BEYOND THE IRON AGE: THE ECOLOGICAL RELEVANCE OF BIOACTIVE TRACE METALS OTHER THAN Fe AND ORGANIC GROWTH FACTORS IN AQUATIC SYSTEMS

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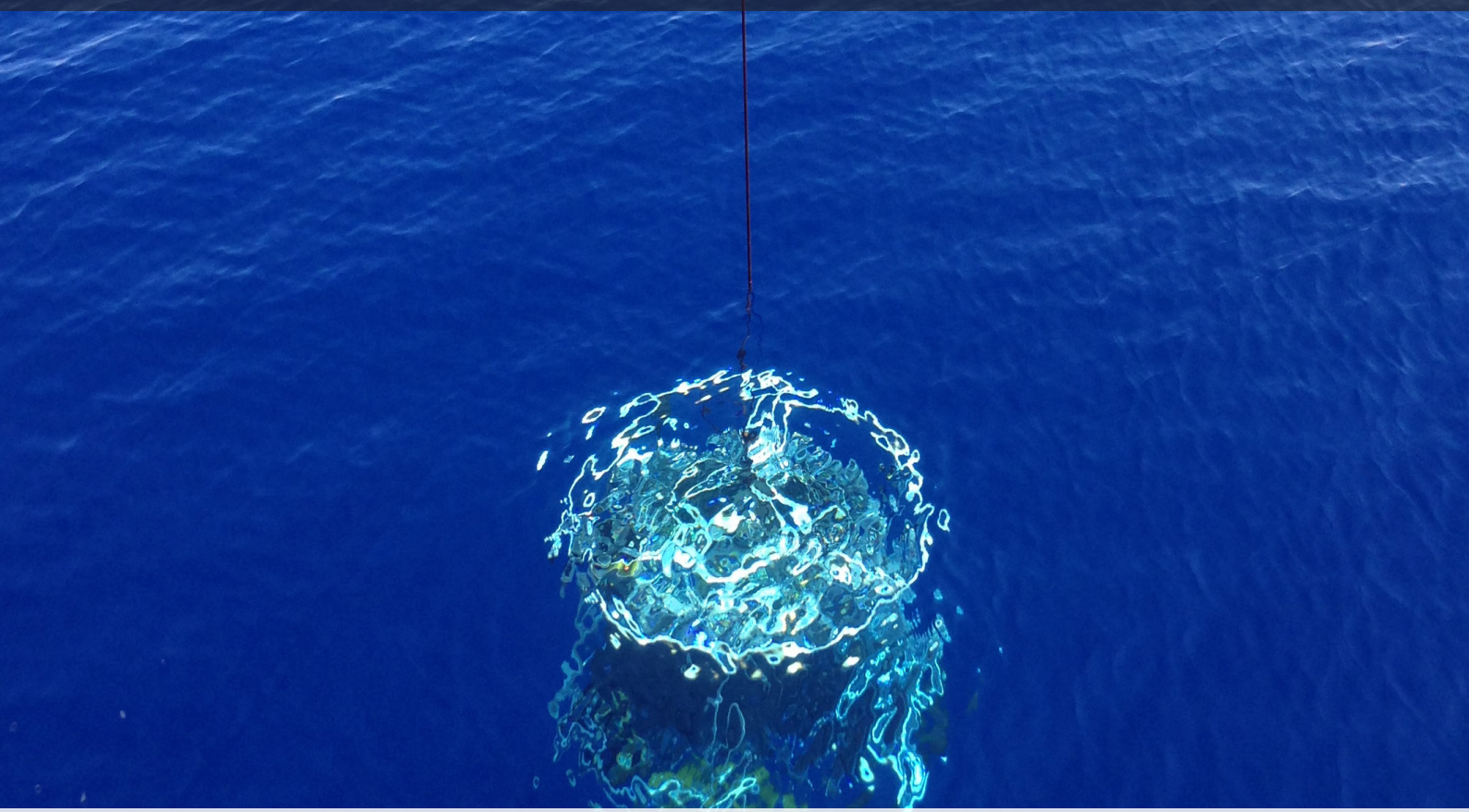

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BEYOND THE IRON AGE: THE ECOLOGICAL RELEVANCE OF BIOACTIVE TRACE METALS OTHER THAN Fe AND ORGANIC GROWTH FACTORS IN AQUATIC SYSTEMS

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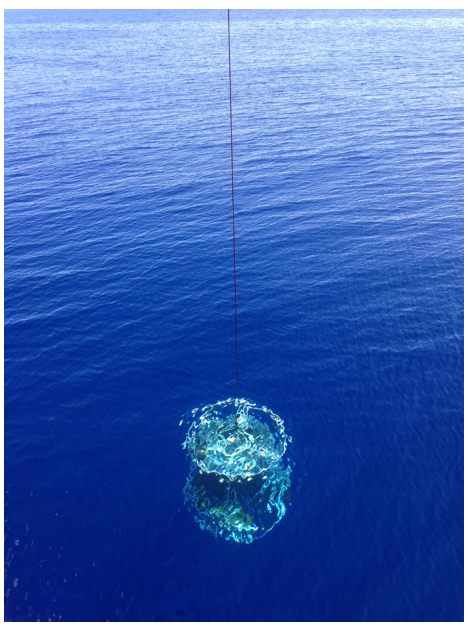

Seawater sample collection in the Mediterranean Sea using a Niskin bottle rosette. (Source: L. Gómez-Consarnau)

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Beyond the iron age: the ecological relevance of non-ferrous bioactive trace metals and organic growth factors in aquatic systems

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The catalysis of chemical reactions is one of the most central processes in biology, as most reactions in living organisms would occur too slowly to play any role in their metabolism. For example, the rate of biologically relevant reactions would take hundreds to millions of years in the absence of a catalyst (Stockbridge et al., 2010). Enzymes are the catalysts of nature, and they can increase the rate of a chemical reaction by as much as 10^{20} times compared to an uncatalyzed reaction in water (Lad et al., 2003). Molecular evidence suggests that enzymatic catalysis evolved very early in life history, even prior to the Archaean genetic expansion (David and Alm, 2010). While some protein functional groups can act as catalysts, they represent a very limited set of reactions as compared to the multitude of enzyme-catalyzed reactions (Price and Stevens, 1999; Bugg, 2012). Enzymes utilize a variety of non-protein molecules or metal ions to increase their versatility in catalytic capacity. Coenzymes and cofactors are the non-protein components of the enzymes that help catalyze the reactions. Coenzymes are typically vitamin B derivatives while cofactors are molecules or metal ions that are covalently bound to the enzyme (Broderick, 2001).

The multiple oxidation states of some metals increase the functionality of the enzymes. Most of the first row transition metals as well as Mo, W, and Mg are known to function as cofactors in enzymatic catalysis (da Silva and Williams, 2001). Fe is the most abundant transition element in the Earth's crust and, although it is very scarce in the ocean, this element was very abundant at the onset of life (Anbar, 2008). Therefore, it is not surprising that Fe is a major cofactor in some of the most central processes in different biological systems (da Silva and Williams, 2001). Starting with the pioneering work of John Martin and collaborators in the early 1990s (Iron limitation?, 2000; Martin et al., 1991), the so-called *iron hypothesis* has produced major breakthroughs in our understanding of the fundamental role it plays in the marine carbon cycle (Smetacek et al., 2012; Thiele et al., 2012). However, biological activity is not entirely sustained by Fe alone as other elements are used as cofactors in relevant metabolic reactions such as Mo, V, and Ni for N and H_2 activation respectively, and Cu and Zn in superoxide dismutase (da Silva and Williams, 2001).

B-vitamins are small organic molecules associated with a large number of biologically important enzymes across all domains of life (Sañudo-Wilhelmy et al., 2014). Decades ago, Carlucci, Droop, Provasoli and others showed that the availability of different B vitamins influenced phytoplankton dynamics in the ocean (Droop, 1957; Carlucci and Silbernagel, 1969; Provasoli and Carlucci, 1974; Swift, 1980). Those initial studies also showed that many marine algae require vitamins B_1(thiamin), B_7 (biotin), and B_{12} (cobalamin) as growth factors (Provasoli and Carlucci, 1974). Those results have recently been validated as field amendments

of different B-vitamins increase phytoplankton growth in many regions of the ocean (Bertrand and Allen, 2012), suggesting that phytoplankton communities in large areas are indeed limited by the availability of these organic metabolites. The initial notion that B-vitamin auxotrophy (i.e., B-vitamin requirement) only applies to phytoplankton species has been reevaluated as it is now well accepted that some bacterial taxa are also auxotrophic (Giovannoni et al., 2005; Croft et al., 2006; Carini et al., 2014; Sañudo-Wilhelmy et al., 2014). Therefore, both vitamin-producing and -consuming bacteria and algae seem to coexist in the ocean. However, more studies are needed to understand the ecological role of the B-vitamins in the marine environment.

A total of 10 manuscripts are included in this e-book being published in Frontiers in Microbiology; 7 are original research (3 on vitamins and 4 on trace metals), 2 reviews and 1 methods article that address some aspect of bioactive trace elements other than Fe, as well as B-vitamins in aquatic environments. The two review articles published focused on the transition element Mo. Glass et al. (2012) reviewed the current state of knowledge regarding Mo limitation in aquatic systems (Glass et al., 2012). One of their major conclusions is that Mo availability seems to influence the N cycle in some freshwater and soil environments, although the authors also concluded that the impact of this element in open ocean waters have not been thoroughly studied. The mini-review by Wang (2012) seeks to elucidate a connection between the evolution of different Mo-requiring enzymes with historical changes in the redox chemistry of Mo in the ocean (Wang, 2012). Those two reviews are complemented by the work of Romero et al. (2013) showing that, in some lakes with different trophic status, field amendments of a specific chemical form of Mo enhanced nitrogen fixation and biomass concentrations (Romero et al., 2013). The biological role of Mo and its geochemical analog, V, in open ocean waters was evaluated in the article of Klein et al. (2013). They found statistical correlations between intracellular levels of Mo measured in natural phytoplankton communities and dimethyl sulfide (DMS) concentrations as well as between intracellular V concentrations and chlorophyll a in the North East Atlantic. While the authors stated that the Mo-DMS relationship was not unexpected as the synthesis of DMS is catalyzed by the Mo-containing enzyme dimethyl sulfoxide reductase, they did not put forward an explanation for the biological role of V in their study. Indeed, V availability could enhance chlorophyll-a biosynthesis by increasing the synthesis of the chlorophyll-precursor, 5-aminolevulinic acid, via the C5 pathway as has previously been observed (Meisch and Bielig, 1975). However, further studies are needed to test the V-chlorophyll connection. The biological importance of another under-studied transition element, Ni, is explored in the article of Ho et al. (2013) who studied the effect of light intensity and Ni availability on *Trichodesmium* growth. The authors concluded that high intracellular levels of Ni in the diazotroph measured under illumination were probably associated with the activity of the Ni-containing superoxide dismutase enzyme that catalyzes the disproportionation of the superoxide radical formed during photosynthesis. The article by Mackey et al. (2012) explores how the atmospheric deposition of different metals could selectively affect phytoplankton community dynamics in coastal and open ocean waters of the Sargasso Sea. Their work showed that the biological response to the aerosol-derived transition trace metal additions was spatially dependent, as the greater biological drawdown of dissolved Co, Mn, and Ni occurred in the open ocean. Therefore, although aerosol metal additions did not cause a shift in the phytoplankton communities, the growth responses were different across ocean regimes. Mackey et al. (2012) hypothesized that the varying biological response was dependent on the metal-nutritional status of the resident phytoplankton prior to the aerosols input, although establishing background metal concentrations prior to addition experiments has faced analytical difficulties due to the complexity of metal analyses at the levels found in open ocean waters. The article of Durand et al. (2012) is a step-forward in the direction to solve that problem. They developed a new microplate-reader method for the rapid analysis of Cu in natural waters that requires less than 1 milliliter of sample and can process about 100 samples per hour. Although Durand et al. (2012) recognized that the analytical method has to be optimized prior to use in open ocean waters, they provide a series of recommendations that could increase the usefulness of their technique. Three articles published in this ebook concentrated on B-vitamins in different marine environments. The articles of Barada et al. (2013) and Bonnet et al. (2013) established spatial gradients in the Mediterranean Sea and in the Amazon River plume respectively. These two studies significantly increased the geographical database of ambient B-vitamin concentrations in the ocean. As observed in other regions (Sañudo-Wilhelmy et al., 2012), those two studies also showed that large areas in Mediterranean and in the Western Tropical North Atlantic are devoid of vitamins. However, Barada et al. (2013) and Bonnet et al. (2013) found significant correlations between different vitamin levels and chlorophyll a concentrations as well as with C and N fixation respectively at some locations, suggesting a potential role of those coenzymes on ecosystem dynamics. The effect of vitamins B_1 and B_{12} on coastal ecosystem dynamics was the main objective of the article of Koch et al. (2012). The authors established the effect of vitamin additions on the planktonic community composition, carbon fixation, and B-vitamin assimilation in two costal sites off Long Island, New York with different trophic characteristics. The reported results were counterintuitive, as dissolved vitamin concentrations and uptake rates were higher in the most eutrophic environment. Koch et al. (2012) concluded that the major B-vitamin consumers in their study sites were heterotrophic bacteria, consistent with genomic results indicating that some heterotrophs are B-vitamin auxotrophs (Sañudo-Wilhelmy et al., 2014). In summary the articles published in this ebook represent a significant advance in our understanding of the roles that trace metals other than Fe and B-vitamins play in the marine environment.

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Molybdenum limitation of microbial nitrogen assimilation in aquatic ecosystems and pure cultures

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Molybdenum (Mo) is an essential micronutrient for biological assimilation of nitrogen gas and nitrate because it is present in the cofactors of nitrogenase and nitrate reductase enzymes. Although Mo is the most abundant transition metal in seawater (107 nM), it is present in low concentrations in most freshwaters, typically <20 nM. In 1960, it was discovered that primary productivity was limited by Mo scarcity (2–4 nM) in Castle Lake, a small, meso-oligotrophic lake in northern California. Follow up studies demonstrated that Mo also limited primary productivity in lakes in New Zealand, Alaska, and the Sierra Nevada. Research in the 1970s and 1980s showed that Mo limited primary productivity and nitrate uptake in Castle Lake only during periods of the growing season when nitrate concentrations were relatively high because ammonium assimilation does not require Mo. In the years since, research has shifted to investigate whether Mo limitation also occurs in marine and soil environments. Here we review studies of Mo limitation of nitrogen assimilation in natural microbial communities and pure cultures. We also summarize new data showing that the simultaneous addition of Mo and nitrate causes increased activity of proteins involved in nitrogen assimilation in the hypolimnion of Castle Lake when ammonium is scarce. Furthermore, we suggest that meter-scale Mo and oxygen depth profiles from Castle Lake are consistent with the hypothesis that nitrogen-fixing cyanobacteria in freshwater periphyton communities have higher Mo requirements than other microbial communities. Finally, we present topics for future research related to Mo bioavailability through time and with changing oxidation state.

Keywords: molybdenum, nitrate reductase, nitrogen fixation, limnology, trace elements, microbes, nutrient limitation, enzyme activity

INTRODUCTION

Molybdenum (Mo) is an essential micronutrient in all three domains of life. Mo is particularly important for microbial nitrogen (N) assimilation due to its presence in nitrogenase, the enzyme that performs N_2 fixation and in nitrate reductase, the enzyme that performs the first step in nitrate (NO_3^-) assimilation, reduction of NO_3^- to nitrite (NO_2^-). For other biological functions of Mo, see Sigel and Sigel (2002). When ammonium (NH_4^+) is present over a certain threshold, it is either preferred over NO_3^- or inhibits NO_3^- uptake (Dortch, 1990), and Mo requirements decrease because NH_4^+ assimilation does not require Mo. While Mo is the most abundant transition metal in seawater (107 nM; Collier, 1985), Mo concentrations are low in most freshwaters (<20 nM), which raises the question of whether N assimilation in low-Mo freshwater ecosystems is limited by Mo availability when NH_4^+ concentrations are scarce enough that organisms must rely on N_2 and NO_3^- for their N sources.

In this paper, we review both laboratory and field experiments in aquatic (and briefly, soil) environments that have tested the effect of varying Mo concentration on N assimilation via N_2 fixation and NO_3^- reduction. This research dates back to the first half of the twentieth century, when studies with pure cultures showed that removal of Mo from growth media induced symptoms of N-limitation in N_2-fixing bacteria (Bortels, 1930, 1940) and NO_3^--assimilating fungi (Steinberg, 1937). Since then, it has been shown that N_2 fixation requires more Mo than NO_3^- assimilation, scarce Mo limits N assimilation in a wide range of natural environments, and Mo bioavailability may decrease with increasing atmospheric CO_2, leading to suppressed terrestrial N_2 fixation (Hungate et al., 2004). We focus particular attention on previous and current research of Mo limitation of N assimilation at Castle Lake, CA, the only aquatic habitat in the world that has been the subject of multiple decades of Mo limitation research. We summarize new results on the effect of Mo additions on total protein abundance and activity of enzymes involved in NO_3^- assimilation in Castle Lake, as well as high-resolution Mo depth profiles that suggest Mo may be limiting N_2 fixation by lake periphyton communities. Finally, we discuss future research directions and novel techniques that hold promise for advancing research on this topic.

PURE CULTURE STUDIES

Research on Mo requirements for N assimilation in aquatic microbes has largely focused on heterocystous cyanobacteria for N_2 fixation studies and green algae for NO_3^- assimilation studies

(**Table 1**). In instances where growth of heterocystous cyanobacteria was compared on different N sources, Mo requirements were consistently higher in the N_2-fixing than NO_3^--grown condition (**Table 1**), as predicted based on specific activity of the nitrogenase vs. nitrate reductase enzymes (see below).

NITROGEN FIXATION

The first reports that Mo was a bioessential element for N_2 fixation were published by the German microbiologist Hermann Bortels on the soil bacterium *Azotobacter chroococum* (Bortels, 1930) and the aquatic heterocystous cyanobacterium *Nostoc*/*Anabaena* (Bortels, 1940). Since Bortels' early studies, *Nostoc*/*Anabaena* and *Azotobacter* strains have been model organisms for the study of Mo requirements for N_2 fixation. Subsequent studies showed that *Anabaena* had optimal growth at dissolved Mo concentrations in the range of 50–2000 nM (Wolfe, 1954; Jacob and Lind, 1977; ter Steeg et al., 1986; Attridge and Rowell, 1997) and that Mo limitation of N_2 fixation occurred at 1–5 nM Mo (Fay and Vasconcelos, 1974; Attridge and Rowell, 1997; Zerkle et al., 2006; Glass et al., 2010). The onset of Mo limitation requires several transfers to ensue, likely due to the expression of high-affinity ModABC MoO_4^{2-} uptake systems, which are widely distributed in bacteria and archaea (Self et al., 2001; Zhang and Gladyshev, 2008) and have been characterized in *Nostoc/Anabaena* (Thiel et al., 2002; Zahalak et al., 2004).

All of the studies mentioned above were performed on freshwater cyanobacteria (*Nostoc/Anabaena*). To our knowledge, no previous studies have explored the effect of varying Mo concentration on marine microbes, although one previous study looked at such an effect on coastal heterocystous cyanobacteria (Glass et al., 2010). In recent years there have been a number of reports of intracellular Mo abundances in marine cyanobacteria that enable us to compare approximate Mo requirements between freshwater, coastal, and marine cyanobacteria (**Table 1**). When grown at Mo concentrations typical of freshwaters (<20 nM), freshwater heterocystous cyanobacteria have Mo:C ratios of 0.1–3 μmol mol^{-1} (ter Steeg et al., 1986; Glass et al., 2010). When grown at higher Mo concentrations, they can accumulate Mo up to extremely high levels (>100 μmol mol^{-1}; Glass et al., 2010). Cultured coastal heterocystous cyanobacteria have Mo:C ratios of 2–4 μmol

Table 1 | Previous studies of Mo dependence of N_2 fixation and nitrate assimilation in cyanobacteria and algae.

Species name	Organism type	Aquatic habitat	Mo requirements by N source	Mo:C (μmol mol^{-1})	References
Nostoc/Anabaena sp.	Cyanobacterium	Freshwater	N_2	–	Bortels, 1940
Chlorella pyrendoisa	Green alga	Freshwater	NO_3^-	–	Walker, 1953; Loneragan and Arnon, 1954
Anabaena cylindrica	Cyanobacterium	Freshwater	$N_2 > NO_3^- > NH_4^+$	–	Fogg and Wolfe, 1954; Wolfe, 1954; Fay and Vasconcelos, 1974; Jacob and Lind, 1977; Attridge and Rowell, 1997
Anabaena oscillarioides	Cyanobacterium	Freshwater	N_2	0.1–3[a]	ter Steeg et al., 1986
Scenedesmus obliquus	Green alga	Freshwater	$NO_3^- > NH_4^+ \cong$ urea	–	Arnon et al., 1955; Ichioka and Arnon, 1955
Scenedesmus acutus	Green alga	Freshwater	NO_3^-	–	Glass, 2011
Navicula pelliculosa	Diatom	Freshwater	NO_3^-	–	Wallen and Cartier, 1975
Chlamydomonas reinhardtii	Green alga	Freshwater	NO_3^-	–	Wallen and Cartier, 1975
Anacystis nidulans[b]	Cyanobacterium	Freshwater	$NO_3^- > NO_2^- > NH_4^+$	–	Peschek, 1979
Anabaena variabilis ATCC 29413	Cyanobacterium	Freshwater	N_2	–	Attridge and Rowell, 1997; Zerkle et al., 2006
Nostoc sp. PCC 7120	Cyanobacterium	Freshwater	$N_2 > NO_3^-$	0.2–132	Glass et al., 2010
Nostoc sp. CCMP 2511	Cyanobacterium	Coastal	$N_2 > NO_3^-$	0.5–5.8	Glass et al., 2010
Aphanizomenon sp.	Cyanobacterium	Coastal	N_2	0.8–2.0	Walve and Larsson, 2007
Nodularia spumigena	Cyanobacterium	Coastal	N_2	0.8–2.0	Walve and Larsson, 2007
Trichodesmium erythraeum strain IMS101	Cyanobacterium	Marine	N_2	1.0–6.6	Tuit et al., 2004
Trichodesmium (field samples from North Atlantic)	Cyanobacterium	Marine	N_2	9–54	Tuit et al., 2004; Nuester et al., 2012
Crocosphaera watsonii strain WH8501	Cyanobacterium	Marine	N_2	0.6–0.9	Tuit et al., 2004

[a] *Calculated by assuming that 45% of dry biomass weight is carbon.*

[b] *Renamed Synechococcus elongatus PCC 7942.*

mol^{-1} when grown at seawater Mo concentrations, similar to the natural range observed in the heterocystous cyanobacteria *Aphanizomenon* sp. and *Nodularia spumigena* sampled from the Baltic Sea (Walve and Larsson, 2007). Interestingly, marine non-heterocystous cyanobacteria *Trichodesmium erythraeum* strain IMS101 and *Crocosphaera watsonii* strain WH8501 have similar Mo quotas to freshwater and coastal cyanobacteria when cultivated in the laboratory (0.6–6.6 μmol mol^{-1} Mo:C), but *Trichodesmium* sp. sampled in the North Atlantic have much higher intracellular Mo (9–54 μmol mol^{-1} Mo:C; Tuit et al., 2004; Nuester et al., 2012), suggesting that they may have higher Mo requirements than other cyanobacteria and/or that they have a mechanism for Mo storage.

Organisms with alternative nitrogenases (containing vanadium (V) or iron (Fe) in place of Mo) express them instead of Mo nitrogenase under Mo limitation (Robson et al., 1986). Such alternative nitrogenases are rare in heterocystous cyanobacteria but are more common in soil bacteria such as *Azotobacter* (Betancourt et al., 2008), likely because the average V concentration in soils is ∼100-fold higher than Mo (Gupta, 1997). Alternative nitrogenases are less efficient at N_2 fixation than Mo nitrogenases and thus Mo nitrogenases are expressed when sufficient Mo is available (Eady, 1996). Recently, it was discovered that *Azotobacter vinelandii* produces organic compounds under Mo and V limitation that form strong complexes with Mo and V and are available for cellular uptake (Liermann et al., 2005; Bellenger et al., 2008, 2011). Such "molybdophores" are one of several mechanisms that N_2 fixers possess for combatting Mo limitation, with other examples including high affinity Mo uptake systems and Mo storage proteins, including MoSto (Pienkos and Brill, 1981; Fenske et al., 2005) and Mop (Hinton and Merritt, 1986; Schüttelkopf et al., 2002; Pau, 2004).

NITRATE ASSIMILATION

Mo was first reported to be essential for NO_3^- assimilation in the fungus *Aspergillus niger* (Steinberg, 1937), which required additional Mo when cultivated on media containing NO_3^- than NH_4^+. Follow-up studies showed that this higher Mo demand for growth on NO_3^- than NH_4^+ was also present in green algae *Chlorella pyrendoisa* (Walker, 1953) and *Scenedesmus obliquus* (Arnon et al., 1955; Ichioka and Arnon, 1955) and the cyanobacterium *Anacystis nidulans* (later renamed *Synechococcus elongatus* PCC 7942; Peschek, 1979). We found that the green alga *Scenedesmus acutus* grown in chemostats contained 60% higher chlorophyll *a*, 3000% higher nitrate reductase activity and 80% higher cellular Mo when grown on high concentrations of Mo (90 μM) than under Mo limitation (1 nM; Glass, 2011). Studies of the purified assimilatory nitrate reductase verified that the enzyme requires Mo for activity (Nicholas and Nason, 1954; Nicholas et al., 1954; Vega et al., 1971) and contains one molybdopterin cofactor per active site (Solomonson et al., 1986).

Different organisms vary in how much Mo they require for NO_3^- assimilation. For instance, the freshwater diatom *Navicula pelliculosa* is more susceptible to Mo limitation of photosynthesis and NO_3^- uptake than the freshwater green alga *Chlamydomonas reinhardtii* (Wallen and Cartier, 1975). This may be due to the presence of a high-affinity eukaryotic MoO_4^{2-} uptake system (MOT1) in *C. reinhardtii* that is absent from diatoms (Tejada-Jimenez et al., 2007). The spotty distribution of MOT1 in eukaryotes was confirmed by a recent bioinformatic survey (Zhang and Gladyshev, 2010). It is possible that eukaryotes containing MOT1 or other unknown high-affinity Mo transporters can cope with Mo limitation for longer time periods than other organisms that lack such transporters.

COMPARISON OF MO REQUIREMENTS

Models and experiments have demonstrated that N_2 fixation requires more Mo than NO_3^- assimilation, while other more chemically-reduced forms of N, such as NH_4^+, do not require Mo for assimilation. This is because the specific reaction rate of nitrate reductase (mol N assimilation per mol Mo s^{-1}) is 2–3 orders of magnitude higher than nitrogenase (Sprent and Raven, 1985). Based on this difference in activity, it has been calculated that a N_2-fixing cell requires ∼125× more Mo per cell than a NO_3^--grown cell (Sprent and Raven, 1985; Raven, 1988). The greater need for Mo during N_2 fixation vs. NO_3^- assimilation was experimentally demonstrated for both cyanobacteria and legumes containing symbiotic N_2 fixers in their root nodules (Anderson and Spencer, 1949, 1950; Fogg and Wolfe, 1954; Wolfe, 1954). In accordance with this higher need, cellular Mo quotas are also higher when cyanobacteria are grown on N_2 than NO_3^- (Glass et al., 2010).

FIELD STUDIES

Dissolved Mo concentrations have been measured in a large number of globally distributed freshwater lakes, and range from <0.1–13 nM (**Table 2**). Mo concentrations increase with salinity, and therefore saline lakes often contain much higher concentrations of Mo than freshwater lakes (Marino et al., 1990; Johannesson et al., 2000). In this section we review previous studies on Mo limitation in freshwater lakes, with particular emphasis on Castle Lake in northern California, USA. We summarize new data for Castle Lake, including results of experiments exploring the influence of Mo and NO_3^- additions on the activity of enzymes involved in NO_3^- assimilation, and dissolved Mo depth profiles. In later sections we discuss the possibility of Mo limitation of N assimilation in marine and soil environments. Mo has been clearly shown to limit N_2 fixation in temperate and tropical soil ecosystems, whereas the evidence is more ambiguous for marine environments.

MO LIMITATION IN CASTLE LAKE AND OTHER FRESHWATERS

Previous studies

Early studies tested the response of Mo additions to oligotrophic lakes in Alaska, California, and New Zealand (Goldman, 1964). Mo additions to bottle incubations were performed for 3 lakes in Alaska, 13 lakes in the Klamath Mountains of California, 2 lakes in the Sierra Nevada Mountains of California and 2 lakes in New Zealand (**Figure 1**). Mo concentrations in Lake Aleknagik (Alaska) and Lakes Coleridge and Lyndon (New Zealand) were <0.7 nM (Goldman, 1964) and lakes in the Sierra Nevada averaged 4 nM (Bradford et al., 1968). Response to Mo additions was measured by comparing photosynthetic $H^{14}CO_3^-$ uptake between control bottles and bottles amended with 40 nM Mo. The majority of lakes showed a significant increase in ^{14}C uptake after Mo addition: 0.1–10% increase over controls (7 lakes),

Table 2 | Dissolved Mo concentrations for Castle Lake and other freshwater lakes around the world.

Lake name and location (number of lakes in study)	Dissolved Mo (nM)	References
Castle Lake, California, USA	2–4	Bachmann and Goldman, 1964; Glass, 2011
Clear Lake, Colorado, USA	1–4	Elser and Glass, unpublished data
Esthwaite Water, England	0.1–2.6	Achterberg et al., 1997
Linsley Pond, Connecticut, USA	0.4–2.7	Cowgill, 1977
New Zealand lakes (3)	<0.7	Goldman, 1964
Alaska lakes (3)	≤0.6	Goldman, 1964
Mirror Lake, New Hampshire, USA	0.1–0.3	Cole et al., 1986
Japan lakes (13)	0.5–13	Sugawara et al., 1961
Lake Greifen, Switzerland	3–5	Magyar et al., 1993
Lake Insjön, Sweden	6.4	Lithner et al., 2000
Lake Lundsjön, Sweden	0.8	Lithner et al., 2000
Eastern Canadian lakes (4)	0.1–3.4	Chappaz et al., 2008
Hall Lake, Washington, USA	1–2	Balistrieri et al., 1994
Sierra Nevada lakes (170), California, USA	0.03–10	Bradford et al., 1968
Northern Germany lakes (8)	0.5–10	Groth, 1971
Amazonas, Brazil lakes (3)	4–8	Groth, 1971

10.1–20% increase over controls (9 lakes), 20.1–30% increase over controls (1 lake) and 40.1–50% increase over controls (1 lake; **Figure 1**). Only 2 lakes of the 20 tested did not respond to Mo additions.

The lake with the most positive response to Mo addition of the 20 tested was Castle Lake, a small (0.2 km^2) meso-oligotrophic, subalpine lake in the Klamath Mountains of northern California with low dissolved Mo (2–4 nM). These low Mo concentrations are likely a result of the ultramafic deposits underlying the lake's watershed, which contain very low concentrations of Mo (Kuroda and Sandell, 1954; Glass, 2011; Glass, J. B., Chappaz, A., Eustis, B., Heyvaert, A. C., Waetjen, D., Hartnett, H. E., and Anbar, A. D., Molybdenum geochemistry in Castle Lake, California, in review, *Geochim. Cosmochim. Acta*). Addition of 1 μM Mo increased the rate of photosynthesis in lake surface phytoplankton communities in the summer, fall, and winter months (Goldman, 1960). Lower concentrations of Mo stimulated primary productivity in January than in June or October, suggesting that planktonic communities were more Mo-limited during the winter than the summer. This explanation is reasonable given that Castle Lake water sampled from under the ice had lower dissolved Mo concentrations (0.1–0.9 nM; Glass, 2011; Glass et al., in review) than surface lake water sampled in the mid-summer 2008 and 2009 (2.4–2.9 nM; **Figure 3A**; see below). Similarly, pronounced winter depletions of Mo have been measured in Lake Donk, Belgium (Dumont, 1972) and Linsley Pond, Connecticut (Cowgill, 1977). For more on the geochemical cycling of Mo in Castle Lake, see Glass et al., (in review).

Two experimental whole-lake additions of Mo were performed in Castle Lake in the 1960s. In July 1963 and again in June 1969, 6.3 kg of Mo was added as solid-phase sodium molybdate (Na_2MoO_4) to the lake's epilimnion to stimulate primary productivity (Goldman, 1966, 1972). These Mo additions increased dissolved Mo in the epilimnion from ∼2–5 nM to 50–80 nM. Following the 1963 experimental addition of Mo to the lake, primary productivity in the epilimnion increased 40% over the previous year (Goldman, 1966). The golden alga *Dinobryon sertularia* exhibited the largest increase in cell numbers (Goldman, 1972; Jassby and Goldman, 1974). Trout, cladoceran, and copepod yields also increased.

Subsequent studies showed that Mo limitation of primary productivity in Castle Lake was likely a result of insufficient Mo for maximal rates of NO_3^- assimilation. Additions of 50 nM Mo to bottle incubations of Castle Lake water led to increased NO_3^- uptake (up to 60%), but only when NO_3^--N concentrations were >1 μM and NH_4^+-N was <0.1 μM (Axler et al., 1980). Regeneration of NH_4^+ by zooplankton excretion and microbial mineralization are important N sources for the Castle Lake phytoplankton community (Axler et al., 1981; Zehr et al., 1985). In the epilimnion, NH_4^+ assimilation rates were significantly higher than those of NO_3^- throughout the summer (Axler et al., 1981, 1982) and mid to late-summer regeneration of NH_4^+ contributed more than 50% of the total N assimilated (Axler et al., 1981). Small amounts of NH_4^+-N (0.3–0.4 μM) were shown to inhibit NO_3^- assimilation by more than 75% in the Castle Lake epilimnion (Priscu et al., 1985), whereas higher NH_4^+ concentrations were required for inhibition at greater depths. At 20 m, ∼3 μM NH_4^+-N was required to significantly inhibit NO_3^- assimilation, whereas at 25 m, enrichment with 5.4 μM NH_4^+-N resulted in minimal inhibition (Priscu et al., 1985). From 1976–2010, mean NH_4^+-N during the ice-free growing season (June through mid-September) was 0.3 ± 0.2 (mean±1 s.d.) μM in the epilimnion at 3 m, 0.3 ± 0.2μM in the upper hypolimnion at 15 m and 1.0 ± 0.9μM in the mid-hypolimnion at 25 m (CLEREP, 2011). Therefore, the epilimnetic phytoplankton community is probably more susceptible to NH_4^+ suppression of NO_3^- assimilation than the hypolimnetic community, where mean NH_4^+ levels are typically below the inhibition threshold (Axler et al., 1980; Axler and Goldman, 1981; Priscu et al., 1985). Thus, it is reasonable to expect that Mo requirements increase with depth in Castle Lake if NO_3^- is a more important N source in the hypolimnion than in the epilimnion.

CURRENT STUDY: ENZYME BIOASSAYS

Building on previous isotopic tracer (^{15}N, ^{13}N, and ^{14}C) uptake experiments in Castle Lake (Goldman, 1960; Axler et al., 1980, 1982; Axler and Goldman, 1981; Axler and Reuter, 1996), we investigated the effect of Mo availability on the enzyme activity of key N assimilation proteins and total protein abundances at three depths in Castle Lake. In July 2008, we assayed the activity of the first and third enzymes involved in NO_3^- assimilation (the Mo-containing eukaryotic nitrate reductase and the non-metal-containing enzyme glutamine synthetase) and total protein content after the addition of 100 μM NO_3^- as $NaNO_3$ and 100 nM Mo as Na_2MoO_4 to bottle experiments (background NO_3^--N

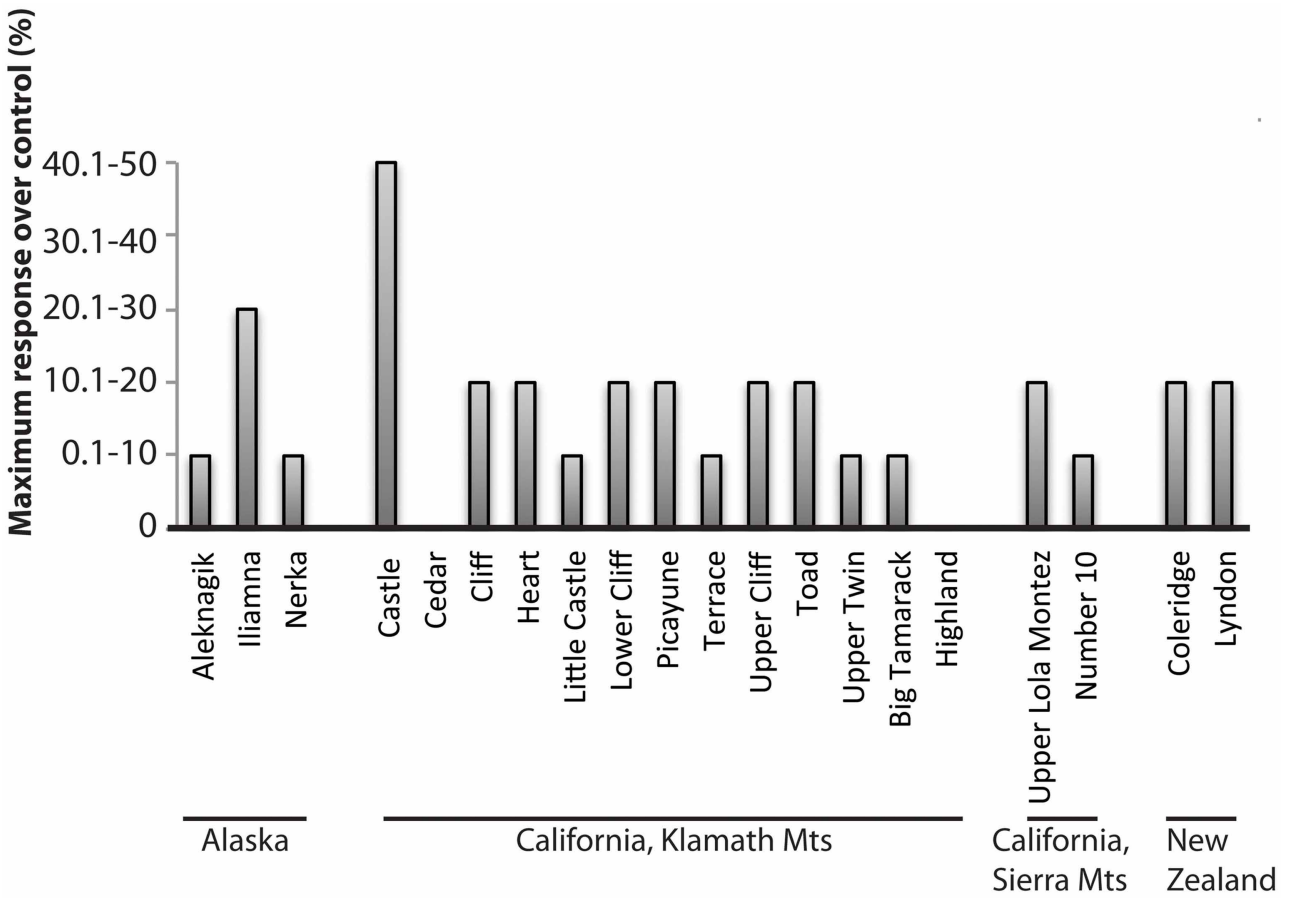

FIGURE 1 | Maximum response of bottle incubations after addition of 40 nM Mo in 20 lakes, in units of percent increase over control. Compiled from Goldman (1964).

concentrations at the start of the experiment were 0.1 μM at 3 m, 0.2 μM at 15 m, and 1.6 μM at 25 m; background Mo was 2–4 nM; see Glass, 2011 for experimental details). These experiments showed a consistent positive response at the lower two depths (15 and 25 m), but only in the +N +Mo treatments (**Figure 2**). This hypolimnetic response occurred for all three measurements: nitrate reductase activity (**Figure 2A**), glutamine synthetase activity (**Figure 2B**), and soluble protein content (**Figure 2C**), suggesting that supplemental Mo enabled added NO_3^- to be assimilated into protein. Ambient NH_4^+-N concentrations were 0.2 μM at 3 m and ≤0.02 μM at 15 and 25 m. The lack of response to +N +Mo treatments at 3 m suggests that 0.2 μM NH_4^+-N, slightly lower than the inhibition threshold of 0.3–0.4 μM NH_4^+-N reported by Priscu et al. (1985), was inhibiting NO_3^- uptake. The strong response to +N +Mo additions at 15 and 25 m suggests that hypolimnetic microbial communities were Mo-NO_3^- co-limited; that is, addition of only one nutrient did not stimulate enzyme activity and protein synthesis because the other nutrient was limiting—only the simultaneous addition of both nutrients produced a positive response. This finding is in accordance with scarce NH_4^+ (≤0.02 μM NH_4^+-N) in the hypolimnion, reducing the possibility of NH_4^+ inhibition.

CURRENT STUDY: MO DEPTH PROFILES

We extended previous studies of Castle Lake Mo bioavailability (Bachmann and Goldman, 1964; Goldman, 1966) by obtaining high-resolution dissolved Mo depth profiles from the center of Castle Lake on 16 July 2008 and 27 June 2009 (see Glass, 2011 for details). The dissolved Mo content of the Castle Lake water column ranged from 2 to 4 nM (**Figure 3**), similar to values reported from the 1960s using different analytical methods. Surface water Mo concentrations were 2.9 and 2.4 nM in 2008 and 2009, respectively. Both years, Mo concentration minima occurred near the thermocline, where Mo was lower by 0.5–0.8 nM than in shallower and deeper waters. The concentration minimum was offset ∼5 m deeper in 2008 than 2009. The Mo minimum was located at a shallower depth (5–10 m) than the deep chlorophyll *a* maximum (15–20 m), whereas Mo minima and dissolved O_2 maxima occurred at similar depths (**Figures 3A**,**B**). Dissolved Mo was relatively constant down to about 20 m, and then increased toward the lake bottom, suggesting that sediments are a source of Mo to the lake in the mid-summer (Glass et al., in review; **Figure 3A**).

The finding of Mo minima in the Castle Lake epilimnion suggests that dissolved Mo is being taken up biologically in excess of that supplied by watershed and sediment inputs. The concurrence

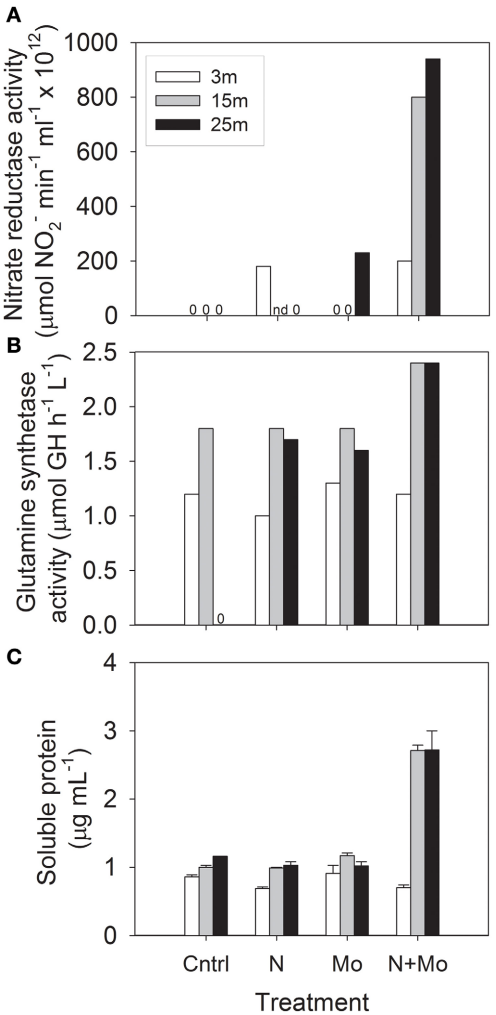

FIGURE 2 | Results of bioassay experiments at three depths in Castle Lake in July 2008 for control (Cntrl), +nitrate (N), +molybdenum (Mo), and +nitrate +molybdenum (N + Mo) treatments: (A) nitrate reductase activity; (B) glutamine synthetase activity; (C) soluble protein content ("0" stands for undetectable activity and "nd" stands for "no data" "GH" stands for γ-glutamyl hydroxamate). After Glass (2011).

of dissolved Mo minima and dissolved O_2 maxima near the thermocline may provide clues as to which Castle Lake microbial community is responsible for the prominent Mo drawdown between 5 and 10 m depth. Positive heterograde O_2 profiles like those of Castle Lake, exhibiting an O_2 maximum at the thermocline, can arise from high phytoplankton photosynthesis and/or diffusion of O_2 (produced by submerged macrophytes, periphyton, or phytoplankton) from the littoral zone into the thermocline (Wetzel, 2001). The littoral zone of Castle Lake is an extensive shallow (3–5 m) platform with a well-studied benthic periphyton community that includes an expansive epipelic (i.e., living freely on sediment surfaces) plain comprised of a mixed community of diatoms, chlorophytes, and non-heterocystous cyanobacteria, and a fringing epilithic (i.e., growing on a rock surface) community dominated by N_2-fixing heterocystous cyanobacteria (Sanders, 1976; Loeb and Reuter, 1981; Reuter et al., 1985; Reuter and Axler, 1988, 1992; Axler and Reuter, 1996), that have high Mo requirements (see above). Even when epilimnetic water is severely depleted in NO_3^-, the overlying epipelic periphyton can still obtain a significant amount of their N-nutrition by fixing N_2 in the dark when they are not producing O_2 (Bergman et al., 1997; Berman-Frank et al., 2003) or by assimilating interstitial porewater NO_3^- that diffuses upward from the sediments (Reuter et al., 1985; Reuter and Axler, 1992). Therefore, this microbial community represents a potentially important sink for available Mo. Neither heterocystous cyanobacteria, nor N_2-fixation, have been found in the planktonic communities of the lake over the many years of intensive monitoring (Reuter et al., 1985; Goldman, unpublished). This fact, together with the extremely low rates of planktonic NO_3^- assimilation rates previously measured in the epilimnion (Gersberg et al., 1978; Axler and Goldman, 1981; Axler et al., 1982), suggests that the Mo minimum at the thermocline likely represents demand by littoral periphyton communities that fix N_2 and also assimilate NO_3^-. This raises the question of whether these periphyton communities are limited for Mo. One previous experiment provides support for this hypothesis: N_2-fixation increased 210% when 50 nM Mo was added to a lake containing <10 nM Mo in the Great Salt Basin during a cyanobacterium (*Aphanizomenon flos-aquae*) bloom (Wurtsbaugh, 1988). However, Mo requirements for periphyton microbial communities remain to be determined.

MARINE AND SALINE ENVIRONMENTS

A long-standing question in oceanography is the cause for widespread N limitation in marine environments (Vitousek and Howarth, 1991). Mo was long dismissed as a potential limiter of marine N assimilation due to its high seawater concentration and conservative behavior with depth (∼107 nM; Collier, 1985). Robert Howarth and colleagues challenged that assumption by suggesting that high levels of seawater sulfate may compete with Mo uptake (Howarth and Cole, 1985; Howarth et al., 1988). Sulfate (SO_4^{2-}) and molybdate (MoO_4^{2-}, the dominant species of Mo in oxic waters) have very similar size and stereochemistry, but sulfate (SO_4^{2-}; 28 mM) is $\sim 10^5$-fold more abundant than molybdate (MoO_4^2; 100 nM) in seawater. Amendment experiments in the Baltic Sea (with ambient Mo of 20 nM and sulfate of 4.8 mM, a S:Mo ratio of 240,000) showed that additions of 100 nM Mo (S:Mo ratio of 40,000) led to 50% higher N_2 fixation rates than the control, whereas additions of 5 mM sulfate (S:Mo ratio of 490,000; roughly 2-fold higher than seawater) led to N_2 fixation rates 25% lower than the control (Howarth and Cole, 1985). However, N_2 fixation rates were low in this experiment and the authors warned against extrapolation to marine systems. In follow-up studies with pure cultures and freshwater systems, Mo uptake was inhibited at S:Mo ratios 50–100-fold lower than those present in seawater (Cole et al., 1986, 1993) and addition of sulfate to seawater levels greatly reduced N_2 fixation rates (Marino et al., 2003). Also consistent with this hypothesis, the S:Mo ratio in 13 saline lakes in Alberta, Canada was negatively correlated with the abundance of N_2-fixing cyanobacteria (Marino et al., 1990).

While these studies lent support to the S/Mo inhibition hypothesis, others challenged it. *In situ* bioassays performed off

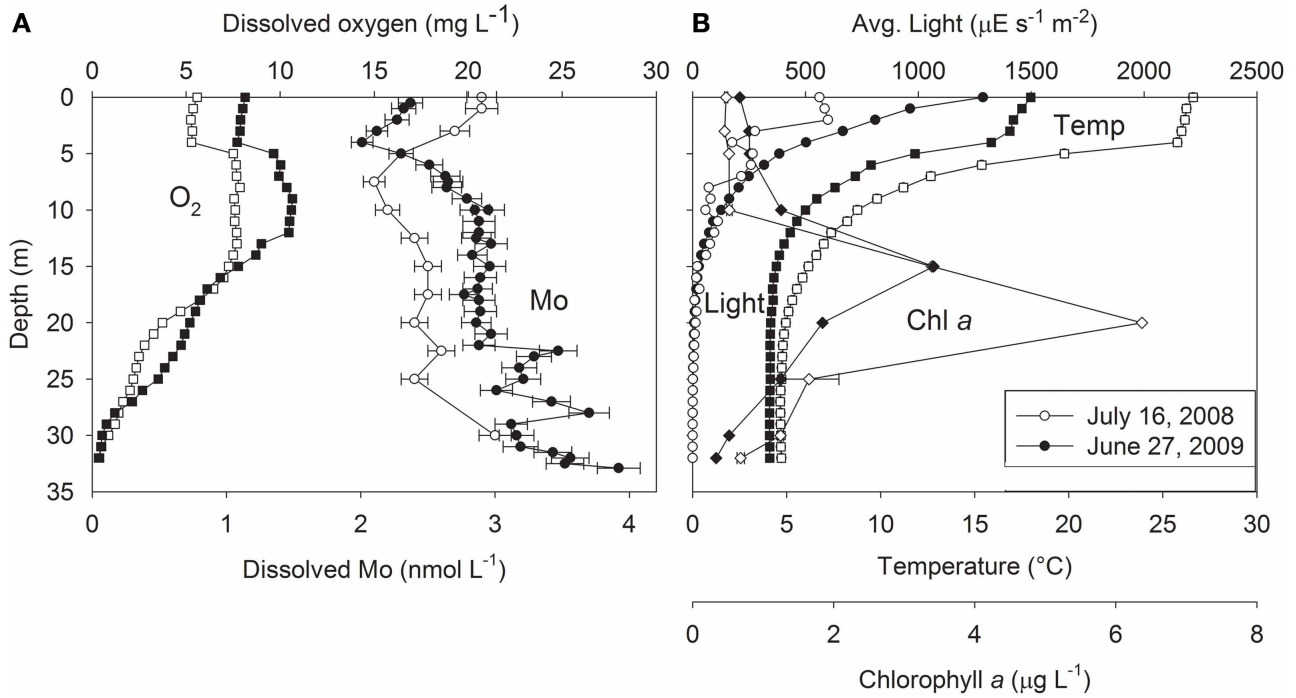

FIGURE 3 | (A) Dissolved oxygen (O_2; squares) and dissolved molybdenum (Mo; circles), and **(B)** average light (circles), temperature (squares), and chlorophyll *a* concentrations (Chl *a*; diamonds) with depth in Castle Lake, from water column samples collected on 16 July 2008 (white symbols) and 27 June 2009 (black symbols). After Glass (2011).

the North Carolina coast showed that addition of 0.1–5 μM Mo resulted in no significant change in N_2 fixation rates (Paerl et al., 1987; Paulsen et al., 1991). Similarly, mesocosm studies with water sampled from Narragansett Bay, Rhode Island produced no significant change in heterocyst abundance (a proxy for N_2-fixing cell abundance) when Mo was increased from ambient levels (98 nM) to 1.23 μM under highly N-limited conditions (Marino et al., 2003). Additions of Mo to the Great Salt Lake, which lowered S:Mo ratios from 299,00:1 to 83,000:1, did not significantly stimulate N_2 fixation (Wurtsbaugh, 1988). Similarly, Mo additions to 8 of the 13 saline lakes studied by Marino et al. (1990) had no significant effect on phytoplankton biomass (Evans and Prepas, 1997). Finally, Mo uptake by *Azotobacter vinelandii* was not inhibited at S:Mo ratios up to eight times that of seawater (Paulsen et al., 1991). Overall, it is still unclear whether high SO_4^{2-} inhibits Mo uptake in marine environments, as this question has never been tested in open ocean conditions. However, it seems that marine microbes are adapted to discriminate between SO_4^{2-} and MoO_4^{2-} in a manner that freshwater microbes are not.

SOIL HABITATS

Since this paper is focused on Mo limitation of aquatic environments, we will touch only briefly on Mo limitation of soil ecosystems. For a much more thorough review of Mo as it relates to soils, crops, and livestock, see Gupta (1997). Studies on Mo deficiencies in soils date back to the 1930s, when Arnon and Stout (1939) first showed that Mo was an essential micronutrient for higher plants. Nitrate was found to accumulate in plant leaf tissues when Mo was omitted from the nutrient medium, presumably because it could not be assimilated into organic matter (Hewitt and Jones, 1947). Mo limitation is especially prevalent on serpentine soils which contain less Mo than other soil types (Walker, 1948). The importance of Mo for symbiotic N_2 fixation in alders containing N_2-fixing bacteria in their root nodules was first shown by Becking (1961a,b). Goldman (1960, 1972) proposed that alders growing along the shore of Castle Lake may scrub soluble Mo from soil porewaters, thereby competing with the aquatic ecosystem for scarce Mo. More recently, N_2 fixation has been found to be limited by Mo availability in both temperate and tropical soils (Silvester, 1989; Barron et al., 2009; Wurzburger et al., 2012). This effect may be exacerbated by rising atmospheric CO_2: Hungate et al. (2004) found that rising atmospheric CO_2 led to increased legume N_2 fixation during the first year of treatment of an oak woodland, but this response declined and disappeared by the third year and N_2 fixation was depressed in the fifth, sixth, and seventh years. This effect was best explained by declines in foliar Mo concentrations with increased atmospheric CO_2, suggesting that the bioavailability of Mo in soils declined, likely either by decreased pH or accumulation of organic matter leading to increased sorption of Mo onto particles. Therefore, Mo limitation of N assimilation may limit the ability of both terrestrial environments (and possibly aquatic ones as well) to serve as sinks for anthropogenic CO_2 emissions.

CONCLUSIONS AND FUTURE DIRECTIONS

Research on Mo requirements for microbial N assimilation dates back to the 1930s and has recently been rejuvenated by the important finding that N_2 fixation may be limited by decreased Mo

bioavailability under increased atmospheric CO_2 (see above). Studies of pure cultures of cyanobacteria, algae, and soil bacteria have shown that Mo requirements are highest when organisms are fixing N_2, lower but still significant when grown on NO_3^-, and negligible when grown on NH_4^+. Furthermore, there are differences in Mo requirements among species for a given N source, likely due to the presence or absence of high-affinity Mo uptake systems and/or other Mo scavenging mechanisms. *In situ* experiments with natural populations of microbes in lakes have shown that Mo limitation of primary productivity may be widespread, particularly in freshwaters underlain by geologic deposits containing scarce Mo, such as Castle Lake in northern California. Data presented here support the theory, originally put forth in previous studies (Goldman, 1960; Axler et al., 1980; Axler and Goldman, 1981), that Mo availability can limit NO_3^- assimilation in Castle Lake when NH_4^+ is scarce, and show that this limitation manifests itself in suppressed enzyme activity and protein content. Furthermore, we found circumstantial evidence in Castle Lake Mo and O_2 depth profiles that N_2 fixation by periphyton communities leads to appreciable dissolved Mo drawdown and thus may also be Mo-limited, an idea that requires future testing. Below we discuss up-and-coming research topics related to Mo-N co-limitation: the bioavailability of different redox states of Mo, the use of Mo isotope systematics to elucidate microbial Mo uptake mechanisms, the possibility of Mo limitation of N_2 fixation in the marine environment, and the co-evolution of microbial metabolisms with changing availability of Mo in the ocean through time.

BIOAVAILABILITY OF MO IN DIFFERENT OXIDATION STATES

While the major Mo species in oxic waters is $Mo(VI)O_4^{2-}$, Mo(V) was found to comprise up to 15% of total Mo concentrations in estuarine waters and likely a significant portion of total Mo in many aquatic systems (Wang et al., 2009). It has been proposed that reduced Mo(V) is more bioavailable than oxidized Mo(VI) as MoO_4^{2-} because Mo^{5+} is the redox state of Mo in some enzymes (see references in Wang et al., 2009) and does not compete with SO_4^{2-} during uptake (Howarth and Cole, 1985; Howarth et al., 1988). Recent experiments support this hypothesis: in California lakes of varying trophic status (Lake Tahoe, Walker Lake, and Clear Lake), N_2 fixation rates and chlorophyll concentrations were positively correlated with Mo(V) concentrations, which increased with trophic status from Lake Tahoe to Lake Walker to Clear Lake (Romero et al., 2011). Under reducing conditions often present in sediments and bottom waters, a higher proportion of Mo will be reduced (Wang et al., 2011) or converted to particle-reactive thiomolybdate ($Mo(VI)O_xS_{4-x}^{2-}$) if H_2S is present in relatively high concentrations (Helz et al., 1996, 2011; Erickson and Helz, 2000; Vorlicek and Helz, 2002; Vorlicek et al., 2004). Very little is known about the bioavailability of thiomolybdate (and other chemical species of Mo besides $Mo(VI)O_4^{2-}$); this is a topic that deserves more attention.

MO ISOTOPIC FRACTIONATION

Recently, studies revealed that *Nostoc/Anabaena* and *Azotobacter* fractionate Mo isotopes with distinct fractionation factors when fixing N_2 (Wasylenki et al., 2007; Zerkle et al., 2011). This fractionation likely occurs during uptake by the ModABC system (see above). Although this research is still in its early phases, future studies may benefit from using Mo isotopes to discern the intricacies of Mo biological pools in microbial species.

MO LIMITATION IN THE MARINE ENVIRONMENT?

Considering the importance of N_2 fixation in the marine environment (Sohm et al., 2011 and references therein), surprisingly few studies have investigated Mo requirements for N_2-fixing marine microbes compared to the greater number of studies on freshwater microbes. The reason for this gap is likely due to the assumption that dissolved Mo is too abundant in seawater to be a limiting micronutrient (see above). However, non-conservative behavior of Mo in coastal regions has been observed; dissolved Mo drops as low as 30 nM in the Wadden Sea (Dellwig et al., 2007). Although this concentration is still higher than has been shown for Mo requirements in freshwater and (one strain of) coastal cyanobacteria, it is possible that other coastal and open ocean strains require higher Mo, a hypothesis that requires future testing. Furthermore, it is quite possible that N_2 fixation in marine sediments (e.g., Dekas et al., 2009) is Mo-limited, as dissolved Mo in marine sediment porewaters may be reduced or converted to particle-reactive thiomolybdate (see above), leaving low levels of bioavailable Mo for microbial N_2 fixation. Alternatively, microbes may have evolved yet-unknown mechanisms to access Mo in sulfidic sediment porewaters. Finally, the S/Mo inhibition hypothesis still needs to be tested in open ocean conditions. If no inhibition is identified, a thorough study of the differences in MoO_4^{2-}/SO_4^{2-} uptake pathways is needed, as that would imply that marine microbial uptake systems have more specificity for Mo than those of freshwater microbes.

