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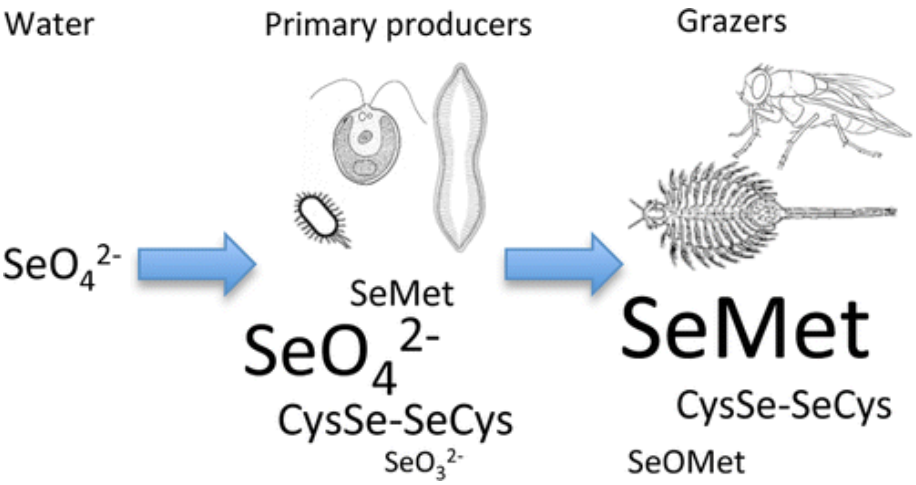
Abstract



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An engineered aquatic ecosystem was specifically designed to bioremediate selenium (Se), occurring as oxidized inorganic selenate from hypersalinized agricultural drainage water while producing brine shrimp enriched in organic Se and omega-3 and omega-6 fatty acids for use in value added nutraceutical food supplements. Selenate was successfully bioremediated by microalgal metabolism into organic Se (seleno-amino acids) and partially removed via gaseous volatile Se formation. Furthermore, filter-feeding brine shrimp that accumulated this organic Se were removed by net harvest. Thriving in this engineered pond system, brine shrimp (*Artemia franciscana* Kellogg) and brine fly (*Ephydridae* sp.) have major ecological relevance as important food sources for large populations of waterfowl, breeding, and migratory shore birds. This aquatic ecosystem was an ideal model for study because it mimics trophic interactions in a Se polluted wetland. Inorganic selenate in drainage water was metabolized differently in microalgae, bacteria, and diatoms where it was accumulated and reduced into various inorganic forms (selenite, selenide, or elemental Se) or partially incorporated into organic Se mainly as selenomethionine. Brine shrimp and brine fly larva then bioaccumulated Se from ingesting aquatic microorganisms and further metabolized Se predominately into organic Se forms. Importantly, adult brine flies, which hatched from aquatic larva, bioaccumulated the highest Se concentrations of all organisms tested.

Introduction

Selenium is a naturally occurring trace element mainly found in soils derived from marine sediments, including Cretaceous shale deposits in western North America.[1,2] Selenium biochemical properties lead to a narrow boundary between deficiency and toxicity,[3] a phenomenon further complicated by a large variety of Se chemical forms with differing levels of biological activity. Human Se deficiency causes multiple diseases, and it is estimated that more than a billion people worldwide ingest insufficient amounts of Se in their diet (less than 10 μg Se day^{-1}).[4,5] In central California, marine, pyritic sedimentary rocks in the Coast Ranges are a major source of soluble selenate, sodium chloride, and magnesium and calcium sulfate salts that can cause hyper-salinization of the Western San Joaquin valley (WSJV) soils.[6] Water-soluble inorganic selenate (SeO_4^{2-}) is often leached from naturally enriched soils by irrigation and winter rains into surface runoff or effluent waters, accumulated by catch basins or reservoirs and in nearby wetland marshes where Se is evapo-concentrated during the summer.[6] Phytomanagement is a strategy for mitigating soluble selenate in soil from entering waters of the WSJV that utilizes *Brassica* plants (mustard or canola) to accumulate soluble Se from soils.[7-9] Seeds from these plants are in turn used to produce

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biodiesel fuel and Se-enriched seed meals are used to supplement livestock animal feed.[7-9] The increased accumulation of naturally occurring salts, boron (B), and SeO_4^{2-} has worsened in some agricultural areas due to limited freshwater supplies, low winter rainfall, and drought conditions, which reduce the leaching of salts away from the root zone. In Se polluted aquatic environments, such as Kesterson Wildlife Reservoir in WSJV, Se is present primarily as water-soluble selenate (SeO_4^{2-}) ≥ 98% and ~2% selenite (SeO_3^{2-}).[2] Due to its relative abundance in these environments, ecotoxicity concerns center around SeO_4^{2-} and its biomagnification up the aquatic food chain.[10,11] Selenium evapoconcentration in aquatic ecosystems has been documented to cause deformity and mortality in fish, waterfowl, shore birds, and mammals.[12-15]

The food chain transfer and bioaccumulation of organic Se represents another exposure route in addition to the direct uptake of SeO_4^{2-}.[11,12] Possible biochemical transformations, variability in food chain accumulation, and a strong dependence on the physical site characteristics make it difficult to predict Se pollution risks based on waterborne, inorganic Se concentrations alone.[11,12] In addition to accumulating Se in biomass, aquatic primary producers are main drivers for Se volatilization in aquatic ecosystems via production of methylated selenides, including dimethylselenide (DMSe) and dimethyldiselenide (DMDSe). These methylated selenides exit the water column into the atmosphere or can be oxidized back to SeO_3^{2-}.[16]

