The relationship between cellular protein content and selenium accumulation in freshwater micro-algae

by

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Abstract

In algal cells, an apparent threshold ceiling for selenium incorporation into amino acids suggests that protein content may determine the amount of selenium accumulated in these organisms at the base of the food web. The amount of selenium taken up at the base of the food web directly impacts bioaccumulation and subsequent toxicity observed in higher trophic level organisms. In this research, the protein content of four different Chlorophyta species (*Parachlorella kessleri*, *Chlorella vulgaris*, *Raphidocelis subcapitata*, and *Tetradesmus obliquus*) was determined during the different phases of algal growth (lag, exponential and stationary), and for *R. subcapitata* grown under different lighting intensities. Results from tissue analysis for selenium and protein following exposure to 40 µg/L showed no relationship between protein and selenium accumulation in the exponential phase, but a strong relationship in the stationary phase for *P. kessleri*, *C. vulgaris*, and *R. subcapitata*, as well as under different lighting intensities for *R. subcapitata*. These results suggest that considering protein content in site-specific primary producers is important in predicting bioaccumulation of selenium in higher trophic levels.

Keywords: Selenium; Selenate; Protein; Micro-algae; Biomagnification; Bioaccumulation; Primary producer; Normalization

This work is dedicated to Mum, Dad and Dakota, my unwavering sources of support and encouragement.

Acknowledgements

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List of Acronyms

SeO_4^{2-}	Selenate
SeO_3^{2-}	Selenite
DMSe	Dimethyl selenide
DMDSe	Dimethyl diselenide
EF	Enrichment factor
TTF	Trophic transfer factor
CCME	Canadian Council of Ministers of the Environment
BBM	Bold's basal medium
HR	High range
MR	Medium range
DRO	Deionized reverse osmosis
PCBs	Polychlorinated biphenyls

Chapter 1.

General Introduction

1.1. Selenium

1.1.1. Properties

Discovered in 1817 by Jöns Jacob Berzelius as an impurity during a sulfuric acid preparation, selenium (Se) has since become an element of anthropogenic use and subsequent environmental concern (Arnér, 2011). Selenium is number 34 on the periodic table; it is a metalloid located between the non-metal sulfur and the metal tellurium, and thus has properties of both metals and non-metals (Gojkovic et al., 2015). As a non-metal, Se is hydrolyzed to form oxyanions (SeO_4^{2-} and SeO_3^{2-}) rather than forming cations in aqueous solution as seen with metals (Young et al., 2010). In contrast to metals, the non-metal oxyanions selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) typically increase in solubility and mobility with an increase in pH (Young et al., 2010). Selenium occurs as: the inorganic forms selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), selenide (Se^{2-}) and elemental selenium (Se^0); the organic forms selenomethionine (SetMet) and selenocysteine (SeCys); and volatile methylated forms such as dimethyl selenide and dimethyl diselenide. The common Se species and associated valence states are presented in Table 1-1.

Table 1-1.   Common valence states of selenium species

Selenium Species	Valence State	Formula
Selenate	+6	SeO_4^{2-}
Selenite	+4	SeO_3^{2-}
Elemental selenium	0	Se^0
Selenide	-2	Se^{2-}
Dimethyl selenide (DMSe)	NC	$(CH_3)_2Se$
Dimethyl diselenide (DMDSe)	NC	$(CH_3)_2Se_2$
Selenomethionine (SeMet)	NC	SeMet
Selenocysteine (SeCys)	NC	SeCys

NC: no charge

1.1.2. Sources

Selenium is widely deposited throughout the Earth's crust, it parallels the geochemistry of sulfur and is largely associated with sulfide ores (Shamberger, 1981). Due to increased volcanic activity during the Cretaceous period, Se is primarily found in organic-carbon enriched sedimentary basins, most notably in phosphate bearing rocks and coal (Young et al., 2010). Additionally, Se is found in mildly organic-carbon and oil-enriched shales (Shamberger, 1981; Presser, 1994; Etteieb et al., 2020). Volcanic eruptions and weathering of these selenium-rich sediments results in natural Se mobilization. However, anthropogenic activities including irrigation, mining and coal combustion can concentrate the Se present in the environment (Schneider et al., 2015; Etteieb et al., 2020).

Elevated aquatic Se levels have been attributed to coal, phosphate, and various other metal mining. Within the mining process, Se can enter the aquatic environment through Se-rich tailings resulting from the weathering of waste rocks as well as from ore processing leachate (Etteieb et al., 2020). In the case of coal mining, Se is not only leached from coal *via* rainwater percolation through exposed rock but can also be released from both the combustion and fly ash disposal processes (Khamkhash et al., 2017). In power generation via coal combustion, the fly ash waste product, which contains selenium, has an increased surface area available for weathering. Therefore, fly ash is a significant contributor to selenium mobilization even in streams several miles from the mine site.

Irrigation and industrial discharges are major anthropogenic sources of mobilized Se. Where seleniferous soils may already be in abundance, oxic wastewater from agricultural drainage systems deliver Se and other trace elements to aquatic ecosystems (Presser and Ohlendorf, 1987). Receiving waters from industrial discharges can contain between 10-100 µg/L Se, which is significantly higher than reference sites containing on average <1 µg/L Se (Wayland and Crosley, 2006; Simmons and Wallschläger, 2011).

1.1.3. Microbial and chemical Interactions

Se speciation is dependent on a variety of biotic and abiotic factors. The different species of Se present in aquatic environments include the highly toxic selenate, selenite,

selenomethionine and selenocysteine as well as the relatively non-toxic selenide and elemental selenium (Eswayah et al., 2016). Additionally, methylated selenium compounds such as dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe) are relatively non-toxic forms; however, these forms exist often as the result of microbial processes (Eswayah et al., 2016). Several bacteria can reduce selenate and/or selenite to elemental Se (Herbel et al., 2003). In addition, methylation of selenate or selenite results in the volatile DMSe or DMDSe both of which are up to 700 times less toxic than the oxyanions (Ranjard et al., 2003).

The dominant Se species in an aqueous environment is dependent on environmental conditions. Lotic habitats have flowing water, such as creeks and rivers, and in these systems, selenate is the dominant Se species present (Ponton et al., 2020). In contrast, lentic habitats have relatively still water including lakes, ponds and bogs, and selenite is generally the dominant Se species (Ponton et al., 2020). In water systems where reducing conditions are prevalent (lentic) whether by biotic factors such as bacterial and periphyton communities or by abiotic factors such as low oxygen, the selenium bioaccumulation into biota is more pronounced than in oxidizing or fast-moving water systems (Orr et al., 2006; Simmons and Wallschläger, 2011).

1.2. Selenium in the Aquatic Environment

1.2.1. Essentiality

In 1973, the discovery of Se-containing enzyme glutathione peroxidase revealed the essentiality of Se to mammalian health (Rotruck et al., 1973; Chow and Tappel, 1974). Glutathione peroxidase protects tissues against peroxidation from hydrogen peroxide and organic hydroperoxide destruction in animals (Janz et al., 2010). Since then, >20 proteins containing Se, known as selenoproteins, with various functions including antioxidant activities and metal binding or chelation (as proposed for the more recently discovered SelP [Moghadaszadeh and Beggs, 2006]), have been identified. Se-deficiency in humans is observed in several regions worldwide while in others Se excess is of greater concern (Zhu et al., 2009). In regions where the human diet is considered Se-deficient, Keshan Disease is prevalent – a condition characterized by cardiac dysfunction and arrhythmia (Sun et al., 2019). While any potential essentiality of Se to higher-order plants has yet to be elucidated, there is evidence of Se uptake from the soil

by many aquatic and terrestrial plants (Garousi, 2017). Since trace amounts of Se are ubiquitous in natural environments, determining the potential effect of Se-deficiency in some plants would be difficult if not impossible to determine under natural conditions (Garousi, 2017).

Non-essential elements such as chromium and lead do not have a therapeutic window, or range of concentrations/doses that are optimal to organism health. The adverse dose-response curve for Se is biphasic or hormetic, with stimulatory effects (indicating essentiality) at very small doses and adverse effect at higher doses (Mattson and Calabrese, 2010). Adverse effects are observed in organisms in the absence of Se, however there is a threshold after which adverse effects occur again resulting in a U-shaped dose-response curve. What remains undisputed is that the dose-response window (or range of non-toxic concentrations) for Se remains narrow as the balance between essentiality and toxicity is specific.

1.2.2. Bioaccumulation

The ecological risk of Se toxicity to higher trophic level organisms is driven by bioaccumulation. Bioaccumulation refers to the increase in tissue concentration of a chemical/element in an organism relative to that of the ambient medium from all potential sources of exposure including inhalation, dermal contact, and ingestion; biomagnification describes the increased chemical concentration in higher trophic levels relative to that of the trophic level below as the result of dietary exposure; this is critical in modeling Se risk (Jasonsmith et al., 2008). Bioaccumulation is distinct from bioconcentration which is the concentration of a chemical in an organism when the source is exclusively surrounding air or water (Alexander, 1999). Phytoplankton rapidly and efficiently take up inorganic forms of Se and can biotransform them into organic forms including the Se-containing amino acids selenomethionine and selenocysteine (Ponton et al., 2020). These can be transferred *via* dietary exposure pathways to higher trophic level organisms (Janz et al., 2010; Ponton et al., 2020). Since algae show high bioaccumulation potential from the surrounding water, they play an important role in the Se bioaccumulation process in subsequent higher trophic levels and therefore dictate the amount of Se available to those higher level organisms (Simmons and Wallschläger, 2011). Comprehending the bioaccumulation potential in prey is critical to identifying predators that may be at risk for Se toxicity. Therefore, a key factor in how

anthropogenically released Se bioaccumulates in an aquatic environment begins with understanding how the nature and composition of the periphyton community (Simmons and Wallschläger, 2011) affects Se uptake.

Site specific conditions including lotic *v*. lentic systems, water chemistry, and prey/predator community composition affect Se concentrations found in higher trophic level organisms (Ohlendorf et al., 2010). The residence time of Se in lentic systems is comparatively longer than lotic systems. Increased residence time within lentic systems results in greater algal accumulation of Se and, ultimately, greater bioaccumulation of Se in higher tropic levels as compared to lotic systems (Simmons and Wallschläger, 2011). The hydrogeology and biogeochemistry of aquatic environments affect the extent of Se bioaccumulation in the primary producer community (Ponton et al., 2020). In general, there is greater Se accumulation in algae found in lentic compared to lotic water bodies. For example, in the Elk Valley, British Columbia watershed, there is a mixture of fast-moving streams and drainage rivers, as well as some oxbow lakes and ponds (Orr et al., 2006). The lentic environments of the Elk Valley exhibit higher selenium accumulation in biota compared to lotic environments in the same watershed (Orr et al., 2006). Since increased Se in surrounding water bodies can be attributed to weathering of Se-bearing rocks and soils, large variations in Se concentrations between different water systems can be correlated to the surrounding geology (Ponton et al., 2020). The presence of organic matter also impacts the bioavailability of selenium in a particular aquatic environment and can explain some of the variability in selenium bioaccumulation between different sites. The fast-flowing conditions in a lotic system prevents settling of organic rich sediment, the opposite is true of lentic systems (Ponton et al., 2020). In the case of lentic water systems, Se-bound to organic matter, particulate, or sediment, is an ideal substrate for the microbial community and can therefore be recycled back into the water column through redox reactions to be taken up by algae (Lemly, 1998). In the case of ecosystem composition, dietary preference appears to drive the differences in Se uptake of consumers (Sappington, 2002). For example, benthic invertebrates have been observed to accumulate higher tissue Se than their filter-feeding equivalents (Schneider et al., 2015). Preference or prevalence of either by secondary consumers would contribute to differences in Se bioaccumulation between sites with similar water column concentrations. Water composition also plays a key role in Se bioavailability. For example, high sulfate (SO_4^{-2}) concentrations in the water column inhibit Se uptake (Lo et

al., 2015). In freshwater, the relationship between SO_4^{-2} concentration and Se accumulation is tightly related (Lo et al., 2015; Ponton et al., 2020). SO_4^{-2} competitively inhibits selenate uptake (Paquin et al., 2002; Lo et al., 2015). Therefore, it is important to report SO_4^{-2} concentrations when comparing Se uptake across field sites or laboratory conditions.