ANCIENT OCEAN MO CHEMISTRY AND EVOLUTION

While Mo is the most abundant transition metal in the modern ocean, it was likely much scarcer in seawater earlier in earth history and may have been an important limiting micronutrient of marine primary productivity. Before the Great Oxidation Event (GOE) ~2.4 billion years ago, the riverine flux of Mo to the ocean would have been small due to minimal oxidative weathering of sulfides, the major source of Mo on the continents (Scott et al., 2008). Nevertheless, Mo proteins seem to have very ancient roots and likely evolved in a low-Mo ocean (Raymond et al., 2004; Boyd et al., 2011; Schoepp-Cothenet et al., 2012). After the GOE up until ~800 million years ago, the presence of sulfide in the deep ocean (even if present over a relatively small areal extent) may have kept Mo concentrations low enough to limit N_2 fixation and perhaps stall the evolution of eukaryotic life (Anbar and Knoll, 2002; Glass et al., 2009; Reinhard, C. T., Planavsky, N. J., Robbins, J., Partin, C., Gill, B. C., Lalonde, S. V., Bekker, A., Konhauser, K. O., and Lyons, T. W. Proterozoic ocean redox and evolutionary stasis, in review, *Proc. Natl. Acad. Sci.*). Marine Mo concentrations likely rose to near their present values ~600 million years ago when the ocean became fully oxic (Scott et al., 2008), but during episodes of extreme global warming or mass extinction events that resulted in marine anoxic events, the return of sulfidic conditions to widespread areas of the deep sea would have caused

seawater Mo depletion (Algeo, 2004). While it is not possible to directly extrapolate from the physiology of modern organisms to those of their ancestors, studying the Mo (and other trace metal) requirements of modern microbes living in environments with scarce Mo can provide us with a possible analogue to ancient ecosystems.

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Redox chemistry of molybdenum in natural waters and its involvement in biological evolution

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The transition element molybdenum (Mo) possesses diverse valances (+II to +VI), and is involved in forming cofactors in more than 60 enzymes in biology. Redox switching of the element in these enzymes catalyzes a series of metabolic reactions in both prokaryotes and eukaryotes, and the element therefore plays a fundamental role in the global carbon, nitrogen, and sulfur cycling. In the present oxygenated waters, oxidized Mo(VI) predominates thermodynamically, whilst reduced Mo species are mainly confined within specific niches including cytoplasm. Only recently has the reduced Mo(V) been separated from Mo(VI) in sulfidic mats and even in some reducing waters. Given the presence of reduced Mo(V) in contemporary anaerobic habitats, it seems that reduced Mo species were present in the ancient reducing ocean (probably under both ferruginous and sulfidic conditions), prompting the involvement of Mo in enzymes including nitrogenase and nitrate reductase. During the global transition to oxic conditions, reduced Mo species were constrained to specific anaerobic habitats, and efficient uptake systems of oxidized Mo(VI) became a selective advantage for current prokaryotic and eukaryotic cells. Some prokaryotes are still able to directly utilize reduced Mo if any exists in ambient environments. In total, this mini-review describes the redox chemistry and biogeochemistry of Mo over the Earth's history.

Keywords: molybdenum, redox speciation, enzymes, ancient ocean, biological evolution

INTRODUCTION

Only a few transition elements (e.g., Fe, Mo, and Cu) were selected in the evolution of life and play a fundamental role in the global cycling of carbon, nitrogen, and sulfur (e.g., Kisker et al., 1999; Bittner and Mendel, 2010). Molybdenum (Mo) is an essential trace element for archea, bacteria, and eukaryotes (e.g., Williams and Fraústo da Silva, 2002; Zhang and Gladyshev, 2008; Hernandez et al., 2009). More than 60 metalloenzymes and proteins have been identified containing Mo (Lippard et al., 1994; Hille, 1996; Kisker et al., 1997; Stiefel, 1997, 1998; Kroneck and Abt, 2002; Boll et al., 2005; NC-IUB, 2012; ExPASy, 2012), including nitrogenase and nitrate reductase, which tie the element to the nitrogen cycle (e.g., Kroneck and Abt, 2002).

Although Mo is relatively scare in the Earth's crust (1.1 ppm, Wedepohl, 1995), it is more available to biological processes than many other abundant metals in the crust (e.g., Al of 7.96%, Sr of 333 ppm, and Ti of 4,010 ppm; Wedepohl, 1995) based on water concentrations. The total dissolved Mo concentrations are relatively low in river waters (~5 nM; Martin and Meyback, 1979), whereas this trace element is the most abundant transition metal in the oxygenated ocean (dissolved Mo: 105 nM; Collier, 1985). Generally, those low-level trace elements such as Fe may potentially limit the growth of phytoplankton in the ocean, and particularly in the high nutrients and low chlorophyll *a* areas (e.g., Martin and Fitzwater, 1988; Boyd et al., 2000). Mo deficiency is not common in natural environments, and it did, however, occur for many terrestrial plants (e.g., Hewitt and Bolle-Jones, 1952; Gupta, 1997; Kaiser et al., 2005), and even for freshwater phytoplankton (Dumont, 1972; Romero et al., 2011; Glass et al., 2012). Recently, Barron et al. (2009) reported that lack of Mo may limit atmospheric N_2 fixation in tropical forests with highly weathered acidic soils. Glass et al. (2010) further demonstrated that extremely low Mo levels (<1 nmol/L) can induce N-limitation for freshwater and coastal filamentous heterocystous cyanobacteria. Indeed, the unavailability of Mo has been long observed as limiting N_2 fixation or nitrate assimilation in coastal waters (e.g., Brattberg, 1977). Howarth and Cole (1985) hypothesized that high levels of sulfate in seawater might competitively inhibit algal Mo uptake in coastal waters.

In enclosed basins, e.g., the Cariaco Trench, the Black Sea, and the Saanich Inlet, Mo may be depleted with concentrations of as low as 3 nmol/L, whereas the sediments there accumulated Mo as high as 140 μg/g (Berrang and Grill, 1974; Emerson and Huested, 1991). It seems that total dissolved Mo concentrations in the ancient reducing ocean might be similarly low (e.g., ~10% of the present oceanic levels, Anbar and Knoll, 2002). Furthermore, reduced Mo probably existed in the ancient reducing ocean too. This mini review summarizes the recent advances regarding the redox chemistry of Mo in natural waters. The biological involvements of reduced Mo over the Earth's history are discussed.

REDOX SPECIATION OF Mo IN NATURAL WATERS

The transition element Mo possesses a wide range of different redox species (+II to +VI). Under the current atmospheric pO_2 of 0.2 atm, molybdate ions (e.g., MoO_4^{2-} and $HMoO_4^-$) are the most abundant chemical forms of Mo in oxygenated freshwater and seawater systems, whilst reduced Mo(V), likely as MoO_2^+, MoO_3^+, and $Mo_2O_4^{2+}$ (e.g., Szilágyi, 1967; Loach, 1970; Bertine,

1972; Coughlan, 1980), are expected to coexist in reducing environments (Brookins, 1988). Under strongly reducing conditions, Mo is expected to be further reduced to its less soluble forms (e.g., MoO_2 and MoS_2). As Mo(II) and Mo(III) have never been reported in the aquatic systems (e.g., Mendel, 2005), their natural occurrences will not be discussed here.

The O atoms in MoO_4^{2-} may be replaced by S in the presence of HS^-, creating a series of thiomolybdate complexes (Helz et al., 1996). Mo in these compounds can be further reduced to Mo(IV) and Mo(V), forming a series of sulfido species (Miller et al., 1980; Vorlicek and Helz, 2002). Wang et al. (2009), using a new technique for separating Mo(V) from Mo(VI) in natural waters, quantified the Mo(V) levels in sulfidic microbial mats and even in some reducing waters (Wang et al., 2009, 2011). As a significant transient intermediate during reductive diagenesis, reduced Mo(V) can be present in specific niches, e.g., in reducing porewater. Indeed, Mo(V) might range from 5–20 nM, accounting for up to ∼20% of ΣMo under low-sulfide conditions (<100 μmol/L; Wang et al., 2011). Mo(V) may be further reduced to Mo(IV), as in MoS_2 under strongly sulfidic conditions (HS^- > 100 μmol/L; Wang et al., 2011).

In the present ocean, the redox switching of Mo can only occur in specific niches under bacterial mediation. For example, Mo(VI) can be reduced to the intermediate state of Mo(V), and the reduced state of Mo(IV) as molybdenite [MoS_2(s)] by sulfate-reducing bacteria in the presence of sulfide (e.g., Tucker et al., 1997, 1998; Biswas et al., 2009). Some microorganisms can also oxidize the reduced Mo (e.g., Sugio et al., 1992), and, indeed, reduced Mo can serve as the electron donor to sustain autotrophic growth (e.g., Lyalikova and Lebedeva, 1984). In particular, Mo(V) may be produced from bio-oxidization of mineral molybdenite (MoS_2; e.g., Brierley, 1974). Once produced, the reduced Mo(V) may be complexed and stabilized with organic ligands naturally for a long while (Szilágyi, 1967; Bertine, 1972).

The existence of reduced Mo(V) was proposed later on as a potential limiting factor for cyanobacterial productivity in coastal and oceanic surface waters (Howarth and Cole, 1985; Yamazaki and Gohda, 1990). Griffin (1975) pointed out that nitrogenase in nitrogen fixers will not be active unless a trace amount of Mo(V) complexes is present. Howarth and Cole (1985) speculated that molybdate might be reduced extracellularly, and the reduced Mo(V), instead of the total, might be responsible for cyanobacterial blooms. Indeed, specific niches like anoxic microzones widely exist in the present oxygenated ocean due to cellular exudation of reduced substances and organic colloids (e.g., Carpenter and Price, 1976; Bryceson and Fay, 1981; Paerl and Bland, 1982; Paerl, 1985; Paerl and Prufert, 1987; Ploug et al., 1997). The diazotrophic cyanobacteria *Trichodesmium* could also form anoxic microzones by aggregating together. Inside these anoxic microzomes, the reduced Mo(V) was produced and actively involved in N_2 fixation (Howarth and Cole, 1985; Paerl, 1985; Paerl et al., 1987).

BIOLOGICAL UPTAKE AND ASSOCIATED REDOX CHANGES OF Mo IN CELLS

In contrast to its higher abundance in the present ocean (105 nM; Collier, 1985), the biological requirement of Mo is relatively lower than many other essential elements including Fe and Cu (e.g., Finkel et al., 2006). The molar Mo/Fe ratio is only 0.03 in bacteria (Barton et al., 2007), and 0.005 in some eukaryotic phytoplankton (Ho et al., 2003). Such a relatively lower requirement of the element may be attributable to the limited numbers of Mo containing enzymes in biology (e.g., Zerkle et al., 2005; Finkel et al., 2006), though these are essential to basic biological processes (e.g., nitrogen metabolism).

Until now, both less-specific and high-affinity molybdate uptake systems have been identified in biology (**Figure 1**). Previous work has confirmed that current prokaryotic and eukaryotic cells possess efficient uptake systems to utilize this element (e.g., high-affinity molybdate transporter; Tejada-Jimenez et al., 2007; Tomatsu et al., 2007; Baxter et al., 2008; Bittner and Mendel, 2010) including ABC transporter. Eukaryotic molybdate transport might involve more complex systems. In contrast, less-specific uptake of oxidized Mo widely exists in the present prokaryotic and eukaryotic cells, which utilizes other anion transporters: phosphate (Heuwinkel et al., 1992) or sulfate transporters (Tweedie and Segel, 1970; Marschner, 1995). Work has further shown an alternative: some soil bacteria are able to excrete siderophores (aminochelin) to complex extracellular Mo, and utilize trace amount of the element from ambient environments (Liermann et al., 2005).

Once inside cells, Mo cofactors are synthesized, and then allocated to the appropriate apo-enzymes via carrier proteins (Aguilar et al., 1992; **Figure 1**). These cofactors can be chaperoned to target proteins, into which they are inserted by specific trafficking proteins in prokaryotes (e.g., Ba et al., 2009; Mendel and Schwarz, 2011). Pau and Lawson (2002) reported that some bacteria possess specific molybdate-binding protein with a capacity of storing up to eight molybdate oxyanions for later use by the cells. As Mo in enzymes is extremely sensitive to intracellular oxidations such as reactive oxo species (Rajagopalan and Johnson, 1992), it is well protected within the storage proteins (e.g., Massey et al., 1970; Aguilar et al., 1992; Ichimori et al., 1999; Fenske et al., 2005; Schemberg et al., 2008; Hernandez et al., 2009; **Figure 1**). With the protection, Mo can easily switch redox states, and be actively involved in transferring electron/proton and even oxygen (e.g., Swedo and Enemark, 1979).

The Mo enzymes generally include two types of cofactors on the basis of the structure: Mo-co and Fe-Mo-co. Fe-Mo-co is a unique poly-metallic compound ($MoFe_7S_6$), which has been found only in Mo nitrogenase (e.g., Howard and Rees, 1996; Einsle et al., 2002). Two alternative nitrogenases (Fe and V) will not be discussed here. Mo nitrogenase catalyzes the ATP-dependent reduction of atmospheric dinitrogen to bioavailable ammonia, which represents the key point of entry of reduced nitrogen into the food chain (Burris, 1991; Burgess and Lowe, 1996; Hu et al., 2008). In the catalytic reaction, the N≡N triple bond is broken and therefore N_2 is being reduced at a sterically protected, single Mo center (Fe-Mo-co; Yandulov and Schrock, 2003). Mo-co is a mononuclear Mo atom coordinated to the sulfur atoms of a pterin. The task of the pterin is to position the catalytic Mo atom correctly within the active center, to control its redox behavior, and to participate in the electron transfer to and from the Mo atom (Mendel and Bittner, 2006). Mo-co containing enzymes are ubiquitous in archae, bacteria, and eukaryotes (Williams and Fraústo da Silva, 2002; Zhang and

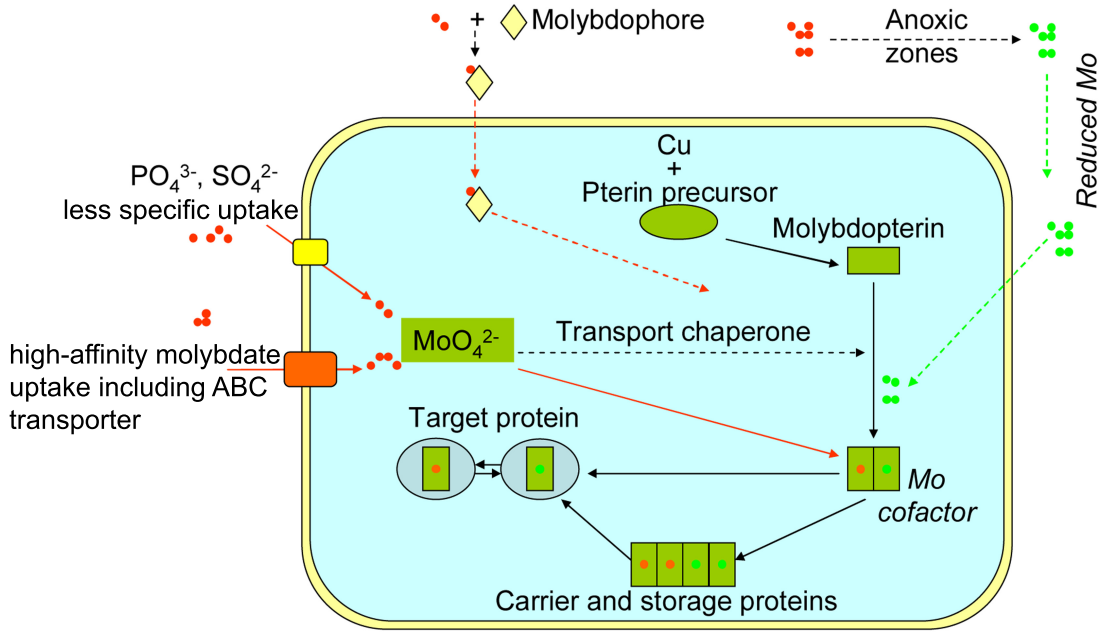

FIGURE 1 | Schematics of Mo uptake and storage in prokaryotic and eukaryotic cells. Routes represented by dashed lines were only identified in prokaryotes, while routes represented by solid lines occurred in both current prokaryotes and eukaryotes. Green dots refer to reduced Mo species, and red dots refer to oxidized Mo (molybdate).

Gladyshev, 2008; Hernandez et al., 2009), including four families: xanthine oxidase, aldehyde oxidoreductase, sulfite oxidase, and dimethylsulfoxide reductase. This mini review will only discuss a few Mo-co-containing enzymes critical in the cycling of sulfur and nitrogen. Among them, sulfite oxidase catalyzes the conversion of sulfite to sulfate, which is the terminal step in the metabolism of sulfur-containing compounds (e.g., cysteine and methionine) in bacteria, plants, and mammals (Cramer et al., 1979). Polysulfide reductase, another group of Mo-containing enzymes, converts polysulfide (as sulfur) to H_2S (Stiefel, 1993). Nitrate reductase catalyzes the first step of nitrate reduction during nitrate assimilation for all autotrophs including higher plants and algae (e.g., Eppley et al., 1969; Butler et al., 1999; Campbell, 1999; Morozkina and Zvyagilskaya, 2007).

Mo may exist in several different redox states in these enzymes: e.g., oxidized Mo(VI), intermediate Mo(V), and reduced Mo(IV) forms. Two electron transfer or one oxygen transfer reactions are coupled with Mo(IV) oxidation to Mo(VI), and the active Mo(IV) state is regenerated by two subsequent one-electron transfer reactions through the intermediate Mo(V) state (Kisker et al., 1997). Those one-electron transfer reactions are carried out by switching the redox pairs: Mo(IV)/(V) or Mo(V)/(VI). The intermediate Mo(V) can act as an interface between one- and two-electron redox reactions, and catalyzes a variety of reactions using water or H_2S as the electron donor (Hille, 1999, 2002). Mo(V) is generally produced from Mo(IV) by transferring a reducing equivalent or from Mo(VI) by accepting an electron (e.g., Barber et al., 1987). Hence, Mo(V) levels in cells could account for as high as 50% of the total intracellular Mo (Hille and Massey, 1985).

INVOLVEMENT OF REDOX SPECIATION OF Mo IN THE EVOLUTION OF LIFE

In the present oxygenated ocean, reduced Mo species have only been confined to specific niches including cytoplasm (e.g., Hille and Massey, 1985), sulfidic mats, and some reducing waters (Wang et al., 2009, 2011). These reduced Mo species might, however, be abundant in the ancient reducing ocean, e.g., in the ferruginous Archaean and the sulfidic Proterozoic (**Figure 2**). The existences of diverse Mo redox species probably facilitated the emergences of Mo enzymes (or prototypes) catalyzing metabolic reactions in the cycling of carbon, nitrogen, and sulfur, and finally the evolution of bacteria and eukaryotes, which possess Mo enzymes (**Figure 2**).

The Archaean ocean (3.5 $\sim$ 2.2 billions years ago) was generally characterized by reduced species including NH_4^+, Fe(II), and a small amount of HS^- (Zerkle et al., 2005; Fani and Fondi, 2009). At this stage, Mo was mostly released from volcanoes and/or hydrothermal vents (Nisbet, 2000). Different redox species (IV, V, and VI) probably coexisted together under such reducing conditions. MoS_2 has an extremely low solubility in aqueous solutions (Ksp $= 10^{-43}$, Garrels and Christ, 1965), and MoS_4^{2-} is easily adsorbed onto mineral particles and organic materials (Helz et al., 1996). Both processes resulted in low levels of the total dissolved Mo, and instead increased the proportion of Mo(V). The existence of intermediate Mo(V), and redox switching of Mo, therefore, faciliated the elctron transfer at this stage, and were essential in functioning of Mo-co containing enzymes, catalyzing certain reactions for carbon, nitrogen, and sulfur cycling since reduced Mo(V) are essential in N_2 fixation (Griffin, 1975; Howarth and Cole, 1985; Yamazaki and Gohda, 1990). Yandulov and Schrock (2003) reported that all reduced species of Mo (+II

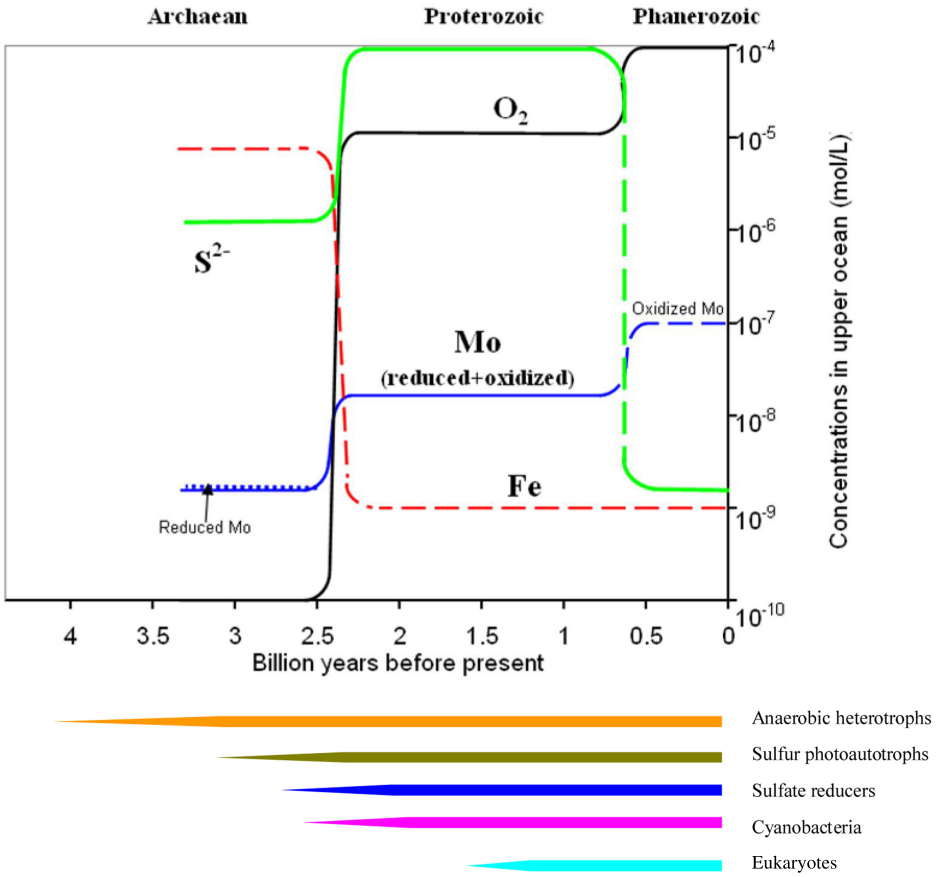

FIGURE 2 | Evolution of the Mo redox dynamics along with key elements of O, S, and Fe in the ocean over the Earth's history. Evolutionary sequence of major life forms is shown at the lower panel. Note: the detailed and complex evolution of both chemical composition and life forms are smoothed out for simplicity.

to +V) are involved in catalytic reactions of N_2 fixation in nitrogenase. As all Mo redox species (+II to +VI) probably existed in the Archaean ocean, the abundance of CO_2 and N_2, and lack of NH_4^+ finally prompted the emergence of Mo nitrogenase, which efficiently fixes atmospheric N_2 to bioavailable NH_4^+. In the late Archaean ocean, as the source of electrons for the photosynthesis switched from HS^- to H_2O for increased energy production, photosynthetically produced O_2 increased accordingly (e.g., Anbar and Knoll, 2002; **Figure 2**) until a slight oxygenation of the atmosphere occurred gradually between 2.4 and 2.2 billions years ago (e.g., Farquhar et al., 2000; Kasting and Siefert, 2001; **Figure 2**).

In the Proterozoic ocean (2.2 ~ 0.6 billions years ago), terrestrial input of SO_4^{2-} along with MoO_4^{2-} predominated due to the slightly increased atmospheric pO_2 (e.g., Anbar and Knoll, 2002). Paradoxically, sulfate-reducing bacteria also developed, and a sulfidic Proterozoic ocean was, therefore, formed at least near the shelves (e.g., Saito et al., 2003). The redox reactions of Mo between Mo(VI) and Mo(IV) at this stage were likely mediated by sulfur photoautotrophs and sulfate reducers (Anderson and Spencer, 1949). With the further increase of atmospheric pO_2 in the late Proterozoic, reduced species of Mo were only confined to limited niches including sulfidic waters/sediments and microzones, whilst all oxyanions including nitrate and sulfate became abundant in the ocean. New Mo uptake and storage systems evolved in order to efficiently utilize the ambient molybdate via either high-affinity uptake or less-specific uptake. A series of Mo-co-containing enzymes were newly formed (e.g., Zerkle et al., 2005) to utilize the abundant sulfate and nitrate (e.g., Nicholas et al., 1963; Scott et al., 2008; Wille et al., 2008). New eukaryotes with more efficient molybdate uptake systems (Thiel et al., 2002; Zahalak et al., 2004) and specific storage proteins protecting the sensitive reduced Mo within cytoplasm eventually evolved along with eukaryotes on Earth probably about 1.5–1.0 billion years ago (e.g., Ba et al., 2009).

SUMMARY

Mo has been considered as one of the most important elements dictating the evolution of life. This mini-review summarized the current findings regarding redox speciation of Mo in natural waters. The contemporary observations of reduced Mo led to the hypothesis that these reduced Mo also existed in the ancient reducing ocean (e.g., in the ferruginous Archaean and sulfidic Proterozoic). The versatile redox chemistry of Mo ranging from

+II to +VI facilitates electron transfer and even oxygen transfer in reactions of carbon, nitrogen, and sulfur in biology. Similarly, redox switching of Mo might be essential in the evolution of Mo enzymes catalyzing different electron and oxygen transfer reactions.

In the ferruginous Archaean, reduced Mo such as Mo(V) might fundamentally contribute to the metabolic reactions of nitrogen and sulfur by forming nitrogenases and other Mo-containing enzymes. In the sulfidic Proterozoic, redox switching of Mo probably coupled with the sulfur cycling initially. The further increasing of photosynthetically produced O_2 constrained reduced Mo only within specific niches including microzones, cytoplasm, and reducing sediments/waters. New eukaryotes with active uptake and storage systems developed in order to utilize oxidized molybdate, and a series of Mo-containing enzymes for nitrate assimilation and sulfur detoxification also evolved later on.

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Potential trace metal co-limitation controls on N_2 fixation and NO_3^- uptake in lakes with varying trophic status

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The response of N_2 fixation and NO_3^- uptake to environmental conditions and nutrient enrichment experiments in three western U.S. lake systems was studied (eutrophic Clear Lake; mesotrophic Walker Lake; oligotrophic Lake Tahoe). We tested the effect of additions of bioactive trace metals molybdenum as Mo(V) and iron (Fe) as well as phosphate (P), N_2 fixation, NO_3^-, carbon (C) fixation, chlorophyll *a* (Chl*a*), and bacterial cell counts under both natural conditions and in mesocosm experiments. We found distinct background N_2 fixation and NO_3^- uptake rates: highest at Clear Lake (N_2 fixation: 44.7 ± 1.8 nmol N L^{-1} h^{-1}), intermediate at Walker Lake (N_2 fixation: 1.7 ± 1.1 nmol N L^{-1} h^{-1}; NO_3^- uptake: 113 ± 37 nmol N L^{-1} h^{-1}), and lowest at Lake Tahoe (N_2 fixation: 0.1 ± 0.07 nmol N L^{-1} h^{-1}; NO_3^- uptake: 37.2 ± 10.0 nmol N L^{-1} h^{-1}). N_2 fixation was stimulated above control values with the addition of Fe and Pin Clear Lake (up to 50 and 63%, respectively); with Mo(V), Fe, and P in Walker Lake (up to 121, 990, and 85%, respectively); and with Mo(V) and P in Lake Tahoe (up to 475 and 21%, respectively). NO_3^- uptake showed the highest stimulation in Lake Tahoe during September 2010, with the addition of P and Mo(V) (~84% for both). High responses to Mo(V) additions were also observed at some sites for C fixation (Lake Tahoe: 141%), Chl*a* (Walker Lake: 54% and Clear Lake: 102%), and bacterial cell counts (Lake Tahoe: 61%). Overall our results suggest that co-limitation of nutrients is probably a common feature in lakes, and that some trace metals may play a crucial role in limiting N_2 fixation and NO_3^- uptake activity, though primarily in non-eutrophic lakes.

Keywords: N_2 fixation, NO_3^- uptake, lake, trace metals, molybdenum

INTRODUCTION

Nitrogen (N) plays a critical role in the productivity of many aquatic ecosystems. N occurs in a variety of inorganic forms in the environment (e.g., N_2, NH_4^+, NO_3^-) and as various organic molecules in living cells (e.g., amino and nucleic acids). Although N is the most abundant element in the atmosphere, its bioavailability depends largely on a suite of transformations catalyzed primarily by microbes (Glass et al., 2009). Two of the most important processes in the N cycle of lakes are N_2 fixation and NO_3^- assimilation. Only certain prokaryotes (at a large energetic expense of 16 ATP) can fix N_2 into $2NH_3$, which is subsequently assimilated through the Glutamine Synthetase-Glutamine OxoGlutarate Aminotransferase (GS – GOGAT) pathway (Meeks et al., 1978; Carpenter et al., 1992; Glass et al., 2009). Alternatively, both prokaryotes and some eukaryotes can enzymatically reduce NO_3^- to NO_2^- (Stolz and Basu, 2002) followed by reduction to NH_4^+which is also assimilated through GS – GOGAT (Muro-Pastor et al., 2005).

It has been postulated that cyanobacterial N_2 fixation in lakes is largely controlled by dissolved inorganic N availability (Hyenstrand et al., 1998; Ferber et al., 2004). However, in many eutrophic lakes, it is the relative availability of phosphorus (P) and N which has generally been found to control N_2-fixing populations (Schindler, 1977; Tilman et al., 1982), with substantial N_2 fixation occurring where N is more severely limiting than P. However, other studies have found no relationship between N_2 fixation and N:P levels (Toetz and McFarland, 1987; Smith, 1990). NO_3^- assimilation is thought to be regulated primarily by light (for energy and as a reductant) and nutrients such as P and/or iron (Fe) (Dodds and Priscu, 1990; Tanigawa et al., 2002; Vasquez-Bermudez et al., 2003; Gardner et al., 2004; Nydick et al., 2004; Ivanikova et al., 2007).

Many of the key enzymatic reactions in the N cycle require not only major nutrients (e.g., P) but also trace metals such as Fe and molybdenum (Mo) for their synthesis and activity (Falkowski, 1983; Blanco et al., 1989; Dos Santos et al., 2004; Berges and Mulholland, 2008; Glass et al., 2009). Biological N_2 fixation and NO_3^- assimilation are catalyzed by Mo- and Fe-containing enzymes (nitrogenases and NO_3^- reductases, respectively; Sigel and Sigel, 2002). Although non-Mo nitrogenases are known (Bishop and Joergert, 1990; Bishop and Premakumar, 1992), Mo-containing nitrogenases are predominant in most environments studied. However, more than a single trace element can limit different biochemical reactions (Saito et al., 2008), suggesting that co-limitation by trace metals, in addition to or in combination with some inorganic nutrients, can regulate biological processes.

In freshwater environments Fe is usually found at higher concentrations (in μM to mM concentration range; Vrede and Tranvik, 2006; Lofts et al., 2008; Warnken and Santschi, 2009) whereas

Mo is generally very low (in the nM range; Cole et al., 1993; Magyar et al., 1993; Johannesson et al., 2000; Wang et al., 2009; Glass et al., 2012). The availability of Mo has been previously shown to be important in controlling primary productivity and NO_3^- reduction in a meso-oligotrophic lake (Goldman, 1960; Axler et al., 1980; Glass et al., 2012) in contrast to P-sufficient lakes where Mo-additions have little effect on phytoplankton standing crop (Evans and Prepas, 1997). Thus, there is some evidence for a relationship between Mo availability and N_2 fixation and NO_3^- assimilation in lakes. However, in none of these studies was the speciation of Mo considered, and experimental Mo amendments were always made with the hypothetically less-bioavailable chemical form of Mo, as Mo(VI) molybdate (Howarth et al., 1988b). Co-limitation by reduced Mo [i.e., Mo(V)] may explain why N_2 fixation often does not occur in oligotrophic lakes, despite the presence of potentially N_2-fixing cyanobacteria (Ferber et al., 2004).

The main objective of our study was to determine major nutrient controls on N_2 fixation and NO_3^- uptake in three western U.S. lakes with varying trophic status (oligotrophic Lake Tahoe; mesotrophic Walker Lake; and eutrophic Clear Lake). We also studied nutrient controls on bacterial cell counts, chlorophyll *a* (Chl*a*), and carbon (C) fixation as proxies for bacterial growth, phytoplankton biomass, and total CO_2 fixation, respectively. Although many factors can control the N cycle (e.g., wind, turbulence, temperature, grazing), here we concentrate on the nutrient that can regulate the biochemical reactions of the N_2 fixation and NO_3^- uptake pathways. While previous studies have shown the important role of Fe and P in the N cycle of lakes, the dynamics of these nutrients cannot always explain observed rates of N_2 fixation (e.g., Axler et al., 1980; Ferber et al., 2004). Therefore, our study investigates not only Fe and P controls on N_2 fixation and NO_3^- uptake but also trace metal co-limitation, including the potentially most-bioavailable form of Mo, Mo(V). The results obtained in our study provide a new perspective on nutrient co-limitation in the N cycle of lakes.

MATERIALS AND METHODS

STUDY SITES

The lakes studied are situated in California and Nevada, USA (**Figure 1**; **Table 1**). They were chosen to represent contrasting limnological conditions, algal assemblages, and diazotrophic populations with varying trophic status expected to influence the N cycling. Lake Tahoe (39°03′N, 122°48′W) is ultra-oligotrophic with the smallest forested watershed (800 km^2), greatest maximum depth (505 m), and largest area (500 km^2) of the lakes studied. It is located in the Sierra Nevada range at an elevation of 1898 m. Over the last several decades, human development in the Tahoe Basin has increased its primary productivity (5% annually), decreased its clarity (0.25 $m\,y^{-1}$) and shifted it from a strongly N limited system toward more P limited as NO_3^- in the lake has

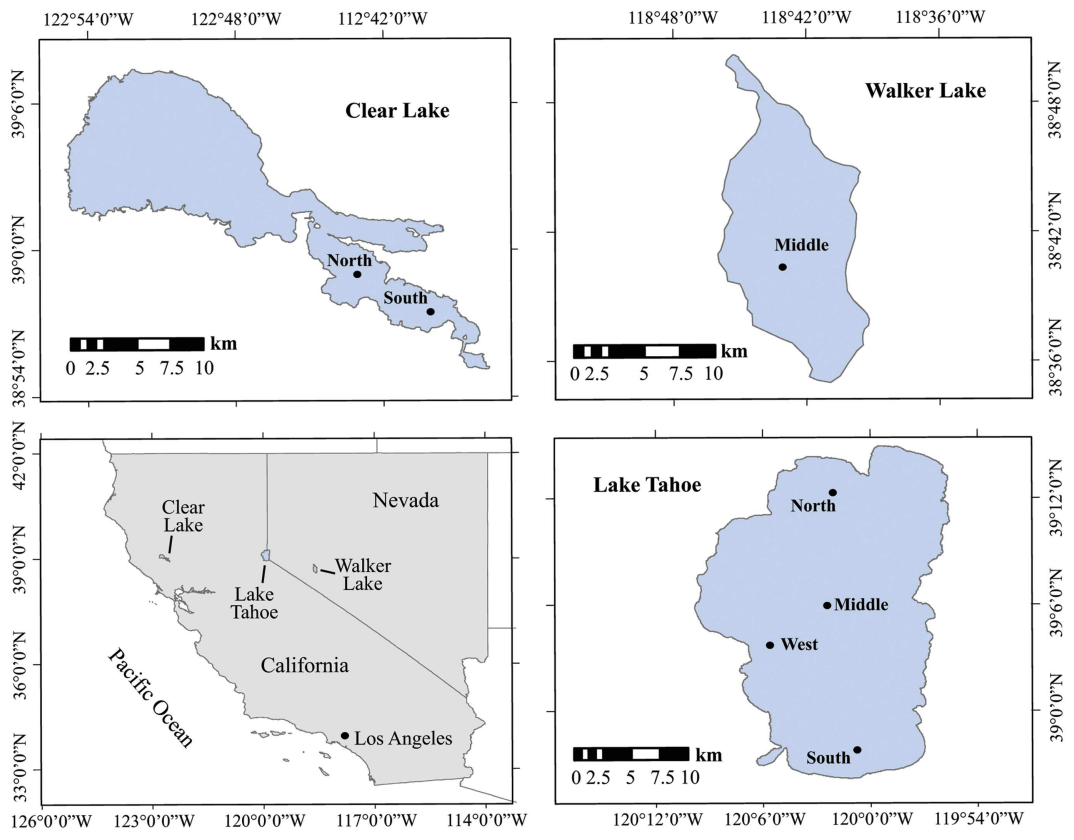

FIGURE 1 | Location and sampling stations of the lakes studied in California and Nevada. Black dots represent the sampling stations.

increased (Goldman, 1988, 2000; Reuter et al., 2003). N_2 fixation research in Lake Tahoe has been focused on periphyton communities estimated to account for about 72% of the total dissolved inorganic nitrogen assimilated (Reuter et al., 1983). Walker Lake (39°05′N, 120°03′W) is a terminal monomictic and mesotrophic lake (Sharpe et al., 2008) with the lowest O_2 concentration values of the lakes studied. It is located within a topographically closed basin in west-central Nevada at an elevation of 1300 m. Walker Lake is P rich and N limited. Thermal stratification occurs during summer with blooms of the cyanobacterium *Nodularia spumigena*, which dominates the phytoplankton (Horne et al., 1994). Clear Lake (38°41′N, 118°44′W) is eutrophic with the shallowest depth of the lakes studied. It is located in the northern Coastal Range of California at an elevation of 402 m. Previous limnological studies and N_2 fixation measurements have been conducted in this lake since the early 1970s. N_2 fixation is largely associated with cyanobacteria of the genera *Aphanizomenon* and *Anabaena* (Horne and Goldman, 1972).

Table 1 | General characteristics of the lakes studied (Lake Tahoe, Walker Lake, and Clear Lake).

Feature	Lake Tahoe	Walker lake	Clear lake
Surface area (km^2)	500	130	170
Mean depth (m)	313	22	10
Max. depth (m)	505	26	18
Elevation (m)	1898	1300	402
Trophic status	Oligotrophic	Mesotrophic	Eutrophic
O_2 ($mg\,L^{-1}$)	9	8	9
Salinity ($g\,kg^{-1}$)	0	17	0
TP (μM)	21 (10–40)	5030 (4729–5331)	275 (246–303)

TP, total phosphorus.

Data shown as arithmetic mean (range).

FIELD SAMPLING

From 2009 to 2010 water was collected from the three lakes at different times with distinct limnological conditions likely to influence trace metal availability, as well as N_2 fixation and NO_3^- uptake. Sampling campaigns were conducted from April (late spring) to October (early fall) to capture periods of high abundance of phytoplankton and nutrients in the water column. Due to logistical constraints, most of our sampling effort was limited to the first 10 m depth (Lake Tahoe: October 2009, September 2010; Walker Lake: April 2010, September 2010; Clear Lake: July 2010). On several occasions we were able to obtain samples from >10 m depth (Lake Tahoe: October 2009; Walker Lake: October 2009).

Surface water samples were collected using a trace-metal-clean Teflon pumping system followed by in-line filtration through a 0.45 μm acid-washed polypropylene capsule filter. In Lake Tahoe, samples from depth profiles were also collected in September 2010 using a pre-programed rosette with trace-metal-clean Niskin bottles. Separately, hydrographic profiles were obtained using a YSI temperature/conductivity sensor (September 2010). The analyses of background water chemistry allowed us to establish the ambient levels of major and minor nutrients.

NUTRIENT ENRICHMENT EXPERIMENTS

The potential limitation of C and N_2 fixation, and NO_3^- uptake by Mo(V), Fe, and/or P was assessed in 45 h incubations, relative to unenriched controls. Incubations for 24 h were conducted as well but no response was observed in any treatment or lake tested (data not shown). Total incubation volumes for the sampling campaigns were about 18 L (trace-metal-cleaned cubitainers) per treatment, including two to three replicates. All cubitainers were acid-cleaned (10% HCl) and rinsed with *in situ* water before use. We spiked with Mo(V) (see Wang et al., 2009 for details on solution preparation), Fe (as iron sulfate; $FeSO_4$), and P (as monobasic potassium phosphate; KH_2PO_4). Final concentrations for the additions were 240–300 nMMo(V), 242–276 nM Fe, and 292–316 nM PO_4^- which varied according to background concentrations measured previously and environmental conditions at the time of sampling in each lake (**Table 2**). Samples were incubated under ambient temperature conditions (**Table 2**) in a temperature-controlled incubator (Lake Tahoe and Walker Lake) or performed *in situ* with cubitainers suspended in the water column at an approximate depth of 0.5 m (Clear Lake). All samples for experiments were collected from the surface of the water column (0.5 m). Because incubation conditions varied among experiments conducted at each lake, treatment responses were normalized to the control (Ctrl) samples (see below for details). At the beginning (Ctrl treatment) and end (all treatments) of each experiment, samples for C and N metabolism, Chl*a*, and bacterial counts were taken from all incubation vessels and processed as described below.

TRACE METALS AND NUTRIENT ANALYSES

Water samples were collected in the field following trace-metal-clean protocols, acidified to pH < 2 with 6 N quartz-distilled HCl (Optima-grade) and stored for at least a month prior to analysis (Sañudo-Wilhelmy and Flegal, 1996) by inductively coupled plasma-mass spectrometry (ICP-MS). By ICP-MS, we determined the total dissolved concentrations of total P (hereafter abbreviated TP) and trace metals (Al, Ti, V, Mn, Fe, Co, Ni, Cu, Cd, Ba, and Pb). The chemical speciation of molybdenum [Mo(V) and Mo(VI)] was measured according to the technique described by Wang et al. (2009). ICP-MS limits of detection (LOD) ranged from 0.8 pM for Cd to 250 pM for TP, and individual element concentrations for all samples were a minimum of one order of magnitude greater than their respective LODs. The LOD for Mo and Mo(V) was 2.8 pM.

Major nutrient samples were collected from the field (1–2 replicates per depth) and the nutrient enrichment experiments (three replicates per treatment), and stored at −20°C. NO_3^- plus NO_2^- were determined spectrophotometrically after reduction of NO_3^- with spongy cadmium using a 10-cm optical path length (detection limit of 0.03 μM NO_3^-; Jones, 1984). PO_4^- was determined spectrophotometrically with a 10-cm optical path (detection limit of 0.03 μM PO_4^-; Strickland and Parsons, 1972).

Water samples for particulate C and N analysis were collected from the field sampling (2–3 replicates per depth) and the nutrient enrichment experiments (three replicates per treatment), filtered (pre-combusted GF/F, 0.7-μm pore size) and stored frozen (−20°C). GF/F filters were dried for 24 h at 60°C. Particulate C and N analyses were performed on a mass spectrometer (see below) at the University of Southern California.

Table 2 | Parameters measured at different stations during the sampling campaigns in Lake Tahoe, Walker Lake, and Clear Lake.

	Lake Tahoe										Walker lake			Clear lake		
	October 2009								September 2010		April 2010		September 2010	June 2010		
	North	South		Middle		West			North	South	Middle		Middle	North		South
Depth (m)	0.5	0.5	0.5	50	70	0.5	50	70	0.5	0.5	0.5	20	0.5	0.5	10	0.5
Salinity (ppt)	0	0	0	0	0	0	0	0	0	0	22	22		0	0	0
Temp. (C)	14	14	14			14			21	19	12	11	21	23	20	23
TP (nM)	28	48	9.9 (0.20)	31 (8.1)	9.2 (1.2)	10 (1.5)	14 (1.2)	15 (1.2)	25 (1.0)	40 (0.80)	5300 (21)	5600 (27)	4700 (49)	250 (5.6)	910 (20)	300 (11)
PO_4^{3-} (μM)	0.77	1.1	0.80	0.82 (0.040)	0.85 (0.010)	0.83	0.85 (0.030)	0.85 (0.040)	0.86 (0.080)	1.0 (0.11)	25	27	23 (1.2)	0.30	2.1	0.48
NO_3^- (μM)	0.14		0.35	0.38	1.5				0.37 (0.17)	0.55 (0.040)	0.61 (0.030)	1.3 (0.010)	0.37 (0.41)	0.54	0.39	0.20 (0.13)
NO_2^-(μM)	<LOD		0.026	0.020	<LOD				0.0059 (0.0048)	0	0.022 (0.11)	0.043 (0.046)	0.060 (0.0061)	0.037	0.041	0.0075 (0.0028)
C:N (mol)			12 (1.8)			8.9			8.3 (0.70)	8.5 (0.60)	9.9 (0.10)		11 (0.90)			
Total Mo (nM)	35.5	28.1	35.6 (0.102)	36.4 (1.77)	33.5 (0.696)	34.3 (0.535)	33.4 (0.536)	34.7 (0.633)	32.3 (0.102)	32.7 (0.128)	3020 (15.0)	3100 (27.3)	33300 (29.6)	3.16 (0.0230)	3.19 (0.0623)	3.44
Mo(V) (pM)	<LOD	<LOD	<LOD	514 (11.4)	512 (22.8)	<LOD	484 (30.2)		<LOD	<LOD	410 (283)	6190 (180)	<LOD	388 (10.8)	305 (8.71)	321 (11.1)
Mo(VI) (nm)	35.5	28.1	35.6	35.9	33.0	34.3	32.9	34.7	32.3	32.7	3020	3090	33300	2.77	2.89	3.12
Al (nm)	45	78	5.6 (0.61)	4.9 (2.6)	2.7 (5.9)	15 (5.4)	20 (6.2)	20 (2.2)	6.5 (0.10)	19 (0.16)	1400 (12)	2700 (3.7)	110 (1.1)	24 (0.47)	17	5300 (56)
Ti (nm)	0.050	1100	0.080 (0.0050)	0.12 (0.010)	0.030 (0.024)	0.18 (0.066)	0.26 (0.080)	0.34 (0.089)	0.25 (0.017)	1.6 (0.21)	22 (0.70)	25 (0.77)	17 (1.3)	3.1 (0.29)	2.4 (0.50)	15 (2.2)
V (nm)	13	9.0	13 (0.074)	12 (0.39)	11 (0.33)	12 (0.35)	12 (0.26)	12 (0.44)	12 (0.20)	12 (0.12)	96 (0.40)	96 (0.70)	98 (0.74)	23 (0.19)	20 (0.21)	23 (0.49)
Mn (nm)	4.7	10	0.78 (0.082)	0.34 (0.034)	0.25 (0.15)	1.2 (0.19)	0.42 (0.050)	0.39 (0.026)	1.9 (0.022)	9.2 (0.055)	36 (0.26)	36 (0.15)	14 (0.43)	52 (0.70)	38 (0.43)	35 (0.20)

(Continued)

Table 2 | Continued

	Lake Tahoe										Walker lake			Clear lake		
	October 2009								September 2010		April 2010		September 2010	June 2010		
	North	South		Middle		West			North	South	Middle		Middle		North	South
Fe (nm)	18	150	2.3 (0.18)	1.6 (0.29)	0.91 (0.31)	9.4 (0.15)	14 (5.6)	18 (7.6)	4.1 (0.076)	120 (2.6)	150 (1.2)	110 (1.0)	92 (0.21)	110 (2.9)	74 (1.7)	180 (5.9)
Co (nm)	0.066	0.10	0.024 (0.0016)	0.021 (0.011)	0.011 (0.0036)	0.020 (0.0042)	0.026 (0.044)	0.027 (0.0060)	0.13 (0.0078)	0.12 (0.0080)	2.1 (0.046)	2.2 (0.25)	1.7 (0.12)	0.57 (0.10)	0.50 (0.16)	0.74 (0.21)
Ni (nm)	0.51	0	0.66 (0.067)	0.54 (0.16)	0.29 (0.016)	0.088 (0.083)	0.34 (0.11)	0.44 (0.11)	1.5 (0.16)	6.9 (0.21)	1.5 (0.056)	2.2 (0.053)	1.0 (0.061)	16 (0.084)	16 (0.41)	17 (0.28)
Cu (nm)	2.2	1.7	0.75 (0.10)	0.71 (0.4)	0.65 (0.38)	0.71 (0.38)	0.87 (0.11)	1.0 (0.16)	5.9 (0.047)	20 (0.42)	5.6 (0.15)	6.0 (0.07)	4.4 (0.14)	8.5 (0.22)	3.2 (0.30)	16 (0.023)
Cd (pm)	0.032	0.044	0.080 (0.0033)	0.056 (0.015)	0.042 (0.48)	0.052 (0.41)	0.060 (0.11)	0.056 (0.16)	0.039 (0.0012)	0.044 (0.0029)	3.4 (0.10)	3.5 (0.020)	3.8 (0.064)	0.024 (0.026)	0.023 (0.0012)	0.072 (0.05)
Ba (nM)	440	230	100 (35)	130 (13)	120 (7.4)	140 (24)	110 (1.2)	110 (4.5)	80 (1.1)	81 (0.90)	630 (0.11)	340 (0.13)	820 (0.12)	520 (4.1)	660 (13)	1100 (96)
pb (pm)	0.054	0.059	0.0075 (0.0016)	0.031 (0064)	0.0089 (0.0026)	0.011 (0.038)	0.088 (0.035)	0.074 (0.033)	0.13 (0.10)	0.44 (0.14)	0.69 (5.2)	0.56 (0.50)	0.18 (1.5)	0.14 (0.10)	0.47 (0.0016)	0.81 (0.0060)
NO_3^- uptake nmol C L^{-1} h^{-1}			30.3 (0.629)			30.4			26.7 (0.924)	49.1 (16.8)	69.4 (15.3)		136 (18.7)			
N2 fixation nmol C L^{-1} h^{-1}	0.0071 (0.012)	0.022 (0.025)	0.051 (0.088)			0.0020 (0.0031)			0.051 (0.044)	0.28 (0.24)	1.2 (0.088)		2.4 (1.2)	41 (2.6)		48 (3.1)
C Fixation nmol C L^{-1} h^{-1}			0.44 (0.00042)			11			94 (15)	210 (72)	34 (4.4)		520 (61)			
Chla L^{-1}	0.81 (0.68)		0.48 (0.15)	1.3 (0.20)	2.2 (0.15)	0.35 (0.01)	2.0 (0.11)	1.0 (0.045)	0.38 (0.02)	0.38 (0.066)	5.5 (0.075)		4.6 (0.65)	32 (7.3)		26 (9.5)
Bacteria (μg) (10^6 cells L^{-1})	0.7 (0.14)		1.1 (0.12)	1.2 (0.11)	0.85 (0.022)	0.99	1.0	0.56	0.20 (0.57)	0.20 (0.036)	2.9		3.8 (0.22)	2.4		4.0

Data shown as arithmetic mean (SE).

<LOD, below the limit of detection (see Materials and Methods for details).

PHYTOPLANKTON CHL*a* AND BACTERIAL COUNTS

Water samples for phytoplankton Chl*a* and bacterial counts were collected from the field sampling (1–2 replicates per depth) and nutrient enrichment experiments (three replicates per treatment). Samples for phytoplankton Chl*a* (0.5–1.0 L) were collected and immediately filtered (pre-combusted GF/F, 0.7-μm pore size) and stored at −20°C. Fluorometric measurements of extracted Chl*a* were taken following established protocols (Arar and Collins, 1997).

Samples for bacterial counts (40 mL) were collected in sterile centrifuge tubes and stored at 4°C with 2% formalin (pre-filtered 0.02-μm pore size). Samples were processed for SYBR Green I microscopy following established protocols (Noble and Fuhrman, 1998; Patel et al., 2007). Slides (2 mL preserved sample) were observed using epifluorescence microscopy, and 10 microscopic fields were counted for each slide. Samples were counted in duplicate.

C AND N METABOLISM

Water samples for C and N metabolism were collected from the field sampling (2–3 replicates per depth) and the nutrient enrichment experiments (three replicates per treatment). N_2 fixation was determined by the acetylene reduction approach (Montoya et al., 1996; Capone and Montoya, 2001). Briefly, water samples (100 mL) were incubated in 160 mL bottles with acetylene (20%) for ∼8 h, and ethylene concentration in the gas samples was determined by comparison with standards on a FID Shimadzu GC-9A gas chromatograph. The acetylene reduction rates calculated were converted to N_2 fixation rates assuming the conversion factor of 4:1 (C_2H_2:N_2; Montoya et al., 1996).

NO_3^- uptake was determined by $^{15}NO_3^-$ incorporation (Glibert and Capone, 1993) in 1-L polycarbonate bottles that were amended with $^{15}NO_3^-$ (99 atom%, Sigma-Aldrich/Isotec $^{15}NO_3^-$ was 4–12% of NO_3^- background) and capped. Also, in the same incubation bottles, C fixation was determined by ^{13}C-bicarbonate incorporation (Hamersley et al., 2011) by adding $NaH^{13}CO_3$ (99 atom%, Sigma-Aldrich/Isotec $NaH^{13}CO_3$ was 0.02–3% of dissolved inorganic background) into the capped 2-L and 1-L polycarbonate bottles. Twenty-four hour incubations were ended by filtration into pre-combusted GF/F filters (0.7-μm pore size) and stored at −20°C. Additional duplicate bottles were filtered immediately after adding the tracers as time zero controls. No significant change was observed in the isotopic composition of C and N of particulate matter immediately after tracer injection. GF/F filters were dried for 24 h at 60°C. Mass spectrometric analysis was performed on a Micromass IsoPrime mass spectrometer at the University of Southern California. Rates were calculated by isotope mass balance as described by Montoya et al. (1996). NO_3^- uptake and C fixation rates were not measured in Clear Lake due to technical problems.

DATA ANALYSIS

Experimental responses to nutrient additions for C fixation, N_2 fixation, NO_3^- uptake, Chl*a*, and bacterial counts were normalized to the Ctrl values in each experiment to facilitate comparison among the lakes at each sampling campaign. Responses were calculated as: ((treatment mean−Ctrl mean) × (Ctrl mean)$^{-1}$ × 100).

Linear regression analysis was used to examine the influence of *in situ* environmental parameters on C and N_2 fixation, NO_3^- uptake, Chl*a*, and bacteria counts in the lakes studied (JMP Software version 8.0, SAS Institute Inc., Cary, NC, USA). All data were transformed ($\log_{10}$) to stabilize the variance and normalize the distribution of each variable. Level of statistical significance was set to <0.05.

RESULTS

LAKE-SPECIFIC ENVIRONMENTAL CONDITIONS

Sampling campaigns from April (late spring) to October (early fall) captured the periods of high abundance of phytoplankton and nutrients in the water column in the lakes studied. Distinct limnological conditions among the lakes were observed that were likely influenced trace metal availability, as well as biological activity in the surface waters (**Table 2**).

Lake Tahoe is ultra-oligotrophic and during this study it showed the lowest values among all lakes studied for concentrations of TP, nitrogen ($NO_3^- + NO_2^-$), Mo(V), and all other metals analyzed (**Table 2**). Spatial variability was observed in this lake, with higher concentrations of TP and some metals (Al, Ti, Mn, Fe, Co, Cu, and Ba) in the north and south stations. Although water temperature ranged from 14 to 20°C (October 2009 and September 2010, respectively), only NO_3^-, Ni, and Cu increased in September 2010 (**Table 2**).

Walker Lake showed the highest values for TP, PO_4^{3-}, NO_3^-, total Mo, Mo(V), Ti, V, Co, and Cd of the three lakes studied (**Table 2**). During April 2010, samples were collected after a storm which influenced the chemistry of the lake through strong mixing of the water column. This event could explain our finding of higher concentrations of NO_3^- (39%), Mo(V) (100%), Al (92%), Mn (62%), Fe (41%) in April compared to September of 2010.

Although Clear Lake is eutrophic, we observed the lowest values among the lakes studied of $PO_4^{3-}$and total Mo (**Table 2**). A large bloom of diazotrophic cyanobacteria (*Lyngbya*, *Gloeotrichia*, *Aphanizomenon*, and *Anabaena*) and the non-diazotrophic cyanobacterium *Microcystis* was present during June 2010, potentially explaining the observed low values of these nutrients.

BIOLOGICAL VARIABILITY AMONG LAKES

As was expected, oligotrophic Lake Tahoe, having the lowest content of major and minor nutrients, also exhibited the lowest levels of biomass and biological activity (**Table 2**; **Figure 2**). On average bacteria abundance in Walker Lake (3.5×10^6 cells L^{-1}) and Clear Lake (3.2×10^6 cells L^{-1}) was 8× higher than in Lake Tahoe (0.4×10^6 cells L^{-1}). Walker and Clear Lakes also had higher Chl*a* concentrations (5.1 and 29.1 μg L^{-1}, respectively) than Lake Tahoe (0.7 μg L^{-1}) by 10× and 60×, respectively. The highest Chl*a* concentrations observed in Clear Lake (up to 35 μg L^{-1}) were due to the large cyanobacterial bloom during the sampling campaign in June 2010 as noted above.

Background N_2 fixation, NO_3^- uptake, and C fixation rates differed among the lakes studied (**Table 2**; **Figure 2**). Lowest rates were observed at Lake Tahoe (N_2 fixation: 0.10 ± 0.07 nmol N L^{-1} h^{-1}; NO_3^- uptake: 37.2 ± 10.0 nmol N L^{-1} h^{-1}, C fixation: 79.5 ± 18.9 nmol C L^{-1} h^{-1}). Intermediate rates were recorded at Walker Lake (N_2 fixation: 1.7 ± 1.1 nmol N L^{-1} h^{-1};

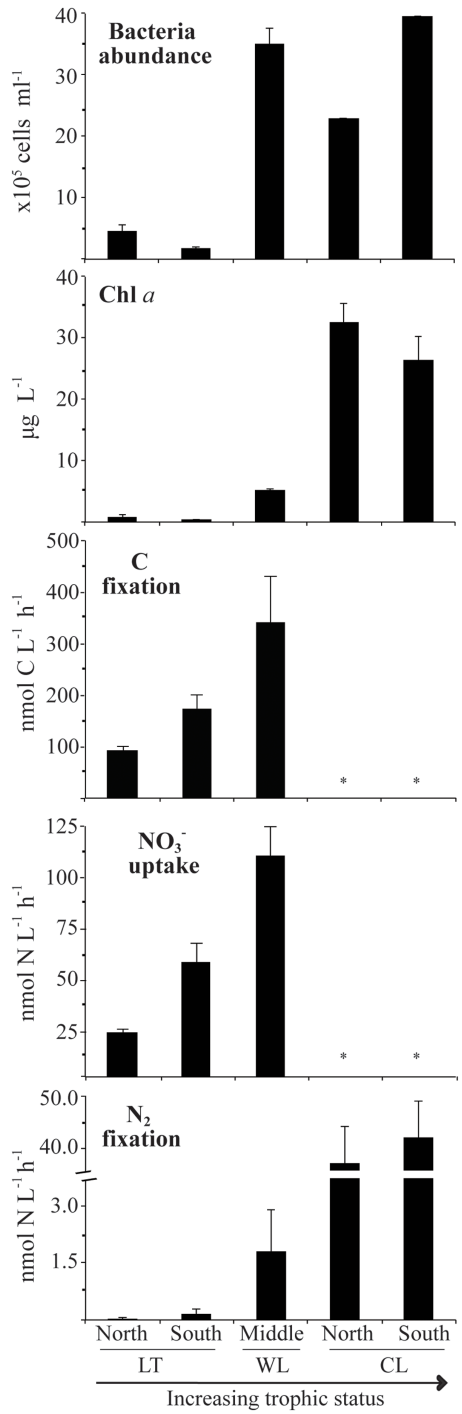

FIGURE 2 | Variability of biological parameters during the period studied from 2009 to 2010 (LT: Lake Tahoe; WL: Walker Lake; CL: Clear Lake). Measurements not determined denoted by *. Water samples were taken from 0.5 m below the surface. Data shown as arithmetic mean ± SE ($N = 6$–9).