The engineered aquatic ecosystem and the drainage waters at Red Rock Ranch (RRR) mimic the conditions present in brackish saline marshes, which are often impacted by selenium, when near low lying agricultural areas adjacent shale derived Se enriched soils. Cyanobacteria, diatoms, and microalgae are the primary producers in this open-air pond system and are the main food source for both brine shrimp and larvae of the brine fly. These same brine shrimp and brine fly are also the two major groups of invertebrates that flourish in highly saline environments and thus play a major role in associated aquatic food webs.[17,18] *Artemia franciscana* Kellogg (brine shrimp) are Brachiopods. *Ephydra cinerea* (brine fly) larvae and adults occupy diverse habitats such as salt pools, alkaline lakes, and marshes and both typically graze mostly on the algal and bacterial communities.

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The RRR pond is completely enclosed by netting to prevent incidental exposure of birds.

As part of a larger integrated on-farm drainage management system (IFDM) used to manage excess saline irrigation water, we studied the bioremediation of SeO_4^{2-} laden wastewater in the RRR pond by analyzing the internal Se concentrations of the various food web organisms present in the engineered aquatic ecosystem pond designed to produce brine shrimp enriched in organic Se and omega-3 and omega-6 fatty acids for use in value added nutraceutical food supplements (Supporting Information Figures 1 and 2).

Furthermore, the biological fate of Se was determined with high resolution in mixed bacteria, diatoms, *Picocystis* sp. (microalgae), and *Cladophora* (macro-algae), and the macroinvertebrates brine fly and brine shrimp. We achieved this by directly collecting specimens (brine shrimp and brine fly) or isolating individual microscopic strains and microorganism groups using a variety of controlled growth and culture systems. These sample isolation methods were then combined with the use of advanced analytical techniques including strong anion exchange, high performance liquid chromatography (HPLC), inductively coupled plasma mass spectrometry (SAX-HPLC-ICPMS), X-ray absorption near edge structure (bulk XANES), micro-focused X-ray fluorescence (μXRF), and micro-X-ray absorption near edge structure (μXANES) (Supporting Information Table 1). The total Se accumulation, chemical Se speciation, and Se atom mapping results yielded high molecular resolution into the accumulation and biotransformation of inorganic Se into organic Se in addition to the localization of Se inside aquatic organisms living in the engineered aquatic remediation system.

Experimental Section

System Design, Location, and Water Chemistry Parameters
At RRR near Five Points, California, the IFDM water has been used in several irrigation cycles by irrigating progressively more salt tolerant crops (Supporting Information Figure 2). The reduced volume of drainage water was collected in the final sump system and the resulting drainage water used in the racetrack pond (Supporting

Information Figure 1). The agricultural drainage water was very high in dissolved salts and metals: Se (6–12 mg L^{-1}), SO_4^{2-} (approximately 65 g L^{-1}), Na^+ (17 g L^{-1}), Mg^{2+} (1 g L^{-1}), Ca^{2+} (0.5 g L^{-1}), K^+ (51 mg L^{-1}), and B (142 mg L^{-1}). The water also typically contained approximately 300 mg NO_3^{2-} L^{-1}, but was low in NH_4^+ (1–3 mg L^{-1}), Fe (0.5 mg L^{-1}), P (0.8 mg L^{-1}), Si (0.9 mg L^{-1}), and V (0.01 mg L^{-1}). Other dissolved metals were below their respective detection limits: Zn, Mn, Cd, Cr, Ni, and Al (<0.05 mg L^{-1}); Cu and Pb (<0.1 mg L^{-1}). Sequential application of the drainage water reused 90% of the wastewater produced at RRR, and there was no disposal into rivers or evaporation basins.

Sample Growth and Preparation

Microorganisms. Bacteria and Bacteria Plus Diatom Growth

Mixed bacterial culture medium consisted of 0.2 μm filter (Millipore) sterilized RRR drainage water at approximately 80 g L^{-1} salinity equivalent amended with Na_2SeO_4 to 40 mg Se L^{-1}, 1% v/v glycerol (Fischer), 2% w/v tryptone (Difco), 20 mg L^{-1} Fe-gluconate (Sigma-Aldrich), 6 mg L^{-1} NaH_2PO_4 (Sigma-Aldrich). Initial tests indicated cultures of bacteria, diatoms, and microalgae at ambient Se concentrations did not produce sufficiently high internal Se concentrations for chemical speciation via X-ray absorption near edge spectroscopy (XANES). For bacteria-only culture, 10 mL RRR drainage water filtered through 5 μm sterile syringe filter (Millipore) was added to 90 mL of media. Cultures were incubated overnight at 25 °C in the dark on an Orbit shaker-incubator (Lab-line) at 100 rpm.

Microalgae Growth

For culturing *Picocystis* sp., the medium consisted of RRR agricultural drainage water at approximately 80 g L^{-1} salinity equivalent amended with Na_2SeO_4 to 40 mg Se L^{-1}. 500 mL culture flasks containing 100 mL culture media were incubated for two weeks under 30W compact fluorescent lights on a MaxQ2000 orbital shaker (Thermo Scientific) at 90 rpm.

