BIOTRANSFORMATION AND DISTRIBUTION OF SELENIUM IN CULTURES OF A SALT-MARSH YEAST

by

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ABSTRACT

The essentiality and toxicity of selenium makes its biogeochemical cycling and global distribution of great interest for human health. Selenium-reducing yeast and fungi are important contributors to the selenium cycle, yet there have been few studies of both particulate Se accumulation and Se volatilization by an environmental yeast isolate. This study quantified the distribution of selenium in liquid, solid (cell-associated), and volatile fractions of cultures of a salt-marsh yeast isolate, *Rhodotorula mucilaginosa*-13B. More than 20% of amended selenite accumulated in cell pellets, while 1% was dispersed in volatile form. Elemental selenium only accumulated during the exponential and stationary growth phases of live cultures. Electron microscopy images indicated the location of particles both within and outside of cells and suggested changes to cellular ultrastructure resulting from internal particulate Se accumulation. Additionally, distribution results indicated several other minor interactions among cells, media and the various species of selenium present in the cultures. We conclude that strain 13B may play a complex and substantial role in the distribution and speciation of selenium in its environment, with its primary contributions to the selenium cycle clearly resulting from its ability to reduce selenium oxyanions to elemental selenium.

Chapter 1

BACKGROUND

1.1 General Introduction and Scope of Study

The duality of selenium as a micronutrient and a toxin makes its biogeochemical cycle both necessary and challenging to study. Its distribution and bioavailability is controlled by several chemical, geological, and geographical factors, but also by microbial interactions, including the biologically-facilitated reduction of Se. In some parts of the world, selenium deficiency compromises the health of rural populations and necessitates dietary supplementation (Gao et al., 2011; Tan et al., 1994). Elsewhere, selenium contamination has become an increasing concern (Bajaj et al., 2011; Ohlendorf, 2002), and has enhanced the need to understand the processes by which selenium is distributed and transformed. The discovery of dozens of microbial species capable of selenate and selenite reduction has brought bioremediation to the forefront of selenium research during the last two to three decades (Flury et al., 1997; Frankenberger and Arshad, 2001; Zhang et al., 2008a). In order to successfully balance human health and Se distribution, research must expand upon bioremediation research to encompass all of the geochemical factors governing selenium distribution and consider the diversity of biological interactions with its cycling.

Yeast and microfungi are important contributors to selenium cycling, particularly in soil environments (Barkes and Fleming, 1974; Doran and Alexander, 1977). However, few pure isolates have been studied for their ability to reduce or

volatilize selenium (Brady et al., 1996; Falcone and Nickerson, 1963). This study will be among the few that describe selenium transformations by a pure yeast strain isolated from an environmental soil, and also the only study so far to combine quantitative analyses with imaging techniques in the investigation of elemental selenium accumulation by a yeast. The organism used, *Rhodotorula mucilaginosa* Strain 13B, was isolated from slurried sediments sampled from a tidal salt marsh on the Mid-Atlantic coast of the USA. The ubiquitous and adaptable nature of this *R. mucilaginosa* (Lahav et al., 2002; Libkind et al., 2008) suggest that interactions between Strain 13B and various selenium species are representative of the role of yeasts in soil selenium cycling.

1.2 The Importance, Cycling, and Distribution of Selenium

Selenium is a multi-faceted component of human physiological processes. The human body assimilates it via the amino acid selenocysteine and requires it for redox signaling, thyroid metabolism, immunity and antioxidative defense (Papp et al., 2007). Dietary deficiency induces susceptibility to infertility, liver necrosis, nutritional muscular dystrophy, Keshan disease (a reversible form of cardiomyopathy), and oxidative stress-related diseases (Tinggi, 2003; Wu, 2004). Depending on the form and concentration of selenium in the body, it can be involved in both the formation and prevention of reactive oxygen species. Once the body's production of oxidants exceeds its production of antioxidants, further selenium intake can become toxic, and chronic exposure damages major organs such as the liver, kidneys, spleen, heart, and pancreas, and may also lead to cancer (Letavayova et al., 2006; Spallholz, 1994). Most dietary selenium is obtained from the ingestion of selenium-accumulating plants in the form of selenomethionine, but the human body also has

mechanisms for assimilating selenite. Animals also have detoxification mechanisms by which excess selenium is released via breath and urine. Some extensive reviews of selenium assimilation and metabolism in humans and animals can be found elsewhere. (Wessjohann et al., 2007).

The speciation and distribution of selenium in the environment depends on such geochemical factors as reduction-oxidation conditions, mineral adsorption and complexation to organic matter. The dominant oxidation states of selenium in aerobic environments are Se(IV) and (VI), which exist as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), respectively, either in minerals or as the free oxyanions. Selenium oxyanions are the most soluble forms of selenium and comprise the majority of Se available for biological uptake, but their mobility can be reduced via sorption to aluminum oxides and iron oxides, or via substitution in carbonate- or phosphate-rich mineral lattices (Fernandez-Martinez and Charlet, 2009). Fulvic acid and humic substances form exceptionally stable complexes with selenate and selenite, while free phosphate, sulfate, oxalate and citrate compete with them for mineral adsorption sites. Selenite commonly forms stable inner-sphere complexes, while selenate forms outer-sphere complexes of low stability and short residence time and is generally more mobile (Fernandez-Martinez and Charlet, 2009).

The reduction of oxyanions to elemental selenium depends on the concentration of reducing agents such as Fe(II) and Mn(II), and is closely related to iron chemistry in natural ecosystems (Masscheleyn et al., 1990; Myneni et al., 1997; Velinsky and Cutter, 1991). Elemental selenium is most stable in its metallic gray trigonal phase (Fernandez-Martinez and Charlet, 2009), but biogenic selenium exists primarily in the red amorphous phase (Oremland et al., 2004; Tam et al., 2010).

Elemental Se is insoluble, stable under acidic and reducing conditions, and is inert to most biological systems. Inorganic reduction to Se(-II) results in the precipitation of metal selenides (Fernandez-Martinez and Charlet, 2009). Selenium (-II) is also the major oxidation state of organic selenium compounds, which are usually produced and accumulated by microorganisms or in biological tissues.

Several geographical factors affect global selenium distribution and bioavailability, often in association with the cycling of sulfur and phosphorus. Soils derived from shales and other phosphatic rocks are high in selenate and selenite, while soils high in sulfide minerals such as pyrite are enriched in selenide (Fernandez-Martinez and Charlet, 2009). Sedimentary rocks are low in selenium, and are also the most common in the Earth's crust, making selenium-deficient soils more widespread than seleniferous ones. Selenium is considered a trace element in the crust with an average worldwide crustal concentration of 0.05 mg kg^{-1} (Fernandez-Martinez and Charlet, 2009). Anthropogenic sources of selenium include sulfur-rich coal combustion and the application of phosphate-rich fertilizers (Fernandez-Martinez and Charlet, 2009). Climate plays an important role because rainwater contains dissolved ions that can release selenium oxyanions from mineral lattices and enrich groundwaters. On the other hand, dry soils may be categorized as "deficient" with regard to bioavailability even if they are rich in selenium minerals, as in parts of China (Tan et al., 1994). Hydrologic changes can thus upset the balance of selenium cycling, and are one of the causes for selenium contamination in parts of California (White and Dubrovsky, 1994).

1.3 Microbial Transformations and Bioremediation

Many microorganisms with high selenium tolerance and the capacity to reduce or oxidize selenium have been identified in the last half-century, representing all three domains and at least three dozen genera (Milne, 1998). They have been found in almost all environments, including pond and wetland sediments, marine and fresh waters, wastewater streams, and in areas of both high and low selenium concentration, ranging several orders of magnitude (Chasteen and Bentley, 2003; Haygarth, 1994). Both selenium reduction and oxidation by microbes have been reported, but only a few microbes believed to oxidize selenium have been isolated (Sarathchandra and Watkinson, 1981; Torma and Habashi, 1972).

Bacteria have been studied extensively for their ability to reduce selenate and selenite. Most selenate reduction is dissimilatory and requires unique molybdenum-containing enzymes (Bebien et al., 2002; Schroder et al., 1997; Watts et al., 2003). In contrast, the mechanisms of selenite reduction are varied and may be carried out by independent enzymes (Kessi, 2006) or by multi-substrate enzymes such as nitrite reductase (Basaglia et al., 2007). Some organisms respire selenite while others simply reduce it as a detoxification mechanism. Many organisms further reduce and methylate selenium to produce methylated volatile selenides (MVSe), most commonly dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe). Most studies of microbial volatilization were performed on environmental samples or field sites rather than pure isolates (Doran and Alexander, 1977; Frankenberger and Karlson, 1994), and the mechanisms of this process are speculative, but several intermediates have been proposed (Challenger, 1945; Cooke and Bruland, 1987; Fan et al., 1998; Reamer and Zoller, 1980; Zhang and Chasteen, 1994). The production of MVSe from soils is primarily associated with microbial processes, as demonstrated by

comparisons between autoclave-sterilized and non-sterilized environmental soil samples (Barkes and Fleming, 1974; Frankenberger and Karlson, 1989).

In the 1980s, increasing concerns about selenium-contaminated sites such as the Kesterson Wildlife Refuge in the San Joaquin Valley of California triggered a great interest in selenium bioremediation research. Many factors have been studied during attempts to optimize bioremediation results. These include ionic competition for reduction (Losi and Frankenberger, 1998; White and Dubrovsky, 1994), growth substrate, moisture and aeration (Flury et al., 1997; Zhang et al., 2008b), and inorganic redox enhancement (Zhang et al., 2008a). Disposal or natural dispersal of reduction products must also be considered. One group proposed a constructed system in which algal blooms provided the carbon and energy source for denitrifying bacteria, which in turn removed nitrate from the water and eliminated competition for microbial selenium reduction (Lundquist et al., 1994). A later study reported 98% removal of selenium from agricultural drainage waters using a biological reactor system (Cantafio et al., 1996). In both cases, simple methods were proposed for the collection and removal of precipitated Se^0.

Volatilization is an attractive method of natural selenium dispersal since the released compounds are rapidly decomposed and are not known to display the same level of oxidative damage in animals as selenite. Since the vapor pressure of DMSe is positively correlated with temperature (Karlson et al., 1994), volatilization should be most efficient during the warmer parts of the day or year, and this has been found to be the case in more than one study (Flury et al., 1997; Velinsky and Cutter, 1991). However, the release of MVSe can be hindered by high moisture levels and microbial oxidation (Dungan et al., 2002; Zhang and Frankenberger, 2002; Zhang et

al., 1999). Although no studies have reported volatilization rates high enough to be the sole remediation effort, optimized Se volatilization might be a cost-effective means of long-term bioremediation in areas with low levels of contamination.

Chapter 2

BIOTRANSFORMATIONS AND DISTRUBUTION OF SELENIUM IN CULTURES OF A SALT-MARSH YEAST

2.1 Abstract

Selenium-reducing yeast and fungi are important contributors to the selenium cycle, yet quantitative studies of both particulate selenium accumulation and selenium volatilization by environmental isolates are few. In this study, we compared the elemental composition, location, and quantity of Se accumulation during each growth stage of a salt-marsh yeast isolate, *Rhodotorula mucilaginosa*-13B. We also quantified the distribution of selenium between aqueous, solid and volatile phases in cultures grown with continuous aeration. Elemental selenium only accumulated during the exponential and stationary growth phases of live cultures. More than 20% of amended selenite accumulated in cell pellets of aerated cultures, while 1% was dispersed in volatile form, and volatile compounds underwent minor interactions with both cultures and sterile media. We conclude that strain 13B may play a complex and important role in the distribution and speciation of selenium in its environment, with its primary contributions to the selenium cycle clearly resulting from its ability to reduce selenium oxyanions to elemental selenium.

2.2 Introduction

Selenium is both an essential micronutrient and a toxin to almost all life. Its availability to organisms depends upon its solubility and environmental mobility, and consequently, on its chemical speciation. Selenium exists in four oxidation states (-II, 0, IV, and VI) and is most soluble as the oxyanions selenite (IV) and selenate (VI), which are stable in aerobic environments and dominate selenium-enriched surface waters (Fernandez-Martinez and Charlet, 2009). Due to its bioavailability, coupled with its ability to form reactive oxygen species in the body, selenite is among the most toxic species of selenium to humans and animals (Spallholz, 1994). A variety of bacteria and yeast isolates have been shown to reduce and sometimes methylate selenium (Gupta et al., 2010; Losi and Frankenberger, 1997; Milne, 1998; Zahir et al., 2003) to products that are less toxic than selenite (Spallholz, 1994), and may do so either to obtain energy or to detoxify their environment (Chasteen and Bentley, 2003; Gharieb and Gadd, 1998; Oremland et al., 2004). Reduction to elemental selenium (Se^0) results in the immobilization of Se as a solid precipitate (Fernandez-Martinez and Charlet, 2009). The production of methylated volatile selenides (MVSe), most commonly dimethyl selenide (DMSe) and dimethyldiselenide (DMDSe), leads to the potential dispersion of organic selenium into the atmosphere, where it is rapidly decomposed (Atkinson et al., 1990). Microbes are thus important controllers of selenium cycling and bioavailability.

Some selenium-reducing microorganisms have shown great promise for bioremediation of selenium-contaminated areas (Frankenberger and Arshad, 2001), such as the San Joaquin Valley, CA, where Se toxicity was responsible for damage to wildlife populations in the 1980s (Ohlendorf, 2002). Several processes for reducing aqueous oxyanions to elemental selenium have been developed with various

approaches to reducing the cost or ensuring complete conversion, and with suggested means of collecting and permanently removing the Se^0 product (Cantafio et al., 1996; Chung et al., 2010; Green et al., 2003; Zhang et al., 2008a**;**Zhang et al., 2008b). Alternatively, microbial selenium volatilization is an attractive means of natural dispersal if conditions can be optimized for the effective release of volatile compounds. Significant Se removal via volatilization has been achieved both in the laboratory and in the field (Flury et al., 1997; Zhang and Frankenberger, 2006), though none have been as complete as removal via elemental selenium precipitation.

