The Synthesis of Elemental Selenium Particles by the 
Cyanobacterium *Synechococcus leopoliensis*

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the degree of Masters of Science

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Abstract

In the present study the light dependent biosynthesis of spherical red elemental selenium $\text{Se}(0)$ granules (average size of 220 nm) by the cyanobacterium *Synechococcus leopoliensis* when exposed to high selenite concentrations for 9 days was investigated. The particles were identified as $\text{Se}(0)$ by energy dispersive x-ray spectroscopy (EDX) analysis and were localized between the periplasmic space and the cell surface through light and environmental scanning electron microscopy (ESEM). The amount of selenite reduced to $\text{Se}(0)$ increased with increasing culture density and less selenite remained in the cultures accordingly. Selenite treated cultures lost their characteristic pigments and turned from green to orange by day 5 and a concomitant increase in the buoyant cell density was observed over the treatment period. The potential abiotic and biotic reactions that may have produced $\text{Se}(0)$ in the cyanobacteria are discussed.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSO</td>
<td>S-n-butyl homocysteine sulfoximine</td>
</tr>
<tr>
<td>EDX</td>
<td>Energy dispersive x-ray spectroscopy</td>
</tr>
<tr>
<td>ESEM</td>
<td>Environmental scanning electron microscope</td>
</tr>
<tr>
<td>Fd</td>
<td>Ferredoxin</td>
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<tr>
<td>FTR</td>
<td>Ferredoxin dependent thioredoxin reductase</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin NADP reductase</td>
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<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GS-Se-SG</td>
<td>Selenodiglutathione</td>
</tr>
<tr>
<td>GS-Se⁻</td>
<td>Selenopersulfide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PNS</td>
<td>Purple Non-Sulfur</td>
</tr>
<tr>
<td>PSI</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>SAM</td>
<td>S-Adenosylmethionine</td>
</tr>
<tr>
<td>TR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>TRX</td>
<td>Thioredoxin</td>
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Chapter 1: General Introduction and Review

The four common oxidation states of selenium (-2, 0, +4 and +6) that are pH and redox potential (pE) dependent can occur in both inorganic and organic forms (Cutter and Bruland, 1984, Cooke and Bruland, 1987; Risher et al. 2003). The -2 oxidation state of selenium exists as free selenide ions (Se\(^2^-\)) or HSe\(^-\) ions under basic and neutral aqueous conditions, respectively, and as H\(_2\)Se gas under acidic conditions. Selenides also exist as insoluble selenide minerals in association with metal cations. The most abundant organic selenides include biologically derived selenocysteine, selenomethionine, and the volatile gases, dimethyl selenide and dimethyl diselenide. Elemental selenium, Se(0) is highly stable, insoluble in water and rarely occurs in nature. It can, however, be formed under acidic conditions and can exist in one of three allotropes: red or orange, black and gray forms. Oxidation states +4 and +6 occur as selenite (Se IV) and selenate (Se VI) respectively, which are commonly found in neutral aqueous environments.

1.1 Toxicity of selenite and selenate to living organisms

The toxicity of selenite and selenate in the environment usually depends on their concentration and bioavailability. Under laboratory conditions, selenate is usually more toxic to living organisms than selenite; i.e. selenate has a lower lethal dose than selenite. However, selenate has lower bio-affinity because it does not adsorb well to minerals and organic matter (Reddy et al. 1995; Sharmasarkar and Vance, 2002; Risher et al. 2003; Martens, 2003). The toxicity of selenate may be attributed to the fact that it is transported via an active sulfate transport system and assimilated like sulfate (Saggu et al. 2010). Stated differently, sulfate deficient conditions can enhance the uptake and possibly the toxicity of selenate to some organisms (Neumann et al. 2003; Umysova et al 2009). The presence of sulfate in the environment generally prevents the uptake of selenite by organisms through substrate
competition via sulfate transporters (Shrift, 1954; 1958; Shrift and Ulrich, 1969; Gouget et al. 2005). Therefore, the reduced bioavailability and presence of sulfate in natural fresh water environments may reduce the toxicity of selenate to organisms.

Selenite, on the other hand, is less stable and is a stronger oxidizing agent than selenate, which makes it more reactive (Shrift, 1958). Selenite also adsorbs more readily to minerals, and living or dead organic matter which increases its bioavailability (Riedel et al. 1991; Reddy et al. 1995; Sharmasarkar and Vance, 2002; Risher et al. 2003; Martens, 2003). Selenite may also react with functional groups on extracellular membranes and with intracellular macromolecules such as lipids and proteins, causing cellular damage (Gennity et al. 1984; Hightower and McCready, 1991; Riedel et al. 1991; Wang et al. 2003; Li et al. 2003). Based on reactivity and bioavailability, selenite is more likely to be toxic to living organisms under natural conditions than selenate (Lemly et al. 1993; Germ et al. 2007). The transport mechanism of selenite into organisms is less clearly understood than for selenate. For selenite, there is evidence for both specific and nonspecific, active and passive mechanisms of selenite transport which may include sulfate transporters as well as other transporters (Riedel et al. 1991; Bebien et al. 2001; Obata et al. 2004; Gouget et al. 2005; Morlon et al. 2006; Jasenec et al. 2009; McDermott et al. 2010; Zhao et al. 2010; Araie et al. 2011).

1.2 Selenium in living organisms

Selenium, in trace amounts, is important for living organisms. In some marine microalgae, selenium is required for growth (Araie and Shiraiwa, 2009). Selenocysteine containing proteins have been found throughout the major families of the animal kingdom, including archaea, bacteria and eukaryotes. Selenocysteine can be incorporated into selenoenzymes and other selenoproteins that have a wide range of metabolic functions including antioxidant enzymes such
as glutathione peroxidase that are responsible for detoxifying a variety of peroxides in living cells (Axley and Stadtman, 1989; Zhang et al. 2001; Gladyshev, 2006). Selenocysteine is also a precursor to the formation of selenomethionine, which is primarily found in plants and, like selenocysteine, can be incorporated into antioxidant selenoenzymes (Schrauzer, 2000). Higher, non-nutritional concentrations of selenium may become toxic to living organisms. As a result, most of the literature on selenium toxicity is devoted to understanding how selenite and selenate exert their toxic effects on organisms and how organisms combat this toxicity.

Living organisms have two different ways in which they may detoxify high concentrations of inorganic and organic forms of selenium as well as other non-metals and metalloids which include arsenic, tellurium, sulfur and antimony from their environment. Bioreduction through biomethylation seems to be a common strategy used among both prokaryotes and eukaryotes (Ridley et al. 1977; Bentley and Chasteen, 2002; Chasteen and Bentley, 2003; Stolz et al. 2006; Mirko et al. 2010; Schilling et al. 2011; Yin et al. 2011; Ye et al. 2012). Both thiol and methionine metabolism are strongly implicated in biomethylation, which first proceed through chemical reactions with thiols and then through S-adenosylmethionine (SAM) and the action of methyl transferase enzymes.

