UNIVERSITY OF CALIFORNIA

Santa Barbara

Ecological feedbacks and engineered nanomaterials in freshwater environments

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution and Marine Biology

by

Louise Mote Stevenson

Committee in charge:

Professor Roger M. Nisbet, Chair

Professor Patricia A. Holden

Professor Cheryl J. Briggs

Professor Edward McCauley

June 2016

The dissertation of Louise Mote Stevenson is approved.

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Edward McCauley

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Patricia A. Holden

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Cheryl J. Briggs

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Roger M. Nisbet, Committee Chair

June 2016

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T-rex marching band, and answering my stupid energy-related questions without judgment. Thank you to both of y'all for responding, usually within seconds, to all Cruise-related text messages. Dad – thank you for your support, wise guidance, and for taking the brunt of the Mominator's fines. I think you owe her approximately a hundred billion dollars. Mom – thank you for your strength and for always answering my calls with a "no, I wasn't busy" even though I know you always are. Please transfer Dad's fines to the Louise Fund. I love you all so much!

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> "In folly's world-wide wind
> Our shoulders shield from the weather
> The calm we now beget together,
> Like a flame held between hand and hand.
> …
> Meanwhile, O load of stress and bother,
> Lie on the shells of our backs in a great heap:
> It will but press us closer, one to the other."
>
> - "Dead Still" by Andrei Voznesensky, translated by Richard Wilbur

That is what you have done and continue to do for me. I love you.

VITA OF LOUISE MOTE STEVENSON
June 2016

Education & Appointments

University of California, Santa Barbara, *(2010-2016)*
PhD Candidate in Ecology, Evolution, and Marine Biology; Advisor: Dr. Roger Nisbet

UC Center for Environmental Implications of Nanotechnology, *Graduate Student Researcher* *(2013-2016)*

Center for Nanotechnology and Society, *Graduate Research Fellow* *(2013-2016)*
CNS Science Fellow working on a project investigating the public risk perception of hydraulic fracturing; responsible for collection, synthesis, and dissemination of scientific and popular media on fracking

Amherst College Class of 2009, *Amherst, MA (2005-2009)*
Graduated Cum Laude, BA in Biology; Senior Honors Thesis with Dr. Ethan Clotfelter

Publications

Adeleye, AS, **Stevenson, LM,** Su, Y, Nisbet, RM, Zhang, Y, & Keller, AA 2016. Influence of phytoplankton on fate and effects of modified zero-valent iron nanoparticles. *Environmental science & technology*.

AC Brown, **Stevenson LM**, Leonard HM, Nieves-Puigdoller K, and ED Clotfelter, 2014. Phytoestrogens β-Sitosterol and Genistein Have Limited Effects on Reproductive Endpoints in a Female Fish, *Betta splendens*. *BioMed Research International*, vol. 2014, Article ID 681396. doi:10.1155/2014/681396

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LM Stevenson, Brown, AC, Montgomery, TM, and ED Clotfelter. 2011. Reproductive consequences of exposure to waterborne phytoestrogens in male fighting fish *Betta splendens*. *Archives of Environmental Contamination and Toxicology* 60:501-510.

GM Anhalzer , Fournier M, O'Connor T, **Stevenson**, **LM,** and M. Yglesias. 2010. Traits for predator selection on *Pentaclethra macroloba* seeds. *American Journal of Undergraduate Research* 8(4):1-8.

Awards, Grants, & Fellowships

2014 & 2015 UCSB Instructional Improvement Grant, "Population Ecology in the Introductory Biology Laboratories": Part 1 (2014) and Part 2 (2015)

2013 **Young Environmental Scientists Meeting Travel Grant,** Krakow, Poland

2012 & 2014 **Worster Summer Research Fellowship Program,** UCSB

2012 **International Conference on the Environmental Effects of Nanomaterials Student Travel Award,** Alberta, Canada

2010-2013 **National Science Foundation Graduate Research Fellow**

2010 **The Lloyd I. Rosenblum Memorial Fellowship for Graduate Studies, Amherst College**

Teaching and Mentoring Experience

2010-2016 **UCSB, Nisbet/McCauley Lab, Graduate Student**
Mentoring undergraduate lab assistants who work 10 hours a week in the lab processing samples, aiding ongoing experiments, and conducting independent research; currently mentoring two students and have worked with nine lab assistants as a graduate student

2015 **Science Buddies Science Fair Project, Author**
Co-developed and wrote a project based on my dissertation research for non-profit organization that provides free science fair project ideas and help to build science literacy. Project title: "Looking Downstream: Could Nanosilver in Consumer Products Affect Pond Life?" Link: www.sciencebuddies.org/science-fair-projects/project_ideas/EnvSci_p064.shtml

2013-15 **UCSB, Ecological Modeling, *Teaching Assistant*** *(January-March)*
Teaching computer laboratory sections (MATLAB) for an upper level undergraduate and graduate course on Ecological Modeling

2014 & 2015 **UCSB Instructional Improvement Grant**
Received Instructional Improvement Grant to redesign computational ecology exercises of Introductory Biology lab; reworked all lab exercises including coding all computational models, incorporating data from the scientific literature, and rewriting the lab narrative to include modern day ecological problems and how models can be used to help solve them

2012-15 **UCSB, Toxics in the Environment, *Invited Lecturer*** *(March)*
Gave invited lecture on environmental effects of nanomaterials on lakes and streams in introduction to ecotoxicology class for multiple terms.

2014 **UCSB, Toxics in the Environment, *Reader*** *(January-March)*

2012 & 2014 **UCSB, Worster Summer Research Fellowship Program** *(June-September)*
Developed independent projects with undergraduate students and received Worster Fellowships for two summers – these fellowships are awarded to undergraduate-graduate student teams to encourage mentorship

2013 & 2014 **UCSB, Freshmen Summer Start Program, *Invited Lecturer*** *(August)*
Lecture on ecotoxicology and my research to incoming first year students interested in science.

2013 **UCSB, Introduction to Ecology, *Teaching Assistant*** *(June-July)*
Taught laboratory/discussion sections for introductory course in Ecology

Presentations

2015 Society of Environmental Toxicology and Chemistry Young Environmental Scientists Meeting. Gainesville, FL

2015 Society of Environmental Toxicology and Chemistry. Salt Lake City, UT

2014 Democratizing Technologies: Assessing the Roles of NGOs in Shaping Technological Futures. Santa Barbara, CA (Rapporteur and helped write Conference Report)

2013 Sustainable Nanotechnology Organization Conference. Santa Barbara, CA (poster)

2013 UC CEIN Nanotechnology EH&S Forum. Los Angeles, CA (poster)

2013 Young Environmental Scientists Meeting (SETAC). Krakow, Poland

2012 Society of Environmental Toxicology and Chemistry. Long Beach, CA

2012 International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, Alberta, Canada (poster)

2012 Ecology, Evolution and Marine Biology Graduate Student Symposium. Santa Barbara, CA

Service

2013-2015 **Society for Environmental Toxicology and Chemistry (SETAC), North America Student Advisory Council,** Outreach Subcommittee chair

2013-2015 **SETAC, Southern California Regional Chapter**, Member of Board of Directors

2010-2014 **UCSB Ecology, Evolution, and Marine Biology (EEMB) Graduate Student Advisory Committee**, Funding Peer

2013-present **Amherst College, Amherst Pathways Program**, Alumni Mentor

2013-present **The Hockaday School, Girls Advancing in Science, Technology and Mathematics (GAINS) Network**, Alumni Mentor

2011 **UCSB EEMB Departmental Graduate Student Symposium,** Coordinator

Ecological feedbacks and engineered nanomaterials in freshwater environments

by

Louise Mote Stevenson

Nanoparticles have had a large impact and driven a growing commercial industry of nano-enabled products. Nanoparticles' small size causes large changes in physicochemical properties compared to their "bulk" states, and this has been utilized for a wide variety of applications, ranging from biomedical to electronic, to cosmetic use. However, these novel properties also carry with them unknown effects on our environment. Numerous laboratory studies have found deleterious effects of nanoparticles on aquatic organisms, but we are missing key knowledge on how these toxic effects amplify in ecological systems. The aim of my dissertation is to investigate the effects of nanoparticles on freshwater systems through a series of experiments on phyto- and zooplankton and the development of quantitative models to explain these empirical results and extrapolate effects to other systems. My work has identified the importance of ecological feedbacks in nanotoxicology, specifically two novel feedbacks: algae produce dissolved organic carbon (DOC) that mitigates the toxicity of nanomaterials (silver and iron nanoparticles) to the cells themselves and a concentration of silver nanoparticles (AgNPs) that is toxic to individual *Daphnia* has no effect on populations of zooplankton due to population-level feedbacks.

The development of quantitative models of both of these feedbacks has enabled the estimation of the strength of both nanomaterial toxicity and of the mitigating feedback. Through the development of models of algal growth, DOC production, and nanoparticle

toxicity, I estimated the strength of both the inactivation of toxicity by DOC as well as the toxic strength of the various contributors to nanotoxicity (nano versus ionic, different transformations of nanoparticles). Estimating the relative contributions of different forms or products of nanoparticles to their overall toxic effect can be useful in ecological risk assessment, as it could identify toxic factors that are already regulated (such as ionic silver) along with those that are currently unregulated (nanosilver) and allow for direct comparison of their toxicity. The development of models of daphnid growth and reproduction, parameterized with individual-level data of AgNP exposure of *Daphnia* at multiple food rations, allowed me to identify the feedback that seemingly disrupts extrapolation between levels of biological organization. A concentration of AgNPs that is toxic to individual *Daphnia* has no effect on small populations of *Daphnia* due to population-level feedbacks in which the zooplankton population equilibrates at a lower consumer and higher resource biomass. This increase in the amount of food per individual allows the zooplankters to survive AgNP exposure. Overall, my dissertation work highlights the importance of ecological and environmental complexity when estimating the impacts of nanoparticles on freshwater systems.

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I. Environmental feedbacks and engineered nanoparticles: mitigation of silver nanoparticle toxicity to *Chlamydomonas reinhardtii* by algal-produced organic compounds[1]

Authors: Louise M. Stevenson, Helen Dickson, Tin Klanjscek, Arturo A. Keller, Edward McCauley, Roger M. Nisbet

Abstract

The vast majority of nanotoxicity studies measures the effect of exposure to a toxicant on an organism and ignores the potentially important effects of the organism on the toxicant. We investigated the effect of citrate-coated silver nanoparticles (AgNPs) on populations of the freshwater alga Chlamydomonas reinhardtii at different phases of batch culture growth and show that the AgNPs are most toxic to cultures in the early phases of growth. We offer strong evidence that reduced toxicity occurs because extracellular dissolved organic carbon (DOC) compounds produced by the algal cells themselves mitigate the toxicity of AgNPs. We analyzed this feedback with a dynamic model incorporating algal growth, nanoparticle dissolution, bioaccumulation of silver, DOC production and DOC-mediated inactivation of nanoparticles and ionic silver. Our findings demonstrate how the feedback between aquatic organisms and their environment may impact the toxicity and ecological effects of engineered nanoparticles.

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Introduction

Natural populations exert feedbacks on their environment through consumption, production and excretion. By modifying environments, organisms could significantly impact the fate and toxicity of nanomaterials. While direct impacts of toxicity have been widely assessed[1], understanding effects of environmental modifications on subsequent organismal responses to nanomaterials has been neglected.

Silver nanoparticles (AgNPs) comprise one of the fastest growing areas of nanotechnology[2] and are used in a broad range of consumer applications from home appliances to textiles, increasing their potential for environmental release. Studies have found considerable leaching of silver from consumer products containing AgNPs[3,4]. These particles are utilized for their well-studied antimicrobial properties[5-7] through mechanisms such as cell wall damage[7,8] and free radical production[9]. Studies have identified a toxic effect of AgNPs on marine and freshwater algal species[10-14], but these ignore the crucial feedback effect of algal species on the particles themselves.

We investigated the effect of citrate-coated AgNPs on the freshwater algae Chlamydomonas reinhardtii in different stages of growth in batch cultures. We found a nano-specific toxic effect of the AgNPs that cannot be explained by the presence of silver ions, a result that differs from past studies that have found that AgNP toxicity is mediated entirely through ionic silver (Ag+)[11,12,14]. Further, we found that extracellular molecules produced by the algal cells themselves mitigate both the nanoparticle-specific and ionic toxicity of AgNPs. This finding highlights how the feedback between freshwater organisms and their environment may impact the potential toxicity of AgNPs.

Materials and Methods

1. Batch culture setups and treatment groups

Batch cultures of Chlamydomonas reinhardtii were grown in 500 mL Erlenmeyer flasks. New cultures were inoculated with a cell concentration of 106 cells/L. Cultures used for inoculation were counted using a hemocytometer and then diluted into 250 mL of fresh COMBO media[33] to 106 cells/L. One and two weeks prior to the start of the experiment, new batch cultures were started and grown undisturbed in the experimental setup used during the experiment itself. To distinguish the effect of AgNPs on different stages of algal growth, the experiment began when these cultures were one and two weeks old. New cultures were inoculated with algal cells the day the experiment started. New cultures were in fast growth phase, one week old cultures were in slowing growth phase, and two week old cultures were in stationary growth phase.

2. Measurement of chlorophyll a concentrations

We measured concentrations of chlorophyll a with a Gemini XPS Fluorescence Microplate Reader (Molecular Devices). We measured the fluorescence of four 200 μL samples of each culture, averaged these values, and converted to concentrations of chlorophyll a (μg/L) using a standard curve calibrated for our instrument with Turner Designs Liquid Primary Chlorophyll A Standards.

3. Dissolved organic carbon removal experiment

To remove DOC, we centrifuged samples of a two-week-old algal batch culture on 7,000 rpm for 8 minutes on an Eppendorf 5430R Centrifuge two times, pouring off the

supernatant and resuspending the pellet in COMBO media without nitrogen or phosphorus after each spin. We spun the samples twice because we found that a large concentration of DOC was removed after two sequential spins, while a third spin removed a negligible amount of DOC (unpublished data). We then diluted this sample to 107 cells/L in media without nitrogen and phosphorus. We decreased cell concentration because pilot experiments with this protocol showed that the initial, higher algal cell concentrations (109 cells/L) rapidly produced a significant amount of DOC that caused AgNPs to aggregate within a day. Control and 5 mg/L AgNPs exposed cultures were sampled using the same experimental setup described in the Supplementary Information Section 1. These late-stage cells were exposed to AgNPs that did not aggregate (mean particle size remained around 40 nm; see Supplementary Information Section 5 for AgNP size measurement protocol).

4. Measurements of the dissolution of silver ions from the AgNPs

To avoid underestimating the dissolution of free silver ions by missing Ag+ absorbed by the algal cells themselves, we filtered all of the algal cells out of new (fast growth phase), one week old (slowing growth phase) and two week old (stationary growth phase) cultures using 5 micron filters (Millipore MF-Mixed Cellulose Ester Membrane filters). We added 5 mg/L of 40 nm citrate-coated AgNPs and took samples at the same frequency we sampled the initial AgNP experiment using the same batch culture sampling apparatus described previously (see Supplementary Information Section 1). We removed 15 mL of the culture for every sample and spun these samples down in acid-washed Amicon centrifugal filter units (Amicon Ultra-15, 10,000 NMWL) for 30 minutes at 5,550 rpm. We added 0.1% nitric acid to the sample and stored it in the dark until digestion. For the digestion process, we

added 3 parts HNO3 and 1 part HCl to every sample and heated the samples in a Hach Reactor (DRB 200 Reactor) for 30 minutes at 85o F. We then measured the final volume. These samples were analyzed by the Marine Science Analytical Lab at UCSB using Atomic Absorption Spectrophotometers with graphite furnace atomization (Varian Instruments AA240Z).

5. Dynamic Model

The model simulations and the parameter estimation were performed using proprietary code written in MATLAB.

Results and Discussion

AgNPs are most toxic to earlier stages of algal batch culture growth

We exposed algal batch cultures to 5 mg/L of 40 nm Citrate BioPure™ AgNPs (NanoComposix) during fast, slowing, and stationary phases of growth. The toxic response in an algal culture to 5 mg/L AgNPs depends on its growth stage. AgNPs were significantly more toxic to cultures in fast growth phase than cultures in later stages (Figure 1). We discovered that shaking algal cultures did not affect algal growth or response to AgNPs (Figure S1), so we continued our experimental analysis with unshaken cultures only. Cultures in slowing growth phase declined in population size over the first three days of exposure, partially recovered for a day, and then experienced a second decline. AgNPs caused a slight decline of cultures in stationary growth phase over the first three days of exposure, after which the cultures recovered for a day and then experienced a second decline similar to, but not as extreme as, cultures in slowing growth phase. Sondi & Salopek-Sondi

(2004) found a similar effect of AgNPs on bacterial colonies, as AgNPs had greater bactericidal effects when the starting concentrations of colony-forming units was lower[7]. The response of algal batch cultures to an equimolar concentration of Ag+ in the form of AgNO3 was toxic to all cultures regardless of growth stage (Figure S2).

Nano- or ionic-specific toxicity?

While AgNP toxicity is well studied, a large question still remains as to whether the observed effect of AgNPs is due to some toxic mechanism of the particle itself (a nano-specific toxic effect) or due to the deleterious effects of Ag+ that can dissolve from AgNPs (an ionic toxic effect). The AgNP literature is divided on this question – some past studies have found that AgNP toxicity to marine and freshwater algae is mediated entirely through dissolution of silver ions from the particles[10,12,14] while others have found a nano-specific toxic effect of AgNPs on bacteria[5,15] and on marine and freshwater algal species[10,13]. In our experiment, introduced AgNPs were initially toxic to all stages of algal growth (Figure 1). To investigate whether this could be explained by the presence of Ag+ in the stock solutions of AgNPs that can be confused with a nanoparticle effect[16], we measured the Ag+ concentration in our stock AgNP solution and exposed algal batch cultures at the same three stages of growth to the measured Ag+ concentration in the form of AgNO3. The concentration, measured using Atomic Absorption Spectroscopy to be 3.5 μg/L Ag+, had little to no effect: later stages of growth experienced no decline, and cultures in fast growth phase only declined initially and were able to recover completely (Figure 2).

In addition to measuring the concentration of silver ions present in the stock solution of AgNPs, we measured dissolution of AgNPs in algal cultures in the three growth stages. Algal cells were removed to minimize loss of measurable Ag+ due to association with algal

cells. The dissolved silver concentration was below 50 μg/L Ag+ for the first three days of introduction of the particles to the media (Figure S3); this slow dissolution is consistent with earlier studies of citrate-coated AgNP dissolution in environmentally-relevant media[17]. Since silver ions up to 100 μg/L do not have a significant toxic effect on cultures in late stages of growth (Figure S4), we conclude that Ag+ could not have caused the initial toxicity of all cultures. The initial decline is most likely due to a nano-specific effect of the AgNPs. A previous study on the effect of carbonate-coated AgNPs on the same freshwater algal species (C. reinhardtii) concluded that AgNP toxicity is mediated by Ag+[12]. The authors also found, as we did, that the free Ag+ concentration could not account for the AgNP toxicity observed, but they characterized the AgNP toxicity as driven by Ag+ because the presence of cysteine, a strong Ag+ ligand, greatly reduced toxicity[12]. However, cysteine may be mitigating toxicity by binding to silver ions that have resorbed to the particle surface[18], a phenomenon that occurs with even citrate-coated particles[19]. This toxic mechanism would be considered a particle-specific effect in terms of DOC mitigation of Ag+ and AgNP toxicity incorporated in our model described later in this paper.

Previous researchers have expended a lot of effort to identify specific mechanisms of toxicity of AgNPs to microorganisms. Our study does not identify a specific toxic mechanism of AgNPs, however we did find that the nanoparticles themselves exert a toxic effect in addition to producing toxic silver ions. This finding corroborates other studies such as a different freshwater algal species found to accumulate AgNPs, and the particles exerted toxic effects intracellularly[10]. Intracellular uptake of AgNPs has been reported in bacteria[5,15] and one study found limited uptake of AgNPs by C. reinhardtii[20]. Nanoparticle uptake may be greater for coated particles, like the citrate-coated AgNPs we

used, due to an interaction between the polymer coating and the cell surface[21]. Intracellular accumulation of AgNPs may enhance dissolution[22] or facilitate damage by reactive oxygen species (ROS) produced by the nanoparticles[13].

Algal-produced dissolved organic carbon mitigates the toxicity of AgNPs

None of these mechanisms of toxicity recognize the environmental feedback of the organisms on the nanomaterials themselves, and the mechanisms alone cannot explain the differential toxicity we observed. Intracellular differences and/or differences in the external environment of algal cultures during the three investigated growth phases might explain the patterns of response to AgNP exposure. Intracellular differences could arise because late-stage cells are no longer absorbing limiting nutrients and are dividing slowly. They also experience a different extracellular environment because they have produced more organic products, especially dissolved organic carbon (DOC), produced by the algae during photosynthesis[23]. For example, we measured extracellular concentrations of dissolved organic carbon and found an increase from 8.94 ± 0.004 mg-C/L in cultures on day 7 of growth to 22.5 ± 0.003 mg-C/L in cultures on day 19 of growth (average of three measurements ± standard error).

We favor the hypothesis that extracellular differences are the primary cause of the differential toxicity observed using exposure experiments that manipulated the DOC concentration of the environment. We centrifuged a culture in stationary growth phase, removed the supernatant containing organic material, and re-suspended the algal cells in synthetic freshwater media without nitrogen or phosphorus to reduce nutrient uptake. We then exposed the algal cells in this "new" stationary growth phase to 5 mg/L AgNPs and

found that the algal cells died within two days (Figure 3) – the same toxicity pattern as cultures in fast growth phase seen previously (Figure 1). Control cultures, which had also been centrifuged and re-suspended, persisted for at least 5 days (Figure 3). The differential response in initial toxicity between the stages of algal growth is explained primarily by differences in the extracellular environment, such as extracellular DOC.

DOC can mitigate the toxicity of AgNPs directly or indirectly. DOC could be promoting the formation of less-toxic aggregates[7] – AgNPs remained as single, unassociated particles in cultures in fast growth phase but aggregated in cultures in slowing and stationary growth (Figure S5). DOC has also been shown to physically interact with nanoparticles[5] and complex with Ag+[24], decreasing their toxicity[11,25], or by interrupting the mode of toxicity of both forms of silver. DOC could prevent a toxic effect on the algal cells by limiting particle-cell interactions[5,26] or uptake, or by acting as a sink for ROS[27]. Humic acids decreased the toxicity of AgNPs to Oryzias latipes embryos by coating the surfaces of the AgNPs and forming bridges between particles; an interaction that may disrupt the release of Ag+ from the particles or prevent the AgNPs from penetrating the embryos[28]. Algal-produced expolymeric substances from a marine diatom mitigated the toxic effect of Ag+, and the natural organic compounds used to complex with Ag+ may have actually coated the AgNPs themselves, protecting the diatom from AgNPs[11].

Dynamic model of feedback

We developed a dynamic, process-oriented model that demonstrates how the processes identified through our experiments and the feedbacks shown in Figure 4 could lead to the observed patterns in phytoplankton growth (Figure 1), in particular the “double

dip" in algal density that followed exposure in the later stages of batch culture. The model includes phytoplankton growth, DOC production, toxicity and dissolution of nanoparticles, bioaccumulation and the associated toxicity, and feedback on toxicity through two mechanisms: inactivation of ionic- and nano-silver by the phytoplankton-produced DOC. Toxicity is characterized as additional mortality with contributions from exposure to both bioaccumulated (ionic) and nano-silver. We used the model to illustrate the effects of the two inactivation mechanisms acting in concert and separately (Figure 5). Further details, model equations, fitting methodology, and parameter values are in the Supplementary Section 6 and Tables 1 and 2.

The model makes the following assumptions on processes, with the formulae implementing them detailed in the "Model Functions" section of Table 1:

- Phytoplankton growth. Chlorophyll a (Chl-a) is used as a surrogate for phytoplankton population size or biomass. The growth curve of the control population represents the combined effects of primary production and natural mortality; we use an empirical fit to this curve as the baseline for phytoplankton dynamics and then model additional mortality due to toxicity.
- DOC excretion. The rate of excretion of DOC from cells to the environment depends on the rates of photosynthesis, maintenance, and growth. In the absence of time resolved empirical data, we assume that the rate of DOC production can be described as a sum of a terms proportional to population size and growth rate.
- Dissolution of AgNPs and Bioaccumulation of dissolved silver. Both processes are described by first order kinetics.

- Toxicity of silver. Toxic effects are represented as additional mortality terms proportional to the concentration of AgNPs in the environment and to bioaccumulated ionic silver above a minimum no-effect "quota".
- Inactivation of nanoparticles and ions. DOC affects toxicity by reducing effective exposure. The inactivation rates are proportional to DOC concentration.

The model has a minimal representation of chemical processes, yet captures the dynamics remarkably well with a single parameter set (Figure 1) and enables us to distinguish effects of nano- and ionic toxicity (Figure 5). Seven parameters (dissolution of NPs (δ), deactivation rates of NPs and ions $(\gamma_{UN}, \gamma_{UI})$, nano-particle and ionic toxicities $(k_{NP,} k_q)$, no-effect quota (q_{NE}), and bioaccumulation rate (γ_q)) were estimated by minimizing the residual sum of squares between model output and Chl-a data for all three treatments simultaneously (see Table 2 for parameter values). The model captures the essentials of the dynamics of the system: phytoplankton populations recover if high DOC levels are present, and then - after a few days - decline again, with the slope of the decline smaller for higher DOC levels. It is sufficiently simple to be coupled as a module to existing nutrient-phytoplankton-zooplankton models[29], and thus to contribute to predicting effects of nanoparticle exposure in more complex food webs.

Conclusion

Through empirical and quantitative analyses, we found that AgNPs are more toxic to algal batch cultures in earlier stages than later stages of growth due to the protective effect of algal-produced DOC. It is worth noting that even though the concentration of AgNPs we

used (5 mg/L) is high compared to predicted environmental concentrations[3,4], we expect the mitigating effect of algal-produced DOC to operate at lower concentrations and lessen the toxicity of AgNPs to other freshwater organisms that can be more susceptible, such as zooplankton[30]. One study identified a mitigating effect of DOC on AgNP toxicity to Daphnia[31], and we found a qualitatively consistent protective effect of algal-produced DOC on the toxic effect of AgNPs to Daphnia pulicaria in preliminary studies (unpublished data). Algal productivity driving AgNP toxicity is particularly important considering the natural cycling of algal and zooplankton populations[32]. Our work emphasizes the importance of the effect of the focal organism on the toxicant and highlights the need for experiments exposing multiple species from the same environment to nanoparticles, since byproducts of one species may influence nanoparticle toxicity on all organisms in that environment.

Tables

Table 1. Simple dynamic model describing the feedbacks.

State	Φ	Chlorophyll-a in algal population (µg-Chl-a L^{-1})
	D	Dissolved organic carbon (DOC) concentration
	C_I	Concentration of dissolved silver (mg-Ag L^{-1})
	C_N	Concentration of bioavailable AgNPs (mg-Ag L^{-}
	C_U	Concentration of inactivated AgNPs (mg-Ag L^{-}
	q	"quota" of bioaccumulated Ag in algae (mg-Ag
Rates	μ	exposure-related specific mortality rate of algae
	J_{IN}	rate of dissolution of AgNPs (mg-Ag L^{-1} day^{-1})
	J_{UI}	rate of inactivation of ionic silver (mg-Ag L^{-1}
	J_{UN}	rate of inactivation of AgNPs (mg-Ag L^{-1} day^{-1})
	P	rate of production of DOC by algae (mg-C day^{-}
Balance Equations	$\frac{d\Phi}{dt} = \left(\frac{1}{\Phi_C} \frac{d\Phi_C(t)}{dt} - \Phi \right) \Phi$	Algae
	$\frac{dD}{dt} = P_D$	Dissolved organic carbon (DOC)
	$\frac{dC_N}{dt} = -J_{IN} - J_{UN}$	Bioavailable AgNPs
	$\frac{dC_U}{dt} = J_{UN}$	Inactivated AgNPs
	$\frac{dC_I}{dt} = J_{IN} - J_{UI}$	Ionic silver
	$\frac{dq}{dt} = \gamma_q C_I - q \frac{1}{\Phi} \frac{d\Phi}{dt}$	Quota
Model functions	$\mu = k_{NP} C_{NP} + k_q \left(q - q_{NE} \right)_+$	Exposure-induced mortality
	$J_{IN} = \delta C_N$	AgNP dissolution

$$J_{UN} = \gamma_{UN} DC_N$$ AgNP inactivation by DOC

$$J_{UI} = \gamma_{UI} DC_I$$ Inactivation of ionic silver

$$P_D = k_{D\Phi} \Phi_C + h_{D\Phi} \frac{d\Phi_C}{dt}$$ DOC production

$$\Phi_C = \frac{\alpha_1 t}{\alpha_3 + e^{\alpha_2 t}} e^{\alpha_2 t}$$ Chl-a in control population

Subscripts used are N:silver nanoparticles; I: ionic silver; U: non-bioavailable (inactivated) silver. In the balance equations, $J_{mn}(t)$ denotes the rate of transformation of silver in state *n* to state *m*. Parameters are defined in Table 2. Note: the notation $[x]_+$ means use the value of x if it is positive, otherwise set to zero.

Table 2. Initial conditions and parameter values for the model fits shown in Figures 1 and 5.

symbol	name	value
$C_N(0)$	Initial total silver	4.9965 mg-Ag L^{-1}
$C_I(0)$	Initial ionic silver	0.0035 mg-Ag L^{-1}
α_1	Parameter in phytoplankton production rate	19.34 μg-Chl-a day^{-1}
α_2	Parameter in phytoplankton production rate	1.287 day^{-1}
α_3	Parameter in phytoplankton production rate	240.9
$k_{D\Phi}$	Parameter in DOC production rate	0.0837 mg-C $(\mu g\text{-Chl-a})^{-1}$ day^{-1}
$h_{D\Phi}$	Parameter in DOC production rate	$9.77 \cdot 10^{-4}$ mg-C $(\mu g\text{-Chl-a})^{-1}$
δ	AgNP dissolution rate	0.0091 day^{-1}
γ_q	Silver bioaccumulation rate	$1.56 \cdot 10^{-4}$ L $(\mu g\text{-Chl-a})^{-1}$ day^{-1}
k_q	Toxicity parameter for bioaccumulated silver	$1.14 \cdot 10^{4}$ (μg-Chl-a) $(mg\text{-Ag})^{-1}$ day^{-1}
k_{NP}	AgNP toxicity parameter	0.692 L $(mg\text{-Ag})^{-1}$ day^{-1}
q_{NE}	No-effect quota	$8 \cdot 10^{-5}$ (mg-Ag) $(\mu g\text{-Chl-a})^{-1}$
γ_{UI}	Silver ion inactivation rate	0.0081 day^{-1}
γ_{UN}	AgNP inactivation rate	0.1788 day^{-1}

Parameters α_1, α_2, α_3 were estimated from the growth curves of the control cultures. Parameters $k_{D\Phi}$ and $h_{D\Phi}$ were calculated from measured DOC values. Other parameters were minimizing the residual sum of squares between model output and chlorophyll a data for all three treatments simultaneously.

Figures:

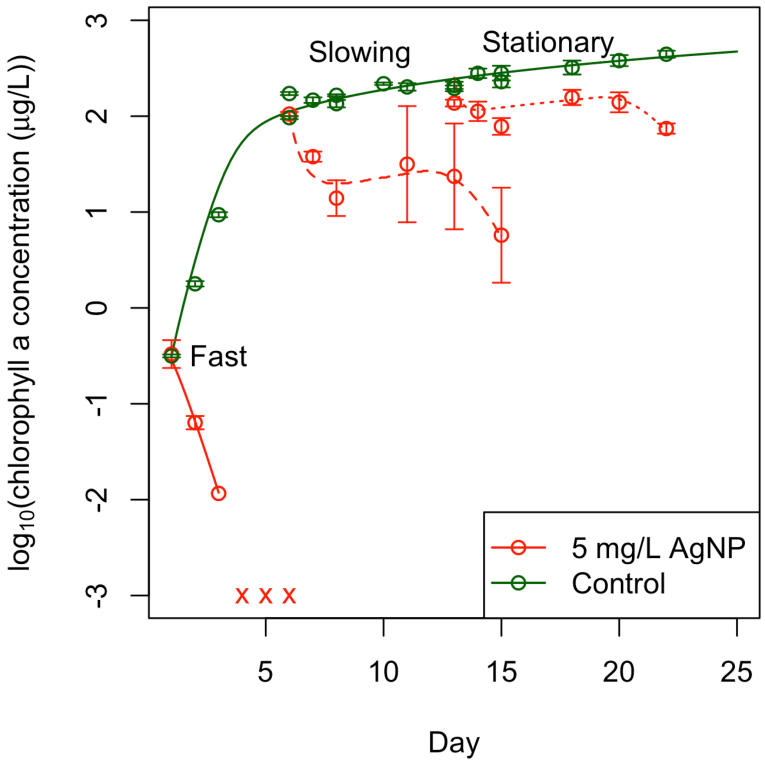

Figure 1. Citrate-AgNPs are more toxic to cultures in earlier stages of growth than in later stages and our dynamic model captures the algal dynamics. 5 mg/L citrate-coated AgNPs were introduced to *C. reinhardii* batch cultures at three different stages of growth: fast growth (solid lines), slowing growth (large dashes), and stationary growth (small dashes). The dynamic model developed through analyses of these data captures the algal dynamics well with a single parameter set (lines). Batch cultures in slowing and stationary growth phases had grown for one and two weeks, respectively, prior to the start of the experiment and before the introduction of AgNPs. Time in this figure is represented as the absolute day of growth of the culture – all cultures were exposed on the same day of the experiment but on different days of growth (cultures in fast growth phase were dosed with AgNPs on day 1 of growth, cultures in slowing growth phase were dosed on day 6 of growth, and cultures in stationary growth phase were dosed on day 13 of growth). AgNPs caused complete mortality of cultures in fast growth phase within two days of introduction. For these cultures, chlorophyll measurements were below detectable limits (denoted by x) by day 3 but the culture was sampled through day 6. We measured concentrations of chlorophyll a to indicate algal cell viability and response to AgNPs because we empirically confirmed that chlorophyll a/cell ratios remain constant after day 5 of growth in algal cultures grown in the light and temperature environments used in this experiment. However, AgNPs had an initial toxic effect on cultures in slowing and stationary growth phases from which the cultures were able to recover until they declined again on days 8 and 10 of the experiment (days 13 and 15 of growth for cultures in slowing phase and days 20 and 22 of growth for cultures in stationary growth phase). The data points are averages from three replicate cultures and the error bars reflect their standard error.

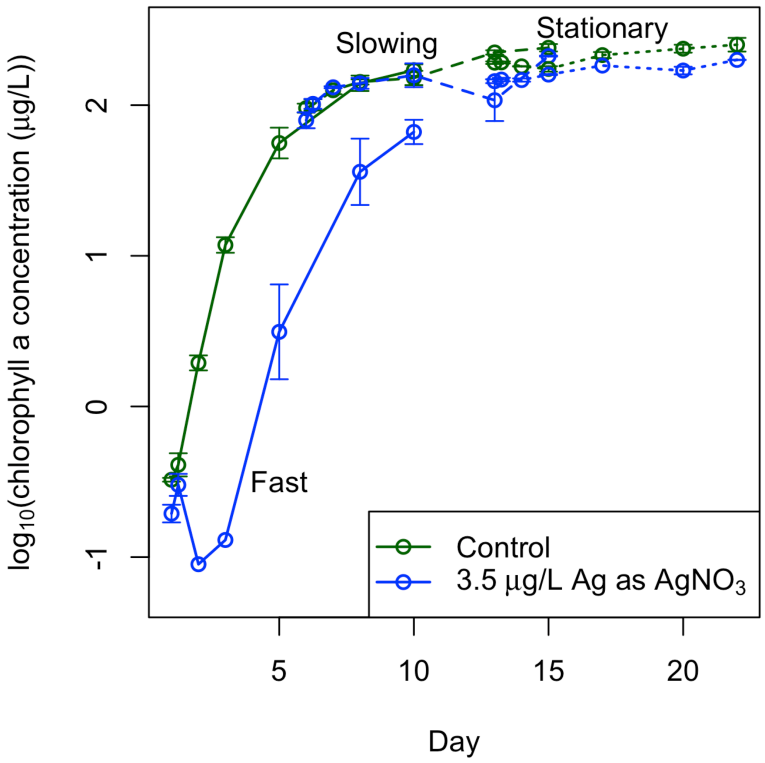

Figure 2. 3.5 µg/L Ag^+ has little to no effect on late stages of batch culture growth. 3.5 µg/L Ag^+ in the form of $AgNO_3$ was introduced to batch cultures in the same way as described in Figure 1. This concentration caused initial toxicity to cultures in fast growth phase, but these cultures were able to recover to the level of the control cultures in fast growth phase. This concentration had no visible effect on cultures in slowing and stationary growth phases. The data points are averages from three replicate cultures and the error bars reflect their standard error. The lines just connect the data points and help differentiate between culture in fast (solid), slowing (large dashes), and stationary (short dashes) growth phases.

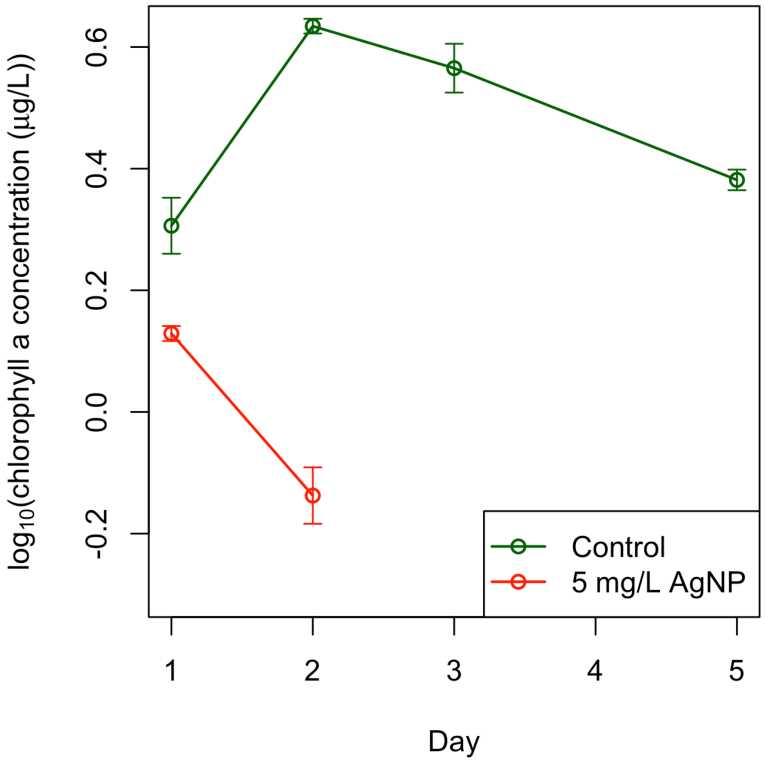

Figure 3. Removal of DOC from algal cultures in stationary growth restores the toxicity of AgNPs. AgNPs cause complete mortality of cells in stationary growth (red) after removal of organic material and resuspension of algal cells in media without nutrients. Control cultures (green), which were also centrifuged and resuspended in media without nutrients, persisted for at least 5 days. This pattern of toxicity is very similar to the rate of decline of cultures in fast growth phase exposed to 5 mg/L AgNP (Figure 1). This finding indicates that the difference in toxicity observed between growth stages of the algae is most likely due to differences in the extracellular environments of the growth stages. The data points are averages from three replicate cultures and the error bars reflect their standard error.

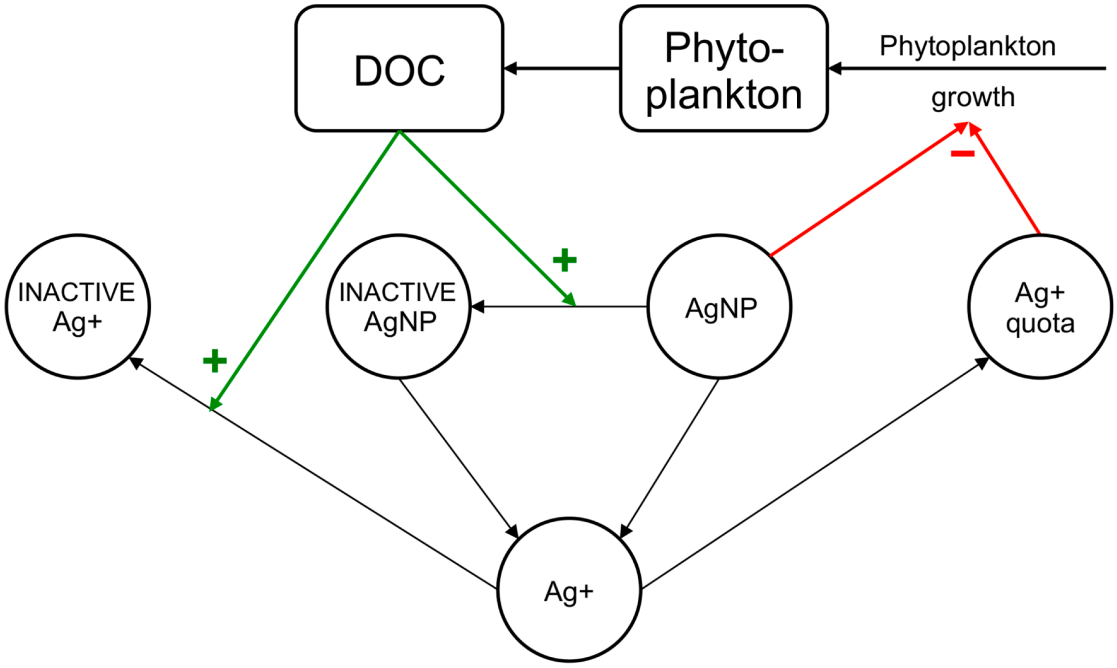

Figure 4. Schematic of dynamic model of environmental feedback. Phytoplankton grow and produce DOC, which inactivates AgNPs and silver ions (Ag^+). Both active and inactive AgNPs dissolve, introducing Ag^+ into the environment. Environmental Ag^+ is either made inaccessible to phytoplankton (inactivated Ag^+) or bioaccumulated by the phytoplankton (entering the Ag^+ quota). The bioaccumulated Ag^+ and the still active AgNPs exert toxic effects on the phytoplankton.

