Aqueous speciation of selenium during its uptake by green algae *Chlamydomonas reinhardtii*

by

Xu Zhang

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Xu Zhang

ABSTRACT

Selenium (Se) is a micronutrient, yet elevated Se can be toxic to aquatic organisms. The range of Se concentrations within which Se uptake goes from insufficient to toxic is very narrow. It is thus important to understand the Se biogeochemical cycle in aquatic systems. In this thesis, the study focuses on changes in Se speciation during uptake by green algae. An optimized method was adopted to quantify and speciate Se in water using flow-injection atomic fluorescence spectroscopy coupled with high-pressure liquid chromatography. Details on the method are given here. For the uptake experiments, the uptakes of four Se species (selenite (Se-IV), selenate (Se-VI), selenocystine (Se-Cys) and selenomethionine (Se-Met)) by the green algae *Chlamydomonas reinhardtii* were compared. This thesis reports that the algae take up higher amounts of organic Se than inorganic Se. Selenomethionine (Se-Met) had the most rapid uptake, during which Se-Cys was produced. For all experiments, Se-IV was produced and found to sorb onto the algae cells, revealing that Se-IV is an important intermediate compound. Mass balance calculations revealed that more than 90% of Se was lost during uptake, probably to the atmosphere. This study also investigated the release of Se during algae decay to simulate the fate of Se during early-diagenesis. Selenium-rich algae cells were mixed with estuarine sediments at the sediment–water interface in a series of column incubations experiments. During the 7-week incubations, Se speciation was measured at the water–sediment interface and in pore water samples. We found that all the Se released to the pore water was in the form of Se-Cys. Although preliminary, these results highlight the key role of organic-Se species in the biogeochemical cycle of Se in the aquatic environment.

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1.0 Introduction

1.1 Selenium in the aquatic environment

Selenium (Se) was first identified in 1817 by the Swedish chemist, Jöns Jacob Berzelius, who isolated it from pyrite and then named it after Selene, which means “moon goddess” in Greek.

This element is widely occurring in the Earth from the crust to the soil, from the water to the atmosphere, in various chemical forms. The Se biogeochemical cycling process includes both the physical transportation and the chemical transformation of Se in the Earth system. The Se biogeochemical cycle has been well studied (Mackenzie, 1979; Lemly, 1999; Hamilton, 2004; Winkel et al., 2012). In the past, the scientific community commonly showed concern about Se deficiency and its impact in humans; however, recently, concerns about Se toxicity have been significantly promoted due to human activities.

Sometimes the Se biogeochemical cycle can be comparable with that of sulphur (S) (Mitchell, et al., 2012; Zehr and Oremland, 1987; Stolz et al., 2002; Hoefs, 2009) since both Se and S are elements within group 16 and these two elements have several similar chemical properties. For example, there are chemical similarities between elemental Se and elemental S, as well as between ionic selenide and sulfide (Goldschmidt and Hefter, 1933; Howard, 1977). As a result, the Se biogeochemical cycle itself is closely related to the S cycle.

Se can be incorporated into sulfides such as the sulfides of iron, lead, zinc and copper (Mackenzie et al., 1979). Due to both the Se incorporation into iron sulfides and the abundance of iron (Fe) in the Earth’s crust, the geochemistry of Se is principally ruled by that of Fe. Specifically, Se geochemistry is associated with pyrite in reducing sediments and with the hydroxylated surfaces of Fe oxides under oxidizing conditions (Howard, 1977).

On the other hand, Se and S also demonstrate different geochemical behaviors during weathering. This separation is mainly caused by the different solubility of Se and S oxyanions. Se is usually found adsorbed on solids because of the low solubility of Se salts and their affinity for Fe oxides, whereas S is generally found dissolved as highly soluble sulphate (SO_4^{2-}) (Mackenzie et al., 1979).

In summary, Se naturally occurs on Earth within sediments, soils, atmosphere and aquatic systems at four major oxidation states as shown in Table 1.1, and its geochemistry is mainly governed by that of Fe.

Table 1.1 Selenium species at four major oxidation states

Oxidation state	Chemical form
+6	Se^{6+} (selenate, Se-VI)
+4	Se^{4+} (selenite, Se-IV)
0	Se^{0} (elemental selenium)
-2	Se^{2-}(selenide), Organoselenium

For the past few decades, Se has been drawing attention from human societies due to elevated Se in the environment and its impact on ecosystems. The major anthropogenic sources of Se include the mining of (1) fossil fuels which contain Se and (2) sulfides ores in which Se is incorporated. Meanwhile the elevated Se in the environment can also be caused by several other anthropogenic activities such as metal mining and smelting, waste disposal and fertilizer use in agriculture.

There are two major compartments of the Se biogeochemical cycle within sediments, soils, atmosphere and aquatic systems: a terrestrial cycle and an aquatic cycle. In the terrestrial environment, Se cycle starts in the soils. Se distribution in the soil is uneven. Soil types vary from Se deficient soils in which Se content is less than 0.1 mg total Se per kg to seleniferous soils in which Se content is larger than 0.5 mg total Se per kg (Dhillon and Dhillon, 2003;Winkel et al., 2012). Because of Se's low solubility, soils do not contain significant soluble Se, and Se is usually found in the forms of elemental Se (Se^0) incorporated with pyrite, or adsorbed to iron oxide (Mackenzie et al., 1979). Therefore most plants take little Se directly up from soils; however, under conditions of alkaline pH values and high redox values, Se in the forms of Selenite (Se-IV) and Selenate (Se-VI) are available in the soils and can be utilized by plants (Mackenzie et al., 1979).

In addition to plants, microorganisms such as bacteria and fungi in the soils utilize soluble Se as well. The Se used by plants and microorganisms can either enter the food chain and be accumulated in upper organisms (e.g. Longchamp et al., 2012), or be released to atmosphere by the production of volatile Se (Lewis et al., 1966; Francis et al., 1974).

The remaining insoluble fraction of Se in the soils can be transported to aquatic systems and finally to the oceans through abotic pathways such as weathering. In the soils, Se sorbs to Fe oxides and clay minerals forming insoluble Se compounds including FeSe and $FeSe_2$ (Belzile et al., 2000). Also, a large part of Se stays in the form of Se^0, which is stable and insoluble (Makcenzie et al., 1979; Bowie et al., 1996; Chen et al., 2006). As water flows through the watershed, the insoluble Se fraction is usually transported by suspended solids. Insoluble Se associated with suspended loads accounts for the most of the Se transported to the oceans through rivers; and it is about 9 times larger than that of the total amount of soluble Se (Kharkar et al., 1968; Mackenzie et al., 1979). Figure 1.1, adopted from Winkel et al., (2012), exhibits the scheme of the Se cycle on the terrestrial environment.

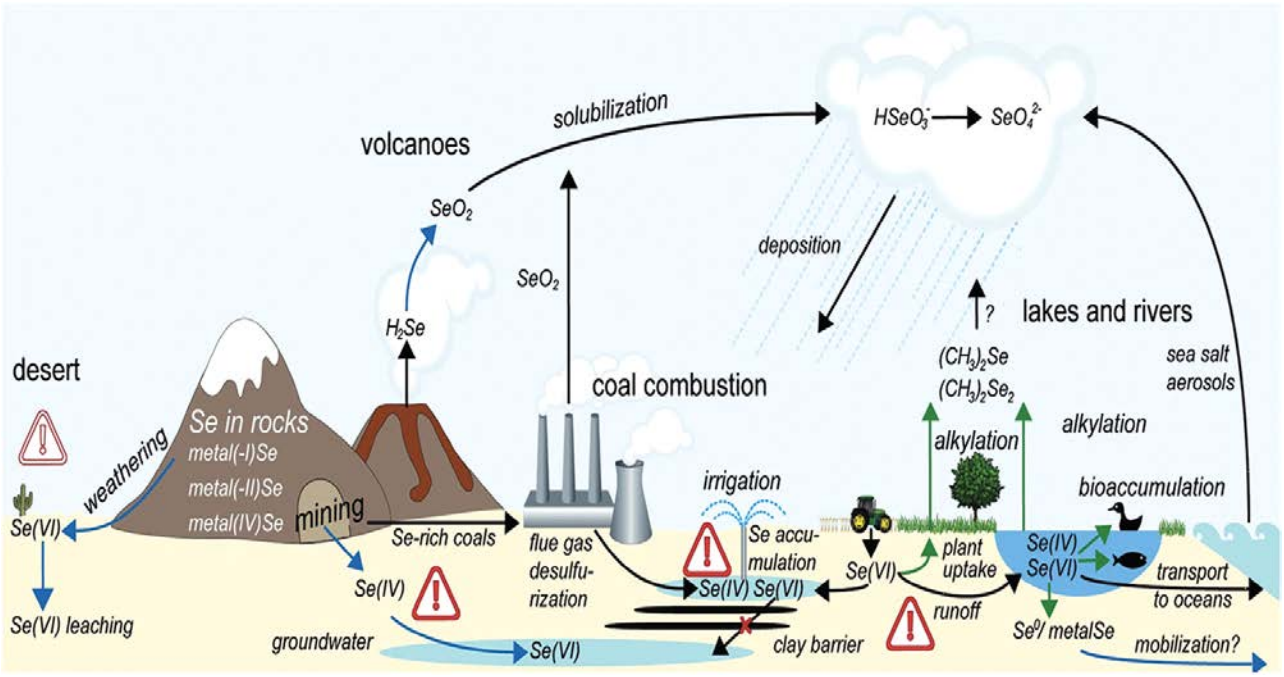

Figure 1.1 Schematic global cycle of Se with the main focus on the terrestrial environment. Blue arrows indicate processes that involve oxidation of Se species and green arrows indicate processes that involve reduction of Se species. Warning symbols indicate specific environmental settings that are at risk of either developing Se deficiency (open warning symbol) or Se excess (shaded warning symbol). Adapted from Winkel et al., (2012) Environmental Selenium Research: From Microscopic Processes to Global Understanding. *Environ. Sci. Technol.*, ***46*** (2), 571–579. © copyright (2012) with permission from American Chemical Society

Regarding the Se biogeochemical cycle in aquatic systems, soluble Se plays a major role in Se transformation and transportation. There are three main alternative pathways of dissolved Se once it enters aquatic systems: (1) it can remain as free ions under the forms of Se-IV and Se-VI in the water; (2) it can bind or complex with particulate matter in the water column or surface sediments; or (3) it can be absorbed to or utilized by organisms in the water column (Lemly, 1999).

Figure 1.2, adapted from Lemly (1999), demonstrates a Se biogeochemical cycle in a highly dynamic aquatic system. The soluble Se can be directly utilized by microorganisms in the water and thereby enter the food web. Simultaneously, aquatic biological processes produce new Se compounds while utilizing ambient Se and part of the newly synthesized Se compounds are

released back to the water column. This released Se has a different speciation than the forms of Se initially taken up by plants and microorganisms (Lemly, 1999; Simmons and Wallschläger, 2011). For instance, Simmons and Wallschläger (2011) found that algae take up Se-VI from the environment and release it back to the water column as selenocyanate and Se-IV. Aquatic biological activities also utilize ambient Se from the water and then release volatile Se to the atmosphere. For example, methylation of inorganic and organic Se by plants and microorganisms converts Se to organic Se containing one or more methyl groups which are usually volatile (Lemly, 1999).

Meanwhile, through a series of physical, chemical and biological processes, part of the soluble Se in aquatic systems is scavenged and adsorbed onto organic detritus and clays incorporated either in Fe oxyhydroxides or within dead biological organisms that settle to the sediment surface. Over time, most Se within aquatic organisms is deposited in the sediments as detritus (Lemly, 1999). In fact, there could be as much as 90% of the total Se in an aquatic system occurring in the upper few centimeters of sediments and above detritus (Lemly and Smith, 1987). Once Se settles at the sediment surface, further chemical and microbial reduction processes allow it to be converted to Se^0, insoluble organoselenium, or to be adsorbed onto Fe oxides (Lemly and Smith, 1987; Lemly, 1999).

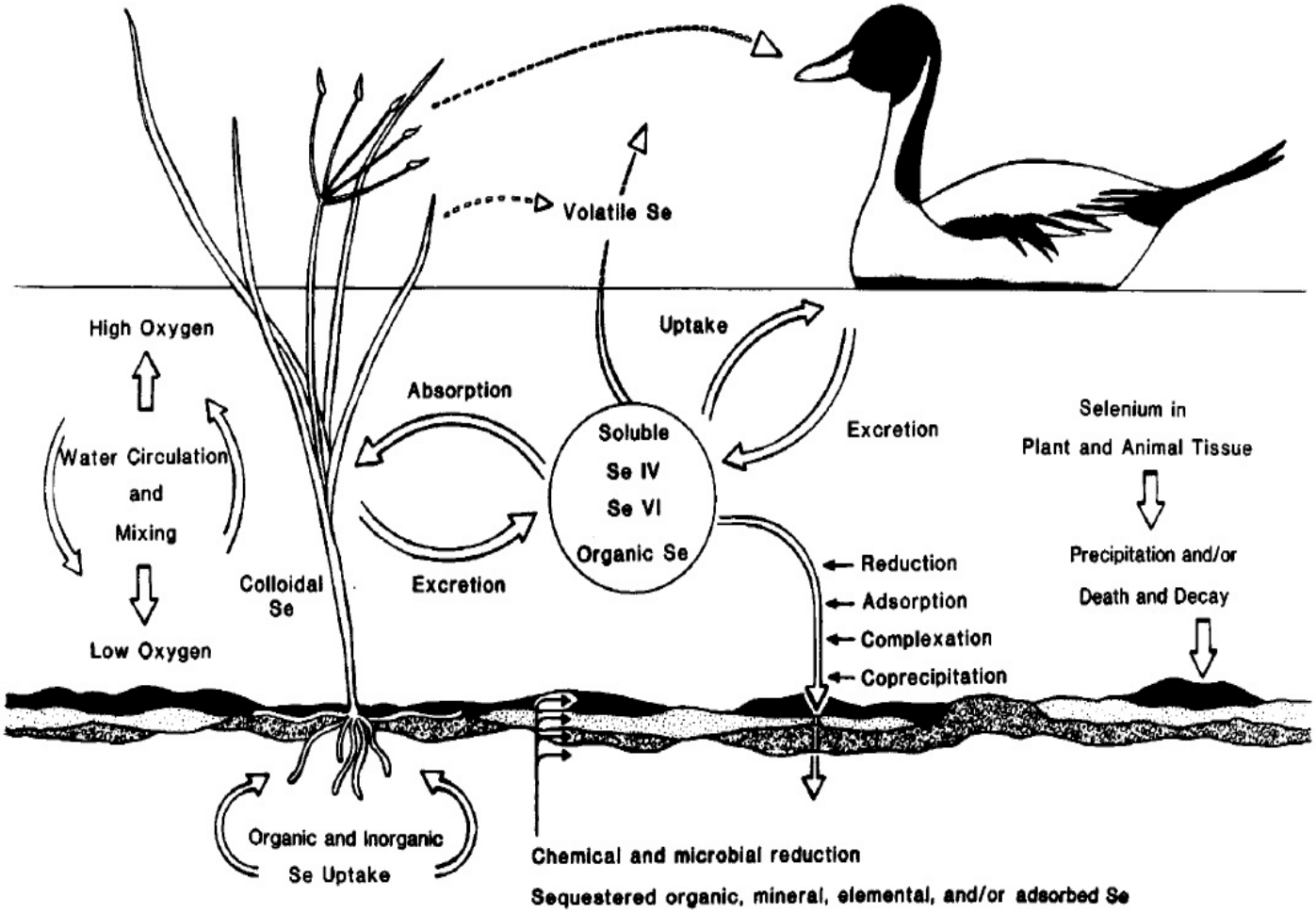

Figure 1.2 Se cycling in a dynamic aquatic system. Reprinted from Se transport and bioaccumulation in aquatic ecosystems: A proposal for water quality criteria based on hydrological units., 42, Lemly A. D. *Ecotox. Environ. Safe*, 150-156., © Copyright (1999), with permission from Elsevier.

Se is a pivotal element for ecosystem health because it is an essential micronutrient for organisms. One of its reduced forms, selenocysteine, is an essential amino acid and is considered by some to be the 21st natural amino acid (Itoh et al., 2012; Nunes et al., 2012). Selenocysteine has a similar chemical structure to that of cysteine with the only difference being that the S atom in cysteine is replaced by a Se atom in selenocysteine (Figure 1.3). Selenocysteine can be easily converted to selenocystine (Se-Cys) which is a di-Se molecule contain two selenocysteine.

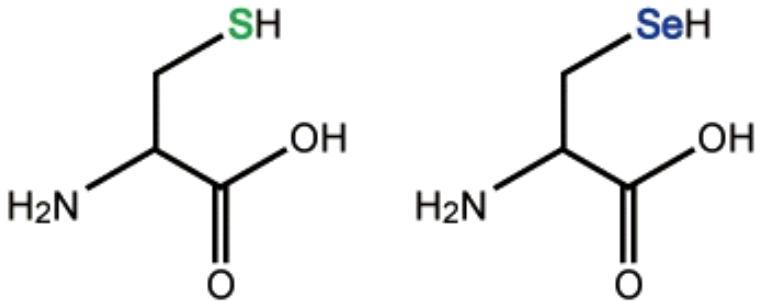

Figure 1.3. Comparison of the structures of cysteine (left) and selenocysteine (right)

Selenoprotein, which contains the amino acid Se-Cys, has been shown to be an essential nutrient in both prokaryotes and eukaryotes (Pinsent, 1954; Schwarz and Foltz, 1957; Flohé et al., 1973; Cone et al., 1976; Böck et al, 1991). As a result, the presence of Se as Se-Cys is widespread in all major domains of life (Hatfield and Gladyshev 2002; Burk and Hill, 2009). Se-Cys can be used for biosynthesis of selenomethionine (Se-Met), which is a major natural-food form of Se supplement for human beings, and widely found in plants, marine algae and brewer yeasts (Schrauzer, 1998).

In animals, dietary Se deficiency (<40 µg day^{-1}) has been linked to various diseases (Levander and Burk, 2006) such as white muscle diseases and Keshan disease in human beings (Fordyce, 2005). Additionally, recent research has shown that Se is also required for optimal growth of plants such as algae (Novoselov et al., 2002).

Although trace amounts of Se are essential, elevated Se concentrations (>400 µg day^{-1}) can be toxic (Fordyce, 2005; Levander and Burk, 2006). Recently, high Se intake and its possible health effects have concerned scientists (Winkel et al., 2012; Fordyce, 2005; Moreno-Reyes et al., 1998). High Se concentration causes phytotoxicity in plants (Fordyce, 2005). Moreover, in animals, subacute concentrations of Se can lead to problems in hair, nail and skin, as well as cause neurological problems (Winkel et al., 2012; Moreno-Reyes et al., 1998). Acute Se poisoning can kill both animals and humans (Fordyce, 2005). In aquatic systems, there are increasing concerns about Se elevation as a result of anthropogenic activities.

In aquatic systems, Se accumulates in aquatic organisms and this bioaccumulation can be significantly greater than the ambient Se levels (Lemly and Smith, 1987), threatening animals and humans higher in the food with Se toxicity (Hamilton, 2004). Previous studies have shown that algae and microorganisms bioaccumulate Se from the water column by factors of up to 10^6 (Stewart et al., 2010), and that the bioaccumulation factors of Se are smaller at higher trophic levels (Stewart et al., 2010; Bowie et al., 1996). The impact of elevated Se on aquatic systems has been heavily studied and reviewed in recent years (Hamilton, 2004; Lemly, 1999). Notably, several fish kills as a result of Se toxicity have been reported (Sorensen, 1988; Lemly, 1985; Nakamoto and Hassler, 1992; Lemly and Ohlendorf, 2002).

Overall, Se in the aquatic system acts as a micronutrient, but it has a very narrow range between deficiency and toxicity. It is therefore crucial to understand the processes that control the Se biogeochemical cycle in the environment. Because of the importance of algae in aquatic food webs, algae have been used as a model organism to study the Se biogeochemical cycle in aquatic ecosystems, and the results have been used to assess the Se uptake process and its environmental impacts. Previous studies exploring the Se cycle in aquatic systems using algae have been conducted by different groups with various scientific objectives including: 1) The growth requirement of Se for different algae species; 2) The toxicity effect of Se on algae; 3) The impact of Se speciation on Se uptake by algae; and 4) The competition of Se uptake with other compounds such as SO_4^{2-}. These studies are summarized below.

Se growth requirements vary among algae species. Harrison et al., (1988) examined the growth requirement of Se for various algae species. The authors of this research conducted a survey of 27 species of marine phytoplankton representing 4 algal classes including Bacillariophyceae, Dinophyceae, Prymnesiophyceae and Cyanophyceae. These marine phytoplanktons' Se requirements were determined by growing each species in artificial seawater with or without 10^{-8} M Se as Se-IV. The results from this survey confirm that Se is an essential element for most species in the Bacillariophyceae class; however, it is not mandatory for the growth of most other phytoplanktons in the other three classes. Doblin et al. (1999) also studied the effect of Se on the growth of *Chaetoceros calcitrans* (Bacillariophyceae class), but their results showed that *C. calcitrans* has a negligible requirement for Se. In contrast, researchers have shown the need of Se for *Chlamydomona reinhardtii* (Fu et al., 2002). Selenoprotein was detected in *C. reinhardtii* and the need of Se for its optimal growth was demonstrated as well (Novoselov et al., 2002).

In the terms of Se toxicity, Umysová et al. (2009) used the green algae *Scenedesmus quadricauda* to determine the Se toxic effect of Se-IV and Se-VI. In both cases, Se was taken up by the algae and the detrimental effects of high Se concentrations on algae cells were observed. Se-IV was found to be more toxic than Se-VI to *S. quadricauda*. During uptake, Se is chemically transformed from inorganic Se to organic Se and Se-Met was found to accumulate in the biomass of *S. quadricauda*.

Toxicities of Se-IV and Se-VI on *C. reinhardtii,* respectively, have also been explored (Morlon et al., 2005; Morlon et al., 2006; Geoffroy et al., 2007). With the addition of Se-IV, damage to the ultrastructure, at the sub-cellular level, was observed in *C. reinhardii* cells. An inhibition effect on cell population was also observed (Morlon et al., 2006; Morlon et al., 2005). Under Se-VI addition, similar observations of ultrastructure damage and impaired photosynthesis were observed (Geoffroy et al., 2007).

Different algae species demonstrate different preferences and uptake rates of different Se species. Hu et al. (1997) found the preference of Se-IV over Se-VI during the uptake by *Chaetoceros calcitrans*. Moreover, Riedel et al. (1991) examined the uptake of Se species including Se-IV, Se-VI and Se-Met by three different algae species, namely, *Anabaena flos-aquae, Chlamydomonas reinhardtii* and *Cyclotella meneghiania.* This study demonstrated a significantly faster uptake of Se-Met than that of Se-IV and Se-VI. Furthermore, an adsorption of Se-IV to heat killed algae cells was also observed, suggesting passive uptake. Morlon et al. (2006) conducted a study to explain that the Se-IV uptake process is concentration dependent. At low concentrations (< nM), Se-IV is transported in a rapid saturated mechanism in which the uptake of Se-IV is specific and saturates readily ; however the Se-IV uptake demonstrates a liner relationship in intermediate Se concentration range (nM-µM) and it saturates at high Se concentration levels (~mM).

Competition uptake between Se and other chemicals was also studied. Fresh water green algae *Chlorella vulgaris* was used to study the effect of SO_4^{2-} concentration on Se uptake (Simmons and Emery, 2011). In this study, 70 nM of Se-VI was added to mediums containing 31.2 nM and 312 nM of SO_4^{2-}. Most of the Se-VI was digested by the algae under the condition of 31.2 nM SO_4^{2-}; however, the Se level remained stable under the condition of 312 nM of SO_4^{2-}. The results indicated the uptake of Se-VI by *C. vulgaris* was significantly inhibited by high concentration of SO_4^{2-}.

Although lots of studies have been carried out to understand Se behaviors in aquatic environments, our knowledge of the Se biogeochemical cycle is still incomplete. For example, little is known about the impact of organoselenium compounds such as Se-Cys at low concentrations in aquatic ecosystems. Additionally, it is still not clearly known what species organisms are taking up versus what species they are releasing back to the water column. Organoseleniums uptake, especially Se-Cys, requires further study. Furthermore, the changes in Se speciation during Se uptake have rarely been studied. Simmons and Wallschläger (2011) briefly examined the Se speciation of the Se released back to water during Se uptake by *C. vulgaris*. This research showed that when Se-VI was utilized by *C. vulgaris*, selenocyanate and Se-IV were released back to the medium.

Here, in this research, similar experiments were performed to determine the evolution of Se speciation in the aqueous phase during the uptake and the release of Se by algae species. Both

inorganic (Se-IV and Se-VI) and organic (Se-Cys and Se-Met) Se species were added to selected algae cultures to study their effect on Se uptake and speciation changes.

Many researchers have studied Se uptake by algae using the model organism *C. reinhardtii* (Fu et al., 2002; Novoselov et al., 2002; Morlon et al., 2005; Morlon et al., 2006; Geoffroy et al., 2007).

C. reinhardtii is ubiquitous in the aquatic environment, being presenting both freshwater and saltwater (Harris, 2004). Moreover, it is resistant to stress and easy to cultivate in the laboratory *C. reinhardtii* grows in defined medium at neutral pH without additional supplementary vitamins (Harris, 1989). *C. reinhardtii* is also heterotrophic, and thus can be used as the carbon source for *C. reinhardtii* in place of CO_2 to grow in the dark. Yet, *C. reinhardtii* grow faster with light source whether or not acetate is present in the growth medium. (Harris, 1989). Nitrate assimilation by *C. reinhardtii* is limited to reduced nitrogen such as ammonia (NH_4^+).

C. reinhardtii is usually haploid which means its cells contain a single set of unpaired chromosomes and there are two genetically fixed mating types designated as mate plus (mt^+) and minus (mt^-) (Harris, 2001). The sexual cycle of *C. reinhardtii* starts from the pairing of both genetic mates after nitrogen deprivation and then the paired cells release cell walls and activate mating structures (Harris, 2001). Next, cell fusion occurs between the paired cells and it is followed by meiosis which produces vegetative cells (Harris, 2001). The new produced vegetative cells undergo asexual reproduction and new vegetative cells are produced through mitosis (Harris, 2001).Based on the robustness of this species and its ubiquity, we chose *C. Reinhardtii* as the model organism to study Se uptake in a saline medium, which is close to marine environment, and to explore the fate of Se utilized by this algae species.

1.2 Se redistribution in sediments during early diagenesis.

During uptake of Se by algae, a fraction of Se is released back to the water (Simmons and Wallschläger, 2011) and the atmosphere (Lemly, 1999), and the rest is retained and stored, within the cell wall. This has been shown for the nutrient element phosphorus (P), where the organism stores P both as a functional molecule (i.e., ATP) and as a nodule (i.e., phosphate minerals) (Diaz et al., 2008). Although the exact speciation and location of the stored Se in the algae cells is not known with certainty, results from the Se uptake experiment presented in this thesis provides a first estimate of the fate and speciation of algae-bound Se.

Once an algae cell dies, Se retained in the cell is likely to settle at the sediment surface. In fact, in the specific case of Se, it is hypothesized that one of the main sources of Se to sediment is the settling of dead phytoplankton, along with deposition of clays and detritus, carrying Se sequestered from the water column (Bowie et al., 1996). As the sediments undergo diagenesis, Se is chemically transformed through a series of chemical and microbial reactions.

Diagenesis is the succession of physical, chemical and biological changes occurring in aquatic sediments after deposition. The sequence of chemical and biological reactions occurring during early diagenesis is fuelled by the microbial degradation of organic carbon and in this process that microbial community in the sediments takes up energies stored in organic compounds which were originally formed from photosynthesis at surface water. Carbon in organic compounds is oxidized to CO_2 in the sediments and energies were released. During the oxidation of organic compounds, several chemicals, including O_2, NO_3^-, Mn^{2+}, Fe^{3+} and SO_4^{2-}, act as oxidants to accept electrons (Emerson and Hedges, 2008). Under oxic condition, O_2 reduction governs the early diagenesis process and when O_2 is consumed, NO_3^-, Mn^{2+}, Fe^{3+} and SO_4^{2-} reductions are responsible to diagenesis for anoxic zone (Emerson and Hedges, 2008). It is estimated that 80% to 90% of the organic compounds are buried in upper 1000 m in river deltas and continental margins. In these areas, anoxic diagnesis becomes important after oxygen has been quickly consumed due to the large input of organic matters (Emerson and Hedges, 2008).

The fate of Se retained in dead algae tissues after it settles at the sediment surface has major implications for Se recycling to the water column in freshwater basins as well as for Se burial over long time scales in oceanic basins. In a dynamic aquatic system, Se settling at the sediment surface can either (i) be cycled back to the biota (Lemly, 1999; Lemly, 1997) through uptake by rooted plants and detritus-feeding wildlife (Lemly and Smith, 1987; Lemly, 1999) or (ii) be buried (Figure 1.2). The buried Se fraction can occur as organic Se (for example, the Se comes from the settlement of phytoplankton and detritus) and inorganic Se (such as Se-IV and Se-IV absorbed on clays and Fe minerals). During early diagenesis organic Se can be released to pore water and the local redox conditions can cause organic Se to oxidize to Se-IV and Se-VI or to Se^0 (Bowie et al., 1996).

