

# The bioavailability of colloidal and dissolved organic phosphorus to the alga *Pseudokirchneriella subcapitata* in relation to analytical phosphorus measurements

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**Abstract** The speciation of phosphorus (P) in freshwater affects its bioavailability. Analytical detection methods were compared for different colloidal and dissolved organic forms of phosphorus (P) in relation to the potential P bioavailability to *Pseudokirchneriella subcapitata*, determined with a 14 day growth response to P. Growth on these P-forms was referenced to supplies of inorganic P ( $P_i$ ) as operational definition of the relative potential bioavailability. The bioavailability of ten organic P molecules ranged 1–70% of  $P_i$  while these forms were generally not detected by colorimetric method (CM, malachite green) or ion chromatography (IC). The bioavailability of P associated with Fe- and Al oxides ranged 55–85% of  $P_i$  and these forms were completely detected by CM and partially by IC. The bioavailability of total dissolved P in the environmental samples ranged 7–85% (mean 43%) of  $P_i$ . The P detected by IC underestimates bioavailable P while CM and total dissolved P (inductively coupled plasma, ICP) overestimate P bioavailability by, on average 44% (CM) or 57% (ICP) in the environmental samples. We

conclude that CM is the best index among the three tested for predicting long-term availability of environmental dissolved P in which colloidal P contributes more importantly than organic P.

**Keywords** Phosphorus · Algae · Bioassay · Long-term bioavailability · Ion chromatography · Colorimetry

## Introduction

Phosphorus (P) is generally the most limiting macronutrient for primary production in freshwater systems (Correll, 1998, 1999; Hudson et al., 2000). Phosphorus might enter aquatic systems in a variety of species in the dissolved, colloidal, or particulate fractions. Dissolved and particulate P fractions are operationally distinguished by filtration (e.g., 0.45  $\mu\text{m}$  membrane), a procedure that separates most bacteria, algae, and mineral particles from the dissolved phase, but fails to completely exclude colloidal particles. Colloidal particles commonly refer to both inorganic (e.g., ferrihydrite colloids) and organic compounds in the size range of 1–1,000 nm and can consequently be present in either dissolved or particulate fractions (Buffle & Leppard, 1995; Zhang & Oldham, 2001; Turner et al. 2005).

Bioavailable P (BAP) is generally defined as the sum of immediately available P and the P that can be transformed into an available form by naturally

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occurring processes (Boström et al., 1988). The immediately available P (within minutes or hours) is often assessed in assays using radiotracers (Brown et al., 1978; Bentzen et al., 1992; Cotner & Wetzel, 1992). Phosphate turnover times and uptake constants are typically determined in this type of experiments. Inorganic P (i.e., orthophosphate) and very labile organic P forms constitute these fractions (Björkman & Karl, 1994). The long-term BAP is expected to be higher than the short-term because phosphatases and/or exposure to UV irradiation promote hydrolysis of dissolved organic P (DOP) (OrmazaGonzalez & Statham, 1996). Phosphatase production is species- or cell-specific (dependent on internal P concentration of phytoplankton) (Rengefors et al., 2003; Štrojsová & Vrba, 2006; Litchman & Nguyen, 2008) and is likely increasing with decreasing  $P_i$  concentrations (Huang & Hong, 1999; Hoppe, 2003; Labry et al., 2005). Potential bioavailability can be estimated from the algal yield after several weeks of culturing when the plateau level of the growth curve is attained. This method yields a larger fraction of bioavailable P compared to the immediately available P fraction (Lee et al., 1980; Boström et al., 1988). The growth response to DOP can be compared with that to equal concentrations of  $P_i$  as a definition of the potentially bioavailable fraction of DOP.

Phosphorus concentrations in natural water samples can be determined using different analytical methods including ion chromatography, IC (Masson et al., 2001; Hens & Merckx, 2002; Ruiz-Calero & Galceran, 2005), colorimetric methods (CM) before or after digestion (Murphy & Riley, 1962; Vanveldhoven & Mannaerts, 1987) or inductively coupled plasma, ICP (Masson et al., 2001; Van Moorlegheem et al., 2011) with each of these techniques measuring (a combination of) different P species and ICP the total dissolved P, i.e., the TDP (Van Moorlegheem et al., 2011). The long-term bioavailability of operationally determined P forms present in environmental samples has been examined for both axenic algal cultures (Ekholm & Krogerus, 1998; Ekholm & Krogerus, 2003; Huang et al., 2005) and natural communities of bacteria and phytoplankton (Bentzen et al., 1992; Björkman & Karl, 1994). The P available to algae proved to be highly variable, ranging from 0 to 100% of the total P concentration, i.e., dissolved + particulate P. None of the operationally determined P fractions cited above could be correlated consistently to the potentially bioavailable fraction (Ekholm & Krogerus, 2003).

The P detected by CM in the dissolved fraction, also termed dissolved reactive P, is the common method to measure bioavailable P but can underestimate the potentially available P (Bradford & Peters, 1987; Ekholm & Krogerus, 1998; Reynolds & Davies, 2001). The bioavailability of the P in the dissolved fraction which cannot be measured by CM without digestion (unreactive P) varied from 0 to 55% for river systems (Ekholm & Krogerus, 2003). The utilization of DOP by phytoplankton is controlled by its chemical composition (Björkman & Karl, 2003). Some of the organic P forms, e.g., nucleic acids, are readily available to algae while more resistant forms would be complexed with humic acids (Reynolds & Davies, 2001). It has been demonstrated that DOP compounds, in particular nucleotides, appear to be highly bioavailable to the microbial community in an oligotrophic marine environment (Björkman & Karl, 1994). The availability of colloidal P, e.g., P associated with colloidal oxyhydroxides of Fe and Al, is not well established. It has been recently demonstrated that P associated with Al-oxide nanoparticles was readily available to plant roots in nutrient solution, largely because P can be desorbed from the particles near the plant roots. The P uptake was higher in the presence compared to the absence of Al colloids because of diffusion-limited P uptake by the plant roots (Santner et al., 2012). The colloidal fraction might be the most important part of TDP in environmental freshwater samples (Van Moorlegheem et al., 2011).

Analytical P measurements neither correspond unambiguously to (a combination of) well-defined P species (Van Moorlegheem et al., 2011), nor to the bioavailable P (Ekholm & Krogerus, 2003). The CM method without prior digestion include  $P_i$  but generally does not detect DOP, in contrast with its bioavailability (Björkman & Karl, 1994; Huang et al., 2005). Almost all colloidal P is likely to be measured completely using CM while the availability of these species remains unknown (Van Moorlegheem et al., 2011). Analytical techniques used to detect the bioavailable forms have rarely been compared between model systems and environmental samples.

