

Alkaline phosphatase activity in symbiotic dinoflagellates (zooxanthellae) as a biological indicator of environmental phosphate exposure

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ABSTRACT: Alkaline phosphatase activity (APA) is an indicator of phosphorus status in marine plants. We examined APA in the symbiotic dinoflagellate *Symbiodinium bermudense*, which occurs with the sea anemone *Aiptasia pallida*. We have developed an assay for APA that serves as a biological indicator of inorganic phosphate (P_i) exposure in coral reef environments and phosphorus deficiency in zooxanthellae. Zooxanthellae maintained in culture responded to a lack of P_i with elevated levels of APA. Laboratory experiments with *A. pallida* demonstrated that this effect occurs in symbiosis, although at a longer timescale. The addition of P_i to ambient sea water resulted in higher zooxanthellae density in unfed anemones compared to unfed controls maintained without additional P_i , indicating that *S. bermudense* can utilize exogenous sources of P_i . APA was measured in zooxanthellae from the coral *Montastraea faveolata* collected from 2 reef sites with different phosphorus exposure. Zooxanthellae from corals at the low P_i site had significantly higher APA levels than those from the high P_i site. These results indicate the potential of this assay as an indicator of phosphorus exposure in a coral reef environment.

KEY WORDS: Alkaline phosphatase · Acid phosphatase · Nutrient deficiency · Symbiosis · *Aiptasia* · *Montastraea* · *Symbiodinium*

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INTRODUCTION

Alkaline phosphatase activity (APA) has been used as an indicator of phosphorus deficiency in a wide range of marine plants. APA hydrolyzes organic phosphorus compounds resulting in the liberation of inorganic phosphate (P_i) and an alcohol moiety (reviewed in Feder 1973). This provides a means of regenerating P_i for marine plants in a P_i -deficient environment. There is an inverse relationship between environmental P_i and APA which is the basis for using phosphatase activity as an indicator of phosphorus deficiency. APA assays were first applied to intact marine algae by

Kuenzler & Perras (1965). They demonstrated that APA in cultured marine algae increases as the algae become phosphorus-deficient, but is repressed in media containing ample P_i . These results were further supported by the work of Fitzgerald & Nelson (1966), which indicated that APA in freshwater phytoplankton was elevated 25-fold in response to phosphorus deficiency. Ecological studies involving APA (Fitzgerald & Nelson 1966, Reichhart et al. 1967, Berman 1970, Wynne 1981, Rivkin & Swift 1982, Jansson et al. 1988, Berman et al. 1990) have shown that natural assemblages of phytoplankton exhibit similar responses to phosphorus deficiency. APA assays have also been applied as indicators of phosphorus deficiency in a variety of marine plants, including cyanobacteria (Yentsch et al. 1972), marine diatoms (Möller et al. 1975), sea grasses (Short et al. 1985, Perez & Romero 1993) and benthic algae (Lapointe 1987, Lapointe &

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O'Connell 1989). All of these studies indicate that phosphatases are repressible enzymes expressed in response to low environmental P_i .

In this paper, we apply an APA assay to zooxanthellae symbiotic with sea anemones and reef corals to address the question of phosphate exposure in coral reef environments. Reef-building corals thrive in oligotrophic tropical waters, and elevated nutrient levels may have deleterious effects on corals that include light limitation by phytoplankton blooms, increased competition from otherwise nutrient limited benthic macroalgae (Johannes 1975, Bell 1992, Lapointe & Clark 1992, but see Hughes et al. 1999), and sub-lethal impacts on growth and reproduction (Marubini & Davies 1996, Koop et al. 2001). Using zooxanthellae as biological indicators of environmental nutrients has the advantage of integrating nutrient exposure over time to include fluctuations and episodic events that might be missed when using conventional nutrient analysis (Cook et al. 1997). The nutrient status of these algae has been assessed by indices such as elemental ratios, internal amino acid pools and ammonium enhancement of dark carbon fixation. However, these techniques have been used largely to assess nitrogen status rather than phosphorus, and have received only limited use in the field.

In corals and sea anemones, zooxanthellae occur in individual membrane-bound vacuoles (symbiosomes or perialgal vacuoles) within the endodermal cells of the host. Thus, exogenous nutrients must pass through at least 2 animal membranes before being assimilated by the algae. Despite these barriers to the external environment, uptake of P_i has been observed in corals and sea anemones, and is dependent upon the presence of zooxanthellae (Yonge & Nicholls 1931, D'Elia & Webb 1977, Jackson & Yellowlees 1990, Muller-Parker et al. 1990).

