Environmental Toxicology

Setting a Protective Threshold Value for Silver Toward Freshwater Organisms

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Abstract: Driven by Regulation (EC) No. 1272/2008 and the European Water Framework Directive 2000/60/EC, we have re-evaluated the available chronic freshwater ecotoxicity data for ionic silver (Ag) using strict data quality criteria. In addition, we generated new chronic ecotoxicity data for species potentially sensitive to Ag (the rotifer *Brachionus calyciflorus*, the cyanobacteria *Anabaena flos-aquae*, and the aquatic plant *Lemna minor*) using Ag nitrate as the test substance. The 10% effect concentrations for the most sensitive endpoint per test species were 0.31 µg dissolved Ag/L for *B. calyciflorus* (population size), 0.41 µg dissolved Ag/L for *A. flos-aquae* (growth rate), and 1.40 µg dissolved Ag/L for *L. minor* (root length). We included these values in the set of reliable chronic freshwater data, subsequently covering a total of 12 taxonomic groups and 15 species. Finally, we applied a species sensitivity distribution approach to the data set using various models. The best-fitting model (Rayleigh distribution) resulted in a threshold value protective for 95% of the species of 0.116 µg dissolved Ag/L. This value is considered reliable and conservative in terms of species protection and can be used as a solid basis for setting thresholds for Ag in freshwater after application of an appropriate assessment factor. Furthermore, this value represents reasonable worst-case conditions for bioavailability in European Union surface waters (low hardness and low dissolved organic carbon). *Environ Toxicol Chem* 2021;40:1678–1693. © 2021 European Precious Metals Federation. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Silver (Ag) has long been valued as a precious metal and is used for many applications, including electronics, batteries, catalysts, and jewelry, but also for its natural antimicrobial properties. In its massive form, Ag is not hazardous to the environment. In ionic form, however, Ag (as Ag^{1+}) is known to be a highly potent ecotoxic substance. Low chronic toxicity thresholds have been reported for numerous aquatic organisms belonging to different trophic levels. For example, a chronic 10% effect concentration (EC10) of 0.84 µg dissolved Ag/L was determined for the freshwater clam *Corbicula*

fluminea (based on growth [Diamond et al. 1990]), chronic no-observed-effect concentrations (NOECs) of 0.59 to 4 µg dissolved Ag/L were derived for the crustacean *Hyalella azteca* (based on mortality [Diamond et al. 1990; Rodgers et al. 1997]), and a chronic NOEC of 1.0 µg dissolved Ag/L was derived for the mayfly *Stenonema modestum* (based on molting [Diamond et al. 1992]). Fish also tend to be very sensitive to elevated dissolved Ag concentrations, likely because of the disruption of the physiological ion balance (Wood et al. 1996). As an example, Davies et al. (1998) derived 10% lethal concentration (LC10) values of 0.17 µg dissolved Ag/L for *Oncorhynchus mykiss* and 0.23 µg dissolved Ag/L for *Salmo trutta* in 196- and 217-d exposure tests, respectively. Algae and cyanobacteria have been suggested to be the most sensitive taxonomic groups to Ag toxicity. Mertens et al. (2019) derived an EC10 value of 0.10 µg dissolved Ag/L for the alga *Pseudokirchneriella subcapitata* (based on growth rate). The growth inhibition of algae by Ag is attributed to various mechanisms, such as the interaction of ionic Ag with cell proteins, enzymes, or the photosynthetic apparatus (Hiriart-Baer et al. 2006). For the

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cyanobacterium *Nostoc muscorum*, nominal EC10 values of 0.16 to 0.67 µg Ag/L have been derived (Rai and Raizada 1985, 1987; Rai et al. 1990). The German Umweltbundesambt (2013) suggested a strong toxic activity of Ag against the cyanobacterium *Anabaena flos-aquae*. By exposing this test organism to increasing Ag concentrations, a steep concentration–response behavior was observed, with EC10 and EC50 values (expressed as nominal Ag) of 0.032 and 0.043 µg Ag/L, respectively. However, because analytical verification of the soluble Ag concentration in the Umweltbundesamt (2013) study was lacking, we consider these data unreliable.

Because of its high ecotoxic potential, Ag has been assessed by many authorities worldwide over the last decades. For instance, the US Environmental Protection Agency concluded in 1980 that chronic Ag toxicity in freshwater environments may occur from 0.12 µg Ag/L (*O. mykiss* [Davies et al. 1978]), but no environmental threshold concentration had been derived for chronic effects (as opposed to acute effects). In Australia, a freshwater trigger value of 0.05 µg Ag/L was calculated using a species sensitivity distribution (SSD) approach with a 95% species protection level (Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand 2000). Seven species were considered, and the trigger value was below the lowest NOEC (0.07 µg Ag/L for *O. mykiss*) of the underlying data set. More recently, the long-term Canadian Water Quality Guideline for the protection of aquatic life was set at 0.25 µg Ag/L using an SSD approach (Canadian Council of Ministers of the Environment 2015). Nine taxonomic groups were considered, with toxic levels varying between 0.24 µg Ag/L for *O. mykiss* (Davies et al. 1978) and 23 µg Ag/L for *Micropterus salmoides* (Coleman and Cearley 1974). In The Netherlands, the National Institute for Public Health and the Environment (2012) has derived an environmental risk limit for Ag for potential implementation under the European Union Water Framework Directive, and the proposed environmental quality standard (EQS) for freshwater was 0.01 µg Ag/L. This value was derived by taking the lowest chronic threshold value of 0.1 µg nominal Ag/L for *O. mykiss* (Brauner et al. 2003) and applying an assessment factor of 10. This approach can be considered scientifically less robust because it did not make use of the full knowledge of the interspecies variation in toxicity, as provided by the availability of data for 13 species (of which only 9 were retained after data quality screening by the Dutch authorities) from 6 different taxonomic groups. This approach could result in a less scientifically sound EQS compared with the SSD approach (Wheeler et al. 2002). Also, the quality of the Brauner et al. (2003) study, which is the driver behind that proposed EQS, is questionable. Indeed, high control mortality of 40% after 58 h of exposure was noted in this experiment, which, according to the authors, could be related to the suboptimal chloride concentration in the test medium. In addition, the study is not in line with current standards because only 2 Ag concentrations (0.1 and 1 µg nominal Ag/L) were tested with a spacing factor of 10 exceeding today's Organisation for Economic Cooperation and Development (OECD) recommendation of 3.2, and the threshold value is expressed as nominal Ag.

The variability of the silver EQS values in different countries is further demonstrated in Table 1. The main reasons behind this variability are differences in the underlying ecotoxicity database, differences in the approach used (deterministic or SSD), and the assessment factors applied.

Considering all these initiatives for setting safe ecological thresholds for Ag and the differences between them, as well as the multitude of publications on the chronic freshwater toxicity of Ag that becomes available on a yearly basis, it is recommended to update the database of chronic freshwater toxicity studies on a regular basis. Identified reliable studies should be reviewed and the threshold value reassessed so that it remains protective for the freshwater environment while ensuring that it is being derived according to the currently recognized practices for metal risk assessments (Metals Environmental Risk Assessment Guidance 2016; European Commission 2018).

Of particular importance is the consideration of factors affecting bioavailability and toxicity. As has been demonstrated for other metals, for Ag it is the ionic form, Ag^{1+}, which is of main interest as the driver behind the environmental effects (Hogstrand and Wood 1998). This concept is the basis behind the development of the free ion activity model (Morel 1983) and the biotic ligand model (BLM). Consequently, the toxic effects of metals (including Ag) might change with water chemistry. For Ag, it has been shown that acute toxicity to aquatic organisms changes with varying ionic composition of the test media,

TABLE 1: List of available chronic freshwater threshold values for silver

Country	Ag chronic freshwater threshold value (µg/L)	Approach	Reference
The Netherlands	0.01	Deterministic	National Institute for Public Health and the Environment (2012)
Denmark	0.017	SSD	Vorkamp and Sanderson (2016)
Germany	0.02	SSD	Vorkamp and Sanderson (2016)
Australia	0.05	SSD	Australian and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand (2000)
Belgium (Flanders)	0.08	SSD	Vorkamp and Sanderson (2016)
Austria	0.1	SSD	Vorkamp and Sanderson (2016)
Canada	0.25	SSD	Canadian Council of Ministers of the Environment (2015)
Czech Republic	3.5	Deterministic	Vorkamp and Sanderson (2016)

SSD = species sensitivity distribution.

including dissolved organic carbon (DOC), chloride, and calcium. Silver is less toxic to fish and crustaceans under conditions of increasing water pH, hardness, sulfide concentration, and DOC and particulate organic carbon concentrations (Galvez and Wood 1997; Erickson et al. 1998; Bury et al. 1999; Karen et al. 1999; Van Genderen et al. 2003). These concepts have been reflected in the BLM developed for acute toxicity of Ag in freshwater environments (Paquin and Di Toro 2008). The effects of water chemistry on chronic Ag toxicity are still not entirely clear, but there are indications that increasing hardness, DOC, and sulfide concentration can mitigate chronic Ag toxicity (Bianchini and Wood 2008; Naddy et al. 2007a). There are no reliable data on the effect of pH on chronic Ag toxicity.