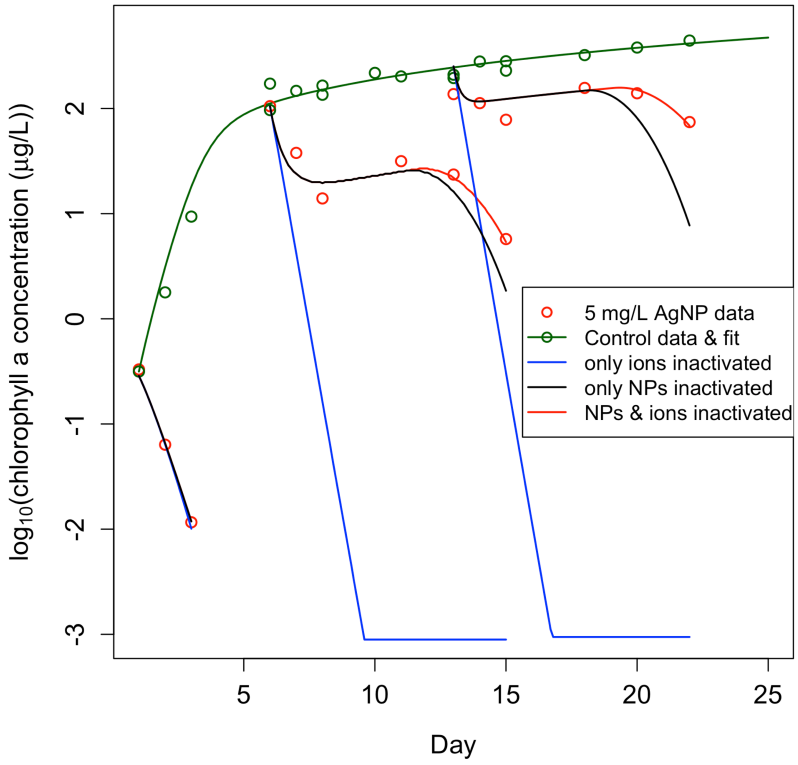

Figure 5. Model predictions with inactivation mechanisms of DOC on AgNP and Ag^+ separately and in unison. Model simulations demonstrate the significance of DOC inactivation of AgNPs *and* Ag^+ (red lines). The simulations suggest that DOC mitigation of nanotoxicity provides a much stronger feedback than mitigation of ionic toxicity: while the model without ionic mitigation (black lines) generally follows the observations and only predicts the second dip slightly sooner, the model without AgNP inactivation (blue lines) radically departs from the observations, with the population going extinct by day three of the exposure**.**

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II. Standardized toxicity testing may underestimate nanotoxicity: Environmentally-relevant food rations increase silver nanoparticle toxicity to *Daphnia*

Authors: Louise M. Stevenson, Katherine E. Krattenmaker, Erica Johnson, Alexandra J. Bowers, Adeyemi S. Adeleye, Edward McCauley, Roger M. Nisbet

Abstract

Daphnia in the natural environment experience fluctuations in algal food supply, such as periods when algal populations bloom and decline or seasons of the year when *Daphnia* have very little algal food. Standardized chronic toxicity tests, used for ecological risk assessment, dictate that *Daphnia* must be fed up to 400 times more food than they would experience in the natural environment (outside of algal blooms) in order for a toxicity test to be valid. This disconnect can lead to underestimating the toxicity of a contaminant. We followed the growth, reproduction, and survival of *Daphnia* exposed to 75 and 200 μg/L silver nanoparticles (AgNPs) at four food rations for up to 99 days and found that AgNP exposure at low, environmentally-relevant food rations increased AgNP toxicity. AgNP exposure at low food rations decreased the survival and/or reproduction of individual *Daphnia*, and these concentrations are predicted to have consequences at the population level (based on calculated specific population growth rates, r). We also found tentative evidence that a sublethal concentration of AgNPs (75 μg/L) caused *Daphnia* to alter energy allocation away from reproduction and towards survival and growth. Although AgNP toxicity to *Daphnia* has been widely studied, our study is the first to

consider AgNP exposure in the ecological context of environmentally-relevant food rations. Our findings emphasize the need to consider feedbacks that determine the density of food an organism may experience in the environment when estimating the predicted effect of a toxicant in natural systems.

Introduction

Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials in consumer products (Vance et al., 2015) and are predicted to contaminate aquatic environments (Keller and Lazareva, 2014). AgNPs are used for their antimicrobial toxicity, and so concern about their environmental presence is high – a recent meta-analysis of nano-ecotoxicity studies of silver, copper, and zinc nanoparticles found that AgNPs were the most commonly studied "by far", and most studies are freshwater exposures (Notter et al., 2014). Our study adds to the growing body of work on AgNP toxicity on freshwater systems, however unlike most other studies we consider AgNP toxicity in an ecological context by feeding the *Daphnia* environmentally-relevant concentrations of algae. While there are many studies investigating AgNP toxicity to *Daphnia*, ours is the first to use food rations that fall in the range of algal concentrations *Daphnia* typically experience in nature (McCauley and Murdoch, 1987; Murdoch et al., 1998).

The amount of food *Daphnia* eats directly affects growth and reproduction – during periods of starvation, a daphnid will slow or halt reproduction (Bradley et al., 1991) but can also produce hundreds of eggs over its lifetime given abundant food. During the summer, average algal densities can vary between 0.009 (McCauley and Murdoch, 1987) -

0.07 mgC/L (Murdoch et al., 1998) and the average summer clutch size of an adult daphnid is less than 1 egg (McCauley and Murdoch, 1987). OECD (Organisation for Economic Co-operation and Development) guidelines for a standard test on the effect of a chemical on *Daphnia magna* reproduction require that adults produce, on average, at least 60 neonates over 21 days in the control treatment for the test to be valid and recommend food rations between 0.1-0.2 mgC/daphnid/day (OECD, 2012). These food rations correspond to between 1-4 mgC/L of algae fed to *Daphnia* that must sustain an average brood size of 6-16 eggs/clutch in order for the test to be valid (*Daphnia* molt on average every 1.5-3 days and typically reproduce after 6-10 days of growth in lab experiments (Ananthasubramaniam et al, 2015)). Outside of periods of algal blooms, *Daphnia* in the natural environment experience as low as 400 times less food than those used in OECD toxicity tests. This disconnect between experimental protocols and the natural environment is troubling, especially considering that the NOEC/LC/EC50 values that are calculated as a result of these tests are used in ecological risk assessment to predict a no-effect concentration or species sensitivity distribution of a potential contaminant (Calow and Forbes 2003). Further, metrics such as LC/EC50 values are dependent on the experimental setup, such as the length of the experiment (Khan, 2015) and amount or type of food given (Allen, 2010; Naddy, 2011).

There is an abundance of studies on the effect of silver to *Daphnia* and other zooplankton in the scientific literature. Most studies have found that silver ions (Ag^{+}) (Bianchini and Wood, 2003; Erickson et al., 1998; Griffitt et al., 2008; Hook and Fisher, 2001; Khangarot and Ray, 1989; Li et al., 2010; Naddy et al., 2007; Peckova et al., 2009; Ribeiro et al., 2013; Rodgers et al., 1997) and AgNPs (Griffitt et al., 2008; Hoheisel et al.,

2012; Qin et al., 2015; Ribeiro et al., 2013; Stensberg et al., 2014; Ulm et al., 2015) are toxic at ppb concentrations to *Daphnia*, and there is agreement in the literature that Ag^+ is more toxic at equimolar silver concentrations than AgNPs (see Ivask et al., 2014 for a review of the literature). Longer (chronic) experiments have observed reductions in growth and/or reproduction of *Daphnia* exposed to Ag^+ (Bianchini and Wood, 2002) and AgNPs (Bianchini and Wood, 2002; Mackevica et al., 2015; Ribeiro et al., 2013; Sakamoto et al., 2015; Volker et al., 2013; Zhao and Wang, 2011). However, all of these studies fed *Daphnia* unrealistically high concentrations of food. Most studies on the effects of silver toxicity to *Daphnia* follow OECD guidelines (OECD, 2012) and feed *Daphnia* the recommended amount of carbon as algae (Ribeiro et al., 2013; Sakamoto et al., 2015; Volker et al., 2013; Zhao and Wang, 2011). Even more studies feed the *Daphnia* a mix of algae and other additives such as trout chow, yeast, alfalfa, wheat grass, and/or *Daphnia* food pellets (Allen et al., 2010; Bianchini and Wood, 2002; Glover and Wood, 2004; Hook and Fisher, 2001; Mackevica et al., 2015; Naddy et al., 2011; Pokhrel and Dubey, 2012; Qin et al., 2015). All of these studies reported the amount of food given as a volume (e.g. a defined volume of a "mixture of *Chlorella autotrophica* and *Daphnia* pellets" (Hook and Fisher, 2001) or of "a 3:5 mixture of yeast, wheat grass, and trout chow (YCT) and algae" (Naddy et al., 2011)), such that calculation of the amount of carbon fed is impossible, while another study simply failed to report the amount of food given altogether (Kolkmeier and Brooks, 2013). However, we can get an idea of the carbon load these *Daphnia* are receiving based on the average reproduction of control individuals – these individuals produced 2.3 - 45 neonates/brood (Bianchini and Wood, 2002; Glover and Wood, 2004; Hook and Fisher, 2001; Qin et al., 2015) compared to an average summer

clutch size of an adult daphnid in the natural environment of less than 1 egg per brood (Mccauley and Murdoch, 1987).

In addition to the fact that these high food rations are environmentally unrealistic, the amount of food present can have effects on AgNP toxicity, further complicating the extrapolation of results from these laboratory studies to the effects of AgNPs in the environment. Studies have found that the presence of high food levels drastically reduces the acute toxicity of AgNPs to *Daphnia* (Allen et al., 2010; Naddy et al., 2011), potentially due to decreased bioavailablity of the particles. Further, our past work identified a feedback in which algal-produced dissolved organic carbon decreased the toxic effect of AgNPs to the algal cells themselves (Stevenson et al., 2013), and this mitigating effect could extend to zooplankton (Kennedy et al., 2012). However, algae can also provide another route for AgNP toxicity – algae accumulate silver from Ag^+ and AgNP exposure, which can lead to dietary exposure (Lam and Wang, 2006; Zhao and Wang, 2010). One study found that over 80% of the silver from AgNPs were found in *Chlamydomonas reinhardtii* cells after 5 days of exposure (McTeer et al., 2014). Dietborne silver is an important exposure route for *Daphnia* – one study found that more than 70% of AgNPs that accumulated in *Daphnia* did so through algal ingestion (Zhao and Wang, 2010). Further, AgNPs can decrease daphnid feeding rates (Ribeiro et al., 2013), potentially increasing stress at low, environmentally-relevant food rations.

In our study, we expose daphnid individuals to AgNPs at environmentally-relevant food rations. We follow the survival, growth, and reproduction of individuals at four food rations, the highest of which is close to an OECD-accepted food ration. Our data show that environmentally-relevant food rations increase the effect of the nanoparticles on *Daphnia*.

Our work emphasizes the need to consider the ecology of the test organism when conducting toxicity tests, since these data may be used in ecological risk assessment.

Methods

Test organisms and media

We used *Daphnia pulicaria* clones drawn from shallow lakes in Alberta, Canada. Laboratory stocks of *Chlamydomonas reinhardtii* (UTEX 90, purchased from the Chlamydomonas Center) were used to feed the *Daphnia*. COMBO media (Kilham et al., 1998) was used to grow algae and "low-P COMBO media" (Kilham et al., 1998) was used to maintain daphnid cultures and for experiments involving *Daphnia*. COMBO media and "low-P COMBO media" have the same base media recipe, however there is less phosphorus and nitrogen in the low-P recipe (Kilham et al., 1998).

Silver nanoparticle characterization

We purchased 40 nm BioPure™ citrate-coated silver nanoparticles from NanoComposix (San Diego, CA). To determine the behavior of AgNPs in these *Daphnia* experiments, we measured the size, dissolution and abiotic reactive oxygen species (ROS) production. We had to conduct separate experiments for these measurements because we had to use higher concentrations of AgNPs than those we used for our daphnid exposures, since the ppb exposures we used in these studies are too low to ensure reliable estimates. The methods and results of these experiments are reported in the Supplementary Information Section 1. One of the aims of these measurements was to test whether AgNPs act differently in the dark versus in the light (form smaller or larger aggregates, produce

more or less ROS, or dissolve more or less Ag^{+}), since we kept our daphnid exposures in the dark to limit phytoplankton growth (which would change the food ration between transfer intervals). AgNPs are smaller in diameter when kept in the light, however they do not produce ROS in the light or in the dark and dissolve at approximately the same rate when kept in the dark versus under lights (see SI Figures 1-3).

Exposure of individual *Daphnia* to AgNPs

We exposed individual *Daphnia* to 75 and 200 μg/L AgNPs and followed their growth, reproduction, and survival. We removed *Daphnia* from our daphnid stock tanks 5 days before the start of the experiment and placed them in clean tanks with autoclaved "low P" COMBO media. Two days later, we moved these individuals to a second clean tank to ensure that the individuals were not carrying additional food or other detritus into the experimental containers at the start of the experiment. We selected the individuals going into the experiments from these "clean" tanks based on size – *Daphnia* started the experiment as neonates (less than 24 hours old, all 0.695-0.705 mm) or as adults (1.95-2.05 mm). At the three lowest food rations, all of the individuals started as neonates and at the highest food ration, half of the individuals started as neonates and half as adults.

During the experiment, individual *Daphnia* were kept in 30 mL of autoclaved "low P" COMBO media with transfers to fresh media on a Monday-Wednesday-Friday schedule. On transfer days, we poured *Daphnia* out of the experimental chambers onto 60 micron nylon mesh (Millipore nylon net filter) and then visually inspected each individual using a dissecting scope (Leica M80) to confirm survival, count eggs or embryos present in the brood pouch, and count free-swimming neonates. Any offspring that were produced

were removed. We measured the length of the *Daphnia* (from the bottom of the antennae [top of the head] to the base of the spine) using Leica microscope software every transfer day for the three lowest food rations tested, and at three points during the experiment at the highest food ration. We then transferred the *Daphnia* into fresh media dosed with AgNPs and added algae. The *Daphnia* were kept in the dark to minimize phytoplankton growth between transfers and were maintained at an average temperature of 22 deg C ± 1 deg C (standard deviation). We also measured the amount of food not consumed by the *Daphnia* by analyzing the chlorophyll a concentration in the media after transferring the daphnid (chlorophyll a measurement methods in Stevenson et al., 2013).

We fed the *Daphnia C. reinhardtii* from standardized cultures (10-12 days old - the algal batch cultures are entering stationary growth phase during this time period), since per cell nutrient and lipid concentrations may vary across algal growth stages. We concentrated the algal cells from a batch culture by centrifuging the 500 mL culture for 4 minutes at 7,000 rpm (Eppendorf 5430R Centrifuge), pouring off the supernatant, and re-suspending the pellet in water. We then measured the concentration of chlorophyll a fluorometrically using a microplate reader as described in Stevenson et al., 2013. We converted this chlorophyll a concentration to a carbon concentration based on a measured carbon::chlorophyll a ratio of *C. reinhardtii cells* determined empirically in our lab: 0.22 ± 0.02 mgC/µg chlorophyll a (standard error) and calculated the volume of cells to add to get the desired carbon ration. We then multiplied this volume by the number of transfer days that were to elapse before the next sampling day and fed the *Daphnia* food rations of 0.0005, 0.001, 0.0025, or 0.01 mgC/daphnid/day. We also measured the carbon content of the food for a few sampling days using a Flash 2000 CN analyzer (Thermo Scientific) to

confirm we were feeding the *Daphnia* the carbon rations were intended (unpublished data).

The data reported in this paper are from two separate experiments with the same AgNP exposures (75 and 200 μg/L AgNPs) but fed different food rations. During the first experiment, the *Daphnia* started as neonates (less than 24 hours old, all around 0.7 ± 0.05 mm) or as adults (2.0 ± 0.05 mm) and we fed them 0.01 mgC/daphnid/day. All of the *Daphnia* in the second experiment started as neonates (less than 24 hours old, all 0.7 ± 0.05 mm) and we fed them 0.0005, 0.001 and 0.0025 mgC/daphnid/day. We conducted the first experiment (0.01 mgC/daphnid/day food ration) for 26 days and we continued the second experiment (all other food rations) for the entire lifetime of the individuals (99 days for the longest living individual).

Neonate survival experiments

All neonates produced by the *Daphnia* in our experiments were removed from the experimental chambers each transfer day, and neonates from the lowest food rations were disposed of. Neonates of individuals fed the highest food ration (0.01 mgC/daphnid/day) were transferred to a survival experiment to test for differences in reproductive investment of daphnid mothers exposed to different concentrations of AgNPs. Neonates were placed into sterilized tubes containing 5 mL of "low P" COMBO media and placed in the dark without food. The individuals were visually checked every day, and mortality was confirmed using a dissecting scope.

Calculation of *r*

We estimated the intrinsic rate of increase (r) of a daphnid population at each food ration and AgNP exposure by solving the Euler equation (Equation 1):

$$1 = \sum_{x=0}^{k} e^{-rx} l(x) b(x) \qquad (1)$$

We calculated the probability an individual survives from birth to age x ($l(x)$) by dividing the number of individuals that are alive at age x by the size of the initial cohort (S(x) / S(0)) (Gotelli, 1995). The fecundity schedule at age x ($b(x)$) is the average number of offspring born per day to a female of age x (Gotelli, 1995), which we found by averaging the number of neonates produced by each individual for each sampling day and dividing by the number of days that had elapsed since the last transfer. We found the value of r by numerically solving the Euler equation (Equation 1) using SOLVER in Excel.

Statistic analysis

We calculated the mean survival time and its standard error from our data using a Kaplan-Meier estimator, a nonparametric statistical routine that fits a survival function to data using maximum likelihood (Kaplan and Meier 1958). We chose to use Kaplan-Meier because it can take censored data into account, such as our survival data at the highest food ration (0.01 mgC/daphnid/day). The data set at our highest food ration is right-censored because we did not follow the individuals until they died (the experiment ran for 26 days), unlike the other food rations. We conducted this analysis using the "survival" package in R (version 3.2.3).

We analyzed the time it took for neonates to succumb to starvation from mothers exposed to 0 or 200 μg/L AgNP in two separate tests – we compared survival time of neonates from mothers that started the experiment as neonates and the survival time of

neonates from mothers that started as adults separately. We did not statistically analyze the difference in neonate survival times from mothers exposed to 0 and 75 μg/L AgNP because they are the same (SI Figure 10). We tested for significant differences between these groups using a Mann-Whitney U-test because these are discrete data (we checked once daily, so a neonate could not survive 6.5 days since we only checked for survival on days 6 and 7) and the sample size for one of the groups was less than 30. We conducted this analysis using the base "statistics" package in R (version 3.2.3).

Results

Less food leads to slower growth and depressed reproduction

Less food led to lower survival rates, slower growth to smaller maximum sizes, a delay in reproduction, and less reproduction comparing control individuals across food treatments (Figures 1-3, SI Figure 9). *Daphnia* did not reproduce at the two lowest food rations (0.0005 and 0.001 mgC/daphnid/day) we used in our experiment (except for one individual exposed to 75 μg/L AgNP who laid two clutches of three neonates total, and only one clutch developed into viable offspring). In addition to these direct effects on survival, growth and reproduction, low food rations increased the negative effects of AgNP exposure on *Daphnia*.

200 μg/L AgNPs decrease survival but individuals exposed to 75 μg/L live longer and grow to a larger size

200 μg/L AgNPs cause mortality at all food rations (Figure 1), however this toxicity is much stronger at lower food rations – the average lifetime of individuals

exposed to 200 μg/L at the lowest food rations (0.0005 and 0.001 mgC/daphnid/day) is 8 days compared to 13.5 and 14.3 days at the higher food rations (0.0025 and 0.01 mgC/daphnid/day) (Table 1). As a result of this shortened lifetime, individuals exposed to 200 μg/L grew to a much smaller maximum size (Figure 2). Individuals exposed to 75 μg/L AgNP lived *longer* and grew to a *larger* size than control individuals (Table 1, Figures 1 and 2). 75 μg/L AgNP exposed individuals grew more slowly than controls, however this increased survival allowed them to grow to a larger maximum size (Figure 2).

AgNPs decreased reproduction at lower food rations that is partially restored by higher food

Exposure to 200 μg/L almost completely halted reproduction – only three individuals out of the 29 exposed to this concentration reproduced across all food rations (Figure 4), all at the highest food ration, and only one individual produced more than 2 viable neonates (Figure 3). Exposure to a lower concentration of AgNPs, 75 μg/L, also had a negative effect on reproduction (Figures 3 and 4) that was relieved by increasing amounts of food. This pattern is striking when comparing the proportion of individuals that reproduced (had eggs present in brood pouch) at the two highest food rations (Figure 4). The negative impact of AgNPs on daphnid reproduction increases at lower food rations at both exposure concentrations – for 200 μg/L exposure, no individuals reproduced except at the highest food ration (Figure 4) and increased food restored reproduction at the highest food ration of individuals exposed 75 μg/L AgNPs on (Figures 3 and 4). Individuals exposed to 75 μg/L AgNPs reproduced less and later at lower food rations – at

0.0025 mgC/daphnid/day, control individuals first produced eggs on day 18 while 75 μg/L-exposed individuals did not reproduce until day 22 (SI Figure 9).

AgNPs have no effect on survival, growth, or reproduction of adult individuals fed highest food ration

At the lowest food rations, all individuals started the experiment as neonates, however individuals fed 0.01 mgC/daphnid/day either started the experiment as neonates or adults. Individuals that started as adults were much less susceptible to the effects of AgNPs than those that started as neonates – survival (SI Figure 6), growth (SI Figure 7), and reproduction (SI Figure 8) were similar across all AgNP concentrations. Other studies have found that organisms are more vulnerable to toxicity at younger life stages, including AgNPs (Sakamoto et al., 2015), and this information is crucial when considering population-level consequences of AgNP toxicity.

AgNP exposure does not a significantly change investment per offspring

We tested how long it would take offspring of *Daphnia* exposed to AgNPs and fed the highest food ration (0.01 mgC/daphnid/day) to starve, and we found a trend that neonates of mothers exposed to the highest concentration of AgNPs (200 μg/L) starved, on average, a half to almost a full day sooner than those of mothers in the control or lower AgNP (75 μg/L) treatment (SI Figure 10). This effect is not statistically significant when comparing survival times of neonates from mothers that started the experiment as adults ($p = 0.06$) or from mothers that started the experiment as neonates ($p = 0.11$). Offspring of *Daphnia* that began the experiment as neonates and were exposed to 200 μg/L starved the

fastest, further demonstrating that AgNPs have a greater effect on younger life stages (SI Figure 10). The difference between the survival time of neonates from mothers that began the experiment as adults exposed to 0, 75, and 200 μg/L is on the cusp of statistical significance ($p = 0.06$), and this is particularly interesting considering that AgNPs did not have an effect on adult survival, growth, or reproduction (SI Figures 6-8) but we do see a marginal effect on the ability of their offspring to survive starvation. This could indicate daphnid mothers exposed to 200 μg/L AgNPs may be investing a little bit less energy in their eggs, meaning that their offspring cannot survive starvation as long as those of mothers at lower AgNP exposures. This is in agreement with a study that found that silver exposure caused daphnid mothers to produce eggs with a lower protein concentration than control individuals (Hook and Fisher, 2001). This finding also indicates the potential for multi-generational effects of AgNPs, which has been found in other studies (Naddy et al., 2011; Volker et al., 2013).

Discussion

Lower food rations increase AgNP toxicity

Low, environmentally-relevant food rations exacerbate the negative effects of AgNP exposure on *Daphnia*. 200 μg/L AgNPs are more toxic and have a stronger negative effect on reproduction at lower food rations (Figures 1 and 3). 75 μg/L AgNPs depress reproduction more at lower food rations, however they also increase survival and growth (see section 4.2 for discussion of this seemingly positive effect).

One hypothesis for the mechanism through which AgNP toxicity was stronger at lower food rations could be that AgNPs somehow hinders uptake of food by *Daphnia*,

which has a greater effect on the survival of individuals fed lower food rations than those fed higher food rations. Multiple studies have found that AgNPs decrease the feeding rate of *Daphnia*, either due to sedimentation of their algal food, decreased palatability of the algae from Ag exposure, or accumulation of nanoparticles in the gut or on appendages of *Daphnia* making it harder to feed or eliminate particles (McTeer et al., 2014; Ribeiro et al., 2013; Zhao and Wang, 2011). However, we measured the amount of food not consumed by each daphnid individual and, while there is a lot of variation through time, there is not a significant pattern that would suggest that the AgNPs systematically affect feeding rates of the *Daphnia* (SI Figures 4 and 5).

Many other studies have found that very low concentrations of silver (0.05 - 6 μg/L) decrease daphnid reproduction (Sakamoto et al., 2015; Volker et al., 2013), and some of these report decreased reproduction at concentrations that do not cause significant mortality or have any observable effect on zooplankton growth (Bianchini and Wood, 2002; Hook and Fisher, 2001; Qin et al., 2015; Ribeiro et al., 2013), similar to our results for the highest food rations studied.

Like us, Mackevica et al. (2015) found that lower concentrations of food increased the effect of citrate-coated AgNPs. They fed Daphnia at two food rations described as "low food" and "high food", but the "low food" treatment was higher than the amount of minimum food recommended by the OECD protocol at 0.25 mgC/daphnid/day. Their "high food" treatment was three times that low food ration and was sufficiently high that the Daphnia in the AgNP exposed treatments "had algal cells adhered to their exoskeleton, which resulted in some immobility" (Mackenvica et al. 2015). The authors found that at their low food treatment, *Daphnia* exposed up to 50 μg/L AgNPs can still survive,

however they do not reproduce at the highest AgNP concentration (Mackevica et al., 2015). Individuals at the high food treatment were able to reproduce at all AgNP exposures (10-50 μg/L of 30 nm citrate-AgNPs), similar to our finding that increased food concentration restores reproductive suppression by AgNPs (Mackevica et al., 2015). However, the food rations in Mackevica et al. (2015) resulted in the *Daphnia* producing an estimated 14 – 34 neonates/brood at "low" and "high" food respectively, compared to the average summer clutch size of less than 1 egg per daphnid in natural freshwater systems (Mccauley and Murdoch, 1987).

The effect of low food on individual daphnid reproduction can also have significant effects on populations of *Daphnia*. We calculated the specific population growth rate (*r*) of *Daphnia* at every food ration and AgNP concentration. This metric predicts the growth of a population at each AgNP exposure and food ration. If r is greater than 0, the population will grow exponentially. If r is less than 0, the population will decline to extinction (Gotelli 1995). At 0.0025 mgC/daphnid/day, we predict that populations exposed to 75 and 200 μg/L AgNP would decline to extinction while a control population at this food ration would grow (Figure 5). It is impossible to calculate a value for r when we did not observe any reproduction (such as the 200 μg/L AgNP exposure at 0.0025 mgC/daphnid/day), however the lack of reproduction indicates that a population would go extinct. At the highest food ration, 200 μg/L AgNP exposure still has a negative effect on population growth but the population is still predicted to grow ($r = 0.084$ day^{-1}). At this food ration, control populations are predicted to grow twice as fast as 200 μg/L-exposed populations ($r = 0.168$ day^{-1} for controls) and the 75 μg/L exposure group now marginally surpasses the controls ($r = 0.173$ day^{-1} for controls; Figure 5). It is important to

note that the data for the 0.0025 mgC food ration covers the entire lifetime of the *Daphnia,* while the 0.01 mgC food ration experiment only lasted 26 days. However, Porter (1983) demonstrated by truncating a complete data set at intervals that a reliable estimate of r can be calculated after only 21 days after high food rations, as opposed to low food rations which needed at least 25-30 days of data for a reliable estimate of r. According to these estimates of population growth, lower food rations can doom a daphnid population to extinction from low concentrations of AgNPs. However, there are feedbacks at the population level that complicate this extrapolation, which we discuss briefly at the end of this manuscript.

75 µg/L AgNP exposure – hormetic effect or allocation shift?

Individuals exposed to 75 µg/L of AgNPs had higher survival and grew to a larger maximum size than controls (Figures 1 and 2). The only negative effect of 75 µg/L is on reproduction – at the 0.0025 mgC/daphnid/day fewer individuals exposed to 75 µg/L AgNPs reproduced (Figure 4) and they reproduced less (Figure 3) and later (SI Figure 9).

Increased survival and growth at 75 µg/L AgNPs could be due to a hormetic response (a positive response to a low concentration of a toxicant) either through the addition of a small amount of carbon from the citrate-coating of the particles or a hormetic effect of the silver itself. At the low food rations at which we fed these *Daphnia,* the citrate coating on the AgNPs could serve as a significant carbon source. The 75 µg/L AgNP exposure added 0.326 µg C at each transfer, which may be a significant amount of carbon, especially for individuals at our lowest food rations (0.5 and 1 µg C/daphnid/day). Another hypothesis is that low levels of silver may have a hormetic response in *Daphnia*.

Previous studies have found a positive response of *Daphnia* to low levels of Ag^+ and AgNPs (Glover and Wood, 2004; Hoheisel et al., 2012; Pokhrel and Dubey, 2012) and that reproduction and survival decrease when silver is removed (Rangwala and Keating, 2008). The authors suggest that silver is a "nutritional requirement…at nanomolar concentrations" (Rangwala and Keating, 2008) and we measured dissolved silver concentrations in our media to be at the nanomolar level and below (see SI Section 1).

However, hormesis does not explain why exposure at 75 μg/L AgNP levels decreased reproduction along with increasing survival and growth. This implies that individuals diverted some energy from reproduction to growth and to enhancing survival. Organisms are able to shift energy allocations based on environmental conditions – in response to changes in photoperiod, pond snails altered reproduction by either reproducing until they died or suppressing reproduction to extend their own survival (Zonneveld and Kooijman 1989). From the start of the exposure, *Daphnia* exposed to 75 μg/L AgNPs appear to shift energy away from reproduction (causing them to reproduce later and produce fewer offspring) and towards survival (see the slower initial mortality of 75 μg/L exposed individuals compared to controls in the first 20-40 days in Figure 1). Interestingly, this allocation shift seems to disappear at higher food rations – at 0.01 mgC/daphnid/day, individuals exposed to 75 μg/L had only slightly higher survival (the difference between controls and 75 μg/L is one individual in Figure 1), similar growth to controls, and reproduced at the same time as control individuals with a slightly *higher* proportion of individuals reproducing. This indicates that the stress associated with 75 μg/L AgNP exposure that induces a shift in energy is alleviated by higher food concentrations.

The positive effect on survival and growth and the negative effect on reproduction of 75 μg/L may be the result of alterations of the daphnid microbiome, either through changes in the bacterial community due to AgNP toxicity or through a change in the microbiome influencing how *Daphnia* allocate energy. *Daphnia* are able to quickly respond to subtle changes in the environment, such as moving from areas of low quality to high quality food in less than ten minutes (Schatz and McCauley, 2007). This rapid awareness of environmental factors could influence how *Daphnia* allocate energy and the daphnid microbiome may be crucial in these decisions. *Daphnia* have a bacterial community that resides in its gut and aid digestion that has recently been characterized (Freese and Schink, 2011) and AgNPs have been shown to accumulate in daphnid digestive tracts (Khan et al., 2015). AgNPs may be exerting toxicity on the bacteria there, altering the composition of the bacterial community due to either differential sensitivity between bacterial species or by changing the competitive balance between bacteria. Altered bacterial composition of the daphnid microbiome could lead to differential nutrient uptake compared to the microbiome of control individuals. Glover and Wood (2004) found that low concentrations of silver increased reproduction and proposed that the anti-bacterial activity of silver altered the microbiological fauna in the daphnid gut. The authors proposed that altering the community of bacteria could result in "enhanced nutritional status" that could lead to higher energy input, causing higher reproduction in silver-exposed individuals (Glover and Wood, 2004). Alternatively, a recent study found that antibiotic exposure changed the bacteria in the daphnid gut from a balanced community to one dominated by a few species, leading to lower incorporation and digestion efficiencies in exposed individuals (Gorokhova et al., 2015). Our knowledge of

the daphnid microbiome, its influence on energy allocation or assimilation of food, and how toxicants can affect it and thus the physiology of *Daphnia* is in an early stage but is an exciting new field of study.

Nano or ionic-specific toxicity of AgNPs?

We measured the dissolution of 5 and 20 mg/L AgNPs after three days in the dark to mimic the longest transfer interval in our experiment (see SI for methods and expanded results and discussion of these data). After three days, AgNPs kept in the dark dissolved 0.37 ± 0.06% (standard error) Ag^+ of the concentration of AgNPs. Given these data, we predict that 0.28 and 0.74 μg/L of Ag^+ to be present in our 75 and 200 μg/L exposures, respectively. These concentrations alone are not high enough to explain the effects we observed (a recent meta-analysis found the median LC/EC50 for Ag^+ to be 0.85 μg/L (Ivask et al. 2014)), however we suspect that the dissolved silver concentration may have been higher than what we measured (see SI for discussion) so we cannot conclude that the effects we observed are due to a nano-specific effect alone. The effects observed may be the result of a combination of ionic and nano-specific effects of AgNPs. In *Daphnia,* silver ions disrupt ionoregulation within an hour of exposure by blocking sodium uptake (Bianchini and Wood, 2003), and AgNPs may act through this toxic mode of action as well (Allen et al., 2010; Hoheisel et al., 2012; Stensberg et al., 2014; Zhao and Wang, 2012a). AgNPs and Ag^+ may also exert toxicity through different modes of action. AgNPs have been found to cause mitochondrial damage and produce ROS (McShan et al., 2014; Ulm et al., 2015), although our data indicate that AgNPs did not produce ROS in our experiments (SI Figure 2). Finally, these two forms of silver may be toxic through a

synergistic combination of Ag^+ and AgNP-specific toxic mechanisms (Stensberg et al., 2014; Ulm et al., 2015). Overall, we cannot conclude if the effects observed are solely due to ionic- or nano-specific effects of AgNPs. However, due to the low dissolution of Ag^+ over three days observed in our media and the evidence from the literature that citrate-coated AgNPs dissolve slowly in freshwater media (Zhao and Wang, 2012), we suspect that nano-specific toxicity of AgNPs may play a role as has been found in a few other studies of AgNP-toxicty to *Daphnia* (Griffitt et al., 2008; Pokhrel and Dubey, 2012; Ribeiro et al. 2013).

Ecological consequences of AgNP exposure

Our study focused on the effect of AgNPs on *Daphnia* experiencing the low food rations typically observed during the "clear water" phase in many temperate lakes and ponds. Even though *Daphnia* did not reproduce in our controls at the two lowest food rations (except for one 75 μg/L exposed individual), the effects of AgNPs at those two food rations may still be ecologically relevant. *Daphnia* experience very low food rations during summer (McCauley and Murdoch 1987), so *Daphnia* will experience these food rations in their natural environment. Further, Kooijman and Metz (1984) calculated the population growth rate from data on daphnid individuals fed different amounts of food and found an "extremely narrow range of food densities" in which r is predicted to change from negative to positive. This indicates that the shift of a daphnid population from growing to declining exponentially occurs rapidly as you decrease food concentrations (Kooijman and Metz 1984). This finding stresses the importance of estimating the effect of a contaminant on populations during periods when the toxicant's effect would be most

significant, such as in or near this narrow window of food densities (Kooijman and Metz 1984).

Population growth rate estimates from this work predict that exposure at the second highest food ration (0.0025 mgC/daphnid/day) to an even sublethal concentration of AgNPs (75 μg/L) would drive a population to extinction. This is a tempting conclusion, however it ignores important population-level feedbacks that occur when *Daphnia* impact the amount of food available per individual through resource-consumer interactions (Murdoch et al., 1998; Martin et al. 2014). In individual experiments, the experimenter defines the food ration by feeding a set amount of carbon to each individual – *the experimenter controls the food supply rate per individua*l. However, if AgNP exposure impacts the fitness of a daphnid in a population, then an AgNP-exposed individual requires more food than an unexposed individual to replace itself during its lifetime (a conclusion consistent with our data). A population exposed to AgNPs could still persist, albeit at a lower equilibrium population size of *Daphnia* than a control population because the reduced *Daphnia* population would lead to elevation in the food density in the environment (Martin et al. 2014). This reasoning of course neglects all other population interactions. Understanding feedbacks in exposed populations is an area for future work empirically (there are few experiments exposing populations of organisms to nanoparticles) and theoretically, highlighting the importance of quantitative models to link individual-level data to population-level responses. Data on toxicant exposure at multiple food rations strengthens our understanding of that toxicant's effect and also our ability to predict the effect of a contaminant on the sustainability of natural populations. A future

aim of this work is to use the data reported here to parameterize an individual-based model with the goal to make population-level predictions.

Tables:

Food ration (mgC/daphnid/day)	[AgNP] ppb	Mean survival time ± standard error (days)
0.0005	0	19.6 ± 3.01
	75	24.6 ± 3.17
	200	8 ± 1.16
0.001	0	17.2 ± 3.15
	75	27.7 ± 3.37
	200	8 ± 1.96
0.0025	0	22.6 ± 3.51
	75	25.5 ± 3.68
	200	13.5 ± 3.47
0.01	0	28.6 ± 5.28
	75	34.5 ± 0
	200	14.3 ± 4.84

Table 1: Mean lifetime (days) calculated by fitting a survival function to our data using a Kaplan-Meier estimator.

Figures:

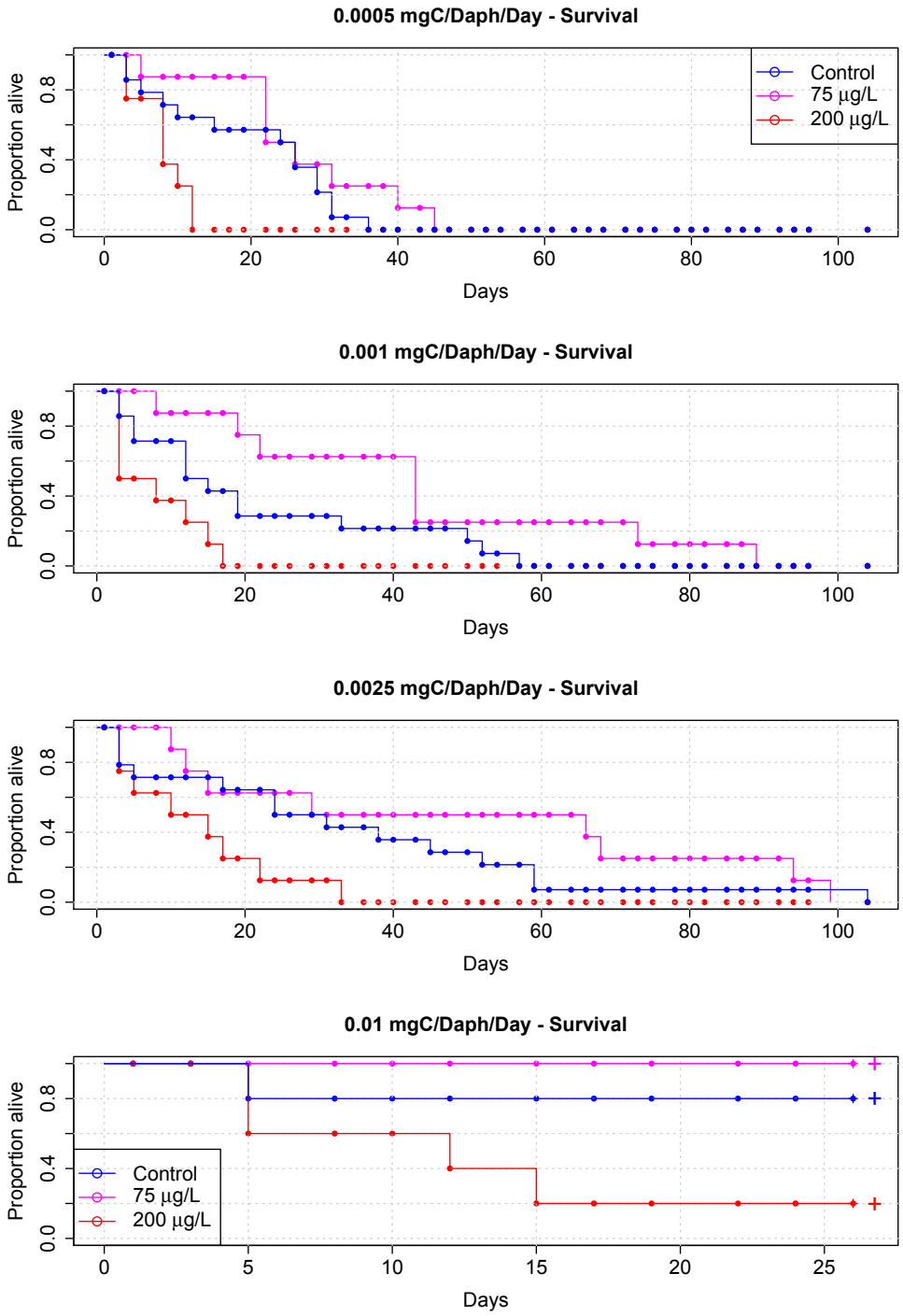

Figure 1: Survival of individual *Daphnia* through time at four food rations exposed to 0, 75, and 200 µg/L AgNP. Lines are Kaplan-Meier survival curves. Sample sizes for the three lowest food rations are 14, 8, and 8 for 0, 75, and 200 µg/L AgNP and n=5 for each treatment at the highest food ration.

