

DESIGN AND APPLICATION OF A DYNAMIC HEADSPACE  
SAMPLING SYSTEM FOR THE STUDY OF BIOREMEDIATION  
OF TOXIC METALLOIDS BY BACTERIA

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Master of Science

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by

Steven L. McCarty

December, 1994

DESIGN AND APPLICATION OF A DYNAMIC HEADSPACE  
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# Abstract

**McCARTY, STEVEN L.** Design and Application of a Dynamic Headspace Sampling System for the Study of Bioremediation of Toxic Metalloids by Bacteria.

Master of Science (Chemistry ), December, 1994, Sam Houston State University  
Huntsville, Texas.

## **Purpose**

The purpose of the experiments describe here was to follow the output of reduced chemical species produced by metalloid-resistant bacteria.

## **Methods**

In order 1) to remove the volatile biological wastes produced as these organisms biodegrade selenium oxyanions and 2) to maintain an anaerobic environment, the bacterial cultures studied had to be continuously purged with an inert gas. This therefore required the trapping and concentration of the gas phase components before analyses. Variable trapping times allowed for the changes in headspace component concentrations associated with the continuous fluctuation in the bacterial efficiency of bioremediation. The trapping apparatus consisted of a double focussing cryogenic system with an initial trap external to the gas chromatograph. The second cryogenic trapping was performed by the oven of the gas chromatograph.

A bioreactor was designed that interfaced with the purge and trap system. It allowed for anaerobic sampling and continuous purging and mixing of the bacterial culture with sterile N<sub>2</sub>.

The methylated selenium and sulfur compounds released by these bacteria were separated by capillary gas chromatography and detected by fluorine-induced chemiluminescence.

## **Findings**

A dynamic headspace sampling apparatus was designed and constructed. Headspace gases above anaerobic bacterial cultures were sampled over long times course experiments. Large volumes of headspace gases could be collected, cryogenically concentrated, and then cryofocussed in an external subambient cryotrap and again in the GC oven. Organosulfide and organoselenide compounds could be determined from that headspace. Repeated sampling of the same culture over time permitted a time based examination of the bioremediation of the oxyanions of selenium by anaerobic bacteria.

The high boiling organo-chalcogens were examined routinely over experiments up to 100 hours long using the same culture. Plotted over time, these analyses showed that the production of dimethyl disulfide and dimethyl diselenide tracked the growth curve of the facultative anaerobe examined, *Pseudomonas fluorescens* K27.

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Thomas G. Chasteen  
Thesis Director

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A special thanks is reserved for my parents for their assistance in everyway there is possible.

This thesis and work is deciated to my best friend Kim Sassie. Her friendship allowed me the peace of mind to follow through and complete the work.

# Table of Contents

Abstract.....	iii
Acknowledgments .....	v
Chapter I. Introduction .....	1
Chapter II. Experimental Methods .....	6
Chapter III. Data .....	20
Chapter IV. Results and Discussion .....	30
Conclusions .....	33
Bibliography .....	34
Vita.....	35

# CHAPTER 1

## INTRODUCTION

In 1860 the first US case of selenium poisoning was recorded by the army doctor at Fort Randle Nebraska. He reported that if their horses grazed in a particular area around the fort, they would suffer from severe loss of hair, deterioration of the hoofs and finally loss of life. He also noted that the cattlemen's livestock also suffered the same fate (Muth, 1967). The disease was called alkali disease and the blind staggers. In 1929 K. W. Franke discovered that the cause for alkali disease was high concentrations of selenium. In 1934 Franke made a very important discovery that correlated plants and selenium to alkali disease. He found that certain plants would accumulate selenium at levels higher than the surrounding soils: biomagnification or bioaccumulation (Muth, 1967). This is an extremely important aspect of the work of bioremediation of selenium contaminated soils.

Selenium is one of many trace elements that is essential for life; however it can be deadly at higher than trace levels. It is very rarely found in the environment at concentrations higher than trace levels. Above or below this range can have serious consequences. Selenium is considered a trace material yet it is the 9<sup>th</sup> most abundant element on the planet. Geologically, selenium occurs often where sulfur is abundant. It tends to be rare in volcanic rocks, however it also tends to concentrate in sedimentary rocks. Shale, a sedimentary rock, has higher than average Se concentrations. Shales from the Cretaceous period range from 0 to 103 ppm in concentration of selenium. The shales of the Permian period in the Wyoming area range in concentration from 100 to 675 ppm of selenium. These shales are weathered away leaving soils that have unusually high concentrations of selenium (Muth, 1967).

The range grasses that occur on high selenium soils have detrimental effects upon the livestock that feed up on the plants. The plants can functionally concentrate selenium

in the plant body, but the selenium seems to concentrate primarily in the seeds (Hamilton and Beath, 1963). The animals that eat these plants can develop alkali disease or the blind staggers along with a multitude of internal and external maladies: loss of hair, elongation of hoofs and in higher concentrations the blind staggers. Internal effects are atrophy and cirrhosis of the liver, enlarged gallbladder, an enlarged heart (Rosenfeld and Beath, 1964). These diseases are dependent upon the concentration of selenium that build up in the animals system. The blind staggers results from the diseased closure of the animals eyes causing the animal to stagger blindly in a circle usually followed by sudden death. Acute poisoning is also a problem; the largest recorded loss of livestock occurred in Elk Mountain Wyoming. Some 340 sheep died within 24 hours after consuming unknown quantities of the seleniferous plant *Astragalus bisulcatus* (Rosenfeld and Beath, 1964; Muth, 1967). Seleniferous plants have the ability to concentrate selenium within parts of the plants. These plants tend to be rather odorous due to the releases of reduced selenides from the plants' leaves. Many of these same reduced gases are also released by certain bacteria in soils and water with high selenium content (Burton *et al.*, 1987). The plants present a danger to any animal consuming them or to people consuming animals that feed upon these plants. The problems presented by toxicity and bio-magnification of selenium have lead to extensive research in the San Joaquin Valley in California, an area rich in selenium (Burton *et al.*, 1987). The San Joaquin Valley is one of the largest producers of vegetable produce for the United States. To put it another way, the food that many of the people of the USA consume every day is produced in this valley.

The San Joaquin Valley is surrounded by mountains with a selenium rich Marino shale of the Cretaceous period. This shale is rich in selenium in its various oxidized forms (Burau, 1985; Tanji *et al.*, 1986). The runoff from the mountains brings the salts of selenium down into the valley. Normally this is not a problem however valuable outside water is brought in to irrigate the crops (from as far away as Colorado). The excess water

trickles down through the porous upper soil to encounter an impermeable clay layer underneath the agriculture crops. This water was originally diverted by a drainage system to the Kesterson Reservoir that collected the surface and subsurface waters. These runoff waters were diverted to holding ponds for evaporation because environmental restrictions did not allow for movement of this water to the Pacific Ocean for disposal. In addition to selenium it contains arsenic, boron, cadmium, chromium and copper (Marshall, 1985). In the two primary oxyanionic forms, Se is toxic to most organisms in high concentrations. Sodium selenate has an LD<sub>50</sub> of approximately 15 mg/kg and sodium selenite has an LD<sub>50</sub> of 7 mg/kg (both 48 hour tests; Ingersoll *et al.*, 1990). These evaporation ponds set up a situation that was biologically selective for bacteria and other organisms resistant to the toxic metals and metalloids present. Another way of looking at this is that this environment enhances the growth of these particular bacteria over non-resistant forms.

There has been an extensive amount of research in the Chasteen research group on the effects of the selenium on facultative anaerobic bacteria, primarily focused on determining the gases released by the bacteria, relative distribution of selenium in bioremediation cultures (Jiang, 1994; Jiang and Chasteen, 1994) and the synthesis and toxicity of proposed intermediates (Coffman and Chasteen, 1994; Zhang, 1994; Zhang and Chasteen, 1994). Most of the research has used static headspace sampling of anaerobic bacteria (Chasteen *et al.*, 1990; McCarty *et al.*, 1993; Stalder *et al.*, 1994; Zhang and Chasteen, 1994). To isolate the selenium resistant bacteria, water or soil samples were added to selenium enriched media plates, spears, and in broths to select for bacteria able to grow in the enhanced environment. Then the resistant bacteria were separated by performing colony isolations on plates with the same type of media. Once the bacteria were isolated, for example *Pseudomonas Fluorescens* K27 from the Kesterson reservoir (isolated by Ray Fall, University of Colorado, Boulder, USA), experiments were carried out in a series of Hungate tubes with a media of tryptic soy broth with potassium nitrate as the

terminal electron acceptor. The tubes were capped with open top caps and Teflon<sup>®</sup> septa. The bacteria were grown for 24 hours or longer. The septa were pierced with a gas sampling syringe and a one mL of headspace gas was pulled and injected into the GC for the analysis. This type of sampling has several advantages: The analyst is able to sample exact volumes at a constant temperature, and the headspace gases reflect in some manner the concentrations of dissolved gases. The injection is quick and allows for reasonable gas chromatographic focusing.

Unfortunately several problems are associated with the use of septa sealed Hungate tubes. The size of sample injection is limited by the volume of the gas syringe. After an injection is made, an elaborate process has to be performed on the syringe to clean out the residues of the sulfides and selenides. From our experience large amounts of residues can be obtained from the contaminated syringe for days after the injection if it is not carefully cleaned. For example if a highly concentrated headspace is sampled, after the sample is injected and the chromatographic run completed, an uncleaned syringe can be filled with 1 mL of clean nitrogen then injected into the GC and it will give results almost equal to the original injection. This makes quantitation extremely difficult or almost impossible.

Most waste by-products of metabolism are toxic to an organism—especially nitrogenous wastes (Campbell, 1990). Although little is actually known about the biological effects of reduced sulfur or selenium compounds created *in vivo*, it would be a reasonable assumption that these waste products also are toxic to the organism that produces the waste, although dimethyl selenide is known to be less toxic to test organisms than selenium oxyanions like selenate and selenite (Wilber, 1990; Ingersoll *et al.*, 1990; Sandholm, 1993). If this is true then the metabolism and growth of the bacteria might be affected as the concentrations of the metabolic wastes dissolved in solution increase with time. The most probable effect would be a decrease (in growth and) in the production of the reduced gases due to the weakening of the organism.