The present study has been conducted in support of Regulation (EC) No. 1272/2008 (Registration, Evaluation, Authorisation and Restriction of Chemicals [REACH] [European Commission 2008]). The REACH regulation requires assessment of the environmental risks of chemicals put on the European Union market. This includes comparison of the expected exposure level with a critical threshold level such as a predicted-no-effect concentration (PNEC). In cases where sufficient reliable experimental data are available, the PNEC may be estimated as a lower 5th percentile of an SSD, corrected with an assessment factor of 1 to 5 (European Chemicals Agency 2008). The SSD approach is being increasingly used in ecological risk assessment and the derivation of EQSs for metals (European Commission 2018). This is largely driven by the multitude of experimental data available for many metals like zinc, copper, cobalt, lead, and nickel but also because SSD-based values are community-based thresholds and are scientifically more robust than deterministic assessments that rely solely on the most sensitive species (Wheeler et al. 2002).

For the registrations of Ag and Ag compounds under REACH, a chronic freshwater threshold value of 0.04 µg dissolved Ag/L had been derived in 2013 using an SSD approach, similar to the PNEC derived by the Environment Agency (2010) of England and Wales. We have reassessed the chronic freshwater threshold value based on more robust data quality criteria and using an SSD approach. Further, we included in the SSD the recent literature on chronic freshwater toxicity of Ag as well as the results of additional chronic freshwater toxicity tests we performed with species potentially sensitive to Ag (*Lemna minor* [higher plant], *Brachionus calyciflorus* [rotifer], and *A. flos-aquae* [cyanobacterium]).

Although assessing the freshwater toxicity of nanosilver was outside the scope of the present study, Mertens et al. (2019) have experimentally confirmed that the aquatic effects of Ag, measured with Ag nitrate as the test compound, are a conservative estimate for the effects caused by nanosilver.

MATERIALS AND METHODS

Review of available chronic freshwater toxicity data

We performed an extensive review of the available literature on the chronic freshwater toxicity of Ag and selected reliable data for an SSD. The quality criteria used for selection of chronic freshwater toxicity values were in line with available guidances from REACH and the European Water Framework Directive 2000/60/EC and with the criteria for reporting and evaluating ecotoxicity data (Moermond et al. 2016). Evaluation criteria for the reliability of the toxicity studies were as follows: 1) only chronic toxicity values based on measured dissolved Ag concentrations (mostly filtered over a 0.45-µm filter) and using an ionic Ag test substance (usually Ag nitrate) were selected; 2) considering the strong influence of water physicochemistry on metal toxicity, the test conditions that could influence the bioavailability and toxicity (pH, hardness, DOC, chloride) of Ag should be adequately described and should be within the tolerance limits of the test organisms as indicated in the corresponding test guidelines; and 3) concentration–response modeling (e.g., regression methods) were preferred over hypothesis-testing methods (NOEC values), with the use of EC10 (i.e., the modeled concentration causing a 10% decrease in response) as the preferred endpoint for deriving safe thresholds.

We also (re)applied evaluation criteria for the relevance of chronic toxicity. Relevance covers the extent to which a test is appropriate for a particular risk assessment and the evaluation of the choice of test species, the test duration, and the test substance used. Regarding test species, we have considered all freshwater species for which Ag toxicity data are available including species not usually tested in standardized test procedures. Regarding test duration, a relevant chronic test duration is a function of the life cycle of the test organism. Recommendations from standard ecotoxicity protocols were followed. In the present study, chronic toxicity tests were generally defined as >4 d for all invertebrates and fish. It is noted, though, that whether or not an effect concentration is considered chronic is not determined exclusively by the exposure duration limit of 4 d. For unicellular algae but also for specific invertebrates (e.g., rotifers), an exposure time of <4 d usually already covers one or more generations. Thus, for these organisms, chronic effect concentrations may be derived from experiments of <4 d. For algae, the minimum required exposure time is 48 h (Organisation for Economic Co-operation and Development 2011). The relevance of specific exposure durations for the estimation of chronic effects for organisms with relatively long life cycles (e.g., fish) was evaluated on a case-by-case basis (e.g., by considering the use of sensitive life stages in the test).

To further develop the SSD for Ag and to resolve uncertainties related to some of the existing toxicity data, we investigated the chronic toxicity of ionic Ag (tested as Ag nitrate) to 3 additional and potentially sensitive freshwater species: *L. minor* (higher plant), *B. calyciflorus* (rotifer), and *A. flos-aquae* (cyanobacterium).

Ecotoxicity assays

The ionic Ag test substance used was Ag nitrate (AgNO₃; Heraeus, 63.49% Ag, purity >99.9%). All experiments were

conducted following standard protocols, unless explained otherwise. The strong metal complexing organic ligand ethylenediaminetetraacetic acid (EDTA) was omitted from all test media and was replaced by 1 mg/L DOC (from a natural origin: Suwannee River standard natural organic matter; ID 2R101N). The presence of natural DOC in the test medium results in a more realistic representation of a natural medium, in comparison with an EDTA-containing medium. In addition, the presence of some natural DOC in test media at test initiation in static and semistatic tests "buffers" metal speciation; that is, it reduces relative changes in free ionic metal concentrations during the exposure (resulting in a more constant free ionic metal concentration). This is because the excretion of DOC by test organisms results in a smaller relative change in DOC concentration during the exposure compared to a medium without added natural DOC (Van Regenmortel et al. 2017; Mebane et al. 2020). The low added concentration of DOC is also ecologically more relevant than the complete absence of DOC and still ensures test acceptability for regulatory purposes according to OECD test guidelines. For each species, a range-finding and definitive test were performed.

All experiments and the storage of spiked solutions were conducted in polypropylene or polycarbonate test vessels to minimize adsorption of Ag to the walls of the test vessels (Sekine et al. 2015). Spiked solutions were left to equilibrate in total darkness in a 24 °C incubation chamber 24 h before use.

Both DOC and dissolved inorganic carbon were measured in all assays with a total organic carbon analyzer following the nonpurgeable organic carbon (NPOC) method (TOC-5000; Shimadzu; limit of quantification 0.7 mg DOC/L, detection limit 0.2 mg DOC/L). The NPOC method entails that after purging the sample with air (to remove inorganic carbon), the remaining organic carbon is measured. Chloride and sulfate samples were measured by spectrophotometry (Aquamate; Thermo Electron; chloride from Merck; Spectroquant 1.14897.001; sulfate from Merck, Spectroquant 1.14548.001). Major cations (Ca, Mg, Na, and K) were measured using inductively coupled plasma optical emission spectrometry (ICP-OES; ICAP 7200 DUA; Thermo Fisher Scientific); pH was measured with a Microcomputer Solution Analyzer (CONSORT), and temperature and oxygen were both measured with the Oxi 3210 Portable Dissolved Oxygen Meter (WTW Profiline).

L. minor *growth inhibition test*

The *L. minor* 7-d growth inhibition test was conducted according to OECD test guideline 221 (2006). A modified Swedish Institute for Standards *Lemna* growth medium was used for the preculture and ecotoxicity testing. Key characteristics of the medium are shown in Table 2. The pH buffer 3-(*N*-morpholino) propanesulfonic acid (MOPS) was omitted because its presence precludes DOC analyses, an important parameter in metal toxicity determination. For the reasons explained in the previous section, EDTA was replaced with 1 mg DOC/L. The aquatic freshwater plant *L. minor* originated from an in-house culture which was initiated in 2015 from plants

purchased at Blades Biological. Plants were acclimated for 2 wk prior to test initiation in the modified growth medium at 24 °C.

Test concentrations were determined after a range-finding experiment (details not shown). For the definitive experiment, nominal concentrations were control medium and 0.32, 1, 3.2, 10, 32, 100, 320, and 1000 µg Ag/L. Each test concentration was assayed in 3 replicates and the control in 6 replicates. The assays were conducted in acid-washed food-grade polypropylene vessels of 300 mL filled with 100 mL of the test medium and covered with transparent punctured polypropylene covers. The pH of the fresh test medium was 7.1 ± 0.1, and no pH manipulation of the media was needed throughout the test.

At test initiation, each test unit received 4 plants, each with 3 fronds (i.e., 12 fronds per vessel). The toxicity test was incubated in a 24 °C growth chamber under continuous light (99–112 µmol/m^2/s). The test was semistatic, with complete test medium renewals on days 3 and 5. Growth of *L. minor* was monitored during the exposure period as number of fronds and total frond area. Total frond area was determined based on image analysis using a Nikon D5300 camera under strong backlighting and linking the pixel density of *L. minor* leaves to the actual frond area using the photo software Image-J. At test termination, the root length was measured using a slide caliper (VWR) on 10 randomly selected plants per test vessel, and the dry weight (after 3 d drying at 55 °C) of all plant colonies (fronds and roots) was measured using a Mettler Toledo balance (AX 105). Growth rate was expressed on the basis of the total frond numbers and total frond area. Growth rate was calculated based on the slope of the relation between $\ln(X_{ti})$ and t_i, where X_{ti} is the number of fronds or total frond area (in square millimeters) at time t_i and t_i is the time since the test initiation.

