A QUANTITATIVE ANALYSIS OF ALKALINE PHOSPHATASE ACTIVITY IN HE‘EIA FISHPOND

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Abstract

Dissolved organic phosphorus (DOP) is a potentially significant source of bioavailable P to primary producers in aquatic systems. The presence of the alkaline phosphatase (APase) enzyme indicates inorganic P deficiency and the potential for utilization of the DOP pool. Seasonal and spatial variability of alkaline phosphatase activity (APA) was quantified in He'eia Fishpond, a coastal brackish-water pond adjacent to Kaneohe Bay, Hawaii. Whole community APA (> 0.7 μm), normalized to chlorophyll *a*, revealed changes in APA in concert with varying nutrient inventories and ratios. Specifically, we observed elevated dissolved organic nitrogen to phosphate ratios (DIN:DIP) following a major storm event, accompanied by elevated APA and alterations in phytoplankton community. Taken together, these data suggest that storm pulses of fluvial material into the coastal ocean can significantly perturb the resident phytoplankton community. Ultimately, investigating the potential bioavailability of DOP increases our understanding of controls on primary production and phytoplankton community structure.

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Chapter 1

Introduction

1.1 Nutrient Limitation: A Brief Review

Primary production in tropical aquatic environments is typically limited by nutrient availability. Redfield (1934, 1958) observed that globally, phytoplankton intracellular nutrient concentrations remained at a surprisingly constant ratio of 106 Carbon (C):16 Nitrogen (N):1 Phosphorus (P). This constant internal ratio, known as the "Redfield Ratio" is a result of preferred nutrient uptake through photosynthesis (Eq. 1).

$$106CO_2 + 16NO_3^- + HPO_4^{2-} + 122H_2O + 18H^+ \xrightarrow{light} (CH_2O)_{106}(NH_3)_{16}(H_3PO_4) + 138O_2 \qquad (1)$$

In marine systems carbon (C) is usually available in excess causing nitrogen (N) and phosphorus (P) availability to restrict primary productivity (Schlesinger 1997). P has been defined as the limiting nutrient in limnic environments and, over geologic time scales, P functions as the limiting nutrient in marine systems as well (Codispoti 1989; Kolowith et al. 2001). On shorter time scales nutrient limitation in the sea becomes more complex. Originally, there was a common bias towards N limitation in marine environments. However, an increasing number of studies over the past two decades have described marine systems that appear to be P limited including: the Mediterranean Sea, Sargasso Sea, Chesapeake Bay, subtropical North Pacific (Palenik & Dyhrman 1998), the Oregon coastal upwelling system (Ruttenberg and Dyhrman 2005; Dyhrman and Ruttenberg 2006) and the central Atlantic (Vidal et al. 2003). Defining a limiting nutrient can prove difficult and is more complicated than determining which nutrient falls below analytical detection limits first. Arrigo (2005) discusses the potential for multiple resource co-limitation, and defines three specific forms of co-limitation including: 1) multi-nutrient, 2) biochemical, and 3) community co-limitation (Fig. 1.1). In addition, the bioavailability of nutrients present and the ability of biological organisms to adapt to low nutrient conditions should also be considered. Nutrient deficiency indicators such as: the presence of

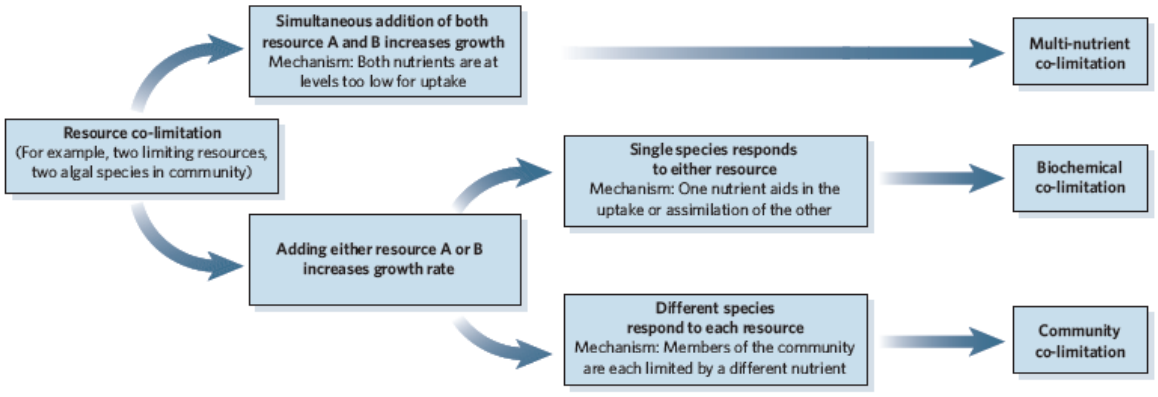

Figure 1.1: A schematic summarizing the three forms of resource co-limitation (Arrigo 2005)

enzymes produced under nutrient deficient conditions, dissolved nutrient ratios, and the composition of intracellular nutrients in biogenic material are all ways to increase the validity of a defined limiting nutrient (Healey and Hendzel 1980; Hecky and Kilham 1988; Howarth 1988). Defining growth limiting factors enables an understanding of the effects that changes in nutrient inputs will have on primary productivity in an ecosystem, and ultimately how this will impact the productivity of higher trophic levels in that ecosystem. Understanding nutrient limitation helps form effective environmental regulations such as the ban of phosphorus based fertilizers in lake watershed areas that ultimately prevented harmful algal blooms sparked by an addition of the limiting resource- P.

In this study bioassay experiments, and dissolved nutrient concentrations will be used to investigate the nutritional status of a coastal Hawaiian environment, He'eia Fishpond and the potential utilization of dissolved organic phosphorus (DOP) as a source of bioavailable P.

1.2 Role of Phosphorus in Marine Systems

Phosphorus (P) is an essential nutrient required for all living organisms. Within the cell P is used for metabolic functions including: structure (cell lipids), energy transfer (nucleotides), and cellular coordination (nucleic acids) (Karl and Yanagi 1997). In marine environments, the P pool is comprised of dissolved inorganic

phosphorus (DIP), dissolved organic phosphorus (DOP), and particulate bound phosphorus (P_p). DIP represents the bioavailable component of the P pool, specifically the ion HPO_4^{2-} (orthophosphate), which can be directly assimilated by phytoplankton (Chróst and Overbeck 1987). Dissolved organic phosphorus (DOP) is not directly bioavailable and is often found at higher concentrations in the photic zone than DIP (Ruttenberg and Dyhrman 2005; Shan 1994). Phytoplankton preferentially uptake DIP, as no additional expenditure of energy required, the DOP pool can be accessed under deplete DIP conditions through the use of phosphatases.

1.3 Alkaline Phosphatase as a Nutrient Deficiency Indicator

Phosphatases are biologically produced enzymes that facilitate the production of DIP through catalyzing the hydrolytic cleavage of orthophosphate from a dissolved organic phosphorus (DOP) molecule. A variety of enzymes are capable of the decomposition of DOP (e.g. diesterase, phytase, C-P lyase, 5' nucleotidase and alkaline phosphatase) and can be produced by bacteria, algae (phytoplankton), and zooplankton (Dhyrman and Ruttenberg 2006). Once orthophosphate is hydrolyzed it can be directly assimilated by organisms to meet their nutritional P demands (Sebastián et al. 2004).

Factors controlling phosphatase production are dependent upon which organism is producing the phosphatase, and what form the phosphatase is in. Phosphatase regulation can be separated into three categories: 1) constitutive- enzymes constantly produced by a cell regardless of nutrient availability, 2) inducible- enzymes produced when exposed to specific substrates and, 3) repressible- enzymes synthesized when nutrient availability becomes low (Kuenzler and Perras 1965; Jansson et al. 1988). Alkaline phosphatase (APase) is both inducible and repressible, as its production is regulated by inorganic phosphate concentrations; therefore, as DIP is diminished the production of APase will increase and as DIP increase APase production will be repressed (Dyhrman and Ruttenberg 2006). This quality allows APase to be an effective nutrient deficiency indicator (NDI) and it has commonly been used as such in studies of both limnic and marine systems (Chróst and Overbeck 1987; Ruttenberg and Dyhrman 2005; Rose and Axler 1998; Koch et al. 2009).

The cellular location of alkaline phosphatase is also an important contributor to its use as a NDI. APase is extracellular, found either bound to the cell surface or as a free enzyme within the water column. This characteristic allows APase to be easily measured without the requirement of cell lysis. APase has a maximum activity in a pH range of 8.5-9.5 (Hoppe 2003), and its activity can also be affected by temperature, ionic strength, and metal ions (Jannson et al. 1988). APase is a metallo-enzyme, and requires Zinc (Zn^{2+}) and Magnesium (Mg^{2+}) at its active bond sites for the hydrolysis reaction to proceed (Shaked et al. 2006). APase has an affinity for specific substrates, in particular phosphomonoesters (Fig. 1.2), but does not discriminate between the organic component of the organic P compound it hydrolyzes.

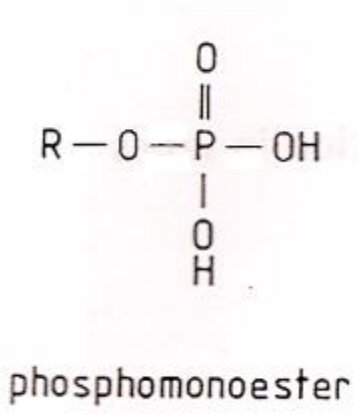

Figure 1.2: Schematic of a phosphomonoester

1.4 Measuring Alkaline Phosphatase Activity: Enzyme Kinetics & Reaction Mechanism

Alkaline Phosphatase catalytically hydrolyzes dissolved organic phosphorus compounds producing orthophosphate and an organic moiety, and includes four steps (Jannson et al. 1988) (Fig. 1.3):

1. The enzyme (EH) bonds to the phosphomonoester (the substrate) through a non-covalent bond (•).

2. An alcohol is released from the enzyme-substrate complex, and orthophosphate becomes covalently bound to the enzyme, producing a phosphoryl-enzyme compound.

3. Hydrolytic conversion of the phosphoryl-enzyme compound to a non-covalent complex occurs and,

4. Orthophosphate is released and the free enzyme is regenerated.

The rate at which APase cleaves orthophosphate from DOP compounds is known as its enzymatic activity. In an experimental setting, alkaline phosphatase activity (APA) can be measured through the use of an artificial, fluorogenic substrate that mimics DOP compounds found in nature. When APase in an environmental sample is exposed to the artificial substrate, the hydrolysis reaction occurs producing dissolved orthophosphate and a fluorogenic compound. The production of the fluorogenic compound can be measured over time. This reaction proceeds in accordance to the Michaelis-Menten theory, which describes enzyme kinetics using equation 2.

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

In which, V_0 is the catalytic rate of hydrolysis, V_{max} is the maximum velocity at which the reaction proceeds in relation to [S], the substrate concentration. K_m is the Michaelis constant, and is defined as the concentration of substrate that allows the reaction to occur at half of its maximum velocity. K_m indicates the affinity of the enzyme for a particular substrate.

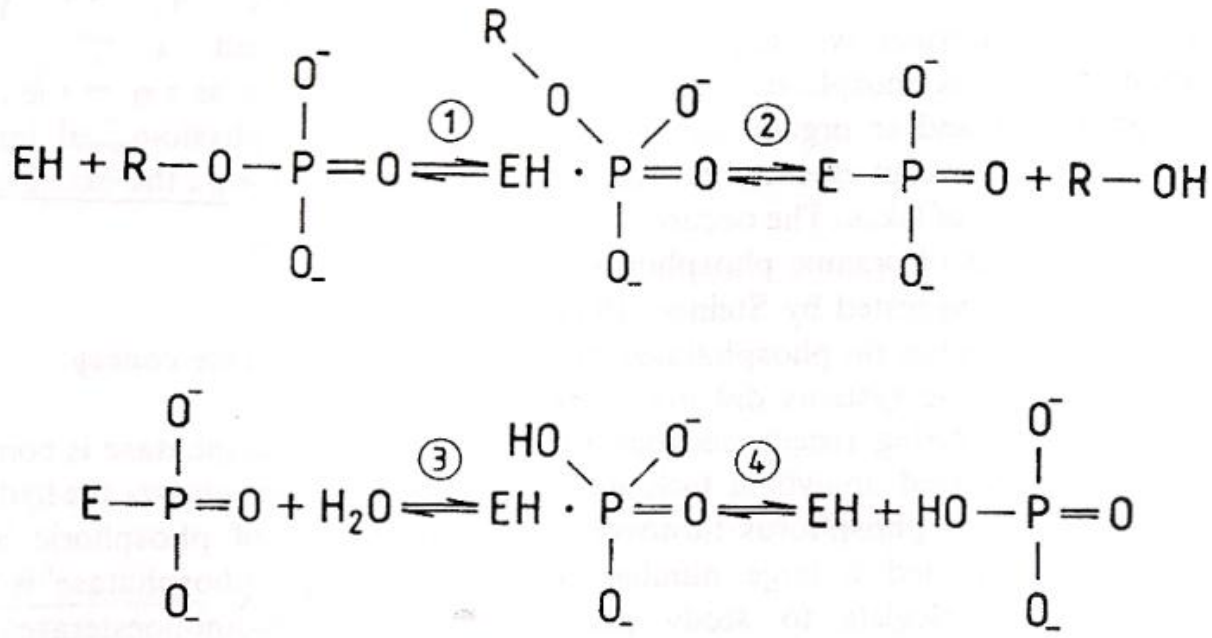

Figure1.3: Enzymatic hydrolysis of phosphate from an organic compound as described by Jansson et al. (1988)

At saturating substrate concentrations, the hydrolysis reaction will occur at its maximum rate. When saturated, V_{max} will be equivalent to the concentration of enzyme present (Jansson et al. 1988). In order for APA measurements from field studies to be comparable between different environments, the artificial substrate concentration must be added to samples at saturating levels to ensure all APase present will react. The substrate saturation concentration can be determined by plotting the initial velocity (V_0) against substrate concentration [S] for a range of [S] until the curve asymptotically levels off indicating substrate saturation (Fig. 1.4). In this study, Michaelis-Menten saturation plots will be used to determine the saturating concentration of the substrate used for the analysis of APA in He'eia Fishpond samples.

1.5 An Introduction to He‘eia Fishpond

He‘eia Fishpond (HFP), located in Kaneohe Bay on the windward (East) side of O‘ahu, is the area of interest for this study. He’eia Fishpond is comprised of a walled area totaling 0.356 Km^2, of which .054 Km^2 is covered by invasive mangrove. The wall is segmented by makahas (gates) that control fresh and marine inputs from He‘eia Stream and Kaneohe Bay, respectively (Fig. 1.5). The makahas allow fishpond managers to control freshwater and marine inputs such that water composition can be manipulated to promote growth of a desired crop. At the present time this control also allows the fishpond to act as a natural laboratory for environmental research.

The composition and depth of the water column within He‘eia Fishpond is variable and driven by tidal changes with an average depth of < 2 m. A sediment layer (5-35 cm thick) covers the floor of the fishpond, with a gradient ranging from course sandy sediment in areas adjacent to ocean makahas to silty/mud proximal to the river inputs.

He‘eia Fishpond is located at the base of a steep, mountainous drainage basin that responds quickly to rainfall, with rainfall intensity ultimately controlling terrestrial inputs. Hawaii’s precipitation is characterized by a small steady base flow

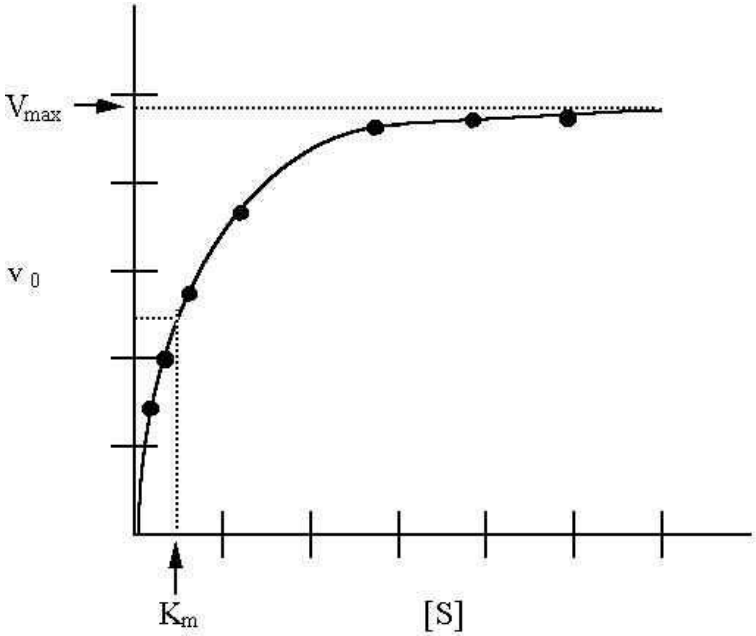

Figure 1.4: Idealized Michaelis-Menten Saturation Plot

Figure 1.5: Location of He'eia Fishpond in relation to O'ahu and Kaneohe Bay.

punctuated by large inputs from storm events (Ringuet and Mackenzie 2005). Storm events can account for up to 80% of the total yearly nutrient and sediment inputs to coastal environments (Milliman and Meade 1983). For this reason, storm driven fluvial inputs can dramatically change the nutrient budget: potentially perturbing the phytoplankton community structure of He‘eia Fishpond and ultimately its productivity.

Historically, Hawaiian fishponds were used for aquaculture, providing the surrounding population with a sustainable food source. Since the mid-1800’s changes in land use have increased erosion in He‘eia Fishpond’s watershed, negatively impacting the ecology of the fishpond. Today, He’eia Fishpond remains of cultural importance and provides insight to historical resource management practices. The fishpond is currently managed by a non-profit organization, Paepae o He‘eia, whose primary objective is restoring the fishpond to its non-impacted state. One of goals of this thesis research is to investigate the biogeochemical processes effecting primary productivity of the fishpond to aid in the restoration of He’eia.

Chapter 2

Initial Kinetic Experiments: Michaelis-Menten Saturation Plots

2.1 Background

Measuring the maximum potential alkaline phosphatase activity allows comparisons of APA between sample sites to be made. Maximum potential activity occurs when the artificial substrate is added at a saturating concentration. The purpose of the initial kinetic experiment was to determine the saturating concentration of the artificial substrate for three distinct regions of He'eia Fishpond: (1) He'eia Stream (HS), (2) He'eia Fishpond (HFP) itself, and (3) adjacent Kaneohe Bay (KB) (Fig. 2.1). Due to known differences in freshwater verse marine P limitation these sites, with distinct salinities: 0 ppt, 28.8 ppt, and 31.3 ppt, respectively, were expected to exhibit diverse levels of APA that might require different concentrations of substrate to reach maximum potential activity. These regions were chosen because they encompass the extreme characteristics found throughout the entire field site.

At each site samples were collected in replicate. Varying amounts of substrate (0-14 μM) were added to each set of replicates and the velocity of the hydrolysis of the fluorogenic compound was measured fluorometrically on a plate reader. The velocity was then plotted against the substrate concentration to attain a Michaelis-Menten (M-M) saturation plot, from which the saturating substrate concentration can be derived.

