Phosphate release due to excess alkaline phosphatase activity in *Trichodesmium erythraeum*

TAMAHÀ YAMAGUCHI, KEN FURUYA*, MITSUIHIDE SATÔ & KAZUTAKA TAKAHASHI

Department of Aquatic Bioscience, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

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**Abstract:** We investigated the dependence of alkaline phosphatase activity (APA) on temperature and light intensity in cultured strains of the diazotroph *Trichodesmium erythraeum* isolated from the Kuroshio Current in the East China Sea. In contrast to the low levels of APA observed under phosphorus (P)-replete conditions, cultures grown under P-limited conditions exhibited strong APA, and the levels steadily increased with increases in temperature and irradiance from 22–30°C and 40–260 µmol m⁻² s⁻¹, respectively. Based on the chlorophyll *a* and APA levels in actively growing *T. erythraeum*, the mean hydrolyzing time required to double the cellular P quota was estimated to be 0.68 and 9.7 times shorter than the reported values under P-replete and P-limited conditions, respectively. This strongly suggests that APA under P-limited conditions is excessively active compared with the cellular P requirement. Furthermore, under P limitation intense bacterial aggregations were observed with fluorescence microscopy around the *T. erythraeum* trichomes in the same location as the visualized APA, suggesting enhancement of bacterial growth by surplus phosphate due to the excess APA, as well as possible release of dissolved organic matter from *T. erythraeum*. The present study suggests an important role for *T. erythraeum* as a phosphate supplier to other organisms in the oligotrophic ocean.

**Key words:** alkaline phosphatase, diazotroph, phosphate, *Trichodesmium erythraeum*

**Introduction**

Marine diazotrophs are characterized by their ability to fix nitrogen gases by using nitrogenase as a catalyst, and by their ability to flourish in the oligotrophic ocean, where dissolved inorganic nitrogen (DIN) is extensively depleted at the surface (Capone et al. 2005, Shiozaki et al. 2009). Generally, not only DIN, but also dissolved inorganic phosphorus (DIP) is depleted in most of the vast oligotrophic areas (Cavendar-Bares et al. 2001, Hashihama et al. 2009). In such areas, DIP is often the major limiting factor for marine diazotrophs because their nitrogen-fixing ability allows them to overcome DIN depletion.

When DIN, the most bioavailable form of P, is depleted in these habitats, dissolved organic phosphorus (DOP) is utilized as the P source instead of DIP. In such conditions, alkaline phosphatase (AP) is expressed in order to catalyze the cleaving of inorganic phosphate (Pᵢ) from phosphoric esters (Yentsch et al. 1972, Stihl et al. 2001). In this context, research on AP activity (APA) is essential to determine how the nitrogen fixers overcome phosphorus limitation by utilizing bioavailable DOP.

*Trichodesmium* is a major diazotrophic cyanobacterium that is distributed in many tropical and subtropical regions and often forms extensive seasonal blooms (Capone et al. 1998). *Trichodesmium* blooms are estimated to produce nearly 80 Tg of fixed nitrogen per year (Capone et al. 1997), thereby playing a substantial role in global nitrogen circulation (Capone & Carpenter 1982). Although it is well known that *Trichodesmium* shows APA under phosphate-depleted circumstances (Yentsch et al. 1972), and some reports discuss *Trichodesmium* APA in natural environments (Nausch 1996, Stihl et al. 2001, Dyhrman et al. 2002), little is known about the influence of environmental factors on the relationship between the bacterium’s growth and APA.

This study aims to reveal the effects of two major environmental factors—water temperature and irradiance—on APA and its relationship to the growth of *Trichodesmium erythraeum* isolated from the East China Sea. We performed microscopic analysis of APA (Dyhrman et al. 2002) by using Enzyme-Labeled Fluorescence 97 (ELF97), as well as conventional measurements of bulk APA (Li et al. 1998, Fu & Bell 2003). ELF97 forms an insoluble fluorescent particle after hydrolysis, which enables visualiza-
tion of APA. Since AP is not unique to *Trichodesmium*, the bulk measurement does not discriminate *Trichodesmium* APA from that of other microbes present in the culture medium. Thus, APA visualization was expected to provide more detailed information about P utilization in *Trichodesmium* and to enhance the data collected from bulk APA measurements.