Samples for analysis of total and dissolved Ag (filtered over a 0.45-µm filter; Acrodisc; Pall Life Science) in fresh media (samples taken before medium renewal) were taken on days 0, 3, and 5 and in old media (samples taken after medium renewal) on days 3, 5, and 7. Samples for Ag and major cation (Ca, Mg, Na, and K) analysis were acidified with 1% HNO_3 and 1% HCl (both Normatom quality; VWR Prolabo). Samples with nominal Ag concentrations <10 µg Ag/L were measured using inductively coupled plasma mass spectrometry (ICP-MS;

TABLE 2: Selected properties of ecotoxicity test media[a]

	Lemna minor test	*Brachionus calyciflorus* test	*Anabaena flos-aquae* test
Temperature (°C)	24.7 ± 0.9	24.9 ± 0.2	22.8 ± 0.6
pH	7.4 ± 0.25	7.9 ± 0.1	7.8 ± 0.2
Na (mg/L)	34.5 ± 2.3	25.4 ± 0.6	12.8 ± 0.9
Mg (mg/L)	3.8 ± 0.3	5.2 ± 0.1	2.9 ± 0.1
K (mg/L)	8.8 ± 0.3	2.1 ± 0.2	0.5 ± 0.2
Ca (mg/L)	10.4 ± 0.5	10.6 ± 0.4	4.7 ± 0.2
Cl (mg/L)	21.9 ± 5.9	2.1 ± 0.2	21.8 ± 0.8
SO_4^{2-} (mg/L)	27.9 ± 7.1	53.9 ± 2.0	6.6 ± 1.1
IC (mg/L)	4.8 ± 0.1	15.9 ± 0.3	6.3 ± 0.2
DOC (mg/L)	1.6 ± 0.2	1.6 ± 0.7	1.8 ± 0.1
Oxygen content	85 ± 4%	85 ± 4%	9.4 ± 0.5 mg/L

[a]Average measured values ± standard deviation are reported.
IC = inorganic carbon; DOC = dissolved organic carbon.

Nexion 350 D; PerkinElmer; detection limit 0.035 µg Ag/L, limit of quantification 0.12 µg Ag/L), whereas Ag in samples of nominal Ag concentrations >10 µg Ag/L was measured using ICP-OES (ICAP 7200 DUA; Thermo Fisher Scientific; detection limit 1.1 µg Ag/L, limit of quantification 3.7 µg Ag/L).

B. calyciflorus *reproduction test*

The *B. calyciflorus* 48-h reproduction test (Snell and Moffat 1992) was conducted following the guidelines included in the Rotoxkit *F* test (MicroBioTests 2018), which follows procedure 8420 of the American Public Health Association (2017) methods. The 48-h test period covers approximately 3 full generations of this rotifer and can therefore be considered a true chronic test. Toxicity tests were performed in moderately hard US Environmental Protection Agency (USEPA) reconstituted water with addition of 1 mg DOC/L (for reasons previously explained). Key characteristics of the medium are shown in Table 2. The *B. calyciflorus* cysts were provided in the Rotoxkit. Prior to test initiation, the cysts were hatched in 10 mL of medium hard USEPA reconstituted water at 25 °C on a light box. After hatching, rotifers were fed with RotiRich food 2 h before test initiation.

Test concentrations were determined after a range-finding experiment (details not shown). For the definitive experiment, nominal concentrations were control and 5, 10, 20, 40, 80, 160, and 320 µg Ag/L. Each test concentration and the control was assayed in 16 replicates. The assays were conducted in acid-washed polycarbonate 54-multiwell plates provided in the Rotoxkit. The pH of the fresh test media was 7.8 ± 0.1, and no pH manipulation was needed throughout the test. Before dividing the test media over the exposure wells, 2×10^6 *P. subcapitata* (delivered in the Rotoxkit as algal beads) cells/mL were added to the exposure media as a food source for the rotifers during the toxicity test. Each exposure well received 1 mL of the appropriate algae-containing exposure medium.

At test initiation, one rotifer (<2 h old) was placed in each well, and the plates were sealed with Parafilm and placed in a dark 25 °C growth cabinet. Two endpoints were used for determining the ecotoxicity of dissolved Ag to *B. calyciflorus*: population size and population growth rate. Population size (n) is the number of live rotifers after 48-h exposure (counted with the aid of a dissection microscope, within 30 min after test ending). The population growth rate was calculated based on the slope of the relation between $\ln(X_{ti})$ and t_i, where X_{ti} is the number of live rotifers after 48-h exposure at time t_i and t_i is the time since the test initiation.

Samples for analysis of total and dissolved Ag (filtered over a 0.45-µm filter; Acrodisc; Pall Life Science) of fresh media were taken for all test concentrations at test initiation (both before and after addition of *P. subcapitata* to the exposure medium), after 24-h exposure, and at test termination (48 h). To have an adequate volume of exposure medium for sampling, the test was set up in quadruplicate. Samples for Ag and major cation (Ca, Mg, Na, and K) analysis were acidified with 1% HNO_3 and 1% HCl (both Normatom quality; VWR Prolabo). All Ag

concentrations were in the first instance measured using ICP-OES (ICAP 7200 DUA; Thermo Fisher Scientific; detection limit 1.1 µg Ag/L, limit of quantification 3.7 µg Ag/L). However, the samples for which measured dissolved Ag concentrations were below the limit of quantification were later also measured using ICP-MS (Nexion 350 D; PerkinElmer; detection limit 0.012 µg Ag/L, limit of quantification 0.04 µg Ag/L).

A. flos-aquae *growth inhibition test*

The *A. flos-aquae* 72-h growth inhibition test was conducted according to OECD test guideline 201 (2011) in modified OECD medium. For the reasons explained in the previous section (see *L. minor growth inhibition test*), the pH buffer MOPS was omitted, and EDTA was replaced with 1 mg DOC/L. Key characteristics of the medium are shown in Table 2. The *A. flos-aquae* strain UTEX 1444 was purchased from the Culture Collection of Algae at the University of Texas (Austin). On arrival, the cyanobacteria culture was transferred to BG11 medium (Culture Collection of Algae at the University of Texas 2009) and incubated at 21 °C under continuous light. The inoculum culture was prepared by transferring an aliquot of the *A. flos-aquae* stock culture kept in BG11 medium to the modified OECD medium and incubated under the same conditions as the actual growth inhibition test. Before test initiation, the cell density of the inoculum culture was determined using a Sedge-Wick Rafter counting chamber and a light microscope. To enable the calculation of cell density from fluorescence measurements in the growth inhibition test, a calibration curve was established based on the relationship between fluorescence intensity and cell density (details not shown). Fluorescence intensity was measured using a TECAN Infinite M200 multiwell reader. An excitation wavelength of 590 nm and an emission wavelength of 683 nm were selected based on Simis et al. (2012). This excitation wavelength in the orange-red spectrum showed the highest correlation between fluorescence and cyanobacteria cell densities and targets specifically the phycobilipigments which are the most important light-harvesting pigments in cyanobacteria. The "optimal gain function" (optimal gain 191) was used for determination of fluorescence intensity in the *A. flos-aquae* exposures.

Test concentrations were determined after a range-finding experiment (details not shown). For the main experiment, nominal concentrations were control and 0.22, 0.46, 1.0, 2.2, 4.6, 10, and 22 µg Ag/L. Each test concentration was assayed in 3 replicates and the control in 6 replicates. The assays were conducted in acid-washed 125-mL polycarbonate Erlenmeyer flasks (Corning) filled with 60 mL of the test solutions. The pH of the fresh test media was 7.8 ± 0.1, and no pH manipulation was needed throughout the test.

At test initiation, each test vessel was inoculated with 10^4 cells/mL (=cell density N_0 at t_0); these were the "algae exposures." In addition to the algae exposures, one chemistry replicate per test concentration was used, which received the same treatment during the toxicity test (test conditions,

manipulation, and sampling) as the algae exposures; but these chemistry replicates were not inoculated with algae. These chemistry replicates allowed monitoring of the Ag chemistry in the absence of algae and served as background conditions during the fluorescence intensity measurements.

All test vessels were incubated at 24 °C under continuous light (24 h light, 45 μmol/m^2/s) and were manually shaken 3 times per day. Cell densities were determined daily (N_1, N_2, and N_3 after 24 [t_1], 48 [t_2], and 72 [t_3] h) based on fluorescence intensity measurements. Growth rate (microns per day) was determined in each replicate of each treatment as the slope of the linear regression of the natural logarithm of cell density versus time.

Samples for analysis of total and dissolved Ag (filtered over a 0.45-μm filter; Acrodisc; Pall Life Science) were taken at test initiation (before inoculation with *A. flos-aquae*) and after 1, 24, 48, and 72 h in both the algae exposures and the chemistry controls. Samples for Ag and major cation (Ca, Mg, Na, and K) analysis were acidified with 1% HNO_3 and 1% HCl (both Normatom quality; VWR Prolabo). All Ag samples were measured using ICP-MS (Nexion 350 D; PerkinElmer; detection limit 0.008 μg Ag/L, limit of quantification 0.029 μg Ag/L).