The development of an APA assay for zooxanthellae also provides an opportunity to examine phosphorus interactions between the host and symbiont. Cook & D'Elia (1987) as well as Miller & Yellowlees (1989) proposed that cnidarian hosts use nutrient limitation to control the growth of symbiotic zooxanthellae populations. Nitrogen has been suggested as limiting the growth of zooxanthellae in several corals (Hoegh-Guldberg & Smith 1989, Muscatine et al. 1989, Muller-Parker et al. 1994), but there is evidence for P-limitation of zooxanthellae in the coral *Acropora formosa* (Jackson et al. 1989, Jackson & Yellowlees 1990). Jackson et al. (1989) isolated 2 acid phosphatases from the zooxanthellae in *A. formosa* and demonstrated the repressibility of these enzymes in freshly isolated zooxanthellae. The nutrient status of *Symbiodinium bermudense* in symbiosis with the sea anemone *Aiptasia pallida* has not been determined with respect to phos-

phorus. Zooxanthellae from well-fed and field-collected *A. pallida* were nitrogen-sufficient, but became N-limited when the host was starved (Cook et al. 1997). Increased uptake rates of P_i by symbiotic *A. pallida* during starvation (Muller-Parker et al. 1990) indicated P-limitation, but the P-status of zooxanthellae was not examined.

Here, we demonstrate that *Symbiodinium bermudense* becomes P-limited with starvation of the host and exhibits APA levels which reflect P_i levels in the ambient seawater. We also report significant differences in the APA of zooxanthellae from the coral *Montastraea faveolata* that reflect ambient P_i levels *in situ*. These results suggest that APA may be a useful indicator of environmental P_i exposure in coral reef environments.

MATERIALS AND METHODS

Organisms. The sea anemone *Aiptasia pallida* and its dinoflagellate symbiont *Symbiodinium bermudense* (Banaszak et al. 1993) were obtained from a clonal culture of *A. pallida* originally collected from Walsingham Pond, Bermuda (Cook et al. 1988). The symbiont was originally isolated from the host and cultured by G. Muller-Parker in 1984 (pers. comm.). Both the anemones and the cultured zooxanthellae (CZ) were maintained in an incubator at 25°C with a 12:12 h light:dark photoperiod (illumination $\sim 70 \mu\text{mol m}^{-2} \text{s}^{-1}$). The anemones were fed freshly hatched *Artemia* spp. daily for at least 5 d prior to use. CZ had been maintained in batch culture with Provasoli's ES medium (Provasoli 1968) since their initial isolation. For this work, a modified ES medium was used with an inorganic source of phosphate ($[\text{NaH}_2\text{PO}_4]_{\text{final}} = 56 \mu\text{M}$) in place of the standard organic phosphorus source (β -glycerophosphate). Cultures were inoculated with sterile technique, using acid-washed, autoclaved glassware and autoclaved filtered (0.22 μm) seawater.

The field component of this work utilized zooxanthellae (*Symbiodinium* sp.) from the coral *Montastraea faveolata*. Corals were collected from 2 sites: the 'Coral Gardens' located approximately 1.5 km from shore on the south side of Lower Matecumbe Key, and 'Bock Cay' near Lee Stocking Island in the Bahamas. All coral colonies were located in 1 to 3 m of water, and coral samples were obtained by chiseling off 10 to 25 cm^2 pieces from the skirt of colonies. The Bahamian corals were collected under a CITES permit issued to C.B.C. Florida Keys corals were collected under federal (FKNMS-225-97) and state (Florida 96S-046) permits issued to C.B.C.

Isolation of zooxanthellae. Freshly isolated zooxanthellae (FIZ) were obtained from *Aiptasia pallida* and *Montastraea faveolata* through homogenization of ani-

mal tissues, and separation of zooxanthellae through repeated centrifugation and suspension in seawater (Johannes & Wiebe 1970). CZ in late log phase growth (2 to 9×10^5 cells ml^{-1}) were isolated from their culture medium by centrifugation and were resuspended in seawater. Gulf Stream filtered ($0.22 \mu\text{m}$) seawater (GSFSW) was used in these preparations and had a background concentration of soluble reactive phosphorus below the level of detection for our assay ($<0.030 \mu\text{M}$; Strickland & Parsons 1972). Final suspensions of algae from the 3 different sources were made up to concentrations between 5 and 10×10^5 cells ml^{-1} for phosphatase assays, and aliquots were frozen for subsequent cell counts.