1.3 Biomethylation of selenium and formation of Se(0) in eukaryotes

Plants and algae (Evans et al. 1968; Fan et al. 1997; Chasteen and Bentley, 2003; Neumann et al. 2003; Germ et al. 2007) have only been shown to biomethylate selenium oxyanions while heterotrophic and photosynthetic bacteria as well as some fungi are also able to bioreduce selenium oxyanions into Se(0). In fungi, bioreduction of selenium oxyanions to Se(0) commonly occurs under aerobic conditions and has been tested with both selenite and selenate (Gharieb and Gadd, 1998; Sarkar et al. 2011). Very little work has been done on selenite and
selenate transport into fungi. Fungi grown in sulfate deficient media are more sensitive to selenate than selenite, potentially because selenate may mimic sulfate in the media (Gharieb et al. 1995; Dilworth and Bandurski, 1977). Selenite may be transported into some fungi via monocarboxylate transporters (McDermott et al. 2010) as the result of molecular mimicry where monocarboxylates, like selenite, are negatively charged in solution. Information on the enzymatic bioreduction of selenite and selenate, not to mention other biosynthesized metal particles is still rudimentary. The role of vacuoles as well as the possibility that enzymes are being secreted into the fungal media requires further characterization (Gharieb and Gadd, 1998; Dhillon et al. 2012).

1.4 Biomethylation of selenium and formation of Se(0) in prokaryotes

Van Fleet-Stalder et al. 1997; Yamada et al. 1997; Van Fleet-Stalder et al. 2000; Bebien et al. 2001; Kessi et al. 2006). The biggest difference between Se(0) formation under aerobic compared to anaerobic conditions is that under aerobic conditions the formation of Se(0) is due to an antioxidant response of bacteria to selenium oxyanions and some amount of assimilation into selenoamino acids and selenoproteins while under anaerobic conditions the bacteria may be using dissimilatory reduction, where energy is liberated from the reduction of alternative electron acceptors through various reductases, which by chance may reduce selenate or selenite. For example a few studies under aerobic conditions have attempted to block the formation of Se(0) from selenite and selenate by adding common final electron acceptors such as nitrate, nitrite, sulfate and sulfite as well as blockers of sulfur oxyanion reduction such as molybdate, chromate and tungstate. As expected when using aerobic conditions, most researchers found that Se(0) formation from selenite was not blocked by these chemical amendments (Lortie et al. 1992; Garbisu et al. 1996; Hunter and Manter, 2008; Hunter and Manter, 2009) while selenate may yield mixed results. In one report using *Pseudomonas sp.*, nitrate blocked Se(0) formation and implicated the action of a nitrate reductase enzyme (Hunter and Manter, 2009) while another report, also using a *Pseudomonas sp.* isolate, reported no inhibition (Lortie et al. 1992). In general, aerobic bacteria may first passively or actively transport the selenium oxyanions through molecular mimicry into cells. Early in exposure, selenate is usually assimilated by cells through thiol and methionine metabolism. The assimilation of selenate may cause a build-up of toxic selenoamino acids such as selenocysteine and selenomethionine. Selenocysteine may be broken down by L-cysteine desulphhydrases into hydrogen selenide that may spontaneously form Se(0). Selenomethionine may be removed by biomethylation through SAM and methyl transferases to produce reduced methyl selenides. The presence of excess selenite, on the other hand, may react
with glutathione and produce reaction products that can act as substrates for enzymes such as thioredoxin and glutathione reductases as well as reduced thioredoxins to form Se(0) (Bjornstedt et al. 1995; Garbisu et al. 1996; Turner et al. 1998; Garbisu et al. 1999). Also, for both selenate and selenite, superoxide dismutases, catalases and peroxidases may be employed to deal with the production of oxygen radicals and hydrogen peroxide produced directly or indirectly by cellular reactions with selenium oxyanions.

On the other hand, it is common for bacteria grown under anaerobic conditions to be able to use more than one of the above alternative electron acceptors which may also be coupled with the oxidation of an organic substrate. Again, depending on which alternative electron acceptor blocks the formation of Se(0) from selenate or selenite may determine which particular reductase is catalyzing the reaction. In sulfate reducing bacteria, there is contradictory evidence for the involvement of sulfate reducing enzymes in the formation of Se(0). In one study, the presence of sulfate with selenate enhances the reduction of selenate to Se(0) in Desulfomicrobium sp. (Zehr and Oremland, 1987) while in Desulfovibrio desulfuricans subsp. aestuarii the presence of sulfate inhibits Se(0) formation (Hockin and Gadd, 2006). The story is even more complicated for some Se-respiring bacteria where some species are able to totally reduce selenate to Se(0) but cannot use selenite as an electron acceptor while others may only reduce selenate to selenite (Oremland et al. 1994; Oremland et al. 2004; Narasingarao and Haggblom, 2007). To date, T. selenatis is the most well characterized selenate-respiring bacteria. For T. selenatis, the presence of nitrate did not block the reduction of selenate because these bacteria have been shown to harbour selenate reductases that function separately from their nitrate reductases. This allows for a two-step reduction of selenate into selenite through selenate reductase and then reduction of selenite to Se(0) via a periplasmic nitrite reductase (Rech and Macy, 1992; DeMoll-Decker and
Macy, 1993; Macy et al. 1993; Schroder et al. 1997). The response of *Rhizobium sp.* to selenium oxyanions presents another interesting but peculiar case. *Rhizobium sp.* can grow in the presence of selenite and selenate under aerobic conditions but Se(0) formed only in the presence of selenite (Hunter et al. 2007; Hunter and Kuykendall, 2007). The presence of nitrate enhanced the rate of Se(0) formation but was not required (Hunter and Kuykendall, 2007). No further characterization of this process under aerobic conditions has been pursued to identify what chemical or enzymatic reactions are responsible for this reduction but the action of an oxygen labile assimilative nitrate or nitrite reductase is possible (Sekiguchi and Maruyama, 1988). Under anaerobic conditions with nitrate (denitrifying conditions, see O'Hara et al. 1983; Halder and Chakrabartty, 1995), the bacteria generally grew with glycerol and nitrate, indicating it is using nitrate as a final electron acceptor but when grown in selenite or selenate alone, the bacteria did not grow at all. This shows that they cannot use the selenium oxyanions as final electron acceptors (Hunter and Kuykendall, 2007). The addition of nitrate to selenite but not selenate treated cultures allowed for better growth and formation of Se(0). The presence of nitrate in selenite treated cultures under anaerobic conditions promoted the activity of a copper or molybdenum containing nitrite reductase that may be responsible for the reduction of selenite to Se(0) (Halder and Chakrabartty, 1995; Basaglia et al. 2007; Hunter and Kuykendall, 2007).

