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Bioconcentration and Biotransformation of Selenite versus Selenate Exposed Periphyton and Subsequent Toxicity to the Mayfly *Centroptilum triangulifer*

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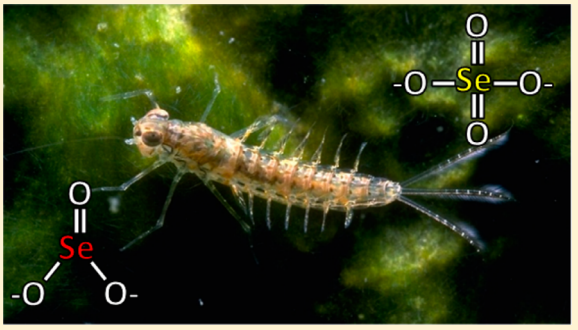
Supporting Information

ABSTRACT: Little is known about the bioaccumulation dynamics, biotransformation processes, or subsequent toxicity to consumers of dissolved selenite (SeO_3) versus selenate (SeO_4) uptake into aquatic primary producer communities. To address these data gaps, we examined SeO_3 and SeO_4 bioconcentration into complex freshwater periphyton communities under static and static-renewal conditions. Further, we explored periphyton biotransformation of Se species using X-ray absorption near edge structure (XANES) spectroscopy analysis and changes in the periphyton associated microbial consortium using denaturing gradient gel electrophoresis (DGGE). Last, we fed differentially treated periphyton to the mayfly *Centroptilum triangulifer* in full life cycle exposures to assess toxicity. Selenite exposed periphyton readily bioconcentrated Se while, in contrast, initial periphyton uptake of SeO_4 was negligible, but over time periphyton [Se] increased steadily in conjunction with the formation of dissolved SeO_3. XANES analyses revealed that both SeO_3 and SeO_4 treated periphyton biotransformed Se similarly with speciation dominated by organo-selenide (~61%). Mayfly survival, secondary production, and time to emergence were similar in both SeO_3 and SeO_4 treated periphyton exposures with significant adverse effects at 12.8 $\mu g\ g^{-1}$ ((d.w.) secondary production) and 36 $\mu g\ g^{-1}$ ((d.w.) survival and development time). Overall, dissolved selenium speciation, residence time, and organisms at the base of aquatic food webs appear to be the principal determinants of Se bioaccumulation and toxicity.



INTRODUCTION

Selenium contaminated sites that contain similar dissolved Se concentrations often vary in their ability to produce toxic effects in fish and wildlife.[1] A major source of variation is the discrepancy in bioconcentration of dissolved Se into the base of aquatic food webs between sites. The movement of Se from the dissolved phase into primary producers is the largest and potentially most variable step (~10^2 to 10^6-fold bioconcentration[2]) in the process of Se bioaccumulation in aquatic food webs.[3] Further, primary producers are considered to be largely responsible for the critical biotransformation step of reducing dissolved Se oxyanions into protein associated organo-selenides (e.g., seleno-cysteine and seleno-methionine),[4] which are regarded as the most toxic forms of Se.[5] The role that dissolved inorganic Se speciation plays in primary producer bioconcentration and biotransformation of Se (i.e., are different inorganic species differentially bioconcentrated and/or bio-transformed?) has not received attention commensurate with its potential importance in determining toxic effects.

Our understanding of these processes is primarily derived from experiments with single algal species in suspension;[6] however the bases of most aquatic food webs are represented by complex assemblages, such as periphyton. Periphyton is a complex benthic community of microorganisms largely found in lotic systems and typically consisting of a range of species of diatoms, green algae, and blue-green algae, plus a whole consortium of associated bacteria and fungi.[7] Experiments with complex primary producer assemblages, such a periphyton, are needed to increase the environmental realism of controlled

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experiments and increase our understanding of how Se behaves at the base of freshwater food webs.

Selenium contaminated freshwater systems are typically dominated by the dissolved inorganic Se oxyanions selenite (SeO_3, Se(IV)), selenate (SeO_4, Se(VI)), or a combination of the two.[8] The relative abundance of one oxyanion versus another is largely a function of source material (e.g., coal fly ash,[9] unconsolidated valley fill rubble from mountaintop removal mining sites,[10] seleniferous agricultural soils[11]), site geochemical factors (e.g., pH, dissolved oxygen, redox potential, ionic composition[8]), and microbial activity.[12,13] Geochemical factors and microbial activity are strongly influenced by the physical characteristics of lentic versus lotic systems. Lentic (still water) systems are characterized by low flow (high hydrologic retention), reduced dissolved oxygen, and increased potential for both biotic and abiotic reduction reactions, whereas lotic (flowing water) systems are characterized by higher flow (low hydrologic retention), increased dissolved oxygen, and less potential for biotic and abiotic reduction reactions.[1] There are a limited number of experiments conducted with primary producers indicating that, generally, the more reduced oxyanion, SeO_3, is more bioavailable than the more oxidized oxyanion, SeO_4[6,14−16] (but also see refs 17 and 18). The more rapid incorporation of SeO_3 at the base of aquatic food webs has also been described on the ecosystem scale.[19] There are, however, no controlled laboratory experiments utilizing complex periphyton biofilms that examine the differential uptake and biotransformation of dissolved SeO_3 versus SeO_4.

Previous work from our lab has indicated the sensitivity of the mayfly *Centroptilum triangulifer* to elevated, but environmentally relevant, exposures to dietary Se.[20,21] Those experiments were conducted with periphyton that had been exclusively exposed to dissolved SeO_3. It has been postulated that regardless of dissolved Se speciation, primary producers largely biotransform dissolved inorganic Se into organoselenides (mostly seleno-methionine) and most likely confer similar toxicity.[4] No studies are available, however, that test this hypothesis using complex periphyton or aquatic insects, which are heavily relied upon as ecological biomonitors.[22]

From an applied perspective, Se monitoring efforts have largely focused on measuring dissolved Se concentrations without attention to speciation.[23] The U. S. EPA is currently moving toward a fish tissue criterion for Se regulation,[24] yet there is still a need for better understanding of how different oxyanions behave in contaminated systems, particularly for risk assessment purposes. Recognizing the differences in the bioavailability, uptake kinetics, bioaccumulation potential, and subsequent toxicity of Se oxyanions will lead to more informed decision making on how to properly address Se contaminated systems.

