Phytoplankton antioxidant systems and their contributions to cellular elemental stoichiometry

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Scientific Significance Statement

Antioxidants are essential for organisms across the tree of life, and organisms connect biogeochemical cycles via their cellular stoichiometry. Despite this, it is unclear how different antioxidants contribute to or influence cellular stoichiometry. We reviewed the mechanisms and elemental composition of phytoplankton antioxidant systems, identifying that they play important roles in influencing elemental stoichiometry in phytoplankton cells.

Abstract

Oxidative stress plays a role in many aspects of cellular metabolism, and as a result, antioxidants have the potential to impact cellular stoichiometry and biogeochemical cycles. We reviewed how antioxidant systems influence macronutrient and micronutrient stoichiometry in marine phytoplankton and identified that antioxidant systems have important implications for micronutrient stoichiometry. By leveraging diatom proteomic data, we empirically estimated the level of micronutrient quota variation that can be attributed to antioxidant systems. Fe-containing antioxidant expression may contribute to 3.3–10 μmol : mol variation in Fe : C, and superoxide dismutases appear to be important contributors to variation in Mn, Ni, Zn, and Cu quotas in phytoplankton. Critical next steps for the study of phytoplankton antioxidant systems are to (1) distinguish between oxidative stress and redox-based gene regulation and (2) determine how antioxidants influence variation or consistency in micronutrient quotas under various environmental conditions.

Elemental stoichiometry connects individual organisms to earth-scale processes, and underpins the connections between all biogeochemical cycles. Redfield (1958) famously connected ratios of nitrogen and phosphorus in surface phytoplankton to dissolved concentrations in the deep ocean, describing a homeostatic system. Comparatively less focus has been on exactly why nutrients have specific stoichiometric ratios. Early work by Elser et al. (1996) connected biochemical composition to life history, providing a causal link between life history and stoichiometry (the biochemical foundations of stoichiometry have been further characterized since then, e.g., in Geider and LaRoche 2002; Elser et al. 2000; Sterner and Elser 2002). Expanding on this body of work, Loladze and Elser (2011) suggest that the ratio of nitrogen to phosphorus is ~16 because of fundamental constraints on protein synthesis by phosphorus-rich ribosomes. Which other cellular processes impact deviations from, and consistency with, the Redfield ratio? In addition to macronutrients, micronutrients...
like iron (Fe) can play a large role in influencing primary productivity and biogeochemical cycling (Martin and Fitzwater 1987; Tagliabue et al. 2017). Yet, attempts to extend the Redfield ratio to micronutrients have uncovered enormous variability. For example, Sunda and Huntsman (1995) found that Fe : C varies by as much as two orders of magnitude, and much of this variability is likely due to high Fe uptake (often called luxury uptake), rather than biochemical responses (e.g., protein production). Which other cellular mechanisms lead to variability in micronutrient stoichiometry?

Oxidative stress influences many aspects of cellular function and metabolism, and thus has the potential to influence cellular stoichiometry. For example, cells invest significant resources in protecting against oxidative stress, shown by the proportion of proteomes invested in antioxidant enzymes (Müller et al. 2020). These resources also include protein chaperone networks (Santra et al. 2018), protective biomolecules (e.g., glutathione [GSH], polyphosphate), and protein synthesis (Nishiyama et al. 2011). Numerous components of metabolism are influenced by oxidative stress. For example, glycolysis is controlled by oxidative stress due to peroxide-induced inactivation of the key protein glyceraldehyde-3-phosphate dehydrogenase (Shenton and Grant 2003). In addition to its influences on cellular stoichiometry, oxidative stress also has large consequences for eco-evolutionary dynamics (Morris et al. 2011; Laman Trip and Youk 2020), cell signaling (Wood et al. 2003; Mittler et al. 2004, 2011; Fomenko et al. 2011; Petrov and Van Breusegem 2012; Rosenwater et al. 2014; Mittler 2017), circadian rhythms (Edgar et al. 2012), marine viruses (Sheyn et al. 2016), and marine cell gravitaxis (Carrara et al. 2021).

Our central goal is to examine how antioxidant systems influence cellular stoichiometry. In doing so, we ask: how might oxidative stress contribute to consistency and variation in cellular stoichiometry? We use the term “contribution” because antioxidants can influence cellular stoichiometry through the processes they mediate, but they themselves form a portion of cellular elemental quotas. We focus on marine phytoplankton, as they are key players in global biogeochemical cycles (Falkowski et al. 2008), and there is motivation to move beyond model organisms and explore oxidative stress in diverse environments (Imlay 2019).

We begin with some definitions and a brief review of conditions that lead to oxidative stress in situ, and then we highlight different antioxidant systems present in phototrophic phytoplankton, and their mechanisms. In discussing these mechanisms, we point to specific examples from different research fields that may have oceanographic relevance. In the following section, we ask: under increased oxidative stress, would increased production of a given antioxidant increase or decrease elemental ratios to carbon? Lastly, we assess the magnitude by which different systems could influence phytoplankton stoichiometry using previously published proteomic data.

What is oxidative stress, and which conditions lead to it in situ?

An antioxidant can be defined as “any substance that delays, prevents, or removes oxidative damage to a target molecule” (Halliwell and Gutteridge 2007). We use the definition from Sies (1991) for oxidative stress, to be associated with “a disturbance in the prooxidant-antioxidant balance in favour of the prooxidant.” Many antioxidant systems are directly involved in gene regulation (Mittler et al. 2011; Sies 2017). In other words, “a disturbance in the prooxidant–antioxidant balance” does not necessarily equate irreversible damage, and this disturbance may directly influence gene expression. Indeed, many reactive oxygen species (ROS; except hydroxyl radicals) have been invoked in some signaling context (singlet oxygen, hydrogen peroxide, and superoxide: Triantaphylides and Havaux 2009; Sies 2017; Case 2017). Therefore, the definition of oxidative stress we use is consistent with gene regulatory functions of antioxidants.