**Materials and Methods**

**Culture and growth conditions**

The nonaxenic culture used herein was that of *T. erythraeum* strain ECS0305. The organism was isolated in 2003 from the East China Sea (Kitajima 2009), where high nitrogen fixation rates and *Trichodesmium* blooms are frequently observed (Shiozaki et al. 2010). Bacteria co-existed with the *Trichodesmium* even at low density in this culture as detected under fluorescence microscopy, and they were presumed to be superficial to the *Trichodesmium* trichomes. These bacteria could not be removed, even after repeated washing procedures, ever since the strain had been first established from a single filament. Because such bacterial associations have also been observed in field samples (Nausch 1996), we regarded this consortium as a natural form of *Trichodesmium*, and we treated it as an ecological unit in the present study.

We cultured the isolate in both P-replete and P-limited conditions in order to compare APA and specific growth rates. For the P-replete condition, TMV culture medium (Prufert-Bebout et al. 1993) was used. Modified TMV medium, with no phosphate added, was used for the P-limited condition.

*Trichodesmium* cultures were grown in 28 mL polycarbonate centrifuge tubes (Nalgene) on a 12L/12D light cycle. Samples were grown in triplicate at five temperatures (22, 25, 28, and 30°C) (26 and 28°C; Sanyo, L), in addition to the dye in the procedure explained above, was also followed. This protocol allowed for observation of the bacteria present around the *Trichodesmium* trichomes. SB fluorescence was observed under a microscope equipped with a blue excitation filter set.

**Results**

**The degree of P-limitation**

Since all culture media in this study were prepared using long-preserved seawater from the western North Pacific, the “P-limited” culture medium was not completely phosphorus-free. Phosphate in this P-limited medium was

**Measurement of APA**

For APA measurement, we used *p*-nitrophenylphosphate (pNPP) as a substrate (Fu & Bell 2003). Ten milliliters of the cultured samples were collected on a GF/F filter (Whatman), and the filter was immersed in a substrate cocktail consisting of 6 mL of sea water filtered using a 0.2 µm pore size filter, 0.3 mL of 10 mM pNPP, 0.81 mL of 50 mM Tris-glycine buffer (pH 8.5) (Tris: Amresco, code 0826; Glycine: Wako, 073-00732), and 0.081 mL of 1 mM MgCl$_2$. After 3 h of incubation at the original culture conditions, the cocktail was mixed gently and absorbance at 410 nm was measured with a spectrometer (Shimadzu; UV-2400PC). A significant standard curve for *p*-nitrophenol (purity >99.0%; Tokyo Chemical Industry Co., Ltd.) was provided by measuring the absorbance of five different concentrations of *p*-nitrophenol solution.

**Chlorophyll a analysis**

Five milliliters of the cultured samples were filtered on a GF/F filter, and pigments were extracted in 6 mL of *N*,*N*-dimethylformamide in the dark for 1 h at room temperature (Suzuki & Ishimaru 1990). The extracts were frozen at −70°C until fluorescence was measured with a fluorometer (Turner Designs; 10AU Fluorometer). In this study, all APA measurements have been presented after normalization to the chlorophyll *a* biomass. Photooinhibition or any other irradiance-related effects on chlorophyll *a* are not considered herein.

**Microscopic observation**

To visualize the cell-specific localization of APA, we applied the fluorescent substrate Enzyme-Labeled Fluorescence 97 (ELF97, Molecular Probes) (Dyhman et al. 2002). One hundred microliters of the cultured samples were mixed with 5 µL of ELF97 (diluted 1 : 20 with pure water) in a microtube. After 45 min of incubation in the dark at the experimental temperature, slides were prepared and observed under a fluorescence microscope (Nikon Labophot-2) equipped with a 4’,6-diamidino-2-phenylindole (DAPI) long-pass filter set.