The final analytical objection to bacterial static headspace analysis is that the gases above the bacteria in static headspaces can only be sampled one time due to the nature of the septa used to seal the tubes. These rubber disks, coated with a thin sheet of Teflon, are not trustworthy enough to reseal after being pierced by a syringe needle. Since many of the bacteria we work with are anaerobic or change their metabolism in the presence of O<sub>2</sub>, leaking septa are not acceptable. In order to have time course studies of these bacteria and to thereby study the production of bioremediation products over extended periods, a complete series of tubes would have to be setup at one time and sampled one time each and then discarded. This requires large numbers of tubes in order to have a significant number of data points.

In order to correct these problems we proposed and built a dynamic headspace sampling system that overcame many of the faults of static tube experiments. The problem of headspace gas buildup was eliminated by continually purging clean nitrogen through the media and the headspace. This shifts the “equilibrium” in such a way that the gases do not built up as readily on the culture container’s glass surfaces, in the culture media itself, or on the enclosure cap’s underside. The sample carry over problems experienced with syringes was eliminated also by using chemically deactivated and continually heated (trace heated) transfer lines between the bioreactor and the cryogenic trapping system and GC injector. This allowed for a more realistic look at the production of gases by the bacteria. The system also allowed for almost an unlimited control in the size of the injected sample because of the design of its cryogenic trap. Sample volumes of bacterial headspace ranged from as large as 180 mL to as small as 6 mL. This meant that headspace gas samples with very small concentrations of headspace analytes could be preconcentrated before being analyzed by gas chromatography.

## CHAPTER 2

# EXPERIMENTAL METHODS

### Reagents

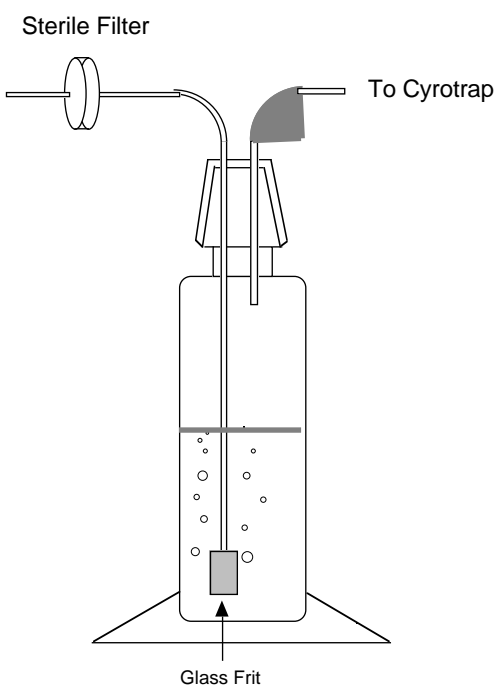
Sodium selenate was purchased from Strem Chemical Company (Newburyport, MA, USA). Tryptic soy broth (TSN) was purchased from Difco (Detroit, MI, USA). Potassium nitrate came from Fisher Scientific (Houston, TX, USA). TSN medium was made by adding 10 g tryptic soy broth and 1 g  $\text{KNO}_3$  to 1 L water. These solutions were filter sterilized before use.

### The Dynamic Headspace Analyzer

#### Bioreactor

The bioreactor used in the experiments reported here consisted of a 250 mL gas washing bottle courtesy of Dr. Rick White. Figure 1 is a drawing of this apparatus. Earlier bioreactor experiments were attempted with a 38 mm by 195 mm test tube (Figure 2) and a 500 mL Erlenmeyer flask (Figure 3) as bioreactors but the chromatographic results pointed to two major problems, sample size and contamination. Both of these problems are discussed briefly below.

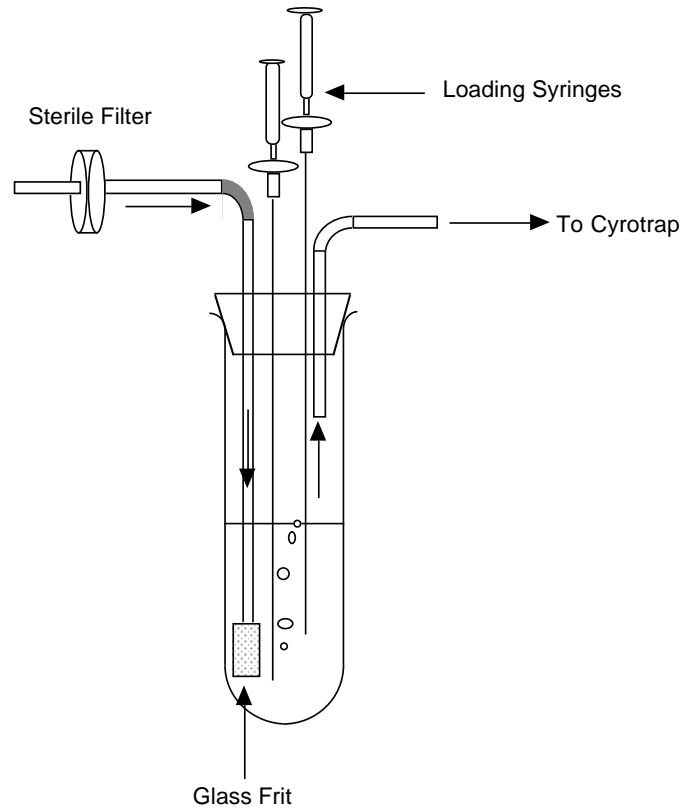
The decision to use the test tube reactor was based upon our previous work with static headspace sampling of Hungate tubes. In these experiments 10 mL of sterile culture media in septa sealable glass test tubes was inoculated with K27 and other various bacteria, then incubated for 24 hours in a water bath at 30° C. These experiments demonstrated the prolific production of reduced sulfide and selenide gases by the bacteria. *Pseudomonas fluorescens* K27 was one of the most productive of the bacteria tested. In a selenium-free TSN media, K27 consistently produced large volumes of methanethiol, dimethyl sulfide, dimethyl disulfide and possibly dimethyl trisulfide. In media poisoned with sodium selenate it also produced the reduced sulfides along with dimethyl selenide,



**Figure 1.** GasWashing Bottle Bioreactor

dimethyl diselenide and dimethyl selenenyl sulfide (Chasteen *et al.*, 1990). In the planned dynamic headspace experiments, the bacterial culture (and headspace) would be continually purged with nitrogen so that the amount of bacteria in the culture had to be increased in order to enlarge the headspace concentrations of organosulfides and organoselenides. Therefore we decided upon a media volume of 10 times that of the Hungate tube, a total of 100 mL.

The test tube reactor consisted of a 38 mm by 195 mm test tube with a rubber stopper (Figure 2). Inserted through the stopper was one 1/4" by 4" glass tube bent at a 90° angle to allow for the exit of gas from the reactor. Adjacent to this, a fritted tube (Fisher Scientific, Pittsburgh, PA, USA) was inserted through the stopper until it touched

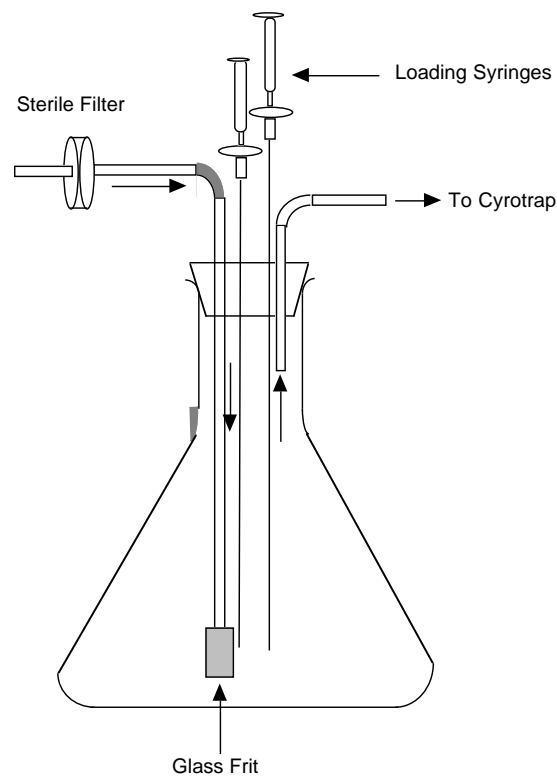


**Figure 2.** Test Tube Bioreactor

the bottom of the reactor. The incoming purge gas flowed down through the tube and bubbled up, purging the media. Finally, two 18 gauge 12 inch needles (Ace Glass, Vineland, NJ, USA) were shoved through the rubber stopper and inserted down into the media. Attached to the needles were 25 mm x 0.2 mm pore size sterile filters (Alltech, Deerfield, IL, USA) and a syringe. These needles allowed for the sterile addition of fresh media, selenate solution and the inoculation of bacteria into the reactor without opening the system to invasion by oxygen.

During the test runs, the average cryogenic trapping time was approximately 30 minutes. In the beginning of the experiment, trapping time ranged as high as 70 minutes. This presented problems of chromatographic non-reproducibility and the disadvantage of the need for large liquid nitrogen volumes for cryogenic cooling. The long trapping times also did not allow for a quick repeat of the sampling. Thus a reasonable verification of the chromatographic (and biological) results was unattainable.

To correct the problems of the test tube reactor, a larger 500 mL Erlenmeyer flask was used to replace the test tube (Figure 3). The same stopper, frit tube, glass tube and needles described above were used on the flask. A total of 300 mL of media was incorporated into the reactor system; however, the size and shape of this reactor presented problems that could not be overcome. The total flow of the nitrogen through the system



**Figure 3.** Erlenmeyer Bioreactor

was limited by this stopper arrangement. If a higher flow rate was attempted by increasing the pressure of the purge gas, the pressure exceeded the ability of the stopper to stay in the flask and it simply popped out of the mouth of the flask or leaked. This limited the ability of system to successfully transport the headspace gases to the cryotrap and thereby allowed for an increase of dissolved gases in the culture media (that is, unpurged remediation products) that would therefore not be successfully purged and trapped. This gave an appearance of a slower increase in headspace gases and slower decline in gases over a longer period of time. Part of this design problem lay in the shape of the Erlenmeyer flask itself. The gases moved up through the center of the flask, failing to expose a large volume of the media to the nitrogen transport gas. This problem was compounded by the fact that the physical mixing of the media was performed only by the passing of purge gases up through the media—no media stirring was attempted.