The uptake and subsequent transformation of dissolved Se to organic forms from the water column *via* microbial processes (known as the "enrichment factor" [EF]), and the successive transfer between trophic levels (trophic transfer factor [TTF]), are critical features in predicting site-specific Se toxicity (Luoma and Presser, 2009; Ohlendorf et al., 2010; Stewart et al., 2010). EFs may differ by orders of magnitude depending on the conditions of a given site (e.g., different SO_4^{-2} concentrations), whereas the TTFs are far less variable (Schlekat et al., 2004; Ohlendorf et al., 2010). Differences in prey type could help explain some of these differences in bioaccumulation as even within a single site, different organisms occupying the same trophic level may rely on different prey items having significantly different tissue Se concentrations (Schlekat et al., 2004). In studies using the ecosystem-scale modeling approach as defined by Presser and Luoma (2010), EFs are calculated and used to model TTFs for invertebrates and fish (Cianciolo et al., 2020). As TTFs are typically consistent (>1) even between laboratory and field studies, EFs are the most variable (Cianciolo et al., 2020). Bioaccumulation of Se at the higher trophic levels is the driving factor of toxicity in most contaminated sites and accurate EF derivation is essential in predicting risk to higher trophic levels without relying exclusively on standard tissue or water guidelines.

1.2.3. Toxicity and Mode of Toxic Action

Current regulations regarding selenium levels in the environment are largely based on water quality guidelines implemented by governmental agencies. According to the BC Water Quality Guideline (WQG), the limit set for Se in the water column protective of aquatic organisms is 2 µg/L (Beatty and Russo, 2014). This value is more conservative than that used in Australia and New Zealand, where there are 4 trigger values (5, 11, 18 and 34 µg/L), which are protective of 99, 95, 90 and 80% of species, respectively (Ohlendorf et al, 2010). In contrast, the Canadian Council of Ministers of the Environment (CCME) guideline is more conservative at 1 µg/L Se (CCME 2007). These water column guidelines are based on specific assumptions about selenium uptake

(from the water into the base of the food web), transfer through the food chain through dietary exposure, and allocation into offspring (*i.e.,* from ovaries to the eggs for fish species). These assumptions include safety factors and conservative values and considered are protective to higher trophic level organisms.

The toxicity of Se to micro-algae and aquatic plants is low in natural water systems and adverse effects are not often observed until water concentrations reach 1 mg/L Se (Gojkovic et al., 2015). In micro-algae, Se toxicity presents as ultrastructural damage including fingerprint-like chloroplasts, less dense stroma, and increased starch production during daylight (Morlon et al., 2005; Gojkovic et al., 2015). Since starch is an energy reserve for cell division and maintenance in micro-algae, its overproduction is normally observed during the dark phase. Therefore, starch overproduction during daylight is suggested as a similar negative response to reduced cell division during exponential growth following Se exposure (Gojkovic et al., 2015; Umysová et al., 2009). As reported by Betty and Russo (2014), growth inhibition, reduced chlorophyll, and protein synthesis as well as wilting and dying of leaves of immature plants are all signs of Se toxicity in aquatic plants. Similarly, the toxicity of Se to invertebrates is low in natural systems and depends on the type of invertebrate (bottom feeders *v*. those in the water column). The signs of Se toxicity at levels exceeding 1-30 µg/g dw in invertebrates include embryonic deformities or mortalities and developmental abnormalities, such as those seen in higher level trophic organisms (Beatty and Russo, 2014).

At elevated selenium levels, sublethal effects (reproductive and teratogenic; particularly in egg-laying vertebrates) are of most concern (Etteieb et al., 2020). Excess selenium in contaminated water bodies primarily affect birds and fish *via* teratogenesis, which is indicative of selenium toxicity in early life-stages (Lemly, 2002). Deformities in fish include concave curvature of the spine (lordosis); convex curvature of the thoracic spinal region (kyphosis); scoliosis; head, mouth, gill, and fin deformities, as well as edema and other heart, brain, and eye issues (Lemly, 2002). According to literature-based Se toxicity thresholds (without cold or warm water fish distinction), egg/ovary selenium levels exceeding 10-20 µg/g dry weight (dw) and whole-body levels exceeding 4-9 µg/g dw can result in juvenile skeletal and cranial deformities, as well as other development malformations (Beatty and Russo, 2014; Etteieb et al., 2020). Warm water fish are typical of lentic and shallow water systems where oxygen levels are lower while cold water fish are associated with lotic systems where oxygen levels are higher

(Hamilton, 2004). There have been some suggestions of alternate toxicity thresholds for Se that are dependent on warm- or cold-water species (DeForest et al, 1999; Brix et al., 2000), however most reported tissue concentrations are without distinction. At levels exceeding 6-15 µg/g dw in bird egg/ovary, lethality or deformation of embryos has been observed (Etteieb et al., 2020). The range of effects reported for Se are broad and inconsistent (Luoma and Rainbow, 2008). Therefore, tissue Se concentrations, which are reflective bioaccumulation from dietary exposure are far more indicative of adverse effect potential to aquatic organisms than background water column concentrations (Luoma and Rainbow, 2008). While background water concentrations are considered conservative for the most part, tissue concentrations are more reflective of Se biomagnification occurring in a specific contaminated water body.

As mentioned previously, Se toxicity is most detrimental in oviparous species occupying higher trophic levels. Teratogenic effects are the dominant effect that occurs in Se toxicity, therefore, in an area with increased Se concentrations there can be largely unaffected, healthy-appearing adult individuals alongside deformed or non-existent juveniles (Lemly, 2002). For example, the contamination of Belews Lake, North Carolina, with Se from the coal-burning facility upstream resulted in sterility of 95% of the freshwater fish populations due to reproductive failures (Lemly, 2002). While terata may not be directly lethal, the resulting deformed spines, fins and gills can negatively impact swimming ability of fish and therefore lead to increased susceptibility to predation – an indirect mortality due to Se toxicity (Lemly, 2002). This has led to complete population collapses of aquatic organisms in areas with elevated Se concentrations (Sorensen et al., 1983; Gillespie and Baumann, 1986; Saiki et al., 1987; Hoffman, 1988; Skorupa, 1998; Lemly, 2002).

Elevated Se levels are observed downstream of anthropogenic activities including mining, power generation, irrigation, and agricultural drainage. Muscatello et al. (2008) noted elevated levels of tissue Se in biota collected downstream of a uranium mine in Saskatchewan, Canada. While the water-column concentrations did not exceed 5 µg/L (the recommended Se water guideline by US EPA), tissue Se in the invertebrates and fish collected at mine-impacted sites were significantly higher than in reference sites (Muscatello et al., 2008). For example, red side shiners and juvenile pike had whole body Se concentrations of 14.98 and 17.02 µg/g dw respectively (Muscatello et al., 2008). As these values exceed the recommended whole body tissue guideline (4-9 µg/g

dw), potential for reproductive impairment of fish or birds in the area has been noted. The Kesterson Reservoir disaster resulted in adverse effects to aquatic life as the area was receiving agricultural drainage from California's San Joaquin Valley in the late 1970s (Ohlendorf, 2002). Tissue Se concentrations in mosquitofish were significantly higher than those in nearby reference sites ranging from 26-31 µg/g (Ohlendorf, 2002). Even more concerning were observations of 347 nests of aquatic birds at Kesterson Reservoir where 40% had >1 embryonic lethality and 20% had embryos with developmental deformities (Ohlendorf et al, 1986). The only trace element at elevated levels at Kesterson Reservoir was Se , which linked the Se contamination of the drainage water to the resulting avian teratogenesis that was observed (Ohlendorf et al, 1986).

Simple substitution of selenium for sulfur (S) in proteins, results in improper protein folding and non-functionality and was the dominant theory (in the 80s and 90s) for the mechanism of Se toxicity (Reddy and Massaro, 1983; Sunde, 1984; Maier and Knight, 1994). This theory may not be current. Selenocysteine and selenomethionine are the two amino acids where Se substitution for S can occur. In the case of selenomethionine, the Se moiety is insulated by the terminal methyl group at the secondary level of the protein structure; therefore, the presence of selenomethionine doesn't appear to alter the functionality of the resultant proteins (Janz et al., 2010). Synthesis of selenocysteine is a highly regulated process at the ribosomal level wherein a UGA codon, containing a specific loop structure, specifies for seleno-cysteinyl-tRNA (Janz et al., 2010). In both cases, simple substitutions are unlikely to cause the observed Se toxicity.

In the past two decades, evidence supporting the role of oxidative stress as the more likely mechanism of toxic action has emerged (Palace et al., 2004). Se is essential to the enzyme glutathione peroxidase; it catalyzes the oxidation of thiols such as glutathione. Normally in the antioxidant process, glutathione peroxidase is required for the redox reaction of reduced glutathione (GSH) to the oxidized form (GSSH) to proceed resulting in the production of water from a reactive oxygen species (ROS; Spallholz et al., 2002). In this process, the ratio of reduced glutathione to the oxidized form exceeds 100:1 for successful reduction of ROS preventing the accumulation of radicals in the cell (Stryer, 1995; Janz et al., 2010). Although many studies remain correlative, supportive evidence for the shift in the reduced to oxidized glutathione ratio and a subsequent

increased presence of oxidative damage occur consistently within cells exposed to elevated Se. In some forms, particularly in the selenide (-2) oxidation state, selenium oxidizes thiols such as glutathione to form thiyl radicals and a selenopersulfide anion, which is responsible for generating superoxide radicals (Spallholz et al., 1998; Palace et al., 2004). Superoxide radicals are potent oxidizing species that can cause significant cellular damage (Janz et al., 2010). The species of selenium is key to the production of these damaging radicals. For example, selenomethionine, the predominant form in the eggs of oviparous vertebrates, has been shown to be not highly reactive with glutathione in that form (Spallholz and Hoffman, 2002). However, certain *in vivo* metabolic processes result in the more reactive Se species from selenomethionine such as the highly reactive methylselenol. Methylselenol generates superoxide *in vitro* as observed by Spallholz et al. (1998) by oxidizing GSH thereby preventing GSH from reducing ROS to water. This indicates the essentiality of Se in the production of superoxide radicals (Janz et al., 2010; Palace et al., 2004). As selenomethionine and/or selenocysteine are the driving species of bioaccumulation, observed metabolic processes in mammals and birds resulting in more reactive Se species provides insight into how oxidative damage is the most likely mode of toxic action. In fact, the cellular damage and resulting lesions observed from oxidative stress in rainbow trout embryos exposed to elevated Se (1 mg/mL selenomethionine) is similar to that observed from exposure to organochlorine contaminants known to cause oxidative stress such as retene (Bauder et al., 2002; Palace et al. 2004).

1.3. Chlorophyta

Chlorophyta consists of green algal species, all of which share certain characteristics: the presence of Chlorophyll a and b, chloroplasts with two-envelope membranes, and plastids containing starch deposits as the primarily polysaccharide reserve (Gojkovic et al., 2015). The micro-algae in this group have standard microbial culture growth cycles under batch culturing conditions consisting of a lag phase, an exponential phase, and then a stationary phase (Nasr et al., 2017). The unicellular green alga *Raphidocelis subcapitata* is used in standard toxicity bioassays worldwide in protocols recommended by EC (2007), USEPA (2002) and OECD (2011). The freshwater algae *Parachlorella kessleri, Chlorella vulgaris* (also recommended in phytotoxicity bioassays), *R.* subcapitata, and *Tetradesmus obiquus* are found

ubiquitously throughout North America (Li et al., 2013; McLarnon-Riches et al., 1998; Oliveria et al., 2021; Simmons and Wallschläger, 2011).