Here, we compared the bioconcentration of SeO_3 and SeO_4 into complex freshwater periphyton communities under static and static-renewal conditions. The objective was to determine whether the initial oxidation state of inorganic Se affects the periphyton uptake rate, biotransformation into other Se species, and ultimately toxicity of Se to the mayfly *C. triangulifer* in full life cycle exposures. Because periphyton consortia are complex, but realistic, we analyzed changes in the periphyton associated microbial consortium using bacterial community fingerprinting.

MATERIALS AND METHODS

Selenium solutions. Selenite stock solution was prepared by dissolving 21.95 mg of Na_2SeO_3 (98% purity; MP Biomedicals, Santa Ana, California, U. S. A.) in 50 mL of deionized water (nominal concentration, 200 mg Se L^{-1}). Selenate stock solution was prepared by dissolving 24.39 mg of Na_2SeO_4 (99% purity; Acros Organics, Morris Plains, New Jersey, U. S. A.) in 50 mL of deionized water (nominal concentration, 203 mg Se L^{-1}). Stock solution concentrations were not analytically verified; however all experimental solutions were analyzed for both dissolved Se concentration and Se speciation.

Periphyton. Natural periphyton biofilms were grown on acrylic plates (6.5 × 23 × 0.15 cm) by allowing fresh streamwater from White Clay Creek, Pennsylvania, U. S. A. (39°51′47″ N, 75°47′07″ W) to flow continuously over the plates in a greenhouse as described previously.[25,26] Periphyton was grown in October 2011 and March 2012 for the static/static-renewal and mayfly exposure experiments, respectively. Colonization was complete when the periphyton reached a thickness of approximately 1 to 2 mm. Periphyton consisted primarily of diatoms with some blue-green and green algae, along with some naturally colonizing consumers (predominantly micro- and meiofauna; see Supporting Information in ref 25) and microbes. All experiments were conducted in 2.0 L glass bottles containing 1.8 L of American Society for Testing and Materials (ASTM) artificial soft water (ASW; 48 mg L^{-1} $NaHCO_3$, 30 mg L^{-1} $CaSO_4{\cdot}2H_2O$, 30 mg L^{-1} $MgSO_4$, and 2 mg L^{-1} KCl, pH 7.9 ± 0.3). Further, all experiments were conducted on the benchtop with natural lighting provided by large laboratory windows and ambient temperature (mean, 21.0 ± 1.1 °C; range, 19.2−24.2 °C).

Periphyton Se Uptake Kinetics. Periphyton SeO_3/SeO_4 bioconcentration kinetics were comparatively measured under static and static-renewal conditions for 8 days. These conditions were employed to observe the differential uptake of Se from solution in which the dissolved [Se] was decreasing over time (static) versus being relatively constant over time (static-renewal). Static exposures were conducted in duplicate using 10 μg Se L^{-1} (initial concentration) of either dissolved SeO_3 or SeO_4 in a single pulse with no Se refreshment. Water and periphyton were sampled at 0, 12, 24, 48, 96, and 192 h. Static-renewal exposures were conducted in duplicate using 10 μg L^{-1} of either dissolved SeO_3 or SeO_4 with plates moved to a new bottle containing fresh solution spiked at 10 μg L^{-1} every 24 h. Periphyton samples were collected at 0, 12, 24, 48, 96, and 192 h, whereas water samples were collected from each bottle prior to moving the plate into the new solution and at the end of each 24 h exposure stage. In both the static and static-renewal experiments, periphyton samples were collected in triplicate, and water samples were collected as a single grab sample and processed as described below. In addition, periphyton samples were collected from each of the static-renewal treatments at 0, 24, and 192 h, placed in microcentrifuge tubes and centrifuged for 15 min at 15 700*g* (4 °C) before removing the supernatant and freezing (−80 °C). Samples were then packed in dry ice and shipped to Stroud Water Research Center (SWRC; Avondale, Pennsylvania, U. S. A.) for bacterial community fingerprinting by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA[27,28] (see Supporting Information for DGGE analysis specifics).

Generating Differentially SeO_3/SeO_4 Treated Periphyton Diets. Toxicity of SeO_3 treated and SeO_4 treated periphyton to the mayfly *C. triangulifer* was assessed using full life cycle exposures. Periphyton was loaded with Se over the course of 8 days in static exposures to either dissolved SeO_3 or SeO_4 at an initial dissolved [Se] of 0 (control), 10, and 30 $\mu g\ L^{-1}$ (n = 4 per treatment). At the end of the 8 day loading period, periphyton plates were moved to new bottles containing fresh ASW and allowed to equilibrate for 24 h. Mayfly dietary Se exposure concentrations were based on the periphyton [Se] at the end of the loading phase/beginning of mayfly exposure.

Test animals. The mayfly *C. triangulifer* (Ephemeroptera: Baetidae; WCC-2 clone) was obtained from culture at SWRC. *C. triangulifer* has been advancing rapidly as a model organism for use in laboratory experiments. This parthenogenetic species has negligible flow requirements and typically inhabits depositional areas of lotic systems, making it particularly amenable to laboratory use. Thus far, *C. triangulifer* has been used in studies of temperature and development[29] and chlordane,[30] aluminum,[31] cadmium,[26] zinc,[32] selenium,[20,21] and total dissolved solids.[33]

Mayfly Life Cycle Exposure to Dietary Se. After the periphyton plates were loaded with Se, sampled, and equilibrated in clean water for 24 h, 15 *C. triangulifer* larvae (<48 h old) were added to each bottle. Each bottle was gently aerated and the light:dark cycle (13L:11D) was natural for the given season with ambient light provided by large laboratory windows and ambient laboratory temperature (mean, 21.0 ± 1.1 °C; range, 19.2−24.2 °C). Subimagos (i.e., subadults) emerged into mesh-lined collection lids during mid to late afternoon and were placed in individually labeled microcentrifuge tubes and frozen at −20 °C. At the completion of the experiment, all subimagos were dried and weighed on a Sartorius CP225D microbalance to the nearest 0.01 mg. Test end points included survival, secondary production (i.e., total insect dry mass), and time to emergence.

