Reduction and Methylation of Various Selenium Anions by *Pseudomonas fluorescens* K27 in Bioreactor-Based Batch Cultures

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by

Lynn A. Eriksen Jr.

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Reduction and Methylation of Various Selenium Anions by *Pseudomonas fluorescens* K27 in Bioreactor-Based Batch Cultures

by

Lynn A. Eriksen Jr.

APPROVED:

____
Dr. Thomas G. Chasteen
Thesis Director

____
Dr. Mary Plishker

____
Dr. Paul A. Loeffler

Approved :

____
Christopher Baldwin, Dean
College of Arts and Sciences

ABSTRACT

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Purpose

The purpose of the experiments in this work was to track the changes in cellular growth, pH and concentrations of specific volatile headspace compounds produced by batch cultures of *Pseudomonas fluorescens* K27 amended with various selenium salts using a commercial bioreactor. Research of this nature has not been carried out before.

Methods

Batch cultures inoculated with *Pseudomonas fluorescens* K27 were grown in a New Brunswick BioFlow III Batch/Continuous Fermentor with a 10% tryptic soy broth, 3% potassium nitrate growth media. Changes in cellular growth were followed by extracting liquid cultures from the fermentor during the experiment and using a spectrometer to analyze for optical density at 526 nm. Changes in pH were monitored by pH probe measurements of the same liquid cultures used to take optical density readings. Bioreactor headspace concentrations were measured by extracting headspace via gas tight syringe from the fermentor. Separation and detection of headspace compounds were carried out by gas chromatography equipped with sulfur chemiluminescence detection. Also, an effort was made to trap gas purged headspace compounds with a 30% hydrogen peroxide solution for analysis of selenium by atomic absorption spectroscopy detection in order to determine the selenium mass balance.

Findings

Batch cultures amended with single or mixed concentrations of selenate and/or selenite produced repeatable results similar to prior established work. In general, control cultures exhibited a specific growth rate near previously reported work for unamended batch cultures: 0.30 h^{-1}. Ten millimolar selenate cultures showed trends that suggest that selenate is less toxic than selenite. Also, results from 2:1 and 1:2 selenate to selenite mixed cultures suggest that a higher concentration of one type of selenium oxyanion in the mix causes a change in specific growth rate based upon the toxicity of the higher concentration selenium oxyanion as compared to 1:1 selenate to selenite amended cultures. Again, selenite appears to be more toxic than selenate to this organism.

Changes in pH over the experimental time course showed the most variability between cultures amended with 1:0, 2:1, and 1:2 selenate to selenite. In general, the 2:1 amendments exhibited a drop in pH while the 1:0, 0:1 and 1:1 selenate to selenite cultures displayed an increase in pH.

The headspace trapping experiments showed that only very small amounts of volatile organoselenium species are purged at the flow rates examined. Less than 0.05% of the added Se was trapped after 10 hours of approximately 2 mL/min continuous nitrogen gas flow.

Approved:

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Dr. Thomas G. Chasteen
Thesis Director

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Chapter I

INTRODUCTION

Background

Selenium contamination event in the San Joaquin Valley

In 1960, authorization was given to construct a 188 mile-long concrete drain from the western San Joaquin Valley of California to the San Francisco Bay area. The expressed use for this drain would be for the removal of saline agricultural drainage from producers in the western half of the San Joaquin Valley. Due to budget constraints and planning problems, only 84 miles of the drainage system was completed. At the northern end of the San Luis Drain was the Kesterson Reservoir, which was intended as a flood control reservoir in the middle of the drainage system (Quinn *et al.*, 1995). When the projected was halted, the Kesterson Reservoir became a drainage disposal facility, consisting of 12 separate ponds. Without drainage, water in the reservoir was left to evaporate or percolate into the soil. With its location at the southern end of the Kesterson National Wildlife Refuge, the reservoir also became a home to migratory and resident water-foul. In the early 1980's, reports of illness and reproductive failure among the water-foul led to investigations which reported that the water in the Kesterson reservoir and the San Luis drain had extremely high level of selenium, predominantly in the form of the selenate anion with a concentration between 200 – 300 ppm selenate. Consequently, the drainage of agricultural wastewater to the San Luis Drain and the Kesterson Reservoir was cut off by the mid 1980's. By the end of the decade the San Luis Drain and the Kesterson Reservoir were closed (Weres *et al.*, 1995). During the period of

extensive research by many groups after the initial reporting of selenium contamination, several selenium resistant bacteria were isolated from the Kesterson Reservoir before its closure. One of these isolates found was *Pseudomonas fluorescens* K27 (Burton *et al.*, 1987).

One of the more interesting aspects of *Pseudomonas fluorescens* K27 is its ability to reduce and methylate selenium salts to volatile organoselenium. It is this ability that qualifies *Pseudomonas fluorescens* K27 as a candidate for use in bioremediation methods of selenium contaminated areas.

Selenium toxicity – a brief history

The effects of toxic selenium concentrations have been well documented for the past 30 years, and work related to this subject goes back more than a century. Initial linking of consumption of toxic levels of selenium with "alkali disease" was first made in 1934. Also know as "blind staggers," this condition causes hair loss, deformation and loss of hooves in cattle, hogs and horses and can also affect numerous internal organs (Spallholz, 1994). As previously mentioned, it can cause reproductive failure as noted in water-foul (Weres *et al.*, 1989). It has also been shown that trace amounts of selenium are necessary as a dietary nutrient in many mammals – including humans. In fact, a Recommended Dietary Allowance (RDA) of selenium for men, women and children was established in the United States in 1989 (Spallholz, 1994). For adult men and women, the RDA was calculated to be 70 micrograms and 55 micrograms respectively (Levander, 1991).

Selenium passes from plants to mammals as a part of the food chain, and selenium compounds in plants (most often found in seleno-amino acids and proteins) are

absorbed from water in the area by the root system. In areas of high selenium contamination, selenium is typically found in sediments as well as in soils, which then mobilizes in drainage. When animals ingest plants that have accumulated high levels of selenium, conditions of "alkali disease" or "blind staggers" can occur.

Plants that are natural accumulators of selenium (which thrive in selenium enriched soils) can also produce volatile organoselenium compounds, which gives some of these plants, such as garlic, its pungent odor (Spallholz, 1994). In the early 1980's it was established that volatile organoselenium compounds could be produced in a laboratory setting by microorganisms in soil amended (poisoned) with selenite or elemental selenium. Headspace analysis of soils amended with selenite showed microbial production of predominantly dimethyl selenide at low selenite concentrations and predominantly dimethyl diselenide at high selenite concentrations. Also noted was the production of dimethyl selenone (Reamer and Zoller, 1980). This component was later shown to be dimethyl selenenyl sulfide (Chasteen, 1993).

Reduction and methylation of organoselenium in an aquatic environment was first established in the 1970's (Chau *et al.*, 1976). As previously discussed, an isolate of *Pseudomonas* species was found in the Kesterson reservoir in an aquatic environment (Burton *et al.*, 1987).

Reduction and methylation of selenium salts to organoselenides

The first work showing the reduction of selenium salts, specifically selenite, was first reported by Challenger in working with fungi. In 1945, Challenger proposed a mechanism for the methylation and reduction of selenite to dimethyl selenide (Figure 1).

This mechanism was suggested by Challenger based upon his prior work with the methylation of arsenic compounds.

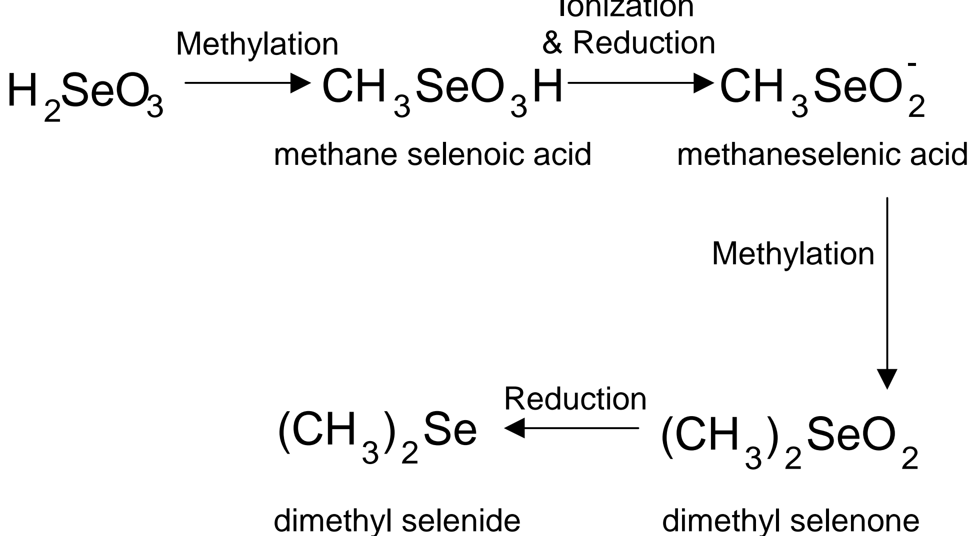


Figure 1. Mechanism for the reduction and methylation of selenite proposed by Challenger.

A different mechanism was proposed by Doran in 1982. He suggested the following based on several criticisms of Challenger's work:

1. None of Challenger's proposed intermediates could be found in culture solution.
2. Dimethyl selenone was never synthesized and tested as a possible intermediate.
3. The potassium salt of methane selenoic acid used in Challenger's studies readily undergoes hydrolysis to form selenite.

Doran's mechanism not only accounts for the production of dimethyl selenide, but dimethyl diselenide as well (Figure 2).

$$SeO_3^{2-} \longrightarrow Se^0 \longrightarrow HSeX \longrightarrow CH_3SeH \longrightarrow (CH_3)_2Se$$

selenite elemental Se selenide methaneselenol dimethyl selenide

Figure 2. Proposed reduction and methylation of selenite by Doran.

In essence, he proposed a direct path from selenite to elemental selenium, and a two step mechanism from elemental selenium to dimethyl diselenide for the methylation of selenite (Doran, 1982). Recently however, dimethyl selenone amended test tube cultures showed production of organoselenides. It is not known if this is accomplished by a biological process or a chemical process (Zhang *et al.*, 1994). This recent development cast uncertainty on the Doran mechanism. It also has been proposed that elemental selenium may not be a part of the mechanism as well (Jiang, 1994). This facultative anaerobe (later known as *Pseudomonas fluorescens* K27) showed production of dimethyl selenenyl sulfide and dimethyl diselenide from the static headspace of test tube cultures amended with selenate and selenite salts via sulfur chemiluminescence detection equipped gas chromatography (Zhang and Chasteen, 1994).

Despite the ongoing work, the reduction and methylation of selenium anions is still not well understood. Recent work with other bacteria suggests that reduction and methylation of selenium oxyanions occurs via enzymatic pathway (Rech and Macy 1992). Regardless of the process, *Pseudomonas fluorescens* K27 consistently and readily produces volatile organoselenides when exposed to various selenium concentrations.

Headspace production problems

The reduction and methylation of selenium anions by *Pseudomonas fluorescens* K27 in the aquatic environment demonstrate promise that has the ability to reduce the concentration of toxic selenium oxyanions in water initially containing selenium oxyanions. This is accomplished by releasing organoselenides gradually into the atmosphere where they would be significantly diluted and mobilized before subsequent oxidation and return to the ground.

The ability of *Pseudomonas fluorescens* K27 to do this is effected by the concentrations and types of the selenium oxyanions present in the water (Yu *et al*., 1997). In general, it has been shown that selenite is more toxic than selenate, and both are more toxic than organoselenides (Spallholz, 1994; Yu *et al.*, 1997). However, other work suggests that selenate is more toxic than selenite for *Pseudomonas fluorescens* K27 based on growth inhibition tests (Ibrahim and Spacie, 1990). Also, work has been done to track the changes in headspace concentrations of selenate and selenite amended test tube cultures over experimental time-course as a function of selenium oxyanions amendment types and concentrations (Yu *et al*., 1997).

One of the problems with measuring static headspace from anaerobic test tube cultures is that each time the Teflon® septa at the top of the test tube is pierced by the sampling syringe, the culture can no longer be considered isolated or anaerobic. The cultures can then grow aerobically which can dramatically change metabolic processes. The use of multiple test tubes can lead to low repeatability in tracking changes in growth and changes in headspace concentration. A way to repeatedly sample headspace above a bacterial culture was implemented by McCarty with an Erlenmeyer-flask based

bioreactor that allowed for repeated headspace sampling while also allowing for isolation of the bacterial culture and gas purging (1993;1994). Although this simple design was ultimately limited in functionality, this work subsequently led to experimentation with the New Brunswick BioFlow III bioreactor (also known as a fermentor) with control or selenate amended cultures of *Pseudomonas fluorescens* K27 (Stone *et al*., 1998).

Bioreactor Implementation

<u>Bioreactor culture types</u>

A bioreactor allows for fine control of culture growth conditions. Control of culture temperature, dissolved oxygen, pH, nutrient inflow and outflow, and agitation (stirring) can all be maintained via probe feed back and computer processing. Liquid samples can also be taken while still maintaining culture isolation. The bioreactor allows for the growth of large cultures; in this work, the average culture volume is 2.7 liters.

When using a bioreactor to grow cultures, two types of cultures are primarily used. The first is the batch culture. An amount of sterilized media in the fermentor is inoculated with a preculture and allowed to grow until the culture exhausts nutrients or some other required component in the growth media and the growth rate levels off or declines. No media is added after the inoculation step. These experiments are usually short — less than 24 hours. Three distinct growth phases are exhibited:

1) Lag phase — little growth is shown as the preculture adapts to the new growth medium conditions in the fermentor.

2) Log phase — rapid growth occurs as the culture grows and consumes the media and greatly exceeds the cell death rate. The growth exhibits an exponential change over time.

3) Stationary phase — the rate of cell growth slows to that of the cell death rate and no further growth is shown. Exhaustion of essential media components by the culture typically causes this to occur, although the limiting nutrient is often hard to determine.

These three phases represent a classical growth curve for batch cultures (Figure3a). One common possible growth curve variation exhibits asynchronous cell replication after several generations — resulting in a growth curve with a sharp change in growth rate soon after inoculation (Figure 3b). Finally, another variation possible is called the *diauxie* (Figure 3c). This growth curve variation shows a slow down in the middle of log phase followed by a resumption of exponential growth, usually at a different rate. This change usually occurs in the middle of the overall log phase and is indicative of a possible change in nutrient or substrate utilization (Pirt, 1975).

The second type of culture commonly used in bioreactors is the continuous culture. In this type of culture, an amount of sterilized growth media is inoculated with a preculture and allowed to grow into log phase. When log phase is reached, dilution of the culture begins with a controlled rate inflow of sterilized growth media. In addition, harvesting of the culture begins by removing amounts of the culture equal to volume of dilution. This results in the culture maintaining a perpetual state of logarithmic growth (Figure 3d) and this type of experiment can go on indefinitely (Pirt, 1975).