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Diatom Growth

A mixed diatom culture was isolated by a modification of the protocols of Bruckner and Kroth.[19] Diatom medium consisted of RRR agricultural drainage water at approximately 80 g L^{-1} salinity equivalent amended with Na_2SeO_4 to 40 mg Se L^{-1}. Chloramphenicol 27 μg mL^{-1}, streptomycin at 135 μg mL^{-1}, and ampicillin at 270 μg mL^{-1} were used as selective agents. The diatom inoculum was prepared by filtering 100 mL RRR water through a 2 μm filter paper. The filter paper was washed with 100 mL 80 g sulfate L^{-1} buffer. The washed filter was placed in 10 mL diatom medium and gently vortexed for 30 s; 5 mL inoculum was used per 100 mL solution. Flasks were incubated for two weeks under 30 W compact fluorescent lights on a MaxQ2000 orbital shaker (Thermo Scientific) at 90 rpm.

Microbial Sample Harvest

Samples (bacteria, microalgae, and diatoms) were harvested by centrifugation at 5856 g for 20 min at 23 °C. Cells were washed three times in 5 mL 80 g L^{-1} sulfate buffer. Pellets in 2 mL microcentrifuge tubes were flash frozen in liquid nitrogen and stored at −80 °C until analysis.

Sample Harvest and Preparation from RRR Racetrack Pond Macroalgae

Cladophora filamentous algae were collected in 50 mL Falcon tubes, washed with sterile 80 mg mL^{-1} $Na_2SO_4^{2-}$ in Büchner filter funnels lined with Whatman No 1. filter papers and flash frozen in liquid nitrogen.

Macroinvertebrates

Brine shrimp and brine fly larvae and pupa were collected in 1 L plastic water sampling bottles. Adult flies were collected by trapping in empty 50 mL Falcon tubes. All macroinvertebrate samples (except for adult fly) were washed with sterile 80 mg mL^{-1} $Na_2SO_4^{2-}$ in Büchner filter funnels lined with Whatman No. 1 filter papers and flash frozen in

liquid nitrogen. Brine fly adults were directly flash frozen in liquid nitrogen. All samples were stored at −80 °C until analysis.

Selenium Analysis

Brief outlines of the various Se analysis methods employed are provided below. Detailed experimental methodology is provided in the Supporting Information.

Quantification of Total Se

Aquatic organisms were removed from the freezer and were digested with HNO_3, H_2O_2, and HCl.[20] Mineral inorganic elements, including Se, were analyzed by an inductively coupled plasma mass spectrometer (Agilent 7500cx, Santa Clara, CA, U.S.A.). The National Institute of Standards and Technology (NIST) wheat flour (SRM 1567; Se content of 1.1 ± 0.2 μg g^{-1} DM) and two internal soil standards (sediment collected from Kesterson Reservoir, CA, with a total Se content of 7.5 ± 0.25 and 25 ± 0.87 mg kg^{-1}) were used as the Se quality control standards. Recovery rates of Se in standard materials after acid digestion were over 94%.

Chemical Speciation of Soluble Se
SAX-HPLC-ICPMS

Aqueous Se compounds were separated from insoluble Se compounds via methanol/chloroform extraction.[21] One vial of each duplicate sample set was treated with Protease XIV as a first step in the extraction. One quarter of each extracted aqueous phase and the entire nonaquaeous chloroform phase were evaporated with a heating block at 50 °C (~300 min), acid digested, and analyzed for total Se by ICPMS. Waters Sep-Pak Classic C18 cartridges were used for final cleanup of the remaining aqueous concentrates. Purified eluent was transferred into Agilent screw-top glass HPLC vials and frozen until SAX-HPLC-ICPMS analysis. (For nonprotease digested sample results see Supporting Information Table 4).

An Agilent 1200 HPLC separations module equipped with a Hamilton PRPX-100 strong anion exchange analytical column (10 mm

particle size; 25 cm length × 4.1 mm internal diameter) and an Agilent 7500 ICPMS was used for Se speciation of aqueous extracts.[22] A single analysis (30 μL injection) was conducted for each of the aqueous extracts. Chromatographic separation of all Se compounds was achieved with an isocratic mobile phase of 5 mM ammonium citrate buffer (pH 5.2) with 2% methanol at a flow rate of 1 mL min^{-1} and ^{78}Se was monitored in real time using ICPMS. Retention times of Se containing peaks were compared to authentic standards (Supporting Information).

Se Distribution and Chemical Speciation μXRF/μXANES

The distribution of Se, Ca, and Zn were determined using micro-focused X-ray fluorescence (μXRF) mapping and the local speciation was determined using micro-Se K-edge X-ray absorption near-edge structure (μXANES) spectroscopy. Washed samples were flash frozen in liquid nitrogen and taken to beamline 10.3.2 of the Advanced Light Source at the Lawrence Berkeley National Laboratory for analyses.[23] Micro-XRF elemental maps were recorded at 13 keV, using a 15 × 6 μm (vertical) beam, a 15 × 15 μm pixel size, and 50-ms dwell time per pixel, except for the brine fly pupa, which was imaged using a 25 × 25 μm pixel size. Se K-edge μXANES spectra were recorded with a seven-element Ge solid-state detector (Canberra). Spectra were deadtime corrected, pre-edge background subtracted, and postedge normalized using standard procedures.[23,24] Red Se (white line maximum set at 12660 eV) was used to calibrate the spectra. Least squares linear combination fitting (LCF) of the Se XANES spectra was performed in the 12630 to 12850 eV range using a library of nine standard selenocompounds (Supporting Information), as previously described.[25] The error on the percentages of species present is estimated to be ±10%. Data processing was performed with LabVIEW.

