# The Effect of Molasses Concentration on Bacterial Treatment of Selenium in Agriculture Waste Water in the San Joaquin Valley

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**Abstract** In the San Joaquin Valley, selenium concentrations in agricultural waste water have become a serious concern as an environmental pollutant causing birth defects and death in birds, small mammals, and fish. One method of lowering the concentration of selenium in the agriculture discharge is to use biological treatment. A pilot treatment system was developed at Panoche Drainage District using local bacterial strains in reduction ponds to minimize the amount of selenium being discharged into the San Joaquin River. In order to keep operation costs down and determine optimal growth for the bacteria, this project examines how molasses substrate concentration and trace nutrient additions for bacterial growth change the total selenium reduction. Three different concentrations of molasses (0.1g/L, 0.2g/L and 0.4g/L) were prepared in triplicate using influent collected at Panoche and processed at Lawrence Berkeley Laboratory. These samples were analyzed periodically (4 or 7 days) in a controlled environment, using atomic absorption spectrometry to determine selenium concentrations. Final results for varying concentration of molasses between 0.1g/L, 0.2g/L and 0.4g/L did not differ for total selenium concentration. Nor did the total selenium concentration of 0.4g/L with trace nutrient buffer differ from the other concentrations of molasses. Total selenium reduction averaged ten percent from the initial concentration. These results did not meet expectations. Previous experiments had a reduction of eighty percent under similar conditions. This implies that at low concentrations of molasses inadequate carbon is available for maximum bacterial growth and therefore selenium reduction.

## Introduction

Sources of selenium pollution are various and include industrial effluents from thermal power plants, oil refineries, smelting plants, and in the production of semiconductors, pigments, and solar batteries (Kashiwa *et al*, 2000). Finding a reliable treatment may be applicable to a broad range of industries. The focus of this project is the analysis of the current selenium laden agricultural waste water problem in California.

In California's San Joaquin Valley, an area of extensive agriculture, high levels of selenium have been found naturally occurring in soils (Oswald *et al*, 2000). For California to maintain its high level of food production, adequate water supplies must be available. Extensive canal systems were built to bring water to the fertile valley, but no canals were built to remove the waste water. Unfortunately, while irrigating fields with selenium rich soils it has been found that selenium leaches into the water. Concentrations between 75 µg/L and 1400 µg/L are measured in the subsurface drainage water (Fan *et al*, 2001). This excess drainage is then pumped up to the surface and sent to lakes or discharged into the San Joaquin River (Quinn *et al*, 2000).

Some of the effects on organisms when selenium is present in aquatic environments are reproductive dysfunction, deformities, anemia, and death in many species of birds, fish and mammals (Amweg *et al*, 2003). Since the discovery of selenium accumulation in vertebrates, law makers have tried to establish safe levels of selenium in discharge waters (Amweg *et al*, 2003). Because much of the selenium cycle is not clearly understood, the establishment of safe levels of selenium in water has been difficult to determine (Fan *et al*, 2001). The result of this situation is that the Environmental Protection Agency (EPA) has tried to reduce the amount of total selenium entering the watershed as a means to reduce the risk to the environment (Quinn *et al*, 2000). In 1987, the EPA set a chronic exposure level for freshwater aquatic life at 5 µg/L of total selenium (Fan *et al*, 2001).

Selenium can be found in four different oxidation states (-II, 0, IV, VI). The chemical form of selenium will determine its solubility and availability to organisms (Zhang, 1999). Selenate (selenium VI), selenite (selenium IV), and selinde (selenium – II) are all water soluble and therefore considered to be the most important sources of

selenium in water (Amweg *et al*, 2003). Though its solubility is agreed upon, there are differing opinions about which forms are most toxic. Zhang, Moore, and Frankenberger cite Mikkelsen, Bingham, and Page (1999) to assert that selenate is generally considered to be the most toxic. Whereas Amweg, Stuart, and Weston (2003) assert that organic forms of selenium are thousands of times more bioavailable than selenate and therefore pose the most important risk to the environment.

