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Nutrient acquisition in dinoflagellates: the role of phosphorus and trace metals on community composition in coastal California and the northern Gulf of Alaska

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NUTRIENT ACQUISITION IN DINOFLAGELLATES: THE ROLE OF PHOSPHORUS AND TRACE METALS ON COMMUNITY COMPOSITION IN COASTAL CALIFORNIA AND THE NORTHERN GULF OF ALASKA

A dissertation submitted in partial satisfaction of the requirements for the degree of DOCTOR OF PHILOSOPHY in OCEAN SCIENCES by Melissa Blakely Peacock

June 2013

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Abstract

Melissa Blakely Peacock

NUTRIENT ACQUISITION IN DINOFLAGELLATES: THE ROLE OF PHOSPHORUS AND TRACE METALS ON COMMUNITY COMPOSITION IN COASTAL CALIFORNIA AND THE NORTHERN GULF OF ALASKA

Dinoflagellates employ a multitude of strategies to competitively assimilate nutrients. These include vertical migration for the assimilation of nutrients at depth and accessing dissolved organic phosphorus (DOP) even when easily accessible inorganic P is available. Identification of these strategies is helpful for characterizing phytoplankton communities and understanding the niche dinoflagellates occupy within that community. Phytoplankton maintain their P status by assimilating dissolved inorganic P (DIP), but when DIP is limiting, or as a preferential utilization, some phytoplankton are able to access dissolve organic P (DOP) by expressing alkaline phosphatase (AP), a metalloenzyme that uses zinc (Zn) as a co-factor.

The first objective of this dissertation was to identify AP activity (APA) in marine phytoplankton as a proxy for determining P-stress amongst communities in the nGoA and coastal Big Sur waters. To this end, a spectrofluorometric method was developed to identify APA in phytoplankton communities with low-cell abundance, like the samples from our study areas. P-stress was identified in dinoflagellates within the communities for both regions, resulting in subtle shifts in phytoplankton composition, even when the entire community was not exhibiting P-stress. In the coastal waters of Big Sur incubation experiments were conducted with the addition of Zn to further
investigate the production of AP has on community structure. We found that the addition of Zn had no significant impact on biomass or growth, but may affect subtle changes in community phytoplankton composition. The second objective of this thesis was to investigate another competitive strategy that dinoflagellates employ: the assimilation of nitrate (N) and iron (Fe) from depth. Active vertical migration of *Akashiwo sanguinea* was observed in laboratory columns under N-replete and N-deplete conditions, as was the significant assimilation of stable isotopes of Fe and N from depth. These observations are the first reported Fe assimilation from depth for any phytoplankton species. Both of these competitive strategies identify tactics dinoflagellates use to access nutrients that are not accessible to other phytoplankton taxon, and the influence this could have on structured phytoplankton communities.
Acknowledgments

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The chapters of this dissertation include reprints of the two following published manuscripts and two submitted publications:


My contribution to this chapter was to perform the method development, sample analysis, and write the presented manuscript. Raphael Kudela supervised the method development and provided assistance with the writing of the manuscript.


I contributed to this chapter by participating in sample collection during the research cruise, analyzing samples, and writing the manuscript. Raphael Kudela directed and supervised the research for this chapter, and gave assistance with the writing of the manuscript.

**Chapter 3:** Peacock, M. B., D. V. Biller, and R. M. Kudela. Submitted. Lack of evidence for direct control of the phytoplankton community by zinc or phosphorus in coastal California. *Continental Shelf Research.*

I designed the experimental set-up for this chapter, collected water, sampled and analyzed all collected data except trace metals, interpreted data, and wrote the manuscript. Dondra Biller contributed by collecting and analyzing trace metal data, figure 1, and assistance with the writing of the manuscript. Raphael Kudela directed and supervised the research for this chapter, and provided assistance with the writing of the manuscript.

**Chapter 4:** Peacock, M. B. and R. M. Kudela. In revision. Evidence for active vertical migration of *Akashiwo sanguinea* experiencing iron and nitrogen limitation. *Limnology and Oceanography.*

I designed the experimental set-up for this chapter, collected, sampled, and analyzed all data, and wrote the manuscript. Raphael Kudela assisted with the design and production of the water column and experimental set-up, analysis of nitrogen data, and provided assistance with the writing of the manuscript.
INTRODUCTION

Phytoplankton account for roughly 50% of global primary productivity, and light, temperature, and nutrients control the rate of primary productivity in the ocean. Limitation of phytoplankton is often presented two ways: rate limitation and biomass limitation. Rate limitation, labeled as “Blackman Limitation”, describes the regulation of growth by the slowest component, or factor (Blackman 1905; Falkowski et al. 1992). For example, if light is increased, growth rate will continue to increase until the rate of chemical reactions (i.e., ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO)) is at its maximum; therefore first light, then the biochemical reactions, are rate limiting. The second form of limitation, “Liebig Limitation” or “Leibig’s Law of the Minimum”, describes the accumulation of biomass being limited by some factor, typically a single nutrient. When the nutrient is not available in sufficient quantities, phytoplankton biomass will not accumulate. This is immediately apparent from satellite chlorophyll-a maps – biomass accumulation patterns follow physical processes that supply nutrients (Fig. 1). In actuality, it is a mixture of both Blackman and Liebig limitation that describes primary productivity and biomass accumulation in the ocean.

Most of the world’s oceans are nutrient-limited either by nitrogen (N) or iron (Fe; Fig 2). A few regions are limited consistently by phosphorus (P), such as the oligotrophic gyre of the north Atlantic (Wu et al. 2000; Mather et al. 2008), and the eastern Mediterranean Sea (Krom et al. 1991), where inorganic P loads are too low to support maximal primary production (Hoppe 2003; Fig. 2). Many other systems are periodically P-limited, usually when the stoichiometric ratio of N:P is much greater than 16:1. These
include coastal areas with riverine input, like the Louisiana basin (Sylvan et al. 2006), estuarine and coastal waters of China (Harrison et al. 1990), and the Gulf of Mexico (Conley et al. 2009). Storm driven systems like Rehoboth Bay, Delaware (Volk et al. 2012) and Kaneohe Bay, Oahu (De Carlo et al. 2007) can also be periodically P-limited. While N- and Fe-limitation may dominate the world’s oceans, geologists view P as the ultimate limiting nutrient over geological time scales, as it cannot be replenished by other means such as direct atmospheric fixation like N (Tyrrell 1999).

Oceanographers often use the canonical molar Redfield ratio of 16:1 (N:P) in the water column, or 106:16:1 (Carbon (C):N:P) in phytoplankton biomass to differentiate N- or P-potential limitation within a system (Redfield 1958; Beardall et al. 2001). If the ratio of dissolved inorganic N (DIN) compared to dissolved inorganic P (DIP) is greater than 16:1, and nutrients are limiting, the system is considered P-limited (Nicholson et al. 2006). This ratio is appropriate for inorganic nutrients, but does not take into account available organic nutrients. These organic nutrients can often be a larger pool than the available inorganic nutrients (Benitez-Nelson 2000). Furthermore, the Redfield ratio is an average for both the water column and phytoplankton biomass, but the stoichiometry of N:P for each phytoplankton species can vary dramatically (Klausmeier et al. 2004; Martiny et al. 2013).

In consistently P-depleted waters, DOP can comprise 25-80% of the P-pool (Hoppe 2003). Not all of this phosphorus is biologically available to phytoplankton (Benitez-Nelson 2000), and most phytoplankton preferentially take up the DIP fraction of available P. In communities with low DIP, the utilization of dissolved organic P (DOP) is immediate and complete. In dynamic coastal environments, which routinely switch
between N-and P-limitation, the DOP pool may be even greater but is difficult to access due to the immediate uptake of DOP by phytoplankton and bacteria. The DOP pool consists almost entirely of two subgroups: phosphomonoesters (~75%) and less-labile phosphonates (~25%). Phosphonates are accessed by C-P utilization enzymes, which are largely produced by hetero- and autotrophic bacteria (Kolowith et al. 2001; Luo et al. 2011). Phosphomonoesters are accessed by alkaline phosphatases (APs), a widely used enzyme produced by phytoplankton, bacteria, and zooplankton in the marine environment (Huang and Hong 1999; Duhamel et al. 2010).

AP is an enzyme used to convert DOP to bio-available DIP by cleaving P from the DOP molecule (Cembella et al. 1984). Some APs are free in the water column, though most are either in the cell or attached to the cell membrane. These enzymes are energetically costly to produce compared to the uptake of DIP (Chróst and Overbeck 1987), and are generally thought to be produced only in instances when DIP is unavailable. Therefore, AP activity (APA) can be used as a proxy to determine P-stress or limitation in the marine environment (Cotner and Wetzel 1991; Vidal et al. 2003). Laboratory studies have determined that the production of AP is inhibited by non-limiting DIP concentrations (González-Gil et al. 1998; Dyhrman and Palenik 1999; Meseck et al. 2009), but field observations are ambiguous. Some studies have observed APA when P is limited, while others determined APA was attributed to specific species within a community, primarily dinoflagellates and coccolithophores, even when the community as a whole was not P-limited, and DIP was not stoichiometrically limiting in the water column (Nicholson et al. 2006; Shaked et al. 2006; Peacock and Kudela 2013). This
suggests that APA more closely tracks individual species’ nutrient stress (Nicholson et al. 2006; Mackey et al. 2012).

Two methods are routinely used to measure APA in the marine environment. The first, a bulk-assay enzyme method that estimates total APA in a given volume of seawater, is based on the amount of phosphomonester substrate that is hydrolyzed in the presence of the AP enzyme (Li et al. 1998). This method delivers reasonably fast results and gives broad estimates as to the amount of APA in the water column. Unfortunately, it can often overestimate APA for primary producers because heterotrophic bacteria signals dwarf the eukaryotic APA (Sebastián and Niell 2004). It is also difficult to routinely compare between studies, as the substrate used to estimate APA can be a variety of phosphomonesters. The second method, an enzyme-labeled fluorescent (ELF) substrate (or probe), can determine species specific APA (González-Gil et al. 1998). The ELF probe attaches to sites of APA and produces a fluorescent precipitate that can be easily viewed with epifluorescent microscopy for eukaryotes (González-Gil et al. 1998) or flow cytometry for prokaryotes (Dignum et al. 2006; Díaz-de-Quijano and Felip 2011). This method separates the heterotrophic bacteria signal, but reports APA as present or absent, regardless of the number of AP sites on the cell (Fig. 3). Therefore, a cell that has one active AP site is considered equally DIP stressed to a cell that has many AP sites, potentially underestimating the amount of APA. Secondly, the ELF method is painstakingly labor- and time-intensive when using microscopy, and samples with low cell abundance are difficult to statistically quantify. Some samples can be counted with flow cytometry, but many eukaryotic cells are too large, or too delicate (i.e., naked flagellates).
The development of a third method, a combination of ELF and bulk spectrofluorometry, was the initial result of this dissertation and is presented in Chapter 1 (Peacock and Kudela 2012; A method for determining alkaline phosphatase activity in marine phytoplankton using spectrofluorometry). This method reduces the amount of labor and time it takes to process samples, reports intensity of APA rather than just presence or absence, and allows samples with low cell abundance to be analyzed. Furthermore, the same samples processed by spectrofluorometry can also (if time and cell abundance permits) be counted within 48 h by microscopy for species specific APA with no measurable decline in fluorescence. The samples for this method are processed similarly to the typical ELF method (González-Gil et al. 1998), but then analyzed with an M2e spectrofluorometer. Spectrofluorometry allows the user to visualize the intensity of APA, capturing the range of fluorescence of the emission curve (470 - 620 nm) compared to just the single emission of 530 ± 30 nm used in epifluorescent microscopy and flow cytometry. To measure intensity with flow cytometry or spectrofluorometry, the sample first needs to be normalized. For flow cytometry, fluorescent beads are used, but the fluorescent signal can sometimes interfere with the peak emission of 525 nm, and does not necessarily relate to the intensity seen in natural populations. The method described in this first chapter improves upon previous APA methods by using a biological standard (Amphidinium carterae) to normalize samples, and was statistically robust, despite the potential variability introduced with a biological standard.

Development of this spectrofluorometric method was necessary for quantification of APA in samples collected from the northern Gulf of Alaska (nGoA), as the quantities of cells in each sample were too low to be enumerated with typical ELF microscopy. The
nGoA is a highly productive coastal environment, even though it is limited by a combination of light and nutrients. The prevailing downwelling winds over the continental shelf reduce potential upwelling of limiting macronutrients, and therefore, the high productivity observed in the nGoA is surprising. This level of productivity is likely due to eddy transport, advection in canyons bringing nutrients to the shelf region, and cross shelf exchange of nutrients (from the interior basin) replenishing limiting nutrients with high-nutrient low-chlorophyll (HNLC) water (Stabeno et al. 2004; Ladd 2005; Ware 2005; Strom et al. 2006). Even though it is macronutrient deficient, the shelf is not considered P-limited (Fig. 2). In Chapter 2 of this thesis (Peacock and Kudela 2013; Alkaline phosphatase activity detected in distinct populations of phytoplankton in the northern Gulf of Alaska), as part of a larger investigation of cross-shelf transport of nutrients and stimulation of biological communities, we investigated the role APA played in nGoA phytoplankton communities using the spectrofluorometric method developed in Chapter 1.

Our results from Chapters 1 and 2 added to the growing body of evidence that not all phytoplankton produce or use AP. Some diatoms are capable of producing AP, but not all, and it is rare that an entire population will be producing AP at the same time. Furthermore, diatoms often show a greater sensitivity to DIP availability than other groups of phytoplankton (i.e., increased APA in diatoms is correlated with decreased DIP concentrations; Mackey et al. 2012; Nicholson et al. 2006) and APA is not observed except under P-stress. The most likely eukaryotes to produce AP and utilize DOP are dinoflagellates, which is what we observed in the nGoA. This suggests that dinoflagellates have a preference for DOP, possibly using it as a competitive strategy to
alleviate P-stress and access a fraction of the P-pool that is not as available to the
dominant phytoplankton within the community.

AP is used to access DOP, and is also a metalloenzyme that requires Zn\(^{2+}\), Ca\(^{2+}\),
or Mn\(^{2+}\) as a cofactor (Cembella et al. 1982). Zinc (Zn) has been hypothesized to be
limiting in oligotrophic environments (Anderson et al. 1978; Sunda and Huntsman 1992;
Morel et al. 1994) and coastal regions with a narrow shelf and HNLC water (Franck et al.
2003), and is reported in low concentrations (< 1 nmol L\(^{-1}\)) along the Big Sur coast of
California. The results from incubations in the field have been inconclusive for Zn-
limitation, and phytoplankton biomass and productivity has only been minimally
stimulated with the addition of Zn (Crawford et al. 2003; Lohan et al. 2005). But, like the
addition of DOP, Zn may be responsible for slight shifts in the phytoplankton
community. Shaked et al. (2006) hypothesized that Zn-P co-limitation may be
responsible for shaping community dynamics of phytoplankton in HNLC regions, and it
is possible that the addition of both Zn and a P-source (either as DIP or DOP) may
produce changes in community composition. Measuring APA may be a reasonable proxy
to determine if the addition of Zn and DOP shifts the community of phytoplankton to AP
producers since the enzyme is closely linked to both nutrients. Chapter 3 of this
dissertation, titled “Lack of evidence for direct control of the phytoplankton community
by zinc or phosphorus in coastal California” and submitted to Continental Shelf Research
for publication, investigates incubations enriched with a suite of nutrients to determine if
the addition of Zn with a P-source influences community dynamics of phytoplankton by
increasing the percentage of dinoflagellates that utilize Zn and DOP to produce AP.
These dinoflagellates would have a competitive advantage over non-AP producing
phytoplankton. Results from Chapter 3 demonstrate the effect that DOP and Zn have on community dynamics of phytoplankton in natural waters, and provide an understanding for the species specific nutrient interactions that may be key to understanding nutrient cycling dynamics.

Chapters 1-3 discuss the competitive advantages gained by dinoflagellates incorporating organic P when the water column is not stoichiometrically limited by DIP, but what happens when all the nutrients from the surface waters have been depleted? Another competitive strategy utilized by dinoflagellates is to vertically migrate to acquire nutrients from subsurface depths (Fig. 4). Dinoflagellate blooms can be persistent, often lasting longer than would be expected based on available nutrients (Smayda 1997). These blooms often follow diatom blooms, when the surface waters have been depleted of all nutrients, including N and Fe. To alleviate nutrient stress, dinoflagellates can vertically migrate to incorporate nitrate (Smayda 2010 and references therein) and ammonium (Hall and Pearl 2011) from depth. Furthermore, they are physiologically adapted to incorporate nutrients during dark cycles, which is more difficult for other groups of phytoplankton (MacIntyre et al. 1997). By vertically migrating, dinoflagellates can produce 5-times the predicted chlorophyll-a from available inorganic nutrients (Doblin et al. 2006). Therefore, when light and nutrients are vertically stratified in the water column with nutrients depleted in the near surface, dinoflagellates have a competitive advantage.

Chapter 4 of this thesis, titled “Evidence for active vertical migration of the dinoflagellate Akashiwo sanguinea experiencing iron and nitrogen limitation”, submitted to Limnology and Oceanography and in revision for publication, investigates nutrient acquisition by the dinoflagellate A. sanguinea.
*A. sanguinea* is a globally ubiquitous dinoflagellate that forms harmful algal blooms (HABs), and has been linked to the mortality of abalone larvae (Botes et al. 2003), coral reef bleaching (Litchman and Nguyen 2008), and seabird death (Jessup et al. 2009; Du et al. 2011). It was one of the first dinoflagellates to be investigated to determine vertical migration for the incorporation of nitrate (Keifer and Lasker 1975; Cullen and Horrigan 1981). When migrating for N, it would presumably be advantageous to also acquire Fe at depth. Much of the world’s oceans are limited for Fe or a combination of Fe- and N (Fig. 2). Fe is an important trace nutrient for phytoplankton, used in nitrate and nitrite reductase enzymes for the assimilation of nitrogen, and in Fe-containing proteins, like ferredoxin, which is an electron receptor during photosynthesis. Fe-deficiency in phytoplankton can also lead to the inability to remove macronutrients from the water column. *In situ* experiments to observe vertical migration for N are difficult, and the difficulty only increases with trace metals, likely explaining why Fe is often assumed to be incorporated during vertical migration, but had not been explicitly tested. Results from Chapter 4 of this thesis present the first observed laboratory Fe incorporation at depth by a vertically migrating dinoflagellate under Fe-limitation and varying levels of N-stress. These results validate the presumption that Fe is being incorporated at depth *in situ* along with N and is enhancing dinoflagellate blooms.