1.4. Selenium and Algae

1.4.1. Uptake and accumulation

Taxonomic differences in selenium uptake potential and bioaccumulation among algal groups are well documented in the literature (Gojkovic et al., 2015; Schneider et al., 2015; Markwart et al., 2019). Physiological requirements for Se in some species *v.* the presence of elimination mechanisms in others, are theorized to be responsible for these taxonomic differences (Ponton et al., 2020). Some algae require Se for metabolic processes (selenoproteins) and have mechanisms for the specific incorporation of Se-amino acids into proteins (Novoselov et al., 2002). Evidence exists that some micro-algae can metabolize inorganic Se into reduced Se-metabolites including the non-toxic elemental Se (Se^0) or to methylated volatile forms resulting in lower steady state levels of Se (Neumann et al., 2003; Vriens et al., 2006; Simmons and Wallschläger, 2011; Schiavon et al., 2017; Kizovsky et al., 2021). The metabolic excretion processes yielding less toxic methylated and elemental Se species are regarded 'detoxifying' mechanisms to the algae by elimination (Gojkovic et al., 2015). Increased Se tolerance can be achieved in algae species that can produce volatile methylated selenium species as intermediates through metabolic processes resulting in decreased accumulation despite elevated Se concentrations in the surrounding media (Pilon-Smits and Quinn, 2010).

Pristine freshwaters generally contain less than 1 µg/L Se (Riedel et al., 1991). In water bodies contaminated with Se, particularly in the forms of selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), micro-algae play a key role in increasing the ecotoxicological risk of Se to higher trophic level organisms (Ponton et al., 2020). Higher Se accumulation in micro-algae can lead to higher biomagnification throughout the food chain leading to higher levels in upper trophic levels and subsequent higher risk to toxicity. In the case of selenate, strong evidence suggests bioaccumulation by a wide range of algal species is a physiologically mediated process, opposed to adsorption (Riedel et al., 1991). This contrasts with selenite, where there is evidence to support both a biotic (transporter-mediated) and abiotic (adsorption) component of uptake by phytoplankton and

subsequent trophic transfer of Se, which can therefore occur by two mechanisms (Riedel et al., 1991).

Saturable protein transporters exist in micro-algae for the uptake of sulfate (SO_4^{2-}) and sulfite (SO_3^{-2}). There has not conclusively been a specific Se transporter identified in algae, suggesting that the uptake of selenium oxyanions is likely mediated by these non-discriminatory proteins that take up either sulfate or sulfite (Umisová et al., 2009). These transporters also recognize selenate (SeO_4^{2-}) and selenite (SeO_3^{-2}) and take them up in lieu of the sulfur compounds, providing a pathway for Se uptake. The direct competition for the transporters results in a negative correlation between S concentration and Se uptake in micro-algae (Lo et al., 2015; Gojkovic et al., 2015). Specifically, due to chemical similarity with S, Se can enter the S reductive assimilation pathway once taken up by micro-algae (Neumann et al., 2003). The S reductive assimilation pathway is where sulfate (SO_4^{2-}) is first reduced to sulfite (SO_3^{2-}) then to sulfide (S^{2-}) for incorporation into cysteine (Neumann et al., 2003 After moving to the chloroplasts, the site of Se/S assimilation into amino acids, Se can be converted to selenide (Se^{2-}) which is the ultimate substrate for Se-amino acids SeCys and SeMet (Neumann et al., 2003; Ponton et al., 2020).

Water chemistry can significantly affect the bioavailability and uptake of Se in surface waters by algae. For example, the effect of pH on selenite uptake and availability to algae is debated. Some research suggests that selenite exists as either the deprotonated or protonated form depending on the pH, potentially affecting its uptake availability as it is preferentially taken up by transporters in the deprotonated form (Ponton et al., 2020). In contrast, selenate does not appear to be affected by pH and instead remains in the deprotonated form regardless of natural water pH fluctuations (Torres et al., 2010; Schiavon et al., 2017). Furthermore, pH can directly alter the algal surface charge and therefore the properties of Se membrane transporters (Schiavon et al., 2017). These transporters require specific pH conditions for optimal uptake efficiency. For example, in in the unicellular green algae species *Chlamydomonas reinhardtii,* optimal selenate uptake occurs at pH values around 8 (Riedel and Sanders, 1996) as this is optimal for the transporters themselves.

1.4.2. Selenoprotein

The ability of algae to alter carbon allocation depending on the environmental conditions indicates changes in cellular composition can occur even within a single species. Under optimal growth conditions, algae synthesize fatty acids primarily for esterification into glycerol-based polar lipids – significant components of intracellular membranes (Fernandes et al., 2013). Alternatively, under stressful environmental conditions, many algae instead form and accumulate neutral lipids in the form of triacylglycerol as an energy reserve (Li et al., 2011; Breuer et al., 2012). Interestingly, micro-algae in the taxon Chlorophyta alter their protein, lipid, and starch compositions in response to various stressors – including lighting intensity (Vendruscolo et al., 2019). Under nitrogen (N) starvation, Chlorophyta often show an increase in starch production sometimes reaching nearly half of the cell composition (Li et al., 2010). Alternatively, some algae instead accumulate both lipids and starches (Li et al., 2011). In either case, stressors such as N depletion tend to result in proportionally less protein.

Micro-algae with biochemistries that require Se contain similar mechanisms as those seen in mammals to control the incorporation of Se-amino acids into selenoproteins (Hatfield and Gladyshev, 2002). The green algae species *Chlamydomonas reinhardtii* contains at least 12 naturally occurring selenoproteins, each with essential roles in maintaining cell viability and in antioxidant defense systems (Gojkovic et al., 2015; Vriens et al., 2016). In micro-algae where Se is considered essential, SeCys is incorporated into the catalytic site of selenoproteins (Schiavon et al., 2017). SeCys is considered the 21st amino acid as it exists naturally in all kingdoms of life (Johansson et al., 2005). SeCys is incorporated into selenoproteins during translation by a UGA codon with special structures to avoid translational termination (Stadtman, 1996). In the case of non-specific incorporation of selenocysteine or selenomethionine into proteins, these are "Se-containing proteins" as opposed to the functional selenoproteins (Gereben et al,, 2008; Bulteau et al., 2015). This process, that has been elucidated in humans and other plants, is likely similar in micro-algae containing selenoproteins (Ponton et al., 2020).

Excess Se can cause saturation of the S/Se protein assimilation machinery. Once algal cells are unable to further produce Se-containing amino acids from reduced inorganic Se, SeCys is metabolized to form Se^0 which is subsequently excreted, thus

indicating a threshold for Se-amino acid incorporation into proteins (Kizovsky et al., 2021). Whether by saturation of the Se/S assimilation machinery or in the translational machinery, this apparent threshold in amino acid Se incorporation suggests that protein may dictate the ability of algae to accumulate Se and by extension, bioaccumulation potential of primary producers.

1.5. Overview of Research

Although an abundance of research exists on the toxic mode-of-action of Se, insight into the driving factors of bioaccumulation in primary producers is warranted. The ecological risk of Se is from its biomagnification throughout the food web, which drives tissue levels of higher trophic levels. Modeling of Se bioaccumulation by Presser and Luoma (2010) and subsequent laboratory and field-based experiments show that TTFs are typically consistent and >1 (Cianciolo et al., 2020). What remains variable are the EFs (i.e. the concentration of Se taken up at the base of the food web over that in the water). Variable EFs between different sites with similar water Se concentrations directly correlate with differences in the biomagnification of Se despite TTFs remaining comparable (Cianciolo et al., 2020). This highlights the importance of primary producers in driving bioaccumulation to higher trophic levels. It has been suggested that differences in the composition of the primary producers of a given area is responsible for the unpredictability in Se tissue concentrations in fish and birds in areas of similar Se contamination (Wayland and Crosley, 2005). It is possible that differences in the cellular composition of the primary producers (e.g., algae) affects bioaccumulation from the surrounding water. Since Se is tightly associated with proteins due to its similarity to S and uptake *via* the same transporters, protein content could be the primary driver of bioaccumulation. To understand trophic transfer and to proactively use Se modeling in risk assessments, it is important to understand the dominant factors in primary producer uptake and accumulation, including protein concentration as it relates to bioaccumulation.

The objective of this study was to evaluate the relationship between the internal protein content and selenium accumulation in micro-algae. Four micro-algae species belonging to Chlorophyta, *P. kessleri*, *C. vulgaris*, *R. subcapitata*, and *T. obliquus* were selected due to their prevalence in freshwater aquatic environments and association with environments with potential Se contamination. Experiments to determine if differences in

protein between these four algae species at different growth phases and lighting levels were conducted. Determined growth phases were used to evaluate tissue selenium accumulation and relate it to total protein content to determine if a relationship existed.

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Chapter 2.

The relationship between cellular protein content and selenium accumulation in freshwater micro-algae

2.1. Abstract

The variability in the bioaccumulation potential at the base of the food web lends to the unpredictability of biomagnification and resulting ecological risks for selenium to higher trophic level organisms. Water flow conditions (lotic/lentic), water chemistry and periphyton community composition have all been suggested as sources for this variation in primary producers. The differences in selenium uptake and accumulation between species of primary producers culminates in highly variable enrichment factors (EFs) that require further investigation. *P. kessleri*, *C. vulgaris*, *R. subcapitata*, and *T. obliquus* were evaluated for total protein content at different growth phases (lag, exponential, and stationary). To determine if a relationship exists between algal protein content and Se accumulation, *P. kessleri*, *C. vulgaris*, and *R. subcapitata* were exposed to 40 µg/L selenium (as selenate) and analyzed for total protein and tissue Se content in both the exponential and stationary growth phases. Protein content and Se accumulation in *R. subcapitata* were additionally evaluated under two different lighting intensities in the stationary phase: moderate (3600-4400 lux) and high (7890-11960 lux). No relationship between cellular protein content and selenium accumulation were found in the exponential phase; however, a strong relationship between the two was seen in the stationary phase, among the different species and within *R. subcapitata* at different light intensities. While the selenium accumulation (expressed on a dry weight basis) between *P. kessleri*, *C. vulgaris*, and *R. subcapitata* in the stationary phase were statistically different, when normalized to protein content values were similar: 0.039, 0.042, and 0.043 µg Se/g protein, respectively, and were not significantly different. These results suggest that cellular protein content in micro-algae is driving Se bioaccumulation and that algal protein content will be useful in bioaccumulation and biomagnification modelling for Se in food webs.

2.2. Introduction

Selenium is naturally found in rocks and soils, most notably black shale, phosphate bearing rocks, and coal (Presser et al., 2004) and release from these natural sources by weathering and erosion processes or from volcanic eruptions (Davis et al., 1988; Etteieb et al., 2020) results in selenium being ubiquitous in the natural environment. However, its mobilization and concentration through anthropogenic activities (e.g. mining [phosphate, uranium, coal], power generation, and irrigation [Schneider et al., 2015; Etteieb et al., 2020]) and subsequent bioaccumulation in aquatic environments are of concern (Young et al., 2010). These anthropogenic activities have resulted in environmental damage from elevated Se levels (Gillespie and Baumann, 1986; Presser and Ohlendorf, 1987; Lemly 2002; Ohlendorf 2002).

The environmental damage that has been attributed to Se-related toxicity has been documented extensively, such as the Se contamination at Hyco Reservoir, NC (Gillespie and Baumann, 1986). The coal-fired power plant that was upstream resulted in high levels of Se in the water column, which were causally linked to reduced larval survival and embryonic deformities present in female bluegills (Gillespie and Baumann, 1986). Another well documented case of toxicity resulting from Se contamination was the deformed embryos and complete reproductive failure of different aquatic birds in the Tulare Basin, CA (Skorupa and Ohlendorf, 1991).