The larger volume of the Erlenmeyer flask and the mixing ability of the test tube were both incorporated in the gas washing bottle finally used for the bioreactor (Figure 1). It had a very tall slender shape allowing for maximum contact of the purging gas with the liquid media and the 250 mL total volume of this vessel allowed for a media volume of 170 mL, an approximate midpoint between the volumes of the two previous reactors. Furthermore changing to the new reactor disposed of the troublesome rubber stopper. It is possible that the rubber itself acted as a sponge for the reduced headspace gases and subsequently acted as a source of sulfur and/or selenium compounds later in the experiment. Unfortunately, the loss of the rubber stopper also meant the loss of the syringe and needles used for injection of additional components to the bioreactor; all the culture components have to be added initially instead of, for instance, inoculating the sterilized medium after *in situ* degassing or poisoning a growing culture with sterile selenate solution. To compensate for this, in the final experiments the culture media was degassed in the bioreactor in a heated vacuum oven and then inoculated with a small amount (~ 20 mL) of growing media.

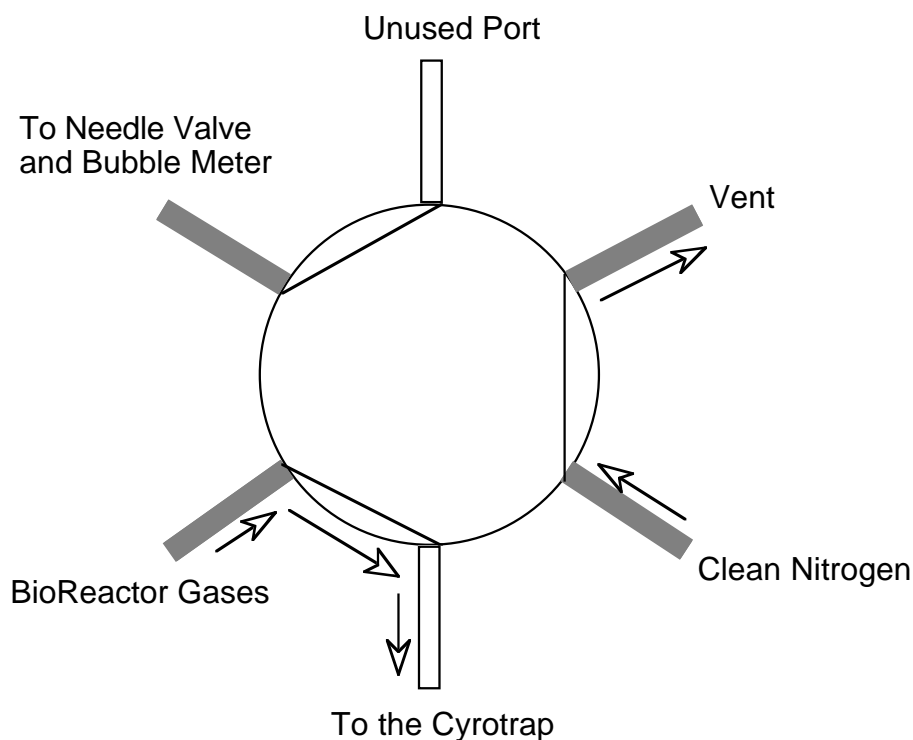
## **Anaerobic Purging of the Bioreactor**

Anaerobic gas purging of the bioreactor was accomplished by passing dry nitrogen through a Bacteria Air Vent filter, 37 mm in diameter with a 0.3 mm pore size (Gelman Sciences, Ann Arbor, MI, USA). The filter was connected to the bioreactor by 2" x 0.25" ID Tygon® tubing. The nitrogen passed through the fritted side of the dispersion tube and bubbled up through the media. The exiting headspace gas passed from the glass tube of the gas dispersion bottle through a 0.025" Swagelock® stainless steel fitting. This was connected to a stainless steel reducer (from 0.25" to 0.0625"). A one sixteenth inch Silcosteel tubing (Restek Corporation, Bellefonte, PA, USA) was connected from the reducer to the six port Valco high temperature stainless steel valve (Valco Instruments, Houston, TX, USA). Silcosteel tubing consists of stainless steel tubing with fused silica coated to the inside of the tube. The fused silica stationary phase inside this tube is deactivated using a silanizing reagent like hexamethyldisilazane which is passed through the column and then evaporated under low pressure and elevated temperature. The result is a very unreactive surface on which (trapped) analytes will not react. This expensive material was used in order to prevent sample carry over between injections and to decrease the chemical reactions of analytes during the movement of these species through the transfer lines.

## **Cryogen Trapping, Six Port Values, and Trapping Time**

The Valco valve performed the function of controlling the trapping time of the sample plus cleaning and purging of the transfer lines and cryotrap. A schematic of this valve in the inject position is shown in Figure 4. In the inject position, the bioreactor gases passed through the Valco valve and on to the cryotrap. The (cleaning) nitrogen, which flowed through the other side of this same valve passed to vent. In the bypass position (Figure 5), after the valve was rotated, the cleaning nitrogen passed through the transfer line to the second valve and the bioreactor gases were vented through a metering needle valve into a bubble meter. A piece of Silcosteel tubing 3' by 1/16" inch con-

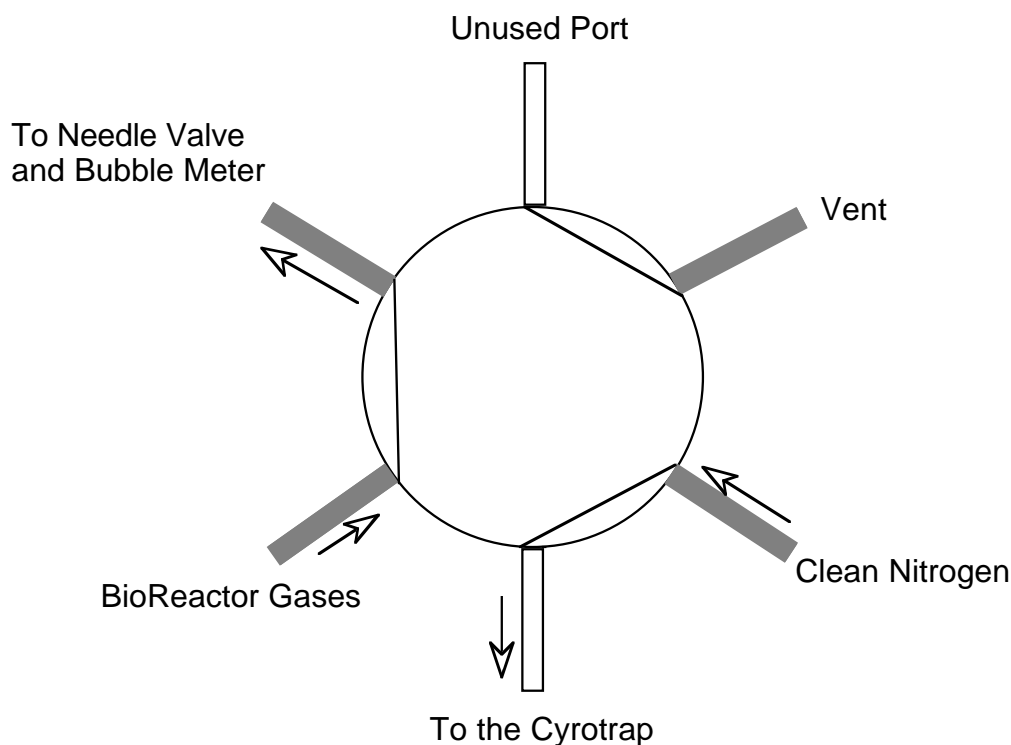
## Inject Position



**Figure 4.** Six Port Valve in Inject Position.

nected the first six port valve to the second six port valve, on the cryotrap. This valve was also a high temperature Valco valve. Two of the ports were connected to a U tube trap placed in a 36.5 cm x 17.8 cm Dewar flask with a metal case (manufacturer unknown). From the valve, two 12" x 1/16" Silcosteel tubes were attached. Each was connected to a Silcosteel reducing adapter reducing (from 1/16" to 1/8"). The adapters were mounted to a steel plate on top of the Dewar flask. Inside the Dewar a 18" x 1/8" U shaped Silcosteel tube was attached to each adapter. The U shaped was specially ordered (Restek) due to the restrictions on the minimum bending radius of 4" necessary to fit inside the Dewar.

## Bypass Position



**Figure 5.** Six Port Valve in Bypass Position.

### Transfer Line Heating and Purging

The cryotrap temperature was controlled by the injection of super cooled nitrogen and superheated air. The cooling apparatus consisted of a coil of 1/4" copper tubing immersed into a 10 L Dewar flask filled with liquid nitrogen. A three foot section of 1/4" Tygon tubing connected the coil to the copper tubing that was mounted to the top of the trap. The incoming and outgoing cooling lines were wrapped together in an insulated foam rubber tube that allowed for precooling of the air before it reached the Dewar. The (gaseous) nitrogen was supplied from the (high pressure) vent valve on a 160 L liquid nitrogen Dewar. This Dewar also acted as liquid nitrogen source for the GC oven. Control of the gaseous nitrogen flow was provided by an electric valve at the tank. Before

the valve was a standard single stage pressure regulator. Heating of the compressed air was performed by a 20' section of 1/4" stainless steel tubing folded into a coiled section 2.5' in length. This coil was wrapped with 3" wide fiberglass insulating tape (Fisher Scientific, Pittsburgh, PA, USA). To provide the heating, 18 gauge Nichrome wire was wrapped on the outside of the insulating tape. The wraps were made with less than 1/4" distance between the loops to provide even heating throughout the heating unit. Fiberglass insulating tape was double wrapped over the wire.

