#)), bioconcentration of silver in macroflora and

1 macrofauna of terrestrial ecosystems, even in areas with silver-spiked sludge applications (Hirsch, 1998,
2 [224892](#)), appears not to have been observed. In this section, plant uptake of silver ions in solution or in
3 soils and whether nano-Ag applied to soils could reach herbivorous animals is discussed. The potential
4 for nano-Ag applied to soils in sewage sludge to reach insectivorous wildlife through soil invertebrates in
5 direct contact with soils is considered. Finally, this section considers the potential for indirect ecological
6 effects of nano-Ag as a result of inhibition of soil microorganisms (e.g., decomposing bacteria, nitrifying
7 bacteria, nitrogen-fixing bacteria, fungi), which could disrupt soil nutrient cycling to support food webs.
8 To date, however, no data on the presence of nano-Ag in soils have been published (Wijnhoven et al.,
9 2009, [180201](#)).

Transfer through Terrestrial Plants

10 A single study of plants exposed to nano-Ag in soils or solution was found, that of zucchini
11 exposed to nano-Ag in hydroponic solutions (Stampoulis et al., 2009, [199839](#)). In this study, some form
12 of silver, possibly silver ions, was transported to shoots (see Section 5.2.4.1).

13 Some studies have demonstrated that most silver ions and complexes dissolved in solution and
14 taken up by metal-tolerant plants accumulate in the roots, possibly in intercellular spaces (e.g., apoplast)
15 (Nowack and Bucheli, 2007, [092294](#)), and are not transported to other parts of the plant (Ratte, 1999,
16 [195564](#)). As noted previously, some metal-tolerant terrestrial plants have been demonstrated to hyper-
17 accumulate silver ions from water (Harris and Bali, 2008, [195522](#)). Young *B. juncea* (a mustard plant)
18 and *M. sativa* (alfalfa) plants exposed to high concentrations of AgNO₃ exhibited BCF values greater than
19 10 (ratio of concentration in fresh, hydrated plants to concentration in water), with silver sequestration
20 occurring in the form of large numbers of silver nanoparticle clusters.

21 There is as yet no evidence that nano-Ag added to soils with sewage sludge amendments is likely
22 to accumulate in the foliage, fruits, or vegetables of plants above ground; sufficiently high concentrations
23 of nano-Ag in soils, however, might not yet have been considered. Nano-Ag or silver ions in soil pore
24 water, if taken up by plant roots, might be sequestered in the roots, possibly as nanoparticles in
25 extracellular spaces such as the apoplast. Some silver, however, probably in the form of silver ions, might
26 be transported to plant shoots and beyond. Whether nano-Ag and excess silver in any form in sewage
27 sludge applied to agricultural fields might accumulate in root and tuber vegetables has not yet been
28 investigated.

29 Among herbivorous wildlife, only two groups might be highly exposed to total silver in plants due
30 to their ecology, although the exposures might be negligible with respect to thresholds for toxic effects.
31 Small burrowing mammals that consume plant roots might ingest quantities of nano-Ag stored in the
32 roots of metal-tolerant plants, and grazing animals might ingest silver that accumulates in stems of a
33 variety of herbaceous plants. Herbivorous insects feeding on plant roots and stems also might be exposed

1 to accumulated silver. The potential for silver exposure via these pathways to affect grazing animals and
2 insects has not been investigated. The potential bioavailability of nano-Ag particles sequestered in plant
3 roots of the hyper-accumulating species has not been examined.

Transfer through Soil Macrofauna

4 Thus far, accumulation of silver by soil macrofauna has not been examined, even at hazardous
5 waste sites (Sample et al., 1999, [081977](#)). That silver ions can remain free in soil pore water is unlikely,
6 given the abundance of inorganic and organic materials with which silver ions can bind or form
7 complexes, but studies confirming this have not been conducted. Earthworms (*Lumbricus terrestris*)
8 exposed to Ag₂S in soils for 28 days did not accumulate silver (i.e., BCF of 1.0) (Ewell et al., 1993,
9 [597397](#)).

Disruption of Ecological Functions of Soil Microorganisms

10 Nano-Ag toxicity in soil microorganisms could adversely affect an ecosystem at large. Of
11 particular concern are the possibilities that silver might inhibit bacteria that fix atmospheric nitrogen (in
12 symbiosis with legumes and other plant species), disrupt denitrifying bacteria (which convert nitrates to
13 nitrogen gas), and impair decomposing and lithotrophic bacteria and other microbes that release essential
14 nutrients from inorganic and organic matter in soils (Panyala et al., 2008, [195554](#)).

15 Senjen (2007, [196134](#)) reported that few studies have been conducted that directly evaluate the
16 community-level effects of nano-Ag exposure for soil microbial communities. Many laboratory studies,
17 however, have evaluated the toxicity of nano-Ag to microorganisms that might be found in soils,
18 particularly bacteria (see Section 6.2.1). In theory, disruption of the nutrient cycling roles of soil
19 microorganisms could result in a cascade of adverse impacts on the structure and composition of
20 terrestrial plant communities, and then on the animal communities as well. A risk assessment based on
21 projections for nano-Ag discharge to the Rhine River in 2010 concluded that most silver released into
22 wastewater would be collected in sewage sludge, which often is spread on agricultural fields (Blaser et
23 al., 2008, [193283](#)). Environmental concentrations were predicted for the river water, river sediment,
24 interstitial water of the sediments, and the wastewater entering sewage treatment plants. Although Blaser
25 et al. (2008, [193283](#)) could not exclude possible risks to benthic organisms in the river, they concluded
26 that the nitrifying bacteria in the sewage treatment plants were at negligible risk of impairment.

5.3. Exposure to Humans

1 One obstacle to measuring or estimating exposure of humans to nanoparticles is the lack of
2 consensus regarding the particle properties that require characterization and on which metric to use to
3 express exposure concentrations or dose. Further complicating human exposure assessments, there are no
4 broadly applied methods for distinguishing background and incidental exposures from source-specific
5 contributions to total nanoparticle exposures. For example, atmospheric particle collection methods that
6 collect ultrafine inorganic particles cannot, without further chemical analyses, separate the fraction
7 comprising manufactured nanoparticles (SCENIHR, 2009, [197723](#)). Data on nanomaterial use in
8 consumer products rely on information provided by the manufacturers and do not account for uses that
9 may be “off-label.” In addition, independent investigations of some nano-enabled products have revealed
10 that actual particle size range, concentration, composition, and purity often differ from what is reported by
11 the manufacturer, and different products with different formulations would result in exposure to a variety
12 of substances in conjunction with the nanomaterials added intentionally to the products. This section
13 describes the current methods and data available for evaluating human exposure to nano-Ag from spray
14 disinfectant use in homes and institutions (Section 5.3.1) and during manufacture (Section 5.3.2).

5.3.1. General Population Exposure

15 Chemical form, shape, concentration, ζ potential, exposure route(s), and media concentration(s)
16 have been highlighted by several reviewers as important parameters when evaluating human exposures to
17 nanoparticles. The application scenario (including how much, how long, how often, and how many
18 people use the product) of a chemical also should be considered (SCENIHR, 2009, [197723](#); Wijnhoven et
19 al., 2009, [195898](#)). Despite the rapid penetration of nanomaterials into the consumer market, little
20 information on their content or the content of other ingredients in consumer products is publicly available.
21 The absence of that information precludes a complete exposure assessment.

22 Hansen et al. (2008, [157560](#)) recently developed a framework for conducting general population
23 exposure assessments of products containing nanomaterials. As part of this framework, they categorized
24 the 580 products listed in the 2007 Woodrow Wilson Center consumer product inventory based on
25 whether the nanoparticles were bound to the surface of the product, suspended in liquid, suspended in
26 solids, or available as free, airborne particles. Using these characteristics, exposures were categorized as
27 “expected” (e.g., due to direct exposure to liquids containing nanoparticles or free, airborne
28 nanoparticles), “possible” (e.g., due to release of surface-bound nanoparticles owing to wear and tear), or
29 “not expected” (e.g., particles are encapsulated in solids and are not released). Based on the types of
30 products in which nanomaterials are used, and the exposure scenarios relating to these products, nano-Ag

1 was determined to have the highest possible consumer exposure of all nanomaterials considered, with
2 roughly 25% of the products categorized as “possible” exposures and 50% categorized as “expected”
3 exposures (Hansen et al., 2008, [157560](#)).

4 Expert elicitation has been used to assess potential for exposure to approximately 50 nanomaterials
5 used frequently in consumer products (Wijnhoven et al., 2009, [195898](#)). A panel of seven experts from
6 the Netherlands National Institute for Public Health and the Environment (RIVM) was assembled to
7 independently identify the most significant exposure characteristics and rank consumer products
8 according to potential exposure. Six product categories in which nanomaterials are purportedly used were
9 assigned the rank of “high” potential for exposure: sunblock cosmetics, oral hygiene products, health
10 products, fuel, coatings and adhesives, and cleaning products. Cleaning products (the category under
11 which nano-Ag spray disinfectants would fall) were generally labeled by Wijnhoven et al. (2009, [195898](#))
12 as “do-it-yourself,” suggesting that products requiring application by the user might lead to higher
13 exposures. Characteristics of each product and the nanomaterial in the product were used to generate
14 rankings of potential exposure (high, medium, or low). For cleaning products containing nanomaterials,
15 the expert panel determined that the characteristics of primary concern are the form of the product (e.g.,
16 spray, powder, liquid, suspension, solid, coating), the form of the nanomaterial (e.g., free particles,
17 particles fixed inside a matrix), the potential for direct versus indirect exposure from application (e.g.,
18 direct exposure to nanoparticles in the product, indirect exposure from particles released from the
19 product), and potential exposure route (e.g., inhalation, dermal, oral). The characteristics of nano-Ag
20 sprays, such as the spray form of the product in which particles are free and not fixed, the potential for
21 direct exposure to nanomaterials in the product through application, and the potential for exposure
22 through multiple routes, indicate that nano-Ag disinfectant sprays would be categorized as “high-
23 potential-exposure” products.

24 In one of the few studies that have modeled exposure to nanoparticles, Mueller and Nowack (2008,
25 [157519](#)) analyzed nano-Ag, nano-TiO₂, and carbon nanotube life-cycle use and release into the
26 environment in Switzerland, albeit using substantially simplified assumptions as discussed in Sections 3.1
27 and 4.5. They estimated release rates for products containing nano-Ag and concluded that, although
28 sprays and cleaners account for only 15% of the nano-Ag in consumer products, the bulk of release (95%)
29 from this use occurs during application when the consumer is likely to be exposed (Mueller and Nowack,
30 2008, [157519](#)).

31 Wardak et al. (2008, [157471](#)) created a hypothetical exposure scenario for the use of an air-
32 freshener spray containing nano-Ag that would also act as a disinfectant. Based on exposure scores on a
33 scale from 1 to 5 (from expert elicitation), Wardak et al. (2008, [157471](#)) concluded that the most
34 significant human exposures would occur via inhalation and dermal pathways and that the most
35 significant environmental exposures would result from water entrainment (i.e., suspension of nano-Ag in
36 water) after disposal. This approach, similar to that used by Wijnhoven et al. (2009, [195898](#)), was

1 reported by the authors to be useful for estimating risk when few or no data on environmental
2 concentrations exist. The authors assumed that the nano-Ag would be contained in a liquid matrix
3 (Wardak et al., 2008, [157471](#)). Exposures during and after application were considered possible.
4 Inhalation was estimated to be the primary exposure route, followed by dermal, and to a lesser degree
5 ingestion. Exposure to workers who manufacture or use nano-Ag spray disinfectant in occupational
6 settings is discussed in Section 5.3.2.

5.3.1.1. Respiratory Exposure

7 Consumers could be exposed to nano-Ag particles as a result of inhaling spray disinfectants during
8 application or disposal. Data on spray use and disposal, and on the concentrations of ingredients other
9 than nano-Ag involved in the manufacturing process and disposal of waste by-products, such as capping
10 agents and stabilizers, were not identified in the literature. The concentrations of nano-Ag in end-use
11 products, the application rates, and the use profiles for these products are not yet known (refer to
12 discussion in Section 2.2), but some hypotheses have been proposed. Wardak et al. (2008, [157471](#))
13 independently surveyed eight experts from five areas of expertise (environmental sciences, toxicology,
14 chemistry, material sciences, and technology policy) and asked them to score (from 1 = low to 5 = high)
15 the exposure and hazard potential of nano-Ag disinfectant air-freshener sprays. The authors concluded
16 that inhalation by users represented the highest potential human exposures. The experts believed that
17 nano-Ag spray uses also might result in elimination of useful bacteria in susceptible populations (Wardak
18 et al., 2008, [157471](#)).

19 Children in the home might inhale aerosols containing nano-Ag formed when others are spraying
20 the material; no data were found, however, on the proximity of children to adult disinfectant spraying
21 activities or how long nano-Ag from the air-freshener sprays would remain airborne. Children might be
22 exposed to higher concentrations of airborne nano-Ag near floor level from original spraying activities
23 and from resuspension of dust and nano-particles containing nano-Ag as they crawl or play on the floor.
24 Again, no information was found to quantify this possibility.

5.3.1.2. Dermal Exposure

25 Dermal exposure to nano-Ag could result from spray deposition on the skin or by contact with a
26 surface that has been sprayed or a cleaning accessory used during application (e.g., cleaning rag, paper
27 towel, sponge). The expert elicitation described by Wardak et al. (2008, [157471](#)) for an air freshener
28 scenario indicated that the potential for exposure via skin contact was medium-high, which was greater

1 than the potential for ingestion exposure but less than the potential for inhalation exposure. Individuals
2 with cuts or abrasions of the skin might be more likely to absorb nano-Ag following dermal contact.

3 Children might be a susceptible population for dermal exposure because the skin of infants and
4 young children and young adults has been shown to be more permeable to some substances than that of
5 older adults (Hostynek, 2003, [193435](#)), although no data for nano-Ag were found. Furthermore, children
6 often have cuts and scratches from play activities.

5.3.1.3. Oral Exposure

7 The potential exists for inadvertent ingestion of nano-Ag through hand-to-mouth contact following
8 dermal exposure in the home (Drake and Hazelwood, 2005, [195504](#)). Wardak et al. (2008, [157471](#))
9 concluded that the potential for exposure via ingestion was medium-low, below the potential for
10 inhalation and dermal exposure, and therefore not of primary concern. Inhalation of nano-Ag could also
11 result in exposure via the GI tract following mucociliary clearance of the lung (SCENIHR, 2009,
12 [197723](#)).

13 For children (e.g., toddlers), mouthing of objects that have been sprayed with nano-Ag, and
14 touching or handling sprayed objects and then mouthing their hands, are possible pathways for oral
15 exposures. Although no studies were identified that examined children's exposure to nano-Ag sprayed in
16 the home, the exposure pathways could be pertinent for children in homes where such sprays are used
17 intensively or for cleaning toys.

5.3.2. Occupational Exposure

18 As the number of products containing nanomaterials in commercial distribution increases, the
19 number of workers involved in the manufacturing of nanomaterials is also likely to increase. Lack of
20 knowledge regarding nanomaterial manufacturing processes requires that conservative assumptions be
21 made about potential exposure routes and likelihood of exposure during manufacturing and by-product
22 disposal; therefore, all possible exposure routes (i.e., respiratory, dermal, oral) are currently considered
23 for occupational settings. The Occupational Safety and Health Administration (OSHA) and the National
24 Institute of Occupational Safety and Health (NIOSH) recommend and set limits on conventional silver
25 concentrations in the workplace environment at 0.01 milligram per cubic meter (mg/m^3) (equivalent to 10
26 micrograms per cubic meter [$\mu\text{g}/\text{m}^3$]) for an 8-hour workday and 40-hour workweek. The American
27 Conference of Government Industrial Hygienists (ACGIH) recommends threshold limit values of 0.01
28 and $0.1 \mu\text{g}/\text{m}^3$ for soluble silver and metallic silver, respectively.

29 The differences, sometimes spanning an order of magnitude, between occupational exposure limits
30 set by different agencies led Drake and Hazelwood (2005, [195504](#)) to review the toxicity literature on

1 chronic exposure to conventional silver. They concluded that the potential effects of chronic exposure to
2 conventional silver depend on its chemical form. Soluble forms of silver are more readily absorbed by
3 the body and therefore can more readily cause adverse health outcomes. For example, in an occupational
4 exposure study of workers employed in silver manufacturing (Armitage et al., 1996, [195476](#)), silver
5 reclamation workers exposed to soluble silver compounds showed the highest blood levels, with an
6 average of 6.8 µg/L (range = 1.3–20 µg/L, $n = 19$), while jewelry makers exposed to metallic silver had
7 the lowest, ranging from 0.2 to 2.8 µg/L ($n = 9$). Blood silver levels ranged from 0.1 to 23 µg/L among
8 workers in all of the factories (no exposure levels were measured), while 11 of 15 agricultural workers
9 with no occupational exposure had blood silver concentrations below the detection limit of 0.1 µg/L, and
10 no blood silver concentrations were higher than 0.2 µg/L.

11 Workers exposed to conventional silver and to silver fumes and dusts, which might contain
12 nanoparticles, have displayed clinical symptoms due to dermal, ocular, and inhalation exposures (Drake
13 and Hazelwood, 2005, [195504](#); Panyala et al., 2008, [195554](#); Rosenman et al., 1987, [195566](#)). The
14 literature commonly reports the effects of these exposures, but few specifics are reported on measured
15 exposure levels. In one study where ambient exposure levels were reported, Pifer et al. (1989, [195559](#))
16 evaluated workers exposed to silver via inhalation. Air sampling indicated an 8-hour
17 time-weighted-average airborne silver concentration of 1–100 µg/m³, with most silver present in insoluble
18 forms. Elevated blood silver concentrations (mean of 0.010 µg/mL among 80% with detectable blood
19 silver levels) and body burdens were reported in these workers relative to controls; however, no instances
20 of argyria were reported.

21 Tsai et al. (2009, [193684](#)) measured exposure resulting from the transfer of nano-Ag and nano-
22 alumina powder from inside fume hoods to the worker breathing zones for three common hood designs in
23 one of the only studies reporting on personal levels of exposure associated with the occupational handling
24 of nanomaterials. Despite the use of common laboratory precautions in addition to the hood, the release
25 of airborne nanoparticles into the laboratory environment and the researchers' breathing zone was
26 significant, with the constant-flow hood resulting in the highest breathing zone concentrations. Tsai et al.
27 (2010, [202131](#)) then compared the efficacy of a recent hood design, the air-curtain hood, to the other hood
28 designs using the same procedures, but with aluminum oxide nanoparticles only 27–56 nm in size.
29 Because the nanoparticles could cluster in the bulk powder, the concentrations of airborne particles with
30 diameters measuring from 5 nm up to 20,000 nm were measured in the breathing zone. Release of
31 particles from the hood was negligible for particle clusters greater than 500 nm in size; releases of clusters
32 in this size range were again highest for the constant-flow hood. Also using the constant-flow hood, when
33 the nanomaterial was poured instead of manually transferred from one beaker to another, particle
34 concentrations (primarily 100- to 200-nm clusters) ranging between approximately 500 and 1,500
35 particles per cubic centimeter (cm³), with a peak at 7,000 particles per cm³, occurred in the breathing
36 zone. Releases were lower for the air-curtain hood, however, ranging from non-detectable to

1 approximately 500 particles per cm³. These studies suggest that procedures generally considered
2 adequate to protect workers during handling of harmful substances might not be sufficient to protect them
3 while handling nanomaterials.

4 Once released in an occupational setting, nanoparticles can be inhaled and might deposit in the
5 lungs (Kreyling et al., 2002, [037332](#); Oberdorster et al., 1995, [044720](#)). A study by Park et al. (2009,
6 [225210](#)) appears to be the only occupational exposure study to date that has examined workplace
7 exposure specifically to nano-Ag during the manufacturing process. For this study, the investigators
8 assessed exposure to nano-Ag in the liquid phase during a wet chemical process at a commercial
9 production facility in Korea. Although most field studies that have analyzed nanomaterial exposure in the
10 workplace have concentrated on the gas-phase production process because of the clear potential for
11 exposure through inhalation of powders and aerosol particles, the investigators argue that the potential
12 remains for exposure to nanomaterials during liquid-phase processes, which are frequently used to
13 manufacture nano-Ag. In this study, Park et al. (2009, [225210](#)) report that the production of nano-Ag at
14 the facility involves a four-stage process: (1) batch reaction based on wet chemical methods, (2) filtering,
15 (3) drying, and (4) grinding. Of these stages, the investigators demonstrated that at least three had the
16 potential for worker exposure (filtering was not explicitly described). During the batch reaction process,
17 the nano-Ag reaction mixture is allowed to age before the filtration stage. The real-time air-particle
18 monitor in the reaction room was located 1 meter from the hatch of the main reactor, which was under a
19 ventilation hood. Nanoparticle concentrations in the room were about 6×10^4 , 5×10^4 , and
20 2×10^4 particles per cm³ for particles with average diameters of 100 ± 5 nm, 200 ± 5 nm, and 20 ± 5 nm,
21 respectively, after about 13 hours. At that point, the reactor hatch was opened for 1 hour to allow for
22 sampling of particles for nano-Ag identification. During that time, the concentrations of the 20- and
23 200-nm nanoparticles in the room air remained relatively stable, but the concentration of 100-nm
24 nanoparticles increased to about 9×10^4 particles per cm³. TEM images indicated that the particles
25 collected in the reaction room were nano-Ag. According to Park et al. (2009, [225210](#)), nano-Ag 50–60
26 nm in diameter formed “agglomerates” in the room air. Concentrations of nano-Ag were estimated by
27 subtracting the background particle concentrations before operations started from the particle
28 concentrations during the silver processing. Similar results were observed during the grinding and drying
29 processes. When the dryer door was opened at the end of the drying period, the number of 60- to 100-nm
30 particles in the air doubled. During the grinding process (1 hour), irregular increases in nanoparticles in
31 the air were observed as workers disturbed particles that had settled on the floor. After the ventilation
32 system was turned on, these irregular increases were reduced. When the grinder hatch was opened,
33 however, the concentration of 30- to 40-nm particles in the air spiked. These results indicate that nano-
34 Ag in a solution can be aerosolized in the workplace air, where workers can inhale the nano-Ag particles
35 (Park et al., 2009, [225210](#)) and that the particles can deposit on exposed skin. Nano-Ag in the air might
36 also settle on clothing or on floors, eventually resulting in a secondary exposure when the nano-Ag is

1 disturbed during clothing removal, sweeping, or walking in a room without an adequate ventilation
2 system.

3 Unlike traditional occupational exposure studies like that of Park et al. (2009, [225210](#)), which
4 focus on workers involved in extracting or refining the material, exposure to a nano-Ag disinfectant spray
5 could involve occupational use by janitorial service workers who might be chronically exposed. No
6 studies measuring or modeling this type of exposure to a nanomaterial were found in the literature.

7 Additional information on exposure to nano-Ag and associated substances during the
8 manufacturing process of nano-Ag and disinfectant sprays and on occupational use of end products would
9 be useful to understand chronic workplace exposures more fully. Traditionally, such information has also
10 proved relevant to the study of subchronic exposure through inference; mechanisms and health effects can
11 be extrapolated to lower exposures in the general population before a complete body of research is
12 available. Occupational exposures historically have been the first indication of toxic effects that
13 ultimately might be occurring more broadly or more subtly within the population.

5.4. Aggregate Exposure to Nano-Ag from Multiple Sources and Pathways

14 Nano-Ag has been advertised as a constituent in at least 259 consumer products currently on the
15 market (Project on Emerging Nanotechnologies, 2009, [196774](#)), although the content of nano-Ag in these
16 products has not been verified. Regardless, humans and biota likely could be exposed to nano-Ag from
17 multiple sources and through multiple pathways. Potential routes of exposure to humans from some
18 possible nano-Ag applications are provided in Table 5-1.

Table 5-1. Nano-Ag applications and potential routes of exposure for humans.

| Product category | Product subcategory | Product examples | Expected exposure route |
|-----------------------------|----------------------------|---|-------------------------|
| Food and beverage | Cleaning | Food product sterilizing sprays | Inhalation, dermal |
| | Cooking utensils, coatings | Cutting and chopping boards, kitchenware and tableware, baby-bottle brushes | Dermal, oral |
| | Storage | Refrigerator fresh boxes, storage bags and containers, baby bottles, mugs | Dermal, oral |
| | Supplements | Colloidal metal in water | Oral |
| Personal care and cosmetics | Skin care | Body creams, hand sanitizers, beauty soaps, face masks | Dermal |
| | Oral hygiene | Tooth brushes, tooth cleaners, toothpastes | Oral |
| | Cleaning | Elimination wipes and sprays | Inhalation, dermal |
| | Hair care | Hair brushes, hair masks | Dermal |
| | Baby care | Pacifiers, tooth developers | Dermal |

Table 5-1. Nano-Ag applications and potential routes of exposure for humans (continued).

| Product category | Product subcategory | Product examples | Expected exposure route |
|--|---------------------------|--|--|
| Personal care and cosmetics | Over-the-counter products | Foams, condoms | Dermal |
| Textiles and shoes | Clothing | Fabrics and fibers, socks, shirts, caps, jackets, gloves, underwear | Dermal, oral |
| | Other textiles | Sheets, towels, shoe care, sleeves and braces | Dermal, oral |
| | Toys | Plush toys | Dermal, oral |
| Electronics | Personal care | Hair dryers, wavers, shavers | Dermal |
| | Household appliances | Refrigerators, washing machines | Dermal |
| | Computer hardware | Notebooks, (laser) mouse, keyboards | Dermal |
| | Mobile devices | Mobile phones, game systems | Dermal |
| Household products/home improvement | Cleaning | Cleaning products for bathroom, kitchen, toilets; detergents, fabric softeners | Inhalation, dermal, oral |
| | Coating | Sprays, paint supplements | Inhalation, dermal |
| | Furnishing | Pillows | Dermal |
| | Furnishing/coating | Showerheads, locks, water taps | Inhalation, dermal |
| Filtration, purification, neutralization, sanitization | Filtration | Air filters, ionic sticks | Inhalation |
| | Cleaning | Disinfectant sprays | Inhalation, dermal |
| Medical products | Anesthesiology | Breathing masks, endotracheal tubes | Inhalation |
| | Neurosurgery, Cardiology | Catheters | Intravascular, intrathecal, intravesical, urethral |
| | Eye care | Contact lenses | Ophthalmic |
| | Patient care | Incontinence materials | Dermal |
| | Orthopedics | Implants, stockings | Intramedullary, dermal |
| | Pharmaceuticals | Dermatitis, acne, ulcerative colitis treatments; HIV-1 replication inhibition | Oral, dermal |
| | Surgery | Gowns, face masks, slings for reconstructive surgery | Inhalation/dermal/ intraperitoneal |
| | Wound care | Hydrogel for wound dressing | Dermal |

Adapted from Wijnhoven et al. (2009, [180201](#)).

1 Because nano-Ag might be used in various applications, numerous pathways exist for human and
2 ecological exposures, some of which could overlap, resulting in aggregate exposures to nano-Ag from
3 many sources. Many of the nano-Ag applications listed in Table 5-1 could be used by the same persons at
4 approximately the same time. For example, an individual might inhale aerosol particles from a nano-Ag
5 spray used to clean and disinfect surfaces in the home, wear a bandage containing nano-Ag on the skin,
6 and consume an oral nano-Ag dietary supplement. Also, a single product could lead to exposure through
7 multiple routes (Wijnhoven et al., 2009, [180201](#)). While using a spray disinfectant, the nano-Ag solution
8 might be sprayed unintentionally on the skin, the aerosol particles inhaled (and possibly coughed up and
9 subsequently swallowed), and ingested from foods in contact with the disinfected surfaces.

10 As one or more nano-Ag products are used in the home, particularly disinfectant sprays, nano-Ag
11 could accumulate on surfaces and in airborne dust. Removal mechanisms for airborne nano-Ag are

1 limited to normal leakage and, perhaps less commonly, slow transfer to outdoor air when windows are
2 open, transfer through a central vacuum system, or capture of particles on heating and cooling system
3 filters, which generally have not been designed for this purpose. High efficiency particulate air (HEPA)
4 filters can remove some proportion of nanoparticles in the air, but are only designed to remove 99.7% of
5 particles 300 nm or larger. Buildup of nano-Ag in carpets, furniture, and floors might lead to higher
6 exposure of children and pets in particular.

7 Biota also could be exposed to nano-Ag through multiple sources and routes, but exposure might
8 be significantly affected by environmental parameters (Luoma, 2008, [157525](#)). Although initially
9 released as nanoparticles, the subsequent fate and transport of the particles depends on many
10 environmental characteristics in the receiving media. Nanoparticles in surface waters might associate to
11 form microparticles or sorb to other materials and fall out of suspension into sediments. Water conditions
12 and chemistry might make the nanoparticles either more or less available for uptake by biota (Navarro et
13 al., 2008, [157517](#)). Fate and transport of nano-Ag in the environment are discussed in Chapter 4 of this
14 document, and bioavailability of nano-Ag in different environmental media is discussed in Section 5.2.1.

15 Although exposure of biota to nano-Ag can be mitigated through various environmental processes,
16 some exposure is likely given that many nanoparticles are engineered to maximize their dispersion in
17 water (Balogh et al., 2001, [196282](#); Lee et al., 2007, [194072](#)). Development of biocidal nano-Ag
18 products for potential use in the home (e.g., clothes washers, surface sprays, cosmetics) or in occupational
19 settings (e.g., industrial misters and foggers, architectural coatings, water filters) could lead to the release
20 of nano-Ag during manufacturing, use, and disposal. Once released into sewer systems, very small
21 nano-Ag particles that escape filtration-capture during wastewater treatment can be released back into
22 aquatic ecosystems, where they could impact biota that are particularly susceptible to aggregate exposure
23 via direct uptake from the water and ingestion of contaminated prey (Navarro et al., 2008, [157517](#)). In
24 cases where sewage sludge from wastewater treatment is applied to land for soil amendments or for
25 disposal, nano-Ag might be absorbed by plants, leached to groundwater, or contained in runoff to surface
26 waters (Blaser et al., 2008, [193283](#)). Terrestrial biota might then be exposed to nano-Ag through
27 ingestion of contaminated soil and prey, as well as through contact with contaminated media.

28 The simplified exposure scenarios mentioned above assume that the nano-Ag entering the
29 environment would remain in its current form; however, the surface chemistry of nano-Ag might be
30 significantly altered as a result of “aging” and transformation processes in complex environmental
31 systems (as discussed in Chapter 4). Chemical and biological transformations of nano-Ag might occur as
32 a result of oxidation-reduction (redox) reactions, particle dissolution, or interactions with pollutants or
33 organic matter, which, in turn, might result in changes in particle form, clustering, transport, and
34 pathways of exposure (Wiesner et al., 2009, [194996](#)). The susceptibility of nano-Ag to transformation

1 and complexation might limit exposure to nano-Ag itself, while increasing exposure to nano-Ag
2 complexes, silver ions released from nanoparticle, and other transformation products (See Section 5.2).

5.5. Cumulative Exposure to Nano-Ag and Other Contaminants

3 Given their high surface area-to-volume ratio and enhanced chemical reactivity, nanoparticles can
4 modify the bioavailability of other toxic agents, such as manufacturing by-products, transformation
5 products, waste products, and other contaminants present in the environment. Moreover, given the
6 possible processes by which nanoparticles sorb to or are absorbed into cell walls and cells (see Section
7 5.7), they also might act as carriers of other chemicals or nanomaterials onto or into cells. Thus, nano-Ag
8 particles serving as carriers could increase exposure of organisms to additional toxic agents.

9 Nano-Ag might be coated with agents that exhibit toxicity during manufacturing or adhere to toxic
10 agents after release into the environment. Navarro et al. (2008, [157517](#)) observed that metallic
11 engineered nanoparticles often are coated with inorganic or organic compounds or surfactants (e.g.,
12 sodium dodecyl sulfate) to maintain a colloidal suspension of the nanoparticles in the end product. In the
13 future, nano-Ag might be combined with other materials to enhance certain properties for specific end
14 uses. Cumulative exposure to other substances released during the manufacturing process and other
15 ingredients of disinfectant sprays might also be a relevant consideration.

16 The potential for nano-Ag releases to result in increased exposure to manufacturing by-products or
17 transformation products in the environment is discussed in Section 5.5.1. Whether nano-Ag specifically
18 might facilitate absorption of other toxic agents or nanomaterials is discussed in Section 5.5.2. Evidence
19 that some other types of nanoparticles facilitate absorption of other contaminants by living organisms is
20 presented in Section 5.5.3.

5.5.1. Nano-Ag By-Products and Transformation Products

21 At this time, no information suggests that nano-Ag manufacturing processes result in the formation
22 of hazardous by-products; however, relatively few data on large-scale manufacturing of nano-Ag are
23 currently available. Information is similarly lacking regarding other materials used in manufacturing
24 other ingredients of disinfectant sprays. Manufacturing of nano-Ag might therefore result in increased
25 exposure to hazardous by-products, and nano-Ag might facilitate the absorption of toxic by-products in
26 living organisms. This consideration is relevant in toxicity testing as well. For example, Samberg et al.

1 (2010, [625612](#)) found that “unwashed” nano-Ag received from a commercial producer of nanomaterials
2 was toxic to human epidermal keratinocytes in vitro, with significant dose-dependent decreases in
3 viability, whereas the same nano-Ag product washed five times did not diminish cell survival. The
4 investigators concluded that the residual formaldehyde solvent and methanol by-product from the
5 production of the silver nanomaterial were probably responsible for the observed toxicity.

6 Once released to the environment, nano-Ag can complex with or sorb to natural dissolved materials
7 (e.g., dissolved organic matter, inorganic compounds) or other nanomaterials in water or soils (Boxall et
8 al., 2007, [157712](#); Nowack and Bucheli, 2007, [092294](#); Wijnhoven et al., 2009, [180201](#)). Once nano-Ag
9 particles, without surface coatings, are released to waters or wastewaters, sorption to macroparticles that
10 settle out of suspension might occur rapidly (Baun et al., 2008, [157598](#); Boxall et al., 2007, [157712](#)).
11 Forming larger clusters with other molecules reduces the specific surface area of the particles and,
12 therefore, their reactivity with other materials (Boxall et al., 2007, [157712](#)). In natural surfacewaters,
13 nanoparticles could also sorb to suspended solids that settle out of the water column (Boxall et al., 2007,
14 [157712](#)), although studies of this phenomenon specific to surfacewaters are lacking. On the other hand,
15 Navarro et al. (2008, [157517](#)) suggested that sorption of nanoparticles to low-molecular-weight NOM
16 might increase bioavailability of nanoparticles and increase the chances of other contaminants “hitching a
17 ride” with the low-molecular-weight organic material and nanoparticles into aquatic organisms in
18 particular.

5.5.2. Examples of Nano-Ag Facilitating Absorption of Other Contaminants

19 Silver nanoparticles act as carriers of silver ions, possibly delivering them directly to a biological
20 surface or into cells, where they might interact directly with the cell machinery (Asharani et al., 2009,
21 [195477](#); Hussain et al., 2005, [088101](#); Lee et al., 2007, [194072](#); Miura and Shinohara, 2009, [098728](#)). If
22 nano-Ag particles adhere to cell surfaces, they can serve as a continuous delivery system for silver ions to
23 the cell. If this is the case, greater human health and ecological risks can be expected from nano-Ag than
24 from comparable quantities of ionic silver because not all silver ions that are free in solution will
25 necessarily come in contact with a biological surface. No reports that nano-Ag facilitates the delivery or
26 entry of other toxic chemicals to or into living organisms were found in the readily available literature;
27 other nanomaterials, however, have been shown to facilitate the absorption of other substances (see
28 Section 5.5.3).

5.5.3. Examples of Nanoparticles Facilitating Absorption of Other Contaminants

1 Nano-Ag spray disinfectant formulations could contain other chemicals with which nano-Ag might
2 react, resulting in a synergistic effect that facilitates the uptake of the other contaminants. Although this
3 has not been demonstrated for nano-Ag, studies have shown synergistic uptake of contaminants occurring
4 with polymer fumes (Johnston et al., 2000, [016073](#)), diesel particulate matter (Wallace et al., 2007,
5 [090137](#)), and some nanoparticles, which are described further in this section. Furthermore, medical
6 applications are being developed using nanoparticles as carriers for targeted drug delivery (McNeil, 2009,
7 [098727](#)). In general, however, the capacity of nanoparticles to sorb and facilitate uptake of other
8 contaminants depends on the structure and composition of the nanoparticle.

9 Zhang et al. (2007, [090114](#)) found an increase in the accumulation of cadmium in the gills and
10 viscera of carp in the presence of nano-TiO₂. Similarly, Sun et al. (2007, [193662](#)) found an increased
11 accumulation of arsenic in carp exposed to arsenate (As[V]) in the presence of TiO₂. The As(V) sorbed
12 quickly to nano-TiO₂ in the water. Both nano-TiO₂ and arsenic concentrations were highest in the
13 intestines, stomach, and gills, and somewhat lower in the liver, muscles, and skin. Much of the internally
14 accumulated arsenic might have been released from nano-TiO₂ at the epithelium of the gills and GI tract.
15 Some nano-TiO₂ reached the liver as well, presumably with sorbed arsenic.

16 Baun et al. (2008, [202667](#)) evaluated the potential effects of C₆₀ nanoparticles (Buckminster
17 fullerenes, or buckyballs) on the bioavailability of this substance, as measured by the toxicity of various
18 organic toxicants to the green alga *Pseudokirchneriella subcapitata* and the freshwater invertebrate
19 *Daphnia magna*. They observed no change in the toxicity of methyl parathion to the algae or the
20 daphnids in the presence of C₆₀, while the toxicity, and by inference the bioavailability and uptake (see
21 Section 5.2.3), of pentachlorophenol was reduced 1.9-fold in the presence of C₆₀. The toxicity of
22 phenanthrene to daphnids, on the other hand, was enhanced by 60% in the presence of C₆₀. Analysis
23 showed 85% sorption of phenanthrene to C₆₀-aggregates (Baun et al., 2008, [202667](#)). In contrast to
24 nano-Ag, however, C₆₀ nanoparticles form spherical molecular cages that can carry molecules trapped to
25 some degree in their interior. This mechanism is not expected for nano-Ag particles.

26 Moore (1997, [202119](#)) reported uptake of sucrose polyester nanoparticles in sea water by the
27 hepatopancreas of whole mussels. The uptake of sucrose polyester nano-“droplets” increased the uptake
28 (160%) and cellular toxicity (122%) of anthracene, a polycyclic aromatic hydrocarbon (PAH).
29 Anthracene damaged the lysosomal system (measured as lysosomal membrane stability) in the
30 hepatopancreatic cells, indicating that although the sucrose polyester was not biodegradable (even in
31 lysosomes), the PAH must have been released into the cell (Moore, 2006, [089839](#)).

32 Despite these examples, however, there are no indications that the structure of nano-Ag particles is
33 likely to facilitate uptake of other contaminants into biota. Clusters of nano-Ag might house other

1 chemical contaminants in the inter-particle spaces; however, clusters of nano-Ag also might be less likely
2 to be absorbed because of their larger size than nano-Ag particles.

5.6. Models to Estimate Exposure

3 Models can be used to provide initial estimates of potential release scenarios, behavior in the
4 environment, exposure pathways, dosimetry, and toxicity, provided that the attributes of nano-Ag particles
5 that influence fate, transport, and dosimetry are adequately considered. Modeling focused on tracking
6 environmental transport, transformation, and fate after release can assist in estimating the potential for
7 human and biotic exposures, linking release estimates with models of uptake and dose (Shatkin, 2008,
8 [180065](#)). EPA uses various models to estimate exposures for chemical assessments, some of which are
9 described on the websites for the Council for Regulatory Environmental Modeling (U.S. EPA, 2009,
10 [196065](#)) and the Center for Exposure Assessment Modeling (U.S. EPA, 2009, [196064](#)). For example, the
11 Exposure and Fate Assessment Screening Tool Version 2.0 (E-FAST V2.0) is a publicly available program
12 that EPA uses for screening-level assessments of conventional industrial chemicals. The tool provides
13 estimates of aquatic, general population, and consumer exposure based on chemical release data
14 (U.S. EPA, 2007, [196060](#)).

15 Quantifying exposure or dose using measured environmental or occupational concentrations is not
16 yet possible because nano-Ag concentrations have not been widely measured in relevant media. Instead,
17 exposure concentrations could be estimated using a fate and transport model (Section 4.5) with inputs
18 based on measured or assumed release scenarios (not covered in detail in this case study). Potential and
19 internal doses can be predicted by models of dosimetry/pulmonary deposition (Section 5.7.3),
20 pharmacokinetics (Section 5.7.1), and bioaccumulation (Section 5.2.1.3). Mode-of-action models can be
21 used to estimate doses delivered to target organs. All models described in these sections use chemical
22 concentrations on a mass basis (e.g., mg/L, mg/kg) as the basis for predicting chemical behavior
23 (e.g., diffusion along concentration gradients). The applicability of this approach for nanomaterials,
24 which exhibit some properties and behaviors that cannot be attributed strictly to changes in mass
25 concentration, has not yet been determined.

5.7. Human Uptake and Dose

26 As described previously in this chapter, internal dose is the amount of a substance that enters an
27 organism by crossing a biological barrier. Quantifying internal dose, or at least administered (i.e.,
28 potential) dose (e.g., quantity inhaled or ingested whether absorbed or not), enables estimation of

1 individual or population-level risk, or both (U.S. EPA, 1992, [090324](#)). Measuring and understanding the
2 dose-response relationship is integral to predicting the human health impacts resulting from an exposure.
3 Because nanoparticles possess unique, size-dependent properties that are not necessarily related to mass,
4 however, their uptake and dose are not understood as well as that of traditional substances, which
5 typically use mass as a dose metric (Borm et al., 2006, [088034](#)). A summary of the various metrics that
6 might be used to best characterize nano-Ag dose is presented in Chapter 2. This current section builds on
7 that summary by presenting information on uptake of nano-Ag and dose levels in laboratory mammals.
8 Current knowledge on uptake and dose of nano-Ag in humans is also presented when available, but most
9 information on this topic is inferred from studies involving laboratory mammals.

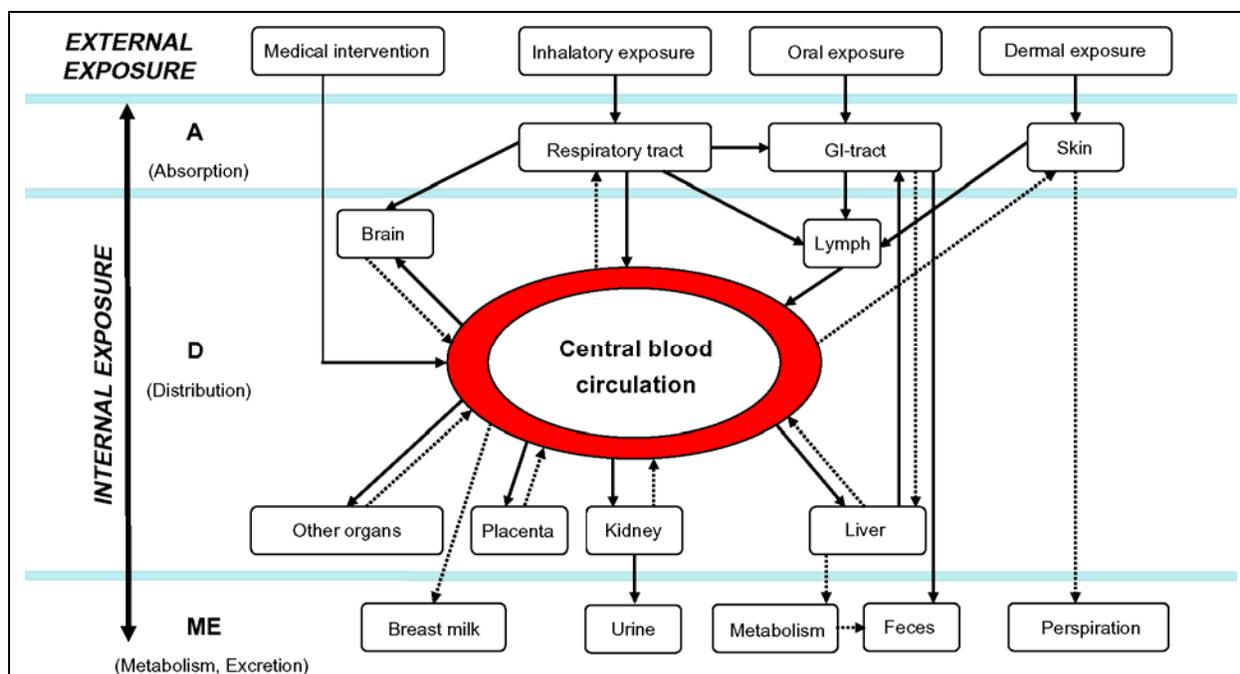
10 This section begins by summarizing what is known regarding the internal behavior (i.e.,
11 pharmacokinetics) of nano-Ag. A discussion of uptake and dose in laboratory mammals then follows.
12 Uptake of nano-Ag through different routes has been investigated predominantly in laboratory rats. For
13 all terrestrial organisms, including laboratory animals used for toxicological studies and as models for
14 human health effects, the route of exposure is critical in determining the dose that ultimately enters the
15 body. Information relevant to nano-Ag uptake and dose to humans is therefore presented here according
16 to the inhalation, ingestion, and dermal routes of uptake. This section concludes with a brief discussion of
17 models for estimating dose of nano-Ag.

5.7.1. Pharmacokinetics

18 Pharmacokinetics, the study of the fate of a substance after it has entered a body, encompasses the
19 absorption, distribution, metabolism, and excretion of a substance. By extension, toxicokinetics focuses
20 on the fate of a toxic substance once present within the body. Understanding toxicokinetics is essential to
21 understanding a toxic substance's mechanism of action and resulting toxicity. Figure 5-1 illustrates an
22 overview developed by Hagens et al. (2007, [157688](#)) of physiological paths by which nanoparticles have
23 been confirmed to travel, and other paths that are hypothesized to be relevant. Although this figure was
24 developed for nanoparticles in general, and thus does not indicate which pathways are more relevant for
25 nano-Ag, it could serve as a framework within which the pharmacokinetics of nano-Ag could be
26 represented once adequate data are available.

5.7.1.1. Absorption

Absorption encompasses the events that lead from external exposure of a substance to its uptake
and transport to the central blood circulatory system. The concept of dose involves the absorption or
uptake of a substance, and in vivo studies of dose often focus on the amount of a substance absorbed by
an organism (i.e., how much of the substance moves from the external environment to the internal space



Source: Hagens et al. (2007, [157688](#)).

Figure 5-1. Absorption and uptake of nanoparticles and transport to the central blood circulatory system.

This figure, developed by Hagens (2007, [157688](#)), depicts the organs and other parts of the body involved in the absorption, distribution, metabolism, and excretion (i.e., the pharmacokinetics) of a nanoparticle that enters the body. Solid lines show paths that have been confirmed to pertain to nanoparticles; dotted lines represent hypothetical routes. Although not all-inclusive, this figure illustrates how nanoparticles might enter and be transported to various parts of the body.

1 of an organism). Penetration into the body depends on the specific properties of the nanoparticle,
 2 including charge, hydrophobicity, surface coating, and the physiology of the particular organ.
 3 Studies identified for this case study that examine the absorption of a substance are discussed later in this
 4 section according to exposure route. An evolving concept relevant to the discussion of absorption of a
 5 nanoparticle into the body is its “corona” (Cedervall et al., 2007, [202112](#)). A corona is a layer of
 6 biomolecules that forms on the surface of a substance once it is absorbed into a physiological system.
 7 This layer can have important implications regarding how the substance interacts with the surrounding
 8 tissue. The concept of a corona is not particularly new or unique to colloid science or the study of
 9 nanoparticles; for example, Lynch and Elder (2009, [195896](#)) note that researchers using medical devices
 10 have been aware of the same phenomenon. When a nanoparticle contacts extracellular bodily fluids,
 11 proteins and other molecules compete to attach to the particle surface, thereby coating the nanoparticle.
 12 Because of the extremely high surface area-to-volume ratio of a nanoparticle, the absorption potential is
 13 significantly greater than for larger particles. Once the particle is encapsulated by these biomolecules, it
 14 is this corona that encounters the cell surfaces and might determine a cell’s initial reaction to the particle
 15 (Lynch and Elder, 2009, [195896](#)). One complicating factor in studying this phenomenon is that the

1 composition of the corona is not static. Instead, its composition is determined largely by competitive
2 binding, and there is a constant tendency toward equilibrium between the corona and its surroundings
3 (Cedervall et al., 2007, [202112](#)). In a recent review, a European Commission scientific committee noted
4 that the composition of the corona is thought to determine, in part, a particle's ability to cross membranes
5 and enter cells or organelles (SCENIHR, 2009, [197723](#)). For example, a particle coated with
6 polyethylene glycol polymer was not available for cellular uptake, thereby increasing the particle's
7 lifetime in the blood, whereas serum albumin (a plasma protein) coatings increased nanoparticle uptake
8 by macrophages. For the current case study, no information specific to the effect of protein coatings on
9 particles of nano-Ag was identified.

5.7.1.2. Distribution

10 It appears that nanoparticles can be distributed via blood circulation following absorption into the
11 body. Anecdotal case reports of medicinal exposures and occupational studies suggest that humans
12 exposed to conventional silver and nano-Ag through various routes in occupational or medicinal settings
13 have shown elevated levels of silver in their blood and urine (Section 6.3.3). Few controlled studies
14 examining the systemic distribution of nano-Ag were identified. Because there appear to be differences
15 in distribution according to the route of exposure, general observations on distribution are presented
16 briefly here and are further described in Section 5.7.2 on human uptake and dose by route.

17 As demonstrated by the studies described in the next section, distribution of silver throughout the
18 body via blood circulation might be widespread following exposure to nano-Ag and subsequent
19 absorption. This pattern would be consistent with the general behavior of nanoparticles following
20 absorption, where they appear to have the potential to distribute to most, if not all, organs throughout the
21 body (Hagens et al., 2007, [157688](#)). Information presented by these authors suggests, however, that the
22 relative extent of distribution to various organs is not well understood for nanoparticles in general, and
23 patterns among substances might vary. For example, the authors note that it is not clear how readily
24 nanoparticles in circulation can cross the blood-brain barrier or how nanoparticles are eliminated from the
25 body.

26 Two studies that demonstrated general distribution of silver in the body following exposure to
27 nano-Ag are summarized here. In a 28-day study of the pharmacokinetics of nano-Ag in rats, Kim et al.
28 (2008, [193473](#)) observed that orally administered nano-Ag resulted in distribution of silver to the
29 stomach, liver, kidneys, lungs, testes, brain, and blood, with dose-dependent accumulation rates reported
30 for all of these tissues (organs are listed in decreasing order of observed silver concentration). In the
31 kidney, accumulation of silver was observed to be gender-specific, with females accumulating about twice
32 the mass of silver as males. In a similar study conducted by Sung et al. (2009, [193664](#)), systemic
33 distribution of silver in rats was also reported for animals exposed to aerosolized nano-Ag via inhalation.

1 Statistically significant ($p < 0.01$) increases in silver concentrations were reported in the lungs, liver,
2 kidneys, brain (excluding the olfactory bulb), and whole blood (organs are listed in order of decreasing
3 silver concentration). Although these studies have been reported in some secondary sources as having
4 demonstrated distribution of nano-Ag to internal organs (e.g., European, 2009, [626420](#); Hussain, 2009,
5 [193440](#)), neither of them actually examined tissues for nano-Ag particles following necropsy. Total silver
6 concentrations in the various tissues were determined after wet digestion using an atomic absorption
7 spectrophotometer with a Zeeman graphite furnace in both studies. Light microscopy was used to
8 identify histopathological changes in various tissues. In their review, Wijnhoven et al. (2009, [180201](#))
9 noted that no studies have determined whether silver distributed to various rat tissues following oral,
10 inhalation, or dermal exposure to nano-Ag remains in nanoparticulate form; all studies measured only
11 total silver concentrations.

12 For substances that are absorbed by the body into the blood, transfer to the brain is generally
13 restricted by the blood-brain barrier. Examples of conventional silver crossing the blood-brain barrier
14 have been identified in the literature and summarized in at least one review (Lansdown, 2007, [195538](#)).
15 However, consensus on this phenomenon has not been reached, and reports of functional consequences
16 are inconsistent, suggesting that penetration of this barrier by conventional silver is low (Lansdown, 2007,
17 [195538](#)). With respect to silver present as nano-Ag, one study used scanning electron microscopy to
18 demonstrate nano-Ag particles in brain tissues of rats following subcutaneous injection of nano-Ag (Tang
19 et al., 2008, [195575](#)). The authors suggested that nano-Ag could penetrate the blood-brain barrier by
20 transcytosis (i.e., the transport of substances into the interior of a cell by way of vesicles or intracellular
21 sacs); only brain tissues, however, were examined for nano-Ag particles. A separate possible route to the
22 central nervous system specific to inhaled substances, however, is via the olfactory nerve, which connects
23 the nasal cavity with the olfactory bulb in the brain. This potential method for distribution of nano-Ag is
24 discussed below in the section describing dose of inhalation exposures.