Bulk XANES

Samples were removed from storage at −80 °C, macerated in liquid nitrogen using a mortar and pestle, packed into 2-mm path length sample cells, and maintained in liquid nitrogen for bulk Se K-edge XANES analysis. Spectra were collected on beamline 9–3 at the

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Stanford Synchrotron Radiation Light source (SSRL). Experimental spectra were calibrated with respect to the spectrum of hexagonal Se. Background subtraction, normalization calibration, and further data analyses were carried out according to standard procedures using the EXAFSPAK program suite. Experimental spectra were analyzed by least-squares linear combination fitting.[26]

Total Se Analysis by Spectrofluorimetry

Total Se in water, tissue, and volatile matrices was analyzed using micronitric acid digestion coupled with fluorescence detection.[27] Fluorescence intensity was measured using a spectrofluorimeter equipped Genios microplate reader (Phenix Research Products). A standard curve consisted of 9 SeO_2 (Fisher Scientific) solution standards.

Results and Discussion

Selenium Water Chemistry

Selenium concentrations in the racetrack pond water (Supporting Information Figure 1) ranged from 6 to 12 mg Se L^{-1}, and the form of Se (determined via SAX-HPLC-ICPMS), in RRR racetrack pond was 97 ± 2% selenate (SeO_4^{2-}) and 3 ± 2% selenite (SeO_3^{2-}). In both freshwater phytoplanktonic and marine algae communities, SeO_4^{2-} is considered to be more ecologically toxic than SeO_3^{2-}.[28,29] In most WSJV soils, Se is predominately present as SeO_4^{2-}, and less abundant SeO_3^{2-}, due to irrigation water leaching out local SeO_4^{2-}-enriched alluvial fan derived soils[2,30] (see Supporting Information Figure 1). The RRR agricultural drainage waters also have high sulfate concentrations of up to 100 g L^{-1} and nitrate concentrations of up to 300 mg L^{-1} . Methylation of Se is one of the primary routes of Se removal from aqueous systems through Se-volatilization,[7,16,31] however, high nitrate concentrations may decrease Se methylation.[32]

Mixture of Bacteria and Diatoms

We initiated our analyses on a mixture of two key Se-utilizing groups of primary producers, the bacteria and diatoms. The average

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total Se for the mixed bacterial diatom sample harvested and rinsed after four days growth in RRR drainage water was 1 μg Se g^{-1} wet weight (ww). Bulk XANES least-squares analysis demonstrated that forms of Se were 26% SeO_4^{2-}, 11% SeO_3^{2-}, 9% elemental (Se^0), and 54% organic Se as C–Se–C forms (Supporting Information Figure 3, Table 2). These Bulk XANES results demonstrated that SeO_4^{2-} in water was actively being reduced by microbes into SeO_3^{2-} and then incorporated into organic C–Se–C forms (e.g., selenocystathionine (SeCyst) and selenomethionine (SeMet), while a portion of SeO_4^{2-} was reduced to elemental Se^0.

Mixed bacteria and diatoms were then protease-XIV digested and extracted using methanol chloroform water (MCW). In the upper aqueous phase (60% methanol + 40% water), 72% of the total Se was recovered due in part to an initial protease-XIV digestion, which led to an 1.7 fold greater recovery of total Se than in nonprotease digested samples. Aqueous phase soluble Se species in mixed bacteria and diatoms (identified by SAX-HPLC-ICPMS) were 78% SeO_4^{2-}, 3% SeO_3^{2-}, and 18% organic Se (C–Se–C; 14% SeCyst and 4% SeMet) (Supporting Information Figure 4, Table 3).

Micro-XRF elemental mapping of Se present in a thin smear of mixed bacteria and diatoms demonstrated numerous hotspots, highly concentrated with Se (Supporting Information Figure 5). Further examination of Se species in hotspots by μXANES revealed they were entirely elemental Se^0, likely in precipitates or granules (Supporting Information Table 5). This is likely due to the reduction of SeO_4^{2-} by sulfate reducing bacteria into SeO_3^{2-} and Se^{2-} intermediates before a final reduction step into elemental Se^0.

Bacteria

Isolation of a bacteria only mixture from the RRR pond allowed the growth and recovery of bacteria from an overnight culture that contained 2.5 μg Se g^{-1} ww. In the soluble aqueous phase of the protease-XIV digested sample 84% of total Se was recovered and a 1.2 fold increase in Se recovery resulted from protease treatment. The aqueous Se forms (identified by SAX-HPLC-ICPMS) inside the washed bacterial samples were 75% SeO_4^{2-}, 3% SeO_3^{2-}, 2% SeCys, 16% free

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C–Se–C forms (SeCyst and SeMet), and 3% SeMet containing proteins (Supporting Information Table 3).

Similar to sulfur (S), bacteria are capable of four possible transformations of Se that include both oxidation and dissimilatory reduction, the reduction and assimilation of Se into amino acids, methylation, and demethylation.[32] Bacteria have also been shown to possess both high and low affinity Se transport systems.[33,34] While RRR pond drainage water contained essentially 95% SeO_4^{2-} and 5% SeO_3^{2-}, these forms made up only 78% of the internal Se concentration in bacteria and the other 21% were organic Se (Supporting Information Table 3). The internal concentration of 2.5 μg Se g^{-1} ww was low compared to the external environment (40 mg Se L^{-1}). The lower Se concentrations in these cells may reflect their initial stage of Se uptake or their ability to efflux Se.