NO_3^- uptake: 114 ± 37 nmol N L^{-1} h^{-1}, C fixation: 340 nmol ± 90.9 C L^{-1} h^{-1}). The highest N_2 fixation rates were noted at Clear Lake (N_2 fixation: 44.7 ± 1.8 nmol N L^{-1} h^{-1}). Among all measured variables, N_2 fixation showed the largest differences among the lakes with rates at Walker Lake and Clear Lake 20× and 490× higher (respectively) than in Lake Tahoe. In contrast, NO_3^- uptake and C fixation were only 2–4× higher in Walker Lake than in Lake Tahoe.

RESPONSES TO NUTRIENT ENRICHMENT EXPERIMENTS

Bacterial cell growth was stimulated above controls by all nutrient enrichment treatments in five out of seven experiments independent of the trophic status of the lakes (**Table 3**). Chl*a* was stimulated above control values by Mo(V) and P in Walker Lake in September 2010 (54 and 9%, respectively) and Clear Lake (north station: 102 and 22%, respectively). Fe was stimulatory only once, in June 2010 at Clear Lake at the northern station. Slight stimulation of Chl*a* occurred on three occasions in Lake Tahoe (October 2009, north station by Mo(V) and P; Sept 2010, south station by P).

C fixation was stimulated above control values by Mo(V), Fe, and P in Lake Tahoe in September 2010 at the southern station (141, 138, and 127%, respectively). Slight stimulation by Mo(V) and P occurred at the northern station on this date. NO_3^- uptake was also stimulated above control values with the addition of Mo(V) and P at the southern station in Lake Tahoe (83% up to 85%, respectively), and by Mo(V) in Walker Lake (only in April: 21%). Strong stimulation of N_2 fixation above control values was observed for Mo(V) in Lake Tahoe in both October 2009 and September 2010 for the southern station (up to 475%) and only in October 2009 for the northern station, and in April 2010 for Walker Lake (121%). Fe and P also stimulated N_2 fixation rates above control values in Walker Lake (up to 989 and 86%, respectively) and Clear Lake (up to 51 and 63%, respectively).

NUTRIENT LIMITATION CONTROLS ON BIOLOGICAL PROCESSES

Correlation analysis between biological parameters and *in situ* major and minor nutrient concentrations revealed significant positive correlations between ambient PO_4^- and/or metals and bacterial counts, Chl*a*, NO_3^- uptake, and N_2 fixation but not C fixation (**Table 4**). Bacterial cell counts were significantly positively correlated to Al, Ti, V, Co, and Ba and not to nutrients used in the enrichment experiments (**Table 4**; $P < 0.05$). Chl*a* was also significantly positively correlated to Al, Ti, and Co, and, also to Mn and the calculated ratio Mo(V):PO_4^- (**Table 4**; $P < 0.05$). A relationship between Chl*a* and nutrients such as Mo(V) and PO_4^- was also observed in the enrichment experiments in Tahoe Lake (October 2009), Walker Lake (only during September 2010 when nutrient concentrations were low), and Clear Lake (primarily at the northern station where concentration of nutrients were high; **Tables 2** and **3**). NO_3^- uptake was significantly positively correlated to PO_4^-, Mo(VI), Ti, V, Co, Cd, Ba, and Mo(VI): PO_4^- (**Table 4**; $P < 0.05$). A relationship between NO_3^- uptake and PO_4^- was also observed in the enrichment experiments in Lake Tahoe where PO_4^- is low (**Tables 2** and **3**). N_2 fixation was significantly positively correlated to Mo(V), Mn, Ni, Ba, and the calculated ratios Mo(V):Fe and Mo(V): PO_4^- (**Table 4**; $P < 0.05$). A relationship between N_2 fixation and nutrients such as Mo(V), Fe, and PO_4^- was also observed in the enrichment experiments with different responses in each lake (**Table 3**).

Table 3 | Normalized responses (%) of biological parameters to nutrient additions [Mo(V), Fe, P] observed in the three lakes studied at different times.

		Bacteria cells			Chl *a*			C fixation			NO_3^- uptake			N_2 fixation		
Lake/date	**Site**	**Mo (V)**	**Fe**	**P**	**Mo (V)**	**Fe**	**P**	**Mo (V)**	**Fe**	**P**	**Mo (V)**	**Fe**	**P**	**Mo (V)**	**Fe**	**P**
LAKE TAHOE																
October 2009	North	62	87	50	11	−18	11	−64	6	−80	−6	−42	2	281	−55	−1
	South													475	−25	21
September 2010	North	25	40	84	−10	−15	−14	12	−2	26	−2	−8	11	−6	−3	−21
	South	−39	34	9	−7	−4	3	141	138	127	83	−30	85	345	−65	−62
WALKER LAKE																
April 2010	Middle	36	54	43	−20	−6	−8	4	4		21	−66		121	169	11
September 2010	Middle	29	31	10	54	−4	9	−52	−44	−48	−11	−14	−18	−65	989	86
CLEAR LAKE																
June 2010	North	−34	−16	23	102	105	22							13	6	41
	South	1	93	124	−22	−8	29							−12	51	62

Values >0 indicate stimulation relative to controls, while values <0 represent suppression relative to controls.

Table 4 | Correlation analysis between biological parameters and *in situ* nutrient conditions.

	Bacteria cells			chl*a*			C fixation			NO_3^- Uptake			N_2 fixation		
	R	***P*-value**	***n***	***R***	***P*-value**	***n***	***R***	***P*-value**	***n***	***R***	***P*-value**	***n***	***R***	***P*-value**	***n***
PO_4^{3-}	0.448	0.125	13	0.124	0.685	13	0.431	0.394	6	0.882	0.020*	6	−0.111	0.761	10
NO_3^-	−0.096	0.793	10	−0.016	0.966	10	0.210	0.734	5	0.247	0.689	5	0.006	0.990	8
NO_2^-	0.639	0.047	10	0.386	0.270	10	0.114	0.856	5	0.738	0.155	5	0.272	0.515	8
Mo(V)	0.399	0.199	12	0.496	0.101	12	−0.045	0.933	6	0.294	0.572	6	0.766	0.010*	10
Mo(VI)	0.245	0.442	12	−0.179	0.579	12	0.413	0.415	6	0.885	0.019*	6	−0.376	0.284	10
Al	0.622	0.023*	13	0.632	0.020*	13	0.311	0.549	6	0.711	0.113	6	0.537	0.110	10
Ti	0.684	0.010*	13	0.679	0.011*	13	0.526	0.284	6	0.911	0.012*	6	0.569	0.086	10
V	0.684	0.010*	13	0.486	0.092	13	0.406	0.425	6	0.882	0.020*	6	0.305	0.391	10
Mn	0.519	0.069	13	0.763	0.002*	13	0.589	0.219	6	0.798	0.057	6	0.762	0.010*	10
Fe	0.406	0.168	13	0.618	0.054	13	0.664	0.150	6	0.795	0.059	6	0.530	0.115	10
Co	0.683	0.010*	13	0.595	0.032*	13	0.448	0.374	6	0.861	0.028*	6	0.423	0.223	10
Ni	0.233	0.444	13	0.733	0.004*	13	0.534	0.275	6	0.171	0.747	6	0.851	0.002*	10
Cu	0.043	0.890	13	0.491	0.088	13	0.754	0.084	6	0.330	0.523	6	0.578	0.080	10
Cd	0.534	0.060	13	0.225	0.461	13	0.420	0.407	6	0.892	0.017*	6	0.004	0.990	10
Ba	0.747	0.003	13	0.594	0.052	13	0.341	0.508	6	0.867	0.025*	6	0.769	0.009*	10
Fe: PO_4^{3-}	0.128	0.676	13	0.596	0.082	13	0.363	0.480	6	−0.029	0.957	6	0.643	0.045	10
Mo(V):Fe	−0.056	0.863	12	−0.099	0.761	12	−0.045	0.933	6	0.294	0.572	6	0.762	0.011*	10
Mo(V): PO_4^{3-}	0.367	0.241	12	0.686	0.014*	11	−0.045	0.933	6	0.294	0.572	6	0.928	0.000*	10
Mo(VI):Fe	0.124	0.702	12	−0.340	0.279	12	−0.077	0.885	6	0.464	0.354	6	−0.393	0.261	10
Mo(VI): PO_4^{3-}	−0.014	0.965	12	−0.508	0.092	12	0.319	0.537	6	0.853	0.031*	6	−0.641	0.046	10

R, linear correlation coefficient.

P-value, level of statistical significance at $P < 0.05$.

N, number samples.

**Significant correlation coefficient.*

DISCUSSION

We studied C and N metabolism in three western U.S. lake systems between late spring and early fall to capture periods of high abundance of phytoplankton and nutrients in the water column. The lakes studied were characterized by different levels of biological activity (**Table 2**; **Figure 2**) each with a distinct trophic status (eutrophic Clear Lake; mesotrophic Walker Lake; oligotrophic Lake Tahoe) as reported previously (e.g., Wurtsbaugh, 1983; Sharp, 2009; Winder, 2009). Upper water column integration (surface to 5 m) of biological

activity was calculated over all stations and months studied for Lake Tahoe (C fixation: ∼9.5 mmol C m^{-2} $days^{-1}$; N_2 fixation: ∼0.01 mmol N m^{-2} $days^{-1}$; NO_3^- uptake: ∼4.1 mmol N m^{-2} $days^{-1}$), Walker Lake (C fixation: ∼33.4 mmol C m^{-2} $days^{-1}$; N_2 fixation: ∼0.2 mmol N m^{-2} $days^{-1}$; NO_3^- uptake: ∼12.3 mmol N m^{-2} $days^{-1}$), and Clear Lake (N_2 fixation: ∼5.4 mmol N m^{-2} $days^{-1}$). Integrated rates are within the values reported in other lakes for N_2 fixation (Howarth et al., 1988a; Ferber et al., 2004), NO_3^- uptake (Ferber et al., 2004; Kumar et al., 2008; Rojo et al., 2008), and C fixation (Kumar et al., 2008; Piehler et al., 2009; Salm et al., 2009). High N_2 fixation rates in Clear Lake were associated with cyanobacterial blooms as observed previously by Horne and Goldman (1972). There are no previously published N_2 fixation rates for Walker Lake and Lake Tahoe for the water column. In Lake Tahoe, studies have focused primarily on periphyton communities showing high rates for N_2 fixation and NO_3^- uptake due to a large supply of nutrients through microbial remineralization of settled particles (Reuter et al., 1983). In Lake Tahoe, Winder (2009) reported C fixation rates for the upper 60 m water column during July to November (2–3 mmol C m^{-2} $days^{-1}$).

We observed large biological variability in stations sampled during different campaigns as well as in the responses to nutrient additions in our experiments. Experimental responses to Mo(V) additions were positive (stimulation) for N_2 fixation and negative (suppression) for C fixation only during the colder months, while bacterial cell counts showed positive responses during all months studied (**Table 3**). Temporally contrasting responses were also observed in experimental P additions for Chl*a*, and C and N_2 fixation (**Table 3**). Changes in temperature and nutrients, as well as community composition in the water column may be the main factors contributing to the biological variability in our study. Multiple limiting nutrients, competition between different planktonic groups for the same pool of nutrients, stronger limitation by nutrient availability for phytoplankton than bacterioplankton, and seasonal changes in limiting nutrients appear to be common in lakes and other in-land systems (Ahmad, 1981; Marcarelli and Wurtsbaugh, 2006; Kumar et al., 2008; Salmaso, 2011). Thus, our observations show that multiple factors influence the response of planktonic communities to nutrient enrichment.

Overall, we found P was limiting phytoplankton C fixation or biomass accumulation (Chl*a*) in all lakes as well as NO_3^- uptake in Lake Tahoe and N_2 fixation in Walker Lake and Clear Lake (**Table 3**). Also, C fixation and N_2 fixation were limited by Fe in Walker Lake and Clear Lake. Additionally, we observed that Mo(V) was limiting C fixation, N_2 fixation, and NO_3^- uptake in some experiments in Tahoe Lake and Walker Lake, and only Chl*a* in Walker Lake and Clear Lake (**Table 3**). Also, significant correlations between Mo(V): PO_4^{3-} and Chl*a* and N_2 fixation, and Mo(VI): PO_4^{3-} and NO_3^- uptake were observed using *in situ* data. In lake systems it is well known that P alone and in combination with other nutrients (e.g., N, Fe) often limits primary production and other processes like N_2 fixation and NO_3^- uptake (Horne and Goldman, 1972; Howarth et al., 1988b; Ferber et al., 2004; Lewis and Wurtsbaugh, 2008; Sterner, 2008). However, other studies have found no relationship between N_2 fixation and N and P levels (Toetz and McFarland, 1987; Smith, 1990), leaving open the possibility of limitation by some factors such as Mo availability, which is known to be required by N_2-fixing cyanobacteria for optimal rates of activity and growth (Glass et al., 2012 and references therein). Therefore, our study supports the notion that nutrient co-limitation of chemical transformations occurring in the N cycle and phytoplankton biomass and/or activity in lakes is common, and also suggests the importance of limitation by trace metals such as the reduced form of Mo, Mo(V).

The hypothesis regarding the involvement of Mo in the N cycle of lakes is not new (Glass et al., 2012 and references therein). Goldman (1960) demonstrated Mo deficiency in Castle Lake using a bioassay approach. Subsequent work in this lake linked Mo limitation to NO_3^- reduction, since the addition of Mo was most effective at increasing primary production during periods of high NO_3^- availability (Axler et al., 1980). In a study of 13 saline Alberta lakes, Marino et al. (1990) found that a low sulfate:Mo ratio was the best predictor of abundant populations of diazotrophic cyanobacteria. Wurtsbaugh (1988) reported that Mo could be a factor limiting N_2 fixation in some lakes and reservoirs in the Great Salt Lake Basin. In contrast, Evans and Prepas (1997) surveyed a series of 11 apparently *P*-sufficient prairie saline lakes for controls on phytoplankton standing crop and found Mo-additions to have little effect in these systems. Thus, these studies reveal that a stronger connection between Mo cycling and N_2 fixation occurs in more nutrient-deficient lakes.

Similarly, in our study we found Mo [specifically Mo(V)] to be a potentially limiting nutrient for N_2 fixation primarily in nutrient-deficient Lake Tahoe (**Figure 3**). Consistent with this hypothesis, Mo-additions in Mo-enriched lakes like Walker Lake did not have any effect on N_2 fixation (**Figure 3**). However, Clear Lake does not fit our hypothesis, as we observed the lowest Mo concentration (∼3.5 nM) and low response to Mo(V) additions (**Table 3**). Based on a comparison of dissolved concentrations at surface vs. depth (unpublished data), the sediments of Clear Lake do not appear to be a source of Mo to the water column, while total *P* is elevated more than threefold at depth. It is possible that the low concentrations of Mo observed in Clear Lake are a result of intensive uptake and recycling by the large biomass of cyanobacteria (due to blooms of *Lyngbya*, *Microcystis*, *Gloeotrichia*, *Aphanizomenon*, and *Anabaena*) coupled with a lack of sediment inputs of Mo to the lake system. Were this the case, it should be reflected by large particulate concentrations of Mo during the bloom and by higher dissolved Mo concentrations in the lake when a bloom was not occurring. Therefore, although further research is needed to understand the temporal and spatial variability of Mo limitation in lakes, and the effect of algal blooms on the relationship between Mo and N_2 fixation (e.g., Clear Lake), our results provide confirmation that primary production, NO_3^- uptake and N_2 fixation can be limited by Mo availability in non-eutrophic lakes with low Mo levels. In addition, further evaluation of other metals limitation and/or co-limitation on biological activity should be considered as some bioactive elements (e.g., Co, Mn, and Ni) also showed a significant correlation with NO_3^- uptake and/or N_2 fixation (**Table 4**).

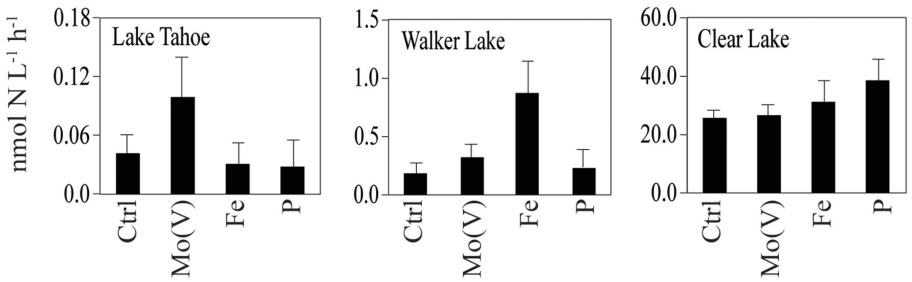

FIGURE 3 | N_2 fixation measured in different lakes under different nutrient enrichment conditions [Ctrl, Mo(V), Fe, P] during the sampling campaigns of 2009 and 2010. Water for incubation experiments was taken from a depth of 0.5 m. Data shown as arithmetic mean ± SE ($N = 4–6$).

While Mo exists as a relatively unreactive oxyanion in the VI oxidation state in seawater (Bruland and Lohan, 2004), thermodynamic calculations show that in freshwater, 30–50% of the dissolved Mo should be present in the V oxidation state – under oxic and suboxic conditions respectively. In fact, the Eh range for the Mo(VI)/Mo(V) couple in freshwater (0.04–0.55 V, depending on the presence of organic ligands) is within the range of potentials reported for As, Mn, and Fe (Turner et al., 1981), metals whose reduced forms have been reported in many lacustrine environments (Anderson and Bruland, 1991; Davison, 1993). These results are not surprising as several ubiquitous mechanisms could result in the formation of Mo(V) species, such as reduction by organic acids and microbial activity (Szilágyi, 1967; Lovley, 1993; Lloyd, 2003). However, these results are based on laboratory experiments and the cycling of the different species of Mo in natural waters is still unknown. Another environmental factor that could influence the concentrations and speciation of dissolved Mo in lakes is seasonal anoxia. This is because under anoxic conditions, Mo can be reduced from the VI oxidation state to the insoluble MoS_2(s) or converted to particle-reactive thiomolybdates (Vorlicek and Helz, 2002). Seasonal anoxia in stratified lakes could potentially reduce the total concentrations of dissolved Mo available to phytoplankton but increase the relative abundance of the reduced form of Mo [e.g., Mo(V)].

Our study provides evidence that Mo(V) stimulates N_2 fixation at Lake Tahoe and to a lesser extent at Walker Lake and Clear Lake. While previous investigators have shown that Mo limitation of N_2 fixation could result from sulfate inhibition of Mo uptake (Howarth et al., 1988a; Cole et al., 1993), they have also established that freshwater molybdate (VI oxidation state) uptake should be inhibited by only 1–5% by the sulfate levels found in most lakes (Marino et al., 1990, 2003). Therefore, as reported for other trace elements (Donat and Bruland, 1995), we hypothesize that Mo availability supporting N_2 fixation and NO_3^- uptake in lakes is mostly controlled by the chemical speciation of this element. The reduced forms of Mo [such as Mo(V)] are likely more bioavailable than Mo(VI), as the biological uptake of the reduced Mo should not be affected by the presence of inhibitors such as sulfate. This hypothesis is consistent with our results and recent studies showing that reduced species of Mo are found in nitrogenases (Howard and Rees, 2006) and in NO_3^- reductases (González et al., 2006). Although further research is needed to understand the temporal and spatial variability of Mo limitation in lakes, the results obtained in our study provide a new perspective on trace metal limitation in lake systems.

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Regression modeling of the North East Atlantic Spring Bloom suggests previously unrecognized biological roles for V and Mo

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In order to identify the biogeochemical parameters controlling pCO_2, total chlorophyll a, and dimethyl sulfide (DMS) concentrations during the North East Atlantic Spring Bloom (NASB), we used previously unpublished particulate and dissolved elemental concentrations to construct several linear regression models; first by hypothesis-testing, and then with exhaustive stepwise linear regression followed by leave-one-out cross-validation. The field data was obtained along a latitudinal transect from the Azores Islands to the North Atlantic, and best-fit models (determined by lowest predictive error) of up to three variables are presented. Total chlorophyll a is predicted best by biomass (POC, PON) parameters and by pigments characteristic of picophytoplankton for the southern section of the sampling transect (from the Azores to the Rockhall-Hatton Plateau) and coccolithophores in the northern portion (from the Rockhall-Hatton Plateau to the Denmark Strait). Both the pCO_2 and DMS models included variables traditionally associated with the development of the NASB such as mixed-layer depth and with Fe, Si, and P-deplete conditions (dissolved Fe, dissolved and biogenic silica, dissolved PO_4^{3-}). However, the regressions for pCO_2 and DMS also include intracellular V and Mo concentrations, respectively. Mo is involved in DMS production as a cofactor in dimethylsulfoxide reductase. No significant biological role for V has yet been determined, although intracellular V is significantly correlated (p-value <0.05) with biogenic silica ($R^2 = 0.72$) and total chlorophyll a ($R^2 = 0.49$) while the same is not true for its biogeochemical analogue Mo, suggesting active uptake of V by phytoplankton. Our statistical analysis suggests these two lesser-studied metals may play more important roles in bloom dynamics than previously thought, and highlights a need for studies focused on determining their potential biological requirements and cell quotas.

Keywords: trace nutrients, North Atlantic Spring Bloom, B-vitamins, vanadium, molybdenum

INTRODUCTION

The North East Atlantic Spring Bloom (NASB) is a large annual phytoplankton bloom event triggered by a decrease in mixed-layer depth in March or April. It is typically characterized by early domination of diatoms, depletion of dissolved Si, and later succession by coccolithophores and other non-silicifying organisms (Sieracki et al., 1993). The dynamics of the NASB strongly influence the partial pressure of carbon dioxide (pCO_2) in the region (Ducklow and Harris, 1993). The bloom is of particular interest in light of global climate change, owing to its status as a significant sink for anthropogenic CO_2 (Gruber, 1996).

The NASB 2005 program set as its goals to describe the phytoplankton community structure during the late stages of the NASB and determine relative contributions of the major phytoplankton taxa (e.g., diatoms and coccolithophores) in export of carbon and biominerals (LeBlanc et al., 2009). The NASB 2005 cruise yielded a large amount of data, including a broad spectrum of phytoplankton pigments, atmospheric CO_2, dimethyl sulfide (DMS), and trace metal and B-vitamins (B_{12} and B_1) concentration data. We present previously unpublished dissolved and P-standardized particulate trace metal data, which are scarce in the literature for that geographical region (Kuss and Kremling, 1999). This publication aims to utilize the trace metal and B-vitamin data in combination with pigment and other environmental data to more fully describe nutrient limitation conditions observed during the 2005 NASB cruise, as well as to employ correlative statistical methods to produce predictive models describing any relationships between pCO_2, chlorophyll a, and DMS with the wealth of other variables in the dataset. The three variables were selected to explore the relationship between primary production (represented by chlorophyll a) and production of the climactically important gases CO_2 and DMS.

Due to the unexpected enrichment of the lesser-studied trace metal nutrients Mo and V in recent phytoplankton metal studies (Tovar-Sanchez and Sañudo-Wilhelmy, 2011; Nuester et al.,

2012), special consideration of the potential roles and importance of these elements is given. Mo and V are the two most abundant transition metals in seawater, with typical average concentrations around 100 nmol L^{-1} (Collier, 1985) and 35 nmol L^{-1} (Dupont et al., 1991). Mo plays important biological roles, particularly in the nitrogen cycle, where it is a metal cofactor in nitrogenase and other enzymes involved in N-fixation and incorporation (Kisker et al., 1997). Mo is also the metal cofactor in dimethylsulfoxide reductase (Schindelin et al., 1996), an enzyme central to production of the modeled gas DMS. The only known biological roles for V in relation to plankton biology is as the metal cofactor of uncommon V-nitrogenases and in V-haloperoxidases (Crans et al., 2004).

AREA OF STUDY

Sampling was conducted from 6 June to 3 July 2005 aboard the R/V Seaward Johnson II along a south–north transect of the northeast Atlantic Ocean (**Figure 1**), generally following the 20°W meridian. Real-time satellite data was monitored during the cruise, and the route adjusted slightly to sample areas where satellite data indicated coccolithophore blooms.

METHODS

Near-surface seawater (5–10 m depth) was pumped onboard using an acid-washed all-Teflon trace-metal clean pumping system (Osmonics Bruiser) extended away from the ship on a boom. Water was pumped directly into a trace metal clean van and

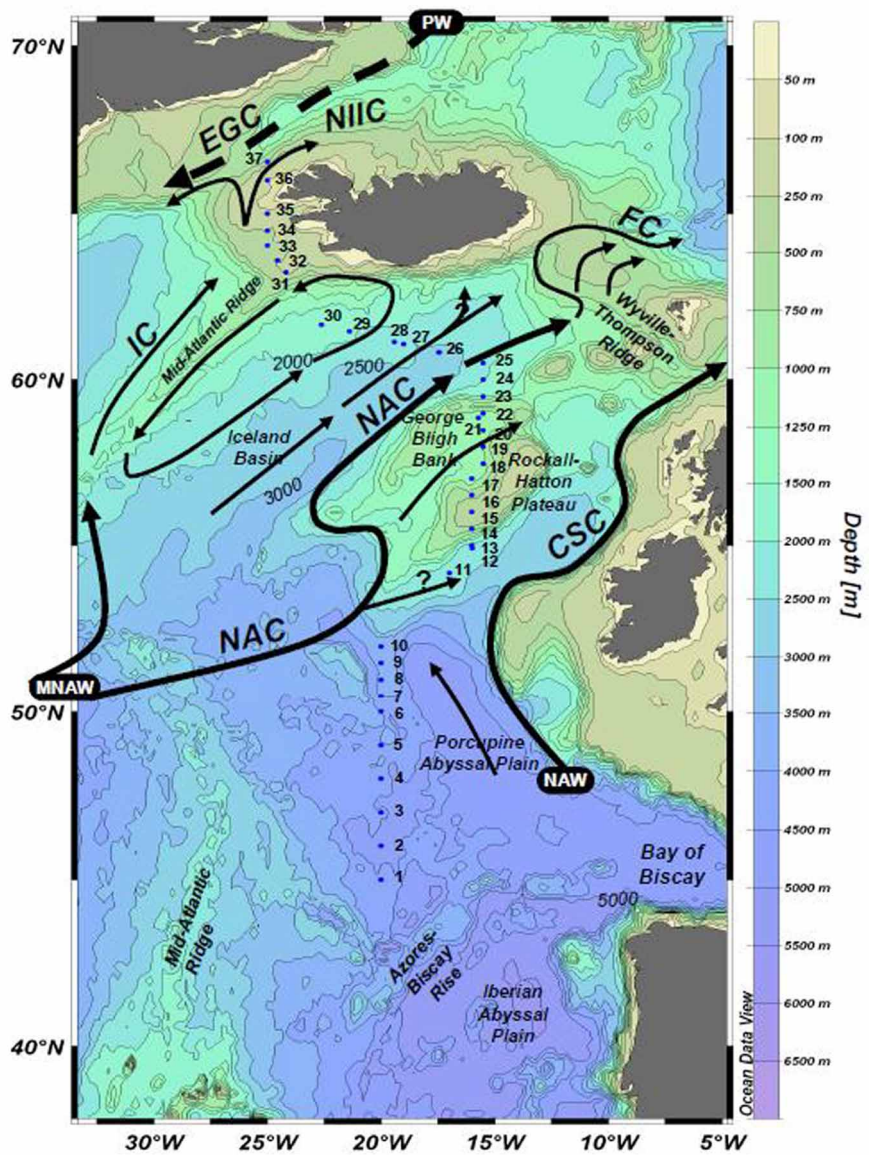

FIGURE 1 | Sampling locations along the NASB transect, shown with major surface currents. Surface sampling stations are numbered 1–37. Figure from LeBlanc et al. (2009).

filtered through a 0.22 μm acid-washed polypropylene capsule filter directly into 1 L acid-washed LDPE bottles. Dissolved trace metal samples were acidified to pH < 2 with 6 N quartz-distilled HCl (Optima-grade) and preconcentrated following Bruland et al. (1985).

Particulate samples for metals determination was filtered onto duplicate acid-washed polycarbonate filter membranes (0.2 μm pore size) from between 0.13 and 4 L of seawater, depending on plankton abundance. For total metals content, particulates collected on one of the filters was rinsed with Chelex-cleaned trace metal-free seawater. For P-standardized particulate metal concentrations, biomass on the second filter was washed to remove surface-adsorbed metals using 10 mL of oxalate reagent (Tovar-Sanchez et al., 2003), although the reagent was not cleaned prior to use. Instead, biomass was rinsed following the oxalate wash with 5 aliquots of 10 mL Chelex-cleaned trace metal-free seawater similar to Tang and Morel (2006). To monitor the rinse efficiency and confirm that there was no contamination from the oxalate reagent, a blank filter was subjected to the oxalate wash and rinse procedure with every sample ($n = 36$). Phytoplankton biomass was digested with 2 mL aqua regia and 50 μL HF (all acids Optima-grade). The digests were evaporated to dryness, and the residue taken up in 2 mL of 1 N Optima-grade HNO_3. Dissolved trace metal extracts and filter digests were analyzed by high resolution inductively-coupled plasma mass spectrometry (ICPMS; Thermo-Fisher Element 2) using indium as an internal standard.

The ancillary dataset was compiled from surface transect data (depth = 10 m) presented in LeBlanc et al. (2009) for a total of 51 variables across 27 surface transect stations.

Dissolved trace metal and nutrient data were compared to published literature stoichiometry to assess potential limitation. All statistical work was performed in the R statistical analysis program (R Development Core Team, 2010). Shorthand abbreviations (e.g. DIC for dissolved inorganic carbon) for each variable are used in the figures presented here, and a key for their interpretation may be found in Appendix.

Missing values (17% of 1404 total) were estimated using nearest-neighbor imputation (Hastie et al., 2010) and the data scaled and centered. This imputation method is not regression-based and does not produce significant bias or smoothing of the data (Chen and Shao, 2000). Hypothesis-driven regression models were constructed from variables of interest identified using existing literature (e.g., mixed-layer depth as a trigger for the NASB) and from the nutrient stoichiometry analyses. Following hypothesis-driven analysis, an exhaustive stepwise linear regression algorithm (Lumley, 2009) was employed and statistically significant regressions of up to three variables were retained for further consideration. As stepwise linear regression amounts to data mining and introduces the risk of Type III statistical errors (formulating hypotheses suggested from the data), leave-one-out cross-validation was performed to aid in selection of linear regression models better reflective of real trends and not data noise (Canty and Ripley, 2010).

RESULTS AND DISCUSSION

Prior to statistical analysis, the dataset was subdivided into two sections on the basis of their distinct hydrographic and biological regimes, a hypothesis confirmed by cluster analyses. A distinct surface salinity and temperature front separated what was subdivided as the southern transect from the northern transect section, and the two regions were observed to have different dominant phytoplankton taxa (see LeBlanc et al., 2009, Figure 2). This hypothesis was tested via application of k-means clustering (R Development Core Team, 2010) on the dataset, which produced two main clusters divided by the observed front, confirming the hypothesis. The following results and discussion consider the northern and southern transect sections separately, with the southern section stations ($n = 13$, station numbers 1–23) corresponding to the waters over the Porcupine Abyssal Plain and Rockhall-Hatton Plateau, while the northern section ($n = 14$, station numbers 24–37) represents those stations from waters overlying the Icelandic Basin and Shelf. Dissolved concentrations and oxalate-washed, P-standardized particulate metal content for bioactive trace metals considered in the nutrient limitation and stoichiometry (section "Nutrient Stoichiometry") calculations (Fe, Cu, Co, Cd, Mo, and V) are presented in **Figures 2**, **3**, respectively. A distinct concentration gradient was observed for dissolved Fe and Co, generally increasing northward (from 0.5 to 1 nmol L^{-1} and 20 to 35 pmol L^{-1} respectively), with a sharp peak observed in the Denmark Straight influenced by ice melt-waters (2 nmol L^{-1} Fe and 80 pmol L^{-1} Co) (**Figure 2**). Dissolved Mo and Cd ranged from 116 to 137 nmol L^{-1} and 0.58 to 0.74 nmol L^{-1}, respectively, with neither element displaying a clear latitudinal trend. Dissolved V ranged from 12 to 32 nmol L^{-1}, with values in the three southernmost stations appearing depleted relative to the remainder of the transect, where V varied between 20 and 30 nmol L^{-1} (**Figure 2**).

P-standardized particulate metal concentrations (**Figure 3**) were plotted with typical literature phytoplankton cellular quota values derived from laboratory culture experiments (Ho et al., 2003) with dashed lines for reference. Though these values are assumed to represent only the biological fraction for purposes of nutrient stoichiometry, the potential for a significant lithogenic contribution cannot be discounted. P-standardized particulate Fe concentrations ranged from less than 0.01 to 0.14 mmol · mol^{-1} P and were generally below or near the typical literature culture value of 7.5 mmol · mol^{-1} P, with stations at the extreme south and north of the transect being enriched by an order of magnitude. P-standardized particulate Cu concentrations ranged from 0.02 to 1.61 mmol · mol^{-1} P, and were generally below the literature value of 0.38 mmol · mol^{-1} P in the northern transect, excepting portions of the southern transect where they were enriched 2-fold. In contrast, observed P-standardized particulate Co concentrations (0.01–0.16 mmol · mol^{-1} P) were below the typical laboratory culture value of 0.19 mmol · mol^{-1} P at all stations. P-standardized particulate Cd levels (0.02–0.35 mmol · mol^{-1} P) are generally below or slightly above the literature value of 0.21 mmol · mol^{-1} P, while Mo (0.01–0.52 mmol · mol^{-1} P) is enriched above the culture value of 0.03 mmol · mol^{-1} P at most stations. Oxalate-washed particulate V:P ranged from 0.076 to 0.87 mmol · mol^{-1} P, but unfortunately no similar laboratory culture data exist with which to compare these values.

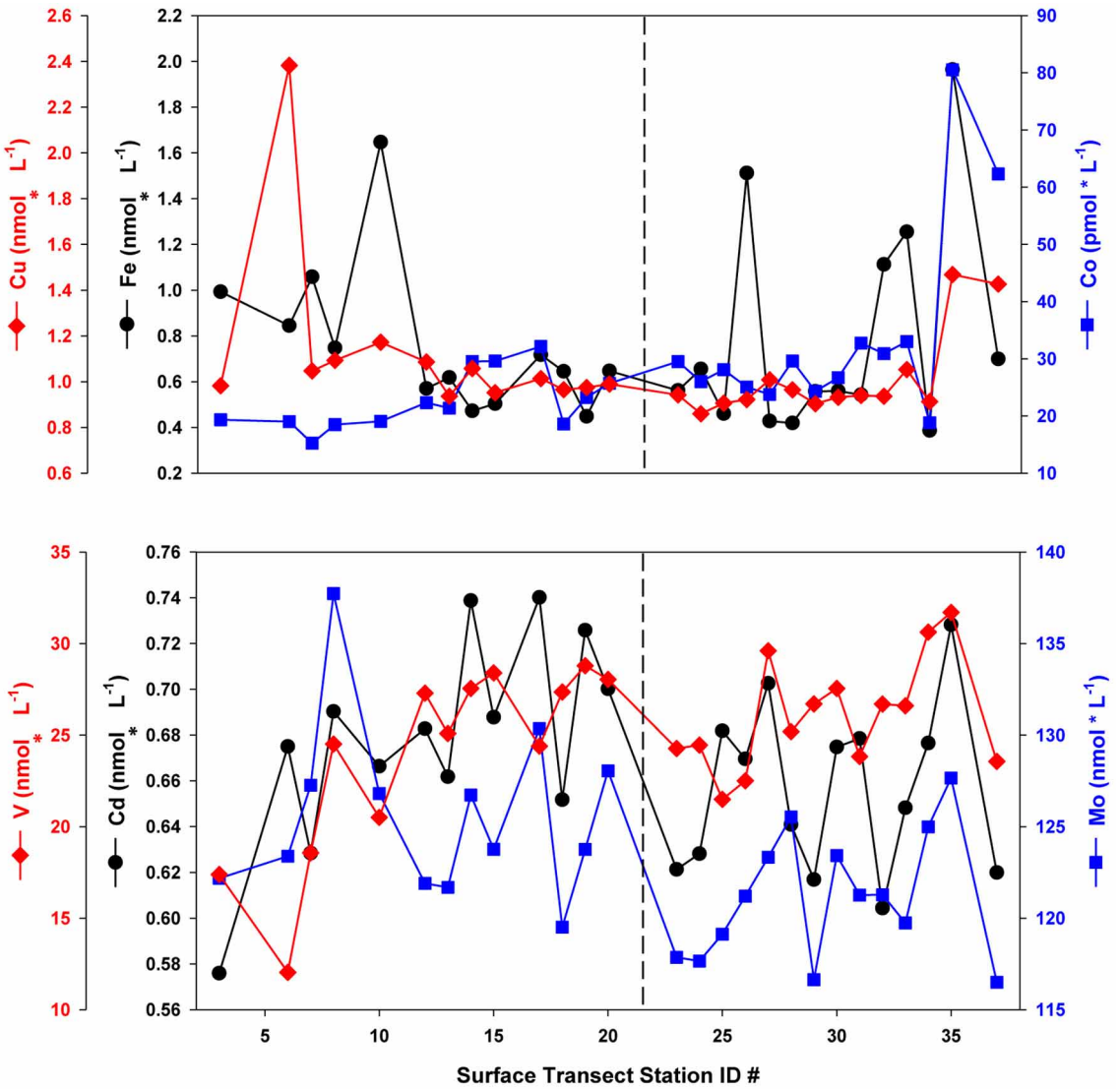

FIGURE 2 | Dissolved trace metal concentrations along the NASB transect (depth = 10 m). Vertical dashed line separates the Southern (left panel, stations 1–23) from Northern transect (right panel, stations 24–37) sections.

NUTRIENT STOICHIOMETRY

To assess the potential for nutrient limitation and the relative importance of the various nutrient elements during the 2005 NASB cruise, the observed range and median value of dissolved and P-standardized particulate concentrations were compared to values derived from laboratory culture experiments (Brzezinski, 1985; Ho et al., 2003; Tang et al., 2010) and are presented in **Table 1**. Though there is significant variability between stations, dissolved inorganic N:P is near the Redfield stoichiometric value of 15 mol · mol^{-1} P for both transect sections with median ratios (**Table 1**) of 15 and 14 mol · mol^{-1} P for the southern and northern sections, respectively (transect values will be presented in the order of southern followed by northern from here onwards). Median particulate ratios of N:P are also near Redfield (Redfield, 1934), with values of 17 mol · mol^{-1} P for both sections of the transect (**Table 1**). In this treatment, the similarity of the measured dissolved and P-standardized particulate values to the laboratory culture data suggest that N is not depleted (relative to P) in either the dissolved or intracellular phase, and therefore is likely not limiting.

Following the same logic, Cu, Cd, Mo, and vitamin B_{12} are enriched in the dissolved phase relative to observed P-standardized particulate and laboratory culture values (**Table 1**) and therefore unlikely to be limiting during the sampling period. Dissolved Cu:P (**Table 1**) is an order of magnitude greater than literature values with observed concentrations of 3.9 and 4.9 vs. 0.38 mmol · mol^{-1} P in laboratory culture (Ho et al., 2003), suggesting it is replete in the dissolved phase. Particulate Cu:P (**Table 1**) generally falls within the range of literature values,

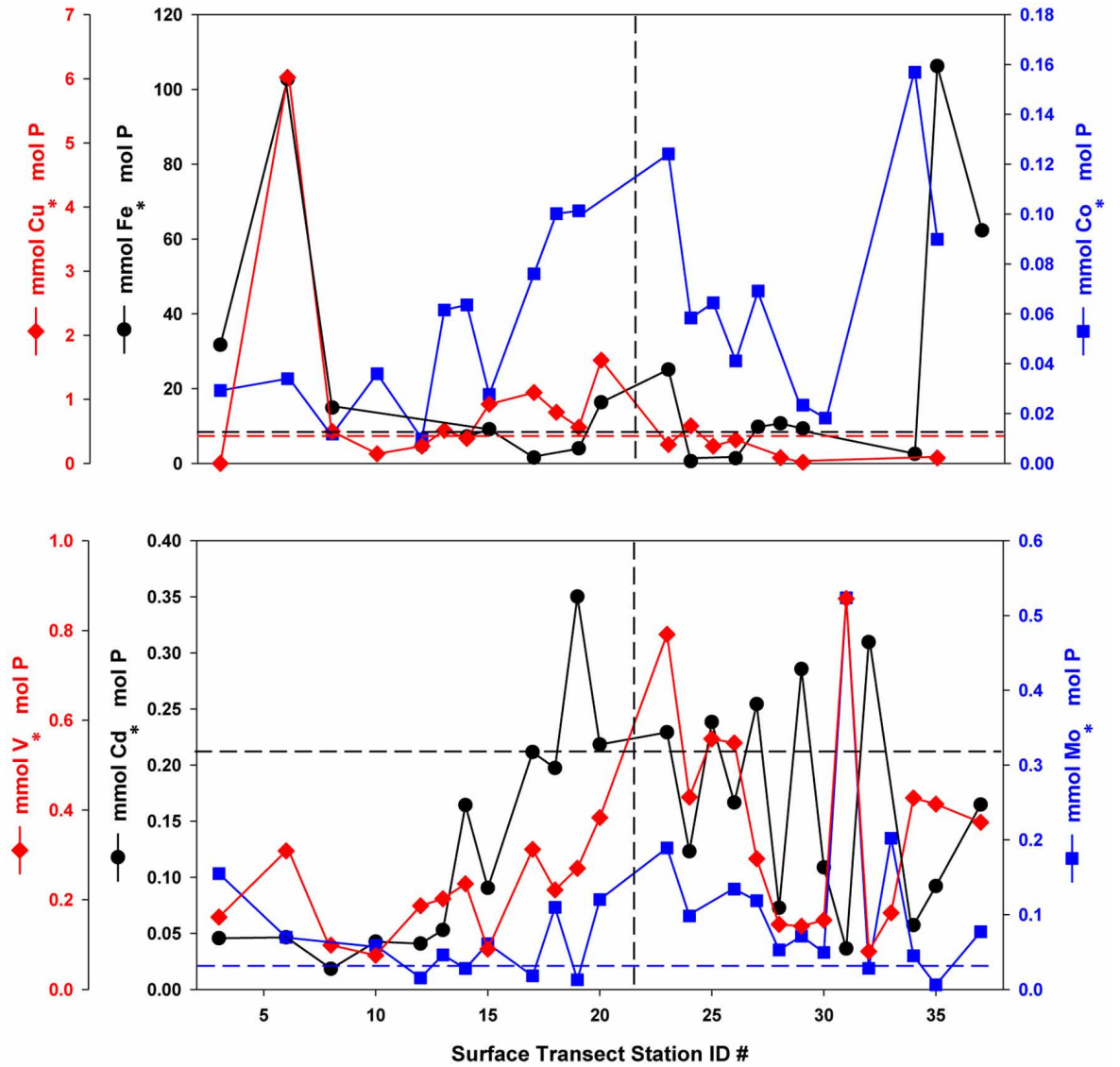

FIGURE 3 | Oxalate-washed P-standardized particulate trace metals along the NASB transect (depth = 10 m). Vertical dashed line separates the Southern (left panel, stations 1–23) from Northern (right panel, stations 24–37) transect sections. Horizontal dashed lines are color-coded by element and correspond to median phytoplankton cellular quotas for that element from Ho et al. (2003). All particulate Co:P values were below the literature value of 0.19. No line is given for V:P due to a lack of laboratory culture data for comparison.

with median values of 0.52 and 0.27 mmol · mol^{-1} P bracketing the median literature value of 0.38 mmol · mol^{-1} P (Ho et al., 2003). Dissolved Cd:P (**Table 1**) is present in ratios of 3.8 and 2.5 mmol · mol^{-1} P, an order of magnitude greater than the laboratory culture stoichiometry value of 0.21 mmol · mol^{-1} P (Ho et al., 2003), and the range and median of Cd:P values fall within the lower range of culture values.

Mo is enriched in the dissolved phase (**Table 1**), with median values of 720 and 450 mmol · mol^{-1} P relative to 0.033 in culture (Ho et al., 2003), Median particulate Mo:P values (**Table 1**) of 0.059 and 0.074 fall in the middle of the laboratory culture concentration range of 0.0090 to 0.11 mmol · mol^{-1} P (**Table 1**). Dissolved V had observed median concentrations of 99 and 94 mmol · mol^{-1} P, while oxalate-washed particulate V:P was a median of 0.20 and 0.39 mmol · mol^{-1} P. There is a lack of similar laboratory algal culture V:P data for comparison as used for the other elements discussed here. However, V is the third most abundant of the metals measured in our samples (behind Fe and Cu), and was one to two orders of magnitude greater in relative abundance than the remaining three metals (Co, Cd, and Mo) at most stations (**Table 1**). In a recent study on field-sampled colonies of the nitrogen-fixing cyanobacterium *Trichodesmium*, Nuester et al. (2012) observed values of 13–63 mmol Fe · mol^{-1} P, which are similar to those reported here. However, in that study V:P was both threefold Fe:P and had the highest relative abundance of all measured metals. When

Table 1 | Comparison of the range and median values (in parentheses) of dissolved and oxalate-washed, particulate nutrients with literature values from laboratory culture experiments, standardized to P.

	Dissolved		Oxalate-washed P-standardized particulate		Laboratory culture
	Southern transect	Northern transect	Southern transect	Northern transect	
N	14–21 (15)	10–36 (14)	9.6–20 (17)	13–25 (17)	5.4–38 (16)
Si	0.2–4.5 (1.9)	0.30–8.0 (1.8)	0.30–1.9 (1.6)	0.80–7.0 (3.7)	15
Fe	1.6–7.9 (2.5)	1.1–16 (2.6)	1.5–100 (9.1)	0.56–110 (9.8)	0.30–15 (7.5)
Cu	2.8–8.0 (4.9)	1.7–11 (3.9)	0.15–1.1 (0.52)	0.018–0.58 (0.27)	0.0060–1.4 (0.38)
Co	0.064–0.17 (0.093)	0.058–0.61 (0.11)	0.010–0.10 (0.036)	0.018–0.12 (0.060)	0.010–0.46 (0.19)
Cd	2.0–5.5 (3.8)	1.2–5.6 (2.5)	0.018–0.35 (0.072)	0.036–0.31 (0.16)	0.068–0.73 (0.21)
Mo	350–1000 (720)	220–980 (450)	0.013–0.15 (0.059)	0.006–0.20 (0.074)	0.0090–0.11 (0.033)
V	70–210 (99)	43–240 (94)	0.076–0.38 (0.20)	0.085–0.87 (0.39)	
B_1	16–150 (37)	2.0–110 (20)			38–740 (150)
B_{12}	1.2–9.8 (5.5)	0.72–17 (3.0)			0.050–500 (4.1)

N and Si are in units of mol · mol^{-1} P, trace metals in mmol · mol^{-1} P, and B-vitamins in nmol · mol^{-1} P, Trace metal and N values are from Ho et al. (2003), B-vitamins are from those complied in Tang et al. (2010), and Si values are from Brzezinski (1985).

compared to the Nuester et al. (2012) study, our data suggest that V content and abundance relative to other metals within algae may vary significantly.

Literature data on B-vitamin requirements for phytoplankton is very limited, but on a stoichiometric basis, B_{12} would appear to be present in excess (**Table 1**), with median B_{12}:P ratios (5.5 and 3.0 nmol · mol^{-1} P) similar to the median laboratory culture stoichiometric value of 4.1 nmol · mol^{-1} P (Tang et al., 2010). When observed B-vitamin concentrations are compared to literature K_s half-saturation constants for growth (**Figure 4**), B_{12} again appears replete while observed B_1 concentrations are an order of magnitude lower than literature K_s values. As B_1 is required for 49% of assayed dinoflagellate species, 15% of diatoms, and 83% of coccolithophores (Tang et al., 2010), it may be limiting to growth rates of those taxa and therefore selectively favor prototrophic species.

Fe, Si, Co, and B_1 all exhibit dissolved nutrient:P ratios (**Table 1**) lower than observed P-standardized particulate and laboratory culture values (**Table 1**), suggesting these nutrients are potentially limiting or co-limiting on the NASB. Although the median oxalate-washed particulate Fe:P values of 9.1 and 9.8 mmol · mol^{-1} P are slightly above the median laboratory culture value of 7.5 (Ho et al., 2003), ratios of 2.5 and 2.6 mmol · mol^{-1} P in the dissolved phase appear significantly depleted. There is other evidence for Fe as a likely limiting or co-limiting element, as previous studies have demonstrated Fe limitation both during the development of the NASB (Moore, 2006) and during post-bloom conditions (Nielsdóttir et al., 2009), and Fe addition experiments during the 2005 NASB cruise stimulated chlorophyll a concentrations above control (LeBlanc et al., 2009).

Si:P is depleted well below the extended Redfield stoichiometry reported for diatoms (Brzezinski, 1985), with values of 1.9 and 1.8 mol · mol^{-1} P in the dissolved phase (**Table 1**) and oxalate-washed P-standardized particulate concentrations of 1.6 and 3.7 (**Table 1**) vs. 15 in the literature (**Table 1**), suggesting it would be limiting on those organisms (such as diatoms) which require Si. Co:P is depleted both in the dissolved (median values 0.093

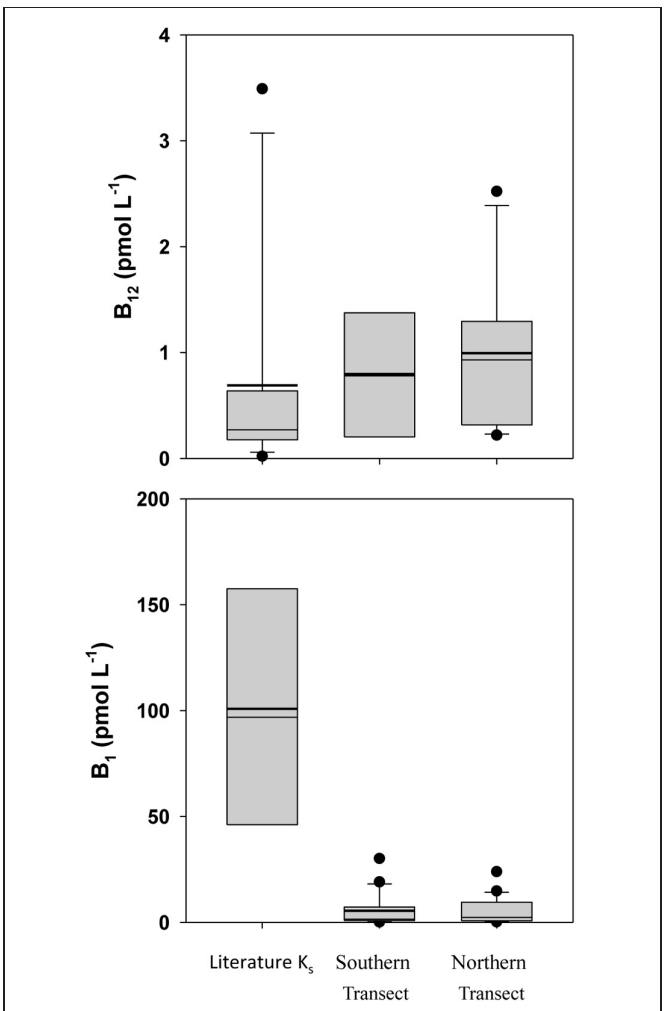

FIGURE 4 | Box-and-whisker plot Comparison of observed B-vitamin concentrations with literature Ks half-saturation constants for growth. Dots represent outlier values (Taylor and Sullivan, 2008; Tang et al., 2010).

and 0.11) and particulate phases (median of 0.036 and 0.060) relative to the median literature value from laboratory cultures of 0.19 nmol · mol^{-1} P (Ho et al., 2003). Despite being lower than the values observed in laboratory culture, oxalate-washed particulate Co:P in this study was several fold less than dissolved Co:P. The Co:P enrichment in the dissolved phase relative to depletion in the particulate phase suggests that a proportion of the dissolved Co is probably unavailable to the phytoplankton, which has been previously reported (Saito and Moffett, 2001). Dissolved vitamin B_1, in addition to being present in quantities below reported K_s half-saturation constants for growth (**Figure 4**), falls on the low end of median laboratory culture stoichiometries (37 and 20 vs. 150 mmol · mol^{-1} P) and is thus a potentially limiting nutrient to B_1 auxotrophs.

Overall, the comparison of nutrient stoichiometric ratios support the conclusions of LeBlanc et al. (2009) that the NASB at the time of sampling was in its late stages and had progressed beyond initial diatom dominance, which is reflected in the strong depletion of and potential limitation for diatom limitation by dissolved Si. The stoichiometry supports previous findings of mid- and post-bloom Fe limitation in the North Atlantic, and indicates that Co and vitamin B_1 may also have the potential to have been limiting or co-limiting at the time of sampling.

LINEAR REGRESSION MODELING OF pCo_2, CHLOROPHYLL a, AND DMS

Linear regression models for pCO_2, chlorophyll a, and DMS were constructed first with hypothesis-testing based on potential nutrient limitation as discussed in section "Nutrient Stoichiometry" (dissolved Si, inorganic N, Fe, B_1, and Co) and with mixed-layer depth, which is classically thought to trigger the NASB (Ducklow and Harris, 1993). Asterisked variable combinations in **Tables 2**, **3**, and **4** (variable abbreviations defined in Appendix) denote statistically significant models constructed from this initial hypothesis-testing.

Following this, a stepwise linear regression algorithm (Lumley, 2009) was employed to exhaustively calculate polynomial regressions vs. the three response variables for all possible combinations of up to three variables. Statistically significant models were retained and leave-one-out cross-validation performed (Canty and Ripley, 2010) to minimize overfitting. These models and relevant statistical metrics are presented in **Tables 2**, **3**, and **4** with the best-fit model bolded. Best-fit regressions for pCO_2, DMS, and chlorophyll a for both Northern and Southern transect sections yield good fits and are plotted vs. observed field data in **Figure 5**. These models and relevant statistical metrics are presented in **Tables 2**, **3**, and **4** with the best-fit model in boldface font.

Table 2 | Linear models and diagnostic statistics for pCO_2, only statistically significant regressions for up to three variables are presented.

	Model variables			R^2	p	PE
Southern transect	POC			0.50	0.00	0.86
	DNi			0.51	0.00	0.75
	DZn			0.55	0.00	0.78
	PON			0.56	0.00	0.83
	*DOC			0.78	0.00	0.98
	DNi	POP		0.81	0.00	0.86
	QFe	DZn		0.84	0.00	1.73
	*DOC	QNi		0.85	0.00	1.61
	DOC	QV		0.85	0.00	1.78
	DNi	QV	QFe	0.93	0.00	3.04
	DOC	QV	QCd	0.94	0.00	1.29
	DOC	**QV**	**PERI**	**0.94**	**0.00**	**0.40**
	DOC	QNi	TEP	0.94	0.00	1.46
	DZn	QV	DV	0.95	0.00	1.98
Northern transect	*DFe			0.38	0.02	0.68
	VIO	Chlb		0.51	0.02	1.10
	POP	DON	DNi	0.72	0.00	0.43
	DFe	Zm	Chlides	0.74	0.00	0.47
	DFe	**Zm**	**BSI**	**0.75**	**0.00**	**0.42**
	VIO	DON	DMo	0.80	0.00	1.00

Models with the lowest predictive error (PE), determined by leave-one-out cross validation, are bolded. Models produced from hypothesis-testing are marked with an asterisk.

pCO_2 MODELING

The best-fit models for pCO_2 (**Table 2**, **Figure 5**) involves DOC (dissolved organic carbon), PERI (peridinin, a pigment characteristic of dinoflagellates), and QV (oxalate-washed P-standardized particulate vanadium concentrations) for the southern transect and DFe (dissolved Fe), Zm (mixed-layer depth), and BSi (biogenic silica) for the northern transect section. For the south, DOC and P-standardized particulate V in particular are present in many of the pCO_2 regression models. DOC alone yields a statistically significant (p-value < 0.05) regression with pCO_2 with an R^2 of 0.78. During the 1989 Joint Global Ocean Flux Study experiment in the North Atlantic, depth-integrated DOC was found to be 10× greater than POC (particulate organic carbon), and bacterial production was 30% of total primary production (Lochte et al., 1992). The authors hypothesized that this bacterial production likely metabolized a significant amount of DOC, and this microbial utilization of the DOC pool could explain the inclusion of DOC in the pCO_2 models. LeBlanc et al. (2009) found dinoflagellates to be a dominant group in the southern portion of the transect, and the fact that dinoflagellates can engage in heterotrophy and osmotrophy (uptake and metabolism of dissolved organic compounds) suggest that they may also contribute to the strong correlation between DOC and pCO_2 in the southern transect (Burkholder et al., 2008).

Biological roles for V are not well-characterized, but the inclusion of P-standardized particulate V in many of the best-fit regression models presented here (**Tables 2**, **3**, **4**, **Figure 5**) as well as statistically significant ($p < 0.05$) correlations between oxalate-washed particulate V:P alone and both biogenic silica and chlorophyll a ($R^2 = 0.72$ and $R^2 = 0.49$, respectively) across the entire transect (**Figure 6**) suggests an important relationship. Vanadium and Mo exist chiefly in seawater as oxyanions

Table 3 | Linear models and diagnostic statistics for chlorophyll a, only statistically significant regressions for up to three variables are presented.

	Model variables			R^2	p	PE
Southern transect	Chlides			0.66	0.00	0.42
	BSi			0.67	0.00	0.39
	QV			0.71	0.00	0.34
	PON			0.77	0.00	0.34
	POC			0.84	0.00	0.24
	POC	QV		0.89	0.00	0.19
	PON	DIADINO		0.89	0.00	0.24
	POC	uChla		0.89	0.00	0.24
	POC	DIADINO		0.90	0.00	0.25
	POC	PFe		0.91	0.00	0.17
	POP	Bact	PFe	0.96	0.00	0.25
	POC	DCu	PFe	0.96	0.00	0.12
	POC	BUT	PFe	0.96	0.00	0.14
	PON	**nChla**	**ALLO**	**0.97**	**0.00**	**0.06**
	POC	nChla	ALLO	0.97	0.00	0.09
Northern transect	*DCo			0.48	0.01	0.46
	*POP			0.48	0.00	0.50
	uChla			0.57	0.00	0.43
	ALLO			0.60	0.00	0.38
	Chlc2			0.63	0.00	0.33
	Chlc2	PFe		0.90	0.00	0.10
	Chlc2	QFe		0.90	0.00	0.09
	Chlc2	DIADINO		0.93	0.00	0.08
	Chlc3	DCo		0.94	0.00	0.08
	Chlc3	QFe		0.94	0.00	0.08
	Chlc3	VIO	nChla	0.97	0.00	0.03
	Chlc3	DCo	uChla	0.98	0.00	0.03
	Chlb	pChla	nChla	0.98	0.00	0.03
	Chlc3	Chlb	pChla	0.98	0.00	0.03
	Chlc3	**HEX**	**VIO**	**0.99**	**0.00**	**0.01**

Models with the lowest predictive error (PE), determined by leave-one-out cross validation, are bolded. Models produced from hypothesis-testing are marked with an asterisk.