In phytoplankton, oxidative stress is typically experienced when photosynthetic electron transport is in excess of that required for CO₂ fixation and nitrate assimilation (Asada 2006). In situ, oxidative stress may correspond with low CO₂, high light, or low Fe. All of these conditions impact the rate of photosynthetic electron transport, more specifically, they typically increase the proportion of electrons leaking from the electron transport chain (producing superoxide). Superoxide is produced via reduction of molecular oxygen mostly at the reducing side of photosystem I (PSI; Asada 2006). Oxygen can therefore act as a sink of electrons, which otherwise would have been donated to NADP⁺. Photosynthetic electron transport is such a dominant source of ROS that even predators of photosynthetic cells have unique adaptations to their prey’s photosynthetic oxidative stress (Uzuka et al. 2019). The unique reactions of different ROS with biomolecules are described in more detail below.

High exogenous hydrogen peroxide is also a direct source of oxidative stress (Cooper et al. 1987; Shaked et al. 2010). However, hydrogen peroxide (H₂O₂) concentrations within cells are not identical to those outside of cells (Seaver and Imlay 2001; Sies 2017), so it is uncertain how much exogenous H₂O₂ (e.g., via rainfall) actually contributes to oxidative stress. These definitions of antioxidants and oxidative stress are broad, which reflects the broad uses (e.g., signaling, protective, etc.) of various antioxidant molecules.

For Fe specifically, it is unclear if there is more oxidative stress under low or high Fe. Under low Fe, photosynthetic electron transport is restricted, thus making the production of superoxide more likely (Niyogi 1999). But, the dominant negative consequences of superoxide and H₂O₂ on biomolecules arise mainly through interactions with Fe (Anjem and Imlay 2012; Imlay 2013), therefore one might expect more oxidative stress under high Fe. Consistent with high Fe leading to oxidative stress, Anand et al. (2019) observed
convergent evolution in several bacteria in the oxidative stress regulator OxyR under a high Fe treatment. Strikingly, van Graaff et al. (2016) showed that chronic Fe starvation leads to more resistance to exogenous H$_2$O$_2$ than Fe replete conditions. They also showed that the chronic-Fe starved proteomic profile resembled in situ conditions observed using metatranscriptomics from Ocean Station Papa (Marchetti et al. 2011), suggesting that an exogenous ROS-tolerant phenotype under low Fe is the norm in iron-limited ocean regimes.

**Antioxidants**

**Enzymatic consumers**

**Superoxide dismutases**

Superoxide dismutases (SODs) are ubiquitous enzymes with metal cofactors (Wolfe-Simon et al. 2005; Miller 2012). They are incredibly efficient enzymes, converting superoxide into dioxygen and H$_2$O$_2$ (Eq. 1) with first-order rate constants approaching diffusion-limited rates:

\[
2O_2^− + 2H^+ \rightarrow H_2O_2 + O_2
\]  

(1)

In addition to being kinetically fast, they are also broadly distributed throughout organisms on earth and evolved billions of years ago (Case 2017). There are three families of SODs containing distinct metal cofactors: nickel SODs (NiSOD); copper/zinc SODs (CuZnSODs); and manganese/iron SODs (MnFeSODs) (Miller 2012).

SODs protect against the deleterious effects of superoxide—but what are the exact effects of superoxide? Interestingly, superoxide reacts with most biomolecules at slow rates (Winterbourn and Metodiewa 1999; Halliwell and Gutteridge 2007). The main targets of superoxide are Fe–S clusters and mononuclear Fe enzymes (Imlay 2013). Gu and Imlay (2013) elegantly showed that superoxide can abstract Fe from mononuclear Fe-containing enzymes in vitro (reversibly), which are then replaced by Zn resulting in a non-functional protein. This then requires re-metallating mononuclear Fe enzymes. SODs are therefore central to mitigating superoxide-induced mismetallation. We hypothesize that SODs play an important role in Southern Ocean phytoplankton in particular, where dissolved Zn levels are high (Vance et al. 2017), and Fe and Mn concentrations can be very low as well (Middag et al. 2011). Mismetallation could therefore strongly influence an organism’s fitness, particularly given low Mn (Imlay 2014). The expression of SOD can also lead to increased levels of H$_2$O$_2$ (Eq. 1; Mittler et al. 2011), which can then have distinct deleterious effects (discussed below). Superoxide also can react directly with H$_2$O$_2$, which produces the hydroxyl radical, but the reaction of superoxide with H$_2$O$_2$ is unlikely under physiological conditions (Haber and Weiss 1932; Wardman and Candela 1996; Imlay 2003).

SODs also have an atypical relationship with temperature, with higher rates of superoxide dismutation under colder temperatures (Perelman et al. 2006), which could increase requirements for trace metal in rapidly warming polar regions. Differential regulation of SODs under various conditions has suggested that superoxide itself is a signaling molecule (Case 2017). So, regulation of SODs may not solely be due to repression of superoxide levels alone, but rather the modulation of superoxide consumption and H$_2$O$_2$ production. Various viruses even encode SODs (e.g., Cao et al. 2002), which may alter the hosts’ regulatory program by interfering with redox signaling. It would be beneficial to empirically quantify the drivers of the SOD expression-fitness landscape. In other words, is superoxide mostly a toxic byproduct of metabolism, or is it used for cell signaling? If phytoplankton SODs are mostly being used to prevent superoxide toxicity, there might be increased metal cofactor requirements in a warming ocean.