Diatoms

Axenic, mixed diatom cultures grew well in the sterilized RRR water, even at the highest Se concentration of 40 mg Se L^{-1}. The average total Se for the diatom sample grown at 40 mg Se L^{-1} was 59.8 ± 1.9 μg Se g^{-1} ww. The percentage of Se recovery in the aqueous phase of the MCW was 18% for the protease-XIV digested sample and protease-XIV treatment increased soluble Se recovery 1.9 fold. In contrast to bacteria, the aqueous Se forms of the mixed diatom sample grown for two weeks contained only 3% SeO_4^{2-} with 24% SeO_3^{2-}, in addition to 6% selenocysteine (SeCys), and 56% organic C–Se–C forms (45% SeCyst and 11% SeMet) as identified by SAX-HPLC-ICPMS (Supporting Information Table 3, Figure 4). SeO_3^{2-} was the highest percentage of inorganic Se measured in diatom samples and was 8 times higher than SeO_4^{2-} concentrations in diatoms. Similarly, a high 6:1 SeO_3^{2-} to SeO_4^{2-} ratio was observed using μXANES analysis, although no organic Se forms were observed (Supporting Information Table 5). Selenium is an essential element for freshwater and marine diatoms,[30,35,36] and μXRF mapping of mixed diatoms isolated from the drainage water showed a relatively uniform Se distribution (data not shown). Unlike higher plants, diatoms utilize selenoenzymes and therefore require a high affinity Se uptake mechanism and specific selenocysteine tRNAs.[37] Mixed diatoms

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isolated from the drainage water were more efficient than the mixed bacteria at reducing selenate into organic forms, whereas the bacteria were better at reducing selenate into elemental Se^0.

Green Microalga

The unicellular green microalga *Picocystis* sp. was originally isolated from RRR water. In hypersaline Mono Lake, CA, which has similar salinity (approximately 81 mg L^{-1}) to RRR pond water and also a significant SO_4^{2-} component (approximately 13 g L^{-1}), a *Picocystis* sp. accounts for 25% to 50% of the primary production and is heavily grazed by endemic brine shrimp.[38] At RRR, the average total Se for the *Picocystis* sp. sample was 1.2 μg Se g^{-1} ww. Bulk XANES analysis of the RRR *Picocystis* sp. demonstrated that after two weeks of growth no Se remained as SeO_4^{2-}, and only 5% was in the inorganic form SeO_3^{2-}, while greater than 95% of the Se was in organic Se forms 71% C–Se–C and 24% CysSe-SeCys (Supporting Information Figure 3 and Table 2). Protease XIV treatment increased soluble Se recovery by 4.1 fold, suggesting a high proportion of selenoamino-acids in proteins; the total soluble Se recovery was 21%. While SAX-HPLC-ICPMS analysis suggested high proportion of inorganic Se forms (36% SeO_4^{2-}, 11% SeO_3^{2-}, 21% CysSe-SeCys, and 32% SeMet (Supporting Information Table 3)), the bulk XANES analysis provided evidence the majority of the unrecoverable Se was in organic forms while essentially all inorganic Se had likely been recovered.

The *Picocystis* sp. analyzed by μXRF mapping had Se distributed homogeneously in a droplet of cells and lacked the hot spots observed in mixed bacteria and diatoms (Supporting Information Figure 5), while the Se concentrations were not high enough to provide good Se signal-to-noise ratios for μXANES analyses.

The low internal inorganic Se concentrations in *Picocystis* sp. was consistent with other unicellular green algae such as *Chlorella,* where >85% Se was present as organic Se.[41] The dominant organic Se form in *Picocystis* sp. in this study was SeMet, followed by CysSe-SeCys (Supporting Information Table 3). Selenocystathionine is a compound often found in secondary Se accumulator plants; thus, it may play a similar role in the Se resistance of the RRR *Picocystis* sp. In

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addition, internal *Picocystis* sp. Se concentrations were approximately 20-fold lower than in the water after two weeks growth, suggesting they have an efficient Se-exclusion or Se-efflux mechanism.

Filamentous Macroalgae (Cladophora)

Bulk XANES spectra of the RRR race track collected *Cladophora* demonstrated Se mainly in the organic forms 39% CysSe-SeCys, 34% SeMet, and 27% methionine selenoxide (SeOMet) (Supporting Information Figure 3 and Table 2). Importantly, SeOMet may be an intermediate molecule in a biochemical pathway protective against cellular damage caused by reactive oxygen species[39,40] and may play a role in scavenging reactive oxygen species in *Cladophora* or other aquatic organisms. This reaction is shown in Supporting Information Figure 6 with hydroperoxide used as an example. The oxidized selenomethionine (SeOMet) can be reduced by other electron donors, such as diglutathione (GSH-GSH → GS-SG) or thioredoxin reductase with NADPH.[39,40] All bulk XANES samples were prepared in anaerobic conditions (ground and stored in liquid nitrogen) and measured at −263 °C and possible radiation damage during measurement was monitored; thus, the potential oxidation of SeMet by exposure to air or radiation damage during measurement can be disregarded.

