Oxidation of the Pnictogens, Antimony and Bismuth, by *Bosea* sp. str. WAO

By

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ABSTRACT OF THE THESIS

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Thesis Director:

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Recently, some bacteria have been observed to grow when exposed to antimony but oxidation is not noted. No bacteria have been tested to grow on or oxidize bismuth. Both of these pnictogens, from group 15 on the periodic table, could support autotrophic growth by serving as an electron donor. *Bosea* sp. str. WAO is able to oxidize arsenic, the lightest metallic pnictogen, for autotrophic growth. *Bosea* sp. str. WAO was given different concentrations of antimony and bismuth in minimal media that would only stimulate autotrophic growth. Concentrations of the redox species of antimony and bismuth were measured by a modified antimony procedure and a novel colorimetric titration method to test for bismuth. The results showed a stoichiometric decrease in the reduced species of antimony and bismuth over time and an increase in the oxidized species. The concentration of *Bosea* sp. str. WAO was observed to increase when either antimony or bismuth had been present, showing that both antimony and bismuth could be used for autotrophic growth.

Acknowledgements

I would like to thank my advising professor Dr. Lily Young for directing me through my thesis, Dr. Nathan Yee for co directing, Dr. Max Haggblom and Dr. Gerben Zylstra for advising, Alexandra Walczak for assisting me in my research, and Maria Rivera for helping obtain the necessities for my experiments.

Dedication

This work is dedicated to my mother, Dr. Sandra Madar, who pushed her sons to achieve in academia and never lost faith in them.

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Chapter I: Introduction and Background

A. Introduction to Metallic Pnictogens

Group XV, or pnictogens meaning "suffocation" in Greek, on the periodic table consists of two elements essential to life, nitrogen (N) and phosphorus (P), two relatively toxic compounds, arsenic (As) and antimony (Sb), and one biological "neutral" element, bismuth (Bi). Bismuth and its compounds have no known biological function, but it is used in cosmetics and medicine (Norman, 1997). Similarities of pnictogens are the electron (e-) configuration of ns^2np^3, oxidation states of +3 and +5, covalent, and/or intermetallic bonding (Encyclopedia Britannica, 2012). Metallic pnictogens: arsenic, antimony, and bismuth, will be defined by their ability to alloy from covalent and intermetallic bonds with corrosion resistant metals (Schafer, 1985). Arsenic, antimony, and bismuth have metallic properties that constitutes them as physically weaker, electrical conductors than biologically essential metals like magnesium (Mg) or iron (Fe) (Arivouli et al., 1988). Regarding electronegativity, phosphorus and arsenic are synonymous, while antimony and bismuth share essentially the same value. These dynamics could be due to the negative thermal expansion property from the 3d and 4f electron shell occupancy, giving antimony and bismuth higher oxidizing properties (Silver, 2005). Higher oxidizing properties could be attractive to microbes that require an electron donor.

Up until the 19th century, the use of metallic pnictogens was limited to cosmetics, anti-microbial remedies, and pyrotechnics. Currently, all three are still used in pyrotechnics and anti-microbial remedies as well as electronics. Arsenic

and antimony are currently banned from cosmetic applications in the United States (Ishiguro, 1992). Although considered toxic, antimony and arsenic have significant use as anti-microbial applications in very small concentrations to fight viral, protozoan, and carcinogenic infections (Zhang et al., 2005).

In terms of specific employment, the metallic pnictogens are used in quite diverse processes in industrial applications. Arsenic is additionally used for industrial applications that include: pesticides, non-ferrous alloys, light emitting diodes (LEDs), solar cells, cancer treatments (arsenicals), livestock feed (roxarsone), and preservatives (Bentley, 2002). Ironically, arsenic's usually toxic properties are useful in treating promyelocytic leukemia where carcinogenic cells were eliminated from bone marrow when trace amounts of arsenic were circulated into the bloodstream (Soignet et al., 1998). However, in chronic exposure to low concentrations of arsenic, will cause cancer to the skin, bladder, kidney, and lungs via the condition, arsenicosis (WHO, 2013).

Antimony is used in different products varying from protozoan antibiotics, veterinary emetics, glazes, flame-retardants, ammunition, and batteries (Reimann et al., 2010). Some of the uses overlap with arsenic since both of the metalloid's physical properties are similar to each other, but it is less toxic since antimony had been used as a human emetic up until the last century (McCallum, 1977). Compounds containing antimony are used for monitoring incineration efficiency because the element is much more abundant in solid waste than in the lithosphere (Paoletti et al., 2001). Regardless, the demand for industrial antimonyl components are rising to replace the more hazardous, arsenic based products (ADROIT, 2012).

With the modern day ban of arsenic and antimony substances in cosmetics, bismuth has become a popular component in eyeliner because of the pink coloration that it forms in its trivalent state. Bismuth thiols are new and upcoming treatments against pathogenic biofilms in plants and animals (Folsom et al., 2011). Trivalent bismuth is used to treat intestinal ailments as well, the most popular being bismuth subsalicylate ($C_7H_5BiO_4$) in Pepto-Bismol (Krebs, 2006). With the economic demand rising for both antimony and bismuth metals (Minor Metals Trade Association, 2012), the mining industry will inevitably introduce more antimony, and bismuth species into water sources.

Arsenic has always been a problem in water supplies, occurring naturally in water, topsoil, and plants (Thornton, 1996). In contaminated areas, detoxifying groundwater contaminated with arsenic to the 10 ppb standard, regulated by the United States Environmental Protection Agency, can be quite difficult (EPA, 2013). Antimony has been recently more problematic to groundwater supplies; from the mobilization of the element from shooting ranges, power plants, and smelting operations as well as the natural abundance of stibnite (Sb_2S_3) that is found up to 0.50 mg/kg of the earth's crust (Reimann et al., 2010). Mining operations have created geological "turnovers" of such antimonyl compounds that introduce the toxin to water sources. Occurring in groundwater sources, antimony is usually paired with a hydroxyl anion in aqueous systems as either $Sb(OH)_3$ or $Sb(OH)_5$/ $Sb(OH)_6^-$ depending on the amount of dissolved oxygen. In an oxygenated form, antimony is very soluble. Antimony and arsenic are similar in terms of valence states and toxicity. The arsenic/antimony (III) valence is more environmentally

hazardous than the (V) state. In reducing, subsurface waters, the trivalent state of both elements is dominant except in very high and very low pHs; nonetheless, both states can exist over the pH range (Krupka, 2002).

Bismuth is regarded as the heaviest, stable element existing in nature as ^{209}Bi and is commonly found in tungsten (W) mining. Also found in the Earth's crust, the average concentration of bismuth is approximately 8 μg/kg, much less abundant than antimony. The element is rarely found by itself, but when paired with other heavier metals, can synergistically act as a neurotoxin in mice (El-Shahawi et al., 2012).

All three metallic pnictogens are capable of being an electron donor, a chemical entity that transfers electrons as a reducing agent in order to be oxidized itself during cellular respiration (Boundless, 2013). In terms of role as an electron donor, arsenic is commonly known as a thermodynamically favorable element for autotrophic growth (Stolz et al., 2010). An autotroph is defined as a life form requiring carbon dioxide (CO_2) or carbonate (CO_3^{2-}) as a source of carbon and simple inorganic nitrogen compounds to metabolically synthesize organic compounds (Merriam-Webster, 2013). In order for this process to carry out, the electrons from the electron donors are stored and generate proton (H^+) gradients for adenosine tri-phosphate (ATP) mobilization. Electron carriers become reduced and fix carbon dioxide according to its reduction potential (Bar-Even et al., 2012). Arsenic can be oxidized by certain prokaryotes, while oxygen (O_2) is reduced in order to fix carbon dioxide as the carbon source via the Calvin Cycle after being paired with oxygen via the electron transport chain. The energy for cell synthesis is

provided by the exergonic thermodynamics as an outcome from the oxidative reaction (Dastidar, 2012).

The enzyme employed in oxidative "arsenotrophy," which uses arsenic for a biological redox reaction, is the arsenite oxidase protein. Arsenite oxidase is controlled by the *aioBA* gene, formerly *aox* (Lett et al., 2012), and is part of a broad family of molybdenum (Mo) reductase enzymes. This hetero-dimer contains the large molybdenum reaction center and the iron-sulfur cluster (Rieske) containing subunit. The arsenic oxidation is then coupled with the reduction of oxygen (Drelich, 2012) where the electrons from the arsenic compounds are brought along an electron transport chain by cytochromes for carbon fixation (Branco et al., 2009). Cytochrome abundance increases when *aioABC* is turned on by the aioD operon (Muller et al., 2007). The energy available is favorable for As(III) oxidation to As(V) according to thermodynamic calculations. By looking at the theoretical energetic estimates for antimony and bismuth, one would presume that other group XV members could also be substituted as an electron donor under standard conditions:

<u>Pnictogen</u> <u>ΔG°_{f}</u>

$4As(s) + 3O_2(g) \rightarrow 2As_2O_3$= -575 KJ/mol (Schuhmann, 1924)

$4Sb(s) + 3O_2(g) \rightarrow 2Sb_2O_3$= -622 KJ/mol (Schuhmann, 1924)

$4Bi(s) + 3O_2(g) \rightarrow 2Bi_2O_3$= -493 KJ/mol

$As_2O_3(s) + O_2(g) \rightarrow As_2O_5$= -205 KJ/mol

$Sb_2O_3(s) + O_2(g) \rightarrow Sb_2O_5$= -200 KJ/mol

$Bi_2O_3(s) + O_2(g) \rightarrow Bi_2O_5$= Literature unavailable

B. Background of *Bosea* sp. str. WAO

The prokaryote used for this study is a recently isolated strain of bacteria named *Bosea* sp. str. White Arsenic Oxidizer (WAO). This alphaproteobacteria was isolated at Rutgers University from an outcrop of Newark Basin black shale. *Bosea* sp. str. WAO is capable of growing autotrophically on arsenic contaminants, behaving as a strict aerobe. The organism can grow on reduced sulfur (S) sources and up to 5 mM of reduced arsenic in the elemental As(0) form, mineral forms, such as arsenopyrite (FeAsS), and trivalent forms, such as sodium arsenite ($NaAsO_2$) for oxidation (Rhine et al., 2007).

The bacterium is capable of arsenic oxidation and possesses the *aio* genes (Rhine et al., 2008). Interestingly, *Bosea* sp. str. WAO has over fifty different types of cytochromes, approximately half with an unassigned function according to genomic analysis [Walczak, 2013 (unpublished data)]. The *aio* gene in *Bosea* sp. str. WAO is over 70% homologous to another Rhizobiales order member, *Agrobacterium tumefaciens*, a strict chemoheterotroph (Rhine et al., 2008). *A. tumefaciens* is able to oxidize 5 μM of antimony as potassium antimonyl tartrate ($K_2Sb_2(C_4H_2O_6)_2$ or PAT), a trivalent form, for detoxification purposes. Other proteobacteria such as *Pseudomonas* and *Acinetobacter* have been shown to grow in the presence of ≤ 5 μM of PAT, where the antimony detoxification gene (*aioBA*) had been identified (Li et al., 2013).

The metalloid detoxifying and energy-utilizing gene, *aioBA*, had been observed within *Bosea* sp. str. WAO's genome [Walczak, 2013 (unpublished data)]. With further observation, the organism is able to withstand much higher concentrations of metals such as iron, zinc (Zn), and lead (Pb), a unique feat for a

facultative haloalkliphilic bacterium. In neutral and alkaline conditions, this organism can oxidize 5 mM As(III) in approximately one week. The combination of metal resistance and arsenic oxidation makes *Bosea.* sp. str. WAO an ideal candidate for metallic pnictogen oxidation and/or utilization [Carey, 2013 (unpublished data)].

C. Hypothesis and specific aims

The hypothesis of this research is that, given the chemical and geochemical similarities to arsenic, *Bosea* sp. str. WAO will grow autotrophically on antimony and bismuth.

To test this hypothesis, experiments were carried out in specific aims that include:

1. Develop and modify chemical procedures for determining the valence states and concentrations of antimony and bismuth.
2. Determine if *Bosea* sp. str. WAO can oxidize antimony and bismuth.
3. Determine if the oxidation of antimony and bismuth can stimulate growth.

Chapter II: Oxidation of Antimony (Sb) by *Bosea* sp. str. WAO

D. Introduction

The *aioBA* gene is the only antimony oxidation gene identified in literature thus far (Li et al., 2013). *AioBA* can represent a gene that is used to either oxidize arsenic for detoxification purposes or autotrophic growth (Lett et al., 2011). This gene turns on an arsenite oxidase efflux pump that oxidizes the trivalent (III) arsenic to a pentavalent (V) form in proteobacteria (Li et al., 2013). The bacteria in the study, however, could all be growing heterotrophically on potassium antimonyl tartrate (PAT) and simply detoxifying the small amounts of antimony. *Bosea* sp. str. WAO has a different arsenite oxidase, so one could speculate that the 30% homology difference from *A. tumefaciens*' detoxification protein might be capable of transporting arsenic or antimony electrons to cytochromes through the electron transport chain and stimulate carbon fixation (Rhine et al., 2008). This difference is most likely the configuration of the Rieske subunit in "arsenotrophic" oxidizing bacteria, which is very similar to the cytochrome bc_1, an electron transport intermediate (Ellis et al., 2001). Cytochrome bc_1 catalyzes the electron source and transfers electrons to other cytochromes across the electron transport chain, thus turning on the Calvin Cycle (Zhang et al., 1998). Because *Bosea* sp. str. WAO can capture electrons with it's arsenite oxidase, the speculated "promiscuous" enzyme, then it could capture the electrons from antimonyl compounds using similar biological mechanisms.