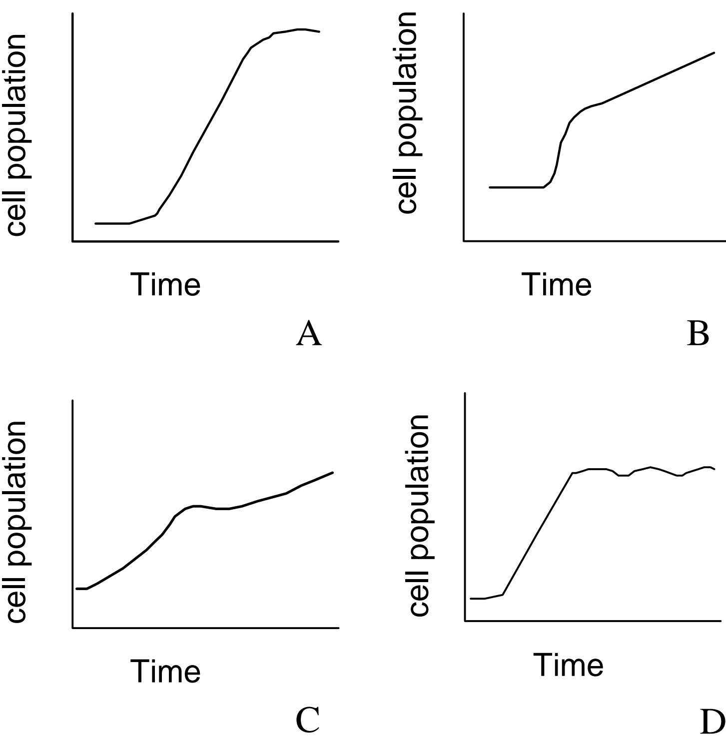


Figure 3. a) example of batch culture growth curve with lag, log and stationary phase, b) variation of batch growth curve showing asynchronous growth, c) *diauxie* growth curve variation, d) example of continuous culture growth with steady state.

The models of the batch and continuous cultures are linked by what is referred to as the specific growth rate. The specific growth rate of both batch and continuous cultures in a bioreactor can be obtained by performing batch cultures and charting change in optical density over time. The natural log of optical density vs. time is plotted, and a linear least squares fit line made of the points in the log phase (Paran, 1990).

The slope of the line is the specific growth rate of the culture. The specific growth rate of a culture (with said amendment) from a batch culture can be used to find the critical dilution rate for the same microorganism growing in a continuous culture under the same conditions (Pirt, 1975; Cherlet and Marc, 1998; Dorofeyev *et al.*, 1997). Thus,

batch culture experiments can be foundational in preparing for continuous cultures, which can be used to simulate a bench scale waste treatment plant (Truax, 1995).

Bioreactor monitoring

A method for selectively detecting organoselenides with high sensitivity was developed using sulfur chemiluminescence detection in the late 1980's (Chasteen *et al.*, 1990). This method has been repeatedly and successfully used for more than a decade in detecting low concentrations of organoselenides in gas phase and is used for this work as well (Chasteen *et al.*, 1990; Chasteen, 1993; McCarty, 1994; Yu *et al.*, 1997).

Liquid sampling of bioreactor cultures can be used to determine cell population and to monitor pH. Light scattering, as outlined by Pirt for the estimation of biomass, can be used to reliably follow changes in optical density via spectrometer or Klett meter. Typically, light in the 540 nm range is used by the instrument for measurement of optical density (1975). Very recent work suggests that light in the 650 nm range may be useful in measuring the optical density of extra-cellular material in the batch culture (Kessi *et al.*, submitted for publication). Light waves of lower wavelength tend to be absorbed and not scattered, which can cause invalid readings. Typically, optical density readings are made and the natural log of these readings calculated for data reporting (Pirt, 1975).

Monitoring liquid samples for pH can also be insightful. It has been shown that changes in growth media pH, due to cellular wastes, can have an effect on the growth rate. These changes in the growth media pH cause the cell to change its intracellular pH as a buffering mechanism. However, extreme changes in intracellular pH can cause metabolic problems, which can dramatically slow or stop culture growth (Cherlet and

Marc, 1998). It is feasible that this may have an affect of *Pseudomonas fluorescens* K27 as well.

Expectations

The use of a bioreactor for bench scale remediation of selenium amended wastes with microorganisms in continuous cultures was done in the early 1990's. However, the microorganism used, *Thauera selenatis*, reduces selenate and selenite to elemental selenium for removal by filtration (Macy *et al.*, 1993). *Pseudomonas fluorescens* K27, however, may present a better bioremediation option due to its established ability to reduce and methylate aqueous selenium anions and release them into the atmosphere in the open environment. This thesis evolves work with *Pseudomonas fluorescens* K27 batch cultures, single and mix amended with a total concentration 10 mM selenate and/or selenite. For these individual cultures, the specific growth rate, change in pH, and change in headspace concentration of organochalcogens over the experimental time course were examined. It is the hope of the author to demonstrate that this work can serve as a first step towards the development of a bench scale continuous culture bioremediation solution.

Chapter II

MATERIALS AND METHODS

Instrumentation

New Brunswick BioFlow III fermentor

Being one of two primary pieces of equipment used for experimentation, the New Brunswick BioFlow III Batch/Continuous Fermentor (Lewisville, TX, USA) bioreactor system allows for control of the environment for batch cultures. As a sealed vessel, it allows for control and monitoring of dissolved oxygen in the culture (for aerobic or anaerobic growth), pH, culture temperature, as well as gas purge and agitation rates. This involves a complex interaction of monitoring and control units on the fermentor, in the fermentor electronic control station, and also by computer software control and monitoring, which was mostly used for monitoring in these experiments. A schematic of the fermentor is provided in Figure 4.

The two main components of the apparatus are the fermentor vessel and the fermentor headplate. Other pieces of equipment for control and monitoring are attached to either of these components. The fermentor vessel consists of two distinct parts that are not separated on a normal basis and is used to contain the growth media. The top of the vessel is made from specially designed high temperature glass that is carefully clamped down to a machined stainless steel base that not only forms the bottom of the vessel, but also allows for heat transfer via warm water that was circulated on demand to maintain a stable temperature in the culture.

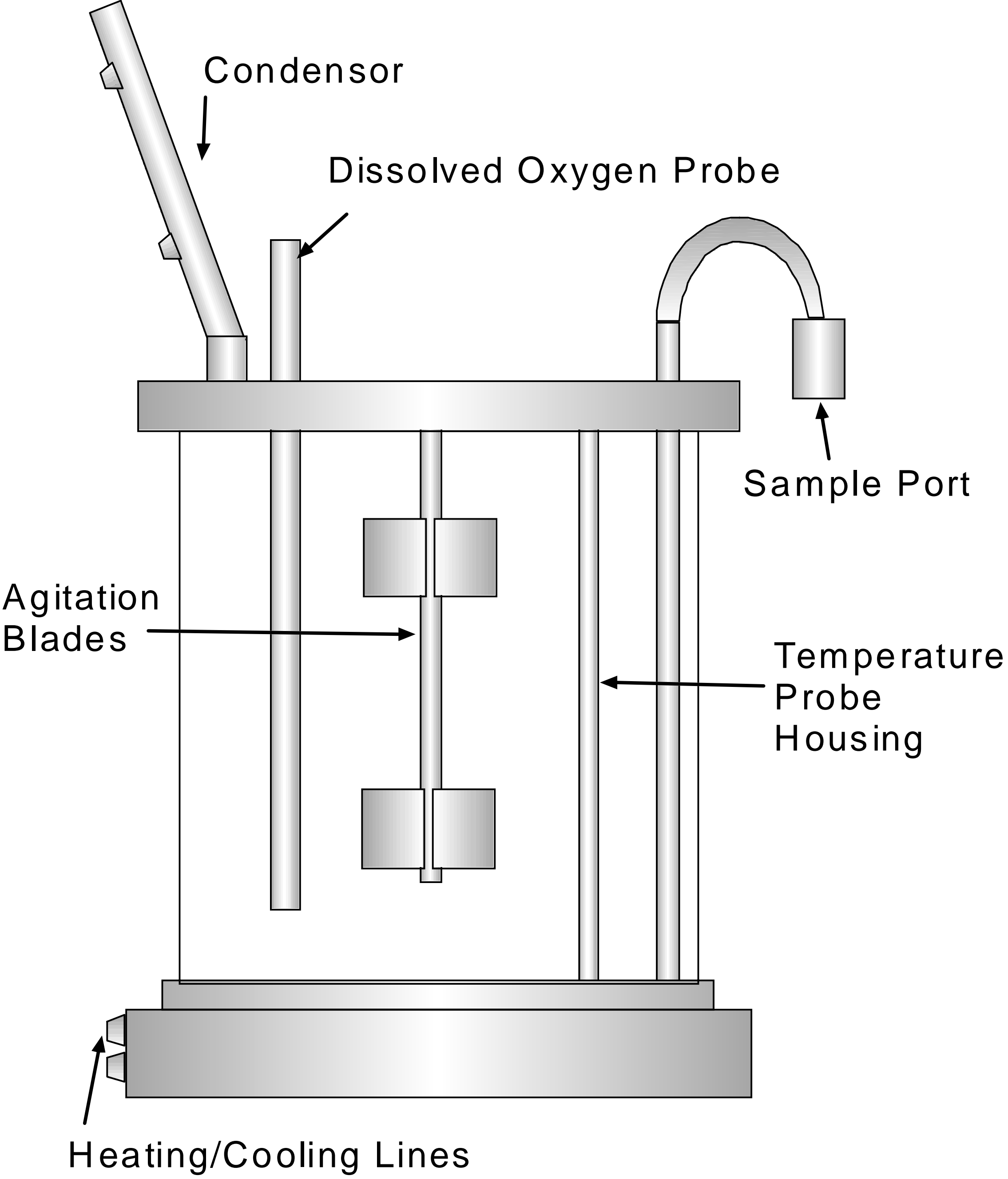


Figure 4. New Brunswick BioFlow III Batch/Continuous fermentor schematic.

The fermentor headplate is made of stainless steel like the vessel base, but is much more complex in design. Of primary interest are the stainless steel tubes and agitation blades attached to the bottom of the plate that extend into the media. Several of the steel tubes

were used to add media or remove culture from the vessel. One of these tubes is used to house the temperature probe, it is not in direct contact with the culture but allows for heat transfer through the tube to the probe by way of a glycerol solution. All of these tubes were permanently attached to the headplate. In addition, the agitation blades were attached to the bottom of the headplate. These blades were turned by a motor that connects to the top of the headplate, and the mixing was controlled via electronic control station and/or computer software.

Several other pieces of equipment were attached to the headplate as well. One of these was the dissolved oxygen probe, which was pushed down into the culture through a gasket-fitted port on the headplate. Also, a rather large port for a screw cap rubber septum was available for adding precultures to the growth media and removing headspace through the septa. For addition of precultures, the screw cap was removed and the preculture poured through the port using sterile technique. Finally, a water-cooled headspace gas condenser connected to 0.2 micron Gelman ACRO 50ST sterile filter (Pall Gelman Laboratory, Ann Arbor, MI USA) was attached to the top of the headplate as well, with a tygon tube line (with clamp) leading to a headspace gas trap. The tygon tubing is fitted with a plastic connector on the end connecting to the condenser to provide a coupling with the condenser's sterile filter. Gas flows through the condenser and the filter, into the tubing and passes into the headspace trap where it bubbles in an oxidizing solution to trap reduced organic compounds in solution.

Sievers 300 sulfur chemiluminescence detection system

The Sievers 300 sulfur chemiluminescence detector (Sievers Instruments, Boulder, CO USA) was the primary detection system used in conjunction with our gas chromatograph for volatile gases in headspace samples. The detector is very selective for volatile sulfur, selenium, tellurium and antimony compounds. A diagram of the detector is shown in Figure 2.

The source of reagent gas was the highly reactive, volatile F_2, which was produced by a high frequency electrical discharge. This was accomplished by applying a high electrical potential across a 1 cm gap, through which sulfur hexafluoride gas (Specialty Products and Equipment, Houston, TX, USA) was passed from an external tank. The production efficiency of F_2 gas using this method was about 10%. From the discharge, gas flows into the reaction chamber, where it meets an analyte (if present) coming off of the gas chromatograph column and reacts to rapidly produce light emissions. These were then detected and amplified by the photomultiplier tube, and a corresponding voltage signal was sent to the integrator to produce a chromatogram. Excess fluorine gas and reacted analyte were removed from the reaction chamber by a constant vacuum by a rotary vacuum pump (Sargent Welch Scientific Co., Skokie, IL USA).

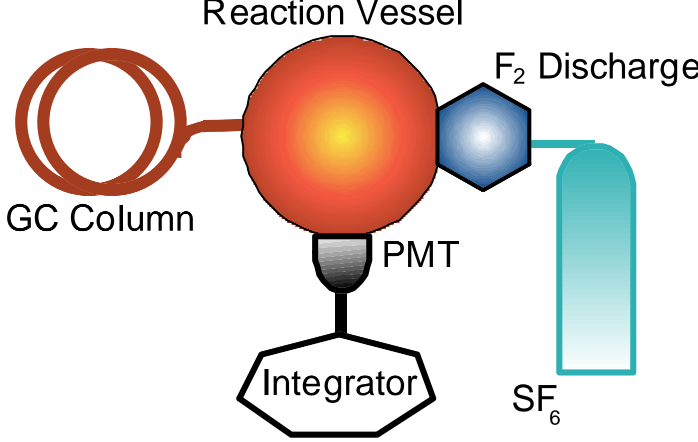


Figure 5. Sievers 300 sulfur chemiluminescence detector schematic.

Media Preparation

Stock solution preparation

Stock solutions for metalloidial amendment of batch cultures were made prior to fermentor inoculation. Two separate solutions were made, each 1 M in concentration. For the 1 M selenate solution, 42 g of sodium selenate 98% (Aldrich Chemical Company, Milwaukee, WI, USA) was dissolved in 250 mL of deionized water and sterile filtered with a 0.2 micron, 250 mL Nalgene disposable filter unit (Nalge Company Rochester, NY, USA) using a vacuum pump (Model 400-1901 - Barnant Company Barrington, IL, USA) to facilitate filtration. After filtration of the solution was complete, the bottom-receiving flask was removed from the 250 mL filter unit, and a sterile cap was placed on the receiving unit for storage. For the production of 1 M selenite solution, 37.5 g of sodium selenite 99% (Aldrich Chemical Company, Milwaukee, WI, USA) was used with the above procedure.

Growth media preparation

The growth media used in all of the experiments for this work consists of 10% tryptic soy broth (DIFCO Laboratories, Detroit, MI USA) and 3% potassium nitrate (certified ACS grade - Fisher Scientific, Houston, TX, USA), hence the term TSN3. Preparation includes making the media solution (10 g tryptic soy broth, and 3 g potassium nitrate per liter water) and sterilizing in a 2540E Autoclave (TuttnauerUSA Co. LTD, NY USA), at 121 ^{o}C for 20 minutes with a secure foil cover over the mouth of the container to prevent contamination during storage (non-refrigerated).