A double staining protocol that involved the dye SYBR Green I (SB, 5 µL), in addition to the dye in the procedure explained above, was also followed. This protocol allowed for observation of the bacteria present around the *Trichodesmium* trichomes. SB fluorescence was observed under a microscope equipped with a blue excitation filter set.
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∼5 nM and it was also assumed to contain some amount of DOP, though the DOP was not measured. Although the *T. erythraeum* strain was able to grow for several days after the first inoculation (from P-replete to P-limited medium), it could not be maintained after the second inoculation (data not shown).

**Specific growth rates of the strain ECS0305**

With a fixed irradiance of 200 µmol m⁻² s⁻¹, the maximum specific growth rate of ECS0305 was at a water temperature of 26°C (Fig. 1a). When water temperature was fixed (28°C), specific growth rates appeared to become saturated at a higher irradiance (Fig. 1b). From these preliminary results, we judged that this strain achieves its optimal growth at 26°C and 260 µmol m⁻² s⁻¹. Note that this optimal growth condition was determined within the range of the settings used herein.

**Effects of temperature and irradiance on APA**

Significant APA was detected under all culture conditions (Figs. 2a & 2b), except for one—that is, 22°C and 260 µmol m⁻² s⁻¹ in P-replete medium—where the culture did not grow sufficiently for measurement. APA was significantly higher in the P-limited medium (*t* test, *p* < 0.05) than in the P-replete medium under all culture conditions. APA and specific growth rates were affected differently by light and temperature. When irradiance was fixed, the APA of P-limited cultures steadily increased as the temperature rose, while specific growth rates peaked at 28°C (Fig. 2a). Likewise, when the temperature was fixed, the APA of P-limited cultures tended to increase with irradiance, while specific growth rates seemed to peak near 180 µmol m⁻² s⁻¹ (Fig. 2b). APA in the P-replete cultures was relatively stable under varying light and temperature conditions (Figs. 2a & 2b). Measured APA in this study covered only the particle fraction of the samples, and the absorption of proteins, such as AP, by the GF/F filters was not considered.
Distribution of APA

Under P-replete conditions, ELF97 fluorescence was not noted on microscopic observation (Fig. 3a). In P-limited cultures, by contrast, green fluorescent particles were observed, and the fluorescence distribution pattern could be classified into two types: (1) fluorescent particles found on or adjacent to the trichomes, and (2) fluorescent particles moving in the medium in no apparent relation to the trichomes (Fig. 3b). The former not only appeared around the trichomes but were also arranged linearly alongside the trichomes in many cases. Moreover, fluorescence was frequently localized intensively at specific spots on the trichomes. Some of these localizations were observed on *Trichodesmium* cells with fewer intracellular structures than other cells (Figs. 3c & 3d). Intense localization was also observed at the boundary between these transparent cells and normal cells (Fig. 3e). Additionally, ELF97 fluorescence was observed at the breaking points of trichomes (Fig. 3f).

**Bacterial aggregation around trichomes**

When trichomes grown in P-limited media were stained solely with ELF97, some transparent particle-like substances were recognized under light microscopy (Figs. 4a & 4b). Under the double staining protocol, such particles could be stained with SB and showed localization patterns similar to the ELF97 fluorescence (Figs. 4c & 4d). Since SB binds with nucleic acids, we judged that these fluo-
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Resistant particles corresponded to bacterial colonies. Neither visible particles nor a recognizable aggregation of SB fluorescence was observed around trichomes grown in P-replete media or stained solely with SB (data not shown). However, bacteria that were tightly associated with the surface of *Trichodesmium* trichomes (Nausch 1996) were excluded from consideration because they were observed regardless of the addition of inorganic phosphate (P<sub>i</sub>) to the culture media.

