

Developing a Method for Determining the Mass Balance of Selenium and
Tellurium Bioprocessed by a Selenium-Resistant Bacterium Grown in the
Presence of Selenite or Tellurite

A Thesis

Presented to

The Faculty of the Department of Chemistry

Sam Houston State University

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

by

Janet Horton Bius

December, 2001

Developing a Method for Determining the Mass Balance of Selenium and
Tellurium Bioprocessed by a Selenium-Resistant Bacterium Grown in the
Presence of Selenite or Tellurite

by
Janet Horton Bius

Approved:

Thomas G. Chasteen

Mary F. Plishker

Rick C. White

Approved:

Brian Chapman, Dean
College of Arts and Sciences

ABSTRACT

Bius, Janet Horton, Developing a Method for Determining the Mass Balance of Selenium and Tellurium Bioprocessed by a Selenium-Resistant Bacterium Grown in the Presence of Selenite or Tellurite, Master of Science (Chemistry), December, 2001. Sam Houston State University, Huntsville, Texas, 68 pp.

Purpose

The purpose of this investigation was to determine: (1) the mass balance of selenium or tellurium that was bio-reduced when a selenium-resistant facultative anaerobe was amended with either selenium or tellurium; and (2) methods to analyze for these metalloids in biological samples.

Methods

Analytical methods were developed for the determination of selenium and tellurium in biological samples by hydride generation-atomic absorption. These methods were developed by: (1) determining the optimal operating conditions for the determination of these metalloids by HGAAS; (2) testing oxidation and reduction processes on known quantities of the metalloid; (3) eliminating interferences in the determination of these metalloids by HGAAS; and (4) applying these analytical methods to biological samples obtained from a culture of *Pseudomonas fluorescens* K27 amended with either selenite or tellurite.

Findings

1. The elimination of positive interference from the growth medium and the reagents used in the oxidation and reduction of these metalloids required dilutions before and after the oxidation by either nitric acid or hydrogen peroxide.

2. The chosen oxidant for selenium in the solid phase was nitric acid, and for the supernatant, it was hydrogen peroxide. The final reduction method used for selenium was 5 mL 12 N HCl and 5 mL of sample with 0.2 mL of 2% ammonium persulfate and heated in a boiling water bath for twenty minutes.

3. The chosen oxidant for tellurium in both the solid and supernatant was nitric acid. The final reduction method used for tellurium was 5 mL 12 N HCl and 5 mL of sample with 0.2 mL of 2% ammonium persulfate heated in a boiling water bath for twenty minutes.

4. When these methods were applied to bioreactor cultures amended with 10 mM selenite, 6.78% of the added selenite was reduced to biologically produced elemental selenium or Se species that had adhered to K27 bacterial cells.

5. In bioreactor cultures that were amended with 0.1 mM tellurite, 26.4% of the tellurite was reduced to elemental tellurium or Te species that had adhered to the cells.

Thomas G. Chasteen
Thesis Director

ACKNOWLEDGMENTS

A special thanks to Dr. Thomas G. Chasteen, I appreciate his patience while I honed my rusty lab skills. I have learned many things from him and greatly admire his ability to share his knowledge of chemistry.

I thank my lab mates: Lynn Erickson, Mehmet Akpolat, Jerry Swearingen, Suminda Hapuarachchi and Rukma Basnayake. For these guys, no question was too small and no request too big. My life is much richer for knowing them.

I thank Dr. Mary Lynn DeShazo for the many years of advice and the confidence that she has had in me.

To Daniel and Will, my wonderful sons, I thank them for supporting my efforts to attend Sam Houston even when they could not conceive of anyone voluntarily going to school. They were patient when mom's mind was full of chemistry and not on what was for dinner.

To Ben, my husband, I thank him for all of his help in achieving a long held goal. I could not have done it without him.

I dedicate this thesis to my family.

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	vii
LIST OF FIGURES	ix
CHAPTERS	
I INTRODUCTION	1
II EXPERIMENTAL	7
III RESULTS	14
IV DISCUSSION AND CONCLUSIONS	41
BIBLIOGRAPHY	51
APPENDIX.	
Chemical Abstract Service Registry Numbers	54
VITA	55

LIST OF TABLES

Table I Comparison of specific growth rates of non-amended bioreactor cultures	15
Table II Comparison of specific growth rate (h^{-1}) of 10 mM Na_2SeO_3 amended bioreactor cultures	16
Table III Oxidation methods used to achieve selenate oxyanion to be analyzed by atomic absorption spectrometry	18
Table IV Fluid from a 10 mM Na_2SeO_3 bioreactor experiment analyzed by atomic absorption spectrometry for selenium in liquid and solid phase combined	19
Table V Selenium concentration of bubbling tubes	20
Table VI Known quantities of dimethyl diselenide oxidized with 30% hydrogen peroxide and determined by AAS	21
Table VII Known quantities of dimethyl diselenide oxidized with 30% hydrogen peroxide over several different time spans and analyzed by AAS	22
Table VIII Known quantities of Se(IV) detected by atomic absorption spectrometry	22
Table IX Oxidation and reduction methods tested on tellurium amended bioreactor fluid	23
Table X Optimized conditions for the detection of tellurium by hydride generation-atomic absorption spectrometry	24
Table XI 0.1 mM Na_2TeO_3 bioreactor fluid: Amount of Te left in supernatant vs amount in solid phase (Te^0 or Te on/or in cells)	25
Table XII Speciation of tellurium compounds in a 0.1 mM $Na_2TeO_4 \cdot 2H_2O$ bioreactor experiment	28
Table XIII The acid concentrations of sample solutions to determine optimum determination of selenium by HGAAS	29
Table XIV Determination of the linear range of calibration standards for hydride generation-atomic absorption spectrometry and optimum concentration of the reducing solution	30
Table XV Comparison of 1.5% (w/w) $NaBH_4$ and 0.6% (w/w) $NaBH_4$ solutions in the determination of selenium concentration	33

Table XVI Testing of calibration standards with background correction on then of	31
Table XVII Various reduction methods applied to samples to test detection by hydride generation-atomic absorption spectrometry	32
Table XVIII Reduction methods tested on a mixture of selenate and selenite	33
Table XIX Testing mixtures of selenate and selenite to detect selenium in Se(IV) ...	34
Table XX a The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the affect of dilutions on the detection of selenium ..	35
Table XX b The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the affect of dilutions on the detection of selenium ..	36
Table XX c The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the affect of dilutions on the detection of selenium ..	37
Table XX d The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the affect of dilutions on the detection of selenium ..	38
Table XXI Results of HGAAS determination of selenium from 10 mM Na ₂ SeO ₃ amended bioreactor experiments	39
Table XXII Parameters used for the determination of selenium by hydride generation-atomic absorption spectrometry	40
Table XXIII Results of the degradation experiments for hydrogen peroxide	40

LIST OF FIGURES

Figure 1. Cell growth vs. time of the K27 bacterium with no amendments added. N ₂ means bioreactor purged with nitrogen gas	14
Figure 2. pH vs. time of control bioreactor experiments	15
Figure 3. Comparison of the cell growth in <i>Pseudomonas fluorescens</i> K27 amended with 10 mM Na ₂ SeO ₃ vs. time	15
Figure 4. pH of a 10 mM Na ₂ SeO ₃ amended K27 bioreactor culture vs. time	17

Chapter I

Introduction

The relationship of selenium to the health of human and animal life presents a dichotomy. As an essential element, selenium has a recommended daily requirement of 70 μg per day. At intake levels above 350 μg per day, signs of overexposure to selenium begin to appear including garlic breath (as dimethyl selenide), a metallic taste in the mouth, pallor and/or irritability (Shamberger, 1983).

Selenium probably occurs in small amounts in most soils (Reilly, 1996). The distribution of selenium in soils is uneven and can range from 0.1 $\mu\text{g}/\text{g}$ in soils deficient in selenium and up to 1 mg/g in soils with high concentrations. Soils containing high levels of selenium are called “seleniferous”. People living in seleniferous areas in South Dakota in the 1930s exhibited signs of selenium exposure such as bad teeth and damaged nails. Children living in seleniferous regions in Venezuela also had symptoms of selenosis, a disease caused by excess dietary selenium. The symptoms that were present in these Venezuelan children included dermatitis, loose hair and damaged nails. Endemic selenosis has appeared in seleniferous regions of China. In the 1960s, a disease of unknown origin was reported in Enshi County, Hubei Province, China. The symptoms of this disease included hair loss, skin lesions, and brittle, damaged nails that fell off. These villagers ate vegetables and grains grown in seleniferous soils and these foods contained high concentrations of selenium (Reilly, 1996).

The first report of a disease caused by a deficient daily intake of selenium was in 1935 from Keshan County, Heilongjiang Province in northeastern China (Reilly, 1996). Symptoms of this disease included chest pain, vomiting and nausea. Characteristics of this disease, called Keshan Disease (after the county in which it was first discovered), included cardiac enlargement and congestive heart failure. It mainly affected young people under the age of 15 years and women of child-bearing age. In this geographical area that includes Keshan County the soils are deficient in selenium (Shamberger, 1983).

Keshan Disease could appear in people within three months of their moving into the Keshan County area, and people who left the area did not seem to incur any more damage to their heart

muscles. Incidents of Keshan disease are almost completely eliminated by treating those at risk with sodium selenite pills (Maas, 1998) or by adding selenised salt to their diets. Selenised salt is prepared by adding 15 mg of sodium selenite to every 1 kg of sodium chloride (Reilly, 1996).

Selenium as an environmental hazard was first recognized at the Kesterson Reservoir in the San Joaquin Valley in California. The Kesterson Reservoir was the termination point of the 85-mile long San Luis Drain and consisted of 12 interconnected holding ponds connected in series. The reservoir was to serve two purposes: as an evaporation basin for agricultural drainage water and as a managed wetland. From

1972-1978 the Kesterson Reservoir system received agricultural spill water that was relatively low in dissolved minerals and salts, but by 1981 the Reservoir was receiving subsurface drainage water high in salinity (Skorupa, 1998). This water contained selenium levels that averaged 300 $\mu\text{g/L}$.

Deformities and death of migratory waterfowl due to high levels of selenium in the holding ponds began to appear, and the number of fish species declined to just one, the mosquitofish, *Gambusia affinis* (Skorupa, 1998). The high levels of selenium were due to the concentration of salts and minerals derived from drainage water from irrigated soils weathered from the Cretaceous rocks of the California Coast Ranges (Parsons, 1987).

Another example of a high levels of selenium found in the environment includes Sweitzer Lake which is located near Delta, Colorado, an area with natural seleniferous geological formations. The lake also receives irrigation water and waters used in mining. It is not known whether the high levels of selenium in the lake are a result of nature or of humans. Water samples taken in the late 1980s showed selenium levels of 10 to 24 $\mu\text{g/L}$. Water samples tested in 1995 contained 24 $\mu\text{g/L}$ of selenium. Aquatic fauna and flora also contained high levels of selenium (Skorupa, 1998).

Selenium can exist in water soluble forms such as selenate or selenite. It can form organoselenium compounds such as dimethyl selenide, a volatile molecule that has a Henry's law constant (K_h) of 0.0879 at 25 °C (Gürleyük, 1996). The elemental, metallic form of selenium is insoluble.

Selenium can exist in several different oxidation states. It can exist as Se(VI) in selenates (SeO_4^{2-}), as Se(IV) in selenites (SeO_3^{2-}), Se(0) in elemental selenium, and as (-II) in selenides such as hydrogen selenide (H_2Se) or organic selenides (CH_3SeCH_3 , $\text{CH}_3\text{SeSeCH}_3$).