The objective of this study is to compare analytical techniques on the dissolved fraction in their capacity to predict P bioavailability to algae. More specifically, the long-term availability of colloidal and organic dissolved P were compared in relationship with P forms detected by analytical techniques in the initial solution,

i.e., colorimetry (malachite green) and ion-chromatography. Algae were first exposed to a suite of model inorganic colloidal P and organic P forms. Subsequently, different environmental freshwater samples were tested. The filtered freshwater samples were first concentrated by reverse osmosis (RO) and subsequently diluted to allow testing all waters at equal concentration of dissolved organic matter, potentially equal DOP. An experiment was included to test the reliability of the RO procedure. All bioavailability assays used the growth response in these P-limiting solutions and were referenced to different initial concentrations of  $P_i$ .

## Methods

### Solutions

Analytical reagent water (18.2 M $\Omega$  cm water provided by a milli-Q water purification system, Millipore) and acid washed glassware were used for the preparation of all solutions. The test media consisted of Guillard's WC medium (Guillard & Lorenzen, 1972) without the  $P_i$  and vitamin solution. The P source consisted of model organic or synthetic colloidal P compounds. To represent a wide variety of P species, taking into account the expected P compounds to be present in environmental water samples, following organic P compounds were incorporated into the test solutions with final concentration of 3 or 10  $\mu\text{mol l}^{-1}$ : D-glucose 6-phosphate disodium (gluP, >98%, Sigma), *o*-phospho-DL-serine (Pser, Sigma-Aldrich), creatine phosphate disodium salt (cP, >98%, Alfa Aesar), calcium glycerol phosphate hydrate (glyP, 97%, Alfa Aesar), adenosine-5'-triphosphoric acid (ATP, Applichem), tri-*N*-butyl phosphate (butP, >99% Fluka), phytic acid sodium salt hydrate (pa, Sigma), adenosine 3'-5'-cyclic monophosphate (cAMP, >99%, Acros Organics), phytic acid sodium salt hydrate (pa, Sigma) and 4-methylumbelliferyl phosphate (mbP, >99%, Fluka) and two inorganic P compound consisting of pentasodium tripolyphosphate (trip, >98%, Fluka) and  $P_i$  as a reference (Merck standard solution). The reference  $P_i$  treatment was supplied at five concentrations between 0 and 10  $\mu\text{mol l}^{-1}$  P as  $\text{KH}_2\text{PO}_4$ .

Two types of inorganic colloidal P forms were prepared. An aluminum oxide nanoparticle dispersion (<50 nm, 20 wt% dispersion, Sigma Aldrich) was

diluted in reagent grade water to 0.1 wt%. Forty millilitre was sonicated for 20 times 1 min at 80 Watt (XL2020 sonifier, Branson Ultrasonic corporation). In addition, ferrihydrite was prepared by titration of  $\text{Fe}(\text{NO}_3)_3$  with 1  $\text{mol l}^{-1}$  NaOH to pH 7. This solution was centrifuged (5 min at 3,500 $\times g$ , Jouan C412 Centrifuge), the supernatant was discarded, reagent grade water was added and the solution homogenized. This procedure was repeated four times to decrease the ionic strength to allow ferrihydrite to be dissolved after which the solution was centrifuged (30 min at 30,000 $\times g$  with Beckman J2-21 centrifuge) to constitute the dissolved fraction. Natural organic matter (Suwannee river NOM, International Humic Substance Society) was added to the supernatant and incubated in an end-over-end shaker for 24 h in a dark room at 21°C. The Al colloidal dispersions were centrifuged (30 min at 30,000 $\times g$  with Beckman J2-21 centrifuge) so that only the dissolved (<0.45  $\mu\text{m}$ ) fraction was retained in the supernatants. A 5  $\mu\text{mol l}^{-1}$   $P_i$  solution ( $\text{KH}_2\text{PO}_4$ , Merck standard solution) was prepared and the two solutions with colloids were added in varying concentration to obtain varying molar ratios of metal: $P_i$ , ranging from 0 to 180. The DOC concentration in the ferrihydrite stabilized by NOM solutions ranged from below detection limit to 9.0  $\text{mg C l}^{-1}$ . The effect of NOM on algae growth was followed up by measuring growth in the same 5  $\mu\text{mol l}^{-1}$   $P_i$  solution containing 16.3  $\text{mg carbon l}^{-1}$  without ferrihydrite additions.

Fourteen natural water samples were obtained from the top 20 cm layer of surface waters (lakes, ditches, and streams). These samples were collected in the autumn of 2010 and were filtered and subsequently concentrated by reverse osmosis (RO). The filtration used two steps (5 and 0.45  $\mu\text{m}$ ) to obtain the dissolved fraction of the sample. The filtered sample was passed over a cation exchange resin (Lewatit cation exchange resin (S 1468 F), Lanxess) to replace cations by Na. This step prevented the dissolved organic matter from precipitation during the concentration process. After pretreatment, the filtered and softened water was collected in a 25-l polyethene reservoir and concentrated in a next step. The concentration cycle existed of a high pressure pump followed by a RO membrane (Filmtec TW30-2521 RO membrane, Dow). The concentrate was directed back to the reservoir while the permeate was discarded. All components of the RO cycle were constructed from plastic components to the greatest extent possible to prevent metal contamination in the concentrate. Concentration factors of about 20

could be obtained for all waters when applying a pressure of 1 MPa over the RO membrane. The 14 samples were diluted with reagent grade water to a final DOC concentrations of  $10 \text{ mg l}^{-1}$ . Each solution was adjusted with concentrated NaCl,  $\text{NaHCO}_3$ ,  $\text{NaNO}_3$ , and  $\text{Na}_2\text{SO}_4$  so that the final concentration of these nutrients equated the highest sample concentration in our sample set. Subsequently,  $\text{KNO}_3$  was added to final concentration  $0.1 \text{ mmol l}^{-1}$ , Hepes buffer (pH 7.4) to  $5 \text{ mmol l}^{-1}$ ,  $\text{Ca}(\text{NO}_3)_2$  to  $1 \text{ mmol l}^{-1}$  and  $\text{MgSO}_4$  to  $0.1 \text{ mmol l}^{-1}$ . The micronutrient solution for the preparation of WC medium were added to all solutions with final concentrations:  $0.01 \text{ mmol l}^{-1}$  FeEDTA,  $0.04 \text{ }\mu\text{mol l}^{-1}$   $\text{CuSO}_4$ ,  $0.08 \text{ }\mu\text{mol l}^{-1}$   $\text{ZnSO}_4$ ,  $0.04 \text{ }\mu\text{mol l}^{-1}$   $\text{CoCl}_2$ ,  $0.8 \text{ }\mu\text{mol l}^{-1}$   $\text{MnCl}_2$ , and  $0.03 \text{ }\mu\text{mol l}^{-1}$   $\text{Na}_2\text{MoO}_4$  to insure that only P is the limiting nutrient in these solutions. The range of the major nutrient concentrations and some characteristics of these test solutions are summarized in Table 1. Reference samples with  $\text{P}_i$  concentrations ranging from 0 to  $5.3 \text{ }\mu\text{mol l}^{-1}$  (Table 1) were prepared in reagent grade water by adding salts and trace elements as described above.