E. Materials and Methods

Antimony oxidation media: The media used for the experiment was set up using elemental Sb(0) that was crushed into a powder and dispersed into water (100 mM) and PAT as [Sb_2O_3], the Sb(III) oxidation state, was dissolved in water (100 mM). Another 100 mM of PAT was then dissolved in concentrated nitric acid (HNO_3^-) in order to oxidize it to [Sb_2O_5], the Sb(V) oxidation state. There were two conditions tested: 0.50 mM elemental Sb(0) or 0.50 mM PAT [Sb_2O_3]. BT is a minimal salts media that was modified by addition of antimony in place of sulfur, which is traditionally used as the electron donor with (DSMZ, 2007). Any organic substrates were omitted from the media. For each of the Sb oxidation states, two flasks were used as background controls (BG) with media and 0.50 mM Sb (0) or (III), two sterile controls (SC) with media, Sb (0) or (III), 100 µL killed cells, and three actives (A-n) with media, Sb(0) or (III), and 100 µL live cells. *Bosea* sp. str. WAO was incubated for two days in 50% normal strength tryptic soy broth (TSB) media with 5 mM sodium thiosulfate ($Na_2S_2O_3$) to generate healthy growth, since *Bosea* sp. str. WAO is capable of using organic carbon for growth as well (Rhine et al., 2007). After growth was obtained, half of the volume was autoclaved to use as inocula for sterile controls. The cells were then centrifuged and washed with phosphate buffer (PO_4^{3-}) three times to remove any residual sodium thiosulfate and TSB. All cultures were incubated for two weeks at 30 °C. One milliliter was sampled on day zero and every three days, thereafter.

Antimony oxidation analyses: A test method had been written for antimony oxide (Sb_2O_n) presence using a standard titration procedure the levels of Sb(III) and Sb(V) can be measured. The analyses are divided into "test A" for Sb(III) presence

and "test B" for the total antimonyl oxidation, Sb_2O_n. Sb(V) can be calculated by the difference, Sb_2O_n (test B)-Sb_2O_3 (test A). "Test A" only measures the trivalent antimony oxidation state by boiling the sample in sulfuric (H_2SO_4) and hydrochloric (HCl) acids and then titrating against potassium permanganate ($KMnO_4$), the dependent variable, to calculate antimony concentration. The faint pink color indicates that the titration is complete. The presence of oxidized antimony species prevents this color change. "Test B" measures both pentavalent and trivalent antimony by using potassium sulfate (K_2SO_4) and sulfuric acid to disintegrate the media and carbon (C) to act as a reducing agent. The sulfur dioxide (SO_2) is expelled by sodium sulfite (Na_2SO_3) while the antimony concentration is measured by the potassium permanganate titration (ASTM, 2010).

The experimental procedure was modified to reduce the reagents to 20% of the original volume and 0.01 M potassium permanganate, a more diluted titrant, was used to measure smaller concentrations of antimonyl oxides and reduce waste. The method was modified further by using methyl red/ methylene blue indicator instead of starch paper for sulfur dioxide presence, which inhibits the reaction and carbon paper to act as a reducing agent (Robinson, 1994). Sodium thiosulfate had been used instead of sodium sulfite since both compounds release sulfide, interference to potassium permanganate according to the test procedure (ASTM, 2010). Sodium thiosulfate releases sulfur dioxide at a slower rate, which would make the test more sensitive for smaller concentrations of antimony (Holleman, 2001). A reference solution, with a known amount of antimony, was used before every run to indicate the faint pink color.

Antimonyl growth analyses: Since PAT is an organic salt, *Bosea* sp. str. WAO is using antimony for growth and not growing heterotrophically on tartrate, a preservative in food used to prevent microbial growth (Robinson, 1994), instead of autotrophically by oxidizing antimony. To determine if it was occuring, a second experiment measured change in population and protein dynamics was set up. The experiment used the conditions previously described: 50 mL of BT media and elemental antimony Sb(0) or antimonite Sb(III) by dissolving Sb(0) in boiling nitric acid (100 mM). The 100 mM antimony trioxide (Sb_2O_3) solution was then buffered in sodium hydroxide (NaOH) to a pH of 9 with a final concentration of 5 mM [Sb_2O_3] as sodium antimonite ($NaSbO_2$).

This time there were two live controls (LC) with 100 µL of cells, absent of Sb or an electron donor, along with two backgrounds (BG), two sterile controls (SC), and three actives with either Sb(0) or Sb(III). Cells were washed three times with 20 mM sodium chloride (NaCl) before the experiment initiated. *Bosea* sp. str. WAO strains were grown in rich TSB media before being inoculated into minimal medium with and without the Sb electron donor. This experiment will measure cell growth based on the media's optical density and the colony forming units (CFU/mL) through a dilution series after being placed in a starved (LC) and Sb-mediated autotrophic growth conditions (A-n). The Sb(0) and Sb(III) biotic actives were pipetted on rich TSB agar while Sb(III) had been pipetted on minimal, autotrophic media without organic substrates after week two (first two weeks were on TSB agar). The concentration of protein in the presence of Sb(III) was determined by digesting albumin protein to measure amino acid concentration using

spectrophotometric analyis (Pierce, 2013). One milliliter of media was selectively taken weekly during a five week experimental run.

F. Results and Discussion

Antimony Titration: The modifications made to the antimony oxidation titration, originally used for paints, determines the concentration of Sb oxidized by measuring how much $KMnO_4$ must be added to show a color change. The results show an increase of $KMnO_4$ volume as the concentration of oxidized Sb increased. The "test A" calibration curve (**Figure 1**) is based off of an average of triplicate Sb(III) standards that showed an accurate curve by reducing the amount of reagents from the original procedure, a linear regression r^2 value of 0.997. By mixing the Sb(III) and Sb(V) standards of equal concentration, the accuracy still maintains acceptional values for the "test B" total oxidized Sb calibration curve. This r^2 value had been 0.983 (**Figure 2**) even after the procedure had replaced sodium sulfite with sodium thiosulfate to liberate sulfide interference from the reaction. The titration standards for "test B" had also been tested in triplicate with little deviation to show how accurate the titration is.

Antimony Oxidation: At the initiation of the experiment there are not any antimony oxides detected by the titrant; however, after three days, antimony oxidation of Sb(0) to Sb(III) and Sb(V) were observed in active cultures. Sterile controls do not show any Sb oxidation whatsoever, indicating that Sb is not oxidized abiotically in these cultures. The active culture average shows a stochiometric oxidation from ≈0.50 mM spike of elemental Sb(0) to ≈0.50 mM Sb(III)+(V) within 12 days. Sb(III) can still be further oxidized to Sb(V), so an additional sample was

taken a month later to show that *Bosea* sp. str. WAO is capable of completely oxidizing 0.50 mM Sb(0) to 0.50 mM Sb(V) after 40 days (**Figure 3**). The amount of Sb(III) increases and drops over time, which shows the cells further oxidizing Sb(III) to Sb(V). Once the Sb(III) is available, it seems to be oxidized much faster than Sb(0) by the cells, oxidizing 0.50 mM Sb(III) to 0.50 mM Sb(V) within a 12 day time period (**Figure 4**). The sterile controls show a constant amount of 0.50 mM Sb(III) since there isn't any biological activity that would allow oxidation to occur. No abiotic oxidation occurs in any of the sterile cultures. This further proves that Sb(III) is oxidized to Sb(V) by *Bosea* sp. str. WAO.

Antimony Growth: After providing an accurate optical density (OD) calibration curve that had shown an increase in absorbance with the albumin standard concentrations (**Figure 5**), cultures containing Sb(III) and cells consistently produced more protein than the cells without any electron donor source over a four week time period (**Figure 6**). This shows that *Bosea* sp. str. WAO is creating protein and replicating in the presence of Sb(III). Optical density reading showed an increase in turbidity when Sb(III) was present, ascending from 0.005 to 0.008, and then to 0.013 OD for cells with an electron donor. Rhizobium species have been observed to double in OD measurements on more biologically favorable, such as nitrogen and sulfur based electron donors, after approximately a week in the presence of carbonate (Pickering, 2008). Cultures without an electron donor remained at approximately 0.002 over the three-week period of time (**Figure 7**). The backgrounds were subtracted from the live cultures containing antimony.

In relationship to Sb(0) and Sb(III) oxidation, the CFU and OD in actives are increasing during the two week time period measured and it appears that the electrons from the autotrophic growth conditions are being stored for further growth due to certain environmental conditions. The Sb(V) valence may inhibit the growth of *Bosea* sp. str. WAO but not enough to notice an increase in cell density. Organic pentavalent antimony, such as sodium stibogluconate, is extremely toxic to certain microbes (Herwaldt, 1992).

Consistent with the turbidity, the CFU/mL from the Sb(III) experiment was counted after one week of inoculation. When cells were pipetted on heterotrophic agar from the autotrophic growth conditions, there was an increase in CFU/ml in both electron donor absent (going from ≈3 CFU/ml to 12 CFU/ml) and antimony present cultures (going from ≈3 CFU to 17 CFU/ml) within two weeks (**Figure 8A**). Autotrophic agar plates were used for weeks three and five when Sb(III) cultures had been sampled. The average colony forming units per mL with the 1 mM Sb_2O_3 source nearly tripled, going from 13 CFU/mL to 38 CFU/mL within two weeks while cells without an electron donor decreased over time (**Figure 8B**). Growth is very slow on these plates promoting autotrophic growth via limited nutrients (BT media, agar, and 5 mM $Na_2S_2O_3$ as the only e- source). A rich agar media needed less time for cells to grow. The CFU from the Sb(0) samples were counted after three days of being pipetted. The average cell population with Sb(0) doubled, going from ≈340 to ≈680 CFU/mL in eleven days. The cell population without an electron donor decreased over that time period (**Figure 9**). This data further explains the concept that *Bosea* sp. str. WAO can oxidize Sb(0) or (III) for autotrophic growth.

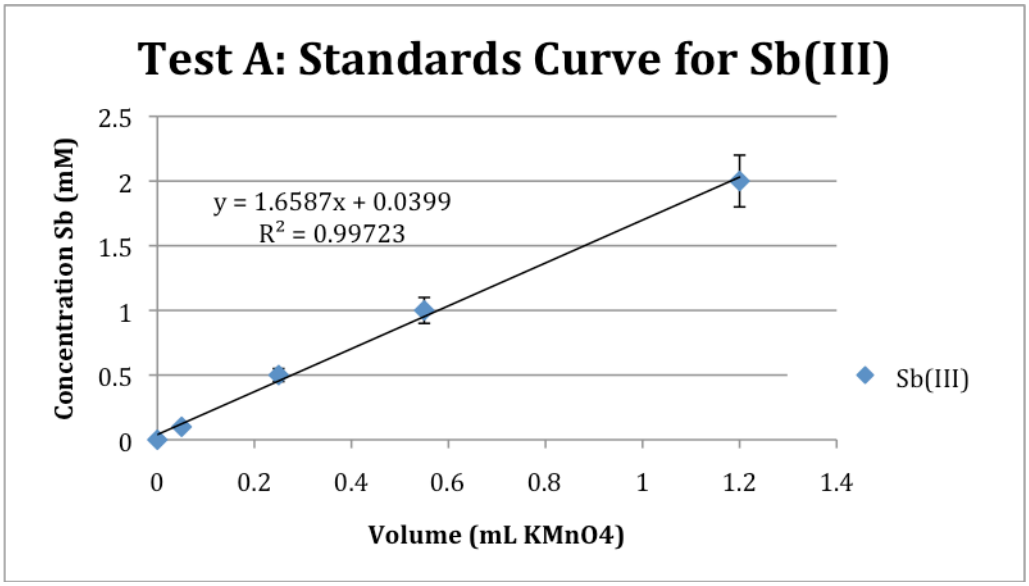

Figure 1- Linear relationship between the concentration of Sb(III) and the volume of $KMnO_4$ titrant used to generate a standards curve. Sb(III) standards concentration increases as the volume of $KMnO_4$ titrated to observe a color change increases. The higher the concentration of Sb(III), the higher amount of $KMnO_4$ is necessary to change the solution to a dull pink color.

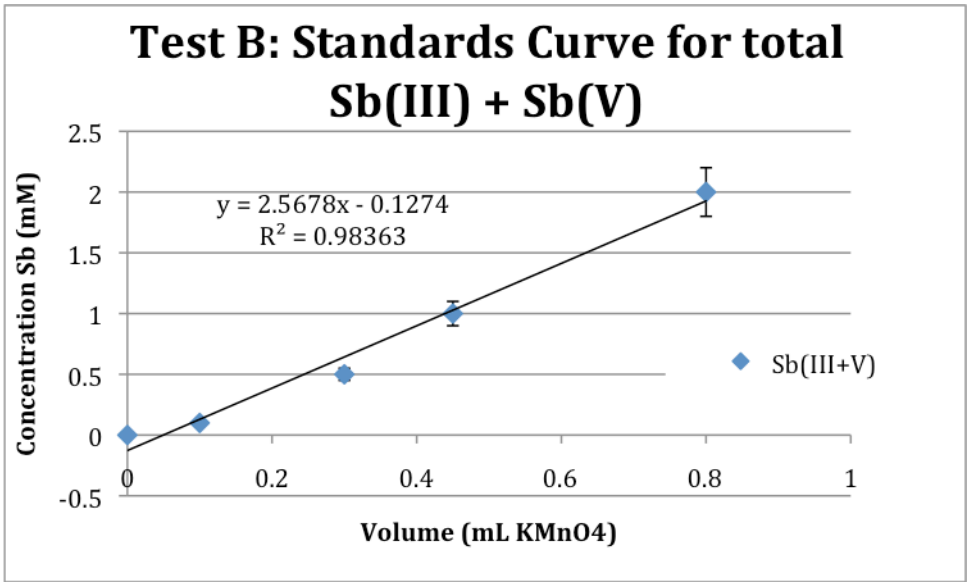

Figure 2- Linear relationship between the concentration of equal amounts of Sb(III)+(V) and the volume of $KMnO_4$ titrant used to generate a standards curve. Concentrations of mixed Sb(III) and Sb(V) standards in equal concentrations (half of the sample volume per valence state) increases as the volume of $KMnO_4$ titrant increases.