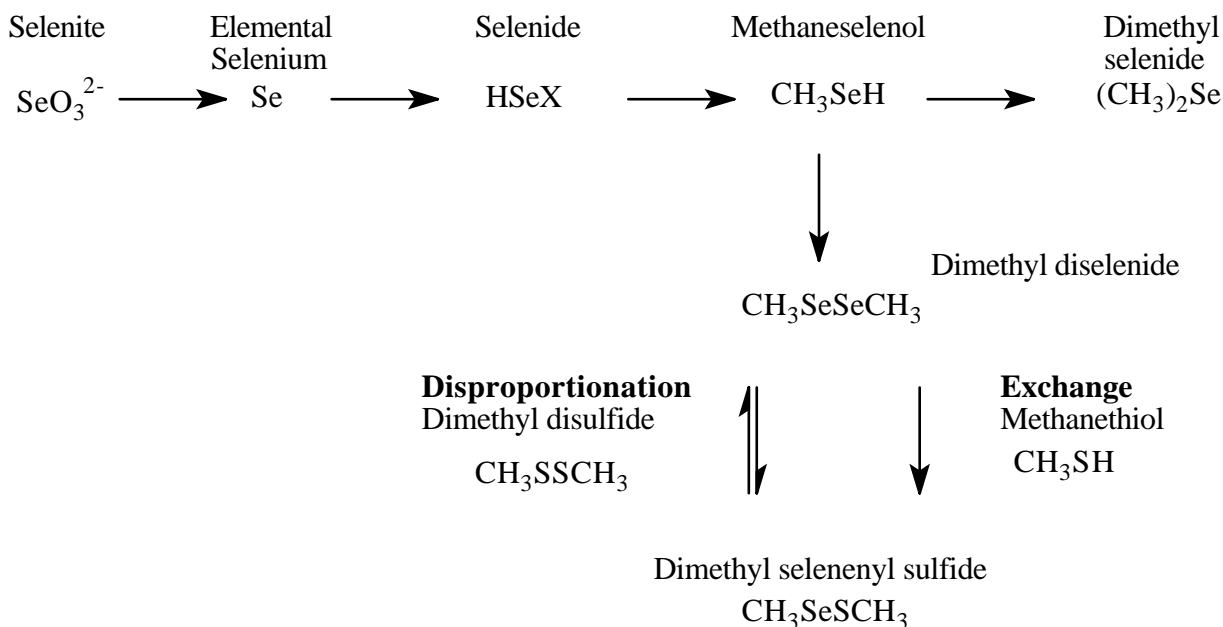
Selenium in the VI oxidation state is considered to be stable under both acidic and alkaline conditions.

Pseudomonas fluorescens K27 is a selenium-resistant bacteria that was isolated from the Kesterson Reservoir in the San Joaquin Valley of California by Ray Fall at the University of Colorado, Boulder in the 1980s. *Pseudomonas fluorescens* K27 is a facultative anaerobe that reduces and methylates selenium under anaerobic conditions (Zhang and Chasteen, 1994). Products of this reduction include elemental selenium and volatile organoselenium compounds such as dimethyl diselenide ($\text{CH}_3\text{SeSeCH}_3$) and dimethyl selenide (CH_3SeCH_3)

When *Pseudomonas fluorescens* K27 is amended with equivalent molar amounts of sodium selenate, higher gas phase concentrations (ppbv) of volatile organoselenium compounds are produced than when it is amended with sodium selenite (Yu, 1996). The reduction of selenite by *Pseudomonas fluorescens* K27 produced more elemental selenium than volatile selenium compounds (Chasteen *et al.*, 1990; Yu, 1996). The mechanism of bioreduction for selenium is not known. Selenium can be used either as an electron acceptor in a detoxification mechanism or possibly as a terminal step in anaerobic respiration by this bacteria as has been determined for *Pseudomonas* sp. AX (Macy *et al.*, 1989; Rech and Macy, 1992).

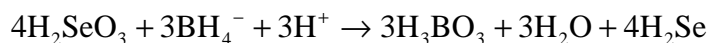
The toxicity of selenium depends upon its chemical form. Selenium in the form of Se(IV) is more toxic to most organisms than selenium in the form of Se(VI). Elemental selenium is not bioavailable; that is, it is not readily absorbed by most organisms. Yu *et al.* (1997) reported the relative toxicity of selenium compounds on *Pseudomonas fluorescens* K27. Selenate is more toxic to K27 bacteria than selenite is (using EC_{50} values: 53 ppm selenate, 412 ppb selenite), but the bacteria can survive at higher concentrations of selenate (15800 ppm) than of selenite (3950 ppm) (Yu *et al.*, 1997).

A proposed mechanism of selenium reduction (shown below) that would explain the presence of elemental selenium and the production of methylated selenium compounds was presented by Doran (1982) and later modified by Chasteen (1993).



Hydride generation-atomic absorption spectrometry (HGAAS) is a generally accepted method for analyzing both geological and water samples to determine selenium concentration. Studies comparing ion chromatography, colorimetry and hydride generation-atomic absorption spectrometry have shown that HGAAS is more sensitive (Blaylock and James, 1993). The low detection levels in HGAAS are analytically useful due to the fact that selenium as a pollutant appears in the environment in the parts per billion range, and therefore, samples do not have to be concentrated for analysis.

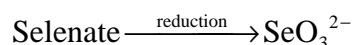
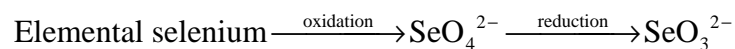
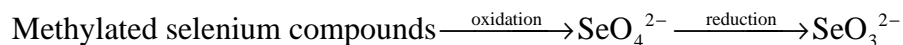
In HGAAS, the volatile selenium hydride is formed when the selenite oxyanion is acidified and reacted with sodium borohydride.



The gaseous selenium hydride is then swept into a heated quartz tube by an inert carrier gas, either argon or nitrogen gas, where it decomposes to produce Se(0) which absorbs light from the hollow cathode lamp. The ability of the sodium borohydride reducing agent to reduce selenium to the hydride depends upon the oxidation state of selenium. This HGAAS process is specific for selenium in the IV oxidation state.

Pseudomonas fluorescens K27 can reduce the selenate oxyanion and selenite oxyanion to elemental selenium and to methylated selenium. To determine the amount of selenium present in a

sample using hydride-generation atomic absorption spectrometry, the selenium in the sample must be in the IV oxidation state. In this work, methods of oxidation and reduction were employed to convert each oxidation state of selenium to the IV oxidation state that can be utilized by hydride generation-atomic absorption spectrometry.



Volatile selenium-containing compounds produced by the K27 bacterium when amended with a selenium oxyanion have been previously determined in this research group and published (Yu *et al.*, 1997). The quantity of the amended selenium oxyanion that has been biotransformed has not been determined. This was one focus of this research.

Methods were developed to determine the amount of selenium added as sodium selenite that was biotransformed by the K27 bacteria into volatile organoselenium compounds and into elemental selenium. Variables that affect the oxidation and reduction processes of the selenium compounds in these methods were investigated. Different oxidizing reagents were tested to determine their ability to oxidize selenium contained in biological samples. Several methods of reducing selenate to selenite also were investigated. Possible interferences in hydride generation atomic absorption spectrometry were also examined. The concentration of the reducing agent and the pH of the acid solution used to generate the hydride of selenium were studied.

Tellurium is a member of the chalcogens, which also includes selenium. Tellurium and selenium have similar chemical properties. Tellurium is used in the semi-conductor industry as a p-type conductor, in thermoelectric devices, in the production of copper and stainless steel, and in blasting caps. As more uses for tellurium are developed and it is harvested, purified and used industrially, the possibility of tellurium becoming an environmental pollutant increases.

Tellurium can exist in several oxidation states. The oxidation states are Te(0) as in elemental tellurium, Te(IV) as in the tellurites, Te(VI) as in the tellurates, and Te(-II) as in the tellurides.

Pseudomonas fluorescens K27 will reduce tellurium oxyanions to elemental tellurium and to organo-tellurium compounds (Basnayake et al., 2001).

Methods were developed to determine the amount of tellurium oxyanion (added as sodium tellurite) reduced by the K27 bacterium. Tellurium must be in the IV oxidation state to be analyzed by hydride generation-atomic absorption spectrometry. Oxidizing reagents and methods of reduction were tested in developing this method to determine tellurium by HGAAS.

To determine the mechanism of bioreduction, the fate of selenium or tellurium oxyanions added to biological samples must be determined. If after amending a K27 bioreactor culture with selenate and determining by hydride generation-atomic absorption spectrometry that only metallic (elemental) selenium is present, the mechanism of reduction would have to favor a step that produces elemental selenium over a step that produces the selenite oxyanion. Before proposing the use of the K27 bacteria as a possible treatment for selenium-polluted sites, the ability of this bacteria to reduce and methylate selenium oxyanions must be determined.

Chapter II

Experimental

Reagents and Laboratory Products

Reagents obtained from Aldrich Chemicals (Milwaukee, WI USA) included selenium atomic absorption standard, tellurium atomic absorption standard, sodium borohydride, ammonium persulfate, sodium selenate, and sodium selenite. Sodium borohydride was obtained from Fisher Scientific (Houston, TX, USA). Tryptic soy broth was obtained from DIFCO Laboratories (Detroit, MI USA). Reagents purchased from VWR Scientific (Sugarland, TX USA) included 30% hydrogen peroxide, and sodium hydroxide. Carrier gases such as argon (UHP) and nitrogen (UHP) were purchased from Conroe Welding (Conroe, TX USA). Materials purchased from VWR Scientific included the culture tubes and culture tube caps, Nalgene® Disposable Filter Unit with a pore size of 0.20 μm and Tygon® tubing. Phenolic culture tube caps were obtained from Fisher Scientific (Houston, TX, USA).

Bioreactor: Stock Solutions and Growth Media

Growth Media

The growth media, TSN3, used in all bioreactor experiments consisted of aqueous solutions of 1% (w/v) tryptic soy broth with 0.3% (w/v) potassium nitrate. All growth media were sterilized by autoclaving at 121 °C for 20 minutes.

Stock Solutions: Selenium

Sodium selenate stock solutions used for amending the batch cultures of K27 were prepared by dissolving 5.1 g of sodium selenate in 100 mL of deionized water then filtering with a Nalgene filter unit using a vacuum-pressure pump (Barnant Company Barrington, IL USA) to facilitate the filtering. Filtering with the Nalgene filter with a 0.2 μm pore size produced a sterile solution. When added to the bioreactor with a total volume of 2.7 L, the concentration of sodium selenate was 10 mM. Sodium selenite stock solutions were prepared by dissolving 4.6 grams of sodium selenite in 100 mL of deionized water and sterile filtering. This resulted in a 10 mM sodium selenite concentration after addition to the bioreactor.

Stock Solutions: Tellurium

Sodium tellurite stock solutions used for amending the batch cultures of K27 were prepared by dissolving 0.629 grams of sodium tellurite in 100 mL of deionized water. This solution was then filter-sterilized as described above. When added to the bioreactor for a total volume of 2.7 L this resulted in a 0.10 mM Na_2TeO_3 concentration.

Flame Atomic Absorption Spectrometry

The atomic absorption spectrometry solutions used as calibration standards were prepared by appropriate dilutions of the commercial selenium atomic absorption standard. This standard contains 1000 ppm selenium as SeO_2 . Calibrations standards were prepared in the 0 ppm Se to 50 ppm Se range with 1% HCl (v/v).

Hydride Generation-Atomic Absorption Spectrometry

Calibration Standards: Selenium

The hydride generation-atomic absorption spectrometry calibration standards were prepared by the appropriate dilutions of the commercial 1000 ppm Se atomic absorption standard and acidified with HCl. The range of calibration standards varied and ranged from 0 ppb Se to 100 ppb Se. A range of HCl concentrations from 1%-50% (v/v) was examined to determine the optimal value.

Calibration Standards: Tellurium

Tellurium calibration standards were produced by the appropriate dilutions of the commercial tellurium atomic absorption standard which contained 1000 ppm Te. Acid concentrations was 50% (v/v). Standards ranged in concentration from 0 ppb Te to 20 ppb Te.

Reducing Solutions

The reducing agent for all hydride generation-atomic absorption spectrometry was sodium borohydride stabilized with sodium hydroxide. The concentration of sodium borohydride needed for optimum production of selenium hydride was a variable tested. The concentrations of sodium

borohydride tested ranged from 0.35% NaBH₄ to 1.5% NaBH₄. The concentration of sodium hydroxide was 0.5% w/w.

Acid Solution for Formation of Selenium Hydride

For the production of selenium hydride by sodium borohydride, the solution must be acidified. Concentrations of 5 M and 10 M HCl were tested.

Instrumentation

Flame Atomic Absorption Spectrometry

A Varian SpectrAA 220 atomic absorption spectrometer was used for all atomic absorption spectrometry and hydride generation-atomic absorption spectrometry. Conditions for flame atomic absorption spectrometry were as follows: lamp current (10 mA), fuel (acetylene), oxidant (air), and flame stoichiometry (highly reducing), Wavelength was 196.0 nm with a slit width of 1.0 nm.

Hydride Generation-Atomic Absorption Spectrometry

A Varian VGA-77 Vapor Generation accessory was used to produce the volatile metalloidal hydrides. When the sample intake tube is placed in the sample, the VGA-77 pumps the sample to the reaction coil where it is acidified and mixed with sodium borohydride pumped from a separate reservoir. The resulting hydride is then measured by atomic absorption upon decomposition in a flame-heated optical cell. Carrier gases used were nitrogen and argon with a flow rate of 100 mL/minute. A Varian Mark V burner was used in conjunction with a fused silica absorption cell. Flow rates of sodium borohydride and hydrochloric acid were 1.0 mL/min to 1.2 mL/min, and these rates were checked periodically. Sample flow rate was 7 mL/min to 10 mL/min. The wavelength used for selenium was 196.0 nm and for tellurium the wavelength was 214.3 nm.

