Phosphate availability controls *Trichodesmium* spp. biomass in the SW Pacific Ocean

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ABSTRACT: Throughout tropical and subtropical seas, *Trichodesmium* spp. contribute significantly to marine fixation of atmospheric di-nitrogen and influence the global carbon cycle. We suggest that dissolved inorganic phosphate (DIP) availability has a predominant role in controlling *Trichodesmium* spp. biomass. From experimental work carried out on cruises in the SW Pacific Ocean, and by re-analysing previous data, we have defined a critical level of DIP needed for single filaments of *Trichodesmium* spp. to grow. Thus, seasonal variations in DIP availability could control *Trichodesmium* spp. growth and decay. As this critical level is below the detection limit of classical DIP measurements obtained during oceanic cruises, we suggest a re-evaluation of the phosphate availability in the oligotrophic ocean in order to determine what ultimately controls di-nitrogen fixation in the sea.

KEY WORDS: *Trichodesmium* spp. · Phosphate availability · Diazotrophy · South Pacific Ocean

INTRODUCTION

In a nitrogen-limited ocean, the input of ‘new’ nitrogen (i.e. not related to organic matter recycling) into the photic zone controls primary production (Codispoti 1989). Nitrogen fixation in the ocean is a source of new nitrogen. Thus, a fundamental question arises as to what factors control nitrogen fixation in the ocean. What are the factors that control N₂ fixation over annual or longer time-scales (Falkowski 1997, Letelier & Karl 1998, Tyrell 1999) and are these distinct from ‘physiological’ factors that may temporarily control the process of nitrogen fixation? Light, temperature (Carpenter et al. 2004) and nutrient availability, particularly that of phosphate (Sañudo-Wilhelmy et al. 2001, Mulholland et al. 2002, Fu & Bell 2003) and iron (Behrenfeld & Kobler 1999, Kustka et al. 2002), could physiologically control the kinetics of nitrogen fixation. These factors could be different from the ‘systemic’ factor (Paasche & Erga 1988) that controls the cumulative biomass over time within a particular oceanic area, and ultimately, when considering all the oceanic provinces, the amount of nitrogen introduced via di-nitrogen fixation in the world’s oceans.

Physiological factors can be investigated using short-term experiments such as selective enrichment experiments showing that there may be co-limitation of diazotrophs by both iron and dissolved inorganic phosphate (DIP) in certain situations (Mills et al. 2004). However, such short-term limitation may not control accumulation of diazotroph biomass over time. For example, if the systemic limiting factor is DIP availability in a particular area, a more or less high iron availability will only drive the system to a more or less rapid consumption of DIP. The cumulative biomass of *Trichodesmium* spp., which depends essentially on the DIP consumption, will not be affected in the long term by iron availability. Considering the cumulative biomass as the end product of the nitrogen fixation process, ‘physiological’ factors act as catalytic factors only. Thus, knowing the systemic controlling factor is of prime necessity and can only be assessed

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using annual or longer-term studies on nutrient availability and uptake kinetics parameters of the species studied.

We studied DIP availability and the response of the DIP uptake system of natural *Trichodesmium* spp. populations to increasing DIP concentrations. The work was carried out in the SW Pacific Ocean near New Caledonia, an area where *Trichodesmium* spp. blooms are frequently observed (Dupouy et al. 2000). Using kinetic experiments, we determined the critical DIP concentration for *Trichodesmium* spp. growth. We then provide further evidence correlating annual patterns of the observed surface accumulations of *Trichodesmium* spp., with decreasing ambient DIP availability. Our results suggest that DIP is the systemic N₂ fixation limiting nutrient in this area.

**MATERIALS AND METHODS**

Surface water samples and *Trichodesmium* spp. were collected on the RV 'L'Alis' from 5 to 12 February 2003 in the open SW Pacific Ocean east of New Caledonia (Diapalis Cruise 7 of the DIAPAZON program: Diazotrophic PACific ZONE; 20–22°S latitude, 166–168°E longitude). Kinetic experiments were carried out on concentrated *Trichodesmium* spp. samples collected using 35 µm mesh nets and on un-concentrated samples.