**Discussion**

In previous studies of *T. erythraeum* nitrogen fixation, strains IMS101 and GBRTRLI101 were often used (Bell & Fu 2005, Breitbarth et al. 2008, Bergman et al. 2013). Studies of strain ECS0305 are quite limited, but its optimal temperature (26°C), estimated from the specific growth rates in this study (Fig. 1a), is similar to those of IMS101 (∼27°C; Breitbarth et al. 2007) and GBRTRLI101 (∼25°C; Fu & Bell 2003, Fu et al. 2005). The specific growth rate of ECS0305 as a function of irradiance showed a pattern similar to that of IMS101 (Breitbarth et al. 2008), with growth saturation around ∼300 μmol m<sup>−2</sup> s<sup>−1</sup> (Fig. 1b), while GBRTRLI101 has a specific growth saturation around ∼50 μmol m<sup>−2</sup> s<sup>−1</sup> (Bell & Fu 2005). From these comparisons, the physiological properties of strain ECS0305 seem to be more similar to those of IMS101 than to those of GBRTRLI101. Thus, the results of previous work on IMS101 can be compared with the present results in the following discussion in order to compensate for the scarcity of knowledge about ECS0305. However, it is notable that there are still some uncertainties regarding the combined effects of different environmental factors (Figs. 1 & 2).

The effect of residual phosphate in the preserved seawater was considered to be negligible, since its concentration in the P-limited media was at an extremely low level (∼5 nM). It has been reported that residual DOP in preserved seawater may affect APA, and the degree of this effect depends on the chemical species (Yamaguchi et al. 2004). However, taking into account the fact that the strain could not be maintained in the P-limited media for more than two transfers (see Results), the residual DOP in the P-limited media was presumed to be refractory and not substantially bioavailable to the *T. erythraeum* strain. Therefore, it is considered that the effect of the residual phosphorus on APA was negligible in the present study.

The different temperature dependencies of APA and specific growth rates (Fig. 2a) imply that, at high temperatures, P<sub>i</sub> may be hydrolyzed in greater amounts than are required for growth maintenance. A high AP optimal temperature of ∼37°C has previously been reported for *Trichodesmium* (Stihl et al. 2001, Mulholland et al. 2002). Additionally, a similar difference in the optimal temperatures for APA and growth has been reported for other cyanobacteria (Singh et al. 2006, Li et al. 2013). Therefore, this difference between growth and phosphoric ester hydrolysis at high temperatures may be a common phenomenon for cyanobacteria.

A similar difference between growth and APA was observed at high irradiance as well, although the underlying mechanism may not be the same as that at high tempera-

![Fig. 4.](a, b) A trichome stained with ELF97, observed (a) under light microscopy, and (b) with ultraviolet excitation. Visible particle-like substances (enclosed with white dashed line) were observed around a trichome that showed ELF97 fluorescence under P limitation. (c, d) A trichome double-stained with ELF97 and SYBR Green I, observed under (c) ultraviolet excitation, and (d) blue excitation. Note the similarly localized fluorescence patterns between (c) and (d) under P limitation.
ture, since it is still not clear how irradiance affects enzymatic activity. One possible explanation for the high APA detected at high irradiance may be the putative increase in the amount of phosphate required for activities such as nitrogen fixation. Under relatively high irradiance, biological activities such as nitrogen fixation are kept high while specific growth rate decreases, as reported for strain IMS101 (Breitbarth et al. 2008).

This hypothesis is also supported by microscopic observations. Cells with a high density of ELF97 fluorescence (Figs. 3c & 3d) displayed diazocyte-like characteristics; that is, those cells appeared to be more transparent than vegetative cells (Fig. 2 in Sandh et al. 2012). Additionally, transmission electron microscope (TEM) analysis of trichomes revealed that diazocytes are distinct in having a reduced size and also fewer gas vacuoles, which make them look more transparent than vegetative cells (Fig. 4 in Sandh et al. 2012, Fig. 2e in Bergman et al. 2013), as observed in this study. In this context, the distribution of APA on trichomes might have been related to the diazocytes. Moreover, ELF97 fluorescence was also localized at the boundary of these diazocyte-like cells (Fig. 3e), and therefore cellular differentiation of diazocytes caused by a putative increase in nitrogen fixation might be linked to intense localized APA.