#### Bioassay

*Pseudokirchneriella subcapitata* (Korschikov) Hindák 1990 was obtained as an axenic culture from the Culture Collection of Algae and Protozoa (CCAP 278/4, Windermere, UK) and was cultivated in sterile freshwater WC medium as described by Guillard & Lorenzen (1972) in autoclaved (20 min at  $121^\circ\text{C}$ ) 250-ml erlenmeyer flasks covered with an autoclaved cotton plug. This culture was shaken continuously on a horizontal shaker in an incubation room and algae were grown under a 12 h/12 h day/night cycle with illumination of  $90 \text{ }\mu\text{E m}^{-2} \text{ s}^{-1}$  (cool white fluorescent lamps) and at constant temperature of  $21 \pm 2^\circ\text{C}$ . After having reached the exponential phase, the algae were centrifuged (15 min at  $6,000\times g$ ) and washed three times with WC medium without P addition prior to inoculation. To detect and quantify a potential contamination of bacteria in our algal cultures, bacterial abundance was measured regularly by microscopy (Olympus BX51,  $400\times$  magnified) with or without DAPI (4',6-diamidino-2-phenylindole dihydrochloride) staining of the culture and subsequently compared to algal abundance. Bacteria were counted using a Helber counting chamber (Hawksley) by pipetting  $2.5 \text{ }\mu\text{l}$  of the culture on the counting chamber. The ratio of bacterial cell surface

**Table 1** Composition of 14 reconstituted environmental freshwater samples (i.e., surface waters) and the model (synthetic) solution used as reference for growth tests with orthophosphate

Component	Unit	Reconstituted freshwater sample		Synthetic solution
		Min	Max	
Na	$\text{mmol l}^{-1}$	27.0	39.4	27.0
Ca		0.75	0.78	0.75
Mg		0.13	0.14	0.13
K		0.09	0.26	0.09
$\text{NO}_3^-$		2.2	3.1	2.2
$\text{SO}_4^{2-}$		2.4	2.7	2.4
$\text{Cl}^-$		12.1	12.8	11.7
$\text{HCO}_3^-$		9.9	13.1	9.5
DOC	$\text{mg C l}^{-1}$	8.8	11.2	0.4
TDP <sup>a</sup>	$\mu\text{mol l}^{-1}$	0.6	5.5	<d.l.–5.3
Fe		0.3	8.1	<d.l.
Al		<d.l.	4.9	<d.l.
SUVA	$1 \text{ mg}^{-1} \text{ cm}^{-1}$	20	41	
pH	–	7.2	7.9	7.2–7.6

SUVA specific UV absorbance at 254 nm, and <d.l. below detection limit

<sup>a</sup> Total dissolved phosphorous detected by ICP, detection limit (d.l.) is  $1.1 \text{ }\mu\text{mol l}^{-1}$

over algal surface was calculated when bacteria were detected. The bacterial surface area was approximated by  $2 \text{ }\mu\text{m}^2 \text{ cell}^{-1}$  (0.8- $\mu\text{m}$  diameter sphere, as microscopically observed) while the algal surface area was approximated by  $21 \text{ }\mu\text{m}^2 \text{ cell}^{-1}$  (OECD, 2006). If any bacteria were detected, a fresh algal culture was started up from the axenically ordered algal strain.

The model organism *Pseudokirchneriella subcapitata* was selected for these bioassays because of the robustness of the system (regarding repetitive lab tests) and the ease of interpretation of the results. These algae were inoculated in the test medium with final cell density of  $10^4 \text{ cell ml}^{-1}$ . All tests were performed in triplicate. The test cultures were placed on a negatoscope and grown under continuous illumination of  $90 \text{ }\mu\text{E m}^{-2} \text{ s}^{-1}$  (cool white fluorescent lamps) and temperature of  $21 \pm 2^\circ\text{C}$ , cultures were not grown in sterile conditions after inoculation. The cultures were homogenized and the pH was measured once per day and adjusted with  $1 \text{ mol l}^{-1}$  HCl to  $\text{pH } 7.4 \pm 0.2$ . The algal cell density was measured once per day during the first 6 days and once every 2 days

after that (particle counter HIAC Royco 9705). As a reference, a test medium was prepared containing the micro and macronutrients of the WC medium with  $P_i$  concentrations ranging from 0 to  $10 \mu\text{mol l}^{-1}$ . These reference tests were conducted simultaneously with the assays of DOP and colloidal P. The reference test medium for environmental P used an adjusted medium (Table 1 and see above) to minimize compositional differences with the environmental samples.

#### Verification of RO isolation procedure

A bioassay on three waters was compared between (i) a filtered ( $0.45 \mu\text{m}$ ) water sample, (ii) the same sample first concentrated (RO) and then diluted to the same DOC concentration and (iii) a synthetic solution with the same inorganic nutrient concentration but without dissolved organic matter (DOM). The bioassays were performed as described above.

#### Analytical methods

Phosphorus was measured in the initial solution of the algal tests using three conventional analytical techniques: Ion chromatography, the malachite green colorimetric method and inductively coupled plasma optical emission spectroscopy (ICP-OES; Perkin-Elmer Optima 3300 DV, Norwalk, CT, USA). For IC, a Dionex ICS2000 system was used, equipped with an AG15  $2 \times 50$  mm guard column and an AS15  $2 \times 250$  mm analytical column and a conductivity detector (CD25) preceded by an anion self-regenerating suppressor (ASRS300). An injection volume of  $50 \mu\text{l}$  was used and the sample was eluted with a gradient concentration of KOH of 35 up to  $65 \text{ mmol l}^{-1}$  over 25 min. The molar absorptivity of our malachite green colorimetric method (Vanveldhoven & Mannaerts, 1987) is up to four times higher than for the more frequently used molybdenum blue colorimetric method, resulting in a higher sensitivity when measuring low P concentrations (Hens, 1999). The absorbance of the batch samples was measured at 630 nm using a Perkin Elmer Lambda 20 spectrophotometer with a 1-cm path length. An ICP with optical emission spectroscopy (Perkin Elmer Optima 3300 DV) was used for measurements of total dissolved P (TDP), and cations (Ca, Na, Mg, K, Fe, and Al). The samples were acidified to pH 1 using a  $5 \text{ mol l}^{-1} \text{HNO}_3$  solution prior to ICP analysis.

The DOP concentration was approximated as TDP minus P detected by CM concentration. Malachite green CM measures all the colloidal P but most organic P

compounds in environmental samples would not be measured using this method (Van Moorleghe et al., 2011). The colloidal P in environmental samples was estimated by subtracting IC from CM measured P, although P associated with Al colloids can be (partially) measured when using IC (Van Moorleghe et al., 2011).