X-Ray Absorption near Edge Structure (XANES) Speciation. Following Se loading and before adding larvae, periphyton samples were collected for Se K-edge XANES analysis. Briefly, a random sample of periphyton was scraped from the plate, quickly blotted on a piece of Kimwipe, placed into an aluminum sample holder, wrapped with kapton film and kapton tape, placed into a microcentrifuge tube, and flash frozen with liquid nitrogen. Samples were kept in a −80 °C freezer for <48 h, then stored on dry ice during shipping and analysis on Beamline X11A at the National Synchrotron Light Source, Brookhaven National Laboratory in Upton, New York, U. S. A. XANES spectra for samples and for standards were all collected at 20 K. Data for the following standards containing 80 to 850 mmol Se kg^{-1} were collected in transmission mode and used for linear combination fitting analysis: sodium selenite, sodium selenate, selenite adsorbed on ferrihydrite (poorly crystalline ferric hydroxide), selenate adsorbed on ferrihydrite, elemental red selenium, elemental gray selenium, seleno-cystine, seleno-cysteine, methyl-seleno-cysteine, seleno-methionine, and trimethylselenonium. No aqueous Se standards were included because residual Se from the periphyton treatment solution was estimated to represent <0.03 mol % of total Se. Data for periphyton samples were collected in fluorescence mode, and self-absorption[34] was considered to be trivial because of the low Se concentration (36 μg Se g^{-1}) and high X-ray energy used (12.7 keV). The Supporting Information contains details on standards, data collection, and fitting analysis.

Selenium Measurement. All measurements of [Se] in water, periphyton, and *C. triangulifer* were performed by ICP-MS, and all measurements of dissolved [SeO_3] and [SeO_4] were conducted using LC-ICP-MS at the Environmental and Agricultural Testing Services Lab (North Carolina State University, Raleigh, North Carolina, U. S. A.). Method detection limits for [total Se], [SeO_3], and [SeO_4] were 0.33, 0.5, and 0.5 $\mu g\ L^{-1}$, respectively. All periphyton samples were collected by scraping a randomly selected ∼1 cm × 1 cm area of the acrylic plate (mean, 26.4 mg w.w./scraping) and placing the scraping onto a piece of dried, preweighed filter paper, then drying it for 48 h at 65 °C, weighing it, and digesting it in PTFE tubes using 2 mL of OmniTrace Ultra high purity HNO_3 (EMD Chemicals, Darmstadt, Germany) for 48 h at 80 °C. All water samples were collected in single 10 mL aliquots, 0.45 μm filtered, and frozen at −20 °C until analysis. Mayflies were collected as subimagos (i.e., subadults), placed in individually labeled microcentrifuge tubes, and frozen at −20 °C until all viable larvae had emerged. Mayflies were then dried at 65 °C for 48 h, weighed, and digested in PTFE tubes using 1 mL of ultrapure HNO_3 at 80 °C for 48 h. All Se concentrations in periphyton and mayflies are reported on a dry weight basis.

All quality control blanks (water and digest blanks (dried and digested filter paper)) were below method detection limits. Standard reference material (NIST 2976 mussel tissue (freeze-dried), 1.8 μg Se g^{-1}) and instrument check standards (2.0 $\mu g\ L^{-1}$) varied by ≤5% of the intended Se concentrations. Further, in all experiments, the initial dissolved [Se] varied by ≤10% from nominal concentrations, and there were no detections of SeO_3 in the SeO_4 exposures, or vise versa, at the beginning of each periphyton exposure.

Statistical Analysis. All data are expressed as mean ± standard deviation, and all data analyses were performed using GraphPad Prism (v5.02; α = 0.05). In the static and static-renewal periphyton uptake experiments, each treatment was represented by two replicate periphyton plates (n = 2) with each plate being sampled via triplicate periphyton scrapings at each time point. The experimental results for each of the two replicates (Rep. 1 and Rep. 2) were strikingly similar; however they are described independently due to the low sample size. Secondary production was calculated as the sum of individual subimago body masses (mg, d.w.) from a given replicate bottle (i.e., total insect biomass produced). Trophic transfer factors (TTFs) were determined by dividing tissue [Se] in *C. triangulifer* (subimagos) by [Se] in periphyton. Survival, secondary production, and development time (time to emergence) were compared to control performance using one way ANOVA with pairwise comparison of significant ANOVA results performed according to Williams.[35]

RESULTS

Periphyton Se Uptake under Static Conditions. To compare the differential uptake of dissolved SeO_3 versus SeO_4, (single pulse dose, 10 $\mu g\ L^{-1}$) into complex periphyton biofilms, we first measured total periphyton [Se] periodically over the course of 192 h under static exposure conditions. Selenite exposed periphyton bioconcentrated Se as a function of dissolved [SeO_3], where the uptake rate declined as the available pool of dissolved [SeO_3] decreased (Figure 1a). In contrast, SeO_4 exposed periphyton did not initially absorb Se as readily as the SeO_3 exposed periphyton, but over time