There is limited knowledge regarding the fate of Se taken up by or adsorbed to aquatic organisms such as algae. There are a few previous studies, however, that took a close look on the behavior of Se at the water–sediment interface. Belzile et al. (2000) studied Se speciation in both pore water and lake sediments. This study showed the predomination of Se-IV over Se-VI in reducing environments. Se-IV can be adsorbed to oxyhydroxides of manganese (Mn) and Fe at the water–sediment interface and the Se can also be released back to water when the oxyhydroxides are reduced. Martin et al. (2011) conducted experiments at two contrasting lacustrine environments to understand the biogeochemical mechanisms of Se exchange between water and sediments. According to this study, the Se speciation is related to the redox condition, the sediments serve as diffusive sinks for Se, and the sedimentary Se is dominated by Se^0 and organoselenium.

This thesis explores the early diagenetic redistribution of Se by representing results from experiments using sediments incubated in the presence of Se-containing algae cells. In particular, this research identifies the biogeochemical processes involved in the fate of Se utilized and retained in algae and how this part of biotic Se is cycled back to abiotic environment once the algae dies. Since the algae cells used in this thesis were grown in a medium similar to a sea–

water environment, the sediment cores used in this research were sampled from a coastal region where the phytoplankton bioactivities are dynamic.

1.3 Problem statements and objectives

The goal of this thesis is to address the lack of information on the speciation of Se in the aquatic environment during the Se uptake by algae, as well as on its release when algae decompose during early diagenesis. This research focused on the following three questions: (1) Which Se specie(s) is (are) the most favorable to *C. reinhardtii* uptake? (2) What is the speciation of Se released to the water column during ambient Se uptake? and (3) What is the fate of Se once the Se-rich algal biomass reaches the sediment–water interface and begins undergoing early diagenesis?

1. Which Se specie(s) is (are) the most favorable to *C. reinhardtii* uptake?

This research compared the uptake of four Se species including two inorganic Se species (Se-IV and Se-VI) and two organoselenium species (Se-Cys and Se-Met).

In terms of inorganic Se species, previous studies have shown a preference of Se-IV over Se-VI on uptake by phytoplankton (Hu et al, 1997; Besser et al., 1993; Araie and Shirawa, 2009; Nishri et al., 1999); however others found the opposite result (Simmons and Wallschläger, 2011; Fournier et al., 2006; Neumann et al., 2003). This difference might be caused by the species of algae, as well as the medium used. Several studies contrasted the adsorption versus the absorption of Se-IV uptake by algae. Some studies found the adsorption of Se-IV is significant (Boisson et al., 1995; Riedel et al., 1991), while others found that it can be neglected (Morlon et al., 2006; Vandermeulen and Foda, 1988). Conflicting results on the adsorption of Se-IV appeared even when both studies were using the same algae *C. reinhardtii* (Morlon et al., 2006; Riedel et al., 1991). Therefore, it remains to be proven whether Se-IV uptake by algae is dominated by active or passive process and which inorganic Se species, Se-IV or Se-VI, is preferred by *C. reinhardtii*.

Regarding organoselenium species, previous studies have determined that the bioaccumulation of Se-Met is greater than that of the two inorganic Se species (Besser et al., 1993; Fournier et al., 2006; U.S. Environmental Protection Agency, 1987; Ingersoll et al., 1990; Sandholm et al., 1973; Kiffney and Knight, 1990; Besser et al, 1989; Sharma and Davis, 1980). Nevertheless some studies report the the opposite phenomenon that algae prefer Se-IV and Se-VI over Se-Met (Neumann et al, 2003) and selenocynate (Se-CN) (Simmons and Wallschläger, 2011). The biosynthesis of Se-Cys and its importance in algae metabolism is depicted on Figure 1.4 (adapted from Arie and Shirawa, 2009). Still, very limited information on the uptake of Se-Cys by aquatic plants and algae can be found in the literature.

This research compared the uptake of inorganic Se and organoselenium at an adjusted concentration of 10 µg Se/L (127 nM Se). The uptake rates of each Se species could be determined and the bioavailability of each Se species could be compared.

2. What is the speciation of Se released to the water column during ambient Se uptake?

As indicated in Figure 1.2, the uptake of ambient Se by organisms can also lead to the release of Se back to the water column. It is then necessary to understand the impact of this released Se on the Se cycle in the aquatic systems. This thesis focuses on both the amount and the speciation of the Se excretion during Se uptake by algae. The literature provides little information about Se released in the aquatic system. Simmons and Wallschläger (2011) examined Se uptake of Se-IV, Se-VI and Se-CN. Se-IV and Se-VI were found more favorable than Se-CN for Se uptake. Se-CN was observed to be released back to the growth medium during Se-VI uptake.

This thesis explores the speciation of Se released back to the water column during the uptake of ambient Se by *C. reinhardtii*. Four species were used as the ambient Se including Se-IV, Se-VI, Se-Cys and Se-Met. The differences between the ambient Se and Se released to water column offer an opportunity to estimate Se chemical transformation for each Se species by organisms in aquatic systems. In addition, the speciation of Se adsorbed on the cell wall and the total amount of internal Se inside the algae cells were also measured. A mass balance was calculated to estimate the fractions of both the Se released to the environment and the Se retained within the algae cells.

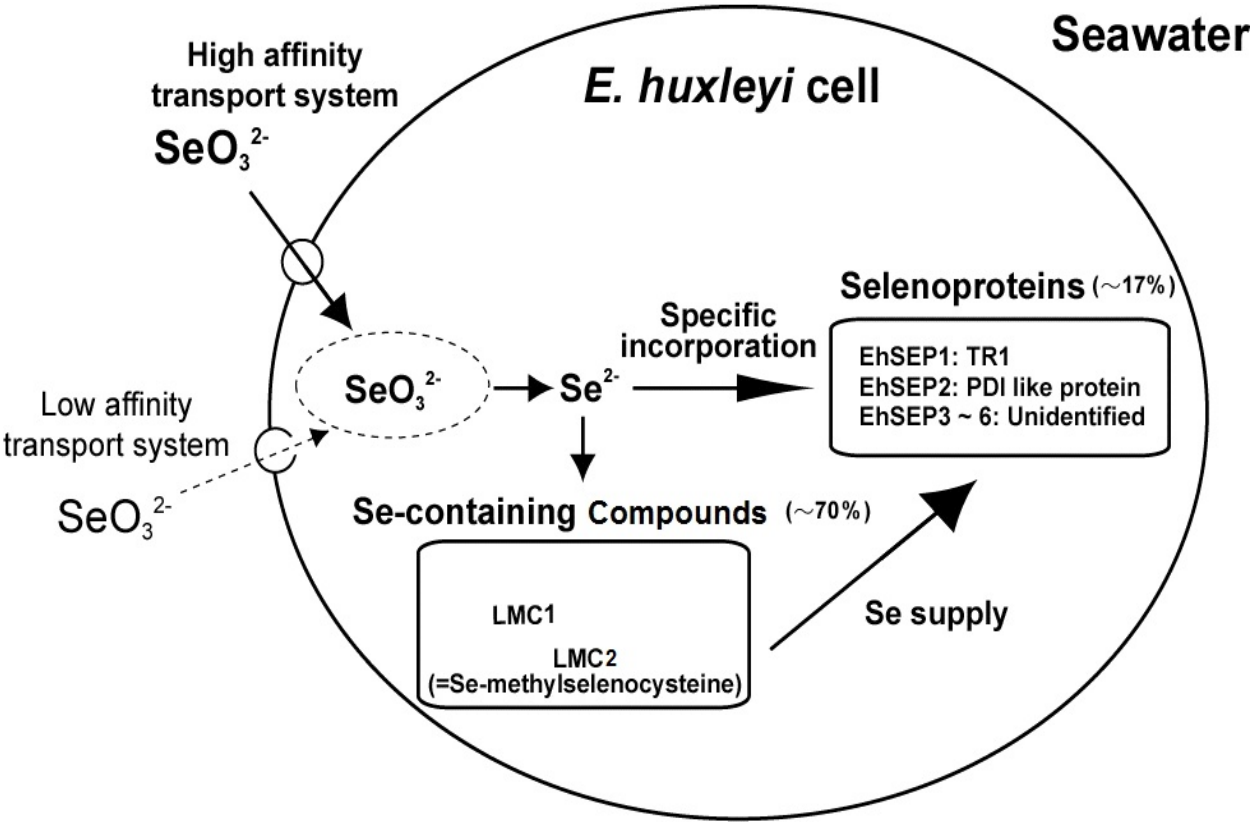

Figure 1.4 Se absorption and metabolic flow in *Emiliania huxleyi*. LMC1, unidentified Se-containing compounds; LMC2, Se-methyl-selenocysteine; EhSEP1, homologous to thioredoxin reductase 1; EhSEP2, homologous to protein-disulfide isomerase; EhSEP3-6, unidentified selenoproteins. Adapted and modified from Araie H., Shirawa Y., (2009) Selenium utilization strategy by microalgae. Molecules. 14, 4880-4891, ©*Multidisciplinary Digital Publishing Institute (open-access)*

3. What is the fate of Se once Se-rich algal biomass reaches the sediment-water interface and begins undergoing early diagenesis?

In the aquatic environment, algae eventually die and settle to the sediment surface. Se precipitated to sediments through dead algae is one of the major sources of Se in the sediments. The fate of Se remaining in the algae is poorly studied.

To answer this question, in this research an early diagenetic redistribution experiment, using marine sediments and dead algae, was designed. Algae grown in a medium containing the most bioavailable Se species were harvested and mixed with the top layer of a sediment column from a salt marsh. The Se concentration and speciation near the water–sediment interface was monitored for 7 weeks to understand the fate of Se released and transformed during diagenesis.

2.0 Method

2.1 Se uptake by *C.reinhardtii*

2.1.0 Experimental design

In this experiment, 1 µg of a selected Se species was added in triplicate to 100 mL of a *C. reinhardtii* culture. The resulting ambient Se had an initial concentration of 10 ppb (127 nM). Then the Se concentration and speciation in the medium were monitored for 168 hours after the Se addition. Harvested algae were collected and rinsed with modified high salt medium (MHSM). Then the harvested algae were washed using alkaline solution to determine Se sorbed on the cell wall and finally the algae pellets were digested using acid and used to determine total internal Se within the algae cells. The experimental design is shown in Figure 2.1

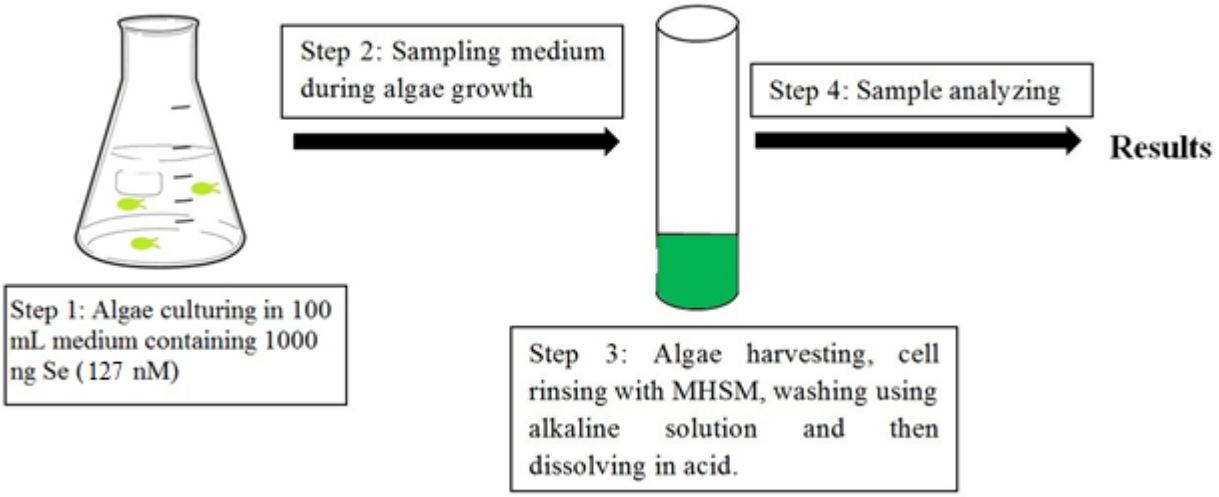

Figure 2.1 The Se uptake experiment design

2.1.1 Algae and medium

Chlamydomonas reinhardtii was chosen as the algal inoculum. Algae cultures (CPCC 11 *C. reinhardtii*) were provided by Canadian Phycological Culture Center (CPCC, Waterloo, Canada). To maintain the growth of *C. reinhardtii*, a modified high salt medium (MHSM) (adopted from Boullemant et al., 2009; Lavoie et al., 2009; Macfie et al., 1994) was used as the medium for the algal culture. Six stock solutions were prepared to make the MHSM (Table 2.1).

Table 2.1 Chemical compositions of the stocking solutions of MHSM

Stock solution	Chemicals	Concentration
Nitrogen solution	NH_4NO_3	15.0 g· L^{-1}
	$MgSO_4 \cdot 7H_2O$	4.00 g· L^{-1}
	$Ca(NO_3) \cdot 4H_2O$	3.21 g· L^{-1}
Phosphor solution	KH_2PO_4	29.6 g· L^{-1}
	K_2HPO_4	57.6 g· L^{-1}
Trace metal solution	H_3BO_3	186 mg· L^{-1}
	$MnCl_2 \cdot 4H_2O$	415 mg· L^{-1}
	$FeCl_3 \cdot 6H_2O$	160 mg· L^{-1}
	$Na_2EDTA \cdot 2H_2O$	300 mg· L^{-1}
	Zn (1 g· L^{-1})*	1.59 mg· L^{-1}
	Co (1 g· L^{-1})*	0.64 mg· L^{-1}
	Mo (1 g· L^{-1})*	2.88 mg· L^{-1}
	Cu (1 g· L^{-1})*	4.47 mg· L^{-1}
pH Buffer	HEPES	23.83 g· L^{-1}
NaOH	NaOH	40 g· L^{-1}
KNO_3	KNO_3	101 g· L^{-1}

*Commercial calibration standards

All solutions were made using ultra-pure water (Milli-Q, resistivity ≥ 18 Ω) in acid-washed lab glassware and polymethylpentene volumetric flasks. All manipulations were conducted under a laminar flow-hood. During the preparation of the trace metal solution, a certain order of chemical additions was strictly followed. $Na_2EDTA \cdot 2H_2O$ was added in Milli-Q water first and allowed to dissolve fully at room temperature for at least 24 hours. $FeCl_3 \cdot 6H_2O$ was added second and it also required at least 24 hours for completed dissolution. Afterwards all other trace metal chemicals were added. All stock solutions were filtered before being stored at 4^oC in the dark. Except for the NaOH solution, all stock solutions were filtered using 0.2-µm polycarbonate filters (Millipore GTTP04700). The NaOH solution was filtered using 0.2-µm polyethersulfone filters (Millipore GPWP04700) to avoid hydrolysis between NaOH and polycarbonate.

To prepare 1 L MHSM medium, 5 mL of the nitrogen solution, 250 µL of phosphor solution, 4.0 mL KNO_3 stock solution, and 100 mL of pH buffer solution were added to 300 mL Milli-Q water. Then the solution was diluted to about 950 mL and was autoclaved at 121^oC for 15 minutes. To avoid precipitation of trace metals, 1 mL of the trace metal stock solution was added after the medium solution was allowed to cool down to room temperature. In the last step, approximate 3 mL of NaOH stock solution was added to adjust the pH of the medium to 7 (VWR, sympHony, SP90M5) and the solution was diluted to 1 L using autoclaved Milli-Q water. The concentrations of each chemical in MHSM are listed in Table 2.2. The MHSM solution was stored at 4^oC in the dark and gently heated in a water bath to room temperature before use.

Table 2.2 Nutrient concentrations in MHSM

Chemical	Concentration in MHSM (M)
NH_4^+	$9.37 \cdot 10^{-4}$
Cl^-	$5.98 \cdot 10^{-6}$
K^+	$4.22 \cdot 10^{-3}$
PO_4^{3-}	$1.37 \cdot 10^{-4}$
CO_3^{2-}	Open to the atmosphere
NO_3^-	$5.07 \cdot 10^{-3}$
SO_4^{2-}	$8.12 \cdot 10^{-5}$
Mg^{2+}	$8.12 \cdot 10^{-5}$
Ca^{2+}	$6.80 \cdot 10^{-5}$
Na^+	$1.02 \cdot 10^{-4}$
BO_3^{3-}	$3.01 \cdot 10^{-6}$
Mn^{2+}	$2.10 \cdot 10^{-6}$
EDTA	$8.06 \cdot 10^{-7}$
Fe^{3+}	$5.92 \cdot 10^{-7}$
MoO_4^{2-}	$3 \cdot 10^{-8}$
Zn^{2+}	$2.43 \cdot 10^{-8}$
Co^{2+}	$1.09 \cdot 10^{-8}$
Cu^{2+}	$7.04 \cdot 10^{-8}$

2.1.2 Se uptake by algae

Six Se exposures with different Se species compositions were tested in *C.reinhardtii* cultures for uptake. Meanwhile, for each Se exposure, a separate series of triplicate control sample containing no algae were also tested to determine the impact of the bioactivity of algae on Se transformation. In addition, this thesis also investigated Se adsorption of Se-VI and Se-Cys on dead algae walls by performing an additional triplicate of Se-VI and Se-Cys exposures on samples containing the same amount of heat-killed algae cells. Arguably, the heat-killed algae cells do not keep exact same cell structures as the live ones, leading different sorption behavior. Despite of this possible discrepenscy, an experiment using heat-killed algae cells still provides an insight of Se absorption to cell walls (e.g. Riedel et al., 1991).

These six Se exposures to *C.reinhardtii*, hereafter, are referred to as experiment 0 to 5 (See Figure 2.2-2.7):

- Experiment 0: no Se in *C. reinhardtii* cultures
- Experiment 1: 10 ppb Se as Se-IV exposure in *C. reinhardtii* cultures and MHSM
- Experiment 2: 10 ppb Se as Se-VI exposure in *C. reinhardtii* cultures, MHSM and heat-killed *C. reinhardtii* cultures

- Experiment 3: 10 ppb Se as Se-Cys exposure in *C. reinhardtii* cultures, MHSM and heat-killed *C. reinhardtii* cultures
- Experiment 4: 10 ppb Se as Se-Met exposure in *C. reinhardtii* cultures and MHSM
- Experiment 5: 10 ppb Se consists of mixed species including 2.5 ppb Se as Se-IV, 2.5 ppb Se as Se-VI, 2.5 ppb Se as Se-Cys and 2.5 ppb Se as Se-Met exposure in *C. reinhardtii* cultures and MHSM

Triplicate experiments were performed for all samples to study the uptake of Se species by algae. *C. reinhardtii* was grown in 500-mL, foam-sealed (Joyce foam plugs), plain-bottom, sterile, polycarbonate flasks (Fisher, PBNV500) containing 100 mL MHSM. Algae were cultured in an incubation chamber (New Brunswick, innova 42R) at 20^{o}C, and shaken at 100 rpm under light. Once the cell density of the *C. reinhardtii* reached 1,500,000 cells per mL, 1 mL of the algal inoculum was subcultured and added into 100 mL MHSM containing either no Se or selected Se species. Meanwhile, the original algae cultures were heat killed at 121^{o}C using the autoclave and the sterilized algae cells were added into 100 mL MHSM containing selected Se species.