Since most of the selenium from agriculture runoff in the San Joaquin Valley is primarily in the form of selenate, a problem arises as to how to appropriately manage selenium discharge without impacting agriculture production. Methods such as chemical precipitation, catalytic reduction, and ion exchange are effective for the removal of selenite but are not effective in removing selenate (Kashiwa *et al*, 2000). These methods are also costly (Kashiwa *et al*, 2000). Due to a lack of affordable treatment of selenium to meet concentration objectives there has been a regulatory shift to reducing the selenium load (Quinn *et al*, 2000). It may prove to be that bioremediation of selenium by bacteria into less toxic and more a stable form (elemental selenium) is the most cost effective method of reducing the selenium load (Quinn *et al*, 2000).

In Panoche Drainage District near Firebaugh in the San Joaquin Valley an algal-bacterial selenium removal system was created to treat drainage water (Oswald *et al*, 2000). The waste water is not only high in selenium but also in nitrate. Algae were originally used to remove the nitrate from the influent before the reduction pond where bacteria reduce the selenium. The old algae could then act as a carbon source for the bacteria and minimize external inputs into the system. However the algae component has since been discontinued due to experiments that showed better selenium reduction with out the drainage first passing though the algae system (T. Lindqust, 2003). The carbon source for the bacteria could be replaced by many sources found from byproducts of food production, and in the San Joaquin Valley molasses is readily available at the price of $60 to $90 per ton (Quinn *et al*, 2000).

Once in the reduction pond local bacteria strains first remove the nitrate. After the nitrate is removed the bacteria reduce selenate to selenite then to elemental selenium in anoxic conditions (Oswald *et al*, 2000). Elemental selenium is non-toxic and insoluble (Kashiwa *et al*, 2000). Removal of elemental selenium from the effluent can then be

accomplished by a physical method such as settling ponds or by filtration. The treatment pond in Panoche Drainage District has effectively removed up to eighty percent of the total selenium by the reduction of selenate by bacteria (Oswald *et al*, 2000). These levels meet regulation needs to reduce the selenium load discharged into surface waters.

This project examines the how bacterial treatment is affected by molasses substrate concentration and trace nutrient additions on the total soluble selenium reduction by using biodigestion with anaerobic bacteria. Does more molasses substrate lead to higher reduction of selenium? Are trace nutrients a limiting factor for bacterial reduction? Does more molasses cause an increase in bacterial growth leading to more organic selenium? These questions will help to define the optimum concentration of molasses. This will save money in treatment costs and may give insight to minimize organic selenium discharged.

The results from these experiments have direct implications on California's water shed. The California Water Quality Board and Bureau of Land Management are desperately looking for reasonable solutions to the selenium problem. If a solution can be found that effectively meets standards at an affordable price then these treatment ponds could save California millions of dollars not only in treating the wastewater but by having clean water and a healthy ecosystem.

## Methods

**Collection** Water samples were collected from the influent to the treatment facility at the Panoche Drainage District located near Los Banos, California. Bacteria were collected from the reduction pond at Panoche. Both water and bacteria samples are kept in one liter plastic bottles and kept cool in a portable cooler for transportation. They are then brought to the Berkeley Lawrence Laboratory and prepared for biological treatment. In the laboratory setting, the reduction pond at Panoche was mimicked by creating an anaerobic environment.

**Incubation of samples** Two tests were run consecutively with the water and bacteria collected from Panoche Drainage District on November 11 2003. The first test had two treatments, addition of 0.1g of molasses per liter of drainage water and 0.2g of molasses per liter of drainage water. The purpose was to test how molasses concentration

as a source of carbon for the bacterial growth affected the rate of selenium reduction. In addition, controls were run with this experiment that included a treatment of drainage water with bacteria but no molasses, and plain drainage water called Panoche Influent (PI). The second test also had two treatments, 0.4g of molasses per liter of drainage water and 0.4g of molasses per liter of drainage water with micronutrients in a phosphate buffer. The second test examined if the lack of additional micronutrients were acting as a limiting factor needed to promote bacteria growth and reduction. Controls ran with this experiment were plain PI and drainage water (PI) with bacteria and micronutrients.