**Kinetics from *Trichodesmium* spp. collected in nets (Exps MT [a] and MT [b]).** The net tows were performed at the stations from a depth of 1 m. The net was slowly lowered and raised 10 times in 10 min. Net tow material (200 ml) was mixed with 800 ml of 0.2 µm-filtered seawater by slow agitation and subsamples were immediately taken up for chemical and biological analyses.

DIP was estimated immediately with the molybdenum blue reaction (Strickland & Parsons 1972) on 50 ml samples using a 10 cm path length cell in a Cecil CE 1011 spectrophotometer. Particulate phosphate (PP) was determined following filtration of 50 ml samples through polycarbonate filters (10 µm; 47 mm) using the standard DIP analysis of high temperature persulfate wet-oxidation at 120°C and 1 bar (Pujo-Pay & Raimbault 1994). Total phosphate (TP) was estimated on 40 ml samples following the same wet-oxidation as for PP. Dissolved organic phosphate: [DOP] = [TP] – [DIP] – [PP].

Biovolumes of *Trichodesmium* spp. and other phytoplankters were estimated with an inverted microscope (Wild M40) using Utermöhl settling chambers (2 ml) from samples fixed with Lugols. Organisms were identified to the species level (trichome width for *Trichodesmium*). Estimates of cell volume for each species (organisms > 5 µm length) were obtained using measurements from 30 to 50 cells for each species and the application of the geometric formula best fitted to the cell shape (Hillebrand et al. 1999) with estimation of the parietal cytoplasm thickness (Smayda 1978).

DIP uptake was measured from 50 ml samples incubated with 185 kBq (5 µCi) carrier-free [33PO₄] (Amersham BF1003) in polycarbonate vials using an on-deck incubator at sea surface temperature. Incubations (t = 1 h) were stopped by the addition of 500 µl of 10 mM non-radioactive KH₂PO₄ and samples were filtered immediately onto 10 µm (25 mm diameter) polycarbonate filters. Radioactivity on the filters (cpm) was measured using scintillation liquid counting and the specific uptake rate $V$ (h⁻¹) was calculated from the equation:

$$ V_{sp} = \frac{[R_t - R_b]/R_b \times \text{DIP}] / (t \times \text{PP}) $$

where $R_t$, $R_b$, and $R_b$ are the radioactivity of the filter, the blank (fixed with ca. 100 µl of 20 g l⁻¹ HgCl₂), and the total tracer added to the sample. DIP is the initial plus the added DIP concentrations (0 to 300 µl of a 100 µM KH₂PO₄ solution). PP and $t$ are the particulate phosphate concentration and the incubation time, respectively. The maximum value of the blank was 2.3% of the filter activity. A time-course experiment conducted for DIP uptake (data not shown) indicated no significant difference in the rates between 5 min and 2 h of incubation time.

**Kinetics using unconcentrated samples (E).** The same protocol as for concentrated *Trichodesmium* spp. was followed for samples collected with a Niskin bottle in the upper surface water. In order to take into account the lower biomass concentrations (no pre-concentration), 1000 ml of water was processed for PP determination. The incubation time for the measurement of DIP uptake was increased to 1.5 h and 25 ml was processed for biomass estimations.

**Seasonal variations of phosphate availability.** Seasonal variations were determined using data from Cruises 1 to 6, see Van Den Broeck et al. (2004) for details on methods, particularly DIP turnover times (TDIP) measurements, and sampling strategy.

**RESULTS**

**Critical DIP availability for *Trichodesmium* spp. growth**

Dissolved inorganic, organic, and particulate phosphate (DIP, DOP and PP) concentrations are presented along with *Trichodesmium* spp. and other phytoplanktonic biomasses (Table 1). As expected in samples
using net tow material, PP and biomass increased. DOP concentrations increased slightly while DIP concentrations exhibited a large increase possibly related to excretion by zooplankton in the net tow material. Experiments were also carried out, for the first time, on unconcentrated samples rich in \textit{Trichodesmium} spp. that had low initial DIP concentrations. \textit{Trichodesmium} spp. represented at least 95\% of the phytoplanktonic biomass estimated by cell volumes, and appeared as single ‘filaments’ (or trichomes) only.