25 No animal studies identified in the literature describe the distribution of nano-Ag following
26 controlled dermal application of nano-Ag, but the use of nano-Ag in topical burn treatment might provide
27 useful information. In one case study, a teenage burn patient was treated with a nano-Ag coated mesh
28 applied over the burned skin, and liver and kidney effects and skin discoloration from silver absorption
29 were observed (Trop et al., 2006, [195576](#)). In another case, a burn patient developed neurological
30 problems following a 2-week exposure to a cream containing silver. Upon autopsy four months later,
31 elevated silver concentrations were detected in the brain, indicating that silver must have crossed the
32 blood-brain barrier, but a complicating factor in this case was a pre-existing kidney condition (Iwasaki et
33 al., 1997, [195527](#)). As described in Section 5.7.2.2, absorption of nano-Ag through damaged dermal
34 tissues (as was present for the burn patients in the aforementioned cases) is greater than for healthy tissue
35 (Larese et al., 2009, [193493](#)).

5.7.1.3. Metabolism

1 The liver typically serves a detoxifying function in the body by removing harmful substances from
2 the blood and metabolizing them to forms that can be more easily excreted from the body. Once nano-Ag
3 particles enter the GI tract, either as a result of absorption or distribution, the particles would presumably
4 enter the portal vein for transport to the liver. No evidence of metabolism of nano-Ag by liver enzymes
5 has been reported (Wijnhoven et al., 2009, [180201](#)). This observation is not unexpected, given that inert
6 metals are typically not transformed by the body into different chemical forms. Wijnhoven et al. (2009,
7 [180201](#)) suggested that nano-Ag might bind to metallothioneins, which are proteins that bind to metals
8 and are involved in metal regulation and transport out of cells; the authors, however, present no specific
9 evidence for this activity.

5.7.1.4. Excretion

10 The European Commission's Scientific Committee on Emerging and Newly Identified Health
11 Risks (SCENIHR) (2009, [197723](#)) suggested that two physiological methods of excretion can occur for
12 nanoparticles that have been absorbed in the body and are present in the circulatory system. Clearance of
13 nanoparticles by urination requires that nanoparticles be absorbed by the gut epithelium and undergo
14 glomerular filtration in the kidneys; the nanoparticles would then be shunted to the bladder and excreted
15 in the urine. Alternatively, nanoparticles could travel in bile from the liver to the intestine and be excreted
16 in feces (SCENIHR, 2009, [197723](#)). Other pathways of excretion might also exist, including transport
17 out of the body through sweat or saliva, but no information was identified regarding these methods.
18 Wijnhoven et al. (2009, [180201](#)) have suggested that these other routes might be less important but
19 provided no firm evidence as to why.

20 Human studies of occupational exposures have shown that exposure to conventional silver results
21 in elevated silver levels in feces and urine (Pifer et al., 1989, [195559](#); Rosenman et al., 1987, [195566](#)).
22 Pifer et al. (1989, [195559](#)) compared fecal silver concentrations in workers exposed for at least five years
23 in positions with high exposure potential in an Eastman Kodak plant to a control set of employees at the
24 plant in positions with low exposure. Measured concentrations in indoor air at the facility were reported
25 as 1–100 $\mu\text{g}/\text{m}^3$, with the majority present in insoluble forms. Although no cases of argyria were
26 reported, 80% of silver workers had detectable blood silver concentrations (mean of 0.010 $\mu\text{g}/\text{mL}$ among
27 those with detectable blood silver levels), and none of the individuals in the low-exposure group had
28 detectable blood silver levels. Fecal concentration among workers was higher than in the control group
29 (i.e., 15 $\mu\text{g}/\text{g}$, compared to 1.5 $\mu\text{g}/\text{g}$ in controls). Body burdens also were calculated and reported to be
30 14 micrograms per kilogram ($\mu\text{g}/\text{kg}$) in workers, which was seven times the level observed in control
31 samples. In one case study, Trop et al. (2006, [195576](#)) reported that the body can clear silver from the

1 blood once the exposure has been terminated. Cases of argyria (silver accumulations in the skin that
2 cause it to turn blue or bluish-gray) and argyrosis (accumulation of silver in the eye), which are generally
3 believed to be irreversible conditions, demonstrate that the body cannot completely clear silver from all
4 organs. How much of an absorbed dose remains as a residual burden, however, is not well understood
5 (Wijnhoven et al., 2009, [180201](#)). Wijnhoven et al. (2009, [180201](#)) also did not identify information in
6 their review regarding whether the silver excreted following exposure to nano-Ag would be released as
7 nano-Ag or as other forms of silver.

5.7.2. Uptake and Dose by Route

5.7.2.1. Respiratory (Inhalation and Instillation)

8 Exposures to nano-Ag and other nanoparticles have been shown to result in nanoparticle deposition
9 in the respiratory system and to both interact with biological material in the lung and penetrate through
10 the lung into the lymph nodes and the circulatory system (Takenaka et al., 2001, [019055](#)). Dosage of
11 particles within the respiratory system depends on particle size and chemistry and on breathing force
12 (Wijnhoven et al., 2009, [180201](#)). Some types of nanoparticles can deposit in all regions of the
13 respiratory tract and can pass through cell walls into cells, through entire cells, or between cells to
14 translocate to other parts of the body (Oberdorster et al., 2005, [090087](#)). The respiratory tract has a very
15 high surface area of alveolar epithelium, which allows for high deposition rates, and is very highly
16 vascularized, which facilitates entry into the bloodstream of some types of particles and substances
17 (Lynch and Elder, 2009, [195896](#)).

18 Particle deposition along the respiratory tract depends on particle size, among other factors. The
19 International Commission on Radiological Protection (ICRP) developed and continues to update the
20 Human Respiratory Tract Model for Radiological Protection. Mark (2007, [202653](#)) used the ICRP 66
21 model to predict nanoparticle deposition in the human respiratory tract to help clarify occupational
22 exposures. The author estimated probabilities of deposition in three regions: (1) the head or
23 nasopharyngeal region, (2) the tracheobronchial region, and (3) the alveolar region in which gas exchange
24 occurs. As expected, as particle size increased beyond 300 nm, the model predicted that particles would
25 be increasingly trapped in the nasopharyngeal region and subject to clearance by the nasal mucociliary
26 epithelium, which moves mucus containing trapped particles toward the glottis where it is swallowed
27 (ingested). The tracheobronchial region also is covered with a mucociliar epithelium, and the cilia move
28 the mucus, deposited particles, and possibly particle-laden macrophages upward toward the oropharynx
29 (mucociliary escalator), where they are swallowed. The model predicted that for a person breathing
30 through the nose, nanoparticles of 100, 10, and 1 nm in diameter had deposition probabilities in the
31 nasopharyngeal region of 0.05, 0.2, and 0.8, respectively (Mark, 2007, [202653](#)). In other words, as the

1 nanoparticles become smaller, they more effectively diffuse into the mucous layers where they can move
2 farther along the respiratory tract (Witschi et al., 2008, [626250](#)). Ninety percent of 1-nm particles were
3 predicted to be deposited in the nasopharyngeal region of the lung, with the remaining 10% deposited in
4 the tracheobronchial region.

5 Mark (2007, [202653](#)) found that the projected probability of nanoparticles reaching the alveoli
6 peaked at a size of approximately 20 nm, with lower probabilities of deposition in the alveoli for both
7 smaller and larger nanoparticles. At 20 nm in diameter, the ICRP 66 model predicted the probability of
8 deposition in the alveolar region to be 0.50, with 0.25 probability of deposition each in the
9 tracheobronchial and nasopharyngeal regions (Mark, 2007, [202653](#)). Nanoparticle deposition, especially
10 for particle sizes 20 nm and smaller, is governed by Brownian motion and diffusion, allowing movement
11 of particles into the alveolar region of the lung, where larger particles (which are transported via bulk air
12 flow) generally are not deposited (Elder et al., 2009, [195897](#)). For nanoparticles between 20 and 100 nm
13 in size, deposition probability dropped for all three regions of the respiratory tract (Mark, 2007, [202653](#)).
14 Nonetheless, the ICRP model indicated that for nanoparticles measuring between 10 and 100 nm, the
15 highest *fractional* deposition would occur in the alveolar region (Lynch and Elder, 2009, [195896](#)).

16 Once present in the alveolar region, nanoparticles can interact with alveolar lining fluid that
17 contains proteins and lipids, with type I or II epithelial cells, and with macrophages (Elder et al., 2009,
18 [195897](#)). From a few studies conducted by others, Elder et al. (2009, [195897](#)) concluded that larger
19 particles and clusters of nanoparticles (especially those greater than 100 nm in size) are more likely to be
20 taken up by alveolar macrophages than single nanoparticles. Takenaka et al. (2000, [195573](#)) examined
21 the fate of 20-nm diameter nano-Ag in rats at 1, 4, and 7 days after intratracheal instillation. They found
22 the nano-Ag particles in larger clusters taken up by alveolar macrophages and inside the alveolar walls at
23 all three time intervals. A small proportion of single particles was also observed. The appearance of
24 clusters of nano-Ag particles in the macrophages seemed unchanged up to 7 days after instillation, and
25 there were no substantial changes in silver concentrations in the lung, liver, and lung-associated lymph
26 nodes over time. Liver silver concentrations remained approximately 3% of that in the lungs. Based on
27 these observations, the investigators suggested that such clusters do not rapidly translocate to other
28 organs. Other researchers have noted that larger particles taken up by macrophages will be removed from
29 the lung by mucociliary transport to the throat, where they are swallowed (Lynch and Elder, 2009,
30 [195896](#)). These particles can then be transported to the digestive system (see Section 5.7.2.3).

31 Based on the findings above, nano-Ag that settles into the alveolar region of the lung could remain
32 there, but as exposure continues and more particles accumulate, clusters are formed that are recognized
33 and engulfed by the alveolar macrophages. The macrophages could eventually transport the silver
34 clusters up the mucociliary escalator or the clusters could be transported to lymph nodes. It is also
35 possible that single nano-Ag particles could pass through the alveolar wall and eventually reach the blood
36 stream.

1 An important feature of nano-Ag particles is their ability to adsorb proteins and other biomolecules
2 (e.g., lipids), which alter the particle's surface properties. For example, biomolecules adsorbed along the
3 way to the circulatory system could influence the ability of the nanoparticles to interact with cells and
4 systems they encounter (Lynch and Elder, 2009, [195896](#)). Because translocation of the nanoparticles
5 depends on their physical and chemical properties, nano-Ag deposited in the lung and translocated to
6 other parts of the body might carry a biological marker of its deposition site until other biomolecules
7 displace those that are initially adsorbed. The surface corona of proteins and other biomolecules
8 surrounding a nanoparticle can affect its solubility, agglomeration, uptake, and distribution in the body
9 (Lynch and Elder, 2009, [195896](#)).

10 Inhalation studies of rats exposed to nano-Ag in air have demonstrated absorption of silver through
11 the lungs into the circulatory system and distribution to other organs as well (Sung et al., 2009, [193664](#);
12 Takenaka et al., 2001, [019055](#)). Takenaka et al. (2001, [019055](#)) exposed rats via inhalation using whole-
13 body exposure chambers and reported a cumulative dose to each rat of approximately 7.2 µg of nano-Ag
14 particles approximately 15 nm in size. Total silver concentrations in various organs and biological
15 systems were monitored, and the distribution of silver was observed. The highest concentration and total
16 silver content were observed in lung tissue. Elevated concentrations also were reported for the liver and
17 blood, with measurable amounts reported in these components 7 days after exposure. Lower levels were
18 measured in other organs, including lymph nodes, kidneys, blood, heart, and brain (listed in order of
19 decreasing concentration).

20 Researchers in Korea administered differing doses of aerosolized nano-Ag to rats and monitored
21 total silver concentrations in organs over 28 days. These researchers found that lung concentrations of
22 silver showed a dose-dependent relationship following exposure (Hyun et al., 2008, [194066](#); Ji et al.,
23 2007, [091301](#)). They suggested that nano-Ag reaches the bloodstream after inhalation dosing via three
24 pathways: (1) ingestion after the mucociliary escalator, (2) passage into the lymph nodes, and (3) direct
25 entry via alveolar epithelial cells (Ji et al., 2007, [091301](#)).

26 Whether nano-Ag particles are distributed in the bloodstream to other organs or only the silver ions
27 reach the circulatory system is not yet known. The eventual fate of the inhaled nano-Ag also is unclear at
28 this time.

Olfactory Nervous System

29 For inhaled substances, the olfactory nerve represents another pathway to the brain. This pathway
30 is treated here as a subtopic of inhalation exposure; it is given special attention because it represents a
31 potential exposure and distribution route to the central nervous system that does not require passage
32 through the blood-brain barrier.

1 The olfactory nerve facilitates the sense of smell by extending from the nasal cavity to the olfactory
2 bulb of the brain, where this sense is processed. As described in detail by Illum (2000, [157897](#)) in a
3 review on the subject, substances that deposit on the nasal olfactory mucosa and are able to enter into the
4 olfactory nerve can be transmitted directly to the brain without encountering the blood-brain barrier.
5 Entry of drugs and other substances to the olfactory bulb of the brain through the olfactory nerve has been
6 demonstrated, and evidence indicates that this pathway might have an upper size limit of 200 nm (Elder et
7 al., 2006, [089253](#); Elder et al., 2009, [195897](#); Oberdörster et al., 2004, [055639](#)). Oberdörster et al. (2004,
8 [055639](#)) noted that results from several studies (including their own research using radiolabeled carbon
9 nanoparticles) suggest that this nerve serves as a pathway to the central nervous system for soluble metals
10 and nanomaterials. In that study, approximately 20% of the carbon nanoparticles deposited on the
11 olfactory mucosa of the rats was translocated to the olfactory bulb in the brain (Oberdörster et al., 2004,
12 [055639](#)). Oberdörster et al. noted that once deposited in the olfactory bulb, nano-Ag might be able to
13 travel to other areas of the brain, a possibility also noted by Lynch and Elder (2009, [195896](#)). Transfer
14 from the olfactory bulb to other parts of the brain, however, was not confirmed by these researchers, and
15 no studies confirming this possibility were identified for this case study. No studies focusing specifically
16 on the transport of nano-Ag to the olfactory bulb via this pathway were identified for this case study.

5.7.2.2. Dermal

17 In their review, Elder et al. (2009, [195897](#)) summarized evidence regarding the interaction of
18 various nanoparticles with skin. The results varied, with different degrees of penetration into and through
19 skin observed in both in vitro and in vivo studies involving human skin and that of other organisms (e.g.,
20 rats, pigs). They noted that nanoparticles of varying composition have been absorbed into the blood in
21 scenarios involving mechanical flexing of the skin and damaged skin patches as well as passage through
22 hair follicles (for particles smaller than ~5 nm).

23 Elder et al. (2009, [195897](#)) noted that nanoparticle penetration of the skin is influenced by surface
24 coatings and geometry of the particles. For example, Monteiro-Riviere and Riviere (2009, [199936](#))
25 reported that skin is “surprisingly permeable” to some nanoparticles, and in particular quantum dot
26 nanoparticles, which are readily absorbed. In addition, the formulation of the nanoparticles that contact
27 the skin might also influence the skin’s permeability by altering its barrier properties. For example,
28 dimethyl sulfoxide facilitates absorption of substances through the skin by removing much of the lipid
29 matrix of the stratum corneum, leaving holes and shunts (Lehman-McKeeman, 2008). The vehicle in
30 which the nanoparticles are dissolved or suspended also might influence partitioning between the stratum
31 corneum and the vehicle. Elder et al. (2009, [195897](#)) concluded that dermal absorption of nanoparticles
32 does not appear to occur readily but can take place under certain conditions, and the factors dictating the
33 extent to which absorption occurs are varied and complex.

1 Only a few experiments on dermal penetration of nano-Ag were identified for this case study.
2 Larese et al. (2009, [193493](#)) reported that nano-Ag can pass through normal human skin (i.e., full-
3 thickness abdominal skin) in vitro at a rate of 0.46 nanograms per square centimeter (ng/cm²) and through
4 damaged skin at a rate five times higher. Nano-Ag particles between 7 and 20 nm can penetrate into the
5 hair follicle, and nano-Ag particles less than 30 nm can passively penetrate the deepest skin layers,
6 probably through the intercellular route (Larese et al., 2009, [193493](#)). Samberg et al. (2010, [625612](#))
7 applied nano-Ag particles 20 and 50 nm in size to the backs of pigs in solutions ranging from 0.34 to
8 34.0 micrograms per milliliter (µg/mL) for 14 days. TEM demonstrated the presence of nano-Ag within
9 the superficial layers of the stratum corneum for the 50-nm particles and on the top layer of the stratum
10 corneum for the 20-nm particles.

5.7.2.3. Ingestion

11 Absorption of conventional silver following ingestion has been reported; for example, Boosalis et
12 al. (1987, [224883](#)) observed that 10–20% of ingested silver metal was absorbed in the GI tract, mainly by
13 the duodenum and small intestines. Nanoparticles, however, do not appear to be readily absorbed. In
14 separate review discussions, Mark (2007, [202653](#)) and Elder et al. (2009, [195897](#)) noted that the few
15 studies investigating the uptake and deposition of various nanoparticles to the GI tract have typically
16 demonstrated that ingested particles pass through without absorption and are eliminated quickly. Specific
17 to nano-Ag, Kim et al. (2008, [193473](#)) reported that ingestion of nano-Ag resulted in distribution of silver
18 in a range of tissues, with dose-dependent deposition of nano-Ag observed in all tissues evaluated.
19 Specifically, in a 28-day study following dosing via nano-Ag ingestion by rats, silver was detected in the
20 blood, stomach, brain, liver, kidneys, lungs, and testes, indicating that nano-Ag was distributed
21 systemically (Kim et al., 2008, [193473](#)). Silver uptake in kidneys was observed to be sex-specific, with
22 twice the silver accumulation in females than in males (Kim et al., 2008, [193473](#)). In another recent
23 evaluation of the ability of nano-Ag to cross the human intestinal wall using an in vitro model,
24 Bouwmeester et al. (2010, [632344](#)) reported limited (0.5%) translocation of nano-Ag across the
25 membrane, with no dependency on size (results not yet published). As is the case with other routes of
26 exposure, surface properties of nano-Ag present in the GI system appear to be determinants of uptake
27 across the biological barrier there, especially given the changes in acidity and the negatively charged
28 mucous layer in the small intestine (Elder et al., 2009, [195897](#)).

5.7.3. Models to Estimate Dose

29 No models for estimating the pharmacokinetics of nano-Ag were identified for this case study.
30 Some models for nanoparticle deposition within the body have been developed that, by extension, could

1 be useful in evaluating dose for nano-Ag. Deposition of nanoparticles can be modeled based on their size
2 (ICRP, 1994, [199638](#); Price et al., 2002, [202677](#)). Price et al. (2002, [202677](#)) developed a mathematical
3 model called the Multiple-Path Particle Dosimetry model, which is used for estimating human and rat
4 airway particle dosimetry. According to this model, between 30% and 80% of inhaled nanoparticles (less
5 than 100 nm in size) might deposit in the lungs; nanoparticles up to 50 nm might also deposit in upper
6 airways (Maynard and Kuempel, 2005, [088094](#)).

5.8. Summary of Exposure, Uptake, and Dose

7 As many as 259 consumer products on the market might contain nano-Ag, suggesting that an
8 understanding of aggregate exposure from numerous sources might be useful to accurately determining
9 exposure pathways and estimating dose levels. Nano-Ag disinfectant spray use alone can result in
10 inhalation, ingestion, and dermal exposure to nano-Ag. Through environmental pathways, nano-Ag
11 might bind to other molecules, which can affect bioavailability to both biota and humans.

Biotic Exposure and Uptake

12 Few data exist to determine the extent to which nano-Ag is present in the environment and whether
13 it is bioavailable to organisms. Most current models for estimating exposure and fate are not suitable for
14 simulating nanoparticles in general or nano-Ag in particular, and therefore require modification and
15 additional research. For biota, the aquatic environment is expected to be a greater source of potential
16 exposure than the terrestrial environment, and sediment also appears to be more a more likely exposure
17 pathway given that nano-Ag preferentially accumulates in sediment. Exposure and bioavailability are
18 strongly affected by environmental factors, such as pH, the presence of other ligands (including sulfides),
19 other particles, and the nature of the environmental medium in which the nano-Ag is present. Ingredients
20 of spray formulations might also alter the behavior of nano-Ag or exhibit increased uptake in the presence
21 of nano-Ag. Some of these factors affect silver in general (e.g., presence of excess sulfides, ligands), and
22 evaluations in the laboratory have confirmed that they also affect the bioavailability of silver present in
23 nanoparticle form. Some environmental factors might more strongly affect nano-Ag in particular because
24 of specific properties and treatments of this form of silver, such as surface coatings.

25 Bacteria and fungi readily take up nano-Ag, which is consistent with the well-known antibacterial
26 properties of silver. Bioaccumulation by aquatic organisms has been studied to a limited extent, with
27 some organisms (e.g., algae, eggs of vertebrates) observed to readily take up nano-Ag. Other biota,
28 including bivalve mollusks and aquatic crustaceans, do bioaccumulate conventional silver and some
29 nanoparticles, but these organisms have not been studied specifically for nano-Ag bioaccumulation.

1 Some microorganisms appear to have the ability to synthesize nano-Ag. Bioaccumulation in fish appears
2 to occur to a limited extent and is more likely in freshwater than saltwater species. Fish appear to adsorb
3 nano-Ag particles to the gill, which could then serve as a pathway for delivering silver ions to the fish. In
4 embryonic zebrafish, nano-Ag particles were absorbed and accumulated in tissues, including the brain,
5 and silver entered the nuclei of cells in diverse organs. Nano-Ag particles can enter via chorion pores.
6 Overall, bioaccumulation of nano-Ag appears to decrease with increasing trophic level in water-column
7 food webs.

8 Some terrestrial plants bioaccumulate silver to a limited extent, although conventional silver is
9 rarely absorbed beyond plant roots. Due to the smaller size and increased surface area of nano-Ag, the
10 potential exists for increased release, and therefore uptake, of silver ions from nano-Ag compared with
11 ions released from conventional silver; however, few data on the uptake of silver of any type are available
12 for terrestrial plants. Limited evidence suggests that invertebrates might absorb nano-Ag that is
13 bioavailable in soil. Bioaccumulation of nano-Ag in larger terrestrial organisms has not been studied.
14 The possibility remains, however, that terrestrial ecosystems could be impacted if microorganisms in soil
15 and elsewhere in terrestrial ecosystems are affected directly by nano-Ag.

Human Exposure and Dose

16 With the growing use of nano-Ag (especially in consumer products), elevated human exposures to
17 nano-Ag through a range of scenarios is increasingly possible. Several expert and modeling exercises
18 have concluded that use of nano-Ag in a spray solution is likely to result in a feasible exposure scenario
19 (including potential exposures to sensitive subpopulations) for consumers via inhalation and dermal
20 exposure; however, no data focusing on nano-Ag were identified for this case study. Occupational
21 exposures to nano-Ag in powders or solutions used in manufacturing might result in inhalation and
22 dermal exposures, with the potential for subsequent ingestion exposures (e.g., from hand-to-mouth or
23 contact with treated surfaces). These exposures appear to differ from those known for conventional
24 silver, because smaller particles have a greater potential to become aerosolized or to penetrate the skin.
25 Occupational studies of conventional silver have not shown clear associations of effects with a particular
26 exposure due to small sample sizes and confounding factors. Perhaps because many of the human studies
27 are retrospective (as described in the following chapter), there are few data on exposure characterization.
28 No occupational exposure studies specific to nano-Ag other than the one by Park et al. (2009, [225210](#))
29 were identified for this case study.

30 With respect to the human uptake of nano-Ag, considerations relevant to understanding uptake and
31 dose include particle properties and route of exposure. Surface properties, such as charge, and surface
32 characteristics, such as the coating and presence of biomolecules that sorb to the surface of the

1 nanoparticle, can affect absorption. Other spray ingredients or materials used in the manufacturing
2 process might associate with nano-Ag and thereby exhibit increased uptake. Current data suggest that
3 nano-Ag crosses biological membranes following oral and inhalation exposure, with resulting
4 accumulation of silver in the lungs, liver, kidneys, stomach, brain, and blood. Conventional silver has not
5 been demonstrated to enter the brain. It is unclear whether soluble silver, silver ions, or nano-Ag particles
6 are the entities entering various tissues after exposure to nano-Ag. No evidence exists regarding the
7 metabolism or transformation of nano-Ag in tissues, nor regarding urinary or fecal excretion pathways
8 and whether they differ from conventional silver excretion.

9 Deposition of nano-Ag in the human lung differs from that of conventional silver. Nano-Ag is
10 more likely to enter the alveolar region and translocate to other tissues, while conventional silver is more
11 likely to be taken up by macrophages in the lung and excreted (Elder et al., 2009, [195897](#); Lynch and
12 Elder, 2009, [195896](#)). Following inhalation, nano-Ag can be translocated from the lung to the
13 bloodstream via the mucociliary escalator and subsequent ingestion, through passage into the lymph
14 nodes, via alveolar epithelial cells, or via absorption to the olfactory bulb (Ji et al., 2007, [091301](#)).

15 Treatment of burn wounds in humans has resulted in kidney, liver, and skin accumulation of silver,
16 and in one case neurological effects were observed, suggesting silver might have entered the brain.
17 Healthy skin exposed to nano-Ag resulted in dermal absorption, which appears to depend on exposure
18 conditions, particle size, and other factors. Conventional silver is not expected to cross the skin barrier.

19 Ingestion exposures to nano-Ag appear to result in lower relative absorption and subsequent dose
20 compared to other exposure pathways. Conventional silver has been demonstrated to cross the intestinal
21 barrier following ingestion exposure. The limited data for nano-Ag suggest particle characteristics,
22 including surface modification, affect whether nano-Ag is absorbed or excreted following ingestion.
23 Models do not currently exist for estimating nano-Ag distribution in the body. Models developed for
24 other particle types could be applied for nano-Ag if such models adequately consider chemistry and
25 surface properties.

26 As expressed by the FIFRA Scientific Advisory Panel, data gaps about potential exposures (and
27 toxicity) related to nano-Ag are broad (U.S. EPA, 2010, [625619](#)). Attempting to follow the risk
28 assessment paradigm for nano-Ag exposures, Christensen et al. (2010, [625598](#)) concluded that available
29 data relevant to exposures and toxicity are inadequate at this time for use in regulatory decision-making.
30 When examining nano-Ag as a hypothetical registration under the Registration, Evaluation, Authorisation
31 and Restriction of Chemicals (REACH) program in Europe, the Netherlands Institute for Public Health
32 [RIVM (2009, [625609](#))] also identified key data gaps in particle characterization, exposure, uptake, and
33 toxicity. These gaps were large enough at that time to prevent implementation of the REACH process for
34 this widely used material.

- 1 Questions reflecting data gaps in the information about exposure, uptake, and dose are listed on the
- 2 following page. They are listed in approximate order of the presentation of information in this chapter.
- 3 The order in no way, however, is intended to reflect the relative importance of the questions.

Questions about Exposure, Uptake, and Dose

- 5.1. Are available methods adequate to characterize nano-Ag concentrations and associated exposure via relevant matrices such as:
 - 5.1.a. air?
 - 5.1.b. water?
 - 5.1.c. food?
- 5.2. To what extent is information on conventional silver applicable to nano-Ag, particularly regarding:
 - 5.2.a. uptake?
 - 5.2.b. biopersistence?
 - 5.2.c. bioaccumulation?
 - 5.2.d. biomagnification?
- 5.3. What effect, if any, do surface treatments of nano-Ag particles have on:
 - 5.3.a. uptake?
 - 5.3.b. biopersistence?
 - 5.3.c. bioaccumulation?
 - 5.3.d. biomagnification?
- 5.4. Which sources, pathways, and routes offer the greatest exposure potential to nano-Ag for humans and biota?
- 5.5. Do particular species of biota and particular human populations have greater potential for exposure to nano-Ag?
- 5.6. By region and environmental segment (e.g., air, water, soil), what are the background concentrations and characteristics of nano-Ag in air, water, and soil due to natural (non-anthropogenic) processes?
- 5.7. Ecologically, is nano-Ag a point-source or regional exposure problem? If a regional distribution issue, what are the exposure concentrations and concentration gradients in key media (e.g., air, water, soil)?
- 5.8. What is the potential for uncoated nano-Ag particles to interact with or form complexes with constituents in water, and what impact do these interactions have on particle bioavailability and release of silver ions?
- 5.9. What is the impact of environmental characteristics such as water chemistry (e.g., pH, ionic strength), the presence of suspended solids, and the concentration of sulfides and other dissolved ligands on:
 - 5.9.a. the potential for uptake of nano-Ag from the environment?
 - 5.9.b. tissue distribution and dose of nano-Ag and silver ions?
- 5.10. To what extent does nano-Ag facilitate the uptake of other contaminants in the environment?
- 5.11. What is the impact of organism characteristics such as physiology (e.g., cell membrane structure for single-celled organisms; respiratory physiology for multicellular organisms), behavior (e.g., filter feeding, habitat), and lifestage on:
 - 5.11.a. the potential for uptake of nano-Ag from the environment?
 - 5.11.b. tissue distribution and dose of nano-Ag and silver ions?
- 5.12. What is the relative bioavailability of nano-Ag and silver ions in aquatic environments, and how might the presence of nano-Ag alter the bioavailability of silver ions in sediments, water, and biota?

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Chapter 6. Characterization of Effects

1 This chapter summarizes the effects of nano-Ag on humans and biota associated with the use of
2 nano-Ag in spray disinfectants. The preceding chapters in this case study have laid a foundation for this
3 chapter by providing an exposure context for characterizing these effects. In this chapter, Section 6.1
4 provides information on the factors that influence the effects of nano-Ag on ecological receptors and
5 human health. The ecological effects resulting from exposure to nano-Ag are discussed in Section 6.2,
6 and relevant results from ecotoxicological studies are summarized for bacteria and fungi (Section 6.2.1),
7 aquatic organisms (Section 6.2.2), and terrestrial organisms (Section 6.2.3). Human health effects
8 resulting from exposure to nano-Ag are discussed in Section 6.3, and relevant information is presented for
9 in vitro studies (Section 6.3.1), in vivo studies (Section 6.3.2), and human health and epidemiological
10 studies (Section 6.3.3). Because nano-Ag releases are likely to result in the formation of silver
11 compounds and discharges of silver ions, the ecological and human health effects from other silver
12 species also are discussed, when appropriate. The technology to differentiate the effects of the silver
13 nanoparticle from those of the silver ions released from the nanoparticles is still developing. As a result,
14 it is not always possible to determine whether the observed effect is due to the nanoparticle per se, the
15 silver ions alone, or the silver ions modulated by the nanoparticle. Few ecological and human health
16 effects studies distinguish between the effects the silver nanoparticle and the silver ions released by the
17 nanoparticle; where this distinction has been made by investigators, it is presented here.

18 The literature on the ecological and human health effects of exposure to silver compounds and
19 silver ions is abundant (Lansdown, 2007, [195538](#); Ratte, 1999, [195564](#)); comparatively few studies,
20 however, are available on the effects of silver nanoparticles. The Scientific Advisory Panel (SAP) for the
21 Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) concluded in its November 3–5, 2009
22 meeting on the evaluation of hazards and exposure of nano-Ag that data gaps about potential hazards of
23 nano-Ag are broad, and that the hazard profile for nano-Ag can differ significantly from that for
24 conventional silver and other silver species (U.S. EPA, 2010, [625619](#)). As noted in Chapter 1, the
25 findings presented in this case study generally support the FIFRA SAP conclusions. Consistent with
26 studies of other nanomaterials (Ostrowski et al., 2009, [193592](#)), most studies of nano-Ag have
27 investigated the ecological or human health effects of various formulations of silver nanoparticles and
28 silver ions, and relatively few have investigated the effects of end-use products containing nano-Ag or
29 their life-cycle by-products. Moreover, the term “nano-Ag” encompasses a variety of materials with a
30 diverse range of physicochemical properties. As a result, not all materials referred to as nano-Ag will
31 necessarily behave the same or cause the same ecological or human health effects. Various members of
32 the scientific community and the FIFRA SAP have cautioned against extrapolating from one nano-Ag

1 formulation to another when assessing hazards. However, the current dearth of information necessitates
2 compiling results from studies using various nano-Ag formulations to detail the current state of
3 knowledge about the potential toxicological properties of nano-Ag. Therefore, for the purposes of this
4 case study, available information from all nano-Ag materials is described together.

5 This chapter focuses on characteristics of the nanoparticle, exposure media, and biological
6 receptors that might influence the degree to which nano-Ag is toxic to humans and biota. In general,
7 Section 6.1 focuses on nanoparticle properties and factors of the exposure environment capable of
8 influencing nano-Ag toxicity, because these data are relevant to both ecological and human health effects.
9 As noted in Chapters 1 and 5, there is no sharp demarcation between exposure-dose and effects, so some
10 overlap is unavoidable between the information on exposure and dose, as presented in Chapter 5, and the
11 information on effects, presented in Chapter 6. To the extent possible, discussion of studies cited in both
12 chapters is limited in Chapter 5 to discussion of exposure-dose and in Chapter 6 to discussion of effects.

13 Evidence is growing that nano-Ag in particular forms and under certain testing conditions can be
14 toxic to bacteria, fungi, algae, aquatic invertebrates, and fish, and to mammalian brain, liver, skin, and
15 stem cells (Panyala et al., 2008, [195554](#)). The breadth of representative species for which nano-Ag
16 toxicity has been studied and the scope of these studies, however, are too narrow to draw definitive
17 conclusions regarding the degree to which nano-Ag might present a threat to the environment (Wijnhoven
18 et al., 2009, [180201](#)). Until recently, the consensus was that the toxicity of silver in the environment
19 depended mainly on the concentration of free silver ions to which an organism is exposed (Khaydarov et
20 al., 2009, [194104](#)). Results from recent studies suggest that some adverse effects on biota can be
21 attributed to properties of the silver nanoparticle itself; furthermore, these effects might be exacerbated by
22 the release of silver ions at the biological interface (Choi et al., 2009, [193317](#); Laban et al., 2009, [199809](#);
23 Lee et al., 2007, [194072](#); Navarro et al., 2008, [157516](#); Roh et al., 2009, [195565](#)). These properties are
24 described in more detail below (Section 6.1).

25 The following sections are not meant to be an exhaustive review of the ecological and human
26 health effects literature for nano-Ag, silver compounds, or silver ions. Instead, this chapter is intended to
27 highlight recent work on the effects of nano-Ag particles and to identify information status and gaps for
28 assessing potential risks of nano-Ag in spray disinfectants.

6.1. Factors that Influence Ecological and Human Health Effects of Nano-Ag

29 Because many variables are associated with synthesis, characterization, and behavior of nano-Ag in
30 experimental and environmental conditions, identifying the primary property(ies) of nano-Ag that
31 contribute to an effect is extremely difficult. The complexity of the factors influencing ecological and

1 human health effects of nano-Ag also makes it extremely difficult to compare the respective influence and
2 importance of the different properties. That many of the novel properties exhibited by nanoparticles
3 result from their small size is widely accepted, but other factors have been noted as variably contributing
4 to the effects of nanoparticles on biota and humans (Luoma, 2008, [157525](#)).

5 For example, in a study comparing nano-gold and nano-Ag toxicity to zebrafish embryos,
6 nano-gold induced minimal sublethal toxic effects at the end of a 120-hour exposure period at the highest
7 concentration tested, while nano-Ag particles of the same size and at the same concentration resulted in
8 nearly 100% mortality (Bar-Ilan et al., 2009, [191176](#)). This study demonstrated that nanoparticle size
9 alone does not dictate toxic effects, and it highlights the potential importance of nanoparticle surface
10 chemistry in eliciting a toxic response. In a study comparing the toxicity of nanoparticles of different
11 metals to fish, aquatic invertebrates, and algae, Griffitt et al. (2008, [157517](#)) concluded by process of
12 elimination that particle chemistry appears to be the most influential characteristic of the nanoparticle
13 because the investigators did not identify a relationship between size, surface area, or zeta (ζ) potential
14 and toxicity. Other physicochemical properties, such as morphology, surface treatments, and solubility of
15 particles, however, also might significantly influence the toxicity of nano-Ag (Choi et al., 2008, [194060](#)).
16 Furthermore, any particle properties that influence bioavailability in general also will influence the effects
17 of nano-Ag on an organism, as interaction between the nanoparticle and the organism is necessary to
18 induce an effect. Factors that influence all substances in the environment might also affect the toxicity of
19 nano-Ag to humans and biota, including exposure medium, type of organism, environmental
20 bioavailability, route(s) of exposure, and the physicochemical properties and size distribution of the nano-
21 Ag particles.

22 Although nanoparticle synthesis, characterization, and detection techniques have advanced
23 considerably in recent years, the influence of various physicochemical properties of nano-Ag has not been
24 fully investigated using well-characterized nano-Ag under controlled conditions (U.S. EPA, 2010,
25 [625619](#)). Nor have experimental or environmental characteristics been the focus of most toxicity studies
26 to date. As a result, the factors and conditions that most influence nano-Ag toxicity have not yet been
27 determined, and data on the influence of physicochemical properties and experimental and environmental
28 conditions are very limited. This section focuses on factors that have been shown to be pertinent to nano-
29 Ag, but findings related to other types of nanomaterials are noted when relevant.

6.1.1. Physicochemical Properties

30 Size, chemical composition, and surface treatment appear to be three of the most critical
31 nanotoxicity metrics (Bar-Ilan et al., 2009, [191176](#)). Other physicochemical properties such as shape,
32 doping, and purity (or impurities) also could influence the outcomes of nano-Ag toxicity tests, but that

1 information is usually not reported in ecological and human health effects studies. Databases describing
2 detailed nanoparticle properties and health effects are being developed (Miller et al., 2007, [092297](#)); these
3 include the National Institute for Occupational Safety and Health/Centers for Disease Control and
4 Prevention (NIOSH/CDC) Nanoparticle Information Library (NIL),¹⁰ Rice University/International
5 Council on Nanotechnology (ICON) Environmental Health and Safety (EHS) Database,¹¹ the Woodrow
6 Wilson Center for Scholars' Environmental Health and Safety Research Inventory,¹² and Oregon State
7 University's Nanomaterial-Biological Interactions Knowledgebase.¹³

8 The need to characterize basic physical and chemical attributes of the nanomaterials used in
9 toxicity studies has been noted in numerous reports and journal articles (Auffan et al., 2009, [199803](#);
10 DEFRA, 2007, [201629](#); Powers et al., 2006, [088783](#); Powers et al., 2007, [090679](#); Warheit et al., 2007,
11 [091075](#)). The Minimum Information for Nanomaterial Characterization (MINChar) Initiative (2008,
12 [594265](#)) has provided recommendations for the minimum required physical and chemical parameters that
13 should be reported for nanomaterials used in toxicological studies. These parameters would establish
14 generally what the material looks like, what the material is made of, and what factors affect how the
15 material interacts with its surroundings. The specific attributes for minimal characterization
16 recommended by MINChar include particle size or size distribution, agglomeration state or aggregation,
17 shape, overall composition (including chemical composition and crystal structure), surface composition,
18 purity (including levels of impurities), surface area, surface chemistry (including reactivity and
19 hydrophobicity), and surface charge (MINChar Initiative, 2008, [594265](#)). For more information on
20 nanomaterial physiochemical properties that could influence ecological and toxicological effects, readers
21 are referred to detailed reports listing recommended information to be included in nanomaterial studies,
22 including publications by the Organisation for Economic Co-operation and Development (OECD) (2008,
23 [157512](#)), Taylor (2008, [157487](#)), and Warheit et al. (2007, [091305](#)).

24 Furthermore, methods for establishing the toxic potential of chemicals, in general, have not been
25 fully standardized internationally. Multiple organizations (e.g., OECD, the U.S. Environmental
26 Protection Agency [EPA], the European Union) have proposed different sets of standard testing protocols,
27 and efforts are currently underway to develop a set of harmonized testing guidelines for establishing
28 chemical toxicity. Because consistent protocols have not yet been developed, not all results from the
29 toxicological studies described in this chapter (and in greater detail in Appendices B and C) can be
30 compared directly. Questions also exist about the suitability of current EPA and other standard testing
31 guidelines for assessing nanomaterials. A general overview of the issues associated with the various

¹⁰ <http://www.cdc.gov/niosh/topics/nanotech/NIL.html>

¹¹ <http://cohesion.rice.edu/centersandinst/icon/virtualjournal.cfm>

¹² <http://www.nanotechproject.org/inventories/ehs/>

¹³ <http://oregonstate.edu/nbi/nanomaterial.php>

1 guidelines for assessing physicochemical properties, human health effects, and ecological effects, as they
2 pertain to nanomaterials, is available in a nano-Ag case study released by the Netherlands National
3 Institute for Public Health and the Environment (RIVM) (Pronk et al., 2009, [625609](#)).

4 In the following subparts of this section, key properties affecting toxicity of nano-Ag are discussed,
5 including size, particle shape and crystal structure, and surface chemical composition and reactivity.

6.1.1.1. Size

6 Although nanoparticles are defined as particles having at least one dimension in the 1- to
7 100-nanometer (nm) range, not all nanoparticles within this range exhibit the same novel properties that
8 set them apart from their conventional counterparts. It has been argued that the unique size-dependent
9 properties that necessitate a classification separate from conventional materials occur primarily in
10 particles 1–30 nm in size, and that larger particles (31–100 nm in size) generally do not exhibit properties
11 that differentiate them from particles greater than 100 nm in size (Auffan et al., 2009, [199803](#)). The
12 smaller size of nano-Ag might allow it to enter an organism more easily than its conventional counterpart.
13 For example, for nanoparticles to penetrate the cell membranes of zebrafish embryos, they must be small
14 enough to diffuse easily through transmembrane porins, which are proteins that facilitate passage of small
15 molecules across the membrane, without attaching to the walls of the pore canal. These porins are
16 approximately 0.5–0.7 micrometer (μm) in diameter. Once through the chorion pore canal, the
17 nanoparticles are inside the cell, where they can interact directly with cellular organelles and potentially
18 disrupt cellular processes (Lee et al., 2007, [194072](#)).

19 The intrinsic properties of materials in the nanoscale size range, such as enhanced reactivity and
20 unique surface structures, can result in higher dissolution rates, reduction and oxidation (redox) reactions,
21 or increased generation of reactive oxygen species (ROS), all of which can in turn affect toxicity in a size-
22 dependent manner (Auffan et al., 2009, [199803](#)). As a result, toxicity studies using the same protocol and
23 test species likely are not directly comparable if the studies do not use nano-Ag within a similar size
24 range. Furthermore, if studies use nano-Ag with average sizes greater than 30 nm (or if the nano-Ag
25 material is not characterized in experimental conditions), the studies might not capture the toxic effects
26 related to unique nanoscale properties if the hypothesis regarding particle size proposed by Auffan et al.
27 (2009, [199803](#)) holds true. For example, Choi et al. (2008, [194061](#)) observed that growth inhibition in
28 nitrifying bacteria correlated strongly with the availability of particles less than 5 nm in diameter and not
29 with silver nanoparticles that were 10 nm or larger. Morones et al. (2005, [196271](#)) also observed that
30 although the bacterium were exposed to silver nanoparticles with an average size of 21 nm, the average
31 size of nano-Ag penetrating the membranes of *Escherichia coli* was about 5 nm.

32 Nano-Ag particle size, however, might be correlated with other properties that affect toxicity, such
33 as the surface area-to-volume ratio, which in turn affects the ratio of reactive silver ions to unavailable

1 silver atoms. If silver ions are the primary agent responsible for observed toxicity, the ratio of silver ions
2 on the exterior of the particle available to react with a biological surface to the silver atoms that are
3 “buried” within the interior of the particle and blocked from interaction might influence nano-Ag toxicity
4 levels. For example, the total mass of silver in larger particles in an exposure medium could indicate an
5 exceedence of an effect level obtained using nano-Ag, based on silver mass alone. Most of the silver in
6 the larger particles, however, will be blocked from interacting with the environment or a biological
7 surface, whereas relatively more of the silver in nano-Ag will be on the surface and readily available. As
8 a result, the methods used now to assess chemical toxicity might not account for a variation in biological
9 responses related to particle size.

10 Lee et al. (2007, [194072](#)) used optical microscopy to observe the uptake of single silver
11 nanoparticles by zebrafish embryos in real time. They reported that nanoparticles can enter cells by
12 passive transport (i.e., Brownian diffusion) through the chorion pore canals and that the diffusion
13 coefficients are inversely proportional to the radius of the nanoparticles. Although most nanoparticles
14 were observed to penetrate the embryo, some that entered the chorion pore canals docked at the chorionic
15 surface and formed clusters with other nanoparticles. Lee et al. (2007, [194072](#)) speculated that larger
16 silver nanoparticles (>31 nm) that embed in the chorion pore canals and act as aggregation sites might
17 eventually block the canals, thus inhibiting normal chemical transport between the egg and its
18 environment.

19 Note that many of the studies investigating nano-Ag effects in humans and biota do not report the
20 average sizes or the size ranges of the nano-Ag materials used. Where size is reported, some investigators
21 report only the size distribution provided by the manufacturer, especially in the case of commercial nano-
22 Ag materials. This size distribution might not be representative of actual particle sizes, given the
23 potential for nano-Ag to form clusters (see Chapter 4). One example was reported by Miao et al. (2009,
24 [196270](#)), where the manufacturer’s information identified the average size of silver nanoparticles in a
25 commercial powder form as 10 nm, but the investigators experimentally determined the size of the nano-
26 Ag primary particles to be between 60 and 70 nm.

6.1.1.2. Morphology

27 Shape and crystal structure also can influence toxicity. A recent review by Auffan et al. (2009,
28 [199803](#)) examined unique properties at the nanoscale and observed that particles with diameters less than
29 30 nm exhibit increased reactivity (e.g., changes in surface reaction rates, redox state, and adsorption
30 capacity) on crystal facets due to size-dependent changes in crystalline structure. It has been shown that
31 {111} facets, which are high-atom-density surfaces, are more reactive than {100} facets in silver crystals
32 (see Section 2.3.2 and Figure 2-2 for explanation of crystal structure notation) (Hatchett and White, 1996,
33 [597401](#)). Pal et al. (2007, [196273](#)) reported the first comparative study on the bactericidal properties of

1 silver nanoparticles of different shapes. The study demonstrated that interactions with gram-negative *E.*
2 *coli* bacteria were shape-dependent, with truncated triangular silver nanoparticles with the {111} basal
3 plane exhibiting higher bactericidal activity than spherical and rod-shaped silver nanoparticles, which are
4 dominated by {100} facets. A dose of 10 micrograms (μg) of truncated triangular silver nanoparticles
5 added to 100 milliliters (mL) of nutrient broth with a bacterial concentration of 10^7 colony forming units
6 per milliliter (CFU/mL) completely inhibited growth even after 24 hours, while 100 μg of silver ions
7 (added as silver nitrate [AgNO_3]¹⁴) or spherical silver nanoparticles resulted in growth inhibition only for
8 up to 10 hours post-exposure, after which bacterial colonies appeared to grow at a normal rate (Pal et al.,
9 2007, [196273](#)). Morones et al. (2005, [196271](#)) observed that silver nanoparticles most likely to be found
10 on the surface of the bacterial membrane are those having more {111} facets. The nanoparticles that
11 interact with the cell membrane, such as those with {111} facets, are those that are most likely to
12 penetrate the cell; interacting with the cell membrane can result in the disruption of membrane processes
13 and the release of silver ions directly to the membrane surface in high concentrations. Therefore,
14 morphology can play a key role in conferring toxicity (Morones et al., 2005, [196271](#)).

6.1.1.3. Surface Chemistry and Reactivity

15 Lok et al. (2007, [196762](#)) found that partially oxidized silver nanoparticles were more toxic to *E.*
16 *coli* than freshly prepared zero-valent (reduced) nano-Ag. Oxidation of zero-valent nano-Ag produces
17 ionic silver, which is likely bound to the surface of the nanoparticle but could become available through
18 desorption or dissolution. The investigators reported that partially oxidizing (i.e., oxidizing the surface)
19 the nano-Ag decreased adenosine triphosphate (ATP) levels in *E. coli* cells by 90%, while exposing *E.*
20 *coli* to reduced nano-Ag did not elicit a response different from that of the controls. Additionally, silver
21 nanoparticles synthesized under an atmosphere of molecular nitrogen (N_2), which precludes surface
22 oxidation, exhibited no antibacterial activity. The investigators also observed that oxidized silver
23 nanoparticles do not appear to elicit a toxic response in silver ion-resistant *E. coli* strains, indicating that
24 the silver nanoparticle likely does not produce a toxic response that is completely independent of silver
25 ion effect (Lok et al., 2007, [196762](#)).

26 The surface chemistry of nanoparticles can be changed by coatings that in turn can influence the
27 particle's toxicity. Nanoparticle surface coatings have been demonstrated to influence cellular uptake, the
28 ability to bind to serum proteins in vivo, ROS generation, and immunosuppression or stimulation
29 response to a high degree in vertebrates (Bar-Ilan et al., 2009, [191176](#)). Surface coatings are frequently
30 applied to nanoparticles to functionalize them to serve a specific purpose or to stabilize them in

¹⁴ Where investigators draw comparisons between the effects of nano-Ag and silver ions added as AgNO_3 , they have generally concluded that the nitrate concentrations in these solutions are too low to elicit a toxic effect and that any observed effects are attributable to the silver ions.

1 suspensions. These coatings can influence the bioavailability or biocompatibility (i.e., the capability of
2 the nanoparticle to coexist with biological tissue without causing adverse effects) of the nanoparticle,
3 which in turn can affect toxicity (Limbach et al., 2005, [088053](#)). Surface coatings that are used to ensure
4 stability can facilitate an interaction between the nanoparticle and the organism. Consequently, such
5 treatments can have a profound effect on the behavior of nano-Ag in the environment and its
6 bioavailability to humans and biota. Metallic nanoparticles can be coated with organic or inorganic
7 compounds that prevent the formation of clusters in solution (Navarro et al., 2008, [157517](#)), which can
8 influence the transport properties of the nanoparticles and maximize the number of individual
9 nanoparticles in suspension. Stability of nano-Ag in suspension in spray disinfectants, for example, is
10 important for product efficacy. When used as a bactericide in water, silver nanoparticles must remain
11 suspended to be effective; therefore, in aquatic environments, coatings that keep nano-Ag in suspension
12 can result in higher concentrations of nano-Ag in the water column and increased exposure of fish and
13 other aquatic biota. Furthermore, the chemicals used to coat nanoparticles could inherently be toxic to
14 certain organisms. For example, Stampoulis et al. (2009, [199839](#)) reported that in the absence of
15 nano-Ag, the surfactant sodium dodecyl sulfate (SDS) significantly inhibited zucchini (*Curcubita pepo*)
16 seed germination and root growth when added to reverse osmosis (RO) water, and the inherent toxicity of
17 the surfactant, when added to the nano-Ag solution, appeared to amplify the toxic effect of nano-Ag.

18 Several examples of the effect of surface-coated nano-Ag on toxicity were identified in the
19 literature. For example, Ahamed et al. (2008, [196100](#)) compared the uptake of uncoated silver
20 nanoparticles and silver nanoparticles coated with the polysaccharide gum arabic by mouse embryonic
21 fibroblasts. After 24 hours at a nano-Ag concentration of 50 micrograms per milliliter ($\mu\text{g}/\text{mL}$), most of
22 the uncoated nano-Ag had formed clusters and had not penetrated cell organelles, while the coated nano-
23 Ag was observed to be distributed throughout the cells. The investigators also reported higher levels of
24 genotoxicity, as determined by measuring levels of the p53 protein (a molecular marker for DNA damage)
25 and two DNA repair proteins, Rad51 and phospho-H2AX. They observed that exposure to coated nano-
26 Ag resulted in more upregulation of these proteins, suggesting that coated nano-Ag causes greater
27 genotoxicity than uncoated nano-Ag (Ahamed et al., 2008, [196100](#)). Kvitek et al. (2009, [195535](#))
28 investigated the effects of surfactant- and polymer-modified nano-Ag on the protozoan *Paramecium*
29 *caudatum* and found that surface modification using a nonionic surfactant, Tween 80, increased the
30 materials' toxicity, while modification using the polymers PVP 360 (polyvinylpyrrolidone with an
31 average molecular weight of 360 kilo-Daltons [kDa]) and PEG 35000 (polyethylene glycol with an
32 average molecular weight of 35,000 Daltons [Da]) did not significantly affect the toxicity of nano-Ag to
33 those organisms.