Brine Shrimp

Artemia franciscana are filter microalgae, diatoms, and bacteria in the water column. The total Se in the RRR brine shrimp sample was 7.2 μg Se g^{-1} ww. Bulk XANES analysis of brine shrimp collected from the RRR pond demonstrated that only 3% Se remained in an inorganic form as SeO_3^{2-}, while the majority of Se was found as the organic Se forms 28% CysSe-SeCys, 59% C–Se–C forms (most likely SeMet), and intriguingly 10% SeOMet, similar to the *Cladophora* (Supporting Information Figure 3 and Table 2). This result further suggests SeMet may either be easily oxidized in aquatic systems or could be playing a role in the scavenging of reactive oxygen species inside aquatic organisms. The percentage of Se recovery into the aqueous phase for the protease-XIV digested MCW extracted brine shrimp sample was 63.8% of total Se and protease-XIV treatment increased soluble Se recovery by a notable 3.9 fold.

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Micro-XRF mapping revealed that, in adult brine shrimp, the muscle along the back, both compound eyes and cysts (eggs) had accumulated the highest Se concentrations (Figure 1). The forms of Se in adult brine shrimp were different from the Se forms in phytoplankton (bacteria, diatoms, and microalgae) that constitute most of their diet. In particular, brine shrimp had very little inorganic SeO_4^{2-} or SeO_3^{2-}. SAX-HPLC-ICPMS showed that brine shrimp mainly contained organic Se forms occurring as 15% CysSe-SeCys, 81% SeMet, and 2% SeOMet (Supporting Information Figure 4 and Table 3).

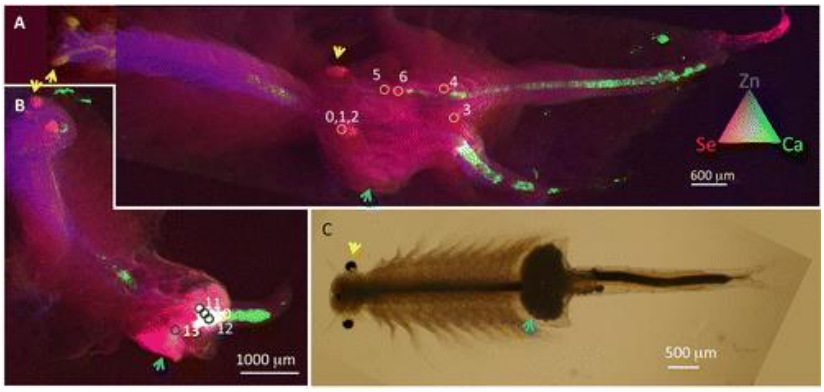

Figure 1. Distribution and speciation of Se in *Artemia franciscana*. µXRF map showing spatial distribution of Se (coded in red) and other selected elements (calcium in green, zinc in blue) in adult female with eggsac and adult male, coupled (A), in single adult female with eggsac (B). Adult female with eggsac shown for comparison (C). Scale bars = 600 µm (A), 1 mm (B), 500 µm (C). Yellow circles in A and B show locations of µXANES spectra whose fitting results are reported in Supporting Information Table 5.

Micro-XANES results (Supporting Information Table 5) also supported the bulk XANES and SAX-HPLC-ICPMS data in that at the specific localized Se hot spots, forms were all C–Se–C. These findings suggest that adult brine shrimp efficiently convert inorganic Se and or accumulate mainly organic Se. The observation of SeMet concentration in brine shrimp eggs (cysts) is of importance to understanding Se in the food web considering these cysts are a major food source for small fish and aquatic insects.

In order to better understand the accumulation of Se in brine shrimp in response to changing water Se concentrations, we analyzed total Se in brine shrimp harvested after controlled laboratory growth under various Se concentrations. Freeze-dried brine shrimp samples

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analyzed by spectrofluorimetry showed proportional increases in total internal Se concentrations in direct correlation with water Se concentrations (Supporting Information Figure 7). This means that at water concentration of 10 mg Se L^{-1}, harvested brine shrimp contained ~10 mg Se kg^{-1}. At least one study has shown aquatic macroinvertebrates unable to get rid of internal Se once acquired,[41] though more recent results indicate slow Se decrease in macroinvertebrates following dose of a Se pulse.[42] The internal Se concentration of brine shrimp in this study appeared to mimic water Se concentration and ratios of total Se were similar to those observed at Kesterson reservoir. Interestingly, these Se ratios were much lower than the ratios observed in waters with low SO_4^{2-} content,[41,43] implicating a synergistic effect of SO_4^{2-} in water on total Se accumulation in aquatic organisms.

Brine Fly (Ephydra cinerea)

The entire brine fly lifecycle takes place in the water or on the surface of brine lakes and *E. cinerea*, tolerating higher salt concentrations than other Ephydrids, thrive in these environments.[44] Adult *E. cinerea* deposit sticky eggs on the water surface, which hatch into larvae that feed on epilithic algae, and show a clear preference for diatoms.[45] The forms of Se in the brine fly (larva and adult) were significantly different from the Se forms in their diet (diatoms, bacteria, and microalgae) (Figure 3, Supporting Information Tables 2 and 3). Bulk XANES analysis of brine fly larva and pupa Se demonstrated that the majority was organic Se occurring as 10% SeCys, 25% CysSe-SeCys, and 52% C–Se–C forms (most likely SeMet). Similar to *Cladophora* and brine shrimp, 13% of Se was SeOMet (Supporting Information Figure 3 and Table 2). The percentage of Se recovery into the aqueous phase for the protease-XIV digested brine fly larva sample was 73.5% of total Se, and protease treatment increased soluble Se recovery by 7.6 fold. The brine fly larva had no inorganic Se as measured by SAX-HPLC-ICPMS, with Se forms occurring as 4% CysSe-SeCys, 92% SeMet, and 4% SeOMet (Supporting Information Figure 4 and Table 3). The average total Se for the aquatic brine fly larva (59.1 ± 3.1 μg Se g^{-1} ww) was elevated when compared to the shrimp and approximately 6 fold greater than in the drainage water.