Statistical analysis

Effect concentrations (NOEC, lowest-observed-effect concentration [LOEC], and EC*x*) for all species were calculated based on measured dissolved Ag concentrations. All effect concentrations were calculated based on relative responses (expressed relative to the mean control response of the respective experiment). The EC10, EC20, and EC50 values and corresponding confidence intervals were determined based on a log-logistic concentration response model with 2 parameters using Statistica software for the *L. minor* and *B. calyciflorus* data:

$$y = \frac{100}{\left(1 + \left(\frac{x}{a}\right)^{\frac{\ln\left(\frac{1}{9}\right)}{b-a}}\right)}$$

In this equation, *y* is the predicted relative response (expressed relative to the average of the controls; percentage), *x* is the measured dissolved Ag concentration (micrograms per liter), *a* is the natural logarithm of the EC50 (micrograms per liter), and *b* is the natural logarithm of the EC10 (micrograms per liter).

For the *A. flos-aquae* data, the EC10, EC20, and EC50 values were calculated based on the 2-parameter Weibull concentration–response model using the "drc" package in R, Ver 3.3.2 (R Development Core Team 2016):

$$y = 100 \times \exp\left(-\frac{x}{k}\right)^b$$

In this equation, *y* is the predicted reproduction (number of offspring per female), *b* is the slope parameter, *k* is the scale parameter, and *x* is the dissolved Ag concentration (micrograms per liter).

The NOECs and LOECs were calculated with the Williams (1971) test, after evaluation of the data for adherence to the underlying assumptions of normality and homogeneity of variances. For the *B. calyciflorus* data, which did not fulfill the assumptions of the parametric Williams test, NOEC and LOECs were calculated with the nonparametric Jonckheere-Terpstra test (Jonckheere 1954).

Derivation of the updated Ag toxicity threshold value

The newly generated data from the ecotoxicity assays were added to the available chronic freshwater toxicity data set. Where more than one endpoint from a test was generated, the most sensitive endpoint was selected for inclusion in the SSD. In the absence of a chronic BLM for Ag, in case toxicity values at different hardness conditions were reported for the same species, only the EC10 from the test at the lowest hardness (potentially representing the highest bioavailability and thus highest toxicity) was retained for Ag threshold derivation purposes. Nine parametric distributions (normal, log-normal, exponential, logistic, Cauchy, Weibull, Rayleigh, Gumbel, and gamma) were fitted to the selected log-transformed toxicity values. The best-fitting distribution was determined based on the Anderson-Darling goodness-of-fit statistic because it puts more emphasis on the tails of the SSDs, which are the regions of interest in the effects assessment (Stephens 1982; Gan et al. 1991). The sampling uncertainty, and therefore calculations of the hazardous concentrations for 5% of the species (HC5-5, HC5-50, and HC5-95), was taken into account using parametric bootstrap simulation of the EC10 values with replacement (Davison and Hinkley 1997). In addition, the HC5-50 and confidence limits were calculated from the "conventional" normal distribution (of log-transformed geometric mean EC10 values) using the ETx 2.0 software, as developed by Van Vlaardingen et al. (2004). This freely available software program calculates a (log) normal distribution through toxicity data entered by the user. This software also includes the Anderson-Darling test for goodness of fit on log-normality, which was evaluated at the 5% significance level. The median hazardous concentration for 5% of the species (HC5-50) is further used for setting environmental effects threshold concentrations of Ag.

The corresponding HC5-50 values are usually derived using well-studied distributions, that is, the normal (Aldenberg and Jaworska 2000) and logistic (Aldenberg and Slob 1993) constructed on the log-transformed toxicity data. However, from the point of toxicity, there is no theoretical justication for any distribution to be the more fundamental to the subject. Therefore, the use of best-fitting distributions, particularly to the tails of the distributions, which is the region of interest for derivation of HC5-50, was also included in this exercise.

RESULTS

Review of ecotoxicity data

An overview of the chronic toxicity values fulfilling the data quality selection criteria is provided in Table 3. The data set

TABLE 3: Overview of the species and toxicity values selected for predicted-no-effect concentration derivation

Species	Exposure duration	Endpoint	pH	Hardness (mg CaCO$_3$/L)	DOC (mg/L)	EC10/NOEC	Value (µg/L)	Test water	Reference
Ceriodaphnia dubia	7 d	Mortality	7.8–8.4	88	4.74–5.11	LC10	10.4	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	7 d	Mortality	7.8–8.4	88	4.76	LC10	14.8	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	7 d	Mortality	7.8–8.4	88	4.76	LC10	10.9	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	7 d	Mortality	7.8–8.4	88	4.76	LC10	11.2	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	10 d	Mortality	7.5–8.5	68–70	1.0[a]	NOEC	0.53	Pond water (with low SS)	Rodgers et al. (1997)
Ceriodaphnia dubia	6–8 d	Reproduction	7.4–7.8	80–100	<2.0[c]	EC10	1.14	Artificial medium	Kolts et al. (2009)
Ceriodaphnia dubia	7 d	Reproduction	7.8–8.4	88	4.74–5.11	EC10	9.5	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	7 d	Reproduction	7.8–8.4	88	4.76	EC10	10.1	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	7 d	Reproduction	7.8–8.4	88	4.76	EC10	10.3	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	7 d	Reproduction	7.8–8.4	88	4.76	NOEC	11.5	Lake water	Naddy et al. (2007a)
Ceriodaphnia dubia	10 d	Reproduction	7.5–8.5	68–70	1.0[a]	NOEC	0.53	Pond water (with low SS)	Rodgers et al. (1997)
Chironomus tentans	10 d	Growth	7.4	52.1	<2.0[c]	EC10	12.54	Tap water (filtered)	Call et al. (1999)
Chironomus tentans	10 d	Mortality	6.9–7.5	10–15	1.0[a]	NOEC	125.0	Pond water (with low SS)	Rodgers et al. (1997)
Corbicula fluminea	21 d	Growth	7.8	34.8	2.0[b]	EC10	0.84	River water	Diamond et al. (1990)
Corbicula fluminea	21 d	Mortality	7.8	34.8	2.0[b]	NOEC	4.4	River water	Diamond et al. (1990)
Daphnia magna	21 d	Growth	7.9–8.6	160	4.52	NOEC	2.37	Lake water	Naddy et al. (2007a)
Daphnia magna	21 d	Growth	8.16	115	4.8	EC10	2.7	Artificial medium	Bianchini and Wood (2008)
Daphnia magna	21 d	Growth	8.16	460	4.8	EC10	3.6	Artificial medium	Bianchini and Wood (2008)
Daphnia magna	21 d	Growth	7.9–9.4	220	0.23	EC10	6.4	Artificial medium	Mertens et al. (2019)
Daphnia magna	21 d	Intrinsic rate	7.9–9.4	220	0.23	EC10	5.0	Artificial medium	Mertens et al. (2019)
Daphnia magna	21 d	Mortality	7.9–8.6	160	4.52	NOEC	2.37	Lake water	Naddy et al. (2007a)
Daphnia magna	21 d	Mortality	7.9–9.4	220	0.23	EC10	3.97	Artificial medium	Mertens et al. (2019)
Daphnia magna	21 d	Reproduction	7.9–8.6	160	4.52	EC10	3.5	Lake water	Naddy et al. (2007a)
Daphnia magna	21 d	Reproduction	8.16	115	4.8	EC10	2.9	Artificial medium	Bianchini and Wood (2008)
Daphnia magna	21 d	Reproduction	8.16	460	4.8	EC10	3.1	Artificial medium	Bianchini and Wood (2008)
Daphnia magna	10 d	Reproduction	7.5–8.5	68–70	1.0[a]	NOEC	0.8	Pond water (with low SS)	Rodgers et al. (1997)
Daphnia magna	21 d	Reproduction	7.9–9.4	220	0.23	EC10	3.5	Artificial medium	Mertens et al. (2019)
Hyalella azteca	21 d	Mortality	7.8	34.8	2.0[b]	NOEC	0.59	River water	Diamond et al. (1990)
Hyalella azteca	10 d	Mortality	6.9–7.5	10–15	1.0[a]	NOEC	4.0	Pond water (with low SS)	Rodgers et al. (1997)
Isonychia bicolor	14 d	Molting	7.8	34.8	2.0[b]	NOEC	0.16	River water	Diamond et al. (1990)
Isonychia bicolor	14 d	Mortality	7.8	34.8	2.0[b]	NOEC	1.67	River water	Diamond et al. (1990)
Lymnaea stagnalis	14 d	Growth rate (weight)	7.8	116	0.76	EC10	1.48	Tap water	Cremazy et al. (2018)
Oncorhynchus mykiss	73 d	Hatching	7.3–8.0	30–36	2.3–3.0	NOEC	>1.25	Pond water (filtered)	Dethloff et al. (2007)
Oncorhynchus mykiss	77 d	Hatching	7.3–8.1	30–34	2.3–3.3	NOEC	>2.26	Pond water (filtered)	Dethloff et al. (2007)
Oncorhynchus mykiss	28 wk	Mortality	6.93	24.7	<0.4	LC10	0.17	Well water (low H)	Davies et al. (1998)
Oncorhynchus mykiss	28 wk	Mortality	7.53	195	0.8	LC10	0.44	Well water (medium hardness)	Davies et al. (1998)
Oncorhynchus mykiss	28 wk	Mortality	7.62	200	1.2	LC10	0.96		Davies et al. (1998)