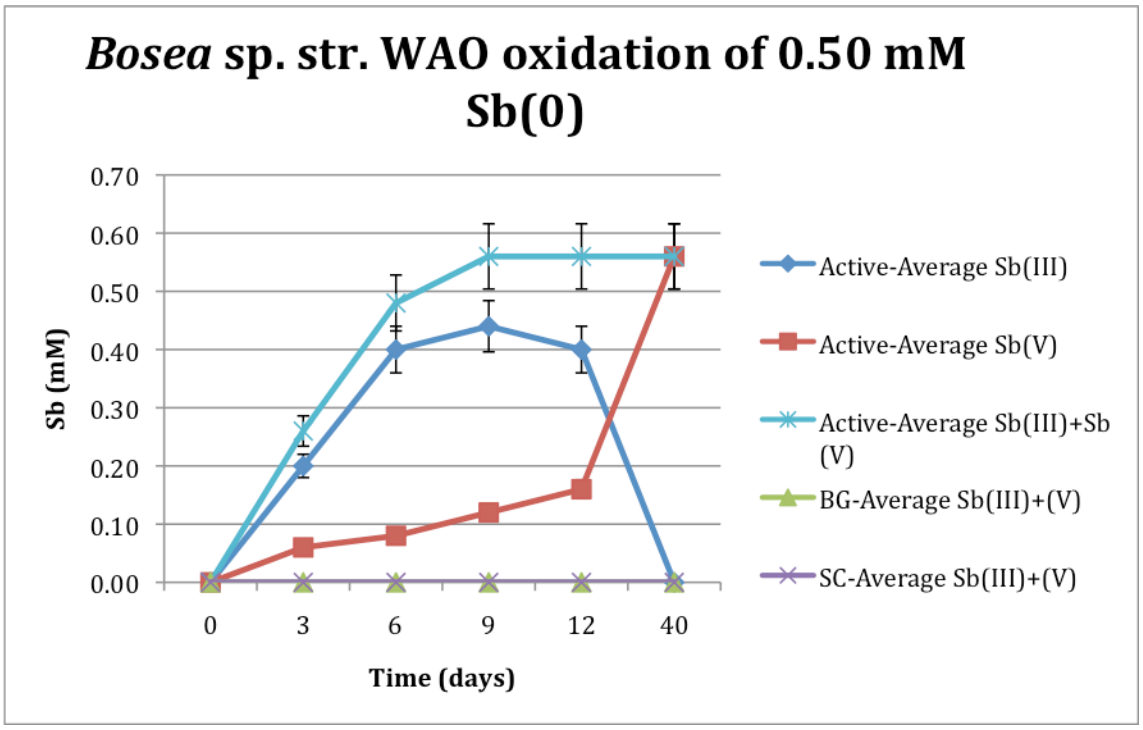

Figure 3- Oxidation of Sb(0) to Sb(III) and ultimately to Sb(V) by active cultures of *Bosea* sp. str. WAO and abiotic controls. The active cells are oxidizing Sb(0) to Sb(III) and ultimately to Sb(V) while controls are not showing oxidation. There is a stoichiometric increase in total oxidized species of ≈0.50 mM Sb(III/V) over the 12 day period and all 0.50 mM of Sb(III/V) is converted to ≈0.50 mM Sb(V). The backgrounds (BG) and sterile controls (SC) do not show any oxidation.

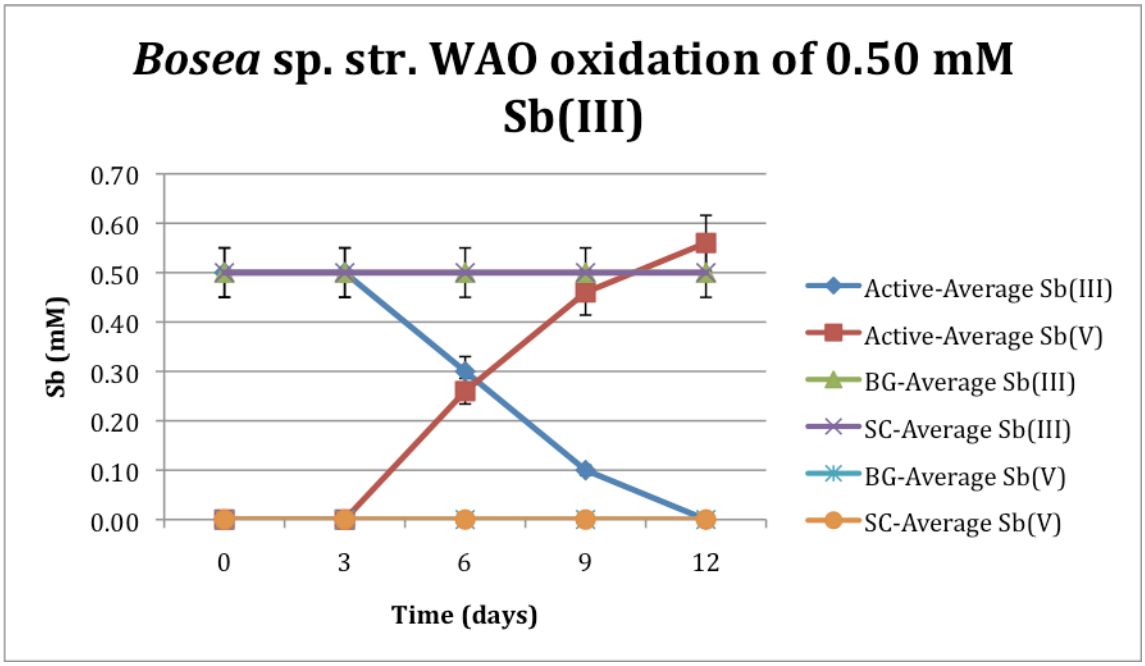

Figure 4- Oxidation of Sb(III) to Sb(V) by active cultures of *Bosea* sp. str. WAO and abiotic controls. The active cells are oxidizing 0.50 mM Sb(III) to Sb(V) within 12 days while the backgrounds (BG) and sterile controls (SC) are not showing any oxidation or reduction of the 0.50 mM Sb(III) throughout the 12 day time period.

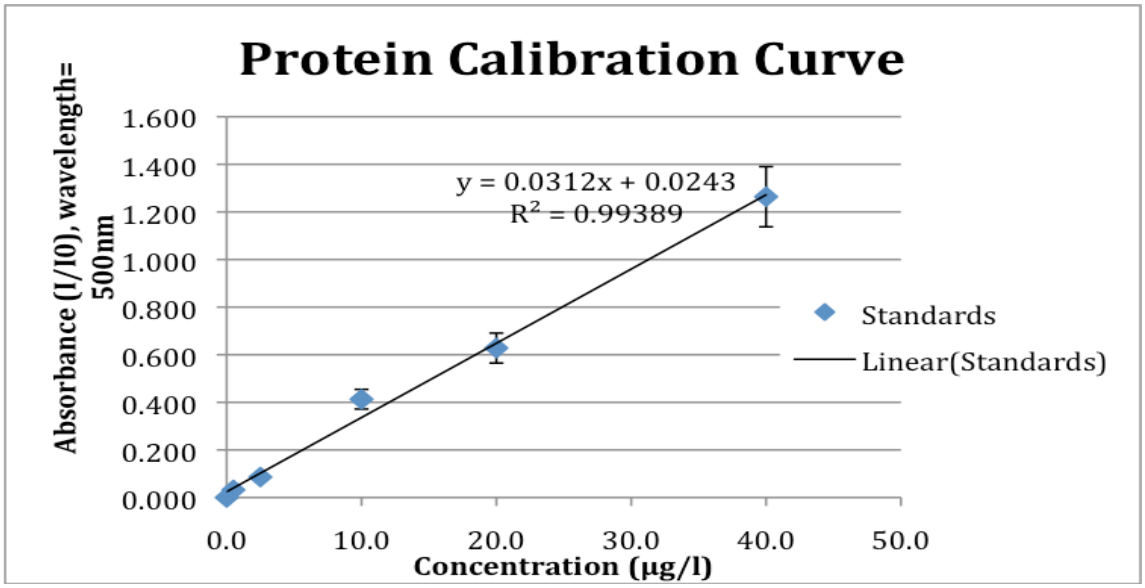

Figure 5- Linear relationship of albumin standard concentrations in the presence of Sb(III) to generate a standards curve. The calibration curve measures the increasing concentration of albumin with respect to the absorbance due to color change. As albumin concentration increases so does the absorbance at 500 nms.

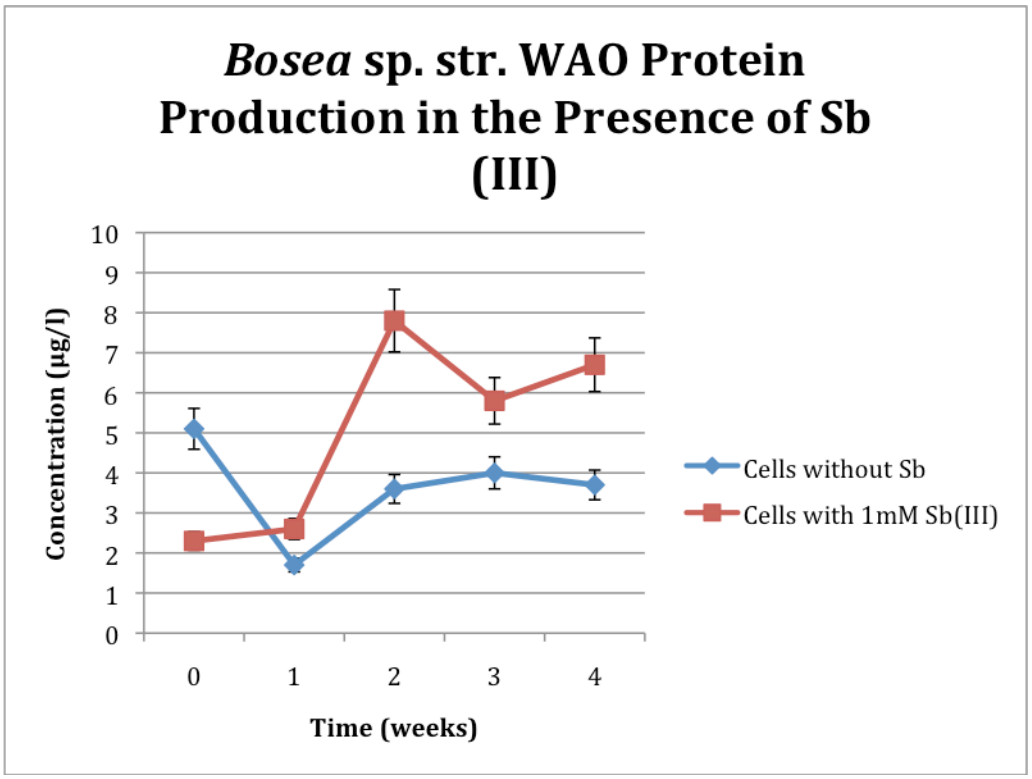

Figure 6- Protein measured in relationship to Sb presence. Active cultures with an Sb(III) source are increasing in protein concentration than cultures without an Sb(III) source, which indicates that there is an increase in biomass. Cultures without Sb(III) constantly have less protein.

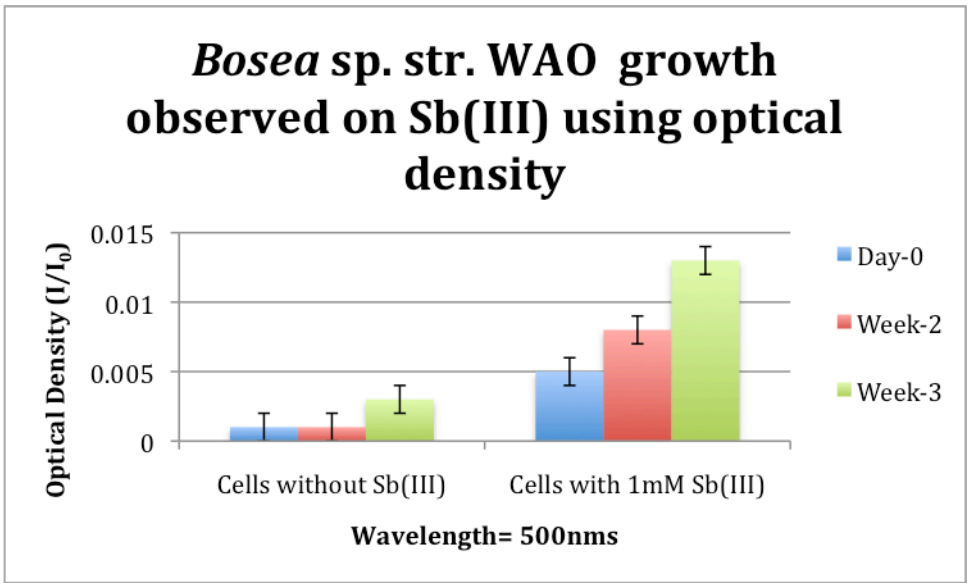

Figure 7- Optical density measurements of biotic cultures with and without Sb(III). Cultures with 1 mM Sb(III) are increasing in OD, indicating an increase in concentration of cells. Where as cultures without Sb(III) are only slightly increasing

in OD. The backgrounds have been subtracted from the cultures with 1 mM Sb(III) since precipitation may occur within the media.