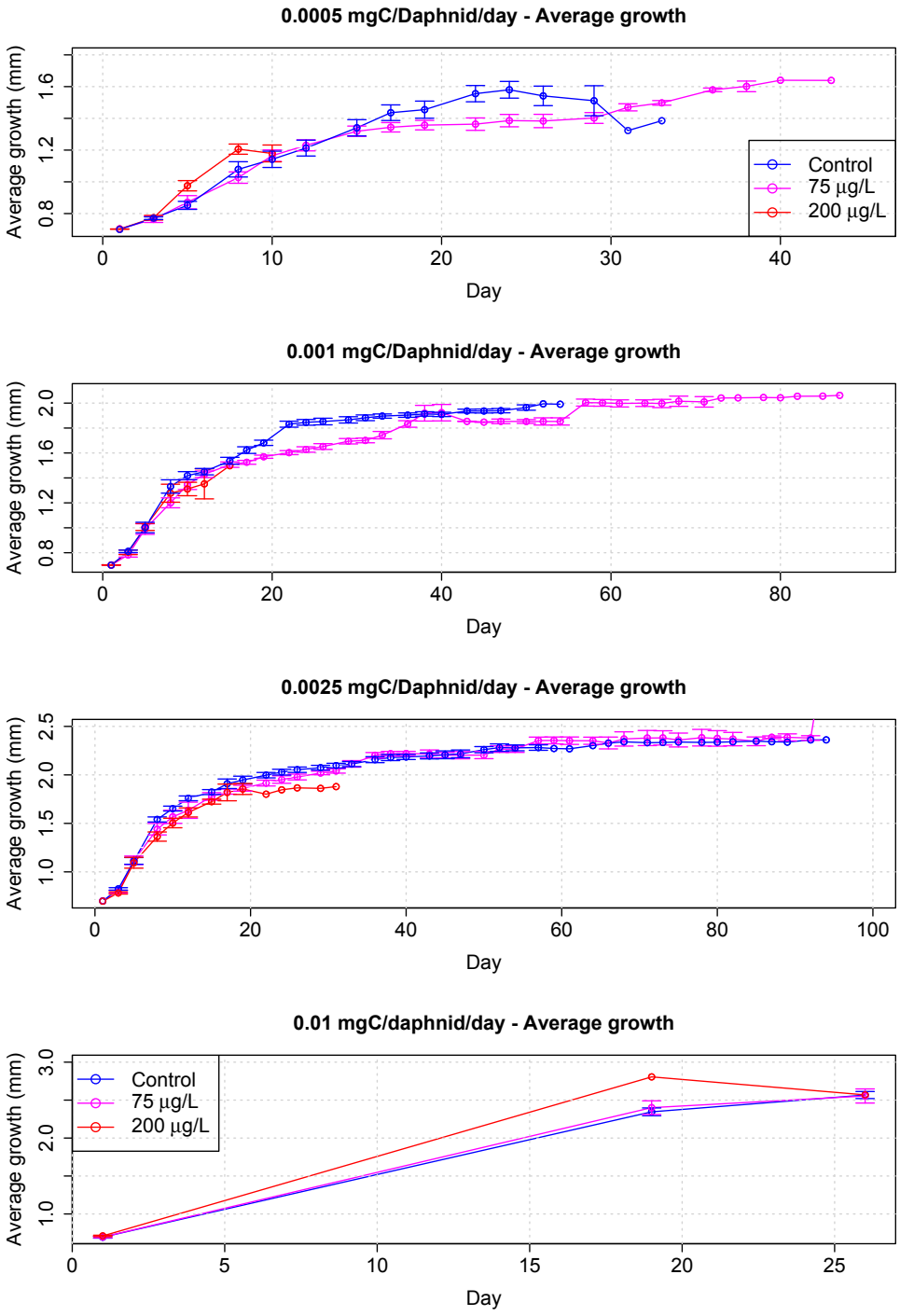

Figure 2: Growth of individual *Daphnia* through time at four food rations exposed to 0, 75, and 200 µg/L AgNP. Error bars reflect standard error of replicates. Sample sizes for the three lowest food rations are 14, 8, and 8 for 0, 75, and 200 µg/L AgNP and n=5 for each treatment at the highest food ration.

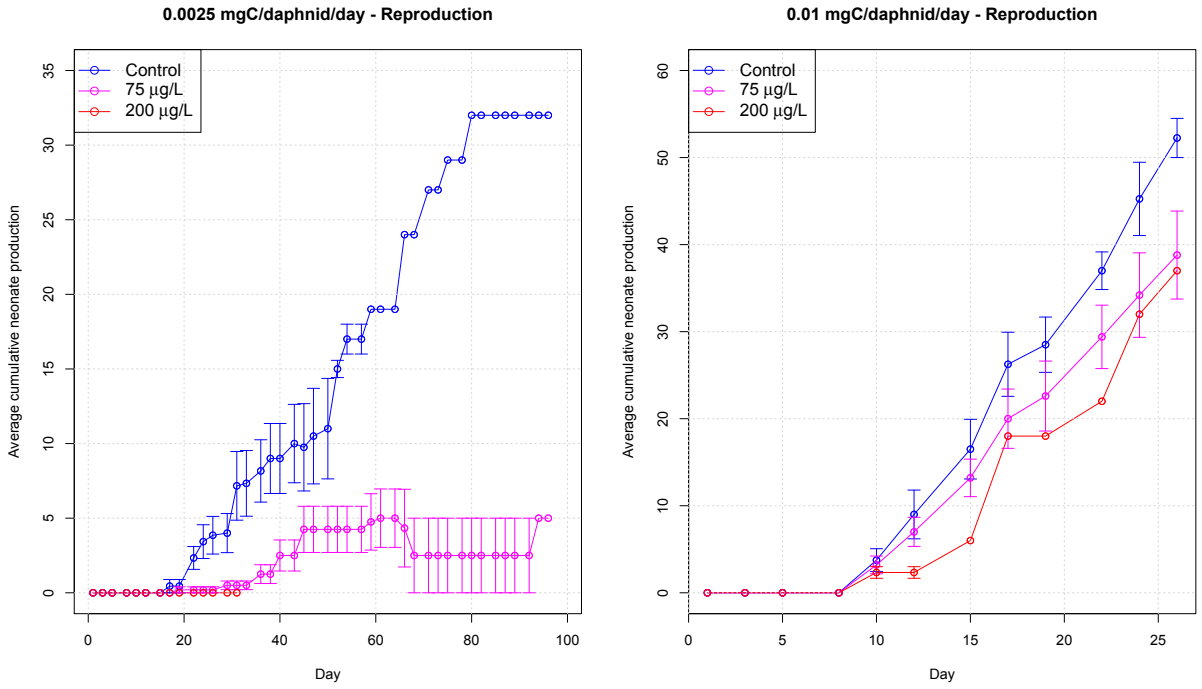

Figure 3: Reproduction (cumulative number of offspring produced) by individual *Daphnia* through time exposed to 0, 75, and 200 µg/L AgNP. Data only shown for food rations at which *Daphnia* produced offspring (0.0025 and 0.01 mgC/Daph/day). Sample sizes for the three lowest food rations are 14, 8, and 8 for 0, 75, and 200 µg/L AgNP and n=5 for each treatment at the highest food ration. For this calculation, we averaged the total number of neonates produced per individual on every sampling day. The average cumulative neonate production of individuals fed 0.0025 mgC/Daph/day exposed to 75 µg/L AgNPs decreases because one reproducing individual died around day 63 and the other surviving individuals were reproducing less on average. Individuals fed 0.0005 and 0.001 mgC/daphnid/day food rations did not produce neonates (two individuals had eggs in the brood pouch but we did not find any neonates). Note that the 200 µg/L reproduction in the right figure is reproduction by one individual after day 12 (two out of five total birthed offspring at 200 µg/L exposure and 0.01 mgC/Daph/day food ration). Error bars reflect standard error of replicates.

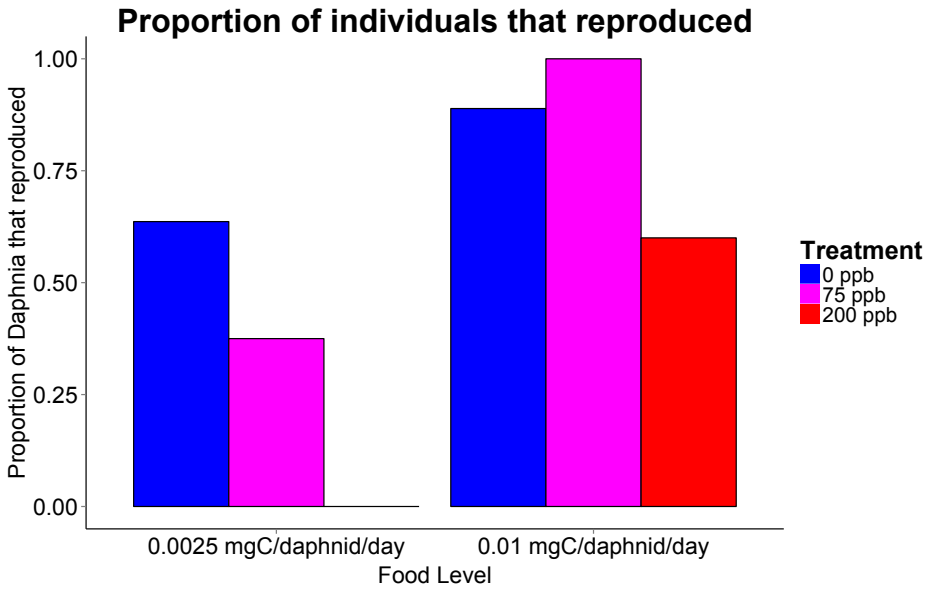

Figure 4: Proportion of individuals that produced eggs at 0.0025 and 0.01 mgC/daphnid/day food rations. As one would predict, higher food rations led to a higher reproduction across all treatments but the negative effect of 75 and 200 µg/L on reproduction decreased with increasing amounts of food. Sample sizes for the three lowest food rations are 14, 8, and 8 for 0, 75, and 200 µg/L AgNP and n=5 for each treatment at the highest food ration.

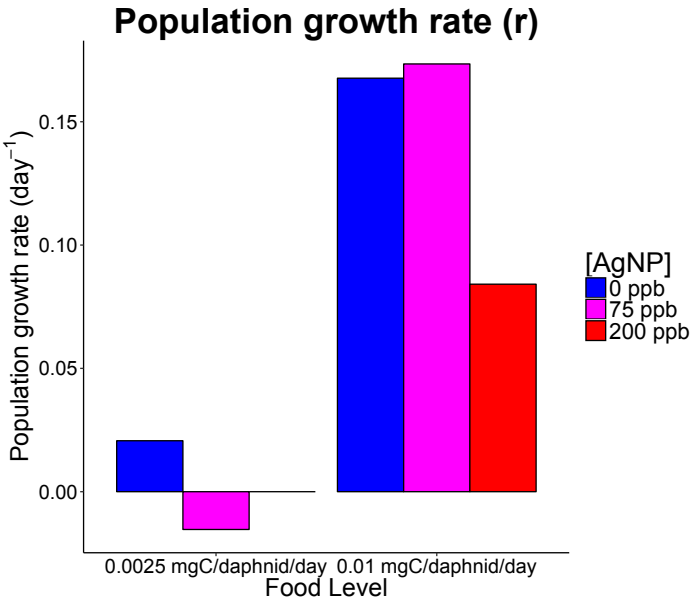

Figure 5: Comparing population growth rate (*r*) of all treatments across the two highest food rations (food rations where we saw significant reproduction). We could not calculate *r* for food rations and AgNP exposures at which the *Daphnia* did not reproduce, which is why there are not any *r* values for 200 µg/L exposure at the 0.0025 mgC/daphnid/day food ration and there are not any *r* values for the two lower food rations (0.0005 and 0.001 mgC/daphnid/day). The only exception at the two lowest food rations is that at 75 µg/L exposure fed 0.001 mgC/daphnid/day had an *r* value of -0.03 day^{-1} (not shown). Note that we followed individuals throughout the duration of their lifetime at the 0.0025 food ration, however the experiment at the 0.01 food ration only ran for 26 days.

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III. AgNPs toxic to individuals have no effect on populations of *Daphnia*: Ecological feedbacks save daphnid populations from AgNP toxicity

Authors: Louise M. Stevenson, Katherine Krattenmaker, Edward McCauley, Roger M. Nisbet

Abstract

Ecological risk assessment (ERA) is charged with assessing the likelihood that a will have adverse environmental or ecological effects. When assessing the risk of a potential contaminant to biological organisms, ecologists are most concerned with the persistence of *populations* of organisms, rather than protecting every *individual*. However, ERA most commonly relies on data on the effect of a potential contaminant on individuals because these experiments are more feasible than costly population-level exposures. In this work, we address the challenge of extrapolating these individual-level results to predict population-level effects. Per-capita population growth rate estimates calculated from individual-level exposures of *Daphnia pulicaria* to silver nanoparticles (AgNPs) at different food rations predict the extinction of daphnid populations exposed to 200 μg/L AgNPs. However, we exposed daphnid populations to the same AgNP concentration at three different food inputs and all populations persisted, except at the lowest food input which goes extinct after 50 days. We demonstrate that we can reconcile this seeming disconnect between individual- and population-level effects by parameterizing an

individual-level biomass model with data on individuals' response to AgNPs and use these parameters to predict the equilibrium biomasses of control and AgNP-exposed populations. This highlights the strength of the ecological feedback between consumer biomass (*Daphnia*) and their resource (algae) that mitigates AgNP toxicity and allows populations to survive AgNP exposure. This work stresses the importance of incorporating ecological complexity into predictions of population-level effects of contaminants.

Introduction

One important goal of ecological risk assessment (ERA) of chemicals is to estimate the effects of a potential contaminant on *populations*, however population experiments are costly, in terms of labor, materials, and organisms. In practice, ERAs end up relying on data from experiments on individuals, and, in the case of ERAs for freshwater systems, they commonly use data from chronic toxicity tests (OECD 2012) in which *Daphnia* are exposed to increasing concentrations of a potential toxicant and their survival and reproduction are measured. From these individual-level data, it is possible to estimate metrics related to population-level responses, such as the long-run population growth rate in a constant environment (per-capita population growth rate, r), an approach that has been employed to predict the effect of toxicants on populations from individuals for many years (Kooijman and Metz 1984). However, such metrics do not permit prediction of population-level impacts, because they ignore potentially important feedbacks between the individuals that comprise the population and their environment. Recognition of this limitation has inspired calls for the use of ecological modeling to incorporate ecological complexity into ERA (Forbes, Hommen et al. 2009, Forbes, Calow et al. 2011). The

feasibility, and challenges, of the use of models to extrapolate ecotoxicological impacts across levels of biological organization was recently demonstrated for *Daphnia* by Martin et al. (2013a,b, 2014).

Our past work (Stevenson et al. in prep) studied the effect of silver nanoparticles (AgNPs) on individual *Daphnia* fed a range of algal food rations (mgC algal food per daphnid per day). We found that low food increased AgNP toxicity, and exposure to two AgNP exposures (75 and 200 μg/L) had direct effects on daphnid survival and reproduction. Specifically, 200 μg/L AgNPs was toxic at all food rations and had effects on reproduction such that no individuals exposed to 200 μg/L reproduced except two individuals at the highest food ration. Both AgNP concentrations were predicted to cause population extinction at all food rations tested except the highest food ration based on calculated long-run population growth rates (*r*) (Stevenson et al. in prep). To test how well population growth rates calculated from individual data in this way can estimate population-level effects of a toxicant, we exposed small populations of *Daphnia pulicaria* to 200 μg/L AgNPs at three food inputs and report the results in this paper. We found that 200 μg/L AgNPs did not have any effect on populations of *Daphnia* except at the lowest food input after 50 days. This disagrees with predictions based on calculations of *r* from the individual-level data of the same *Daphnia* clone exposed to the same concentration of AgNPs.

This seeming disconnect between individual and population-level effects of AgNPs may be the result of two potential feedbacks. The first is the feedback in which organic material produced by the organisms could have mitigating effects on AgNP toxicity, which has been previously found to occur and lessen the toxicity of AgNPs to freshwater

algae (Stevenson et al. 2013) and *Daphnia* (Blinova, Niskanen et al. 2013, Newton, Puppala et al. 2013). The second feedback could involve the interaction between daphnid populations and their algal food – *Daphnia* can equilibrate at lower consumer biomasses in stressful environments which results in a higher resource biomass, thus increasing the individual daphnid ration of food (e.g. Murdoch and Scott, 1984). If a contaminant (such as AgNPs) decreases individual performance such that each exposed individual requires more food to replace itself, a population can equilibrate with fewer individuals and could survive exposure while an individual isolated in an experimental chamber and given a fixed daily ration of food may perish.

To investigate whether either of these two feedbacks may be complicating the extrapolation from individual to population-level effects of AgNPs, we conducted a theoretical experiment in which we parameterized an individual-level biomass model of daphnid growth and reproduction using data from Stevenson et al. in prep. We fit control and 200 µg/L AgNP-exposed individuals separately, such that we constructed two parameter sets describing control and AgNP-exposed individuals. We then used those parameters (estimated from individual-level data) in a population model and predicted the equilibrium biomass of daphnid populations in either control or 200 µg/L AgNP treatments. This population model included feedbacks with algal food but did not include the potential feedback between daphnid-produced DOC mitigating AgNP toxicity. The population model predicted population persistence, indicating a feedback with their algal food in which AgNP-exposed populations equilibrate at a lower biomass to compensate for AgNP toxicity is sufficient for population persistence. In addition to this theoretical experiment, we also conducted experiments on the effect of daphnid-produced DOC on

AgNP toxicity and did not find a protective effect of the organic material. This work highlights the strength of ecological models to aid in the extrapolation of toxicant effects from the individual to population level.

Methods

Silver nanoparticles characterization

We purchased 40 nm BioPure citrate-coated silver nanoparticles (AgNPs) from NanoComposix (San Diego, CA). We measured the size, dissolution, and reactive oxygen species (ROS) production of AgNPs in the light and in the dark (since these daphnid experiments were performed in the dark to control algal growth which would change the food concentration) and these data are reported in Stevenson et al. (in prep). In summary, AgNPs at these exposures concentrations do not produce ROS, do no aggregate, and have very slow dissolution in "low P" COMBO media (Kilham, Kreeger et al. 1998), the same media used for the experiments reported here. Our data indicate that less than 1 μg/L of the 200 μg/L AgNP exposure is present after three days as ionic silver (Stevenson et al. in prep).

Daphnia population experiment

To test the effect of AgNPs at different food inputs to populations of *Daphnia*, we set up twelve small populations, half of which were kept as controls and half of which were dosed with 200 μg/L AgNPs. The populations were fed one of three food inputs (0.07, 0.14, and 0.27 mgC/day), and there were two replicate populations per treatment.

Populations were kept in 400 mL of autoclaved "low P" COMBO media (Kilham, Kreeger et al. 1998) and all glassware was autoclaved prior to the start of the experiment.

We started the populations at the approximate neonate/juvenile:adult ratio of *Daphnia pulicaria* populations at equilibrium, based on data from past in experiments in our lab (unpublished data). This ratio is approximately 3 neonates/juveniles:1 adult and we started the populations with neonates (0.7 ± 0.5 mm) and adults (2.0 ± 0.5 mm). We chose these sizes to attempt to standardize the age of the individuals across treatments – 0.7 mm neonates are approximately 1 day old while the 2.0 mm adults are all approximately equal in age since these individuals all came from the same stock tank and food history. We removed *Daphnia* from our stock tanks 5 days prior to the experiment and placed them in tanks of fresh COMBO media for two days, then transferred them to another tank of fresh COMBO media for another 3 days to clean the individuals and minimize any carryover of algal cells or other detritus into the experimental containers. We started the populations at total populations sizes proportional to the food input; the populations fed the highest food input (0.27 mgC/day) started with the most individuals (34 neonates and 11 adults), the middle food input started with approximately half that number of individuals (18 juveniles and 5 adults), and the lowest food input started with approximately a quarter of the population size as the highest food input (9 neonates and 3 adults).

We sampled each population on a Monday-Wednesday-Friday schedule. For sampling, we poured the daphnid populations onto a 60 micron nylon net filter (Millipore NY60) and counted and identified the stage of all individuals using a dissecting scope (Leica M80). We identified developmental stages of the individuals under the microscopes using circles printed on a transparency film of known diameters to correspond to the stages

of interest. There were two circles: neonates fit into the circle with diameter 1.0 mm, juveniles fit into the circle with diameter 1.8 mm, and any individuals larger than both circles were adults. We also identified pregnant adults and counted the number of eggs and embryos in the brood pouch. We then placed the individuals into fresh COMBO media dosed with 0 or 200 µg/L AgNPs and fed the populations.

We fed the *Daphnia Chlamydomonas reinhardtii* cells from cultures 10-12 days old. We centrifuged a 500 mL algal batch culture on 7,000 rpm for 4 minutes, re-suspended the cells in nanopure water, and then measured the concentration of chlorophyll fluorometrically (see methods in Stevenson et al. 2013). We then converted the chlorophyll a concentration to a carbon concentration using a fixed mg C: µg chlorophyll ratio that was measured empirically in our lab (0.22 mgC/µg chlorophyll a). We measured the concentration of algal carbon fed through time by removing 5 mL of sample and drying the samples down in a drying oven and analyzing them for total carbon and nitrogen on a CN analyzer (Thermo Scientific Flash 200 CN Analyzer). We also measured the amount of food left behind by the populations on a microplate reader and converted these concentrations to chlorophyll a concentrations (see methods in Stevenson et al. 2013).

We placed the experimental populations in the dark for the duration of the experiment to maintain the fed algal food input. The cultures were at a temperature of 21.8 ± 0.6 degrees Celsius (average ± standard deviation of hourly measurements taken by Maxim Integrated iButton DS1921G throughout experiment placed in 400 mL of water next to the experimental cultures).

Bacterial cell counts

Throughout the experiment, we noticed that AgNP-exposed populations had more buildup of detritus, especially shed carapaces, compared to controls, potentially indicating AgNPs may be toxic to the bacterial populations in our experiment and hindering breakdown of organic material. We measured the bacterial population through DAPI staining (4',6-diamidino-2-phenylindole), a commonly used method for counting cells as the stain binds to DNA and fluoresces. We removed 50 mL samples from media post-transfer (we removed the daphnid individuals and then took samples of the media left behind), fixed them with formalin, and then stained the samples within 72 hours of collection. We stained 5 mL of each sample with 0.5 mL of 5 μg/mL DAPI (Acros Organics) for 3 minutes and then filtered via a vacuum filtration system. We filtered the samples through 0.2 micron PC filters (Whatman Nuclepore Polycarbonate Track-Etched Membrane) stained with irgalan black (2 mg/mL) with a 0.8 micron backing filter (Fisherbrand General Filtration Membrane Filter) behind the 0.2 micron filter. We mounted the filters onto glass microscope slides and counted bacterial cells using epifluorescence microscopy (Olympus B202).

DOC measurements

To compare the production of DOC by individual *Daphnia* versus daphnid populations, we collected samples during the population experiment to calculate DOC production after a two-day transfer interval. After sampling, we removed 10 mL of the media left behind and filtered it through a 0.22 micron filter (Millipore mixed cellulose membrane). We dried these samples in a drying oven and analyzed them for total carbon

on a CN analyzer (Thermo Scientific Flash 200 CN Analyzer). We also measured COMBO media alone (blank measurements) and samples of the highest concentration of food fed to calculate the contribution of algal-produced DOC to the DOC present in the media. We also analyzed 3 blank samples via High Temperature Combustion (Shimadzu TOC-V, precision 1-2 μM) to measure whether that the contribution of dissolved inorganic carbon was significant.

For measurements of individual production of DOC, we collected neonates (0.7 – 1 mm), juveniles (1 – 1.8 mm), small adults (1.8 – 2.3 mm), and large adults (> 2.3 mm) from clean tanks (see rinsing procedure described in earlier methods section for cleaning *Daphnia* pre-experiment). Based on preliminary estimates of DOC production, we grouped individuals of the same size class together for the smaller size classes to ensure that the carbon measurement would be within range of the CN Analyzer; we placed 5 neonates, 3 juveniles, 2 small adults, and 1 large adult per tube. There were 12 tubes of neonates, 5 tubes of juveniles, 33 tubes of small adults, and 8 tubes of large adults that were separated approximately evenly between treatment groups. Each tube was autoclaved prior to use and contained 30 mL of autoclaved "low P" COMBO media. We fed all tubes 0.01 mgC/day of *C. reinhardtii.* The tubes were separated into three groups based on the AgNP treatment and transfer interval – two and three day controls and three day AgNP exposures, because we wanted to measure the production of DOC per day, and our sampling regime included 2- and 3-day transfer intervals, and we were also interested in the effect of AgNPs on DOC production by *Daphnia*. We measured the lengths of all individuals at the start and end of the experiment to estimate the average daphnid biomass that produced the measured quantity of DOC. We also checked all individuals at the end of

the experiment for survival. To measure DOC, we removed the *Daphnia* and filtered all 30 mL of media through a 0.22 micron filter. We dried these samples in a drying oven and then analyzed them for total dissolved carbon.

Acute toxicity test with and without organic material

Daphnid-produced organic material may have a mitigating effect on AgNP toxicity. To test this, we performed a standard acute toxicity test assay (a short term exposure to a toxicant without feeding the animals; OECD (1984)) on *Daphnia* exposed to AgNPs in autoclaved "low P" COMBO media and media from a daphnid population. To harvest daphnid-produced organic material for this experiment, we set up daphnid populations at the same population sizes as the middle food input, fed them 0.14 mgC/day, left them for three days, and used the media from these populations for half of the acute toxicity exposure treatments. We set up these populations to simulate the amount of DOC the daphnid populations produced at the start of the long-term experiment. In more detail, we started three populations of *Daphnia* identical to the daphnid population experiment (methods described earlier in this section). We started the populations with 18 neonates and 5 adults each and fed these populations the middle food input (0.14 mgC/day). We did not add any AgNPs to these populations and sampled them after 3 days. We initially wanted to harvest the media and start the acute toxicity test after 2 days, so the population were fed 0.28 mgC each. However we could not find enough neonates in our clean tanks to start the acute toxicity test, so we fed the populations one more days worth of food (another 0.14 mgC) and started the experiment the next day. We counted and identified the stage of all individuals and then removed the media for our experiment. We filtered the

media through 0.22 micron filter and used the media from these populations (mixing the media from the three populations together) for half of the individuals in the toxicity test. We measured the DOC concentration from all three populations separately and also a sample of the mixture using High Temperature Combustion (Shimadzu TOC-V). We exposed less than 24 hour old neonates to 10, 100, 200, 250, 500 and 750 µg/L AgNPs in 30 mL of either COMBO media or media removed from daphnid populations with 5 neonates per tube and 4 tubes per treatment (n = 20 neonates for all treatments). The tubes were checked every 24 hours for 96 hours and visually inspected for survival using a dissecting scope (Leica M80).

Individual and population model descriptions

We fit a very simple biomass-based model of *individual* daphnid growth and reproduction to the individual data in Stevenson et al in prep (Table 1). The model has three state variables – algal food density, weight of an individual, and the cumulative number of eggs produced. The model distinguishes between juveniles and adults – any individual less than the weight at reproductive maturity (estimated from the individual data) is a juvenile, and any individual above that weight is an adult. Since our experiments were transfer cultures (where the media and food was replaced on a Monday-Wednesday-Friday schedule), the algal food densities resets to the food ration every 2, 2, and 3 days. In between the transfer intervals, daphnid individuals consume food at a rate proportional to their weight and the maximum ingestion rate for that stage (I_J and I_A for juveniles and adults, respectively). Juveniles and adults grow at a rate proportional to the amount of carbon assimilated from food. All life stages respire at the same rate per unit of body

weight, which takes energy away from growth. Adults allocate assimilated carbon to either growth and maintenance or to reproduction, and the proportion of carbon allocated to reproduction is defined by the parameter χ, an assumption similar to that used in a dynamic energy budget model known as "DEBkiss" proposed by Jager et al. (2013). Juveniles do not allocate any carbon to reproduction since they have not reached maturity. Adults produce eggs at a rate proportional to the amount of carbon assimilated that is allocated to reproduction, and this amount of carbon is converted to eggs based on the amount of carbon that is required to produce one egg (γ). We fit all food level simultaneously to this model but fit AgNP treatments separately to produce two parameter sets – a set of parameters that describe growth and reproduction of a control individual and a set of parameters for an AgNP-exposed individual (Table 2).

In the population experiments, the food is consumed very quickly, so the analogous population model defines a "ration" such that algal food density is no longer a state variable and the algal food input is assumed to be immediately consumed by the population. Individual rations depend on the population at any time. The population model describes both numbers of individuals and the biomasses of juveniles and adults (model equations in Table 3). It shares many features with previous stage-structured models proposed for *Daphnia* (Nisbet et al. 1989, McCauley et al 1996, McCauley et al 2008, Ananthasubramaniam et al 2011), but differs in the method used to couple biomass and population dynamics, which was based on a previous formalism by Nisbet et al (1985). As in the individual model, juveniles and adults grow at a rate proportional to the amount of carbon assimilated from food and lose carbon to respiration. Adults also partition carbon towards reproduction. Juvenile maturity is described using a delay dependent differential

equation, in which the current state (in this case, maturation) is dependent on the past value of another state (in this case, food) instead of the value of that state at that time point. Juveniles mature into adults with a stage duration that is dependent on the *past* food supply – juvenile development is described using a delay differential equation in which the current state of the system (number of juveniles and adults) depends on the past state of the system (earlier rations of food per individual juvenile). Juveniles and adults die at a constant per capita death rate that was estimated from the response of daphnid individuals to control and 200 μg/L AgNPs treatments across four food levels (Stevenson et al. in prep). We used parameters from the individual model fit to individual data (Stevenson et al. in prep) to simulate the population model. In the present analyses, we only looked at equilibrium states in order to evaluate the qualitative outcomes.

Statistical analyses

We measured bacterial cell densities in the daphnid populations and analyzed for statistically significant differences between food inputs and treatments by performing linear regression using R (version 3.2.3). We fit a Weibull regression model (an accelerated failure time model) with media type and AgNP concentration as predictors to analyze survival data for acute toxicity test with and without organic material. This analysis was done in R (version 3.2.3) using the "survival" package.

We fit the individual model (Table 1) to individual data from Stevenson et al. in prep using a likelihood method coded in the package BYOM ("Bring Your Own Model") platform for parameter estimation, developed by Tjalling Jager for Matlab (http://debtox.info).

Results

200 µg/L AgNPs only toxic after 50 days to lowest food input

200 µg/L AgNPs had no effect on the two highest food inputs, and only had an effect on populations fed the lowest food input after 50 days (Figures 1 – 4). Visual inspection makes it clear that, although there is some variability, there are no apparent large differences between control and AgNP treatments in the two highest food inputs (Figure 2). After 50 days, all AgNP-exposed treatments decline across all food inputs. This effect is stronger at lower food inputs – at 0.27 mgC/day, the population returns to control levels within 10 days and do not decline as sharply, while populations fed 0.14 mgC/day take about 15 days to return to control levels, and populations fed the lowest food input decline to extinction (Figure 2). Populations exposed to AgNPs at the two highest food inputs were able to recover to control levels, driven by boosts in fecundity in populations fed 0.14 and 0.27 mgC/day between days 50 and 70 (Figure 6), however populations fed the lowest food input never recovered. One AgNP-exposed replicate at the lowest food input went extinct immediately on day 54 while the other declined to one adult that continued to produce offspring every transfer interval, however none of her offspring reached adulthood and the adult eventually died. While there are declines in the AgNP-treatments across all stages (Figures 3-5), adults seem to be the stage most affected by this decline in even the control treatments (Figure 5).

200 µg/L AgNPs have a small effect on bacterial communities

We measured the bacterial population in all experimental cultures after a 2 and 3 day transfer interval to analyze for differences between AgNP and control cultures. We found no difference between control and AgNP bacterial populations after the three-day transfer interval (Figure 7A), however samples taken after the two-day transfer interval are significantly different (Figure 7B). The food input ($p = 0.011$) and AgNP treatment ($p = 0.010$) both had significant effects on bacterial abundance.

These samples were taken on days 57 and 59 of the population experiment, as AgNP-exposed populations were recovering from the decline around day 50. It is unclear whether the significant or the not-significant result from the two- and three-day transfer results, respectively, more accurately describes the average state of the cultures throughout the experiment. However, 200 μg/L AgNPs is probably not a high enough concentration to kill most bacterial species – a recent meta-analysis of AgNP toxicity studies to a variety of taxa found that the average L/EC50 of AgNPs to bacteria is 7.10 mg/L, about 35 times higher than our exposure concentration (Bondarenko, Juganson et al. 2013).

Daphnid populations produce more DOC than individuals and AgNPs do not have an effect on DOC production

These measurements are all total dissolved carbon (TDC), so they include dissolved inorganic carbon (DIC) measurements. However, blanks (autoclaved "low P" daphnid media that has never had organisms in it) measured on a TC analyzer were nearly identical to those measured by a DOC analyzer: 2.15 ± 0.08 mgC/L TDC and 2.20 ± 0.63 mgC/L DOC (averages ± standard error). Therefore, these measurements can serve as a proxy for DOC.

Individuals exposed to AgNPs produced a little less DOC per mg of daphnid biomass than unexposed control individuals – control individuals produced 0.19 ± 0.01 mgC DOC/mg daphnid/day and AgNP-exposed individuals produced 0.12 ± 0.04 mgC DOC/mg daphnid/day (Figure 8). However, this was not a statistically significant difference ($p = 0.19$ from two-sample t-test of unequal variance).

Overall, populations produced significantly more DOC than individuals (Figure 9). Of course, multiple daphnid individuals will respire more and thus produce more DIC than daphnid individuals, however DIC from respiration is also included in our measurements of individual dissolved carbon, so this comparison is valid. Again, there does not appear to be a difference between the amount of DOC produced by populations exposed to 200 μg/L AgNPs versus controls, however we do not have enough data to draw a significant conclusion.

Daphnid-produced organic material does not protect the zooplankters from acute AgNP toxicity

We set up the three populations of *Daphnia* at the same population sizes as the middle food input and fed them 0.14 mgC/day to then harvest the media from these populations to use for the acute toxicity test exposing *Daphnia* to AgNPs with and without daphnid-produced DOC. We set up these populations to simulate the amount of DOC the daphnid populations produced at the start of the long-term experiment. These three populations declined a little bit over the transfer interval, similar to the results from the long-term population experiment. Populations for the acute toxicity test declined to an average population size of 20.7 ± 0.7 individuals and the populations from the full daphnid

experiment (Figure 2) declined to population sizes of 18.5 ± 1.2 individuals (averages of 3 and 2 replicates, respectively, ± their standard error) in the first transfer interval. The data shows that we can assume the populations were similar enough such that we can assume the amount of DOC produced by these populations was similar to the amount present during the start of our long-term experiments. We chose to simulate the concentration of daphnid-produced organic material at the *start* of the long-term experiment because since this was the period of the lowest population density (except for those populations that eventually went extinct), we assume the start would be the period of the experiment with the lowest concentration of organic material (assuming more daphnid individuals produce more DOC). At the same time, we did not see a lot of initial mortality in these exposures, so if DOC is protecting the daphnid populations, the concentration at the start should have been enough to mitigate AgNP toxicity to the *Daphnia.*

Daphnia exposed to AgNPs in media from daphnid populations (with daphnid-produced DOC) died *faster* than those exposed to AgNPs in COMBO media (Figure 10). We fit a Weibull regression model (an accelerated failure time) using maximum likelihood estimation and found a significant effect of both AgNP ($p < 1e\text{-}12$) and media ($p < 1e\text{-}12$) treatments. This result is the opposite of our hypothesis that daphnid-produced organic material would have a mitigating effect on AgNP toxicity.

A population model parameterized with individual data correctly predicts population persistence

We fit an individual based model (Table 1) to the individual-level data (Stevenson et al. in prep) on the growth and reproduction of individuals exposed to 0 and 200 μg/L

AgNPs by estimating all of the parameters in Table 2 except F_h, ε and γ. We did not fit these three parameters because we assumed AgNPs did not have an effect on them (therefore the values will be equivalent between both treatments) and used estimates from the literature that were measured more exhaustively (using more data) than our data set could provide (Nisbet et al. 2004). The individual model is a very simple biomass model of the growth and reproduction of daphnid individuals with food as a state variable. In the individual model, the algal food concentration resets every 2, 2, 3 days, meaning it is set to the concentration of algal food at transfer intervals, to simulate our sampling regime.

We could not get a good fit of the simple individual biomass model (Table 1) to both growth and reproduction – the model is able to correctly simulate the data points of the cumulative number of eggs produced, however the model greatly under-predicts growth (Figures 11 and 12). We were able to get a better fit of the model to the growth data but with a much poorer fit to the reproduction data (fits not shown), however we chose to follow-through these analyses using parameters that gave a better fit to the reproduction data, as that could have a large influence on population size and structure. When we use these parameters in the population model (Table 2), we get general agreement between the predicted equilibrium biomasses of the model compared to the data collected from our experiment, except at the lowest food level (Figure 13).

Discussion

AgNP toxicity exacerbated at lower food inputs

Low food enhanced AgNP toxicity to populations of *Daphnia.* 200 µg/L AgNPs did not have a significant effect on the daphnid populations until around Day 50, when all

AgNP-exposed populations declined sharply across all food inputs (Figure 1). Populations at higher food inputs were able to survive the decline in AgNP-exposed populations around day 50 but the populations at the lowest food input went extinct, indicating the compounding effects of nano- and food stress.

Is DOC protecting populations of *Daphnia* from 200 µg/L AgNPs?

One of the most interesting and striking results of this work is that 200 µg/L AgNPs is toxic to individuals exposed as neonates at all food rations (Stevenson et al. in prep) but not when the neonates are part of a larger population (Figure 1), except when the food input is very low (Figure 1c). Our past work found that lower food rations increased the toxicity of 200 µg/L AgNPs (individuals fed less died faster when exposed to 200 µg/L AgNPs), however that concentration of AgNPs was toxic at all food rations tested (Stevenson et al. in prep). At the three lowest food rations, daphnid individuals exposed to that 200 µg/L AgNPs did not produce any offspring (Stevenson et al. in prep). At the highest food ration (0.01 mgC/day) in Stevenson et al. (in prep), only two individuals exposed to 200 µg/L AgNPs lived long enough to produce offspring at the highest food ration.

One explanation for the difference between the effect of AgNPs on individuals and populations is that the daphnid populations produce more DOC that is mitigating the effect of the AgNPs, both from the higher food inputs that come with more DOC and that the populations themselves produce more DOC than individual *Daphnia*. We did find that populations produce significantly more DOC than individuals (Figure 9), however we also found that media taken from daphnid populations not only failed to protect individual

Daphnia from AgNP acute toxicity, but individuals exposed to media containing daphnid-produced DOC actually died *faster* than individuals exposed to AgNPs in COMBO media (Figure 10). Therefore, it is unlikely that the DOC produced by daphnid populations mitigated AgNP toxicity. This contradicts past studies that found a protective effect of natural organic material on silver toxicity to zooplankton: natural organic material has been shown to mitigate ionic silver toxicity (Bury, Galvez et al. 1999, Karen, Ownby et al. 1999, Glover, Sharma et al. 2005, Naddy, Gorsuch et al. 2007) and, more recently, AgNP toxicity (Kennedy, Chappell et al. 2012, Blinova, Niskanen et al. 2013, Newton, Puppala et al. 2013) to zooplankton. Our results contradict a similar study that conducted an acute toxicity test using AgNPs (with different coatings from the citrate-coated AgNPs used here) that found a protective effect of Suwanee River DOC (Newton, Puppala et al. 2013). However, the difference between our empirical results may be a result of the type of DOC used. The strength of the mitigating effect of silver by DOC varies with the type of organic material (artificial versus natural and even between types of artificial organic material) (Glover, Playle et al. 2005). Newton et al. 2013 used artificial DOC (Suwanee River DOC) while our exposures only included daphnid-produced DOC. One way in which DOC could *increase* AgNP toxicity is if the coating of organic material on the particle stabilized it and kept it in suspension, thus increasing interactions between the nanoparticle and aquatic organisms (Ivask, Juganson et al. 2014).

Population-level feedbacks between daphnid consumers and its algal resource compensate for AgNP toxicity

A population model parameterized using individual level data from control and AgNP-exposed individuals was able to broadly match the predicted equilibrium biomass of our experimental populations (Figure 13). This indicates that population-level feedbacks, in which daphnid populations exposed to AgNPs equilibrate at a lower biomass, causing the algal equilibrium to increase, such that each individual daphnid in the population has access to more food per individual. This rescues AgNP-exposed individuals from toxicity, and we have also shown empirically that increased rations can alleviate AgNP toxicity (Stevenson et al. in prep). Through this work, we have demonstrated the strength of ecological modeling to effectively extrapolate individual-level effects of a toxicant to small populations.

Tables

Table 1: Individual model for stage structured and ration model

State variables:	
F	Food density (mgC/L)
W	Weight of an individual (mgC)
C	Cumulative eggs produced
Functions:	
$\phi_J(t) = \frac{I_J F W}{F + F_h}$	Juvenile food ingestion
$\phi_A(t) = \frac{I_A F W}{F + F_h}$	Adult food ingestion
Balance equations:	
Juveniles (If $W < W_p$)	
$\frac{dF}{dt} = -\phi_J(t) V^{-1}$	Food
$\frac{dW}{dt} = \varepsilon \phi_J(t) - b\,W$	Juvenile growth
$\frac{dC}{dt} = 0$	Juvenile egg production
Adults (If $W \geq W_p$)	
$\frac{dF}{dt} = -\phi_A(t) V^{-1}$	Food

$\frac{dW}{dt} = (1-\chi)\varepsilon\phi_A(t) - b\,W$	Adult growth
$\frac{dC}{dt} = \frac{\chi}{\gamma}\varepsilon\phi_A(t)$	Adult egg production

Table 2: Individual model parameters estimated from data in Stevenson et al. (in prep) fit to control and 200 µg/L AgNP exposures. The units mgC-D and mgC-A are milligrams carbon in *Daphnia* and algae respectively.