**Catalases and catalase-peroxidases**

Catalases (CATs) are ancient H$_2$O$_2$ metabolizing enzymes that dismutate two molecules of H$_2$O$_2$ (which is mostly generated from photosynthesis), with overall reaction stoichiometry (Zamocky et al. 2008; Vlasits et al. 2010; Zámócky et al. 2012; Tehrani and Moosavi-Movahedi 2018):

\[
2H_2O_2 \rightarrow 2H_2O + O_2
\]  

(2)

Monofunctional CATs use heme to catalyze the above reaction. Peroxidase-CATs and Mn-CATs have similar catalytic mechanisms, except peroxidase-CATs can use an external reductant to reduce the active site (Vlasits et al. 2010; Tehrani and Moosavi-Movahedi 2018). For peroxidases, the overall stoichiometry follows (where R denotes a reductant):

\[
H_2O_2 + 2RH \rightarrow H_2O + 2R^+ + \cdot O_2
\]  

(3)

CATs are extremely efficient enzymes that are not saturated by H$_2$O$_2$ within most physiological concentration ranges (Aebi 1984), and therefore do not display typical Michaelis–Menten kinetics (described further in Tehrani and Moosavi-Movahedi 2018). Peroxidase-CATs, however, can rely on an external reductant, and have a lower half saturation coefficient (i.e., a higher affinity; Vlasits et al. 2010). This difference in affinity provides a hypothesis for why bacteria have multiple enzymes that metabolize H$_2$O$_2$ (Mishra and Imlay 2012). CATs are typically inhibited by light, although inhibition can be protected against by chlorophyll (Feierabend and Germany 1986).

How does H$_2$O$_2$ damage cells? H$_2$O$_2$ sluggishly reacts with most biomolecules (Winterbourn and Metodiewa 1999; Halliwell and Gutteridge 2007; Imlay 2013). Its toxicity mainly derives from the interaction with Fe (or other metals with Fenton chemistry like copper), where the highly oxidizing hydroxyl radical is formed via Fenton chemistry. Fenton chemistry refers to the reaction of a reduced form of a metal.
reacting with an oxidant like \( H_2O_2 \), to produce an oxidized metal, a hydroxyl radical, and a hydroxide ion (Wardman and Candeias 1996). No known enzyme “metabolizes” hydroxyl radicals, and these ROS react at diffusion-limited rates. Notably, Mn does not have Fenton chemistry (Cheton and Archibald 1988), and several hypotheses have been put forward suggesting that Mn-containing antioxidants have evolved because they lack the ability to produce hydroxyl radicals via this mechanism (Aguirre and Culotta 2012).

**Ascorbate peroxidases and glutathione peroxidases**

CATs and peroxidase-CATS are complemented by several other \( H_2O_2 \) metabolizing enzymes. Glutathione peroxidases (GPXs) are thiol-based and ascorbate peroxidases (APXs) are heme-based enzymes. These two enzyme groups are tied together through a common set of reductants. Ascorbate (AsA) and GSH are used as reductants (as in Eq. 3). The concentrations of AsA and GSH are critical for the kinetics of these two enzyme groups. For example, insufficient AsA will lead to rapid deactivation of APX (under 0.1 \( \mu M \) AsA; Miyake et al. 1991). Detailed descriptions of these systems have been previously reviewed (Asada 2006). The degree of coupling between GSH-based and AsA-based antioxidant systems is complex, but modeling studies showed that coupling is partially dependent on the activity of a key enzyme, monodehydroascorbate radical reductase (Polle 2001; Tuzet et al. 2019). Overall, there is incredible redundancy between these systems, CATs, and other \( H_2O_2 \) metabolizing systems (Mhamdi et al. 2010; Tuzet et al. 2019).

**Peroxiredoxins**

Peroxiredoxins (PRXs) metabolize \( H_2O_2 \) as above, they also first reduce \( H_2O_2 \) to water, and then are reduced by an external reductant (Karplus 2015; Perkins et al. 2015). This reductant typically comes in the form of reduced thioredoxin (e.g., in *Synechocystis*; Pérez-Pérez et al. 2009). PRXs are unique because they are oxidized at moderate concentrations of \( H_2O_2 \); and this oxidation inactivation has been suggested to mediate barrier-free compartmentalization (Wood et al. 2003; Perkins et al. 2015). PRXs also display chaperone behavior (Jang et al. 2004). There is ample evidence that PRXs modulate \( H_2O_2 \) concentrations to control gene expression in highly localized subcellular regions (Brown et al. 2013; Perkins et al. 2015).

**Cytochrome c peroxidases**

Cytochrome c peroxidases (CCPs) are heme-containing and they convert \( H_2O_2 \) into water using reduced cytochrome. In *Escherichia coli*, CCPs can donate electrons to \( H_2O_2 \) to be used as a terminal electron acceptor in respiration, and likely do not metabolize a large fraction of \( H_2O_2 \) (Khademian and Imlay 2017). Jamers et al. (2006) showed that CCP in *Chlamydomonas reinhardii* is differentially expressed under various copper stressors. Compared to the aforementioned antioxidant systems, the role of CCPs in photosynthetic microbes has received much less attention.