The specific uptake rate versus concentration curve (Fig. 1) shows a saturation curve. Michaelis-Menten parameters obtained following the Marquardt-Levenberg minimisation calculation with 4 iterations gives $K_s = 630$ nM (SD = 133) and $V_{\text{max sp.}} = 0.31$ h$^{-1}$ (SD = 0.04). The substrate [DIP] varies from 0.03 to 1.23 of the $K_s$ value and $V_{\text{sp.}}$ varies from 0.03 to 0.52 of the $V_{\text{max sp.}}$ value. This is the first recorded estimate of $K_s$ for \textit{Trichodesmium} spp. at environmentally significant concentrations. The $V_{\text{max}}$ value was only reached at concentrations greater than the maximum concentration already measured in the upper water column in this area (DIP = 120 nM). Interestingly, similar specific uptake rates were measured with and without pre-concentrating \textit{Trichodesmium} spp. A decrease in uptake rates following brief exposure to high DIP concentrations might be expected, but was not observed.

From the Michaelis-Menten relationship, we calculated the time required for the \textit{Trichodesmium} spp. biomass to double constituent P ($t_d$), $t_d = \ln2/(24 V_{\text{sp.}})$, (Table 2), assuming growth in an exponential phase, no excretion, and DIP as the only source of phosphate. The $t_d$ range is calculated using an upper limit of 50\% positive or negative variation in the $V_{\text{sp.}}$ estimates. The 1 to 100 nM range of DIP concentration corresponds to the variations in seasonal DIP concentrations observed in the upper surface water of this area (Van Den Broeck et al. 2004). These \textit{in situ} concentrations were measured by the classical colorimetric method (Detection limit: DL = 30 nM) and by an indirect method (DL = 2 pM) based on turnover times when DIP reached undetectable levels (DIP = TDIP $\times$ DIP uptake). DIP uptake was derived from primary and bacterial productions using C:P ratios. Given the high accuracy of turnover time measurements, the natural variability in the C:P uptake ratios did not affect the order of magni-

\begin{table}
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DIP (nM) & PP (nM) & TP (nM) & DOP (nM) & \textit{Trichodesmium} & Other Phyto. spp. >5 µm \\
\hline
MT (a) & 247 ± 20 (3) & 322 ± 26 (3) & 1158, 1064 & 542 & 1.92; 2.50 & 0.08; 0.08 \\
MT (b) & 177 ± 40 (3) & 365 ± 78 (3) & 1005 ± 70 (3) & 463 & 7.24; 7.28 & 0.07; 0.01 \\
(E) & 20, 20 & 20.4 & 397 ± 12 (3) & 357 & 0.41 & 0.01 \\
\hline
\end{tabular}
\caption{Initial concentrations of dissolved inorganic phosphate (DIP), particulate phosphate (PP), total phosphate (TP), dissolved organic phosphate (DOP) and biomasses of \textit{Trichodesmium} spp. and other phytoplanktonic species for the kinetic experiments from \textit{Trichodesmium} spp. collected with a net (MT(a) and MT(b)) and unconcentrated samples (E). Data are mean ± SD with no. of samples in parentheses, where shown. ; indicates duplicate values}
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DIP (nM) & $V_{\text{sp.}}$ (h$^{-1}$) & $t_d$ (d) \\
\hline
1 & 0.0005 & 59 (39–118) \\
10 & 0.0048 & 6.0 (4.0–11.9) \\
30 & 0.0141 & 2.0 (1.4–4.1) \\
50 & 0.0228 & 1.3 (0.8–2.5) \\
100 & 0.0425 & 0.7 (0.5–1.4) \\
\hline
\end{tabular}
\caption{Dissolved inorganic phosphate (DIP), specific uptake rates ($V_{\text{sp.}}$) and time required to double constituent P ($t_d$) in parentheses $t_d$ calculated with $V_{\text{sp.}} = 50\%$) for DIP concentrations in the range encountered during our observations in the open SW Pacific Ocean near New Caledonia (Diapalis cruises)}
\end{table}