Dissolved organic carbon (DOC), the analytical equivalent of dissolved organic matter (DOM), was measured by TOC analyzer (Analytik Jena multi N/C 2100) for all samples containing ferrihydrite stabilized by NOM and the 14 environmental samples. The samples were acidified to pH 1.9 using 50%  $\text{H}_3\text{PO}_4$  and purged with nitrogen gas prior to analysis. The organic carbon was oxidized by catalytic (Pt) combustion at  $800^\circ\text{C}$  and  $\text{CO}_2$  was measured by IR detection. Total inorganic carbon (TIC) was measured after injecting the sample in 10%  $\text{H}_3\text{PO}_4$  and  $\text{CO}_2$  concentration was consequently measured by IR detection. The  $\text{HCO}_3^-$  concentration can be calculated from TIC based on the theoretical fraction of TIC (92%, VisualMinteq 2.6) in our buffered test solutions at pH 7.4. The UV absorbance was measured at 254 nm (Abs, dimensionless) (Perkin Elmer Lambda 20, 1 cm path length, quartz cells) for the 14 standardized environmental samples and the specific UV absorbance (SUVA,  $\text{l g}^{-1} \text{cm}^{-1}$ ) was calculated as:

$$\text{SUVA} = \text{Abs } b^{-1} [\text{DOC}]^{-1} \quad (1)$$

with Abs the UV absorbance at 254 nm,  $b$  the path length of the spectrophotometer (1 cm) and [DOC] the DOC concentration ( $\text{mg C l}^{-1}$ ).

#### Statistics

The growth response, i.e., maximum cell number obtained during 14 days, to different initial  $P_i$  forms yielded a linear relationship (see below) and was used to define the fraction bioavailable P (BAP, %) of a given P form. This fraction is the equivalent  $P_i$  concentration yielding equal cell numbers relative to the TDP concentration of the sample with the given P form. The equivalent  $P_i$  concentration was derived from the regression curve established simultaneously with the growth test to the unknowns. The confidence limits on the mean BAP was calculated from the summed variance of predicted  $P_i$  for the replicates of the unknown and the variance related to the calibration curve (Fig. 1, below). The latter was derived with standard equations for estimating variance of inverse



predictions in linear regression, i.e., taking into account the variance in the calibration growth  $P_i$  curve. The specific growth rate during the exponential phase ( $\mu_{\text{exp}}$ ) was calculated by linear regression of  $\ln$  transformed cell densities during the exponential phase of each replicate. Duncan's multiple range test (SAS9.3) was used to detect differences among means of analytical measures of P and  $\mu_{\text{exp}}$  among different forms. Pearson correlations between bioavailability and P measured by ICP, CM and IC and correlations between bioavailability and SUVA, initial Fe and Al concentration in the environmental samples were calculated (SAS9.3).

## Results

### $P_i$ solutions

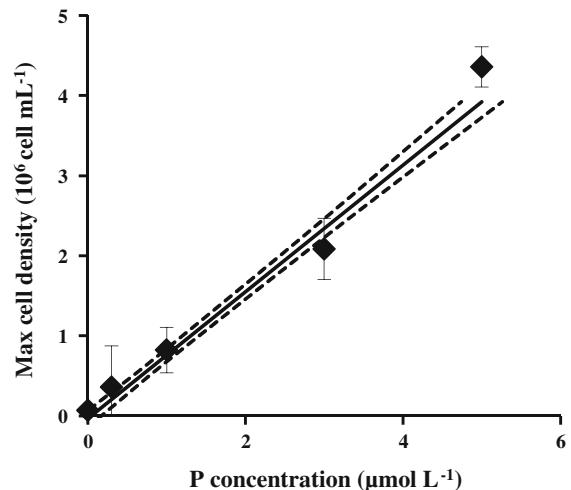
Higher initial  $P_i$  concentrations linearly increased the algal cell density between 0 and  $5 \mu\text{mol l}^{-1}$  P (Figs. 1, 2) according to:

$$N_{\text{max}} = 7.9 \times 10^5 P_i - 0.3 \times 10^5 \quad (2)$$

with  $N_{\text{max}}$  the algal cell density in the test solution ( $\text{cell ml}^{-1}$ ) obtained during 14 days and  $P_i$  the corresponding measured initial  $P_i$  concentration ( $\mu\text{mol l}^{-1}$ ). This equation can be reformed to calculate the relative P availability (BAP, %) according to:

$$\text{BAP} = \frac{(N_{\text{max}} + 0.3 \times 10^5)}{(7.9 \times 10^{-5} \times [\text{TDP}])} \times 100 \quad (3)$$

with [TDP] the total dissolved P concentration ( $\mu\text{mol l}^{-1}$ ) in the corresponding test solution. The coefficients of the linear regression were obtained by fitting the cell numbers versus  $P_i$  concentrations in all the tests carried out in this study. The BAP of the organic P compounds and environmental samples in following sections was calculated using the same equation, however, with slightly adjusted parameters for the slope and intercept, corresponding each time to the simultaneously measured growth response on the  $P_i$  solutions, the  $R^2$  values of the regressions varied from 0.85 to 0.99. Cell densities on  $10 \mu\text{mol l}^{-1}$   $P_i$  growth reached on average  $6.12 \times 10^6 \text{ cell ml}^{-1}$ , corresponding to 78% of the growth that would be expected based on Eq. 2.



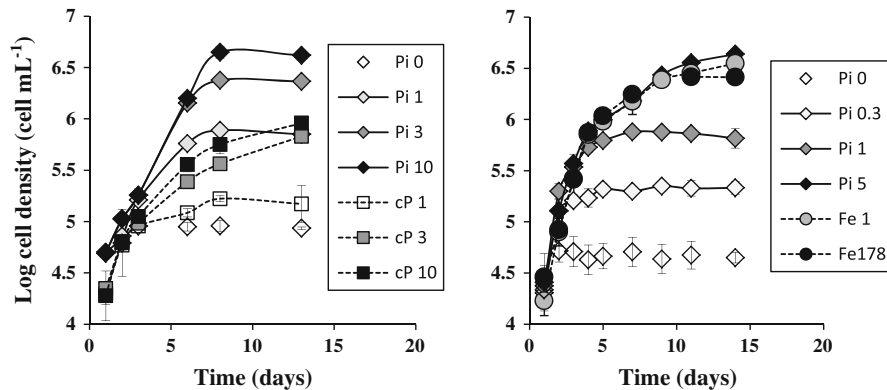
**Fig. 1** Algal growth on orthophosphate ( $P_i$ ). Cell density after 14 days growth in relation to initial  $P_i$  concentration, means, and standard error (*error bars*),  $n = 10$ . Initial cell density was  $0.01 \times 10^6 \text{ cell ml}^{-1}$ . The growth response is linear up to  $5 \mu\text{mol l}^{-1}$  and a regression line (with 95% confidence limit as *dotted lines*) was fitted to data  $0\text{--}5 \mu\text{mol l}^{-1}$

Algae and bacteria were counted by a Helber counting chamber. Algal cell density in our algal culture used for inoculation in the test solutions (inoculation culture) varied generally from  $10^5$  to  $10^6 \text{ cell ml}^{-1}$  while bacterial cells were below detection limit in the inoculation culture throughout all experiments. In some inoculation cultures, however, few bacteria could be detected. If so, the ratio “bacterial cell density/algal cell density” remained below 22.8%, equaling an estimated ratio “bacterial cell surface/algal cell surface” of 2.2%.