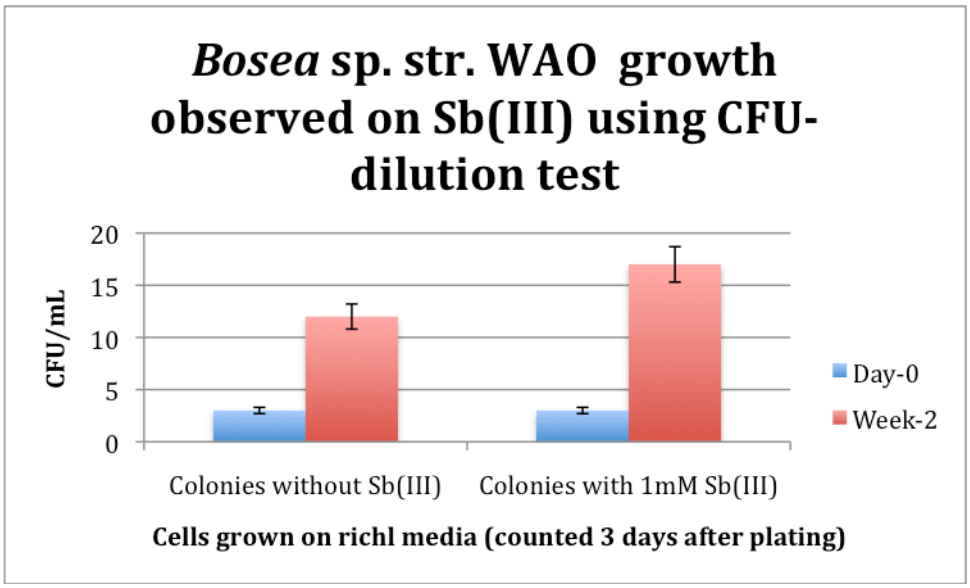

Figure 8A- Colony forming unit measurements of biotic cultures with and without Sb(III) on rich agar. Cells with 1 mM Sb(III) are increasing at a higher rate than cells without Sb(III) over the first two weeks, which could be due to *Bosea* sp. str. WAO's proposed ability to store energy and/or carbon sources. Autotrophic plates were then used to measure CFU/ml for Sb(III) growth (8B).

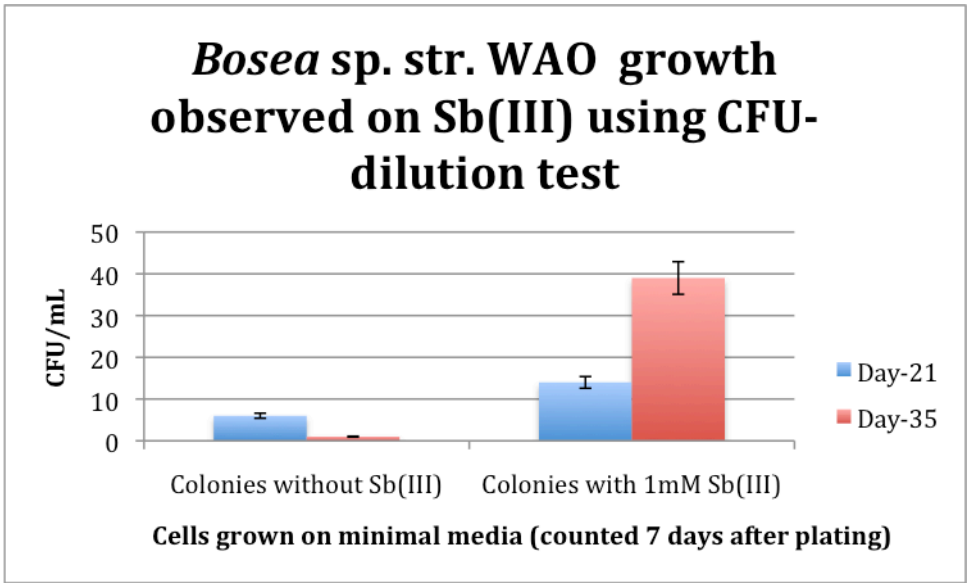

Figure 8B- Colony forming unit measurements of biotic cultures with and without Sb(III) on minimal agar. Cells with Sb(III) are increasing in colony forming units while cells without Sb(III) are decreasing in colony forming units.

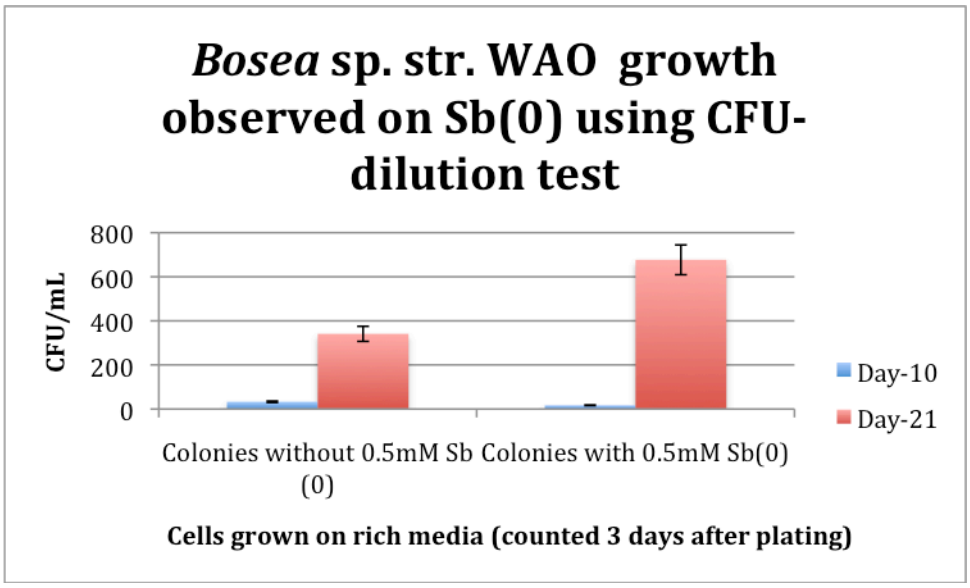

Figure 9- Colony forming unit measurements of biotic cultures with and without Sb(0) on rich agar. Cells with Sb(0) are growing a lot more quickly than cells without Sb(0) as an electron donor. The increase in population could be due to *Bosea* sp. str. WAO's plausible ability to store nutrients. Environmental interfaces terminated the ability to sample after day-21.

G: Conclusion

Based on the results of the experiments that tie in oxidation, growth, and protein production due to presence of antimony, *Bosea* sp. str. WAO is using Sb(0) and/or Sb(III) as an electron donor for autotrophic growth. The increase in biomass and production of Sb(V) demonstrates that *Bosea* sp. str. WAO is using the antimony for autotrophic growth. Not just arsenic and sulfur, among other reduced ions, can be used as an electron donor in autotrophy. The significance of the experiments shown are the first to demonstrate microbial oxidation of antimony for growth. The genetics behind the antimony oxidation still remains a mystery. This would question one to believe that arsenic oxidase may be involved rather than a whole new set of antimony specific genes.

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Chapter III: Assay for Determining Bismuth Concentration and Oxidation State

I. Introduction

Since there is clear evidence of biotic antimony oxidation in the previous chapter, the obvious question arises as to whether oxidation of the heaviest natural pnictogen, bismuth, can be microbially mediated. At the beginning of this project, a major obstacle arose. Namely, there is no readily available method to measure bismuth concentrations or oxidation states. It is likely because the element is considered non-toxic, has a small market for Bi(III), and the difficulty of measuring Bi(V), the literature for bismuth analysis remains almost non-existent. The most economical bismuth measurement tests are decades old and measure only the total bismuth rather than the valence states (Hubbard, 1939; Ward, 1956). Another, recently published method uses a polyurethane foam column to measure Bi(III) and Bi(V) concentrations, but a variety of diverse metallic ions and nitrogen cause interference. This method is quick, but costly in the short run (El Shahawi et al., 2012). In order to carry out the microbial experiments, a bismuth assay had to be developed first. This chapter describes the assay, which was developed based on the fact that bismuth iodide (BiI_3) forms a dark colored precipitate (Bruno, 2003) and manganese (Mn) is bound by Bi(V) (Colins, 1924). The novel titration described is readily carried out and less costly than the procedures described previously.

J. Material and Methods

<u>Total Bismuth Valence Assay (Test B):</u> The analysis to measure Bi(III) and Bi(V) described requires the following chemicals: distilled water, concentrated hydrochloric acid (HCl), potassium iodide (KI) anhydrous, and Bi(III) and/or Bi(V)

standards of various concentrations. The following items are needed: centrifuge, pH paper or meter, hot plate, titration pipette, 50 mL Erlenmeyer flask or beaker, and 25 mL graduated cylinder.

Reagent #1: dissolve 16.600g of KI in 100 mL of distilled water for 1.0M concentrations for trace (5-0.025 mM Bi) detection, 1.660g of KI in 100 mL of distilled water for 0.1 M concentrations for significant (10-5 mM Bi) detection.

Procedure:

1. Add 10 mL of distilled water to the 50 mL flask or beaker and bring pH down to 2.00 +/- 0.10 with HCl.
2. Add 0.50 mL of centrifuged sample or standard to acidified water and place on hot plate and set to boil.
3. As soon as the sample boils, titrate 5 mL of 1.0 M or 0.1 M KI reagent and allow mixture to evaporate until a goldenrod color (110C Pantone, 2008) appears, which indicates that Bi ions are present. The sample will remain clear if Bi(III) or Bi(V) isn't present and white precipitate will appear at the bottom of the flask.
4. Immediately remove sample from hot plate as soon as the goldenrod color appears before a dark purple precipitate appears (in significant concentrations that indicates BiI_3 has formed). Then pour extracted goldenrod colored volume into graduated cylinder and record volume.
5. Calculating Bi(III)+(V) molarity (M) can be done by a linear regression (y=ax+b), curve comparing the standards used for total, Bi(III)+Bi(V),

bismuth valence concentrations. This is done by standard concentration vs. remaining volume, the dependent variable.

Trivalent Bismuth Assay (Test A): The analysis to measure strictly Bi(III) requires the following chemicals: distilled water, concentrated hydrochloric acid (HCl), potassium iodide (KI) anhydrous, manganese chloride tetrahydrate ($MnCl_2 \bullet 4H_2O$) and Bi(III) standards of various concentrations. The following items are needed: 50 mL Erlenmeyer vacuum flask and filtering apparatus, carbon filter paper, hot plate, pH paper or meter, titration pipette, 50 mL Erlenmeyer flask or beaker, and 25 mL graduated cylinder.

Reagent #1: dissolve 16.600g of KI in 100 mL of distilled water for 1.0M concentrations for trace (5-0.025 mM Bi) detection, 1.660g of KI in 100 mL of distilled water for 0.1 M concentrations for significant (10-5 mM Bi).

Reagent #2: dissolve 19.7910g of $MnCl_2 \bullet 4H_2O$ in 100 mL of distilled water for a 1 M concentration.

Procedure:

1. 1. Add 10 mL of distilled water to the 50 ml flask or beaker and bring pH down to 1.00 +/- 0.10 with HCl. Then add the 0.50 mL sample or standard.
2. Add beaker or flask containing sample and acidified water to the hot plate and allow it to boil.
3. Once boiling, add 5 mL of 1 M $MnCl_2$ reagent and wait until color turns dull pink (251C Pantone, 2008), binding Bi(V) (Colins, 1924). Strong ionic presence can prevent this from happening. In this case, wait until approximately half of the volume has evaporated (5 mL).

4. After color change or half the volume has evaporated, take the sample off the hot plate and filter through carbon paper with a vacuum flask. Use 5 mL of distilled water to rinse and swish empty receptacle and filter additionally.
5. Collect the analysis filtrate from vacuum flask with a cleaned 50 mL beaker or flask and put to boil on the hot plate.
6. As soon as the sample boils, titrate 5 mL of 1.0 M or 0.1 M KI reagent and allow mixture to evaporate until a goldenrod color (110C Pantone, 2008) appears, which indicates that Bi(III) ions are present. The sample will remain clear if Bi(III) isn't present and white precipitate will appear at the bottom of the flask.
7. Immediately remove the sample once the goldenrod color appears and measure the extracted goldenrod volume with a 25 mL graduated cylinder.
8. Calculating Bi(III) molarity (M) can be done by a linear regression (y=ax+b) curve, comparing the standards used for trivalent bismuth concentrations. This is done by standard concentration vs. remaining volume, the dependent variable.

<u>Pentavalent Bismuth Calculation:</u> Once molarity (M) is calculated from the "Total Bismuth Valence Assay" (Test B) and the "Trivalent Bismuth Assay" (Test A), the difference in molarity represents the Bi(V) value [i.e. Bi(III)+(V) – Bi(III)= Bi(V)]. This part of the method is based off the standard method to measure Sb(III) and Sb(V) (ASTM, 2010).

K. Results and Discussion

After testing the bismuth assay curves with a combination of elemental bismuth Bi(0), bismuth nitrate ($Bi(NO_3)_3$), bismuth trioxide (Bi_2O_3), and sodium bismuthate ($NaBiO_3$), the assay calibration curves yielded very accurate results. Bi(0) had not interfered with the curve due to the initial centrifugation and $NaBiO_3$, the Bi(V) source, had successfully been inhibited by $MgCl_2$. The higher the concentration of Bi(III) standards used, the more goldenrod volume remained when reacting with the KI titration. The ascension in Bi(III) concentrations (0.1-2.0 mM) created a precise line of best fit model with an $r^2 \cong 1.000$ (**Figure 10**). The trivalent bismuth valence assay (test B) successfully detected Bi(III) from the trivalent bismuth standards (Bi_2O_3 and $Bi(NO_3)_3$) tested in triplicate.