Assay of phosphatase activity. The working parameters for the assay of phosphatase activity in suspensions of zooxanthellae were developed in preliminary studies (Annis 1998). Phosphatase activity was determined using a colorimetric assay modified from that of Kuenzler & Perras (1965), using para-nitrophenyl phosphate (*p*-NPP) as a substrate which yields the yellow hydrolysis product, para-nitrophenol (*p*-NP). The incubation solution consisted of $500 \mu\text{l}$ cell suspension (as above), $500 \mu\text{l}$ *p*-NPP stock solution (7.6 mM *p*-NPP in GSFSW) and $50 \mu\text{l}$ Tris buffer solution (1.0 M prepared in GSFSW). Incubations were conducted in darkness at 25°C for 2 to 5 h and were terminated by the addition of $200 \mu\text{l}$ 1.0 N NaOH. Samples were centrifuged immediately after incubation to remove algal cells and precipitate formed by the addition of NaOH. The absorbance of the supernatant was read at 410 nm on a spectrophotometer using 1.0 cm cuvettes against a blank of $500 \mu\text{l}$ GSFSW, $500 \mu\text{l}$ *p*-NPP, $200 \mu\text{l}$ 1.0 N NaOH and $50 \mu\text{l}$ 1.0 M Tris base. The blank solution was prepared immediately prior to sample reading. Three background samples were run with each incubation using $500 \mu\text{l}$ GSFSW in place of the algal suspension to account for non-enzymatic decomposition of the substrate and any sources of phosphatase activity present in the GSFSW. The average value of the background replicates was subtracted from sample readings before enzyme activity was calculated. A standard curve for absorbance of the reaction product was established by linear regression (absorbance = $0.159[p\text{-NP}] + 0.0005$, $R^2 = 0.9998$) using known concentrations of ACS reagent grade *p*-NP in samples containing $1000 \mu\text{l}$ GSFSW, $50 \mu\text{l}$ 1.0 M Tris base and $200 \mu\text{l}$ 1.0 N NaOH. Enzyme activity was expressed in units of $\text{fmol } p\text{-NP released cell}^{-1} \text{ h}^{-1}$.

Biomass determinations. Total zooxanthellae per anemone was calculated from cell counts of the initial anemone homogenate. Aliquots were frozen at the time of isolation, and cell counts were performed on a hemocytometer and diluted 2- or 4-fold with GSFSW as needed. Total zooxanthellae were reported as

10^6 cells anemone $^{-1}$. Symbiont density (algal cells mg protein^{-1}) was also calculated to compensate for natural variation in the size of anemones. Anemone protein was assayed using the combined supernatants from the first 2 rinses of the algal pellet. Three 1.0 ml aliquots were taken for protein assays. Protein content ($\text{mg protein anemone}^{-1}$) was determined for each aliquot by the Hartree modification of the Lowry procedure (Hartree 1972).

pH optima of phosphatases of *Symbiodinium bermudense*. To determine the optimal pH for the phosphatases of zooxanthellae in culture and in symbiosis, CZ and FIZ were assayed for phosphatase activity over a range of pH values between 4.0 and 11.0 . Assay solutions with pH values of 4.0 , 5.0 and 6.0 were prepared using a 1.0 M stock solution of citrate buffer (prepared after Gomori 1975). To obtain pH 7.0 , 8.0 and 9.0 , 1.0 M Tris buffer was titrated with 6 N HCl, while a 1.0 M carbonate/bicarbonate buffer (Gomori 1975) was titrated with 6 N NaOH to yield pH 10.0 and 11.0 . All buffer solutions were prepared in GSFSW, and $50 \mu\text{l}$ of each was added to the incubation solution in place of the regular Tris base to yield final buffer concentrations of $\sim 0.05 \text{ M}$. Three cultures of algae and 3 preparations of FIZ from *Aiptasia pallida* were assayed over this pH range, using an incubation time of 4.0 h .

Effect of exogenous P_i on APA of *Symbiodinium bermudense*. Cultured zooxanthellae were isolated from eight 150 ml subcultures; 4 were resuspended in a treatment medium containing ES medium with no NaH_2PO_4 (P_i), while the other 4 were resuspended in ES medium with the standard P_i concentration ($56 \mu\text{M}$). APA assays were conducted on these cultures immediately prior to resuspension in treatment medium (Day 0) and periodically for 25 d after the transfer.

To evaluate the relationship between APA of zooxanthellae in symbiosis and external P_i levels, sea anemones from fed cultures were incubated without feeding in GSFSW with P_i concentrations of 0.0 and $0.5 \mu\text{M}$. Anemones were maintained individually in 20 ml glass scintillation vials with 5 ml of experimental medium at 25°C with a $12:12 \text{ h}$ light:dark regime, and the medium was changed every 2 to 3 d . The incubation lasted 35 d with assays conducted at Days 0 , 14 , 21 , 28 and 35 . Anemones were randomly assigned a treatment and incubation period when they were initially isolated in scintillation vials. Six anemones were processed from each treatment on each sampling day. By Day 35 , these unfed anemones were greatly reduced in size so that 2 anemones were combined for each sample to obtain enough algae for APA assays. Total zooxanthellae populations and anemone protein content were determined for Days 0 , 14 , 28 and 35 .