Fig. 1. Dissolved inorganic phosphate (DIP) specific uptake rate by natural populations of \textit{Trichodesmium} spp. versus DIP concentration. Experiments conducted in February 2003 (Diapalis Cruise 7, SW Pacific Ocean near New Caledonia) from net-collected \textit{Trichodesmium} spp. (MT(a): +; MT(b): o) and from unconcentrated samples in triplicates (E(1): c; E(2): t; E(3): δ). Theoretical curve using Michaelis-Menten parameters obtained following the Marquardt-Levenberg minimization: $K_s = 630$ nM and $V_{\text{max sp.}} = 0.31$ h$^{-1}$.
tude of the calculated concentrations and allowed us to reach a sub-nanomolar detection limit (Moutin et al. 2002; Van Den Broeck et al. 2004).

The $t_\text{s}$ values varied from 0.7 to 59 d (Table 2), thus enabling the P-requirement for the *Trichodesmium* spp. biomass to be maintained until the DIP concentration remained above the critical DIP concentration of 9 nM ($t_\text{s} < 7$ d). This indicates that a relatively high DIP concentration (DIP > 9 nM corresponding to an in situ DIP turnover time $T_{\text{DIP}} > 50$ h) may be necessary for *Trichodesmium* spp. to maintain a growth rate above 0.1 d$^{-1}$ (considered the minimum value because it was the lowest *Trichodesmium* spp. growth rate measured in the water column during all the cruises). Another condition, always observed in our study area, is a DIN:DIP < the Redfield ratio (DIN: dissolved inorganic nitrogen) which favours di-nitrogen fixing organisms (Karl et al. 2002).

**DISCUSSION**

According to Mulholland et al. (2002), ‘There are few studies that have investigated the kinetics of non-nitrogenous nutrient uptake by *Trichodesmium*. In the only kinetic study published regarding P uptake, it was demonstrated that *Trichodesmium* has a low affinity for DIP (McCarthy & Carpenter, 1979)’. However, the lowest concentration used in their inorganic P uptake experiment was 100 nM, at least 2× higher than the ambient DIP that they measured in the subtropical N Atlantic Ocean (between Spain and Bermuda). They went on to use a linear extrapolation from their plot (see Fig. 4, p. 79 of McCarthy & Carpenter 1979) for the uptake rate at 50 nM, and approximated a maximum uptake rate of 1.2 pmol colony$^{-1}$ h$^{-1}$ (see line 22, p. 80). Due to an error in units, this value was underestimated by a factor of 1000. The maximum uptake rate was in fact 1.2 nmol colony$^{-1}$ h$^{-1}$ which gives a very different interpretation of their results. Using the C colony$^{-1}$ data and an estimated C:P ratio of 100, they calculated that *Trichodesmium* spp. at 1.2 pmol colony$^{-1}$ h$^{-1}$ required 5000 h to double the constituent P (see line 28, p. 80). Using the same calculation, and the ‘corrected’ uptake rate of 1.2 nmol colony$^{-1}$ h$^{-1}$, this time is reduced to 5 h, which gives a specific uptake rate of 0.14 h$^{-1}$; a value closer to the one we observed (Table 2).

Letelier & Karl (1998) used sinking colonies of *Trichodesmium* spp. at the ALOHA station in the North Pacific subtropical gyre and found an active uptake of DIP. Using a time course experiment with an initial DIP concentration of 180 nM, a concentration not encountered in the upper surface water, it was found that sinking *Trichodesmium* colonies were able to assimilate the equivalent of 35 to 57% of their P content from inorganic P over the first 12 h of a dark incubation. This is in accordance with the 5.0 to 8.3 h, calculated using our results, required to obtain the same P content at this concentration.

Recently, Sañudo-Wilhelmy et al. (2004) and Fu et al. (2005) proposed new $K_s$ values between 0.20 and 0.68 µM for laboratory cultures of *Trichodesmium* IMS101 and GBRTRL101, in agreement with our field results ($K_s = 0.63$ µM). New $K_s$ estimations are largely below the $K_s$ value of 9 µM previously reported by McCarthy & Carpenter (1979).