The total bismuth valence test showed similar results. The concentration of Bi(III)+Bi(V) standards (2-0.1 mM) in equal concentrations were synonymous in comparison to the trivalent bismuth test results. Similarly, the higher the standard concentration of Bi(III)+Bi(V) mixture, the increased amount of goldenrod colored volume remained when reacting with the KI titration. The ascension in Bi(III)+(V) concentrations created another exceedingly precise line of best fit, calculating a linear regression r^2 value of 0.995 (**Figure 11**). The total bismuth valence assay (test B) resulted in accurate detection of Bi(III) and/or Bi(V) from the ionic bismuth standards tested in triplicate ($NaBiO_3$, Bi_2O_3, $Bi(NO_3)_3$). Elemental Bi(0) did not interfere with this assay either.

Fairly few interferences have been acknowledged for this assay that include: higher concentrations >100 mM of biotin, >100 mM folic acid, 0.10 mM antimony (III), and >100 mM phosphate for both assays out of the ≈1 M concentrations of

various organics, halogenated compounds, vitamins, minerals, and transition metals tested. If this is the case, then the interfering components will act synergistically with the bismuth ions. Its speculated that spectrophotometric analysis could also be done to determine bismuth concentrations at a wavelength of 400-450 nms, which would absorb the complimentary color of goldenrod (University of California, 2010). This could be done similarly, boiling with KI to a certain volume and using the absorbance as the dependent variable. The simple titration assays is calculated to cost the operator approximately $1.50 per sample in chemicals for test A and B results.

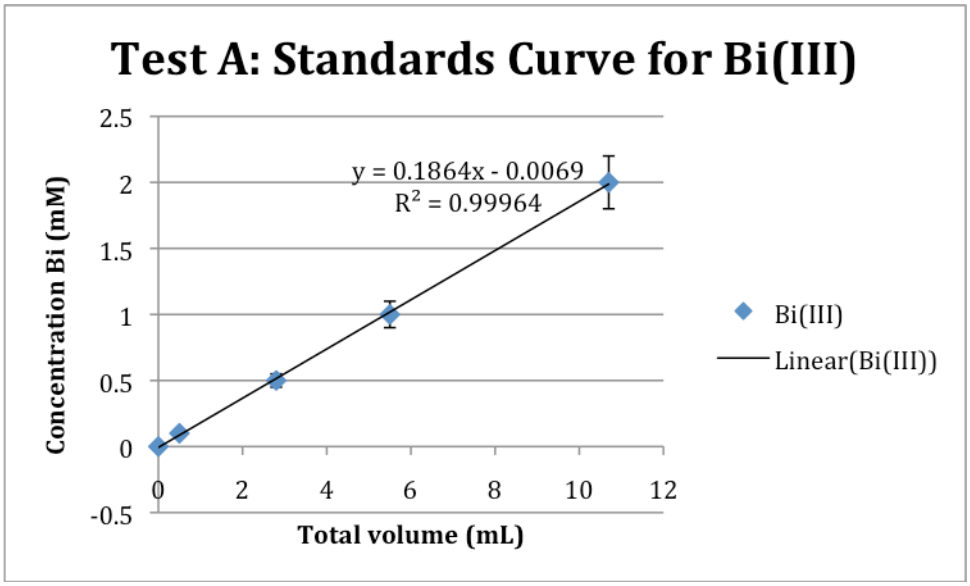

Figure 10- Linear relationship between the concentration of Bi(III) and the total volume used to generate a standards curve. As Bi(III) standards increase in concentration, the more imminent it becomes to react with KI and produce a goldenrod color as volume increases. The correlation is directly related, higher concentrations of Bi(III) yield higher total volumes.

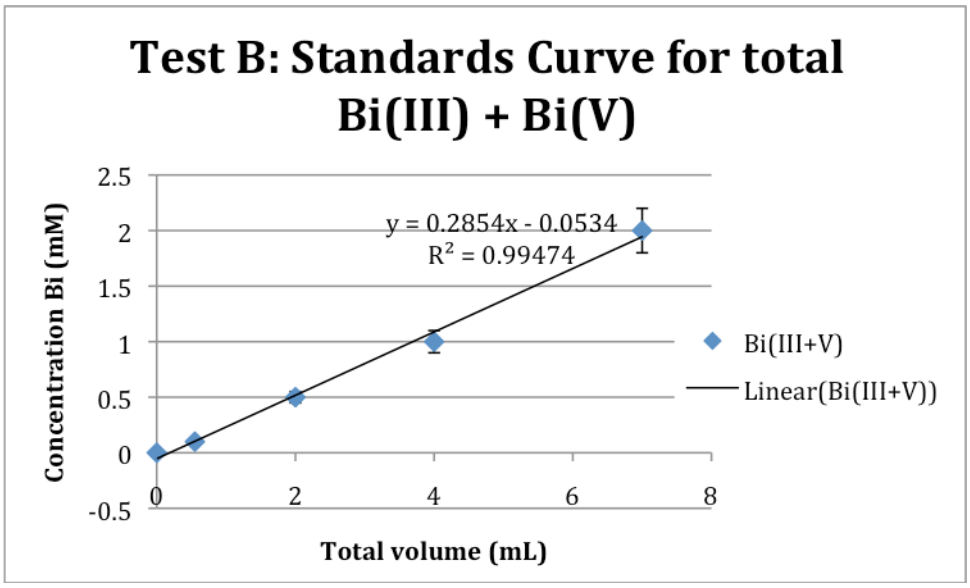

Figure 11- Linear relationship between equal concentrations of Bi(III)+(V) and the total volume used to generate a standards curve. As Bi(III) and Bi(V) standards increase in concentration, the more imminent it becomes to react with KI and produce a goldenrod color as volume increases.

L. Conclusion

The total and trivalent bismuth assays are a quick and simple technique that provides accurate results for measuring the concentration and valence state of the element. The calculations are very simple and based off of the method used in chapter II to determine Sb oxidation states. The method has few interferences and is cost effective with low maintenace and one corrosive chemical to dispose of (HCl). The increase in standard curves used for trivalent bismuth (test A) and total bismuth valence (test B) concentrations show a direct correlation with the increase in total volume remaining.

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Chapter IV: Oxidation of Bismuth (Bi) by *Bosea* sp. str. WAO

N. Introduction

Bismuth has been found in very small concentrations (5 μgs) in prokaryotic, eukaryotic cells, and mutli cellular tissue with little knowledge of its biological function (Hubbard, 1939). Bismuth salts have been important anti-microbial agents in the intestinal tract that are still used today against bacterial pathogens like *Escherichia Coli* and *Campylobacter pylori* (Sox, 1989). The oxidation and reduction of bismuth can determine the survival of bacteria. Elemental bismuth, which is terrestrially rare, has no observed effect on many gut microbiota including *Bacteroides thetaiotaomicron*, but once mobilized by another communal species, *Methanobrevibacter smithii*, bismuth becomes toxic to a number of species of bacteria in the gut (Bialek et al., 2011). Biological methylation has been noted as a detoxification mechanism for bismuth, most notably *Helicobacter pylori*, in order to mobilize bismuth subsalicylate (Bentley, 2002). Biological oxidation of bismuth has not been noted in available literature. *Bosea* sp. str. WAO has successfully oxidized arsenic and antimony for autotrophic growth, both toxic metals to many life forms. The speculated enzyme responsible, arsenic oxidase, from the *aioBA* gene (Li et al., 2013), could be involved with antimony, and therefore bismuth oxidation if it follows the metallic pnictogen pattern. Nonetheless, *Bosea* sp. str. WAO would stand a greater chance of oxidizing bismuth than many prokaryotes.

O. Material and Methods

<u>Bismuth oxidation media:</u> The bismuth oxidation experiment was designated similarly to the antimony oxidation experiment. Since bismuth is non toxic to most

biological life, 1 mM of Bi(0) or Bi(III) would be used as an electron donor. Elemental Bi(0) was crushed into a powder and dispersed into distilled water (100 mM). Bismuth nitrate ($Bi(NO_3)_3$) was dissolved in boiling water as the trivalent sources (100 mM). Sodium bismuthane ($NaBiO_3$) was disolved in hot water as a Bi(V) source for the calibration curves (100 mM). BT media would be used for this experiment with bismuth replacing the electron donors in the ingredients (DSMZ, 2007). For each of the two bismuth oxidation conditions, two flasks were used as backgrounds (BG) with media and 1 mM Bi (0) or (III), two sterile controls (SC) with media, 1 mM Bi (0) or (III), 100 µL killed cells, and three actives (A-n) with media, 1 mM Bi(0) or (III), and 100 µL live cells. Additionally, two live controls (LC) with media and 100 µL healthy cells as a biotic control. No electron donor had been added to them.

Bismuth oxidation analyses: *Bosea* sp. str. WAO was incubated for two days in 50% regular strength tryptic soy broth (TSB) media with 5 mM sodium thiosulfate ($Na_2S_2O_3$) to generate healthy growth, since *Bosea* sp. str. WAO is capable of using organic carbon for growth as well (Rhine et al., 2008). After growth was obtained, half of the volume was autoclaved to use as inocula for sterile controls. The cells were then centrifuged and washed with phosphate buffer three times to get rid of any excess sodium thiosulfate or TSB. The sixteen flasks were then put into a 30 °C incubator, on a shaker, and one mililiter would be sampled once a week for a month. The method used to analyze bismuth oxidation would be the concentration and valence of total, trivalent, and pentavalent bismuth assay

previously described in chapter III. A reference solution, with a known amount of trivalent bismuth, was used before every run to indicate the goldenrod color.

Bismuth growth analyses: After week four of the bismuth oxidation experiment, a sample would be taken at months one, two, and three for optical density (OD) and colony forming unit (CFU)-dilution testing from the autotrophic cultures. The biotic controls (LC) and active cultures with Bi(0) and Bi(III) will be pipetted on rich agar and counted one week after being sampled from the minimal media. The population will then be measured based on their ability to grow on the heterotrophic media from a starved (LC) and Bi mediated (A-n) autotophic growth conditions.

P. Results and Discussion

Bismuth oxidation: The microbial interaction with bismuth species is quite apparent, according to the analytical results. When *Bosea* sp. str. WAO was given 1 mM of Bi(0), after week one, oxidation started to occur compared to sterile controls, which show no oxidation of Bi(0). The 1 mM Bi(0) is being transformed to 0.70 mM of Bi(III) and Bi(V) over the four weeks. This matches the loss of 0.70 mM of Bi(0) over the given time frame (**Figure 12**). Similarly with bismuth nitrate as the electron donor, Bi(III), is being stochiometrically oxidized to Bi(V) within active cultures. There is a loss of ≈0.80 mM Bi(III) and a gain of ≈0.80 mM Bi(V) over the four week time period. Compared to the Bi(0) results, the rate of bismuth oxidation is consistent at 0.70-0.80 mM per month. Sterile controls do not show oxidation of Bi(III), remaining at the 1 mM concentration that was given (**Figure 13**). Bismuth is not being oxidized abiotically.

Bismuth growth: Immediately after the termination of the bismuth oxidation experiment, the test for growth experiment initiated. Background OD value had been subtracted from the actives since precipitates in the media could interfere. Using a spectrophotometer to measure Bi(III), the OD gradually increases between months one and two for biotic controls and active cultures. This is no surprise since some Rhizobium species take approximately a week to double in OD measurements on more biologically essential substrates, such as nitrogen and sulfur, that act as electron donors when bicarbonate was used as a carbon source (Pickering, 2008). Biotic controls show another accretion between months two and three. In active cultures, there is a dramatic increase in population from 0.060-0.350 OD. Cells with Bi(III) had a turbidity rise to almost 600% (**Figure 14**). The Bi(III) OD data and CFU data are consistent with each other. The cell population given Bi(III) tends to flatline from month one to month two, but increases nearly 600% from month two to month three (from ≈15 to 90 CFU/mL). Biotic controls without an electron donor, decrease gradually over time since there is not an energetic source present (**Figure 15**). This type of trend is also seen synonymously with the biotic controls in comparison to the actives with Bi(0) given as an electron donor, both populations showing a negative growth rate. The cells with Bi(0) have ascended their population size even further, increasing 700% (from ≈25 to 175 CFU/mL) between the first month into the second month. The population flatlines from month two into month three (**Figure 16**). The small amount of CFU growth that appears to be delayed in actives from month one in Bi(0) cultures and two for Bi(III) cultures could be from electron storage to support further autotrophic growth (month three)

because of environmental conditions. The Bi(V) state could be toxic to *Bosea* sp. str. WAO and therefore prevent growth until precipitating out of the culture or cells are mobilized a certain distance from the compound. Nonetheless, the data shows oxidation correlating to an increase in population size of *Bosea* sp. str. WAO when Bi(0) or Bi(III) is subsituted as the electron donor. This supports the evidence that *Bosea* sp. str. WAO can use all three metallic pnictogens for autotrophic growth.