Yeast and microfungi are especially important to Se reduction in soils and on minerals (Barkes and Fleming, 1974; Peitzsch et al., 2010). Selenium tolerance and toxicity in fungal species is well-studied (Fujs et al., 2005; Golubev and Golubev, 2002), and the ability to reduce the oxyanions to Se^0 has been associated with higher tolerance levels in some species (Falcone and Nickerson, 1963; Gharieb and Gadd, 1998, 2004). However, few pure isolates have been studied quantitatively for their Se-reducing potential (Brady et al., 1996) and most studied isolates were not cultured from natural water or soils (Brady et al., 1996; Falcone and Nickerson, 1963; (Falcone and Nickerson, 1963; Gharieb and Gadd, 2004), and do not represent the behavior of Se-reducing fungi in their natural environment. Recently, several tellurite-resistant bacterial and yeast isolates were cultured from slurries of tidal salt marsh sediments collected near Rehoboth, Delaware, USA. All strains exhibited signs of elemental tellurium accumulation when grown in tellurite-amended media. Strain 13B, a strain of the carotenogenic yeast *Rhodotorula mucilaginosa*, displayed the highest tolerance for tellurite and produced the greatest amount and diversity of volatile tellurides relative to other isolates studied (Ollivier et al., 2008). This was later shown to be the

case for selenite, as well (Ollivier et al., in progress). Since *R. mucilaginosa* strains are ubiquitous in aquatic and wetland environments, it is possible that 13B is representative of yeast populations contributing to selenium cycling in a variety of soil environments. Thus, knowledge of the interactions between this model strain and various species of selenium would provide insight into the role of yeasts in the biogeochemical cycling of selenium.

The goal of this study was to describe the interactions of selenium with Strain 13B by quantifying the distribution of Se among three phases (aqueous, cell-associated, and volatile) and determining the role of cellular metabolism in selenium reduction. Quantitative analysis of total selenium content, over time and in all three phases, was combined with electron microscopy to give multiple insights into the biotransformations and interactions between this strain and various species of selenium. We concluded that strain 13B may contribute to several facets of the environmental selenium cycle, primarily via reduction of selenium oxyanions to elemental selenium.

2.3 Materials and Methods

2.3.1 Culture and Standard Preparations

The yeast strain *R. mucilaginosa*-13B was previously isolated from the top 2 cm of salt marsh sediments on the fringes of the Indian River Inlet in Rehoboth, DE. Details of its isolation were described elsewhere (Ollivier et al., 2008). The medium used, LB-Marine, was based on Luria-Bertani medium and optimized by Ollivier, et al. (2008) for the recovery of organisms from the sediment samples collected in Rehoboth. It contained 2.0 g/L tryptone, 1.0 g/L yeast extract, 12.5 g/L sodium

chloride, and 1.0 mL/L of Pf-7 Trace Element solution (Wahlund et al., 1991). Sterile 1 M $MgSO_4$ was added to media after autoclaving (20 mL added per liter of media). Starter cultures for inoculating experimental samples were prepared from a plate of pure 13B isolate and incubated at 30 °C and 250 rpm for approximately 48 hours. For killed-cell control experiments, hexadecyl trimethylammonium bromide (CTAB) was added to a concentration of 1%, which was previously shown to be effective at killing cultures of this strain (Bahrou, et al., in preparation). Dried solid sodium selenite was used to prepare a stock selenite solution of 100 mM in ultra-purified water (Barnstead NANOpure purification system). The stock solution was autoclaved before use. Selenium standards for inductively-coupled mass spectrometry (ICPMS) analysis were prepared in a mixed solution of 1% nitric and 0.5% hydrochloric acid from a certified 1000 mg/L stock (Perkin Elmer). New standards were prepared every 1-3 months, and stock selenite solutions were remade several times to avoid using contaminated or degraded standards. All standards and digestions were prepared using trace metal grade acids and NANOpure water.

2.3.2 Culture Incubation

Cultures analyzed for selenium accumulation over time were grown in 15-mL glass vials with phenolic caps (referred to hereafter as vial cultures or vial incubation), using cotton plugs as closures to allow oxygen exchange. Ten-milliliter cultures were harvested in triplicate every 24 to 72 hours for cell counts and total selenium analysis. Experimental cultures were amended with sodium selenite (0.68 mM final concentration) and were paired with control samples that received no selenite amendment. Additionally, two types of CTAB treatments were applied to a batch of samples after four days of growth without selenite amendment. Cultures

receiving the first treatment ("washed-killed cells") were separated from the media, washed twice by centrifugation with sterile NANOpure water, and re-suspended in fresh media containing 1% CTAB and 2 mM sodium selenite. Cultures receiving the second treatment ("killed cells") remained in original media and were amended directly with CTAB and selenite to the same concentrations. Triplicate batches of each treatment were harvested for ICPMS analysis at 3, 5, 8, and 11 days after inhibition.

An apparatus for growing cultures with continuous aeration and for trapping volatile compounds was modified from a previously-developed system (Ollivier et al., 2011) (Figure 1). Air flow carried MVSe produced in Sample A through the apparatus to Acid Traps 1 and 2, where 8N HNO_3 oxidized the volatiles to aqueous species. "Teflon traps" contained Teflon® shavings to impede transfer of liquid aerosol from one sample to the next, and thus preserve the separation of aqueous from volatile phases. Teflon was chosen for the modified system because of its reusability and low chemical reactivity.

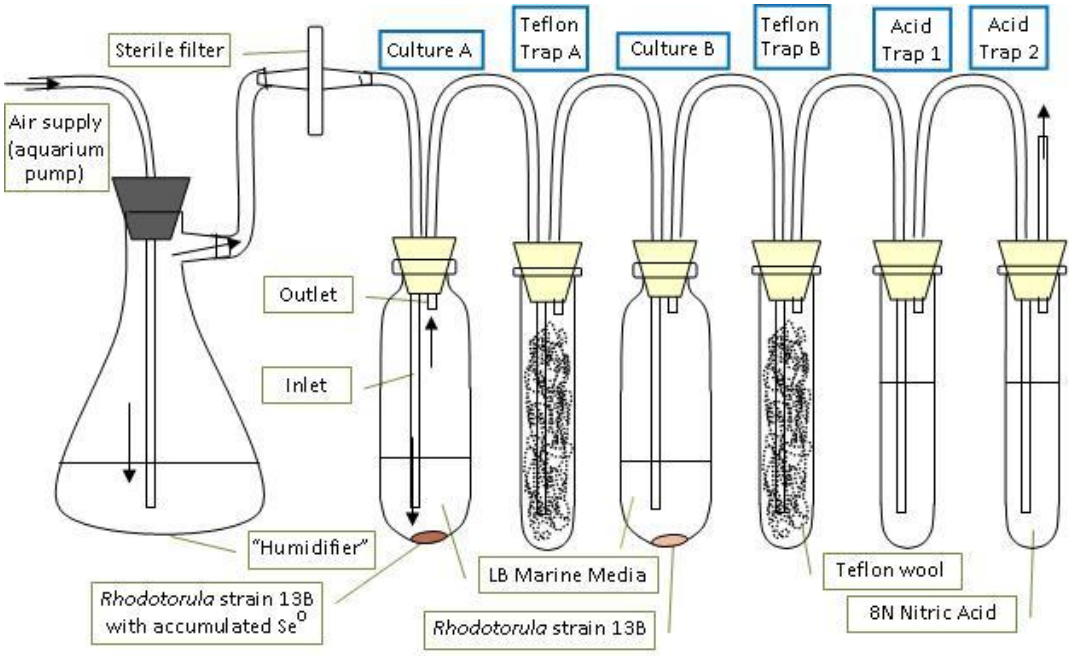

Figure 1 **Two-culture apparatus with continuous aeration for analyzing the distribution of selenium in all three physical phases—aqueous (media), solid (cell-associated), and volatile (acid traps)—with Teflon traps for preventing aerosol carry-over.**

Four experiments were compared for determination of biotic and abiotic processes (Table 1). Samples A of "MVSe-cells" and "MVSe-media" experiments were amended with selenite and inoculated with Strain 13B such that the cultures would produce the volatile selenide species. Sample B was inoculated in the MVSe-cells experiment, but only contained sterile media in the MVSe-media experiment, such that the MVSe produced by A-cultures came into contact with either cells or sterile media. The control contained only sterile media, but was amended with selenite in sample A for quantification of the potential abiotic production of MVSe. A blank, in which no selenite or inoculum was added to either sample, was necessary to

correct all analyses for the small amount of selenite added to all base media. All four experiment types were grown concurrently in duplicate, and the whole experiment set was repeated twice for a total of four replicates of each experiment.

Selenite was added to a final concentration of 2 mM, and cultures were inoculated to an initial cell density of approximately $5x10^5$ cells/mL. At the end of 14 days standard incubation, an aliquot of each culture was counted in a Petroff-Hausser counting chamber using phase contrast microscopy. The remainder was centrifuged to separate the media from cell pellets. Two to four milliliters of media were transferred to tared Teflon digestion vials and weighed, and the remainder was filter-sterilized using 0.45 µm syringe-driven filters (Millipore), weighed, and stored in Nalgene bottles. Cell pellets were also saved for digestion.

Table 1 Four experimental designs compared for determination of biotic and abiotic processes associated with selenium transformations in cultures with continuous aeration.

(b)	MVSe-cells		MVSe-media		Control		Blank	
	Cult. A	Cult. B	Cult. A	Cult. B	Cult. A	Cult. B	Cult. A	Cult. B
Spiked with Se	✓	O	✓	O	O	O	O	O
Inoculated	✓	✓	✓	O	✓	O	O	O

2.3.3 Sample Preparation and Instrumental Analysis

For total selenium analysis, liquid samples (media and acid traps) were first evaporated to near-dryness in Teflon digestion vials, sealed and heated in 5 mL of 8N nitric acid at approximately 80°C for 24 hours, and then evaporated again to near-dryness. Cell pellets were agitated by sonification for ten minutes in 2 mL of concentrated nitric acid and then allowed to dissolve for at least 24 hours in order to digest cells and associated selenium. Pellets that did not fully dissolve were warmed in a water bath until full dissolution was observed. The resulting solutions were rinsed into digestion vials and digested following the procedure for liquid samples. Teflon traps were pre-digested by transferring both accumulated liquid and Teflon wool into digestion vials, adding 5 mL of concentrated HNO_3 and heating for at least 24 hours to desorb material from Teflon shavings. Afterwards, the liquid was transferred into fresh vials and digested as above for liquid samples. All digested samples were brought up to 10-mL final volumes in the mixed acid solution, capped and heated for at least 24 hours. Dilutions were made using the same acid. Total selenium content in each digest was analyzed using an Agilent model 7500CX ICP-MS with octopole reaction system and a Cetac ASX-500 automatic sampler. Carrier gas was scientific-grade argon, and reaction cell gas was scientific-grade helium.

Cultures for microscopic analysis were grown according to the vial incubation procedure described above. Four to five vial cultures were combined at each time point, media was removed, and cells were washed twice by centrifugation with NANOpure water. TEM samples were harvested at 2, 5, and 15 days of incubation and fixed in a solution of 2% glutaraldehyde and 2% paraformaldehyde

solution in 0.1 M cacodylate buffer. They were prepared for analysis by high-pressure freezing, followed by freeze substituting with 2% osmium tetroxide in 99% acetone and 1% water. Samples were embedded in Embed-812 epoxy resin and microtomed into ultrathin sections. Sixty to 70 nm sections were mounted on Formvar/carbon-coated copper grids, stained with methanolic uranyl acetate and lead citrate, and imaged on a Zeiss Libra 120 TEM at 120 kV with a Gatan Ultrascan 1000 camera. Elemental analysis and mapping using electron energy-loss spectroscopy (EELS) and energy-filtered TEM (EF-TEM) were performed on unstained, carbon-coated 40- to 50-nm sections mounted on uncoated copper grids.

Scanning electron microscopy (SEM) samples were fixed in 2% glutaraldehyde in NANOpure water. They were washed and fixed again with 1% osmium tetroxide in water, dehydrated using serial dilutions in ethanol and critical-point dried in an Autosamdri-815B. Dried samples were attached to aluminum stubs using carbon tabs and then gold-palladium coated in a Denton BenchTop Turbo III. Mounted samples were imaged by a Hitachi 4700 Field-Emission SEM at 12 keV with energy dispersive X-ray (EDX) elemental analysis using an Oxford INCAx-act.

2.4 Results

2.4.1 Accumulation of Elemental Selenium

We used total selenium analysis and direct cell counts to examine the accumulation of cellular selenium over time in selenite-amended cultures. The total selenium content of live cultures increased exponentially during the first eight days of cell growth to around 17% of the total amendment. Accumulation continued more slowly until it approached a maximum recovery of 61 ± 2% after 17 days of growth

(Figure 2a). Exponential accumulation corresponded to the exponential (zero to five days) and early stationary phases of cell growth (Figure 2b). Selenium concentrations averaged over cell concentration (pg/cell) did not show a trend over time; however, number of cells with particulate selenium accumulation (as determined by direct counts in phase contrast) were linearly correlated with total cell count ($R^2 = 0.97$), indicating a constant percentage of cells with particulate selenium accumulation for the duration of the culture growth. The average over the experiment was 18 ± 3% of the total cell counts. The doubling time of selenium accumulation, at 21 hours, was almost twice as long as that of cell growth (12 hours).

Two CTAB experiments were performed in which cultures were killed with CTAB after four days of growth in the absence of selenite amendment. Killed cultures were killed by direct CTAB amendment, and any biomolecules or waste excreted into the media during the first four days of growth remained in these cultures. Potential selenium accumulation by these cultures was compared with that by washed cells suspended in fresh CTAB-amended media (washed-killed cultures). CTAB-amended cultures were compared with live cultures that were similarly grown for four days prior to selenite amendment. After ten days in selenite-amended media, only 0.05% of the amendment was recovered in CTAB-killed cells, compared with around 20% in live cells. Accumulations by washed-killed cells and killed cells were both higher than the control ($p < 0.01$ and $p < 0.05$). There was no correlation between cell-associated selenium recovery and time elapsed for washed-killed cells or killed cells. No selenium was detected in non-amended control cells.