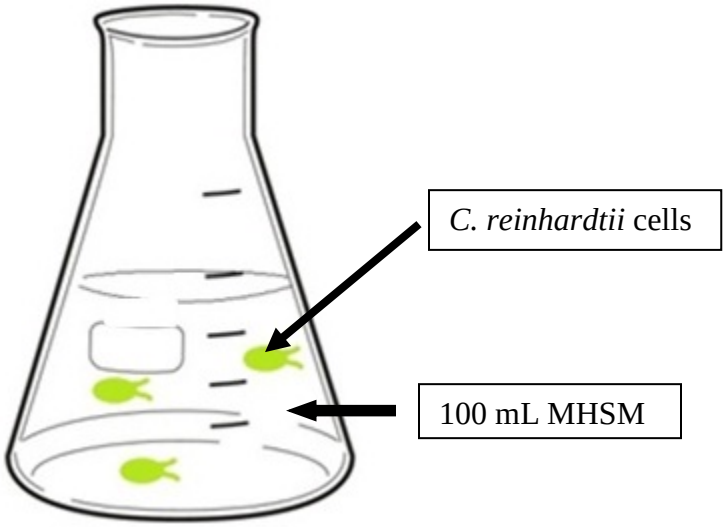

Figure 2.2 Schematic of Experiment 0

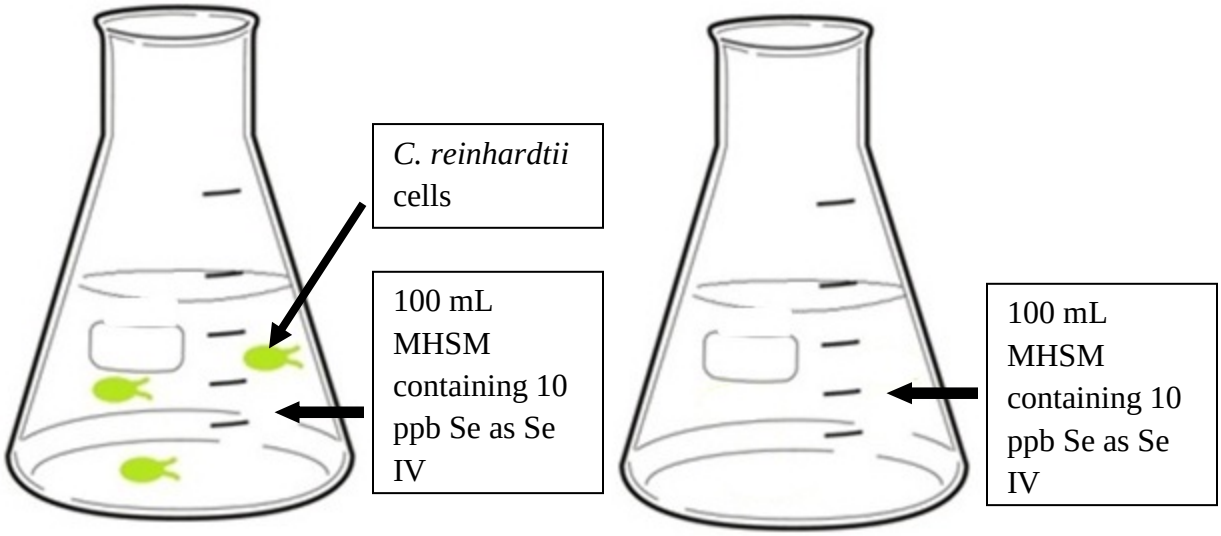

Figure 2.3 Schematic of Experiment 1

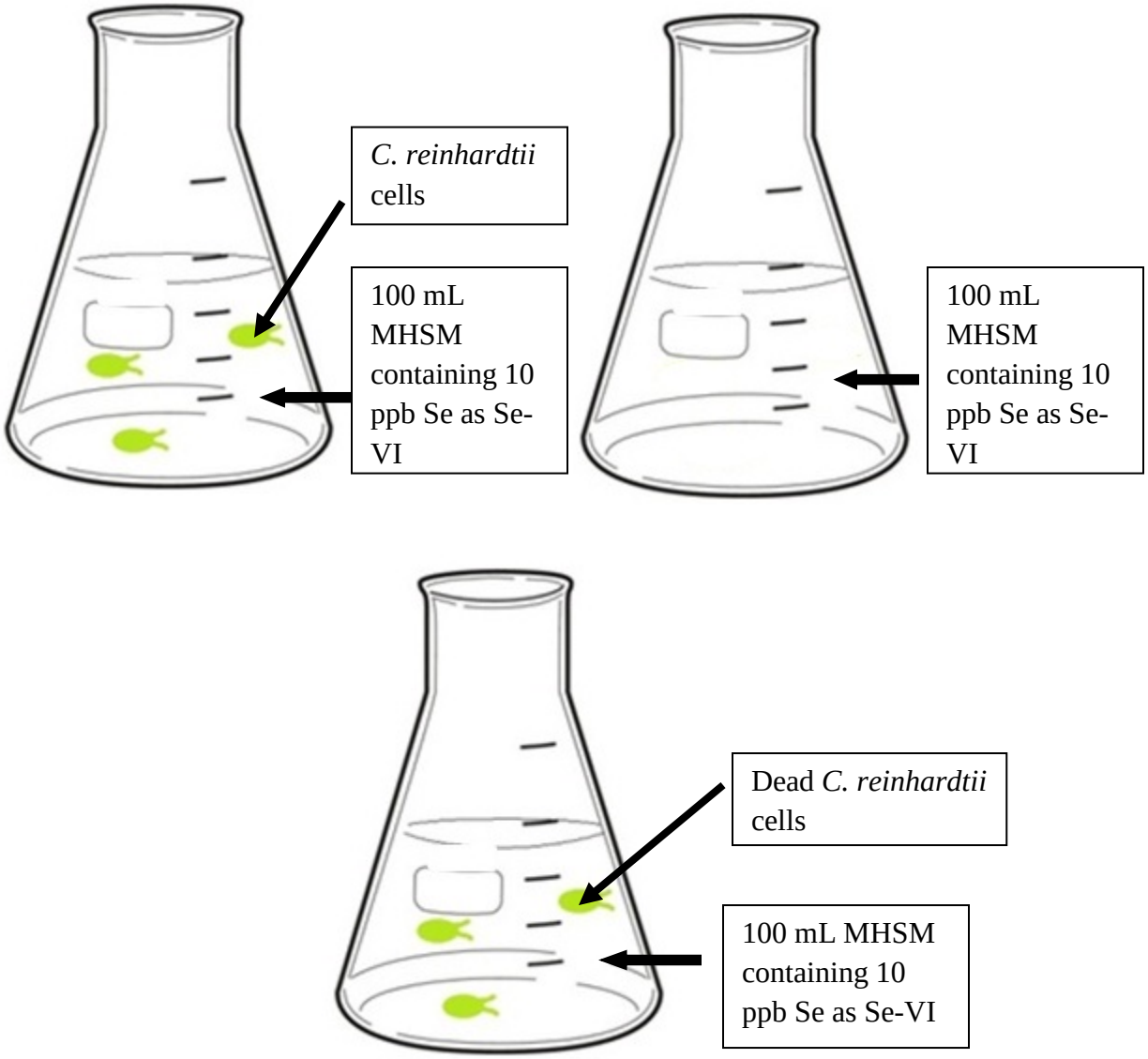

Figure 2.4 Schematic of Experiment 2

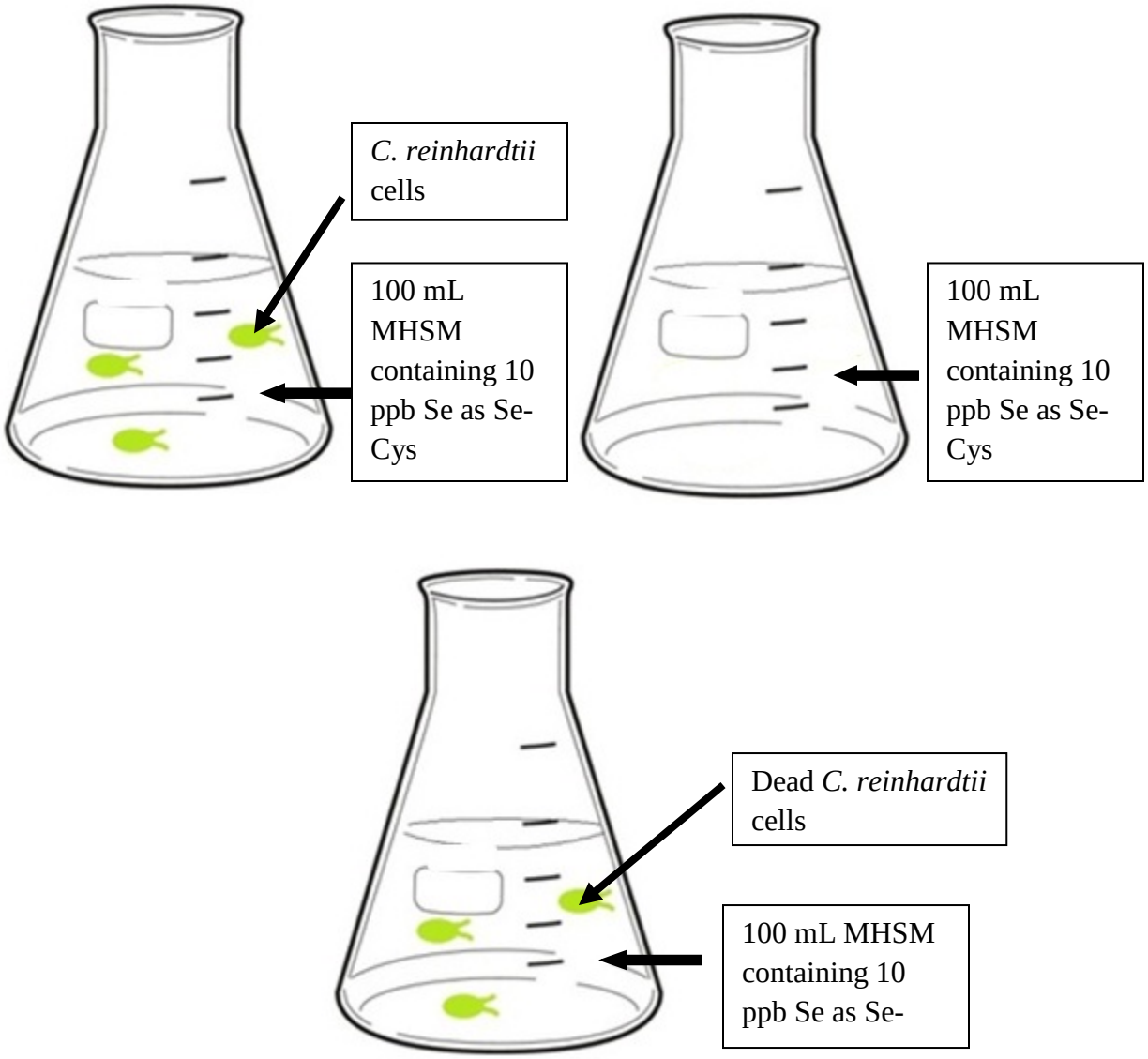

Figure 2.5 Schematic of Experiment 3

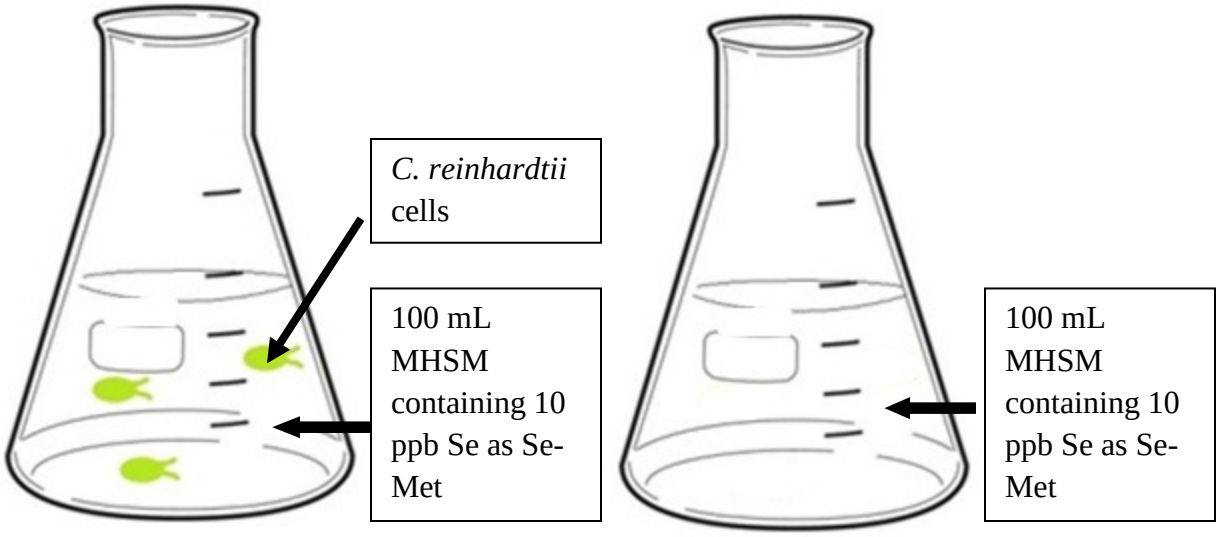

Figure 2.6 Schematic of Experiment 4

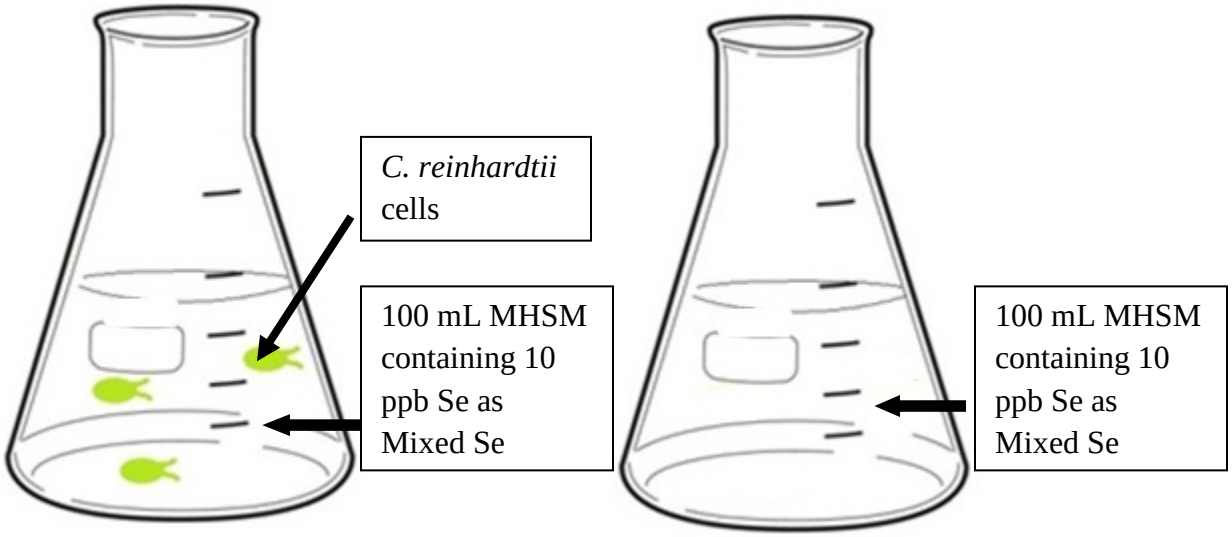

Figure 2.7 Schematic of Experiment 5

The 1000 mg $Se \cdot L^{-1}$ stocking solutions (Section 2.1.4) were diluted to 1:1000 resulting 1000 ppb Se standard solutions. Then 1 mL 1000 ppb Se solutions as Se-IV, Se-VI, Se-Cys and Se-Met were respectively diluted to 100 mL using MHSM in Experiment (1) to (4). For Experiment (5), 250 µL of each 1000 ppb Se solutions were added and diluted to 100 mL. As a result, the total Se concentrations in Experiments (1) to (5) were adjusted to 10 ppb (~127 nM). The algae was then added to each culturing flask and then gently shaken before being sampled under a laminar hood. Subsamples (1 mL of culture solution) were sampled at 0, 1, 2, 3, 6, 9, 12, 15, 24, 48, 72, 120 and 168 hours (a total of 13 samples). At the end of the uptake experiment, the medium has a volume of 87 mL because of subsampling for 13 times. The 1 mL algal inoculum subsamples

were analyzed using ultraviolet-visible spectrometer (Thermo, evolution 260 bio) to determine cell densities immediately after sample collection.

The control samples, in which the same initial total concentration of Se was adjusted using MHSM without algae cells, were incubated under the same conditions as those samples containing algae cells. The quantitative and qualitative changes of Se in the MHSM in the control samples were monitored as well; however, the control samples were sampled less frequently than the samples containing algae cells. The control samples were respectively sampled at 0, 24, 72 and 168 hours after the addition of Se.

For samples containing heat-killed algae cells in Experiment (2) and (3), each culture was treated in the same way as all other cultures except that samples were taken at 0, 1, 2 and 3 hours after the addition of the heat-killed algae cells.

All samples were filtered using 0.2-μm hydrophilic-polyethersulfone-membrane syringe filters (Pall, PN4602). Supernatants were collected in 1.2 ml cryogenic vials (Corning 430658) and stored at – 80^{o}C until analysis by high performance liquid chromatography-ultraviolet treatment-hydride generation-atomic fluorescence spectrometry (HPLC-UV-HG-AFS) to measure Se speciation (See section 2.1.4 for details on Se speciation analysis). Centrifuged algae pellets then were made to determine Se adsorbed on the cell wall (Section 2.1.5) using HPLC-UV-HG-AFS and total internal Se stored inside the algae cells using AFS (Section 2.1.6).

2.1.3 Cell density

The cell densities of algae cultures were monitored throughout the experiments. Once the algae solution was sampled from the culture medium, its absorbance was measured using a UV/Vis spectrophotometer. The absorbance between 650 nm and 750 nm varies with cell density, peaking at 687 nm (Figure 2.2). Therefore the magnitude of the absorbance at 687 nm of each subsample was used to determine the corresponding cell density. A calibration curve was constructed by plotting the absorbance of *C. reinhardtii* cultures at 687 nm vs. cell densities determined by manual counting performed at the CPCC, where 0.5-1 mL of algae cultures were subsampled and preserved using a 1% (i.e., 5-10 μL) aliquot of Lugol's solution (Lund, et al., 1958). Then the 12.5 μL aliquot of preserved algae was loaded onto the haemocytometer, and the number of algal cells in the central counting grid on both sides of the haemocytometer was counted (where possible) at 80–128x magnification on the microscope. The cell density of each subsample during the Se uptake experiments was then estimated using the calibration curve and corrected using a manual method at the CPCC to count the initial cell density.

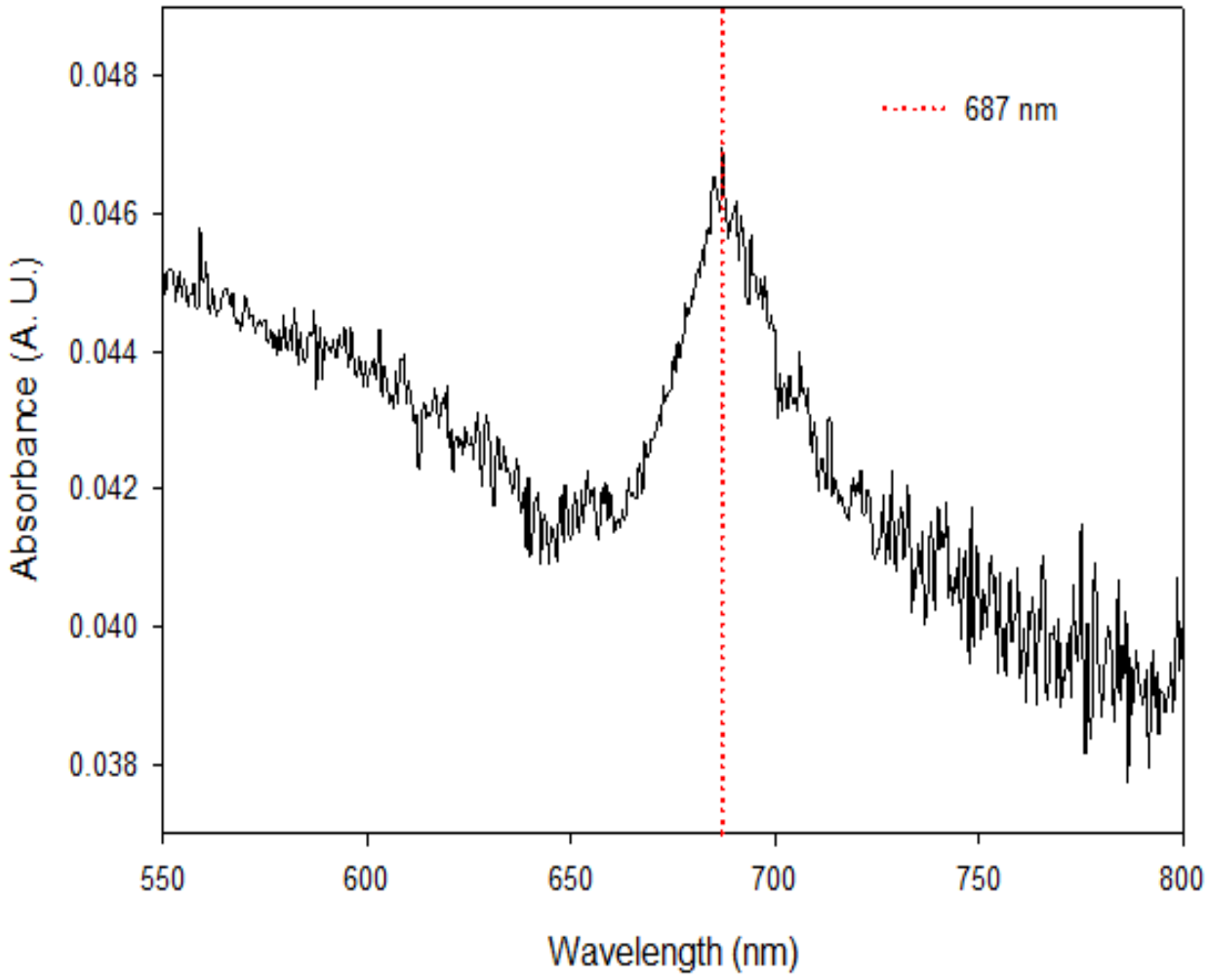

Figure 2.8 A sample UV/Vis absorbance of a *C. reinhardtii* culture sample between 550 to 800 nm, the peak value appeared at 687 nm

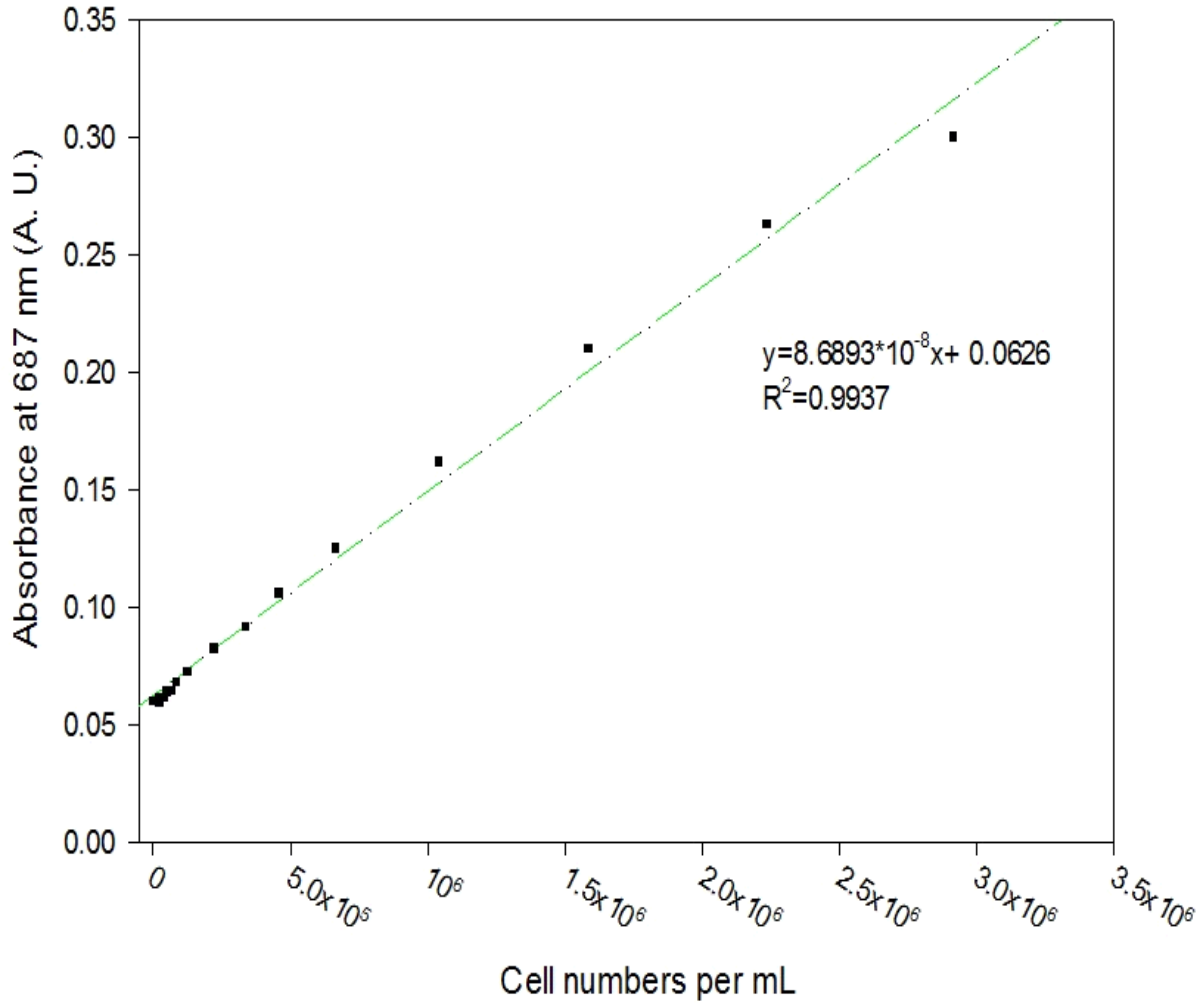

Figure 2.9 Cell density calibration curve of *C. reinhardtii* (dashed line), plotted by using absorbance at 687 nm using UV-Vis (Thermo, evolution 260 bio) against cell density counted under microscope at the CPCC. Algae in the exponential growth phase were sampled and diluted using ultra-pure water. The initial and diluted samples were counted using a manual counting method at the CPCC and the corresponding absorbance at 687 nm of each subsample was measured using UV-Vis.

2.1.4 Se speciation

One of the main goals of this project was to monitor Se speciation as a function of time, during Se uptake by *Chlamydomonas reinhardtii* in order to understand the chemical transformations of Se in an aquatic system. Various methods and instruments have been developed to quantify

soluble Se, for example, inductively coupled plasma - mass spectrometry (ICP-MS) is a popular way to analyze Se (Pedersen and Larsen, 1997; Shamberger, 1983; B'Hymer and Caruso, 2000; Cai, 2000; Uden, 2005). Another method is the conversion of Se to volatile Se hydride and the subsequent analysis of the Se hydride using elemental detection systems such as atomic absorbance spectrometer (AAS) and atomic fluorescent spectrometer (AFS). The latter technique has shown great sensitivity for the chemical analysis of Se at the part per trillion (ppt) level. The most important benefit of the Se conversion method is that the hydride generation process is able to significantly reduce matrix effects from samples (Cai, 2000). Regarding Se qualification, one of the most often used techniques to separate different Se species is high performance liquid chromatography (HPLC). HPLC is able to separate different Se compounds contained in the original sample, and the Se speciation can be determined by analyzing the separated Se species (Uden, 2005; Vilanó and Rubio, 2000; Bird et al., 1997). In this research, we coupled an HPLC to an AFS detector to separate and quantify Se species (HPLC-UV-HG-AFS, Fig. 2.10)

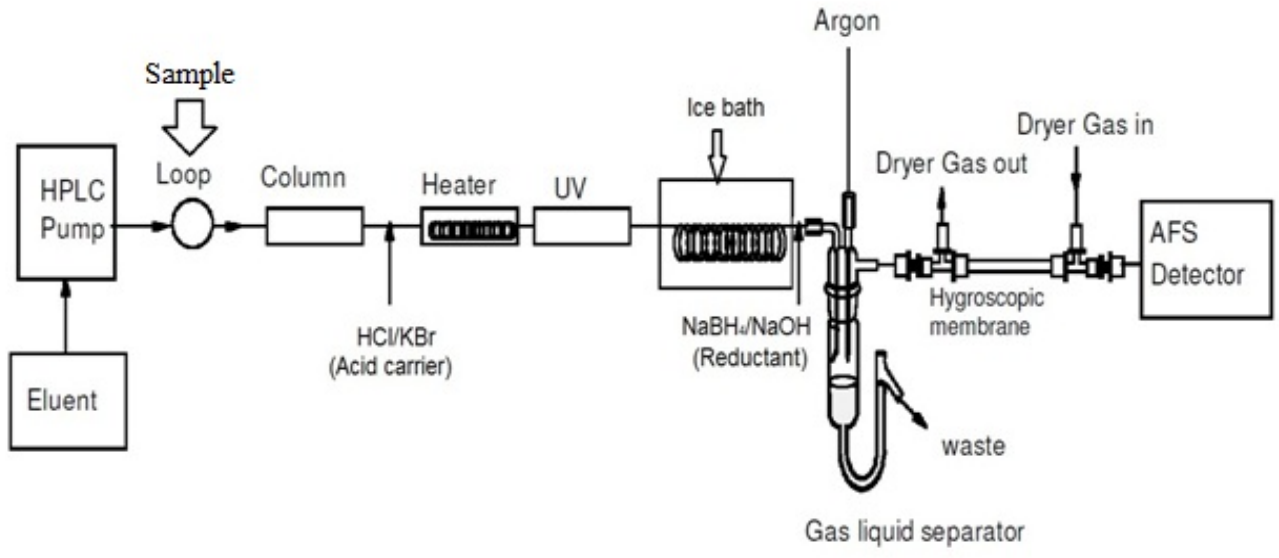

Figure 2.10 Schematic of the instrumental setup: the coupling of HPLC to AFS consists of a UV-heating block, an ice bath, a hydride generation block, a gas liquid separation and a gas dryer block. Adopted and modified from Ipolyi et al., (2001), open access

A 50 µL injection loop was rinsed and filled with at least 200 µL of filtered sample using a pre-rinsed syringe. The sample was then pumped in to the column (Hamilton, PRP-X100 Anion Exchange, 250 x 4.1 mm) by the HPLC pump at a rate of 1 mL per minute. The order of elution was as follows: Se-Cys in the first, then Se-IV, followed by Se-Met, and Se-VI at the end. Because only Se-IV can be transformed to Se hydride, all other Se species had to be converted to Se-IV before the hydride generation step. Se-VI was reduced in-line using hydrochloride acid (HCl) heated to 150^{o}C while organic forms were oxidized using an in-line UV system. All samples underwent both treatments consecutively. After the conversion of all Se species to Se-IV,

Se-IV was then reduced by hydride generation. The Se hydride evolves as gas in the gas liquid separator. The reduction reagent used in the instrument was sodium borohydride ($NaBH_4$, Alfa Aesar, 88983). Before the hydride generation step, the sample extracts were cooled by passing the tubes through an ice bath to avoid sample loss due to the quick vaporization of Se hydrides. Se hydrides were then carried out to the detector using Argon (Ar) gas (Praxair, AR 5.0UH). A constant diffusion of hydrogen was created by hydride generation using $NaBH_4$, and a hydrogen diffusion flame was lit up throughout the Se speciation analysis. Se hydride was then atomised by the hydrogen diffusion flame and the atomic fluorescence was detected at a wavelength of 196 nm using a Se boosted discharge hollow cathode lamp (Se HCL superlamp, Photron Ltd., P849S). Then the light signals were transferred to computer system as electronic signals, and the electronic signals were finally recorded by the software SAMS.

As the four Se species were separated by the HPLC, the signals of each species were separately monitored and measured. All liquid wastes from used chemicals were separated using the gas–liquid separator and collected as liquid waste. The gaseous wastes produced at the hydride generation step were collected by a venting pipe at 10 m^3 hr^{-1}. In order to maintain the performance of the HPLC-UV-HG-AFS system, the HPLC system was flushed using a mobile phase for 10 minutes before and after the operations, and the AFS system was rinsed with deionised water for at least 10 minutes after operation. The details of the Se standards and reagent solution compositions used for instrument performance are given below.

Stock solutions containing 1000 mg $Se \cdot L^{-1}$ as Se-IV, Se-VI, Se-Cys and Se-Met were prepared by respectively by dissolving 0.2190 g of sodium selenite, Na_2SeO_3, 98% (MP Biomedicals, Solon, Ohio, USA); 0.2393 g sodium selenate, Na_2SeO_4, 99.5% (Pfaltz&Bauer, Waterbury, CT, USA); 0.2116 g of L- Se-Cys, $CO_2HCH(NH_2)CH_2(Se)_2CH_2CH(NH_2)CO_2H$, 98%, (Acros, Geel, Belgium); and 0.2484 g DL-Se-Met, 99%, (Acros, Geel, Belgium) in 50 mL 18 Ω Milli-Q water and then diluted to 100 mL. In order to completely dissolve Se-Cys to make the 100 mL stock solution, 1 mL 0.5 % (v/v) HCl was added to the solution. Individual and mixed standards with known concentrations were freshly prepared by diluting the 1000 mg $Se \cdot L^{-1}$ stock solutions each time before using the instruments.

The mobile phase (5 mM citric acid, 2% methanol and pH=5.9) used for the HPLC system was prepared by dissolving 0.960 g citric acid and 20 mL HPLC grade methanol in approximate 950 mL ultra-pure water. Approximate 3 mL of 20% (m/v) NaOH was then used to adjust the pH of the mobile phase to 5.9. Afterwards the solution was diluted to 1 L with ultra-pure water. The mobile phase solution was then filtered through 0.2-µm membrane (polyethersulfone filters, Millipore GPWP04700) to avoid potential blockage from occurring either in the HPLC tubes or in the column. Furthermore, the mobile phase solution was only kept for at most 2 days. Acid carrier solution (50% v/v HCl and 5% KBr) was prepared by dissolving 50 g potassium bromide (KBr) in 250 mL of ultra-pure water, then mixing with 500 mL of concentrated HCl and diluting to 1 L with ultra-pure water. Occasionally the KBr forms precipitates after mixing with HCl, and the precipitates must be re-dissolved in the solution using a sonicator before using the KBr

solution. The reductant solution (0.7% m/v $NaBH_4$ and 0.1 M NaOH) was prepared by dissolving 4.0 g of NaOH in 500 ml of ultra-pure water, then dissolving 7.0 g of $NaBH_4$ in the solution and diluting to 1 L. Air bubbles, produced inside the solution, were removed by using a sonicator for approximate 10 minutes. Typical experimental conditions and settings are listed Appendix I.

The signals obtained from measuring Se using HPLC-UV-HG-AFS were subtracted by the average signals of the background acquired over 200 to 600 data points to obtain the baseline. Each peak in the chromatogram represents a Se species. A sample chromatogram obtained from Se speciation measurement using HPLC-UV-HG-AFS is shown in Figure 2.11. During the operation, the Se-Cys peak appeared around 3.4 minutes after injection, then the Se-IV, Se-Met and Se-VI peaks appeared around 4.7 minutes, 6 minutes and 10 minutes after the sample injection, respectively. The peak heights were used to construct the calibration curve and determine the concentrations of unknown samples. In order to construct a calibration curve, duplicates of mixed standards consisting of Se-IV, Se VI, Se-Cys and Se-Met with five concentrations (0 ppb, 1 ppb, 2 ppb, 5 ppb and 10 ppb) were measured. A sample of calibration curve is shown in Figure 2.12:

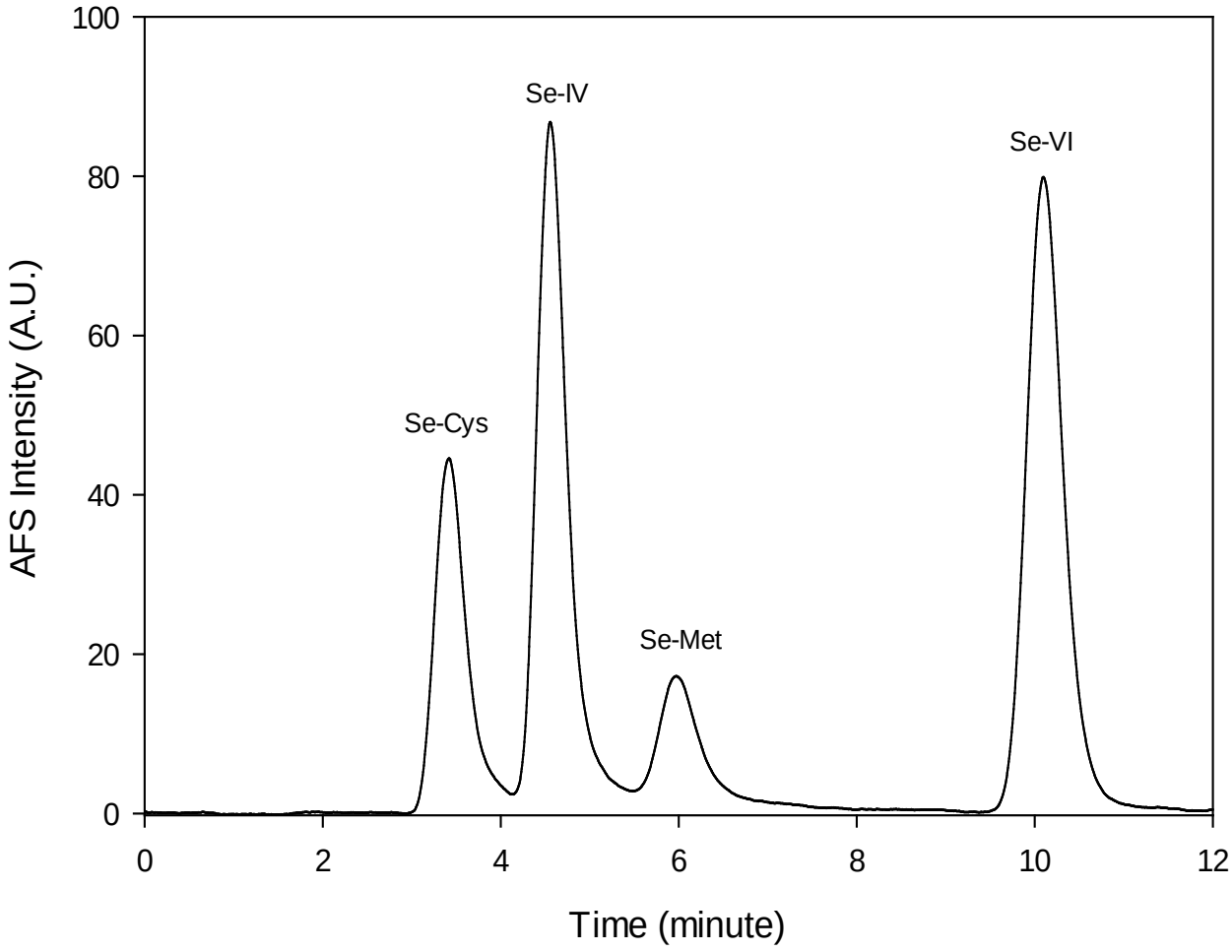

Figure 2.11 A typical chromatogram of a sample containing 10 ppb Se as Se-Cys, 10 ppb Se as Se-Met, 10 ppb Se as Se-IV and 10 ppb Se as Se-VI. The solid line represents the intensity recorded as a function of time by the AFS detector with a gain of 10.