Field-collected corals. *Montastraea faveolata* samples were collected from the Florida Keys and the

Bahamas to compare APA in FIZ from corals in areas of differing ambient P_i levels. The Bahamian site near Lee Stocking Island was a relatively pristine reef habitat with minimal anthropogenic nutrient influences. Bahamian corals were collected on July 29, 1997 and were processed at Lee Stocking Island. The Florida Keys site was in close proximity to a developed island and subject to tidal exchange with Florida Bay water. Corals from the Florida Keys were collected on October 20, 1997, and transported without water in plastic bags to Harbor Branch Oceanographic Institution for analysis. In the Bahamas, 1 piece was collected from each of 5 colonies. In the Florida Keys, 1 piece was collected from each of 4 colonies. Assays were conducted in triplicate for each piece of coral. Unfiltered seawater samples were taken at the time of coral collection in the Bahamas, stored at -40°C for 2 d and assayed for phosphate using the molybdate assay of Strickland & Parsons (1977).

Statistical analysis. APA data exhibited unequal variances as determined by an F_{\max} test and were log-transformed prior to analysis. Biomass data did not exhibit unequal variance and were not transformed. P-limitation experiments with CZ were analyzed using ANOVA for repeated measures. APA and biomass measurements from anemones incubated in P_i treatments were analyzed using a 2-way ANOVA for factors of time and treatment with a Tukey post-hoc pairwise comparison. The Day 0 samples were not included in this analysis as they had not yet been assigned to treatments. APA data from 2 anemones were discarded: one (Day 28) due to a cell counting error, and the other (Day 0) because it was identified as an outlier using a Dixon test (Sokal & Rohlf 1995). Bio-

mass data for each treatment were secondarily analyzed by single-factor ANOVA for changes over time with respect to the Day 0 samples. Field-collected corals were compared using a 2-sample t -test, assuming unequal variance.

RESULTS

pH optima of phosphatase activity in *Symbiodinium bermudense*

Whole cell suspensions of CZ and FIZ were tested using pH values from 4 to 11 in increments of single pH units. The results (Fig. 1) showed that CZ phosphatase activity had a pH optimum of 9, while FIZ had a pH optimum of 5; however, phosphatase activity was higher in the FIZ at every pH value tested. All subsequent experiments were conducted at a pH of 8.1 (corresponding to the pH of GSFSW) and should be considered as assays for APA.

Effect of exogenous P_i on APA of *Symbiodinium bermudense*

Cultured zooxanthellae provided with an ample supply of NaH_2PO_4 showed little change in APA during a 25 d exposure; APA increased by Day 3 and then remained constant for the remainder of the experiment (Fig. 2). In contrast, APA of cultures without added NaH_2PO_4 continued to increase up to Day 13, when APA levels were 3.4 times greater than those of the cultures replete with P_i . APA levels of the P_i -free cultures declined after Day 13, but always exceeded those of cultures with P_i . ANOVA revealed highly significant effects of time and treatment ($p < 0.001$ for both). These results indicate that APA was expressed in CZ in response to P-limitation.

FIZ from unfed anemones in GSFSW with 0.0 and 0.5 μM NaH_2PO_4 exhibited a slight decrease in APA during the first 3 wk (Fig. 3). APA in both groups increased on Day 28, although the increase was 3 times greater in FIZ from anemones maintained without additional P_i . The high variance of APA in the 0.0 μM treatment on Day 28 resulted from large increases in APA in half the anemones, while APA levels of the others remained near baseline values. APA of FIZ from these 'high APA' anemones was 15 times greater than that of the others. APA in the 0.5 μM treatment subsequently returned to baseline levels on Day 35. APA in the 0.0 μM treatment also declined substantially on Day 35, but remained higher than that of the group with added P_i . Two-way ANOVA revealed significant effects both of time ($p < 0.001$) and treatment ($p < 0.05$).

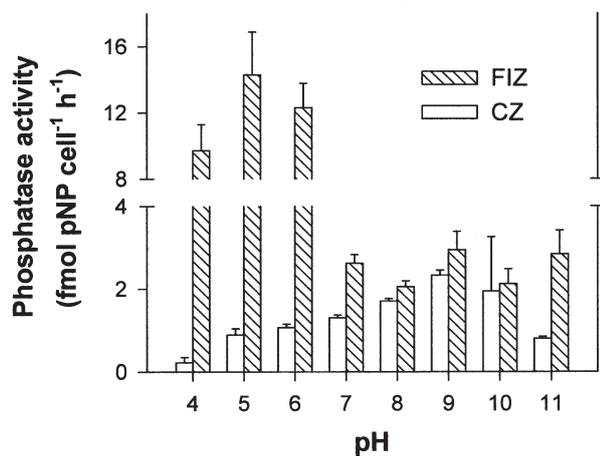


Fig. 1. *Symbiodinium bermudense*. The pH-dependent phosphatase activity of cultured zooxanthellae (CZ) and freshly isolated zooxanthellae (FIZ) from well-fed *Aiptasia pallida*. Enzyme activity of FIZ is reported with a break in the y-axis to facilitate comparison of peak activity. Bars denote ± 1 SE ($n = 3$)