1 Surface coatings also can influence the ζ -potential of the silver nanoparticle, which affects the
2 particle's electrostatic attraction to cell surfaces. Some investigators have claimed that direct contact
3 between the nanoparticle and a bacterial membrane is required for bactericidal activity; however, particle-
4 membrane contact and the influence of ζ -potential have not been fully evaluated in ecotoxicity assays
5 (Neal, 2008, [196069](#)).

6.1.2. Test Conditions

6 Methods of mixing such as sonication and ultrasound can be used in the preparation of nanoparticle
7 suspensions to increase stability and the contact of the nanoparticles with the test organism or cells.
8 These mixing methods can be part of aquatic toxicity testing procedures and can result in an overestimate
9 of toxicity of nanoparticles compared to results under realistic (natural) conditions (Gao et al., 2009,
10 [195514](#)). For example, Laban et al. (2009, [199809](#)) exposed fathead minnow (*Pimephales promelas*)
11 embryos to nano-Ag solutions that had been either sonicated or stirred. Stirring mimics fin movement by
12 males in natural conditions, while sonication is not expected to represent any process in the natural
13 environment. The investigators found that sonicating the nano-Ag solutions from two commercially
14 produced nano-Ag products for 5 minutes before adding the embryos resulted in LC_{50s}¹⁵ that were
15 statistically significantly lower than when nano-Ag solutions were stirred (Laban et al., 2009, [199809](#)).

16 The medium used in experimental studies also can affect the apparent toxicity of nano-Ag. For
17 example, nano-Ag in a liquid medium might only delay bacterial growth, while equivalent mass
18 concentrations of nano-Ag added to plated agar appears to inhibit bacterial growth completely, although
19 results are not always consistent from study to study (Pal et al., 2007, [196273](#); Sondi and Salopek-Sondi,
20 2004, [196277](#)). In water, damaged microbial cells can release intracellular substances that cause
21 nanoparticles to aggregate and fall out of suspension, ultimately resulting in decreased numbers of silver
22 nanoparticles in the water (Sondi and Salopek-Sondi, 2004, [196277](#)). No such microbial-induced
23 aggregation of nanoparticles seems to occur in agar plates.

24 The antibacterial effect of nano-Ag also seems to depend in part on initial bacterial density used in
25 experiments (measured in terms of bacterial CFUs) (Sondi and Salopek-Sondi, 2004, [196277](#)).
26 Antibacterial activity is generally higher at lower bacterial cell concentrations. Because the high CFUs
27 used in many experiments are rarely found in the environment, this suggests that the bactericidal effect of
28 nano-Ag in real-life systems might be underestimated using current experimental techniques (Sondi and
29 Salopek-Sondi, 2004, [196277](#)).

¹⁵ Lethal concentration: the chemical concentration at which 50% of the exposed organisms die; this is a common effect level used to estimate the toxicity of a substance to a specific group of organisms.

6.1.3. Environmental Conditions

1 The characteristics of the environmental medium in which nano-Ag exposure occurs can affect the
2 properties of nano-Ag that ultimately affect toxicity. For example, changes in the pH, ionic strength,
3 dissolved oxygen content, temperature, quantity of natural organic macromolecules, light availability, and
4 quantity of ligands in the environment can significantly affect nano-Ag dissolution, bioavailability, and
5 reactivity, all of which can affect toxicity (Choi and Hu, 2008, [194061](#); Cumberland and Lead, 2009,
6 [199804](#); Gao et al., 2009, [195514](#); Liu and Hurt, 2010, [625606](#)).

7 The behavior of nano-Ag can be affected by the type of liquid medium in which it is present and
8 the characteristics of that medium. For example, nano-Ag released in wastewater can disperse silver ions,
9 form complexes with ligands, cluster to form larger silver particles, or remain as nanoparticles (Choi et
10 al., 2009, [193317](#)). Few studies, however, have examined nano-Ag effects in complex natural media.
11 One recent study compared the effects of nano-Ag on bacteria and aquatic invertebrates in natural waters
12 obtained from different locations in a river-estuarine system (Gao et al., 2009, [195514](#)) (see Sections 6.2.1
13 and 6.2.2.2), and another study investigated the effect of nano-Ag on bacterial diversity in natural
14 estuarine sediments (Bradford et al., 2009, [193294](#)) (see Section 6.2.1). Most toxicity studies, however,
15 have added nano-Ag to deionized water and other experimental media purely to establish the toxic
16 potential of the test material outside of natural systems.

17 Kvittek et al. (2008, [196266](#)) demonstrated that unmodified nano-Ag in deionized water can remain
18 well-dispersed, exhibiting “long-term¹⁶ stability” in solution. As the pH of the system was lowered and
19 the solution became acidic, however, Kvittek et al. (2008, [196266](#)) observed that the nano-Ag particles
20 slowly formed clusters, a condition that can influence particle uptake by aquatic organisms. Liu and Hurt
21 (2010, [625606](#)) also demonstrated that citrate-stabilized nano-Ag phase partitioning was highly dependent
22 on pH, with silver ion release rates increasing with decreasing pH. They noted, however, that changes in
23 pH affected ion release kinetics only in the presence of dissolved oxygen. Because Kvittek et al. (2008,
24 [196266](#)) did not report the dissolved oxygen content of the solution in which the unmodified nano-Ag
25 was reported to be stable, their data are of limited value in determining the effect of pH on nano-Ag
26 properties influencing toxicity.

27 Nano-Ag has been observed to form clusters in media with high salt content, thereby diminishing
28 its antibacterial activity (Gan et al., 2004, [195513](#)). Because of the high ionic strength of sea water,
29 particle association can occur rapidly when nano-Ag solutions are released to estuaries and in coastal
30 environments, thus preventing the large-scale dispersion of nano-Ag in the water column. Lui and Hurt
31 (2010, [625606](#)) offer a different perspective, however, arguing that the inhibition of oxidation (i.e.,
32 formation of silver ions on the surface of nanoparticles) is less dependent on salt content (i.e., ionic

¹⁶ “Long-term” was not defined.

1 strength) and more dependent on the higher pH of sea water when compared to deionized water. They
 2 suggest that the aggregation due to increases in ionic strength results in the formation of larger particle
 3 clusters, but that the amount of available surface area with which oxygen can react is preserved. This
 4 implies that ionic strength has little effect on oxidation of nano-Ag in solution, and might possibly have a
 5 correspondingly small effect on nano-Ag environmental effects if the formation of silver ions on the
 6 surface of the nanoparticle and subsequent release are the principal actions conferring nano-Ag toxicity.

7 Natural organic compounds in sea water can have surfactant and binding qualities that stabilize
 8 nano-Ag suspensions, thus making the particles more available for sorption to or uptake by specific
 9 aquatic organisms (Miao et al., 2009, [196270](#)). Gao et al. (2009, [195514](#)) found that increasing dissolved
 10 organic carbon (DOC) content generally decreased nano-Ag toxicity to both bacteria and *Ceriodaphnia*
 11 *dubia*. Other natural organic compounds, such as humic substances and carboxylic acids, have been
 12 found to adsorb quickly onto nanoparticle surfaces and stabilize them in suspension via repulsion
 13 (Cumberland and Lead, 2009, [199804](#)). Gao et al. (2009, [195514](#)) also experimentally examined the
 14 effects of natural surface-water characteristics on the dispersion, bioavailability, and toxicity of
 15 manufactured nanoparticles, including nano-Ag. Toxicity was examined in the freshwater invertebrate, *C.*
 16 *dubia*, and in bacteria using a 48-hour bioassay and METPLATE analysis, respectively. Characteristics of
 17 the materials used and toxicity test results are provided in Table 6-1.

Table 6-1. Experimental parameters and toxicity of nano-Ag in deionized water and natural surface waters.

| Water characteristics | Deionized water | | River-water samples | | |
|---|-----------------|------------------|---------------------|------------------|--|
| | | Headwater | Midsection | Delta | |
| pH | Not reported | 7.56 | 7.15 | 4.7 | |
| Alkalinity (mg CaCO ₃ /L) | ~0 ^a | 132 | 88 | 6 | |
| Ionic strength (mM) | ~0 ^a | 475 | 3.34 | 0.94 | |
| Dissolved organic carbon (mg C/L) | ~0 ^a | 2.3 | 10.18 | 45.71 | |
| Na ⁺ (mM) | ~0 ^a | 31.38 | <1 | <1 | |
| Ca ²⁺ (mM) | ~0 ^a | 6.61 | <1 | <1 | |
| Mg ²⁺ (mM) | ~0 ^a | 30.94 | <1 | <1 | |
| Ag _{Total} (µg/L) | ~0 ^a | <10 ^b | <10 ^b | <10 ^b | |
| Silver characteristics and toxicity assays | | | | | |
| Diameter nano-Ag (nm) | ~80 | >1000 | ~300 | ~80 | |
| Nominal Ag (mg/L) | 1000 | 1000 | 1000 | 1000 | |
| Measured total Ag (mg/L) ^c | 1.67 | 0.66 | 0.043 | 0.54 | |
| MetPLATE bacterial LC ₅₀ (µg/L) | 47.79 | 112 | No toxicity | No toxicity | |
| <i>Ceriodaphnia dubia</i> 48-hr LC ₅₀ (µg/L) | 0.46 | 0.70 | 0.77 | 6.18 | |
| 95% Confidence limits (µg/L) probit analysis | 0.45–0.47 | 0.66–0.73 | 0.75–0.80 | 5.5–6.7 | |

^a Not measured, but assumed to be approximately 0

^b The detection limit for Ag is 10 µg/L

^c After mixing and filtering to remove particles larger than 1.6 µm

Source: Adapted from Gao et al. (2009, [195514](#)).

1 The manufacturer reported the nominal diameter of the nano-Ag to be in the 20- to 30-nm range,
2 although transmission electron microscopy (TEM) revealed the average size of the nano-Ag in suspension
3 to be approximately 80 nm in deionized water and in one river-water sample and more than 100 nm in
4 two other river-water samples (Table 6-1) (Gao et al., 2009, [195514](#)). The average size of nano-Ag
5 suspended in the water sample taken from the river delta was in the μm range. Note that differences in
6 total silver measured in solution indicate differences in sorption of some of the silver to inorganic ligands
7 and DOC. The bacterial bioassay, known to be sensitive to dissolved metal ions, indicated no toxicity at
8 the two highest DOC concentrations. The nano-Ag solution prepared in deionized water was the most
9 toxic to both the bacteria and *C. dubia*. Gleaning specific conclusions from these experiments, however,
10 is challenging due to the co-variation among some water chemistry parameters.

11 Nano-Ag from spray disinfectants might end up in treated and untreated wastewaters;
12 consequently, other constituents of wastewater might influence nano-Ag toxicity. For example,
13 wastewater often contains an abundance of organic and inorganic ligands with which nano-Ag and silver
14 ions form strong complexes (Blaser et al., 2008, [193283](#); Choi et al., 2009, [193317](#)). Information on how
15 ligands might influence the bioavailability of nano-Ag, which in turn influences the effect of nano-Ag on
16 organisms, is presented in Section 5.2.1.2. Choi et al. (2009, [193317](#)) investigated the influence of
17 ligands on the toxicity of nano-Ag to nitrifying bacteria and found that a range of ligands, including
18 chloride, phosphate, $\text{H}_2\text{EDTA}^{2-}$, and sulfide, reduced toxicity to varying degrees, although sulfide was the
19 only ligand to reduce nano-Ag toxicity by more than 40%. At a 1 milligram Ag per liter (mg/L)
20 concentration in deionized water, nano-Ag inhibited nitrification by 100%. After sulfide was added to
21 achieve a final sulfide concentration of 10 micromoles per liter (μM), toxicity decreased by about 80%.
22 Miao (2009, [196270](#)) reported that adding thiols ($-\text{SH}$) to aqueous suspensions of nano-Ag increased the
23 dispersion of nano-Ag several orders of magnitude beyond levels predicted for the natural environment.
24 No toxicity was observed in the marine diatom *Thalassiosira weissflogii*, however, when exposed to
25 nano-Ag in the presence of thiols. The investigators believed this lack of toxicity might have been due to
26 the large size of the nano-Ag (60–70 nm), the protective layer of natural organic material around the
27 nano-Ag that prevented a direct interaction between nano-Ag and the algal cell, the concentrations of
28 nano-Ag used, or a combination of these factors (Miao et al., 2009, [196270](#)).

6.2. Ecological Effects

29 In its conventional form, silver can be toxic to fish, aquatic invertebrates, algae, some terrestrial
30 plants, fungi, and bacteria (U.S. EPA, 1993, [196772](#)). The ecological effects of conventional silver have
31 been studied extensively, although some data gaps remain; tests and environmental case studies have
32 revealed that conventional silver can be toxic to biota at aqueous concentrations at or below 50

1 nanograms per liter (ng/L) (Wijnhoven et al., 2009, [180201](#)). Some of the organisms most sensitive to
2 conventional silver are freshwater and marine phytoplankton, freshwater salmonids, and marine
3 invertebrates in early life stages (Luoma et al., 1995, [629664](#)). Although conventional silver can be
4 extremely toxic to biota, concentrations of free silver ions are not expected to be high enough in most
5 natural systems to adversely affect the environment (Luoma, 2008, [157525](#)). Nano-Ag, however, might
6 present a higher risk to ecosystems because it could become more bioavailable and provide a reservoir of
7 silver ions that could be delivered directly onto the surface of an organism or to cell constituents. Despite
8 this possibility, relatively few studies have investigated the effects of nano-Ag on organisms other than
9 bacteria and laboratory rodents. Moreover, such single-species tests likely do not capture the influence of
10 nano-Ag on structural and functional complexities at the ecosystem level. In addition, studies have not
11 explored the ecological effects from actual nano-Ag technologies at the product level, although these
12 products are thought to be becoming increasingly prevalent.

13 Although in vitro studies dominate the literature investigating nano-Ag ecological toxicity, a few in
14 vivo studies have been published recently. Ecological effects studies predominantly investigate nano-Ag
15 effects associated with acute exposure (generally 96 hours or less), and only a few studies examine nano-
16 Ag effects over subchronic and chronic exposure periods. Studies of ecological effects indicate that
17 exposure to nano-Ag could lead to adverse effects on higher level endpoints such as survival, growth, and
18 reproduction, and on sublethal endpoints such as phenotypic changes, gene expression, and oxidative
19 stress. Reported indirect effects of nano-Ag include pore clogging, solubilization of toxic compounds,
20 and production of ROS (Navarro et al., 2008, [157517](#)).

21 Because the dose in all studies discussed in this section was given as either mass concentration or
22 nanoparticle number in exposure media, these are the dose metrics provided here. Converting all
23 concentration data to the same units was not possible due to a lack of information provided by many
24 study authors on the factors used to define their units of measurement (e.g., for parts per million [ppm],
25 whether this unit is based on ppm by mass, number, or another metric is not always stated). Nominal
26 nano-Ag concentrations in the studies were based on total silver, silver ions, free silver, or added nano-Ag
27 content. This information is provided in the tables in Appendix B that summarize the ecological effects
28 studies; measured concentrations are also presented when provided in the studies. The studies are
29 presented in Appendix B in alphabetical order by author for each of the ecological effects sections; the
30 reader is referred to this appendix for study details not presented in this chapter. The following sections
31 present the available data on the effects of nano-Ag on bacteria and fungi (Section 6.2.1), aquatic
32 organisms (algae in Section 6.2.2.1, invertebrates in 6.2.2.2, and vertebrates in 6.2.2.3), and
33 nonmammalian terrestrial organisms (plants in 6.2.3.1, invertebrates in 6.2.3.2, and vertebrates in 6.2.3.3).
34 For each group of organisms, the discussion is organized into three parts: known effects of conventional

1 silver exposure, effects of nano-Ag exposure, and nano-Ag mode of action.¹⁷ This organization is
2 intended to capture the potential effects of nano-Ag, silver ions released from the silver nanoparticles, and
3 common silver complexes.

6.2.1. Bacteria and Fungi

4 The effects of silver ions on microbial communities are well-documented, and it is possible that
5 nano-Ag could have similar effects. Investigations of toxic effects on bacterial and fungal communities
6 might be relevant because such communities are key to nutrient decay and recycling processes that
7 support overall ecosystem functioning (Navarro et al., 2008, [157516](#)). In addition, nano-Ag might disrupt
8 gut microbes found within the digestive systems of higher level organisms (Sawosz et al., 2007, [194076](#)).
9 The antibacterial activity of nano-Ag has been demonstrated in laboratory testing of isolated species,
10 including *E. coli* (Hwang et al., 2008, [194065](#); Morones et al., 2005, [196271](#); Pal et al., 2007, [196273](#);
11 Sondi and Salopek-Sondi, 2004, [196277](#)); *Pseudomonas aeruginosa* (Morones et al., 2005, [196271](#));
12 *Vibrio cholerae* (Morones et al., 2005, [196271](#)); *Bacillus subtilis* (Yoon et al., 2007, [196281](#)); and
13 nitrifying cultures (Choi and Hu, 2008, [194061](#); Choi et al., 2008, [194060](#); Choi et al., 2009, [193317](#)).
14 The results from these studies demonstrate that the range of bacteria potentially susceptible to nano-Ag
15 toxicity spans gram-positive, gram-negative, autotrophic, and heterotrophic species. Studies specific to
16 nano-Ag effects on microbes, however, remain limited in number and scope compared to those
17 investigating effects of exposure to other forms of silver. In a recent review, Navarro et al. (2008,
18 [157516](#)) did not identify any studies of the effects of nano-Ag on fungi found in the environment; nano-
19 Ag effects on fungi that are commensal with humans, however, have been studied (Kim et al., 2009,
20 [194069](#)). Furthermore, as mentioned in Section 6.1.3, only two studies to date have attempted to
21 determine nano-Ag bacterial toxicity in more complex natural media (Bradford et al., 2009, [193294](#); Gao
22 et al., 2009, [195514](#)), and even these studies were limited in scope and not necessarily representative of
23 nano-Ag behavior in highly complex ecosystems. As a result, the available studies provide limited insight
24 into potential impacts from intentional and unintentional releases of nano-Ag into the environment.

¹⁷ Mode of action is defined in the US EPA Cancer Guidelines as “a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes and resulting in cancer formation” (US EPA 2005). However, multiple definitions for mode of action exist within the regulatory context. For the purposes of this document, mode of action refers to the key steps in the toxic response at the target site that are responsible for the physiological outcome or pathology of the chemical. Because mode of action is inherently linked to effects, it is sometimes not possible to completely separate the discussion of mode of action from the discussion of adverse effects (e.g., physical disruption of cell membrane is both an effect and mode of action for lethality). Where possible in this chapter, mode of action is discussed in a section following effects to highlight the processes that might be responsible for the observed adverse effects (e.g., reduced reproductive success, increased mortality).

Known Effects of Conventional Silver Exposure

1 Silver is a relatively toxic substance for microbes; for example, when compared to 12 other metals,
2 silver was identified as the most toxic to microbial soil communities (Cornfield, 1977, [060128](#)). Silver
3 can inhibit microbial growth, affecting sensitive communities such as ammonifying, nitrogen-fixing, and
4 chemolithotrophic¹⁸ bacteria (Albright and Wilson, 1974). The bacterial plasma and cytoplasmic
5 membrane are important target sites because silver ions cause the release of ionic potassium (K⁺) from
6 bacteria (Jung, et al., 2008, [199891](#)). Reduced DNA transcription also has been observed in bacteria
7 exposed to silver, resulting in microbial growth often being delayed or inhibited completely. Other
8 evidence suggests that exposure to silver leads to changes in membrane structure and deposition of silver
9 throughout the cell via formation (by combination of silver with cell constituents) of electron-dense
10 granules, as observed by TEM and X-ray microanalysis (Feng et al., 2000, [625629](#)). The bactericidal
11 mode of action of conventional silver is only partially understood, but is believed to be the result of
12 contact of the silver compounds with bacterial cell walls and release of silver ions that combine with –SH
13 groups of enzymes and lead to the deactivation of microbial proteins (Morones et al., 2005, [196271](#); Yoon
14 et al., 2007, [196281](#)).

Effects Specific to Nano-Ag Exposure

15 Examples of recent studies investigating the effects of nano-Ag on bacteria and fungi are presented
16 in detail in Section B.2 of Appendix B. These studies illustrate that exposure of bacteria and fungi to
17 nano-Ag frequently results in growth inhibition, inhibition of nitrifying enzymatic processes, arrest in
18 fungal cell cycles, cell membrane damage (e.g., pitting), cell membrane process disruption, and ROS
19 generation.

20 It is important to note that in experimental conditions, the bacterial cell density is generally high,
21 surface area-to-volume ratio is low, nano-Ag concentration is high, and contact between nanoparticles and
22 bacteria is ensured. In natural systems, significantly more reactive surfaces are available with which both
23 the nanoparticle and bacteria can interact, and concentrations of both might be much lower, which might
24 result in relatively rare contact between nanoparticles and bacteria (Neal, 2008, [196069](#)).

25 Sensitivity to nano-Ag varies among phyla, among species, and even among studies using the same
26 species. Shrivastava et al. (2007, [196276](#)) reported that gram-negative bacteria were more sensitive to
27 nano-Ag than gram-positive bacteria. For the gram-negative species *E. coli* and *Salmonella typhi* (drug-
28 resistant strains), 100% inhibition of growth was observed at a nano-Ag concentration of 25 µg/mL, while
29 no growth inhibition was noted in the gram-positive species *Staphylococcus aureus* at the same
30 concentration. Even at 100 µg/mL, the growth of *S. aureus* was only partially inhibited (Shrivastava et

¹⁸ Chemolithotrophic bacteria are those that derive energy from the oxidation of inorganic materials.

1 al., 2007, [196276](#)). Kvittek et al. (2008, [196266](#)) also reported that *E. coli* was more sensitive to exposure
2 to unmodified silver nanoparticles than *S. aureus*, but further comparisons of six gram-positive bacterial
3 strains to four gram-negative strains indicated that other factors beyond gram-status influence sensitivity.
4 The difference in minimum inhibitory concentrations (MICs) appeared to be species-specific, rather than
5 category-specific, as both the gram-positive and gram-negative bacterial groups displayed the same range
6 of MICs (1.69–6.75 µg/mL).

7 One area of concern resulting from increases in nano-Ag released to the environment is the
8 potential effect of nano-Ag and associated compounds on bacteria used in wastewater treatment processes
9 or located in areas where wastewater effluent is discharged (Bradford et al., 2009, [193294](#); Choi et al.,
10 2008, [194060](#)). Although many different types of bacteria can be used at different stages of the
11 wastewater treatment process, nitrifying bacterial communities (which can include a mixture of species
12 responsible for oxidizing ammonia to nitrite or nitrite to nitrate, including bacteria in the *Nitrospira*,
13 *Nitrosococcus*, *Nitrobacter*, *Nitrospina*, and *Nitrosomonas* genera) are considered especially vulnerable
14 due to their slow growth rate and history of sensitivity to other environmental pollutants (Choi and Hu,
15 2008, [194061](#); Choi et al., 2008, [194060](#); Choi et al., 2009, [193317](#)). Furthermore, nitrifying bacteria are
16 critical to processes involving nutrient removal in wastewater treatment (Neal, 2008, [196069](#)). If
17 nitrifying bacterial concentrations were significantly reduced or eliminated in wastewater treatment
18 bioreactors, chemical nutrients (e.g., ammonia) in the wastewater would not be removed, which might
19 ultimately result in eutrophication in areas where the wastewater is discharged (Grady et al., 1999,
20 [094147](#)). Eutrophication can cause anoxia and other reductions in water quality, leading to adverse
21 effects on aquatic biota.

22 Nitrifying bacteria are sensitive to nano-Ag exposure, but seemingly less so than *E. coli*.
23 Nitrification occurs in nitrifying bacterial communities by a two-step process involving three specific
24 enzymes. Of these three critical enzymes, the enzyme partially responsible for the oxidation of ammonia,
25 ammonia monooxygenase (AMO), appears to be the most sensitive to nano-Ag exposure. A
26 concentration of 1 mg/L nano-Ag inhibited nitrification (measured as change in AMO-related oxygen
27 uptake rates) by 100% in a respirometric assay, while silver ions inhibited growth by 83% at this
28 concentration (Choi et al., 2009, [193317](#)). Choi and Hu (2008, [194061](#)) determined that nano-Ag
29 inhibited the growth of nitrifying cultures ($EC_{50} = 0.14$ mg/L)¹⁹ more than silver chloride (AgCl) colloids
30 and silver ions (as AgNO₃). They also found that intracellular ROS concentrations increased significantly
31 compared to the controls when bacteria were exposed to nano-Ag, and that this increase correlated
32 strongly with growth inhibition ($R^2 = 0.86$). Choi et al. (2008, [194060](#)) demonstrated that at 1 mg Ag/L in

¹⁹ Effective concentration: the chemical concentration at which 50% of the exposed organisms experience a specific effect; this is a common effect level used to estimate the toxicity of a substance to a specific group of organisms.

1 nitrifying suspension, nano-Ag, silver ions, and AgCl colloids inhibited respiration by approximately
2 86%, 42%, and 46%, respectively.

3 Bradford et al. (2009, [193294](#)) examined the effects of nano-Ag concentrations of up to 1 mg/L on
4 bacterial abundance and diversity in an estuarine microcosm study. The investigators reported that the
5 lowest concentration used in this study (0.25 mg/L) was an order of magnitude higher than the
6 concentration expected from the highest estimated release of engineered nanoparticles from nano-enabled
7 products, as estimated by Boxall et al. (2007, [196111](#)). After applying 1/20th of the total nano-Ag dose to
8 20 L of estuarine water over ~3.8 kilograms (kg) of estuarine sediment for 20 days (followed by a 10-day
9 period with no dosing), Bradford et al. determined that nano-Ag exposure at a total concentration of
10 1 mg/L did not affect bacterial abundance and had a small statistically significant effect on bacterial
11 diversity on the sediment surface at the highest concentration tested (1 mg/L) when compared to controls.
12 The authors argued, however, that this difference was likely due to chance (based on a similarity profile
13 permutation procedure [SIMPROF]). To assess bacterial diversity, the investigators used a nested
14 polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method to amplify the
15 16S ribosomal RNA fragment in the bacteria species. They reported that the DNA primers they used were
16 specific to “*Bacteria*,” but which species might be included in this classification is unclear. Nevertheless,
17 multiple studies have used similar methods to establish nitrifying community structures in wastewater
18 treatment processes and to establish bacterial diversity in communities not associated with the
19 nitrification process (Mills et al., 2008, [094150](#); Muhling et al., 2008, [094151](#)), so nitrifying species
20 among others likely were included in this analysis. Because nitrifying bacteria will generally be found in
21 areas where wastewater is discharged into the environment due to higher concentrations of ammonia in
22 these locations, and because the samples were taken from an area that received discharges of wastewater
23 effluent, such bacteria likely were also present in the sediment cores. Without reporting which species
24 comprised the clusters in the estuarine sediment, Bradford et al. (2009, [193294](#)) argued that impacts on
25 bacteria in estuarine sediment will likely be negligible at nano-Ag concentrations expected from
26 estimated future releases (<1 mg/L). The investigators also reported, however, that the transformation of
27 nano-Ag into other forms of silver (e.g., Ag⁺, AgCl, AgCl₂⁻, AgCl₃²⁻) within the experimental tanks was
28 not studied, but such transformations would be expected to influence the potential impacts of nano-Ag in
29 estuarine waters (Bradford et al., 2009, [193294](#)).

30 As stated previously, very little information is available on the effects of nano-Ag on fungi. Based
31 on a study of the model fungus *Candida albicans*, Kim et al. (2009, [194069](#)) observed that exposure to
32 nano-Ag resulted in a loss of membrane potential and an increase of pitting on the cell surface, which was
33 tentatively linked to the formation of large pores through the cell walls and membranes and subsequent
34 cell death. The MIC of nano-Ag for this species was 2 µg/mL. Exposure to 40 µg/mL nano-Ag also
35 resulted in the arrest of the fungal cell cycle, most likely by inhibiting some of the cellular processes

1 necessary for bud growth. Although this fungus is commensal with humans and not commonly found in
2 the “external” environment, this study of *C. albicans* is the only representative fungal study at this time.

Nano-Ag Mode of Action

3 Silver ions released from silver nanoparticles are often purported to be the primary source of
4 toxicity from exposure to nano-Ag, particularly for higher organisms. Several studies have investigated
5 whether the nanoparticles inherently exacerbate the toxic effects or cause them outright. The full
6 sequence of events that causes silver nanoparticles to be toxic to bacteria and fungi is still largely
7 unknown or unconfirmed, although several recent studies have attempted to elucidate such mode(s) of
8 action (Choi and Hu, 2008, [194061](#); Hwang et al., 2008, [194065](#); Kim et al., 2009, [194069](#); Lok et al.,
9 2006, [196268](#); Morones et al., 2005, [196271](#)).

10 Several possible modes of action are discussed in the literature on nano-Ag effects on bacteria and
11 fungi. These are (1) membrane disruption through direct attachment of the nanoparticle to the bacterial
12 membrane, (2) cellular invasion and enzyme disruption by nanoparticles, (3) changes in cell membrane
13 permeability (4) interference with cellular S-containing compounds, and (5) intracellular ROS
14 accumulation (Hwang et al., 2008, [194065](#); Kim et al., 2009, [194069](#); Lok et al., 2006, [196268](#); Morones
15 et al., 2005, [196271](#); Pal et al., 2007, [196273](#); Panáček et al., 2006, [196274](#); Sondi and Salopek-Sondi,
16 2004, [196277](#)). That several of these events might act together to result in cell death is probable, but the
17 specific processes and interactions required for toxicity have not been fully confirmed.

18 Choi and Hu (2008, [194061](#)) present one example of how potential modes of action can work in
19 tandem in a study assessing the effects of nano-Ag on autotrophic bacteria. Although apoptosis occurred
20 and silver nanoparticles adsorbed to the surface of the microbial cell walls, cell membrane leakage was
21 not evident. The investigators believed that cell death occurred partially as a result of intracellular ROS
22 generation (although not proven directly) by the silver ions released at the membrane surface, which have
23 been shown to damage DNA and to induce apoptosis without causing visible damage to the outer bacterial
24 wall or cytoplasmic membrane (Inoue et al., 2002, [196758](#)). The toxic effects cannot be explained
25 completely by ROS generation, however, because silver ions did not induce the same level of toxicity at
26 similar intracellular ROS concentrations and total silver mass concentrations. Thus, the higher degree of
27 toxicity exhibited by nano-Ag may also have been because of the presence of particles smaller than 5 µm
28 that were able to cross the cell membrane and interact directly with cell constituents, releasing silver ions
29 directly to sensitive areas. Choi and Hu (2008) used TEM to identify the nanoparticles and determine
30 their size distributions. They also postulated that nano-Ag in the cell likely disrupted enzyme function,
31 ultimately causing cell death (Choi and Hu, 2008, [194061](#)).

32 Many studies suggest that heightened antibacterial activity of nano-Ag (compared to conventional
33 silver or silver ions alone) is related to a physical disruption of membrane function and cell processes,

1 most likely due to the direct contact of the nanoparticle with the bacterial membrane. Several studies
2 have reported the presence of pits or perforations on microbial surfaces, and Sondi and Salopek-Sondi
3 (2004, [196277](#)) confirmed by energy dispersive X-ray analysis (EDX) that nano-Ag was incorporated into
4 *E. coli* cell membranes. This effect has also been reported in the fungus *C. albicans*, where exposure to
5 nano-Ag resulted in pitting of the cell wall and a breakdown of the cell membrane permeability barrier.
6 The authors postulated that the membrane effects occurred through physical perturbation of the lipid
7 bilayers on the outer membrane, causing ion leakage, pore formation, and dissipation in the electrical
8 membrane potential. The destruction of the membrane integrity also could have inhibited normal fungal
9 budding processes (Kim et al., 2009, [194069](#)).

10 Silver nanoparticles have also been observed to embed themselves in nitrifying cell flocs when
11 added to nitrifying cultures, resulting in toxicity to the AMO enzyme, which is membrane-bound (Choi et
12 al., 2009, [193317](#)). The authors speculated that toxicity occurred as the result of small (<5 nm) silver
13 nanoparticles entering the cell, where they generated ROS or interfered with cellular S-containing
14 compounds in the respiratory path. In a proteomic²⁰ analysis, Lok et al. (2006, [196268](#)) demonstrated that
15 certain envelope protein expressions in *E. coli* were significantly altered after exposure to 0.4 and 0.8
16 nanomoles (nM) of bovine serum albumin-stabilized nano-Ag. This observation suggests that the ATP-
17 dependent preprotein translocase, which is associated with the inner membrane, had ceased to function.
18 In a typical cell, mature proteins are translocated to the outer membrane, but if that process is inhibited,
19 protein precursors simply build up in the cytoplasm. This study also indicated that *E. coli* exposed to
20 nano-Ag experience a decrease in proton motive force, which was observed in the near complete loss of
21 intracellular K⁺. Nano-Ag also decreased cellular ATP levels, which might also have contributed to cell
22 death (Lok et al., 2006, [196268](#)).

23 To demonstrate the effect of membrane disruption on cell invasion, certain antibiotics have been
24 used to increase permeability and porosity in bacterial membranes, which allows the ingress of silver
25 nanoparticles as large as 80 nm (Kyriacou et al., 2004, [196761](#); Xu et al., 2004, [196770](#)). Although many
26 investigators have observed through optical microscopy that nanoparticles can accumulate in the
27 cytoplasm of bacterial cells, there is very little information explaining the process by which cell invasion
28 occurs. For example, Pal et al. (2007, [196273](#)) noted that the cell walls of bacteria treated with nano-Ag
29 were significantly damaged (the type of damage was not specified). They also determined that
30 nanoparticles had accumulated in the cell walls and membrane and inside the cells, but did not report the
31 mode by which the nanoparticles were transported into these areas. Lok et al. (2006, [196268](#)) observed
32 silver nanoparticles attached to the surface of *E. coli* cells and within the cells, but again the mode of
33 transport was not reported. They did, however, observe perforation of the cell walls. Neal et al. (2008,
34 [196069](#)) hypothesized that nano-Ag, like other metals, might lead to the release of lipopolysaccharide

²⁰ A proteomic analysis evaluates the structure, function, interactions, and control of proteins.

1 proteins from gram-negative bacteria, causing the formation of pits in the outer membrane. This
2 morphology change increases permeability and leads to uncontrolled transport through the plasma
3 membrane, finally resulting in overall cell malfunction and death.

4 Bacterial membranes exposed to nano-Ag might be compromised as a result of lipid peroxidation
5 by ROS, which forms as natural by-products of aerobic metabolism but can increase considerably under
6 conditions of stress (Choi and Hu, 2008, [194061](#)). Some support for ROS-mediated adverse cellular
7 effects comes from a study using stress-specific bioluminescent *E. coli* (Hwang et al., 2008, [194065](#)).
8 The bioluminescent response of the bacteria indicated that superoxide radicals, which are a type of ROS,
9 were generated in response to nano-Ag exposure, and that protein and membrane damage also occurred,
10 most markedly at a concentration of 0.4 mg Ag/L. At a concentration of 0.5 mg/L, the reduction in the
11 growth rate of the *E. coli* strains was statistically significant. Silver nanoparticles have also been
12 observed to embed in nitrifying cell flocs when added to nitrifying cultures, resulting in toxicity to the
13 AMO enzyme, which is membrane-bound (Choi et al., 2009, [193317](#)). The authors speculated that
14 toxicity occurred as the result of small (<5 nm) silver nanoparticles entering the cell, where they
15 generated ROS or interfered with cellular S-containing compounds in the respiratory path. Section 5.2.2
16 discusses the findings of various studies on membrane disruption and permeability in gram-negative
17 bacteria, suggesting various ways that nano-Ag enters bacterial cells.

6.2.2. Aquatic Organisms

18 Due to the range of products into which nano-Ag is thought to be incorporated and the fact that
19 wastewater could be one of the most significant release pathways (see Chapter 3), the aquatic
20 environment might act as a substantial reservoir for nano-Ag and ionic silver discharged from silver
21 nanotechnologies or silver complexes formed as a result of those discharges.

22 Ionic silver is the only form of silver that has been broadly tested on aquatic organisms. From
23 these tests, ionic silver was deemed the second most toxic metal to aquatic organisms, after mercury
24 (Luoma, 2008, [157525](#)). Silver ions in the aquatic environment occur only at very low doses, however,
25 suggesting that under natural conditions, contact between silver ions and aquatic organisms might be
26 relatively rare (Blaser et al., 2008, [193283](#)). On the other hand, several studies report that nano-Ag
27 functionalized to remain stable in suspension might pose a significant risk to aquatic species if the
28 nanoparticles are in this state in the aquatic environment (Asharani et al., 2008, [194056](#); Kvittek et al.,
29 2009, [195535](#); Lee et al., 2007, [194072](#)). More data are available on the effects of silver ions than on the
30 effects of nano-Ag on aquatic species. Of the available data on nano-Ag effects, however, most
31 concentrate on effects in either aquatic organisms or bacteria. The majority of the research conducted on
32 nano-Ag effects on aquatic organisms has focused on fish, with very little attention to nano-Ag effects on

1 aquatic plants and invertebrates, despite evidence suggesting that these lower order aquatic organisms can
2 be highly sensitive to nano-Ag exposure (Griffitt et al., 2008, [157565](#); Luoma, 2008, [157525](#)). At this
3 point, the comparative toxicity of silver nanoparticles and conventional silver to aquatic organisms is
4 unclear, with studies reporting both enhanced toxicity of nano-Ag to aquatic organisms (Chae et al., 2009,
5 [196262](#)) and lower toxicity relative to silver ions (e.g., Griffitt et al., 2008, [157565](#); Miao et al., 2009,
6 [196270](#)). Because nano-Ag sheds silver ions, the distinction between nano-Ag toxicity and silver ion
7 toxicity is not always clear, so some studies have focused explicitly on investigating the effects due to
8 exposure to silver ions from AgNO₃ in comparison to silver ion effects as mediated by the silver
9 nanoparticle (Navarro et al., 2008, [157516](#); Navarro et al., 2008, [157517](#)).

10 The silver nanoparticles and silver ions released from nano-Ag are also likely to form complexes
11 with ligands and other materials in the aquatic environment, as discussed in Chapter 4. Silver complexes
12 will be most common in the particulate form, both suspended in the water column and deposited to the
13 sediment; however, no studies have been conducted investigating the effects of silver in the particulate
14 fraction and for silver thiolates in benthic organisms (Blaser et al., 2008, [193283](#)).

6.2.2.1. Algae

15 Most studies examining the effects of nano-Ag on biota have focused on bacteria and higher level
16 organisms, with only a few focused on plants. Even fewer studies are available for marine plants, with
17 the focus for most aquatic plants (e.g., algae) centering on freshwater species.

18 Algae are primary producers, acting as the food base in aquatic ecosystems, and algae in the oceans
19 provide much of Earth's oxygen. In addition to being an ecologically important group of organisms,
20 algae can sometimes act as indicators of aquatic ecosystem change. As such, algal toxicity tests are
21 integral to the investigation of potential effects on the aquatic environment resulting from the release of
22 chemicals.

Known Effects of Conventional Silver Exposure

23 Silver ions are highly algicidal, and various silver compounds (e.g., AgNO₃, sodium-silver
24 thiosulfate [NaAgS₂O₂], silver sulfate [AgSO₄]) can cause toxic effects in both freshwater and marine
25 algae (Ratte, 1999, [195564](#)). Exposure to silver has been shown to reduce freshwater growth rates in
26 *Chlamydomonas eugametos*, *Chlorella vulgaris*, *Haematococcus capensis*, and *Scenedesmus accuminata*
27 at concentrations of 0.01 mg/L or less (Hutchinson and Stokes, 1975, [094149](#)). Conversely, chronic
28 exposure to silver at low concentrations (up to 0.094 mg/L) promoted algal growth in *Selenastrum*
29 *capricornutum*, but high silver concentrations inhibited growth (Schmittschmitt et al., 1996, [094154](#)).

Effects Specific to Nano-Ag Exposure

1 Examples of recent studies investigating the effects of nano-Ag on algae are presented in detail in
2 Section B.3 of Appendix B. Effects on algae are measured at the population level, for example, in terms
3 of population growth. Effects on both freshwater and marine algae species have been investigated, as
4 described below.

5 In a comparative study by Griffitt et al. (2008, [157565](#)), the freshwater green alga
6 *Pseudokirchneriella subcapitata* was reported to be more sensitive to nano-Ag (96-hour EC_{50} =
7 0.19 mg/L) than fish (48-hour EC_{50} = 7.07 and 7.2 mg/L for adult and juvenile zebrafish [*Danio rerio*],
8 respectively), but slightly less sensitive than the aquatic invertebrates that were tested (48-hour EC_{50} =
9 0.040 and 0.067 mg/L for *Daphnia pulex* adults and *C. dubia* neonates, respectively).

10 Exposure to nano-Ag resulted in significant inhibition of growth, chlorophyll *a* production, and
11 photosystem II quantum yield in the marine diatom *Thalassiosira weissflogii* (Miao et al., 2009, [196270](#)).
12 The investigators used photosynthetic yield as a toxicity endpoint because of the importance of this
13 process to aquatic ecosystems. To eliminate the possibility that the direct effect of nano-Ag was being
14 masked by indirect effects from much higher concentrations of free silver ions in the immediate vicinity
15 of the nanoparticles, Miao et al. (2009, [196270](#)) removed the silver ions from solution either by
16 diafiltration or complexation with thiols. No significant toxicity to the diatom was observed following
17 silver ion removal. The authors tentatively concluded that toxicity was mainly due to the release of silver
18 ions, rather than from the direct interaction of nanoparticles with the diatom. However, the authors then
19 challenged this conclusion by pointing out that the silver nanoparticles used might not have been
20 appropriate for eliciting toxic effects because of their large size (60–70 nm), the wide range of
21 concentrations used, or the presence of organic compounds in the sea water that complexed with the
22 nanoparticles making them less bioavailable. These caveats suggest that further research using different
23 sized particles and experimental conditions could be useful to understanding toxic effects of nano-Ag on
24 *T. weissflogii* and other diatoms.

25 In a separate study, photosynthetic yield of the freshwater green alga *Chlamydomonas reinhardtii*
26 also was reduced after exposure to nano-Ag (5-hour EC_{50} = 829 nM [based on total Ag] or 8 nM [based
27 on free silver ions at the beginning of the experiment]) (Navarro et al., 2008, [157516](#)). The EC_{50} values
28 for silver ions determined in this study were 2–13 times higher than those shown to inhibit growth in
29 several algal species, including *C. reinhardtii*. The exposure duration for this study was only five hours,
30 however, while most algal toxicity studies evaluate effects after exposure durations of one or more days.
31 Navarro et al. (2008, [157516](#)) argued, however, that algal toxicity cannot necessarily be attributed to the
32 concentration of silver ions in original suspensions. They observed that nano-Ag was more toxic to *C.*
33 *reinhardtii* than $AgNO_3$ based on free silver ion content at the beginning of the experiment. In other
34 words, the lower free silver ion concentrations measured in the nano-Ag test waters compared with the

1 free silver ion concentrations measured in AgNO₃ solutions could not account for the higher toxicity
2 observed in the nano-Ag test vessels. Silver nanoparticles appear to continue to release silver ions over
3 time, whereas silver ions from AgNO₃, which is highly soluble, are released quickly. Thus, the
4 investigators speculated that the heightened toxicity of silver nanoparticles to algae compared with
5 soluble silver compounds was due in part to the nanoparticles' ability to act as prolonged sources of silver
6 ion delivery. Because the release and uptake of silver ions could depend on interaction between the
7 nanoparticle and the algal cell, assimilation of silver ions into the cell from nano-Ag might be more
8 efficient (Navarro et al., 2008, [157516](#)). Whether silver ions form at the algal surface or in the water
9 following interaction of the nanoparticle with secreted algal products, however, is unclear.

10 In addition to the effects observed in the toxicity studies, there is some speculation that due to the
11 propensity of nano-Ag for agglomeration and complexation, high nano-Ag exposures might lead to
12 increased cell density, shading, and clogging that produce adverse effects that cannot be attributed to the
13 toxicity of the silver nanoparticles (Navarro et al., 2008, [157517](#)). Although this result has not been
14 confirmed for nano-Ag, nanoscale titanium dioxide (nano-TiO₂) was shown to adsorb to algal cell
15 surfaces, increasing cellular weight by more than two-fold (exposure concentration not specified) (Huang
16 et al., 2005, [157801](#)).

Nano-Ag Mode of Action

17 Only one study has specifically explored the nano-Ag mode of action in algae. Depending on
18 whether observed effects are the result of direct nanoparticle effects or indirect effects resulting from the
19 release of silver ions from the nanoparticle on the surface of the algae, the mode of action might differ.

20 In the study by Miao et al. (2009, [196270](#)) investigating the effect of nano-Ag on growth and
21 photosynthetic yield in the marine diatom *T. weissflogii* under different nutrient conditions, investigators
22 distinguished between the direct effects of the silver nanoparticle and the indirect effects of released silver
23 ions, and proposed a potential mode of action. They reported that the silver ions released from the
24 nanoparticles appeared to be driving the toxic responses, and that the growth endpoint was more sensitive
25 than photosynthetic yield to silver ion exposure, indicating that the photosynthetic system was not the
26 primary target of the silver ions. Under nutrient-limited conditions, this diatom seemed to be less
27 susceptible to adverse effects of nano-Ag and produced significantly higher levels of carbohydrates,
28 which indicates the generation of polysaccharide-rich exopolymeric substances (EPS). EPS could be
29 involved in processes that regulate the uptake and subcellular distribution of silver ions, and higher EPS
30 levels might protect algae from oxidative damage associated with exposure to certain metals. As a result,
31 under nutrient-rich conditions, a potential mode of action for nano-Ag toxicity in this species might be
32 ROS accumulation leading to oxidative damage.

6.2.2.2. Aquatic Invertebrates

Known Effects of Conventional Silver Exposure

1 Data for the effects of conventional silver on benthic organisms are highly varied due to the
2 complex processes occurring in the sediment and differences in experimental designs and species
3 characteristics. In general, the amphipod *Hyalella azteca* is believed to be among the most sensitive
4 benthic invertebrates in the limnic environment, exhibiting a 10-day LC₅₀ as low as 1.6 milligrams Ag per
5 kilogram (mg/kg) dry weight when exposed to AgNO₃. Like most aquatic organisms, *H. azteca* is
6 markedly less sensitive to other silver species like silver thiosulfate and silver sulfide; toxicity of these
7 silver complexes is limited, however, primarily due to significantly lower bioavailability in the benthic
8 environment (Hirsch, 1998, [094148](#)).

9 More data are available for silver toxicity to freshwater planktonic invertebrates, such as the water
10 flea, *Daphnia magna*, which is also among the most sensitive planktonic invertebrates identified in
11 laboratory toxicity studies. Acute LC₅₀s for *D. magna* are as low as 5 micrograms total silver per liter
12 (µg/L) when organisms are unfed and exposed to silver added as AgNO₃ (Erickson et al., 1998, [094146](#)).
13 Increased mortality and decreased growth and reproduction in *D. magna* have also been reported
14 following chronic exposure to dissolved silver (inhibitory concentration causing 20% response [IC₂₀] =
15 2.56 µg/L) (Naddya et al., 2007, [195546](#)).

16 A toxic mode of action similar to that in freshwater fish is believed to lead to the toxic effect on
17 freshwater invertebrates from exposure to conventional silver. That sequence of events, discussed further
18 in Section 6.2.2.3, involves the inhibition of branchial ionic sodium/potassium-ATPase (Na⁺/K⁺-ATPase)
19 by silver, which ultimately leads to failure in the organism's ability to regulate ionic transport (Bianchini
20 and Wood, 2003, [195486](#)).

21 Studies on silver toxicity to marine invertebrates are not abundant and often are not comparable to
22 one another due to differences in experimental procedures. The existing data suggest that juvenile
23 bivalves are among the most sensitive marine organisms, with toxicity to silver ions occurring in the
24 <1- to 14-µg/L range. Toxicity endpoints observed in marine invertebrates include increased mortality
25 and delayed or abnormal development (Ratte, 1999, [195564](#)). The primary mode of action dictating silver
26 toxicity to marine invertebrates is suspected to be different from that in freshwater invertebrates and fish.
27 Silver toxicity to marine invertebrates is not associated with osmotic or ionoregulatory disruption at the
28 hemolymph level, but silver still could act on the Na⁺/K⁺-ATPase enzymes at the gill level, only
29 producing different effects (e.g., increased changes in univalent and divalent cations in tissues, change in
30 intracellular ion concentrations) (Bianchini et al., 2005, [195484](#)).

Effects Specific to Nano-Ag Exposure

1 Examples of recent studies investigating the effects of nano-Ag exposure on aquatic invertebrates
2 are presented in detail in Section B.4 of Appendix B. Currently, the only toxic endpoints that have been
3 examined for nano-Ag exposure to aquatic invertebrates are mortality and immobility. These effects have
4 been examined for the unicellular eukaryote, *P. caudatum* and two species of water flea, *D. pulex* and *C.*
5 *dubia*.

6 Exposure of *P. caudatum* to nano-Ag without surface modification resulted in significantly lower
7 toxicity (LC₅₀ = 39 mg/L) than that observed for many bacteria (LC₅₀ values from 1.69 to 13.5 mg/L)
8 (Kvitek et al., 2009, [195535](#)). No toxic effects were observed in *P. caudatum* at nano-Ag concentrations
9 lower than 25 mg/L, but mortality occurred at silver ion concentrations of 0.4 mg/L, indicating that silver
10 ions are more toxic to *P. caudatum* in terms of total silver added.

11 Feeding strategy and other interspecies differences, as well as environmental conditions, could
12 affect toxicity to aquatic invertebrates, as evidenced by the much higher sensitivity of *C. dubia* to
13 nano-Ag added to natural waters than that of paramecia exposed to nano-Ag in deionized water. Under
14 different experimental conditions not using natural water samples, adult *D. pulex* and *C. dubia* neonates
15 exhibited significantly higher sensitivity (Navarro et al., 2008, [157517](#)) than that noted by Kvitek et al.
16 (2009, [195535](#)) in *P. caudatum*, although this observation might be due in part to differences in
17 experimental conditions. Exposure of the filter-feeding water flea *C. dubia* to unspecified concentrations
18 of nano-Ag resulted in significant mortality when added to samples of headwaters, midsection, and delta
19 waters of the Suwannee River, with lower toxicity observed for headwaters (Gao et al., 2009, [195514](#)).
20 Daphnids also appear to be more sensitive to nano-Ag than adult and juvenile zebrafish and algae (Griffitt
21 et al., 2008, [157565](#)). Griffitt et al. (2008, [157565](#)) proposed that the large difference in nano-Ag
22 toxicities exhibited by various aquatic organisms largely depends on feeding strategies. Because
23 daphnids are particulate filter feeders, they might encounter relatively large numbers of nanoparticles over
24 the course of an acute exposure period. Nanoparticles might also adhere to invertebrate exoskeletons,
25 interfering with swimming and appendage movement. Significant changes in mobility and behavior have
26 been observed in *D. magna* on which carbon-60 (C₆₀) aggregates have formed, although these changes
27 were not explicitly linked to particle adhesion (Lovern et al., 2007, [091069](#)).

28 Although no published studies on the effects of nano-Ag in benthic invertebrates or marine
29 invertebrates were identified for this case study, research on the effects of nano-Ag in an estuarine oyster,
30 *Crassostrea virginica*, is currently underway. Preliminary results suggest a significant relationship
31 between nano-Ag exposure and total silver levels in tissues (type not specified) that contribute to toxicity
32 (unpublished, Ringwood et al., 2009, [196767](#)). Although little direct gill and hepatopancreas damage has
33 been observed following short-term exposure to nano-Ag, tissue-specific alterations of antioxidant
34 response have occurred (unpublished, McCarthy et al., 2009, [196763](#)).