This dissertation first focused on understanding dinoflagellate use of DOP as a competitive means to avoid P-stress in mixed communities of phytoplankton. Chapter 1 developed a method that was used in Chapters 2 and 3 to understand phytoplankton community dynamics and preferential use of organic nutrients by dinoflagellates. Results from those chapters add to the growing body of evidence that dinoflagellate’s nutrient
stoichiometry may be vastly different than the phytoplankton community as a whole.

Chapter 4 investigated another competitive dinoflagellate method, vertical migration to alleviate Fe-stress, and provides the first evidence of micronutrient uptake at depth by phytoplankton.
References


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Figure 1. Global annual averaged SeaWiFS satellite ocean color data. Patterns of ocean color match closely with physical properties that inject nutrients into the surface waters.
Figure 2. Modeled nutrient, light, and temperature limitation of three main phytoplankton groups (diatoms, small phytoplankton, and diazotrophs) averaged during the summer season for each hemisphere. Reproduced from Moore et al. 2004.
Figure 3. Differences in labeling of alkaline phosphatase activity for (A) *Rhizosolenia*, (B) *Pseudo-nitzschia*, (C) *Prorocentrum micans* (top) and *P. gracile* (bottom), (D) *P. rostratum*, (E) *Prorocentrum sp.*, (F) *P. minimum*, (G) *Ceratium furca* (left) and *C. balechii* (right), (H) *Dinophysis fortii* with attached bacteria, and (I) *D. fortii*. APA is considered present or absent regardless of the differences in APA placement (either on
the outside of the cell or inside) and the number of APA sites with typical ELF-methods.

Samples are not considered for intensity of APA.  Reproduced from Mackey et al. 2007.
**Figure 4.** Profiler data from October 2010 during a *P. micans* dinoflagellate bloom in Monterey Bay, CA. Panels are (from top) temperature, salinity, density, chlorophyll fluorescence, oxygen and rhodamine (a dye tracer). Notice the vertical migration on fluorescence and the temperature stratification. Vertical migration depth corresponds to the observed nutricline. Data provided courtesy of the LatMix 2 experiment.
CHAPTER 1: A METHOD FOR DETERMINING ALKALINE PHOSPHATASE ACTIVITY IN MARINE PHYTOPLANKTON USING SPECTROFLUOROMETRY

Melissa Blakely, Peacock and Raphael M. Kudela (2012) 

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A method for determining alkaline phosphatase activity in marine phytoplankton using spectrofluorometry

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ABSTRACT

A method for determining relative percent intensity alkaline phosphatase activity (APA) using enzyme labeled fluorescence coupled with spectrofluorometry is presented. Compared to traditional microscopy and flow cytometry, we increase statistical power and reduce sample-handling issues. Combined with a biological standard, our method can quantify APA of natural plankton assemblages.

Field samples were collected from the Santa Cruz Municipal Wharf (36°57.48′ N, 122°31.62′ W). Ancillary measurements included macronutrients (Knepel and Bogren, 2001; Smith and Bogren, 2001a, 2001b), temperature, chlorophyll-a (Welschmeyer, 1994), ammonium (NH4; Holmes et al., 1999), and urea (Mulvanna and Savidge, 1992; Price and Harrison, 1987). Phytoplankton was preserved for species identification using 4% buffered formalin.

ELF-APA samples were filtered (<30 mm Hg) onto 1 or 5 μm polycarbonate filters (Poretics) to minimize the contribution of heterotrophic bacteria, and stored at ~80 °C or in 1 mL reagent-grade 70% ethanol at 4 °C in the dark. A subset of samples (n=6) was analyzed immediately. Cells were gently scraped off filters and placed in epitubes (Cytotech, Inc., Seattle, WA, USA) flow cytometer (A1 microscope equipped with Chroma Technology filter set #31000v2). Cells with ELF-labeling were considered positive for APA.

Ten μL of the prepared sample was suspended in two drops of mounting medium component C (Invitrogen #E6601) on a glass slide. Samples were examined with a Leica Axios Imager microscope equipped with a Chroma Technology filter set #31000v2. Cells with ELF-labeling were considered positive for APA (Dyhrman and Palenik, 1999; González-Gil et al., 1998). Field samples were enumerated as percent labeled cells relative to all enumerated cells (>100 per sample).

A Cytotepea influx (Cytotepea Inc., Seattle, WA, USA) flow cytometer with a blue (488 nm) laser was used for a subset of samples. Each sample was characterized by forward angle light scatter (FSC; relates to particle size), and three fluorescence parameters: ELF (525+/−40 nm), red fluorescence for chlorophyll-a (692+/−20 nm), and SYBR Green I (520+/−25 nm) for heterotrophic bacteria. Samples
were enumerated using ELF-fluorescence intensity versus FSC with a minimum of 45,000 particles per sample. FlowJo software was used to obtain a volumetric estimate of cell density (cells ml⁻¹; Goebel et al., 2008). Histograms were used to determine the geometric mean of the fluorescent peak of each sample (Meseck et al., 2009) and frequency statistics were computed in FlowJo.

Heterotrophic bacteria were counted with flow cytometry after being labeled with SYBR Green I to increase the intensity of the DNA signal. Autotrophic phytoplankton and heterotrophic bacteria were enumerated with red fluorescence versus FSC and green fluorescence versus FSC respectively.

For spectrophotometric analysis, 70-100 μl samples were analyzed on a 96-well opaque plate using an M2e SpectraMax spectrophotometer internally standardized with fluorescein isothiocyanate (FITC; 3 fmol well⁻¹). Excitation/emission was set to 365 nm and 400-700 nm at 5 nm resolution. Automatic mixing (20 s) was used to minimize particle sinking. Temperature control (+/−0.5 °C) was used during the analysis. The 525 nm emission peak corresponds to the emission used for ELF-microscopy (González-Gil et al., 1998). For our intensity analysis we determined the area under the emission curve from 470 to 620 nm. Intensity was measured in arbitrary units (AU).

A. carterae cultured under P-replete and deplete conditions was used to establish a standard curve by serial dilution. The standard curve was created using known percent labeling (from microscopy) versus AU (spectrophotometry) after normalizing AU to 450 nm to account for differences in biomass. Field sample ELF-APA intensity was expressed as percent-labeled intensity by applying the standard curve.

APA from epifluorescent microscopy is performed based on presence or absence of labeling (Fig. 1; Dyhrman and Palenik, 1999; González-Gil et al., 1998). This does not account for intensity of ELF-labeling (i.e., how many AP sites are labeled based on fluorescence), intercellular labeling (Dyhrman and Palenik, 1999; Díaz-de-Quijano and Felip, 2011), or the inability to ‘count’ the number of cell markers when the entire cell wall is not visible (Dyhrman and Ruttenberg, 2006; González-Gil et al., 1998; Meseck et al., 2009; Nicholson et al., 2006). Spectrophotometry and flow cytometry can determine intensity (Duhamel et al., 2010; González-Gil et al., 1998; Nedoma et al., 2003), but results need to be normalized for biomass. For our method we normalized to 450 nm, an application unique to spectrophotometry since epifluorescent microscopes and flow cytometers typically cannot scan the visible light spectrum. Our normalization to 450 nm does not interfere with the ELF-97 emission (maximum 525 nm), and is more accurate than normalizing to chlorophyll-a (data not shown). We quantified detectable differences for cultures with as low as 2% labeling (data not shown), and suggest that modern instruments can be used for routine quantification of APA, in contrast to results from previous studies (González-Gil et al., 1998; Nedoma et al., 2003).

By using a serial dilution of A. carterae we were able to set a repeatable upper limit for ELF-APA intensity (90.3+−0.01% n=17) and for the baseline intensity (−0.001+−0.001%, n=13) allowing us to determine relative intensity of natural samples. For multiple standard curves (separate cultures) the slopes (but not the intercepts) were not significantly different (ANOVA, p<0.05, n=5); it is therefore necessary to adjust for the baseline (intercept) fluorescence from run to run. The close correspondence with microscopic enumeration suggests that, despite the potential variability introduced with a biological standard, our proposed method is robust.

Microscopy cannot statistically quantify APA if a low quantity of labeled cells are present (Nedoma et al., 2003; Nicholson et al., 2006). In contrast, spectrophotometric measurement account for changes in labeling-intensity due to both the proportion of cells labeled and the number of sites per cell. Our P-replete cultures (n=5) exhibited fluorescent intensity 26.1+−3.7 AU. lower than samples grown under P-deplete conditions (n=5). From field samples (n=8) three were negative for APA (0% labeling by microscopy) and the others range from 0.8% to 34.0% (Table 1). Both dinoflagellates and diatoms exhibited positive APA (Table 2). There was excellent correspondence between microscopic and spectrophotometric measurements, both with field samples and with cultures (Tables 1 and 3).

While epifluorescent microscopy can only determine APA in phytoplankton >2 μm (Li et al., 1998), flow cytometry has a typical upper limit of ~70 μm (Dignum et al., 2004; Duhamel et al., 2010). From our field samples, diatoms (Table 2) and the A. sanguinea culture (-90 μm) were unable to be counted by flow cytometry due to clumping despite the use of a 150 μm nozzle. While the flow cytometer may be more quantitative for determining APA-intensity than spectrophotometry, its cell size capabilities limit its use and may better represent prokaryote contributions (Duhamel et al., 2010; Grégoire et al., 2011).

Our A. carterae P-deplete culture (87.4% labeling by flow cytometry, 92.4% labeling by microscopy) exhibited a 12.3% stronger 525 nm fluorescence signal than P-replete media cells (34.4% labeling; Fig. 2; Table 3) as measured by flow cytometry. The two populations were significantly different (p<0.01; X², Kolmogorov-Smirnov). ELF-labeled and un-labeled heterotrophic bacteria could not be distinguished for the P-replete sample; for the P-deplete sample small populations of ELF-labeled and non-ELF-labeled bacteria were present (Fig. 3).

Spectrophotometry exhibits fast acquisition of data without compromises caused by low ELF-labeled cell abundance. But like flow cytometry, does not allow for direct determination of species using APA. We feel this to be an acceptable compromise given the speed, efficiency, and ability to quantify labeling intensity rather than just presence/absence of ELF-labeling. Furthermore, unlike flow cytometry,

![Fig. 1. Amphidinium carterae labeled with ELF-97 precipitate (green) at the sites of alkaline phosphatase activity, visualized using epifluorescence microscopy as described in the text. Light is autofluorescence from endogenous pigments.](image-url)
Tip of the tongue...
References


CHAPTER 2: ALKALINE PHOSPHATASE ACTIVITY DETECTED IN DISTINCT PHYTOPLANKTON COMMUNITIES IN THE NORTHERN GULF OF ALASKA

Melissa B. Peacock and Raphael M. Kudela (2013)

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Alkaline phosphatase activity detected in distinct phytoplankton communities in the northern Gulf of Alaska

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ABSTRACT: Alkaline phosphatase activity (APA) has been proposed as a proxy to determine phosphorus (P) stress in marine phytoplankton. Phytoplankton typically meet P requirements by incorporating dissolved inorganic P (DIP), but can use reservoirs of dissolved organic P (DOP) by expressing alkaline phosphatase (AP). Enzyme-labeled fluorescence (ELF) was used to determine group-specific APA within 4 phytoplankton communities associated with mesoscale eddies, which are common to the northern Gulf of Alaska (nGoA). Nutrient stress in relation to eddies in the nGoA has not been well established and these anti-cyclonic eddies may be influencing P status in phytoplankton by isolating distinct biological communities. In the nGoA (fall 2007), APA was greatest in the northwest transitional waters of the Sitka eddy and the western edge of the Kenai eddy compared to coastal (shelf), eddy core waters, and open ocean waters. The APA signal was dominated by picoplankton (0.6 to 5 µm) and nanoplanckton (5 to 20 µm), primarily nanoflagellates, though AP-producing phytoplankton were not the dominant phytoplankton at any station. APA did not correlate with DIP levels, and macronutrient concentrations suggest that DIP was not limiting for the bulk phytoplankton assemblage. Dissolved organic P (DOP) was not measured. These results suggest that P status within a community is more nuanced than typical Redfield or inorganic nutrient indices indicate and that organic P sources could influence community composition of phytoplankton.

KEY WORDS: Phosphorus limitation · Dissolved organic phosphorus · Spectrofluorometry · Enzyme-labeled fluorescence · Mesoscale eddy

INTRODUCTION

While most ocean systems are not considered phosphorus (P) limited except over geological timescales (Tyrrell 1999), P is an essential nutrient for all phytoplankton growth, energy transfer, and reproduction (Benitez-Nelson 2000). Marine ecosystems are usually limited (sensu Liebig) by nitrogen (N) or iron (Fe) (Moore et al. 2001), but 2 major P-limited environments include coastal areas influenced by seasonal freshwater inputs (Lahry et al. 2002) and oligotrophic gyres that routinely have P levels too low to support maximal phytoplankton growth rates (Li et al. 1998, Hoppe 2003, Oh et al. 2005). Nutrient limitation can be difficult to determine because there are often synergistic or antagonistic effects between individual nutrients (Wei et al. 2003), ecosystems may shift seasonally between N or P limitation, and mixed communities of phytoplankton may have variable nutrient requirements (McComb et al. 1981, González-Gil et al. 1998).

Marine P is typically present as various dissolved organic P (DOP) compounds and as orthophosphate, dissolved inorganic P (DIP). The canonical molar Redfield ratio of 16:1 (N:P) in the water column or the C:N:P ratio (106:16:1) in phytoplankton biomass has traditionally been considered the determining factor for whether there is potential for N or P limitation in phytoplankton for a specific ecosystem (Redfield 1958, Beardall et al. 2001). However, the Redfield...
ratio does not account for organic nutrients, and thus it is not always an accurate assessment of nutrient availability (Benitez-Nelson 2000). Furthermore, while the Redfield ratio may hold true for globally averaged phytoplankton biomass, there can be variable, species-specific P demands (González-Gil et al. 1998, Krause et al. 2004, Nicholson et al. 2006). This may result in potentially subtle shifts in community composition in response to nutrient limitation (Dyhrman & Palenik 2001). Since many phytoplankton are capable of nutrient storage and luxury uptake, each species’ nutrient requirements must be based on species-specific stoichiometry (Rhee & Gotman & Palenik 2001). Since many phytoplankton are capable of nutrient storage and luxury uptake, each species’ nutrient requirements must be based on species-specific stoichiometry (Rhee & Gotman & Palenik 2001). Since many phytoplankton are capable of nutrient storage and luxury uptake, each species’ nutrient requirements must be based on species-specific stoichiometry (Rhee & Gotman & Palenik 2001). Since many phytoplankton are capable of nutrient storage and luxury uptake, each species’ nutrient requirements must be based on species-specific stoichiometry (Rhee & Gotman & Palenik 2001). Since many phytoplankton are capable of nutrient storage and luxury uptake, each species’ nutrient requirements must be based on species-specific stoichiometry (Rhee & Gotman & Palenik 2001).

The nGoA is a highly productive coastal environment despite the low macronutrients over the continental shelf, a result of prevailing downwelling winds. Regardless, it is not classified as a P-limited system, and one would not expect to find P stress in phytoplankton in this region. The prevalence of downwelling winds suggests that other mechanisms for increased productivity over the shelf area are at play (Stabeno et al. 2004, Ware & Thomson 2005). Mesoscale anti-cyclonic eddies often form along the shelf break and act to exchange water from the shelf to the slope (Whitney et al. 2005). Biological productivity is typically enhanced along the tracks of these eddies due to greater mixing and entrainment of nutrients (Fiechter et al. 2009). As part of a larger investigation focusing on the role of eddies in cross-shelf transport and stimulation of biological communities, we utilize an ELF spectrophotometric method to evaluate APA within the nGoA.

**MATERIALS AND METHODS**

**Field collection**

Water samples for nGoA APA were collected from the surface and the chlorophyll a (chl a) maximum (determined in situ by fluorescence) from an instrumented CTD rosette aboard the RV ‘Thomas Thompson’ from August 17 to September 20, 2007 at 32 stations (Fig. 1). Sample locations were chosen based on the paths of 2 anti-cyclonic eddies that were present during the cruise (Fig. 2). Sample sites include the transition edges and cores of the eddies, along with the coastal water and basin water not associated with either eddy. AP samples were filtered through 0.6, 5 and 20 µm polycarbonate filters and stored frozen at −80°C. Ancillary samples collected included nutrients, size-fractionated chl a, flow cytometric samples, and whole water for phytoplankton enumeration. Temperature, salinity, and oxygen profiles for each sample site were obtained from the CTD.

**Laboratory cultures**

Non-axenic cultures of the dinoflagellate *Amphidinium carterae*, obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, strain 1332), were grown in Pyrex 2 l flasks with sterile-filtered artificial seawater (ASW); these cultures were used to create APA standard curves by varying the P stress in the culture conditions (Peacock & Kudela 2012). The cultures were grown under P-replete (CCMP L1 nutrients) and P-deplete (CCMP L1 nutrients without P) conditions. Cells grown under P-deplete conditions were transferred at least twice to fully deplete residual DIP before they were used as standards. Cells were used as standards when they reached stationary phase (2 to 3 wk). Further details of the culture conditions and APA methods are included in a separate manuscript (Peacock & Kudela 2012).