Bioreactor

The bioreactor used for all experimentation was a New Brunswick BioFlow III Batch/Continuous Fermentor (Edison, NJ USA). The bioreactor is connected to a Digital Celebris model 4100 computer that records information from the bioreactor including dissolved oxygen levels, pH, agita-

tion speeds, and temperature. The computer or the bioreactor can control these variables. In all experimentation the bioreactor controlled the temperature at 30 °C and the agitation speed at 200 rpm. The bioreactor and contents were sterilized in a 716 liter autoclave (Wisconsin Aluminum Foundry Co., Inc., Manitowoc, WI USA) before any bioreactor runs.

Procedures

Bioreactor

There were several stages for the preparation of a bioreactor experiment:

- Inoculum Preparation. Two days before the start of an experiment, the preparation of the K27 inoculum was started. A 50 mL aliquot of growth medium was prepared and autoclaved (Tuttnauer USA Co. LTD, NY USA). An isolated culture of *Pseudomonas fluorescens* K27 was transferred from an agar plate to the 50 mL aliquot of sterile media. The top of the flask was double foiled and placed in a water-bath shaker (New Brunswick Scientific Co., Inc., Edison, NJ USA) where it was shaken vigorously and aerobically at 30 °C for 24 hours. The contents of this flask were then transferred in a sterile manner to a 200 mL aliquot of sterilized growth media. This 250 mL aliquot was placed in the water-bath shaker for another 24 hours and incubated aerobically. This was the inoculum for the bioreactor run.
- Preparation of the bioreactor. The bioreactor was disassembled and cleaned with bleach and detergent before each experiment. The bioreactor was reassembled and filled with 2.35 L of growth media and autoclaved. After the bioreactor cooled to room temperature, the K27 bacteria were transferred to it in a sterile manner. Amendments were added in 100 mL aliquots at this time.

Growth Rate Experiments

After the transfer of the bacteria and amendment, the bioreactor was purged for 5 minutes with nitrogen gas to produce anaerobic conditions. A small liquid sample was obtained through the bioreactor's liquid sampling port. This sample was used to measure the optical density and the pH of the culture. The optical density [Spectronic 20D+ Spectrometer at 526 nm (Stone *et*

al., 1998)] and the pH of the sample (Corning Incorporated Science Products Division, Corning, NY USA) were determined. Optical density and pH readings were made every hour during lag and log growth phases until stationary phase was reached; this varied in time from five hours to eight hours.

Headspace gas sampling was done every hour beginning with 0 hours. A gas sample was removed from the space above the bioreactor liquid by using a 1 mL gas-tight syringe through a septum-lined gas sampling port. The gas syringe was inserted into the hot injection port of the gas chromatograph and quickly injected to separate and detect any volatile selenium compounds.

For experiments where the metalloid mass balance was to be determined, no liquid or gas samples were taken during the experiment. The bioreactor was prepared in the same manner as in the growth rate experiments with the addition of gas 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 (A, B & C). Each bubbling tube contained 15 mL of 30% to 3% H₂O₂ with a concentration of 0.5 N NaOH. Nitrogen gas was purged through the bioreactor and through the bubbling tubes at a flow rate of 10 mL/min to 2 mL/min. Even though the flow rate into the bioreactor was controlled with a needle-nose valve, it was difficult to maintain a systematic, controlled flow rate. The error in this setting was approximately 52% to 100%.

At the end of 24 hours (bacteria was then in the stationary growth phase), four 25-mL well-mixed, liquid samples were removed from the bioreactor and transferred to centrifuge tubes. The bioreactor samples were centrifuged at 10000 X *g* for 20 minutes (Rech, 1992). The supernatant was decanted, and the supernatant and solid stored at 5 °C until further processing.

Flame Atomic Absorption Spectrometry (FAAS)

The linear range tested for FAAS was 0 ppm to 100 ppm Se. A linear relationship existed from 0 ppm Se to 50 ppm Se. This range was used for the remainder of FAAS experiments. All samples and standards contained 1% HCl (v/v). FAAS detects selenium in the VI oxidation state. Several methods were tested to determine the optimum oxidation methods for detection of selenium. Known amounts of selenium in differing oxidation states were oxidized to Se(VI) by either boiling

in concentrated nitric acid or boiling in hydrogen peroxide.

Nitric acid was added in a 10 mL aliquot to a selenium sample, and the sample was boiled to dryness. Another 10 mL aliquot of nitric acid was added, and again, the sample boiled to dryness. The sample was diluted to a concentration within the calibration range.

Hydrogen peroxide was added to a selenium sample. The sample was boiled until fine bubbles that represent hydrogen peroxide degradation disappeared. The sample was diluted to the original volume then diluted to a concentration with the calibration range.

Hydride Generation-Atomic Absorption Spectrometry (HGAAS)

Hydride generation-atomic absorption spectrometry detects selenium in $\mu\text{g/L}$ concentrations (ppb). The calibration range for HGAAS was determined to be 0 ppb to 20 ppb Se. The oxidation state of selenium must be IV for the formation of the hydride to occur. Processes had to be developed to find the optimum oxidation and reduction methods for the matrix and bacteria used in these experiments. Several methods used in HGAAS have been reported (Dedina and Tsalev, 1995; American Public Health Association, 1989; Zhang and Frankenberger, Jr., 1999; Reddy *et al.*, 1995; Terry *et al.*, 1992; Narasaki and Ikeda, 1984; Martens and Suarez, 1997; Cutter, 1978; Bujdos *et al.*, 2000; Zhang and Frankenberger, Jr., 2000; Cooke and Bruland, 1987; Zhang *et al.*, 1999; Manning and Bureau, 1995; Diaz *et al.*, 1996; Blaylock and James, 1993; Thompson-Eagle and Frankenberger, Jr., 1990; Krivan *et al.*, 1985). These methods were first tested with known quantities of selenium and optimized methods applied to samples retrieved from the bioreactor. Oxidation methods using either nitric acid or hydrogen peroxide, and variables such as boiling or not boiling, boiling times, etc., were tested.

Several reduction methods were examined and applied to known quantities of selenium. These methods were then tried on selenium obtained from the bioreactor. Again variables such as length of boiling, capped or uncapped test tubes while boiling, etc. were examined. The sequence of oxidation, dilutions, and reduction was also a variable. The methods attempted and the variables tested are listed in the results section.

Tellurium

Bioreactor

The preparation of the bioreactor for the tellurium experiments was the same as for the selenium experiments except that no attempts were made to trap any headspace gases in bubbling tubes. The condensing unit of the bioreactor was attached to a flask by way of tubing. The flask contained bleach water to prevent any organotellurium compounds from escaping into the lab environment. All these experiments were amended with 0.1 mM sodium tellurite.

Hydride Generation-Atomic Absorption Spectrometry

Hydride generation-atomic absorption spectrometry can be used to detect tellurium in the range of 0 ppb to 20 ppb. The oxidation state of tellurium must be IV before the tellurium hydride can be formed. The concentration of the sodium borohydride (reducing solution) was 0.35% stabilized with 0.5% sodium hydroxide. The acid solution used in the formation of the hydride was 6 N hydrochloric acid. The purge gas was either nitrogen or argon.

Methods of oxidation and reduction were tried on samples with known quantities of tellurium. These processes were then tested on biological samples containing tellurium.

Chapter III

Results

Control *Pseudomonas fluorescens* K27 Experiments

Bioreactor experiments of non-amended (containing no selenium) *Pseudomonas fluorescens* K27 were carried out to establish growth rate values, pH ranges, and production of headspace gases. To determine the mass balance of the selenium added, the amount of selenium produced in the headspace gases had to be quantified. Nitrogen gas was used to purge the bioreactor, sweeping any selenium containing headspace gases into the bubbling tubes where these compounds would be trapped and oxidized to selenate. The effect of purging with nitrogen gas on the growth of un-amended K27 bacteria was investigated (Figure 1).

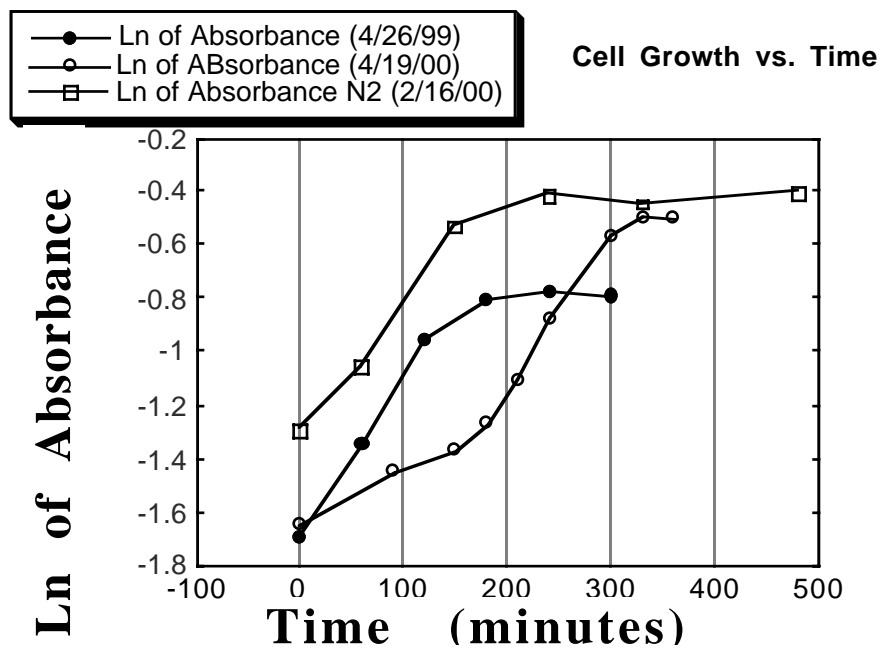


Figure 1. Cell growth vs. time of the K27 bacterium with no amendments added. N₂ means bioreactor purged with nitrogen gas.

The specific growth rates of the control experiments were calculated to determine if purging with nitrogen gas during the time course of the experiment had an effect on the bacterial growth rate.

Specific growth rates (SGR, slope of \ln optical density vs. time plot) were determined in the exponential growth phase in all experiments (Table I).

Table I

Comparison of specific growth rates of non-amended bioreactor cultures.

Date of Bioreactor Experiment	Specific Growth Rate h^{-1}
04/26/99 no purging	0.37
04/19/00 no purging	0.33
2/23/00 continuous N_2 purge	0.31
Previous Research no purging (Erikson, 1999)	0.30

The pH of the non-amended or control K27 bioreactor was measured throughout the lag, log and stationary phases of growth. These experiments where the pH is not set or controlled are called pH drift experiments. Again, the effect of purging with nitrogen gas was investigated (Figure 2).

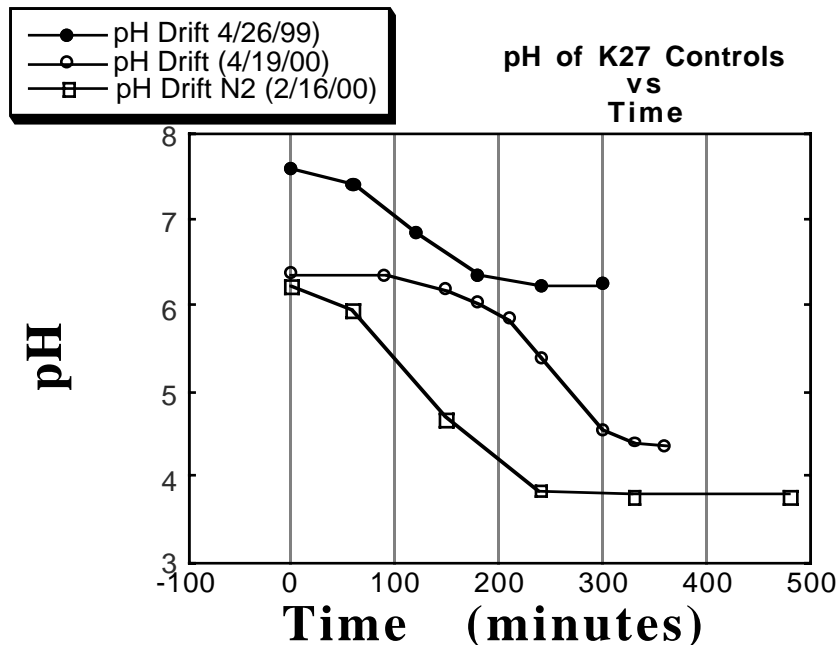


Figure 2. pH vs. time of control bioreactor experiments.