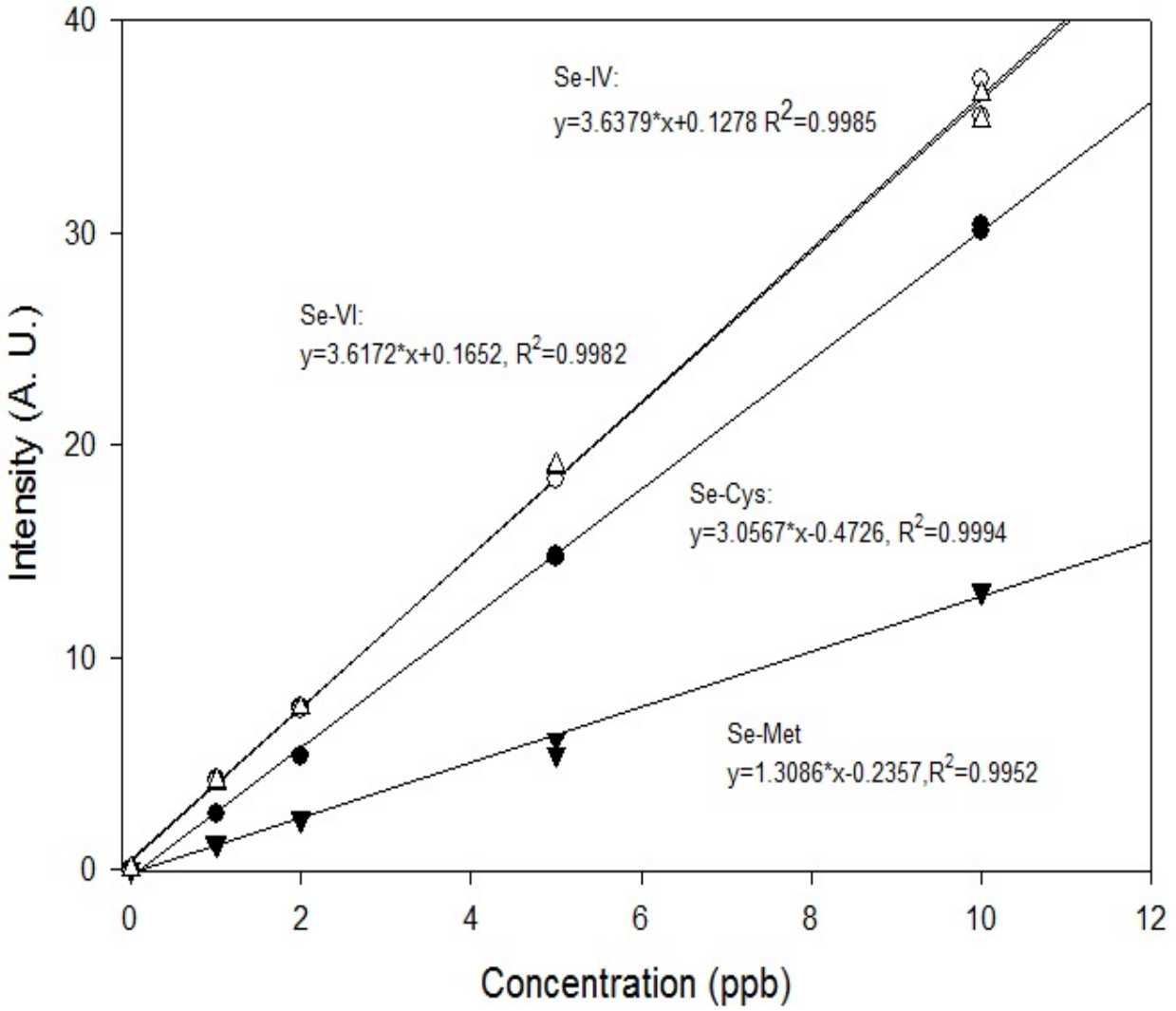

Figure 2.12 Sample calibration curves for Se speciation analysis using HPLC-UV-HG-AFS. The open circles represent Se-IV standards, the open triangles represent Se-VI standards, the solid circles represent Se-Cys standards and the solid triangles represent Se-Met standards. The best-fit-curves for each Se species are calculated and illustrated as calibration curves.

2.1.5 Se sorption on the cell wall of *C. reinhardtii*

A certain amount of Se may be attached on the algae cell wall after incubating the algae in the MHSM with Se for 7 days. Most of the Se that would be attached to the cell walls would be in the form of two negatively charged oxy-anions SeO_3^{2-} and SeO_4^{2-} (Boisson et al., 1995; Riedel et al., 1991; Morlon et al., 2006). Therefore an anionic wash was applied to the algae cells in order to exchange the Se oxy-anions on the cell wall with anions in solution. In this experiment, an

alkaline solution (NaOH) was employed for the anionic wash. The exact procedure is described in the following paragraph.

After growing in 100 mL of the MHSM medium for 168 hours, the algae cells were harvested by centrifugation for 30 minutes at 4500 rpm. The resulting algae pellets then were washed using 2 mL Se-free MHSM, shaken at 100 rpm for 25 minutes, and centrifuged again at 4500 rpm for 10 minutes. This procedure of washing the harvested *C. reinhardtii* was then repeated until the supernatant solution from the last centrifugation contained no Se. The supernatant solution was then analyzed for Se concentrations using HPLC-UV-HG-AFS in order to ensure all Se from the initial medium was completely rinsed away.

The algae pellets were then subjected to anionic wash using 1mL alkaline solution (NaOH, pH=10.5) and shaken afterwards at 100 rpm for 30 minutes. The mixtures were re-centrifuged., and the resulting supernatant solutions were collected and filtered through 0.2-µm polyethersulfone filters. The Se in the supernatant was analyzed to measure the amount of Se complexed on the cell wall of *C. reinhardtii*. The Se speciation of each sample was analyzed using HPLC-UV-HG-AFS. Meanwhile, the centrifuged algae pellets were collected and stored at -80^{o}C for the next step to analyze the total internal Se stored within the algal cells.

2.1.6 Total Se digested inside the cell wall

Se, after being utilized by algae, can appear as different chemical forms in the algae cells (Figure 1.4); however, there is no available tool, in this research, to break down the algae cells to determine the speciation of Se stored inside. In addition, the HPLC-UV-HG-AFS used in this project is able to identify and analyze only certain Se species, including the four Se species used in this thesis. Therefore, the internal Se within algae cell walls was measured as a total Se using AFS.

Among all Se species, AFS can only measure Se-IV. As a result, all possible Se species required to be converted to Se-IV. This conversion was achieved in two steps.

In the first step, algal pellets were digested using concentrated HNO_3 which oxidizes all Se species to Se-VI. The acid digestion step, adopted from a method from Morlon et al. (2006), uses 1 mL of concentrated HNO_3 (65%) to dissolve the algae cells in borosilicate test tubes for 24 hours at room temperature . An immediate colour change from green to dark brown was observed when the concentrated HNO_3 was added to the algae pellets. After 24 hours of acid-digestion, all precipitates were dissolved and the solution exhibited as clear yellow colour. Then the samples were slowly heated on a hot plate and allowed to evaporate until near dryness. The samples were then allowed to cool down to room temperature. At this stage, all Se should exist as Se-VI. In the second step of Se conversion, Se-VI was reduced to Se-IV using HCl:

$$SeO_4^{2-} + 2HCl \rightarrow SeO_3^{2-} + Cl_2\uparrow + H_2O \qquad \text{Equation (1)}$$

A 1 mL aliquot of 30% concentrated HCl was added to the borosilicate tube to dissolve the Se-VI left over, and then 4 ml of concentrated HCl was added to the borosilicate tube. The Se extracts then were heated for 30 minutes at 140^{o}C in the open test tubes, allowing Cl_2 to evaporate. After 30 minutes, samples were allowed to cool down to room temperature and analyzed within 24 hours to minimize further changes in Se speciation.

The procedure of measuring total Se is the same as for measuring individual Se species. With the one difference that only the AFS module was used. Since all Se have been converted to the Se-IV form, there is no need for a UV-heating system. The reductant solution (0.7% m/v $NaBH_4$ and 0.1 M NaOH) was made in the same way as the one used in the Se speciation analysis. A reagent blank was made by diluting concentrated HCl to 30% v/v. During the total Se analysis, the AFS was coupled to an auto sampler system; therefore an additional 3% v/v HCl batch was prepared to rinse the auto sampler system. Pre-reduced Se-IV samples were diluted to 10 mL using the reagent blank and transferred to 50 mL tubes. Then each sample was diluted again to 1:10. Then the diluted samples were introduced to the AFS auto sampling system and the reduction solution reacted with Se-IV in the sample to form Se hydrides, which were then atomised by the hydrogen diffusion flame. The signals were transferred to a computer and analyzed by the software Millennium. Each sample took at least 2 minutes to analyze (Figure 2. 13).

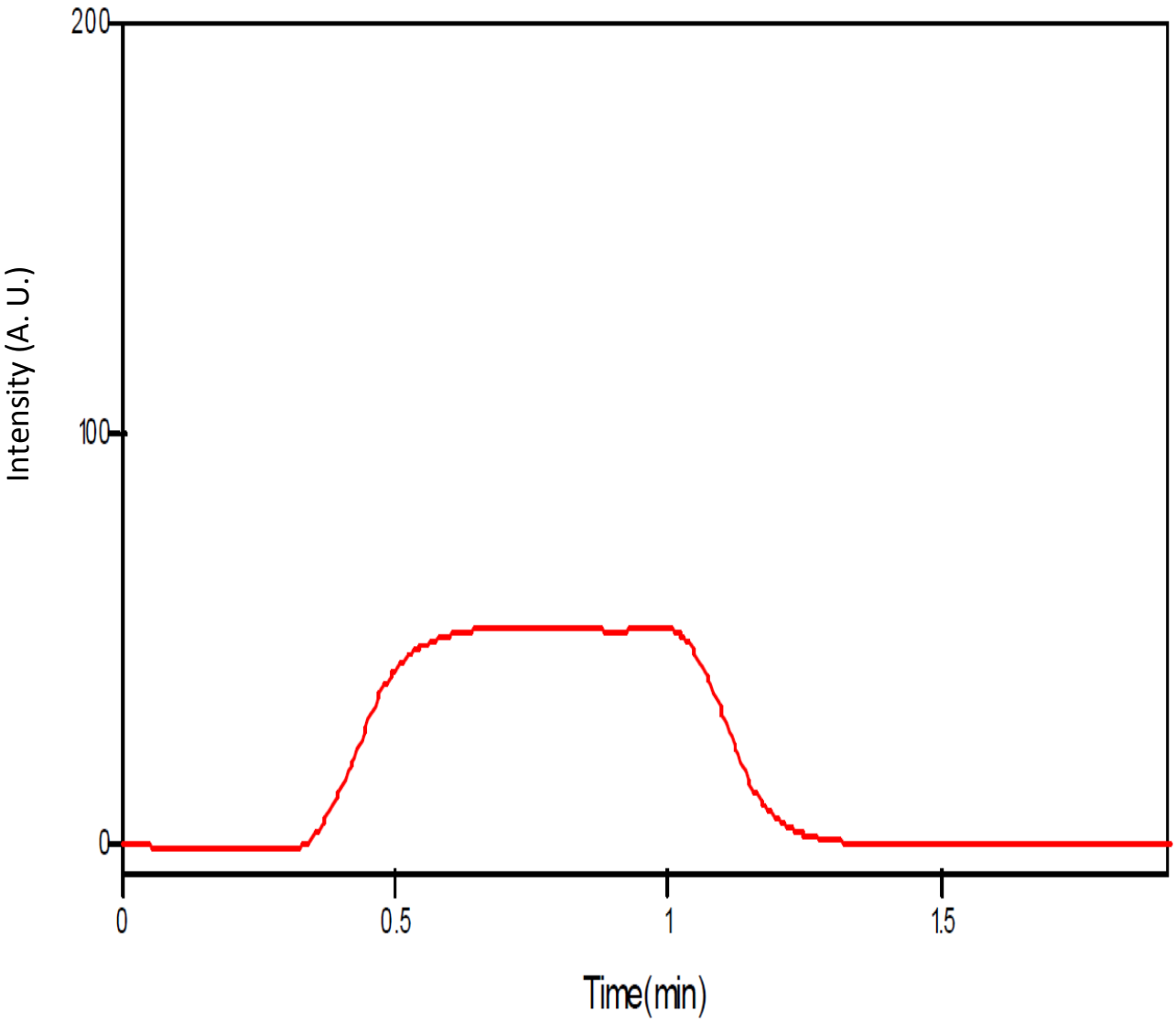

Figure 2.13 A sample of total Se as measured by AFS. The line represents the signals of a 50 ppb Se sample recorded as a function of time. Peak height and area were automatically calculated by computer.

Calibration curve was constructed using standards with the concentrations of 0, 0.5, 1, 5, 10, 20 ppb Se as Se-IV. The stock solution of Se-IV that was used was the same one as the one prepared for Se speciation experiment; however, in order to dilute the 1000 ppm Se-IV stock solution to the required concentrations, the 30% concentrated HCl reagent blank was used as the diluent for each dilution. Likewise, all samples were also diluted with the reagent blank. Furthermore, a 5 ppb Se as Se-IV standard was prepared separately to check the calibration curve. The standard calibration curve is shown in Figure 2.14, and the linear function of the calibration curve is calculated by computer as $y=8.4069x+0.3076$, $R^2=0.9999$.

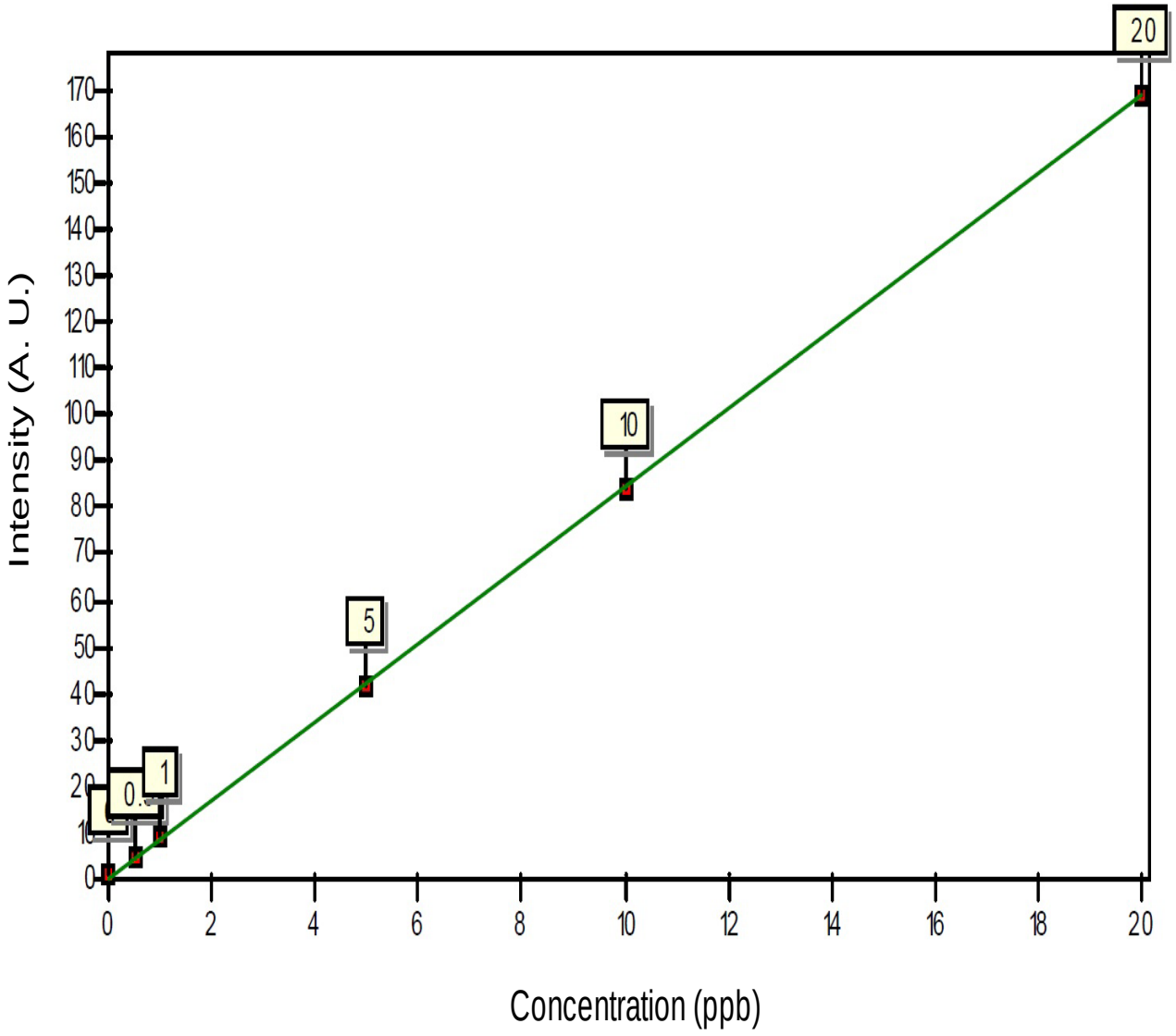

Figure 2.14 Standard calibration curve of total Se measurement using AFS. Each point respectively represents peak area values of 0 ppb, 0.5 ppb, 1 ppb, 5 ppb, 10 ppb and 20 ppb Se standards. The green line represents the best-fit curve and is used as the calibration curve.

With the Se concentration of 10 mL Se digestion, it is then possible to calculate the total amount of Se retained in the algae cells using

$$m_{(\text{Total Se})} = c * V \qquad \text{Equation (2)}$$

In equation (2), $m_{(\text{Total Se})}$ is the total amount of Se retained in harvested algae cells, c is the Se concentration measured in the 10 mL Se digestion solution and V is the volume of the solution (10 mL).

2.2 The diagenetic redistribution experiment

2.2.0 Experimental design

In the diagenetic redistribution experiment (Figure 2.15), the Se released to sediments from precipitated dead *C. reinhardtii* overlying a layer of sediments was measured. Harvested algae incubated in Se-rich medium were mixed at the top of a layer of marine sediments and the sediments were incubated for seven weeks. Pore water in the sediments were sampled and analyzed for Se speciation and concentrations to determine the Se released back to the sediments during the decomposition of the organic carbon from the algae cells.

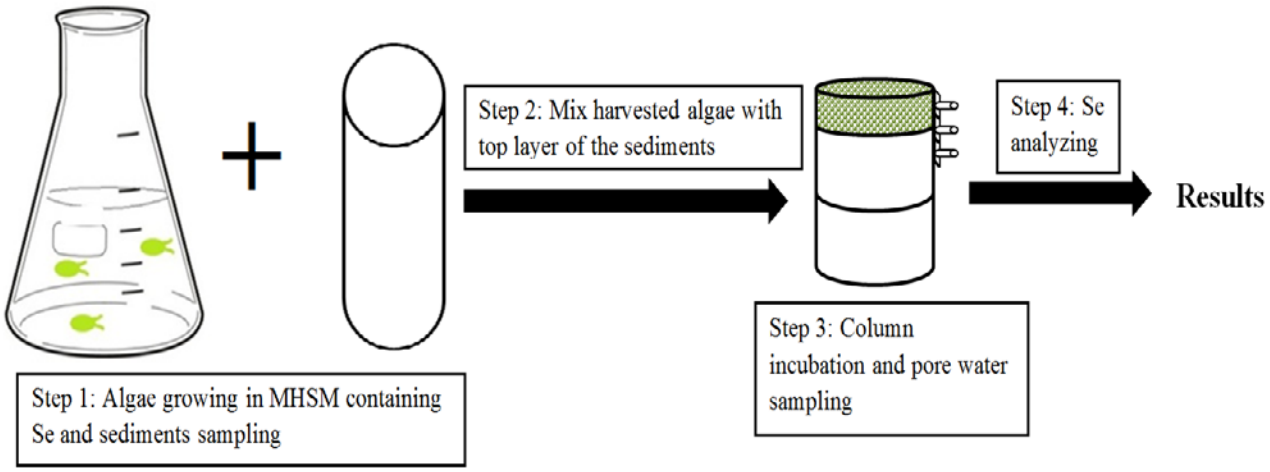

Figure 2.15 The Se diagenesis experiment design

2. 2. 1 Sediment sample collection

Sediment cores were sampled at Penouille (48°51'07.43" N and 64°25'45.92" W), Quebec, Canada in the Gaspé bay. The Gaspé bay is characterized as a fine salt marsh covered by shallow sea water. Sediments were sampled using cores with an inner diameter of 7 cm and a length of 30 cm. Sampling cores were gently inserted into the salt marsh and then sealed from the bottom. Dynamic biological activities including abundant phytoplankton and benthic fauna in the sea water were observed at the sampling site. Three cores were taken from the site for this thesis (labeled as Column 1, 2, and 3). Other organisms, such as unidentified worms were observed in the sample sediments. The worms were found moving through the core vertically and horizontally, and sometimes they moved to the water column. One core was used as the control sample, and the other two were used for diagenetic study. Furthermore, seawater was collected and filtered using 0.45 µm filters (Nylon membrane filter, Millipore HNWP04700). The filtered seawater was added into the column to construct a near-natural environment and re-added often to the column throughout the experiment to keep water level stable.

2. 2. 2 Algae growth

The goal of the diagenesis experiment was to explore the fate of the Se utilized and retained in the algae cells. Therefore this experiment required *C. reinhardtii* cells with significant amount Se retained in the algae cells after Se utilization. In this experiment, *C. reinhardtii* cultures were incubated in selected Se species and the total internal Se measured. Then the *C. reinhardtii* cells containing a known amount of total Se in their cells were harvested.

Nine batches of *C. reinhardtii* were cultured in 100 mL MHSM containing 50 µg Se as Se-Met, therefore the Se concentration was adjusted as 500 µg Se/L (6.33 µM Se). After 7 days growth, the harvested algae cells were collected by centrifugation. Then the algal pellets from all nine cultures were mixed and transferred to a 50 mL test tube. Then the cells were rinsed with Se-free MHSM and centrifuged afterwards. The rinsing process was repeated five times to eliminate the effect of leftover Se from the initial medium. Once no Se was detected in the supernatant using HPLC-UV-HG-AFS, the harvested cells were diluted to 45 mL using MHSM and shaken at 100 rpm for 20 minutes. A 5-mL aliquot of the homogeneous algae solution was sub-sampled to determine the total internal Se content stored in the algae cells (Section 2.1.6). The rest of the algae solution was then divided into two 20-ml sub-samples. In the next step, the sub samples were centrifuged and the wet weight of each sample was recorded.

2. 2. 3 Column incubation

The three sediment cores, labeled as Column 1, 2 and 3 (Section 2.2.1) were incubated in a custom incubation chamber in the dark at 12^{o}C using a water circulation system. Column 1 contained no algae cells and was therefore designated as the control. The first top 1 cm of the sediment in Column 2 and 3 were sliced out and mixed with the two harvested algae samples, and then each sediment sample mixture was respectively placed back to Columns 2 and 3. It needs to be noted that some sediments with unknown amount was spelled from the Colum 2 during the sampler installation and this could affect the experimental results.

Three custom samplers were installed at each column at various locations in order to sample the pore water from the sediments at specific depths. Namely, the samplers were installed in the column near the sediment-water interface: 0.5 cm, 1.0 cm and 1.5 cm depth from the top of the sediments. A 1-mL water sample was weekly sampled from each depth for 7 weeks. To avoid the water from diffusing, the sampling process followed an order of sampling from the top to the bottom. Water samples were stored at -80^{o}C for the further Se analysis. The Se analysis followed the method described in section 2.1.4, providing the information on the speciation and the amount of the Se released to sediments from decomposed algae.

3.0 Results

3.1 Se uptake by C. reinhardtii

3.1.1 Algae growth

The average cell densities of *C. reinhardtii* monitored in triplicates throughout the 168 hours incubation in the MHSM containing selected Se compositions are shown in Figure 3.1. In all algae cultures the cell densities increased, which confirms biomass accumulation during the experiments. After 168 hours, this research found the algae cultures had different cell densities depending on the Se species introduced to the medium. Most notably, the culture incubated in the Se-free medium had the lowest cell density. For the Se-exposed algae cultures, the cell densities found after 168 hours were, in order from low to high: Se-Met, Se-VI, mixed Se species, Se-IV and Se-Cys. Detailed results on cell densities during algae growth of each culture are listed in Appendix II.

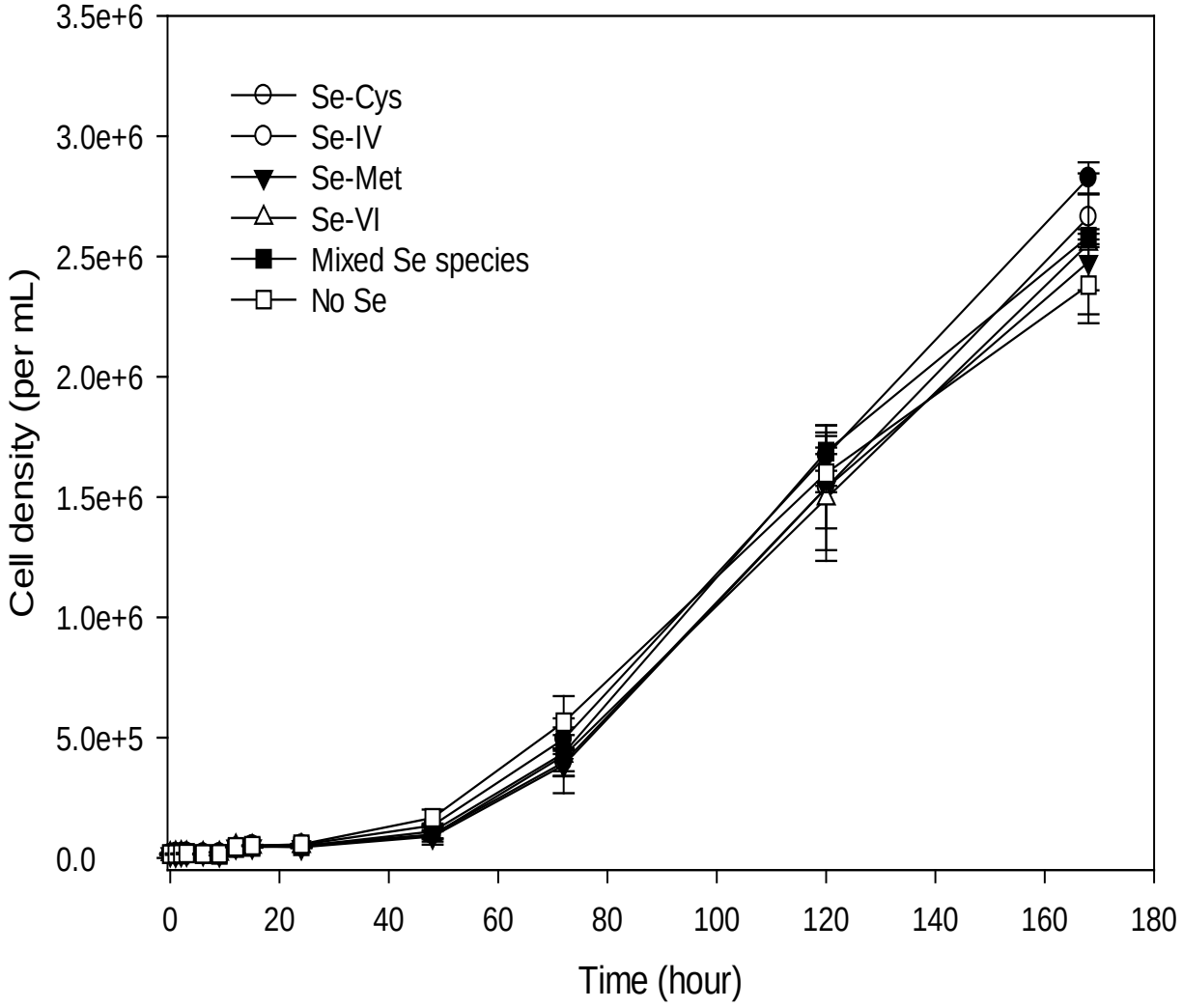

Figure 3.1 Cell densities of 168 hours of *C. reinharditii* grown in modified high salt medium containing 10 ppb Se as Se-Cys (solid circles), 10 ppb Se as Se-IV (open circles), 10 ppb Se as Se-Met (solid triangles), 10 ppb Se as Se-VI (open triangle), 10 ppb Se as mixed Se species (2.5 ppb Se as Se-Cys, 2.5 ppb Se as Se-IV, 2.5 ppb Se as Se-Met and 2.5 ppb Se as Se-VI) (solid squares), and in the Se-free MHSM (open squares). Error bars indicate ±1 Standard Deviation (SD) of triplicate samples.

The algae growth rate was stationary in the first 24 hours, but accelerated (Monod, 1949; Bolier and Donze, 1989) between 24 hour to 72 hour and reached the exponential phase at 72 hours. The growth rate was linear between 72 and 168 hours. In the stationary phase, the *C. reinhardtii* cells started exchanging chemical nutrients with the medium and the net growth of algae cells was negligible. After the stationary phase, algae began growing and biomass started accumulating at an accelerated rate. As the nutrients in the medium were consumed, one or more

nutrients in the medium may have been exhausted, thereby limiting algae culture (Monod, 1949). The culture then grew at a slower rate as observed in the linear growth phase.

3.1.2 Experiment 0: Se speciation in algae culture in MHSM with no addition of Se

Ambient Se remained stable at below detection throughout the background study, Experiment 0. All measured concentrations were lower than the instrument detection limit (0.4 ppb).