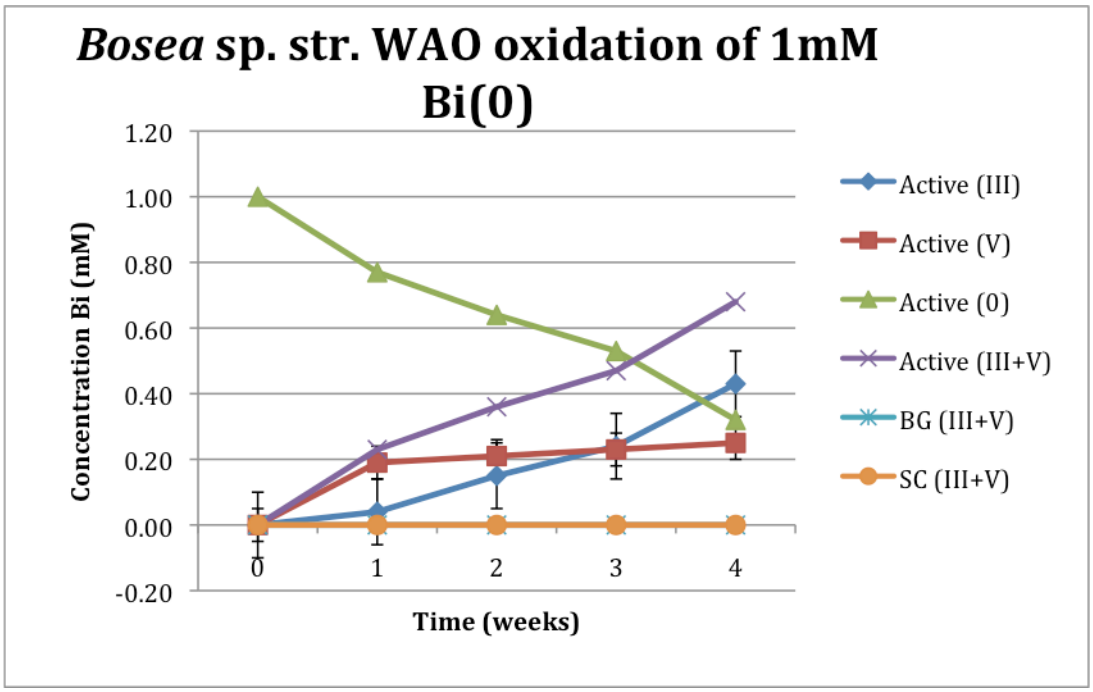

Figure 12- Oxidation of Bi(0) to Bi(III) and ultimately to Bi(V) by active cultures of *Bosea* sp. str. WAO and abiotic controls. The active cells are oxidizing Bi(0) to Bi(III) and ultimately Bi(V) while backgrounds (BG) and sterile controls (SC) are not showing oxidation over the four week time period. The decrease in ≈0.70 mM Bi(0) stoichiometrically agrees with the ≈0.70 mM Bi(III/V) oxidation over the time period.

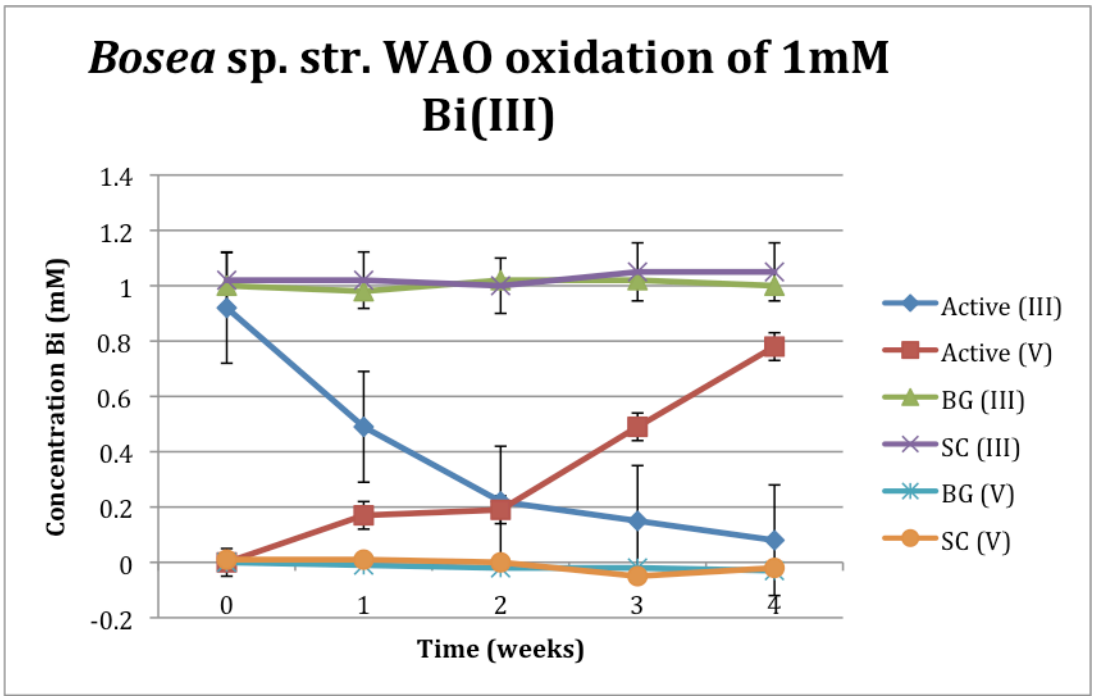

Figure 13- Oxidation of Bi(III) to Bi(V) by active cultures of *Bosea* sp. str. WAO and abiotic controls. The active cells are oxidizing Bi(III) to Bi(V) while backgrounds (BG) and sterile controls (SC) are not showing oxidation over the four week period. There is ≈0.80 mM Bi(III) being stoichiometrically oxidized to Bi(V) over the four week time period.

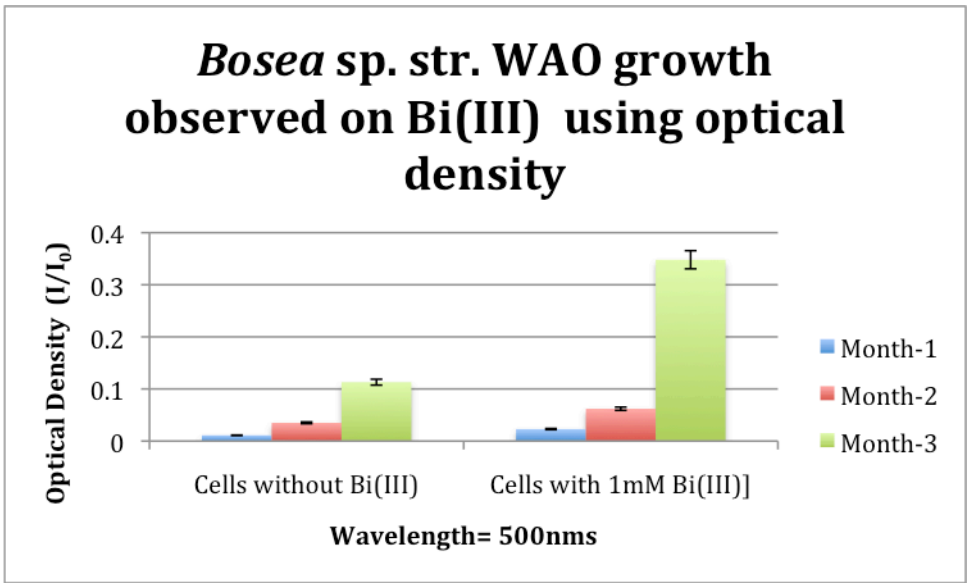

Figure 14- Optical density measurements of biotic cultures with and without Bi(III). Cultures with Bi(III) are are increasing in OD indicating an increase in turbidity while cultures without Bi(III) are not increasing. The backgrounds have been subtracted from the actives containing 1 mM Bi(III) because of precipitation within the media.

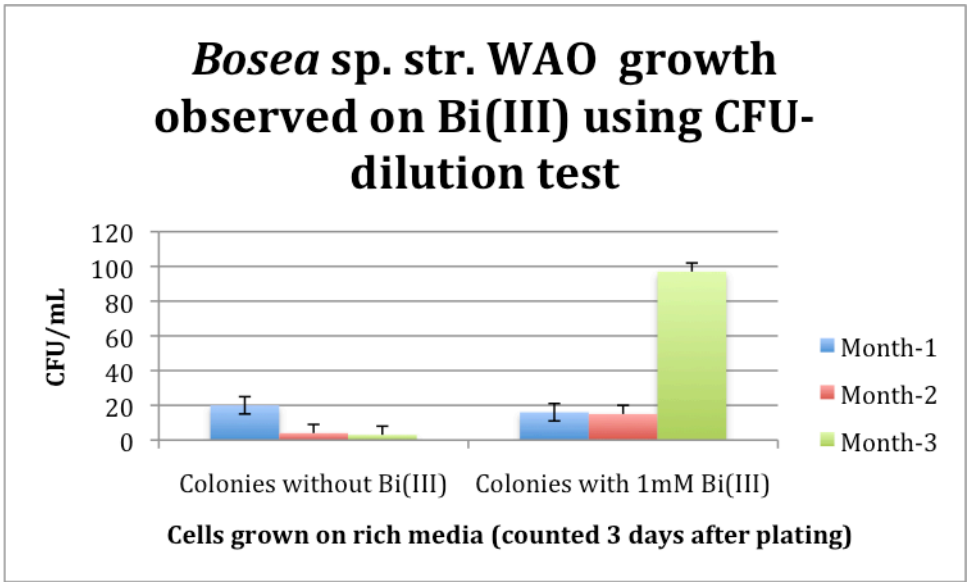

Figure 15- Colony forming unit measurements of biotic cultures with and without Bi(III) on rich agar. Cultures with Bi(III) are increasing in CFU/mL while cultures without Bi(III) are decreasing in CFU/mL. The delay in growth from months one and months two could be a misrepresentation when sampling the actives.

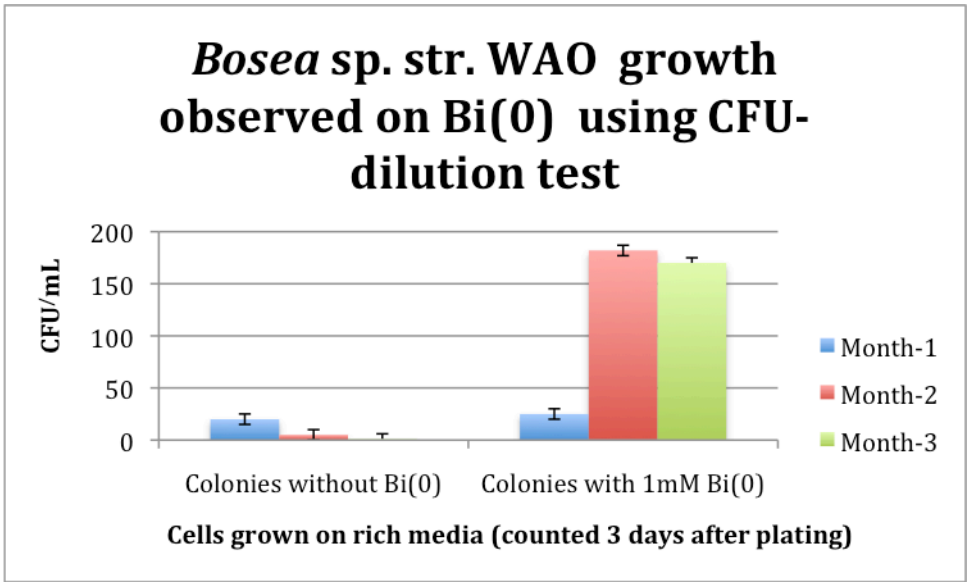

Figure 16- Colony forming unit measurements of biotic cultures with and without Bi(0) on rich agar. Cultures with Bi(0) are increasing in CFU/mL while cultures without Bi(0) are decreasing in CFU/mL. Cells appear to gather the electrons during the first month for later growth since there isn't any other known substrates to stimulate a population boom.

Q. Conclusion

The microbiological significance of bismuth is almost unknown. Trace amounts of data are present on bismuth methylation while bismuth oxidation is non existant in available literature. This study pioneers bismuth oxidation from known literature and has shed some light on the role of bismuth species and their potential role in bacteria. Given the data, *Bosea* sp. str. WAO is able to oxidize Bi(0) and Bi(III), the elctron donors, fully to Bi(V) for autotrophic growth. The mechanism remains completely unknown but is speculated that it may be arsenite oxidase out of any known enzymes. However, this does not dismiss the fact that there could be bismuth oxidation gene sets as well. There is a lot more research that needs to be done regarding the biochemical and physiological interactions of this element.

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Chapter V: Overall Conclusions and Future Work Needed

S. Concluding Remarks

The conclusions obtained from the results of the experimental data indicate that both antimony and bismuth are being oxidized by *Bosea* sp. str. WAO as an electron donor for autotrophic growth. The oxidative chemistry stoichiometrically agrees by means of analysis through a modified antimony and novel bismuth test procedure to determine both speciation and concentration. Thermodynamically, the Gibbs' free energy potential is favorable for these two electron donors in both elemental and trivalent forms for oxidation. The elemental and trivalent forms promote autotrophic behavior that flourishes through carbon fixation via the Calvin Cycle (Berg et al., 2010). However, this only shows chemical evidence that can limit the conclusion of the roles that these metallic pnictogens have. Are these *aio* genes and arsenite oxidase transports mobilizing this group of elements? Certain phosphate transports have been constantly identified with arsenic, which gives the element poisonous characteristics to higher eukaryotes (Wolfe-Simon et al., 2010). Depending on the species of bacteria, phosphate catabolism genes are very similar to arsenic oxidation ones (Muller et al., 2007). There may be intermediates that can use the metallic pnictogens for growth by means of selected evolutionary pressures.