**ELF analysis**

Both culture and field samples were labeled with ELF-97 (Endogenous Phosphatase Detection Kit, Invitrogen) prepared in sterile ASW (González-Gil et al. 1998, Dyhrman & Palenik 1999, Peacock & Kudela 2012). Samples were examined with a Zeiss Axio imager manual A1 microscope using a DAPI filter set with excitation at 350 nm and emission at >450 nm, as well as standard (brightfield) illumination. For culture standards, >400 cells were counted in triplicate to determine presence of APA. Labeled cells were identified by multiple attachment sites of the bright green fluorescing ELF precipitate (González-Gil et al. 1998). Unlabeled cells only displayed autofluorescence (Fig. 3). Standards grown with P-deplete media exhibited >99.9% ELF labeling, as determined...
by labeled cells normalized to total cells, and standards grown under P-replete media exhibited <0.1% ELF labeling. Standards for spectrofluorometric determination of APA were prepared from the cultures using serial dilutions of cells harvested during the stationary phase (Peacock & Kudela 2012).

Samples were run on a 96-well opaque plate using an M2 SpectraMax spectrofluorometer internally standardized with fluorescein isothiocyanate (FITC) to 3 fmol per well. Excitation was set to 365 nm with a wide emission band from 400 to 700 nm at 5 nm resolution. Automatic mixing for 20 s before reading was used to minimize particle sinking. Each well contained 70 to 100 µl of sample and was averaged over 8 readings. Temperature control (±0.5°C) was used during the analysis. The maximum emission wavelength, 525 nm, corresponds to the emission used for ELF microscopy (González-Gil et al. 1998). All samples and standards were adjusted for biomass by normalizing the ELF fluorescence to 450 nm. For our intensity analysis, we determined the area under the emission curve from 470 to 620 nm. Intensity was measured in arbitrary units (A.U.) for the nGoA samples, where 0 is no expression and 1 is maximum expression for the nGoA (i.e. 1 = the highest A.U. for the unknown nGoA field samples). Field samples were also qualitatively examined by microscopy to determine presence/absence of APA, but cell counts were not performed. As per previously published protocols (Dyhrman & Palenik 1999, González-Gil et al. 1998), cells with any ELF labeling (regardless of the number of attached sites per cell) were considered positive for APA.

Ancillary data

Inorganic nutrient samples were collected in the field, 0.7 µm filtered, frozen, and later analyzed using a Lachat autoanalyzer with QuickChem standard co-
lométric methods for nitrate + nitrite (hereafter referred to as nitrate), P, and silicic acid (Knepel & Bogren 2002, Smith & Bogren 2001a, b). External precision was < 0.2 µM for all nutrient species. DOP was not analyzed. Water was filtered from either the surface or the chl a maximum onto GF/F filters for total chl a and using 5 and 20 µm polycarbonate filters for size-fractionated chl a (referred to as pico-, nano-, and microplankton for <5, 5 to 20, and >20 µm fractions), extracted in 90% acetone and analyzed on a Turner 10-AU fluorometer (Welschmeyer 1994). Primary productivity data were obtained using standard 14C deckboard incubation methods of whole water (Kudela et al. 2006). Flow cytometry samples were collected from the surface and chl a max, preserved with 4% paraformaldehyde and stored at −80°C until counted for Synechococcus, picoeukaryotes, and heterotrophic bacteria. Analysis was performed on a Cytopeia Influx flow cytometer with greater than 200,000 events counted per sample. Heterotrophic bacteria samples were prepared by staining the cells with SYBR Green I DNA stain. Qualitative enumeration of microplankton was conducted shipboard with unprepared samples using a Fluid Imaging FlowCam.

RESULTS

Characterization of water masses in the nGoA

Both the Sitka and Kenai eddies, named for their formation region, had hydrographic properties similar to previously described nGoA eddies (Tabata 1982, Ladd et al. 2007, Rovegno et al. 2009). Algorithms developed by Rovegno et al. (2009) using satellite altimetry to identify and track nGoA eddies determined that both eddies were about 8 mo old at our sampling time. Sampling sites were categorized by hydrographic data: basin, coastal, transitional, and eddy core water (Fig. 4). Sea surface height anomaly (SSHa) for both eddies was 30 to 40 cm (Rovegno et al. 2009). There were 2 distinct SSHa maxima for the Sitka eddy during the sampling period, with the northernmost SSHa showing clearly defined eddy core water and the southern lobe having a signature more closely related to transitional water (Rovegno et al. 2009). We identified the southern lobe as the Sitka secondary eddy core for distinction from the primary core water. Core water for both eddies was warmer and fresher than the surrounding transitional water and closely resembled the coastal end members sampled for these eddies. The sampled basin water was outside the influence of the mesoscale anomalies and was high nutrient, lower than expected chlorophyll (HNLC) water associated with the GoA basin.

The N and P concentrations of the Sitka and Kenai eddies were depressed at the surface with concentrations generally increasing at the pycnocline. The transitional water (especially the coastal edges) had enhanced N (~8 µM) and DIP (~1.2 µM) compared to the eddy core. The basin water had >10 µM N and ~1.3 µM DIP. Reactive Fe (bio-available) was depleted in the eddy core and basin waters compared to the shelf waters (Lippiatt et al. 2011). The surface
core water had <1 µM nitrate and <0.4 µM DIP. For both eddies, transitional water had stoichiometric ratios of 8.1 (N:P) indicating N deficiency (Tyrrell 1999, Mackey et al. 2007), while core water was still indicative of potential N limitation (10 to 12:1 N:P), but closer to conventional Redfield proportions.

Phytoplankton assemblages as related to water masses

The dominant phytoplankton for the nGoA in September 2007 were nanoplankton-sized (5 to 20 µm) centric diatoms. Each of the 4 water types identified displayed distinctly different phytoplankton community assemblages based on flow cytometry, FlowCAM imaging, and size-fractionated chl a. On average, the Sitka eddy transitional water had lower depth-integrated biomass (mean ± SD, 38.9 ± 3.4 mg m⁻² chl a, n = 4) compared to the eddy core and coastal water with concentrations of 39.1 ± 9.7 mg m⁻² chl a (n = 3) and 56.9 ± 38.2 mg m⁻² chl a (n = 4), while the HNLC basin water was 48.3 ± 0.9 mg m⁻² chl a (n = 2). The transitional water and the HNLC water depth-integrated biomass were significantly different from each other (p < 0.001, ANOVA). There were also lower rates of depth-integrated primary productivity within the transitional water (52.6 to 59.2 mg C m⁻² d⁻¹; n = 2) compared to the shelf and the basin water (67.8 to 170.0; n = 4 and 49.3 to 164.6 mg C m⁻² d⁻¹; n = 3, respectively; not significantly different). Dinoflagellates were never the dominant phytoplankton, but exhibited greater relative numbers in the transitional water compared to other water masses (as determined by FlowCAM data). Synechococcus was also identified in greater numbers within the transitional water (10⁵ cells ml⁻¹) compared to the other water masses (10³ to 10⁴ cells ml⁻¹) for both eddies (though the Kodiak eddy had 2-fold more Synechococcus than the Yakutat eddy). Picoplankton (0.6 to 5 µm) exhibited the highest biomass within the transition and basin water while nanoplankton (>20 µm) were the dominant size classes in the eddy core and coastal stations.

APA in the nGoA

Of the 32 nGoA stations, 23 exhibited ELF labeling (Table 1). APA was more intense at the chl a maximum regardless of the physical depth. Visual examination by microscopy determined that ELF-labeled diatoms were in the Sitka secondary eddy core and the Yakutat coast (Stns 16, 17, 30; Table 1), but not in any other stations. All other

<table>
<thead>
<tr>
<th>Station no.</th>
<th>Sample depth (m)</th>
<th>Description</th>
<th>ELF (µg/m²)</th>
<th>Relative APA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>Coastal, on shelf</td>
<td>+</td>
<td>0.92</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Coastal, on shelf</td>
<td>+</td>
<td>0.62</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>Coastal, on shelf</td>
<td>+</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>Coastal, on shelf</td>
<td>+</td>
<td>0.88</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>Sitka transitional, eddy edge</td>
<td>+</td>
<td>0.52</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>Sitka transitional, eddy edge</td>
<td>+</td>
<td>0.80</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>Sitka transitional, eddy edge</td>
<td>+</td>
<td>0.98</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>Sitka core</td>
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</tr>
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<td>11</td>
<td>25</td>
<td>Sitka core</td>
<td>+</td>
<td>0.12</td>
</tr>
<tr>
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<td>10</td>
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<tr>
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<td>25</td>
<td>Basin</td>
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<td>0.69</td>
</tr>
<tr>
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</tr>
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<td>0.96</td>
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<tr>
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<td>10</td>
<td>Basin</td>
<td>+</td>
<td>0.80</td>
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</table>
labeled cells were dinoflagellates or unidentified picoplankton, possibly haptophytes. Based on qualitative microscopic examination, dinoflagellates were more likely to express APA compared to either diatoms or unidentified picoplankton.

There were clearly varying amounts of APA with ELF labeling present in all 4 water masses. Multivariate tests and canonical correlation did not identify significant relationships between any measured environmental factors and APA (temperature, salinity, oxygen, inorganic nutrients, chl a, and fluorescence), while the 4 water masses were distinguishable based on the environmental data. Maximum APA was seen in the Kenai eddy source water (Stn 31) while the least APA was observed in both the Sitka eddy primary core and Kenai eddy core (Fig. 5). Stations identified as transitional or coastal water had greater APA compared to either eddy core or basin water. There was a weak, nearly significant relationship between APA and distance from the eddy core ($r^2 = 0.29, p = 0.07$). When samples were grouped by water mass type, transitional waters exhibited significantly higher APA values compared to eddy core waters ($p < 0.05$, 1-way $t$-test).

Size-fractionated APA determined that microplankton contributed to a significant portion of the APA at only the 2 Sitka eddy primary core water stations, a Kenai basin water station, and a coastal station near the Yakutat coast not related to either eddy (Stns 16, 17, 62, and data not shown; Fig. 6). For all other stations APA was documented in the picoplankton or nanoplanckton samples. APA and DIP concentrations were not significantly correlated for size fractions (Fig. 7A–C). There was no correlation between chl a and APA (Fig. 7D–F) or DIP and chl a concentrations (data not shown) for any of the size fractionated data.

**DISCUSSION**

**APA in the nGoA detected by spectrofluorometry**

ELF spectrofluorometric analysis detected APA throughout the nGoA, even though nutrient indices (dissolved inorganic nutrient measurements, stoichiometric ratios compared to the Redfield ratio) indicated that the phytoplankton should not be P-stressed (Fig. 5). The Sitka and Kenai eddy cores exhibited the lowest concentration of P, though on average, stoichiometric proportions were still indicative of N limitation rather than P limitation. Typically, phytoplankton APA has an inverse relationship with inorganic P (Cembella et al. 1984); therefore, one might expect that APA would be most prominent in the eddy cores where DIP was low. In contrast, our results show that APA was highest near the Yakutat coastline, the northwest transitional water of the Sitka eddy, and the western Kenai eddy transitional water, where nutrients were replete (Fig. 5). Compared to the nearby transitional water, APA was low in the eddy cores and HNLC basin water not influenced by the eddies. APA in the secondary Sitka eddy core was greater compared to either the primary Sitka core or the Kenai core water, but this water mass had hydrographic properties more closely attributed to transitional water than to core water (Rovegno et al. 2009).

The transitional waters had N:P ratios that would seemingly preclude expression of AP by phytoplankton, as sufficient DIP concentrations have been reported to stop gene transcription of AP (Ray et al. 1991), yet these stations demonstrated the greatest amount of APA. Dinoflagellates are strong AP producers (González-Gil et al. 1998), and the greatest APA signal was seen in the transitional water masses with a higher number of dinoflagellates. Regardless,
Dinoflagellates never outnumbered diatoms (i.e. diatoms were always the dominant taxon).

There was no correlation between DIP and APA in any of the size fractions (Fig. 7A–C), likely because of the diatom-dominated communities. We did not specifically determine the fraction of total chl a attributed to dinoflagellates, but hypothesize that APA would be correlated with dinoflagellate biomass as has been seen in other studies (Nicholson et al. 2006, Meseck et al. 2009). We did not measure DOP for this study; however, previous DOP estimates in the nGoA indicate that DOP is small relative to DIP (Ridal & Moore 1992). If DOP is rapidly recycled and is being actively hydrolyzed by phytoplankton as well as heterotrophic bacteria, then absolute concentrations of DOP may be low but still important for nutrient acquisition of some phytoplankton groups.

Size-fractionated APA

The transitional water stations, where APA was greatest, had proportionally more microplankton than other stations. The dominant size class at the Sitka eddy transition stations was pico-plankton, an almost equal mixture of picoeukaryotes and Synechococcus (10^3 cells ml^{-1} as determined by flow cytometry); the Sitka eddy transitional water was also the most consistently labeled with ELF, but there was low total biomass (chl a < 0.5 mg m^{-3}). We could not assess APA by flow cytometry, either due to the addition of ethanol during processing of the samples (Dignum et al. 2004, Štrojsová & Vrba 2006) or because labeling of Synechococcus by ELF is rare as some strains of cyanobacteria are not able to cleave P from DOP (Meseck et al. 2009). Unlike some strains of cyanobacteria, many diatoms (the dominant group in the nGoA during our study) do have the ability to hydrolyze DOP. However, diatoms often express AP under extremely low DIP concentrations compared to dinoflagellates (Dyhrman & Palenik 1999, Nicholson et al. 2006, Meseck et al. 2009). The small number of nano- and microplankton cells labeled by ELF made statistical microscopy counts of phytoplankton unreliable. This has been reported in other field studies and can be problematic when the dominant taxon is not the species producing AP (Dyhrman & Ruttenberg 2006, Nicholson et al. 2006, Duhamel et al. 2010). Regardless, based on size-fractionated APA and microscopy it appears nGoA AP production was most often expressed in the pico- and nano-plankton (dinoflagellates) rather than by the dominant diatom assemblage in the nano- and microplankton size classes.

Fig. 6. Size-fractionated relative alkaline phosphatase activity (APA) in arbitrary units (A.U.) for the northern Gulf of Alaska stations in the (A) Sitka eddy and (B) Kenai eddy. Most of the APA was associated with the pico- and nano-plankton size fractions.

Plankton: Pico Nano Micro

costal basin trans. prim. core. sec. core.

[Graph showing size-fractionated APA activity across different stations and depths, indicating the dominance of APA in pico- and nano-plankton fractions.]

35
Community APA

An increasing body of literature confirms the superior ability of dinoflagellates and haptophytes to produce AP compared to other phytoplankton (Cembella et al. 1984, González-Gil et al. 1998, Dyhrman & Palenik 1999, Nicholson et al. 2006, Meseck et al. 2009). While size-fractionation of APA samples has not been used to differentiate between micro- and nanoplankton, it has been used to separate Synechococcus and heterotrophic bacteria (Li et al. 1998, Duhamel et al. 2010) and the principle is the same: to differentiate APA within a phytoplankton assemblage to better understand group dynamics, rather than contributing P stress to the bulk plankton community. Nanoplankton dinoflagellates were synthesizing AP when microplankton dinoflagellates at the same station were not, suggesting that larger cells contain sufficient internal stores of P to avoid synthesizing AP (John & Flynn 2000). It is also possible these non-responding cells may resist synthesizing AP until their internal stores of P are very depleted (Meseck et al. 2009) and assimilation of DOP may be an adaptation based on the cell’s ability to store P internally.

In our study, P deficiency is clearly not attributed to the entire community of phytoplankton. APA in the

Fig. 7. Relative alkaline phosphatase activity (APA) in arbitrary units (A.U.) plotted against (A, B, C) dissolved inorganic P (DIP) and (D, E, F) chlorophyll a for (A, D) picoplankton, (B, E) nanoplankton, and (C, F) microplankton
nGoA appears to be a group- or species-specific response to P stress rather than a response characteristic of the entire assemblage, although we note that we do not have sufficient resolution to identify species-specific patterns of APA. This is consistent with the lack of obvious P limitation using indices such as the stoichiometric ratio (N:P was between 0 and 13 for the nGoA during our sampling period) or DIP concentrations, and classical nutrient criteria do not accurately portray what is happening within the phytoplankton assemblage. The shelf and transitional water in particular indicated N limitation, yet the highest APA was recorded in these water masses. Other coastal high-P systems such as Monterey and San Francisco Bay, California (Nicholson et al. 2006), Tokyo Bay Japan (Kobori & Taga 1979), and off NW Africa (Sebastián et al. 2004) also exhibit APA when classic P deficiency is not present. Furthermore, recent culture studies contradict the classical idea that uptake of DOP is based on a lack of DIP in the media or seawater. APA was detected in uni-algal cultures of 5 species of phytoplankton grown in DIP concentrations >10 µM (Meseck et al. 2009), which is >10x the amount of P we recorded in the nGoA. This study reinforces the idea that AP production is group- or species-specific. Individual cells may also exhibit luxury uptake or internal P pools resulting in differential P limitation under the same environmental conditions (Rengefors et al. 2003), while a possible lag in AP production in response to cellular P stress (Litchman & Nguyen 2008) may make snapshot determination of P stress in populations in dynamic P environments like the nGoA difficult to interpret.

As it appears that AP production is not always linked to depleted DIP concentrations, particularly in coastal environments, other mechanisms for AP regulation should be explored. During our study of the Kenai and Sitka eddies, there was little or no mixing between biological communities of the core water and basin water, except at the transition edges. The transition water for the eddies is a mixture of Fe-deplete, HNLC basin water and Fe-replete, macronutrient-deplete water drawn off the coastal shelf (Okkonen et al. 2003, Crawford et al. 2005). CHl a is often transported off the shelf along with the core eddy water, wrapped around the edges of eddies and carried several hundred km into the interior of the GoA (Crawford et al. 2005, Whitney et al. 2005, Ladd et al. 2007). The original core water of the eddies characterized by this study is similar in physical, nutrient, and biological properties to putative source water on the shelf (Ladd et al. 2007, Rovegno et al. 2009)—but as the eddy ages there is nutrient mixing (Lippiatt et al. 2011) and biological properties change quickly, forming distinct communities of phytoplankton. It has been noted previously in laboratory studies that phytoplankton growth is retarded during periods of AP production (Litchman & Nguyen 2008). This corresponds with observations of transitional water where APA increased while biomass and primary productivity were minimal compared to water masses with little or no APA.