Inoculum preparation

The inoculum for the fermentor was prepared in two steps. In the first step, starting two days before the fermentor experiment, an isolated culture of *Pseudomonas fluorescens* K27 was extracted from a streaked agar plate via sterile wire loop and placed into 40 mL of sterile TSN3 in a 125 or 250 mL Erlenmeyer flask, previously sterilized. These were placed into a water bath shaker (Model R76 - New Brunswick Scientific Co. Inc., Edison, NJ, USA) at 30 ^{O}C with foil cover and shaken vigorously to grow aerobically. These cultures reached stationary phase overnight. The next day, this 40 mL culture was added to sterile 250 mL TSN3, contained in a 500 mL Erlenmeyer flask and this was again vigorously shaken for aerobic growth in a water bath overnight to reach stationary phase. This provided the 10% inoculum for the beginning of a batch culture experiment in the fermentor.

Fermentor preparation

The fermentor was usually prepared the day before the batch experiment was to proceed. Draining of the fermentor was done by clamping off a line between the condenser apparatus and the headspace gas trap, allowing compressed nitrogen gas (Conroe Welding Company, Conroe, TX, USA) to force the liquid through an open valve on a line connected to a port on the fermentor head plate. This line leads to a 20 L waste container; and, in this process, all but several milliliters of the liquid culture were moved to the waste container. The fermentor apparatus was then taken apart, removing the condenser apparatus (gas condenser and sterile filter) and unhooking the condenser's water- cooling lines, the dissolved oxygen probe (which was subsequently placed in a water bath), and the agitation motor, which sits on top of the fermentor. The temperature probe, which was placed in the closed steel tube that extrudes from the headplate into the growth media, was removed. Also, the headspace septa cap was removed, as well as the water lines that control culture temperature connected to the steel base of the fermentor vessel. The condenser apparatus and headspace septa cap were covered with aluminum foil covered and autoclaved separately at 130 ^{O}C for 7 minutes. The fermentor headplate was then removed from the vessel for cleaning. The fermentor vessel was placed in a large sink and a household bleach solution was added to facilitate the oxidation of any remaining organoselenium or organotellurium compounds (which were produced in the previous experiment) into selenate and tellurate salts. After the oxidation, the resulting oxyanions were easily removed by water and bleach. This dramatically reduces the possibility of contamination. The sides of the fermentor vessel were scrubbed with the bleach solution and then thoroughly rinsed from the vessel. Next, liquid soap and water

were used for cleaning, and thoroughly rinsed. The headplate of the fermentor was cleaned in the same manner as the vessel.

Growth media for the fermentor was then prepared. To prepare 2500 mL of TSN3 for the bioreactor, a concentrated solution of TSN3 was made (as outlined in the previous growth media section), added to the fermentor, and then diluted to a final volume of 2500 mL. The headplate of the fermentor was reattached; care was taken that a rubber gasket that fits between the headplate and vessel was aligned correctly between these two pieces. If the gasket was not aligned correctly, the fermentor would not seal and the batch cultures would not grow anaerobically. Sealing problems also prevent liquid samples from being sampled into a receiving vial. Next, the headplate was firmly bolted down to the vessel, hand-tight, to create the necessary seal. The dissolved oxygen probe was then repositioned by insertion in its specific port in the headplate. Foil was then used to cover the condenser opening, headspace septa cap opening, the sample port opening, and a line that leads to the nitrogen gas sparger valve for autoclaving. The nitrogen gas sparger valve line, which has an autoclaveable sterile filter inline and is connected to a steel tube that extends down to the bottom of the vessel, was secured in an upright position to the top of the dissolved oxygen probe to prevent the release of the growth media through the line as it cools. Foil placed over openings to the fermentor helps to prevent contamination after autoclaving. The fermentor was sterilized with a 716-liter autoclave (Wisconsin Aluminum Foundry Co., Inc., Manitowoc, WI, USA) for 50 minutes at 100 ^{O}C. When the newly sterilized fermentor was cool, using a flame for sterile technique the headspace cap and condenser apparatus were replaced. In addition, the water lines to the condenser and vessel were reconnected as well as the line connecting

the condenser apparatus and the headspace trap. Next, the wires leading from the control station to the dissolved oxygen probe were reconnected. Finally, the agitator motor was repositioned at the top of the headplate; the line leading from the headplate to the sparger valve was reconnected, and the temperature probe was replaced with several drops of 50% glycerol solution (Glycerol 99%, Aldrich Chemical Company, Milwaukee, WI, USA) to facilitate adequate thermal conduction.

Experimental Procedure

Inoculation of fermentor

At the beginning of the batch culture experiments, the BioCommand software, used on a Digital Celebris model 4100 computer, was initialized to track the dissolved oxygen and temperature of the culture in the vessel. The water supply to the fermentor was turned on and adjusted to approximately 2 L per minute when the fermentor control station was turned on. The control station was "primed" for water flow, as it has an internal water heater that was used to control the temperature of the water delivered to the fermentor vessel, which in turn controls the temperature of the culture. Temperature and the agitation speed were set on the control panel, 30 ^{O}C and 100 rpm respectively, and the agitation motor turned on. At this point, liquid inoculum was poured into the fermentor vessel through the headspace septa port after the removal of the headspace septa cap to inoculate the culture. Flaming on the port was used to maintain sterile technique. After the cap was replaced, amendments to the culture were made by injecting selenate and/or selenite stock solutions with a 30 mL disposable syringe (Becton Dickinson and Company, Franklin Lakes, NJ, USA) equipped with PrecisionGlide Needle B-D 21g1.5

Sterile (Becton Dickinson, Franklin Lakes, NJ, USA). At this time, the first headspace gas sample, discussed in the next section, was taken. Next, the newly inoculated culture was purged with nitrogen gas to facilitate anaerobic growth conditions. The display panel of the control station was selected to display dissolved oxygen levels, and the nitrogen gas was allowed to flow into the fermentor vessel at a rate of 3 L/min., which produces vigorous bubbling in the culture. The line leading from the condenser apparatus to the headspace trap was opened as well, and vigorous bubbling was seen in the headspace trap. As the nitrogen flow continues, the displayed amount of dissolved oxygen decreased. This was allowed to continue for approximately 15 minutes, after which the dissolved oxygen stops declining. At this point, the dissolved oxygen level indicator was reset to read zero on the display, the nitrogen flow was turned off, and the culture in the fermentor vessel was growing anaerobically.

Dynamic headspace sampling

During the time-course of a batch culture experiment, the headspace gas produced was sampled along with the optical density of the culture. Due to the necessity of a small purge with nitrogen gas for liquid sampling, the headspace samples were taken before liquid sampling. One milliliter samples were taken with a 1 mL gas-tight syringe (Alltech, Deerfield, IL USA) through the septum port on the fermentor. After withdrawing the sample, the gas lock mechanism was pushed to the lock position, and then the sample was injected into the Hewlett Packard 5890 Series II Gas Chromatograph (Palo Alto, CA USA). The chromatographic column used in this work was a DB-1, 5 micron, 30 meter capillary column (J&W Scientific, Folsom, CA USA). Chromatographic data from the detection system was recorded by Hewlett Packard

3396 Series II Integrator. Samples were trapped on column with splitless injection (injector at 275 ^{O}C) with an initial oven temperature at 30 ^{O}C. The initial temperature was maintained 2 minutes, after which a ramp of 15 ^{O}C per minute to 200 ^{O}C was executed. Immediately following, a ramp of 30 ^{O}C per minute heated the oven to 250 ^{O}C, with this final temperature maintained for 3 minutes. Carrier gas flow rates (helium) were maintained at approximately 1 mL per minute. For these experiments, the compounds in Table I were tracked chromatographically throughout the time-course of the batch cultures.

Table I

Headspace Compounds Tracked in Batch Culture Experiments.

Compound	Structure	Boiling Point
dimethyl disulfide (DMDS)	S S	109 ^{o}C[1]
dimethyl selenenyl sulfide (DMSeS)	Se S	~132 ^{o}C[2]
dimethyl diselenide (DMDSe)	Se Se	158 ^{o}C[1]

[1]CRC Handbook of Chemical Physics. [2] Chasteen (1993)

Liquid culture sampling

Liquid culture samples were taken to test for the optical density and pH of the fermentor culture at regular intervals during the lag, log and stationary phases. Liquid

samples were taken through a liquid sample port that allows for metered control. Samples were taken approximately every one to two hours.

Taking samples involved making a small purge of nitrogen gas through the sparger valve. The line leading from the gas condenser of the fermentor to the headspace trap was clamped off to prevent gas flow out, which provides a back pressure in the fermentor that dramatically reduces the amount of purging necessary to take the sample. Next, with the fermentor now gas-sealed, a small 20 mL glass vial, with screw cap removed, was attached to the sample port using the same threading mechanism used to attach the vial screw cap. With the vial secured to the sample port, the in-line flow meter that controls the sparger valve was opened, the resulting pressure allowing liquid sample to flow from the bottom of the fermentor through a steel tube to the sample vial via gas displacement. Approximately 10 mL of sample was taken, and the sample port flow was stopped. The headspace pressure in the fermentor was then re-equilibrated by allowing small amounts of headspace gas to be vented off by reopening the line between the fermentor gas condenser and the headspace trap.

Next, liquid medium sampling optical density measurements were made. Before the experiment began, the Spectronic 20D+ Spectrometer was turned on and remained on through the experiment. The wavelength selected was 526 nm, and the lamp intensity was set to 100%. All optical density readings were taken as absorbance, with a capped test tube filled with sterile TSN3 used to calibrate for zero absorbance. An open test tube, rinsed after each sample, was approximately 80% filled with the sample. Before placing in the sample port of the spectrometer, the sides of the test tube were wiped clean to reduce refraction due to oils on the sides of the test tubes. After placing the sample test

tube in the spectrometer, the port lid was closed to block external ambient light and the absorbance reading was allowed to stabilize. An absorbance reading was taken and the sample was transferred from the test tube back to the sample vial for pH monitoring via Coring pH meter 340 (Corning Incorporated Science Products Division, Corning, NY USA). Biomass per mL liquid medium was determined using a relationship derived earlier by Stone *et al.* (1998). Basically, a linear relationship holds between biomass and optical density.

The pH probe used was kept in a buffer solution at pH 7.0 between readings. When taking samples, the probe was removed from the buffer solution and placed directly into the sample vial. The pH meter was allowed to acquire a stable reading, after which the pH was recorded and the pH probe rinsed with distilled water before replacement in the buffer solution. Finally, the sample was poured into a labeled waste container and rinsed for another sample.

Determination of specific growth rate

To determine the specific growth rate, the natural logs (ln) of the optical density readings were plotted versus time for each culture. From the plot, the data points that best represent the log phase (respective to the culture's growth type) was used to make a linear least squares fit line in the form 'y=mx+b'. From this equation, the slope (m) is the calculated specific growth rate (Paran *et al.*, 1990). Lastly, the specific growth rates for each of the triplicate set are averaged and the standard deviation was found.

Dynamic Headspace Trapping and Analysis

Equipment and reagents

In an effort to determine the mass balance of compounds in the liquid versus the headspace, a different series of experiments was devised to determine the amount of headspace components that can be removed via constant nitrogen purging and subsequent headspace trapping. Analysis was performed with a Perkin-Elmer Model 603 Atomic Absorption Detector (Norwalk, CN USA) equipped with a neon gas lamp (Fischer Scientific Model 14-386-1034, Fischer Scientific, Houston, TX) designed to emit frequencies absorbed by arsenic, selenium, and tellurium. For both the selenium standards and the headspace trapping, a 30% hydrogen peroxide solution (Aldrich Chemical Company, Milwaukee, WI USA), diluted with concentrated HCl (HCl 36.5%-38%, J.T. Baker, Phillipsburg, NJ USA) added after trapping but before analysis, was used for its strong oxidizing ability to convert any reduced organoselenium to back to selenate anions.

Selenium standard preparation and analysis

Selenium standards for calibration were prepared by diluting a 1000 ppm selenium standard (RICCA Chemical Company, Arlington, TX USA) with 30% hydrogen peroxide and a final concentration of 10% HCl. Solutions of 50 ppm, 100 ppm, and 150 ppm were subsequently made. Standards above 200 ppm were outside the linear range of the method, and not examined. Analyses were performed using acetylene, air fuel/oxidant mixture.

The atomic absorption detector was turned on, and the lamp was selected. Power for the lamp was initially set at 0 ma, and gradually raised to 10 ma as specified by the

manufacturer. Next, the flame was ignited by first turning on the air supply, holding a lighter to the flame base, and then turning on the fuel. When the flame was lit, the monochrometer was optimized for selenium. The AA detects light emissions absorbed by ionized selenium at 196 nm. Optimization was made for an emission intensity around 70% of maximum by adjusting the angle of the lamp in the AA and making fine adjustments to the monochrometer which can be monitored by the lamp intensity meter on the AA itself. Water was aspirated between samples. Three readings for each concentration (50 ppm, 100 ppm, and 150 ppm, respectively) were taken. Next, the three data points (absorbance) were averaged and plotted versus concentration. From these three points, a linear least squares fit line was made and the derived equation was used to find concentrations of selenium in the headspace trap solutions based on the absorbance of these solutions.

Headspace trapping and analysis

Batch cultures were prepared and amended according to directions described in a previous section of this chapter, with the noted change of continuous purging of nitrogen gas (~2 mL per minute) during the entire experiment. The physical setup of the fermentor apparatus was the same as well. The culture was allowed to grow into stationary phase and was constantly purged for 24 hours. After this time, the headspace trap (a 250 mL Erlenmeyer flask with 30% hydrogen peroxide) was removed from the gas condenser line and sealed for storage with a firmly placed rubber stopper.

Chapter III

DATA AND RESULTS

The experimental results of several variations of the previously discussed batch culture experiments are detailed in this section. The experimental results are of triplicate batch cultures unless otherwise noted. Error bars on x-y plots represent one standard deviation.

