Availability of different phosphorus forms in agricultural soil to *Microcystis aeruginosa*

Yoko Okubo, Takanobu Inoue, Kuriko Yokota and Nguyen Minh Ngoc

**ABSTRACT**

We investigated the availability of different forms of particulate soil phosphorus (P) to *Microcystis aeruginosa* by sequential extraction and bioassay. We cultured *M. aeruginosa* in media containing, as the sole source of P, soils sequentially extracted with 1 M NH₄Cl, 0.11 M bicarbonate dithionite, 1 M NaOH, and 0.5 M HCl. Analyses of chlorophyll-a, particulate organic carbon, and particulate organic nitrogen showed that *M. aeruginosa* could utilize some of the P remaining in the soil after each extraction. Alkaline phosphatase (AP) assays of sequentially extracted soils showed distinct patterns that depended on the type of co-cultured soil. A direct relationship between cellular P concentrations and the level of alkaline phosphatase activity was observed in only some media, an indication that not all forms of P were equally suitable substrates for AP hydrolysis. These results imply that cyanobacterial-available P included not only HCl-extractable P, which is assumed to consist of carbonate or apatite bound-P and organic P, but also refractory P, which has been considered to be unavailable to algae. Both HCl-extracted P and refractory P enhance the production of chlorophyll a, but did not lead to the storage of P by *M. aeruginosa*.

**Key words** | alkaline phosphatase, cyanobacterial available phosphorus, density gradient centrifugation, *Microcystis aeruginosa*, particulate phosphorus, sequential phosphorus extraction

**INTRODUCTION**

Phosphorus (P) is a major factor responsible for eutrophication and the concomitant occurrence of phytoplankton blooms in freshwater environments (Zhou *et al.* 2008; Lv *et al.* 2014). Total P (TP) concentrations have been used to estimate the degree of eutrophication; however, only some forms of P are utilized by algae (Ellison & Brett 2006). Algal-available P includes nearly all dissolved P (DP) and some fractions of particulate P (PP) (Ellison & Brett 2006; Okubo *et al.* 2011). DP is not always the dominant fraction of TP, and P associated with soil particles eroded by rain contributes a major portion of the annual supply in streams (Ellison & Brett 2006) and rivers (Inoue & Ebise 1991; Pacini & Gächter 1999). Additionally, PP, which encompasses P associated with sediment and organic material eroded during runoff, contributes a variable but long-term source of potentially bioavailable P (Sharpley 1993). Once added to the soil, P in its common form as phosphate is stable and generally immobile. To estimate the potential for eutrophication in freshwater environments it is therefore necessary to quantify the portions of PP actually available for phytoplankton.

The bioavailability of PP has been investigated in many studies by comparison of the chemically determined P fractions in sediments (Williams *et al.* 1980; Dorich *et al.* 1985; Fabre *et al.* 1996) and soils (Okubo *et al.* 2011) with the results of bioassays. A sequential extraction scheme, modified by a number of investigators, is generally used to extract various forms of P from their hosts with reagents such as NaOH and HCl (Psenner *et al.* 1976; Pacini & Gächter 1999). Bioassays have been used to determine and quantify P availability to phytoplankton. To obtain a proper estimate of bioavailable P, it is necessary to compare the amounts of P liberated by this extraction scheme with the results of bioassays. There are many procedures for extracting various forms or fractions of P. The definition of bioavailable P has been debated in several publications; however, NaOH-extractable P has often been found to be equal to the bioavailable P fraction (Dorich *et al.* 1985; Boström *et al.* 1988).
Besides these issues, the question of how the PP is transported into a form available for phytoplankton has important implications for estimating bioavailable P. Cyanobacteria (including *Microcystis* spp.), algae, and bacteria have been shown to express alkaline phosphatase (AP), an enzyme that catalyzes the liberation of orthophosphate from organic P compounds at alkaline pH and that is expressed when cellular P reservoirs are depleted (Litchman & Nguyen 2008). Utilization of P from organic P compounds would allow cells to access a greater range of P forms than just the inorganic form (Dyhrman 2008). Many studies suggest that both zooplankton and bacteria support the growth of phytoplankton by excreting AP to enhance the bioavailability of P in aquatic environments (Chróst & Overbeck 2011), but few studies concern the contribution of AP produced by phytoplankton to their utilization of PP.