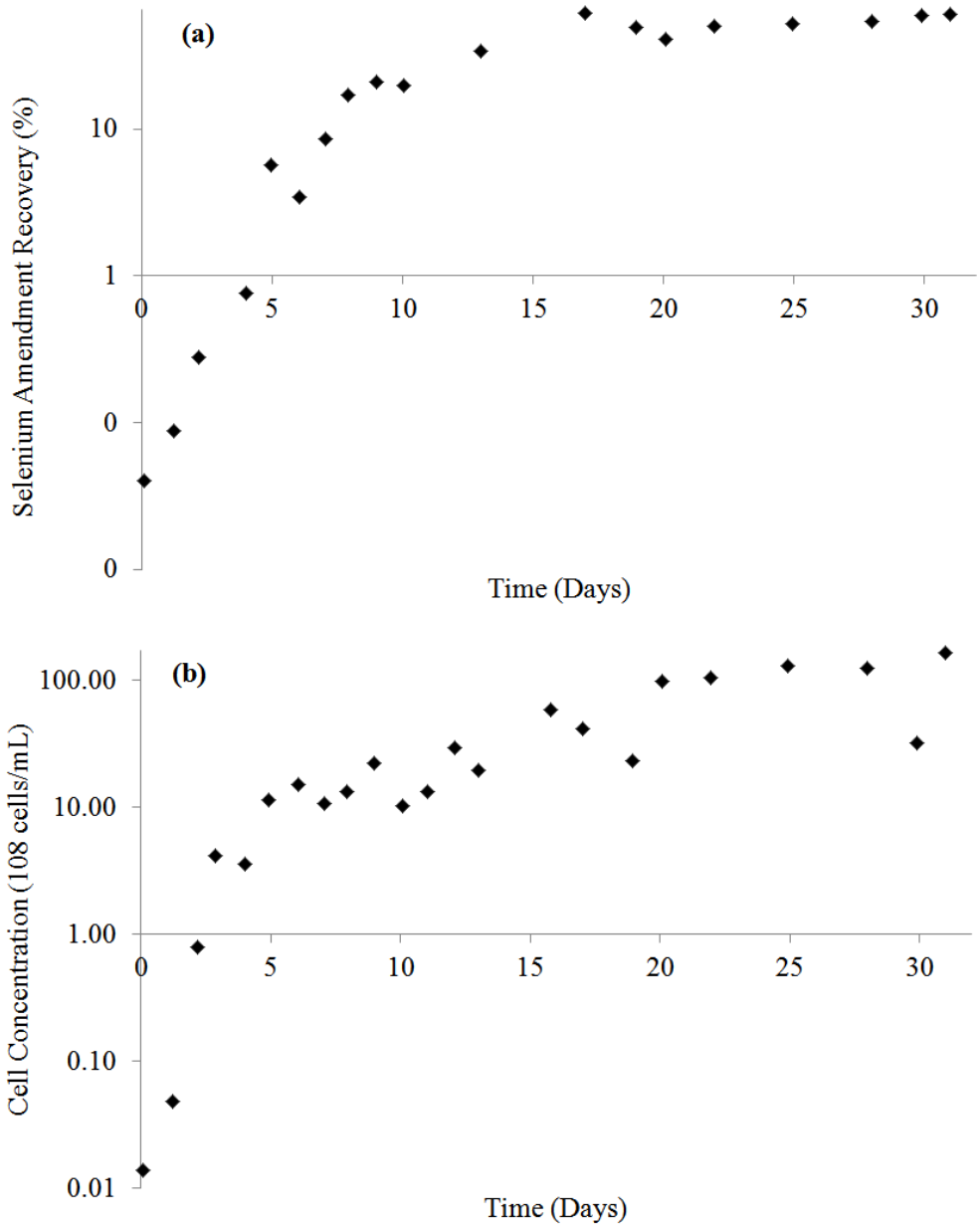

Figure 2 **Comparison of (a) cell-associated selenium recovery over time and (b) cell growth with time in live cultures amended with selenite. Exponential selenium accumulation occurred during log and early stationary phases. All data points represent the average of at least two to three replicates.**

We examined the location and elemental composition of selenium particles associated with strain 13B cells. Scanning electron micrographs in analysis mode (15 kV) suggested the presence of external and internal selenium particles in experimental samples (Figure 3a). Energy dispersive X-ray analysis confirmed the presence of selenium in both external and internal particles, with very little to no selenium detected on the yeast surfaces (Figure 3b). Other elements were detected in selenium particles, including oxygen and zinc; these were also detected on yeast surfaces and in control samples and may be attributed to residual media or extracellular organic material. No other elements commonly associated or precipitated with selenium were found on the surfaces of the particles, and no selenium was detected in control samples.

Elemental selenium particles became visible by both phase contrast and TEM imaging in a few cells after five days of growth, and were relatively common after 15 days of growth (Figure 4a, b). Transmission electron micrographs confirmed the location of electron-dense particles both outside and within cells, including trapped within cell walls or contained within membranous organelles, and in the cytoplasm. Elemental maps were obtained for selenium using EF-TEM by filtering out most of the signal and detecting only electrons of the energy corresponding to the selenium M_5-edge at approximately 53 eV. Signal intensities were highest on the electron-dense particles thought to be elemental selenium (Figure 4c,d). The selenium L_2 and L_3 edges were clearly visible by EELS at 1439 and 1482 keV (Figure 5), and the spectra obtained were consistent with the reference spectrum for elemental selenium (Madwid et al., 2008). The spectra were also consistent with a reference spectrum for selenomethionine, but since selenomethionine and other organic selenides are soluble,

this was discounted as a possible composition of particles. Again, no selenium was detected in the control samples.

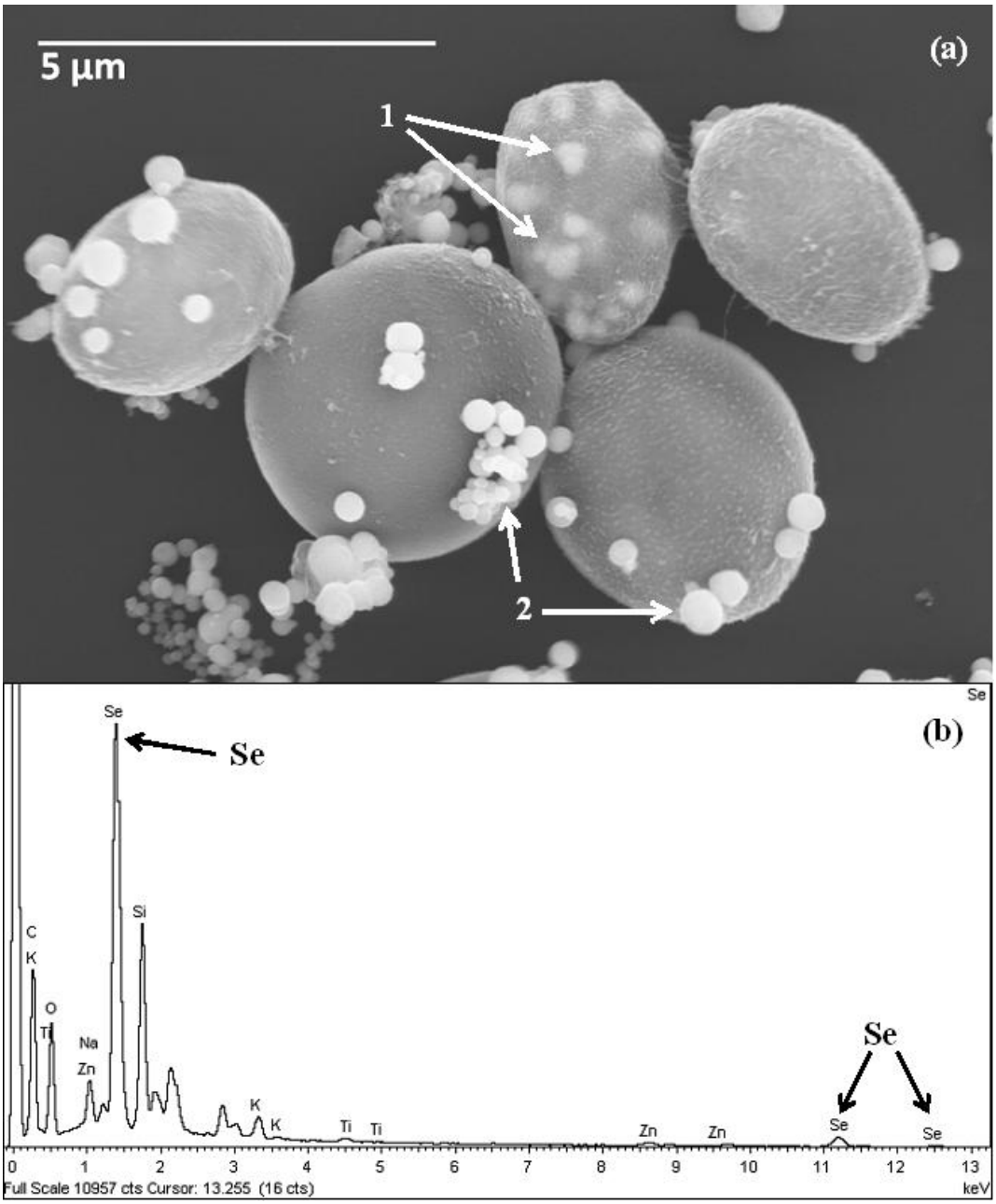

Figure 3 **(a) Scanning electron micrograph of cells with high-density particles. At 15 kV, the large interaction volume of the electron beam with the sample allows internal (1) particles as well as external ones (2) to be visible. (b) EDX spectrum of an external Se particle with selenium procucing the dominant peak at close to 1.5 keV and two smaller peaks at approximately 11.2 and 12.5 keV.**

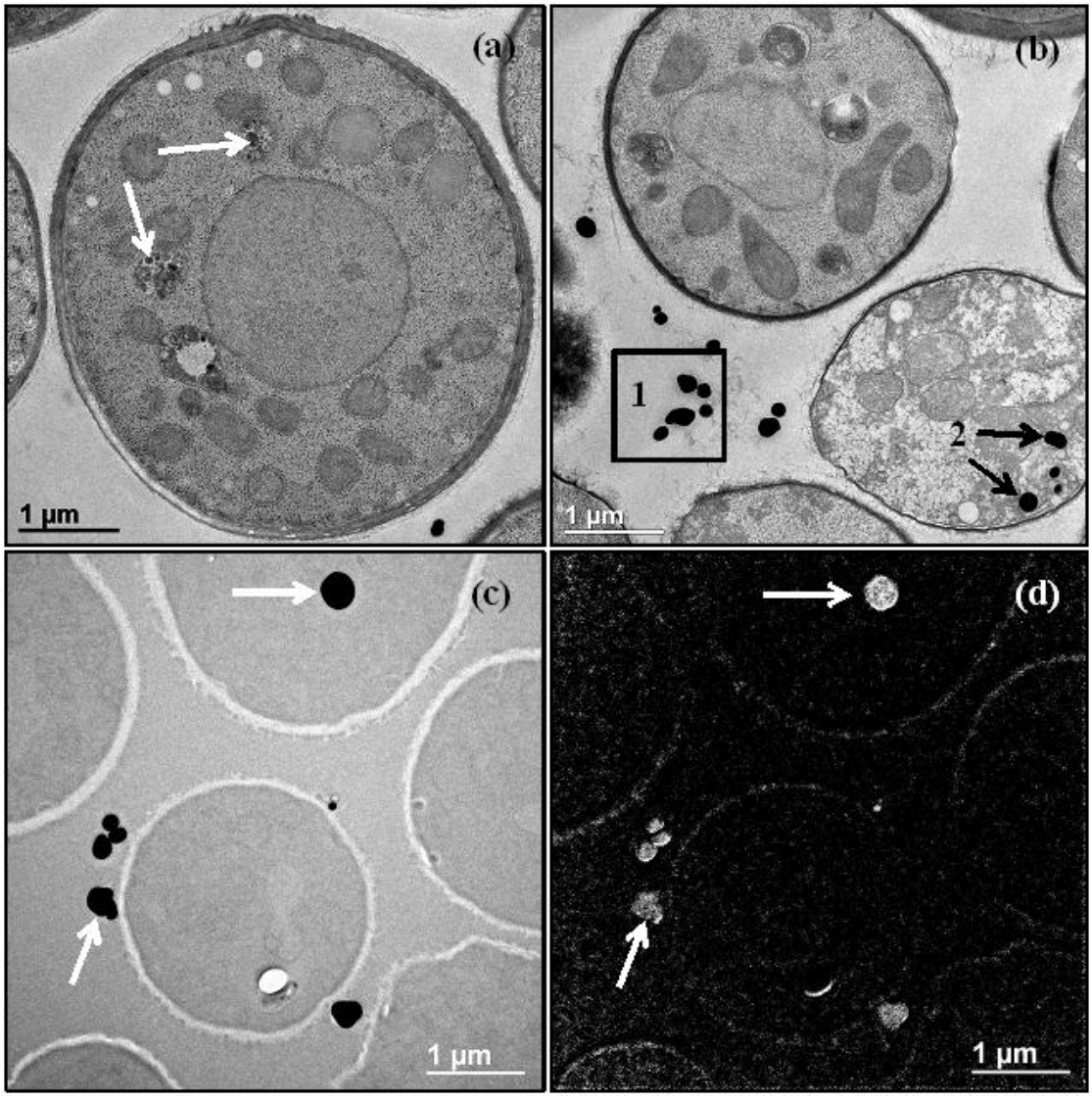

Figure 4 **Transmission electron micrographs of cells with electron-dense particles. (a) Stained image of cells at five days of growth containing some dense areas within their vacuoles. Similar spots also appeared in control cells and did not contain selenium detectable by SPOT-EELS or elemental mapping. (b) Stained image of cells at 15 days of growth containing 1-external and 2-internal particles. Particles at 15 days were shown to be selenium by SPOT-EELS at the selenium M-edge. (c) An image of unstained cells after 15 days of growth. (d) EF-TEM map of the field depicted in (c), showing that selenium was concentrated in the particles.**

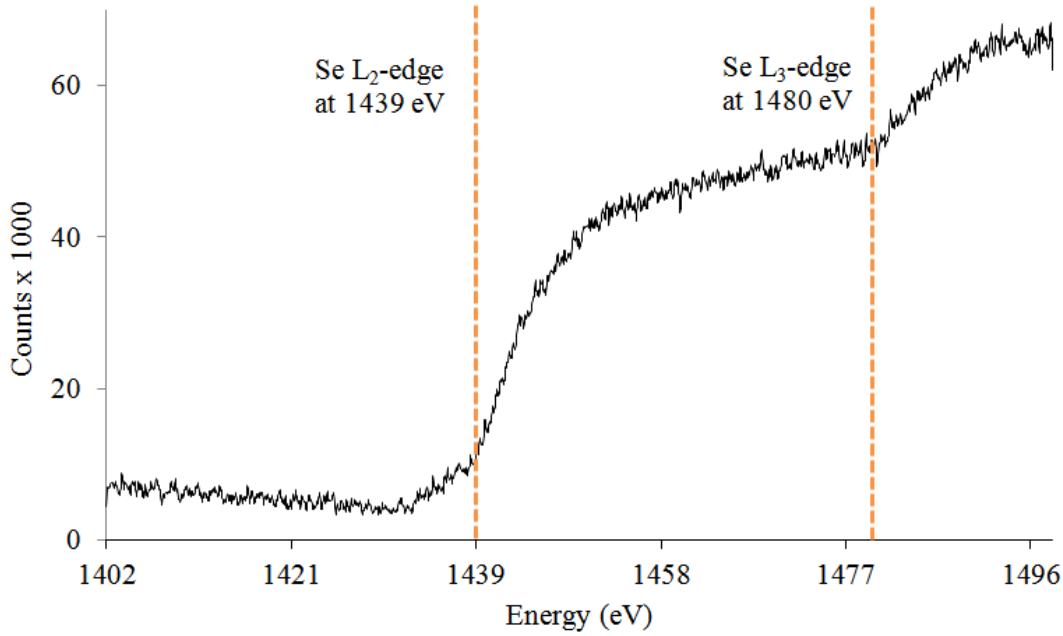

Figure 5 **Electron energy loss spectrum (EELS) at the selenium-L_2 and L_3 edges of an external particle in a 15-day sample. This spectrum closely resembles the reference spectrum of elemental selenium.**

2.4.2 Selenium Distribution among Three Phases

Methylated volatile selenides produced by strain 13B cultures were carried by continuous air flow into fresh cultures or sterile media. The cell pellets from the second samples of all experiments were compared for selenium accumulation. Since no solid material was present in sterile media samples, digestions were performed on residual liquid after removing the bulk of the media. Cell pellets contained statistically significant concentrations of selenium (compared with blank concentrations, $p < 0.001$), while the "solid" concentrations in the sterile media and blank were indistinguishable from each other ($p > 0.10$). Meanwhile, amendment recoveries from the aqueous phase of these samples were higher in the culture (MVSe-cells) than in the sterile sample (MVSe-media) ($p < 0.10$), but both were much higher

than blank concentrations ($p < 0.05$ and $p < 0.001$, respectively), indicating that both abiotic and biotic processes influenced selenium concentrations in the aqueous phase.