### Model organic P species

Algal growth on organic P yielded lower final cell numbers (Table 2) or induced slower growth, compared to growth on equal concentrations of  $P_i$ , as shown for creatine phosphate (Fig. 2, left panel). Algal growth in our experiments was completed after 14 days since no further increase could be detected in algal cell density after 21 and 28 days (data not shown). Algal cell density typically started to decline slightly after or before this 14 day period.

Adenosine 5'-triphosphoric acid supported algal growth to the greatest extent in our test, with algal cell densities up to 72% of growth compared to  $P_i$  at  $3 \mu\text{mol l}^{-1}$  (Table 2). The BAP of the other phosphate



**Fig. 2** Comparison of algal growth curves on orthophosphate ( $P_i$ ), creatine phosphate (cP) as a model organic P compound (left) and ferrihydrite stabilized by natural organic matter as a synthetic colloidal P compound (right). Means and their standard error (error bars),  $n = 3$ . The  $P_i$  0– $P_i$  5 represent test

media with initial  $P_i$  concentrations of 0–5  $\mu\text{mol l}^{-1}$ ; cP 1–cP 10 creatine phosphate of 1–10  $\mu\text{mol l}^{-1}$  as P source and Fe:P 1 and Fe:P 178 represent 5  $\mu\text{mol l}^{-1}$   $P_i$ , equilibrated with colloidal ferrihydrite at a molar ratio Fe:P of, respectively, 1 and 178

monoesters is highly variable, ranging from as low as 2 up to 58%. The specific growth rate in the exponential phase ( $\mu_{\text{exp}}$ ) decreased significantly compared to growth on  $P_i$  as P source apart from adenosine adenosine-5'-triphosphoric acid and triphosphate. A Pearson correlation ( $P > 0.05$ ) between the  $\mu_{\text{exp}}$  and BAP was, however, insignificant.

Increasing initial DOP concentrations from 3 to 10  $\mu\text{mol l}^{-1}$  did not increase the maximum cell number ( $P > 0.05$ ) apart from cAMP (data not shown). This limited or no increase effectively decreases BAP with increasing DOP, dwindling to 25% for ATP and 2% for phytic acid. The ranking of the BAP among DOP forms remained the same for all P compounds except for cAMP for which the BAP increased slightly.

#### Inorganic colloids

We tested two colloidal P solutions in the dissolved phase: Al oxide particles and ferrihydrite stabilized by NOM. Algal growth was unaffected ( $P < 0.05$ ) by NOM itself (data not shown), the NOM was used to stabilize ferrihydrite. The maximum algal cell density and growth rate dropped significantly ( $P < 0.05$ ) upon metal oxide addition in a concentration-dependent manner (Table 3; Fig. 2, right panel).

#### Environmental samples

The RO concentration procedure did not change the colloidal and organic P compounds in the dissolved

fraction with respect to the long term availability of P since the algal cell density was not different ( $P < 0.05$ ) between the original water, the reconstituted water using the RO concentrate and the DOM-free water (data not shown). The initial growth was, however, faster for the samples containing DOM compared to the DOM free water, probably because the Fe associated to DOM is more bioavailable compared to the synthetic Fe-EDTA.

The fraction DOP in our environmental sample set, i.e.,  $(\text{TDP}-P_i)/\text{TDP}$ , the  $P_i$  measured with IC, ranged 0–56%, mean 20%. The estimated colloidal inorganic P fraction, i.e.,  $(\text{CM}-P_i)/\text{TDP}$  was at least 30%, mean 69%. As indicated above, this fraction is somewhat unsure since some DOP is detected by the CM and since the IC method can detect some inorganic colloidal P. The IC measured P concentrations were for 10 out of the 14 samples below the detection limit. The  $P_i$  fraction was consequently negligibly small for these samples compared to the colloidal and organic P.

The BAP ranged 7–84% of TDP (Fig. 3; Table 4). This variation could not be attributed to any or a combination of the three different P species, when these P species were estimated as explained above since no Pearson correlations could be established between BAP and concentrations of  $P_i$  (IC measurement),  $P_i$  + estimated DOP,  $P_i$  + estimated colloidal P, and  $P_i$  + estimated DOP + estimated colloidal P ( $P > 0.14$ ). The P detected by IC underestimates the BAP whereas the reverse is true for the P detected by CM and ICP. The ICP and CM measured P correlate

**Table 2** Bioavailability of model dissolved organic P compounds in relation to analytical P measurements

P compound	Initial solution P			$N_{\max}$ ( $10^6$ Cell $\text{ml}^{-1}$ )	BAP (%)	$\mu_{\text{exp}}$ ( $\text{day}^{-1}$ )
	TDP ( $\mu\text{mol l}^{-1}$ )	CM (% TDP)*	IC (% TDP)*			
Pi	3.0 (0.2)	102.3 (2.9)	97.4 (1.3)	$2.09 \times 10^6$	98 ( $\pm 6$ )	1.38 <sup>ab</sup>
ATP	2.7 (0.1)	1.9 (0.3)	0.2 (3.3)	$1.53 \times 10^6$	72 ( $\pm 4$ )	1.22 <sup>abcd</sup>
Pser	3.0 (0.1)	n.m.	n.m.	$1.35 \times 10^6$	58 ( $\pm 14$ )	0.85 <sup>f</sup>
gluP	3.1 (0.2)	0.4 (0.2)	0.1 (0.1)	$1.28 \times 10^6$	53 ( $\pm 11$ )	0.85 <sup>f</sup>
mbP	3.1 (0.2)	0.3 (0.1)	0.6 (1.1)	$1.19 \times 10^6$	49 ( $\pm 8$ )	1.04 <sup>de</sup>
triP	3.3 (0.1)	3.8 (1.7)	0.5 (0.1)	$1.08 \times 10^6$	48 ( $\pm 7$ )	1.31 <sup>abc</sup>
glyP	3.0 (0.1)	0.1 (0.1)	0.2 (0.1)	$0.81 \times 10^6$	39 ( $\pm 2$ )	n.m.
cP	2.9 (0.1)	102.9 (1.7)	17.9 (3.1)	$0.711 \times 10^6$	31 ( $\pm 2$ )	n.m.
cAMP	0.9 (0.1)	<d.l.	0.1 (0.1)	$0.020 \times 10^6$	9 ( $\pm 3$ )	0.32 <sup>g</sup>
pa	2.3 (0.1)	2.8 (0.5)	1.9 (1.3)	$0.091 \times 10^6$	7 ( $\pm 7$ )	0.14 <sup>g</sup>
butP	3.4 (0.4)	0.2 (0.1)	0.2 (0.2)	$0.023 \times 10^6$	2 ( $\pm 2$ )	n.m.

