

**MASS BALANCE AND TRAPPING OF HEADSPACE  
SAMPLES OF BIOREMEDIATION IN SELENIUM  
AMENDED SAMPLES**

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BY

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## ABSTRACT

Hapuarachchi, Suminda, Mass balance and trapping of headspace samples of bioremediation in selenium amended samples, Master of Science (Chemistry), August 2002, Sam Houston State University, Huntsville, Texas.

One of the purposes of these experiments was to determine the mass balance in the bioremediation of selenium amended samples and to try to improve the efficiency of the bioremediation of selenium. The other purpose was to study the interference of glass containers in selenium determination by atomic absorption spectrometry with hydride generation.

In this research, the first step was to develop a gas phase trapping method and to prove the success of that method using known concentrations of known organo-selenium compounds. Fifty percent nitric solution was a good trapping solution to collect volatile organoselenium compounds purged from live, liquid bacterial cultures. Then, bioreactor experiments were carried out to determine the mass balance of selenium as it was biologically processed by a selenium-resistant bacterium. First, bioreactor experiments were undertaken anaerobically with different amended selenium concentrations and the mass balance of each process measured. After that, bioreactor experiments were carried out under sequential anaerobic/aerobic culture growth conditions and the distribution of selenium among three different physical states was determined. As a percentage of amended selenium, selenite was more effectively reduced to  $\text{Se}^0$  by *Pseudomonas fluorescens* than selenate. When low amounts of selenite were present in the solution, reducing efficiency as a percentage of added Se was higher as measured by the production of  $\text{Se}^0$ . Sequential anaerobic/aerobic growth conditions did not have a big effect in this detoxification process as carried out. We saw no real difference in elemental

Se production between culture grown completely anaerobic as compared to mixed anaerobic and aerobic periods.

To remove the interference of glass containers in selenium determination by HGAAS, glass test tubes were treated with different chemical solutions to minimize interferences and the most successful reagent for this process determined. Rinsing borosilicate test tubes with concentrated nitric acid was most successful at reducing the poor recovery rates of Se in HGAAS. The poor recovery rates of selenium in glass test tubes may be due to adsorption of Se to the glass instead of dissolution and the contribution of interfering ions to sample solution during the analysis.

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Special thanks go to my parents and my family for their love and encouragement given to me and for bringing me to this level. Finally, I thank my wife for being so patient and supportive throughout my studies. Thanks to everyone who wished me luck in my studies.

I dedicate this thesis to my parents and my wife; without them I would not be here.

## TABLE OF CONTENTS

	PAGE
ABSTRACT .....	3
ACKNOWLEDGEMENTS.....	5
LIST OF TABLES .....	7
LIST OF FIGURES.....	9
CHAPTERS	
I.    INTRODUCTION .....	10
II.   EXPERIMENTAL .....	21
III.  DATA AND RESULTS.....	31
IV.  DISCUSSION AND CONCLUSIONS.....	50
BIBLIOGRAPHY .....	61
APPENDIX.....	65
VITA .....	66

## LIST OF TABLES

Table I	Uses of selenium.....	2
Table II	Some commonly used selenium compounds and their applications .....	3
Table III	Chromatographic and detector conditions .....	20
Table IV	Selenium calibration data from the HGAAS.....	22
Table V	Se HGAAS % recoveries for samples in untreated test tubes .....	23
Table VI	Se HGAAS % recoveries for samples in HCl treated test tubes.....	24
Table VII	Se HGAAS % recoveries for samples in H <sub>2</sub> SO <sub>4</sub> treated test tubes.....	24
Table VIII	Se HGAAS % recoveries for samples in distilled water treated test tubes.....	25
Table IX	Se HGAAS % recoveries for samples in 0.2 % Extran treated test tubes.....	25
Table X	Se HGAAS % recoveries for samples in 2 % Extran treated test tubes.....	26
Table XI	Se HGAAS % recoveries for samples in aqua regia treated test tubes.....	26
Table XII	Se HGAAS % recoveries for samples in chromic acid treated test tubes.....	27
Table XIII	Se HGAAS % recoveries for samples in nitric acid treated test tubes.....	27
Table XIV	Se % recovery observed for 50 % HNO <sub>3</sub> trapping solutions.....	29
Table XV	Selenium calibration data from the ICP-AES.....	30
Table XVI	Ten mM of selenite .....	31
Table XVII	One mM of selenite.....	33
Table XVIII	Ten mM of selenate.....	34

Table XIX	Ten mM of selenite .....	35
Table XX	One mM of selenite.....	37
Table XXI	One mM of selenite.....	38
Table XXII	One mM of selenite.....	40

## LIST OF FIGURES

Figure 1	Mechanism for the reduction and methylation of selenite proposed by Challenger.....	7
Figure 2	Proposed mechanism for the reduction and methylation of selenite by Doran .....	7
Figure 3	Proposed selenium conversion mechanism by Chasteen .....	8
Figure 4	Calibration curve for Se analysis by HGAAS .....	23
Figure 5	Calibration curve for Se analysis by ICP-AES.....	30
Figure 6	Anaerobic growth vs time of the K27 bacterium with 10 mM selenite amendment.....	32
Figure 7	Anaerobic growth vs time of the K27 bacterium with one mM selenite amendment.....	33
Figure 8	Anaerobic/aerobic growth vs time of the K27 bacterium amended with one mM selenite .....	36
Figure 9	Anaerobic/aerobic growth vs time of the K27 bacterium with one mM selenite amendment .....	37
Figure 10	Anaerobic/aerobic growth vs time of the K27 bacterium with one mM selenite amendment .....	39
Figure 11	Anaerobic/aerobic growth vs time of the K27 bacterium with one mM selenite amendment .....	40

## CHAPTER I

### INTRODUCTION

Over recent decades, environmental scientists have clearly focused attention towards toxic trace elements like selenium due to the significant part they play in our natural environmental system. Therefore, the understanding and identification of selenium's chemical behavior in our natural systems have been a main focus of some environmental researchers.

Selenium, discovered by Berzelius in 1817 in the flue dust of pyrite burners, was a product of copper refining (Muth et al., 1967). Even though there are selenium production plants in certain parts of the world, those are associated with sulfur or copper ores. Seleniferous ores have not been found alone because of the low abundance of selenium in the earth's crust, about 0.05 ppm (Lee, 1991). Selenium is found in igneous rocks mostly and is present in sedimentary rocks and fossil fuels too (Kessi et al., 1999).

There are three metallic forms of selenium (elemental Se), that is, three different allotropic forms, red amorphous, black amorphous and gray hexagonal. The red and black amorphous allotropes are most often found in soils; whereas red amorphous selenium originates when  $\text{Se}^0$  precipitates in aqueous solution (Kessi et al., 1999). At temperatures higher than 30 °C, red  $\text{Se}^0$  can revert to the black form. In addition to the insoluble, elemental form, there are different soluble forms of selenium such as oxyanions of Se(IV), Se(VI), and molecules containing Se(-II). Selenite [Se(IV)] occurs in oxic to suboxic environments and it is less available to organisms because of its affinity to sorption sites of sediment and soil constituents in natural water (Bar-Yosef and Meek, 1987; Fritz and Hall, 1988). Elemental and selenide [-II] are the stable forms

under anoxic conditions. While elemental selenium is insoluble in water, selenide precipitates as metal selenides of very low solubility. This selenide [-II] oxidation state is also found in organic metalloids as selenomethionine and selenocystine. Therefore, Se(VI), Se(IV) and organic Se(-II) are the most important soluble forms of selenium in natural environments (Zhang et al., 1999a).

Selenium has long been used for various purposes in our society. Due to its photoelectrical properties and semi-conducting characteristics, it is used in photocell devices and xerography, solar batteries, specially transformers and rectifiers. Apart from those uses, selenium's chemical properties have applied in the field of colored glass manufacturing to decorate and to cut down glare and heat transfer as well in chemical production and nutritional supplements. Table I gives general uses of selenium (Craig, 1986).

Table I

Uses of Selenium

Application	% of total	Purpose
Glass manufacture	35	Discolorations, manufacturing of colored glasses
Electronic application	20	Selenium rectifiers, solar batteries
Photocopying	25	Transfer photographic image by static electricity
Pigments	15	Use in plastic, paints, enamels, ink and rubber
Other	5	Additive to poultry and swine feed, fungicide

There are other applications in addition to the ones mentioned above. Organo-selenium compounds are used as antibacterial agents and selenium containing polymers

are used as semi-conducting materials. In the metal industry, selenium is added to increase stability of stainless steel and copper alloys. In the chemical industry, selenium is used in catalysts for petroleum processing and in reagents for organic synthesis. It is used in the rubber industry to increase the wear resistance and elasticity of rubber. Table II gives some of the commonly used selenium compounds and their applications in industry (Craig, 1986).

Table II

Some commonly used selenium compounds and their applications

Compound	Application
Aluminium selenide, $\text{Al}_2\text{Se}_3$	In semiconductor research
Ammonium selenite, $(\text{NH}_4)_2\text{SeO}_3$	In manufacture of red glass
Arsenic hemiselenide, $\text{As}_2\text{Se}$	In manufacture of glass
Bismuth selenide, $\text{Bi}_2\text{Se}_3$	In semiconductor research
Cadmium selenide, $\text{CdSe}$	In photoconductor, semiconductor, rectifiers
Calcium selenide, $\text{CaSe}$	In electron emitters
Cupric selenate, $\text{CuSeO}_4$	In coloring Cu or Cu alloys black
Cupric selenide, $\text{CuSe}$	As catalyst, in semiconductors
Indium selenide, $\text{InSe}$	In semiconductor research
Selenium disulphide, $\text{SeS}_2$	In antidandruff agents and fungicides
Selenium hexafluoride, $\text{SeF}_6$	As gaseous electric insulator
Selenium monosulphide, $\text{SeS}$	In fungicides for animals and people
Sodium selenate, $\text{Na}_2\text{SeO}_4$	As veterinary therapeutic agent

As selenium compounds are more used the more those compounds appear in our environment. Due to excessive inconsiderate use of selenium compounds in the industrial world it has become a threat to our natural environment as well as our modern day society. Increasing amounts of selenium-containing compounds entering into the environment has greatly contributed to contamination of our natural systems (Reddy et al., 1995; Weres et al., 1989).

