DETERMINATION OF SELENIUM AND TELLURIUM OXYANION TOXICITY, DETECTION OF METALLOID-CONTAINING HEADSPACE COMPOUNDS, AND QUANTIFICATION OF METALLOID OXYANIONS IN BACTERIAL CULTURE MEDIA

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ABSTRACT

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Purpose

The purpose of this research was: (1) to determine the extent of bacterial tolerance to grow in the presence of different concentrations of toxic selenium and tellurium oxyanions; (2) to identify organo-selenium compounds in the headspace of metalloid-resistant bacteria; and (3) to develop a simple, quick, and reliable method for selenium and tellurium oxyanion analysis in live bacterial cultures.

Methods

Using optical density to follow bacterial population and growth rate, a *Bacillus* bacterial species was examined in the presence of different concentrations of selenium and tellurium oxyanions. At regular time intervals, the optical density of these samples was measured at 525 nm using a spectrophotometer. Specific growth rates, which reflect relative metalloidal oxyanion toxicity, were determined using the slope of the growth curve. Zone of inhibition experiments were also used to measure the relative toxicity of selenium and tellurium oxyanions to the test organism. Bacteria were grown in Petri dishes with a paper disk placed in its center which was saturated with a metalloid solution of various concentrations. A zone of growth inhibition observed around this disk was used as a measure of relative toxicity of these oxyanions.

The second set of experiments carried out included the identification of organo-selenium compounds in the headspace of metalloid-resistant bacteria and the determination of bacterial ability to reduce and methylate toxic metalloidal salts. Solid

phase micro extraction was used to collect headspace gases above live bacterial cultures. Analysis of the headspace samples was performed by a fluorine-induced sulfur chemiluminescence detector coupled to a gas chromatograph. Gas chromatography/mass spectrometry was used to confirm the identity of the compounds collected above the cultures of the bacteria.

The final set of experiments performed included analysis of selenium and tellurium oxyanions using UV/visible spectrophotometry. Sodium borohydride ($NaBH_4$) was used as a reducing agent to convert soluble selenium and tellurium oxyanions to their elemental forms whose optical density was measured at 500 nm.

Findings

Specific growth rate and zone of inhibition experiments showed that tellurite was the most toxic among the selenium and tellurium oxyanions examined. Headspace experiments resulted in the detection of two new organo-metalloidal components previously unreported in the literature: dimethyl diselenenyl sulfide and dimethyl triselenide. In addition a wide range of organo-sulfur and -selenium compounds were detected. Spectrophotometric determination of selenium and tellurium oxyanions in bacterial growth media using $NaBH_4$ as the reducing agent provided a good linear range for selenate, selenite, and tellurite. The linear range for tellurate analysis using the borohydride method was quite small but this may have been caused by the poor solubility of this oxyanion in growth media. The detection limits of this method for metalloidal oxyanions are modest in relation to other modern instrumental methods; however, borohydride reduction was shown to be a quicker and more straightforward method

compared to most other analytical techniques, with simple instrumental requirements and the need for few reagents.

Keywords: Zone of inhibition, specific growth rate, selenocyanate, borohydride

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CHAPTER 1

INTRODUCTION

Introduction to Environmental Metalloids

Natural geological processes and diverse actions of living beings are the main sources of inorganic trace metalloids in the environment. Severe weathering of minerals and rocks, and release of chemicals from volcanic eruptions are the few natural sources (Ernst, 1998). Human sources of these elements are irrigation, unregulated dumping of industrial wastes, use of hazardous chemicals in agriculture, mining, and production of energy and fuel (Seward and Richardson, 1990; Lasat, 2000). Occurrence of these trace elements where they are not necessary or present beyond the optimum levels causes significant hazards to biological and ecosystem health. These effects have provoked governments worldwide to instigate several environmental practices to deal with the concerns caused by these elements (McIntyre, 2003).

Introduction to Selenium and Tellurium

Selenium and tellurium in the environment and their speciation are major research areas because of their dual role as crucial elements and potential environmental toxicants (Fan and Kizer, 1990). They play significant biological roles which require that we understand their reactivities in biological systems. Extensive use of these elements has resulted in their accumulation in the environment, creating a threat to living organisms.

Various methods have been implemented to eliminate these toxicants from the ecosystem because of their toxicity and environmental concerns. Some of these methods include phytoremediation and bioremediation which will be discussed further. A brief description of their occurrence, uses and adverse effects are necessary before proceeding

to the discussion of the antagonistic activities developed by different organisms to overcome the problems caused by these elements.

Introduction to Selenium

Selenium (Se) was discovered and named by Jöns Jacob Berzelius in the year 1817from a Greek word '*selene'*, meaning moon. It is the 67th most abundant element with a concentration of 50 ppb in the earth's crust. Its concentration is 5 ppm in soil and 0.2 ppb in sea water. Selenium in the atmosphere usually exists in its methylated forms such as $CH_3SeSeCH_3$ or CH_3SeCH_3, with a concentration of 1 nanogram per cubic meter of air. It is chemically analogous to sulfur and usually occurs with sulfides of metals such as copper, zinc and lead (Emsley, 2002). Selenium has six naturally occurring isotopic forms with different relative abundance values (%). These include ^{80}Se, 49.82%; ^{78}Se, 23.52%; ^{82}Se, 9.19%; ^{76}Se, 9.02%; ^{77}Se, 7.58% (CRC Handbook, 1975).

Selenium behaves as an essential micronutrient to biological systems at lower concentrations but becomes toxic at more prominent levels (Kuo and Jiang, 2008). The soluble forms of Se includes selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}). Based on toxicity experiments, selenite has been shown to be relatively more toxic than selenate to most living organisms (Brasher and Ogle, 1993; Boisson et al., 1995; Yu et al., 1997; Chasteen and Bentley, 2003).

Uses of Selenium

Selenium plays an important biological role in the form of Se-containing amino acids selenocysteine and selenomethionine, analogous to sulfur-containing cysteine and methionine. Selenium deficiency in human beings has been found to result in severe adverse effects which can be overcome by its increased uptake (Clark et al., 1996). Sodium selenate and sodium selenite are the inorganic selenium-containing compounds

used as dietary supplements in the prevention of various nutritional-deficiency diseases in mammals (Fan and Kizer, 1990). Selenium has been found to exhibit anticancer properties (Schrauzer, 2000; Rayman, 2000). Se deficiency in animals and humans is even found to be associated with anemia (Semba et al., 2009).

Se also has conductive properties which make it useful in the design and function of photoelectric cells, light meters, solar cells and photocopiers (Emsley, 2002).

Different cellular functions that involve selenium include protection of cell membranes from oxidative damage (Gómez-Ariza et al, 1999b) and antagonistic activity in the form of glutathione peroxidase in reducing the toxicity of metals such as mercury, arsenic, and thallium in biological systems (Emsley, 2002). To achieve all these functions selenium is required in trace amounts.

Selenium can offer the above mentioned advantages only when present in minimal quantity. Upon exceeding the optimum levels, it affects various body functions such as the nervous system (Wrobel et al., 2005). Usually, humans can have 55-70 μg of Se daily in food. If the consumption of Se exceeds, for instance, more than 130 μg L^{-1} in drinking water, toxicity results. According to the World Health Organization, not more than 10 μg L^{-1} of Se is permissible in drinking water (Gómez-Ariza et al., 1999a; WHO, 2009).

Selenium Toxic Effects in the Environment

Governments have imposed restrictions on uptake of selenium because of some natural disasters caused by high selenium concentrations (Engberg et al., 1998; Lemly and Ohlendorf, 2002; Ohlendorf, 2002). The actual cause for this increase in Se concentration in the environment in many cases is human activity and the result is that Se has become an environmental toxicant.

In an attempt to discover a solution for poor rainfall conditions in some parts of the western United States, the government has carried out extensive irrigation projects. Typically selenium in, the form of ores, concentrates in soil and subsoil as a result of weathering of rocks and other geological processes. One main aspect of their irrigation project in California's central valley was to collect salt-contaminated groundwater from the root zone under irrigated fields and discharge that water into natural or artificial channels. This process was carried out to increase the agricultural yield by decreasing crop exposure to the metals in those salts (Engberg et al., 1998). In those drainage ponds, the solubility and bioavailability of Se further increases upon atmospheric exposure for longer periods of time, resulting in the formation of oxidized Se species such as SeO_3^{2-} and SeO_4^{2-}. These chemical species pose a risk to living organisms residing in that selenium-rich environment (Ohlendorf, 1989; Sharmasarkar et al., 1998). Extinction of endangered species and death and deformation of waterfowl at Kesterson reservoir of California's San Joaquin Valley are some of the problems that have made selenium a major environmental toxicant in that region (Ohlendorf et al., 1986; Ohlendorf, 2002).

Introduction to Selenocyanate

In recent times, another Se-containing anion, selenocyanate, has gained much interest. It has been found that the availability of Se from crude oil, upon interaction with cyanides from petroleum refining process and cyanide leaching of selenide ores, results in the formation of $SeCN^-$ (Golub and Skopenko, 1965; Soderback, 1974; Sun, 2008).

Although the toxicity of $SeCN^-$ is not as well-known as that of SeO_4^{2-} and SeO_3^{2-}, selenocyanate bioprocessing by different organisms is being investigated (Vadhanavikit et al., 1987; Burra et al., 2009a). Some of the organo-selenium compounds containing $SeCN^-$ groups have been employed in lots of chemo preventive studies. For example, 1,4-

phenylenebis(methylene) selenocyanate (p-XSC) has been considered a promising chemopreventive agent in treating human non-small cell lung cancer and also in curing human oral squamous cell carcinoma (El-Bayoumy et al., 2006; Chen et al., 2009).

Selenium Treatment

Based on observation of proven toxic effects and relative accessibility, Se and its compounds have been recognized as toxicants of concern in the spectrum of identified environmental pollutants (Ohlendorf et al., 1986; Ohlendorf, 1989; Sharmasarkar et al., 1998; Ohlendorf, 2002). Any technique employed for the removal of Se from wastewater mainly depends on the physical and chemical properties of Se species. Many difficulties must be faced for the removal of Se from oil refinery wastewaters. Particularly in the case of $SeCN^-$ because of its complex behavior in aqueous system, removal of $SeCN^-$ in remediation process is difficult even after using conventional ferric salt removal processes for Se in waste steams (Kapoor et al., 1995; Meng et al., 2002; Miekeley et al., 2005). Moreover, use of chemicals in metalloid treatment does not remove the toxicants completely; in turn, this may result in the production of toxic by-products (Philip et al., 2005).

Introduction to Tellurium

Tellurium (Te) is another metalloid of interest, usually found in association with Se, and both have similar chemical and physical characteristics (Chasteen and Bentley, 2003). Being the 72^{nd} most abundant element in the earth's crust, Te has soil concentrations of 0.05 ppm to 30 ppm and 0.15 ppt (parts per trillion) in sea water. Calaverite ($AuTe_2$), sylvanite ($AgAuTe_4$), and tellurite (TeO_2) are the minerals which contain tellurium (Emsley, 2002). Tellurite (TeO_3^{2-}) and tellurate (TeO_4^{2-}) are the oxyanions of Te among which tellurite is the most toxic form for most organisms

(Emsley, 2002). Stable isotopes of Te include ^{130}Te, 34.48%; ^{128}Te, 31.79%; ^{126}Te, 18.71%; ^{125}Te, 6.99%; ^{124}Te, 4.61%; ^{122}Te, 2.46%; ^{123}Te, 0.87%; ^{120}Te, 0.089% (CRC Handbook, 1975).

Uses and Toxicity of Tellurium

Commercial applications of Te include vulcanization of rubber, in alloys, use to color glass and ceramics, and use in metal-oxidizing solutions to blacken or tarnish metals (Bagnall, 1966). Tellurium and its derivatives such as organo-tellurium compounds have been found to exhibit probable antioxidative and anticancer properties (Jacob et al., 2000; Engman et al., 2003).

Tellurium compounds except hydrogen telluride are, in general, less toxic when compared to the analogous form of Se due to their rapid reduction to the elemental or methylated form in the body, which are then excreted in the form of unpleasant smelling organo-tellurium compounds (Bagnall, 1966). Despite these advantages, acute toxicity of Te was also reported in young children from ingestion of metal-oxidizing solutions that contained substantial concentrations of Te (Yarema and Curry, 2005).

Remediation Methods for Metalloids

In order to handle the very complex matrix of refinery wastewaters for the removal of Se and Te, development of a more efficient and robust method is desirable (Miekeley et al., 2005). Therefore, a novel approach of utilizing the ability of plants and microbes to degrade toxic compounds has been explored. This is the basis for the sprouting field of bioremediation for treating contaminated environments biologically (Philip et al., 2005).

One of the earliest areas of bioremediation includes phytoremediation which involves the use of plants to lower the metalloid concentrations in soil. The high cost of

physical, chemical, and thermal techniques for remediation has led to the development of alternatives such as phytoremediation. The advantages of this method are *in situ* application; inert, solar driven 'green' technology; lower effort and cost in application; and also it can be applied to a wide range of metals (McIntyre, 2003).

This kind of remediation was found in the members of *Brassicaseae* family (commonly called Indian mustard) which were found to accumulate and reduce Se in soil by almost 50%, that is, that plant dropped the Se content in soils in which the plants were grown by ~50%. The chemical similarity between sulfur and selenium was thought to be the main reason for Se reduction by sulfur accumulating plants (Bañuelos et al., 1997; de Souza et al., 1999). Plants with accumulated Se in various parts of their tissue can be harvested and can thereby be used as a means of remediating Se-enriched soils (Kumar et al, 1995). The plants rich in Se can be further used as a source of Se for industrial or nutritional purposes. Also, the plant can volatilize Se to the atmosphere in the form of dimethyl selenide during which the microbes in the rhizosphere (root) may participate (Terry and Zayed, 1998). Therefore, such plants can be employed in the environmental cleanup methods of Se (Meija et al., 2002; Kahakachchi et al., 2004). Several strains of yeast and algae have also been shown to participate in remediation process (Quinn et al., 2000; Umysova et al., 2009).

Another environmental cleanup method for toxic metalloids includes the use of microorganisms such as bacteria and fungi which have the ability to alter the biogeochemical cycling of Se and Te. As part of biodegradation, microbes resistant to metalloids can lessen the metalloid-containing compound's toxicity by reduction of their toxic forms to either elemental or methylated forms which are relatively less toxic (Maiers et al., 1988; Van Fleet-Stalder and Chasteen, 1998; Basnayake et al., 2001;

Swearingen et al., 2004; Burra et al., 2009b). Selenium levels ranging from 5 μg L^{-1} to 2000 μg L^{-1} can be tolerated by many microorganisms, and almost all metalloid-resistant microbes carry out some form of metalloid reduction and methylation (Doran, 1982; Chasteen and Bentley 2003; Burra et al, 2009a). Therefore, by means of bioremediation, microbes can affect the global cycling of selenium (Maiers, et al 1988; Wilber, 1990).

Mechanisms of Biomethylation

Lots of attention is being focused on the thorough understanding of biomethylation and transformation of Se and Te, as these are considered potential environmental toxicants (Yu et al., 1997; Zhang and Frankenberger, 2005; Soudi et al., 2009). As noted above, Se- and Te-resistant strains can be potentially employed for the bioremediation of contaminated soils, sediments, industrial effluents, and agricultural drainage water rich in Se and Te species (Morlon et al., 2005; Soudi et al., 2009). This biotransformation brings some changes in the mobility and toxicity of these metalloids. Microbial biosynthesis of methylated metalloids and their physiological actions are of concern in disciplines such as medicine, toxicology and environmental studies (Doran and Alexander, 1977; Chasteen and Bentley, 2003; Zhang and Frankenberger, 2005). Investigations have been carried out to develop a detailed understanding of the mechanism of biomethylation and transformation of these metalloids. A mechanism for biomethylation of selenium and arsenic was proposed by Challenger (1945) and expanded by Chasteen (1993).

In the 1990's, Van Fleet Stalder and Chasteen reported the methylation of sulfur, selenium and tellurium by phototrophic microorganisms. Volatile sulfur compounds detected above bacterial headspace include methanethiol (MeSH), dimethyl sulfide (DMS), dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) (Ishihara et al.,

1995; Stalder et al., 1995; Van Fleet-Stalder and Chasteen, 1998). Biomethylated, volatile organo-selenium and tellurium compounds were also produced by different metalloid-resistant organisms (Zhang and Chasteen, 1994; Basnayake et al., 2001; Hapuarachchi et al., 2004; Swearingen et al., 2004; Swearingen et al., 2006; Burra et al., 2009b).

Despite the fact that microbes can actively transform Se- and Te-containing compounds, their extent of contribution towards the biogeochemical cycling of metalloids is unknown (Maiers et al., 1988). For many years, the Chasteen research group has been focusing on the aspects of metalloid biomethylation. In part, toxicological studies were carried out in our lab by employing metalloid-resistant bacteria provided by the research group of Dr. Claudio C. Vasquez, at the Universidad de Santiago de Chile. The bacterium used for this research was named LHVE and belongs to the *Bacillus* genus based on classical microscopy and biochemical tests (Bergey et al., 1984). One recent approach to probe bacterial response to exposure to metalloids has involved the insertion of genes specific to metalloid-resistance. For example, the incorporation of the *ubiE* gene from *Geobacillus stearothermophilus* V into a metalloid-sensitive organism like (wild-type)*E. coli* was carried-out to convert *E. coli* into a metalloid-resistant organism (Araya et al., 2004; Swearingen et al., 2006).

Four chapters are included in the thesis following this introductory chapter. Chapter 2 discusses the biological interactions of selenocyanate and its bioprocessing, toxicity, and detection. The presence of $SeCN^-$ in the bacterial culture medium was found to affect the growth rate of LHVE bacteria. To verify this, the specific growth rates (SGR) of bacteria were determined in the presence of different metalloid concentrations. SGR was used as a measure of relative toxicity (Bennett, 1988; Yu et al., 1997; Morlon et al., 2005).

The classical growth curve of bacteria has three phases. The first is the lag phase, the initial phase where the preculture starts adapting to the new growth medium conditions after inoculation. Therefore, little growth is observed. In the second, the log phase, cells start consuming the medium and grow rapidly, exceeding the cell death rate. There is an exponential change in growth over time, and this can be measured by light scattering and absorption, so-called optical density. Bacteria enter the stationary phase due to the buildup of metabolic products or exhaustion of essential media components by the culture, even though the limiting nutrient is often hard to determine.

A lot of variations can be observed in bacterial growth when a soluble form of a metalloid like Se or Te is added to the medium. The bacteria rapidly enter the stationary phase with a relatively short log phase because of the toxic effect of the compound. An example of a growth curve with or without toxicant is depicted in Figure 1.

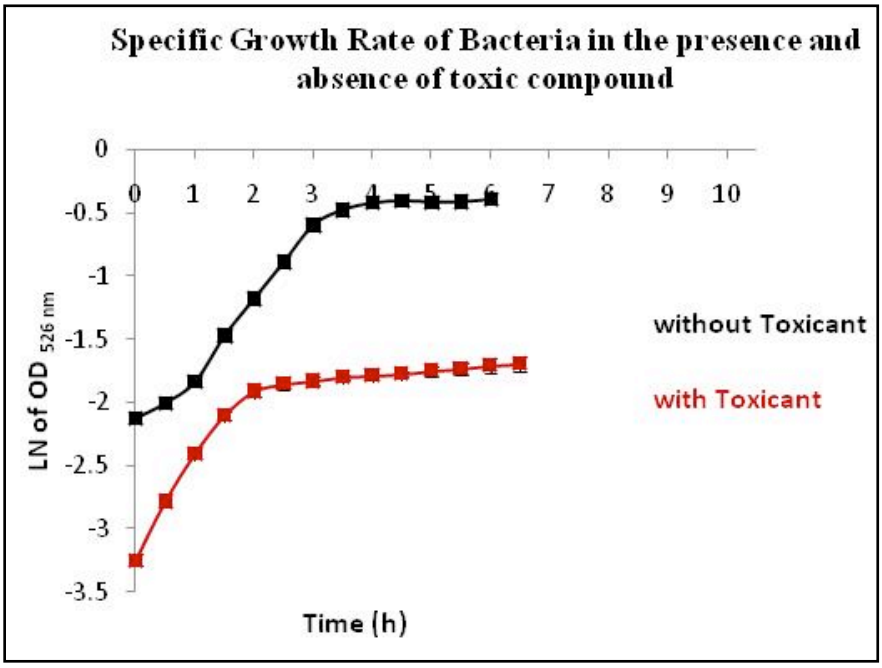

Figure 1. Typical growth curve of bacteria in the presence/absence of a toxic metalloid.

Another set of toxicity experiments performed on LHVE and reported here include zone of inhibition. This technique is used as another method for estimating the relative toxicity of metalloid anions (Speck, 1976). A filter paper disk saturated with metalloid solution is placed in the center of a sterile bacterial Petri plate containing solid agar. The plate is inoculated with a bacterium and, depending upon the toxic range of that particular metalloid, a zone of growth inhibition is formed around the disk which is considered as a measure of metalloid toxicity.

A fast and easy analytical method for the detection of methylated species of selenium and tellurium in bacterial headspace when amended with metalloids has been developed in the Chasteen lab over the past decade and achieved using gas chromatography. In Chapter 3, production of dimethyl triselenide and dimethyl diselenenyl sulfide in the headspace of metalloid-resistant *Bacillus* spp. grown in the presence of selenium oxyanions will be discussed. Analytical techniques such as gas chromatography with fluorine-induced chemiluminescence detection and gas chromatography-mass spectrometry (GC-MS) have been employed for the identification of volatile selenium- and tellurium- species.

Biomethylated or reduced forms of chalcogens such as sulfides, selenides, and tellurides can be analyzed efficiently using gas chromatography coupled with fluorine-induced (sulfur) chemiluminescence detection (GC/SCD) (Van Fleet-Stalder and Chasteen, 1998; Basnayake et al., 2001; Swearingen et al., 2006). High selectivity and sensitivity can be achieved using fluorine-induced chemiluminescence detection. In this technique, sulfur hexafluoride (SF_6) flowing across electrodes with a 1100 V potential produces gaseous, elemental fluorine *in situ* which enters the reaction vessel to react with methylated chalcogens to produce excited state hydrogen fluoride. Upon de-excitation

these excited state HF molecules emit photons that are detected via a red-sensitive photomultiplier tube. Less than one torr pressure is maintained via a vacuum pump to ensure low collisional deactivation in the reaction cell. The detection limits lie in the picogram range for most methylated sulfide, selenides, and tellurides with good selectivity over other compounds normally found in bacterial headspace. Molecules like $CH_3SeSeCH_3$ react very intensely with the F_2 compared to other molecules (such as normal hydrocarbons or alcohols) which results in low background signal (Chasteen, 1993).

The identity of unknown compounds analyzed by SCD is confirmed using GC/MS. To increase the concentration of analytes for GC analysis, a preconcentration technique called solid phase micro extraction (SPME) was employed in this work. SPME is a novel technique that can be used in both liquid and gas phases, unlike manual headspace extraction. For samples with low analyte concentration and for analysis on a less sensitive instrument, pre-concentrating the sample using SPME leads to much improvement in detection limits (Kataoka et al., 2000; Ábalos et al., 2002).

Different parameters like fiber thickness and adsorption time decide the outcome of analysis. For example, the greater the fiber thickness, the greater the proportion of analyte extraction, up to a point (Fritz, 1999). Various SPME fibers with different stationary phases can be selected depending upon the analytes of interest. Industrial applications, headspace analysis, environmental analysis, forensic analysis, toxicology, flavors, and odors are the few areas of SPME applications (Guan et al, 2007; Da Silva et al., 2007; Regueiro et al., 2009a; 2009b).

In this technique, the SPME fiber is exposed to the headspace for a specific period of time (typically 15-30 minutes for SCD and up to 45 minutes for GC/MS), during

which the analytes get adsorbed onto the thin film of the fiber's stationary phase. The extent of the equilibrium between the stationary phase and the analytes purely depends on the affinity of the analytes toward the stationary phase. The fiber can then be directly inserted into the hot GC injector where the (heated) fiber undergoes desorption of the analytes leading to their chromatographic analysis.

Chapter 4 describes a simple and novel method for the tellurite, tellurate, selenite and selenate anions quantification in culture media. Sodium borohydride ($NaBH_4$) is used as the reducing agent for the reduction of Se and Te oxyanions to their elemental forms, whose absorbance is measured at 500 nm using UV/visible spectrophotometer.

The success of speciation analysis of any element depends on the selectivity and sensitivity of the method employed. The progress in speciation analysis has been advanced by the use of hyphenated analytical instrumental techniques. For example: atomic absorption spectrometry (AAS) and gas chromatography (GC). Using these techniques, compounds can be identified and quantified simultaneously because of the combination of a powerful separation technique with an extremely sensitive detector (Pedrero and Madrid, 2009).

Hydride generation atomic absorption spectrometry (HG-AAS) (Basnayake, 2001; Tarin, 2006), gas chromatography (GC) (Meija et al., 2002; Swearingen et al., 2004), inductively coupled plasma (ICP) (Zhang et al., 2000; Darrouzes et al., 2005) and capillary electrophoresis (CE) (Pathem et al., 2007) coupled to various detectors are the widely used hyphenated analytical techniques for the analysis of Se and Te in environmental samples. Some of the disadvantages of these methods include digestion of solid samples with strong acids or the need to convert analytes to specific oxidation states resulting in loss of volatile selenium compounds. Moreover, these are highly time–

consuming, strenuous and require constant attention from operators (Bius, 2001; Tarin, 2006).

For many years, spectrophotometric determination of Se and Te have been carried out as a part of their speciation analysis (Mahaveer and Jaldappa, 2000; Revanasiddappa and Kiran Kumar, 2001). As a part of this, a new method is described in Chapter 4 which is shown to be reliable and rapid, offering a wide linear range without the need for extraction and heating. It does not involve rigorous reaction conditions and is easy when compared to the aforementioned techniques.