Nano-Ag Mode of Action

1 No information specific to nano-Ag on the mode of action causing toxicity to aquatic invertebrates
2 was located. Nano-sized particles, however, have been reported to enter the digestive gland cells of blue
3 mussels and cockles by endocytosis (Moore, 2006, [089839](#)). Once in the cell, nanoparticles could
4 become embedded in cell constituents and contribute to oxidative damage by preventing the cell from
5 extruding the particles, although there is no direct evidence to support this hypothesis. Nano-Ag might
6 also adhere to the surfaces of sperm cells spawned freely into the water by organisms using this
7 reproductive strategy (e.g., seaweed, mussels, clams). Adhesion to the sperm cell surfaces might affect
8 fertilization success in aquatic invertebrates, as was demonstrated in the marine seaweed, *Fucus serratus*,
9 when exposed to carbon black. But whether nano-Ag would be available in suspension in sufficient
10 quantities for this to occur is unclear (Nielsen et al., 2008, [199963](#)). Furthermore, as observed in *D.*
11 *magna* exposed to C₆₀, direct particle adhesion to zooplankton exoskeletons might result in adverse
12 effects on behavior and mobility (Lovern et al., 2007, [091069](#)).

6.2.2.3. Fish

13 Fish are key secondary consumers in aquatic systems. Of the data available on nano-Ag toxicity to
14 aquatic organisms, fish studies are the most abundant. At this point, fish are the only aquatic vertebrates
15 for which the effects of nano-Ag have been reported in published literature. Ongoing research is
16 investigating the effects of nano-Ag on two species of whale (unpublished, Wisel et al., 2009, [196769](#)),
17 but overall, published research on the toxicity of nano-Ag to aquatic mammals, amphibians, and other
18 aquatic vertebrates does not appear to exist at this time.

Known Effects of Conventional Silver Exposure

19 Acute silver LC₅₀s for the most sensitive fish species are between 2.5 and 10 µg/L. Chronic no-
20 observed-effect concentrations ²¹ (NOECs) and maximum acceptable toxic concentrations (MATCs) were
21 between 0.4 and 0.7 µg/L for sensitive fathead minnows exposed to AgNO₃ (Ewell et al., 1993, [597397](#)).
22 Concentrations of AgNO₃ at or above 17 µg/L resulted in premature hatching and 15% reduced growth in
23 rainbow trout (*Salmo gairdneri*) fry (Davies et al., 1978, [094145](#)). Other silver compounds that are less
24 soluble, such as Ag-thiosulfate and AgCl, exhibited very little toxicity to developing *S. gairdneri*
25 (Hogstrand et al., 1996, [098721](#)).

26 Silver toxicity to freshwater fish is believed to result from silver ion interaction at the negatively
27 charged gill surface, where nano-Ag inhibits the basolateral Na⁺/K⁺-ATPase-dependent transport across

²¹ The highest tested concentration at which no adverse effects are observed on the aquatic test organisms at a specific time of observation.

1 the gills. Due to the inhibition of the ionic transport system, normal electrochemical gradients are
2 disrupted, and fish lose the ability to actively control the transport of ions across the gills, which can
3 result in a net loss of ions from the blood plasma, osmoregulatory failure, and ultimately in circulatory
4 collapse causing death (Bar-Ilan et al., 2009, [191176](#)). Although fish bioaccumulate silver, the toxic
5 mode of action in freshwater fish does not appear to be the result of internal silver accumulation, but
6 rather the accumulation of silver at the gill surface (Ratte, 1999, [195564](#)).

7 The mode of action of silver toxicity to marine fish is not well understood, but appears to be very
8 different from that of freshwater species. There is some speculation that toxicity is attributable in equal
9 parts to processes taking place at the gill surface and those occurring in the gut of the fish (Grosell and
10 Wood, 2001, [199806](#)). Silver induces ionoregulatory failure in marine fish, although typically at
11 concentrations that are one to two orders of magnitude higher than in freshwater fish (Pedroso et al.,
12 2007, [195557](#)). Preliminary (unpublished) research underway at Duke University's Center for
13 Environmental Implications of Nanotechnology (CEINT) suggests that silver toxicity to Atlantic killifish
14 (*Fundulus heteroclitus*) embryos and larvae is not due entirely to exposure to silver ions, as previously
15 thought (Matson, 2010, [202113](#)). The investigators reported that conventional silver toxicity did not
16 follow a linear response curve along an increasing salinity gradient. Instead, toxicity decreased up to a
17 certain chloride concentration and then increased again at salinities similar to those in estuarine
18 environments. The reason underlying the U-shaped salinity-toxicity relationship is unknown (this
19 response is not observed in adult *F. heteroclitus* adults under the same conditions), but the investigators
20 proposed that the observed toxicity might be due to the concentration of total dissolved silver in solution.

Effects Specific to Nano-Ag Exposure

21 Examples of recent studies investigating the effects of nano-Ag on fish are presented in detail in
22 Section B.5 of Appendix B. Currently, fish are the only aquatic vertebrates for which the effects of
23 nano-Ag have been investigated. The only species for which nano-Ag toxicity tests have been published
24 are freshwater zebrafish (*D. rerio*) and fathead minnow (*P. promelas*), and the anadromous rainbow trout
25 (*Oncorhynchus mykiss*) and Japanese medaka (*Oryzias latipes*). *Danio rerio*, the most widely used test
26 organism for investigating the effects of nano-Ag in fish, are gaining popularity as model organisms in
27 toxicological studies due to the high degree of homology to the genome of other vertebrates (including
28 humans) and similarities in physiologic responses to various stressors across vertebrate species. They
29 also display rapid ex utero and post-fertilization development and high fecundity. The transparent
30 embryos, with tissues turning opaque upon cell death, allow for real-time analysis of developmental
31 effects in addition to real-time monitoring of nanoparticle transport (Bar-Ilan et al., 2009, [191176](#); Lee et
32 al., 2007, [194072](#)). Fish study endpoints can include tissue and whole-body concentrations of chemicals

1 (e.g., bioaccumulation, as discussed in Section 5.2.3.8), mortality, behavioral markers (e.g., coughing and
2 abnormal swimming), and morphological malformations (e.g., pericardial edema, bent spine, small head).

3 A recent study using *O. latipes* reported that this species is susceptible to nano-Ag with observed
4 effects at concentrations at or above 25 µg/L, but that changes in gene expression possibly indicative of
5 nano-Ag toxicity occurred at concentrations as low as 1 µg/L (Chae et al., 2009, [196262](#)). Moreover, the
6 gene expression patterns observed in *O. latipes* exposed to nano-Ag were distinguishable from those
7 observed following exposure to silver ions (added as AgNO₃), indicating a distinct nanoparticle effect.
8 The genes analyzed were hepatic biomarkers associated with metals detoxification, antioxidant defense,
9 cellular responses to stressors, toxin binding and transport, catalysis, cell-cycle arrest, apoptosis, DNA
10 repair, biotransformation and detoxification of endogenous and exogenous compounds, iron metabolism,
11 and immune system response. Chae et al. (2009, [196262](#)) found that nano-Ag at concentrations of 1 and
12 25 µg/L significantly affected gene expression in the six genes examined at various time points during the
13 10-day exposure period. Although all tested genes responded differently to nano-Ag and silver ions, the
14 largest statistically significant differences were observed in the heat shock protein HSP70, a stress
15 protein; p53, a DNA repair and apoptosis-inducing protein; and transferrin, an iron transport protein. As
16 more ecological toxicity tests attempt to elucidate a mode of action by investigating the induction of such
17 “stress-response genes,” however, it is important to keep in mind that gene induction in response to
18 stressors does not necessarily indicate an adverse effect at the organism level. Genes responding to stress
19 are expected to provide some resistance to adverse effects up to a certain threshold exposure level
20 (Crawford and Davies, 1994, [629931](#)). Furthermore, the impact of changes at the molecular level due to
21 exposure to toxicants has not been fully investigated or correlated to changes that might occur in dynamic
22 populations of these same organisms. This caveat is applicable to all toxicogenomic studies discussed
23 hereafter.

24 In a conference presentation, Wisel et al. (2009, [196769](#)) reported that nano-Ag is highly cytotoxic
25 (in a concentration-dependent manner) and genotoxic to *O. latipes*. Additionally, nano-Ag appears to be
26 more toxic to this species than silver ions released from AgNO₃. Although the 96-hour LC₅₀ for nano-Ag
27 and silver ion exposures to *O. latipes* were comparable (34.6 vs. 36.5 µg/L), significantly more mortality
28 occurred at higher nano-Ag concentrations than at equivalent concentrations of silver ions from AgNO₃,
29 and statistically significant gene induction was less common following exposure to AgNO₃ (Chae et al.,
30 2009, [196262](#)). Laban et al. (2009, [199809](#)) reported that dissolved silver from AgNO₃ was more toxic to
31 *P. promelas* embryos than dissolved silver released from nano-Ag.²² They reported a AgNO₃ LC₅₀ of
32 15 µg/L, which is below the level of dissolved silver released by the silver nanoparticles in any treatment
33 group (18–95 µg/L). The investigators compared the toxicity of nano-Ag solutions that had been briefly
34 sonicated to those that had only been stirred, and measured the concentrations of dissolved silver in each

²² Defined as ionic silver released from silver nanoparticles and dissolved silver nanoparticles.

1 type of solution. Although the dissolved silver concentrations were not significantly different between the
2 stirred and sonicated samples, the sonicated samples were 10 times more toxic than the stirred samples,
3 suggesting that nano-Ag toxicity cannot be attributed purely to the concentration of dissolved silver
4 released from the nanoparticle. The nano-Ag LC₅₀s determined by Laban et al. (2009, [199809](#)) for
5 *P. promelas* in the sonicated samples were 1.25 and 1.36 mg/L for the two commercially produced
6 nano-Ag particles tested, nanoAmor and Sigma-Aldrich, respectively, suggesting that this species is less
7 sensitive to nano-Ag exposure than *O. latipes*. Toxicity, however, could have been affected by a number
8 of experimental conditions, such as pH, water hardness, and dissolved oxygen, all of which were reported
9 by Laban et al. (2009, [199809](#)), but not by Wisel et al. (2009, [196769](#)).

10 Lower sensitivity than described for *O. latipes* was also observed in *O. mykiss* exposed to nano-Ag
11 in the form of Nanocid[®], a water-based colloidal suspension designed by Nano Nasb Pars Co. (Tehran,
12 Iran) for use as a disinfectant in aquaculture. In addition to lethality, reported effects included hypoxia,
13 lethargy, unusual swimming behavior, elevated gill ventilation, and darkening of the body, although the
14 concentrations at which these effects occurred was not reported. The 96-hour LC₅₀ was 2.3 mg/L, which
15 is almost two orders of magnitude higher than that reported for other fish species. Characterization of the
16 test material was not reported, however, and particle clusters could have formed, reducing the number of
17 nanoparticles in suspension (Shahbazzadeh et al., 2009, [195010](#)).

18 Zebrafish (*D. rerio*) have been examined in the embryonic, juvenile, and adult life stages for lethal
19 and sublethal toxic effects, including the influence of nano-Ag on caudal fin regeneration. Bar-Ilan et al.
20 (2009, [191176](#)) investigated the size-dependent toxicity of nano-Ag on *D. rerio* embryonic development
21 using 3-, 10-, 50-, and 100-nm silver nanoparticles. The embryos exhibited almost 100% mortality at
22 nano-Ag concentrations of 250 μM, regardless of the size of the nanoparticle. LC₅₀ values (93.31 μM for
23 3-nm particles to 137.26 μM for 100-nm particles) indicated that toxicity is loosely size-dependent,
24 although only at certain concentrations and time points. In a study by Asharani et al. (2008, [194056](#)),
25 mortality was higher in *D. rerio* embryos exposed during the cleavage period of development (2- to 8-cell
26 stages, up to approximately 1 hour post-fertilization) than in those exposed after the cleavage period when
27 embryos are entering epiboly (4 to 6 hours post-fertilization). Embryos exposed earlier were more
28 sensitive, exhibiting an LC₅₀ of 25 μg/mL, compared to embryos exposed later in development that
29 exhibited an LC₅₀ of 50 μg/mL (Asharani et al., 2008, [194056](#)).

30 Bar-Ilan et al. (2009, [191176](#)) reported that 3-nm and 10-nm silver nanoparticles seemed to
31 produce the greatest amount of statistically significant sublethal toxic effects (based on seven quantified
32 effects) in *D. rerio* embryos at a concentration of 100 μM nano-Ag when compared to a range of other
33 nano-Ag concentrations. Overall, 100 μM nano-Ag exposure resulted in embryos having 31–46% smaller
34 heads, 87–119% larger yolk sacs, 31–68% smaller caudal fins, and 38–55% smaller eyes than the
35 controls. In addition, embryos were 14–25 mm shorter, and had 5–14π more axial curvature and 16–64%
36 larger pericardial sacs, but these were not statistically significantly different from the controls (Bar-Ilan et

1 al., 2009, [191176](#)). At 120 hours post-fertilization, the embryos had not depleted their yolk sacs, which
2 were their only source of food throughout the exposure period. The underdeveloped bodies of the
3 exposed embryos suggest that nano-Ag exposure inhibited the uptake of nutrients from the yolk sac,
4 although how nutrient uptake was impaired was not investigated. Lee et al. (2007, [194072](#)) reported a
5 threshold for *D. rerio* of 0.19 nM nano-Ag, above which no embryos exposed at the eight-cell stage
6 developed normally. At concentrations higher than 0.19 nM, all embryos were either dead or deformed,
7 and the incidence of deformities decreased as the number of dead embryos increased. Mortality ranged
8 from 20% to 90% at nano-Ag concentrations of 0.05 to 0.72 nM, respectively, and deformities ranged
9 from approximately 2% at 0.05 nM nano-Ag to 42% at 0.19 nM (Lee et al., 2007, [194072](#)). Some
10 sublethal toxic effects, such as yolk sac and pericardial edema, were also observed in *P. promelas* exposed
11 to nano-Ag concentrations up to 20 mg/L (Laban et al., 2009, [199809](#)). Several studies have
12 demonstrated that nano-Ag exposure contributes to mortality and sublethal developmental effects on *D.*
13 *rerio* embryos in a concentration-dependent manner; the results from these studies, however, cannot be
14 compared directly due to different experimental designs and dosing methods (Asharani et al., 2008,
15 [194056](#); Bar-Ilan et al., 2009, [191176](#); Lee et al., 2007, [194072](#)).

16 Additional phenotypic and physiological endpoints shown to be affected by *D. rerio* exposure to
17 nano-Ag at concentrations greater than 50 µg/mL are decreased heart rate, hatching delay, accumulation
18 of blood in the blood vessels near the tail, apoptosis, slimy external skin coating, finfold abnormalities,
19 tail and spinal cord flexure and truncation, cardiac malformation, head edema, eye malformation,
20 hemorrhaging, blood clots, and distortion of the yolk sac (Asharani et al., 2008, [194056](#); Bar-Ilan et al.,
21 2009, [191176](#); Lee et al., 2007, [194072](#); Yeo and Kang, 2008, [199841](#)). Laban et al. (2009, [199809](#))
22 observed similar abnormalities in developing *P. promelas* embryos exposed to nano-Ag. One concern in
23 the testing of nano-Ag for toxic effects is that residual silver ions from the nano-Ag feedstock might be
24 present in nano-Ag stock solutions and therefore be responsible for the observed effects. Asharani et al.
25 (2008, [194056](#)) investigated whether exposure to silver ions alone could result in the effects observed in
26 developing zebrafish embryos following exposure to the nano-Ag test material. They reported that
27 exposure of *D. rerio* embryos to concentrations of silver ions equivalent to the range of nano-Ag
28 concentrations shown to result in gross malformations did not affect any of the phenotypic endpoints
29 examined. The highest silver ion concentration tested (20 nM) resulted in 10% mortality and hatching
30 delay in 4% of the embryos, but did not significantly affect overall development of the embryos. The
31 results indicate that the observed toxic effects were not the result of residual silver ions in the exposure
32 medium left over from the synthesizing process.

33 Phenotypic changes such as those described above have been observed in *D. rerio* embryos
34 exposed to toxic chemicals other than nano-Ag. For example, Lee et al. (2007, [194072](#)) noted that finfold
35 abnormalities, tail and spinal cord flexure and truncation, cardiac malformation, and yolk sac edema have
36 all been observed in embryos exposed to dichloroacetic acid and cadmium. The specific eye

1 malformation (no formation of retina or lens), however, resulting from exposure to nano-Ag has not been
2 reported in literature describing exposure of *D. rerio* to any other toxic chemical (Lee et al., 2007,
3 [194072](#)).

4 *Danio rerio* can regenerate many body structures, including the spinal cord, optic nerve, heart, and
5 fins (Yeo and Pak, 2008, [191177](#)). The effect of nano-Ag on caudal fin regeneration was investigated by
6 Yeo and Pak (2008, [191177](#)). At a concentration of 4 ppm, nano-Ag significantly inhibited regeneration,
7 and exposure to 0.4 ppm delayed, but did not completely inhibit, caudal fin regeneration. Exposure to 0.4
8 and 4 ppm nano-Ag resulted in defects in regeneration observed within 10 days following amputation.

9 Griffitt et al. (2009, [199805](#)) investigated the effects of the NOEC of nano-Ag on adult *D. rerio* gill
10 histology and gene expression, and compared these responses to those elicited by soluble silver
11 concentrations (through addition of AgNO₃) equivalent to those released by the nano-Ag after 48 hours.
12 Although the dissolved silver concentrations were comparable between the nano-Ag and AgNO₃
13 solutions, gill filament widths were significantly larger in those zebrafish exposed only to the dissolved
14 silver from the AgNO₃ solutions. The gill tissues and whole carcasses of zebrafish exposed to nano-Ag,
15 however, contained significantly higher concentrations of total silver than those of the zebrafish exposed
16 only to soluble silver.

17 Because the research presented in this section suggests that aquatic organisms are susceptible to
18 nano-Ag toxicity, the effects on fish and other aquatic species at the organism level suggest that impacts
19 from nano-Ag at the population and ecosystem level are possible. Research investigating nano-Ag effects
20 on sperm whales (*Physeter macrocephalus*) and North Atlantic right whales (*Eubalaena glacialis*)
21 (unpublished, Wisel et al., 2009, [196769](#)); Atlantic tomcod (*Microgadus tomcod*) (unpublished, Nichols et
22 al., 2009, [196766](#)); and Atlantic killifish (*F. heteroclitus*) (Matson, 2010, [202113](#)) is currently underway.
23 Preliminary results indicate that nano-Ag is toxic to both cells and genetic constituents of cells in both
24 whale species in a concentration-dependent manner, although whale cells appear to be less sensitive than
25 those of medaka (*O. latipes*) (unpublished, Wisel et al., 2009, [196769](#)). These data can be used as an
26 indication of the body of information that might become available in the next several years.

Nano-Ag Mode of Action

27 The toxic mode of action of nano-Ag in fish has not been fully elucidated. Although silver
28 nanoparticles have been observed inside fish embryos and toxic effects have been quantified, these results
29 serve only to allow speculation concerning the mode of action. Griffitt et al. (2009, [199805](#)) examined
30 the transcriptional profiles of *D. rerio* adults exposed to nano-Ag and soluble silver (from AgNO₃).
31 Despite having similar concentrations of soluble silver in both exposure groups, the zebrafish exposed to
32 nano-Ag exhibited a significantly different gene expression profile than those fish exposed to only the
33 soluble silver, indicating that the nano-Ag mode of action differs significantly from that of silver ions. As

1 examined by Chae et al.(2009, [196262](#)), the independent expression of genes in the *O. latipes* liver that
2 act as indicators for carcinogenesis, mutagenesis, DNA repair, and oxidative damage indicates that rapid
3 biotransformation and detoxification was occurring in the liver. If biotransformation and detoxification
4 were indeed occurring, this suggests that nano-Ag might induce apoptosis, which is assumed to result
5 from nano-Ag-generated ROS. There is no direct evidence, however, linking ROS to toxicity in this case;
6 cytotoxicity from ROS was inferred only from the response of the indicator genes. Furthermore, Griffith
7 et al. (2009, [199805](#)) did not measure induction of any *D. rerio* genes currently mapped to oxidative stress
8 regulation when the fish were exposed to nano-Ag NOEC concentrations, indicating that ROS generation
9 alone might not completely explain toxic effects from nano-Ag exposure.

10 Several studies (Asharani et al., 2008, [194056](#); Laban et al., 2009, [199809](#); Lee et al., 2007,
11 [194072](#)) have demonstrated the ability of fish embryos to take up silver nanoparticles (see Section 5.2.3).
12 Lee et al. (2007, [194072](#)) demonstrated that silver nanoparticles can penetrate the egg chorion²³ through
13 the chorionic pore canals by passive transport. From there, some nanoparticles penetrated the embryo
14 itself and embedded in a number of zebrafish organs (Lee et al., 2007, [194072](#)). Another study showed
15 that nano-Ag deposits on the skin and uniformly within the body, showing a particular affinity for the cell
16 nucleus (Asharani et al., 2008, [194056](#)). One study also reported penetration and accumulation in all
17 organelles – including the nucleus – of the gill, muscle, and regenerated fin tissue of zebrafish (age not
18 specified) (Yeo and Pak, 2008, [191177](#)). Because accumulation in the nucleus can lead to genomic
19 damage, this observation supports other results indicating a genomic response to nano-Ag exposure in *O.*
20 *latipes*. Yeo and Pak (2008, [191177](#)) reported upregulation of five genes involved in apoptosis 50 hours
21 post-fertilization in *D. rerio* embryos exposed to nano-Ag. Increased apoptosis in response to nano-Ag
22 might result from increased production of free radicals. Additional evidence that nano-Ag exposure
23 increases free radicals that could cause DNA damage comes from observations in a study by Yeo and
24 Kang (2008, [199841](#)), in which levels of the enzyme catalase, which is responsible for removing free
25 radicals, increased in *D. rerio* exposed to 10 and 20 parts per trillion (ppt) nano-Ag. Although Rojo et al.
26 (2007, [091296](#)) did not observe any developmental effects or mortality in *D. rerio* at nano-Ag
27 concentrations up to 5 ppm, they did measure increased expression of genes involved in detoxification
28 and regulation of the oxidative stress response.

29 Nano-Ag has been observed to interfere with cardiac muscle function, preventing the flow of blood
30 through the body of the embryo (Asharani et al., 2008, [194056](#)). Nano-Ag might inhibit cardiac function
31 directly by interacting with cardiac cells, disrupting normal cell functioning, and weakening the pumping
32 of the heart. The weakened pumping of the heart results in restricted blood flow, which might indirectly
33 affect the ability of the embryo to access vital energy sources contained in the blood. Following a loss of
34 blood flow, nano-Ag deposits in the brain, which could interfere with signal transduction and other

²³ The chorion is the outermost of two membranes surrounding the embryo; the inner membrane is the amnion.

1 nervous system processes, leading to a loss in neurological function. A loss of neurological function is
2 supported by observed insensitivity of the larvae to touch. The silver nanoparticle, by simply attaching to
3 a biological surface (e.g., a developing zebrafish organ), might act as a foreign body, thereby limiting
4 functionality of the cell or organ to which it has attached (Asharani et al., 2008, [194056](#)). For example,
5 Handy et al. (2008, [157563](#)) noted that although engineered nanoparticles are typically too large to exploit
6 direct uptake channels to nerve cells, they are probably capable of attaching to the epithelium and
7 interacting with receptors. Such interactions might interfere with olfactory function, such as chemical
8 signaling, leading to changes in behavior that affect survival.

6.2.2.4. Model to Estimate Toxicity to Aquatic Biota

9 The computational models that are currently being recommended for use in predicting the toxicity
10 of nano-Ag and related silver species are limited in number and scope. At this time, no models appear to
11 be available for assessing toxicity from nano-Ag particles, but two models can be used to estimate the
12 toxicity of silver ions released from the particles. The models apply only to the aquatic environment,
13 however, and no terrestrial toxicity models appear to be available at this time. The models discussed here
14 are used to estimate predicted no-effect concentrations (PNECs) for aquatic biota in the water column
15 (Mueller and Nowack, 2008, [157519](#)) and to predict metal toxicity to fish (Di Toro et al., 2001, [081226](#)).

Predicted Water Column No-Effect Concentration Model

16 Mueller and Nowack's (2008, [157519](#)) fate and transport model (described in Section 4.5) also
17 estimated a PNEC for aquatic biota based on a published study by Yoon et al. (2007, [196281](#)) of acute
18 nano-Ag toxicity with two bacteria, *B. subtilis* and *E. coli*. The threshold concentrations (equivalent to a
19 NOEC) for these species in water (20 and 40 mg/L, respectively) were divided by an assessment factor of
20 1,000, in accordance with the Technical Guidance Document on Risk Assessment published by the
21 European Chemicals Bureau (European Chemicals Bureau, 2003, [196375](#); Mueller and Nowack, 2008,
22 [157519](#)). The ratios of the PEC of nano-Ag in water of 0.03 µg/L for the "realistic scenario" and
23 0.08 µg/L for the "high scenario," to the PNEC of 20 µg/L (for bacteria) were orders of magnitude less
24 than 1.0 (i.e., less than a hazard quotient of 1.0). The investigators did not, however, consider silver ions
25 potentially released from the nanoparticles. They noted that release of nano-Ag particles is of secondary
26 importance to the release of silver ions from the nanoparticles given the higher toxicity of ionic silver, for
27 which the authors cite Blaser et al. (2008, [193283](#)) (Mueller and Nowack, 2008, [157519](#)).

Biotic Ligand Model

1 A biotic ligand model (BLM) has been developed as a predictive tool to enable estimation of acute
2 metal toxicity in an aqueous environment when several aspects of the water chemistry are known
3 (Di Toro et al., 2001, [081226](#)). The BLM accounts for several water chemistry parameters, including
4 ionic chlorine (Cl^-) concentration and the amount of dissolved organic matter, to predict the amount of
5 free metal ion available to bind to the fish gill. For acute toxic effects on fish, the gill is considered a
6 proximate site of toxic action. The biotic ligand binding site is thought to be one or more sensitive
7 enzyme systems (carbonic anhydrase or Na^+/K^+ -ATPase) (Bielmyer et al., 2008, [195490](#)). Accumulation
8 at the surface of gills is relevant to a possible mode of action of nano-Ag because gill transport of silver
9 and other ions also varies with water chemistry. Notably, however, the BLM was specifically developed
10 to model binding and toxicity of metal cations, and consequently it is unlikely that this model would
11 adequately predict the behavior and toxicity of nano-Ag and other metals when present in nanoparticle
12 form.

6.2.3. Terrestrial Organisms

13 Very few studies have investigated the effects of nano-Ag on terrestrial organisms, with no
14 investigations of nano-Ag toxicity in soils (Wijnhoven et al., 2009, [180201](#)). This section summarizes
15 knowledge regarding conventional silver and nano-Ag effects on several types of terrestrial organisms,
16 including plants, invertebrates, and vertebrates.

6.2.3.1. Terrestrial Plants

17 Only recently have some studies emerged that investigate the effects of plant exposure to nano-Ag.
18 At this point, literature on the effects from environmental exposure to nano-Ag are still lacking, with most
19 studies relying on experimental procedures that do not mimic natural conditions and endpoints that might
20 not be relevant to environmental assessment. Furthermore, the available studies that were identified lack
21 information concerning the characteristics of the nano-Ag materials used, adequate data supporting the
22 study procedure, and in many cases, identification of treatment groups that differ significantly from the
23 controls for the endpoint in question. Nonetheless, these studies provide some insight into the potential
24 responses of higher order plants to nano-Ag exposure.

Known Effects of Conventional Silver Exposure

25 Although data on the effects of silver in higher plants are limited and highly varied, a review by
26 Ratte (1999, [195564](#)) suggests that plants are most sensitive to nano-Ag during germination and growth.

1 Lettuce (*Lactuca sativa*), radish (*Rhaphanus sativas*), and maize (*Zea mays*) seeds exposed to AgNO₃
2 exhibited reduced germination and growth at or below 7.5 mg/L AgNO₃ (Ewell et al., 1993, [597397](#)).
3 Spiking sewage sludge with 5.2 and 120 mg Ag/kg dry weight, however, did not have a statistically
4 significant effect on growth or emergence of lettuce, turnips, oats, and soybeans. In fact, the mean fresh
5 weight of the lettuce leaves increased significantly in the groups exposed to silver, although a change in
6 dry weight was not observed (Hirsch, 1998, [224892](#)).

Effects Specific to Nano-Ag Exposure

7 Summaries of three recent relevant studies investigating the effects of nano-Ag on terrestrial plants
8 are provided in Section B.6 of Appendix B. These studies suggest that nano-Ag might be toxic to higher
9 order plants at concentrations above 10 ppm, although statistical significance was not always reported
10 (Babu et al., 2008, [195479](#); Kumari et al., 2009, [199808](#); Rostami and Shahsavar, 2009, [199810](#)).
11 Rostami and Shahsavar (2009, [199810](#)) demonstrated that submerging olive (*Olea europea* L.) explants,
12 or the part of the plants used to initiate a culture, in nano-Ag solutions with concentrations ranging from
13 100 to 400 ppm effectively eliminated bacterial contamination in the explants, but also resulted in a high
14 percentage of plant mortality. When the investigators added nano-Ag to a prepared medium in lower
15 concentrations (2–6 ppm) and allowed the explants to grow in the contaminated media for 30 days, the
16 nano-Ag exposure seemed to result in more than a 50% decrease in plant mortality up to 4 ppm when
17 compared the controls (statistical significance not reported); mortality then again increased slightly
18 (~20%), although this increase was reported as not statistically significant.

19 Nano-Ag exposure induced a dose- and duration-dependent mitodepressive and cytotoxic effect on
20 onion (*Allium cepa*) meristems (root tips). In a toxicity assay, Babu et al. (2008, [195479](#)) reported that
21 exposure of *A. cepa* meristems to nano-Ag resulted in a reduced frequency of mitotic index, which is a
22 measure of cell proliferation, and increased frequency of chromosomal aberrations. These results
23 occurred at all nano-Ag concentrations tested and at every exposure duration, although results were not
24 always statistically significant for shorter exposure durations. Kumari et al. (2009, [199808](#)) also reported
25 a nano-Ag concentration-dependent effect on *A. cepa* mitotic index, but the decrease was significantly
26 different from the control group only at concentrations at or above 50 ppm. Babu et al. (2008, [195479](#))
27 reported that a significant reduction in frequency of cell division occurred even in the groups exposed to
28 the lowest concentration of nano-Ag (10 ppm) for 2 hours, and at higher concentrations (20–50 ppm)
29 within 1 hour. A significant increase in chromosomal aberrations was observed by Babu et al. (2008,
30 [195479](#)) in all treatment groups after a 0.5-hour exposure and by Kumari et al. (2009, [199808](#)) at all
31 treatment groups except the lowest concentration group (25 ppm) at the end of a 4-hour exposure period.
32 Chromosomal aberrations described in both studies include chromatin bridge, stickiness, disturbed
33 metaphase, and breaks and fragments (Babu et al., 2008, [195479](#); Kumari et al., 2009, [199808](#)).

1 In unpublished research by Cho et al. (2008, [196264](#)) presented at the first meeting of the Asian
2 Horticultural Congress in 2008, the investigators reported that nano-Ag inhibits the growth and
3 elongation of lettuce and pak-choi roots in a concentration-dependent manner. Nano-Ag exposure also
4 reduced lettuce and pak-choi fresh and dry weights with an increase in concentration above the 0.04-ppm
5 treatment level. At 0.04 ppm, however, nano-Ag appeared to optimize growth, resulting in 10% and 20%
6 increases in the weight of lettuce and pak-choi, respectively. This U-shaped concentration-response curve
7 is similar to that reported by Rostami and Shahsavar (2009, [199810](#)) in olive explants, indicating that
8 there is a narrow nano-Ag threshold at which plant performance is optimized, but above and below which
9 plant performance is inhibited.

Nano-Ag Mode of Action

10 Although there are few data on the specific mode of action for nano-Ag in terrestrial plants,
11 comparisons to conventional silver and silver ions suggest that its mode of action differs from its
12 conventional counterparts. Stampoulis et al. (2009, [199839](#)) compared the toxicity of nano-Ag, “bulk”
13 silver, and ionic silver in zucchini (*Curcubita pepo*) and determined that 1,000 mg/L nano-Ag resulted in
14 a 69% reduction in biomass when compared to 1,000 mg/L bulk silver. The authors also found that
15 exposure to 10 mg silver ions/L (from AgNO₃) produced an effect similar to that of nano-Ag, but that
16 exposure to the supernatant containing silver ions released from the 1,000-mg/L nano-Ag solution
17 resulted in significantly more growth when compared to the nano-Ag solution, indicating that toxicity of
18 nano-Ag is not due entirely to dissolution of the silver ion (Stampoulis et al., 2009, [199839](#)).

19 It has been proposed that the reduction of the mitotic index in *A. cepa* meristems results from DNA
20 transcription inhibition at S-phase, and that the observed mitotic abnormalities are indications that mitotic
21 spindle function is impaired, likely due to nano-Ag interactions with tubulin-SH groups (Babu et al.,
22 2008, [195479](#); Kumari et al., 2009, [199808](#)). Babu et al. (2008, [195479](#)) posit that the observed
23 chromosome stickiness might be the result of “intermingling” chromatin fibers leading to connections
24 between chromosomes at the sub-chromatic level. They also argue that nano-Ag has a clastogenic effect,
25 as observed in the induction of the chromosomal breaks and micronuclei, which might result in a loss of
26 genetic material.

6.2.3.2. Terrestrial Invertebrates

27 Terrestrial invertebrates include those living in soils and aboveground. Few studies have been
28 conducted on nanomaterial toxicity to terrestrial invertebrates, and even fewer have specifically focused
29 on nano-Ag. Because the terrestrial environment has not been thoroughly investigated for pathways of
30 concern, very little information is available on suspected routes of exposure. Nano-Ag in soils is likely to
31 form complexes with organic matter and thiols, which might render it largely unavailable for uptake, as

1 discussed in Section 5.2.4.2. If nano-Ag were applied in sprays directly to the surface of plants, it is
2 possible that plant-dwelling invertebrates might ingest particles unbound to organic matter. This type of
3 application, however, is considered “off-label”, or not in accordance with product instructions for indoor
4 spray disinfectants.

Known Effects of Conventional Silver Exposure

5 Silver toxicity has not been studied extensively in terrestrial invertebrates. Some data presented by
6 Ewell et al. (1993, [597397](#)) at the 1st Argentum International Conference on the Transport, Fate, and
7 Effects of Silver in the Environment indicate that nano-Ag is toxic to the earthworm *Lumbricus terrestris*
8 at concentrations above 62 mg Ag/kg when chronically exposed to artificial soil contaminated with AgS.
9 Because the worms did not bioaccumulate silver after the 28-day exposure period, the investigators
10 determined that direct contact of the dermal tissues with silver in the soil particles resulted in the observed
11 reduction in growth, although the mode of action has not been elucidated.

Effects Specific to Nano-Ag Exposure

12 Only one study was found that explicitly examined nano-Ag toxicity to a soil invertebrate; study
13 details are provided in Section B.7 of Appendix B. Roh et al. (2009, [195565](#)) examined the effect of
14 nano-Ag on DNA transcription, survival, growth, and reproduction in wild type and mutant strains of the
15 soil nematode *Caenorhabditis elegans*. Using microarray analysis, the investigators observed that
16 exposure to nano-Ag resulted in the significant upregulation of 415 gene probes²⁴ and significant
17 downregulation of 1,217 gene probes. The investigators found that survival and growth were not affected
18 by exposure to nano-Ag in any of the treatment groups for the wild type and mutant *C. elegans* strains
19 tested. Reproduction, however, decreased significantly in all strains at all concentrations, with only one
20 exception: reproduction was not statistically significantly affected in one *C. elegans* type evaluated at the
21 lowest concentration. Exposure to silver ions from AgNO₃ did not result in any significant effect on
22 survival or growth, but did significantly reduce reproduction potential. The degree to which reproduction
23 potential decreased, however, was greater in *C. elegans* exposed to nano-Ag than to silver ions. Exposure
24 to silver ions resulted in a different gene expression pattern than that of nano-Ag. Silver ion
25 concentrations of 0.1 and 0.5 mg/L resulted in the statistically significant induction of four *hsp* gene
26 groups, which are heat shock proteins, but did not result in the upregulation of the gene probes
27 significantly affected by exposure to nano-Ag (Roh et al., 2009, [195565](#)).

²⁴ A probe is a specific sequence of single-stranded DNA or RNA, usually labelled with a radioactive atom, that is designed to bind to, and therefore single out, a particular segment of DNA to which it is complementary.

Nano-Ag Mode of Action

1 The mode of action for nano-Ag effects on terrestrial invertebrates is not yet known. Roh et al.
2 (2009, [195565](#)) attempted to elucidate part of the mode of action for nano-Ag toxicity by analyzing
3 expression in genes mapped to specific metabolic processes in the wild type and mutant *C. elegans*
4 strains. The investigators reported that the significant induction of the *sod-3* gene, which is a superoxide
5 dismutase protein, in *C. elegans* exposed to nano-Ag confirms that oxidative stress contributes to nano-
6 Ag toxicity. These gene expressions are correlated with reproduction; this does not, however, necessarily
7 support a causal relationship. The investigators also propose that the loss in function of certain genes
8 might improve the reproductive potential of *C. elegans* when exposed to nano-Ag, possibly related to
9 antioxidant response. The significant upregulation of these genes could have occurred as a compensatory
10 mechanism in the absence of this primary antioxidant enzyme gene. The sequence of processes or events
11 by which the *sod-3* gene contributes to a decrease in reproductive potential, however, was not explored
12 (Roh et al., 2009, [195565](#)).

6.2.3.3. Terrestrial Vertebrates

13 Because silver is not considered a significant risk to higher order organisms, ecotoxicological
14 studies have traditionally focused on more sensitive and more susceptible (i.e., having greater potential
15 for exposure) lower order organisms. Many studies investigating human health toxicity rely on
16 mammalian bioassays from which a human response to nano-Ag is inferred; these studies are covered in
17 detail in Section 6.3 on human health effects. Non-mammalian terrestrial vertebrates are discussed in this
18 section, although the available data are limited to avian species.

Known Effects of Conventional Silver Exposure

19 No studies were identified that have investigated conventional silver toxicity to non-mammalian
20 terrestrial vertebrates. The 1992 data call-in for EPA's Silver Reregistration Eligibility Decision for silver
21 and silver compounds in pesticides, however, required that one avian study be conducted using the
22 formulated product under consideration for reregistration (U.S. EPA, 1993, [196772](#)). As a result,
23 proprietary studies might exist that have investigated effects on avian species.

Effects Specific to Nano-Ag Exposure

24 Only two studies examining nano-Ag toxicity to terrestrial vertebrates were identified, and only
25 one of these examined the direct effects of nano-Ag. These studies are presented in detail in Section B.8
26 of Appendix B. In a study by Grodzik and Sawosz (2006, [196265](#)), chicken eggs were injected with
27 nano-Ag to investigate its effect on the development of chicken embryos. Special attention was paid to

1 the bursae of Fabricius (lymphoid glands contributing to immune system development) in the embryos.
2 Although nano-Ag exposure did not affect the weight of the embryos or the weights of the hearts, livers,
3 and eyes of the chicks, some effects were observed in the bursae of Fabricius. The investigators reported
4 that embryos exposed to nano-Ag developed fewer and smaller lymph follicles in the bursae of Fabricius
5 than in the control groups; statistical significance was not reported. They also observed that the surfaces
6 of the primary and secondary canals extending between the lymphoid follicles in the bursae were larger
7 and more wrinkled than in the controls (Grodzik and Sawosz, 2006, [196265](#)). What effect, if any, the
8 abnormalities observed in the bursae of Fabricius might have on normal development of the chicks is
9 unclear. Furthermore, the nano-Ag material used in the study was not well-characterized, so the size,
10 shape, stability, and other properties that might affect toxicity are not known.

11 The other study examining nano-Ag effects on terrestrial organisms is primarily a study of the
12 caecum microflora²⁵ and secondarily an examination of the histological effects of nano-Ag in tissues in
13 the duodena of 10-day-old quail (*Coturnix coturnix japonica*) free-fed nano-Ag in drinking water
14 (Sawosz et al., 2007, [194076](#)). Gut flora are sometimes likened to a virtual organ within an organ because
15 of the high level of metabolic activity produced by these organisms (O'Hara and Shanahan, 2006,
16 [633151](#)). Bacterial colonization in the gut has been shown to heighten immunological function in
17 animals, and the bacterial composition in the gut might influence variations in immunological response.

18 The only significant effect reported in the study by Sawosz et al. (2007, [194076](#)) was on the
19 content of the gut microflora in the quail exposed at the highest nano-Ag concentration. Of the nine
20 bacterial species included in the microbial caecum profile examined in the study, four significantly
21 increased in density at the 25-mg/kg nano-Ag level. Why the concentrations were reported in terms of
22 mg/kg, when the nano-Ag was dispersed in water, is not clear from the report. The four affected bacterial
23 species were the gram-positive lactic acid bacteria *Lactobacillus salivarius*, *Lactobacillus fermentum*,
24 *Leuconostoc lactis*, and *Actinomyces naeslundii*. Sawosz et al. (2007, [194076](#)) could not explain why the
25 densities of these bacteria increased while the other species remained unchanged when exposed to nano-
26 Ag, a known antimicrobial agent. Furthermore, the investigators note that there are currently no data
27 suggesting that nano-Ag interacts with constituents of the digestive tract or that it can be absorbed from
28 the digestive tract. In this experiment, they hypothesized that nano-Ag successfully penetrated the gastric
29 acid barrier to the stomach and passed through to the duodenum (Sawosz et al., 2007, [194076](#)).

Nano-Ag Mode of Action

30 Because significant toxicity to non-mammalian terrestrial vertebrates exposed to nano-Ag has not
31 been reported, modes of action for toxicity have not been explored.

²⁵ The caecum microflora is the natural bacterial population in the gut organs of animals and humans.

6.3. Human Health Effects

1 The bactericidal effects of conventional silver have led to the incorporation of conventional silver
2 into a range of consumer products, and as described in Chapter 2, the use of nano-Ag in antiseptic
3 products has increased markedly in recent years. As described in Chapters 2, 4, and 5, and Section 6.1,
4 differences in the behavior of conventional silver and nano-Ag appear to be attributable to differences in
5 key properties, including surface area, reactivity, and quantum behavior (ACHS, 2009, [224955](#)). This
6 section examines and summarizes the evidence for nano-Ag-induced health effects from in vitro studies
7 (Section 6.3.1), in vivo studies (Section 6.3.2), and human health and epidemiological studies (Section
8 6.3.3) as they pertain to the use of nano-Ag in spray disinfectants. In each section, the effects of
9 conventional silver are described first, followed by information on the effects relevant to the nano-Ag life
10 cycle specific to this use scenario. For more comprehensive information regarding the health effects of
11 nano-Ag in general, the reader is referred to reviews by Wijnhoven et al. (2009, [180201](#)) and Panyala et
12 al. (2008, [195554](#)).

6.3.1. In Vitro Studies

13 Separating the physical properties affecting nano-Ag toxicity from experimental factors has proven
14 to be an ongoing challenge in the field of nanoparticle exposure. In vitro studies can provide a useful
15 evaluation of controlled dose and exposure scenarios and material characteristics to help identify the
16 processes and factors potentially contributing to nano-Ag toxicity. Because testing environments for in
17 vitro studies, however, are not identical to those for in vivo systems, in vitro studies cannot be compared
18 directly to real-world exposures. Despite such limitations, in vitro studies can be a useful approach for
19 exploring possible mechanisms of action at the cellular and molecular levels, as well as a tool in deciding
20 whether or what further testing is appropriate to pursue.

21 Nano-Ag properties and relevant effects of nano-Ag exposure on different cell types and endpoints
22 observed in key in vitro studies are presented in detail in Section C.2 of Appendix C, with studies
23 presented in alphabetical order by author. Many studies have demonstrated the ability of nanoparticles to
24 penetrate cells, although the mechanism appears to depend on the cell type, particular particle type, and
25 exposure method. Additionally, many researchers have reported that exposures can be cytotoxic. Other
26 endpoints observed in association with exposure to nano-Ag include oxidative stress, induction of
27 cytokines and chemokines as markers of inflammation, DNA and molecular damage, growth inhibition,
28 mitochondrial perturbation, and changes in cellular morphology (see Section C.2 of Appendix C for
29 citations and study details).

Known Effects of Conventional Silver Exposure

1 The potential destabilizing effect of metal ions, including silver ions, on the mitochondrial electron
2 transport chain has been well understood for some time. Chappell et al. (1954, [195499](#)) demonstrated that
3 using conventional silver electrodes to pass electricity through rabbit cerebral cortex cells increased
4 ATPase activity, which could not be replicated with the same currents using different electrodes.
5 Application of AgNO₃ to the cells similarly increased activity, and experiments using pigeon breast
6 muscle mitochondria resulted in the same effects following exposure to AgNO₃. This study indicated that
7 silver ions could increase mitochondrial respiration.

8 Almost four decades after the work of Chapell et al. (1954, [195499](#)), Almofti et al. (2003, [195475](#))
9 showed that, when isolated mitochondria from rat liver cells were similarly treated with silver ions, the
10 mitochondria immediately swelled and metabolism accelerated. These mitochondrial reactions have been
11 shown to be a preliminary step along the mitochondrial permeability transition (PT) path, a cascade of
12 events resulting from increased permeability of proteinaceous pores (PTP) in the inner mitochondrial
13 membrane. Certain conditions including the presence of calcium and inorganic phosphate increase the
14 likelihood of PT, which is characterized by the subsequent release of apoptogenic proteins into the
15 cytoplasm. Silver ions induced PTP opening, resulting in the release of the apoptogenic proteins
16 cytochrome c and apoptosis-inducing factor from the mitochondrial intermembrane space, thereby leading
17 to programmed cell death. Notably, increased respiration and mitochondrial swelling occurred in a dose-
18 dependent pattern correlated with silver ion concentration, and the effects were more profound in the
19 presence of inorganic phosphate. The kinetics of silver ion effects on the mitochondria were markedly
20 different from the classical calcium and inorganic phosphate PT. Mitochondrial respiration and swelling
21 were immediate and independent of inorganic phosphate concentration, and known inhibitors of classical
22 PT could not block the effect of conventional silver. The pore opened by silver ion-PT was also larger in
23 diameter, although whether this pore was distinct or one associated with classical PT is unclear; the pore,
24 however, did not remain open as it does in classical PT. Additionally, the conventional silver effect was
25 blocked by (but could be reversed following) treatment with glutathione (GSH) or dithiothreitol. These
26 substances keep the sulfhydryl groups from being reduced during oxidative stress, suggesting that silver
27 ions were causing PT by binding to the sulfhydryl groups on mitochondrial membrane proteins. Taken
28 together, these results suggest that silver ions induce non-classical PT, characterized by increased
29 mitochondrial respiration and cytochrome c signaling due to binding of silver ions to mitochondrial
30 membrane proteins.

31 More recent work has pointed to the potential for silver ions to cause developmental neurotoxicity
32 after crossing the placenta and accumulating in the fetus. Powers et al. (2010, [197808](#)) examined effects
33 of silver ions on the viability, division, and differentiation of neuronotypic PC12 cells in vitro, using
34 chlorpyrifos (CPF), a known developmental neurotoxicant, as a positive control. After 1 hour of

1 exposure, DNA synthesis was inhibited more by exposure to 10 μM silver ions than by exposure to 50 μM
2 CPF. Longer exposures to 10 μM silver ions reduced cell viability. With onset of cell differentiation,
3 DNA synthesis was inhibited even further, and the acetylcholine phenotype was preferentially expressed
4 over the dopamine phenotype.²⁶ Exposing the PC12 cells to 1 μM silver ions, on the other hand,
5 enhanced cell numbers by suppressing ongoing cell death and impaired differentiation for both
6 neurotransmitter phenotypes.

Effects Specific to Nano-Ag Exposure

7 Information on health effects specific to nano-Ag that have been observed in vitro is presented in
8 the following sections according to notable toxicity endpoints, including oxidative stress (Section
9 6.3.1.1), damage to DNA (Section 6.3.1.2), and cytokine induction (Section 6.3.1.3).

6.3.1.1. Oxidative Stress

10 Oxidative stress is a state of imbalance between radical-generating and radical-scavenging
11 activities within a cell's mitochondrial metabolism. During the metabolism of oxygen by the electron
12 transport chain, the production of ROS occurs. Studies have demonstrated that the ability of
13 nanoparticles to generate ROS plays a key role in inducing toxicity. Elevated ROS production
14 overpowers the cellular antioxidant defenses and decreases mitochondrial function. These events enhance
15 oxidative stress, resulting in cellular damage including mitochondrial apoptosis and necrosis (Xia et al.,
16 2006, [089620](#)). Nano-Ag appears to generate ROS by disrupting ion and electron flux across the
17 mitochondrial membrane, thereby interfering with the electrochemical gradient (Almofiti et al., 2003,
18 [195475](#)). ROS can react with critical cellular molecules (lipids, proteins, nucleic acids, and
19 carbohydrates) and generate additional radicals. Cellular defense mechanisms such as the production of
20 GSH peroxidase, which scavenges radicals, can counteract ROS generation, at least to some extent.

21 Many studies have focused on the effect of nano-Ag on skin cells due to its use in treating wounds.
22 These studies might be useful in estimating the potential effects of dermal exposure to nano-Ag in
23 disinfectant spray, despite the differences in the exposure scenarios. Arora et al. (2009, [196104](#)) studied
24 dermal fibroblasts and primary liver cells to examine possible cellular responses following dermal
25 exposure to an antimicrobial gel for wound treatment. They showed that exposure to spherical nano-Ag
26 particles with diameters 7–20 nm did not cause cell death despite intracellular incorporation of the
27 particles, and that cellular antioxidant defenses were upregulated in both primary fibroblasts and primary
28 liver cells. In a similar model of therapeutic treatment, starch-coated nano-Ag caused mitochondrial

²⁶ “Phenotype” in this case refers to a distinct behavior profile resulting from interacting neuronal networks modulated by different nerve centers in the brain.

1 damage and dose-dependent ROS damage in lung fibroblast and glioblastoma cell lines. Separately,
2 Asharani et al. (2009, [195477](#)) measured DNA damage, presumably from ROS, and observed G2/M cell-
3 cycle arrest possibly due to DNA damage repair following exposure to starch-capped globular particles of
4 nano-Ag 6–20 nm in size. Although cell death was observed by Asharani et al. but not by Arora et al.
5 (2009, [196104](#)), both studies concluded that nano-Ag resulted in increased ROS production. Possible
6 explanations for differing toxicities include differences in doses, properties of particles, and cellular
7 sensitivity.

8 Two additional in vitro studies involving oxidative stress might be relevant when evaluating effects
9 from exposure to spray disinfectants containing nano-Ag. Carlson et al. (2008, [195497](#)) and Hussain et
10 al. (2005, [088101](#)) observed decreased mitochondrial function in alveolar macrophages and liver cells,
11 respectively, in response to nano-Ag exposure. Alveolar macrophages might be vulnerable upon exposure
12 to sprays because their generation is the primary response in the deep lung following insult (macrophages
13 act to phagocytose or endocytose foreign matter). And the liver is one organ known to be affected in
14 people exposed to conventional silver (Venugopal and Luckey, 1978, [191844](#)). Carlson et al. (2008,
15 [195497](#)) used many of the same protocols in their study as Hussain et al. (2005, [088101](#)), making
16 comparisons between these two studies feasible and appropriate.

17 Carlson et al. (2008, [195497](#)) observed that a loss of mitochondrial function was associated with
18 exposure to a range of sizes of spherical nano-Ag particles (15 nm, 30 nm, and 55 nm in diameter;
19 reported by the manufacturer), with the greatest effect observed for the 15-nm size. The toxic effect on
20 mitochondrial function was measured by the degree of mitochondrial reduction of the tetrazolium salt
21 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For smaller particle sizes,
22 lower doses of nano-Ag were sufficient to achieve a significant decrease compared to controls. In the
23 same assay, no significant MTT reduction was observed when the cells were exposed to nano-TiO₂. In
24 liver cells, Hussain et al. (2005, [088101](#)) observed that nano-Ag 15 nm and 100 nm in diameter showed a
25 similar ability to impede mitochondrial function, as measured by MTT reduction, and to increase ROS
26 production. Unlike Carlson et al. (2008, [195497](#)), however, no significant difference was observed
27 between the effects at different particle sizes. These studies also measured levels of the potent antioxidant
28 GSH because it plays a key role in maintaining oxidation-reduction equilibrium within cells. Both studies
29 found that GSH was dramatically reduced following increased exposure to nano-Ag, with a size-
30 dependent trend, in macrophages and independently in the liver cells. Both studies also noted a dose-
31 dependent loss of cell viability that the authors assumed was due to oxidative stress (Carlson et al., 2008,
32 [195497](#); Hussain et al., 2005, [088101](#)).