To accomplish these tests the appropriate concentration of molasses was first added to 2000mL of drainage water for each treatment of molasses. In the case of the micronutrient treatment 1mL of phosphate buffer was added to each liter. The phosphate buffer solution was made by combining 2.0g $KH_2PO_4$, 2.1g $K_2HPO_4$, and 2.0g $NH_4Cl$ brought up to 500mL with deionized water. Then the samples were thoroughly shaken and 80mL of each treatment was added into nine 100mL glass bottles to allow for three different testing dates in triplicate.

Next, the bacteria were mixed and added to the samples. This was done in an anaerobic environment to keep the local bacteria from being exposed to oxygen, which slows down the reduction process. One gram of concentrated bacteria flocks were broken up and mixed with 100ml of drainage water. This mixture was then decanted to remove large flocks in order to keep the bacterial solution homogeneous. Two milliliters of bacterial solution was then added to each 80mL sample in a 100mL glass bottle. This was done in an anaerobic hood, (5% $H_2$, 5% $CO_2$, 90% $N_2$ that has an autovacuum, triple sealed and pressurized), and then sealed with rubber tops. The bacteria will only reduce the selenium after the oxygen and nitrate is removed from the water. At that point the samples are placed into a temperature controlled environment, of 28 degrees Celsius, for 4, 7, 14, or 21 days. Everyday the bottles were vigorously shaken for 30sec to prevent bacteria from settling to the bottom of the bottle or sticking to the sides which would reduce the bacteria's ability to reduce the selenium. The samples are then tested for selenium content by hydride generation atomic absorption spectrometry (AA), using two different processes to determine soluble selenium and total selenium concentrations.

**Preparation of Samples** The AA uses two types of preparation of the samples before analyses, Alkaline Digest or Acid digest. Since the AA can only read selenite it is necessary to convert other forms of selenium into selenite by one of these methods. The Acid Digest uses the filtered sample treated with hydrogen chloride and persulfate to determine the total soluble selenium. The soluble selenium is what is currently the focus of reduction in the watershed as set by the EPA (Oswald, 2000). To filter the samples a 0.22 micron glass filter was used. This is small enough to remove most of the particulates from the sample. Two and a half milliliters of the sample is added into two test tubes. Two and a half milliliters of 12M HCl was added to each test tube. Two percent ammonium persulfate was added, 0.1mL or 0.2mL, into one of the two test tubes for each sample. Ammonium persulfate levels can have an effect on the selenium readings therefore the highest reading of multiple concentrations is accepted. Next the test tubes are set on the heating block for 30 minutes at 98 degrees Celsius. The Alkaline Digest uses an unfiltered sample treated with sodium hydroxide and hydrogen peroxide. The method is similar as above; hydrogen peroxide is added at 1.5mL or 2.0mL to 2.5mL of sample. The test tubes are then set on the heating block for an hour and a half. Next the samples are followed by a hydrogen chloride digest to determine the total selenium concentration in the water.

To assure that the AA is running with precision and accuracy the last four test tubes are for quality assurance and quality control (QA/QC). Two split test tubes are prepared from a randomly selected sample and are prepared in the same manner as the rest of the samples. Two spike test tubes are prepared with 2.25mL of randomly selected sample with the addition of 0.25mL of 1000ppb standard selenium stock solution then the process is the same as the rest of the samples.