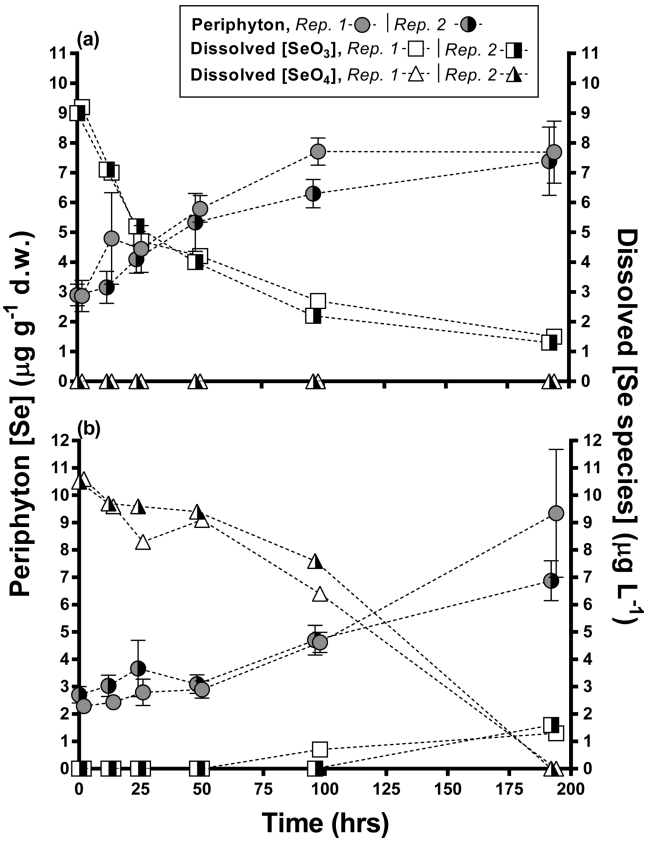

Figure 1. Change in dissolved [Se species] (right *y* axis) and periphyton [Se] (left *y* axis; background periphyton [Se] = 2.9 μg g^{-1}) in duplicate (Rep. 1 = replicate 1; Rep. 2 = replicate 2) static, single pulse dose exposures (10 μg L^{-1}) of either SeO_3 (panel a) or SeO_4 (panel b) over 192 h. Each periphyton data point represents the mean ± s.d. of triplicate periphyton subsamples, and each dissolved Se data point represents a single grab sample. Panel b displays the formation of SeO_3 in the SeO_4 treated replicates. Open symbols were offset 2 h to avoid visual overlap.

periphyton [Se] increased steadily in conjunction with the unexpected appearance of dissolved SeO_3 (Figure 1b).

Specifically, after 48 h of exposure, the two SeO_3 treated periphyton plates concentrated Se 2.0-fold and 1.8-fold (average uptake rate, 1.27 μg Se g^{-1} d^{-1}) above their respective background concentrations while dissolved [SeO_3] had decreased by 57% and 56% in the two replicates. Over the remaining 144 h, SeO_3 treated periphyton only increased in [Se] by an additional 0.33-fold (Rep. 1) and 0.39-fold (Rep. 2) as a result of the depletion of dissolved SeO_3. In contrast, after the initial 48 h of exposure, the two SeO_4 treated periphyton plates concentrated Se only 0.27-fold and 0.14-fold (average uptake rate, 0.26 μg Se g^{-1} d^{-1}; 4.9-fold less than SeO_3 uptake rate) above their respective background concentrations and dissolved [SeO_4] decreased by 12% and 9% in the two replicates. Interestingly, SeO_4 treated periphyton concentrated Se more rapidly over the final 144 h of exposure (3.1-fold (Rep. 1) and 1.5-fold (Rep. 2)), which occurred in conjunction with the appearance of dissolved SeO_3. SeO_3 was not observed during the initial phases of the experiment where periphyton bioconcentration was relatively slow but was measured at concentrations up to 1.3 μg L^{-1} (Rep. 1) and 1.6 μg L^{-1} (Rep. 2) in the later stages of the experiment when periphyton bioconcentration was more rapid. Even though the pattern and rates of uptake were different, by the end of the experiment there were no significant differences in [Se] between the SeO_3 exposed periphyton (7.7 ± 1.0 μg g^{-1}, Rep. 1; 7.4 ± 1.2 μg g^{-1}, Rep. 2) and the SeO_4 exposed periphyton (9.4 ± 2.3 μg g^{-1}, Rep. 1; 6.9 ± 0.7 μg g^{-1}, Rep. 2). These results indicate that the complex periphyton absorbed SeO_3 much more rapidly than SeO_4 and that SeO_4 reduction to SeO_3 enhanced Se uptake in the SeO_4 treatments.

Periphyton Se Uptake under Static-Renewal Conditions. On the basis of the results of the static exposures, we hypothesized that differences in Se uptake between SeO_3 and SeO_4 exposed periphyton would be accentuated under static-renewal conditions, in which these oxyanions were replenished every 24 h. Selenite exposed periphyton readily absorbed Se with a linear increase ($y = 0.0887x + 2.66$; $r^2 = 0.99$) over the full exposure period (Figure 2a). In contrast, static-renewal SeO_4 exposed periphyton conformed to an exponential model ($y = 2.10^{(0.00994x)}$, $r^2 = 0.97$; Figure 2b) with little change in periphyton [Se] until the later stages of the exposure (i.e., after 96 h). In general, initial uptake rates (up to 96 h) were 3.2-fold greater in SeO_3 exposed periphyton (1.98 μg Se g^{-1} d^{-1}) than SeO_4 exposed periphyton (0.62 μg Se g^{-1} d^{-1}) under static renewal conditions.

The initial uptake of SeO_3 was similar between the static and static-renewal exposed periphyton (Figure 2a). However, by 48 h the data began to diverge, and by the end of the exposure (192 h), [Se] in both of the SeO_3 static-renewal periphyton replicates (20.3 ± 5.1 and 19.5 ± 3.4 μg g^{-1}; Rep. 1 and 2, respectively) was significantly greater (ANOVA, $p = 0.001$; 2.6-fold greater, on average) than both of the static periphyton replicates (7.7 ± 1.0 and 7.4 ± 1.2 μg g^{-1}; Rep. 1 and 2, respectively), largely due to the discrepancy in dissolved [SeO_3] available for absorption. Differences in periphyton Se bioconcentration were less pronounced between the static (9.4 ± 2.3 and 6.9 ± 0.7 μg g^{-1}; Rep. 1 and 2, respectively) and static-renewal (15.8 ± 6.0 and 12.7 ± 2.6 μg g^{-1}; Rep. 1 and 2, respectively) SeO_4 exposures (Figure 2b). The increase in periphyton [Se] in the static-renewal system with SeO_4 was also influenced by the production of dissolved SeO_3 (similar to the static exposure) that became detectable after 96 h. Qualitative community fingerprinting via PCR-DGGE of the bacterial 16S rRNA gene indicated that a member of the family *Comamonadaceae* appeared and dominated in SeO_4 treated periphyton (see SI for tables and figures). Bacterial species within this family are known to reduce SeO_4.[36] This suggests a potentially important role for SeO_4-reducing bacteria in the incorporation of Se into periphyton and may explain the appearance of SeO_3 in SeO_4 exposures, which corresponded with increased Se uptake into periphyton.