6.3.1.2. DNA Damage

1 As described in Section 6.2 for biota, the antiseptic properties of nano-Ag are in part due to its
2 ability to bind to and alter the cell membrane and for silver ions to alter the cell's DNA, which in turn
3 interrupts cell proliferation. Nano-Ag might cause DNA damage within mammalian cells by several
4 modes of action. By affecting the mitochondria, nano-Ag can cause an increase in ROS (as described in
5 the previous section), which can interact with and damage proteins or DNA (Asharani et al., 2009,
6 [195477](#)). Additionally, silver ions have been observed in vitro to bind directly with DNA and RNA (Chi
7 et al., 2009, [196122](#)). Furthermore, DNA repair mechanisms, which operate in normal functioning of the
8 cell, depend on ATP. Reduction in cellular ATP levels through interference with mitochondrial respiration
9 could hamper the essential enzymes for DNA repair, leading to damage (Asharani et al., 2009, [195477](#)).
10 DNA damage or a reduction in ATP can interfere with the cell cycle, and thus cellular proliferation
11 (Asharani et al., 2009, [195477](#); Sweet and Singh, 1995, [225222](#)).

12 Surface coating is one property of nanoparticles that can influence toxicity (see Section 6.1.1.3); the
13 genotoxicity of nano-Ag coated with a detergent (such as might occur in an environmental exposure) was
14 examined by Chi et al. (2009, [196122](#)). In this study, the investigators exposed calf thymus DNA to
15 nano-Ag (20–50 nm, spherical) and demonstrated genotoxicity, but only in combination with a detergent.
16 No effect was observed when the DNA was exposed to detergent alone or to nano-Ag alone. The
17 researchers concluded that the detergent cetylpyridine bromide (CPB) formed a complex, reducing the
18 distance between the nano-Ag particles and the calf thymus DNA. This effect was maintained until the
19 concentration exceeded 3.3 µg/mL, when the electrostatic repulsive forces between the DNA and the
20 particle apparently overcame the attraction from the CPB and the genotoxicity decreased.

21 In an exposure system using mouse embryonic and fibroblast cells, Ahamed et al. (2008, [196100](#))
22 showed the DNA damage repair proteins to be upregulated upon exposure to nano-Ag. Exposures were
23 conducted with both uncoated and polysaccharide-coated nano-Ag. Similar effects on the expression of
24 DNA damage proteins Rad51 and phosphorylated-H2AX were observed for coated and uncoated
25 particles, including upregulation of the cell-cycle checkpoint protein p53. The result of p53 activation is
26 cell-cycle arrest, senescence, or apoptosis. Both uncoated and coated particles induced cell death in
27 mouse embryonic and fibroblast cells, as measured by annexin V and MTT assays. Annexin V expression
28 was lower in mouse fibroblast cells treated with coated nano-Ag than in those treated with uncoated
29 nano-Ag. Mouse embryonic cells, however, did not display much difference when treated with coated or
30 uncoated nano-Ag, suggesting mouse embryonic cells are more sensitive than fibroblast cells to both
31 types of nano-Ag used in this study. Despite initiating these common responses, nano-Ag coated with the
32 polysaccharide gum arabic appeared to be more genotoxic than uncoated nano-Ag (statistical significance
33 was not reported). The coating of the particles was believed to prevent the formation of particle clusters,

1 allowing coated nano-Ag to disperse throughout the cell. Uncoated nano-Ag tended to form clusters,
2 resulting in exclusion from some organelles such as the nucleus and mitochondria.

3 Activation of p53 has been linked to ROS production; the mechanism by which p53 is upregulated
4 following nano-Ag exposure, however, is unclear. Some evidence indicates that nano-Ag can directly
5 interact with DNA and the DNA replication machinery. Yang et al. (2009, [193733](#)) demonstrated in a
6 cell-free assay that nano-Ag (30–50 nm in size) in various forms can influence DNA replication as
7 measured by PCR fidelity. The authors observed similar results in *E. coli* bacteria in which the particles
8 became bound to the genomic DNA and influenced replication. Cha et al. (2008, [195498](#)) measured a
9 decrease in DNA content of cells exposed to nano-Ag. Microscale silver also resulted in a decrease, but
10 neither exposure was observed to affect mitochondria or GSH. ROS production can also lead to
11 genotoxicity, as described in the previous section. Research investigating whether either DNA damage or
12 p53 activation is caused directly by nano-Ag or indirectly through ROS generation is lacking. The
13 limitations in data available on genotoxicity of nano-Ag in vitro are due in part to the limited types of in
14 vitro assays that have been used so far to evaluate genotoxic endpoints. Specifically, common in vitro
15 tests, such as the Single Cell Gel (Comet) assay, chromosome damage assays, and micronuclei assays
16 have not yet been used to evaluate the genotoxicity of the nano-Ag agent.

6.3.1.3. Cytokine Induction

17 Several in vitro studies have investigated the effects of nano-Ag on pro-inflammatory cytokine
18 induction within the cell. These cytokines are considered to be classic indicators of toxic effects and have
19 been implicated in effects from exposure to other nanoparticles, particularly in the vascular system
20 (Kreyling et al., 2006, [067261](#)). Lung cell models have shown upregulation of pro-inflammatory
21 cytokines following exposure to ultrafine (nano-sized) particulate matter (Brown et al., 2007, [156300](#)).
22 Brown et al. (2007, [156300](#)) suggested that this induction is caused by intracellular ROS generation. On
23 the other hand, chromium and manganese exposure each cause induction of the pro-inflammatory
24 cytokines Interleukin-8 (IL-8) and Interleukin-6 (IL-6) in airway epithelial cells, effects that are thought
25 to be mediated through the epidermal growth factor receptor pathway (Pascal and Tessier, 2004, [054182](#)).
26 Although several signals could trigger cytokine production, including ROS, the specific signal resulting
27 from nano-Ag exposure remains unclear.

28 Carlson et al. (2008, [195497](#)) evaluated the production of several cytokines in alveolar macrophages
29 and found that at all doses evaluated (5, 10, and 25 µg/mL) for a range of sizes of nano-Ag (15, 30, and
30 55 nm), the cells produced significantly increased amounts of tumor necrosis factor- α (TNF- α), a central
31 mediator of immune response; macrophage inhibitory protein-2 (MIP-2), a signal that recruits neutrophils
32 to sites of inflammation; and Interleukin 1 β (IL-1 β), a mediator in the inflammatory response. IL-6,
33 another pro-inflammatory signal, was not induced. Similarly, Greulich et al. (2009, [195516](#)) observed

1 that human mesenchymal stem cells exposed to nano-Ag concentrations ranging from 5 to 50 µg/mL
2 decreased production of IL-8, a neutrophil attractant, and IL-6. At concentrations less than 5 µg/mL,
3 however, statistically significant increases in IL-8 production were observed when compared with
4 controls. Samberg et al. (2010, [625612](#)) reported a statistically significant dose-dependent decrease in
5 viability of human epidermal keratinocytes (HEKs) and statistically significant increases in IL-1 β, IL-8,
6 TNF- α, and IL-6 production following exposure to 0.34 µg/mL unwashed nano-Ag of various sizes.
7 Nano-Ag from the same source that was washed several times, however, had no effect on cell viability.
8 The investigators believe that the toxicity of the unwashed nano-Ag preparation could be attributed to
9 residual contamination of the nano-Ag with formaldehyde and methanol by-products of the nanomaterial
10 production.

11 Shin et al. (2007, [195568](#)) obtained peripheral blood mononuclear cells (PBMCs), including
12 lymphocyte and monocyte cells, from blood drawn from healthy human volunteers. The cells were
13 incubated in the presence of 1, 5, 10, 15, 20, or 30 ppm nano-Ag for 72 hours. The PBMCs then were
14 resuspended in a complete medium supplemented with phytohemagglutinin (PHA, 5 µg/L) in the absence
15 or presence of 1, 3, 5, 10, or 20 ppm nano-Ag. PHA was used to stimulate the PBMCs to divide and
16 produce cytokine. Nano-Ag levels greater than 15 ppm were observed to be cytotoxic and inhibited the
17 PHA-induced cytokine production in a dose-dependent manner. The PHA effect on cell division was not
18 affected.

6.3.2. In Vivo Studies

19 In vitro studies, discussed above, can improve the understanding of factors potentially contributing
20 to the effects of nano-Ag. In vivo studies elucidate the whole-animal exposure response. Both in vitro
21 and in vivo studies can provide insight on possible modes of action that can be used in interspecies
22 extrapolation, with the goal of better understanding the effect of nano-Ag in a human exposure scenario
23 (Sutter, 1995, [094155](#)). As previously mentioned, however, the recent review conducted by the FIFRA
24 SAP cautions that differences in the formulation of nano-Ag (e.g., generated in a laboratory vs.
25 commercially available) reduce the reliability of extrapolating effects from experimental studies to effects
26 of exposures to commercial products (U.S. EPA, 2010, [625619](#)). This section summarizes the effects of
27 in vivo exposures of nano-Ag in animal studies. The subsections that follow describe noteworthy points,
28 followed by a summary of the key studies. Nano-Ag properties and relevant effects of nano-Ag exposure
29 on different cell types and a range of endpoints observed in key in vivo studies are presented in detail in
30 Section C.3 of Appendix C, with studies presented in alphabetical order by author.

Known Effects of Conventional Silver Exposure

1 The Agency for Toxic Substances and Disease Registry (ATSDR) provided a comprehensive
2 overview of the known effects of exposure to conventional silver in their 1990 publication, *Toxicological*
3 *Profile for Silver*, citing numerous scientific publications reporting health effects in animals exposed to
4 conventional silver by inhalation, ingestion, or dermal exposure (ATSDR, 1990, [196773](#)). ATSDR
5 determined a no observed adverse effect level (NOAEL) of 181.2 milligrams Ag per kilogram per day
6 (mg/kg-day) based on animal mortality from a study by Walker et al. (1971, [597526](#)) of rats ingesting
7 AgNO₃ in drinking water over a 2-week exposure period. The lowest observed adverse effect level
8 (LOAEL) for this study was 362.4 mg Ag/kg-day. In a chronic 37-week study of rats also ingesting
9 AgNO₃ in drinking water, the LOAEL associated with decreased weight gain was 222.2 mg Ag/kg-day
10 (Matuk et al., 1981, [597514](#)). ATSDR did not report specific immunological, developmental,
11 reproductive, carcinogenic, or systemic (e.g., respiratory or gastrointestinal) effects in animals resulting
12 from ingestion of conventional silver. Oral exposure studies also demonstrated that conventional silver
13 was deposited in the liver, spleen, bone marrow, lymph nodes, skin, and kidneys of rats (ATSDR, 1990,
14 [196773](#)). Dermal exposure of guinea pigs to conventional silver resulted in a LOAEL of 137.13 mg
15 Ag/kg-day associated with decreased weight gain compared to unexposed animals; the treated animals
16 were given 2 mL of 0.239 M AgNO₃ solution on an area of skin measuring 3.1 square centimeters (cm²)
17 for eight weeks (Wahlberg, 1965, [009686](#)).

Effects Specific to Nano-Ag Exposure

18 Health effects specific to nano-Ag exposure and possibly relevant to exposures associated with the
19 use of spray disinfectants are discussed in this section. The following sections present information on
20 effects on the central nervous and respiratory systems (Sections 6.3.2.1–6.3.2.2); effects on the liver,
21 kidney, and urinary system (Section 6.3.2.3); effects on the cardiovascular system (Section 6.3.2.4);
22 effects on hematology (Section 6.3.2.5); DNA damage (Section 6.3.2.6); and effects on skin (Section
23 6.3.2.7).

6.3.2.1. Central Nervous System Effects

24 Several studies have examined the translocation of nano-Ag particles into the brain and the
25 potential for effects. In rats, Takenaka et al. (2001, [019055](#)) observed accumulation of nano-Ag in the
26 brain seven days after an acute (6-hour) inhalation exposure to nano-Ag; however, no adverse pulmonary
27 effects were reported (see Section 5.7.2.1 for study details). Tang et al. (2008, [195575](#)) subcutaneously
28 injected nanoscale and microscale silver at 62.8 mg/kg into rats and observed that nano-Ag crossed the
29 blood-brain barrier and accumulated in the brain, while silver microparticles did not. Unlike other

1 studies, like that conducted by Takenaka et al. (2001, [019055](#)), which simply measured accumulation, the
2 neuronal degeneration and necrosis endpoints were measured as indicated by pyknotic, necrotic neurons.
3 Incidences of electron-dense globular substances in both normal and pyknotic neurons were observed in
4 vascular endothelial cells of the rats treated with nano-Ag, but not in those treated with microscale silver.
5 Tang et al. (2008, [195575](#)) concluded that nano-Ag accumulation in neurons over time increased the
6 incidence of necrosis in these cells.

6.3.2.2. Respiratory System Effects

7 The most complete analysis of effects of nano-Ag on the respiratory system appears to have been
8 conducted by Sung et al. (2008, [195571](#)), who observed changes in rat lung function and inflammation
9 parameters following exposure to nano-Ag (average size 18–19 nm) concentrations ranging from
10 0.7×10^6 to 2.9×10^6 particles per cubic centimeter (cm^3), although statistically significant ($p < 0.01$ –
11 0.05) changes were only observed at the highest dose. Statistically significant increases in inflammatory
12 markers (albumin, lactate dehydrogenase, and total protein levels) in the bronchoalveolar lavage fluid
13 were observed in females at the highest dose. Decreased tidal volume, minute volume, and peak
14 inspiration flow and incidences of alveolitis, granulomatous lesions, alveolar wall thickening, and
15 alveolar macrophage accumulation were observed in both male and female rats exposed to the highest
16 dose of nano-Ag in whole-body exposure chambers for 90 days, though not all effects were statistically
17 significant. Although Ji et al. (2007, [091301](#)) and Takenaka et al. (2001, [019055](#)) contributed to the
18 knowledge of potential health effects from inhalation of nano-Ag, they did not specifically examine
19 respiratory endpoints. The 28-day inhalation exposure paradigm and analysis from Ji et al. quantified
20 silver concentration in tissues but did not report any significant respiratory effects of exposure. In an
21 acute exposure paradigm, Takenaka et al. (2001, [019055](#)) measured silver in the lungs and found that only
22 4% of the initial silver burden remained seven days after exposure; however, the functional effects of this
23 accumulation were not examined. Takenaka et al. (2001, [019055](#)) suggested that the rapid clearance of
24 silver from the lungs could be due to phagocytosis of fine particles by macrophages or direct access of
25 ultrafine particles in the alveolar wall to blood capillaries.

26 The study of respiratory-related health effects from exposure to nanoparticles has grown out of the
27 study of ambient ultrafine particulate matter exposure studies. Exposure to ultrafine particles has been
28 shown to have greater health consequences for susceptible populations, such as those with pre-existing
29 respiratory disorders (Pietropaoli et al., 2004, [156025](#); U.S. EPA, 2009, [179916](#)). No studies were
30 identified in the current literature examining nano-Ag health effects in models of susceptible populations.

6.3.2.3. Liver, Kidney, and Urinary System Effects

1 Consistent with its role in detoxification, the liver has been shown to accumulate a disproportionate
2 amount of silver following exposure to nano-Ag (Kim et al., 2008, [193473](#); Sung et al., 2008, [195571](#);
3 Sung et al., 2009, [193664](#)). An oral 28-day toxicology study (Kim et al., 2008, [193473](#)) dosed rats with a
4 0.5% aqueous carboxymethyl-cellulose vehicle and low (30 mg/kg), middle (300 mg/kg), and high (1,000
5 mg/kg) levels of 60-nm diameter nano-Ag powder suspended in the vehicle. The study was said to have
6 been performed according to OECD test guideline 407, but the exact method of oral administration was
7 not reported. Tissue damage was primarily observed in the liver, as indicated by incidences of bile-duct
8 hyperplasia and inflammatory cell infiltration. Incidences of bile-duct hyperplasia, dilation of the central
9 vein, and increased foci also were recorded for the kidneys.

10 In an acute oral dose study by Cha et al. (2008, [195498](#)), the livers of mice fed either microscale or
11 nanoscale silver were examined for RNA upregulation and for pathology. As observed using RNA
12 analysis, seven genes in the apoptotic pathway and five in the inflammatory pathway were induced in the
13 livers of nanoparticle-exposed mice. Histopathology of livers exposed to both micro- and nano-Ag
14 showed infiltration of lymphocyte immune cells, suggesting inflammation. Mice treated with nano-Ag
15 exhibited additional pathologies that were not observed in those exposed to microscale silver, including
16 hemorrhages in the heart, lymphocyte infiltration in the intestine, and congestion in the spleen.

17 Ji et al. (2007, [091301](#)) conducted a 28-day inhalation study in which concentrations of spherical
18 aerosolized nano-Ag were delivered at multiples of 100 $\mu\text{g}/\text{m}^3$ (the American Conference of
19 Governmental Industrial Hygienists [ACGIH] occupational exposure limit) for dust generated from
20 conventional silver. The doses represented one-half, one, and five times the limit. The geometric mean
21 diameters of nano-Ag particles in the low-, mid-, and high-concentration chambers were reported to be
22 12.61, 12.60, and 15.38 nm, respectively. Significant effects (vacuolization and hepatic focal necrosis) in
23 this study were found not to be dose-related.

24 These findings support the conclusions of the subchronic oral study conducted by Kim et al. (2008,
25 [193473](#)) and the subchronic inhalation study conducted by Sung et al. (2009, [193664](#)) that the liver and
26 bile ducts are targets for nano-Ag toxicity. Christensen et al. (2010, [625598](#)) investigated the feasibility
27 of conducting a human health risk assessment for nano-Ag by conducting a literature review and similarly
28 concluded that “the liver is expected to be a (or the) major target organ of systemic toxicity of nano-
29 silver.”

6.3.2.4. Cardiovascular System Effects

30 Only one known study has evaluated effects of nano-Ag on the cardiovascular system. In this 24-
31 hour study, Rosas-Hernández et al. (2009, [625611](#)) exposed rat coronary endothelial cells to 0, 0.1, 0.5, 1,

1 5, 10, 50, or 100 µg/mL of nano-Ag in aqueous solution. Mitochondrial function decreased at
2 concentrations at or less than 10 µg/mL and was associated with an increase of LDH activity, an indicator
3 of membrane disruption. At higher concentrations, the investigators observed an increase in cell
4 proliferation that was dependent on increased production of nitric oxide. At a low concentration
5 (5 µg/mL), nano-Ag had a vasoconstrictive effect on isolated rat aortic rings, while a vasodilative effect
6 was observed at a high concentration (100 µg/mL). No effects were observed when the endothelium was
7 removed from the aortic ring. These findings suggest that cardiovascular effects of nano-Ag target the
8 vascular endothelium and could have opposite effects depending on particle size.

6.3.2.5. Hematology

9 Studies suggest that silver is transported to the blood of rats exposed orally (Kim et al., 2008,
10 [193473](#)) and via inhalation (Sung et al., 2009, [193664](#)) to nano-Ag, but whether nano-Ag particles or just
11 silver ions are responsible is not known. If nano-Ag is absorbed by the blood, functional consequences to
12 the circulatory system might result. To evaluate potential medical uses in the treatment of thrombotic
13 disorders, nano-Ag was injected into mice to evaluate the behavior of platelets. Shrivastava et al. (2009,
14 [200846](#)) found that the nano-Ag inhibited platelet aggregation in a dose-dependent manner in the whole
15 blood. Results from cellular assays suggested that the particles might have affected the signaling
16 necessary for aggregation. Many modes of action are possible.

6.3.2.6. DNA Damage

17 Although genotoxicity of nano-Ag has been observed in vitro, only one study is known to have
18 evaluated DNA damage in vivo. In this 28-day oral study, Kim et al. (2008, [193473](#)) exposed rats
19 repeatedly for one month to 30, 300, or 1,000 mg/kg-day of nano-Ag at 10 mL/kg dosing volumes. The
20 bone marrow of exposed rats was evaluated for chromosomal damage using a micronucleus test, and the
21 investigators reported that no significant difference compared to controls was found. An incomplete
22 description of the statistical procedures was provided in this study, however, and no positive controls were
23 used to evaluate the production of micronuclei. The data presented by Kim et al. (2008, [193473](#)) for the
24 treated male animals showed a modest trend toward increased micronuclei as a function of dose, using 10
25 animals per dose and 3 doses plus the control, but no statistical analysis was described to demonstrate
26 either a statistically significant or nonsignificant trend. The investigators indicated that nano-Ag was
27 found in the blood, but no information was provided to demonstrate that the nano-Ag was actually
28 detected in the bone marrow. In summary, the investigators indicate that no genetic toxicity in bone
29 marrow in vivo was caused by nano-Ag, but the data might actually show a weak effect.

6.3.2.7. Skin

1 As part of the study discussed in Section 6.3.1.3 that examined the effects of nano-Ag in primary
2 human epidermal keratinocytes in vitro, Samberg et al. (2010, [625612](#)) examined whether nano-Ag
3 particles (~20–50 nm in diameter) could penetrate porcine skin in vivo and cause morphological
4 alterations of the skin cells. Because of its comparable thickness and absorption rates, porcine skin is
5 considered to be a good model for human skin (Monteiro-Riviere and Riviere, 1996, [625774](#)). Pigs were
6 topically dosed daily for 14 days with nano-Ag concentration ranging from 0.34 to 34 µg/mL, after which
7 skin samples were examined both macroscopically and microscopically. Although macroscopic
8 observations revealed no gross edema or erythema at any tested dose, microscopic observations showed
9 dose-dependent increases in morphological changes. Common morphological changes observed at the
10 highest dose of nano-Ag were edema, focal inflammation, and epidermal hyperplasia with rete pegs
11 extending below into the dermis. This reaction was reported to be typical irritation, which is also
12 observed following exposure to jet fuels (Monteiro-Riviere et al., 2001, [625773](#)).

6.3.3. Human and Epidemiological Studies

13 Some scientists believe that nano-Ag, although not purified and produced extensively until
14 recently, has long been present as a fraction in conventional silver, and particularly in colloidal silver,
15 though not as intentionally engineered particles (SNWG, 2009, [225301](#)). Muller (1926, [196765](#))
16 investigated exposure effects from a colloidal silver medical product, Collargol, in which the average
17 particle size was determined to be 10–20 nm. Most studies do not characterize the particles in the
18 exposure thoroughly, and the specific presence of nano-Ag is often difficult to determine. The following
19 sections are intended as an overview of the state of the science of health effects from conventional silver
20 exposure only. Particle properties of the exposures are reported, as available.

21 Chronic exposure to conventional silver has been shown to commonly result in argyria
22 (discoloration of the skin) and argyrosis (discoloration of the eyes), due to tissue incorporation of soluble
23 forms of conventional silver (Wijnhoven et al., 2009, [180201](#)). AgNO₃ is associated with lowered blood
24 pressure, diarrhea, stomach irritation, and respiratory irritation. Inhalation or ingestion of conventional
25 silver salts can result in fatty degeneration of the liver and kidneys, as well as blood cell abnormalities
26 (Venugopal and Luckey, 1978, [191844](#)). Studies have shown that soluble silver compounds can
27 accumulate in organs, muscle tissue, and the brain, while elemental silver appears to have no known
28 severe health effects (Drake and Hazelwood, 2005, [195504](#)). This difference in absorption is thought to
29 be due to differences in solubility, which is the reasoning supporting the differing threshold limits for
30 soluble silver (0.01 mg/m³) and metallic silver (0.1 mg/m³) proposed by ACGIH. The following sections
31 summarize several key human studies on the health effects of exposure to different silver forms (given the

1 limited data available for these studies, and that no relevant studies specific to nano-Ag were available, a
2 study table appendix was not developed for this section). Taken together, they function to provide a
3 context for considering the human health consequences of nano-Ag.

6.3.3.1. Medical Use Studies

Known Effects of Conventional Silver Exposure

4 Conventional silver has long been used for medicinal purposes. Colloidal silver, which contains a
5 percentage of nano-Ag, has been used as a dietary supplement to treat illness and, in combination with
6 sulfadiazine, as a treatment for burns (Drake and Hazelwood, 2005, [195504](#)). The Nanotechnology
7 Project inventory of commercially available products reported to contain nanomaterials includes
8 examples of these types of nano-Ag products, although the presence of nano-Ag in these products has not
9 been verified (Project on Emerging Nanotechnologies, 2009, [196774](#)). Germ Slayer by Aluwe, LLC, for
10 example, is a colloidal silver, liquid dietary supplement that is reported to contain 20 ppm nano-Ag. This
11 product is intended to kill viruses and bacteria while not harming the body, and it is recommended by the
12 manufacturer to be taken upon sign of infection.

13 Ingestion of dietary supplements can lead to extremely high exposures, such as an estimated 70–90
14 µg/day (Wijnhoven et al., 2009, [180201](#)). In vitro studies of burn creams, such as Acticoat, show
15 cytotoxicity upon exposure (Fraser et al., 2004, [195510](#); Lam, 2004, [195536](#); Paddle-Ledinek et al., 2006,
16 [195552](#)) and raise concerns that these products might cause health effects. Acticoat is described as a
17 nano-Ag-coated, high-density polyethylene mesh developed by Smith & Nephew, Inc., with
18 approximately 0.2–0.3 mg of silver per mg of mesh (Trop et al., 2006, [195576](#)). Although the magnitude
19 of these exposures is unlikely to occur in the case of a spray disinfectant, the health effects associated
20 with exposure via dietary supplements and burn creams might illustrate worst-case scenarios for ingestion
21 and dermal contact, respectively.

22 Mirsattari et al. (2004, [195543](#)) described the case of a 71-year-old man who, upon taking a
23 homemade colloidal silver dietary supplement for four months, developed seizures, followed by coma,
24 and death. His blood-plasma-silver level was 41.7 nM/L, as compared to the normal range of 1.0–
25 2.3 nM/L, and his urinary level for a 24-hour period was 47.28 nM/L, as compared to the normal range of
26 0.0–0.46 nM/L. Additionally, silver levels in erythrocytes and in cerebral spinal fluid were extremely
27 high. No other significant contaminants were found in the body. Blood purification attempts successfully
28 removed silver from the blood, reducing the plasma-silver level from 41.7 to 1.9 nanomoles per liter
29 (nmol/L) over six days. The silver concentration in the brain measured at autopsy, however, was elevated,
30 with 0.068 micrograms per gram (µg/g) reported in the cerebrum (compared to 0.029 micrograms per
31 gram [µg/g] in a control sample). The cellular mechanism of conventional silver neurotoxicity in this

1 case is not clear (Györi et al., 1991, [225171](#); Rungby and Danscher, 1983, [195567](#); Rungby et al., 1987,
2 [224910](#)).

3 Two other clinical cases documenting neurological effects following conventional silver exposure
4 have been reported by Ohbo et al. (1996, [195551](#)) and Iwasaki et al. (1997, [195527](#)). The exposure
5 scenario described by Ohbo et al. (1996, [195551](#)) involved a schizophrenic patient who had been addicted
6 to antismoking pills containing conventional silver and displayed convulsive seizures and argyria. The
7 patient reportedly ingested more than 20 mg of silver per day for 40 years. Blood-serum-silver levels
8 were elevated at 1.2 micrograms per deciliter ($\mu\text{g}/\text{dL}$) (normal levels are less than 0.05 $\mu\text{g}/\text{dL}$). Levels of
9 silver in blood plasma observed by Mirsattari et al. (2004, [195543](#)) were more than three times those
10 found in this study. Sixty-three days following treatment, serum-silver levels had been reduced to 0.2
11 $\mu\text{g}/\text{dL}$ and seizure activity ceased. Iwasaki (1997, [195527](#)) studied a case involving a burn patient treated
12 with a silver sulfadiazine cream; the male patient developed severe neurotoxicity that reduced brain tissue
13 weight (determined post mortem), decreased mental ability, and increased blood-silver levels. Although
14 the patient eventually died, his renal function had previously been compromised, which is thought to have
15 contributed to the dramatic toxicity observed in this case.

16 Other investigations of asymptomatic general argyria from medical applications are reported by
17 Kakurai et al. (2003, [195528](#)) and Van de Voorde et al. (2005, [225230](#)). Exposures in these cases were
18 associated with silver acupuncture needles and argyrophedrine nose drops, respectively. Argyria is the
19 most common health effect from exposure to conventional silver in general; as the cases above illustrate,
20 confounding and small sample sizes impair the ability to demonstrate statistically significant associations
21 between the exposures and outcomes (Drake and Hazelwood, 2005, [195504](#)).

Effects Specific to Nano-Ag Exposure

22 Nano-Ag toxicity has not been extensively studied with regard to medical use; however, several
23 studies of nano-Ag wound dressings provide insight on the potential effects resulting from dermal
24 exposure. For example, Trop et al. (2006, [195576](#)) documented the case of a previously healthy, 17-year-
25 old patient who suffered burns over 30% of his body as the result of an accident. After cleaning and
26 debriding the patient's wounds under anesthesia, the burns were covered with Acticoat and moistened
27 with sterile water and the areas were wrapped with sterile gauze. The Project on Emerging Technologies'
28 nanomaterial database reports that the concentration of nano-Ag in Acticoat is 70–100 ppm and the
29 particle size ranges from 1 to 100 nm (Project on Emerging Nanotechnologies, 2009, [196774](#)).

30 The patient's Acticoat dressings were changed on days 4 and 6 following surgery. On day 6, the
31 patient presented with argyria-like skin discoloration, lack of energy, loss of appetite, elevated liver
32 enzymes, a slightly enlarged liver and spleen, and normal renal function. On day 7, laboratory tests
33 revealed concentrations of 107 micrograms silver per kilogram ($\mu\text{g}/\text{kg}$) in the blood and 28 $\mu\text{g}/\text{kg}$ silver in

1 the urine. The patient reportedly was not exposed to any other form of silver. The Acticoat dressings
2 were immediately removed, and the patient's facial discoloration reversed. At 7 weeks, however, the
3 levels of silver in the blood and urine, although four-fold lower, remained elevated; 10 months following
4 treatment, silver concentration had returned to within normal levels (0.9 µg/kg in the blood and 0.4 µg/kg
5 in the urine) (Trop et al., 2006, [195576](#)).

6.3.3.2. Occupational Studies

Known Effects of Conventional Silver Exposure

6 Although studies of human exposure to conventional silver are common, no studies were identified
7 that specifically report effects from exposure during the manufacture or use of nano-Ag spray
8 disinfectants. A few studies evaluated the effects of exposure to nano-Ag or substances containing nano-
9 Ag, where the exposure route could be similar to possible exposures to commercial spray disinfectants.
10 These studies are described below and are also mentioned in Section 5.7.1.4.

11 Rosenman et al. (1987, [195566](#)) conducted a cross-sectional study of workers in a plant producing
12 AgNO₃, silver oxide, AgCl, and silver cadmium oxide powders, as well as silver ingots. Air sampling in
13 the factory by the Occupational Safety and Health Administration (OSHA) resulted in calculated time-
14 weighted averages, assuming an 8-hour exposure period, of 0.04–0.35 mg/m³. Particle size and other
15 dosimetric factors were not measured. Of the 27 workers in the study, 6 had general argyria and 20 had
16 argyrosis. Most study participants had high levels of silver in the blood (mean of 1.0 µg/100 mL, with
17 range 0.05–6.2 µg/100 mL) and in the urine (mean 11.3 µg/L, with range 0.5–52.0 µg/L), and 30% of the
18 workers complained of nose bleeds and respiratory irritation. Kidney dysfunction, indicated by levels of
19 the urinary enzyme N-acetyl-B-D glucosaminidase (NAG), was significantly correlated with blood-silver
20 levels ($p < 0.05$); yet blood-silver levels were not a significant predictor of NAG levels when normalized
21 by age. Cadmium was also found in significant levels in the urine of the workers, and this additional
22 exposure, and exposure to solvents, makes definitive associations difficult.

23 Pifer et al. (1989, [195559](#)) compared workers exposed for at least five years in positions with high
24 exposure potential in an Eastman Kodak plant to employees at the plant in positions with low potential for
25 exposure. Air sampling provided 8-hour time-weighted average air-borne silver concentrations of 1–100
26 µg/m³, with most silver in insoluble forms. Although no cases of argyria were noted in the study, 80% of
27 silver workers had detectible blood-silver levels, while none of the study controls had detectible levels.
28 No organ function tests were performed as part of the study.

29 Case reports of individuals occupationally exposed to conventional silver complement the
30 epidemiology studies by providing a more in-depth view of the response at the individual level. Williams
31 et al. (1999, [225234](#)) described the case of a 51-year-old man who displayed corneal and conjunctival

1 argyrosis following seven years of employment in a silver refinery. Concentrations of silver in air were
2 between 0.11 and 0.17 mg/m³. No other functional abnormalities were observed. Similarly, Cho et al.
3 (2008, [195501](#)) described the case of a 27-year-old employee of the mobile telephone industry whose job
4 was to apply plating to mobile telephone subunits with aerosolized silver. After four years of
5 occupational exposure, the employee developed general argyria. The employee's blood-silver
6 concentration was 15.44 µg/dL, as compared to normal values between 1.1 and 2.5 µg/dL, and the urinary
7 silver concentration was 243.2 µg/L, as compared to normal values between 0.4 and 1.4 µg/L. Despite
8 these high internal silver levels, a complete blood count, chemistry panel, liver function test, and routine
9 urinary analysis did not demonstrate any adverse functional effects.

10 Similarly, clinical examinations, both general and neurological, reported by Williams and Gardner
11 (1995, [195584](#)) demonstrated no negative health outcomes in the cases of two conventional silver
12 reclamation workers with blood-silver levels of 49 and 74 µg/L. One of these workers, a 42-year-old
13 process engineer exposed for 2 years, mostly through shoveling insoluble silver halide and silver
14 oxide-containing ash, showed no argyria or other negative signs of exposure. Personal air samples in
15 different areas of the plant measured air-silver compound concentrations of 0.0085, 1.03, and 1.36 mg/m³.
16 The other reclamation worker, a 51-year-old engineer working in a refinery dominated by soluble AgNO₃
17 and metallic silver species for 7 years, presented argyrosis and fingernail discoloration, but no other
18 effects of exposure were noted following clinical examination. Personal air sampling in the refinery area
19 measured the highest concentrations ranging from 0.10 to 0.17 mg/m³ atmospheric silver. Following
20 improved safety measures to reduce exposure, both men's blood-silver levels decreased as measured at 6,
21 12, and 18 months.

Effects Specific to Nano-Ag Exposure

22 No studies investigating nano-Ag toxicity resulting from occupational exposure were identified for
23 this case study.

6.4. Summary of Ecological and Human Health Effects

1 As described in earlier chapters, the behavior of engineered nanoparticles is greatly influenced by
2 the properties of the particles and the composition and chemistry of the surrounding environment. This
3 influence also extends to the toxicity of nanoparticles, and some evidence exists that particle size and
4 surface properties affect nano-Ag toxicity. No conclusive determinations have been made concerning the
5 degree to which specific particle properties or environmental properties influence nano-Ag toxicity.
6 Particular emphasis, however, has been placed on surface coatings, which can affect the degree to which
7 particles form clusters thereby influencing the level of uptake into the organism and cells, and
8 subsequently organelles, including the cell nucleus.

9 Most information available on the effects of conventional silver and nano-Ag on biota is from
10 bacterial studies. A robust database exists regarding the toxicity of colloidal silver and silver salts in
11 aquatic organisms, but information on the toxicity of nano-Ag in the aquatic environment is relatively
12 scarce. Even fewer data are available for terrestrial organisms on the effects of exposure to conventional
13 or nano-Ag.

14 Although the effects of silver ions from nano-Ag particles are presumed to be similar to effects of
15 silver ions from conventional silver, the rate of ion release appears to affect toxicity, a phenomenon that
16 has been demonstrated in bacterial assays. These studies also have demonstrated that nano-Ag can result
17 in adverse effects on gram-negative, gram-positive, autotrophic, heterotrophic, and nitrifying species of
18 bacteria. Microbial assays suggest that nano-Ag also can result in greater toxicity to bacteria than silver
19 ions alone. Studies on embryonic zebrafish, a freshwater vertebrate, indicate that nano-Ag can be taken
20 up and can affect development. Studies in which nano-Ag was sonicated demonstrate higher toxicity,
21 suggesting that the form of the silver (i.e., as particles or ions) plays a role in inducing a toxic response.

22 Silver ions and complexes interfere with ion transport pathways in freshwater invertebrates, and
23 appear to affect marine organisms by altering ionic tissue concentrations. Differences in the toxic
24 responses from exposure to ionic silver, silver complexes, and nano-Ag have been observed, and these
25 responses tend to vary with water quality conditions. Overall, many model aquatic organisms (e.g.,
26 *C. reinhardtii*, *D. magna*, *D. rerio*) are sensitive to nano-Ag exposure, but they appear to be less sensitive
27 than bacteria. Some evidence from studies on fish indicates that exposure to nano-Ag activates certain
28 “stress-response” genes and that the particles can enter key organs and organelles, resulting in physical
29 and toxic effects. Some evidence, although limited, indicates that nano-Ag is cytotoxic, inhibits growth,
30 and alters the genome in some plants and soil macroinvertebrates.

31 Conventional silver affects mitochondrial and cytochrome c signaling in mammalian cells, which
32 can cause cell death. Exposure to conventional silver also causes argyria and argyrosis, which are

1 cosmetic effects associated with the distribution of silver in the body following circulation in the blood.
2 Gastrointestinal distress, seizures, and neurotoxicity have been reported in humans ingesting very high
3 levels of colloidal silver. At the cellular level, nano-Ag has been shown to bind to and enter cells,
4 generate ROS, affect mitochondrial function, and result in genotoxicity.

5 In vivo mammalian studies with conventional silver suggest systemic toxicity, reported as
6 decreased weight gain following ingestion and dermal exposure, and cell death following inhalation
7 exposure. Nano-Ag has been demonstrated to cause upregulation of gene expression pathways for cell
8 death, inflammation in the liver and kidney following ingestion exposure, and adverse effects in the heart,
9 intestine, and spleen. Inhalation exposure to nano-Ag was also demonstrated to affect liver, kidney, and
10 lung function. Nano-Ag has been shown to accumulate in the olfactory bulb and brain following
11 inhalation exposure, although the toxic effects have not been fully elucidated. Genotoxicity has not been
12 demonstrated in whole-animal studies.

13 For spray disinfectants, the potential for human and biotic nano-Ag toxicity depends on the level of
14 exposure to nano-Ag and related silver compounds from these products, and also aggregate exposure to
15 nano-Ag from other products containing nano-Ag. Toxicity of nano-Ag is dictated by the abundance and
16 bioavailability of nano-Ag in environmental compartments, suggesting that factors influencing release
17 scenarios, fate and transport, and exposure potential of nano-Ag all influence toxicity.

18 Questions reflecting data gaps in the information about ecological and human health effects are
19 listed on the following page. They are listed in approximate order of the presentation of information in
20 this chapter. The order in no way, however, is intended to reflect the relative importance of the questions.

Questions about Ecological and Human Health Effects

- 6.1. To what extent do particle properties (e.g., size, shape, chemical composition, surface treatments) determine biological responses to nano-Ag?
- 6.2. Are there physicochemical properties of nano-Ag that could change significantly between the initiation and termination of toxicity studies, thereby affecting biological responses?
- 6.3. Are the effects observed for exposure to nano-Ag due to silver ion release or the presence of nanoparticles? Can this be distinguished?
- 6.4. Do nano-Ag particle size and phase partitioning (i.e., nano-Ag particle, nano-Ag clustering, dissolved silver ions from nano-Ag) affect organ distribution and biological effects?
- 6.5. Is the available ecological effects evidence adequate to support ecological risk assessment for nano-Ag? If no, what research is needed to make an assessment possible?
- 6.6. At a minimum, what assays could be considered in a harmonized test guideline for determination of the ecological effects of nano-Ag?
- 6.7. How do abiotic factors in the environment affect nano-Ag effects in biota? These include but are not limited to:
 - 6.7.a. UV light
 - 6.7.b. Water quality
 - 6.7.c. Other chemicals
- 6.8. What are the most sensitive ecological endpoints to nano-Ag exposure? Are there sufficient data/analytical techniques to determine how sensitive specific endpoints and organisms are to nano-Ag exposure, including:
 - 6.8.a. Benthic invertebrates
 - 6.8.b. Marine invertebrates
 - 6.8.c. Freshwater invertebrates
- 6.9. Are there secondary human health effects resulting from the ecological impacts of nano-Ag exposure? For example, exposure of terrestrial biota to sewage sludge contaminated with nano-Ag?
- 6.10. At a minimum, what assays could be considered in a harmonized test guideline for determination of the human health effects of nano-Ag?
- 6.11. Is there sufficient information available to determine appropriate standard reference materials for use in analysis of nano-Ag ecological and human health effects?
- 6.12. What is the primary mechanism of action for nano-Ag in different species?
- 6.13. What are the fundamental biological responses to and associated mechanisms of nano-Ag exposure at the cell, organ, and whole-animal levels?
- 6.14. What are the biological responses observed at current nano-Ag occupational exposure levels?
- 6.15. Do current publications describing the health effects of nano-Ag particles and laboratory-generated nano-Ag particles accurately depict the toxicity of commercially available nano-Ag materials?

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Appendix A. Common Analytical Methods for Characterization of Nanomaterials

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Appendix A. Common Analytical Methods for Characterization of Nanomaterials

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A.1. Introduction

1 Presented in this appendix is a compilation of analytical methods that have been used to
2 characterize nanoparticles, including nano-Ag. This information is not intended to be exhaustive in
3 reporting every applicable method, nor to be comprehensive in describing available methods; rather, it is a
4 summary of relatively common or known methods for characterizing nanoparticles based on the U.S
5 Environmental Protection Agency's (EPA) experience and knowledge at the time this case study was
6 developed. Because of the rapid pace at which the field of nanotechnology continues to evolve and grow,
7 undoubtedly there are additional methods that are not included here, and some information presented
8 about specific methods that might not fully reflect the current state of the science. Furthermore, the most
9 appropriate methods for characterizing certain nanomaterials or the best methods for specific contexts
10 could vary, and having such a general compilation cover each of these nuances is not possible. This
11 appendix is expected to be useful, however, in listing some of the more commonly used methods and in
12 providing general information relevant to evaluating the research needs regarding nano-Ag.

13 The methods summarized here are grouped into 19 tables, presented alphabetically according to the
14 properties and characteristics being analyzed. Within each table, information is presented regarding the
15 approximate detection range and advantages and disadvantages for each technique. Although the
16 techniques included in each table have not been prioritized according to accuracy, cost, or other
17 characteristics (methods are listed alphabetically within each table), the most commonly employed
18 techniques are listed in bold font.

19 Research staff in EPA's Office of Pesticide Programs compiled this information. Although citations
20 are not provided for individual techniques, several important sources used to develop these tables, such as
21 some Web sites of manufacturers that produce equipment used to characterize nanomaterials are cited
22 here: (AZoNano, 2010, [625594](#); Becker, 2008, [625595](#); Bruker Corporation, 2010, [625596](#); Coulson et
23 al., 2002, [625599](#); GE Healthcare, 2010, [625602](#); Imaging Technology Group, 2010, [625603](#); NCEM,
24 2010, [625607](#); Oberdorster et al., 2005, [090087](#); PerkinElmer, 2010, [625608](#); Quantachrome Instruments,
25 2010, [625610](#); Shimadzu Scientific Instruments, 2010, [625613](#); Tiede et al., 2008, [196278](#); TSI, 2010,
26 [625616](#); Tsuda and Tanaka, 1996, [625617](#); Varian Inc, 2010, [625620](#); Zuin et al., 2007, [625622](#)). Full
27 citations are listed at the end of this appendix.

A.2. Aggregation

| Technique | Detection Range | Advantages | Disadvantages |
|---|------------------------------|--|---|
| Analytical Ultracentrifugation (ANUC) | >nm range | | |
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> • Artifacts can result from tip smearing • Prone to overestimations |
| Chemical Force Microscopy (CFM) | >0.1 nm | <ul style="list-style-type: none"> • Used in biology • Many modifications to AFM tip | |
| Confocal Laser Scanning Microscopy (CLSM) | | | |
| Differential Interference Contrast Microscopy (DIC) | | <ul style="list-style-type: none"> • Can be used to analyze unstained biological samples • High resolution with no artifacts | <ul style="list-style-type: none"> • Calls for transparent specimen with refractive index similar to its surroundings • Expensive |
| Differential Mobility Analyzer (DMA)¹ | 3 nm– μ m range | <ul style="list-style-type: none"> • Can be used in combination with many techniques | <ul style="list-style-type: none"> • Possible sample degradation |
| Dynamic Light Scattering (DLS) | 3 nm– μ m range | <ul style="list-style-type: none"> • Allows in situ measurement • Fast and simple • Convenient for analyzing aggregation | <ul style="list-style-type: none"> • Dust particles can ruin measurement • High particle interactions |
| Flow Field-Flow Fractionation (FIFFF) | 1 nm–1 μ m | | |
| Fluorescence Microscopy (FLM) | >10 nm | | |
| Nuclear Magnetic Resonance (NMR) | mM range | <ul style="list-style-type: none"> • Used to analyze solid or liquid samples • Convenient temperature range | <ul style="list-style-type: none"> • Solid-state experiments are more difficult |
| Scanning Electron Microscopy (SEM)² | 1 nm–1 μ m | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Scanning Transmission Electron Microscopy (STEM)³ | resolution: <0.1 nm | <ul style="list-style-type: none"> • Used to analyze low concentrations (ppm) | |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> • Allows three-dimensional characterization | |
| Size Exclusion Chromatography (SEC) | 5 nm–100 μ m | <ul style="list-style-type: none"> • Good separation efficiency | <ul style="list-style-type: none"> • Interactions of solute and solid phase |
| Small Angle Neutron Scattering (SANS) | 1 nm–1 μ m | <ul style="list-style-type: none"> • Used to analyze liquids • Used to characterize structural details of pores of all types (open, blind, and closed) | <ul style="list-style-type: none"> • Careful data analysis needed |
| Transmission Electron Microscopy (TEM)⁴ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Ultracentrifugation (UC) | nm range | <ul style="list-style-type: none"> • Currently used in carbon nanotubes | <ul style="list-style-type: none"> • More qualitative than quantitative • Requires homogeneous sample preparation |
| X-ray Diffraction (XRD) | 1–3 wt % | | |
| Zeta Potential | 5 nm–10 μ m | | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and Aerosol Time-of-Flight Mass Spectrometry (ATOF-MS).

²Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

³Can be used in combination with X-ray Diffraction (XRD), High Angle Annular Dark-Field Imaging (HAADF), Coherent Electron Nanodiffraction (CEND), Annular Dark Field Imaging (ADF), Thermophilic Aerobic Digestion (TAD), Analytical Electron Microscopy (AEM), and Convergent Beam Electron Diffraction (CBED).

⁴Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.3. Chemical Composition

| Technique | Detection Range | Advantages | Disadvantages |
|---|----------------------------------|--|--|
| Aerosol Time-of-Flight Mass Spectroscopy (ATOF-MS) | 0.32–1.8 μm | | |
| Analytical Electron Microscopy (AEM)¹ | >0.5 nm | <ul style="list-style-type: none"> • Electron Energy Loss Spectroscopy (EELS) can be used (< Zn) | |
| Atomic Absorption Spectroscopy (AAS) | ppm range | | |
| Auger Electron Microscopy (AES) ² | 1–2 nm | | |
| Chemical Force Microscopy (CFM) | >0.1 nm | <ul style="list-style-type: none"> • Used in biology • Many modifications to Atomic Force Microscopy (AFM) tip | |
| Electron Backscattered Diffraction (EBSD) | 20–100 nm | | |
| Electron Paramagnetic Resonance (EPR) | mM range | <ul style="list-style-type: none"> • Can be used to analyze paramagnetic samples | <ul style="list-style-type: none"> • Data interpretation can be difficult |
| Flow Field-Flow Fractionation (FIFFF) | 1 nm–1 μm | | |
| Fourier Transform Infrared Spectroscopy (FT-IR) | ppm range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples | |
| Gel Permeation Chromatography (GPC) | 5 nm–100 μm | <ul style="list-style-type: none"> • Used to determine molecular weight and distribution of polymers | |
| High Performance Liquid Chromatography (HPLC) | $\mu\text{g/mL}$ range | | |
| Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) | 1 ppt–0.1 ppb | | <ul style="list-style-type: none"> • Sample must be soluble in suitable solvent |
| Mossbauer Spectroscopy (MS) | mM range | | |
| Nuclear Magnetic Resonance (NMR) | mM range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples • Good temperature range | <ul style="list-style-type: none"> • Solid state experiments are more difficult |
| Raman Spectroscopy | ppm range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples | |
| Scanning Electron Microscopy (SEM)³ | 1 nm–1 μm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Secondary Ion Mass Spectrometry (SIMS) | 1,012–1,016 atom/cm ³ | <ul style="list-style-type: none"> • Small sample size | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation • Possible sample degradation |
| Transmission Electron Microscopy (TEM)⁴ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Ultraviolet/Visible Spectroscopy (UV/Vis) | mM range | <ul style="list-style-type: none"> • Fast | |
| X-ray Diffraction (XRD) | 1–3 wt % | | |
| X-ray Photoelectron Spectroscopy (XPS) | >1 μm | <ul style="list-style-type: none"> • Reveals atomic composition of layers (1–10 μm) | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Scanning Transmission Electron Microscopy (STEM).

²Can be used in combination with Scanning Electron Microscopy (SEM).

³Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

⁴Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.4. Crystal Structure

| Technique | Detection Range | Advantages | Disadvantages |
|---|-------------------|--|--|
| Differential Scanning Calorimetry (DSC) | mg range | <ul style="list-style-type: none"> Allows the study of phase transitions | |
| Electron Paramagnetic Resonance (EPR) | mM range | <ul style="list-style-type: none"> Can be used to analyze paramagnetic samples | <ul style="list-style-type: none"> Data interpretation can be difficult |
| Fourier Transform Infrared Spectroscopy (FTIR) | ppm range | <ul style="list-style-type: none"> Can be used to analyze solid or liquid samples | |
| Nuclear Magnetic Resonance (NMR) | mM range | <ul style="list-style-type: none"> Can be used to analyze solid or liquid samples Good temperature range | <ul style="list-style-type: none"> Solid-state experiments more difficult |
| Raman Spectroscopy | ppm range | <ul style="list-style-type: none"> Can be used to analyze solid or liquid samples | |
| Scanning Electron Microscopy (SEM)¹ | 1 nm–1 μm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| Thermo-Gravimetric Analysis (TGA) | mg range | <ul style="list-style-type: none"> Allows the study of weight loss in samples | |
| Transmission Electron Microscopy (TEM)² | >0.1 nm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| X-ray Diffraction (XRD) | 1–3 wt % | | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

²Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.5. Dissolution

| Technique | Detection Range | Advantages | Disadvantages |
|--|------------------|---|---|
| Cross Flow Ultrafiltration (CFUF) | 1 nm–1 μm | <ul style="list-style-type: none"> High speed High volume Low concentration | <ul style="list-style-type: none"> Not good for high concentrations Not well defined size fractionation Not fully quantitative Separation is based only on size Titration limits: micromolar to millimolar lower limits |
| Dialysis | <5 nm | | |
| Diffusive gradients in thin films | nM–μM range | <ul style="list-style-type: none"> Simple Concentrating effect helps lower detection limits | |
| Voltammetry | mM–ppm range | | |

Bold font indicates most commonly employed techniques.

A.6. Heterogeneity

| Technique | Detection Range | Advantages | Disadvantages |
|---|------------------------------|--|---|
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> • Artifacts can result from tip smearing |
| Differential Mobility Analyzer (DMA)¹ | 3 nm– μm range | <ul style="list-style-type: none"> • Can be used in combination with many techniques | <ul style="list-style-type: none"> • Possible sample degradation |
| Infrared Spectroscopy (IR) | ppm range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples | |
| Nuclear Magnetic Resonance (NMR) | mM range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples • Good temperature range | <ul style="list-style-type: none"> • Solid-state experiments more difficult |
| Raman Spectroscopy | ppm range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples | |
| Scanning Electron Microscopy (SEM)² | 1 nm–1 μm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> • Allows three-dimensional characterization | |
| Transmission Electron Microscopy (TEM)³ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Ultraviolet/Visible Spectroscopy (UV/Vis) | mM range | <ul style="list-style-type: none"> • Fast | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectroscopy (ICP-MS), and Aerosol Time-of-Flight Mass Spectroscopy (ATOF-MS).

²Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

³Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.7. Mass Concentration

| Technique | Detection Range | Advantages | Disadvantages |
|---|-----------------|--|---|
| Analytical Electron Microscopy (AEM)¹ | >0.5 nm | <ul style="list-style-type: none"> • Electron Energy Loss Spectroscopy (EELS) can be used (< Zn) | |
| Chemical Force Microscopy (CFM) | >0.1 nm | <ul style="list-style-type: none"> • Used in biology • Many modifications to Atomic Force Microscopy (AFM) tip | |
| Gravimetrics | ppb range | <ul style="list-style-type: none"> • Precise measurements • Stable • Inexpensive | <ul style="list-style-type: none"> • Gravity difference measurements are site dependent and require calibration • Less efficient than spectrophotometry |
| Thermal Analysis | mg range | | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Scanning Transmission Electron Microscopy (STEM).