**CONCLUSION**

Conditions that influence regulation of AP by dinoflagellates are not well understood on a community level, and laboratory experiments to establish P threshold limits for AP production have only been conducted with uni-algal cultures (González-Gil et al. 1998, Dyhrman & Palenik 1999, Meseck et al. 2009). Laboratory studies indicate that APA is activated only when under P stress (González-Gil et al. 1998, Dyhrman & Palenik 1999, Dignum et al. 2004, Meseck et al. 2009), but our investigation and previous work by others indicate that AP is actively produced by dinoflagellates (Nicholson et al. 2006, Dyhrman 2008) or haptophytes (Ranhofer et al. 2009) in diatom-dominated assemblages, when P stress is not evident. It is unclear what role AP production plays in modulating the community structure in these mixed assemblages. Using classical nutrient indices, the phytoplankton communities in the nGoA should not have been P stressed, yet small dinoflagellates were clearly synthesizing AP. This suggests that it may be more informative to assess nutrient stress at a group or species level rather than at a bulk community level.

Nutrient concentrations (inorganic and organic) and CHl a measurements without corresponding APA data make the P status of the community difficult to interpret (Dyhrman & Ruttenberg 2006). The same can be said for measuring only dissolved APA (bulk enzyme assays), a common APA measurement, and assigning P status to a community without determining group- or species-specific contributions. ELF labeling has improved understanding of AP production in marine phytoplankton but it can be difficult to interpret for samples with low biomasses (Nicholson et al. 2006, Štrojsova & Vrba 2006). P status of eukaryotic communities in waters influenced by mesoscale eddies appears to be complex and variable; if APA is indicative of P stress, then some portions of the nGoA phytoplankton assemblage are likely P stressed under typical summertime conditions. The near-
ubiquitous (but low percentage) ELF labeling of cells throughout the nGoA, with enhanced APA in transn- 

tional waters, strongly suggests that a more nuanced 

approach to nutrient acquisition and limitation in 

these systems needs to be applied as part of future 

studies.

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CHAPTER 3: LACK OF EVIDENCE FOR DIRECT CONTROL OF THE
PHYTOPLANKTON COMMUNITY BY ZINC OR PHOSPHORUS IN COASTAL
CALIFORNIA

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Abstract

Zinc (Zn) can act as a co-factor for phosphatase enzymes, such as alkaline phosphatase (AP), which eukaryotic algae use to access phosphate (P) from dissolved organic phosphorus (DOP). An increase in available Zn may allow greater access to DOP and shift phytoplankton community composition toward species able to produce AP. Deck-board enrichment experiments were conducted with water collected off the coast of Big Sur, California to determine community response to the addition of a suite of nutrients, their effect on phytoplankton growth, and to explore Zn-P co-limitation for selected taxa within the phytoplankton community. Nutrient enrichments included nitrate (N), P, DOP, iron (Fe), and Zn. Biomass increased with addition of N, but there was no significant difference between the treatments and the control for nutrient drawdown, chlorophyll-a, growth rate, or total cell counts. AP was significantly higher than the control in the +P and +DOP +Zn incubations, but it was not correlated with any specific taxa or size fraction of chlorophyll-a. There were slight differences in the percent relative abundance of three major taxa (diatoms, dinoflagellates, nanoflagellates) depending on the enrichment, indicating a short-term shift in community structure. We did not find any indication of P-Zn co-limitation for any group of phytoplankton, and surmise that compared to N and Fe, addition of P, DOP, or Zn has limited influence on traditional indices for phytoplankton growth and biomass. However, availability of P and Zn may subtly influence the phytoplankton community in the high-nutrient, low-chlorophyll-a waters of coastal Big Sur, California.
1. Introduction

Phosphorus (P) is an essential nutrient for the growth of marine phytoplankton (Karl 2000), and can limit primary productivity in oligotrophic gyres (Björkman et al. 2000; Wu et al. 2000; Duhamel et al. 2011) and dynamic coastal environments such as the Bay of Biscay (Labry et al. 2002) and the Louisiana Shelf (Sylvan et al. 2006). Marine P is available to phytoplankton in two major reservoirs: dissolved inorganic P (orthophosphate; DIP) and various dissolved organic phosphorus (DOP) compounds (Cembella et al. 1984). DIP is assumed to be the P-fraction preferred by marine organisms, and is easily utilized, but, when not available, phytoplankton can rapidly become P-limited (Benitez-Nelson 2000). Phytoplankton circumvent P-limitation in a variety of ways. These include luxury uptake of DIP (Falkner et al. 1998; Ou et al. 2008; Mackey et al. 2012) and maximizing P-uptake transport efficiency (Moore et al. 2005; Orchard et al. 2009). When DIP is scarce, DOP is often the only available P-source.

DOP can account for as much as 80% of available marine P (Hoppe 2003; Suzumura et al. 2012), but it is not all biologically available (Björkman and Karl 1994). Alkaline phosphatase (AP) is a P-cleaving enzyme (Cembella et al. 1984), thought to be the most widely used enzyme by marine phytoplankton and heterotrophic bacteria to hydrolyze many different DOP compounds (Huang and Hong 1999; Labry et al. 2002; Duhamel et al. 2010; 2011). The DOP pool is varied but comprised of two main groups: phosphoesters (~75%), which are used by marine organisms with AP, and less labile phosphonates (~25%), which are used by organisms with C-P utilization enzymes (Clark et al. 1998; Kolowith et al. 2001; Luo et al. 2011). The prevailing theory, supported by culture and in situ studies (González-Gil et al. 1998; Dyhrman and Palenik 2001; Sylvan
et al. 2006; Meseck et al. 2009; Duhamel et al. 2010), attributes DOP utilization when DIP is not in Redfield ratios with N. Contrary to culture and some in situ studies it appears DOP utilization may be more closely related to species’ stoichiometric nutrient ratio than DIP availability (Li et al. 1998; Nicholson et al. 2006; Duhamel et al. 2009; Peacock and Kudela 2013). The utilization of DOP by eukaryotic phytoplankton may therefore be responsible for subtle changes in phytoplankton composition (Shaked et al. 2005; Nicholson et al. 2006; Peacock and Kudela 2013).

AP and other phosphatases are metalloenzymes with Zn$^{2+}$, Ca$^{2+}$, or Mg$^{2+}$ as co-factors (Cembella et al. 1982). Relatively little is known about eukaryotic AP (Lin et al. 2012a), but the handful of AP enzymes that have been sequenced most often have a Zn$^{2+}$ metal co-factor (Lin et al. 2011; Sun 2011; Lin et al. 2012a; 2013). Zn is also an important trace metal in the regulation of silicic acid (Si), N, (Franck et al. 2003) and carbon (C) uptake (Morel et al. 1994; Tortell et al. 2000). Zn availability may be particularly important for diatoms (Tortell et al. 1997; 2000). It has been hypothesized that Zn can be limiting in oligotrophic environments (Anderson et al. 1978; Morel et al. 1994; Sunda and Huntsman 1995; Lohan et al. 2002), and coastal environments with decreased CO$ _2$ (Morel et al. 1994; Sunda and Huntsman 1995; Cullen 1999; Varela et al. 2011; Xu et al. 2012). Zn also may be limiting in coastal regions with a narrow shelf where high-nutrient low-chlorophyll-a (HNLC) conditions exist (Franck et al. 2003), but direct evidence for Zn-limitation is conflicting, as the response to Zn is not consistent across experiments (Lohan et al. 2005; Croot et al. 2011). Zn is typically in the subnanomolar range for the open ocean (Bruland 1980; Lohan et al. 2002), and has been found to limit phytoplankton growth in cultures at picomolar concentrations (Anderson et
al. 1978; Morel et al. 1994; Sunda and Huntsman 1995). At present there have been few Zn-limitation experiments (Croot et al. 2011 and references therein), but experiments conducted in HNLC waters have only minimally stimulated phytoplankton growth, slightly increasing chlorophyll-a (Crawford et al. 2003; Coale et al. 2003; Lohan et al. 2005; Leblanc et al. 2005; Shaked et al. 2006). This has led to the hypothesis that similar to DOP, additions of Zn may lead to shifts in community composition rather than stimulation of total biomass (Lohan et al. 2005; Shaked et al. 2006).

Although phytoplankton nutrient dynamics are often described as limited by a single nutrient (i.e. Liebig limitation) some HNLC regions exhibit co-limitation by Fe and Si (Boyd et al. 1999; Hutchins et al. 2001; Franck 2000). Co-limitation of Zn and P has similarly been observed in cultures of the coccolithophore Emiliania huxleyi, one of the most efficient eukaryotic AP producers that utilize a Zn co-factor (Riegman et al. 2000). The utilization of DOP facilitated by Zn has been suggested as a mechanism for alleviating P-limitation. Shaked et al. (2006) speculated that phytoplankton with less efficient AP production become Zn-P co-limited faster than E. huxleyi, providing a mechanism for shifts in community composition controlled by the availability of both Zn and P.

Zn-limitation, in theory, should decrease growth in phytoplankton because it is also a co-factor for carbonic anhydrase, which is used in uptake and assimilation of inorganic C (Anderson et al. 1978; Morel et al. 1994). Yet, laboratory studies have found that coastal diatoms are not limited at coastal Zn concentrations, nor are oceanic diatoms limited at oceanic Zn concentrations, either through adaptation to ambient Zn concentrations, or use of Co$^{2+}$ or Mg$^{2+}$ in place of Zn (Sunda and Huntsman 1992; 1995).
Along the Big Sur coast of central California Franck et al. (2003) found that the addition of Zn to natural waters increased the uptake of both N and Si in diatoms, but not in two other HNLC regions. Studies in Monterey Bay (Nicholson et al. 2006; Mackey et al. 2012; Peacock and Kudela 2012) and the Gulf of Alaska (Shaked et al. 2006; Peacock and Kudela 2013) have indicated that, as a whole, these areas are not P-limited, yet some phytoplankton groups (dinoflagellates and coccolithophores) may have a greater P-requirement and utilize DOP more readily than diatoms through the production of AP. Similarly, the addition of Zn, or Zn and DOP, even when Zn and P are not stoichiometrically limiting, may alter the community composition by selecting for species that produce AP. In this study we conducted incubation enrichment experiments with a variety of nutrient treatments to determine if Zn-P co-limitation is detectable in waters off the coast of Big Sur and whether a subsequent shift in community composition was apparent, to directly test these hypotheses.

2. Methods

2.1 Study site. Shipboard incubation experiments were conducted from 28 August to 1 September 2011 aboard the R/V Point Sur. Water was collected for 4 hours over an 11 km transect 2-3 km off the coast of Big Sur, California (36.20° N, 121.79° W to 36.13° N, 121.70° W). The ship travelled at 3 knots during collection, repeating the same transect twice (Fig. 1).

2.2 Trace metal clean procedures and experimental setup. Seawater was collected 2 m below the surface using a trace metal clean surface tow fish system pumped into a Class-100 clean area (Bruland et al. 2005). Whole seawater was collected in 4-L low-density polyethylene (LDPE) flexible cubic containers (“cubitainers”; VWR Canlab).
Cubitainers had been filled with 6N hydrochloric acid (HCl) for more than three weeks, rinsed thoroughly with Milli-Q deionized water (>18 MΩ), filled with 0.1% quartz-grade acetic acid for two weeks, then rinsed with whole seawater from the pump five times prior to sampling. Cubitainers were filled simultaneously at two sampling clean areas, and the water was not pre-filtered to exclude grazers. The collection period did not exceed four hours. It is likely there were some differences in the species composition and small changes in nutrient concentration. Therefore to minimize bias, 56 cubitainers were randomly assigned to seven treatments and the control. Initial samples were taken at the start and end of the sampling period and homogenized for analysis.

The experiment included four time points in addition to the initial sample, with each time point performed using a fresh cubitainer to limit contamination. Treatments included addition of N (as NaNO₃), Fe (as FeCl₃), P (as NaPO₄), Zn (as ZnCl₂), or DOP (as α-glycerophosphate; C₆H₉O₆P) in different combinations (Table 1). Each treatment consisted of eight cubitainers (four time points sampled in duplicate) and were as follows: unaltered controls, 20 µmol L⁻¹ N (+N), 5 µmol L⁻¹ P (+P), µmol L⁻¹ DOP (+DOP), 5 nmol L⁻¹ Zn (+Zn), 5 µmol L⁻¹ P and 5 nmol L⁻¹ Zn (+P +Zn), 5 µmol L⁻¹ DOP and 5 nmol L⁻¹ Zn (+DOP +Zn). All amended cubitainers included the addition of 5 µmol L⁻¹ N and 3 nmol L⁻¹ Fe as experiments from the same area on a previous cruise were depleted of N after only 1 d (data not shown) and to alleviate potential iron limitation from this HNLC region (Hutchins et al. 1998; Bruland et al. 2001; Biller and Bruland, in review) since this experiment was to investigate the effects of Zn and P addition, not Fe-limitation. Each cubitainer was triple-sealed with electrical tape and incubated until the entire cubitainer was sampled at 1.4, 2.4, 3.4, and 4 d. The cubitainers
were incubated in on-deck incubators supplied with surface seawater to maintain ambient temperature (~12°C). Neutral density screening provided shading to ~50% of the ambient light level, estimated to correspond to the irradiance level at the depth of collection. Before sampling, cubitainers were thoroughly scrubbed under filtered seawater to remove any contaminant algae on the outside of the cubitainer.

2.3 Nutrients and trace metals. All nutrient samples were analyzed with a QuickChem Lachat 8000 using colorimetric methods (Smith and Brogen 2001a,b; Knepel and Brogen 2002). Initial samples for dissolved N (nitrate + nitrite), P, and Si were measured onboard after filtration with a 0.4 µm polycarbonate (PC; Poretics) filter in near-real time. Nutrient samples from all other time points were filtered with a 0.7 µm GF/F filter (Whatman) and stored at -20°C until analysis. Samples for trace metals were filtered using acid cleaned 0.4 µm Nulepore PC filters. Initial concentrations of Fe were measured onboard inside a portable analytical clean lab using a flow injection method (Lohan and Bruland 2006). Samples for Zn analysis were acidified to ~1.8 pH with quartz distilled 6N HCl until post-cruise analysis (Biller and Bruland 2012).

2.4 Alkaline phosphatase collection and analysis. Incubated water samples (between 0.5 – 2 L) from each time point were processed for AP activity (APA) as described by Peacock and Kudela (2012). Samples were prepared for analysis with ELF (Endogenous Detection Kit Invitrogen #E6601; (González-Gil et al. 1998; Dyhrman and Palenik 1999; Peacock and Kudela 2012), and 70-100 µL of sample was analyzed on a 96-well opaque plate with a M2e SpectraMax spectrofluorometer internally standardized with fluorescein isothiocyanate (FITC; 3 fmol well⁻¹). Intensity was measured in arbitrary units and normalized to chlorophyll-a (Peacock and Kudela 2012).
2.5 Domoic acid collection and analysis. Domoic acid (DA) samples were collected on 0.7 GF/F filters (Whatman), stored in liquid nitrogen during the cruise, and then transferred to -80 °C until analysis. Samples were analyzed following the method of Wang et al. (2007) modified as described in Lane et al. (2010).

2.6 Chlorophyll-a and cell counts. Chlorophyll-a samples were collected onto 0.7 GF/F filters (bulk chlorophyll) for all time points, 5 µm PC filters for all time points, and 20 µm PC filters for the initial time point and at 2.4 and 4 d. Bulk chlorophyll-a was collected in duplicate from each cubitainer, while size-fractions were collected without replication because of volume constraints. Samples were extracted in 90% acetone for 24 hours at -20 °C and analyzed onboard using a Turner Designs 10-AU fluorometer with a non-acidic method (Welschmeyer 1994). Chlorophyll-a samples were averaged for each treatment and are reported with standard deviation. Phytoplankton samples (50 mL) of whole water were collected at each time point for each treatment, preserved with 0.5 mL of non-acid Lugol’s iodine, and stored in the dark at 4°C. Samples were counted following the Utermöhl method (Utermöhl 1958; Edler and Elbrächter 2010) using either a 10 or 50 mL settling chamber. Counts were performed for diatoms, dinoflagellates, nanoflagellates (non-dinoflagellates), and dominant genera on a Zeiss Axio Imager microscope. Greater than 400 cells were enumerated for each sample, three times for each cubitainer, then averaged between treatments and reported with standard deviation.

3. Results

3.1 Chlorophyll-a. Bulk chlorophyll-a was not significantly different for any of the treatments compared to the control. The peak bulk chlorophyll-a concentrations were recorded at 3.4 d (10.6 ± 4.4 - 36.9 ± 9.8 µg L⁻¹) for all treatments except the +P +Zn
treatment, which peaked at 2.4 d with 27.6 ± 2.5 µg L\(^{-1}\) (Fig. 2). The greatest increase compared to the initial concentration of 3.5 µg L\(^{-1}\) was observed in the +N treatment (36.9 ± 9.8 µg L\(^{-1}\)). The control exhibited a 6-fold increase (21.5 ± 8.7 µg L\(^{-1}\)) in comparison to the initial concentration, and the +DOP treatment exhibited the least increase, 3-fold with 10.5 ± 4.4 µg L\(^{-1}\) of chlorophyll-a. Figure 2b-d presents size-fractionated chlorophyll-a data. Peak chlorophyll-a is likely underestimated for the bulk of the treatments in the size-fractionated samples given the limited sampling. The greatest increase compared to the initial concentration of 1.8 µg L\(^{-1}\) in the <5 µm size class was also observed in the +N treatment (9.6 ± 3.1 µg L\(^{-1}\)). The least increase was exhibited in the +Zn treatment with 2.3 ± 0.3 µg L\(^{-1}\) (Fig. 2b). The trends for the 5-20 µm and the >20 µm size class were similar and the greatest increase was exhibited in the +DOP +Zn treatment for the 5 - 20 µm size fraction with 3.5 ± 4.4 µg L\(^{-1}\), a 6-fold increase from the initial concentration of 0.6 µg L\(^{-1}\), and the least increase was exhibited in the +Zn treatment with 0.5 ± 0.02 µg L\(^{-1}\), a decrease of ~0.1 µg L\(^{-1}\) (Fig. 2c). The >20 µm fraction had the greatest increase in the +P +Zn treatment with 17.7 ± 8.2 µg L\(^{-1}\), a 17-fold increase from the initial concentration of 1.05 µg L\(^{-1}\), and the least increase for the >20 µm size-fraction was also exhibited in the +Zn treatment with 2.1 ± 0.9 µg L\(^{-1}\) (Fig. 2d).