Initially, manufactured heating tapes were used to perform the heating tasks (Barnstead/Thermolyne, Dubuque, IA, USA); however, they proved to be very unreliable due to the difficulty of wrapping the very small lines. Every heating tape initially used burned out within a very short time. Therefore all subsequent heating tapes were hand-made by the same method as the heating coil trap described above. Power was supplied to the heating element for the trap by a variable transformer (Powerstat, Superior Electric Co., Bristol, CT, USA) set in the 140 V position at maximum output. The power for the valve and homemade transfer line heaters was supplied by controllers that were built under the direction of Dr. Calvin Banta. These controllers were made of simple dimmer switches (General Electric) used normally for lighting purposes.

The size restriction on the Silcosteel materials required longer transfer lines and extensive loops for the trap to valve connections. A three foot transfer line of 1/16" Silcosteel extend from the trap valve to an adapter fixed above the GC injector. The temperatures of all of the transfer lines were maintained at approximately 200° C during the entire experiment to prevent the condensation of organosulfur (and more probably) organoselenium species in the lines. This high temperature was accomplished by using heat tape axially wrapped around all metal transfer lines and six port valves. When this heating process failed, the result was seen in the chromatograms as missing or badly misshapen chromatographic peaks.

The flow rate for all experiments was 3.0 mL of nitrogen per minute through the bioreactor. This flow was maintained during the entire experiment; even when no headspace sampling was taking place and the transfer lines were being cleaned 3.0 mL/min N<sub>2</sub> flowed through the culture, through the headspace and through to vent. This flow was monitored periodically with a bubble meter.

Between sample cryogenic trapping or injection, the transfer lines between the bioreactor and the first 6 port valve, the transfer lines connected to the steel plate above the Dewar, and the transfer lines between the second 6 port valve and the GC were purged with cleaning nitrogen to remove any traces (carry over) from the last sample. Chromatographic blanks (long cryogenic trapping times of cleaning gas) were run periodically to determine the integrity of this cleaning process.

### **Sample Injection**

Nitrogen was plumbed to the second 6 port valve to purge the cryogenically trapped headspace gases into the GC. The final valve port of this valve was a vent connected to a bubble meter for determination of bioreactor flow rates during cryogenic trapping. The cryotrap was purged by 1.0 mL/min N<sub>2</sub> instead of 3.0 mL/min in order to maintain the necessary head pressure in the GC injector (see below). This flow was monitored by a bubble meter periodically.

### **Gas Chromatographic Adapter**

The connection from the transfer line from the second 6 port valve to the GC was made by a adapter from the Silcosteel tubing to a 0.32 mm deactivated capillary column. The total length of the deactivated capillary was 4" with 3" pierced through the injector's septa into the injector. One inch of the column was pushed back into the adapter to just making contact with the Silcosteel. The capillary was held in place by a fused silica adapter (1/16" to a 0.4 mm reducer; Valco).

A sample injection into the GC injector was accomplished by passing clean nitrogen through the cryotrap at a flow of 1.0 mL per minute. This prevented sample

backing up into the line connected to the injector. Since the head pressure inside the GC injector was 50 kpa, the gas pressure coming from the (sample) transfer line had to be larger than this in order for gas to be successfully transferred onto the column—that is, to make an injection. Experiments were carried out to determine the pressure necessary to accomplish this and a flow of 1.0 mL/min was ultimately used.

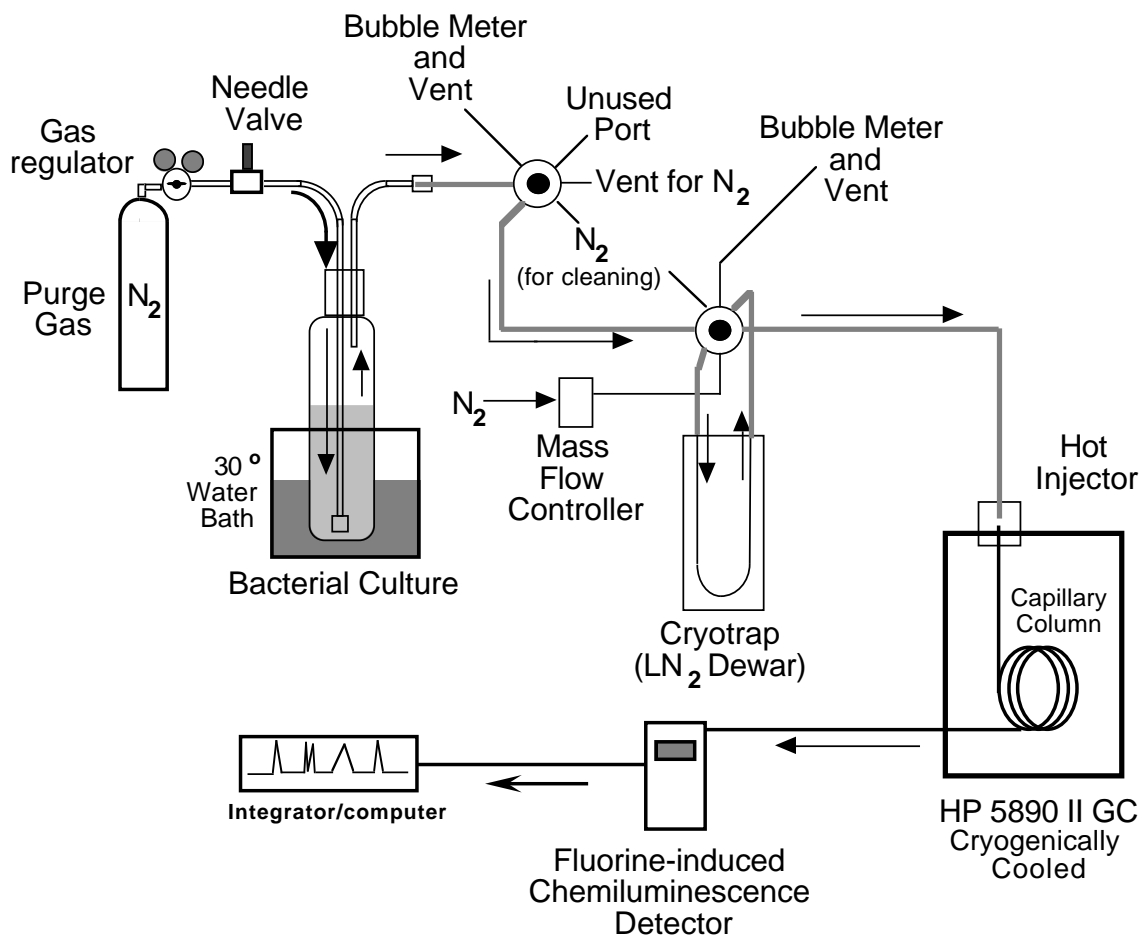
### **Gas Chromatography with F<sub>2</sub>-induced Chemiluminescence Detection**

The chromatographic system used for analysis of all headspace samples was a Hewlett Packard 5890 Series II gas chromatograph coupled with a chemiluminescence detector. The specialized detection system included a Sievers Instruments Model 300 Fluorine-Induced Chemiluminescence Detector (Sievers Instruments, Boulder, CO, USA) and one stage vacuum pump (Model 8811; Sargent-Welch Scientific Co, Skokie, IL, USA). The sulfur hexafluoride used in the detector's electrical discharge came from Air Products (Deerpark, TX, USA). The chromatographic column was a 0.32 mm x 30 m capillary (EconCap DB-5 chromatographic phase; 1 µm thickness; Alltech, Austin, TX, USA). The carrier gas was helium at approximately 1 mL/min flow. The temperature program used in all runs is detailed in Table 1 below.

**Table 1**  
Chromatographic Temperature Program

<b>Injector temperature</b>	275° C
<b>Initial temp</b>	-20° C for 1 minute
<b>Ramp A</b>	8° degrees per minute
<b>Final temperature A</b>	20° C
<b>Final time A</b>	0 minutes
<b>Ramp B</b>	15° C
<b>Final temperature B</b>	200° C
<b>Final time B</b>	3 minutes

A complete schematic diagram of the dynamic headspace apparatus is shown in Figure 6. The external liquid nitrogen Dewar for cooling the nitrogen entering the cryogenic trap is not detailed.



Pure flow rate = 3.0 mL/min  $N_2$

Cryogenic trapping temperature =  $-60^\circ C$

Transfer line temperature =  $200^\circ C$

● = 6 Port Valves

— = Heated Transfer Lines

**Figure 6.** Complete Schematic of Dynamic Headspace Sampling Apparatus, Gas Chromatograph, and Fluorine-induced Chemiluminescence Detector

## A Complete Time Course Experiment

What follows is a description of one time course experiment, complete with all of the steps for operation of the bioreactor, cryogenic trap, sample transfer and injection, and gas chromatography.

If the run was to be poisoned with  $\text{SeO}_4^{2-}$ , sodium selenate was added to 150 mL growth medium so that the final concentration at 170 mL total volume would be 10 millimolar. For the control runs, 150 mL of unpoisoned medium was used. The media (TSN) was filter sterilized using 0.20 mm pore size x 25 mm diameter LUER-LOK® filter (Alltech, Austin, TX, USA). The media was added to the bioreactor which had been sterilized by autoclaving. After the addition of the media, the reactor was placed into a vacuum oven to degas the oxygen from the media. The bacteria *Pseudomonas fluorescens* K27 used for inoculation was grown for 48 hours (into stationary phase) in TSN media and 20 mL of this culture was added to the rest of the bioreactor media at the start of the experiment—bringing the total volume to 170 mL. This left 80 mL of open head-space gas above the media. The bioreactor was placed into a water bath for the entire experiment and kept at a temperature of  $29 \pm 2$  °C.

A flow of nitrogen gas (99.99 %, Trigas Inc., Irving TX, USA) was immediately started through the reactor to prevent the movement of bacteria and oxygen back into the reactor through the exit port. At this point the transfer lines had already been preheated to 250 degrees. Valve A was placed in the bypass position (Figure 5) and clean nitrogen was allowed to flow through the transfer lines and the hot cryogenic trap (~ 180 °C) for 30 minutes initially or until the next injection. At the end of the preparation time, valve B (in the load position) and the cryotrap were cooled down to a temperature of -40° C by flowing cooled air from the externally cooled Dewar described above, and the clean nitrogen was trapped for 30 minutes to check for background residuals within the system.