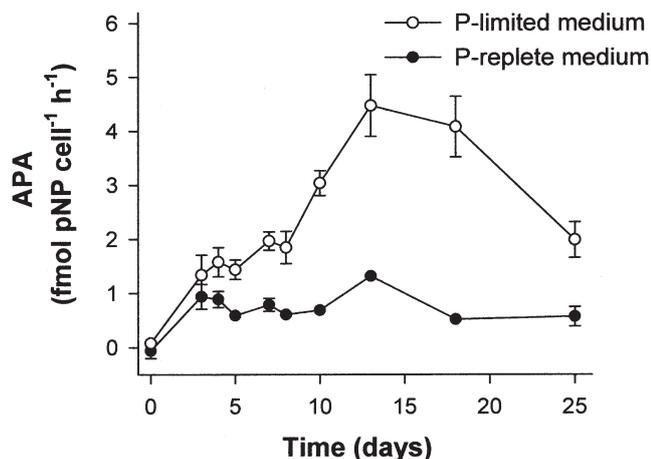


Fig. 2. *Symbiodinium bermudense*. Alkaline phosphatase activity (APA) levels of cultured zooxanthellae (CZ) in response to P-limitation. CZ were maintained in ES media with initial PO_4 concentrations of 0 μM (P-limited) and 56 μM (P-replete). Bars denote ± 1 SE ($n = 4$)

Biomass measurements

The total number of zooxanthellae in anemones maintained without food or added P_i decreased steadily during the 5 wk of the experiment (Fig. 4A). In contrast, the total number of zooxanthellae in the anemones kept in 0.5 μM P_i increased slightly during the first 14 d, then slowly decreased to just below the initial population level. After 35 d, these anemones contained more than 10 times as many symbionts as those without added P_i . Two-way ANOVA revealed highly significant effects of time ($p < 0.01$) and treatment ($p < 0.001$). Single-factor ANOVA showed a significant reduction of zooxanthellae populations over time in the 0.0 μM treatment ($p < 0.01$), while there was no significant change in the 0.5 μM treatment ($p > 0.05$) over the 5 wk period.

Host protein declined in both treatments during the experiment (Fig. 4B), but 2-way ANOVA indicated no overall effect of either time or treatment ($p > 0.05$ for both). During the first 2 wk, the density of zooxanthellae (algal cells per mg host protein) increased 1.6-fold in the 0.5 μM anemones and 1.4-fold in the 0.0 μM anemones (Fig. 4C). After the first 2 wk, zooxanthellae density in the 0.0 μM treatment declined steadily. Two-way ANOVA showed a significant effect of treatment ($p < 0.01$) and time ($p < 0.05$). The results of a single-factor ANOVA on each treatment over time show that the ratio of zooxanthellae to anemone protein decreased significantly in the 0.0 μM treatment ($p < 0.05$), but did not change significantly in the 0.5 μM treatment ($p > 0.05$).

Field-collected corals

Zooxanthellae from the Lee Stocking Island corals had APA levels 3.3 times higher than zooxanthellae from the Florida corals (Fig. 5). The difference in APA between the sites was highly significant ($p < 0.01$, $t = 5.223$, $df = 5$). Analysis of water samples from the Lee Stocking Island site at the time of collection indicated that the average soluble reactive phosphorus concentration was 0.018 μM . This is below the 0.030 μM lower limit of detection for the molybdate assay (Strickland & Parsons 1972). One sample was discarded due to probable phosphorus contamination. Water samples were not taken at the time of collection at the Florida Keys site, but we recorded values ranging between 0.08 and 0.23 μM from this site during the previous year (Cook et al. 2002). Szmant & Forrester (1996) reported a mean soluble reactive phosphorus concentration of 0.17 μM near the collection site. Thus, it is likely that corals from the Florida Keys were exposed to higher concentrations of P_i .

DISCUSSION

pH optima of phosphatases in *Symbiodinium bermudense*

Our results indicate the presence of both acid (AcPA) and alkaline (APA) phosphatases in cell suspensions of *Symbiodinium bermudense*. The phosphatase activity of FIZ from *Aiptasia pallida* was predominantly acidic, while the activity in CZ was predominantly alkaline,

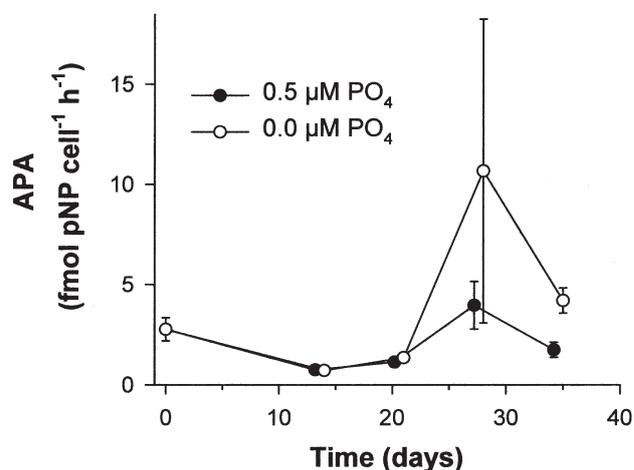


Fig. 3. *Symbiodinium bermudense*. Alkaline phosphatase activity (APA) levels of freshly isolated zooxanthellae from *Aiptasia pallida* incubated in seawater containing 0.0 or 0.5 μM PO_4 . The t_0 point is data from 5 anemones which were maintained in unenriched seawater and fed daily. Bars denote ± 1 SE ($n = 6$)

