# Micronutrients

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## 1 Introduction

In the 40 years since the chapter 'Inorganic nutrients' was written by O'Kelley in the book '*Algal Physiology and Biochemistry*' (Stewart 1974), our understanding of the types, amounts, and roles of micronutrients in microalgae has expanded enormously, as has our ability to measure and decipher their activities, fate and behavior in cells and the surrounding environment. This chapter aims to provide a state-of-the art account of micronutrients in microalgae. Unlike the original chapter by O'Kelley, which included the macronutrient elements Sulfur, Potassium, Calcium and Magnesium, the reader is referred to other chapters in this book for an update on those elements. Given the extensive literature towards our understanding of some micronutrients, the reader is also referred to chapters "Iron" (Fe) (Marchetti and Maldonado 2016), "Selenium in Algae" (Se) (Araie and Shiraiwa 2016), and "Silicification in the Microalgae" (Si) (Finkel 2016) which are dedicated exclusively to each of these micronutrient elements.

Although each micronutrient is considered on an element-by-element basis briefly below, following O'Kelley, it is recognized that each functions in the presence of others and is affected by them, such that these interactions, as we know them, will also be examined. The reader is referred to excellent reviews and/or treatises published over the decades since O'Kelley's chapter, particularly the book by Frausto da Silva and Williams (2001) and papers by Raven (1988, 1990, Raven et al. (1999), Whitfield (2001), Morel et al. (2003), Worms et al. (2006), Glass et al. (2009), and Sunda (1994, 2012). More recent papers have begun to reveal the nature of yet-to-be-discovered metalloproteins involved in biochemistry and physiology (e.g. Cvetkovic et al. 2010) and these understudied and unknown roles and activities of micronutrients will be the subject of future research efforts.

Unlike other groups of organisms, microalgae have polyphyletic origins (e.g., Delwiche 1999; Falkowski et al. 2004). They are not only morphologically but also physiologically and biochemically heterogeneous, making generalizations about their micronutrient requirements challenging. As O'Kelley pointed out in 1974, whereas higher plants are thought to have essentially the same elemental requirements, there appear to be differences in elemental requirements between algal species. This includes the obvious fundamental requirements for Si in diatoms and some chrysophytes (see chapter "Silicification in the Microalgae", Finkel 2016) and Ca in coccolithophores (see chapter "Calcification", Taylor and Brownlee 2016), but also the lesser known and understood differences in micronutrient requirements between prokaryotes and eukaryotes as well as between eukaryotes.

In addition, micronutrient requirements are known to differ between oceanic, coastal (neritic) and freshwater microalgae. The majority of examples in this Chapter will be microalgae that have a coastal and oceanic origin; this is in no way intended to discount the importance of freshwater systems. Anthropogenic inputs of micronutrients to the environment exceed inputs from natural sources by 10- to 100-fold, particularly to lakes, rivers, and the coastal ocean. There has been a concurrent steady increase in their concentrations in the biota, altering ecological stoichiometries, food webs and trophic movement of these elements. We also raise concerns of a new emergent pollutant (engineered nanoparticles), which will likely also be the focus of future studies of micronutrient effects on microalgae.

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## 2 Evolution of Micronutrient Requirements in Microalgae

Throughout Earth's history, it can be (and has been) argued that the environment and microalgae have influenced each other's composition (e.g., Redfield 1934; Falkowski and Raven 1997; Falkowski 1997; Frausto da Silva and Williams 2001; Anbar and Knoll 2002; Morel and Price 2003; Quigg et al. 2003a, 2011; Falkowski et al. 2004; Martin et al. 2008; Sunda 2012; Martin and Quigg 2013). In order to do so, microalgae appear to have evolved simultaneously mechanisms that maximize use of available micronutrient fluxes (e.g., by releasing strong complexing agents) and strategies to promote more efficient recycling of some elements compared to others (e.g., by catalyzing redox reactions that modify the bioavailability of micronutrients). Microalgal uptake of some essential elements results in their extraordinarily low concentrations in surface seawater (except for Mo). This in turn, controls the rate of photosynthetic fixation of carbon (primary production) and the transformation and uptake of major nutrients, particularly nitrogen (see e.g. Falkowski 1997; Anbar and Knoll 2002; Martin et al. 2008). In this way, the extremely low concentrations of essential micronutrients results in ultra-efficient uptake systems in microalgae. It may also explain the widespread replacement of micronutrients in metallo-centers of enzymes by one another in important biochemical reactions (e.g., nitrogenase, superoxide dismutase, carbonic anhydrase). Recent studies have shown that the ability to acquire (uptake) and eliminate (efflux) micronutrients is a physiological trait that varies between taxa and can be linked to evolutionary histories and changes in ocean chemistry. Such species-specific traits play an important role in determining the micronutrient quota's (intracellular concentrations), their response to different environmental perturbations including upwelling and pollution, and consequently successional patterns, community composition and/or competition.

The growth of marine microalgae in the environment was thought for a very long time to be primarily limited by the availability of the major or macro-nutrients (nitrogen and phosphorus, and to a lesser extent silicate in diatoms) (see chapters "Combined Nitrogen", "Nutrients and Their Acquisition: Phosphorus Physiology in Microalgae", and "Sulphur and Algae: Metabolism, Ecology and Evolution"; Raven and Giordano 2016; Dyhrman 2016; Finkel 2016). Although some early laboratory studies suggested that Fe may limit microalgal growth in the oceans (see chapter "Iron", Marchetti and Maldonado 2016), it was not until Martin and Fitzwater (1988) and Martin et al. (1991) provided the first evidence of a micronutrient (Fe) playing a major role in microalgal growth and primary productivity that scientists more seriously considered the role of micronutrients. Despite being the fourth most abundant element in the earth's crust, Fe is relatively scarce (in terms of bioavailability) in today's oxygenated oceans (0.02–1 nmol $L^{-1}$) (Bruland et al. 1991). We now know that Fe limits microalgae in as much as 30–40 % of the world oceans, particularly in high nitrate-low chlorophyll regions (see chapter "Iron"; Marchetti and Maldonado 2016). Studies have also found that Fe limits cyanobacterial $N_2$ fixation and thereby controls oceanic inventories of biologically available fixed nitrogen over long (geological) time scales (see e.g., Falkowski 1997; Anbar and Knoll 2002; Sunda 2012). The interaction between N and Fe and microalgae is now so well recognized and studied that these are discussed in greater detail in other parts of this book (see Raven and Giordano 2016; Marchetti and Maldonado 2016).

By contrast, during the Archean, some 3.8–2.5 billions of years ago, as a result of widespread ocean anoxia Fe is thought to have been relatively abundant (50 μmol $L^{-1}$) in the surface and deep oceans (Anbar and Knoll 2002). This may explain why it is used in so many important proteins associated with the photosynthetic pathways (Raven 1988, 1990; Raven et al. 1999; Larkum 2016). The opposite is thought to be true for Mo with prebiotic concentrations ten-times less than present values of ~100 nmol $L^{-1}$ in surface oceans (Collier 1985; Anbar and Knoll 2002; Scott et al. 2008). Anbar and Knoll (2002) hypothesized that Mo–N co-limitation likely influenced the early evolution of cyanobacteria. Heterocystous cyanobacteria, which diversified over 2 billion years ago (Tomitani et al. 2006), evolved during a period when bioavailable Mo was low in the oceans (Anbar and Knoll 2002; Scott et al. 2008). This would have created strong selection pressures for Mo acquisition and storage, especially for the $N_2$ fixation enzyme, nitrogenase, which has both high Fe and high Mo requirements (in a $Fe_7MoS_9$ cluster). As Mo levels rose in the sea, ca. 500 million years ago (Scott et al. 2008), Mo no longer limited nitrogen assimilation pathways (nitrate reduction) thereby allowing eukaryotes to diversify (Anbar and Knoll 2002). Frausto da Silva and Williams (2001) reported that there was a proliferation of Zn enzymes in microalgae that evolved only after the establishment of an oxidizing environment; however, biological utilization of Co shows the opposite trend. The significance of this will be discussed below in the section describing enzymes which use a variety of metallo-centers and in particular can use Zn or Co for the same catalysis reaction. Consistent with this is the observation of generally lower Zn quotas in prokaryotes because they produce fewer Zn finger proteins and are less likely to use Zn as a metallo-center in the enzyme superoxide dismutase relative to eukaryotes (Saito et al. 2002; Dupont et al. 2010; Quigg et al. 2011). Comparable shifts in micronutrient utilization are thought to have occurred for other micronutrients with redox-sensitive environmental chemis-

tries but supporting evidence for these less abundant elements is not easy to gain from the geologic record.

Laboratory studies have suggested that there is an evolutionary inheritance hypothesis which can be used to predict similarities in elemental composition within related taxonomic lineages of microalgae; driven at least in part by changing ocean chemistry at the time of their evolution and radiation (Quigg et al. 2003a, 2011; Falkowski et al. 2004). Twenty-nine species of microalgae from ten taxa were grown under identical conditions (light, temperature, nutrient-replete media) to examine macro- and micronutrient composition. Initially it was found that eukaryotic algae of the green (chlorophyll $a+b$ plastids) and red (chlorophyll $a+c$ plastids) lineages (see definitions of lineage in Delwiche 1999; Falkowski et al. 2004) had stoichiometrically distinct elemental signatures. Diatoms, dinoflagellates and coccolithophores of the red lineage generally had lower Fe:P, Zn:P and Mn:P but higher Co:P, Mo:P and Cd:P relative to chlorophytes and prasinophytes in the green lineage (Quigg et al. 2003a). Dinoflagellates have plastids (chloroplasts) derived from tertiary endosymbiotic events such that both green and red types are present in extant species. Quigg et al. (2003a) found that the dinoflagellate with the green plastid (*Gymnodinium chlorophorum* (= *Lepidodinium chlorophorum*))[1] clustered with the green lineage in terms of micronutrient utilization while those with red plastids (*Prorocentrum minimum* (= *Prorocentrum cordatum*), *Amphidinium carterae* and *Thoracosphaera heimii*) clustered with the red lineage, suggesting the micronutrient requirements of the plastids plays a significant role in determining micronutrient requirements of microalgae. In a later study, cyanobacteria, Glaucocystophyta, and additional members from both of the green and red lineages were included in a further analysis (Falkowski et al. 2004; Quigg et al. 2011). The largest differences in the elemental profiles distinguished prokaryotic cyanobacteria and primary endosymbiotic events that resulted in the green and red plastid lineages (Quigg et al. 2011). Smaller differences in micronutrient stoichiometry within the red and green plastid lineages were consistent with changes in micronutrient stoichiometry owing to the processes associated with secondary endosymbioses and inheritance by descent with modification. The authors hypothesized that differences in elemental composition among species primarily represents phylogenetic differences in biochemical requirements and the ability of the organisms to take up and store these elements, not environmental or culture conditions. Hence, phenotypic variation in micronutrient stoichiometry is much smaller than genetic differences between phylogenetic groups. Studies by Ho et al. (2003) and Finkel et al. (2006, 2007) have supported these contentions, finding that phenotypic differences in elemental composition due to environmental conditions rarely result in more than two to fivefold variability in element to phosphorus ratios in response to an order-of-magnitude variation in irradiance or macronutrient concentration, or an order-of magnitude variability for over three orders-of-magnitude variation in trace metal concentrations in the external media.