A.8. Melting Point

| Technique | Detection Range | Advantages | Disadvantages |
|---|-----------------|------------|---------------|
| Differential Scanning Calorimetry (DSC) | mg range | | |

A.9. Particle Number Concentration

| Technique | Detection Range | Advantages | Disadvantages |
|---|------------------|------------|--|
| Condensation Particle Counter (CPC) ¹ | 5–1,100 nm | | |
| Particle Counter | >1 μm | | <ul style="list-style-type: none"> • Meets clean room standards |

Bold font indicates most commonly employed technique.

¹Can be used in combination with a Differential Mobility Analyzer (DMA).

A.10. Porosity

| Technique | Detection Range | Advantages | Disadvantages |
|--|------------------------------|---|---|
| Brunauer Emmett Teller (BET) | >1,000 m^2/g | | |
| Differential Mobility Analyzer (DMA) ¹ | 3 nm– μm range | <ul style="list-style-type: none"> • Can be used in combination with many techniques | <ul style="list-style-type: none"> • Possible sample degradation |
| Transmission Electron Microscopy (TEM) ² | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |

Bold font indicates most commonly employed techniques (all three techniques are common).

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and Aerosol Time-of-Flight Mass Spectrometry (ATOF-MS).

²Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.11. Shape

| Technique | Detection Range | Advantages | Disadvantages |
|--|-------------------------------------|--|---|
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> • Artifacts can result from tip smearing |
| Confocal Laser Scanning Microscopy (CLSM) | | | |
| Differential Interference Contrast Microscopy (DIC) | | <ul style="list-style-type: none"> • Can be used to analyze unstained biological samples • High resolution with no artifacts | <ul style="list-style-type: none"> • Calls for transparent specimen with refractive index similar to its surroundings • Expensive |
| Differential Mobility Analyzer (DMA)¹ | 3 nm–μm range | <ul style="list-style-type: none"> • Can be used in combination with many techniques | <ul style="list-style-type: none"> • Possible sample degradation |
| Dynamic Light Scattering (DLS) | 3 nm–μm range | <ul style="list-style-type: none"> • Allows in situ measurement • Fast and simple • Convenient for analyzing aggregation | <ul style="list-style-type: none"> • Dust particles can ruin measurement • Higher particle interactions |
| Flow Field-Flow Fractionation Static Light Scattering (FFFF-SLS) | 1 nm–1 μ m | | |
| Fluorescence Microscopy (FLM) | >10 nm | | |
| Scanning Electron Microscopy (SEM)² | 1 nm–1 μm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Scanning Transmission Electron Microscopy (STEM)³ | resolution: <0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze low concentrations (ppm) | |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> • Allows three-dimensional characterization | |
| Sedimentation Field-Flow Fractionation Dynamic Light Scattering (SedFFF-DLS) | 1 nm–1 μ m | | |
| Transmission Electron Microscopy (TEM)⁴ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Ultracentrifugation (UC) | nm range | <ul style="list-style-type: none"> • Currently used in carbon nanotubes | <ul style="list-style-type: none"> • More qualitative than quantitative • Requires homogeneous sample preparation |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and Aerosol Time-of-Flight Mass Spectrometry (ATOF-MS).

²Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectrometry (EDS).

³Can be used in combination with X-ray Diffraction (XRD), High Angle Annular Dark-Field Imaging (HAADF), Coherent Electron Nanodiffraction (CEND), Annular Dark Field Imaging (ADF), Thermophilic Aerobic Digestion (TAD), Analytical Electron Microscopy (AEM), and Convergent Beam Electron Diffraction (CBED).

⁴Can be used in combination with Electron Energy Loss Spectrometry (EELS) and Energy-Dispersive X-ray Spectrometry (EDS).

A.12. Size

| Technique | Detection Range | Advantages | Disadvantages |
|---|-------------------------------------|--|---|
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> • Artifacts can result from tip smearing |
| Confocal Laser Scanning Microscopy (CLSM) | | | |
| Differential Interference Contrast Microscopy (DIC) | | <ul style="list-style-type: none"> • Can be used to analyze unstained biological samples • High resolution with no artifacts | <ul style="list-style-type: none"> • Calls for transparent specimen with refractive index similar to its surroundings • Expensive |
| Differential Mobility Analyzer (DMA)¹ | 3 nm–μm range | <ul style="list-style-type: none"> • Can be used in combination with many techniques | <ul style="list-style-type: none"> • Possible sample degradation |
| Dynamic Light Scattering (DLS) | 3 nm–μm range | <ul style="list-style-type: none"> • Allows in situ measurement • Fast and simple • Convenient for analyzing aggregation | <ul style="list-style-type: none"> • Dust particles can ruin measurement • Higher particle interactions |
| Fluorescence Microscopy (FLM) | >10 nm | | |
| Scanning Electron Microscopy (SEM)² | 1 nm–1 μm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Scanning Transmission Electron Microscopy (STEM)³ | resolution: <0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze samples of low concentrations (ppm) | |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> • Allows three-dimensional characterization | |
| Size Exclusion Chromatography (SEC) | 5 nm–100 μ m | <ul style="list-style-type: none"> • Can be used to determine molecular weight and distribution of polymers | |
| Transmission Electron Microscopy (TEM)⁴ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and Aerosol Time-of-Flight Mass Spectrometry (ATOF-MS).

²Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectrometry (EDS).

³Can be used in combination with X-ray Diffraction (XRD), High Angle Annular Dark-Field Imaging (HAADF), Coherent Electron Nanodiffraction (CEND), Annular Dark Field Imaging (ADF), Thermophilic Aerobic Digestion (TAD), Analytical Electron Microscopy (AEM), and Convergent Beam Electron Diffraction (CBED).

⁴Can be used in combination with Electron Energy Loss Spectrometry (EELS) and Energy-Dispersive X-ray Spectrometry (EDS).

A.13. Size Distribution

| Technique | Detection Range | Advantages | Disadvantages |
|---|--|---|---|
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> • Artifacts can result from tip smearing |
| Cross Flow Ultrafiltration (CFUF) | 1 nm–1 μ m | <ul style="list-style-type: none"> • High speed • Higher volume • Less clogging than piston filtration or stirred cells | <ul style="list-style-type: none"> • Potential alterations due to increased particle concentration • Turbulent flow • Large surface exposure • Size fractionation is not well defined |
| Cross-Flow Filtration (CFF) | 7 nm–2 μ m | <ul style="list-style-type: none"> • Can be used to separate several compounds based on size | |
| Differential Mobility Analyzer (DMA) ¹ | 3 nm– μ m range | <ul style="list-style-type: none"> • Can be used in combination with many techniques | <ul style="list-style-type: none"> • Possible sample degradation |
| Dynamic Light Scattering (DLS) | 3 nm– μ m range | <ul style="list-style-type: none"> • Allows in situ measurement • Fast and simple • Convenient for analyzing aggregation | <ul style="list-style-type: none"> • Dust particles can ruin measurement • Higher particle interactions |
| Field Flow Fractionation (FFF) ² | Flow FFF: 1 nm–1 μ m Sedimentation FFF: 50 nm–1 μ m | <ul style="list-style-type: none"> • Good size range • Direct relation between retention time and size | <ul style="list-style-type: none"> • Experienced operator needed |
| Flow Field-Flow Fractionation (FIFFF) | 1 nm–1 μ m | | |
| High Performance Liquid Chromatography (HPLC) | μ g/mL range | <ul style="list-style-type: none"> • Can be used to separate and analyze several compounds | |
| Hydrodynamic Chromatography (HDC) ³ | 5–1,200 nm | | <ul style="list-style-type: none"> • Mobile phase interactions |
| Scanning Electron Microscopy (SEM) ⁴ | 1 nm–1 μ m | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Scanning Mobility Particle Sizer (SMPS) ⁵ | 3–1,000 nm | <ul style="list-style-type: none"> • Particle Concentration Range 20–10,000,000 particles/cc • Higher resolution than DMPS | |
| Scanning Transmission Electron Microscopy (STEM) ⁶ | resolution: <0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze low concentrations (ppm) | |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> • Allows three dimensional characterization | |
| Single Particle Mass Spectrometry (SPMS) ⁵ | 3–1,000 nm | <ul style="list-style-type: none"> • Particle Concentration Range 20–10,000,000 particles/cc • Higher resolution than DMPS | |
| Size Exclusion Chromatography (SEC) ⁷ | 5 nm–100 μ m | <ul style="list-style-type: none"> • Simple; good separation efficiency • Can be used to determine molecular weight and distribution of polymers | <ul style="list-style-type: none"> • Limited size separation range |
| Small-Angle X-ray Scattering (SAXS) ⁸ | 5–25 nm | <ul style="list-style-type: none"> • Averaged particle sizes • Shapes, distribution, and surface-to-volume ratio can be determined. • Can be used to analyze liquids or solids | |
| Transmission Electron Microscopy (TEM) ⁹ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Ultracentrifugation (UC) | nm range | <ul style="list-style-type: none"> • Currently used in carbon nanotubes | <ul style="list-style-type: none"> • More qualitative than quantitative • Requires homogeneous sample preparation |

A.13. Size Distribution (continued)

| Technique | Detection Range | Advantages | Disadvantages |
|--|-----------------|------------|---------------|
| Ultrafine Condensation Particle Counter (UCPC) | 2.7–10 nm | | |
| X-ray Diffraction (XRD) | 1–3 wt% | | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and Aerosol Time-of-Flight Mass Spectrometry (ATOF-MS).

²Can be used in combination with Ultraviolet/Visible Spectroscopy (UV/Vis) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) on line; and Atomic Force Microscopy (AFM) off line.

³Can be used in combination with Ultraviolet/Visible Spectroscopy (UV/Vis) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

⁴Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

⁵A Differential Mobility Analyzer (DMA) can be used in combination.

⁶Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

⁷Can be used in combination with Ultraviolet/Visible Spectroscopy (UV/Vis) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

⁸Ultra-Small Angle X-ray Scattering (USAXS) can be used in combination.

⁹Can be used in combination with X-ray Diffraction (XRD), High Angle Annular Dark-Field Imaging (HAADF), Coherent Electron Nanodiffraction (CEND), Annular Dark Field Imaging (ADF), Thermophilic Aerobic Digestion (TAD), Analytical Electron Microscopy (AEM), and Convergent Beam Electron Diffraction (CBED).

A.14. Speciation

| Technique | Detection Range | Advantages | Disadvantages |
|---|------------------|--|--|
| Size Exclusion Chromatography with Inductively Coupled Plasma Mass Spectroscopy (SEC-ICP-MS) | | <ul style="list-style-type: none"> • Element-specific separation | |
| Titration | μM–mM range | <ul style="list-style-type: none"> • Simple | <ul style="list-style-type: none"> • Prevalent human errors • Sample must be soluble |
| X-ray Absorption Fine Structure (XAFS) | ppm to ppb range | <ul style="list-style-type: none"> • Nearly all elements have binding energies in range of X-rays | <ul style="list-style-type: none"> • Inadequate for lighter elements |
| X-ray Diffraction (XRD) | 1–3 wt% | | |

Bold font indicates most commonly employed techniques.

A.15. Structure

| Technique | Detection Range | Advantages | Disadvantages |
|---|---|---|--|
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> Artifacts can result from tip smearing |
| Scanning Electron Microscopy (SEM)¹ | 1 nm–1 μm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| Scanning Transmission Electron Microscopy (STEM)² | resolution: <0.1 nm | <ul style="list-style-type: none"> Can be used to analyze low concentrations (ppm) | |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> Allows three-dimensional characterization | |
| Secondary Ion Mass Spectrometry (SIMS) | 10 ¹² –10 ¹⁶ atom/cm ³ | <ul style="list-style-type: none"> Small sample size | <ul style="list-style-type: none"> Requires high vacuum, which can degrade sample |
| Small Angle Neutron Scattering (SANS) | nm–μm range | <ul style="list-style-type: none"> Can be used to analyze liquids | |
| Transmission Electron Microscopy (TEM)³ | >0.1 nm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| X-ray Diffraction (XRD) | 1–3 wt% | | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

²Can be used in combination with X-ray Diffraction (XRD), High Angle Annular Dark-Field Imaging (HAADF), Coherent Electron Nanodiffraction (CEND), Annular Dark Field Imaging (ADF), Thermophilic Aerobic Digestion (TAD), Analytical Electron Microscopy (AEM), and Convergent Beam Electron Diffraction (CBED).

³Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.16. Surface Area

| Technique | Detection Range | Advantages | Disadvantages |
|---|------------------------------|---|---|
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> Artifacts can result from tip smearing |
| Brunauer Emmett Teller (BET) | >1,000 m ² /g | | |
| Differential Mobility Analyzer (DMA)¹ | 3 nm–μm range | <ul style="list-style-type: none"> Can be used in combination with many techniques | <ul style="list-style-type: none"> Possible sample degradation |
| Dynamic Light Scattering (DLS) | 3 nm–μm range | | |
| Scanning Electron Microscopy (SEM)² | 1 nm–1 μm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> Allows three-dimensional characterization | |
| Transmission Electron Microscopy (TEM)³ | >0.1 nm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| Ultracentrifugation (UC) | nm range | <ul style="list-style-type: none"> Currently used in carbon nanotubes | <ul style="list-style-type: none"> More qualitative than quantitative Requires homogeneous sample preparation |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Electron Spectroscopy (ES), Condensation Particle Counter (CPC), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), and Aerosol Time-of-Flight Mass Spectrometry (ATOF-MS).

²Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

³Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.17. Surface Charge

| Technique | Detection Range | Advantages | Disadvantages |
|--------------------------------|---|---|---------------|
| Capillary Electrophoresis (CE) | 25–100 μm diameter | <ul style="list-style-type: none"> • Run time is short | |
| Zeta Potential | 5 nm–10 μm | | |

Bold font indicates most commonly employed technique.

A.18. Surface Chemistry

| Technique | Detection Range | Advantages | Disadvantages |
|---|--|--|--|
| Analytical Electron Microscopy (AEM)¹ | >0.5 nm | | |
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> • Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> • Artifacts can result from tip smearing |
| Auger Electron Microscopy (AES) ² | 1–2 nm | | |
| Chemical Force Microscopy (CFM) | >0.1 nm | <ul style="list-style-type: none"> • Used in biology • Many modifications to AFM tip | |
| Differential Scanning Calorimetry (DSC) | mg range | <ul style="list-style-type: none"> • Allows the study of phase transitions | |
| Electron Paramagnetic Resonance (EPR) | mM range | <ul style="list-style-type: none"> • Can be used to analyze paramagnetic samples | <ul style="list-style-type: none"> • Data interpretation can be difficult |
| Flow Field-Flow Fractionation (FIFFF) | | | |
| Fourier Transform Infrared Spectroscopy (FT-IR) | ppm range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples | |
| High Performance Liquid Chromatography (HPLC) | $\mu\text{g/mL}$ range | <ul style="list-style-type: none"> • Can separate and analyze several compounds | |
| Nuclear Magnetic Resonance (NMR) | mM range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples • Good temperature range | <ul style="list-style-type: none"> • Solid-state experiments more difficult |
| Raman Spectroscopy | ppm range | <ul style="list-style-type: none"> • Can be used to analyze solid or liquid samples | |
| Scanning Electron Microscopy (SEM)³ | 1 nm–1 μm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> • Allows three-dimensional characterization | |
| Secondary Ion Mass Spectrometry (SIMS) | 10^{12}–10^{16} atom/cm^3 | <ul style="list-style-type: none"> • Small amount of sample necessary for analysis | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation • Possible sample degradation |
| Size Exclusion Chromatography (SEC) ⁴ | 5 nm–100 μm | <ul style="list-style-type: none"> • Good separation efficiency • Simple • Can be used to determine molecular weight and distribution of polymers | <ul style="list-style-type: none"> • Limited size separation range |
| Surface Enhanced Raman Spectroscopy (SERS) | Can detect single molecules | | <ul style="list-style-type: none"> • Sensitive to the surface on which the experiment is conducted |
| Thermo-Gravimetric Analysis (TGA) | mg range | <ul style="list-style-type: none"> • Allows the study of weight loss in samples | |
| Transmission Electron Microscopy (TEM)⁵ | >0.1 nm | <ul style="list-style-type: none"> • High resolution | <ul style="list-style-type: none"> • Requires high vacuum for sample preparation |

A.18. Surface Chemistry (continued)

| Technique | Detection Range | Advantages | Disadvantages |
|---|---|---|---------------|
| Ultraviolet/Visible Spectroscopy (UV/Vis) | mM range | <ul style="list-style-type: none"> Fast | |
| X-ray Photoelectron Spectroscopy (XPS) | >1 μm | <ul style="list-style-type: none"> Can be used to determine atomic composition of layers (1–10 μm) | |
| Zeta Potential | 5 nm–10 μm | | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Transmission Electron Energy Loss Spectroscopy (EELS) (<Zn), Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Scanning Transmission Electron Microscopy (STEM).

²Can be used in combination with Scanning Electron Microscopy (SEM).

³Can be used in combination with Auger Electron Microscopy (AES) and Energy-Dispersive X-ray Spectroscopy (EDS).

⁴Can be used in combination with Ultraviolet/Visible Spectroscopy (UV/Vis) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

⁵Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

A.19. Surface Contamination

| Technique | Detection Range | Advantages | Disadvantages |
|---|-------------------------------------|---|--|
| Aerosol Time-of-Flight Mass Spectroscopy (ATOF-MS) | 0.32–1.8 μm | | <ul style="list-style-type: none"> Efficiencies decrease as particle gets smaller |
| Atomic Force Microscopy (AFM) | >0.1 nm | <ul style="list-style-type: none"> Can be used to analyze dry, moist, and liquid samples | <ul style="list-style-type: none"> Artifacts can result from tip smearing |
| Auger Electron Microscopy (AES) ¹ | 1–2 nm | | |
| Differential Scanning Calorimetry (DSC) | mg range | <ul style="list-style-type: none"> Allows the study of phase transitions | |
| Infrared Spectroscopy (IR) | ppm range | <ul style="list-style-type: none"> Can be used to analyze solid or liquid samples | |
| Nuclear Magnetic Resonance (NMR) | mM range | <ul style="list-style-type: none"> Can be used to analyze solid or liquid samples Good temperature range | <ul style="list-style-type: none"> Solid-state experiments more difficult |
| Raman Spectroscopy | ppm range | <ul style="list-style-type: none"> Can be used to analyze solid or liquid samples | |
| Scanning Tunneling Electron Microscopy (STM) | nm in x, y, and z directions | <ul style="list-style-type: none"> Allows three-dimensional characterization | |
| Thermo-Gravimetric Analysis (TGA) | mg range | <ul style="list-style-type: none"> Allows the study of weight loss in samples | |
| Transmission Electron Microscopy (TEM)² | >0.1 nm | <ul style="list-style-type: none"> High resolution | <ul style="list-style-type: none"> Requires high vacuum for sample preparation |
| Ultraviolet/Visible Spectroscopy (UV/Vis) | mM range | <ul style="list-style-type: none"> Fast | |
| X-ray Photoelectron Spectroscopy (XPS) | >1 μm | <ul style="list-style-type: none"> Can be used to determine the atomic composition of layers (1–10 μm) | |

Bold font indicates most commonly employed techniques.

¹Can be used in combination with Scanning Electron Microscopy (SEM).

²Can be used in combination with Electron Energy Loss Spectroscopy (EELS) and Energy-Dispersive X-ray Spectroscopy (EDS).

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Appendix B. Summary of Ecological Effects Studies of Nano-Ag

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Appendix B. Summary of Ecological Effects Studies of Nano-Ag

| | |
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| B.2. Summary of Nano-Ag Effects in Bacteria and Fungi _____ | B-4 |
| Bradford et al. (2009, 193294) Impact of silver nanoparticle contamination on the genetic diversity of natural bacterial assemblages in estuarine sediments _____ | B-4 |
| Choi and Hu (2008, 194061) Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. _____ | B-5 |
| Choi et al. (2008, 194060) The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. _____ | B-6 |
| Choi et al. (2009, 193317) Role of sulfide and ligand strength in controlling nanosilver toxicity. _____ | B-7 |
| Hwang et al. (2008, 194065) Analysis of the toxic mode of action of silver nanoparticles using stress-specific bioluminescent bacteria. _____ | B-8 |
| Kim et al. (2009, 194069) Antifungal activity and mode of action of silver nano-particles on <i>Candida albicans</i> . _____ | B-9 |
| Kvitek et al. (2008, 196266) Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). _____ | B-10 |
| Lok et al. (2006, 196268) Proteomic analysis of the mode of antibacterial action of silver nanoparticles. _____ | B-11 |
| Morones et al. (2005, 196271) Bactericidal effect of silver nanoparticles. _____ | B-12 |
| Pal et al. (2007, 196273) Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium <i>Escherichia coli</i> . _____ | B-13 |
| Shrivastava et al. (2007, 196276) Characterization of enhanced antibacterial effects of novel silver nanoparticles. _____ | B-14 |
| Sondi and Salopek-Sondi (2004, 196277) Silver nanoparticles as antimicrobial agent: a case study on <i>E. coli</i> as a model for Gram-negative bacteria. _____ | B-15 |
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| Griffitt et al. (2008, 157565) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. _____ | B-16 |
| Miao et al. (2009, 196270) The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. _____ | B-17 |
| Navarro et al. (2008, 157516) Toxicity of silver nanoparticle to <i>Chlamydomonas reinhardtii</i> . _____ | B-18 |
| B.4. Summary of Nano-Ag Effects in Aquatic Invertebrates _____ | B-19 |
| Gao et al. (2009, 195514) Dispersion and toxicity of selected manufactured nanomaterials in natural river-water samples: Effects of water chemical composition. _____ | B-19 |
| Griffitt et al. (2008, 157565) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. _____ | B-20 |
| Kvitek et al. (2009, 195535) Initial study on the toxicity of silver nanoparticles (NPs) against <i>Paramecium caudatum</i> . _____ | B-21 |
| B.5. Summary of Nano-Ag Effects on Fish _____ | B-22 |
| Asharani et al. (2008, 194056) Toxicity of silver nanoparticles in zebrafish models. _____ | B-22 |
| Bar-Ilan et al. (2009, 191176) Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos. _____ | B-23 |
| Chae et al. (2009, 196262) Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (<i>Oryzias latipes</i>). _____ | B-24 |

| | |
|---|-------------|
| Griffitt et al. (2009, 199805) Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. _____ | B-25 |
| Laban et al. (2009, 199809) The effects of silver nanoparticles on fathead minnow (<i>Pimephales promelas</i>) embryos. _____ | B-26 |
| Lee et al. (2007, 194072) In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. _____ | B-27 |
| Shahbazzadeh et al. (2009, 195010) The effects of nanosilver (Nanocid®) on survival percentage of rainbow trout (<i>Oncorhynchus mykiss</i>). _____ | B-28 |
| Yeo and Kang (2008, 199841) Effects of nanometer-sized silver materials on biological toxicity during zebrafish embryogenesis. _____ | B-29 |
| Yeo and Pak (2008, 191177) Exposing zebrafish to silver nanoparticles during caudal fin regeneration disrupts caudal fin growth and p53 signaling. _____ | B-30 |
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| Babu et al. (2008, 195479) Effect of nano-silver on cell division and mitotic chromosomes: A prefatory siren. _____ | B-31 |
| Kumari et al. (2009, 199808) Genotoxicity of silver nanoparticles in <i>Allium cepa</i> . _____ | B-32 |
| Rostami and Shahstavar (2009, 199810) Nano-silver particles eliminate the in vitro contamination of olive 'Mission' explants. _____ | B-33 |
| Stampoulis et al. (2009, 199839) Assay-dependent phytotoxicity of nanoparticles to plants. _____ | B-34 |
| B.7. Summary of Nano-Ag Effects on Terrestrial Invertebrates _____ | B-35 |
| Roh et al. (2009, 195565) Ecotoxicity of silver nanoparticles on the soil nematode <i>Caenorhabditis elegans</i> using functional ecotoxicogenomics. _____ | B-35 |
| B.8. Summary of Nano-Ag Effects on Non-mammalian Terrestrial Vertebrates _____ | B-36 |
| Grodzik and Sawosz (2006, 196265) The influence of silver nanoparticles on chicken embryo development and bursa of Fabricius morphology. _____ | B-36 |
| Sawosz et al. (2007, 194076) Influence of hydrocolloidal silver nanoparticles on gastrointestinal microflora and morphology of enterocytes of quail. _____ | B-37 |
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B.1. Study Selection Criteria

1 The process used to select studies for inclusion in the ecological effects tables differed for each
2 category of organism based on the quantity and quality of available ecotoxicological data. In general,
3 literature searches were conducted for each category of organism (e.g., bacteria and fungi, aquatic plants,
4 terrestrial vertebrates). To reflect the most current state of the science, the tables in this appendix include
5 only studies published in or after 2000. For those categories for which a substantial amount of
6 ecotoxicity data was available (e.g., bacteria, fish), studies examining relevant endpoints were selected
7 based on the data quality and the relative contribution of the results to the state of the science (determined
8 largely by examining the number of articles in which the study was later cited). Also, studies were
9 included if the investigators examined an endpoint for which there was otherwise little information, used
10 a novel technique to assess toxicity, or compared the relative toxicities of nano-Ag possessing different
11 sets of characteristics (e.g., nano-Ag of different sizes, surface areas, shapes). For categories with very
12 little available ecotoxicological information (e.g., terrestrial organisms), all identified studies were
13 included unless they were judged to be of poor quality.

14 Information in the tables is organized to take into account the minimum requirements for
15 physicochemical characterization proposed by the Minimum Information for Nanomaterial
16 Characterization (MINChar) Initiative (MINChar Initiative, 2008, [594265](#)). The infancy in understanding
17 toxicity of nano-Ag and its mechanisms of toxicity, and the equivocal nature of some studies that give
18 conflicting results, preclude the direct comparison of results for many studies. To emphasize that caution
19 is warranted in interpreting the results of the available nano-Ag toxicological studies, these tables are
20 organized in a way that emphasizes each study's relevant attributes in the context of this case study –
21 especially characterization of the nano-Ag used in the study – rather than to facilitate direct comparison of
22 results among studies.

B.2. Summary of Nano-Ag Effects in Bacteria and Fungi

Bradford et al. (2009, [193294](#)) Impact of silver nanoparticle contamination on the genetic diversity of natural bacterial assemblages in estuarine sediments

Test Species

Natural bacterial assemblages

Material

Commercial nano-Ag (form not reported) supplied by Sigma-Aldrich (location not reported).

| | |
|--|---------------------------------|
| Shape: Assumed to be spherical (not verified experimentally) | Solubility: Not reported |
| Composition: Not reported | Surface Area: Not reported |
| Crystal Structure: Not reported | Surface Treatment: Not reported |
| Average Size: 58.6 ± 18.6 nm (determined using TEM) | Surface Charge: Not reported |

Protocol

| | |
|--|---|
| Exposure Duration: 30 days | Exposure Media: Estuarine sediment and water from Tamar Estuary in Plymouth Sound ("St John's Lake" mud flats OSGB grid ref SX412539) |
| Endpoints: Prokaryotic abundance and genetic diversity | Bacterial Density: Not reported (natural assemblage) |
| Exposure Concentrations: 0, 25, and 1000 $\mu\text{g/L}$ | |

Methods: To achieve final concentrations of 25 and 1000 $\mu\text{g/L}$ in the experimental tanks, daily doses $1/20^{\text{th}}$ of the final concentration were added for 20 days to estuarine water overlying estuarine sediment, followed by 10 days in which no dose was administered. Prokaryotic abundance in the water column of each of the experimental tanks was determined using a Becton Dickinson flow cytometer for 10-mL subsamples from experimental tanks. Clades were defined on cytogram plots of side scatter vs. green fluorescence to define high and low nucleic acid cells. Environmental DNA was extracted from sediment samples and a two-step nested PCR-denaturing gradient gel electrophoresis (DGGE) approach, using PCR primers specific to the "phylum Bacteria," was adopted to assess bacterial diversity. Fragments of the 16S rRNA gene were amplified from the environmental DNA. DGGE profiles of PCR-amplified 16S rRNA gene fragments were converted to binary (presence/absence) data and analyzed using analysis of similarities.

Study Outcome

Abundance. Flow cytometric analysis of samples from the overlying water revealed that mean prokaryotic cell counts did not change significantly between treatments over time but were highly correlated ($p = 0.725$), indicating that bacterial and archaeal abundance in the water was not affected by the presence of nano-Ag.

Diversity. Independent of the nano-Ag-dosing, there were no changes in bacterial diversity in the surface of the sediment over the 30-day exposure. DGGE-PCR results for sediment samples taken at the start and finish of the dosing period (Day 1–20) for the control tank and the 1000 $\mu\text{g/L}$ tank differed slightly but significantly ($p = 0.04$). However, similarity profile permutation analysis revealed that most of the clustering of the bacterial diversity in these samples could have arisen by chance.

Choi and Hu (2008, [194061](#)) Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria.

Test Species

Nitrifying bacteria (species not reported)

Material

Nano-Ag synthesized using AgNO_3 by varying the molar ratios (R) of BH_4^- to Ag^+ due to changes in NaBH_4 concentration.

Shape: Not reported

Size Distribution: 5–70 nm

Composition: Not reported

Solubility: Not reported

Crystal Structure: Not reported

Surface Area: Not reported

Average Size: 9 ± 5 nm (R = 0.1), 15 ± 9 nm (R = 0.2),
 14 ± 6 nm (R = 0.36), 12 ± 4 nm (R = 0.6), or 21 ± 14 nm
(R = 1.2) (determined using TEM)

Surface Treatment: Capped with polyvinyl alcohol (PVA)

Surface Charge: Not reported

Protocol

Exposure Duration: 30 minutes

Exposure Concentrations: 0.05–1 mg Ag/L

Endpoints: Growth and reactive oxygen species (ROS)
generation

Exposure Medium: Nitrifying biomass from tank reactor

Bacterial Density: Not reported

Methods: Growth inhibition of nitrifying bacteria was inferred from oxygen uptake rates due to ammonia oxidation and measured using batch extant respirometric assay. EC_{50} s were determined using a saturation-type biological toxicity model. Intracellular ROS concentrations were determined using fluorescence assays following exposure to nano-Ag with an average size of 15 nm. Photocatalytic ROS concentrations were determined using the same method but in the absence of nitrifying cultures to determine exogenous influence of ROS generation.

Study Outcome

Growth Inhibition.

EC_{50} (nano-Ag): 0.14 mg/L

EC_{50} (silver chloride [AgCl] colloid): 0.25 mg/L

EC_{50} (Ag^+): 0.27 mg/L

ROS Generation. Exposure to nano-Ag resulted in an increase of intracellular ROS concentrations, which correlated strongly with the degree of growth inhibition ($R^2 = 0.86$). Photocatalytic ROS concentrations did not correlate strongly with observed inhibition and were therefore not deemed a good predictor of growth inhibition by nano-Ag.

Choi et al. (2008, [194060](#)) The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth.

Test Species

Autotrophic bacteria (nitrifying; species not reported) and heterotrophic bacteria (*Escherichia coli* PHL628-gfp)

Material

Nano-Ag synthesized through reduction of AgNO₃ with NaBH₄.

Shape: Polydisperse (spherical and ellipsoidal)

Composition: Not reported

Crystal Structure: Not reported

Average Size: 14 ± 6 nm (determined using STEM)

Size Distribution: 10–40 nm (determined using STEM)

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Capped with polyvinyl alcohol (PVA)

Surface Charge: Not reported

Protocol

Exposure Duration: 24 hours

Endpoint: Growth

Exposure Concentrations: 0.1–1 mg Ag/L

Exposure Media: Mixed liquor from sludge tank reactor

(autotrophic) or BBL™ medium containing Gelysate peptone and beef extract (heterotrophic)

Bacterial Density: Not reported

Methods: Antibacterial activity of nano-Ag was assessed using LIVE/DEAD BacLight™ bacterial viability kit. The degree of growth inhibition in autotrophic bacteria was inferred from oxygen uptake rates due to ammonia oxidation and measured using a batch extant respirometric assay. The degree of growth inhibition in heterotrophic bacteria was determined using an automated microtiter assay using hourly fluorescence intensity measurements to derive a microbial growth rate.

Study Outcome

Autotrophic Bacterial Growth. At 1 mg Ag/L in the nitrifying suspension, nano-Ag inhibited growth by 86%, while Ag⁺ and AgCl colloids inhibited growth by 42% and 46%, respectively.

Heterotrophic Bacterial Growth. IC₅₀ = 4.0 μM. No inhibitory effect was observed at concentrations below 1 μM. The inhibitory effect of nano-Ag increased to 55% at 4.2 μM (~0.5 mg/L Ag), while Ag⁺ and AgCl colloids inhibited growth by 100% and 66%, respectively. At 100 mg Ag/L, nano-Ag inhibited growth completely.

Choi et al. (2009, [193317](#)) Role of sulfide and ligand strength in controlling nanosilver toxicity.

Test Species

Nitrifying bacteria (species not reported)

Material

Nano-Ag suspension synthesized through reduction of AgNO₃ with NaBH and capped with polyvinyl alcohol (PVA); in suspension with Ag⁺.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 15 ± 9 nm (method not reported)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: PVA-capped

Surface Charge: Not reported

Protocol

Exposure Duration: 300 seconds (inhibition test) or 8 or 18 hours (Ag₂S stability test)

Endpoints: Enzyme inhibition and uptake

Exposure Concentrations: 1 mg Ag/L

Exposure Media: Nitrifying enriched culture to which MOPS was added

Bacterial Density: Nitrifying biomass concentrations of 540 mg/L chemical oxygen demand (COD) or 210 mg/L COD

Methods: Following nano-Ag exposure, inhibition of ammonia monooxygenase (AMO), hydroxylamine oxidoreductase (HAO), and nitrite oxidoreductase (NOR), three critical enzymes involved in nitrification, was calculated by relative oxygen uptake rate after the addition of aliquots of ammonium, hydroxylamine, and nitrate to the respirometric bottles. Effects of ligands on nano-Ag toxicity were determined through a one-time addition of Ag-ligand complexes as well as sequential addition of a specific ligand followed by nano-Ag to the nitrifying cultures. The ligands tested were chloride, sulfate, phosphate, EDTA⁴⁻, and sulfide. Toxicity was determined using a respirometric assay which also determined stability of Ag₂S complexes made from nano-Ag and sulfide. Nano-Ag attachment to cells was determined using a back-scattered electron detector coupled with a secondary electron detector, and elemental composition of specimens was determined using energy dispersive X-ray spectroscopy.

Study Outcome

Inhibition of Enzyme Activity. AMO was the most sensitive of the three enzymes tested when exposed to both nano-Ag and Ag⁺ at nitrifying biomass concentrations of 540 mg/L COD.

Influence of Ligand Complexation. At 1 mg Ag/L and nitrifying biomass concentrations of 210 mg/L COD, nano-Ag inhibited nitrification by 100%. A 10 μM concentration of sulfide reduced nano-Ag toxicity by 80%, while the other ligands also reduced toxicity, but to a lesser degree. Additional phosphate concentrations up to 0.3 mM had little effect on toxicity, while chloride concentrations of 2.8 mM reduced nano-Ag toxicity up to 20%, and a sulfide concentration of 15 μM reduced nano-Ag toxicity from 86% to 15%.

Adsorption to Cell Floccs. Nano-Ag embeds in cell floccs. When sulfide was added prior to nano-Ag exposure, embedding largely decreased.

Hwang et al. (2008, [194065](#)) Analysis of the toxic mode of action of silver nanoparticles using stress-specific bioluminescent bacteria.

Test Species

Wild-type bacteria (*Escherichia coli* RFM443) and recombinant bioluminescent bacteria (All *E. coli* strains: DS1 [*yoda::luxCDABE*], DK1 [*katG::luxCDABE*], DC1 [*clpB::luxCDABE*], DPD2794 [*recA::luxCDABE*])

Material

Commercial nano-Ag purchased from Nanopoly Company (Republic of Korea), and synthesized by the company through reduction of AgNO₃ with hydrazine hydrate, formaldehyde, and sodium formaldehydesulfoxylate.

| | |
|--|---|
| Shape: Not reported | Average Size: 10 nm (provided by the manufacturer and supported by TEM) |
| Composition: Pure silver (provided by the manufacturer based on X-ray diffraction pattern and thermogravimetric/differential thermal analyzer [TG/DTA] curves) | Size Distribution: Not reported |
| Crystal Structure: Not reported | Solubility: Not reported |
| | Surface Area: Not reported |
| | Surface Treatment: Not reported |
| | Surface Charge: Not reported |

Protocol

| | |
|--|---|
| Exposure Duration: 2 hours or 60 minutes (RT-PCR analysis) | Exposure Concentrations: 0.1–1 mg/L |
| Endpoints: Growth, oxidative stress, protein/membrane and DNA damage | Exposure Media: Luria-Bertani culture media |
| | Bacterial Density: 0.08 O.D. ₆₀₀ |

Methods: Growth inhibition was determined in the wild-type *E. coli* based on O.D.₆₀₀. DS1 and DK1 were exposed to incremental concentrations of nano-Ag and 15 units/mL catalase and superoxide dismutase to measure response to superoxide radicals and hydroxyl radicals, respectively, based on maximum relative bioluminescence (RBL). DC1 was exposed to incremental concentrations of nano-Ag to measure response to protein/membrane damage based on RBL. DPD2794 was exposed to incremental concentrations of nano-Ag to measure response to DNA damage based on RBL and real-time quantitative RT-PCR.

Study Outcome

Growth Inhibition. Growth rates declined significantly after exposure to concentrations above 0.5 mg/L.

Oxidative Stress Damage. Nano-Ag led to production of superoxide radicals but little or no production of hydroxyl radicals.

Protein/Membrane Damage. Nano-Ag induced a bioluminescent response indicative of protein/membrane damage seen most strongly at the 0.4 mg/L nano-Ag. Greater concentrations led to a reduction in bioluminescence due to toxic conditions (as seen in growth inhibition test). DC1 did not discriminate between toxicity caused by nano-Ag or Ag⁺.

DNA Damage. Nano-Ag did not induce a response; therefore, no DNA damage was inferred.

Kim et al. (2009, [194069](#)) Antifungal activity and mode of action of silver nano-particles on *Candida albicans*.

Test Species

Diploid fungus (*Candida albicans* ATCC 90028)

Material

Nano-Ag synthesized by dissolving solid silver in nitric acid and adding sodium chloride.

Shape: Spherical

Composition: Not reported

Crystal Structure: Not reported

Average Size: 3 nm (determined using TEM)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Durations: 2 hours (membrane dynamics and released glucose and trehalose), 3 hours (membrane integrity), 8 hours (cell cycle), 24 hours (envelope structure), 48 hours (growth inhibition)

Endpoints: Growth, membrane damage/disruption, and cell-cycle arrest

Exposure Concentrations: 20, 40, 60, and 80 µg/mL (membrane dynamics); 20 µg/mL (released glucose and trehalose), 30 µg/mL (membrane integrity); 40 µg/mL (cell cycle); and not reported for envelope structure or growth inhibition tests.

Exposure Media: Yeast extract, peptone, and dextrose broth for all tests but released glucose and trehalose, which used phosphate-buffered saline

Bacterial Density: 2×10^4 cells/mL (growth inhibition) or 1×10^8 cells/mL

Methods: Growth was assayed with a microtiter enzyme-linked immunosorbent assay (ELISA) reader by monitoring absorption at the 580-nm wavelength. Minimum inhibitory concentrations (MICs) were defined as the lowest concentrations that inhibited 90% of fungal growth when compared to the control. MICs were determined by a series of 2-fold dilutions. Membrane integrity was assessed using flow cytometric analysis, and membrane dynamics were determined by steady-state fluorescence anisotropy using spectrofluorometry. Released glucose and trehalose were measured by weighing dry fungal pellets and measuring color formations in supernatants. Fungal envelope structure was examined using TEM, and cell cycle arrest was assessed using flow cytometric analysis.

Study Outcome

Growth Inhibition. MIC: 2 µg/mL

Membrane Damage/Disruption. Membrane depolarization occurred. Plasma membrane 1,6-diphenyl-1,3,5-hexatriene (DPH) significantly decreased with increasing concentrations of nano-Ag. Nano-Ag-treated cells both accumulated more intracellular and more extracellular glucose and trehalose than untreated cells. Extracellular glucose and trehalose amounts were 30.3 µg/mg fungal dry wt. Treated fungal cells showed significant damage characterized by pits in the cell walls and pores in the plasma membranes.

Arrest of Cell Cycle. The percentage of cells in the gap 2/mitosis (G₂/M) phase increased by 15%, while cells in gap 1 (G₁) phase significantly decreased by about 20%. This indicates that the budding process was inhibited.

Kvitek et al. (2008, [196266](#)) Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs).

Test Species

Gram-positive bacteria (*Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 3953, *Staphylococcus aureus* MRSA, *Staphylococcus epidermidis* [methicillin-susceptible], *Staphylococcus epidermidis* [methicillin-resistant], *Enterococcus faecium* VRE) and Gram-negative bacteria (*Escherichia coli* CCM 3954, *Pseudomonas aeruginosa* CCM 3955, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* ESBL)

Material

Nano-Ag synthesized by modified Tollens process, prepared by reduction of the $\text{Ag}(\text{NH}_3)_2^+$ with D(+)-maltose monohydrate.

| | |
|--|--|
| Shape: Spherical | Surface Area: Not reported |
| Composition: Not reported | Surface Treatment: Unmodified or modified with anionic sodium dodecyl sulfate (SDS), nonionic polyoxyethylene-sorbitan monooleat (Tween 80), or polyvinylpyrrolidone (PVP 360) |
| Crystal Structure: Not reported | |
| Average Size: 26 nm with 2.3% polydispersity (determined using DLS and a Zeta plus analyzer) | Surface Charge: -25 mV (ζ potential in aqueous dispersion) |
| Size Distribution: Not reported | |
| Solubility: Not reported | |

Protocol

| | |
|---|---|
| Exposure Duration: 24 hours | Exposure Medium: Mueller-Hinton broth |
| Endpoint: Growth | Bacterial Density: 10^5 – 10^6 CFU/mL |
| Exposure Concentrations: 0.84 to 54 $\mu\text{g}/\text{mL}$ | |

Methods: Modifiers were added to dispersions of nano-Ag prior to titration in the final amount of 1% (w/w). Stability of unmodified and modified nano-Ag was tested using serial additions of a destabilizer, cationic polyelectrolyte poly(diallyldimethylammonium) chloride (PDDA, 20% [w/w] aqueous solution), and was confirmed using DLS and UV/vis absorption spectra.

Study Outcome

Minimum inhibitory concentrations (MICs) in $\mu\text{g}/\text{mL}$ listed below for unmodified nano-Ag, and nano-Ag modified with SDS, Tween 80, and PVP 360, respectively.

| | |
|---|---|
| <i>E. faecalis</i> CCM 4224: 6.75, 3.38, 6.75, 6.75 | <i>E. faecium</i> VRE: 6.75, 3.38, 3.38, 3.38 |
| <i>S. aureus</i> CCM 3953: 3.38, 1.69, 3.38, 3.38 | <i>E. coli</i> CCM 3954: 1.69, 1.69, 1.69, 3.38 |
| <i>S. aureus</i> MRSA: 3.38, 1.69, 3.38, 1.69 | <i>P. aeruginosa</i> CCM 3955: 3.38, 1.69, 3.38, 1.69 |
| <i>S. epidermidis</i> (methicillin-susceptible): 1.69, 0.84, 1.69, 1.69 | <i>P. aeruginosa</i> : 3.38, 3.38, 1.69, 1.69 |
| <i>S. epidermidis</i> (methicillin-resistant): 1.69, 1.69, 1.69, 1.69 | <i>K. pneumoniae</i> ESBL: 6.75, 6.75, 3.38, 6.75 |

Lok et al. (2006, [196268](#)) Proteomic analysis of the mode of antibacterial action of silver nanoparticles.

Test Species

Wild-type gram-negative bacteria (*Escherichia coli* K12, MG1655)

Material

Nano-Ag synthesized by borohydride reduction of AgNO₃ in the presence of citrate as a stabilizing agent.

Shape: Spherical

Composition: Not reported

Crystal Structure: Not reported

Average Size: 9.3 ± 2.8 nm (determined using TEM)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Stabilized with bovine serum albumin (BSA) when in M9 medium, but uncoated when in HEPES buffer

Surface Charge: Not reported

Protocol

Exposure Duration: ≥600 minutes (growth) or 30 minutes

Endpoints: Growth, protein expression, membrane damage/disruption

Exposure Concentrations: 0.4 and 0.8 nM

Exposure Medium: M9 defined medium for growth inhibition and proteomic analyses and sodium or potassium HEPES buffers containing glucose for membrane analyses.

Bacterial Density: 0.15 O.D.₆₅₀ for growth inhibition and proteomic analyses and 0.1 O.D.₆₅₀ for membrane analyses.

Methods: Growth inhibition was assessed by monitoring the O.D.₆₅₀. Proteomes were analyzed using two-dimensional electrophoresis (2-DE) followed by silver staining. Proteins stimulated by nano-Ag were identified using matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) and tandem mass spectrometry (MS/MS) on the tryptic digests of protein spots of interest. Expression of cell envelope protein OmpA was examined using immunoblots. Membrane damage was determined by pretreating *E. coli* with 1 nM nano-Ag and followed by exposure to 0.1% sodium dodecyl sulfate (SDS). Effects of nano-Ag at the minimum inhibitory concentration (MIC: 1 nM) on the cytoplasmic membrane potential were examined using fluorescence.

Study Outcome

Growth Inhibition. Inhibition became apparent at concentrations of 0.4 nM and 6 μM, for nano-Ag and AgNO₃, respectively. Statistical significance of the results was not reported.

Protein Expression. No global changes in proteomes due to nano-Ag exposure were found. Expressions of “at least” 8 proteins were stimulated by nano-Ag and by AgNO₃. Expressions of a number of cell envelope proteins (OmpA, OmpC, OmpF, and MetQ) were stimulated by nano-Ag. The 37 kDa band was enhanced by nano-Ag. Nano-Ag resulted in accumulation of precursor forms of OmpA.

Membrane Damage. Rapid cell lysis occurred in cells pretreated with nano-Ag and subsequently exposed to SDS, where no cell lysis occurred with only exposure to SDS or nano-Ag alone. DiSC₃(5) fluorescence decreased upon addition to *E. coli* cells and stabilized. After nano-Ag was added, fluorescence rapidly recovered, indicating a dissipation of membrane potential. Also, an almost complete loss of potassium from the cell and depletion of ATP was observed after 5 minutes.

Morones et al. (2005, [196271](#)) Bactericidal effect of silver nanoparticles.

Test Species

Gram-negative bacteria (*Escherichia coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Salmonella typhi*)

Material

Commercial nano-Ag powder inside a carbon matrix, supplied by Nanotechnologies, Inc. (location not reported).

| | |
|--|---------------------------------|
| Shape: Cuboctahedral and multiple-twinned icosahedral and decahedral | Size Distribution: Not reported |
| Composition: Not reported | Solubility: Not reported |
| Crystal Structure: {111} lattice plane | Surface Area: Not reported |
| Average Size: 16 ± 8 nm (determined using TEM) | Surface Treatment: Not reported |
| | Surface Charge: Not reported |

Protocol

| | |
|--|--|
| Exposure Duration: 30 minutes | Exposure Media: Agar plates with Luria-Bertani medium broth |
| Endpoints: Growth and membrane damage | Bacterial Density: 0.5 O.D. ₅₉₅ which corresponds to $\sim 5 \times 10^7$ CFU/mL solution |
| Exposure Concentrations: 0, 25, 50, 75, and 100 $\mu\text{g/mL}$ | |

Methods: Growth inhibition was assessed by monitoring the O.D.₅₉₅. Effects on the bacterial membrane were examined using high angle annular dark field scanning transmission electron microscopy (HAADF-STEM).

Study Outcome

Growth Inhibition. At nano-Ag concentrations above 75 $\mu\text{g/mL}$, there was no bacterial growth for any species. *E. coli* and *S. typhi* were more sensitive to nano-Ag exposure than *P. aeruginosa* and *V. cholerae*.

Membrane Damage. Individual silver nanoparticles were attached to the cell membrane and distributed throughout the cell. Only particles of the sizes attached to the membrane were observed inside the cell. The mean size of the nano-Ag interacting with bacteria was 5 nm, even though the mean nano-Ag size in solution was 16 nm.

Pal et al. (2007, [196273](#)) Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium *Escherichia coli*.

Test Species

Gram-negative bacteria (*Escherichia coli* ATCC 10536)

Material

Nano-Ag powder reduced from aqueous AgNO₃ with sodium citrate or produced via large-scale preparation in particle growth solution containing AgNO₃, ascorbic acid, CTAB, silver seeds, and NaOH.

Shape: Rod, truncated triangular plate, or spherical
Composition: Pure silver (triangular), composition of other shapes was not reported
Crystal Structure: {111} basal lattice plane (triangular)
Average Size: 133–192 nm (rod edge), 16 nm (rod diameter), 40 nm (triangle edge), 39 nm (spherical) (determined using EFTEM)

Size Distribution: Not reported
Solubility: Not reported
Surface Area: Not reported
Surface Treatment: CTAB, a cationic quaternary ammonium surfactant, was used in synthesis of truncated triangular and rod-shaped nano-Ag
Surface Charge: Not reported

Protocol

Exposure Duration: Not reported (nutrient broth) or 24 hours (agar plate)
Endpoint: Growth and membrane damage
Exposure Concentrations: 1–100 µg (delivered to either 100 mL nutrient broth or onto agar plates; concentrations not reported)

Exposure Media: Difco nutrient broth or Difco nutrient agar
Bacterial Density: 10⁸ CFU/mL (in broth) or 10⁷ and 10⁵ CFU/mL (on agar plate)

Methods: Colonies were exposed to concentrations of nano-Ag of different shapes and counted at the end of the exposure period. Membranes were examined using EFTEM.

Study Outcome

Growth. A dose of 10 µg of triangular particles added to 100 mL of the nutrient broth inhibited growth after 24 hours, and 100 µg of AgNO₃ and spherical nano-Ag delayed growth up to 10 hours. At cell concentrations of 10⁷ CFU/mL, almost complete growth inhibition was observed at a triangular nano-Ag content of 1 µg on the agar plate. An amount of 12.5 µg spherical nano-Ag reduced bacterial colonies significantly on the agar plate, and 100% inhibition was seen at contents of 50–100 µg. Even at 100 µg, rod-shaped nano-Ag and AgNO₃ did not inhibit growth completely on the agar plate. Nano-Ag inhibition of bacterial growth was also dependant on the initial number of bacterial cells. At cell concentrations of 10⁵ CFU/mL, spherical nano-Ag almost completely prevented growth at 6 µg, and 12 µg of AgNO₃ inhibited growth completely on the agar plate.

Membrane Damage. Nano-Ag-treated bacterial cells were significantly changed, and major damage was observed on the outer membrane, characterized by pitting. Nanoparticles also accumulated in both the membrane and within the cells.

Shrivastava et al. (2007, [196276](#)) Characterization of enhanced antibacterial effects of novel silver nanoparticles.

Test Species

Gram-negative bacteria (*Escherichia coli* ATCC 25922, ampicillin-resistant *E. coli*, multi- drug-resistant strain of *Salmonella typhus*) and Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923)

Material

Nano-Ag synthesized through reduction of AgNO₃ with a blend of reducing agents including D-glucose and hydrazine.

| | |
|---|--|
| Shape: Spherical or polyhedral | Size Distribution: Approximately 5–47 nm |
| Composition: Not reported | Solubility: Not reported |
| Crystal Structure: Face centered cubic | Surface Area: Not reported |
| Average Size: Approximately 10–15 nm (determined using TEM) | Surface Treatment: Not reported |
| | Surface Charge: Not reported |

Protocol

| | |
|--|--|
| Exposure Duration: 24 hours (agar plates) | Exposure Media: Luria-Bertani agar plates or liquid broth |
| Endpoints: Growth and membrane damage | Bacterial Density: 10 ⁶ CFU per agar plate or 10 ⁸ CFU per mL liquid broth |
| Exposure Concentrations: 10, 25, 35, 50 (only <i>S. aureus</i>), and 100 (only <i>S. aureus</i>) µg/mL | |

Methods: Growth inhibition on the agar plates was assessed by counting the colonies following exposure, and growth rate in the liquid broth was determined by measuring the optical density of bacterial cultures at 600 nm (O.D.₆₀₀). Bacterial cells were also cultured for 60 minutes in the presence of nano-Ag, after which they were re-cultured in a fresh medium without nano-Ag. Growth was then measured in these cells and compared to controls that were not exposed initially to nano-Ag. The effect on bacterial signal transduction was explored by measuring phosphotyrosine content of proteins. Membrane damage was examined using transmission electron microphotographs.