	Parameters	**Control value**	**200 µg/L AgNP value**	**Units**
q	I_A/I_J – Ratio of adult and juvenile mass-specific ingestion rates	0.88	0.75	
F_h	Half saturation constant in function response	0.16	0.16	mgC-A/L
ε	Assimilation efficiency	0.6	0.6	mgC-D/mgC-A
χ	Proportion of net production allocated to reproduction	0.74	0.65	dimensionless
γ	Carbon required to produce one new offspring (carbon per egg)	0.0017	0.0017	mgCD/egg
b	Maintenance rate	0.13	0.15	1/day

Table 3: Structure of the stage-structured "ration" model. The terms "mass" and "biomass" throughout relate to carbon mass. The units mgC-D and mgC-A are milligrams carbon in *Daphnia* and algae respectively.

Populations	$N_J(t)$	= Number of juveniles at time t (Ind.)
	$N_A(t)$	= Numer of adults at time t (Ind.)
Carbon Masses	$B_J(t)$	= Carbon biomass of juveniles at time t (mgC-D)
	$B_A(t)$	= Carbon biomass of adults at time t (mgC-D)
Rates	$R_J(t)$	= Juvenile recruitment rate at time t (ind d^{-1})
	$M_J(t)$	= Maturation rate from juvenile stage at time t (ind d^{-1})
	$g_J(t)$	= Juvenile specific growth rate (d^{-1})
	$\beta(t)$	= Adult fecundity (neonates per adult per day) at time t (d^{-1})
	$\tau_J(t)$	= Juvenile development time for individual maturing at time t (d)
	$\rho_J(t)$	= Daily food ration per unit biomass for juveniles (mgC-A $mgC\text{-}D^{-1}$ d^{-1})
	$\rho_A(t)$	= Daily food ration per unit biomass for juveniles (mgC-A $mgC\text{-}D^{-1}$ d^{-1})
Parameters for Individuals	m_J	= Juvenile per capita death rate (constant) (d^{-1})
	m_A	= Adult per capita death rate (constant) (d^{-1})
	b	= Respiration rate (d^{-1})
	w_B	= Carbon mass at birth (mgC)
	w_M	= Carbon mass at maturity (mgC)
	χ	= Fraction of adult assimilate allocated to reproduction
	γ	= Amount of carbon needed to produce one egg (mgC-D/egg)

q = Ratio of adult and juvenile mass-specific ingestion rates

ε = Assimilation efficiency (mgC-D mgC-A^{-1})

Experimental setup

V = System volume (L)

F_R = Food density at transfers (mgC-A/L)

T_R = Average time interval between transfers

Balance Equations

$$\frac{dN_J(t)}{dt} = R_J(t) - M_J(t) - m_J N_J(t)$$ Juveniles

$$\frac{dN_A(t)}{dt} = M_J(t) - m_A\ N_A(t) - M_A(t)$$ Adults

$$\frac{dB_J(t)}{dt} = w_B R_J(t) + g_J(t) B_J(t) - w_M M_J(t) - \left(m_J + b\right) B_J(t)$$ Juv. Biomass

$$\frac{dB_A(t)}{dt} = w_M M_J(t) + \left(1-\chi\right) g_A(t) B_A(t) - \left(m_A + b\right) B_A(t)$$ Adult Biomass

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Expressions for Rates and delay

$$R_J(t) = \beta(t) N_A(t)$$ Juvenile recruitment

$$\beta(t) = \frac{\varepsilon\chi\rho_A(t) B_A(t)}{\gamma N_A(t)}$$ Fecundity

$$g_J(t) = \varepsilon\rho_J(t) - b$$ Juvenile growth

$$g_A(t) = \varepsilon\left(1-\chi\right)\rho_A(t) - b$$ Adult growth

$$M_J(t) = R_J(t-\tau_J(t))e^{-m_J\tau_J(t)}\frac{g_J(t)}{g_J(t-\tau_J(t))}$$ Adult recruitment

$$w_B - w_M = \int_{t-\tau(t)}^{t} g_J(x)dx \Rightarrow \frac{d\tau_J(t)}{dt} = 1 - \frac{g_J(t)}{g_J(t-\tau(t))}$$ Juv. development

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Rations

$$\rho_J(t) = \frac{\left(\frac{F_R V}{T_R}\right)}{\left(B_J + qB_A\right)}$$ Juvenile Ration

$$\rho_A(t) = \frac{q\left(\frac{F_R V}{T_R}\right)}{\left(B_J + qB_A\right)}$$ Adult Ration

---

Table 3: Parameters for population model.

Parameter	Units	Interpretation	Source	Value Control/Ag NP
m_J	1/day	Juvenile per capita death rate	Individual data	0.039/0.097
m_A	1/day	Adult per capita death rate	Individual data	0.139/0.159
w_B	mgC-D	Carbon mass at birth	Individual data	1.06e-3
w_M	mgC-D	Carbon mass at maturity	Individual data	1.02e-2
V	L	System volume	Population experiment	0.4
F_R	$mgCL^{-1}$	Food concentration at transfer	Population experiment	0.41, 0.82, 1.57
T_F	day	Average transfer interval	Population experiment	2.33

Figures

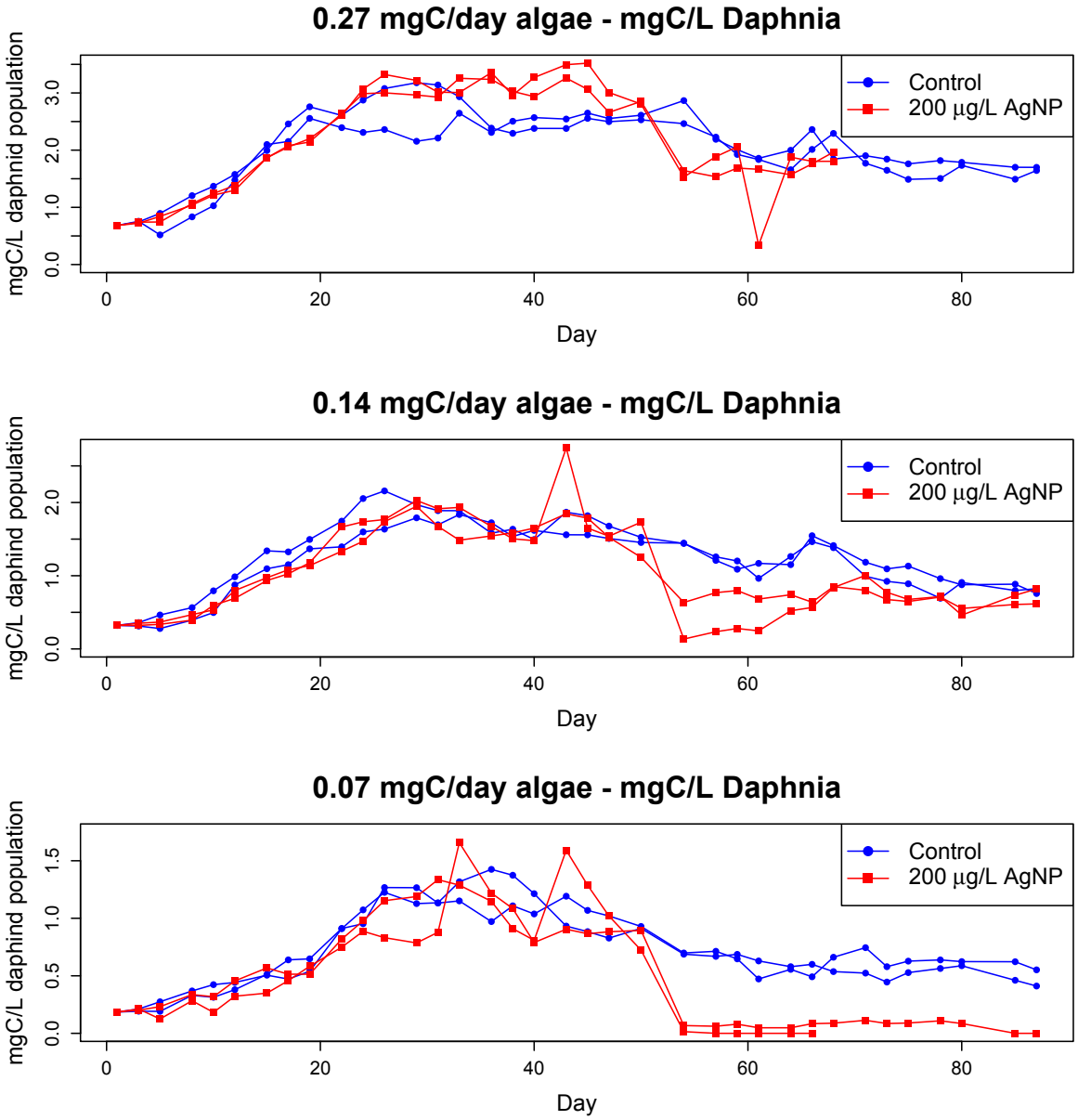

Figure 1: Carbon biomass per L of daphnid populations fed different food inputs. Each treatment (nano and food concentration) had 2 replicates.

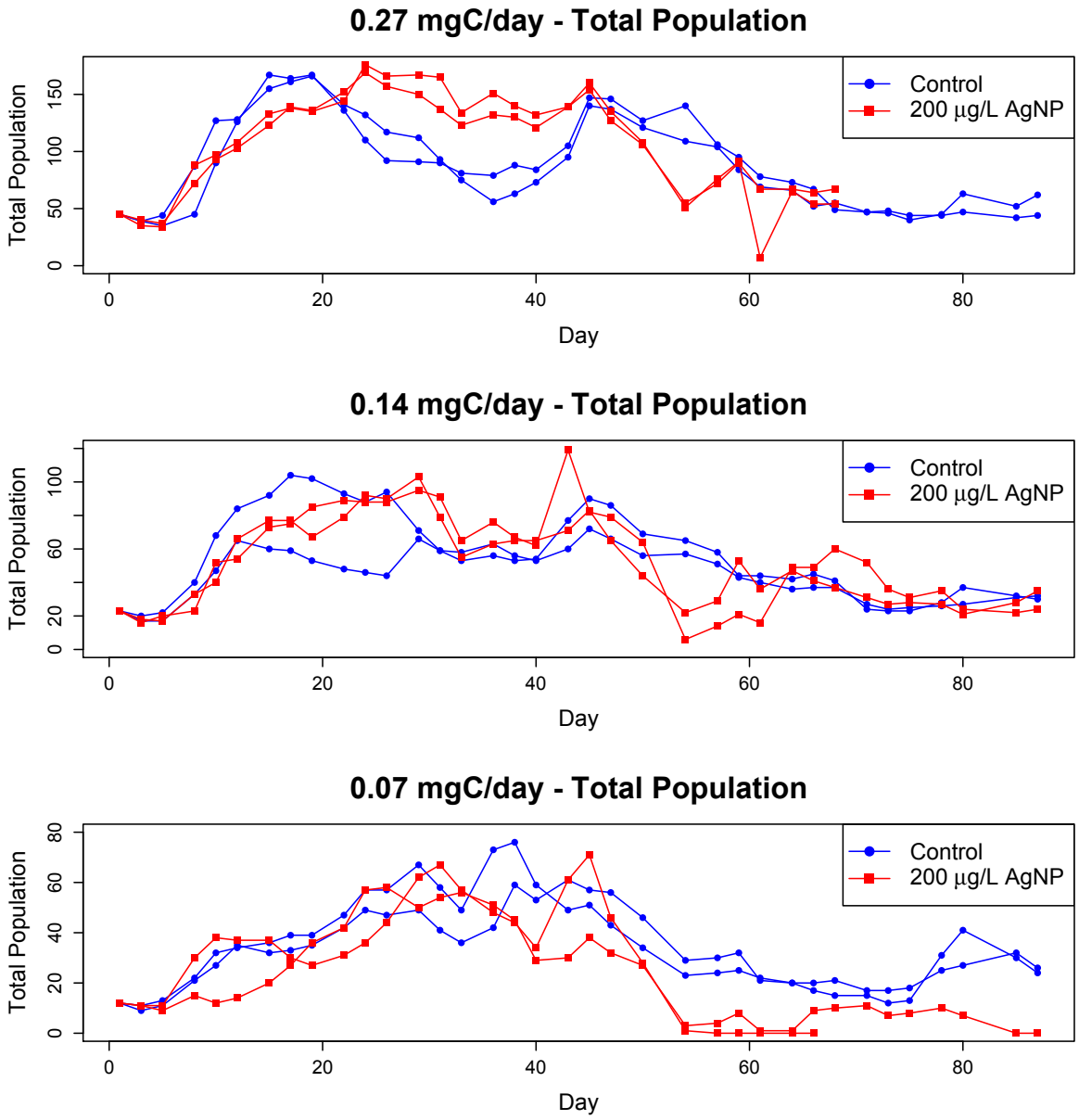

Figure 2: Total number of individuals in each of the daphnid populations fed different food inputs. Each treatment (nano and food concentration) had 2 replicates.

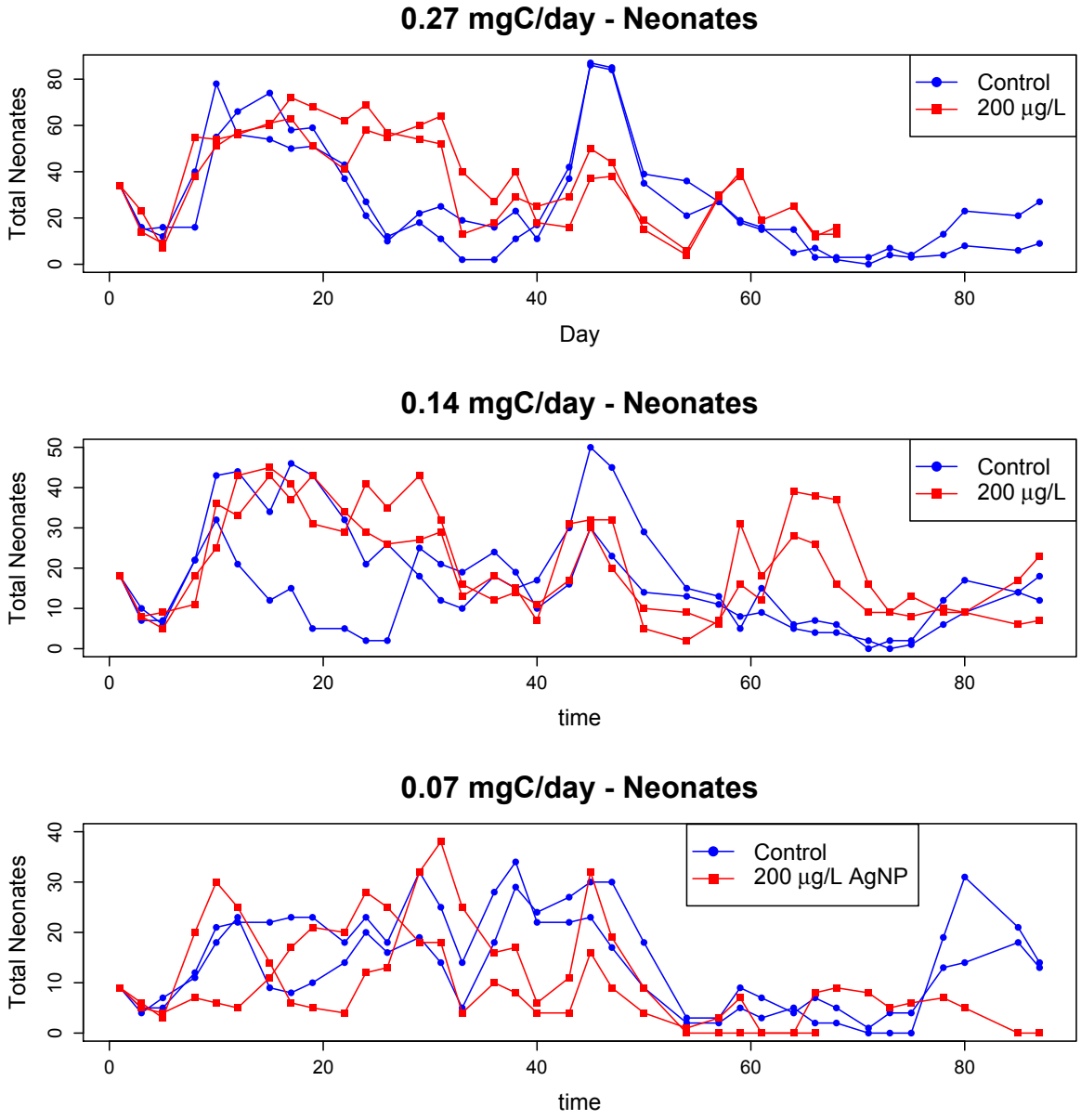

Figure 3: Total number of neonates in each of the daphnid populations fed different food inputs. Each treatment (nano and food concentration) had 2 replicates.

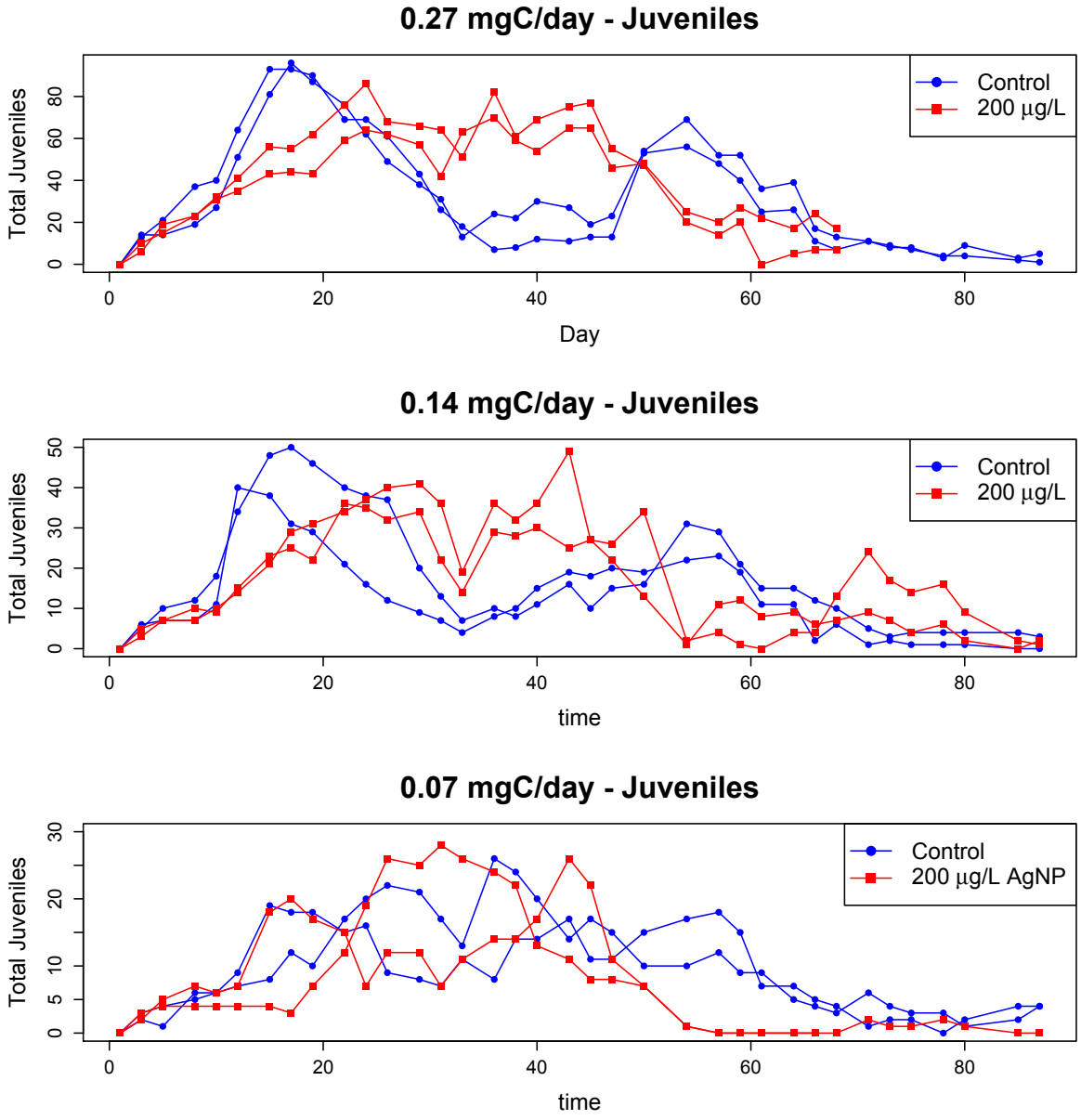

Figure 4: Total number of juveniles in each of the daphnid populations fed different food inputs. Each treatment (nano and food concentration) had 2 replicates.

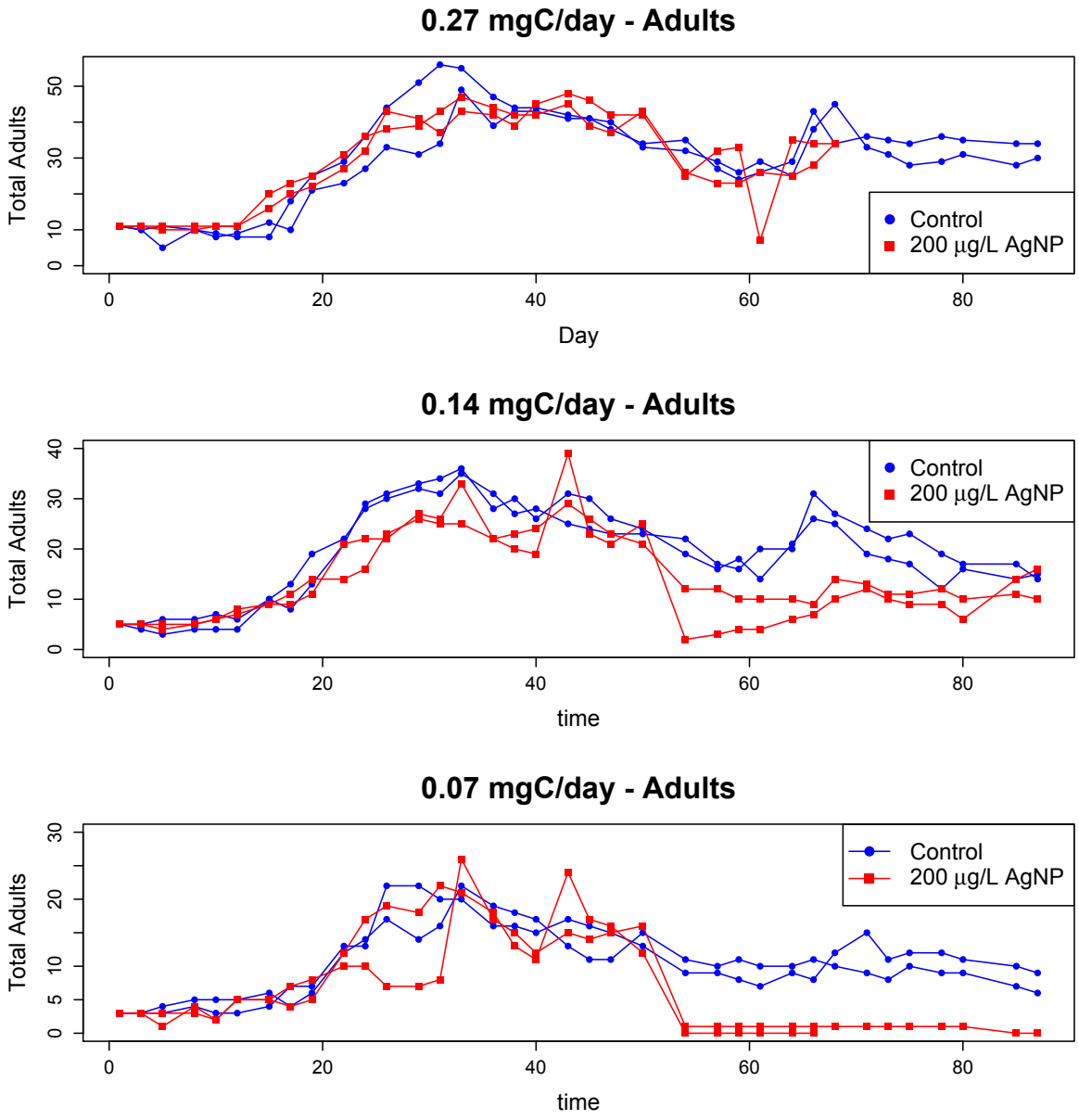

Figure 5: Total number of adults in each of the daphnid populations fed different food inputs. Each treatment (nano and food concentration) had 2 replicates.

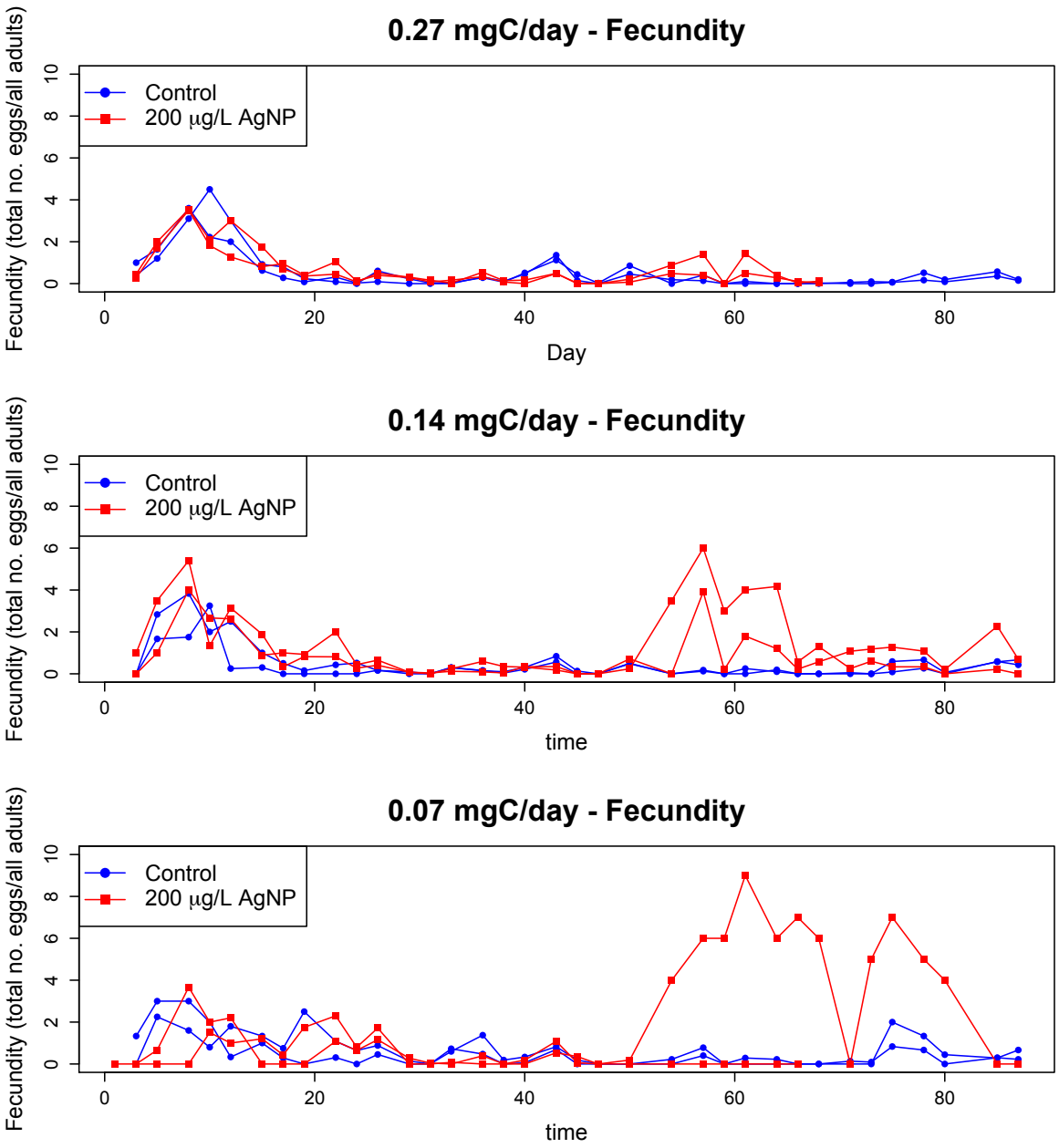

Figure 6: Fecundity (total number of eggs divided by total number of adults results in number of eggs per adult) in each of the daphnid populations fed different food inputs. Each treatment (nano and food concentration) had 2 replicates. Note that the giant spikes in fecundity in panel C from days 50-85 are from the one surviving adult producing neonates at every moult, however none of these offspring developed into adults.

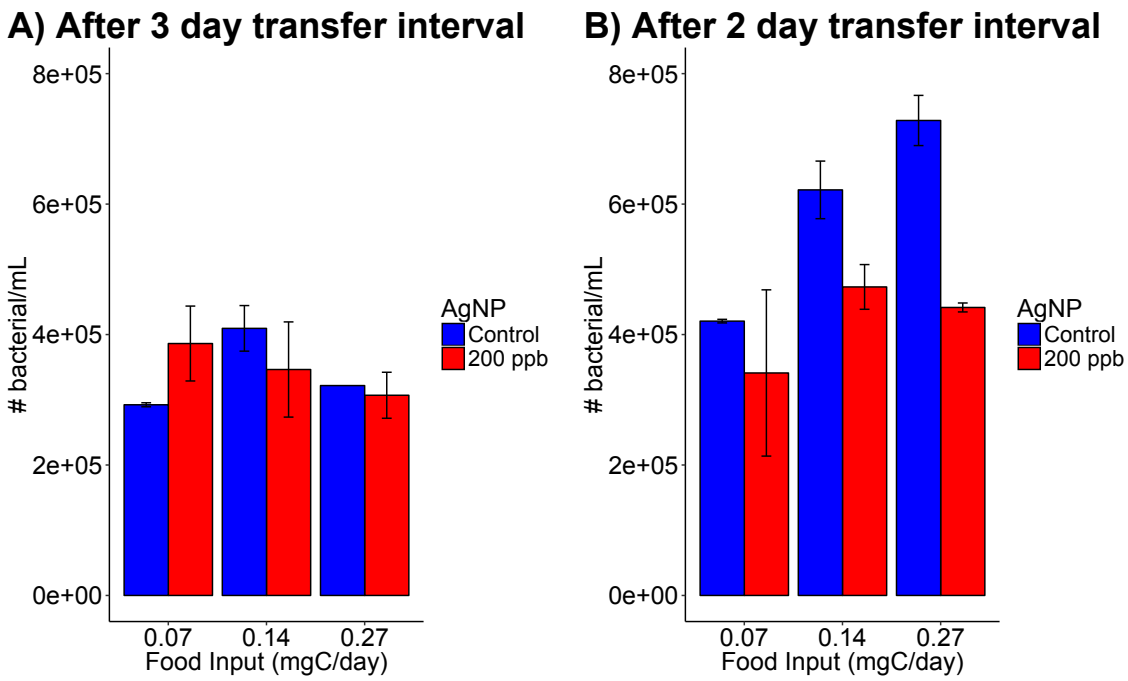

Figure 7: Bacterial populations after a 3 day (A) and 2 day (B) transfer interval. Samples were taken on days 57 and 59 of the population experiment. Data represent averages of all replicate samples (n=2 per treatment as one sample was taken per experimental culture) and error bars reflect their standard error.

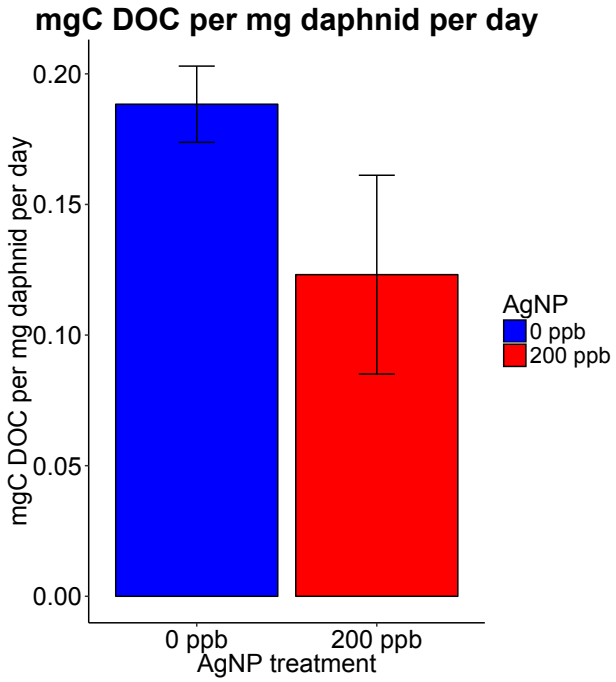

Figure 8: DOC produced per day per mg of total daphnid biomass in the tube at the start of the experiment. These data include those samples in which daphnid individuals died because that did not have a significant effect on the results. Overall, 7 individuals in control treatments and 1 individual in the AgNP treatment died. Samples are from 58 tubes containing 127 total *Daphnia*.

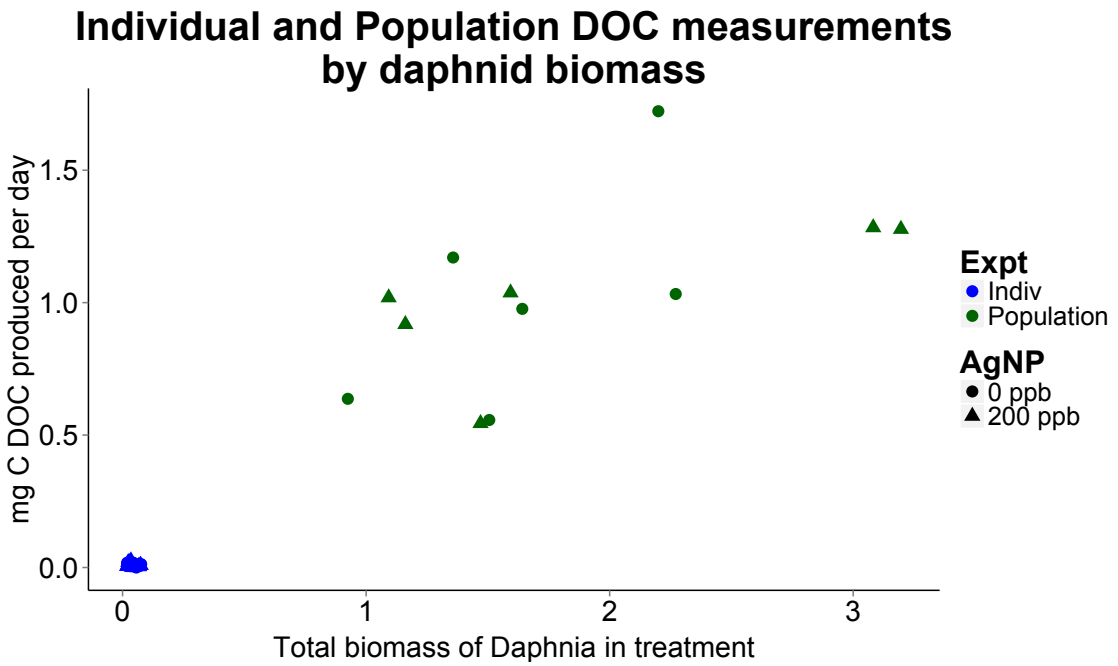

Figure 9: DOC produced per day by daphnid individuals and populations. Daphnid populations produce more DOC than individuals and there does not appear to be an effect of AgNPs on daphnid DOC production.

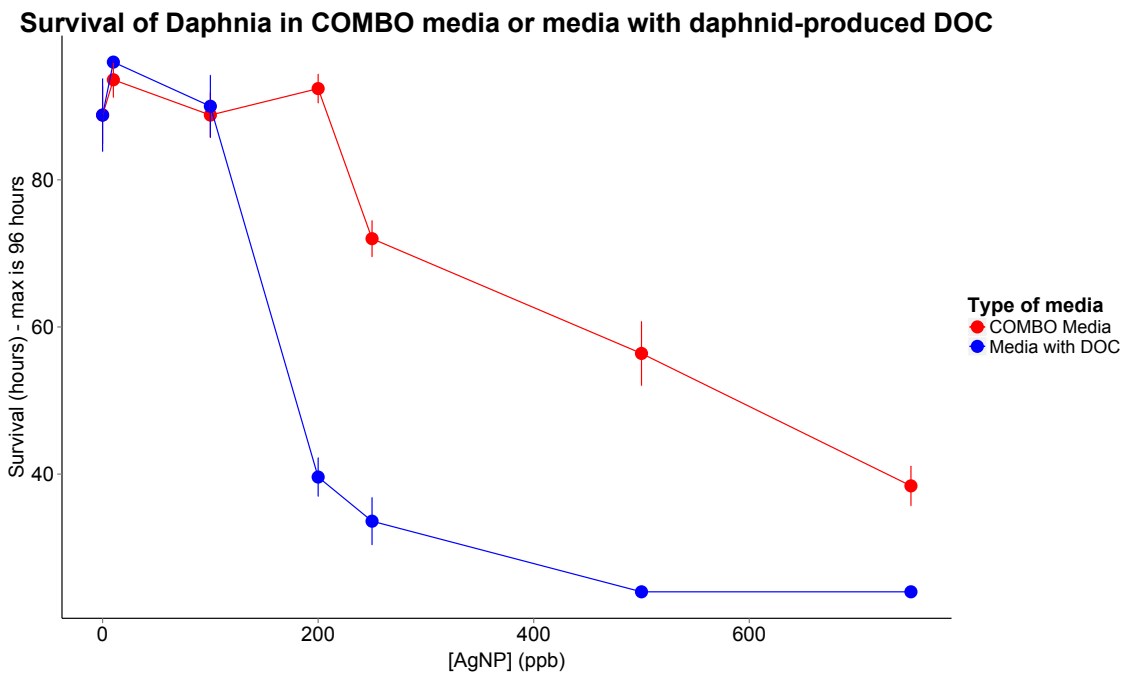

Figure 10: Average survival time (in hours) of less than 24-hour-old *Daphnia* exposed to 0, 10, 100, 200, 250, 500 and 750 µg/L AgNPs in COMBO media versus those exposed in media containing daphnid-produced DOC. The data points reflect averages and the error bars are their standard error (n=20 for all treatments).

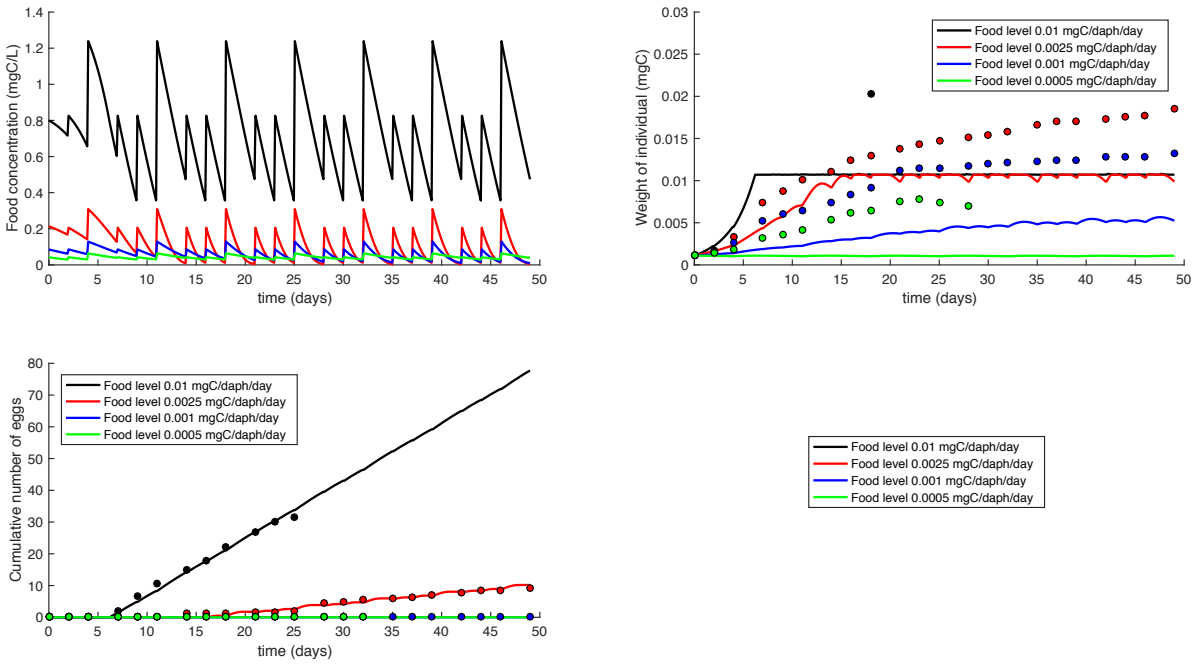

Figure 11: Fit of individual model to control data (Stevenson et al. in prep).

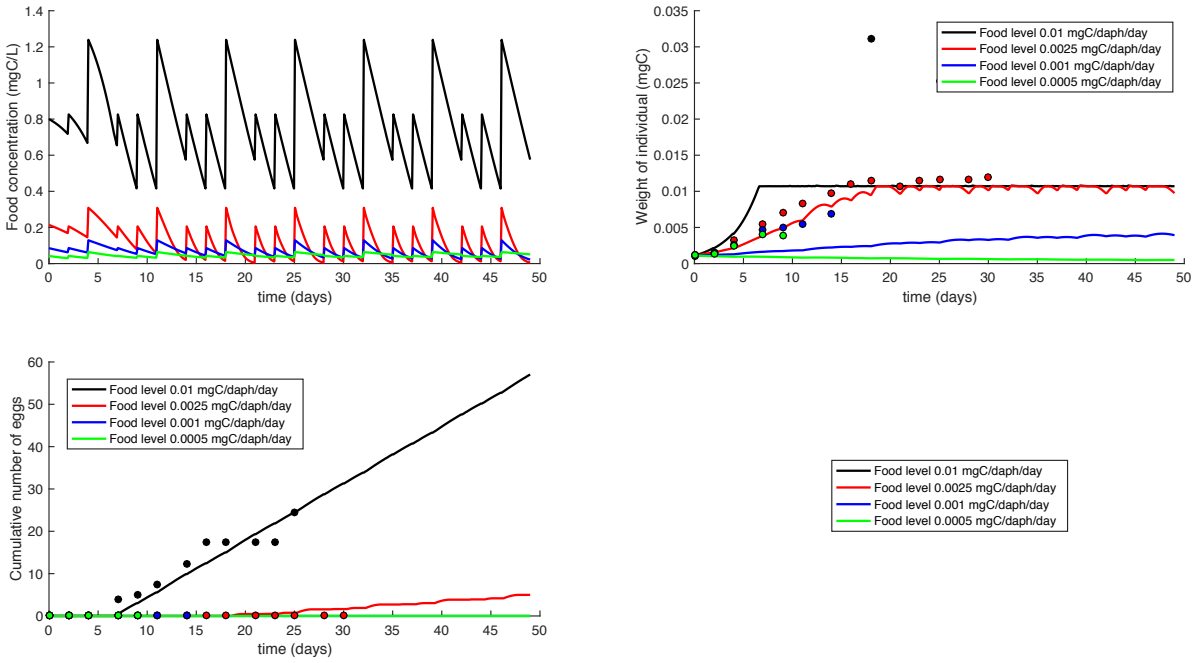

Figure 12: Fit of individual model to 200 µg/L AgNP data (Stevenson et al. in prep).