The flow through the bioreactor was set throughout the entire experiment at 3.0 mL per minute. At this flow sufficient bubbles were produced in the bioreactor to allow

for reasonable purging of the media and the flow was easily controlled with needle valves and pressure regulators. Between runs the cleaning nitrogen was set to a flow of 30 mL per minute through the trap allowing total cleaning of the transfer system and valves. Once the system was purged, the trap's temperature was decreased to approximately  $-60^{\circ}$  C. Once the trapping temperature was reached, valve A was switched to the inject position (Figure 4) and the headspace gases were sent to the trap via valve B. The GC's microprocessor timer was used to time the manual switching of all valves. The trapping time was dependent upon the growth stage of the bacteria: in the early stages of growth the trapping time periods could range as high as 1 hour (180 mL) because of low bacterial population. However in the period where the bacteria reach the log phase (fastest growth), trapping time could be as short as 1 minute.

After the required cryogenic trapping time was completed, valve A was switched to the bypass position (Figure 5). The GC oven was now cooled to  $-20^{\circ}$  C with  $\text{LN}_2$ . After a 1 min thermal equilibration period at this temperature the super cooled nitrogen valve was switched off; valve B was switched to the inject position; the valve controlling the superheated air for the cryotrap was switched on and this air was fed into the cryotrap Dewar, raising the temperature. At this point the cryogenically trapped headspace gases vaporized off the trap and were injected into the GC. The GC temperature program was started after the measure temperature in the trap reached  $180^{\circ}$  C. After the chromatographic run was complete the GC integrator (Hewlett Packard 3396) recorded all peak retention times and integrated all the peaks.

During the days, the bioreactor's headspace was sampled in this way approximately every hour; however, during the night only two or three runs were made (once every 3 or 4 hours). The final K27 selenate poisoned experiment had a duration of 106 hours.

# CHAPTER 3

## DATA

All of the chromatographic data from two unpoisoned control runs and one poisoned run of *Pseudomonas fluorescens* K27 (10 mM Na<sub>2</sub>SeO<sub>4</sub>) are shown in Table 2, 3, and 4 respectively. All integrated peak areas are normalized to one minute trapping time. For instance, a peak area generated from a peak from a 20 minute trapping run was divided by 20 to obtain an area normalized to a one minute trapping time (3.0 mL).

**Table 2**  
Unpoisoned Bioreactor Experiment #1  
TSN media and *Pseudomonas fluorescens*

Comments	run #	hours	Trapping Time	DMDS
	2001	0	30	3602800
	2004	2.32	20	6738500
	2005	3.3	20	9498100
	2007	12.98	7.5	14922000
	2011	17.15	14	19147000
Color change from white	2012	19.65	8	20971000
to a dull brown	2013	20.77	5	49940000
and Bubbles appeared	2014	21.93	5	61942000
before run 2013	2015	22.63	5	60231000
and a peak at 1.777 appeared	2016	23.35	4	63306000
	2017	26.97	5	51693000
	2018	27.63	5	49144000
	2019	28.3	5	39890000
	2020	36.38	4	60173000
	2022	38.21	4	58032000
	2023	41.45	4	76292000
	2026	42.92	5	71596000
The Foam is begining	2027	44.05	4	54415000
decrease	2028	46.67	4	56368000
	2029	47.38	4	58323000
	2030	48.17	4	58177000
	2031	50.85	4	57013000
	2032	51.74	4	5701270.5
	2033	61.24	4	6308275.25

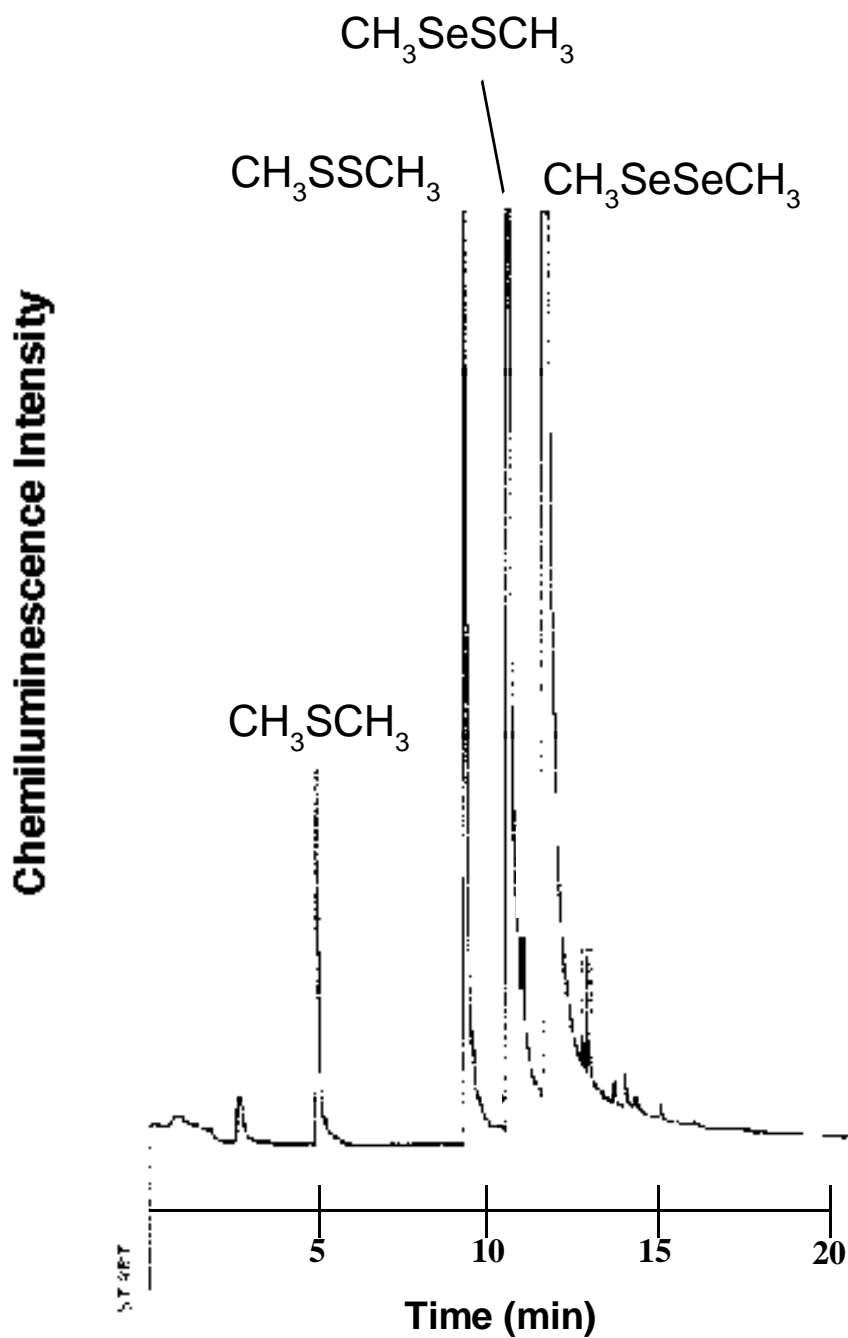
**Table 3**  
 Unpoisoned Bioreactor Experiment #2  
 TSN media and *Pseudomonas fluorescens* K27

Comments	Run number	Time	Trap Time	nDMDS
		hours	min	
	2109	<b>0</b>	4	7260168
	2110	<b>1.35</b>	4	4703112
	2112	<b>3.65</b>	4	10957072
Foam appeared	2113	<b>4.65</b>	4	4234800
Very light foam	2114	<b>5.83</b>	4	7567712
	2115	<b>9.83</b>	4	7413992
Foam disappeared	2116	<b>10.51</b>	4	7084060
Foam appeared	2117	<b>22.08</b>	4	6855736
	2119	<b>23.58</b>	4	13444064
Foam disappeared	2121	<b>24.33</b>	4	17459520
	2122	<b>25.91</b>	4	14886400
Foam appeared	2123	<b>27.24</b>	8	15376464
	2124	<b>27.89</b>	4	13437032
Large amounts	2125	<b>33.17</b>	4	11855728
of Foam built up	2126	<b>34.24</b>	4	12664384
	2127	<b>34.99</b>	4	10786560
large amt of foam	2128	<b>35.94</b>	4	11181832
	2129	<b>44.07</b>	4	9520216
foam decreasing	2130	<b>44.82</b>	4	13087296
	2131	<b>45.37</b>	4	3035268
light foam	2132	<b>51.8</b>	4	10727744
`1/4 foam	2133	<b>52.47</b>	4	10931728
`1/8" foam	2134	<b>52.46</b>	4	11547392
	2135	<b>53.26</b>	4	17345248
`1/16"foam	2136	<b>56.99</b>	4	18584992
	2137	<b>57.64</b>	4	18068448
foam on edges	2139	<b>67.32</b>	4	15177496
	2141	<b>72.72</b>	4	17241072
	2142	<b>73.37</b>	4	17119088
no foam	2143	<b>81.25</b>	4	14843048

**Table 4**  
 Selenium Poisoned Bioreactor Experiment  
 10 mM Sodium Selenate Added and TSN Media  
 Inoculated with *Pseudomonas fluorescens* K27 Bacteria

<b>Run number</b>	<b>Trapping Time min.</b>	<b>Time hours</b>	<b>nDMDS</b>	<b>nDMDS<sub>Se</sub></b>
2145	4.25	<b>0</b>	5608173.18	606693.176
2146	4	<b>0.68</b>	6930196	0
2147	4	<b>1.28</b>	6895588	2809894
2148	4	<b>3.08</b>	11468600	55400800
2149	4	<b>3.78</b>	19375136	66180420
2150	4	<b>9.42</b>	66312384	208875360
2152	4	<b>10.53</b>	100816000	310721440
2153	2	<b>11.15</b>	155646976	403422720
2154	2	<b>18.12</b>	143451136	319201792
2155	2.08	<b>19.15</b>	147740062	313469785
2156	2	<b>20.17</b>	165172224	296319488
2157	2	<b>21.77</b>	151239808	245649408
2159	2	<b>25.89</b>	124166144	178359168
2160	2	<b>26.7</b>	138320384	168974592
2161	2	<b>27.7</b>	139142144	156122240
2162	2	<b>29.88</b>	136830848	142627584
2163	2	<b>30.49</b>	135696512	151913344
2164	2	<b>31.24</b>	139237760	154441472
2165	2	<b>31.89</b>	157233792	161005952
2166	2	<b>32.57</b>	141931520	147519616
2167	2	<b>34.55</b>	144756352	151413632
2168	2	<b>44.78</b>	147827072	120078272
2169	2	<b>46.08</b>	139495168	123793920
2170	2	<b>47.1</b>	138441984	123913856
2171	2	<b>57.65</b>	130711424	110330816
2172	2	<b>69.85</b>	136377600	107288192
2173	2	<b>81.97</b>	117555520	85799872
2174	2	<b>82.05</b>	124428288	90651136
2175	2	<b>91.95</b>	74911936	57463712
2176	2	<b>105.95</b>	54265312	35370816

Figure 7 is a chromatogram of the headspace from *Pseudomonas fluorescens* K27 after 13 hours of anaerobic growth. The culture in the bioreactor was continually purged with nitrogen at 3.0 mL/min.



