Selenium Speciation in *Salicornia bigelovii*: Plant-Mediated Reduction of Selenate for the Remediation of Selenium Contaminated Soil

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Abstract

Selenium contamination of soil in the San Joaquin Valley, California poses a threat to wildlife and to agricultural productivity. Traditional phytoremediation methods use the process of phytoextraction to remove selenium from soil by sequestering selenium in plant tissues, potentially allowing the re-release of selenium to the soil if the plants are not harvested after senescence. The volatilization of selenium by plants is an attractive alternative to phytoextraction because selenium is detoxified and transported out of the contaminated system as dimethyl selenide. Previous field studies have shown that the soil-pickleweed (*Salicornia bigelovii*) system volatilizes selenium at significantly higher rates than other plants tested for *in situ* selenium remediation. In order to increase the efficiency of selenium remediation through volatilization, an understanding of the factor controlling the rate-limiting step for dimethyl selenide formation is necessary. The objective of this research was to determine whether microbes were necessary in pickleweed in order to overcome the rate-limiting step of selenium volatilization: the conversion of inorganic selenate to a reduced selenium species, such as organic selenomethionine. To elucidate the role of microbes in selenate reduction, X-ray Absorption Spectroscopy (XAS) was used to determine selenium speciation in tissues of sterile and non-sterile pickleweed plants supplied with 50μM selenate. The results from this research indicate that both sterile and non-sterile pickleweed plants can convert selenate into organic selenium, and that microbes are not necessary for the reduction of selenium in pickleweed plants. Comparisons with other plant species examined for the ability to overcome the rate-limiting step for selenium volatilization show that pickleweed is unique in its ability to convert selenate into organic selenium in both shoot and root tissues of sterile and non-sterile plants.

Introduction

Selenium is an essential trace element for many organisms, including humans and other mammals (NRC, 1983), that becomes toxic at high concentrations (Wilbur, 1980). Weathering and runoff from the selenium-rich Coast Range Mountains, combined with current agricultural irrigation practices, has concentrated selenium in Central Valley soils by allowing selenium-rich agricultural drainage water to evaporate in ponds (Presser, 1994). In California during the mid 1980's, elevated concentrations of selenium in soil and water caused the deaths and deformities of aquatic birds and fish at the Kesterson Reservoir, a marsh receiving agricultural runoff water (Ohlendorf *et al.*, 1986). Elevated concentrations of selenium remain a concern to many parts of the western United States (Ohlendorf *et al.*, 1986). The threat of selenium contamination to wildlife and agricultural productivity in the San Joaquin Valley of California has spurred much research on the remediation of selenium contaminated soil and water in the Central Valley (Frankenberger and Karlson, 1988; Terry and Zayed, 1994; Terry and Lin, 1998).

Selenium occurs in five different oxidation states, Se^{+6} as selenate, Se^{+4} as selenite, Se^{+2} as selenium dioxide, Se^{0} in the elemental form, and Se^{-2} as selenide, that differ in toxicity (Eisler, 1985). The dominant selenium species in agriculturally contaminated soil occurs in the most oxidized form, as inorganic selenate (McNeal and Balisteri, 1989). Attempts to reclaim selenium contaminated waters and soils often utilize a process known as phytoextraction: the use of plants to remove selenium from soil. However, the accumulation of selenium and other elements within the tissues of plants may become toxic to wildlife upon consumption, or may be re-released into the soil upon senescence if the plants are not harvested. The adverse effects of phytoextraction may be avoided when the plant-soil system releases volatile selenium into the atmosphere, rather than sequestering selenium in its tissues.

The volatilization of selenium by plant-soil systems is especially attractive because it releases selenium as a gas, largely in the form of dimethyl selenide ($(CH_3)_2Se$) (Eisler, 1985), into the atmosphere, where decomposition reactions with either ozone or the nitrate and hydroxyl radicals will contribute to its short atmospheric lifetime (Atkinson *et al.*, 1990).