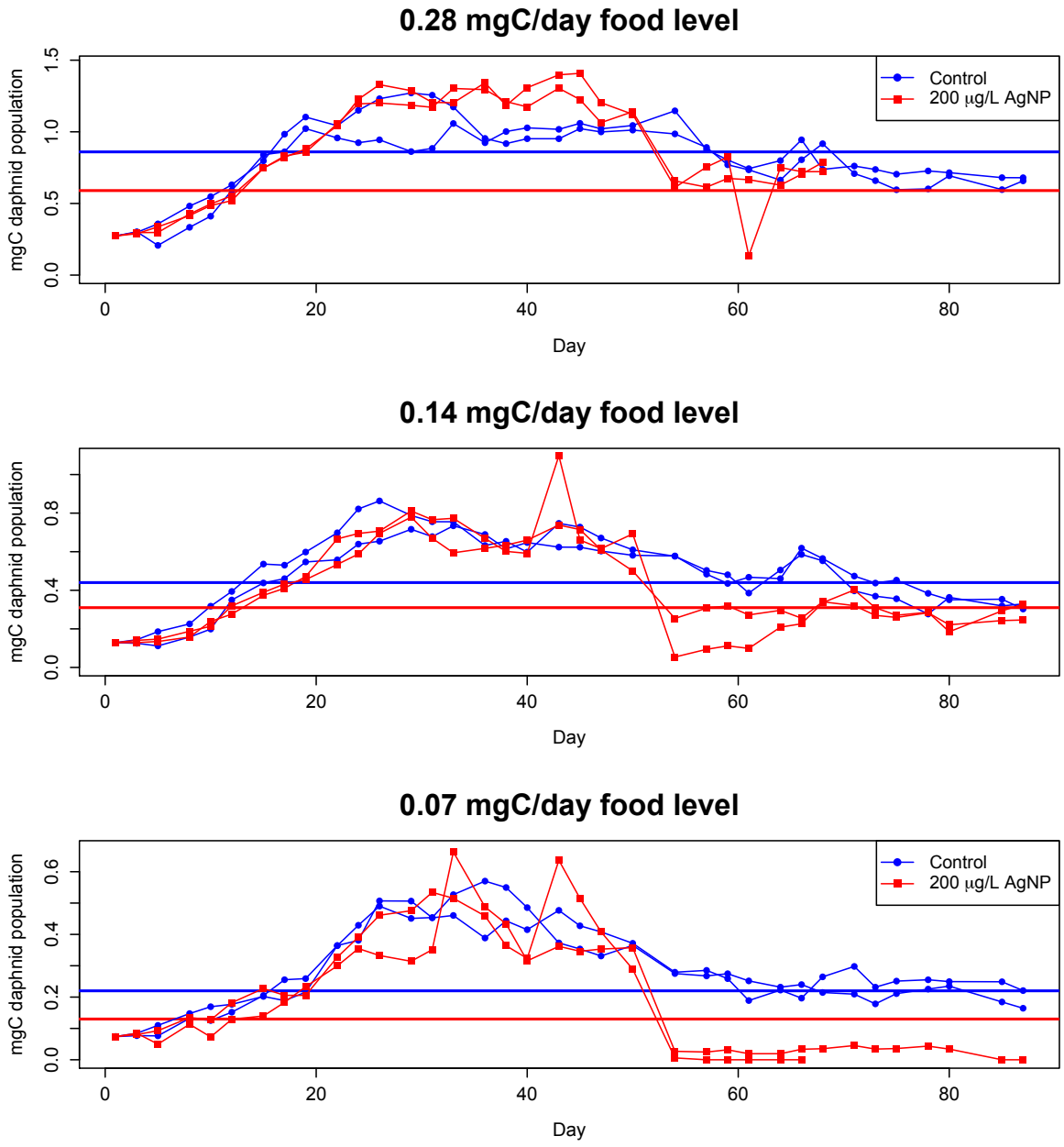

Figure 13: Equilibrium biomass predictions (horizontal lines) compared to population data.

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IV. Modified nano-zerovalent iron (FeSSi) mitigates cadmium toxicity to a freshwater alga

Authors: Louise M. Stevenson[1,4], Adeyemi S. Adeleye[2,4], Yiming Su[3], A. Keller[2, 4], Roger M. Nisbet[1,4]

Abstract

Nano-zerovalent iron (nZVI) and its derivatives are widely used for their ability to remove environmental contaminants in lab and field studies. However, to our knowledge, the ability of nZVI and derivatives to remove the toxicity of its target contaminant to a biological organism has not been demonstrated. To address this void in the literature, we used a derivative of nZVI modified with sulfur and silica (FeSSi) to sorb cadmium from aqueous media and remove Cd toxicity to a model freshwater alga, *Chlamydomonas reinhardtii*. FeSSi itself is toxic to the algae, however the particle's toxicity is mitigated by dissolved organic carbon (DOC) produced by the algal cells themselves. Further, we developed a quantitative model that allowed us to estimate the relative toxicity of cadmium and FeSSi to the algae, and this analysis also demonstrated that FeSSi particles with cadmium sorbed onto them are actually *more* toxic than FeSSi alone. Overall, our study demonstrates the effectiveness of FeSSi as an environmental remediator and also the strength of a generalizable quantitative model of the mitigation of nanoparticle toxicity by algal-produced organic material.

Introduction

Nano-zerovalent iron (nZVI) and its derivatives are currently the most commonly applied technology for nanoremediation (Lefevre, Bossa et al. , Karn, Kuiken et al. 2009, Adeleye, Conway et al. 2016, Stefaniuk, Oleszczuk et al. 2016) and can remove a wide array of metals and organic compounds in the lab (Su, Adeleye et al. 2014, Phenrat, Thongboot et al. 2016) and in the field (Wei, Wu et al. 2010, Su, Puls et al. 2012, Su, Puls et al. 2013). nZVI-based nanoremediation technology is appealing both because of its high reactivity (due to its small size and thus high surface area to volume ratio) and because it can be applied in situ, without the need for excavation and/or waste disposal (Karn, Kuiken et al. 2009, Su, Puls et al. 2012, Su, Puls et al. 2013, Adeleye, Conway et al. 2016). However, it is unclear how the direct introduction of nZVI and derivatives for environmental remediation will affect natural ecosystems. Many studies have investigated the potential fate and transport of nZVI in natural systems (Phenrat, Cihan et al. 2010, Crane and Scott 2012, Adeleye, Keller et al. 2013, Su, Puls et al. 2013, Shi, Fan et al. 2015) and others have found that nZVI and derivatives alone can be toxic to bacteria (Auffan, Achouak et al. 2008, Diao and Yao 2009, Sevcu, El-Temsah et al. 2011, Lefevre, Bossa et al. 2016), phtyo- and zooplankton (Keller, Garner et al. 2012), fish (Li, Zhou et al. 2009, Chen, Su et al. 2011, Chen, Wu et al. 2013), and earthworms (El-Temsah and Joner 2012).

In all of these studies on the toxicity of nZVI and derivatives, the iron nanoparticles are exposed to test organisms as pristine nZVI. However, these nanoparticles are released into the environment for remediation purposes and will probably be bound to the contaminants (Su, Adeleye et al. 2015, Su, Adeleye et al. 2016) or will have reacted

with some environmental factor since they transform quite rapidly in the environment (Crane and Scott 2012, Adeleye, Keller et al. 2013, Su, Puls et al. 2013, Liu, Liu et al. 2014, Liu, Liu et al. 2015). To the best of our knowledge, our study is the first to investigate the effects of nZVI or its derivatives bound to its target contaminant on an organism.

In this study, a derivative of nZVI (modified with sulfur and silica (Su, Adeleye et al. 2016)) named FeSSi was used to remove cadmium (Cd) from cultures of *Chlamydomonas reinhardtii* (a model freshwater algae). Cadmium is a potent environmental contaminant and second in toxicity only to mercury to algae (Trevors, Stratton et al. 1986). We employed a step-wise approach to both the experimental and modeling aspects of this work. We began by exposing algal cultures to cadmium alone ("Cd only experiments") to characterize Cd toxicity and developed a model of Cd toxicity to *C. reinhardtii* using these data. Secondly, we exposed algal cultures to FeSSi alone ("FeSSi only experiments", published in Adeleye et al. 2016) and adapted the model of Stevenson, Dickson et al. (2013) to describe the mitigating effect of algal dissolved organic carbon (DOC) on FeSSi toxicity (Adeleye, Stevenson et al. 2016). Along with these exposures of FeSSi alone, we exposed additional algal cultures to FeSSi with Cd ("FeSSi+Cd experiments") and adapted the model in Adeleye et al. (2016) to include the effect of Cd (characterized by the Cd only experiments and Cd model) and the effect of FeSSi with Cd sorbed onto it. In all experiments, we exposed algal cultures at two different growth stages: new cultures (entering exponential phase) and 11-day old cultures (entering stationary phase). These two stages of algal growth differ from each other in the amount of dissolved organic carbon (DOC) that has accumulated in the cultures. In the

FeSSi+Cd experiments, we monitored algal growth and changes in the nanoparticle-contaminant composites over time for 24 days through the use of an array of analytical methods including X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), and inductively coupled plasma atomic emission spectroscopy (ICP-AES). Our study is the first to demonstrate that a nZVI derivative can bind and reduce the toxicity of its target contaminant to an organism. Through the development of a quantitative model, we are able to identify the relative toxic strength of Cd, FeSSi and FeSSi with Cd sorbed onto it and demonstrate that FeSSi with the contaminant sorbed onto its surface is more toxic than FeSSi alone. Further, our model can be used to make environmental remediation decisions by predicting the effect of other concentrations of Cd and FeSSi on *C. reinhardtii.* Using the model, we can estimate the amount of FeSSi needed to effectively remediate a contaminated environment and then predict the effect of FeSSi and FeSSi with Cd sorbed onto it on the alga. Overall, our study provides important insights into the potential environmental implications of nanoremediation technology.

Methods

We performed three sets of experiments, exposing new and 11-day old cultures to: 1) Cd only ("Cd only experiments"), 2) FeSSi with and without Cd (FeSSi-only exposures are reported in Adeleye et al. 2016 and denoted as "FeSSi only experiments"; FeSSi with Cd exposures are reported here and denoted as "FeSSi+Cd experiments") and 3) additional exposures to FeSSi and FeSSi+Cd for XRD and XPS ("XRD and XPS exposures"). In all of these experiments, algal cultures were exposed to the toxicants as new and 11-day old cultures in order to test for the effect of dissolved organic carbon (DOC) on toxicity. New

cultures were inoculated on the first day of the experiment and 11-day old cultures were grown for 11 days prior to the experiment's start such that exposures and sampling of both ages occurred at the same time.

Synthesis and characterization of nanoparticles

We synthesized sulfide-modified nZVI seeded with silica (FeSSi) according to the method described in Su et al. (2016). Briefly, we mixed 7.6 g sodium borohydride ($NaBH_4$) purchased from Oakwood Chemical (Estill, SC), 1.5 g sodium dithionite ($Na_2S_2O_4$) purchase from Sigma-Aldrich (St. Louis, MO), and 0.2 mL of colloidal silica (30 wt. %) together in an Erlenmeyer flask and made up the volume to 250 ml with deionized water (DI, 18.2 MΩ.cm, Barnstead Nanopure Diamond). This solution was titrated into another 250 ml solution containing 4.9 g $FeCl_3$ (Fisher Scientific). After the reduction reaction, we collected the FeSSi nanoparticles and triple-washed them with nitrogen-purged DI water. We then separated FeSSi from the aqueous phase with a neodymium-iron-boron magnet, and stored them in 30% ethanol at 4 °C until use.

The physicochemical properties of FeSSi were published in previous studies (Adeleye, Stevenson et al. 2016, Su, Adeleye et al. 2016), and we present the major ones here: FeSSi is mainly spherical with an average particle size of 90 nm (Figure SI 6). Mössbauer analyses revealed that FeSSi is made up of 55.6% Fe(0), 9.9% Fe(II), and 34.5% (Fe(III). The hydrodynamic diameter of FeSSi was determined as 340 nm in DI water (adjusted to pH 7.5 using phosphate buffer) while its zeta (ζ) potential in the same media was -38 mV. The hydrodynamic diameter of FeSSi increased to 369 nm in new algal COMBO media (Kilham, Kreeger et al. 1998) (ζ potential of FeSSi in media = -17

mV), and 390 nm in media from 11-day old cultures with the algal cells removed (ζ potential of FeSSi in media = -11.4 mV).

Toxicity of cadmium to *Chlamydomonas*

We exposed new and 11-day old cultures (algal cultures that had grown for 11 days prior to the experiment) to 0.1, 1, 5 and 10 mg/L cadmium as cadmium chloride ($CdCl_{2;}$ "Cd only experiment"). Algal cultures were 250 mL in COMBO media (Kilham, Kreeger et al. 1998) and all glassware and sampling apparatus were autoclaved prior to the experiment. We measured the concentration of chlorophyll a fluorometrically on a microplate reader and converted the relative fluorescent units to chlorophyll concentrations using a standard curve developed in our lab using chlorophyll a standard (see methods in Stevenson, Dickson et al. (2013)).We also measured the concentration of Cd throughout the Cd only experiment by taking samples from the cultures at a few time points, filtering (0.45 μm) them to remove cells, digesting the samples with trace-metal grade HNO_3 (Fisher Scientific), and analyzing them for Cd via ICP-AES (Thermo Scientific iCAP 6300). To confirm whether or not 5 and 10 ppm cadmium was toxic to 11-day old cultures or if the populations were just at low cell densities, we spun down all 11-day old cultures after 19 days of exposure and re-suspended them in fresh, cadmium-free COMBO media. We concentrated the cells by centrifuging them for 4 minutes at 7,000 rpm, re-suspending the pelleted cells in COMBO media, and centrifuging them for another 4 minutes. We then re-suspended the pelleted cells and added them to media to dilute the cultures to 250 mL and measured the chlorophyll a concentrations of these cultures for another 25 days to see if the algal cultures could recover from cadmium toxicity.

Influence of FeSSi on toxicity of cadmium to *Chlamydomonas*

To investigate the effect of FeSSi-cadmium (Cd) composites in freshwater systems ("FeSSi+Cd experiment"), we added 180 mg/L of FeSSi and 4.5 mg/L Cd (as $CdCl_2$) to new algal COMBO media or media from 11-day old cultures with the algal cells removed (with DOC present) and allowed them to interact for 1 hr by shaking on a Dayton-6Z412A roller-mixer (80 rpm). A previous study showed that adsorption of Cd onto FeSSi from the aqueous phase mostly occurs within 1 hour (Su, Adeleye et al. 2016). We then dosed three dilutions (dilution factors of 1, 10, and 100) of this FeSSi-Cd composite to both new and 11-day old cultures to obtain (1) 180 mg/L FeSSi + 4.5 mg/L Cd, (2) 18 mg/L FeSSi + 0.45 mg/L Cd, and (3) 1.8 mg/L FeSSi + 0.045 mg/L Cd. We incubated algal cultures at 20 °C on a diurnal light cycle (12:12 light:dark) under fluorescent growing lights. All glassware was autoclaved prior to the experiment and all algal cultures were 250 mL of COMBO media. We monitored the effect of FeSSi on algal populations through time by measuring chlorophyll a concentrations (see methods in Stevenson, Dickson et al. (2013)). We also measured dissolution of FeSSi and availability of Cd by taking aliquots from the supernatant of cultures at time points, filtered (0.45 μm) them to remove cells and undissolved particles, then digested the samples with trace-metal grade HNO_3 (Fisher Scientific), and analyzed them for iron (Fe), silicon (Si), sulfur (S), and Cd via ICP-AES (Thermo Scientific iCAP 6300). As we reported previously, FeSSi aggregates in COMBO media to sizes > 0.45 μm within 3 min so it is not expected to pass through the filter (Adeleye, Stevenson et al. 2016, Adeleye, Stevenson et al. In review). In addition, we visualized the interactions between FeSSi-Cd composites and algal cells using a Phillips

FEI XL30 FEG environmental scanning electron microscope (ESEM) equipped with a Bruker XFlash 6160 energy dispersive spectrometer (EDS). Imaging was done in wet mode at 2.2 Torr, 4 °C, and an accelerating voltage of 10 kV. ESEM images were taken at the end of the experiments by fixing 1 mL of cultures (dosed with 180 mg/L FeSSi + 4.5 mg/L Cd) with formalin (5%). The fixed cultures were deposited on a JEOL aluminum specimen mount, and then imaged using the ESEM.

Transformation of FeSSi in culture

We performed X-ray diffraction and X-ray photoelectron spectroscopy (XRD and XPS), the two most common techniques for determining the chemical state of solids and powders, to investigate the transformation of FeSSi and the fate of Cd during these experiments. We conducted an additional algal exposure experiment ("XRD and XPS exposures") so that we could sacrifice entire algal cultures at various time points to collect a large enough mass of FeSSi particles for XRD and XPS. We prepared stocks of FeSSi-Cd composites (180 mg/L of FeSSi and 4.5 mg/L Cd) in new algal COMBO media and media from 11-day old cultures and dosed new and 11-day old cultures as described in the last section. The response of these algal cultures (chlorophyll a concentrations measured fluorometrically) was comparable to the results of our FeSSi+Cd experiment (SI Figure 10), so we are confident that the XRD results reflect the transformation of FeSSi during our the FeSSi+Cd experiments. At certain time points (2, 10, and 30 days), we sacrificed an entire 250 mL culture and separated the solid fractions from suspension via centrifugation (10,000 *g*, 30 min; Sorvall RC 5B Plus). After centrifugation, we decanted the supernatant and immediately vacuum-dried the particles (Yamato ADP-21). The solid

fractions obtained thereafter were then analyzed via X-ray diffraction (XRD, fluorescence mode; Bruker D8 Advance) and X-ray photoelectron spectroscopy (XPS; Kratos Axis Ultra). For XRD analyses, step scans were performed from 10 to 90° 2θ, and a step size of 0.02°.

Model description

To interpret our results, we first developed a model of the effect of cadmium on *C. reinhardtii* (Model 1, see SI for details). We then incorporated this model of Cd toxicity into a mechanistic model based on concepts from Stevenson et al. (2013) and developed for FeSSi-only exposure in Adeleye et al. (2016). The state variables of the model are algal biomass, DOC concentration, Cd concentration, and the various forms of FeSSi – F (unbound FeSSi), F_D (FeSSi inactivated by DOC), F_C (FeSSi with Cd sorbed onto it), and F_{CD} (FeSSi with Cd sorbed onto it that is inactivated by DOC) (see Table 1 for model equations and Table 2 for parameter values). Algal growth is modeled using a logistic growth curve and DOC production is assumed to be proportional to algal biomass and growth rate. FeSSi (with and without DOC; F and F_D) sorbs Cd (turning F into F_C and F_D into F_{CD}) at the same rate (*b*) (Table 2). Cd, F and F_C are all toxic at different rates (described by k_C, k_F, and k_{FC}, respectively) and DOC inactivates F and F_C (turning F into F_D and F_C into F_{CD}) at the same rate (γ_{UN}) (Table 2). We fit parameters specific to Cd exposure by fitting the Cd-only model to the Cd only experimental data (Model 1; see SI) and used parameter values from Adeleye et al. (2016) for parameters related to FeSSi only toxicity (Table 2). All other parameters (k_{FC}, *b*, and α_{CF}) were fit to the FeSSi+Cd data: algal biomass, Cd concentration, and DOC concentration (Table 2). We fit parameters

using BYOM ("Bring Your Own Model") platform for parameter estimation, developed by Tjalling Jager for Matlab (http://debtox.info). Further model details are in the SI.

Results/Discussion

Cadmium toxicity to *C. reinhardtii*

Dissolved Cd is toxic to *Chlamydomonas reinhardtii* at low ppm concentrations (Figure 2). 5 and 10 ppm cadmium causes complete mortality of new cultures and reduces populations of 11-day old cultures to a very low population size (Figure 2). We re-suspended 11-day old algal cultures exposed to 5 and 10 ppm Cd to see if the populations were dead or at low cell numbers and found that cultures exposed to 5 ppm Cd grew to population sizes similar to controls after 25 days in new, Cd-free media, however cultures exposed to 10 ppm did not grow even after being moved to the new media (Figure S1). This may indicate that 11-day old cultures were able to either survive or even adapt to 5 ppm cadmium. Our finding that 5 and 10 ppm Cd is toxic to *C. reinhardtii* is in agreement with some studies in the literature (Cain and Allen 1980, Prasad, Drej et al. 1998, Mosulen, Dominguez et al. 2003, Jamers, Blust et al. 2013) while other studies have found that lower concentrations are toxic (Trevors, Stratton et al. 1986, Macfie, Tarmohamed et al. 1994) or that the concentration must be much higher to exert toxicity to *C. reinhardtii* (Aguilera and Amils 2005, Gillet, Decottignies et al. 2006). These discrepancies may be due to differences in experimental conditions, such as pH, which can have a large effect on the toxicity of Cd to algae (Macfie, Tarmohamed et al. 1994). Cadmium exerts toxicity through oxidative stress (Gillet, Decottignies et al. 2006, Jamers, Blust et al. 2013), disruption of photosynthesis (Trevors, Stratton et al. 1986, Nagel, Adelmeier et al. 1996,

Gillet, Decottignies et al. 2006), and/or nitrate uptake inhibition (Mosulen, Dominguez et al. 2003). Further, numerous studies have found that *C. reinhardtii* accumulates cadmium within the cell itself (Hu, Lau et al. 2001, Aguilera and Amils 2005), primarily in the chloroplast (Nagel, Adelmeier et al. 1996).

We fit a simple model of cadmium toxicity to the data from our cadmium-only experiment (Model 1 in SI). The model only has four parameters – two that describe algal growth (growth rate r, and algal carrying capacity K from the logistic growth equation) and two that describe cadmium toxicity (cadmium-specific toxicity k_C and the no effect concentration of cadmium NEC). This model is very simple but gives a good fit to our data (see SI Figure 2) and the estimated NEC value (0.5126 mg Cd/L) is in agreement with some studies (Cain and Allen 1980, Jamers, Blust et al. 2013). The development and parameterization of this model allows us to predict the effect of cadmium concentrations beyond the concentrations we used in our experiments, which is crucial in evaluating whether or not FeSSi is able to mitigate the toxicity of cadmium to algae.

FeSSi removes Cd from interacting with the algae

FeSSi sorbed cadmium within the first hour of FeSSi-Cd interaction and held onto the cadmium throughout the experiment. This is because, prior to exposure in our FeSSi+Cd experiment (results in Figure 3), 180 ppm FeSSi and 4.5 ppm cadmium were added to new algal media or media from 11-day old cultures with the algal cells removed (with DOC present) and interacted for an hour before dosing three concentrations to *C. reinhardtii*. Our aim was to show that FeSSi removed cadmium from the media using qualitative and quantitate measures, such as XPS, XRD, ICP measurements, and ESEM

imaging, and we have data throughout the experiment to show that FeSSi sorbed cadmium for the duration of the algal exposure.

We quantified the amount of cadmium present in the media (not bound to FeSSi or taken up by the algal cells) throughout the algal experiment (SI Figure 4). The ICP data show that at the start of the experiment (after FeSSi had interacted with Cd for an hour pre-exposure), the FeSSi particles had bound 87.6 ± 1.0 % and 81.2 ± 0.7% (averages ± standard error of 3 treatments) of the cadmium introduced in new media (which was then dosed to new cultures) and media from 11-day old cultures, respectively (SI Figure 4). Su et al (2016) reported that the Cd binding potential of FeSSi is 105 mg Cd per g FeSSi, however the amount of Cd leftover after an hour of binding indicates that the maximum initial Cd binding was 18.3 mg Cd per g FeSSi when exposure occurs in media as opposed to pure water (as in Su, Adeleye et al. (2016)). This is probably due to the sorption of nutrients on FeSSi, potentially outcompeting the cadmium for binding sites. We measured the concentration of nutrients that can potentially limit algal growth (PO_4, NO_3, NO_2, and NH_4) and found that the initial concentration of phosphate decreased with increasing concentrations of FeSSi particles (SI Figure 7). Interestingly, FeSSi particles appear to sorb the same amount of phosphate with or without cadmium present, potentially indicating that phosphate sorprtion is faster or somehow outcompetes sorption of cadmium onto the particles (SI Figure 7). Further, XPS data also showed that the strong adsorption properties of FeSSi led to the removal of some essential nutrients (such as N, P, and Ca) from algal media. For instance, in the XPS quantification data obtained after FeSSi-Cd composites had been exposed to 1-day cultures for 30 days, we found that 1.3% of the atoms analyzed were N while 0.13% were P (SI Table 1). Adsorption of essential nutrients

from media by FeSSi may explain more subtle deleterious effects of FeSSi on algae, such as the delayed growth of new cultures exposed to 18 ppm FeSSi, however the removal of essential nutrients likely did not cause the complete toxicity observed in some treatments. Our finding that FeSSi can sorb significantly less cadmium in synthetic freshwater media than when the particle is tested with Cd in pure water emphasizes the need to analyze the removal capacity of potential remediators in environmentally relevant media instead of only testing in pure water.

The XPS data we obtained agreed well with the ICP analyses that FeSSi particles removed Cd from the aqueous phase in both new and 11-day algal cultures. This is evident by the peaks for Cd in the XPS survey scan presented in Figure 1. The primary XPS peak for metals may be one or more of 1s, 2p, 3d, or 4f regions. The main peak for Cd is the 3d peak, which we found at BE = 405 eV in the spectra obtained from samples dosed with FeSSi-Cd composites (Figure 1a). For comparison, the survey scan of FeSSi only in algal culture was also presented, which clearly shows no peaks for Cd. Since XPS typically probes the top 10 nm of samples, this data shows that the Cd adsorbed from the aqueous phase are mainly sequestered on the surface of the nanoparticles.

FeSSi sorbed, in some treatments, nearly 90% of the Cd at the start of the experiment, and our data demonstrate that FeSSi held on to most of the cadmium throughout the duration of the experiment (SI Figure 4). There is an interesting pattern at the start of the experiment in which the cadmium concentration actually *increases* over the first few days in new algal cultures. The opposite occurs in 11-day old cultures – the cadmium concentration actually declines through time in this exposure. This decline is probably due to uptake of cadmium by *C. reinhardtii* into the cells themselves, which has

been well documented in the literature (Nagel, Adelmeier et al. 1996, Hu, Lau et al. 2001, Aguilera and Amils 2005). On the whole, Cd measurements taken throughout the duration of the experiment confirm that FeSSi sorbs cadmium and holds on to it for over 30 days (SI Figure 4). Further, ESEM imaging of algal treatments at the end of the FeSSi+Cd experiment qualitatively confirms that cadmium is sorbed onto the FeSSi particles and not onto the algal cells (Figure 5).

Additional XPS analyses were done up to 30 days after the FeSSi-Cd composites were introduced to algal cultures. As can be seen in Figure 1b, peaks for Cd were present in the spectra obtained from the undissolved FeSSi-Cd composites analyzed after 30 days, which shows that Cd mostly remained strongly adsorbed to the surface of FeSSi particles. The stable interactions between FeSSi and Cd reduced the bioavailability of the toxic metal (Cd) to the algal cells during the course of this study.

The XRD diffractograms showed that Fe^0 in FeSSi is rapidly transformed to higher oxidation states, mainly maghemite (γ-Fe_2O_3) and/or magnetite (Fe_3O_4) corresponding to $2\theta = 35.7°$, 63.1° and γ-FeOOH ($2\theta = 54.2°$) (Adeleye, Keller et al. 2013, Liu, Liu et al. 2015). In fact, the peak for Fe^0 ($2\theta = 44.7°$) was not detected in the FeSSi particles obtained from new cultures after 24 h due to rapid oxidation (SI Figure 8). This is in contrast to the observation of Liu and coworkers (Liu, Liu et al. 2015) who reported finding Fe^0 after 90 days when nZVI was suspended in static water. The main difference between that study and this may be that the presence of algae (in this study) provided a constant supply of oxygen that rapidly oxidized all the Fe^0 present in FeSSi according to Equation 1.

$$4Fe^0 + 3O_2 + 2H_2O \rightarrow 4FeOOH \quad (1)$$

In 11-day cultures, however, peaks for Fe^0 were observed up to the last sampling done after 30 days of exposing FeSSi-cadmium composite to the cells. In fact, peaks for Fe^0 were also detected after 30 days when FeSSi alone (without Cd) was released into 11-day cultures (SI Figure 9). The persistence of Fe^0 in 11-day algal cultures is attributed to the presence of dissolved organic carbon (DOC) in the media, which binds to the surface of the nanoparticles and slow their reactivity. This is in agreement with several previous studies, including ours, that show that DOC is able to bind to the surface of iron (including FeSSi) (Johnson, Johnson et al. 2009, Chen, Xiu et al. 2011, Adeleye, Stevenson et al. 2016). Peaks for Cd were not detected in the XRD analyses. This is probably due to the relatively small amount of Cd present on the surface of FeSSi (experimental molar ratio of Fe/Cd ≈ 80). In addition, XRD probes samples to depths up to hundreds of microns but Cd is mainly located within the outer 10 nm of the surface of FeSSi (as shown by XPS data).

FeSSi mitigates the toxicity of cadmium to *C. reinhardtii*

We dosed three dilutions of FeSSi and cadmium to both new and 11-day old cultures – 180 ppm FeSSi + 4.5 ppm Cd (full concentration), 18 ppm FeSSi + 0.45 ppm Cd (10-fold dilution), and 1.8 ppm FeSSi + 0.045 ppm Cd (100-fold dilution). We found that none of these concentrations has any effect on new or 11-day old cultures, except that 180 ppm FeSSi + 4.5 ppm Cd was completely toxic to new cultures (Figure 3).

If FeSSi did not bind the cadmium at all, we can predict whether or not these concentrations of cadmium (0.045, 0.45, and 4.5 ppm Cd) would be toxic to new and 11-

day old algal cultures using the model we developed and parameterized to our data on algal response to cadmium toxicity. Our model predicts exposure to just the two lowest concentrations (0.045 and 0.45 ppm Cd) would not have an effect on algal cultures while 4.5 ppm cadmium alone would be toxic to new and 11-day old cultures (see model simulation in SI Figure 3). However, we found that 180 ppm FeSSi and 4.5 ppm Cd has no effect on 11 day old cultures, indicating that FeSSi is binding cadmium and mitigating its toxicity, since that amount of Cd alone would be toxic (Figure 3). This result is consistent with measurements of the extracellular concentration of cadmium throughout the algal experiment (SI Figure 4) and XRD and XPS analysis. However, cadmium toxicity alone does not explain why this same concentration (180 ppm FeSSi and 4.5 ppm Cd) is toxic to new cultures. Nearly the same amount of cadmium is present in new cultures as 11-day old cultures at the start of the experiment (SI Figure 4), however 11-day old cultures are able to grow almost indistinguishably from control cultures while new cultures die.

FeSSi with cadmium sorbed onto it is more toxic than FeSSi alone

High concentrations of FeSSi are toxic to *C. reinhardtii*, however we found in a past study that DOC produced by the algae themselves can mitigate this toxicity (Adeleye, Stevenson et al. 2016), in agreement with other studies that found that natural organic material can mitigate nZVI toxicity (Li, Greden et al. 2010, Chen, Su et al. 2011). In exposures to FeSSi alone, 180 ppm of FeSSi delayed the growth of new cultures for over a week while not having an effect on 11-day old cultures (Adeleye, Stevenson et al. 2016). This is a similar result to our past work that identified the feedback in which algal-produced organic material mitigates ionic and nano-specific toxicity of silver nanoparticles

to the algae themselves (Stevenson, Dickson et al. 2013). We adapted the model developed in Stevenson et al. (2013) and fit it to the response of *C. reinhardtii* to FeSSi exposures in Adeleye, Stevenson et al. (2016). We found that DOC again explained the patterns of toxicity observed – 11-day old cultures had produced enough DOC to mitigate FeSSi's toxicity, however FeSSi delayed growth of new cultures until enough FeSSi particles had been inactivated by DOC that the cultures were able to grow (Adeleye, Stevenson et al. 2016). Similar to this study, FeSSi particles were introduced into the media for an hour before dosing, which allowed some of the particles to be inactivated by DOC before introduction into the algal cultures. FeSSi did not have a significant toxic effect on 11-day old cultures because 30% of the total dosage of FeSSi particles were no longer bioavailable due to DOC upon introduction to the 11-day old algal cultures (Adeleye, Stevenson et al. 2016).

We built on the model developed for Adeleye, Stevenson et al. (2016) to explain how FeSSi and cadmium exposure has no effect on 11-day old cultures but is toxic to new cultures at the highest exposure concentration. We expanded the model in Adeleye, Stevenson et al. (2016) to include cadmium toxicity (Model 1), the sorption of cadmium onto FeSSi, and the binding of DOC to FeSSi and FeSSi particles with cadmium sorbed (see Table 1 for model equations and Table 2 for parameter estimates). As in Adeleye, Stevenson et al. (2016), the model describes algal growth (using the logistic growth model), DOC production, FeSSi toxicity, and the inactivation of FeSSi particles by DOC. We expanded this model to include the different forms of FeSSi present in the FeSSi and cadmium exposures (Table 1). FeSSi particles in the exposures were either "free" (bioavailable, with neither cadmium nor DOC on the particle, variable F), bound and

inactivated by DOC (F_D), with cadmium sorbed on the particle's surface (F_C), or bound by DOC with cadmium sorbed on the particle's surface (F_{DC}). We assume FeSSi sorbs Cd at a rate *b* (fit to the data on the free cadmium concentration present in the algal exposures, SI Figure 4) and that DOC binds to FeSSi at a rate γ_{UN} (the same value as in Adeleye, Stevenson et al. (2016)). We assumed that FeSSi sorbs Cd at the same rate whether or not DOC is bound to FeSSi and that DOC binds to FeSSi at the same rate regardless of whether or not the particle has sorbed cadmium. These were necessary simplifying assumptions in the absence of data on cadmium sorption to FeSSi with or without DOC.

In these exposures, algal cultures experience toxicity from three possible sources – FeSSi with and without cadmium and from free cadmium (not sorbed to FeSSi). Model 1 effectively describes free cadmium toxicity to *C. reinhardtii* in batch cultures. Both F and F_C are toxic to the algae – our past work (Adeleye, Stevenson et al. 2016) identified the toxicity of FeSSi alone (F) and we assume that FeSSi with cadmium sorbed to the surface of the particle (F_C) is at least as toxic as FeSSi alone (F). Our first hypothesis is that the toxicity of F is equivalent to F_C (Tables 1 and 2, $k_F = k_{FC}$) and FeSSi particle-specific toxicity combined with the concentration of cadmium that is not bound by FeSSi is great enough to cause new cultures to never grow while 11-day old cultures have produced enough DOC to mitigate the toxic effect of FeSSi particles. However, we can simulate this scenario by setting $k_F = k_{FC}$ in Model 2, and the model predicts that new cultures would be able to growth after a week of exposure (SI Figure 5). The model correctly predicts that there is no effect on 11-day old cultures due to DOC binding and inactivating FeSSi, but the mismatch between our empirical results and the model's prediction indicates that we are underestimating toxicity in the new cultures.

FeSSi particles with cadmium sorbed onto the particles' surface could also be *more* toxic than FeSSi particles alone ($k_{FC} > k_F$). This version of the model gives a good fit to the data (Figure 4) when the toxicity of FeSSi particles with cadmium is over ten times greater than FeSSi particles alone (see Table 2 for parameter values). The model still correctly predicts that this concentration does not have an effect on 11-day old cultures due to DOC inactivation of the FeSSi particles for the hour prior to dosing the algal cultures. We found a similar result in Adeleye, Stevenson et al. (2016) – in the hour before dosing, the model predicts that almost 30% of the FeSSi particles in that experiment had been inactivated by DOC before dosing the 11-day old cultures (Adeleye, Stevenson et al. 2016). The model in this paper predicts a greater inactivation by DOC – half of the total amount of FeSSi particles are inactivated by DOC (F_D) and all of the cadmium is sorbed onto FeSSi particles that are also inactivated by DOC (F_{DC}).

One of the biggest strengths of the modeling approach taken here is that through the incorporation of a simple cadmium toxicity model (Model 1) into the model developed for Adeleye, Stevenson et al. (2016), we are able to predict the toxicity of other FeSSi and Cd concentrations such that the model could be used to make environmental remediation decisions. Since we know the toxicity of Cd, FeSSi alone, and FeSSi with Cd sorbed onto it (FeSSi+Cd), we can use the model to predict the FeSSi concentration needed to successfully remediate an environmental concentration of Cd (remove enough Cd such that it is no longer toxic to the alga) and test to see if the necessary concentration of FeSSi is toxic in and of itself (both as FeSSi and FeSSi+Cd). In this way, we can predict the maximum Cd concentration FeSSi can remediate without replacing Cd toxicity with FeSSi and FeSSi+Cd toxicity. The model predicts that concentrations above 8 ppm Cd cannot be

mitigated by FeSSi – at this concentration, an environmental manager would need to use 1.2e14 FeSSi particles/L (around 386 ppm FeSSi) to remove enough of the 8 ppm Cd such that it was no longer toxic to algae. This concentration of FeSSi (386 ppm) delays growth of new and 11-day old cultures by 70 and 50 days, respectively, however the populations do grow. The concentrations of FeSSi needed to remediate more than 8 ppm of Cd are toxic on their own, and this is actually due to the higher toxicity of FeSSi+Cd than FeSSi alone. If FeSSi and FeSSi+Cd were toxic at the same concentrations, FeSSi could be applied to mitigate up to 55 ppm Cd. These predictions emphasize the importance of our finding that the toxicity of the nZVI derivative increases when the contaminant of interest (Cd) is sorbed onto it. In addition to explaining our experimental results, the model presented here could be used for environmental management decisions by predicting the effect of FeSSi on biological organisms once applied for remediation of Cd.

Environmental implications

We have demonstrated the ability of nano-zerovalent iron to mitigate the toxicity of its target contaminant, in this case Cd through the systematic exposure of organisms to the contaminant (Cd), nZVI iron alone, and nZVI with the contaminant. While FeSSi does exert toxicity to the algae (Adeleye, Stevenson et al. 2016), our study confirms that it is able to remove the toxicity of Cd to all algal cultures except new cultures at the highest FeSSi and Cd concentrations. Our study also found that FeSSi with Cd sorbed onto it is more toxic than FeSSi alone, which is a novel finding and important when considering the implications of using nZVI as an environmental remediator. Finally, the development of a quantitate model to quantify the strength of FeSSi, Cd, and FeSSi with Cd toxicity to algae

emphasizes the importance of the mitigating effect of DOC on FeSSi toxicity, which adds to the growing body of work that has identified the detoxifying effect of organic material, especially algal-produced organic material (Stevenson, Dickson et al. 2013, Adeleye, Stevenson et al. 2016), on nanoparticle toxicity (Hall, Bradley et al. 2009, Miao, Schwehr et al. 2009, Lee, Kim et al. 2011, Kennedy, Chappell et al. 2012, Kim, Kim et al. 2013, Seitz, Rosenfeldt et al. 2015).

Tables

Table 1: State variables, functions, and balance equations for Model 2 – FeSSi-Cd-DOC model.

State Variables	
N	Algal biomass (μg chl a/L)
D	DOC concentration (mgC/L)
C	Unbound (free and toxic) cadmium (mg Cd/L)
F	Bioavailable FeSSi (FeSSi particles/L)
F_D	FeSSi inactivated by DOC (FeSSi particles/L)
F_C	FeSSi + Cd (FeSSi particles/L)
F_{DC}	FeSSi + Cd + DOC (FeSSi particles/L)
Functions	
$\mu = k_F F + k_{FC} F_C + k_C (C - NE($	Mortality due to FeSSi and cadmium exposure
$P_D = j_{DN} N + h_{DN} \frac{dN}{dt}$	DOC production
$J_{FD} = \gamma_{UN} FD$	Inactivation of FeSSi by DOC
$J_{FCD} = \gamma_{UN} F_C D$	Inactivation of FeSSi + cadmium by DOC
$B_{FC} = bFC$	Binding of cadmium by FeSSi
$B_{FDC} = bF_D C$	Binding of cadmium by FeSSi + DOC
Balance equations	

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) - \mu N$$ Algal biomass

$$\frac{dD}{dt} = P_D - \beta(J_{FD} + J_{FCD})$$ DOC

$$\frac{dC}{dt} = -\alpha_{CF}(B_{FC} + B_{FDC})$$ Unbound cadmium

$$\frac{dF}{dt} = -B_{FC} - J_{FD}$$ Bioavailable FeSSi

$$\frac{dF_C}{dt} = B_{FC} - J_{FCD}$$ FeSSi with Cd bound to it

$$\frac{dF_D}{dt} = J_{FD} - B_{FDC}$$ FeSSi inactivated by DOC

$$\frac{dF_{DC}}{dt} = J_{FCD} + B_{FDC}$$ FeSSi with Cd bound inactivated by DOC

Table 2: Parameters for Model 2 – FeSSi-Cd-DOC model. Algal growth and DOC production parameters were fit to control cultures (Adeleye, Stevenson et al. 2016). FeSSi toxicity parameters, DOC inactivation rate, and loss of DOC due to heteroaggregation with FeSSi and binding rates were fit previously from FeSSi-only exposures (Adeleye, Stevenson et al. 2016). Parameters pertaining to Cd toxicity are from the fit of the Cd-only experiment and are identical to the values in SI Table 3. Parameters pertaining to FeSSi and Cd toxicity (FeSSi binding Cd rate, toxicity of FeSSi with Cd bound, and the maximum amount of Cd bound per particle) were fit to data on FeSSi+Cd exposure (empricial results in Figure 3 in the main text).