Headspace Chromatography

Headspace samples for all of the bacterial cultures in the bioreactor were analyzed over the time-course by the previously discussed gas chromatography with sulfur chemiluminescence detection (SCD) system. Detection with SCD is very selective for sulfur and selenium compounds. Other compounds separated by the gas chromatograph pass through the detector's reaction vessel and probably react with the reagent gas, but do not emit light detectable by the photomultiplier tube used. The SCD responds linearly over a wide range, from the low pictogram to nanogram range for organoselenium compounds (Chasteen, 1990). This is made possible by several integration time settings available via the detectors' photon counting electronics that determine how long data from the PMT is collected before sending that data to the integrator. Data sent from the detector to the integrator is a varying voltage signal — the more data collected by the photon counting electronics, the higher the voltage signal sent to the integrator. Initial integration times are set at 2 seconds. If the detector readings go above 999 mv, the detector's integration time should be lowed by half, to 1 second. The integration setting can be halved repeatedly, if necessary, down to 0.06 seconds. When changing the

integration time from 2 to 1 seconds, the peak area reported by the integrator is also halved due the photon counting time being halved. When analyzing the data, this can be accounted for by doubling the integrators' reported peak area to obtain actual peak areas normalized to the 2 second integration time. Failure to reduce integration time when analyte emissions in the detector rise above 999 mv for a particular setting causes a "chopping off" effect of peak height with minimal affects on peak width. For this work, integration times were not changed when analyte concentrations went above the threshold for an integration time of 2 seconds in some runs. Therefore, a chopping off effect occurred in chromatograms whose data were used to produce x-y graphs seen in this chapter. Consequently, these invalid data points are displayed in the headspace time course with a dashed line, with a solid line connecting valid points. However, these data points can serve as a minimum concentration. Work in determining the percent deviation from actual values shows that the percent variation between actual peak area and "chopped off" peak area increases with concentration and is no more than 30 percent higher than the highest reported minimum. This was determined by analyzing a series of six similar headspace samples half an hour apart, with alternating detector integration time settings of 2 and 1 seconds. The peak area vs. time for the 2 second integration time headspace samples was plotted and a linear least squares regression line fitted. The same was done for the samples analyzed at a detector integration time of 1 second with normalization to 2 second integration time. From these two line equations, the point where they crossed was determined to be the point past which chopping off occurred as measured by peak area units. Using calibration plots, this resulting peak area was converted to gas phase units ppbv. Using this method, the average concentration past

which concentrations could not be quantitatively known is 80.0 ppbv in this work, because of the integration adjustment error explained above. Finally, peaks above 65.0 ppbv with a standard deviation above 25.0 ppbv are graphically connected with a dashed line as previously discussed due to the error introduced by peak areas that were not accurately measured. That said, discussion of concentration minimums will be given for many of the graphs to show a qualitative contrast in headspace production for various types of amended cultures. Statistical analysis was used to determine the uncertainty in gas phase concentrations calculated via inverse prediction using the standard calibration curves (Harris, 1995). The average uncertainty in headspace concentrations reported here is ± 0.1 ppbv for the three measured components, DMDS, DMSeS, and DMDSe. This takes into account that the calculated slope and intercept of the calibration plots were not independent of each other; therefore, both contributed to the uncertainty in the headspace concentrations determined in that process (Neter et al., 1990). These uncertainties do not apply for the minimum concentration values reported as discussed above.

A representative chromatogram of tracked headspace compounds is shown in Figure 6. With only the exception of the control cultures, changes in concentration of dimethyl disulfide (DMDS), dimethyl selenenyl sulfide (DMSeS), and dimethyl diselenide (DMDSe) are tracked over the time-course. For control cultures, only DMDS concentration changes are tracked.

Detection limits

To find the detection limit for the tracked headspace compounds analyzed by the gas chromatography/sulfur chemiluminescence detection system, chromatographic peaks were found from various chromatograms that were approximately 3:1 signal:noise ratio.

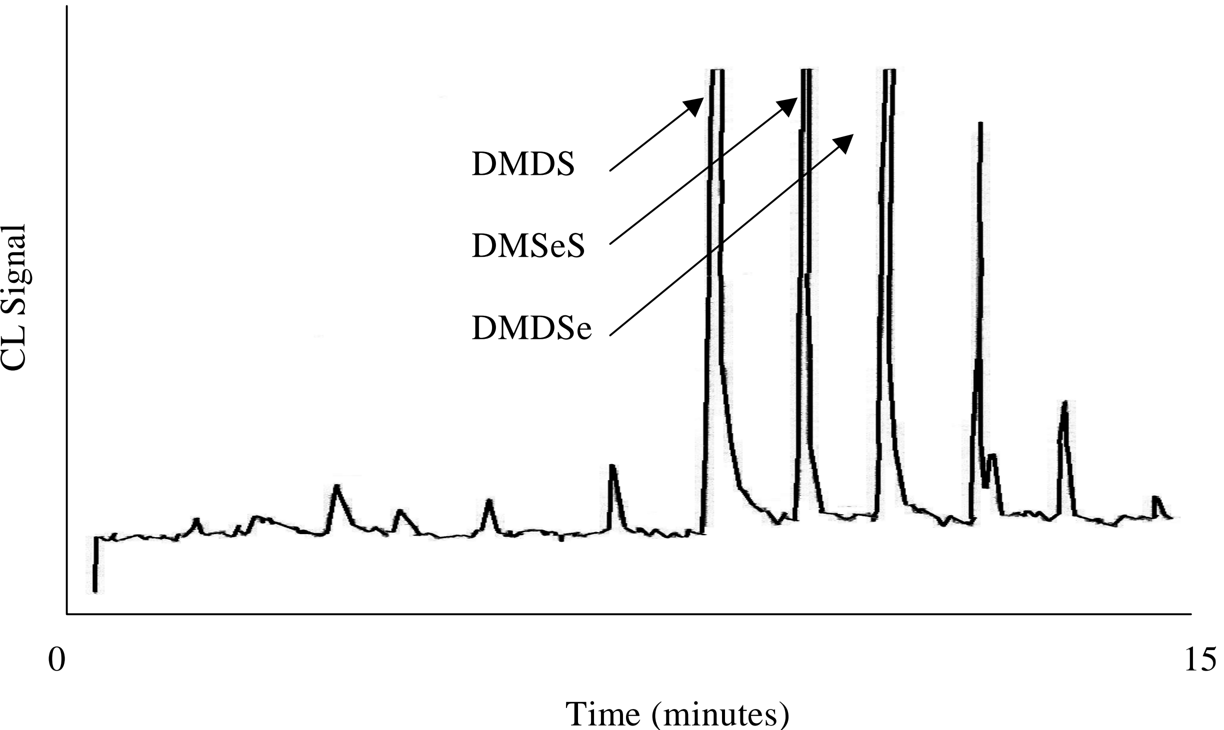


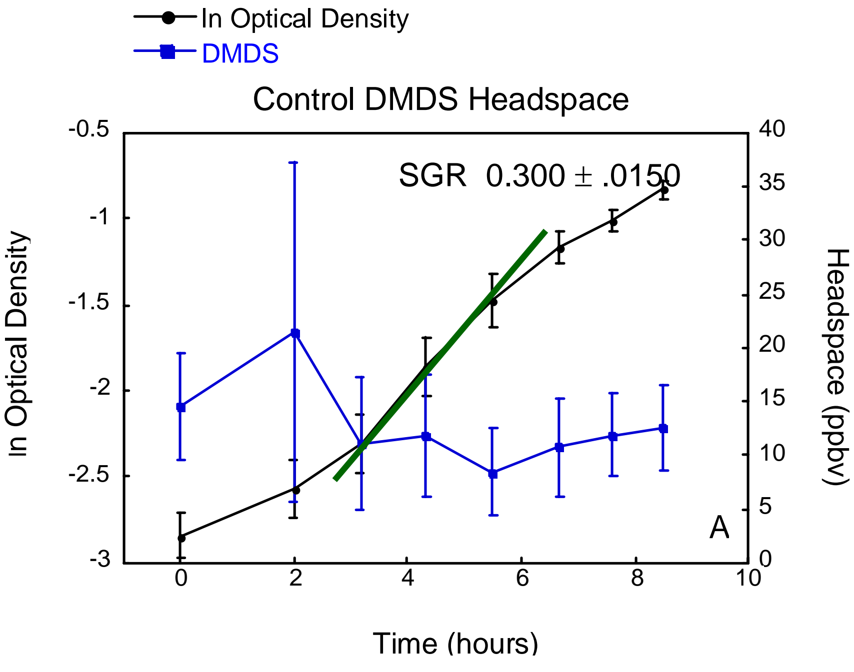
Figure 6. Sample headspace chromatogram. CL signal represents chemiluminescence signal.

When the peak areas were converted to ppbv, the approximate detection limit for DMDS was 1.05 ppbv, DMSeS was 0.412 ppbv, and DMDSe was 1.25 ppbv. Peak identity was determined by injecting known compounds or identified based on GC/MS.

Control Culture Experiments

Experimental work began with investigation of the growth rate, headspace production and pH changes in a 2.7 L batch culture inoculated with 10% by volume (250 mL) *Pseudomonas fluorescens* K27 preculture. No amendments with selenium salts were made.

Two sets of triplicate experiments are reported here. Initially carried out to determine DMDS headspace production or pH change, these two experiments are essentially identical. Experimental results show a classical growth curve with definite lag, log and stationary phases. The optical density readings recorded for these experiments in the stationary phase were quite low when compared to stationary phase optical density readings recorded in other control experiments, which indicates a low cell population relative to culture volume. It is most likely that these readings were a result of precultures not given enough time grow to stationary phase, which was a common problem with other early research experiments (data not shown). Allowing a minimum of 12 hours with vigorous shaking in a waterbath provided for precultures that reached stationary phase and thus higher initial optical density readings for the batch cultures. The specific growth rate average (SGR) for the three control batch cultures was determined to be $0.30\ h^{-1} \pm .0150$ (n=3) for the cultures used to find the control headspace measurements (Figure 7a). A different triplicate set of control cultures used when examining pH readings had a SGR of $0.290\ h^{-1} \pm .0190$ (n=3) (Figure 7b). As expected, these specific growth rates are very similar. Headspace production of dimethyl disulfide (DMDS) in these controls appeared to be relatively high in the lag phase, peaking at 21.5 parts per billion by volume (ppbv). After a reproducible decline in the beginning of the log phase, DMDS headspace concentration steadily increased (with the exception of a peak at 4 hours) to stationary phase with a final ppbv of 12.5 (Figure 7a). In the lag phase, the initial pH readings were around 7.40, with a steady drop to 6.60 during log phase. A slight increase is seen to 6.65 at the beginning of stationary phase (Figure 7b).



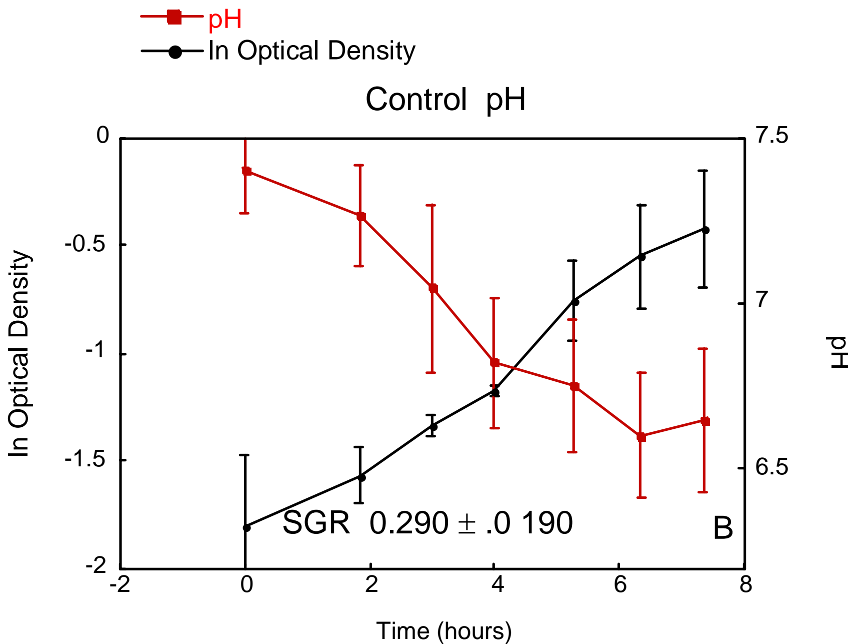


Figure 7. Control growth vs. a) DMDS headspace concentration, b) pH.

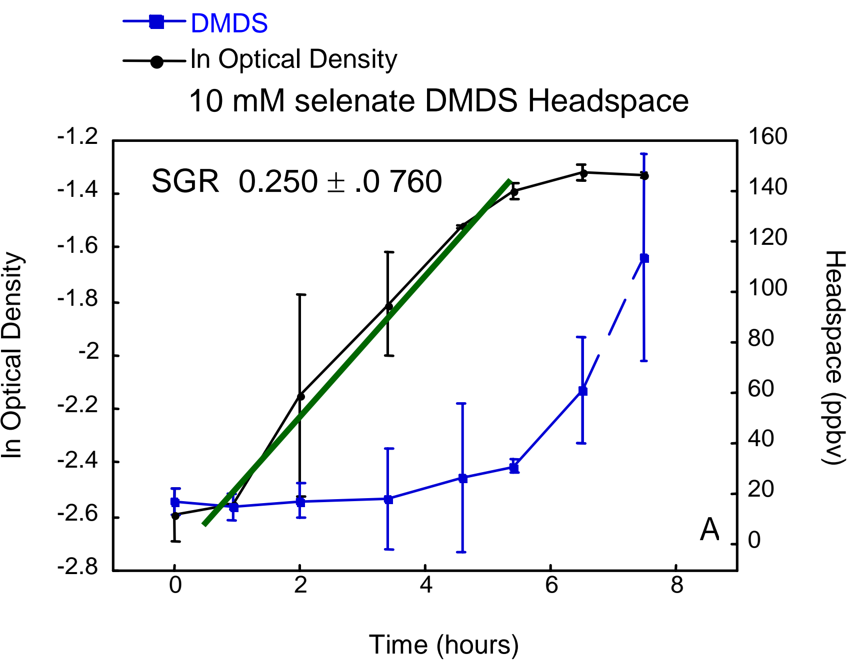
Single Amendment Batch Cultures

The initial research focus for this work was not only to monitor time-course changes in control cultures, but in 10 mM sodium selenate and 10 mM sodium selenite (1:0 and 0:1 selenate to selenite) amended cultures as well. Amendment concentrations were chosen based upon prior work that yielded substantial growth and headspace concentration of volatile organometalloids. These cultures seem to exhibit log phases with a modified growth curve.

Ten millimolar sodium selenate amended experiments

Sodium selenate amended batch cultures differ from the control experiments in that only the growth media was amended to a final 10 mM sodium selenate concentration. The growth curves for these experiments were very similar to the control cultures, but with a lower final biomass attained and smaller specific growth rates. In the presence of 10 mM sodium selenate, *Pseudomonas fluorescens* readily entered log phase without a detectable lag phase. The average specific growth rate was 0.250 ± .0760 (n=3) (Figure 8a). Variations in headspace gas production were also seen. Not only were dimethyl selenenyl sulfide (DMSeS) and dimethyl diselenide (DMDSe) present with DMDS, but also substantial concentration increases were seen beginning in the middle of the log growth phase, with a steady rise in concentration towards stationary phase (Figure 8 a,b; Figure 9a). The highest concentration headspace compound found was DMDS with a minimum concentration of 114 ppbv at the beginning of stationary phase (Figure 8a). The highest concentration of DMDSe was a minimum concentration of 99.0 ppbv at the beginning of stationary phase (Figure 9a). Finally, DMSeS had the lowest headspace concentration of 49.0 ppbv at the beginning of stationary phase (Figure 8b). However, the

change in pH as compared to the control was very different. The pH of the culture increased from 7.44 at the beginning of log phase with a gradual climb to 7.58 just before stationary phase. A slight decline from this peak pH occurs as the stationary phase progressed. The significance of these pH changes are made less important by the large deviations in the pH error bars, which suggest a wide possibility of pH change between runs for these cultures (Figure 9b).