**Analysis** The AA is turned on and optimized for an hour before samples are ran. Before the samples can be run a new selenium concentration curve must be established. Standards are run at 1, 5, and 10ppb prepared from 1000ppb stock solution. In addition to accurately test the concentration of selenium the samples may have to be diluted to read inside of this curve. Two common dilution factors are 1:7 and 1:40.

| | |
|---|---|
| 1 in 7 Dilution | 0.50mL sample and 3.0mL of DDI |
| 1 in 40 Dilution | 0.25mL sample and 9.75mL of DDI |

The results from the two experiments were entered into Microsoft Excel spreadsheet and the progression of selenium was graphed over time. A t-test was run to determine if there was any significant difference between the initial concentration and the final values.

**Results**

Due to limited time and money, this experiment was missing some of the components that I had hoped that it would have. Namely, the selenite and organic selenium concentrations would give a fuller understanding of the species in the drainage water over time of treatment. I was only able to test for soluble and total selenium.

The first experiment which examined the molasses substrate concentrations had showed no difference in the final concentration of total soluble selenium and total selenium. See figure 1. Initial concentration of selenium was 466ppb ± 60ppb. Final results for varying concentration of molasses between 0.1g/L, and 0.2g/L and did not differ for total selenium concentration (426ppb ± 15ppb, and 405ppb ± 20ppb respectively). The final concentration of total soluble selenium in varying concentration of molasses between 0.1g/L, and 0.2g/L did not show any difference (initial concentration of 466ppb ± 60ppb, to 405ppb ±8, and 418ppb ±13ppb respectively).

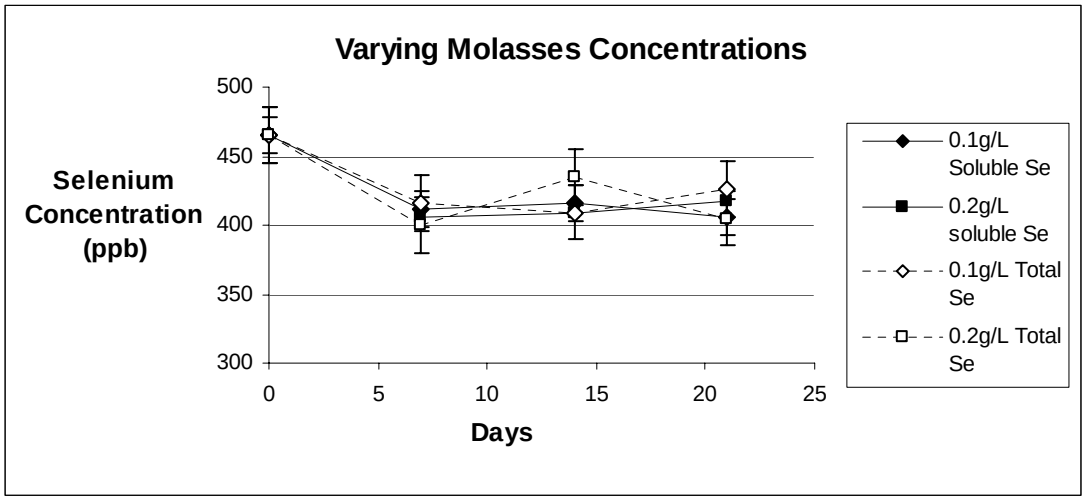


Figure 1. The results for varying concentrations of molasses at 0.1g/L and 0.2g/L over the course of the experiment. Soluble Se includes selenate, selenide and selenite. Total Se includes selenite, selenate, selenide and elemental selenium. Standard deviation bars illustrate final concentration was the same regardless of molasses concentration.

There appeared to be an average of a ten percent reduction in the selenium concentration. Further analysis showed that it was not a significant reduction in selenium concentration when using an unpaired T- test. For example the results for total selenium between the final values .2g/L (405ppb) and the initial concentration (466ppb) had a two-tailed P value equal to 0.1728, and the t static was 1.6572.