Parameters			Data used for parameterization
r	algal intrinsic growth rate	0.44 1/day	Control cultures during FeSSi exposure
K	algal carrying capacity	453 µg chl a / L	Control cultures during FeSSi exposure
$\mathbf{k_F}$	FeSSi toxicity parameter	$5.576*10^{-6}$ L / (FeSSi particles * day)	Control cultures during FeSSi exposure
$\mathbf{j_{DN}}$	parameter in DOC production rate	0.003484 mgC / (ug chl a * day)	Control cultures during FeSSi exposure
$\mathbf{h_{DN}}$	parameter in DOC production rate	0.0072 mgC / ug chl a	Control cultures during FeSSi exposure
γ_{UN}	FeSSi inactivation rate	0.2484 L / (mgC * day)	FeSSi-only exposure
β	Loss of DOC due to	$4.069*10^{-7}$ mgC /	FeSSi-only exposure

	heteroaggregation with FeSSi	FeSSi particles	
$\mathbf{k_C}$	Cadmium-specific toxicity	0.2283 L / (mg Cd * day)	Cadmium-only expt
NEC	No effect concentration of cadmium	0.5126 mg Cd/L	Cadmium-only expt
$\mathbf{k_{FC}}$	Toxicity of FeSSi particles with Cd bound	7e-5 L / (FeSSi particles * day)	Fit from FeSSi+Cd exposures
b	Rate of FeSSi binding Cd	0.4211 L / (mg Cd * day)	Fit from FeSSi+Cd exposures
α_{CF}	Maximum mg cadmium bound per FeSSi particle	1.005e-6 mg Cd / FeSSi particle	Fit from FeSSi+Cd exposures

Figures

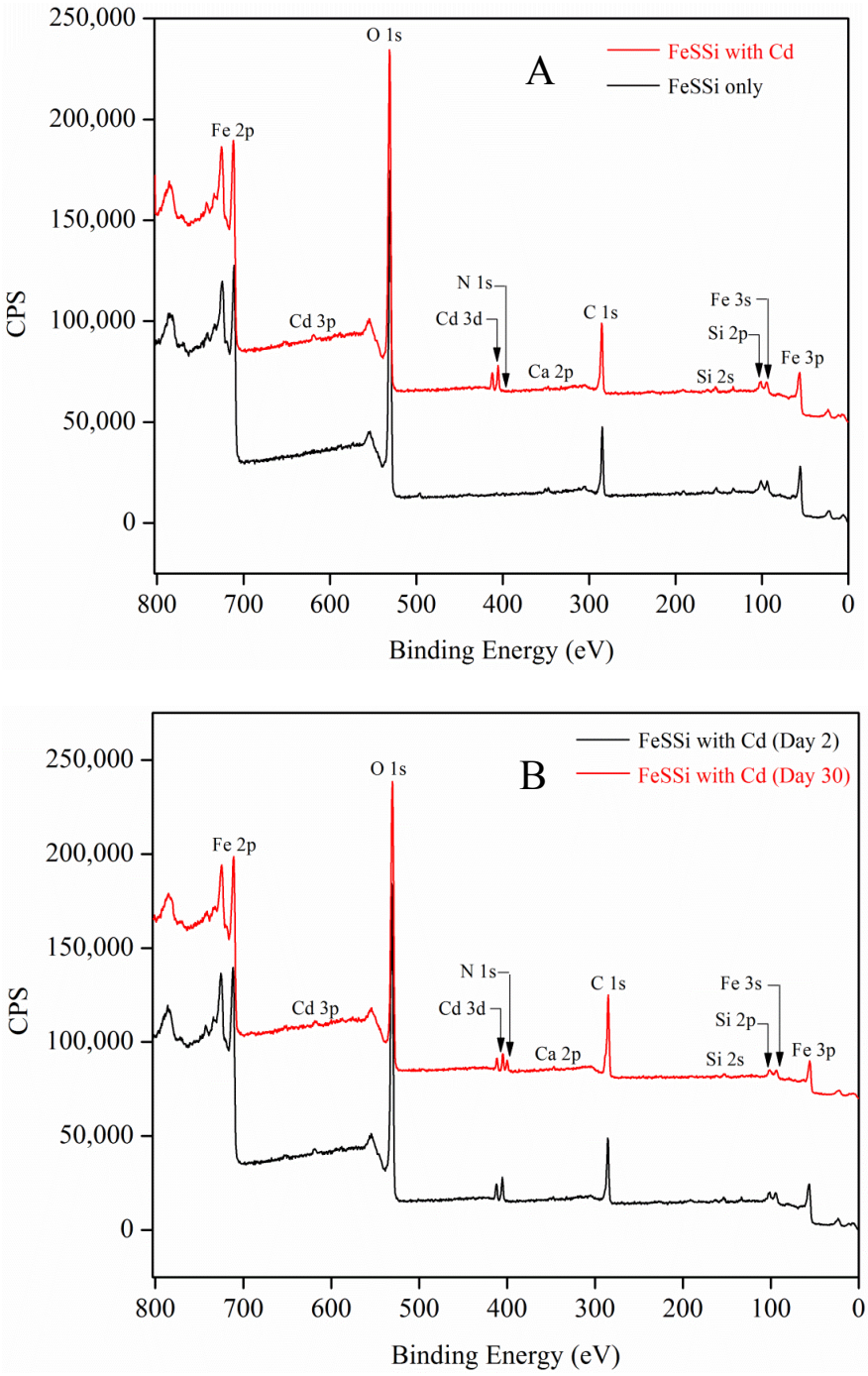


Figure 1. XPS analyses of FeSSi. (A) shows the survey scan of FeSSi with and without cadmium (Cd) obtained after 24 h in 1-day culture, while (B) compares the survey spectra of FeSSi with cadmium obtained after 2 and 30 days.

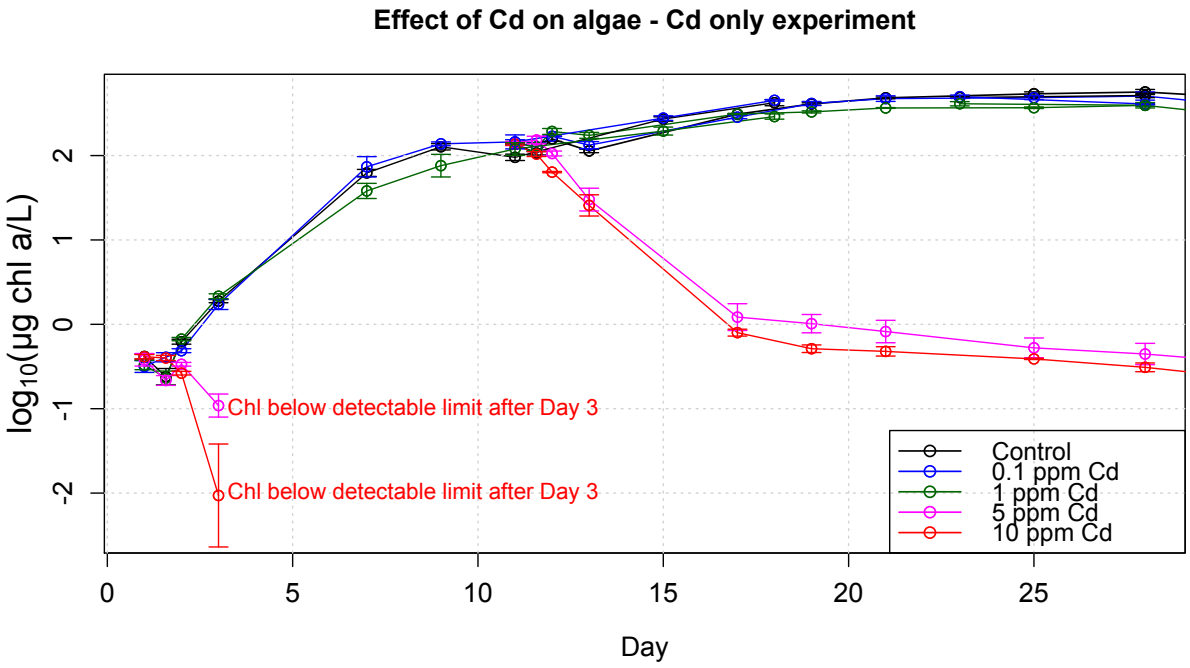


Figure 2: 5 and 10 ppm cadmium (in the form of cadmium chloride) is toxic to new and 11-day old cultures. The data points are averages of that treatment's replicates (n=3 for all treatments) and the error bars reflect their standard error.

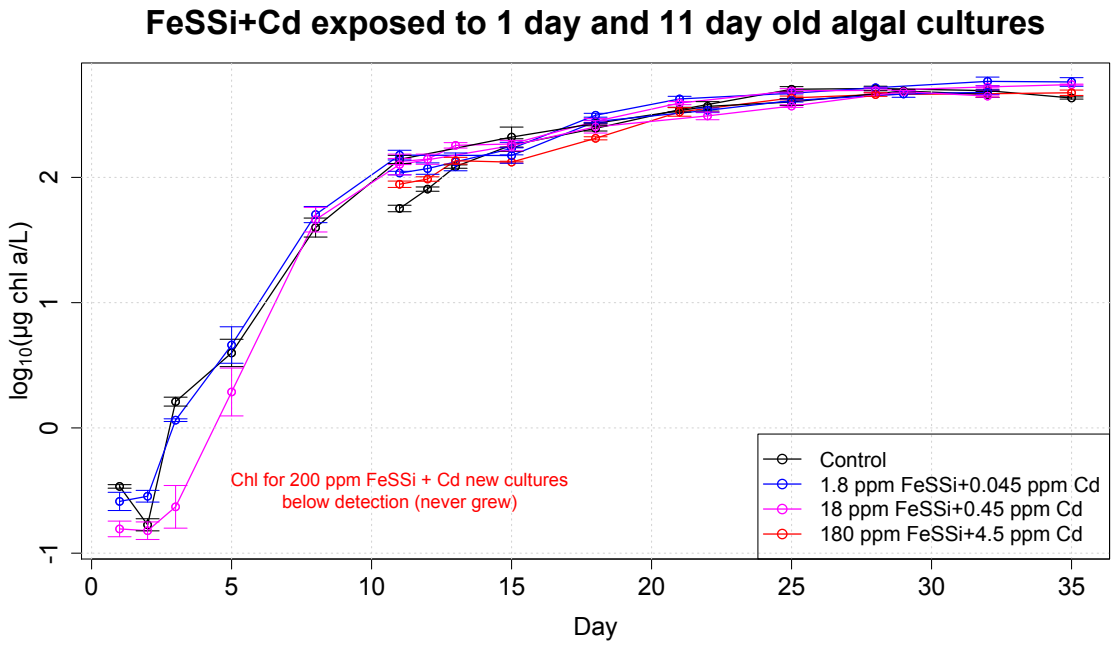


Figure 3: FeSSi removes the toxicity of cadmium to all cultures except new cultures at the highest concentrations of FeSSi and cadmium, which never grow. The data points are averages of that treatment's replicates (n=3 for all treatments) and the error bars reflect their standard error.

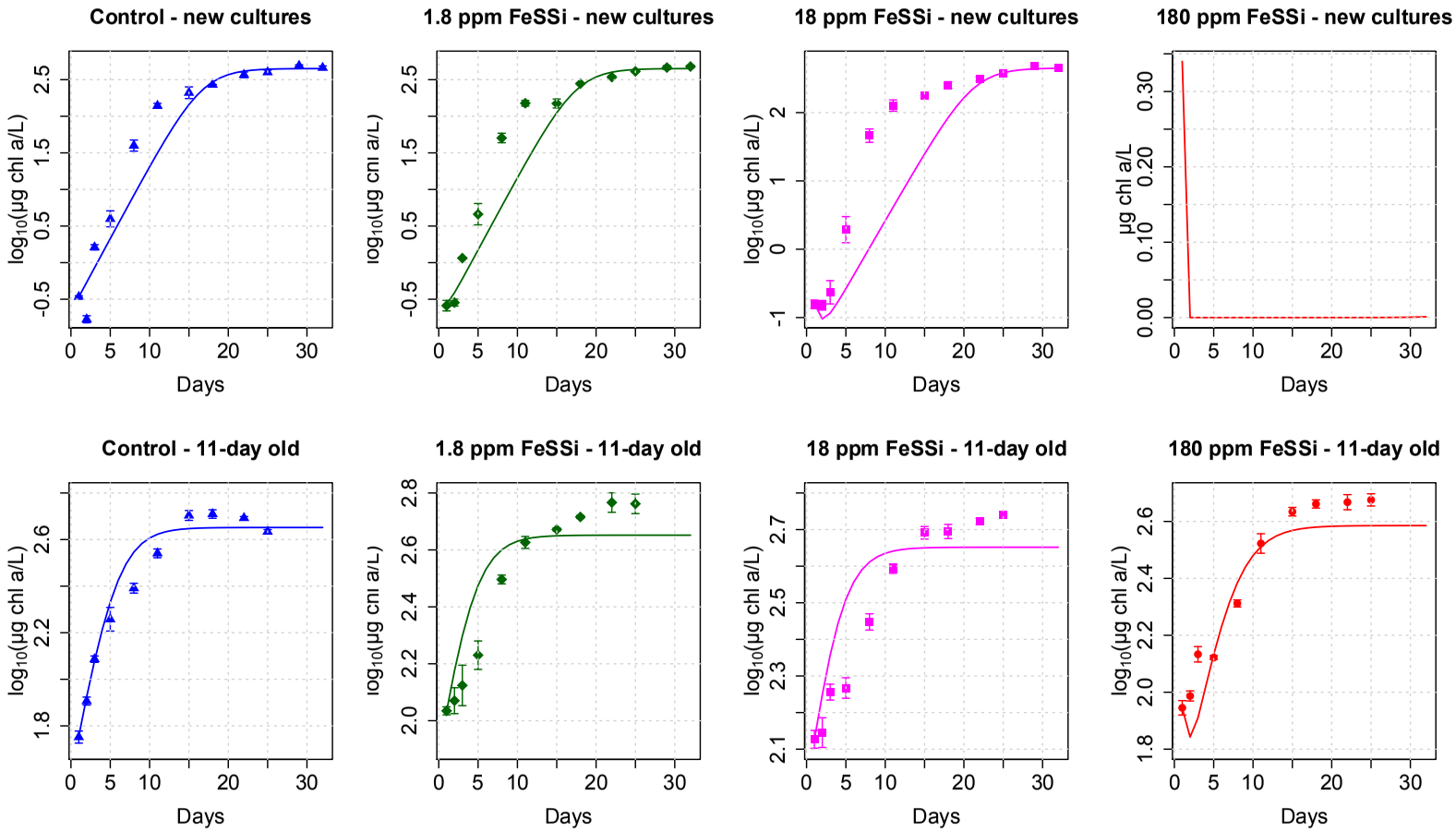


Figure 4: Fit of FeSSi, cadmium and DOC model (lines; Model 2) when FeSSi particles that have sorbed cadmium (F_C) are more toxic than FeSSi particles alone (F). Note that new cultures never grew at the highest FeSSi and cadmium concentrations (top right panel) so that model simulation is not shown on a log scale to demonstrate that the model predicts complete toxicity to these cultures. The data points are averages of that treatment's replicates (n=3 for all treatments) and the error bars reflect their standard error.

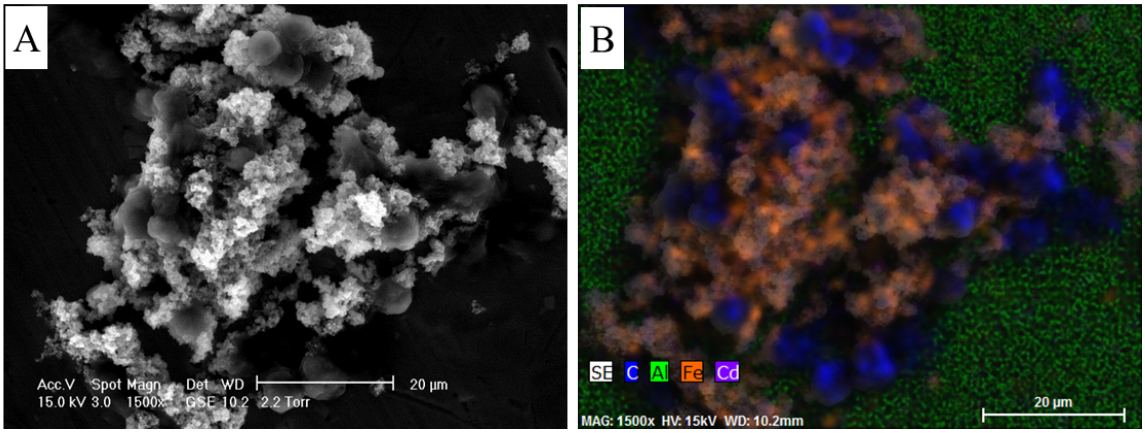


Figure 5. A representative ESEM micrograph with EDS hypermaps showing interactions between FeSSi-Cd composites and 11-day old culture algal cells. (A) Heteroaggregation between FeSSi-Cd aggregates and *C. reinhardtii* cells, with (B) showing an overlay of carbon (C, from algae), iron (Fe, from FeSSi), cadmium (Cd), and aluminum (Al, from the specimen mount) distribution on micrograph. The hypermap shows that Cd (purple color) was mostly adsorbed to FeSSi (orange color), and not algae (blue color).

Appendix

Chapter 1 – Supplementary Information

The overall description of our algal batch culture experiments is presented in the Methods section. In Sections 1-5 of the SI we describe the sampling and experimental analyses. In Section 6 we detail potential expansions of the dynamic model that demonstrates the feedback mechanism.

1. Batch culture setups for all algal batch culture experiments

Samples were taken directly from the algal batch cultures every day for the first 3 days, and on a Monday-Wednesday-Friday schedule for the rest of the experiments. The sampling setup allowed us to sample directly from the flasks without opening them, thus minimizing contamination. The setup consists of a 9-inch glass pipette held in place by a foam stopper at the top of the 500 mL Erlenmeyer flask with Tygon tubing attached to the end of the pipette. We secured two plastic 10 mL syringes (Exel International Luer Lock syringe) at the other end of the tube with a polypropylene T-valve (Thermo Scientific Nalgene Tubing T-type connectors). By sucking air out with one syringe, we were able to extract a sample with the other plastic syringe. We swirled cultures prior to sampling them to suspend algal cells and ensure that we were taking a homogenous sample. The whole setup was autoclaved prior to the experiment to ensure sterility.

Batch cultures of *C. reinhardtii* were grown under constant light provided by fluorescent "growing" lights (Philips T8 Natural Light 32 W fluorescent lamps) suspended above the cultures on wooden sawhorses. We measured photosynthetically active radiation (PAR) levels across the entire experimental setup using a digital light meter (Extech

Instruments Model 401025) to ensure that each flask grew in approximately equivalent PAR levels. PAR readings across the experiment varied by approximately 10% Lux.

2. The effect of 5 mg/L AgNP on three stages of batch culture growth

Nanoparticles in stationary cultures could sink to the bottom and not homogenously interact with algal cells. To examine the effect of shaking on particle behavior and algal toxicity, we placed half of the cultures on shaker tables (Thermo Scientific MaxQ 3000 orbital shaker) for the duration of the experiment and the other half on stationary platforms of the same height as the shaker tables. We did not find an effect of shaking the cultures on control or AgNP-treated cultures (Figure S1).

The first algal batch culture experiment involved four treatment groups: 1) citrate-coated AgNPs, 2) an equimolar concentration of silver nitrate ($AgNO_3$), 3) an equimolar concentration of nitrate (NO_3) to the $AgNO_3$ treatment, and 4) control. Treatment 1 tested the effect of 5 mg/L citrate-coated silver nanoparticles (40 nm Citrate BioPure™ Silver from NanoComposix, Inc). The aim of treatment 2 was to investigate a "nano" versus "silver" effect of these particles; we therefore dosed the algal cultures with an equimolar silver concentration to the 5 mg/L concentration of AgNPs in the form of $AgNO_3$. Treatment 3 controlled for possible effects of the addition of nitrate in treatment 2 that could affect algal growth; we exposed a set of algal cultures to an equimolar concentration of NO_3 to the $AgNO_3$ treatment.

The equimolar to 5 mg/L silver concentration of Ag^+ in the form of $AgNO_3$ was toxic to all algal batch cultures regardless of growth stage and we did not see a difference between control cultures and cultures with an NO_3 addition (Figure S2).

3. Measurements of the dissolution of silver ions from the AgNPs

The concentration of Ag^+ in our 40 nm citrate-coated AgNP stock was measured by first separating the Ag^+ from the AgNPs through ultracentrifugation and then measuring the concentration of this separated ionic fraction on an Atomic Absorbance Spectrophotometer.

In order to estimate the total dissolution of silver ions from the AgNPs throughout the duration of the experiment, we conducted a follow up experiment to measure the dissolution of Ag^+ from the nanoparticles in the organic environment of the three stages of batch culture growth. Please refer to the Materials & Methods section in the main text of this study for details on the experimental setup and sample collection and processing. Dissolution of the AgNPs in the organic environment of algal batch cultures was relatively slow (Figure S3), with a maximum Ag^+ concentration of 90 μg/L after 10 days of dissolution in the organic material from a culture growing in stationary growth phase. We also found an interesting pattern in which the concentration of dissolved ions actually decreased initially for AgNPs in fast growth phase and cultures in stationary growth phase. Numerous other studies of AgNP dissolution have also observed this decrease in the dissolved ionic fraction and it is thought to be due to either released Ag^+ rejoining existing AgNPs [1-3] and/or complexation of Ag^+ with various ligands, including chloride and DOC [4], which may or may not have been removed by the Amicon filter. However, measurements from algal cultures in slowing growth phase do not reflect this pattern for which we do not have an explanation.

4. The effect of 10, 50 and 100 μg/L Ag^+ in the form of $AgNO_3$

New, one week, and two week old cultures were inoculated for growth prior to the experiment in the same manner as described in Materials & Methods. The same batch

culture sampling setup was employed and cultures were sampled for chlorophyll a measurements at the same frequency as previously described (see Supplementary Section 1). We looked at the effect of 3.5 (Figure 2), 10, 50, and 100 μg/L Ag^+ in the form of $AgNO_3$ (Figure S4). We found little to no effect of 3.5 μg/L Ag^+ (Figure 2). All other concentrations tested were toxic to cultures in fast growth phase, but only 100 μg/L Ag^+ had an effect on later stages of batch culture growth (Figure S4).

5. Measurement of AgNP size

We conducted an experiment to measure the size of the AgNPs in algal batch culture using a higher concentration of AgNPs than was used in our original experiment, since samples of the 5 mg/L concentration used for our original experiment were not of a high enough concentration for the Malvern Instruments Zetasizer Nano ZS90 to read accurately (the small attenuator value meant we could not have confidence in these measurements). We grew batch cultures of *C. reinhardtii* for one, two, and three weeks prior to this follow-up experiment. We employed two different filter techniques to estimate if filtration (necessary to remove algal cells prior to nanoparticle size measurements) removed large aggregates of nanoparticles. AgNPs in samples from which algal cells had already been removed still experienced the level of organic material produced by an algal culture. On the day of the experiment, we drew two 1.5 mL samples of new (fast growth phase), one week old (slowing growth phase), two week old (stationary growth phase), and three week old (late stationary growth phase) cultures. We then added 20 mg/L AgNPs to half of these samples, which were left for an hour under fluorescent lights and then filtered. For the other half, we filtered algal cells out of these samples using a 5 micron filter (Millipore MF-Mixed Cellulose Ester Membrane filters)

prior to the addition of 20 mg/L AgNPs. We left all of these samples under fluorescent growing lights for an hour, filtered the half of the samples that still contained algal cells, and ran all of the samples on the zetasizer. We did not find a significant difference between samples that were filtered before AgNP addition and those filtered after AgNP addition (unpublished data). We found that the particles aggregated in later stages of algal batch culture growth, with a maximum size around 130 nm (Figure S5; data from algal culture that were filtered after AgNP addition).

6. Dynamic model of feedback

The model is deliberately simplistic, as its aim was to find a minimal suite of mechanisms consistent with our observations. Potentially important elaborations and their likely consequences include:

- variable strength and types of silver ion complexation by the DOC (affects effective exposure),
- dependence of NP dissolution rate on DOC (faster dissolution would lead to faster bioaccumulation),
- DOC introduced to the environment as the result of phytoplankton mortality (additional DOC would accelerate inactivation of both AgNPs and ions),
- a possible no-effect concentration for nano-particle exposure (transition between the initial population decline and recovery phases would be more abrupt),
- bioaccumulation of AgNPs into the algal cells themselves where they could exert toxic effects intracellularly

Figures:

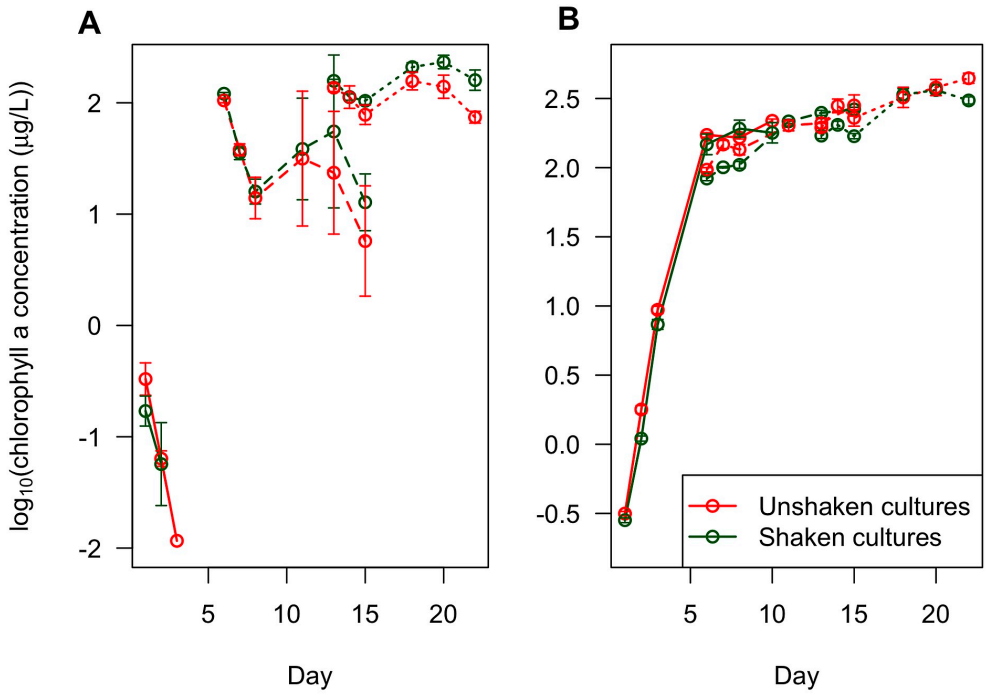


Figure S1. Shaking algal cultures has no effect on control or AgNP cultures. There was no difference between AgNP (a) and control (b) cultures on shaker tables (red) and kept stationary (green). The data points are averages from three replicate cultures and the error bars reflect their standard error.

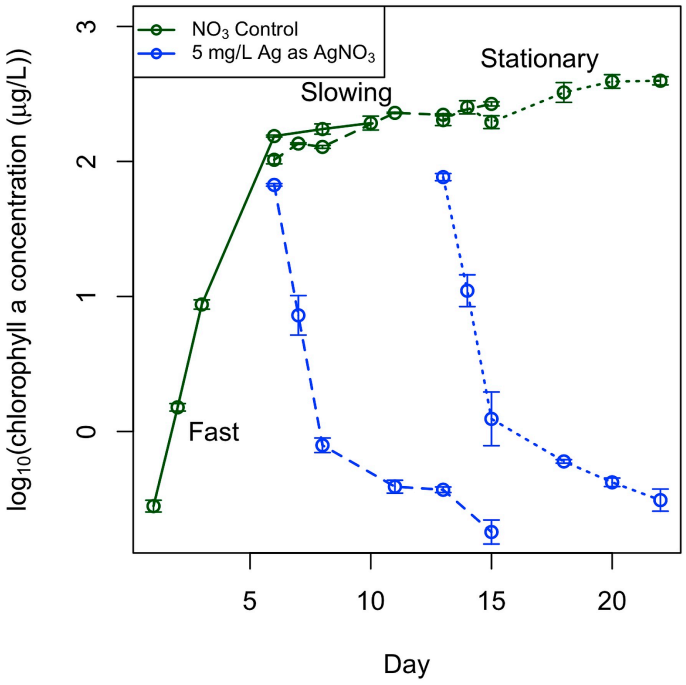


Figure S2. An equimolar to 5 mg/L silver concentration of Ag^+ was toxic to all algal batch cultures. An equimolar concentration of Ag^+ in the form of $AgNO_3$ was toxic to algal cultures in all growth stages (blue). Cultures in fast growth phase never registered a positive chlorophyll reading so the $AgNO_3$ treatment is not represented on this graph. We also exposed cultures to an equimolar concentration of NO_3 as the $AgNO_3$ treatments to compare to control for this addition of nitrogen (green), which algal cells can use for growth. We did not see a difference between control cultures and cultures with an NO_3 addition. The data points are averages from three replicate cultures and the error bars reflect their standard error.

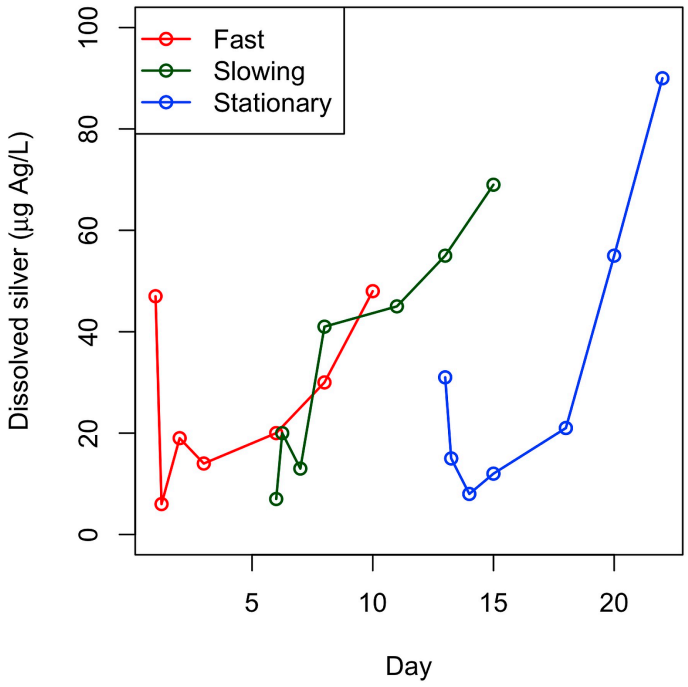


Figure S3. Concentration of dissolved silver from AgNPs introduced to algal cultures with algal cells removed. We removed the algal cells from cultures in fast, slowing, and stationary growth phases in order to minimize loss of Ag^{+} in our measurement due to association with algal cells.

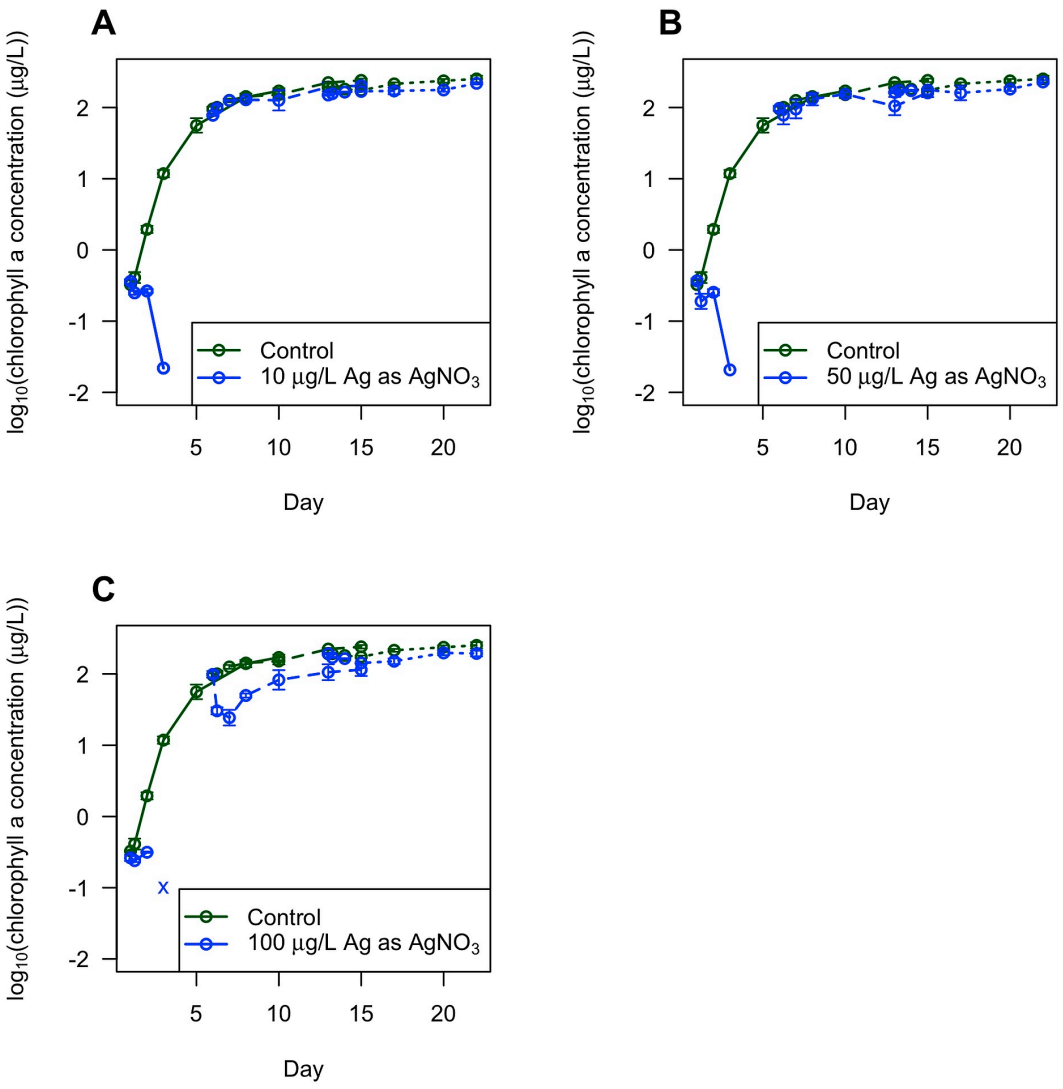


Figure S4. The effect of 10 (a), 50 (b), and 100 (c) µg/L Ag^+ on algal cultures. These concentrations of Ag^+ were introduced to batch cultures in the form of $AgNO_3$ (blue) in the same way described in Supplementary Section 1. All three concentrations caused complete mortality of cultures growing in fast growth phase within two days of introduction (chlorophyll measurements were below detectable limits, denoted by x, on day 3 of new cultures exposed to 100 µg/L $AgNO_3$). However, 10 and 50 µg/L Ag^+ in the form of $AgNO_3$ had negligible effect on cultures growing in slowing and stationary growth phases (a and b). 100 µg/L Ag^+ in the form of $AgNO_3$ had no effect on cultures in stationary growth phase but was initially toxic to cultures growing in slowing growth phase, however the cultures were able to partially recover (c). The data points are averages from three replicate cultures and the error bars reflect their standard error.

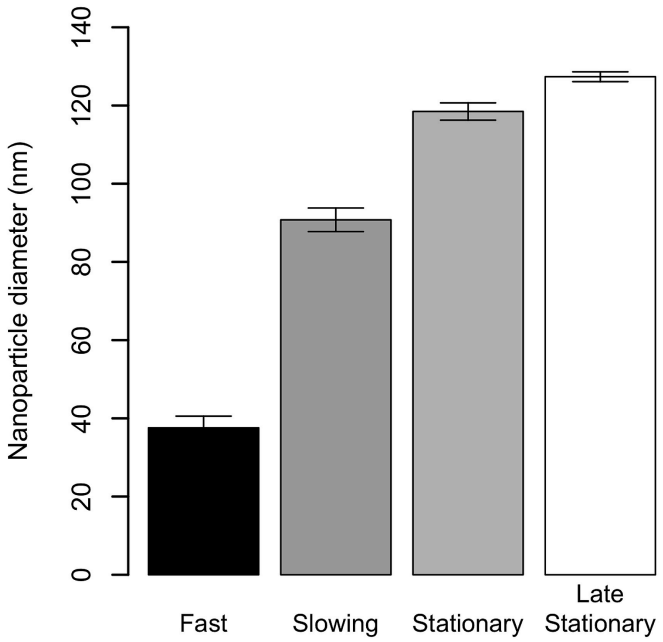


Figure S5. AgNPs remained as single particles in cultures in fast growth phase and aggregated in later stages. The 40 nm particles remained unassociated in cultures in earlier stages of growth but aggregated up to 130 nm in later stages of growth. The data points are averages from three replicate samples and the error bars reflect their standard error.

References:

1. Lee YJ, Kim J, Oh J, Bae S, Lee S, et al. (2012) Ion-release kinetics and ecotoxicity effects of silver nanoparticles. Environ Toxicol Chem 31: 155-159.

2. Li X, Lenhart JJ (2012) Aggregation and dissolution of silver nanoparticles in natural surface water. Environ Sci Technol 46: 5378-5386.

3. Liu J, Hurt RH (2010) Ion release kinetics and particle persistence in aqueous nano-silver colloids. Environ Sci Technol 44: 2169-2175.

4. Janes N, Playle RC (1995) Modeling silver binding to gills of Rainbow trout (Oncorhynchus mykiss). Environmental toxicology and chemistry.

Chapter 2 – Supplementary Information

1. AgNP size, dissolution and ROS production

1.1 Experimental methods

We conducted separate experiments to examine the size, dissolution, and ROS production of the AgNPs used in this experiment. We could not take these measurements during the *Daphnia* experiments because the exposure concentrations used (75 and 200 μg/L) are too low to be detected reliably by any of the methods used for these measurements. For example, previous experimentation indicated that concentrations below 20 mg/L AgNPs resulted in small attenuator values for Zetasizer analysis indicating unreliable measurements. Therefore we had to use higher concentrations for reliable estimates. We also compared AgNP size, dissolution, and ROS production between samples kept in the light (a diurnal cycle of fluorescent growing lights) versus samples kept in the dark (similar to our *Daphnia* experiments) to identify whether these particles act differently in light versus dark, which could have implications for our experimental results.

To measure nanoparticle size and dissolution, we dosed four replicate beakers of 150 mL autoclaved low-P COMBO (Kilham, Kreeger et al. 1998) with 20 mg/L AgNPs (without any experimental organisms), mixed them, and placed two underneath fluorescent "growing" lights (Philips T8 Natural Light 32 W fluorescent lamps) on a 12:12 cycle and placed the other two beakers in complete darkness. We took samples on Day 0 (right after dosing), 1 (24 hours after dosing), 2 and 3 to investigate the dynamics of nanoparticle size and dissolution of silver ions from the AgNPs through time. We followed nanoparticle size

and dissolution for up to 3 days because our *Daphnia* experiments were sampled on a 2-2-3 day transfer interval schedule and AgNP treatments were refreshed after sampling.

We measured nanoparticle size using a Malvern Instruments Zetasizer Nano ZS90. We measured dissolution of Ag^+ by removing 15 mL from each suspension at each time point (with replicates on Day 0, 2, and 3) and centrifuging the samples using Amicon centrifugal filter units (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 [3 kDa] membrane; maximum pore size ~ 2 nm) at 5,000 *g* for 40 minutes. We chose this filter size and spinning speed and duration in accordance with past studies investigating AgNP dissolution (Navarro, Piccapietra et al. 2008, Liu and Hurt 2010, Ma, Levard et al. 2011, Zhang, Yao et al. 2011). We also tested dissolution further with a smaller concentration (5 mg/L) of AgNPs since the concentration of nanoparticles can affect its dissolution (Fairbairn, Keller et al. 2011, Zhao and Wang 2012). We added HNO_3 such that the acid concentration was 0.1% by volume and stored samples in the dark until digestion. We digested all samples for dissolution according to EPA Microwave Digestion Method 3051A, using a Multiwave GO microwave digestion system (Anton Paar) after adding 9 mL of 3:1 HNO_3:HCl acid mixture to each sample. We then diluted all samples to 100 mL and they were analyzed using an Agilent 7900 ICP-MS.

While the technique of using Amicon filters is widely used in the literature to measure dissolution of Ag^+ from AgNPs, we found that Amicon filters retain about 99 ± 0.12% (standard error) Ag^+ when we test the filter's ability to allow the dissolved fraction to pass through by adding 5 mg/L $AgNO_3$ to "low P" COMBO media. This is probably due to complexation of free Ag^+ with salts, especially chloride, in COMBO media, and these complexes/precipitates may have been large enough to be caught by the 3 kDa filter.

Further, the Amicon tube only allows about 22 ± 14% (standard error) Ag^+ to pass through by adding 5 mg/L $AgNO_3$ to nanopure water. While this complication makes it difficult to decipher the absolute bioavailable concentration of Ag^+ in our experiments, we are still able to compare light versus dark samples to test if the light treatment has an effect on AgNP dissolution in our test media and discuss whether or not we expect the observed toxicity is due to an effect of the AgNPs themselves or due to the presence of Ag^+.