These minor interactions did not affect the overall distribution of selenium. Overall distribution of selenium did not differ between the MVSe-cells and MVSe-media experiments in any of the three fractions: aqueous, cell-associated, or volatile ($p > 0.10$). The total distribution of selenium amounted to $23 \pm 3\%$ in the cells and $1.0 \pm 0.1\%$ in the volatile phases of these two experiments averaged together. No selenium at all was recovered from the solid or volatile phases of the control, indicating that the production of MVSe and precipitation of selenium could only occur in the cultures. Together, these results suggested biotic and abiotic influences on the solubility or reactivity of MVSe that did not significantly change the average Se distribution by cultures of strain 13B.

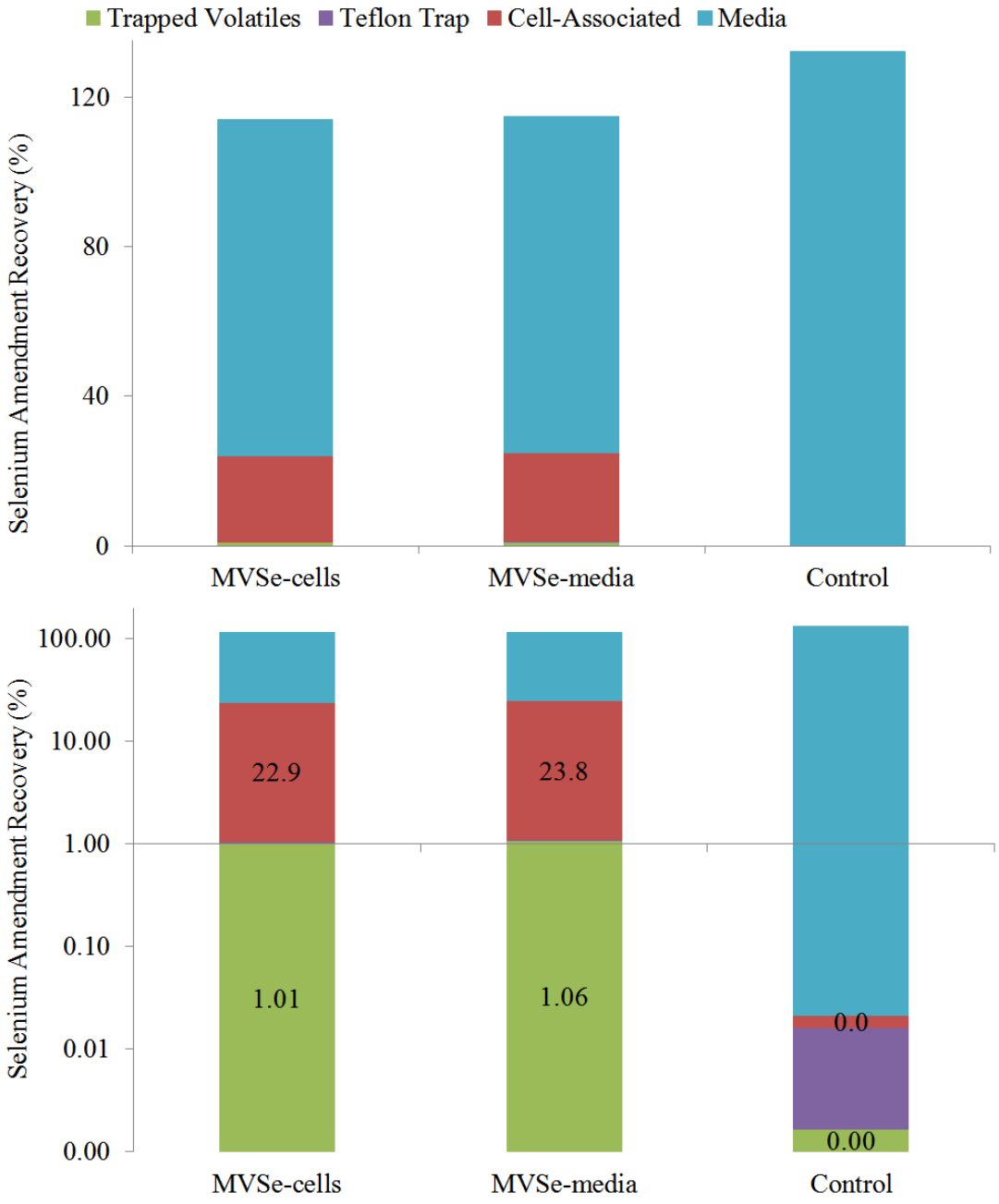

Figure 6 **Distribution of selenium amendments in three aerated experiments in (a) linear scaling and (b) in logarithmic scaling for comparison of smaller fractions.**

2.5 Discussion and Conclusions

Our results described both the transformations of selenite by strain 13B and some interactions of 13B with resulting selenium products. Selenium accumulated rapidly in the first eight days of cell growth, and both accumulation and elemental selenium precipitation only occurred in live cultures. Precipitation began after five days of growth and increased substantially after one week of growth. Our cellular accumulation results are consistent with previous studies that indicated selenium uptake by some yeast occurs most rapidly during early exponential cell growth (Falcone and Nickerson, 1963; Gharieb and Gadd, 2004). Most previously studied yeast species precipitate elemental selenium primarily during resting and decline stages. For example, a *Penicillium* sp did not display the red coloration indicative of this precipitation until they entered decline phase (Brady et al., 1996), while selenite was rapidly reduced in resting cell suspensions of *C. albicans* (Falcone and Nickerson, 1963). In contrast, strain 13B began to accumulate elemental selenium particles during late exponential growth and continued to do so well into stationary phase. This implies that strain 13B has adapted to influence aqueous selenium concentrations in its environment for a relatively long period of time and with more control than would be possible after cell death.

Based on the overall distribution of selenium in strain 13B cultures, we conclude that the reduction of selenite to elemental selenium was the primary contribution of this strain to the removal of selenite from the media. This is consistent with earlier findings in which a *Penicillium* species volatilized 8% of amended selenite and accumulated 36% of it within cells after 14 days of growth (Brady et al., 1996). It is also consistent with results of earlier studies in cultures of strain 13B with tellurite amendment, although this strain appears to precipitate twice as much

selenium as it does tellurium after two weeks of growth with continuous aeration (Ollivier et al., 2011). On the other hand, tellurium volatilization by these cultures was two to three times higher than selenium volatilization. This suggests the importance of volatilization for detoxification, since tellurium is more toxic than selenium. It is worth noting that the average selenium amendment recovery in the cellular fraction of vial cultures was 46% higher than that of aerated cultures. It was found previously that oxygen stress produced similar results in tellurite-amended cultures of strain 13B. Tellurium precipitates reached their maximum accumulation of approximately 12% of the total amendment after only four days of growth with aeration, while in sealed vials, precipitation continued at a steady rate until it accounted for 98% of the original amendment after five weeks of growth (Ollivier et al., 2011). The vial cultures in this study fell in between the two conditions studied by Ollivier et al. (2011), and it is possible that under greater oxygen tension, selenium precipitation could have approached significantly greater levels.

Our TEM images provided clues to the process and effects of particle formation in and around the cells. Most of the cells containing internal selenium particles appeared to be less healthy than cells without, as judged visually by decreased homogeneity and density in the cytoplasm, with internal structures that were less well-defined than in particle-free cells (Figure 7). In some cells, the particles appeared to be contained within membranes, such as in vacuoles or even mitochondria. The sequestration of toxic metals in yeast vacuoles has been described before as a mechanism of detoxification (Culotta et al., 2005; Gharieb and Gadd, 1998). A correlation between unhealthy cells and the occurrence of internal particles could suggest several possible relationships that cannot be elucidated using the data

collected in this study. Decline in health could result from toxic levels of elemental selenium precluding the precipitation of particles; or the decline could occur only after precipitation began, as a direct result of the stress imposed by excessive accumulation of foreign bodies. Alternatively, the correlation could be explained by the age of the cells, rather than any cause-effect relationships. Live confocal imaging of cells in their later stages of selenium accumulation might discern the relationship if an in-situ method of determining the health of individual cells could be employed under the microscope.

Our most surprising discovery was the accumulation of selenium particles within the walls of some cells. Because these particles are hundreds of times larger in diameter than the pores of a yeast cell wall (Scherrer et al., 1974), it is impossible for the aggregates to have formed in the cytoplasm before diffusing into the cell wall. It is possible that instead the elemental selenium diffused into the cell walls before precipitating, and then nanoclusters acted as nuclei upon which the larger particles grew. Whether or not this nucleation and precipitation occurred independently of biological influence cannot be determined from this data, but could help to elucidate the potential causal relationship between toxicity and selenium precipitation in the cells. Amorphous elemental selenium precipitation was previously observed to occur on the surface of cells of *Shewanella* sp HN-41, and it was proposed in this case that while the reduction of selenite to elemental selenium was biological in nature, the precipitation of particles was an abiotic process (Tam et al., 2010). Particles located outside of the strain 13B cells may have been formed similarly, but could also have originated in cells that had lysed during sample preparation, particularly since the cells were washed with water (hypotonic to the cell interior), rather than with media. We

also considered the possibility that the apparent location of particles in organelles and within the cell wall was an artifact of preparation. This is probably not the case in the walls since they bulged where particle growth stretched the cell wall material (arrow 2 of Figure 7). Other particles are more clearly superimposed on the cross-section (arrow 3), and were thus considered artifacts. The difference is less clear in the case of particles in vacuoles and mitochondria, but the presence of particles within any cell fraction could be confirmed or refuted by isolating intact fractions for further imaging and selenium content analysis (Bacon et al., 1969; Diekert et al., 2001; Horst et al., 1999).

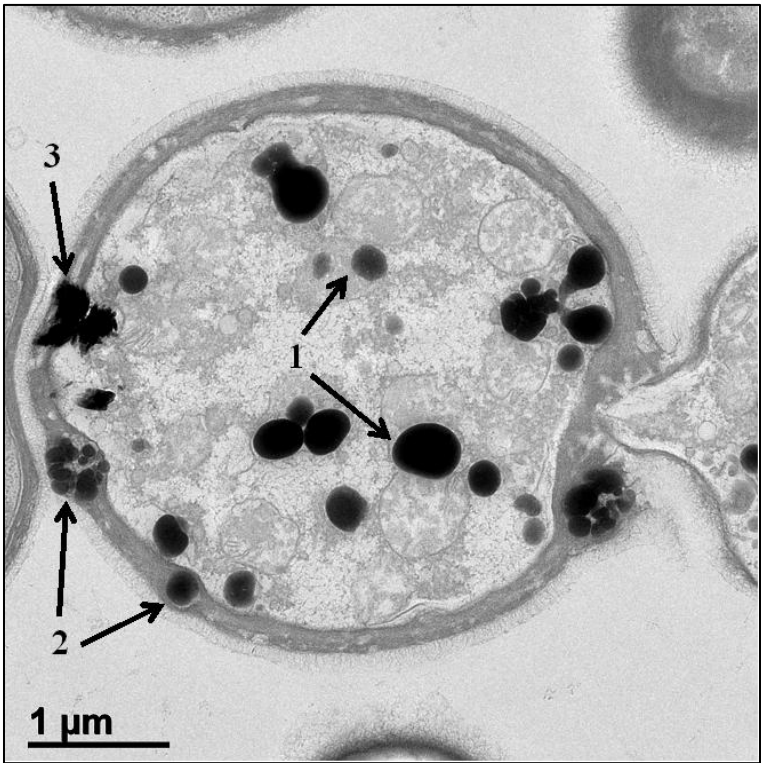

Figure 7 **Transmission electron micrograph (stained section) of an unhealthy yeast cell containing selenium particles in 1-membranous organelles and 2-cell wall. The bulging of the cell wall around some particles indicates that these particles were formed inside the wall.**

Several potential interactions between MVSe and either cells or media could provide information about the complex nature of biogeochemical Se cycling and the multi-faceted role of microbes. No volatile selenium was found in sterile cultures; thus, selenium volatilization was biologically mediated. The recovery of small but significant selenium concentrations in cell pellets of the second cultures of MVSe-cells experiments indicate minor interactions between the MVSe and cells that were not large enough to be detected as a percentage of the total amendment. Since these organisms require selenium to survive, a very small amount of selenite (not detected

by ICP-MS in blank media) was added to all media, and this would be concentrated in the cells by assimilation. However, the amount of selenium present in the media accounts for only 6% of the average amount of selenium detected in cells. This is indicative of another interaction which could not be described using these experiments. Comparisons of the aqueous recoveries in the second samples of each experiment indicated the influence of both abiotic and biotic processes on the solubility or reactivity of MVSe under the studied conditions. Methylated volatile tellurides were already reported to be redistributed into both aqueous and solid (precipitated) fractions in cultures of this strain (Ollivier et al., 2011). Future exploration of the speciation of selenium and tellurium in the aqueous and solid fractions of cultures would provide further insights into the interactions among cells, media and the volatile compounds, and potentially into the mechanisms by which strain 13B transforms various selenium and tellurium species. Although we have not described these in detail, it is clear from the above results that multiple types and pathways of interaction are possible among the cells and selenium species present.