References

Ábalos, M.; Prieto, X.; Bayona, J.M. *J. Chromatog.A* **2002,** *936*, 249-257.

Araya, M.A.; Swearingen Jr., J.W.; Plishker, M.F.; Saavedra, C.P.; Chasteen, T.G.; Vásquez, C.C. *J. Biol. Inorg. Chem*. **2004**, *9*, 609-615.

Bagnall, K.W. *The chemistry of selenium, tellurium and polonium*; Elsevier Press: Netherlands, **1966**; Chapter 1.

Bañuelos, G.S.; Ajwa, H.A.; Terry, N.; Zayed, A. *J. Soil Water Conserv.* **1997**, *52*, 426-430.

Basnayake, R.T. M.S. Thesis, Sam Houston State University, Huntsville, TX, **2001**.

Basnayake, R. T.; Bius, J. H.; Akpolat, O.M.; Chasteen, T.G. *Appl. Organomet. Chem.* **2001**, *15*, 499-510.

Bennett, W. N. *Water Res.* **1988***,22*, 939–942.

Bergey, D. H.; Holt, J. G.; Krieg, N.R.; *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins Publishers, Baltimore, MD, **1984.**

Bius, J. H. M. S.Thesis, Sam Houston State University, Huntsville, TX, **2001**.

Boisson, F.; Gnassia-Barelli, M.; Romeo, M. *Arch. Environ. Contam. Toxicol*. **1995**, *28*, 487-493.

Brasher, A.M.; Ogle, S. *Arch. Environ. Contam. Toxicol*. **1993**, *24*, 182-186.

Burra, R.; Fox, J. D.; Pradenas, G. A.; Vasquez, C. C.; Chasteen, T.G. *Environ. Technol.***2009a**, in press.

Burra, R.; Pradenas, G. A.; Montes, R. A.; Vasquez, C. C.; Chasteen, T.G. *Anal. Biochem*. **2009b**, in press;

Challenger, F. *Chem. Rev.* **1945**, *36*, 315-361.

Chasteen, T.G. *Appl. Organomet. Chem.* **1993**, *7*, 335-342.

Chasteen, T.G.; Bentley, R. *Chem. Rev.* **2003**, *103*, 1-26.

Chen, K.M.; Sacks, P.G.; Spratt, T.E.; Lin, J.M.; Boyiri, T.; Schwartz, J.; Richie, J.P.; Calcagnotto, A.; Das, A.; Bortner, J.; Zhao, Z.; Amin, S.; Guttenplan, J.; El-Bayoumy, K. *Biochem. Biophys. Res. Commun*. **2009**, *383*, 151-155.

Clark, L.C.; Combs, G. F; Turnball, B.W.; Slate, E.H.; Chalker, D.K.; Chow, J.; Davis, L.S.; Glover, R.A.; Graham, G.F.; Gross, E.G.; Krongrad, A.; Lesher, J.L.; Park, H.K.; Sanders, B.B.; Smith, C.L.; Taylor, J.R.; Alberts, D.S.; Allison, R.J.; Bradshaw, J.C.; Curtus, D.; Deal, D.R.; Dellasega, M.; Hendrix, J.D.; Herlong, J.H.; Hixon, L.J.; Knight, J.; Moore, J.; Rice, J.S.; Rogers, A.I. ; Schuman, B.; Smith, E.H.; Woodward, J.C. *J. Am. Med. Assoc*, **1996**, *276*, 1957-1963.

CRC *Handbook of Chemistry and Physics*, CRC Press: Cleveland, OH, **1975**.

Da Silva, G.A.; Augusto, F.; Poppi, R.J. *J. Chromatogr. A*, **2007**, 1138, 251-261.

Darrouzes, J.; Bueno, M.; Lespes, G.; Potin-Gautier, M. *J. Anal. At. Spectrom*. **2005**, *20*, 88-94.

de Souza, M. P; Chu, D.; Zhao, M.; Zayed, A. M.; Ruzin, S. E.; Schichnes, D.; Terry, N. *Plant Physiol*., **1999**, *119*, 565-573.

Doran, J.W. *Adv. Microb. Ecol.*1**982**, *6,* 1-32.

Doran, J. W.; Alexander, M. *Appl. Environ. Microbiol*. **1977**, *33*, 31-37.

El-Bayoumy, K.; Das, A.; Narayanan, B.; , Narayanan, N.; Fiala, E.S.; Desai, D.; Rao, C.V.; Amin, S.; Sinha, R. *Carcinogenesis*. **2006**, *27*, 1369-1376.

Emsley, J. *Nature's Building Blocks*: *An A-Z Guide to the Elements*; Oxford University Press: New York, **2002**.

Engberg, R. A.; Westcot, W.D.; Delamore, M.; Holz, D.D. *Environmental Chemistry of Selenium*, Marcel Dekker, New York, **1998**.

Engman, L.; Al-Maharik, N.; McNaughton, M.; Birmingham, A.; Powis, G. *Anticancer Drugs,* **2003***, 14*, 153–161.

Ernst, W.H. Metal Contaminated Soils; In: Vangronsveld J.; Cunningham S.C. eds. In Situ Inactivation and Phytorestoration, Springer: New York, **1998**, 17.

Fan, A. M.; Kizer, W. K., *West J. Med.* **1990**, *153*, 160-167.

Fritz, J. S. *Analytical Solid Phase Extraction*, Wiley-VCH: New York, **1999**, 161-171.

Golub, A. M.; Skopenko, V. V. *Russ. Chem. Rev.* **1965**, *34*, 901- 908.

Gómez-Ariza, J. L.; Pozas, J.A.; Giráldez, I; Morales, E. *Analyst*. **1999a**,*124*, 75-78.

Gómez-Ariza, J. L.; Pozas, J.A.; Morales, E. *J. Chromatogr. Sci*. **1999b**, *37*, 436-442.

Guan, W.; Xu, F.; Liu, W.; Zhao, J.; Guan,Y. *J. Chromatogr. A,* **2007***,* 1147, 59-65.

Hapuarachchi, S.; Swearingen Jr., J.W.; Chasteen, T. G. *Process Biochem*. **2004**, *39*, 1607-1613.

Ishihara, K.; Honma, N.; Uchiyama, T, *J. Ferment. Bioeng.* **1995**, *80*, 633.

Jacob, C.; Arteel, G. E.; Kanda, T.; Engman, L.; Sies, H. *Chem. Res. Toxicol.* **2000**,*13,* 3-9.

Kahakachchi, C.; Boakye, H.T.; Uden, P.C.; Tyson, J.F. *J. Chromatogr.* A**2004**, *1054*, 303-312.

Kapoor, A.; Tanjore, T.; Viraghavan, T. *Envion. Sci. Technol*. **1995**, *49*, 137-147.

Kataoka, H.; Lord, H. L.; Pawliszyn, J. *J. Chromatogr.* A**2000**, *880*, 35-62.

Kumar, P. B. A. N.; Dushkenov, V.; Motto, H.; Raskin, I. *Environ. Sci. Technol*. **1995**, *29*, 1232–1238.

Kuo, C. Y.; Jiang, S. J, *J. Chromatogr. A***2008**, *1181*, 60-66.

Lasat, M. Unpublished manuscript from the USEPAl Am. Assoc. Adv. Sci. Fellowship Prog., **2000**, 34.

Lemly, A. D.; Ohlendorf, H. M. *Ecotoxicol. Environ. Saf*. **2002**, *52*, 46-56.

Mahaveer, B. M.; Jaldappa, S. *Turk. J. Chem*. **2000**, *24*, 287-290.

Maiers, D. T.; Wichlacz, P. L.; Thompson, D. L.; Bruhn, D. F, *Appl. Environ. Microbiol.* **1988**, *54*, 2591-2593.

McIntyre, T. *Adv. Biochem. Eng. Biotechnol*. **2003**, *78*, 97-123.

Meija, J.; Montes-Bayón, M.; Le Duc, D.L.; Terry, N.; Caruso, J. A. *Anal. Chem.* **2002**, *72*, 5837-5844.

Meng, X.; Bang, S.; Korfiatis, G.P.; *Water Res*. **2002**, *36*, 3867-3873.

Miekeley, N.; Pereira, R.C.; Casartelli, E. A.; Almeida, A. C.; Carvalho, M. F. B. *Spectrochim. Acta B* **2005**, *60*, 633-641.

Morlon, H.; Fortin, C.; Adam, C.; Garnier, L. J., *Radioprot.* **2005**, *40,* 101-106.

Ohlendorf, H. M.; Hoffman, D. J.; Saiki, M. K.; Aldrich, T. W. *Sci. Total Environ*. **1986**, *52*, 49-63.

Ohlendorf, H. M. Soil Science Society of America; Madison, WI,; Special Publication No. 23. **1989**, 133-177.

Ohlendorf, H. M. *Aquat Toxicol.* **2002**, *57*, 1-10.

Pathem, B. K.; Pradenas, G. A.; Castro, M. E.; Vasquez, C. C.; Chasteen, T. G. *Anal. Biochem*. **2007**, *364*, 138-144.

Pedrero, Z.; Madrid, Y.*Anal. Chim. Acta*. **2009**, *634*, 135–152.

Philip, J. C.; Bamforth, S. M.; Singleton, I.; Atlas, R. M. Environmental pollution and restoration: A role for bioremediation. In: Atlas R. M., Philip J. eds. Bioremediation: applied microbial solutions for real-world environmental cleanup; ASM Press: Washington; 2005; 1-48.

Quinn, N. W. T.; Lundquist, T. J.; Green, F. B.; Zarate, M. A.; Oswald, W. J.; Leoghton, T. *Cal. Agri*. **2000**, *54*, 53-56.

Rayman, M. P. *Lancet*, **2000**, *356*, 233-241.

Regueiro, J.; Becerril, E.; Garcia-Jaresa, C.; Llomparta, M. *J. Chromatogr. A*, **2009a**, *1216*, 4693–4702.

Regueiro, J.; Llompart, M.; Carmen Garcia-Jares, C.; Cela, R. *J. Chromatogr. A*, **2009b**, *1216*, 2816–2824.

Revanasiddappa, H. D.; Kiran Kumar, T. N. *Anal. Sci*. **2001**, *17*, 1309-1312.

Schrauzer, G. N. *Cell Mol. Life Sci*. **2000**, *57*, 1864-1873.

Semba, R. D.; Ricks, M. O.; Ferrucci, L.; Xue, Q-L.; Guralnik, J. M.; Fried, LP, *Eur. J. Clin. Nutr.,* **2009**, *63*, 93-99.

Seward, M.R.; Richardson, D.H. In: Shaw, A. J. ed.; *Heavy Metal Tolerance in Plants: Evolutionary Aspects*, CRC Press: Boca Raton, FL, **1990**, 75.

Sharmasarkar, S.; Vance, G.F.; Cassel-Sharmasarkar, F, *Environ. Geol*. **1998**, *34*, 31-38.

Soderback, E, *Acta Chem. Scand.* **1974**, *28*, 116-117.

Soudi, M. R.; Ghazvini, P. T. M.; Khajeh, K.; Gharavi, S. *J. Hazard. Mater.* **2009**, *165*, 71-77.

Speck, M. L. Compendium of Methods for the Microbial Examination of Foods; American Public Health Association: New York, **1976**.

Stalder, V.; Bernard, N.; Hanselmann, K.W.; Bachofen, R.; Chasteen, T.G, *Anal. Chim. Acta* **1995**, *303*, 91-97.

Sun, P.P., Shell Oil Company, personal communication, 2008.

Swearingen Jr., J.W.; Araya, M.A.; Plishker, M.F.; Saavedra, C.P.; Vasquez, C.C.; Chasteen, T.G. *Anal. Biochem.***2004**, *331*, 106-114.

Swearingen Jr., J.W.; Fuentes, D.E.; Araya, M.A.; Plishker, M.F.; Saavedra, C.P.; Chasteen, T.G.; Vasquez, C.C. *Appl. Environ. Microbiol*. **2006**, *72*, 963-967.

Tarin, P. Ph.D. Thesis, University of Wollongong, New South Wales, Australia, **2006**.

Terry, N; Zayed, A. *Environmental Chemistry of Selenium*, Marcel Dekker, New York, **1998**. 633-656.

Umysová, D.; Vítová, M.; Douškova, I.; Bišová, K.; Hlavová, M.; Čížková, M.; Machát, J.; Doucha, J.; Zachleder, V. *BMC Plant Biol,* **2009**, *9*, 58.

Vadhanavikit, S.; Kraus, R. J.; Ganther, H. E. *Arch. Biochem. Biophys.* **1987**, *258*, 1-6.

Van Fleet-Stalder, V.; Chasteen, T.G, *J. Photochem. Photobiol. B*.**1998**, *43*, 193-203.

Wilber, C.G. *Clin. Toxicol*. **1980**, *17*, 171-230.

World Health Organization; "Chemical hazards in drinking-water–selenium"; http://www.who.int/water_sanitation_health/dwq/chemicals/selenium/en/; **2009.**

Wrobel, K.; Wrobel, K.; Caruso, J.A. *Anal. Bioanal. Chem.* **2005**, *381*, 317-331.

Yarema, M.C.; Curry, S.C. *Pediatrics*. **2005**, *116*, 319-321.

Yu, R.; Coffman, J.P.; Van Fleet-Stalder, V.; Chasteen, T.G.; *Environ. Toxicol. Chem*. **1997**, *16*, 140-145.

Zhang, L.; Chasteen, T.G. *Appl. Organomet. Chem*. **1994**, *8*, 501-508.

Zhang, Y.; Frankenberger, W. T., Jr. *Sci. Total Environ*. **2005**, *346*, 280-285.

Zhang, Y.; Kharel, Y.; Koyama, T. *J. Mol. Catal. B Enzym.* **2000**, *10*, 623-630.

CHAPTER 2

BIOLOGICAL INTERACTIONS OF SELENOCYANATE: BIOPROCESSING, DETECTION AND TOXICITY

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ABSTRACT

Selenocyanate anion, $SeCN^-$, has been reported in wastewater from refineries whose petroleum comes from Se-rich marine shales. A metalloid-resistant bacterium was exposed to aqueous solutions of $SeCN^-$ to examine the relative toxicity of $SeCN^-$ and results compared to the toxicity of selenate and selenite and another G16 metalloid's oxyanion, tellurite. We also determined the volatile organo-selenium species produced by bacterial cultures amended with selenocyanate anion; and we investigated a solid phase preconcentration technique for collecting $SeCN^-$ from aqueous samples with different ionic strengths and subsequent detection using capillary electrophoresis. The relative toxicity of $SeCN^-$ is comparable to selenate and selenite using the metalloid-resistant bacterium LHVE as the test organism. Tellurite was more toxic at all concentrations examined than all three selenium-containing anions, SeO_4^{2-}, SeO_3^{2-}, $SeCN^-$. Live cultures of LHVE amended with 1 mM NaSeCN produced volatile organo-sulfides and organo-selenides that could be collected in headspace using a solid phase microextraction fiber. The bioprocessing, that is reduction and methylation of $SeCN^-$, is similar to that of selenate and selenite by other metalloid-resistant bacteria. Aqueous 1.0 mM solution of $SeCN^-$ could be captured from solution on solid phase extraction (SPE) cartridges using aminopropyl-based stationary phase. Selenocyanate anions slowly pumped into a prewet SPE cartridge were trapped on the cartridge's solid phase and were subsequently eluted, thereby providing an increase in concentration above the original $SeCN^-$-containing solution. Preconcentration factors of 3.9 were achieved using a mixed sodium hydroxide/methanol elution solvent and by adding NaCl to aqueous $SeCN^-$ before loading on the SPE cartridge.

Keywords: $SeCN^-$; environmental selenium; petrochemical wastewater; solid phase extraction of metalloid anions; specific growth rate

INTRODUCTION

The two Group 16 elements immediately below sulfur are selenium (Se) and tellurium (Te). Although selenium (Se) is a toxic metalloid, for humans it is also a required nutrient; however, the range between required and toxic levels is surprisingly small [1]. Highly toxic selenium oxyanions, usually as selenate (SeO_4^{2-}) salts, are added to multivitamin formulations to provide trace amounts of Se as a dietary supplement. Determination of selenate and selenite (SeO_3^{2-}) in the environment has been on-going for a long time, because, in some settings, Se deficiencies are prominent in humans and animals in regions with low Se-containing soils and foods grown there, and in some cases toxic effects on mammals are noted because of Se enrichments of plants and soils [1,2].

In contrast to the long historical investigation of the environmental effects of selenate and selenite in aqueous systems, recently another water soluble selenium anion has been reported in the literature: Selenocyanate ($SeCN^-$) is produced in the process of petrochemical refining of crude oils that arise from marine shales, and minute amounts of selenocyanate are discharged in aqueous wastewater [3,4], escaping the last coagulation and precipitation step, usually involving iron salts. Meng et al. reported in 2002 that elemental iron could be used at a pH of 6 to reduce and precipitate $SeCN^-$ in these waste streams [5], but analysis of trace amounts of $SeCN^-$ is still required.

Bioprocessing (biological transformation from one chemical form to another) of selenium oxyanions is an area that has received a lot of attention as a means of detoxifying natural pollution events such as Se salt buildup in the central valley of California USA [6,7]. Extensive field tests have been carried out to determine the

viability of using either Se reduction and methylation to dimethyl selenide or to precipitate elemental Se as a means of bioremediation of agricultural irrigation drainage-water [8]. Volatile organo-selenides produced by bacterial response when exposed to selenium oxyanions besides dimethyl selenide include dimethyl diselenide, dimethyl selenenyl sulfide, and most recently dimethyl selenodisulfide ($CH_3SeSSCH_3$) [9].

Again in contrast to SeO_4^{2-} and SeO_3^{2-}, whose relative toxicity is well established, the toxicity and fate of selenocyanate in aqueous systems has not been studied, nor has the result of the interactions of dissolved $SeCN^-$ with Se-resistant bacteria. Only a single evaluation of selenocyanate bioprocessing has been reported using mice as the model organism [10]; however, organic molecules with selenocyanate moieties have been investigated as chemopreventive agents. While selenite is, in general, considered more toxic than selenate for most organisms [11-14], where selenocyanate falls is still unknown. In this report, we examined the relative toxicity to metalloid-resistant bacteria of $SeCN^-$ as compared to that of selenate and selenite and another G16 metalloid's oxyanion, tellurite (TeO_3^{2-}); we also determined the volatile organo-selenium species produced by live bacterial cultures amended with selenocyanate anion; and we investigated a solid phase extraction technique for collecting $SeCN^-$ from aqueous samples with different ionic strengths.

MATERIALS AND METHODS

Reagents

The sources for the chemical reagents used in these experiments are as follows: bacto ™ tryptone (pancreatic digest of casein) from Becton Dickson and company (Sparks, MD, USA); yeast extract from Difco Laboratories (Detroit, MI, USA); sodium selenate, sodium selenite, sodium tellurite trihydrate from Sigma-Aldrich (Milwaukee, WI, USA);

potassium selenocyanate and tetradecyl trimethyl ammonium bromide (TTAB) from Acros Organics (New Jersey, USA); sodium chloride from BDH (West Chester, PA, USA); agar from Amresco (Solon, OH, USA). A RiOs 3 water purification system from Millipore (Billerica, MA, USA) was employed in the purification of water, necessary for these experiments. Isolute® solid phase extraction cartridges (aminopropyl, stationary phase) were purchased from International Sorbent Technology (Glamorgan, UK).

Metalloid resistant organism

The bacterium used in this study was isolated from sediment and water from Tinguilico Lake, 9th Region, Chile, as follows [15,16]: 1 mL of sediment suspension was serially diluted in Luria-Bertani (LB) media, incubated under aerobic conditions at 37, 25 and 15 ºC for 24 h. Growth was achieved in samples incubated at 25 and 37 ºC. Serial dilutions were then seeded in LB-agar plates amended with 40 μM of K_2TeO_3 and incubated for 24 h at the respective temperatures. Several colonies belonging to the same organism were obtained at both temperatures. A purified, single colony was grown in LB media and tested for metalloid resistance. Classical microscopy and biochemical tests [15,16] indicated that the isolated bacterium belongs to the *Bacillus* genus and was named LHVE.

Bacterial cultures and growth media

Bacterial cells were grown anaerobically in LB medium at 37 ^{o}C which was prepared by adding 10.0 g of pancreatic digest of casein, 5.0 g of yeast extract and 5.0 g of sodium chloride to 1.0 L of water and mixed thoroughly. The pH of the solution was adjusted to 7.0 with 1.0 M sodium hydroxide, if necessary. This medium was sterilized at 121 ^{o}C and 15 psi for 20 min.

Preparation of precultures was carried out by inoculating sterile LB medium with a single LHVE colony and the liquid culture incubated anaerobically for 24 h. Cultures for growth curve analysis and headspace analysis were made by distributing sterile LB medium into 16-mL test tubes and inoculating with a 1:10 v/v inoculum from the liquid preculture whose final volume was 10 mL. Sterile filtered metalloid solutions (0.2 μm pore size) were then added to these test tubes to achieve required concentration. Tubes for growth analysis were screw capped; tubes for headspace analysis were capped with open-top screw caps with Teflon®/silicon-lined septa (Alltech, Deerfield, IL, USA). After 48 h of selenium amendment and anaerobic incubation at 37 °C, headspace sampling was carried out.

Growth curves

The patterns of bacterial growth determined in the presence of different metalloid concentrations were used as a measure of relative toxicity [11, 17-19]. Optical density/scattering readings at 525 nm were taken periodically on replicate cultures incubated at 37 °C until the bacterium reached the stationary phase. A plot of the natural log of the optical density versus growth time in hours was plotted.

Zone of inhibition

Zone of inhibition experiments [20, 21] were used as a second method of estimating the relative toxicity for the metalloid anions. LHVE bacteria were grown in liquid LB medium to the stationary phase at 37 °C. Fresh, sterilized LB media in test tubes were inoculated with a 10% vol/vol inoculum and incubated to 0.1 $OD_{525\ nm}$. Culture dilutions of 10^4 were made with sterilized water and evenly spread on solidified agar plates. Metalloid solutions of various concentrations ranging from 25–100 mM and volumes of 1–5 microliters were pipetted directly onto a sterile filter paper disk in the center of the

plate. These plates were covered and incubated at 37 °C for 9-12 hours when the diameter of the zones of inhibition was measured.

Capillary electrophoresis

All CE samples were run according to the methods developed by Pathem et al. [22]. The run buffer was made by adding 0.204 g KH_2PO_4 and 0.036 g TTAB to 100 mL of water. This was mixed thoroughly and then the pH of the buffer was adjusted to 10.5 using 1.0 M NaOH. All fluids (samples, buffers, wash liquids, etc.) were sterile filtered using 0.2-µm syringe filters (Pall Corporation, Ann Arbor, MI, USA) before introduction into the CE. The capillary was conditioned by injecting 1.0 M NaOH for 5 min at 40 ºC, then rinsed with water for 5 min at 25 ºC. For all samples, the capillary was maintained at 25 ºC. Before each sample, the capillary was rinsed with water for 1 min, methanol for 2 min, water for 1 min, 0.1 M HCl for 2 min, water for 1 min, 1.0 M NaOH for 2 min and then with run buffer for 2 min. This ensured that the capillary was thoroughly cleaned and prepped for the sample. Samples were injected using hydrodynamic injection at 0.5 psi for 5 sec. The separation was performed using -25 kV over the course of five minutes. Data acquisition was accomplished using a photodiode array to simultaneously monitor absorbance from 190-300 nm. Selenate and selenite were monitored at 190 nm and selenocyanate was monitored at 200 nm. Quantification of the peaks was accomplished with peak integration based on a standard curve.

Solid phase extraction for $SeCN^-$ preconcentration

Cartridges

Isolute® solid phase extraction cartridges contained 500 mg of sorbent material (housed in a 10-mL reservoir). In order to effectively solvate and extract analytes using the SPE cartridges, buffers and samples were required to pass over the sorbent at approximately

1 mL/min. This was accomplished by assembling a rudimentary device (Figure 2) that used compressed air to achieve the desired flow rate. The cartridges were solvated with 10 mL of a solution of 15 mM KH_2PO_4 in a 50/50 mix of water and methanol. Then the sample was loaded into the reservoir and eluted using compressed air. The cartridge was removed from the device and fitted with a 0.2-µm sterile filter. Elution buffer was loaded into the reservoir and 2.0 mL of the buffer (pH = 11.8) were passed through the sorbent and filter directly into CE sample vials for analysis. This process is detailed in Figure 3. In step A, the cartridge was solvated with the buffer solution. This protonated the amine groups, creating a positive charge in the solid phase surface that was balanced by the phosphate anions. The extraction of selenocyanate occurred in step B, causing selenocyanate to displace the phosphate ions and accumulate on the cartridge's solid phase. The subsequent elution in step C (performed with sodium hydroxide) deprotonated the amino groups, causing the selenocyanate to continue through the column and elute. Two different elution buffers were examined. The first contained only the sodium hydroxide used to adjust the pH. The second contained 86 mM sodium chloride in addition to the sodium hydroxide. The sodium chloride was used in attempt to actively displace selenocyanate bound to the solid phase.

Headspace sampling of bacterial cultures

SPME culture headspace sampling

The collection of headspace was carried out using 75 µm Carboxen^{TM} - polydimethylsiloxane (PDMS) solid-phase microextraction (SPME) fiber from Supelco (Bellfonte, PA, USA). The concentrations of analyte gases were brought up to the detectable range for GC/MS analysis by the use of SPME. The adsorption timings of SPME in the headspace ranged between 15 and 50 min at 37 ^{o}C. The fiber exposure was

carried out by piercing the septum of the tube with the needle which contained the SPME fiber. The collected samples were then introduced into the hot injector of the GC where the SPME fiber undergoes desorption process. The fiber was left in the injector for the entire run, but the septum purge valve was opened at 1 min.

Gas chromatography with F_2-induced chemiluminescence analysis

This chromatographic method has been published elsewhere [23], but briefly a splitless injection mode was used for the sample analysis. The GC injector temperature was 275 ^{o}C. The initial oven temperature was 30 ^{o}C, held for 2 min and then ramped to 250 ^{o}C at 15 ^{o}C/min. The final temperature was held for 5 min.