Selenium contamination of our potable water has been a major concern over the past 20 years or so. Ground water is a major natural resource in United States because it is one of the main sources of drinking water in many communities. Therefore, contamination of ground water could lead to severe consequences. High levels of selenium were recently found in the Power River Basin, Wyoming (Reddy et al., 1995). Selenium concentration in the river basin was reported from 3 to 330  $\mu\text{g/L}$ , well above the allowed Se concentration in drinking water imposed by federal regulations, which is 10  $\mu\text{g/L}$  (U.S. EPA, 1986).

There is a major environmental problem in the Kesterson Reservoir of California. Accumulation of selenium in this area has been serious and has become a threat to the regional wildlife population, causing deformities and deaths. Additionally, a very large area of agricultural land in California's San Joaquin Valley has been affected by high levels of selenium (Terry et al., 1992). Measurements of selenium in the drainage water from the west side of the San Joaquin Valley have shown Se concentrations that reach as high as 3.8 mg/L and that the selenium is predominantly in the form of the most oxidized Se form, the selenate anion. These concentrations can lead to accumulation of Se in plants which is potentially harmful to the animals in that area when they ingest those

plants. This can result in a condition referred to as “alkali disease” or “blind staggers” (Eriksen, 1999; Nakamuro et al., 2000).

Under natural conditions, biospheric selenium distribution remains relatively constant in the environment because of natural biological processes that effect the degradation and synthesis of organometalloids; however, when industrial use of selenium has disrupted the natural action of organisms in such a way that the balance between degradation and synthesis can no longer be maintained, problems arise (Wood, 1974). Scientists have been trying to reduce the toxic nature of these selenium contaminated environmental sites by introducing detoxification methods for remediation of polluted sites (Cantafio et al., 1996; Frankenberger and Arshad, 2001).

The metabolic activity of microorganisms plays a significant part in the mobility of toxic selenium in the environment. Bacteria are exceedingly versatile in the way they metabolize natural substances. This nature of microorganisms is the leading factor for choosing them to work as detoxification agents in natural systems. Therefore, one of the detoxification methods currently practiced is bioremediation. Microorganisms can be grouped according to their oxygen requirements:

1. Aerobes

They use oxygen as terminal electron acceptor.

2. Anaerobes

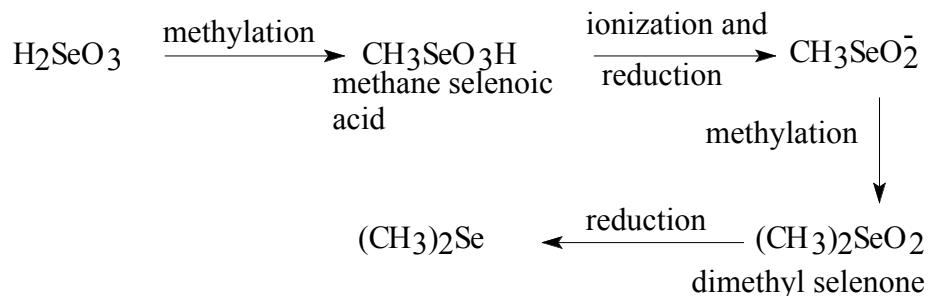
They grow in the absence of oxygen and use nitrate as terminal electron acceptor.

3. Facultative anaerobes

They can grow either the presence or the absence of oxygen (Chapelle, 2000; Avers, 1981).

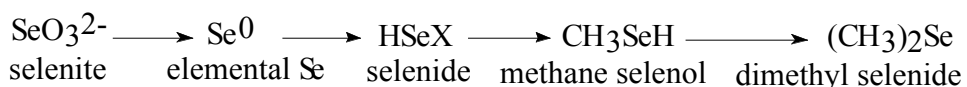
The ability of these different microbial pathways to metabolize toxic compounds facilitates the removal of these compounds from the environment. When these microorganisms grow in the presence of a toxic substance, like selenium, these organisms can attempt to detoxify this metalloid by using their metabolic pathways (Wood, 1974). The biological reducing power of bacteria such as *Pseudomonas fluorescens* had been used as early as 1925 to reduce the toxicity of soluble forms of selenium (Levine, 1925). As a result of the interaction of selenium-resistant microorganisms with soluble selenium oxyanions, it has been reported that these oxyanions could be reduced to elemental and volatile organo-selenium compounds such as dimethyl selenide (DMSe), dimethyl diselenide (DMDS<sub>2</sub>) and dimethyl selenenyl sulfide (DMSeS) (Zhang and Chasteen, 1994; Chasteen, 1990). Therefore, studies of microbial inter-conversion of selenium compounds can contribute substantially to a possible understanding and solution of environmental problems by volatilization, transport and, gas phase dilution of this toxic element. The *Pseudomonas fluorescens* K27 bacterium that was isolated from the San Joaquin Valley of California (Burton et al., 1987) was used in this study to investigate biological remediation of selenium.

These experiments involved cultures of K27 bacterium and the study of biological reduction of the selenium oxyanions  $\text{SeO}_4^{2-}$  and  $\text{SeO}_3^{2-}$ . The first published work showing the reduction of selenium salts, especially selenite, was reported by Challenger while working with fungi grown on bread. He proposed a mechanism (Figure 1) for the methylation and reduction of selenite to dimethyl selenide (Challenger, 1945).



**Figure 1.** Mechanism for the reduction and methylation of selenite proposed by Challenger.

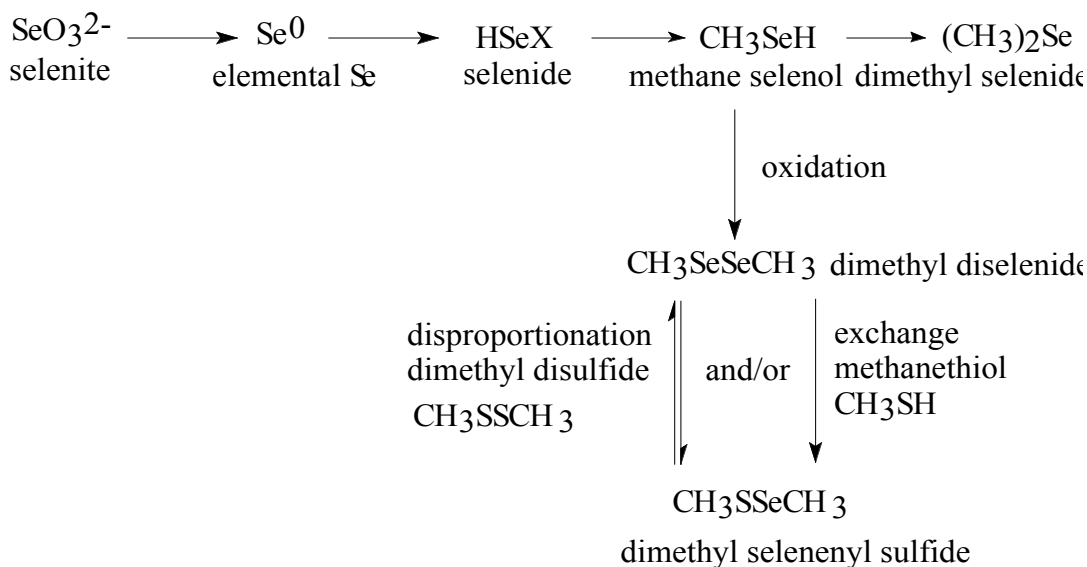
Apart from Challenger's reports about biological reduction of selenium compounds, there are other discussions of this process by Doran and Chasteen (Doran, 1982; Chasteen, 1993). Doran proposed a different mechanism than that of Challenger (Figure 2). He proposed a direct path from selenite to elemental selenium followed by methylation of elemental selenium to yield dimethyl selenide. Not only did he account for the production of dimethyl selenide in his mechanism but dimethyl diselenide too.



**Figure 2.** Proposed mechanism for the reduction and methylation of selenite by Doran.

Though little evidence existed for his mechanism at the time (1982), later work with selenium-resistant phototrophic bacteria showed that organoselenium metabolites were produced by these obligate anaerobes in cultures grown with only solid selenium added. Zhang and Chasteen reported the production of DMS<sub>2</sub>S from the static headspace of test tube culture amended with selenite and selenate salts via chemiluminescence detection equipped-gas chromatography (Zhang and Chasteen, 1994). Therefore, production of dimethyl selenenyl sulfide was included in Chasteen's mechanism (Chasteen, 1993) and he extended Doran's mechanism to illustrate it (Figure 3).

Mechanistically all this information gives useful insight to the biological process involved in bioremediation.



**Figure 3.** Proposed selenium conversion mechanism by Chasteen.

A thorough understanding of selenium bioremediation would not only help to optimize the selenium removal process but, more importantly, would help to minimize the ecotoxicological risk to wildlife, the latter being the real goal of selenium remediation (Fan et al., 1997). Understanding the pathway of the bioremediation process is the key to the improvement of this process. Therefore, the purpose of this work is to determine a mass balance for the bioremediation process as it is carried out in a 2-liter bioreactor. This determination involved finding the amounts of selenium evolved as organo-selenium compounds, amounts of elemental selenium and the amount of dissolved selenium, which was not reduced by bacteria over the time of the experiment.

Selenium volatilization and the trapping of those volatile compounds have been discussed by several reports in the scientific literature (Zhang and Frankenberger, 2000;

Abu-Erreish et al., 1968; Fan et al., 1997; Karlson and Frankenberger, 1988). Volatile organo-selenium compounds have been collected using different trapping solutions especially alkaline hydrogen peroxide (Terry et al., 1992). Zhang et al. have collected gaseous selenium compounds in a denuder coating solution containing 2% HNO<sub>3</sub> and 2% glycerin (Zhang et al., 2002). Abu-Erreish discussed how to use concentrated nitric acid to collect volatile organo-selenium compounds but in this study, one of the main purposes was to find an effective trapping technique to overcome various constraints of other methods. Because the concentration of selenium released to the gas phase is very small in the biological system under study we needed a good method to collect all volatiles produced by the bacteria.

