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Dissolved Organic Phosphorus Uptake by Marine Phytoplankton is enhanced by the presence of Dissolved Organic Nitrogen

Mark F. Fitzsimons[1*], Ian Probert[2], Fanny Gaillard[2], Andrew P. Rees[3]

[1]Biogeochemistry Research Centre, Marine Institute, Plymouth University, Plymouth PL4 8AA, UK

[2]Station Biologique de Roscoff, Place Georges Tessier, Roscoff 29680, France

[3]Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH, UK

[*]Corresponding author tel: +44 (0)1752 584555; fax: +44 (0)1752 584710; email:

mfitzsimons@plymouth.ac.uk

apre@pml.ac.uk

probert@sb-roscoff.fr

fanny.gaillard@sb-roscoff.fr

Abstract

Organic nutrients can constitute the major fractions (up to 70 %) of aquatic nitrogen (N) and phosphorus (P), but their cycling is poorly understood relative to the inorganic pools. Some phytoplankton species access P from the dissolved organic phosphorus (DOP) pool through expression of alkaline phosphatase (AP), which hydrolyses orthophosphate from organic molecules, and is thought to occur either at low concentrations of dissolved inorganic P (DIP), or elevated ratios of dissolved inorganic N (DIN) to DIP. Three algal strains native to the North-East Atlantic Ocean (coccolithophore, dinoflagellate and diatom species) were grown under representative, temperate conditions, and the dissolved N and P components amended to include dissolved organic N (DON) and DOP. The activity of AP was measured to determine the rate of DOP uptake by each algal species. The addition of DON and DOP enhanced the growth of the algal species, regardless of DIN and DIP concentrations. In cultures where the total concentrations and absolute N : P ratio was unchanged but the N pool included both DON and DIN, an increase in alkaline phosphatase activity (APA) was measured. This suggested that the presence of DON triggered the selective uptake of DOP. The uptake of organic P was confirmed by detection of adenosine in DOP-amended culture media, indicating that P had been cleaved from ADP and ATP added to the media as DOP, and cellular P concentration in these cultures exceeded the calculated concentration based on uptake of DIP only. Our data demonstrates that organic nutrients can enhance and sustain marine algal productivity. The findings have implications for marine ecosystem function and health, since climate change scenarios predict variable riverine inputs to coastal areas, altered N : P ratios, and changes in the inorganic to organic balance of the nutrient pools.

Key-words: alkaline phosphatase; dissolved organic nitrogen; dissolved organic phosphorus; coastal waters; marine algae; P-limitation;

Introduction

As limiting nutrients for algal growth, phosphorus (P) and nitrogen (N) play an essential role in the biological productivity of aquatic ecosystems (Redfield 1958; Hecky & Kilham 1988). Most nutrient cycling studies have focussed on dissolved inorganic N and P (DIN and DIP, respectively). However, recent studies show that the dissolved organic pools (DON and DOP, respectively) also merit consideration. For example, DON frequently comprises the largest part (60–69 %) of total dissolved N in rivers, estuaries and surface ocean waters (Bronk 2002), while DOP was shown to account for at least 40 % of the total dissolved phosphorus pool in an estuary (McKelvie 2005; Monbet *et al.* 2009), and 70-90 % in oligotrophic waters (Ruttenberg & Dyhrman 2012). Although bacteria are primarily responsible for the processing of DON in aquatic environments (Berman & Bronk 2003), a variety of phytoplankton species have been shown to utilise DON to meet their N needs (Antia *et al.* 1991; Moschonas *et al.* 2017). Studies of DON uptake by phytoplankton (Gobler & Boneillo 2003; Mulholland & Lee 2009) demonstrated that both external hydrolysis and direct assimilation occurred, depending on molecular size, with highest rates measured in the size fraction containing the dominant phytoplankter.