Sodium Selenite Amended *Pseudomonas fluorescens* K27 Experiments

The pH and specific growth rate of *Pseudomonas fluorescens* K27 amended with a final concentration of 10 mM Na₂SeO₃ were determined for both N₂ purged and non-purged bioreactors. The specific growth rates are shown in Table II, and the graphs of ln of optical density vs. time are in Figure 3. The change in the pH in the bioreactor with time is displayed in Figure 4.

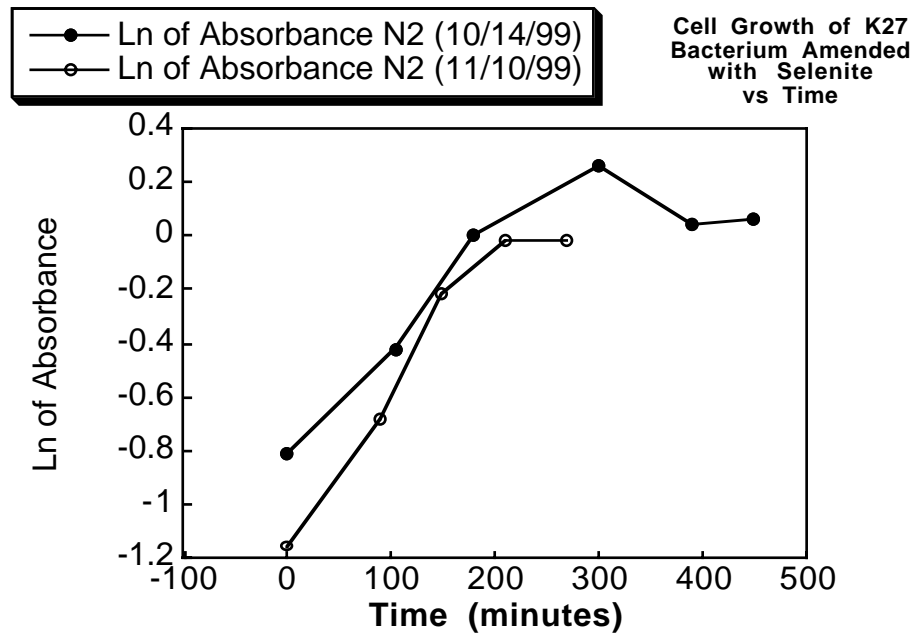


Figure 3. Comparison of the cell growth in *Pseudomonas fluorescens* K27 amended with 10 mM Na₂SeO₃ vs. time.

Table II

Comparison of specific growth rate (h⁻¹) of 10 mM Na₂SeO₃ amended bioreactor cultures.

Bioreactor Experiment	Specific Growth Rate h ⁻¹
09/22/99 10 mM Na ₂ SeO ₃ no purging	0.10
10/14/99 10 mM Na ₂ SeO ₃ continuous N ₂ purging	0.19
11/10/99 10 mM Na ₂ SeO ₃ continuous N ₂ purging	0.34
Previous Research amended but no purging (Erikson, 1999)	0.19

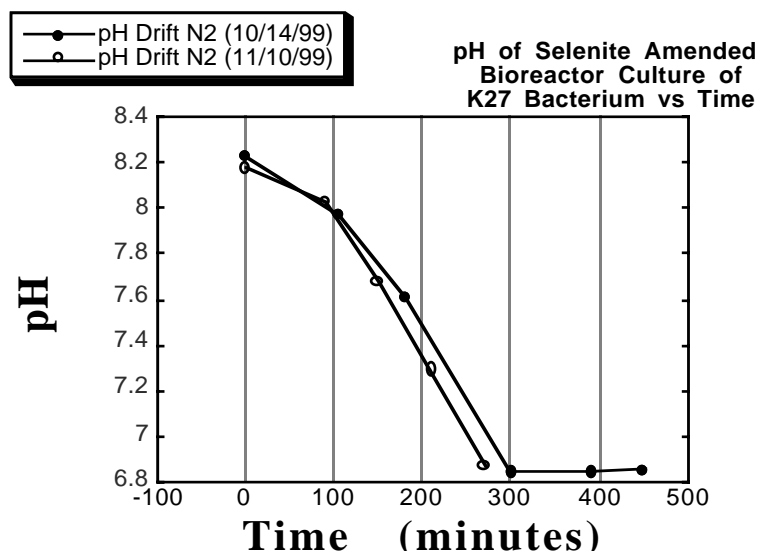


Figure 4. pH of a 10 mM Na_2SeO_3 amended K27 bioreactor culture vs. time.

Atomic Absorption Spectrometry

Varian (1989) states that 1.00 g of selenium metal dissolved in 80 mL of 50% (v/v) nitric acid diluted to 1 liter produces a selenium standard for atomic absorption spectrometry. The treatment of selenium metal with nitric acid produces the selenate oxyanion. The optimum working range is 5-250 $\mu\text{g}/\text{mL}$ (ppm) as per Varian.

The standards used for the atomic absorption spectrometry were produced by using a 1000 ppm Se atomic absorption standard in 1% (v/v) HCl. Dilutions were made to produce standards with the concentration range of 0 ppm to 50 ppm Se with 1% (v/v) of HCl.

Atomic absorption spectrometry with nitric acid as the oxidant has been reported as useful in the detection of selenium in biological samples (Lortie *et al.*, 1992). Several techniques were used to increase the accuracy of this detection method (Table III).

Hydrogen peroxide was used in the bubbling tubes of the bioreactor apparatus (Weres *et al.*, 1989). Hydrogen peroxide oxidizes the organoselenium compounds to Se(VI). Experiments were performed that tested hydrogen peroxide as the oxidant (Table III). Percentage error was calculated as $100 - [(\text{determined}/\text{known}) \times 100]$.

A sample from a bioreactor experiment which was amended to a 10 mM Na_2SeO_3 final concentration was analyzed by atomic absorption spectrometry. The results are listed in Table IV.

Table III

Oxidation methods used to achieve selenate oxyanion to be analyzed by atomic absorption spectrometry.

Sample	Oxidant	Method	Results		
			Known Conc. ppm	Determined Conc. ppm	% Error
Se(0)	Nitric Acid	Add 10 mL of nitric acid & boil to dryness; add 10 mL of nitric acid & boil to dryness then dilutions to ppm range. Boil uncovered.	25	4.6	82
Se(0)	Nitric Acid	Add 10 mL of nitric acid & boil to dryness; add 10 mL of nitric acid & boil to dryness then dilutions to ppm range. Boil in covered beaker.	25	0.7	97
Se(0)	Nitric Acid	Boil but not to dryness	30	26.8	11
Se(IV)	Nitric Acid	Boil but not to dryness	45	33.7	25
Se(VI)	Nitric Acid	Boil but not to dryness	15	14.1	6
Se(IV)	30% H ₂ O ₂	Boil until H ₂ O ₂ bubbles disappear	25	25	0
Se(IV)	15% H ₂ O ₂	Boil until H ₂ O ₂ bubbles disappear	25	27.4	10
Se(IV)	5% H ₂ O ₂	boil until H ₂ O ₂ bubbles disappear	25	28	12

The amount of selenium detected was total selenium; no quantification of the various oxidation states of selenium could be attempted because all selenium was oxidized to Se(VI).

Bubbling tubes were attached by silicon tubing to the end of the condensing unit of the bioreactor. Headspace gases which include organoselenium compounds were swept into the tubes by purging the bioreactor with nitrogen gas. The hydrogen peroxide oxidized the organoselenium compounds to selenate (Zhang and Frankenberger, 2000) which was then detected by atomic absorption spectrometry. These experimental results are shown in Table V.

Table IV

Fluid from a 10 mM Na₂SeO₃ bioreactor experiment analyzed by atomic absorption spectrometry for selenium in liquid and solid phase combined.

Sample	Oxidation	Method	Results		
			Bioreactor Conc. Known ppm	Bioreactor Conc. Determined ppm	% Recovery
10 mM SeO ₃ ²⁻	Nitric Acid	Boil but not to dryness	789.6	754.2	95

The results of the bubbling tube (trapping) analysis were inconsistent with expected results, i.e., Se content of tube A (the first in the series) should be greater than the Se content of tube B and the Se content of tube B should be greater than the Se content of tube C because the purge gas entered tube A then tube B then tube C. The oxidizing power of 30% hydrogen peroxide was tested with various concentrations of dimethyl diselenide (CH₃SeSeCH₃) that was pipetted directly into 100 mL of 30% hydrogen peroxide (Table VI).

These samples were stored in the lab at room temperature. Over various time periods, these samples were read by atomic absorption spectrometry. The results are listed in Table VII.

The results of Se(VI) determination by atomic absorption spectrometry had large analytical error ranges. Detection of Se(IV) by atomic absorption spectrometry was attempted. Selenium as Se(-II) (dimethyl diselenide) was oxidized to selenate then reduced to selenite (Weres *et al.*, 1989) and analyzed by atomic absorption. Selenium as Se(IV) (selenite) was treated by boiling with 12 N HCl and 2% (NH₄)₂S₂O₈ and determined by AAS (Table VIII).

Hydride Generation-Atomic Absorption Spectrometry

Tellurium by HGAAS

The oxidation state required for detection by hydride generation-atomic absorption spectrometry is IV for tellurium. Dedina and Tsalev (1995) used many of the same oxidation and reduction

Table V

Selenium concentration of bubbling tubes.

Experiment	Method	Results		
		Tube	Determined by AA ppb	Calculated Se in Each Tube (mg)
10 mM Na ₂ SeO ₃ pH 6.45 53 hours N ₂ purge	30% H ₂ O ₂ Recycled tubing	A	143.75	2.156
		B	1.2	0.0180
		C	0.5	0.00750
10 mM Na ₂ SeO ₄ pH drift 27.5 hours N ₂ purge	30% H ₂ O ₂ Recycled tubing	A	0.4	0.00600
		B	0.1	0.00150
		C	0.7	0.0105
10 mM Na ₂ SeO ₄ pH 7.55 56.5 hours N ₂ purge	30% H ₂ O ₂ 1% HCl Recycled tubing	A	8.8	0.0133
		B	0.3	0.00450
		C	0.2	0.00300
No amendments pH drift 51 hours N ₂ purge	30% H ₂ O ₂ 1% HCl Tubes (A,B &C) cleaned with bleach & rinsed/recycled tubing	A	2.7	0.0409
		B	0.3	0.00450
		C	0.1	0.00150
No amendments pH drift 51 hours N ₂ purge	30% H ₂ O ₂ 1% HCl Tubes (A,B &C) cleaned with bleach & rinsed/new tubing	A	2.8	0.0424
		B	3.9	0.0591
		C	3.0	0.0454
10 mM Na ₂ SeO ₃ pH drift 51.5 hours N ₂ purge	30% H ₂ O ₂ 1% HCl Tubes (A, B & C) cleaned with bleach & rinsed/new tubing	A	1.6	0.0242
		B	0.4	0.00610
		C	0.7	0.0106
10 mM Na ₂ SeO ₄ pH 6.24	30% H ₂ O ₂ 1% HCl	A	0.3	0.00450
		B	0.1	0.00150

Table VI

Known quantities of dimethyl diselenide oxidized with 30% hydrogen peroxide and determined by AAS.

Known Concentration ppm	Determined Concentration ppm	% Error
40	23	42
30	21	30
10	3.3	67
0	1.2	

methods for tellurium and selenium. Methods that were tested on selenium were applied to bioreactor samples that were amended with tellurium. Two methods of reduction were tested: 1) 5 mL of sample plus 5 mL of 12 N HCl boiled in a hot water bath in loosely capped test tubes for 30 minutes (labeled $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{wo}$); 2) 5 mL of sample plus 5 mL of 12 N HCl plus 0.2 mL of 2% ammonium persulfate boiled in a hot water bath in loosely capped test tubes for 30 minutes (labeled $(\text{NH}_4)_2\text{S}_2\text{O}_8/\text{w}$). The benefits of boiling versus not boiling were tested. The results are presented in Table IX.

The operating parameters used in the determination of tellurium by hydride generation-atomic absorption are presented in Table X.

Four separate bioreactor experiments were completed. Each experiment was amended with Na_2TeO_3 for a final concentration of 10 mM Te. A 25 mL-sample of bioreactor fluid was centrifuged as stated previously. The solid and liquid phases were separated by decantation.

Nitric acid was used as the oxidant for each experiment. The solid phase was oxidized by adding 1 to 2 mL of concentrated nitric acid and gently heating until the pellet dissolved. This solution of tellurate was then diluted to 100 mL with deionized water. Appropriate dilutions were then made until the approximate concentration of tellurium was within the calibration range of the HGAAS.