One aim of this work was to improve the efficiency of the bioremediation process, which will help the usefulness of bacteria like K27 in addressing environmental problems like selenium contamination. In order to improve the low removal efficiency of bioremediation processes on other chemical substances in the environment, sequential anaerobic-aerobic bioreactor-based culturing has been tested by other researchers. For example, to remove phosphate in biological wastewater, sequential anaerobic-aerobic culturing has been successful and helped to improve phosphate removal efficiency up to 90 % from 20-30 % (Choi and Yoo, 2000). In addition, this method has been tested on other bioremediation applications such as chlorophenol remediation (Singh and Viraraghavan, 1997) and combined removal of arsenic and non-chlorinated volatile organic compounds (Lipton et al., 1994). Therefore, one of our goals was to adopt this technique to improve our selenium bioremediation process and try to improve the efficiency of this method. In this experiment, cultures were grown under an

environmental stress of alternating anaerobic and aerobic conditions and the mass balance was calculated after a 72-hour time course.

A bioreactor allows for fine control of culture conditions such as temperature, dissolved oxygen, pH, nutrient inflow, outflow and agitation. It makes it easier to collect headspace samples by gas purging while allowing for isolation of the bacteria culture. In these experiments batch-type cultures were used for the study of bioremediation. A bioreactor with culture medium was sterilized and was inoculated with a pre-culture and selenium sterile solution. Then, it was allowed to grow until the culture reached stationary phase. Once the bioreactor was started, neither media nor pre-culture was added (Eriksen, 1999) and therefore, bioreactor volumes were always constant throughout the experiment. The average volume was 2.7 liters. This type of culture shows three distinct growth phases. Those are the lag phase, the log phase and the stationary phase. The lag phase shows little growth as the bacterium adapts to the new growth medium conditions in the bioreactor; whereas, the log phase shows rapid growth as the culture grows and consumes the media. The stationary phase shows no growth and most of the cells are dead as this phase progresses.

Inductively coupled plasma (ICP) spectrometry is a good analytical technique to determine selenium concentration in biological samples (Zhang et al., 2000). The low detection limits in ICP are analytically useful due to the fact that environmental samples contain low levels of selenium. Therefore, it is easy to analyze those samples with good reproducibility. In ICP, samples are introduced in solution form through a spray chamber/nebulizer to the plasma, which has a core temperature of about 9000 to 10000 K. The high temperature of the plasma eliminates chemical interference (Christian,

1994). One of the advantages of ICP is that the oxidation state of the selenium analyte is not crucial as in flame methods. Therefore, samples can be in any soluble form of selenium but the matrix of the solution and the standards should be the same.

One other purpose of this study which is quite different from the aims mentioned earlier, was to study the interference of glass containers in selenium determination by atomic absorption spectrometry with hydride generation (HGAAS), a commonly used method to analyze selenium due to the method's high sensitivity and selectivity (Murphy, 2001; Stripekis, 2000; Chatterjee et al., 2001). There are numerous studies, which discuss different types of interference in determination of selenium by this technique. However, little previous work addresses the role that glass surfaces in sample containers play in these analyses. To convert selenium(VI) to selenium(IV)—the most sensitive oxidation state in hydride generation atomic absorption spectrometry—selenate is reduced to selenite, most commonly using boiling 50% HCl (Zhang et al., 1999b; Martens and Saurez, 1997; Blaylock and James, 1993) as reducing agent. In our research group's work with biological samples containing metalloids for analysis (Basnayake et al., 2001), this process of reduction is carried out in a borosilicate glass test tube, which we have found, caused poor recoveries in our sample analysis by HGAAS. We have also discussed this problem with two commercial laboratories which carry out Se determination with this method and which are also having difficulty with Se recoveries in biological samples (Tompkins, 2001) and food samples (Duffy, 2002). Therefore, in this study the aim was to determine the magnitude of interference from our glassware and proper pretreatment to remove or minimize that interference in determination of selenium in hydride generation atomic absorption spectrometry.

## **CHAPTER II**

### **EXPERIMENTAL**

#### **PART 1. INTERFERENCE OF GLASS CONTAINERS IN SELENIUM**

##### **DETERMINATION BY HGAAS**

##### **Reagents and laboratory products for glass experiment**

The reagents used throughout this research were analytical grade chemicals and were used as received.

Selenium dioxide ( $\text{SeO}_2$ ), selenium standard solution for both HGAAS and ICP, and concentrated hydrochloric acid (12 M HCl) were purchased from Fisher Scientific (Houston, TX, USA). Nitric acid (15.9 M  $\text{HNO}_3$ ) and sodium borohydride were ordered from Aldrich Chemicals (Milwaukee, WI, USA). Sulfuric acid was purchased from J. T. Baker Inc. (New Jersey, USA) and Extran<sup>®</sup>, commercial detergent, was ordered from EM Science (Darmstadt, Germany). Borosilicate glass test tubes (Corning Inc., Corning, NY, USA) used for selenium reduction (screw cap; 16 mm x 125 mm) were purchased from VWR scientific (Sugarland, TX, USA).

##### **HGAAS Reagent**

##### **Selenium calibration standards**

The calibration standards were prepared by appropriate dilution of the commercial selenium atomic absorption standard, which is 1000 ppm selenium as  $\text{SeO}_2$ . One hundred mL of 5 ppb, 10 ppb, 15 ppb and 20 ppb were prepared in 50 % HCl. This is 50 % concentrated HCl v/v (Clesceri et al., 1998). All subsequent dilutions will use this notation. For an example, the 1 ppm Se standard solution was prepared by diluting 0.1

mL of 1000 ppm Se standard solution to 100.0 mL with DI water. From that solution, 0.5 mL was diluted to 100.0 mL with 50 mL concentrated HCl and DI water to prepare 5 ppb solution. Other standards were prepared in a similar manner.

## **Acid**

A ten molar hydrochloric acid (HCl) solution was prepared to acidify the sample and to act as the reducing agent for selenate oxyanions. Two hundred fifty mL of 10 M HCl were prepared using 208 mL of concentrated (12N) HCl acid and deionized water.

## **Hydride generation reducing agent**

A solution of 0.6% sodium borohydride ( $\text{NaBH}_4$ ) in 0.5 % sodium hydroxide (NaOH) was used as a reducing agent in HGAAS. This solution was prepared by dissolving 0.5 g of NaOH in less than 50 mL of deionized water and adding 0.6 g of  $\text{NaBH}_4$  into the solution, followed by dilution up to 100 mL with deionized water.

## **Tube preparation for sample analysis**

To convert selenium(VI) to selenium(IV), the most oxidized state in hydride generation atomic absorption spectrometry, borosilicate glass test tubes were used. In all cases the tubes were used new for each of the analysis described below and were treated with different chemical acids or commercially available detergents to remove glass interference in determination of selenium with HGAAS as described. The test tube glass composition was  $\text{SiO}_2$ ,  $\text{B}_2\text{O}_3$ ,  $\text{Na}_2\text{O}$ ,  $\text{Al}_2\text{O}_3$  and  $\text{K}_2\text{O}$  80.6%, 13.0%, 4.0%, 2.3%, and 0.1% respectively as determined by the manufacturer (Type I, Class A borosilicate) (Corning, 2002). The following solutions were used to treat test tubes:

1. untreated—directly from shipping container
2. deionized water
3. concentrated HCl
4. concentrated H<sub>2</sub>SO<sub>4</sub>
5. 0.2% Extran<sup>®</sup> solution in deionized water (Granchinho et al., 2001)
6. 2% Extran soaked for 24 hrs.
7. aqua regia (3 HCl:1 HNO<sub>3</sub> v/v) (Harris, 1995)
8. chromic acid (2.5 g of Na<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> dissolved in 250 Con. H<sub>2</sub>SO<sub>4</sub>)
9. concentrated HNO<sub>3</sub>

Test tubes were rinsed with the above solutions followed by rinsing with distilled water thoroughly and then air-drying before use; the untreated tubes were used without any washing whatsoever. All rinsing—except the overnight Extran soaking—involved soaking tubes for 15 minutes in the specified reagent, followed by rinsing in deionized water.

### **Sample preparation in tubes**

Standard selenium-containing solutions prepared for instrument calibration (in 50% HCl) were used as samples in this study of Se interferences. Those samples were heated in a water bath (30 min) in capped, treated and untreated test tubes then analyzed in HGAAS.

### **HGAAS analysis**

Samples prepared in test tubes, as described above, were analyzed by HGAAS (Dedina and Tsalev, 1995; Clesceri et al., 1998). A Varian FS 220 AAS with a hydride generation module was used following user manual guidelines: 196.0 nm Se line, 0.2 nm

slit width, air/acetylene flame to heat the quartz tube atomizer, and N<sub>2</sub> as carrier gas (Operation manual, 1997).

Ten molar HCl and 0.6% NaBH<sub>4</sub> (prepared in 0.5% NaOH) were used as hydride generation reagents. Sample, acid and borohydride solution flow rates were maintained respectively at 8 mL, 1.2 mL and 1.2 mL min<sup>-1</sup>. Recalibration (reslope) was carried out after every fifth sample to minimize instrumental drift. Sample blanks were 50% HCl. The working range was up to 20 ppb Se and R<sup>2</sup> values for calibrations were routinely > 0.99.

## **PART 2. MASS BALANCE EXPERIMENTS**

### **Reagents for the bioreactor experiments**

Tryptic soy broth (TSB) was obtained from DIFCO Laboratories (Detroit, MI USA) and sodium selenite and sodium selenate were purchased from Aldrich Chemicals (Milwaukee, WI USA). Sodium nitrate was purchased from EM Science (Darmstadt, Germany).

### **Calibration standards for ICP-AES**

Five calibration standards were prepared. One hundred mL 0.2 ppm, 0.6 ppm, 5.0 ppm, 10.0 ppm and 20.0 ppm solutions were prepared in 50% HNO<sub>3</sub> acid using 1000 ppm standard selenium solution. This is 50 % concentrated HNO<sub>3</sub> v/v (Clesceri et al., 1998). All subsequent dilutions will use this notation. The following method was used to prepare solutions. Diluted standard solutions of 4 ppm were prepared by taking 1 mL of 1000 ppm standard and diluting it up to 250 mL with DI water. Then that solution was used to prepared the first two calibration standards. One hundred milliliter volumes of 0.2

ppm and 0.6 ppm standards in 50% HNO<sub>3</sub> were prepared using 5 and 15 mL of the 4 ppm standard solution, respectively.

### **Growth medium for bioreactor experiments**

The growth media tryptic soy broth with 0.3 % nitrate (TSN3) was used through out all the bioreactor experiments. This was prepared by dissolving 10.0 g TSN3 and 3.0 g potassium nitrate per 1.0 L of deionized water. The medium was always sterilized by autoclave (50 min @ 121<sup>0</sup> C).