The story only gets more interesting when examining metabolically diverse prokaryotes such as the purple-non sulfur (PNS) bacteria. These bacteria are able to grow under various growth conditions (Hadicke et al. 2011). They include: 1) anaerobic photoautotrophic growth (CO₂ is used as a carbon source and H₂ as an electron donor), 2) anaerobic photoheterotrophic growth (electrons are provided by an organic carbon source and light is used to generate ATP), 3) fermentative heterotrophic growth on a carbon source in the dark and 4) they may also grow
aerobically in the dark on a carbon source. Most studies have tested and compared how selenite and selenate are biotransformed under photoheterotrophic, photoautotrophic and dark heterotrophic conditions. PNS bacteria reduce high selenite concentrations to Se(0) at a higher rate when grown under photoheterotrophic conditions than when grown under photoautotrophic conditions (Yamada et al. 1997). This may be because the photoautotrophic bacteria grew more slowly as a result of not having a carbon source. Under dark heterotrophic conditions, little to no selenite is converted to Se(0) (Yamada et al. 1997; Kessi et al. 1999; Bebien et al. 2001). When PNS bacteria are exposed to high concentrations of selenate under photoheterotrophic conditions, it is predominantly converted to selenomethionine and methylated selenide gases (McCarty et al. 1993; Van Fleet-Stalder et al. 1997; Van Fleet-Stalder et al. 2000). Dark heterotrophic conditions with selenate produce very little methylated selenium and no Se(0) (Fleet-Stalder et al. 1997; Bebien et al. 2001). Exposure to selenite and selenate under dark heterotrophic conditions lead to the expression of heat shock proteins, superoxide dismutases, glutathione, thioredoxin and other xenobiotic reductases in response to oxidative stress, similar to bacteria treated under aerobic conditions (Bebien et al. 2001). To date, there have been no attempts to test the response of PNS bacteria with selenate under photoautotrophic conditions nor have there been attempts to test the response of the bacteria under fermentative conditions with selenite or selenate. Only recently, has there been an interest in trying to discover what enzymes and metabolic activities may lead to the formation of Se(0) from selenite, so far, only under photoheterotrophic conditions. As with other anaerobic bacteria, the potential association of sulfite and nitrite reductases with the reduction of selenite to Se(0) in PNS bacteria has been tested by adding sulfite and nitrite to media with selenite. A much more complex and intricate picture arises. First and foremost, there are differences in how different species of PNS bacteria respond to the above media amendments.
*Rhodospirillum rubrum* separated its reduction of nitrite or sulfite from reduction of selenite in a successive fashion. Nitrite or sulfite were reduced first during the exponential phase of growth whereas only selenite was reduced during the stationary phase implying that these species may have specific recognition and transport systems for these ions (Kessi et al. 2006). On the other hand, *Rhodobacter capsulatus* reduced nitrite or sulfite simultaneously with selenite during the exponential growth phase (Kessi et al. 2006). Selenite may be transported into the cell in a non-specific manner through ABC transporters of sugar alcohols as shown in *Rhodobacter sphaeroides* (Bebien et al. 2001). In both species the combination of nitrite and selenite caused the highest suppression of growth and slowest rate of selenite reduction. The data suggests that the PNS bacteria are not using selenite as a final electron acceptor and that nitrite and sulfite reductases are not responsible for reducing selenite to Se(0). The authors then blocked the synthesis of GSH with S-n-butyl homocysteine sulfoximine (BSO) in both species during stationary phase and found that selenite reduction to Se(0) was delayed (Kessi et al. 2006). This may indicate that initial abiotic reactions of GSH with selenite which forms selenodiglutathione may act as a substrate for enzymes such as glutathione or thioredoxin reductases which may aid in the formation of Se(0). Intriguingly, from an evolutionary point of view, the PNS bacteria can be considered metabolically versatile organisms that employ selenite detoxification strategies that are more common to prokaryotes treated under aerobic conditions. These prokaryotes may include the closely related cyanobacteria.

The production of Se(0) particles using aerobic photosynthetic cyanobacteria may have a distinct advantage over using aerobic heterotrophic bacteria. Unlike heterotrophic bacteria, cyanobacteria do not need an external carbon source for growth and only require a simple mineral nutrient media, light and adequate aeration.
Most of the studies which expose selenium of various oxidation states to cyanobacteria have focused on growth promoting to growth inhibiting concentrations (Kumar and Prakash, 1971; Sielicki and Burnham, 1973; Kiffeney and Knight, 1990; Abdel-Hamid and Skulberg, 1995; Li et al. 2003; Gouget et al. 2005). At growth promoting concentrations these studies examine how selenium is incorporated into the cyanobacteria and in what form. At growth inhibiting concentrations, the studies tend to only focus on the effects of selenium toxicity by measuring the lack of normal growth, the loss of photosynthetic pigments and changes in cellular ultrastructure and amounts of macromolecules. A few studies on selenite toxicity in cyanobacteria have anecdotally noted the formation of granules, the compositions of which were not determined (Kumar and Prakash, 1971; Sielicki and Burnham, 1973; Pronina et al. 2002; Li et al. 2003).

In the present study the production of orange Se(0) particles by the cyanobacterium *Synechococcus leopoliensis* as a product of high exposure levels to selenite was investigated. The cellular localization, shape and size of the orange granules are characterized using light and environmental scanning electron microscopy (ESEM) and identified through energy dispersive x-ray spectroscopy (EDX). The amount of selenite reduced by the cells and the amount of selenite remaining in the culture media is also examined over a 9 day period as is how the buoyant cell density of the treated cells changes with the production of Se(0).

1.5 Future applications

The information obtained from this study may have direct applications in bioremediation of selenite from contaminated soil and water (Gadd, 2010). The Se(0) particles produced by *Synechococcus leopoliensis* may have indirect applications for the development of safer selenium vitamins or food additives (Wang et al. 2007), novel antibiotic coatings (Wang and
Webster, 2012), new chemo-preventative (Zhang et al. 2008) or anti-cancer treatments (Kong et al. 2011), in vivo fluorescent dyes for bioimaging applications (Gu et al. 2012) and finally Se(0) particles may be used in electronic devices which may include solar cells (Robel et al. 2006), photo sensors (Niu et al. 2012) as well as chemical and biological sensors (Wang et al. 2010).
Chapter 2: Materials and Methods

Cyanobacteria and growth conditions

*Synechococcus leopoliensis* UTEX 2434 was obtained from the University Of Texas Culture Collection Of Algae. The cyanobacteria were grown axenically under autotrophic conditions, starting at an O.D.\(_{665}\) = 0.1 (3.3 x10\(^6\) cells/ml) in three 3 L bioreactors containing sterilized BG-11 medium (Sigma, Mississauga, Canada) under constant white fluorescent light (T12, 34W; 90 mol/m\(^2\)/s) and aeration at 35 °C. The cyanobacteria were grown for 7-10 days to stationary phase (O.D.\(_{665}\) = 0.8 - 1.0; 2.7 - 3.3 x 10\(^7\) cells / ml) and were harvested for experimentation. To maintain a healthy stock culture, the bioreactors were renewed to an O.D.\(_{665}\) of 0.1 every 7 days by centrifuging an appropriate amount of culture at 2,200 x g for 10 min at 15ºC (Beckman Coulter Allegra 6R, GH-3.8A) and resuspending the cells in fresh BG-11 medium.

Selenite reduction experiments

Cells were harvested in sufficient quantities from the reactors to make three sets of treatments at culture densities of O.D.\(_{665}\) equal to 0.5 (1.7x10\(^7\) cells/ml), 1.0 (3.3 x10\(^7\) cells/ml) and 3.0 (1.0 x10\(^8\) cells/ml). This was performed by centrifuging the cells in sterilized 500 ml Nalgene centrifuge polypropylene copolymer bottles (Fisher Scientific, Ottawa, Canada) at 1,592 x g for 10 min at 15 ºC (Beckman Coulter Avanti J-E, JA-10) and discarding the old media. Prior to resuspending the cells in BG-11 medium with 5 mM of Na\(_2\)SeO\(_3\) and dispensing 100 ml into sterilized glass jars, 6 x 1 ml samples were taken to determine the initial amount of selenite in the medium as mentioned above. Then the jars were placed on a rotary shaker at 150 rpm (VWR Standard Analogue Shaker 3500) under continuous white fluorescent light (T5015, 54W; 166
µmol/m²/s) at a temperature of 22 ºC. Of three independent experiments that were performed, one representative experiment is presented.