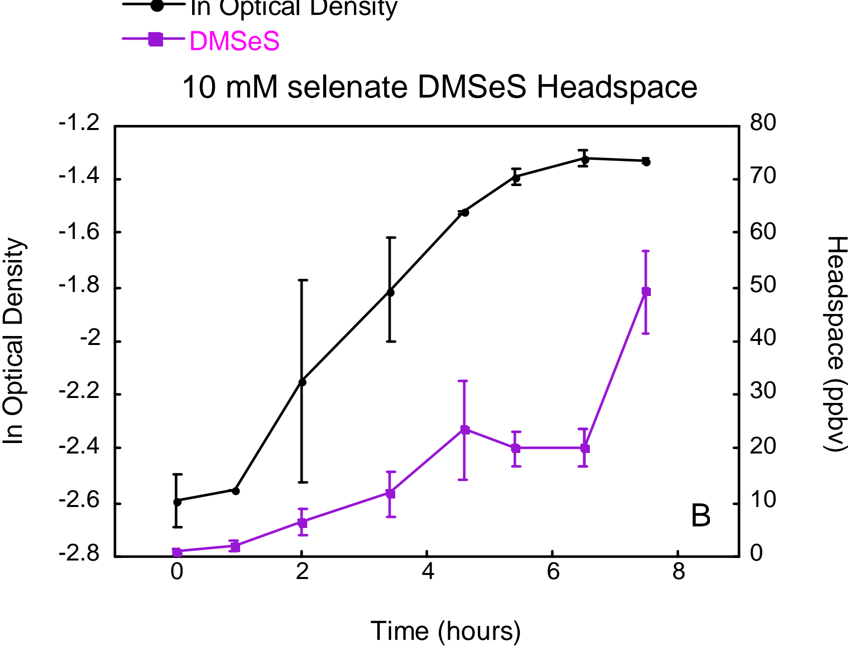
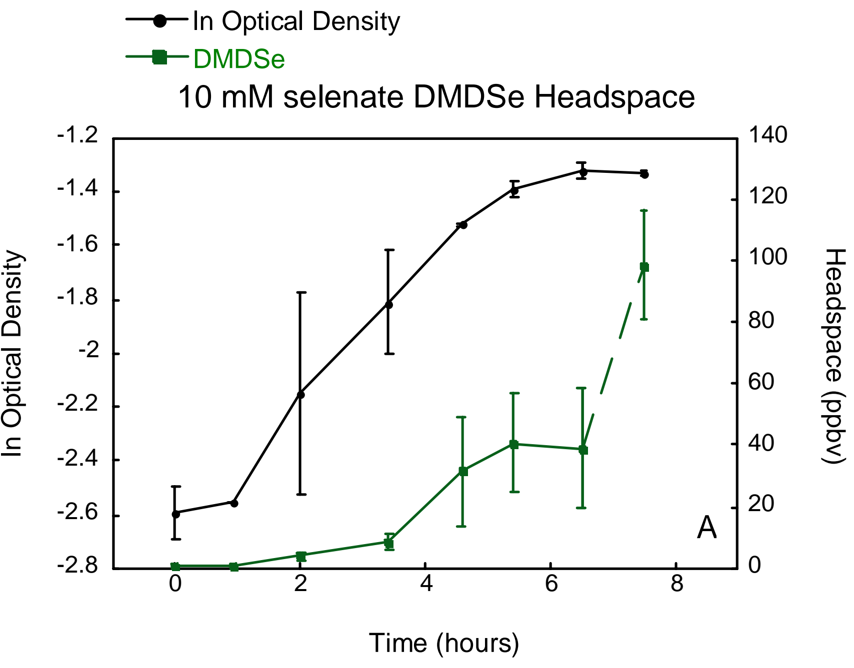


Figure 8. 10 mM selenate amended growth vs. a) DMDS headspace concentration, b) DMSeS headspace concentration.



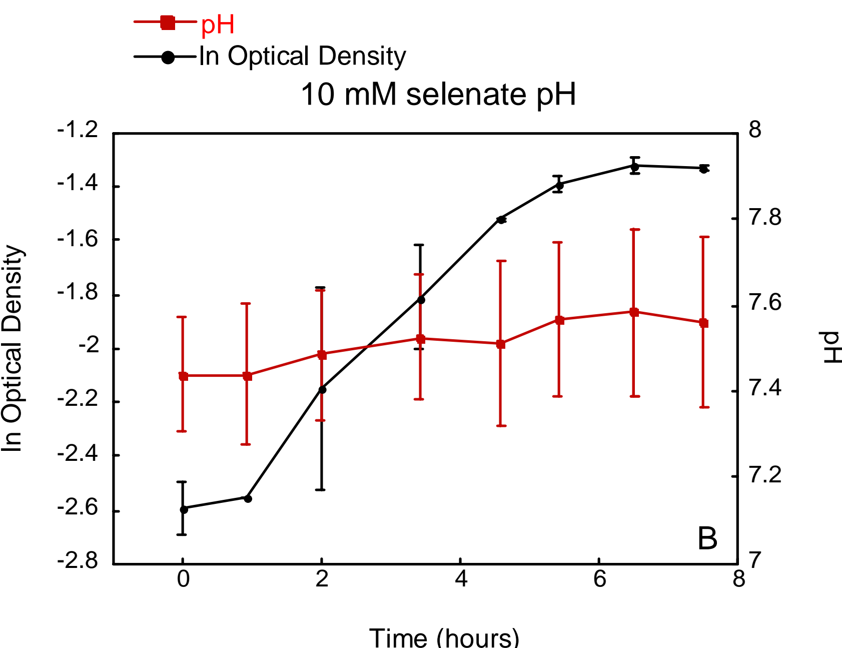


Figure 9. 10 mM selenate amended growth vs. a) DMDSe headspace concentration, b) pH.

Ten millimolar sodium selenite amended experiments

These experiments were performed under the same parameters as the selenate experiments with 10 mM sodium selenite substituted for 10 mM sodium selenite. Growth rates and headspace production were very different. The average specific growth rates was 0.190 h^{-1} ± .015 (n=3) for these systems, substantially lower than the control and 10 mM selenate amended cultures (Figure 10a). Variations in headspace gas production were also seen. Unlike the selenate amended cultures, substantial headspace concentration increases were seen at the end of log phase and into stationary phase (Figure 10 a,b; Figure 11a). The highest concentration headspace compound found was DMDS with a minimum concentration of 75.1 ppbv at the beginning of stationary phase (Figure 10a). DMDSe hit a peak concentration of 35.0 ppbv at the beginning of stationary phase (Figure 10b). Lastly, DMSeS had a minimum concentration of 54.2 ppbv at the beginning of stationary phase (Figure 11a). The change in pH as compared to the control was significantly different. The pH of the culture increased from 7.86 at the beginning of log phase with a gradual climb to 8.06 after 3.5 hours. A slight decline from this peak to 7.01 occurs before climbing to 8.02 at the beginning of stationary phase (Figure 11b).

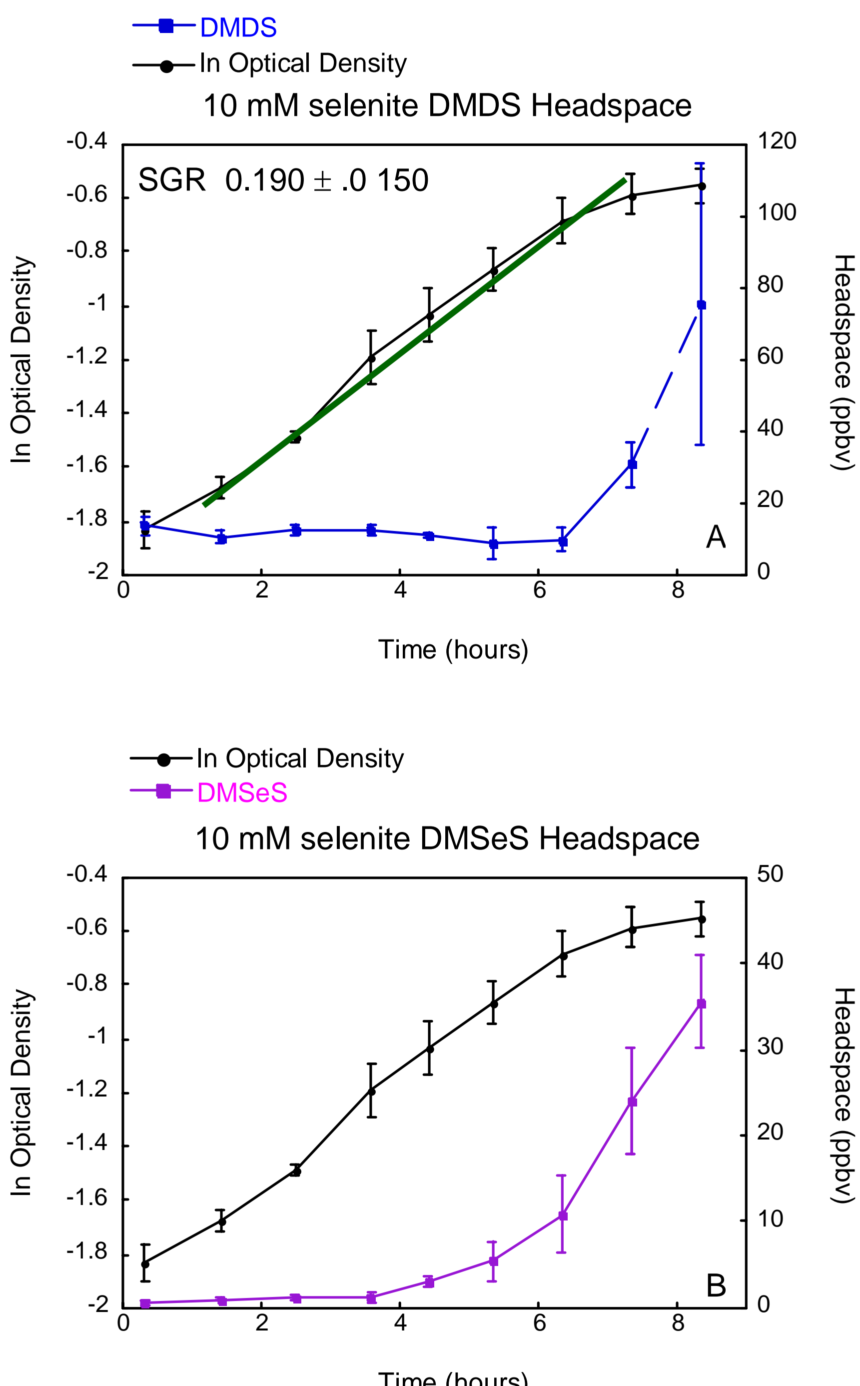


Figure 10. 10 mM selenite amended growth vs. a) DMDS headspace concentration, b) DMSeS headspace concentration**.**

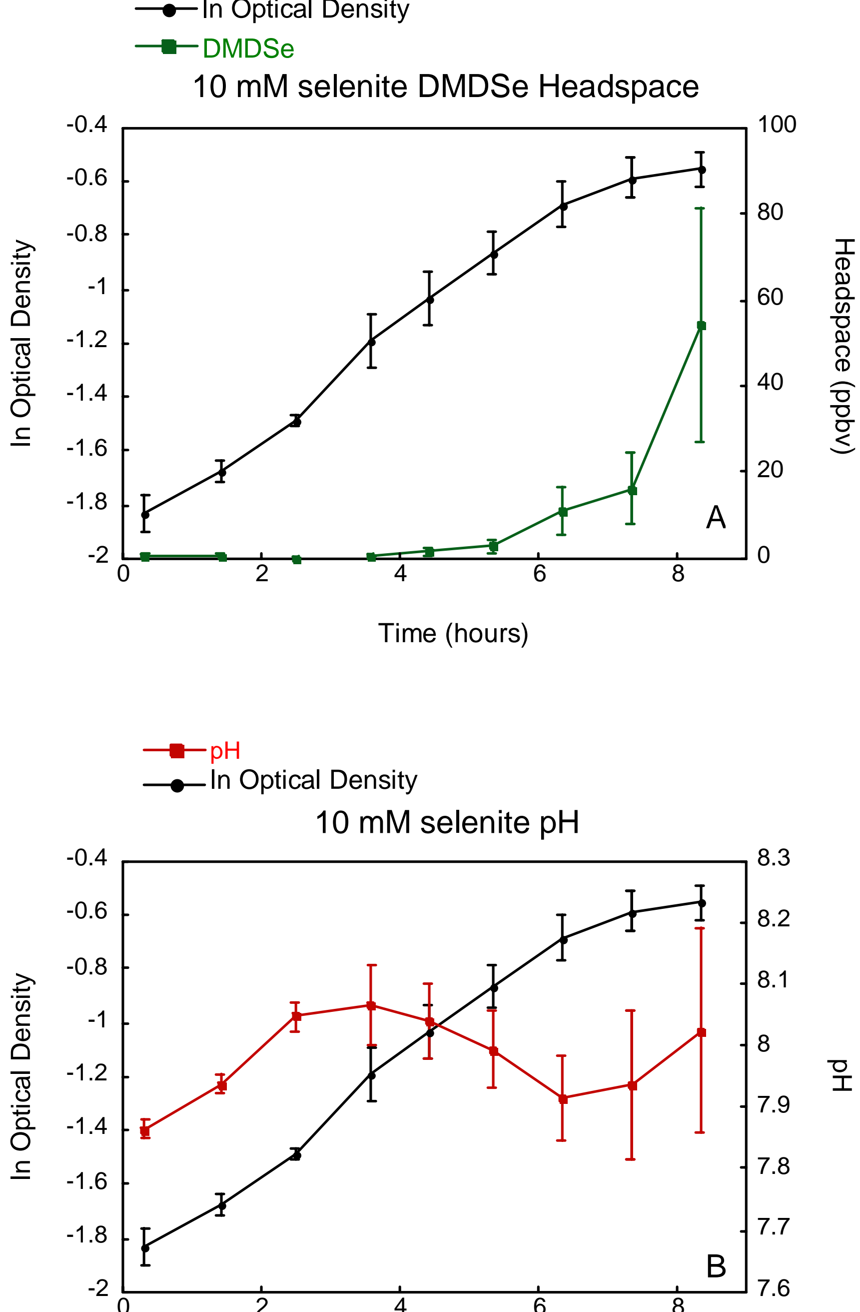


Figure 11. 10 mM selenite amended growth vs. a) DMDSe headspace concentration, b) pH.