### **Selenium amendment solutions**

#### Ten mM selenite

A 10 mM stock solution of selenite was prepared by dissolving 4.671 g of Na<sub>2</sub>SeO<sub>3</sub> in 100 mL deionized water. That solution was sterile-filtered with a disposable filter unit (0.2 μm pore size: Nalgene company Rochester, NY USA) using a vacuum-pressure pump (Barnant company Barrington, IL USA).

#### One mM selenite

This stock solution was prepared by dissolving 0.467 g of Na<sub>2</sub>SeO<sub>3</sub> in 100 mL deionized water, followed by sterile-filtering.

#### Ten mM selenate

A 10 mM stock solution of selenate was prepared by dissolving 5.101 g of Na<sub>2</sub>SeO<sub>4</sub> in 100 mL deionized water, followed by sterile-filtering.

#### One mM selenate

A 1 mM stock solution of selenate was prepared as above using 0.510 g of Na<sub>2</sub>SeO<sub>4</sub>.

## **Inoculum preparation**

Precultures of growing bacteria for bioreactor experiment were started 48 hours before each bioreactor experiment was started. A K27 culture was grown in the same growth medium described above. A 50 mL sample of growth medium was prepared and autoclaved. Once the solution came to room temperature, an isolated culture of *Pseudomonas fluorescens* K27 was transferred from an agar plate to the medium with a sterile loop. Then the solution was placed in a water-bath shaker (New Brunswick Scientific Co., Inc., Edison, NJ USA), where it was kept at 30 °C and shaken aerobically. After 24 hours growing, this solution was transferred into 200 mL of sterilized growth media and was grown for another 24 hours aerobically.

## **Trapping solution**

The trapping solution used in all three bubbling tubes was 50% HNO<sub>3</sub> acid. A 100 mL of this solution was prepared using 50 mL concentrated HNO<sub>3</sub> and diluted with deionized water.

## **Instrumentation for bioreactor experiments**

### **New Brunswick BioFlow III fermentor**

A New Brunswick BioFlow III batch/continuous fermentor (Edison, NJ USA) was used for bioremediation experiments. This apparatus allows the controlling of the culture conditions of a relatively large volume of bacteria culture. Experimental parameters such as dissolved oxygen in the culture (for aerobic or anaerobic growth), pH, and culture temperature and agitation rates can be monitored using this fermentor (Eriksen, 1999; Bius, 2001). The computer or the bioreactor's microprocessor itself can

control these experimental parameters. In these experiments, the controlled temperature was 30 °C and agitation speed was maintained at 200 rpm throughout all experiments.

### Centrifuge

To separate bacteria cells and elemental selenium from the liquid, a centrifuge was used (Eppendorf 5810-R). Three 25-mL aliquots of samples were removed from the bioreactor and centrifuged in 50 mL tubes. The samples were centrifuged for 30 min at 5 °C and 10,000xg.

### Inductively coupled plasma-atomic emission spectrometry (ICP-AES)

Inductively coupled plasma (ICP) is an excitation technique for the detection of trace metals in environmental samples. The main goal of ICP is to get sample elements to emit—in high temperature plasma—characteristic wavelengths, which can then be measured by AES (Atomic Emission Spectrometry). This technology was first employed in the early 1960s. In our experiments, a Leeman Labs PS 1000 UV inductively coupled plasma-atomic emission spectrometer was used following the manufacture's guidelines: 196.0 nm Se line, 1.4 mL/min sample flow rate, argon plasma (PS series ICP manual, 1990).

### Bioreactor Experiments

- The fermentor was cleaned very well before starting the experiment with bleach and other detergents to minimize contaminants.
- Growth medium for the fermentor was prepared as mentioned previously in this chapter and added to the glass bioreactor vessel.
- The third step was to sterilize the bioreactor in a 716-liter autoclave (Wisconsin Aluminium Foundry Co. Inc., Manitowoc, WI USA) (50 min @ 121°C).

- Once the bioreactor cooled to room temperature, inoculum was transferred to it in sterile manner followed by adding 100-mL aliquots of the sterile selenium salts at known final concentrations.
- After that, bioreactor agitation was started and the bioreactor vessel was purged with sterile nitrogen, which forced the bacteria to grow anaerobically.
- In the initial experiments, nitrogen flow rates were set to 50 mL/min and headspace samples were collected using the trapping apparatus connected to the end of the gas condenser of the bioreactor. The gas trapping apparatus consisted of three bubbling tubes connected in series. Each bubbling tube contained 15 mL of 50% HNO<sub>3</sub>.
- Gas flow rates were checked every 24 hours and one milliliter sample of headspace gas was taken every 24 hours with a gas-tight syringe (Alltech, Deerfield, IL USA) through the septum port on the fermentor to determine the identity of the organosulfur and organoselenium metabolites produced in culture headspace.
- After cultures reached the stationary phase (on average at 72 hours after the inoculation), three replicate 25-mL solution-phase samples from the bioreactor were harvested for further analysis.

Bioreactor oxic conditions were controlled in the following manner.

- Anaerobic culture growth

These experiments were anaerobically executed in the presence of sterile nitrogen gas, which was purged through the bioreactor for 72 hours and flow rate was maintained 50 mL/min as mentioned in the bioreactor experimental method section.

- Sequential anaerobic/aerobic culture growth

In these bioreactor experiments, the same method was used except gas purging involved N<sub>2</sub> or air. The bioreactor was operated with anaerobic (N<sub>2</sub> purge) alternating with aerobic growth (air purge). Therefore, by introducing different combinations and lengths of purge cycles, various types of anaerobic/aerobic culture growth were tested.

## Sample preparation and analysis

### Dynamic headspace samples

One-milliliter headspace samples taken from the bioreactor were immediately injected into a Hewlett Packard 5890 series II gas chromatograph (Palo Alto, CA USA). The chromatographic column used in this analysis was a DB-1, 5-micron, 30-meter capillary column (J&W Scientific, Folsom, CA USA). Table III gives the chromatographic and detector conditions used in this analysis.

Table III

#### Chromatographic and detector conditions

Gas chromatograph carrier	He, 1 mL min <sup>-1</sup>
Injector	275°C
Temperature program	30°C for 1 min 30-250°C at 15°C min <sup>-1</sup>
Injection mode	Splitless
Transfer line	150°C
Reaction cell pressure	< 1 Torr
Source of F <sub>2</sub>	DC discharged of SF <sub>6</sub> reagent gas

## Bioreactor liquid samples

The liquid samples removed from the bioreactor were transferred to centrifuge tubes. Then samples were centrifuged at 5°C (10,000xg; 30 min) and the supernatant was separated from the solid (cells + red elemental selenium). Supernatant and solid samples were analyzed separately using ICP-AES.

## **Analysis by inductively coupled plasma-atomic emission spectrometry**

Solid and liquid samples were prepared for analysis separately. The solid samples were dissolved in concentrated nitric acid (5 mL) and diluted with deionized water. The samples with higher concentrations were diluted until the concentration was within the calibration range. Liquid samples too were diluted to get required concentration ranges. All the samples had a 50% HNO<sub>3</sub> matrix by the time they were analyzed. These samples were analyzed using ICP-AES. Calibration samples (0.2 ppm–20 ppm) were also prepared in 50% HNO<sub>3</sub>.

## **Headspace trapping and samples analysis**

Headspace samples were collected in 50 % HNO<sub>3</sub> solutions contained in gas bubbling tubes, which were connected in series using 1/8-inch diameter Tygon<sup>®</sup> sanitary silicone tubing. Each of these trapping tubes contained 15 mL of 50 % HNO<sub>3</sub>. Analysis of headspace trapping samples was also accomplished using ICP-AES. These samples were already in 50% HNO<sub>3</sub>. Therefore, they were tested without any dilutions or additions.

## CHAPTER III

### DATA AND RESULTS

#### PART 1. INTERFERENCE OF GLASS CONTAINERS IN SELENIUM

#### DETERMINATION BY HGAAS

#### HGAAS analysis

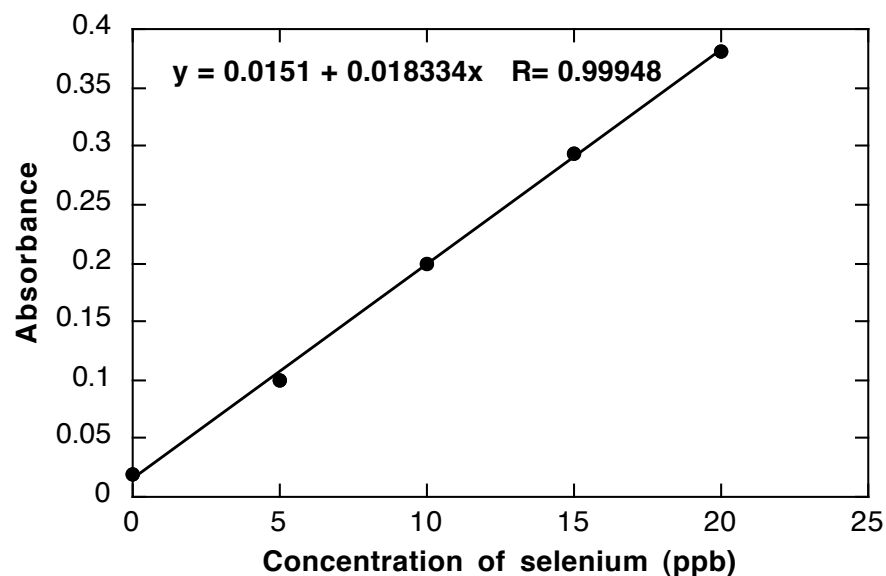
Samples prepared in borosilicate glass test tubes, which were treated with different chemical acids or commercially available detergents to remove glass interference, were analyzed by HGAAS.

The calibration curve for these HGAAS analyses was constructed using 0 ppb to 20 ppb of selenium standards. Then known concentrations of samples from glass tubes treated in various ways were determined according to the calibration curve. Table IV gives absorbance of an example set of selenium standards and Figure 4 gives a typical calibration curve for selenium analysis.