Environmental selenium exists in various forms; most commonly as selenate (SeO_4^-) and selenite (SeO_3^{2-}) in water (Young et al, 2010), but also as elemental selenium, selenide, and methylated selenium (Orr et al. 2006). The SeO_4^- and SeO_3^{2-} anions are converted by primary producers to organic forms through active uptake and substitution of sulfur in amino acids, creating the amino acids selenocysteine and selenomethionine. As an essential trace element, Se is incorporated into enzymes that play important roles in protecting against oxidative damage (e.g. plasma glutathione peroxidase, thioredoxin reductase 1, type 1 deiodinase, selenoprotein P). The more recently discovered selenoprotein (SelP), which appears to be highly conserved across taxa, seems to serve a function in metal binding or chelation (Mostert, 2000). Despite its essentiality as a trace element for the health and survival of aquatic organisms, selenium has a very narrow dose range between essentiality and toxicity (Mattson and Calabrese, 2010). The dose-response curve for Se is U-shaped as adverse effects are observed in

organisms when it is absent, however due to bioaccumulation once it enters the food chain, Se levels quickly become toxic.

The most widely accepted hypothesis for the toxic mechanism of action is through oxidative stress (Palace et al., 2003). With increasing levels of Se, interference with the normal process of antioxidation involving glutathione is observed (Spallholz and Hoffman, 2002). Certain species of Se such as selenium dioxide (SeO_2) are strong oxidizing agents that can result in increased levels of oxidized glutathione (GSSG) and lower levels of reduced glutathione (GSH), resulting in reactive oxygen species (ROS) accumulation (Spallholz and Hoffman, 2002). An increased level of ROS and subsequent radical formation results in oxidative damage to the surrounding cells. For example, toxic effects, particularly in oviparous organisms, are observed in actively growing embryos after Se has been assimilated from the yolk, likely because of oxidative stress (Palace et al., 2003). In a study examining rainbow trout (*Oncorhynchus mykiss*) fry, development of the yolk sac and pericardial edema was followed by hemorrhaging, which could be mediated by the superoxide radical production and resulting oxidative stress in the myocardial tissue (Palace et al., 2003).

Acute lethality of Se exposures to adult aquatic vertebrates are not observed until levels exceeding 1 mg/L in the water column or (Cardwell et al., 1976; Kumar et al., 2018). In a study by Bennett et al. (1986), whole body tissue concentrations as high as 61.1 µg Se/g dw in fathead minnow larvae did not result in mortality after 7 d of Se dietary exposure. Sublethal effects such as reduced growth, embryo-larval developmental effects (teratogenesis) and diminished immunity at elevated water Se levels (>2 µg/L) are of concern for egg-laying vertebrates (Etteieb *et al*., 2020). Teratogenesis as the result of Se toxicity is observed in both birds and fish at the embryo-larval stage (Hamilton SJ, 2004). Deformities in fish include concave curvature of the spine (lordosis), convex curvature of the thoracic spinal region (kyphosis), scoliosis and head, mouth gill and fin deformities as well as edema and other heart brain and eye issue (Hamilton SJ, 2004). In birds, similar deformities as those in fish are observed in addition to decreased immune function and reduced growth of ducklings (Hoffman, 2002). In fish, egg/ovary selenium levels exceeding 10-20 µg/g dry weight (dw) and whole-body levels exceeding 4-9 µg/g dw have been observed to result in juvenile skeletal and cranial deformities as well as other development malformations

(Etteieb *et al*., 2020). At levels exceeding 6-15 µg/g dw in bird egg/ovary, lethality or deformation of embryos is observed (Etteieb *et al*., 2020).

Tissue Se levels in higher trophic level organisms such as fish and birds are the result of the biomagnification of Se driven by Se-containing amino acid transfer between trophic levels (Ponton et al., 2020). The conversion of inorganic forms into organic forms is completed in primary producers at the base of the food web. Se bioaccumulation at the primary producer level is variable and not easily predicted (Conly et al., 2013), which is reflected in calculated enrichment factors (EFs; [tissue Se in primary producers]/[Se in water column]), which range from 10^2-10^6 (Stewart et al., 2010). The incorporation of selenium containing amino acids into proteins by primary producers promotes its entry into the food web (Sunde, 1997). Se moves into the higher trophic levels *via* the diet and biomagnifies to levels far greater than those that would result from water uptake alone (Schneider et al., 2015). This is reflected in the trophic transfer factors (TTF) >1.0 between subsequent trophic levels or concentration of tissue Se in the predator over that of its prey (Stewart et al., 2010). While EFs may range in orders of magnitude between sites, TTFs remain relatively consistent: 0.94-3.2 (3.4-fold) in invertebrates and 0.8-1.7 (2.1-fold) in fish (Presser and Luoma, 2010). TTFs are species specific due to differences in physiology across organisms occupying the same trophic level. What is consistent are TTFs for a specific species whether in lab or field exposures (Presser and Luoma, 2010). The physiology of a given organism is not variable for the most part and variation in TTFs of the same species can be attributed in slight differences in assimilation efficiency depending on the exact prey source at a contaminated site (Luoma and Presser, 2010).

Predictions of tissue selenium concentrations in organisms at higher trophic levels based on water concentrations are inconsistent and extending these predictions to the risk of toxic effects are not accurate (Holm et al., 2005; Wayland and Crosley, 2005). In the study by Wayland and Crosley (2005), Se was significantly greater in mayflies and caddisflies compared to stoneflies at a mine-affected site in the Rocky Mountains despite all insect taxa seemingly sharing similar habitats. In 2002, the water concentration at the mine-impacted site was 4.23 µg/L resulting in tissue concentrations of 7.06, 7.94. 4.89 µg/g dw for mayflies, caddisflies, and stoneflies respectively (Wayland and Crosley, 2005). It was concluded that differences in dietary preference in primary producer prey within the same ecosystem was responsible for the difference in tissue Se

concentrations among the taxa (Wayland and Crosley, 2005). There is a large data bank of TTFs for various predators with some variability in invertebrates depending on site-specific conditions but with relative consistency in the TTFs for fish or bird species (Presser and Luoma, 2010). Findings such as these further emphasize the knowledge gap that exists in understanding the mechanism of selenium assimilation and subsequently toxicity.

Given the propensity for bioaccumulation and biomagnification and the potential for adverse effects, understanding the factors that influence Se accumulation in primary producers is critical for effective regulatory management. The ecosystem-scale modeling of Se described by Presser and Luoma (2010) emphasizes that empirical observations of Se of partitioning from the water to the base of the food web are recommended due to the wide variability of EFs. If translation of water-column concentrations into the base of the food web is required, the EFs are suggested to be 1000 (Presser and Luoma, 2010). Despite being a main driver for Se bioaccumulation into the food web, there are many unknowns regarding the factors that determine Se uptake in algae. Due to their chemical similarity and ability to move throught the same transporters and incorporate into similar amino acids there is a tight relationship between Se and S uptake indicating a potential relationship between Se uptake and cellular protein (Sunde, 1997; Lo et al., 2015). If cellular protein drives Se uptake and bioaccumulation at the base of the food web (e.g., algae) then this would potentially aid in predicting EFs more accurately from theoretical water-column Se concentrations.

The aim of this study was to determine if total protein content in green micro-algal species changed with each phase of growth and with light intensity and to determine if Se uptake and accumulation were correlated with protein concentrations. The freshwater unicellular Chlorophyta species used in this study (*Parachlorella kessleri*; *C. vulgaris; R. subcapitata*; and *Tetrasdesmus obliquus)* are found ubiquitously in Se contaminated water systems in North America; making up a significant proportion of prey to higher trophic level organisms (McLarnon-Riches et al., 1998; Simmons and Wallschläger, 2011).

2.3. Methods

2.3.1. Culturing micro-algae and capturing growth phases

Exposures were carried out at Nautilus Environmental (Calgary, AB) in a temperature and photoperiod controlled environmental chamber. The chemicals used were reagent grade. Micro-algae species (Table 1) were obtained from the Canadian Phycological Culture Centre (CPCC, Waterloo, ON). The media for all exposures was a modified recipe for Bold's Basal Medium (BBM) (Stein, 1973). The modified BBM was composed of: KH_2PO_4, 0.13 mM; $CaCl_2 \cdot 2H_2O$, 0.17 mM; $MgSO_4 \cdot 7H_2O$, 0.30 mM; $NaNO_3$, 2.94 mM; K_2HPO_4, 0.43 mM; NaCl, 0.43 mM; $Na_2EDTA \cdot 2H_2O$, 0.03 mM; $FeCl_3 \cdot 6H_2O$, 0.59 µM; H_3BO_3, 0.39 mM; $MnCl_2 \cdot 4H_2O$, 9.15 µM; $ZnSO_4 \cdot 7H_2O$, 0.77 nM; $Na_2MoO_4 \cdot 2H_2O$, 1.61 nM; $CuSO_4 \cdot 5H_2O$, 0.32 nM; $Co(NO_3)_2 \cdot 6H_2O$, 0.17 nM.

Table 2-1. **Micro-algae strains used in this study. Algae were obtained from the Canadian Phycological Culture Centre (CPCC, Waterloo, ON, Canada).**

Genus	Species	CPCC No.	Isolator (date)
Parachlorella	*kessleri*	266	Pratt (pre-1986)
Chlorella	*vulgaris*	90	Unknown
Raphidocelis	*subcapitata*	37	Skulberg (1959)
Tetradesmus	*obliquus*	5	Unknown

Aliquots of starter cultures obtained from the CPCC were initially placed onto solid medium slants and stored at 4 ± 2°C in darkness according to Environment Canada methods for culturing algae (2007). Axenic cultures of 200 mL were established by inoculating the contents of the slants into the BBM media. Flasks were placed on an orbital shaker plate and shaken at 100 rpm. Across all experiments, cell counts were performed using a coulter-counter (Beckman-coulter, L10). Prior to using the coulter-counter on selected species, cell size thresholds (maximums) were performed using the count comparison method provided by the coulter-counter particle size analyzer User Manual (2011). This was done first by using a hemocytometer and microscope to manually count cells in a subsample of cells after 3 d of incubation. These cell counts were taken from a 100, 25, and 12.5% dilution of the subsample and were used as reference counts. The same dilution series from the same sample was concurrently measured using the Z series coulter-counter and a Chi^2 test was conducted using the

observed (Z series) and expected (reference) cell counts. An example of this process is provided in Appendix A.

The cultures were maintained and checked for health (visual assessment of color, cell shape and integrity) using a compound microscope as per the guideline for algae culturing by Environment Canada (2007). Cultures were turned over every 7 d by transferring 1 mL of culture containing ~3 x 10^6 cells of each species to a 1 L baffled flask containing 200 mL of fresh sterile growth media. Growth curves for each species were generated by performing daily cell counts using the coulter-counter on a single flask (n=1). The three growth phases (lag, exponential and stationary) were visually identified on the resulting growth curve figure *via* the methods described by Environment Canada (2007) and the timing for each phase was assumed with cell counts used as confirmation for subsequent experiments.

2.3.2. Experimental Design

The first experiment was performed to determine the total protein content of each species (*P. kessleri*, *C. vulgaris*, *R. subcapitata*, and *T. obliquus)* within each growth phase established during culture. Experiments were conducted in 1 L glass Erlenmeyer test flasks containing 792 mL of modified BBM. 8 mL of sterile culture were transferred to the test flasks. Test flasks were exposed to identical conditions as those stated above for culturing with the exception that flasks were not placed on a shaker plate and instead supplied with continuous aeration with shaking to prevent algae from settling and daily rotations. Protein analysis was performed at each growth phase (lag, exponential, and stationary), which were on days 2, 3 and 7 for *P. kessleri* and *C. vulgaris*, and on days 3, 7 and 10 for *R. subcapitata* and *T. obliquus* as confirmed by cell counts. Two biological replicates (initiated at different points in time using inoculum from different starter cultures) were used for protein analysis: the first with four flasks (technical replicates) and the second with three flasks (technical replicates) resulting in n=7 of *P. kessleri*, *C. vulgaris*, *R. subcapitata*, and *T. obliquus*. 40 mL was subsampled for protein analysis, and a separate 40 mL was subsampled for dry weight at lag, exponential and stationary growth for all replicates. Subsamples were removed using 50 mL serological pipettes with an electronic pipettor.