Mixed Amendment Batch Cultures

Based upon data from the single amended experiments and with the understanding that in natural or industrial remediation conditions that *Pseudomonas fluorescens* will probable encounter a mix of both selenate and selenite anions, it was decided to explore mixed amended cultures. Mixtures of 1:1, 2:1, and 1:2 selenate to selenite amendments were carried out. Like the "singly" amended cultures, these cultures also seem to exhibit log phases without as much classical growth curve character.

Mixed 5 mM sodium selenate / 5 mM sodium selenite amended experiments

Cultures amended with 1:1 selenate to selenite produced very interesting results when compared with the single amended cultures. The average specific growth rate was $0.118 \pm .010$ (n=3), an SGR lower than all previously reported cultures of any amendment concentration up to 10 mM selenium oxyanions, this though the summed Se concentration is once again 10mM. Headspace concentrations were dramatically different when compared to the singly amended selenate or selenite amended cultures. The highest concentration headspace compound found was DMDS with a minimum concentration of 222 ppbv at the beginning of stationary phase (Figure 12a), which is more than the concentration of DMDS of the 1:0 and 0:1 selenate to selenite amended cultures combined (Figure 8a; Figure 10a). The concentration of DMDSe was a minimum concentration of 156 ppbv at the beginning of stationary phase (Figure 13a). Again, the headspace concentration minimum at the beginning of stationary phase was more than double the concentration of DMDSe of the selenate and selenite amended cultures combined cultures (Figure 9a; Figure 11a). Lastly, DMSeS showed a peak concentration

of 56.0 ppbv at the beginning of stationary phase (Figure 12b). Unlike the concentrations of DMDS and DMDSe, the final concentration minimum for DMSeS was similar to the final concentration for DMSeS of both the selenate and selenite amended cultures (Figure 8b; Figure 10b). The change in pH as compared to the control was very different. The pH of the culture increased from 7.34 at the beginning of log phase with a gradual climb to 7.65 at the beginning of stationary phase (Figure 11b). The small standard deviation in the error bars shows this to be a reproducible trend.

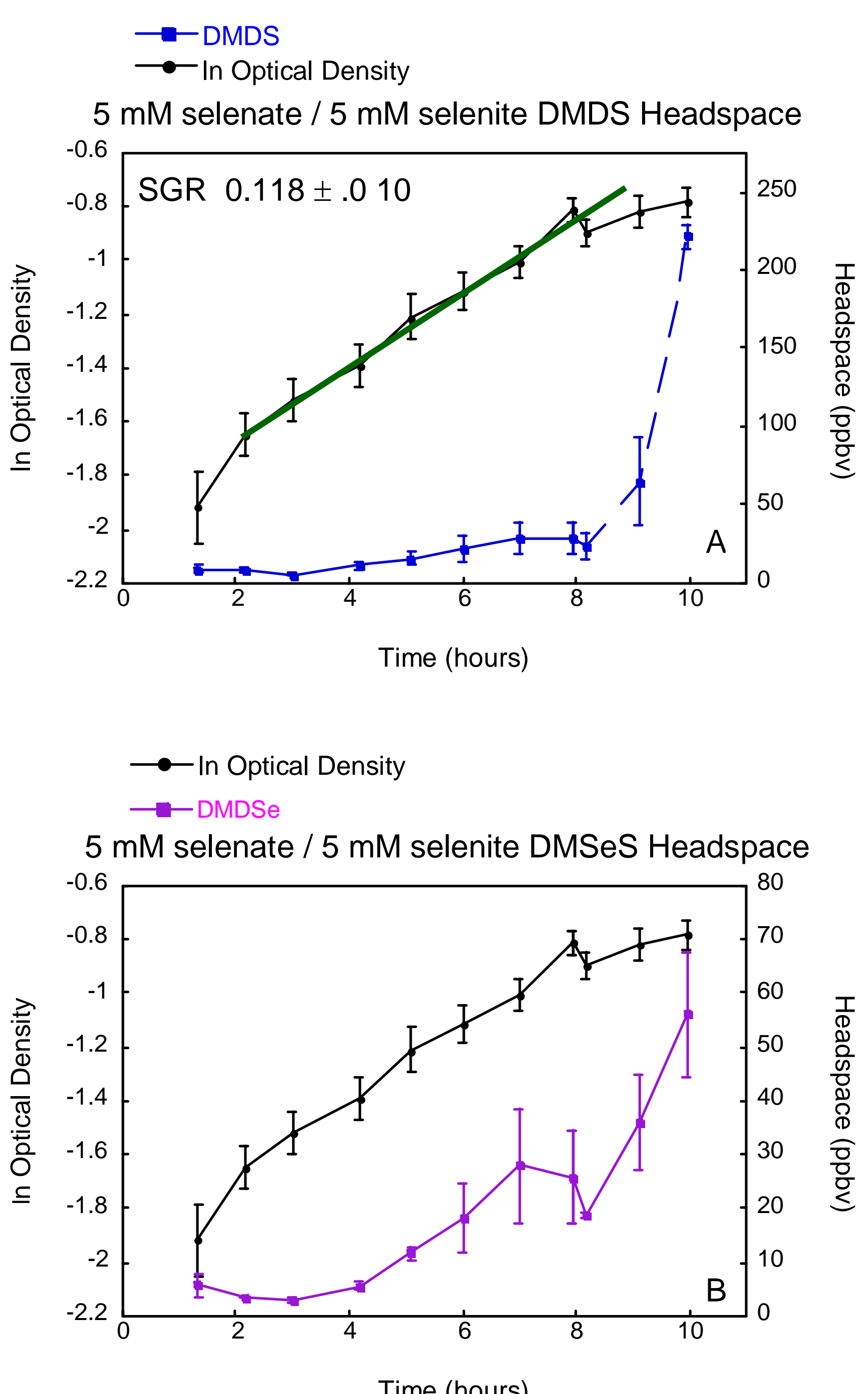
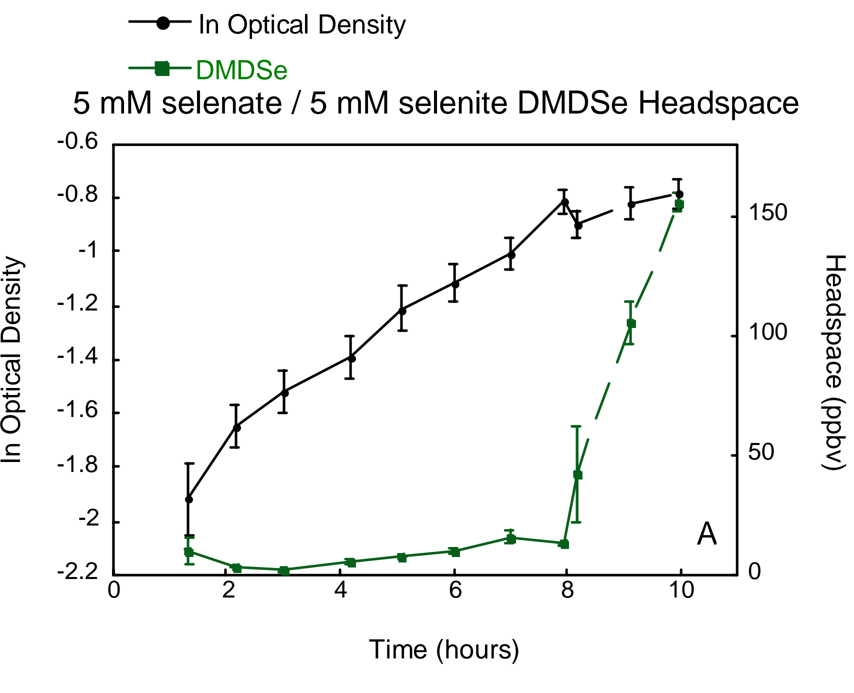


Figure 12. 5 mM selenate / 5mM selenite amended growth vs. a) DMDS headspace concentration, b) DMSeS headspace concentration.



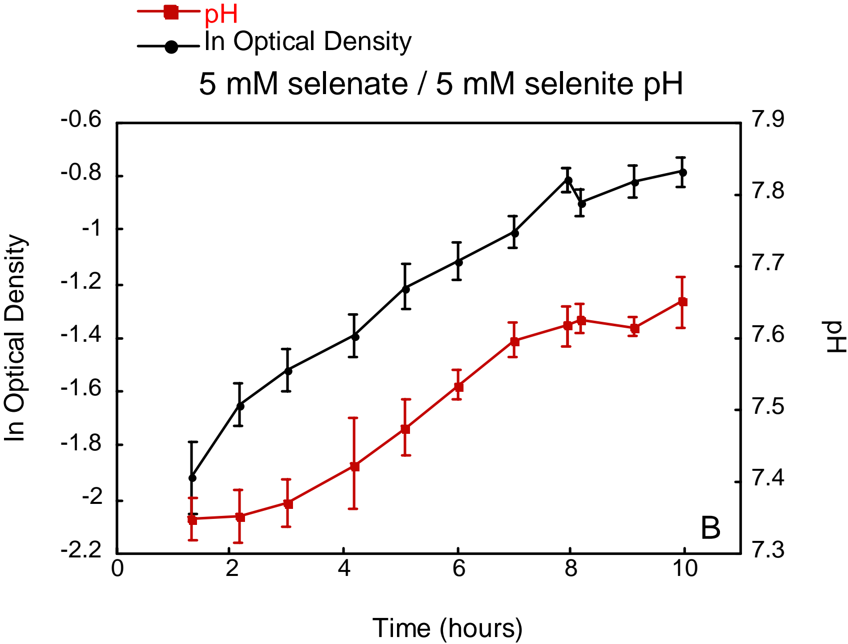


Figure 13. 5 mM selenate / 5mM selenite amended growth vs. a) DMDSe headspace concentration, b) pH.

Mixed 6.66 mM sodium selenate / 3.34 mM sodium selenite amended experiments

With the interesting results from the 1:1 selenate to selenite amended cultures, it was decided to explore uneven mixed concentrations to see if the higher concentration of one anion would influence the growth, headspace production, and pH. The first uneven mixed selected was 2:1 selenate to selenite.

These cultures, again, were quite different when compared with 1:1 selenate to selenite amended cultures and the single amended cultures. The average specific growth rate was 0.160 ± .045 (n=3), an SGR just below the average of the 1:1 and 1:0 selenate to selenite cultures' SGR. It is very hard to place the amounts of headspace gas produced with any repeatability because large deviations occurred between runs as noted by the error bars on the graphs. In general, headspace concentrations were quite low, with the unusual trend of a near continually high production of DMDS when compared to DMSeS and DMDSe (Figure 14 a,b; Figure 15a). Usually, the change in DMDS over the time-course was very similar to the changes in DMSeS and DMDSe concentrations. However, in these cultures, the concentration of DMDS was consistently higher before stationary phase than previous amended cultures, showing up at 40.0 ± 10 ppbv until stationary phase (Figure 14a). At the beginning of stationary phase, DMDSe has a minimum concentration at 83.3 ppbv, but with a standard deviation of 42.3 ppbv. Of interest is that the pH at the beginning of log phase starts at 7.34 and declines to 6.53 at the beginning of stationary phase (Figure 15b). Though large standard deviations were reported, the trend does appear as a decline in pH.

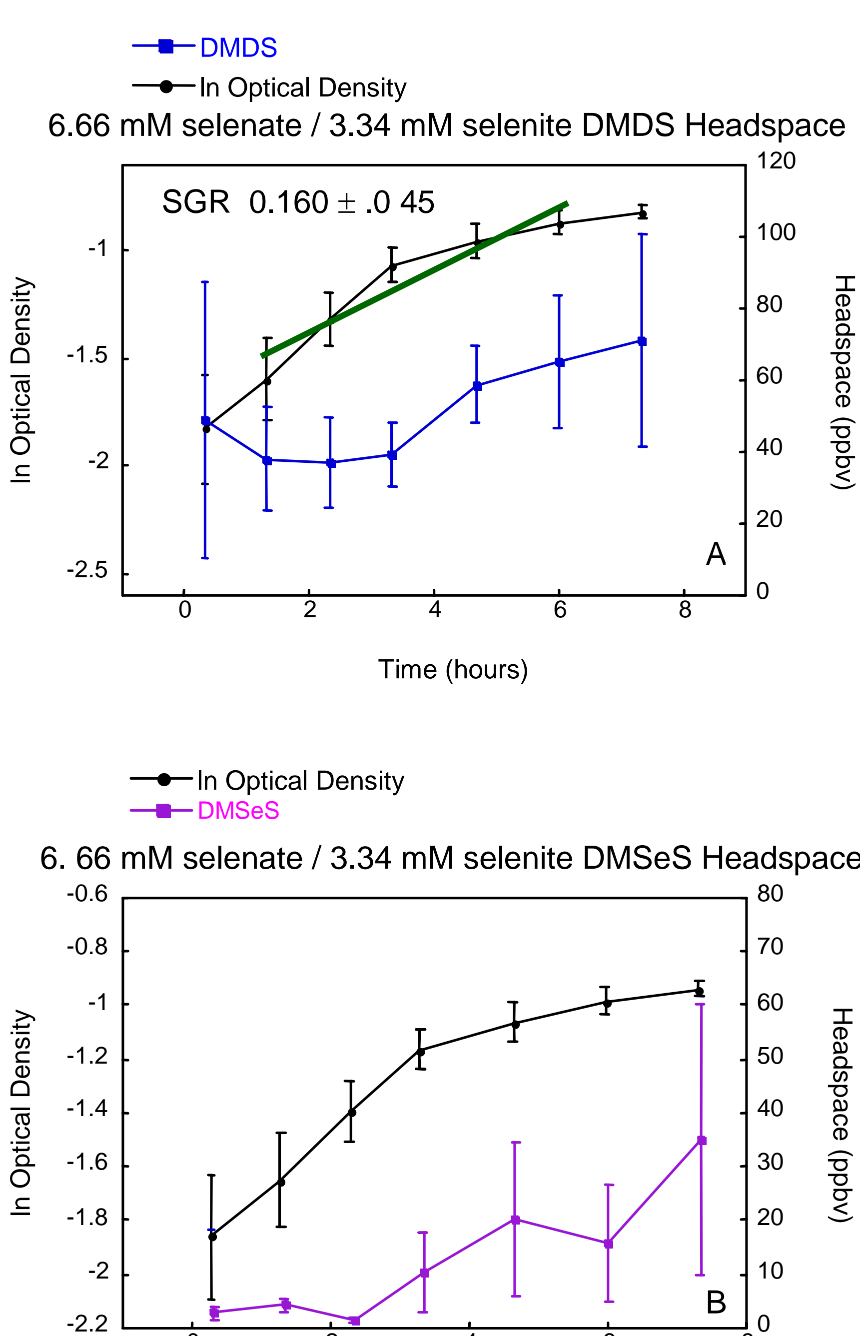
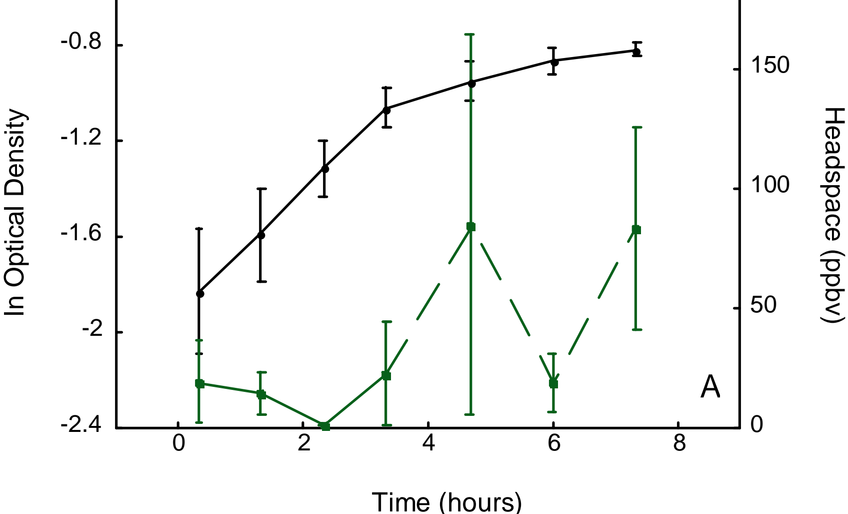


Figure 14. 6.66 mM selenate / 3.34 mM selenite amended growth vs. a) DMDS headspace concentration, b) DMSeS headspace concentration.