Local meteorological conditions would allow for the transport of dimethyl selenide out of the San Joaquin Valley within 24 hours, either to the ocean, or to terrestrial areas where high soil selenium concentrations do not occur (Lin *et al.*, 2000). Additionally, dimethyl selenide has been found to be 500 times less toxic than inorganic selenate to laboratory rats (Wilbur, 1980) and previous studies have shown that atmospheric concentrations of dimethyl selenide, as a result of enhanced volatilization rates, are not expected to exceed concentrations that are harmful to humans or wildlife (CH2M Hill, 1988). Thus, the formation of dimethyl selenide not only reduces the concentration of selenate in the soil through volatilization, but also reduces the toxicity of selenium by converting selenate into a considerably less toxic compound.

Previous studies at Red Rock Ranch, a managed agroforestry field site located at Five Points, CA, measured selenium volatilization rates from eleven different plant species. The soil-plant system involving a particular species, pickleweed (*Salicornia bigelovii*), was found to volatilize selenium at an annual average rate of 155±25 μg Se m^{-2} d^{-1}, which was ten times higher than cordgrass (*Spartina spp.)*, the next best volatilizer (Terry and Lin, 1998). Pickleweed is a succulent, C_3 annual plant with a high transpiration rate that thrives in the hot, salty environments (Ayala and O'Leary, 1995) characteristic of the San Joaquin Valley and of soils contaminated with selenium and other trace element salts. Thus, the ability of pickleweed to volatilize selenium at elevated rates, combined with its requirements for high salt concentrations and high temperatures, makes this plant species ideally suited for studies on phytoremediation in the Central Valley.

Although a mass balance calculation from the agroforestry site in the San Joaquin Valley showed that volatilization from the soil-pickleweed system was significant, removing 6.5% of the total annual selenium input into the study site, it is not high enough to be maintained as an efficient remediation system (Terry and Lin, 1998). However, a one-time, maximum volatilization rate from pickleweed of 576 μg Se m^{-2} d^{-1}, observed in the study under *unmanipulated* environmental conditions, illustrates the huge potential that exists to increase the efficiency of the volatilization technology. If the maximum rate of volatilization could be maintained throughout the year, then selenium removal could increase to 22% of the total annual selenium input (Terry and Lin, 1998).

In order to enhance the efficacy of *in situ* selenium remediation of agriculturally contaminated sites, increasing and sustaining the highest rate of selenium volatilization from the soil-pickleweed system would be necessary. Previous research has focused on the role of microbial activity on the production of dimethyl selenide (Frankenberger and Karlson, 1988). However, volatilization experiments at the agroforestry site in the San Joaquin Valley showed the soil-pickleweed system produced higher rates of selenium volatilization than the bare soil system, suggesting that the presence of pickleweed enhanced the formation of dimethyl selenide (Terry and Lin, 1998). Because pickleweed increased volatilization rates, dimethyl selenide formation was likely facilitated by selenium-reducing enzymes in the pickleweed plant itself, by microbial populations unique to the pickleweed rhizosphere environment, or a combination of the two factors, rather than by soil microbial populations. In order to achieve and maintain elevated rates of selenium volatilization for enhanced and effective *in situ* remediation, it is first necessary to understand why the volatilization rates from the soil-pickleweed system are so high. Elucidating the dominant mechanism controlling the volatilization of selenium, whether microbial or enzymatic, can allow for appropriate manipulations that sustain high rates of dimethyl selenide formation.

Zayed *et al.* (1998) reported that the rate-limiting step in the formation of dimethyl selenide is the chemical reduction of inorganic selenate into a selenium species that is easily volatilized, such as selenite or an organic selenium species such as selenomethionine. The goal of this study was to determine the controlling factor on the rate-limiting step for selenium volatilization in order to illuminate possible target sites for physical or physiological manipulations of the soil-pickleweed system. Because it is believed that the controlling factor in selenium reduction and volatilization could be an internal, enzymatic process or an external, rhizosphere microbial process, the objective of this experiment is to resolve whether the presence of microbes is necessary in the chemical reduction of selenate. If microbes are not necessary in overcoming the rate-limiting step for dimethyl selenide formation, it suggests that the pickleweed plant itself facilitated the reduction of selenate.

Methods and Materials

The experiment was designed to determine whether microbes were necessary for the reduction of selenate. The experimental design involves the exposure of sterile and non-

sterile pickleweed plants to selenate and a determination of the resulting selenium speciation in the pickleweed tissues using X-ray Absorption Spectroscopy (XAS).