The oxidation of tellurium in the liquid phase was achieved by taking a 0.1 mL sample of the liquid phase and diluting to 50 mL with deionized water. An 18 mL sample of this solution was removed and 2 mL of concentration nitric acid was added to it. This solution was boiled and then

Table VII

Known quantities of dimethyl diselenide oxidized with 30% hydrogen peroxide over several different time spans and analyzed by AAS.

Sample	AA (1 hour)		AA (45 hours)		AA (117 hours)		AA (381 hours)	
	Conc.	%Error	Conc.	%Error	Conc.	%Error	Conc.	%Error
40 ppm	23.1	42	25.6	36	26.6	33	28.5	29
30 ppm	21.0	30	23.9	20	25.2	16	27.2	9.3
10 ppm	3.3	67	4.3	57	2.7	73	1.8	82
0 ppm	1.2		2.3		0.8		0.0	

Table VIII

Known quantities of Se(IV) detected by atomic absorption spectrometry.

Sample	Oxidation	Reduction	Results	
			Known Sample (ppm)	Determined (ppm)
Dimethyl diselenide	H ₂ O ₂	5 mL sample		
		5 mL 12 N HCl		
		0.2 mL 2% (NH ₄) ₂ S ₂ O ₈	39.2	57.2
Sodium selenite	None		20.0	6.12
			10.0	6.12
		5 mL sample	40.0	35.6
		5 mL 12 N HCl	19.6	16.8
		0.2 mL 2% (NH ₄) ₂ S ₂ O ₈	9.8	9.8
		36.1	35.9	
				11
				14
				0.0
				0.5

Table IX

Oxidation and reduction methods tested on tellurium amended bioreactor fluid.

Sample	Oxidation	Reduction	Results		
			Tube	Boiled Conc. (mg)	Not Boiled Conc. (mg)
0.1 mM Na ₂ TeO ₃ solid phase	2 mL nitric acid boiling vs. non boiling	5 mL Sample 5 mL 12 N HCl 0.2 mL of 2% ammonium persulfate	C	0.0156	0.0110
			C'	0.0081	0.0095
0.1 mM Na ₂ TeO ₃ solid phase	2 mL nitric acid	5 mL Sample 5 mL 12 N HCl 0.2 mL of 2% ammonium persulfate (5/10.2) vs. 5 mL Sample 5 mL 12 N HCl (5/10)	Tube	(NH₄)₂S₂O₈ /w	(NH₄)₂S₂O₈ /wo
			C	0.1397	0.1208
			C'	0.1491	0.1527

Table X

Optimized conditions for the detection of tellurium by hydride generation-atomic absorption spectrometry.

Parameter	Optimal Setting
Oxidation State	Te(IV)
Oxidation Method	nitric acid
Reduction Method	5 mL sample 5 mL 12 N HCl
Reducing Solution	0.2 mL 2% (NH ₄) ₂ S ₂ O ₈ 0.35% NaBH ₄ 0.50% NaOH
Acid Solution	6 N HCl
Standard Range	0 ppb to 20 ppb
Acid Concentration of Standards and Samples	50% HCl

diluted to 20 mL. Appropriate dilutions were then made until the concentration of tellurium was within the calibration range of HGAAS.

Two methods of reduction were used. One method was 5 mL of sample plus 5 mL of 12 N HCl boiled in a hot water bath in loosely capped test tubes for 30 minutes (labeled (NH₄)₂S₂O₈/wo). The second method of reduction was the same as above but 0.2 mL of 2% ammonium persulfate was added (labeled (NH₄)₂S₂O₈/w).

In Table XI, each bioreactor experiment is listed by date and the amount of tellurium added. More than one sample from a specific bioreactor experiment was analyzed. Percent recovery was calculated by (determined/known) x 100.

Since hydride generation-atomic absorption spectrometry is specific for the IV oxidation state it should be possible to detect all oxidation states present in a sample (Cutter, 1985). These principles should apply to tellurium.

Manipulation of these results will give the amount of tellurium in each oxidation state:

$$(\text{Te}^{6+} + \text{Te}^{4+}) - \text{Te}^{4+} = \text{Te}^{6+}$$

$$\text{Te}_{\text{total}} - (\text{Te}^{6+} + \text{Te}^{4+}) = \text{Te}^0$$

One attempt was made to determine each oxidation state of tellurium present in a 0.1 mM Na₂TeO₄·2H₂O amended bioreactor experiment. The oxidation step used nitric acid and the reduction step used concentrated hydrochloric acid and ammonium persulfate. The results are presented in Table XII.

Table XI

0.1 mM Na₂TeO₃ bioreactor fluid: Amount of Te left in supernatant vs. amount in solid phase (Te⁰ or Te on/or in cells).

07/31/00: 0.0598 g Na ₂ TeO ₃ (0.319 mg/25 mL Te) 72 hours	Tube A Oxidation step includes boiling		
	Phase	Te (mg)	% in Phase
	Solid	0.0156	17
	Liquid	0.0749	83
	Total/ 25 mL	0.0906	100
	% Error = 71.6		% Recovery = 28.4
	Tube A Oxidation step does not include boiling		
	Phase	Te (mg)	% in Phase
	Solid	0.0110	13
	Liquid	0.0749	87
Total/ 25 mL	0.0859	100	
% Error = 73		% Recovery = 27	
Tube B Oxidation step includes boiling			
Phase	Te (mg)	% in Phase	
Solid	0.00806	5	
Liquid	0.155	95	
Total/25 mL	0.163	100	
% Error = 49		% Recovery = 51	

Selenium by HGAAS

The oxidation state of selenium detected by hydride generation-atomic absorption spectrometry is IV. The linear range of calibration standards, concentration of reducing agent, acid levels of standards, and oxidation/reduction methods were optimized for this method.

In Table XIII, the acid concentration of the samples was tested for optimum detection of selenium. The standards were prepared by the appropriate dilutions of the atomic absorption standard and acidified to 1% (v/v) HCl. The samples were prepared by the appropriate dilutions of the atomic absorption standard and acidified from 1% (v/v) to 50% (v/v) HCl.

The linear range for the calibration standards was determined by examining two Se ranges, 0 - 25 and 0 - 50 ppb Se. The standards were produced by diluting the atomic absorption standard to the appropriate concentration and acidifying to 1% (v/v) HCl (Table XIV).

Table XI continued

0.1 mM Na₂TeO₃ bioreactor fluid: Amount of Te left in supernatant vs. amount in solid phase (Te⁰ or Te on/or in cells).

Experiment	Results		
07/31/01: 0.0598 g Na ₂ TeO ₃ continued	Tube B Oxidation step does not include boiling		
	Phase	Te (mg)	% in Phase
	Solid	0.00949	6
	Liquid	0.155	94
	Total/25 mL	0.164	100
	% Error = 49	% Recovery = 51	
	<hr/>		
08/01/00: 0.0618 g Na ₂ TeO ₃ (0.324g mg/25 mL) 72 hours	Tube A Reduction includes ammonium persulfate		
	Phase	Te (mg)	% in Phase
	Solid	0.149	37
	Liquid	0.236	63
	Total/25 mL	0.385	100
	% Error = 19	% Recovery = 119	
	Tube A Reduction without ammonium persulfate		
	Phase	Te (mg)	% in phase
	Solid	0.121	34
	Liquid	0.236	66
	Total/25 mL	0.357	100
	% Error = 10	% Recovery = 110	
	Tube B Reduction includes ammonium persulfate		
	Phase	Te (mg)	% in Phase
	Solid	0.149	39
Liquid	0.236	61	
Total/25 mL	0.385	100	
% Error = 19	% Recovery = 119		
Tube B Reduction without ammonium persulfate			
Phase	Te (mg)	% in Phase	
Solid	0.153	39	
Liquid	0.236	61	
Total/25 mL	0.389	100	
% Error = 20	% Recovery = 120		
<hr/>			
08/08/00: 0.0629 g Na ₂ TeO ₃ (0.366 mg/25 mL Te) 72 hours	Tube A		
	Phase	Te (mg)	% in Phase
	Solid	0.0520	19
	Liquid	0.226	81
	Total/25 mL	0.278	100
	% Error = 24	% Recovery = 76	

Table XI continued

0.1 mM Na₂TeO₃ bioreactor fluid: Amount of Te left in supernatant vs amount in solid phase (Te⁰ or Te on/or in cells).

Experiment	Results		
08/08/00: 0.0629 g Na ₂ TeO ₃ (0.366 mg/25 mL Te) 72 hours	Tube A		
	Phase	Te (mg)	% in Phase
	Solid	0.0552	20
	Liquid	0.226	80
	Total/25 mL	0.281	100
	% Error = 23	% Recovery = 77	
	Tube B		
	Phase	Te (mg)	% in Phase
	Solid	0.0483	16
	Liquid	0.246	84
	Total/25 mL	0.294	100
	% Error = 20	% Recovery = 80	
	Tube B		
	Phase	Te (mg)	% in Phase
Solid	0.0514	17	
Liquid	0.246	83	
Total/25 mL	0.297	100	
% Error = 19	% Recovery = 81		
08/16/01: 0.0678 g Na ₂ TeO ₃ (0.3615 mg/25 mL) 72 hours	Tube A		
	Phase	Te (mg)	% in Phase
	Solid	0.202	54
	Liquid	0.174	46
	Total/25 mL	0.376	100
	% Error = 4	% Recovery = 104	
	Tube B		
	Phase	Te (mg)	% in Phase
	Solid	0.106	26
	Liquid	0.307	74
	Total/25 mL	0.413	100
	% Error = 14	% Recovery = 114	

Table XII

Speciation of tellurium compounds in a 0.1 mM Na₂TeO₄·2H₂O bioreactor experiment.

Oxidation State	Concentration (ppm)	Percent of Each Oxyanion Present in Sample
Tellurite	13.4	59.5
Tellurite + tellurate	15.0	
Total tellurium	22.5	
Tellurate (tellurite + tellurate - tellurite)	1.61	7.1
Tellurium (total tellurium - tellurite - tellurate)	7.49	33.3
Tellurium added	13.5	
% Recovery	167	

The concentration of reducing agent was determined by testing the same calibration standards under different concentrations of the sodium borohydride solution (Table XV).

For vapor generation work, Varian (1997), the manufacturer of the instrument, suggested that using the background correction was not necessary. The standards were used as samples, and they were tested with the background correction on and then with the background correction off (Table XVI).

There are several selenium reduction methods reported for use with HGAAS (Standard Methods for the Examination of Water and Wastewater, 1989; Dedina and Tsalev, 1995). Many of these methods were tested and the results are presented in Table XVII.

The reduction method that gave the best results was the method that employed 5 mL of sample plus 5 mL of 12 N HCl with 0.2 mL of 2% ammonium persulfate then boiling. This method was further refined by testing the reducing power in loosely capped tubes versus uncapped tubes. Also, the time of boiling in regards to reduction was tested (Table XVIII).

The following important question was posed: Do mixtures of selenate and selenite oxyanions interfere with the detection of the selenite oxyanion by HGAAS? Table XIX addresses this question.

Table XIII

The acid concentrations of sample solutions to determine optimum determination of selenium by HGAAS.

Test Date	Known ppb	%HCl v/v	Determined ppb	%Error
05/24/00	25	1	10.8	57
	25	10	11.8	53
	25	20	12.4	50
	25	30	34.0	36
	25	40	17.0	32
	25	50	15.8	37
06/01/00	50	1	47.2	5.6
	40	1	25.1	37
	30	1	17.2	43
	20	1	12.2	39
	10	1	6.1	39
	5	1	2.8	44
	25	1	19.5	22
	25	10	17.5	30
	25	20	19.4	22
	25	30	19.9	20
	25	40	20.5	18
25	50	19.2	23	

Pseudomonas fluorescens K27 reduces selenite to elemental selenium and to volatile, organoselenium compounds. HGAAS cannot detect these compounds so methods of oxidation to selenate then reduction to Se(IV) were developed to allow application of HGAAS for these samples. Nitric acid has been used as an oxidant in atomic absorption (Lortie *et al.*, 1992), in colorimetric determination (Magin *et al.*, 1960), and in HGAAS (Martens and Suarez, 1997). Hydrogen peroxide as an oxidant has reported in the literature (Weres *et al.*, 1989; Zhang and Frankenberger, Jr., 2000; Zhang *et al.*, 1999; Reddy *et al.*, 1995; Terry *et al.*, 1992; Manning and Burau, 1995). Both of these oxidizing agents were tested and the results are compared in Table XX (a, b, c, and d).

Table XIV

Determination of the linear range of calibration standards for hydride generation-atomic absorption spectrometry and optimum concentration of the reducing solution.