The second experiment examined whether higher molasses concentrations of 0.4g/L and the addition of micronutrients in the form of a phosphate buffer had better reduction. See figure 2. Results found that there was no difference in final results for varying concentration of molasses between 0.1g/L, 0.2g/L and 0.4g/L for total selenium concentration (426ppb ± 15ppb, 405ppb ± 20ppb, and 408ppb ±14ppb respectively). Nor did the total selenium concentration of 0.4g/L with buffer, 422ppb ±14ppb differ from the other concentrations of molasses in total selenium reduction.

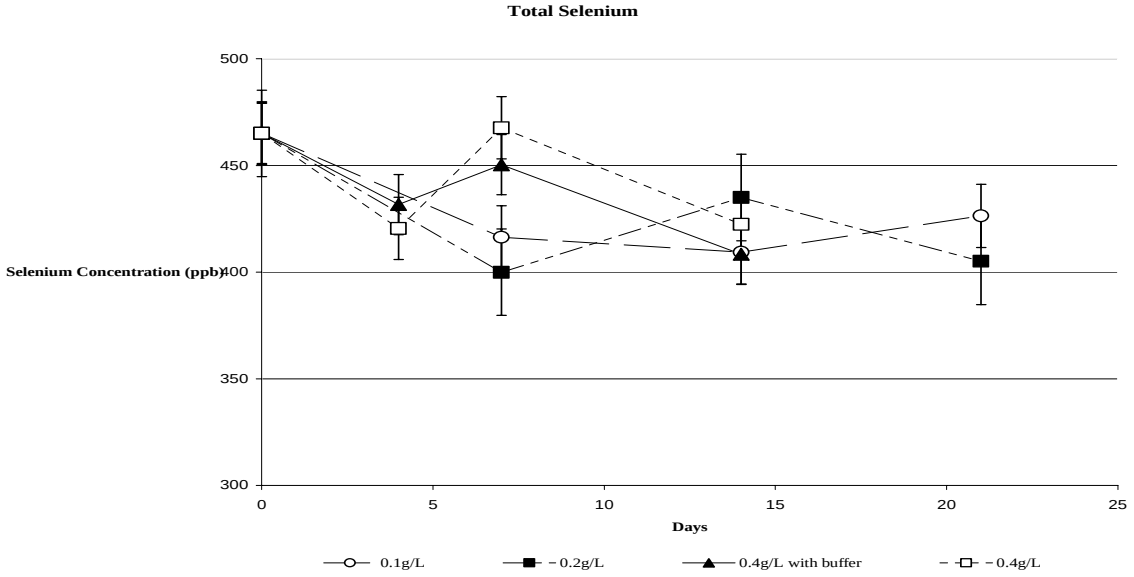


Figure 2. Results for total selenium in both experiments. Standard deviation shows that there is no difference between final concentrations.

In general the second experiment proved to be more variable. See figure 3. There was no apparent reduction in the initial selenium concentration 466ppb ± 60ppb. Final results for total soluble selenium at 0.4g/L of molasses were 430ppb ± 45ppb. Final

results for total soluble selenium at 0.4g/L molasses with phosphate buffer was 454ppb ± 7ppb respectively).