(Continued)

TABLE 3: (Continued)

Species	Exposure duration	Endpoint	pH	Hardness (mg CaCO₃/L)	DOC (mg/L)	EC10/ NOEC	Value (µg/L)	Test water	Reference
Oncorhynchus mykiss	77 d	Mortality	7.3–8.1	30–34	2.3–3.3	LC10	1.24	Pond water (filtered)	Dethloff et al. (2007)
Oncorhynchus mykiss	77 d	Weight	7.3–8.1	30–34	2.3–3.3	EC10	0.89	Pond water (filtered)	Dethloff et al. (2007)
Pimephales promelas	32–34 d	Weight	7.3–8.2	30.5	2.4	NOEC	0.351	Lake water (filtered)	Naddy et al. (2007b)
Pimephales promelas	32–34 d	Weight	7.3–8.2	30.5	2.4	EC10	0.32	Lake water (filtered)	Naddy et al. (2007b)
Pimephales promelas	32–34 d	Weight	7.3–8.2	30.5	2.4	EC10	0.86	Lake water (filtered)	Naddy et al. (2007b)
Pimephales promelas	32–34 d	Hatching	7.3–8.2	30.5	2.4	EC10	0.38	Lake water (filtered)	Naddy et al. (2007b)
Pimephales promelas	32–34 d	Mortality	7.3–8.2	30.5	2.4	NOEC	0.351	Lake water (filtered)	Naddy et al. (2007b)
Pimephales promelas	32–34 d	Mortality	7.3–8.2	30.5	2.4	NOEC	0.27	Lake water (filtered)	Naddy et al. (2007b)
Pimephales promelas	32–34 d	Mortality	7.3–8.2	30.5	2.4	NOEC	1.0	Lake water (filtered)	Naddy et al. (2007b)
Pseudokirchneriella subcapitata	72 h	Growth rate	7.2–8.5	10.0	0.38–0.88	EC10	0.1	Artificial medium	Mertens et al. (2019)
Pseudokirchneriella subcapitata	72 h	Yield	7.2–8.5	10.0	0.38–0.88	EC10	0.1	Artificial medium	Mertens et al. (2019)
Salmo trutta	31 wk	Mortality	6.88	27.9	0.8	LC10	0.23	Well water (low hardness)	Davies et al. (1998)
Salmo trutta	31 wk	Mortality	7.53	200.0	0.9	LC10	0.8	Well water (medium hardness)	Davies et al. (1998)
Salmo trutta	31 wk	Mortality	7.56	460.0	1.0	LC10	1.39	Well water (high hardness)	Davies et al. (1998)
Stenonema modestum	14 d	Molting	7.7	48.5	<2.0[c]	NOEC	1.0	Tap water (filtered)	Diamond et al. (1992)
Stenonema modestum	14 d	Mortality	7.7	48.5	<2.0[c]	LC10	4.1	Tap water (filtered)	Diamond et al. (1992)

[a] The DOC of University of Mississippi Biological Field Station pond water is reported to be 1.0 mg/L (European Copper Institute 2008).
[b] The DOC of New River water was provided by Don Cherry (Virginia Polytechnic Institute and State University, Blacksburg, VA) and analyzed by Environmental Health Laboratories. Values presented are from a grab sample collected in August 2000 (US Environmental Protection Agency 2007).
[c] The DOC of artificial water and tap water is assumed to be <2.0 mg/L.
DOC = dissolved organic carbon; EC10 = 10% effect concentration; LC10 = 10% lethal concentration; NOEC = no-observed-effect concentration; SS = suspended solids.

consists of 12 different species covering 9 different taxonomic groups.

For further assessment, only studies using a soluble Ag salt as test substance (being $AgNO_3$ in all studies) and reporting filtered (<0.45 μm) Ag concentrations have been selected (because monitoring data are also reported as 0.45 μm–filtered concentrations). As such, several studies did not meet these criteria and were not considered further, for example, Schäfers and Weil (2013), Nebeker et al. (1983), Kolkmeier and Brooks (2013), Ribeiro et al. (2014), Nebeker (1982), Davies et al. (1978), Holcombe et al. (1983), and Taylor et al. (2016). These studies reported Ag not as dissolved Ag (i.e., filtered <0.45 μm) but as dissolved Ag after centrifugation or ultrafiltration, total Ag, or free ionic Ag or used nanosilver as a test substance.

Ecotoxicity assays

L. minor growth inhibition test.
Total Ag concentrations in fresh medium were 0 to 30% lower than nominal concentrations. Losses of Ag in the fresh solutions are most likely due to adsorption of Ag to storage vessels despite the use of polypropylene vessels (Sekine et al. 2015). At the highest Ag dose level (1000 μg nominal Ag/L solution), only 54% of nominal Ag was recovered in the total fraction, suggesting that Ag solubility may have been exceeded in this solution and that Ag precipitation may have occurred. This hypothesis is consistent with the yellowish color at this dose level compared to uncolored solutions in the other treatments and with speciation modeling using Visual MINTEQ, suggesting that a significant fraction of Ag is precipitating as AgCl. Therefore, this treatment was not further considered in any of the concentration–response fittings. Dissolved concentrations in fresh solutions of the 0.32 to 320 μg nominal Ag/L treatments were on average $17 \pm 7\%$ lower than total concentrations in fresh solutions. Total concentrations decreased during the exposure to $55 \pm 8\%$ (average concentrations in old solutions ± standard deviation) of the total Ag concentrations in fresh solutions. Dissolved Ag concentrations in old solutions were on average $15 \pm 7\%$ lower than the total Ag concentrations in old

solutions and $44 \pm 7\%$ lower than dissolved Ag concentrations in fresh solutions. Hence, as recommended in the Organisation for Economic Co-operation and Development (2006) protocol, concentration–response analysis was based on the geometric mean of the measured dissolved Ag concentrations in fresh and old solutions. Geometric mean dissolved Ag concentrations were on average $51 \pm 14\%$ lower than nominal concentrations (Table 4).

The average doubling time in the control treatments of the *L. minor* test was 1.75 ± 0.10 d. This value is <2.5 d, and the test was therefore considered valid (Organisation for Economic Co-operation and Development 2006). The average control growth rate was $0.40 \pm 0.02\,d^{-1}$ for the total number of fronds (r_n) and $0.30 \pm 0.02\,d^{-1}$ for the frond area endpoint (r_a). At the end of the exposure, the average root length in the control treatments was 9.8 ± 1.8 mm, and the average control dry weight was 5.2 ± 1.0 mg.

All endpoints showed a clear concentration–response behavior with increasing Ag dosing levels (Table 4).

The concentration response of the dry weight endpoint showed a significant hormesis effect. This effect was not observed for any of the other endpoints. The corresponding effect concentrations are reported in Table 5.

Based on the ECx values, root length was the most sensitive endpoint. The EC10 and EC50 values for root length were 1.4 and 41.6 μg dissolved Ag/L, respectively. The growth rate $(r_n$ and $r_a)$ had the lowest NOEC and LOECs: 1.3 (NOEC) and 4.3 (LOEC) μg dissolved Ag/L. The intratreatment variation was generally higher for the root length and dry weight endpoints.

B. calyciflorus reproduction test.
Total Ag concentrations in the fresh medium were in the 2 lowest Ag treatments up to 26% lower compared to nominal concentrations, whereas total Ag concentrations in the other Ag treatments were 4 to 16% higher than nominal concentrations. Dissolved Ag concentrations in fresh solutions were on average $12 \pm 10\%$ lower than total concentrations in fresh solutions. A strong decrease in dissolved Ag was observed immediately after addition of the algae food source to the fresh solution, probably because of

TABLE 4: Overview of average[a] growth rate in the different exposure treatments of the 7-d *Lemna minor* tests for the growth rate endpoints (r_n and r_a), root length, and dry weight

Nominal Ag (μg/L)	Dissolved Ag in fresh solutions[b] (μg/L)	Dissolved Ag in old solutions[b] (μg/L)	Geometric mean dissolved Ag (μg/L)	Growth rate (frond number), r_n (d^{-1})	Growth rate (frond area), r_a (d^{-1})	Root length (mm)	Dry wt (mg)
0	<0.035	<0.035	<0.035	0.40 ± 0.02	0.30 ± 0.02	9.8 ± 1.8	5.2 ± 1.0
0.32	0.18 ± 0.01	0.09 ± 0.01[c]	0.13	0.41 ± 0.01	0.31 ± 0.01	10.8 ± 0.9	6.4 ± 0.2
1	0.59 ± 0.04	0.30 ± 0.04	0.42	0.40 ± 0.003	0.30 ± 0.004	10.2 ± 1.0	6.4 ± 0.6
3.2	1.8 ± 0.2	0.97 ± 0.07	1.30	0.41 ± 0.02	0.30 ± 0.01	9.2 ± 0.6	6.3 ± 0.8
10	6.0 ± 1.6	3.4 ± 0.5	4.31	0.36 ± 0.02	0.28 ± 0.02	8.2 ± 0.7	5.4 ± 0.8
32	28 ± 4	14 ± 5	18.3	0.36 ± 0.002	0.24 ± 0.02	5.2 ± 0.5	4.8 ± 0.3
100	84 ± 6	48 ± 13	60.4	0.31 ± 0.01	0.20 ± 0.01	3.8 ± 0.4	3.3 ± 0.3
320	292 ± 17	206 ± 27	243	0.27 ± 0.02	0.14 ± 0.02	3.7 ± 0.7	2.3 ± 0.3
(1000)[d]	351 ± 9	333 ± 55	336	(0.29 ± 0.01)	(0.17 ± 0.004)	(3.4 ± 0.6)	(3.1 ± 0.3)

[a]Average of all replicates ± standard deviation is reported.
[b]Average of 3 samples ± standard deviation is reported.
[c]Below limit of quantification (0.12 μg/L) of inductively coupled plasma mass spectrometry.
[d]The responses of the 1000 μg nominal Ag/L treatment were not taken into account for concentration response fitting because measurements of actual Ag concentrations indicate that Ag precipitation likely occurred in the exposure solution.