Our study indicates that inorganic phosphate uptake by single filaments of *Trichodesmium* spp. in the upper surface water may sustain *Trichodesmium* spp. growth when DIP > 9 nM. Thus, growth may not require uptake of dissolved organic phosphate nor entail vertical migrations. Letelier & Karl (1998) hypothesized that the vertical migration of *Trichodesmium* spp. could contribute to their P-requirement. They admitted, though, that their hypothesis did not explain the large concentration of non-migratory single trichomes observed in the upper water column, unless the colony versus free trichomes morphology was a transient condition under cellular control.

Direct cross-membrane transport is known for a few DOP compounds; however, this pathway is probably minor compared with ectoenzyme hydrolysis of DOP prior to DIP uptake (Björkman & Karl 2003). Assuming that DOP compounds are hydrolyzed outside the cells (Thingstad et al. 1996), phosphate required to create new biomass is then taken up in the form of orthophosphate only. According to this classical hypothesis, organisms compete for DIP in order to obtain their P-requirements. In this case, DIP uptake measurements correspond to the utilization of the DIP + DOP pool and remain the key process when studying P control on *Trichodesmium* spp. growth and biomass. The small differences observed during our seasonal observations in the DOP pool (Van Den Broeck et al. 2004) could indicate that it was mainly composed of refractory materials. This is not in contradiction with the rapid turnover times of a small fraction of the DOP pool. The data of McCarthy & Carpenter (1979) show that *Trichodesmium* spp. has a low affinity for DIP but a high potential for utilizing phosphomonoesters (DOP). As a result, recent studies have only focused on the utilization of DOP compounds when attempting to explain *Trichodesmium* spp. abundance (Stihl et al. 2001, Mulholland et al. 2002). Mulholland et al. (2002) argued that DOP can represent an important P source for *Trichodesmium* spp. growth; however, DIP uptake was not measured. This conclusion may need to be re-examined considering the results presented here.
The relationship between P-limited *Trichodesmium* spp. growth and the cellular-P quota is currently unknown (Kustka et al. 2003). *Trichodesmium* spp. growth is obviously related to the P internal quota rather than to the direct uptake of phosphate. This implies that the critical DIP availability could correspond to the end of the P-storage ability that may precede the decrease in *Trichodesmium* spp. growth rate.

Studies of nutrient dynamics in marine systems have begun to focus on turnover times (Benitez Nelson 2000). DIP concentrations, recently estimated by indirect methods in oligotrophic systems (Moutin et al. 2002), are often below the detection limit of the classical blue molybdenum method currently in use during oceanic cruises (30 nM) even when using the MAGIC procedure for pre-concentrating (5 nM) (Benitez Nelson 2000). DIP turnover time appears to be the most useful variable for characterizing DIP availability. The annual seasonal variations observed for DIP turnover times are plotted against seawater temperature (Fig. 2), indicating favourable and unfavourable growth conditions for *Trichodesmium* spp.

Large \( T_{DIP} \) variations were observed when DIP concentrations were undetectable. \( T_{DIP} \) reached 4 h during summer, which is characteristic of extremely P-depleted areas, such as the Mediterranean Sea (Moutin et al. 2002). Summer \( T_{DIP} \) values were lower than the \( T_{DIP} \) value of 48 h measured in the summer of 1997 at the climax station (Björkman et al. 2000). The latter station is close to the ALOHA station in the North Pacific Ocean where a shift from nitrogen to phosphate limitation was observed (Letelier & Karl 1998). A very simple scheme which considers both the input of new phosphate from deep layers during winter mixing (homogeneous temperature around 23 to 24°C from the surface to the upper phosphacline), and the decrease in DIP availability corresponding to the beginning of summer stratification (temperature >25°C), could explain why most *Trichodesmium* spp. surface accumulations (observed between November 1998 and April 2003 in the SW subtropical Pacific Ocean, 18–25° S latitude; 160–174° E longitude) occur from November to January (Fig. 3). This observation zone is the Caledonian Economical Zone (CEZ) which was under surveillance throughout the year and entirely supervised twice a month by the French Navy (no geographical parcel was better supervised than another). Winter DIP enrichment requires a previous cooling of the surface waters, which may explain the low occurrence of *Trichodesmium* spp. accumulations during fall. Indeed, temperatures above 26°C are necessary for *Trichodesmium* spp. bloom development (Carpenter et al. 2004).