Gas chromatography with mass spectrometric analysis

GC/MS analysis was accomplished by a Hewlett-Packard 5973 Mass Selective Detector (using 70 eV electron impact mode). The carrier gas was the ultra-high-purity helium with a flow rate of 1 mL/min. An Agilent HP-5 (0.5 μm film, 30 m length) chromatographic column was used for GC/MS. The temperature program used for GC/MS was similar to that used for GC-SCD. Splitless injections were carried out using the SPME fiber inserted into the 275 °C injector.

RESULTS AND DISCUSSION

Selenocyanate toxicity

The relative toxicity of three selenium-containing anions and tellurite anion were examined at 1, 5, and 10 mM toxicant concentrations (Figure 4) in growth culture experiments. Optical density (light scattering at 525 nm) was used as a measure of cell population. At the lowest concentration, 1 mM, little growth rate differences were determined over the 10 h period of the experiment and the final optical density of all cultures were close except for the 1 mM tellurite amendments which were clearly lower.

Our 5 and 10 mM amendments showed more obvious differences in both final optical density and the progress of the earlier growth phase. While tellurite is still the most toxic of the metalloid anions examined, using these experiments selenocyanate exhibits toxicity comparable to that of selenate and selenite. Similar patterns of bacterial growth for cultures exposed to other toxicant has been reported before, including lower final biomass and especially the biphasic log phase growth patterns [17] seen in here for higher concentrations of added metalloidal anions (Figure 4C).

The zone of inhibition experiments confirmed the growth curve experiment's relative toxicity of the selenium-containing anions as compared to tellurite. On plates amended with either 1 or 5 microliter of toxicant, only the plate with 25 mM TeO_3^{2-} showed a zone of inhibition (diameter 52 mm) while analogous selenate, selenite, and selenocyanate at 25 mM exposure experiments showed LHVE bacterial growth uniformly across the entire surface of the plate. Subsequent plates prepared with 100 mM concentrations of the selenium-containing anions and with 5 μL volumes added to the plate also yielded uniform growth with no apparent growth inhibition. Selenite and tellurite exposed plates examined after 10 hours showed the formation of elemental selenium, as a red deposit and elemental tellurium, a black deposit, respectively, at the interface of bacteria growing closest to the plate's center. Tellurite-amended plates showed more elemental Te production for the 5 μL amended plates as compared to the 1 μL-amended plates.

Volatile headspace compounds

GC with fluorine-induced chemiluminescence and GC-MS analysis of selenocyanate-amended cultures yielded bacterial-produced organo-sulfur and organo-selenium compounds released into culture headspace. Volatile compound identities were

determined by comparison of retention times of purchased standards or mass spectra. Figure 5 is a time shot of a $SeCN^-$-amended culture after 72 h using a 15 min SPME fiber headspace exposure. Along with volatile organo-sulfurs normally produced by a growing culture, organo-selenide detected included dimethyl selenide (DMSe), dimethyl selenenyl sulfide (DMSeS), and dimethyl diselenide (DMDSe). Dimethyl selenodisulfide was also detected in $SeCN^-$-amended LHVE cultures with longer SPME fiber exposure times (data not shown). Se-free cultures produced only volatile organo-sulfides. Sterile controls handled identically yielded no detectable headspace compounds. Although volatile organo-selenium compounds have long been reported in cultures amended with selenium oxyanions, this is the first report of reduction and methylation of selenium in selenocyanate by Se-resistant bacteria. Although quantification was not attempted for the compounds reported here, compared to our work with other metalloid resistant bacteria under similar growing conditions [9, 23, 24], the headspace production of LHVE amended with selenocyanate was similar to the headspace production of this bacterium amended with selenite or selenate. Vadhanavikit et al. [10] reported that mice dosed subcutaneously with $SeCN^-$ produced DMSe in exhaled breath. Studies to determine whether endogenous bacterial reduction and methylation are the source of volatile organo-metalloids that have been reported in mammalian breath have yet to be carried out.

Selenocyanate preconcentration

Preconcentration of $SeCN^-$ by SPE in these experiments was measured by loading a solvated (prewet) SPE cartridge with a known volume of a solution of sodium selenocyanate (usually 10 mL) of known concentration (1.0 mM). After loading, the cartridge contents was eluted using 2 mL of solvent and the concentration of $SeCN^-$ in

that 2 mL subsequently determined via capillary electrophoresis. Increases in $SeCN^-$ concentration in the eluted solution verses that used to load the cartridge were considered preconcentration.

Two different SPE cartridge solvating buffer solutions were considered. Solvation or prewetting of the SPE cartridge was examined using a buffer solution of 50/50 (vol/vol) mixture of methanol/water with 15 mM potassium dihydrogen phosphate (KDP, pH 6) or a 50/50 mixture of acetonitrile/water with 15 mM potassium dihydrogen phosphate (pH 6.5). Using the same concentration of selenocyanate in test samples loaded onto the cartridge, pre-wetting with methanol/water/KDP yielded 2.5 times the preconcentration factor as compared to acetonitrile/water/KDP. The methanol/water/KDP solvation buffer was used in all subsequent experiments. See Table 1.

Table 1. Solid phase extraction parameters.

[$SeCN^-$]	Solvation buffer	Eluting solvent	Preconcentration factor
1.0 mM	Acetonitrile/water/KDP	NaOH	1.0
1.0 mM	Methanol/water/KDP	NaOH	2.5
1.0 mM	Methanol/water/KDP	Methanol/water/pH 11.8	3.3
1.0 mM	Methanol/water/KDP	NaOH/85.5 mM NaCl	3.9

KDP is potassium dihydrogen phosphate.

Three different eluting solvents were used to release trapped $SeCN^-$ on the cartridges solid phase: sodium hydroxide (1 mM), a 50/50 water/methanol solution with

pH adjusted to 11.8, and an 85.5 mM NaCl solution in 1 mM sodium hydroxide. Concentration factors were highest with the NaCl/NaOH eluting solvent.

The effect of the ionic strength of other anions in selenocyanate-containing solutions on $SeCN^-$ preconcentration was examined by adding sodium chloride (85.5 mM) to solutions containing known amounts of selenocyanate (1.0 mM) and carrying out the SPE preconcentration method. While a preconcentration factor of 3.3 was achieved for the pH adjusted 50/50 methanol/water elution solvent, a maximum preconcentration factor of 3.9 was achieved with the NaCl/NaOH elution solvent. Table 1 summarizes all SPE experiments. Given the CE method's detection limit for $SeCN^-$ in complex growth medium [22], this preconcentration factor would allow detection limits (3S/N) of a few hundred ppb.

CONCLUSIONS

The relative toxicity of selenocyanate anion is comparable to selenate and selenite using the metalloid-resistant bacterium LHVE as the test organism. Tellurite was more toxic at all concentrations examined than all three selenium-containing anions using growth curve and zone of inhibition methodology with a metalloid-resistant bacterium.

Live cultures of LHVE amended with 1 mM sodium selenocyanate produced volatile organo-sulfides and organo-selenides that could be collected in headspace using an SPME fiber. Organo-selenium species produced included DMSe, DMSeS, and DMDSe. The reduction and methylation of $SeCN^-$ is similar to that of selenate and selenite by other metalloid-resistant bacteria.

Aqueous 1.0 mM solution of $SeCN^-$ can be captured from solution on solid phase cartridges using aminopropyl-based stationary phase. Selenocyanate anions slowly flowed in solution (1 mL/min) into a prewet cartridge were trapped on the cartridge's

solid phase and were subsequently eluted, thereby providing an increase in concentration above the original $SeCN^-$–containing solution. Preconcentration factors of 3.9 were achieved using a mixed sodium hydroxide/methanol elution solvent and by adding sodium chloride to aqueous $SeCN^-$ before loading on the SPE cartridge.

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Figures

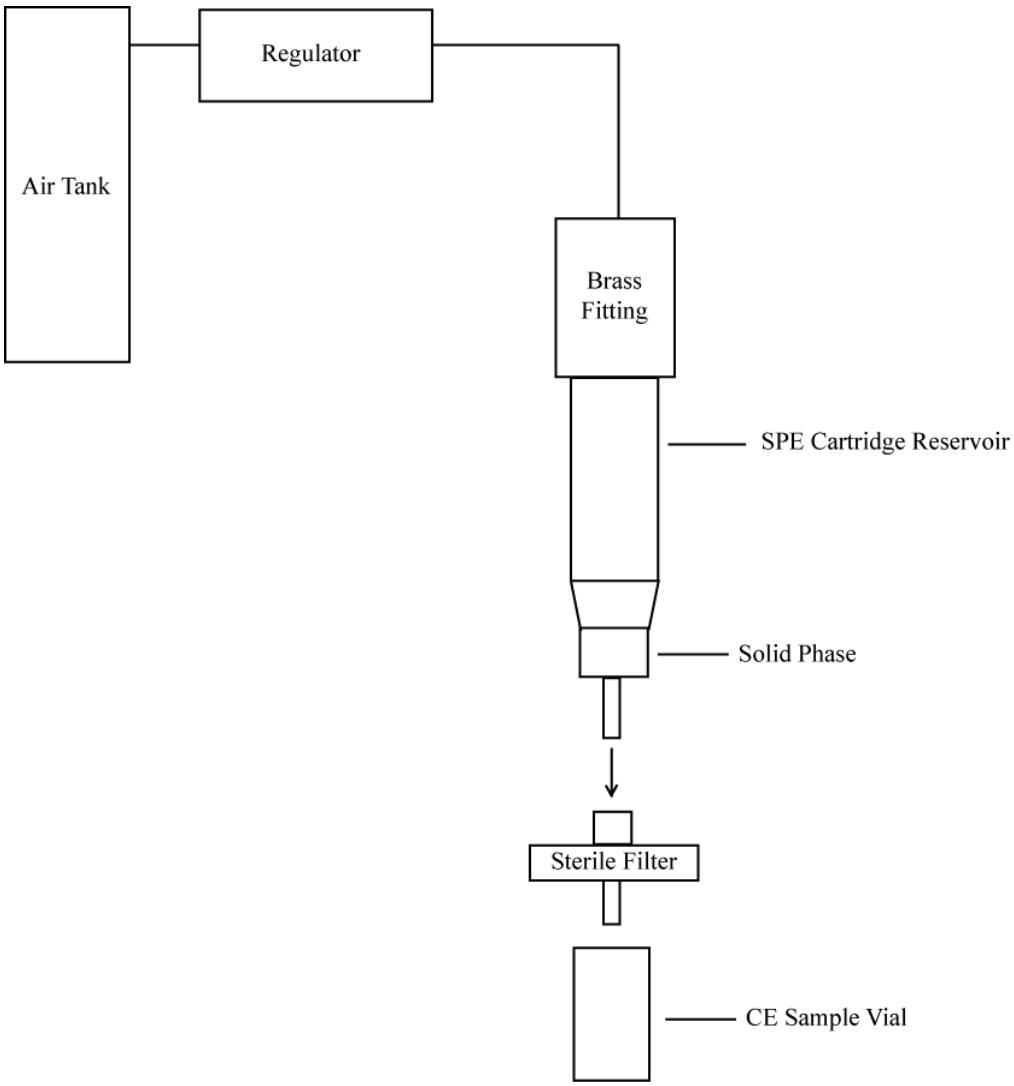

Figure 2. Solid Phase Extraction Apparatus

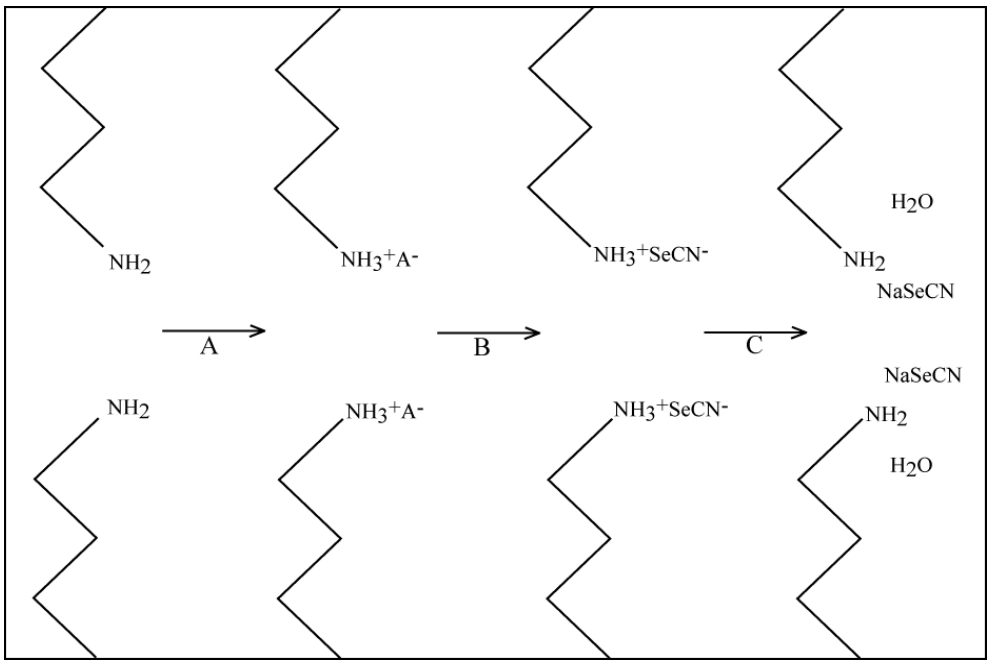

Figure 3. Extraction process on solid phase cartridge used in $SeCN^-$ preconcentration.

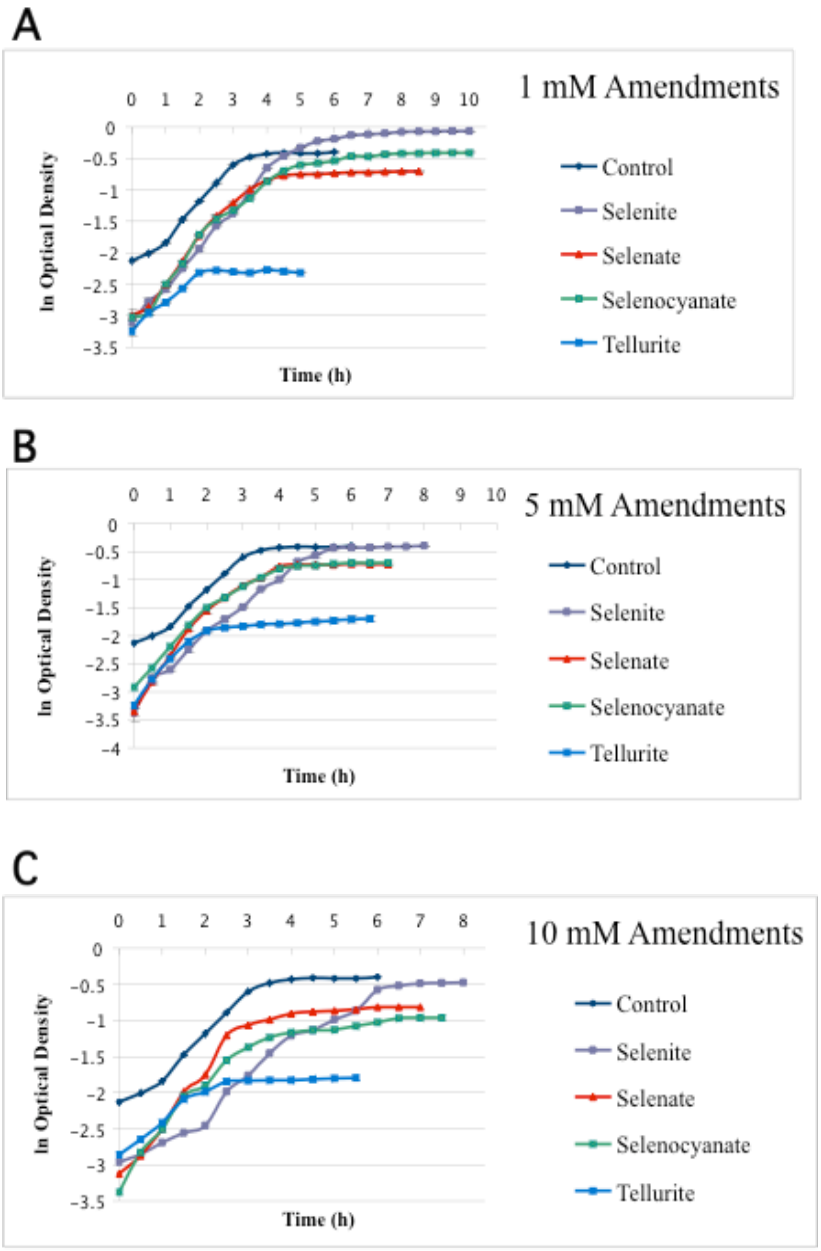

Figure 4. Bacterial Growth Curves in the presence of metalloids. A: Bacterial growth curves of 1 mM toxicant amendment to live LHVE cultures. B: Bacterial growth curves of 5 mM toxicant amendment to live LHVE cultures. C: Bacterial growth curves of 10 mM toxicant amendment to live LHVE cultures. Error bars are one standard deviation of 3 replicates.

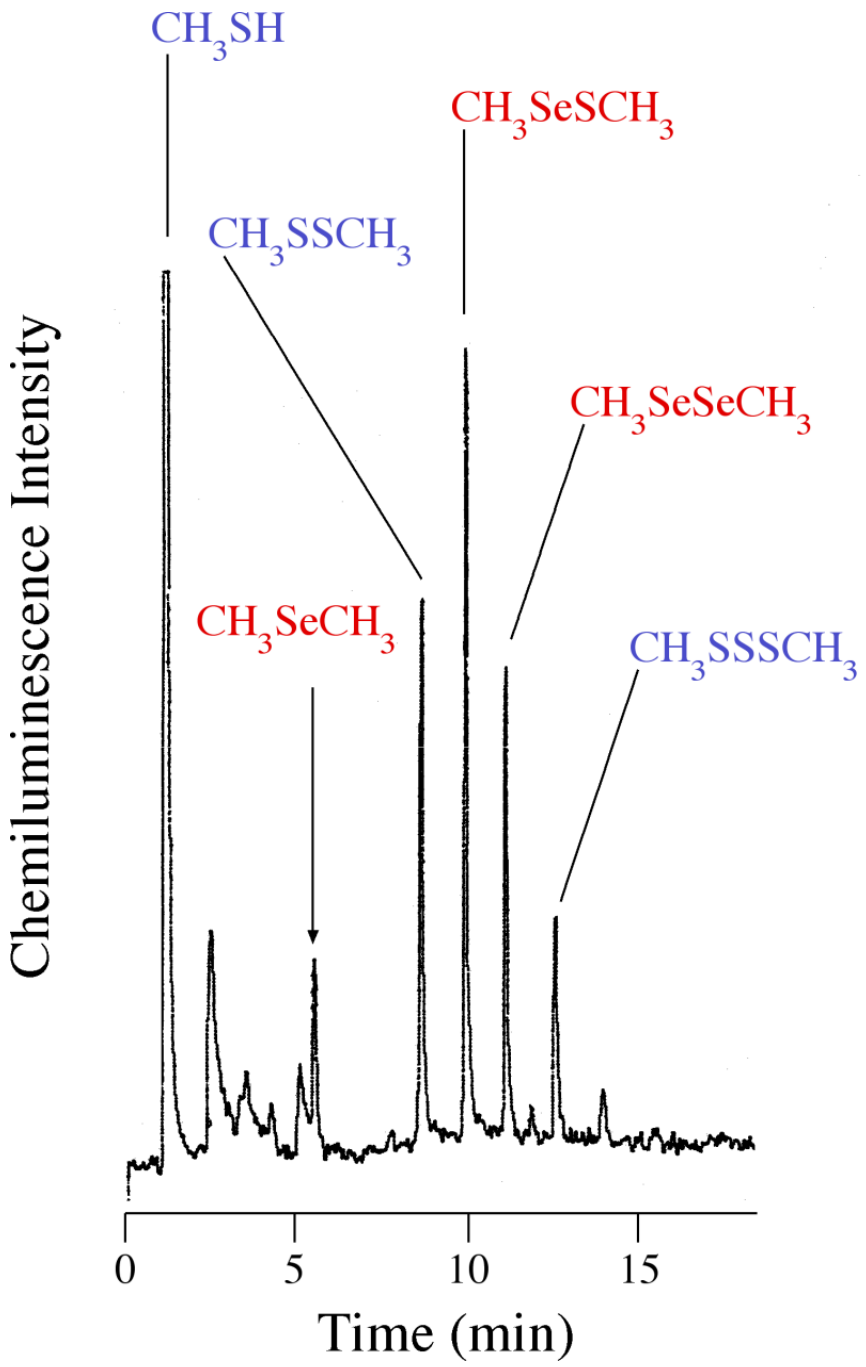

Figure 5. Gas chromatography with fluorine-induced chemiluminescence chromatogram of headspace above a live, anaerobic culture of LHVE 72 hours after inoculation, amended with 1 mM sodium selenocyanate.

REFERENCES

[1] T.G. Chasteen and R. Bentley, *Chalcogens (S, Se, Te) in Microorganisms and Plants*, in *Handbook of Chalcogen Chemistry*, F. A. Devillanova, ed., Royal Society of Chemistry Publishing, Cambridge, UK, 2007, pp. 671-713.

[2] T.G. Chasteen and R. Bentley, *Biomethylation of Selenium and Tellurium: Microorganisms and Plants*, Chem. Rev. 103 (2003), pp. 1-26.

[3] A. Manceau and D.L. Gallup, *Removal of selenocyanate in water by precipitation: characterization of copper-selenium precipitate by x-ray diffraction, infrared, and x-ray absorption spectroscopy*, Environ. Sci. Tech. 31 (1997), pp. 967-976.

[4] A.R. Appleton and C.B. Cain, *Selenium removal from refinery wastewaters: biological field testing report*, Western State Petroleum Association Report, Concord California, 1995.

[5] X. Meng, S. Bang, and G.P. Korfiatis, *Removal of selenocyanate from water using elemental iron*, Water Res. 36 (2002), pp. 3867-3873.

[6] G.S. Banuelos and Z.-Q. Lin, *Phytoremediation management of selenium-laden drainage sediments in the San Luis Drain: a greenhouse feasibility study*, Ecotoxicol. Environ. Saf. 62 (2005) pp. 309-316.

[7] L. Wu, *Review of 15 years of research on ecotoxicology and remediation of land contaminated by agricultural drainage sediment rich in selenium*, Ecotoxicol. Environ. Saf. 57 (2002) pp. 257-269.

[8] W.T. Frankenberger, Jr. and M. Arshad, *Bioremediation of selenium-contaminated sediments and water*, Biofactors 14 (2001), pp. 241-254.

[9] J.W. Swearingen Jr., D.P. Frankel, D.E. Fuentes, C. Saavedra, C.C. Vásquez, and T.G. Chasteen, *Identification of biogenic dimethyl selenodisulfide in the headspace*

gases above genetically modifiedEscherichia coli, Anal. Biochem. 348 (2006), pp. 115-122.

[10] S. Vadhanavikit, R.J. Kraus, and H.E. Ganther, *Metabolism of selenocyanate in the rat*, Arch. Biochem. Biophys. 258, (1987) pp. 1-6.

[11] R. Yu, J.P. Coffman, V. Van Fleet-Stalder, and T.G. Chasteen, *Toxicity of Oxyanions of selenium and of a proposed bioremediation intermediate, dimethyl selenone*, Environ. Toxicol. Chem. 16 (1997) pp. 140-145.

[12] Z. Somogyi, I. Kiss, I. Kadar, and G. Bakonyi*, Toxicity of selenate and selenite to the potworm Enchytraeus albidus (Annelida: Enchytraeidae) A laboratory study*, Ecotoxicol. 16 (2007), pp. 379-384.

[13] S.P. Canton, *Acute aquatic life criteria for selenium*, Environ. Toxicol. Chem. 18 (1999), pp. 1425-1432.

[14] K.V. Brix, J.S. Volosin, W.J. Adams, R.J. Reash, R.G. Carlton, and D.O. McIntyre, *Effects of sulfate on the acute toxicity of selenate to freshwater organisms*, Environ. Toxicol. Chem. 20 (2001), pp. 1037-1045.

[15] M.L. Speck, *Compendium of Methods for the Microbiological Examination of Foods*, American Public Health Association, New York, 1976.

[16] D.H. Bergey, J.G. Holt, and N.R. Krieg, *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins Publishers, Baltimore, MD, 1984.

[17] U.J. Strotmann, H. Eglsäer, and U. Pagga, *Development and evaluation of a growth inhibition test with sewage bacteria for assessing bacterial toxicity of chemical compounds*, Chemosphere 28 (1994) pp. 755-766.

[18] H. Morlon, C. Fortin, C. Adam, and J. Garnier-Laplace, *Cellular quotas and induced toxicity of selenite in the unicellular green alga Chlamydomonas reinhardtii*. Radioprot. 40 (2005), pp. 101–106.

[19] W.N. Bennett, *Assessment of selenium toxicity in algae using turbidostat culture*, Water Res. 22 (1988), pp. 939–942.

[20] V.P. Daniel, B. Murukan, B.S. Kumari, and K. Mohanan, *Synthesis, spectroscopic characterization, electrochemical behaviour, reactivity and antibacterial activity of some transition metal complexes with 2-(N-salicylideneamino)-3-carboxyethyl-4,5-dimethylthiophene*, Spectrochimica Acta A 70 (2008) pp. 403-410.

[21] M. Stawiecka-Mirota, J. Kaminska, D. Urban-Grimal, D.S. Haines, T. Zoladek, *Nedd4, a human ubiquitin ligase, affects actin cytoskeleton in yeast cells*, Exp. Cell Res., 314 (2008), pp. 3318-3325.