There is obviously a mechanism in certain autotrophic prokaryotes that benefits from toxic elements like arsenic. Since arsenic, antimony, and bismuth do not have the microbiological role that other metallic ions have including iron in heme or cobalt (Co) in vitamin B12, can these or other speculated "detoxification" genes or proteins conjugate energy from thermodynamically favorable elements for

autotrophic growth? This could be connected with the theory that energy class is changing simultaneously as a conveyed electron donor reaches its receptor. Only has this behavior been noted in photoautotrophs using photons (e+) to create NADP for later ATP production, not inorganic electron donors (e-), which would be considered lithoautotrophy (Cramer, 2002). Both speculations can be answered with further research.

The results from the research have successfully pioneered antimony and bismuth's potential place in microbiological life forms and the capacity the element has as an energy source substituent for cell growth. *Bosea* sp. str. WAO is the only known organism with this characteristic so far, but surely enough other bacteria and presumably archaeons will be identified. Similar biological arsenic transports could be synonymous in higher life forms like fish and certain flora's ability to transform hazardous pnictogens into betaine, a nitrogen group based cation zwitterion (electron valence "isomer") that acts as a supplement for cells by attaching glycine to render the arsenic ion harmless (Nickon, 1987). Regardless, genetics and the protein interactions of both elements, antimony and bismuth, are lacking and have a lot of room for expanding knowledge for potential bioremediation and health purposes.

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K. Supplementary Data

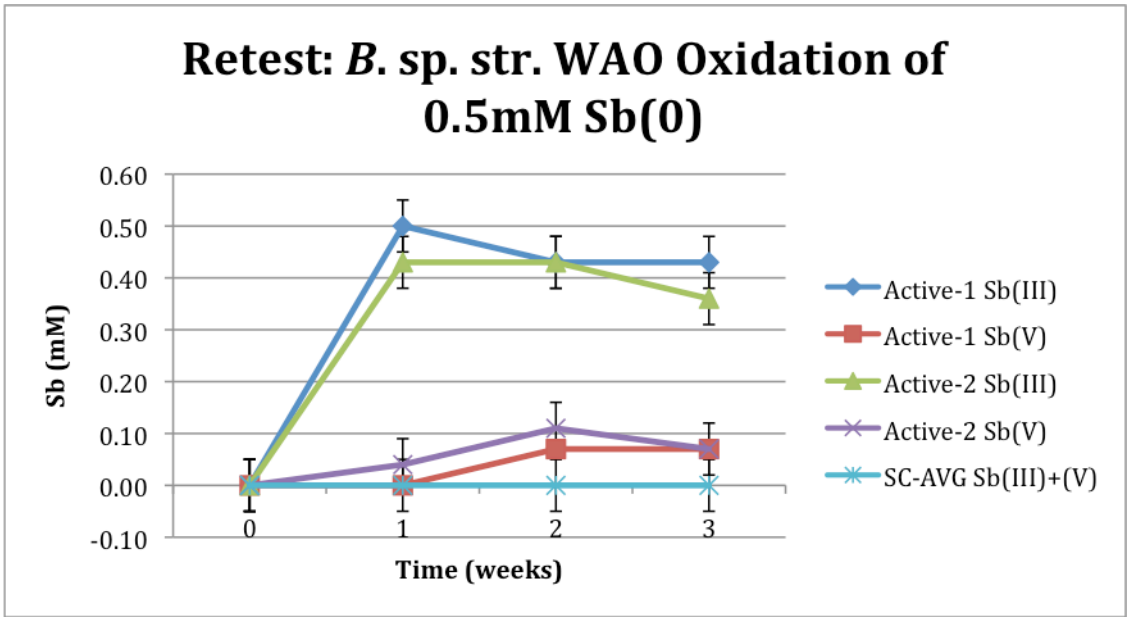


Figure S-1

Retested *Bosea* sp. str. WAO's antimonyl oxidation of 0.50 mM Sb(0) using the same, described test method.

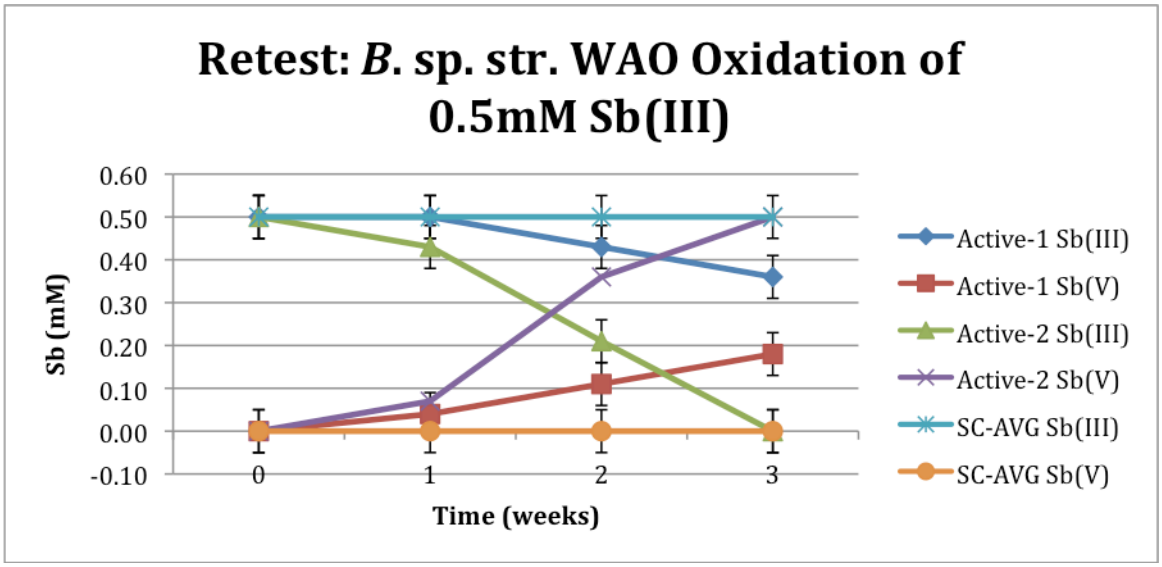


Figure S-2

Retested *Bosea* sp. str. WAO's antimonyl oxidation of 0.50 mM Sb(III) using the same, described test method.

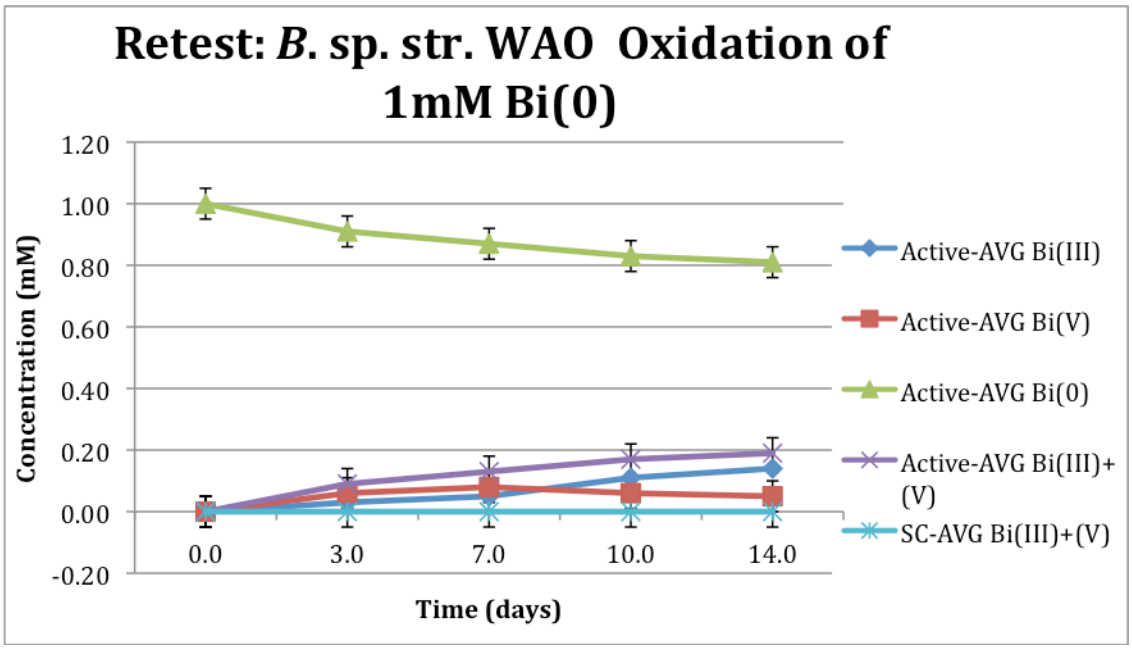


Figure S-3
Retested *Bosea* sp. str. WAO's bismuth oxidation of 1 mM Bi(0) using the same, described test method.

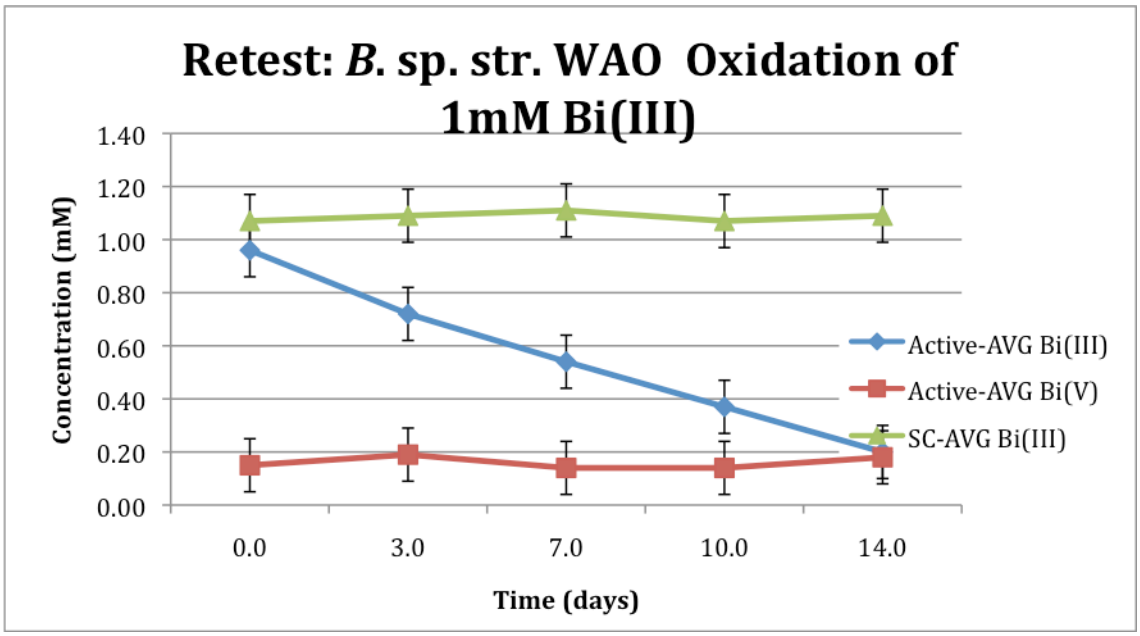


Figure S-4
Retested *Bosea* sp. str. WAO's bismuth oxidation of 1 mM Bi(III) using the same, described test method.