The second experiment evaluated whether Se exposure altered the total protein content of *P. kessleri*, *C. vulgaris, R. subcapitata*, and *T. obliquus* in exponential and stationary phase. Growth phases were confirmed by cell counts. Test flasks (as described above) contained modified BBM and Se was added to achieve a final concentration of 40 µg/L Se. 4 mL of a 10 mg/L Se stock solution was added (as Na_2SeO_4) to 788 mL of modified BBM prior to algae inoculation into the test flasks. In a negative control, only modified BBM was added to the test flasks prior to algae inoculation. Negative controls and Se exposures were performed with *P. kessleri*, *C. vulgaris, R. subcapitata*, and *T. obliquus* in triplicate. Chamber conditions for this experiment were identical to those described in experiment 1. 40 mL was subsampled for protein analysis, and a separate 40 mL was subsampled for dry weight at exponential and stationary growth for all replicates. Subsamples were removed using 50 mL serological pipettes with an electronic pipettor.

The third experiment evaluated total protein content and tissue Se of *P. kessleri*, *C. vulgaris,* and *R. subcapitata* exposed to 40 µg/L Se (added as Na_2SeO_4) in each of the exponential and stationary phases. Growth phases were confirmed by cell counts. Selenium exposure with modified BBM + 40 µg/L Se (added as a 10 mg Se/L Na_2SeO_4 stock solution) was performed with *P. kessleri*, *C. vulgaris,* and *R. subcapitata* in triplicate. Experimental conditions were identical to those described above for experiment 2. 40 mL was subsampled for protein analysis, second 40 mL was subsampled for dry weight, and a third 40 mL was subsampled for tissue Se analysis (for a total of 120 mL) at exponential and stationary growth for all replicates. Subsamples were removed using 50 mL serological pipettes with an electronic pipettor.

In experiment 4, the accumulation of Se in *R. subcapitata* was examined in the stationary phase at different cellular protein contents. *R. subcapitata* was cultured in triplicate under two light conditions: high range (HR) at 7890 – 11960 lux cool white and medium range (MR) 3600-4400 lux cool. The range for MR lighting is provided by Environment Canada (2007), and HR lighting was chosen based on the optimal range suggested for *Chlorella* sp. (He et al., 2015). Prior to algal inoculation, Se was added using a 10 mg Se/L stock (as Na₂SeO4) to achieve a final concentration of 40 µg/L Se. Stationary phase was confirmed by cell counts. 40 mL was subsampled for protein analysis, second 40 mL was subsampled for dry weight, and a third 40 mL was subsampled for tissue Se analysis (for a total of 120 mL) at stationary growth for all

replicates. Subsamples were removed using 50 mL serological pipettes with an electronic pipettor.

2.3.3. Dry weight

The 40 mL subsample was placed into pre-weighed and dried aluminum weigh boats and placed in a 60° C oven for 48 h. The final weight minus the initial weight was used as the dry weight to normalize protein values.

2.3.4. Protein Extraction and Analysis

For protein extraction, a combination of sonication methods described by Meijer and Wijffels (1998) and Lee et al. (2017) were used. 40 mL aliquots of samples taken from cultures were pipetted into 50 mL centrifuge tubes and centrifuged at 3500 $x\ g$ for 15 min. The supernatant was decanted without disturbing the pellet and the algae were then washed with deionized reverse osmosis water (DRO). The centrifugation and washing steps were repeated two additional times. The final algal pellet was resuspended in 0.08 M potassium chloride to be used for protein analysis and cooled to $4 \pm 2°C$ for 30 min prior to protein extraction.

The pellet resuspended in potassium chloride was placed on ice and then cells were lysed via sonication at 8 Watts (RMS) with a frequency of 20 kHz for 15 sec. The tube was placed on ice for a minimum of 2 min before the sonication cycle was repeated twice more, for a total sonication time of 45 sec. Following cell lysis, samples were centrifuged at 3500 $x\ g$ for 10 min and the supernatant was used for protein content determination, with care taken as to not disturb the pellet at the bottom of the centrifuge tube.

The Bradford microplate method with Quick Start^{TM} reagent was used for total protein content analysis using a commercially available kit (Bio-rad, Ontario, CA). In this method, 150 μL of sample was added to the wells followed by 150 μL of Bradford reagent. The sample and reagent incubated at room temperature for 5 min. The microplate was read using a UV spectrophotometer micro-plate reader (Bio-rad) at 595 nm. A standard curve using bovine serum albumin (Barbarino and Lourenço, 2005) and a blank control were run on each plate.

2.3.5. Tissue Selenium Concentration

The sample sonication methods used for protein extraction were used for tissue Se sample preparation. 40 mL aliquots of samples taken from cultures were pipetted into 50 mL centrifuge tubes and centrifuged at 3500 $x\ g$ for 15 min. The supernatant was decanted without disturbing the pellet and the algae were then washed with deionized reverse osmosis water (DRO). The centrifugation and washing steps were repeated two additional times. The resulting pellet in the 50 mL centrifuge tube was stored frozen at -20°C. The samples were sent with ice for total selenium content to be measured in the tissue of *P. kessleri*, *C. vulgaris*, and *R. subcapitata* by Trichanalytics (Victoria, BC). This was done via a custom-made laser ablation inductively coupled plasma mass spectrometer (LA-ICP-MS) combination. Samples were quantified using algae calibration standards prepared by Nautilus Environmental (16 Jul 2018), where theoretical concentrations were reported by a third-party laboratory. Limits of detection were 257 µg/L. All tissue concentrations in this study are presented on a dry weight basis.

2.3.6. Statistical Analysis

All statistical analyses were performed using Rstudio (Rstudio Team, version 2021.2.382). A two-way analysis of variance (ANOVA) followed by Tukey's post-hoc method for pairwise multiple comparisons was used to analyze for differences between total cellular protein content between the four algae species at each of the three growth phases. If a significant interaction was detected, a one-way ANOVA was performed followed by Tukey's post-hoc test to determine the significance of one factor. The untransformed data met the assumption for normality however it did not meet the assumption of equal variance. A log transformation of the protein concentrations (normalized to dry wt) was necessary to meet the assumptions of ANOVA. A t-test was performed to determine whether protein content differed in control algae and those exposed to 40 µg/L Se in the four algae species at the exponential and stationary growth phases. Linear regression analysis was performed to determine if a relationship existed between total protein content and tissue selenium for *C. vulgaris*, *R. subcapitata* and *P. kessleri* at either the exponential or stationary growth phases. Additionally, a linear regression was performed to determine if a relationship between total protein content and tissue selenium for *R. subcapitata* at each of the light regimes existed. A chow test, to analyze the similarity between the two coefficients of regression was run to determine

whether they were significantly different from each other. Significance was established at α= 0.05 for all statistical analyses.

2.4. Results

All algal species were successfully cultured under the conditions described and exhibited all typical growth phases. The growth curves for *P. kessleri*, *C. vulgaris*, *R. subcapitata*, and *T. obliquus* are shown in Figure 2-1. All algal cells appeared healthy up to the end of the stationary phase based on daily observations of cell shape and integrity. An additional growth phase is typical of micro-algae following the stationary phase and is termed the decline phase; it was not evaluated in this study. Differences in the initiation and length of each of these phases was noted for each species (Table 2-2).

Table 2-2. **Days *Parachlorella kessleri*, *Chlorella vulgaris*, *Raphidocelis subcapitata*, and *Tetradesmus obliquus* were in each phase (lag, exponential, and staionary) of growth as determined visually from growth curves.**

Species	Lag phase (d)	Exponential phase (d)	Stationary phase (d)
Parachlorella kessleri	0-2	3-5	6-7
Chlorella vulgaris	0-2	3-4	5-7
Raphidocelis subcapitata	0-4	5-8	9-10
Tetradesmus obliquus	0-4	5-8	9-10

All 4 species of algae were evaluated for total protein content (in μg protein/mg dry weight) at each phase (lag, exponential, stationary) in the absence of Se (Figure 2-2). All algal cells appeared healthy in all phases according to established criteria as described above. Total cellular protein content (on a dry weight basis) increased in each growth phase, but not to the same extent. The highest levels of protein were seen for all species in the stationary phase. A two-way ANOVA showed that species, growth phase and the interaction between the two factors were significant for total protein content (species: $F_{(3, 84)} = 49.43$, $p < 0.0001$, growth phase: $F_{(2, 84)} = 480.00$, $p < 0.0001$, and interaction: $F_{(6, 84)} = 37.60$, $p < 0.0001$). A one-way ANOVA and Tukey's post-hoc test for multiple pairwise comparisons indicated that there were significant differences

(p<0.05) between different species within each phase (Figure 2-2). The minimum increase in protein content was in *T. obliquus* which showed an increase of 3.6-fold between the lag and stationary phases, followed by *C. vulgaris* (4.4-fold), *R. subcapitata* (8.9-fold), and the greatest increase in *P. kessleri* (25.1-fold) µg/mg, dry wt, respectively. The largest increases generally occurred between the lag phase and the exponential phase (except for *P. kessleri*).
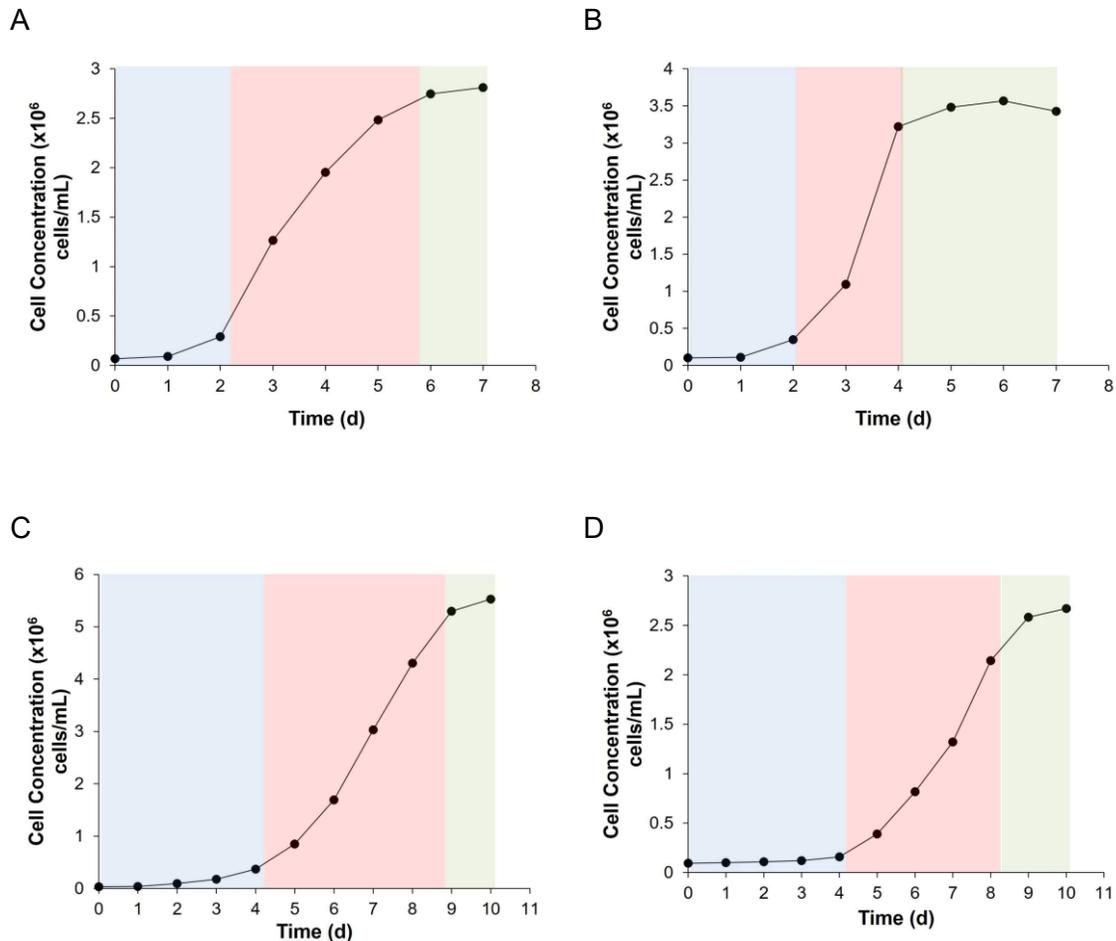
Figure 2.1. **Daily cell counts (cells/mL) of *Parachlorella kessleri* (A), *Chlorella vulgaris* (B), *Raphidocelis subcapitata* (C), and *Tetradesmus obliquus* (D) grown in 200 mL modified BBM at 3600-4400 lux. The different phases are indicated for each species by background color: blue=lag phase, red=exponential phase, green=stationary phase. Each point is the cell counts taken from a single flask (n=1) over an incubation period of 7-10 d.**