**Selenium particle purification procedure**

Two hundred ml of high density cultures (O.D.₆₆₅ = 3.0) treated for 9 days with 5mM selenite were aliquoted into 50 ml sterile tubes and centrifuged for 10 min at 2,200 x g, 15 ºC (Beckman Coulter Allegra 6R, GH-3.8A). The supernatant was discarded and the cell pellets were resuspended in 10 ml of ddH₂O. The solution was then vortexed to resuspend the cells followed by sonication for 1 min at power level 10 on a Virtis Virsonic 60 sonicator (The Virtis Company Inc., NY, USA). Vortexing and sonication was repeated two more times. Each tube was then made up to 50 ml with ddH₂O and shaken well by inversions. The contents of all the tubes were then pooled in a 250 ml Erlenmeyer flask and filtered through Grade 3 Whatman (6 µm pore size) filter paper under suction using a Sartorius Type 16510 filter apparatus (Sartorius AG, Weender Landstr, Germany). The filtrate was then passed through Grade 5 Whatman (2.5 µm pore size) filter paper using the same filter apparatus. The resulting filtrate was then passed through a Corning 500 ml filter system (0.22 µm pore size) to obtain mostly cell-free selenium particle samples. The selenium particles were then concentrated by centrifuging at 15,000 x g for 60 min at 4ºC and transferred into 1.5 ml tubes using plastic disposable transfer pipettes with enlarged mouths. Finally, 400 µl of ddH₂O were added to the enriched selenium particles preparations and the samples were stored in a fridge at 4ºC.

**Scanning electron microscopy and elemental analysis**

Purified selenium particles and day 9 cells were dried overnight on glass disks inside a desiccation chamber under vacuum. The particles and cells were then gold sputtered using a
Denton Vacuum Desk V sputter coater (Denton Vacuum LLC, New Jersey) and an environmental scanning electron microscope (FEI-MLA Quanta 650 FEG-ESEM) equipped with an energy dispersive x-ray spectrometer (EDX) was used to localize the particles on the cells, identify the composition of the particles, and determine their size and shape.

**Cell imaging**

Control and selenite treated cell cultures were initiated at a culture density of $O.D_{665} = 3.0$. Five microliter samples were examined on day 1, 3, 5, 7 and 9 under 1000 x magnification using oil immersion and a condenser phase plate 2 (Ph2) with a Nikon Eclipse E600 microscope. Images were taken using QImaging MicroPublisher 5.0 RTV camera attached to the microscope and processed with QCapture Pro 7.0 software.

**Standard curve for the determination of elemental selenium**

Selenium was made by placing 10 to 100 µl of a 0.1 M sodium selenite (Sigma) stock solution in 1.5 ml tubes and precipitating the elemental selenium by adding 160 mM of ascorbic acid (Fisher Scientific, Ottawa, Canada) to a total volume of 1.0 ml, and vortexing (Levine 1936, Oluwafemi et al. 2010). The tubes were then centrifuged at 8500 x g for 10 min in an IEC MicroMax microfuge and the supernatant removed. The precipitated selenium was then washed with 1 ml of ddH$_2$O water and vortexed followed by centrifugation at 8500 x g for 5 min, and removal of the supernatant. To convert the insoluble elemental selenium to a red-brown solution, 1 ml of a 1 M of sodium sulfide hydrate (Alfa Aesar, Ward Hill, USA) stock solution was added. Two hundred µl aliquots were transferred to clear 96 well plates (VWR International, Mississauga, Canada) and read at an absorbance of 500 nm using a SpectraMax Plus 384 UV-Visible Spectrophotometer (Biswas et al. 2011). The blank control contained only the sodium sulfide
hydrate solution. The standard curve was created from a total of \( n=4 \) determinations as seen in the figure Appendix I.

**Determination of elemental selenium produced by cells**

Cell sampling was performed at day 1, 3, 5, 7 and 9 during which 10 ml of culture from each of the jars were centrifuged at 15,000 x g (Beckman Coulter Avanti J-E, JA-17 rotor) for 60 min at 4 ºC. One ml of culture supernatant was removed to determine the amount of selenite remaining in the cell-free medium. The cell pellets were washed in 1 ml of ddH\(_2\)O, transferred to 1.5 ml tubes and vortexed. The tubes were then centrifuged at 8,500 x g for 5 min (IEC MicroMax) and the supernatant discarded. Then the cell pellet was crushed with a glass rod and 1 ml of 1 M of sodium sulfide hydrate (Alfa Aesar) added to the tubes followed by vortexing. The tubes were then centrifuged again and the resulting supernatant is assayed for selenium as described above. Then the amount of selenium is determined by using the standard curve Appendix I.

**Determination of selenite remaining in culture supernatant**

One ml samples of culture supernatant obtained as described in the previous section was placed in 1.5 ml tubes then 500 ul of 160 mM ascorbic acid added and the tubes were vortexed to precipitate the remaining selenite into elemental selenium. Selenium was then measured as described above. Again the amount of selenium was determined by using the standard curve Appendix I.

**UV-Visible absorption spectra**

A SpectraMax Plus 384 UV-Visible Spectrophotometer was used to scan the absorbance of culture samples between wavelengths of 250 -1000 nm at 10 nm intervals. Two hundred \( \mu l \)
aliquots from each culture jar were measured in clear 96 well UV plates (VWR) on days 1, 3, 5, 7 and 9.

**Cell density experiments**

The density of cells was determined using discontinuous sucrose gradient centrifugation as described by Kessi et al. (1999). Cultures were initiated at O.D.\textsubscript{665} = 3.0 as previously described. Six ml of culture were taken from each jar on days 1, 3, 5, 7 and 9, and this volume was layered on a discontinuous density gradient containing 6 ml of 4.0 M, 4.25 M, 4.50 M, 4.75 M and 5 M of sucrose layered from the top to bottom in 38.5 ml thin walled Nalgene UltraTubes. The samples were then spun at 60,000 x g for 30 min and 4 °C (Beckman Coulter Avanti J-30 I, JS-24.38 rotor).

**Statistical analysis**

All statistical analyses were performed using Microsoft Excel 2010. Data presented graphically are means and S.D. Two sample, one-tailed student t-tests with unequal variance were used on the data where appropriate.
Chapter 3: Results

ESEM visualization and EDX analysis

The micrograph of uncoated *S. leopoliensis* cells shown in Fig. 1A was obtained using an environmental scanning electron microscope. The particles on the surface and outside of the cells appear as white spheres, and those located just below the cell surface, possibly in the periplasm, are grey. In Fig. 1A, cell associated particles had an average size of about 254 ± 52 nm (n=15) with a size range of 174-348 nm while particles not associated with cells had an average size of about 200 ± 37 nm (n=15) with a size range of 174-290 nm. The procedure used to isolate selenium particles was aimed at obtaining pure samples at the expense of yield. The average particle size in the purified sample had a mean and S.D. of 143 ± 30 nm and a range of 92-192 nm (n= 30 particles). The closed black circle in Fig. 1C shows the sampling point on purified particles where EDX analysis was performed. The resultant spectrum (Fig. 1D) reveals a peak wavelength at approx. 1.4 keV, indicative of Se. When the electron beam was used to analyze areas away from the particles, the Se signal was absent, but the other signals remained the same. This indicated that these other elements were present as background, including for example, the contents of the glass disks (data not shown).

Light Microscopy

Digital images were acquired using light microscopy of the control and selenite treated cells, at an initial culture density (O.D. 665) of 3.0 (Fig. 2). Control cells sampled throughout the experiment (Fig. 2 F-J) appear green and look otherwise normal while cells treated for 1 day in selenite are similarly green but with a slightly grainy appearance. By day 3, treated cells began to form round opaque Se(0) granules extracellularly and on their surfaces (Fig. 2B black arrows).
From day 5 and onwards, the cells had lost their green pigmentation and continued to form larger round opaque granules both on the surface and outside of the cells (Fig. 2 C-E black arrows). These occurred in higher numbers and densities, i.e. on average 6 particles/cell on day 3 compared to 8 particles/cell on day 9. The extracellular granules were sometimes observed to clump together in aggregates as seen by the black arrow in Fig. 2C.