[1]Wherever possible the currently accepted names for species are used. The name used in the paper cited is also indicated. For details of names see chapter "Systematics, Taxonomy and Species Names: Do They Matter?" of this book (Borowitzka 2016).

## 3 Essential, Required, Replaceable?

The following inorganic elements were reported by O'Kelley (1974) as being found in microalgae: N, P, K, Mg, Ca, S, Fe, Cu, Mn, Zn, Mo, Na, Co, V, Si, Se, Cr, Cd, Cl, B, I. Of these, N, P, Mg, Fe, Cu, Mn, Zn and Mo were thought to be required by all microalgae. Early studies on micronutrients focused on the roles of essential versus required elements. The presence of a particular element in an organism is not to be mistaken with that element being essential, as some elements, particularly micronutrients, may be absorbed at levels in excess of requirements or may be absorbed but not required by microalgae (see Quigg 2008). The essential transition metal ions (referred to as micronutrients in this chapter) used in enzymes include vanadium to zinc (first-row transition metal series) and molybdenum (second-row series). Because these elements exist in nature in multiple oxidation states, they are ubiquitously utilized as micronutrients.

The accepted criteria for confirming a micronutrient as essential were: (1) growth ceases in its absence or is optimal (or close to) in its presence (Arnon 1953) and (2) the demonstration by in vitro experiments that an element has a non-replaceable role in a fundamental life process (O'Kelley 1974). According to Liebig's law of the minimum, when all other factors are favorable (e.g., light, temperature), the nutrient available in the smallest quantity will limit the growth of an organism. Given that such studies require axenic cultures and the ability to exclude the micronutrient of interest; they are very difficult to perform (e.g., presence of contaminants in 'pure' chemicals, non-selective binding or complexation which in turn alters bioavailability). Thirdly it was thought that a comparison of the composition of microalgae and that of seawater may provide insights into which micronutrients may be most limiting for growth (O'Kelley 1974). This approach however was challenged once we had a greater understanding of the differences in micronutrient concentrations in coastal versus open ocean environments and the corresponding microalgae present in these distinct ecosystems. Further, a deeper understanding of the complex nature of micronutrient chemistry has challenged this latter approach for studying micronutrient requirements by microalgae (more below), specifically the

ability of some micronutrients to replace one another in times of limitation or as a result of synergistic/antagonistic behaviors when one element is present in excess of another.

## 4 Micronutrient Elements Considered Essential to All Microalgae

Arguably, the most important micronutrients in microalgae are those used in redox reactions (e.g. Fe, Mn, Cu), acid-base catalysis (e.g. Zn, Ni), transmission and storage of information and energy (K, Ca), and in structural cross-links (e.g. S, Si) (Table 1). While this selection may have been biased from life's beginnings by abundance, later in evolution energy costs and functional advantages would have become more important. The bioavailability of these micronutrients influences the flow of energy and nutrients in ecosystems impacting both biogeochemical processes and community structure. Various biotic and abiotic factors affect accumulation of micronutrients in microalgae, including their size, water quality and environmental contamination. Further, the organic ligands (chelators) produced by microalgae play an important role in mediating the positive (and negative) impacts of micronutrients. Intracellularly, micronutrient quotas in microalgae are driven largely by the biochemical demands of the cells as they respond to various external forces.

In oceans, macro- and micronutrients essentially have the same profiles: surface waters have depleted concentrations (close to or below detection limits) which then increase with depth (e.g., Bruland 1980; Bruland et al. 1991) with one exception, Mo. Similarities between vertical profiles of micronutrients with those of macronutrients have been taken to suggest that similar biological uptake and regeneration processes are occurring. Examples of these will be given below and are discussed in more detail in reviews such as Whitfield (2001), Morel et al. (2003), Sunda (2012) and Twining and Baines (2013). Similar profiles are not found in coastal and freshwater environments; this is a result of the complex chemistries and physical interactions at play in these systems. Mo concentration is roughly proportional to salinity, hence the same concentration (~105 nmol $L^{-1}$) has been measured at all depths and ocean basins (Collier 1985; Bruland et al. 1991). The lack of a nutrient-like profile

**Table 1** Roles of micronutrients in microalgal cells

| Element | Functions | Examples of compounds |
|---|---|---|
| Fe | Photosynthetic pathways, active groups in porphyrin molecules and enzymes; component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions, reactions with oxygen and nitrogen (e.g. $N_2$ fixation, N uptake, photosystems), cytochrome oxidase, cytochrome P-450, chlororespiration and cyclic electron flow around PS I, Mehler reaction (production of superoxide), | PS II complex (cytochrome *b*559 and one additional Fe which is not in haem or part of a FeS complex), the cytochrome *b*6-*f* complex (cytochrome *b*563, cytochrome *f*, Rieske non-haem iron-sulfur complex containing 2 Fe), cytochrome *c*6 (an alternate to Cu containing plastocyanin), PS I complex (3 non-haem iron-sulfur complexes FX, FB and FA, each containing 4 Fe) and ferredoxin (a protein with one non-haem iron-sulfur complex containing 2 Fe, an alternate to flavodoxin which contains no metals), nitrate reductase, nitrite reductase, catalase, cytochromes (e.g. *b* and *c* for electron transport in photosynthesis and respiration; *f* for photosynthetic electron transport), superoxide dismutase (alternatively with Mn or Zn), NAD(P)H dehydrogenase, |
| Mn | Electron transport in PSII, maintenance of chloroplast membrane structure, breakdown reactions and those involving halogens, Mehler reaction (production of superoxide) | $O_2$ evolving complex, catalases, peroxidases (alternatively with Fe, Se, or V), manganin |
| Zn | Enzymes (ca. 150 known), ribosome structure, nucleic acid replication and polymerization, hydration and dehydration of $CO_2$, hydrolysis of phosphate esters, Mehler reaction (production of superoxide) | DNA and RNA polymerases, carbonic anhydrase |
| Cu | Electron transport (photosynthesis and respiration) enzymes, disproportionation of $O_2$ radicals to $O_2$ and $H_2O_2$ in reaction | Plastocyanin, cytochrome oxidases, superoxide dismutase (alternatively with Fe or Mn), at trace element levels, has a role in the thylakoid lumen in facilitating $H_2O$ dehydrogenation and $O_2$ evolution |
| Mo | Nitrogen reduction (nitrate and nitrite reduction to ammonium), ion absorption | Nitrate and nitrite reductase, nitrogenase enzyme |
| Co | Component of Vitamin $B_{12}$, C4 photosynthesis pathway, C and H transfer reactions with glycols and ribose | Vitamin $B_{12}$, carbonic anhydrase |
| V | May be a component of nitrogenase | Nitrogenase enzyme |
| Ni | Hydrolysis of urea; co-factor in enzymes | Urease |
| Cd | May substitute into enzymes that typically use Zn | Carbonic anhydrase in diatoms |

suggests minimal removal of Mo by microalgae relative to its seawater concentration (Sunda 2012).

As mentioned earlier, the reader is referred to other Chapters in the book for details on Fe (Marchetti and Maldonado 2016). Below the focus is on a small sub-set of important micronutrients following O'Kelley's original list: Mn, Zn, Cu, Mo, Co, V and Ni. The level of detail reflects, perhaps coincidentally, our current interest in understanding of the roles and activities of these micronutrients.

## 4.1 Manganese

Hopkins (1930) was the first report a Mn requirement for microalgae. Like higher plants, microalgae become chlorotic when Mn is deficient as it alters chlorophyll formation, galactosyldiglyceride content and/or glycolate synthesis (see O'Kelley 1974). More recent studies have found Mn (II) forms strong nitrogen (N)- and S-ligands and is a cofactor in several Krebs-cycle enzymes. Mn (III) is used in superoxide dismutases, acid phosphatases, and ribonucleotide reductases and in transfers across membranes (Table 1; Raven et al. 1999; Frausto da Silva and Williams 2001). While earliest micronutrient studies revealed Mn was associated with PSII but not PSI (Cheniae and Martin 1969; Teicheler-Zallen 1969), today the most studied role of Mn in algal metabolism is its function in the oxygen-evolving complex of photosynthesis. Mn is second only to iron in its quantitative role in the thylakoids reactions of all microalgae; the reader is referred to the excellent reviews of Raven (1990), Raven et al. (1999) and Morel et al. (2003) for very specific details.

Evolutionarily, Mn was thought to be selected for the oxygen-evolving complex (and some other functions) because of its greater overall availability (reduced and oxidized) relative to Fe, rather than because of its unique chemistry (Raven et al. 1999; Frausto da Silva and Williams 2001). While it is recognized that four atoms of Mn (in oxidation states ranging from +3 to +5) and one $Ca^{2+}$ are required to split water in the protein heterodimer, there is still a great deal of attention directed at understanding the exact process by which this occurs at a molecular level (e.g. see Ferreira et al. 2004). The Mn complex provides the redox system for the accumulation of the four oxidizing equivalents required for water oxidation, providing a gating mechanism between the single electron turnover of the reaction centers and the oxidation of water. Three of the five oxidation states of the water oxidizing complex are known to involve different Mn redox states. There are some inefficiencies thought to be associated with the process such as backward transitions (double misses) which lead to the formation of super reduced states (Messinger et al. 1997; Quigg et al. 2003b); current evidence however suggests that these may have a more photoprotective role allowing the cells to dissipate excess photon energy.