**Nonenzymatic consumers**

Nonenzymatic consumers of ROS are important factors in the defense and modulation of redox status in cells (Noctor 2006). We should not only consider reaction rates between these compounds and ROS directly, but more importantly, the rate constants of regenerating oxidized compounds. Davies and Holt (2018) concluded this exact issue underpins why dietary antioxidants have failed clinical trials for their antioxidative effect—they require a kinetically fast system for regenerating the oxidized compound (also described in Imlay 2013). This same argument may be applied to enzymatic antioxidant systems described above that require a reductant (e.g., APX).

**Ascorbate and Glutathione**

AsA and GSH are both small molecule antioxidants. Yet, they are both key players in reducing antioxidative enzymes, and therefore essential components of different antioxidant systems. Are these two small molecules important in reacting with ROS alone? It seems unlikely, because the first-order reaction rate constants of AsA and GSH are several orders of magnitude lower than those of enzymes which directly metabolize \( H_2O_2 \) or superoxide (Rahantaniaina et al. 2013).

There are many unknowns regarding the in situ role of GSH in particular—even in highly studied systems, the main routes of GSH oxidation are unclear (Rahantaniaina et al. 2013). Several studies have shown intriguing results. For example, GSH can chelate metals like Cu, therefore inhibiting production of hydroxyl radicals via Fenton chemistry (Halliwell and Gutteridge 2007). Perhaps GSH can modulate Fe-induced oxidative stress in this manner, similar to ferritin. GSH is intertwined in multiple antioxidant enzyme systems (GPX, APX, PRXs). This may explain why GSH displays diurnal variations in concentration in phytoplankton (Dupont et al. 2004), with higher concentrations during the day.

**Tocopherols and carotenoids**

Tocopherols and carotenoids protect against singlet oxygen, a unique ROS. This ROS is produced from the transfer of energy from a photosensitized chlorophyll to ground-state triplet dioxygen to form highly reactive singlet dioxygen. This transfer of energy changes the electron configuration of oxygen, which then substantially alters its reactivity (Laing 1989). There are no known enzymes that metabolize singlet oxygen, but tocopherols and carotenoids can protect cells from singlet oxygen through two mechanisms: physical and chemical quenching (Ledford and Niyogi 2005; Krieger-Liszka and Trebst 2006). Physical quenching occurs after the transfer of energy from singlet oxygen to a carotenoid, after which the energy is dissipated as heat. Chemical quenching is simply the reaction of singlet oxygen with either tocopherols or
carotenoids (Ramel et al. 2012). After chemical quenching, the oxidized molecule is typically resynthesized (Ramel et al. 2012).

**Other**

Several other compounds have received some attention as antioxidants. For example, the highly studied marine metabolite DMSP displays antioxidant activity (Sunda et al. 2002). Manganese phosphate can also act as a SOD (albeit with lower catalytic activity; Barnese et al. 2008). Interestingly, cobalamin (a cobalt-containing micronutrient) can also react with superoxide with rates similar to SODs (Suarez-Moreira et al. 2009). As mentioned above, antioxidant activity in vitro does not necessarily equate with activity in vivo, and it is unclear how these various compounds contribute to antioxidant system capacity in marine phytoplankton. However, these three examples may play important roles in sulfur, manganese, and cobalt quotas in photosynthetic microbes.

**Protective biomolecules**

Several other antioxidant biomolecules have evolved that prevent reactions of ROS with target biomolecules, rather than destroying ROS. Protein chaperones, for example, act as protectors of unfolded proteins, which are particularly sensitive to oxidative stress (Dahl et al. 2015; Santra et al. 2018). Ferro-tin, a large multi-unit protein that sequesters Fe, can play an important role in preventing interactions between H₂O₂ and Fe (Marchetti et al. 2009). Polyphosphates, which contribute varying amounts to total cell P (Lin et al. 2016) and have roles in phosphate and energy storage, can also protect proteins from ROS (Dahl et al. 2015). Previous estimates suggest that polyphosphates can comprise of up to 40% of total P (Rhee 1974; Geider and LaRoche 2002). The requirement for polyphosphates as a protective antioxidant may specifically contribute to high variation in P quotas (Galbraith et al. 2013).

**Antioxidant influences on cellular stoichiometry**

How do antioxidants impact macronutrient stoichiometry? If oxidative stress leads to increases in total protein per unit of cell biomass (via increased enzymatic antioxidants), this would increase N per cell, as protein is a large proportion of cellular N (therefore increasing N : C in cells, Geider and LaRoche 2002). However, it is also possible that only the proportion of protein in antioxidants is shifted, which would then lead to no change in C : N : P ratios, but could influence metal or sulfur stoichiometry (depending on the composition and function of the antioxidant). This uncertainty is shown in Table 1, where we hypothesize how oxidative stress would influence stoichiometric ratios with carbon (only directions of influence are considered here). For other macronutrients, non-protein antioxidants may influence cell stoichiometry. For example, beta-carotenes might alter C per cell, polyphosphate might alter P per cell, and GSH might alter S per cell (Table 1).

Overall, antioxidants would probably have the largest impacts on micronutrient stoichiometry, because previous work has suggested they are a large fraction of the total quota (e.g., MnSOD and NiSOD impacts on Mn and Ni quotas; Wolfe-Simon et al. 2006; Twining and Baines 2013). The focus from this point on is specifically looking at antioxidant impacts on micronutrient quotas.

We expect that increased oxidative stress would result in increased expression of antioxidants (Table 1). For example, increased oxidative stress resulting from excess light would produce a saturated electron transport chain and increased superoxide production. This superoxide increase would then be met with increased amounts of NiSOD. In this case, oxidative stress would increase the cellular Ni quota. Using a similar logic, we predicted how increased oxidative stress would change total cellular stoichiometry of N, P, S, Fe, Mn, Cu, Zn, and Ni (Table 1). An increase in superoxide production could be met with no change in antioxidant production, but we posit that this would eventually lead to deleterious effects from excess superoxide. The responses of antioxidants to oxidative stress are complex, and here we only aim to make first-order predictions.