**Selenium production from selenite by S. leopoliensis**

When cells of *S. leopoliensis* grown to stationary phase were treated with 5 mM sodium selenite in fresh BG-11 medium, the cyanobacteria were observed to turn from green to orange over a 9 day period whereas control cultures remained green. The orange colour of the culture suggested the formation of elemental selenium. The addition of selenite to the medium in the absence of cells resulted in no colour change (data not shown). To measure the amount of biosynthesized Se(0), cellular samples were compared to a standard curve generated by reducing known quantities of selenite into selenium with ascorbic acid (see Methods and materials). Cultures with higher culture densities produced more selenium at the same concentration of applied selenite over a 9 day period (Fig. 3). For selenite treatments at low and medium culture densities the amount of Se(0) produced reached a peak on day 3 of 23.4 ± 3.3 and 36.7 ± 1.8 µmoles and slowly decreased to 17.0 ± 0.4 and 29.8 ± 0.4 µmoles by day 9, respectively. Throughout the 9 day experiment, the amount of Se(0) produced by the medium culture-density treatment was significantly higher than the low culture-density treatment (P < 0.05). Furthermore, the high culture-density treatment continued to increase in Se(0) production reaching a peak of 99.5 ± 10.6 µmoles by day 5 followed by a slight drop to 88.4 ± 8.1 µmoles by day 9. Throughout the 9 day experiment, the amount of Se(0) produced by the high culture-density treatment was significantly higher than both the low and medium culture-density treatments (P < 0.05).
Amount of selenite remaining in the culture media

When assaying cell-free culture media to measure the amount of selenite remaining via ascorbic acid reduction to Se(0), a reverse pattern to selenium synthesis was observed (Figure 4). From day 1 to 5, the amount of selenite remaining in the culture for the low and medium culture-density treatments decreased slowly and then the amount of selenite dropped more quickly from day 5 to 9. By day 9, the low and medium culture-density treatments had reduced 13.2 and 31.2 µmoles of selenite into elemental selenium, respectively. During the high culture-density treatment, the amount of selenite decreased more quickly than in the lower density treatments. By day 9, 97.1 µmoles of selenite was reduced to elemental selenium. Again, throughout the 9 day experiment, the amount of selenite remaining in the high culture-density treatments was significantly lower than the low and medium culture-density treatments (P < 0.05).

UV-Visible absorption spectra

The three main photosynthetic pigments in *Synechococcus* species include carotenoids (absorption peak at 420 nm), phycocyanins (620 nm) and chlorophyll a (670 nm). The spectral peaks of the pigments are evident at days 1 and 3 (Fig. 5 A and B), but are not discernible by day 5 (Fig. 5C) in the selenite treated cells. The disappearance of the pigment peaks for selenite treated cultures was concurrent with the gradual colour change from green to orange in these cultures. The intensity of the orange colour also increased with increasing culture densities. Control cultures did not lose their photosynthetic pigments, as the absorption spectra remained virtually unchanged throughout the time course of the experiment (Fig. 5 F-J).

Discontinuous Sucrose Gradient Analysis
Control (Figs. 6 F-J) and selenite (Figs. 6 A-E) treated cultures at an initial culture density (O.D.₆₆₅) of 3.0 were subjected to centrifugation through a discontinuous sucrose gradient, layered from top to bottom with 6 ml of 0.00, 4.00, 4.25, 4.50, 4.75 and 5.00 M sucrose for each layer. Throughout the time course of the experiment, the control sample cyanobacteria formed a dark green band at the top of the 4.25 M (2.946 g/cm³) sucrose layer (Fig. 6 F-J). In contrast to the control cells, after 1 day the majority of the selenite treated cells (Fig. 6A) migrated into the 4.25 M sucrose layer. After 3 days, there were two green bands located at the top of the 4.50 M (3.119 g/cm³) and 4.75 M (3.293 g/cm³) sucrose layers (Fig. 6B). It was also observed that a small amount of these cells also passed through the 5.00 M (3.466 g/cm³) sucrose and formed a pellet at the bottom of the tube (data not shown), but there was no noticeable accumulation of cells at the 4.75-5.00 M sucrose interface. For samples taken from day 5 to 9 (Fig. 6 C-E), the majority of cells were present within the 4.5 M layer with a higher proportion of cells passing through the 4.75 and 5.0 M layers as the treatment progressed. Orange cell pellets at the bottom of the tubes also became larger with time of exposure to selenite (data not shown).
Figure 1. ESEM and EDX analysis of elemental selenium particles produced from selenite by *S. leopoliensis* after 9 days. ESEM images of gold sputtered cells with spherical Se(0) particles located on the sub-surface (grey particles), surface and outside (white particles) of the cells (A); gold sputtered spherical Se(0) particles isolated from the cyanobacteria (B); spherical Se(0) particles isolated from the cyanobacteria with no gold sputtering (C); and the EDX spectra showing the qualitative identification of Se and background elements (D). Black dot of panel C indicates EDX sample point.
Figure 2. Light microscopy of *S. leopoliensis* over a 9 day period. Cells treated with 5 mM sodium selenite (A-E) compared to untreated control cells (F-J) started at an initial culture-density (O.D.\textsubscript{665}) of 3.0, after 1, 3, 5, 7, and 9 days (top to bottom). Arrows indicate extracellular Se(0) particles. Scale bar = 1.5 µm.
Figure 3. Production of Se(0) by *S. leopoliensis*. Initial culture densities (O.D.₆₆₅) of 0.5 (■■), 1.0 (▲▲) and 3.0 (●●) treated with 5 mM of sodium selenite over a 9 day period. Means ± SD, n = 4.

Figure 4. Selenite remaining in culture media after removal of *S. leopoliensis*. Initial culture densities (O.D.₆₆₅) of 0.5 (■■), 1.0 (▲▲) and 3.0 (●●) treated with 5 mM of sodium selenite over a 9 day period. Means ± SD, n = 4.
Figure 5. UV-Visible absorption spectra of *S. leopoliensis* cultures. Initial culture densities (O.D.₆₆₅) of 0.5 (-----), 1.0 (----) and 3.0 (-----) treated with 5 mM sodium selenite (A-E) compared to untreated control cells (F-J) after 1, 3, 5, 7, and 9 days (top to bottom). Values are means, n = 4. SD always less than 0.07, 0.09, and 0.20 for selenite treated, and less than 0.06, 0.06, and 0.30 for untreated cells with starting densities (O.D.₆₆₅) of 0.5, 1.0 and 3.0, respectively. Caro= Carotenoids, Phyc= Phycocyanins, Chl a = Chlorophyll a
Figure 6. Sucrose density gradient analysis of *S. leopoliensis* cultures. Initial culture density (O.D.₆₆₅) of 3.0 treated with 5 mM selenite (A-E) compared to untreated cultures (F-J) sampled on days 1, 3, 5, 7 and 9 (left-right). The discontinuous gradient layers are shown in panels A and F. Marker lines on sides of remaining tubes indicate interfaces between layers of the discontinuous sucrose gradient (top to bottom; 0.00-4.00, 4.00-4.25, 4.25-4.50, 4.50-4.75 and 4.75-5.00 M sucrose).
Chapter 4: Discussion