Pickleweed seeds were harvested from Red Rock Ranch, an agroforestry field site located at Five Points, CA (36°23'03"N, 120°13'62"W). Pickleweed plants were cultured hydroponically in Half-Hoagland's solution (Zayed *et al.*, 1998). Pickleweed seeds were sterilized using ethanol and hypochlorite as described by de Souza, *et al.* (1999). Six autoclave-sterilized, airtight, clear acrylic Magenta boxes (Sigma) were each filled with 300mL of autoclave-sterilized Half-Hoagland's solution. A sterilized wire mesh was placed on the surface of the liquid growth media. Sterilized cotton fibers were spread across the wire, and fifty pickleweed seeds were sown on top of the cotton-wire mesh. Two sterilized glass tubes, sealed and held in place with silicone sealant, were inserted into the top of each Magenta box to allow for gas exchange. Airborne bacteria were removed from the air entering the boxes using autoclaved air filters (Millipore 0.22 μm) that were connected to the glass tubes with sterile Nalgene tubing and autoclave tape. All equipment and growth media used were autoclave-sterilized.

Six Magenta boxes, three replicates each of the sterilized and unsterilized pickleweed seeds, were placed in a growth chamber maintained at 25°C. The pickleweed seeds were allowed to germinate. After ten weeks, aqueous sodium selenate (Sigma) was passed through a filter (Millex 0.22μm) to remove bacteria from the solution, and added to the sterile growth media to a concentration of 50 μM, which is comparable to typical selenium concentrations found in Red Rock Ranch soil. After ten days, shoots and roots from all pickleweed plants (numbering from five to eight seedlings), from each of the three replicates, were immediately separated and freeze-dried using liquid nitrogen, ground, and stored at -80°C, for analysis using XAS at the Stanford Synchrotron Radiation Laboratory to determine selenium speciation in plant tissues. Individual pickleweed plants from each replicate were pooled into one sample for XAS analysis, but each of the three replicates from the sterile and non-sterile plants remained independent. Selenium K-edge analysis was conducted at beamline 4-3, using a Si (111) double-crystal monochromometer. X-rays passed through a 1 mm entrance slit. Frozen samples were placed in a sample chamber at a 45° angle to the x-ray beam. A series of replicate scans were used to determine the fluorescent x-ray absorption

spectra for selenium in the sample (Lytle *et al.*, 1998). Data analysis of the absorption spectra was performed using EXAFSPAK.

To ensure that sterile conditions were achieved, samples taken from the nutrient solution and from pickleweed plants from the sterile and non-sterile treatments were plated onto a tryptic soy agar growth media as a qualitative test for the presence of bacteria (Personal comm., M. de Souza, 1999). Plates were checked daily for bacterial growth over a two-week period.

Results

X-ray Absorption Spectroscopy was used to determine whether the presence of microbes was necessary for pickleweed plants to facilitate the reduction of selenate, the rate-limiting step for the formation of dimethyl selenide. Figure 1 shows the selenium K-edge absorption spectra for the non-sterile pickleweed samples. The top and bottom curves represent the selenate and selenomethionine standards, respectively. The shoots of the non-sterile pickleweed plants supplied with selenate in the growth medium show an accumulation of largely selenomethionine (Fig.1). Under a conservative approximation, selenomethionine represents at least 70% of the accumulated selenium in the shoot tissues, with elemental selenium, selenite, and selenate accounting for the remaining accumulated selenium. The spectrum for non-sterile pickleweed roots is similar to the spectra for pickleweed shoots, where selenomethionine is the dominant species of selenium accumulated, comprising at least 45% of the accumulated selenium. However, the percentage of selenate is greater in non-sterile roots than in shoots, comprising the second most abundant selenium species, followed by elemental selenium and selenite. Pickleweed shoots and roots, grown with the presence of microbes, can convert selenate into reduced selenium compounds such as selenite and selenomethionine.