For each antioxidant system, we estimated macronutrient stoichiometry from amino acid composition with a large dataset of photosynthetic phytoplankton metagenome assembled genomes (Delmont et al. 2021). Protein sequences (n = 2767) were subsetted using their Enzyme Commission (EC) numbers (Table 1), and then stoichiometric composition was empirically estimated, summarized using median values (Table 1, Fig. 1; Goloborodko et al. 2013). Notably, there were no large differences in H : N, O : N, C : N, or even S : N ratios across antioxidants. Therefore, the major connections between antioxidant system use and cellular stoichiometry would arise from antioxidants that have unique cofactors. For example, GPXs and CATs were quite similar in their macromolecular stoichiometry, but differ because CAT contains Fe (as heme). This analysis also showed that thiol-based antioxidant systems were not enriched in sulfur compared to non-thiol-based antioxidant systems (Fig. 1; Table 1).

**Quantifying antioxidant contributions to cellular stoichiometry**

Methods for quantifying antioxidant contributions to cellular stoichiometry

How much variation in micronutrient stoichiometry is due to antioxidants, or more specifically to metal containing antioxidant enzymes? In this section, we use several data sources and some simplifying assumptions to examine the range of stoichiometric contributions antioxidants could have, with a focus on diatoms. Note that the range estimates we produce are not necessarily realized, as antioxidant expression could contribute to either the variation or consistency in micronutrient stoichiometry.
Table 1. Summary table of antioxidant systems in phytoplankton and their stoichiometric composition. Median stoichiometric composition of proteins is given with respect to nitrogen. Proteins were subsetted from photosynthetic phytoplankton metagenome assembled genomes (Delmont et al. 2021), and then categorized using their Enzyme Commission (EC) numbers. Stoichiometry of macronutrients was determined from the amino acid sequence using pyteomics (Goloborodko et al. 2013). For micronutrient stoichiometry, we examined literature sources and the Protein Data Bank for structural information on ligands. On the right side of the table, we indicate the direction that various antioxidant systems might influence cellular stoichiometry, by asking how increased production of a given antioxidant would change stoichiometric composition (see the text for rationale).

| Antioxidant category | Antioxidant | Stoichiometry | Median monomer length (SD) | Multimer | EC number | N : C | P : C | S : C | Fe : C | (Zn, Cu, Mn, Ni) : C |
|----------------------|-------------|---------------|----------------------------|----------|-----------|-------|-------|-------|-------|-------------------|
| Enzymatic consumers  | Mn superoxide dismutase | C₆₄₋₆₅ : H₅₀₋₆₇ : O₁₀₋₁₉ : N₁ : S₀₋₁₂ : Mn₀₋₁₆ | 235.5 (148) | Homodimer or homotetramer, each with 1 Mn atom | 1.15.1.1 | 1 - - - 1 |
|                      | Fe superoxide dismutase | C₆₄₋₆₅ : H₅₀₋₆₇ : O₁₀₋₁₉ : N₁ : S₀₋₁₂ : Fe₀₋₁₂ | 235.5 (148) | Homodimer or homotetramer, each with 1 Fe atom | 1.15.1.1 | 1 - - 1 - |
|                      | CuZn superoxide dismutase | C₆₄₋₆₅ : H₅₀₋₆₇ : O₁₀₋₁₉ : N₁ : S₀₋₁₂ : Cu₀₋₂ : Zn₀₋₁₂ | 235.5 (148) | Homodimer, each with 1 Zn and 1 Cu atom | 1.15.1.1 | 1 - - 1 |
|                      | Ni superoxide dismutase | C₆₄₋₆₅ : H₅₀₋₆₇ : O₁₀₋₁₉ : N₁ : S₀₋₁₂ : Ni₀₋₁₂ | 235.5 (148) | Homohexamer, each with 1 Ni atom | 1.15.1.1 | 1 - - - 1 |
|                      | Ascorbate peroxidase | C₅₈ : H₅₀ : O₁₀ : N₁ : S₀₋₁₂ : Fe₀₋₂ | 336 (134) | Monomer, with 1 Fe atom (in a heme group) | 1.11.1.11 | 1 - - - 1 |
|                      | Glutathione peroxidase | C₇₂ : H₇₅ : O₁₁ : N₁ : S₀₋₁₂ | 197 (163) | Monomer or dimer | 1.11.1.9 | 1 - - - - |
|                      | Catalase | C₅₁ : H₆₁ : O₁₀ : S₀₋₁₂ : Fe₀₋₂ | 367 (114) | Homotetramer, each with 1 Fe atom (in a heme group) | 1.11.1.6 | 1 - - - 1 |
|                      | Peroxiredoxin | C₃₋₄ : H₅₋₆ : O₁₀ : N₁ : S₀₋₁₂ : Fe₀₋₂ | 221 (133) | Monomer to homo-12-mer | 1.11.1.15 | 1 - - - 1 |
|                      | Cytochrome c peroxidase | C₄₋₄ : H₅₋₆ : O₁₀ : N₁ : S₀₋₁₂ : Fe₀₋₂ | 459.5 (833) | Dimer, 2 Fe atoms per monomer (in a heme group) | 1.11.1.5 | 1 - - - 1 |
| Nonenzymatic consumers | Manganese phosphate | Mn₀ : H₁ : P : O₄ | NA | NA | NA | NA | - - - - - |
|                      | DMSP | C₅ : H₁₀ : O₂ : S₁ | NA | NA | NA | NA | - - 1 - - |
|                      | Glutathione | C₁₀₋₁₂ : N₁ : O₆ : S₁ | NA | NA | NA | NA | - - - 1 - |
|                      | Ascorbate | C₆ : H₇ : O₆ | NA | NA | NA | NA | 1 - - - 1 |
|                      | Tocopherols | C₂₉ : H₅₀ : O₁₂ | NA | NA | NA | NA | 1 - - - 1 |
|                      | Carotenoids | C₄₀ : H₇₆ | NA | NA | NA | NA | 1 - - - 1 |
| Protective biomolecules | Chaperones | NA | NA | NA | NA | 1 - - - - |
|                      | Polyphosphate | P₁ : O₃ | NA | NA | NA | NA | - 1 - - - |
|                      | Ferritin | C₅₋₇ : H₅₋₂₀ : O₁₇ : N₁ : S₀₋₁₂ : Fe₀₋₁₂ : O₈₄ | 230 (NA) | Homo-24-mer | 1.16.3.1 | 1 - - - - |