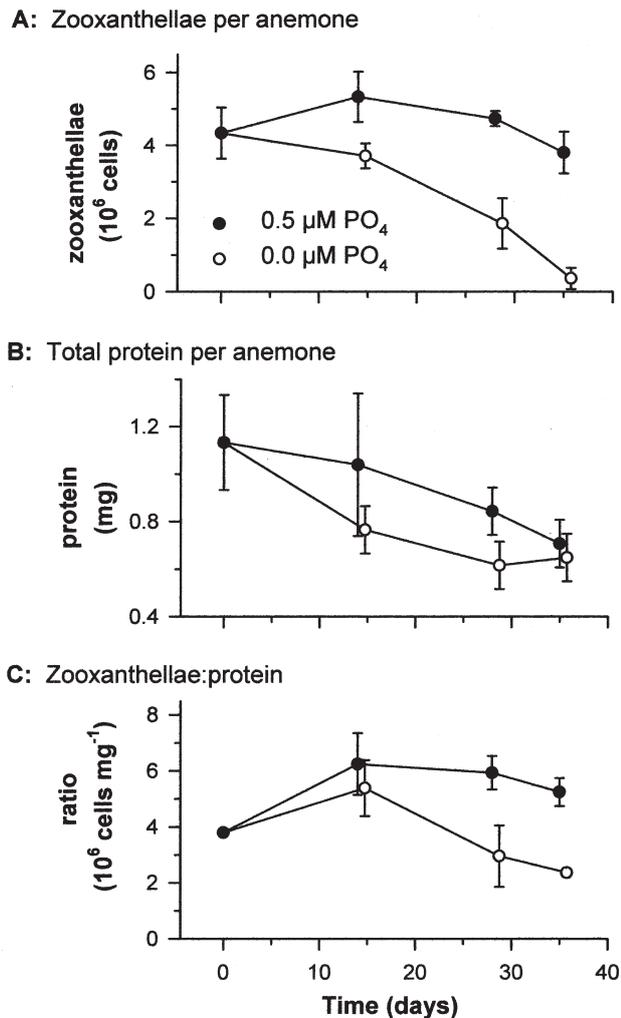


Fig. 4. *Aiptasia pallida*. Biomass parameters during a 35 d exposure to PO_4 -enriched seawater without feeding. PO_4 concentrations were 0.0 and 0.5 μM . The t_0 data were collected from 6 anemones maintained in unenriched seawater with daily feeding, and are representative of the initial populations and protein content. (A) Total zooxanthellae population per anemone is expressed in 10^6 cells. (B) Total protein per anemone reported in μg . (C) The ratio of zooxanthellae to host protein expressed as cells $\mu\text{g protein}^{-1}$. Symbols as in Fig. 3. Data points have been slightly shifted in the x-axis to prevent overlap. Bars denote ± 1 SE ($n = 6$)

with little activity at acidic pH (Fig. 1). Jackson et al. (1989) reported that phosphatase activity in cell-free extracts of FIZ from the coral *Acropora formosa* at pH 5 was 20 times greater than at pH 8. However, it is not clear from their study if this activity resulted from surface or internal enzymes. Since we used intact cells for our study, we believe that the bulk of our activity was from surface enzymes. The presence of both AcPA and APA has been reported in cell suspensions of marine phytoplankton (Kuenzler & Perras 1965, Aaronson &

Patri 1976), but it is intriguing that *S. bermudense* appears to express predominately one or the other. We suggest that this is a function of the pH environment in which the algae are maintained, depending on whether the algae are grown in the host or in culture. The pH inside the perialgal vacuole is not known, but histochemical evidence suggests that the environment is mildly acidic (Jackson et al. 1989, Rands et al. 1993). Jackson et al. (1989) made a similar suggestion based on their finding of AcPA in FIZ. By contrast, the CZ in our study were maintained in a mildly alkaline seawater medium with a pH of between 7.8 and 8.0. Thus, it appears that *S. bermudense* can express either AcPA or APA in response to ambient pH levels.