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Figure 2.2. **Total protein content of *Parachlorella kessleri* (■), *Chlorella vulgaris* (■), *Raphidocelis subcapitata* (■), and *T. obliquus* (■) at the lag, exponential, and stationary phases of the algae growth cycle. Values are means ± SEM, n=7. Different letters denote significance of protein content within a given phase.**

P. kessleri, *C. vulgaris*, *R. subcapitata*, and *T. obliquus* were examined for total protein content following an exposure to 40 µg/L Se (as Na_2SeO_4) in the exponential and stationary phases (Figure 2-3). No significant differences (t-test, $p > 0.05$) were observed in protein content between controls and the exposed groups.

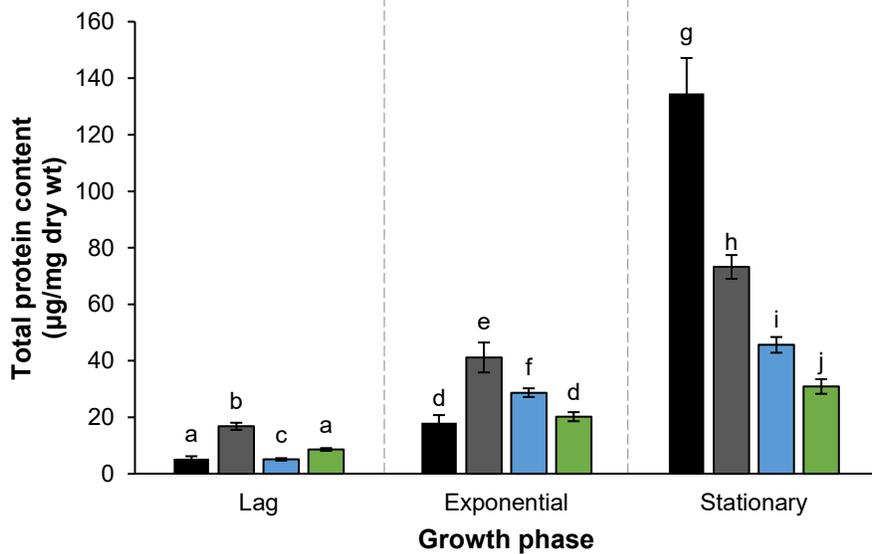A   B
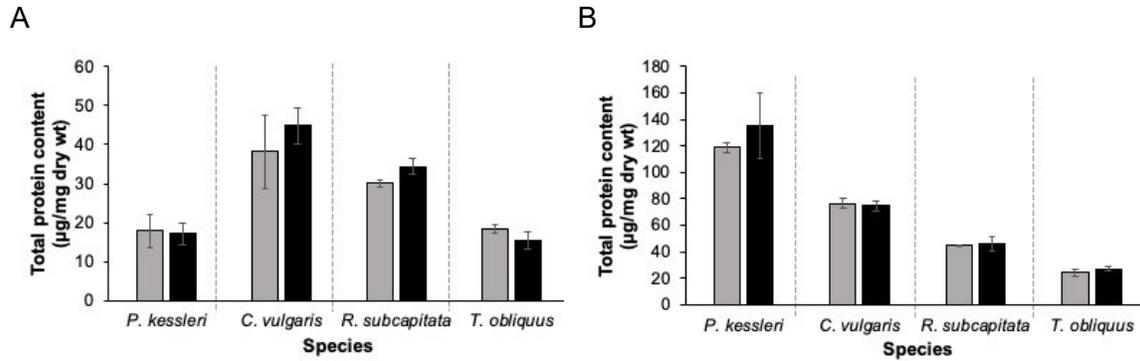
Figure 2.3. **Total protein content of Parachlorella kessleri, Chlorella vulgaris, Raphidocelis subcapitata, and Tetradesmus obliquus at the exponential (A) and stationary (B) phases of the algae growth cycle with 0 (negative control; ▨) or 40 µg/L Se (■). Values are means ± SEM, n=3. Different letters denote significance.**

The relationship between protein content and tissue Se accumulation was examined in *P. kessleri*, *C. vulgaris*, and *R. subcapitata* exposed to 40 µg/L Se in the exponential phase (Figure 2-4). No correlation in the relationship between total protein content and tissue Se was identified (R^2 value of 0.02; $p>0.05$). In the stationary phase, the same exposures for *P. kessleri*, *C. vulgaris*, and *R. subcapitata* (experiment 3) as well as an additional two treatments of *R. subcapitata* (experiment 4): one exposed in MR lighting (3600 – 4400 cool lux) and the other HR lighting (7890 – 11960 cool lux), were examined for a relationship between total protein content and tissue Se concentrations (Figure 2-5). A strong correlation in the relationship between total protein content and tissue Se was detected among different species in the stationary phase with an R^2 value of 0.95 ($p<0.0001$). A strong correlation in the relationship between total protein content and tissue Se was detected among different light intensity within *R. subcapitata* with an R^2 value of 0.83 ($p<0.05$). The regression coefficients from both experiments in the stationary phase were not significantly different from each other (chow test ($F_{(2,11)} = 2.2$, $p=0.16$)).
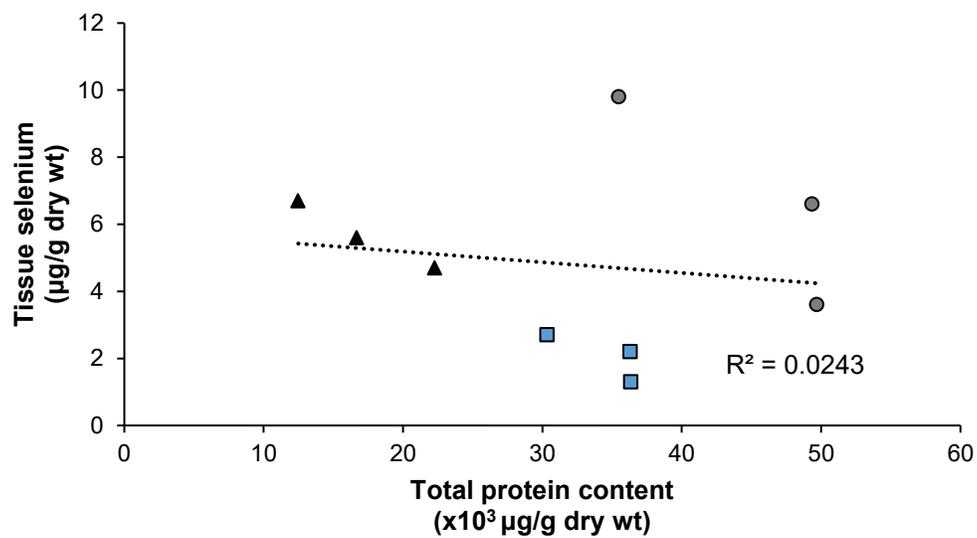
Figure 2.4.   **Algal uptake of selenium from exposure to 40 µg/L Se as a function of total protein content during the exponential growth phase. Algal species were: Parachlorella kessleri (▲), Chlorella vulgaris (◉), and Raphidocelis subcapitata (▣). Relationship analyzed by linear regression ($p<0.05$).**
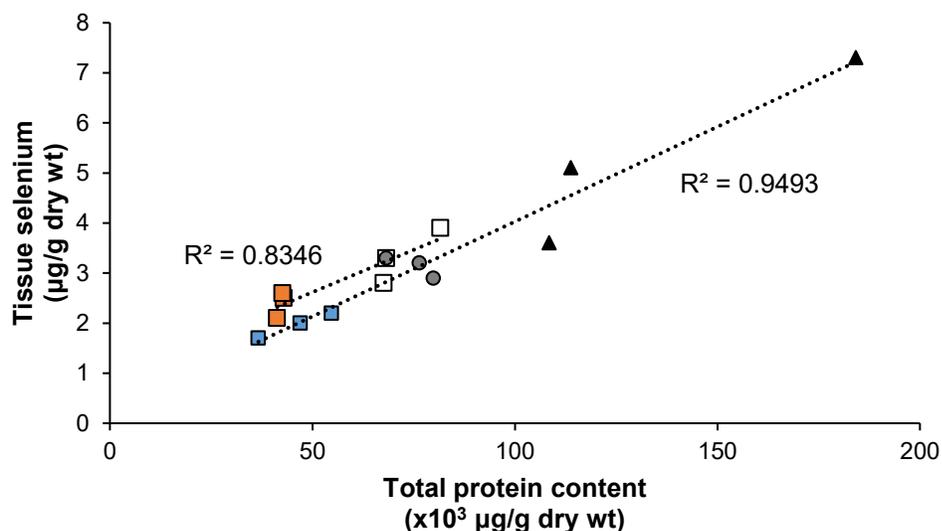
Figure 2.5. Algal uptake of selenium from exposure to 40 µg/L Se as a function of total protein content during the stationary growth phase at different light levels. Two experiments are presented. In the first, algal treatments/species were: *Parachlorella kessleri* at medium range lighting (3600-4400 lux; ▲), *Chlorella vulgaris* at medium range lighting (3600-4400 lux; ●), and *Raphidocelis subcapitata* at medium range lighting (3600-4400 lux; ■). In the second treatments were: *Raphidocelis subcapitata* at medium range lighting (3600-4400 lux; ■) and *Raphidocelis subcapitata* at high range lighting (7890-11960; □). Relationships analyzed by linear regression ($p < 0.05$).

Since both total protein content and tissue Se were normalized to dry weight, Se can be presented per g protein by dividing an individual Se concentration by its respective protein content. When selenium content is normalized to total protein content in the stationary phase (from experiment 3), between *P. kessleri*, *C. vulgaris*, and *R. subcapitata*, the following values are obtained: 0.039±0.0034, 0.042±0.0035, and 0.043±0.0018 µg Se/g protein (n=3), respectively. A one-way ANOVA demonstrated that there was no significant difference detected between these normalized values ($F_{(4,4)} = 0.916$, $p = 0.533$).