Table 4 | Linear models and diagnostic statistics for DMS, only statistically significant regressions for up to three variables are presented.

	Model variables			R^2	p	PE
Southern transect	*Si			0.31	0.05	0.58
	DON			0.35	0.03	0.52
	*DIN			0.41	0.02	0.51
	PO4	Chlides		0.70	0.00	0.63
	PO4	BUT		0.71	0.00	0.59
	TEP	Si		0.72	0.00	0.82
	DIN	QV		0.74	0.00	0.41
	PO4	Chla		0.81	0.00	0.48
	PO4	Chla	QNi	0.88	0.00	0.39
	Si	**Chia**	**Zm**	**0.89**	**0.00**	**0.22**
	PO4	Chla	QCd	0.90	0.00	0.29
	PO4	Chla	QCo	0.90	0.00	0.35
	PO4	BUT	PFe	0.90	0.00	0.50
Northern transect	nChla			0.63	0.00	0.34
	HEX			0.67	0.00	0.30
	PON			0.68	0.00	0.29
	POC			0.71	0.00	0.25
	BUT			0.74	0.00	0.23
	ALLO	POC		0.81	0.00	0.21
	QMo	PON		0.81	0.00	0.23
	QMo	POC		0.84	0.00	0.21
	QMo	BUT		0.84	0.00	0.26
	QMo	PON	DCd	0.91	0.00	0.14
	QMo	POC	DCd	0.91	0.00	0.14
	PMn	POC	DFe	0.92	0.00	0.57
	B12	FUCO	Chlc2	0.92	0.00	0.12
	QMo	**POC**	**QV**	**0.92**	**0.00**	**0.09**

Models with the lowest predictive error (PE), determined by leave-one-out cross validation, are bolded. Models produced from hypothesis-testing are marked with an asterisk.

chemically analogous to PO_4^{3-} (Crans et al., 2004), so correlations with particulate V:P but not Mo:P (as shown) implies selective uptake of V by the phytoplankton. Tovar-Sanchez and Sañudo-Wilhelmy (2011) and Nuester et al. (2012) both observed high intracellular concentrations of V in sampled *Trichodesmium* colonies (with the latter study finding it to be the most abundant intracellular trace metal), and hypothesized that this enrichment of V might be due to a biochemical role in elimination of reactive oxygen species via V-haloperoxidases and/or passive uptake as an analogue of PO_4^{3-} during P-limited conditions.

V-containing haloperoxidase activity has been identified in a number of polar and temperate diatoms (Hill and Manley, 2009) and the non-diazotrophic cyanobacterial strain *Synechococcus* CC9311 (Johnson et al., 2011), but the enzyme's function and thus potential relation to pCO_2 is not well-understood. Since our results indicate both active uptake of V and relate particulate V to photosynthetic biomass variables (chlorophyll a and biogenic silica, **Figure 5**), they are consistent with an important biological role for this element. However, caution must be taken in inferring causation from correlation models such as those presented here. Further experimentation to verify a direct causal relationship between V and pCO_2 or algal biomass (such as V-amendments of laboratory cultures or field samples) is necessary before any strong assertions about the role and importance of V can be made.

The best-fit model for pCO_2 in the north contains variables more typically associated with bloom development (mixed-layer depth, biogenic silica) as well as dissolved Fe, which is likely limiting based on stoichiometric ratios presented here. Regression with likely-limiting dissolved Fe yields a statistically significant regression with an R^2 of 0.38. The two regressions with VIO (violaxanthin, a pigment characteristic of coccolithophores) have predictive errors much greater than the other models and as such are not considered further here.

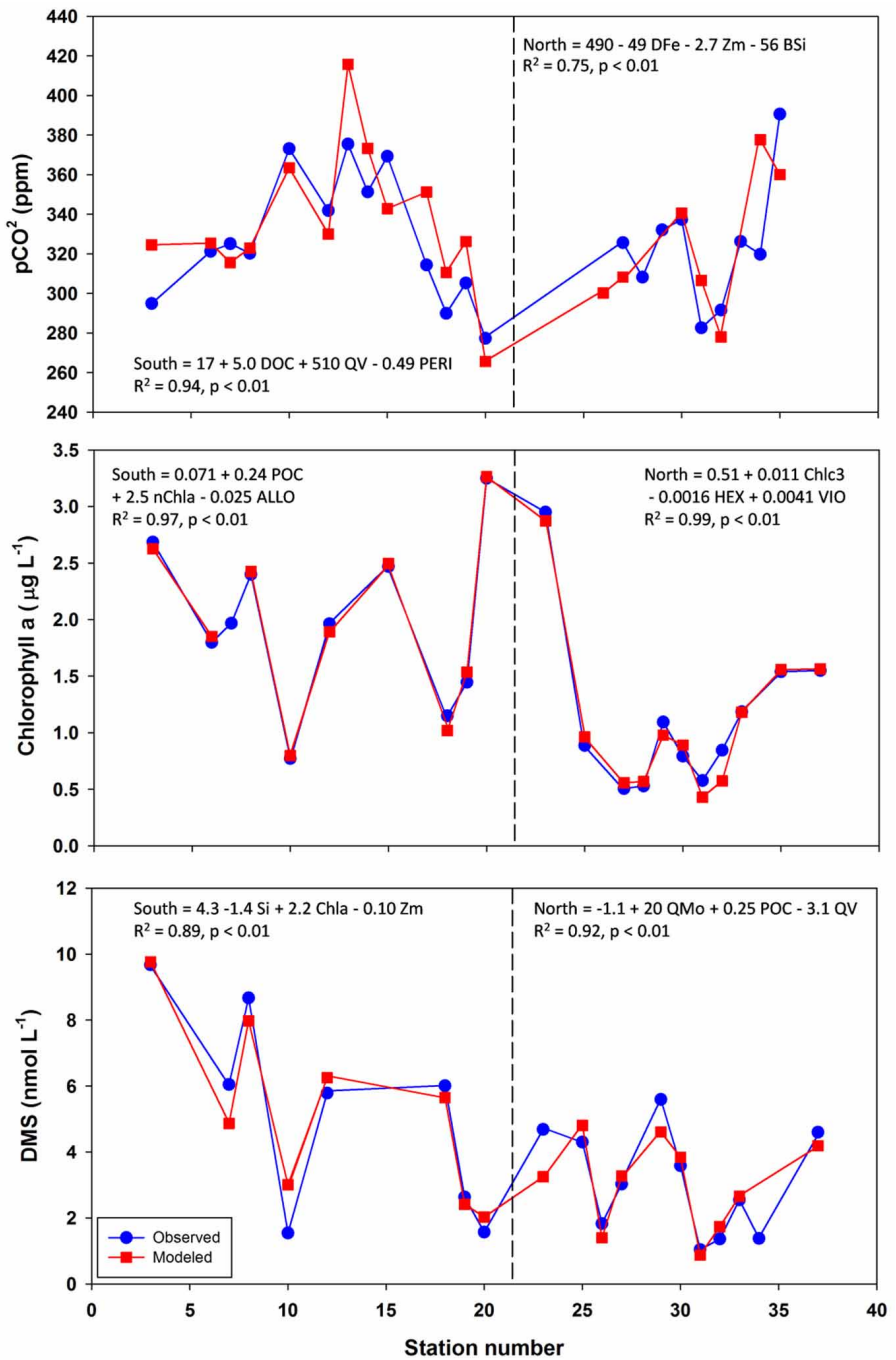

FIGURE 5 | Observed vs. modeled pCO_2, chlorophyll a, and DMS along the NASB transect. Models graphed are those with lowest predictive error as determined by leave-one-out cross-validation. Formulas, R^2, and *p*-values are given for each regression. Vertical dashed line separates the Southern (left panel, stations 1–23) from Northern transect (right panel, stations 24–37) sections.

CHLOROPHYLL A MODELING

Models for chlorophyll a (**Table 3**, **Figure 5**) contain mostly biomass variables in the southern transect section (PON, POC, POP, BSi) and chiefly other pigments in the northern transect section (size-fractionated chlorophyll a, eukaryotic accessory pigments chlorophyll c2 and chlorophyll c3). The best-fit model for the southern transect includes particulate organic nitrogen, the nanophytoplankton fraction of chlorophyll a, and alloxanthin, which is a pigment characteristic of cryptophytes (Roy et al., 2006). Regressions containing oxalate-washed particulate V:P concentrations also occur here, also. The best-fit model for chlorophyll a in the northern transect section includes

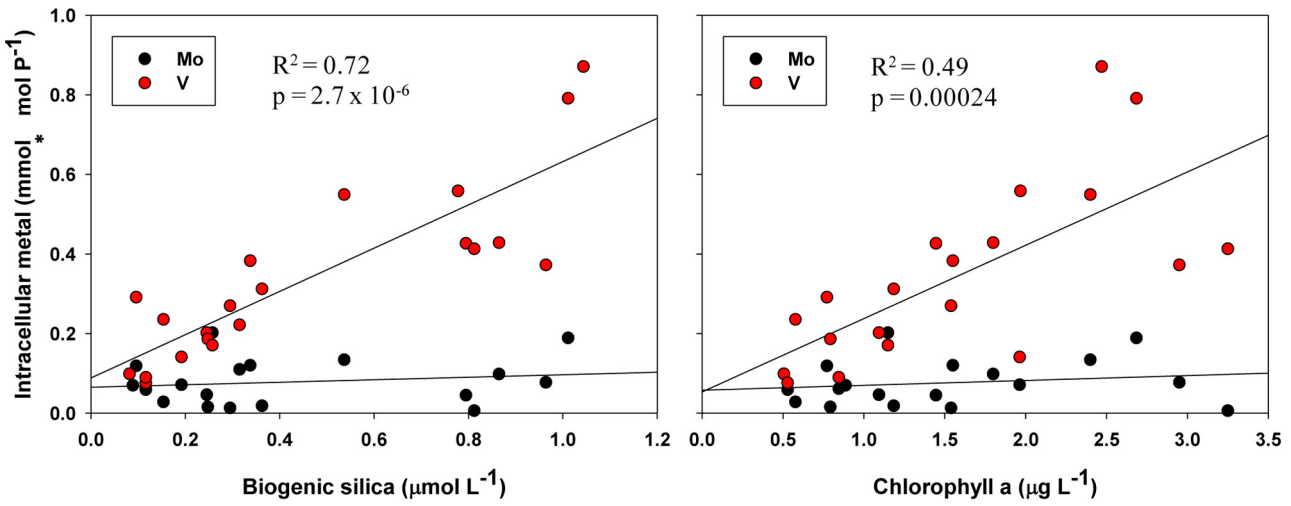

FIGURE 6 | Oxalate-washed particulate V:P and Mo:P vs. biogenic silica and total chlorophyll a across the entire NASB transect. R^2 and *p*-values are given for linear regressions of the independent variable vs. intracellular V. Regressions against particulate Mo:P were not statistically significant.

chlorophyll c3 and 19′hexanoyloxyfucoxanthin (both characteristic of the then-dominant coccolithophores) and violaxanthin, a pigment characteristic of dinoflagellates.

DMS MODELING

DMS linear regression models (**Table 4**, **Figure 5**) for the southern transect subset include dissolved inorganic nutrients (phosphate, DIN, Si) as well as chlorophyll a. For the northern section, they involve mostly biomass indicators (PON, POC) and oxalate-washed Mo:P concentrations. The best-fit model for the southern section comprises dissolved silica, chlorophyll a, and mixed-layer depth—all variables associated with the classical NASB progression. For the north, the best-fit model involves oxalate-washed Mo:P and V:P as well as POC. As referenced earlier, Mo is a cofactor in DMSO reductase (Schindelin et al., 1996). Mo is relatively more depleted stoichiometrically (**Table 1**) in the northern transect section than in the south. This along with the inclusion of particulate Mo:P in many of the DMS regressions for the north suggests that Mo may be important for the production of DMS. No relationship between V and DMS production has been previously suggested in the literature, and a better understanding of the biological role of V is needed to understand the relationship between V and DMS implied by the inclusion of oxalate-washed particulate V:P in the best-fit regression for the northern transect.

CONCLUSIONS

The 2005 NASB data analyzed here indicate, on the basis of nutrient stoichiometry, that the bloom could have been both Si and Fe-limited at the time of sampling, and Co and B_1 concentrations were also potentially limiting. With the caveat that correlation models do not imply causation, linear regression modeling suggest the importance of mixed-layer depth and dissolved Si and Fe concentrations in relation to pCO_2 and DMS concentrations. The inclusion of oxalate-washed particulate Mo:P and V:P concentrations alongside parameters traditionally of importance in the NASB (mixed layer depth, dissolved Fe, Si) in the models for DMS and pCO_2, respectively, hint at unknown and potentially important roles for these lesser-studied trace metals, perhaps particularly in the case of V where biological functions are not well elucidated. Further investigations are needed into the possible linkages between V and phytoplankton biology, and between particulate Mo:P and DMS production in the oceans.

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APPENDIX

Variable abbreviations used in linear regression modeling (**Tables 2–4**, **Figure 5**).

Abbreviation	Variable
ALLO	Alloxanthin
B1	Dissolved vitamin B1 (thiamin)
B12	Dissolved vitamin B12 (cobalamin)
Bact	Bacterial abundance
BSi	Biogenic silica
BUT	19′-butanoyloxyfucoxanthin
Chla	Chlorophyll a
Chlb	Chlorophyll b
Chlc2	Chlorophyll c2
Chlc3	Chlorophyll c3
Chlides	Total chlorophyllides
DCd	Dissolved Cd
DCo	Dissolved Co
DCu	Dissolved Cu
DFe	Dissolved Fe
DIADINO	Diadinoxanthin
DIN	Dissolve dinorganic nitrogen
DMo	Dissolved Mo
DMS	Dissolved dimethyl sulfide
DNi	Dissolved Ni
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
DV	Dissolved vanadium
DZn	Dissolved zinc
FUCO	Fucoxanthin
HEX	19′Hexanoyloxyfucoxanthin
pChla	pico fraction of chlorophyll a
pCO_2	Partial pressure of CO_2
PERI	Peridinin
PFe	Particulate Fe
PIC	Particulate inorganic carbon
PMn	Particulate Mn
PO4	Dissolved ortho-phosphate
POC	Particulate organic carbon
PON	Particulate organic nitrogen
POP	Particulate organic phosphorus
QCd	P-standardized particulate quotas of Cd
QCo	P-standardized particulate quotas of Co
QCu	P-standardized particulate quotas of Cu
QFe	P-standardized particulate quotas of Fe
QMn	P-standardized particulate quotas of Mn
QMo	P-standardized particulate quotas of Mo
QNi	P-standardized particulate quotas of Ni
QV	P-standardized particulate quotas of V
Si	Dissolved silicic acid
TEP	Transparent exopolymer particles
uChla	Micro fraction of chlorophyll a
VIO	Violoaxanthin
ZEA	Zeaxanthin
Zm	Depth of the mixed layer
Zn	Depth of the nitracline

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Interrelated influence of light and Ni on *Trichodesmium* growth

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Our previous laboratory study revealed that insufficient Ni supply can limit nitrogen fixation in *Trichodesmium*, a primary diazotrophic phytoplankton in the tropical and subtropical oceans. Here we show that light intensity and Ni availability interrelate to influence *Trichodesmium* growth. *Trichodesmium* growth is severely inhibited under high light (670 μE m^{-2} s^{-1}) and insufficient Ni condition. On the contrary, the sufficient supply of Ni in seawater can sustain the growth of *Trichodesmium* under either high or low light conditions. We also observed elevated intracellular Ni uptake in *Trichodesmium* grown under relatively high light condition, supporting that the Ni requirement is used for removing superoxide generated through photosynthetic electron transport. This study shows that light saturation condition for *Trichodesmium* growth is Ni concentration dependent. This finding may exhibit implications for interpreting temporal and spatial distributions and activities of *Trichodesmium* in both modern and ancient oceans when light intensity and Ni concentrations have significantly varied.

Keywords: *Trichodesmium*, light intensity, Ni, superoxide, superoxide dismutase

INTRODUCTION

Biological pump is mainly driven by the supply of bioavailable nitrogen, a major limiting factor for phytoplankton growth in the ocean. Nitrogen fixation is a crucial process providing bioavailable nitrogen to marine ecosystem and thus plays an important role on influencing material cycling globally (Capone et al., 1997; Karl et al., 1997; Zehr and Kudela, 2011). Understanding how nitrogen fixation is regulated in the oceans may shed light on mechanisms controlling global carbon dioxide cycling and climate change (Falkowski, 1997; Sigman and Boyle, 2000; Canfield et al., 2010). One of the major diazotrophs in the tropical and subtropical ocean, *Trichodesmium*, has received intensive studies due to their dominant abundance and quantitative importance on nitrogen fixation in the ocean region (Davis and McGillicuddy, 2006; Westberry and Siegel, 2006). Previous studies show that the environmental factors, including temperature, light intensity, P and Fe concentrations, can be important parameters on influencing the growth of *Trichodesmium* (Capone et al., 1997; Karl et al., 1997; Berman-Frank et al., 2001; Sañudo-Wilhelmy et al., 2001; Mills et al., 2004). However, the major mechanisms for controlling the global distribution and activities of *Trichodesmium* are still not fully understood (Hood et al., 2004). *Trichodesmium* contains gene encoding Ni-containing superoxide dismutase (SOD; Palenik et al., 2003; Dupont et al., 2008), indicating that Ni can be essential metal in the cyanobacterium for removing superoxides. As nitrogenase is known to be irreversibly inactivated by oxygen and reactive oxygen species (ROS; Gallon, 1981), Ni and Ni-SOD can be particularly important for *Trichodesmium*, a non-heterocystous diazotroph carrying out oxygen-producing photosynthesis and nitrogen fixation simultaneously. Our previous study demonstrates that insufficient Ni supply can limit nitrogen fixation in *Trichodesmium* in both natural and artificial seawater when the supply of P and Fe is sufficient (Ho, 2013). We also found that increasing Ni concentrations elevates cellular SOD activities and nitrogen fixation rates, suggesting that Ni-SOD may be involved in the protection of nitrogenase from ROS production during photosynthesis in *Trichodesmium* (Ho, 2013). Indeed, studies on other diazotrophs showed that cellular SOD levels were elevated when they carried out nitrogen fixation (Gallon, 1981; Puppo and Rigaud, 1986), supporting that SOD might be involved in protecting the overall process of nitrogen fixation from the inhibition of superoxides in the diazotrophs (Puppo and Rigaud, 1986).

It is well-known that ROS are inevitably generated by photosynthetic electron transport in photosystem II in plant, particularly under high light condition (Asada, 1999, 2006; Mittler et al., 2004; Latifi et al., 2009). *Trichodesmium* dwells in the surface water of tropical and subtropical regions where light intensity of photosynthetic active radiation (PAR) may reach hundreds to a couple of thousands μE m^{-2} s^{-1} in the surface water during day time period (Carpenter et al., 1993). Field and laboratory studies also observed that the growth of *Trichodesmium* was light intensity dependent (Carpenter et al., 1993; Breitbarth et al., 2008). The photosynthetic rates of *Trichodesmium* were proportional to light intensities up to 2,500 μE m^{-2} s^{-1}. In theory, cellular ROS production and accumulation would induce SOD expression and simultaneously elevate cellular uptake on the SOD co-factor metals to maintain cellular reproduction and growth. Here, we hypothesize that *Trichodesmium* require additional Ni intracellularly to maintain normal reproduction and growth under elevated light intensity due to the requirement to over-express SOD. We carried out laboratory culture experiments by varying light intensities and Ni concentrations to investigate the interrelated influence of light and

Ni on the growth of *Trichodesmium*. We also determined the SOD activities and intracellular trace metal quotas of *Trichodesmium* grown in the diverse treatments to evaluate our hypothesis.

MATERIALS AND METHODS

Trichodesmium erythraeum IMS101 was purchased from the NCMA, Provasoli-Guillard National Center for Marine Algae and Microbiota, USA. Cultures of *Trichodesmium* were grown in trace metal-clean polycarbonate bottles in a temperature-controlled growth chamber at 26°C with illumination varying from 30 to 670 $\mu E\ m^{-2}\ s^{-1}$ (PAR) under a 12:12 h light–dark cycle. Photon irradiances were created by using different distance from light source and were verified by measuring light penetration PAR into a seawater-filled polycarbonate culture bottle using a submersible radiometer (Biospherical Instruments Inc. Model: QSL 2100).

The *Trichodesmium* IMS-101 strain was maintained in trace metal-defined culture media prepared by modified YBCII culture medium without containing fixed nitrogen (Chen et al., 1996; Ho, 2013). To prepare a trace metal-defined culture medium, we remove trace metal impurities from the salt solution of the YBCII medium by passing the artificial seawater through quartz column filled with Chelex-100 (Biorad, CA, USA) chelating resins. The background concentrations of trace metals were determined (Ho, 2013). Medium sterilization, medium preparation, and trace metal control and manipulation for the trace metal-defined YBCII media generally follows the procedures for preparing trace metal-defined culture media (Ho, 2013).

Two culture experiments were designed and carried out in this study to test the hypothesis (**Figures 1** and **2**). The first experiment was designed to vary both light and Ni to two relatively extreme conditions (**Figure 1**). The light intensities and the total dissolved Ni concentrations were set to be 100 and 670 $\mu E\ m^{-2}\ S^{-1}$ and 10 and 100 nM, respectively (**Figure 1**). The second experiment was to vary the light intensities and Ni concentrations to multiple conditions to further examine the relationship of the variations of the growth rates and intracellular trace metal quotas. The information of the light intensities and total dissolved and inorganic Ni concentrations ranged from 30 to 670 $\mu E\ m^{-2}\ S^{-1}$ and from 20 to 100 nM, respectively. The details are shown in the **Figure 2**. Hereafter, we would mention the experiments in this study with the abbreviated terms, the two-condition or the multiple-condition experiments.

The ethylenediaminetetraacetic acid (EDTA) concentrations in the culture media for all treatments were 20 μM. The total dissolved Ni concentrations in the multiple-condition experiments were 20, 50, or 100 nM, which are estimated to be equivalent to 13, 33, and 67 pmol L^{-1} correspondingly as inorganic concentrations (Westall et al., 1976). The concentrations of phosphate and Fe were 50 μM and 500 nM for the two-condition experiment. To avoid precipitation and to accurately determine intracellular Fe quota (Ho et al., 2003), we decreased total dissolved Fe concentrations in the culture media to 100 nM (or 500 pM as inorganic form) for the multiple-condition experiment. Other trace metal concentrations in the medium were 20, 4, 1, 2.5, and 11 nM for Mn, Zn, Cu, Co, and Mo, respectively. The inorganic concentrations were 8.3 nM, 5 pM, 0.05 pM, and 5 pM for Mn, Zn, Cu, and Co, respectively (Westall et al., 1976). The two-condition experiment was carried out with duplicate bottles and the average deviation of the biomass for the duplicate bottles was generally less than 20% (**Figure 1**). The multiple-condition experiment included 21 different treatments so that we only used single bottle for the experiment due to the large sample number. The systematic trend observed for the growth curve and trace metal quotas indicates that the results obtained from single bottle were reliable (**Figures 2** and **3**). By using the average value obtained from the same light but different Ni treatments, the differences of the specific growth rates and the maximum sustainable biomass were statistically significant between the high light and relatively low light treatments. The detailed statistical results are shown in the **Figure 2**.

The biomass curves and the specific growth rates of *Trichodesmium* were determined by total cell volume per unit volume of seawater ($\mu m^3\ mL^{-1}$), which was validated in previous studies (Goebel et al., 2008; Ho, 2013). The accuracy of the cell volume determined by particle counter was also validated by microscope (Ho, 2013). The SOD activities were determined using a cytochrome c with xanthine-xanthine oxidase method based on the ability of SOD to inhibit the reduction of cytochrome c by scavenging superoxide anion radicals produced by the xanthine-xanthine oxidase system (Flohe and Otting, 1984). One unit of SOD activity is defined as the amount of enzyme that produced a 50% inhibition of cytochrome c reduction. The calibration curve, with an r^2 of more than 0.99 ($p < 0.01$), was first established between the reciprocal of the absorbance of ferrous cytochrome c and the enzymatic activity by using seven SOD standards. For each bottle, a linear correlation was observed between biomass and SOD activities. The SOD activity of each sample was obtained using the SOD assay for four different sample volumes. We then collected *Trichodesmium* samples through filtration, resuspended the cells in 1 mL seawater and determined the cell volume. We broke the cells using sonication under an ice-water slurry. After observing pigment release, we centrifuged the samples and removed the particulate material. Then, 100 μL of cell sample was added into a 3 mL quartz cuvette prefilled with 2.89 mL phosphate buffer solution, which contained xanthine and cytochrome C. Then, 10 μL xanthine oxidase was added into the cuvette for analysis (Flohe and Otting, 1984).

To determine intracellular trace metal quotas, cultured cells were harvested onto acid-washed 25-mm polycarbonate filters with 10 μm pore size in class 100 trace metal-clean laboratory. The filtered cells were rinsed with trace metal-clean seawater to remove the culture medium residue. The trace metal-clean seawater is the seawater passed through column filled with Chelex 100 chelating resins (Ho, 2013). Trace metal quotas in the phytoplankton were then determined using a HR-ICPMS (Thermo Scientific Element XR) fitted with a de-solvation system (Elemental Scientific). The details of the analytical precision, accuracy, and detection limits of the ICPMS (inductively coupled plasma mass spectrometry) method for seawater and phytoplankton were described in our previous studies (Ho et al., 2003, 2007).

RESULTS

In the two-condition experiment, *Trichodesmium* were grown under 100 or 670 $\mu E\ m^{-2}\ s^{-1}$ and in the culture media with 10 or 100 nM total dissolved Ni. The specific growth rates for

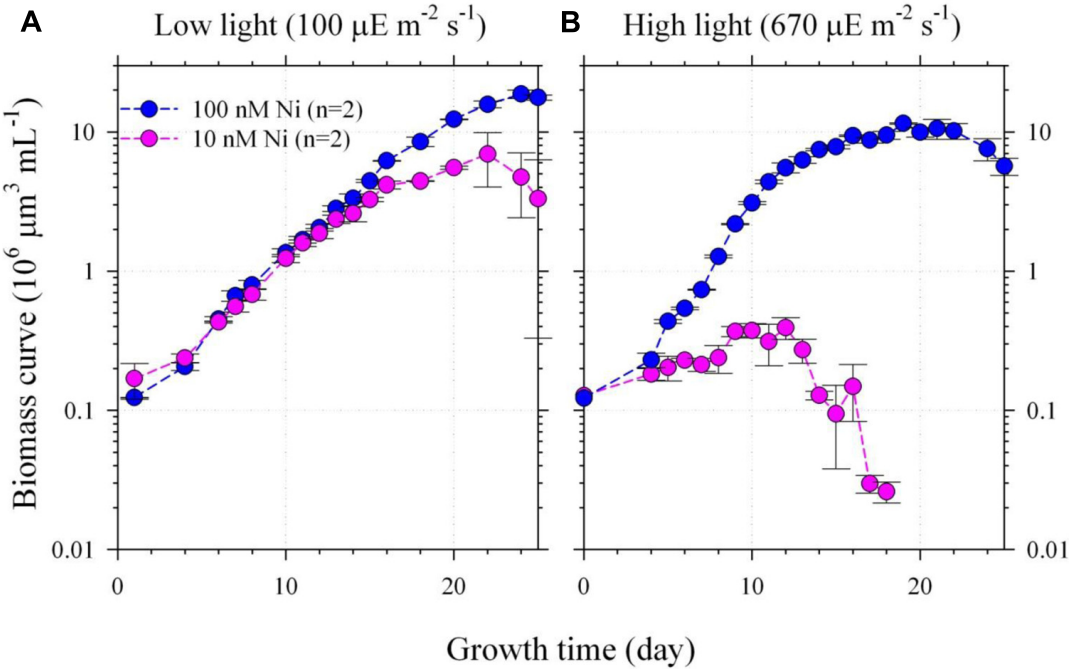

FIGURE 1 | The growth curves of *Trichodesmium* grown under the low (A) or high (B) light intensities (100 and 670 μE m^{-2} s^{-1}) and low or high Ni concentrations (10 and 100 nM Ni). The inorganic Ni concentrations for 10 and 100 total dissolved Ni concentrations with 20 μM EDTA are equivalent to 6.7 and 67 pmol L^{-1} correspondingly (Westall et al., 1976). The biomass was presented by cellular volume measured by particle counter. The average deviation of the biomass measurement for duplicate bottles was generally less than 20%. The cellular SOD activities in the two-condition experiment were also determined during their late exponential growth period. Assuming the averaged cellular volume to be 250 μm^3, the SOD activities measured during the late exponential growth period were 2.3 ± 0.2 μU per cell in the high light and high Ni treatment and the activities were only 1.1 ± 0.2 and 1.8 ± 0.2 μU per cell in the low light low Ni and low light high Ni treatments during the late exponential growth period, respectively. The biomass of the high light and low Ni treatment was too low to obtain the SOD activity.

the treatments under the low light–high Ni and the low light–low Ni conditions were similar, 0.26 and 0.23 d^{-1} (**Figure 1A**), respectively. The maximum sustainable biomass of low light and high Ni treatment reached 20 × 10^6 μm^3 mL^{-1} (**Figure 1A**). However, under the high light–low Ni treatment (**Figure 1B**), the growth of *Trichodesmium* was significantly inhibited after slow growth for 10 days ($p < 0.05$). We have mainly used single-tailed and un-equal variances Student's *t* test to examine if two samples are significantly different from each other in this study. The biomass only slowly increased from the initial biomass 0.1–0.5 (×10^6 μm^3 mL^{-1}) in the first 10 days with specific growth rate (μ) to be 0.10 d^{-1} then the non-heterocystous diazotroph collapsed (**Figure 1B**). However, under the high light–high Ni condition (**Figure 1B**), *Trichodesmium* grew normally and the biomass exponentially increased from 0.1 to 2.0 (×10^6 μm^3 mL^{-1}) in 10 days with specific growth rate up to 0.42 d^{-1} then gradually increased to 10 × 10^6 μm^3 mL^{-1}. Under the low light–low Ni treatment (**Figure 1A**), the growth rate was similar to the low light–high Ni treatment and its biomass reached 7 × 10^6 μm^3 mL^{-1} (**Figure 1A**), which was 14-fold of the biomass for the high light–low Ni treatment (**Figure 1**). These results show that sufficient Ni supply is required for *Trichodesmium* to efficiently grow and reproduce under the high light condition.

We further carried out light and Ni interrelated experiment by using multiple light intensities and Ni concentrations. The results of their specific growth rates and the maximum sustainable biomass were presented in **Figure 2**. The variations of intracellular trace metal quotas of Ni, Fe, and Mn were also determined (**Figure 3**). Overall, **Figure 2** shows that the growth rates of *Trichodesmium* were both light intensity and Ni availability dependent (**Figure 2A**). Independent to Ni concentrations, the growth rates increased from 0.10 to 0.35 d^{-1} with increasing light intensities from 30 to 370 μE m^{-1} s^{-1} (**Figure 2A**). The growth rates mainly ranged from 0.42 to 0.57 d^{-1} for the treatments with light intensity higher than 370 μE m^{-2} s^{-1}. With Ni concentrations higher than 20 nM, the specific growth rates under high light condition (≥370 μE m^{-2} s^{-1}) were generally high and comparable.

DISCUSSIONS

The results of **Figures 1** and **2** show that sufficient Ni supply is essential for *Trichodesmium* to grow efficiently under high light condition. With total dissolved Ni to be 100 nM or Ni′ to be 67 pM, the growth of *Trichodesmium* was light saturated at 370 μE m^{-2} s^{-1} and the growth rates reached 0.5 d^{-1} (**Figure 2**). The light saturated intensity and the corresponding growth rates observed in this study are both higher than previous studies reported for the same *Trichodesmium* strain. For example, Breitbarth et al. (2008) observed that the light saturated intensity of the species to be 180 μE m^{-2} s^{-1} with μ to be 0.26 d^{-1}.

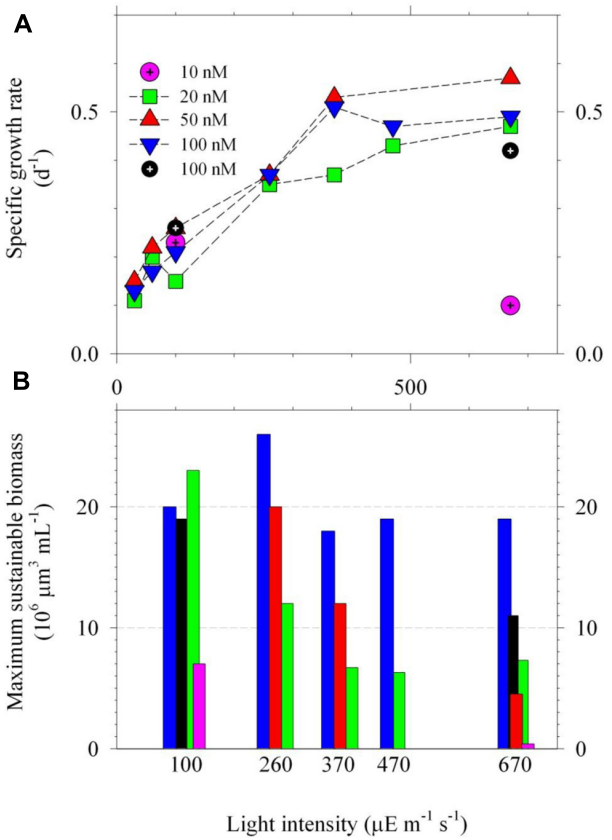

FIGURE 2 | The specific growth rates (A) and maximum sustainable biomass (B) of *Trichodesmium* grown under multiple-condition light and Ni treatments. The light intensities ranged from 30 to 670 µE m^{-2} s^{-1} with intensities to be 30, 60, 100, 260, 370, 470, or 670 µE m^{-2} s^{-1} (PAR). The total dissolved Ni concentrations were 20, 50, or 100 nM, which are equivalent to 13, 33, and 67 pmol L^{-1} as inorganic concentrations, correspondingly. The specific growth rates were estimated by using data obtained from the exponential growth period. The *r*-square of linearity between growth time and the natural log of biomass were better than 0.98. The results of the growth rates of **Figure 1** are shown here by using black and pink circle symbols. Excluding the data of the treatments with 10 nM Ni, the growth rates in the low light treatments (<370 µE m^{-2} s^{-1}) were significantly lower ($p < 0.01$) than the high light treatments (≥370 µE m^{-2} s^{-1}). The biomass data for treatments with light intensities lower than 100 µE m^{-2} s^{-1} were not reported due to their low growth rates. The growth rate for the treatment with 470 µE m^{-2} s^{-1} and 50 nM Ni and the biomass data for the treatment with 100 µE m^{-2} s^{-1} and 50 nM Ni are not available due to operational issues.

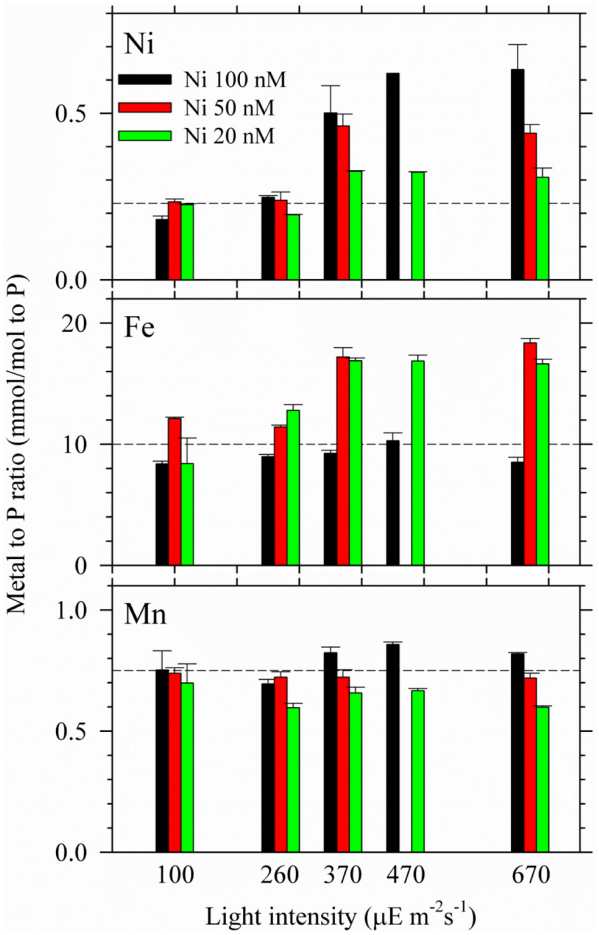

FIGURE 3 | Intracellular Ni, Fe, and Mn quotas in *Trichodesmium* grown under the multiple light and Ni conditions. The trace metal quotas were all normalized to intracellular P concentrations. Cells were harvested during the late exponential phase. The error bar stands for analytical precision (one standard deviation of three replicate samples).

It should be noted that most of previous culture studies commonly grew *Trichodesmium* under relatively low light condition, 90 or 100 µE m^{-2} s^{-1}, by following the growth condition used in the study of Chen et al. (1996). These light intensities used in the culture studies are much lower than the light intensities *Trichodesmium* generally encounters in the surface water of the tropical and subtropical oceans and may result in low growth rates. We show that increasing Ni concentrations and light intensity would obtain elevated growth rates for *Trichodesmium* culture in laboratory. This finding also suggests that *Trichodesmium* blooming in the ocean may require Ni supply under high light condition.

Superoxide dismutase activities were measured in the two-condition experiments. Assuming the averaged cellular volume to be 250 µm^3, the SOD activities measured during the late exponential growth period were 2.3 ± 0.2 µU per cell in the high light–high Ni treatment and the activities were only 1.1 ± 0.2 and 1.8 ± 0.2 µU per cell in the low light–low Ni and low light–high Ni treatments during the late exponential growth period, respectively. The biomass of the high light and low Ni treatment was too low to obtain the SOD activity. The SOD activities we obtained in the experiments were consistent to our previous study (Ho, 2013), in which the SOD activities and nitrogen fixation rates were determined under various Ni concentrations. The elevated SOD activities with elevated Ni availability validated the important role of Ni on the SOD expression in *Trichodesmium*.

In terms of the maximum sustainable biomass, the biomass ranged from 4 × 10^6 to 26 × 10^6 µm^3 mL^{-1} for the treatments with Ni concentration over 20 nM (**Figure 2B**). With sufficient Ni supply, the maximum sustainable biomass seems to be independent to light intensities in the multiple-condition experiment

(**Figure 2B**). In terms of Ni availability, 10 nM dissolved Ni concentrations (Ni′ = 6.7 pM) appears to be insufficient to maintain the growth rates and to sustain the biomass for all of the light treatments. Under light intensity to be 670 μE m^{-2} s^{-1}, *Trichodesmium* barely grew in the medium with 10 nM Ni and the biomass only reached 4×10^6 μm^3 mL^{-1} in medium with 20 nM Ni. However, under 100 μE m^{-2} s^{-1}, the biomass of the treatments with 10 and 20 nM Ni were much higher than the high light treatment, reaching 7×10^6 to 23×10^6 μm^3 mL^{-1}, respectively. For relatively high light treatments, the total sustainable biomass for the treatments with 20 nM Ni was generally lower than the treatments with 100 nM Ni. For treatments with 20 and 50 nM Ni, the maximum cellular volumes at 670 μE m^{-2} s^{-1} were only 4.5 and 7.3 μm^3 mL^{-1}, possibly showing growth stress induced by the combined effect of strong light and the lack of bioavailable Ni. Although the 20 and 50 nM total Ni concentrations may be high enough for *Trichodesmium* to grow normally at the beginning stage the bioavailable concentrations may not be high enough to sustain the growth rates at the later period with relatively high biomass (**Figure 2**). Our previous study has demonstrated that nitrogen fixation rates in *Trichodesmium* are Ni availability dependent (Ho, 2013). The inconsistency between the growth rates and the maximum sustainable biomass may be attributed to the influences of Ni availability on its nitrogen fixation rates. Overall, the results of **Figures 1** and **2** indicate that the growth of *Trichodesmium* under relatively high intensity is Ni availability dependent and light saturating or inhibiting condition in *Trichodesmium* is Ni concentration dependent. When Ni supply is sufficient in culture medium (Ni > 20 nM or Ni′ > 13.4 pM), the specific growth rate of *Trichodesmium* can be up to 0.5 d^{-1} at light intensity higher than 370 μE m^{-2} s^{-1}.

Intracellular trace metal quotas were presented by normalizing the cellular concentrations to phosphorus (**Table 1**; **Figure 3**). It should be noted that *Trichodesmium* also contains gene encoding Mn-containing SOD (Dupont et al., 2008). However, no significant evidence has been found for the mutual replacement between Ni and Mn in *Trichodesmium*. We did not observe considerable Mn quota changes in *Trichodesmium* grown in the media varying Ni concentrations from 10 to 200 nM in our previous study either (Ho, 2013), suggesting that Mn-SOD may not be involved in removing the superoxide generated in chloroplasts. Similarly, the Mn quota did not significantly change among different light intensities and Ni concentrations in this study (**Figure 3**). The *p* value of the *t* test for the Mn quota between any two light intensities are all larger than 0.2. Indeed, the Mn quota seemed to slightly increase with increasing Ni concentrations at the same light intensity for the high light treatments (≥370). Both Ni and Fe quotas varied significantly with light intensities and Ni availability. The Ni quota for the cells grown under the high light treatments (≥370) were significantly higher than the quota of the cells grown in the treatments with lighter intensity lower than 370 μE m^{-1} s^{-1} ($p < 0.05$). The Ni quota increased from 0.18 to 0.62 mmol/mol P with increasing light intensity for the 100 nM treatments; increased from 0.23 to 0.45 mmol/mol P for the 50 nM treatment; and only increased from 0.23 to 0.30 mmol/mol P in the treatment of 20 nM Ni. The increasing trend of the Ni quota indicates that intracellular Ni requirement was elevated in the cells with increasing light intensity or ROS production. Both treatments with 20 and 50 nM Ni in the media probably were not high enough to provide *Trichodesmium* sufficient Ni to form Ni-SOD for removing superoxide, which is consistent with the relatively low biomass observed for the high light treatments (≥370) shown in **Figure 2B**. In terms of Fe quota, although total Fe concentration was lowered down to 100 nM in the culture medium, we did not see significant change of Fe quotas with varying light intensity when Ni was 100 nM ($p = 0.11$), suggesting that the 100 nM Fe may be adequate for *Trichodesmium* at high light and 100 nM Ni condition. For the treatments with 20 and 50 nM Ni, the cellular Fe quotas were significantly higher ($p < 0.05$) for the high light treatments (≥370) than the relatively low light treatments (<370). We assume that the variations of Fe quotas with light intensity and Ni availability may be related to the associated interaction with Fe catalases or uptake hydrogenases. For example, the accumulation of the superoxide due to insufficient Ni-SOD activity may enhance the over-expression of catalases and elevate Fe uptake or intracellular Fe quota.

Table 1 | Trace metal quotas to P (mmol/mol) in *Trichodesmium* grown under various Ni availability and light intensity (μE m^{-2} s^{-1}). The total dissolved and the estimated inorganic Ni (Ni′) concentrations in the culture media are shown in the first column.

Ni (Ni′) (nM/pM)	Light intensity	Mn/P	Fe/P	Ni/P
20 (13)	100	0.70 ± 0.08	8.4 ± 2.1	0.23 ± 0.01
	260	0.60 ± 0.02	12.8 ± 0.5	0.20 ± 0.01
	370	0.66 ± 0.02	16.9 ± 0.2	0.33 ± 0.01
	470	0.67 ± 0.01	16.9 ± 0.5	0.32 ± 0.01
	670	0.60 ± 0.01	16.6 ± 0.4	0.31 ± 0.03
50 (33)	100	0.74 ± 0.02	12.1 ± 0.1	0.23 ± 0.01
	260	0.72 ± 0.02	11.4 ± 0.1	0.24 ± 0.02
	370	0.72 ± 0.03	17.2 ± 0.8	0.46 ± 0.04
	670	0.72 ± 0.02	18.4 ± 0.4	0.44 ± 0.03
100 (67)	100	0.75 ± 0.03	8.4 ± 0.2	0.18 ± 0.01
	260	0.70 ± 0.02	9.0 ± 0.2	0.25 ± 0.01
	370	0.82 ± 0.01	9.3 ± 0.2	0.50 ± 0.08
	470	0.86 ± 0.06	10.3 ± 0.6	0.62 ± na
	670	0.82 ± 0.03	8.5 ± 0.4	0.63 ± 0.07

In conclusion, the data of this study show that light intensity and Ni availability interrelate with each other in controlling the growth and activities of *Trichodesmium*. We have demonstrated that *Trichodesmium* requires sufficient Ni to maintain elevated reproduction and growth rates under elevated light intensity conditions. The condition of photo-saturation for *Trichodesmium* growth is Ni concentrations dependent. As light intensity varies dramatically temporally and spatially in oceanic surface water of the tropical and subtropical region our finding might exhibit major implications for modeling temporal and spatial distributions and activities of *Trichodesmium* in both modern and

ancient oceans when light intensity and Ni concentrations have changed.

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Phytoplankton responses to atmospheric metal deposition in the coastal and open-ocean Sargasso Sea

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This study investigated the impact of atmospheric metal deposition on natural phytoplankton communities at open-ocean and coastal sites in the Sargasso Sea during the spring bloom. Locally collected aerosols with different metal contents were added to natural phytoplankton assemblages from each site, and changes in nitrate, dissolved metal concentration, and phytoplankton abundance and carbon content were monitored. Addition of aerosol doubled the concentrations of cadmium (Cd), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), and nickel (Ni) in the incubation water. Over the 3-day experiments, greater drawdown of dissolved metals occurred in the open ocean water, whereas little metal drawdown occurred in the coastal water. Two populations of picoeukaryotic algae and *Synechococcus* grew in response to aerosol additions in both experiments. Particulate organic carbon increased and was most sensitive to changes in picoeukaryote abundance. Phytoplankton community composition differed depending on the chemistry of the aerosol added. Enrichment with aerosol that had higher metal content led to a 10-fold increase in *Synechococcus* abundance in the oceanic experiment but not in the coastal experiment. Enrichment of aerosol-derived Co, Mn, and Ni were particularly enhanced in the oceanic experiment, suggesting the *Synechococcus* population may have been fertilized by these aerosol metals. Cu-binding ligand concentrations were in excess of dissolved Cu in both experiments, and increased with aerosol additions. Bioavailable free hydrated Cu^{2+} concentrations were below toxicity thresholds throughout both experiments. These experiments show (1) atmospheric deposition contributes biologically important metals to seawater, (2) these metals are consumed over time scales commensurate with cell growth, and (3) growth responses can differ between distinct *Synechococcus* or eukaryotic algal populations despite their relatively close geographic proximity and taxonomic similarity.

Keywords: atmospheric metal deposition, colimitation, copper toxicity, incubation, nutrient addition experiment, picoeukaryote, *Prochlorococcus*, *Synechococcus*

INTRODUCTION

The growth of phytoplankton in the ocean is directly influenced by the availability of macro- and micronutrients required for the synthesis of new cells. Compared to micronutrients, macronutrients such as nitrogen (N) and phosphorus (P) are required by cells in higher quantities and at relatively similar ratios across different taxa (Redfield et al., 1963; Bertilsson et al., 2003). In contrast, for trace metal micronutrients, phytoplankton display a broad range of cellular quotas and stoichiometries that can span orders of magnitude (Sunda and Huntsman, 1995b; Bruland et al., 2001). Historically much emphasis has been placed on understanding macronutrient limitation of phytoplankton communities; however, with the development of methods to accurately measure very low metal concentrations in seawater, and improved methods to monitor the growth responses of specific phytoplankton taxa within a mixed population, the effects of trace metals on phytoplankton in the marine environment has become an active field of research (Bruland et al., 1991; Sunda and Huntsman, 1995a,b; Saito et al., 2005; Moore et al., 2006; Paytan et al., 2009).

Atmospheric deposition is a known source of macronutrients to the surface ocean, and the effect of deposition of N (Prospero and Savoie, 1989; Prospero et al., 1996; Paerl, 1997; Bange et al., 2000; Duce et al., 2008; Mackey et al., 2010), Fe (Duce and Tindale, 1991; Mills et al., 2004; Jickells et al., 2005; Moore et al., 2006), and P (Bergametti et al., 1992; Herut et al., 1999; Mackey et al., 2007, 2012) on phytoplankton growth have been documented. While atmospheric deposition is known to supply other biologically important trace metals to the surface ocean, the effect of these trace micronutrients on resident phytoplankton communities remains largely unexplored. A wealth of information is available about the trace metal content of atmospheric aerosols

collected at locations around the globe (Duce et al., 1991; Chen et al., 2008; Trapp et al., 2010), and anthropogenic sources appear to have higher fractional metal solubility (and potentially bioavailability) than mineral sources (Sedwick et al., 2007; Sholkovitz et al., 2010, 2012). For some locations, atmospheric deposition may be the main source of certain biologically important metals, such as in the open ocean during seasonal stratification where other potential metal sources like upwelling and discharge from rivers and groundwater are scarce.

There is mounting evidence that the different metal requirements among diverse phytoplankton taxa help determine their distribution, abundance, and activity in the ocean, highlighting a potentially important ecological role for atmospheric metal deposition. For example, addition of Sahara aerosol stimulates N_2 fixation by diazotrophs in the North Atlantic by providing Fe and P (Mills et al., 2004). Enzymatic requirements for specific metal cofactors can also lead to niche-defining metal requirements for certain groups of phytoplankton. For example, in contrast to eukaryotes, the cyanobacteria *Synechococcus* and *Prochlorococcus* have an absolute cobalt (Co) requirement for which Zn cannot substitute (Sunda and Huntsman, 1995a; Saito et al., 2002), suggesting their distributions in the ocean could be limited by Co availability (Saito and Moffett, 2001; Saito et al., 2005). On the other hand, different toxicity thresholds may influence phytoplankton community composition when metal concentrations are high. Variable toxicity thresholds to copper (Cu) and other trace metals in picophytoplankton has been suggested to influence how phytoplankton community structure responds to atmospheric deposition in the North Atlantic Ocean (Mann et al., 2002), Red Sea (Paytan et al., 2009), and western North Pacific Ocean (Guo et al., 2011). The unique metal requirements and toxicity thresholds of different phytoplankton taxa are therefore important in determining whether segments of the overall population will respond to trace metal availability from atmospheric metal deposition.

The Sargasso Sea is an oligotrophic region in the western North Atlantic Ocean where (co)limitation between macronutrients and trace metals has been identified (Mills et al., 2004; Moore et al., 2008). While atmospheric deposition relieves P and Fe (co)limitation in the Sargasso (Mills et al., 2004), atmospheric N input may have a smaller influence on phytoplankton growth. Michaels et al. (1993) showed that the amount of N contributed by wet deposition is negligible compared to the overall N budget for the region, although it could be important under specific circumstances, such as after large rain events that are followed by calm conditions. Under these conditions, the N-rich rainwater could be retained in the surface ocean for sufficient time to be exploited by phytoplankton before getting diluted through mixing.

Less is known about the effect of dry deposition on phytoplankton in the region and particularly the response of phytoplankton to N and trace metals from this source. The Sargasso Sea receives a mixture of mineral dust from the Sahara Desert and anthropogenic aerosols from North America (Prospero et al., 1996), and receives a moderate amount of dry deposition relative to other open ocean waters (Mahowald et al., 2005). If atmospheric dry deposition provides a similar magnitude of N as wet deposition in the region, as is the case for other areas in the Western Atlantic (Paerl, 1997), then it is likewise doubtful that aerosol N plays a major role in supporting productivity in the Sargasso Sea throughout the year.

In the Sargasso Sea a spring bloom occurs from March to April, when increased irradiance throughout the spring warms surface waters, leading to stratification and trapping relatively high levels of nitrate (NO_3^-) from winter mixing above the critical depth. During the bloom, Fe availability affects both the rate of NO_3^- consumption and the photosynthetic response to changing light regimes (Moore et al., 2006). Rapid phytoplankton growth during the bloom could lead to higher demands for metal micronutrients, and indeed the extent of atmospheric Fe deposition is believed to influence bloom dynamics at some locations in the Sargasso Sea (Moore et al., 2006). However, in light of the knowledge that atmospheric deposition can be an important source of other biologically important metals to remote ocean locations, it seems feasible that deposition of these metals could also shape phytoplankton communities through selective fertilization and toxicity in the Sargasso Sea during the bloom. The greater input of anthropogenic aerosol sources during the spring supports this possibility, because these aerosols generally have higher metal solubility compared to Sahara dust, which dominates during the summer. The responses of phytoplankton to atmospheric metal deposition during the bloom could differ over relatively small spatial scales based on distance from land, depending on the ubiquity and magnitude of terrestrial metal sources such as runoff, groundwater, fluvial discharge, sediment resuspension, mixing, and upwelling.

The goal of this study was to compare the growth responses of phytoplankton in the open-ocean Sargasso Sea and coastal Bermuda (**Figure 1**) to atmospheric deposition during the spring bloom, with an emphasis on the effect of aerosol metal content. To address this goal, we conducted incubation experiments with distinct locally collected aerosols that had different levels of biologically important metals. We monitored changes in phytoplankton growth and trace metal concentration, and show that metals were drawn down more sharply in the open ocean as compared to the coastal site. Moreover, phytoplankton subpopulations responded differently to aerosol additions between the two sites, likely due to different trace metal requirements and metal speciation. We explore the responses of phytoplankton to metal availability in the coastal and open, and discuss how atmospheric deposition may influence biogeochemical cycles by providing trace metals to metal limited phytoplankton communities.

MATERIALS AND METHODS

AEROSOL COLLECTION AND ANALYSIS

Weekly integrated (over ∼7 day periods) atmospheric particulate samples were collected at the Tudor Hill observatory in southwestern tip of Bermuda on top a 15 m tall sampling tower as described in (Arimoto et al., 1995; Sholkovitz and Sedwick, 2006). Air was pumped and aerosols collected for all wind directions except when the wind traversed Bermuda directly [contingent on wind speed (>1 m/s) and direction (210–315°)], thereby eliminating potentially contaminating air masses from local emissions on the island. Aerosol samples were collected on acid cleaned quartz filters and stored frozen until analysis. The soluble metal content of each aerosol sample was determined following dissolution as described

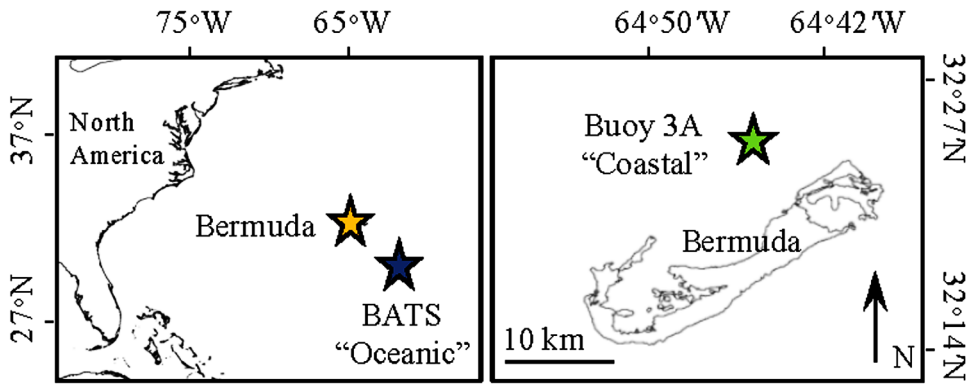

FIGURE 1 | Map showing the location of the Bermuda Atlantic Time-series Station (BATS) where the oceanic incubation water was collected (blue star) and Buoy 3A where the coastal water was collected (green star). Orange star in the left panel shows the location of Bermuda.

in Buck et al. (2006). A 100 mL volume of Milli-Q water was passed using vacuum filtration through a 47 mm diameter subsection of the sample filter, and the filtrate was acidified to (pH 2) using ultrapure trace metal grade nitric acid (Optima). Metal concentrations in the filtrate were measured on an Element 2 inductively coupled plasma-mass spectrometer (ICP-MS). Scandium and Rhodium were added to each sample for internal standardization. Mixed trace metal standards were prepared over a range of concentrations (1–100 ppb) from concentrated certified trace metal stock solutions diluted in 2% nitric acid. The mass of total suspended particles (TSP) on the filter was calculated from the aluminum (Al) content, assuming 10% solubility and an Al:TSP ratio of 0.101, which is typical for Bermuda aerosols (Prospero et al., 1996).

INCUBATION EXPERIMENTS

Nutrient and aerosol addition incubation experiments were conducted in April 2010 with surface water collected at open ocean ("oceanic") and coastal locations in the Sargasso Sea (**Figure 1**). The oceanic experiment used water collected at the Bermuda Atlantic Time-series Station (BATS; 31°40′N, 64°10′W). The coastal experiment used water collected at Buoy 3A (32°24.531′N, 64°44.769′W) located within the Bermuda platform, inside of the Rim Reef Zone that encircles the waters north of the island. In 2010, deep mixing to 300–400 m occurred at BATS during February, and stratification began to occur in March. By April at the time of our sampling the mixed layer depth had shoaled to ∼80 m, though stratification was punctuated by periods of deeper mixing down to ∼120 m in the weeks leading up to our sampling. For both experiments, water was collected on windy days reaching 4–6 on the Beaufort scale.

Water was transported back to the Bermuda Institute of Ocean Science (BIOS), where both experiments were conducted. Water was dispensed into acid cleaned, sample rinsed 500 mL clear polycarbonate incubation bottles, and treatments were made as described below (12 bottles per treatment). Bottles were incubated under shading material (50% light attenuation) in a tank with circulating seawater to maintain surface ocean temperature. Three bottles from each treatment were collected at each time point, including time zero (t0), which was processed immediately and completed within 1 h after the nutrient or aerosol treatments were added, as well as after 1, 2, and 3 days of incubation. Samples for monitoring phytoplankton growth and nutrient and metal analysis were collected as described below.

In each experiment, treatments included inorganic nutrient additions, aerosol additions, and controls (no nutrient or aerosol additions). Inorganic nutrient treatments included single addition or a combination of 0.2 μM PO_4^{3-}, 10 μM NO_3^-, and 5 nM Fe. The following inorganic nutrient treatments were included: PO_4^{3-} alone, NO_3^- alone, Fe alone, and PO_4^{3-}, NO_3^-, and Fe together (hereafter referred to as the "N,P,Fe" treatment). For the aerosol treatments, we selected two aerosol samples (Aerosol 1 and Aerosol 2) based on the chemical composition of the dissolved fraction in each sample as previously determined in the lab (see **Figure 2**; **Table 1**). Each type of aerosol was added at two concentrations, for a total of four aerosol treatments. The higher deposition treatments (referred to as "Aerosol 1" and Aerosol 2" for simplicity) simulated a concentration of aerosol that would occur in the surface ocean upper 10 m mixed layer following 10 days of deposition after a moderately strong deposition event typical of the central North Atlantic (20 g m^{-2} y^{-1}). Lower aerosol concentration treatments were also tested, simulating a typical annual average deposition rate for the Sargasso Sea near Bermuda (2 g m^{-2} y^{-1}; Mahowald et al., 2005), and these treatments are referred to as "Aerosol 1 low" and "Aerosol 2 low" to differentiate them from the high treatments.