Periphyton Loading and XANES Speciation Analysis. To compare the dietary bioavailability of SeO_3 and SeO_4 treated periphyton to mayflies, we exposed periphyton to low (10 μg L^{-1}) and high (30 μg L^{-1}) dissolved SeO_3 and SeO_4 for 8 days. Periphyton bioconcentration of Se was similar within a given exposure level between the two treatments. Periphyton [Se] in the low exposure levels was 12.8 ± 1.5 and 12.8 ± 1.7 μg g^{-1} in the SeO_3 and SeO_4 treated periphyton, respectively. Similarly, periphyton [Se] in the high exposure levels was 36.7 ± 7.5 and 36.8 ± 5.5 μg g^{-1} in the SeO_3 and SeO_4 treated periphyton, respectively.

We collected periphyton samples from both SeO_3 and SeO_4 treatments at the high exposure level at the end of the 8 day loading phase and analyzed them for Se speciation using Se K-

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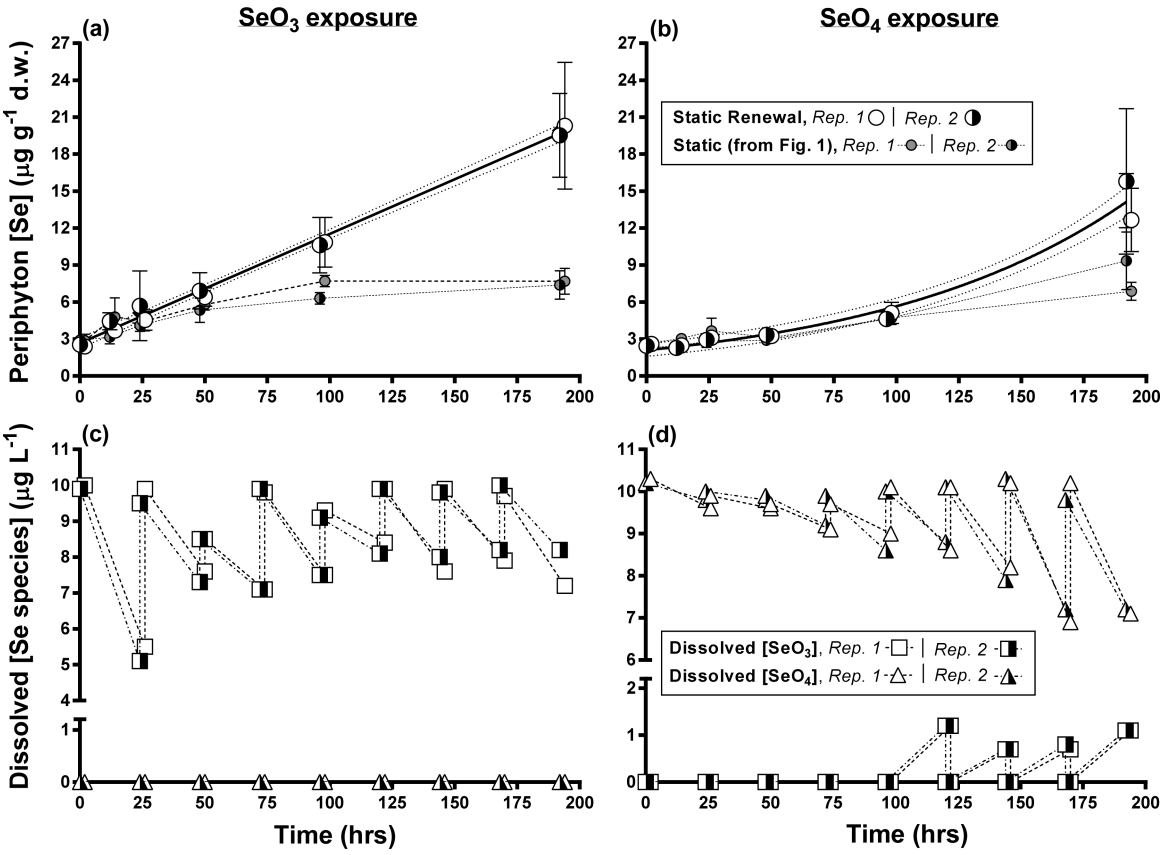

Figure 2. Change in periphyton [Se] (panels a and b) and [dissolved Se] (panels c and d) in static (single pulse dose 10 μg L^{-1}, borrowed from Figure 1) and duplicated (Rep. 1 = replicate 1; Rep. 2 = replicate 2) static-renewal (renewed every 24 h with 10 μg L^{-1}) exposures to either dissolved SeO_3 (panels a and c) or SeO_4 (panels b and d) over 192 h. Each periphyton data point represents the mean ± s.d. of triplicate periphyton subsamples, and each dissolved data point represents a single grab sample. Static-renewal periphyton [Se] exposed to SeO_3 increased linearly (r^2 = 0.99) while SeO_4 exposed periphyton increased exponentially (r^2 = 0.97). Open symbols were offset 2 h to avoid visual overlap.

edge XANES spectroscopy. XANES analyses revealed that both SeO_3 and SeO_4 treated periphyton biotransformed Se into a similar combination of species (Figure 3). The combination of our standards that yielded the best fit to the periphyton spectra included 58−64 mol % methyl-seleno-cysteine (Se(II−)), 14−20 mol % selenite adsorbed to ferrihydrite (Se(IV)), and 22−24 mol % elemental gray selenium (Se(0)). The presence of elemental Se along with selenite, selenides, diselenides, and trimethylselenonium was found previously in Se-exposed field collected biofilms.[37]

Mayfly Exposure to SeO_3/SeO_4 Treated Periphyton. The low and high periphyton diets described above were then fed to *C. triangulifer* larvae in full life cycle exposures. In addition to the low and high exposure levels, a control diet with a background periphyton [Se] of 2.2 ± 0.3 μg g^{-1} was utilized. In general, there were no differences in mayfly tissue Se bioaccumulation between the SeO_3 and SeO_4 treatments (Figure 4a). Overall, mayfly tissue [Se] increased with periphyton [Se] but showed signs of saturation at the high exposure levels. This trend was further reflected in the trophic transfer factors (TTF; Figure 4b), which appeared to decline with the increase in dietary Se exposure level. Overall, across all treatments and exposure levels, mayflies displayed a mean TTF of 2.1 ± 0.5.