Study Outcome

Growth Inhibition. For non-resistant *E. coli*, 60% growth inhibition was observed at the 5-µg/mL level, which increased to 90% inhibition at 10 µg/mL and complete inhibition at 25 µg/mL. For ampicillin-resistant *E. coli* and *S. typhi*, 70–75% inhibition was observed at 10 µg/mL and complete inhibition at 25 µg/mL. Similar effects were observed in the liquid medium, where lag time before growth was 8 hours at 25 µg/mL. No reduction in *S. aureus* growth was observed at 25 µg/mL on the agar plate. Cells that were re-cultured in fresh medium following exposure to nano-Ag (concentration not reported) exhibited significant retardation in growth relative to controls. There was very little change in the tyrosine phosphotyrosine profile in *S. aureus*, but noticeable dephosphorylation of two peptides (unidentified) occurred in *E. coli*.

Membrane Damage. Clusters of nano-Ag were observed anchored to bacterial cell wall, which perforated the cell membrane, and accumulated inside the cell.

Sondi and Salopek-Sondi (2004, [196277](#)) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria.

Test Species

Gram-negative bacteria (*Escherichia coli* Strain B)

Material

Nano-Ag powder synthesized by reduction of AgNO₃ with ascorbic acid and Daxad 19, a dispersing and thinning agent.

Shape: Not reported

Solubility: Not reported

Composition: Not reported

Surface Area: 158 m²/g (dried powder)

Crystal Structure: Not reported

Surface Treatment: Daxad 19 reportedly removed prior to bacterial exposure

Average Size: 12.3 ± 4.2 nm (determined using TEM)

Surface Charge: Not reported

Size Distribution: 4–29 nm

Protocol

Exposure Duration: 24 hours (agar plates)

Exposure Media: : Luria-Bertani agar plates or liquid broth

Endpoints: Growth and membrane damage

Bacterial Density: 10⁵ CFU per agar plate or 10⁷ CFU per

Exposure Concentrations: 10–100 µg/cm³

mL liquid broth

Methods: Growth inhibition was assessed by counting colonies on agar plates following exposure. Growth rate was determined by measuring O.D.₆₀₀ in the liquid broth. SEM and TEM were used to evaluate the surface morphology of the cells. The qualitative chemical composition of the membranes was assayed by energy dispersive X-ray analysis (EDX).

Study Outcome

Growth Inhibition. Nano-Ag at a concentration of 10 µg/cm³ inhibited growth on the agar plates by 70%. The number of colonies was reduced when compared to controls and most were located on edges of agar plates at exposure concentrations of 20 µg/cm³ nano-Ag, and concentrations of 50–60 µg/cm³ nano-Ag inhibited growth completely. The number of cells applied to the plate was related to the degree of antibacterial activity (i.e., less cells resulted in more antibacterial activity). All concentrations of nano-Ag applied to the liquid medium resulted in delayed growth, with the higher concentrations resulting in longer delays.

Membrane Damage. Nano-Ag-treated cells were significantly changed, showing major damage, characterized by formation of pits in the cell walls. EDX showed that nano-Ag was incorporated into the bacterial membrane, which was confirmed by TEM. TEM also revealed penetration of nano-Ag into cells and intracellular substances, in addition to coagulated nano-Ag on the bacterial surface.

B.3. Summary of Nano-Ag Effects in Algae

Griffitt et al. (2008, [157565](#)) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms.

Test Species

Freshwater green algae (*Pseudokirchneriella subcapitata*)

Material

Commercial nano-Ag powder produced by gas-phase condensation and coated with a thin layer (2–3 nm) of metal oxide; supplied by Quantum Sphere (Santa Ana, CA, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 26.6 ± 8.8 nm (determined using a laser diffraction particle size analyzer)

Size Distribution: Approximately 20–1000 nm

Solubility: Dissolution 48 hours after resuspension was 0.07% of total mass

Surface Area: $14.53 \text{ m}^2/\text{g}$ (determined using the Brunauer, Emmett, and Teller method)

Surface Treatment: Sodium citrate stabilizer

Surface Charge: -27.0 mV (ζ potential in moderately hard freshwater with pH 8.2, determined using a Zeta Reader Mk 21-II)

Protocol

Exposure Duration: 96 hours

Endpoint: Growth

Exposure Concentrations: EC_{50} from the ranging-finding test, and concentrations 0.6-, 0.36-, 1.67-, and 2.78-times the estimated EC_{50} from the range-finding test

Exposure Medium: Moderately hard freshwater (dissolved oxygen 8.5–8.9 mg/L; pH 8.2 ± 1 , hardness 142 ± 2 mg/L CaCO_3 ; conductivity 395 μS ; un-ionized ammonia <0.5 mg/L)
Cell Density: Not reported

Methods: Algal growth media were prepared to produce a concentration gradient before being inoculated with a similar volume of algal culture. Algal growth was assessed by measurement of chlorophyll *a*. These results from exposure to nanometals of copper, aluminum, and nickel were compared to controls

Study Outcome

EC_{50} : 0.19 mg/L

P. subcapitata was more susceptible to nano-Ag than to any of the other nanometals tested. The reported test concentrations are nominal; therefore actual concentrations to which the organisms were exposed may be lower or higher than reported.

Miao et al. (2009, [196270](#)) The algal toxicity of silver engineered nanoparticles and detoxification by copolymeric substances.

Test Species

Coastal marine diatom (*Thalassiosira weissflogii* CCMP 1336)

Material

Commercial nano-Ag powder supplied by Nanostructured & Amorphous Materials Inc. (Houston, TX, USA).

| | |
|---|--|
| Shape: Not reported | Size Distribution: Not reported |
| Composition: Not reported | Solubility: Not reported |
| Crystal Structure: Not reported | Surface Area: 9–11 m ² /g (provided by manufacturer) |
| Average Size: 60–70 nm (determined using TEM) | Surface Treatment: polyvinylpyrrolidone (PVP) surfactant and suspended with Suwannee River Fulvic Acid |
| | Surface Charge: Not reported |

Protocol

Exposure Duration: 48 hours
 Endpoints: Growth, quantum yield, and chlorophyll *a* production
 Exposure Concentrations: Dispersed in the 0.22 μM fraction as (1) Total Ag: 2.12 × 10⁻¹⁰, 1.06 × 10⁻⁹, 5.30 × 10⁻⁹, 2.65 × 10⁻⁹, and 1.03 × 10⁻⁷ M; (2) Total dissolved Ag: 2.28 × 10⁻⁹, 4.56 × 10⁻⁸, 2.28 × 10⁻⁷, 1.14 × 10⁻⁶, 5.70 × 10⁻⁶, and 2.21 × 10⁻⁵ M; or (3) Free Ag⁺: 1.23 × 10⁻¹⁴, 2.46 × 10⁻¹³, 1.23 × 10⁻¹², 6.14 × 10⁻¹², 3.07 × 10⁻¹¹, and 1.19 × 10⁻¹⁰ M.

Exposure Media: f/2 Medium with a basis of artificial seawater containing different nutrient conditions (nutrient-enriched [+NE], nitrogen-limited [-N], and phosphorous-limited [-P])
 Cell Density: 15,000 to 65,000 cells/mL, with higher cell density used in nutrient-limited and higher nano-Ag concentration treatments.

Methods: In +NE experiment, diatoms were harvested after acclimating in f/2 medium and arriving at mid-exponential growth phase. Cells were then suspended in toxicity media to which a range of nano-Ag concentrations had been added. In -N and -P test, +NE cells further incubated in f/2 medium without any addition of N or P for 2 and 4 days, respectively, before resuspension in the -N (or -P) toxicity media. Quantum yield was determined by fluorescence induction and relaxation system, and chlorophyll *a* content was quantified using a fluorometer. To separate effects of Ag⁺ from Ag nanoparticles, 4 additional tests were conducted with +NE culture in f/2 medium. (1) nano-Ag was removed by ultrafiltration through 1 kiloDalton (kDa) membrane to examine indirect effects on nano-Ag (<1 kDa). (2) Diafiltration was performed to compare photosynthesis, chlorophyll *a*, and growth in treatments and control. (3) Glutathione (GSH) and cysteine were added to the nano-Ag stock to assess direct effects from nano-Ag. (4) Nano-Ag aggregate toxicity was assessed by mixing 4.5 × 10⁻⁴ M GSH with 4.63 × 10⁻⁴ M nano-Ag and adding to nutrient enriched cells.

Study Outcome

Because the concentration of Ag was so much higher in the <1 kDa fraction than in the < 1 kDa – 0.22 μm fraction (2.21 × 10⁻⁵ versus 1.03 × 10⁻⁷ M), and because the cellular concentration of Ag⁺ was 10-fold higher than maximum possible nano-Ag concentrations, it was deemed that the direct effects from the nanoparticles were negligible compared to the indirect effects from the released Ag⁺. Results are therefore presented in terms of indirect effects from free and cellularly accumulated Ag⁺.

| IC ₅₀ for Free Ag ⁺ (M) | | | | IC ₅₀ for Ag ⁺ Accumulated in the Cell (M) | | | |
|---|-----------------------------|--------------------------|--------------------------|--|-----------------------------|------------------------|------------------------|
| +NE | -N | -P | <1kDa | +NE | -N | -P | <1kDa |
| Growth Inhibition | | | | Growth Inhibition | | | |
| 2.16 × 10 ⁻¹² | 1.02 × 10 ⁻¹¹ | 2.14 × 10 ⁻¹¹ | 1.03 × 10 ⁻¹² | 3.11 × 10 ³ | 1.61 × 10 ⁴ | 2.08 × 10 ⁴ | 1.37 × 10 ³ |
| Quantum Yield Inhibition | | | | Quantum Yield Inhibition | | | |
| 8.83 × 10 ⁻¹¹ | Not inhibited more than 50% | | 6.36 × 10 ⁻¹¹ | 1.84 × 10 ⁵ | Not inhibited more than 50% | | 1.72 × 10 ⁵ |
| Chlorophyll <i>a</i> Inhibition | | | | Chlorophyll <i>a</i> Inhibition | | | |
| 5.82 × 10 ⁻¹² | Not inhibited more than 50% | | 4.13 × 10 ⁻¹² | 7.39 × 10 ³ | Not inhibited more than 50% | | 6.29 × 10 ³ |

Navarro et al. (2008, [157516](#)) Toxicity of silver nanoparticle to *Chlamydomonas reinhardtii*.

Test Species

Freshwater green algae (*Chlamydomonas reinhardtii*)

Material

Commercial nano-Ag suspension supplied by Nanosys GmbH (Wolfhaldon, Switzerland).

| | |
|--|---|
| Shape: Not reported | Solubility: Not reported |
| Composition: Not reported | Surface Area: Not reported |
| Crystal Structure: Not reported | Surface Treatment: Carbonate-coated |
| Average Size: 44 nm (determined using DLS and TEM) | Surface Charge: -36.6 ± 3.2 mV (ζ potential at pH 7.52 determined using DLS with Zeta Sizer) |
| Size Distribution: <10–200 nm | |

Protocol

| | |
|--|--|
| Exposure Duration: 1–5 hours | Exposure Media: MOPS media |
| Endpoint: Photosynthetic yield | Cell Density: 2×10^5 cells/mL |
| Exposure Concentrations: 10–100,000 nM | |

Methods: Toxicity of nano-Ag and AgNO₃ to algal photosynthesis was assessed by dose-response experiments, and photosynthetic yield was measured periodically. To examine effects of Ag⁺, cysteine, an amino acid, was added in varying concentrations to 100 nM AgNO₃ solution to which algae were exposed for 1 hour, and photosynthetic yield was recorded. The role of Ag⁺ in toxicity of nano-Ag was examined by exposing algae for 1 hour to 5 or 10 μM nano-Ag and cysteine concentrations ranging from 10 to 500 nM, and results were plotted as a function of calculated Ag⁺. Photosynthetic values were reported as percent of the controls, and values were plotted as a function of measured values of total Ag and Ag⁺ to obtain EC_{50s}.

Study Outcome

EC_{50s} for nano-Ag are presented as a function of total Ag content and free Ag⁺ concentrations, respectively, at the beginning of the experiment. The EC_{50s} for nano-Ag complexed with cysteine are also presented. Based on total Ag concentration, AgNO₃ appeared to be more toxic than nano-Ag, but based on Ag⁺, nano-Ag appeared to be more toxic than AgNO₃.

| Total Ag | Free Ag ⁺ | Nano-Ag + cysteine (expressed as free Ag ⁺) |
|------------------|----------------------|---|
| 1-hour: 3,300 nM | 1-hour: 33 nM | 1-hour (5 μM nano-Ag + cysteine): 57 nM |
| 2-hour: 1,049 nM | 2-hour: 10 nM | 1-hour (10 μM nano-Ag + cysteine): 61 nM |
| 3-hour: 879 nM | 3-hour: 9 nM | |
| 4-hour: 801 nM | 4-hour: 8 nM | |
| 5-hour: 829 nM | 5-hour: 8 nM | |

B.4. Summary of Nano-Ag Effects in Aquatic Invertebrates

Gao et al. (2009, [195514](#)) Dispersion and toxicity of selected manufactured nanomaterials in natural river-water samples: Effects of water chemical composition.

Test Species

Water flea neonates (*Ceriodaphnia dubia*)

Material

Commercial nano-Ag powder produced by gas-phase condensation and coated with a thin layer (2–3 nm) of metal oxide; supplied by Quantum Sphere (Santa Ana, CA, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: ~80 nm (DI water and SR-1 sample), ~300 nm (SR-2 sample), >1000 nm (SR-3 sample), all determined using DLS)

Size Distribution: 20–30 nm (nominal; provided by manufacturer), but larger in solution

Solubility: The highest analyzed total dissolved and particulate Ag concentration in the original suspensions was in DI water, with concentrations in SR-1 and SR-3 an order of magnitude lower. The concentrations in SR-2 was an order of magnitude lower than SR-1 and SR-3.

Surface Area: 14.53 m²/g [reported by Griffitt et al. (2008, [157565](#))]

Surface Treatment: Not reported

Surface Charge: -27.0 mV [ζ potential in moderately hard freshwater with pH 8.2, determined using a Zeta Reader Mk 21-II by Griffitt et al. (2008, [157565](#))]

Protocol

Exposure Duration: 48 hours

Endpoint: Mortality/immobility

Exposure Concentrations: Not reported

Exposure Media: DI water, Suwannee River headwater sample (SR-1), midsection water sample (SR-2), or delta water sample (SR-3) diluted with moderately hard water used as culture medium.

Organisms per Replicate: 5 neonates × 3 replicates

Methods: Neonates were exposed to 5 incremental concentrations of nano-Ag (not reported) and survival was assessed visually after 48 hours.

Study Outcome

LC₅₀ (DI-water): 0.46 µg/L

LC₅₀ (SR-1): 6.18 µg/L

LC₅₀ (SR-2): 0.771 µg/L

LC₅₀ (SR-3): 0.696 µg/L

Griffitt et al. (2008, [157565](#)) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms.

Test Species

Adult water fleas (*Daphnia pulex*) and water flea neonate (*Ceriodaphnia dubia*)

Material

Commercial nano-Ag powder produced by gas-phase condensation and coated with a thin layer (2–3 nm) of metal oxide; supplied by Quantum Sphere (Santa Ana, CA, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 26.6 ± 8.8 nm (determined using laser diffraction particle size analyzer)

Size Distribution: Approximately 20–1000 nm

Solubility: Dissolution 48 hours after resuspension was 0.07% of total mass

Surface Area: 14.53 m²/g (determined using the Brunauer, Emmett, and Teller method)

Surface Treatment: Sodium citrate stabilizer

Surface Charge: -27.0 mV (ζ potential in moderately hard freshwater with pH 8.2, determined using a Zeta Reader Mk 21-II)

Protocol

Exposure Duration: 48 hours

Endpoint: Mortality/Immobility

Exposure Concentrations: The estimated LC₅₀ from the range-finding test and concentrations 0.6-, 0.36-, 1.67-, and 2.78-times the estimated LC₅₀ (approximately 0.01, 0.02, 0.04, 0.07, and 0.1 mg/L for *D. pulex* and 0.02, 0.04, 0.07, 0.1, and 0.2 mg/L for *C. dubia*)

Exposure Medium: Moderately hard freshwater (dissolved oxygen 8.5 to 8.9 mg/L; pH 8.2 \pm 1; hardness 142 \pm 2 mg/L as CaCO₃; conductivity 395 μ S; un-ionized ammonia <0.5 mg/L)

Organisms per Replicate: 5 adult *D. pulex* or 10 *C. dubia* neonates \times 4 replicates

Methods: Death was assessed by lack of movement or response to gentle prodding. Significance of these results were determined by comparing nanometal-induced (copper, aluminum, and nickel) effects relative to control

Study Outcome

LC₅₀ (*D. pulex* adults): 0.04 mg/L

LC₅₀ (*C. dubia* neonates): 0.067 mg/L

Daphnid species were more susceptible to nano-Ag than to any of the other nanometals tested. The concentration data in this test are nominal, and the actual concentrations to which the organisms were exposed may be lower than reported, with the nanometals being correspondingly more toxic.

Kvitek et al. (2009, [195535](#)) Initial study on the toxicity of silver nanoparticles (NPs) against *Paramecium caudatum*.

Test Species

Unicellular eukaryote (*Paramecium caudatum*)

Material

Nano-Ag synthesized through a modified Tollens process using AgNO₃, NH₃, NaOH, and D(+)-maltose monohydrate.

Shape: Spherical

Composition: Not reported

Crystal Structure: Not reported

Average Size: 27 nm (determined using TEM and UV/vis absorption spectra)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Unmodified and modified with nonionic surfactant polyethylene (2) sorbitan mono oleate (Tween 80), polyethylene glycol with molecular weight 35,000 (PEG 35000), and polyvinylpyrrolidone with a molecular weight of 360,000 (PVP 360)

Surface Charge: -37 mV (ζ potential determined using dynamic light scattering in diluted solution with pH 11.5), 1.23 mS/cm (conductivity)

Protocol

Exposure Duration: 1 hour

Endpoint: Mortality

Exposure Concentrations: Approximately 0.1–100 mg/L

Exposure Media: Culture medium (type not reported)

Organisms per Replicate: 1–5 mL containing 200–300 organisms/mL × 3 replicates

Methods: A set of about 50 organisms was monitored over an area of 1 cm². LT₅₀ was measured from the moment of the addition of the nano-Ag into the culture up to the point when 50% of the organisms died. LC₅₀s were then determined from the dependence of the LT₅₀ on nano-Ag concentration.

Study Outcome

LC₅₀ (unmodified nano-Ag): 39 mg/L

LC₅₀ (modified with Tween 80): 16 mg/L

LC₅₀ (modified with PEG 35000): ~39 mg/L

LC₅₀ (modified with PVP 360): ~39 mg/L

B.5. Summary of Nano-Ag Effects on Fish

Asharani et al. (2008, [194056](#)) Toxicity of silver nanoparticles in zebrafish models.

Test Species

Zebrafish embryos (*Danio rerio*)

Material

Nano-Ag synthesized through reduction of AgNO₃ with NaBH₄.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: Not reported

Size Distribution: 5–20 nm (for both types of surface-treated nano-Ag, determined using TEM)

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Capped with soluble potato starch or bovine serum albumin (BSA)

Surface Charge: Not reported

Protocol

Exposure Duration: 72 hours

Endpoints: Mortality, hatching rate, heart rate, phenotypic changes, and apoptosis

Exposure Concentrations: 5, 10, 25, 50, and 100 µg/mL

Exposure Medium: Embryo water (60 mg sea salt per L ultrapure water)

Organisms per Replicate: 10 embryos × 6 replicates

Methods: To differentiate between dead and malformed embryos, opaque embryos were transferred to well plates with 4 mL medium and incubated for 24 hours. Mortality was determined by counting the number dead after 72 hours. Hatching rate was determined by the number of embryos hatched by 72 hours, and heart rate was recorded using a stopwatch at various stages post-fertilization. Other phenotypic deformities were also recorded. Embryos were examined using a photographic method. To assess the level of apoptic cells, acridine orange was added to all embryos exposed to nano-Ag above 50 µg/mL and samples were examined under a microscope. Embryos at various stages were collected and the chorion poked to aspirate fluid containing unidentified brown flakes. Flakes were examined using DAPI staining.

Study Outcome

Nano-Ag was uniformly distributed throughout the embryos, on the skin, and in brain and heart cells, showing affinity for the cell nucleus. Nano-Ag in the brain was well-dispersed, but clumping was observed elsewhere.

Mortality. LC₅₀s varied from 25 to 50 µg/mL, and were dependant on the growth stage (64–128 cell stage) of the embryo, with the later embryonic stages exhibiting more resistance to nano-Ag.

Hatching Rate and Heart Rate. Delay was observed with increasing nano-Ag concentrations, with 15% of embryos exposed to BSA-capped nano-Ag and 33% of embryos exposed to starch-capped nano-Ag hatching at the 100 µg/mL level. Heart rate decreased with increasing nano-Ag concentration, reached an average of 39 beats/minute above a concentration of 50 µg/mL (versus 150 beats/minute in the controls).

Phenotypic Changes and Apoptosis. Above 50 µg/mL concentrations of BSA-capped nano-Ag and starch-capped nano-Ag, 60–90% of embryos exhibited severe phenotypic changes characterized by bent and twisted notochord, accumulation of blood in blood vessels near the tail, low heart rate, pericardial edema, degeneration of body parts, and distorted yolk sacs. About 40–50% of embryos displayed apoptosis spots all over the body. Decay was observed primarily near the head and tail.

Bar-Ilan et al. (2009, [191176](#)) Toxicity assessments of multisized gold and silver nanoparticles in zebrafish embryos.

Test Species

Zebrafish embryos (*Danio rerio*)

Material

Nano-Ag of various sizes synthesized using commonly used methods utilizing various strengths and types of reducing agents (no specific details provided).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Sizes: 5.9 nm (nominally 3 nm), 15.3 nm (nominally 10 nm), 51.2 nm (nominally 50 nm), and 108.9 nm (nominally 100 nm) (determined using TEM)

Size Distribution: <4.5 to >7.5 nm (3 nm group), <12 to >20.1 nm (10 nm group), <31 to >71 nm (50 nm group), and <85 to >120 nm (100 nm group)

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 120 hours (5 days)

Endpoints: Mortality and morphology

Exposure Concentrations: 0.25, 2.5, 25, 100, and 250 μ M

Exposure Medium: Egg water

Organisms per Replicate: 12 embryos \times 3 replicates

Methods: Dosing solutions were prepared by transferring nano-Ag in reverse osmosis water to egg water, which was changed daily to prevent destabilization of nano-Ag in solution. Using a scoring system, embryos were evaluated for severity of morphological defects, survival, and toxic adverse effects. The 100 μ M treatment group was used to analyze sublethal toxic effects. At 120-h post fertilization, embryos exposed to 75 μ M nano-Ag were examined by instrumental neutron activation analysis (INAA) to determine whether nano-Ag were taken up or adsorbed.

Study Outcome

Mortality. Almost 100% mortality occurred in all size groups at 250 μ M nano-Ag 120-h post-fertilization.

LC₅₀ (3 nm nano-Ag): 93.31 μ M

LC₅₀ (10 nm nano-Ag): 125.66 μ M

LC₅₀ (50 nm nano-Ag): 126.96 μ M

LC₅₀ (100 nm nano-Ag): 137.26 μ M

Phenotypic Effects. Sublethal endpoints that were statistically significant from controls were opaque and nondepleted yolk; small head; jaw and snout malformations; stunted growth; circulatory malformations, such as hemorrhages and blood clots; tail malformations; body degradation, such as bubble-like formations on yolk sac and decaying tail tissue; pericardial edema; bent spine; and not hatching. Preliminary findings suggest that there was embryonic uptake and/or adsorption of nano-Ag.

Griffitt et al. (2009, [199805](#)) Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles.

Test Species

Adult female zebrafish (*Danio rerio*)

Material

Commercial nano-Ag powder produced by gas-phase condensation and coated with a thin layer (2–3 nm) of metal oxide; supplied by Quantum Sphere (Santa Ana, CA, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 26.6 ± 8.8 nm (determined using laser diffraction particle size analyzer)

Size Distribution: Approximately 20–1000 nm

Solubility: After 24 and 48 hours, only 5.1% of initial nano-Ag dose remained, with the rest aggregating and settling out. Soluble Ag decreased over time, reaching a peak concentration of 4.9 $\mu\text{g/L}$ at 2 hours, and decreasing to 0.2 $\mu\text{g/L}$ at 48 hours.

Surface Area: 14.53 m^2/g (determined using the Brunauer, Emmett, and Teller method)

Surface Treatment: Not reported

Surface Charge: -27.0 mV (ζ potential in moderately hard freshwater with pH 8.2, determined using a Zeta Reader Mk 21-II)

Protocol

Exposure Duration: 48 hours

Endpoints: Mortality, gill histopathology, and gene expression

Exposure Concentration: 1000 $\mu\text{g/L}$ nano-Ag (corresponding to 0.014 m^2/L if monodispersed)

Exposure Medium: 0.22 μm filtered moderately hard water
Organisms per Replicate: 4 fish \times 3 replicates

Methods:

Static renewal assays were conducted using the no observed effect concentration (NOEC) of nano-Ag. Gills and whole carcasses were analyzed for metal concentration at 24 and 48 hours, and lethality was assessed. After 48 hours, gills were examined for structural changes in filament and lamellae, characterized by increased cellularity in intramellar space. RNA from gill tissue samples was also analyzed by microarray to determine gene response.

Study Outcome

Lethality. NOEC concentrations were used, and no mortality occurred. There were no changes in appearance or behavior.

Gill Histopathology. No significant change in gill filament width resulted from exposure to nano-Ag.

Gene Expression. At 24 hours, nano-Ag-exposed fish exhibited 66 significantly upregulated genes and 82 downregulated. At 48 hours, there were 126 significantly upregulated and 336 downregulated genes.

Laban et al. (2009, [199809](#)) The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos.

Test Species

Fathead minnow embryos (*Pimephales promelas*)

Material

Commercial nano-Ag supplied by either NanoAmor (Houston, TX, USA) or Sigma-Aldrich (St. Louis, MO, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: approximately 31–50 nm (NanoAmor) and 21–60 nm (Sigma-Aldrich) (determined using TEM)

Size Distribution 29–100 nm (NanoAmor) and 21 to >300 nm (Sigma-Aldrich)

Solubility: Increasing concentrations of nano-Ag (Sigma-Aldrich) resulted in a decreased percentage of dissolved silver

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 96 hours

Endpoints: Mortality and morphology

Exposure Concentrations: 0.625, 1.25, 2.5, 5, 7.5, 10, 15, 20, and 25 mg/L

Exposure Medium: Test water with dissolved oxygen content of 7.5 mg/L, pH 8.3–8.5, and water hardness 215-240 mg/L as CaCO₃

Organisms per Replicate: 10 embryos × 3 replicates

Methods: Static nonrenewal tests were conducted using both commercial nano-Ag products and AgNO₃. To assess mortality and developmental changes, dead and abnormal embryos were counted, and uptake was analyzed by TEM. Toxicity was assessed using both stirred and sonicated samples.

Study Outcome

LC₅₀ (sonicated NanoAmor): 1.25 mg/L LC₅₀ (sonicated Sigma-Aldrich): 1.36 mg/L LC₅₀ (AgNO₃): 15 µg/L

LC₅₀ (stirred NanoAmor): 9.4 mg/L LC₅₀ (stirred Sigma-Aldrich): 10.6 mg/L

Significant abnormalities included absence of air sac, pericardial and yolk sac edema, hemorrhages to head and pericardial area, and lordosis (upward bending of vertebral column).

Lee et al. (2007, [194072](#)) In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos.

Test Species

Zebrafish embryos (*Danio rerio*), at the 8–64-cell stages and 0.75–2.25 hours post fertilization

Material

Nano-Ag synthesized by sAgClO₄ reduction with sodium citrate and NaBH₄.

Shape: Spherical

Composition: Not reported

Crystal Structure: Not reported

Average Size: 11.6 ± 3.5 nm (determined using high-resolution TEM)

Size Distribution: Approximately 5–46 nm

Solubility: Stable in egg water

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 120 hours (5 days)

Endpoints: Mortality and morphology

Exposure Concentrations: 0.04, 0.06, 0.07, 0.08, 0.29, 0.38, 0.57, 0.66, and 0.71 nM

Exposure Medium: Egg water

Organisms per Replicate: 35–40 embryos × 3 replicates

Methods: Nano-Ag effects on embryonic development and survival were determined through direct observation. Single nanoparticle transport was analyzed in vivo in embryos exposed to nano-Ag in real-time using dark-field single-nanoparticle optical microscopy and spectroscopy (SNOMS).

Study Outcome

Ag nanoparticles were observed embedded in retina, brain, heart, gill arches, and tail. The number of dead and deformed zebrafish increased with increasing dose, with no normal development occurring after the 0.19 nM concentration level (i.e., all zebrafish were either dead or deformed at concentrations higher than 0.19 nM). Finfold abnormality and tail/spinal cord flexure and truncation were observed at all concentrations. Cardiac malformation and yolk sac edema were observed in the 0.07 to 0.71 nM concentration range. Head edema and eye deformity were observed only at the higher concentrations ranging from 0.44–0.71 and 0.66–0.71, respectively. Multiple deformities in a single embryo were observed concentrations higher than 0.38 nM.

Shahbazzadeh et al. (2009, [195010](#)) The effects of nanosilver (Nanocid®) on survival percentage of rainbow trout (*Oncorhynchus mykiss*).

Test Species

Rainbow trout (*Oncorhynchus mykiss*), median weight 1.049 g

Material

Commercial colloidal Ag suspension (Nanocid L-series colloidal product) provided by Nasb Pars Co. (Iran).

Shape: Not reported

Solubility: Not reported

Composition: Not reported

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: Not reported

Average Size: Not reported

Surface Charge: Not reported

Size Distribution Not reported

Protocol

Exposure Duration: 96 hours

Exposure Medium: Tap water maintained at $16 \pm 1^\circ\text{C}$,

Endpoint: Mortality

dissolved oxygen >8 mg/L, carbon dioxide <6 mg/L,

Exposure Concentrations: 1.25, 2.5, 5, 10, 20, 30-50, and 60–70 ppm

ammonia <0.01 mg/L, nitrite <0.1 mg/L, water hardness <200 mg/L as CaCO_3 , conductivity 780 μS , pH 7.5–8.4

Organisms per Replicate: 30 fish \times 3 replicates

Methods: Partial mortality data at 24 and 96 hours were used to determine LC_{50} values, and all calculations were based on mean measured concentrations in the aquarium, rather than nominal concentrations.

Study Outcome

LC_{50} (48-hour): 3.5 mg/L

LC_{50} (72-hour): 3 mg/L

LC_{50} (96-hour): 2.3 mg/L

Yeo and Kang (2008, [199841](#)) Effects of nanometer-sized silver materials on biological toxicity during zebrafish embryogenesis.

Test Species

Zebrafish embryos, 64- to 265-cell stages and 2.5 hours post fertilization (*Danio rerio*)

Material

Commercial nano-Ag supported by titanium oxide purchased from N Corporation (Korea).

Shape: Not reported

Solubility: Not reported

Composition: Ag₃O, Ag₄H, and TiO

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: Supported by TiO

Average Size: Not reported

Surface Charge: Not reported

Size Distribution: Approximately 10–20 nm (determined using TEM)

Protocol

Exposure Duration: 72 hours

Exposure Medium: Carbon-filtered city water

Endpoints: Hatching rate, morphology, and gene expression

Organisms per Replicate: 300 embryos × 3 replicates

Exposure Concentrations: 10 and 20 ppt

Methods: Hatching rates were determined based on the number of hatched embryos 72 hours post-fertilization. RNA was isolated from nano-Ag exposed zebrafish 72 hours post-fertilization and expression of SEL N1 and N2 genes were analyzed by RT PCR.

Study Outcome

Hatching Rate. Hatching rates were significantly decreased in both 10 and 20 ppt treatment groups, and catalase activity increased significantly in the 20-ppt groups.

Phenotypic Changes. Almost all individuals in the 10- and 20-ppt nano-Ag groups exhibited abnormal properties, with more observed in the 20-ppt compared to the 10-ppt groups (significance was not reported). Observed phenotypic changes included abnormal notochord development, undeveloped eyes, weak heartbeats, and edema.

SEL N Gene Expression. SEL N1 and N2 gene expression reduced in a concentration-dependent manner (significance not reported). SEL N2 gene expression was 38% that of control group.

Yeo and Pak (2008, [191177](#)) Exposing zebrafish to silver nanoparticles during caudal fin regeneration disrupts caudal fin growth and p53 signaling.

Test Species

Zebrafish (*Danio rerio*), age not specified

Material

Commercial nano-Ag supported by titanium oxide purchased from N Corporation (Korea).

Shape: Not reported

Composition: Ag₃O, Ag₄H, and TiO

Crystal Structure: Not reported

Average Size: Not reported

Size Distribution: Approximately 10–20 nm (determined using TEM)

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Supported by TiO

Surface Charge: Not reported

Protocol

Exposure Duration: 36 days

Endpoints: Caudal fin regeneration, histology, and gene expression

Exposure Concentrations: 0.4 and 4 ppm

Exposure Medium: Distilled water, supplemented with 0.3 g/L Instant Ocean Sea Salt, filtered through 0.45 µm mesh, denitrified by bacterial filtration, and disinfected by ultraviolet light exposure

Organisms per Replicate: 1 fish × 5 replicates

Methods: Regeneration experiments were performed on caudal fins amputated at approximately 50% proximal-distal level. Phenotypic comparisons were made between fish challenged to regenerate in the presence of nano-Ag when compared to the controls. Photographs of the regeneration area were taken intermittently. To examine histological effects from exposure to nano-Ag, tissues were fixed, post-fixed, dehydrated, and then embedded in Embed 812-Araldite 502 resin. Ultra-thin sections were then mounted, stained, and examined using a field emission TEM. RNA was isolated in nano-Ag-exposed zebrafish 52 hours post-fertilization, and gene expression profiles were analyzed by microarray analysis.

Study Outcome

Caudal Fin Regeneration. Caudal fin regeneration was significantly inhibited at 4 ppm of nano-Ag while only a delay in regeneration was observed at 0.4 ppm.

Histological Effects. Nano-Ag penetrated all organelles, including the nucleus, and accumulated in blood vessels in both treatment groups. Destroyed or swollen mitochondria with empty matrices were observed in fin, gill, and muscle tissue.

Gene Expression. Genes coding for tumor protein p53; bc12-associated X protein; phosphatidylinositol glycan, class C; phosphatidylinositol glycan, class P; and insulin-like growth factor binding protein 3 were upregulated (range: 2.05- to 3.08-fold). Gene coding for insulin-like growth factor 1 was significantly downregulated (0.38-fold).

B.6. Summary of Nano-Ag Effects on Terrestrial Plants

Babu et al. (2008, [195479](#)) Effect of nano-silver on cell division and mitotic chromosomes: A prefatory siren.

Test Species

Onion (*Allium cepa*)

Material

Commercial nano-Ag (source not reported).

Shape: Not reported

Composition: Pure Ag (method not reported)

Crystal Structure: Not reported

Average Size: 2 nm (method not reported)

Size Distribution: Not reported

Solubility: Approximately 80% nano-Ag and 20% ionic Ag in solution (method not reported)

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 0.5, 1, 2, or 4 hours

Endpoints: Cell proliferation and chromosomal damage

Exposure Concentrations: 10, 20, 40, and 50 ppm

Exposure Medium: Distilled water

Plants per Replicate: 8 meristems

Methods: Root tips (meristems) from *A. cepa* bulbs were treated with nano-Ag concentrations of 10, 20, 40, or 50 ppm. Chromosomal preparations were made and approximately 10,000 cells from 10 root tips and 5 bulbs were analyzed to score the frequency of mitotic index and chromosomal aberrations.

Study Outcome

Significantly reduced frequency in mitotic index was observed at all concentrations and exposure durations except 10 ppm concentration for 0.5- and 1-hour exposure periods and 40 and 50 ppm for the shortest exposure period (0.5 hour).

Chromosomal aberrations increased in a dose-and duration-dependent manner, and were significantly different from the controls for all concentrations and exposure durations. Structural aberrations included C-metaphase, disturbed metaphase, fragments, sticky metaphase, laggards, anaphasic bridge, disturbed anaphase, and micronuclei.

Kumari et al. (2009, [199808](#)) Genotoxicity of silver nanoparticles in *Allium cepa*.

Test Species

Onion (*Allium cepa*)

Material

Commercial nano-Ag purchased from Sigma-Aldrich (USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: <100 nm (provided by the manufacturer)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: 5.0 m²/g (provided by the manufacturer)

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 4 hours

Endpoints: Cell proliferation and chromosomal damage

Exposure Concentrations: 25, 50, 75, or 100 ppm

Exposure Medium: Deionized water

Plants per Replicate: 4 bulbs and 8 root tips × 3 replicates

Methods: Roots 2–3 cm in length were treated with nano-Ag, and mitotic index and aberrant cells were quantified after microscopic observation.

Study Outcome

There was a concentration-dependent decrease in mitotic index (MI), with the lowest MI (27%) occurring in the 100 ppm group. MI was significantly different from the controls at the 50-, 75-, and 100-ppm levels. Different chromosomal aberrations occurred at the different nano-Ag concentrations. At 50 ppm, chromatin bridge, stickiness, and disturbed metaphase were observed; at 75 ppm, chromosomal breaks were observed; complete disintegration of the cell walls was observed at 100 ppm.

Rostami and Shahstavar ([2009, 199810](#)) Nano-silver particles eliminate the in vitro contamination of olive 'Mission' explants.

Test Species

Olive (*Olea europea L.*)

Material

Commercial nano-Ag (L2000 Series) purchased from NanoCid (Iran).

Shape: Not reported

Solubility: Not reported

Composition: Not reported

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: Not reported

Average Size: Not reported

Surface Charge: Not reported

Size Distribution: Not reported

Protocol

Exposure Duration: 1 hour (submerged in nano-Ag solution) or 30 days (amended media)

Exposure Medium: Sterile distilled water or Murashige and Skoog half strength media

Endpoints: Decontamination and mortality

Organisms per Replicate: 20 explants × 4 replicates

Exposure Concentrations: 100, 200, 300, and 400 mg/L (submerged) or 0, 4, 8, and 16 mg/L (amended media)

Methods: Explants with nodes and shoot apices were submerged in 100 mg/L ascorbic acid and 100 mg/L citric acid to control phenolic compounds. Explants were then submerged in 70% ethylic ethanol for 0, 0.5, or 1 min, rinsed, then submerged in 0.1% Clorox solution for 0, 5, or 10 min, then washed. Explants were then submerged in nano-Ag solution for 1 hour, after which they were placed in uncontaminated media and monitored for 30 days. In a separate experiment, some explants were placed in Murashige and Skoog half strength media amended with concentrations of 2, 4, or 6 mg/L nano-Ag and grew for 30 days in the amended media. After 30 days, infected and developed plants were recorded, but the method by which these endpoints were quantified was not reported.

Study Outcome

Nano-Ag at all concentrations appeared to effectively reduce or eliminate internal bacterial contamination in both submerged explants and those in contaminated media, though significance was not reported and the method by which contamination was assessed was not reported. Nano-Ag resulted in increased mortality in explants submerged in nano-Ag solutions in a dose-dependent manner, with no explants surviving in the 300 and 400 mg/L treatment groups (significance not reported). Mortality decreased with increasing nano-Ag concentrations in media up to 4 mg/L, after which mortality increased (significance not reported).

Stampoulis et al. (2009, [199839](#)) Assay-dependent phytotoxicity of nanoparticles to plants.

Test Species

Zucchini (*Cucurbita pepo* cv Costata Romanesco)

Material

Commercial nano-Ag purchased from Sigma-Aldrich (St. Louis, MO, USA).

| | |
|--|---|
| Shape: Not reported | Solubility: Not reported |
| Composition: Not reported | Surface Area: Not reported |
| Crystal Structure: Not reported | Surface Treatment: Sodium dodecyl sulfate (SDS) |
| Average Size: <100 nm (provided by the manufacturer) | surfactant |
| Size Distribution: Not reported | Surface Charge: Not reported |

Protocol

| | |
|---|--|
| Exposure Duration: 5 days (elongation), 12 days (germination), 15 days (biomass), 17 days (transpiration) | Exposure Medium: Reverse osmosis water (elongation and germination) or Hoagland solution (biomass and transpiration) |
| Endpoints: Changes in time to germination, root elongation, biomass, and transpiration volume | Organisms per Replicate: 3 seeds × 5 replicates (germination and elongation) or 1 plant × 6 replicates (biomass and transpiration) |
| Exposure Concentrations: 1000 mg/L (elongation and germination) or 1.0, 10, 50, 100, 500, and 1000 mg/L (biomass and transpiration) | |

Methods: Pre-germinated seeds with radicals of 0.5 mm were selected for the root elongation assay. Root elongation was measured after a 5-day exposure to nano-Ag. To assess time to germination, seeds were placed on Petri dishes and amended with 1000 mg/L nano-Ag with or without 0.2% SDS. A batch hydroponic experiment was conducted to determine the effect of nano-Ag on biomass of seedlings. 18-day-old seedlings were exposed to 1000 mg/L nano-Ag in Hoagland solution after which biomass was monitored for 15 days. In addition, 4-day-old seedlings were exposed to concentrations of nano-Ag ranging from 0 to 1000 mg/L nano-Ag in Hoagland solution and biomass and transpiration volume (determined by mass change of solution) were measured over a 17-day exposure period.

Study Outcome

Seed Germination. There was no significant impact on seed germination in the nano-Ag or nano-Ag plus SDS treatment groups when compared to controls. Nor did conventional Ag affect seed germination.

Root Elongation. There was no significant impact on root growth in the nano-Ag or nano-Ag plus SDS treatment groups when compared to controls. Nor did conventional Ag affect root growth.

Biomass. Nano-Ag exposure resulted in 69% reduction in plant biomass compared to the control and 1000 mg/L conventional Ag groups.

Transpiration. Exposure to 500 and 1000 mg/L nano-Ag resulted in 51 and 70% reduction in biomass when compared to controls and conventional Ag, respectively. Transpiration volume decreased significantly at and above 100 mg/L nano-Ag.

B.7. Summary of Nano-Ag Effects on Terrestrial Invertebrates

Roh et al. (2009, [195565](#)) Ecotoxicity of silver nanoparticles on the soil nematode *Caenorhabditis elegans* using functional ecotoxicogenomics.

Test Species

Soil nematode (3 days old), wild type and 3 mutants (*Caenorhabditis elegans* N2 var. Bristol, *mtl-2* [gk125], *sod-3* [gk235], and *daf-12* [rh286])

Material

Commercial nano-Ag purchased from Sigma-Aldrich (St. Louis, MO, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: Approximately 14–20 nm (determined using DLS)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 24 (gene expression, survival, and growth) or 72 hours (reproduction)

Endpoints: Gene expression, survival, growth, and reproduction

Exposure Concentrations: 0.05, 0.1, and 0.5 mg/L

Exposure Medium: K-media

Organisms per Replicate: 800–1000 × 3 replicates (gene expression), 10 × 5 replicates (survival), 150 × 5 replicates (growth), and 1 × 5 replicates (reproduction)

Methods: To determine effects on gene expression following exposure to nano-Ag, RNA was prepared and analyzed by microarray analysis and 26 genes were analyzed by real-time RT-PCR. At the end of the exposure period, survival was assessed by counting live and dead individuals, and growth was assessed in heat-killed samples by measuring body length. Reproduction was assessed by counting the number of offspring at all developmental stages beyond the egg following exposure of young adults to nano-Ag.

Study Outcome

Gene Expression. Microarray analysis revealed upregulation of 415 gene probes and downregulation of 1,217 by more than 2-fold compared to the controls. Thirteen gene ontology categories were significantly represented within upregulated genes and 149 in downregulated genes. Four genes analyzed by PCR were significantly upregulated (*M162.5*, nonannotated; *mtl-2*, stress-response metallothionein; *sod-3*, stress-response superoxide dismutase; and *daf-12*, stress-response abnormal dauer formation protein).

Survival and Growth. Survival and growth were not significantly affected in either the wild type or mutant *C. elegans*.

Reproduction. Reproduction decreased significantly in both wild type and mutant strains at all treatment levels except in *sod-3*(gk235) mutant exposed to 0.05 mg/L nano-Ag. Wild type and *daf-12*(rh286) were the most sensitive of the four types tested.

B.8. Summary of Nano-Ag Effects on Non-mammalian Terrestrial Vertebrates

Grodzik and Sawosz (2006, [196265](#)) The influence of silver nanoparticles on chicken embryo development and bursa of Fabricius morphology.

Test Species

Ross hen embryos

Material

Commercial hydrocolloidal Ag suspension purchased from NanoTech (Poland) and diluted with NaCl.

Shape: Not reported

Solubility: Not reported

Composition: Not reported

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: Not reported

Average Size: Not reported

Surface Charge: Not reported

Size Distribution: Not reported

Protocol

Exposure Duration: 13 days

Exposure Medium: Egg interior

Endpoint: Development

Organisms per Replicate: 30 eggs × 1 replicate

Dose: 10 ppm administered on incubation days 5, 11, and 17

Methods: Eggs were injected with 10 ppm nano-Ag on incubation days 5, 11, and 17. Eggs were weighed on day 18, opened and sacrificed. Embryos' livers, hearts, and eyes were weighed and examined according to Hamburger and Hamilton standard (1951). Bursae of Fabricius (BF) were collected and treated for examination by microscope.

Study Outcome

Exposure to nano-Ag did not affect embryo weight or weights of liver, heart, or eyes. There was no significant difference in apoptotic signs between control and nano-Ag groups, and no necrosis was observed. The number and luminal intensity of cell nuclei in BFs of nano-Ag group were decreased and a slight increase was observed in the population of cells with peripherally deep staining nuclei (significance not reported).

Sawosz et al. (2007, [194076](#)) Influence of hydrocolloidal silver nanoparticles on gastrointestinal microflora and morphology of enterocytes of quail.

Test Species

Quail (*Coturnix coturnix japonica*), approximately 10 days old, and quail gut microflora (*Escherichia coli*, *Enterobacter*, *Streptococcus bovis*, *Enterococcus faecium*, *Bacteroides* spp., *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Lactobacillus fermentum*, and *Leuconostoc lactis*)

Material

Commercial hydrocolloidal Ag suspension purchased from NanoTech (Poland) and produced by solid-liquid phase discharge method.

Shape: Not reported

Solubility: Not reported

Composition: Not reported

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: Not reported

Average Size: 52% of Ag nanoparticles were 3–4 nm in size.

Surface Charge: Not reported

Size Distribution: 2 to >7nm (determined using TEM)

Protocol

Exposure Duration: 12 days

Exposure Medium: Drinking water

Endpoints: Bacterial content and tissue damage

Organisms per Replicate: 15 birds × 1 replicate

Exposure Concentrations: 0, 5, 15, and 25 mg/kg

Methods: Quail were allowed to drink freely from water containing nano-Ag. Following exposure for 12 days, quail were killed and caeca were opened. The mucosa was scraped and the number of total culturable anaerobic bacteria was enumerated along with other enterobacteria. Tissues samples of the duodenum were also analyzed.

Study Outcome

No pathological changes or behavioral changes were observed in quail exposed to nano-Ag. Composition of the gut microflora was significantly altered in the 25 mg/kg nano-Ag group, with significant increases in the *Lactobacillus* spp., *L. lactis*, and *A. naeslundii*. No significant changes were observed in the other bacteria tested. There were no changes in the structure of enterocytes, glands, or connective tissue of intestinal villi. There was also no change in number of leucocytes.

Appendix B References

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Appendix C. Summary of Human Health Effects Studies of Nano-Ag

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Appendix C. Summary of Human Health Effects Studies of Nano-Ag

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| Arora et al. (2009, 196104) Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells. | C-5 |
| AshaRani et al. (2009, 195477) Cytotoxicity and genotoxicity of silver nanoparticles in human cells. | C-6 |
| Braydich-Stolle et al. (2005, 088105) In vitro cytotoxicity of nanoparticles in mammalian germline stem cells. | C-7 |
| Carlson et al. (2008, 195497) Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. | C-8 |
| Greulich et al. (2009, 195516) Studies on the biocompatibility and the interaction of silver nanoparticles with human mesenchymal stem cells (hMSCs). | C-9 |
| Hsin et al. (2008, 196156) The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells. | C-10 |
| Hussain et al. (2005, 088101) In vitro toxicity of nanoparticles in BRL 3A rat liver cells. | C-11 |
| Kittler et al. (2009, 195530) Synthesis of PVP-coated silver nanoparticles and their biological activity towards human mesenchymal stem cells. | C-12 |
| Paddle-Ledinek et al. (2006, 195552) Effect of different wound dressings on cell viability and proliferation. | C-13 |
| Samberg et al. (2010, 625612) Evaluation of silver nanoparticle toxicity in vivo and keratinocytes in vitro. | C-14 |
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| Shrivastava et al. (2009, 200846) Characterization of antiplatelet properties of silver nanoparticles. | C-16 |
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| Ji et al. (2007, 091301) Twenty-eight-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats. | C-19 |
| Kim et al. (2008, 193473) Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. | C-20 |
| Rosas-Hernández et al. (2009, 625611) Effects of 45-nm silver nanoparticles on coronary endothelial cells and isolated rat aortic rings. | C-21 |
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| Sung et al. (2009, 193664) Subchronic inhalation toxicity of silver nanoparticles. | C-25 |
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Tang et al. (2008, 195575) Influence of silver nanoparticles on neurons and blood-brain barrier via subcutaneous injection in rats _____ C-27

Appendix C References _____ C-28

C.1. Study Selection Criteria

1 The process by which studies were selected for inclusion in the human health effects tables differed
2 for each category of study (e.g., in vitro, in vivo, occupational) based on the quantity and quality of
3 available toxicological data. In general, literature searches were conducted for specific human and rodent
4 cell types, and later for specific toxicological endpoints, to identify relevant in vitro studies, and literature
5 searches were conducted for exposure pathway (e.g., inhalation, oral, dermal) to identify relevant rodent
6 in vivo studies. To reflect the most current state of the science, the tables in this appendix include only
7 studies published in or after 2000. For those toxicological endpoints for which a substantial amount of
8 data was available (e.g., mitochondrial function, reactive oxidative stress), studies examining relevant
9 endpoints were selected based on the data quality and the relative contribution of the results to the state of
10 the science (determined largely by examining the number of articles in which the study was later cited).
11 Studies were also included if the investigators examined an endpoint for which there was otherwise little
12 information, used a novel technique to assess toxicity, or compared the relative toxicities of nano-Ag with
13 different sets of characteristics (e.g., nano-Ag of different sizes, surface areas, shapes). For study
14 categories with very little available toxicological information, all identified studies were included unless
15 they were judged to be of poor quality. In this case, no studies were included for toxicity due to
16 occupational exposure because no occupational effects studies specific to nano-Ag were identified.

17 Information in the tables is organized to take into account the minimum requirements for
18 physicochemical characterization proposed by the Minimum Information for Nanomaterial
19 Characterization (MINChar) Initiative and others (MINChar Initiative, 2008, [594265](#)). The infancy in
20 understanding toxicity of nano-Ag and its mechanisms of toxicity, and the equivocal nature of some
21 studies that give conflicting results, preclude the direct comparison of study results. To emphasize that,
22 caution is warranted in interpreting the results of the available toxicological studies of nano-Ag. These
23 tables are organized in a way that emphasizes each study's relevant attributes in the context of this case
24 study – especially characterization of the nano-Ag used in the study – rather than to facilitate direct
25 comparison of results among studies.

C.2. Summary of Key In Vitro Studies

Ahamed et al. (2008, [196100](#)) DNA damage response to different surface chemistry of silver nanoparticles in mammalian cells.

Test Species

Mouse embryonic stem cells (mES) and mouse embryonic fibroblasts (MEF)

Material

Commercial uncoated nano-Ag, supplied by Novacentrix, Austin, Texas, and polysaccharide-coated nano-Ag, supplied by Clark University, Potsdam, NY. Nano-Ag dispersions were vortexed.