In marine waters, the supply of P to phytoplankton to meet their cellular demands is thought to be mainly in the form of orthophosphate (Cembella *et al.* 1984; Nicholson *et al.* 2006; Mahaffey *et al.* 2014) rather than DOP. During times of DIP depletion, relative to other nutrients, microbial activity and phytoplankton growth are often considered to be P-limited (Karl *et al.* 1995; Shaked *et al.* 2006), even though the concentration of marine DOP can be 5–10 times higher than DIP (Mather *et al.* 2008). However, when DIP is depleted, a number of marine organisms, including dinoflagellates (Dyhrman & Palenik 1999; Lin *et al.* 2012), coccolithophores (Dyhrman & Palenik 2003), diatoms (Dyhrman & Ruttenberg 2006) and bacteria (Huang & Hong 1999) are known to synthesize hydrolytic enzymes in order to access the DOP pool to derive their P requirement (Monaghan & Ruttenberg 1999; Ruttenberg & Dyhrman 2005) via expression of the alkaline phosphatase (AP) enzyme (Perry 1972; Cembella *et al.* 1984), which hydrolyzes orthophosphate from the DOP compound.

The use of AP by phytoplankton is believed to occur at either low concentrations of DIP, or elevated ratios of dissolved inorganic N to DIP (DIN : DIP). Studies from a range of marine environments indicate variable inorganic phosphate concentration thresholds, below which alkaline phosphatase activity (APA) is induced; specifically, below 10 nM in the Sargasso Sea (Lomas *et al.* 2010), ~20 nM in the subtropical Pacific (Suzumura *et al.* 2012) and ~100 nM in the northwest African upwelling region (Sebastian *et al.* 2004). As such, APA has been used to determine phytoplankton community P status (Sebastián *et al.*, 2004; Nicholson *et al.* 2006; Suzumura *et al.* 2012; Mahaffey *et al.* 2014). However, several studies have shown that P from DOP can be taken up by bacteria or phytoplankton, even in the presence of DIP, via enzymatic hydrolysis, depending on their competing strengths, substrate concentrations, saturation, storage capacity and the availability of other nutrients such as organic carbon (Cotner *et al.* 1997; Labry *et al.* 2005; Luo *et al.* 2011). The combination of inducible and constitutive behaviour of AP means that its relationship with phosphate may be complex when considered across a spectrum of marine environments.

Climate change scenarios predict both episodic conditions of elevated rainfall and extended periods of dry conditions (Stocker *et al.* 2013), leading to variable riverine inputs to coastal areas, altered N : P ratios, and changes in the inorganic to organic balance of the nutrient pools. Organic nutrients can constitute up to 69 and 90 % of the N and P pools, respectively (Bronk 2002; McKelvie 2005; Monbet *et al.* 2009), but their cycling is still poorly understood relative to the well-characterised inorganic fractions. It is crucial, therefore, to understand the cycling of organic nutrients in coastal waters and how changes in the composition of the N and P pools could impact on marine ecosystem function and health.

This study was undertaken to: 1) investigate algal growth rates using culture media containing mixtures of N and P components; 2) examine the effect of culture media macronutrient compositions on alkaline phosphatase activity; 3) Monitor uptake of P by algal species. The experimental conditions were designed to facilitate a comparison of the growth of algal species in media containing both inorganic and organic forms of N and P so that uptake was not governed by the lack of alternative forms of each macronutrient.

Materials and methods

CLEANING PROCEDURE

Glass- and plasticware were first degreased (2% Nutracon solution, 24 h), then acid-washed (10 % HCl, 24 h) and thoroughly rinsed with high purity water (HPW; Millipore, 18.2 MΩ cm). Cleaned items were stored in resealable plastic bags. Glass fibre filters (GF/F) were cleaned by combustion in a muffle furnace (450 ^{o}C, 6 h). Clean techniques were used throughout the study and critical handling steps were performed in a laminar flow cabinet.