The monthly mean sea surface temperature was plotted for the central Chenal des Loyauté station (21° 30’S latitude; 167° E longitude) together with the DIP turnover times \( (T_{DIP}) \) calculated from the relationship between \( T_{DIP} \) and temperature \( (\theta) \) measured in the mixed layer during Diapalis Cruises 1 to 6 (Fig. 2: \( T_{DIP} = 1.39 \times 10^{11} \times e^{-0.86 \theta}, r^2 = 0.76, n = 45 \)). Mean \( T_{DIP} \) versus mean temperatures also displayed a good agreement between calculated and measured values. Due to the ability of *Trichodesmium* spp. to store phosphate, phosphate deficiency in the upper water column and therefore *Trichodesmium* spp. decay may be delayed. This could explain why most of the observed *Trichodesmium* spp. accumulation occurred over a period of 3 mo. It might also be due to geographical variations in decreasing phosphate availability. Indeed, the CEZ spread out over 800 km from south to north. In late summer and fall when DIP from the mixed layer was almost exhausted, the occurrence of *Trichodesmium* spp. accumulations was reduced. This demonstrates their poor competitiveness at very low DIP concentrations. The significance of accumulating *Trichodesmium* spp. at the sea surface is not well understood. It could be related to *Trichodesmium* spp. blooms or may be only the result of physical currents, which concentrate somewhere at the surface, the biomass being produced elsewhere in the water column. In any case, it does correspond with the end of biomass production.
and is observed when the available DIP decreases at the sea surface. This 'bottom up' control hypothesis for the termination of the *Trichodesmium* 'blooms' differs from the 'top down' controls of grazers (O’Neil 1998) or by bacteriophage infections (Ohki 1999) already mentioned. Others have suggested that phosphate starvation is one of the key factors controlling *Trichodesmium* spp. mortality, based on laboratory experiments (Berman-Frank et al. 2004) but no direct links between P-availability and early summer sea surface *Trichodesmium* spp. accumulations have been previously mentioned. Others have suggested that phosphate starvation is one of the key factors controlling *Trichodesmium* spp. mortality, based on laboratory experiments (Berman-Frank et al. 2004) but no direct links between P-availability and early summer sea surface *Trichodesmium* spp. accumulations have been previously shown. The observations of some *Trichodesmium* spp. accumulations, at other time periods, require different explanations. Could these observations be related to mixing events that occasionally provide phosphate from deep nutrient rich waters?

The sharp decrease of DIP availability in the early summer season could explain most of the numerous and periodic sea-surface accumulations of *Trichodesmium* spp. observed, without taking into account the role of iron, the other major factor controlling nitrogen fixation in the ocean (Falkowski 1997). This phosphate control may be related to a high iron availability in this area (Van den Broeck et al. 2004), as it has been suggested for the North Atlantic Ocean (Wu et al. 2001). It runs against current ideas that the Pacific Ocean is more severely iron limited (Wu et al. 2001) with the S Pacific having the greatest degree of Fe limitation of diazotrophy (Behrenfeld & Kohler 1999). Our results suggest a re-evaluation of DIP availability in oligotrophic areas where *Trichodesmium* spp. are common, in order to study geographical trends of nutrient limitation on N2 fixation, particularly from the west to the east South Pacific Ocean. Our results are also important in understanding how the N/P balance is controlled in the ocean. If the N-level adjusts to the P-level via nitrogen-fixation and denitrification, a model with nitrogen fixers that will be controlled by iron (Falkowski 1997) or phosphate (Tyrell 1999) would predict different ultimate controls on primary production, i.e. different responses of the ocean to global warming.

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**LITERATURE CITED**


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