The primary objective of this study was to evaluate which forms of PP in soil collected from a Chinese cabbage field were available to the cyanobacterium *M. aeruginosa* by using a sequential extraction and bioassay. Bioavailable P was calculated directly from the results of bioassays in which sequentially extracted soils were used as a sole source of P. A secondary objective was to clarify the relationship between PP availability and AP synthesis by *M. aeruginosa* under the same conditions.

**METHODS**

**Study site and sample collection**

Soil samples were collected from a fertilized Chinese cabbage field located approximately 200 m southwest of the Umeda River, Aichi prefecture, in central Japan in January 2011. The depth of soil sampled (0–50 mm) represents an average effective depth of surface soil–runoff interaction for a range of soils, rainfall intensities, slopes, and soil management characteristics (Sharpley et al. 1995). The field is located in an area that consists predominantly of agricultural fields in which leafy vegetables are grown throughout the year. Phosphate fertilizer in the form of fermented poultry manure was added to the fields to replace the P depleted by cropping during autumn. For chemical extractions, soils were air dried at 40°C and sieved through a 0.149-mm mesh screen to remove small particles such as plant fibers. The samples were stored at 4°C until use.

**Characterization of extractable phosphorus in soils**

Soil characteristics were determined by a sequential four-step extraction scheme according to Pacini & Gächter (1999). The extractants were applied in the following sequence: 1 M NH4Cl (4 h), (repeat for 1 h), (H2O wash); 0.11 M BD (bicarbonate dithionite) (2 h), (repeat for 2 h), (1 M NH4Cl 20 min), (H2O wash); 1 M NaOH (17 h), (repeat for 2 h), (1 M NH4Cl 20 min), (H2O wash); 0.5 M HCl (17 h), (repeat for 2 h), (1 M NH4Cl 20 min), (H2O wash). The extractants were separated from the residues by centrifugation at 750 × g for 20 min. After each extraction the soils were washed with 1 M NH4Cl for 20 min to avoid the secondary adsorption of the liberated P onto the residual soils and then with H2O to prevent chemical interaction between successive extractants. The TP of the remaining soils was analyzed as refractory P. In each step, an extractant-to-solid ratio of 500:1 (v/w) was used to avoid the risk of saturating the solution with the extracted P phase. Soluble reactive P (SRP) was analyzed in each raw extract. All extracts and the solid residue left after the last extraction step were then digested in an autoclave with peroxodisulfate according to Menzel & Corwin (1965) to convert all forms of P to SRP. The SRP concentration following digestion was then analyzed by the molybdenum-blue method of Murphy & Riley (1962) and equated to TP. Non-reactive P (NRP) was equated to the difference between this TP and the SRP in the raw extract. The BD extracts were diluted before analysis to eliminate a chemical interference with the autoanalyzer reagents. Forty milligrams of one soil sample introduced into 50-mL centrifuge vials was sequentially extracted with 1 M NH4Cl, 0.11 M bicarbonate dithionite (BD), 1 M NaOH, and 0.5 M HCl on a shaker to produce the following fractions: NH4Cl-P (immediately available inorganic P and P loosely adsorbed on surfaces of particles such as Fe(OX), clays, and CaCO3), BD-SRP (redox-sensitive P bound to Fe and Mn), BD-NRP (organic P), NaOH-SRP (P bound to Al(OX) exchangeable against OH-, inorganic P compounds soluble in bases, and clay bound-P), NaOH-NRP (microorganism-P, humic-P, detrital organic-P, and polyphosphates), HCl-SRP (carbonate or apatite bound-P), and HCl-NRP (organic P), respectively. Sequential extraction was carried out in triplicate.