*aSheng et al. (2014).
bProtein Data Bank structure 1V0H.
cNavrot et al. (2013).
dBorges et al. (2014).
ePoole and Nelson (2016).
fProtein Data Bank structure 2VHD.
gFormula for alpha-tocopherol is given.
hFormula for beta-carotene is given.
iMarchetti et al. (2009); we did not use this EC number for assessing protein stoichiometry, but rather this *Pseudo-nitzschia multiseries* specific protein.
To illustrate these calculations (see Fig. 2), consider the example: how much does FeSOD expression contribute to Fe : C variation? Fe : C ratios are particularly variable (Sunda and Huntsman 1995; Twining et al. 2020), but it is unclear what underpins this variability. FeSOD is a dimeric protein, with each monomer containing one Fe cofactor. This is divided by the number of amino acids per enzyme molecule, converting this to Fe per amino acid. These two parameters

\[
\frac{\text{Fe per antioxidant}}{\text{C}} = \frac{\text{Fe atoms}}{\text{Amino Acids}} \times \frac{\text{Antioxidant Protein}}{\text{Total Protein}} \times \frac{\text{Antioxidant Protein}}{\text{Total Protein}} \times \frac{\text{Nitrogen Atoms}}{\text{Amino Acid}} \times \frac{\text{N}_{\text{protein}}}{\text{N}_{\text{total}}} \times \frac{\text{Nitrogen}}{\text{Carbon}}
\]

where \(N_{\text{protein}} \sim U(0.5, 0.85)\) and \(\text{Nitrogen} \sim N(\mu = 16, \sigma^2 = 5^2)\).
(metal cofactor atoms per antioxidant molecule and amino acids per antioxidant molecule) are well constrained using genomic data and data on protein cofactors. We then multiply this value by the proportion of the proteome that is made up by FeSOD (details given below), now with units of Fe (from FeSOD) per total protein. Converting protein to N, we divide by the average number of N atoms per amino acid, and then multiply this value by the ratio of N in protein to N total. We incorporate variation in the ratio of N in protein : total N by sampling from a uniform distribution bounded by 0.5 and 0.85 (Geider and LaRoche 2002). Lastly, we convert this ratio to Fe : C by multiplying by the Redfield ratio (16N : 106C Redfield 1958), but variation in N : C is incorporated by sampling from a truncated normal distribution (mean = 16, SD = 5, lower bound at 0 and no upper bound), and then adjusting the numerator, assuming a constant denominator.

One key parameter is the proportion of the proteome attributable to a given antioxidant protein. We used two previously published pennate diatom proteomes (Fragilariopsis cylindrus, Phaeodactylum tricornutum; Kennedy et al. 2019; Müller et al. 2020), and reanalyzed their data to obtain a range of proteomic proportion estimates for each micronutrient-containing enzymatic antioxidant. In brief, we converted mass spectrometry raw files with ThermoRawFileParser (Hulstaert et al. 2020), appended a database of common contaminants (Global Proteome Machine Organization common Repository of Adventitious Proteins), searched mass spectra against a database of proteins (using published genomes, with MSGF+ and OpenMS; Mock et al. 2017; Bowler et al. 2008; Kim et al. 2014; Röst et al. 2016), and then quantified proteomic mass fraction by summing quantified peptides (quantified at the MS1 level with FeatureFinderIdentification; Weisser and Choudhary 2017; Weisser et al. 2013). We then obtained the mean expression value across taxa to give a representative proteomic proportion for these pennate diatoms. One disadvantage of averaging over different diatoms is that their repertoire of antioxidants are slightly different. This becomes particularly important for the MnFeSOD family, because our predictions are different if the protein considered contains an Mn or an Fe cofactor. To address this, we show all calculations assuming that the observed MnFeSOD expression value is from a MnSOD or from a FeSOD. Code for all analyses is provided at: https://github.com/bertrand-lab/antiox-review.