Known Concentration ppb	Standard Range 0-40 ppb Se		Standard Range 0-25 ppb	
	Determined ppb	%Error	Determined ppb	%Error
	40	36.4	9	-
30	28.2	6	-	-
25	25.8	3	-	-
20	21.6	8	20.9	4
15	16.1	7	14.8	1
10	12.6	26	11.3	13
5	7.75	55	6.1	22
23	35.3	53	>25	-
11	19.3	75	16.2	47
1	4.1	310	2.3	130
6	12.3	105	9.5	175
17	28.9	70	24	41

The steps outlined below were determined to be the analytical method that gave the best percent recoveries when tested on K27 bioreactor cultures amended with 10 mM Na₂SeO₃. After the bacteria reached stationary phase, 25 mL samples were removed. These samples were then centrifuged and the solid and liquid phases were separated by decantation. The liquid samples were treated as follows: A 0.75 mL aliquot was removed then diluted to 100 mL with deionized water. This sample was then oxidized with 6% hydrogen peroxide by boiling in a covered beaker for 45 minutes. The sample was diluted to 30 mL, and 1.0 mL of this solution was diluted to a total volume of 100 mL. Six 5 mL-aliquots were then reduced; three by boiling in loosely capped test tubes with 5 mL of 12 N HCl, and the other 3 samples were boiled in loosely capped test tubes with 5 mL of 12 N HCl and 0.2 mL of 2% ammonium persulfate. These samples were analyzed by HGAAS.

For the solid samples, 1 to 2 mL of concentrated nitric acid was added and the sample was heated gently until the sample dissolved. This oxidized sample was diluted to 100 mL with deionized water. A 0.1 mL aliquot was diluted to 100 mL with deionized water. Six 5 mL-portions of this sample were reduced by the above reduction methods.

Table XV

Comparison of 1.5% (w/w) NaBH₄ and 0.6% (w/w) NaBH₄ solutions in the determination of selenium concentration.

Known Concentration ppb	1.5% Sodium Borohydride		0.6% Sodium Borohydride	
	Determined ppb	% Error	Determined ppb	% Error
20	20.3	2	21.6	8
15	14.8	1	14.9	0.7
10	11.5	15	11.1	11
5	6.5	30	5.7	14
11	16.1	46	16.3	48
1	2.5	150	2.1	110
6	9.5	58	9.5	58
17	24.2	42	23.9	40

Results of these optimized methods applied to bioreactor cultures are presented in Table XXI. Calcium chloride was added to only one of these experiments to evaluate the report (Lueschow and Mackenthun, 1962) that this would minimize volatilization of selenium during the oxidation step.

The operating conditions for the optimum detection of selenium by HGAAS are presented in Table XXII.

Table XVI

Testing of calibration standards with background correction on then off.

Known Concentration ppb	Background Correction On		Background Correction Off	
	Determined ppb	% Error	Determined ppb	% Error
15	19	26.7	14.8	1.3
10	13	30	9.9	1
5	6.6	32	4.2	16

In experiments where the H₂O₂ oxidation of selenium occurred directly before the reduction step, bubbling of the sample occurred when the hydrochloric acid was added. This indicated that the hydrogen peroxide degradation was not complete. Mauzanner *et al.* (2001) described a method to

Table XVII

Various reduction methods applied to samples to test detection by hydride generation-atomic absorption spectrometry.

Sample n=3	Method	Results		
		Known ppb	Deter- mined ppb	% Error
Selenate in deionized water	15 mL 12 N HCl + 30 mL sample : 4 N HCl final concentration then boil to dryness	5.8	4.96	14
	15 mL 12 N HCl + 15 mL sample: 6 N HCl final concentration then boil for 10 minutes	4.4	-5.21	218
Selenate in deionized water	5 mL 12 N HCl + 5 mL sample + 0.2 mL 2% (NH ₄) ₂ S ₂ O ₈ then boil for 30 minutes	49	48.9	0.2
	15 mL 12 N HCl + 15 mL sample then boil in beaker for 15 minutes	49.97	73.8	47.7
	15 mL 12 N HCl + 30 mL sample then boil in beaker for 30 minutes	66.6	24.4	63.4
	5 mL 12 N HCl + 5 mL sample then autoclave for 121 °C for 60 minutes	49.97	22.9	54
Mixture of selenate and selenite	15 mL 12 N HCl + 15 mL sample then boil in covered beaker for 30 minutes	8.89	0.76	91.45
	15 mL 12 N HCl + 15 mL sample then boil in uncovered beaker for 20 minutes	8.89	4.85	45.4
	5 mL 12 N HCl + 5 mL sample in loosely capped test tubes boiled for 30 minutes in hot water bath/tested within 1 hour of reduction	8.89	10.04	12.9
	5 mL 12 N HCl + 5 mL sample in loosely capped test tubes boiled for 30 minutes in hot water bath/tested within 24 hours of reduction	8.89	11.64	30.9
	5 mL 12 N HCl + 5 mL sample in loosely capped test tubes then autoclaved for 60 minutes @ 121 °C	8.89	2.51	71

Table XVIII

Reduction methods tested on a mixture of selenate and selenite.

Sample n=3	Method	Results			
		Known Conc. ppb	Deter- mined ppb	% Error	
Mixture of selenate and selenite	5 mL 12 N HCl + 5 mL sample in loosely capped test tubes boiled:	10 minutes	8.89	12.05	35.5
		20 minutes	8.89	10.6	19.6
		30 minutes	8.89	11.07	24.6
		40 minutes	8.89	9.29	4.49
		50 minutes	8.89	9.24	3.9
		Mixture of selenate and selenite	5 mL 12 N HCl + 5 mL sample in uncapped test tubes boiled:	10 minutes	8.89
		20 minutes	8.89	9.4	5.74
		30 minutes	8.89	8.89	0.0
		40 minutes	8.89	8.53	4.05
		50 minutes	8.89	9.52	7.1

determine the concentration of hydrogen peroxide in a sample by using a spectrophotometer. The wavelength used was 240 nm and the extinction coefficient was 43.7 M/cm. Standards and samples containing various concentrations of hydrogen peroxide were prepared and the absorbance determined by UV/VIS spectrophotometer (Jasco V-550). The results were deemed not acceptable because percent errors ranged from -26.08 to 35.29. Each sample and standard was then scanned to find the optimum wavelength for absorbance. The optimum wavelength for each solution was 258 nm. The solutions and standards were tested again but at wavelength 258 nm.

Fresh standards and eight samples containing 6% hydrogen peroxide were prepared. Four of the hydrogen peroxide solutions also contained 0.05 N sodium hydroxide, a reagent suggested by others (Zhang and Frankenberger, 2000; Thompson-Eagle and Frankenberger, Jr., 1990; Weres *et al.*, 1989; Terry *et al.*, 1992) to cause faster H₂O₂ decomposition. The method presented in the literature

Table XIX

Testing mixtures of selenate and selenite to detect selenium in Se(IV).

Sample	Results		
	Known	Determined	%
	Conc. of Selenite ppb	ppb	Error
Mixture of selenate (1.85 ppb) and selenite (10 ppb) n=3	10	10.68	6.8
Mixture of selenate (1.85 ppb) and selenite (10 ppb) n=3	10	11.67	16.7
Mixture of selenate (1.5 ppb) and selenite (1.56 ppb) n=3	1.56	1.19	23.7

to degrade hydrogen peroxide was to boil the sample until the fine bubbles of hydrogen peroxide disappear. This method plus boiling for a specific time period were tested and the results are presented in Table XXIII.

Table XX a

The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the affect of dilutions on the detection of selenium.

Sample	Method	Results		
		Known Conc. ppb	Deter- mined ppb	% Error
Mixture of selenite and selenate; selenate; selenite; blank; All solutions are 1% TSN3	Selenate + Selenite	14.93	17.8	19.2
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate			
	Selenate	14.86	>20	
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate		>20	
	Selenite	15.01	16.51	8.6
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate		>20	
	Blank	0	6.34	
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate		16.10	
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate			

Table XX b

The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the effect of dilutions on the detection of selenium.

Sample	Method	Results		
		Known Conc. ppb	Deter- mined ppb	% Error
Mixture of selenite and selenate; selenate; selenite; blank; All solutions are 1% TSN3	Selenate + Selenite	14.93	17.8	19.2
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate			
	Selenate	14.86	>20	
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate		>20	
	Selenite	15.01	16.51	8.6
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate		>20	
Blank		0	6.34	
	Dilution 1; dilution 2; oxidation with hydrogen peroxide then reduction with ammonium persulfate		16.10	

Table XX c

The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the effect of dilutions on the detection of selenium.

Sample	Method	Results		
		Known Conc. ppb	Deter- mined ppb	% Error
Selenite in TSN3;	Selenite in TSN3	14.8	16.45	11
blank TSN3,	Dilute then oxidize with			
selenite in TSN3;	hydrogen peroxide then dilute			
deionized water	then reduction without			
	ammonium persulfate			
	Selenite in TSN3	14.5	18.97	30.8
	Dilute then oxidize with			
	hydrogen peroxide then dilute			
	then reduction with ammonium			
	persulfate			
	TSN3	0	-2.01	
	Dilute then oxidize with			
	hydrogen peroxide then dilute			
	then reduction without			
	ammonium persulfate			
	Deionized water	0	-1.47	
	Dilute then oxidize with			
	hydrogen peroxide then dilute			
	then reduction without			
	ammonium persulfate			
	Selenite in TSN3: no oxidation	5.4	6.04	11.8
	nor reduction			

Table XX d

The results of oxidizing selenium compounds with either nitric acid or hydrogen peroxide and the effect of dilutions on the detection of selenium.

Sample	Method	Results		
		Known Conc. ppb	Deter- mined ppb	% Error
Se(0); mixture of selenate + selenite; selenate; selenite; TSN3	Se(0)	10	15.97	59.7
	Oxidize with hydrogen peroxide then reduction without ammonium persulfate			
	Selenite + Selenate	15.04	19.41	29
	Dilution 1; dilution 2; oxidize with hydrogen peroxide then reduction without ammonium persulfate			
	Selenite	15.11	19.81	31
	Dilution 1; dilution 2; oxidize with hydrogen peroxide then reduction without ammonium persulfate			
	Selenate	14.97	>20	
	Dilution 1; dilution 2; oxidize with hydrogen peroxide then reduction without ammonium persulfate			
	TSN3	0	1.11	
	Oxidation with hydrogen peroxide then reduction without ammonium persulfate			

Table XXI

Results of HGAAS determination of selenium from 10 mM Na₂SeO₃ amended bioreactor experiments.

Sample	Results			% Recovery
	Phase	Se Determined grams	% Se	
01/01/01	Solid	0.11	7.3	72
2.1 g Se	Liquid	1.4	93.3	
72 hours n=6 (both reduction methods)	Total	1.5	100	
01/19/01	Solid	0.171	7.2	111.7
2.1 g Se	Liquid	2.21	92.8	
72 hours n=6 (both reduction methods)	Total	2.38	100	
03/26/01	Solid	0.117	2.2	250
2.1 g Se	Liquid	5.13	97.0	
24 hours CaCl ₂ added to solid oxidation n=6 (both reduction methods)	Total	5.25	100	
05/02/01	Solid	0.236	11.4	98
2.1 g Se	Liquid	1.834	88.4	
24 hours n=6 (both reduction methods)	Total	2.07	100	
06/04/01	Solid	0.141	6.2	108.6
2.1 g Se	Liquid	2.14	93.8	
72 hours n=6 (both reduction methods)	Total	2.28	100	

Table XXII

Parameters used for the determination of selenium by hydride generation-atomic absorption spectrometry.

Parameter	Optimal Setting
Oxidation State	Se(IV)
Reducing Solution	0.6 % NaBH ₄ 0.5 % NaOH
Acid Solution	10 N HCl
Standard Range	0 ppb to 20 ppb Se
Acid Concentration of Standards and Samples	50% HCl

Table XXIII

Results of the degradation experiments for hydrogen peroxide.

Known Concentration M	Decomposition Method	Determined M	% Decomposed
0.0176	boil for 10 minutes 0.05 N NaOH	0.00266	85
0.0176	boil for 20 minutes 0.05 N NaOH	0.00699	60
0.0176	boil until bubbles disappear 0.05 N NaOH	-0.00241	
0.0176	no boiling 0.05 N NaOH	0.0191	
0.0176	boil for 10 minutes	0.0324	
0.0176	boil for 20 minutes	0.0117	34
0.0176	boil until bubbles disappear	0.0162	8
0.0176	no boiling	0.0163	8

Chapter IV

Discussion and Conclusions

Many attempts were made to duplicate the analytical methods found in the literature. A method was developed and tested on known quantities of selenium, tried on biological samples containing selenium, and the % recoveries evaluated. The method was modified and again tested on known quantities before being tested on a biological sample. Reported operating conditions for hydride generation-atomic absorption were widely disparate. Each parameter was tested and values optimized for the HGAAS in our laboratory.