**Cyanobacterial bioassays**

*Microcystis aeruginosa* clone NIES 44 was obtained from the National Institute for Environmental Studies Collection and was grown in 200 mL of CB medium (Kasai et al. 2004), as shown in Table 1.
Microcystis is the most common cyanobacterium responsible for cyanobacterial blooms around the world. When the cultures of *M. aeruginosa* reached the late logarithmic growth phase, the cells were harvested by centrifugation at 670 × g for 20 min. The cells were washed twice with P-free CB medium to remove excess P in the medium. To evaluate the P fractions available to *M. aeruginosa* culture, we prepared four types of extracted soil samples as above. The samples were adjusted to 4 mL with 1 M Tris-hydrochloride buffer (pH 7.6) for 1 h. At the end of the incubation, 1 mL of 1 N NaOH was added to stop the reaction. After centrifugation, the absorbance of the samples was measured at 400 nm against a blank containing buffer, substrate, and p-nitrophenol (p-NP). The samples were adjusted to 4 mL with 1 M Tris-hydrochloride buffer (pH 8.6) and placed in a 37°C water bath with 1 mL of p-nitrophenylphosphate (1 mg mL⁻¹) in 0.2 M Tris buffer (pH 7.6) for 1 h. At the end of the incubation, 1 mL of 1 N NaOH was added to stop the reaction. After centrifugation, the absorbance of the samples was measured on a spectrophotometer. APA was expressed as the amount of p-NP produced per hour per Chl-α concentration. Triplicate samples were prepared for the AP assay. APA in soil cultures without the *M. aeruginosa* inoculum was used to check for indigenous bacterial or algal contamination under a microscope. The pH of the medium was monitored during the experience.

**AP assay**

Alkaline phosphatase activity (APA) was measured according to Sayler et al. (1979), except that the absorbance of the samples was measured at 400 nm against a blank containing buffer, substrate, and p-nitrophenol (p-NP). The samples were adjusted to 4 mL with 1 M Tris-hydrochloride buffer (pH 8.6) and placed in a 37°C water bath with 1 mL of p-nitrophenylphosphate (1 mg mL⁻¹) in 0.2 M Tris buffer (pH 7.6) for 1 h. At the end of the incubation, 1 mL of 1 N NaOH was added to stop the reaction. After centrifugation, the absorbance of the samples was measured on a spectrophotometer. APA was expressed as the amount of p-NP produced per hour per Chl-α concentration. Triplicate samples were prepared for the AP assay. APA in soil cultures without the *M. aeruginosa* inoculum was used to clarify the effect of indigenous microorganisms.

**Cellular phosphorus analysis**

To determine the P content of *M. aeruginosa* cells, we separated the cells from the soil in the medium by density gradient centrifugation. We used Percoll medium (GE Healthcare) with a density of 1.13 g mL⁻¹. Among various media used for density gradient centrifugation, Percoll eliminates any problems associated with cell lysis or dehydration during the separation procedure (Pertoff 2000). Approximately 3 mL of concentrated sample solutions were layered on 4 mL Percoll in 15-mL centrifuge tubes and centrifuged at 120 × g for 5 min. As the cyanobacteria

<table>
<thead>
<tr>
<th>CB medium</th>
<th>15 mg</th>
<th>10 mg</th>
<th>5 mg</th>
<th>4 mg</th>
<th>0.01 μg</th>
<th>1 μg</th>
<th>0.3 mL</th>
<th>50 mg</th>
<th>99.7 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-Na₂glycerophosphate·5H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIV metals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 mg</td>
<td>19.6 mg</td>
<td>3.6 mg</td>
<td>1.04 mg</td>
<td>0.4 mg</td>
<td>0.25 mg</td>
<td>100 mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1 | CB medium for *M. aeruginosa* (pH 9.0)**

Growth of *M. aeruginosa* was monitored by measuring concentrations of chlorophyll-a (Chl-a), particulate organic nitrogen (PON), and particulate organic carbon (POC) throughout the assay. Chl-a was determined spectrophotometrically after extraction with 10 mL methanol at 4°C in the dark, according to Otsuki et al. (1987). Samples for DP analyses were filtered through Whatman GF/F filters and analyzed as above. For PON and POC determination, 15 mL of well mixed sample solution was filtered through a Whatman GF/F filter, and the residue retained on the filter was dried at 105°C for 2.5 h and then analyzed with an NC analyzer. Triplicate tubes of each treatment were prepared and swirled once daily. All analyses were done using samples taken on days 0, 10, 17, 24, and 31. Soil medium without the *M. aeruginosa* inoculum was used to check for indigenous bacterial or algal contamination under a microscope. The pH of the medium was monitored during the experience.
are less dense than soil, they floated to the top after centrifugation, whereas the soil particles sediments on the bottom. The cells were harvested with a pipette and then filtered through a pre-weighed Whatman GF/F filter. This process was repeated. The residue retained on the filter was washed with P-free CB medium to remove excess Percoll and dried at 105°C for 2.5 h. The dry weight of *M. aeruginosa* was determined as the difference in filter weight before and after filtration. These samples were also used for cellular P analysis. Preliminary experiments showed that there was no notable loss of *M. aeruginosa* cells during the separation procedure.