Figure 2 shows the absorption spectra for the sterile pickleweed plants. The top and bottom curves represent the selenate and selenomethionine standards, and the middle curves represent the sterile pickleweed samples. The dominant form of selenium in both sterile shoots and roots is selenomethionine, with an approximate contribution of at least 50% of the total accumulated selenium in both shoots and roots. In both samples, selenate is the second most abundant form of selenium, comprising roughly 20% of samples, followed by selenite

and organic selenium. Pickleweed plants, grown without the presence of microbes, show the same ability to overcome the rate-limiting step in dimethyl selenide formation, as do the pickleweed plants grown with the microbes associated with their seed coat. Selenium speciation in sterile roots and shoots show roughly similar percentages of selenomethionine and selenate, whereas the non-sterile shoots show a greater dominance of selenomethionine over selenate than do the non-sterile roots of pickleweed.

The sterile and non-sterile pickleweed and hydroponic solution samples were plated onto a tryptic soy agar media. All three replicate non-sterile samples showed growth of an array of bacterial colony types after one day of incubation (Table 1). Of the three replicate sterile samples, two samples showed no bacterial growth throughout the incubation period and one sample showed growth of one bacterial colony type. Because pickleweed plants from the three replicates were not pooled for the XAS analysis, the contamination of the third sterile replicate did not affect the results of the other two sterile samples. Sterile conditions were maintained in two replicates of the Magenta box system of the sterilized pickleweed seedlings and the unsterilized seeds maintained a non-sterile growing environment.

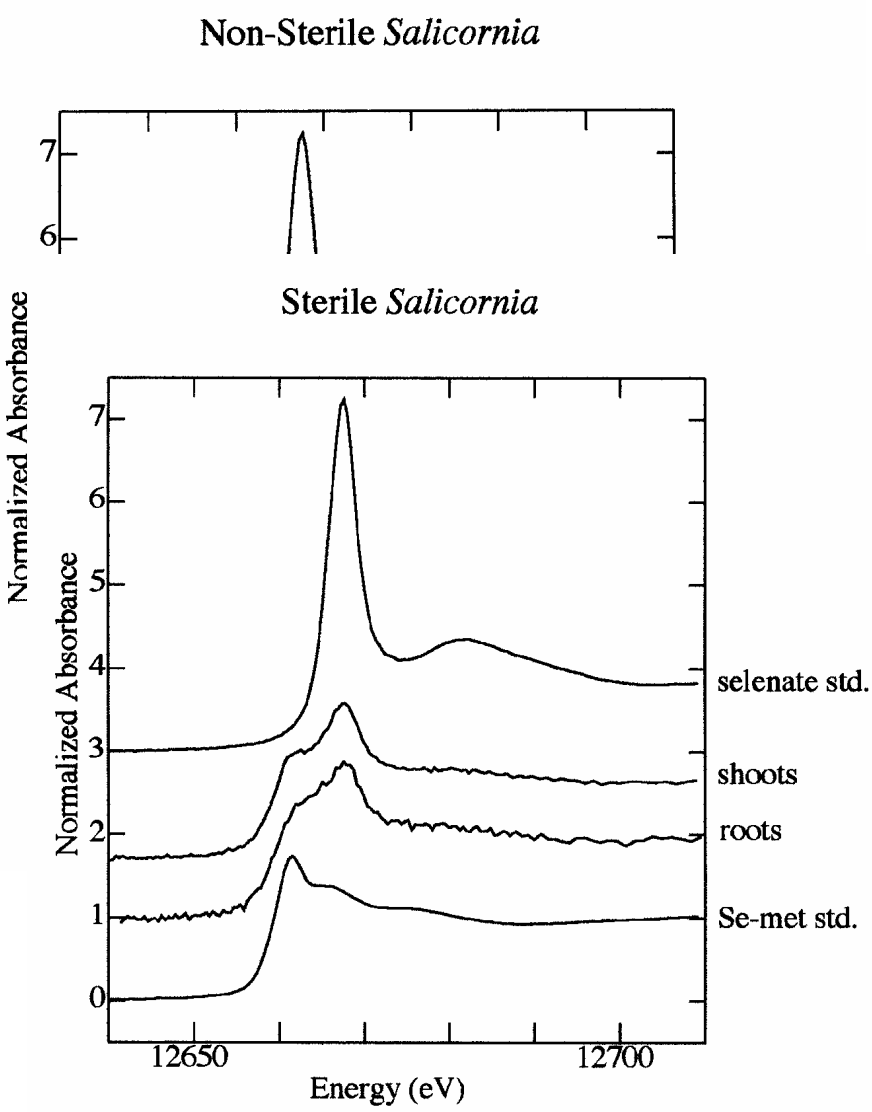

Figure 1: X-ray absorption spectra for 10-week-old non-sterile pickleweed plants supplied with 50 μM selenate for 10 days.