**Figure 7.** Chromatogram of the headspace of *P. fluorescens* K27 after 13 hours of anaerobic growth. Four minutes of cryogenic trapping was used.

Figure 8 is a chromatogram of the headspace from *Pseudomonas fluorescens* K27 after 20 hours of anaerobic growth. The culture in the bioreactor was continually purged with nitrogen at 3.0 mL/min.

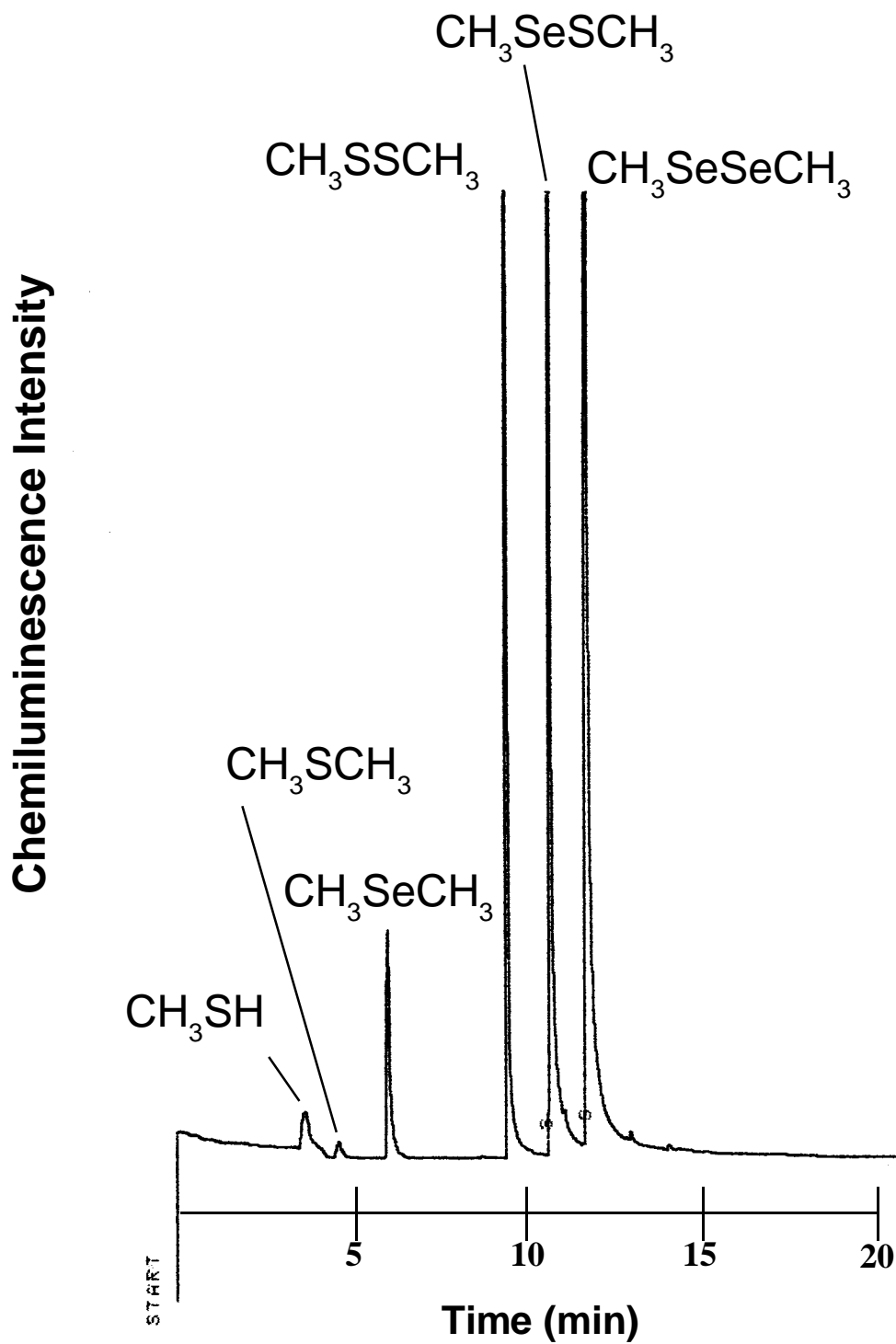
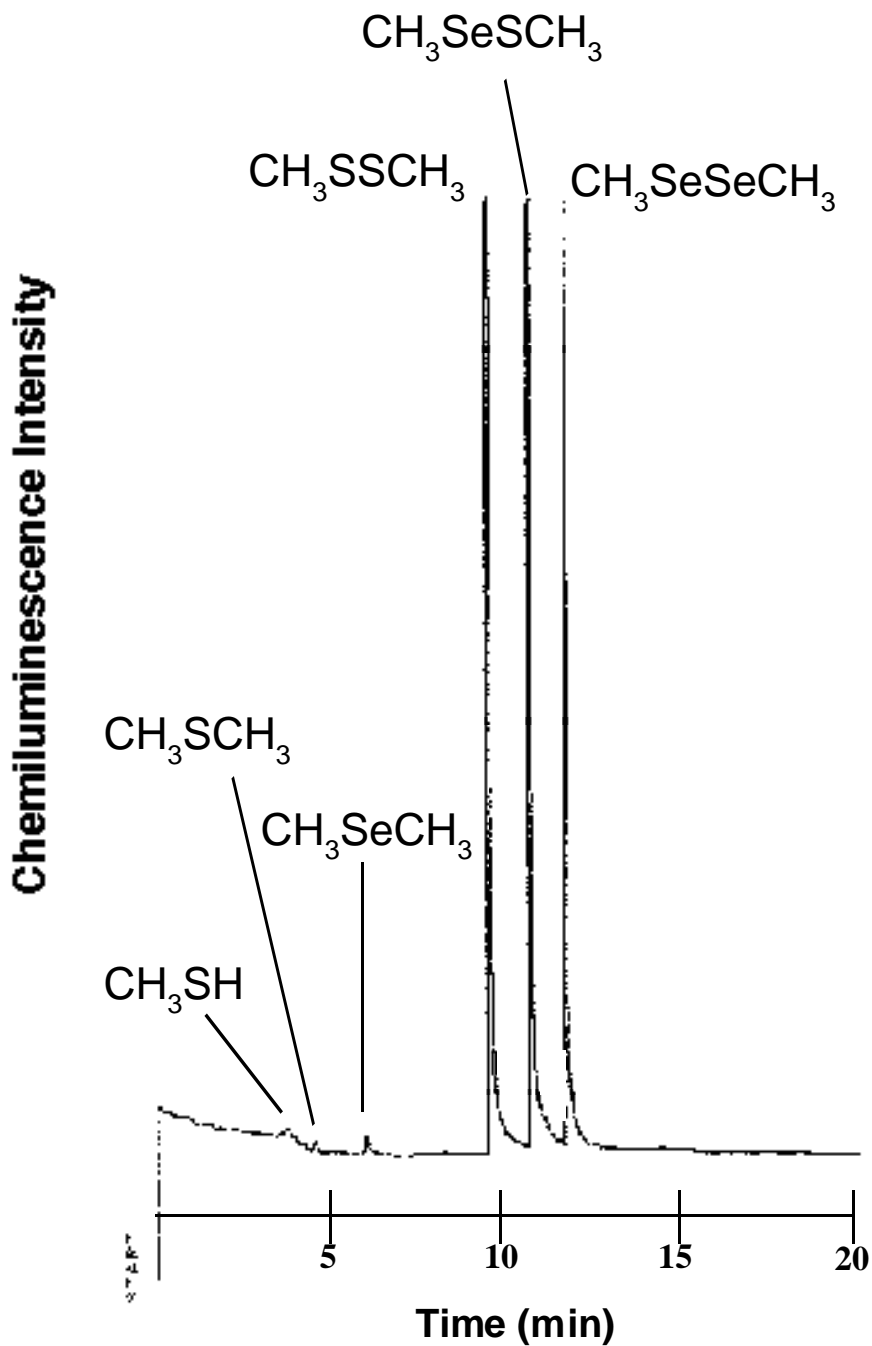