PHYTOPLANKTON GROWTH RESPONSES

Aliquots (1.5 mL) for flow cytometry were preserved with 75 μL 10% paraformaldehyde solution, incubated in the dark at room temperature for 10 min, and frozen at −80°C until analysis. Samples were analyzed on an Influx flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) triggering on forward angle light scatter (FSC). Cell abundances and particulate organic carbon (POC) contribution were determined for each group (**Figures 4A–H**). Populations of *Synechococcus* and eukaryotic algae were identified in all samples and discriminated based on their characteristic fluorescence and scattering properties (**Figure 4I**). Two subpopulations of eukaryotic algae lacking orange/yellow (580/20 nm

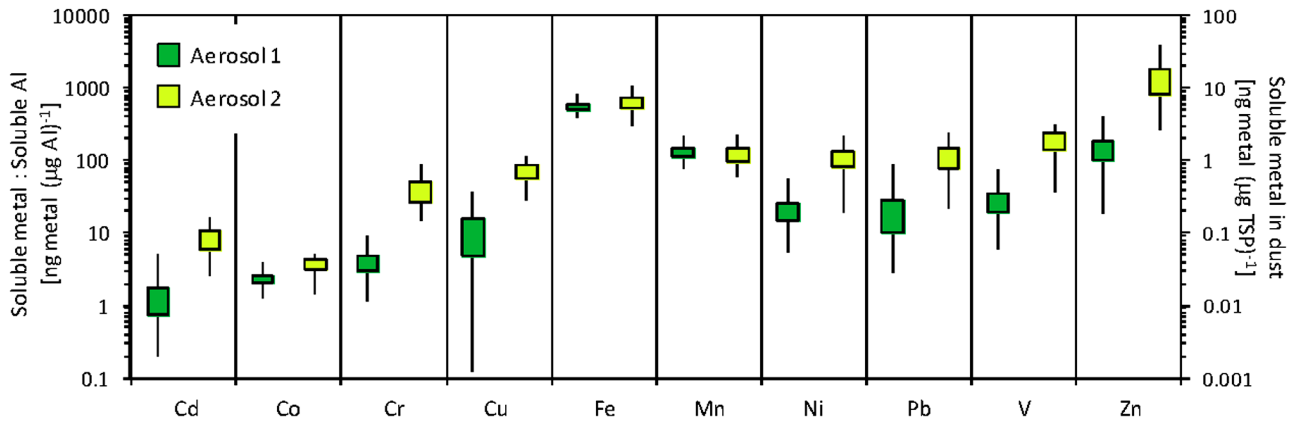

FIGURE 2 | Freshwater-dissolved trace metals in the two types of aerosol used in the incubation experiments. Primary *y*-axis shows the ratio of dissolved metal to dissolved Al, which tends to be higher for anthropogenic aerosols. Secondary *y*-axis shows the amount of freshwater dissolvable metal per mass of aerosol. The mean is at the center of each box, the upper and lower edges of the box depict ± SE, and lines show range. Of the 15 samples extracted, nine were classified as Aerosol type 1 and six were classified as Aerosol type 2. The individual aerosol samples used in the incubation experiment were selected from among these samples, and their metal contents are given in **Table 1**.

Table 1 | Freshwater-dissolved trace metal content of the aerosol samples used in the incubation experiment.

Metal	Aerosol 1 (ng metal/μg TSP)	Aerosol 2 (ng metal/μg TSP)
Cd	0.051	0.123
Co	0.026	0.051
Cr	0.094	0.508
Cu	0.361	1.057
Fe	8.100	6.711
Mn	1.786	1.864
Ni	0.571	1.745
Pb	0.885	1.505
V	0.737	2.766
Zn	4.075	10.95

bandpass) autofluorescence were also detected in both coastal and oceanic samples, and were discriminated by FSC-H amplitude (eukaryotic algae 3A and 3B, herein). *Prochlorococcus* was found only in very low abundances in coastal waters, and was a minor community member in terms of cell abundance and POC contribution; accordingly, we focus our discussion on the eukaryote and *Synechococcus* subpopulations. The low *Prochlorococcus* abundances are not likely due to the low autofluorescence of these cells being below detection limits of the flow cytometer, since the same protocol and flow cytometer used to enumerate these cells for routine monitoring at BATS was employed in this study. Possible explanations for the low *Prochlorococcus* abundances are explored in the discussion. Quality flagging during batch processing was defined to reject gated populations which failed the following criteria: events <100, kurtosis <0, skewness >|5|, median absolute deviation >50%. The cellular carbon (C) content of *Synechococcus* and picoeukaryote cells during each experiment was determined based on forward scatter signal following Casey et al. (2012). The contribution of these cells to particulate organic C was determined by multiplying cellular C content by cell abundance.

For chlorophyll *a* (chl *a*) analysis, 250 mL seawater was filtered through GFF filters (Whatman). Filters were frozen at −20°C until analysis. Chl *a* concentration was determined fluorometrically following 24 h 90% acetone extraction at −20°C. Fluorescence was measured on an AU10 fluorometer (Turner Designs) calibrated with chl *a* standard solution derived from *Anacystis nidulans* cyanobacteria following (JGOFS, 1994).

SEAWATER NITRATE AND TRACE METAL ANALYSES

Seawater samples for nutrient analysis were collected by syringe filtration (0.45 μm PES) and stored frozen until analysis. NO_3^- concentrations were measured on a flow injection autoanalyzer (FIA, Lachat Instruments Model QuickChem 8000) using standards prepared in Milli-Q water. Blanks were prepared in aged, low nutrient seawater. The detection limit based on three-times the standard deviation of the blanks was determined to be 0.1 μM for NO_3^-. PO_4^{3-} was measured as described above for NO_3^-, with a detection limit of ∼0.01 μmol L^{-1}, and is discussed in Mackey et al. (2012). We report here only NO_3^- (including trace amounts of nitrite and ammonium) data as growth in the PO_4^{3-} treatment was not appreciably different than controls in any of the experiments.

To measure trace metals dissolved in seawater, 50 mL subsamples were collected by syringe filtration (0.2 μm PES) in a laminar flow hood. For simplicity we only report data for Aerosol 2 "high" in comparison to the control and N,P,Fe treatments, as phytoplankton growth indicated that trace metal limitation may have been relieved by this treatment (see below). Samples were acidified with four drops of ultrapure trace metal grade hydrochloric acid (Optima) to pH < 2. Trace metals were separated from the major seawater ions and pre-concentrated by a factor of ∼3 using NOBIAS Chelate PA-1, a non-swelling resin with ethylenediaminetriacetic acid functional groups (Sohrin et al., 2008; Sohrin

and Bruland, 2011). Metal concentrations in the eluant were determined on an Elan DRC II ICP-MS. Metal drawdown was quantified by calculating the difference between mean concentrations at t0 and t3, and uncertainty in the difference was calculated by propagating the error according to the standard error propagation formula: $(A \pm a) - (B \pm b) = (A - B) \pm \sqrt{(a^2 + b^2)}$.

Statistical significance was evaluated at $p < 0.05$ and $p < 0.10$ performing a one-way analysis of variance (ANOVA) to detect differences between the mean values, followed by Dunnett's test to compare control values with other treatments for each experiment.

COPPER SPECIATION MEASUREMENTS

Copper speciation measurements were performed at two analytical windows in the control, Aerosol 1, and Aerosol 2 treatments in the coastal and oceanic experiments. Samples were collected at t0, as well as t3. To obtain a sufficient volume of sample, 200 mL of sample water was combined from each of the three replicate bottles at each time point. The composite sample was passed through an in-line cartridge filter (0.45 μm, Osmonics, PTFE membrane) using a peristaltic pump, and stored frozen (−20°C) until analysis.

Copper speciation was measured using competitive ligand exchange- adsorptive cathodic stripping voltammetry (CLE-ACSV) using the added ligand salicylaldoxime (SA) as described previously (Buck and Bruland, 2005; Buck et al., 2010). Briefly, thawed speciation samples were buffered with 7.5 mM ammonium-borate buffer (final pH 8.2, NBS scale), titrated with 0–15 nM of dissolved Cu and allowed to equilibrate for at least 2 h before the addition of SA as the competing ligand. Following an additional equilibration time of 15 min, each vial in the titration was analyzed by ACSV. Two analytical windows were employed in analyses using SA additions of 2.5 μM and 0.5 μM. Titration data were interpreted using van den Berg/Ružic (Ružic, 1982; van den Berg, 1982) and Scatchard (Scatchard, 1949) linearization techniques, and the results from both linearizations were averaged to provide final ligand concentrations and conditional stability constants, with standard deviations of these values reflecting the variability between interpretation method output for the titrations (Buck et al., 2010). One ligand class was determined within each analytical window with the ligand determined in the higher analytical window denoted "stronger" while the lower analytical window defined the "weaker" ligand class. Free, hydrated Cu^{2+} concentrations were determined using the higher analytical window data, which better represents the ambient speciation, following the calculations presented by Moffett and Dupont (2007).

RESULTS

AEROSOLS CHEMISTRY

To identify unique aerosol "types" to be used in the incubation experiment, the concentration of dissolved trace metals in Milli-Q water was measured in aerosol samples collected in Bermuda. Of the 15 samples analyzed, two qualitatively defined types of aerosol were identified based on the relative amounts of dissolved trace metals in the sample (Aerosol type 1 $n = 9$; and Aerosol type 2 $n = 6$). The concentration ranges for each metal in these aerosol samples are shown in **Figure 2**. We selected individual filters from each of these types to use as the aerosol additions in the incubation experiments. Aerosol 1 was collected over November 2–9, 2009 and Aerosol 2 was collected over September 28, 2009 to October 12, 2009 and October 26, 2009 to November 2, 2009. Aerosol 2 had relatively higher amounts of dissolved Cd, Cr, Cu, nickel (Ni), Pb, V, and Zn than Aerosol 1, while dissolved Fe and manganese (Mn) concentrations were similar between the two types (**Table 1**). Aerosol 2 had twice as much dissolved Co (0.051 ng Co $(\mu g\ TSP)^{-1}$) as Aerosol 1 (0.026 ng Co $(\mu g\ TSP)^{-1}$).

Vanadium (V) enrichment relative to Al has been used to identify anthropogenic influence in aerosol samples, because anthropogenic aerosols are enriched in V while the main source of Al in aerosols is from the earth's crust (Chen and Duce, 1983). In this study, the ratio of dissolved V to dissolved Al was higher in Aerosol type 2 (192 ± 49 ng V $(\mu g\ Al)^{-1}$) than Aerosol type 1 (27 ± 7 ng V $(\mu g\ Al)^{-1}$), which indicates a higher fraction of anthropogenic material in Aerosol type 2. The higher content of other dissolved trace metals in Aerosol type 2 (**Figure 2**) is also consistent with greater anthropogenic influence.

PHYTOPLANKTON GROWTH

The initial chl *a* concentration in the oceanic experiment was 0.17 mg m^{-3}. The greatest increases in chl *a* after 3 days of incubation were observed in the N,P,Fe, Aerosol 2, and Aerosol 1 treatments, reaching 4.7, 2.5, and 2.0 mg m^{-3}, respectively (**Figure 3A**). All other treatments showed levels close to the control (range 0.50–0.69 mg m^{-3} in day 3). In the coastal experiment, the initial chl *a* concentration was 0.35 mg m^{-3}, and the largest increases were observed for the N,P,Fe and Aerosol 2 treatments, which reached 2.7 and 1.3 mg m^{-3}, respectively (**Figure 3B**). All other treatments had levels close to the control (range 0.21–0.59 mg m^{-3}). Because substantial growth responses were only observed in the "N,P,Fe" and both the "Aerosol 1 high" and "Aerosol 2 high" treatments, the remainder of our discussion focuses on these treatments, along with the controls for each experiment. For simplicity, we refer to these treatments as "Aerosol 1" and "Aerosol 2" as described above.

Initial cell abundances, cellular carbon content and fluorescence were statistically indistinguishable between treatments for each population and subpopulation reported ($p > 0.05$). In the oceanic experiment, the initial phytoplankton population was dominated by *Synechococcus* ($31.7 \pm 0.5 \times 10^3$ cells mL^{-1}; **Figure 4A**), followed by picoeukaryotes ($8.6 \pm 0.2 \times 10^3$ cells mL^{-1}; **Figures 4C,E**). *Synechococcus* abundance increased 10-fold in the Aerosol 2 treatment (reaching $339.2 \pm 9.6 \times 10^3$ cells mL^{-1}), and approximately fourfold in the Aerosol 1 ($135.5 \pm 7.5 \times 10^3$ cells mL^{-1}) and N,P,Fe treatments ($96.0 \pm 1.1 \times 10^3$ cells mL^{-1}). Two subpopulations of eukaryotes, 3A and 3B, were initially present at 5.6 ± 0.2 and $1.2 \pm 0.1 \times 10^3$ cells mL^{-1}, respectively. Total picoeukaryote growth of both subpopulations responded most strongly in the N,P,Fe treatment, increasing sixfold ($51.9 \pm 1.3 \times 10^3$ cells mL^{-1}). The Aerosol 1 and Aerosol 2 treatments also resulted in increased total picoeukaryote growth, reaching $39.1 \pm 0.2 \times 10^3$ and $41.2 \pm 4.0 \times 10^3$ cells mL^{-1}, respectively. Eukaryotic subpopulations 3A and 3B reached the highest concentrations at t3 in the N,P,Fe treatment (20.1 ± 1.6 and $30.0 \pm 0.7 \times 10^3$ cells mL^{-1}, respectively). While subpopulation 3A responded identically between Aerosol 1 and Aerosol

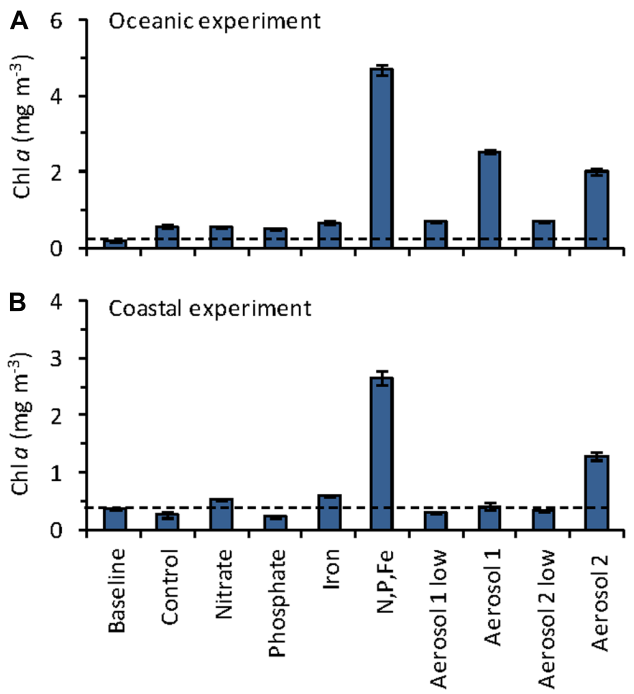

FIGURE 3 | Mean chlorophyll *a* concentrations on day 3 of the (A) oceanic, and (B) coastal incubation experiments. Dashed line shows the initial (baseline) chl *a* level. Error bars show standard error for triplicate bottles.

2 treatments (16.5 ± 1.2 and 17.0 ± 2.9 × 10^3 cells mL^{-1}, respectively; $p = 0.88$), subpopulation 3B concentrations were significantly higher in Aerosol 1 (23.5 ± 0.8 × 10^3 cells mL^{-1}) than Aerosol 2 (10.8 ± 1.6 × 10^3 cells mL^{-1}; $p = 0.006$).

The initial cellular C content in the oceanic experiment for *Synechococcus* was 223 ± 4 fg C $cell^{-1}$. Both Aerosol 1 and Aerosol 2 treatments resulted in decreased carbon quotas at the end of the incubations (205 ± 7.5 and 181 ± 6.6 fg C $cell^{-1}$, respectively) while N,P,Fe treatment resulted in the highest cellular quotas (250 ± 1.1 fg C $cell^{-1}$). Carbon quotas for eukaryotic subpopulations 3A and 3B increased significantly throughout all incubation treatments (initially 829 ± 3.5 and 2953 ± 11 fg C $cell^{-1}$). Subpopulation 3A increased similarly, in the control, Aerosol 2, and N,P,Fe treatments to 1019 ± 4.0 fg C $cell^{-1}$ at t3, each significantly higher than Aerosol 1 (963 ± 5.2 fg C $cell^{-1}$; $p < 0.05$). Subpopulation 3B also increased significantly but less than subpopulation 3A for all treatments (2809 ± 61 fg C $cell^{-1}$) compared to the control 3140 ± 27 fg C $cell^{-1}$; $p < 0.05$).

The initial amount of POC contributed by *Synechococcus* and picoeukaryotes was 15.3 ± 0.6 mg C m^{-3}, and by the end of the experiment the control remained close to this level (16.7 ± 0.5 mg C m^{-3}; **Figure 4G**). Aerosol 1 and Aerosol 2 treatments caused similar increases in POC, reaching 143 ± 4.4 mg C m^{-3} and 136 ± 17.8 mg C m^{-3} by t3, respectively. The greatest increase was observed in the N,P,Fe treatment, where POC increased to 204 ± 29.3 mg C m^{-3}. As reflected in cell abundance data, the highest biomass increases for *Synechococcus* were 10-fold for Aerosol 2 treatment. Although total eukaryotic algae biomass

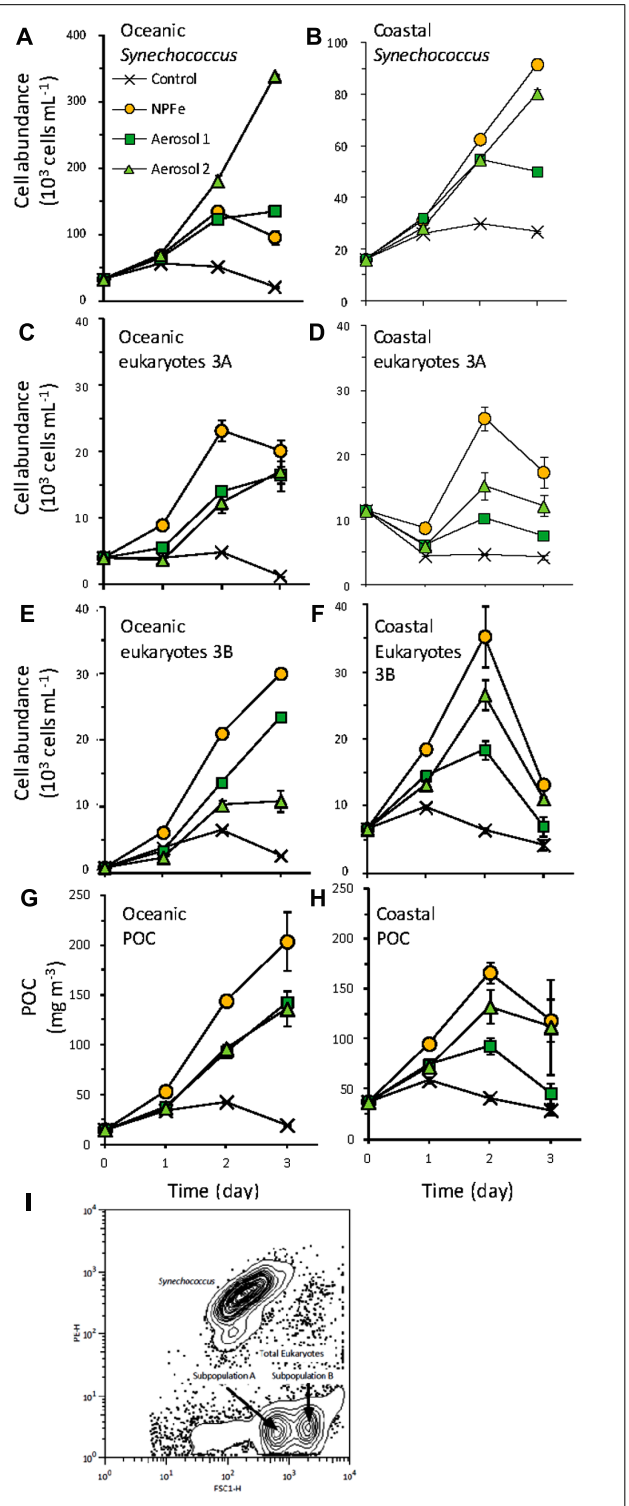

FIGURE 4 | Cell growth for (A,B) *Synechococcus* (C,D) picoeukaryote subpopulation 3A, and (E,F) picoeukaryote subpopulation 3A during the oceanic (A,C,E) and coastal (B,D,F) experiments. (G,H) Total particulate C concentration contributed from *Synechococcus* and picoeukaryotes in the **(E)** oceanic, and **(F)** coastal experiments. Error bars show standard error for triplicate bottles. **(I)** Distributions of *Synechococcus* and picoeukaryote subpopulations based on forward scatter (FSC-H) and orange fluorescence (PE-H).

increases were again highest (15-fold) for the N,P,Fe treatment, subpopulation responses differed considerably; subpopulation 3A increased fourfold while subpopulation 3B increased 23-fold.

In the coastal experiment *Synechococcus* and picoeukaryotes were present at more similar abundances (17.8 ± 0.7 and $18.0 \pm 0.4 \times 10^3$ cells mL^{-1}) at the start of the experiment (**Figure 4**). Over 3 days, *Synechococcus* numbers increased fivefold in the Aerosol 2 and N,P,Fe treatments (80.3 ± 2.1 and $91.4 \pm 4.4 \times 10^3$ cells mL^{-1}), and threefold in the Aerosol 1 treatment ($49.9 \pm 0.9 \times 10^3$ cells mL^{-1}). Picoeukaryote abundances increase for the first 2 days of incubation but then decreased during the final day for all treatments. Total picoeukaryotes also responded most strongly in the Aerosol 2 and N,P,Fe treatments, reaching $41.8 \pm 3.1 \times 10^3$ and $60.9 \pm 4.8 \times 10^3$ cells mL^{-1}, respectively. Total picoeukaryotes in the Aerosol 1 treatment reached only $28.6 \pm 1.5 \times 10^3$ cells mL^{-1} by the last day of the experiment.

The initial cellular C content in the coastal experiment was 211 ± 1 fg C $cell^{-1}$ for *Synechococcus*. Carbon quotas at the end of the experiment were statistically indistinguishable between control and both Aerosol treatments (209 ± 6 fg C $cell^{-1}$; $p > 0.05$). Carbon quotas increased to 257 ± 6 fg C $cell^{-1}$ by t3 in the N,P,Fe treatment, significantly higher than other treatments ($p < 0.001$). The initial picoeukaryote cellular C content was 953 ± 14 fg C $cell^{-1}$ for subpopulation 3A and 3177 ± 25 fg C $cell^{-1}$ for subpopulation 3B. In all treatments carbon quotas decreased throughout the incubation to 885 ± 10 fg C $cell^{-1}$ for subpopulation 3A and 2787 ± 83 fg C $cell^{-1}$ for subpopulation 3B at t3.

The initial POC contributed by *Synechococcus* and picoeukaryotes in the coastal experiment was 47.5 ± 0.5 mg C m^{-3} (**Figure 4H**). Autotrophic biomass declined slightly in the control by the end of the experiment (30.5 ± 3.7 mg C m^{-3}). For the treatments, biomass increased for the first 2 days of incubation (maximum for N,P,Fe treatment reached 167 ± 10.3 mg C m^{-3}) and then declined in the last day of the experiment at t3, reaching final POC concentrations of 45.7 ± 10.3 mg C m^{-3}, 112 ± 48.0 mg C m^{-3}, and 119 ± 21.1 mg C m^{-3} for Aerosol 1, Aerosol 2, and N,P,Fe treatments, respectively. At t3 subpopulation 3A biomass was significantly higher in the N,P,Fe experiment ($p = 0.043$).

NITRATE AND TRACE METAL DRAWDOWN

The ambient NO_3^- concentrations in the untreated control bottles were 0.91 ± 0.23 μM in the oceanic experiment and 0.11 ± 0.02 μM in the coastal experiment (**Figure 5**). Addition of aerosol to the incubation water increased the concentration of NO_3^- above background levels. In the oceanic experiment, the NO_3^- concentration almost doubled increasing to 1.9 ± 0.23 μM in the Aerosol 1 treatment and increased fivefold to 4.8 ± 0.11 μM in the Aerosol 2 treatment. About 2 μM NO_3^- was drawn down by the final time point for both treatments (**Figure 5**). In the coastal experiment NO_3^- was enriched to 0.63 ± 0.07 μM in the Aerosol 1 treatment and to 1.5 ± 0.07 μM in the Aerosol 2 treatment. The lower apparent enrichment in the coastal samples could be due to rapid NO_3^- uptake during the 1 h it took to process the samples after aerosol additions were made. By the end of the coastal experiment, nearly threefold more NO_3^- was drawn down in the Aerosol 2 treatment (1.4 μM) as compared to the Aerosol 1 treatment (0.55 μM).

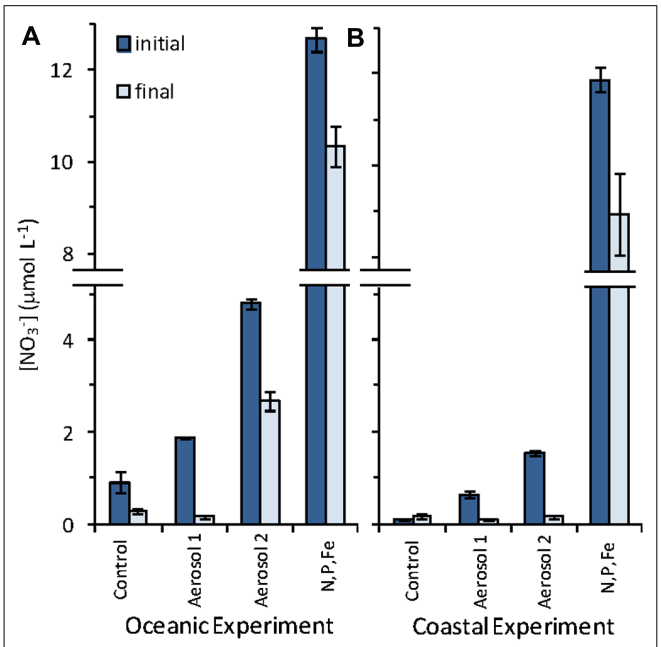

FIGURE 5 | Initial and final NO_3^- concentrations in the (A) oceanic experiment, and (B) coastal experiment. Error bars show standard error for triplicate bottles.

Background trace metal concentrations in the coastal and oceanic incubation water were within the range of concentrations typically observed in the open ocean (**Table 2;** Sohrin and Bruland, 2011; Jickells and Burton, 1988; Sedwick et al., 2005). Aerosol 2 had higher dissolved metal content that Aerosol 1 (**Table 1**; **Figure 2**). Because the Aerosol 2 treatment induced the strongest growth response in *Synechococcus* in the oceanic experiment (**Figure 4A**) despite showing similar NO_3^- drawdown as Aerosol 1 (**Figure 5A**), we measured the concentrations of seawater-soluble trace metals only in the Aerosol 2 incubation water (both open ocean and coastal) and compared that to the levels in the control and the N,P,Fe treatment (which also induced growth) with the goal of identifying which metal(s) might have been responsible for this growth response. Addition of Aerosol 2 doubled the concentrations of Fe, Cd, Co, Cu, Mn, and Ni above background levels at t0 (**Figure 6**). These concentrations however, are still within the typical range for open ocean waters (**Table 2**). Enrichment of metals other than Fe was also evident in the N,P,Fe treatment at t0; these were likely introduced from trace amounts of these metals in the salts used to prepare the nutrient amendments.

Drawdown of dissolved trace metals during the experiment was determined by calculating the difference between t0 and t3 concentrations, and propagation of error was done to determine standard errors of the differences. Drawdown of Fe, Cd, Co, Cu, Mn, and Ni was apparent in the oceanic experiment in the Aerosol 2 and N,P,Fe treatments (**Figure 7**). The largest variability was seen in dissolved Fe content for bottles treated with Aerosol 2 in

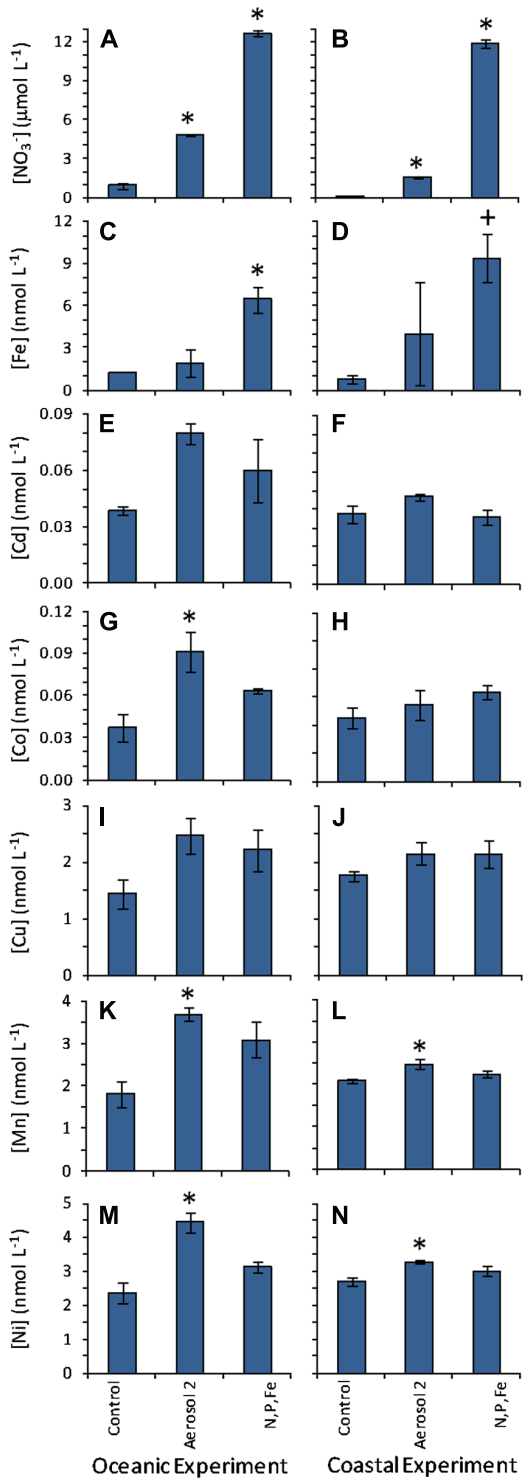

FIGURE 6 | Initial NO_3^- and seawater-dissolved metal concentrations measured at t0. Left-hand column shows values in the oceanic experiment and right-hand column shows values in the coastal experiment for **(A,B)** NO_3^-; **(C,D)** Fe; **(E,F)** Cd; **(G,H)** Co; **(I,J)** Cu; **(K,L)** Mn; **(M,N)** Ni. NO_3^- data is re-plotted from **Figure 5** for easier comparison with the trace metal data. Error bars show standard error. Levels that were statistically different from the initial control incubation water at time zero are indicated by "*" for $p < 0.05$, and "+" for $p < 0.10$.

the coastal experiment (**Figure 6D**), and is typical of an incubation study using non-homogenized natural aerosol samples due to natural variability in the size and composition of the aerosol particles as distributed on the filter (Cahill and Wakabayashi, 1993; Mackey et al., 2010). In contrast to the oceanic experiment, there was less change over time in the concentration of dissolved metals in the coastal experiment (**Figure 7**), indicating little net drawdown of these metals during the incubation. In a few instances (e.g., for Cu and Ni) final concentrations were higher than initial concentrations, and could be the result of low biological demand for these metals together with continuous release of metals from the added aerosols over time, as has been observed for aerosol PO_4^{3-} (Ridame and Guieu, 2002; Mackey et al., 2012).

COPPER SPECIATION

The initial (t0) soluble Cu concentration in the oceanic experiment was 1.14 ± 0.18 nM, and final concentrations at t3 were similar to initial levels in the control (0.92 ± 0.53), and enriched for both Aerosol 1 and 2 (∼1.9 nM; **Figure 8A**). In the coastal experiment, the initial Cu concentration was 1.41 ± 0.10 nM. The final Cu concentration in the control also did not change significantly by t3 (1.43 ± 0.19 nM), whereas enrichment occurred in Aerosol 1 (1.60 ± 0.18 nM) and Aerosol 2 (1.88 ± 0.18 nM; **Figure 8B**).

Two ligand classes were identified in the experiments, a stronger ligand class (average log $K^{cond}_{CuL_1,Cu^{2+}} = 14.38 \pm 0.17$, $n = 16$) in the higher analytical window and a weaker ligand class (average log $K^{cond}_{CuL_2,Cu^{2+}} = 13.52 \pm 0.29$, $n = 16$) in the lower analytical window. Both ligand classes were present in concentrations in excess of dissolved copper in all samples. Excess stronger Cu-binding ligands ($[L_1]$–$[Cu]$) increased in the Aerosol 1 addition above initial and control samples in both the oceanic and coastal experiments, while excess L_1 in the Aerosol 2 treatments were similar to t3 control concentrations in both experiments (**Figure 8**). Excess L_1 concentrations were similar between initial and t3 control bottles in the oceanic experiment, but decreased between initial and t3 control samples in the coastal experiment. Excess weaker Cu-binding ligands ($[L_2]$–$[Cu]$) increased in the Aerosol 2 treatment for the open ocean experiments, but were similar or lower than the control in the coastal experiment. Excess L_2 concentrations were similar between t0 initial, t3 controls and the Aerosol 1 treatment in the open ocean experiment. In the coastal experiment, excess L_2 concentrations more than doubled between the initial t0 and the control t3, Aerosol 1 and Aerosol 2 bottles (**Figure 8**).

Bioavailable free, hydrated Cu^{2+} (hereafter referred to simply as free Cu^{2+}) concentrations were low ($<10^{-13}$ M) and well below toxicity thresholds for phytoplankton in all samples. Free Cu^{2+} levels differed markedly between the oceanic and coastal experiments (**Figures 8C,D**). In the oceanic experiment (**Figure 8C**), the initial free Cu^{2+} concentration and the final concentration in the control were both ∼4 fM. Aerosol 1 caused a slight enrichment in Cu^{2+} (∼5 fM). The highest free Cu^{2+} was observed in the Aerosol 2 treatment, where Cu^{2+} nearly doubled relative to initial levels (∼7 fM). In contrast, the amount of free Cu^{2+} decreased in aerosol treatments in the coastal experiment (**Figure 8D**). Initial and final control levels were high (∼16 fM), and decreased by half in both Aerosol 1 and 2 (∼8 fM).

Table 2 | Trace metal concentrations in seawater used in the oceanic and coastal incubation experiments before any nutrient or aerosol additions were made.

Metal	Background concentration in oceanic experiment water (mean ± SE)	Background concentration in coastal experiment water (mean ± SE)	Estimated mean concentration in open ocean seawater*	Concentration range in open ocean seawater*
Cd	0.04 ± 0.002	0.04 ± 0.005	0.6	0.001–1.05
Co	0.04 ± 0.010	0.05 ± 0.007	0.04	0.003–0.3
Cu	1.4 ± 0.26	1.8 ± 0.084	3	0.4–5
Fe	1.3 ± 0.047	0.73 ± 0.30	0.5	0.03–3
Mn	1.8 ± 0.31	2.1 ± 0.055	0.3	0.06–10
Ni	2.4 ± 0.30	2.7 ± 0.12	8	2–12

The concentrations were within the range of typical values for open ocean water (Sohrin and Bruland, 2011; ranges include surface and deep waters). Units are nmol L^{-1}.
**Values from Sohrin and Bruland (2011).*

DISCUSSION

PHYTOPLANKTON COMMUNITY DYNAMICS

The potential for atmospheric deposition to alter phytoplankton community composition via selective fertilization (Mills et al., 2004; Moore et al., 2006) and toxicity (Mann et al., 2002; Paytan et al., 2009) of different taxonomic groups has been demonstrated in various ocean waters. In the present study, we sought to evaluate the range of responses that coastal and open ocean phytoplankton communities in the Sargasso Sea would have following simulated atmospheric deposition events during the spring bloom period. Based on chl *a* levels, the greatest increases in autotrophic biomass occurred in the N,P,Fe and the Aerosol 1 and 2 treatments. Drawdown of NO_3^- occurred in both oceanic and coastal experiments for Aerosol 1 and Aerosol 2 (**Figure 5**). In contrast, PO_4^{3-} levels for Aerosol 1 and Aerosol 2 stayed the same in the coastal experiment (not shown), and actually increased in the oceanic experiment due to gradual aerosol P dissolution from the aerosols and DOP remineralization over the course of the experiment (Mackey et al., 2012). Chl *a* levels remained similar to the control at t3 following treatment with N alone, suggesting that autotrophic biomass at the coastal and oceanic sites required Fe and possibly additional metals to show maximal growth at the time of our experiments.

We compared the growth of different members of the phytoplankton community to determine differences in taxon-specific responses to atmospheric deposition and how this varied between sites, or within the same site for aerosols with different chemical compositions. At the time of our experiments the phytoplankton community at both locations was dominated by picoeukaryotes and *Synechococcus*. The absence of oceanic *Prochlorococcus* is notable, as it is typically the numerically dominant phytoplankter at the BATS site throughout much of the year. *Prochlorococcus* population crashes are observed annually following the maximal thermal convective mixing depth event (DuRand et al., 2001), depending on the phase and amplitude of the North Atlantic Oscillation index (NAOi) and associated regional weather (Hurrell and Deser, 2009). A particularly cold winter and strong negative anomaly of the NAOi was perhaps responsible for late winter 2010 mixing depths to exceed 400 m (not shown), leading to enhanced *Prochlorococcus* population crashes preceding our sampling.

Picoeukaryotes at both oceanic and coastal locations responded most strongly to the N,P,Fe addition (**Figure 4**), consistent with chl *a* responses. Aerosol 1 and Aerosol 2 both induced moderate increases in picoeukaryote abundances in the oceanic experiment (**Figures 4C,E**), whereas Aerosol 2 induced more picoeukaryote growth than Aerosol 1 in the coastal experiment (**Figures 4D,F**). Important differences in the response of picoeukaryote subpopulations to aerosol treatments were observed in both oceanic and coastal experiments. For instance, initial subpopulation 3B abundances were lower than for subpopulation 3A, but the rapid growth of subpopulation 3B allowed it to out-compete subpopulation 3A by the end of the oceanic experiment, such that their abundances were more comparable. This shift in the dominance of picoeukaryote subpopulations suggests that atmospheric deposition induces competition among picoeukaryotes causing changes in population distribution and abundance. Additionally, atmospheric deposition influenced the carbon dynamics within this group. Picoeukaryotes, as identified through flow cytometry, are an operationally defined group, so the increase in abundance of the larger, subpopulation 3B cells in the oceanic experiment tended to increase the overall cellular POC content of picoeukaryotes relative to initial values.

Coastal *Synechococcus* abundance also responded differently to different treatments. Like the overall picoeukaryote community, *Synechococcus* abundance increased more in response to Aerosol 2 (fivefold increase) than Aerosol 1 (threefold increase), reaching levels comparable to the N,P,Fe treatment (**Figure 4B**). The stronger growth responses of picoeukaryotes and coastal *Synechococcus* to Aerosol 2 than Aerosol 1 could stem from the greater content and drawdown of NO_3^- in the coastal experiment for Aerosol 2 (1.9 μM added and ∼1.5 μM drawdown) compared to Aerosol 1 (0.6 μM added and ∼0.5 μM drawdown, **Figure 5B**). Treatment with N alone, P alone, or Fe alone did not stimulate growth (**Figure 3B**), whereas treatment with N,P,Fe induced strong growth responses, although no P drawdown was observed in these samples (not shown). Together these results suggest that oceanic and coastal picoeukaryotes and coastal *Synechococcus*

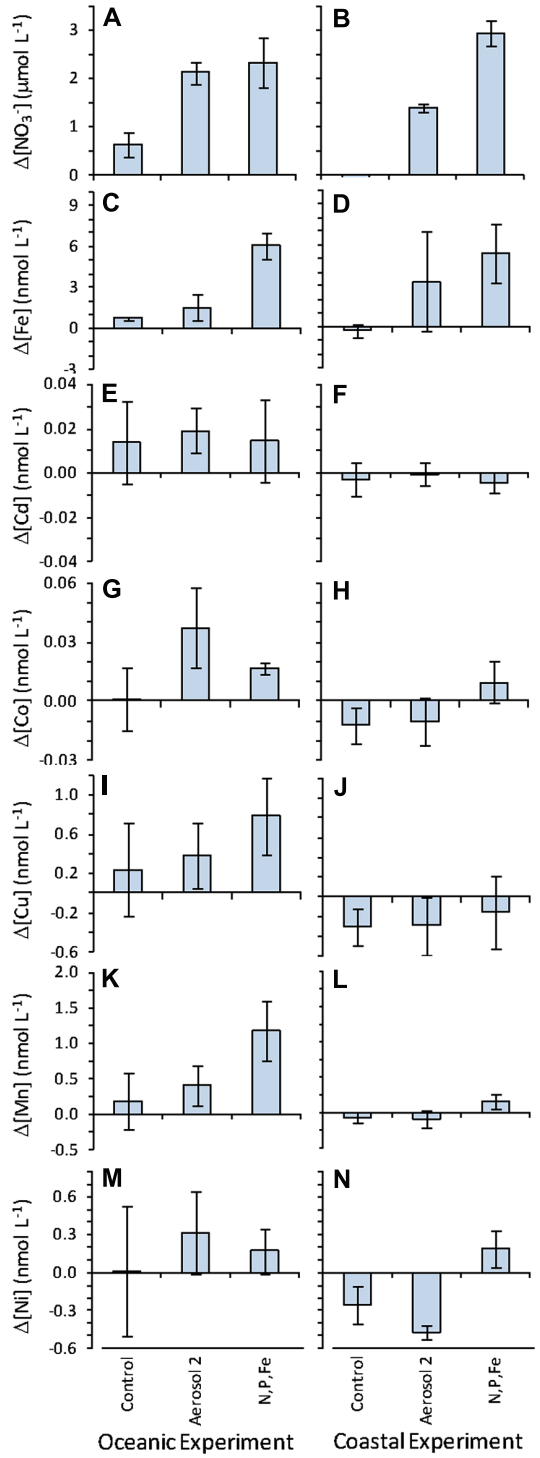

FIGURE 7 | Change in seawater-dissolved NO_3^- and metal concentrations over 72 h measured as the difference between values at t0 and t3. Positive values indicate the nutrient was drawn down over the course of the experiment, and values close to zero indicate little change over the course of the experiment. Left-hand column shows values in the oceanic experiment and right-hand column shows values in the coastal experiment for **(A,B)** NO_3^-; **(C,D)** Fe; **(E,F)** Cd; **(G,H)** Co; **(I,J)** Cu; **(K,L)** Mn; **(M,N)** Ni. Error bars show standard error for the difference between mean concentrations at t0 and t3.

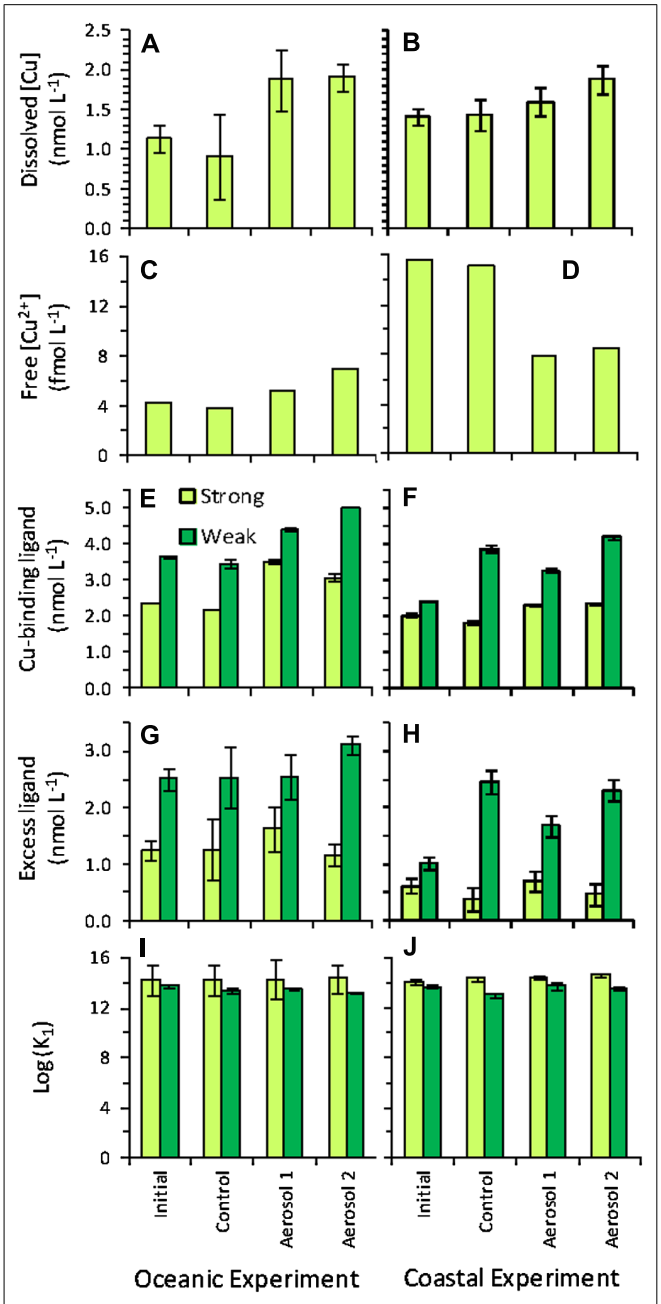

FIGURE 8 | Copper chemistry for initial (baseline) samples, and final (t3) samples for control, Aerosol 1, and Aerosol 2. Left-hand column shows values in the oceanic experiment and right-hand column shows values in the coastal experiment for **(A,B)** dissolved Cu concentration; **(C,D)** free Cu^{2+} concentration; **(E,F)** Cu-binding ligand concentration; **(G,H)** excess ligand concentration; **(I,J)** log K.

growth responded strongly to N and Fe, and that atmospheric aerosols were able to relieve this limitation.

The growth response of oceanic *Synechococcus* to aerosol additions differed markedly from coastal *Synechococcus* populations (**Figures 4A and B**). Unlike the coastal population, oceanic *Synechococcus* did not respond as strongly to the inorganic nutrient addition of N,P,Fe, in keeping with the observation that oceanic

Synechococcus respond more favorably to smaller (nanomolar) NO_3^- additions (Glover et al., 2007). The largest oceanic *Synechococcus* growth response occurred when Aerosol 2 was added, which resulted in a nearly 10-fold increase in *Synechococcus* abundance. Unlike the coastal experiment, the drawdown of NO_3^- was similar in the Aerosol 1, Aerosol 2, and N,P,Fe treatments in the oceanic experiment (∼2 μM drawdown, **Figure 5**), suggesting that the stronger growth of *Synechococcus* was probably not due to differences in NO_3^- uptake. Likewise, the greater growth response in Aerosol 2 was likely not due to Fe since Aerosol 1 and Aerosol 2 contributed similar dissolved Fe. Rather, our results indicate that the growth may have been due to relief from limitation by a trace metal micronutrient, since Aerosol 2 had a higher compliment of most dissolved metals than Aerosol 1 (**Table 1**; **Figure 2**).

To determine which micronutrients in Aerosol 2 might have contributed to the strong growth response of *Synechococcus* in the oceanic experiment, we compared the initial levels and calculated the drawdown of several biologically important metals to see which had been drawn down as the *Synechococcus* bloomed (**Figure 7**). In the oceanic experiment, drawdown of Fe, Cd, Co, Cu, Mn, and Ni all occurred following addition of Aerosol 2. Additionally, initial levels of Co, Mn, and Ni were significantly higher in the aerosol 2 treatment compared to the control (**Figure 6**), whereas they were not significantly higher than the control in the N,P,Fe treatment. This suggests that aerosol-derived Co, Mn, or Ni may have contributed to the strong *Synechococcus* growth response to Aerosol 2.

Unlike eukaryotic phytoplankton, *Synechococcus* has an absolute requirement for Co that cannot be satisfied by other metals. Eukaryotic phytoplankton, including diatoms and coccolithophores, are able to substitute Zn for Co, thereby maintaining growth even when Co levels are vanishingly low (Sunda and Huntsman, 1995a; Saito and Goepfert, 2008). In contrast, *Synechococcus* cells cannot substitute Zn for Co, and their populations have been shown to correlate with dissolved Co concentrations in the Sargasso Sea (Saito and Moffett, 2001) and the Costa Rica Upwelling Dome in the Equatorial Pacific Ocean (Saito et al., 2005). Moreover, concentrations of Co-binding ligands also co-vary with *Synechococcus* cell abundances at these locations, leaving open the possibility that *Synechococcus* produces these organic chelating compounds to help acquire Co where Co concentrations are in the low picomolar range. The high affinity of *Synechococcus* for Co is one of many adaptations that could contribute to high *Synechococcus* abundance during the spring bloom in the Sargasso Sea. The preference of *Synechococcus* to utilize NO_3^- as a N source may also promote *Synechococcus* growth over that of *Prochlorococcus*, which tends to dominate this region during the stratified season when NO_3^- levels are very low (Moore et al., 2008; Martiny et al., 2009).

Relief from Co limitation is a possible explanation for the strong *Synechococcus* growth response observed in response to the Aerosol 2 addition. However, this explanation does not rule out the possibility that *Synechococcus* were driven to Co limitation during the experiment by the high levels of NO_3^- in Aerosol 2, or that they benefited from other metals that were introduced by Aerosol 2. Additionally, it is possible that other phytoplankton and bacteria may have contributed to the drawdown of Co we observed in the Aerosol 2 treatment. The cellular Co quota for cultured *Synechococcus bacillaris* ranges from 0.08 to 1.43 μmolCo $molC^{-1}$, similar to the Co content of particulate material in the Sargasso Sea in which picoplankton dominate (∼1.5 μmolCo $molC^{-1}$; Sherrell and Boyle, 1992). In the oceanic experiment, *Synechococcus* POC increased ∼70 mgC m^{-3} in response to the Aerosol 2 treatment (**Figure 4G**). Taking the higher cellular Co quota estimate of 1.5 μmolCo $molC^{-1}$, this increase in *Synechococcus* POC would have only required ∼9 pM Co uptake, suggesting that picoeukaryotes and other microbes may have also contributed to the 40 ± 20 pM Co drawdown we observed.

Other biologically relevant metals, such as Mn and Ni, were also drawn down over the course of the experiment, suggesting that they too fueled the growth of the phytoplankton community. Mn is an important plant micronutrient, and is a central component of the oxygen evolving complex of photosystem II in all photosynthetic organisms, including phytoplankton (Falkowski and Raven, 2007). Mn is also a cofactor in the superoxide dismutase (SOD) enzyme of diatoms that protects cells from oxidative stress (Wolfe-Simon et al., 2006). Similarly, Ni is a cofactor in some (cyano)bacterial SOD enzymes (Palenik et al., 2003), as well as a cofactor for the urease enzymes of many marine phytoplankton (Price and Morel, 1991; Palinska et al., 2000). The sharp drawdown of these metals during the oceanic incubation experiment suggests that aerosol-associated Mn and Ni enrichment has the potential to supply phytoplankton with these vital micronutrients in the open ocean.

In addition to requiring metals for nutritional purposes, the higher resistance to Cu toxicity in *Synechococcus* compared to *Prochlorococcus* has also been proposed to shape phytoplankton community structure in the Sargasso Sea, particularly during the summer-stratified period (Mann et al., 2002). The bioavailability of dissolved Cu has been shown to be a function of free Cu^{2+} concentrations, and not total dissolved Cu concentrations (Sunda and Guillard, 1975). As dissolved Cu is typically complexed by strong organic ligands in seawater, Cu bioavailability is dependent upon ambient Cu speciation. In our oceanic experiment, Cu^{2+} concentrations were similar between the initial and t3 control bottles, increased slightly in the Aerosol 1 treatment, and nearly doubled over control concentrations in the Aerosol 2 treatment. In contrast, in the coastal experiment, Cu^{2+} concentrations in the initial and t3 control were roughly fourfold higher than observed in the initial and control bottles of the oceanic experiment, and decreased by nearly half in both the Aerosol 1 and Aerosol 2 treatments.

Cu^{2+} concentrations in the oceanic and coastal experiments were well below toxicity thresholds for marine phytoplankton, including *Synechococcus* species (toxicity threshold $\sim 10^{-11}$ M; Brand et al., 1986) in all samples and treatments. Thus, Cu toxicity is not likely to have had an effect on phytoplankton growth in these experiments. This is consistent with previous Cu addition experiments in the Sargasso Sea showing no decrease in the growth of *Synechococcus* following 2 nM Cu additions (Mann et al., 2002).

The changes in Cu^{2+} concentrations in the oceanic and coastal experiments reflect the changes in total dissolved Cu and strong Cu-binding ligands measured in the experiments. In the absence of a change in L_1 concentrations, the increase in dissolved Cu

measured from the aerosol treatments would have increased Cu^{2+} concentrations in both the coastal and oceanic experiments. However, only in the Aerosol 2 treatment of coastal waters, where dissolved Cu concentrations (1.88 nM) would have exceeded the L_1 concentrations measured in the t3 control (1.82 nM), would Cu^{2+} concentrations have increased dramatically and approached toxicity thresholds for *Synechococcus* in the absence of Cu-binding ligand production. *Synechococcus* have been shown to produce Cu-binding ligands under Cu toxicity stress in the laboratory (Moffett and Brand, 1996), but there is no indication of Cu toxicity in any of the treatments and *Synechococcus* growth was much higher in the Aerosol 2 treatment than Aerosol 1. It is not obvious, then, why excess Cu-binding ligands in both experiments were highest in the Aerosol 1 treatments, and similar between initial, t3 control, and t3 Aerosol 2 treatments.

It is also notable that initial Cu-binding ligand concentrations were in greater excess of dissolved Cu in the oceanic waters than in the coastal waters. This is counterintuitive if Cu-binding ligands are primarily produced by cyanobacteria to alleviate Cu toxicity, as Cu^{2+} concentrations were well below toxicity thresholds throughout both of these experiments, even after aerosol-associated Cu additions. The sources and function of Cu-binding ligands, particularly in oceanic waters, merits further attention, as the conventional wisdom linking Cu toxicity with ligand production does not fit well here. Recent studies have highlighted the role of Cu as a micronutrient in the ocean, limiting large diatom growth under low Fe conditions (Peers et al., 2005; Peers and Price, 2006). However, experiments in this study were dominated by picoeukaryotes and cyanobacteria under relatively Fe replete conditions, hence regulation of nutritional Cu uptake may be more complicated than previously believed.

Prochlorococcus was not abundant in our incubation water, so we were unable to directly assess how aerosol-derived Cu would affect this population. The results of Mann et al. (2002) show that Cu toxicity to *Prochlorococcus* in this region can occur following 2 nM or less Cu enrichment. Our results show that following moderate amounts of aerosol enrichment, Cu^{2+} levels remained well below the toxicity threshold for *Prochlorococcus* during the spring. However, the summer-stratified period generally has more large atmospheric deposition events, and Cu-binding ligand concentrations are ~twofold lower than during winter mixing (Moffett, 1995; Mann et al., 2002). This leaves open the possibility that aerosol-Cu enrichment could potentially curb *Prochlorococcus* growth in the summer-stratified period, but this would likely be limited to exceptionally large deposition events of aerosols with high fractions of soluble Cu.

THE IMPACTS OF ATMOSPHERIC METALS IN THE SURFACE OCEAN

During the spring in the Sargasso Sea, nutrients introduced during winter mixing are trapped within the sunlit layer as the surface ocean stratifies, providing conditions necessary for the spring bloom to occur. NO_3^- availability is high in the spring relative to the rest of the year, and has been shown to drive the system toward Fe limitation (Moore et al., 2006). Moore et al. (2006) show that the supply of Fe from atmospheric deposition could influence the initiation, duration, and magnitude of the spring bloom by regulating phytoplankton NO_3^- uptake, thereby stimulating new primary production. The results of the incubation experiments in this study support the hypothesis of Moore et al. (2006) regarding the importance of atmospheric deposition as a source of Fe that regulates the spring bloom, as the greatest increases in photosynthetic biomass were observed in treatments where Fe (and N) were available.

In addition to supplying Fe, our results also suggest an additional role for atmospheric deposition during the spring bloom. Atmospheric deposition can influence phytoplankton community composition during the spring bloom by providing other trace metal micronutrients, such as Co, that could limit growth as the bloom progresses. These findings are in agreement with those of Shelley et al. (2010), who observed that the nutrient-like profile for dissolved Co displayed a surface maximum following a period of strong aeolian deposition in the Sargasso Sea. The results presented here suggest that aerosol-derived Co is bioavailable and can support the growth of phytoplankton. Our results also show that in addition to Fe and Co, aerosols provide many biologically important trace metals needed for phytoplankton growth (**Figure 6**), and that these nutrients get consumed as cells grow (**Figure 7**). The selective enhancement of *Synechococcus* abundance in the oceanic experiment demonstrates the potential for these aerosol-derived metals to cause shifts in community composition by relieving the nutrient limitation of certain taxa and altering competition among different subpopulations.

The influence and fate of atmospheric metals may differ spatially in the Sargasso Sea. In the oceanic experiment, metals were consumed rapidly as phytoplankton grew. In contrast, little drawdown occurred following aerosol additions in the coastal experiment, suggesting that coastal phytoplankton assemblages may have been less stressed for these metals. This difference between sites may stem from a greater input of metals to coastal waters from terrigenous sources (e.g., runoff, groundwater) and resuspension of shelf sediments, compared to the open ocean that has fewer sources of trace metals. Alternately, the results could point toward a higher degree of NO_3^- limitation in the coastal population, which may have precluded greater uptake of trace metal. Indeed, all of the NO_3^- contributed by Aerosol 2 was consumed by the end of the coastal experiment, whereas >2 μM residual NO_3^- persisted in the oceanic experiment (**Figure 5**). If coastal phytoplankton became NO_3^- limited during the experiment, this may have precluded efficient uptake of aerosol-derived trace metals.

The effect of atmospheric metal deposition on bloom development depends on the nutrient status of the resident phytoplankton assemblage, the aerosol depositional flux to the ocean's surface, and the chemical composition of the aerosol. The Sargasso Sea receives a substantial input of Sahara dust during the summer months, whereas in the winter aerosols from North America predominate (Prospero et al., 1996). In this study both Aerosol samples contained a mixture of material from Sahara and North American sources (not shown); however, Aerosol 2 had more anthropogenic material than Aerosol 1 and caused a stronger growth response in the oceanic phytoplankton community that was commensurate with metal drawdown. The flux of soluble metal (i.e., the fraction that is likely available to phytoplankton) depends strongly on the relative proportions of anthropogenic versus mineral particles a region receives. Anthropogenic aerosols can be >10-fold

enriched relative to average crustal abundances in metals such as Ni, Cu, Zn, Pb, and Cd (Chen et al., 2008; Chase et al., 2011). More variability exists in the total Fe content in atmospheric deposition with respect to anthropogenic and mineral sources; however, anthropogenic aerosols tend to have much higher fractional metal solubility compared to mineral aerosol (i.e., a greater proportion of the total Fe is water soluble, Sholkovitz et al., 2012). Similar solubility trends have been observed for Cu in the North Atlantic, where the fractional solubility of Cu in anthropogenic sources ranges from 10 to 100%, while Cu in mineral aerosol from the Sahara desert is less soluble (1–7%; Sholkovitz et al., 2010). Accordingly, the amount of soluble, and presumably bioavailable, metals arriving at a location may be strongly influenced by even small inputs of anthropogenic aerosol.