To examine the authenticity of Pi surplus due to high APA, we compared Pi supply and demand using estimates based on certain assumptions. The P-specific doubling time ($T_d$) is defined as the period necessary to double the cell P quota. $T_d$ values previously reported for strains IMS101 and GBRTRL101 varied substantially between the strains and decreased inversely with the ambient Pi concentration, showing saturation at the level of 5.0 µM (Fu et al. 2005). At this saturation level, $T_d$ values were similar between strains, and therefore we treated this value (~0.32 d) as the shortest potential $T_d$ ($T_{sd}$) of ECS0305. We then calculated the hydrolyzing time ($T_h$) required to compensate for the P demand required to double cell P quota using the following formula:

$$T_h = \frac{cell\ P\ quota\ (\mu mol\ P\ L^{-1})}{APA\ (\mu mol\ pNP\ L^{-1}\ h^{-1})} = \frac{2.6\ (nmol\ P\ colony^{-1}) \cdot Chl\ a\ (\mu g\ L^{-1})}{27(ng\ Chl\ a\ colony^{-1}) \cdot APA\ (\mu mol\ pNP\ L^{-1}\ h^{-1})}$$

The cell P quota was estimated from the chlorophyll a values determined herein, assuming a cell P quota of 2.6 nmol P per colony (Sañudo-Wilhelmy et al. 2004) and an average of 27 ng of chlorophyll a per colony (Orchard et al. 2010a).

$T_h$ values were shorter under P-limited conditions than under P-replete conditions, and were shorter than $T_{sd}$ (~0.32 d = ~7.68 h) in many cases (Table 1). The average $T_{sd}/T_h$ ratios were 0.68 and 9.7 under P-replete and P-limited conditions, respectively (Table 1). This finding suggests that, under P-limited circumstances, P demand would

### Table 1. Hydrolyzing time necessary to meet the P-specific requirement for doubling the estimated cell P quota ($T_h$), and its ratio to the published P-specific doubling time ($T_{sd}$) of 0.32 d.

| Pi addition | Temperature (°C, irradiance = 260 µmol m⁻² s⁻¹) | Irradiance (µmol m⁻² s⁻¹, temperature = 26°C) | Chl a (µg L⁻¹) | APA (µmol pNP L⁻¹ h⁻¹) | $T_h$ (h) | $T_{sd}/T_h$ |
|-------------|-----------------------------------------------|-----------------------------------------------|-----------------|--------------------------|---------|---------------|
|             | Temperature (°C, irradiance = 260 µmol m⁻² s⁻¹) |                   |                 |                          |         |               |
|             | 22 +                                        | -                              | nd              | nd                       | nd      | nd            |
|             | -                                          | 25 +                          | 3.09            | 0.17                     | 1.9     | 4.0           |
|             |                                             | -                              | 54.0            | 0.39                     | 13      | 0.58          |
|             |                                             | -                              | 24.0            | 3.11                     | 0.72    | 11            |
|             |                                             | 28 +                          | 19.7            | 0.24                     | 7.8     | 0.99          |
|             |                                             | -                              | 29.7            | 4.78                     | 0.60    | 13            |
|             |                                             | 30 +                          | 48.6            | 0.53                     | 8.8     | 0.87          |
|             |                                             | -                              | 18.9            | 4.21                     | 0.40    | 19            |
|             | Irradiance (µmol m⁻² s⁻¹, temperature = 26°C)|                   |                 |                          |         |               |
|             | 40 +                                        | -                              | 18.2            | 0.089                    | 20      | 0.38          |
|             |                                             | -                              | 11.4            | 0.12                     | 9.0     | 0.85          |
|             |                                             | 100 +                         | 45.4            | 0.26                     | 17      | 0.45          |
|             |                                             | -                              | 37.1            | 3.28                     | 1.1     | 7.1           |
|             |                                             | 180 +                         | 35.5            | 0.23                     | 16      | 0.48          |
|             |                                             | -                              | 33.9            | 3.64                     | 0.90    | 8.6           |
|             |                                             | 260 +                         | 25.2            | 0.33                     | 7.4     | 1.0           |
|             |                                             | -                              | 34.4            | 6.34                     | 0.53    | 15            |