Figure 2: X-ray absorption spectra for 10-week-old sterile pickleweed plants supplied with 50 μM selenate for 10 days.

	Non-Sterile			*Sterile*		
	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
Pickleweed	G	G	G	--	--	G
Media	G	G	G	--	--	G

Table 1: Bacterial growth results from a two-week incubation period of pickleweed and growth media samples plated on tryptic soy agar media. "G" indicates growth of bacteria and "—" indicates no growth of bacteria.

Discussion

Pickleweed plants grown from both sterilized and unsterilized seeds were able to overcome the rate-limiting step in the volatilization of selenium by converting selenate supplied in growth media into organic selenium stored in roots and shoots. These results show that the presence of microbes with pickleweed plants was not necessary for the reduction of selenate into selenite or organic selenomethionine.

Pilon-Smits *et al.* (1999) examined selenium speciation in wildtype and transgenic Indian mustard (*Brassica juncea*) grown hydroponically in media supplied with selenate. The transgenic Indian mustard plants were genetically engineered with the APS1 gene from *Arabidopsis thaliana,* for the over-expression of ATP-sulfurylase, the rate-limiting enzyme controlling sulfur assimilation and reduction (Pilon-Smits, 1999). Because sulfur and selenium behave analagously, the ATP-sulfurylase enzyme can also control selenium assimilation and reduction. While selenium speciation in pickleweed showed a dominance of selenomethionine over selenate in both sterile and non-sterile samples, speciation in wildtype Indian mustard showed a dominance of selenate in both shoots and roots, with minimal reduction of selenate to organic selenium. However, transgenic Indian mustard shoots with increased ATP-sulfurylase activity showed the ability to reduce selenate and accumulate predominantly organic selenium, exhibiting speciation patterns similar to both the non-sterile and sterile pickleweed plants. Additional experiments with transgenic Indian mustard involving the de-topping of the plant prior to the addition of selenate to the hydroponic media, exhibited the same speciation patterns of wildtype Indian mustard, where selenate was not converted to organic selenium, suggesting that selenate reduction is either carried out almost exclusively in the shoots, or that selenate reduction in the roots of transgenic plants is dependent on shoot-generated metabolites or signals (Pilon-Smits *et al.*, 1999).

Previous Se speciation studies by Zayed *et al.* (1998) have shown that selenate supplied to the hydroponic media of broccoli (*Brassica oleracea*) plants under non-sterile conditions resulted in the accumulation of selenate in the leaves, and a mixture of selenate and selenomethionine in the roots. Those results indicate that with the presence of bacteria in the hydroponic media, the bacteria-broccoli system was unable to reduce selenate in the shoots, but able to convert selenate in the roots to organic selenium. The importance of the

rhizosphere environment of plants and its associated microbial populations is highlighted by the conversion of selenate into organic selenium in the roots. The accumulation of selenate in the shoots suggests that broccoli shoots, like wildtype Indian mustard, do not contain enzymes that can convert selenate to organic selenium.

The ability of non-sterile pickleweed plants to convert selenate in roots and shoots into organic selenium is distinctive compared to Indian mustard and broccoli. The results of this research show that microbes are not required in pickleweed to overcome the rate-limiting step of selenium volatilization because the sterile pickleweed plants also reduced selenate into selenomethionine. Microbes were not necessary in pickleweed for the reduction of selenate, and the absorption spectra for the sterile pickleweed plants was similar to the transgenic Indian mustard plants (Pilon-Smits *et al.*, 1999), suggesting that enzymatic activity in the shoots of pickleweed is responsible for overcoming the rate-limiting step for selenium volatilization.