2.5. Discussion

Identifying the factors that influence Se uptake and accumulation at the primary producer level is critical in predicting bioaccumulation to higher trophic levels. In this study, *P. kessleri*, *C. vulgaris*, *R. subcapitata* and *T. obliquus* were cultured and the timing of each growth phase was captured. These results were used to establish sampling intervals for protein content and to determine if differences in total protein content existed between species at each growth phase. Following protein analysis, *P. kessleri*, *C. vulgaris*, and *R. subcapitata* were exposed to SeO_4^{2-} to determine if a relationship existed between total protein content and the tissue Se. Evaluating the effects of lighting intensity on cellular protein content and how that altered Se accumulation was also examined in *R. subcapitata*. There are several studies that have investigated the bioaccumulation of Se at the lowest trophic level (e.g., Sandholm et al., 1973; Orr et al., 2005; Lo et al., 2015; Schneider et al., 2015; Oldach et al., 2022), however, the potential role of total protein content as a driver of Se uptake has not been examined.

All of the species in this study were selected due to their prevalence in North American freshwater systems. Despite the similarities among species, the optimal culture conditions, either nutritional (chemical) or environmental (physical), may vary. For example, *C. vulgaris* achieves maximum density under high lighting intensity with dark periods (opposed to continuous illumination) as well as temperatures near 30°C (Daliry et al., 2017). *R. subcapitata* however, is recommended by Environment Canada (2007) to be cultured under continuous illumination at temperatures closer to 25°C. Since cellular composition may be affected by differences in physical conditions, maintaining similar environmental culture parameters was imperative to ensuring results were comparable across treatments and species. Metabolic responses including degradation of nitrogenous compounds including protein, can be triggered in micro-algae by nitrogen starvation (Pancha et al., 2014). Therefore, the highly enriched Bold's Basal Media (BBM) was selected for this study to ensure differences in protein content were not the result of nutrient depletion. Since each species had variable times spent in each growth phase, this led to different sampling times for different species. This contrasts with some Se uptake studies involving micro-algae that used a single exposure duration, where all subsampling occurred on a single day (Simmons and Wallschläger, 2011; Lo

et al., 2015; Kizovský et al., 2021). For example, *P. kessleri* and *C. vulgaris* were in stationary phase after 7 d of exposure while *R. subcapitata* and *T. obliquus* reached the stationary phase between 8 and 10 d.

Protein analysis at each growth phase for the tested species indicated that total protein content changed with growth phases. Similar results were reported by Lourenço et al. (2004); cellular composition (proportion of protein, lipid, and carbohydrate) changed with changing phases of growth. In the exponential growth phase, protein content was significantly different between *P. kessleri*, *C. vulgaris*, and *R. subcapitata*, whereas *T. obliquus* had a similar protein content to *P. kessleri*. In the stationary phase, protein content was significantly different between all species with *P. kessleri* > *C. vulgaris* > *R.subcapitata* > *T.obliquus*. Across all species, protein content was greatest in the stationary phase. Lourenço et al. (2004) reported the highest cellular protein in the exponential growth phase of several different marine micro-algae species. Differences in exposure conditions such as continuous lighting and the use of a highly N-enriched media in the present study could explain these different results. For example, large changes (depletion) of N or phosphorus (P) following micro-algae inoculation triggers an increase in cellular lipids and decrease in protein (Lourenço et al., 2004). If a sufficient concentration of N and P is not provided at the start of algae culturing or added soon after initiation, it seems reasonable to expect protein would begin to decrease after the exponential phase under some culturing conditions. The highly N-enriched BBM media used in our study could explain the continued increase in cellular protein into the stationary phase.

The potential effect of Se on cellular composition (specifically protein content) was explored in the exponential and stationary phases for *P. kessleri*, *C. vulgaris*, *R. subcapitata*, and *T. obliquus* . Protein content was not affected by Se exposure to 40 µg/L and no evidence of toxicity was observed either in cell density or in cell health. In a study examining Se toxicity to the micro-algae *Scenedesmus quadricauda*, increased lipid production (change in cellular composition), starch accumulation and reduced stroma density were noted as signs of toxicity (Umysová et al., 2009). The authors of this study identified that Se toxicity to micro-algae was both Se concentration and species dependent and at higher concentrations no cell growth/division was observed and cells appeared bleached (Umysová et al., 2009). However, the selenium

concentration (10 -100 mg/L) used by these authors was significantly higher than that used in this study.

The method of cell lysis used in this study was selected as sonication provides consistent results with sufficient percent recovery (Meijer and Wijffels, 1998; Barbarino and Lourenço, 2005; Lee et al., 2017). Differences in cell structure across growth phases might influence the effectiveness of chemical methods of protein extraction in some cases. For instance, López et al. (2010) reported that cell robustness contributes to the difficulty of complete cell disruption during cell lysis prior to protein analysis. Since cells are more robust in the stationary phase, this could impact the protein results in certain studies depending on the growth phase and protein extraction methods used. In addition, micro-algae cell wall composition and the intracellular concentration of nitrogen (in cases where protein is calculated using the Kjeldahl method) impact the results for protein extraction and should be considered when comparing with other studies (Lourenço et al., 2004). Additionally, methods for cell lysis are specific to the cell type (e.g., unicellular). In the case of *Tetradesmus* which is considered pleomorphic, the formation of coenobia, a colony or aggregate of unicellular organisms, has been reported to influence cell lysis effectiveness and subsequent protein recovery (Oliveria et al., 2021), The mucilaginous sheath wrapped around the coenobia is resistant to adverse environmental conditions and therefore cell lysis is difficult and requires additional methods than those used in this study and as such, *T. obliquus* was not carried forward in subsequent experiments.

Evaluating the relationship between total protein content and tissue Se content indicated that no relationship existed between *P. kessleri*, *C. vulgaris*, and *R. subcapitata* in the exponential phase. However, there was a strong relationship in the stationary phase. A strong relationship was also present within *R. subcapitata* at different protein contents (achieved by changes in lighting intensity) in the stationary phase. Changes in algal cellular composition have been achieved in nutrient deprivation studies and a similar response has been identified in light intensity alteration (Nzayisenga et al., 2020). There was greater protein content in the HL (7890 – 11960 lux) exposure compared to that in the ML (3600-4400 lux) for *R.* subcapitata. This is consistent with the findings by He et al. (2015), where the protein content for *Chlorella* increased at 10,800 lux lighting prior to declining after photoinhibition occurred at 21,000 lux.

The tissue Se content in each species in the exponential phase was higher compared to that in stationary based on dry wt. These findings are consistent with the study by Vriens et al. (2015) where the rate of SeO_4^{2-} uptake was highest immediately following exposure and subsequently decreased until equilibrium was established in the stationary phase. Although less studied in micro-algae than in higher order plants, methylation and subsequent volatilization of Se has been observed in several species of micro-algae (Oyamada et al., 1991; Neumann et al., 2003; Vriens et al., 2015). Volatilization of Se following methylation is considered a detoxification mechanism as this process excretes excess intracellular Se as was observed in the study examining Se uptake by *Chlamydomonas reinhardtii* (Vriens et al., 2015). Similar observations of *Chlorella* species producing volatile Se were observed by Neumann et al. (2003); consistent with the Se methylation and volatilization observed in *C. vulgaris*, *Tetradesmus* sp. (previously *Ankistrodesmus*) and *Raphidocelis* sp. (previously *Selenastrum*) reported by Oyamada et al. (1991). The exposure times in these studies were varied but methylation was observed in all cases in the lag and exponential phases. While the present study did not include observations of methylated Se production, for the most part, our results are consistent with observations of others studied, and show that lower tissue Se was observed in the stationary phase on a dry wt basis after equilibrium was reached, compared to exponential.

The tissue Se of *P. kessleri*, *C. vulgaris*, and *R. subcapitata* in the stationary phase expressed per g protein are not significantly different among species. Therefore, expressing tissue Se normalized to protein could have a similar environmental importance as expressing polychlorinated biphenyls (PCBs) normalized to tissue lipid content (Hebert and Keenleyside, 1995). Differences in Se uptake at the base of the food web has been identified as a driver of bioaccumulation at higher trophic levels between otherwise similar sites of contamination (Ponton et al., 2020). Thus, the results of this study indicate that protein content of micro-algae may contribute to the variations in tissue Se between different primary producers, and subsequently to higher order organisms.

Batch culturing conditions, such as those utilized in this study, are specific to a closed-system laboratory environment. Natural environments are considered open systems and so distinct growth phases as seen in batch culturing are not observed for micro-algae in natural water systems (Wanner and Egli, 1990). Different types of growth

strategies are utilized by micro-algae in natural systems depending on the availability of nutrients, lighting and temperature at a given time (Gorry et al., 2021). Feast-famine cycles are commonly observed in situations where there are sharp influxes of nutrients resulting in period of exponential growth followed by long periods of stationary growth (Gorry et al., 2021). Significant periods of exponential growth in micro-algae are associated mostly with an algal bloom (Schleyer and Vardi, 2020). Since physiologically, stationary phase is more environmentally relevant under consistent environmental conditions, the relationship between protein content and tissue Se observed only in the stationary phase could be applied to most systems.

The results of the present study are currently limited to the micro-algae tested; therefore, further studies are needed to confirm whether protein normalization applied to the wider primary producer community could be appropriate for Se bioaccumulation modeling at contaminated sites. At present, extrapolating the relationship between protein and tissue Se to higher trophic levels would be inappropriate as additional considerations such as regulatory mechanisms and complex partitioning would need to be explored. However, integrating protein analysis and normalization into the calculation of EFs into the base of the food web could be useful in current Se modeling. The relationship between total protein content and tissue Se found in this study provides groundwork for further experiments of different primary producers to determine the bioaccumulation potential of Se contamination at the base of the food web. Improving the quality of predicted EFs (rather than using theoretical conservative values) could improve the models for risk assessments of contaminated areas to oviparous vertebrates.

2.6. References

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Chapter 3.

General discussion and conclusions

3.1. General conclusions

The findings described in Chapter 2 indicate that in the exponential growth phase, there was no strong relationship between protein content and tissue Se across *P. kessleri*, C*. vulgaris*, and *R. subcapitata*, whereas in the stationary phase a strong relationship was present. In a separate experiment, a relationship was also detected within *R. subcapitata* when protein was altered by growing algae at different lighting intensities. Only one concentration of Se was tested at 40 μg/L Se (added as Na_2SeO_4). Differences were observed in protein content among the three growth phases (lag, exponential, and stationary). Within each growth phase, there were also differences between *P. kessleri*, *C. vulgaris*, and *R. subcapitata*.

This is one of the first studies specifically looking at the relationship between total protein content and tissue Se accumulation in freshwater algae. The closest study where incorporation of Se into proteins was examined was with the Coccolithophorid *Emiliania huxleyi* – a unicellular calcifying marine algae (Obata et al., 2004). In that study, the authors reported that 70% of ^{75}Se was incorporated into the low-molecular weight compounds (LMCs) and 17% into the proteins (Obata et al., 2004). Further, ^{75}Se in the Se-containing LMCs was transferred into the selenoproteins (Obata et al., 2004). The apparent partitioning of Se into the proteins by *E. huxleyi* provides a potential mechanism to explain the relationship between protein content and tissue Se observed in our study. In our study, while we did not trace the Se as it was taken up by the micro-algae, we did see a strong relationship between the Se accumulated in the stationary phase and the total cellular protein of *P. kessleri*, C*. vulgaris*, and *R. subcapitata* indicating protein dictated Se bioaccumulation to an extent. The apparent partitioning of Se into the protein fraction in the cells observed by Obata et al. (2004) is consistent with the results described in Chapter 2.

The purpose of this research was to characterize the growth of four green mico-algae species, determine if Se exposure altered the protein content within various growth in these species, and to investigate the relationship between total protein content

and tissue Se accumulation. Se is an element of ecological concern as it is found in concentrations greatly exceeding 1 µg/L in coal and phosphate mine-affected waters as well as in agricultural drainage waters (Young et al., 2010). The concern for Se contamination is that it bioaccumulates in the food chain leading to levels in fish and birds that may cause teratogenic and developmental defects. While TTFs in contaminated areas have been accurately predicted, the EFs or partitioning of Se from the water column into the base of the food chain are often highly variable and dependent on factors including flow conditions, biogeochemistry, and primary producer community composition. Since micro-algae make up a significant proportion of the primary producer community in most aquatic ecosystems, elucidating the mechanism driving EF variability in these species is critical in understanding biomagnification potential differences among phytoplankton.