2.2 Methods

2.2.1 Sample Collection and Processing

Three sample sites were chosen for the initial kinetic experiments including; 1) He'eia Stream (HS, RIVER), 2) within He'eia Fishpond (HFP, 9SFC) itself and 3) adjacent Kaneohe Bay (KB, OCN2) (Fig.6). At each site bulk water sample was

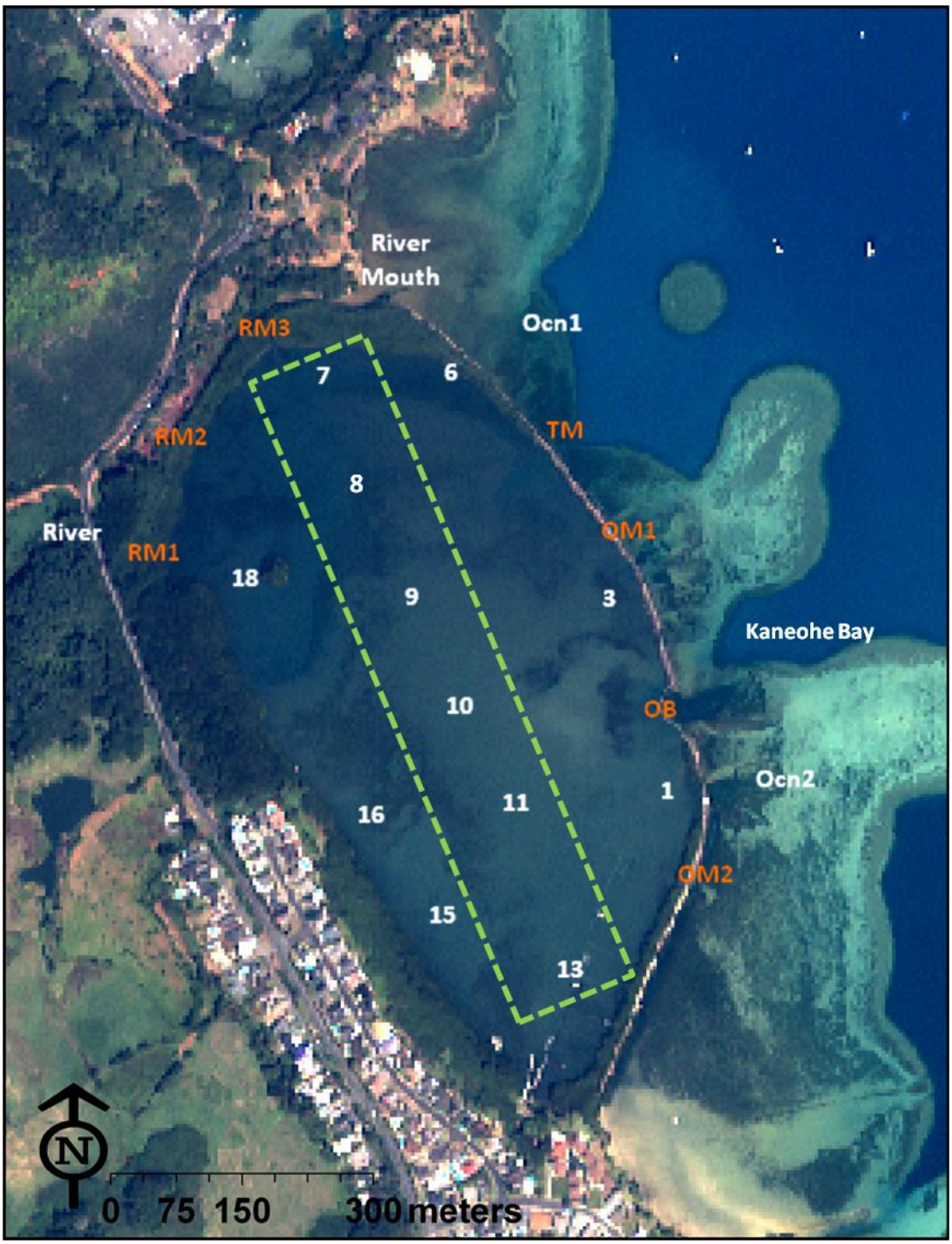

Figure 2.1: An aerial photograph of He'eia Fishpond. All sampling locations are labeled appropriately. Storm transect is boxed in green. Stake 8 is not part of the storm sampled transect.

collected in 6 L carboys and stored on ice until filtered, within two hours after collection. Carboys were homogenized by gently inverting, three times, before sampling. Replicate APA samples were collected by filtering 220 mL aliquots through muffled (550°C) 0.7 μm GF/F (Whatman®) filters. Samples were stored frozen (-30°C) in plastic Petri dishes until analyzed.

2.2.2 Analytical Methods

Replicate samples were incubated at room temperature in 2 mL of artificial seawater (30 ppt) (ASW) on a shaker table (200 rpm) for 10 minutes prior to the start of the assay. Variable amounts of the artificial substrate 6,8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP, Molecular Probes®) were added to final concentrations ranging from 0-14 μM . Directly after the addition of DiFMUP, 150 μL splits were transferred to wells in a 96-well, half-area, solid black (Co-Star®) plate. Hydrolysis of DiFMUP to the fluorescent product 6,8-difluro-7-hydroxy-4-methylcoumarin (DiFMU) was analyzed immediately on a temperature controlled (25°C) Synergy HT multi-well plate reader (BioTek®) (360/460, excitation/emission) using the KC4 software package. Additional fluorescence measurements were made every 10 minutes over a 60 minute time course.

2.3 Results

A time course of fluorescence build up due to enzymatic hydrolysis of DiFMUP to the fluorogenic compound, DiFMU allows for the velocity of the hydrolysis reaction to be determined. Hydrolysis occurred at a constant velocity over a 60 minute time course, producing a slope with an average R^2 of 0.998 for all samples analyzed (Fig.2.2-2.4).

The velocity of hydrolysis for different substrate concentrations was then plotted against the substrate [DiFMUP] concentrations to generate a M-M saturation plot.

In KB (Fig. 2.5) samples, the velocity of enzymatic hydrolysis increased with an increase in substrate [DiFMUP] concentration until asymptotically reaching a maximum velocity (V_{max}) at a substrate concentration of 8 μM DiFMUP. Hydrolysis velocities for HS (Fig. 2.6) and HFP (Fig.2.7) followed a similar trend, reaching V_{max} at a substrate concentration of 10 μM DiFMUP.

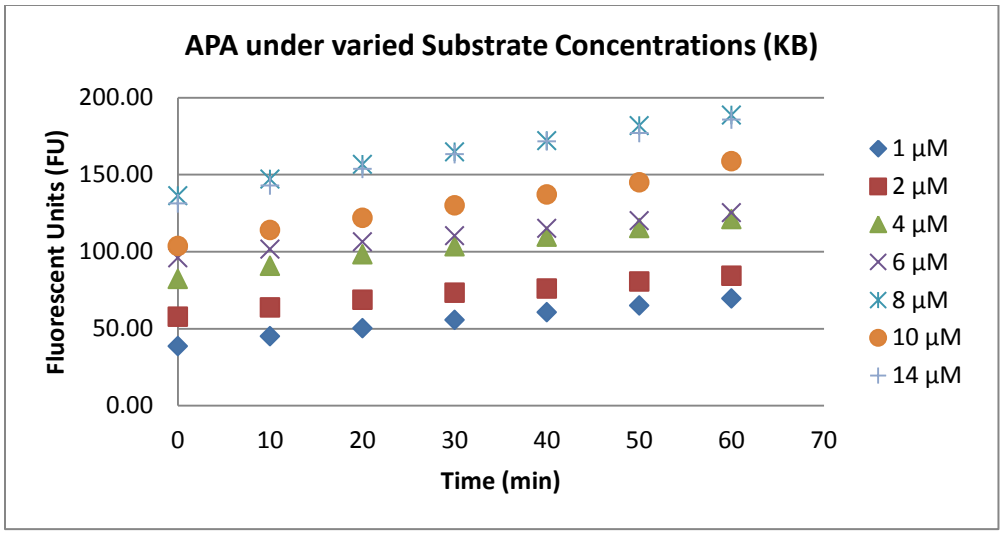

Figure 2.2: Kaneohe Bay (KB): Enzymatic hydrolysis of DiFMUP to the fluorescent product DiFMU results in increased fluorescence with time. Different substrate concentrations were assayed (1-14 µM) in order to provide variable hydrolysis rates for construction of Michaelis-Menten Plots.

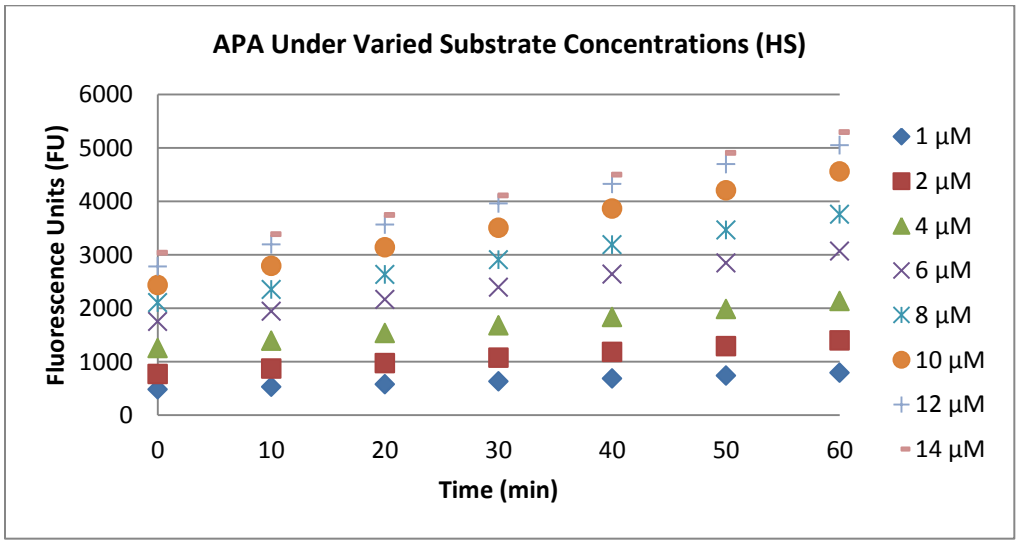

Figure 2.3: He'eia Stream (HS): Enzymatic hydrolysis of DiFMUP to the fluorescent product DiFMU results in increased fluorescence with time. Different substrate concentrations were assayed (1-14 µM) in order to provide variable hydrolysis rates for construction of Michaelis-Menten Plots.

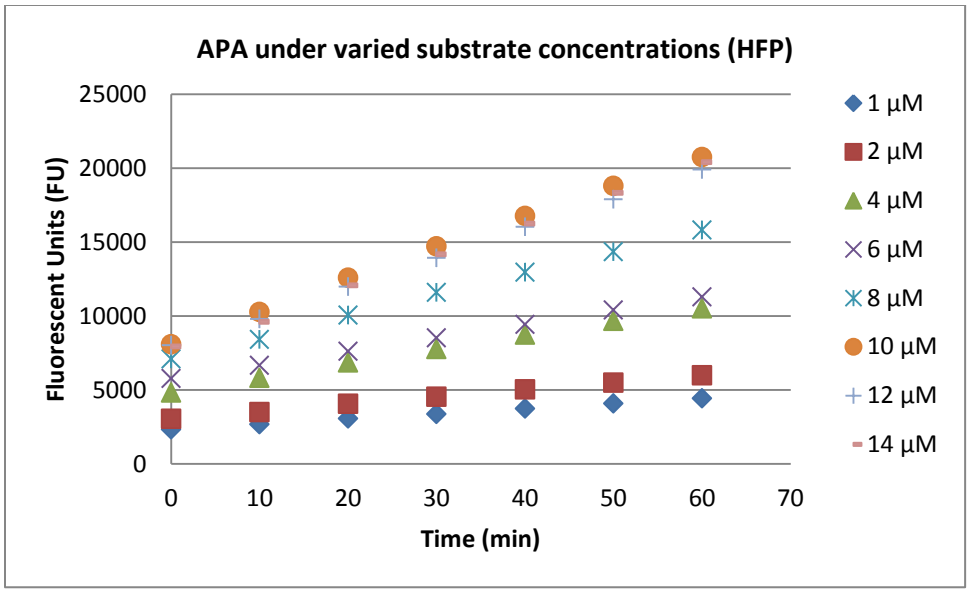

Figure 2.4: He'eia Fishpond (HFP): Enzymatic hydrolysis of DiFMUP to the fluorescent product DiFMU results in increased fluorescence with time. Different substrate concentrations were assayed (1-14 µM) in order to provide variable hydrolysis rates for construction of Michaelis-Menten Plots.

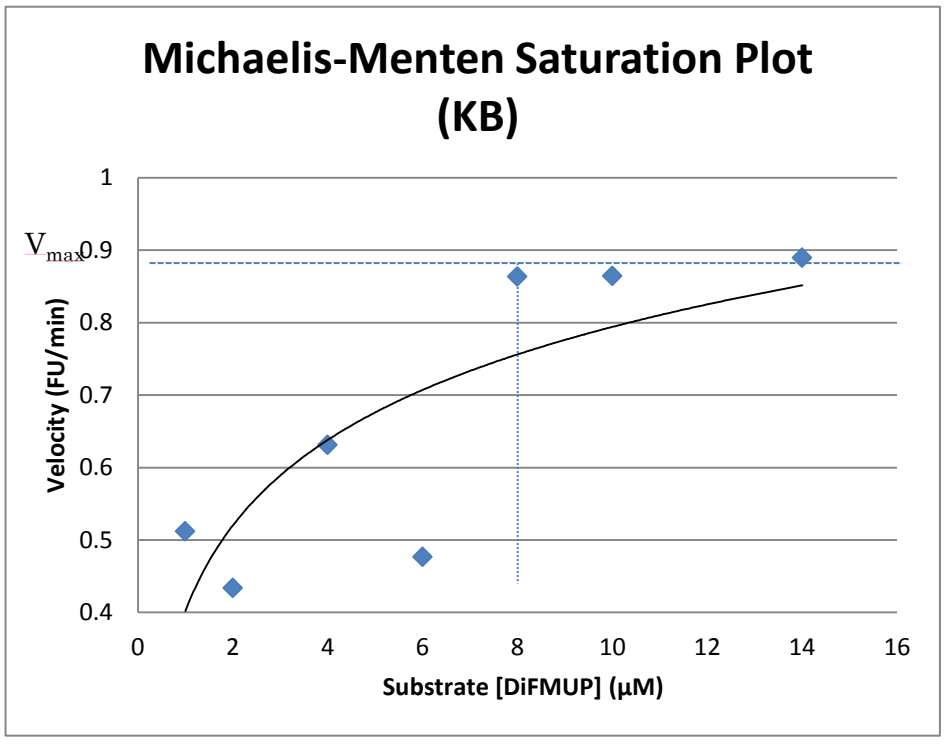

Figure 2.5: M-M saturation plot for Kaneohe Bay samples

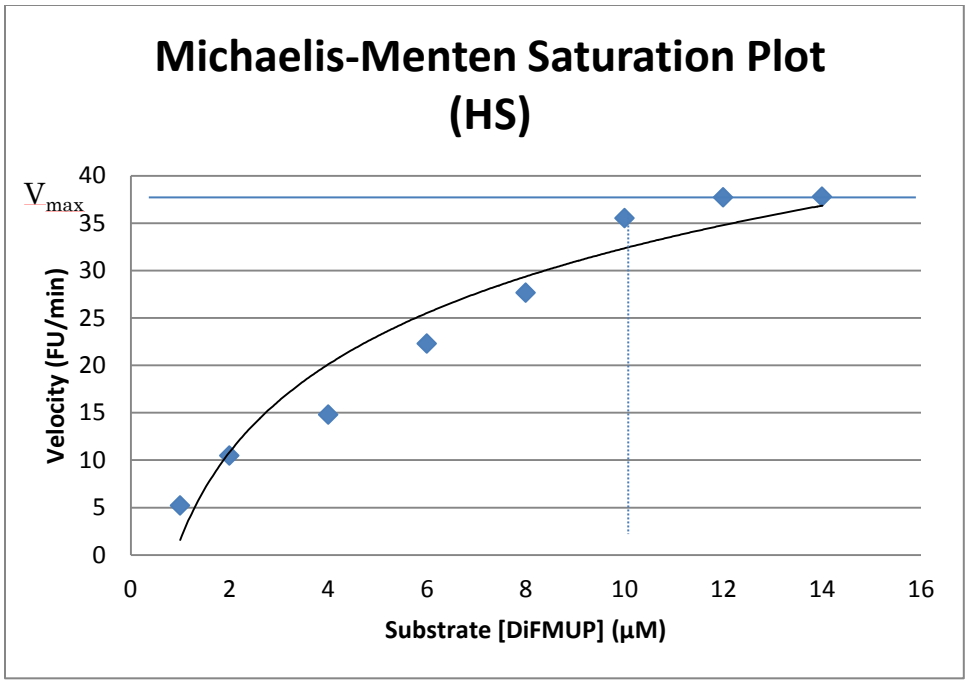

Figure 2.6: M-M saturation plot for He'eia Stream samples

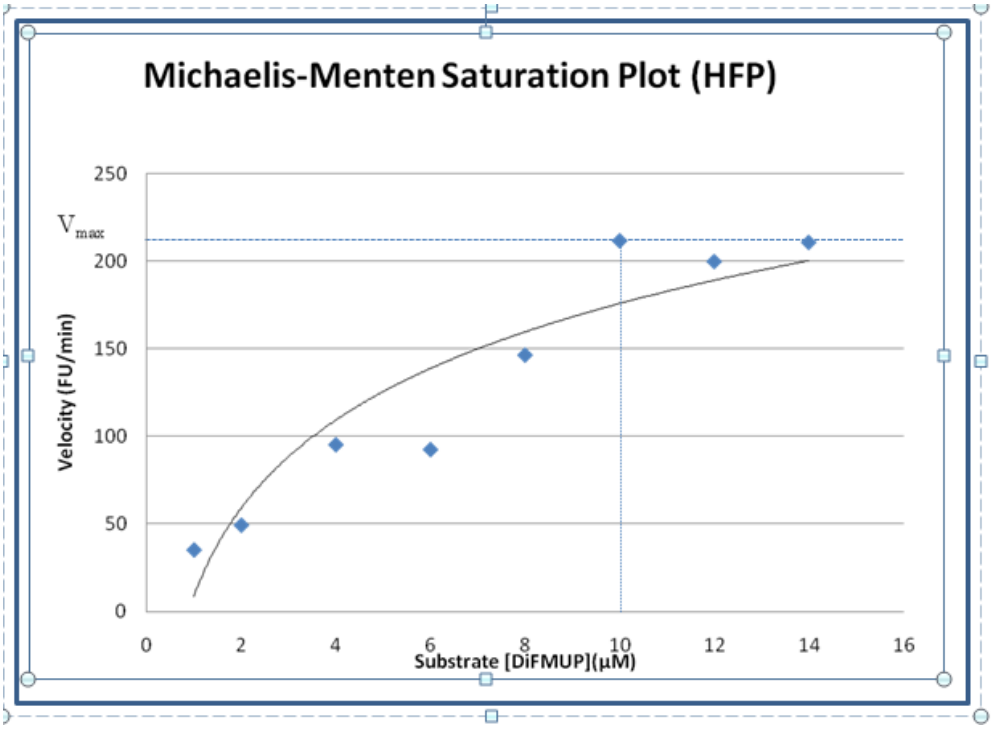

Figure 2.7: M-M saturation plot for He'eia Fishpond samples

2.4 Discussion

Michaelis-Menten plots can be used to define substrate saturation. Saturation occurs at $V_{max,}$ where any further addition of substrate will not promote further enzymatic hydrolysis. V_{max} is reached at 8 μM DiFMUP in KB and 10μM DiFMUP in HS and HFP.

DiFMUP should be added to all samples for analysis of APA at a saturating concentration in order to measure maximum potential activity, making comparison between sites possible. However, it is also important to avoid adding substrate at extreme excess as high substrate concentrations produce increased background fluorescence (Rose and Axler 1998).

Due to a large quantity of APA samples to be analyzed, substrate was added to a final concentration of 10 μM DiFMUP to all samples for the purpose of simplicity and to ensure direct comparison between sites.

Chapter 3

Spatial Variability and Storm Driven Changes in APA in He'eia Fishpond

3.1 Background

Hawaii is characterized by two distinct seasons, a dry season (May- October) and a wet season (October –April). Reduced river flow during the dry season allows organic material and nutrients to build up in soil waters. The first storm event of the wet season is typically referred to as a "first flush" event, because rivers and streams flush out the material that has built up since the end of the previous wet season, transporting it to the coastal ocean. As a result, coastal waters, which have not received significant terrestrial inputs for the duration of the previous dry season quickly become enriched in nutrients. For the purposes of this study, a 'storm event' is classified as more than 5.08 cm of rainfall observed within a 24 hour period. This criteria for identifying a major storm was defined previously by Hoover et al. (2006), and by Ringuet and Mackenzie (2005), who conducted storm event research in Kaneohe Bay.

Rivers typically are enriched in nutrients relative to coastal marine waters (e.g., Schlesinger 2003), so that increases in phytoplankton biomass often follows episodic riverine input to the coastal ocean (Ringuet and Mackenzie 2005; Hoover et al. 2006). Besides having higher nutrients in general, riverine input typically has a high concentration of nitrate (NO_3^-) relative to phosphate (PO_4^{3-}) and, consequently, is characterized by a high N:P ratio. An increase in stream inputs following a storm event is thus expected to increase the N:P ratio of coastal waters, in this case of He'eia Fishpond, promoting phytoplankton blooms and the rapid assimilation of bioavailable N and P. Due to the high N:P ratio of storm input, a draw down in available P is expected to drive the phytoplankton into a phosphate stress response, and thus promote the production of alkaline phosphatase (APase). The increase in phytoplankton primary productivity that is expected to follow a storm event should be reflected by an increase in chlorophyll *a*, a proxy for biomass. If the phytoplankton community is driven to a P-stress response due to the high N:P ratio of storm input, an increase in APA should be observed.