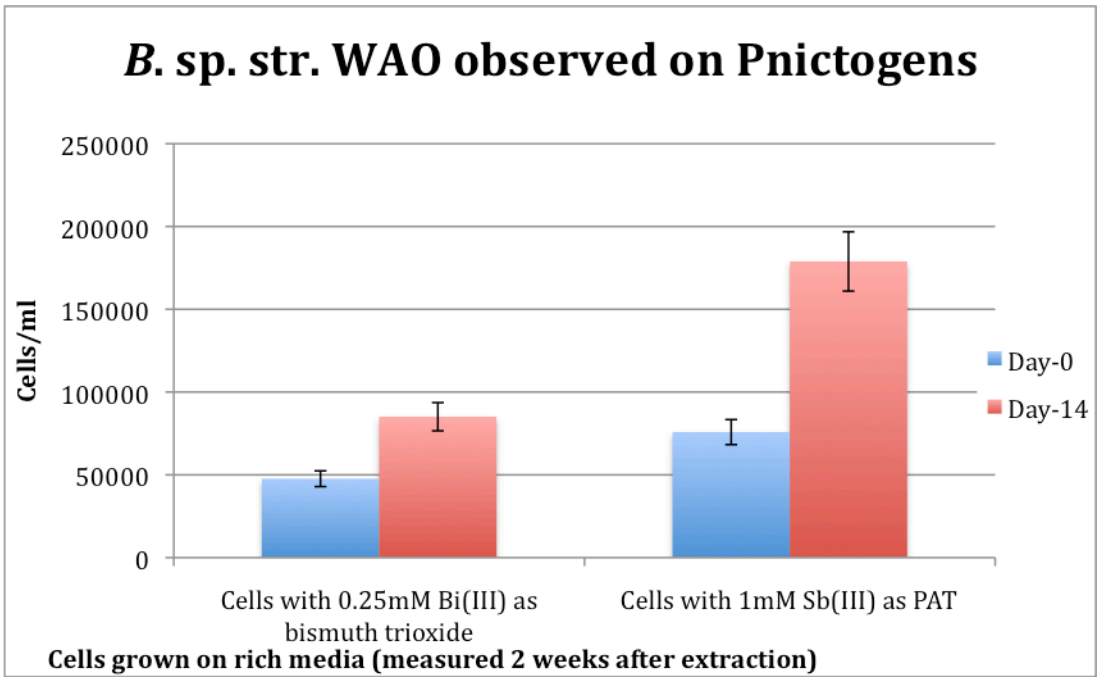
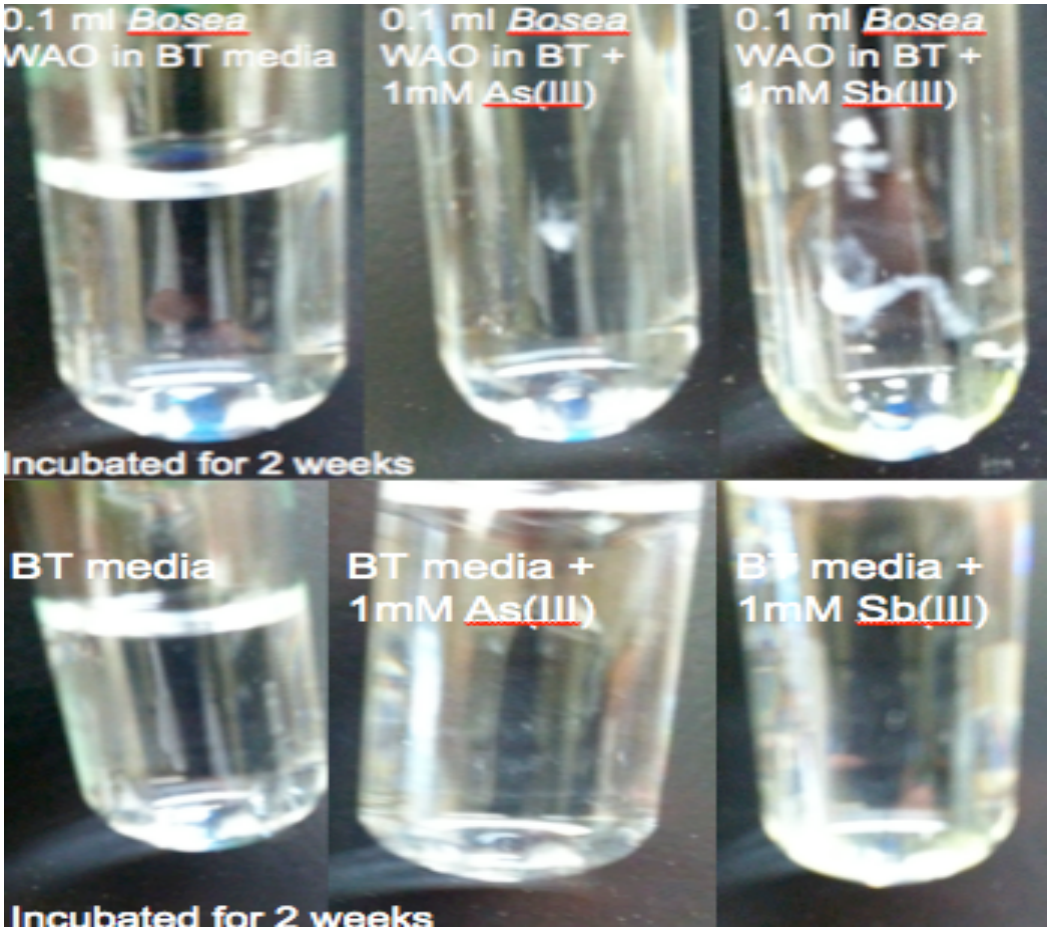
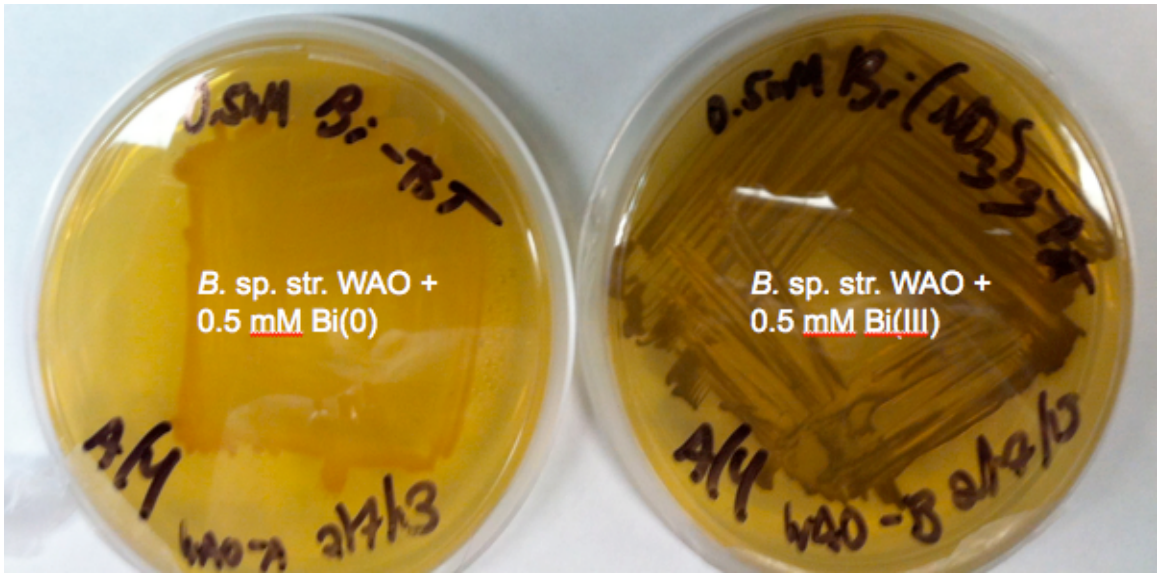


Figure S-5

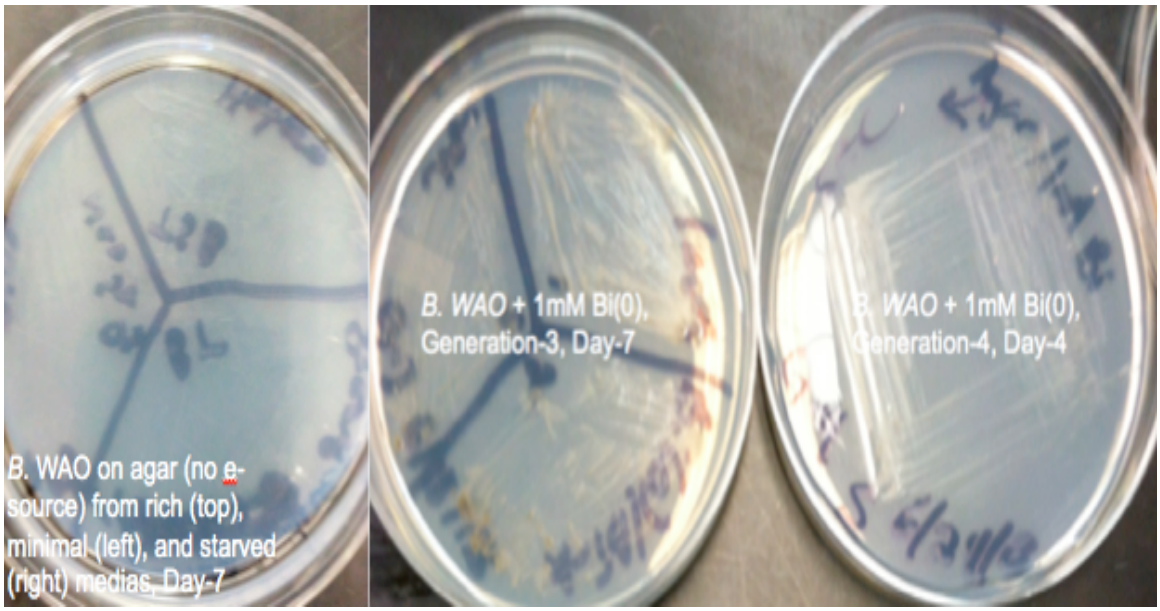
Tested *Bosea* sp. str. WAO's ability to grow on other bismuth and antimonyl species.



Picture S-1
Bosea sp. str. WAO growing autotrophically on minimal media with either 1 mM As_2O_3 (top center) or 1 mM Sb_2O_3 (top right) as the sole electron donor. Abiotic controls (bottom) and BT biotic control (top left) do not show turbidity after being inoculated for 2 weeks.

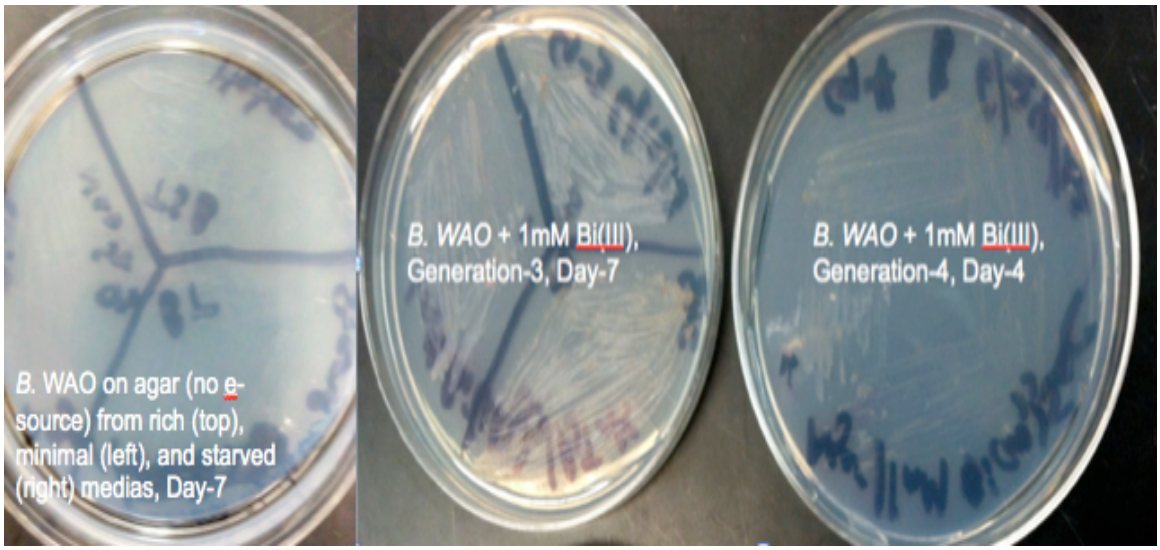


Picture S-2
Bosea sp. str. WAO has a unique ability to change color from the traditional white colony to a pink color when oxidizing Bi(0), shown on the right, the color of some Bi(III) species. A brown color appears when Bi(III) is oxidized to Bi(V), shown on the right, another color of certain Bi(V) species.



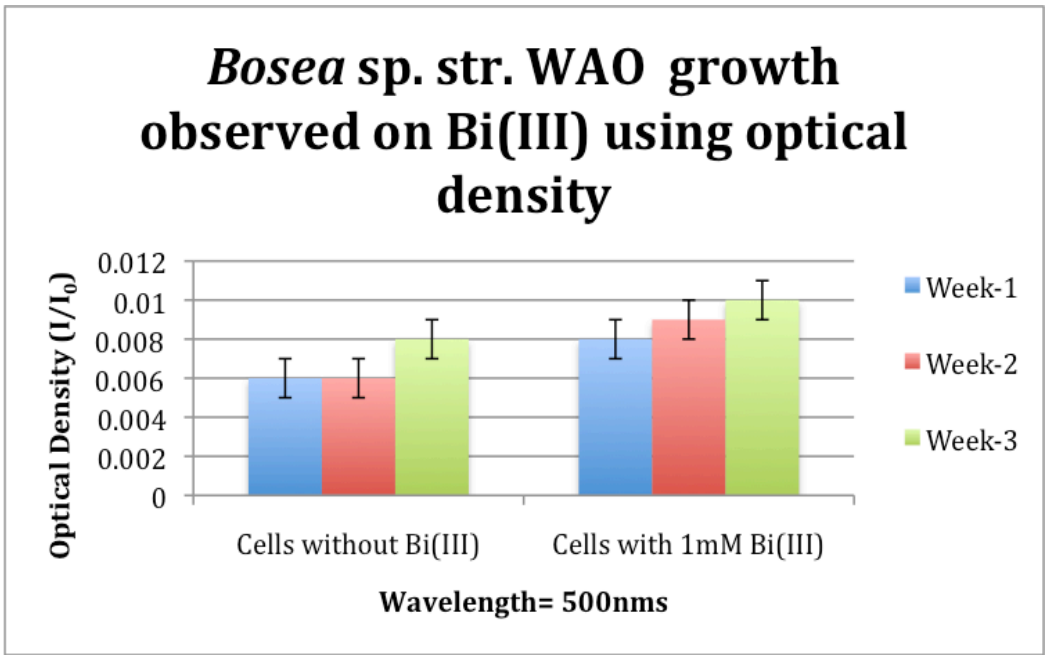
Picture S-3

Bosea sp. str. WAO grew using 1 mM of Bi(0) as the only e- donor for 4 generations, which took approximately 6 weeks after. Each generation took less time. Generation 1 took 3 weeks, 2 took 2 weeks, 3 took 6 days, and 4 took 3 days selecting for only autotrophic growth. The agar control only showed minimal growth when cells were taken from a rich media. This would lead one to believe that *B.* sp. str. WAO can "horde" elements to store for future growth.



Picture S-4

Bosea sp. str. WAO grew using 1 mM of Bi(III) as the only e- donor for 4 generations, which took approximately a 6 weeks. Each generation took less time. Generation 1 took 3 weeks, 2 took 2 weeks, 3 took 6 days, and 4 took 3 days selecting for only autotrophic growth. This was approximately the same amount of time as the 1 mM Bi(0) plates. The cell growth eventually showed a dark brown coloration. The agar control only showed minimal growth when cells were taken from a rich media.



Picture S-5

OD measurement of cells with and without 1mM Bi(III) in the first weeks of growth. The backgrounds have been subtracted from cultures containing Bi(III), but there still is not a significant amount of growth.