Figure 3 presents the relative abundance of size-fractionated chlorophyll-a data expressed as the percentage of the total occurring in each sample. The initial sample had 51.4% in the <5 µm fraction, 18.3% in the 5 - 20 µm fraction, and 30.3% in the >20 µm fraction. For the +P, +DOP, and +P +Zn treatments the <5 µm fraction remained relatively unchanged (48.4 – 56.6%; Fig. 3c-d,f), but increased for the +Zn (59.7%; Fig. 3d), +N (62.2%; Fig. 3b), and the control (72.8%; Fig. 3a). The 5 - 20 µm fraction
remained relatively unchanged (15.7%) for the +P treatment (Fig. 3c), but was
dramatically and steadily reduced for all other treatments and the control by 4 d (1.7 –
9.9%; Fig. 3a-b, d-g). The >20 µm fraction remained relatively unchanged for most of the
treatments (31.4 - 35.2 %) at 4 d (Fig. 3b-e, g), except the +P +Zn treatment increased to
49.9% (Fig. 3f), and the control decreased to 21.6% (Fig. 3a).

3.2 Macronutrients. Nutrient drawdown for N, P, and Si was not significantly
different for any of the treatments compared to the control. Figure 4 presents
macronutrient data for all of the experiments. At 4 d, N in all treatments was depleted to
<1 µmol L⁻¹ compared to the initial concentration of ~16.9 - 35 µmol L⁻¹, except for the
+N treatment with 1.7 ± 1.3 µmol L⁻¹, and the +DOP +Zn treatment with 1.3 ± 0 mol L⁻¹
(Table 1). At 3.4 d N in the +DOP +Zn treatment was not detected, and the apparent
increase at 4 d is due either to remineralization or to variability introduced in the random
assignment of cubitainers (Fig. 4a). By the fourth day P was ≤0.1 µmol L⁻¹ for all
treatments compared to the initial concentration of 1.1 µmol L⁻¹, except for the +DOP
+Zn (0.6 ± 0.2 µmol L⁻¹), +P (3.6 ± 0.8 µmol L⁻¹), and +P +Zn treatments (4.8 ± 0.9 µmol
L⁻¹; Table 1; Fig. 4b). Si was <2 µmol L⁻¹ for all treatments at 4 d, except for the +P and
+DOP +Zn treatments (6.3 ± 6.1 and 10.8 ±3.1 µmol L⁻¹ respectively; Fig 4c).

3.3 Trace metals. Lohan et al. (2005) reported no significant adsorption for either
Zn or Fe (3.4% and 2.6% respectively after 8 d) onto cubitainer walls for an experiment
using similar methods, so we assume that adsorption was minimal. Zn is one of the most
contamination-prone trace metals, and there appears to have been some slight
contamination during this experiment. The initial concentration of Zn was 0.46 ± 0.23
µmol L⁻¹ from the beginning and end of the collection period. The initial Zn
concentration was not sampled from the cubitainers. The Zn samples at 1.4 d were on average ~1-1.5 nmol L\(^{-1}\) higher than we would expect from the initial concentration. Whether the contamination is from the cubitainers or the filtration of the subsamples is unclear. Four time points were eliminated due to clear contamination: both replicates of the +N treatment (24.9 and 8.2 nmol L\(^{-1}\)), one replicate of the +P +Zn treatment (15.1 nmol L\(^{-1}\)) at 1.4 d, and one replicate of +P (3.6 nmol L\(^{-1}\)) at 4 d.

Total dissolved Zn concentrations generally decreased for all treatments as shown in Figure 5. The +Zn treatment exhibited the smallest decrease (0.2 nmol L\(^{-1}\)) and the +P treatment exhibited the greatest decrease (1.8 nmol L\(^{-1}\); Fig. 4). The +P +Zn and +DOP +Zn treatments each exhibited 1.3 nmol L\(^{-1}\) drawdown over 4 d. All other treatments exhibited <1.0 nmol L\(^{-1}\) drawdown over 4 d (Fig. 5). The drawdown for all treatments was not significantly different from the control.

3.4 Growth rates. Growth rates, presented in Figure 6, were calculated from linear regressions of the semi-log transformed chlorophyll-a data for the first 3.4 d of the experiment. The +N treatment exhibited the fastest growth rate (0.82 ± 0.14 d\(^{-1}\)) while the +DOP treatment had the slowest (0.27 ± 0.02 d\(^{-1}\)). For <5 µm size fraction the fastest growth rates were the +N and the +P treatments (0.68 ± 0.18 and 0.68 ± 0.08 d\(^{-1}\) respectively) and the slowest was the +Zn treatment (0.04 ± 0.05 d\(^{-1}\)). For the 5 - 20 µm size fraction the fastest growth rate was the +DOP +Zn treatment (0.46 ± 0.73 d\(^{-1}\)) and the slowest was +Zn (-0.13 ± 0.02 d\(^{-1}\)). The >20 µm size fraction exhibited the fastest growth rate (for all the size fractions) in the +P +Zn treatment (1.18 ± 0.10 d\(^{-1}\)) and the +Zn treatment was the slowest (0.33 ± 0.11 d\(^{-1}\); Fig. 6).
3.5 Broad taxonomic groups and dominant genera, cell counts, and relative percent abundance. The most abundant taxonomic group for all the treatments on all days was diatoms (Table 2). The centric diatoms Chaetoceros and Skeletonema were similarly abundant and the two dominant genera for the control, +Zn, and +P +Zn treatments at 3.4 d, which corresponded to the peak cells mL⁻¹. The dominant genus for the +N treatment was the centric diatom Coscinodiscus. The pennate diatom Pseudo-nitzschia was the dominant genus for the +P and +DOP treatments, and split almost evenly with Skeletonema for the +DOP +Zn treatment. Most dinoflagellates and flagellates were rare (<20 per 400 counted cells) and were never the dominant taxa (Table 2).

Cell count data from the initial sample and from 3.4 d for all treatments are presented in Table 2. Total cells ranged from 205 cells mL⁻¹ for the initial sample to 2339 ± 523 cells mL⁻¹ for the +P +Zn treatment at 2.4 d. All total cell counts for each treatment peaked at 3.4 d except for the +P +Zn treatment, which peaked at 2.4 d, and the +DOP treatment, which peaked at 4 d. There was no statistical difference between the control and the other treatments for cell counts.

Figure 7 presents the relative abundance of taxonomic groups for all of the experimental treatments. The initial relative percent abundance of cells was 87.9% diatoms, 10.9% dinoflagellates, and 1.2% nanoflagellates. The +Zn treatment had the highest percentage of diatoms (92 ± 2% at 4 d; Fig. 7e) and the +DOP +Zn treatment had the lowest (52.9 ± 3.0% at 4 d; Fig. 7g). The +DOP +Zn treatment had the highest percentage of dinoflagellates (43.7 ± 1.7% at 4 d; Fig. 7g), and the +P treatment had the lowest (4.7 ± 0.4% at 4 d; Fig. 7c). The +DOP treatment had the highest percentage of
flagellates (12.6 ± 13.6% at 4 d; Fig. 7d), and the +Zn treatment had the lowest (0% at 2.4 d; Fig. 7e).

3.5 Alkaline phosphatase and domoic acid. The biomass from the filters for one replicate of +N and +DOP +Zn treatments at 1.4 d and one replicate of +P treatment at 4 d was too low (<0.6 µg L⁻¹ chlorophyll-a) to provide an accurate APA measurement, and were not included in the calculations. The +N, +P, +DOP, +Zn, and +P +Zn treatments exhibited their respective highest APA normalized to chlorophyll-a at 4 d, the control at 1.4 d, and the +P +Zn treatment at 3.4 d (Fig. 8). The +P treatment, with a 13-fold increase from the initial APA normalized to chlorophyll-a, and +DOP +Zn treatments were significantly different from the control on the fourth day (t-test; p<0.05). The control, +N, and +P +Zn treatment did not significantly increase in APA normalized to chlorophyll-a by 4 d. Most of the treatments had a similar ratio compared to the initial for the first 3.4 d, and then a dramatic increase at 4 d (Fig. 8).

The amount of particulate DA (pDA) per cell and growth rates for *Pseudo-nitzschia* were calculated and found to be not well correlated, but toxin generally increased with decreasing growth rate. Cellular pDA at 3.4 d and growth rates for *Pseudo-nitzschia* significantly increased in treatments with added Zn (+Zn, +P +Zn, and +DOP +Zn) compared to treatments without addition Zn (t-test; p<0.05).

4. Discussion

4.1 Macronutrients, Fe, and biomass response. Initially N was ~16.9 - 35 µmol L⁻¹ but by the fourth day was depleted to below detection limits (≤0.2 µmol L⁻¹) except for the control, +N, and +DOP +Zn treatments (Fig. 4a). These results are comparable to nitrate uptake rates seen in other upwelling systems (Dugdale and Wilkerson 1991). The
amount of N depleted was variable because of the different initial N concentrations, but essentially all available N was removed from the incubations by 4 d. In past studies, the addition of Fe enhanced N-uptake in the Gulf of Alaska (Banse 1991; Lohan et al. 2005; Boyd et al. 1996) and the Big Sur coast of California (Franck et al. 2003). Our experiments included 3 nmol L⁻¹ of Fe, and we expect that our N-drawdown rates were similarly enhanced. Interestingly, the rate of drawdown was fastest in the first 1.4 d, and the treatments with additional N and P (+N, +P, and +P +Zn) had 3-4 fold more N drawdown (9-11 µmol L⁻¹; Fig. 4b-c, f) compared to the control or other treatments (2-3 µmol L⁻¹; Fig. a, d-e, g). This suggests that the addition of P enhanced N drawdown (Inanikova et al. 2007) and that there could have been luxury uptake of N once additional P was available (Elrifi and Turpin 1985).

Similarly to N, P was depleted from 1.1 µmol L⁻¹ to below detection (≤0.02 µmol L⁻¹) for most treatments except the +P and +P +Zn treatments, both of which had an additional 5 µmol L⁻¹ added initially, as well as the +DOP +Zn treatment (Fig. 4b). Although there was ~4 µmol L⁻¹ residual P in +P and +P +Zn treatments, the drawdown was roughly twice that of the other treatments over 4 d. The stoichiometric ratio of N: P initially was 16: 1, suggesting that neither nutrient was limiting (Klausmeier et al. 2004). Drawdown for Si was similar for all the treatments except for the +P treatment, which was 1.5 times less than the other treatments (Fig. 4c). This did not appear to have an effect on the biomass, but may have influenced the community composition as the +P treatment had a greater percentage of flagellates compared to the other treatments, as well as fewer chain-forming diatoms (Fig. 7; Table 2).
The greatest increase in biomass was observed in the +N treatment, with changes in biomass generally inversely related to nutrient drawdown. The coast of Big Sur is characterized by its narrow shelf, HNLC water, and frequently Fe-limited biological community (Bruland et al. 2001). Fe is a co-factor for both nitrate and nitrite reductase (Milligan and Harrison 2000), and Fe-limited cells have decreased rates of N-uptake (Timmermans et al. 1994; Milligan and Harrison 2000). The initial concentration of Fe was 0.88 ± 0.13 nmol L\(^{-1}\), which is not normally limiting for the Big Sur coast, as the threshold for Fe-limitation was found to be \(\leq 0.3\) nmol L\(^{-1}\) (Bruland et al. 2001).

Regardless, prior enrichment experiments conducted in the same region, with \(~1\) nmol L\(^{-1}\) Fe and \(<10\) \(\mu\)mol L\(^{-1}\) N, exhibited clear signs of both Fe and N limitation with limited biomass increase during similar incubation experiments (data not shown). Therefore, we added additional N and Fe to all of our treatments, except the control, to better understand the synergistic or antagonistic effects that P, DOP, and Zn would have on the treatments in the absence of strong Fe- and N-limitation. The increase in biomass for the control was unexpected but may have been influenced by other factors, such as the exclusion of vertically migrating zooplankton, which have been hypothesized to control biomass accumulation (Martin et al. 1989; Coale 1991). The control may also have been influenced by differences in species composition or nutrient concentrations, based on our collection transect of 11 km, though the control and all treatment incubations were randomly assigned to minimize these differences.

Chlorophyll-a is commonly used as a proxy for both biomass and growth rate (Arrigo et al. 2000; Smith et al. 2000; Landry et al. 2001), as are primary productivity measurements (Cloern et al. 1995) and cell counts (Holligan et al. 1984), but each of
those proxies will provide different results, as they are fundamentally different measurements. For example, Landry et al. (2001) determined that estimates of net growth rate from chlorophyll-a and autotrophic cell counts were not in agreement. For this experiment we also found large variation between the growth rates based on chlorophyll-a and cell counts (Fig. 9). The correlation between total chlorophyll-a and total cells mL\(^{-1}\) was poor \((r^2 = 0.45)\), slightly better for total chlorophyll-a and total diatoms \((r^2 = 0.49)\) and uncorrelated for total chlorophyll-a and total dinoflagellates \((r^2 = 0.11)\). The >20 \(\mu\)m size fraction chlorophyll-a and total diatoms exhibited the highest correlation \((r^2 = 0.53)\). However, regardless of the metric used we did not find significant differences in bulk growth rates within or between treatments and the control.

4.2 Trace metals. Addition of Zn to phytoplankton assemblages in laboratory and field conditions have produced conflicting results as to whether it enhances N-, Si-, and C-uptake (Coale 1991; Sunda and Huntsman 1992; Crawford et al. 2003; Franck et al. 2003; Lohan et al. 2005). Lohan et al. (2005) observed no significant difference of N or Si drawdown with the addition of Zn alone or with Fe at Ocean Station Papa in the Gulf of Alaska (Crawford et al. 2003; Lohan et al. 2005). Other incubation experiments have recorded no increase in biomass, cell number, or cell concentration upon Zn addition (Franck et al. 2000; Coale et al. 2003). In HNLC waters in the Southern Ocean, two out of three experiments demonstrated increased uptake with the addition of both Fe and Zn compared to just Fe, but whether this is a synergistic effect between Fe and Zn or due to Fe is unclear (Franck et al. 2003). It is hypothesized that when input of Fe and Zn to surface waters is decoupled, as in the case of atmospheric dust that is deficient in Zn, phytoplankton would bloom and deplete Fe until the system became Zn-limited (Coale et
al. 2003). The experimental results presented here did not demonstrate any Zn-limitation despite the addition of Fe to all treatments, but the addition of Zn without an added P-source appeared to inhibit phytoplankton growth in the >20 µm size fraction (Fig 6). The only noticeable enhancement in the biomass and growth rate with the addition of Zn was in the 5-20 µm and >20 µm size fractions for the +DOP +Zn and +P +Zn, treatments, respectively. Comparing these results with the slow growth rate and low biomass of the +Zn treatment, the enhancement of biomass and growth rate are more likely due to the addition of a P-source in the +DOP +Zn and +P +Zn treatments. Our experiments did have slight contamination of Zn, but the trends present from the Zn drawdown are accurate and the 1-3 nmol L⁻¹ Zn concentration range determined in the experiments is comparable to upwelling waters from the central California Current (Biller and Bruland, in review) and stoichiometrically balanced by addition of N and Fe in concentrations equivalent to typical upwelling conditions. Zn was clearly drawn down in all experiments, with the greatest drawdown in the +P +Zn and +DOP +Zn treatments.

4.3 Nutrient limitation. In laboratory settings there is compelling evidence that there is a threshold for Zn-limitation (Sunda and Huntsman 1992; Morel et al. 1994; Sunda and Huntsman 1995), but the limited Zn-enrichment experiments in situ rarely show an increase in biomass or chlorophyll-a (Crawford et al. 2003; Coale et al. 2003; Lohan et al. 2005). Our study site does not appear to be Zn-limited and there was no significant difference between the treatments and the control for either chlorophyll-a, nutrient drawdown, or growth rate with additional Zn (Fig. 2,4,6). There is some evidence for Zn toxicity with the addition of ~ 10 nmol L⁻¹ in oceanic diatoms (Sunda and Huntsman 1992; Martin et al. 1993), but our incubations were well below that
threshold, and coastal diatoms in culture were not inhibited by Zn (Sunda and Huntsman 1992). Furthermore, the potentially inhibitory effects seen in the +Zn treatment were not noticeable in the +P +Zn and +DOP +Zn treatments.

The Big Sur coast is thought to be predominantly Fe-limited and secondarily N-limited (Hutchins et al. 1998; Bruland et al. 2001; Franck et al. 2003; Biller et al. submitted). Despite the addition of both Fe and N, our incubations do not appear to be limited for P (DIP or DOP) or Zn, or a combination of P-Zn co-limitation, in contrast to previous reports for Zn (Crawford et al. 2003; Lohan et al. 2005; Shaked et al. 2006), P (Kudela and Dugdale 2000; Dyhrman and Ruttenberg 2006; Nicholson et al. 2006), and DOP (Nicholson et al. 2006) for other regions. The additions of P, DOP, and Zn in this study had no significant effect on biomass or growth rate, but did have a slight effect on community composition (Table 2, Fig. 7). The addition of P, DOP, and Zn caused the phytoplankton community to shift toward a higher percentage of dinoflagellates, though every treatment was still diatom-dominated.