## 4.2 Zinc

Zn is an essential element (Table 1) with a long list of important metabolic functions (over 150 enzymes are known) as a result of its ability to function as a Lewis acid and its bioavailability in aquatic habitats (Frausto da Silva and Williams 2001; Morel et al. 2003). But it can also inhibit the growth of microalgae if present at elevated concentrations or as a result of antagonistic behaviors in the presence of some other micronutrients (Anderson et al. 1978; Sunda and Huntsman 1992, 1995a, 2000; Sunda 1994, 2012). The earliest demonstration of a Zn requirement in microalgae was in 1926 in the green alga *Stichococcus bacillaris* (Eilers 1926). Early studies focused on its role in the nucleic apparatus (such as DNA and RNA polymerases), finding the RNA content was low in Zn deficient algal cells (e.g., Wacker 1962; Altman et al. 1968; Prask and Plocke 1971; Frausto da Silva and Williams 2001). Because of Zn's essential role in DNA metabolism, zinc-limited *Euglena* cells were found to be incapable of completing their mitotic cycle and were unable to undergo cell division (Falchuk et al. 1975). Since then studies have shown its requirement in important proteins and enzymes such as Zn finger transcription factors, reverse transcriptase, alkaline phosphatases, superoxide dismutase, lactate dehydrogenase and in phycobilin production (Table 1; Frausto da Silva and Williams 2001). Zn may also be involved in silica uptake and frustule deposition in diatoms, as evidenced by decreased silica uptake rates and Si:P ratios at low dissolved inorganic zinc concentrations (see Sunda and Huntsman 2005 for references).

Because of its importance in cellular process, Zn internalization can be up regulated to the point where Zn diffusion becomes limiting and thermodynamically strong Zn complexes become bioavailable (Hassler and Wilkinson 2003; Hassler et al. 2005). In natural waters, Zn has a nutrient type profile and is present as the free ion or in relatively weak complexes (Bruland 1980; references in Sunda 1994, 2012). Further, Zn uptake is tightly regulated such that Zn internalization and intracellular Zn contents can be maintained relatively constant despite external variations of several orders of magnitude: $10^{-8.5}$ to $10^{-11}$ M (Sunda and Huntsman 1992; Hassler and Wilkinson 2003). There is evidence both that Zn limits productivity in some regions of the ocean (e.g., sub-Arctic Pacific) and coastal waters (e.g., California and Costa Rica) (see references in Sunda and Huntsman 2005) and that this limitation affects the composition and structure of marine microalgal communities because of differences in Zn requirements among microalgal species (Brand et al. 1983; Sunda and Huntsman 1995a, 2000; Quigg et al. 2003a, 2011). Support for this also comes from Sunda and Huntsman (1992) who found that the ability of the oceanic species (*Thalassiosira oceanica* and *Emiliania huxleyi*) to outgrow coastal ones (*Thalassiosira pseudonana* and *Conticribra* (*Thalassiosira*) *weissflogii*) at low Zn ion concentrations was

due almost entirely to a reduced growth requirement for cellular Zn rather than to an increased capability for uptake.

In terms of photosynthesis in microalgae, Zn plays a critical role in the enzyme carbonic anhydrase, involved in $CO_2$ supply to RUBISCO for C fixation (see reviews such as Raven et al. 1999; Morel et al. 2003; Beardall et al. 2009; Sunda 2012), one of the most catalytically active enzymes known across plant and animal kingdoms. In marine microalgae, carbonic anhydrase catalyzes the following reversible reaction:

$$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$$

In the last decade, the activity, function and micronutrient requirements of this enzyme have been the focus of many studies including those addressing questions of the carbon concentrating mechanism, C4 versus C3 photosynthesis pathways, C acquisition (see chapter "Carbon Acquisition by Microalgae", Beardall and Raven 2016), and partially in order to understand how microalgae will respond to climate change factors such as ocean acidification. In terms of micronutrients, predications of a ~40 % increase in $CO_2$ in the atmosphere and surface ocean waters suggests that co-limitation by Zn and $CO_2$ are unlikely as lower quantities of this enzyme are needed at high $CO_2$ concentrations (more below). In eukaryotes, there is evidence from both laboratory and field measurements that Zn limitation may at least be partially alleviated by the use of Co as a metallo-center in the enzyme carbonic anhydrase (Morel et al. 1994; Sunda and Huntsman 1995a, 2000), and in diatoms Cd has been found to be replaceable for Zn or Co in this important enzyme (Lee and Morel 1995; Yee and Morel 1996; Lane et al. 2005; Xu and Morel 2013).

## 4.3 Copper

The importance of Cu as a micronutrient was first established by Guseva (1940). Its activity may be related to the three accessible oxidation states (I, II, III) of Cu in biological systems resulting in this micronutrient having a redox potential range from +0.2 to 0.8 V (Falkowski and Raven 1997). Cu is known to play a role in the photosynthetic electron transport chain of microalgae that use the metallo-protein plastocyanin (Katoh et al. 1961; Hill et al. 1996), in PSI activity (Bishop 1964) and the respiratory electron transport chain, as well as in the Cu-Zn form of superoxide dismutase, and in the transmembrane uptake of Fe (La Fontaine et al. 2002; Maldonado et al. 2006) (Table 1).

Cu-plastocyanin it is not used ubiquitously in all microalgae (Raven et al. 1999). In some species, in times of differential Fe/Cu availabilities, cells can switch from using the Fe-containing enzyme cytochrome $c_6$ to plastocyanin in the photosynthetic electron transport pathways without compromising photosynthetic activity (Hill et al. 1996; Raven et al. 1999; Peers and Price 2006). In the respiratory electron transport pathway, cytochrome oxidase reduces oxygen to water (Frausto da Silva and Williams 2001).

A variety of adverse effects in the presence of elevated Cu have been reported, including reductions in growth, photosynthesis and respiration. The severity of the effect is dependent on pH, ambient light, the presence of other ions (e.g., Fe) and whether cells are grown under light or dark, aerobic or anaerobic conditions (see O'Kelley 1974; Brand et al. 1986; Frausto da Silva and Williams 2001). Not surprisingly, the internal concentration (homeostasis) of copper is tightly regulated by a range of cellular processes. These can quickly internalize Cu to vesicles or externalize it; high Cu efflux rates have been measured within seconds of elevated Cu exposures in microalgae (Croot et al. 2000, 2003; Quigg et al. 2006). Unlike the response to other micronutrients, the response to Cu seems to vary with microalgal species such that there is an evolutionary imprint of ocean chemistries and physiologies (more below).

Cu is present in total concentrations of 0.5–4.5 nmol $L^{-1}$ in open ocean surface waters (Bruland 1980) whereas coastal Cu concentrations can reach 50 nmol $L^{-1}$ (references in Glass et al. 2009; Sunda 2012). The free metal of Cu is more toxic than the complexed form of the metal because the former is considered more available (Brand et al. 1986; Moffett and Brand 1996; Croot et al. 2000, 2003). Cu is heavily complexed in the euphotic zone by a group of organic ligands (weak and strong) (Moffett et al. 1990; Moffett and Dupont 2007); the ligand concentration generally covaries with that of Cu, resulting in a relatively constant free cupric ion concentration of $10^{-13}$ M in coastal and near-surface oceanic waters. It is not known whether ligands bind Cu ions intracellularly and then are released as a detoxification method or if the ligands are first released and then bind Cu ions extracellularly. In both scenarios, complexation to Cu decreases the amount of biologically available Cu around the cell (Moffett and Brand 1996). It is however known that chemical speciation in the environment is dominated by complexed Cu, which may elevate or reduce Cu uptake in regions with low and high Cu concentrations respectively (Moffett and Dupont 2007).

## 4.4 Molybdenum

Bortels (1940) was the first to demonstrate the importance of Mo in $N_2$ fixation by heterocystous cyanobacteria. Wolfe (1954) then showed that the N content of $N_2$ fixing *Anabaena cylindrica* was positively correlated with the Mo concentration of the growth media. Shortly thereafter, studies found Mo essential for the biological assimilation of N either as dinitrogen ($N_2$) gas by nitrogenase (Hallenbeck et al. 1979)

or nitrate (e.g., Arnon et al. 1955; Arnon and Ichioka 1955; Rao 1963) because of its role in the nicotinamide adenine dinucleotide nitrate-reducing complex (Vega et al. 1971). Since then, the role of Mo in the nitrogen fixation and reduction processes has been the subject of many investigations (see Raven 1988; Glass et al. 2010; Hoffman et al. 2014) including those focused on evolutionary aspects of micronutrients and microalgal evolution (Anbar and Knoll 2002; Martin et al. 2008; Martin and Quigg 2013).

Nitrogenase is the oxygen-sensitive protein in cyanobacteria, which reduces $N_2$ to ammonia. It is composed of two subunits: a Mo-Fe containing multi-subunit dinitrogenase reductase protein (NifDK) (contained in a cofactor called MoFe-cofactor or MoFe-co, believed to be the substrate binding site) and a Fe-containing dinitrogenase protein (NifH) (see reviews such by Raven 1988; Raven et al. 1999; Hoffman et al. 2014). The process is both energetically and nutrient expensive. The Mo content of purified nitrogenase from *Anabaena cylindrica* is 2 atoms per enzyme complex (and per 20 Fe atoms) with a specific activity of 1.2 mmol $C_2H_2$ mg $NifDK^{-1}$ $min^{-1}$ (Hallenbeck et al. 1979). The specific activity of MoFe-nitrogenase for $N_2$ reduction is 1.5 times that of VFe-nitrogenase at 30 °C and it is at least this much more efficient than Fe-nitrogenase. This difference in specificity of the known nitrogenase metalloenzyme systems has been used to explain the prevalence of MoFe-nitrogenase relative to other forms (see references in Anbar and Knoll 2002; Hoffman et al. 2014).

Nitrate reductase, used ubiquitously by all organisms in N metabolism for reducing nitrate to nitrite, also has a Mo metallo-center (Mikami and Ida 1984). Cyanobacterial nitrate reductase (NArB) contains only one Mo atom and has a specific activity of ~300 μmol $NO_2^-$ $mg^{-1}$ NarB protein $min^{-1}$ in *Plectonema boryanum* (= *Leptolyngbya boryana*) (Mikami and Ida 1984). In terms of N assimilation, NarB is 37 times more efficient per mol N than NifD (nitrogenase in *Nostoc* sp.). This indicates that significantly less cellular Mo is required to support $NO_3^-$ assimilation than $N_2$ fixation, consistent with calculations by Raven (1988).

Early studies showed that pigment content and nitrogenase activity was found to decline after 7–10 days of growth in Mo-deficient media for *Anabaena cylindrica* (Fay and Vasconcelos 1974; Jacob and Lind 1977) but these physiological changes could be reversed by Mo resupply to the growth media of *Anabaena oscillarioides* (Ter Steeg et al. 1986). Intracellular Mo and Fe concentrations concurrently increase with nitrogenase activity (Tuit et al. 2004), reflecting the greater need for these micronutrients when $N_2$ fixation is taking place in cyanobacteria. In general, $N_2$-fixation requires 10 nmol Mo $L^{-1}$ in the medium for growth of heterocystous cyanobacteria such as *Anabaena cylindrica* (Jacob and Lind 1977), *Trichormus* (*Anabaena*) *variabilis* (Zerkle et al. 2006), and both freshwater and coastal strains of *Nostoc* sp. (Glass et al. 2010). Similar findings were found by Quigg et al. (2011) for the growth of a variety of both marine and freshwater cyanobacteria.