[22] B.K. Pathem, G.A. Pradenas, M.E. Castro, C.C. Vasquez, and T.G. Chasteen, *Capillary Electrophoretic Determination of Selenocyanate and Selenium and Tellurium Oxyanions in Bacterial Cultures*, Anal. Biochem. 364 (2007), pp. 138-144.

[23] J. W. Swearingen, Jr., M. A. Araya, M. F. Plishker, C. P. Saavedra, C. C. Vasquez, and T. G. Chasteen, *Identification of biogenic organotellurides in* Escherichia coli *K-12 headspace gases using solid phase microextraction and gas chromatography*, Anal. Biochem. 331 (2004) pp. 106-114.

[24] J. W. Swearingen, Jr., D. E. Fuentes, M. A. Araya, M. F. Plishker, C. P. Saavedra, T. G. Chasteen, and C. C. Vasquez, *The expression of the ubiE gene of* Geobacillus stearothermophilus *V in* Escherichia coli *K-12 mediates the evolution of selenium compounds into the headspace of selenite- and selenate-amended cultures*, Appl. Environ. Microbiol. 72 (2006), pp. 963-967.

CHAPTER 3

PRODUCTION OF DIMETHYL TRISELENIDE AND DIMETHYL DISELENENYL SULFIDE IN THE HEADSPACE OF METALLOID-RESISTANT *BACILLUS* SPP. GROWN IN THE PRESENCE OF SELENIUM OXYANIONS

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Abstract

A *Bacillus* species harvested from the environment is metalloid-resistant and when grown anaerobically in complex growth medium and amended with selenium oxyanions selenate or selenite or selenocyanate produces volatile organo-selenium compounds in bacterial culture headspace. Two novel compounds, $CH_3Se_2SCH_3$ and $CH_3SeSeSeCH_3$, so far undetected in bacterial culture headspace, are produced and can be detected using solid phase microextraction and gas chromatography with either fluorine-induced chemiluminescence or mass spectrometric detection. Differences in the electron impact fragmentation pattern of the mixed sulfur/selenide compounds allow for the tentative differentiation between the symmetric and asymmetric isomers in this bacterium's headspace in favor of the asymmetric, $CH_3SeSeSCH_3$ isomer.

Keywords: bioremediation, volatilization, organo-selenium, polyselenides, polysulfides

Introduction

Selenium (Se) is an essential trace element occurring naturally in the environment. Its presence in soil and ground water may be attributed to either natural or anthropogenic activities [1]. It is considered as the sixty-sixth most abundant element in the earth's crust. At controlled and defined concentrations, selenium plays a crucial role in cellular metabolism; however, at elevated levels, it is toxic. Among selenium oxyanions, in general, selenite (SeO_3^{2-}) is more toxic and exhibits higher bioaccumulation than selenate (SeO_4^{2-}) [2, 3, 4].

Selenium plays an important biological role in the form of amino acids selenocysteine and selenomethionine, analogous to sulfur-containing cysteine and methionine. Sodium selenate and sodium selenite are the inorganic selenium containing compounds used as dietary supplements in the prevention of various nutritional

deficiency diseases in mammals [5]. There is a narrow range between the essentiality and toxicity levels for selenium, with the nervous system a target for selenium toxicity [6, 7]. Widely reported death and deformation of waterfowl in California's San Joaquin Valley was due to selenium contamination of agricultural drainage water [8, 9]; and therefore, it has been considered a major environmental pollutant [2].

The concentration and toxicity of selenium compounds in the environment can be decreased by the reduction of selenium oxyanions to elemental selenium and/or methylated, volatile Se products by selenium-resistant microbes [10, 11]. Along with weathering and erosion, global cycling of selenium is carried out by this kind of biological activity [1, 12]. Selenium levels ranging from 5 μg L^{-1} to 2,000 μg L^{-1} can be tolerated by many microorganisms [2, 13, 14].

The earliest reports of biologically-produced organo-selenium compounds were in the 19th century when researchers dosed animals with salts of selenium or tellurium and noted the production of unpleasant or garlic-like smells [15, 16, 17]. Frederick Challenger's mid-20th century review of periodic table Group 15 and 16 biomethylation [18] is an excellent springboard into those fascinating historical reports, many of which were incorrect in their assumption of the alkylated metalloid investigated. In fact, the true identity of dimethyl selenide (CH_3SeCH_3, DMSe), the first organo-selenide positively identified as a biological product of an organism exposed to selenium salts, was established by Challenger and North in 1934[19] . Their analytical method was to trap gases produced by a fungus, *Penicillium brevicaule* (*Scopulariopsis brevicaulis*) growing on bread in flasks and exposed to either selenate or selenite by purging air through the flask into aqueous solutions of $HgCl_2$, $HgBr_2$, $PtCl_2$, or alcoholic benzyl chloride. DMSe

as the appropriate derivative was identified by melting point of the products as compared to authentic derivatives [19].

The list of organo-selenides that originate from biological sources grew as toxicological interest and analytical methods improved. Hydrogen selenide [20, 21], methaneselenol [22, 23], and dimethyl diselenide ($CH_3SeSeCH_3$, DMDSe) [23, 24] were all detected before the end of the last century. Volatile, mixed sulfur/selenium compounds have also been determined in the headspace of bacterial cultures. In addition, dimethyl selenenyl sulfide (CH_3SeSCH_3, DMSeS) [23, 24] and dimethyl selenenyl disulfide ($CH_3SeSSCH_3$, DMSeDS) [25] have been reported in the headspaces of bacterial cultures.

As part of our continuous examination of both naturally occurring and genetically-modified bacteria for metalloid resistance and their biological response to exposure to the toxic oxyanions of Te and Se, here we report two new mixed organoselenide/sulfides biologically produced by an environmental metalloid-resistant organism exposed to toxic Se salts in liquid culture.

Materials and Methods

Reagents

Chemicals used in this study included bacto™ tryptone (Becton Dickson, Sparks, MD, USA), yeast extract (Difco Laboratories, Detroit, MI, USA), sodium selenate and sodium selenite (Sigma-Aldrich, Milwaukee, WI, USA), potassium selenocyanate (Acros Organics, Morris Plains, NJ, USA), sodium chloride (BDH, West Chester, PA, USA), agar (Amresco, Solon, OH, USA) and DL-dithiothreitol, DTT (Sigma, St. Louis, MO, USA). A RiOs 3 water purification system from Millipore (Billerica, MA, USA) was employed for water deionization.

Bacterial strain and growth conditions

A metalloid-resistant, Gram positive, spore-forming, gelatinase-positive bacterium identified as *Bacillus spp.* LHVE (hereafter LHVE) was isolated from Huerquehue National Park, southern Chile and grown anaerobically in Luria Bertani (LB) medium [26]. Precultures were prepared by inoculating LB medium from a single plate colony followed by incubation for 24 h at 37 ^{0}C.

Samples for headspace analysis were made by distributing sterile LB medium into 16-mL test tubes and inoculating with a 1:10 v/v inoculum from the preculture. The total volume of liquid culture was 10 mL. Sterile-filtered (0.2-µm) selenium solutions were then added to these test tubes. The concentration of selenium species in the liquid culture (selenate or selenite) was 1 mM. Bacteria were allowed to grow in 16-mL test tubes, enclosed with open-top screw caps with Teflon®/silicon-lined septa (Alltech, Deerfield, IL, USA) followed by subsequent headspace sampling. Sampling was carried out at 48 h after selenium amendment.

Headspace analysis with SPME

The collection of volatile components from bacterial headspace was carried out using 75-µm Carboxen™- polydimethylsiloxane (PDMS) solid-phase microextraction (SPME) fiber from Supelco (Bellfonte, PA, USA). The adsorption timings of SPME in the headspace ranged between 15 and 50 min at 37 ^{0}C. The fiber exposure was carried out by piercing the septum of the 16-mL test tubes with a needle which contained the SPME fiber and exposing the fiber for a specific adsorption time. The fiber's adsorbed sample was then introduced into the hot injector of the GC by piercing the GC injector's septum with the SPME needle and pushing down and locking the SPME plunger to expose the fiber. The SPME fiber quickly desorbed its sample but the GC split/splitless

valve was left open for 1 min. The exposed SPME fiber was left in the GC hot injector for the entire GC run and subsequently checked between each sample run for carry over.

Gas chromatography with F_2-induced chemiluminescence analysis

A Hewlett-Packard Model 5890 Series II gas chromatograph (GC) with a Sievers 300 fluorine-induced sulfur chemiluminescence detector (SCD) (GE Analytical Instruments, Boulder, CO, USA) was used for the analysis with data integrated by a Hewlett-Packard 3396 Series III integrator. A 30-m 0.32 μm i.d. capillary column with a DB-1 stationary phase and a 5.0-μm chromatographic film was used (J&W Scientific, Folsom, CA, USA). Helium was the carrier gas with a flow rate of 1 mL/min. A splitless injection mode was used for the sample analysis, 275 ^{0}C injector, with the split valve opened at 1 min. Two different temperature programs were used in the chemiluminescence work. In the short program, the initial oven temperature was 30 ^{0}C, maintained for 2 min and then ramped to 250 ^{0}C at 15 ^{0}C/min. In the long temperature program, the initial temperature was 30 ^{0}C for 1 min followed by a 5 ^{0}C/min ramp.

Gas chromatography with mass spectrometric analysis

GC/MS analysis was accomplished by a Hewlett-Packard 5973 Mass Selective Detector (using positive electron impact mode, EI^+, at 70 eV). The carrier gas was ultra-high-purity helium with a flow rate of 1 mL/min. An Agilent DB-5 (0.32 μm i.d, 1.4-μm film thickness, 30-m length) chromatographic column was used for GC/MS and the temperature program used was 30 ^{0}C initial, 10 °C/min ramp to 200 ^{0}C. The injector temperature was 275 ^{0}C. SPME sampling was identical with fiber-exposure times of 45 min.

Results

LHVE is a metalloid-resistant *Bacillus* spp. which can grow in LB medium in concentrations up to 500 mM SeO_3^{2-}. LHVE cells, growing in LB medium with no added selenium, yielded GC-SCD chromatograms with commonly detected organo-sulfur compounds; headspace analysis of bacterial cultures often exhibit the presence of methylated organo-sulfur compounds [25]. Sulfur-containing components in the growth medium are the S source and biological reduction and methylation yields organo-sulfides which are then released into the headspace where gas phase concentrations vary relative to the bacterial growth phase [27, 28, 29]. Methanethiol (CH_3SH, MeSH), dimethyl disulfide (CH_3SSCH_3, DMDS) and dimethyl trisulfide (CH_3SSSCH_3, DMTS) are the volatile organo-sulfides most often detected in the bacterial culture headspace [25, 30]. Fig. 6 depicts a typical GC-SCD chromatogram of LHVE bacterial culture headspace 48 h after inoculation (short temperature program).

When LHVE cultures were amended with selenite as the sodium salt, the bacterial culture produced the usual organo-sulfides and also organo-selenides including DMSe and DMSeS. The latter is a mixed sulfur/selenium compound [31] that we have often detected in Se-resistant bacterial culture headspace [25, 32, 33] but that has also been detected in North Atlantic ocean water [34] and most recently in duck manure [35]. Fig. 7 is a chromatogram of an LHVE culture amended with 1 mM selenite with all identified organo-sulfides and selenides indicated; however, two compounds elute after DMTS, a compound with a boiling point of approximately 168 ^{0}C. Initially these late eluting peaks were detected in LHVE cultures that had been resampled by piercing the caps' septum a second time and exposing the SPME fiber. Since the polysulfides/selenides, for instance, DMTS or dimethyl diselenenyl sulfide ($CH_3SeSeSCH_3$, DMDSeS) have heteroatoms that

are more oxidized than the lower analogues, we considered the possibility that reduced headspace compounds were being oxidized by atmospheric oxygen to yield the higher boiling point compounds. To test this idea, we subsequently sampled only fresh, sealed cultures but found the same headspace compounds in replicate cultures.

Analysis of the headspace of a 1 mM selenite-amended LHVE culture by GC/MS yielded the reconstructed total-ion-current chromatogram (RTICC) shown in Fig. 8. The major differences between the chemiluminescence detector and GC/MS figures are the delay in the retention times of all compounds in case of GC/MS when compared to GC-SCD and, most pronounced, the difference in detector sensitivity.

There were four initially unknown chromatographic peaks in the RTICC, compounds which were thought to originate from the bacterial headspace because the peaks disappeared when a clean fiber was exposed to lab air only and then analyzed; however, subsequent chromatograms generated by analysis of a clean fiber exposed to the headspace of either LB medium or sterile water at 37 ^{0}C exhibited these unknowns. The mass spectra of each of these (not shown) contain *m/z* fragments characteristic of dimethyl polysiloxane [36] and are therefore assigned as originating from the SPME fiber. This fiber bleed has been reported before [36, 37]. Since the chemiluminescence detector does not respond to this family of compounds they do not appear in the SCD chromatograms (Figs. 6 and 7).

In addition to confirming the identity of the selenium-containing compounds determined by GC/SCD, the mass spectra of the two late eluting peaks in Fig. 7 from selenite-amended cultures were also generated and are shown in EI^{+} mass spectra of Figs. 9 and 10.

Discussion

Metalloid-resistant bacteria have long been found to be involved in the biological transformation of selenium into methylated and reduced chemical forms [18]. While the recent use of SPME fiber sampling has helped to broaden the family of organo-metalloids determined in biological headspace [25, 38, 39, 40] this sensitive sampling technique cannot, obviously, be used to detect analytes that are not volatile enough to be released into the headspace. With that said, we have previously reported the detection of dimethyl selenenyl disulfide ($CH_3SeSSCH_3$, DMSeDS) [25] and two new organo-tellurides [38] in bacterial cultures amended with Se or Te oxyanions. Two of these compounds exhibited boiling points higher than DMTS and therefore their gas-phase concentrations above a growing culture would expect to be lower than those of more volatile components given equal Henry's Law constants. When LHVE cells were amended with 1 mM selenite, sampled by SPME and analyzed by GC/SCD two very late eluting chromatographic peaks were detected (Fig. 7), both with boiling points beyond DMSeDS (~192 ^{0}C) given the characteristics of the GC column used (DB-1, nonpolar 100% dimethylpolysiloxane). The chromatographic conditions in our method led us to assume the two late-eluting peaks were either higher poly-sulfides, -selenides, or mixed sulfur/selenium compounds since the fluorine-induced chemiluminescence detector exhibits such selective response to alkyl compounds with chalcogen or metalloidal heteroatoms with almost no response to analytes not containing both these moieties [28]. Sampling of selenite-amended LHVE cultures that had been grown into stationary phase and whose culture headspace was only sampled once using the SPME fiber gave similar headspace results. One possibility is that these two new compounds might have formed by the rearrangement of the early eluting compounds on SPME fiber's stationary phase. This can be overruled by the fact that

these compounds were not detected in the cultures amended with 1 mM selenate (data not shown) which also produced a wide range and relatively high concentrations of early eluting compounds. Moreover, in previous work by Swearingen et al. in this lab and using this method, although metalloid-amended bacteria produced a variety of organo-sulfur and -selenium compounds, these two new high boiling compounds were absent [25]. Therefore, we propose that the unidentified, late-eluting compounds are clearly biochemical products of this specific bacterium under the conditions studied.

As the chemical members of the series of polyselenides/sulfides molecular weights get heavier, these compounds' boiling points increase in a predictable manner [41, 42]. Therefore given the known retention times of DMTS (12.7 min, short program) and DMSeDS (13.7 min, short program) from our previous work and based on the temperature program used in Fig. 7 we examined the GC/MS mass spectra for the late eluting 15.6 and 17.3 min peaks (short program) considering these bioanalytes to be DMDSeS or dimethyl triselenide ($CH_3SeSeSeCH_3$, DMTSe). These analytes appeared at 29.2 and 32.0 min respectively in the long temperature program chromatogram in Fig. 7. This is the theoretically predicted elution order [41, 42] and the elution order found by Kubachka et al. (2007) [43] and Meija and Caruso (2004) [42] in similar chromatography. While dimethyl diselenenyl sulfide has been reported in the headspace of green onions, dimethyl triselenide has not been detected as originating from a bacterial source; however, DMTSe has been reported as released as a "minor headspace Se-containing volatile" by a genetically-modified selenium hyper-accumulator plant, an Indian mustard, *Brassica juncea* [43]. Figs. 9 and 10 show the mass spectra of these two late eluting chromatographic peaks. The diselenenyl compound has a symmetric ($CH_3SeSSeCH_3$) and asymmetric ($CH_3SeSeSCH_3$) isomer. Selenium has five isotopes

that account for 99.13% of natural abundance: ^{80}Se; 49.82%; ^{78}Se; 23.52%; ^{82}Se; 9.19%; ^{76}Se; 9.02%; and ^{77}Se; 7.58% and so the trichalcogen-containing spectra are relatively complex. Based on work by Shah and coworkers (2007) [39], it appeared that the earlier of the two late-eluting compounds (Fig. 9) was the asymmetric diselenenyl sulfide; however, this comparison was made difficult for at least two reasons. The first is that the differences in the mass spectra of the two $CH_3Se_2SCH_3$ isomers-as determined by Shah et al.—varies only in the relative intensities of the *m/z* fragmentation, specifically clusters around *m/z* 142 and 190. Nominally these groupings stem from CH_3SeSCH_3 and Se_2S fragments respectively, the first of these formed by methyl migration in the MS ionization source. The symmetrical isomer from synthetic chemical exchange mixtures has very low relative intensities in the *m/z* 142 cluster and much higher intensities around *m/z* 190 in the positive electron impact spectra, EI^+ [39, 43, 44], the ionization method reported in this work too. A close look at Fig. 9 shows both of these *m/z* groupings; however, the intensity around *m/z* 142 is very low, evidence pointing to the symmetric isomer. However, the interpretation is further made difficult by the presence of fragments in our Fig. 9 spectrum around *m/z* 160. These are Se-Se fragment clusters whose presence in the mass spectra of the asymmetric spectrum would logically follow from the asymmetric structure: $CH_3SeSeSCH_3$ → Se-Se. The presence of the *m/z* 160 cluster could reliably be used as the differences between these isomers if the process of self-exchange that Meija and Caruso have reported (2004) [42] did not occur. In this event SeSe, CH_3SeSe (cluster around *m/z* 190) and $CH_3SeSeCH_3$ (*m/z* 205) adducts could be expected to form from the asymmetric isomer, and that is also seen in Fig. 9's mass spectrum. In addition the 160 m/z cluster seen in Figure 9 is completely missing in Meija and Caruso's EI^+ mass spectrum of the symmetric spectrum but are 20% of the base peak in those

authors' asymmetric isomer's spectrum, published in their papers supplemental data available online [44]. Finally, the mass spectral fragment cluster around *m/z* 47 is also found in Fig. 9, indicative of CH_3S, a logical fragment of the asymmetric diselenenyl sulfide molecule. And furthermore the *m/z* 47 ion is over 70% of the base peak and therefore less likely as an exchange product. While both of the published EI^+ spectra of these isomers show the *m/z* 47 cluster, neither of these rise above 20% in those published spectra while Figure 9's *m/z* 47 cluster is, again, over 70% of our spectrum's base peak. This therefore gives, we believe, strong but not ambiguous evidence that the bacterium LHVE has produced the asymmetric polyselenide isomer $CH_3SeSeSCH_3$ and not the symmetric isomer ($CH_3SeSSeCH_3$).

Experiments were carried out using DTT in an effort to further differentiate between the two possible $CH_3Se_2SCH_3$ isomers found in LHVE headspace based on a technique published by Meija and Caruso and others [42, 45]. If diselenide bond-containing compounds-such as the asymmetric $CH_3Se_2SCH_3$ isomer are present in a sample amended with DTT, those compounds will be reduced, and that process can be detected by a decrease in chromatographic peak area of the compounds with Se-Se bonds. Se-S bond containing compounds, however, are substantially unaffected by DTT addition, at least as suggested by previous work [42, 45]. Growing LHVE cultures amended with 1 mM selenite were treated in this manner using concentrations ranging from 5 mM to 200 mM DTT; however, DTT preferentially reduced disulfide bond-containing species in these cultures (basically CH_3SSCH_3 and CH_3SSSCH_3 disappeared), producing chromatograms showing no significant decrease in selenium-containing species (data not shown). DTT reduction of disulfide bonds is also well known [38].

Therefore this method will not function for a means of differentiating between these isomers in these samples.

The mass spectrum in Fig. 10 less unambiguously confirms that the last eluting compound in both chromatograms in Fig. 7 is dimethyl triselenide, $CH_3SeSeSeCH_3$. Dimethyl triselenide has never been reported in bacterial culture headspace before.

In our previous work with selenium and tellurium resistant bacteria using SPME sample we have not detected dimethyl triselenide, even in bacterial cultures that were rich in the other organo-selenides [25]. Selenate- and selenocyanate-amended cultures produce similar headspace results (data not shown) but none of the polyselenides. The bacterial production of mixed sulfur/selenium and polyselenide compounds might play a role in the geochemical cycling of selenium given that an estimated 90% of all volatile Se emissions to the atmosphere are biogenic [46]. It might be proposed that the biochemical source of the polysulfides/selenides in this bacterium's culture headspace comes most probably from the expression of genes not present in our former bacterial models that await further identification.

Conclusions

In the present work, headspace analysis of the cultures of the metalloid-resistant bacterium *Bacillus* spp. LHVE resulted in the production of organo-sulfides and mixed organo-selenide/sulfides in addition to two new compounds when amended with 1 mM selenite. Based on retention time, boiling point comparisons, and mass spectral analysis, these two compounds were identified as dimethyl diselenenyl sulfide ($CH_3SeSeSCH_3$) and dimethyl triselenide ($CH_3SeSeSeCH_3$); however, the first of these can only be tentatively assigned as the asymmetric isomer at this time. This is the first report of the bacterial headspace production of these two compounds.

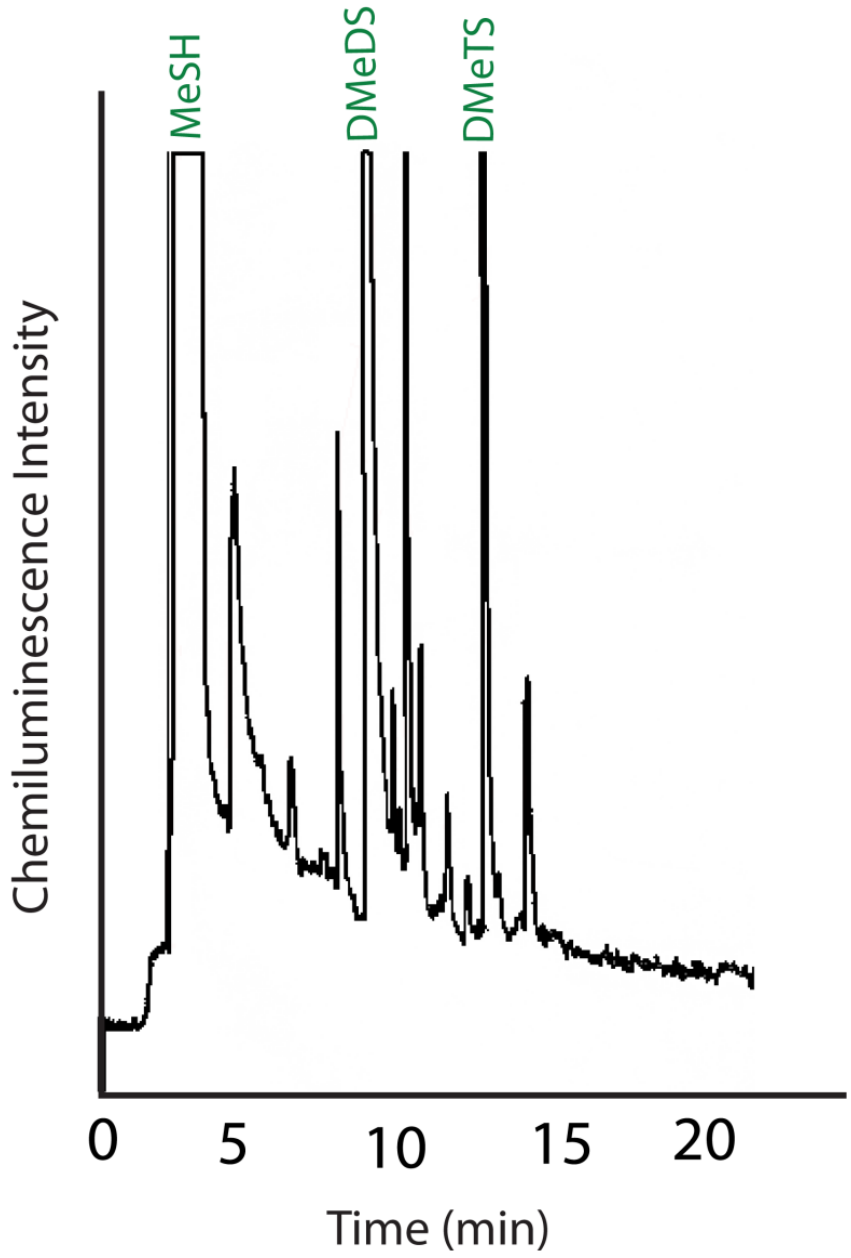

Figure 6. GC-SCD chromatogram of LHVE control. MeSH, DMDS and DMTS are the organo-sulfides detected in the bacterial headspace in the absence of metalloid amendment. The SPME fiber exposure time was 20 min. Compound abbreviations are defined in the text.