Because strain 13B and a few close relatives were isolated from sediment samples (Ollivier et al., 2008), their role in the selenium cycle must be partly governed by the effects of soil conditions on the mobility and reactivity of their reduction products. For example, the efficacy of selenium volatilization for selenite removal depends on how effectively volatile compounds are released into the atmosphere. Of the two major compounds produced by strain 13B and the other strains isolated with it (Ollivier et al., 2008), DMSe and DMDSe, DMSe is the more stable in soils since up to 93% of DMDSe may be adsorbed to soil particles, dissolved, or degraded by microbial activity to non-volatile organic selenium compounds or to DMSe (Zhang

and Frankenberger, 2002). The primary controls on the release of DMSe from soils are moisture (Zhang et al., 1999), wherein their release may be hindered by dissolution, and microbial activity, which may degrade the compounds into non-volatile forms (Dungan et al., 2002). Factors such as bioturbation (mixing of sediments by macro-organisms), turbulence at the soil-water interface, and periodic exposure of sediments to the atmosphere (as in tidal marshes) may alleviate these hindrances, since periodic tillage was previously observed to stimulate DMSe volatilization in field plots (Flury et al., 1997). Since aeration can enhance the release of MVSe from soils, by extension, we considered the possibility that continuous aeration of our cultures may have artificially enhanced the release of MVSe from our media. The average residence times in the second samples of our experiments were 2.4 ± 0.1 minutes in the media and 4.8 ± 0.2 minutes in the headspace. Although this may not be enough time for the volatiles to have completely reacted with media components or reach their equilibrium vapor pressure, soil residence times of around six to eight minutes allowed for the retention of upwards of 80% of DMDSe and DMSe in soil column studies (Zhang and Frankenberger, 2002; Zhang et al., 1999). This implies that the residence times in our experiments should have allowed enough culture interaction to support a statistically significant difference between the distribution of selenium in MVSe-cells and MVSe-media experiments, albeit much less than 80% retention. Since this difference was not found, we maintain the conclusion that despite minor interactions between MVSe and strain 13B, cultures of this strain do not degrade the volatiles substantially. Additionally, periodic disturbances of the soil in tidal marshes should enhance the release of volatile

compounds, so that volatilization may be a relatively important contribution of strains like 13B in its environment.

The major product of selenite reduction by strain 13B, elemental selenium, may also be subject to degradation, and its fate in the environment is primarily controlled by reduction-oxidation conditions. The electrode potential for the reduction of selenite to crystalline elemental selenium at pH = 7 (E_h) (Bard et al., 1985; Filep, 1999) is 0.261 V. This is very low compared with the reduction potentials of oxygen (E_h = 0.800 V) and nitrate (E_h = 0.350 V), but it is high compared with iron (III) and manganese (IV) reductions (E_h = 0.120 and -0.196 V, respectively) (White and Dubrovsky, 1994). Thus, in soils high in iron (II) and manganese (II), Se^0 is stable, while high nitrate and oxygen concentrations promote oxidation back to selenite or selenate (Masscheleyn et al., 1990). Substantial evidence suggests that some microbes are also capable of oxidizing elemental selenium to selenite or selenate (Dowdle and Oremland, 1998; Losi and Frakenberger, 1998; Zhang et al., 2004). Since amorphous nanoparticles and colloids are generally more reactive than large crystalline structures, the elemental selenium particles accumulated by microbial reduction may be especially reactive. However, since most microbial methylation appears to require selenium oxyanions as starting material, (Masscheleyn et al., 1990), microorganisms may yet remove the re-oxidized selenium from the aqueous phase. Since strain 13B is capable of producing both elemental selenium and MVSe, its contribution to local Se cycling is potentially multi-faceted and continuous.

Selenium reduction and volatilization by strain 13B and its closest relatives may additionally have implications for bioremediation. *Rhodotorula mucilaginosa*-13B was isolated from tidal salt-marsh sediments, where it must tolerate

seasonal to hourly fluctuations in temperature, pH, and salinity (Velinsky and Cutter, 1991). This condition makes it likely that strain 13B is highly adaptable, and indeed, strains of *R. mucilaginosa* have been isolated from a variety of environments and shown to tolerate pH levels between 2 and 10, as well as salinities between 3% and saturation (Lahav et al., 2002; Libkind et al., 2008). Strain 13B grew slowly even in the absence of excess selenite, but continued to grow for over four weeks, and it is evidently tolerant of a wide range of oxygen concentrations. With growth observed at selenium concentrations up to 2 mM, it is also more resistant than almost all yeast isolates studied for tolerance in the past (Fujs et al., 2005; Gharieb and Gadd, 1998; Golubev and Golubev, 2002). More than one bioremediation study has succeeded in close to 100% reduction and precipitation of elemental selenium, retrievable by chemical or physical means (Cantafio et al., 1996; Chung et al., 2010); while others have achieved substantial methylation and volatile dispersal (Flury et al., 1997). However, the exceptional tolerance of strain 13B may allow it to provide an advantage as a first-response organism at especially contaminated sites; while its capacity for long-term growth and selenium reduction may allow it to enhance the long-term results of bioremediation efforts. Additionally, while some organisms may be more effective in aqueous environments, this organism could be used to reduce sediment contamination. Thus, bioaugmentation with strain 13B may serve as an advantageous compliment to other strains at sites with especially high selenium contamination.

Chapter 3

ADDITIONAL AND FUTURE WORK

3.1 Additional VIA Experiments

Single-culture VIA experiments were prepared to investigate the distribution of selenium among all three phases in two types of CTAB-inhibited cultures(see Appendix A for methods).The distribution of selenium in live cultures appeared to diverge from that in inhibited cultures (Figure 8), but results were not all conclusive. Cellular selenium recovery was significantly higher in live cultures than in either CTAB-inhibited treatment ($p < 0.05$), and did not differ between killed and washed-killed cells ($p > 0.10$). Washed-killed cell concentrations were statistically different from blank concentrations ($p < 0.10$), while killed cell concentrations were not. This contrasts intuitively with the result that these two concentrations were not different from each other. The only conclusion to be taken from these results is that neither value is substantial, and the inconsistencies are probably a result of high variation in results and analytical matrix interferences. Despite this, the results clearly support the conclusion that substantial volatilization requires live cultures.

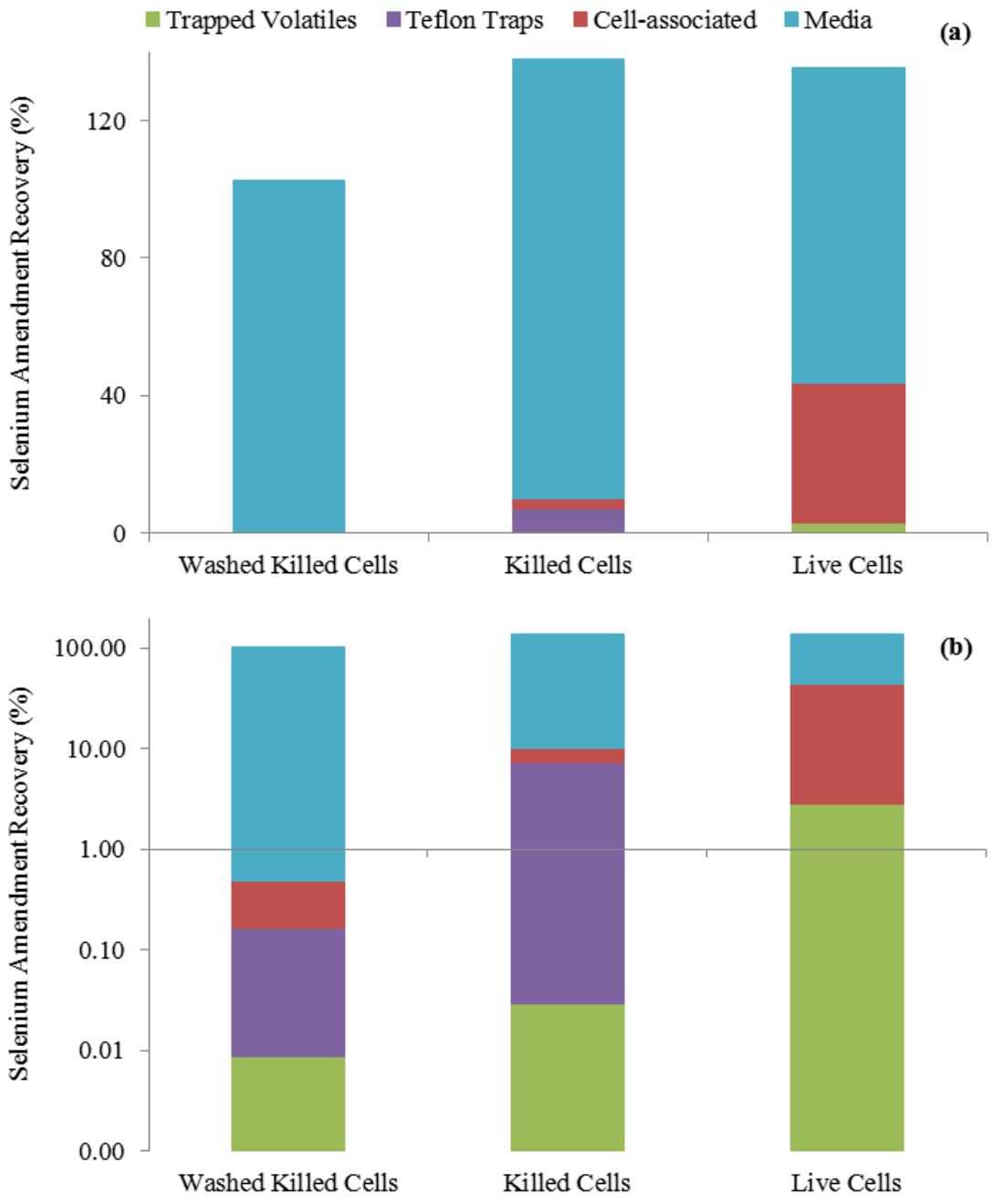

Figure 8 **Selenium amendment distributions in live and killed-cell aerated cultures, shown with (a) linear scaling, and (b) logarithmic scaling for comparison of smaller fractions.**

The results for individual replicates of the single-culture VIA experiments were highly variable. In the case of killed-cells and live cultures, selenium and CTAB amendments were added directly without removing samples from the apparatus. The difficulty of mixing cultures while they were in the apparatus may have resulted in uneven distribution of amendments and thus incomplete or inconsistent exposure of cultures to CTAB and selenite. There may also have been additional matrix interferences during ICP-MS analysis in samples treated with CTAB, which gave the media a thick, soapy consistency and was difficult to completely wash from cell pellets. Although some results appeared unreasonable, the variability of results prevented the identification of outliers and conveyed a need for substantially more than four replicates of each Scheme. Care must be taken with future experiments to ensure cultures are well-mixed, cell pellets are washed thoroughly, and a substantial number of replicate experiments are performed. In repeating this experiment, I would also replace all tubes and apparatus materials, in order to ensure ease of complete mixing while maintaining clean and sterile conditions.

3.2 Method Development and Complications

I attempted to determine the speciation of selenium in VIA sample media via HPLC-ICPMS, but found that this was not practical without further development of analytical methods(see Appendix A for methods). Small amounts of selenocysteine were eluted from combined standard solutions at 3.5 minutes, almost simultaneously with selenite at 3.4 minutes (Figure 9). Selenomethionine and selenourea were also eluted as one peak with retention time 4.7 to 4.8 minutes. Thus, the method used was unable to resolve all species, and did not detect any other selenium peaks. In the media of some B-cultures, very small peaks eluted at the selenite/selenocystine

retention time. These were inconsistent in size and appeared randomly in all three selenite-amended schemes, and were originally attributed to carry-over from the selenite-amended cultures. However, ICP-MS results later showed that Schemes 1 and 2 B-culture media contained a detectable amount of selenium, whereas Scheme 3 did not. Therefore, I conclude that the sensitivity of our instrumentation was not sufficient to distinguish the selenium content of Scheme 1-B media from that of Scheme 2-B media or 3-B media, and no further attempts were made to refine analytical methods.

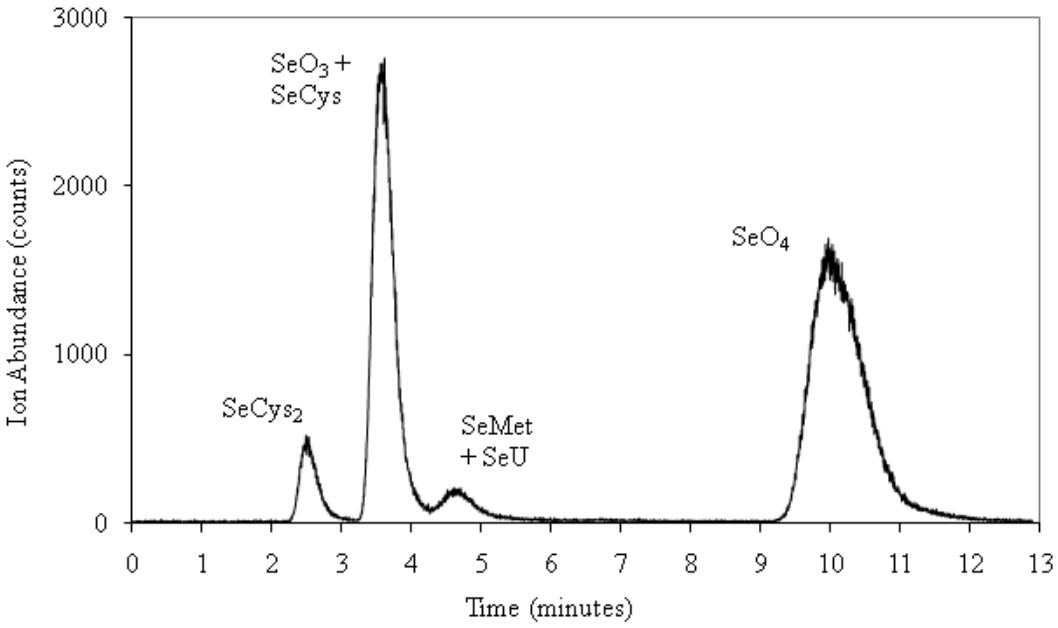

Figure 9 **HPLC-ICP-MS chromatogram of combined organic selenium standards with peak overlap. Compounds eluted were selenocystine ($SeCys_2$), selenocysteine (SeCys), selenite (SeO_3), selenomethionine (SeMet), selenourea (SeU), and selenate (SeO_4).**

Toward the end of my research, I reconsidered the calculations used in total selenium content analysis. Early in my experimentation, I prepared additional standards from the selenite stock amendment in optimized LB-Marine media and digested them according to the sample digestion procedure. I compared calibration curves of these and certified standards to check for matrix interferences. I noted that calibrations using digested selenite standards and the calculations using Perkin Elmer certified standards commonly differed by 10-30% or more. Calculations were thus initially made using both calibration curves. A linear calibration (A) was first obtained for the digested selenite standards, assuming that instrumental response to these standards were representative of instrumental response to the sample matrix. The regression equation "A" was used to obtain apparent concentrations from the raw absorbance values of the Perkin Elmer certified standard set. These apparent concentrations (x-axis) were then plotted with certified concentrations (y-axis) to obtain a second linear calibration (B). Both calibration curves were always well-correlated ($R^2 > 0.995$). Sample calculations were made similarly, first obtaining apparent concentrations from raw absorbance values using "A", and then calculating final concentrations from apparent concentrations using "B". Using this method of calculation, the values obtained for very low-concentration samples (such as sterile controls and Teflon-wool traps) were often negative, while the very concentrated samples (such as the media) appeared to be overestimated, and total amendment recoveries were frequently greater than 115%.