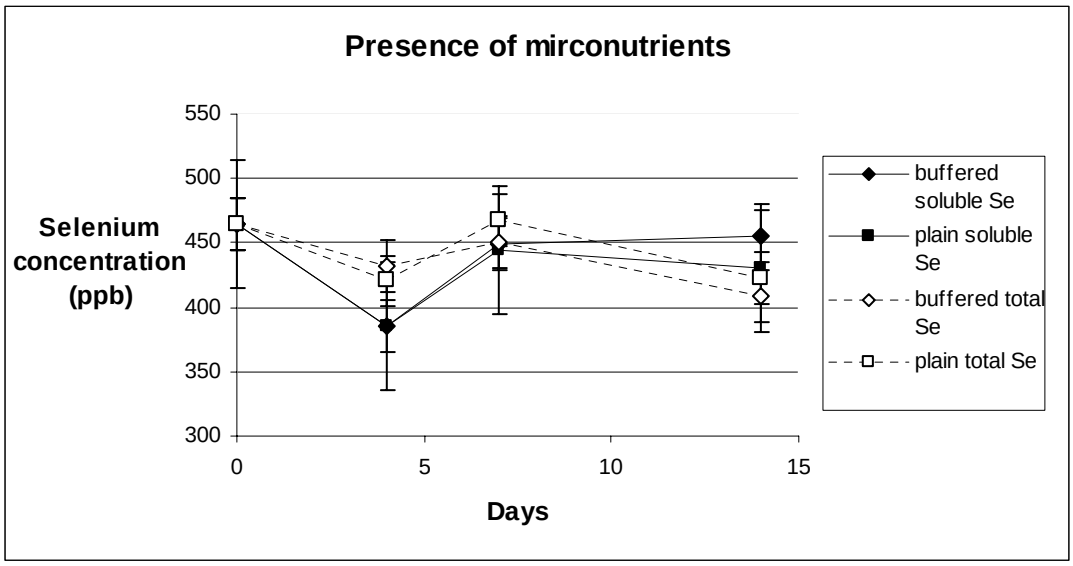


Figure 3. Results for the presence of micronutrients in the form of a phosphate buffer in 0.4g/L molasses solution over the course the experiment. Plain indicates no buffer was added. Soluble Se includes selenate and selenite. Total Se includes selenite, selenate, selenide and elemental selenium. Standard deviation bars illustrate final concentration was the same regardless of molasses concentration.

The controls to the experiments show that without bacteria, regardless of molasses concentrations, the final selenium concentration is close to the initial level of selenium for both soluble and total selenium. It also shows that even in the absence of molasses, total selenium reduction occurred at the same rate as those samples that had molasses.

**Table of Total Selenium Controls**

| | [Se] t=0 (ppb) | expected [Se] t=end (ppb) | measured [Se] t=end (ppb) |
|---|---|---|---|
| 0.1g/L, 0.2g/L, no bacteria | 466 ± 60 | 465 ± 20 | 466 ± 62 |
| only bacteria | 465 ± 60 | 465 ± 20 | 412 ± 16 |
| 0.4g/L, no bacteria | 465 ± 60 | 465 ± 20 | 436 ± 17 |
| 0.4g/L plus buffer, no bacteria | 465 ± 60 | 465 ± 20 | 599 ± 30 |

**Discussion**

I found that there was no significant decrease of selenium after the biodigestion of anaerobic bacteria. This was surprising because literature suggested a dramatic decrease of 80% (Oswald *et al*, 2000). These results are not favorable and suggest several failings of the experiment that could be explained by low molasses concentrations, inaccuracy in analytical equipment (AA), poor experimental design, and presence of limiting factors being produced by the bacteria.

The bacteria will first convert nitrate to nitrogen gas because of favorable redox potential (Gerhart and Oswald, 1990). In the San Joaquin Valley nitrate concentrations have been found to be in the range of 20-120mg/L (Oswald *et al*, 2000). It is possible that the molasses concentrations that I used may not have been enough to sustain the bacteria through the reduction of the nitrate and nitrite and on to reduce the selenium. Other successful trials with selenium reducing bacteria used more substrate; Herbel used between 0.7-1.8g of carbon per liter in the form of lactate. For similar methods I used much lower doses. The methods used in this project were developed and have been successfully implemented at Lawrence Berkeley Laboratory. Previous experiments at the LBL used 0.4g/L of molasses and contained 40-80mg/L of nitrate, yet saw a reduction of selenium (Huang, 2003). Seasonality may have played an important role in the nitrate concentrations. I collected the drainage water shortly after the first rains in fall. This might have caused an unusual spike in the nitrate levels. I do not currently have the results for the nitrate levels but have learned through Tryg Lundquist that the final nitrate levels at the end of the experiments were about 40mg/L. This indicates that very little to no selenium reduction would have occurred under these conditions.