Concentrations of Mo vary widely from <20 nmol $L^{-1}$ in freshwater, to 30–160 nmol $L^{-1}$ in coastal areas, to ~105 nmol $L^{-1}$ in the open ocean were its profile is dictated by salinity concentrations (see Collier 1985; Bruland et al. 1991; Glass et al. 2010; Sunda 2012). Howarth et al. (1988) hypothesized that the difference in Mo bioavailability in aquatic environments influences the expression and the activity of Mo-containing enzymes involved in N assimilation. These authors found low Mo concentrations associated with freshwaters could limit the function of Mo-based nitrogenase and nitrate reductase, slowing cyanobacterial growth due N-limitation. Sulfate has been found to inhibit Mo assimilation by microalgae (Raven 1988; Frausto da Silva and Williams 2001), making Mo less available in seawater than in freshwater despite its higher concentration because of analogue differences in sulfate distributions in these water masses. As a result, N assimilation may require greater energy expenditure in seawater than freshwater.

In a comparison of Mo requirements for $N_2$ fixation between freshwater and coastal strains of the heterocystous cyanobacterium *Nostoc* sp., Glass et al. (2010) set out to test the hypothesis that the aquatic environment plays a role in Mo requirements. In the coastal strain, 3 days were required for chlorophyll *a* concentrations to fall below those of corresponding Mo replete cultures but it was not until 12 days had elapsed that $N_2$ fixation rates began to decline. By comparison, 7 and 11 days, respectively, were required for $N_2$ fixation rates and chlorophyll levels to decline in Mo limited freshwater cultures. Contrary to their hypothesis, at <1 nmol Mo $L^{-1}$, N-limitation was induced (increased C:N ratios, decreased nitrogenase expression and activity). Further, when Mo in the medium was >1 μmol $L^{-1}$, the freshwater strain stored Mo (>100 μmol $mol^{-1}$ Mo:C) but this did not occur the coastal strain. The high Mo content and extended time required for $N_2$ fixation to decrease in the freshwater strain was thought to be due to expression of the gene *mop*, which encodes a putative molybdate-storage protein, coded just upstream of the *nif* operon in freshwater heterocystous cyanobacteria (Markowitz et al. 2008). Studies have also investigated the role of Fe requirements for $N_2$ fixation (Berman-Frank et al. 2007). These fundamentally different biochemical and physiological strategies exhibited by strains of the same species found in different aquatic environments reflects both acclimation (phenotypic) and adaptation (genotypic) in response to micronutrient acquisition and utilization. Similar studies are required to understand such strategies for other micronutrients and in other microalgae.

## 5 Microelements Required by Some Microalgae

### 5.1 Cobalt

Co is known to be required in vitamin $B_{12}$ (metallo-centre) in flagellates such as *Euglena* (Hutner et al. 1949) or in the inorganic form by some cyanobacteria (Holm-Hansen et al. 1954) and in a variety of other microalgae (see references in O'Kelley 1974). These early studies revealed that in DNA synthesis, vitamin $B_{12}$ plays an important role in the reduction of ribotides to deoxyribotides, and in the synthesis of thymine, through the conversion of glutamate to beta-methyl aspartate (see summary of Provasoli and Carlucci 1974). Cyanobacteria such as *Synechococcus bacillaris* (= *Cyanobium bacillare*) have an absolute Co requirement (Sunda and Huntsman 1995a; Saito et al. 2002). Eukaryotic cells however do not, this maybe because they cannot make their own vitamin $B_{12}$ but are dependent on bacteria for this and other vitamins (Frausto da Silva and Williams 2001).

The coccolithophore *Emiliania huxleyi* has a Co requirement that can be partly met by Zn while the diatoms *Thalassiosira pseudonana* and *Thalassiosira oceanica* have Zn requirements that can be largely met by Co and/or Cd (Sunda and Huntsman 1995a). Eukaryotes, particularly diatoms, are able to switch between these micronutrients in the enzyme carbonic anhydrase. Associated with this replacement, there was a 700-fold increase in cellular Co uptake rates with decreasing Zn ion concentration, indicating that Zn will have a major influence on biological scavenging of Co.

While it has long been known that Zn is typically the metallo-center for alkaline phosphatase, recent studies suggest Co may substitute under a range of conditions (Wojciechowski et al. 2002; Gong et al. 2005; Jakuba et al. 2008). Further, the Zn, Co, and Cd contents of cells appear to increase under low inorganic phosphorus availability (Ji and Sherrell 2008; Twining et al. 2010), implying a potential for Cd substitution in alkaline phosphatase, but there is as yet no evidence for this taking place.

### 5.2 Vanadium

Early studies revealed a requirement for vanadium (V) in a green alga (*Scenedesmus obliquus*; Arnon and Wessel 1953) and the $N_2$ fixing cyanobacterium, *Anabaena cylindrica* (Allen and Arnon 1955). *Anabaena cylindrica* was also found to require $Ca^{2+}$ in both the presence and absence of a nitrogen source, strontium could not replace $Ca^{2+}$ and Mo could not replace V (Allen and Arnon 1955). Some freshwater diazotrophs have one or two nitrogenase isoforms with V or Fe taking the place of Mo (e.g., Raven 1988; Thiel 1993; Raven et al. 1999; Boison et al. 2006). V is thought to stimulate $CO_2$ uptake in photosynthesis by serving as a catalyst for $CO_2$ reduction (Warburg et al. 1955). The requirement for V was ×1,000 higher than for Mo in the green alga (*Acutodesmus* (*Scenedesmus*) *obliquus*; Arnon and Wessel 1953).

Vanadium enzymes which catalyze peroxidative halogenation reactions (haloperoxidases) are found in virtually all classes of marine algae (Butler 1998); these are thought to be responsible for the vast array of halogenated marine natural products (Lee and Morel 1995; Yee and Morel 1996). On a global level, algal blooms produce massive quantities of volatile chlorinated and brominated hydrocarbons; the significance of these halogenated compounds is not yet entirely understood and requires further investigation.

### 5.3 Nickel

Ni is present in total concentrations of 2–12 nmol $L^{-1}$ in open ocean surface waters (Bruland 1980) and amol ($10^{-18}$) or lower concentrations in microalgae (see references in Quigg 2008; Quigg et al. 2011). It is often below detection limits in microalgal biomass and requires concentrating cells before analysis, especially in cells grown on nitrate. Little is known about the precise catalytic activity of Ni in biology. Its redox functions are replaceable by Fe, Cu or Mn. An example of this would be in the enzyme superoxide dismutase which has a Ni metallo-center in cyanobacteria but typically Fe or Mn in eukaryotes (Table 1; Frausto da Silva and Williams 2001; Morel et al. 2003). Most work has focused on investigating the role of Ni as the cofactor for the enzyme urease which catalyzes the hydrolysis of urea to ammonium (Frausto da Silva and Williams 2001; Dyhrman and Anderson 2003). Microalgae which can take advantage of this N-source are thought to have a competitive advantage over those which cannot. More recently, it has been speculated that Ni scarcity limits urea utilization in the marine environment as a result of organic complexation and slow uptake kinetics (see references in Dupont et al. 2007).

## 6 Microelements Which Are Known to Be Replaceable in Some but Not All Microalgae

Given that photosynthetic pathways are key to survival, micronutrient stress in microalgae (limitations as well as excess, synergistic as well as antagonistic behaviors) to meet the demands for proteins with metallo-centers has been addressed using a variety biochemically and physiologically sophisticated strategies. Here we will focus on the enzyme carbonic anhydrase as is studied in this respect. For observations related to the roles and functions of Fe in the photosyn-

thetic process and other activities, see details in Larkum (2016). One example which considers Fe/Cu is: in low Fe waters, microalgae may substitute the Cu-containing enzyme plastocyanin for the Fe-containing enzyme cytochrome $c_6$ (Hill et al. 1996; Raven et al. 1999; Peers and Price 2006). As a result, low-Fe conditions may increase Cu requirements in microalgae. Another example is the strategy which involves substitution of the metal-free protein flavodoxin under conditions of low Fe for the Fe-S protein ferredoxin in photosystem I (LaRoche et al. 1996; McKay et al. 1999). Further, some microalgae are able to reduce the abundance of Fe-rich photosystem I relative to photosystem II under conditions of Fe limitation (Strzepek and Harrison 2004).

Co, Cd and Zn are known to functionally substitute for each other in the enzyme carbonic anhydrase of the marine diatom *Conticribra* (*Thalassiosira*) *weissflogii* to maintain optimal growth under Zn limitation (e.g., Lane et al. 2005; Xu and Morel 2013). In *Conticribra weissflogii*, Cd is exported as a phytochelatin complex (Lee et al. 1996) suggesting cells recognize this metal ion as a nutrient and/or as potentially toxic. Lee and Morel (1995) and Finkel et al. (2006, 2007) found biological uptake of Cd (as measured by Cd quotas in cells) in a majority of species tested. This is thought possible as Zn and Co have similar size/charge ratios and that Zn and Cd have similar chemical natures such that Zn and Co transporters will also mobilize Cd (Xu and Morel 2013). Many studies have also linked biogeochemical cycles of Cd and P through microalgae in surface waters, export into the deep sea and remineralization at depth (see Sunda and Huntsman 2000; Morel et al. 2003; Finkel et al. 2007). Sunda and Huntsman, (2005) determined from measured relationships between cellular Zn:C ratios and Zn concentrations and depth profiles for Zn and $PO_4^{3-}$, that Zn chelation in the nutricline of the North Pacific provide evidence that concentrations of this micronutrient are controlled by biological uptake and regeneration. A similar relationship to Zn but for Cu was reported in Sunda and Huntsman (1995b). Despite Cd having a nutrient-like distribution profile in the ocean, that is, depleted at the surface and remineralized at depth, and being complexed by organic ligands (Bruland 1980; Xu and Morel 2013), present findings do not prove there is ubiquitous biological utilization of Cd.