In an attempt to obtain values that gave closer to 100% total recovery, I prepared several sets of digest standards and one set of undigested selenite standards (prepared directly in acid rather than media), as well as the certified standard set.

Each digest standard set was prepared slightly differently, with dilutions performed at different points in the preparation and digestion process (see Appendix A for details of preparations). Along with these, I re-diluted and reanalyzed a selection of samples. Total amendment recoveries for these samples were reassessed using several combinations of standard curves. Recoveries varied by a factor of three depending on the digest-standard set used and whether "apparent" concentrations were corrected using the certified or selenite standard set, or were not corrected at all. If "apparent" concentrations were calculated using the certified standard set and final concentrations calculated using the selenite standards, the total amendment recovery was approximately 100% and all values were positive, even when concentrations were near the detection limit.

I repeated the analysis several weeks later on the same certified standards, undigested selenite standards, and samples. This time, the same calculations produced results with the same aberrant qualities as the original calculation method. Additionally, amendment recoveries differed between the two analyses by approximately 30-50% for cell-associated and volatile fractions. Ultimately, the only way to achieve consistent results for these two fractions was to calculate concentrations using only the PE certified standard calibration. As before, low concentrations were ND and total recoveries varied and exceeded 100%. Since the cell-associated and volatile recoveries were consistent and are the most relevant to understanding the role of 13B in the selenium cycle, I finally chose to recalculate all results using the PE calibration curve alone. Other than the Teflon traps, which were expected to give variable results, the major contributor to variation in and overestimation of total amendment recovery appeared to be the aqueous fractions of

cultures. I suggest that the unusual results were due to matrix interferences, which might be attributable to the high salt content and undefined organic composition of the media.

A previous study in which selenium was quantified in the liquid, solid, and volatile phases reported a total selenium amendment recovery of 95.6% from cultures of a *Penicillium* species (Brady et al., 1996). Inevitable losses during sample transfers and digestion make this value the maximum that could be reasonably expected. Hydride generation atomic absorption spectroscopy (HGAAS) was used to analyze total selenium, rather than ICP-MS. Additionally, these researchers found the capture of volatiles to be incomplete in room-temperature acid traps. They used activated charcoal traps instead and harvested volatiles using methanol extraction and a boiling acid mixture. Their total digestion procedure also used both concentrated hydrochloric and nitric acids, rather than 50% nitric acid alone. This suggests that my digestion and analytical procedures may not have been rigorous enough to obtain reproducible results.

3.3 Proposed Future Studies

A comprehensive comparison of the latest methods of selenium determination would be helpful for future environmental studies. HGAAS has long been a popular method of both selenium speciation and quantification (Haygarth et al., 1993), and continues to be coupled with atomic fluorescence spectroscopy (Zhao et al., 2010), HPLC-ICPMS (Darrouzes et al., 2008), and other instrumentation. Inductively coupled plasma-mass spectrometers are efficient and versatile, as well as capable of analyzing many elements simultaneously, making them convenient and popular for elemental analysis today; however, they experience significant

interferences for several elements and in many matrices. Matrix interferences with selenium determination by ICPMS can be improved while maintaining good sensitivity with the use of a pressurized hydrogen collision cell (Chen et al., 2008). Digestion techniques such as microwave digestion and UV-photolysis were each reported to assist in the complete digestion of organic samples and reduction of matrix interferences (Manjusha et al., 2007; Zhao et al., 2010), but both studies used atomic absorption instruments, and the coupling of these digestion techniques to ICP-MS analysis have not yet been explored.

Selenium speciation by HPLC-ICP-MS is relatively common, but generally is generally performed using reversed-phase rather than strong ion exchange HPLC (Afton et al., 2009; Cankur et al., 2006). Separating all of the selenium species commonly associated with biological samples is difficult because of the range in polarity of biologically relevant species. Separation of all of the compounds selenite, selenate, selenoethionine, selenomethionine, selenourea, and DMSe and DMDSe was successful using reverse-phase liquid chromatography (Ochsenkuhn-Petropoulou et al., 2003). No studies have resolved DMSeO or $DMSeO_2$ by HPLC-ICP-MS, but (Zhang et al., 1999) separated oxidized DMSe species (DMSeO and $DMSeO_2$) collectively from Se(IV) on a strong basic anion-exchange column prior to separation by HGAAS. Improved sensitivity could have been obtained for the speciation of selenium compounds in B-sample media had I used HGAAS, or been able to replace helium with hydrogen in the collision cell of the ICP-MS. This data would have allowed for further speculation on the biotic and abiotic transformations that governed selenium distribution in the cultures.

Controlled laboratory studies of pure isolates are important for understanding the basic physiology of cellular interactions with selenium in the environment. The next step is to study the more complex interactions with actual environmental samples. Bioreactors are a versatile tool for both controlled and environmental studies. Access to a bioreactor would have given me better control over aeration rates and culture volumes over time. Additionally, the large volume capacity of a bioreactor would have eliminated the need to analyze whole cultures at each time point, thus reducing the variability of my quantitative results. Future studies using environmental samples could also be performed in solid-phase bioreactors, recently developed for the investigation of soil processes (Abdulsalam et al., 2011; Mohan et al., 2008). This would allow convenient laboratory studies of the distribution of selenium by strain 13B in conditions more closely resembling its native environment, with controlled variations in factors that fluctuate regularly in salt marshes, such as temperature, oxygen levels, salinity, and pH.

Appendix A

MATERIAL SPECIFICATIONS AND ADDITIONAL METHODS

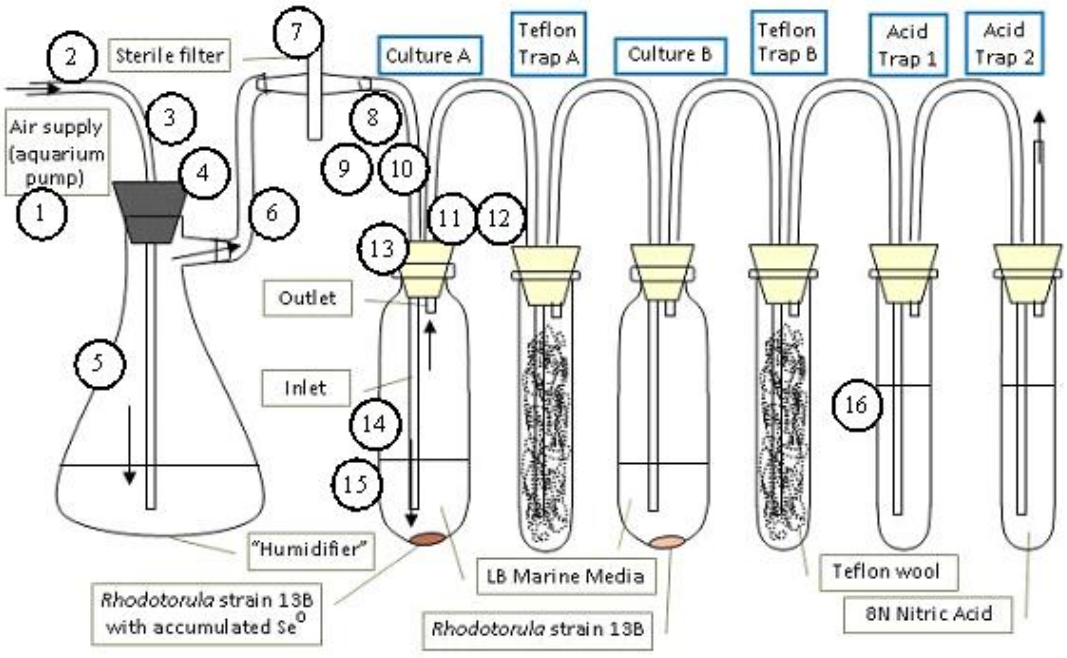

Figure A.1 Volatile-trapping incubation apparatus for growth of cultures with continuous aeration. Each material or component is labeled for reference to specifications listed below.

The Volatile Incubation Apparatus contained the following materials:

1. Aquarium pump: Air – Tech 2K4, Penn-Plax, Inc. (not shown)
2. 3/16" PVC tubing, Fisherbrand
3. 6 mm OD glass rod
4. #7 rubber stopper
5. 500 mL filtering flask, Pyrex®

6. 5/16” PVC tubing, Fisherbrand
7. Millipore Aervent 0.2 Micron Sterilizing Grade Filter
8. 3/16” PVC tubing, Fisherbrand
9. Lok-Tite 4 Way Brass Gang Valve, Penn-Plax, Inc. (not shown)
10. 1/8” ID 180 PVC (autoclavable) tubing, Nalgene, Fisher part no. 14-176-12
11. 1/16” ID, 1/8” OD, 1/32” wall 890 FEP tubing, Nalgene, Fisher part no. 14-176-178
12. 1/4” OD Autoclavable pinch clamps, Small Parts, Inc.
13. Size 24D silicone stoppers, Lab Pure, Saint-Gobain, Fisher Scientific
14. 50 mL Oak Ridge FEP rounded-bottom centrifuge tube, Nalgene, Fisher part no. 05-562-16B
15. WBS Water bath and heating element, Fried Electric (not shown)
16. Glass test tube, 20 x 150 mm, Pyrex® (for both Teflon and Acid Traps)

Ultra-pure water was produced in-house using a Barnstead NANOpure model D4741 (Thermo Scientific). PFA Teflon digestion vials were purchased from Savillex, CTAB (99+% solid) was purchased from Acros Organics, and selenite was purchased from Sigma-Aldrich. Samples for HPLC analysis and sterile storage were filtered through 0.45 μm Millex-HV PVDF low-protein binding filters from Millipore.

Digest standards prepared for exploring total selenium content analysis and matrix interferences were categorized as follows. Set “A” digestion began with 2 to 4 mL of a selenite standard dilution equal to around 500 times the desired final concentration. (The volume of aqueous samples evaporated at the start of digestion was also 2 to 4 mL.) Set “B” began with 10 mL at 100 times the desired final concentration. Set “C” began with 10 mL of selenite standard at the desired concentration. Set “D” was essentially a duplicate of C, beginning with 50 mL of the same concentration. At the end of the digestion, sets A, B, and C were all brought to final volumes of 10 mL, capped and heated for 24 hours, while set D was brought to final digest volume of 50 mL. Sets A and B were then diluted 100 times to achieve the desired final concentrations. All standards had the same final concentrations when

analyzed, but set A was digested at the highest concentration, followed by B, with sets C and D being digested at full dilution.

Killed-cell VIA experiments were prepared in single-culture trains using the same materials used in double-culture VIA experiments. Since no non-amended cultures were being analyzed, these experiments did not require the 2 mM concentration of double-VIA experiments. Cultures were instead amended with the same CTAB (1%) and selenite (0.68 mM) concentrations as vial experiments. Cultures were also treated similarly to vial experiments and three conditions (live, washed-killed, and killed cells) were studied. Twelve replicate sample trains were incubated in the water baths for 6 days free of inhibitor and selenite, then were split into four replicates of each condition and incubated for another 14 days.

Selenium speciation was attempted using a Hamilton PRPX-100 analytical column and an ammonium citrate buffer in 2% methanol. Details of the method were described elsewhere (Bueno et al., 2007). Samples were eluted through an Agilent 1200 Series HPLC coupled to the ICPMS used for total content analyses. Combined standards in diluted media contained selenite, selenate, selenomethionine, and selenocystine in ranges between 1 and 1000 ppb. A separate solution of selenocystine was prepared in TCEP in an attempt to cleave the dipeptide into its monopeptide component amino acid, selenocysteine. Aliquots to be added to the final standards were calculated based on the assumption that conversion was quantitative. Media samples were diluted 100x, and all samples and standards were filtered through the syringe-driven filters before eluting through the HPLC column.

Appendix B

RAW DATA

Table B.1 Cellular selenium accumulation in (a) live and (b) killed cells. All errors are calculated from 95% confidence intervals.