To investigate the biogeochemical changes induced by storm input into He'eia Fishpond, the following sampling scheme was devised: (1) monthly sampling to determine base-line nutrient, chl-A and APA levels, and (2) intensive storm sampling to document and quantify perturbations from baseline conditions (Fig. 2.1).

3.2 Methods

3.2.1 Study Site

He'eia Fishpond receives water from the land, through input from He'eia Stream, and from Kaneohe Bay, through the gates (makaha) in the seawall that separate the fishpond from the stream and bay. During monthly sampling, He'eia Stream (RIVER) and Kaneohe Bay (OCN2) sites were sampled to evaluate the composition of the fresh and marine water end members (Fig. 2.1). OCN1 is located just bayward of the point where He'eia Stream discharges into Kaneohe Bay (Fig. 2.1), and was sampled to observe the effects of freshwater being directly discharged into Kaneohe Bay without the buffer of the fishpond. Sampling sites included all makahas, which are the points at which freshwater and seawater enter and leave the pond, as well as the large broken portion of the fishpond wall proximal to Kaneohe Bay (OB), to evaluate all sources of nutrient inputs and outputs. Within the fishpond, surface and bottom water sampling was conducted at ten stakes to observe spatial variations throughout He'eia Fishpond, as well as differences between surface and deep water to characterize vertical changes in the water column at each site. Intensive storm sampling was conducted along six-station transect within He'eia Fishpond (Fig.2. 1) for several days after the first-flush storm event to document biogeochemical responses to storm nutrient inputs.

3.2.2 Sample Collection and Processing

Monthly sampling occurred over the course of one year (August 2007- August 2008) at designated sites (Fig.2.1). Bulk water samples were collected in 1 L HDPE and 250 mL amber HDPE bottles, and were stored on ice until filtered, typically within two hours after collection. All bottles and carboys used for sample collection were acid-cleaned (10% HCl) and rinsed three times with sample water prior to collection.

For the purpose of this study samples for the analysis of dissolved inorganic nutrients (NO_3^-, NO_2^-, PO_4^{3-}, and NH_4^+), chlorophyll *a* (Chl *a*),and alkaline phosphatase activity (APA) were collected.

After gently inverting the bottle three times to homogenize, splits of sample collected in the 1-L HDPE bottles were filtered through muffled (550°C) 0.7 µm GF/F (Whatman®) filters to collect cell concentrates for analysis of APA, and through 0.2 µm acid-clean (10% HCl) GHP (Pall®) filters to collect filtrate for dissolved nutrient analysis. Filters for APA analysis were folded, wrapped in aluminum foil, and stored frozen (-30°C) until analyzed. Filtrate (<0.2 µm) was collected in HDPE bottles and segregated into two subsamples: a frozen (-30°C), untreated split and a refrigerated-acidified split (pH=1) for later nutrient analysis.

Splits of sample collected in the 250-mL amber HDPE bottles were filtered through 0.7 µm GF/F (Whatman) filters, after first gently inverting the bottle three times to homogenize, for analysis of Chl *a*. Cell concentrates (filters) were stored frozen (-30°C) in aluminum foil wrapped borosilicate test tubes until analyzed.

3.2.3 Analytical Methods

3.2.3.1 Alkaline Phosphatase Activity (APA)

Aluminum foil wrapped cell concentrates were carefully unfolded using acid-clean (10% HCl) forceps and placed in plastic Petri dishes. APA was analyzed using a fluorometric method described by Dyhrman & Ruttenberg (2006) with only slight modification. Samples were incubated at room temperature in 2 mL of 30 ppt artificial seawater (ASW) on a shaker table (200 rpm) for 10 minutes prior to the start of the assay. The fluorogenic DOP substrate, DiFMUP, was added to a final concentration of 10 µM, which had previously been determined to be saturating in the kinetic experiments (see description of kinetic experiments in Chapter 2). Directly after the addition of DiFMUP,150 µL splits were transferred to wells in a 96-well, half-area, solid black (Co-Star®) plate. Hydrolysis of DiFMUP to the fluorescent product 6,8-difluro-7-hydroxy-4-methylcoumarin (DiFMU) was quantified immediately on a temperature controlled (25°C) Synergy HT multi-well plate reader (BioTek®) using the Gen5 software package. At time intervals adjusted

to the rate of DiFMU production, 150 µL splits were added to a new well in the same plate and analyzed. Between fluorescence measurements samples were continuously shaken on a shaker table (200 rpm). Production of the DiFMU fluorescent compound was compared to triplicate 0-5 µM DiFMU (Molecular Probes®) in ASW standard curves. Blanks were prepared by filtering 100 mL aliquots of ASW (30 ppt) through muffled (550°C) 0.7 µm GFF (Whatman®) filters or acid-clean (10% HCl) 0.2 µm GHP (Pall®) filters, depending upon which filter was used for sample collection. Blanks were prepared prior to assays and stored frozen (-30°C) in plastic Petri dishes until used. At the time of the assay, blanks received equivalent addition of ASW and DiFMUP substrate and were analyzed alongside samples. A detailed protocol for the analysis of APA samples can be found in appendix A.

3.2.3.2 Chlorophyll a (Chl a)

Chlorophyll *a* (Chl *a*) samples were analyzed using two methods, fluorometry and High Performance Liquid Chromatography (HPLC) as desribed below.

Fluorometer- 5 mL of acetone (≥99.9%, ACS certified, Fisher Scientific®) was added to each sample in borosilicate test tubes. Samples were centrifuged to ensure the entire filter was submerged in acetone. Chl *a* was extracted for 24 hours in a freezer (-30°C). After 24 hours, samples were removed from the freezer and brought to room temperature prior to analysis. A 1:1 dilution (1 mL 99.9% acetone: 1 mL sample) was performed on all samples in 75 mm lime glass culture tubes. Samples were analyzed in duplicate on a Trilogy (Turner Designs®) fluorometer equipped with a Non-Acidified module. Sample fluorescence was compared to a 0-185 µg L^{-1} chlorophyll *a* standard curve (Turner Designs®).

High Performance Liquid Chromatography (HPLC)- Techniques to detect and quantify photopigments were employed using a modified version of the reverse-phase C-18 HPLC method of Wright et al. (1991). These analyses were performed on a Varian 9300 auto sampler in-line with a Beckman DU800 spectrophotometer. In this study, photopigment analysis was used for the quantification of Chl *a*.

3.2.3.3 Dissolved Inorganic Nutrients

Water column samples were analyzed for PO_4^{2-}, NO_3^-, NO_2^-, and NH_4^+ on a Seal Analytical AA3 Auto-analyzer at the USF/USGS Nutrient Biogeochemistry Laboratory in St. Petersburg, Florida using standard, well-established colorimetric methods.

3.3 Results

3.3.1 Blanks and Standards

APA- Fluorescence measurements of APA samples were compared to a linear (R^2=0.99) 0-5 μM DiFMU standard curve in ASW (30ppt) (Fig.3.1). APA of blank filters varied between undetectable- 0.162 nM-P h^{-1}, with an average activity of 0.019 ± 0.034 nM-P h^{-1}.

Chlorophyll a- Fluorescence measurements of Chl *a* samples were compared to a linear (R^2=1) 0- 185 μg L^{-1} standard curve (Fig.3.2). Fluorescence of 100% acetone was used as a blank prior to measurement of samples.

3.3.2 Relationship between Chlorophyll a and APA

APA is positively correlated to the abundance of Chl *a*. Freshwater dominated samples (River, RM1, RM2, and R3) deviate from this trend with higher APA at lower Chl *a* levels (Fig.3.3).

3.3.3 Variability of Baseline APA in He'eia Fishpond

Horizontal Variability- During the one-year, monthly sampling effort that formed the basis of this study, APA was detected in 391 of 407 samples assayed (Fig.3.4). The 16 samples that fell below detection were predominately collected within Kaneohe Bay. APA levels range from undetectable to 30.67 nM-P h^{-1} throughout the field site. Average baseline APA ranges from a high of 9.98 nM-P h^{-1} at RM2 to a low of 0.49 nM-P h^{-1} at OCN2. Higher APA levels were typically observed in freshwater dominated sites (River, RM1, RM2, and RM3); marine dominated sites usually exhibited lower APA levels. Among the marine sites, OCN1 displayed the highest annual average APA. Pockets of increased APA were observed in the northeast

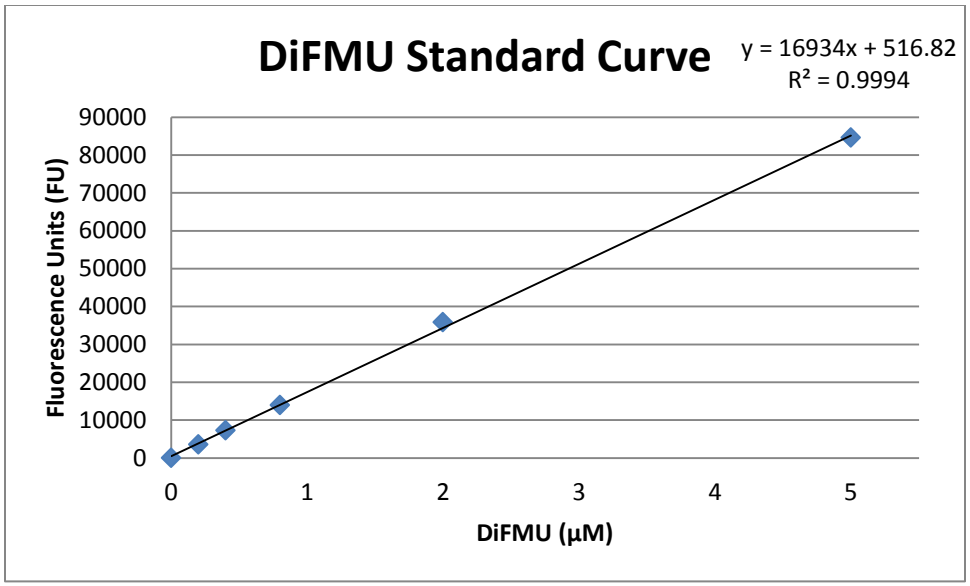

Figure 3.1: DiFMU Standard Curve

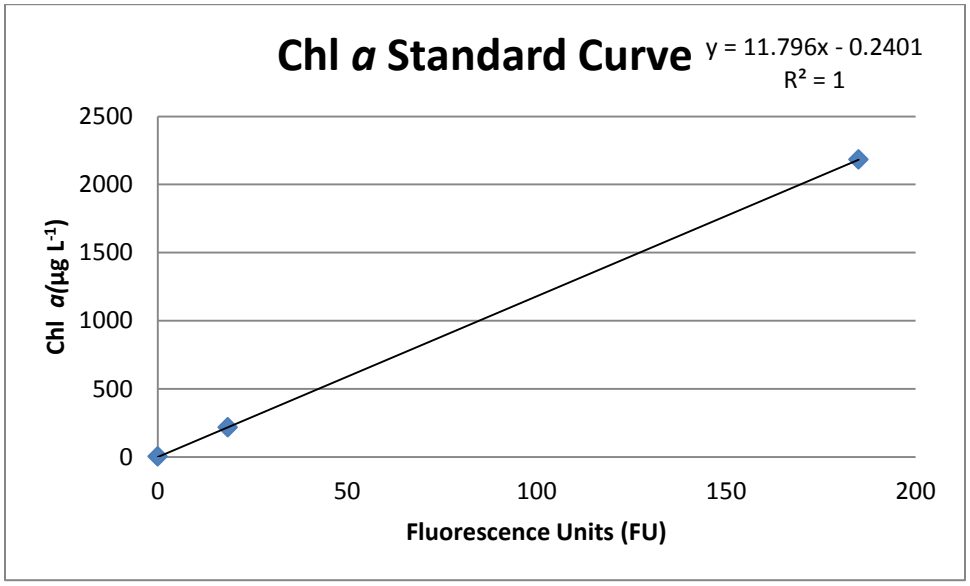

Figure 3.2: Chlorophyll *a* Standard Curve

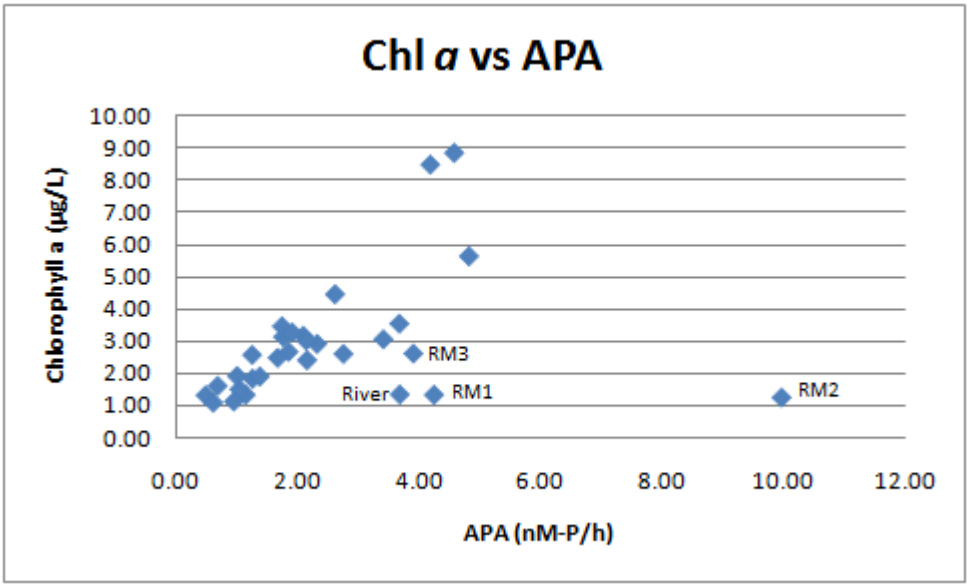

Figure 3.3: Correlation of Chlorophyll *a* and APA. Freshwater outliers are labeled accordingly.

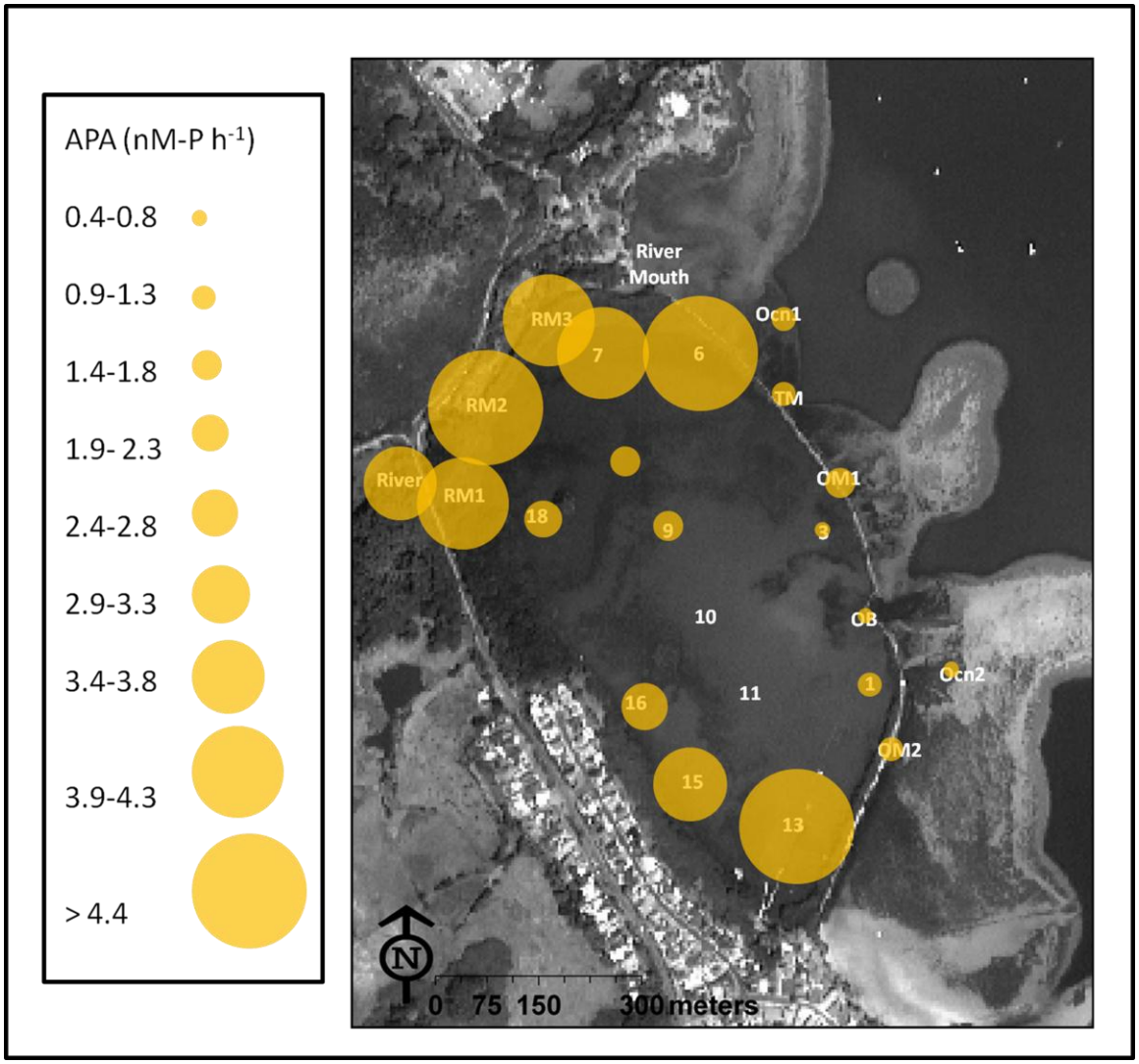

Figure 3.4: Baseline spatial variation of APA. Each circle represents baseline activities at each site over the course of one year during a period free of perturbations.

corner (Stakes 6 and 7) and in the southwest corner (Stakes 13, 15, and 16) of the pond.

Specific APA, e.g., APA normalized to Chl *a* (a proxy for biomass), followed trends similar to those exhibited by non-normalized APA. Specific APA ranged from a baseline high of 9.0 nM-P h^{-1} at RM2 to a low of 0.62 nM-P h^{-1} at OCN2. Regionally, average baseline APA was highest in freshwater dominated sites (4.60 nM-P μg-chla^{-1} h^{-1}), while He'eia Fishpond (0.98 nM-P μg-chla^{-1} h^{-1}) and Kaneohe Bay (1.04 nM-P μg-chla^{-1} h^{-1}) levels were nearly equivalent.

Variability with Depth- Baseline APA levels at depth were higher than in the overlying surface water at 70% of sites within HFP. Differences in surface and bottom water APA range from 0.83% - 36.1% (Fig.3.5).

3.3.4 Storm Driven Changes in Alkaline Phosphatase Activity

The first storm event of the winter season occurred on November 4th, 2007. Storm event sampling occurred daily for 4 days following the storm and then once again 7 days after the storm. Analysis of APA over the post-storm time course shows an increase in activity on days 2 and 3 following the storm event, followed by a return to initial APA levels on days 4-7 post-storm. Spatially, APA was highest at Stake 6 and remained at baseline levels at Stake 18 (Fig.3.6).

Transect samples (Fig. 2.1) collected during post-storm sampling effort show variable time-response at different stations within the pond. APA increases at Stake 7 and Stake 9 two days after the event, with APA levels decreasing by day 3. Stake 11 and Stake 13 display elevated increases in APA on day 3, returning to pre-storm levels the following day (Fig.3.7).