Ideally, we would have antioxidant proteomic proportions observed across all realistic environmental conditions, which would give the exact contribution to variation in elemental stoichiometry. These data are currently unavailable (if they did exist, these calculations would not be necessary!). However, we can estimate how most proteins vary using the distribution of fold changes for proteins across different environmental conditions. In other words, how much might antioxidant expression change across all environmental conditions? We compared protein expression data from high and low Fe treatments in the coastal diatom Thalassiosira pseudonana (Nunn et al. 2013), and then examined the distribution of fold-changes. This distribution showed that most proteins (~75%) change between 2- and 20-fold across different environmental conditions (from 0.05 to 2 times). In using this distribution of fold changes, we make two key assumptions: (1) high and low Fe treatments with T. pseudonana represent typical variation in protein fold changes across taxa and conditions; and (2) antioxidants can be considered as “average” proteins following this distribution. To assess the former assumption, we reanalyzed the fold-change distribution from an E. coli proteomic experiment which examined 22 different growth conditions (ranging from pH, to media, to growth phase; Schmidt et al. 2016). By comparing every condition with every other condition to calculate fold changes, we intriguingly found an almost identical distribution of fold changes. Using protein concentrations inferred from protein synthesis rates in E. coli under three conditions also revealed a similar distribution (Li et al. 2014). Without specific data on antioxidant expression in phytoplankton across environmental gradients, the second assumption is difficult to rigorously assess. Of the antioxidants, Nunn et al. (2013) observed varying across Fe concentrations, the magnitude of variation is uncommon. Most Fe : C ratios vary greater than 100 (11–33.3%, based on the effective Ocean. There are two main conclusions from this analysis: antioxidant contributions to Fe : C may explain some variation (11–33.3%, based on the effective range of 30 Fe : C). But, antioxidant systems are unlikely to

**Antioxidants can contribute important variation to micronutrient : C**

**Fe-containing antioxidants**

Overall, we estimated that Fe-containing antioxidant expression can account for variation in Fe : C of between 3.3 and 10 (µmol : mol; depending on whether FeSOD or MnSOD is present; Fig. 2, 2.5–97.5 quantile range). Median values of antioxidant contribution to Fe : C ratios were 0.7–2.2 (again dependent on MnSOD vs. FeSOD; Fig. 3). Fe : C can vary even greater than 100 µmol : mol (Twining et al. 2020), but this magnitude of variation is uncommon. Most Fe : C ratios vary around 30 Fe : C (µmol : mol; Twining et al. 2020; Strzepek et al. 2011, 2012; Sunda and Huntsman 1995); however, see Twining et al. (2020) for estimated variation across a wide gradient in the South Pacific Ocean. There are two main conclusions from this analysis: antioxidant contributions to Fe : C may explain some variation (11–33.3%, based on the effective range of 30 Fe : C). But, antioxidant systems are unlikely to
explain the enormous variation sometimes observed (e.g., the >100 μmol : mol changes in Fe : C for some taxonomic groups; Twining et al. 2020).

Is this calculated range of antioxidant Fe : C important? In many areas of the ocean, Fe is a limiting resource, and it would seem reasonable to assume that 11–33.3% variation in the cellular Fe : C ratio would significantly affect growth. In low-Fe conditions, Fe-containing antioxidant expression would play a larger role in influencing Fe : C compared to high-Fe conditions. For example, diatoms in low Fe conditions can have Fe : C below 10 μmol : mol, and therefore Fe-containing antioxidants would impart a very significant stoichiometric signal. However, remember that antioxidant systems display extreme redundancy. All of these Fe-containing antioxidants have non-Fe counterparts: for example, MnSOD, CuZnSOD, or NiSOD instead of FeSOD, or GPX instead of APX. However, it is unclear how interchangeable these enzymes are, particularly given some of them are only expressed in certain subcellular compartments (see “Antioxidant system redundancy and the implications for cellular elemental stoichiometry” section). Perhaps organisms retain these different antioxidants to respond to environmental conditions, such that these non-Fe counterparts would be used under low Fe conditions.

Antioxidants are important in mediating the negative effects of a high Fe quota, which would be particularly relevant with dramatic changes in Fe : C (Twining et al. 2020). This magnitude of change is likely due to high uptake of Fe, sometimes referred to as “luxury” uptake (Twining et al. 2020). However, high uptake of Fe comes with a cost: free Fe can react with H₂O₂ to produce hydroxyl radicals (as discussed above). So high amounts of Fe must be met with either a system for metabolizing H₂O₂ to limit this reaction, or storing Fe to prevent contact with H₂O₂.

Fig 3. The potential contribution of antioxidant expression to Fe : C ratios, showing the kernel density estimates. (a) CCP; (b) CAT; (c) MnFeSOD; and (d) APX are all shown individually. (e) The distribution of each Fe-containing antioxidant is summed. Note that the summed distribution (panel e) includes two distributions are shown: (1) assuming the MnFeSOD is FeSOD, and (2) assuming it is MnSOD. Similarly, the x-axis label for panel c is either Mn : C or Fe : C, depending on if this enzyme is MnSOD or FeSOD. Calculation underpinning the Monte Carlo estimates is shown in Fig. 2.
sources (or both). In this case, antioxidant systems indirectly influence Fe stoichiometry.

**Ni-, Cu-, Zn-, and Mn-containing antioxidants**

Other micronutrients (Ni, Cu, Zn, and Mn) play important roles in global biogeochemistry (Twining et al. 2012; Richon and Tagliabue 2019), and there is increasing evidence that some limit primary production (e.g., Mn; Buma et al. 1991; Wu et al. 2019; Browning et al. 2021). Comparing across cultures and field observations, Mn and Zn displayed similar amounts of variation across conditions compared to Fe (Twining et al. 2004). We found that MnSOD contributions are unlikely to exceed 8.7 Mn : C (μmol : mol; 2.5–97.5 quantile range). Considering a range of Mn : C observations (3.4–46.7 μmol : mol for diatoms; Twining et al. 2004), the variation in MnSOD expression that we calculated could account for ~20% in Mn : C variation. Despite using very different approaches, our calculations complemented Wolfe-Simon et al. (2006) who found that chloroplast MnSOD accounted for 10–20% of cellular Mn. As with Fe, the contribution of MnSOD to Mn : C ratios would be even more important under low Mn. Consider the investment of Mn in MnSOD vs. PSII (two dominant components of the Mn cellular quota in diatoms; Peers and Price 2004; Wolfe-Simon et al. 2006). Under low Mn, we hypothesize that this SOD can be replaced by another SOD with a different metal cofactor, as there is no replacement for the Mn in PSII (see “Antioxidant system redundancy and the implications for cellular elemental stoichiometry” section).