3.2. Environmental relevance

The most oxidized form and most prevalent in lotic systems; selenate (SeO_4^{2-}) was the only Se species added to the exposures described in Chapter 2. It has been suggested that in addition to active/biological carrier-mediated uptake, non-biological/passive diffusion can occur for selenite (SeO_3^{2-}) as observed in heat-killed algal cells (Riedel et al., 1991). In this case, the selenite is adsorbed onto the algal cells and therefore is not biotransformed into organic Se (as it is not truly being incorporated by the algae), however it is still available to be passed through the food web as inorganic Se (personal communication, Mikayla Oldach). The objective of this research was to examine the connection between protein and Se uptake and therefore Se incorporated into the algae cells was of greatest interest. Since algal uptake of SeO_4^{2-} appears to be dominantly by biotic processes it was applicable for the objective here. In the field, Se is present in a variety of states simultaneously as discussed in Chapter 1. However, only some Se species are considered toxic in contaminated sites, including SeO_4^{2-}, and therefore the results of this study could be considered in the context of a contaminated freshwater lotic site (Orr et al., 2005).

The objective of this study was to examine the role of total protein content in Se uptake by micro-algae in a laboratory exposure between 7 and 10 d. The results of this study indicated that there was a strong positive relationship between total protein content and Se uptake in the micro-algae species examined in the stationary phase. At

aquatic sites with comparable water Se concentrations, differences in tissue Se concentrations in organisms occupying similar trophic levels have been suggested to be the result of different bioaccumulation potential in the periphyton community (Stewart et al., 2004; Schlekat et al., 2004; Wayland and Crosley, 2005; Holm et al., 2005; Schneider et al., 2015). Bioaccumulation of Se and mercury are similar in that the variability in biomagnification across systems is dependent on the partitioning from the water into the primary producers and initial consumers (Beneditto et al., 2011). In the study by Wayland and Crosley (2005), three different insect taxa: mayflies, caddisflies, and stoneflies aquatic were monitored for tissue Se between a reference site and coal mine-affected site. The results of this study found that caddisflies at coal mine-affected sites had significantly greater tissue Se than those in the reference sites while the mayflies and stoneflies had no difference between the two sites. In addition, there was a difference in Se bioaccumulation between different taxa as stoneflies had lower Se at both sites than both mayflies and caddisflies (Wayland and Crosley, 2005). Large variations in the trace elements, such as Se, found in invertebrates have been attributed to physiological regulation as well as different habitat and feeding zones (Schneider et al., 2015). However, these differences may be an indirect result driven by differences in protein content among primary producers inhabiting these areas. Thus, our results have potential environmental relevance as they provide a possible alternative explanation for site-to-site variability in Se biomagnification potential of different primary producers in the field.

In the field, the poor predictability of Se toxicity in higher trophic level organisms has been attributed to interaction with other trace elements, differences in bioaccumulation, sensitivity among species, and differences in ecosystems such as lotic or lentic systems and water temperatures (Hamilton, 2004). In the case of aquatic insects, tissue concentrations can range from <1 to 200 µg/g dry wt at coal and phosphate mine-impacted sites (McDonald and Strosher, 2002; Hamilton, 2004). As Presser and Luoma (2010) reported, the TTF specifically for fish is similar between that derived in the laboratory and that from field research (ranging from 0.5-1.6), further emphasizing that the initial uptake from water and/or sediment to the primary producer community is important for risk assessment of a given area of concern. As noted in the study by Schneider et al. (2015) and modelled previously by Presser and Luoma (2010), the ratio of Se taken up by primary producers from the water column (EF) determines

the differences in biomagnification magnitude in the field. Polychaetes (benthic invertebrates) have higher Se concentrations than those observed in *Corbula truncate*, a filter-feeding bivalve, both sampled from the same aquatic ecosystem (Schneider et al., 2015). In the same study, the diet or feeding zone was established to be more significant than trophic level in predicting Se concentrations in Lake Macquarie (Schneider et al., 2015). Our research contributes to the knowledge scope modeling Se uptake at the base of the food web (the mechanism driving EFs) and is critical in estimating ecological risk of Se contamination on a site-to-site basis. The model by Presser and Luoma (2010) predicts TTFs provided the EFs can be calculated. The relationship between protein and tissue Se in primary producers would add to the existing model. If protein correlates to Se uptake in micro-algae in the field, this could help explain the discrepancies between similar aquatic ecosystems and the further differences observed in organisms of similar trophic level.

3.3. Future directions

3.3.1. Laboratory based research

The research presented herein expands on the current knowledge regarding factors affecting Se uptake in micro-algae, however continued research is merited. The relationship established in Chapter 2 between protein content and tissue Se involved only three species of micro-algae all belonging to the Chlorophya taxa. Since the periphyton community includes a complex association of primary producers, examining the relationship between protein and tissue Se among other species is warranted. The Chlorophyte *C. reinhardtii*, the Coccolithophorid *E. huxleyi*, and the diatom *Cyclotella meneghiania* have been used in Se uptake studies and could expand the scope of knowledge associated with the present study (Riedel et al., 1991; Novoselov et al., 2002; Obata et al., 2004). Under similar culturing and growth conditions to those in this research, examining different species could be compared to the results presented in Chapter 2 and if appropriate, added to the stationary phase regression presented in our results.

Food chain transfer of Se resulting in toxic concentrations at higher trophic levels is through dietary exposure. To establish whether protein differences in the primary producers is driving differences in bioaccumulation, a food chain exposure could be

conducted. For example, *P. kessleri* and *R. subcapitata*, representing the highest and lowest protein and tissue Se species, could be cultured, and exposed to Se under identical conditions as those in the present study. These Se-containing algae could be used as a feeding source for a freshwater invertebrate such as *Daphnia magna*. Tissue Se of the invertebrate could elucidate the variable biomagnification potentials of site-specific primary producer communities of different compositions. Furthermore, assessment of the protein content and tissue Se of different primary consumers exposed to identical food sources would provide insight into whether protein and tissue Se are correlated to some extent at higher trophic levels. In the report by Orr et al. (2005), it was suggested that a different pathway (uptake directly from the water column by consumers related to Se speciation) aside from consumption of primary producers was responsible for the differences in Se uptake among consumers in lotic and lentic systems. However, the authors determined that dietary exposure was still the dominant Se exposure route for aquatic consumers (Orr et al., 2005). If the relationship between protein and Se uptake exists to some degree at higher trophic levels, this would also explain some of the discrepancies between expected and observed results in the field (Bender et al., 1991; Hamilton and Buhl, 2003; Wayland and Crosley, 2005; Schneider et al., 2015).

3.3.2. Site-specific normalization

This research demonstrated that there was a strong relationship between protein content and tissue Se. Such a relationship would suggest that protein normalization may be appropriate for determining selenium uptake in algae and by extension in predicting EFs. A similar relationship exists and allows for lipid-normalization of polychlorinated biphenyl (PCB) data and has been valuable in modeling food webs (Thomann and Connolly, 1984) as well as establishing lipophilic contaminant guidelines (Schlechtriem et al., 2012; Webster et al., 2013; Arblaster et al., 2015). Lipophilic contaminants such as PCBs, bioaccumulate in proportion to tissue lipid content. Therefore, normalization either by the ratio approach (dividing the contaminant concentration by the concentration of lipid) or the ANCOVA approach (adjusting tissue contaminant concentrations by lipid covariation) can be effective in removing the effect of lipid on the concentration of contaminant (Hebert and Keenleyside, 1995). Normalization by the ANCOVA approach was also used in assessing the effect of calcium (Ca) on spatial and temporal lead (Pb)

bioaccumulation in a study by Schmitt and McKee (2016). Although additional information and assessment is required in aquatic systems with Pb contamination, Ca-normalization of Pb data has been helpful in monitoring fish populations in contaminated sites (Schmitt and McKee, 2016). In the cases of PCB and Pb normalization, the correlation between lipid and Ca, respectively, has been established in higher level organisms (those of greatest ecological risk). As our research is limited to freshwater micro-algae, the relationship between protein and Se uptake would need to be established for the greater primary producer community before normalization could be considered in risk assessment. Since selenium is often monitored by water column concentrations, but known thresholds are available for tissue concentrations (Beatty and Russo 2014; CCME, 2007), understanding the relationship between selenium and protein could be used in future selenium bioaccumulation modelling.

3.4. References

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Appendix A.

Example of threshold determination

Table A.1.   Coulter counter summary for *R. subcapitata* (observed values; O)

Threshold (µm)	Cell Density (x10,000 / mL)			ΣO
	100%	25%	12.5%	
2.75	107.9	28.3	14.6	151
3.00	105.1	27.5	14.6	147
3.25	104.4	24.4	12.3	141
3.50	83.8	21.4	10.7	116
3.75	68.1	17.5	8.8	94
4.00	50.6	12.8	6.3	70
4.25	34.5	8.4	4.3	47
4.50	21.2	5.3	2.5	29
4.75	11.9	2.9	1.4	16
5.00	6.4	1.5	0.7	9

Table A.2.   Hemacytometer summary for *R. subcapitata* (expected values; E)

Calculations	100%	25%	12.5%	ΣE
Mean	5.9	2.3	0.6	-
SD	2.2	1.1	0.7	-
CV (%)	37	49	107	-
Cells/mL (x10,000)	**94**	**36**	**10**	**140**

Table A.3. Threshold evaluation summary for *R. subcapitata*. Determined cell size highlighted in grey.

Threshold (μm)	$(ΣE - ΣO)^2$	$(E - O)^2$				$\sqrt{((ΣE - ΣO)^2 \times Σ(E - O)^2)}$
		100%	25%	12.5%	Σ	
2.75	116	192	59	21	272	178
3.00	52	124	73	21	218	106
3.25	1	108	134	5	247	18
3.50	577	103	212	0	316	427
3.75	2089	672	344	2	1018	1458
4.00	4946	1886	539	14	2438	3473
4.25	8614	3536	764	33	4332	6109
4.50	12307	5298	940	56	6295	8802
4.75	15311	6733	1097	73	7904	11001
5.00	17268	7665	1192	87	8944	12428

Appendix B.

Media Composition

Table B.1.　　Analytical results of BBM taken prior to algae inoculation

Parameter	Result (mgL)[1]
SO_4^{2-}	62.8
NO_3^-	86.5
pH	7.22
Conductivity	1220
HCO_3^-	60.8
Alkalinity (mg/L $CaCO_3$)	49.8
Hardness (mg/L $CaCO_3$)	91.9
Selenium (Se) - total	0.0398

[1]Results provided by ALS Environmental, Calgary, AB

Appendix C.

Raw protein and tissue selenium

Table C.1. Measured concentrations from exponential phase (grey) and stationary phase at 3600-4400 lux

P. kessleri		C. vulgaris		R. subcapitata	
Tissue Se (ppm)	Total protein (µg/mg dry wt)	Tissue Se (ppm)	Total protein (µg/mg dry wt)	Tissue Se (ppm)	Total protein (µg/mg dry wt)
6.7	12.5	3.6	49.7	2.2	36.3
5.6	16.7	6.6	49.4	1.3	36.4
4.7	22.3	9.8	35.5	2.7	30.3
5.1	113.8	2.9	79.8	2.0	47.0
3.6	108.5	3.3	68.2	1.7	36.7
7.3	184.2	3.2	76.4	2.2	54.7
				2.5	42.9
				2.1	41.3
				2.6	42.6
				3.9[1]	81.6[1]
				3.3[1]	68.2[1]
				2.8[1]	67.6[1]

[1]7890-11960 lux