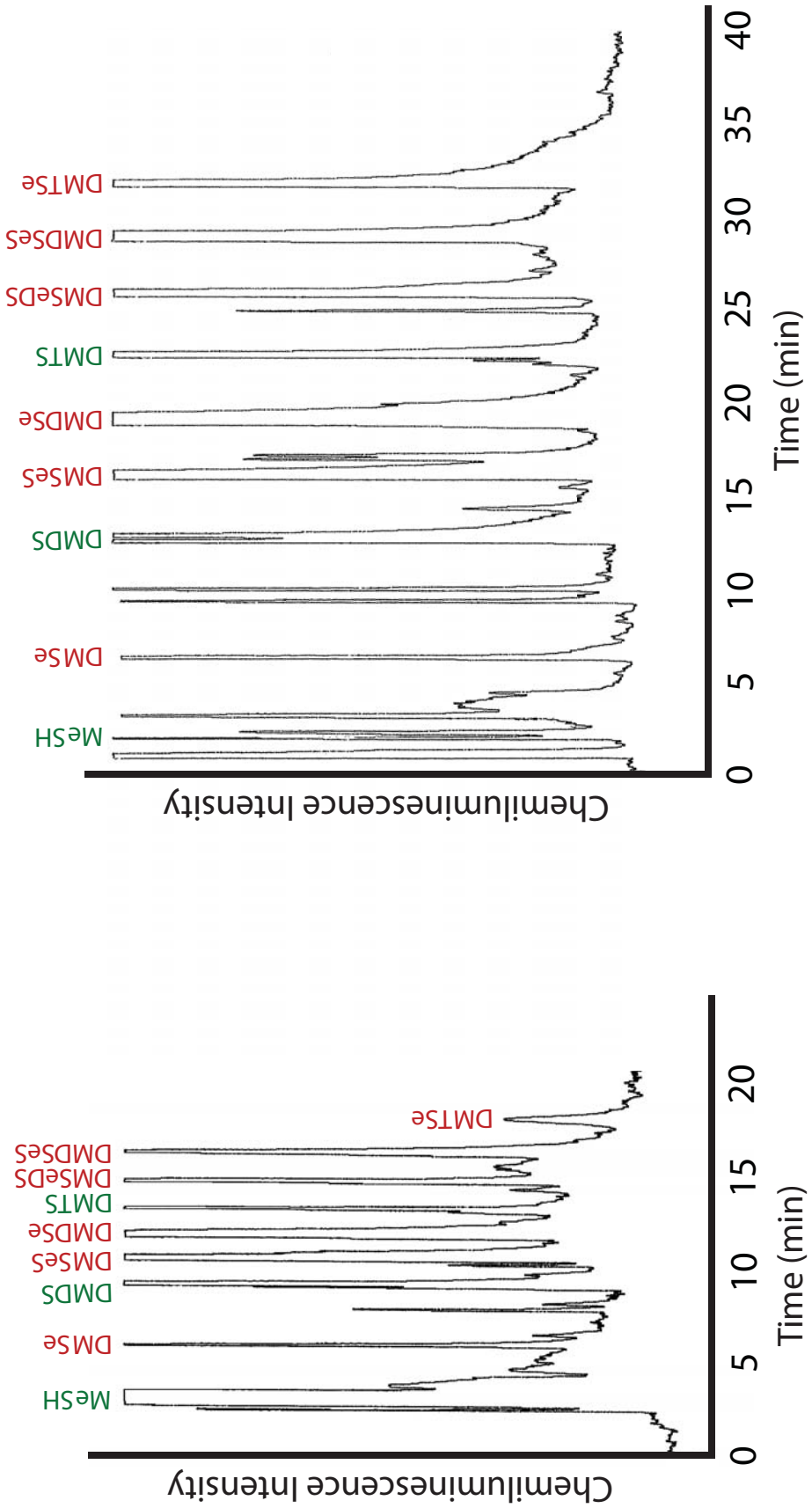

Figure 7. SCD chromatograms of LHVE headspace amended with 1 mM selenite, using short and long temperature programs. The SPME fiber exposure time was 20 min for each run.

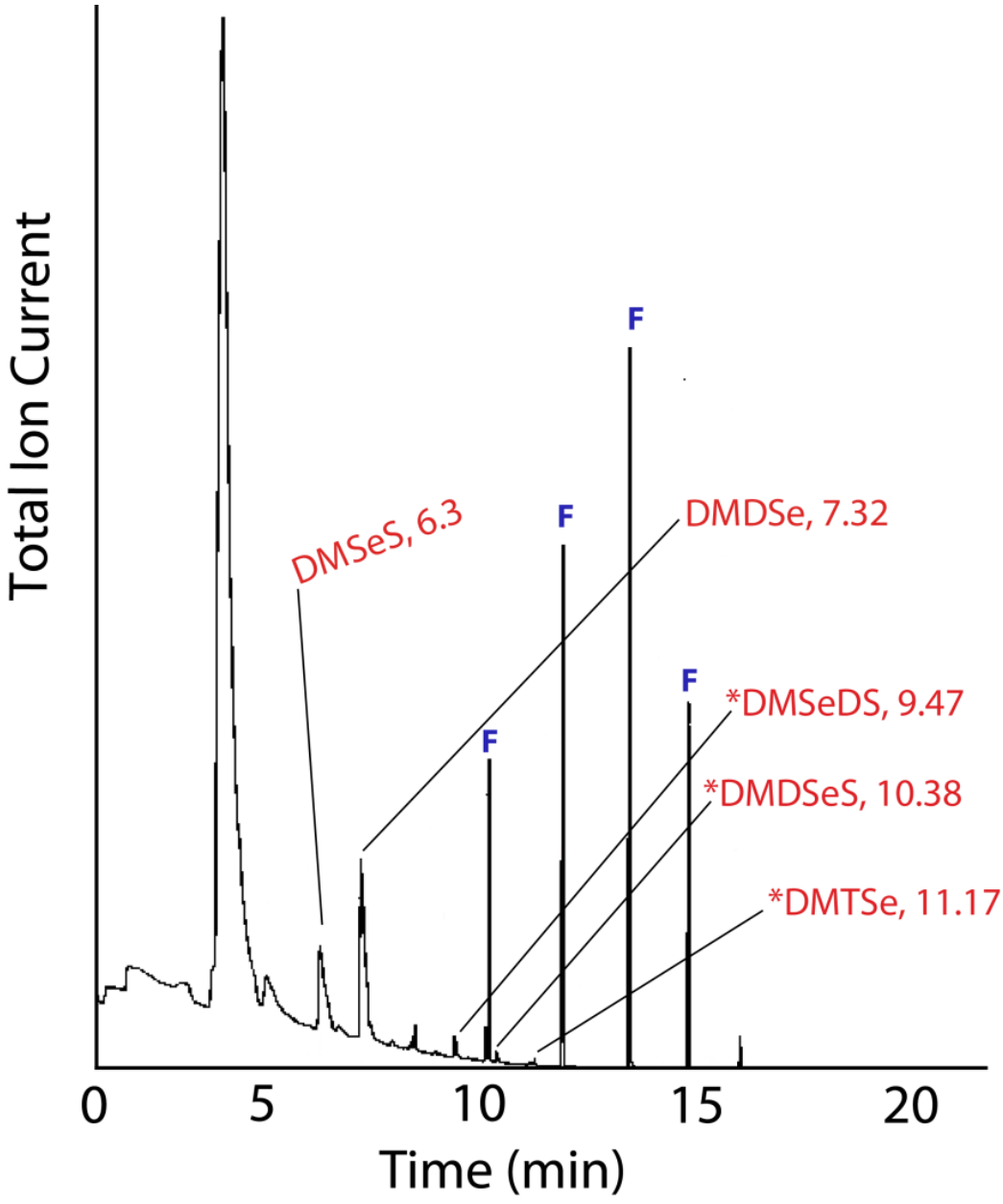

Figure 8. Reconstructed total-ion-current chromatogram of headspace sample of an LHVE culture amended with 1 mM selenite. Sampling was performed at 72 h. Organo-selenium peaks are labeled. Peaks marked F originated from SPME fiber bleed. The SPME fiber exposure time was 45 min.

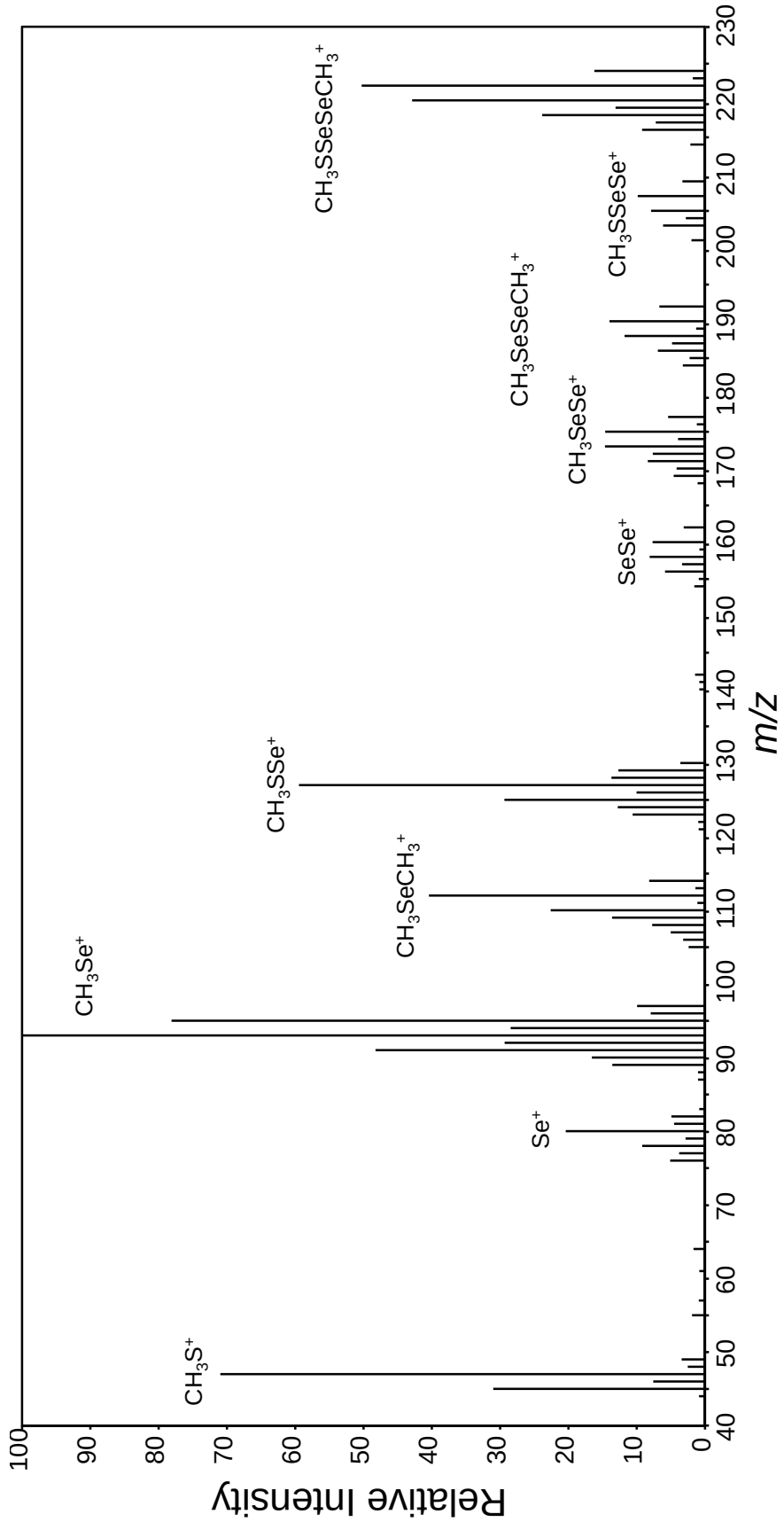

Figure 9. Mass spectrum of the asymmetrical dimethyl diselenenyl sulfide isomer ($CH_3SeSeSCH_3$) from headspace sample of an LHVE culture amended with 1 mM selenite, from a GC/MS chromatographic peak eluting at 10.38 min.

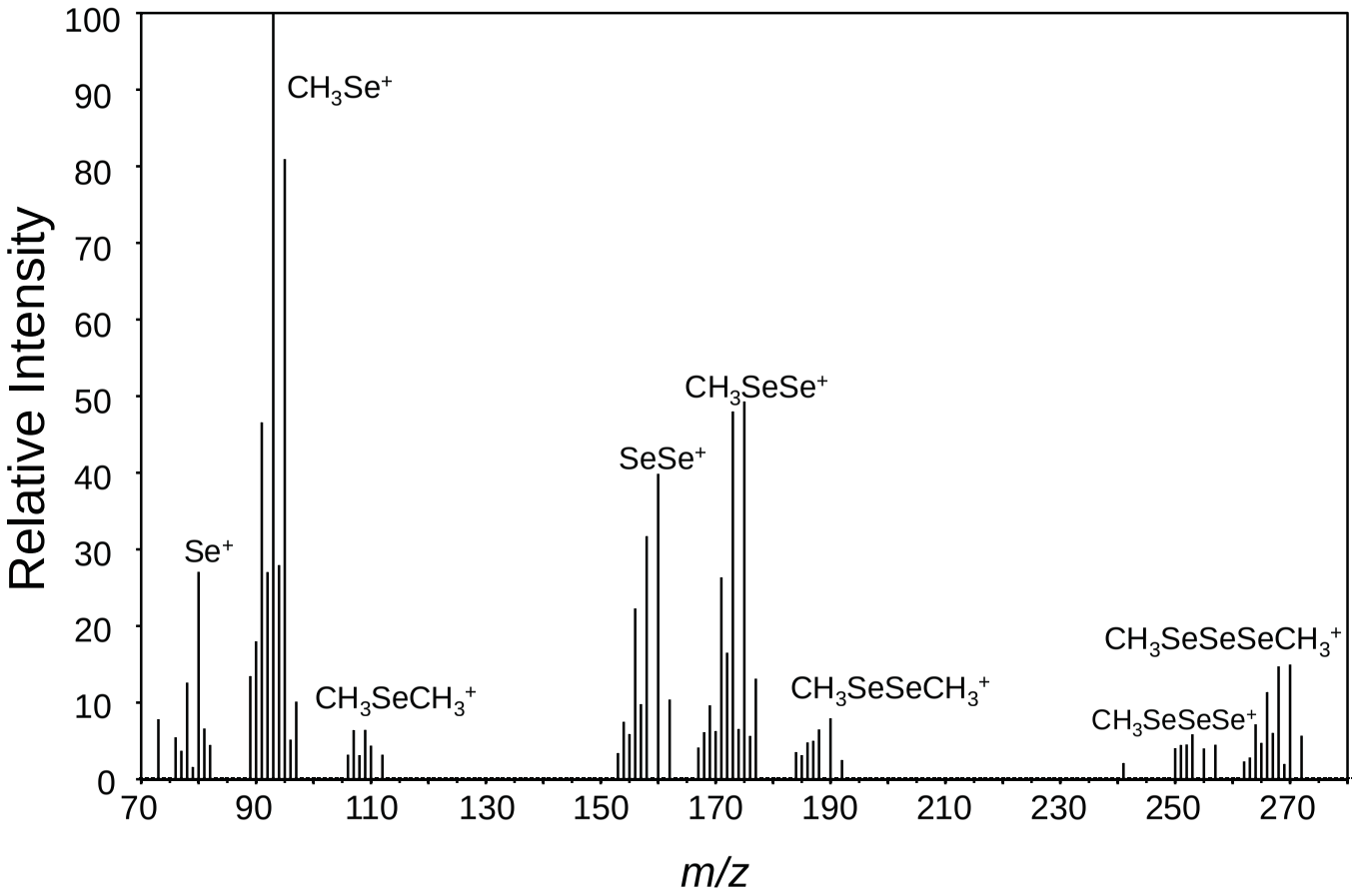

Figure 10. Mass spectrum of dimethyl triselenide (DMTSe) produced by LHVE amended with 1 mM selenite from a GC/MS chromatographic peak eluting at 11.17 min.

References

[1] W. Hunter, L. Kuykendall, Determination of dimethylselenide and dimethyldiselenide by gas chromatography–photoionization detection, *J. Chromatogr. A* 1038 (2004), 295-297.

[2] W. Frankenberger, Jr., R. Engberg, R. (Eds.). Environmental Chemistry of Selenium, Marcel Dekker, Inc., New York, 1998.

[3] USEPA, 2004. United States Environmental Protection Agency [USEPA], 2004. Draft aquatic life water quality criteria for selenium. Washington, DC, USEPA Office of Water. EPA EPA-822-D-04-001D.

[4] J. Skorupa, In Environmental Chemistry of Selenium, Frankenberger, W.T., Jr. and Engberg, R.A. (Eds.), Marcel Dekker, Inc., New York, 1998, Chapter 18.

[5] A. Fan, W. Kizer, Selenium: Nutritional, toxicological, and clinical aspects, *West. J. Med*. 153 (1990), 160-167.

[6] A. Lemly, In Environmental Chemistry of Selenium, Frankenberger, W.T., Jr. and Engberg, R.A. (Eds.), Marcel Dekker, Inc., New York, 1998; Chapter 16.

[7] K. Wrobel, K. Wrobel, J. Caruso, Pretreatment procedures for characterization of arsenic and selenium species in complex samples utilizing coupled techniques with mass spectrometric detection, *Anal. Bioanal. Chem.* 381 (2005), 317-331.

[8] M. Losi, W. Frankenberger, Reduction of selenium oxyanions by *Enterobacter cloacae* SLD1a-1: isolation and growth of the bacterium and its expulsion of selenium particles, *Appl. Environ. Microbiol.* 63 (1997), 3079-3084.

[9] D. O'Toole, M.F. Raisbeck. In Environmental Chemistry of Selenium, Frankenberger, W.T., Jr. and Engberg, R.A. (Eds.), Marcel Dekker, Inc., New York, 1998, Chapter 19.

[10] N. Terry, A. Zayed In Environmental Chemistry of Selenium, Frankenberger, W.T., Jr. and Engberg, R.A. (Eds.), Marcel Dekker, Inc., New York, 1998, Chapter 31.

[11] J. Milne, In Environmental Chemistry of Selenium, Frankenberger, W.T., Jr. and Engberg, R.A. (Eds.), Marcel Dekker, Inc., New York, 1998; Chapter 23.

[12] P. Haygarth, Global importance and global cycling of selenium, p. 1-27. In W. T. Frankenberger, Jr., and S. Benson (ed.), in Selenium in the environment, Marcel Dekker, New York, 1994.

[13] T. Chasteen, R. Bentley, Biomethylation of selenium and tellurium: Microorganisms and plants, *Chem. Rev.* 103 (2003), 1-26.

[14] J. Doran, Microorganisms and the biological cycling of selenium, *Adv. Microbial Ecol.* 6 (1982), 1-32.

[15] C. Gmelin, Versuche über die Wirkungen des Baryts, Strontians, Chroms, Molybdaens, Wolframs, Tellurs, Titans, Osmiums, Platins, Iridiums, Rhodiums, Palladiums, Nickels, Kobalts, Urans, Ceriums, Eisens und Mangans auf den tierischen Organismus. 1824, Tübingen, *43*.

[16] K. Hansen, Versuche über die Wirkung des Tellürs auf den lebenden Organismus. *Annalen* 86 (1853), 208-215.

[17] F. Hofmeister, Uber Methylirung im. Tierköerper, *Arch. Exp. Pathol. Pharmakol*. 33 (1894), 198-215.

[18] F. Challenger, *Chem. Rev.*, Biological Methylation, 36 (1945), 315-361.

[19] F. Challenger, H.E. North, The production of organo-metalloid compounds by micro-organisms. Part II. Dimethyl selenide, *J. Chem. Soc*. 1934, 68-71.

[20] J. Karle, K. Wilson, A. Shrift, Selenocysteine lyase activity in a cysteine-requiring mutant of *Escherichia coli* K-12, *Biol. Trace Elem. Res*., 10 (1986), 307-315.

[21] J. Doran, M. Alexander, Microbial transformations of selenium, *Appl. Environ. Microbiol*. 33 (1977), 31-37.

[22] T. Chasteen, Ph.D. Dissertation, University of Colorado, Boulder, C.O., 1990.

[23] T. Chasteen, G. Silver, J. Birks, R. Fall, Fluorine-induced Chemiluminescence detection of biologically methylated tellurium, selenium, and sulfur compounds, *Chromatographia*, 30 (1990), 181-185.

[24] D. Amouroux, E. Tessier, C. Pecheyran, O. Donard, Sampling and probing volatile metal(loid) species in natural waters by in-situ purge and cryogenic trapping followed by gas chromatography and inductively coupled plasma mass spectrometry (P-CT–GC–ICP/MS) *Anal. Chim. Acta*, 377 (1998), 241-254.

[25] J. Swearingen Jr., D. Frankel, D. Fuentes, C. Saavedra, C. Vasquez, T. Chasteen, Identification of biogenic dimethyl selenodisulfide in the headspace gases above genetically-modified *Escherichia coli*, *Anal. Biochem*., 348 (2006), 115-122.

[26] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory, New York, 1989.

[27] S. McCarty, T. Chasteen, M. Marshall, R. Fall, R. Bachofen, Phototrophic bacteria produce volatile, methylated sulfur and selenium compounds,*FEMS Lett.* 112 (1993), 93-98.

[28] V. Stalder, T. Chasteen, Using fluorine-induced chemiluminescence to detect organo-metalloids in the headspace of phototrophic bacterial cultures amended with selenium and tellurium, *J. Photochem. Photobiol. B: Biol.* 43 (1998),193-203.

[29] L. Zhang L, T. Chasteen, Amending cultures of selenium-resistant bacteria with dimethyl selenone *Appl. Organomet. Chem.*8 (1994), 501-508.

[30] V. Stalder, N. Bernard, K. Hanselmann, R. Bachofen, T. Chasteen, A method of repeated sampling of static headspace above anaerobic bacterial cultures, *Anal. Chim. Acta* 303 (1995), 91-97.

[31] T. Chasteen, Confusion between dimethyl selenenyl sulfide and dimethyl selenone released by bacteria, *Appl. Organomet. Chem*. 7 (1993), 335-342.

[32] V. Van Fleet-Stalder, T. Chasteen, Using fluorine-induced chemiluminescence to detect organo-metalloids in the headspace of phototrophic bacterial cultures amended with selenium and tellurium, *J. Photochem. Photobiol*. 43 (1998), 193-203.

[33] V. Van Fleet-Stalder, H. Gürleyük, R. Bachofen, T. Chasteen. Effects of growth conditions on production of methyl selenides in cultures of *Rhodobacter sphaeroides* J. *Ind. Microbiol. Biotech*. 19 (1997), 98-103.

[34] D. Amouroux, P. Liss, E. Tessier, M. Hamren-Larsson, O. Donard, Role of oceans as biogenic sources of selenium *Earth Planet Sc. Lett*., 189 (2001), 277-283.

[35] P. Pinel-Raffaitin, C. Pécheyran, D. Amouroux, New volatile selenium and tellurium species in fermentation gases produced by composting duck manure, *Atmos. Environ*., 42 (2008), 7786-7794.

[36] M. McMaster, GC/MS A Practical User's Guide, Wiley, New York, 1998.

[37] T. Satchwill, MS Thesis, University of Calgary, Alberta, Canada, 2001, http://dspace.ucalgary.ca/bitstream/1880/40765/1/64977Satchwill.pdf; accessed June 12, 2009.

[38] J. Swearingen, Jr., M. Araya, M. Plishker, C. Saavedra, C. Vasquez, and T. Chasteen, Identification of biogenic organotellurides in *Escherichia coli* K-12 headspace gases using solid phase microextraction and gas chromatography, *Anal. Biochem.*, 331 (2004), 106-114.

[39] M. Shah, J. Meija, J. Caruso, Relative mass defect filtering of high-resolution mass spectra for exploring minor selenium volatiles in selenium-enriched green onions, *Anal. Chem*. 79 (2007), 846-853.

[40] P. Ollivier, A. Bahrou, S. Marcus, T. Cox, T. Church, and T. Hanson, Volatilization and precipitation of tellurium by aerobic, tellurite-resistant marine microbes, *Appl. Environ. Microbiol*. 74 (2008), 7163-7173.

[41] L. Hillen, R. Werner, Correlation of retention index data for polysulphides, polyselenides and related thiaselena-alkanes, *J. Chromatogr*. 79 (1973), 318-321.

[42] J. Meija, J. Caruso, Selenium and sulfur trichalcogenides from the chalcogenide exchange reaction, *Inorg. Chem*. 43 (2004), 7486-7492.

[43] K. Kubachka, K. Meija, D. LeDuc, N. Terry, J. Caruso, Selenium volatiles as proxy to the metabolic pathways of selenium in genetically modified *Brassica juncea*, *Environ. Sci. Tech*. 41 (2007) 1863-1869.

[44] J. Meija, J. Caruso, Selenium and sulfur trichalcogenides from the chalcogenide exchange reaction, *Inorg. Chem*. 43 (2004), 7486-7492; supplemental material URL: http://pubs.acs.org/doi/suppl/10.1021/ic0494206/suppl_file/ic0494206si20040504_022022.pdf.

[45] M. Montes-Bayón, E.G. Yanes, C. Ponce de León, K. Jayasimhulu, A. Stalcup, J. Shann, J.A. Caruso, Initial studies of selenium speciation in *Brassica juncea* by LC with ICPMS and ES-MS detection: an approach for phytoremediation studies, *Anal. Chem*. 74 (2002), 107-113.

[46] T. Cahill, R. Eldred, In Environmental Chemistry of Selenium, Frankenberger, W.T., Jr. and Engberg, R.A. (Eds.), Marcel Dekker, Inc., New York, 1998; Chapter 30.

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CHAPTER 4

NOVEL SPECTROPHOTOMETRIC METHOD FOR THE QUANTITATIVE ANALYSIS OF SELENIUM AND TELLURIUM OXYANIONS IN CULTURE MEDIA

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Introduction

Agricultural irrigation with water containing selenium extracted from soils and subsoils enhanced the concentration and mobility of selenium (Se) species in the surface waters of the western United States. The biological consequences of elevated Se in these surface waters have resulted in the extinction of endangered species near Kesterson Reservoir, designating Se as a major environmental contamination event (Engberg et al., 1998). On the other hand, the presence of Se in glutathione peroxidase, an antioxidant enzyme designates Se as an essential element for some living organisms (Emsley, 2002). The dual role of Se as a potential environmental toxicant and an essential element makes it a unique element for speciation analysis.