Moving to the other micronutrients we considered, NiSOD is likely to play a dominant role in Ni cell stoichiometry in diatoms (Fig. 4; Twining et al. 2012). Twining et al. (2004) observed that the maximum variation in Ni : C within taxa was 1.6 Ni : C (μmol : mol), and we can attribute up to 1.37 Ni : C (μmol : mol; 2.5–97.5 quantile range) to NiSOD. It is interesting to note that some SODs are membrane associated (Ogawa et al. 1995; Regelsberger et al. 2002), so perhaps the high Ni content of diatom frustules is related to frustule-associated NiSOD (Twining et al. 2012). Another important Ni-dependent metalloenzyme is urease (Boer et al. 2014). As with the MnSOD and PSII pair, we hypothesize that under low Ni conditions, NiSOD could be replaced with a different SOD but urease would not be.

**Antioxidant system redundancy and the implications for cellular elemental stoichiometry**

Antioxidant systems display a lot of functional redundancy; what are the implications for cellular elemental stoichiometry? If various antioxidants are interchangeable, then environmental scarcity of an element would cause the production of another similar antioxidant that does not contain this scarce element. For example, under low Fe, as occurs in much of the ocean, MnSOD might replace FeSOD, therefore implicating both Fe and Mn cellular stoichiometry. This prediction requires two assumptions: (1) “nutritional coherence” and (2) functional similarity. We define “nutritional coherence” as a characteristic of protein expression, such that environmental availability of an element would negatively correlate with protein expression, if a given protein uses this element as a cofactor. Are these reasonable assumptions? Page et al. (2012) provide evidence that FeSOD behaves in a nutritionally incoherent fashion. They found that FeSOD expression in *Chlamydomonas reinhardtii* increased under low Fe rather than decreased. Functional similarity is somewhat easier to assess. For example, all SODs catalyze the same reaction. However, even SOD isoforms with the same cofactors display unique expression patterns (Gallie and Chen 2019; Najmuldeen et al. 2019), suggesting that even though functionally similar proteins can mediate the same reaction, that does not mean they actually do in vivo. Furthermore, this suggests that
protein expression patterns, at least for SODs, are entrenched (Shah et al. 2015; Lalanne et al. 2021) and perhaps less interchangeable than would be anticipated. Overall, the degree to which different antioxidants are functionally interchangeable in phytoplankton is a key unknown. We require empirical observations of antioxidant protein expression to first determine if a given antioxidant behaves in a “nutritionally coherent” way. Then, we would be able to assess if functionally similar proteins are interchanged under conditions of elemental scarcity. These types of observations would (1) help determine the relative costs of Mn in PSII vs. MnSOD, or Ni in urease vs. NiSOD, and so on, and (2) determine the elemental stoichiometric consequences of antioxidant system redundancy.

Conclusions and next steps

We reviewed the central antioxidant systems present in phytoplankton, and used several approaches to identify and quantify how antioxidant system use may contribute to phytoplankton cell stoichiometry. Throughout, we have discussed various ways that antioxidant systems could influence cell stoichiometry, and concluded that they most likely have the largest impacts on micronutrient quotas.

Our original goal was to outline how antioxidant systems contribute to both variation and consistency in elemental stoichiometry. Using a series of simulations, we quantified how antioxidant systems may contribute to variation in trace metal quotas. A critical next step is quantifying how these systems are behaving in situ, to then determine the exact contribution under various environmental conditions. We were unable to assess how antioxidants influence consistency in elemental stoichiometry, and large-scale proteomic characterization of phytoplankton across diverse environmental conditions would achieve this goal. In terms of macronutrient stoichiometry, it is less likely that antioxidant systems play a dominant role, but a notable exception is polyphosphates for P quotas.

This leads us to synthesize two major unknowns and next steps for studying antioxidant systems in phytoplankton:

1. Is differential production of antioxidants a sign of oxidative stress in phytoplankton leading to damaged biomolecules, or of redox-based regulatory mechanisms? By extension, when cells are challenged with H2O2, for example, is this mainly inducing irreversible damage or interfering with regulatory networks? Quantifying what underpins protein expression-fitness landscapes is challenging, but promising new tools and techniques may suit these questions (Parker et al. 2020).

2. What are the environmental controls on specific antioxidants? There are many antioxidant systems in prokaryotes and eukaryotes (Mishra and Imlay 2012). Quantifying how these superoxide- and hydrogen-peroxide metabolizing enzymes are produced in tandem may provide more insight into both the selective pressure on antioxidant production (regulatory or metabolic), as well as their contributions to stoichiometry in situ. Also, antioxidants sometimes behave counterintuitively (Page et al. 2012), so direct measurements of antioxidants under various environmental conditions is necessary.

Oxidative stress has shaped many facets of life. Describing and quantifying how the mediators of oxidative stress—antioxidants—affect cellular stoichiometry is important for connecting cellular processes to ocean biogeochemistry.

Data Availability Statement

We provide code for all analyses at https://github.com/bertrand-lab/antiox-review. All data used here are previously published.

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