Because periphyton [Se] and speciation profiles were not significantly different between SeO_3 and SeO_4 treated periphyton, we assessed mayfly survival, secondary production, and development time both individually (SeO_3 and SeO_4 treatments; Figure 5) and pooled across treatments (figure in SI). Mayfly survival was significantly reduced at the high exposure level in both SeO_3 (20 ± 12%) and SeO_4 (27 ± 23%)

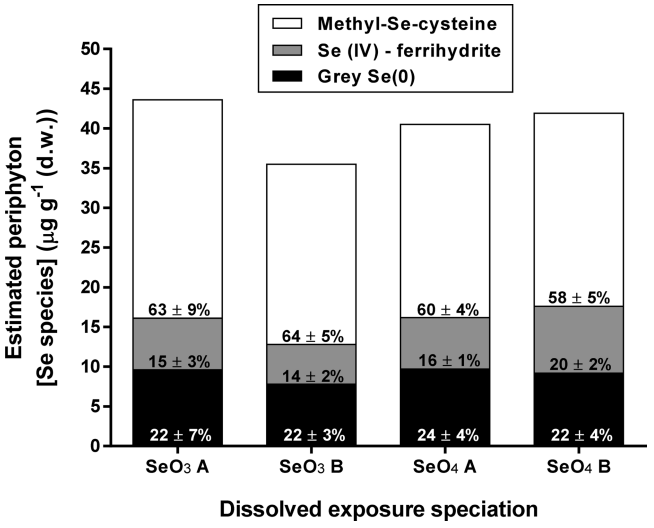

Figure 3. Results of linear-combination fitting of Se K-edge XANES spectra showing the best-fit combinations of standards to duplicate samples of SeO_3 (left bars) and SeO_4 (right bars) treated periphyton exposed for 8 days. Species segments (within bars) indicate the proportion (%) of each species out of the total [Se species], which is represented by total bar height.

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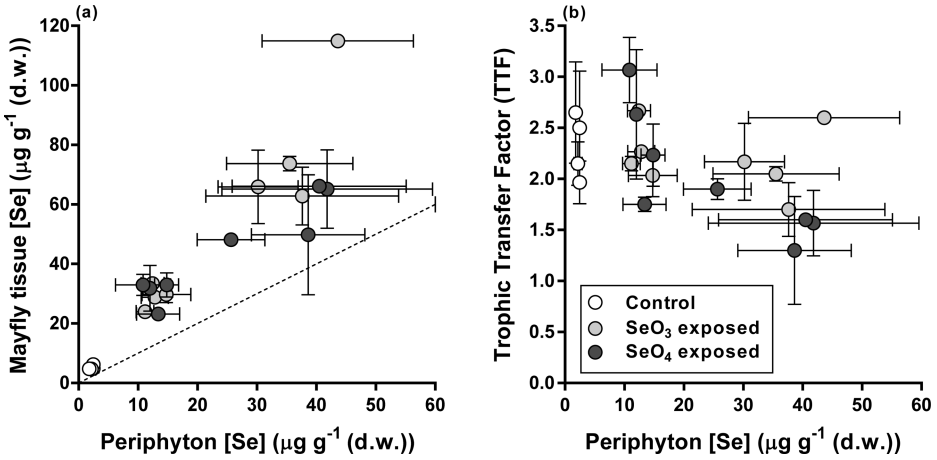

Figure 4. Mayfly tissue [Se] (panel a) and trophic transfer factors (TTF, panel b) as a function of dietary Se exposure. Data points represent mean ± s.d. for mayflies fed control diets (open circles), SeO_3 treated periphyton diets (light gray), and SeO_4 treated periphyton diets (dark gray). Panel a displays a 1:1 dashed line for visual reference.

treated periphyton as compared to control mayflies (73 ± 14%, $p < 0.01$; Figure 5a). Survival at the low exposure level was not significantly different from controls in the SeO_3 (54 ± 16%) or SeO_4 (70 ± 17%) treatments. Similarly, analysis of the pooled data indicated that mayflies exposed to the high Se treatment suffered significant reductions in survival (23 ± 17%, $p < 0.01$), while those in the low exposure did not (62 ± 18%).

Secondary production (total mayfly biomass produced) was significantly reduced at the high exposure level in both SeO_3 (2.1 ± 1.4 mg) and SeO_4 (2.6 ± 1.4 mg) treated periphyton when compared to control production (8.2 ± 1.4 mg, $p < 0.01$; Figure 5b). At the low exposure level, mayflies fed SeO_4 treated periphyton (5.0 ± 1.6 mg) were significantly different from controls ($p < 0.05$), while those fed SeO_3 treated periphyton (6.5 ± 3.0 mg) were not. However, when the data were pooled, secondary production was significantly lower than control in both the low (5.8 ± 2.4 mg, $p < 0.05$) and high exposure levels (2.3 ± 1.3 mg, $p < 0.01$).

Finally, control mayflies emerged 26 ± 2 days posthatch, which was significantly faster than those fed SeO_3 (35 ± 5 days, $p < 0.01$) and SeO_4 (36 ± 9 days, $p < 0.01$) treated periphyton at the high exposure level (Figure 5c). Emergence time was not significantly different from controls at the low exposure level in SeO_3 (26 ± 2 days) or SeO_4 (26 ± 2 days) treated periphyton. Similar to the individual analysis, pooled emergence was significantly delayed (35 ± 5 days, $p < 0.05$) at the high exposure level but not in the low exposure level (26 ± 2 days).