3.4 Discussion

3.4.1 Relationship between Chlorophyll a and APA

Chlorophyll *a* is a robust proxy for phytoplankton biomass in aquatic systems (citation). In this study, a positive correlation between APA and Chl *a* abundance was observed (Fig. 3.3). It is useful to normalize APA to biomass in order to

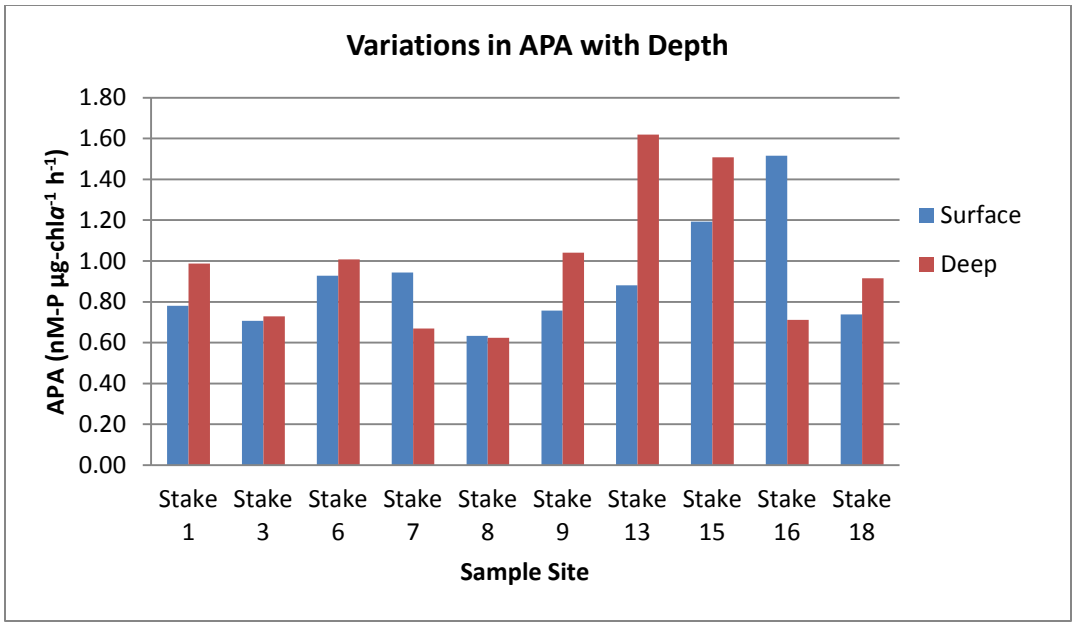

Figure 3.5: Variability in APA with depth at sample site within He'eia Fishpond.

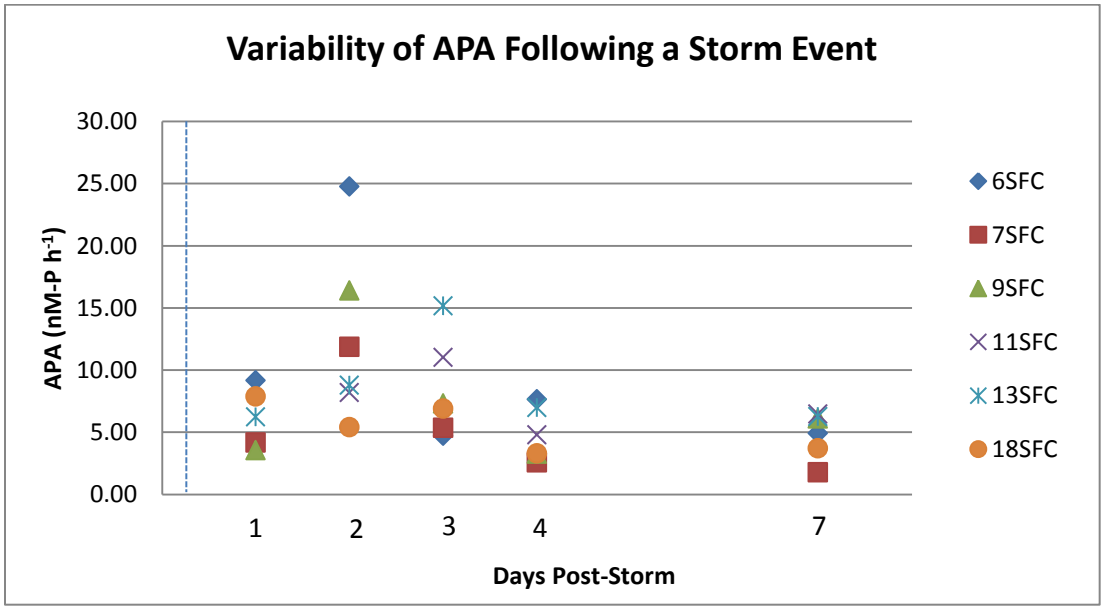

Figure 3.6: Variability of APA following a storm event (indicated with a blue dashed line) over the course of 7 days. Sample locations are indicated in the legend.

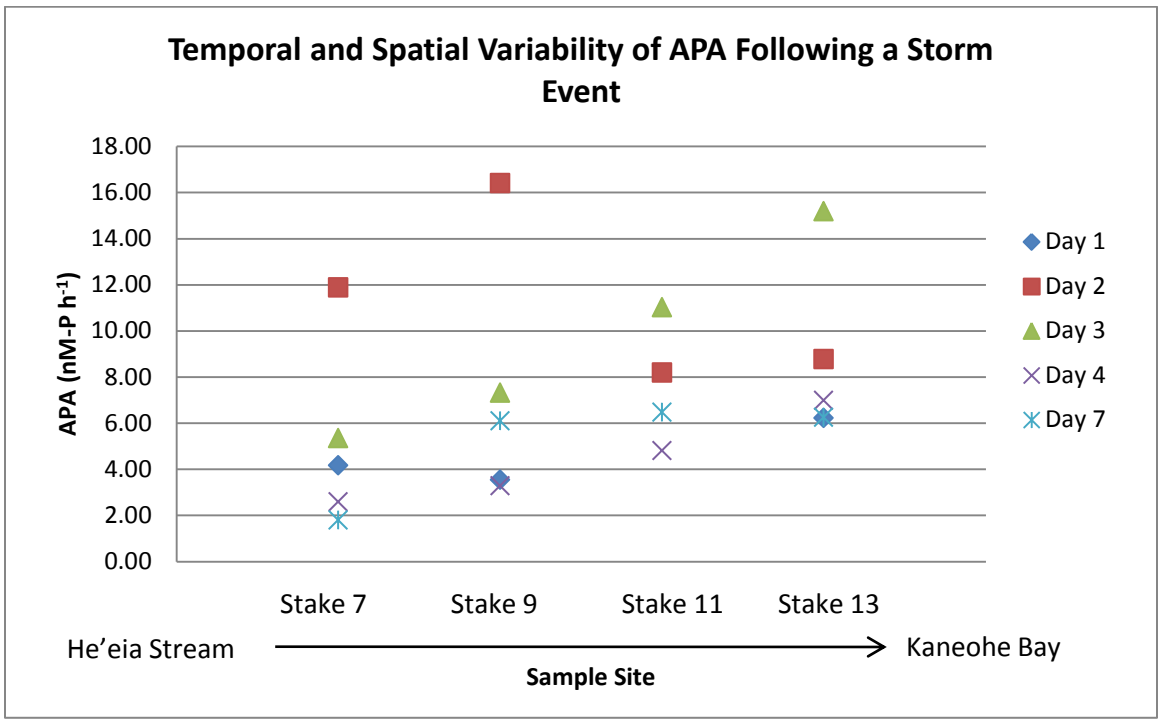

Figure 3.7: Development of APA following a storm event. Sample sites are part of a transect with proximity to stream inputs decreasing from left to right.

determine whether observed variations in APA result from different phytoplankton biomass levels with populations expressing similar APA levels, as opposed to variations in APA resulting from differences in nutrient stress levels. When APA is normalized to Chl *a*, the quantity obtained is referred to as the specific APase enzyme activity, or specific APA. Thus, for example, determination of specific APA permits differentiation between high APA due to a high concentration of organisms producing APA at relatively low levels (in this case, specific APA would be low), as contrasted with high APA due to a more profound P-stress response (in this case specific APA would be high).

Samples from freshwater dominated sites (River, RM1, RM2, and RM3), however, deviated from this general trend, often exhibiting higher APA at lower Chl *a* levels. Given observed differences in biogeochemical parameters between freshwater and brackish or marine sites in the field area, and what is generally known about how

nutrient cycling processes differ in freshwater versus marine systems, it is not surprising that the freshwater-dominated sites display a distinct APA vs. Chl *a* trend. For example, biomass production in freshwater sites is more likely to be limited by light due to higher turbidity caused by stream inputs (Young, unpubl.). Lower Chl *a* levels at freshwater sites are consistent with the higher observed turbidity at these sites. The higher turbidity in stream-influenced sites is also likely to result in lower dissolved phosphate concentrations, owing to sorption onto particles. This effect might be particularly severe in environments such as He'eia Fishpond, owing to the fact that Hawaiian soils, strongly enriched in iron oxyhydroxides, are a source of reactive iron minerals to local streams (De Carlo et al. 2007). Reactive iron oxyhydroxides have an extremely high affinity for phosphate sorption (Fox, 1985; De Carlo et al. 2007, Ruttenberg and Sulak 2010), and thus are expected to efficiently transform bioavailable P (phosphate) into particulate bound P (Pomeroy et al. 1965; Ruttenberg and Sulak 2010). The loss of bioavailable P through sorption to iron minerals is a possible explanation for the observed increase in APA in freshwater sites, and for the deviation of freshwater samples from the linear Chl *a* verse APA trend.

3.4.2 Relationship between APA and Dissolved Inorganic Phosphorus (DIP)

Production of APase is generally triggered by low cellular phosphate levels, which typically correlate to low DIP concentrations in the surrounding water. Thus, it is common to observe a negative correlation between APA and DIP concentrations in aquatic systems in which phytoplankton are P-stressed (Cembella et al. 1984; Chróst and Overbeck 1987; Jansson et al. 1988; Naush 1993). However, no significant correlation (R^2= 0.0004) was observed in the He'eia Fishpond data set as a whole (data not shown).

In a study conducted by Ruttenberg and Dyhrman (2005) of two contrasting shore-perpendicular transects on the Oregon coast, one transect showed a negative correlation (R^2= 0.77) between APA and DIP while the other showed no correlation (R^2= 0.13). Ruttenberg and Dyhrman (2005) argued that this difference was arose from the different residence times of the water characteristic of the two transects. The site that displayed a negative correlation had a relatively longer water

residence time of approximately 10 days, as opposed to the site that displayed no correlation, which was characterized by rapid flushing (order of hours). Thus, there was insufficient time to deplete phosphate levels, and to generate an observable APA response to low phosphate levels, at the well-flushed site (Ruttenberg and Dyhrman 2005). He'eia Fishpond, located on the windward side of Oahu, is exposed to persistent trade winds which, in combination with a shallow water column and vigorous tidal flushing (Young, unpubl.) results in a well-mixed system. As observed in the Ruttenberg and Dyhrman (2005) study, a highly mixed system may not allow sufficient time to develop a negative correlation between APA and DIP.

It is also possible that the lack of correlation between APA and DIP could be caused by micro-variations in DIP concentrations, as follows. In that in the vicinity of an individual cell, on a scale of microns, the DIP levels may have been low enough to promote production of APase as a phosphate stress response, despite the fact that the water body as whole did not exhibit low DIP concentrations. Micro-variations in DIP would be resolvable with bulk the sort of bulk water and analysis typical of chemical oceanographic studies (Dyhrman and Palenik 2003; Ruttenberg and Dyhrman 2005; Dyhrman and Ruttenberg 2006). As the DIP data for this study was obtained from bulk water samples, any micro-variations in DIP are not resolvable for this study, as well.

The absence of a correlation between APA and DIP could also be a reflection of the nutritional history of the cell. To a large extent, He'eia Fishpond is a transport dominated system, with transport of water and particles due to both freshwater input and tidal flushing. Thus, for example, the APA of cells isolated from one location (with high DIP) could actually have been produced at another location (one characterized by low DIP). The observation of cells with high APA in relatively high-DIP waters thus may not reflect *in situ* production of APA, but instead the past history of the cells (Ruttenberg and Dyhrman 2005; Dyhrman and Ruttenberg 2006).

3.4.3 Spatial Variability in Baseline APA in He'eia Fishpond

APA levels are higher at freshwater dominated sites (RIVER, RM1, RM2, RM3), and lower at marine dominated sites (Fig. 3.4). A pocket of elevated APA levels occurs in the northeast corner of the fishpond at Stake 6 and 7. While Stake 6, by virtue of its location, might be expected to have lower APA more consistent with a marine-dominated site, this region of the pond is surrounded by a mangrove forest which, from empirical physical observations from repeated excursions to He'eia Fishpond, acts as a wind-block to the prevailing northeastern trade winds, and thus limits wind driven mixing at this site. Limited mixing results in a relatively stagnant water column characterized by a longer residence time, allowing APase to accumulate, resulting in higher observed APA.

An second pocket of high APA was observed in the southwest corner of the fishpond, at stakes 13, 15, and 16 (Fig. 3.4). The higher APase observed in this region could be the result of enhanced runoff entering this region from the developed land surrounding it, and particle-DIP interactions as described in the previous section. The potential for artificial fertilization at these sites also exists, brought about by the addition of fish food to fish pens located proximal to Stake 11. Finally, the higher APA observed in this region of the pond could also be the result of groundwater inputs with high N:P ratios. At the present time, based on the data set in hand, none of these possible explanations for the observed spatial distribution of APA can be ruled out.

Of the two sites located outside the perimeter of the pond wall (OCN1 and OCN2), OCN1 exhibits the highest APA. Located near the mouth of He'eia Stream, OCN1 is is influenced by freshwater stream input (Young, unpubl.). Thus, the higher APA observed at this site is likely driven by stream inputs, either high N:P promoting a P-stress response in phytoplankton at OCN1, or by transport of P-stressed phytoplankton from the stream to site OCN1. These two possible explanations are equally likely based upon existing data.

At pond sites where both surface and deep water samples were collected and assayed for APA, deep water samples generally displayed higher APA than surface water

samples. A possible explanation for higher APA at depth is that cells in surface waters may experience photoinhibition, resulting in a reduction of primary productivity that would subsequently decreases P demands. A reduced P-demand could bring about a reduction in observed APA, which would be consistent with the lower APA observed in surface waters of the pond.

Overall, 96% of He'eia Fishpond samples assayed expressed APA. Photosynthetic organisms can directly assimilate orthophosphate with no additional expenditure of energy. When orthophosphate is unavailable at optimal levels, photosynthetic organisms can produce enzymes to access the DOP pool, the most common of these enzymes is APase (Ruttenberg and Dyhrman 2005; Dyhrman and Ruttenberg 2006). The production of enzymes is not preferred as it requires energy to execute; consequently APase is only produced when low DIP concentrations render it necessary. The large presence of APA throughout He'eia Fishpond indicates that the biological community is experiencing some degree of P-stress.

3.4.4 *Storm Driven Impacts on Temporal and Spatial Variability of APA*

Kaneohe Bay is typified by a dissolved N:P ratio ranging from 2-4, characteristic of an N-limited environment. However, during storm events large fluxes of dissolved inorganic nitrogen increase surface water N:P ratios to 25-29, driving surface waters toward P-limitation (Ringuet and Mackenzie 2005; De Carlo et al. 2007). Ringuet and Mackenzie (2005) observed phosphate depletion after all monitored storm events sampled in their study. During the November 2007 storm event that occurred during the present study, He'eia Fishpond exhibited characteristics analogous to those observed in previous Kaneohe Bay studies, as follows. He'eia Fishpond has an average baseline N:P ratio of 17 (this study), nearly equivalent to the preferred N:P ratio of 16 as described by Redfield (1934). Following the November 2007 first-flush storm event, the average N:P ratio in He'eia Fishpond increased to 33, an N:P ration that might be expected to promote P-stress or P-limitation. As a consequence of the storm-driven increase in pond N:P ratios, APase production was induced (Figs. 3.6 and 3.7).

Intensive sampling following the storm event, focusing on a pond transect arrayed perpendicular to the site of dominant stream input (Fig. 2.1), provided insight on the spatial and temporal development of APA. By the second day post-storm (Fig. 3.6) a significant elevation of APA was observed at most sites along the transect, indicating that phytoplankton within the pond mounted the P-stress response of synthesizing APA in reaction to storm input of high N:P stream waters. Already by day-3 levels of APA began to diminish (Fig. 3.6), reaching pre-storm baseline levels by the 7th day post storm.

Spatial variability in response to the pulse of storm driven stream-input can also clearly be seen along the transect (Fig.3.7). The first day following the storm APA at all transect locations remained relatively low. On day 2 APA increased at Stake 7 and 9, the sites in closest proximity to river inputs. On day 3 APA at Stake 7 and 9 decreased as the storm pulse moved through the fishpond to Stake 11 and 13 where APA began to increase. On day 4 APA decreased throughout the transect and returned to initial conditions by day 7. The progression of APA development spatially across the pond is completely consistent with movement of the storm pulse through the pond, and reveals that the response time of resident phytoplankton to storm-input induced P-stress is on the order of 1- to 2-days. This response time is further substantiated by results of the nutrient addition experiments discussed in the next chapter.

Chapter 4

Response of Phytoplankton Community to a Shifting Nutrient Regime: An Experimental Study

4.1 Background

Like many river-dominated coastal marine environments, He'eia Fishpond is subject to dramatic shifts in nutrient influx as a consequence of episodic storm input (Ringuet and Mackenzie 2005; De Carlo et al. 2007; §3.3.4;§3.4.4). River nutrient concentrations generally exceed nutrient concentrations in coastal waters (Milliman and Meade 1983), and the relative concentrations of different nutrients can vary between rivers and the coastal ocean, as well. Nutrient shifts caused by freshwater input can perturb coastal phytoplankton communities. For example, after storm input to Kaneohe Bay, elevated dissolved N:P ratios implied that the phytoplankton community may have been driven toward phosphate limitation (Ringuet and Mackenzie 2005).

It can be challenging to monitor phytoplankton response to nutrient shifts in natural systems. A common approach to studying phytoplankton response to nutrient additions, and shifting nutrient ratios, is to conduct bioassay experiments in enclosures. The simplest and most widely employed form of this type of experimental approach are bottle nutrient addition experiments (e.g., Hecky and Kilham 1988, Healey and Hendzel, 1980). In such experiments, replicate bottles are incubated under ambient light and temperature conditions over a time course of hours to days, after amending them with added nutrients. A set of controls, bottles with no nutrients added, are typically run in parallel to amended bottles. The biological and chemical responses of the resident phytyoplankton community isolated in the bottles to changes in nutrient concentrations are observed, providing valuable information on how a phytoplankton community will respond to nutrient shifts in the natural environment. Results of small scale nutrient addition experiments such as these can be extrapolated and applied to larger ecosystems.

The nutrient addition experiment conducted in this study is the first of several planned experiments, and was designed to provide information that will guide planning for future studies. The purpose of this nutrient addition experiment was to: (1) deploy the experimental apparatus to ensure its stability under natural conditions, (2) determine when, during a 24-hour period, maximum photosynthesis occurs to guide selection of sampling times, (3) ensure the concentration of added nutrients (nitrate (NO_3^-) & phosphate (PO_4^{3-})) stimulated a detectable change in phytoplankton biomass, (4) determine on what time scale a biological response to nutrient addition could be observed and (5). investigate variations in APA in the > 0.7 μm particle size class (dominated by phytoplankton), as contrasted with the < 0.7 μm particle size class (dominated by bacteria), by sequential filtration of incubation subsamples on 0.7 and 0.2 μm filters.

4.2 Methods

4.2.1 Overview of Experimental Design

Bulk water was collected at the sample site and filtered through a 200 μm mesh to remove zooplankton. 3.75 L of pre-screened sample water was added to each of the 4-L clear incubation bottles. Head space was left in each bottle to prevent hypoxia within the bottle that would impact phytoplankton growth. Nutrient (NO_3^- and PO_4^{3-}) spikes were added to final concentrations of 10 μM and 0.5 μM, respectively. These concentrations were chosen to replicate nutrient concentrations observed within He'eia Fishpond during the November, 2007 storm event. Incubation bottles were prepared in triplicate, for each of three treatments (+N, +P, and a control (no nutrient addition)). Incubation bottles were secured to a plastic (polyvinylchloride, or PVC) and nylon rope grid and suspended just below the surface of the HFP water column. A sensor for determining photosynthetically active radiation (a PAR sensor) was deployed at the same depth as the incubation bottles to measure solar radiation levels. Radiation levels were used to determine the optimal time of day for sample collection. Ideally, sample collection should take place during the time of day at which maximum photosynthesis, which is typically directly before or after maximum light intensity (Steele 1962). Sampling during maximum light levels should be avoided due to potential photoinhibition (Trivedi et al. 1997).

At the start of the incubation triplicate chlorophyll *a* and APA samples were collected from the pre-screened sample water to determine initial levels (t=0), prior to any nutrient addition. Samples for analysis of chlorophyll *a* and APA were then collected from each bottle each day of the 5-day incubation at approximately the same time (11 am). Splits from one bottle for each nutrient treatment collected on days 3 and 4 of the incubation were subjected to size fractionation via sequential filtration in order to evaluate whether different size classes of organisms expressed different levels of APA.