**RESULTS AND DISCUSSION**

**Fractional composition**

The sequentially extracted P fractions averaged 37.8% (NH₄Cl-P), 28.5% (BD-P), 20.3% (NaOH-P), 11.6% (HCl-P), and 1.8% (refractory P) of the TP in the soils (Figure 1). Therefore, the NH₄Cl, BD, and NaOH treatments extracted approximately 86.6% of the P from the soils. The HCl- and refractory-P fractions accounted for 13.4% of the TP. NH₄Cl-SRP constituted the largest percentage of the TP (34.4%), followed by BD-SRP (24.8%), NaOH-SRP (14.3%), and HCl-SRP (6.2%). The P concentrations in the NRP fraction decreased in the order of NaOH-NRP (6.0%), HCl-NRP (5.4%), BD-NRP (3.7), and NH₄Cl-NRP (3.4%).

**Algal available phosphorus**

Chl-a concentrations peaked after 10–17 days at 0.84 (control), 1.01 (NH₄Cl), 0.94 (NH₄Cl-BD), 0.87 (NH₄Cl-NaOH), and 0.87 (NH₄Cl-HCl) mg L⁻¹, respectively (Figure 2). Both PON and POC concentrations in all media increased as *M. aeruginosa* grew (Figure 2). The C/N ratio is based on the means of increased PON and POC (n = 3) during incubation. The C/N ratios were 4.93 (control), 4.60 (NH₄Cl), 5.12 (NH₄Cl-BD), 6.24 (NH₄Cl-NaOH), and 6.00 (NH₄Cl-HCl), respectively. Microscopic observation revealed that indigenous cyanobacteria did not grow, whereas a small number of indigenous bacillus-like bacteria were found in the media.

**Figure 1** Sequential extraction of soils collected from a Chinese cabbage field (the standard errors for each fraction are <5%).

**Figure 2** Chl-a contents (upper) and PON (○) and POC (Δ) concentrations (lower) in cultures of *M. aeruginosa* incubated with (a) control soil, and with soil extracted with (b) NH₄Cl, (c) NH₄Cl and BD, (d) NH₄Cl-BD, and NaOH, and (e) NH₄Cl, BD, NaOH, and HCl. Each value is the mean of three replicates; bars represent standard deviations.
APA and cellular phosphorus contents

The APA of *M. aeruginosa* responded differently to each type of co-cultured soil (Figure 3). In control, NH$_4$Cl-extracted and NH$_4$Cl-BD-extracted soil media, APA increased when the cellular P concentrations fell to <2.5 mg P/g dry weight on day 17. In contrast, APA in NH$_4$Cl-NaOH- and NH$_4$Cl-HCl-extracted soil media remained low even after cellular P levels reached 1.0–1.4 mg P/g dry weight on day 17. Cyanobacteria grown with control and NH$_4$Cl-extracted soil had up to 12 times as much APA as cyanobacteria grown with NH$_4$Cl-NaOH- and NH$_4$Cl-HCl-extracted soils. Cellular P in all media decreased for the first 17 days. It then increased in the control, NH$_4$Cl-extracted, NH$_4$Cl-BD-extracted soil media but remained more or less constant after day 17 in the NH$_4$Cl-NaOH- and NH$_4$Cl-HCl-extracted soil media. The pH of each media was between 8.8 and 9.1 during the experiments.

CONCLUSIONS

In previous studies on the bioavailability of PP, sequential extraction and bioassays were performed independently (see above). A likely explanation is that it is difficult to differentiate between P held within phytoplankton cells and P associated with soil or sediment particles (Dorich et al. 1985).

Sequential P fractionation of the tested soils revealed that the largest single fraction was NH$_4$Cl-P, and that SRP exceeded NRP in all extracts. The NH$_4$Cl-SRP extract was the largest single SRP extract, whereas NaOH-NRP was the largest nonreactive extract. NH$_4$Cl-P usually constitutes only a small percentage of TP (Golterman 2001), but increases when excess fertilizer P is applied (Kuo et al. 2005), as in the field we sampled.