We measured hydroxyl radical (•OH) production through the hydroxylation of coumarin-3-carboxylic-acid (3CCA) into the fluorescent 7-hydroxy-coumarin-3-carboxylic acid (7OH-3CCA) (Bennett and Keller 2011). We measured 7OH-3CCA production from 0, 0.2, and 10 mg/L AgNPs in COMBO media through UV-Vis spectrophotometry at 280 nm (Shimadzu BioSpec-1601, Shimadzu Inc., Japan). We wanted to compare the effect of light on hydroxyl production, so half of the treatments were kept in the dark and the other half were kept on a 10:14 light cycle under fluorescent growing lights, all at 20 degrees Celsius. The initial concentration of coumarin was 0.1 mM, which was suggested by (Czili and Horvath 2008) as the most effective concentration of coumarin for photocatalytic studies. We sampled for hydroxyl production after 6, 24, and 120 hours. To sample, we removed 1.2 mL aliquot from each sample and centrifuged for 20 min at 10,000 rpm to separate AgNPs from the aqueous phase. We then removed 1 mL of the supernatant, diluted it by a factor of two with DI water (Barnstead nanopure), and analyzed it spectrophotometrically, using Fisher Scientific quartz cuvettes.

1.2 Visual MINTEQ analysis

We modeled the transformation of 75 and 200 µg/L AgNPs in "low P" COMBO media using Visual MINTEQ version 3.1, similar to (Adeleye, Conway et al. 2014). We downloaded the software from http://vminteq.lwr.kth.se/ and used it without modification. We input AgNPs as finite solids at concentrations of 75 and 200 µg/L. We set the model parameters as follows: pH was to be calculated from mass balance of chemicals present (and determined to be 7.6), temperature was set at 25 C°, ionic strength was to be calculated depending on constituents of media, and AgNP was entered as Ag metal.

1.3 Results/Discussion

1.3.1 AgNP behavior similar in dark and light

Overall, there were differences in AgNP behavior between samples in the light versus those kept in the dark, however we do not think these differences would correspond to differential toxic effects. Nanoparticles kept under fluorescent lights on a 12:12 light cycle were significantly smaller after 24 hours (SI Figure 1) – samples in the dark were around 22 nm in size compared to those in the light which were around 12 nm. This is similar to other studies that have found that light can induce disagglomeration of nanoparticles (Bennett, Zhou et al. 2012, Zhou, Bennett et al. 2012), or the smaller size of AgNPs in the light could be due to the slightly higher rate of dissolution (SI Figure 3). However, samples kept in the light did not produce significantly more hydroxyl radicals compared to those in the dark and both were very similar to the control (0 AgNPs) treatment (SI Figure 2). This result agrees with studies that have found that low concentrations of AgNPs do not produce ROS (Ivask, Kurvet et al. 2014). Further, one study concluded that since AgNP EC50 concentrations are usually in the ppb range, ROS

are probably not involved in AgNP toxicity (Ivask, Kurvet et al. 2014). Lastly, samples kept in the dark seem to dissolve Ag^+ more slowly over the first two days (samples in the light contained about 12 μg free Ag^+ more than dark samples), however the two samples are indistinguishable by the third day (SI Figure 3). Overall, there are no significant differences between AgNP ROS production and dissolution in samples kept in the light or in the dark, therefore we conclude that AgNP behavior that could influence toxicity does not differ between dark and light samples.

1.3.2 AgNP dissolution

Our data indicate that as little as 1% of dissolved silver (as $AgNO_3$) actually passes through the filters we used to measure dissolution, meaning that the data we collected may underestimate the actual concentration of Ag^+ in our experiments. We can get an idea of the overall potential of 200 μg/L AgNPs to dissolve by modeling our reaction system using Visual MINTEQ, however this method has the major drawback that it predicts the *equilibrium* distribution of silver into various species, therefore we cannot use these data to estimate the concentration of Ag^+ after only 3 days (the longest transfer interval in our experiment). According to MINTEQ analysis, silver will be almost evenly divided between an aqueous complexation with chloride and as free ions (SI Table 1). This means that at equilibrium, 52% of the silver of 200 μg/L AgNPs will be present as free ions in our media. It is not possible that 104 μg/L Ag^+ was present in our experiment after 2-3 days, both because citrate-coated AgNPs has been found to dissolve slowly (Zhao and Wang 2012) and 104 μg/L Ag^+ is incredibly toxic to *Daphnia* and we would have seen much higher initial mortality (a recent meta-analysis found the median LC/EC50 for Ag^+ to be

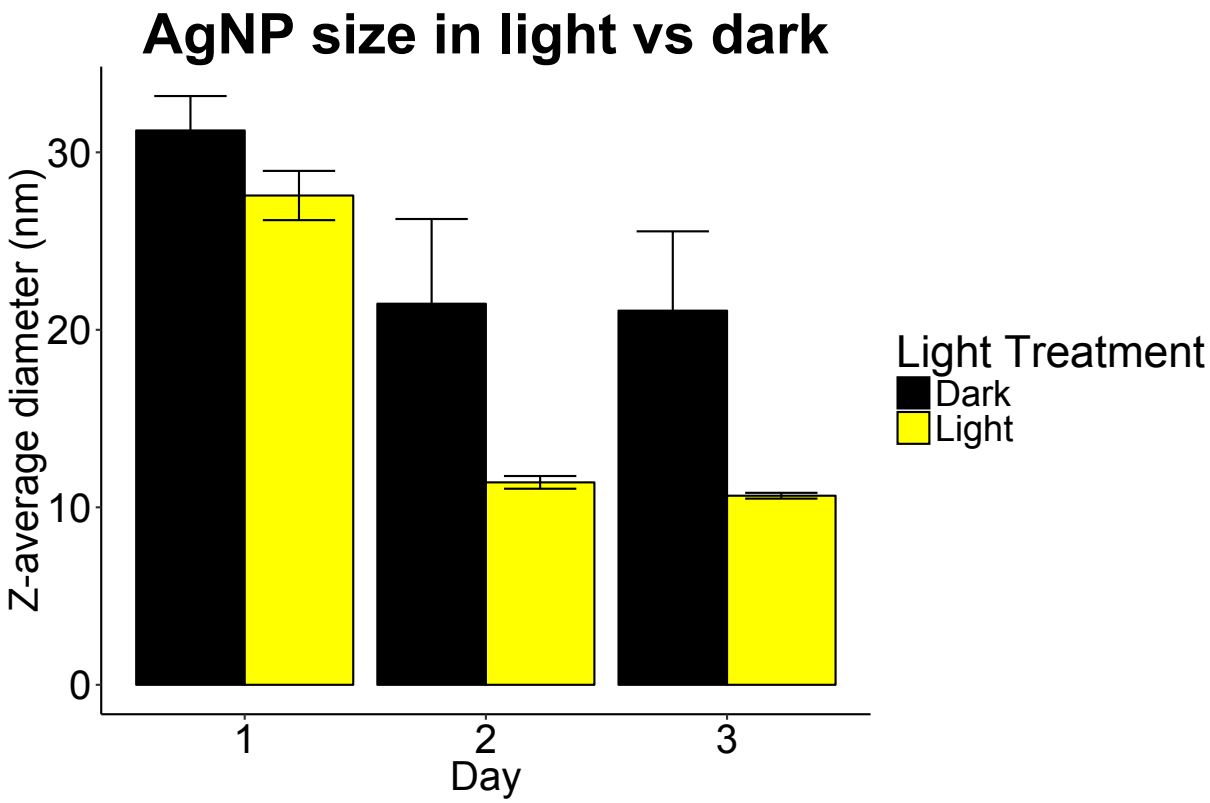
0.85 µg/L (Ivask, Juganson et al. 2014)). According to our dissolution data measured using Amicon filters, 5 and 20 mg/L AgNPs released 0.37 ± 0.06% (standard error) Ag^+ of the concentration of AgNPs, which would give a concentration of 0.28 and 0.74 µg/L of Ag^+ in our 75 and 200 µg/L exposures (8.4 and 22.2 ng of Ag^+ per 30 mL experimental chamber). These dissolution measurements are our best estimates, however we expect that dissolution will be faster at lower concentrations of nanoparticles. These data agree with those of Zhao and Wang (2012) who followed the dissolution of 50 nm citrate-coated AgNPs over 24 hours in freshwater media and found only 0.2 and 3 µg/L Ag^+ dissolved from 5 and 500 µg/L AgNPs, respectively, after 24 hours. We do not predict that this concentration is high enough to explain the effects we observed in our experiments, leading us to believe that nano-specific toxicity may be involved along with the ionic toxicity of Ag^+ (see main text for discussion).

Tables:

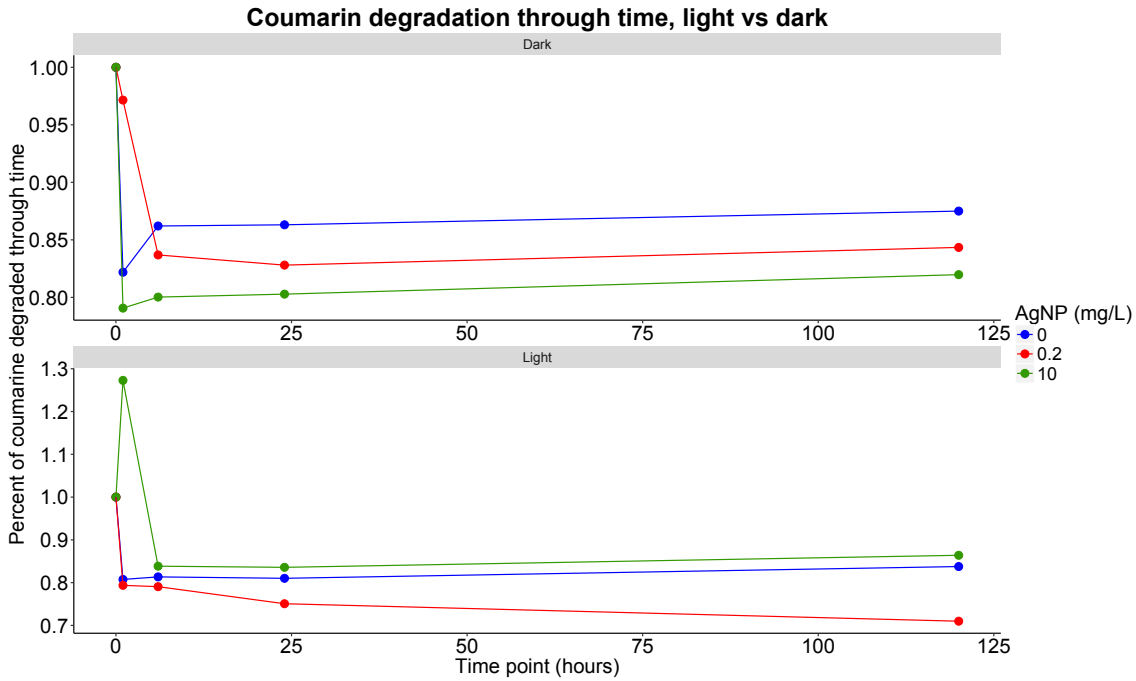
Ag species	µmol/L	Percent of total Ag
Ag^{+1}	9.59E-01	51.79
AgCl (aq)	8.34E-01	45.03
$AgCl^{2-}$	3.39E-02	1.83
AgI (aq)	1.54E-02	0.83
AgBr (aq)	5.24E-03	0.28
$AgSO^{4-}$	2.26E-03	0.12

SI Table 1: MINTEQ analysis results for the fate of Ag from 200 µg/L AgNP in "low P" COMBO media. We do not report Ag species that comprised less than 0.1% of the total Ag in this table.

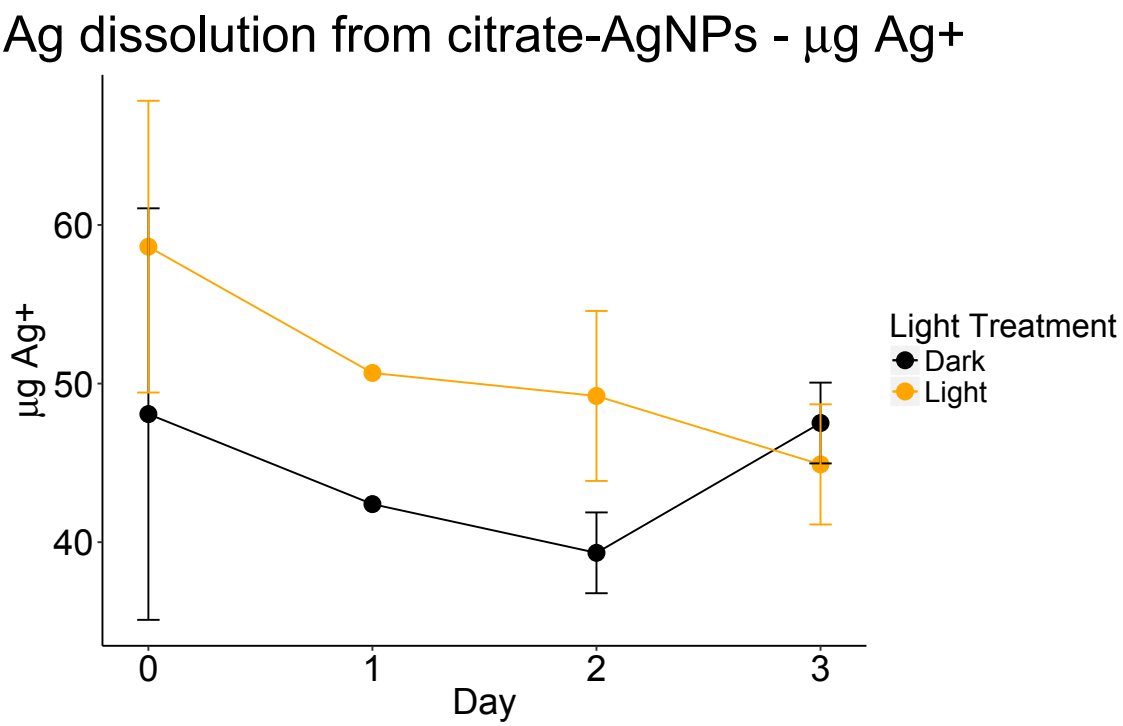
Figures:



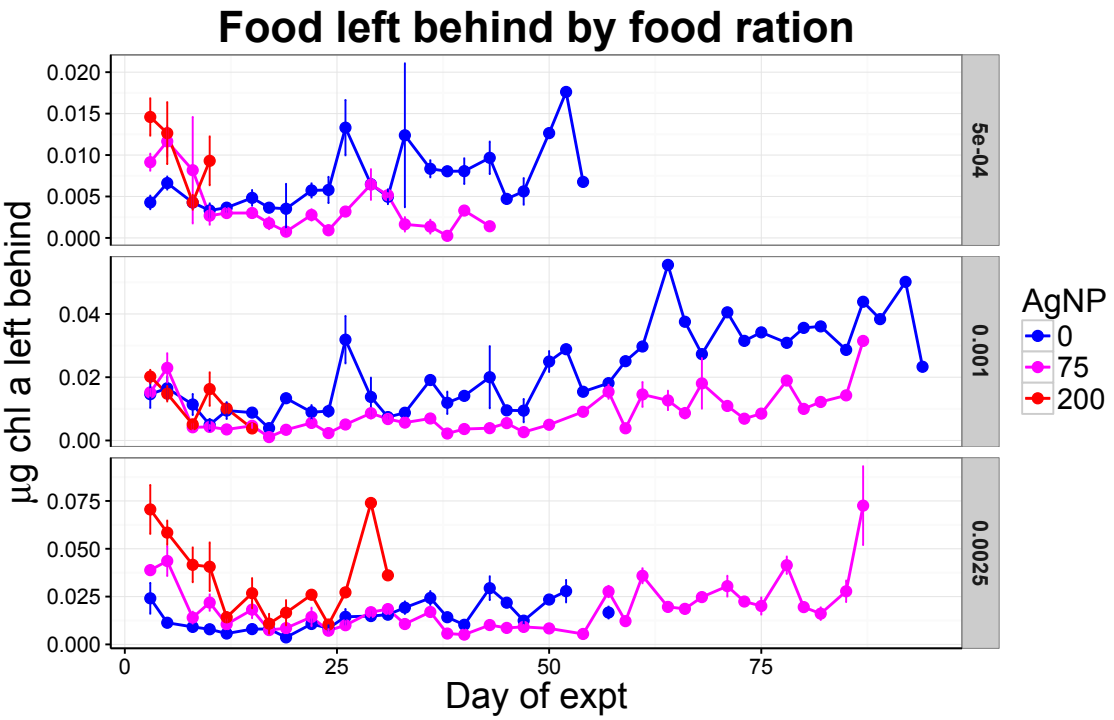
SI Figure 1: Size of 40 nm citrate-coated AgNPs in freshwater media kept in the dark versus under fluorescent growing lights on a diurnal light cycle.



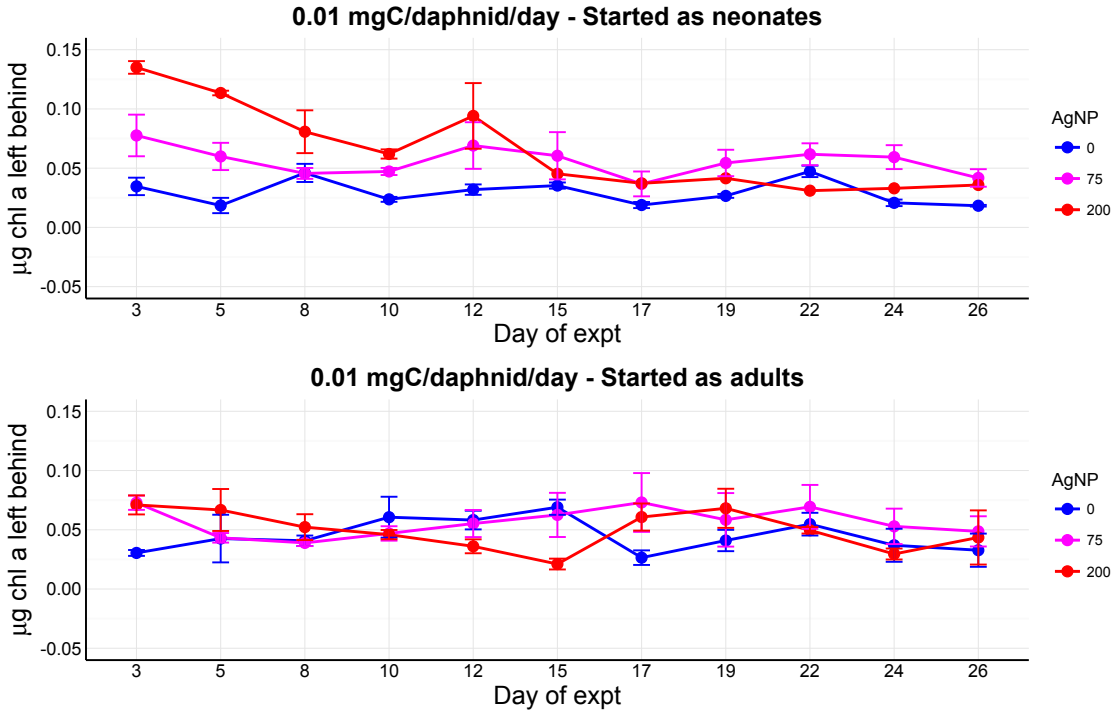
SI Figure 2: Coumarin degradation kinetics by 0, 0.2 and 10 mg/L 40 nm citrate-coated AgNPs kept in the dark (top panel) and under fluorescent growing lights on a diurnal light cycle.



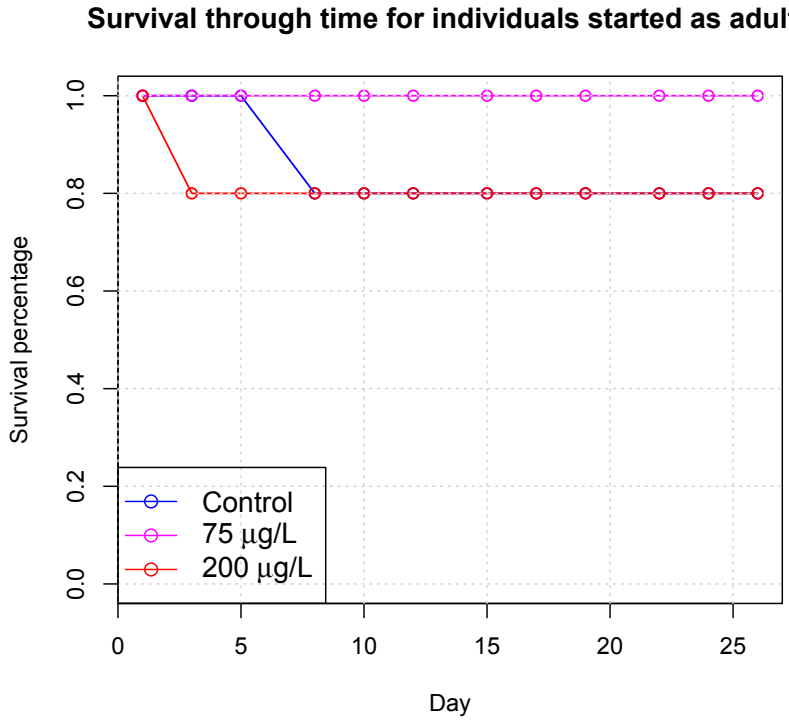
SI Figure 3: 20 mg/L AgNPs released approximately the same concentration of Ag^+ over 3 days if the samples were kept in the dark or in the light. The data points are the average of all replicates and the error bars reflect their standard error.



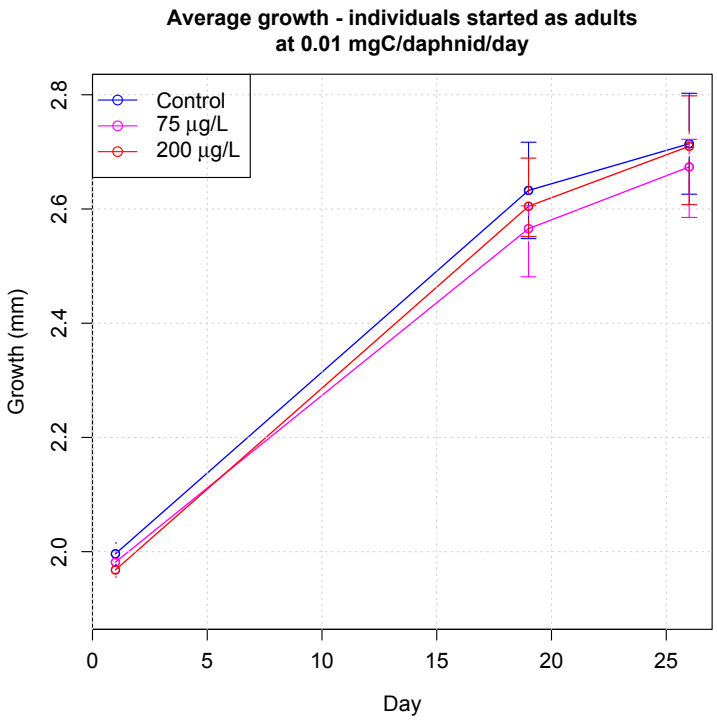
SI Figure 4: The amount of algal food not eaten by individuals exposed to 0 (blue), 75 (green), and 200 (red) µg/L AgNPs at the three lowest food levels – 0.0005 (top panel), 0.001 (middle panel), and 0.0025 (bottom panel) mgC/daphnid/day. The data points are the average of all replicates and the error bars reflect their standard error.



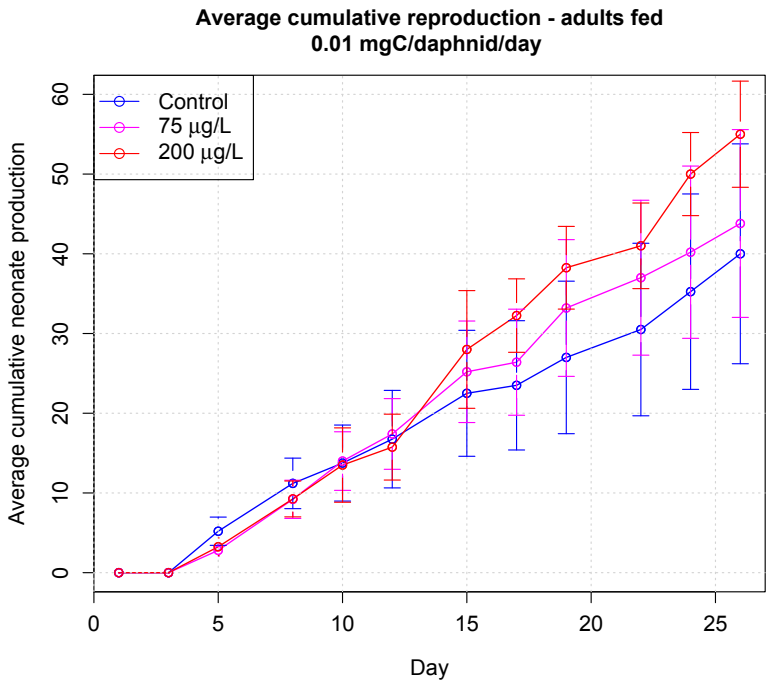
SI Figure 5: The amount of algal food not eaten by individuals exposed to 0 (blue), 75 (green), and 200 (red) µg/L AgNPs fed 0.01 mgC/daphnid/day that started the experiment as neonates (top panel) or adults (bottom panel). The data points are the average of all replicates and the error bars reflect their standard error.



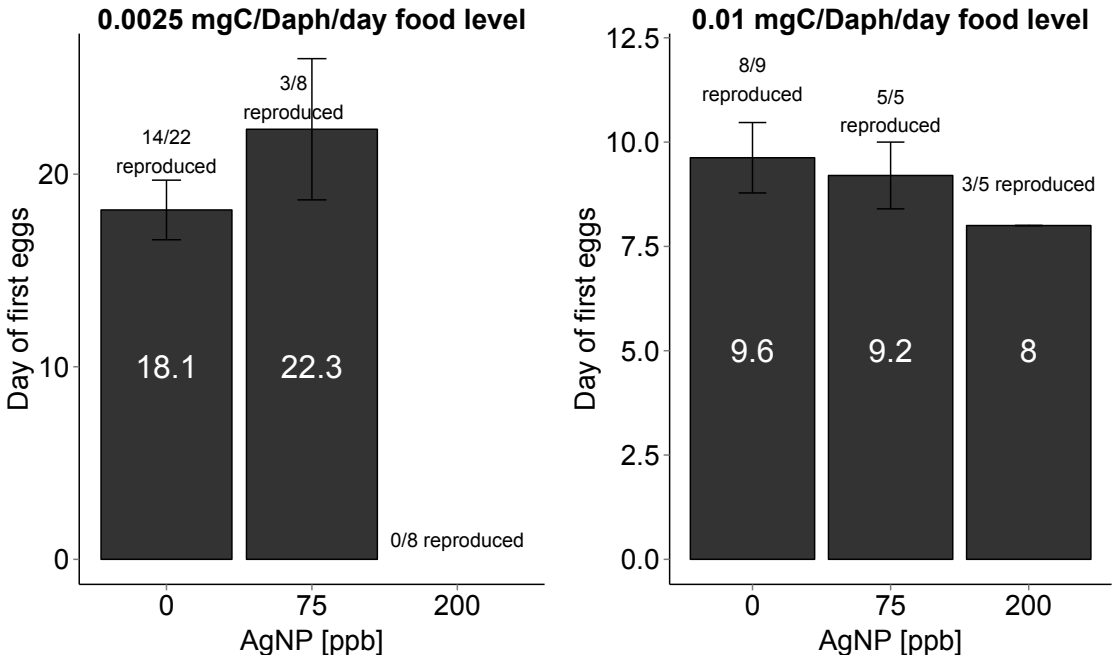
SI Figure 6: Survival of *Daphnia* that started as adults and were fed 0.01 mgC/daphnid day are similar across all AgNP treatments at the highest food level.



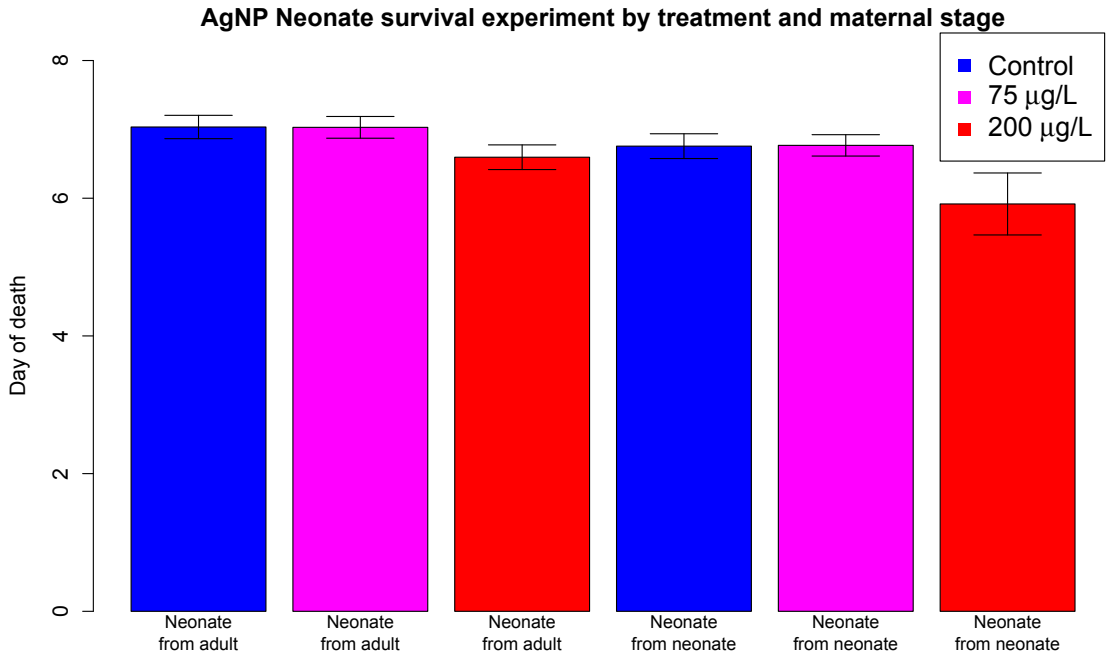
SI Figure 7: Growth of *Daphnia* that started as adults and were fed 0.01 mgC/daphnid day are similar across all AgNP treatments at the highest food level. The data points are the average of all replicates and the error bars reflect their standard error.



SI Figure 8: Cumulative neonate production of *Daphnia* that started as adults and were fed 0.01 mgC/daphnid day are similar across all AgNP treatments at the highest food level. The data points are the average of all replicates and the error bars reflect their standard error.



SI Figure 9: Time to first egg clutch (the day at which eggs were first observed in the brood pouch) at the two food rations at which the *Daphnia* reproduced viable neonates.



SI Figure 10: The average survival time of neonates removed from mothers fed 0.01 mgC/daphnid/day and exposed to 0, 75, and 200 µg/L. The first three bars represent offspring from *Daphnia* that began the experiment as adults (2.0 mm) and the three bars on the right represent offspring from *Daphnia* that began the experiment as neonates (0.7 mm). The samples sizes are (from left to right): 87, 102, 89, 78, 99, and 24 neonates.

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Chapter 4 – Supplementary Information

Section 1: Model details – Cd only model (Model 1) & FeSSi-Cd-DOC model (Model 2)

1.1 Model 1: Cadmium-only model

The model's state variable and equations are defined in Table S2. The parameters and their fitted values are in Table S3.

1.1.1 Model details

This is a very simple model of cadmium toxicity, in which algal cultures grow logistically in the absence of the toxicant. Cadmium-specific mortality decreases algal density at a constant rate until the Cd concentrations reaches the No Effect Concentration (NEC).

1.1.2 Parameter fitting

All four parameters in Table S3 were estimated simultaneously for all treatments by fitting the model to our chlorophyll a data (algal biomass) from our experiment on the response of new and 11-day old algal cultures to cadmium (Figure 2 in main text). We assumed that the cadmium concentration is constant and used the measured concentrations of Cd as opposed to the nominal doses (Figure S13). We used the BYOM ("Bring Your Own Model") platform for parameter estimation, developed by Tjalling Jager for Matlab (http://debtox.info). BYOM finds parameters that minimize the value of the negative log(likelihood) function.

1.2 Model 2: FeSSi-Cd-DOC model

The model's state variable and equations are defined in Table S4. The initial values of the state variables are defined in Table S5 and the parameters and their fitted values are in Table S6.

1.2.1 Model details

Algal biomass

We used chlorophyll a concentration as a proxy for algal biomass. Algal growth is modeled using the logistic equation.

DOC concentration

DOC was used as a proxy for algal organic matter. As in (Stevenson, Dickson et al. 2013, Adeleye, Stevenson et al. 2016), we assumed that DOC production rate is the sum of rates proportional to growth rate and algal population size, since DOC excretion depends on photosynthesis, maintenance, and growth. DOC can be lost due to heteroaggregation of DOC with FeSSi (F) and FeSSi with cadmium sorbed onto it (F_C). We assume that the rate of heteroaggregation of DOC is the same whether DOC is binding with F or F_C.

Bioavailable and inactivated FeSSi

Bioavailable FeSSi (F) and FeSSi with Cd sorbed onto it (F_C) are inactivated when coated by DOC. We assume that DOC inactivates F and F_C at the same rate. In exponentially growing cultures that started at a very low cell concentration, all the added

FeSSi particles were bioavailable when introduced into the cultures as either F or F_C. However, FeSSi particles were a mix of bioavailable (F and F_C) and inactivated particles (FeSSi with DOC and FeSSi with Cd sorbed onto it with DOC, F_D and F_{CD}, respectively) when introduced to 11-day old cultures (see next section).

Fitting of parameters and initial conditions

All initial conditions and parameters in Tables S5 and S6 were estimated for all treatments by fitting the model to chlorophyll a (algal biomass), DOC, and/or cadmium concentrations (see the end of this section of details on which data sets were used to estimate which parameters). We again used the BYOM ("Bring Your Own Model") platform for parameter estimation.

We fit the initial population size of the algal cultures and the initial DOC concentrations (new and 11-day old cultures) to the control cultures first, and then allowed them to change +/- 5-15% during subsequent fits. FeSSi started in the model as either unbound FeSSi (F), FeSSi with Cd sorbed onto it (F_C), FeSSi inactivated by DOC (F_D), or FeSSi with Cd sorbed onto it inactivated by DOC (F_{CD}). All of these forms of FeSSi could be present in the algal cultures at the start because we allowed FeSSi to interact with Cd in new or 11-day old media for an hour before dosing the algal cultures. Because new media did not contain any DOC, FeSSi at the start of the new culture exposures was either unbound FeSSi (F) or FeSSi bound with Cd (F_C). We fit F_C using the Cd data and the initial value of F was just the total concentration of FeSSi particles in the dosage minus F_C. The best fit of the initial conditions predicts that 36% of the total FeSSi concentration were F_C in new culture exposures.

In 11-day old cultures, FeSSi could be in any of the four forms upon dosage. We fit the initial values of F_C, F_D, and F_{DC} to the algal chlorophyll a, DOC, and Cd concentration data and the initial value of F is just the total concentration of FeSSi particles in the dosage minus, F_C, F_D, and F_{DC}. Due to the higher concentration of DOC in 11-day old cultures that interacted with FeSSi particles and Cd for an hour before exposure, the model predicts that 86% of the FeSSi particles introduced into the algal cultures were inactivated by DOC – 50% of the total FeSSi concentration was F_D and 36% of the total FeSSi concentration was F_{CD}. None of the FeSSi particles were introduced into the 11-day old algal cultures as F_C (all had been inactivated by DOC). Overall, the initial values give a good fit to Cd data at the start to all cultures except at the lowest exposures to new cultures (Figure S15). We are not sure why the concentration of Cd appears to *increase* for the first few days of exposure in these cultures (for some reason the Cd appears to be desorbing from the FeSSi). However, since these Cd concentrations are well below the NEC of Cd (0.5126 mg/L), the mismatch in the fit does not have an affect on the toxicity predicted by the model at these concentrations and we felt it was more important to fit the high concentrations of Cd, which would exert toxicity, than lower concentrations of Cd. See the next section on possible model refinements for a discussion on ways we could improve fits of the model to the Cd data.

Parameters relating to algal growth and DOC production (r, K, h_{DN} and k_{DN}) were fit to control data only and then fixed (parameter values were not allowed to change). FeSSi-related parameters (k_F, γ_{UN}, and β) were fit to FeSSi-only exposure treatments in Adeleye, Stevenson et al. (2016) and we used those values here. Parameters pertaining to Cd toxicity are from the fit of the Cd-only experiment and are identical to the values in SI

Table 3. Parameters pertaining to FeSSi and Cd toxicity (k_{FC}, b, α_{CF}) were fit to FeSSi-Cd exposures (Figure 3 in the main text). Overall, even though this model has 12 parameters, only 3 were fit to the FeSSi and Cd exposures and the rest come from other data sets of similar exposures. In this way, we employed a step-wise modeling technique: we started by fitting Cd toxicity alone to data on Cd exposures and combined that with a FeSSi toxicity model fit to FeSSi-only exposures (Adeleye, Stevenson et al. 2016) and then combined these two models with a representation of FeSSi with Cd toxicity to produce our full model (Model 2). The fact that we get a good fit of this model to the data encourages us that this technique was able to piece together the various contributors of toxicity (Cd, FeSSi, FeSSi+Cd) to explain the patterns observed in our experiments.

1.2.2 Potential model refinements

1) Mechanistic algal model – we used the logistic growth equation to model phytoplankton, an oversimplification of algal growth. We could use a mechanistic model of nutrient limited algal growth, but this would involve at least 3 additional parameters.

2) DOC addition from algal mortality – dead algal cells could add to the DOC pool.

3) Nanoparticle exposure altering DOC production – nanomaterial exposure may alter algal DOC production, which could help explain the mismatch between our model's prediction and DOC concentrations of algal cultures exposed to the highest concentration of FeSSi, especially the 11-day old cultures. The effect of nanomaterials on organic material production is an unexplored but very interesting area for future research.

4) Explicit cadmium dynamics, such as desorbing from FeSSi and/or being taken up by algal cells – the fit of our model to the Cd data (Figure S15) is poor but able to capture the

basic dynamics, especially at the highest Cd concentrations that exert toxicity to the algae. The model fit to the two lower FeSSi concentrations to new cultures is particularly poor, because the Cd concentration actually *increases* in the first few days and then declines. It is unclear why this occurred, however it is probably due to Cd desorbing from FeSSi. Further, the Cd concentration slowly decreases through time in 11-day old cultures, and this is probably due to uptake of Cd by the algae, as has been shown in the literature (Nagel, Adelmeier et al. 1996, Hu, Lau et al. 2001, Aguilera and Amils 2005). Since these two processes (Cd desorbing from FeSSi and algal cells taking up Cd) occur simultaneously and we do not have data measuring FeSSi uptake of Cd beyond 2 hours (Su, Adeleye et al. 2016), we cannot reliably estimate parameters describing algal uptake and Cd desorption from FeSSi. However, this emphasizes the need to measure the removal capacity of FeSSi and other nZVI and derivatives for longer than a few hours in order to estimate the effect of these particles on organisms.

Tables

Table S1: XPS quantification of FeSSi-cadmium composite obtained after 30 days of exposure to 1-day algal culture

Name	Position	% Atomic Conc	% Mass Conc
Fe 2p	710.5	6.77	21.06
O 1s	530.5	55.57	49.54
Si 2s	153.5	1.21	1.89
C 1s	285	34.14	22.85
Ca 2p	346.5	0.26	0.58
P 2s	190	0.13	0.22
Sr 3d	132.5	0.06	0.29
Cd 3d	405	0.34	2.15
Cl 2p	197.5	0.11	0.21
N 1s	400	1.32	1.03
S 2p	162	0.1	0.18

Table S2: State variables, functions, and balance equations for Model 1 – Cadmium only model.

State Variables	
N	Algal biomass (µg chl a/L)
Functions	
$\mu = k_{Cd}(C_d - NEC)$	Mortality due to FeSSi and cadmium exposure
Balance equations	
$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) - \mu$	Algal biomass

Table S3: Parameters for Model 1 (Cd-only model). We used data from the cadmium-only exposure (Figure 2 in main text) and used the *measured* cadmium exposure (Figure S13).

		Value	Units
r	Intrinsic rate of increase	0.5668	1/day
K	Carrying capacity	454.5	ug chl a/L
k_{Cd}	Cadmium-specific toxicity	0.2283	L / (mg Cd * day)
NEC	No effect concentration of cadmium	0.5126	mg Cd/L
C_d	*Measured* cadmium exposure (Figure S13)	New cultures: 0, 0.1, 0.98, 4.56, 9.4 11-day old cultures: 0, 0.09, 0.93, 3.86, 7.85	mg Cd/L

Table S4: Initial values for Model 2 – FeSSi-Cd-DOC model. Due to the higher concentration of DOC in 11-day old cultures that interacted with FeSSi particles and Cd for an hour before exposure, 86% of the FeSSi particles introduced into the algal cultures were inactivated by DOC – 50% of the total FeSSi concentration was FeSSI bound to DOC and 36% of the total FeSSi concentration was FeSSI with Cd sorbed onto it bound to DOC. None of the FeSSi particles were introduced into the 11-day old algal cultures as FeSSi with Cd sorbed onto it (all had been inactivated by DOC), however 36% of the total FeSSi concentration were F_C in new culture exposures.