3.1.3 Experiment 1: Se-IV uptake by *C.reinhardtii*

The results from control experiment (no algae added) show that the Se-IV concentration in the MHSM remained relatively stable and that there was no chemical transformation between Se-IV and any other Se species observed during the 168 hours incubation (Figure 3.2). The result of Se-IV uptake by *C. reinhardtii* is shown in Figure 3.3.

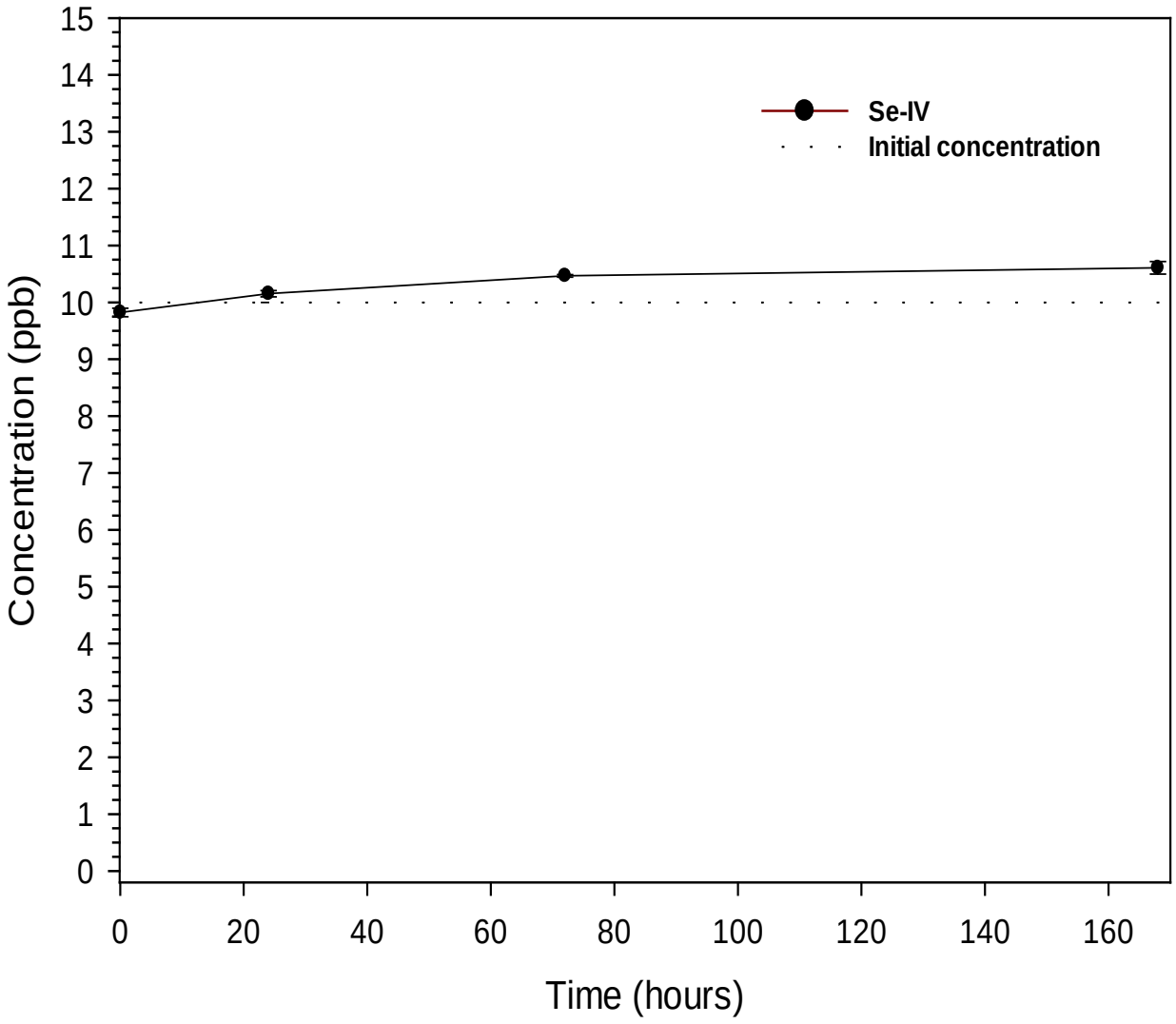

Figure 3.2 Se concentrations of Se-IV in modified high salt medium containing 10 ppb Se as Se-IV throughout 168 hours. Average results of the triplicate experiments are presented; error bars indicate ±1 SD. Solid circles represent Se-IV results from each sample dates and the short dashed line represents the initial Se concentration.

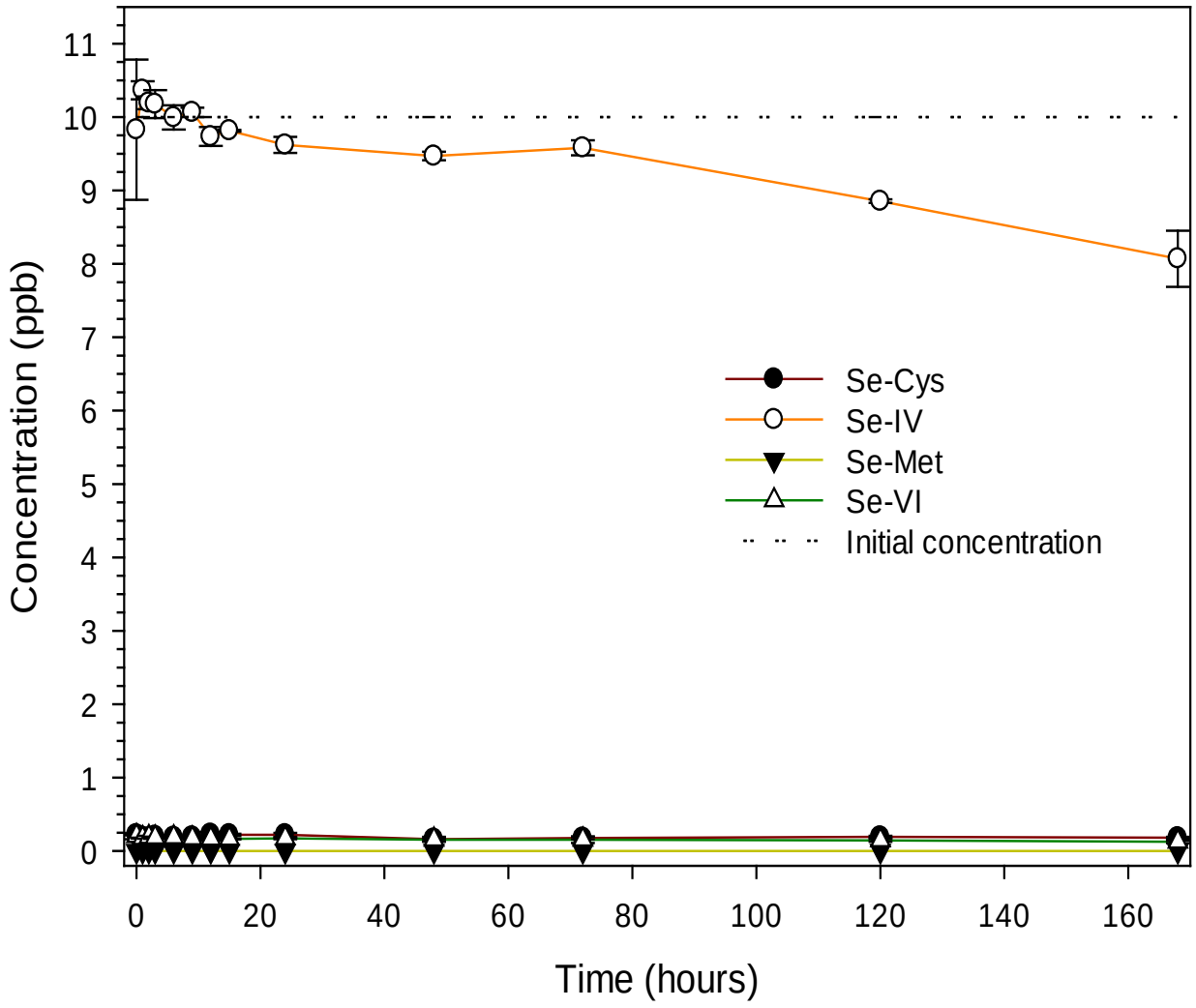

Figure 3.3 Se concentrations of Se species in the medium throughout 168 hours growth of *C. reinhardtii* in modified high salt medium containing 10 ppb Se as Se-IV. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. The solid circles represent Se-Cys, the open circles represent Se-IV, the solid triangles represent Se-Met and the triangles represent Se-VI concentrations. The dash-dot line represents the initial Se concentration of Se-IV in the medium and the straight lines connect the data points of each Se species represent the changing trend of Se species as a function of time.

Two observations can be made from the results presented in Figure 3.3. Firstly, the Se-IV in the medium slowly decreased over the 168 hours. For the first 9 hours of the experiment, Se-IV concentration in the medium stayed relatively stable. After the first 9 hours, Se-IV started to decrease in the medium. After the first 48 hours, the decrease of Se-IV became slightly faster. At the end of the experiment, 2 ppb Se as Se-IV were removed from the medium. Secondly, there

were no obvious speciation changes occurring in the medium during the exposure of Se-IV to *C. reinhardtii*. All other three Se species concentrations remained under the detection limit (0.4 ppb Se) throughout the entire experiment.

3.1.4 Experiment 2: Se-VI uptake by *C. reinhardtii*

Three sets of results were obtained from Experiment 2: control, uptake and adsorption by heat killed cells. Results from the control samples are shown in Figure 3.4. Se-VI remained stable with slight fluctuations; however, there was no significant change in Se speciation during the 168 hours incubation.

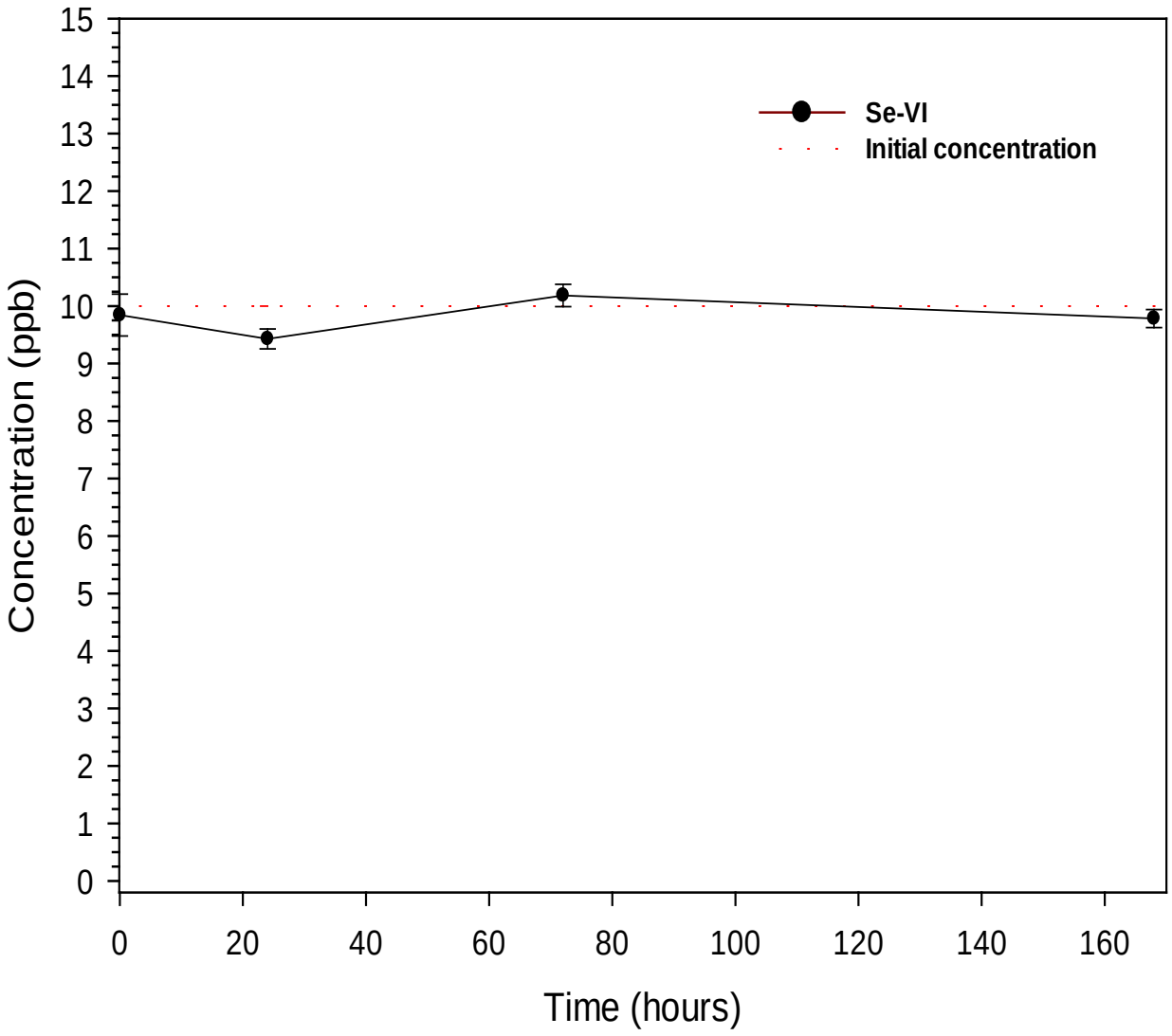

Figure 3.4 Se concentration of Se-IV in modified high salt medium containing 10 ppb Se as Se-VI throughout 168 hours. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. Solid circles represent the concentration of Se as Se-VI results obtained from each sample point and the short dashed line represent the initial Se concentration.

Results obtained from the Se-VI uptake experiment are shown in Figure 3.5. The concentration of Se as Se-VI in the medium dropped immediately from the initial concentration of 10 ppb to near 7.1 ppb at the beginning of this experiment (0 hours). The concentration of Se as Se-VI subsequently increased during the first 12 hours and then decreased continuously afterwards. At 168 hours, the concentration of Se as Se-VI in the medium was 4.9 ppb. The concentrations of Se-Cys, Se-Met and Se-IV remained under the detection limit throughout the experiment.

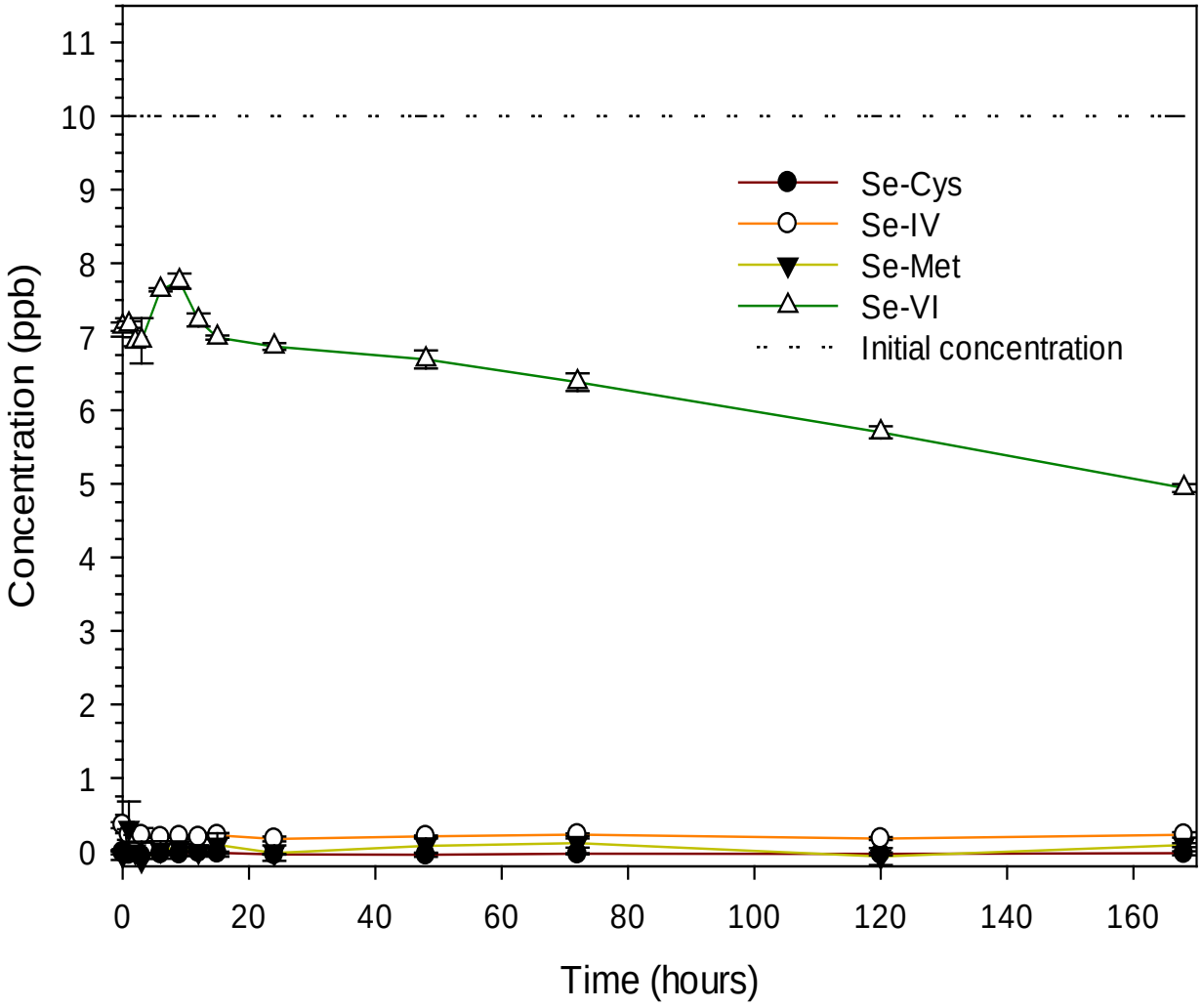

Figure 3.5 Se concentrations of Se species in the medium throughout 168 hours incubation of *C. reinhardtii* in modified high salt medium containing 10 ppb Se as Se-VI. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. The solid circles represent Se as Se-Cys, the open circles represent Se as Se-IV, the solid triangles represent Se as Se-Met and the triangles represent Se as Se-VI concentrations at each sampling point. The dash-dot line represents the initial Se concentration.

The results of experiment with heat-killed algae showed that there was no instantaneous loss of Se-VI in the medium for the first three hours (Figure 3.6).

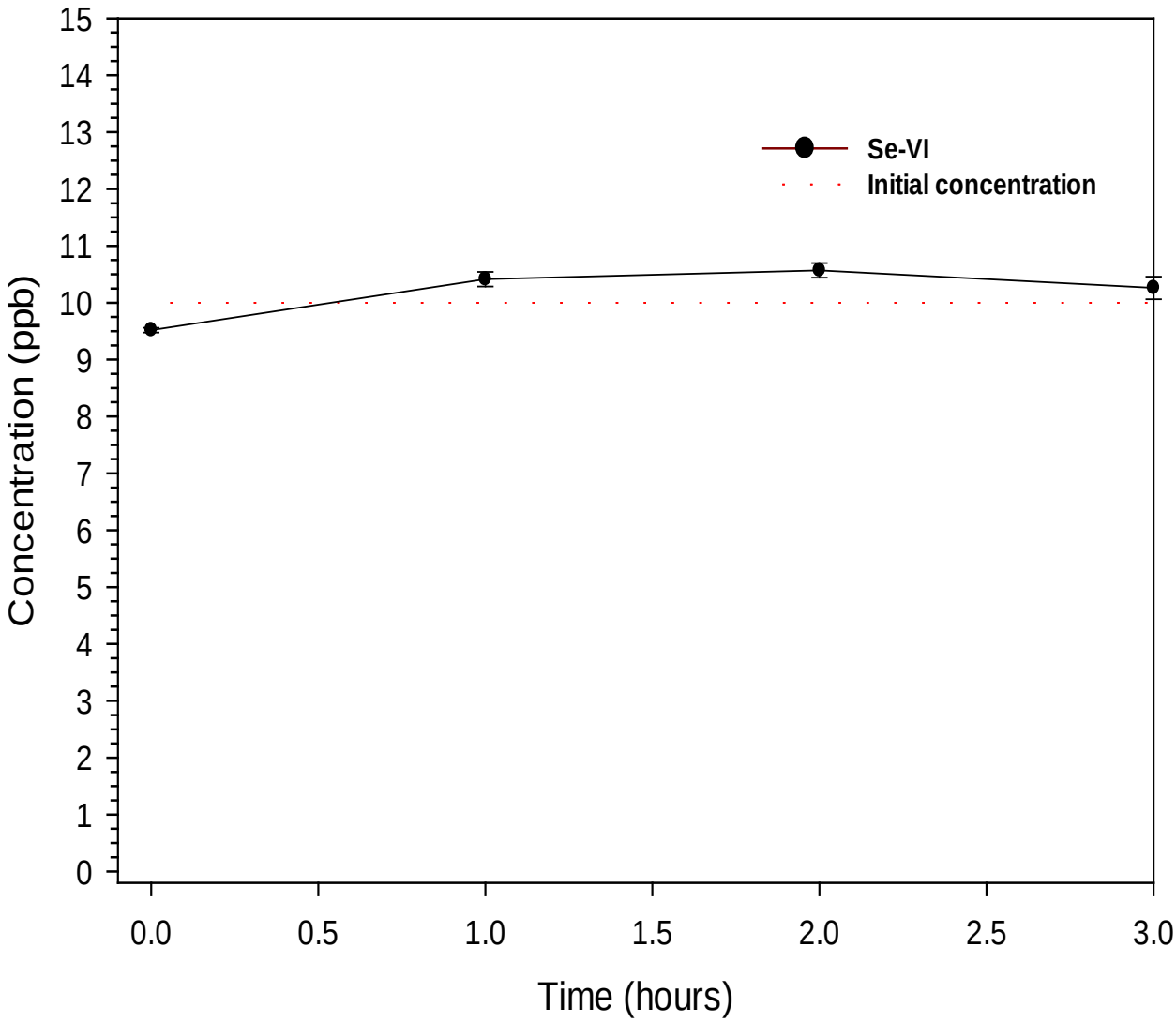

Figure 3.6 Se concentration of Se-VI in hot killed *C. reinhardtii* cells added modified high salt medium. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. Solid circles represent the concentration of Se as Se-VI in the medium from each sample dates and the short-dashed line represents the initial Se concentration in the medium.

3.1.5 Experiment 3: Se-Cys uptake by *C. reinhardtii*

Three sets of results were obtained from Experiment 3: control, Se uptake and Se adsorption by heat killed cells. The control experiment shows that the total concentration of Se as Se-Cys in the MHSM decreased in the experiment with no algae present by approximately 30% (Figure 3.7). Additionally, during the analysis, an unidentified peak appeared on the chromatogram. The peak heights and peak areas of this unidentified peak increased with the decrease of the Se-Cys peak. Figure 3.8 indicates the appearance, increase and the location of this unidentified peak on the chromatogram using the HPLC-UV-HG-AFS. The unidentified peak appeared at 4.2 minutes on

the chromatogram, which is located between the Se-Cys peak (3.7 minutes) and the Se-IV peak (4.8 minutes).

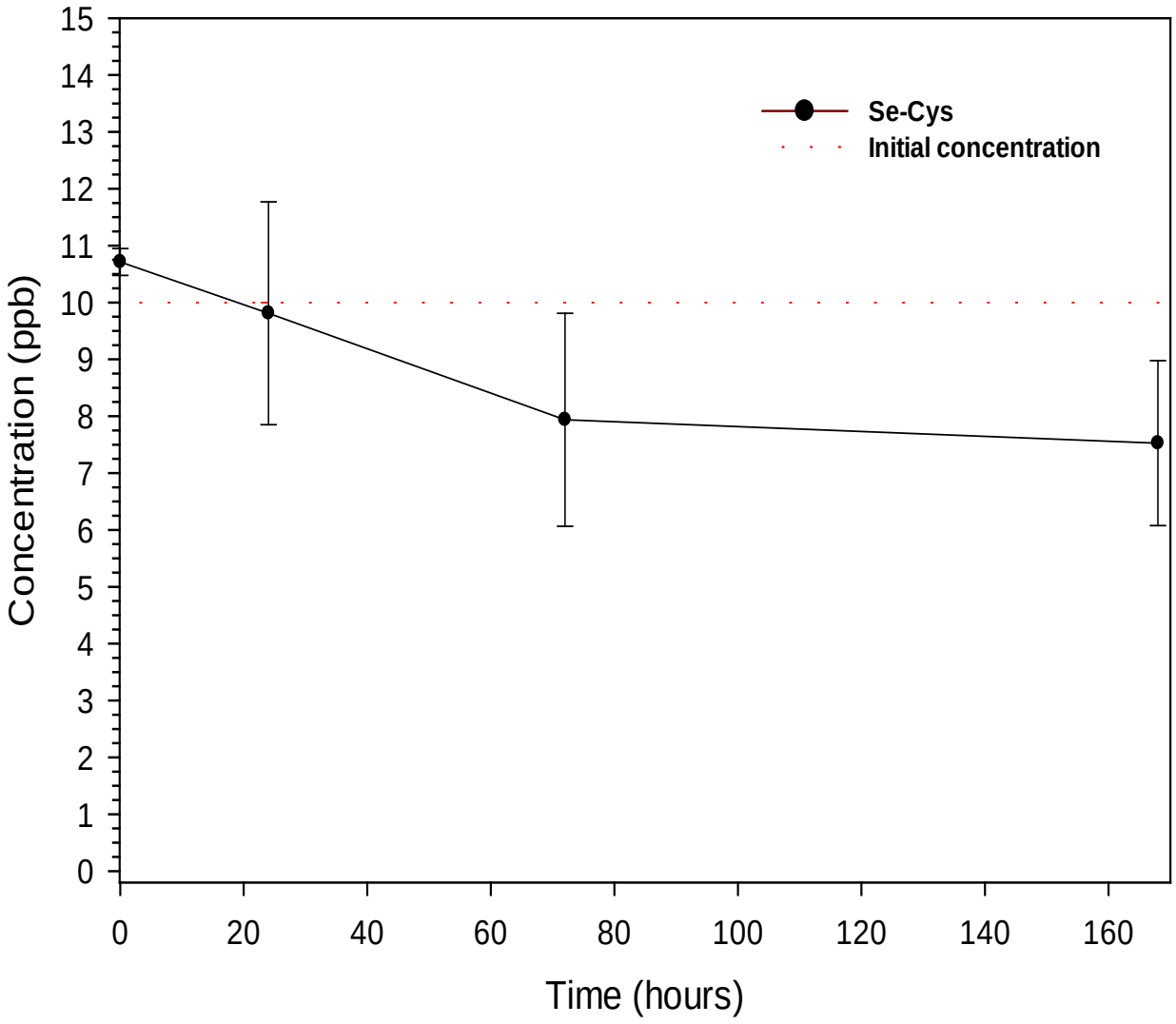

Figure 3.7 Se concentration of Se-Cys in modified high salt medium containing 10 ppb Se as Se-Cys throughout 168 hours. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. Solid circles represent the concentration of Se as Se-Cys from each sample dates and the short dashed line represent the initial Se concentration.

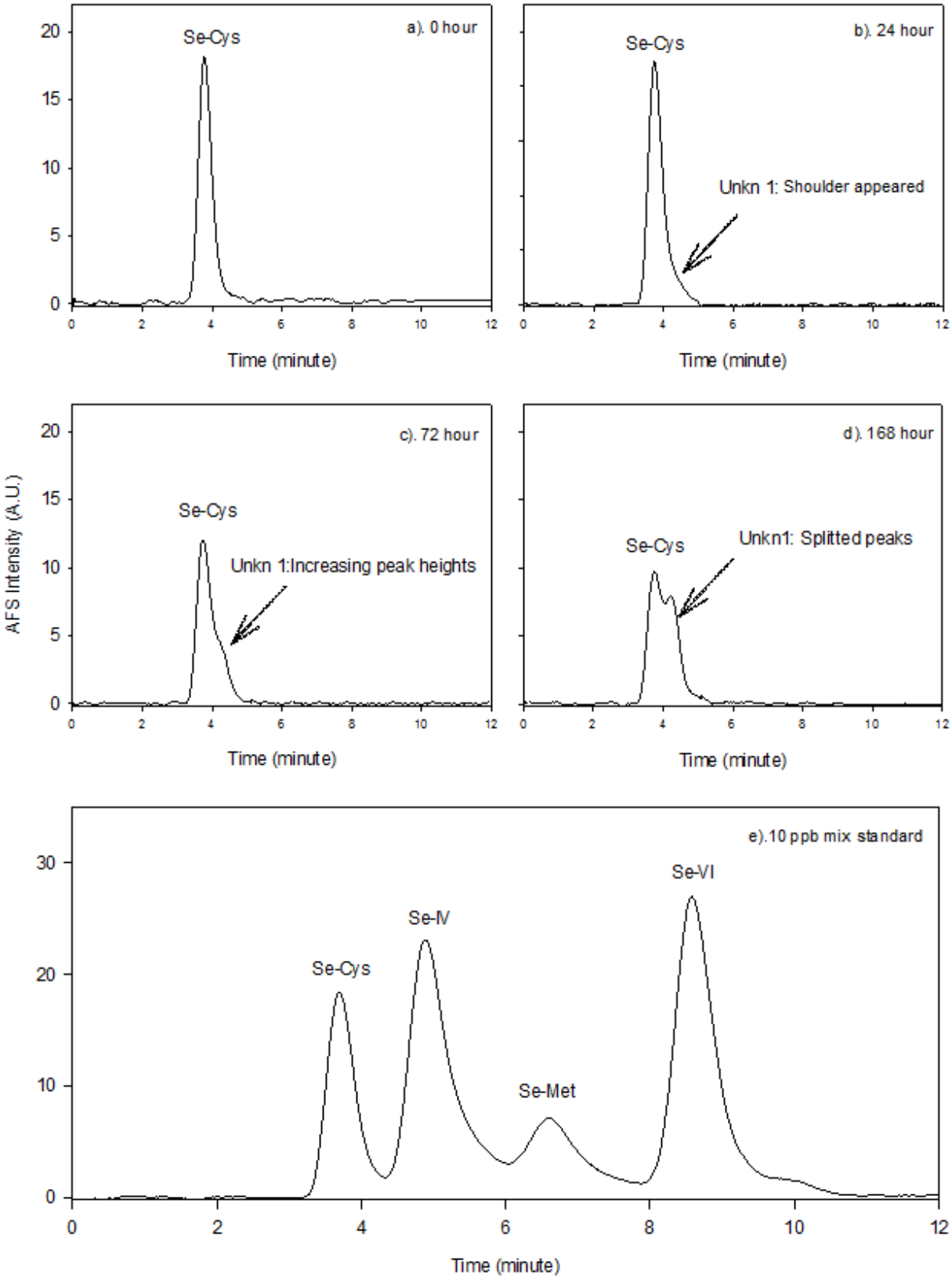
a). 0 hour
Se-Cys
b). 24 hour
Se-Cys
Unkn 1: Shoulder appeared
c). 72 hour
Se-Cys
Unkn 1:Increasing peak heights
d). 168 hour
Se-Cys
Unkn1: Splitted peaks
AFS Intensity (A.U.)
Time (minute)
e).10 ppb mix standard
Se-Cys
Se-IV
Se-Met
Se-VI
Time (minute)

Figure 3.8 Chromatograms of Se-Cys control samples from a) 0 hour, b) 24 hour, c) 72 hour, d) 168 hour and e) mixed Se standard containing 10 ppb Se as Se-Cys, 10 ppb Se as Se-Met, 10 ppb Se as Se-IV and 10 ppb Se as Se-VI. The signal of the unknown species is labeled as “Unkn 1”.