4.2.2 Nutrient Addition Preparation, Sample Collection and Processing

Water from 15 SFC (Fig. 2.1) was collected into a 50-L HDPE carboy. A 200 µm mesh was attached to the spigot of the carboy and 3.75 L aliquots of pre-screened sample were dispensed into 4-L clear polycarbonate bottles. Nutrient treatments were applied to triplicate bottles as follows: 1) no nutrient addition (control) 2) an addition of phosphate (+P) to a final concentration of 0.5 µM PO_4^{3-} and 3) an addition of nitrate (+N) to a final concentration of 10 µM NO_3^-. Incubation bottles were secured to a nylon grid. Bottles remained near the surface of the water column throughout the incubation however; the exact depth fluctuated with changing tides. A Photosynthetic Active Radiation (PAR) sensor (Oddysey®,New Zealand) was suspended from the nylon grid at the same depth as incubation bottles.

Incubation bottles were sampled daily at approximately the same time over the course of four days for APA and Chl *a*. All samples were collected in triplicate from triplicate bottles. Incubation bottles were homogenized, by gently inverting three times, prior to sampling. 100 mL aliquots were filtered through acid-clean (10% HCl) 0.2 µm GHP (Pall®) filters for analysis of APA. On day 3 and 4 size fractionation of APA was conducted by filtering 100 mL aliquots through muffled (550°C) 0.7 µm GF/F (Whatman®)filters. Filtrate (< 0.7 µm) was then collected and subsequently filtered through acid-clean (10% HCl) 0.2 µm GHP (Pall®) filters. Cell concentrates for analysis of APA were then stored frozen (-30°C) in plastic Petri dishes, until analyzed. Samples collected for analysis of Chl *a* were processed by filtering 150 mL aliquots through muffled (550°C) GF/F 0.7 µm (Whatman®) filters. Cell concentrates

for analysis of Chl *a* were stored frozen (-30°C) in aluminum foil wrapped, borosilicate test tubes, until analyzed.

4.2.3 Analytical Methods

APA and Chl *a* samples were analyzed as discussed in sections 3.2.3.1 and 3.2.3.2, respectively.

4.3 Results

4.3.1 PAR Sensor: Radiation Levels

Radiation levels from the PAR sensor depict typical diurnal cycles of light intensities (Fig.4.1). Scatter occurs on a smaller time scale throughout the larger diurnal cycle; averaging 30 minute increments of radiation measurements smoothes this scatter (Fig. 4.2). Maximum light levels occur between 11:30 am – 1:30 pm.

4.3.2 APA Response to Nutrient Addition

Samples analyzed for APA at t=0 exhibited a starting activity of 0.407 ± 0.038 μM-P h^{-1}. The addition of nitrate produced an increase of APA over the four day incubation to a final activity of 4.890 ± 0.334 μM-P h^{-1}. Addition of phosphate produced a decrease in APA to a final activity of 0.237 ± 0.029 μM-P h^{-1}. APA samples analyzed from control bottles exhibited an increase in APA to 1.67 ± 0.320 μM-P h^{-1} (Fig.4.3).

4.3.3 Size Fractionation of APA

Alkaline phosphatase activity (APA) was observed in both the > 0.7 μ and the < 0.7 μm size fractions for all three treatments. Significantly more APA was observed in the 0.7 μm filtrate for all treatments (Fig. 4.4). The largest difference was observed in the +N treatment, with 541% higher activity in the larger size fraction. In both the control and +P treatments, the > 0.7 μm size fraction displayed approximately 22% more activity than the < 0.7 μm fraction. There was no significant difference in APA observed between the +N and control treatments for the <0.7 μm size fraction.

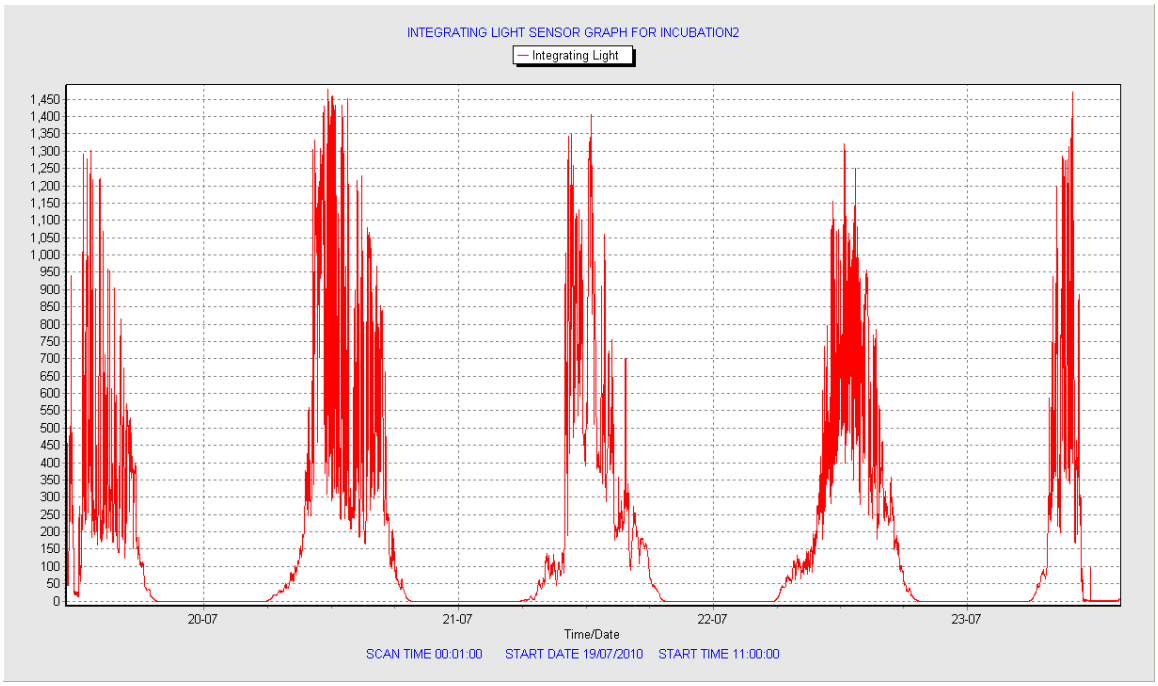

Figure 4.1: Light Radiation over the four day incubation.

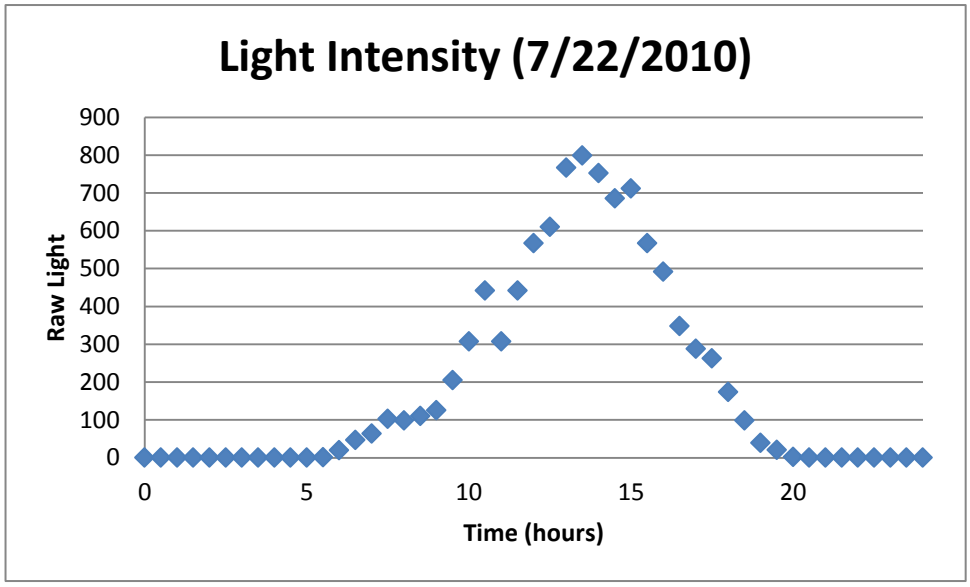

Figure 4.2: Thirty-minute averages of light measurements on the 3rd day of incubation.

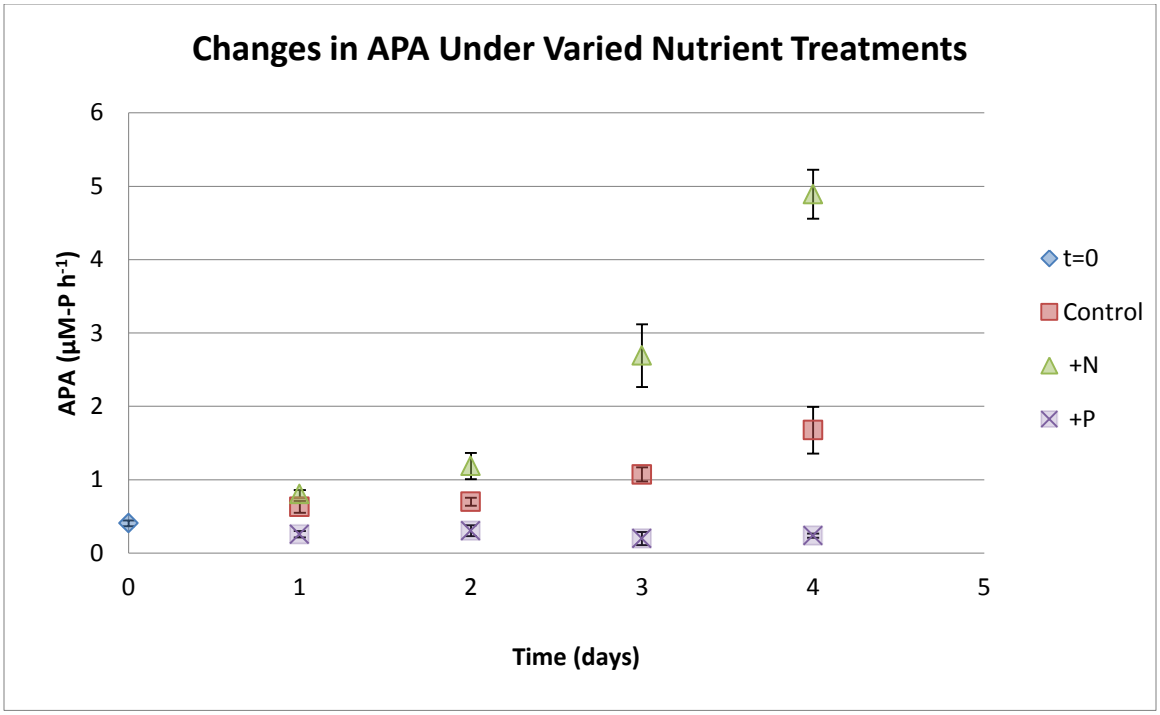

Figure 4.3: Induction and repression of alkaline phosphatase activity following varied nutrient additions. Error bars represent the standard deviation over triplicate samples from triplicate bottles.

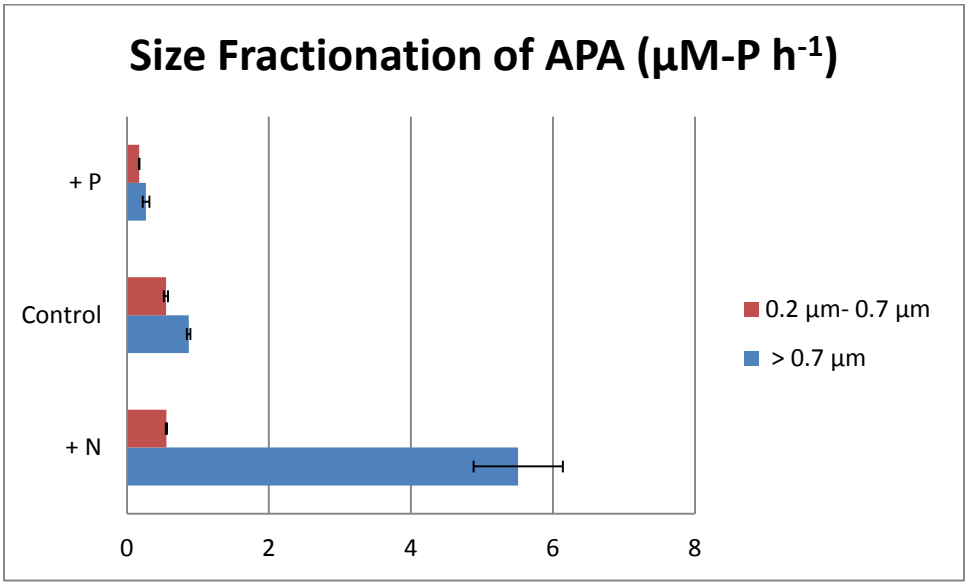

Figure 4.4: Size fractionation through successive filtration. The 0.2 µm- 0.7 µm portion is representative of the bacteria community. >0.7 µm is representative of the phytoplankton community. Error bars represent the standard deviation over triplicate samples.

4.4 Discussion

4.4.1 Evaluation of the Stability and Suitability of the Incubation Apparatus

The PVC and nylon rope grid was weighed down at the corners by large metal weights that rested securely on the bottom of the fishpond. Bottles were secured to the nylon rope by zinc plated lobster-claw clips and reinforced with plastic zip-ties. Lobster-claw clips allowed for secure fastening of incubation bottles to the grid, while at the same time allowing easy removal for sampling. The PAR sensor was connected to the grid with nylon rope and zip-ties. The design of the apparatus allowed for all bottles and the PAR sensor to remain suspended just below the surface, with no obstruction of light, for the majority of the experiment. On occasion, however, at maximum tidal lows, the upper-most surfaces of the incubation bottles were exposed above the water line.

The apparatus remained in place for the entirety of the 5 day incubation. No incubation bottles were lost or damaged. While periodic exposure of the bottles due to tidal variations was unanticipated, this was a short-term problem (lasting 2-3 hours), the apparatus proved to be a secure and convenient way of incubating the nutrient addition experiment bottles within the pond for several days.

4.4.2 Choice of Optimal Sampling Time: Analysis of PAR Data

It is generally considered optimal to sample nutrient addition experiments at a time when the phytoplankton exhibit maximum photosynthesis, so that the largest growth response signal can be observed. Sampling at maximum light levels should be avoided as this is the time where photoinhibition is most likely to occur. Photoinhibition takes places when high light intensities increase the rate of transpiration, potentially causing stomata to close and photosynthesis to be reduced (Trivedi et al. 1997). Maximum photosynthesis is likely to occur either an hour before or after maximum light intensities. Maximum light levels occurred, on average, at 12 noon during our experiment. On this basis, a sampling time of 11 a.m. was chosen as optimal for increasing the likelihood of capturing the phytoplankton community exhibiting maximum photosynthetic rates.

4.4.3 APA Response to Nutrient Addition

Measureable APA is observed in the t=0 sample, indicating that the phytoplankton sampled at Station 15 in He'eia Fishpond are potentially phosphate stressed. Phosphate stress is a condition that occurs when the phytoplankton to not have access to levels of phosphate that are optimal for their physiological demands. Under these circumstances, phytoplankton may produce APase, a hydrolytic enzyme that allows them to produce phosphate from dissolved organic phosphorus, by cleaving the phosphate from phosphomonoesters (Dyhrman and Ruttenberg 2006; Ruttenberg 2004).

Addition of NO_3^- elevated the N:P ratio from initial conditions. The daily increase in APA observed in the +N treatment shows a clear response to the elevated N:P ratio. The high N:P ratio creates a situation where excess bioavailable N (as NO_3^-) is present in the growing medium, relative to P. Low P in relation to N causes P-stress in the phytoplankton and promotes APase. An increase in APA as a consequence of low PO_4^{3-} concentrations is consistent with physiologically induced production of APA.

An increase in APA is observed in the control treatments, bottles to which no nutrients were added. This indicates that under these experimental conditions, the evolving N:P ratio drives a P-stress response. Although there is an increase in APA it is not as significant as the increase driven by the artificially elevated N:P ratio in +N treated bottles. The decrease in observed APA in the +P treatment relative to the control is an indication of repression of APase.

The nutrient addition experiment clearly indicates that the phytoplankton community in He'eia Fishpond is operating under conditions of P-stress under natural conditions at the time of sampling. The fact that APA was induced under +N conditions, and repressed under +P conditions (Fig.4. 3), is confirmation that the APase in these organisms is phosphate regulated. Phosphate regulation of APase is a necessary condition for the presence of APase to be confidently used as a P-stress indicator.

4.4.4 Size Fractionation of APA

Phytoplankton are classified as > 0.7 µm fraction. Bacteria are classified as 0.2 µm – 0.7 µm fraction (Nausch et al. 2004). Slight variations and deviations from these size fraction cut-offs are acknowledged. For example, phytoplankton may be smaller than 0.7 µm (Nausch et al. 2004). These smaller phytoplankton will not be retained on the 0.7 µm filter, and will be counted with the < 0.7 µm, or the bacterial fraction. The filtration rate through 0.7 µm filters was high, with 100 mL of sample filtered within several seconds. Due to high filtration rates it can be assumed that the 0.7 µm filter did not become clogged at any point during filtration, and it thus can be confidently assumed that little to no < 0.7 µm cells are included in the > 0.7 µ fraction. The larger size class thus likely provides an accurate size segregation of phytoplankton cells, with the caveat that some smaller phytoplankton cells may not be included.

APA is clearly repressed in both size fractions in the +P treatment, indicating that cells in both size classes are producing APase that is phosphate regulated. The < 0.7 µm size class did not show a clear increase in APA in the +N treatment, relative to the control, however. This may indicate that these smaller cells did not experience increased P stress, beyond what they already were experiencing in the pond at the time the experiment was initiated. The largest response to +N treatment is exhibited by the phytoplankton in the >0.7 µm size class, indicating that they were driven to significant P stress by the elevated N:P ratios in the +N treatment.

Chapter 5

Conclusions

Phosphorus (P) is an essential nutrient for primary productivity and may be growth limiting in certain marine environments. The bioavailability of phosphorus is significantly influenced by the production of phosphatases. Alkaline phosphatase, produced under deplete DIP conditions; can access DOP through the hydrolytic cleavage of inorganic P from the organic compound. In this study, the production of alkaline phosphatase in He'eia Fishpond was quantified, and used as a nutrient deficiency indicator.

Analysis of APA in He'eia Fishpond showed higher activities at sites with strong freshwater inputs and lower activities at sites proximal to Kaneohe Bay. APA was found at all locations in He'eia Fishpond indicating that some degree of P stress occurs. APA compared to DIP concentrations showed no correlation. Poor correlation is potentially a function of the short (on the order of days) residence time of water in He'eia Fishpond. Rapid flushing of the system does not allow for the development of the anticipated negative correlation between APA and DIP.

A short residence time of water in He'eia Fishpond is illustrated in the temporal and spatial changes in alkaline phosphatase activity following the November 2007 storm event. The storm event was characterized by enhanced stream inputs and the subsequent increase in the N:P ratio of the fishpond waters from 17 to 33, promoting the production of alkaline phosphatase. A storm pulse of increased APA is observed during intensive storm sampling marking the effect of freshwater inputs as the plume moves through the fishpond toward Kaneohe Bay within the matter of 7 days.

Nitrate and phosphate were artificially added to the nutrient addition experiment incubation bottles at concentrations representative of inputs during the November 2007 storm event. Changes in APA observed in the nutrient addition experiment were analogous to changes observed during the storm event. The results of the nutrient addition experiment clearly depict the inducible and repressible qualities of APase, verifying its regulation by PO_4^{3-} concentrations. While APA correlates poorly

to DIP in He‘eia Fishpond during baseline conditions its production and repression during the storm event and nutrient addition experiment clearly validates its use as a nutrient deficiency indicator.