Much of the preceding research in selenium volatilization have measured the rate of formation of dimethyl selenide using enclosed chamber studies (Duckart *et al.*, 1992; Frankenberger and Karlson, 1994; Hansen *et al.*, 1998; Lin *et al.*, 1999). Although chamber studies are convenient for selenium volatilization measurements, one disadvantage is the enclosure of the soil-plant system in an environment that may differ in humidity, temperature, and concentration gradients compared to conditions experienced *in situ*. This is an important confounding factor in quantifying volatilization rates because the release of dimethyl selenide is dependent on concentration gradients determined by Fick's Law of diffusion (Dungan *et al.*, 2000). To avoid anomalous measurements of selenium volatilization due to chamber effects, rather than directly measuring rates of selenium volatilization in the sterile and non-sterile pickleweed, XAS was used to determine if pickleweed plants could overcome the rate-limiting step in dimethyl selenide formation. Selenium speciation analysis in plants can be used to qualitatively predict relative rates of selenium volatilization from different plant species by determining the reduction of selenate to selenium forms that are readily volatilized, such as selenite or organic selenium. In this experiment, testing the role of microbes on selenate reduction in pickleweed also required a chamber to maintain an environment that excluded ambient airborne microbes. Although inaccurate measurements of selenium volatilization from the Magenta box system were

avoided by using XAS, chamber effects, such as temperature, humidity, and concentration gradients, on selenate reduction in pickleweed tissues are currently uncertain. However, because previous XAS analyses on Indian mustard and broccoli used similar enclosure systems as was used in this experiment, any confounding factors from the chamber would not hinder a comparison between the three plant species, and would not seem to affect the unique selenate reduction results achieved from pickleweed when compared to Indian mustard and broccoli.

Comparisons of the speciation results obtained here from pickleweed with previous studies on Indian mustard and broccoli may be affected by differences in the ages and sizes of the plants. The Indian mustard used by Pilon-Smits *et al.* (1999) and the broccoli plants used by Zayed *et al.* (1998) were five and four weeks old respectively, younger than the ten-week-old pickleweed plants used in this experiment. However, the slow growth rate of pickleweed, compared to the fast growth rate of Indian mustard (Pilon-Smits *et al.*, 1999) could possibly result in disparities in size and maturity between the different plant species. The pickleweed seedlings used in this research were not yet fully mature, and the ability of seedlings to overcome the rate-limiting step in selenium volatilization may or may not be expressed in mature pickleweed plants. However, the extraordinarily high rates of selenium volatilization rates achieved *in situ* from mature pickleweed plantations suggest that the selenate-reducing capability could also be present in mature plants because high volatilization rates require overcoming the rate-limiting step of dimethyl selenide formation. The results of this study are still relevant to phytoremediation research because the physiological or enzymatic properties of pickleweed seedlings, whether or not they are present in mature plants, can still be identified and isolated to enhance selenate reduction in other plant species using bioengineering technologies.

Although much of the past research has focused on the role of microbes in selenium volatilization (Frankenberger and Karlson, 1988; de Souza *et al.*, 1999), this research has shown that another mechanism exists in pickleweed for the reduction of selenate to organic selenium that is likely facilitated by enzymatic activity in the shoots. Future research can test the idea that shoot enzymatic activity is the dominant controlling factor in the reduction of selenate to organic selenium either by conducting de-topping experiments on pickleweed, or determining whether selenate reduction also results from exposure to pickleweed shoot

homogenates that contain the suspected selenate-reducing enzymes. Future research can also work towards isolating the enzyme or enzymes responsible for selenate reduction in pickleweed and identifying the gene controlling for the expression of this enzyme so that this ability to reduce selenate can be genetically engineered in other plants, or can be modified for the over-expression in pickleweed itself. In order to enhance the efficiency of selenium volatilization to improve its applicability for the remediation of *in situ* contamination, the rate-limiting step for the formation of dimethyl selenide, the reduction of selenate to organic selenium, must be overcome at a higher rate in order to increase the rate of dimethyl selenide release into the atmosphere. An understanding of the mechanism and controlling factors in overcoming the rate-limiting step must be achieved in effectively target the relevant steps in the volatilization process.

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