4.4 APA, DA, and community composition. Chlorophyll-a normalized APA increased in all the treatments with time, which is to be expected as DIP concentrations were depleted over the course of the experiment (Fig. 8). Also, by day 4, N was depleted to zero for most of the treatments, and it is possible that communities were becoming mixotrophic and utilizing dissolved organic matter to supplement their nutritional requirements. The APA for the +P and +DOP +Zn treatments were significantly different at 4 d from the control. To our knowledge, this is the first reported field enrichment experiment including both DOP and Zn. Laboratory experiments with *E. huxleyi* observed no significant difference in APA between treatments with low DIP and DOP,
but found high DIP inhibited APA (Shaked et al. 2006). Our +P treatment exhibited the greatest APA at 4 d, and also had ~ 4 \( \mu \text{mol L}^{-1} \) of residual DIP. While 4 \( \mu \text{mol L}^{-1} \) of DIP in laboratory culture usually inhibits AP production (Dyhrman and Palenik 2001; Meseck et al. 2009) fieldwork observations imply that APA may be closely related to the composition of the community rather than to the concentration of available DIP (Nicholson et al. 2006; Shaked et al. 2006; Peacock and Kudela 2013).

Flagellates were in low abundance for all treatments; only the +P and +DOP treatments exhibited a strong response for this group, almost entirely composed of *Heterosigma*. Raphidophytes, including *Heterosigma*, do not produce AP and have not been found to utilize phosphomonoesters to supplement their P needs (Luo et al. 2009; Lin et al. 2013). Therefore, the increased flagellates observed in the +P treatment would not contribute to the increased APA we observed at 4 d of the experiment (Fig. 8). The eukaryotic APA in the central California Current would likely be associated with dinoflagellates (Nicholson et al. 2006; Mackey et al. 2012; Peacock and Kudela 2012), so the observed APA in the +P treatment is surprising. The +P treatment does not have a greater percentage of dinoflagellates (compared to the other treatments) and less than the +DOP and +DOP +Zn treatments (Fig. 7; Table 2). Some diatoms do produce AP, including *Pseudo-nitzschia*, *Chaetoceros*, and *Skeletonema* (Nicholson et al. 2006; Meseck et al. 2009; Peacock and Kudela 2012; Lin et al. 2013). The +P treatment did have increased *Pseudo-nitzschia* and *Chaetoceros* compared to the other treatments, but diatom APA is not usually ubiquitous within a sample, and we would not expect all of the diatoms capable of producing AP to be active (Nicholson et al. 2006; Peacock and Kudela 2012). A unique application of the spectrofluorometric APA assay is the ability to
assess relative intensity (Peacock and Kudela 2012). The high APA observed in the +P treatment could therefore be caused by cells producing many AP enzymes, rather than many cells having APA, which would account for the seemingly contradictory low percentage of dinoflagellates and elevated APA.

The elevated cellular quota of pDA in *Pseudo-nitzschia* cells exhibited in the treatments with additional Zn is expected, as decreased growth rates are negatively correlated with cellular pDA (Pan et al. 1996a; b). The total growth rates for the treatments with additional Zn were on average lower than the growth rates for the treatments without Zn, as were our growth rates for *Pseudo-nitzschia* cell counts specifically. Rhodes et al. (2006) observed that the addition of Zn increased cellular pDA in cultures of *P. australis*, though cell counts barely increased compared to the control. Our results are similar, where the addition of Zn significantly increased the cellular pDA compared to the non-Zn treatments, but cell counts were not enhanced (Table 2). To our knowledge, this is the first reported field study reporting increased domoic acid toxin production with the addition of Zn.

4.6. **Implications for Zinc addition as a stimulant for community shift.** Very few eukaryotic AP gene sequences have been published, and recent results suggest that APs use varied metal co-factors. Marine bacteria often utilize $\text{Ca}^{2+}$ instead of $\text{Zn}^{2+}$ as a co-factor for AP (Lin et al. 2012b; a), but it is widely believed that most eukaryotic AP use $\text{Zn}^{2+}$. For example, *E. huxleyi*, the first eukaryotic phytoplankton with sequenced AP genes (Xu et al. 2006; 2012), has an atypical AP (EHAP1) with $\text{Zn}^{2+}$ as a co-factor. However, other eukaryotic phytoplankton can produce multiple putative APs simultaneously, and not all of them use $\text{Zn}^{2+}$ as a co-factor but may substitute $\text{Ca}^{2+}$ or
The genera we observed in our incubations have the capability to produce AP (Luo et al. 2009; Meseck et al. 2009; Lin et al. 2013), and we observed APA in all of our treatments (Fig. 8). Given the recent discovery of non-Zn APs, the link between Zn availability and APA may be less obvious than previously hypothesized.

5. Conclusion

The addition of N, P, DOP, and Zn to the water off the coast of Big Sur did not significantly change biomass, growth rate, or cell counts relative to the control. Furthermore, there was no shift in the dominant taxa with the addition of DOP and Zn. The addition of P, DOP and Zn caused slight shifts in species composition toward a higher percentage of dinoflagellates, but this was most likely a short-term response, as was previously seen in studies when the P-fraction shifted toward more available DOP. There was a statistically significant increase in APA in the +P and +DOP +Zn, but these are not correlated to the percentage of dinoflagellates. The slight shifts in community structure seen at Ocean Station Papa in the Gulf of Alaska with additional Zn (Crawford et al. 2003) were reflected in this study, but we were not able to force a shift from the dominant taxa (diatoms) to dinoflagellates, which are better able to utilize DOP. Zinc plays a role in more than 300 enzymes (Anderson et al. 1978) that both diatoms and dinoflagellates use, and until further APs are identified in eukaryotic phytoplankton there is no way to determine if Zn is the primary co-factor for AP in this study. The recent determination of eukaryotic AP pathways hint at complicated metal interactions, and the future of understanding community APA may lie in deciphering the structure of eukaryotic AP. We conclude that the weak and circumstantial support for Zn or P-Zn co-
limitation in coastal waters was not corroborated for the Big Sur region, which remain strongly regulated by N and Fe availability. Nonetheless, more subtle changes in response to addition of Zn and P, including apparent enhancement of domoic acid by *Pseudo-nitzschia*, suggest that multiple nutrient interactions exist and are ecologically relevant, despite the lack of strong control on community composition and biomass.
References


Tables

Table 1. Nutrient additions to treatments at experimental set-up

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nutrient Additions</th>
<th>NaNO₃ (µmol L⁻¹)</th>
<th>NaPO₄ (µmol L⁻¹)</th>
<th>C₆H₉O₆P (DOP) (µmol L⁻¹)</th>
<th>FeCl₃ (nmol L⁻¹)</th>
<th>ZnCl₂ (nmol L⁻¹)</th>
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Table 2. Cell counts with standard deviation for broad taxonomic groups and dominant genera for the initial time point and 3.4 d for all treatments. Rare counts are <20 cells per 400 counted. nd = not detected.
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<th>Phytoplankton Taxa</th>
<th>Initial (cells mL(^{-1}))</th>
<th>Control (cells mL(^{-1}))</th>
<th>+N (cells mL(^{-1}))</th>
<th>+P (cells mL(^{-1}))</th>
<th>+DOP (cells mL(^{-1}))</th>
<th>+Zn (cells mL(^{-1}))</th>
<th>+P +Zn (cells mL(^{-1}))</th>
<th>+DOP +Zn (cells mL(^{-1}))</th>
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<td>Chaetoceros</td>
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**Figure Legends**

**Figure 1.** Location and 11 km transect (blue line) of waters collected near Big Sur, CA for enrichment experiments.

**Figure 2.** Chlorophyll-a for (A) bulk chlorophyll-a, (B) <5 µm size fraction, (C) 5 – 20 µm size fraction, and (D) 20 µm size fraction. Error bars represent duplicate cubitainers.

**Figure 3.** Relative percent abundance of size fractionated chlorophyll-a for at 0, 2.4, and 4 days. (A) control (B) +N, (C) +P, (D) +DOP, (E) +Zn, (F) +P +Zn, (G) +DOP +Zn

**Figure 4.** Macronutrients for all treatments over 4 days. (A) Nitrate, (B) phosphate, and (C) silicic acid

**Figure 5.** Zinc for all treatments over 4 days.

**Figure 6.** Growth rates for all treatments, calculated from chlorophyll-a size fractions calculated from linear regressions of the semi-log transformed chlorophyll-a data for the first 3.4 d of the experiment.

**Figure 7.** Relative abundance of taxonomic groups for all treatments. (A) control (B) +N, (C) +P, (D) +DOP, (E) +Zn, (F) +P +Zn, (G) +DOP +Zn

**Figure 8.** APA normalized to chlorophyll-a for all treatments. The +P and +DZn treatment are significantly different from the control (t-test, p<0.05).

**Figure 9.** Comparison of growth rates for chlorophyll-a and cell counts calculated from linear regressions of the semi-log transformed chlorophyll-a data for the first 3.4 d of the experiment.
Figures

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
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Figure 7
Figure 8
Figure 9
CHAPTER 4: EVIDENCE FOR ACTIVE VERTICAL MIGRATION OF AKASHIWO SANGUINEA EXPERIENCING IRON AND NITROGEN LIMITATION

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\textbf{Running Head:} Akashiwo sanguinea acquires Fe and N during vertical migration
Acknowledgements

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Abstract

Vertical migration and subsequent assimilation of iron and nitrate at depth by the dinoflagellate *Akashiwo sanguinea* was investigated using laboratory vertically-stratified water columns. Active migration was observed in both nitrate-replete and nitrate-deficient experiments. Stable isotopes of iron ($^{57}$FeCl$_3$) and nitrogen (Na$^{15}$NO$_3$) were assimilated from the bottom 0.4 m of the column during migration, with significant enrichment of particulate samples collected from the surface compared to the initial *A. sanguinea* culture. Under nitrogen-deficient conditions, there was a significant decrease in total nitrogen over 120 hours, while both experiments exhibited > 20 µmol L$^{-1}$ m$^{-2}$ drawdown of nitrate. These experiments are the first to demonstrate dinoflagellate assimilation of iron at depth. Harmful algal blooms (HABs) of *A. sanguinea* and other vertically migrating dinoflagellates are often associated with macro- and micronutrient depletion in surface waters; assimilation of iron as well as nitrate at depth could be an important ecological advantage in environments where light and nutrients are spatially separated.
1. Introduction

Blooms of the dinoflagellate *Akashiwo sanguinea* are periodic and widespread along the west coast of the United States (Horner et al. 1997; Ryan et al. 2010; Lewitus et al. 2012), Ireland (O'Boyle and McDermott 2012), the Black Sea (Gómez and Boicenco 2004), and Hong Kong (Lu and Hodgkiss 2004). Until recently, *A. sanguinea* was known as *Gymnodinium sanguineum* Hirasaka, and is synonymous with *Gymnodinium splendens* Lebour and *Gymnodinium nelsonii* Martin (Daugbjerg et al. 2000). *A. sanguinea* is an autotrophic dinoflagellate classified as a harmful alga (Trainer et al. 2010) and has been linked to the mortality of abalone larvae (Botes et al. 2003) and coral bleaching (Litchman et al. 2002), although the exact mechanism for these impacts is not clear. These blooms have also been coupled to seabird deaths by the production of large amounts of mycosporine-like amino acids released during bloom senescence. This release, in combination with wind-driven turbulence, creates a surfactant-like foam that can interfere with the waterproofing capacity of seabird feathers leading to death by hypothermia (Jessup et al. 2009; Du et al. 2011).

*A. sanguinea* blooms are initiated under various physical-chemical conditions (Matsubara et al. 2007; Ryan et al. 2010; Du et al. 2011) and significant bloom biomass often accumulates when the water column is vertically stratified. Vertical migration typically occurs under conditions with low surface nutrients (Ryan et al. 2010; Du et al. 2011) and is a common, competitive strategy employed by dinoflagellates (Smayda 2010 and references therein) in a vertically stratified water column to acquire nutrients from depth (Eppley et al. 1968; Lieberman et al. 2004; Kudela et al. 2010). This has been documented for natural populations of *A. sanguinea* (Keifer and Lasker 1975; Ryan et al.
Vertically migrating phytoplankton incorporate nitrate (Cullen and Horrigan 1981; Katano et al. 2011), ammonium (Hall and Pearl 2011), and phosphorus (Watanabe et al. 1988; 1991) from depth, providing a competitive benefit over non-motile phytoplankton (Smayda 2010).

Nutrient data suggest that Monterey Bay, California, USA is not only episodically nitrate-limited but can also be seasonally iron-limited (Johnson et al. 1999; Bruland et al. 2001; McGaraghan and Kudela 2012), similar to other ecosystems that support dinoflagellate blooms (Hutchins et al. 2002). During relaxation of upwelling, the vertical gradients of both nitrate and iron are intensified, leaving limited surface nutrients and a nutricline about 10-15 m below the surface (Egli et al. 2009; Ryan et al. 2010). These conditions are common immediately following a diatom bloom, when *A. sanguinea* blooms are expected to occur (Matsubara et al. 2007; Du et al. 2011). Given the potential for limitation or co-limitation of iron in addition to nitrate and phosphorus, it would presumably be advantageous for vertically migrating phytoplankton to acquire both macro-and micronutrients at depth. Here we present the first evidence for *A. sanguinea* (or any phytoplankton) actively migrating to acquire iron from depth in stratified water columns under both nitrate-replete and nitrate-deficient conditions.

2. Methods

**Culture**

Non-axenic cultures of *A. sanguinea* isolated from Monterey Bay were grown in 1-L containers with sterile-filtered artificial seawater (ASW). ASW consisted of “Instant Ocean” basal salts at a salinity of 29.5. Cultures were maintained under 12h: 12h light:
dark conditions using ~100 μmol photons m⁻² s⁻¹ GE “white light” fluorescent bulbs at a temperature of 16.5°C within an environmental chamber.

**Experiment 1 – Nitrate-replete column**

*A. sanguinea* MB1206 cultures were grown with L1 media from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; salinity 29). Once stationary growth was reached (3 weeks), cultures were diluted every 5-7 days for 6 weeks by 1:1 dilution with ASW (no nutrient addition). Before the initiation of the vertical migration experiments (36 hours) 50 nmol L⁻¹ desferoximine-B (DFB, or desferral; an iron chelator) was added to the cultures to bind any bio-available iron.

**Experiment 2 – Nitrate-deficient column**

Cultures were grown in 250 mL sterile containers with CCMP L1 media with nitrate, phosphorus, and iron additions diluted to 1:100 standard L1 concentrations (all other nutrients kept the same; salinity 29). Once in stationary growth, cultures were diluted 1:1 every 2-3 days with ASW and sampled daily for analysis of nitrate and phosphorus on a Lachat autoanalyzer (Smith and Brogren 2001; Knepel and Brogren 2002) until nitrate was <2 μmol L⁻¹. Before the initiation of the vertical migration experiments (36 hours) 50 nmol L⁻¹ DFB was added to the cultures.

**Experimental set-up**

The experimental system consists of a Plexiglas column 0.3 m diameter by 1.8 m height. The column was washed with 2 mol L⁻¹ trace-metal grade hydrochloric acid (HCl), rinsed with purified (>18 MΩ cm⁻¹) Milli-Q deionized water several times, then filled with pH 2 Milli-Q water until use. Six luer-lock ports with attached silicone tubing were spaced 0.14-0.23 m apart, with port 1 at the bottom and port 6 at the surface (Fig.
1). Ports and tubing were cleaned with a series of acid baths (6 mol L\(^{-1}\) HCl for 48 hours, then 3 mol L\(^{-1}\) nitric acid for 48 hours) and rinsed and stored in pH 2 Milli-Q. For the experiments, the column was partitioned into three distinct density layers (modulating temperature and salinity; Fig. 1) by filling the column with ASW amended with nutrients as described below. The column was kept upright in an opaque plastic container filled with 5°C water covering the bottom 0.7 m (~8 L; Fig. 1). The experiments were conducted in a temperature-regulated environmental chamber at 16°C on a 12h:12h light:dark cycle. The bottom 0.8 m of the column was covered with black plastic to eliminate direct light. The gradient of light from the surface to the covered portion of the column ranged from 50-175 µmol photons m\(^{-2}\) s\(^{-1}\) with the highest light levels near the top of the chamber.

*Experiment 1 – Nitrate-replete column*

The column was filled with nutrient-deplete ASW by a peristaltic pump with acid cleaned tubing at 20 mL min\(^{-1}\) to minimize mixing. There were 3 distinct layers: bottom (0.5 m; salinity = 32), middle (0.5 – 1.2 m; salinity = 31.5 - 29.5), and surface (1.2 – 1.4 m; salinity = 29; Table 1; Fig. 1). The pycnocline was maintained by adding ice to the outside water bath every two hours for the first 24 hours, and subsequently every 4-6 hours, keeping a 7-9°C gradient between the surface and the bottom layer. The pycnocline was stable for 72 hours as confirmed with periodic checks of salinity and temperature from the luer-lock ports. Nutrient concentrations in the bottom layer were 21.3 µmol L\(^{-1}\) for nitrate (20 µmol L\(^{-1}\) Na\(^{15}\)NO\(_3\)) and 17.8 nmol L\(^{-1}\) for iron (10 nmol L\(^{-1}\) \(^{57}\)FeCl\(_3\)). In the middle and surface layers, nitrate measured 1.3 µmol L\(^{-1}\) with the
addition of 30 nmol L$^{-1}$ DFB at 0.7 m. After the addition of the culture, the surface layer concentrations were 58 µmol L$^{-1}$ nitrate and 50 nmol L$^{-1}$ DFB (Table 1).

**Experiment 2 – Nitrate-deficient column**

The column was filled according to experiment 1, with 3 distinct layers: bottom (0.5 m; salinity = 34.5), middle (0.5 – 1.2 m; salinity = 34.5-29.5), and surface (1.2 – 1.6 m; salinity = 29; Table 1). The pycnocline was maintained with an external circulating chiller bath keeping a 6-8°C gradient between the surface and the bottom. The pycnocline remained stable under these conditions for 120 hours. At 72 hours the column was replenished by peristaltic pump at 50 mL min$^{-1}$ with 6 L of nutrient-deplete ASW (salinity = 29). Nutrient concentrations in the bottom layer were 21.8 µmol L$^{-1}$ for nitrate (20 µmol L$^{-1}$ Na$^{15}$NO$_3$) and 26.9 nmol L$^{-1}$ for iron (20 nmol L$^{-1}$ FeCl$_3$). In the middle layer nitrate measured 1.8 µmol L$^{-1}$ with 30 nmol L$^{-1}$ DFB added at 0.7 m, and the surface layer concentrations were 2.4 µmol L$^{-1}$ for nitrate and 50 nmol L$^{-1}$ DFB. After the addition of the culture, the surface measured 3.4 µmol L$^{-1}$ nitrate and 50 nmol L$^{-1}$ DFB (Table 1).