Effects of N₂ Purging on Growth of K27 Bacterium

Figures 1, 2, 3, and 4 (pH and growth curves) and Tables I and II (SGR) were presented to compare N₂ purged bioreactor experiments against bioreactor experiments that were not purged. Both selenium amended and non-amended bioreactor experiments were compared. These experiments were designed to investigate the affect of inert gas purging on the growth of the *Pseudomonas fluorescens* K27 bacterium. Since formation and removal of volatile selenium compounds could be an important detoxification process for this organism, the removal of these volatile gases at higher rates needed to be investigated. The SGR values and the growth curves values are within the value range found in other experiments. Purging with N₂ does not enhance or inhibit the growth of the bacteria under the conditions studied.

Non-amended bioreactor cultures have higher SGR values than amended cultures. In Table II, the experiment carried out on 11/10/99 has a SGR value that is similar to values found with non-amended bioreactor cultures. This result is questionable because this culture was amended with 10 mM SeO₃²⁻. Possible error in this reading could include errors in using the Spectronic 20 (reading or calibrating errors).

Further testing of the optical density (SGR) of bioreactor experiments was not done because a change of emphasis towards efforts to determine the mass balance of selenium. Each of these experiments where the SGR was not determined appeared to follow other experiments in the timing and appearance of the brick-red color of elemental selenium.

Atomic Absorption Determination of Selenium

Detection of selenium by atomic absorption requires that all selenium be oxidized to Se(VI). Oxidation by either nitric acid or hydrogen peroxide was tested, and the results were compared in Table III.

Biologically produced elemental selenium is present in biological samples as indicated by the red color of the precipitate (Doran, 1982). Analysis of selenium in these samples requires dissolution of the precipitate and oxidation to Se(VI). Oxidation of elemental selenium was attempted by two methods using nitric acid. One method employed repeated additions of nitric acid and boiling that solution to dryness. The results of these experiments showed poor % recoveries due to possible volatilization of selenium (Table III). Better results were obtained when nitric acid was added in one aliquot and then boiled only to near dryness. This method was tried on several oxyanions of selenium plus elemental selenium. Errors ranged from 6 to 25%. The lowest error was for selenium that was already in the VI oxidation state. The oxidation of elemental selenium by nitric acid was chosen due to the lower % error.

Also shown in Table III are data related to the treatment of selenium AA standards with various strengths of hydrogen peroxide as an oxidant. This method of oxidation gave errors in a range of 0 to 12%. While these results are acceptable analytically, other experiments were performed using hydrogen peroxide for the oxidation of selenium, and these results differed greatly.

The results from the bubbling tube experiments in which volatile organoselenium compounds are trapped in hydrogen peroxide and thereby oxidized to Se(VI) are presented in Table V. The results are inconsistent and not reproducible. The bubbling tubes labeled A, B and C were connected in series with tube A connected directly to the bioreactor, then tube B connected "down stream" of tube A, then tube C connected to tube B. The quantity of selenium found in tube A should be the greatest value if the trapping procedure is effective. Tube B should contain little oxidized selenium and then tube C would have zero to very little oxidized selenium. Out of seven experiments, only two gave reasonable results. Selenium was detected in the bubbling tubes of non-amended bioreactor experiments. Contamination of the tubing from previous experiments or the inefficient cleaning of the bubbling tubes could be the source of the selenium detected by HGAAS. New tubing and bubbling tubes that had been washed with soap and water then soaked in water containing bleach (a highly oxidizing hypochlorite solution) and then rinsed in deionized water was tested with a non-

amended bioreactor experiment. Analysis by AAS detected selenium present in unexpected tube order and in non-amended cultures. The H_2O_2 that was not destroyed in the decomposition step could be the cause of the high readings for selenium in this section.

Known quantities of dimethyl diselenide were pipetted directly into 30% H_2O_2 and the concentration of selenium was analyzed by AAS (Table VI). The error range is high. These samples were left in sealed but not air-tight volumetric flasks and the samples were analyzed by AAS for selenium at various time periods. The results in Table VII show that as time passed, the error for the 30 ppm and 40 ppm samples decreased. The data in Table VII, work done in HGAAS, and bubbling tube experiments where Se was detected when none was present, indicate that the increase in accuracy could possibly come from the decomposition of hydrogen peroxide. As time passed, the hydrogen peroxide decomposed and no longer interfered in the detection of selenium. An alternative interpretation is that Se oxidation proceeds too slowly and more time is required to oxidize all trapped volatile selenium compounds.

The data presented in Table VIII were recorded when Se(-II) in the form of dimethyl diselenide was oxidized with hydrogen peroxide and then reduced to Se(IV); the percent error was high and similar to other results using hydrogen peroxide as the oxidant. Again, the possibility that hydrogen peroxide was not completely degraded and was causing high percentage errors must be considered.

It is not clear why the results of the detection of sodium selenite (Table VIII) are so much better than other results using AAS. Selenium is atomized in AAS in the VI oxidation state, but the oxidation state of selenium in these experiment is IV.

Mass Balance Attempt Using AAS

One attempt was made to find the mass balance of a Se-amended bioreactor experiment using atomic absorption, and the results are presented in Table IV. Of the amount of selenium added as Se(IV), 95% of it was detected by atomic absorption (% recovery = 95%). Since there is no way to determine the different oxidation states produced by the K27 bacterium in the bioreduction of selenium when using AAS, the use of other instruments for the detection of selenium was investigated (see page 57).

Determination of Tellurium using Hydride Generation-Atomic Absorption

The results of the tellurium and selenium HGAAS experiments are not presented in chronological order. Some of the parameters set for the determination of tellurium were first determined for selenium and then applied to tellurium. The calibration standard range (0-20 ppb) and the acid content of the calibration standards and samples (50% v/v) were determined for selenium and then applied to tellurium analysis. The reductant concentration and acid solution concentration were determined from the operating manual for the Varian AA.

Table IX presents the results of tests that were performed to refine the oxidation and reduction processes of the solid phase of bioreactor fluid amended with tellurium. The variable tested was whether boiling the solid phase in nitric acid or not boiling in nitric acid had an effect on the amount of tellurium detected. Also, the reduction method was tested to see the effect of ammonium persulfate on the reduction of biological samples. Weres *et al.* (1989) suggested 6 N HCl as the reducing agent with 0.2 mL of 2% ammonium persulfate as a viable method for increasing accuracy of metalloid analysis in biological samples. Neither process was consistently better nor worse than the other process in detecting tellurium. The method including the oxidation step with nitric acid (not boiled) was chosen as the preferred method to decrease any chances of volatilization of tellurium (analogous to Se).

Table X gives the optimal operating conditions for the determination of tellurium using hydride generation-atomic absorption. These parameters were used to determine tellurium in biological samples.

Table XI presents data obtained from four separate bioreactor experiments. For some of the bioreactor experiments, more than one tube was processed. The average amount of tellurium found in the solid phase was 26.4% with $n=4$ and a standard deviation of 14.5. At stationary phase 26.4% of the tellurium added as water soluble sodium tellurite (IV) had been reduced to Te(0) or had adhered to the cells in some manner since both cells and elemental tellurium were harvested by the collection method used (Basnayake *et al.*, 2001).

Table XII gives information on an attempt to speciate the tellurium present at stationary phase from bacteria amended with 0.1 mM sodium tellurate (VI). Water soluble forms of tellurium were composed of 59.5% of tellurite (IV) and 7.1% of tellurate for a total of 66.6%. The amount of tellurium in the solid phase was 33.3%.

Further work in this area would include determining the specific oxyanions present at stationary phase, and the concentration of these oxyanions at various time periods during the log phase of growth using sequential sampling of a live bacterial culture.

Determination of Selenium by Hydride Generation-Atomic Absorption

Each operating condition for the determination of selenium was determined for the analysis by hydride generation-atomic absorption. Table XIII shows the effect of acid concentration of the samples and standards. The recovery results for samples and standards containing from 20 to 50% hydrochloric acid (v/v) were the lowest. The decision to use 50% HCL (v/v) was determined by these results and from those of the literature. Conditions that were chosen through this optimization process ultimately led to samples with standard deviations of less than 10%

Table XIV shows the calibration range for the standards. The optimum range was determined to be 0 - 20 ppb. Although this range is relatively narrow, it is still analytically useful.

Table XV shows the comparison of the concentration of reducing solutions used in HGAAS. The 0.6% (w/w) sodium borohydride solution produced the results with the lowest errors.

To determine whether the background correction feature of the HGAAS instrument should be on or off was addressed in experiments presented in Table XVI. The most accurate results were obtained by turning the background correction feature off.

Table XVII is a tabulation of results obtained to determine the best selenium reduction method from those found in the literature (Standard Methods for the Examination of Water and Wastewater, 1989). These methods were tested on known quantities of selenium in deionized water. The method that gave the most accurate results was the reduction method employing 5 mL of sample plus 5 mL of conc. HCl with 0.2 mL of 2% ammonium persulfate.

Further refinement of this process was investigated and data are reported in Table XVIII. The most accurate method in this tabulation was boiling the sample for 30 minutes in uncapped tubes; the decision was made not to use the uncapped methods because of experiments where the water in the boiling water baths contaminated the samples.

Results of testing for Se(IV) in a mixture of Se(IV) and Se(VI) are presented Table XIX. The results indicate that Se(IV) can be analyzed by HGAAS in the presence of Se(VI).

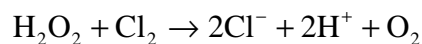
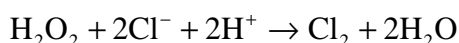
Two methods of selenium oxidation found in the literature were oxidation with hydrogen peroxide or with nitric acid. Tables XX a, XX b, XX c, and XX d present the results of Se analyses when these methods were tested. The impetus behind these experiments was to determine the best method for oxidation of selenium in samples obtained from the bioreactor that were to be analyzed by HGAAS. These results are of experiments done over several months and are not presented in chronological order. In Table XXI a, nitric acid and hydrogen peroxide were tested on sodium selenite in water. The nitric acid results for the determination for water-soluble selenium were not acceptable (recoveries > 10%). Nitric acid has been used to digest organic materials containing selenium (Narasaki and Ikeda, 1984; Bujdos and Kubova, 2000). The decision was made to use nitric acid on only the solid phase of the bioreactor fluid. Schumb *et al.* (1955) stated that hydrogen peroxide slowly oxidizes Se⁰ to selenium dioxide which reinforces this decision.

Many reports in the literature referred to hydrogen peroxide as an oxidant for water-soluble forms of selenium and for volatile organoselenium compounds (Weres, 1989; Thompson-Eagle and Frankenberger, Jr., 1990; Zhang and Frankenberger, Jr., 2000; Reddy *et al.*, 1995; Standard Methods for the Examination of Water and Wastewater, 1989). This oxidant was tested and the data from these tests were presented in Tables XX a, XX b, XX c and XX d. The difference between the two processes in Table XX a, was that in one experiment the hydrogen peroxide was boiled until O₂

bubbles disappeared versus boiling until dryness. The results for the process where boiling until dryness was employed are within 10% of the known concentration. The results for boiling until the oxygen bubbles disappear show an error of 27%. The only difference in these two methods is the time allowed for the decomposition of the hydrogen peroxide. The significance of these data was not recognized at the time.

The concentration of selenium added to the bioreactor in a 10 mM amendment experiment is approximately 789 ppm. Dilutions were made to reduce the selenium concentration to levels within the calibration range of the HGAAS method (~ 10 ppb range). The timing of these dilutions in regards to where they appear in the oxidation and reduction steps was investigated in Tables XX a, XX b, XX c and XX d. The oxidation of various oxyanions of selenium in TSN3 was tested with hydrogen peroxide and dilutions were made in different steps of the oxidation and reduction process.

In Table XX b, the dilutions occurred at the beginning of the process before oxidation. The thinking behind this step was that any interferents that might be present in the TSN3 growth media could be reduced in concentration when diluted. One observation made during these experiments was that when the HCl was added for the reduction step uncontrolled bubbling occurred. So much bubbling occurred that some of the sample was lost in several tubes. A possible explanation for the bubbling could be the following reactions involving undecomposed H₂O₂ from the degradation step (Jones, 1999):



Whatever the source of the bubbles, the recovery of selenium in these experiments was poor. The calculated or known concentrations of these solutions were within the calibration range but in several of the tests the determined concentration was over the calibration range. The hydrogen peroxide may not have degraded completely in the boiling stage and may have interfered positively. The proposed method for determining that all H₂O₂ was degraded was when the O₂ bubbles disappeared (Weres *et al.*, 1989, Standard Methods for the Examination of Water and Wastewater, 1989). These experiments were repeated in XX d and again the results were high.