Initial Values – after 1 hour of FeSSi, Cd, and DOC (for 11 day old cultures) interaction	
1 day old cultures	
N	0.34 ug chl a/L
D	5.5 mg C/L
C	0, 0.012, 0.12, 1.2 mg Cd/L
F	0, 3.8e11, 3.8e12, 3.8e13 particles/L
F_D	0, 0, 0, 0 particles/L
F_C	0, 2.18e11, 2.18e12, 2.18e13 particles/L
F_{DC}	0, 0, 0, 0 particles/L
11 day old cultures	
N	57 ug chl a/L
D	8.8 mg C/L
C	0, 0.012, 0.12, 1.2 mg Cd/L
F	0, 8.18e10, 8.18e11, 8.18e12 particles/L
F_D	0, 3e11, 3e12, 3e13 particles/L
F_C	1.81e8, 1.81e9, 1.81e10, 0 particles/L
F_{DC}	0, 2.18e11, 2.18e12, 2.18e13 particles/L

Figures:

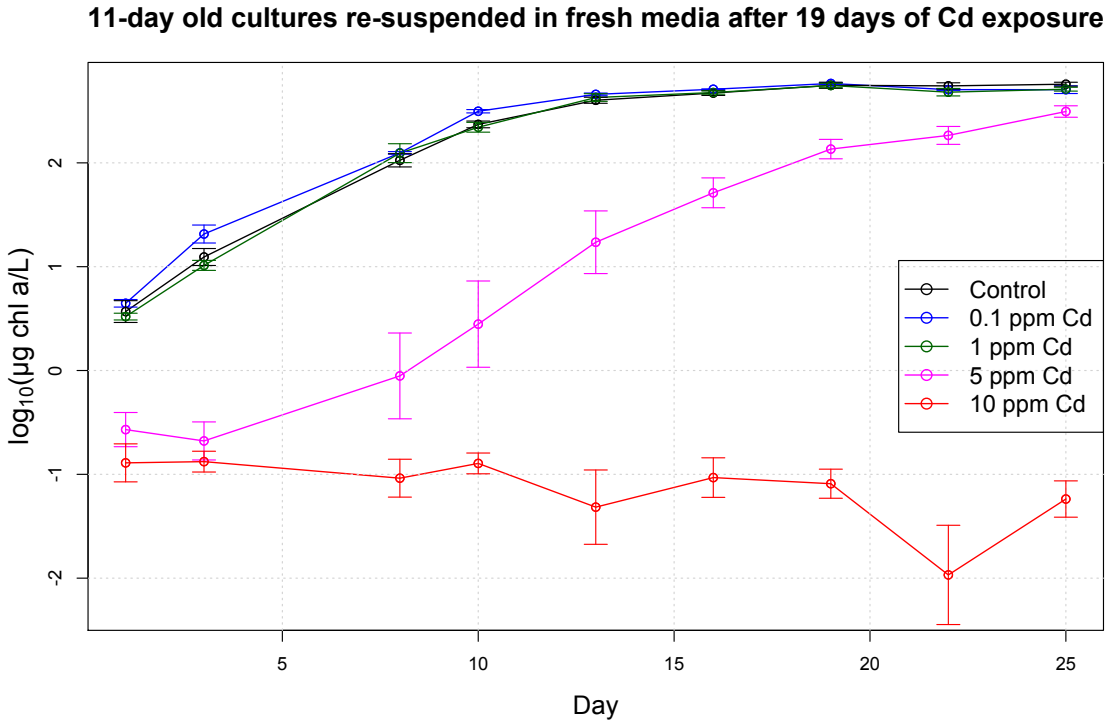

Figure S1: We re-suspended 11-day old algal cultures that had been exposed to cadmium for 19 days (results in Figure 2) in new, cadmium-free algal media to see if cultures exposed to 5 and 10 ppm cadmium were able to recover from cadmium toxicity. Algal cultures exposed to 5 ppm for 19 days were able to recover while cultures exposed to 10 ppm were not. The data points are averages of that treatment's replicates (n=3 for all treatments) and the error bars reflect their standard error.

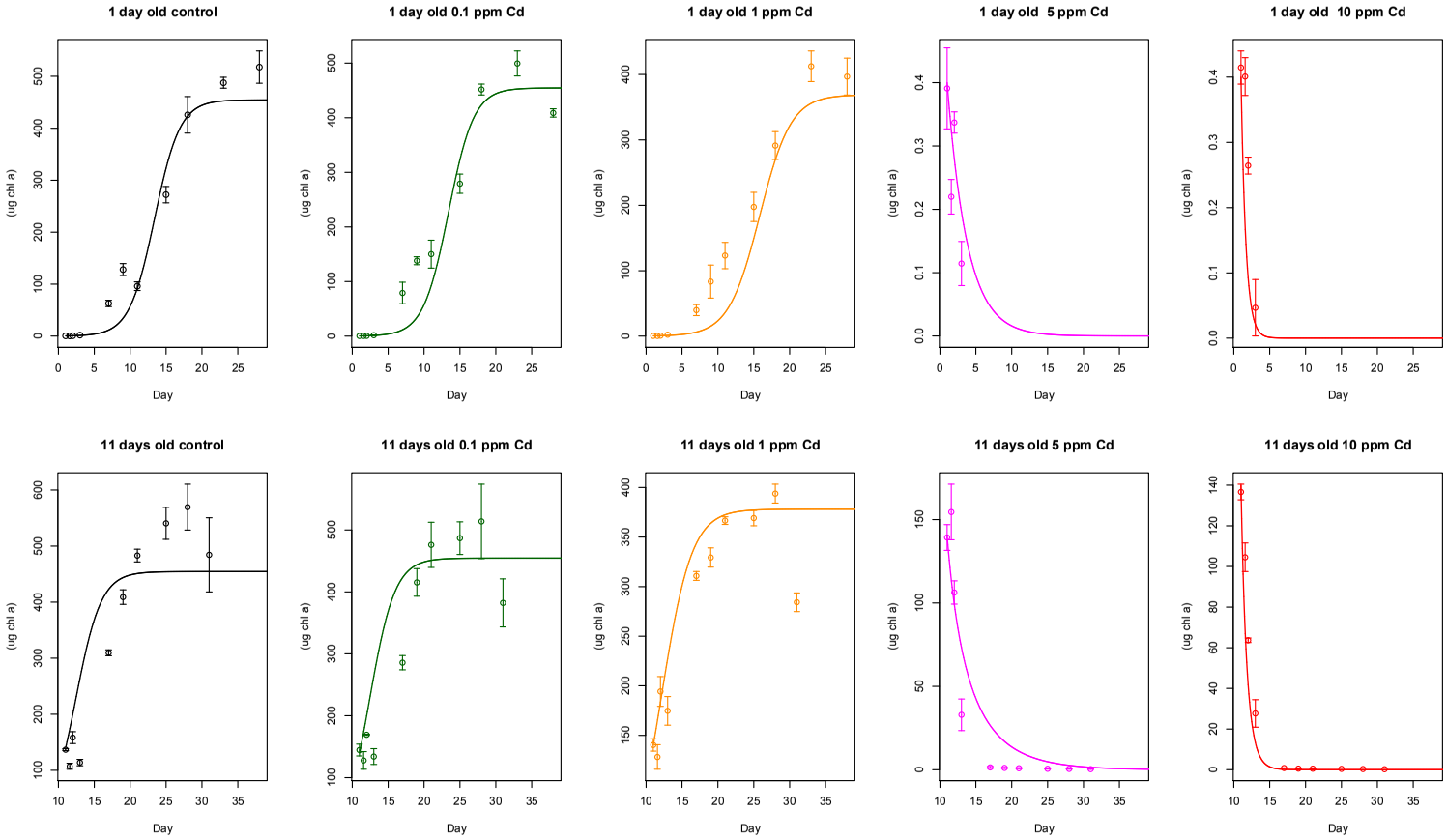

Figure S2: Fit of cadmium toxicity model to cadmium-only data. The data points are averages of that treatment's replicates (n=3 for all treatments) and the error bars reflect their standard error.

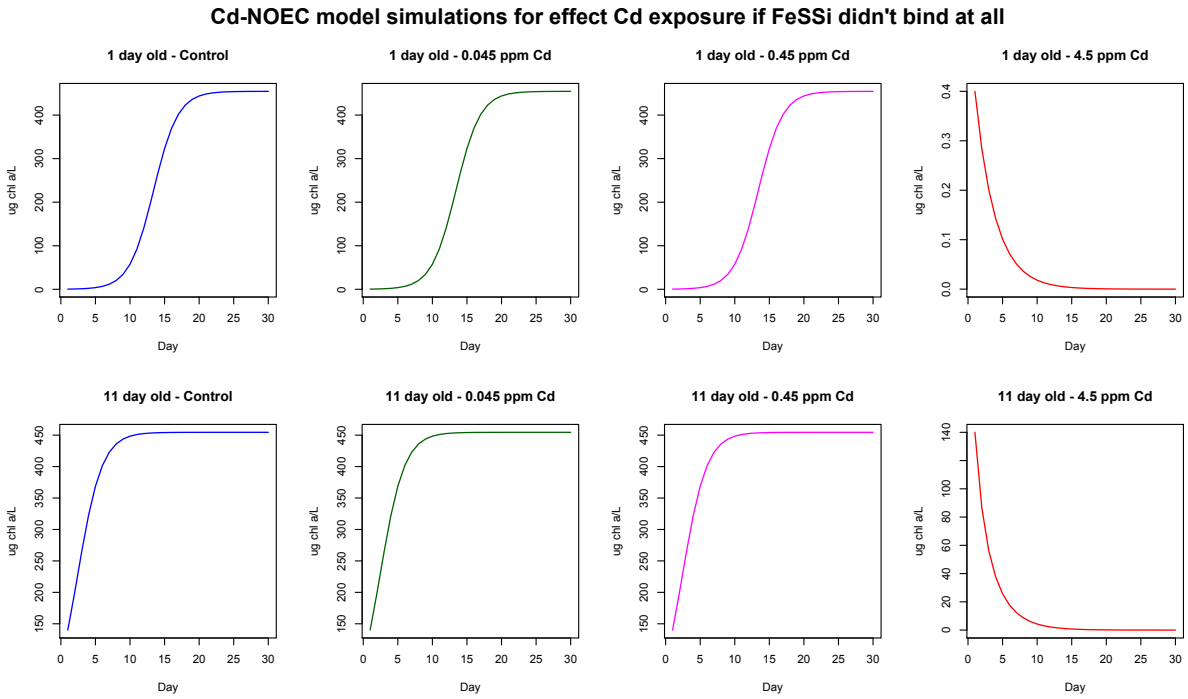

Figure S3: Predicting the effect of 0.045, 0.045, and 4.5 ppm cadmium exposure if FeSSi did not bind cadmium at all.

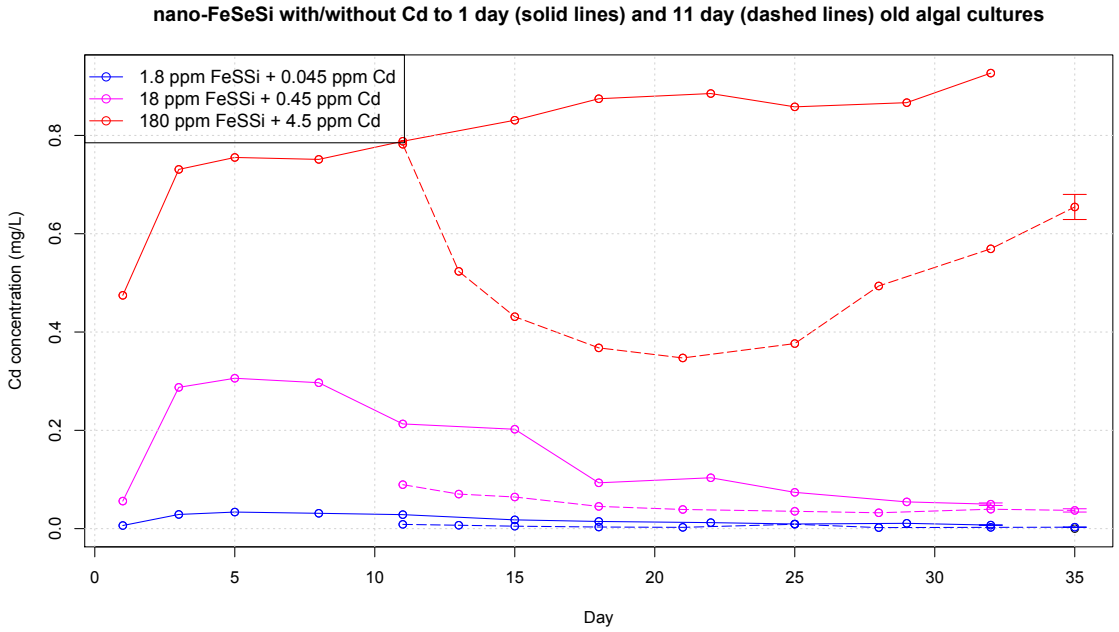

Figure S4: Cd ICP data – extracellular cadmium measured through FeSSi + Cd experiment in new cultures (solid lines) and 11 day old cultures.

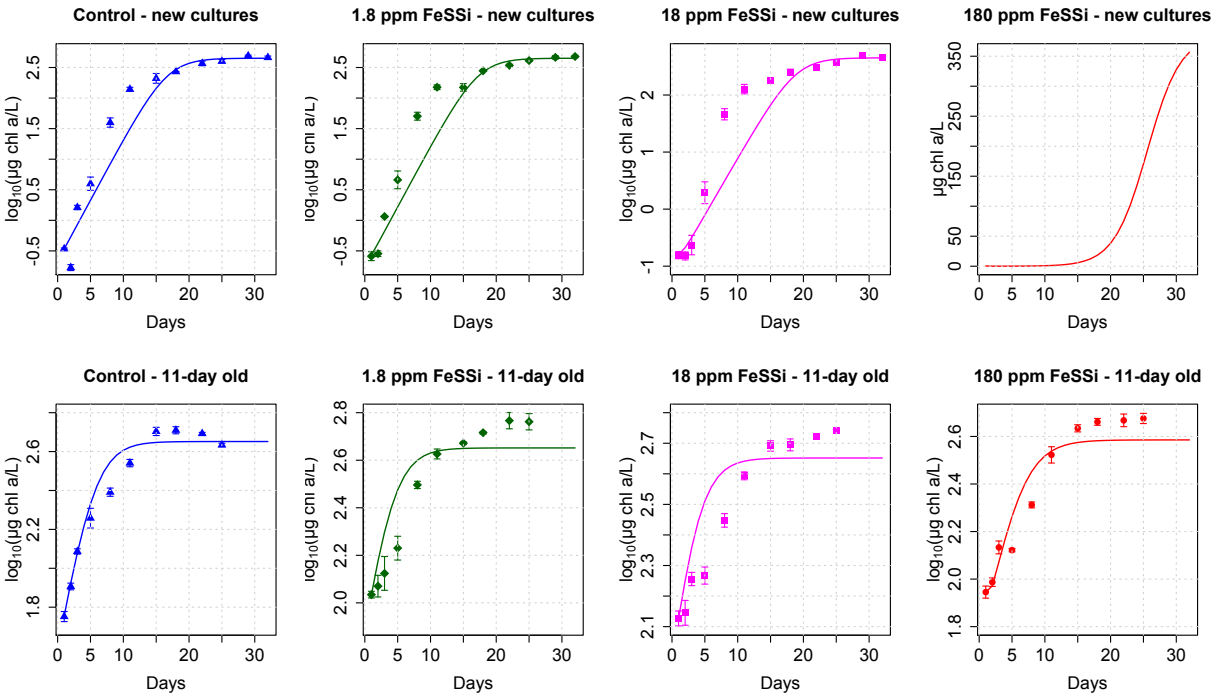

Figure S5: Model 2 simulation if FeSSi and FeSS+Cd had the same toxicity ($k_F = k_{FC}$). This simulation incorrectly predicts that 180 ppm FeSSi + 4.5 ppm cadmium would only delay the growth of new cultures (top right panel); these cultures never grew in the experiment. The data points are averages of that treatment's replicates (n=3 for all treatments) and the error bars reflect their standard error.

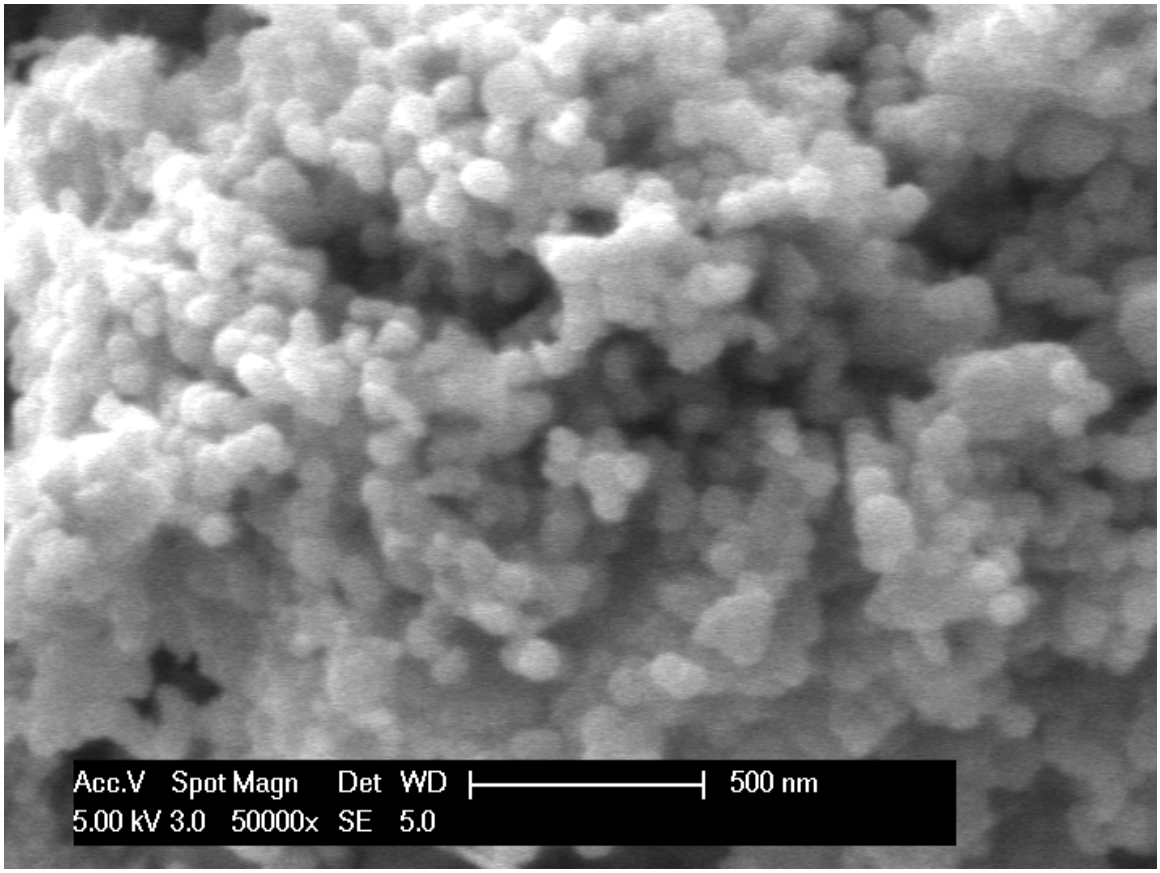

Figure S6: A scanning electron micrograph of FeSSi particles showing spherical particles with an average diameter of 90 nm

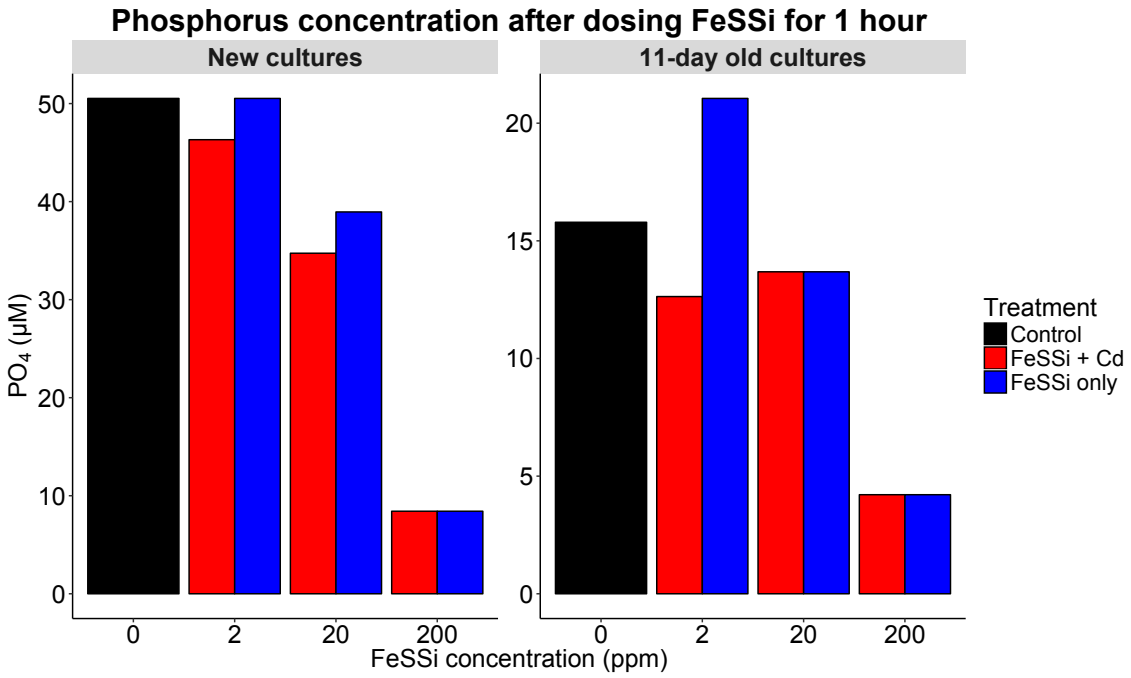

Figure S7: Initial concentration of phosphate in algal cultures after being dosed with FeSSi and cadmium (for FeSSi + Cd treatments) compared to control concentrations. FeSSi sorbed phosphate onto the particles, shown through the decrease in phosphate concentrations with increasing FeSSi concentrations. Further, there does not appear to be a difference between the amount of phosphate FeSSi particles sorb when cadmium is present, potentially indicating that phosphate sorprtion is faster or somehow outcompetes sorption of cadmium onto the particles.

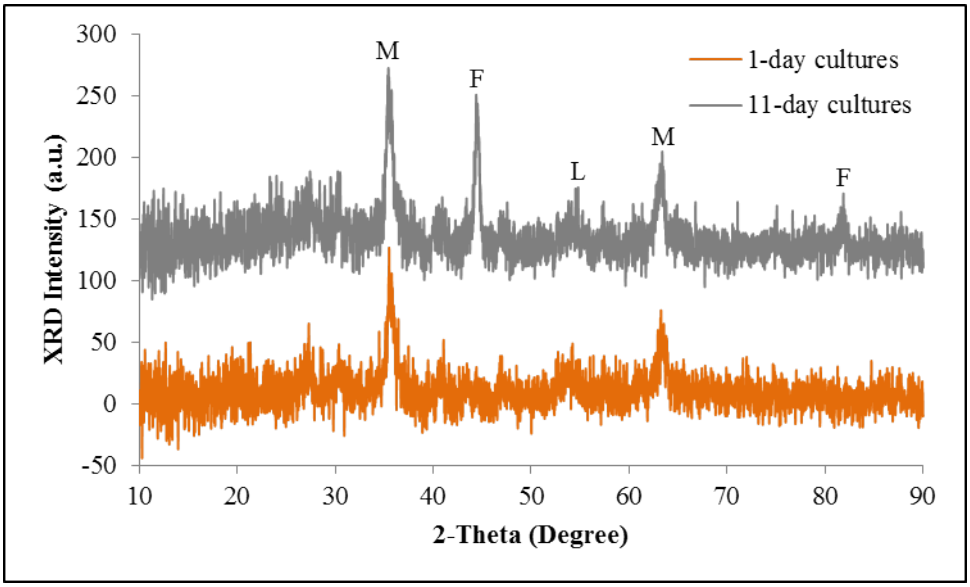

Figure S8. XRD diffractograms of FeSSi with cadmium in 1-day and 11-day *Chlamydomonas* cultures. M = maghemite/magnetite (Fe_2O_3/Fe_3O_4), F = zerovalent iron (Fe^0), L = lepidocrocite (ɣ-FeOOH)

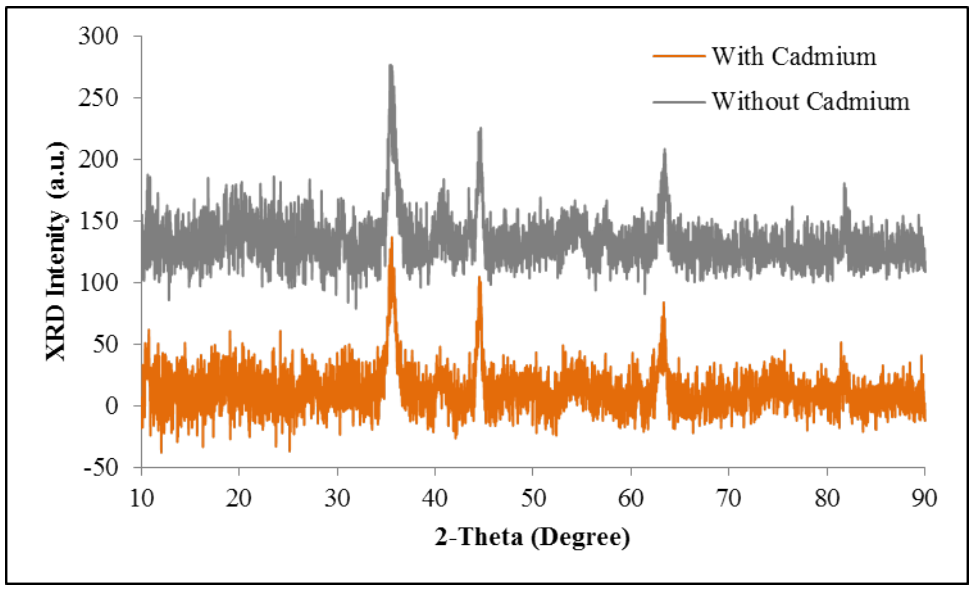

Figure S9. XRD diffractogram showing transformations of of FeSSi nanoparticles after 30 days in 11-day algal cultures

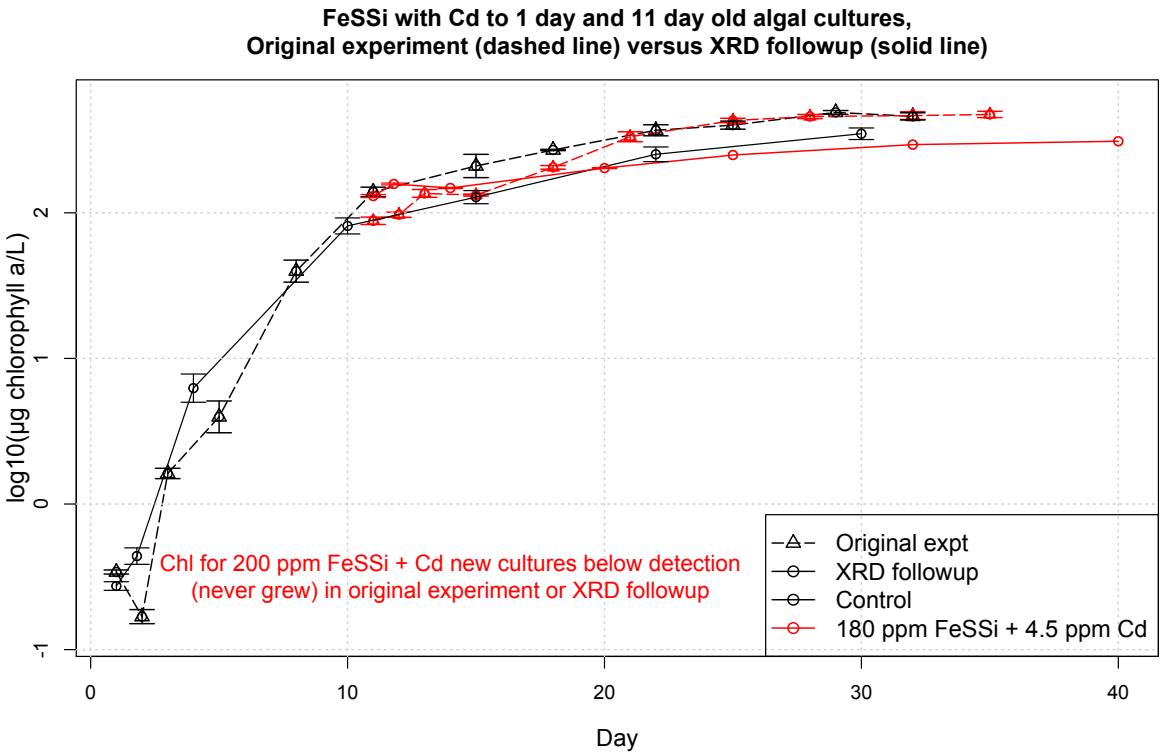

Figure S10: Comparing the effect of 180 ppm FeSSi + 4.5 ppm Cd in the FeSSi+Cd experiment (Figure 3) and the response of cultures during follow-up experiment for XRD measurements (XRD and XPS exposures). The data points are averages of that treatment's replicates (n=3 for all treatments at the start, FeSSi-exposed cultures were destructively sampled through time for XRD analysis) and the error bars reflect their standard error.

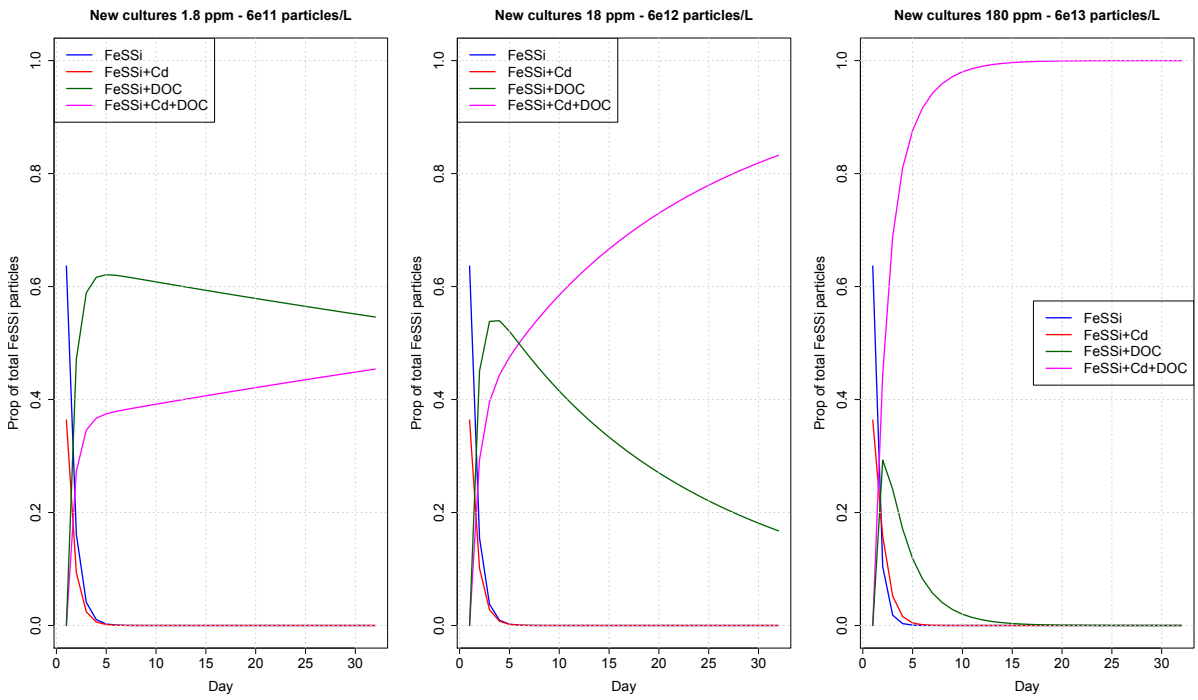

Figure S11: Model simulation (Model 2) of FeSSi particles through time in new algal cultures, displayed as the proportion of the total concentration of FeSSi particles in that current FeSSi state, either unbound FeSSi (FeSSi without DOC or Cd; "FeSSi"), FeSSi with Cd sorbed onto it ("FeSSi+DOC"), FeSSi inactivated by DOC ("FeSSi+DOC"), or FeSSi with Cd sorbed onto it inactivated by DOC ("FeSSi+Cd+DOC").

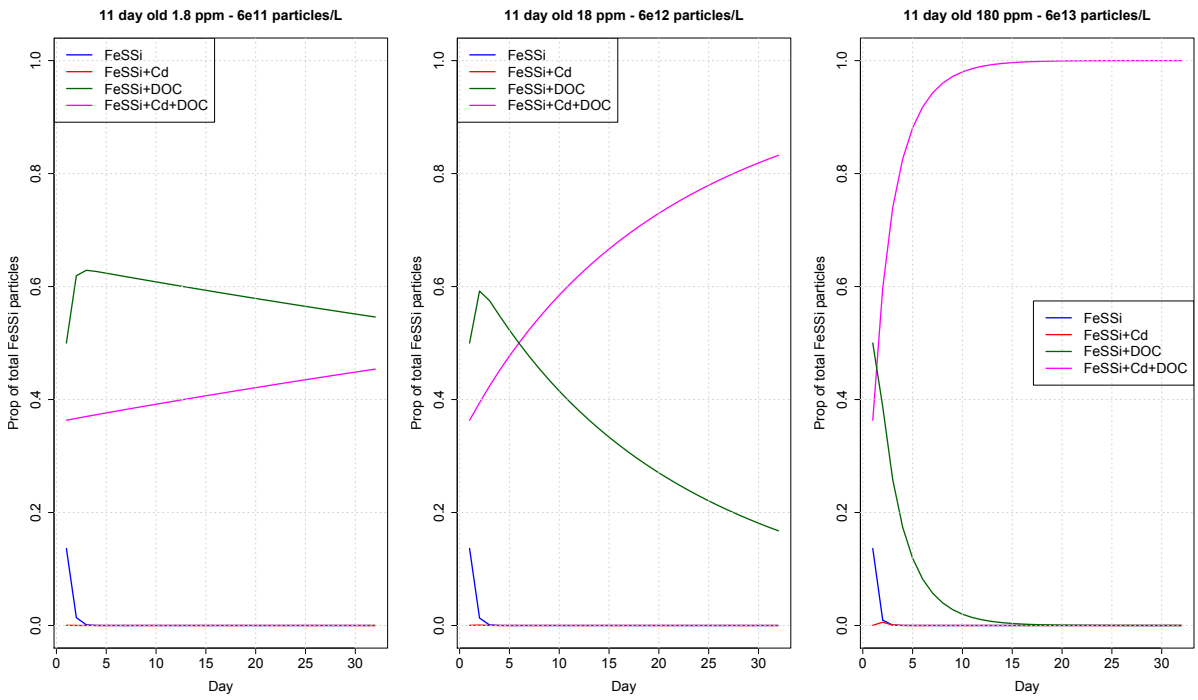

Figure S12: Model simulation (Model 2) of FeSSi particles through time in 11-day old algal cultures, displayed as the proportion of the total concentration of FeSSi particles in that current FeSSi state, either unbound FeSSi (FeSSi without DOC or Cd; "FeSSi"), FeSSi with Cd sorbed onto it ("FeSSi+DOC"), FeSSi inactivated by DOC ("FeSSi+DOC"), or FeSSi with Cd sorbed onto it inactivated by DOC ("FeSSi+Cd+DOC").

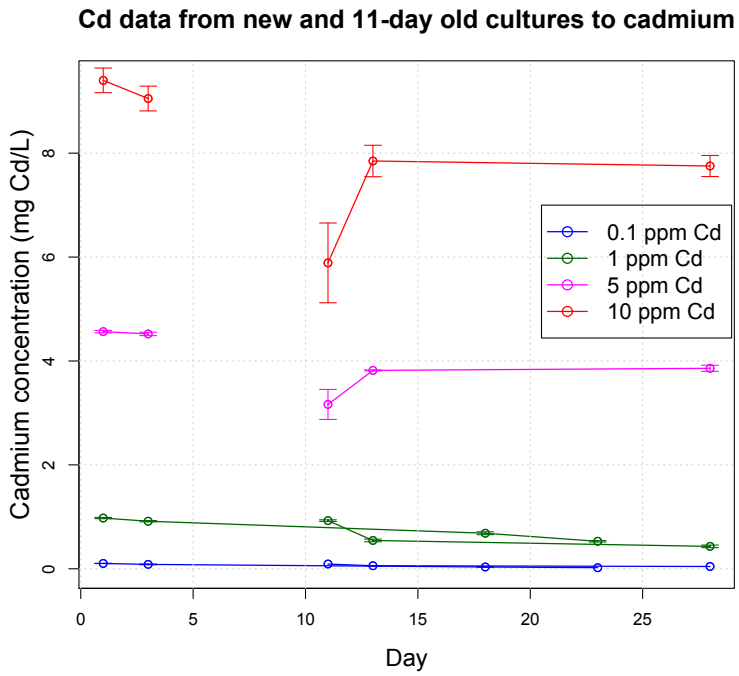

Figure S13: Cadmium concentration during Cd-only exposure. We used the average measured exposure value of Cd for each treatment when fitting Model 1 (Cd only model).

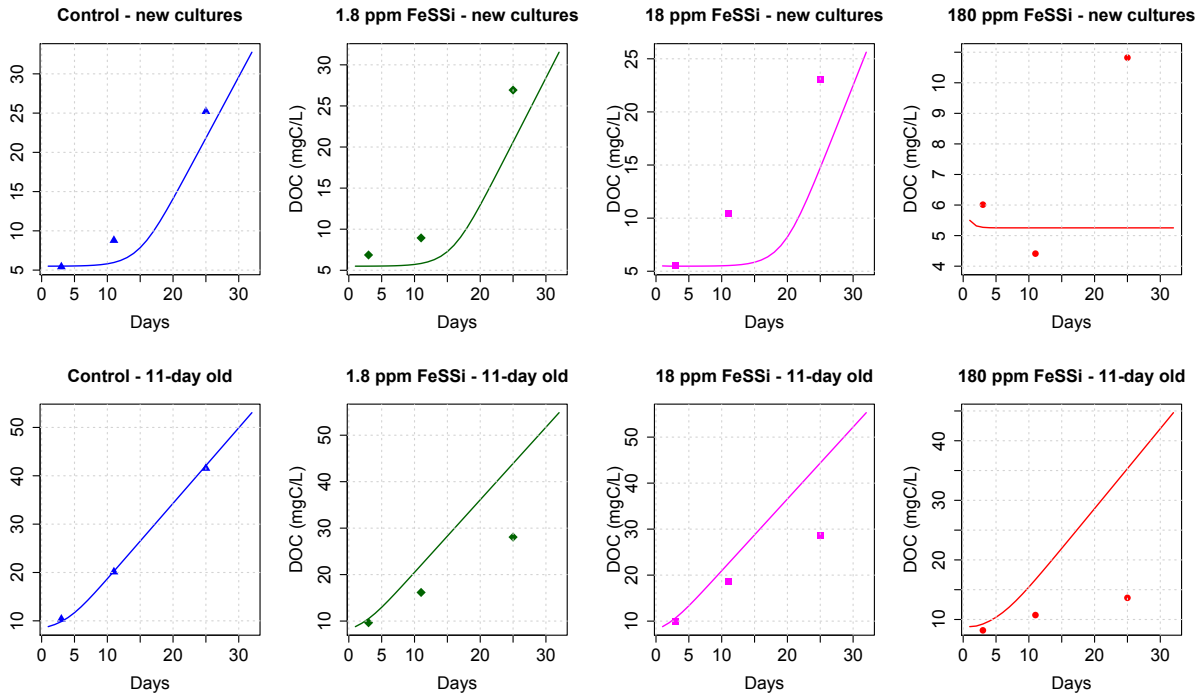

Figure S14: Fit of Model 2 to DOC (mg C/L) data.

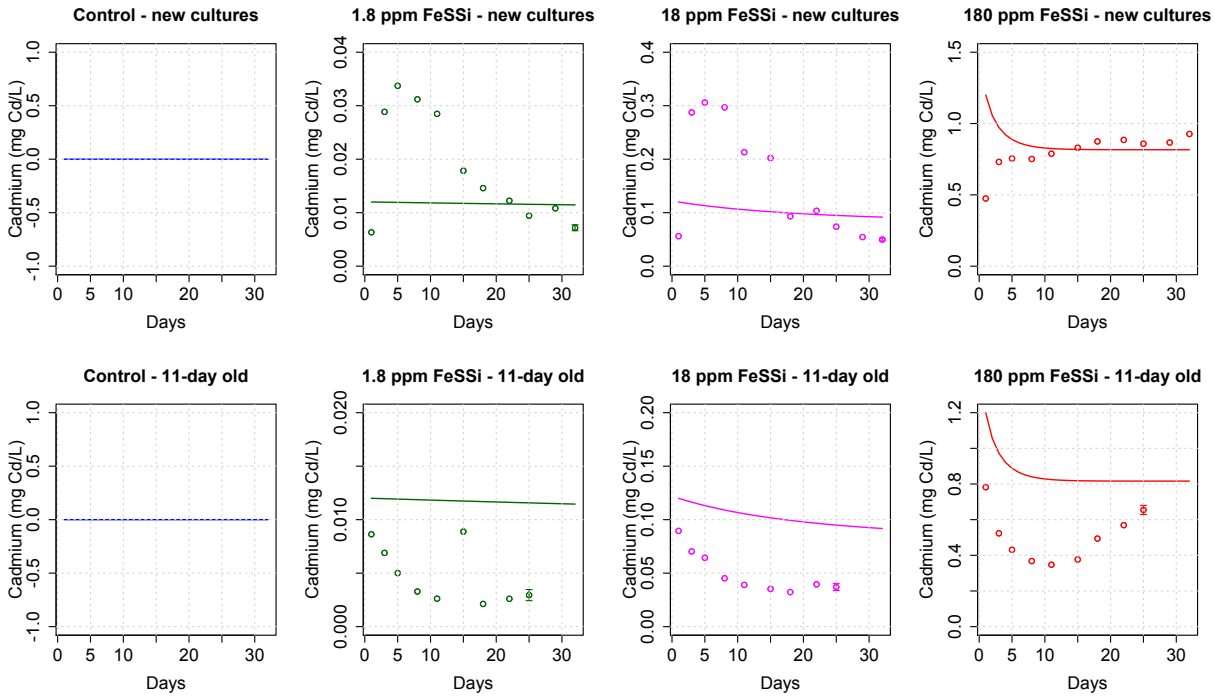

Figure S15: Fit of Model 2 to cadmium (mg Cd/L) data.

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