Table IV

#### Selenium calibration data from the HGAAS

Concentration of Se (ppb)	Absorbance
0	0.0195
5	0.0991
10	0.1998
15	0.2928
20	0.3810



**Figure 4.** Calibration curve for Se analysis by HGAAS.

The percent recovery for glass experiments using untreated tubes was calculated for each selenium sample at three different concentrations. All the % recoveries, which are tabulated in Table V, are an average of three replicate samples with standard deviation in parenthesis.

Table V

Se HGAAS % recoveries for samples in untreated test tubes

Expected Se ppb	Determined Se ppb	% Recovery
5	1.85(±1.73)	37.0
10	3.47(±4.77)	34.7
15	4.54(±6.03)	30.3
	<b>Average</b>	34%

Results from the treated test tube experiments are presented in Table VI through XIII. The reagents used to treat a group of tubes are detailed on page 14. As above, % recovery, standard deviation and the average recovery are included.

Table VI

Se HGAAS % recoveries for samples in HCl treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	0.51( $\pm$ 0.09)	10.1
10	0.83( $\pm$ 1.36)	8.3
15	2.53( $\pm$ 1.88)	16.7
	<b>Average</b>	11.7%

Table VII

Se HGAAS % recoveries for samples in H<sub>2</sub>SO<sub>4</sub> treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	3.01( $\pm$ 2.68)	60.2
10	9.93( $\pm$ 0.18)	99.3
15	3.64( $\pm$ 3.85)	24.2
	<b>Average</b>	61.2%

Table VIII

Se HGAAS % recoveries for samples in distilled water treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	2.76( $\pm$ 2.48)	55.2
10	3.17( $\pm$ 4.62)	31.0
15	5.35( $\pm$ 6.02)	35.7
	<b>Average</b>	40.6%

Table IX

Se HGAAS % recoveries for samples in 0.2 % Extran treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	4.18( $\pm$ 2.04)	83.6
10	1.94( $\pm$ 1.34)	18.4
15	1.10( $\pm$ 0.45)	7.3
	<b>Average</b>	36.4%

Table X

Se HGAAS % recoveries for samples in 2 % Extran treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	5.16( $\pm$ 0.32)	103.2
10	6.65( $\pm$ 4.64)	66.5
15	1.06( $\pm$ 0.66)	7.1
	<b>Average</b>	58.9%

Table XI

Se HGAAS % recoveries for samples in aqua regia treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	5.52( $\pm$ 1.32)	110.5
10	10.17( $\pm$ 0.48)	101.7
15	10.82( $\pm$ 6.32)	72.1
	<b>Average</b>	94.8%

Table XII

Se HGAAS % recoveries for samples in chromic acid treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	6.84( $\pm$ 0.91)	136.8
10	6.20( $\pm$ 3.18)	62.0
15	9.52( $\pm$ 3.3)	63.5
	<b>Average</b>	87.4%

Table XIII

Se HGAAS % recoveries for samples in nitric acid treated test tubes

Expected Se ppb	Determined Se ppb (SD)	% Recovery
5	6.06( $\pm$ 0.90)	121
10	10.99( $\pm$ 0.46)	109
15	15.49( $\pm$ 0.36)	103
	<b>Average</b>	111%

## PART 2. MASS BALANCE EXPERIMENTS

### Proving the volatile organoselenium trapping method

As previously discussed, there are several headspace-trapping solutions/methods available but in this work a new trapping solution was tested and used in bioreactor experiments. One of the advantages of using 50 % HNO<sub>3</sub> over 30 % H<sub>2</sub>O<sub>2</sub> (Bius, 2001) was to address the chemical matrix problem. The use of this acid solution eliminated the matrix problems because samples were prepared in 50 % HNO<sub>3</sub> in ICP-AES analysis and so trapping solutions did not require matrix adjustments. Before using 50 % HNO<sub>3</sub> as trapping solution, it was tested with known amounts of volatile dimethyl diselenide added to samples. In this experiment, three bubbling tubes were connected in series and 315.81 µg of selenium in the form of dimethyl diselenide were put into 15 mL of 50 % HNO<sub>3</sub> trapping solution in the first bubbling tube then followed by purging with nitrogen gas (50 mL/min) for 24 hours. (No DMSe was added to the second or third tubes.) After that, trapping solutions from these three bubbling tubes were analyzed for selenium using ICP-AES. Table XIV gives the recoveries for selenium in this experiment and these data showed that 50 % HNO<sub>3</sub> effectively retained all the selenium put in the first trapping tube.

Table XIV

Se % Recovery observed for 50 % HNO<sub>3</sub> trapping solution

Run	Trap-1	Trap-2	Trap-3	Total	% Recovery
	Se $\mu$ g	Se $\mu$ g	Se $\mu$ g	Se $\mu$ g	
1	276.21	25.58	22.28	324.07	102.6
2	271.32	23.84	22.56	317.72	100.6
3	291.04	24.74	19.95	335.73	106.3
Average	279.52	24.72	21.60	325.84	103.17

**Selenium analysis by ICP-AES**

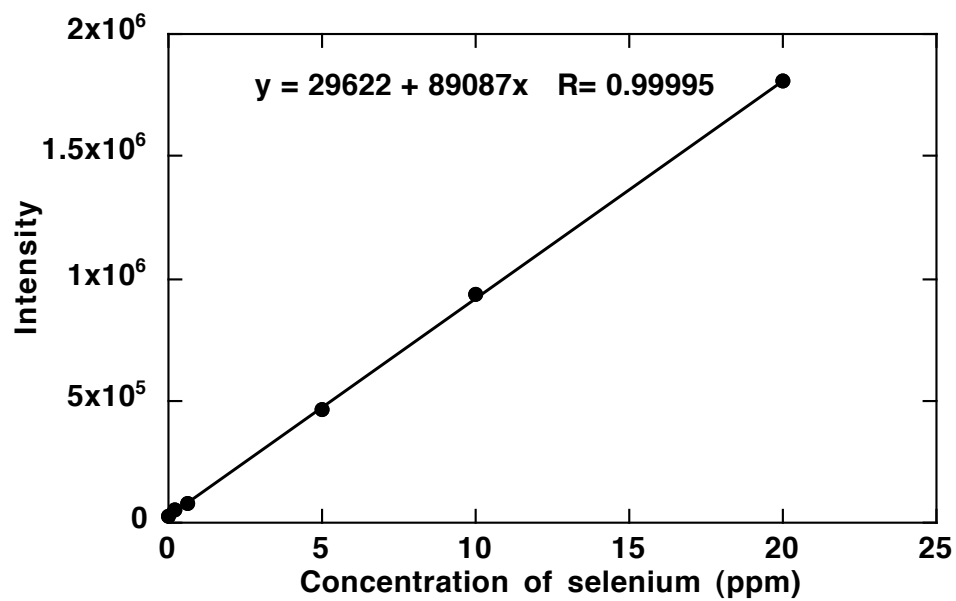
One of the advantages of analysis of selenium by ICP-AES is the unimportance of the oxidation state of the sample. This technique is sensitive to both selenate and selenite oxidation states. Therefore, sample matrix was the only major concern. Both samples and calibration standard were prepared in the same matrix, which was 50 % HNO<sub>3</sub>.

To calculate unknown concentrations of samples, the experimental calibration curve was used. According to Beer's law, emission intensity is directly proportional to the concentration of the selenium over an analytical range that differs for each method but which is most strongly dependent upon the emission line chosen. Therefore, using the emission intensity of the unknown sample, the concentration of the unknown can be calculated using the calibration curve (Boss and Fredeen, 1989). In this method, the linear range was approximately 0.2 ppm to 20 ppm of selenium. Table XV gives emission intensities of selenium standards and Figure 5 gives a typical calibration curve for selenium analysis.

Table XV

Selenium calibration data from the ICP-AES

Concentration of Se (ppm)	Intensity
0	27738
0.2	49922
0.6	81845
5.0	467594
10.0	932926
20.0	1807024

**Figure 5.** Calibration curve for Se analysis by ICP-AES.

## Mass balance for anaerobic culture growth

The calculation of mass balance was done using different amended selenium concentrations. Selenium concentrations for bioreactor amendments were chosen based on previous studies (Bius, 2001; Eriksen, 1999).

### Ten millimolar sodium selenite amended experiments

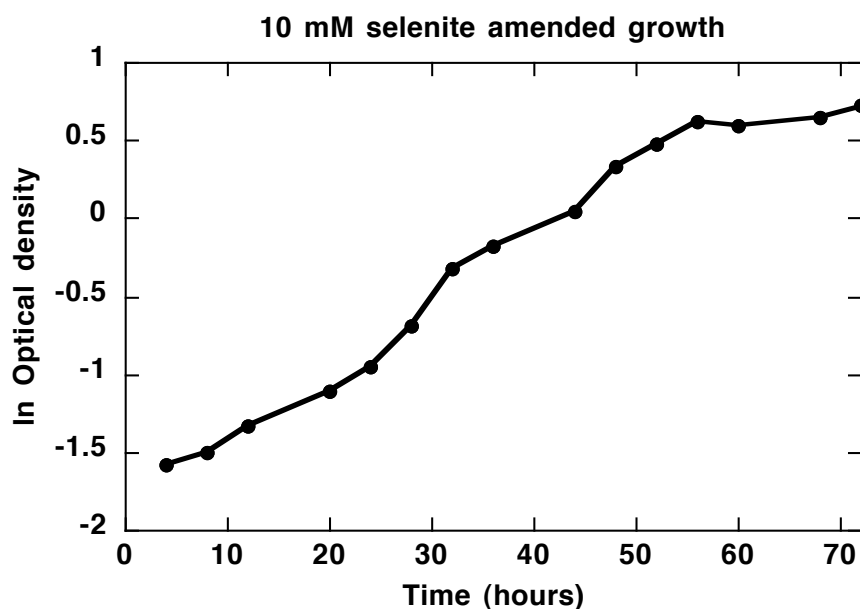
One set of sodium selenite-amended batch culture experiments were purged with nitrogen gas throughout 72 hours of growth. Gas flow rates were maintained at 50 mL/min and readjusted every 24-hour if there was a significant change in flow rate ( $\Delta > 10\%$ ). The headspace sample chromatographic analysis for every 24 hour showed that no detectable amounts of headspace gas were present (LOD  $\approx 15$  pg on column). We attribute this to the effective removal of headspace gas by continuous purging. The mass balance calculation (Table XVI) for this amendment concentration showed that most of the selenite was left in solution and much smaller amounts were reduced to elemental and gaseous forms. The headspace gas trapped sample analysis showed that amounts of gaseous selenium-containing produced were very low.