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He'eia Fishpond Data (2007-2008)

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chla^{-1} h^{-1})
1	OM2	8/11/2007	3.44	0.20	0.29	3.65	12.61	1.86	0.97	1.92
2	OCN2	8/11/2007	0.61	0.17	0.19	0.78	4.11	0.76	1.15	0.66
3	OB	8/11/2007	3.78	0.40	0.20	4.17	20.44	0.90	0.51	1.76
4	OM1	8/11/2007	1.91	0.16	0.16	2.07	12.57	0.88	0.43	2.07
5	TM	8/11/2007	4.45	0.51	0.32	4.95	15.49	1.61	0.85	1.89
6	OCN1	8/11/2007	3.16	0.57	0.31	3.73	12.17	1.50	0.90	1.66
7	RM3	8/11/2007	25.87	0.24	0.86	26.10	30.49	3.36	0.99	3.40
8	RM2	8/11/2007	6.09	0.09	0.88	6.18	7.02	7.81	0.79	9.93
9	RM1	8/11/2007	10.01	0.22	0.98	10.23	10.39	2.58	2.04	1.26
10	River	8/11/2007	9.89	0.04	0.91	9.93	10.87	5.47	0.47	11.66
11	Stk1sfc	8/11/2007	1.06	0.21	0.10	1.27	12.72	1.78	1.25	1.43
12	Stk1deep	8/11/2007	1.66	0.23	0.18	1.89	10.38	1.06	0.48	2.20
13	Stk3sfc	8/11/2007	1.50	0.36	0.20	1.86	9.49	0.64	0.24	2.66
14	Stk3deep	8/11/2007	1.81	0.26	0.17	2.07	12.00	0.58	0.67	0.87
15	Stk6sfc	8/11/2007	1.47	0.15	0.16	1.62	10.34	2.46	2.66	0.92
16	Stk6deep	8/11/2007	1.34	0.34	0.20	1.68	8.60	1.85	2.08	0.89
17	Stk7sfc	8/11/2007	2.04	0.11	0.25	2.15	8.73	7.33	2.60	2.81
18	Stk7deep	8/11/2007	0.84	0.24	0.28	1.08	3.80	3.18	2.64	1.20
19	Stk8sfc	8/11/2007	2.04	0.18	0.18	2.23	12.58	3.25	1.57	2.08
20	Stk8deep	8/11/2007	0.72	0.24	0.16	0.97	6.07	1.12	1.56	0.72
21	Stk9sfc	8/11/2007	1.07	0.18	0.08	1.25	14.66	1.72	1.31	1.31
22	Stk9deep	8/11/2007	1.11	0.28	0.19	1.39	7.44	4.46	2.35	1.90
23	Stk13sfc	8/11/2007	1.82	0.13	0.19	1.95	10.15	3.97	2.20	1.81
24	Stk13deep	8/11/2007	1.54	0.21	0.23	1.74	7.52	3.31	1.02	3.25
25	Stk15sfc	8/11/2007	1.56	0.17	0.19	1.73	8.92	2.86	1.39	2.06
26	Stk15deep	8/11/2007						2.92	1.12	2.61
27	Stk16sfc	8/11/2007	1.20	0.65	0.22	1.85	8.49	1.90	1.38	1.37
28	Stk16deep	8/11/2007	1.19	0.23	0.24	1.42	6.01	2.11	1.97	1.07
29	Stk18sfc	8/11/2007	4.59	0.10	0.31	4.70	15.17	8.95	2.28	3.93
30	Stk18deep	8/11/2007						2.89	2.56	1.13

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chla^{-1} h^{-1})
31	OM2	9/15/2007	3.23	0.23	0.22	3.46	15.54	2.88	0.64	4.49
32	OCN2	9/15/2007	1.35	0.38	0.21	1.73	8.24	0.55	0.56	0.98
33	OB	9/15/2007	4.20	0.53	0.30	4.73	15.96	1.91	0.64	2.98
34	OM1	9/15/2007	3.41	0.64	0.34	4.06	11.87	2.51	0.28	8.93
35	TM	9/15/2007	3.80	0.46	0.33	4.26	12.95	1.57	0.80	1.96
36	OCN1	9/15/2007	4.70	0.64	0.40	5.34	13.35	4.57	0.56	8.14
37	RM3	9/15/2007	6.37	0.54	0.56	6.91	12.41	5.36	1.12	4.78
38	RM2	9/15/2007	6.09	0.20	0.88	6.28	7.16	7.82	1.20	6.51
39	RM1	9/15/2007	9.33	0.23	1.06	9.57	9.06	1.90	0.72	2.63
40	River	9/15/2007	5.14	0.02	0.91	5.16	5.66	8.12	4.33	1.88
41	Stk1sfc	9/15/2007	3.12	0.09	0.22	3.21	14.84	2.32	1.12	2.07
42	Stk1deep	9/15/2007	2.02	0.20	0.30	2.21	7.37	1.74	0.96	1.81
43	Stk3sfc	9/15/2007	2.32	0.22	0.22	2.54	11.73	1.05	0.56	1.87
44	Stk3deep	9/15/2007	1.95	0.19	0.26	2.14	8.28	0.95	0.40	2.37
45	Stk6sfc	9/15/2007	2.17	0.14	0.28	2.30	8.27	3.42	0.96	3.56
46	Stk6deep	9/15/2007	0.38	0.26	0.33	0.65	1.98	2.73	1.44	1.89
47	Stk7sfc	9/15/2007	2.64	0.14	0.26	2.78	10.82	2.02	1.92	1.05
48	Stk7deep	9/15/2007	0.90	0.18	0.33	1.09	3.29	2.19	1.76	1.24
49	Stk8sfc	9/15/2007	2.82	0.16	0.16	2.98	18.96	2.00	2.56	0.78
50	Stk8deep	9/15/2007	0.20	0.24	0.29	0.44	1.52	2.57	2.40	1.07
51	Stk9sfc	9/15/2007	3.04	0.28	0.17	3.32	19.09	2.22	1.12	1.98
52	Stk9deep	9/15/2007	2.07	0.17	0.29	2.23	7.84	2.57	1.12	2.29
53	Stk13sfc	9/15/2007	2.79	0.33	0.23	3.11	13.50	2.88	0.64	4.49
54	Stk13deep	9/15/2007	1.86	0.26	0.32	2.12	6.53	4.34	0.56	7.74
55	Stk15sfc	9/15/2007	2.76	0.17	0.17	2.93	17.62	5.99	1.44	4.15
56	Stk15deep	9/15/2007	0.87	0.19	0.31	1.06	3.46	3.83	1.52	2.52
57	Stk16sfc	9/15/2007	1.39	1.13	0.14	2.52	18.53	3.91	1.20	3.26
58	Stk16deep	9/15/2007	0.96	0.27	0.26	1.23	4.74	4.04	2.89	1.40
59	Stk18sfc	9/15/2007	2.83	0.13	0.58	2.95	5.11	2.40	1.92	1.25
60	Stk18deep	9/15/2007	1.50	0.19	0.36	1.68	4.68	2.44	1.28	1.90

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
61	OM2	10/13/2007	3.48	0.20	0.00	3.69		0.62	0.81	0.76
62	OCN2	10/13/2007	1.41	0.15	0.12	1.56	12.98	0.73	0.40	1.85
63	OB	10/13/2007	6.16	0.37	0.13	6.53	49.25	0.62	0.75	0.83
64	OM1	10/13/2007	2.55	0.27	0.17	2.82	16.59	1.49	1.30	1.15
65	TM	10/13/2007	3.86	0.28	0.26	4.14	15.63	2.01	1.38	1.46
66	OCN1	10/13/2007	4.59	0.25	0.44	4.84	11.07	1.07	0.37	2.90
67	RM3	10/13/2007	3.44	0.29	0.31	3.73	12.06	1.91	3.11	0.61
68	RM2	10/13/2007	8.26	0.06	0.87	8.32	9.61	7.53	1.49	5.04
69	RM1	10/13/2007	8.40	0.19	0.79	8.58	10.85	1.78	0.69	2.58
70	River	10/13/2007	7.99	0.09	0.89	8.08	9.06	4.97	2.98	1.66
71	Stk1sfc	10/13/2007	4.46	0.11	0.15	4.57	30.90	1.02	2.42	0.42
72	Stk1deep	10/13/2007	1.00	0.17	0.28	1.17	4.23	3.27	3.19	1.03
73	Stk3sfc	10/13/2007	4.05	0.13	0.08	4.18	50.97	1.36	1.41	0.97
74	Stk3deep	10/13/2007	2.12	0.20	0.26	2.32	8.92	1.48	1.34	1.11
75	Stk6sfc	10/13/2007	4.18	0.10	0.24	4.28	17.67	1.09	1.19	0.92
76	Stk6deep	10/13/2007	3.20	0.23	0.29	3.43	11.70	3.33	3.07	1.08
77	Stk7sfc	10/13/2007	5.95	0.11	0.27	6.06	22.35	1.43	1.41	1.01
78	Stk7deep	10/13/2007	1.82	0.17	0.26	1.99	7.67	1.71	2.09	0.82
79	Stk8sfc	10/13/2007	3.91	0.14	0.28	4.05	14.22	1.18	2.20	0.54
80	Stk8deep	10/13/2007	2.94	0.23	0.27	3.16	11.58	2.73	2.17	1.26
81	Stk9sfc	10/13/2007	3.78	0.13	0.16	3.91	24.92	2.22	1.39	1.60
82	Stk9deep	10/13/2007	2.03	0.31	0.28	2.34	8.34	3.34	1.33	2.52
83	Stk13sfc	10/13/2007	5.14	0.04	0.46	5.18	11.22	5.88	4.66	1.26
84	Stk13deep	10/13/2007	2.31	0.13	0.45	2.44	5.43	10.82	4.20	2.58
85	Stk15sfc	10/13/2007	3.23	0.11	0.43	3.34	7.69	2.40	2.58	0.93
86	Stk15deep	10/13/2007	2.45	0.19	0.43	2.64	6.08	1.40	2.60	0.54
87	Stk16sfc	10/13/2007	4.73	0.18	0.42	4.91	11.80	3.33	3.26	1.02
88	Stk16deep	10/13/2007	2.82	0.24	0.35	3.06	8.80	1.47	3.01	0.49
89	Stk18sfc	10/13/2007	5.54	0.22	0.42	5.76	13.68	1.56	2.49	0.63
90	Stk18deep	10/13/2007	3.93	0.18	0.34	4.11	11.97	2.18	1.86	1.18

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
91	River	11/4/2007	7.45	6.17	0.91	13.62	14.96	13.36	0.62	21.45
92	Stk10sfc	11/4/2007	20.76	40.19	0.93	60.95	65.85	14.24	0.85	16.77
93	Stk18sfc	11/4/2007	8.80	12.44	0.94	21.24	22.68	13.16	0.42	31.12
94	OM2	11/6/2007	4.38	0.26	0.04	4.65	110.65	0.30	1.08	0.28
95	OCN2	11/6/2007	3.64	1.15	0.15	4.79	32.01	0.56	0.85	0.65
96	OB	11/6/2007	3.26	1.78	0.47	5.05	10.79	0.53	0.81	0.65
97	OM1	11/6/2007	4.01	1.83	0.46	5.84	12.64	0.17	0.74	0.24
98	TM	11/6/2007	6.42	2.16	0.58	8.58	14.86	0.23	0.92	0.26
99	OCN1	11/6/2007	2.75	1.69	0.49	4.44	9.01	0.42	0.59	0.71
100	RM3	11/6/2007	9.08	9.03	0.86	18.11	21.04	3.75	1.03	3.64
101	RM2	11/6/2007	10.57	6.22	0.88	16.79	19.03	6.66	0.68	9.79
102	RM1	11/6/2007	26.98	9.98	0.52	36.96	70.72	2.50	0.32	7.93
103	River	11/6/2007	8.13	1.19	0.91	9.31	10.25	3.30	0.31	10.82
104	Stk1sfc	11/6/2007	11.52	22.00	0.92	33.52	36.46	5.89	2.52	2.34
105	Stk1deep	11/6/2007	17.34	7.61	0.56	24.95	44.73	3.14	5.07	0.62
106	Stk3sfc	11/6/2007	16.82	22.49	0.93	39.31	42.10	10.18	1.98	5.14
107	Stk3deep	11/6/2007	16.32	9.05	0.88	25.37	28.88	2.15	2.72	0.79
108	Stk6sfc	11/6/2007	15.60	19.99	0.93	35.59	38.38	9.16	10.09	0.91
109	Stk6deep	11/6/2007	10.09	10.24	1.31	20.34	15.55	2.58	2.24	1.15
110	Stk7sfc	11/6/2007	16.55	19.07	0.93	35.62	38.23	4.16	2.78	1.50
111	Stk7deep	11/6/2007	18.35	10.72	0.97	29.07	29.97	4.49	3.70	1.21
112	Stk8sfc	11/6/2007						4.69	9.46	0.50
113	Stk8deep	11/6/2007						2.38	1.41	1.69
114	Stk9sfc	11/6/2007	19.74	18.34	0.92	38.08	41.21	3.54	0.86	4.13
115	Stk9deep	11/6/2007	9.92	6.85	0.47	16.78	35.51	4.95	2.44	2.03
116	Stk13sfc	11/6/2007	25.37	8.71	0.79	34.07	42.98	6.22	1.28	4.85
117	Stk13deep	11/6/2007	10.27	4.03	1.97	14.30	7.27	5.18	3.59	1.44
118	Stk15sfc	11/6/2007	18.45	19.21	0.92	37.66	40.82	4.43	1.15	3.85
119	Stk15deep	11/6/2007	9.87	9.76	1.15	19.63	17.13	3.91	2.28	1.71
120	Stk16sfc	11/6/2007						3.92	2.05	1.91
121	Stk16deep	11/6/2007	10.04	11.43	1.03	21.47	20.83	4.11	5.57	0.74
122	Stk18sfc	11/6/2007	9.21	7.72	0.93	16.93	18.11	7.87	1.02	7.73
123	Stk18deep	11/6/2007	20.30	13.70	0.91	34.00	37.16	6.65	4.60	1.45
124	Stk6sfc	11/6/2007		3.24	0.69			24.78	12.71	1.95
125	Stk7sfc	11/6/2007	12.64	2.50	0.78	15.14	19.51	11.87	333.33	0.04
126	Stk9sfc	11/6/2007	35.22	5.56	0.80	40.78	50.74	16.40	67.68	0.24
127	Stk11sfc	11/6/2007	27.36	2.14	0.80	29.51	36.92	8.19	40.08	0.20
128	Stk13sfc	11/6/2007	16.01	6.53	0.84	22.54	26.85	8.78	77.95	0.20
129	Stk18sfc	11/6/2007	11.25	0.49	0.93	11.74	12.62	5.40	184.08	0.11

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
130	Stk6sfc	11/7/2007	6.90	0.60	0.10	7.50	71.73	4.72	13.57	0.35
131	Stk7sfc	11/7/2007	12.20	0.33	0.61	12.53	20.54	5.35	3.51	1.52
132	Stk9sfc	11/7/2007	13.70	0.70	0.03	14.40	546.63	7.31	20.08	0.36
133	Stk11sfc	11/7/2007	10.84	1.34	0.25	12.18	48.53	11.02	10.55	1.05
134	Stk13sfc	11/7/2007	14.38	1.78	0.69	16.15	23.38	15.18	19.64	0.77
135	Stk18sfc	11/7/2007		0.38	0.78			6.89	114.97	0.06
136	Stk6sfc	11/8/2007	3.64	0.15	0.23	3.79	16.18	7.64	6.50	1.18
137	Stk7sfc	11/8/2007	8.67	0.20	0.75	8.87	11.82	2.59	4.27	0.61
138	Stk9sfc	11/8/2007	2.55	0.15	0.11	2.70	23.57	3.27	3.84	0.85
139	Stk11sfc	11/8/2007	2.11	0.12	0.07	2.22	31.42	4.79	7.70	0.62
140	Stk13sfc	11/8/2007	2.10	0.14	0.05	2.24	43.88	6.98	8.48	0.82
141	Stk18sfc	11/8/2007	9.21	0.07	0.92	9.28	10.12	3.29	1.80	1.83
142	Stk6sfc	11/11/2007	3.10	0.04	0.29	3.13	10.88	4.92	3.81	1.29
143	Stk7sfc	11/11/2007	12.61	0.09	0.05	12.70	264.51	1.79	3.12	0.57
144	Stk9sfc	11/11/2007	3.23	0.03	0.41	3.26	7.95	6.09	6.99	0.87
145	Stk11sfc	11/11/2007	2.96	0.05	0.15	3.01	20.25	6.46	3.70	1.75
146	Stk13sfc	11/11/2007	2.69	0.06	0.14	2.75	19.18	6.25	3.71	1.69
147	Stk18sfc	11/11/2007	5.14	0.04	0.56	5.18	9.23	3.71	7.31	0.51

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
148	OM2	11/17/2007	0.68	0.20	0.11	0.88	8.15	0.00	1.82	0.00
149	OCN2	11/17/2007	1.05	0.29	0.20	1.34	6.61	0.00	1.53	0.00
150	OB	11/17/2007	1.22	0.25	0.21	1.47	6.91	0.00	1.03	0.00
151	OM1	11/17/2007	1.57	0.41	0.27	1.98	7.21	1.16	0.89	1.30
152	TM	11/17/2007	2.15	0.39	0.25	2.54	10.06	0.48	0.95	0.51
153	OCN1	11/17/2007	1.80	0.27	0.17	2.07	12.45	0.14	0.60	0.23
154	RM3	11/17/2007	5.99	0.16	0.97	6.14	6.34	4.15	2.80	1.48
155	RM2	11/17/2007	6.36	0.07	0.88	6.43	7.33	30.68	1.44	21.30
156	RM1	11/17/2007	12.20	0.09	0.80	12.29	15.43	0.88	0.77	1.15
157	River	11/17/2007	10.84	0.09	0.91	10.93	12.05	1.16	2.12	0.55
158	Stk1sfc	11/17/2007	2.29	0.10	0.07	2.39	33.99	0.00	1.24	0.00
159	Stk1deep	11/17/2007	1.44	0.17	0.13	1.61	12.52	0.00	1.35	0.00
160	Stk3sfc	11/17/2007	3.22	0.15	0.15	3.37	22.46	0.00	2.22	0.00
161	Stk3deep	11/17/2007	2.03	0.22	0.19	2.26	11.93	0.00	1.31	0.00
162	Stk6sfc	11/17/2007	4.45	0.12	0.36	4.57	12.75	0.44	3.29	0.13
163	Stk6deep	11/17/2007	2.01	0.17	0.30	2.18	7.21	0.16	3.52	0.05
164	Stk7sfc	11/17/2007	6.77	0.05	0.36	6.81	18.83	0.36	2.49	0.14
165	Stk7deep	11/17/2007	0.71	0.14	0.30	0.85	2.81	0.20	2.04	0.10
166	Stk8sfc	11/17/2007	4.32	0.05	0.25	4.37	17.43	0.52	2.83	0.18
167	Stk8deep	11/17/2007	1.71	0.20	0.27	1.91	7.09	0.17	1.68	0.10
168	Stk9sfc	11/17/2007	4.44	0.12	0.21	4.56	21.94	0.20	1.84	0.11
169	Stk9deep	11/17/2007	0.61	0.16	0.24	0.77	3.25	0.08	1.46	0.00
170	Stk13sfc	11/17/2007	3.42	0.17	0.12	3.59	30.68	0.35	1.28	0.27
171	Stk13deep	11/17/2007	2.07	0.20	0.19	2.27	12.00	2.10	1.83	1.15
172	Stk15sfc	11/17/2007	3.51	0.04	0.51	3.54	6.97	0.62	3.10	0.20
173	Stk15deep	11/17/2007	1.37	0.17	0.26	1.54	5.97	0.00	1.66	0.00
174	Stk16sfc	11/17/2007	6.49	0.05	0.81	6.54	8.03	0.81	4.68	0.17
175	Stk16deep	11/17/2007	3.20	0.20	0.28	3.39	12.07	0.08	2.57	0.03
176	Stk18sfc	11/17/2007	7.04	0.07	0.81	7.11	8.76	0.43	2.26	0.19
177	Stk18deep	11/17/2007	0.76	0.19	0.25	0.95	3.74	0.00	1.68	0.00