The formation of orange granules in *S. leopoliensis* was examined in detail by observing cultures with high densities that were treated with 5 mM selenite over a 9 day period. Particles purified from cells treated for 9 days (black dot in Fig. 1C) were shown by energy dispersive X-ray spectroscopy to be composed of Se as shown by a detected peak wavelength of approx. 1.4 keV (Fig. 1D; black closed circle in Fig. 1C indicates sampling point). Other studies that exposed bacteria to selenite also formed Se granules with similar peak wavelengths (Dhanjal et al. 2010; Fesharaki et al. 2010). Because the granules are orange in colour and composed of Se, it can be assumed that they are comprised of elemental selenium, Se(0). Figure 1A is an ESEM image of gold sputtered *S. leopoliensis* cells with mostly spherical Se(0) particles found either on or within the cell surface in association with the cells, or disassociated extracellularly. Cell associated Se(0) spherical particles had an average size of about 254 ± 52 nm with a size range of 174-348 nm. Those that were not associated with cells had an average size of about 200 ± 37 nm with a size range of 174-290 nm. These extracellular particles often formed aggregates of up to and greater than ten particles. Although the majority of the particles were spheres, there were also other shapes such as fused spheres and elongated rods. The process for particle isolation used filters with 200 nm pores in order to prepare pure samples for EDX analysis which accounts for their smaller average size of 143 ± 30 nm and a range of 92-192 nm. The high level of purity of these particles is shown in Fig. 1B. Other studies using bacteria have reported cell associated spherical Se(0) particles with a size range between 10-400 nm after treatment with selenite (Prakash et al. 2009; Dhanjal et al. 2010).

A time course study of the development of the selenium particles revealed their gradual formation by cells treated with selenite over a 9 day period (Fig. 2). Over the time course the
selenite treated cells formed particles (Fig 2. B-E) while control cells did not (Fig 2. F-J). The formation of these particles began on day 1 as the treated cells assumed a distinctive grainy texture (Fig 2. A) and an orange tint by comparison with the control cells. This indicates that the particles first form within the cells, possibly in the periplasmic space. By day 3, the selenite treated cultures started to lose their green pigmentation (Fig 2. B) and began to form Se(0) particles both attached to the surface and free in the medium. The green coloration of the cells was entirely lost by day 5 and larger opaque Se(0) particles had formed (Fig 2. C). This has been previously observed in aerobically grown gram negative heterotrophic bacteria treated with selenite (Gerrard et al. 1974; Garbisu et al. 1996; Turner et al. 1998; Roux et al. 2001; Yadav et al. 2008; Prakash et al. 2009; Dhanjal et al. 2010). As with the cyanobacterium used in this study, the Se(0) particles formed in the periplasmic space of bacteria also ended up in the extracellular media (Yadav et al. 2008; Prakash et al. 2009; Dhanjal et al. 2010; Wang et al. 2010; Biswas et al. 2011). It remains to be determined if the process is active or passive. Most active processes of excretion involve vacuolar activity and eventual exocytosis while particle release via cell lysis may be considered passive. The current study favors an active excretion process by the cyanobacteria because very few cyanobacteria in my experiments lysed as a result of the particle formation or possible selenite toxicity. The clumping of the extracellular Se(0) particles may be due to their negative surface charges (Dhanjal et al. 2010).

The mechanisms of Se(0) particle synthesis and why they begin forming close to the cell surface is not understood. Investigations were undertaken to determine if S. leopoliensis is able to reduce selenite into Se(0) in the dark. Under these conditions no particles were produced and the cultures remained green (data not shown). This may indicate that the uptake of selenite and formation of the Se(0) particles is an active light dependent process which relies directly on
electrons or reducing equivalents derived from an active photosynthetic electron transport chain. In *S. leopoliensis* the Se(0) particles may begin to form close to the thylakoid layers which are intimately associated with the plasma membrane. This is where biochemical and enzymatic reactions involving glutathione (GSH) are likely to occur (Allen, 1968a; Allen, 1968b; Edwards et al. 1968). Glutathione is the most abundant cellular thiol in cyanobacteria, reaching concentrations of 2 to 5 mM (Tel-or et al.1985; Tel-or et al. 1986; Fahey et al. 1987), especially when cells are in stationary phase as in the present experiments (Fahey et al. 1978; Kessi and Hanselmann, 2004; Smirnova and Oktyabrsky, 2005; Kessi, 2006). Glutathione has also been localized to the cytosol and interthylakoid spaces (Zechmann et al. 2010).

To better understand how Se(0) may form in *S. leopoliensis* exposed to selenite, the abiotic reactions of selenite with GSH must be considered first. Under neutral conditions with a large excess of GSH (Cui et al. 2008), the reactions are as follows:

(1) \(4\text{GSH} + \text{SeO}_3^{2-} \rightarrow \text{GSSG} + \text{GS-Se-SG} + 2\text{OH}^- + \text{H}_2\text{O}\)

(2) \(2\text{GSH} + \text{GS-Se-SG} + 2\text{OH}^- \rightarrow 2\text{GSSG} + \text{Se}(0) + 2\text{H}_2\text{O}\)

The sum of reactions 1 and 2 is:

(3) \(6\text{GSH} + \text{SeO}_3^{2-} \rightarrow 3\text{GSSG} + \text{Se}(0) + 3\text{H}_2\text{O}\)

If GSH is not in great excess where the reactants GSH: selenite are in a 2:1 ratio the reaction according to Kessi and Hannselmann (2004) proceeds as follows:

(4) \(6\text{GSH} + 3\text{SeO}_3^{2-} + 4\text{H}^+ \rightarrow 3\text{GS-Se-SG} + 2\text{O}_2^- + 5\text{H}_2\text{O}\)

(5) \(\text{GS-Se-SG} + 2\text{O}_2^- + 4\text{H}_2\text{O} + \text{H}^+ \rightarrow \text{GS-Se}^- + \text{GSH} + 4\text{H}_2\text{O}_2\)
The reaction involving GSH and selenite produces oxygen radicals in reaction 4 and subsequently hydrogen peroxide in reaction 5, both of which may oxidize unreacted GSH to GSSG (reactions 7 and 8, respectively) or may re-oxidize Se(0) back into selenite (reaction 9), preventing the formation of selenopersulfide (GS-Se⁻) and its spontaneous dismutation into Se(0) as in reaction 6 (Turner et al. 1998; Gao et al. 2002; Kessi and Hannselmann 2004). These impeding reactions are shown below:

\[
(6) \quad \text{GS-Se}^- + \text{H}^+ \rightarrow \text{GSH} + \text{Se}(0)
\]

\[
(7) \quad \text{O}_2^- + 4\text{GSH} + \text{O}_2 \rightarrow 2\text{GSSG} + 2\text{H}_2\text{O} + \text{O}_2^-
\]

\[
(8) \quad 2\text{GSH} + \text{H}_2\text{O}_2 \leftrightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

\[
(9) \quad \text{Se}(0) + 4\text{H}_2\text{O}_2 \rightarrow \text{SeO}_3^{2-} + 3\text{H}_2\text{O} + \text{O}_2 + 2\text{H}^+
\]