Table XVI

#### Ten mM of selenite (n=3)

Phase	% Recovery
Liquid	92.167( $\pm 8.13$ )
Solid	6.900( $\pm 1.32$ )
Gas	0.004( $\pm 0.002$ )
Total Recovery	99.071( $\pm 8.07$ )

Taking absorbance readings periodically throughout the experiment as a measure of cell population (Stone, 1997), the growth curve (Figure 6) was plotted for this experiment and it can be used to compare growth of K27 in the anaerobic/aerobic experiments.



**Figure 6.** Anaerobic growth vs time of the K27 bacterium with 10 mM selenite amendment.

### **One millimolar sodium selenite-amended experiments**

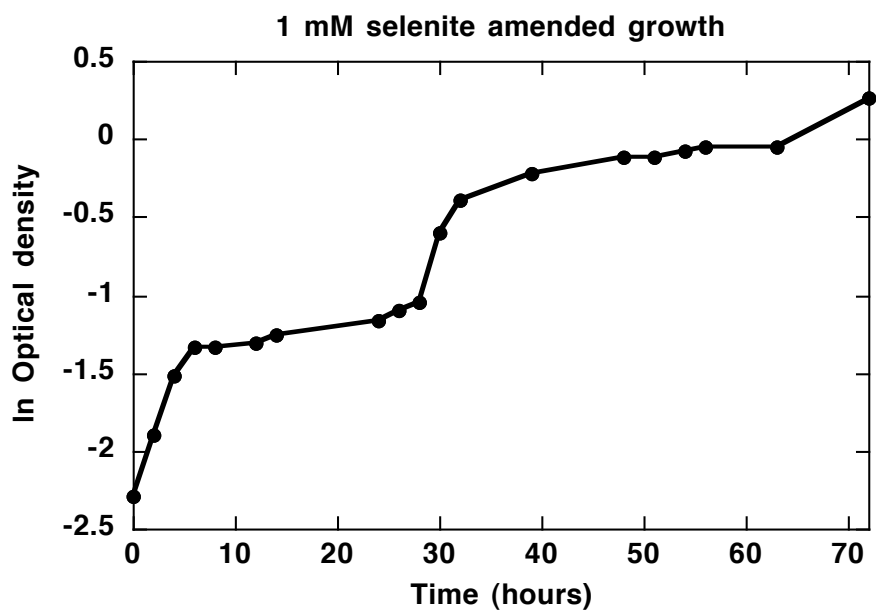
These experiments were performed under the same conditions as the 10 mM selenite amended experiments with 1 mM sodium selenite amendments used instead. From these mass balance data (Table XVII), it is obvious that as a percentage the reduction of selenite to elemental selenium is higher than 10 mM amended bioreactor data although the absolute amounts were less. Percent conversion to the solid elemental form had been increased about 5 times when compared to the 6.9 % conversion of 10 mM amended bioreactor experiments. The amounts of gas produced in this experiment

are also higher as compared to the previous culture with high amounts of selenite. The growth of the K27 under these conditions is given in Figure 7.

Table XVII

One mM of selenite (n=5 )

Phase	% Recovery
Liquid	66.680( $\pm$ 18.29)
Solid	32.440( $\pm$ 19.81)
Gas	0.041( $\pm$ 0.07)
Total Recovery	96.161( $\pm$ 0.62)



**Figure 7.** Anaerobic growth vs time of the K27 bacterium with one mM selenite amendment.

### Ten millimolar sodium selenate-amended experiments

These experiments were also performed under the same parameters as the selenite experiments. The mass balance calculations (Table XVIII) showed that effectiveness of bioremediation process was very low because selenate was not effectively reduced to elemental selenium by *Pseudomonas fluorescens*. The amount of selenate reduced to elemental selenium was very low.

Table XVIII

Ten mM of selenate (n= 3)

Phase	% Recovery
Liquid	95.067 ( $\pm 6.98$ )
Solid	0.733( $\pm 0.06$ )
Gas	0.001( $\pm 0.001$ )
Total Recovery	95.801( $\pm 6.93$ )

### Mass balance for anaerobic/aerobic culture growth

In these experiments, bioreactor cultures were grown under mixed anaerobic and aerobic conditions. This technique was introduced to test whether there is an improvement in bioremediation of selenium in stressed conditions that are more environmentally realistic. First cultures were forced to grow anaerobically followed by changing to aerobic conditions by introducing air into the bioreactor. Time durations for anaerobic and aerobic growth were changed according to different experiments but the total time course of all cultures were maintained at 72 hours.

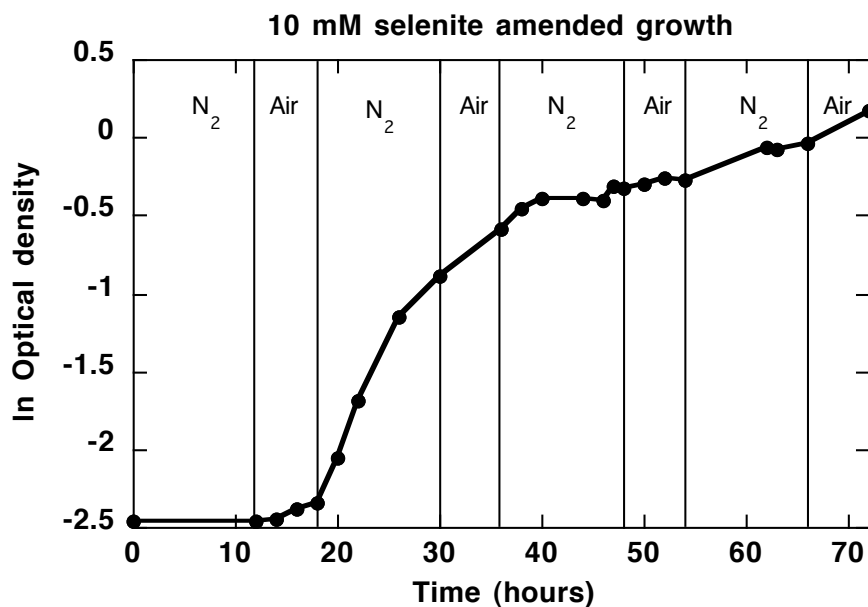
### Ten millimolar sodium selenite-amended experiments

This experiment was carried out with four cycles: 12 hours of anaerobic growth followed by 6 hours of aerobic growth. The nitrogen and airflow rates were alternately maintained at 50 mL/min in this experiment. The following table (Table XIX) gives the mass balance of the experiment and growth curve for this experiment is shown in Figure 8 with the gas purge cycle indicated.

Table XIX

Ten mM of selenite (n=3)

<b>Phase</b>	<b>% Recovery</b>
Liquid	83.048( $\pm$ 3.043)
Solid	8.532 ( $\pm$ 1.902)
Gas	0.002 ( $\pm$ 0.001)
Total Recovery	91.583 ( $\pm$ 4.429)



**Figure 8.** Anaerobic/aerobic growth vs time of the K27 bacterium amended with one mM selenite.

### One millimolar sodium selenite-amended experiments

A series of one-millimolar experiments was undertaken with mixed anaerobic/aerobic growth by changing the amounts of air introduced into the bioreactor in aerobic growth.

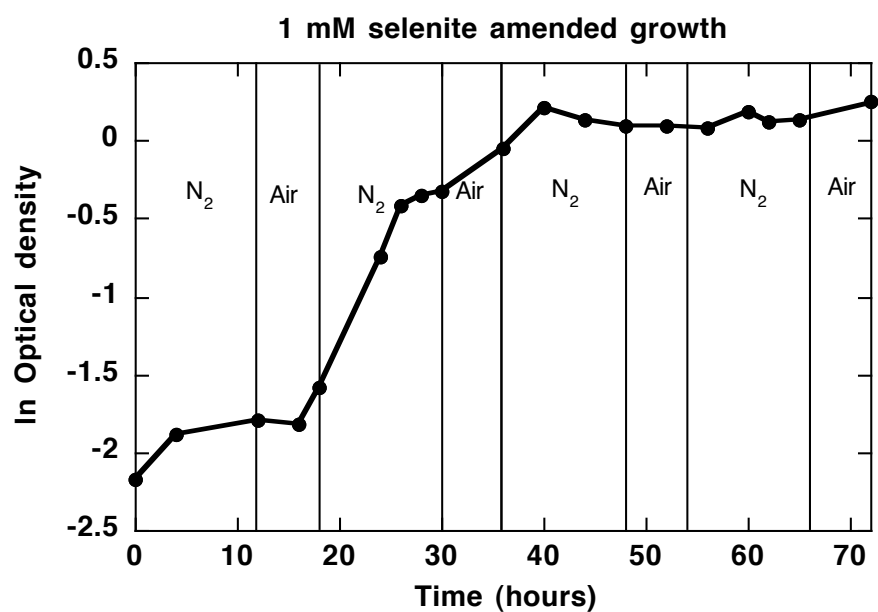
#### Experiment 1

This experiment's conditions were with mixed anaerobic /aerobic growth but low amounts of sodium selenite (1 mM) and involved four cycles: 12 hours of anaerobic growth followed by six hours of aerobic growth. Both nitrogen and air flow rates were maintained at 50 mL/min. Table XX gives the mass balance of the experiment and the growth of K27 is given in Figure 9 with each purge cycle indicated.