## 7 Uptake, Homeostasis, Toxicity and Efflux of Micronutrients

The acquisition and retention of micronutrients in microalgae is under the influence of internal (physiological) and external (physio-chemical) factors as well as by the size and nature of the cell (Hudson and Morel 1993; Hudson 1998; Raven et al. 1999; Pinheiro and van Leeuwen 2001; Franklin et al. 2002; Morel and Price 2003; Wilkinson and Buffle 2004; Sunda 1994; Finkel et al. 2006, 2007). If available, microalgae may concentrate micronutrients up to $10^3$–$10^5$ higher than in their environment. This process, known as *bioaccumulation*, concerns essential micronutrients such as Fe, Cu, Se, and Zn, but also toxic elements such as Cd or Hg (see also Quigg 2008). Physico-chemical factors (e.g., pH, salinity, temperature, light, and particulate and organic matter concentrations) influence their accumulation inside cells (*bioconcentration*).

Major pathways for the uptake of micronutrients by microalgae are described in excellent reviews such as those by Raven et al. (1999), Worms et al. (2006), Glass et al. (2009), and Sunda (1994, 2012). Most recent studies have focused attention on the biological (transport across membrane), physical (diffusion) and chemical (dissociation kinetics of metal complexes) reactions occurring immediately on biological surfaces. Major questions still to be addressed were summarized by Worms et al. (2006) as: what is the behavior of micronutrients species during their transport from the bulk solution (i.e. >few microns from the biological surface) to the biological interface? How does transfer of the chemical occur across the biological membrane? And, what is the role of the organism in modifying the chemistry and biology of the uptake process? These questions are not entirely new (see e.g. Hudson and Morel 1993; Hudson 1998; and others) but in some cases reflects that a lack of understanding of some of these process still requires concurrent technological developments which are only now emerging.

### 7.1 Uptake of Micronutrients

Briefly, unlike macronutrients, the key to determining the concentrations of micronutrients which are available to microalgae for growth and protein function, and the uptake mechanisms themselves, is determining the *bioavailable* concentration of a specific micronutrient, denoted commonly as M′ and not simply its total concentration in the medium/environment (Sunda and Guillard 1976; Anderson et al. 1978; Morel et al. 2003; Worms et al. 2006). Rates of micronutrient uptake from solution have subsequently been found to be directly proportional to M′, which in turn, is highly dependent on the redox potential of the element in the environment (directly or indirectly powered in part by photochemistry) and the presence and type of chelator and/or complexation process(es) (Morel and Hudson 1985; Hudson and Morel 1993; Sunda and Huntsman 1992, 1995a, b, 2000; Hudson 1998). It is now known that chemical speciation must be taken into account when predicting bioavailability of micronutrients to microalgae.

Over the past 25 years, equilibrium models including the free ion activity model (FIAM) and the biotic ligand model

(BLM) have used extensively to describe micronutrient bioavailability in environmental systems (e.g., Sunda and Guillard 1976; Sunda 1994; Campbell 1995; Campbell et al. 2002). Synthetic chelators such as EDTA have been used extensively to control M′ in laboratory experiments. With knowledge of complexation constants, the free metal ion concentration in a media can be determined (Hudson and Morel 1993; Morel and Hudson 1985; Hudson 1998). FIAM and BLM predict the role of chemical speciation on micronutrient bioavailability. In this respect, they are often (qualitatively) successful in predicting a reduction in micronutrient effects due to complexation (inorganic and organic ligands), competition or other reactions which may reduce micronutrient bioaccumulation; these processes result in a decreased interaction of the micronutrient with uptake sites on the surface of the cells. Yet environmental systems are rarely at equilibrium. These models and the challenges have been recently reviewed by Morel et al. (2003) and Worms et al. (2006) and the reader is referred to these for details.

Arguably, it has been more challenging, and ongoing efforts have been directed towards understanding the types, roles and activities of the naturally occurring complexing agents. As with the synthetic chelators, the availability and uptake of micronutrients is controlled by complexation with organic ligands. These phenomena are currently best studied for Fe (see chapter "Iron", Marchetti and Maldonado 2016) but less so for other micronutrients. Organic ligands acting as chelators can release micronutrient ions into the medium while internalizing others into cells. Siderophores (low molecular weight compounds) are a type of natural chelator produced by bacteria and cyanobacteria that are thought to aid in the assimilation of otherwise difficult to obtain micronutrients, especially Fe (see e.g., Butler 1998).

In response to recently upwelled waters, bacteria and microalgae produce specific chelators to reduce concentrations of Cu ions in their immediate surroundings, thereby diminishing their immediate potential toxicity (Moffett and Brand 1996; Croot et al. 2000, 2003; Quigg et al. 2006). For more on the important role of ligands in controlling micronutrients in the natural environment, see reviews by Vraspir and Butler (2009) and Sunda (2012).

## 7.2 Internalization of Micronutrients

The vast majority of micronutrients are hydrophilic and their transport through biological membranes is mediated by specific proteins. In some cases, a single transport site may be used by several micronutrients or several transport sites may be used by a single micronutrient. If micronutrient internalization occurs via anionic transporters (Campbell et al. 2002), they essentially are "piggy-backed" across transporters meant for low molecular weight ligands such as citrate, thiosulfate or phosphate (e.g., Errecalde and Campbell 2000; Fortin and Campbell 2001). Such transport is dependent on micronutrient speciation and cell nutrition. Other highly specific ligands such as siderophores may be produced by the cell specifically to facilitate the transport of essential cations that are present at low concentrations in the environment (see above). This further complicates the prediction of uptake or effects when using simple chemical models such as FIAM or BLM.

The majority of micronutrients are thought to be transported as the free ion. While transport sites often demonstrate a high affinity for required micronutrients, they are not always highly selectivity. Toxicity may occur when a potentially toxic micronutrient binds to the site of an essential micronutrient with a similar ionic radius or coordination geometry. Calcium has been shown to reduce the internalization of Cd and Zn (e.g., Kola and Wilkinson 2005; Heijerick et al. 2002) and Cd has been shown to replace Zn or Co (e.g., Lane et al. 2005) while Mn and Zn directly compete for the same transporters in some diatoms (Sunda and Huntsman 1992, 1998a, b, c, d).

If microalgae are adapted to low micronutrient concentrations, especially open ocean species, micronutrient uptake can be enhanced by increasing the production of transporters (e.g. Mn; *Thalassiosira pseudonana*; Sunda and Huntsman 1998b, c) or by increasing the affinity of the transporters (e.g. Zn; *T. pseudonana*; Sunda and Huntsman 1992). Internalization has also been linked to low and high affinity transporters (e.g. Sunda and Huntsman 1992; for Zn). As a result, maximum internalization fluxes will be sufficiently different (most often by several orders of magnitude) so that these two uptake mechanisms can be distinguished as two distinct and saturable processes (see e.g. Sunda and Huntsman 1998a; Sunda 1994, 2012). In a direct comparison of Mn′ classical saturation kinetics for an oceanic species and an estuarine species, Sunda and Huntsman (2008) found the half-saturation constant[2] $K_s$ of Mn uptake in *Thalassiosira oceanica* was one-seventh of that for *T. pseudonana*. $V_{max}$ appeared to be under negative feedback control in both species. Six- to 12-fold variations in this parameter allowed cells to regulate intracellular Mn at nearly constant values. While Mn′ and $V_{max}$ varied in relation to each other, $K_s$ did not. They found that the range in Mn′ concentrations over which regulation occurred was different for the two species, and coincided with the Mn′ concentrations of the natural habitat of each species.

For micronutrients which are both essential but can be toxic if over accumulated, microalgae have developed at set of processes to manage their utilization whilst preventing harmful consequences. Cu is such a micronutrient. Studies of Cu accumulation have revealed that Cu ions bind to

[2]See Harrison et al. (1989) for definition.

specialized transport ligands associated with the cell membrane. Cu uptake is light- and ATP-dependent (Verma and Singh 1990, 1991) as well as temperature dependent (Hill et al. 1996), and follows Michaelis–Menten kinetics for facilitated and active transport (Sunda and Huntsman 1995b; Hill et al. 1996). The Cu transport rate is also known to be determined by the activity of the free metal ion in the medium (FIAM model applies) and not the total complexed Cu concentration (Sunda 1994; Van Leeuwen 1999). Knauer et al. (1997) found that Cu uptake is mediated by two systems in the freshwater chlorophyte *Desmodesmus* (*Scenedesmus*) *subspicatus*: a high-affinity and a low affinity system operating at Cu′ of $10^{-14–12}$ M and $10^{<12}$ M, respectively.

This management of micronutrient internalization can also result in species-specific uptake rates (e.g. for Cu). When comparing short-term (2–20 min) uptake rates in the laboratory, Quigg et al. (2006) found cellular net uptake of Cu was two to three orders of magnitude higher in the cyanobacterium *Synechococcus* sp., on the basis of carbon and surface-area–normalized Cu-accumulation rates (46 μmol Cu $mol^{-1}$ C $min^{-1}$ and 1,100 zmol Cu $\mu m^{-2}$ $min^{-1}$) (zmol = $10^{-21}$ mol) relative to those measured in diatoms, chlorophytes, a dinoflagellate, and a coccolithophore. Cu-accumulation rates for *Conticribra* (*Thalassiosira*) *weissflogii* were found to be three times faster in natural seawater than in EDTA-buffered artificial seawater containing an inorganic Cu concentration of 28 pmol $L^{-1}$. Calculations showing that the diffusive flux of inorganic Cu was insufficient to account for observed short-term uptake rates suggest that some of the Cu bound to naturally occurring organic ligands is released through the rapid dissociation of those complexes in the cell's boundary layer.

## 7.3 Homeostasis of Micronutrients

Homeostasis (Cannon 1932) of element composition is one of the central concepts of ecological stoichiometry (see below for more) but while much has been done to understand C, N, P (Redfield 1934; Sterner and Elser 2002) and even Si, less is known about the controls on homeostasis for micronutrients in microalgae. Understanding implications of physiological homeostasis, how microalgae (and other organisms) regulate and maintain their fundamental biochemical functions despite continuous internal and external changes in their environment, is certainly a challenge for future generations. Increased anthropogenic activities since the start of the industrial revolution have resulted in environmental issues such as eutrophication, acidification, carbon dioxide increase, and climate warming. The ecological consequences of these can be measured by examining the response(s) of microalgae. Hence, the ability of microalgae to maintain themselves against recognized changes in environmental conditions depends on their ability to regulate internal conditions despite changes in external elemental supplies.