DISCUSSION

Here, we provide an example of a complex, natural freshwater periphyton assemblage which clearly absorbed SeO_3 much more rapidly than SeO_4. However, organisms within this natural periphyton assemblage were able to reduce SeO_4, apparently via a dissimilatory pathway (i.e., enzymatic reduction that is independent of Se assimilation[38]), generating dissolved SeO_3 and leading to enhanced primary producer bioconcentration. The end result was a similar distribution of Se species in the periphyton, regardless of initial oxidation state of inorganic Se in the exposure. This process appears to be affected by changes in the microbial consortia as a response to water chemistry (e.g., increased density of SeO_4 reducing bacteria as found here). This process in turn may be affected by residence time. To our knowledge, there are no relevant laboratory experiments with complex periphyton assemblages and selenium to compare these results, however a number of studies have reported the preference of SeO_3 uptake in monospecific phytoplankton cultures.[6,14−16] In contrast, Simmons and Wallschläger[17] recently reported rapid SeO_4 uptake in the freshwater algae *Chlorella vulgaris* (4−5-fold greater than SeO_3 or selenocyanate uptake). In general, it seems that the majority of available research, as well as our study, supports the notion that SeO_3 is more readily bioconcentrated by primary producers than SeO_4 (both of which are less readily bioconcentrated than organo-selenides).[6,8,15,16,39] It has been speculated that SeO_4 is bioconcentrated more slowly than SeO_3 because Se is an essential element, utilized in Se-containing enzymes in the fully reduced oxidation state (Se(-II)) making SeO_3 (Se(IV), the more reduced of the two oxyanions) thermodynamically favorable for metabolic processing and protein incorporation as compared to SeO_4 (Se(VI), the more oxidized of the two oxyanions).[16]

Experiments utilizing complex periphyton assemblages provide a considerable advantage in ecological realism given the inclusion of naturally occurring microbial populations. Microbes are environmentally ubiquitous, highly diverse, and critical to Se biogeochemical cycling because they mediate the oxidation/reduction reactions of SeO_3/SeO_4, which do not spontaneously occur in nature at physiological pH and temperature.[13] A decade of research in this area led Stolz and Oremland[13] to conclude that microbial reduction of Se oxyanions largely occurs via dissimilatory reduction. Microbes, therefore, are responsible for (among myriad other processes) utilizing SeO_4 as a terminal electron acceptor and producing reduced Se species into the surrounding media, such as SeO_3, elemental Se_0, and organo-selenides. For example, the periplasmic selenate reductase of *Thaurea selenatis* is known to specifically reduce SeO_4 to SeO_3 in a dissimilatory process.[40] Microbes are also speculated to reduce Se oxyanions as a toxic defense mechanism.[12] Further, it should be noted that the literature also contains evidence of dissimilatory SeO_4 reduction (and SeO_3 release) by algal cells.[16] We cannot rule out algal-mediated SeO_4 reduction in the current experiments; however we did find evidence that known SeO_4 reducing bacteria (*Comamonadaceae*) increased in density in response to SeO_4 exposure. Overall, the interplay of microbial (and possibly algal) mediated reduction of SeO_4 and subsequent absorption

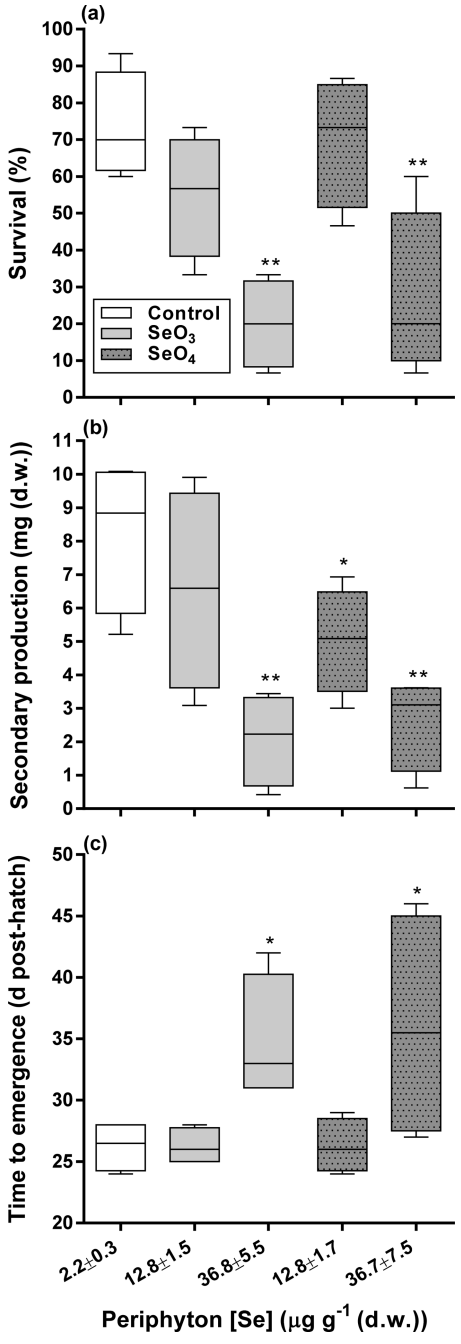

Figure 5. Performance of *C. triangulifer* mayflies in full life-cycle exposures to control (white boxes) and low and high SeO_3 treated (light gray boxes) and SeO_4 treated (dark gray dotted boxes) periphyton diets. Boxplots display 25th, 50th, and 75th quartiles with whiskers representing min−max ($n = 4$). Survival (panel a), secondary production (panel b), and development (panel c) were significantly impaired in both SeO_3 and SeO_4 treated periphyton exposures (**, $p < 0.01$; *, $p < 0.05$).

of SeO_3 by primary producers found in the current experiment would not have been realized in a more simplified experimental food web that did not contain naturally occurring microbes.