Shape: Not reported

Solubility: Not reported

Composition: Not reported

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: Uncoated and polysaccharide-coated

Average Size: 25 nm (reported by manufacturer)

Surface Charge: Not reported

Size Distribution: Not reported

Protocol

Exposure Duration: 4, 24, 48, and 72 hours

Exposure Medium: Ham's F12 Dulbecco's modified eagle medium (DMEM)

Endpoint: Uptake, morphology, viability, and apoptosis

Exposure Concentrations: 50 µg/mL

Cell Density: Not reported

Methods: Uptake and morphology were measured by fluorescence and confocal microscopy. Morphology was also analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) immunoblotting. Cell viability was assessed by 3-MTT-based cell viability test and by measuring annexin V protein.

Study Outcome

Uptake. The uncoated particles tended to agglomerate, suggesting they may not be found in some organelles (e.g., nucleus and mitochondria), while the coated particles appeared dispersed.

Membrane Integrity. Exposure to 50 µg/mL of uncoated or coated nano-Ag up-regulated cell cycle checkpoint protein p53 in mES and MEF cells. After 4 hours, p53 was phosphorylated only by coated nano-Ag, and only in mES cells. After 4 and 24 hours, both types of nano-Ag resulted in increased levels of the DNA damage repair proteins Rad51 and phosphorylated-H2AX expression in mES cells. Though significance was not reported, they report that cellular response to coated nano-Ag appears greater than uncoated nano-Ag.

Mitochondrial Function. Exposure to 50 µg/mL of uncoated and coated nano-Ag induced mES and MEF cell death (annexin V protein expression and MTT assay). The annexin V expression was higher in MEF cells treated with coated nano-Ag.

Arora et al. (2009, [196104](#)) Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells.

Test Species

Primary mouse fibroblasts and liver cells

Material

Nano-Ag synthesized by photo-assisted reduction of Ag⁺ to metallic nanoparticles.

Shape: Spherical

Composition: Not reported

Crystal Structure: Not reported

Average Size: 16.6 nm (determined using DLS)

Size Distribution: 6.5–43.8 nm (determined using DLS)

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 24 hours

Endpoint: Morphology, viability, antioxidant defense, and apoptosis

Exposure Concentrations: 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 150, 200, 250, 300, 350, 400, and 500 µg/mL

Exposure Medium: Colloidal aqueous solution

Cell Density: 2 × 10⁴ cells/2 mL growth medium/plate (morphology and apoptosis); 1 × 10⁴ cells/200 mL growth medium/well (cytotoxicity)

Methods: Cell morphology was examined by phase contrast microscopy and cell viability was assessed by XTT-based cell viability test. Glutathione (GSH) reduction and antioxidant defense were used in the estimation of enzyme activity. Apoptosis was measured by using a colorimetric assay and fluorescence microscopy.

Study Outcome

Morphology. Cells less polyhedral, more fusiform, and shrunken with treatment concentration from 50 µg/mL to 100 µg/mL. Primary liver cells displayed no changes relative to control cells at concentrations of 100 µg/mL and below. At 200 µg/mL and above, primary liver cells displayed damaged irregular cell membranes.

Membrane Integrity. IC₅₀: 61 µg/mL primary fibroblasts; 449 µg/mL primary liver cells; spherical assemblages were found inside the mitochondria of both treated fibroblasts and liver cells, also in the vacuoles of liver cells.

Superoxide Dismutase (SOD). In primary fibroblasts, changes in SOD levels were statistically insignificant. In primary liver cells, SOD levels increased from 9.2 micromoles per milligram (µM/mg) protein in untreated cells to 13 µM/mg protein in treated cells, a factor of 1.4

Lipid Peroxidation. In primary fibroblasts, lipid peroxidation decreased from 0.31 µM/mg protein in untreated cells to 0.22 µM/mg protein in treated cells, a factor of 1.4. In primary liver cells, changes in lipid peroxidation were statistically insignificant.

GSH Reduction. In primary fibroblasts, GSH levels increased from 0.82 µM/mg protein in untreated cells to 0.95 µM/mg protein in treated cells, a factor of 1.2. In primary liver cells, GSH levels increased from 72.3 µM/mg protein in untreated cells to 79 µM/mg protein in treated cells, a factor of 1.1.

Apoptosis. Nano-Ag induced apoptosis at concentrations in the ranges 3.12–50 µg/mL and 12.5–400 µg/mL for primary fibroblasts and primary liver cells, respectively. For primary fibroblasts, 69% live cells, 24% apoptotic cells, and 7% necrotic cells were observed at nano-Ag concentration 30 µg/mL (~ ½ IC₅₀) whereas, at 4-fold higher nano-Ag concentration (~2 × IC₅₀), 37% live cells, 17% apoptotic cells, and 46% necrotic cells were observed. In the case of primary liver cells, 71% live cells, 24% apoptotic cells, and 5% necrotic cells and 32% live cells, 14% apoptotic cells, and 54% necrotic cells for ~1/2 IC₅₀ and ~2 × IC₅₀, respectively.

Asha Rani et al. (2009, [195477](#)) Cytotoxicity and genotoxicity of silver nanoparticles in human cells.

Test Species

Normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251)

Material

Nano-Ag synthesized by reducing AgNO₃ solution using NaBH₄.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 6 to 20 nm (determined using TEM and UV absorption)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Coated with starch

Surface Charge: Not reported

Protocol

Exposure Duration: 24, 48, and 72 hours

Endpoint: Morphology, viability, reactive oxygen species (ROS) generation, cell cycle, apoptosis, genotoxicity, and uptake

Exposure Concentrations: 0, 25, 50, 100, 200, and 400 µg/mL

Exposure Media: Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (U251); glutamine with 15% FBS, 1% each of penicillin streptomycin, nonessential amino acids, vitamins, and 2% essential amino acids

Cell Density: 1×10^4 cells

Methods: Morphology was assessed by TEM and STEM; ATP concentration was measured using Cell-Titer glow luminescent cell viability assay; mitochondrial function was analyzed using a CellTiter blue cell viability assay; ROS generation was assessed using DCF-DA and DHE staining methods; apoptosis was measured using Annexin-V staining; genotoxicity was analyzed using comet assay and cytokinesis-blocked micronucleus assay; and uptake was studied using TEM.

Study Outcome

Cell Morphology. Treated cells were clustered with few cellular extensions and displayed restricted spreading patterns.

ATP Concentration. ATP assays demonstrated a concentration- and time-dependent decrease in luminescence intensity in both IMR-90 and U251 cells, with ATP content declining statistically after 48 hours.

Mitochondrial Function. CellTiter blue cell viability assays demonstrated a concentration-dependent decrease in mitochondrial activity in both IMR-90 and U251 cells.

ROS Generation. Statistically significant increase in hydrogen peroxide and superoxide production in cells treated with 25 and 50 µg/mL; however no significant increase was observed beyond 100 µg/mL.

Cell Cycle. Possible mechanism of toxicity is proposed which involves disruption of the mitochondrial respiratory chain by Ag-NP leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage. It is anticipated that DNA damage is augmented by deposition, followed by interactions of nano-Ag to the DNA leading to cell cycle arrest in the gap-2/mitosis (G2/M) phase.

Apoptosis. Apoptotic cells populations increased from 25 to 100 µg/mL in IMR-90 cells. Late apoptosis and necrosis caused 16% (± 5) of cell death.

Chromosomal Aberrations. Chromosomal breaks were observed in cells treated with nano-Ag.

DNA Damage. A concentration-dependent increase was observed in tail momentum. A concentration-dependent increase in DNA damage was observed in U251 cells, while DNA damage did not increase beyond 100 µg/mL in IMR-90 cells.

Uptake. Treated cells displayed endosomes containing many nanoparticles near the cell and nuclear membrane, suggesting nanoparticles more likely entered through endocytosis than diffusion. Nanoparticles were found throughout the cytoplasm, as well as inside lysosomes, mitochondria, nucleolus, and nucleus.

Braydich-Stolle et al. (2005, [088105](#)) In vitro cytotoxicity of nanoparticles in mammalian germline stem cells.

Test Species

Mouse spermatogenic cell line (C18-4)

Material

Nano-Ag synthesized in commercial pulsed-plasma reactor at Air Force Research Laboratory, Brooks Air Force Base, TX. All characteristics reported after synthesis, not measured by study authors.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 15 nm

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 48 hours

Endpoint: Morphology and viability

Exposure Concentrations: 0, 5, 10, 25, 50, and 100 µg/mL

Exposure Medium: Phosphate buffered solution

Cell Density: 60% confluency; 3×10^5 cells/well for

morphology, 1×10^4 cells/well MTS and lactate

dehydrogenase (LDH), 1×10^5 cells/well for apoptosis

Methods: Cell morphology assessed via light microscopy. Cell viability and cytotoxicity assessed with MTS assay for mitochondrial function, LDH leakage; apoptosis measured by cell membrane permeability assay.

Study Outcome

Cell Morphology. Necrotic areas and intact plasma membranes indicating apoptosis observed at concentrations 10 µg/mL and greater.

Mitochondrial Function. EC_{50} : 8.75 µg/mL; concentrations greater than 10 µg/mL not tested because of clumping and precipitation of test solution.

Membrane Integrity. EC_{50} : 2.5 µg/mL; concentrations greater than 10 µg/mL not tested because of clumping and precipitation of test solution.

Apoptosis. Increase in percent of apoptotic cells with increasing concentrations of nano-Ag. 5 µg/mL dose statistically significantly different from control. Found C18-4 cells to be more sensitive to nanoparticle concentrations than previously tested BRL 3A cells.

Carlson et al. (2008, [195497](#)) Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species.

Test Species

Rat alveolar macrophages (NR8383 CRL-2192)

Material

Commercially-available hydrocarbon-coated silver (NovaCentrix, formerly Nanotechnologies, Inc.).

| | |
|---|---|
| Shape: Spherical (confirmed by SEM) | Solubility: Insoluble |
| Composition: Not reported | Solubility: Insoluble |
| Crystal Structure: Not reported | Surface Area: Not reported |
| Average Size: 15, 20, and 50 nm (reported by manufacturer); 15, 30, and 55 nm (determined using microscopy) | Surface Treatment: Hydrocarbon coated (~2 nm thick) to prevent sintering during plasma synthesis and maintain constant coating in aqueous solutions |
| Size Distribution: Not reported | Surface Charge: Not reported |

Protocol

| | |
|---|---|
| Exposure Duration: 24 hours | Exposure Concentrations: 0, 5, 10, 25, 50, and 75 µg/mL |
| Endpoints: Uptake, morphology, viability, inflammatory response, reactive oxygen species (ROS) generation | Exposure Medium: Ham's Nutrient Mixture F12K |
| | Cell Density: 80% confluency |

Methods: Following 24-hour incubation of treated samples, uptake, and morphology examined by light microscopy and TEM (uptake only). Cell viability assessed by MTT-based cell viability test, membrane integrity assessed using fluorescence to measure lactate dehydrogenase (LDH) released, and mitochondrial membrane potential assay measured by fluorescence. Glutathione (GSH) levels and inflammatory responses measured by GSH assay and enzyme-linked immunosorbent assay (ELISA), respectively. ROS generation determined using dichlorofluorescein diacetate (DCFH-DA).

Study Outcome

Uptake. Nanoparticles of all sizes taken up into cells.

Cell Morphology. Cells dosed with 15-nm particles (only 0, 25, and 75 µg/mL tested) appeared shrunken and lacked defined plasma membrane. Agglomerated nano-Ag observed inside and outside cells dosed with 30-nm particles, and macrophages appeared larger. 55-nm particles agglomerated and were not observed inside the cells.

Mitochondrial Function. EC₅₀: 27.87 ± 12.23 µg/mL 15-nm particles; EC₅₀: 33.38 ± 11.48 µg/mL 30-nm particles; EC₅₀ greater than 75 µg/mL 55-nm particles; tests with silver nitrate (AgNO₃) produced results similar to 15-nm nano-Ag.

Mitochondrial Membrane Potential (MMP). Statistically significant loss of MMP observed with increasing dose for 15- and 30-nm nano-Ag. Loss of MMP may be size dependent and warrants further study to determine if cell death is result of mitochondrial-instigated apoptosis.

Membrane Integrity. Statistically significant, dose-dependent decrease in cell viability for 15-nm and 30-nm nano-Ag at concentrations between 10 and 75 µg/mL. Decrease in cell viability as compared to control not statistically significant until 75 µg/mL dose for samples treated with 55-nm nano-Ag.

GSH Reduction. GSH levels decreased with increasing dose for both 15-nm and 30-nm, and the GSH level was undetectable at 50 µg/mL. However, the same responses were not seen for the 55-nm doses.

ROS Generation. For 15-nm particles, statistically significant increases in ROS with increasing dose from 10 µg/mL to 50 µg/mL. Results for 30- and 55-nm particles not statistically different from control.

Inflammatory Response. Statistically significant increases in the cytokines, TNF-α, MIP-2, and IL-1β at all doses (5, 10, and 25 µg/mL tested), but responses showed no trend with size or concentration. No increases occurred in IL-6 cytokine, but this was unexplained in the study.

Greulich et al. (2009, [195516](#)) Studies on the biocompatibility and the interaction of silver nanoparticles with human mesenchymal stem cells (hMSCs).

Test Species

Human mesenchymal stem cells (hMSC)

Material

Nano-Ag particles prepared by polyol process using AgNO₃ in C₂H₆O₂ and polyvinylpyrrolidone (PVP).

Shape: Spherical

Solubility: Not reported

Composition: Pure silver

Surface Area: Not reported

Crystal Structure: Not reported

Surface Treatment: PVP

Average Size: 100 nm (determined using DLS)

Surface Charge: Not reported

Size Distribution: 35 nm to 350 nm with median of 100 nm (determined using DLS)

Protocol

Exposure Duration: 7 days

Exposure Medium: Cell culture medium RPMI1640 with 10% fetal calf serum

Endpoint: Morphology, viability, inflammatory response, and chemotaxis

Cell Density: Not reported

Exposure Concentrations: 0, 0.05, 0.5, 1, 2.5, 3, 3.5, 4, 5, and 50 µg/mL

Methods: hMSCs treated with nano-Ag/ultrapure water solution or silver acetate/ultrapure water solution. Cell viability assessed by fluorescence staining and microscopy after 7 days of incubation. Morphology of cells in same samples observed by phase-contrast microscopy. Chemotaxis analyzed by transwell assay using peripheral blood mononuclear cells as chemoattractants. Inflammatory response assessed via enzyme-linked immunosorbent assay (ELISA).

Study Outcome

Preliminary experiment with nano-Ag in different fluids and cell media showed no agglomeration of nano-Ag particles in cell culture media with fetal calf serum but agglomeration when cells were incubated in phosphate-buffered saline or culture medium alone.

Viability. No viable cells detected in samples treated at concentrations between 3.5 and 50 µg/mL nano-Ag particles and between 2.5 and 50 µg/mL silver acetate.

Chemotaxis. Observed decreasing chemotactic response with increasing silver concentration for both nano-Ag particles and silver acetate solution. Response statistically significant compared to control for nano-Ag doses of 3.5, 4, and 5 µg/mL and for silver acetate doses of 2.5, 3, 3.5, 4, and 5 µg/mL.

Inflammatory Response. Observed decrease in the release of interleukin-6 (IL-6), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) from cells for Ag particle and Ag ion doses of 0.05–50 µg/mL. Release of interleukin-11 (IL-11) in adherent cells was not affected following silver treatment; however IL-11 release resembled that of IL-6 or VEGF when silver was added during cell seeding.

Hsin et al. (2008, [196156](#)) The apoptotic effect of nanosilver is mediated by a ROS- and JNK-dependent mechanism involving the mitochondrial pathway in NIH3T3 cells.

Test Species

NIH3T3 (mouse fibroblast), A10 (rat vascular smooth muscle), and HCT116 (human colon cancer)

Material

Commercial nano-Ag powders, supplied by Ching-Tai Resin (Taichung, Taiwan, ROC) and Sun-Lan International Biotechnology (Taipei, Taiwan, ROC) and commercial silver powder (<250 µm), supplied by the Sigma-Aldrich (St. Louis, MO, USA).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 1–100 nm (determined using TEM)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 24 hours

Endpoint: Uptake, viability, apoptosis, reactive oxygen species (ROS) generation

Exposure Concentrations: 0, 0.0005, 0.005, 0.05, 5, 50, and 500 µg/mL

Exposure Media: Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (NIH3T3 and A10) or McCoy's 5A medium with 10% fetal bovine serum (HCT116)

Cell Density: 8×10^3 cells/well (viability); 1×10^5 cells (apoptosis and ROS generation)

Methods: Cell viability assessed by MTT-based cell viability test, apoptosis, and ROS generation were measured using flow cytometry and immunofluorescence microscopy, and cell uptake was determined using an atomic absorption spectrophotometer.

Study Outcome

Membrane Integrity. Statistically significant cytotoxicity at concentrations of 50 µg/mL and greater in NIH3T3 and A10 cells, decreasing viability to less than 50% and approximately 60% of control values at 50 µg/mL for Ching-Tai and Sun-Lan nano-Ag, respectively. HCT116 cells were less sensitive to Sun-Lan nano-Ag. Commercial Ag powder did not demonstrate cytotoxicity at concentrations of 50 µg/mL.

Apoptosis. Treatment with 50 µg/mL Sun-Lan and Ching-Tai increased the percentage of early apoptotic NIH3T3 cells to 12.9% and 43.4%, respectively, while 100 µg/mL Ching-Tai increased apoptotic HCT116 cells to only 6.5%. Commercial Ag powder did not induce apoptosis. Immunofluorescence microscopy demonstrated that cytochrome c was released from mitochondria of NIH3T3 cells into the cytosol with 50 µg/mL Ching-Tai, which was associated with translocation of Bax from the cytosol to mitochondria. In HCT116 cells, cytochrome c remained in the mitochondria without or without treatment.

Uptake. Increased intracellular Ag was observed with treatments of 50 µg/mL and 100 µg/mL in NIH3T3 and HCT116 cells, respectively. Poly(ADP-ribose) polymerase (PARP) cleavage, c-Jun N-terminal kinases (JNK) phosphorylation, and p53 phosphorylation increased in NIH3T3 cells treated with 50 µg/mL Ching-Tai. Unlike NIH3T3 cells, HCT116 cells did not demonstrate PARP cleavage; however, an increased RNA level of B-cell leukemia/lymphoma (Bcl-2) observed in HCT116 cells but not in NIH3T3 cells.

ROS Generation. Increased ROS levels in both NIH3T3 and HCT116 cells observed with Ching-Tai treatment, with NIH3T3 cells demonstrating a 48.1% increase in intracellular ROS. Pre-treatment of cells with N-acetyl cysteine and 5 mM cyanide resulted in 7.14% and 40% inhibition of Ching-Tai induced ROS generation. Data suggest activation of JNK and intracellular ROS produced by mitochondria in NIH3T3 cells contributes to the apoptotic effect of nanosilver.

Hussain et al. (2005, [088101](#)) In vitro toxicity of nanoparticles in BRL 3A rat liver cells.

Test Species

BRL 3A (ATCC, CRL-1442) immortalized rat liver

Material

Commercial nano-Ag, supplied by Air Force Research Laboratory, Brooks AFB, Texas.

| | |
|--|---|
| Shape: Not reported | Solubility: Ag-100 nm was not homogeneously suspended in solution |
| Composition: Not reported | Surface Area: Not reported |
| Crystal Structure: Not reported | Surface Treatment: Not reported |
| Average Size: 15 and 100 nm (reported by manufacturer) | Surface Charge: Not reported |
| Size Distribution: Not reported | |

Protocol

| | |
|---|--|
| Exposure Duration: 24 hours | Exposure Concentrations: 0, 2.5, 5, 10, 25, 50 and 150 µg/mL |
| Endpoint: Morphology, viability, and reactive oxygen species (ROS) generation | Exposure Medium: Culture media with 5% fetal bovine serum |
| | Cell Density: Confluent |

Methods: After exposure, morphology was observed by microscopy. Membrane integrity was measured by measuring lactate dehydrogenase (LDH) leakage, cell viability was assessed spectrophotometrically by MTT-based cell viability test, ROS generation was determined using dichlorodihydrofluorescein diacetate (H₂ DCFDA), mitochondrial membrane potential was determined by the uptake of rhodamine 123, reduced glutathione (GSH) measured by GSH assay.

Study Outcome

Cell Morphology. Cells began to shrink and became irregular in shape with increasing doses of nano-Ag.

Membrane Integrity. LDH EC₅₀: 24 ± 9.25 µg/mL 100-nm particles; LDH EC₅₀: 50 ± 10.25 µg/mL 15-nm particles; statistically significant concentration-dependent decrease in cell viability for 100-nm and 15-nm particles at concentrations between 10 and 50 µg/mL, with 100-nm particles demonstrating higher toxicity at 25 and 50 µg/mL.

Mitochondrial Function. MTT EC₅₀: 19 ± 5.2 µg/mL 100-nm particles; MTT EC₅₀: 24 ± 7.25 µg/mL 15-nm particles; Ag exposure demonstrated a significant cytotoxicity from 5 to 50 µg/mL.

GSH Reduction. Statistically significant decrease of GSH (70%) at 25 µg/mL, relative to controls.

ROS Generation. Statistically significant concentration-dependent increase in ROS generation from 10 µg/mL, with an approximately 10-fold increase in ROS generation at 25 and 50 µg/mL over control levels.

Mitochondrial Membrane Potential (MMP). Statistically significant decrease (80%) of MMP at 25 and 50 µg/mL

Kittler et al. (2009, [195530](#)) Synthesis of PVP-coated silver nanoparticles and their biological activity towards human mesenchymal stem cells.

Test Species

Human mesenchymal stem cells (hMSCs)

Material

Nano-Ag prepared by the polyol process (reduction of AgNO₃ with C₂H₆O₂ in the presence of polyvinylpyrrolidone [PVP]).

Shape: Cube, rod, and sphere

Size Distribution: Not reported

Composition: Not reported

Solubility: Not reported

Crystal Structure: Not reported

Surface Area: Not reported

Average Size: 200 nm (cube), 100–5000 nm (rod), and 20–50 nm (sphere) (determined using SEM); 80–100 nm (determined using DLS)

Surface Treatment: Coated with a shell of polymer to reduce agglomeration and aggregation

Surface Charge: Not reported

Protocol

Exposure Duration: 7 days

Exposure Medium: Cell culture medium RPMI1640 containing 10% fetal calf serum and L-glutamine

Endpoint: Morphology, viability, and proliferation

Cell Density: Not reported

Exposure Concentrations: 0, 50, 5, 4, 3.5, 3, 2.5, 1, and 500 µg/mL

Methods: Cell viability and morphology were observed using fluorescence staining and phase-contrast microscopy. Cell proliferation was assessed by a fluorogenic proliferation assay.

Study Outcome

Cell Morphology. Adherent and growing hMSCs demonstrated concentration-dependent adverse cell reactions (e.g., rounded cells) in treated cells at 50 and 5 µg/mL.

Viability. No signals of cell viability were observed in treated cells at 5 µg/mL and above and 2.5 µg/mL and above for cells treated with nano-Ag and Ag ions, respectively.

Proliferation. Statistically significant decrease in proliferation was observed in treated cells at 5 µg/mL and above and 2.5 µg/mL and above for cells treated with nano-Ag and Ag ions, respectively. However, the fluorogenic proliferation assay is analogous to a redox indicator; thus the redox state (reduction of silver ions to elemental silver) rather than the cell number may have influenced the readout of the assay.

Paddle-Ledinek et al. (2006, [195552](#)) Effect of different wound dressings on cell viability and proliferation.

Test Species

Human keratinocytes

Material

Acticoat (nanocrystalline Ag/Polyethylene mesh), manufactured by Smith & Nephew; Aquacel-Ag (Ag ions/cm cellulose), manufactured by ConvaTec; Avance (Ag/Polyurethane foam), manufactured by SSL; and Constreet-H (Ag/Hydrocolloid/alginate), manufactured by Coloplast.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: Not reported

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 40 hours

Endpoint: Viability, proliferation, morphology

Exposure Concentrations: Acticoat (109 mg/100 cm²);

Aquacel-Ag(19.7 mg/100 cm²); Avance (1.6 mg/100 cm²);

Constreet-H (31–32 mg/100 cm²)

Exposure Medium: Basal keratinocyte serum-free medium supplemented with L-glutamine, bovine pituitary extract, and recombinant epidermal growth factor

Cell Density: 6,000 cells/well

Methods: Cell viability was assessed by estimation of mitochondrial ability to reduce MTT and cell proliferation was estimated by measuring the incorporation of bromodeoxyuridine (Br-dU) into nuclear DNA, both using a microplate reader. Phase contrast microscopy was used to assess cell morphology.

Study Outcome

Viability and Proliferation. Percent MTT reduction and percent Br-dU incorporation listed below.

| Sample | Percent MTT Reduction | | Percent Br-dU Incorporation | |
|------------|-----------------------|-------------------|-----------------------------|-------------------|
| | % Control (mean ± SE) | % Control (range) | % Control (mean ± SE) | % Control (range) |
| Acticoat | 1.3 ± 0.3 | 0.5–2.5 | 8.5 ± 0.8 | 5.2–11.4 |
| Aquacel-Ag | 0.6 ± 0.1 | 0.3–1.4 | 5.6 ± 1.6 | 0.0–8.7 |
| Avance | 15.2 ± 6.3 | 1.9–46.0 | 12.5 ± 1.8 | 4.7–18.4 |
| Contreet-H | 1.0 ± 0.2 | 0.2–1.9 | 9.8 ± 3.0 | 0–34.2 |

Cell Morphology. In no extracts was there any evidence of a monolayer, and cell apposition did not follow the normal pattern. All treated cells lacked conspicuous nuclei, had missing nucleoli, and cultures displayed particulate and irregularly shaped cell debris. Cells treated with Contreet-H and Avance extracts were three times larger than control cells and displayed abundant polygonal cytoplasm with many cells showing cytoplasmic projections. Cells treated with Aquacel-Ag and Acticoat extracts had a round to ovoid cytoplasm.

Samberg et al. (2010, [625612](#)) Evaluation of silver nanoparticle toxicity in vivo and keratinocytes in vitro.

Test Species

Primary neonatal human epidermal keratinocytes (HEKs)

Material

Three sizes (reported by manufacturer as 20, 50, and 80 nm in diameter) of commercial unwashed/uncoated and washed/uncoated nano-Ag in deionized water and two sizes (reported by manufacturer as 25 and 35 nm in diameter) of dried/carbon-coated nano-Ag powder, all supplied by nanoComposix, San Diego, CA. Unwashed and washed nano-Ag synthesized by manufacturer using ammonium hydroxide-catalyzed growth onto 5-nm gold seed particles. Carbon-coated nano-Ag synthesized by manufacturer using pulsed plasma reactor and coated with polyaromatic graphitic carbon.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: Unwashed: 30.8 ± 0.6 , 47.7 ± 0.5 , and 75.5 ± 1.0 nm (determined using DLS) or 22.4 ± 2.6 , 49.4 ± 6.2 , and 79.2 ± 8.0 nm (determined using TEM).

Washed: 25.5 ± 0.4 , 43.7 ± 1.1 , and 79.9 ± 28.0 nm (determined using DLS) or 21.4 ± 3.1 , 50.0 ± 5.9 , and 77.0 ± 6.0 nm (determined using TEM). Carbon-coated: 149.0 ± 89 and 167.0 ± 110 nm (determined using DLS) or 27.2 ± 10.3 and 37.0 ± 11.6 nm (determined using TEM).

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: None (unwashed and washed), polyaromatic graphite carbon (carbon-coated)

Surface Charge: Unwashed: -29.7 mV (~ 20 -nm size), -27.8 mV (~ 50 -nm size), and -33.2 mV (~ 80 -nm size).

Washed; -46.0 mV (~ 20 -nm size), -44.3 mV (~ 50 -nm size), and -43.7 mV (~ 80 -nm size). Carbon-coated: -24.0 mV (~ 25 -nm size) and -29.0 mV (~ 35 -nm size) (expressed as ζ potential in deionized water)

Protocol

Exposure Duration: 24 hours

Endpoint:s: Viability and cytokine release

Exposure Concentrations: 0.000544 to 1.7 $\mu\text{g}/\text{mL}$ for all nano-Ag types, and up to 45 $\mu\text{g}/\text{mL}$ for washed and carbon-coated nano-Ag

Exposure Medium: Keratinocyte Growth Medium-2 (KGM-2)

Cell Density: Initial density of 12,500 cells/well grown over 18–24 hours to 80% confluency

Methods: HEKs were exposed to either KGM-2 (control) or to serial dilution of each of the eight types of nano-Ag. To assess viability, HEKs were exposed to nano-Ag diluted in KGM-2 at concentrations ranging from 0.000544 to 1.7 $\mu\text{g}/\text{mL}$ for 24 hours using MTT, alamarBlue (aB), and Celltiter 96 Aqueous One (96AQ) assays. Concentrations up to 42.5 $\mu\text{g}/\text{mL}$ of the washed and carbon-coated nano-Ag particles were also tested. To determine if the supernatants or washing permeates resulting from the synthesis and washing of the unwashed/uncoated and washed/uncoated particles, respectively, were contributing to observed cytotoxicity, HEKs were treated with either the supernatant or permeates for 24 hours at concentrations ranging from 0.068 to 1.7 $\mu\text{g}/\text{mL}$. For those nano-Ag concentrations resulting in cytotoxicity, cytokine analysis was conducted by assessing release of interleukin (IL)-8, IL-6, tumor necrosisfactor- α (TNF- α), IL-10, and IL-1 β . Results were presented with those from an in vivo porcine skin test conducted as part of the same study. These results are presented in presented in Section C.3 of this appendix.

Study Outcome

Viability. Exposure of HEKs to unwashed/uncoated nano-Ag resulted in a dose-dependent decrease in cell viability in all three assays, with significant ($p < 0.05$) decreases occurring at the 0.34- $\mu\text{g}/\text{mL}$ level in the ~ 20 - and ~ 50 -nm groups using the aB and 96AQ assays and at the 1.7- $\mu\text{g}/\text{mL}$ level for these size groups using the MTT assay. The ~ 80 -nm group resulting in a significant decrease in viability at the 0.34- $\mu\text{g}/\text{mL}$ level using all three assays. No significant decrease in viability were observed in any assay for the washed/uncoated or carbon-coated nano-Ag samples. The unwashed nano-Ag supernatant contained 5.55 mg/mL formaldehyde solvent and methanol by-product from synthesis. Exposure to this supernatant resulted in significantly decreased viability at the 0.34- $\mu\text{g}/\text{mL}$ level using the aB and MTT assays and at the 1.7- $\mu\text{g}/\text{mL}$ level using the 96AQ assay. Exposure to the washing permeates from the washed and uncoated samples did not result in any loss in viability.

Cytokine Release. Following exposure to 0.34 $\mu\text{g}/\text{mL}$ unwashed nano-Ag of all sizes, significant ($p < 0.05$) increases were observed in IL-1 β , IL-6, IL-8, and TNF- α .

Shin et al. (2007, [195568](#)) The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells.

Test Species

Human peripheral blood mononuclear cells (PMBCs)

Material

Nano-Ag colloidal solution synthesized from AgNO₃.

Shape: Not Reported

Composition: Not Reported

Crystal Structure: Not Reported

Average Size: 1.3 nm

Size Distribution: 1–2.5 nm

Solubility: Not Reported

Surface Area: Not Reported

Surface Treatment: Not Reported

Surface Charge: Not Reported

Protocol

Exposure Duration: 72 hours

Endpoint: Cytotoxic effects on PMBCs

Exposure Concentrations: 0, 1, 3, 5, 10, 20, 30 ppm nano-Ag

Exposure Medium: Cell proliferation assay

Cell Density: 2×10^6 cells/mL

Methods: PMBCs from healthy human volunteers were stimulated with 5 µg/ml phytohaemagglutinin (PHA) in the presence of varying concentrations of nano-Ag. PMBC proliferations were measured using an aqueous cell proliferation assay kit and supernatants were analyzed using enzyme-linked immunosorbent assays.

Study Outcome

Inflammatory Response. Proliferation of PMBCs showed cytotoxicity levels of nano-Ag over 15 ppm. At low levels nano-Ag decreased cytokine production (PHA-induced IL-5: at 10 ppm, interferon (INF)-gamma and tumor necrosis factor (TNF)-alpha at 3 ppm).

Shrivastava et al. (2009, [200846](#)) Characterization of antiplatelet properties of silver nanoparticles.

Test Species

Human primary platelets

Material

Nano-Ag synthesized from AgNO₃ using deionized water, NaOH, and NH₃.

Shape: Spherical

Solubility: Not reported

Composition: Not reported

Surface Area: Not reported

Crystal Structure: Face centered cubic

Surface Treatment: Not reported

Average Size: 10–15 nm (determined using diffraction)

Surface Charge: Not reported

Size Distribution: Monodispersed

Protocol

Exposure Duration: 10 minutes

Exposure Medium: Aqueous

Endpoint: Morphology and membrane integrity

Cell Density: Not reported

Exposure Concentrations: 0, 25, 50, 100, 150, 200, 250, and 500 µM

Methods: Morphology was assessed by labeling cells with ANS and recording fluorescence emission spectra. Membrane integrity was measured by assaying lactate dehydrogenase (LDH) activity from the decrease in reduced NADH absorbance.

Study Outcome

Morphology. Fluorescence intensity declined in a dose-dependence manner, suggesting nano-Ag contributes significantly to platelet membrane disorder.

Viability. Nano-Ag did not cause significant release of LDH from platelet cytosol. Platelet membrane integrity was not compromised nor did cell lysis occur.

Soto et al. (2007, [090880](#)) Cytotoxic effects of aggregated nanomaterials.

Test Species

Mouse alveolar macrophage cell line (RAW 264.7) and human macrophage (THB-1) and epithelial lung cell lines (A549)

Material

Commercially manufactured inorganic nano-Ag (manufacturer not reported).

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 3–100 nm particle; 25 nm–1 μ m assemblage (determined using TEM)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: 15 m²/g (determined using the Brunauer, Emmett, and Teller method)

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 48 hours

Endpoint: Viability

Exposure Concentrations: 5 μ g/mL

Exposure Medium: Dimethyl sulfoxide

Cell Density: 50,000 cells/well

Methods:

Cell viability and mitochondrial activity was assessed by measuring the relative absorbance or optical density for mitochondrial dehydrogenase-transformed formazan.

Study Outcome

At 5 μ g/mL, nano-Ag was the most cytotoxic, even more than the asbestos positive control (Cytotoxicity index = 1.8). Nano-Ag was particularly toxic to murine macrophages, with similar results to the human macrophage line. Generally, the epithelial cells were most sensitive.

C.3. Summary of Key In Vivo Studies

Cha et al. (2008, [195498](#)) Comparison of acute responses of mice livers to short-term exposure to nano-sized or micro-sized silver particles.

Test Species

Balb/c mouse (male), 7 weeks old

Material

Nano-Ag, synthesized by the reduction of AgNO₃ with NaBH₄.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 13 nm, or 2–3.5 μm (determined using TEM)

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 24 hours

Endpoint: Acute liver effects

Exposure Concentrations: 2.5 g

Exposure Route: Gastric intubation

Methods: Livers, hearts, intestines, and spleens from test species were obtained 3 days after treatment, fixed in 10% formalin, and subjected to histopathological analysis. A total RNA isolation kit was used to isolate the RNA from the livers and results were confirmed by a semi-quantitative RT-PCR.

Study Outcome

Nanoparticle and microparticle-treated livers showed lymphocyte infiltration, which indicated inflammation. Nonspecific focal hemorrhages in the heart, focal lymphocyte infiltration in the intestine, and nonspecific medullary congestion in the spleen were also observed in mice treated with nano-Ag. RNA microarray analysis of livers showed altered apoptosis and inflammatory responses in the livers of nanoparticle-exposed mice, as indicated by the up-regulation of seven genes in the apoptotic pathway and five in the inflammatory pathway gene expression.

Ji et al. (2007, [091301](#)) Twenty-eight-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats.

Test Species

Sprague-Dawley rat, 40 males and females (10 rats per dosing group), 10 weeks old at start of experiment (~283-g males, ~169-g females)

Material

Nano-Ag; particles generated by evaporation/condensation using a small ceramic heater within a quartz tube case.

Shape: Spherical

Composition: Not Reported

Crystal Structure: Not Reported

Average Size: 16 nm (determined using differential mobility analyzer and a condensation particle counter)

Size Distribution: 1.98–64.9 nm; geometric mean diameter and geometric standard deviation of 15.38 nm and 1.58, 12.60 nm and 1.53, and 12.61 nm and 1.52 in the high-, middle-, and low-concentration chambers, respectively

Solubility: Not Reported

Surface Area: 1.41×10^9 , 9.68×10^7 , and 1.32×10^7 nm²/cm³ in the high-, middle-, and low-concentration chambers, respectively

Surface Treatment: Not Reported

Surface Charge: Not Reported

Protocol

Exposure Duration: Subchronic 28-day inhalation

Endpoint: Inflammatory response

Exposure Concentrations: 1.32×10^6 particles/cm³ (high dose), 1.27×10^5 particles/cm³ (middle dose), and 1.73×10^4 particles/cm³ (low dose) for 6 hours/day, 5 days/week

Exposure Route: Whole-body inhalation chamber

Methods: Exposure-related effects including respiratory, dermal, behavioral, nasal, and genitourinary changes suggestive of irritancy were studied daily on weekdays. Body weights were measured after purchase, grouping, weekly during exposure, and at study termination. Hematological analysis was conducted 24 hours after study termination. Organ weights were then measured, followed by a complete histopathological analysis and determination of tissue silver using an atomic absorption spectrophotometer.

Study Outcome

Despite deposition of nano-Ag particles in the liver, olfactory bulb, and brain, no significant exposure-related adverse health effects were observed.

Kim et al. (2008, [193473](#)) Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats.

Test Species

Sprague-Dawley rat, 40 males and females (10 rats per dosing group), 6 weeks old at start of experiment (~283-g males, ~192-g females)

Material

Commercial nano-Ag, micro-sized Ag; purchased from NAMATECH Co., Ltd.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: 60 nm

Size Distribution: 52.7–70.9 nm

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 28 days

Endpoint: Effects on hematology and blood biochemistry; genotoxic effect on rat bone marrow; and Ag distribution in tissue.

Exposure Concentrations: Vehicle control (0.5% carboxymethylcellulose), low-dose group (30 mg/kg), middle-dose group, (300 mg/kg), and high-dose group (1000 mg/kg).

Exposure Route: Daily oral gavage

Methods:

After exposure, the blood biochemistry and hematology were investigated using a blood cell counter, along with a histopathological examination and Ag distribution study using spectrophotometry.

Study Outcome

Hematology and Blood Biochemistry. Dose-dependent changes in serum alkaline phosphatase, with a significant increase in high- and middle-dose male rats and high-dose female rats. Similarly, high-dose male rats and high- and middle-dose female rats displayed significant increases in cholesterol. Total protein significantly decreased in high-dose male rats. Mean corpuscular volume significantly increased in high-dose male rats, while the red blood cell count, hemoglobin content, and hematocrit significantly increased in high- and middle-dose female rats.

Inflammation. Dose-dependent bile duct hyperplasia with increase in inflammatory cells.

Genotoxic Effect on Rat Bone Marrow. Nano-Ag did not induce genetic toxicity in bone marrow.

Ag Accumulation in Tissues. All tissues revealed significant dose-dependent accumulation of Ag in tissues. Two-fold increase in the female kidneys compared with the male kidneys.

Rosas-Hernández et al. (2009, [625611](#)) Effects of 45-nm silver nanoparticles on coronary endothelial cells and isolated rat aortic rings.

Test Species

Rat coronary endothelial cells (CECs)

Material

Commercial nano-Ag, supplied by Novacentrix, Austin, Texas.

Shape: Irregular spheres

Composition: Not reported

Crystal Structure: Not reported

Average Size: 45 nm (reported by manufacturer);

35.75 ± 13.1 nm (determined using TEM)

Size Distribution: 10–90 nm (determined using TEM)

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 24 hours

Endpoint: Proliferation, nitric oxide production, cytotoxicity, aortic ring vascular tone

Exposure Concentrations: 0, 0.1, 0.5, 1, 5, 10, 50, and 100 µg/mL

Exposure Medium: Dulbecco's modified eagle medium (DMEM) for cultured cells; Krebs-Henseleit (K-H) for rat aortic ring

Cell Density: Not reported

Methods: Proliferation was measured by MTT assay and nitric oxide production was measured using the Griess reaction to determine the ratio of total nitrites to total nitrates. Cytotoxicity was assessed using a cytotoxicity assay kit to detect the level of lactate dehydrogenase (LDH). Aortic ring vascular tone was analyzed by pre-contracting rat aortic vessels with 5 and 100 µg/mL nano-Ag.

Study Outcome

Proliferation. Mitochondrial function was significantly decreased at concentrations of 1, 5, and 10 µg/mL and significantly increased at concentrations of 50 and 100 µg/mL.

Nitric Oxide Production. Concentrations of 10, 50, and 100 µg/mL were associated with significantly increased production of nitric oxide.

Cytotoxicity. LDH activity was significantly increased at concentrations of 1, 5, and 10 µg/mL.

Aortic Ring Vascular Tone. Rat aortic rings were constricted at 5 µg/mL and relaxed at 100 µg/mL.

Samberg et al. (2010, [625612](#)) Evaluation of silver nanoparticle toxicity in vivo and keratinocytes in vitro.

Test Species

Pig, two weanling females (20–30 kg)

Material

Two sizes (reported by manufacturer as 20 and 50 nm in diameter) of commercial unwashed/uncoated and washed/uncoated nano-Ag in deionized water, both supplied by nanoComposix, San Diego, CA. Unwashed and washed nano-Ag synthesized by manufacturer using ammonium hydroxide-catalyzed growth onto 5-nm gold seed particles.

Shape: Not reported

Composition: Not reported

Crystal Structure: Not reported

Average Size: Unwashed: 30.8 ± 0.6 , 47.7 ± 0.5 , and 75.5 ± 1.0 nm (determined using DLS) or 22.4 ± 2.6 , 49.4 ± 6.2 , and 79.2 ± 8.0 nm (determined using TEM).

Washed: 25.5 ± 0.4 , 43.7 ± 1.1 , and 79.9 ± 28.0 nm (determined using DLS) or 21.4 ± 3.1 , 50.0 ± 5.9 , and 77.0 ± 6.0 (determined using TEM).

Size Distribution: Not reported

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: None

Surface Charge: Unwashed: -29.7 mV (~20-nm size), -27.8 mV (~50-nm size), and -33.2 mV (~80-nm size).

Washed: -46.0 mV (~20-nm size), -44.3 mV (~50-nm size), and -43.7 mV (~80-nm size).

Protocol

Exposure Duration: 14 days

Endpoint:s: Morphological alterations

Exposure Concentrations: 0.34 to 34 $\mu\text{g/mL}$

Exposure Route: Daily dermal application

Methods: Pigs were topically dosed once per day for 14 days at 14 sites on back skin with nano-Ag concentrations ranging from 0.34 to 34 $\mu\text{g/mL}$. The Draize system was then used to evaluate the skin for erythema and edema. Microscopic observations were taken on harvested skin samples after pigs were euthanized on Day 14. Samples were analyzed for intercellular and intracellular edema, dermal edema, and inflammation. Results were presented with those from an in vitro test using human epidermal keratinocytes conducted as part of the same study. These results are presented in Appendix C.2.

Study Outcome

Macroscopic observations of porcine skin exposed topically with nano-Ag of all types and sizes revealed no gross erythema or edema. Microscopic observations of the exposed skin samples revealed a concentration-dependent response that was not related to particle size or washing. Effects observed following exposure to the washed ~20-nm nano-Ag solutions were intracellular and intercellular epidermal edema (all doses), focal epidermal and dermal inflammation (mid dose), epidermal hyperplasia (high dose), parakeratosis (high dose), and extension of rete pegs into the superficial papillary layer of the dermis (high dose). Effects observed following exposure to the unwashed ~20-nm nano-Ag solutions were intracellular epidermal edema (all doses), intercellular edema (mid and high dose), and focal areas of intraepidermal infiltrates and superficial papillary dermal inflammation (high dose).

Shrivastava et al. (2009, [200846](#)) Characterization of antiplatelet properties of silver nanoparticles.

Test Species

AKR and PARKES mouse, 50 males (25 from each strain), 7–8 weeks

Material

Nano-Ag synthesized from AgNO₃ using deionized water, NaOH, and NH₃.

Shape: Spherical

Composition: Not reported

Crystal Structure: Face centered cubic

Average Size: 10–15 nm (determined using TEM)

Size Distribution: Monodispersed

Solubility: Not reported

Surface Area: Not reported

Surface Treatment: Not reported

Surface Charge: Not reported

Protocol

Exposure Duration: 10 minutes

Endpoint: Hematology and mortality

Exposure Concentrations: 2, 4, 6, and 8 mg/kg body weight

Exposure Media/Route: Intravenous injection into tail veins

Methods: Mice were divided into 10 groups of 5 animals each. Aggregation was measured in whole blood by electronic impedance. Tail bleeding was monitored at 15-minute increments until no blood was observed on the filter paper.

Study Outcome

Hematology. Intravenous nano-Ag (2–8 mg/kg) inhibited platelet aggregation in whole blood. No adverse effect on bleeding time was observed.

Mortality. Survival was unaffected.

Sung et al. (2008, [195571](#)) Lung function changes in Sprague-Dawley rats after prolonged inhalation exposure to silver nanoparticles.

Test Species

Sprague-Dawley rat, 40 males and females (10 rats per dosing group), 8 weeks old at start of experiment (~253-g males, ~162-g females)

Material

Nano-Ag; particle generation described in Ji et al. (2007, [091301](#)).

Shape: Spherical; non-aggregated/agglomerated forms with diameters under 55 nm (determined using TEM)

Composition: Not Reported

Crystal Structure: Not Reported

Average Size: 18–19 nm (determined using differential mobility analyzer and a condensation particle counter)

Size Distribution: 1.98–64.9 nm

Solubility: Not Reported

Surface Area: 1.08×10^9 , 2.37×10^9 , and 6.61×10^9 nm²/cm³ in the low-, middle-, and high-concentration chambers, respectively

Surface Treatment: Not Reported

Surface Charge: Not Reported

Protocol

Exposure Duration: Subchronic 90-day inhalation

Endpoint: Inflammatory response; pulmonary function changes

Exposure Concentrations: 0.7×10^6 particles/cm³ (low dose), 1.4×10^6 particles /cm³ (middle dose), and 2.9×10^6 particles /cm³ (high dose) for 6 hours/day

Exposure Media/Route: Whole-body inhalation chamber

Methods: The lung function was measured every week after the daily exposure, and the animals were sacrificed after the 90-day exposure period. Cellular differential counts and inflammatory measurements, such as albumin, lactate dehydrogenase (LDH), and total protein, were also monitored in the acellular bronchoalveolar lavage (BAL) fluid of the rats.

Study Outcome

Lung Function. Tidal volume and minute volume showed a statistically significant decrease during the 90 days of nano-Ag exposure.

Inflammatory Response. Although BAL fluid cellular differential counts were not found to be statistically significant, increased inflammation measurements were observed in the high-dose female rats. Dose-dependent increases in lesions related to nano-Ag exposure, such as infiltrate mixed cell and chronic alveolar inflammation, including thickened alveolar walls and small granulomatous lesions.

Sung et al. (2009, [193664](#)) Subchronic inhalation toxicity of silver nanoparticles.

Test Species

Sprague-Dawley rat, 40 males and females (10 rats per dosing group), 8 weeks old at start of experiment (~253-g males, ~162-g females)

Material

Nano-Ag; particle generation described in Ji et al. (2007, [091301](#)).

Shape: Spherical; non-aggregated/agglomerated forms with diameters under 55 nm (determined using TEM).

Composition: Not Reported

Crystal Structure: Not Reported

Average Size: 18–19 nm (determined using differential mobility analyzer and a condensation particle counter)

Size Distribution: 1.98–64.9 nm

Solubility: Not Reported

Surface Area: 1.08×10^9 , 2.37×10^9 , and 6.61×10^9 nm²/cm³ in the low-, middle-, and high-concentration chambers, respectively

Surface Treatment: Not Reported

Surface Charge: Not Reported

Protocol

Exposure Duration: Subchronic 13-week inhalation

Endpoint: Inflammatory response; pulmonary function changes; liver toxicity; and Ag distribution in tissue.

Exposure Concentrations: 0.6×10^6 particles/cm³; 49 µg/m³ (low dose), 1.4×10^6 particles/cm³; 133 µg/m³ (middle dose), and 2.9×10^6 particles/cm³; 515 µg/m³ (high dose) for 6 h/day, 5 days/week

Exposure Media/Route: Whole-body inhalation chamber

Methods: At the end of the study, the rats were subjected to a full necropsy, blood samples were collected for hematology and clinical chemistry tests, and the organ weights were measured.

Study Outcome

NOAEL: 100 µg/m³.

Inhalation Toxicity. Dose-dependent increases in lesions related to nano-Ag exposure, including mixed inflammatory cell infiltrate, chronic alveolar inflammation, and small granulomatous lesions.

Liver Toxicity. Dose-dependent bile duct hyperplasia in liver.

Ag Distribution in Tissue. Statistically significant dose-dependent increases in Ag concentration in lung tissue. Dose-dependent increase in the Ag concentration in the blood. Dose-dependent increase in the liver Ag concentration. Ag concentration in the olfactory bulb was higher than in brain, and increased in a dose dependent manner in both genders ($p < 0.01$). Ag concentrations in the kidneys showed a gender difference, with female kidneys containing 2–3 times more Ag accumulation than in male kidneys.

Takenaka et al. (2001, [019055](#)) Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats.

Test Species

Female Fischer 344 rat, (150–200 g)

Material

Nano-Ag; particles generated by spark discharging through an argon atmosphere.

Shape: Spherical

Composition: Not Reported

Crystal Structure: Not Reported

Average Size: 14.6 nm (determined using differential mobility analyzer)

Size Distribution: Not Reported

Solubility: Not Reported

Surface Area: Not Reported

Surface Treatment: Not Reported

Surface Charge: Not Reported

Protocol

Exposure Duration: Acute 6-hour inhalation; intratracheal instillation

Endpoint: Accumulation in the lungs and brain

Exposure Concentrations: Inhalation mass concentration of 133 $\mu\text{g Ag}/\text{m}^3$ and a particle number concentration of $3 \times 10^6 \text{ cm}^{-3}$ for 6 hours; injected with 150 μL aqueous solution of 7 $\mu\text{g AgNO}_3$ (4.4 $\mu\text{g Ag}$) or 150 μL aqueous suspension of 50 μg elemental Ag.

Exposure Media/Route: Whole-body inhalation chamber; intratracheal instillation

Methods: Rats were sacrificed 0, 1, 4, and 7 days after exposure or intratracheal instillation for morphology and elemental analysis. The ultrastructure of nano-Ag particles was examined using a transmission electron microscope.

Study Outcome

After inhalation exposure, silver accumulation was largest in the lungs, followed by the nasal cavities, in particular the posterior portion, and the lung-associated lymph nodes. Only 4% of the initial body burden in the lungs remained on day 7. Low concentrations of silver were observed in the brain at day 0 and day 1, but no data were collected for the brain on days 4 and 7. Following intratracheal instillation, particles were observed within the alveolar walls, and the rate of clearance was seen to be much slower than following inhalation.

Tang et al. (2008, [195575](#)) Influence of silver nanoparticles on neurons and blood-brain barrier via subcutaneous injection in rats

Test Species

Wistar rat, 90 females (110–130 g), divided into 3 groups (control, nano-Ag, micro-sized Ag)

Material

Commercial nano-Ag, micro-sized Ag; obtained from Sigma-Aldrich (USA).

| | |
|---|---------------------------------|
| Shape: Globular (nano-Ag); irregular cubes (micro-sized Ag) | Solubility: Not Reported |
| Composition: Not Reported | Surface Area: Not Reported |
| Crystal Structure: Not Reported | Surface Treatment: Not Reported |
| Average Size: Not Reported | Surface Charge: Not Reported |
| Size Distribution: 50–100 nm (nano-Ag); 2–20 μm (micro-sized Ag) | |

Protocol

| | |
|---|--|
| Exposure Duration: Single injection | Exposure Concentrations: 1 mL suspension, 62.8 mg/kg |
| Endpoint: Accumulation in the brain and blood-brain barrier effects | Exposure Media/Route: Subcutaneous injection |

Methods: Rat brains were obtained for ultra-structural observation and Ag level detection. Five rats from each group were sacrificed at weeks 2, 4, 8, 12, 18, and 24 to obtain brain tissue. The remaining brain tissue was digested to measure Ag levels.

Study Outcome

Ag levels were significantly higher in the nano-Ag group than in the micro-Ag and control groups. Results show that micro-sized Ag did not traverse into the brain, whereas nano-Ag did and accumulated in brain at least 24 weeks. Nano-Ag can induce neuronal degeneration and necrosis by accumulating in the brain over a long period of time.

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