Under the experimental conditions used to produce Se(0) in this study, it may be assumed that GSH concentrations in *S. leopoliensis* at all culture densities were not in excess. Even at the highest culture densities approx. 400 of the original 500 µmol of selenite remained in the culture medium after 9 days of treatment (Fig. 4). My results also indicate that the presence of GSH alone in the absence of light did not produce Se(0) particles since it is known that *S. leopoliensis* cells grown in the dark increase their GSH levels (Bermudes, 1985). This supports the notion that the cellular biochemical conversions, *i.e.* biotic conversions, occurring between GSH and selenite follow those of the abiotic reactions 4 and 5. Subsequently, light driven enzymatic reactions could drive the reduction of selenodiglutathione (GS-Se-SG) into Se(0). The possible reactions are as follows:
Cyanobacteria contain plant-like chloroplast associated thioredoxins (Trx), including the m-type version (TrxA), found in the vicinity of the thylakoid membrane that are reduced by light driven ferredoxin dependent thioredoxin reductase (FTR) of photosystem I (PSI) (Schmidt, 1980; Whittaker and Gleason, 1984; Cossar et al. 1985; Muller and Buchanan, 1989; Heping et al. 1992; Bjornstedt et al. 1995; Florencio et al. 2006; Perez-Perez et al. 2009a; Perez-Perez et al. 2009b). They may also contain other Trxs (TrxB, TrxC, TrxQ) which can be reduced by NADPH through light driven ferredoxin NADP reductase (FNR) also found in association with PSI (Florencio et al. 2006; Perez-Perez et al. 2009a). As seen in reactions 10.1 and 10.2, and 11.1 and 11.2, the reduction of selenodiglutathione to Se(0) via thioredoxins reduced either by FTR or NADPH from FNR is possible, especially if we consider that the cells may be regenerating GSH for further reactions with selenite. Surprisingly, only two studies with *Bacillus subtilis* have implicated the formation of Se(0) via NADPH dependent thioredoxins (Garbisu et al. 1996; Garbisu et al. 1999). The other possibility shown in equation 12 is that high light on top of the oxidative stress conditions caused by selenite in the cultures may have stimulated the expression of glutathione reductase (GR) using NADPH from FNR to reduce selenodiglutathione to Se(0) and GSH (Serrano et al. 1984). The generation of oxygen radicles and hydrogen peroxide from

\[
\begin{align*}
(10.1) \ 2e \text{ from PSI} & \rightarrow 2Fd_{\text{red}} + \text{Trx}_{\text{ox}} \xrightarrow{\text{FTR}} 2Fd_{\text{ox}} + \text{Trx}_{\text{red}} + 2H^+ \\
(10.2) \ 2\text{GS-Se-SG} + \text{Trx}_{\text{red}} & \rightarrow 2\text{GSH} + \text{Se}(0) + \text{Trx}_{\text{ox}} \\
(11.1) \ NADPH \text{ from PSI FNR} + H^+ + \text{Trx}_{\text{ox}} & \xrightarrow{\text{TR}} \text{Trx}_{\text{red}} + \text{NADP}^+ \\
(11.2) \ 2\text{GS-Se-SG} + \text{Trx}_{\text{red}} & \rightarrow 2\text{GSH} + \text{Se}(0) + \text{Trx}_{\text{ox}} \\
(12) \ NADPH \text{ from PSI FNR} + H^+ + \text{GS-Se-SG} & \xrightarrow{\text{GR}} 2\text{GSH} + \text{Se}(0) + \text{NADP}^+
\end{align*}
\]
reactions 7 through 9 could be inhibited by superoxide dismutases, peroxidases, peroxiredoxins, and catalase in *S. leopoliensis* (Tel-or et al. 1986; Mittler and Tel-or, 1991; Miller et al. 2000; Perelman et al. 2003; Stork et al. 2005; Stork et al. 2009).

In Figure 3, the relationship between increasing culture densities (O.D$_{665}$ = 0.5, 1.0 and 3.0, hereafter referred to as low, medium and high, respectively) and the amount of Se(0) produced as a result of treating *S. leopoliensis* with 5 mM selenite over a 9 day period is shown. The production of Se(0) does not occur when the cyanobacteria are treated with 5 mM selenate in the light and these cells lost their pigmentation and died by day 5. Furthermore, when cells were treated with selenate in the dark, no Se(0) was formed, but the cells remained green. This suggests that like sulfate, selenate accumulation and assimilation is an active process requiring light (Ritchie, 1996; Saggu et al. 2010). It has been shown that selenate is more toxic to cyanobacteria despite the fact it does not bioaccumulate as much as selenite does (Kiffeney and Knight, 1990; Gouget et al. 2005; Saggu et al. 2010). For low and medium culture densities, the amount of Se(0) produced in the selenite treatments reached a maximum on day 3 and decreased slowly until day 9. For the high culture-density treatments, the amount of Se(0) produced by the cells continued to increase until day 5. On day 7 the cells may have started to die since the formation of Se(0) decreased until day 9. It is evident that increasing the culture-density produces a dilution effect by the cyanobacteria. At a fixed amount of selenite in the culture, increasing the amount of cells per unit volume of medium would lower the amount of selenite that is bound, accumulated and transformed per cell. For the high culture-density treatment, the dilution effect may have allowed for more cells to survive for longer and convert more selenite than the medium and low density treatments, which may have succumbed shortly after day 1. In the cyanobacterium *Anabaena flos-aquae*, it was shown that selenite bioaccumulation at sub-
lethal levels (5.8 and 17.3 μM) was regulated by the cells, but at higher concentrations (29.0 and 58.0 μM) the regulation of selenite entering the cells broke down and disrupted metabolic activities (Kiffeney and Knight, 1990). In the sub-lethal treatments, they observed their highest accumulation of selenite by day 2 with no change throughout the rest of the 10 day experiment. As expected in the present study, by day 9, the amount of Se(0) produced depended on culture-density. That of the high density was approx. three and six times the amount found in the low and medium density treatments.

The amount of Se(0) produced by the cells (Fig. 3) is accompanied by the consumption of selenite from the culture media (Fig. 4). However, all of the loss of selenite from the low and medium culture-density treatments between day 1 and 9 could not be accounted for by the increases in the amount of Se(0) produced. Gouget et al. 2005 found that selenite treated Synechocystis sp. PCC 6803 may be incorporating the selenite into cells through two competing reduction mechanisms. One is specific and reduces selenite to be incorporated into selenoproteins and the other is a non-specific mechanism where selenite interferes with thiols and enzymes in general. It is unlikely that all the unaccounted for selenite was assimilated into selenoproteins because this would require a very large pool of target proteins. It is more likely that the difference between Se(0) biosynthesis and selenite loss is due to volatile hydrogen selenide or methylated selenium. Although this requires further investigation, an unpleasant odour was produced by the selenite treated cultures that was reminiscent of horseradish, possibly because of evolved H₂Se. This likely explains the deficit in the amount of selenite remaining in the culture media. Synechococcus sp. have been known to produce hydrogen sulfide under normal growth conditions (Sheridan, 1973; Fahey et al. 1987). There are two ways in which hydrogen selenide may be produced. If selenite is incorporated into toxic selenocysteine, it is
possible that hydrogen selenide may be produced via L-cysteine desulphhydrase (Tarze et al. 2007). It may also form as a result of selenol (GS-SeH) reactions with GSH or TR/GR (Bjornstedt et al. 1995; Turner et al. 1998). The hydrogen selenide gas may become volatilized and lost, or it may form hydrogen selenide ions (HSe-) under physiological conditions and react with GSH or oxygen to form Se(0) (Nuttall and Allen, 1984; Turner et al. 1998). Selenols may also be used to form methyl selenide gases through methyl transferases (Turner et al. 1998; Ranjard et al. 2002; Ranjard et al. 2003). Biomethylation of non-metals and metalloids has only recently been shown in cyanobacteria (Yin et al. 2011; Chu et al. 2012; Ye et al. 2012; Yin et al. 2012), but is well documented in prokaryotes (Ridley et al. 1977; Chasteen, 1993; Ranjard et al. 2002; Ranjard et al. 2003; Stolz et al. 2006) and eukaryotic organisms (Evans et al. 1968; Zieve et al. 1985; Thompson-Eagle et al. 1989; Brady et al. 1996; Neumann et al. 2003). It is important to note that most studies that use bacteria tend to focus only on the formation one of either Se(0) particles or methylated selenium. There has been very little work done to discern when Se(0) formation is favoured over biomethylation, or vice versa, and whether the two processes are linked. Only a single report by Chasteen (1993) suggested that Se(0) formation may be a precursor to the formation of methylated selenium.