Table XX

One mM of selenite (n=3)

Phase	% Recovery
Liquid	59.496( $\pm$ 19.654)
Solid	32.988( $\pm$ 18.710)
Gas	0.011( $\pm$ 0.014)
Total Recovery	92.495( $\pm$ 0.985)



**Figure 9.** Anaerobic/aerobic growth vs time of the K27 bacterium with one mM selenite amendment.

## Experiment 2

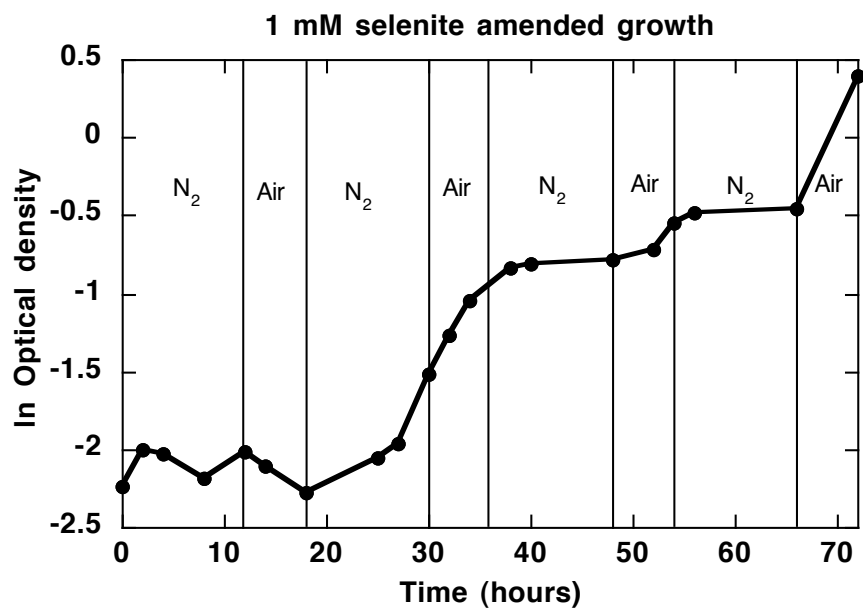
In this experiment the same conditions were used as in the previous bioreactor experiment except airflow rate was increased to 250 mL/min during aerobic growth. The change in airflow rate was introduced to see whether there was a change in growth of K27 and increased conversion of selenite to elemental selenium. Table XXI gives the mass balance of this single bioreactor experiment. In this experiment headspace gas was not collected due to high gas flow; it was impossible to use small (15 mL) bubbling tubes to collect headspace samples with this flow rate of purge gas.

Table XXI

One mM of selenite (n= 3)

<b>Phase</b>	<b>% Recovery</b>
Liquid	45.759(±10.796)
Solid	43.152((±10.859)
Total Recovery	88.911((±4.373)

Figure 10 gives the growth curve for this experiment. It includes indicated purge cycles in these experiments.



**Figure 10.** Anaerobic/aerobic growth vs time of the K27 bacterium with one mM selenite amendment.

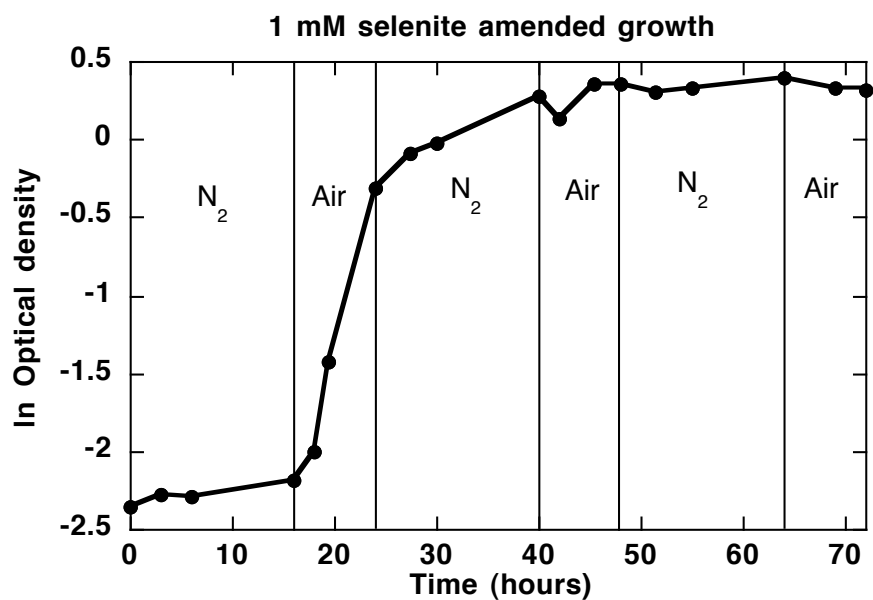
### Experiment 3

In this experiment (Table XXII and Figure 11) the same conditions were used as in the previous bioreactor experiments except the lengths of purge cycles were modified. These involved three cycles of 16 hours of anaerobic growth and 8 hours of aerobic growth.

Table XXII

One mM of selenite (n=3)

Phase	% Recovery
Liquid	52.311( $\pm$ 4.429)
Solid	37.583( $\pm$ 7.992)
Total Recovery	89.894( $\pm$ 11.220)



**Figure 11.** Anaerobic/aerobic growth vs time of the K27 bacterium with one mM selenite amendment.

## CHAPTER IV

### DISCUSSION AND CONCLUSIONS

#### PART 1. INTERFERENCE OF GLASS CONTAINERS IN SELENIUM

##### DETERMINATION BY HGAAS

Since similar sample handling, reduction reagents and procedures, and hydride generation reagents and procedures are widely used in published analytical methods (Dedina and Tsalev, 1995; Zhang et al., 1999b; Martens and Saurez, 1997; Blaylock and James, 1993; Clesceri et al., 1998), our efforts to minimize our poor Se recovery rates ultimately focused on our glass containers. The first clue to the mystery of poor recoveries was excellent recoveries and linearly for our selenite standards in heavily reused glass (also borosilicate), volumetric flasks; however, when glass test tubes were used in our selenium analysis without any kind of pretreatment, that is, straight out of the manufacturer's shipping container, a large experimental error was seen. (As will be described below, most of the cleaning procedures we tried also yield poor recovery also.) Samples were reduced in test tubes without washing to determine the magnitude of this interference (Table V).

Next we tried to treat the glass test tubes with a commercially available detergent, Extran, which is a powder, forms a mildly basic solution in water, and has been used for cleaning glassware in metalloid analysis by others (Granchinho et al., 2001). Different concentrations of Extran solutions were evaluated: tube were rinsed with 0.2% Extran or 2.0% Extran and tubes were soaked in 2.0% Extran for 24 hr and then rinsed with deionized water. These data (Table IX, Table X) show that this reagent was not capable

of substantially affecting the negative recovery on selenium analysis. In fact, Extran may be good for cleaning metalloids from glass, but also may keep the glass surface receptive to adsorption of metalloids and therefore defeat their purpose in this work.

Aqua regia was used to treat glass tubes due to its highly oxidative and metal dissolving ability (Cotton and Wilkinson, 1972; Christian, 1994). These results display some kind of improvement with the % recovery approaching 100% except in the 15 ppb samples with only 72 % recovery (Table XI).

Similar to aqua regia, chromic acid is also a strong oxidant and therefore possibly a useful means of cleaning glass surfaces for these sorts of analyses (Christian, 1994).  $H_2CrO_4$  was evaluated to see whether it could remove the problematic glass effects, but data (Table XII) from that experiment too show it is not capable of removing glass interference; results showed large positive *and* negative errors.

According to data in Table XIII, nitric acid is capable of removing glass interference in selenium determination in HGAAS. The % recovery range for nitric acid treated tubes was the smallest of the ten treatments studied, with an average recovery rate of 111%. The 5 ppb Se samples showed the largest positive deviation (121% recovery) for the  $HNO_3$  treatment. This can be rationalized by noting the larger amounts of analytical noise at this, the lowest concentration studied and only at about 3 times the detection limit, and some contribution from surface and residual oxidant back oxidation (see below).

Even though the composition of the glass in the test tubes used in these experiments is known (see above), it is not at all clear what elements in the glass makeup would interfere with selenium analysis given the interfering ions reported in the literature

(Maher, 1983; Dedina and Tsalev, 1995). The chemical interferences of  $\text{Hg}^{2+}$ ,  $\text{Sn}^{4+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Bi}^{3+}$ , and  $\text{Te}^{4+}$  (among others) in HGAAS determination of selenium have been studied (Naraski and Ikeda, 1984); however, none of these interferents is a major component in the borosilicate glass used in these studies. And possibly most important, the concentrations of interfering ions that are known to cause Se in HGASS, as reported in the literature, are orders of magnitude lower in our tubes' solutions than would be present from their dissolution out/off of the glass surfaces were they present as glass surface contaminants (Dedina and Tsalev, 1995).

Another possible mechanism to explain poor recoveries is the oxidation of selenite to selenate on the container glass surface itself after the HCl boiling/reduction step, so called back oxidation; however, our work with mixed selenite and selenate samples—in which ppb-level final concentrations of  $\text{SeO}_4^{2-}$  were intentionally introduced after the HCl reduction step or in which selenate samples were purposely not reduced at all—showed anomalously high recoveries (+50 to 100 %), not low recoveries (data not shown). Therefore the glass surfaces apparently aren't contributing significantly to back oxidation of selenite in the results we report here.

Instead the poor recovery rates (almost always low for all but  $\text{HNO}_3$ -treated glass) suggest **adsorption of Se to the glass** instead of dissolution and the contribution of interfering ions to sample solution during the analysis or back oxidation.

According to the glass treatment data reported here, rinsing borosilicate test tubes with concentrated nitric acid is most successful at reducing the poor recovery rates. From average recovery rate of 34% for untreated tubes, surfaces treated with concentrated  $\text{HNO}_3$  averaged 111% recovery rates. Higher recoveries in the final successful glass

treatment of low Se-containing samples (5 ppb; 121% recovery) may be partially explained by the creation of low amounts of selenate via back oxidation on glass surfaces which, based on past experiments, contribute to erroneously high recoveries. And logically this high recovery is most prominent in the lowest concentration samples using the nitric acid treatment (Table XIII).

It is still not clear why other oxidants like chromic acid are not is job well, but this suggests that an oxidizing acid wash of the surface is, in a simple way, not the mechanism that reduces adsorption. Furthermore simply filling empty chemical sites in the glass surface with anions also does not appear a reasonable answer because high  $\text{Cl}^-$  or  $\text{HSO}_4^-$  (and  $\text{SO}_4^{2-}$ ) in  $\text{HCl}$  or  $\text{H}_2\text{SO}_4$  treatments don't solve the low recovery problem either (Table VI and Table VII).

Shendrikar and West (1975) reported that storing selenium samples in nitric acid prevented sample loss; however, they did not address pretreatment issues and since  $\text{HNO}_3$  is itself an interferant in the hydride generation process that we and most others use, this is not an alternative for our analysis; although a useful pretreatment reagent, nitric acid must be washed away with deionized water or decomposed before the  $\text{HCl}$  reduction step common to almost all Se analyses. Gómez-Ariza et al. (1998) do suggest rinsing glassware with 0.08 M nitric acid; however, our use of 30% nitric acid solution (aqua regia) while successful in the 5 and 10 ppb samples did not reduce recoveries successfully for the 15 ppb range. Most recently Gómez-Ariza et al. in other work (2000) have determined storage of inorganic Se species in borosilicate to be more stable than that storage in quartz. While we have not worked with quartz test tubes this makes us even less inclined to do so.