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Micro-XRF mapping showed that brine fly larva mostly had a uniform Se distribution throughout tissues, except for the concentrated Se hot spots associated with the intestinal tract, which is visible due to the presence of high Zn levels, while the midgut accumulated high concentrations of Ca (Figure 2 A). Micro-XANES analysis of the localized area on the brine fly larva (Figure 2 A) showed it contained only C–Se–C forms and based on SAX-HPLC-ICPMS the C–Se–C was all in SeMet (Supporting Information Table 5).

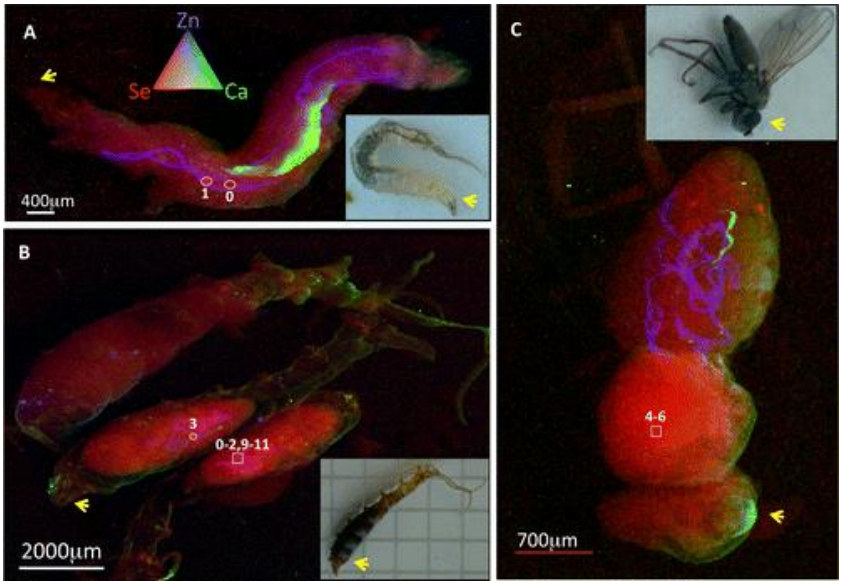

Figure 2. Distribution and speciation of Se in the brine fly *Ephydra cinerea*. μXRF map showing spatial distribution of Se (coded in red) and other selected elements (calcium in green, zinc in blue) in fly larva (A), three pupae (B), and adult fly (C). Circles in A, B, and C show locations of μXANES spectra whose fitting results are reported in Supporting Information Table 5. Light micrographs of similar individuals are shown as insets.

Ephydra cinerea pupae showed accumulation of Se within the developing fly tissues with very little Se associated with the pupa cuticle, and Se was homogeneously concentrated in the pupa intestine (Figure 2B). Micro-XANES analysis of the localized areas on the brine fly pupa (Figure 2B) showed it also contained all C–Se–C forms (Supporting Information Table 5).

The total Se for the brine fly adult was highly elevated compared to all the other samples at 169 μg Se g^{-1} ww (approximately 3 fold higher than the second highest sample brine fly larva). The

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highest Se concentrations in the brine fly adult were located in the thorax, with multiple Se enriched areas associated with organs in the abdomen (Figure 2 C). In the protease-XIV digested MCW extracted brine fly adult sample, Se recovery in the aqueous phase was 78%. Analysis by SAX-HPLC-ICPMS showed the brine fly adult contained mainly organic Se at 4% CysSe-SeCys, 94% SeMet, and only 2% SeO_3^{2-} (Supporting Information Table 3 and Figure 4).

In comparison, both SeMet and CysSe-SeCys were the dominant species in all insects studied in mine-impacted streams near Alberta, Canada.[46] A large scale test of Se speciation effects on invertebrate Se levels in WSJV in 1998–99 showed that aquatic animals obtain a majority of their Se directly from organic Se forms, while the inorganic forms of Se in water played a relatively minor role in animal toxicity[47] and these results agree with characterization of the Se speciation of insects living in the RRR pond.

The distribution of Se in the brine fly shows that Se is mostly accumulated in muscle tissues and intestines, which suggests Se accumulation and biotransformation is associated with either the forms of Se originally in food sources or as a result of metabolism by microbial gut microflora in the fly. Zinc was predominantly localized in larval and adult excretory organs, the Malpighian tubules (Figure 2D), as also observed in other fly such as *Drosophila*.[48] Calcium appeared to accumulate in the larval brine fly midgut and to lesser extent in the adult fly midgut (Figure 2).

Selenium Contaminated Water and Aquatic Ecosystem Management

Summary Figure 3 illustrates our findings and complete biochemical profiling of the biotransformation of Se in the different organisms in our engineered RRR drainage water aquatic ecosystem, as quantified by two different complementary analytical techniques that are contrasted in Supporting Information Table 1. Brine shrimp and brine fly at the top tropic level contained low concentrations of (~1%) SeO_4^{2-} or (1–2%) SeO_3^{2-}, while the predominant forms of Se accumulated were (82–94%) SeMet and (4–15%) SeCyst. Little or no trimethylselenonium was detected in brine shrimp or in brine flies,

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although interestingly some MeSeCys was detected in brine fly larvae. Future studies should investigate how microbial gut flora might be influencing the accumulation of these organic Se forms in these two aquatic organisms.