TABLE 5: Effect concentrations (expressed as measured dissolved Ag concentrations) of ionic Ag to the aquatic species *Lemna minor*, *Brachionus calciflorus*, and *Anabaena flos-aquae*[a]

Species	Endpoint	EC10[b] (µg Ag/L)	EC20[b] (µg Ag/L)	EC50[b] (µg Ag/L)	NOEC[c] (µg Ag/L)	LOEC[c] (µg Ag/L)
Lemna minor[d]	Growth rate (frond number)	14 (7–29)	62 (42–92)	769[f] (381–1550)	1.3 (–1.8 ± 5.4)	4.3 (9.9 ± 5.0)
	Growth rate (frond area)	5.2 (3.2–8.5)	18 (13–25)	159 (124–205)	1.3 (0.4 ± 3.5)	4.3 (9.5 ± 5.3)
	Root length	**1.4 (0.4–4.2)**	4.8 (2.2–10.5)	42 (25.1–68.9)	4.31 (16.1 ± 7.5)	18.3 (47.5 ± 5.4)
	Dry weight	19.0 (3.9–91.9)	41.8 (14.5–120.4)	162 (78–336)	18.3 (6.6 ± 4.9)	60.4 (35.8 ± 4.9)
Brachionus	Population size	**0.31 (0.13–0.73)**	1.0 (0.6–1.9)	8.2 (5.9–11.3)	0.27 (6.8 ± 7.8)	0.52 (17.0 ± 8.6)
calciflorus[d]	Population growth rate	2.6 (1.5–4.7)	5.2 (3.5–7.7)	16.7 (13.5–20.7)	0.27 (0.2 ± 4.6)	0.52 (6.9 ± 5.3)
Anabaena flos-aquae[e]	Growth rate	**0.41 (0.29–0.52)**	0.46 (0.25–0.67)	0.56 (0.16–0.96)	0.35 (4 ± 10)	0.84 (100 ± 0)

[a]The toxicity value selected for use in the species sensitivity distribution (most sensitive endpoint) is indicated in bold.
[b]The ECx values were calculated using a log-logistic concentration response model with 2 parameters for *L. minor* and *B. calciflorus* and using a Weibull function with 2 parameters for *A. flos-aquae*. The 95% confidence intervals are reported in parentheses.
[c]The NOEC and LOECs were calculated using the Williams test for *L. minor* and *B. calciflorus* and using the nonparametric Jonckheere-Terpstra test for *B. calciflorus*. The average growth rate inhibition ± standard deviation (percentage) relative to the control at the NOEC or LOEC is reported in parentheses.
[d]Effect concentrations are expressed as the geometric mean of the measured dissolved Ag concentrations in fresh and old solutions.
[e]Effect concentrations are expressed as measured dissolved Ag concentrations.
[f]Extrapolated outside the tested concentration range (geometric mean of the highest Ag treatment used for concentration–response fitting was 243 µg/L).
ECx = x% effect concentration; NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration.

surface sorption of Ag to the algae. Dissolved samples taken just after the addition of the algae to the fresh solution contained on average only 21 ± 4% of the Ag that was present in the dissolved fraction before addition of the algae. Dissolved Ag concentrations in the 3 lowest Ag treatments could not be accurately determined (concentrations below quantification limit of the ICP-OES analysis and an insufficient sample volume remained for ICP-MS analysis). In the other Ag treatments, dissolved Ag concentrations after 24-h exposure were on average 34 ± 12% lower than dissolved Ag concentrations in fresh solutions after the algae addition. After 48-h exposure, dissolved Ag concentrations further decreased and were on average 70 ± 29% lower than dissolved Ag concentrations in fresh solutions after the algae addition. The highest decreases in dissolved Ag concentrations were observed at the 3 lowest Ag concentrations, where dissolved concentrations decreased up to 95% compared to dissolved concentrations in fresh solutions (Table 6).

Concentration–response analysis was based on the time-weighted average of dissolved Ag concentrations measured in fresh medium after algae addition (*t* 0 h) and those measured in

the old solutions after the 24 (when available) and 48-h exposures. These time-weighted average dissolved Ag concentrations were on average 90 ± 4% lower than nominal concentrations.

The average control population size at the end of the test was 7.4 ± 0.7 rotifers. Reproduction occurred in 94% of the control replicates, and the control population growth rate was 0.95 ± 0.07 d⁻¹. As such, the test is considered valid (reproduction in at least 88% of the control replicates and mean control growth rate of at least 0.55 d⁻¹ [MicroBioTests 2018]).

A clear concentration–response behavior of population size and population growth rate was observed (Table 6). Corresponding effect concentrations for the *B. calciflorus* test are shown in Table 5. The EC10 and EC50 values for population size are lower than for population growth rate, and NOEC and LOEC levels for both endpoints are similar.

A. flos-aquae growth inhibition test. Total Ag concentrations in fresh medium were on average 27 ± 15% lower compared to the targeted nominal concentrations. Dissolved Ag concentrations in fresh solutions were on average 11 ± 8%

TABLE 6: Overview of average[a] population size (at end of test) and population growth rate in the different exposure treatments of the 48-h *Brachionus calciflorus* reproduction test

Nominal Ag concentration (µg/L)	Dissolved Ag (µg/L)				Time-weighted dissolved Ag[b] (µg/L)	Population size	Population growth rate (d⁻¹)	Mortality (%)
	t 0 h before algae addition	*t* 0 h after algae addition	*t* 24 h	*t* 48 h				
Control	<0.01	<0.01	<0.01	<0.01	<0.01	7.4 ± 0.7	0.95 ± 0.07	6
5	2.8	0.7	—[c]	0.07	0.27	6.9 ± 0.4	0.95 ± 0.03	0
10	6.6	1.4	—[c]	0.12	0.52	6.1 ± 0.4	0.89 ± 0.04	0
20	20	4.5	—[c]	0.23	1.4	5.3 ± 0.4	0.82 ± 0.03	0
40	38	9.8	5.0	2.1	5.2	4.3 ± 0.4	0.70 ± 0.04	0
80	89	18	11	4.5	11	3.6 ± 0.2	0.62 ± 0.04	0
160	172	30	21	18	23	4.0 ± 0.4	0.65 ± 0.06	0
320	296	42	33	34	35	0.3 ± 0.2	0.02 ± 0.02	75

[a]Average of all replicates ± standard error is reported.
[b]Time-weighted average of dissolved Ag concentrations in fresh solutions at *t* 0 h (after addition of algae) and in old solutions at *t* 24 h (when available) and *t* 48 h.
[c]Below limit of quantification (3.7 µg/L) of inductively coupled plasma optical emission spectrometry. Not enough sample left for analysis on inductively coupled plasma mass spectrometry.

lower than total concentrations in fresh solutions and did not change considerably 1 h after inoculation of the solutions with cyanobacteria (dissolved Ag concentrations on average $11 \pm 7\%$ lower than in the fresh solutions). During the test period, total Ag concentrations in the algae exposures decreased by $56 \pm 7\%$ in the 4 lowest Ag treatments and by $33 \pm 2\%$ in the higher Ag treatments. At test termination, dissolved Ag concentrations in the algae exposures were on average $24 \pm 6\%$ lower than total concentrations at the same sampling occasion. During the test period, dissolved Ag concentrations decreased by $55 \pm 13\%$, with differences between fresh solutions and exposure solutions at test termination being highest in the lowest Ag treatments (Table 7). In a previous experiment, Ag concentrations measured in the chemistry controls (not inoculated with algae) decreased on average only $40 \pm 20\%$ compared to the dissolved concentrations of the fresh solutions. The latter indicates that part of the decrease in dissolved Ag in the algae exposures is due to algal adsorption and/or uptake. Further analysis of some of the Ag measurements indicates that, especially at lower Ag doses, adsorption of Ag to the test container walls contributes significantly (see Supplemental Data).

The calibration curve used to determine cell densities in the algae exposures shows a clear linear relationship between cell densities and fluorescence intensity ($R^2 = 0.998$). The control in the *A. flos-aquae* test increased 28-fold over 72 h, which is in line with the validity criteria of the OECD guideline (i.e., $\geq$16-fold). Average control growth rate was $1.03 \pm 0.05 \, d^{-1}$. The coefficient of variation for the control growth rate (5%) and the coefficient of variation among the sectional (day-by-day) growth rates in the control (33%) were also in line with the validity criteria mentioned in the OECD protocol.