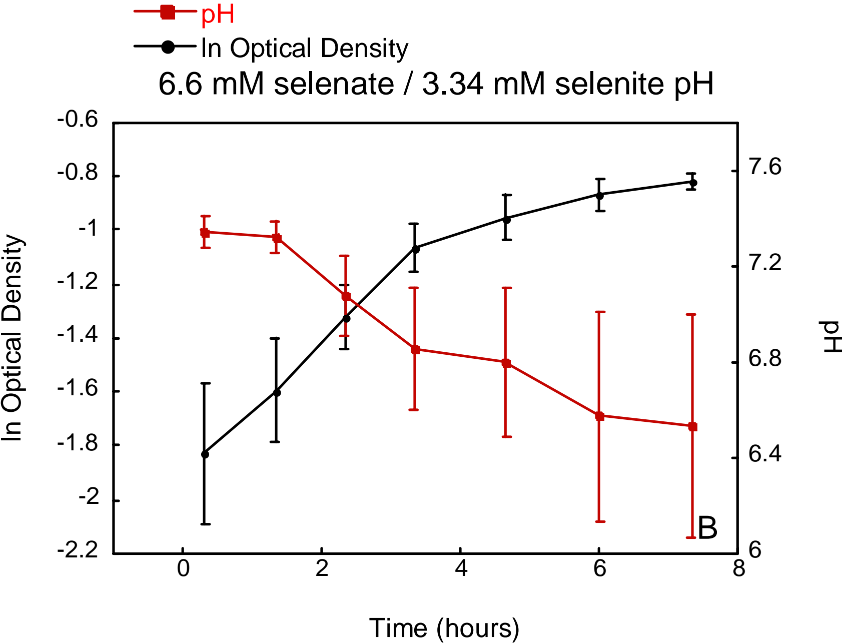


Figure 15. 6.66 mM selenate / 3.34 mM selenite amended growth vs. a) DMDSe headspace concentration, b) pH.

Mixed 6.66 mM sodium selenite / 3.34 mM sodium selenate amended experiments

Results of the 1:2 selenate to selenite cultures were similar to 2:1 selenate to selenite batch cultures. However, the average specific growth rate was $0.078\ h^{-1} \pm .038$ (n=3) for the majority selenite mixture, the lowest specific growth rate of all cultures reported. Concentrations of DMDS were so high that only concentration minimums could be reported, therefore no graph data is shown. Again, concentrations of DMSeS and DMDSe were low and the standard deviations high such that almost no repeatability is shown (Figure 16 a,b). The pH did decline similar to the previous "unevenly mixed" culture, but from a high of 7.97 at the beginning of log phase to a low of 7.50 at the beginning of stationary phase (Figure 17), and again with large variability between runs.

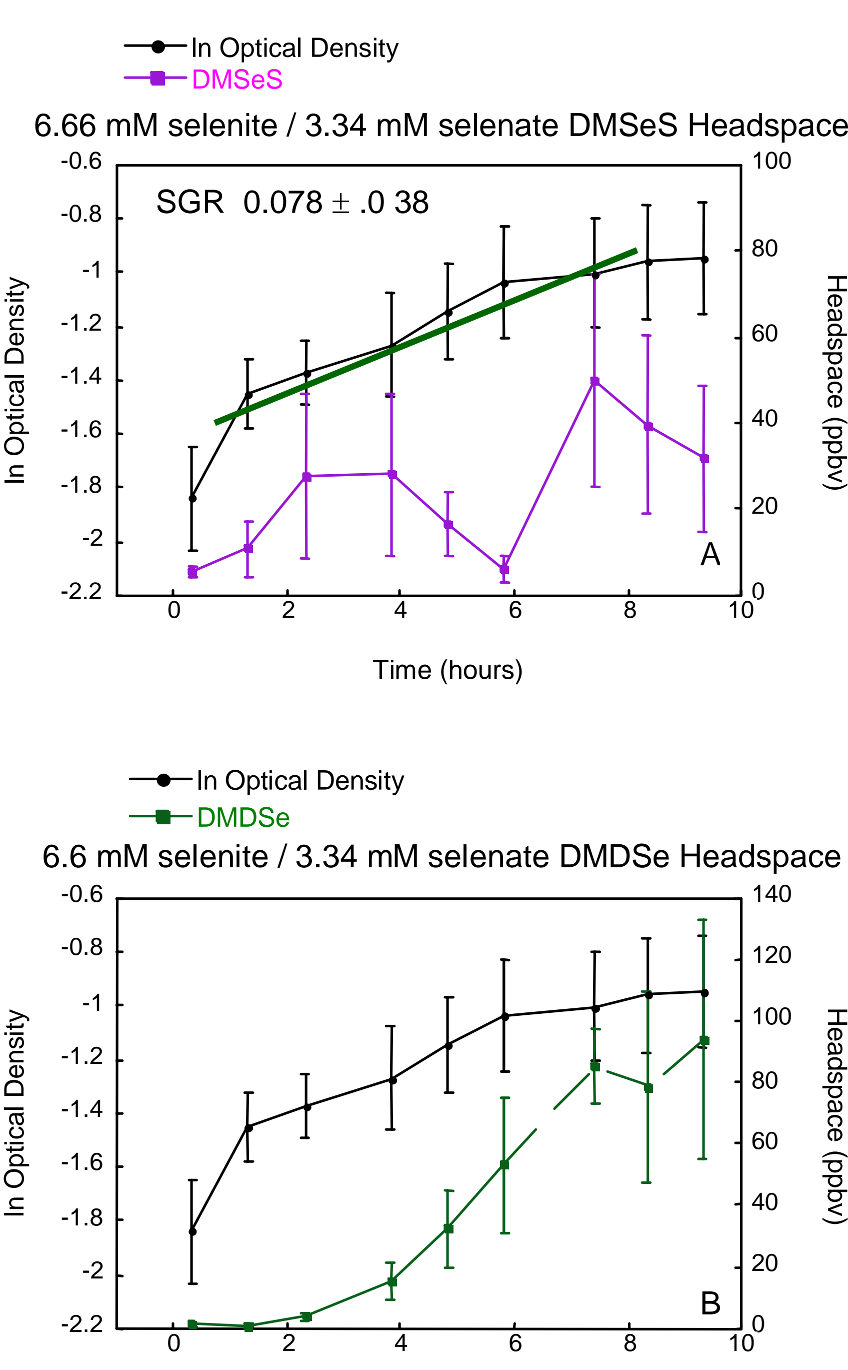


Figure 16. 6.66 mM selenite / 3.34 mM selenate amended growth vs. a) DMDS headspace concentration, b) DMSeS headspace concentration.

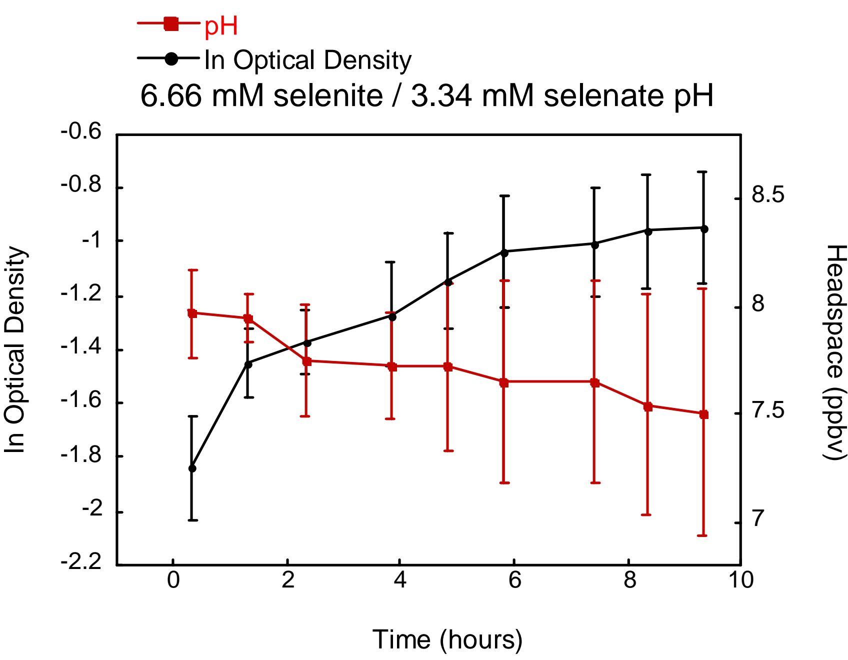


Figure 17. 6.66 mM selenite / 3.34 mM selenate amended growth vs. pH.

Headspace Trapping

During all of the batch culture experiments, the build up of gases in the bioreactor creates a positive outflow from the bioreactor with increasing concentration as stationary phase is reached. To augment this positive flow in the experiments described below, nitrogen gas was purged through the bioreactor, from the bottom of the liquid medium. This constant purging forced gas phase compounds into the external headspace trap over the time-course of the experiment (Figure 18). It was the belief that the nitrogen gas purge would remove the more volatile organoselenide compounds from the fermentor.

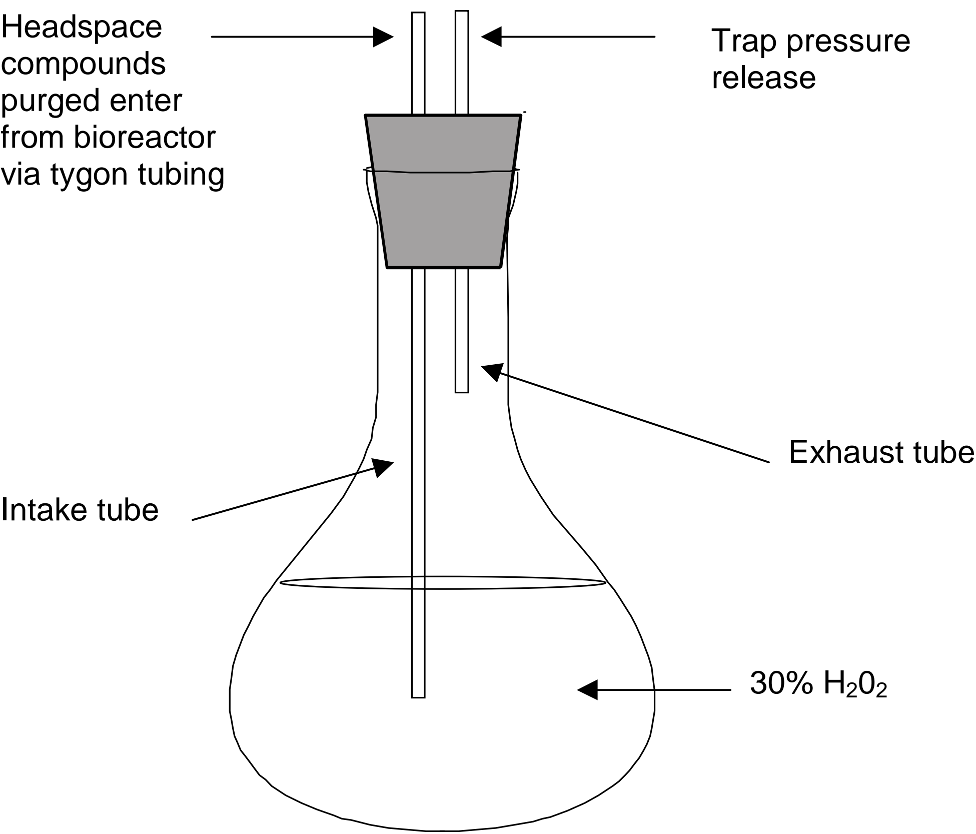


Figure 18. Headspace trap schematic.

In the previously discussed experiments, these gases were trapped in household bleach solution as a method to prevent laboratory contamination. However, using a hypochlorite solution for a headspace trap for atomic absorption could present matrix problems. Therefore, thirty-percent hydrogen peroxide provided a strong oxidative solution for the affective trapping of headspace gases, and 100 mL of H_2O_2 was used for trapping. To create a calibration curve, 5 ppm, 10 ppm, and 15 ppm Se standards were used (Figure 19). Analyses were performed on batch cultures that were allowed a time course of 24 hours (with constant purging) before analysis of the headspace trap sample for selenium. Before analysis, liquid trap samples were boiled so that the hydrogen peroxide concentrations, which have an affect on the AA flame, could be reduced by the release of oxygen gas from the solution while boiling. Individual batch cultures of 10 mM selenate and selenite along with a 5 mM selenate/5 mM selenite culture with a nitrogen purge of approximately 2 ml per minute were performed. Also, a 5 mM selenate/5 mM selenite batch culture was grown and sampled. To determine a percent recovery of Se, the concentration of recovered selenium in the liquid trap was converted to total mass entrapped. This was then divided by the initial mass present in the10 mM selenium oxyanion amended cultures, which was 2132 milligrams. Final recovery results are shown as a percentage of initial Se. Tabulated data is shown in Table II.

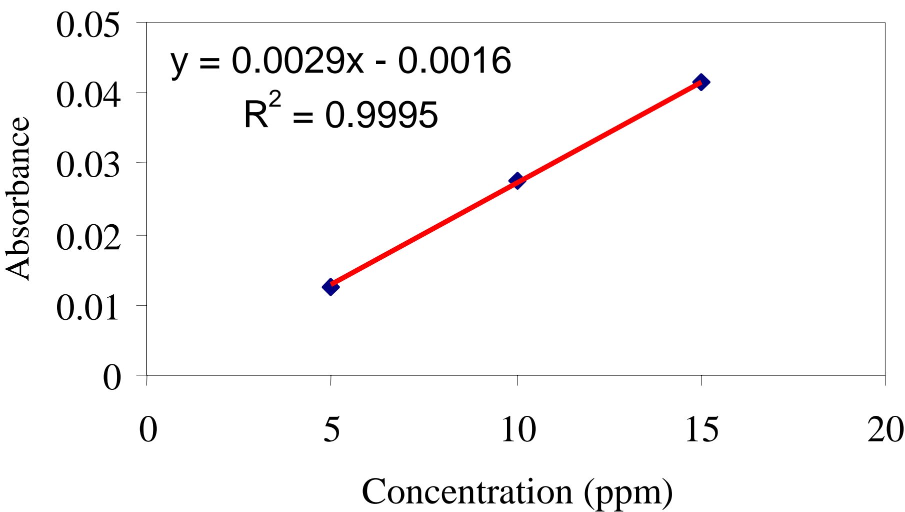


Figure 19. Selenium calibration curve from selenium standards.