Another concern is the inaccuracy attributed to the difficulties experienced with the atomic absorption machine (AA). Initially, the AA was not able to produce a concentration curve. A repairman came to service the machine which proved to be beneficial. A concentration curve was obtained but it took some time before we could use methods devised at the lab (6M acid flush). Some methods were never able to be adapted, such as the selenite reads or the amount of organic selenium present in the water. The machine has approximately 5% variability in concentration reads. However

our quality control methods to ensure precision and accuracy, done while processing the samples in the form of splits and spikes, (chosen randomly) showed up to 15% read variability. This means that a sample with a true concentration of 500ppb should be read with a standard deviation of 25ppb but might really have a standard deviation of 75ppb. In my results, because the samples where processed in triplicate, I used the standard deviation of the reads and not of the maximum 15% deviation from the quality control. However this larger deviation could explain some of the poor results by proving that there is no real difference between the initial values and the final values. It also helps to explain an otherwise unexplainable difference between the soluble selenium being higher than the total selenium.

Experimental design is the most important part of any experiment. There are many ways to process waste water with bacteria. I used a batch system that may have intrinsic problems. Often bacteria are incubated at controlled temperatures (I did that) while being constantly shaken to prevent settling and sticking to the side of the bottles. I did not have the apparatus to continuously shake the bottles, so once a day they were shaken vigorously for ten seconds. Also since the bottles were sealed until the time of analyzing, it is possible that the bacteria were releasing toxic substances into the sample or head space inhibiting further reduction under conditions that had higher concentrations of salts. It is worth noting here that the drainage water is also very high in sulfate. When preparing some of the samples for analysis there was an odor of rotten eggs. Normally this would indicate that all the selenium has been reduced since sulfate has a lower redox potential then selenate (Gerhart and Oswald, 1990). Other designs incubate the bacteria in an apparatus that the head space is a vacuum to prevent the build up of unwanted gases.

My results strongly suggest that at low concentrations of molasses selenium reduction does not occur. It does not matter at low concentrations of molasses if trace nutrients are added or if the solution is buffered. The practical application of this is that to reduce selenium sufficient quantity of molasses must be added or the system cannot perpetuate itself.

My project also suggests that further research needs to be conducted. Since my results were inconclusive as to why the experiments failed, I would suggest researching the following.

Future research includes:

1. Test bacteria or pond conditions over time and how that leads to different populations of bacteria present. My results could have been due to lack of the selenium reducing bacteria. A change in the microorganisms' composition in the reduction pond would provide interesting results as to how to manage a dynamic biodigestion system.
2. Establish a nitrate-selenate-molasses matrix. In other words, examine nitrate levels in correlation to selenium reduction. Finding appropriate concentration of molasses particularly during spring and fall, when application rates vary from fertilizers, and run off is exaggerated by rain and irrigation. This will not only help to ensure continuous reduction of selenium but reduce operation costs of inputs.
3. Determine if the presences of micronutrients are necessary. Molasses has a number of trace nutrients and the additional cost of supplements may not be necessary.
4. How bioremediation effects the concentration of organic selenium and its bioavilablity is perhaps the most important topic that needs to be further developed. The legislation is currently based on the belief that less selenium in the water is better. In fact what kind of species is present may have the largest impact on toxic it is to wildlife.

**Acknowledgments**

This project would not have been possible if not for the patience and support of my mentor Tryg Lundquist at the Lawrence Berkeley Laboratories. His vision helped me to overcome many of the obstacles in this project such as malfunctioning equipment, and hence he included a subproject that became the focus this paper. I would also like to thank Clemet Hseih and Matt Takata who offered me guidance and expertise in operating the atomic absorption spectrometer at the LBL. I would like to thank Donna Green and

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