(a) Live Cell Selenium Accumulation with Growth				
Days of Cell Growth	Cell Concentration (10^7 cells/mL)	Error	Selenium Amendment Recovery (%)	Error
0	0.014	± 0.010	0.040	± 0.060
1	0.048	± 0.016	0.088	± 0.011
2	0.789	± 0.517	0.278	± 0.162
3	4.13	± 2.04		
4	3.56	± 2.66	0.762	± 0.174
5	11.5	± 4.75	5.68	± 0.863
6	15.2	± 8.23	3.46	± 0.657
7	10.7	± 1.74	8.55	± 0.621
8	13.2	± 2.74	17.1	± 3.34
9	22.1	± 2.68	20.8	± 2.54
10	10.3	± 5.95	19.9	± 2.29
11	13.2	± 2.14		
12	29.5	± 7.85		
13	19.6	± 3.92	46.5	± 25.4
16	58.1	± 17.8		
17	41.4	± 8.29	61.1	± 13.6
19	23.4	± 7.37	49.2	± 8.07
20	99.1	± 30.8	41.1	± 6.52
22	104	± 37.0	49.9	± 0.60
25	130	± 85.7	52.4	± 2.63
28	125	± 30.2	53.8	± 4.78
30	32.3	± 16.9	59.5	± 6.72
31	163	± 54.1	60.5	± 2.42

Table B.1 Cont'd

(b) CTAB-inhibited Cellular Selenium Accumulation				
Days after inhibition	"Washed-Killed Cells" Amendment Recovery (%)	Error	"Killed Cells" Selenium Amendment Recovery (%)	Error
3	0.019	± 0.020	0.041	± 0.012
5	0.012	± 0.016	0.019	± 0.003
8	0.019	± 0.014	0.025	± 0.008
11	0.021	± 0.009	0.022	± 0.012

Table B.2 Selenium distribution in live and sterile cultures: double-VIA experiments. Sample concentration and percent amendment recovery are given for (a) aqueous fractions, (b) cell-associated fractions, and (c) volatile and Teflon-trapped fractions.

(a)

			Aqueous Selenium				
			A Sample		**B Sample**		**Total**
Exp Name	Experiment Set	Rep-licate	Concen-tration (ppb)	Amend-ment Recovery (%)	Concen-tration (ppb)	Amend-ment Recovery (%)	Amend-ment Recovery (%)
MVSe-cells	July 2010	1	128	90.3	0.078	0.056	90.4
		2	142	102	0.099	0.069	102
	September 2010	1	111	82.8	0.173	0.128	82.9
		2	115	85.0	0.052	0.037	85.1
	Average		**124**	**90.0**	**0.101**	**0.073**	**90.0**
	± Error		**13.8**	**8.25**	**0.051**	**0.038**	**8.23**
MVSe-media	July 2010	1	155	105	0.031	0.020	105
		2	134	55.8	0.034	0.023	55.9
	September 2010	1	136	103	0.028	0.020	103
		2	129	95.5	0.023	0.016	95.5
	Average		**139**	**89.9**	**0.029**	**0.020**	**89.9**
	± Error		**11**	**22.6**	**0.005**	**0.003**	**22.6**
Control	July 2010	1	194	130	0.003	0.002	130
		2	195	135	0.008	0.005	135
	September 2010	1	179	132	0.007	0.005	132
		2	177	131	0.009	0.006	131
	Average		**186**	**132**	**0.007**	**0.005**	**132**
	± Error		**9.29**	**1.97**	**0.002**	**0.002**	**1.97**
Blank	July 2010	1	0.002	--	0.004	--	--
		2	0.010	--	0.003	--	--
	September 2010	1	0.006	--	0.008	--	--
		2	0.002	--	0.001	--	--
	Average		**0.005**	--	**0.004**	--	--
	± Error		**0.004**	--	**0.003**	--	--

Table B.2 Cont'd

(b)

			Cell-Associated Selenium				
			A Sample		B Sample		Total
Exp Name	Experiment Set	Rep-licate	Concen-tration (ppb)	Amend-ment Recovery (%)	Concen-tration (ppb)	Amend-ment Recovery (%)	Amend-ment Recovery (%)
MVSe-cells	July 2010	1	128	90.3	0.078	0.056	90.4
		2	142	102	0.099	0.069	102
	September 2010	1	111	82.8	0.173	0.128	82.9
		2	115	85.0	0.052	0.037	85.1
	Average		**31.9**	**22.9**	**0.015**	**0.011**	**22.9**
	± Error		**4.96**	**3.36**	**0.002**	**0.002**	**3.36**
MVSe-media	July 2010	1	155	105	0.031	0.020	105
		2	134	55.8	0.034	0.023	55.9
	September 2010	1	136	103	0.028	0.020	103
		2	129	95.5	0.023	0.016	95.5
	Average		**32.6**	**23.8**	**0.000**	**0.000**	**23.8**
	± Error		**5.44**	**4.72**	**0.001**	**0.001**	**4.72**
Control	July 2010	1	194	130	0.003	0.002	130
		2	195	135	0.008	0.005	135
	September 2010	1	179	132	0.007	0.005	132
		2	177	131	0.009	0.006	131
	Average		**0.008**	**0.006**	**-0.001**	**-0.001**	**0.005**
	± Error		**0.002**	**0.002**	**0.000**	**0.000**	**0.002**
Blank	July 2010	1	0.002	--	0.004	--	--
		2	0.010	--	0.003	--	--
	September 2010	1	0.006	--	0.008	--	--
		2	0.002	--	0.001	--	--
	Average		**0.001**	--	**-0.001**	--	--
	± Error		**0.004**	--	**0.000**	--	--

Table B.2 Cont'd

(c)

			Volatile and Trapped Selenium				
			Trapped Volatiles			Teflon Trap	
Exp Name	Experiment Set	Replicate	Concentration (ppb)	Amendment Recovery (%)		Concentration (ppb)	Amendment Recovery (%)
MVSe-cells	July 2010	1	128	90.3		0.056	90.4
		2	142	102		0.069	102
	September 2010	1	111	82.8		0.128	82.9
		2	115	85.0		0.037	85.1
	Average		**0.735**	**1.01**		**8.54**	**0.044**
	± Error		**0.115**	**0.144**		**3.91**	**0.019**
MVSe-media	July 2010	1	155	105		0.020	105
		2	134	55.8		0.023	55.9
	September 2010	1	136	103		0.020	103
		2	129	95.5		0.016	95.5
	Average		**0.769**	**1.06**		**5.04**	**0.042**
	± Error		**0.175**	**0.242**		**2.80**	**0.016**
Control	July 2010	1	194	130		0.002	130
		2	195	135		0.005	135
	September 2010	1	179	132		0.005	132
		2	177	131		0.006	131
	Average		**0.001**	**0.002**		**1.72**	**0.014**
	± Error		**0.002**	**0.002**		**0.678**	**0.003**
Blank	July 2010	1	0.002	--			--
		2	0.010	--			--
	September 2010	1	0.006	--			--
		2	0.002	--			--
	Average		**0.001**	--		**2.08**	--
	± Error		**0.003**	--		**2.49**	--

Table B.3 Selenium distribution in live and metabolically-inhibited cultures: single-culture experiments with continuous aeration. Sample concentration and percent amendment recovery are given for (a) aqueous and cell-associated fractions, and (b) volatile and Teflon-trapped fractions.

(a)

			Aqueous and Solid Selenium			
			Aqueous Selenium		Cell-Assoc. Se	
Scheme	Experiment Set	Rep-licate	Concen-tration (ppb)	Amend-ment Recovery (%)	Concen-tration (ppb)	Amend-ment Recovery (%)
"live"	December 2010	1	28.4	57.8	27.4	63.4
		2	55.6	113	9.86	23.4
		3	44.7	90.8	18.1	45.0
		4	52.3	107	12.7	30.9
		Avg	**45.3**	**96.2**	**17.0**	**40.7**
		± Err	**11.9**	**24.1**	**7.54**	**17.1**
"washed-killed"		1	55.4	105	0.198	0.422
		2	53.5	101	0.195	0.363
		3	54.5	103	0.103	0.189
		4	52.5	99.2	0.149	0.286
		Avg	**54.0**	**108**	**0.161**	**0.331**
		± Err	**1.24**	**2.57**	**0.044**	**0.099**
"killed"		1	62.7	129	0.063	0.134
		2	65.4	131	0.061	0.220
		3	60.8	127	2.36	5.79
		4	64.7	132	6.75	13.8
		Avg	**63.4**	**136**	**2.31**	**4.99**
		± Err	**2.02**	**2.01**	**3.09**	**6.29**

Table B.3 Cont'd

(b)

			Volatile and Trapped Selenium			
			Trapped Volatiles		Teflon Trap	
Scheme	Experiment Set	Rep-licate	Concen-tration (ppb)	Amend-ment Recovery (%)	Concen-tration (ppb)	Amend-ment Recovery (%)
"live"	December 2010	1	28.4	57.8	27.4	63.4
		2	55.6	113	9.86	23.4
		3	44.7	90.8	18.1	45.0
		4	52.3	107	12.7	30.9
		Avg	**0.597**	**2.80**	**0.015**	**0.060**
		± Err	**0.396**	**1.88**	**0.005**	**0.013**
"washed-killed"		1	55.4	105	0.198	0.422
		2	53.5	101	0.195	0.363
		3	54.5	103	0.103	0.189
		4	52.5	99.2	0.149	0.286
		Avg	**0.002**	**0.044**	**0.084**	**0.171**
		± Err	**0.001**	**0.004**	**0.046**	**0.079**
"killed"		1	62.7	129	0.063	0.134
		2	65.4	131	0.061	0.220
		3	60.8	127	2.36	5.79
		4	64.7	132	6.75	13.8
		Avg	**0.006**	**0.074**	**10.8**	**46.1**
		± Err	**0.003**	**0.010**	**17.1**	**80.3**

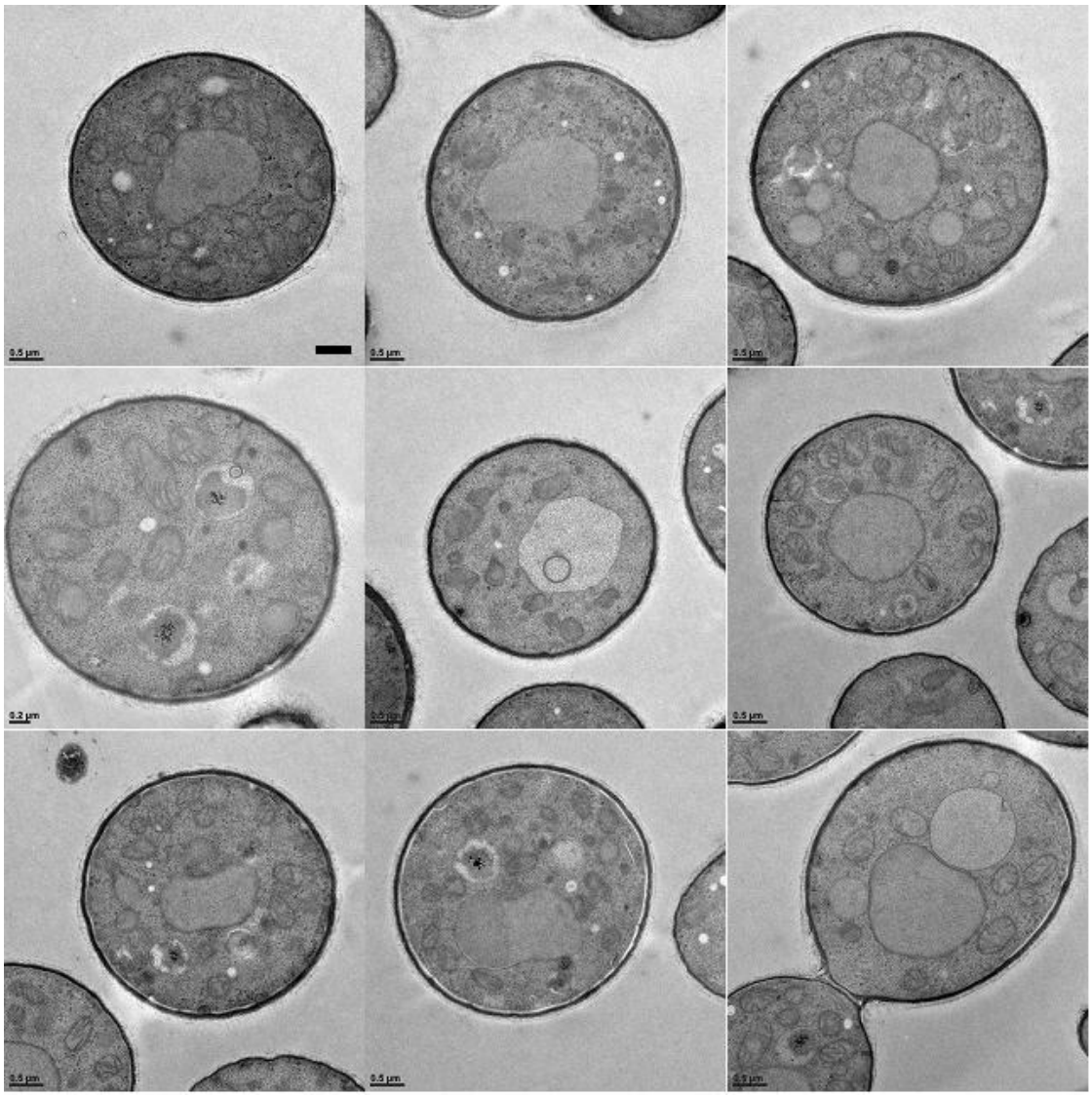

Figure B.1 Transmission electron micrographs of stained sections of cells grown with selenite amendment for two days. Scale is the same for all panels; scale bar (lower right of top left panel) equals 0.5 µm.