The UV-Visible absorption spectra of control and cells treated with 5 mM of selenite were monitored over a 9 day period (Fig. 5). This revealed the gradual change in colour of the cultures from green to orange in the treated cells. On day 1 (Fig. 5 A and F), the predominant pigments were identified as carotenoids, phycocyanins and chlorophyll a with absorption maxima at 420 nm, 620 nm and 670 nm, respectively (Rabinowitch and Govindjee, 1969; Biswal et al. 1994; Six et al. 2004; Alvey et al. 2011). The spectra for the major pigments were more prominent for higher culture densities than the lower culture densities. At day 1, all selenite
treated cultures remained green in colour but had a slight orange tinge (Fig. 5A). The amount of
pigment as discerned by absorption at their respective maxima, was much reduced by day 3, and
on day 5 all of these peaks had disappeared from the absorption spectra (Fig. 5C). The
disappearance of the pigments appears to be due to the toxicity of selenite to the photosynthetic
machinery. Oxidative stress caused by selenite toxicity is known to lower chlorophyll a levels
and disrupt oxygenic photosynthesis in cyanobacteria (Sielicki and Burnham, 1973; Kiffeney and
Knight, 1990; Pronina et al. 2002; Li et al. 2003). At day 9 for the high culture-density treatment
the absorption spectrum values slightly increased between 400 and 600 nm, indicating an
increase in the amount or size of the Se(0) particles formed. The day 9 absorption spectra look
very similar to absorption spectra taken for aerobic heterotrophic bacteria that formed Se(0)
particles after treatment with selenite (Dhanjal et al. 2010; Fesharaki et al. 2010; Wang et al.
2010). On the other hand the pigment spectrum of control cells for all culture densities remained
the same throughout the 9 day experiment (Fig. 5 F-J). The control spectra also show that the
cultures did not grow since there was no change in the absorbance peaks of the pigments over the
9 day period. This may be because the cells started the treatment in stationary phase and could
have rapidly depleted their nutrients at such high culture densities, thereby preventing normal
cell growth and division (Schwabe and Hickel, 1978; Kullberg, 1981; Wang et al. 2011; Goclaw-
Binder et al. 2012).

The buoyant density of control and selenite-treated cells were assessed using
discontinuous gradient centrifugation (Fig. 6). The control samples equilibrated above the 4.25
M sucrose layer (2.946 g/cm³) throughout the 9 day experiment, indicating no change in the
buoyant density of the cells (Fig. 6 F-J). This is in agreement with previous estimates of
buoyant densities between 1.100 - 1.368 g/cm³ for the cells of *Synechococcus sp*. (Guerrero,
1985; Matsumoto et al. 2002). The reason there are cells above and below this layer may be due to the fact that *Synechococcus sp.* cells in general are not uniform in size and form short chains of colonies in liquid culture (Rippka et al. 1979). Also, my cells formed elongated cells as a result of high light exposure (Kullberg, 1981) and nutrient deprivation (Schwabe and Hickel, 1978; Goclaw-Binder et al. 2012), due to an unnaturally high culture-density (Wang et al. 2011).

On day 1 for the selenite treated samples, a deep green layer within the 4.25 M layer (2.946 g/cm³) which extended to the top of the 4.5 M layer (3.119 g/cm³) indicated an increase in the buoyant cell density of the cells and marked the beginning of intracellular formation of the Se(0) particles (Fig. 6A). By day 3, the cells had formed larger Se(0) particles (see also Fig. 2B) and the cells formed two prominent layers located at the top of the 4.5 M (3.119 g/cm³) and 4.75 M (3.293 g/cm³) layers. The day 5 to 9 (Fig. 6 C-E) selenite treated samples looked very similar to each other, with a majority of the cells trapped in the 4.5 M layer. Again, for all selenite treated samples, the lighter green layers of cells appearing above or below the prominent bands may be due to cell elongation which may be caused by a combination of treatment with high selenite concentrations and relatively high light exposure (McCready et al. 1965; Kullberg, 1981; Hunter and Manter, 2008). This may cause larger cells to form more Se(0) than smaller cells. These larger cells along with Se(0) particles that have a density of 4.79 g/cm³ passed through both the 4.75 M and 5.0 M layers, and formed cell pellets at the bottom of the tubes during days 3-9. My results agree well with a study done with *Rhodospirillum rubrum* where the selenite reduction phase caused an increase in the buoyant density of the cells but unlike my study, the researchers found that the cells returned to the buoyant density of control cells after expulsion of the Se(0) particles. It appears that the majority of the *S. leopoliensis* cells in this study died before the expulsion phase (Kessi et al. 1999).
In summary, this study has shown that *S. leopoliensis* produces elemental selenium, Se(0) particles when exposed to high concentrations of selenite in the light. These particles appeared to be formed within the periplasmic space between the thylakoids and cell membrane. This may be a result of abiotic and enzymatic reactions involving light dependent thioredoxins or glutathione reductases. Unbound and bound particles were on average 200-254 nm in diameter and generally spherical in shape. Throughout the treatment period, more Se(0) was accumulated at higher culture densities and although selenite was consumed from the media, selenium particle formation could not account for all of it. When *S. leopoliensis* cultures were exposed to selenite the cultures turned orange by day 5 because of Se(0) particle formation, and cyanobacterial organic pigment loss as a result of selenite toxicity. The cells also become heavier as they produced and accumulated more Se(0) particles throughout the treatment period.

There are a few research questions that remain unanswered which may be tested in future experiments. They may include questions like: 1) Does blocking electron flow to photosystem I using photosynthetic inhibitors such as Diuron prevent the formation of Se(0) particles by the cyanobacteria? 2) What other growth conditions or parameters improve or impede the formation of Se(0) particles in the cyanobacteria? 3) How much selenite is converted to volatile selenide gases? 4) What proteins are involved in Se(0) formation when the cyanobacteria are exposed to selenite? 5) What concentration of selenite marks the transition between nutritional, sub-lethal and toxic levels for the cyanobacteria?
Summary

- *S. leopoliensis* produced spherical elemental selenium, Se(0) particles when exposed to high concentrations of selenite in the light by day 5.

- These particles appeared to be formed within the periplasmic space between the thylakoids and cell membrane potentially as a result of abiotic and enzymatic reactions involving light dependent thioredoxins or glutathione reductases.

- As the culture densities increased more selenite was reduced to Se(0) and less selenite remained in the media.

- *S. leopoliensis* turned orange by day 5 when exposed to selenite due to the formation of Se(0) and there was a loss of photosynthetic pigments due to selenite toxicity.

- As cells produced more Se(0) particles when treated with selenite during the 9 day treatment period, their buoyant densities increased.

Literature Cited


Kessi, J. 2006. Enzymatic systems proposed to be involved in the dissimilatory reduction of selenite in the purple non-sulfur bacteria *Rhodospirillum rubrum* and *Rhodobacter capsulatus*. Microbiology 152:731-743.


Appendix

Appendix I: Elemental selenium standard curve produced by reducing known amounts of sodium selenite in 160 mM of ascorbic acid and reading the absorbance at 500 nm. Means ± SD, n = 4.