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chla^{-1} h^{-1})
178	OM2	12/9/2007	4.65	0.65	0.31	5.30	17.18	0.00	1.86	0.00
179	OCN2	12/9/2007	3.92	0.42	0.22	4.35	19.60	0.47	2.11	0.22
180	OB	12/9/2007	21.77	0.42	0.40	22.19	54.81	0.16	1.25	0.13
181	OM1	12/9/2007	11.72	0.50	0.79	12.22	15.53	0.20	2.01	0.10
182	TM	12/9/2007	10.33	1.00	1.10	11.33	10.30	0.66	0.92	0.72
183	OCN1	12/9/2007	10.85	0.77	0.91	11.62	12.77	0.49	1.07	0.46
184	RM3	12/9/2007	15.65	0.35	0.96	16.00	16.70	1.00	2.10	0.48
185	RM2	12/9/2007	7.72	0.13	0.87	7.84	8.97	5.71	0.44	12.97
186	RM1	12/9/2007	12.86	0.32	1.90	13.18	6.94	0.36	0.54	0.66
187	River	12/9/2007	8.94	0.31	0.91	9.25	10.22	1.75	0.29	6.00
188	Stk1sfc	12/9/2007	7.45	0.20	0.81	7.64	9.40	0.14	3.02	0.05
189	Stk1deep	12/9/2007	3.13	0.29	0.39	3.42	8.70	0.00	4.96	0.00
190	Stk3sfc	12/9/2007	8.80	0.23	0.69	9.03	13.01	0.20	3.78	0.05
191	Stk3deep	12/9/2007	1.11	0.19	0.41	1.29	3.15	0.03	3.27	0.01
192	Stk6sfc	12/9/2007	9.08	0.13	0.73	9.21	12.61	0.04	2.37	0.02
193	Stk6deep	12/9/2007	5.35	0.18	0.40	5.53	13.96	3.67	6.26	0.59
194	Stk7sfc	12/9/2007	6.63	0.13	0.67	6.76	10.12	0.63	2.28	0.28
195	Stk7deep	12/9/2007	3.79	0.16	0.33	3.95	12.01	1.58	6.34	0.25
196	Stk8sfc	12/9/2007	9.89	0.15	1.15	10.05	8.71	0.21	3.40	0.06
197	Stk8deep	12/9/2007	3.02	0.18	0.28	3.21	11.53	3.72	4.81	0.77
198	Stk9sfc	12/9/2007	5.68	0.14	0.74	5.82	7.90	0.45	2.90	0.16
199	Stk9deep	12/9/2007	1.17	0.29	0.29	1.46	4.97	0.62	3.93	0.16
200	Stk13sfc	12/9/2007	6.77	0.19	0.83	6.96	8.41	0.00	3.03	0.00
201	Stk13deep	12/9/2007	3.78	0.14	0.11	3.92	34.64	1.68	6.49	0.26
202	Stk15sfc	12/9/2007	7.45	0.21	0.83	7.65	9.24	0.69	1.38	0.50
203	Stk15deep	12/9/2007	9.24	0.30	0.34	9.53	27.84	2.03	3.45	0.59
204	Stk16sfc	12/9/2007	7.58	0.07	0.92	7.65	8.34	0.83	1.50	0.56
205	Stk16deep	12/9/2007	4.22	0.38	0.25	4.60	18.29	11.19	3.85	2.91
206	Stk18sfc	12/9/2007	10.71	0.10	0.93	10.80	11.63	1.30	2.09	0.63
207	Stk18deep	12/9/2007	4.30	0.26	0.38	4.57	11.99	0.81		

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chla^{-1} h^{-1})
208	OM2	1/12/2007	8.54	0.33	1.38	8.87	6.42	1.43	1.77	0.81
209	OCN2	1/12/2008	2.79	0.58	0.35	3.37	9.57	0.82	1.25	0.66
210	OB	1/12/2008	21.77	0.23	0.35	22.00	63.19	1.05	1.42	0.74
211	OM1	1/12/2007	3.37	0.21	0.42	3.58	8.51	1.60	2.13	0.75
212	TM	1/12/2008	21.77	0.18	0.34	21.95	63.91	1.42	1.70	0.84
213	OCN1	1/12/2008	4.18	0.29	0.73	4.47	6.11	1.28	0.91	1.41
214	RM3	1/12/2007	9.21	0.22	0.92	9.43	10.29	1.76	2.17	0.81
215	RM2	1/12/2008	9.48	0.26	0.87	9.74	11.15	10.94	0.83	13.18
216	RM1	1/12/2008	10.42	0.54	0.26	10.95	42.02	2.52	0.51	4.99
217	River	1/12/2007	7.04	0.61	0.90	7.65	8.46	6.05		
218	Stk1sfc	1/12/2008	5.82	0.18	1.06	6.00	5.66	1.69	2.92	0.58
219	Stk1deep	1/12/2008	1.14	0.17	0.24	1.32	5.58	1.52	4.39	0.35
220	Stk3sfc	1/12/2007	3.23	0.08	0.44	3.32	7.56	0.59	1.98	0.30
221	Stk3deep	1/12/2008	0.00	0.10	0.27	0.10	0.39	1.40	3.81	0.37
222	Stk6sfc	1/12/2008	4.84	0.08	0.42	4.92	11.61	0.53	0.36	1.46
223	Stk6deep	1/12/2007	1.96	0.16	0.26	2.12	8.25	1.38	3.56	0.39
224	Stk7sfc	1/12/2008	2.59	0.08	0.37	2.67	7.22	0.31	2.26	0.14
225	Stk7deep	1/12/2008	4.39	0.24	0.32	4.63	14.54	0.05	3.96	0.01
226	Stk8sfc	1/12/2007	4.71	0.14	0.46	4.85	10.65	0.02	2.85	0.01
227	Stk8deep	1/12/2008	3.08	0.15	0.35	3.23	9.18	0.05	5.91	0.01
228	Stk9sfc	1/12/2008	3.91	0.11	0.28	4.02	14.18	0.01	2.05	0.01
229	Stk9deep	1/12/2007	1.04	0.13	0.21	1.17	5.60	0.09	3.62	0.03
230	Stk13sfc	1/12/2008	6.63	0.06	0.82	6.69	8.14	0.08	2.59	0.03
231	Stk13deep	1/12/2008	1.75	0.44	0.28	2.19	7.93	0.02	2.70	0.01
232	Stk15sfc	1/12/2007	8.67	0.67	0.82	9.33	11.39	1.15	0.27	4.33
233	Stk15deep	1/12/2008	1.64	0.16	0.27	1.80	6.63	2.76	3.17	0.87
234	Stk16sfc	1/12/2008	8.80	0.54	1.47	9.35	6.36	5.29	0.52	10.18
235	Stk16deep	1/12/2007	1.58	0.28	0.30	1.86	6.20	3.72	10.56	0.35
236	Stk18sfc	1/12/2008	8.26	0.35	1.06	8.61	8.11		1.10	0.00
237	Stk18deep	1/12/2008	1.88	0.16	0.30	2.04	6.75	4.17	9.45	0.44

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
238	OM2	2/16/2008	5.93	0.19	0.15	6.12	40.66	0.01	1.48	0.01
239	OCN2	2/16/2008	4.29	0.39	0.33	4.67	14.37	0.06	1.00	0.06
240	OB	2/16/2008	4.54	0.81	0.42	5.35	12.70	0.04	1.08	0.04
241	OM1	2/16/2008	5.79	0.28	0.23	6.07	26.19	0.56	0.79	0.70
242	TM	2/16/2008	0.38	0.22	0.17	0.60	3.51	0.28	1.77	0.16
243	OCN1	2/16/2008	2.21	0.19	0.28	2.40	8.50	0.19	1.25	0.15
244	RM3	2/16/2008	5.71	0.29	0.29	6.00	20.88	1.87	2.89	0.65
245	RM2	2/16/2008	10.57	0.29	0.87	10.86	12.46	9.76	1.85	5.26
246	RM1	2/16/2008	11.26	0.31	1.00	11.58	11.61	0.80	0.47	1.72
247	River	2/16/2008	8.67	0.43	0.90	9.09	10.08	2.16	0.60	3.59
248	Stk1sfc	2/16/2008	2.85	0.10	0.14	2.95	20.66	0.27	2.61	0.10
249	Stk1deep	2/16/2008	1.80	0.25	0.22	2.06	9.21	0.24	1.81	0.13
250	Stk3sfc	2/16/2008	2.09	0.09	0.12	2.18	18.70	0.29	1.53	0.19
251	Stk3deep	2/16/2008	1.12	0.17	0.13	1.29	9.94	0.26	2.30	0.11
252	Stk6sfc	2/16/2008	3.54	0.17	0.21	3.71	17.93	0.92	1.80	0.51
253	Stk6deep	2/16/2008	3.45	0.17	0.30	3.62	12.11	2.04	3.38	0.60
254	Stk7sfc	2/16/2008	2.72	0.12	0.23	2.84	12.10	0.38	1.95	0.19
255	Stk7deep	2/16/2008	0.22	0.19	0.19	0.41	2.19	0.51	3.16	0.16
256	Stk8sfc	2/16/2008	3.03	0.15	0.21	3.17	15.23	0.12	1.77	0.07
257	Stk8deep	2/16/2008	2.10	0.14	0.23	2.24	9.93	0.29	1.52	0.19
258	Stk9sfc	2/16/2008	1.49	0.15	0.11	1.63	15.30	0.41	1.75	0.23
259	Stk9deep	2/16/2008	1.01	0.16	0.14	1.17	8.12	0.64	3.46	0.19
260	Stk13sfc	2/16/2008	7.04	0.08	0.74	7.12	9.62	0.49	6.16	0.08
261	Stk13deep	2/16/2008	1.90	0.21	0.24	2.11	8.96	16.39	77.16	0.21
262	Stk15sfc	2/16/2008	3.51	0.10	0.25	3.60	14.17	0.14	2.89	0.05
263	Stk15deep	2/16/2008	1.05	0.16	0.19	1.21	6.40	1.14	2.65	0.43
264	Stk16sfc	2/16/2008	4.86	0.02	0.44	4.89	11.06	0.19	2.19	0.09
265	Stk16deep	2/16/2008	1.38	0.15	0.20	1.53	7.74	1.24	2.22	0.56
266	Stk18sfc	2/16/2008	5.09	0.16	0.58	5.25	9.08	0.42	1.59	0.27
267	Stk18deep	2/16/2008	0.86	0.18	0.23	1.04	4.54	2.21	1.95	1.13

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
268	OM2	3/15/2008	2.40	0.22	0.26	2.62	9.94	1.30	3.19	0.41
269	OCN2	3/15/2008	0.17	0.18	0.18	0.34	1.94	0.00	2.14	0.00
270	OB	3/15/2008	2.76	0.33	0.35	3.09	8.94	0.01	0.94	0.01
271	OM1	3/15/2008	3.23	0.27	0.32	3.50	10.95	1.24	1.73	0.72
272	TM	3/15/2008	2.00	0.10	0.26	2.09	8.01	0.91	3.00	0.30
273	OCN1	3/15/2008	8.32	0.23	0.93	8.56	9.25	0.13	1.47	0.09
274	RM3	3/15/2008	5.54	0.03	0.73	5.58	7.63	4.14	2.79	1.49
275	RM2	3/15/2008	9.62	0.33	0.87	9.95	11.44	11.14	1.29	8.66
276	RM1	3/15/2008	15.73	0.19	0.86	15.92	18.56	1.57	1.56	1.01
277	River	3/15/2008	4.59	0.04	0.90	4.63	5.14	2.33	1.74	1.34
278	Stk1sfc	3/15/2008						0.88	1.60	0.55
279	Stk1deep	3/15/2008	0.41	0.12	0.16	0.53	3.35	0.49	2.12	0.23
280	Stk3sfc	3/15/2008	2.15	0.09	0.11	2.24	21.29	0.32	1.75	0.18
281	Stk3deep	3/15/2008	2.55	0.07	0.02	2.63	143.46	3.56	2.24	1.59
282	Stk6sfc	3/15/2008							1.84	
283	Stk6deep	3/15/2008	2.83	0.08	0.22	2.91	13.17	0.25	2.51	0.10
284	Stk7sfc	3/15/2008	5.82	0.09	0.82	5.91	7.20		2.46	
285	Stk7deep	3/15/2008	4.59	0.06	0.50	4.66	9.39	4.00	22.95	0.17
286	Stk8sfc	3/15/2008							1.83	
287	Stk8deep	3/15/2008	1.74	0.04	0.05	1.78	33.03	0.06	5.37	0.01
288	Stk9sfc	3/15/2008							1.38	
289	Stk9deep	3/15/2008	2.01	0.09	0.01	2.10	151.17	0.42	1.60	0.26
290	Stk13sfc	3/15/2008							1.80	
291	Stk13deep	3/15/2008	3.91	0.09	0.05	4.00	77.22	5.12	3.47	1.48
292	Stk15sfc	3/15/2008							1.76	
293	Stk15deep	3/15/2008	2.42	0.05	0.03	2.47	78.81	24.59	2.89	8.50
294	Stk16sfc	3/15/2008							4.27	
295	Stk16deep	3/15/2008	3.51	0.07	0.28	3.57	12.70		4.31	
296	Stk18sfc	3/15/2008							1.72	
297	Stk18deep	3/15/2008	4.32	0.03	0.71	4.35	6.16	2.67	3.15	0.85

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
298	OM2	4/19/2008	11.11	0.26	0.14	11.37	81.37	0.24		
299	OCN2	4/19/2008	2.56	0.24	0.22	2.80	12.92	0.00		
300	OB	4/19/2008	2.57	0.65	0.19	3.22	17.07	0.19		
301	OM1	4/19/2008	2.10	0.12	0.19	2.23	11.45	0.95		
302	TM	4/19/2008	3.49	0.22	0.23	3.71	16.40	0.79		
303	OCN1	4/19/2008	2.70	0.14	0.20	2.84	14.34	0.00		
304	RM3	4/19/2008	5.06	0.09	1.05	5.15	4.92	4.85		
305	RM2	4/19/2008						5.59		
306	RM1	4/19/2008						2.82		
307	River	4/19/2008	6.36	0.03	0.90	6.39	7.10	2.57		
308	Stk1sfc	4/19/2008						0.52		
309	Stk1deep	4/19/2008	3.44	0.19	0.26	3.64	13.82	18.99		
310	Stk3sfc	4/19/2008						0.57	0.57	1.00
311	Stk3deep	4/19/2008	2.34	0.30	0.28	2.64	9.38	0.40	0.54	0.75
312	Stk6sfc	4/19/2008						0.86	1.74	0.49
313	Stk6deep	4/19/2008						0.80		
314	Stk7sfc	4/19/2008						1.13	1.10	1.03
315	Stk7deep	4/19/2008						0.71	36.35	0.02
316	Stk8sfc	4/19/2008						1.05	1.99	0.53
317	Stk8deep	4/19/2008						1.18	1.54	0.77
318	Stk9sfc	4/19/2008						1.73		
319	Stk9deep	4/19/2008						0.61	7.73	0.08
320	Stk13sfc	4/19/2008						1.93		
321	Stk13deep	4/19/2008						3.23	2.75	1.17
322	Stk15sfc	4/19/2008						1.14		
323	Stk15deep	4/19/2008						2.11		
324	Stk16sfc	4/19/2008						0.54	1.18	0.46
325	Stk16deep	4/19/2008						0.47		
326	Stk18sfc	4/19/2008						1.40	6.36	0.22
327	Stk18deep	4/19/2008						2.96	1.86	1.59

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
328	OM2	5/17/2008	5.76	0.33	0.27	6.10	22.77	3.46	2.76	1.25
329	OCN2	5/17/2008	3.63	0.51	0.45	4.15	9.32	0.00	1.40	0.00
330	OB	5/17/2008	2.82	1.12	0.45	3.94	8.80	0.00	1.06	0.00
331	OM1	5/17/2008	3.32	0.47	0.53	3.80	7.11	0.00	0.93	0.00
332	TM	5/17/2008	2.97	0.46	0.53	3.43	6.41	0.02	1.35	0.02
333	OCN1	5/17/2008	6.03	0.35	0.72	6.38	8.86	0.02	0.97	0.02
334	RM3	5/17/2008	11.65	0.26	0.95	11.91	12.51	3.57	2.72	1.31
335	RM2	5/17/2008	6.49	0.12	0.88	6.61	7.51	14.83	1.91	7.76
336	RM1	5/17/2008	21.55	0.33	1.07	21.89	20.48	0.44	1.07	0.41
337	River	5/17/2008	5.00	0.13	0.90	5.13	5.72	3.06	0.63	4.89
338	Stk1sfc	5/17/2008	3.11	0.18	0.32	3.29	10.27	0.20	3.27	0.06
339	Stk1deep	5/17/2008	5.74	0.19	0.36	5.93	16.52	2.73	3.97	0.69
340	Stk3sfc	5/17/2008	3.10	0.57	0.37	3.66	9.97	0.00	0.94	0.00
341	Stk3deep	5/17/2008	2.49	0.59	0.27	3.08	11.23	0.00	1.37	0.00
342	Stk6sfc	5/17/2008	3.38	0.17	0.15	3.55	23.39	2.38	2.71	0.88
343	Stk6deep	5/17/2008	3.29	0.40	0.22	3.69	16.97	23.09	5.48	4.21
344	Stk7sfc	5/17/2008	1.31	0.14	0.24	1.46	6.07	2.11	2.00	1.06
345	Stk7deep	5/17/2008	4.02	0.19	0.32	4.21	13.21	0.71	5.51	0.13
346	Stk8sfc	5/17/2008	5.83	0.22	0.19	6.05	32.20	1.20	2.25	0.53
347	Stk8deep	5/17/2008	3.57	0.20	0.32	3.76	11.94	1.08	2.75	0.39
348	Stk9sfc	5/17/2008	6.68	0.17	0.14	6.85	47.57	0.38	0.73	0.52
349	Stk9deep	5/17/2008	3.51	0.22	0.21	3.73	17.84	3.09	0.96	3.22
350	Stk13sfc	5/17/2008	6.18	0.26	0.37	6.44	17.51	2.14	2.82	0.76
351	Stk13deep	5/17/2008	6.84	0.32	0.36	7.16	19.82	1.83	3.49	0.53
352	Stk15sfc	5/17/2008	4.43	0.21	0.37	4.64	12.55	0.19	2.03	0.09
353	Stk15deep	5/17/2008	5.38	0.31	0.27	5.69	21.35	0.10	2.67	0.04
354	Stk16sfc	5/17/2008	4.18	0.15	0.28	4.34	15.23	0.02	3.44	0.00
355	Stk16deep	5/17/2008	13.19	0.32	0.32	13.51	42.60	1.16	2.56	0.45
356	Stk18sfc	5/17/2008	2.56	0.13	0.36	2.70	7.51	0.55	3.15	0.18
357	Stk18deep	5/17/2008	2.01	0.20	0.31	2.20	7.08	0.00	3.03	0.00

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chla^{-1} h^{-1})
358	OM2	6/14/2008	4.71	1.19	0.48	5.90	12.19	2.41	4.12	0.58
359	OCN2	6/14/2008	21.77	0.21	0.63	21.98	34.68	1.44	2.63	0.55
360	OB	6/14/2008	7.39	0.13	0.79	7.52	9.56	2.06	2.33	0.88
361	OM1	6/14/2008	5.66	0.10	0.92	5.76	6.28	2.75	3.21	0.86
362	TM	6/14/2008	6.47	0.53	0.81	7.00	8.70	2.45	2.23	1.10
363	OCN1	6/14/2008	5.59	0.14	0.79	5.73	7.22	1.21	2.92	0.42
364	RM3	6/14/2008	16.53	1.62	0.79	18.15	22.84	4.03	3.49	1.15
365	RM2	6/14/2008						8.95	0.83	10.83
366	RM1	6/14/2008	6.09	1.19	0.86	7.27	8.46	3.40	2.13	1.59
367	River	6/14/2008	4.59	0.04	0.90	4.64	5.17	2.01	0.39	5.22
368	Stk1sfc	6/14/2008	4.05	0.22	0.39	4.27	10.99	1.02	4.81	0.21
369	Stk1deep	6/14/2008	6.68	0.48	0.34	7.16	20.77	3.76	6.53	0.58
370	Stk3sfc	6/14/2008	10.84	0.45	0.05	11.29	246.34	1.17	1.58	0.74
371	Stk3deep	6/14/2008	7.79	1.41	0.39	9.20	23.78	0.51	2.68	0.19
372	Stk6sfc	6/14/2008	8.53	0.95	0.83	9.48	11.40	2.43	3.91	0.62
373	Stk6deep	6/14/2008	2.89	1.47	0.53	4.36	8.26		3.74	0.00
374	Stk7sfc	6/14/2008	6.36	0.05	0.82	6.41	7.77	2.78	6.47	0.43
375	Stk7deep	6/14/2008	2.08	0.70	0.50	2.78	5.61	25.72	13.56	1.90
376	Stk8sfc	6/14/2008	6.36	5.98	0.83	12.34	14.86	2.71	4.09	0.66
377	Stk8deep	6/14/2008	2.32	1.00	0.38	3.33	8.76	3.00	4.27	0.70
378	Stk9sfc	6/14/2008	5.95	4.31	0.84	10.26	12.20	2.55	4.40	0.58
379	Stk9deep	6/14/2008	2.18	0.10	0.22	2.27	10.24	0.63	1.91	0.33
380	Stk13sfc	6/14/2008	10.16	4.65	0.84	14.81	17.72	2.32	6.62	0.35
381	Stk13deep	6/14/2008	2.83	0.73	0.43	3.56	8.27	0.84	3.04	0.28
382	Stk15sfc	6/14/2008	14.10	5.82	0.83	19.92	23.87	1.99	4.93	0.40
383	Stk15deep	6/14/2008	3.88	0.93	0.36	4.81	13.51	1.70	4.54	0.38
384	Stk16sfc	6/14/2008	7.58	6.14	0.83	13.72	16.47	2.21	5.23	0.42
385	Stk16deep	6/14/2008	2.96	1.24	0.33	4.20	12.73	1.32	3.31	0.40
386	Stk18sfc	6/14/2008	13.56	4.93	0.92	18.49	20.08	2.01	3.66	0.55
387	Stk18deep	6/14/2008						1.13	2.75	0.41