The Se-Cys uptake results with live algae are shown in Figure 3.9. A loss of 3 ppb Se occurred immediately once Se-Cys was added to the algal culture. The Se concentration of Se-Cys in the medium remained relatively stable for the first 12 hours during the algae growth and then decreased gradually afterwards until it reached below the instrumental detection limit (0.4 ppb Se) at the end of the 168-hours experiment. As Se-Cys decreased, Se as Se-IV peaked at 1.0 ppb at 96 hours. Both Se-Met and Se-VI remained below detection. It should be noted that the unidentified Se peak, which was found in the control experiment, was not observed in the medium throughout the Se-Cys uptake experiment.

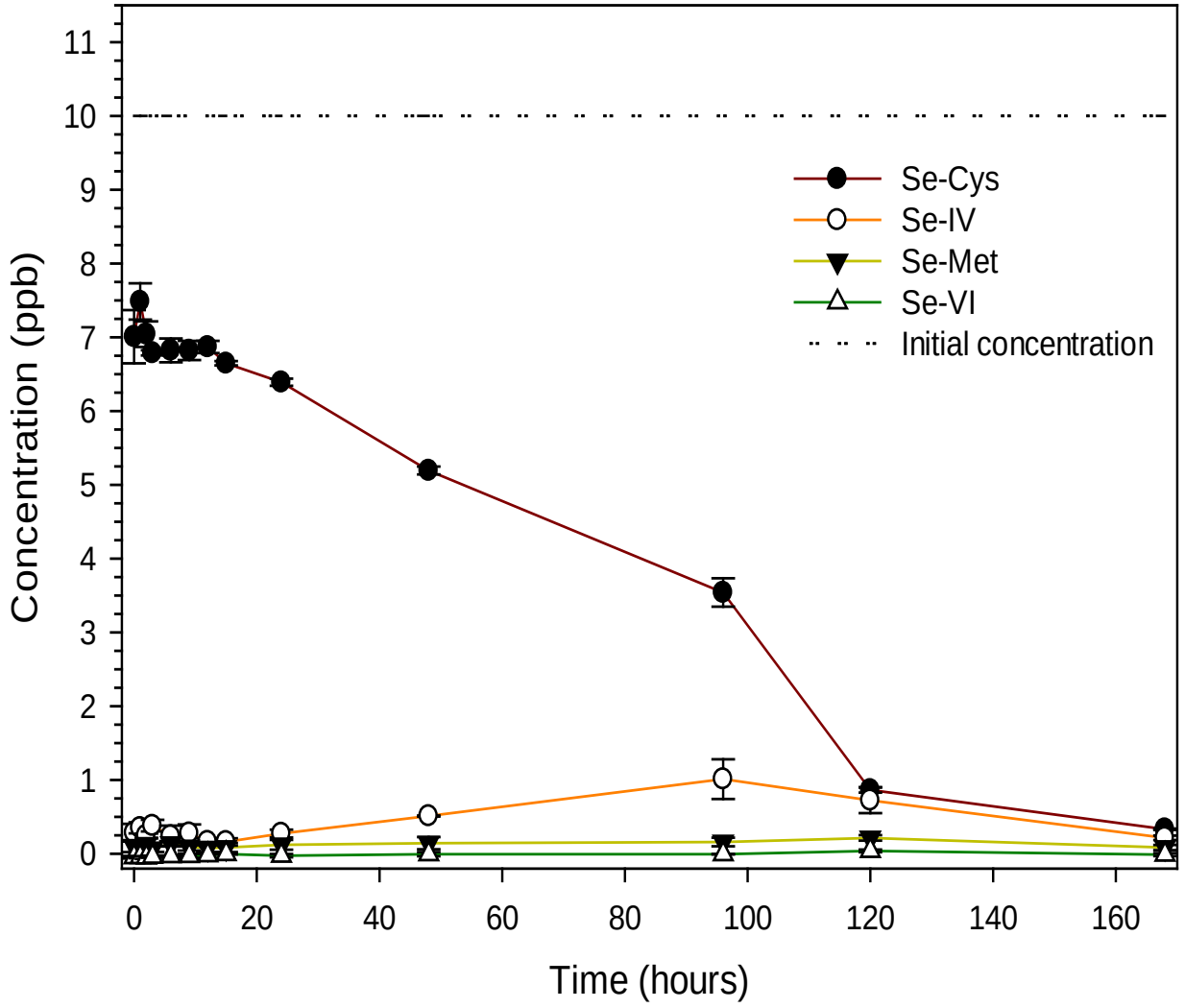

Figure 3.9 Se concentrations of Se species in the medium throughout 168 hours incubation of *C. reinhardtii* in modified high salt medium containing 10 ppb Se as Se-VI. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. The solid circles represent Se as Se-Cys, the open circles represent Se as Se-IV, the solid triangles represent Se as Se-Met and the triangles represent Se as Se-VI concentrations at each sampling point. The dash-dot line represents the initial Se concentration.

The results of experiment with heat-killed algae are shown in Figure 3.10. A loss of 2 ppb Se occurred in the medium after the addition of heat-killed algae cells, mirroring what was also observed in the experiment with live algae cells.

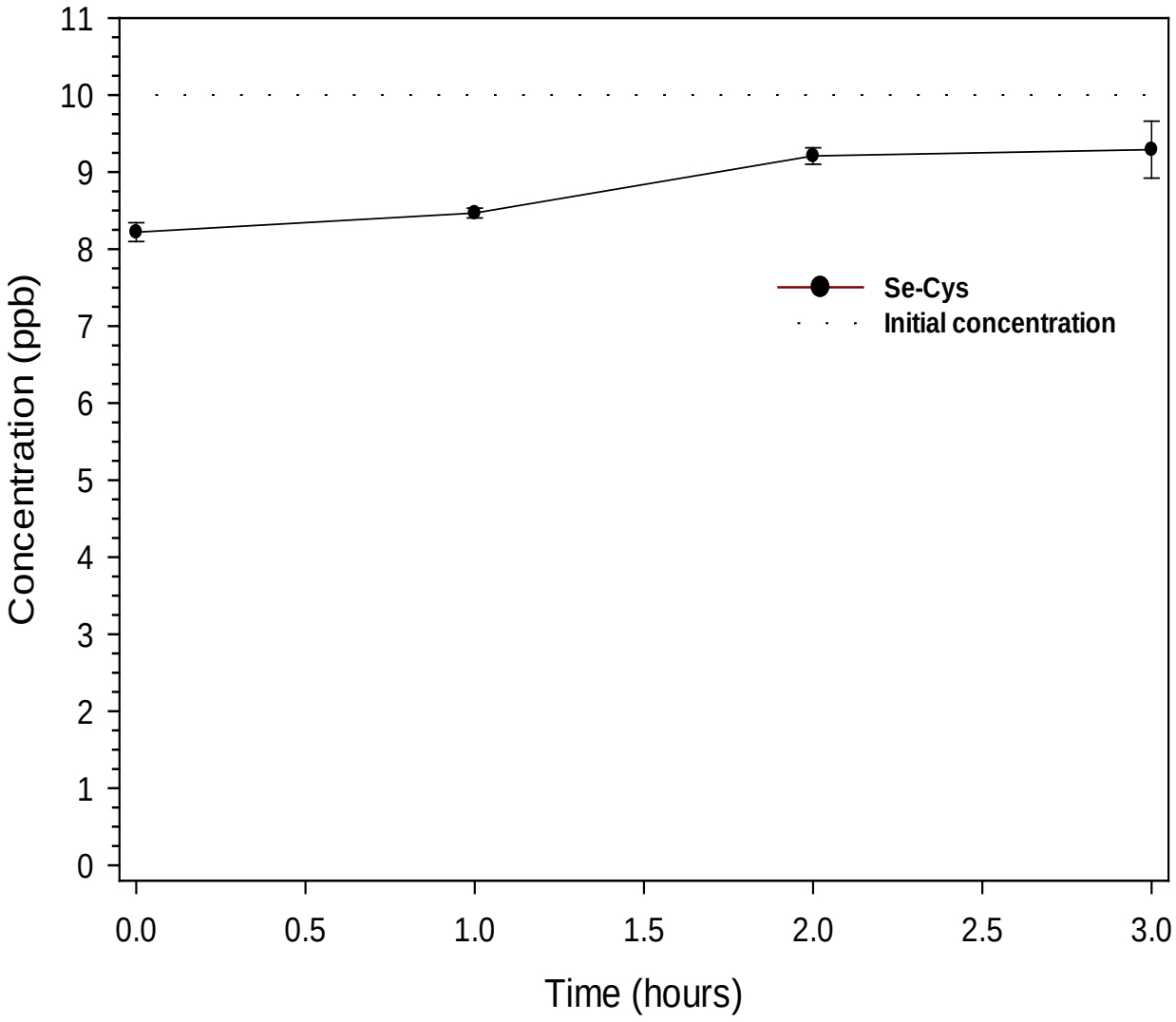

Figure 3.10 Se concentration of Se-VI in heat killed *C. reinhardtii* cells added modified high salt medium. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. Solid circles represent the concentration of Se as Se-Cys from each sample points and the short dashed line represents the initial Se concentration.

3.1.6 Experiment 4: Se-Met uptake by *C. reinhardtii*

Figure 3.11 presents the results from the control experiment. Three significant results were found. First, the Se as Se-Met concentration in the medium decreased 3 ppb during the first 24 hours. Then it remained relatively stable for the next 48 hours. Afterwards, Se as Se-Met slowly increased back to 9 ppb. Second, once Se-Met was added into MHSM, Se-Cys was produced, which might be caused by reactions between Se-Met and constituents of the MHSM. Last, Se-

Cys was constantly increasing, although the end Se concentration of Se-Cys was low (1.6 ppb). According to Figure 3.11, the production of Se-Cys was linear over time.

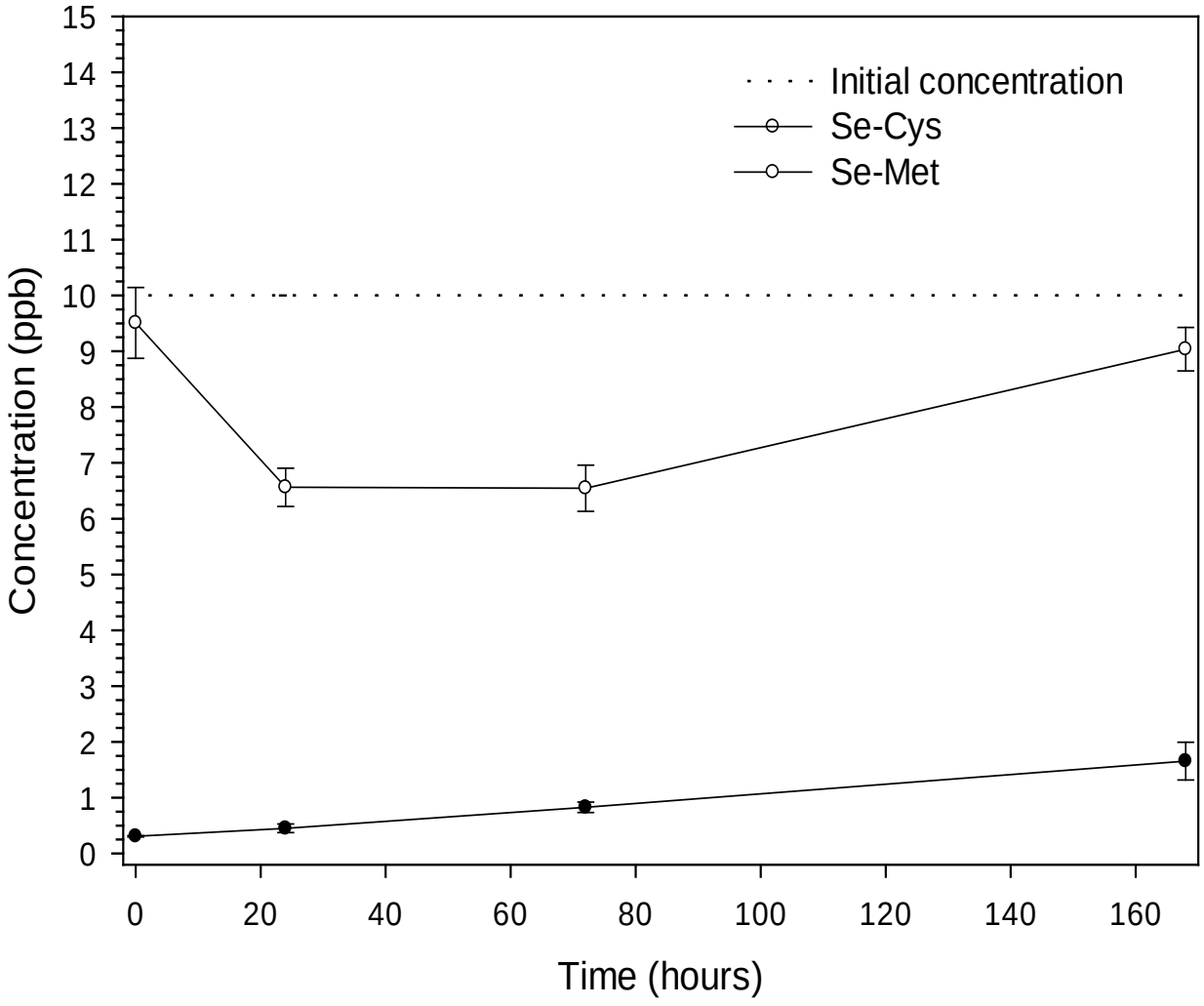

Figure 3.11 Se concentration of Se species in modified high salt medium containing 10 ppb Se as Se-Met throughout 168 hours. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. Solid circles represent the Se concentration of Se-Cys and open circles represent Se concentration of Se-Met from each sample point. The short-dashed line represents the initial Se concentration.

During the Se-Met uptake by *C. reinhardtii* experiment, it is observed that Se-Met decreased quickly to 1 ppb during the first 6 hours (Figure 3.12). Afterwards, Se-Met stayed stable with minor fluctuations. Se-Cys was identified in the medium and its Se concentration peaked at 4 ppb after 8 hours. After the peak value, Se as Se-Cys in the medium slowly decreased over the

next 120 hours; at the end of this experiment, it dropped to 0.5 ppb. Both Se-IV and Se-VI remained under the detection limit.

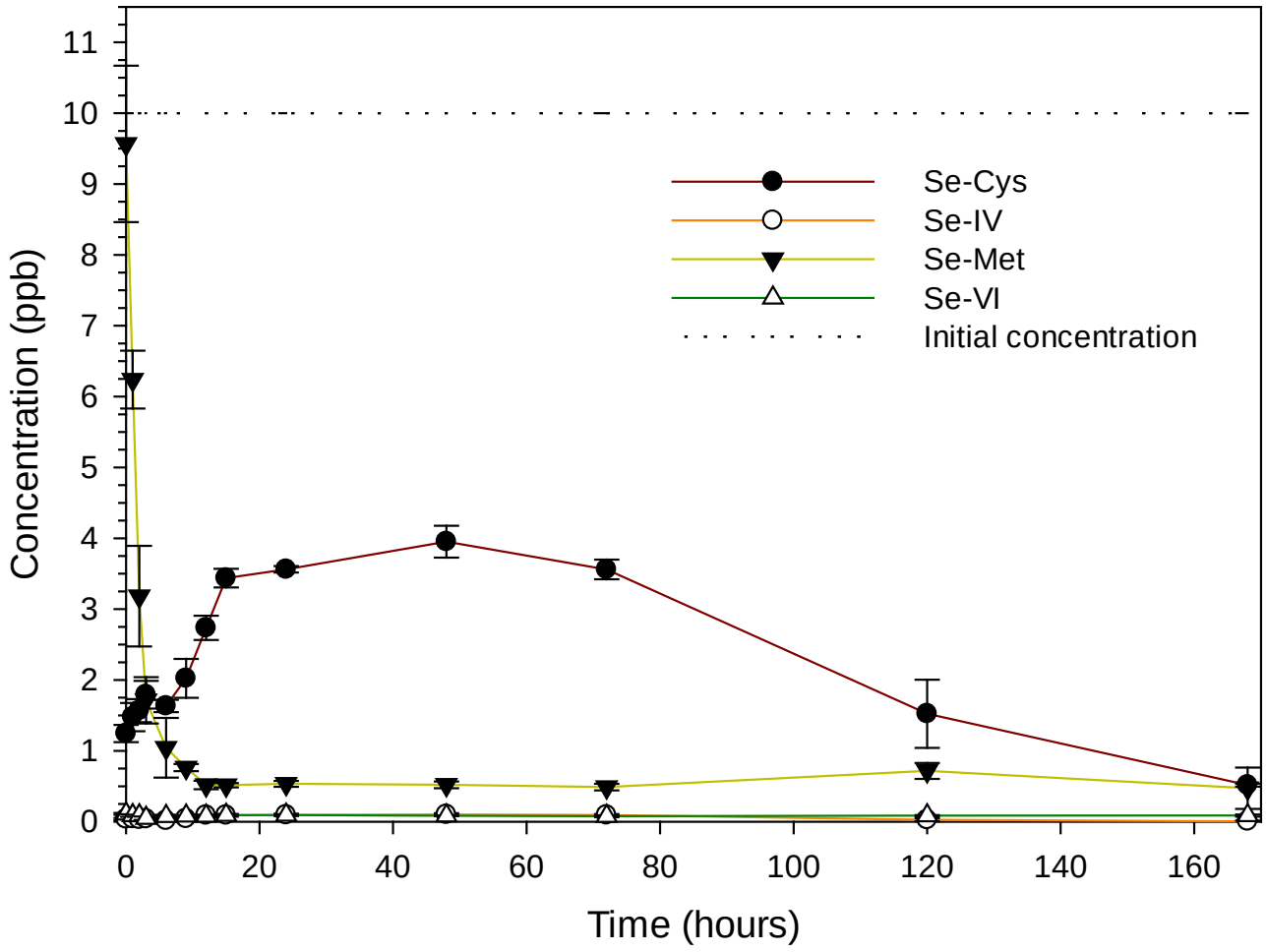

Figure 3.12 Se concentrations of Se species in the medium throughout 168 hours incubation of *C. reinhardtii* in modified high salt medium containing 10 ppb Se as Se-Met. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. The solid circles represent Se as Se-Cys, the open circles represent Se as Se-IV, the solid triangles represent Se as Se-Met and the triangles represent Se as Se-VI concentrations at each sampling point. The dash-dot line represents the initial Se concentration.

3.1.7 Experiment 5: Mixed species (Se-IV, Se-VI, Se-Cys and Se-Met) uptake by *C. reinhardtii*

The control results show that there was a 1 ppb increase of Se as Se-IV (Figure 3.13); however, Se as Se-VI remained stable in the medium. Meanwhile, the concentration of Se as Se-Cys

decreased 1 ppb; however, the concentration of Se as Se-Met in the MHSM stayed relatively stable.

Results from the uptake experiments with *C. reinhardtii* added are shown in Figure 3.14. When all four species were mixed, the concentrations of each species in the medium behaved similarly in contrast to when the species were added separately. For both Se-Cys and Se-VI, the concentrations declined immediately once *C. reinhardtii* was added to the medium. The Se-Cys concentration in the medium declined linearly with time and then dropped under the detection limit at the end of the experiment. Se-Met concentration in the medium decreased rapidly in the first 9 hours and then stayed relatively stable. About 1 ppb Se as Se-Met detected in the medium at the end of the 168 hours experiment.

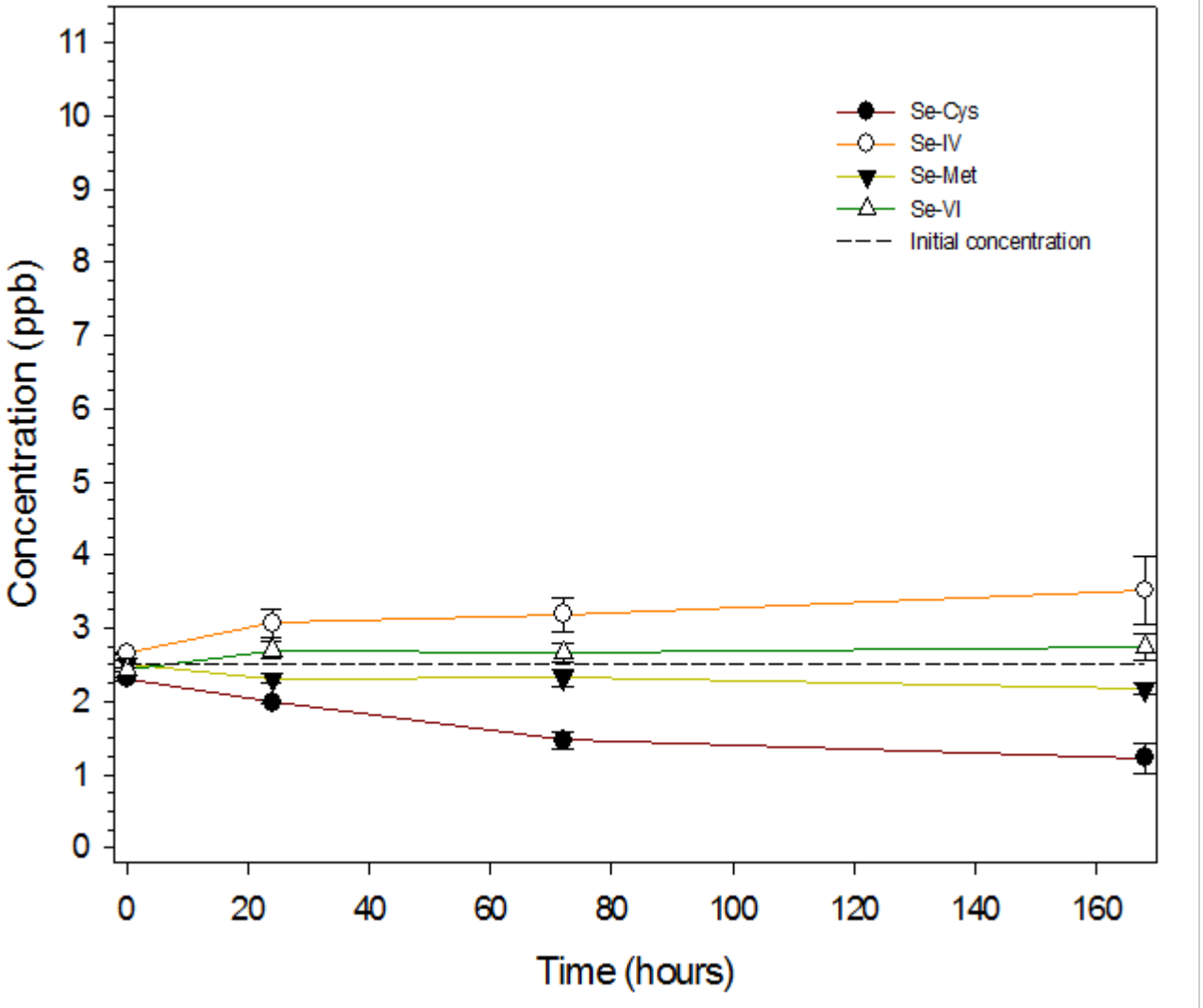

Figure 3.13 Se concentration of Se species in modified high salt medium containing 2.5 ppb Se as Se-Cys, 2.5 ppb Se as Se-Met, 2.5 ppb Se as Se-IV and 2.5 ppb Se as Se-VI. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. The solid circles

represent Se as Se-Cys, the open circles represent Se as Se-IV, the solid triangles represent Se as Se-Met and the open triangles represent Se as Se-VI concentrations at each sampling point. The short-dashed line represents the initial Se concentration of each Se species (2.5 ppb).

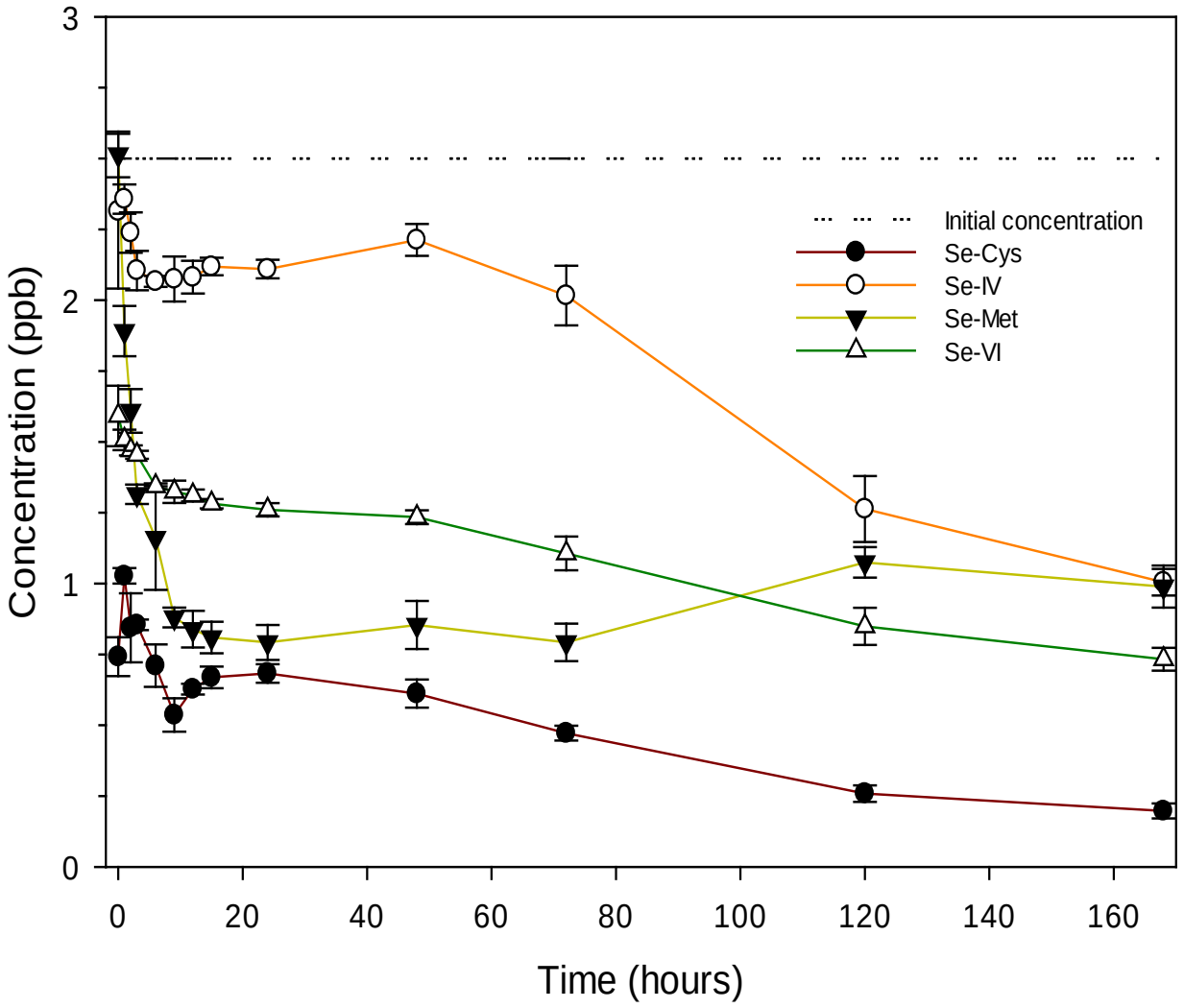

Figure 3.14 Se concentrations of Se species in the medium throughout 168 hours incubation of *C. reinhardtii* in modified high salt medium containing 10 ppb Se as mixed species including 2.5 ppb Se as Se-IV, Se-VI, Se-Cys and Se-Met. Average results from the triplicate experiments are presented; error bars indicate ±1 SD. The solid circles represent Se as Se-Cys, the open circles represent Se as Se-IV, the solid triangles represent Se as Se-Met and the triangles represent Se as Se-VI concentrations at each sampling point. The dash-dot line represents the initial Se concentration (2.5 ppb).