The pH of coral tissues exhibit a strong diel pattern resulting from the photosynthetic activity of their symbiotic zooxanthellae (Fitt et al. 1995, Kühl et al. 1995). This raises the possibility that expression of APA and AcPA may alternate with diel changes in tissue pH. Although we did not test this hypothesis explicitly, data were collected from anemones held in light or dark depending on the time of day the assays were run. In the P-incubated anemone experiment (Fig. 3), Day 14 anemones were processed during daylight hours, while Day 21 anemones were processed at night. A Tukey post-hoc pairwise comparison showed no significant difference in APA in either treatment between these 2 days ($n = 6$, $p = 0.71$ for the 0.0 μM treatment and $p = 0.97$ for the 0.5 μM treatment). This suggests that the APA levels probably fluctuate on time scales longer than the daily pH cycles.

Effect of P_i availability on APA of zooxanthellae in culture and in symbiosis

The results of P-limitation experiments with CZ demonstrated that APA is elevated in P-limited cultures as in other microalgae (Kuenzler & Perras 1965). Jackson et al. (1989) studied the effect of P_i on AcPA of *Symbiodinium kawagutii* cultured from the coral *Montipora verrucosa*. They found a similar temporal pattern of expression in P-limited cells as we did with cultured *Symbiodinium bermudense*: a lag period followed by an increase in activity and then a return to lower levels. The elevation of APA in CZ in response to low levels of exogenous P_i provides the basis for using APA as an indicator of phosphorus sufficiency in zooxanthellae.

Assays of APA in FIZ from *Aiptasia pallida* showed that activity increased significantly in response to starvation of the host and that this effect was heightened by the absence of P_i in the ambient water (Fig. 3). It appears that in the absence of host feeding, zooxanthellae in both treatments became phosphorus-

deficient and expressed APA. The zooxanthellae from the 0.0 μM treatment experienced the combined effect of host starvation and minimal ambient P_i . Accordingly, they were more P-deficient than those in the 0.5 μM treatment and responded with higher levels of APA. Lower APA levels in the 0.5 μM treatment indicate that the zooxanthellae were able to utilize the exogenous phosphorus, resulting in reduced P-deficiency. In a broader context, this establishes a relationship between APA of symbiotic zooxanthellae and P_i levels outside the host, which is fundamental to the use of APA as a biological indicator of environmental P_i . As with P_i fluxes in symbiotic *A. pallida* (Muller-Parker et al. 1990), symbiont APA most likely reflects P_i levels in host tissue, which depend upon both exogenous P_i and host feeding. The P_i levels in the host tissue may also affect the timing of peak APA. We note that CZ exhibited peak APA 13 d after the removal of P_i from the medium, while zooxanthellae in symbiosis did not reach peak APA until 28 d of starvation. Zooxanthellae also exhibit higher growth rates in culture than in symbiosis (Cook & D'Elia 1987, Cook et al. 1997, Smith & Muscatine 1999). This may result in faster depletion of P_i in CZ, and earlier onset of P-deficiency and elevation of APA.

The major difference between zooxanthellae from anemones in the 2 P_i treatment groups was observed on Days 28 and 35, when FIZ from anemones without added P_i had higher APA. The high variance observed in this group probably resulted from using a fixed 7 d interval to assess a brief and variable period of elevated APA. We speculate that those FIZ in the 0.0 μM treatment with low activity on Day 28 had already passed the period of peak activity or had not yet

increased APA, and we suggest this reflects initial differences in host or algal reserves between anemones. In both CZ and FIZ, APA was turned on in P-limited cells but declined substantially after reaching peak values. The reason for this decline in APA is not known. It may reflect the attainment of sufficient P_i levels through APA, repression of APA in the absence of available substrate, or a general decline in metabolism.

Biomass parameters from anemones exposed to elevated P_i

This is the first study to demonstrate that the reduction of zooxanthellae density typically associated with starvation of the host may be countered by elevated exogenous P_i . Anemones exposed to 0.5 μM P_i maintained higher symbiont density than those in the 0.0 μM P_i treatment throughout the experiment. Anemones in the 0.5 μM treatment experienced a 1.6-fold increase in zooxanthellae density that remained elevated for the remainder of the experiment, while density in anemones without P_i declined after Day 14 (Fig. 4C). These results indicate that *Symbiodinium bermudense* becomes P-limited in unfed *Aiptasia pallida* and that it is able to utilize exogenous sources of P_i . These results are consistent with increases in zooxanthellae density in response to elevated exogenous P_i observed in several coral species during the ENCORE experiments on the Great Barrier Reef (Koop et al. 2001). In contrast, 2 μM P_i had no effect on the symbiont density of the coral *Stylophora pistillata* (Muscatine et al. 1989), and densities and growth rates of the dinoflagellate symbionts of the hydroid *Myrionema amoiboense* declined at this concentration (Fitt & Cook 2001). The addition of 60 μM P_i had no effect on zooxanthellae densities in unfed *Aiptasia pulchella* (Smith & Muscatine 1999). The reasons for these differences are not clear, but may involve genetic differences between the symbionts, or that high initial P_i concentrations (3.5 to 5.0 μM) in the artificial seawater used in the latter study provided sufficient levels of P_i .