**Sampling**

**Experiment 1 – Nitrate-replete column**

Samples of 10 – 420 mL were collected in acid cleaned syringes one half hour before the experiment start from the surface, port 4 (middle), port 1 (bottom; Fig. 1), as well as from the *A. sanguinea* culture. Chlorophyll-a and inorganic nutrient (nitrate and phosphorus) samples were collected hourly for the first 24 hours, and at 48, and 72 hours from ports 1 – 5 and the surface. Samples were also collected hourly for the first 19 hours from port 6, but from hours 20 – 72 the water level was below port 6. A Turner Designs
Cyclops-7 fluorometer was used to measure raw fluorescence through the Plexiglas column and sampled as described for chlorophyll-a. Particulate iron and nitrogen samples were taken at time zero from the surface, port 1, and the *A. sanguinea* culture and again at 6, 12, 18, 24, 48, and 72 hours from the surface and port 1. Dissolved iron samples were collected from port 1 at zero, 24, and 72 hours.

**Experiment 2 – Nitrate-deficient column**

Experiment 2 was sampled at time zero as per experiment 1. Samples were collected with the same protocol as experiment 1 from ports 1 – 5 and the surface hourly for the first 24 hours and again at hour 48, 72, 96, 102, and 120. Samples were also taken hourly for the first 13 hours and for 72 and 96 hours from port 6, but from hours 13 – 72 and 102 – 120 the water level was below port 6. At 72 hours 6 L of nutrient deplete ASW was added to the column (29 salinity) to increase the water level. Dissolved iron samples were collected from port 1 at time zero, 24, and 120 hours.

**Analysis**

Chlorophyll-a samples were filtered onto glass-fiber filters (GF/F; Whatman), extracted in 90% acetone and analyzed on a Turner 10-AU fluorometer (Welschmeyer 1994). Nitrate and phosphorus samples were collected from the chlorophyll-a filtrate, frozen at -20°C for 48 hours, then analyzed using a Lachat autoanalyzer (Smith and Brogren 2001; Kneple and Bogren 2002). Particulate nitrogen samples were filtered onto combusted (450°C for 5 hours) GF/F filters and frozen at -20°C until analyzed using a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer for particulate nitrogen and isotopes (Dugdale and Wilkerson 1986). Particulate iron samples were filtered onto 0.4 µm polycarbonate (Poretics) 45 mm acid cleaned filters and frozen at -20°C until
analysis for leachable particulate iron (bio-available) and iron isotopes by Thermo
Element XR Magnetic Sector inductively coupled plasma mass spectrometry (ICP-MS;
Berger et al. 2008; Lippiatt et al. 2010). Dissolved iron samples were collected by acid-
cleaned syringe, stored in acid clean HDPE bottles, acidified to pH 2, and refrigerated at
4°C until analysis by ICP-MS (Biller and Bruland 2012).

3. Results

Experiment 1 – Nitrate-replete column

*A. sanguinea* at an initial concentration of 62 µg L⁻¹ chlorophyll-a was added to
the surface of the column. The cells began migration immediately, reaching port 1 after 3
hours and almost half the measured chlorophyll-a was at port 1 by hour 5. Chlorophyll-a
samples were collected hourly, but were lost during sampling and storage after hour 6.
The Cyclops was calibrated to the collected chlorophyll (from both experiments) and raw
fluorescence was converted to chlorophyll-a. We report the fluorescence data since this is
the most complete record of biomass from the experiments, and because the fluorometer
provided fluorescence readings between ports, removing the error of “disappearing”
chlorophyll-a (cells between sampling ports; Cullen and Horrigan 1981; Doblin et al.
2006). After hour 6, the bulk of the fluorescence was evenly distributed throughout the
column, except for a fraction that stayed below the nutricline for the duration of the
experiment (Fig. 2). Bulk migration to the surface at hour 16 was evident, three hours
before the light cycle; following this initial migration a fraction of the cells (chlorophyll-
a) remained at the bottom for the remainder of the experiment.

Drawdown of nitrate was accelerated from hours 12 – 72, most evident from
samples collected at port 2, within the isotope-enriched water (Fig. 1, 3a). Trapezoidal
integration of the total column nitrate provided drawdown estimates of 30.0 µmol L⁻¹ m⁻², but drawdown based on analysis of nutrient samples from individual ports was not statistically significant (Fig. 3b). The particulate nitrogen increased with time at the surface while port 1 was variable with no clear trend (Fig. 3c). The particulate atom % enrichment of $^{15}$N at the surface was 0.384, slightly enriched after 1 hour compared to the natural abundance of 0.367 (the initial *A. sanguinea* culture value). Enrichment increased until hour 6 and stayed at a similar level of enrichment for the rest of the experiment (Fig. 3d). Particulate $^{15}$N samples taken from port 1 were enriched from the experiment start at 0.936 and increased as the experiment progressed to 1.020 atom % $^{15}$N. Enrichment peaked at hour 18 for both port 1 and the surface samples.

The concentration of dissolved iron was 7.8 nmol L⁻¹ in the column at time zero. After the addition of 10 nmol L⁻¹ of $^{57}$FeCl₃, the bottom layer increased to 18 nmol L⁻¹ (Table 1). Dissolved iron samples taken at 24 and 72 hours were contaminated. The amount of particulate iron in the surface increased over the course of the experiment (Fig. 4a). At hour 6 the particulate atom % enrichment of $^{57}$Fe at the surface was 0.031, enriched compared to the natural abundance (for phytoplankton) of 0.022. Samples for hours 12 and 24 were also enriched, but samples from hours 48 and 72 returned to natural abundance (Fig. 4b). Samples from port 1 did not show any enrichment, but the volume of water collected may have been insufficient for accurate measurement. The ratio of total particulate nitrogen to chlorophyll-a was similar throughout the experiment compared to the initial culture ratio and the ratio of particulate iron to chlorophyll-a increased slightly over time (Fig 5a,b).

*Experiment 2 – Nitrate-deficient column*
A. sanguinea at a concentration of 128 µg L\(^{-1}\) chlorophyll-a was added to the surface of the column and cells began migrating immediately. Chlorophyll-a was measured at port 1 by hour 3 (Fig. 2). In the first 7 hours the cells accumulated at the top of the pycnocline (0.6-0.7 m) before the bulk of the cells migrated back to the surface 3 hours before the light cycled on. The highest fluorescence at port 1 was at hour 72.

During the light period, cells generally accumulated in the top 1 – 1.4 m, above the dark interface, though there was always some fluorescence at the top of the pycnocline (Fig. 2). By 102 hours the temperature had increased by 1°C at the bottom and the halocline had marginally relaxed, but the pycnocline remained stable through the duration of the experiment.

Phosphorus was 0.2 µmol L\(^{-1}\) throughout the column during the experiment. Nitrate was 2 µmol L\(^{-1}\) between port 4 and the surface at the start of the experiment (Table 1). Surface nitrate concentration increased to 3.5 µmol L\(^{-1}\) with the addition of the A. sanguinea culture and was depleted after 3 hours to 2 µmol L\(^{-1}\). The drawdown of nitrate at the surface and ports 3, 2, and 1 was significant (p<0.001; t-test; n = 31 for each port; Fig. 3e). Trapezoidal integration of the total column nitrate provided drawdown estimates of 20.8 µmol L\(^{-1}\) m\(^{-2}\) (Fig. 3f). Particulate nitrogen in the surface increased with time (Fig. 3g) but port 1 samples were variable with no clear trend (data not shown). The particulate atom % enrichment of \(^{15}\)N at the surface was enriched at 0.391 by hour 6 compared to the initial A. sanguinea culture value of 0.362 atom % \(^{15}\)N and increased until hour 120 where the surface atom % enrichment was 1.267 (Fig. 3h). The samples were significantly enriched compared to the culture enrichment (p<0.001; t-test; n= 10).
Particulate $^{15}$N samples taken from port 1 were highly enriched from time zero with enrichment peaking at hour 18 at 9.365 (data not shown).

Dissolved iron concentration was 6.9 nmol L$^{-1}$ in the column at time zero. After the addition of 20 nmol L$^{-1}$ $^{57}$FeCl$_3$, the bottom layer was 27 nmol L$^{-1}$ (Table 1). Dissolved iron samples at 24 and 120 hours were contaminated. Particulate iron in the surface samples increased with time (Fig. 4c). The particulate surface atom % enrichment of $^{57}$Fe was 0.025 by hour 6, enriched relative to the culture value of 0.023, and continued to increase during the experiment (Fig. 4d). The port 1 particulate enrichment oscillated between 0.049-0.070 atom % throughout the experiment (data not shown). The nitrogen to chlorophyll-a ratio increased with time, as did the iron to chlorophyll-a ratio in the surface (Fig. 5c,d).

4. Discussion

Many dinoflagellates vertically migrate to assimilate nitrate from depth, and our results are consistent with previous reports for *A. sanguinea* (Cullen and Horrigan 1981; Kamykowski 1981). Here, we document for the first time the assimilation of iron during vertical migration by a dinoflagellate. Previous nitrogen assimilation experiments have been supported by in situ observations (Cullen and Horrigan 1981; MacIntyre et al. 1997; Smayda 2010), and we assume that laboratory-based iron assimilation during vertical migration is similarly representative of dinoflagellates in the environment. The atom % enrichment of iron and nitrate at the surface and the drawdown of nitrate at depth offers convincing evidence that *A. sanguinea* is assimilating iron and nitrate before returning to the surface. These results therefore suggest that *A. sanguinea* under nitrate- and iron-deficient surface conditions is capable of sustaining growth through vertical migration.
**Vertical Migration**

A combination of factors can influence the start and completion of vertical migration while still allowing for the assimilation of nitrate from depth (Flynn and Fasham 2002). These include changes in irradiance (Kamykowski 1981; Heaney and Furnass 2006), anoxia (George and Heaney 1978; Taylor et al. 1988; Whittington et al. 2000), density gradients (Kamykowski 1981), influence of other particulates (Doblin et al. 2006), toxins (MacIntyre et al. 1997), sexual reproduction (Persson et al. 2008), and predator avoidance (Park et al. 2002; Bollens et al. 2012). The mechanism is further complicated by internal factors such as circadian rhythm, growth stages, and internal orientation (e.g. phototaxis (Kamykowski et al. 1999) and geotaxis (Eggersdorfer and Häder 1991; Kamykowski et al. 1999)). For these experiments not all the cells were migrating to depth, or were not migrating synchronously, indicating that under these conditions vertical migration was not solely regulated by nitrate concentration. This has been reported with *A. sanguinea* (Cullen and Horrigan 1981) and other dinoflagellates such as *Lingulodinium polyhedrum* (Heaney and Eppley 1981), *Heterocapsa niel* (Eppley et al. 1968; Kamykowski 1981; Cullen 1985), *Prorocentrum micans* (Kamykowski 1981), and *Alexandrium tamarense* (MacIntyre et al. 1997).

*A. sanguinea* migrates in the field (Lu and Hodgkiss 2004; Ryan et al. 2010; Du et al. 2011) and under various conditions in the lab (Cullen and Horrigan 1981; Kamykowski et al. 1992). Five separate experiments (the first three not reported here for brevity, including a natural *A. sanguinea* nitrate-limited bloom from Monterey Bay), established that *A. sanguinea* in culture and field samples migrated over a range of pycnocline, light, and nutrient conditions. Swimming speeds for *A. sanguinea* vary from
0.41 - 1.08 m h\(^{-1}\) (Kamykowski et al. 1992; Smayda 2010), with the descent faster than the ascent (Kamykowski et al. 1992) and with no explicit light: dark pattern to the migration (Cullen and Horrigan 1981). Swimming descent speeds documented here, at least 0.53 m h\(^{-1}\), are consistent with this reported range. The pattern of cell migration varied with the nutrient concentration in the column, with more active migration when the column was depleted of nitrate, consistent with prior studies (Cullen 1985; Doblin et al. 2006).

Strong vertical migration was observed in both experiments, under nitrogen-replete and –deplete conditions. The only apparent difference was that for the nitrate-deficient experiment, cells (fluorescence) were not observed in abundance at the bottom port until after 72 hours. This is likely due to a combination of a strong halocline (Olsson and Granéli 1991) and phosphorus stress, which could reduce the production of ATP and decrease the ability of the cells to migrate (Heaney and Eppley 1981). Even though the nitrate-deficient migration to the bottom port was delayed, much of the fluorescence was in the nutricline where drawdown of nitrate was also observed (Fig. 3a-b,e-f). Once the halocline had relaxed, fluorescence increased at port 1. This flexible adaptation to changing conditions presumably allows A. sanguinea to thrive and enhance its growth potential in a temporally and spatially dynamic environment where light and nutrients are separated, but accessible.

**Nutrient acquisition at depth**

Our results indicate that both nitrate and iron were assimilated at depth with subsequent transfer to the surface as enriched particles. It is well established that dinoflagellates migrate and incorporate inorganic nutrients at depth (Smayda 2010), and
it has been speculated that along with nitrate and phosphorus, iron is also acquired (Salonen and Rosenberg 2000; Naito et al. 2005). In situ experiments to determine macronutrient uptake during migration are difficult to execute (MacIntyre et al. 1997; Matsubara et al. 2007) and this difficulty increases for trace metals. Our results indicate that assimilation of nitrate and iron occurred even when *A. sanguinea* was not strongly nutrient limited (Fig. 3, 4). This has been documented for nitrate with other dinoflagellates (Harrison 1976; Olsson and Granéli 1991; Doblin et al. 2006), but some reports suggest nutrient-limitation is required before vertical migration to depth is triggered (Paasche et al. 1984; Cullen 1985). We found *A. sanguinea* migrated regardless of nitrogen deficiency, and while there was nitrate and iron assimilation at depth for both experiments, assimilation increased with the extreme nutrient stress seen in our nitrate-deficient experiment (Fig. 3g-h; 4c-d). This difference is presumably due to the pre-experiment nutrient starvation and the duration of the experiment, 120 hours compared to 72 hours. The particulate samples from the bottom port exhibited enhanced atom % enrichment compared to the initial culture, though it was not possible to clearly identify this enrichment as intracellular since iron may attach extracellularly (Hudson and Morel 1989; Tang and Morel 2006). Loosely bound extracellular iron would react with the DFB in the upper column, so surface particulate samples (after vertical migration) are presumably representative of intracellular enrichment (Hudson and Morel 1989). Sinking particles (i.e. senescent or dead cells) introduced with the addition of the culture at time zero may also explain enhanced enrichment seen at the bottom port if these particles served to adsorb and concentrate the isotopes.
Evidence for vertical migration under iron stress has been based primarily on conjecture and iron requirements for phytoplankton, combined with their documented ability to incorporate nitrogen and phosphorous from depth (McKay et al. 2000; Salonen and Rosenberg 2000; Naito et al. 2005). Naito et al. (2005) determined growth yields with insoluble iron (FePO$_4$ and FeS) for high biomass ("red tide") species (including 5 species belonging to Dinophyceae), demonstrating that the phytoplankton were able to utilize species of insoluble iron that are associated with re-suspended sediments or bottom water. This result suggests that motile phytoplankton with access to bottom water may use insoluble iron, supporting blooms when soluble iron is insufficient to support the often very high surface biomass. A. sanguinea has a comparatively large cellular iron requirement compared to neritic phytoplankton of similar size (Doucette and Harrison 1990), and if iron is not available in the surface, it must be acquired from another source.

Our experimental enrichment rates were variable, based on a multitude of factors. Both experiments had a marginal relaxation of the pycnocline, at 48 hours for the nitrate-replete experiment and 102 hours of the nitrate-deficient experiment. For the nitrate-replete experiment, it is possible that the 48 and 72 hour surface time points were dominated by cells that had not migrated, as most of the fluorescence was seen at the bottom ports (Fig. 2). This result would be consistent with nitrate stress acting as a migratory trigger for some cells (Cullen 1985) as the column was not nitrate-deficient and the cells were nitrate-replete prior to the experiment. The presence of a strong halocline can trigger vertical migration, or in the nitrate-deficient experiment, it may have slowed the rate of vertical migration as cells will often form in tight bands above or below the halocline (Bjørnsen and Nielsen 1991; Olsson and Granéli 1991; Hall and
Pearl 2011), which could explain the slower rates of atom % enrichment for both nitrate and iron for the nitrate-deficient experiment (Fig. 3d,h; 4b,d). It could also be a result of cell health, as the cells were phosphorus- and iron-limited, which can slow migratory rates (Heaney and Eppley 1981) and nitrate assimilation since iron is needed for many of the nitrate and nitrite reductase assimilatory enzymes (Falkowski 1983; Doucette and Harrison 1991). For both experiments the ratios of iron to chlorophyll-a (Fig. 5b,d) and the concentration of particulate iron increased with time for the surface samples; the longer duration of the nitrate-deficient experiment (120 hours) provided clearer results (Fig. 4 b,d). Nitrogen to chlorophyll-a (Fig. 5a,c) and particulate nitrogen increased for the nitrate-deficient experiment with time, but not for the nitrate-replete experiment. This increase is consistent with alleviation of nitrate- and iron-stress (Fig. 4c,g). The clear drawdown of nitrate from depth for both experiments is in agreement with these observations, consistent with previous experiments that determined assimilation of intracellular nitrate during migration (Fig. 4a-b,e-f; Doblin et al. 2006; Schaeffer et al. 2009).