Tellurium (Te) has physical and chemical properties similar to that selenium. Te is mainly used for commercial purposes such as in vulcanizing rubber and coloring of glass and ceramics in the semiconductor industry (Bagnall, 1966). Some organo-tellurium compounds have been found to have anticancer and antioxidant properties (Jacob et al., 2000; Engman et al., 2003). Also, Yarema and Curry (2005) reported the acute toxicity of tellurium in young children. Recent reports suggest that ~1000 tons of tellurium is produced annually for photovoltaic solar cell industries. Because of the extensive use of Te industrially, the probability of finding Te as an environmental pollutant increases. It is detected at a concentration of mg L^{-1} in waste waters released by industries and at μg L^{-1} in natural waters (Bruzzoniti et al., 1997).

Selenite (SeO_3^{2-}), selenate (SeO_4^{2-}), tellurite (TeO_3^{2-}) and tellurate (TeO_4^{2-})are the water soluble oxyanions of selenium and tellurium. Tellurate is the least soluble of these four. The less oxidized forms are generally more toxic (Brasher and Ogle, 1993; Boisson et al., 1995). Keeping in view the adverse effects of Se and Te, the development of

analytical methods for their detection and quantification in the aqueous samples is, therefore, needed (Bruzzoniti et al., 1997).

Existence of these elements in a variety of chemical forms in the environment provoked interest in many scientists to determine their total element content and also to establish how they occur in the sample of interest. Normally, speciation of Se and Te involves several steps including sample pretreatment, separation and identification of species. The accuracy and precision of the final results depend on exactly how these steps are carried out. Moreover, the technique employed for speciation should be capable of offering highly efficient separation from interferences and sensitive detection (Engberg et al., 1998; Pedrero and Madrid, 2009).

Lack of sufficient sensitivity and selectivity in the methods for metalloid oxyanions determination such as gravimetry, volumetry and colorimetry for the analysis of Se and Te has led to the introduction of several hyphenated instrumental techniques, a major turning point in the progress of Se and Te speciation (Palmer, 1998; Pedrero and Madrid, 2009). Because of the combination of both separation technique and sensitive detection, these methods permit the identification and quantification of each element simultaneously (Pedrero and Madrid, 2009).

Hydride generation atomic absorption spectrometry (HG-AAS) is one of the most common techniques widely used for the analysis of Se and Te in environmental samples because of those methods' high sensitivity and selectivity. It is one of the recognized methods for water analysis by the Environmental Protection Agency (EPA) (Palmer, 1998). During this analysis, Se or Te oxyanions are chemically reduced to H_2Se or H_2Te using $NaBH_4$, in a strongly acidic solution. Subsequent thermal decomposition of H_2Se or H_2Te purged from solution produces their elemental forms whose absorption is recorded

at 196 nm (Se^0) or 214.3 nm (Te^0) (Tarin, 2006; Basnayake, 2001). Several disadvantages are associated with the HG-AAS method. For example, the Se and Te must be converted to + 4 oxidation state because + 6 can't be analyzed in this hydride generation method. The more oxidized forms must be converted to selenite and tellurite in a second analysis and subsequently determined by subtraction. Moreover, the accuracy of the method depends on the complete digestion of the sample. Interferences from different elements in hydride generation are also possible (Hershey and Keliher, 1986). For instance, iron, copper and nickel were found to decrease the formation of hydride in HG-AAS analysis (Goulden and Brooksbank, 1974; Vijan and Wood, 1976; Brown et al., 1981). However, it was also found that increasing the concentration of acid could reduce the interferences (Vijan and Leung, 1980). This hydride method is highly time–consuming and cannot determine all the chemical forms of Se and Te such as selenate and tellurate as mentioned before.

Inductively coupled plasma (ICP) in combination with atomic emission spectrometry (AES) has proved the most sensitive and powerful analytical tool for the speciation analysis of Se, and it has the advantage of analyzing more than one element at a time in each sample (Hwang et al., 1989; Tracey and Möller, 1990). ICP coupled to detectors like mass spectrometry (MS) have also been used for the successful determination of selenium concentration in biological samples (Darrouzes et al.,2005; Vanhaecke, 2002; Zhang et al., 2000). The ICP-AES technique involves the excitation of samples in the presence of a high temperature plasma with elements emitting radiation at their characteristic wavelengths, which can then be measured by AES. Complications because of the oxidation state of the sample can be ruled out using this technique, as it is sensitive to both selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) oxidation states, and interference

from the sample medium are eliminated at the plasma's higher temperatures (Christian, 1994). Low levels of selenium in environmental samples can be easily analyzed using ICP with good reproducibility because of its low detection limits. Trace amounts of water-soluble selenium species have been detected using ion chromatography-inductively coupled plasma-mass spectrometry (IC-ICP-MS) (Miekeley et al., 2005).

Capillary electrophoresis (CE) is another method which has recently been used for the determination of Se and Te oxyanions in bacterial cultures (Pathem et al., 2007). This method requires a low quantity of sample (less than 1 ng) and provides good absolute detection limits (pg levels). Relatively wide variety of species can be analyzed using this technique. For example, cations, anions and neutral species and also, non-covalently bound and colloidal systems (Michalke, 2003). Also in CE, stationary phase is absent, and therefore sample integrity modification by stationary phase can be avoided. CE coupled with ICP-MS was used in speciation analysis but this technique was associated with many drawbacks such as difficulty in adapting the sample flow rates required for both techniques e.g., nL min^{-1} for CE and mL min^{-1} for ICP-MS. Also, interface construction between the two components for this technique seems to be critical (Uden, 2002).

For many years, spectrophotometric determination of Se and Te has been carried out as a part of their speciation analysis. This technique is very simple when compared to the complicated hyphenated techniques described above. In a study performed by Mahaveer and Jaldappa (2000), selenite (SeO_3^{2-}) was allowed to react with methdilazine hydrochloride in the presence of HCl leading to the formation of a red radical cation via oxidation by Se (IV)which absorbs at 513 nm. This method was used for the determination of Se in alloys.

In another method, trace amounts of Se were determined spectrophotometrically using Variamine Blue (VB) as a chromogenic reagent (Revanasiddappa and Kiran Kumar, 2001). This method was based on the reaction of Se with potassium iodide in acidic medium generating iodine which oxidizes VB to form aviolet-colored species. Another recent method for the spectrophotometric determination of the tellurium (IV) include the conversion of leuco methylene greento its blue form of methylene green via oxidation by tellurium in acidic medium. This dye absorbs at 650 nm in acetate buffer medium (Prasad et al., 2007).

In summary, several methods have been implemented for the spectrophotometric determination of Se and Te with the use of different dyes. Although these method sare able to detect trace amounts of Se, these analyses require heating of the sample and required a long time for color formation, and the dyes used for the detection are carcinogenic. To overcome these inadequacies, development of a new method is necessary (Revanasiddappa and Kiran Kumar, 2001; Mahaveer and Jaldappa, 2000).

In the present work, we introduce a simple and quick method for the quantification of Se and Te anions in the culture media. Sodium borohydride ($NaBH_4$) is used as the reducing agent for the reduction of Se and Te oxyanions to their elemental forms whose absorbance/scattering is measured at 500 nm using a UV/vis spectrophotometer. This method is straightforward and fast, with relatively less expensive instrumentation, and involves the use of a single reagent. The method's linear range and detection limit for Se and Te oxyanions and use to determine oxyanion bioprocessing by metalloid- resistant bacteria are reported here.

Materials and Methods

Apparatus and Reagents

For quantitative analysis of Se and Te oxyanions in aqueous solutions, a UV/vis spectrophotometer, model V-550 (Jasco Corp., Tokyo, USA) was used. The sources of chemicals used are as follows: bacto^{TM} tryptone (Becton Dickson, Sparks, MD, USA), yeast extract (Difco Laboratories, Detroit, MI, USA), potassium tellurite hydrate, sodium tellurate dihydrate, sodium selenate and sodium selenite, sodium borohydride (Sigma-Aldrich, Milwaukee, WI, USA) and sodium chloride (BDH, West Chester, PA, USA). Deionized water is prepared using a RiOs 3 water purification system from Millipore (Billerica, MA, USA).

Bacterial Strain used in the experiments

A metalloid-resistant organism was isolated from sediment and water from Tinguilico Lake, 9th Region, Chile. This organism named LHVE, was classified into *Bacillus* genus based on classical microscopy and biochemical tests (Speck, 1976; Bergey et al., 1984).

Sample Preparation

Luria-Bertani (LB) growth medium was prepared by dissolving 10.0 g of tryptone, 10.0 g of sodium chloride, 5.0 g of yeast extract in 1 liter of deionized water. The pH was adjusted to 7 using sodium hydroxide. LB medium was autoclaved for the experiments carried out with LHVE. Fresh metalloid stock solutions of required concentration were prepared initially by dissolving the sodium or potassium salts of Se and Te in LB medium. A series of standard solutions for calibration experiments were prepared from this stock solution. The total volume in the reduction reaction vessel, a test tube, was 10 mL.

To determine the optimum borohydride concentration, different concentrations of borohydride ranging from 40 mM down to 2.5 mM were prepared and added to metalloid oxyanion-containing standards. Based on visual observation of low effervescence and metalloid detection limits, a 3.5 mM borohydride concentration in the solution was used for all the subsequent experiments carried out.

The solution of borohydride (3.5 mM) was prepared in water and then added to each of the standards to reduce the anions of Se and Te in solution to their elemental forms. After 5 min, optical density (OD, absorbance/scattering) of the standard solutions was read at 500 nm using UV/visible spectrophotometer.

LB medium with borohydride alone was used in the reference cuvette in these experiments. Upon addition of borohydride the initial colorless solutions changed to red or black indicating the formation of Se and Te elemental forms, respectively. The absorbance/scattering readings correspond (in the linear range) to the concentration of elemental form of Se and Te produced after reduction. The absorbance of the analyte samples which were beyond the linear range (*i.e.,* where the proportionality between absorbance and concentration is lost) were not recorded. For instance, tellurite has a linear range that ends at ~220 μg/mL. Beyond this range (300 μg/mL), the linearity is lost, that is, the optical density reading taken was at the instrument's maximum output reading and no longer increased with concentration.

Spectral (light scattering) measurements for selenium and tellurium

Experiments were carried out to determine the UV/vis (absorbance/scattering) spectrum of the elemental forms of Se and Te produced by the reduction of borohydride in LB media. Solutions were reduced for 10 minutes and then spectral measurements

within a range of 200 to 800 nm were carried out at metalloid oxyanion concentrations that produced a maximum OD reading not larger than 3.5.

Results

Elemental Se and Te spectral measurements

Figures 11 and 12 display the spectral measurements for Se and Te formed upon reduction with borohydride. Since the reference beam's cuvette contained LB medium with 3.5 mM sodium borohydride, the features in Figures 11 and 12 represent only the absorbance and scattering of the metalloid-containing particles formed by the reduction of selenite and tellurite, respectively.

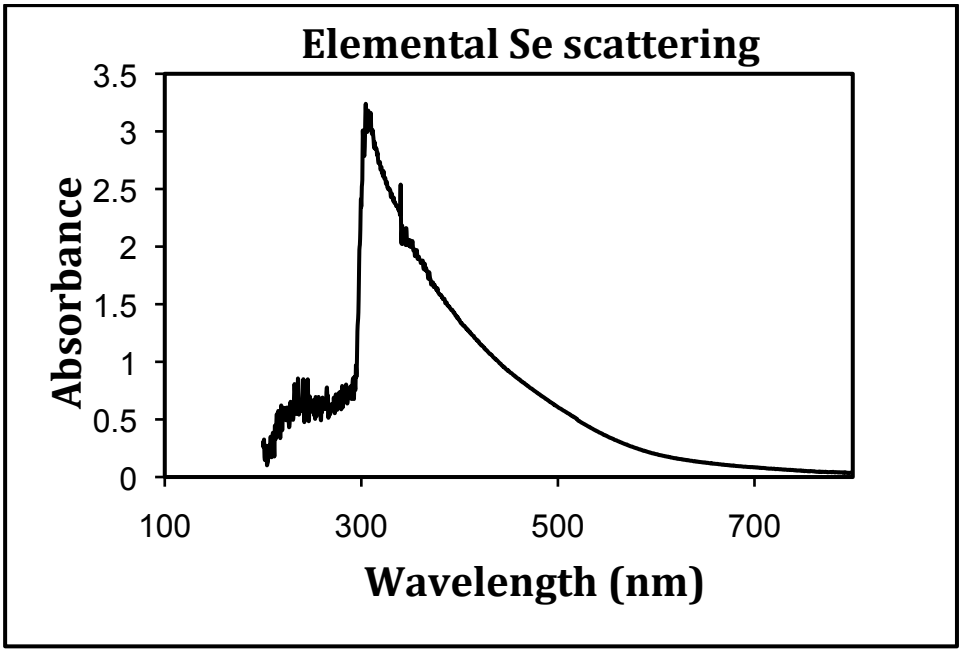

Figure 11. Scattering of elemental selenium formed by the 3.5 mM borohydride reduction of selenite.

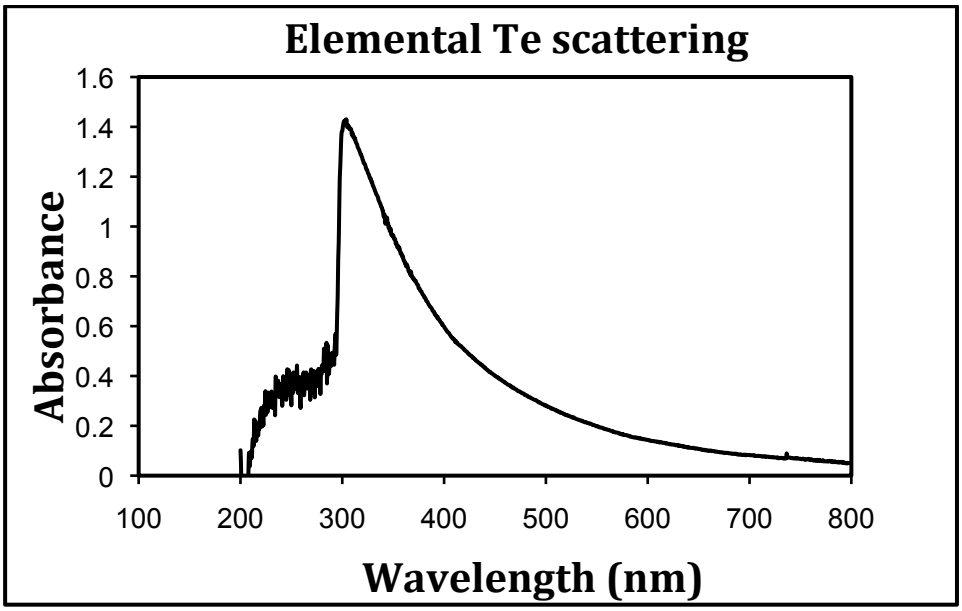

Figure 12. Scattering of elemental tellurium formed by the reduction of tellurite by 3.5 mM borohydride.

Calibration Experiments

In an attempt to develop a method for the quantification of Se and Te oxyanions in aqueous solution, a set of calibration experiments were performed. Different sets of solutions containing increased concentrations of Se and Te anions in LB media were prepared and then reduced using a final concentration of 3.5 mM $NaBH_4$ as determined from the borohydride optimization experiments described above. The absorption readings taken were used to develop a linear range for these anions' analyses. The calibration curve developed was used to determine whether the increase in absorption at 500 nm caused by the reduction of anions present in the solution was proportional to Se^0 or Te^0 concentration or not and over what range. The results obtained can be used to correlate the bacterial ability of reducing these anions in grown cultures with oxyanion concentration in those cultures.

Selenite and Selenate Calibration

Using borohydride as reducing agent, the linear ranges for selenite and selenate were determined. Figure 13 and 14 represent the calibration graphs for selenite and selenate determined spectrophotometrically. Formation of brick red precipitate indicated the reduction of Se oxyanions to elemental Se. The detection limit was determined as an analyte concentration that would produce 3 times the standard deviation of multiple blank readings (n=5; 95% confidence), and the bottom of the linear range for each of the anions studied was determined as the concentration that would yield a signal 10 times the standard deviation of multiple blank readings (Skoog et al., 2007). A typical linear calibration graph obtained had a correlation coefficient of 0.991 for selenite and 0.993 for selenate.

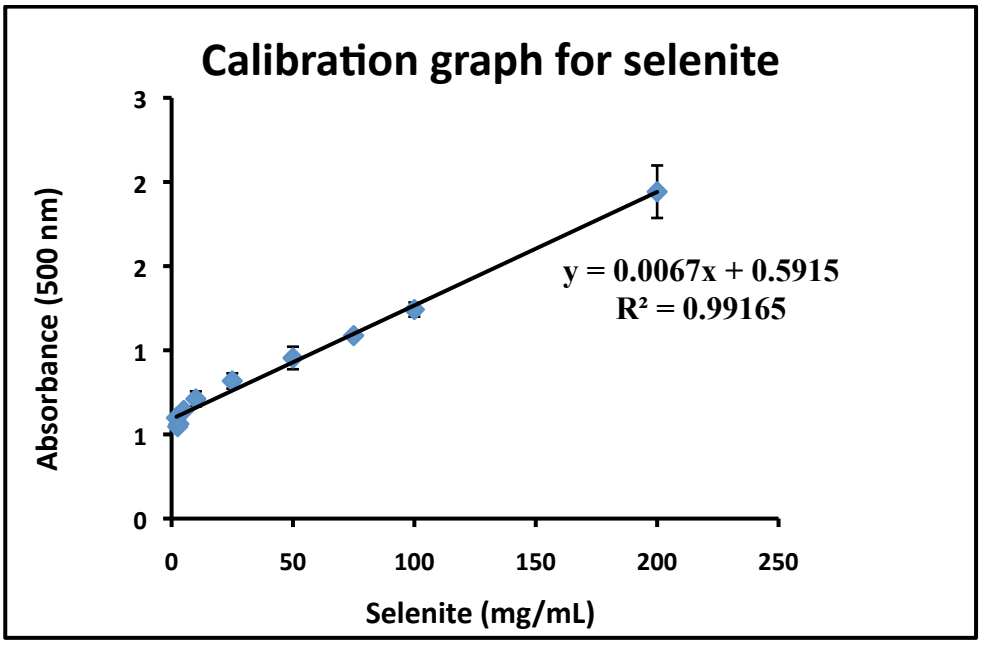

Figure 13. Calibration curve for selenite. Three replicates were prepared for each standard and error bars represent the standard deviation of those three replicates.

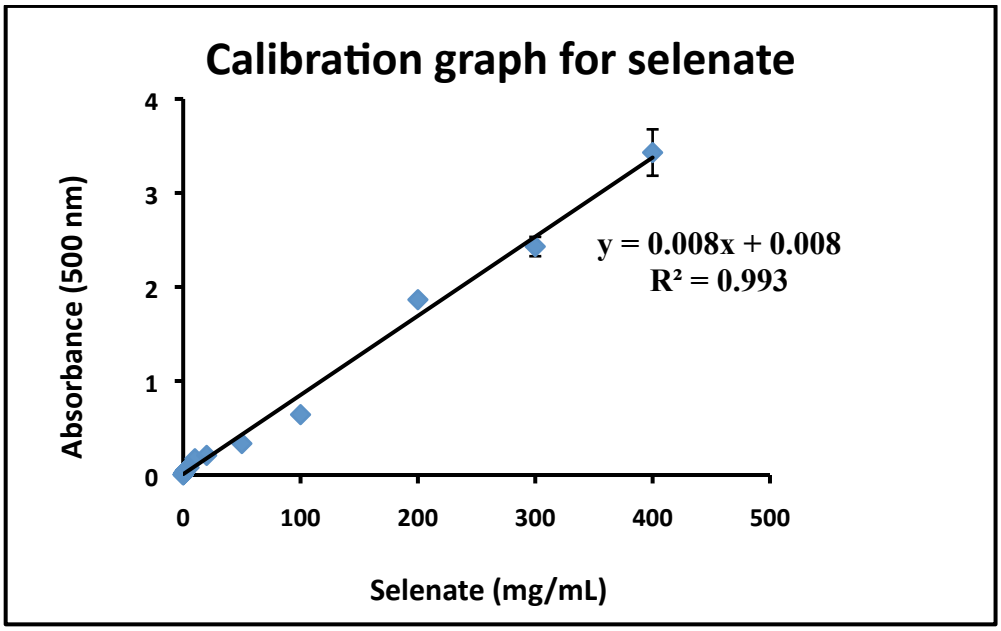

Figure 14. Calibration curve for selenate quantification by this method. Error bars represent 1 standard deviation of 3 replicates

Tellurite and Tellurate Calibration

A good correlation coefficient of 0.996 was typically obtained for tellurite, and for tellurate analysis, R^2 value was 0.963 with a much narrower linear range (Figures 15 and 16). The reason for this was probably the insolubility of tellurate at higher concentrations: the highest concentration of dissolved TeO_4^{2-} was not much beyond 6 mg/mL in LB medium. The determined concentration ranges for all metalloidal oxyanions examined were in the range of the Minimal Inhibitory Concentrations (MICs) for this oxyanions of metalloid-resistant bacteria we have been studying in our lab. Therefore, this method can reasonably be used as a means of determining metalloid concentrations in bacterial culture supernatants.

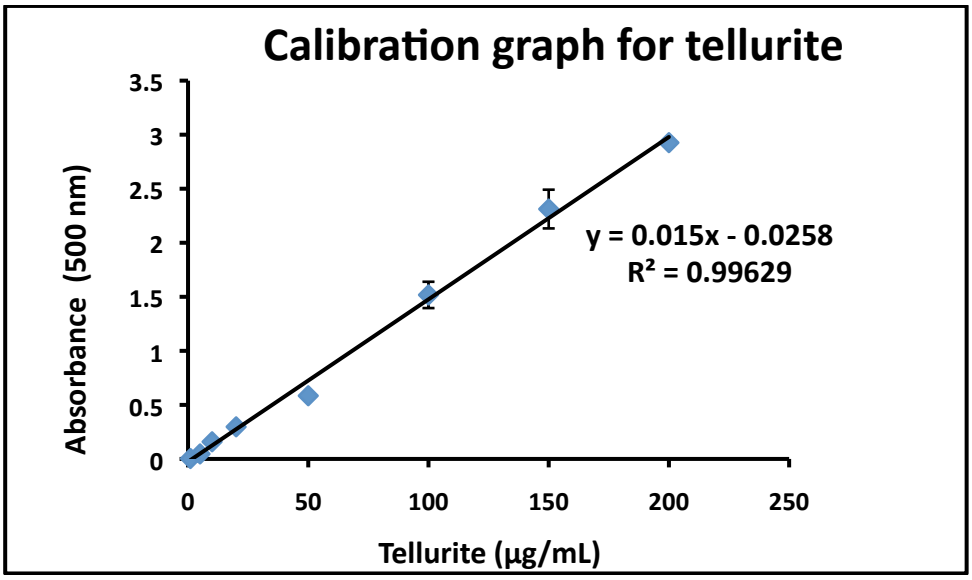

Figure 15. Tellurite calibration curve. Error bars represent 1 standard deviation of 3 replicates.

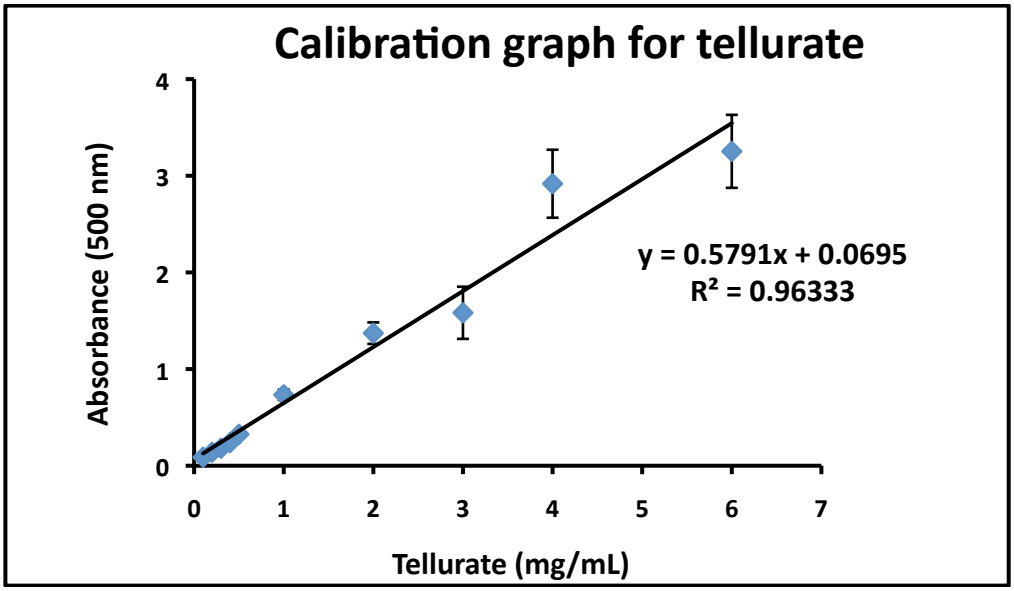

Figure 16. Calibration curve for tellurate with a narrow linear range compared to tellurite. Error bars represent 1 standard deviation of 3 replicates.

Time Course Experiments for Reduction Reaction

To estimate the range of time over which the elemental forms of Se or Te that formed as a result of the reduction reaction were stable, measurements of OD for reduced oxyanion solutions at regular intervals of time for a particular period were performed. Figure 17 and 18 depict the time course measurement of absorbance for selenite and tellurite standard solutions. The stability of elemental Se or Te formed in the solution by borohydride reduction was longer at lower metalloid anion concentration. The stability was shorter at higher metalloid anion concentration probably because of the presence of more metalloid available for reduction: for instance, the OD_{2hours} of a 200 mg/mL selenite sample was 37% different from the OD_{10min} ($\frac{2.37-1.72}{1.72}$ x100) while for a 25 mg/mL selenite sample this difference was 0.77% ($\frac{0.785-0.779}{0.779}$ x100). For tellurate this reduction was much slower, reaching a stable maximum after only about 60 minutes.