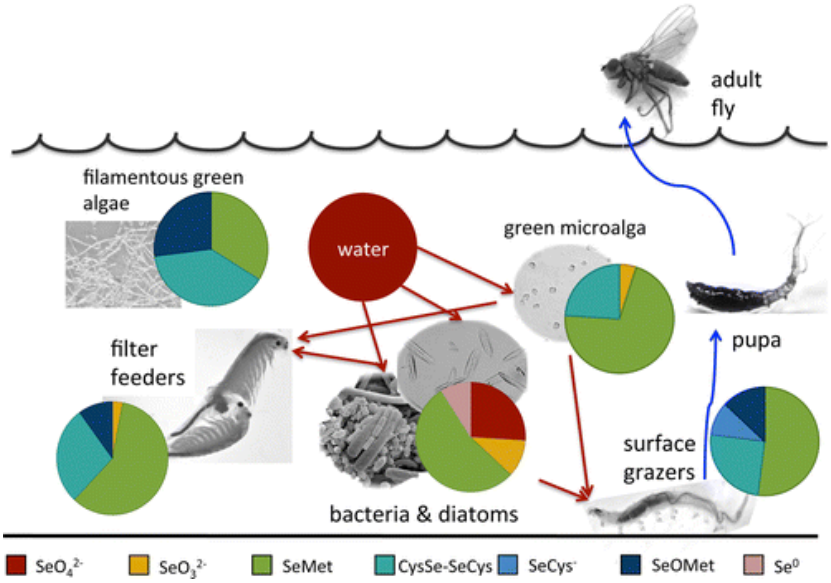

Figure 3. Community Se distribution—conceptual Se trophic levels model. Se speciation for all trophic levels except bacteria based on bulk XANES analysis. Note that bacteria and Picocystis samples were harvested from media with an ambient concentration of 40 mg Se L^{-1}. All other samples were harvested from water with ambient concentration of approximately 9 mg Se L^{-1}.

Selenium is very similar to sulfur in terms of its biochemical behavior in biological ecosystems. High SO_4^{2-} concentrations reduce the bioavailability of SeO_4^{2-} to a variety of organisms, including algae, bacteria, midges, daphnids, and brine shrimp.[28,49,50] High SO_4^{2-} concentrations in the RRR pond water likely contribute to the survival of brine shrimp and brine fly in this water. There is some evidence that invertebrates do not have efficient Se removal excretion pathways, leading to toxic levels of organic Se bioconcentration and high levels of organic Se that are in turn bioavailable to fish and birds in these environments.[41]

Our data elucidating all the individual forms of Se in these specific aquatic invertebrates is important because different forms have various levels of toxicity with some organic Se forms reported as nutritionally beneficial and anticarcinogenic.[51-55] Selenomethionine, for example, has been shown to cause higher toxicity rates in birds and

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fish during laboratory feeding studies than observed using SeO_3^{2-} or CysSe-SeCys.[11,56] In hyper-saline ecosystems, brine flies and brine shrimp are essential components of shore birds' diet.[45,57,58] Brine fly larvae, pupa, and adults have an even higher nutrient value per individual than brine shrimp and dominate shore bird diets at certain times of the year.[57,59]

On the other hand, organic Se is a required micronutrient for vertebrates, including livestock and humans. A well-established fish farm industry for growing brine shrimp for harvest already exists in the United States and in Asia. An understanding of the biochemistry associated with Se-enriched brine shrimp production in order to efficiently supplement organic Se from healthful brine shrimp meals in animal diets, could lead to the development of these Se-polluted environments into sources for producing valuable bioavailable Se supplements. The closed pond system could safely provide organic Se enriched foods containing essential oils, omega 3 fatty acids, and proteins especially valuable in those areas where people are currently suffering from Se deficiency or as a Se enriched animal feed for rearing healthy livestock.

Supporting Information

Details of experimental methods and additional experimental data tables and figures. This information is available free of charge via the Internet at http://pubs.acs.org/.

○Author Contributions

R.S., G.S.B., K.R.H., and J.L.F contributed equally to this paper.

The authors declare no competing financial interest.

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Abbreviations

C–Se–C	carbon-selenium-carbon
γGMeSeCys	gamma-glutamyl-methyl-selenocysteine
MeSeCys	methyl-seleno-cysteine
μXRF	micro-focused X-ray fluorescence
μXANES	micro-K-edge X-ray absorption near-edge structure
bulk XANES	X-ray absorption near-edge structure
SeO_4^{2-}	selenate
Se^{2-}	selenide
SeO_3^{2-}	selenite
SeCyst	seleno-cystathionine
SeCys	seleno-cysteine
$SeGSH_2$	seleno-diglutathione
SeMet	seleno-methionine
CysSe-SeCys	selenocystine
Na_2SeO_4	sodium selenate
Na_2SeO_3	sodium selenite
SAX-HPLC-ICPMS	strong anion exchange high performance chromatography coupled to real time inductively coupled plasma mass spectrometry
ww	wet weight
DMSe	dimethylselenide
DMDSe	dimethyldiselenide

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