The cyanobacteria grew in all the media prepared with sequential extracts. Chl-a ranged from 0.84 to 1.01 mg L$^{-1}$, lower than the concentration in CB medium containing β-glycerophosphate as a sole P source (Okubo et al. 2011). This result suggests that *M. aeruginosa* was able to utilize some forms of P, such as carbonate or apatite bound-P, organic P, and refractory P, all of which have generally been considered to be unavailable. The increased C/N ratios in NH$_4$Cl-NaOH- and NH$_4$Cl-HCl-extracted soil media indicate that *M. aeruginosa* grew under P-limited conditions although each soil was added to the tubes, equivalent to 0.1 mg L$^{-1}$. Wang et al. (2010) reported that carbohydrates were higher in P limited cells than in control cells of *M. aeruginosa*.

APA depended on the soil extract. APA in NH$_4$Cl-NaOH- and NH$_4$Cl-HCl-extracted soil media was not enhanced by decreases in cellular P stores. This result implies that not all forms of organic P were equally available for AP hydrolysis. A phosphatase enzyme other than AP may supply some of the P to *M. aeruginosa*, because the only form of P that can be taken up by phytoplankton is orthophosphate, and thus utilization of organic P compounds necessitates enzymatic action to liberate orthophosphate (Williams et al. 1980). Percoll density gradient centrifugation was effective in the discrimination of cellular P and P associated with soil particles. Cellular P concentrations in *M. aeruginosa* grown in control, NH$_4$Cl-extracted, and NH$_4$Cl-BD-extracted soil media decreased as the cells grew. However, once APA was induced, cellular P levels increased again. In contrast, the cellular P in *M. aeruginosa* grown in NH$_4$Cl-NaOH- and NH$_4$Cl-HCl-extracted soil media did not increase noticeably when APA was low. These results suggest that the relationship between APA and cellular P contents varies with the form of P. More direct measurements of the hydrolysis of organic P by AP may be necessary to clarify the role of AP in mediating the P supply to microorganisms in aquatic environments.

![Figure 3](https://iwaponline.com/wst/article-pdf/69/6/1205/472350/1205.pdf)

**Figure 3** | APA (○) and cellular P content (Δ) in cultures of *M. aeruginosa* incubated with (a) control soil, and with soil extracted with (b) NH$_4$Cl, (c) NH$_4$Cl and BD, (d) NH$_4$Cl, BD, and NaOH, and (e) NH$_4$Cl, BD, NaOH, and HCl. APA is expressed as the amount of p-NP produced per hour per Chl-a concentration of *M. aeruginosa*. Each value is the mean of three replicates; bars represent standard deviations.
We conducted this study to estimate the forms of P in agricultural soil that are available to *M. aeruginosa*. Our results are not in full agreement with the suppositions that: (1) all the non-apatite PP (NH₄Cl-PP, BD-PP, NaOH-PP) may be considered potentially bioavailable (Pacini & Gächter 1999); (2) HCl-P, which is assumed to estimate calcium bound-P, is negatively correlated with the P that is extractable by microorganisms; and (3) particulate material, in which apatite P is the dominant fraction, supports microbial growth poorly (Boström et al. 1988). Our results imply that not only HCl-extractable P, which is assumed to estimate carbonate or apatite-bound P and refractory P, but also refractory P, which has been considered to be unavailable to algae, are potentially available to *M. aeruginosa*. The similar maximum Chl-a concentrations were obtained in all the media, while the cellular P values varied with the type of co-cultured soil. The cellular P in *M. aeruginosa* grown in NH₄Cl-NaOH- and NH₄Cl-HCl-extracted soil media did not increase as they grew. These results show that *M. aeruginosa* were not capable of luxury uptake and storing P when P needed for growth was supplied as carbonate or apatite-bound P and refractory P. Our conclusion, based on soil from a single location and a single cyanobacterial species, should not be applied to available P in aquatic environments in which the composition of P is highly variable. However, the bioassay involving sequentially extracted soils may offer an alternative way to estimate forms of PP available to microorganisms.

ACKNOWLEDGEMENT

This research was financially supported by the Environment Research and Technology Development Fund (B-0908) of the Ministry of the Environment, Japan.

REFERENCES


Saylor, G. S., Puszis, M. & Silver, M. 1979 Alkaline phosphatase assay for freshwater sediments: application to perturbed...
sediment systems. Applied and Environmental Microbiology 38, 922–927.


First received 2 October 2013; accepted in revised form 16 December 2013. Available online 28 December 2013