A clear concentration–response behavior was observed with increasing Ag doses (Table 7). At the 3 lowest silver doses, growth was comparable to the control growth. In the higher Ag doses, a negative growth was observed during the 72-h growth inhibition test (i.e., lower cell densities than the inoculum density at test initiation), suggesting that *A. flos-aquae* was dying in these Ag treatments. Algal clusters were observed at the control and 3 lowest Ag doses but were absent in the higher Ag doses.

Corresponding effect concentrations for the *A. flos-aquae* test are shown in Table 5.

Assessment of the data set for chronic aquatic toxicity

As a next step, we collated the chronic toxicity data of Table 3 and the data described for *L. minor*, *B. calyciflorus*, and *A. flos-aquae* (Table 5) to obtain only a single value per species. This was always the value for the most sensitive endpoint (expressed as 0.45 µm–filtered fraction). If toxicity values at different hardness were reported for the same species and endpoint, only the EC10 from the test at the lowest hardness was retained (e.g., *Salmo trutta* [Davies et al. 1998] and *Daphnia magna* [Rodgers et al. 1997; Naddy et al. 2007a; Bianchini and Wood 2008; Mertens et al. 2019]). If multiple values at a similar hardness were available, then the geometric mean value was used (e.g., *O. mykiss* [Davies et al. 1998; Dethloff et al. 2007]). An overview of the selected NOEC and EC10 values with additional information on DOC and hardness is provided in Table 8.

The selected toxicity values vary between 0.1 µg Ag/L (*P. subcapitata*) and 12.54 µg Ag/L (*Chironomus tentans*). The reported DOC concentrations for the selected toxicity values vary between 0.63 and 3.4 mg C/L, with an average of 1.7 mg/L. The hardness varies between 10 and 116 mg/L CaCO₃, with an average of 42.9 mg/L CaCO₃. The toxicity values for the most sensitive species are all derived in test media with <50 mg/L CaCO₃ (Table 8). The use of these chronic toxicity data therefore aims at deriving Ag threshold values that are representative for soft (<50 mg/L CaCO₃) waters with low DOC (<2.0 mg/L).

The data are modeled using an SSD approach. The SSDs are derived using the chronic toxicity data as reported in Table 8. The Rayleigh distribution resulted in the best fit of the toxicity data. An overview of the HC5-50 values using the "conventional" distributions (i.e., normal and logistic) and the best-fitting distribution is provided (Table 9 and Figure 1).

The HC5-50 values vary between 0.088 (using conventional SSD) and 0.116 (using best-fitting SSD) µg dissolved Ag/L.

TABLE 7: Overview of cell density and growth rates[a] in the different exposure treatments of the 72-h *Anabaena flos-aquae* growth inhibition test

Nominal Ag (µg/L)	Dissolved Ag (µg/L)					Time-weighted average dissolved Ag[c] (µg/L)	Cell density (×10⁴ cells/mL)			Growth rate (d⁻¹)
	t 0 h	t 1 h[b]	t 24 h	t 48 h	t 72 h		Day 1	Day 2	Day 3	t 0–72 h
Control	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	3.91 ± 0.34	10.4 ± 1.6	22.5 ± 4.4	1.03 ± 0.05
0.22	0.16	0.16	0.12	0.10	0.09	0.11	3.66 ± 1.00	10.4 ± 2.4	23.4 ± 4.1	1.05 ± 0.06
0.46	0.28	0.28	0.17	0.12	0.10	0.16	3.52 ± 0.38	9.29 ± 1.36	24.4 ± 4.2	1.05 ± 0.05
1	0.6	0.5	0.41	0.27	0.22	0.35	1.90 ± 0.18	6.11 ± 1.58	18.5 ± 3.9	0.99 ± 0.10
2.2	1.4	1.1	1.1	0.66	0.44	0.84	1.99 ± 0.28	1.27 ± 1.21	0.63 ± 0.4	−0.24 ± 0.18
4.6	2.9	2.4	2.1	2.0	1.8	2.0	1.57 ± 0.29	0.40 ± 0.09	0.15 ± 0.03	−0.71 ± 0.06
10	7.5	6.5	4.1	3.7	4.0	4.3	1.20 ± 0.20	0.42 ± 0.08	0.08 ± 0.06	−0.96 ± 0.31
22	11	10	6.5	5.6	5.6	6.6	1.32 ± 0.17	0.56 ± 0.04	0.16 ± 0.05	−0.66 ± 0.10

[a]Average of all replicates ± standard deviation is reported.
[b]Sample taken in the exposure vessels approximately 1 h after inoculation with cyanobacteria.
[c]Time-weighted average of dissolved Ag concentrations in fresh solutions at t 0 h and those measured in algae exposures (1, 24, 48, and 72 h).

TABLE 8: Overview of selected NOEC/EC10 values ranked per taxonomic group[a]

Taxonomic group		Species	Endpoint	NOEC or EC10 (µg Ag/L)	DOC (mg/L)	Hardness (mg CaCO$_3$/L)	Reference(s)
Fish	Cyprinidae	*Pimephales promelas*	Hatching	0.38	2.4	30.5	Naddy et al. (2007b)
	Salmonidae	*Oncorhynchus mykiss*	Mortality	0.46[b]	1.4[c]	28.5[c]	Davies et al. (1998), Dethloff et al. (2007)
		Salmo trutta	Mortality	0.23	0.8	27.9	Davies et al. (1998)
Crustaceans	Cladocera	*Ceriodaphnia dubia*	Reproduction	4.36[b]	3.4[c]	85.2[c]	Kolts et al. (2009), Naddy et al. (2007a), Rodgers et al. (1997)
		Daphnia magna	Reproduction	0.80	1.0	69.0	Rodgers et al. (1997)
	Amphipoda	*Hyalella azteca*	Mortality	1.54[b]	1.5[c]	23.4[c]	Diamond et al. (1990), Rodgers et al. (1997)
Insects	Ephemeroptera	*Isonychia bicolor*	Molting	0.16	2.0	34.8	Diamond et al. (1990)
		Stenonema modestum	Molting	1.00	<2.0	48.5	Diamond et al. (1992)
	Diptera	*Chironomus tentans*	Growth	12.54	<2.0	52.1	Call et al. (1999)
Rotifera		*Brachionus calyciflorus*		**0.31**	**1.6**	**48.0**	
Mollusks	Bivalvia	*Corbicula fluminea*	Growth	0.84	2.0	34.8	Diamond et al. (1990)
	Gastropoda	*Lymnaea stagnalis*	Growth	1.48	0.76	116	Cremazy et al. (2018)
Cyanobacteria		*Anabaena flos-aquae*		**0.41**	**1.8**	**25.0**	
Algae	Chlorophyceae	*Pseudokirchneriella subcapitata*	Yield, growth rate	0.10	0.63	10.0	Mertens et al. (2019)
Higher plants	Tracheophyta	*Lemna minor*		**1.40**	**1.6**	**10.4**	

[a]Newly generated data are shown in bold.
[b]Geometric mean of multiple values.
[c]Mean of multiple values.

DISCUSSION

Silver has been shown to be a highly potent toxicant to freshwater organisms, but EQS vary considerably across jurisdictions and legislative frameworks. This is related to differences in the data used, data quality requirements, and approaches to translating single-species data to protective values for ecosystems. In some cases, data quality requirements have not or not fully been in line with scientific guidance for metals. In the present study, we therefore assessed existing available aquatic toxicity data, applied robust data selection criteria, generated additional toxicity values for taxonomic groups for which no or no reliable data were available, and
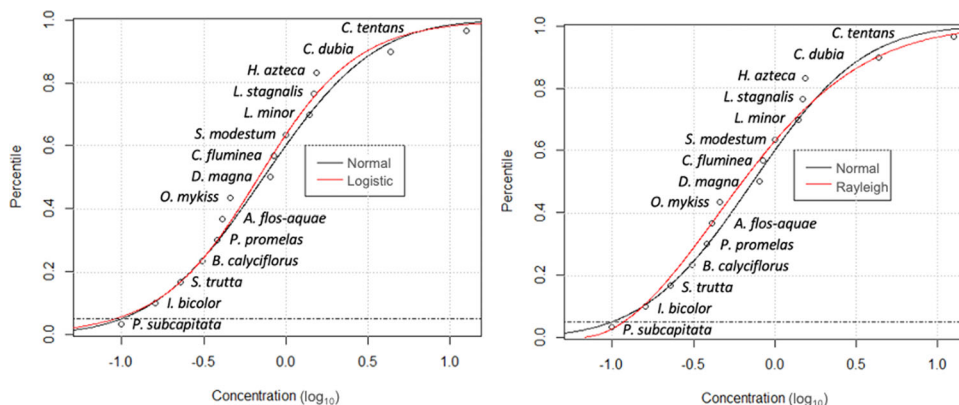
TABLE 9: Summary of the derived HC5-50 values (micrograms of dissolved Ag per liter) using the conventional and best-fitting distributions

Distribution	HC5-50 (HC5-5–HC5-95; µg/L)
Normal using ETx[a]	0.088 (0.029–0.18)
Normal using bootstrapping[b]	0.110 (0.048–0.24)
Logistic using bootstrapping[b]	0.099 (0.039–0.22)
Best-fitting distribution (Rayleigh)	0.116 (0.065–0.23)

[a]Calculated using ETx 2.0 software (Van Vlaardingen et al. 2004).
[b]Calculated using bootstrapping statistical methods.
HC5-50/HC5-5/HC5-95 = hazardous concentrations for 5% of the species.