A general observation has been that the cellular concentrations of micronutrients vary sigmoidally with the concentration of the unchelated micronutrient (i.e., M′) in the medium of microalgae cultured with chelators such as EDTA or NTA (see FIAM model above) (reviewed in Sunda 1994). Evidence for this comes from Sunda and Huntsman (1992) who grew the diatoms *Conticribra* (*Thalassiosira*) *weissflogii*, *Thalassiosira pseudonana* and *Thalassiosira oceanica* and the coccolithophore *Emiliana huxleyi* across a range of Zn concentrations ($10^{-11}$ to $10^{-8.5}$ M). Sunda and Huntsman (1992) found that all isolates exhibited similar sigmoidal relationships between cellular Zn:C ratios and Zn concentrations with minimal slopes at $10^{-10.5}$ to ~$10^{-9.5}$ M and increasing slopes above and below this range. The minimal slopes at intermediate Zn concentrations were explained by negative feedback regulation of a high-affinity Zn uptake system, while increased slopes at high Zn concentrations appeared to be related to uptake by a low-affinity site. By balancing micronutrient uptake and export rates, and all other conditions being optimal, these microalgae were able to sustain maximum growth rates.

While cellular concentrations may increase in direct proportion to free metal ion concentration for Zn, cells regulate concentrations of other micronutrients (e.g., Fe, Mn, Cu) much more tightly (e.g., see Marchetti and Maldonado 2016 for Fe, see Twining and Baines 2013; for other micronutrients). Sunda and Huntsman (1995b) did find a similar sigmoidal relationship between cellular Cu:C ratios and free cupric ion concentration for *Thalassiosira pseudonana*, *Thalassiosira oceanica* and *Emiliania huxleyi* grown in trace metal ion buffered media. Only five to ninefold variations in cellular Cu:C were measured despite $10^{-15}$ to $10^{-12}$ M Cu ions in the growth medium. There is clearly a strong internal economy for the use and re-use such that microalgal cells have a *homeostatic system* to maintain appropriate cellular concentrations of micronutrients (Sunda and Huntsman 1992; Sunda 1994).

When quotas increase above what is needed to sustain maximal growth, luxury uptake appears a strategy for storage of potentially important limiting micronutrients (e.g., for Fe, see chapter "Iron"; Marchetti and Maldonado 2016) while rapid efflux is used for potentially toxic micronutrients (see above for Cu). Little is still known about micronutrient storage in microalgae (Morel et al. 2003). Bioaccumulation and bioconcentration of micronutrients affects their ecology as a consequence of the energy and costs associated with excreting and/or detoxifying. If the costs are significant, then this may reduce growth, reproduction and/or competitive ability. This also influences which and how micronutrients may be biotransferred or biomagnified to higher trophic levels (see Quigg 2008).

## 7.4 Toxicity Caused by Some but Not All Micronutrients

Toxic effects may be observed when proteins (enzymes) are inactivated as a result of their interaction with micronutrients or due to a competitive interference on the uptake of one micronutrient by another or by the induction of an oxidative stress with subsequent cell damage (Sunda and Huntsman 1998a; Payne and Price 1999; Worms et al. 2006). Microalgae are known to use the following protective mechanisms: intracellular binding or sequestration by metal-complexing agents; compartmentalization and transport of micronutrient to subcellular compartments; efflux; and/or extracellular sequestration. Once complexed, toxic micronutrients may be stored in internal compartments such as vacuoles (Nagel et al. 1996; Cobbett 2000). The transport of chelated metal ion from the cytosol into the vacuole is mediated by transporter proteins.

In terms of intracellular binding or sequestration, microalgal strategies to preserve low intracellular concentrations of potentially toxic micronutrients include (i) biomethylation and transport through cell membranes of metal alkyl compounds by diffusion controlled processes, (ii) the biosynthesis of intracellular polymers that serve as traps for the removal of metal ions from solution, (iii) the sequestration of metal ions to cell surfaces, (iv) the precipitation of insoluble metal complexes (e.g., metal sulfides), and (v) metal exclusion from cells (Foster 1977; Frausto da Silva and Williams 2001; Whitfield 2001). While biomethylation appears to be limited to nonessential metals (e.g., HgII ), the other strategies are pertinent to our understanding of Cu accumulation and homeostasis in algal cells (see Quigg et al. 2006).

Once inside cells, intracellular sequestration is an effective mechanism used by eukaryotic microalgae to reduce the bioavailability of toxic ions. Chelators produced include glutathione, amino acids, phytochelatins, metallothioneins, organic acids and thioredoxins (e.g., Ahner and Morel 1995; Mendez-Alvarez et al. 1999; Lemaire et al. 1999; Le Faucheur et al. 2005). Induction of these compounds depends on both the concentration and nature of the micronutrients present in excess (mainly Cd, Cu, Ni). For example, thioredoxins are small but ubiquitous proteins with highly reactive exposed disulfide sites that can be reduced to thiols under stressful conditions (Lemaire et al. 1999). Cd is known to be very effective at inducing the production of phytochelatins and other thiols in microalgae; they use this both as a mechanism to control Cd homeostasis but also as a detoxification mechanism (Payne and Price 1999; Xu and Morel 2013).

Two of the most extensive studies on toxicity, in terms of number of marine microalgae and micronutrients examined, were performed by Brand et al. (1983, 1986). In Brand et al. (1983), alterations to the growth rate of 21 species by a range of Zn′, Mn′ and Fe′ concentrations were measured using an EDTA-buffered medium. Coastal species tended to be limited by Zn′ below $10^{-11.5}$ M whereas Zn′ at $10^{-13}$ M did not limit all oceanic species. This gradient of Zn requirements is consistent with distributions of both species and micronutrient availability. Mn and Fe were not found to be toxic but ultra-low levels reduced growth even in oceanic species. We now know that the oceanic species of eukaryotic microalgae in the study by Brand et al. (1983) and Sunda and Huntsman (1992) (and others) likely could not be limited even at these very low Zn ion concentrations as Co and Cd in the media could alleviate Zn limitation.

The sensitivity of 20 species of marine microalgae to free cadmium ion activity was measured by Brand et al. (1986) using a NTA-cadmium ion buffer system that controlled free Cd ion activity. Prokaryotic cyanobacteria were the most sensitive to Cd toxicity, diatoms were the least sensitive, and coccolithophores and dinoflagellates were intermediate in their response. Cyanobacteria were killed by Cd′ of $10^{-9.3}$ M whereas reproduction rates of most of the eukaryotic algae were not reduced significantly until $10^{-8.3}$ Payne and Price (1999), Sunda and Huntsman (1998d, 2000), and Finkel et al. (2007) found similar patterns, suggesting an apparent metabolic demand for Cd in diatoms, no functional response in Cd:P ratios of prasinophyte and dinoflagellate species, and a metabolic sensitivity to Cd by cyanobacteria. Several potential physiological mechanisms have been proposed to explain these phylogenetic differences in Cd:P regulation (see Finkel et al. 2007). For example, there are taxonomic differences in elemental transport systems and their specificity for Cd versus other elements in response to environmental conditions in microalgae. Or, it may be more energetically costly to restrict and select entry of different ions than induce efflux, which would require a direct input of ATP. Finkel et al. (2007) added a new hypothesis, that the fundamental and striking differences in the regulation of Cd:P in cyanobacteria versus eukaryotes, especially the diatoms, may reflect the active selection of different biochemical regulatory networks in response to the changing availability of elements over Earth's history (Ho et al. 2003; Quigg et al. 2003a, 2011; Falkowski et al. 2004).

Cu toxicity (measured as a decrease in growth rate) of 38 marine microalgae, measured using a NTA-cupric ion buffer system that controlled free cupric ion activity, was found to be dependent on the phylogenetic origin of the species (Brand et al. 1986). On a smaller subset of microalgae, Croot et al. (2000, 2003) and Quigg et al. (2006) found the response to Cu could be related to the phylogenetic origins of the species investigated. Cyanobacteria were typically found to be the most sensitive (with reproduction inhibited at Cu′ of $10^{-12}$ M), diatoms the least sensitive, with coccolithophores and dinoflagellates having an intermediate sensitivity. *Emiliania huxleyi*, *Skeletonema costatum*, *Thalassiosira pseudonana* and *Thalassiosira oceanica* were most resistant

to Cu, with growth continuing at the highest Cu′ tested ($10^{-9.5}$ M and $10^{-9.2}$ M). Brand et al. (1986) did not find differences between coastal and oceanic species in their terms of their sensitivity to Cu; more recent studies concur with this finding. It appears that Cu′ influences seasonal succession of species, particularly in regions of upwelling (Moffett and Brand 1996; Moffett and Dupont 2007), by species specific toxicity, which can be ameliorated by the production of Cu complexing ligands that reduce Cu′ extracellularly.

### 7.5 Efflux of Micronutrients

The effects of excess micronutrients in the cytosol may also be reduced by efflux. In addition to decreasing toxicity, efflux reduces the net micronutrient flux and may modify the chemical speciation of the micronutrient through the expulsion of complexes. Microalgae can also excrete compounds that complex with micronutrients in the extracellular medium in order to reduce their bioavailability and therefore, entry to microalgal cells (see references in Chen et al. 2011; Quigg et al. 2013). These complexing agents include polysaccharides, proteins, peptides and small organic acids that are able to decrease the concentration of bioavailable micronutrients in the immediate vicinity of the cell. In marine systems, Cu, Cd and Zn tend to be strongly complexed by exudates produced by microalgae. See reviews by Worms et al. (2006) and Quigg et al. (2013) for more details.

## 8 Relationships among Individual Micronutrients Used by Microalgae

There is a developing understanding of antagonistic and/or synergistic relationships among micronutrients in microalgae and in aquatic environments. How different microalgae respond to and interact with bioavailable micronutrients (M′) in various combinations is still poorly understood. What we do know is that the cellular concentration of one micronutrient can strongly influence or be influenced by others via competition for transport through transmembrane channels, coordination chemistry, or charge (Bruland et al. 1991; Hudson and Morel 1993; Sunda and Huntsman 1998a, b, c, d; Morel et al. 2003; Worms et al. 2006).