Compare all uptake experiments, the total Se in the medium decrease as a function of time and it is illustrated in Figure 3.15

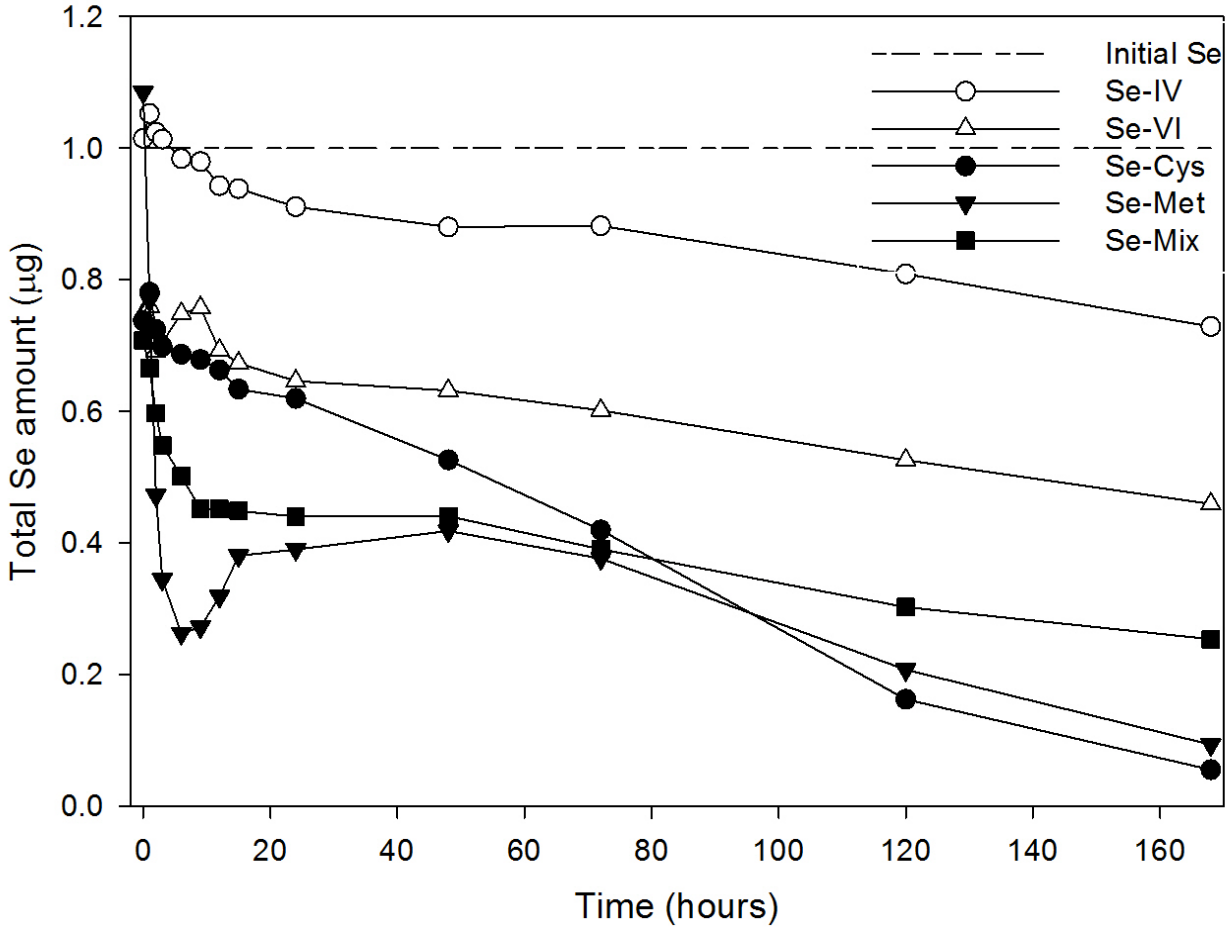

Figure 3.15 Change of total Se concentrations of all Se uptake experiment throughout 168 hours incubation of *C. reinhardtii* in modified high salt medium. The open circles represent total Se in Se-IV uptake experiment, the open triangles represent Se-VI uptake experiment, the solid circles represent Se-Cys uptake experiment, the solid triangles represent Se-Met uptake experiment, the solid squares represent mixed Se uptake experiment, and the dashed line represents the initial total Se.

3.1.8 Complementary experiments: Sorption of Se to cell walls and dead algaes

After each Se sorption experiment, the *C. reinhardtii* cultures were harvested and the total Se adsorbed to the cell walls was measured. Results show that there was no Se on the *C. reinhardtii* grown in the Se-free MHSM. A low amount of Se was detected on all algae cell walls exposed to Se. In the terms of Se speciation, only Se-IV was detected; all other species were below the detection limit. The calculated Se-IV concentrations in the 1 mL alkaline solution are shown in Figure 3.16.

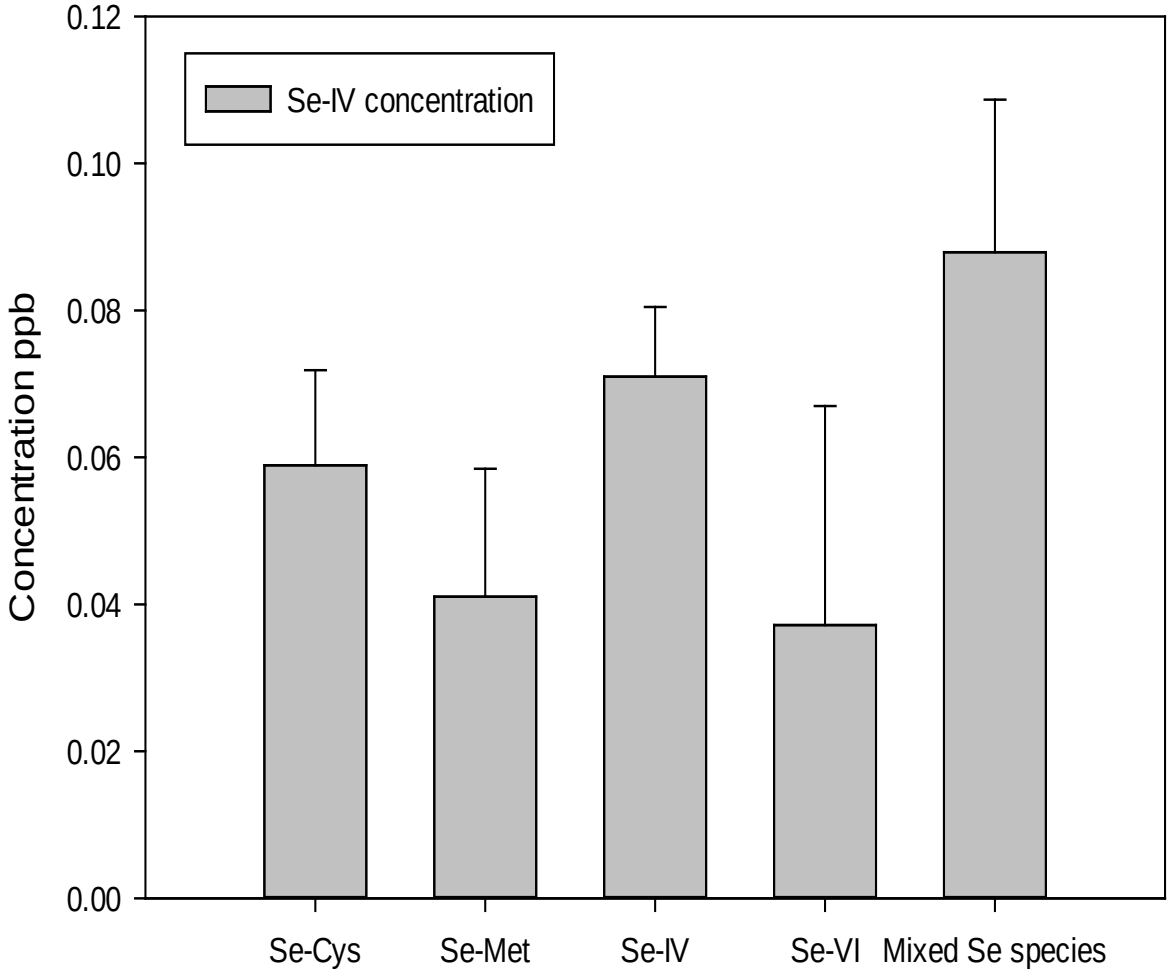

Figure 3.16 Se adsorption on the cell wall. Each bar represents the average Se-IV measured from the triplicate experiments of the solution recovered after washing centrifuged algae with NaOH (pH=10.5). Y-axis represents the calculated concentration of Se as Se-IV using Se-IV peak heights and calibration curve. X-axis represents different Se exposure experiments using Se-Cys, Se-Met, Se-IV, Se-VI or mixed Se species (all four Se species) Error bars indicate ±1 SD.

The values of the concentrations shown on Figure 3.16 are estimated using Se-IV peak height values and they are very low, i.e., they are lower than the quantification limit of the method (0.4 ppb). Nevertheless, as shown on Figure 3.17, a distinct Se-IV peak can be observed on the chromatogram for all algae cells harvested from MHSM with Se exposures.

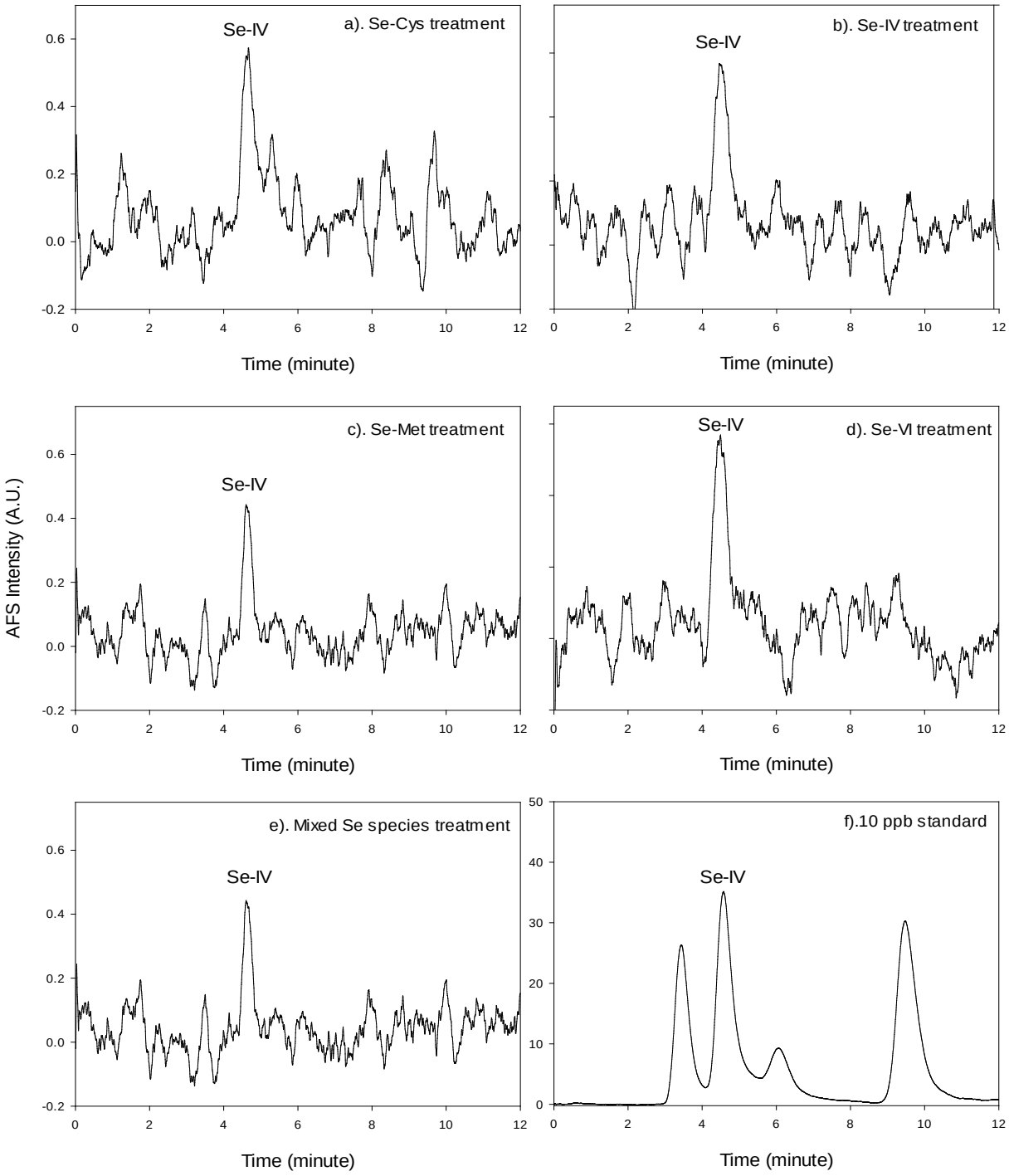

Figure 3.17 Se-IV sorbed on algae cell walls harvested from the uptake experiments: Sample chromatograms indicate Se sorption on the cell wall from incubating in MHSM containing a) 10 ppb Se as Se-Cys, b) 10 ppb Se as Se-IV, c)10 ppb Se as Se-Met, d)10 ppb Se as Se-VI and e) 10 ppb Se as mixed Se species including all species in a) to d). 10 ppb mixed Se standard

chromatogram is shown in f) and used to compare and determine the species of the peaks shown in a) to e).

3.1.9 Total internal Se stored inside cell wall

The Se concentrations of 10 mL Se digestion solution obtained from each Se exposure experiment are listed in Table 3.1.

Table 3.1 Total Se retained in algae cells

Se species exposed to algae culture	Total Se concentration in 10 mL HCl (ppb)	Total Se (µg)
Se-IV	1.53	0.0153
Se-VI	1.68	0.0168
Se-Cys	1.34	0.0134
Se-Met	2.41	0.0241
Mixed Se	2.25	0.0225

Table 3.1 suggests that only a small fraction (less than 3%) of the Se was retained within the algae cell. Particularly, algae cultured in MHSM containing Se as Se-Met stored the most amount of Se within the algae cells, with about 0.025 µg internal Se detected. On the other hand, algae grew in MHSM containing Se as Se-Cys stored the least amount of Se within algae cells, with about 0.013 µg internal Se detected.

We use the following equation to calculate, through mass balance, the amount of Se lost during the uptake experiment:

Se loss = Initial Se in the medium - (Se in algae cells + Se left in the medium) Equation (3)

We further assume that the Se is lost to the volatile phase, as suggested by Winkel et al. (2011). Since the initial Se in the medium is 1 µg, Se retained in algae cells and Se left in the medium can be measured, then the Se loss can be estimated using equation (3). A detailed mass balance is listed in Table 3.2. This Se loss is hypothesized as Se volatile.

Table 3.2 Se mass balance of each Se uptake experiment. Se loss is estimated by subtracting the total internal Se in algae cells and the Se left in the medium at the end of the experiment from the initial ambient Se.

Se species exposed to algae culture	Initial Se in the medium (µg)	Total Se retained in algae cells (µg)	Se left in the medium after 168 hours incubation (µg)	Se loss (µg)	Se loss(%)
Se-IV	1	0.0153	0.70	0.28	94.90%
Se-VI	1	0.0168	0.43	0.55	97.05%
Se-Cys	1	0.0134	0	0.99	98.66%
Se-Met	1	0.0241	0.04	0.94	97.49%
Mixed Se	1	0.0225	0.24	0.74	97.04%

3.2 Diagenetic redistribution of Se

A separate set of *C. reinhardtii* cultures were grown in MHSM containing 500 ppb Se as Se-Met. Cells were harvested and the internal Se stored within the algae cells was determined as 4.65±0.20 µg Se per 1 gram of wet algae paste. A known weight of harvested algae cells were mixed with the top 1 cm layer of sediments in Columns 2 and 3: 0.5301 g algae paste in Column 2 and 0.5242 g algae paste in Column 3. In another words, there were 2.4670 µg Se added to the top 1 cm layer of sediments in Column 2 and 2.440 µg Se to Column 3. The Se concentration in the seawater sample from the sampling site was measured using the speciation method and all detectable Se species had concentrations lower than the detection limit.

Figure 3.18 shows the Se released to the pore water in Column 1 (control experiment), in which no algae cells (Se free) were added. Speciation results indicated that only Se-Cys was released, at a low level, to the pore water. At the 0.5 cm depth, Se-Cys increased from to 1.5 ppb during the first week and then slowly declined to below detection. Similar behavior of Se-Cys was observed in the sediment at 1.0 cm. About 1.2 ppb Se as Se-Cys was released to the pore water in the first week and then it slowly dropped to below the detection limit. Meanwhile, at 1.5 cm depth from the interface, the concentration of Se as Se-Cys kept decreasing from 2.5 ppb on the first day to below the detection limit at the end of the seven-week experiment.

In the columns with Se-rich algae cells added (Columns 2 and 3), only Se-Cys was detected while the other Se species remained below the detection limit (Figure 3.19). During the first week of incubation, concentrations of Se as Se-Cys in the pore water at 0.5 cm depth climbed from below detection to 6.6 and 2.2 ppb in Columns 2 and 3, respectively. The Se as Se-Cys concentration peaked at the end of the first week, and then during the next 5 weeks of incubation, it slowly declined to below the detection limit. At 1.0 cm depth, pore-water concentrations of Se-Cys increased during the first week and declined during the second week. This process repeated again during the third and fourth week. Afterwards, pore-water concentrations of Se as Se-Cys dropped to below the detection limit. Lastly, at 1.5 cm depth, the concentration of Se as Se-Cys was to the pore water peaked at the end of week 1: 1.7 ppb Se as Se-Cys in Column 2 and 6.7

ppb Se as Se-Cys in Column 3. Then both pore-water concentrations gradually decreased to below the detection limit by the end of the seven-week incubation.

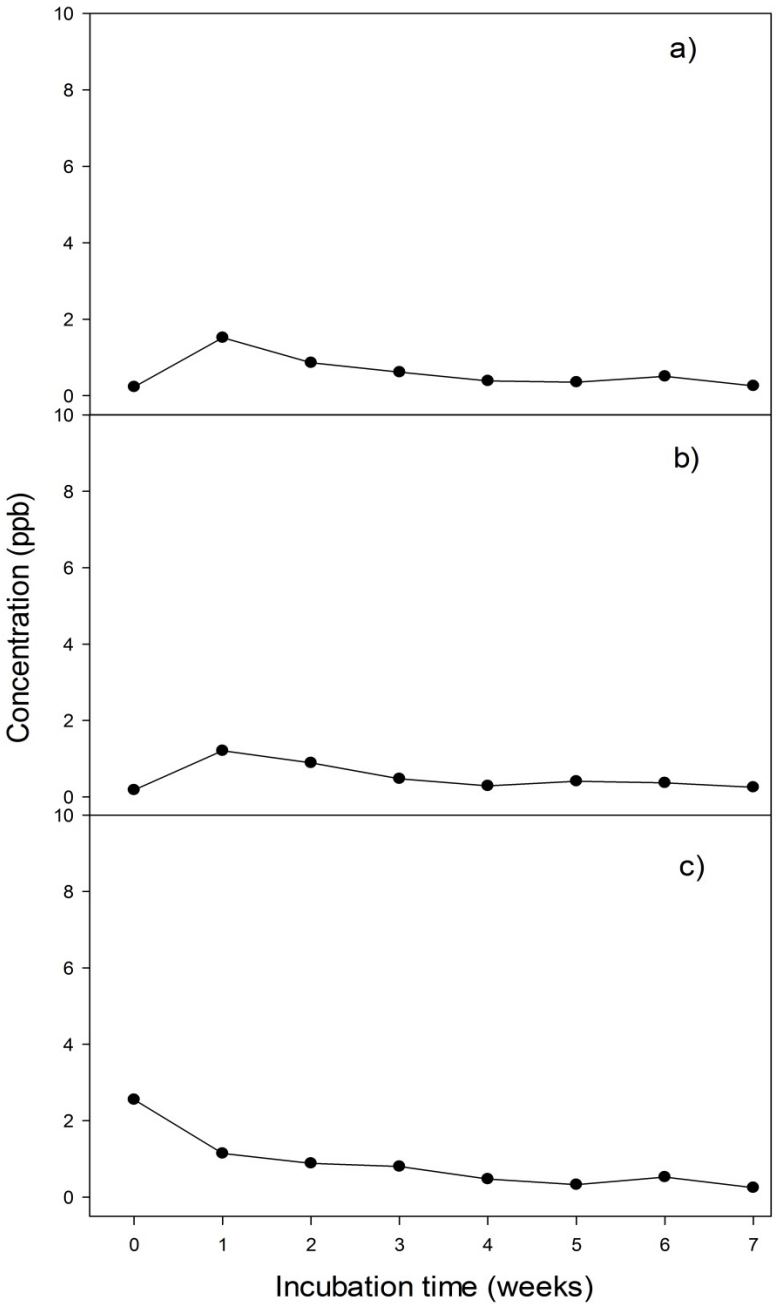

Figure 3.18 Se releasing to pore water in the form of Se-Cys in column 1 (data represented as solid circles) at three depths from the water-sediment interface: a) 0.5 cm; b) 1 cm; c) 1.5 cm. The straight line indicates the change of the concentration of Se as Se-Cys

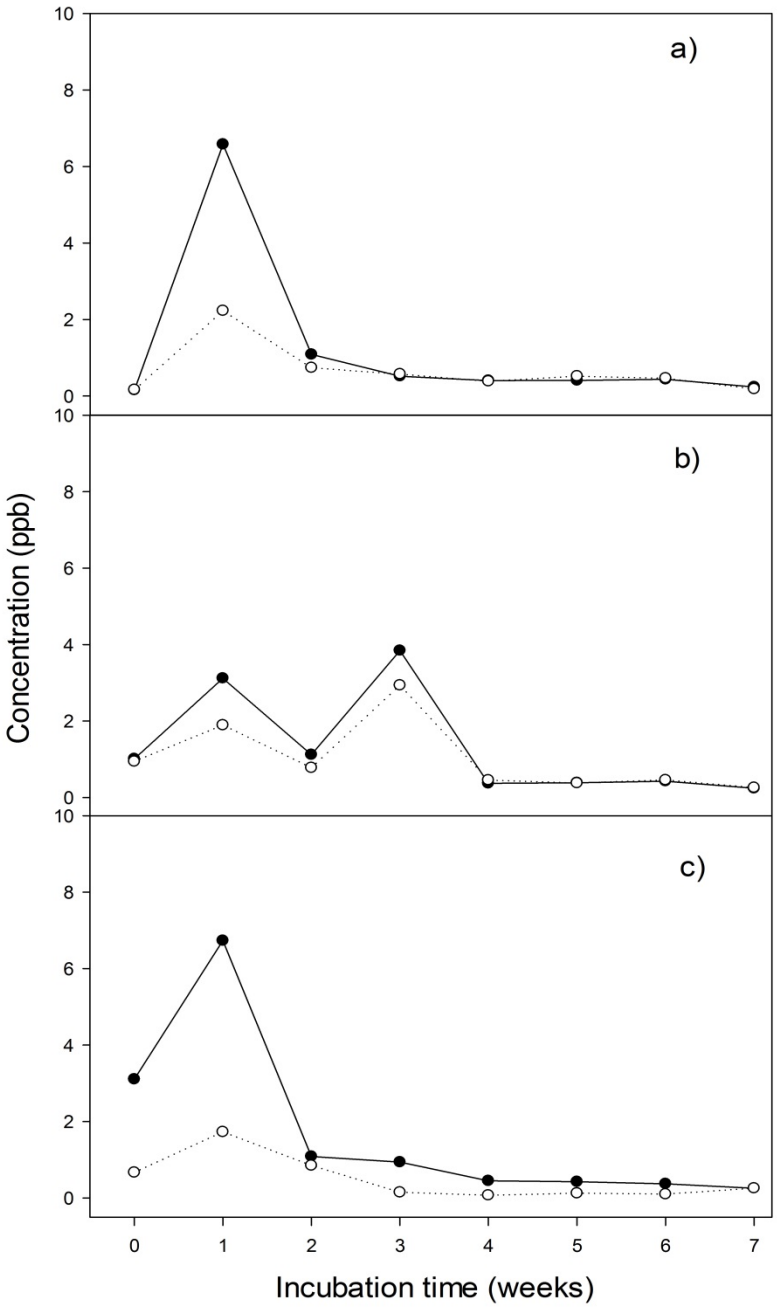

Figure 3.19 Se released to pore water in the form of Se-Cys in Column 2 (solid circles) and Column 3 (open circles) at three depths from the water-sediment interface: a) 0.5 cm; b) 1 cm; and c) 1.5 cm. The straight line and the dashed lines respectively represent the change in the Se as Se-Cys pore water concentration in Column 2 and Column 3.

4.0 Discussion

The purpose of this thesis is to study the utilization of Se in aquatic systems by algae and to explore the fate of the Se retained in algae cells after Se utilization. The ultimate goal of this thesis is to contribute to a better understanding of the biogeochemical cycle of Se in aquatic system.

4.1 Se speciation during *C.reinhardtii* uptake experiments

The goal of the Se uptake experiment was to determine which Se species were excreted during Se uptake by *C. reinhardtii*.

Experiment 0 (control), in which *C. reinhardtii* was grown in MHSM containing no Se, showed that no Se in any chemical forms was found in either the MHSM or the algae culture obtained from CPCC. Therefore, neither the MHSM nor the *C. reinhardtii* cells contributed Se that would have affected the Se uptake experiments.

In each uptake experiment, inorganic Se (Se-IV and Se-VI) and organoselenium (Se-Cys and Se-Met) species were added as an external source of Se. In general, results from the uptake experiments demonstrate *C. reinhardtii* shows a preference for taking up organoselenium over inorganic Se. Ambient Se-IV or Se-VI was only partially utilized by *C. reinhardtii* and no significant change in Se speciation was measured during the 168-hour incubation; however algae growing in the medium containing either Se-Cys or Se-Met, utilized most of the ambient Se and the chemical transformations of Se were observed in the Se released back to the medium in both cases. The detailed behaviors of Se species in the artificial aquatic system are discussed below.

Results from the uptake experiments of Se-IV and Se-VI indicated a slow uptake process of inorganic Se species at low concentrations (10 ppb Se) and no release of any other Se species by *C.reinhardtii* to the aquatic environment. Se-IV uptake by *C.reinhardtii* followed a cell density dependent process. Se-VI uptake, on the other hand, proceeded in two steps process: an instantaneous uptake followed by a cell density dependent process. In the cell density dependent process for both Se-IV and Se-VI uptake, the utilization of inorganic Se by *C. reinhardtii* is relatively modest at low cell density stage compared to Se-Cys and Se-Met uptake; however when the cell density became greater, the inorganic Se utilizations from the medium become more significant. In another word, the uptake of 10 ppb inorganic Se is proportional to the cell densities in the medium.

In the stationary growing phase, during the first 15 hours, Se-IV remained relatively stable in the medium. Se-IV in the medium began decreasing after the first 12 hours as the cell number started noticeably increasing. Once the algae began growing exponentially, ambient Se-IV had the most significant decrease. In total, approximate 20% of the initial Se-IV in the medium was utilized by *C. reinhardtii*.

Previous studies report faster and greater uptake of Se-IV by *C. reinhardtii* (Morlon et al., 2006; Fournier et al., 2006; Riedel et al., 1991) compared with the results obtained from this thesis.

The difference may be explained by the different initial ambient Se-IV concentrations and the different initial cell densities. The results reported by Riedel et al. (1991) used an initial Se-IV of 50 ppb, and Fournier et al. (2006) used Se-IV ranging from 200 to 2000 ppb while the initial Se concentrations of Se-IV in this thesis were 10 ppb. With a higher concentration of ambient Se-IV, there is a higher Se-IV uptake (Fournier et al., 2006). Morlon et al. (2006) investigated Se-IV uptake by using a wild range of Se-IV concentrations between nM and µM levels. Se-IV was utilized in both low concentrations and high concentrations; however, the Morlon et al. (2006) study used an initial cell density of 100,000 cells/mL which is an order of magnitude higher than the initial cell density used in this research (16,000 cells/mL). Consequently, the Se-IV uptake rate from this thesis is lower compared with literature results due to a lower initial ambient Se-IV concentration and low initial cell density. Nevertheless, Morlon et al. (2006) also concluded that the uptake of Se-IV in a high salt environment depends on cell density.

Furthermore, just as Se-IV uptake increased with increasing cell density, ambient Se-IV remained relatively stable throughout the stationary phase of algae growth in agreement with several previous studies (Morlon et al., 2006; Vandermeulen and Foda, 1988). These same studies also found that the absorption of Se-IV on the cell wall is negligible. In contrast, Boisson et al. (1995) and Riedel et al. (1991) conclude that the absorption of Se-IV to algae cell walls is a major part of Se-IV uptake. Results from this thesis show that the adsorption of Se-IV to algae cells does not occur at low Se concentration (10 ppb).