Comparing static and static-renewal exposures provided additional support for the importance of SeO_4 reduction in periphyton Se bioconcentration. It appears the similarity in periphyton bioconcentration between SeO_4 exposure conditions through 96 h could largely be explained by the lack of difference in dissolved SeO_4 concentrations. However, once the density of SeO_4-reducing bacteria was sufficient to begin decreasing SeO_4 concentrations (~96 h), static and static renewal treatments apparently began diverging (i.e., with the static-renewal periphyton [Se] increasing more rapidly). Whether this change was due to an increased density of SeO_4-reducing bacteria because of the higher average SeO_4 concentrations in the static-renewal exposures or the maintenance of higher SeO_4 concentrations available for microbial reduction (or both) remains unclear. Nonetheless, the production of measurable SeO_3 was commensurate with increased rates of Se bioconcentration by periphyton in both static and static-renewal SeO_4 exposures and is clearly an important process at the base of food webs in SeO_4 contaminated systems.

The experimental design used here (aerated bottle test) is likely more comparable to lentic systems that have high hydrologic retention and generally favor reducing conditions as opposed to lotic systems (better approximated by flow-through experimental designs). However, many lotic systems also contain depositional areas that share characteristics more similar to lentic sites than those found in flowing streams. Low-flow depositional areas of lotic systems are the preferred habitat for *C. triangulifer*.[30] Overall, natural systems are typically spatially heterogeneous, and different zones (inhabited by different flora and fauna) may be subject to greater or lesser risk of Se bioaccumulation and toxic effects. On the basis of the current results, however, we speculate that under true lotic conditions the risk of Se bioaccumulation would be much lower in streams that are dominated by SeO_4 than those dominated by SeO_3 and that in true lentic conditions the risk is more similar between the two species.

Only one relevant study of XANES Se speciation in freshwater biofilms exists for comparison of the present results.[37] Interestingly, the speciation of field collected biofilms from Se contaminated streams in Alberta, Canada (52% R−Se−R, 16.5% R_3−Se, 16.5% Se^0, 15% SeO_3[37]) was strikingly similar to the periphyton speciation found here (61% R−Se−R, 23% Se^0, 16% SeO_3). In both sample analyses, the periphyton/biofilm speciation contained ~60−70% organo-Se, supporting the hypothesis that primary producers tend to biotransform the majority of their pool of absorbed Se into organo-selenides.[4] There were some notable differences reported for the biofilm Se speciation from Andrahennadi et al.[37] and the speciation results found here. The R−Se−R standard was selenomethionine in the field study, and here it was methyl-selenocysteine. Here, we found no evidence of R_3−Se, but it was 17% of the field sample speciation. Our SeO_3 was modeled using SeO_3 adsorbed to ferrihydrite (SeO_3 is known to strongly sorb to ferrihydrite[41]) as opposed to nonadsorbed, dissolved SeO_3 in the field study.

Similar to the results of our previous work with *C. triangulifer* exposure to dietary Se, we observed significant adverse effects on all life cycle performance end points at the high dietary exposure level (36.7 $\mu g\ g^{-1}$) and significantly reduced secondary production at the low exposure level (12.8 $\mu g\ g^{-1}$).[20,21] In Conley et al.,[20] we reported reductions in body mass and fecundity in *C. triangulifer* exposed to 11 $\mu g\ g^{-1}$ dietary Se. In Conley et al.,[21] we reported similar effects on survival (significant reductions at dietary exposure to 11.9 $\mu g\ g^{-1}$) as well as body mass (11.9 $\mu g\ g^{-1}$) and fecundity (4.2 $\mu g\ g^{-1}$) when dietary resources were limited. The life cycle exposures here were conducted under similar resource

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limitation conditions as Conley et al.,[21] and the results were largely repeated. Overall, the combined results from these three experiments highlight the potential for adverse effects to occur in aquatic insects exposed to dietary Se and support the suggestion by deBruyn and Chapman[42] that some invertebrate species may suffer adverse effects of Se exposure at levels considered to be safe for higher trophic level consumers.

Interestingly, despite being clonal, we observed a few individual *C. triangulifer* subimagos that were resistant to elevated tissue Se concentrations (as high as 114 $\mu g\ g^{-1}$) that were successful at completing their aquatic larval stage and able to emerge with very high tissue Se concentrations. Such interindividual variation has been reported in other species, including dietary Se experiments with mallards where liver [Se] was indistinguishable between individuals that died as a result of exposure and those that survived.[43,44] It is generally believed that oxidative damage from disruption of the glutathione peroxidase/glutathione system is the primary mechanism of action for toxic effects from dietary Se exposure.[5] This suggests that some individuals are potentially able to cope with oxidative stress more readily than others, making them more fit for survival with elevated tissue [Se]; however the true mechanism of tolerance is unknown.

The Se uptake and biotransformation activities of microbes and primary producers are both highly complex and variable. The literature is replete with examples of both microbial and algal species that differ in their rates of SeO_3 and SeO_4 bioconcentration,[6,8,12−18] yet here we observed a clear dominance of SeO_3 uptake into periphyton (up to 4.9-fold greater uptake rate than SeO_4). Further, there is evidence for a broad range of biotransformation processes that produce a variety of Se metabolites (i.e., SeO_3 (from SeO_4);[17,45] elemental Se_0 (from SeO_3[46] and SeO_4); as well as dissolved, volatile, and protein bound organo-selenides; and other small molecular weight Se compounds[17,47]) through both dissimilatory and assimilatory metabolic pathways. Our results support the hypothesis of Fan et al.[4] that regardless of dissolved speciation, once absorbed by primary producers, Se is largely converted to organo-selenide and produces similar effects in higher trophic levels. Experiments with complex periphyton assemblages and ecologically relevant primary consumers improve our understanding of basal food web Se activity and provide a more environmentally realistic representation of the behavior of Se in aquatic systems.

■ ASSOCIATED CONTENT

Supporting Information

Additional information concerning XANES speciation analyses, specifically source and synthesis of Se standards and linear combination fitting methodology, as well as details on bacterial community fingerprinting via DGGE of PCR-amplified 16S rRNA including sample preparation methodology and taxonomic identification of selected DGGE bands. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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