Since the spring bloom is a transitional time for air masses reaching the Sargasso Sea (Prospero et al., 1996), the amount of bioavailable metals delivered via atmospheric deposition would depend strongly on the relative proportion of anthropogenic aerosols in a given deposition event. In this study, Aerosol 2 was enriched in V relative to Al, a property that indicates greater anthropogenic input. The higher soluble metal content and greater fertilization potential of Aerosol 2 (**Figures 6 and 4**), may therefore be linked to an anthropogenic source. Our results suggest that the effect of atmospheric deposition during the spring bloom therefore depends not only on the amount of deposition, but the specific source and chemical composition of the aerosol deposited. The greater input of anthropogenic aerosols in the late winter/early spring as the bloom develops could be important in determining the magnitude of NO_3^- drawdown, as has been proposed for aerosol Fe (Moore et al., 2006), as well as in shaping phytoplankton community composition, as observed for Aerosol 2 in this study. Coupled ecosystem-biogeochemical models that include atmospheric deposition should include this distinction where possible to explore its effect on productivity.

CONCLUSION

In this study we sought to understand the effect of atmospheric metal deposition on oceanic and coastal Sargasso Sea phytoplankton communities. This study shows that aerosol deposition contributes biologically important constituents in addition to Fe and N to seawater, and that these bioavailable metals get consumed over time scales commensurate with cell growth. In particular, aerosol-derived micronutrients like Co, for which certain taxa have absolute requirements (Sunda and Huntsman, 1995a), may favor the growth of certain subpopulations and alter phytoplankton community composition. In contrast, during the spring season when these experiments were conducted, Cu toxicity did not appear to be an important factor influencing phytoplankton community composition.

The solubility of atmospheric metals is an important factor affecting metal bioavailability, and the relative proportions of anthropogenic and mineral sources in atmospheric deposition has the potential to influence phytoplankton growth and community composition in a given region. Accordingly, different types of aerosol can elicit very different growth responses from phytoplankton communities. These responses can differ between distinct populations despite their relatively close geographic proximity, and can reflect different antecedent conditions to which the cells were exposed with respect to metal availability.

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Microplate-reader method for the rapid analysis of copper in natural waters with chemiluminescence detection

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We have developed a method for the determination of copper in natural waters at nanomolar levels. The use of a microplate-reader minimizes sample processing time (~25 s per sample), reagent consumption (~120 μL per sample), and sample volume (~700 μL). Copper is detected by chemiluminescence. This technique is based on the formation of a complex between copper and 1,10-phenanthroline and the subsequent emission of light during the oxidation of the complex by hydrogen peroxide. Samples are acidified to pH 1.7 and then introduced directly into a 24-well plate. Reagents are added during data acquisition via two reagent injectors. When trace metal clean protocols are employed, the reproducibility is generally less than 7% on blanks and the detection limit is 0.7 nM for seawater and 0.4 nM for freshwater. More than 100 samples per hour can be analyzed with this technique, which is simple, robust, and amenable to at-sea analysis. Seawater samples from Storm Bay in Tasmania illustrate the utility of the method for environmental science. Indeed other trace metals for which optical detection methods exist (e.g., chemiluminescence, fluorescence, and absorbance) could be adapted to the microplate-reader.

Keywords: microplate-reader, copper detection, chemiluminescence, 1,10-phenanthroline, seawater

INTRODUCTION

Copper, like many trace metals, is an essential micronutrient at very low concentrations or availability, but may be toxic and have deleterious effects at elevated concentrations. Copper concentrations in natural waters vary greatly depending on water type and location. Open ocean surface water concentrations can be as low as 0.5 nM, while deep ocean concentrations are as high as 5 nM (Boyle et al., 1977; Bruland, 1980). Coastal waters generally have higher copper concentrations, from between 1 nM in pristine waters to as high as 755 nM in highly contaminated coastal area such as Chañaral in northern Chile (Stauber et al., 2005) or 170 nM for the Erhjin Chi Esturay in China (Han et al., 1994). The average concentration of copper in world rivers is 160 nM (Sarmiento and Gruber, 2006), with concentrations as high as 1.2 mM found in the Odiel river, Spain (Olias et al., 2004).

Overexposure to high copper concentrations is associated with myriad biological and ecological impacts, including salmon physiology (Baldwin et al., 2003), domoic acid production by toxigenic pennate diatoms (Maldonado et al., 2002), and the growth and relative abundance of phytoplankton species (Moffett et al., 1997; Mann et al., 2002; Paytan et al., 2009). Mining, fossil fuel combustion, industrial processes, and other anthropogenic activities have greatly accelerated the release of this toxic trace metal to the oceans (Newman and Unger, 2003) resulting in increased efforts to determine the sources, transport and fate of Cu (and other metals) in the aquatic environment (Taylor and Shiller, 1995).

Current methods for low-level copper analysis in natural waters include flow injection analysis with chemiluminescence detection (Coale et al., 1992) based on the luminescence produced by the complexation of copper with 1,10-phenanthroline (Yamada and Suzuki, 1984), *in-situ* analysis using chemiluminescence (Holm et al., 2008) and colorimetry (Callahan et al., 2003), ICP-MS (Field et al., 1999) and electrochemical methods (Achterberg and Braungardt, 1999; Wang, 2002). The complexity of these methods and their relatively large sample size requirements (generally at least 40 mL) has limited their use and contributes directly to the lack of regular monitoring of environmental copper concentrations. Such measurements would increase our understanding of the sources of copper to the aquatic environment and their impact. A simple, low-volume, and low-cost method for copper analysis would also be useful in manipulative biological experiments such as *in-vitro* culturing work (Brand et al., 1986; Peers et al., 2005) and mesocosm incubations (Paytan et al., 2009) where sample volumes are often limited.

Microplate-readers, or plate-readers, are instruments designed to measure the absorbance, fluorescence, or luminescence of samples in microtitre plates. The plates typically consist of 96 wells, with a volume of 100–200 μL per well. Their main advantages are small sample volume, high-throughput of samples and ease of use. They have been widely used in biological sciences for many years (Ashour et al., 1987), but their use in environmental chemistry is limited. Methods have been published for macro-nutrient

analysis in seawater (Ringuet et al., 2011) and pore-water constituents (Laskov et al., 2007). To our knowledge there have been no plate-reader methods developed for low-level trace metal analysis.

In this paper we describe the development of a plate-reader method to detect copper by chemiluminescence via the reaction with 1,10-phenanthroline and hydrogen peroxide. The plate reader is well suited to this application. Many chemiluminescent reactions are kinetically fast, resulting in optimal detection immediately after reagent-sample mixing. The plate reader uses two precision injectors (two needles placed around the detector) to introduce reagent into the sample and is able to begin detection before or during reagent injection. Furthermore, the small size of the instrument makes it practical for use at sea or in the field.

MATERIALS AND METHODS

MATERIALS

This work used a FLUOstar OPTIMA microplate-reader equipped with two reagent injectors. The reagent needles are made of stainless steel, the tubing and valve housing are made of Teflon and Kel-F, and the syringe barrel is made of glass. Each syringe has a volume of 500 µL, and can inject a minimum of 50 µL and maximum of 450 µL in each cell. The detector present on the FLUOstar OPTIMA is a photomultiplier tube (PMT). No filters are used in this method.

All microplate measurements were made in a lab with an air conditioning system set to 20°C. No further temperature control was employed.

Low Density Polyethylene (LDPE) bottles were used to store reagents, stock solutions and samples. Plates used to determine copper concentration were the CELLSTAR Cell Culture Multiwell Plates 24 and (grenier bio-one) with a physical surface treatment, made in crystal Clear® polystyrene, sterile and individually packed.

MEASURES TO MINIMIZE CONTAMINATION

The preparation and manipulation of the reagents, standards, and samples was carried out in a class 100 laminar flow hood. All the materials used for the experiments (e.g., reagents, samples, standards, tips) were stored in two ziplock bags inside the flow hood except for the hydrogen peroxide, which was stored in three ziplock bags in a fridge dedicated to trace metal analysis. Not ideally, the microplate reader instrument was not in a laminar flow hood, so plates and reagents were placed in a ziplock bag for transport and kept covered until analysis. During the analysis reagent bottles were partially capped and kept in closed ziplock bags. During manipulations the operator wore non-sterile nitrile gloves.

New and used bottles were rinsed five times with Milli-Q water, and then soaked in a 6 N HCl solution (Aldrich ACS reagent). Then the bottles were rinsed five times with Milli-Q water and soaked for 7 days in a 6 N trace metal grade nitric acid (J. T. Baker) solution. Then bottles were rinsed 10 times with Milli-Q water, dried in a laminar flow hood, and stored capped in ziplock bags. Acid baths were changed after 6 months or 200 bottles washed. Pipet tips were cleaned immediately prior to use with two rinses of 6 N Ultrex® II HCl, one rinse of Milli-Q water then 1–3 complete volumes of the intended solution.

Plates were rinsed first with 50% acetone (Aldrich ACS reagent), and then rinsed five times with Milli-Q water. The cells were then filled with 6 N trace metal HCl (J. T. Baker) for two days. Finally they were rinsed five times with Milli-Q water and dried in a laminar flow hood. Plates were used only once because during prolonged exposure the hydrogen peroxide attacked the plastic.

The two precision injectors of the plate reader, used to inject reagents, were washed each day before use. The first step was to rinse the syringes, needles, and tubes three times with 4.5 mL (this is the maximum volume of the syringe) of 6 N HCl (J. T. Baker "trace metal" grade). Then injectors were rinsed three times with 4.5 mL of Milli-Q. These steps were repeated and finally the injectors were flushed with 4.5 mL of each reagent. At the end of each analytical session the system was rinsed two times with 4.5 mL of Milli-Q water.

ANALYTICAL METHOD

A chemiluminescence method for flow-through analysis of copper (II) in seawater using 1,10-phenanthroline (Coale et al., 1992; Zamzow et al., 1998), was adapted to the microplate-reader. This method involves the production of luminescence during the catalytic decomposition of hydrogen peroxide by the copper-1,10-phenanthroline complex, at a pH ∼9.5 (Yamada and Suzuki, 1984). The addition of cetylethyldiethylammonium bromide (CEDAB) introduces surfactant micelles in solution (Yamada and Suzuki, 1984) which increases the sensitivity of the method by increasing the probability of contact between the reagent and the dissolved copper in the solution. In addition, a stable complexing agent for copper, tetraethylenepentamine (TEPA), is added to remove the background signal attributed to copper impurities in the reagents themselves (Yamada and Suzuki, 1984).

REAGENT PREPARATION

Reagent 1 consists of 30% (in volume) hydrogen peroxide solution (Stabilized ACS reagent grade).

Reagent 2 contains 0.180 M 1,10-phenanthroline, 0.06 M TEPA, 0.225 M NaOH and 0.06 M CDAB in purified Milli-Q water (see below). This reagent is made by adding 2.4 g of CDAB (Reagent grade, Sigma) and 0.9 g of NaOH (ACS Reagent grade, J. T. Baker) to 100 mL of purified Milli-Q water and allowing for complete dissolution. Then 30 µL of a 4 mM stock solution of TEPA (97%, Fluka) and 1.5 mL of a 12 mM stock solution of 1,10-phenanthroline (99%, Aldrich) are added.

CREATION OF STANDARDS AND BLANKS

All standard and blank solutions are made from "copper free" seawater or Milli-Q water. Seawater is filtered using a 0.2 µm poly-ester-sulphone membrane filter (Pall Acropak). The filtered water is brought to pH 6 with ∼300 µL/L of 6 N Ultrex® II HCl and passed through a 5 mL iminodiacetic acid column (HiTrap Chelating HP, Amersham Biosciences) at a flow rate of <5 mL/min. The column is prepared by first washing it with 50 mL of 0.1 N HCl, then 100 mL of Milli-Q water, and finally

with 50 mL of seawater before collection. Acidified (pH ∼1.7) standards are made from pH 6 seawater which is then acidified by adding 3.7 mL/L of 6 N Ultrex® II HCl. Standards are prepared by gravimetric dilution from a 1 g/L copper certified reference solution for trace metal analysis and were stable for at least 1 month. Milli-Q water cleaned with the HiTrap column ("purified Milli-Q"), was used to make pure water standards and blanks.

PROCEDURE

The cleaned and dried 24-well plate is filled with 700 µL of pH 1.7 sample, blanks and standards, using a 100–1000 µL micropipette and clear polypropylene tips. Generally five pH 1.7 standards and a blank, bracketing the range of expected concentrations, are used, with four replicates of each standard. We generally run each sample in triplicate. The plate is covered and placed inside two ziplock bags for transport to the plate reader. The plate reader injectors are flushed (see cleaning process) and finally the plate is loaded into the plate reader and analyzed. Generally samples are inside the plate for less then an hour before their analysis.

One hundred and twenty microliter of each reagent is injected to produce the luminescence reaction. A pump speed of 310 µL/sec was used, as recommended by the manufacturer. This speed permits good mixing between the reagents and the sample and limits well-to-well contamination. The positioning delay refers to a waiting period after a well of the microplate moves to the measurement position and before the measurement begins. The positioning delay allows the liquid to settle and the surface to become stable so that the measurement is more accurate. The recommended delay time from BMG LABTECH of 0.2 s was used. An end-point mode was used for all analyses, meaning each cell was measured once before moving to the next cell. The instrument was programmed to record light emission after 25 s, in order to maximize the signal (see "Results and Discussion").

SAMPLE COLLECTION

Surface seawater was collected from six stations in Storm Bay, Tasmania, from a 15 m aluminum ship. A nylon net was braided around a 60 mL acid-washed LDPE bottle to create a holder and the net was linked to the operator with a 5 m nylon line. Eight 30 g lead weights placed inside ziplock bags were attached to the net to sink the bottle. To keep the bottle as far as possible from the boat hull a boat hook was used to catch the line, separated from the sample bottle by at least a meter in order to not contaminate the sample. Contact between sample and air never exceed 30 s (the time to catch and close the bottle). All samples were individually packed inside two ziplock bags and stored in a closed HDPE box for transport back to the lab. The nylon net was rinsed with 1 M HCl and Milli-Q water between samples. Samples were refrigerated for two days, then acidified to pH 1.7 with ultra-pure HCl and then filtered through acid-cleaned 0.2 µm poly-ester-sulphone membrane filters (pall Acropak). This sequence (acidification followed by filtration) is not ideal, and consequently the copper concentrations reported here should be interpreted with caution. They most likely represent a fraction equivalent to slightly less than the total dissolvable copper.

UV IRRADIATION

We investigated the impact of UV irradiation on copper analysis. Samples were dispensed into 100 mL teflon bottles and placed between two commercial GPH843TSL/4 ultraviolet lamps inside a black PVC chamber for 1–3 h.

CALCULATION

The detection limit is calculated as three times the standard deviation of the concentration measured on blanks. Precision is calculated as the relative standard deviation (%) (RSD) of the standard concentration. Generally analyses were performed on four replicates, except where noted.

RESULTS AND DISCUSSION

REPEATABILITY

The repeatability of the method was assessed using a 24-well plate filled with 700 µL of either a 50 nM seawater standard or copper-free seawater. The Cu-free seawater was obtained by passing a low-Cu, UV-irradiated open ocean surface sample through the chelating column. For each plate the first three replicates were discarded because of poor precision on start-up. Well-to-well reproducibility is good (**Figure 1**), with a relative standard deviation of 4.46% on the 50 nM standard or a standard deviation equivalent to 2.23 nM. On blanks the standard deviation is equivalent to 0.23 nM. Generally the %RSD of replicates run on the same plate is about 5% and never above 10%.

SENSITIVITY, LINEAR RANGE, DETECTION LIMITS, AND ACCURACY

Detection limits were 0.4 nM in pure water and 0.7 nM in seawater (**Figure 2**). Linearity was observed to 200 nM in pure water and 100 nM in seawater (data not shown). Sensitivity, represented by the slope of the calibration curve, was always greater in pure water then in seawater by a factor of 3–5, consistent with previous studies (Holm et al., 2008). It is therefore important to match the standard matrix to that of the samples. Sensitivity varied by 25% day-to-day and appeared random, possibly linked to factors such as room temperature, humidity, oxygen, and CO_2 levels (Xiao et al., 2002). It is therefore important to perform a calibration with each plate analysed.

Accuracy was assessed with four replicates of the standard reference material CASS-5 (5.9 ± 0.44 nM). The measured value for CASS-5 (7.1 ± 1.1 nM) is consistent with the certified value (**Figure 2**), confirming the accuracy of the technique.

OPTIMIZATION OF INJECTED REAGENT CONCENTRATIONS

Previous studies (Zamzow et al., 1998; Holm et al., 2008) have optimized reaction pH, and concentrations of 1,10-phenanthroline, TEPA, CDAB, and hydrogen peroxide for maximum signal intensity. We wanted to see whether the concentration of reagents injected into the cells had an impact on signal intensity. We were interested in minimizing contamination and reagent volume injected. In this test we maintained optimized reagent concentrations and pH in the reaction cells but adjusted the volume and concentration of injected reagents; when the reagent concentrations were increased the volume injected was decreased proportionally within the capabilities of the plate reader's injectors (**Table 1**). Sample volume was held

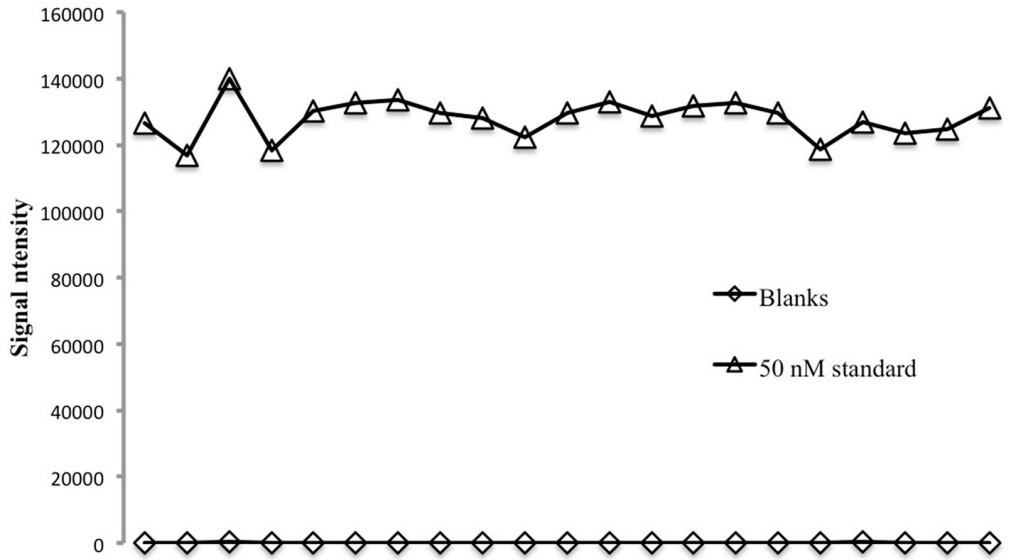

FIGURE 1 | Reproducibility on 21 replicates analyzed in a 24-well plate for 50 nM seawater consistency standard and seawater blanks.

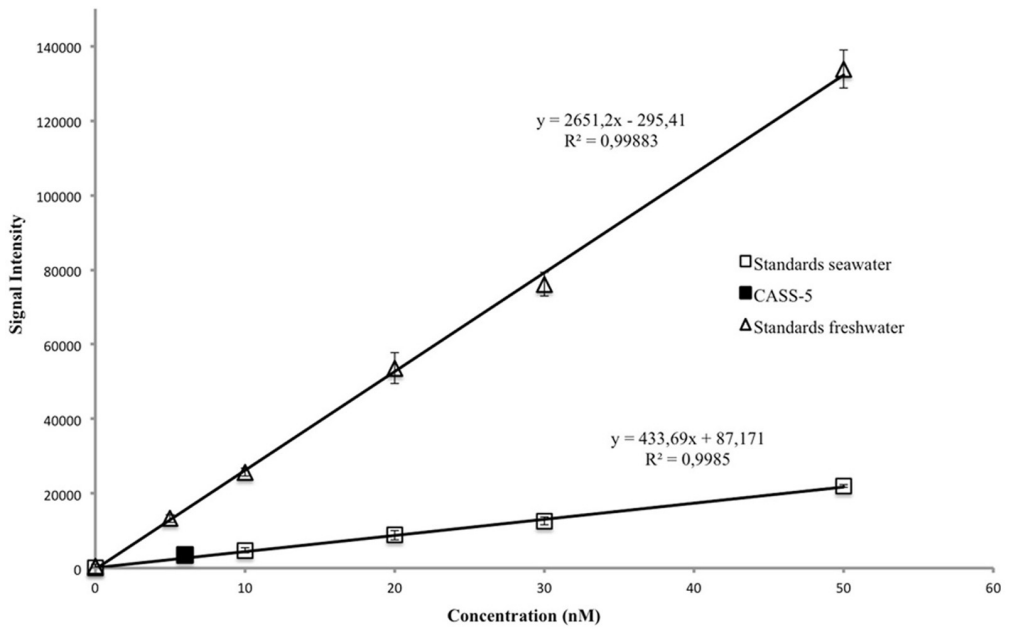

FIGURE 2 | Calibration curves in seawater and freshwater with CASS-5 reference for seawater.

constant at 700 μL. In pure water, the signal intensity increased with decreasing reagent volume, reaching a maximum at 130 μL (**Figure 3**), corresponding to a hydrogen peroxide concentration of 30%. This result most likely reflects the effect of a decreased dilution of the sample. In seawater the signal intensity was not as sensitive to reagent volume (**Figure 3**). However, using a 30% hydrogen peroxide solution and 130 μL reagent injection for both pure water and seawater reduces the risk of contamination by eliminating a dilution step during reagent preparation.

OPTIMIZATION OF REAGENT VOLUME INJECTED

An optimization was made to determine whether the assay response was sensitive to small variations in reagent volume. For this experiment reagent concentrations corresponding to 30% hydrogen peroxide were used (see **Table 1**) and the concentration of reagents in the well was not maintained constant; when the volume injected increased the concentration was not adjusted. For both seawater and freshwater, an injected volume of 120 μL produced the best signal

Table 1 | Reagent and sample volumes used in the experiment to optimize reagent concentrations and volumes.

Reagent volume injected (μL)	Sample volume (μL)	R1 concentration in injectors (% V/V)	R1 amount of substance in cell (mmol)	R2 concentration in injectors (M)	R2 amount of substance in cell (μmol)
130	700	30	1.3	0.18	24
200	700	20	1.3	0.12	24
400	700	10	1.3	0.06	24
450	700	5	0.75	0.03	13

The amount of substance of each reagent stays constant in the well by adjusting the volume and concentration injected.

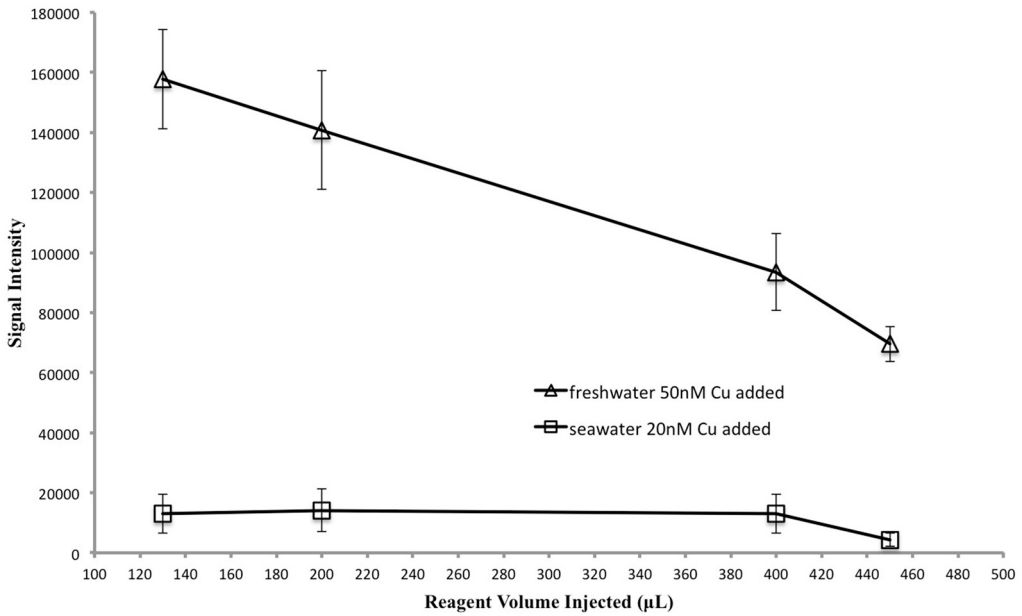

FIGURE 3 | Reagent volume vs. signal intensity to test the optimum reagent concentration and volume. Sample volume was held constant. As reagent volume was adjusted so too were reagent concentrations, in order to maintain a constant amount of each reagent in the cell. See **Table 1** for details.

intensity (**Figure 4**), so this volume was chosen for all experiments.

We also tried pre-mixing the two reagents in 1-1 proportions and using only one mixed reagent with twice the volume injected, as other studies have done. A mixed reagent did not work with the plate reader, as the two tensioactives (CDAB and TEPA) present in the phenanthroline reagent produced bubbles and foam inside the injector syringes in the presence of hydrogen peroxide.

OPTIMIZATION OF DETECTION TIME

The kinetics of the luminescence reaction between copper and 1,10-phenanthroline are very fast, less then a second (Eigen, 1963). However, we observed that signal intensity increased for 25 s after reagent injection (**Figure 5**). This delay probably occurs because there is no mechanical mixing in the well, so it takes time for convection and diffusion to achieve a homogenous solution and for complete reaction to occur. The optimal detection time was 25 s. For each cell, the luminescence signal at 25 s was recorded as the final signal. With these settings a 24-well plate can be analysed in 10 min.

PLATE CONTAMINATION

We found that when new, sterile plates are used unwashed the signal intensity for blanks is as much as 35 nM and the standard deviation is more than 86% of the signal intensity. After 12 h in a cleaned plate the signal intensity of a 20 nM seawater standard (pH ∼1.7) had increased by 45% and the standard deviation increased more than 4-fold. These data suggest that polystyrene continues to release copper into solution even if plates are acid-cleaned, consistent with previous studies (Batley and Gardner, 1977; Howard and Statham, 1997). Consequently plates need to be analysed as soon as possible after filling. The use of alternative plate materials such as PTFE or polyethylene may minimize this source of contamination.

UV IRRADIATION

UV irradiation is normally not used with flow-through copper detection via the luminescence reaction with 1,10-phenanthroline (Coale et al., 1992; Holm et al., 2008). An acidification step to pH ∼1.7 for at least 24 h prior to analysis was assumed to release all ligand-bound copper to the solution. However, other studies

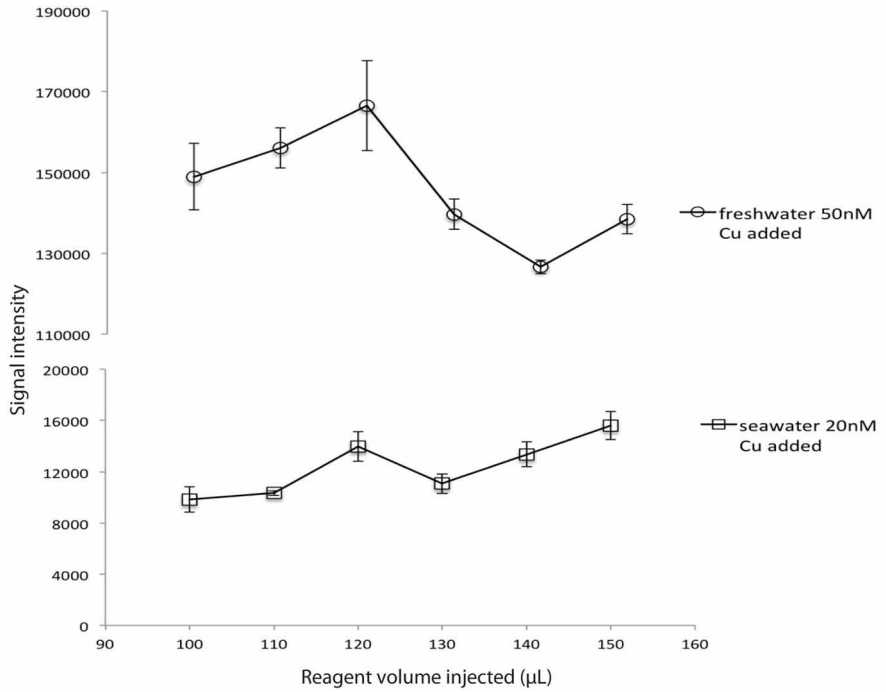

FIGURE 4 | Volume of reagents injected vs. signal intensity for a constant sample volume and a constant reagent concentration (R1 30% H_2O_2; R2 0.18 M).

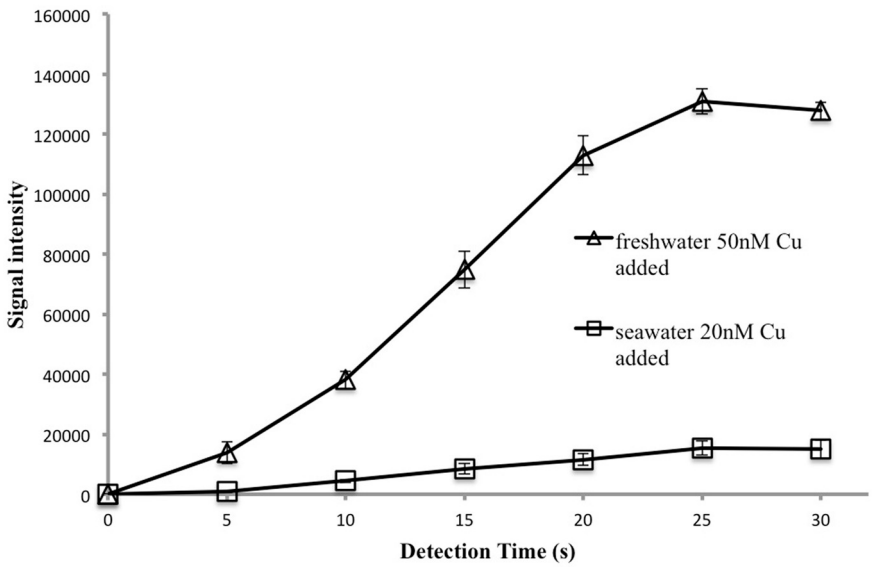

FIGURE 5 | Signal intensity as a function of detection time. Each data point represents the average and standard deviation of 4 replicates analyzed after the time indicated. Thus four samples were analyzed after 5 s, another four after 10 s and so on.

have suggested UV irradiation may be necessary to dissociate some metal-ligand complexes (Van Den Berg, 1984; Achterberg et al., 2001a).

We found evidence to support the need for UV irradiation with a seawater sample collected off the coast of Bruny Island, Tasmania. This water was sampled without respecting trace metal protocols, filtered, "copper-cleaned" with the Hi-Trap column, and acidified, after which copper was added to produce a range of standards. However, we were unable to obtain a linear calibration curve, with the number of photons emitted staying approximately constant across copper additions. Furthermore, the signal intensity for the un-amended sample was very high. It is clear that

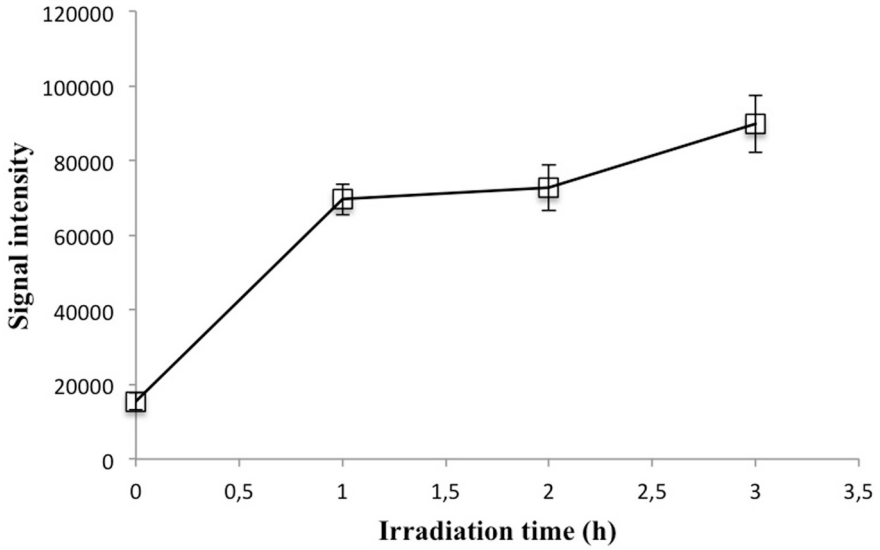

FIGURE 6 | Signal intensity vs. irradiation time for a 20 nM coastal seawater standard. Each point represents the mean and standard deviation of four subsamples independently irradiated and analyzed.

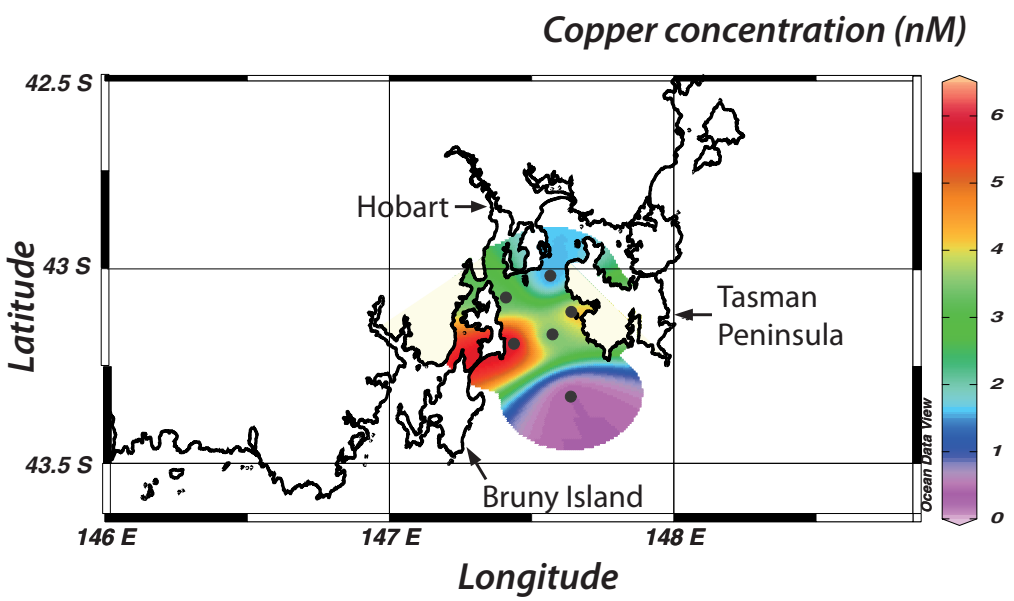

FIGURE 7 | Copper concentrations in Storm Bay, concentrations are given in nM. Concentrations starting from the north east and moving south and east are 1.37 ± 0.4; 3.26 ± 0.7; 4.25 ± 0.8; 3.18 ± 1; 6.18 ± 0.4; 0.39 ± 0.1.

in this case the chelating column was not effective at removing copper ions, possibly due to presence of a strong ligand, as only free copper is removed by the column. **Figure 6** shows the effect of UV irradiation on the signal intensity of a 20 nM addition made to this water. After 1 h of irradiation the signal intensity is increased 4-fold and stays approximately constant past this time, suggesting successful release from organic copper-binding ligands.

ANALYSIS OF COASTAL SEAWATER SAMPLES

The analysis of six seawater samples collected from Storm Bay, Tasmania, illustrates the application of the plate-reader method to real samples (**Figure 7**). The sampling sites are directly downstream from the Derwent estuary, which is highly contaminated by trace metals including copper (Butler and Wangersky, 2006). Despite the proximity to the Derwent estuary, copper concentrations in surface waters of Storm Bay are relatively low, and

similar to oceanic concentrations (0.3–3 nM) (Buckley and Van Den Berg, 1986; Coale and Bruland, 1990). One exception is a sample adjacent to Bruny Island with a concentration of 6.2 nM. We are not aware of any previous measurements of Cu in Storm Bay, but Butler and Wangersky (2006) report Cu concentrations in the Derwent River and estuary of 1.8–232 nM, consistent with our results. In the Macquarie estuary on the west coast of Tasmania, copper concentrations are between 10 and 50 nM (Carpenter et al., 1991). The relatively low copper concentrations measured in Storm Bay may be due to a strong oceanic influence, as a storm had passed through the area 3 days before sampling. This example demonstrates the efficiency of the plate reader method, as sample analysis took only 3 h, including acidification and filtration steps. The microplate-reader technique is particularly well suited to large sample sets (more than 100 samples); the absence of a pre-concentration step and the short detection time minimizes sample processing time.

POTENTIAL APPLICATIONS

The plate-reader method for copper analysis described here provides a rapid, simple, low-volume method with good precision at relatively low concentrations suitable for work in freshwater and saltwater. These characteristics of the method make it applicable to a range of applied and basic research in the biological and environmental sciences. The method could be useful in culture, mesocosm and aquaculture studies and in the analysis of pore-waters or samples from benthic flux chambers. The method would also be useful for environmental monitoring of surface waters at high spatial and temporal resolution, or monitoring of wastewater streams and storm water overflow. Significantly, the detection limits of the method in freshwater and seawater are 13 and 7 times greater than the respective ANZECC guidelines for freshwater and marine ecosystems, 5.2 and 5.0 nM (ANZECC/ARMCANZ, 2000).

The detection limits reported here are not suitable for determining copper in seawater at the lowest concentrations found in the literature for the open ocean (e.g., <1 nM). This problem could be solved by the use of a dedicated, trace metal clean instrument placed inside a laminar flow hood to protect plates during their analysis and the use of other types of plates, for example polyethylene or teflon. The method could also be coupled with off-line pre-concentration (Biller and Bruland, 2012) for measurement of ultra-trace level samples.

Other optical methods for trace metal analysis using chemiluminescence, fluorescence, or spectrophotometric detection may be amenable to adaptation to the microplate reader. Iron (Achterberg et al., 2001b), cobalt (Sakamoto-Arnold and Johnson, 1987), aluminum (Hydes and Liss, 1976), and zinc (Grand et al., 2011), are good candidates for this method. A plate-reader method could also be used for Cu speciation, following the titration method of Zamzow et al. (1998).

CONCLUSIONS

With the use of our optimization conditions, which include detection after 25 s, concentrated reagents with appropriate volume injected, and plate cleaning, we have obtained a low detection limit of 0.4 nM in freshwater and 0.7 nM in seawater for a 700 µL sample with a precision of 7%. These detection limits permit a rapid, simple, and inexpensive determination of copper concentration suitable for coastal seawater and river water.

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Geographical gradients of dissolved Vitamin B_{12} in the Mediterranean Sea

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Most eukaryotic phytoplankton require vitamin B_{12} to grow. However, the cycling of this organic growth factor has received substantially less attention than other bioactive substances such as trace metals in the marine environment. This is especially true in the Mediterranean Sea, where direct measurements of dissolved vitamins have never been reported. We report here the first direct measurements of dissolved vitamin B_{12} across longitudinal gradients in Mediterranean waters. The range of vitamin B_{12} concentrations measured over the whole transect was 0.5–6.2 pM, which is slightly higher than the range (undetectable—4 pM) of ambient concentrations measured in other open ocean basins in the Pacific and Atlantic oceans. The concentrations measured in the western basin were significantly higher ($p < 0.05$) than those of the eastern basin. They were positively correlated with chlorophyll concentrations in the most western part of the basin, and did not show any significant correlation with any other biological variables in other regions of the sampling transect.

Keywords: vitamin B_{12}, Mediterranean Sea, growth factor, phytoplankton, bacteria

INTRODUCTION

The Mediterranean Sea is an oligotrophic ecosystem (Mc Gill, 1965; Krom et al., 1991), with a west to east gradient of increasing oligotrophy. It is characterized by a strong thermal stratification with a sharp thermocline (10–20 m deep) during late spring to fall, and a mixing period in winter, leading to a phytoplankton bloom in the early Spring (e.g., Marty et al., 2002; Moutin et al., 2012).

Surface macro-nutrient concentrations also depend on the exchanges with the Atlantic Ocean (through the Strait of Gibraltar), the Black Sea (through the Bosphorus Strait and Marmara Sea), and depend on river discharge. In addition, the Mediterranean Sea receives the highest rate of aeolian dust deposition of the world's oceans (Guerzoni et al., 1999) as well as anthropogenic aerosols from industrial and domestic activities from the highly populated areas around the basin (Chester et al., 1996; Guieu et al., 1997). Consequently, Mediterranean surface waters exhibit relatively high dissolved iron (Fe) concentrations that are linked to the dynamics of atmospheric deposition and water column stratification (Bonnet and Guieu, 2006). Therefore, Fe availability rarely limits primary and prokaryotic heterotrophic production in those waters (Bonnet et al., 2005; Pulido-Villena et al., 2008). However, the elemental stoichiometry measured in different pools (i.e., particulate and dissolved, inorganic and organic) reveals a deficiency of phosphorus (P) relative to nitrogen (N) (Bethoux et al., 2002), and phosphorus (P) availability has been seen to limit primary production, prokaryotic heterotrophic production and N_2 fixation along the Mediterranean basin (e.g., Thingstad and Rassoulzadegan, 1995; Lasternas et al., 2010; Ridame et al., 2011).

Besides inorganic bioactive elements such as Fe and P, most eukaryotic phytoplankton require organic nutrients such as vitamin B_{12} (Provasoli and Carlucci, 1974; Droop, 2007). Vitamin B_{12} is a cobalt-containing organometallic compound involved in several vital enzymes in the central metabolism of algae (Raux et al., 2000; Martens et al., 2002). Many eukaryotic phytoplankton lack the biosynthetic pathway for vitamin B_{12}. Croft et al. (2005, 2006) recently showed that over 50% of the 326 algal species tested in culture collections were unable to grow without any vitamin B_{12} additions, showing that they have an absolute requirement for this co-factor and thus depend on an exogenous pool. In the open and coastal ocean, the ambient pool may be insufficient to support maximum primary productivity as vitamin B_{12} amendments have been shown to stimulate phytoplankton growth in the Atlantic, Pacific and Southern Ocean (Panzeca et al., 2006; Sañudo-Wilhelmy et al., 2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011). The ambient pool of vitamin B_{12} depends upon prokaryots (Archaea, Bacteria, Guillard, 1968) as they possess the biosynthetic pathway to produce this vitamin, and eukaryotic algae would acquire vitamin B_{12} either from symbiotic bacteria or directly from the dissolved pool (Provasoli, 1963; Croft et al., 2005; Droop, 2007). Furthermore, vitamin concentrations have been linked to shifts in plankton community composition (Koch et al., 2011). Recent laboratory

studies also showed that ubiquitous picocyanobacteria, such as *Synechococcus* and unicellular diazotrophic cyanobacteria such as *Crocosphaera*, were also able to produce and release vitamin B_{12}, with higher production rates compared to heterotrophic bacteria (Bonnet et al., 2010).

Despite the recognized biogeochemical importance of vitamin B_{12} in the Ocean, the cycling of this organic growth factor has received substantially less attention than other bioactive substances such as trace metals in the marine environment. This is especially true in the Mediterranean Sea, where direct measurements of dissolved vitamins have never been reported. Measuring vitamin B_{12} concentrations in seawater represents a technical challenge, as this cofactor is found at picomolar (10^{-12} M) levels in open ocean waters (e.g., Panzeca et al., 2008, 2009). The development of direct and efficient methods, in contrast to the indirect microbiological assays, to measure low levels of dissolved vitamin B_{12} (Okbamichael and Sañudo-Wilhelmy, 2004) now allows us to expand our knowledge on the biogeochemical cycling of this growth factor in the ocean (e.g., Panzeca et al., 2006, 2008, 2009; Sañudo-Wilhelmy et al., 2006, 2012; Suárez-Suárez et al., 2011).

In late spring 2007, we measured dissolved vitamin B_{12} concentrations along a 3000 km west-east transect in the Mediterranean Sea (**Figure 1**), that exhibited nutrient and chlorophyll gradients, to characterize the vertical distribution of vitamin B_{12} concentrations along with other relevant hydrological and biogeochemical factors.

MATERIALS AND METHODS

Dissolved water samples for vitamin B_{12} and other biological and chemical parameters were collected during May 2007 along a longitudinal transect in the Mediterranean Sea onboard the Spanish ship R/V Garcia del Cid. A total of 13 stations were sampled across a west-to-east transect (**Figure 1**), starting southwest of Sardinia and finishing in the Aegean Sea (Station 13). The cruise track also covered the eastern Mediterranean basin, near the Egyptian coast (Station 10).

HYDROLOGICAL AND BIOGEOCHEMICAL MEASUREMENTS

Vertical profiles of temperature and salinity were obtained using a Seabird 911 plus CTD. Seawater samples were collected at 6 depths between the surface and 200 m depth using 12 l Niskin bottles mounted on a rosette sampler.

Chlorophyll *a* (Chl *a*) concentrations were determined fluorometrically according to the method of Parsons et al. (1984). At each depth, 50 ml of seawater were filtered through 25 mm glass fiber filters (Whatman GF/F), extracted into 10 ml of 90% acetone for 24 h in the dark at 4°C. The fluorescence of the extracts was then measured on a calibrated Turner Designs fluorometer (Parsons et al., 1984).

For inorganic nutrients (nitrate, phosphate, silicate), samples were collected in acid-washed 20 ml plastic flasks. Concentrations were determined using standard colorimetric techniques on a Bran Luebbe autoanalyser AA3. Detection limits for the procedures were 0.05 μM, 0.01 μM, and 0.1 μM for $NO_2^- + NO_3^-$, PO_4^{3-} and $Si(OH)_4$, respectively.

VITAMIN B_{12} CONCENTRATION MEASUREMENTS

At each depth, 2 l of seawater were sampled for dissolved vitamin B_{12} concentration determination. Briefly, immediately after collection, samples were acidified to pH 6 using 12 N trace metal grade HCl, transferred to sterile 2 l intravenous (IV) bags, and pumped through 5 g of Bondesil C18 resin (pre-conditioned with methanol) at a flow rate controlled at

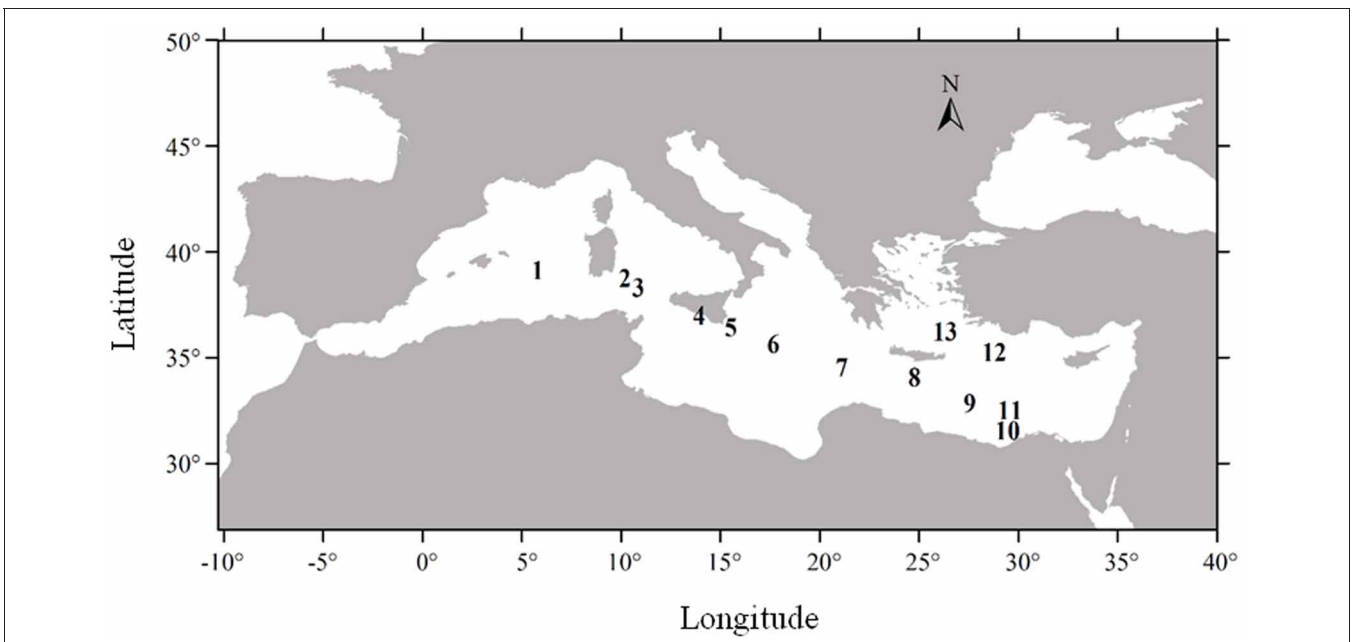

FIGURE 1 | Location of the thirteen stations of the cruise.

1 ml min^{-1}. Columns were then rinsed with 20 ml of MilliQ water and eluted with 5 ml HPLC grade methanol. The eluent was then evaporated under vacuum (Labconco Rapid-Vac), redissolved in 200 µl of MilliQ water, and analyzed by High Performance Reverse Phase Liquid Chromatography (Shimadzu 10AD-vp) according to Okbamichael and Sañudo-Wilhelmy (2004). Samples were filtered again through 0.45 µm small-volume syringe filters (Millex®- FH) to further purify the sample before injection into the HPLC The method and filter type was tested using B_{12} standard recovery (Panzeca, 2007).

PROKARYOTIC ABUNDANCES (PA) AND PROKARYOTIC HETEROTROPHIC PRODUCTION (PHP) MEASUREMENTS

PA was determined by flow cytometry according to Trousselier et al. (1995) after fixation of samples (4 ml) using a mixture of paraformaldehyde and glutaraldehyde (1%) and freezing in liquid nitrogen. Briefly, 200 µl of each sample was stained with 4 µl of 5 µmol l^{-1} SYBR Green (Molecular Probes) for 10 minutes in the dark, and run through a FACS calibur™ flow cytometer (BD Biosciences) fitted with a laser emitting at 488 nm. Samples were run at a low flow rate and data were acquired in log mode until around 10,000 events were acquired. A stock solution (5 µl) of yellow–green 0.92 µm Polysciences latex beads was added as an internal standard per 200 µl of sample. The concentration of the fluorescent beads was calibrated every 2 days by direct microscope enumeration. Prokaryotic cells were detected by their signature in bivariate plots of side scatter (SSC) vs. green fluorescence (FL1). Data were gated and counted in the SSC vs. FL1 plot using the Paint-a-Gate software (del Giorgio et al., 1996; Gasol and Del Giorgio, 2000). PA was expressed in cells per liter.

PHP was estimated from 3H-leucine–protein synthesis following the microcentrifugation technique described in Smith and Azam (1992). Briefly, 5 µl of L-[4,5-3H] leucine were added to 1.5 ml samples, yielding a final concentration of 28.8 nM, and were incubated for 3 h. Incubations were stopped by addition of trichloroacetic acid (5% final concentration) and samples were stored at −20°C until processing at the home laboratory. We used a conversion factor from leucine to carbon incorporation of 1.5 kg C mol leu^{-1} (Simon and Azam, 1989).

Ocean Data View software was used for graphical presentation of all the data mentioned above. For constructing of the charts, one of the ODV interpolation methods—VG Gridding (X and Y scale length: 150) was used.

STATISTICS

Vitamin B_{12} concentrations in the eastern and western basins were compared using a 2-tailed non parametric mean comparison test ($n = 3$, $\alpha = 0.05$, unpaired samples). Surface (0–100 m) and deep (100–200 m) vitamin B_{12} concentrations were compared using a 2-tailed non parametric mean comparison test ($n = 3$, $\alpha = 0.05$, paired samples). To examine the relationship between vitamin B_{12} concentrations, PA, PHP, and Chl *a* concentrations, Pearson's correlation coefficients were calculated and tested between each variable of interest (degree of freedom = $n - 2$, $\alpha = 0.05$).

RESULTS

HYDROLOGICAL AND BIOGEOCHEMICAL BACKGROUND CONDITIONS

Temperature ranged from 15.95–20.24°C across the Mediterranean basin over the first 200 m (**Figure 2A**). These figures show the occurrence of thermal stratification along the whole transect, but with a shallower thermocline depth in the western basin (around 40 m depth) compared to the eastern basin (50–200 m depth). There was also a strong horizontal salinity gradient from west to east with a marked halocline in the Ionian Sea (stations 5 and 6) (**Figures 1**, **2B**). This gradient separates western Atlantic waters entering the Mediterranean Sea through the Strait of Gibraltar, from the high salinity waters of the eastern Mediterranean Sea. Salinity was lower on the western side of the transect (down to 37 in surface waters), and gradually increased eastwards to reach up to 39 in the top 200 meters.

Surface waters were depleted in nutrients (**Figures 2C–E**). $NO_2^- + NO_3^-$ (hereafter NO_x) concentrations were low (around 0.1 µM) along the whole transect. The thickness of this depleted layer increased towards the east from about 50–80 m in the western part of the transect to more than 180 m in the eastern basin. Phosphate concentrations followed the same geographical trend as NO_x, with surface concentrations close to the detection limit of conventional micromolar methods (0.01 µM), and a progressive deepening of the phosphacline going eastward. Surface phosphate concentrations increased in the Aegean Sea (stations 12 and 13) to reach around 0.06–0.07 µM. The NO_x:PO_4 molar ratio was 21 over the whole cruise suggesting that the Mediterranean was potentially phosphate limited with respect to nitrate during our sampling campaign.

Silicate concentrations decreased from west to east to reach up to 0.18 µM in the eastern basin (**Figure 2E**), and increased again to 0.5 µM in surface waters of the Aegean Sea (stations 12 and 13). As seen with NO_x and PO_4, the depth of the silicacline deepened in the east (**Figures 2C–E**). The average stoechiometric Si:NO_x molar ratio was 2.5 over the whole transect, which suggested that silicate was in sufficient supply relative to nitrate.

Chl *a* concentrations (**Figure 3A**) ranged from 0.02 to 1.6 µg l^{-1} over the 0–200 m layer of the studied transect. The whole section was characterized by a deepening of the Deep Chlorophyll Maximum (DCM) from west (40–80 m) to east (80–125 m), associated with the increasing oligotrophy. Chl *a* concentrations in the DCM also decreased eastward. In the last 2 stations of the transect located in the Aegean Sea (stations 12 and 13), Chl *a* concentrations increased to 0.97 µg Chl *a* l^{-1} in the DCM, and the depth of the DCM was shallower (around 60 m) as in the western side of the Mediterranean.

VITAMIN B_{12} CONCENTRATIONS

The range of vitamin B_{12} concentrations (**Figures 3B**, **4**) measured over the Mediterranean transect was 0.5–6.2 pM. A slight longitudinal gradient was present; the concentrations measured in the western basin (stations 1–6: 2.44 ± 1.64 pM; mean of all profiles from the western basin ± standard deviation) were significantly higher ($p < 0.05$) than those of the eastern basin (stations 7–13: 1.67 ± 0.92 pM) (2-tailed non parametric mean

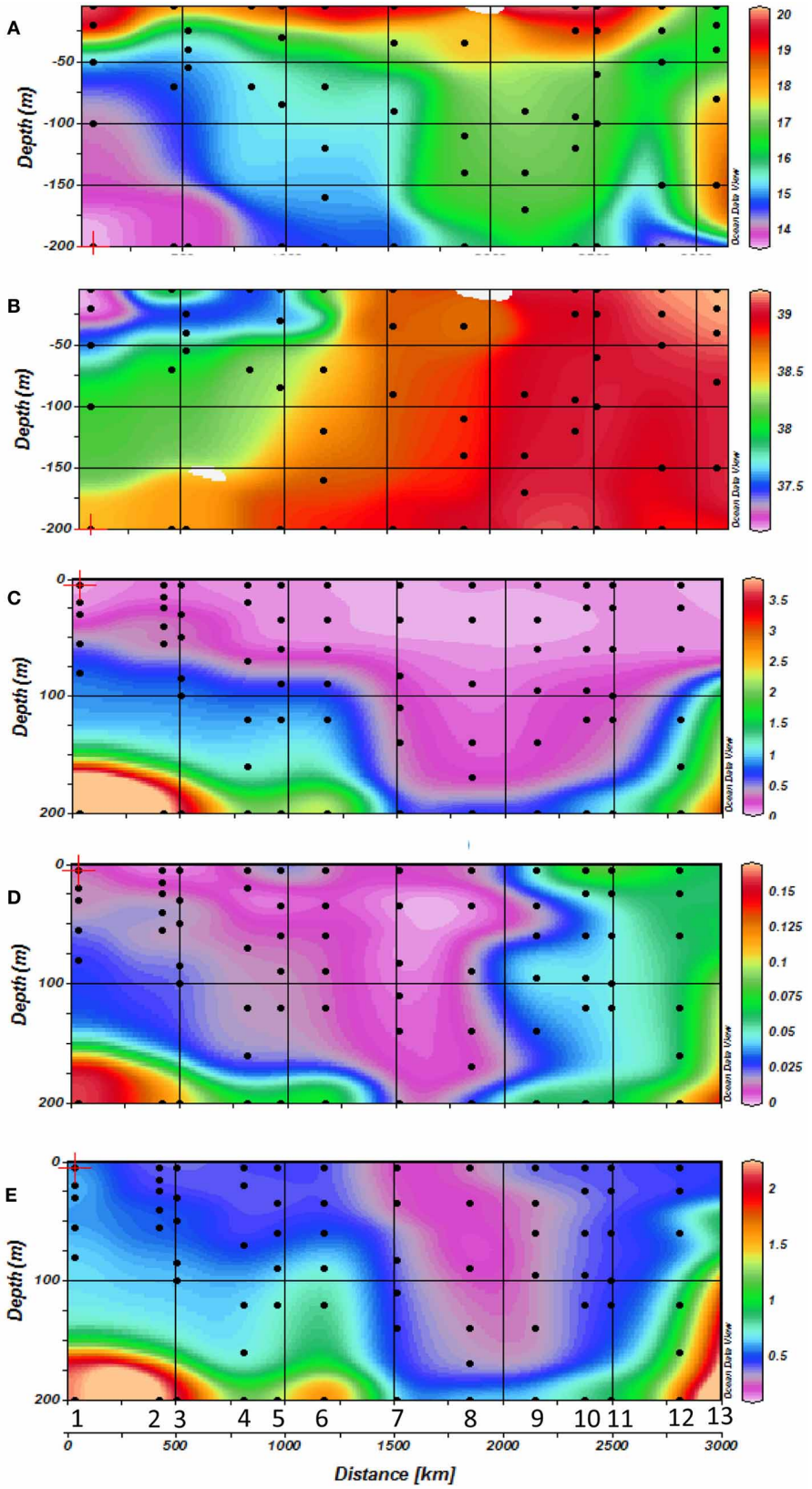

FIGURE 2 | Horizontal and vertical distribution of (A) temperature, (B) salinity, (C) NOx concentrations, (D) PO_4 concentrations and (E) Silicate concentrations along the transect with station numbers (1–13) and sampling depths indicated by dots during cruise.