An example of this behavior comes from examining the response of microalgae to different concentrations of the micronutrients Zn, Co and Cd. This provides a good example of what is called "biochemical substitution" (Morel et al. 2003). If all three micronutrients are at bioavailable concentrations, Zn is used preferentially (Sunda and Huntsman 1995a, 2000). However, when Zn′ is depleted but Co is available, the coccolithophore *Emiliania huxleyi* is able to utilize the Co to reach their maximum growth rates (Sunda and Huntsman 1995a). If Zn′ is increased from 1 to 25 pM, then Co quotas decrease twofold in *Emiliania huxleyi* or if Zn′ is increased from 10 to 100 pM, the Cd quota of *Conticribra* (*Thalassiosira*) *weissflogii* is decreased fivefold (Sunda and Huntsman 1995a, 2000). In another study, high levels of Cu and Zn were shown to antagonize Mn nutrition by inhibiting cellular Mn uptake in the diatom *Thalassiosira pseudonana* (Sunda and Huntsman 1998c). Cu and Zn inhibition of growth rate occurs only at low Mn′ (independent of the irradiance used for growth). Studies with coastal diatoms have shown that algal Cd concentrations are not only controlled by aqueous Cd ion concentrations but are also inversely related to Mn and Zn ion concentrations (Lee and Morel 1995; Sunda and Huntsman 1996, 1998d, 2000). Hence, a second example comes from investigating the antagonistic relationships between the micronutrient Mn and Zn and Cd – while Zn can be a micronutrient, it is also known to be toxic at elevated concentrations whereas Cd is generally consider to be toxic and not a micronutrient by Sunda and Huntsman (1996). Zn and Cd where found to inhibit Mn uptake by interfering with intracellular feedback regulation of Mn uptake capacity and by competitively blocking Mn binding to membrane uptake sites. Further, Cd was found to be transported into the cell via the Mn uptake system, an effect Sunda and Huntsman (1996) found to be accentuated at low Mn free ion concentrations by negative feedback which increases uptake capacity and by lowering Mn competition for binding to the uptake sites. The net result of these two effects was higher intracellular Cd:Mn ratios at low Mn free ion concentrations which exacerbates Mn limitation due to competitive binding of Cd to Mn nutritional sites. To alleviate such problems in the natural environment, phytochelatins and efflux mechanisms are activated. If these processes do not work, Mn limitation may occur. Enhanced cellular Cd concentrations at low ionic Mn lead to reduced biodilution rates associated with Mn limitation of growth rate.

In some situations, toxicity is observed even for micronutrients which are typically consider primarily nutritive in nature. Mn can be inhibitory or toxic in excess amounts to cyanobacteria (*Microcystis* sp.; Velichko, 1968 in O'Kelley 1974) and a negative fertility factor for diatoms (*Ditylum brightwellii*; Steele 1965). In this situation, what typically occurs is that the concentration of the micronutrient becomes elevated beyond the homeostatic range required by a microalgal cell. In this situation, the microalgae will accumulate the micronutrient uncontrollably by simple diffusion (usually electrically uncharged complexes) through membranes or by leakage through the transport system of another micronutrient (Morel et al. 2003). Sunda and Huntsman (1998b) found that Zn enters diatoms cells through Mn transport systems, if present in elevated concentrations. This is an example of "competitive inhibition" (Morel et al. 2003). Zn will then become toxic to the cell and a corresponding decrease in

growth rate is observed. This in turn results in an even faster rate of accumulation (i.e., the micronutrient quota is no longer being diluted by growth) so that growth stops abruptly as M′ increases above a critical threshold in the growth medium (Morel et al. 2003). In the studies by Brand et al. (1983, 1986), authors examined this phenomenon in a range of microalgae with different phylogenetic origins and with a range of Zn′, Mn′, Fe′, Cu′ and Cd′ in buffered media. They were able to define the "tipping point", i.e., the concentration where these micronutrients switched from being nutritional to toxic; further studies are required to elucidate the molecular mechanisms in most microalgae.

## 9 Importance of Light

Changing the irradiance for growth changes the cellular concentration of elements required for light harvesting and photosynthesis: while N, Fe, and to a lesser extent, Mn increase with decreasing irradiance in many species (Raven 1988, 1990; Sunda and Huntsman 1997; 1998c; Raven et al. 1999; Morel et al. 2003) the opposite pattern has been observed for Zn in the coastal diatom *Thalassiosira pseudonana* (Sunda and Huntsman 2004, 2005). The increased need for cellular Fe:P and Mn:P at low irradiances is triggered by requirements for these elements in the photosynthetic reaction centers, photosynthetic electron transport, and the water splitting in the oxygen evolving complex of photosystem II. This is described in great detail in Raven (1988, 1990) and Raven et al. (1999). When microalgae are photoacclimated to extremely low irradiances, they have a larger ratio of light-harvesting pigments to thylakoid protein complexes, that is, an increase in photosynthetic unit size. This requirement for the photosynthetic pathways is so significant that when the irradiance for the growth of the dinoflagellate *Prorocentrum minimum* ( = *P. cordatum*) was increased from 50 to 500 µmol quanta $m^{-2}$ $s^{-1}$, the Fe quota decreased by a factor of 2 (Sunda and Huntsman 1997). In contrast, a tenfold rise in irradiance increased the Zn requirements in *Thalassiosira pseudonana*. Sunda and Huntsman (2005) associated this with a 2.8-fold increase in the maximum specific growth rate, and resultant increased demand for carbonic anhydrase and other biosynthetic Zn enzymes. Miao and Wang (2004) also observed increases in the uptake of Cd and Zn in the coastal diatom *Thalassiosira pseudonana* with increases in irradiance. Cellular micronutrient concentrations in general are directly related to the uptake rate and inversely related to the specific growth rate. Consequently, the reduced growth rate under low irradiances allow cells to accumulate the increased Fe, Mn and other elements needed for synthesis of additional photosynthetic units.

On the basis of the changing elemental composition of five species (*Cyanothece* sp., diazotroph; *Pycnococcus provasolii*, prasinophyte; *Amphidinium carterae*, dinoflagellate; *Conticribra* (*Thalassiosira*) *weissflogii*, diatom; *Chaetoceros calcitrans*, diatom) examined when growing a five light intensities from 15 to 500 µmol quanta $m^{-2}$ $s^{-1}$, Finkel et al. (2006) concluded that phenotypic responses to light were responsible for less than one to about three orders of magnitude in micronutrient:P ratios within species, comparable to the phylogenetic differences observed in Quigg et al. (2003a). Finkel et al. (2006) found that many of the micronutrients were present in highest concentrations at very low irradiance (<30 µmol quanta $m^{-2}$ $s^{-1}$): Fe:P, Mn:P, Zn:P, Cu:P Co:P, Mo:P, and Ni:P. However, under saturating irradiances, and in a few species, micronutrients were enriched relative to P. This included Fe, Mn, and Zn in the coastal diatom *C. weissflogii*, and Co in the diazotroph *Cyanothece* sp. and the prasinophyte, *Pycnococcus provasolii*. Further, they found evidence of storage of cellular micronutrients under 50 µmol $m^{-2}$ $s^{-1}$. These authors found a facultative increase in micronutrients not related to photosynthesis. There is little known about micronutrient storage (aside from perhaps Fe). Finkel et al. (2006) hypothesized that dilution of cellular micronutrients could occur in response to the release of cells from light limitation, allowing microalgal growth to increase rapidly. This warrants further investigation as we need to better understand how microalgae modulate micronutrients for metabolic requirements.

Micronutrients interact with light within the euphotic zone, thereby controlling algal growth rates (see Sunda and Huntsman 1998a, c; Finkel et al. 2006). This occurs as a result of several processes. Complexation is known to be a highly light dependent process (see Worms et al. 2006; Sunda 1994, 2012). Given microalgae growing at very low irradiances (growth-limiting) have higher concentrations of Mn and chlorophyll *a*, needed for the synthesis of additional photosynthetic units (Raven 1990; Raven et al. 1999), there is a concurrent need for more Mn. Sunda and Huntsman (1998c) found this additional Mn was not provided by higher cellular Mn uptake rates, since these rates were found to be the same in light-saturated and light-limited cells of *Thalassiosira pseudonana*. Rather, the steady-state cell Mn was equal to the uptake rate divided by the specific growth rate such that cells accumulated the additional Mn needed for photoacclimation once light limitation caused growth rate to decline, an inherent negative feedback relationship. In addition, the fractionation of micronutrients relative to phosphorus into microalgal biomass under low light is consistent with depleted levels of $Cu^{2+}$ and $Mn^{2+}$ in deep chlorophyll maxima (Finkel et al. 2006), suggesting that the export of low light-acclimated microalgae is a major source of trace element flux to the deep ocean and an important factor in the biogeochemical cycles of many of the biologically limiting elements in the oceans.

Also to be considered is the diel photoperiod which regulates daily rates of carbon-fixation, growth, and cell division.

Because photosynthesis occurs only during the day, the growth and composition of cells must vary over the diel cycle. While this is well documented for cell carbon, nitrogen, carbohydrate, protein, chlorophyll *a* in cultured and natural microalgal populations, very few studies have investigated changes on micronutrients. One such study is that of Sunda and Huntsman (2004) in which they examined variations in cellular Fe and Zn in the diatom *Thalassiosira pseudonana*. Cellular concentrations of carbon, chlorophyll *a*, zinc, and iron varied during the light period in response to day–night differences in rates of C-fixation, chlorophyll *a* synthesis, growth, and metal uptake. Higher cellular Fe intracellular levels measured increased concurrently with rates of C-fixation thereby allowing the diatom cells to synthesis additional iron-rich proteins (e.g., those utilized in photosynthetic electron transport – See Raven et al. 1999). Cellular Zn concentrations decreased by 25 % during the light period because of higher daytime specific growth rates, which led to higher rates of biodilution. Mean chlorophyll *a* concentration decreased linearly with decreasing specific growth rate under Fe and Zn limitation, thereby allowing the cells to maintain a balance between light harvesting and biosynthesis. The diel cycle also influenced cellular Fe concentrations through day–night differences in iron chelation, linked to photochemical redox cycling. Differences between Fe and Zn can be accounted for by complexation, that is, for Zn this process this is unaffected by light, but for iron chelates photo-redox cycling increases the concentration of dissolved inorganic Fe(II) and Fe(III) species and thus increases algal iron uptake rates in the light (see e.g., Sunda and Huntsman 1995b).

## 10 Importance of $CO_2$

Given the current interest in global increases in atmospheric $CO_2$ and temperature and the associated with changes in ocean chemistry (not only acidification) and circulation, light and nutrient regimes, there are concurrent changes in the availability of macronutrients (reviewed by Finkel et al. 2010 and elsewhere in this book) and micronutrients for microalgae. The consequences include, but are not limited to, alterations in marine microalgal communities which in turn will affect primary and export production, food web dynamics and structure and biogeochemical cycling of carbon and nutrients in the oceans. While it is well known that carbonic anhydrase is required for the uptake and fixation of inorganic carbon, especially at low $CO_2$ concentrations at the cell surface (Morel et al. 1994; Raven et al. 1999; Beardall et al. 2009), microalgae are known to activate carbon concentrating mechanisms in order to increase $CO_2$ concentrations at the active site of RUBISCO (see chapter "Carbon Acquisition by Microalgae", Beardall and Raven 2016). Few studies to date have however investigated the relationships between changes in micronutrients and $CO_2$ concentrations.