Zooxanthellae in symbiosis derive phosphorus from host feeding, host metabolites and ambient water (Johannes et al. 1970, D'Elia 1977, Muscatine & Porter 1977, Cates & McLaughlin 1979). Accordingly, starvation of the host removes a potential source of nutrients for the algae. *Aiptasia pallida* typically responds to starvation with a reduction in the symbiont population (Clayton & Lasker 1984, Cook et al. 1988), and zooxanthellae populations in our 0.0 μM treatment anemones declined steadily throughout the incubation. The presence of elevated P_i levels in the ambient water offset the effects of host starvation, and anemones in

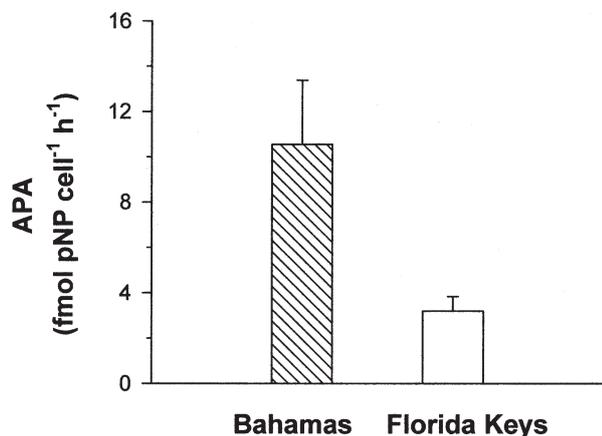


Fig. 5. *Symbiodinium* spp. Comparison of phosphatase activity in freshly isolated zooxanthellae from *Montastraea faveolata* collected from the Bahamas and Florida Keys. Bars denote ± 1 SE ($n = 5$ for Bahamian corals and $n = 4$ for Florida corals)

the 0.5 μM treatment exhibited no net loss of zooxanthellae during the 35 d incubation period. The maintenance of zooxanthellae populations in this treatment is remarkable given the loss of nearly 40% of the host's protein biomass over the course of the experiment.

Host protein content declined in both treatments as a result of starvation, and it was evident by Day 35 that the anemones in both groups were reduced in size. There were no differences between the treatments, but the loss of host protein appeared to be more gradual in the 0.5 μM treatment. This may have resulted from symbiont contribution to host metabolism. When reserves of carbohydrate and lipid are exhausted, unfed anemones resort to protein catabolism to support metabolic functions (Fitt & Pardy 1981). Under starvation conditions, photosynthates translocated from the zooxanthellae to the host provide an alternate source of energy and reduce catabolism of host proteins (Szmant et al. 1990). Anemones in the 0.5 μM treatment maintained larger zooxanthellae populations and exhibited a more gradual loss of protein. These results are consistent with the results of Fitt & Pardy (1981), who demonstrated that the presence of symbiotic zooxanthellae enhanced the survival of unfed anemones.

Field-collected corals

APA of FIZ from *Montastraea faveolata* was more than 3 times higher in Bahamas samples than in those from the Florida Keys, indicating that zooxanthellae in Bahamas corals were more P-limited than zooxanthellae in Florida Keys corals. This corresponded to what was likely a 5-fold difference in the ambient P_i level between sites. While our field data are preliminary, they indicate that APA levels do reflect environmental P_i levels and demonstrate the potential for using this APA assay as an indicator of P_i exposure of reef corals. The use of intact algae rather than a purified enzyme makes this a relatively simple and convenient assay. Additionally, the methodology circumvents some of the confounding factors commonly associated with phosphatase assays of natural phytoplankton populations. Populations of zooxanthellae in a single coral host may be maintained with minimal spatial and temporal variation, although the genetic composition of populations within a host may vary (Rowan et al. 1997). Thus, the relatively long-term association of zooxanthellae with a cnidarian host essentially integrates P_i exposure over time. Extrapolations from our laboratory experiments with unfed *Aiptasia pallida* suggest that the APA in field samples might integrate P_i exposure over a period of 1 to 2 mo. This provides an

alternative to APA levels that fluctuate on the scale of days to weeks in benthic algae and phytoplankton (Kuenzler & Perras 1965, Weich & Graneli 1989, Urnezis 1995). Potentially, this assay would provide a means of monitoring long-term changes in P_i in a coral reef environment. The integration of APA response over time would allow bimonthly or quarterly sampling to determine shifts in P_i exposure, resulting from seasonal changes or prolonged anthropogenic impacts. Clearly, additional research is needed to establish the efficacy of this assay in the field. Future work should address regional differences in APA expression, and examine local effects of environmental parameters such as turbidity, irradiance, temperature and depth with respect to APA expression. Most importantly, local baseline APA values need to be established and correlated with ambient PO_4 levels. Developing a more complete understanding of this relationship in the field will allow the assay of APA in zooxanthellae to be used effectively as a biological indicator of P_i exposure in the coral reef environment.

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