Table II

<u>Results of Batch Culture Headspace Trap Experiments.</u>

Batch Culture Type	N_2 Flow Rate (mL / min)	Headspace Trap Concentration (ppm)	Percent selenium recovery
1:0 selenate to selenite	2	10	0.047%
0:1 selenate to selenite	2	10	0.047%
1:1 selenate to selenite	2	9.4	0.044%
1:1 selenate to selenite	10	6.4	0.030%

Chapter IV

DISCUSSION AND OBSERVATIONS

Discussion

Overall, *Pseudomonas fluorescens* K27 in control and amended batch cultures yielded mixed results. Some of the cultures show high repeatability between different runs — such as the control, 1:0, 0:1 and 1:1 selenate to selenite amended cultures. The 2:1and 1:2 selenate to selenite cultures showed less repeatability. The effort to show mass balance with nitrogen purged headspace trapping was ineffective.

Comparison with stationary phase headspace concentration

A comparison of culture type, specific growth rate, optical density at stationary phase (related to final biomass), and headspace concentrations at stationary phase for DMDS, DMSeS, and DMDSe is shown in Table III for all batch cultures in this work. Biomass was calculated based on previous work by Stone *et al.* (1998) and involved calibrations of measured dry weights of harvested cells and optical density. The control attained a final biomass content of 0.35 g/mL and a DMDS headspace concentration of 35.0 ppbv at stationary phase. It also had the highest specific growth rate at approximately 0.30 h^{-1} when the specific growth rates for the two sets of control cultures are averaged (Figure 7 a,b). This specific growth rate is also similar to the one reported by Stone *et al.*, which was 0.266 h^{-1} (1998). The difference in specific growth rate may be attributed to the increase in percent nitrate in the growth media, which was increased from 1% to 3% to overcome nitrate limitation.

Table III

Comparison Culture Type, Final Biomass and Headspace Concentrations at Stationary Phase.

Culture Type selenateto selenite ratio (amended cultures 10 mM)	**Specific Growth Rate (h^{-1})**	**Stationary Phase Biomass g / mL ** (ln Optical Density)**		**Headspace (ppbv) at Stationary Phase**		
				DMDS	**DMSeS**	**DMDSe**
Control (no amendment)	0.300 ± .0150	0.35	(-.77)	35.0	—	—
1:0	0.250 ± .0760	0.30	(-1.37)	114.*	49.0	99.0*
0:1	0.190 ± .0150	0.37	(-.56)	75.1*	24.0	35.0
1:1	0.120 ± 0.0100	0.35	(-.81)	222*	56.0	156*
2:1	0.160 ±. 0.0450	0.34	(-.85)	71.0*	34.0	83.0*
1:2	0.078 ± 0.0380	0.34	(-.96)	too high	45.0	90.0*

*estimated minimum concentration
** using Stone *et al.*, 1998

The 1:0 selenate to selenite amended cultures had the second highest specific growth rate (0.250 h^{-1}), but had the lowest final biomass of 0.30 g/mL. The 0:1 selenate to selenite cultures had a slower average specific growth rate (0.190 h^{-1}) but attained the highest final biomass content of 0.37 g /mL. The headspace concentrations measured at stationary phase for 0:1 selenate to selenite cultures were between 40% − 60% lower than then corresponding measurements made for the 1:0 selenate to selenite cultures. As a comparison of final biomass to stationary phase headspace production, the 1:0 selenate to selenite cultures produced a much higher headspace concentration per unit of biomass. This suggests that selenate is more toxic than selenite when the cultures are exposed individually to the same concentrations of the different anions. The lower optical density at stationary phase for the 1:0 selenate to selenite cultures as compared to the control and 0:1 selenate to selenite cultures, along with higher headspace concentrations produced, may suggest that reduction and methylation of selenate anions is very efficient when compared to selenite anions. This also suggests that *Pseudomonas fluorescens* K27 cultures may actually transfer metabolic and reproductive resources to reduction and methylation of selenate at certain biomass density or when confronted with a threshold Se concentration. That said, toxicity is more often determined via a measurement of changes in specific growth rate, and this would therefore connote that selenite is more toxic than selenate, results that are inline with previous work with this microbe (Chasteen, 1998).

As previously discussed, it is unlikely that *Pseudomonas fluorescens* K27 would encounter one type of anion exclusively in environmental or industrial remediation situations, which necessitates experimentation with mixed amendment cultures. The evenly mixed cultures containing 1:1 selenate to selenite exhibited an average specific

growth rate (0.120 h^{-1}) much lower than any of the control or single amended cultures, but had a final biomass content of 0.35 g/mL similar to the control at 0.35 g/mL. Most interesting were the results that showed that the headspace concentrations in stationary phase for the individual compounds tracked in the 1:1 selenate to selenite mixed culture were just above or below the sum of the stationary phase headspace concentrations for the 1:0 selenate to selenite and 0:1 selenate to selenite cultures. This may suggest that there are separate mechanisms for conversion of selenite and selenate to volatile organoselenides working at the same time. Another possible conclusion is that the presence of both anions speeds the detoxification response.

Mixed amendment cultures with unequal anion concentrations were also performed to see if 2:1 or 1:2 selenate to selenite amended cultures would influence growth rate, headspace production and pH. In average specific growth rate measurements, 2:1 selenate to selenite amended cultures showed a growth rate approximately 0.04 h^{-1} *faster* (SGR of 0.160 h^{-1} ± 0.45) than the 1:1 selenate to selenite average specific growth rate. The 1:2 selenate to selenite cultures' growth rate was approximately 0.04 h^{-1} *slower* (SGR of 0.78 h^{-1} ± 0.038) than the 1:1 selenate to selenite average specific growth rate. That the 2:1 selenate to selenite amended cultures grew faster than the 1:1 selenate to selenite suggests that in this amendment mixture, the abundance of the selenate anion actually increases the growth rate for mixed cultures, using the 1:1 selenate to selenite amended cultures as a "mixed control." And therefore, as expected, the abundance of the selenite anion in the 1:2 selenate to selenite mix actually decreases the specific growth rate as compared to the mixed control. This also supports that the conclusion that selenite is more toxic than selenate. Final biomass

results differ however. Unlike the large difference between the 1:0 and 0:1 selenate to selenite cultures, the 2:1 and the 1:2 selenate to selenite cultures' final biomass results were only 0.001 g biomass / mL apart and near that of the control.

Headspace readings were also varied. For DMDSe, the headspace concentrations at stationary phase for the 2:1 and 1:2 selenate to selenite cultures had similar final minimums of 83.0 ppbv and 90.0 ppbv respectively at the beginning of stationary phase. The other compounds concentrations varied greatly. For the 2:1 selenate to selenite cultures, DMDS concentration at stationary phase was very similar to the control, and the DMSeS concentration was low. For the 1:2 selenate to selenite cultures however, the DMDS concentrations were so high over the time course as to be non-reportable but also with a low DMSeS concentration. From these results, a 2:1 or 1:2 selenate to selenite mix does seem to affect specific growth rate definitively, but no systematically observable conclusion can be based on headspace concentrations.

Comparison Between pH Change over Time Course

A comparison of culture type, specific growth rate, pH change, and relative pH change is shown in Table IV for all batch cultures in this work. For the control cultures, the average starting pH was about 7.4 and ended at 6.6. Prior work by Stone *et al.* showed an increase in pH over time-course for control cultures from 7.2 to 7.5 for experiments with this same microorganism (1998). A slowing in pH drop or increase seemed to occur when stationary phase was reached and this would be expected if the pH change were biologically driven as opposed to a response from an external source. Unlike the control, the 1:0, 0:1 and 1:1 selenate to selenite cultures all showed a *slight increase*

Table IV

Comparison of Culture Type, Specific Growth Rate, and Change in pH.

Culture Type selenateto selenite ratio (amended cultures 10 mM)	**Specific Growth Rate (h^{-1})**	**Initial pH**	**Final pH**	**pH change**
Control	0.300 ± .0150	7.4	6.60	decrease
1:0	0.250 ± .0760	7.46	7.49	no change
0:1	0.190 ± .0150	7.85	8.02	increase
1:1	0.118 ± 0.0100	7.35	7.63	increase
2:1	0.160 ±. 0.0450	7.35	6.48	decrease
1:2	0.078 ± 0.0380	7.86	7.5	decrease

in pH. The 1:0 and 1:1 selenate to selenite cultures all started near the pH of the control, at 7.46 and 7.35 respectively. The 1:0 selenate to selenite culture only showed a climb to 7.49, and the 1:1 selenate to selenite cultures showed a climb to 7.65. Both seemed to change linearly as opposed to the variable response seen with the 0:1 selenate to selenite amended cultures (Figure 11). The 0:1 selenate to selenite seemed to start higher than the control at a pH of 7.85. The 1:2 selenate to selenite cultures also seemed to start near the same value at 7.86. This increase in culture pH for these two cultures may be due to the interaction of the selenite anion with water to slightly increase the hydroxide concentration, and thus the pH, of the culture. The 0:1 selenate to selenite amendment had an overall positive trend exhibited in a sine curve manner with small deviation; whereas, the 1:2 selenate to selenite cultures declined to an average of 7.5, but had increasingly large variability over the time course of multiple runs. The 2:1 selenate to selenite cultures started near the control norm at 7.4 and decreased to 6.48 in a manner very similar to the control. Although the standard deviation for pH measurements between runs increased over time, the final pH is still well below the starting pH.

In general, a drop in culture pH is indicative of acidic extracellular wastes in the culture, such as lactic acid. Increases in culture pH can be due release of basic components such as ammonia produced via deamination (Kurtz, H., Sam Houston State University, personal communication, 1999). Since most bacteria have an optimal pH range in which they grow, changes in extracellular pH outside of this range typically causes the cell to change its *intracellular* pH. If the extracellular pH is too low the culture cells may increase their intracellular pH, or vice versa. Large changes in intracellular pH may dramatically slow or stop cell growth (Cherlet and Marc, 1998). The

drop in pH with the control and 2:1 selenate to selenite amended cultures suggests a build up of acidic wastes that may bring about a sudden decline in cell growth due to the cultures' inability to intracellularly buffer the extracellular pH. This suggests that achievement of stationary phase due to pH growth inhibition might be reached before essential components of the growth media are exhausted and instead would be brought about solely by pH changes. The 1:2 selenate to selenite cultures do show a pH drop over the 10 hours of a typical experiment, but not as large as that of the control and the 2:1 selenate to selenite amended cultures. Along with the singly amended cultures and the 1:1 mixed amendment culture, the 2:1 selenite to selenate may actually reach stationary phase due to exhaustion of essential components of the growth media. It is apparent that maintaining an optimal culture pH may be important for attainment of high biomass and a sustainable culture growth. From this work, it is difficult to reach a conclusion on the effect of pH change due to the large amounts of variability in the 10 mM selenate and both 2:1 mixed cultures.

Headspace Trapping Results

Results from the headspace trapping experiment showed that very little selenium is transferred out of the bioreactor in the trapping method used. The results show that for all cultures tested, less than 0.05% of added Se was recovered in the trap. It was anticipated that the nitrogen purging would remove volatile organoselenides in the liquid culture phase and transport it to the trap. Purging is necessary since both DMDSe and DMSe (not tracked in this work) at equilibrium have a water concentration approximately 17 times greater than the headspace concentrations (Gürleyük, 1996). No established

physicochemical properties for DMSeS are known, the last volatile selenium compound reported here. Since only very low flow rates were used in these trapping experiments (2 mL/min) the amounts of volatile organoselenium removed appears to be insignificant. This may very well have been due to the low flow rates and high reactivity of these highly reduced compounds removed by the purging process. No effort was made to passivate the tubing or glass tubes used in the purge apparatus (Figure 18).

Observations

- Selenite is more toxic than selenate to K27 in this system based on 1) specific growth rate of the 0:1 selenate to selenite cultures compared to the 1:0 selenate to selenite cultures, and 2) change in specific growth rate in the 1:2 selenate to selenite cultures as compared to the 1:1 selenate to selenite cultures' specific growth rate.
- Selenate amended cultures produce more headspace per unit biomass than selenite based upon a comparison of stationary phase optical density vs. stationary phase headspace compound concentrations for 1:0 selenate to selenite cultures with 0:1 selenate to selenite cultures.
- An equal mix of selenate with selenite anions produces the most organoselenide headspace compound concentrations when comparing the 1:1selenate to selenite cultures' headspace compound concentrations with the all other selenium oxyanion amended batch cultures.
- In mixed amended batch cultures, concentrations of selenate and selenite effect specific growth rate when comparing the 2:1 selenate to selenite cultures' and the 1:2 selenate to selenite culture' specific growth rate with the 1:1 selenate to selenite cultures' specific growth rate. The "uneven" mixed amendment cultures of 1:2 selenate to selenite exhibited to lowest SGR determined (0.078 h^{-1}).
- More investigation into the effect that change in pH has on batch cultures of *Pseudomonas fluorescens* K27 needs to be made due to the large amount of variability shown in many different culture amendment types and the disagreement in control pH as reported by Stone *et al.* (1998). Finding an ideal pH range for optimal

and continual growth is necessary for sustaining optimal culture growth in both batch and, eventually, continuous cultures.

- The method used to trap purged culture headspace compounds was ineffective. This may be accounted for by the inefficiency of the low flow rate and reactive surfaces in the trapping apparatus.

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VITA

Lynn A. Eriksen Jr. was born on July 9th, 1971 in Galveston, TX. After graduating from Spring High School in the spring of 1989, he began undergraduate studies at North Harris County College in the fall of the same year. An accomplished swimmer in high school, he also began coaching for summer recreational swim teams during the summer of 1989. While continuing to teach swimming while attending college, he transferred to Sam Houston State University in the fall of 1991. In December 1995, Lynn graduated with a bachelors degree in All-level Kinesiology, a minor in Chemistry, and completing teaching certification. Certified in All-level Kinesiology, Composite Sciences, and Chemistry — he taught Chemistry I and Physical Science during the 1996-1997 school year. After returning in the Fall of 1997 to Sam Houston State University to pursue a Masters of Science in Chemistry, he graduated in the May 1999. His interests range from teaching swimming and chemistry at the secondary and junior college level, to developing internet-based learning web sites for a variety of subjects.