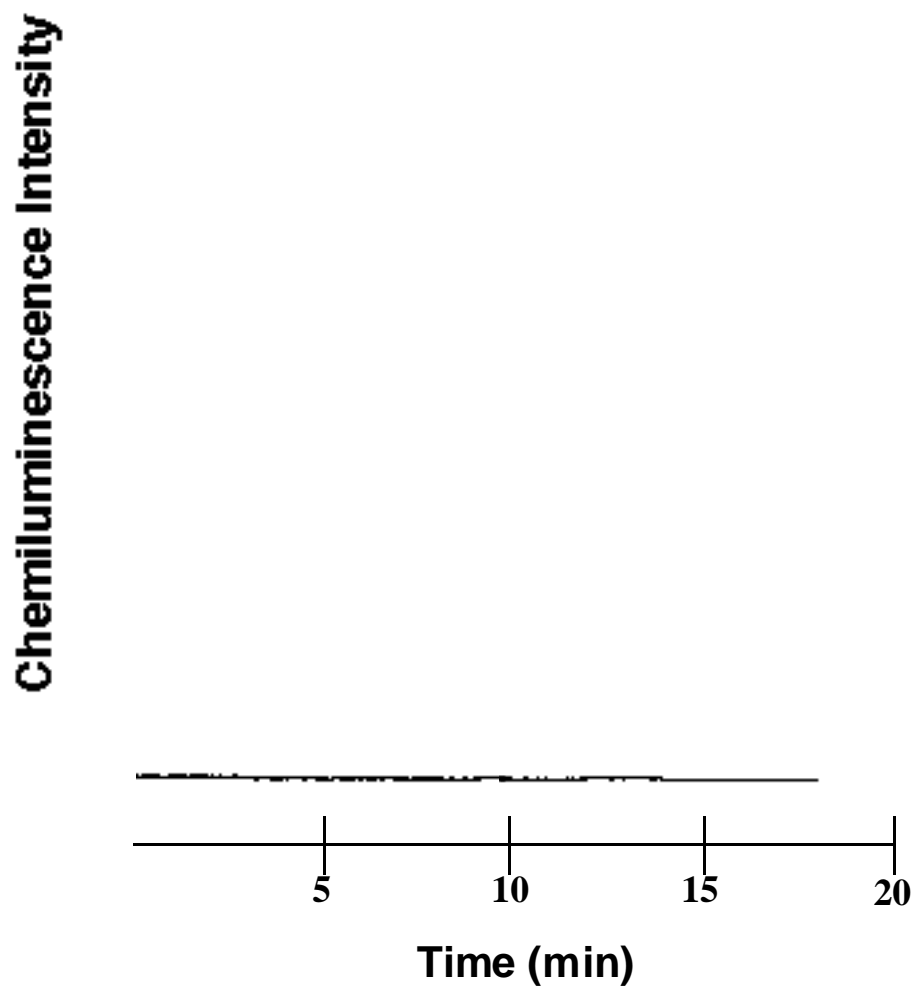
Figure 8. Chromatogram of the headspace of *P. fluorescens* K27 after 20 hours of anaerobic growth. Two minutes of cryogenic trapping gas used.

Figure 9 is a chromatogram of the headspace from *Pseudomonas fluorescens* K27 after 106 hours of anaerobic growth. The culture was continually purged with nitrogen at 3.0 mL/min.



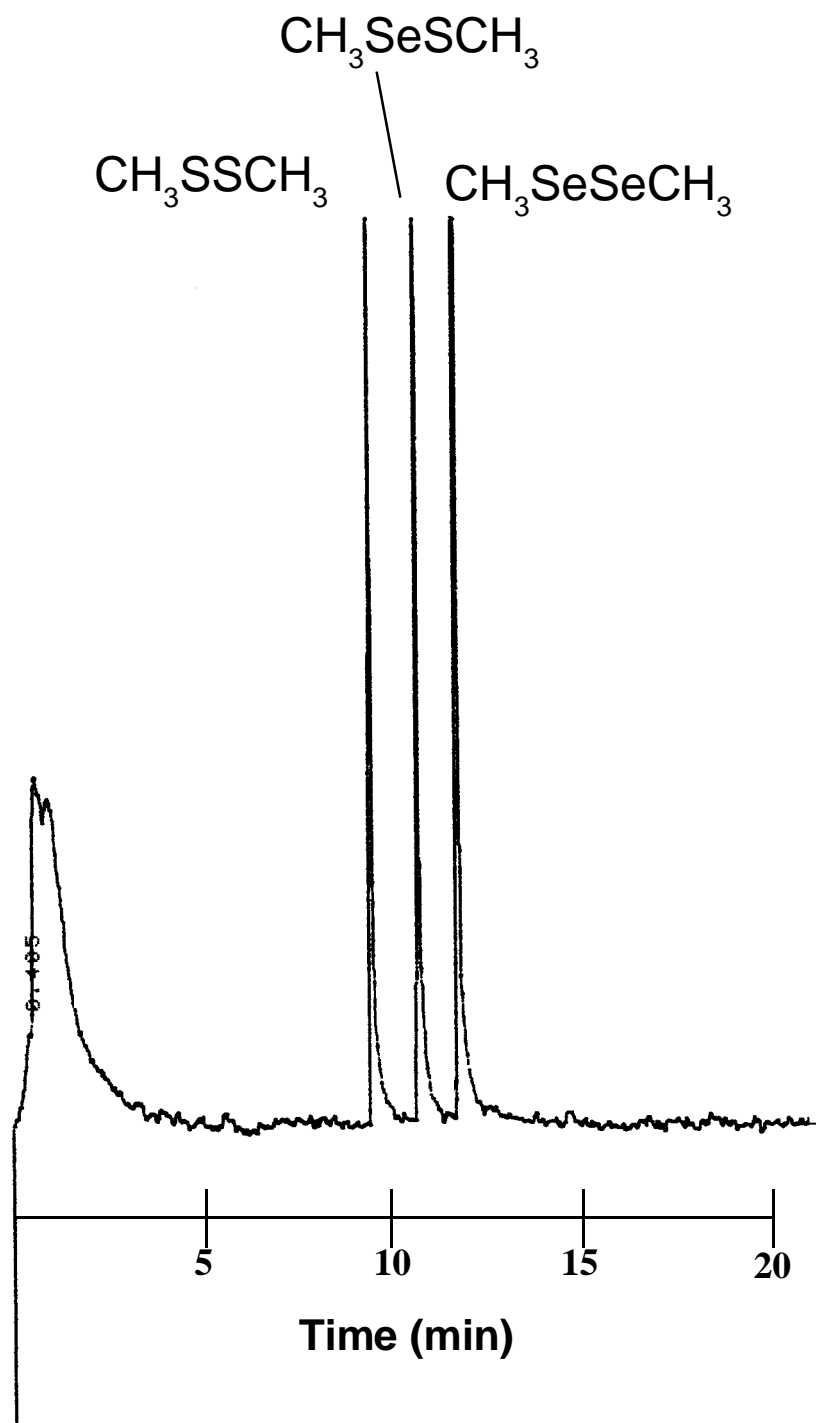
**Figure 9.** Chromatogram of the headspace of *P. fluorescens* K27 after 106 hours of anaerobic growth. Two minutes of cryogenic trapping was used.

Figure 10 is a chromatogram of a quality control blank. For fifteen minutes, clean nitrogen was flowed through transfer lines and valves and was trapped exactly like a headspace sample. This was performed periodically between sample runs to test the cleaning process and the purity of the nitrogen purge gas.



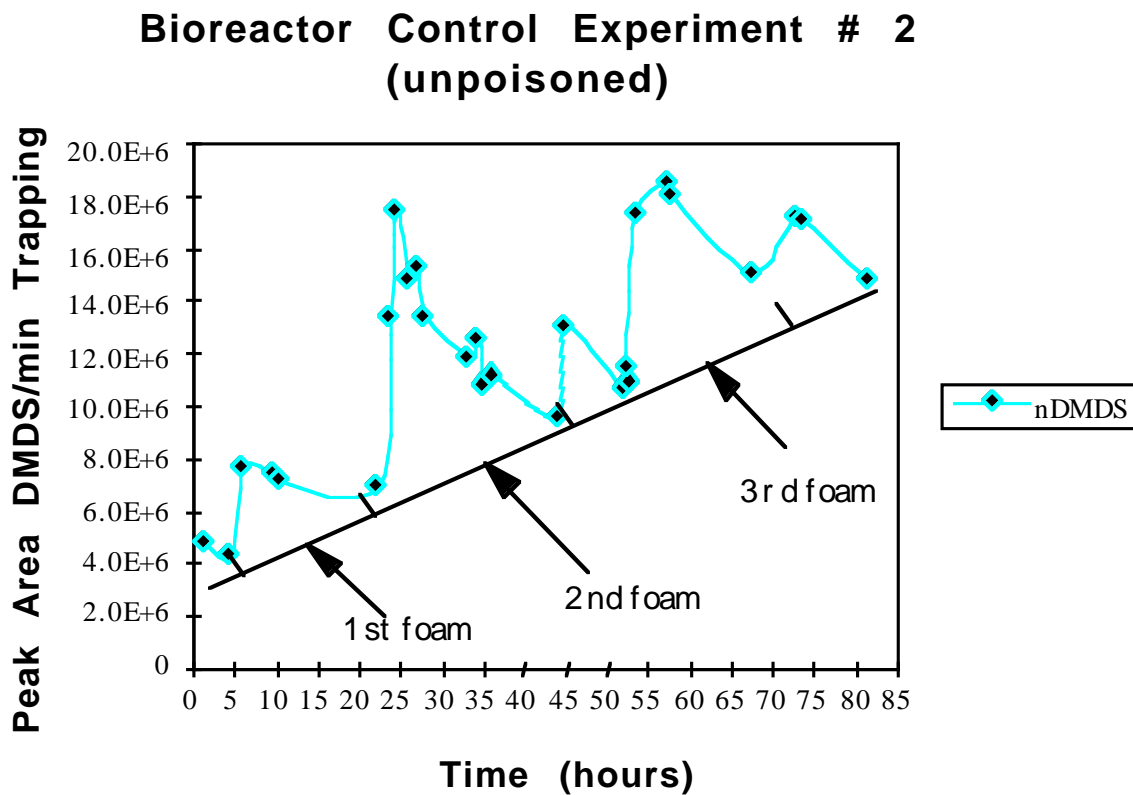
**Figure 10.** Fifteen minute cryogenic trapping of purge gas.

Figure 11 is a chromatogram from the test tube bioreactor preliminary experiments. The large unresolved early eluting peak is probably methanethiol, DMS, and DMSe.



**Figure 11.** Chromatogram from the headspace above the test tube bioreactor containing live K27 bacteria poisoned with 10 mM sodium selenite. The trapping time was 12 minutes.

Figure 12 is a time course experiment using the gas washing bioreactor and a live, unpoisoned culture of K27 bacteria. The points where headspace foam appeared are noted.