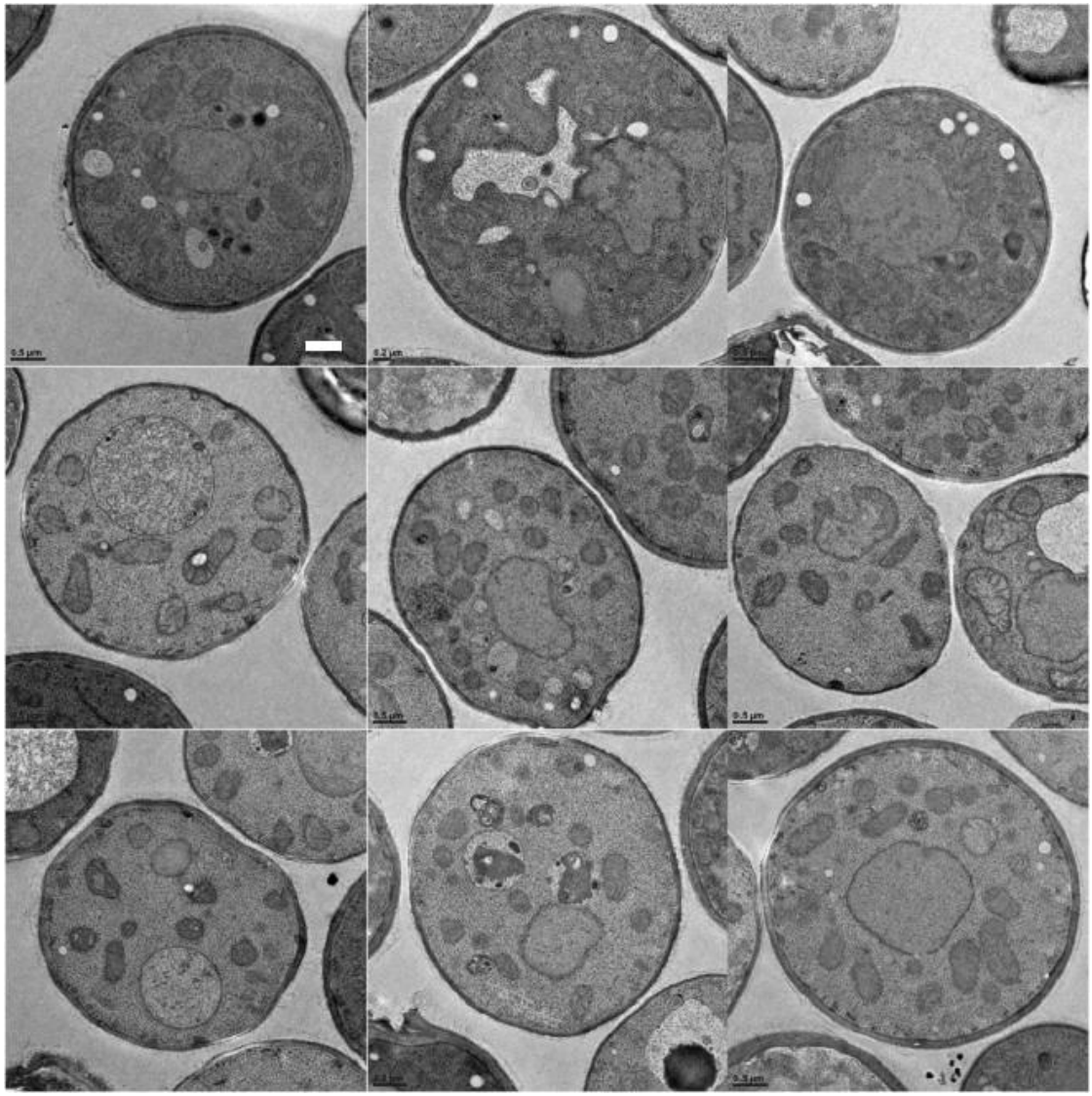

Figure B.2 Transmission electron micrographs of stained sections of cells grown with selenite amendment for five days. Scale is the same for all panels; scale bar (lower right of top left panel) equals 0.5 µm.

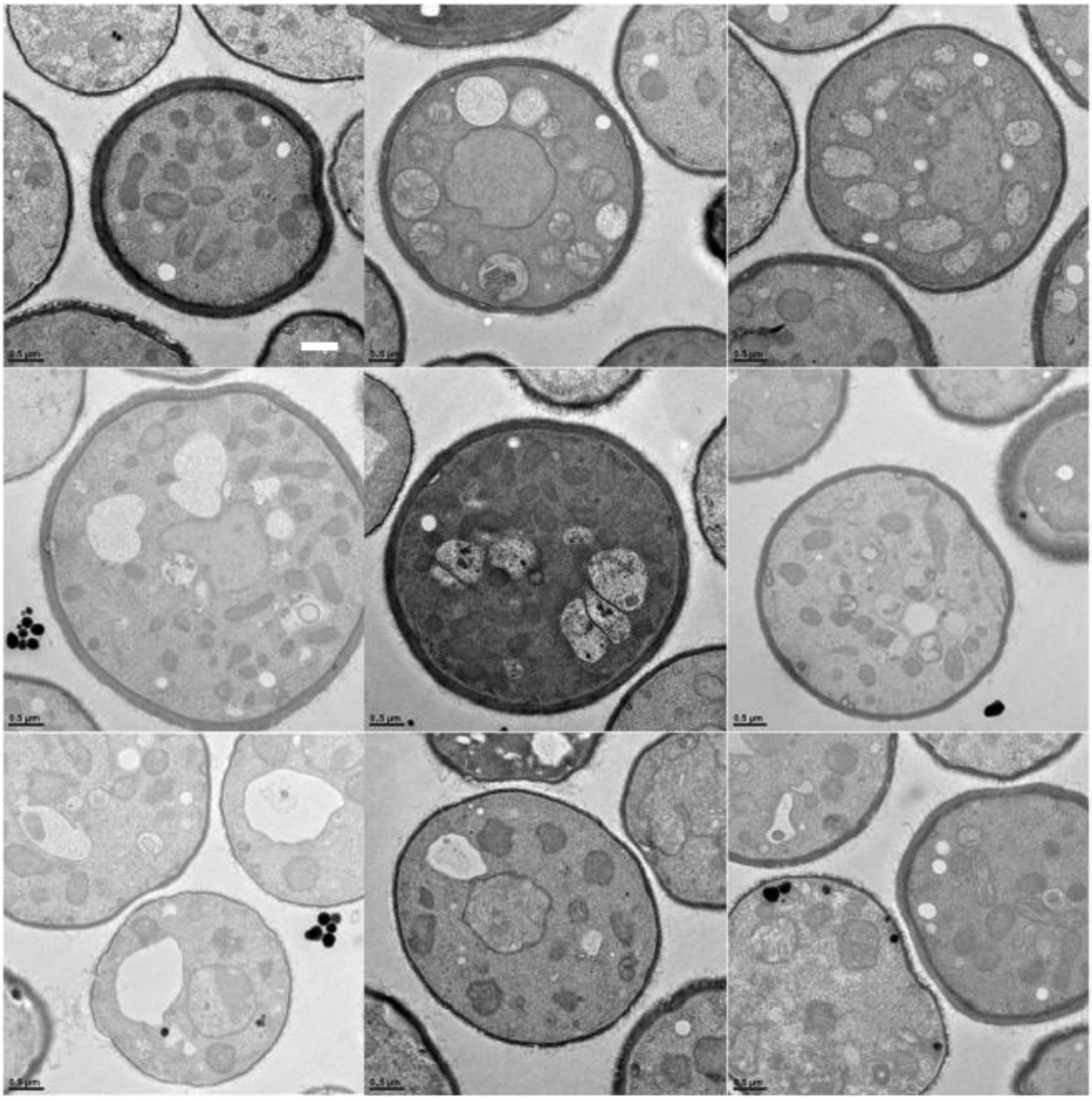

Figure B.3 Transmission electron micrographs of stained sections of cells grown with selenite amendment for 15 days. Scale is the same for all panels; scale bar (lower right of top left panel) equals 0.5 µm.

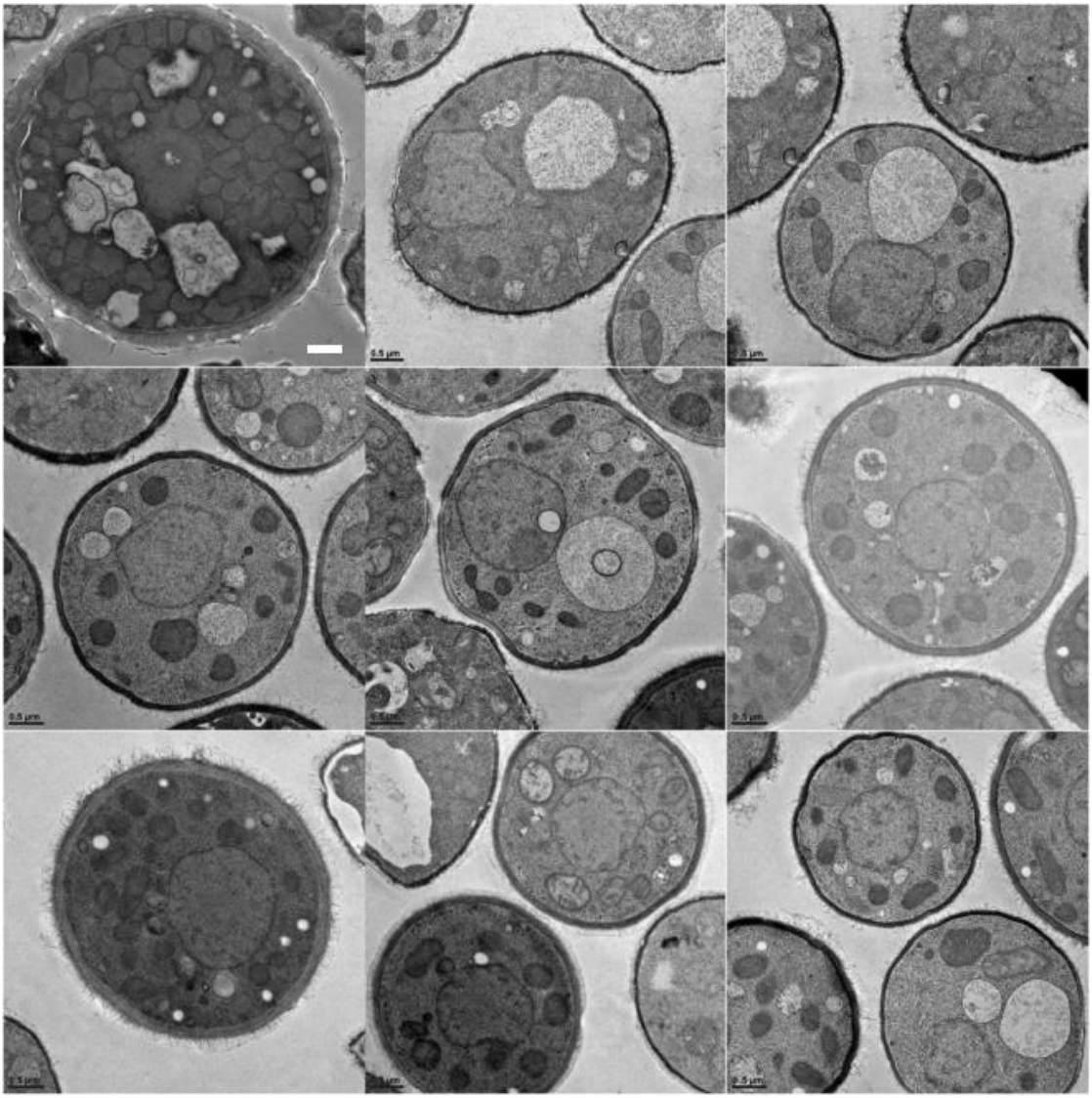

Figure B.4 Transmission electron micrographs of stained sections of cells grown for 15 days without selenite amendment. Scale is the same for all panels; scale bar (lower right of top left panel) equals 0.5 µm.

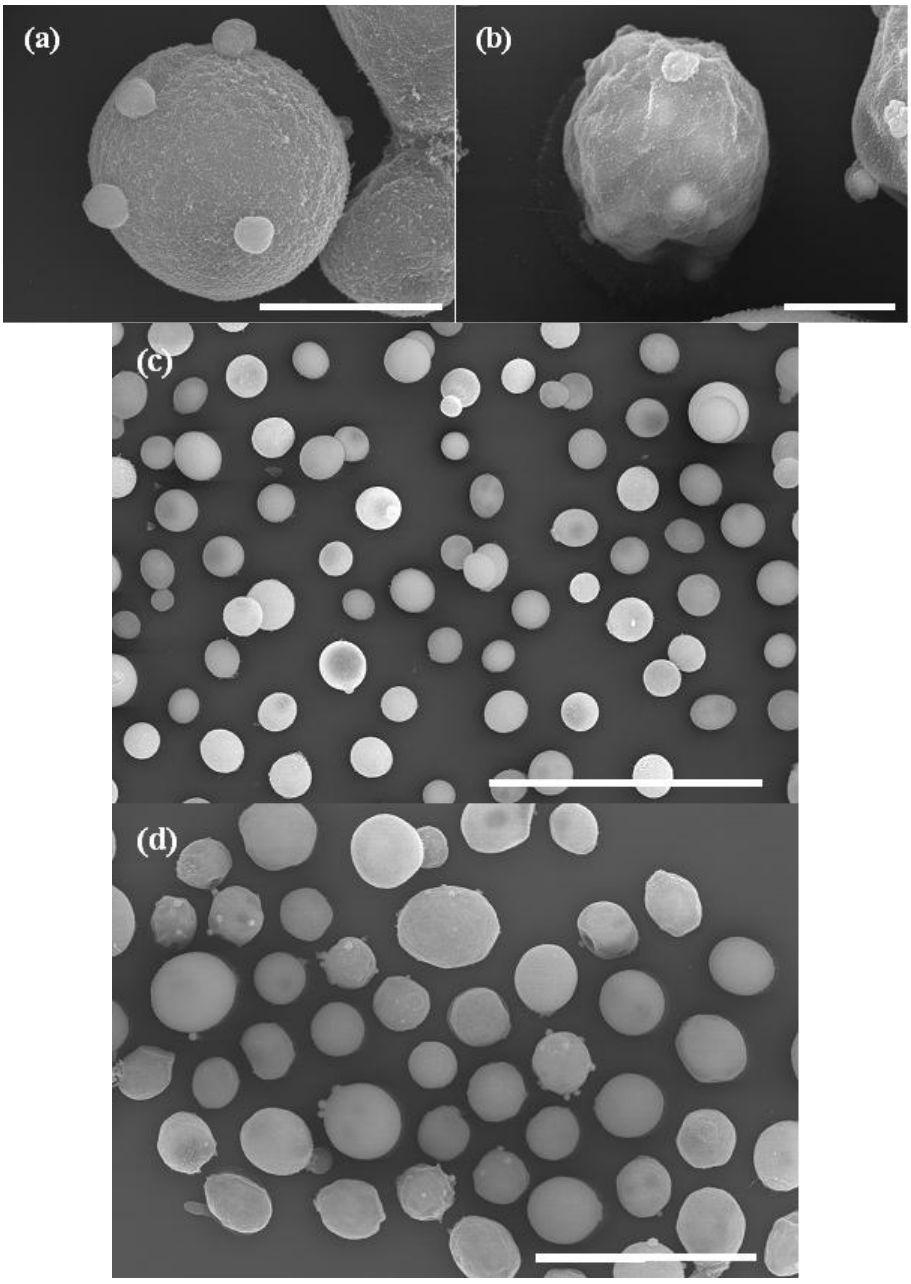

Figure B.5 **(a), (b) Scanning electron micrographs of cells grown for 15 days with selenite amendment. Scale bars equal 2.0 and 1.0 µm. (c) Larger field of view showing some cells with and some without particulate selenium accumulation (scale bar = 10 µm). (d) Field of view showing many control cells, grown without selenite amendment, and lacking selenium particles (scale bar = 20 µm).**

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