In Table XX c, 1) dilution before the oxidation step to dilute any possible TSN3 interferents, then 2) oxidation with hydrogen peroxide, 3) dilution to negate any remaining hydrogen peroxide and then 4) reduction, was the method tested. This process gave better results as measured by % error when compared to the analysis of Se(IV) in TSN3 with no oxidation or reduction methods applied. This series of dilutions was the final method applied to the supernatant of bioreactor experiments that were amended with selenium.

Table XXI gives the results obtained when the fluid from five different bioreactor experiments were analyzed for selenium using the dilution-oxidation-dilution-reduction-analysis method. For the 10 mM Na_2SeO_3 amended bioreactor experiments, 6.78% (n=5 with a standard deviation of 3.14) of the water-soluble selenium was reduced to elemental selenium or adhered to the K27 cells and was collected in the solid.

Table XXII gives the operating parameters for the determination of selenium. The standard range and the acid concentration are similar to that of tellurium. The concentration of the reducing solution and the acid for hydride production are different.

Data in Table XXIII show the investigation of the best process to degrade hydrogen peroxide after the oxidation step. The best method was to boil for at least 20 minutes with 0.05 N NaOH. The final method applied to biological samples was to boil the sample for 45 minutes with 0.05 N NaOH.

Reduction of Possible Interferents in Determining Se by HGAAS

Each step of the determination of selenium was fraught with possible sources of interference. The following outlines these possible contributing factors and describes steps used to reduce any possible interferences.

The use of nitric acid could erroneously enhance selenium readings by producing NO or NO_2 gases by the oxidation of any organic matter present (Bujdos and Kubova, 2000). The NO_3^- ion causes suppression of the selenium signal in concentrations of 4000 ppm (Narasaki and Ikeda, 1984). These possible interferences of selenium in the solid phase were reduced by dilutions by a factor of 10000.

Humic substances can cause interferences by reacting with sodium borohydride (the reductant) to produce effervescence which perturbs selenium measurements (Zhang, *et al.*, 1999; Roden and Tallman, 1982). The first dilution in the analytical process for the supernatant would reduce the concentration of any humic substances to a value of 0.075% of the sample. Further steps would dilute this value by a factor of 400 before analysis by HGAAS. For the solid phase, any humic substances would also be diluted by a factor of 10000.

Hydrogen peroxide is listed as an interferent by Narasaki and Ikeda (1984) in levels of 1400 ppm to 2800 ppm for determination of selenium at 8 ppb and 4 ppb levels respectively. Any production of gas besides H_2Se during analysis distorts the detection of selenium. Weres *et al.* (1989) described the process to degrade hydrogen peroxide as boiling until the fine bubbles of hydrogen peroxide decomposition are replaced by the ordinary bubbles of boiling. Zhang *et al.* (1999) reported that several drops of 1 N NaOH added to boiling hydrogen peroxide would aid in the decomposition of the hydrogen peroxide. The stability of H_2O_2 was not expected after reading these descriptions of how to degrade hydrogen peroxide. In the experiments presented in Tables XX a, XX b and XX d, hydrogen peroxide was used as the oxidant, and the oxidized samples were boiled until the fine bubbles disappeared. After the research in reported in Table XX was done, the oxidized samples were boiled for 45 minutes with 0.05 N NaOH present. The final dilution before reduction was to dilute any remaining H_2O_2 to below levels that could interfere with the detection of selenium.

Back oxidation of Se(IV) to Se(VI) can be caused by Cl_2 generated in the reduction process with HCl (Krivan *et al.*, 1985). To address this possibility, samples were analyzed immediately after the reduction step.

There are many interesting avenues to pursue in research related to the material presented here. Some have already been mentioned. One avenue I would like to pursue is the bioreduction of selenium at controlled pH values.

Conclusions

- For biological samples containing tellurium, the best recoveries were obtained by oxidation of both the liquid and solid phases by nitric acid, and reduction by 5 mL of sample and 5 mL of 12 N HCl boiled for twenty minutes.
- Operating parameters for HGAAS determination of tellurium were determined to be 0-20 ppb calibration standards, samples and standards of 50% HCl (v/v), sodium borohydride concentration of 0.35% (w/w) stabilized by 0.50% NaOH (w/w), and an acid solution for the production of the hydride as 6 N HCl.
- For the determination of selenium in biological samples, oxidation of the solid phase by nitric acid and oxidation of the liquid phase by hydrogen peroxide were determined to be the methods that gave the best recoveries. The reduction method that was determined to give the best results was 5 mL of sample and 5 mL of 12 N HCl boiled for twenty minutes.
- Both oxidants and the reducing agent used in this method for HGAAS determination of selenium can cause erroneous analysis. Steps must be taken to minimize these possible errors. Steps used in this research were to use dilutions to minimize these interferents, and in the case of interference by the reducing agent, analysis was performed within one hour of reducing the samples.
- Operating parameters for the detection of selenium by HGAAS are 0-20 ppb calibration range, 50% HCl (v/v) for samples and standards, sodium borohydride concentration of 0.60% (w/w) stabilized with 0.050% NaOH (w/w), and acid solution for hydride production 10 N HCl.

Bibliography

- Basnayake, R. T.; Bius, J. H.; Akpolat, O. M.; Chasteen, T.G. *Appl. Organomet. Chem.* **2001**, *15*, 499-510.
- Blaylock, M. J.; James, B. R. *J. Environ. Qual.* **1993**, *22*, 851-857.
- Bujodos, M.; Kubova, J.; Stresko, V. *Anal. Chim. Acta* **2000**, *408*, 103-109.
- Chasteen, T. G. , Silver, G. M., Birks, J. W. , Fall, R. *Chroma.* **1990**, *30*, 181-185.
- Chasteen, T. G. *Appl. Organomet. Chem.* **1993**, *7*, 335-342.
- Cooke, T. D.; Bruland, K. W. *Environ. Sci. Technol.* **1987**, *21*, 1214-1219.
- Cutter, G. A. *Anal. Chim. Acta* **1978**, *98*, 59-66.
- Cutter, G. A. *Anal. Chem.* **1985**, *57*, 2951-2955.
- Dedina, K.; Tsalev, D.L. "Hydride Generation Atomic Absorption Spectrometry"; John Wiley & Sons: Chichester, 1995; 308-354; 355-370.
- Diaz, J. P.; Navarro, M.; Lopez, H.; Lopex, M. C. *Sci. Total Environ.* **1996**, *186*, 213-236.
- Doran, J. W. In "Advances in Microbial Ecology"; Marshall, K. C., Ed.; Plenum Press: New York, 1982: Vol. 6, Chapter 1.
- Eriksen, Jr., L. A., M.S. Thesis, Sam Houston State University, Huntsville, TX, U.S.A., 1999.
- Gürlyük, H., M.S. Thesis, Sam Houston State University, Huntsville TX, USA, 1996.
- Jones, C. W. "Applications of Hydrogen Peroxide and Derivatives"; The Royal Society of Chemistry: Cambridge, 1999; 156.
- Krivan, V.; Petrick, K.; Welz, B.; Melcher, M. *Anal. Chem.* **1985**, *57*, 1703-1706.
- Lortie, L.; Gould, W. D.; Rajan, S.; McCready, R. G. L.; Cheng, K. J. *Appl. Environ. Microbiol.* **1992**, *58*, 4042-4044.
- Lueschow, L. A., Mackenthun, K. M. *J. AWWA* **1962**, *Jun.*, 746-750.
- Maas, J., In "Environmental Chemistry of Selenium", Engberg, R., Frankenberger, Jr., W., Eds.; Marcel Dekker: New York, 1998: 113-128.
- Macy, J. M.; Michel, T. A.; Kirsch, D. G. *FEMS Microbiol. Lett.* **1989**, *61*, 195-198.

- Magin, Jr., G. B.; Thatcher, L. L.; Rettig, S.; Levine, H. *J. AWWA*. **1960**, *52*, 1199-1205.
- Manning, B. A.; Burau, R. G. *Environ. Sci. Technol.* **1995**, *29*, 2639-2646.
- Martens, D. A.; Suarez, D. L. *Environ. Sci. Technol.* **1997**, *31*, 133-139.
- Mauzannar, R.; Miric, S. J.; Wiggins, R. C.; Konat, G. W. *Neurochemistry International* **2001**, *38*, 9-15.
- Narasaki, H.; Ikeda, M. *Anal. Chem.* **1984**, *56*, 2059-2063.
- Parsons, J. J., "A Geographer Looks at the San Joaquin Valley"; Departments of Geography, University of California at Berkeley; 1987 Carl Sauer Memorial Lecture; http://geography.berkeley.edu/ProjectsResources/Publications/Parsons_SauerLect.html
- Rech, S. A.; Macy, J. M. *J. Bacteriol.* **1992**, *174*, 7316-7320.
- Reddy, K. J.; Zhang, Z.; Blaylock, M. J.; Vance, G. F. *Environ. Sci. Technol.* **1995**, *29*, 1754-1759.
- Reilly, C. "Selenium in Food and Health"; Blackie Academic & Professional: London, 1996; 5-7; 110-114; 117-122.
- Roden, D. R.; Tallman, D. E. *Anal. Chem.* **1982**, *54*, 307-309.
- Schumb, W. C.; Satterfield, C. N.; Wentworth, R. L. "Hydrogen Peroxide"; Reinhold: New York, 1955; 400.
- Skorupa, J. In "Environmental Chemistry of Selenium"; Engberg, R., Frankenberger, Jr., W., Eds.; Marcel Dekker: New York, 1998: 315-354.
- Shamberger, R. J. "Biochemistry of Selenium"; Plenum Press: New York, 1983, 202-204, 252-254.
- "Standard Methods for the Examination of Water and Wastewater", 17th ed., Clesceri, L. S., Greenberg, A. E., Trussell, R.R., Eds., American Public Health Association, Washington DC, 1989, 3-43-53, 3-128-134, 3-137-141.
- Stone, A. J.; Van Fleet-Stalder, V.; Chasteen, T. G., 215 Nat. A.C.S. meeting, Dallas TX, March 1998; abst. EVNR 46.
- Terry, N.; Carlson, C.; Raab, T. K.; Zayed, A. M. *J. Environ. Qual.* **1992**, *21*, 341-344.
- Thompson-Eagle, E. T.; Frankenberger, Jr., W. T. *J. Environ. Qual.* **1990**, *19*, 125-131.
- Varian, "Analytical Methods Flame Atomic Absorption Spectrometry", Publication No 85-100009-00, Varian Australia Pty Ltd, Mulgrave Victoria, Australia, 1989.

Varian, Vapor Generation Accessory VGA-77", Publication No. 85 101047 00, Varian Australia Pty Ltd, Mulgrave Victoria, Australia, 1997.

Weres, O.; Jaouni, A.; Tsao, L. *Appl. Geochem.* **1989**, *4*, 543-563.

Yu, R., M.S. Master Thesis, Sam Houston State University, Huntsville, TX, U.S.A., 1996.

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, Jr., W. T. *Environ. Sci. Technol.* **1999**, *33*, 1652-1656.

Zhang, Y., Frankenberger, Jr., W. T. *Environ. Sci. Technol.* **2000**, *34*, 776-783.

Zhang, Y.; Frankenberger, Jr., W. T.; Moore, J. N. *Sci. Total Environ.* **1999**, *229*, 183-193.

AppendixG
E
}**CHEMICAL ABSTRACT SERVICE REGISTRY NUMBERS**

Compound Name	CAS Registry Number
Hydrochloric acid	7647-01-0
Hydrogen peroxide	7722-84-1
Nitric acid	13587-52-5
Potassium nitrate	7757-79-1
Selenium powder	7782-49-2
Sodium borohydride	16940-66-2
Sodium hydroxide	1310-73-2
Sodium selenate	13410-01-0
Sodium selenite	10102-18-8
Sodium tellurate	26006-71-3
Sodium tellurite	10102-20-2

VITA

Janet Kay Horton Bius was born in Houston, Texas but spent most of her childhood in Palestine, Texas. She graduated from Palestine High School in 1977. She received a Bachelor of Science in chemistry with a minor in mathematics in August 1981.

In 1985, Mrs. Bius received her State of Texas teaching certificate in science and English. She taught physical science at Huntsville High from 1985 until 1988 when she left to work in the business sector.

In January 1999 Mrs. Bius entered the chemistry graduate program at Sam Houston State University in Huntsville, Texas. She received her Master of Science degree in chemistry in December 2001.