| † Calculated from a cell quota of 2.6 nmol P per colony (Sañudo-Wilhelmy et al. 2004) and a mean chlorophyll a content of 27 ng per colony (Orchard et al. 2010a). | * nd = no data |
mostly be fulfilled within 1 h by intensely active APA. Furthermore, since measured APA in this study covered only the particle fraction of the samples, it might still be an underestimate of the total APA expressed by the *Trichodesmium*-bacteria consortium.

Although the calculation assumes constant APA and sufficient substrate supply during $T_{\text{ap}}$, this crude estimate revealed that $P_i$ surplus due to excess APA may have occurred not only at high temperature or irradiance, but also under other culture conditions. A possible explanation for this presumed high APA might be the long persistence of AP itself; AP has a half-life of 3–6 weeks (Li et al. 1998). Thus, once AP is expressed, its activity would be sustained regardless of whether or not $P$ limitation is resolved. In other words, AP may be regarded as a biologically low-cost approach to attenuate $P$ limitation, as it retains relatively long-term activity.

While the detailed mechanism of excess hydrolysis by *Trichodesmium*-derived AP remains to be verified, another issue is the question of which organisms actually utilize the surplus $P_i$. One possible explanation is utilization by environmental bacteria, which might be inferred from the aggregation of bacteria observed in samples that were double-stained with ELF97 and SB (Figs. 4c & 4d). Because epibionts and neighboring bacteria are generally likely to benefit from nitrogen fixed by *Trichodesmium*, it was expected that a similar pattern of aggregation would be observed under $P$-replete conditions as well. However, such aggregation was not observed. Additionally, taking into account the absence of bacterial aggregation in samples stained only with SB, the existence of ELF97 as a substrate for AP might have been necessary for the aggregation observed. This inference was probable since the ECS0305 strain was nonaxenic and therefore our results evaluated the entire APA of the combined *Trichodesmium*-bacteria consortium.

In large oligotrophic oceans such as the Sargasso Sea and the western North Pacific, where *Trichodesmium* often blooms, bioavailable DOP is depleted at nanomolar levels (Orchard et al. 2010a, Suzumura et al. 2012). In such environments, by apparently excessive APA, *Trichodesmium* can efficiently utilize not only the ambient labile DOP at low concentrations, but also the sporadically supplied bioavailable DOP, as previously proposed (Orchard et al. 2010b). This potential adaptability to occasional influxes of bioavailable DOP may be profitable not only for *Trichodesmium* itself but also for ambient microbial assemblages, as supported by our observations of bacterial aggregation. Our observations together with the above inference suggest that *Trichodesmium* supplies oligotrophic areas with $P_i$ through excess APA, and with combined nitrogen through its nitrogen fixation ability, while the present study does not eliminate the possibility of release of organic matter from *T. erythraeum*.

To test this hypothesis, further analyses such as measurements of $P_i$ uptake and nitrogen fixation rates will be required. Determining the time series of the cellular $P$ quota would also be indispensable. Additionally, we should also consider the APA in the dissolved fraction, not only in the particle fraction, since AP is an ectoenzyme. A better understanding of $P$ utilization by this major diazotroph would certainly improve our overall understanding of biogeochemical phosphorus and nitrogen cycling.

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