Very recently we have been in contact with a commercial analytical laboratory which has also suffered poor recoveries/reproducibilities in Se standards for HGAAS using borosilicate glass flasks (Duffy, 2002). They also traced this problem to loss on glass surfaces and have switched to Nalgene<sup>®</sup> plastic flasks as a solution yielding < 5% loss over 5 days storage. This confirms, in our minds the importance that untreated borosilicate glass plays in poor recovery rates for this analytical method.

## **PART 2. MASS BALANCE EXPERIMENTS**

The determination of selenium in environmental samples is considered to be a challenging task because of matrix problems due to the number of foreign ions present. There are various analytical techniques to determine selenium content in environmental samples such as HGAAS, colorimetry, fluorometry (Blaylock and James, 1993) and ICP-AES. Colorimetric methods (Magin et al., 1960) and fluorometric methods are considered to be reliable methods but time consuming. Analysis of solid elemental selenium with colorimetry was not successful because of oxidation and reduction steps involved with analysis. HGAAS is one of the best methods for analysis of Se due to its good detection limits (~5 ppb) and high accuracy but it requires sample preparation involving reduction of selenate to selenite. In our analysis, ICP was used for several practical reasons such as short analysis time, good detection limits, and equal applicability for selenate or selenite containing sample. There is no need of having a specific oxidation state for ICP-AES experiments. Therefore, samples can have Se(IV) , Se(VI) or mixtures of Se(IV) and Se(VI) (Basnayake, 2001).

The calculation of the selenium mass balance in these bioreactor experiments was carried out using different amended selenium concentrations. When high amounts of

selenite were added (10 mM final concentration) conversion to solid Se by K27 was low, 6.9 % (Table XVI), but for 1 mM amended experiments, the percentage conversion of selenite to solid was higher than in the 10 mM experiments, 32.44 % (Table XVII). These data suggest that when low amounts of selenite were present in the solution, reducing efficiency as measured by the percentage of added Se was higher. Less selenite present may allow more detoxification to occur because of lower overall toxicity of the metalloid amendment.

Even though we chose these concentrations (10 mM and 1 mM) in our bioreactor experiments based on previous works (Bius, 2001; Eriksen, 1999), environmental sites like the San Joaquin Valley contain only low concentrations of selenium. When comparing the concentrations of selenium that we studied in lab-scale experiments to the concentrations in environmental samples, concentrations in environmental samples are very low. Agricultural water samples from the San Joaquin Valley have a maximum selenium concentration of 1400  $\mu\text{g/L}$  (Frankenberger and Arshad, 2001), which is 0.017 mM. Therefore, we can, in theory, use K27 for the bioremediation of selenium from agricultural drainage water: using low concentrations of selenium in bioreactor experiments comparable to environmental samples, high % conversion to solid elemental form might be achieved. Now that we have proven that K27 can be used in bioremediation of selenium, it will be good a next step to use environmental samples for doing lab scale bioremediation experiments to test the effectiveness of this method on natural samples.

From the data presented in Table XVIII, we can conclude that selenate was less effectively reduced to elemental selenium by K27, than was selenite. This may be because selenite is more toxic to this microbe (Eriksen, 1999).

The purpose of introducing sequential anaerobic-aerobic culturing was to attempt to improve the removal efficiency of the bioremediation process, but sequential anaerobic/aerobic culture growth as we carried it out did not have a big effect on this detoxification process. According to the data from Table XIX, ten millimolar sodium selenite-amended experiments had very similar elemental selenium production as compared to the same experiment performed under strict anaerobic growth conditions (Table XVI). When comparing 1 mM selenite amended experiments, which were carried out under anaerobic conditions, with experiments carried out with sequential anaerobic-aerobic culture experiments, we can clearly see that the amounts of solid Se formation were not affected significantly: The percent solid selenium formed in all of these experiments were very close (Table XVII, Table XX, Table XXI and Table XXII). Therefore, those results showed that introducing aerobic growth conditions in these experiments has not substantially changed the reducing power of these bacteria.

An average total percent recovery of selenium in all of these experiments was 93.417 % ( $\pm 3.699$ ). In most of the cases, the total percent recovery was less than 100 %. This low Se recovery may be caused by the loss of elemental selenium when sampling from the bioreactor vessel. To get homogeneous samples for analysis, it was necessary to mix the bioreactor liquid and solid very well before harvesting the samples via the bioreactor's liquid sampling port. It was sometimes difficult to get all the solid particles, which were adsorbed onto the walls of bioreactor vessel, into the solution. That may be

one of the errors of sampling, which could lead to low percent recoveries. There may be other possibilities of losing elemental selenium: when separating liquid from solid after centrifugation, it was inevitably difficult to prevent solid from going, in small part, into the liquid phase. This may have resulted in the loss of solid selenium in the analysis and this would contribute to lower recoveries.

A comparison of biomass produced at the end of each experiment gives an idea about how bacteria biomass production was affected by each of the different experimental conditions. Therefore, biomass for each experiment was calculated based on the previous studies done by Stone (1997). In that work, a relationship between total biomass and optical density of liquid cultures was determined. The 10 mM sodium selenite-amended experiments attained a final biomass content of 0.47 g/L and at the same point in time for the analogous experiment, done under anaerobic/aerobic growth conditions, the final biomass was 0.43 g/L. These data suggest that there are no big differences in final biomass attained under these different growth conditions. This probably explains why we observed similar elemental solid formation in ten millimolar experiments (Table XVI, Table XIX) despite different growth conditions. The final biomass for the one millimolar sodium selenite-amended experiments with anaerobic growth was 0.44 g/L and the final biomass for the other one millimolar sodium selenite-amended experiments with anaerobic/aerobic growth conditions were also quite similar: 0.43 g/L, 0.45 g/L and 0.45 g/L respectively for experiment 1, experiment 2 and experiment 3 (see page 36-40). These data also suggest that final biomass for anaerobic and anaerobic/aerobic low selenite amendment experiments were very close. These results again explain why we observed similar elemental solid formation for each of these

experiments with different growth conditions (Table XVII, Table XX, Table XXI and Table XXII).

The volatile organoselenium trapping method we finally settled upon was successful. Even though 30 % H<sub>2</sub>O<sub>2</sub> had been used in headspace trapping of volatile organoselenium compounds, there were difficulties proving this method (Bius, 2001). One of the advantages of using 50 % HNO<sub>3</sub> over 30 % H<sub>2</sub>O<sub>2</sub> is matrix matching. When hydrogen peroxide was used in that previous work, it was necessary to destroy excess H<sub>2</sub>O<sub>2</sub> before sample analysis, but it was observed that removing excess H<sub>2</sub>O<sub>2</sub> was not always easy to do and errors subsequently occurred. Therefore, 50 % HNO<sub>3</sub> is a good trapping solution that solves most of these complications. The results from Table XIV—proving the validity of the volatile organoselenium method—show that trapping of volatile selenium compounds was effective and % recoveries of that experiment show that almost all the volatile compounds, which passed into trapping solutions, was collected using three bubblers containing 50 % HNO<sub>3</sub> solutions connected in series. From the data (Table XIV), it is obvious that the largest amounts of volatile organoselenium compounds were retained in the first trapping solution followed by the second highest amount in the second solution and the rest in the third solution. As a result of that, three 50 % HNO<sub>3</sub> solutions were used in our subsequent experiments.

Results from the headspace trapping experiments involving bioreactor cultures showed that very little selenium was biologically reduced to Se[-II] because very small amounts of added Se were recovered in the traps. When ten millimolar sodium selenite amended experiments were carried out (Table XVI), a very small amount of gaseous selenium was trapped, (0.004% sum of all three traps). This may be due to the fact that

the amounts of selenite converted to solid selenium were relatively small (Table XVI) compared to the amounts of solid formed from one millimolar sodium selenite experiments (Table XVII). Results from one millimolar amendment experiments showed that the amounts of organoselenium collected was ten times higher than from the ten millimolar sodium selenite amended experiments (Table XVI) and analogously solid formation was also higher in these experiments. Therefore, the chances of bio-formation of the organoselenium compounds are higher in experiments where more elemental Se was also formed. It is clear that, overall, selenite reduction to volatile organoselenium compounds by K27 was very small.

### Conclusions

- Rinsing borosilicate test tubes with concentrated nitric acid is the most successful method examined at reducing the poor recovery rates of Se in HGAAS.
- The poor recovery rates of selenium in glass test tubes may be due to adsorption of Se to the glass instead of dissolution or the contribution of interfering ions to the sample solution during the analysis.
- As a percentage of amended selenium, selenite was more effectively reduced to Se<sup>0</sup> by *Pseudomonas fluorescens* than was selenate.
- When low amounts of selenite were present in the solution, reducing efficiency as a percentage of added Se was higher as measured by the production of Se<sup>0</sup>.
- Sequential anaerobic/aerobic growth conditions did not have a big effect in this detoxification process as carried out. We saw no real difference in elemental Se

production between culture grown completely anaerobic as compared to mixed anaerobic and aerobic periods.

- 50 % HNO<sub>3</sub> solution is a good trapping solution to collect volatile organoselenium compounds in gas purging experiments.

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**APPENDIX****CHEMICAL ABSTRACT SERVICE REGISTRY NUMBERS**

<b>Compound Name</b>	<b>CAS Registry Number</b>
Hydrochloric acid	7647-01-0
Nitric acid	7697-37-2
Potassium nitrate	7757-79-1
Selenium dioxide	7783-00-8
Sodium borohydride	16940-66-2
Sodium dichromate	10588-01-9
Sodium hydroxide	1310-73-2
Sodium selenate	13410-01-0
Sodium selenite	10102-18-8
Sulfuric acid	7664-93-9

## VITA

Suminda Hapuarachchi was born in Kurunegala, Sri Lanka, in 1972. He attended the Dharmaraja College and graduated in 1991. He received a Bachelor of Science in Special Degree in Chemistry in 1998. After two years working in the private sector, he entered Sam Houston State University in 2000 and received his Masters Degree in Chemistry in 2002.