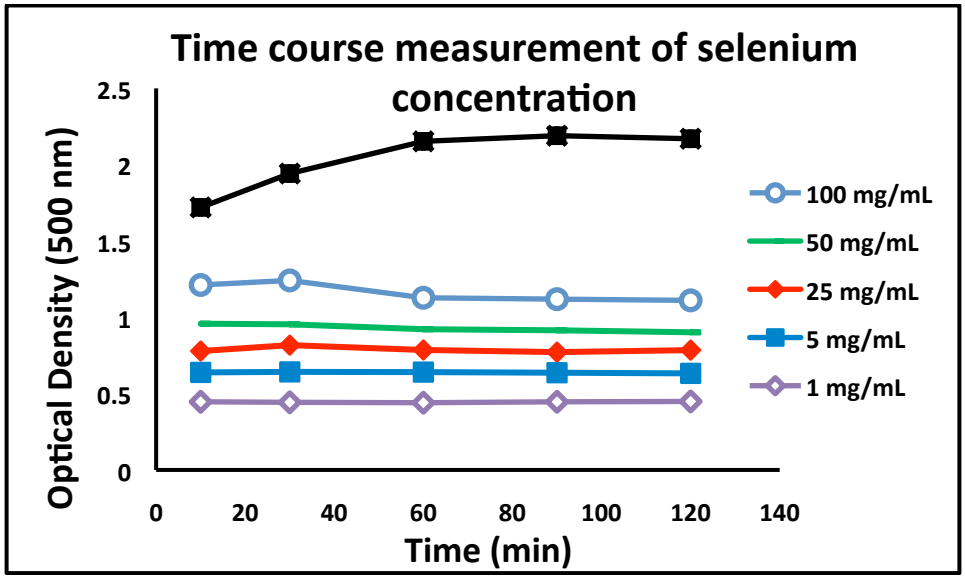

Figure 17. Time course measurement of selenium concentration at 500 nm formed as a result of reduction of selenite by sodium borohydride.

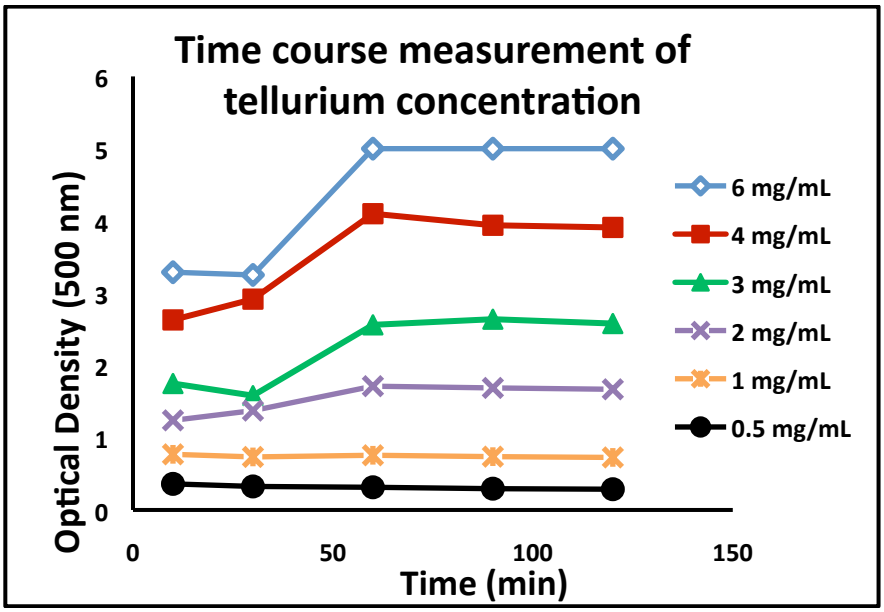

Figure 18. Measurement of absorbance at regular intervals of time for the concentration of tellurium formed in the reaction between tellurate and borohydride at 500 nm.

Short time course experiments were also performed with 20 µg/mL tellurite to determine the time necessary to develop the metalloid's elemental form. The formation of the elemental particles in solution increased gradually in the first five minutes of reduction (see minimized graph in Figure 19) and then maintained stability thereafter for a considerable amount of time (maximized graph in figure 19). The $OD_{120\ min}$ of a 20 µg/mL TeO_3^{2-} sample was only 2.7 % different ($\frac{0.225 - 0.219}{0.225} x100$) from the OD averaged from 30 to 120 minutes and only 4.8% different ($\frac{.0219 - .0209}{0.209} x100$) from the sample at 5 minutes.

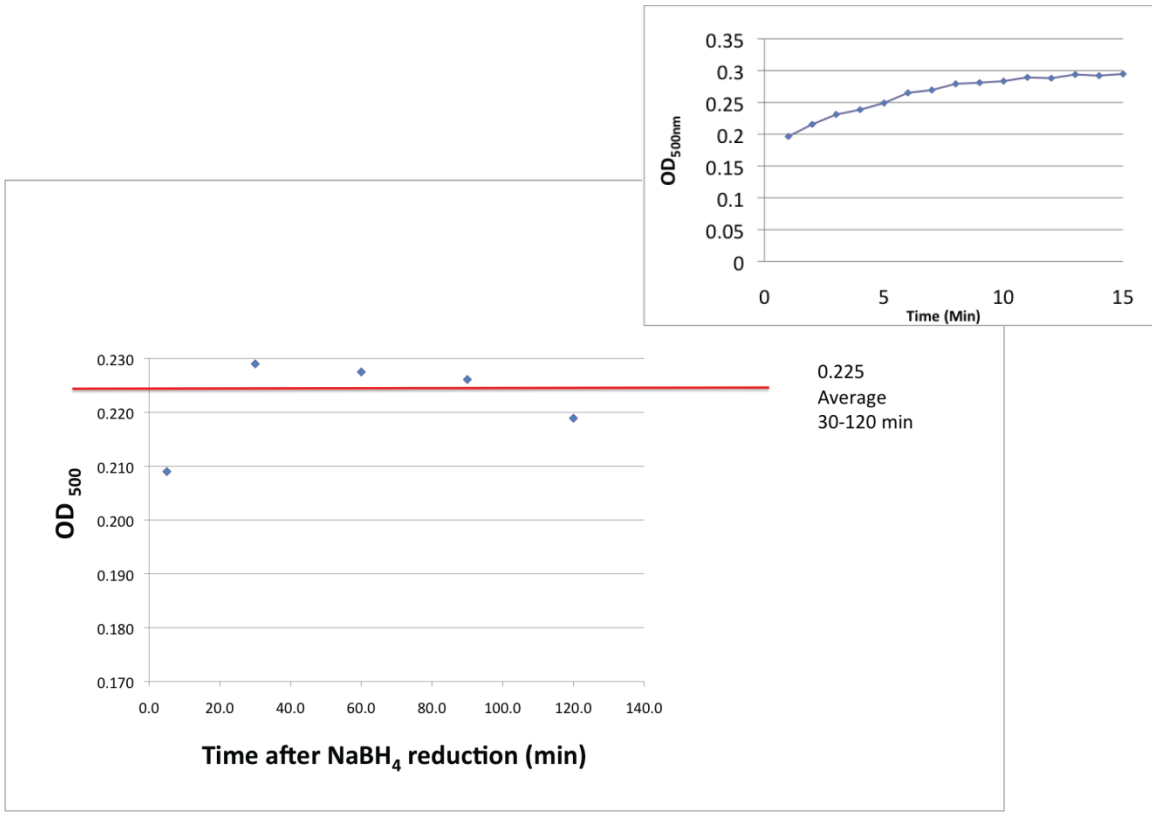

Figure 19. Short (minimized graph) and long (maximized graph) course experiment for a 20 µg/mL tellurite sample reduced with sodium borohydride.

Following Bacterial Bioprocessing of Tellurite Using This Method

The proposed borohydride method was implemented for the quantification of Te and Se in culture media to compare the extent of bacterial reduction in a live culture. In this experiment, LB medium was inoculated with a 1 % LHVE preculture and amended with 25 µg tellurite/mL in a conical flask. These were allowed to incubate anaerobically at 37 ^{0}C. At regular intervals of time, 10 mL culture was taken out of this solution. Five mL were used to measure the growth rate of the bacteria at 526 nm and another five mL were used to determine the tellurite concentration using the borohydride method. For this, that second 5 mL sample was centrifuged for 3 min at 13000xg, then 3.5 mM $NaBH_4$ was added to the supernatant solution and the optical density was measured at 500 nm to estimate the amount of Te^0 formed by borohydride reduction and interpolation from the tellurite calibration curve.

Similar procedures were carried out for the cultures amended with 0.86 mg/mL (5 mM) selenite in culture media. The results from these experiments are depicted in Figures 20 and 21.

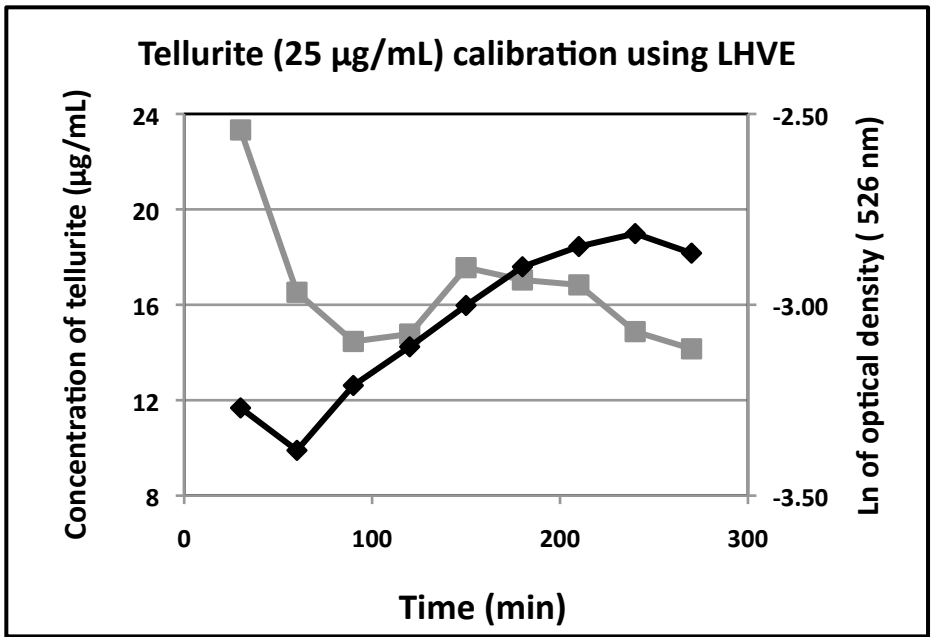

Figure 20. LHVE amended with 25 µg/mL tellurite. The black curve represents the growth rate of LHVE in the presence of 25 µg/mL tellurite over a particular period of time whereas the grey curve gives the tellurite content using the borohydride reduction method. These results are from a single trial without any replicate analysis.

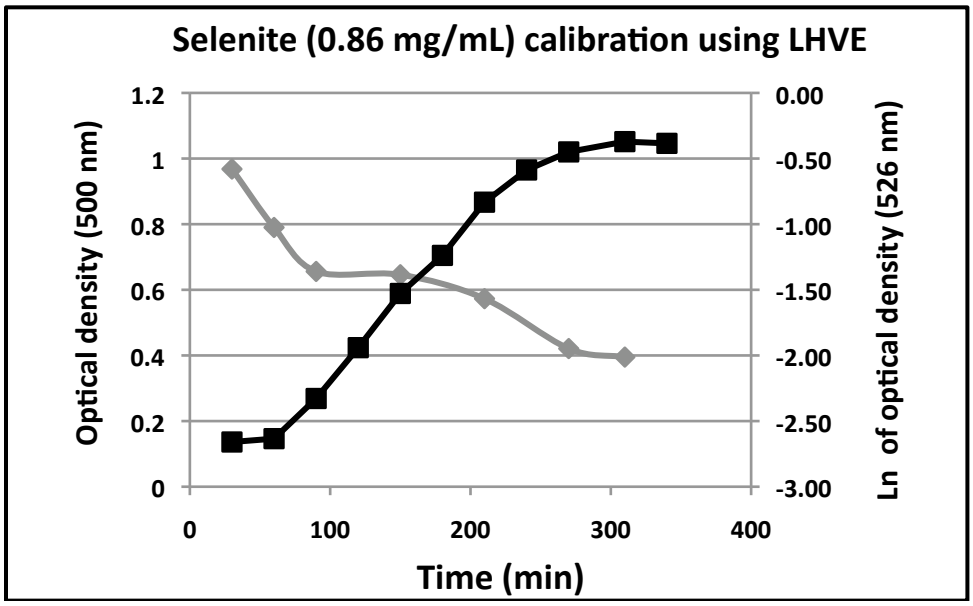

Figure 21. LHVE amended with 0.86 mg/mL selenite. The black line indicates the cell population of LHVE in the presence of selenite and the grey line represents the decrease in optical density because of bioprocessing by LHVE. These results are from a single trial without any replicate analysis.

Discussion

A simple and novel method was developed for the quantification of Se and Te in aqueous culture solutions. Compared to the much more complicated techniques which require high-cost investments, this method is a useful replac7ement for some types of samples. Good linear ranges were exhibited for Se and Te oxyanions. During the initial stages of method development, there was a major problem with bubbling of solutions immediately after adding $NaBH_4$ to the standards. Different parameters were adjusted such as the pH of deionized water and the nature of borohydride added (solid/liquid).

In order to minimize the runaway bubbling during sample reduction, the optimum borohydride concentration was determined by reducing a 200 μg/mL tellurite solution with varying concentrations of sodium borohydride in the reduction/reaction vessel's solution. Our initial experiments showed that the worse the borohydride concentration, the higher the bubbling problem. Different sets of experiments were performed with borohydride concentrations ranging from 40 mM to 2.5 mM. There was extreme bubbling at high $NaBH_4$ concentration and the bubbling decreased at lower borohydride levels. Based on the observations of low effervescence and good linear range for the standards, 3.5 mM was determined as the optimum borohydride concentration for these experiments. The detection limits and linear ranges for all the oxyanions studied are in Table 2.

Table 2. Detection limits and linear ranges for the oxyanions under study using 3.5 mM $NaBH_4$ as the reducing reagent.

Oxyanion	*Detection limit*	*Linear Range*
Selenite	0.13 mg/mL	0.77- 200 mg/mL
Selenate	2.88 mg/mL	47.25-400 mg/mL
Tellurite	1.61 μg/mL	28.24-200 μg/mL
Tellurate	0.026 mg/mL	0.39-6 mg/mL

A broad linear range was determined for selenate, selenite and tellurite whereas for tellurate, the range was narrow. The linear range includes most of the MICs of tellurite for the microorganism we have been studying recently (Burra et al., 2009a; 2009b). Therefore, this method can be employed successfully to estimate the metalloid concentrations in culture media.

From an analytical point of view, any new method employed should produce analytical conditions which are stable for a considerable amount of time. To confirm the stability of the elemental metalloid formation in this method, time course experiments were carried out. The elemental form of Se or Te formed after borohydride reduction for concentrations lower in the linear range seems to be stable for an analytically useful period from about 10 minutes to longer than an hour and quite a bit longer time at lower metalloid concentration. At higher analyte concentrations, samples exhibited some fluctuations with sample ODs rising until about 60 min before stabilizing; however, the differences over time may still be viable for some analyses. This might be due to the lower $NaBH_4$/analyte ratio affecting the time necessary to reduce the metalloid present in

solution. At lower analyte concentrations, excess reducing power almost certainly stabilized the samples.

In the experiments carried out to determine the metalloid concentration in bacterial culture using this borohydride method, a time dependent decrease in metalloid anion concentration was observed (Figure 20 and 21, grey curve). The decrease in supernatants' oxyanion concentration can be related to an increase in the number of cells as determined by bacterial growth rate (Figure 20 and 21, black curve). The decrease in anion concentration might be attributed to the bioprocessing of those anions by metalloid-resistant LHVE resulting in the production of other forms of metalloids in solution or forms escaping into bacterial headspace. For instance, this organism produces methylated forms of both selenium and tellurium when amended with metalloidal salts. We have detected dimethyl selenide, dimethyl diselenide, and dimethyl telluride in LHVE headspace (data not shown). The bacteria can adopt several different ways to cope with the toxic environment and may convert these toxic anions to less toxic forms.

Conclusion

Quantification of Se and Te oxyanions in aqueous solutions was shown to be possible using this simple and novel technique. This method uses only one reagent and simple instrumentation for analysis, and has a relatively wide linear range for all the oxyanions under study except tellurate. We have demonstrated the use of this technique to determine Se and Te oxyanions in growing bacterial cultures amended with metalloid-containing salts. The interference of species other than the target ions in this method of Se and Te anions determination is under investigation in our lab.

References

Bagnall, K.W. *The Chemistry of Selenium, Tellurium and Polonium,*Elsevier Press: Netherlands, **1966**; Chapter 1.

Basnayake, R.T. M.S. Thesis, Sam Houston State University, Huntsville, TX, **2001**.

Bergey, D. H.; Holt, J. G.; Krieg, N. R.*Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins Publishers, Baltimore, MD, **1984**.

Boisson, F.; Gnassia-Barelli, M.; Romeo, M. *Arch. Environ. Contam. Toxicol*. **1995**, *28*, 487-493.

Brown, R. M. Jr.; Fry, R. C.; Moyers, J. L.; Northway, S. J.; Denton, M. B.; Wilson, G. S. *Anal. Chem*. **1981**, *53*, 1560-1566.

Brasher, A.M.; Ogle, R.S. *Arch. Environ. Contam. Toxicol*. **1993**, *24*, 182-186.

Bruzzoniti, M.C.; Mentasti, E.; Sarzanini, C.; Cavalli, S. *Chromatographia***1997**, *46*, 49-56.

Burra, R; Fox, J.D.; G.A. Pradenas, G.A.; Vasquez, C.C; Chasteen, T.G. *Environ. Technol*., **2009a**, in press.

Burra, R.; Pradenas, G.A.; Montes, R.A.; Vasquez, C.C.; Chasteen, T.G. *Anal. Biochem*. 2009b, in press.

Christian, G.D. *Analytical Chemistry,* 5th ed.; Wiley: New York, **1994**, 465.

Darrouzes, J.; Bueno, M.; Lespes, G.; Potin-Gautier, M. *J. Anal. At. Spectrom*. **2005**, *20*, 88-94.

Emsley, J. *Nature's Building Blocks*: *An A-Z Guide to the Elements*; Oxford University Press: New York, **2002**.

Engberg, R. A.; Westcot, W.D.; Delamore, M.; Holz, D.D. *Environmental Chemistry of Selenium*, Marcel Dekker: New York, **1998**.

Engman, L.; Al-Maharik, N.; McNaughton, M.; Birmingham, A.; Powis, G. *Anticancer Drugs,* **2003***, 14*, 153–161.

Goulden, P. D.; Brooksbank, P. *Anal. Chem*. **1974**, *46*, 1431-1436.

Hershey, J.W.; Keliher, P.N. *Spectrochim. Acta B* **1986**, *41*, 713-723.

Hwang, J.D.; Guenther, G.D.; Diomiguardi, J.P. *Anal. Chem*. **1989**, *61*, 285-288.

Jacob, C.; Arteel, G.E.; Kanda, T.; Engman, L.; Sies, H. *Chem. Res. Toxicol.* **2000**,*13,* 3-9.

Mahaveer, B.M.; Jaldappa, S. *Turk. J. Chem*. **2000**, *24*, 287-290.

Michalke, B. *Handbook of Elemental Speciation,* Wiley Interscience: London, **2003**; Chapter 4.

Miekeley, N.; Pereira, R.C.; Casartelli, E.A.; Almeida, A.C.; Carvalho, M.F.B. *Spectrochim. Acta B* **2005**, *60*, 633-641.

Palmer, I.S. *Environmental Chemistry of Selenium*, Marcel Dekker: New York, **1998**; Chapter 3.

Pathem, B.K.; Pradenas, G.A.; Castro, M.E.; Vasquez, C.C.; Chasteen, T.G. *Anal. Biochem*. **2007**, *364*, 138-144.

Pedrero, Z.; Madrid, Y. *Anal. Chim. Acta*. **2009**, *634*, 135–152.

Prasad, P. R.; Kumar, J. D.; Priya, B. K.; Subrahmanyam, P.; Ramanaiah, S.; Chiranjeevi, P. *E. J. Chem*. **2007**, *4*, 354-362.

Revanasiddappa, H.D.; Kiran Kumar, T.N. *Anal. Sci*. **2001**, *17*, 1309-1312.

Skoog, D.A; Hooler, F.J.; Crouch, S.R. *Principles of Instrumental Analysis*, 6th ed. Thomson: Belmont, CA, **2007**; Chapter 1.

Speck, M. L.*Compendium of Methods for the Microbiological Examination of Foods*, American Public Health Association: New York, **1976**.

Tarin, P. Ph. D. Thesis, University of Wollongong, New South Wales, Australia, **2006.**

Tracey, M.L.; Möller, G. *J. Assoc. Off. Anal. Chem*. **1990**, *73*, 404-410.

Uden, P. *Anal. Bioanal. Chem*. **2002**, *373*, 422-431.

Vanhaecke, F. *Anal. Bioanal. Chem*. **2002**, *372*, 20-21.

Vijan, P.N.; Leung, D. *Anal. Chim. Acta* **1980**, *120*, 141-146.

Vijan, P. N.; Wood, G. R. *Talanta* **1976**, *23*, 89-94.

Yarema, M.C.; Curry, S.C. *Pediatrics*. **2005**, *116*, e319-321.

Zhang, Y.; Kharel, Y.; Koyama, T. *J. Mol. Catal. B Enzym.*, **2000**, *10*, 623- 630.

CHAPTER 5

CONCLUSIONS

Three different projects were carried out in this research. First, toxicity experiments including specific growth rate (SGR) and zone of inhibition (ZOI) were used to determine the relative effect of oxyanions of selenium and tellurium on a *Bacillus* species isolated from Chile. Second, volatile organo-selenium compounds were detected in the headspace of the metalloid-resistant bacteria using gas chromatography coupled to fluorine-induced chemiluminescence detection. Third, selenium (Se) and tellurium(Te) oxyanions in the bacterial culture media were quantified using sodium borohydride reduction followed by UV/visible spectrophotometry.

Toxicity experiments have determined LHVE to be a metalloid-resistant organism compared to other environmental bacteria such as to *E. coli* (wild type) and demonstrated LHVE's ability to grow in the presence of different concentrations of Se and Te oxyanions. Based on SGR and ZOI experiments, tellurite (TeO_3^{2-}) was shown to be more toxic than all the anions examined. A ZOI of 52 mm was observed on the plates amended 5 microliters of 25 mM TeO_3^{2-}. No inhibition zone was observed for the Se anions at concentrations up to 200 mM; however, the relative toxicity of selenocyanate anion was comparable to selenate and selenite.

Headspace analysis of the selenite amended LHVE culture led to the detection of dimethyl diselenenyl sulfide and dimethyl triselenide ($CH_3SeSeSeCH_3$). These compounds were previously unreported in the literature as occurring in bacterial headspace and their identities were confirmed based on retention time, boiling point comparisons, and mass spectral analysis. DL-Dithiothreitol(DTT) experiments tentatively assigned the dimethyl diselenenyl sulfide asymmetric isomer,$CH_3SeSeSCH_3$, as the form

found in headspace above LHVE bacterial cultures amended with sodium selenite. In addition to these two new compounds, a wide range of other organo-sulfur and –selenium, compounds were also detected in the bacterial headspace.

In contrast to the most prolonged and strenuous methods, a straightforward and easy technique was developed for the quantification of Se and Te oxyanions using sodium borohydride ($NaBH_4$) as a reducing agent. Simple instrumentation and a single reagent were required for this analysis. Except for tellurate, relatively wide linear ranges were obtained for all the oxyanions examined. This method was employed for the determination of Se and Te oxyanions in growing bacterial cultures amended with metalloid-containing salts.

Ongoing research involves the determination of interferences from chemical species other than target ions during analysis of Se and Te anions and future work will include the quantification of mixed Se and Te oxyanions in the bacterial culture media with LHVE.

REFERENCES

Ábalos, M.; Prieto, X.; Bayona, J.M. *J. Chromatog.* A **2002,** *936*, 249-257.

Amouroux, D.; Liss, P.; Tessier, E.; Hamren-Larsson, M.; Donard, O. *Earth Planet Sc. Lett*., **2001**, *189*, 277-283.

Amouroux, D.; Tessier, E.; Pecheyran, C.; Donard, O. *Anal. Chim. Acta*, **1998**, *377*, 241-254.

Appleton, A. R.; Cain, C. B. Western State Petroleum Association Report, Concord California, **1995**.

Araya, M.A.; Swearingen Jr., J.W.; Plishker, M.F.; Saavedra, C.P.; Chasteen, T.G.; Vásquez, C.C. *J. Biol. Inorg. Chem*. **2004**, *9*, 609-615.

Bagnall, K.W. *The chemistry of selenium, tellurium and polonium*; Elsevier Press: Netherlands, **1966**; Chapter 1.

Bañuelos, G.S.; Ajwa, H.A.; Terry, N.; Zayed, A. *J. Soil Water Conserv.***1997**, *52*, 426-430.

Banuelos, G. S.; Lin, Z. Q. *Ecotoxicol. Environ. Saf.* **2005**, *62,* 309-316.

Basnayake, R.T. M.S. Thesis, Sam Houston State University, Huntsville, TX, **2001**.

Basnayake, R.T.; Bius, J.H.; Akpolate, O.M.; Chasteen, T.G. *Appl. Organomet. Chem.* **2001**, *15*, 499-510.

Bennett, W. N. *Water Res.* **1988***,22*, 939–942.

Bergey, D.H.; Holt, J.G.; Krieg, N.R.; *Bergey's Manual of Systematic Bacteriology*, Williams and Wilkins Publishers, Baltimore, MD, **1984.**

Bius, J.H. M. S. Thesis, Sam Houston State University, Huntsville, TX, **2001**.