FIGURE 1: Species sensitivity distributions (SSD) for the most sensitive endpoint per species: normal and logistic SSD (left); normal and Rayleigh SSD (right). *C. tentans* = *Chironomus tentans*; *C. dubia* = *Ceriodaphnia dubia*; *H. azteca* = *Hyalella azteca*; *L. stagnalis* = *Lymnaea stagnalis*; *L. minor* = *Lemna minor*; *S. modestum* = *Stenonema modestum*; *C. fluminea* = *Corbicula fluminea*; *D. magna* = *Daphnia magna*; *O. mykiss* = *Oncorhynchus mykiss*; *A. flos-aquae* = *Anabaena flos-aquae*; *P. promelas* = *Pimephales promelas*; *B. calyciflorus* = *Brachionus calyciflorus*; *S. trutta* = *Salmo trutta*; *I. bicolor* = *Isonychia bicolor*; *P. subcapitata* = *Pseudokirchneriella subcapitata*.

derived a protective HC5 value for European waters with low hardness and low DOC.

The present study confirmed the steepness of the concentration–response curve of *A. flos-aquae* growth to Ag demonstrated by the Umweltbundesamt (2013), but its sensitivity to Ag was approximately 10-fold lower in the present experiment.

The acute toxicity of Ag to the rotifer *B. calyciflorus* has previously been reported, with a 24-h LC50 of 7.5 µg Ag/L (Snell et al. 1991); but no chronic toxicity data for Ag were identified. It is nevertheless considered a very useful test organism for chronic aquatic toxicity testing, with data being generated for a multitude of organic and inorganic chemicals (Rico-Martínez et al. 2016). From the present study, it is concluded that *B. calyciflorus* was comparatively sensitive to Ag with regard to *A. flos-aquae* but less sensitive than *P. subcapitata*.

Also for aquatic plants, no reliable Ag test data were previously available. For *L. minor*, the Umweltbundesamt (2013) reported EC10 and EC50 values for dry weight of 4 and 43 µg measured Ag/L, respectively. However, these authors indicated that recovery of $AgNO_3$ was insufficient in their test and therefore considered their test not suited for use in an SSD data set. In other studies, EC10 values of 1.8 µg Ag/L for *Lemna paucicostata* (Nasu and Kugimoto 1981) and 6 µg Ag/L for *L. minor* (Naumann et al. 2007; Gubbins et al. 2011) were derived. However, in these studies Ag was expressed as nominal Ag concentration, and details on the experimental setup and key physicochemical conditions were lacking. Our guideline-conforming test showed comparable thresholds to the Umweltbundesamt (2013) study but for root length as an endpoint (EC10 = 1.4 µg Ag/L and EC50 = 42 µg Ag/L). Using dry weight as an endpoint, our test showed a lower sensitivity, with EC10 and EC50 values of 19 and 162 µg dissolved Ag/L, respectively.

All chronic freshwater toxicity data that have been identified via an extensive literature search were reviewed and quality-assessed. Key physicochemical parameters considered and known to influence the bioavailability and toxicity of metals were the availability of measured dissolved Ag concentrations and pH, DOC, and hardness being reported and within the tolerance limits of the test organisms. This approach has been applied to and internationally accepted for other metals before and is included in the Metals Environmental Risk Assessment Guidance (2016) guidance. The retained toxicity values range from 0.1 (*P. subcapitata*) to 12.54 (*Chironomus tentans*) µg Ag/L. The DOC concentrations (known to mitigate acute toxicity of Ag toward freshwater organisms and presumably chronic toxicity) in the test media are low (average 1.7 mg/L, corresponding to the 20th percentile in the FOREGS database). For hardness, the average is 42.9 mg $CaCO_3$/L (corresponding to the 32nd percentile of the FOREGS database). For comparative purposes, and according to the FOREGS database (Salminen et al. 2005), the median DOC concentration in European Union surface waters is 5.5 mg/L and the median hardness is 125 mg/L $CaCO_3$. The use of hardness as a criterion for the selection of toxicity data (i.e., only the data point generated for the lowest hardness is retained in case multiple data points are available for a specific species) assumes that hardness influences the chronic toxicity of Ag toward freshwater organisms. The latter is supported by some evidence from chronic toxicity assays with *D. magna* (Bianchini and Wood 2008). In further work, a hardness correction could be considered for the derivation of an Ag threshold value for moderately hard or hard surface waters. In the present study, the derived environmental threshold for Ag represents reasonable worst-case conditions for bioavailability in European Union surface waters (low hardness and low DOC). This ensures that the derived threshold value is protective for the freshwater environment using today's scientific data set for Ag.

Toxicity data for 15 species, representing 12 taxonomic groups, have been retained in the silver SSD data set (Table 8). This data set covers more species and taxonomic groups than required in current regulatory guidance, in which at least 10 species covering at least 8 taxonomic groups are required (European Chemicals Agency 2008). The data set for Ag covers key taxonomic groups (including insects, mollusks, and cyanobacteria) and sensitive life forms and feeding strategies. In particular, the data set includes algae (including cyanobacteria), which were considered to be particularly at risk from Ag exposure in the environment because of their well-known antimicrobial properties.

The Rayleigh distribution resulted in the best fit of the toxicity data. The alternative distributions give slightly lower threshold values for Ag. However, these distributions provide poor fits of the extreme values. Selection of the best-fitting SSD also revealed a lower uncertainty around the 5th percentile: the 90% confidence limits of the HC5 using the Rayleigh distribution were 0.065 (HC5-5) and 0.23 (HC5-95) µg Ag/L; that is, they were separated by a factor of 3.5, whereas a factor of 6.2 separation between them was observed when using the log-normal distribution (HC5-5 of 0.029 and HC5-95 of 0.18 µg Ag/L). The lower statistical uncertainty around the HC5 supports the selection of the HC5-50 derived from the Rayleigh SSD.

When considering the complete Ag ecotoxicity data set (Table 3), it is noted that only one value of the database is below the HC5-50. This is the value for the alga *P. subcapitata* (Mertens et al. 2019). The next most sensitive species in the Ag SSD data set are *Isonychia bicolor* (Diamond et al. 1990) and *Salmo trutta* (Davies et al. 1998), with a NOEC of 0.16 and an EC10 of 0.23 µg Ag/L, respectively. Another expected sensitive organism, like the cyanobacterium *A. flos-aquae*, has an EC10 of 0.41 µg Ag/L.

In a regulatory context, it is often a prerequisite to consider if an additional assessment factor needs to be applied. This factor is supposed to cover the remaining uncertainties in the data set to ensure the protectiveness of the threshold value derived. For Ag, however, there is little uncertainty left, considering the high quality and taxonomic diversity of the Ag SSD data set and the species and endpoints covered. Also, there is a strong inherent conservatism in the threshold value selection per species; that is, the selected chronic toxicity data typically reflect conditions of high bioavailability, with hardness and DOC well below median values in European Union natural waters. The main remaining uncertainty is related to the limited

availability of field and mesocosm data. There are a number of microcosm/mesocosm studies available for Ag, but most are either in marine water or specifically with nanosilver. However, Jiang et al. (2017) examined the distribution and toxicity of $AgNO_3$ (~500 µg Ag/L nominal) in a series of freshwater microcosms for 90 d. Total measured Ag concentration in the water column ranged from >500 µg Ag/L at test initiation to approximately 9 µg Ag/L after 90 d. Although it was demonstrated that aquatic plants (*Hydrilla verticillata*), fish (*Gambusia affinis*), and snails (*Radix* spp.) significantly accumulated Ag, the biomass of phytoplankton, aquatic plants, and animals was not significantly different between control and samples treated with $AgNO_3$ for 90 d. Dissolved Ag concentrations were not measured in this experiment, but the minimum total measured Ag concentration (9 µg Ag/L) was well above the statistically derived HC5-50 of 0.116 µg Ag/L, with no effects being observed. Also, it must be repeated that the derived threshold value in the present study is considered conservative in terms of "bioavailability." Limited to no scientifically robust guidance exists on how to select an appropriate assessment factor, but for other metals with similar data sets, assessment factors of 1 to 3 have typically been applied. For Ag, this would result in a threshold level of 0.039 to 0.116 µg Ag/L. Considering the observations of the 90-d microcosm study combined with the conservatism in the physicochemical selection criteria of the Ag ecotoxicity data set, it is expected that this threshold value is conservative and protective for ecologically relevant natural freshwater environments. Furthermore, because secondary poisoning from Ag is not relevant for the derivation of the EQS (National Institute for Public Health and the Environment 2012) and derivation of a biota standard for humans is not triggered for Ag because none of the toxicological triggers are met, the threshold value for protection of aquatic life against direct toxic effects can be considered as a protective EQS (as outlined in European Commission 2018).

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