The bioavailability of dissolved P compounds (BAP, in %) is the maximum cell number obtained during 14 days growth ( $N_{\max}$ ) relative to the cell number obtained at corresponding initial P concentrations of orthophosphate ( $P_i$ ) calculated using regression lines corresponding to Fig. 2 and Eq. 3. The 95% confidence interval of BAP is given in *brackets*. The values followed by the same letter within the columns are not significantly ( $P > 0.05$ ) different according to Duncan's multiple range test. The standard deviations of the three repetitions are given in parentheses for TDP, CM, and IC measurements. (CM colorimetric method P, IC ion chromatography, TDP total dissolved P with standard deviation in *brackets*,  $\mu_{\text{exp}}$  specific growth rate during exponential phase, <d.l. below detection limit, and n.m. not measured)

ATP adenosine-5'-triphosphoric acid, Pser o-phospho-DL-serine, gluP D-glucose 6-phosphate, mbP 4-methylumbelliferyl phosphate, triP triphosphate, glyP glycerol phosphate, cP creatine phosphate, pa phytic acid, cAMP adenosine 3'-5'-cyclic monophosphate, butP tri-N-butyl phosphate

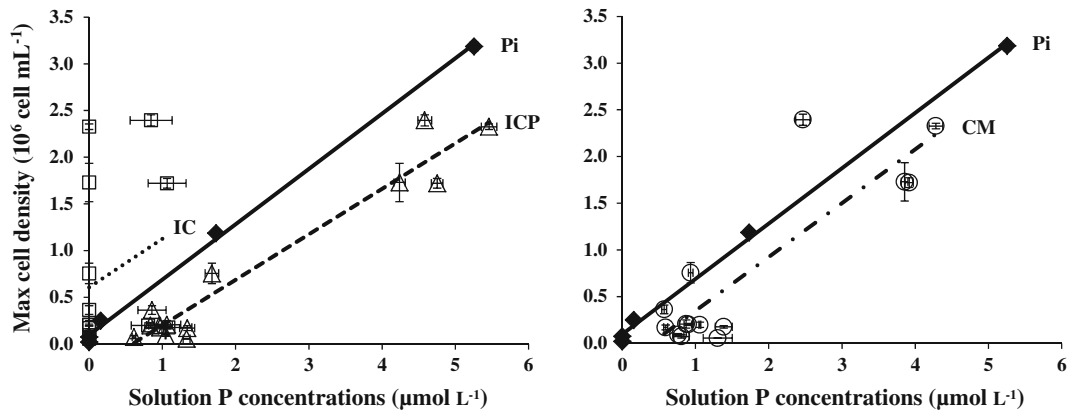
\* Measured and reported in Van Moorlehem et al. (2011)

**Table 3** Bioavailability of model colloidal P compounds in relation to analytical P measurements

P compound	Molar ratio Metal/ $P_i$	Initial solution P			$N_{\max}$ (Cell $\text{ml}^{-1}$ )	BAP (%)	$\mu_{\text{exp}}$ ( $\text{day}^{-1}$ )
		(% of $P_i$ without colloids)					
		TDP	CM	IC			
$P_i$	0	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	$4.16 \times 10^6$	100 <sup>a</sup>	1.36 <sup>a</sup>
AlOx	0.3	94 <sup>a</sup>	97 <sup>ab</sup>	89 <sup>a</sup>	$2.94 \times 10^6$	71 <sup>b</sup>	1.24 <sup>ab</sup>
	2.5	104 <sup>a</sup>	98 <sup>ab</sup>	95 <sup>a</sup>	$3.30 \times 10^6$	79 <sup>b</sup>	1.29 <sup>ab</sup>
	31.8	98 <sup>a</sup>	96 <sup>b</sup>	83 <sup>a</sup>	$3.37 \times 10^6$	81 <sup>b</sup>	1.34 <sup>a</sup>
	102.3	102 <sup>a</sup>	92 <sup>c</sup>	86 <sup>a</sup>	$2.23 \times 10^6$	54 <sup>c</sup>	0.83 <sup>c</sup>
FeSRNOM	1.3	103 <sup>a</sup>	100 <sup>a</sup>	96 <sup>a</sup>	$3.54 \times 10^6$	85 <sup>a</sup>	1.37 <sup>a</sup>
	12.7	90 <sup>b</sup>	93 <sup>a</sup>	73 <sup>ab</sup>	$2.25 \times 10^6$	54 <sup>b</sup>	1.24 <sup>ab</sup>
	51.2	97 <sup>ab</sup>	95 <sup>a</sup>	68 <sup>b</sup>	$2.81 \times 10^6$	68 <sup>b</sup>	1.45 <sup>a</sup>
	178.1	103 <sup>a</sup>	96 <sup>a</sup>	56 <sup>b</sup>	$2.74 \times 10^6$	66 <sup>b</sup>	1.10 <sup>abc</sup>

The bioavailability of colloidal P (BAP, in %) is the maximum cell number obtained during 14 days growth ( $N_{\max}$ ) relative to that in the  $P_i$  solution. All experiments used  $5 \mu\text{mol l}^{-1}$  of initial P and colloids were AlOx Al oxide nanopowder, FeSRNOM ferrihydrite stabilized by natural organic matter. Values followed by the *same letter* in the same column (per metal) do not differ significantly ( $P > 0.05$ ) according to Duncan's multiple range test (TDP total dissolved P, CM colorimetric method P, IC ion chromatography,  $\mu_{\text{exp}}$  specific growth rate during exponential phase)





**Fig. 3** Algal cell density (after 14 days) in 14 standardized environmental freshwater samples in relation to the initial P concentration measured by ICP (=total dissolved P, *triangle*), ion chromatography (IC, *square*, left panel) or colorimetry (CM, *open circle* right panel). Means and standard errors (*vertical and*

*horizontal bars*),  $n = 3$ . Algal growth at five different orthophosphate ( $P_i$ ) concentrations is represented as reference (*filled diamond*), these concentrations are identically detected by ICP, IC, or CM. The data illustrate that the ICP and CM overestimate bioavailable P whereas IC largely underestimates it

with the maximum algal cell density (Pearson correlation of respectively 0.97 and 0.88 and  $P < 0.0001$ ), in contrast to IC measured P ( $P = 0.35$ ).