Sample #	Site:	Date	NH_4^+	NO_2^-+ NO_3^-	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
388	OM2	7/26/2008	0.98	0.20	0.11	1.17	10.67	1.13	0.53	2.13
389	OCN2	7/26/2008	3.36	0.23	0.19	3.58	19.19	1.15	0.54	2.13
390	OB	7/26/2008	0.85	0.23	0.22	1.08	4.95	0.74	0.80	0.92
391	OM1	7/26/2008	1.81	0.21	0.26	2.02	7.83	1.20	1.06	1.13
392	TM	7/26/2008	2.40	0.21	0.17	2.62	15.49	1.28	0.82	1.57
393	OCN1	7/26/2008	3.39	0.28	0.29	3.68	12.85	1.33	0.92	1.44
394	RM3	7/26/2008	6.00	0.35	0.59	6.34	10.79	10.93	4.29	2.55
395	RM2	7/26/2008	7.04	0.05	0.86	7.09	8.22	2.21	1.50	1.47
396	RM1	7/26/2008	6.77	0.03	0.79	6.80	8.57	22.45	4.40	5.10
397	River	7/26/2008	7.17	0.11	0.89	7.29	8.14	4.59	0.66	6.95
398	Stk1sfc	7/26/2008	3.09	0.16	0.01	3.25	241.83	4.01	1.17	3.42
399	Stk1deep	7/26/2008	4.46	0.22	0.09	4.69	52.81	5.87	1.48	3.97
400	Stk3sfc	7/26/2008	3.44	0.17	0.15	3.61	24.71	1.96	2.64	0.74
401	Stk3deep	7/26/2008	1.76	0.30	0.10	2.06	20.14	1.63	1.09	1.50
402	Stk6sfc	7/26/2008	2.94	0.09	0.35	3.03	8.71	9.07	6.36	1.43
403	Stk6deep	7/26/2008	0.73	0.18	0.29	0.91	3.10	4.68	2.57	1.82
404	Stk7sfc	7/26/2008	3.78	0.16	0.23	3.93	16.75	10.46	3.82	2.74
405	Stk7deep	7/26/2008	0.38	0.14	0.30	0.51	1.72	5.55	4.71	1.18
406	Stk8sfc	7/26/2008	3.37	0.03	0.28	3.40	12.27	5.94	3.34	1.78
407	Stk8deep	7/26/2008	0.67	0.20	0.20	0.87	4.25	3.40	2.96	1.15
408	Stk9sfc	7/26/2008	1.86	0.09	0.28	1.95	6.85		2.07	
409	Stk9deep	7/26/2008	1.09	0.18	0.20	1.27	6.30		4.42	
410	Stk13sfc	7/26/2008	2.48	13.27	0.05	15.75	326.42		1.38	
411	Stk13deep	7/26/2008	2.50	0.21	0.22	2.71	12.14		1.91	
412	Stk15sfc	7/26/2008	5.68	0.03	0.33	5.71	17.06		4.80	
413	Stk15deep	7/26/2008	1.16	0.20	0.18	1.36	7.64		4.02	
414	Stk16sfc	7/26/2008	4.73	0.02	0.73	4.74	6.53		10.51	
415	Stk16deep	7/26/2008	0.93	0.17	0.22	1.10	4.94		3.69	
416	Stk18sfc	7/26/2008	5.68	0.03	0.82	5.71	6.99		5.15	
417	Stk18deep	7/26/2008	0.50	0.14	0.19	0.64	3.31		3.66	

Sample #	Site:	Date	NH_4^+	$NO_2^- + NO_3^-$	PO_4^{3-}	DIN	DIN:DIP	APA	Chl *a*	Specific APA
		M/D/Y	(µM)	(µM)	(µM)	(µM)		(nM-P h^{-1})	µg L^{-1}	(nM-P µg-chl*a*$^{-1}$ h^{-1})
418	OM2	8/30/2008	2.90	0.18	0.20	3.08	15.69	1.08	1.99	0.54
419	OCN2	8/30/2008	0.33	0.11	0.16	0.44	2.75	0.41	1.07	0.38
420	OB	8/30/2008	2.81	0.59	0.13	3.39	26.98	0.31	1.17	0.27
421	OM1	8/30/2008	3.75	1.02	0.19	4.77	24.60	0.40	1.14	0.36
422	TM	8/30/2008	3.46	0.45	0.41	3.90	9.52	0.14	2.21	0.06
423	OCN1	8/30/2008	3.25	0.26	0.41	3.51	8.61	0.53	1.47	0.36
424	RM3	8/30/2008	9.18	0.26	1.51	9.44	6.24	3.95	2.88	1.37
425	RM2	8/30/2008	7.85	0.06	0.87	7.91	9.11	6.81	1.33	5.12
426	RM1	8/30/2008	8.41	0.20	0.99	8.61	8.73	13.81	1.02	13.61
427	River	8/30/2008	7.17	0.10	0.89	7.27	8.14	3.74	0.50	7.48
428	Stk1sfc	8/30/2008	1.93	0.24	0.15	2.17	14.30	2.50	5.42	0.46
429	Stk1deep	8/30/2008	2.06	0.27	0.22	2.33	10.35	4.79	5.46	0.88
430	Stk3sfc	8/30/2008	2.27	0.17	0.16	2.45	15.77	0.82	1.69	0.49
431	Stk3deep	8/30/2008	0.99	0.16	0.15	1.15	7.58	2.31	3.83	0.60
432	Stk6sfc	8/30/2008	5.57	0.17	0.37	5.74	15.37	2.30	2.08	1.11
433	Stk6deep	8/30/2008	1.83	0.13	0.34	1.96	5.78	13.97	30.15	0.46
434	Stk7sfc	8/30/2008	8.40	0.08	0.97	8.48	8.78	4.21	3.05	1.38
435	Stk7deep	8/30/2008	0.33	0.11	0.39	0.44	1.13	8.39	5.52	1.52
436	Stk8sfc	8/30/2008	7.45	0.12	0.34	7.58	22.18	4.07	4.00	1.02
437	Stk8deep	8/30/2008	2.20	0.20	0.32	2.39	7.37	3.59	3.77	0.95
438	Stk9sfc	8/30/2008	1.71	0.13	0.19	1.84	9.53	3.34	1.83	1.83
439	Stk9deep	8/30/2008	0.61	0.19	0.26	0.79	3.07	4.44	9.11	0.49
440	Stk13sfc	8/30/2008	1.92	0.15	0.19	2.07	11.03	3.06	4.85	0.63
441	Stk13deep	8/30/2008	1.07	0.14	0.26	1.20	4.64	5.37	6.72	0.80
442	Stk15sfc	8/30/2008	1.46	0.73	0.13	2.19	16.54	1.27	3.18	0.40
443	Stk15deep	8/30/2008	1.05	0.25	0.20	1.30	6.47	1.60	12.22	0.13
444	Stk16sfc	8/30/2008	4.90	0.18	0.48	5.08	10.69	2.05	3.16	0.65
445	Stk16deep	8/30/2008	1.75	0.15	0.20	1.90	9.51	2.07	12.61	0.16
446	Stk18sfc	8/30/2008	3.78	0.06	0.18	3.83	20.81	4.23	4.13	1.02
447	Stk18deep	8/30/2008	2.45	0.18	0.84	2.63	3.13	4.30	3.01	1.43

Overview of Method

1) DiFMU (Standard) Stock Solution 942.9 μM

Materials Needed:

- 6,8-difluro-7-hydroxyl-4-methylcoumarin ($C_{10}H_6F_2O_3$) [DiFMU] 10 mg (Molecular Probes) Cat # D6566
- Methyl sulfoxide ($C_{10}H_6OS$) [DMSO]
- 50 mL volumetric flask
- Glass stopper
- 60 mL HDPE amber bottle

Procedure:

- Dissolve the entire contents (10 mg) of DiFMU into 50 mL of DMSO. This produces a 942.9 μM DiFMU stock solution.
- Advice:
    - To dissolve entire contents, add 1 mL aliquots to the 10 mg vial of DiFMU.
    - After each addition shake well and pipette contents of vial into 50 mL volumetric flask.
    - Continue doing this until vial is thoroughly rinsed of DiFMU [a yellow powder].
    - Once thoroughly rinsed you may bring up in DMSO to a final volume of 50 mL
- Insert glass stopper into 50 mL volumetric flask and invert multiple times to homogenize.
- Rinse a 60 mL HDPE amber bottle with a small amount of solution
- Pour the remaining solution into amber bottle and store at room temperature
- If stored properly the solution is stable indefinitely

2) DiFMU (Standard) Working Solution 20 μM

Materials Needed:

- 942.9 μM DiFMU stock solution [DiFMU Stock]
- Methyl sulfoxide ($C_{10}H_6OS$) [DMSO]
- 250 mL volumetric flask
- Glass stopper
- 250 mL amber bottle

Procedure:

- In an acid clean, 3 times DMSO rinsed, 250 mL volumetric flask add 2650 μL of DiFMU Stock, add remaining volume of DMSO to a final volume of 250 mL
- Glass stopper and invert several times to homogenize
- Rinse an amber bottle 3 times with the 20 μM DiFMU standard
- Store in a plastic amber bottle at room temperature
- If stored properly the solution is stable indefinitely

3) DiFMU-P (Substrate) Stock Solution 10 mM

Materials Needed:

- 6,8-Difluoro-4-Methylumbelliferyl phosphate ($C_{10}H_2F_2O_6P$) [DiFMU-P] (Molecular Probes) Cat # D6567
- Methyl sulfoxide ($C_{10}H_6OS$) [DMSO]
- 1.5 mL amber microtubes (Fisher Scientific) Cat # 05-406-134

Procedure:

- Dissolve entire contents of DiFMU-P vial (5 mg) by adding 1.71 mL of DMSO directly to vial. Cap and shake to dissolve and homogenize.
- Remove 40 µL aliquots from vial and store frozen (-30°C) in 1.5 mL amber microtubes.
- 40 µL aliquots are stable for approximately 3 months but this should be monitored

4) 30 ppt Artificial Seawater (ASW)

Materials Needed:

- Sigma Sea Salts (Sigma- Life Sciences) Cat # S9883-500G
- Milli-Q Deionized water [MQ]
- 0.2 µm GHP filter (47 mm diameter)
- Glass filtration rig and pump
- Balance
- Glass beaker

Procedure:

- Add 30 g of Sigma Sea Salt to 970 ml of MQ in a 2-L acid clean beaker
- Dissolve sea salts completely by placing beaker on stir plate, heating slightly to ensure that salts are completely dissolved (< 5 minutes)
    - Please Note: Avoid heating excessively to prevent evaporation
- Filter through a 0.2 µm GHP filter
- Rinse an acid clean bottle 3 times with prepared ASW
- Store at room temperature

5) To Prepare DiFMU Standards

Materials Needed:

- 20 µM DiFMU (prepared in section 2)
- 30 ppt. ASW (prepared in section 4)

Procedure:

- Label small (at least 2 mL), acid clean, reaction vessels with the final intended standard concentration
- Add 20 µM DiFMU and ASW as follows:

Final DiFMU Concentration (μM)	20 μM DiFMU (μL)	30 ppt. ASW (μL)	Total Volume (μL)
0	0	500	500
0.2	10	490	500
0.4	20	480	500
0.8	40	460	500
2.0	100	400	500
5.0	250	250	500
	For Final Volume	of 1000 μL	
0	0	1000	1000
0.2	20	980	1000
0.4	40	960	1000
0.8	80	920	1000
2.0	200	800	1000
5.0	500	500	1000

- Vortex each standard for 3 sec at 800 rpm to homogenize
- Add 150 μL of each standard (preferably in triplicate) to a half-area 96-well solid black plate

6) DiFMU-P (Substrate) Working Solution 0.8 mM

Materials Needed:

- 40 μL frozen aliquot of 10 mM DiFMU-P (prepared in section 3)
- 30 ppt. ASW (prepared in section 4)

Procedure:

- Add 460 μL ASW to frozen 40 μL aliquot of DiFMU-P directly into microtube
- Invert microtube several times to homogenize

7a) Sample Preparation:

Note: Samples should be placed into new Petri dishes (47mm) if not stored there originally

Materials Needed:

- 30 ppt ASW (prepared in section 4)
- 0.8 mM DiFMU-P (prepared in section 6)
- 96-well half-area solid black plate (Costar) Cat # 3694
- NOTE: Solid black plates are used to reduce interference between wells.

Procedure:

- Pipette 2 ml ASW directly onto filters in petri dish
- Incubate on a shaker table (250 rpm) for 10 minutes
    - This allows for the re-suspension of cells
- Prepare Standards and Blanks
    - Each plate being run should be accompanied by a standard curve between 0-5 μM DiFMU (see section 5)
    - Each plate should also be accompanied by a filter with an equivalent volume as samples of DIW filtered through it as a blank

        - These filters should be run the same way as the sample filters
        - A minimum of 1 blank should be run for each plate
- Add 25 µl of 0.8 mM DiFMU-P
    - This results in a final concentration of 10 µM DiFMU-P
- A 150 µl split should be removed from the petri-dish and added to a 96-well half-area solid black plate that has been prepared with standards and blanks
    - Using the Gen5 software as described in section 8 allows the computer to keep track of read times. The accuracy of these read times are of great importance
- Read plate as described in section 8
- Samples should continue to shake in between splits
- A minimum of 8 splits should be analyzed within a 3 hour period
    - Samples with higher activity should be analyzed more rapidly, while lower activity samples should be analyzed more slowly.

7b) Preparation of Samples For Initial Kinetic Experiments

Note: Initial Kinetic Experiments are completed to determine the saturating concentration of DiFMU-P for a given field site.

Materials Needed:

- Identical to those listed in section 7a

Procedure:

- Pipette 2 mL ASW directly onto filter in petri dish.
- Incubate on a shaker table (250 rpm) for 10 minutes
- Add varying concentrations to each replicate sample as follows:

Final DiFMU-P (µM)	0.8 mM DiFMU-P (µL)
1.0	2.5
2.0	5.0
4.0	10.0
6.0	15.0
8.0	20.0
10.0	25.0
12.0	30.0
14.0	35.0

- Shake (250 rpm) for an additional 3 minutes to homogenize
    - Please note: an orbital shaker is preferable. If a reciprocal shaker is all that is available it is beneficial to rotate samples periodically during shaking to ensure even re-suspension of cells and distribution of DiFMU-P.
- Add 150 µl of each sample (preferably in triplicate) to the 96-well plate
- Read plate as detailed in section 8

8a) To Read Plate on Synergy HT Plate Reader with KC4 v3.4

PLEASE NOTE: The following should be set up prior to reacting samples

- Select “New”
- Select “Data”
    - Select “New Plate”
- Select “Protocol”
    - Select “New”
- Select “Settings”
    - Note: The following instructions will be moving from top left to bottom right
    - Section name: Reading Type
        - Mark “End Point”
    - Section name: Detection Method
        - Mark “Fluorescence”
    - Section name: Filters
        - Mark “Filter Set 1”
            - Note: It will be the only filter set needed
        - Set Excitation to “360/40”
        - Set Emission to “460/40”
        - Set Optics to “Top”
        - Set Sensitivity
            - Sensitivity will vary depending on the APA of the sample. “25” is the lowest sensitivity “255” is the highest. There are a few things to keep in mind when setting your sensitivity, they are as follows:
                - Samples and standards must be read at the same sensitivity setting
                - Setting sensitivity too low may produce 1-2 digit results, drastically increasing percent relative error
                - Setting sensitivity too high can cause readings to exceed the limits of the instrument. The highest raw number measurement is “99998”
                - Typically a sensitivity setting around 50 is sufficient and is recommended.
    - Section name: Plate
        - Set Type to: “Costar 96 half area black opaque”
            - Note: If using a different type of plate adjust accordingly, select best fit of types offered
        - Set First Well to: The top left well that is filled
            - Ex: A1
        - Set Last Well to: The bottom right well that is filled
            - Ex: H12
                - A1-H12 will read every well on the entire plate
                - NOTE: Reducing the number of wells being read decreases the required run-time of a plate. During kinetics you want to reduce run-time as much as possible.

    - Section name: Temperature Control
        - Mark "Yes"
        - Mark "Pre-Heating"
        - Set Temperature to: "25°C"
        - There will be NO
            - Lag time
            - Shaking
            - Pre-Reading
            - General Options
    - Select "Ok" at the very bottom to save and close "Settings"
- Select "Layout"
    - Note: The layout is a reflection of the layout of your plate
    - In the top right corner there are a variety of settings for Standards, Samples, and Blanks
        - Label Appropriately
    - Select "Ok" at the bottom to save and close "Layout"
- Select "Read"
    - Select "Start Reading"
        - Insert plate and press "Ok"
        - Allow ample time for the plate reader to come up. A pre-heating screen will appear. When pre-determined temperature is met (25°C) "Reached" will appear. When "Reached" had become illuminated select "Read Plate"
        - A screen for warming will appear with a 170 second countdown, allow the warming to proceed. When warming is complete the plate will be read automatically.
- To Export Data
    - When reading is complete select "Data"
        - Select "Export"
            - An excel spreadsheet will open
                - Select "Plates"
                    - This will give a variety of different options; choose the data you would like to export. The following are some recommendations:
                        - Plate Layout
                        - M360/460
                        - M360/460 Cor.
        - Save data in a systematic way. Consider a uniform file name that incorporates the date and the users initials.

8b) To Read Plate on Synergy HT Plate Reader with Gen5 v 1.10 Software

- Ensure that the plate reader is switched on prior to opening the software
    - This will resolve any connectivity issues
- Click on the Gen5 icon on the desktop

To Create an APA protocol

- In the "Create a New Item" box click "Protocol"

- A list will appear in the screen (Procedure, Plate Layout, Data Reduction, etc)
- Click "Procedure"
    - A new window will appear "Procedure-Synergy (Com 4)
    - On the left side of the window there is a box titled "Add Step"
        - Click on "Read"
        - A new window "Read Step" will appear
        - Select the following (in this order):
            - Detection Method: Fluorescence
            - Read Type: Endpoint
            - Under Filter Sets
                - Excitation: 360/40
                - Emission: 460/40
                - Optics Position: Top
                - Sensitivity: 50*
                - Click "OK"

*A Note on Sensitivity: The sensitivity will change depending on the activity of your samples. I recommend choosing standard concentrations that seem reasonable (typically between 0-5 μM). Add standards to your plate and run with the selected sensitivity to ensure that your standard curve is within range. If not in range you will receive an "OVERFLW" reading. Then, to ensure that the activity of your samples falls within this range analyze sample splits at a faster or slower pace to utilize the most dynamic range while staying within the upper limit.

- You will return to the "Procedure" window. In the "Description" box you will see that you will "Read: (F) 360/40,460/40
- Select "Set Temperature" in the add step box
    - Select "Incubator On"
    - Set temperature to "35" °C
    - Check the "Preheat before continuing with next step box"
    - Click "OK"
- You will return to the "Procedure" window.
- In the "Description" box drag the "Temperature: Setpoint 25°C" command to be above the "Read: (F) 360/40,460/40" command
- Select "Advanced Options" button in the upper right corner of the "Procedure" window
    - A new window "Advanced Options" will appear
    - Check the "Discontinuous Kinetic Procedure" box
    - Set the following:
        - Estimated total time: 00:03:00
        - Estimated interval: 0:00:25
        - This will give "Number of runs": 8
        - Click "OK"
- This will bring you back to the "Procedure" box
    - Click "OK"

- Select "Plate Layout" from the list
- A "Plate Layout" box will appear

- This box enables you to set up a template that matches your plate, fill in accordingly. These area is self-explanatory
- When done, click the "OK" button

You are now all done in setting up your protocol
Save this protocol

Running Plates:

Select "File" then "New Experiment"
A window entitled "New Experiment" select the protocol that you just created, then click "OK"

Step 1: Test your standards and sensitivity
- You should have your plate loaded with only your standards
- In the left hand control box click "Plate 1"
- Select "Read" from the drop down menu
- A new window entitled "Plate 1 Reading" will appear, select "READ"
- Allow bulb to warm up and make sure that the 25°C temperature is met
- Read plate

If the standards to not "OVERFLW" you may proceed

Step 2: Read samples
- Because you want to keep track of your time and the first plate was used to check your standards you want to add a new plate. Left click "Plate 1" and select "Add plates"
- Click "OK" in the "Add Plates" window
- Add 150 µL of sample and blank into plate as described in previous sections
- Left click "Plate 2" and clich "Read"
- Read plate
- Repeat this until all 8 "reads" have been completed.

Step 3: Export Data
- In the left window left click "Plate 2"
- From the dropdown menu select "File Export"
- This will export the file into an Microsoft Excel document, save data as desired