Two features of Se-VI uptake need to be noted. First, there was a rapid instantaneous Se loss in the medium once the algae were exposed to Se-VI. Approximate 30% of Se-VI was not recovered. Possible contributions to this Se loss could be caused by rapid active uptake of Se-VI and passive absorption of Se-VI to cell walls. This rapid Se loss was not observed when heat-killed algae cells were exposed to the same initial medium. Therefore, this uptake cannot be explained by passive absorption. In fact, this rapid loss of Se-VI agrees with the results from Simmons and Wallschläger (2011) and Simmons and Emery (2011). In these two studies, *C. vulgaris* were incubated in a medium containing Se-VI and they both had an immediate loss from the initial Se-VI concentration at the start of the experiments. Those author note, however, that algal uptake of different Se oxyanions depends strongly on the algal species present and probably also on the chemical composition of the water in which they grow.

Second, after the rapid initial Se decrease, a slow uptake of Se-VI was observed. Simmons and Emery (2011) propose that Se-VI uptake is inhibited by the presence of SO_4^{2-}. But in this thesis Se-VI uptake was observed, even though the initial MHSM contained adequate SO_4^{2-}, and most of the Se-VI uptake was completed during the exponential growth of *C. reinhardtii*. This result suggests that once the cell density is high enough, Se-VI uptake will still occur instead of being completely inhibited by SO_4^{2-}.

Regarding organoselenium, the results demonstrate that the organoselenium species are more bioavailable than inorganic Se species in terms of uptake rate. In addition, both Se-Cys and Se-Met undergo chemical transformation and the new Se species are released back to the medium.

In terms of Se-Cys, there are several studies reporting that synthesis of Se-Cys by organisms (Turlo et al., 2007; Ng and Anderson, 1978; Forchhammer and Böck, 1991); however only few studies looked at the utilization of Se-Cys. Using *Escherichia coli*, it was found that Se-Cys can act as a Se source to provide Se in the synthesis of selenophosphate which is required for selenoprotein synthesis (Lacourciere and Stadtman, 2001). In the control experiment, the instability of the Se-Cys compound in the MHSM was observed. It can be ascribed to light-induced degradation, or to the oxidation of the molecule. It should be noted that cysteine and selenocysteine are both subject to quick oxidation, forming di-S and di-Se bound to produce cystine and selenocystine, respectively. The latter form of Se was used in this study.

Meanwhile an unknown Se species appeared which is illustrated by the unidentified peak in Figure 3.8. This unknown Se species was only observed in the medium at the end of the experiment during the algae uptake. Almost all Se-Cys were either digested or adsorbed by the algae cells after the 168 hours incubation.

The Se-Cys uptake by *C. reinhardtii* consists of three steps: (1) an immediate rapid absorption of Se-Cys by algae cells once *C. reinhardtii* cells were added; (2) Se-Cys remained stable in the medium at the stationary phase of the algae culture; and (3) when algae began growing quickly, Se-Cys in the medium was quickly consumed by algae cells. At the same time, the utilization of Se-Cys by *C. reinhardtii* led to the chemical transformation of Se-Cys to Se-IV (which was not added in the initial medium but found during the Se-Cys uptake). The excretion of Se-IV back to the environment occurred before the exponential growth phase and then this part of Se-IV was re-cycled back to algae cultures as a result of increasing number of algae cells. The result from this study verifies that at low concentrations (10 ppb Se), Se-Cys can be readily used by *C. reinhardtii* compared with inorganic Se species including Se-IV and Se-VI. On the other hand, it is less bioavailable compared with Se-Met.

Among the four Se species used for the Se uptake experiment, Se-Met was found to be the most readily taken up. Although the control samples indicated a slow degration of Se-Met in the medium, it still can be concluded that algae rapidly use Se-Met , because most of the Se-Met in the medium disappeared in the first 12 hours of incubation. I Most previous results agree with conclusion in this thesis that Se-Met is the most bioavailable Se species (Besser et al., 1993; Fournier et al., 2006; U.S. Environmental Protection Agency, 1987; Ingersoll et al., 1990; Sandholm et al., 1973; Kiffney and Knight, 1990; Besser et al, 1989; Sharma and Davis, 1980).

In terms of Se speciation, there was considerable amount of Se-Cys synthesized by the algae and released back to the medium. In control samples, about 1.6 ppb Se as Se-Cys was produced at the end of hour 168; however, 4 ppb Se as Se-Cys was released to the medium at hour 48, and this

part of Se-Cys was then re-used by the algae later. Uptake of Se-Met did not result in a release Se-IV or Se-VI back to the medium, although the release of Se-IV was found in the Se-Cys uptake experiment.

To the best of our knowledge, this is the first time that the uptake of Se-Cys and its release back to the medium is reported in the presence of algae. In summary, the Se-Met uptake experiment suggests that a favorable precursor for selenoproteins would be Se-Met, since it is the most readily available Se species in the aquatic environment.

Only Se-IV was found on the cell wall for all uptake experiments (Se-Cys, Se-Met, Se-IV, Se-VI and mixed Se species); however, Se transformation from the Se species of interest to Se-IV and the release of Se-IV back to medium was found only in the Se-Cys uptake experiment. This observation that Se-IV found on the cell wall for all uptake experiment suggests that Se-IV is an important intermediate Se species in the Se biochemistry. If the ambient Se concentration is large enough, an excretion of Se-IV back to the medium may be observed.

Regarding the total internal Se retained within algae cells, the results point to two observations. First, most Se utilized during the uptake did not stay within the algae cells. This leads to the hypothesis that gaseous Se such as methylated Se was produced and released back to the atmosphere

Second, there is a greater recovery of total Se (Se retained in the algae cells over Se utilized from ambient water) harvested in inorganic Se uptake experiments (>3%) than in organic Se uptake experiments (<3%). This difference once again confirms that organic Se species are more bioavailable than inorganic Se species.

The results from the Se uptake experiment offer a better understanding of Se uptake by green algae *Chlamydomanos reinhardtii.* Particularly, the results show that organic selenium is more bioavailable, uptaken faster and possibly released to the atmosphere. This behavior could be due to the fact that the cells expends energy to detoxifies Se to form methyl-Se, and that organic Se can be transformed faster into gaseous phase than inorganic Se, which has to be reduced first within the cell. This is concurrent with previous findings showing the large proportion of Se being volatilized in field studies (e.g., Cook and Bruland, 1987). This result once again confirms that Se can be cycled from aquatic environment to the air through biological uptake and this Se flux to the air can be fairly efficient.

We identify several drawbacks to the experiments that were performed here. For instance: 1) The Se absorption experiments using sterilized cells could not fully represent Se absorption by algae cell walls because of the possible cell structure change under high temperature; 2) Unknown Se species were found in Se-Cys control samples and this Se species was not able to be quantified; 3) Total Se loss, especially volatile Se to the atmosphere was not measured in this research; 4). Elemental Se in both medium and algae cells could have formed (Winkel et al., 2012) but were

not measured in this study. Nevertheless, this exploration of the fate of Se species in the presence of algae is unique and sets the stage for more detailed experiment.

4.2 Se redistribution in sediments during early diagenesis.

The purpose of the Se diagenesis experiment was to explore the fate of Se-Met utilized by *C. reinhardtii* and to understand how this part of Se is released back to sediments once the algae dies and settles on the sediment surface. As mentioned in the introduction, there are studies on Se in sediment and pore water (Belzile et al. 2000, Martin et al. 2011); however, little knowledge of the fate of the Se retained within organisms once they die is available. This experiment of Se redistribution in sediments was therefore exploratory and novel. Only Se-Met was tested as Se-Met was found to be the most bioavailable form of Se and easily concentrated in the algae culture used in this study.

Results of this study clearly demonstrate an elevation of Se in the sediments and pore water with the addition of Se-rich algae cells at the water–sediment interface. The released Se moved down the column, increasing with concentration at 0.5 cm, 1.0 cm and 1.5 cm over a period of 7 weeks. A big difference of Se concentrations in pore water between Column 2 and Column 3 is likely due to the loss of material from the column during the setup. Alternately, the important bioturbation observed throughout the experiment could be responsible for the discrepancies between both columns. Yet, bioturbation is an important natural mechanism that distributes fresh organic carbon within the sedimentary column.

In terms of the Se speciation, only Se-Cys was released to the pore water in the sediments. This is consistent with the uptake experiment results on Se-Met. Se-Met utilized from the medium was used to synthesize Se-Cys, which was incorporated by the cells as selenoprotein. When the cells decomposed, the selenoprotein released Se-Cys from the cell tissues to pore water. There were significant amounts of Se released to the pore water in the form of Se-Cys at all three depths in the first week, and this could be caused by the consumption of algae cells by microbes in the sediments and the release of Se that had been incorporated with organic carbon. The released Se could be recycled back to the water body, adsorbed on the surface of sediment materials or dissolved in pore water.

This thesis showed that in a dynamic system, the microbial activity is able to significantly decompose organic carbon and rapidly release the incorporated Se. Most Se was released to the pore water in the first week and then the Se level kept declining at 0.5 cm and 1.5 cm for the remainder of the experiment. At 1.0 cm, the pore water underwent two cycles of Se release. This could be ascribed to bioturbation of the worms in sediments, which was dynamic throughout the experiment.

Although there are defects in this diagenesis experiment, this part of the thesis was of exploratory nature, in order to contribute to a better understanding of the cycling of Se during early diagenesis. It shows that organic Se governs Se speciation in the pore water in the

sediments and Se-Cys is particularly the dominant species when phytoplankton cells have been grown in Se-Met rich environment.

4.3 Future study

It is apparent that future study on Se uptake experiment should also focus on elemental Se and volatile Se species. It is possible that there were elemental Se produced by algae cells; however its concentration was not monitored in this study. In addition, the details of Se escaped to the atmosphere remains unclear. Limited knowledge is known about these two possible Se pathways, and further studies are therefore on demand. In terms of elemental Se, which could stay in the medium as colloidal Se in the form of nanoparticles, instruments have a function of size fractionation, for example field flow fractionation (FFF) combined with an ICP-MS, can be employed to determine the colloidal Se. By studying volatile Se, techniques used for gas chemistry such as Solid-Phase Microextraction (SPME) and Gas Chromatography (GC) can be applied.

Regarding to redistribution of Se in early diagenesis, very little was done on that topic so far. In the future, the solid phase sediments can be analyzed using X-Ray Fluorescence or synchrotron-based techniques. Also, additional experiments can be performed to determine Se release to pore water by adding algae cultured in inorganic Se riched and Se-Cys riched medium, although they would contain very little Se according to our results.

5.0 Conclusion

This thesis identifies and explores questions about Se cycling in the aquatic system, namely 1) Se speciation following Se uptake and excretion by the green algae *C. reinhardtii* in an aquatic system; and 2) the fate of the utilized Se after the death of *C. reinhardtii* and under which form Se is remobilized during the early diagenesis. We show that, at low concentrations (≤10 ppb), organic Se is favored over inorganic Se during Se uptake by algae. More specifically, Se-Met has the highest bioavailability among Se-IV, Se-VI, Se-Cys and Se-Met. Although previous studies have demonstrated the greater bioavailability of Se-Met compare to inorganic Se, to our knowledge the bioavailability of Se-Cys was not included in their investigation. We thus present the most up-to-date survey of the bioavailability of key Se species. Mass balance reveals that a significant proportion of Se is lost. We hypothesize this Se to be methylated to the atmosphere: 94.90%, 97.05%, 98.66% and 97.49% for Se-IV, Se-VI, Se-Cys and Se-Met, respectively. Although this volatilization mechanisms is thought to be a dominant pathway of Se loss to the atmosphere in natural environment (e.g., Winkel et al., 2012), laboratory studies do not report it to the extent that we have observed (e.g., Simmons and Wallschleager, 2011). Assuming that these authors did not, in fact, observe Se losses, we hypothesize that Se volatilization might not be performed by all species of algae, or that this mechanisms maybe not be trigged under all experimental conditions. Here, we have used low Se and low algal density, analogous to the natural environment, and thus our results are more comparable to field-derived studies than

previous studies. Se-Cys, predominantly released to the sediment pore water upon decomposition of fresh, Se-enriched algae biomass, suggesting again that this species is more mobile in the aquatic environment.

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Appendix I

Conditions of the HPLC-UV-HG-AFS system used for Se speciation analyzing

Item	Specification
Column	Hamilton,PRP-X100 Anion Exchange,250 x 4.1 mm
HPLC pressure (open)	0~3 bar
HPLC pressure (in-line)	50~55 bar
Injection volume	200 -1000 µL
HPLC flow rate	1 ml $minute^{-1}$
Mobile phase	5 mM Citric Acid, 2% Methanol, pH=5.9
Acid carrier	50% v/v HCl. 5% KBr
Reductant	0.7% m/v $NaBH_4$ in 0.1 M NaOH
Heating temperature	150^oC
Se lamp warming up time	20 minutes
AFS rinsing	Flowing water for at least 10 minutes
Column flushing	Mobile phase for at least 10 minutes

SAMS setting for Se speciation analyzing during HPLC-UV-HG-AFS operation

Name	Settings
Mode	Emission
Time of measurement	12 min
Filter factor	×64
Gain	×10
Primary lamp current	20 mA
Boost lamp current	25 mA
Pumps speed	50%

Appendix II
Cell densities and UV-vis absorption

Time (hours)	*C.reinhardtii* growing in MHSM containing 10 ppb Se as Se-Cys		*C.reinhardtii* growing in MHSM containing 10 ppb Se as Se-IV		*C.reinhardtii* growing in MHSM containing 10 ppb Se as Se-Met		*C.reinhardtii* growing in MHSM containing 10 ppb Se as Se-VI		*C.reinhardtii* growing in MHSM containing 10 ppb Se equivalently as Se-IV, Se-VI, Se-Cys and Se-Met		*C.reinhardtii* growing in MHSM	
	Cell numbers (per mL)	standard deviation	Cell numbers (per mL)	standard deviation	Cell numbers (per mL)	standard deviation	Cell numbers (per mL)	standard deviation	Cell numbers (per mL)	standard deviation	Cell numbers (per mL)	standard deviation
0	16042	3181	16042	188	16042	2386	16042	1082	16042	522	16042	3267
1	20705	1721	18695	2186	13862	1894	18155	2254	16256	2972	16156	3111
2	17427	2731	20150	1781	16151	1866	19559	3459	14502	8000	16869	2099
3	22097	2223	20828	1872	17540	2148	18255	3872	21186	10271	18625	2605
6	18723	2233	16333	1189	19591	2310	16598	510	16085	2109	16409	1608
9	19037	2109	17274	2116	15196	1892	17193	2202	13212	2318	17228	6430
12	41783	1772	39910	743	47603	4481	47533	2858	41372	417	46076	1354
15	51726	3321	51557	1542	48089	2143	46342	354	46269	3369	51182	1377
24	55073	1125	51348	6320	44629	2625	49547	8164	48986	3002	57020	1873
48	134855	8253	97741	18553	89586	21105	95079	39409	110080	28374	166793	34651
72	494149	48634	397297	55719	385868	46430	424658	155519	435704	75295	564119	108731
120	1672306	125615	1538936	259518	1537866	167800	1494194	259282	1688802	79539	1599418	79163
168	2826532	64905	2664040	93490	2477092	117524	2552407	293009	2581842	31049	2380448	158189

Appendix III

The results of uptake experiment

III.I The results of Experiment 0: Se speciation in algae culture in MHSM with no addition of Se

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	0.05	0.02	0.13	0.10	0.01	0.03	0.03	0.01
1	0.04	0.05	0.03	0.01	0.05	0.14	0.04	0.02
2	0.06	0.04	0.05	0.01	0.08	0.21	0.06	0.04
3	0.07	0.00	0.03	0.02	0.12	0.16	0.04	0.00
6	0.05	0.04	0.05	0.01	0.02	0.06	0.04	0.00
9	0.07	0.05	0.05	0.01	0.08	0.11	0.06	0.01
12	0.10	0.03	0.05	0.01	0.16	0.07	0.06	0.01
15	0.06	0.02	0.04	0.00	0.02	0.10	0.05	0.02
24	0.06	0.01	0.04	0.01	0.02	0.05	0.03	0.01
48	0.06	0.03	0.05	0.02	0.01	0.03	0.03	0.02
72	0.05	0.01	0.04	0.01	0.06	0.12	0.05	0.02
120	0.08	0.01	0.06	0.01	0.00	0.20	0.05	0.02
168	0.06	0.01	0.04	0.00	0.07	0.08	0.06	0.02

III.II

The results of Experiment 1: Se-IV uptake control samples in which no algae was added

Time	Se as Se-IV	
	Concentration (ppb)	Standard Deviation
0	9.82	0.08
24	10.15	0.06
72	10.47	0.02
168	10.61	0.11

Se-IV uptake by *C.reinhardtii*

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	0.22	0.01	9.83	0.96	0.00	0.00	0.20	0.04
1	0.19	0.00	10.37	0.12	0.00	0.00	0.18	0.01
2	0.18	0.02	10.19	0.09	0.00	0.00	0.19	0.02
3	0.20	0.01	10.18	0.19	0.00	0.00	0.18	0.02
6	0.19	0.01	9.99	0.17	0.00	0.00	0.18	0.01
9	0.19	0.02	10.06	0.06	0.00	0.00	0.17	0.01
12	0.23	0.03	9.74	0.13	0.00	0.00	0.17	0.02
15	0.22	0.01	9.81	0.01	0.00	0.00	0.17	0.01
24	0.22	0.03	9.62	0.11	0.00	0.00	0.17	0.01
48	0.16	0.03	9.47	0.06	0.00	0.00	0.15	0.02
72	0.18	0.02	9.58	0.10	0.00	0.00	0.15	0.04
120	0.19	0.01	8.85	0.02	0.00	0.00	0.15	0.02
168	0.18	0.01	8.07	0.38	0.00	0.00	0.13	0.02

III.III

The results of Experiment 2: Se-VI uptake control samples

Time	Se as Se-VI	
	Concentration (ppb)	Standard Deviation
0	9.84	0.36
24	9.43	0.17
72	10.18	0.19
168	9.78	0.16

The results of Se-VI uptake by *C. reinhardtii*

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	0.01	0.03	0.36	0.04	0.05	0.07	7.14	0.06
1	0.03	0.01	0.23	0.07	0.32	0.36	7.17	0.05
2	0.02	0.04	0.18	0.05	0.00	0.04	6.93	0.06
3	0.06	0.04	0.22	0.10	0.11	0.25	6.95	0.31
6	0.03	0.01	0.19	0.01	0.02	0.12	7.64	0.02
9	0.03	0.03	0.21	0.01	0.06	0.05	7.76	0.10
12	0.01	0.05	0.20	0.03	0.01	0.06	7.23	0.09
15	0.02	0.02	0.22	0.03	0.09	0.16	6.99	0.03
24	0.04	0.00	0.17	0.03	0.02	0.11	6.87	0.05
48	0.04	0.03	0.21	0.01	0.08	0.10	6.69	0.12
72	0.03	0.01	0.23	0.02	0.12	0.06	6.38	0.12
120	0.03	0.02	0.18	0.02	0.07	0.11	5.70	0.08
168	0.02	0.03	0.23	0.04	0.09	0.03	4.94	0.05

The results of Se-VI uptake by sterilized *C. reinhardtii* cells

Time	Se as Se-VI	
	Concentration (ppb)	Standard Deviation
0	9.52	0.04
1	10.41	0.13
2	10.57	0.13
3	10.26	0.20

III.IV

The results of Experiment 3: Se-Cys uptake control samples

Time	Se as Se-Cys	
	Concentration (ppb)	Standard Deviation
0	10.71	0.24
24	9.81	1.96
72	7.94	1.87
168	7.53	1.45

The results of Se-Cys uptake by *C. reinhardtii*

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	7.01	0.36	0.29	0.12	0.10	0.08	0.05	0.02
1	7.49	0.25	0.35	0.07	0.09	0.04	0.04	0.01
2	7.04	0.17	0.25	0.03	0.13	0.08	0.05	0.02
3	6.79	0.04	0.38	0.08	0.07	0.04	0.04	0.01
6	6.82	0.16	0.24	0.14	0.14	0.03	0.03	0.01
9	6.82	0.13	0.28	0.12	0.09	0.05	0.03	0.03
12	6.87	0.08	0.16	0.05	0.08	0.06	0.02	0.04
15	6.65	0.03	0.16	0.05	0.08	0.06	0.00	0.01
24	6.39	0.05	0.27	0.05	0.12	0.07	0.03	0.02
48	5.19	0.05	0.51	0.01	0.14	0.08	0.01	0.03
96	3.54	0.19	1.01	0.27	0.16	0.06	0.01	0.00
120	0.87	0.04	0.72	0.17	0.22	0.04	0.04	0.02
168	0.33	0.01	0.22	0.03	0.08	0.03	0.01	0.02

The results of Se-Cys uptake by sterilized *C. reinhardtii* cells

Time	Se as Se-Cys	
	Concentration (ppb)	Standard Deviation
0	8.22	0.12
1	8.47	0.06
2	9.21	0.11
3	9.29	0.37

III.V

The results of Experiment 4: Se-Met uptake control samples

Time	Se as Se-Cys		Se as Se-Met	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	0.31	0.01	9.51	0.63
24	0.45	0.08	6.56	0.34
72	0.83	0.09	6.54	0.41
168	1.66	0.34	9.04	0.39

The results of Se-Met uptake by *C. reinhardtii*

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	1.24	0.12	0.04	0.01	9.57	1.10	0.11	0.01
1	1.48	0.20	0.04	0.01	6.24	0.41	0.09	0.02
2	1.57	0.16	0.03	0.02	3.18	0.71	0.09	0.02
3	1.79	0.20	0.04	0.01	1.71	0.33	0.05	0.02
6	1.63	0.09	0.02	0.02	1.04	0.42	0.08	0.02
9	2.02	0.27	0.04	0.01	0.76	0.05	0.08	0.00
12	2.74	0.17	0.09	0.01	0.52	0.06	0.08	0.02
15	3.44	0.13	0.09	0.01	0.52	0.03	0.09	0.02
24	3.56	0.05	0.10	0.02	0.53	0.04	0.10	0.01
48	3.95	0.22	0.10	0.02	0.52	0.05	0.08	0.00
72	3.56	0.14	0.10	0.01	0.49	0.05	0.08	0.01
120	1.52	0.48	0.03	0.02	0.72	0.11	0.09	0.01
168	0.52	0.02	0.00	0.01	0.47	0.29	0.09	0.02

Appendix III.VI

The results of Experiment 4: Mixed Se species uptake control samples

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	2.31	0.01	2.66	0.02	2.51	0.06	2.43	0.02
24	1.99	0.02	3.07	0.20	2.31	0.06	2.70	0.11
72	1.47	0.12	3.19	0.23	2.32	0.12	2.67	0.13
168	1.22	0.21	3.52	0.48	2.17	0.08	2.74	0.17

The results of mixed Se species uptake by *C. reinhardtii*

Time	Se as Se-Cys		Se as Se-IV		Se as Se-Met		Se as Se-VI	
	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation	Concentration (ppb)	Standard Deviation
0	0.74	0.07	2.31	0.27	2.51	0.08	1.59	0.11
1	1.03	0.03	2.36	0.05	1.89	0.09	1.51	0.04
2	0.84	0.12	2.24	0.07	1.61	0.08	1.47	0.02
3	0.85	0.02	2.10	0.07	1.31	0.03	1.45	0.01
6	0.71	0.07	2.07	0.02	1.16	0.18	1.34	0.01
9	0.54	0.06	2.07	0.08	0.88	0.03	1.32	0.04
12	0.63	0.02	2.08	0.06	0.84	0.06	1.31	0.02
15	0.67	0.04	2.12	0.03	0.81	0.06	1.28	0.02
24	0.68	0.03	2.11	0.03	0.79	0.06	1.26	0.02
48	0.61	0.05	2.21	0.06	0.85	0.08	1.23	0.02
72	0.47	0.03	2.02	0.11	0.79	0.07	1.11	0.06
120	0.26	0.03	1.26	0.12	1.07	0.05	0.85	0.07
168	0.20	0.03	1.00	0.05	0.99	0.07	0.73	0.04

Appendix IV

Results of the complementary experiments: Sorption of Se to cell walls and dead algaes

Ambient Se species	Se as Se-IV	
	Concentration (ppb)	Standard Deviation
Se-Cys	0.06	0.01
Se-Met	0.04	0.02
Se-IV	0.07	0.01
Se-VI	0.04	0.03
Mixed Se species	0.09	0.02

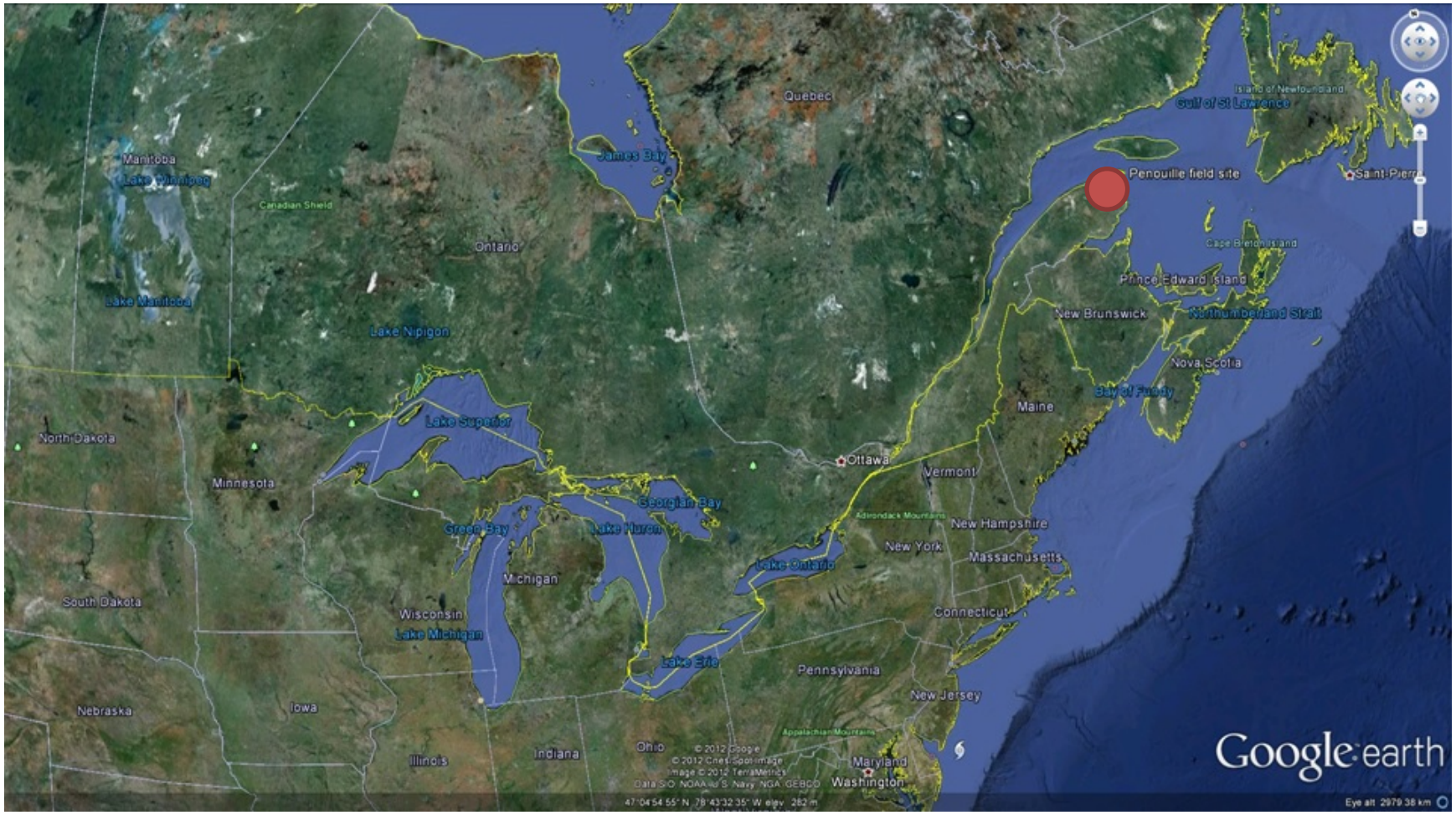
Appendix V

Results of total internal Se stored within the algae cells

Ambient Se species	Se as Se-IV	
	Total amount (μg)	Standard Deviation
Se-Cys	0.0134	0.0078
Se-Met	0.0241	0.0139
Se-IV	0.0153	0.0076
Se-VI	0.0168	0.0120
Mixed Se species	0.0225	0.0018

Appendix VI

VI.IThe location of the sampling site of the sediments used for the early diagenesis experiment



VI.II The map of the sampling site of the sediments used for the early diagenesis experiment

Appendix VII

Results of 7 weeks early diagenesis experiment

Time (weeks)	Concentration of Se as Se-Cys at each location								
	Column 1			Column 2			Column 3		
	Top (0.5 cm)	Middle (1.0 cm)	Bottom (1.5 cm)	Top (0.5 cm)	Middle (1.0 cm)	Bottom (1.5 cm)	Top (0.5 cm)	Middle (1.0 cm)	Bottom (1.5 cm)
0	0.23	0.18	2.56	0.16	1.01	3.11	0.16	0.95	0.67
1	1.52	1.21	1.15	6.58	3.12	6.73	2.23	1.89	1.73
2	0.86	0.89	0.89	1.09	1.12	1.09	0.74	0.78	0.85
3	0.61	0.48	0.80	0.52	3.84	0.94	0.57	2.94	0.15
4	0.39	0.29	0.48	0.40	0.37	0.45	0.39	0.45	0.07
5	0.35	0.41	0.33	0.41	0.39	0.43	0.52	0.38	0.13
6	0.51	0.37	0.53	0.44	0.43	0.37	0.46	0.46	0.10
7	0.26	0.25	0.25	0.24	0.24	0.26	0.18	0.26	0.26