The SUVA, a measure for DOM aromaticity, is negatively correlated to biodegradable DOM (Saadi et al., 2006). We could, however, not detect a correlation between SUVA of the environmental samples and algal growth on these P limited samples (Pearson correlation  $-0.27$  with  $P = 0.37$ ; Fig. 4), possibly due to a rather low overall contribution of organic P in our environmental sample set. The initial Fe concentration in our test solutions ranged from 0.2 to 8.1  $\mu\text{mol l}^{-1}$  and Al concentrations from 0.1 to 5.0  $\mu\text{mol l}^{-1}$ . Again, no correlation could be established between algal available P and initial Fe and Al concentrations (Pearson correlation of, respectively,  $-0.39$  and  $-0.14$  with  $P$  0.16 and 0.64) (Fig. 4).

## Discussion

In this study, we examined the bioavailability of phosphorus in the dissolved fraction in relationship with analytical techniques. Higher initial  $P_i$  concentrations linearly increased the algal cell density between 0 and 5  $\mu\text{mol l}^{-1}$  P. The cell density after 14 days obtained with 10  $\mu\text{mol l}^{-1}$   $P_i$  deviated from the linear fit between 0 and 5  $\mu\text{mol l}^{-1}$ , probably due to another limiting factor than P, most likely a decrease in light absorption due to an excessive algal

cell density. The cell density obtained on the model organic P compounds, colloidal P and environmental samples were all lower than the cell density obtained on 5  $\mu\text{mol l}^{-1}$   $P_i$ , justifying the use of Eq. 2.

Bacterial abundance in our inoculation cultures was measured using both light microscopy and fluorescence microscopy after DAPI staining. The DAPI was only able to stain free bacteria in the medium but bacteria associated with the algae could not be detected since the fluorescence of algal chlorophyll masks the DAPI stain (Croft et al., 2005). When comparing algal and bacterial effects regarding P uptake and enzyme excretion, the cell surface ratio of bacteria over algae is likely the most reliable parameter since phosphatases are mainly associated with intact or lysed cell membranes (Jansson, 1988) and the hydrolyzed P is taken up actively or passively through the membrane. This ratio remained below 2.2% at all times at the start of the experiment. Since phosphatases can also be released as a free molecule by bacteria and algae, the use of a volume ratio bacteria/algae could possibly be used, rendering an even lower contribution to bacteria in the organic P hydrolysis and P uptake processes.

However, algal and bacterial abundance is only a vague indication while ideally it is the activity of both bacteria and algae which matters to conclusively distinguish between algal and bacterial effects. Moreover, the role of bacterial activity regarding enzyme production and P uptake is potentially becoming even

**Table 4** Bioavailability of dissolved P compounds in 14 environmental samples in relation to analytical P measurements

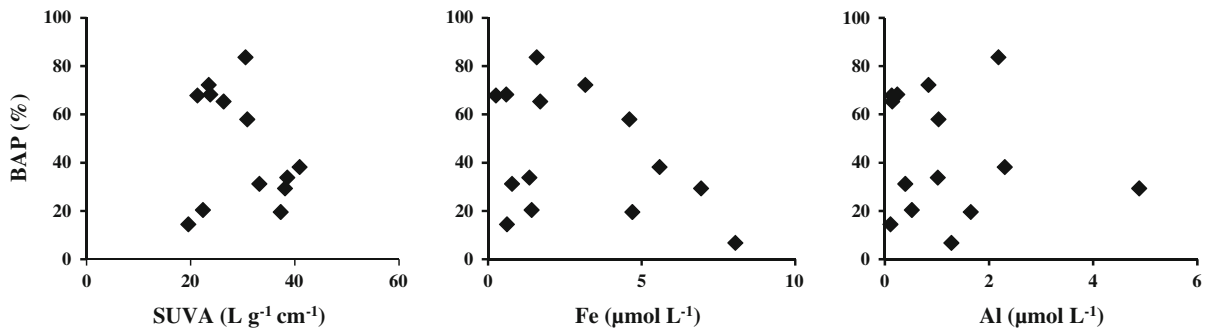
TDP ( $\mu\text{mol l}^{-1}$ )	CM (% TDP)	IC (% TDP)	BAP (%)
<i>P<sub>i</sub></i>			
5.3 (0.5)	100 (0)	107 (7)	104 ( $\pm$ 6)
1.7 (0.1)	97 (1)	111 (18)	99 ( $\pm$ 4)
<i>Environmental samples</i>			
5.5 (0.6)	78 (12)	<d.l.	68 ( $\pm$ 14)
4.8 (0.2)	82 (10)	22 (9)	58 ( $\pm$ 13)
4.6 (0.2)	54 (10)	18 (11)	84 ( $\pm$ 4)
4.3 (0.1)	91 (19)	<d.l.	65 ( $\pm$ 33)
1.7 (0.2)	56 (3)	<d.l.	72 ( $\pm$ 23)
1.3 (0.2)	44 (3)	<d.l.	20 ( $\pm$ 23)
1.3 (0.1)	98 (26)	<d.l.	7 ( $\pm$ 38)
1.1 (0.3)	82 (15)	<d.l.	31 ( $\pm$ 29)
1.0 (0.1)	73 (13)	<d.l.	14 ( $\pm$ 27)
1.0 (0.1)	143 (20)	116 (57)	29 ( $\pm$ 20)
1.0 (0.3)	94 (10)	<d.l.	34 ( $\pm$ 25)
0.9 (0.3)	67 (7)	<d.l.	68 ( $\pm$ 30)
0.8 (0.1)	127 (2)	88 (48)	38 ( $\pm$ 61)
0.6 (0.1)	132 (30)	<d.l.	20 ( $\pm$ 78)

All environmental samples were standardized for DOC concentration and inorganic compounds (except P; Table 1). The bioavailability of dissolved P (BAP, in %) is the maximum cell number obtained during 14 days growth ( $N_{\text{max}}$ ) relative to the cell number obtained at corresponding initial P concentrations of orthophosphate ( $P_i$ ) calculated using the regression line corresponding to Fig. 2 and Eq. 3. The standard deviation is given in parentheses for TDP, CM, and IC measurements while the 95% confidence limits are given in parentheses for BAP (TDP total dissolved P, CM colorimetric method P, IC ion chromatography, <d.l. below detection limit ( $0.1 \mu\text{mol l}^{-1}$ ))

more important in the course of the experiment since we did not performed the bioassay itself axenically. On one hand, bacteria may be superior competitors for P (especially  $P_i$  and nucleotide DOP) over phytoplankton (Currie & Kalff, 1984) and, on the other hand, bacteria may be significant producers of extracellular enzymes catalyzing the hydrolysis of organic P compounds (Cotner & Wetzel, 1992; Hoppe, 2003). Moreover, the competition for P by algae and bacteria is dependent on the P concentrations in the medium and therefore, is likely to be variable in time as bacteria become better competitors for P with decreasing P concentrations (Mindl et al., 2005). The term BAP in this study refers to algal available P, potentially enhanced during the bioassay by the action

of algal enzymes which is possibly becoming more important as the long-term experiments proceed.