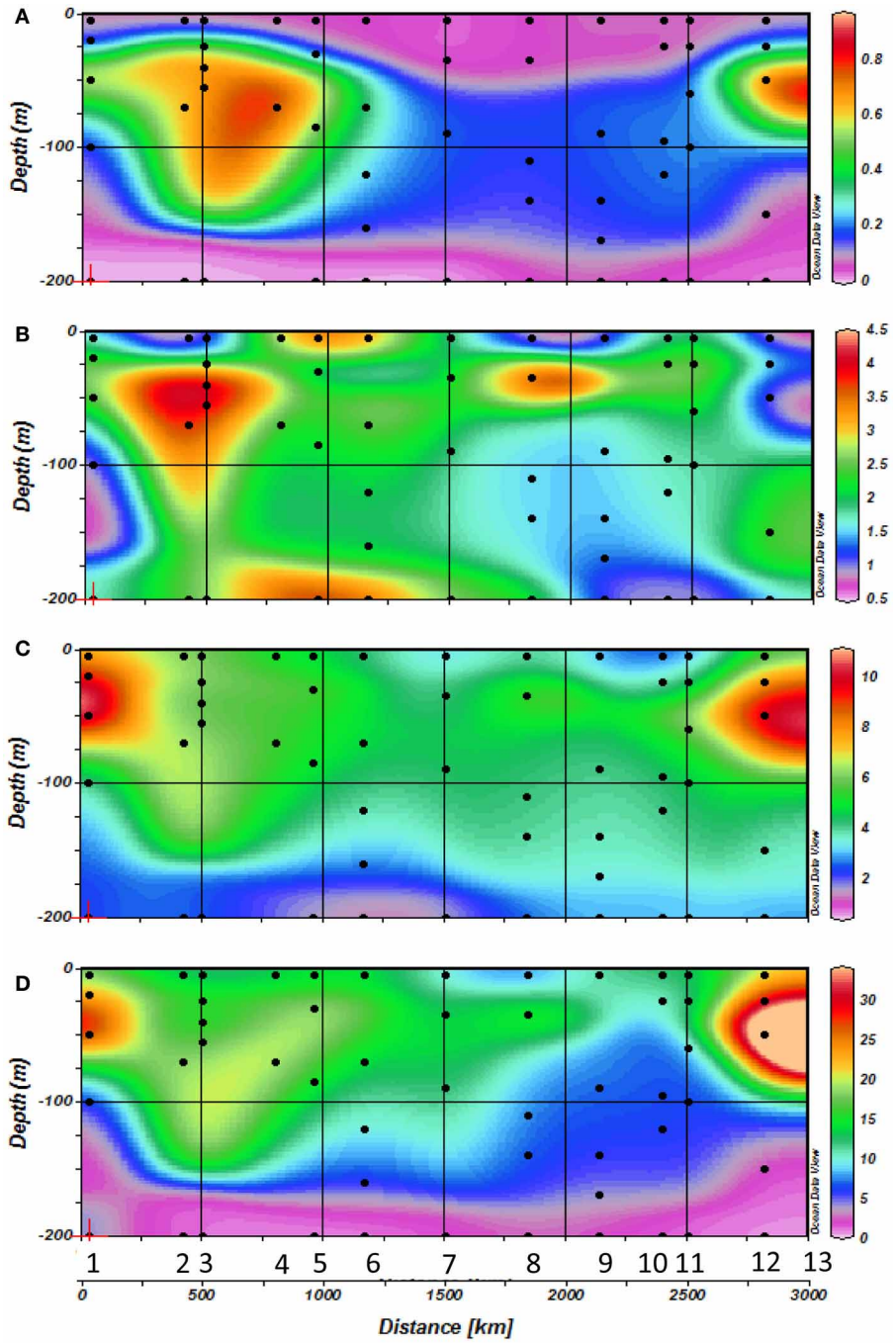

FIGURE 3 | Horizontal and vertical distribution of (A) Vitamin B_{12} concentrations, (B) Chlorophyll *a* concentrations, (C) Prokaryotic abundances (PA) and (D) Prokaryotic heterotrophic production (<php) rates along the transect with station numbers (1–13) and sampling depths indicated by dots during cruise.

comparison test). Maximum values were reached at 40 m depth at station 3 (6.15 pM) and in surface at station 5 (6.20 pM). Most vertical profiles exhibited maximum vitamin B_{12} concentrations close to the DCM (**Figure 4**), except at station 5. Other B_{12} depth profiles were more homogeneous vertically (Stations 7 and 10). The two stations where samples were collected at 1000 m (i.e., stations 5 and 7) exhibited concentrations of 1.19 and 2.7 pM at this depth, respectively.

PROKARYOTIC ABUNDANCES AND PRODUCTION

Prokaryotic abundance (**Figure 3C**) declined markedly from west to east. Maximum abundances were measured at station 1 at

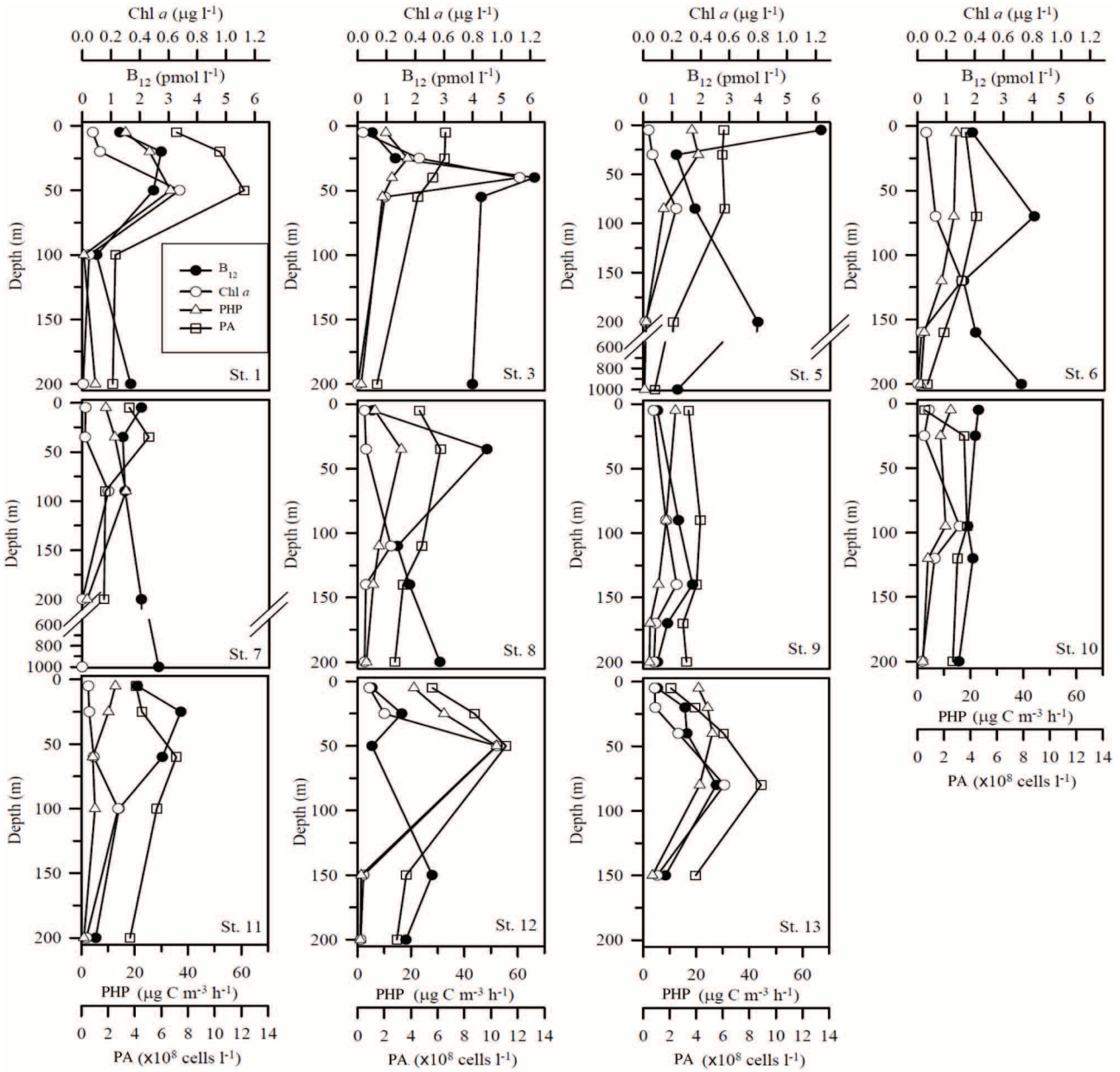

FIGURE 4 | Vertical profiles of vitamin B_{12} (pM), Chl *a*, Prokaryotic abundances (PA) and Prokaryotic heterotrophic production (PHP). Units for B_{12} and Chl *a* concentrations, PA and PHP are pmol l^{-1}, µg l^{-1}, $\times 10^8$ cells l^{-1} and µg C m^{-3} h^{-1}, respectively. Note that profiles for stations where only two data points were available are not plotted as vertical profiles.

20-50 m depth with 12×10^8 cells l^{-1}. The abundance maxima then decreased towards the east and was located deeper within the water column (around 75 m depth). Abundances increased again at the Aegean Sea at the end of the transect to reach concentrations around 11×10^8 cells l^{-1} at station 12 at 50 m depth.

Prokaryotic heterotrophic production (**Figure 3D**) followed approximately the same trend observed for prokaryotic abundance with maximum rates measured at station 1 at 20–50 m depth (25–33 µg C m^{-3} h^{-1}); it then decreased toward the eastern basin to reach minimum rates at stations 10 and 11 (10–12 µg C m^{-3} h^{-1}), increasing again in the Aegean Sea, reaching up to 52 µg C m^{-3} h^{-1}.

DISCUSSION

The Mediterranean Sea displayed a large variety of hydrological conditions during the stratification period, covering a large range of trophic conditions from the oligotrophic western basin to the ultra-oligotrophic eastern basin (**Figures 2**, **3**). The west-to-east gradient in hydrological and biogeochemical conditions

encountered during our cruise (thermal stratification, nutrient depletion in surface waters and the deepening of the nutriclines and the DCM) are typical conditions for the Mediterranean Sea during the so-called stratification period (e.g., Moutin et al., 2012).

Vitamin B_{12} is known to be a labile organic molecule with a short half-life (days) in seawater (Carlucci et al., 1969). Thus, elevated concentrations are generally associated with local production. Bacteria are the primary producers of this organic growth factor (Raux et al., 2000; Martens et al., 2002), but as soon as it is produced, it is consumed by eukaryotic phytoplankton and some ubiquitous heterotrophic bacteria lacking biosynthetic pathways for vitamin B_{12} (Giovannoni et al., 2005; Bertrand et al., 2007). The relation between production and stocks is thus sometimes more complex. In this study, vitamin B_{12} distributions do not show any significant correlation with spatial patterns observed for prokaryotic heterotrophic production and abundance (**Figure 3**, $r = 0.04$ and -0.04 respectively, $p > 0.05$), probably because vitamin B_{12} stocks are the net result of vitamin production and consumption by micro-organisms.

The depth distributions of vitamin B_{12} were correlated to the chlorophyll maximum in the western part of the transect (stations 1–3, $r = 0.66$, $p < 0.05$). This trend is contrary to what has been observed in some eutrophic coastal systems (Sañudo-Wilhelmy et al., 2006) where chlorophyll stocks in the fraction $>5\,\mu m$ were inversely correlated with vitamin B_{12} concentrations; these inverse distributions were interpreted as vitamin consumption by large size phytoplankton species in these coastal areas, as most large phytoplanktonic species are auxotrophic for vitamin B_{12} (Croft et al., 2005, 2006). In the Mediterranean Sea, during the stratification period, large-size phytoplankton are scarce and the system is dominated by prokaryotic phytoplankton which are pico-cyanobacteria *Synechococcus* and *Prochlorococcus* (Marty et al., 2002; Lasternas et al., 2010). Contrary to eukarytotic phytoplankton who are B_{12} consumers, recent studies indicate that *Synechococcus* is able to produce and excrete large amounts of vitamin B_{12} (Bonnet et al., 2010), and *Prochlorococcus* also possess the biosynthetic pathway for vitamin B_{12} production. Cyanobacterial counts are not available for this study but recent studies indicate that they are maximum at the DCM in Mediterranean waters during the season studied (Mella-Flores et al., 2011); vitamin B_{12} production by these cyanobacteria may explain the large vitamin B_{12} stocks measured at stations 2 and 3 in the DCM. It has to be noted that relatively high vitamin B_{12} concentrations were also measured in surface waters above the DCM at stations 4 and 5. These concentrations may be attributed either to prokaryotic heterotrophic production, or to synthesis by small cyanobacteria located in surface waters. Recent studies have reported an unusual near surface *Synechococcus* abundance maximum close to the Sicily Strait (Mella-Flores et al., 2011) during the stratification period in the Mediterranean Sea, which might explain high production rates of vitamin B_{12}. This cyanobacterial maximum is unusual compared to other oligotrophic areas (Olson et al., 1988; Li et al., 1992) and has been attributed to surface nutrient enrichment by atmospheric dust deposition (Mella-Flores et al., 2011). In fact, it has been shown that dust inputs cause an increase predominantly in the cyanobacteria in Mediterranean waters (Bonnet et al., 2005). At stations 4 and 5, nutrient inputs could also be from land sources as those stations are located near the Sicilian coast and usually that region is less oligotrophic than the rest of the Mediterranean Sea (e.g., Moutin et al., 2012). This hypothesis is consistent with the slight increase in phosphate concentrations observed in the present study (**Figure 2**).

Vitamin B_{12} concentrations were lower in deeper waters (100–200 m) compared to surface and subsurface (0–100 m) waters ($p < 0.05$), except at stations 3, 5, and 6 where they reached 3.99, 3.99, and 3.51 pM, respectively at 200 m depth. Stations 5 and 6 are located close to the Sicily strait, which separates the Mediterranean eastern and western basins, leading to active and complex hydrodynamic features (Pinardi and Masetti, 2000); we could hypothesize that vitamin B_{12} has been upwelled from deeper waters, potentially explaining the maxima seen at those stations. The two stations for which data are available below 200 m (stations 5 and 7) show concentrations of 1.19 and 2.67 pM at 1000 m, which are not very high but the lack of data between 200 and 1000 m makes it difficult to interpret. The only report of deep vitamin B_{12} concentrations (surface to 800 m depth) available (Sañudo-Wilhelmy et al., 2012) indicate that maximal concentrations were found in the upper mesopelagic zone, around 300 m and surface maxima can be the result of vitamin transport by different water masses. In the Mediterranean Sea, future studies including a deeper vertical resolution are needed to better understand B_{12} distributions.

Depth-averaged vitamin concentrations were significantly ($p < 0.05$) higher in the western basin (2.44 ± 1.64 pM) and decreased slightly going eastward towards more oligotrophic waters (1.67 ± 0.92 pM). This trend is consistent with the survey

Table 1 | Ranges of vitamin B_{12} concentrations reported in different marine areas of the world.

Studied area*	Range of vitamin B_{12} concentrations (pmol l^{-1})	References
Sargasso Sea	0–0.3	Menzel and Spaeth (1962)
San Pedro Basin, California, USA	0.2–1.8	Panzeca et al. (2009)
North Atlantic surface waters	0.1–2.5	Panzeca et al. (2008)
Northeast Pacific Ocean	0–2.7	Carlucci and Silbemagel (1966)
Southern part of the Indian Ocean	0.1–3.0	Fiala and Oriol (1984)
Bay of Biscay	0.1–3.7	Daisley and Fisher (1958)
Gerlache Strait, Southern Ocean	0.4–4	Panzeca et al. (2009)
Mediterranean Sea	0.5–6.2	This study

**The studied areas are ranked by the high limit of reported vitamin B_{12} concentration range.*

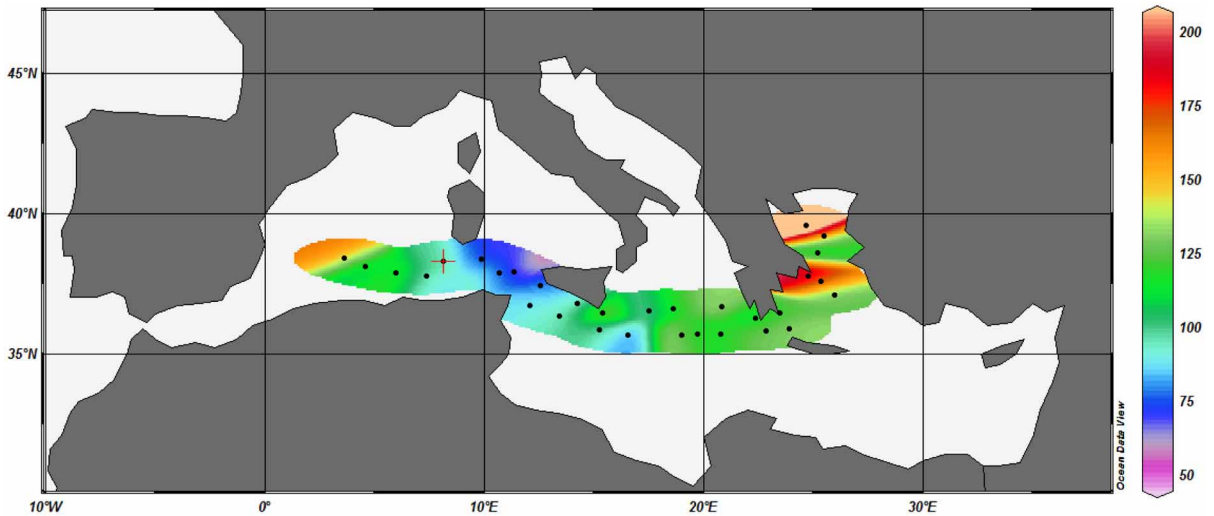

FIGURE 5 | Surface dissolved cobalt concentrations (pM) along the Mediterranean transect. Surface seawater (<0.22 μm) was collected during the cruise THRESHOLDS I (from June 5 to June 30, 2006; 35.6–41.9° N, 3.6–30.1° E) according to (Tovar-Sánchez, 2012). Cobalt was pre-concentrated by the APDC/DDDC organic extraction method and analyzed by ICP-MS according to protocols described in (Tovar-Sánchez, 2012).

performed by Moschopoulou and Ignatiades (1993), who measured the seasonal and spatial distribution of vitamin B_{12} in the Saronicos Gulf (Aegean Sea). They reported concentrations ranging between 0.8 and 5.8 pM, which are higher than those measured in the present study within the same area (1.39 ± 1.79 pM). However, those results were obtained indirectly using the microbiological assay method that may not accurately reflect ambient conditions, as they were also conducted in 0.45–0.8 μm filtered seawater that may have included B-vitamin–producing bacteria in the filtrate (Menzel and Spaeth, 1962; Carlucci and Bowes, 1970). Despite the few vitamin B_{12} data available in Mediterranean waters, the west-to-east gradient of B_{12} observed in our study is consistent with previous studies. For example, Martin and Vacelet (1975) and Fiala (1982) reported higher values in the western basin (French coast) compared to those reported for the eastern basin by Moschopoulou and Ignatiades (1993).

The overall range of vitamin B_{12} concentrations measured in the Mediterranean Sea in the present study (0.5–6.2 pM) are slightly higher than the range of ambient concentrations (undetectable—4 pM) measured in other open ocean locations in the world ocean (**Table 1**). For example, vitamin B_{12} concentrations at stations located off the coast of Baja California were undetectable for 100 s of kilometers (Sañudo-Wilhelmy et al., 2012). In the Southern Ocean, the North Atlantic and the South Indian Ocean, vitamin B_{12} concentrations ranged from 0.4 to 4 pM (Panzeca et al., 2009), 0.1–2.5 pM (Panzeca et al., 2008) and 0.1–3 pM (Fiala and Oriol, 1984) respectively (**Table 1**). The reasons for such relatively high B_{12} concentrations in Mediterranean waters despite oligo- to ultra-oligotrophic conditions are unclear. We can hypothesize that vitamin B_{12} accumulates in surface waters because the growth of the main consumers (eukaryotic phytoplankton) is limited by macronutrients (nitrate and phosphate, e.g., Lasternas et al., 2010; Ridame et al., 2011; Tanaka et al., 2011) in this area. Another hypothesis is that the high cobalt concentrations in Mediterranean waters stimulate de novo synthesis of vitamin B_{12} as cobalt is the central metal ion in the B_{12} molecule (Raux et al., 2000; Martens et al., 2002). Panzeca et al. (2008, 2009) have shown that the spatial distribution of vitamin B_{12} in various coastal and open ocean waters followed the abundance of total dissolved cobalt. Surface dissolved cobalt concentrations measured in surface Mediterranean waters are high (45–291 pM, **Figure 5** and Heimbürger et al., 2009) compared to those of the Atlantic (5–87 pM, Saito and Moffett, 2002; Panzeca et al., 2008) and the Pacific Ocean (30–105 pM, Knauer et al., 1982). They have been attributed to the high atmospheric dust inputs (Heimbürger et al., 2009) as cobalt is a crustal constituent of dust (1.8%) and is soluble in seawater (Thuróczy et al., 2010). Future studies combining simultaneous analysis of dissolved cobalt and vitamin B_{12} will be necessary to substantiate their possible relationship in Mediterranean waters.

This study reports the first direct measurements of dissolved vitamin B_{12} concentrations across longitudinal and trophic gradients in Mediterranean waters. Because vitamin B_{12} is an essential nutrient for most algal species (Croft et al., 2005), the availability of this growth factor could play a significant role on phytoplankton successions and carbon export in Mediterranean waters. Whereas our study could not resolve the possibility of B_{12} limitation in the Mediterranean, it did provide insights onto the possible mechanisms influencing the geographical distribution of vitamin B_{12}. Future studies will need to address the factors, such as photochemical degradation, production, excretion, and uptake rates by microorganisms, responsible for the cycling of vitamin B_{12} in Mediterranean waters.

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The distribution of thiamin and pyridoxine in the western tropical North Atlantic Amazon River plume

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B-vitamins are recognized as essential organic growth factors for many organisms, although little is known about their abundance and distribution in marine ecosystems. Despite their metabolic functions regulating important enzymatic reactions, the methodology to directly measure different B-vitamins in aquatic environments has only recently been developed. Here, we present the first direct measurements of two B-vitamins, thiamin (B_1), and pyridoxine (B_6), in the Amazon River plume-influenced western tropical North Atlantic (WTNA) Ocean, an area known to have high productivity, carbon (C) and dinitrogen (N_2) fixation, and C sequestration. The vitamins B_1 and B_6 ranged in concentrations from undetectable to 230 and 40 pM, respectively. Significantly higher concentrations were measured in the surface plume water at some stations and variation with salinity was observed, suggesting a possible riverine influence on those B-vitamins. The influences of vitamins B_1 and B_6 on biogeochemical processes such as C and N_2 fixation were investigated using a linear regression model that indicated the availability of those organic factors could affect these rates in the WTNA. In fact, significant increases in C fixation and N_2 fixation were observed with increasing vitamin B_1 concentrations at some low and mesohaline stations (stations 9.1 and 1; p value <0.017 and <0.03, respectively). N_2 fixation was also found to have a significant positive correlation with B_1 concentrations at station 1 (p value 0.029), as well as vitamin B_6 at station 9.1 (p value <0.017). This work suggests that there can be a dynamic interplay between essential biogeochemical rates (C and N_2 fixation) and B-vitamins, drawing attention to potential roles of B-vitamins in ecosystem dynamics, community structure, and global biogeochemistry.

Keywords: B-vitamin, thiamin, thiamine, pyridoxine, Atlantic, biogeochemical cycles, organic growth factors

INTRODUCTION

The Amazon River has the largest freshwater discharge of any river into the world ocean, resulting in an influx of low-salinity, nutrient-rich water into the western tropical North Atlantic (WTNA) Ocean (Subramaniam et al., 2008). The environmental conditions resulting from the river plume, influencing approximately two million km^2 in the WTNA, contributes to phytoplankton species succession, high rates of primary production, and a significant carbon (C) sink (Subramaniam et al., 2008). The neritic, high-nutrient areas of the plume are dominated by diatoms that utilize the nitrogen (N) and silicate (Si) supplied by the river leading to nutrient depletion in the water column. Following nutrient draw down, a shift in community composition occurs beginning with diatom-diazotroph associations (DDAs) as N becomes limited but sufficient supplies of Si are still present. This is followed by a subsequent community shift to more typical oceanic N_2 fixing organisms such as *Trichodesmium* spp. (Wood, 1966; Capone et al., 1997; Carpenter et al., 1999; Foster et al., 2007). The succession of phytoplankton species supports an extensive area of increased C and dinitrogen (N_2) fixation resulting in a C sink of approximately 1.7 Tmol annually (Subramaniam et al., 2008). Although many of the factors that limit C and N_2 fixation in this region have been extensively studied, the roles of organic growth factors such as B-vitamins have not been investigated despite their biological importance. With recent advances in analytical methodologies that directly measure B-vitamins in marine systems (Sañudo-Wilhelmy et al., 2012), we can now start understanding the processes influencing the distribution and concentrations of B-vitamins in the world ocean. River and groundwater inputs are thought to be sources of B-vitamins as their concentrations have been inversely correlated with salinity (Gobler et al., 2007) and river plumes have previously been shown to transport macronutrients and trace metals to the ocean (Boyle et al., 1982; Tovar-Sanchez and Sañudo-Wilhelmy, 2011). However, the transport of dissolved B-vitamins from rivers to the coastal ocean has never been evaluated. This study represents the first attempt to establish the importance of the Amazon River as a source of some B-vitamins to the WTNA Ocean.

B-vitamins are essential coenzymes for many diverse biochemical reactions, including enzymes in the Calvin cycle, amino acid biosynthesis, the tricarboxylic acid cycle (TCA cycle), and nucleic acid metabolism (Voet et al., 2001). Fitting with their central role in metabolism, B-vitamins were recognized as important promoters of bacterial growth as early as the 1930s (McDaniel et al.,

1939) and by the 1950s were found to be essential for the cultivation of many marine and freshwater algae (Provasoli and Pintner, 1953). Recent studies have confirmed the ecological relevance of B-vitamins in the environment by demonstrating their ability to limit or co-limit phytoplankton growth and biomass (Panzeca et al., 2006; Bertrand et al., 2007), including harmful algal blooms (Tang et al., 2010). Furthermore, Sañudo-Wilhelmy et al. (2012) recently showed that large areas of the ocean are vitamin depleted. However, no study has addressed the influence of some B-vitamins on C and N_2 fixation in the Atlantic Ocean, and herein we describe the potential relationship between two B-vitamins, thiamin (B_1), and pyridoxine (B_6), concentrations and biogeochemical rates in the Amazon-influenced WTNA Ocean.

Vitamin B_1 is an essential organic growth factor required by most organisms, and plays an integral role in biogeochemical reactions involving C transformations (Henkes et al., 2001; Jordan, 2003; Pohl, 2004). It functions by associating with a number of important enzymes including pyruvate dehydrogenase, which bridges glycolysis and the citric acid cycle, as well as transketolase, which plays a critical role in the Calvin cycle (C fixation reactions of photosynthesis) and the pentose phosphate pathway (Henkes et al., 2001; Jordan, 2003). Many bacteria and Protista have been shown to require vitamins.

Vitamin B_6 was first identified in 1932 by Ohdake (Ohdake, 1932), and is now known to catalyze over 160 biochemical reactions that mainly involve amino acid transformations (Snell, 1953; Percudani and Peracchi, 2009). Because the role that the amino acids glutamine and glutamate have in the assimilation of ammonia (NH_3), the product of N_2 fixation, which is incorporated into two amino acids (Staley et al., 2007), we hypothesized that vitamin B_6 concentrations and availability could therefore also influence the N cycle.

Previous field and laboratory studies have focused on the vitamins B_1, B_7, and B_{12} as they were thought to be required for growth, while other B-vitamins (e.g., B_6) were largely ignored (Provasoli and Pintner, 1953; Droop, 1957; Burkholder and Burkholder, 1958; Carlucci and Bowes, 1970, 1972; Strickland, 2009). This paradigm shifted when the genome of one of the most abundant bacteria in the ocean, *Pelagibacter ubique*, was first published revealing the absence of the genes required for the biosynthetic pathways of vitamins B_1 and B_6 (Giovannoni et al., 2005). *P. ubique* belongs to the SAR11 clade, which accounts for a third of all heterotrophic cells present in surface waters (Morris et al., 2002), and thus plays a large role in the global carbon cycle (C cycle). Subsequently, the genes required for the *de novo* synthesis of B-vitamins were found to be absent from bacteria belonging to the SAR86 clade, which are highly abundant uncultured members of marine surface bacterial populations (Dupont et al., 2011). In fact, over half of marine phytoplanktonic species investigated thus far are auxotrophic, which includes some of the most abundant and ubiquitous marine species (Croft et al., 2006), highlighting the importance of external sources of B-vitamins, including vitamin B_1. These genomic data suggest that exogenous B-vitamin pools are essential for the survival of some marine plankton, as they rely solely on the environment to meet their B-vitamin requirements. The availability of vitamins B_1 and B_6 may therefore play a significant role in N and C cycling, and may be a previously unknown factors contributing to the regulation of the "biological carbon pump." However, little is known about the sources and sinks of B-vitamins in marine systems, or how they cycle between vitamin producers and consumers.

Despite the biologically important role vitamins B_1 and B_6 play in ecologically relevant enzymes involved in C and N cycling, primarily carbohydrate and amino acid metabolism, little is known about their concentrations or distributions in marine systems. The objectives of this study were (1) to provide the first directly measured depth profiles of vitamins B_1 and B_6 in a highly productive region of the WTNA, (2) to determine the spatial distributions of those vitamins in that region, (3) to determine the influence of the Amazon River Plume on that spatial gradient, and (4) to determine the importance of these vitamins in C and N cycles.

MATERIALS AND METHODS

Samples were collected in the WTNA on board the R/V Knorr as part of the Amazon influence on the Atlantic: carbon export from nitrogen fixation by diatom symbioses (ANACONDAS) project from May 23 to June 22, 2010. Sampling stations were between longitude −56.8°E and −45.0°E and latitude 4.3°N and 12.4°N (**Figure 1**). Stations were grouped by sea surface salinity (SSS) and designated as low-salinity (SSS < 30, stations 4, 9.1, 10, and 11), mesohaline (30 < SSS > 35, stations 1–3, and 9), and oceanic (SSS > 35, stations 7, 8, and 27).

Vitamin samples were collected from the top 150 ms using a Niskin bottle rosette sampler and filtered through a 0.2-μm Supor filter (PALL, Life Sciences) using a peristaltic pump. The filtrate was collected in 250 ml acid cleaned high density polyethylene (HDPE) bottles and frozen until analysis. Vitamin samples were extracted and pre-concentrated according to the method of Sañudo-Wilhelmy et al. (2012). Briefly, samples were passed through solid-phase C18 resin at a flow rate of 1 mL/min to concentrate vitamins. Samples were adjusted to pH 6.5 before being passed through the resin, and then adjusted to pH 2.0 to obtain

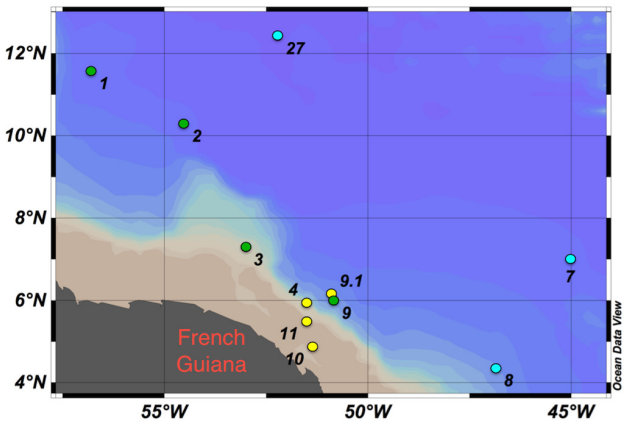

FIGURE 1 | Study sites in the western tropical North Atlantic (WTNA) Ocean with degrees latitude north and degrees longitude west shown. Stations clustered by sea surface salinity (SSS): low-salinity stations (SSS < 30, yellow circles), mesohaline stations (30 <SSS>35, green circles), and oceanic/open ocean stations (SSS > 35, blue circles). Ocean data view (Schlitzer, 2011).

maximum vitamin recovery. Vitamins were subsequently eluted off the columns with methanol, dried, and dissolved in 200 μl of MilliQ water. Vitamin concentrations were then quantified using liquid chromatography/tandem mass spectrometry (LC/MS/MS). Each extraction included a blank and spiked positive control to test for contamination and extraction efficiency. Detection limit of vitamins B_1 and B_6 were 0.81 pM and 0.61 pM, respectively (Sañudo-Wilhelmy et al., 2012). Some controls used for estimating extraction efficiency with a vitamin spike were compromised by vitamin-contaminated DI water yielding in some cases efficiency greater than 100%. However, for most of the samples, extraction efficiency was close to 100%.

Chlorophyll a (Chl a) samples were collected from a Niskin bottle rosette into 1L amber bottles, filtered onto 25 mm GF/F filters and analyzed according to the EPA modified fluorometric method 445.0 (Arar and Collins, 1997) in a Turner Designs Fluorometer. Sample volumes ranged from 500 mL to 1 L depending on biomass. In general, oceanic stations utilized 1L volumes, while mesohaline and low-salinity stations had higher biomass allowing only 500 mL volumes to be filtered.

N_2 fixation and C fixation were performed according to the method of Montoya et al. (1996) and Montoya and Voss (2006) using 4 L polycarbonate bottles completely filled and equipped with silicone rubber caps. Bottles were enriched with 3 mL of 99% $^{15}N_2$ (Isotec) and 250 μL of 0.1 M $NaH^{13}CO_3$ (Sigma). After on-deck incubation for 24 h at surface seawater temperature and simulated conditions of light for the collection depth, bottles were pre-filtered through 10-μm Nitex mesh onto pre-combusted GF/F filters. Material on the 10-μm filter was washed onto GF/F filters. Filters were dried and stored until mass spectrometric analysis in the laboratory. Isotope abundances were measured by continuous-flow isotope ratio mass spectrometry using a CE NA2500 elemental analyzer interfaced to a Micromass Optima mass spectrometer.

Statistical analysis was performed using SigmaPlot's (Systat Software Inc.) *T*-test except when assumptions of normality and equal variance were violated resulting in the use of the non-parametric Mann–Whitney rank sum test. The degree to which C and N_2 fixation correlated with each of the B-vitamins was evaluated by means of a Pearson product moment correlation test. Linear regression models were performed using R v2.12.2 statistical programming language (R Development Core Team, 2012). Exhaustive step-wise general linear regression models and leave one out cross validation for generalized linear models utilized the following packages: boot (Canty and Ripley, 2012), leaps (Lumley and Miller, 2009), random Forest (Liaw and Wiener, 2002), and data analysis and graphics (DAAG; Maindonald and Braun, 2012). Due to missing data, the parameters omitted from this analysis were PAR, Chl a, and cell counts.

RESULTS

CONCENTRATIONS OF B-VITAMINS

Vitamin B_1 in the WTNA varied widely among stations and ranged from undetectable to 229 pM (**Figure 2**), except for the surface sample at station 11 measuring 964 pM and was suspected to be compromised by sample contamination. The lowest concentrations of vitamin B_1 were measured at the oceanic stations (undetectable to 50 pM) followed by low-salinity stations (2.5–184 pM), and the highest concentrations were observed at mesohaline stations (undetectable to 229 pM, **Figure 2**). Vitamin B_6 concentrations also varied widely among stations ranging from undetectable to 36 pM. B_6 concentrations were lowest at the mesohaline stations (undetectable to 7 pM) followed by oceanic stations (undetectable to 20 pM), and were highest at low-salinity stations (undetectable to 36 pM, **Figure 2**). In general, higher concentrations of B-vitamins were found at lower salinity stations and were significantly higher in the surface plume water at some stations suggesting a riverine source (**Table 1**). There was no clear spatial trend observed between the two vitamins suggesting they function and behave differently from one another, and the high variability suggests a dynamic behavior influenced by sources and sinks.

POTENTIAL EFFECT OF B-VITAMINS ON BIOLOGICAL PROCESSES

N_2 fixation rates were positively correlated with vitamin B_1 concentrations at station 7, 8 (in the small size class), 9.1, and 10 (**Table 2**). N_2 fixation rates were inversely correlated with vitamin B_1 at stations 1, 4, and 8 (in the large size fraction, **Table 2**). N_2 fixation rates were positively correlated to vitamin B_6 concentrations at station 1 (in the small size fraction), 8, 9.1, and 10 (in the large size fraction, **Table 2**). N_2 fixation rates were negatively correlated to vitamin B_6 at stations 1 (in the large size fraction), 4, 7, and 10 (in the small size fraction, **Table 2**). However, significant relationships between increases in N_2 fixation rates and vitamin B_1 concentrations were only observed at station 7 in the small size class (*p* value 0.045, **Figure 3A**). A significant inverse relationship was observed at station 1 in the large size fraction (*p* value 0.029, **Figure 3B**). Significant relationships between increases in N_2 fixation rates and vitamin B_6 concentrations were only observed at station 9.1 in the large size class (*p* value 0.017, **Figure 3C**).

Carbon fixation rates were positively correlated with vitamin B_1 at stations 1 (in the small size fraction), 4, 7, 9.1, and 10 (**Table 2**). Carbon fixation rates were inversely correlated to vitamin B_1 at stations 1 (in the large size class) and 8 (**Table 2**). Carbon fixation rates were positively correlated with vitamin B_6 at stations 1, 9.1, and 10 (**Table 2**). Carbon fixation rates were inversely correlated with vitamin B_6 at stations 4, 7, and 8 (**Table 2**). However, significant increases in C fixation rates with increasing B_1 concentrations were only observed at station 9.1 in both size classes (*p* values 0.000008 and 0.004, respectively, **Figure 3D**). No significant relationships between vitamin B_6 concentrations and rates of C fixation were observed.

LINEAR REGRESSION MODELS

Linear regression models included data from all stations except for station 9 where N_2 and C fixation data were not available. Tests were performed omitting Chl a, photosynthetically active radiation (PAR), and/or cell counts due to missing data. The linear model showed that the factors correlating with C fixation in the small size class included Si, vitamin B_1, and water temperature (**Figure 4**). The model was significant with a *p* value of 8.83×10^{-11}, predictive error (the average deviation between the known values and the models predicted values) of 721, and an R^2 value of 0.522 (**Table 3**). The model predicting N_2 fixation in the

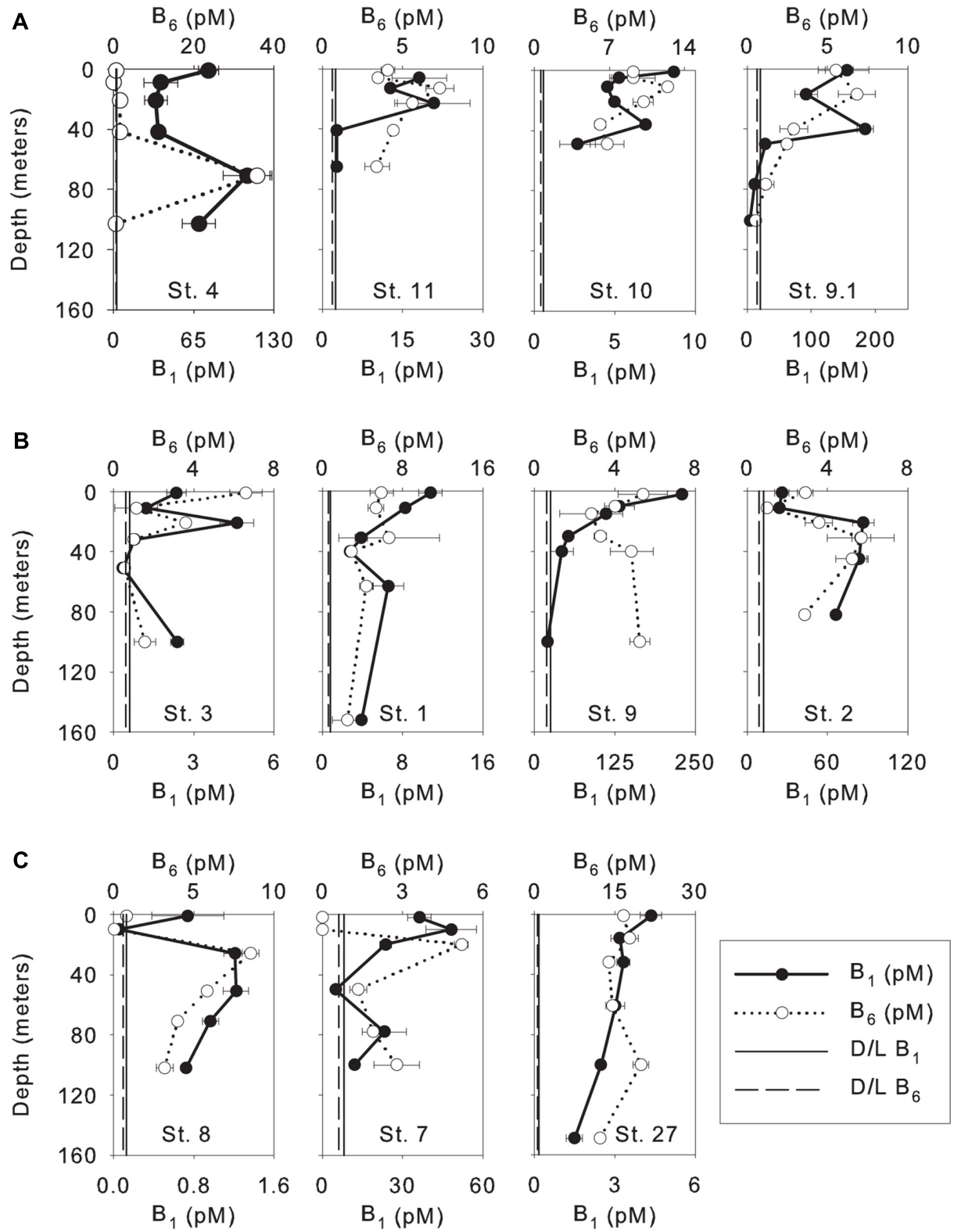

FIGURE 2 | Depth profiles of dissolved vitamins (B_1 and B_6) measured in the WTNA Ocean; (A) low-salinity stations; (B) mesohaline stations; (C) oceanic/open ocean stations. Stations are ordered by sea surface salinity (SSS) moving from the lowest to highest SSS. Surface concentration of vitamin B_1 for station 11 (964.8 ± 426 pM) omitted due to concerns with possible contamination and for visualization of variation within the depth profile (average concentrations ± 1 standard deviation). Vertical lines show the detection limit (D/L) of vitamin B_1 (solid line) and B_6 (dashed line).

Table 1 | Statistical test results comparing B vitamin concentrations at surface depths with below surface or halocline depths. Vitamin B_1 followed by B_6, the specific statistical test, failed *T*-test assumptions, and *p* value for each station is listed.

Station	Station type	Surface concentrations	Test	Failed assumption	*p* value
B_1					
1	Mesohaline	Higher	Mann–Whitney	Normality	0.002
2	Mesohaline	Lower	Mann–Whitney	Equal variance	<0.001
7	Oceanic	Higher	*T*-test		<0.001
9	Mesohaline	Higher	*T*-test		<0.001
9.1	Low-salinity	Higher	Mann–Whitney	Normality	0.044
10	Low-salinity	Higher	*T*-test		0.018
11	Low-salinity	Higher	Mann–Whitney	Normality	0.009
27	Oceanic	Higher	*T*-test		0.006
B_6					
2	Mesohaline	Higher	*T*-test		0.001
7	Oceanic	Lower	Mann–Whitney	Normality	0.005

Table 2 | Correlation coefficients of vitamins B_1 and B_6 with nitrogen and carbon fixation in the less than and greater than 10-μm size classes, direction of relationships, correlation coefficients, and *p* values.

	Correlation coefficients (*p* value)			
Station	*N* fix <10	*N* fix >10	*C* fix <10	*C* fix >10
B_1				
1	−0.581 (0.304)	−0.916 (0.029)	0.236 (0.703)	−0.694 (0.194)
4	−0.072 (0.86)	−0.358 (0.35)	0.183 (0.64)	0.073 (0.85)
7	0.820 (0.045)	0.215 (0.68)	0.597 (0.21)	0.103 (0.85)
8	0.288 (0.580)	−0.189 (0.760)	−0.174 (0.742)	−0.416 (0.413)
9.1	0.430 (0.40)	0.395 (0.44)	0.998 (<0.001)	0.95 (0.004)
10	0.691 (0.129)	0.547 (0.261)	0.430 (0.394)	0.609 (0.200)
B_6				
1	0.315 (0.606)	−0.697 (0.191)	0.659 (0.227)	0.177 (0.776)
4	−0.200 (0.61)	−0.273 (0.48)	−0.416 (0.27)	−0.290 (0.449)
7	−0.392 (0.44)	−0.301 (0.56)	−0.394 (0.44)	−0.024 (0.964)
8	0.627 (0.183)	0.307 (0.616)	−0.0728 (0.891)	−0.0547 (0.918)
9.1	0.780 (0.067)	0.892 (0.017)	0.58 (0.227)	0.645 (0.167)
10	−0.0741 (0.889)	0.133 (0.801)	0.190 (0.718)	0.195 (0.711)

larger size class showed the most important factors were temperature, mixed layer depth (MLD), and vitamin B_6 (**Figure 4**). The model was significant with a p value of 3.92×10^{-4}, predictive error of 6.7×10^{-4}, and an R^2 value of 0.241 (**Table 3**).

DISCUSSION

This is the first study to measure directly the B-vitamins, B_1 and B_6, in the WTNA euphotic zone within the influence of the Amazon River plume. We observed high variability in the concentrations and distributions of these vitamins in the area of study. Vitamin B_1 was found below the limit of detection at mesohaline station 3 (31 and 51 m) and oceanic station 8 (2, 10, and 100 m), and vitamin B_6 was found to be below the limit of detection at low-salinity station 4 (8 m), mesohaline stations 3 and 9 (51 and 100 m, respectively, and oceanic station 8 (10 m). The low concentrations of B-vitamins and high spatial variability observed were consistent with previous studies. In fact, in large regions of the Eastern Pacific Ocean between 24°N and 34°N, B-vitamins were found to be below the limit of detection (Sañudo-Wilhelmy et al., 2012). The ranges of vitamin B_1 concentrations measured in this study (0.05 to ∼1000 pM) are consistent with previously published results from both bioassays and direct measurements

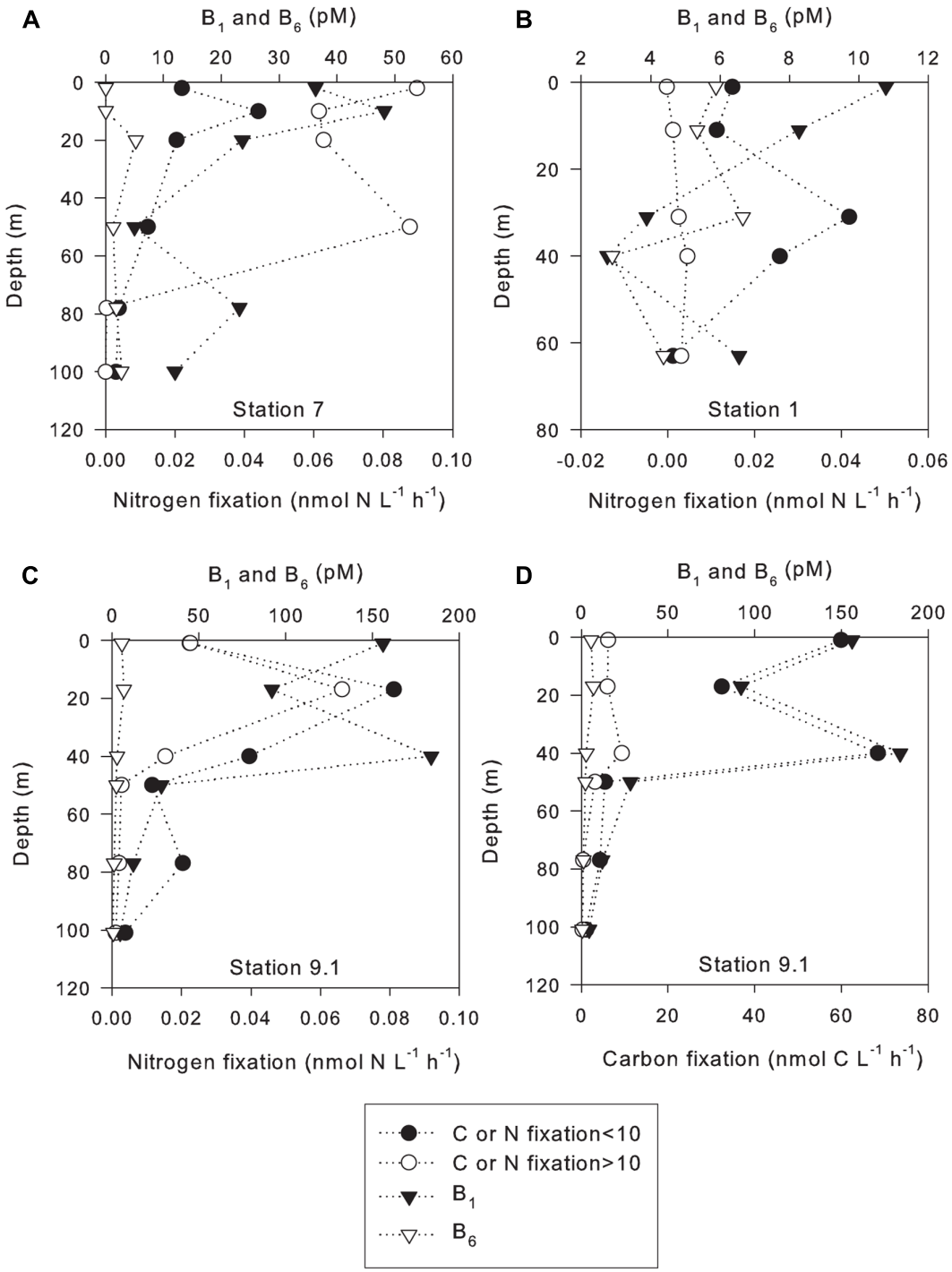

FIGURE 3 | Carbon and nitrogen fixation rates in the less than and greater than 10-μm size classes with B vitamin depth profiles. (A) Station 7; **(B)** Station 1; **(C)** Station 9.1; **(D)** Station 9.1.

(**Table 4**). The concentration of B_1 measured using bioassays ranged from 33 to 1633 pM in the North Pacific Ocean (Natarajan and Dugdale, 1966), 36 to 1500 pM in the subarctic Pacific Ocean (Natarajan, 1970), 16 to 133 pM in the Southern California Coastal zone (Eppley et al., 1972), and direct measurements of vitamin B_1 ranged from 200 to 600 pM in the Stony Brook Harbor channel and Peconic River (Okabamichael and Sañudo-Wilhelmy, 2005), 0.7 to 30 pM in the North Atlantic ocean (Panzeca et al., 2008), and from undetectable to 500 pM in the Southern California-Baja California coast (Sañudo-Wilhelmy et al., 2012). Vitamin B_6 concentrations

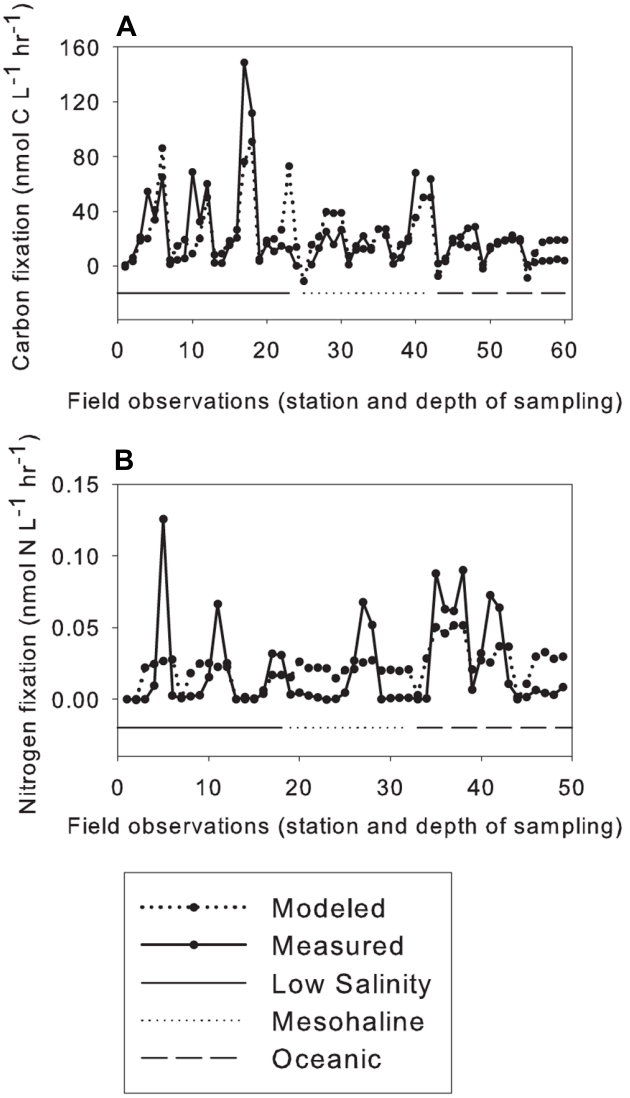

FIGURE 4 | Multiple linear regression models. Nutrients; phosphate, PO_4^{3-}; silicate, dSi; thiamin, B_1; mixed layer depth, MLD; photosynthetically active radiation, PAR; fluorescence, fluor.; and temperature (°C), Temp. Stations are separated by sea surface salinity (SSS), solid line below low-salinity stations, dotted line below mesohaline stations, and dashed line below oceanic stations, **(A)** Carbon fixation <10-μm size class, and (**B**) Nitrogen fixation >10-μm size class.

in the WTNA ranged from undetectable to 40 pM and were generally lower than previous measurements from the North Pacific Ocean, specifically from the Santa Monica Basin (70–284 pM), Rosario (255–386 pM), and Magdalena (40–393 pM) from the upper 150 m, and Vizcaino (159–360 pM) from the upper 120 m of the water column (Sañudo-Wilhelmy et al., 2012). However, they fell within the range measured at Soledad (3.5–49 pM) from the upper 150 m and Pescadero (4.6–180 pM) from the upper 180 m of the water column (Sañudo-Wilhelmy et al., 2012). In summary, the concentrations of B-vitamins observed in this study were consistent with previous results showing they vary spatially, and are often found below the limit of detection.

Recent studies on the role that B-vitamins play in marine ecosystems have shown that they can limit or co-limit primary production (Panzeca et al., 2006, 2008; Bertrand et al., 2007; Gobler et al., 2007; Tang et al., 2010). Although this study did not directly investigate the effects of vitamin additions on biological processes, some conclusions can be drawn from the correlations between vitamin concentrations and rates of N_2 and C fixation. This study found a significant increase in C fixation with increasing ambient B_1 concentrations at low-salinity station 9.1 in both size classes (p value $\leq$0.004, **Table 2**). At low-salinity station 4, the lack of correlation between C fixation and B_1 concentrations could be explained by the high abundance of the diatom *Coscinodiscus* sp. Based on isolates that have been studied it appears that this diatom species does not require vitamin B_1 (Croft et al., 2006) and likely contributed to the majority of C fixation at this station. Significant increases in N_2 fixation were also found with increasing B_1 in the large size class at station 1 (p value 0.029) and oceanic station 7 in the small size class (p value <0.045). These data suggest that B_1 may be limiting or co-limiting N_2 fixation in some areas of the WTNA since low PO_4^{3-} concentrations were also measured at station 7, and PO_4^{3-} has been previously shown to limit N_2 fixation (Sañudo-Wilhelmy et al., 2001; Mills et al., 2004; Webb et al., 2007; Moutin et al., 2008; Van Mooy et al., 2009). Hence, vitamin B_1 appears to be playing a role in C and N_2 fixation in both riverine influenced and open ocean stations. These results are consistent with the role of B_1 in C metabolism but the role B_1 plays in N metabolism is less clear. However, pyruvate-ferredoxin oxidoreductase, an enzyme crucial for electron transfer to nitrogenase, requires thiamin (Brostedt and Nordlund, 1991; Bothe et al., 2010) and some diazotrophs have been shown to be B_1 auxotrophs, suggesting that B_1 availability in the environment may be limiting the N biogeochemical cycle. N_2 fixation was found to increase with increasing vitamin B_1 at one low-salinity station; however, this was not observed at other stations. Therefore, further investigations such as vitamin addition experiments which show an increase of N_2 fixation with B_1 amendments, are required to fully understand the role of this vitamin in the WTNA N and C cycles. However, the tight correlation between B_1 and C fixation observed at station 9.1 (**Figure 3D**) suggests that this vitamin may also be important for C fixation in the WTNA, and argues for further study.

N_2 fixation co-varied with vitamin B_6 at low-salinity station 9.1; significant positive relationships were found in larger size class between vitamin B_6 and N_2 fixation (p value < 0.017). However, there was not a significant relationship between N_2 fixation and concentrations of vitamin B_6 at the other stations. No significant relationships were observed between C fixation and vitamin B_6 concentrations at any stations. Independence of vitamin B_6 and N_2 fixation can be explained by other factors; for instance, at station 7, low nutrient concentrations were observed and dissolved P may have limited N_2 fixation, while station 4 was dominated with the diatom *Coscinodiscus* sp. whose requirements for B_6 are currently unknown. Thus, at some stations N_2 fixation appears dependent on B-vitamins, which appears to be limiting or co-limiting biogeochemical cycles in the WTNA. Since there were few correlations between vitamin concentration and rate measurements, either standing concentrations are

Table 3 | Multiple linear regression model factor coefficients and statistical results for carbon fixation in the less than 10-μm size class and nitrogen fixation in the greater than 10-μm size class.

Model	Factor coefficients			Intercept	Predictive error	Adjusted *R*-squared	*p* value
C							
	Si	B_1	Temp				
	1.81E+00	−7.43E−02	4.25E+00	−1.04E +02	7.21E +02	5.22E−01	8.83E−11
N							
	Temp	MLD	B_6				
	7.03E−03	4.72E−04	−1.21E−03	−1.80E−01	6.70E−04	2.41E−01	3.92E−04

Table 4 | Global B vitamin concentrations including current and previous studies. n/a not available, n/d not detectable.

Location	Lat.	Long	Depth (m)	Method	Range (pM)	Reference
B_1						
North Atlantic Ocean	4.3–12.4	−56.8 to −45	150	direct	0.05–1000	This study
North Pacific Ocean	58	134 to 137	150	bioassay	33–1633	Natarajan and Dugdale (1966)
Subarctic Pacific Ocean	52–58	153 to 170	150	bioassay	36–1500	Natarajan (1970)
Southern CA Coastal zone	−117 to −119	32 to 34	80	bioassay	16–133	Eppley et al. (1972)
Stony Brook Harbor	n/a	n/a	surface	direct	230–310	Okabamichael and Sañudo-Wilhelmy (2005)
North Atlantic ocean	45–66	−14 to −24	surface	direct	0.7–30	Panzeca et al. (2008)
Southern CA-Baja	113–119	34–23	180	direct	0.34–122	Sañudo-Wilhelmy et al. (2012)
B_6						
North Atlantic Ocean	4.3–12.4	−56.8 to −45	150	direct	n/d to 40	This study
Santa Monica Basin	−119.03	33.84	150	direct	70–284	Sañudo-Wilhelmy et al. (2012)
Rosario	−116.08	29.8	150	direct	255–368	Sañudo-Wilhelmy et al. (2012)
Vizcaino	−114.52	27.01	120	direct	159–360	Sañudo-Wilhelmy et al. (2012)
Soledad	−112.71	25.22	150	direct	3.5–49	Sañudo-Wilhelmy et al. (2012)
Magdalena	−111.57	23.2	150	direct	40–393	Sañudo-Wilhelmy et al. (2012)
Pescadero	−108.2	24.28	180	direct	4.6–180	Sañudo-Wilhelmy et al. (2012)

a poor measure, auxotrophic phytoplankton are not commonly abundant, or they are getting their vitamins through symbiosis (Croft et al., 2005). However, to determine the extent that N and C cycles are actually dependent on vitamin B_6, more extensive studies including vitamin addition experiments will be required.