Boisson, F.; Gnassia-Barelli, M.; Romeo, M. *Arch. Environ. Contam. Toxicol*. **1995**, *28*, 487-493.

Brasher, A.M.; Ogle, R.S. *Arch. Environ. Contam. Toxicol*. **1993**, *24*, 182-186.

Brix, K. V.; Volosin, J. S.; Adams, W. J.; Reash, R. J.; Carlton, R. G.; McIntyre, D. O. *Environ. Toxicol. Chem*. **2001**, *20,* 1037-1045.

Brown, R. M.; Fry, R. C. Jr., ; Moyers, J. L.; Northway, S. J.; Denton, M. B.; Wilson, G. S. *Anal. Chem*. **1981**, *53*, 1560-1566.

Bruzzoniti, M.C.; Mentasti, E.; Sarzanini, C.; Cavalli, S. *Chromatographia***1997**, *46*, 49-56.

Burra, R.; Fox, J.D.; Pradenas, G.A.; Vasquez, C.C.; Chasteen, T.G. *Environ. Technol.***2009a**, in press.

Burra, R.; Pradenas, G.A.; Montes, R.A.; Vasquez, C.C.; Chasteen, T.G. *Anal. Biochem*. **2009b**, in press.

Cahill, T.; Eldred, R. Marcel Dekker, Inc., New York, **1998**; Chapter 30.

Canton, S. P. *Environ. Toxicol. Chem.***1999**, *18*, 1425-1432.

Challenger, F. *Chem. Rev.* **1945**, *36*, 315-361.

Challenger, F.; H.E. North, H. E. *J. Chem. Soc*. **1934**, 68-71.

Chasteen, T, G. Ph.D. Dissertation, University of Colorado, Boulder, C.O., **1990**.

Chasteen, T.G. *Appl. Organomet. Chem.* **1993**, *7*, 335-342.

Chasteen, T.G.; Bentley, R. *Chem. Rev.* **2003**, *103*, 1-26.

Chasteen, T. G.; Silver, G.; Birks, J.; Fall, R. *Chromatographia*, **1990**, *30*, 181-185.

Chasteen, T. G.; Bentley, R. F. A. Devillanova, ed., Royal Society of Chemistry Publishing, Cambridge, UK, **2007**, 671-713.

Chen, K.M.; Sacks, P.G.; Spratt, T.E.; Lin, J.M.; Boyiri, T.; Schwartz, J.; Richie, J.P.; Calcagnotto, A.; Das, A.; Bortner, J.; Zhao, Z.; Amin, S.; Guttenplan, J.; El-Bayoumy, K. *Biochem. Biophys. Res. Commun*. **2009**, *383*, 151-155.

Christian, G.D. *Anal. Chem.* **1994**, 465.

Clark, L.C.; Turnball, B.W.; Slate, E.H.; Chalker, D.K.; Chow, J.; Davis, L.S.; Glover, R.A.; Graham, D.K.; Gross, E.G.; Krongrad, A.; Lesher, J.L.; Park, H.K.; Sanders, B.B.; Smith, C.L.; Taylor, J.R.; Alberts, D.S.; Allison, R.J.; Bradshaw, J.C.; Curtus, D.; Deal, D.R.; Dellasega, M.; Hendrix, J.D.; Herlong, J.H.; Hixon, L.J.; Knight, J.; Moore, J.; Rice, J.S.; Rogers, A.I. ; Schuman, B.; Smith, E.H.; Woodward, J.C. *J. AM. Med. Assoc*, **1996**, *276*, 1957.

CRC *Handbook of Chemistry and Physics*, CRC Press: Cleveland, OH, **1975**.

Daniel, V. P.; Murukan, B.; Kumari, B. S.; Mohanan, K. *Spectrochimica Acta* A **2008**, *70,* 403-410.

Da Silva, G.A.; Augusto, F.; Poppi, R.J. *J. Chromatogr. A*, **2007**, **1138,** 251-261.

Darrouzes, J.; Bueno, M.; Lespes, G.; Potin, G. M. *J. Anal. At. Spectrom*. **2005**, *20*, 88.

de Souza, M. P; Chu, D.; Zhao, M.; Zayed, A. M.; Ruzin, S. E.; Schichnes, D.; Terry, N. *Plant Physiol*., **1999**, *119*, 565-573.

Doran, J.W. *Adv. Microb. Ecol.* 1**982**, *6,* 1-32.

Doran, J. W; Alexander, M. *Appl. Environ. Microbiol*. **1977**, *33*, 31-37.

El-Bayoumy, K.; Das, A.; Narayanan, B.; , Narayanan, N.; Fiala, E.S.; Desai, D.; Rao, C.V.; Amin, S.; Sinha, R. *Carcinogenesis*. **2006**, *27*, 1369-1376.

Emsley, J. *Nature's Building Blocks*: *An A-Z Guide to the Elements*; Oxford University Press: New York, **2002**.

Engberg, A.R.; Westcot, W.D.; Delamore, M.; Holz, D.D. *Environmental Chemistry of Selenium*, Marcel Dekker, New York, **1998**.

Engman, L.; Al-Maharik, N.; McNaughton, M.; Birmingham, A.; Powis, G. *Anticancer Drugs,* **2003***, 14*, 153–161.

Ernst, W.H. Metal Contaminated Soils; In: Vangronsveld J.; Cunningham S.C. Eds. In Situ Inactivation and Phytorestoration, Springer: New York, **1998**, 17.

Fan, A.M.; Kizer, W. K., *West J. Med.* **1990**, *153*, 160-167.

Fiala, E.S.; Staretz, M.E.; Pandya, G.A.; El-Bayoumy, K.; Hamilton, S. *Carcinogenesis,***1998**, *19*, 597-604.

Frankenberger, Jr., W. T.; Arshad, M. *Biofactors* **2001**, *14,* 241-254.

Frankenberger, Jr., W.; Engberg, R. (Eds.). Marcel Dekker, Inc., New York, N.Y. **1998**.

Fritz, J.S. *Analytical Solid Phase Extraction*, Wiley-VCH: New York, **1999**, 161-171.

Gmelin, C. **1824**, Tübingen, *43*.

Golub, A.M.; Skopenko, V.V. *Russ. Chem. Rev.* **1965**, *34*, 901- 908.

Gómez-Ariza, J.L.; Pozas, J.A.; Giráldez, I; Morales, E. *Analyst*. **1999a**, *124*, 75-78.

Gómez-Ariza, J.L.; Pozas, J.A.; Morales, E. *J. Chromatogr. Sci*. **1999b**, *37*, 436-442.

Goulden, P. D.; Brooksbank, P. *Anal. Chem*. **1974**, *46*, 1431-1436.

Guan, W.; Xu, F.; Liu, W.; Zhao, J.; Guan,Y. *J. Chromatogr. A,* **2007***, 1147*, 59-65.

Hansen, K. *Annalen.***1853**, *86,* 208-215.

Hapuarachchi, S.; Swearingen Jr., J.W.; Chasteen, T.G. *Process Biochem*. **2004**, *39*, 1607-1613.

Haygarth, P. Marcel Dekker, New York, **1994**.

Hershey, J.W.; Keliher, P.N. *Spectrochim. Acta B* **1986**, *41*, 713-723.

Hillen, L.; Werner, R. *J. Chromatogr*. **1973**, *79*, 318-321.

Hofmeister, F. *Arch. Exp. Pathol. Pharmakol*. **1894**, *33,* 198-215.

Hunter, W.; Kuykendall, L. *J. Chromatogr. A* **2004**, *1038*, 295-297.

Hwang, J.D.; Guenther, G.D.; Diomiguardi, J.P. *Anal. Chem*. **1989**, *61*, 285-288.

Ishihara, K.; Honma, N.; Uchiyama T, *J. Ferment. Bioeng.* **1995**, *80*, 633.

Jacob, C.; Arteel, G. E.; Kanda, T.; Engman, L.; Sies, H. *Chem. Res. Toxicol.* **2000**, *13,* 3-9.

Kahakachchi, C.; Boakye, H. T.; Uden, P. C.; Tyson, J. F. *J. Chromatogr.* A **2004**, *1054*, 303-312.

Kapoor, A.; Tanjore, T.; Viraghavan, T.; *Envion. Sci. Technol*. **1995**, *49*, 137-147.

Karle, J.; Wilson, K.; Shrift, A. *Biol. Trace Elem. Res*., **1986**, *10*, 307-315.

Kataoka, H.; Lord, H.L.; Pawliszyn, J. *J. Chromatogr.* A **2000**, *880*, 35-62.

Kubachka, K.; Meija, K.; LeDuc, D.; Terry, N.; Caruso, J. *Environ. Sci. Tech*. **2007**, *41*, 1863-1869.

Kumar, P. B. A. N.; Dushkenov, V.; Motto, H.; Raskin, I. *Environ. Sci. Technol*. **1995**, *29*, 1232–1238.

Kuo, C.Y.; Jiang, S.J, *J. Chromatogr. A* **2008**, *1181*, 60-66.

Lasat, M. Unpublished manuscript from the USEPAl Am. Assoc. Adv. Sci. Fellowship Prog., **2000**, 34.

Lemly, A. Marcel Dekker, Inc., New York, **1998**; Chapter 16.

Lemly, A.D.; Ohlendorf, H.M. *Ecotoxicol. Environ. Saf*. **2002**, *52*, 46-56.

Losi, M.; Frankenberger, W. *Appl. Environ. Microbiol.* **1997**, *63*, 3079-3084.

Mahaveer, B.M.; Jaldappa, S.*Turk. J. Chem*. **2000**, *24*, 287-290.

Maiers, D.T.; Wichlacz, P.L.; Thompson, D.L.; Bruhn, D.F, *Appl. Environ. Microbiol* **1988**, *54*, 2591-2593.

Manceau, A.; Gallup, D. L. *Environ. Sci. Tech.* **1997**, *31*, 967-976.

McCarty, S.; Chasteen, T.; Marshall, M.; Fall, R.; Bachofen, R. *FEMS Lett.* **1993**, *112*, 93-98.

McIntyre, T. *Adv. Biochem. Eng. Biotechnol*. **2003**, *78*, 97-123.

McMaster, M. Wiley, New York, **1998**.

Meija, J.; Caruso, J. *Inorg. Chem*. **2004**, *43*, 7486-7492.

Meija, J.; Montes-Bayón, M.; Le Duc, D.L.; Terry, N.; Caruso, J.A. *Anal. Chem.* **2002**, *72*, 5837-5844.

Meng, X.; Bang, S.; Korfiatis, G.P.; *Water Res*. **2002**, *36*, 3867-3873.

Michalke, B. *Handbook of Elemental Speciation,* Wiley Interscience: London, **2003**; Chapter 4.

Miekeley, N.; Pereira, R.C.; Casartelli, E.A.; Almeida, A.C.; Carvalho, M.F.B. *Spectrochim. Acta B* **2005**, *60*, 633-641.

Milne, J. Marcel Dekker, Inc., New York, **1998**; Chapter 23.

Montes-Bayón, M.; Yanes, E. G.; Ponce de León, C.; Jayasimhulu, K.; Stalcup, A.; Shann, J.; Caruso, J. A. *Anal. Chem*. **2002**, *74*, 107-113.

Morlon, H.; Fortin, C.; Adam, C.; Garnier, L.J., *Radioprot.* **2005**, *40,* 101-106.

Ohlendorf, H.M. Soil Science Society of America; Madison, WI; Special Publication No. 23. **1989**, 133-177.

Ohlendorf, H.M. *Aquat Toxicol.* **2002**, *57*, 1-10.

Ohlendorf, H.M.; Hoffman, D.J.; Saiki, M.K.; Aldrich, T.W. *Sci. Total Environ*. **1986**, *52*, 49-63.

Ollivier, P.; Bahrou, A.; Marcus, S.; Cox, T.; Church, T.; Hanson, T. *Appl. Environ. Microbiol*. **2008**, *74*, 7163-7173.

O'Toole, D.; Raisbeck, M. F. Marcel Dekker, Inc., New York, N.Y., **1998**, Chapter 19.

Palmer, I.S. *Environmental Chemistry of Selenium*, Marcel Dekker: New York, **1998**; Chapter 3.

Pathem, B.K.; Pradenas, G.A.; Castro, M.E.; Vasquez, C.C.; Chasteen, T.G. *Anal. Biochem*. **2007**, *364*, 138-144.

Pedrero, Z.; Madrid, Y. *Anal. Chim. Acta*. **2009**, *634*, 135–152.

Philip, J.C.; Bamforth, S.M.; Singleton, I.; Atlas, R.M. *Environmental pollution and restoration: A role for bioremediation.* In: Atlas R.M., Philip J. eds. *Bioremediation: applied microbial solutions for real-world environmental cleanup*; ASM Press: Washington; **2005**; 1-48.

Pinel-Raffaitin, P.; Pécheyran, C.; Amouroux, D. *Atmos. Environ*., **2008**, *42*, 7786-7794.

Prasad, P. R.; Kumar, J. D.; Priya, B. K.; Subrahmanyam, P.; Ramanaiah, S.; Chiranjeevi, P. *E. J. Chem*. **2007**, *4*, 354-362.

Quinn, N.W.T.; Lundquist, T.J.; Green, F.B.; Zarate, M.A.; Oswald, W.J.; Leoghton, T. *Cal. Agri*. **2000**, *54*, 53-56.

Rayman, M.P. *Lancet*, **2000**, *356*, 233-241.

Regueiro, J.; Becerril, E.; Garcia-Jaresa, C.; Llomparta, M. *J. Chromatogr. A*, **2009a**, *1216*, 4693–4702.

Regueiro, J.; Llompart, M.; Carmen Garcia-Jares, C.; Cela, R. *J. Chromatogr. A*, **2009b**, *1216*, 2816–2824.

Revanasiddappa, H.D.; Kiran Kumar, T.N. *Anal. Sci*. **2001**, *17*, 1309-1312.

Sambrook, J.; Fritsch, E.; Maniatis, T. Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory, New York, **1989**.

Satchwill, T. M. S. Thesis, University of Calgary, Alberta, Canada, **2001**.

Schrauzer, G.N. *Cell Mol. Life Sci*. **2000**, *57*, 1864-1873.

Semba, RD.; Ricks, MO.; Ferrucci, L.; Xue, Q-L.; Guralnik, JM.; Fried, LP, *Eur. J. Clin. Nutr.,***2009**, *63*, 93-99.

Seward, M.R.; Richardson, D.H. In: Shaw, A. J. ed.; *Heavy Metal Tolerance in Plants: Evolutionary Aspects*, CRC Press: Boca Raton, FL, **1990**, 75.

Shah, M.; Meija, J.; Caruso, J. *Anal. Chem*. **2007**, *79*, 846-853.

Sharmasarkar, S.; Vance, G.F.; Cassel-Sharmasarkar, F, *Environ. Geol*. **1998**, *34*, 31-38.

Skoog, D.A; Hooler, F.J.; Crouch, S.R. *Principles of Instrumental Analysis*, 6th ed. Thomson: Belmont, CA, **2007**; Chapter 1.

Skorupa, J. Marcel Dekker, Inc., New York, **1998**, Chapter 18.

Soderback, E, *Acta Chem. Scand.* **1974**, *28*, 116-117.

Somogyi, Z.; Kiss, I.; kadar, I.; Bakonyi, G. *Ecotoxicol.* **2007**, *16,* 379-384.

Soudi, M.R.; Ghazvini, P.T.M.; Khajeh, K.; Gharavi, S. *J. Hazard. Mater.* **2009**, *165*, 71-77.

Speck, M.L. *Compendium of Methods for the Microbial Examination of Foods*; American Public Health Association: New York, **1976**.

Stalder, V.; Bernard, N.; Hanselmann, K.W.; Bachofen, R.; Chasteen, T.G, *Anal. Chim. Acta.* **1995**, *303*, 91-97.

Stalder, V.; Chasteen, T. *J. Photochem. Photobiol. B: Biol.* **1998***, 43,* 193-203.

Stawiecka-Mirota, M.; Kaminska, M. J.; Urban-Grimal, D.; Haines, D. S.; Zoladek, T. *Exp. Cell Res*., **2008**, *314*, 3318-3325.

Strotmann, U. J.; Eglsäer, H.; Pagga, U. *Chemosphere* **1994**, *28*, 755-766.

Sun, P.P., Shell Oil Company, personal communication, **2008**.

Swearingen Jr., J.W.; Araya, M.A.; Plishker, M.F.; Saavedra, C.P.; Vasquez, C.C.; Chasteen, T.G. *Anal. Biochem.***2004**, *331*, 106-114.

Swearingen Jr., J. W.; Frankel, D. P.; Fuentes, D. E.; Saavedra, C.; Vásquez, C. C.; Chasteen, T. G. *Anal. Biochem.***2006**, *348,* 115-122.

Swearingen Jr., J.W.; Fuentes, D.E.; Araya, M.A.; Plishker, M.F.; Saavedra, C.P.; Chasteen, T.G.; Vasquez, C.C. *Appl. Environ. Microbiol*. **2006**, *72*, 963-967.

Tarin, P. Ph.D. Thesis, University of Wollongong, New South Wales, Australia, **2006**.

Terry, N; Zayed, A. *Environmental Chemistry of Selenium*, Marcel Dekker, New York, **1998**. 633-656.

Terry, N.; Zayed, A.; Marcel Dekker, Inc., New York, N.Y., **1998**, Chapter 31.

Tracey, M.L.; Möller, G. *J. Assoc. Off. Anal. Chem*. **1990**, *73*, 404-410.

Uden, P. *Anal. Bioanal. Chem*. **2002**, *373*, 422.

Umysová, D.; Vítová, M.; Douškova, I.; Bišová, K.; Hlavová, M.; Čížková, M.; Machát, J.; Doucha, J.; Zachleder, V. *BMC Plant Biol,* **2009**, *9*, 58.

USEPA, 2004. United States Environmental Protection Agency [USEPA], 2004. Draft aquatic life water quality criteria for selenium. Washington, DC, USEPA Office of Water. EPA EPA-822-D-04-001.

Vadhanavikit, S.; Kraus, R. J.; Ganther, H. E. *Arch. Biochem. Biophys.***1987**, *258*, 1-6.

Van Fleet-Stalder, V.; Chasteen, T.G,*J. Photochem. Photobiol. B.* **1998**, *43*, 193-203.

Van Fleet-Stalder, V.; Gürleyük, H.; Bachofen, R.; Chasteen, T. G. J. *Ind. Microbiol. Biotech*. **1997**, *19*, 98-103.

Vanhaecke, F. *Anal. Bioanal. Chem*. **2002**, *372*, 20

Vijan, P.N.; Leung, D. *Anal. Chim. Acta* **1980**, *120*, 141-146.

Vijan, P. N.; Wood, G. R. *Talanta*, **1976**, *23*, 89-94.

Wilber, C.G. *Clin. Toxicol*. **1990**, *17*, 171.

World Health Organization; "Chemical hazards in drinking-water–selenium"; http://www.who.int/water_sanitation_health/dwq/chemicals/selenium/en/; **2009.**

Wrobel, K.; Wrobel, K.; Caruso, J.A. *Anal. Bioanal. Chem.* **2005**, *381*, 317-331.

Wu, L. *Ecotoxicol. Environ. Saf.* **2002**, *57,* 257-269.

Yarema, M.C.; Curry, S.C. *Pediatrics*. **2005**, *116*, 319-321.

Yu, R.; Coffman, J.P.; Van Fleet-Stalder, V.; Chasteen, T.G.; *Environ. Toxicol. Chem*. **1997**,*16*,140-145.

Zhang, L.; Chasteen, T.G. *Appl. Organomet. Chem*. **1994**, *8*, 501-508.

Zhang, Y.; Frankenberger, W. T., Jr. *Sci. Total Environ*. **2005**, *346*, 280-285.

Zhang, Y.; Kharel, Y.; Koyama, T. *J. Mol. Catal. B Enzym.*, **2000**, *10*, 623- 630.

APPENDIX A

ASSIGNMENT OF WORK CARRIED OUT IN THIS RESEARCH

Radhika Burra: LHVE toxicity experiments for selenium and tellurium compounds, borohydride production of elemental metalloids, and LHVE headspace experiments

Alex O. Elias (in Chile): Borohydride production of elemental metalloids

James D. Fox (in Texas): Determination of selenocyanate by capillary electrophoresis

Roberto C. Molina (in Chile): Borohydride production of elemental metalloids

Rebecca Montes (in Texas): Borohydride production of elemental metalloids, LHVE headspace analyses, and DTT work

Claudia Munoz (in Chile): Borohydride production of elemental metalloids

Jose M. Perez (in Chile): Borohydride production of elemental metalloids

Gonzalo Predenas (in Texas and Chile): Isolation and morphology of the *Bacillus* sp. (LHVE)

Claudio Vásquez (in Chile): Isolation and morphology of the *Bacillus* sp., borohydride production of elemental metalloids

APPENDIX B

CHEMICAL ABSTRACT SERVICE REGISTRY NUMBERS

Compound Name	CAS Number
agar	9002-18-0
potassium selenocyanate	3425-46-5
potassium tellurite hydrate	123333-66-4
sodium borohydride	16940-66-2
sodium chloride	7647-14-5
sodium selenate	13410-01-0
sodium selenite	101202-18-8
sodium tellurate dihydrate	26006-71-3
tryptone	73049-73-7
yeast extract	8013-01-2

VITA

RADHIKA BURRA
Email: radhika.burra@gmail.com

OBJECTIVE

Seeking a progressive and challenging position in the field of analytical/pharmaceutical/organic chemistry to utilize my skills in separation and extraction techniques in an outstanding organization.

SKILLS SUMMARY

- industrial and academic exposure in research and development, bioanalytical techniques
- areas of expertise include qualitative analysis using GC/SCD, GC/MS
- ability to manage time in the most efficient manner relative to timeframe and budgetary targets
- ability to be comfortable in a nonhierarchical, flat organization and, if required, operate under a dual reporting relationship
- ability to adopt new technologies with minimal or no support in learning and performing the duties
- strong analytical, problem solving, project management, communication and interpersonal skills

EDUCATION

M.S., in Chemistry, Sam Houston State University, TX,USA, Dec' 2009 GPA: 3.75
M.S., in Medicinal Chemistry, Osmania University, India, May 2006 GPA: 3.9
B.S., in Kakatiya University, India May 2004 GPA: 3.9

AREA OF RESEARCH

Bioanalytical chemistry, Research and development in organic chemistry, Plant tissue culture

RESEARCH PROJECTS

- Triclosan analysisusing gas chromatography- flame ionization detection (GC-FID)
- Identification of organoselenium and organotellurium in the headspace of metalloid resistant bacteria using gas chromatography-sulphur chemiluminescence detection (GC-SCD)
- Towards the total synthesis of 2-cyclopentene 1- carboxamide, 1, 4- dihydroxy (*1S, 4R*)
- Diastereoselective synthesis of *cis*-fused pyranobenzopyrans
- Micropropagation of *Lycopersicum esculatam*

WORK EXPERIENCE

Graduate Teaching Assistant (August 2007 – Present)
Chemistry Department, Sam Houston State University, Huntsville, Texas

- taught general chemistry, organic chemistry
- taught instrumental analytical chemistry for senior undergraduates
  - taught the operation/ theory of GC, GC/MS, GC/FID, UV/Vis., AAS, ICP, CE
- set-up laboratory for upcoming laboratory exercises

Research Student (August 2007- Present)
Chemistry Department, Sam Houston State University, Huntsville, Texas

- Develop bioremediation techniques
  - designed experiments that involve analysis and identification of volatiles in the headspace of metalloid-resistant bacteria
  - designed experiments that monitor the specific growth rate of bacteria using Spectrophotometer
  - performed experiments such as zone of inhibition, cup-borer methods to examine the relative toxicity of selenium and tellurium on bacteria
- Instruments operated
  - GC/F_2-induced chemiluminescence detection, GC/FID, GC/MS

PUBLICATIONS

"Cloning, purification and characterization of Geobacillus stearothermophilus V uroporphyrinogen-III C-methyltransferase: evaluation of its role in resistance to potassium tellurite in Escherichia coli", ***Research in Microbiology***, 2009, **160**, 125-133, (doi:10.1016/j.resmic.2008.12.004),
Manuel A. Araya, Juan C. Tantaleán, José M. Pérez, Derie E. Fuentes, Iván L. Calderón, Claudia P. Saavedra, Radhika Burra, Thomas G. Chasteen, and Claudio C. Vásquez

*"Biological interactions of selenocyanate: bioprocessing, detection, and toxicity**",*** Manuscript accepted, ***Environmental Technology,*** April 2009
Radhika Burra, James D. Fox, and Thomas G. Chasteen

"*Production of dimethyl triselenide and dimethyl diselenenyl sulfide in the headspace of metalloid-resistant Bacillus spp. grown in the presence of selenium oxyanions"*, Manuscript accepted, ***Analytical Biochemistry,*** September 2009
Radhika Burra, Gonzalo A. Pradenas, Rebecca A. Montes, Claudio C. Vásquez and Thomas G. Chasteen

PRESENTATIONS

Oral presentation at 64th Regional ACS Meeting in Little Rock, October 1, 2008 on *"Identification of organoselenium and organotellurium compounds in the headspace of metalloid-resistant bacteria using gas chromatography with fluorine-induced chemiluminescence detection"* Abstract 60392

Oral presentation at 12th Annual University Wide Graduate Research Exchange, Sam Houston State University, April 30, 2009 (an award winning paper) on *"Biological interactions of selenium and tellurium: bioprocessing, detection, and toxicity**".***

HONORS

- Gold Medalist from Osmania University
- College of Arts and Sciences Academic Scholarship, SHSU

COMPUTER KNOWLEDGE

Package : MS Office
Operating System: windows 98, NT, 2000, XP, Vista
Software : HP and Agilent Chemstation, 33 Karat (capillary electrophoresis)

REFERENCE:

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Other references available upon request.