The BAP of the organic P compounds was highly variable and lower compared to the  $P_i$  treatment, likely due to slow or no release of P from DOP (Aaronson & Patni, 1976). Phosphorus incorporated in nucleotides are likely more bioavailable compared to other monophosphate esters because of the action of general phosphatases plus some substrate-specific enzymes, such as 5'-nucleotidases, catalyzing nucleotide DOP more efficiently than nonnucleotide DOP (Björkman & Karl, 1994; Nedoma et al., 2003). This is reflected in our long-term test, in which the nucleotide ATP has the highest BAP. Most of the selected model organic P compounds in our test are phosphate monoesters, reflecting the most abundant P species present in surface waters (Kolowitz et al., 2001; Turner et al., 2005). These model organic P compounds are generally not susceptible to acid or alkaline catalyzed hydrolysis in the short run (minutes) as they are measured in only negligible amounts by the alkaline IC or acid CM (Van Moorlegheem et al., 2011). Even a phosphate triester, tri-*N*-butyl phosphate, was measured for less than 0.2% by CM and IC although phosphate triesters are supposed to be the most reactive of all phosphate esters (Turner et al., 2005). Some organic compounds, including creatine phosphate and acetylphosphate, prove to be (partially) detectable by CM and IC. Creatine phosphate had, however, in this bioassay a lower BAP (31%), compared to the more stable phosphate esters. Acid or alkaline induced hydrolysis of low molecular weight organic P compounds is, hence, not always reflected in the ability to be hydrolyzed biologically during our assay. For phytic acid, cAMP and tri-*N*-butyl phosphate, algal growth was limited to maximally 9% of the growth expected on  $P_i$  as P source. Although the last one might be toxic towards aquatic organisms, no growth inhibitory effects have been noticed for different algae species for concentrations below  $6 \mu\text{mol l}^{-1}$  (Michel et al., 2004). Phytic acid is known to be very stable and only the phosphates in the 3 and 6 position can be hydrolyzed by mainly two classes of specialized enzymes (Turner et al., 2005), resulting in limited growth in our test. Algal growth on cyclic adenosine-5'-phosphate (cAMP) remained limited to 12%. Although cyclic nucleotides proved to be less labile than phosphate monoesters in phytoplankton uptake (Cotner & Wetzel, 1992), it has been



**Fig. 4** The bioavailability of dissolved P (relative to orthophosphate, %) in 14 environmental freshwater samples as a function of the initial specific UV absorbance at 254 nm

(SUVA) or dissolved Fe and Al concentration in these waters. (BAP (%) bioavailable P, Fe Fe concentration, and Al Al concentration)

demonstrated that cAMP can rapidly be assimilated by marine bacteria which can take up the entire molecule through high-specificity and high-affinity transport systems (Hansell & Carlson, 2002). Growth on glucose-6-phosphate as P source varied largely among different algae cultures as inocula (Aaronson & Patni, 1976). A more diverse community of bacteria and phytoplankton might result in the excretion of more varied types of enzymes, catalyzing the hydrolysis of DOP more efficiently.

Increasing initial DOP concentrations by a factor 3 did not significantly increase the maximum cell number apart from cAMP, resulting in a decrease of the P Avail. Hence, in general, the decreasing P Avail of DOP with increasing TDP suggests that the concentration of hydrolyzing enzymes and not the substrate concentration is the growth limiting factor. Alternatively, bacterial consumption of DOP might also be stimulated by increasing DOP concentrations, thereby rendering relatively less to algae.

The  $\mu_{\text{exp}}$  generally decreased for the organic P compounds compared to the  $P_i$  treatment. Note, however, that  $\mu_{\text{exp}}$  only refers to the (initial) exponential phase that is comparable between DOP or  $P_i$  and growth differences only appear at a later stage. This initial fast growth phase was not induced by  $P_i$  contamination of the chemicals since colorimetric P measurements were mostly below the detection limit ( $0.2 \mu\text{mol l}^{-1}$ ). This initial growth can (partially) result from the P reserves inside the algal cells since algae can store a large amount of P as polyphosphates in their vacuoles (Nishikawa et al., 2006).

The bioavailability of particulate matter bound P (Ekholm & Krogerus, 2003) and (model) organic P

compounds (Björkman & Karl, 1994, 2003; Huang et al., 2005) has already been investigated. However, the availability of P associated with colloidal Fe- and Al-oxhydroxydes remains largely unknown, although this P form might contribute more importantly than the organic and  $P_i$  forms in environmental water samples (Van Moorleghe et al., 2011). To obtain a better understanding of the bioavailability of the colloidal P fraction, algal growth was tested on  $P_i$  in the presence of synthesized Fe- and Al colloids. Growth inhibition effects of alumina and aluminium oxide nanoparticles to algae are observed with  $\text{EC}_{50}$  values above  $400 \mu\text{mol l}^{-1}$  (Sadiq et al., 2011). Consequently, no growth inhibition was expected with Al concentrations as used in our test ( $<7 \mu\text{mol Al l}^{-1}$ ).

The IC measured P did not differ significantly from BAP for the ferrihydrite stabilized by SRNOM addition, suggesting that the algal available P is limited to the  $P_i$  fraction. The P associated with Al-oxide particles is (partially) prone to hydrolysis during IC measurements (Van Moorleghe et al., 2011) and may explain why the BAP is lower than that measured by IC.

The BAP in our environmental samples was highly variable and no correlation could be established between BAP and (a combination) of the analytically measured P fractions. The malachite green colorimetric was preferred above the molybdenum blue colorimetric method in this study because of its higher sensitivity. Both colorimetric methods are likely to measure an equal fraction of organic P forms while the malachite green method might measure a larger colloidal P fraction than the molybdenum blue because the sample is acidified to a lower pH for a

longer time during measurement (Hens, 1999). Most likely, the  $P_i$  fraction (IC detected P) is completely available whereas DOP and colloidal P contribute in varying proportions, depending on the sample. Both the model DOP and the model inorganic colloids also showed contrasting fractions of available P, and the variable sources and properties of the natural P forms may explain the scatter in the available P fractions. Measurement of Al and Fe in the environmental samples—as an approximation of the quantity of Fe and Al colloids and associated colloidal P—were not useful in the prediction of BAP since no correlations could be established.

Taken together, our study did show that DOP is generally not detected by CM in contrast with the bioavailability and, conversely, that colloidal P is completely measured by CM whereas its bioavailability is limited. The overestimated bioavailability of P detected by CM suggests that the latter factor is more important, i.e., inorganic colloids likely dominate the P in solution. From a practical point of view, however, the CM on the dissolved fraction of water is the most defensible among the three methods to predict potential available P: the correlation with cell numbers is large (Fig. 3) and the overestimation of available P compared to  $P_i$  is smaller than for the TDP method (respectively factors 1.44 and 1.56). No such correlation may appear in waters where DOP forms dominate dissolved P. However, such waters are often found in oligotrophic conditions for which the legislations are likely less important.

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