**Figure 12.** Time course experiment of *Pseudomonas fluorescens* K27 grown anaerobically at 30 C in TSN medium. This culture was not poisoned with a metalloidal salt.

Figure 13 shows the results of a single 106 hour time course experiment tracking the production of DMDS and DMDS<sub>e</sub> in the headspace of a single culture of *Pseudomonas fluorescens* K27. Throughout the entire experiment the culture was purged with 3.0 mL/min nitrogen.

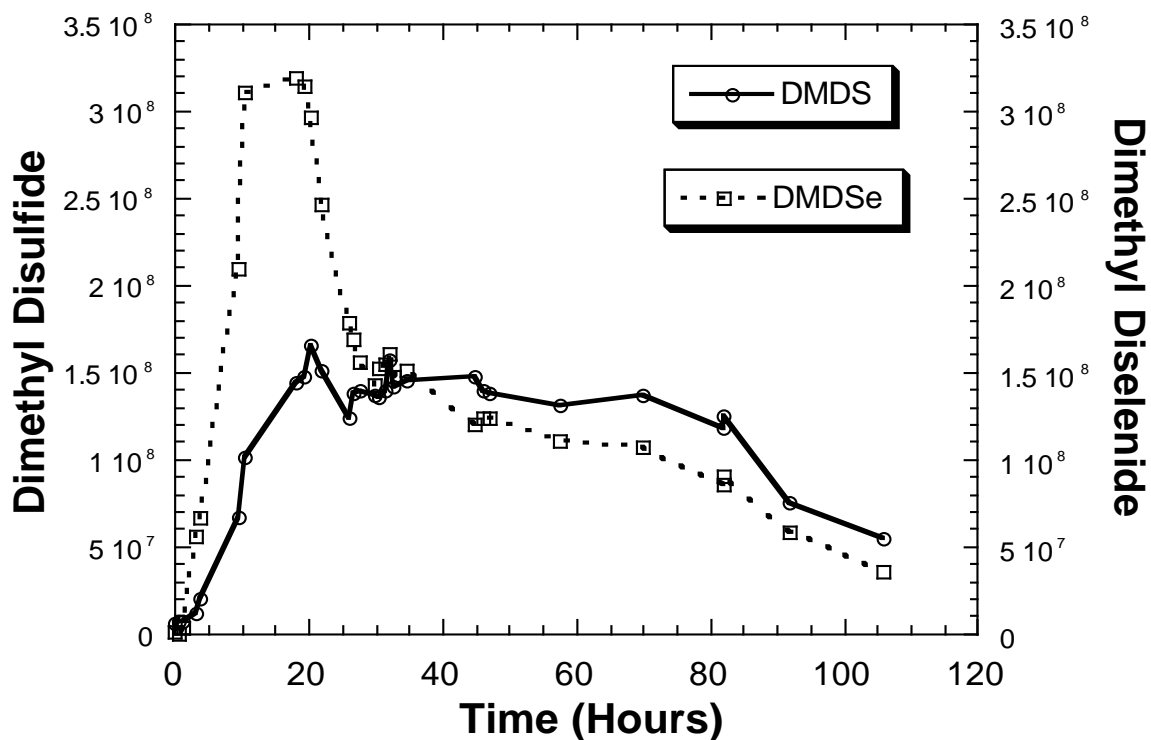


Figure 13. Time course experiment of *Pseudomonas fluorescens* K27 grown anaerobically at 30 C in TSN medium. Culture was initially poisoned with 10 mM sodium selenate.

## CHAPTER 4

### RESULTS AND DISCUSSION

All of the past headspace analysis experiments in the Chasteen group were carried out with static headspace sampling. Furthermore these have mostly been a one time sample because the septa available could not be expected to maintain an anaerobic environment after being pierced by a syringe's needle. Therefore, in order to develop a time course of the metabolic production of gases by bacteria, a series of tube (and replicates) had to be setup with the same bacterial constituents and the headspace sampled from each tube at different time intervals. This provided a good representation of the patterns of the gases released by the bacteria over time. A specially designed enclosure cap has also been used to allow multiple sampling of the same culture with no contamination from oxygen (Stalder *et al.*, 1994). It should be noted that those experiments were carried out in sealed Hungate tubes or larger volume Schott® flasks where there was no means for headspace gases to escape or be purged from culture solution. This presented several limitations. The headspace environment was obviously closed and static and thereby failed to give a true representation of real life circumstances, that is conditions that would exist in more dynamic conditions in the natural environment where gases are exchanged, replaced, and diluted by gas phase pressure and temperature changes. It is believed that the waste products from an organism are, to a degree, poisonous to that organism. The question is: would the removal of these poisons enhance the growth and ability of the organism to remove selenium from its environment? What we were primarily looking at in these experiments were the metabolic selenium compounds; but there are reduced sulfides and other various hydrogenated gases among others that, if concentrated in culture, perhaps might also alter the growth patterns of the bacteria as well.

In the design of this experiment we had two primary objectives: first, to remove the gaseous products produced by the bacteria thereby decreasing the biological effects

that they might have upon the bacteria and second to have the ability to continuously monitor the production of headspace gases over extended periods of time without affecting the bacteria by exposing them to atmospheric oxygen during sampling.

These experiments were successful to a certain degree. First we successfully designed and built a dynamic headspace sampling system that could 1) repeatedly remove the headspace gases above an anaerobic bacterial culture, 2) trap and cryogenically concentrate and then 3) inject the sample successfully into the GC. Experiments were carried out over periods as long as 106 hours. Figures 7, 8, and 9 show chromatograms at various points in a very long term experiment *with a single culture*. Figure 10 shows that the cleaning procedure developed was wholly adequate to assure no sample carry over between injections and that the nitrogen purge gas was free from impurities.

However, we were unable to separate the early eluting chemical species of methanethiol, dimethyl sulfide, and dimethyl disulfide that *were* seen in some runs and that we have routinely found in static headspace sampling experiments (Chasteen *et al.*, 1990; McCarty *et al.*, 1993; Zhang and Chasteen, 1994). These species appeared in the chromatogram as large undefined peaks whose retention times changed run to run (Figure 11). We can offer no explanation other than this a possible effect of the double cryotrapping process and that these lower boiling components ( $\text{CH}_3\text{SH}$ —bp 5 °C; DMS—bp 38 °C; DMSE—bp 58 °C) were not successfully captured by the first cryogenic trap. Another explanation is that these more polar species (compared to less polar DMDS and DMDS<sub>e</sub>) were somehow lost in the transfer lines or valves.

The initial dynamic headspace experiments (data in Tables 2 and 3) were control experiments with only TSN media and *Pseudomonas fluorescens* K27. The purpose of these was twofold: to develop a baseline for the upcoming selenium poisoned experiments and to show the history of the production of sulfides by the unstressed metabolism of the bacteria. This proved difficult for a variety of reasons. Even though we had had excellent experience growing these bacteria in many different situations, these control

experiments were hampered by poor bacterial growth or no growth, culture foaming, and foam contamination of the gas transfer lines of the dynamic sampling system. Although the appearance of foam in these cultures was not unexpected—we had seen signs of small amounts of foam in our static headspace samples—the *magnitude* of foaming was quite unexpected. Our best guess as to the original of this foam centers on the time of its occurrence (Figure 12). It often appeared first near the end of the log phase of growth and could have possibly been the effects of bacterial production of proteins released at the onset of the stationary growth phase. This said, the foaming came and went multiple times during the experiment as shown in Figure 12. Foaming at the beginning of the stationary phase is obviously not the whole answer. We have seen no reference to this in the literature.

As Figure 13 shows, the long term determination of the headspace products of this anaerobic bacteria displayed that the highest concentrations produced by the live culture, continually purged with nitrogen, were reached soon after stationary phase was achieved (about 8–10 hours as determined by optical density experiments; Zhang 1993). The concentrations of DMDS and DMDS<sub>e</sub> dropped off continually from this point until the end of the experiment some 80 hours later. These results can be contrasted with those of some of our static experiments with phototrophic bacteria grown anaerobically in light. In one case, *Rhodobacter sphaeroides* 2.4.1 *increased* the headspace concentrations of DMSe and DMS after 80 hours of growth and late in the stationary phase (McCarty et al., 1995).

## Conclusions

1. A dynamic headspace sampling apparatus was designed and constructed. Headspace gases above anaerobic bacterial cultures were sampled over long times course experiments. Large volumes (up to 180 mL) of headspace gases could be collected, cryogenically concentrated, and then cryofocussed in an external subambient cryotrap and again in the GC oven. Organosulfide and organoselenide compounds could be determined from that headspace. Repeated sampling of the same culture over time permitted a time based examination of the bioremediation of the toxic salts of selenium by anaerobic bacteria.

2. High boiling organo-chalcogens (DMDS and DMDS<sub>2</sub>) could be examined routinely over experiments up to 100 hours long using the same culture. Plotted over time these analyses showed that the production of these disulfides and diselenides tracked the growth curve of the facultative anaerobe examined, *Pseudomonas fluorescens* K27. Peaking soon after the log phase of growth ended (about 10 to 15 hours), the culture's production of headspace chemical species dropped off over the subsequent 80 hours as the organism entered death phase. No significant increases in headspace concentration were seen in this latter stage of the experiment in any of the species determined.

3. Lower boiling headspace components, routinely detected in static headspace experiments were not successfully trapped and chromatographed using this apparatus. Methanethiol, DMS, and DMSe, whether lost during transfer and cryotrapping or coeluting in extremely poorly resolved and completely nonreproducible chromatographic peaks, could not be determined in these experiments.

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# Vita

Steven McCarty was born in Colorado City, Texas on November 10, 1956. He graduated from Snyder High School in Snyder, Texas in 1975. Steve received a Associate Arts degree in Metal technology from Western Texas Jr. College in 1977. He afterward went to work in the oilfields of West Texas as a wireline engineer. As the result of the great depression of the middle 1980s, in 1988 he entered in the Chemistry and Environmental Science programs at Sam Houston State University. He received a Bachelor of Science degree with a double major in Chemistry and Environmental Science. In 1992 he entered into the graduate chemistry program at Sam Houston State. He received his Master of Science degree in chemistry in the fall of 1994. He is currently working as a chemist for Phibro Energy.