**Ecological implications**

Understanding dinoflagellate behavior and autecology is key to predicting dinoflagellate dominance and particularly important for those species considered HAB organisms. Our results advance our understanding of both *A. sanguinea* and dinoflagellate adaptations leading to a competitive advantage during low-nutrient, stratified conditions when vertical migration provides a favorable strategy for acquisition of both nitrogen and iron. HAB organisms from upwelling systems can often be prolific during periods of both low and high nitrate pulses (Kudela et al. 2010), making it difficult
to determine the exact nutrient requirements that precede bloom events. Matsubara et al. (2007) reported optimal growth for *A. sanguinea* at a salinity of 20, under high light, and temperatures of 25°C, producing a growth rate of 0.4 m day$^{-1}$. This indicates that the species does well in warm, stratified, lower salinity water conditions. The strain MB1206 used in this experiment exhibits similar growth rates of 0.4 m day$^{-1}$ when grown in a salinity of 35 at 25°C (Boyd et al. *in press*). In the California Current System, *A. sanguinea* is associated with stratified, low salinity, warm surface waters (Kudela et al. 2008; Ryan et al. 2010), and a relaxation of upwelling (Du et al. 2011), which typically occurs seasonally in the late summer and autumn. *A. sanguinea* often blooms immediately after diatom blooms (Cloern 2005; Kudela et al. 2008; Du et al. 2011) and Matsubara et al. (2007) described *A. sanguinea* as being suppressed when grown in cultures with *Chaetoceros* and *Skeletonema* spp.

Current modeling efforts include the average swimming patterns of dinoflagellates (Shulman et al. 2012; Yamazaki et al. 2013), but it is important to model the actual complex swimming behaviors which can be influenced by internal as well as external factors. Modeling behavior of dinoflagellates demonstrates that they will often undertake vertical migration when nitrogen-stressed (Kamykowski and Yamazaki 1997; Ji and Franks 2007) but models including vertical migration for iron requirements have not been tested, even though the importance of including iron in modeling bulk primary productivity is well established. While most vertical migration nutrient experiments have focused on the assimilation of nitrate, studies have shown that phytoplankton are able to acquire phosphorus (Watanabe et al. 1988; 1991) and ammonium (Hall and Pearl 2011) under nutrient-limiting conditions. Therefore it is not surprising that the dinoflagellate *A.*
*sanguinea* would actively migrate and assimilate iron from depth under iron-limiting conditions, providing an additional competitive advantage for these slow-growing dinoflagellates in seasonally high nutrient low chlorophyll-a waters.
References


Matsubara, T., S. Nagasoe, Y. Yamasaki, T. Shikata, Y. Shimasaki, Y. Oshima, and T. Honjo. 2007. Effects of temperature, salinity, and irradiance on the growth of the


Tables

Table 1. Nutrient concentrations and physical parameters for experimental set-up (time zero) of Experiment 1: nitrate-replete column and Experiment 2: nitrate-deficient column.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Experiment 1: Nitrate-replete</th>
<th></th>
<th>Experiment 2: Nitrate-deficient</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bottom (0 - 0.4m)</td>
<td>Middle (0.4 – 1.2 m)</td>
<td>Top (1.2-1.4 m)</td>
<td>Bottom (0 - 0.4 m)</td>
</tr>
<tr>
<td><strong>Temp (°C)</strong></td>
<td>6</td>
<td>13</td>
<td>15</td>
<td>7</td>
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<tr>
<td><strong>Salinity</strong></td>
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<td>31.5-29.5</td>
<td>29</td>
<td>34.5</td>
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<td><strong>Phosphorus</strong></td>
<td>2.8</td>
<td>3.2</td>
<td>4.3</td>
<td>0.2</td>
</tr>
<tr>
<td>(µmol L⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nitrate</strong></td>
<td>1.8 (¹⁴N)/22.0 (¹⁵N)</td>
<td>1.3 (¹⁴N)</td>
<td>58.0 (¹⁴N)</td>
<td>1.8 (¹⁴N)/24.0 (¹⁵N)</td>
</tr>
<tr>
<td>(µmol L⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Iron</strong></td>
<td>7.8 (⁵⁶Fe)/10.0 (⁵⁷Fe)</td>
<td>-</td>
<td>-</td>
<td>6.9 (⁵⁶Fe)/20.0 (⁵⁷Fe)</td>
</tr>
<tr>
<td>(nmol L⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>DFB</strong></td>
<td>0.0</td>
<td>30.0</td>
<td>50.0</td>
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</table>
Figure Legends

**Figure 1.** Diagram of laboratory column used in these experiments. The density profile is taken from observational data for hour zero of Experiment 2. Density stratification was maintained either by the addition of ice (Experiment 1: nitrate-replete) or by a circulating water chiller (Experiment 2: nitrate-deficient). Stable isotopes are present in the bottom 5 L of water. Ports are labeled with distances (m). Surface water level varied but was initiated as indicated.

**Figure 2.** Distribution of *A. sanguinea* biomass for Experiment 1: nitrate-replete (gray) and Experiment 2: nitrate-deficient (black). Experiment 1 was sampled for 72 hours and Experiment 2 was sampled for 120 hours. Stable isotopes were present below 0.4 m.

**Figure 3.** (a-d) Nitrogen data for Experiment 1: nitrate-replete (gray circles; 72 hours) and (e-h) Experiment 2: nitrate-deficient (black squares; 120 hours). (a,e) Nitrate concentrations [µg L⁻¹] for port 1 (solid line), port 2 (long dash), port 3 (medium dash), port 4 (short dash), port 5 (dot), port 6 (line-dot), and the surface (line-2 dots). Drawdown from ports 1, 2, 3, and the surface for Experiment 2 was significant (n= 31). (b,f) Integrated nitrate [µg L⁻¹ m⁻²] drawdown for each experiment. (c,g) Particulate nitrogen [µg L⁻¹] collected from the surface. (d,h) Particulate ¹⁵N % atom enrichment collected from the surface particulate nitrogen samples. The dashed line is the initial enrichment for the *A. sanguinea* culture.

**Figure 4.** (a,b) Iron data for Experiment 1: nitrate-replete (gray circles; 72 hours) and (c,d) Experiment 2: nitrate-deficient (black squares; 120 hours). (a,c) Particulate iron
[ng L\(^{-1}\)] collected from the surface. (b,d) Particulate \(^{57}\)Fe % atom enrichment collected from the surface. The dashed line is the initial enrichment for the *A. sanguinea* culture.

**Figure 5.** (a) Total particulate nitrogen normalized by chlorophyll-\(a\) from the surface for Experiment 1: nitrate-replete (gray circles; 72 hours). (b) Total particulate iron normalized by chlorophyll-\(a\) [ng: \(\mu\)g] from the surface for Experiment 1: nitrate-replete (gray circles; 72 hours). (c) Total particulate nitrogen normalized by chlorophyll-\(a\) from the surface for Experiment 2: nitrate-deficient (black squares; 120 hours). (d) Total particulate iron normalized by chlorophyll-\(a\) [ng: \(\mu\)g] from the surface for Experiment 2: nitrate-deficient (black squares; 120 hours). For all panels the dashed line is the initial ratio for the *A. sanguinea* culture.
Figures

Figure 1
Figure 2
Figure 3

Figure 4
CONCLUSION
This dissertation resulted initially in the development of a method to determine alkaline phosphatase activity (APA) in marine phytoplankton, combining the sensitive enzyme-labeled fluorescence (ELF) probe with time-saving spectrofluorometry. This method was used to identify APA in eukaryotic phytoplankton in the northern Gulf of Alaska (nGoA) and off the coast of Big Sur, California. Phytoplankton community structure was also investigated to determine if increased APA was a competitive strategy used by dinoflagellates to access organic phosphorus (P) and the trace metal zinc (Zn) in the California Current System. A second competitive strategy of dinoflagellates, vertical migration, was investigated with laboratory cultures and water columns to determine if nitrogen (N) and iron (Fe) were being assimilated at depth.

In Chapter 1 of this dissertation a method was developed to determine APA by spectrofluorometry (Peacock and Kudela 2012). This method adapted and improved upon the ELF-microscopy and -flow cytometry methods of (González-Gil et al. 1998) and (Dignum et al. 2006). The preparation of samples for analysis is similar to the previous methods, but instead of being analyzed by microscopy or flow cytometry the samples are analyzed by spectrofluorometry. Spectrofluorometry allows the user to integrate the area under the emission curve (470 - 620 nm) for the fluorescent signal of APA, where typical ELF-methods only use the peak emission of 530 ± 25 nm. Our method also utilizes a biological standard (*Amphidinium carterae*) to normalize samples instead of fluorescent beads, which can interfere with the peak emission signal on flow cytometry. This method cuts down on the time- and labor intensiveness of ELF-microscopy, can provide accurate results for samples with low cell abundance, and reports results as intensity of APA rather than presence or absence. Lastly, the same sample can be analyzed with both
spectrofluorometry and ELF-microscopy, as we saw no decline in fluorescence within 48 h. The spectrofluorometry method is a bulk-labeling method, and does not allow for determination of species’ specific APA, but we feel this compromise is reasonable given the described advantages.

This method was originally developed to determine APA in the nGoA, as cell abundance for our samples was too low for ELF-microscopy, and cell size was too large for ELF-flow cytometry. Observations from the Bering Sea and Monterey Bay, California, suggested that even when P was not stoichiometrically limiting in the water column, some species within the phytoplankton community were P-stressed (Nicholson et al. 2006; Shaked et al. 2006). The continental shelf of the nGoA is considered macronutrient stressed, though it is N- rather than P-limited, and an ideal location to investigate community structure under P-replete conditions. APA is often used as a proxy to determine P-stress in phytoplankton (Vidal et al. 2003), and the original hypothesis for Chapter 2 was to determine if phytoplankton were using dissolved organic P (DOP) to alleviate P-stress in the nGoA. Our results indicated that portions of the phytoplankton community, namely 5 – 20 µm dinoflagellates and haptophytes, were producing AP, indicating that there was either P-stress within the community, or that the dinoflagellates were preferentially accessing DOP as a competitive strategy, even though dissolved inorganic P (DIP) was not limiting (Peacock and Kudela 2013). In the nGoA, mesoscale eddies cross from the macronutrient deplete (but iron (Fe) replete) shelf waters to the high-nutrient low-chlorophyll-a (HNLC; Fe deplete) basin waters, stimulating biological production in the eddy tracks as entrained shelf water mixes with basin water (Whitney et al. 2005; Fiechter et al. 2009). The transitional water related to the edges of two eddies in
the nGoA during this study recorded the highest DIP concentrations, yet also the most APA observed. The results from this chapter document that the P-requirement for the phytoplankton community in the nGoA is variable and complex, and a more nuanced approach to determining nutrient acquisition and limitation for phytoplankton communities in these systems is appropriate.

With the indication that dinoflagellates are utilizing DOP through the production of AP, even when DIP is not limiting, it is logical to investigate the variation in community structure that can occur when more complex nutrient interactions are introduced. The AP enzyme allows phytoplankton to access DOP for nutrient uptake, but it also requires (or is stimulated by) a metal co-factor. In eukaryotic phytoplankton, this is typically Zn$^{2+}$. Zn is hypothesized to be limiting in HNLC waters, though the addition of Zn only minimally stimulates chlorophyll-a production (Croot et al. 2011 and references therein). The coast of Big Sur, California is an upwelling HNLC coastal region, associated with high macronutrient concentrations, but low Fe and Zn and phytoplankton biomass (Bruland et al. 2001; Franck et al. 2003). The relatively narrow shelf (a few km) in comparison to the rest of central California, and the negligible river inputs into this region are responsible for low micronutrient concentration in the surface (Bruland et al. 2001).

Chapter 3 examined the effect that a suite of nutrients added as incubation enrichments had on phytoplankton communities from the upwelling HNLC region. In particular, this chapter was focused on how the addition of Zn and a P source (either DIP or DOP) would influence community dynamics. We determined that the addition of N, DIP, DOP, and Zn (in different combinations) did not significantly increase biomass,
growth rate, or cell counts compared to the control, but that there was a noticeable shift in the phytoplankton community with different nutrient combinations. We did observe a significant increase in APA in the enrichments with additional DIP (only) and DOP and Zn together, but the increase was not correlated to dinoflagellates as has been reported in previous studies (Nicholson et al. 2006). Of particular note is the fact that APA was increased compared to the other treatments with the addition of DIP, which implies that there was preferential uptake of DOP, as the community was not DIP-stressed. Previous studies have reported the addition of Zn has had no or minimal effect on phytoplankton growth rate (Crawford et al. 2003; Coale et al. 2003; Lohan et al. 2005), and we found in this study that the addition of Zn without other nutrient enrichment may have actually inhibited growth rate and chlorophyll-a.

Recent advances in determining AP-pathways in eukaryotic phytoplankton report that a variety of metals, including Co$^{2+}$ and Mg$^{2+}$, instead of Zn$^{2+}$ may be used as a co-factor with AP (Lin et al. 2013). These metals were not limiting during this experiment, but may be important to deciphering the role Zn plays in AP production and subsequent community shifts. Future work should investigate the structure of eukaryotic AP, as complicated metal interactions could play a role in community phytoplankton dynamics.

Chapter 4 of this dissertation investigated an alternate competitive strategy to access nutrients. Vertical migration is a complicated process, and some phytoplankton, namely dinoflagellates, migrate from nutrient-deplete surface waters to nutrient-replete waters at depth (Flynn and Fasham 2002). Nutrient imbalance within the cell is often the reason for the initiation of migration (Yamazaki and Kamykowski 2000). At depth, dinoflagellates assimilate N in darkness, then migrate to the surface for photosynthesis.
during daylight. There have been many studies observing N-assimilation at depth, both in
the field and in laboratory experiments, including with the dinoflagellate Akashiwo
sanguinea, which was studied in this dissertation. In Chapter 4 we conducted two
experiments: (1) the cells were initially N-replete and Fe-deplete, and (2) the cells
initially were N- and Fe-deplete. Under both conditions A. sanguinea migrated in a
laboratory water column and assimilated Fe along with N at depth, and then migrated
back to the surface. There was also an increase in the ratio of Fe to chlorophyll-a with
time for both experiments, reinforcing that Fe was being assimilated into the cell. This
experiment is the first reported Fe-assimilation at depth by any phytoplankton during
migration, and is significant validation of the assumed Fe-assimilation hypothesized as a
competitive strategy to decrease nutrient dependence of surface nutrients. Future research
to determine Fe uptake rates and to quantify the amount of Fe per cell should be
undertaken. Those results will be invaluable to current modeling efforts that are
investigating dinoflagellate migratory behavior for N-assimilation (Shulman et al. 2012;
Yamazaki et al. 2013).

This dissertation is focused on dinoflagellate’s unique ability to assimilate
nutrients. Recent decadal trends have observed taxon shifts from diatom- to
dinoflagellate-dominated systems, including the California Current (Alheit 2007; Jester et
al. 2009; Widdicombe et al. 2010; Warns et al. 2013), highlighting the need to better
understand the ecology of coastal dinoflagellate populations. This shift could have long-
reaching effects on ecosystem dynamics and carbon export, as diatoms are often
associated with high carbon export efficiency while dinoflagellates are most often
associated with noxious, toxic, and harmful impacts. The shift in dominant phytoplankton
is also highly correlated with regime shifts due to recent climate change (Alheit 2007; Jester et al. 2009) and entire ecosystems can be restructured based on these regime shifts with lasting consequences for higher trophic levels (including reduced biomass for zooplankton, fish, and seabirds).

It is projected that global warming will greatly affect eastern boundary currents, like the California Current System (CCS; Doney et al. 2012), where this dissertation research was conducted. With increased ocean warming, projections for the CCS include reduced nutrient inputs (reduced upwelling), deepening of the thermocline, delayed timing and intensity of the spring bloom, and increased stratification of the surface water (Doney et al. 2012). The CCS covers a large area, and warming is not heterogeneous within the system. From 1982 - 2008 the central CCS (cCCS) from Monterey Bay to Bodega Bay, CA exhibited increased upwelling (due to an increase in upwelling favorable winds) and increased shoaling of the nutricline, a reversal of what is predicted for the CCS in regards to climate shift (García-Reyes and Largier 2010; Chavez et al. 2011). But, most recently, the cCCS, including Monterey Bay and Big Sur, CA has exhibited short-term anomalous oceanographic conditions (from 2004-2005), including delayed upwelling and anomalous warming. This time period corresponds with the onset of the shift from diatoms to dinoflagellates in the cCCS, and it is likely that the regime shift, predictive of current trends in climate change, is responsible for the increased dinoflagellates.

The physical oceanographic conditions predicted for the CCS with climate change are consistent with the hypothesis that physical parameters will favor dinoflagellate dominance, as previous research and this dissertation have indicated that dinoflagellates’
migration for nutrients from depth offers a distinct advantage over non-motile phytoplankton. This dissertation has addressed a long-unanswered assumption that Fe, arguably one of the most important nutrients for phytoplankton, is available to migrating dinoflagellates. Also, with a predicted decrease in inorganic nutrient input, the ability to utilize DOP compounds would be advantageous, as DIP is introduced primarily through upwelling in the CCS. Dinoflagellates are therefore well adapted to predicted future climate regime shifts within the CCS; documenting dinoflagellate nutrient assimilation and preference may yield an understanding of the causal relationship seen in the cCCS and other regimes of recent decadal trends in increased dinoflagellates.

Future research must include modeling of these regime shifts and the observed changes in primary productivity. These long-term and spatially extensive trends are difficult or impossible to study in the laboratory or through the use of short-term mesocosm experiments. Ecosystem models have historically not addressed phytoplankton diversity (Goebel et al. 2010), and dinoflagellates are inherently difficult to model, based on their complex life cycle and unorthodox nutrient acquisition. It is apparent that these regime shifts include dramatic changes in the biogeochemical cycling of nutrients; preferential uptake of organic nutrients and vertical migration, are not addressed in typical numerical models. An advanced approach to coupled physical-biological ecosystem modeling, as proposed by Follows et al. (2007) and Goebel et al. (2010) allows the modeler to input reasonable ranges for rate-controlling parameters, and this, in addition with modeled swimming behavior of dinoflagellates (Yamazaki et al. 2013) could be key to predicting basin scale oscillations and global warming outcomes in the
CCS, which would lead to better understanding of the relationships observed within these increasingly dinoflagellate-dominated regimes.
References