Multiple linear regression models were used to identify the environmental variables that correlated with biogeochemical cycles in the WTNA Ocean during our study. Variables correlating to C fixation in the small size class included Si, vitamin B_1, and temperature. Two of these variables, Si and water temperature, were also identified as factors affecting the distribution of N_2 and C fixing organisms in previous studies (Coles and Hood, 2007; Foster et al., 2007; Webb et al., 2007; Sohm and Capone, 2008; Hynes et al., 2009; Van Mooy et al., 2009; Sohm et al., 2011a,b). Model results were consistent with the role that vitamin B_1 plays in the Calvin cycle and C metabolism (Natarajan, 1970; Jordan, 2003). Our analysis showed that temperature, MLD, and vitamin B_6 correlated to N_2 fixation in the greater size fraction. Measured N_2 fixation rates were on average an order of magnitude less than modeled rates except at depths where the highest rates of N_2 fixation were measured. When the highest rates of N_2 fixation were observed, measured rates were an order of magnitude greater than the modeled rates (**Figure 4**). This pattern was observed across all station types and resulted in the models low R^2 value. However, this is consistent with the role vitamin B_6 plays in catalyzing many diverse amino acid transformations (Percudani and Peracchi, 2009), specifically with the assimilation of NH_3 into the amino acids glutamine and glutamate. Collectively, these results suggest that vitamin B_1 and B_6 could be important organic growth factors affecting biologically mediated C and N_2 fixation in the WTNA Ocean.

Insights into the potential ecological importance of B-vitamins have been investigated by determining half-saturation constants (K_s) for maximal growth for vitamins B_1 and B_{12} for some phytoplankton species (Tang et al., 2010). However, the K_s for diazotrophic microorganisms and B-vitamins have yet to be determined. The K_s of maximal growth rates for different phytoplankton species for vitamin B_1 ranged from 6 to 184 pM. Some of our measured concentrations of B_1 were below the K_s

suggesting that vitamin B_1 may be a limiting growth factor in the WTNA. Future studies are needed to determine the K_s for maximal growth on different B-vitamins of endemic WTNA plankton species, which will help to establish the ecological framework and importance of directly measured environmental B-vitamin concentrations.

The influence of the Amazon River plume on B-vitamin concentrations and the sources of B-vitamins in the WTNA are still unresolved. Although it has been hypothesized that fresh water inputs from rivers and groundwater can be a source of B-vitamins to marine systems (Gobler et al., 2007), clear patterns were not observed to support this in the WTNA Ocean. As a general trend, there was an increase in B-vitamins as salinity decreased but no linear relationship was observed, suggesting that mixing of river and seawater did not solely control it. An inverse correlation was observed with vitamin B_1 concentration and SSS (R value 0.25, data not shown), but no correlation was observed between vitamin B_6 and SSS (R value 0.002, data not shown). The surface water sampled during this cruise was estimated to be nearly 30 days out from the mouth of the river, and may explain the weak correlations found between SSS and B-vitamin concentrations. Further studies investigating B vitamin concentrations near the discharge point of the Amazon River should help resolve whether the river is a source of vitamins to the WTNA. In addition, the removal processes of B-vitamins are poorly understood, and the half-life of these vitamins has yet to be determined. However, the half-life of some vitamins (B_1 and B_{12}) in seawater has been shown to occur on time scales from days to weeks (Gold et al., 1966; Carlucci et al., 1969), suggesting that they are highly dynamic and that local production may be an important biologically available source of B-vitamins. Our understanding of the ecological importance of B-vitamins in marine systems is continuing to increase, with the current study demonstrating that B-vitamins are highly variable and could significantly influence both N_2 and C fixation in the WTNA Ocean. However, further studies are needed to determine the sources, sinks, and cycling of B-vitamins in oceanographic sensitive marine systems, such as the WTNA.

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Vitamin B_1 and B_{12} uptake and cycling by plankton communities in coastal ecosystems

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While vitamin B_{12} has recently been shown to co-limit the growth of coastal phytoplankton assemblages, the cycling of B-vitamins in coastal ecosystems is poorly understood as planktonic uptake rates of vitamins B_1 and B_{12} have never been quantified in tandem in any aquatic ecosystem. The goal of this study was to establish the relationships between plankton community composition, carbon fixation, and B-vitamin assimilation in two contrasting estuarine systems. We show that, although B-vitamin concentrations were low (pM), vitamin concentrations and uptake rates were higher within a more eutrophic estuary and that vitamin B_{12} uptake rates were significantly correlated with rates of primary production. Eutrophic sites hosted larger bacterial and picoplankton abundances with larger carbon normalized vitamin uptake rates. Although the $>2\,\mu m$ phytoplankton biomass was often dominated by groups with a high incidence of vitamin auxotrophy (dinoflagellates and diatoms), picoplankton ($<2\,\mu m$) were always responsible for the majority of B_{12}-vitamin uptake. Multiple lines of evidence suggest that heterotrophic bacteria were the primary users of vitamins among the picoplankton during this study. Nutrient/vitamin amendment experiments demonstrated that, in the Summer and Fall, vitamin B_{12} occasionally limited or co-limited the accumulation of phytoplankton biomass together with nitrogen. Combined with prior studies, these findings suggest that picoplankton are the primary producers and users of B-vitamins in some coastal ecosystems and that rapid uptake of B-vitamins by heterotrophic bacteria may sometimes deprive larger phytoplankton of these micronutrients and thus influence phytoplankton species succession.

Keywords: phytoplankton dynamics, heterotrophic bacteria, vitamins, B_{12} limitation, co-limitation, vitamin to carbon ratio

INTRODUCTION

Coastal marine ecosystems are amongst the most ecologically and economically productive areas on the planet, providing an estimated US$14 trillion, or about 43% of the global total, worth in ecosystem goods and services, annually (Costanza et al., 1997). While coastal areas comprise only 8% of the world's ocean surface they account for over 28% of the annual ocean primary production (Holligan and de Boois, 1993). In a manner paralleling global trends, nearly 75% of the US population lives within 75 km of the coastline, making these regions subject to a suite of anthropogenic influences including intense nutrient loading (de Jonge et al., 2002; Valiela, 2006) which in turn can lead to ecological perturbations such as harmful algal blooms and hypoxia (Cloern, 2001; Heisler et al., 2008). Coastal zone management efforts typically focus on minimizing total nitrogen input since primary production in most coastal marine systems are typically nitrogen-limited (Nixon, 1995). While the absolute magnitude of nitrogen entering coastal zones often controls the amount of phytoplankton biomass, the availability, and/or type (e.g., inorganic vs. organic) of nutrients can also influence the algal community composition of coastal environments (Smayda, 1997; Koch and Gobler, 2009). For example discharge from salt marsh ditches, rich in dissolved organic N, elicited a community shift in the adjacent estuarine plankton community favoring dinoflagellates over cryptophytes and cyanobacteria, likely attributable to the mixotrophic tendencies of many dinoflagellates (Taylor, 1987; Anderson et al., 2008; Burkholder et al., 2008). B-vitamins such as thiamin (B_1), biotin (B_7), and cobalamin (B_{12}) are important cofactors in a number of cellular processes such as the biosynthesis of methionine (B_{12}), the decarboxylation of pyruvic acid (B_1), and fatty acid synthesis (B_7). While vitamins have long been implicated as growth regulators for microalgae (Droop, 1955; Provasoli and Carlucci, 1974) their ecological importance has received little attention since early surveys (Vishniac and Riley, 1961; Menzel and Spaeth, 1962) and laboratory experiments (Droop, 1968) suggested that ambient concentration were sufficient to satisfy micro-algal demands (Swift, 1980). Newly developed methods to directly measure B-vitamins in seawater (Okbamichael and Sanudo-Wilhelmy, 2004, 2005), have facilitated surveys in several open ocean and coastal ecosystems and have revealed that vitamin concentrations are low, ranging from <0.1–40 to <0.1–100 pmol L^{-1} (Gobler et al., 2007;

Panzeca et al., 2008, 2009; Koch et al., 2011) for B_{12} and B_1, respectively.

Surveys of the literature as well as novel laboratory experiments indicate that over half of all phytoplankton species surveyed have an obligate requirement for an exogenous supply of one or more of the B-vitamins with B_{12} being required by the largest number of algae (Croft et al., 2005; Tang et al., 2010). Vitamin enrichment studies in coastal (Sanudo-Wilhelmy et al., 2006; Gobler et al., 2007) and open ocean environments (Panzeca et al., 2006; Bertrand et al., 2007; Koch et al., 2011) have shown that B-vitamins can co-limit phytoplankton biomass along with a primary limiting nutrient (i.e., nitrogen or iron). Vitamin availability can also shape coastal phytoplankton community structure. Many have argued that B_{12} concentrations might influence the dynamics and composition of the spring bloom (Carlucci and Bowes, 1970; Swift, 1980) since the majority of centric diatom species which comprise the spring bloom are vitamin auxotrophs (Droop, 1955; Guillard and Ryther, 1962). Recently, Koch et al. (2011) demonstrated that vitamin B_{12} concentrations strongly influence algal communities in the coastal Gulf of Alaska, with high concentrations favoring dinoflagellates over diatoms, affirming that B-vitamins can indeed play an important ecological role in plankton succession. Despite this renewed interest in B-vitamins, very little is known regarding how the trophic state of coastal ecosystems influences the cycling of vitamins and only one study has investigated B-vitamin concentrations and uptake rates by plankton communities in an aquatic ecosystem (Koch et al., 2011).

The goal of this study was to elucidate the relationships between phytoplankton community composition, carbon fixation, and B-vitamin (B_1 and B_{12}) assimilation by coastal plankton assemblages. This field study was performed over 2 years in two contrasting, coastal, marine systems: a hypereutrophic and a mesotrophic estuary. In support of the primary objectives of this study, nutrient, and vitamin amendment experiments were performed to explore the extent to which the availability of vitamins affected phytoplankton biomass and community assemblages.

MATERIALS AND METHODS

STUDY SITES

Two estuaries were investigated during this study and were sampled on 18 occasions on a bi-weekly to monthly basis from March 2007 until November 2008 to capture a complete annual cycle of plankton and vitamin dynamics. Water was collected from the mesotrophic portion of Long Island Sound (LIS) near Mount Sinai Harbor (40.97°N, 73.04°W; **Figure 1**) and a hypereutrophic, tidal tributary on eastern Shinnecock Bay, Old Fort Pond (OFP, 40.87°N, 72.45°W, **Figure 1**). LIS is a large, urban estuary bordered on the western end by New York City and in the east exchanges with the North Atlantic Ocean, thus displaying a strong east-west eutrophication gradient (Gobler et al., 2006). Our sampling location was located within the eastern, mesotrophic half of this estuary. OFP exchanges tidally with Shinnecock Bay and is a shallow (<2 m), well-mixed, hypereutrophic body of water which, during the summer months, experiences dense algal blooms that create a large demand for micro- and macro-nutrients and may be influenced by the availability of vitamins (Gobler et al., 2007). This inland, hypereutrophic tributary with high levels of mixed algal biomass

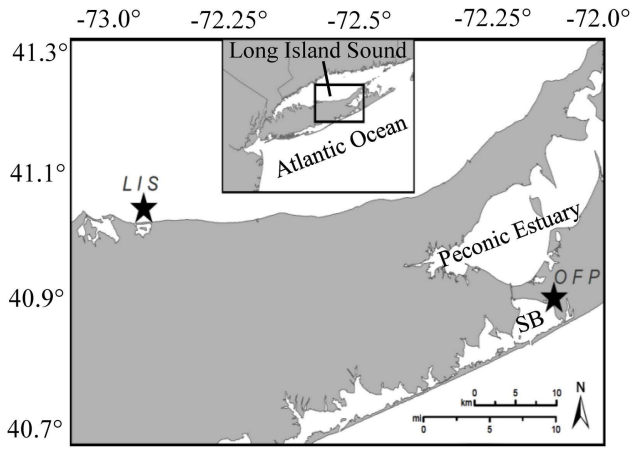

FIGURE 1 | A map of Long Island, NY showing the two study sites (stars). OFP denotes Old Fort Pond, a hypereutrophic systems which exchanges tidally with the Atlantic Ocean through Shinnecock Bay (SB). The second study site, Long Island Sound (LIS), was sampled at the incoming tide from a dock at the Mount Sinai Harbor entrance and represents a mesoeutrophic system.

(up to 50 μg Chl a L^{-1}; Gobler et al., 2007) contrasts with the LIS location which typically displays lower levels of algal biomass (mesotrophic; ~3 μg Chl a L^{-1}; Gibson et al., 2000).

CHEMICAL ANALYSIS

Water for nutrient analysis was filtered through pre-combusted GF/F filters. Concentrations of nitrate, nitrite, ammonium, and phosphate were determined in duplicate by standard spectrophotometric methods (Jones, 1984; Parsons et al., 1984). Total dissolved nitrogen and phosphorus (TDN, TDP) were analyzed in duplicate by persulfate oxidation techniques (Valderrama, 1981) and dissolved organic nitrogen and phosphorus (DON and DOP) calculated by subtracting levels of nitrate, nitrite and ammonium, or orthophosphate from concentrations of TDN and TDP, respectively. Full recoveries (mean ± 1 SD) of samples spiked with SPEX Certi-Prep^{INC} standard reference material for TDN, TDP, nitrate, nitrite and ammonium, and orthophosphate were obtained at environmentally representative concentrations. Vitamin samples were collected and analyzed according to Okbamichael and Sanudo-Wilhelmy (2004, 2005). Briefly, water was filtered through 0.2 μm capsule filters (GE Osmonics, DCP0200006) into 1 L LDPE bottles and stored frozen and in the dark. The samples were then acidified and concentrated at 1 mL min^{-1} onto columns containing 17% High Capacity C_{18} (Varian, HF BONDESIL), stored frozen, and analyzed via reverse phase HPLC.

CHARACTERIZATION OF THE PLANKTON COMMUNITY

Several approaches were utilized to characterize resident plankton communities. Size fractionated chlorophyll a (Chl a) samples were collected by filtering triplicate samples onto 0.2 and 2 μm polycarbonate filters. These filters were stored frozen until analysis via standard fluorometric methods (Welschmeyer, 1994). Whole seawater was also preserved in 5% Lugols iodine solution for enumeration of plankton (>5 μm) under an inverted microscope.

Organisms were identified to the lowest taxonomic level possible and were generally grouped as diatoms, dinoflagellates, ciliates, and autotrophic nanoplankton. A minimum of 200 organisms or 100 grids were be counted per sample (Omori and Ikeda, 1984). Whole water samples were preserved with 10% buffered formalin (0.5% v/v final) and analyzed flow cytometrically to assess picoplankton densities (Olson et al., 1991). Abundance of heterotrophic bacteria (stained with SYBR Green I; Jochem, 2001), phycoerythrin-containing picocyanobacteria, and photosynthetic picoeukaryotes were quantified using a FACScan (BD®) flow cytometer using fluorescence patterns and particle size from side angle light scatter (Olson et al., 1991).

PRIMARY PRODUCTION AND VITAMIN UPTAKE

A ^{57}Co-labeled vitamin B_{12} from MP-Biomedicals (specific activity 7.84 MBq μg^{-1}; half-life 272 days) and a ^{3}H-labeled B_1 (specific activity 0.37 MBq mmol^{-1}; half-life 12.3 years) were used to measure planktonic uptake rates of these vitamins largely following the methods described by Koch et al. (2011). While vitamin B_{12} assimilation was measured throughout this study, vitamin B_1 assimilation was measured for the second half of this study only (3/7/08 to 11/4/08). Since preliminary studies with these tracers revealed linear uptake rates by multiple types of coastal marine plankton assemblages over 24 h without displaying a diel pattern, incubations were carried out for 1 day. Measured uptake rates never depleted more than 15 ± 6.3 and 7.7 ± 2.2% of vitamin B_1 and B_{12} standing stocks during these 24 h incubations. Trace amounts (0.5 pM B_{12}, 1.48 kBq) of ^{57}Co-cyanocobalamin and ^{3}H-thiamine (2 pM, 2.22 kBq) were added to separate sets of triplicate, 300 mL polycarbonate bottles. To assess abiotic binding of radiolabeled vitamin B_1 and B_{12} to particles and, thus, establish a "blank," several approaches were explored including incubations of natural plankton communities from LIS for 24 h in the dark at 1°C, and with the addition of mercuric chloride and glutaraldehyde at final concentrations of 1%. All of these approaches exhibited similarly low levels of abiotic binding and 1% glutaraldehyde was ultimately chosen as a "killed control" method with one such bottle being spiked with tracer and incubated along the "live" bottles during all experiments. The background activity for detection of ^{57}Co was generally between 20 and 40 counts per minute while samples ranged from 80 to 1500 counts per minute.

To determine primary productivity rates, 0.37 MBq of ^{14}C-bicarbonate (MP-Biomedicals©, specific activity 2035 MBq mmol^{-1}) was added to triplicate bottles according to Joint Global Ocean Flux Study (JGOFS) protocols (1994). All bottles were incubated in an incubator set to mimic ambient light and temperature conditions. Incubations were terminated after 24 h by filtering up to 100 mL from both live and dead bottles onto 0.2 and 2 μm pore size polycarbonate filters, allowing for the determination of size fractionated uptake of the tracers. At the beginning and end of the incubation, a small aliquot of each bottle (250 μL for ^{14}C and 1 mL for ^{3}H and ^{57}Co) was removed to quantify total activity. The ^{57}Co containing experimental filters and total activities were analyzed on a LKB Wallac 1282 Compugamma gamma counter equipped with a NaI(Tl) well detector, while the ^{14}C and ^{3}H samples were measured with a scintillation counter (Packard-Carb2100TR). Uptake rates of vitamins B_1 and B_{12} were calculated by using the equation: $[(A_f - A_D/A_{tot}) \times (\text{vitamin})]/t$ where A_f is the activity on the live filters, A_D is the activity on the dead ("killed control") filters, A_{tot} is the total activity added, (vitamin) is the ambient B_1 or B_{12} concentration and t equals the length of the incubation in days. Similarly, uptake of ^{14}C-bicarbonate was determined according to the JGOFS (1994) protocol.

Using cell counts obtained via flow cytometry, size fractionated Chl *a* concentrations, estimated carbon contents of heterotrophic bacteria and cyanobacteria and previously published C: Chl *a* ratios, carbon-specific vitamin uptake rates were calculated for both size classes and systems. For the >2 μm plankton, a carbon: Chl *a* ratio of 60 obtained from estuaries including LIS was used (Lorenzen, 1968; Riemann et al., 1989; Boissonneault-Cellineri et al., 2001) while previously published values of average carbon contents of 20 Fg cell^{-1} for heterotrophic bacteria (Fukuda et al., 1998; Ducklow, 2000), and 200 and 250 Fg cell^{-1} for cyanobacteria and picoeukaryotes (Kana and Glibert, 1987; Liu et al., 1999; Worden et al., 2004), respectively were used for the <2 μm size class.

VITAMIN AMENDMENT EXPERIMENTS

On each sampling date, water from both sites was used to conduct nutrient/vitamin amendment experiments. Acid-washed, polycarbonate bottles (1.1 L) were filled and amended in triplicate with either 20 μM nitrate, 100 pM B_{12}, or both nutrients while another triplicate set was left unamended as a control treatment. The bottles were then incubated for 48 h in OFP mimicking ambient light and temperature conditions (Gobler et al., 2007). To assess phytoplankton responses, bottles were analyzed for size fractionated Chl *a* (0.2 and 2 μm polycarbonate filters) and net growth rates were estimated for the different size fractions based on changes in pigment concentrations using the formula: $(\mu = \ln B_{final}/B_{initial})$/incubation time where B_{final} is the Chl *a* in bottles at the end of experiments, $B_{initial}$ is the Chl *a* in bottles at the beginning of the experiments, and the incubation time is days.

DATA ANALYSIS

Relationships between environmental parameters were evaluated by means of a Spearman rank order correlation matrix. *p*-values <0.05 were deemed to be significantly correlated and the correlation coefficient is reported as *R*. For nutrient amendment experiments, differences in growth rates among treatments for each size class of plankton pigments were statistically evaluated using two-way analyses of variance (ANOVA) with N and B_{12} as the main effects. $p < 0.05$ was used to establish significant differences among treatments. To assess seasonal trends, the data was also grouped seasonally into spring (March 21–June 20), summer (June 21–September 20), fall (September 21–December 20), and winter (December 21–March 20).

RESULTS

NUTRIENT AND VITAMIN DYNAMICS

Dissolved inorganic nutrients and vitamin concentrations in OFP were seasonally dynamic (**Figure 2**; **Table 1**). DIN (nitrate + nitrite + ammonium) concentrations varied inversely with water temperature being significantly ($p < 0.001$) higher in the Winter and Spring than in Summer and Fall. In contrast, vitamin B_{12} concentrations (range: <0.1–148 pmol L^{-1};

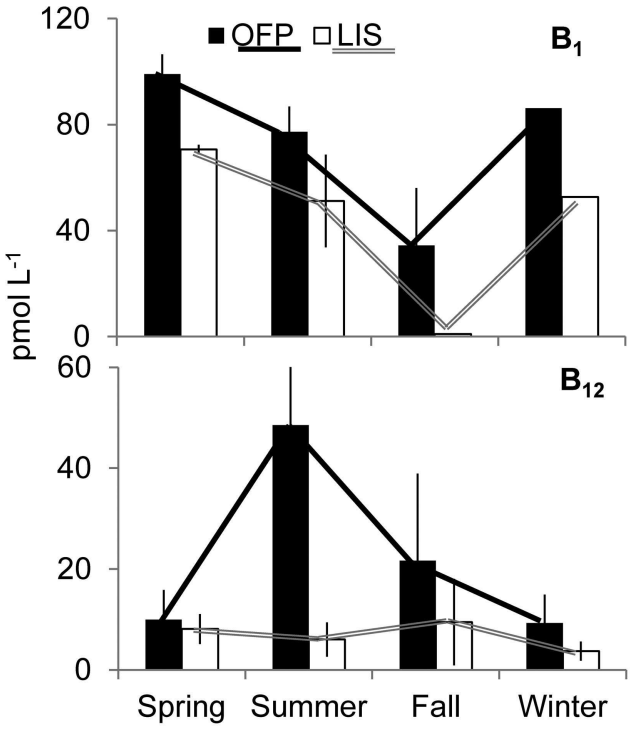

FIGURE 2 | Seasonally averaged concentrations of B_1 (top) and B_{12} (bottom) in Old Fort Pond and Long Island Sound, NY. The solid and hollow lines accentuate the seasonal trend of vitamins in Old Fort Pond and Long Island Sound, respectively. Concentrations are shown in pmol L^{-1}.

Table 1) were significantly ($p < 0.05$) higher in the Summer and Fall (48.6 ± 32.7 and 21.7 ± 17.3 pmol L^{-1}; seasonal average ± SE) than the Spring and Winter (9.95 ± 5.89 and 9.29 ± 4.36 pmol L^{-1}, respectively). Concentrations of B_1 were, on average, higher than vitamin B_{12} (34.9 ± 21.7–99.1 ± 7.46; **Table 1**) and displayed no clear seasonality (**Figure 2**). Vitamin B_{12} concentrations were significantly higher in 2008 (55.2 ± 30.7 pmol L^{-1}) than 2007 (7.07 ± 1.61 pmol L^{-1}; **Table 1**). In LIS, levels of DIN, DIP, and vitamins were all significantly lower than OFP (**Table 1**, $p < 0.05$ for all) and generally highest in Winter and Spring. Vitamin B_{12} concentrations in LIS ranged from <0.1–43 pmol L^{-1} and B_1 concentrations ranged from <0.1 to 99 pmol L^{-1} (**Table 1**). Unlike OFP, seasonal averages of B_{12} in LIS were higher in Spring and Fall, while levels of B_1 were highest in the Spring and low (<0.1 pmol L^{-1}) in the Fall (**Figure 2**; **Table 1**).

PLANKTON COMMUNITY SUCCESSION

Chl *a* concentrations in OFP varied from 1.12 ± 0.04 to 38.9 ± 0.89 μg L^{-1} (mean = 12.7 ± 2.78; **Figure 3A**). Size fractionation of Chl *a* revealed a seasonal succession with larger photoautotrophs being most dominant in the Spring, Summer, and Fall and all size classes contributing equally to the algal biomass during Winter (**Figure 3A**). Heterotrophic bacteria abundances were high, ranging from 0.6 to 11.6 × 10^6 cells mL^{-1} while densities of picocyanobacteria were lower, 0.11–98.2 × 10^3 cells mL^{-1} (**Figure 3B**). Both populations displayed maximal densities during Summer and Fall months with densities of both populations being significantly correlated to water temperature ($p < 0.01$; **Figure 3B**). The microphytoplankton community in OFP was dominated by autotrophic nanoflagellates and dinoflagellates in the Summer and Fall with diatoms present during the Winter and early Spring only (**Figure 3C**). In contrast to OFP, the annual mean of plankton biomass in LIS was significantly lower throughout the year (6.55 ± 1.33 vs. 12.7 ± 2.78 μg Chl *a* L^{-1} for OFP), ranging from <1 μg L^{-1} in winter to 18.7 ± 1.79 μg L^{-1} during the spring bloom in 2007 and, with a few exceptions, was dominated by large (>5 μm) diatoms (**Figures 3A,C**). Heterotrophic bacteria and cyanobacterial abundances in LIS were substantially lower than in OFP with abundances correlating strongly with phytoplankton biomass (Chl *a*, $p < 0.001$) and temperature ($p = 0.001$; **Figure 3B**).

PRIMARY PRODUCTION AND VITAMIN UPTAKE

Primary production rates in OFP displayed a strongly seasonal signal with extremely high rates (607 ± 118 mg C m^{-3} day^{-1}) in the Summer of both years (**Figures 4A** and **5**). With few exceptions, the majority of this productivity occurred in the >2 μm size fraction (**Figure 4A**). Vitamin B_{12} uptake followed a similar seasonal trend with uptake rates ranging from <0.1 to 27.4 ± 2.32 pmol L^{-1} day^{-1} and the highest rates observed in Summer and Fall (mean = 3.07 ± 0.57; **Figures 4B** and **5**). In contrast, B_1 uptake rates, which were higher, displayed little seasonality, ranging from 0.32 ± 0.01 to 29.5 ± 2.45 pmol L^{-1} day^{-1} (mean = 14.4 ± 0.79; **Figures 4C** and **5**). In stark contrast to primary production, picoplankton (0.2–2 μm) were responsible for the majority of vitamin B_1 and B_{12} uptake in OFP (>65% from Spring-Fall; **Figures 4** and **5**). While there was no relationship between vitamin uptake and primary production among plankton <2 μm, among larger plankton (>2 μm) these rates were significantly correlated ($r = 0.88$; **Figure 6**). Notably, the scale of vitamin uptake for the picoplankton was about 10-fold higher than in the >2 μm size fraction.

Mean primary production rates in LIS (343.4 ± 96.8 mg C m^{-3} day^{-1}) were twofold lower than OFP, and peak rates were confined to Spring and Fall of both years (**Figures 4A** and **5**). Like in OFP, larger phytoplankton (>2 μm) accounted for the majority of primary production in LIS (**Figure 4**). Vitamin B_{12} (0.22 ± 0.08 pmol L^{-1} day^{-1}) and B_1 (4.39 ± 1.96 pmol L^{-1} day^{-1}) uptake rates were 10- and 4-fold lower in LIS than OFP, respectively (**Figures 4B,C**). Like OFP, picoplankton were responsible for the majority of vitamin assimilation in LIS (>55% for all seasons, **Figures 4** and **5**). Carbon-specific vitamin uptake rates calculated for both size classes and systems revealed striking differences between picoplankton and the >2 μm size class. Specifically, picoplankton utilized an order of magnitude more B_1 and B_{12} per gram of carbon (**Figure 7**). In addition, there were 200 and 550% higher vitamin uptake for B_1 and B_{12} normalized to carbon, respectively in OFP compared to LIS.

VITAMIN AMENDMENT EXPERIMENTS

Experiments conducted using water from OFP and LIS revealed that nitrogen frequently stimulated phytoplankton biomass (56% of experiments, **Table 2**) and generally did so for both large and

Table 1 | Physical and chemical characteristics of old fort pond and long island sound over the course of the study.

	Temperature °C	Salinity	DIN μmol L^{-1}	PO_4^- μmol L^{-1}	$Si(OH)_4$ μmol L^{-1}	B_{12} pmol L^{-1}	B_1 pmol L^{-1}
OFP							
3/26/2007	7.9	29.6	11.2 ± 1.9	0.8 ± 0.1	8.4 ± 1.5	0.3	ND
4/16/2007	9.1	24.4	18.9 ± 1.4	0.2 ± 0.1	12.1 ± 0.0	6.8	ND
4/30/2007	12.5	24.6	21.3 ± 1.6	0.3 ± 0.1	17.2 ± 2.1	7.9	ND
5/21/2007	14.8	28.7	2.2 ± 0.2	0.2 ± 0.0	12.9 ± 0.2	2.7	ND
6/19/2007	21.1	20.5	11.9 ± 2.0	0.1 ± 0.0	43.7 ± 0.1	9.0	ND
7/10/2007	24.6	24.3	0.0 ± 0.2	0.2 ± 0.0	54.8 ± 0.4	3.7	ND
8/8/2007	25.5	26.9	3.5 ± 0.5	0.5 ± 0.0	41.8 ± 0.4	15.5	ND
8/29/2007	ND	ND	1.7 ± 0.3	1.3 ± 0.3	39.1 ± 3.3	17.0	ND
9/26/2007	21.8	28.1	3.5 ± 0.2	0.2 ± 0.0	21.2 ± 6.4	13.0	ND
11/5/2007	11.2	28.5	7.1 ± 2.6	0.3 ± 0.1	10.2 ± 1.3	4.3	ND
12/4/2007	3.9	27.0	3.7 ± 0.2	0.2 ± 0.0	6.0 ± 0.3	1.0	ND
1/8/2008	3.4	25.4	36.2 ± 6.2	0.1 ± 0.0	46.3 ± 4.6	3.7	ND
3/17/2008	5.6	28.2	0.8 ± 0.4	0.1 ± 0.0	3.9 ± 0.5	14.9	86.3
4/14/2008	10.4	27.5	0.8 ± 0.4	0.1 ± 0.0	7.4 ± 0.2	3.1	112.0
5/19/2008	16.2	26.5	29.3 ± 2.5	0.4 ± 0.1	10.9 ± 2.8	38.9	86.1
6/25/2008	23.1	27.8	7.2 ± 0.7	0.9 ± 0.1	30.7 ± 4.9	158.0	79.0
7/30/2008	27.0	25.6	0.8 ± 0.4	0.7 ± 0.1	10.7 ± 0.2	25.1	52.1
9/4/2008	ND	ND	0.4 ± 0.2	0.4 ± 0.1	10.5 ± 0.2	ND	114.6
9/30/2008	20.2	27.2	3.4 ± 0.6	0.4 ± 0.0	21.9 ± 1.1	90.1	68.7
11/4/2008	10.7	28.4	8.9 ± 0.5	0.3 ± 0.0	18.9 ± 0.3	<0.10	0.1
LIS							
3/26/2007	3.9	26.0	13.7 ± 0.9	0.3 ± 0.1	3.1 ± 0.6	5.7	ND
4/16/2007	6.2	25.9	13.4 ± 0.9	0.3 ± 0.1	5.4 ± 1.0	0.3	ND
4/30/2007	9.8	24.0	2.8 ± 0.2	0.2 ± 0.0	8.9 ± 3.2	6.0	ND
5/21/2007	11.6	25.0	2.2 ± 0.2	0.5 ± 0.1	13.2 ± 0.6	10.7	ND
6/19/2007	18.2	25.3	2.1 ± 0.6	0.6 ± 0.0	18.0 ± 2.0	2.8	ND
7/10/2007	20.5	26.8	0.8 ± 0.2	0.4 ± 0.1	22.1 ± 0.3	4.0	ND
8/8/2007	23.1	27.1	3.1 ± 0.3	0.9 ± 0.0	35.9 ± 0.4	4.3	ND
8/29/2007	ND	ND	6.4 ± 0.6	2.1 ± 0.3	66.9 ± 3.0	1.9	ND
9/26/2007	21.8	28.1	1.7 ± 0.4	1.3 ± 0.2	26.3 ± 0.6	0.3	ND
11/5/2007	15.1	28.3	12.1 ± 0.9	1.8 ± 0.1	43.3 ± 1.9	2.5	ND
12/4/2007	7.6	28.2	14.1 ± 1.7	1.7 ± 0.1	42.4 ± 5.3	0.9	ND
1/8/2008	4.8	28.2	14.5 ± 3.5	1.9 ± 0.2	50.7 ± 0.9	1.8	ND
3/17/2008	3.7	26.3	1.9 ± 0.2	0.9 ± 0.0	2.4 ± 0.1	5.6	52.7
4/14/2008	6.9	25.7	0.7 ± 0.1	0.6 ± 0.1	2.7 ± 0.2	21.3	73.7
5/19/2008	12.1	24.7	4.1 ± 0.3	0.4 ± 0.0	4.7 ± 0.3	4.7	67.5
6/25/2008	20.0	25.9	1.1 ± 0.5	0.2 ± 0.0	5.3 ± 0.2	0.6	<0.10
7/30/2008	22.1	26.8	2.3 ± 0.4	0.9 ± 0.0	34.5 ± 0.3	27.5	54.8
8/12/2008	21.8	26.7	3.2 ± 0.2	1.0 ± 0.1	35.9 ± 1.2	1.0	98.8
9/30/2008	19.7	25.7	15.8 ± 1.0	1.6 ± 0.0	33.5 ± 12.5	<0.10	<0.10
11/4/2008	11.9	26.7	9.7 ± 0.9	1.7 ± 0.1	38.9 ± 0.5	43.7	<0.10

Note that B_1 concentrations were not measured until the second part of the study (3/17/08–11/4/08).

small phytoplankton (> and <2 μm). While B_{12} additions only occasionally enhanced larger algal biomass (>2 μm; 11% of experiments) it more frequently enhanced the growth rate of the 0.2- to 2-μm size fraction (28% of experiments; **Table 2**). When added together, nitrogen and B_{12} enhanced total algal biomass more than each individual treatment in 20% of experiments, suggesting a co-limitation of the community by both compounds. The >2 μm size class was more frequently enhanced in this treatment and most of these effects were observed in the Summer and Fall.

DISCUSSION

Although the potential for B-vitamins to influence the structure and productivity of phytoplankton communities has been recognized for decades, this study is the first to investigate vitamin B_1

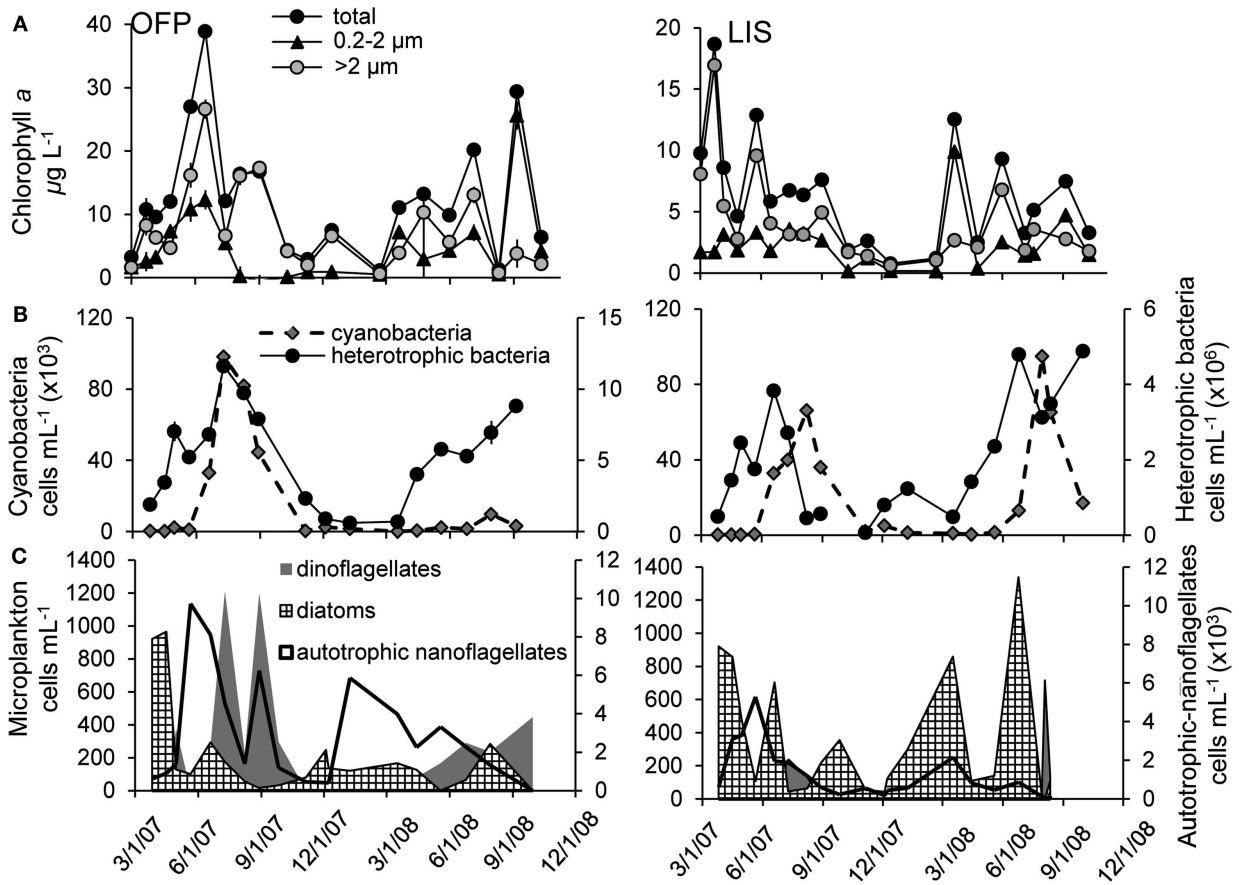

FIGURE 3 | Planktonic community composition from 2007 to 2008 in Old Fort Pond (left) and Long Island Sound (right), NY. Size fractionated chlorophyll *a* **(A)** was obtained using varying pore size polycarbonate filters, cyano-, and heterotrophic bacteria concentrations were measured via flow cytometry **(B)** and the nano- and microplankton community was determined via light microscopy **(C)**.

and B_{12} uptake by plankton communities in an aquatic ecosystem. Performing this investigation in two contrasting estuaries (mesotrophic vs. hypereutrophic), this study specifically documented high vitamin concentrations and uptake rates in eutrophic regions that host elevated primary production and heterotrophic bacterial abundances. Although phytoplankton communities were occasionally stimulated by the addition of vitamin B_{12} alone and in tandem with nitrogen, it was the picoplankton community that was responsible for most of the vitamin uptake during this study. The sum of the data collected suggests that within this size class, heterotrophic bacteria were responsible for the majority of the vitamin uptake in both systems and in all seasons.

VITAMIN AVAILABILITY

The two systems studied here displayed vastly different chemical and biological characteristics, with the OFP containing higher nutrient (**Table 1**), Chl *a*, and vitamin concentrations than LIS (**Figures 2–4**). While seasonally averaged primary production values did not vary significantly between the sites (**Figure 5**), OFP had much larger sustained primary productivity rates throughout the Summer and Fall as evidenced by the reduced variability among seasonal averages. Only prokaryotes possess the genes necessary to synthesize vitamin B_{12} (Warren et al., 2002; Rocap et al., 2003; Newton et al., 2010) and the twofold higher heterotrophic bacterial densities in OFP likely resulted in higher rates of *de novo* vitamin synthesis and concentrations. Gobler et al. (2007) found a strong linear relationship between bacterial abundance and B_{12} concentrations in OFP in summer, a time when the highest vitamin concentrations and uptake rates were observed. Cyanobacteria populations can also produce vitamins (Parker, 1977; Palenik et al., 2003; Bonnet et al., 2010) and were also present at higher concentrations in OFP (**Figure 3**) compared to LIS. Abundances of both groups of picoplankton were strongly correlated with water temperature ($p < 0.01$) a fact which likely contributed to the seasonal peaks in B_{12} observed in OFP during Summer and Fall (**Figure 2**) but not in LIS where B_{12} concentrations and primary production were less dynamic (**Figure 2**). For both systems, primary productivity and B_{12} concentrations were strongly correlated ($r = 0.61$; $p < 0.005$) suggesting a tight coupling between photoautotrophs and B_{12} producing prokaryotes. In contrast, vitamin B_1 was inversely correlated with bacterial biomass ($r = -0.63$, $p < 0.05$) and primary production ($r = -0.74$, $p < 0.05$). Given that vitamin B_1 requirements of phytoplankton generally exceed those of vitamin B_{12} (Tang et al., 2010) and that vitamin B_1 uptake

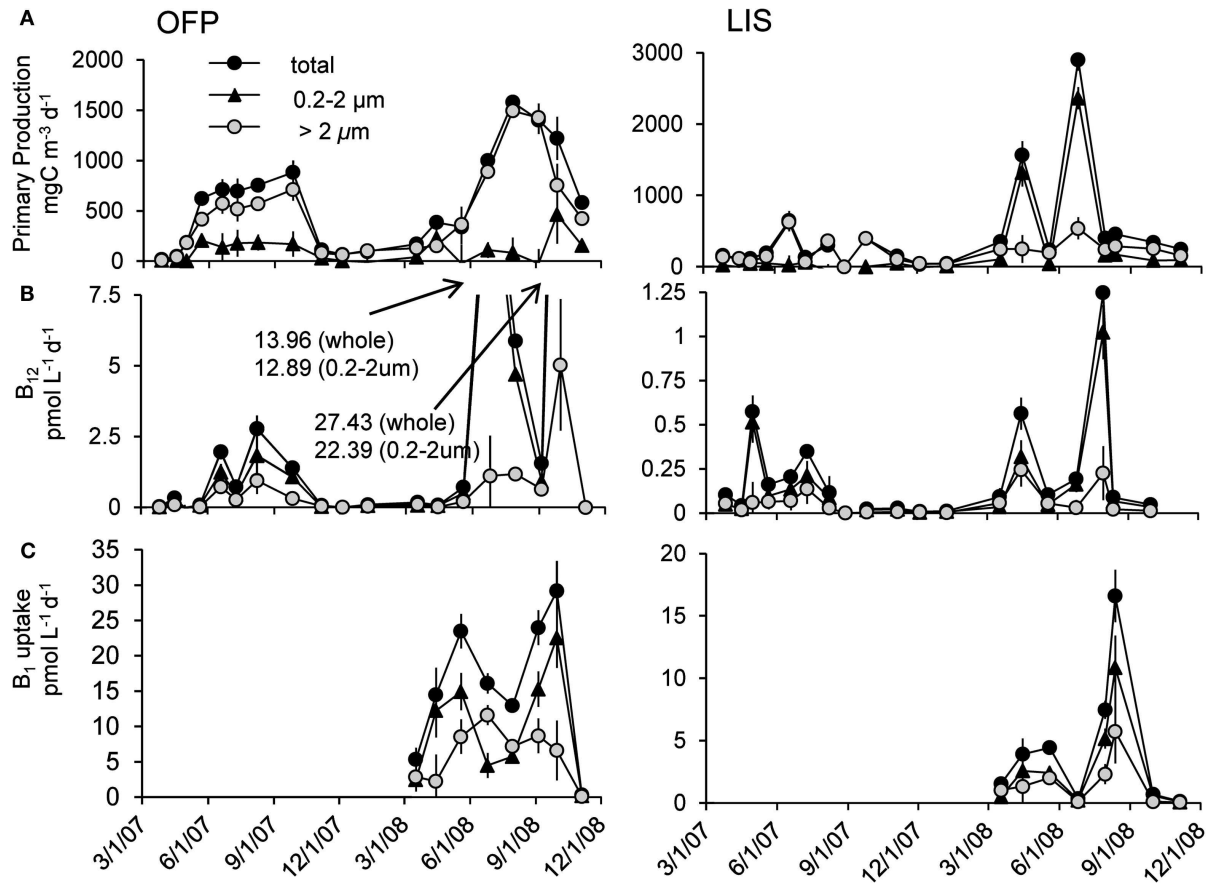

FIGURE 4 | Time series of primary productivity (A), vitamin B_{12} (B), and B_1 (C) uptake dynamics in Old Fort Pond (left) and Long Island Sound (right), NY. Size fractionated primary production and uptake measurements were obtained via the use of polycarbonate filters and are reported in mg C m^{-3} day^{-1} and pmol L^{-1} day^{-1}, respectively.

rates exceeded those of vitamin B_{12}, these inverse correlations may reflect a larger net microbial (phytoplankton and bacteria) uptake of this vitamin.

VITAMIN UPTAKE

B-vitamins were actively utilized by phytoplankton as total uptake rates of B_{12} and total primary production in both systems were highly correlated ($r = 0.80$, $p < 0.001$). Examining this trend among the different size fractions indicated that while primary production and vitamin uptake in the $>2\,\mu m$ size were also positively correlated ($r = 0.83$, $p < 0.001$), this was not true for the picoplankton ($<2\,\mu m$) despite 10-fold higher vitamin uptake rates in this size class (**Figure 6**). The strong correlation between vitamin uptake and primary production in the $>2\,\mu m$ size class is consistent with the fact that most phytoplankton are B_{12} auxotrophs (Croft et al., 2005). The absence of such a relationship among picoplankton suggests auxotrophic, heterotrophic bacteria were responsible for the majority of the vitamin use. Peak vitamin uptake rates in OFP exceeded those of LIS by an order of magnitude (**Figure 5**) and OFP microphytoplankton were dominated by dinoflagellates through much of this study, a group with a high incidence of vitamin B_{12} and B_1 auxotrophy (91 and 41%, respectively; (Tang et al., 2010). In LIS, phytoplankton communities were generally dominated by diatoms, another group with widespread vitamin B_{12} auxotrophy (Provasoli and Carlucci, 1974; Croft et al., 2005) and diatom abundances in LIS were also positively correlated with vitamin B_{12} uptake by the $>2\,\mu m$ size class ($r = 0.65$, $p < 0.02$).

This study reports the first vitamin B_1 uptake rates by aquatic plankton communities and reveals important similarities and differences between these rates and those of vitamin B_{12} uptake. Similar to the ambient B_1 concentrations (**Figure 2**), uptake rates of that vitamin were an order of magnitude higher than B_{12} uptake rates (**Figure 5**). Like vitamin B_{12}, B_1 assimilation also occurred primarily in the picoplankton size class (60.0 ± 5.60 and $62.8 \pm 5.10\%$ for OFP and LIS respectively; **Figure 5**) suggesting that bacteria may hold a competitive advantage over larger phytoplankton for access of this micronutrient. Vitamin B_1 uptake rates in LIS and OFP were generally higher in the Spring, Summer, and Fall, paralleling patterns for vitamin B_1 concentration (**Figure 2**), likely due to temperature dependency of microbial metabolisms. Unlike vitamin B_{12}, B_1 uptake rates were not correlated with any plankton group suggesting that multiple plankton groups were important for B_1 uptake or that groups not

definitively documented by this study were important vitamin B_1 users.

A lack of correlation between primary production and vitamin uptake in the picoplankton (**Figure 6**), 10- to 100-fold greater heterotrophic bacteria biomass (104 ± 16.0 and $42.2 \pm 7.35\,\mu g\,C\,L^{-1}$ for OFP and LIS, respectively) compared to phototrophic picoplankton (3.34 ± 1.48 and $4.44 \pm 1.42\,\mu g\,C\,L^{-1}$ for OFP and LIS, respectively), and a strong correlation between heterotrophic bacterial abundances and B_{12} uptake ($p < 0.008$), all suggest that heterotrophic bacteria were the group responsible for the majority of vitamin uptake during this study. Several recently sequenced marine bacterial genomes possess B_{12} dependent enzymes while lacking genes for B_{12} synthesis (Medigue et al., 2005), suggesting that in addition to being B_{12} synthesizers (Warren et al., 2002; Rocap et al., 2003; Newton et al., 2010) marine heterotrophic bacteria might also be important vitamin consumers. Cyanobacteria and picoeukaryote populations in OFP for 2008 were low ($<10^4$ cells mL^{-1}) when vitamin uptake rates were the highest recorded for the study (**Figures 3** and **4**), again implicating heterotrophic bacterial community as the most important group for vitamin assimilation. This study highlights the importance of picoplankton in vitamin consumption and suggests that vitamins are primarily assimilated by heterotrophic bacteria, even when larger ($>2\,\mu m$), eukaryotic cells dominate plankton biomass (>85% of the total POC during spring and summer). As such, previously reported paradoxically low vitamin concentrations in areas of high bacterial activity such as the deeper LIS waters during summer hypoxia (Panzeca et al., 2009) may be due to a large vitamin demand by actively growing heterotrophic bacteria outpacing the vitamin supply or low vitamin production.

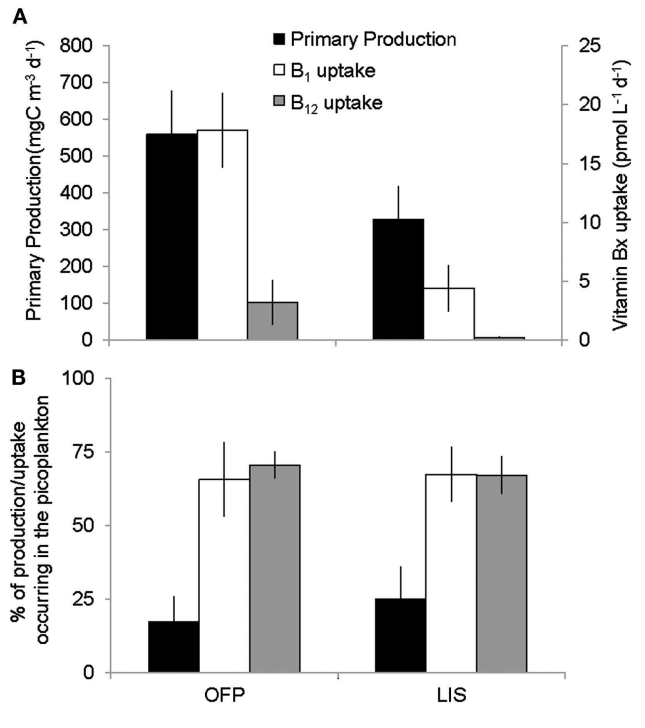

FIGURE 5 | Seasonal averages of primary production and vitamin B_{12} and B_1 uptake (A) and the percent of uptake occurring in the picoplankton (0.2–2 μm) size fraction (B) in the two systems studied. OFP stands for Old Fort Pond while LIS denotes Long Island Sound. Values shown are seasonal means ± SE.

VITAMINS ALTER PLANKTON COMMUNITY COMPOSITION

In both OFP and LIS, vitamin B_{12} amendments stimulated total Chl *a* production in 12% of experiments (**Table 2**) while eliciting size structure changes in nearly half of experiments (47%). In OFP, B_{12} limited phytoplankton solely in the Winter and Spring times when vitamin concentrations were lowest (**Table 1**). In contrast to OFP, phytoplankton in LIS were most limited by vitamins in the summer and fall. The significant correlation between primary production and B_{12} concentrations ($r = 0.83$, $p < 0.005$) as well as between heterotrophic bacterial densities and B_{12} concentrations ($r = 0.47$, $p = 0.05$) suggest that increases in heterotrophic bacteria, fueled by the high primary production, likely led to increased vitamin production and the alleviation of vitamin limitation. As such, the supply of vitamins by bacteria likely influenced vitamin limitation of the phytoplankton in both systems. This conclusion is consistent with Bertrand et al. (2007, 2011) who found vitamins limited phytoplankton communities in coastal regions of the Ross Sea only when bacterial densities were low. As has been previously

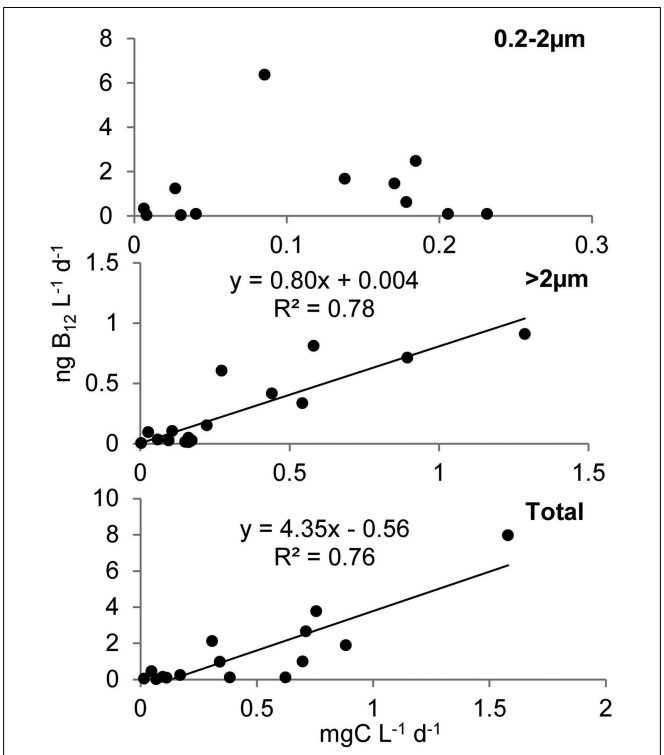

FIGURE 6 | Relationship between B_{12} and carbon uptake in Old Fort Pond. Uptake of B_{12} and bicarbonate are reported as ng L^{-1} day^{-1} and mg L^{-1} day^{-1} respectively. Regressions show that in the larger size class ($>2\,\mu m$), uptake of B_{12} was driven by the photosynthetic organisms in contrast to the $<2\,\mu m$ size class where B_{12} uptake was not correlated to primary production, with heterotrophic bacteria most likely responsible for the majority of vitamin assimilation.

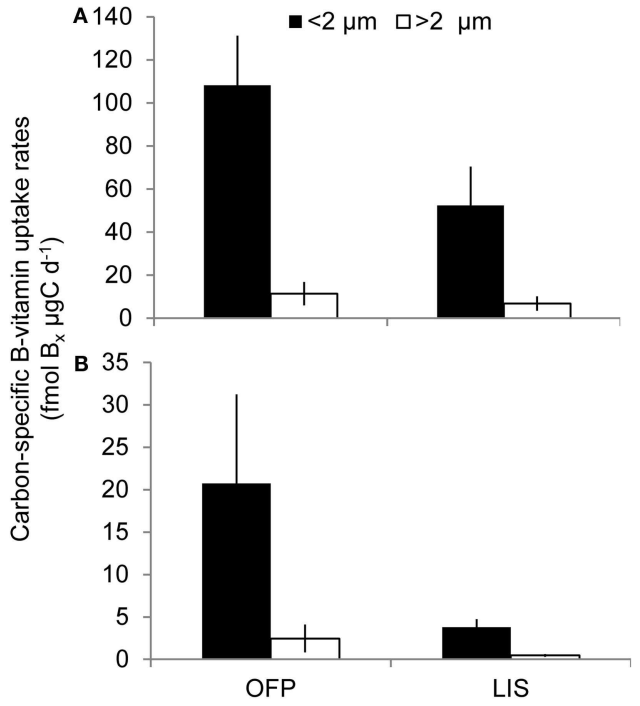

FIGURE 7 | Average B1 (A) and B12 (B) uptake rates normalized to particulate organic carbon (see Materials and Methods) for each size class and location over the course of the study. Values are shown as fmol of Bx $\mu g\,C^{-1}$ day^{-1}, where Bx stands for the corresponding B-vitamin.

Table 2 | Responses to nitrogen and vitamin B_{12} amendments by different size classes of phytoplankton collected from OFP and LIS.

		Total	0.2–2 μm	>2 μm
OFP ($n = 18$)	B_{12}	2	4	5
	N	9	5	8
	N + B_{12}	2	4	4
LIS ($n = 18$)	B_{12}	2	6	4
	N	11	11	11
	N + B_{12}	4	2	6
ALL ($n = 36$)	B_{12}	4	10	9
	N	20	16	18
	N + B_{12}	6	6	9

Responses for each size fraction were measured via changes in chlorophyll a over time and are shown for each sample location. In addition overall effects were examined by looking at all the experiments conducted for each size class (ALL). An effect of the $NO_3 + B_{12}$ treatments was deemed significant when $p < 0.05$ compared to the NO_3 and B_{12} only additions. Values represent the number of times a treatment resulted in a positive growth response in that size class.

observed (Gobler et al., 2007; Koch et al., 2011), the addition of B_{12} in conjunction with nitrogen led to increased growth rates over B_{12} or nitrogen alone, a result that may be partly elicited by an increase in B_{12} demand due in the presence of extra nitrogen. This occurred most frequently in the >2 μm size fraction. While uptake rates of vitamin B_{12} were tightly coupled to primary production in this larger size class (**Figure 6**), the rate of vitamin uptake for that size class was <35% of the total vitamin consumption suggesting that the uptake of the low concentrations of vitamins is dominated by the picoplankton (**Figure 4**), but that both groups are occasionally limited by the availability of vitamin B_{12}.

A REVISED NOTION OF B-VITAMIN CYCLING

Karl (2002) hypothesized that in marine ecosystems, vitamins are produced by bacteria and utilized by larger phytoplankton and while the importance of B-vitamins in phytoplankton physiology has been well-established, the ecological relevance of these micronutrients in the field has been questioned (Droop, 1961, 1968, 1970, 2007). Although prior culture studies suggested that vitamins are present at high enough concentrations to satisfy algal demand, these studies relied on bioassays to estimate the levels of vitamins in the world's oceans. Furthermore phytoplankton are often stimulated experimentally by the addition of B-vitamins (Panzeca et al., 2006; Sanudo-Wilhelmy et al., 2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011). This paradigm may be best explained by the revelation that, in addition to being the source of B-vitamins (Medigue et al., 2005; Bonnet et al., 2010; Bertrand et al., 2011), prokaryotes are also the main sink for these micronutrients in marine systems (**Figures 4** and **5**; Koch et al., 2011) and vitamin uptake by these microbes may, on occasion, deprive eukaryotic phytoplankton of a sufficient vitamin supply. The higher vitamin demand by the plankton communities in OFP point to a microbial community adapted to higher vitamin concentrations in this more eutrophic systems with higher ambient vitamin concentrations. The higher carbon normalized B_{12}-vitamin demand in OFP may be caused by the denser heterotrophic bacterial populations with a higher percentage of vitamin auxotrophs. Similarly, the dominance of dinoflagellates (**Figure 3**), a group comprised almost exclusively of auxotrophs (Tang et al., 2010), in OFP likely accounted for the large >2 μm B_{12} demand per unit carbon there (**Figure 7**). This group was rare in LIS.

Recent work exploring natural ecosystems (Bertrand et al., 2011), cyanobacterial cultures (Bonnet et al., 2010), and prokaryotic genomes (Raux et al., 2000; Palenik et al., 2003; Newton et al., 2010) have highlighted the important role of prokaryotes in ocean vitamin production and consumption. While it is unknown whether specific groups of vitamin producers are also responsible for vitamin assimilation, this would seem counterintuitive since vitamin biosynthesis is complicated and, in the case of B12 utilities numerous genes and enzymatic steps (Raux et al., 2000; Warren et al., 2002). Due to the demanding nature of this process, B-vitamin auxotrophic bacteria may harbor an energetic advantage over vitamin-producing bacteria and seem to hold a kinetic advantage in assimilating picomolar levels of vitamins over larger, vitamin auxotrophic phytoplankton likely due to their larger surface to volume ratio (Raven and Kubler, 2002). In addition, the faster doubling times and higher biomass of heterotrophic bacteria (Giovannoni et al., 2005) likely leads to a much higher vitamin demand compared to larger, slower growing phytoplankton and the development of vitamin depleted surface oceans observed by Sañudo-Wilhelmy et al. (2012). Regardless, we hypothesize that vitamin cycling (uptake and production) occurs

primarily within the prokaryotic picoplankton community, a factor which may limit the growth of some eukaryotic phytoplankton cells.

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