Sunda and Huntsman (2005) found decreasing the $CO_2$ concentration (via an increase in pH from 8.2 to 9.0; $CO_2$ values not reported) in the culture medium for the diatom *Thalassiosira pseudonana* resulted in an increased cellular Zn requirement to achieve a given growth rate and also that needed to achieve maximum growth. This increase was linked to a greater demand for Zn in the enzyme carbonic anhydrase, which is needed for cellular $CO_2$ acquisition. The increased demand for cellular Zn in *T. pseudonana* was possibly because of a decrease in the daily specific growth rate, which increased cellular Zn concentrations by decreasing biodilution rates. The coastal diatom *Conticribra weissflogii* responds similarly to changes in $CO_2$ and Zn concentrations in its environment (Morel et al. 1994). Particulate Cd:P in field samples of microalgae have been shown to respond to changes in $CO_2$ (Cullen et al. 1999). In the study by Morel et al. (1994), a change in cellular demand for Cd (or Zn and Co) in carbonic anhydrase of diatoms was measured, the only taxonomic group with a demonstrated use for Cd, in response to the availability of inorganic carbon.

As a result of the high concentrations of carbonic anhydrase, the increased demand for cellular Zn needed for algal growth under conditions of low $CO_2$ availability may lead to $CO_2$ and zinc co-limitation in some oceanic environments (Morel et al. 1994). To test this hypothesis, Zn additions to bottle incubation experiments were performed in the different parts of the Pacific (see references in Sunda 2012). Consistently results showed a modest stimulation of algal growth (compared to Fe additions), but a correspondingly significant effect on microalgal species composition. The growth of the coccolithophore *Emiliania huxleyi* was preferentially stimulated over other species (Crawford et al. 2003). Sunda and Huntsman (1995a) found this alga has an unusually large cellular uptake and growth requirement for Zn/Co compared to the diatoms *Thalassiosira oceanica* and *Thalassiosira pseudonana*. Given *Emiliania huxleyi* and other coccolithophores are largely responsible for calcium carbonate formation and regulation of ocean water alkalinity, which in turn influences the air-sea exchange of $CO_2$, it was hypothesized that Zn (and possibly Co) could indirectly affect atmospheric $CO_2$ and global climate in oceans. Further studies are however required.

## 11 Emergent Concepts and Issues

### 11.1 Ecological Stoichiometry

One of the biggest ecological challenges is linking molecular biomarkers of pollution with ecologically relevant life history characteristics including growth, survival and reproduction

of aquatic organisms. The study of the balance of chemical elements in components, interactions, and processes in ecosystems is called ecological stoichiometry, which seeks to track the mass flow of elements from genes to ecosystems (Sterner and Elser 2002). For micronutrients and microalgae, this involves an examination of the bioaccumulation, biotransference and/or biomagnification of elements through food webs (see Quigg 2008; Bradshaw et al. 2012).

An implicit assumption associated with ecological stoichiometry is that the organism of interest can maintain homeostasis, that is, maintain constant "body" concentrations of elements despite changing concentrations in the environment and/or their resource supply (Sterner and Elser 2002). Stoichiometric homeostasis for a range of micronutrients has been shown in microalgae (see section above) such that a multi-elemental signature may be defined. Stoichiometrically, the most abundant micronutrient in microalgae is Fe, followed by Mn and Zn (Quigg et al. 2003a, 2011; Ho et al. 2003; Quigg 2008; Twining and Baines 2013). These are present in concentrations at least one to two orders of magnitude higher than those for the other essential elements (Cu, Co, Ni, Se, Mo), and several orders of magnitude higher than elements which are replaceable (e.g., V) or whose roles remain poorly defined (e.g. Cd). Differences in terms of cellular concentrations of Cu, Co, Ni, Se and Mo may be explained in terms of the evolutionary histories of microalgae (see above) and their corresponding cellular physiology and biochemistry, but in most cases, the known specialized roles for these elements do not readily explain their cellular concentrations or stoichiometric relationships with other nutrients – micro or macro – in microalgae (Quigg 2008).

Expanding the Redfield ratio ($C_{106}N_{16}P_1$) (Redfield 1934) to include micronutrients has the potential to improve our understanding of elemental cycling in ecosystems and food webs, homeostatic regulation of micronutrients, and consumer-resource mismatches. In essence, it will provide a mechanism to explore relationships between macro- and micro-nutrients from an ecological perspective. Early efforts to determine the multi-elemental composition of microalgae and expand the Redfield ratio began in the 1970s (e.g., Martin and Knauer 1973; Morel and Hudson 1985; Kuss and Kremling 1999; Ho et al. 2003) with ratios such as the following: $P_{1000}(Fe,Mn)_{10}(Zn, Cu, Ni, Cd)_1$ providing very basic information on the relatively proportions of micronutrients in microalgae. There are significant challenges to overcome to find a set of stoichiometries that may have the same kind of appeal as the Redfield ratio but new technologies and approaches may make that feasible in the future. Advances in genomics and proteomics will be key to characterizing microalgal genomes, and more specifically metalloproteomes, many of which remain largely uncharacterized at this time (see paper of Cvetkovic et al. 2010).

## 11.2 Micronutrients in Natural Populations of Microalgae

Analytical techniques to determine the micronutrient composition of natural populations, and more specifically, single cells in situ, have been developed in recent decades. At the population level (that is, bulk particulate matter), inductively coupled plasma mass spectrometry approaches (e.g., ICP-AES, ICP-SFMS) have enabled the simultaneous multi-elemental measurement of natural populations (e.g., Kumblad and Bradshaw 2008; Ho et al. 2010; Bradshaw et al. 2012). The major challenge with this approach however is subtracting interference of elements which maybe acting as scavengers on the organic material (e.g. Al, Pb) or be scavenged (e.g. Fe, Co), and/or the interference of lithogenic and detrital matter. Nonetheless, these studies compliment earlier findings such as those by Bruland (1980) and Bruland et al. (1991) for micronutrient profiles in seawater. Further, these studies have shown how the relationship between intra- and extra-cellular micronutrients change with depth – surface, mixed layer, below photic zone (e.g., Ho et al. 2010) or during an algal bloom (e.g., Bradshaw et al. 2012). The study by Bradshaw et al. (2012) also showed for the first time how high rates of incorporation of micronutrients (Co, Fe, Mn and Zn) to microalgae during a bloom can temporarily lower corresponding micronutrient concentrations in the surrounding seawater and thereby alter competition for these resources.

Synchrotron X-ray fluorescence (>6 keV) measures the concentrations of micronutrients such as Fe, Mn, Zn, Ni and P, at the single (nano- or pico-plankton) cell level. Studies using this approach (summarized in the review of Twining and Baines 2013) have revealed the localization and distribution of specific micronutrients in microalgal cells. Using this approach, Fe, Mn, Zn and Ni can be clearly seen to be localized in the chloroplasts of diatom and flagellate cells. Such studies also reveal regional (for temperate, equatorial, and Antarctic waters in the Pacific and Atlantic Oceans) variability in micronutrient concentrations, which have also been found to match trends in dissolved metal availability for micronutrients (e.g., Fe, Zn, Cu, Mn). An example from the review of Twining and Baines (2013) is that of Zn:P ratios – these were found to be differ for autotrophic flagellates depending on their presence south versus north of the polar front in the Southern Ocean; and in parallel with corresponding dissolved Zn concentrations in those waters.

One of the challenges with these new techniques is that although they can measure P concentrations simultaneously with micronutrients, they cannot do so for C or N. Hence, efforts at mass balance modeling cannot be directly based on element:C ratios or on carbon fluxes, unless additional assumptions are made. It is well understood that the Redfield ratio of C:N:P is affected by both biotic and abiotic factors

(Redfield 1934); hence model imbalances of micronutrient:P ratios will also be influenced by intra- and extra-cellular factors. Strategies to address this will be the focus of future studies.

## 11.3 Emergent Pollutants

Micronutrient concentrations occur in picomolar to nanomolar quantities in the aquatic environment. Enrichment of seawater may occur naturally through local environmental processes, such as volcanism or upwelling, but it may also occur as a result of human activities. Nanomaterials are diverse substances that are characterized by having one dimension that is <100 nm (Luoma 2008). They may be natural, incidental, or engineered and may be released through point and nonpoint sources, or introduced directly to the environment. Engineered nanoparticles are a class of nanomaterials with at least two dimensions between 1 and 100 nm (Nel et al. 2006; Luoma 2008); these are being used in a wide variety of applications including environmental remediation, pollution sensors, photovoltaics, medical imaging, and drug delivery. Of concern for microalgae (and other trophic levels) are nanoparticles formed from metal oxides ($TiO_2$, ZnO, CeO; natural and engineered), zero-valent metals (Fe, Ni, Au, engineered) and quantum dots such as those made with cores of cadmium selenide (CdSe), cadmium telluride (CdTe), and/or zinc selenide (ZnSe) (Nel et al. 2006; Klaine et al. 2008).

Research reveals that these emergent pollutants may cause toxicity to cells either directly (by becoming incorporated into the members or cellular space) or indirectly (by releasing chemical ions on and inside of cells) (reviewed in Navarro et al. 2008; Quigg et al. 2013). Silver engineered nanoparticles are the arguably the best studied nanomaterials to date in terms of their environmental impacts. It is known that with microalgae, it is less frequently the nanoparticles themselves causing toxicity and more frequently the released Ag ions that interact with cell membranes, proteins, DNA, RNA and inactivate vital cellular functions (e.g., Miao et al. 2009, 2010a). The cadmium used in quantum dots is known to have a similar effect on microalgae (e.g., Werlin et al. 2011; Zhang et al. 2012) as is the case for the zinc in metal oxides (e.g., Miao et al. 2010b; Ma et al. 2013). In a number of studies, it has been shown that microalgae have the ability to "protect themselves" from these potentially toxic materials by producing exopolymeric substances (see Chen et al. 2011; Quigg et al. 2013). Nonetheless, once inside cells, these materials have the potential to be bioaccumulated and/or biomagnified to higher trophic levels as described above for micronutrients. In a recent review, Hou et al. (2013) found that daphnia, fish, aquatic worms and earthworms are the most commonly studied ecological receptors of nanomaterials in freshwater systems. Hence, going forward, when studying the interactions of micronutrients and microalgae in the aquatic environment, an additional challenge will be the presence of these and other emergent pollutants which alter nutrient availability and/or may be toxic.

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