ALGAL CULTURING

Three species of algae isolated from the English Channel were obtained from the Roscoff Culture Collection; a coccolithophore, *Emiliania huxleyi* (BIO 8), a dinoflagellate, *Prorocentrum minimum* (RCC 2563), and a diatom, *Chaetoceros sp.* (RCC 2565). Stock cultures of *Emiliania huxleyi* and *Prorocentrum minimum* were maintained in k/2 medium, whilst *Chaetoceros sp.*, a diatom requiring Si, was grown in k/2 media with added Si; full details of the culture media are provided as supporting information. The N and P component of the media was adjusted to include media containing DON and DOP. The DON component comprised protein-forming amino acids and urea, while DOP was prepared using adenosine di- and tri-phosphate (ADP and ATP, respectively). ATP is a labile form of DOP, in the low molecular weight fraction (< 10 kDa); algal uptake of ATP has been studied in marine systems and it was reported to be the preferred DOP source, after DIP, in recent comparison studies (Diaz et al., 2018, Nausch et al., 2018). Details of the N and P components of the culture media are shown in Table 1. All cultures were maintained at 15 ^{o}C under a 16 : 8 h light : dark cycle. For the first inoculation, 100 mL of culture medium was transferred to a 125 mL sterile culture flask and 5 mL of stock culture added. Cell growth was subsequently monitored visually and under a light microscope, and sub-culturing was performed every 3-7 days prior to inoculation of larger volumes of culture media. Culture volumes of 2 L were maintained over 21 days; these cultures were maintained through addition of fresh medium (25 % addition by volume) weekly over this period. Larger volume (6 L) cultures of *E. huxleyi* were prepared from sub-cultures of established 2 L volume cultures, to provide adequate volume for the extraction and detection of organic molecules via solid phase extraction and analyses of dissolved nitrate and phosphate. The 6 L cultures were not refreshed over the experimental period.

The cultures were sampled for cell counts during the culture period and when harvested; aliquots of sample were collected in a clean-air laminar-flow cabinet. Cells were enumerated using a BD Accuri™ C6 Cytometer on *in vivo* samples, with a typical analysis time of 1 minute at a flow rate of 35 µL min^{-1}, and a threshold of 10000 events on the chl *a* fluorescence side scatter. For 6 L *E. huxleyi* cultures, cell counts for k/2 DON and k/2 DON+DOP were compared with those for k/2 when harvested.

DISSOLVED INORGANIC NUTRIENTS

Dissolved NO_3^- and PO_4^{3-} concentrations were measured in water samples (100 mL), which had been gravity-filtered through combusted GF/F filters (0.7 µm nominal pore-size) into pre-cleaned polycarbonate bottles then stored frozen at -20 °C before analysis. Analyses were performed colorimetrically on an auto-analyser (AXFLOW SEAL AA3 AAHR) within one month of sampling according to the national protocol within the SOMLIT (Service d’Observation en Milieu LITtoral) based on Aminot and Kérouel (2004). Filters were immediately frozen (-20 °C) for subsequent analysis of total particulate P content.

ALKALINE PHOSPHATASE ACTIVITY

Kinetic assays of APA were performed using a sensitive fluorometric protocol similar to Perry (1972). The APA in culture samples was assayed as a change in fluorescence through enzymatic hydrolysis of the artificial P substrate, 4-methylumbelliferyl phosphate (MUF-P) releasing the fluorescent product methylumbelliferone (MUF). A 4 mL aliquot of unfiltered culture water was distributed into triplicate series (blank control plus 5 concentrations between 12.5 and 200 nM MUF-P in 10 nM Tris buffer) in 12 mL glass tubes. These samples were incubated at 15 ºC in the dark for 1 hour and their fluorescence determined (Turner Designs Laboratory Trilogy Fluorometer) at excitation and emission wavelengths of 365 and 455 nm, respectively. The procedure was calibrated on each occasion against six MUF standards (concentration range 5-40 nM), measured in triplicate, which was sufficient to account for all samples. Kinetic data were estimated using the Lineweaver-Burk transformation of the Michaelis-Menten equation.

TOTAL PHOSPHATE

Frozen GF/F filters were thawed, wrapped in a double-layer of aluminium foil then combusted at 450 °C for 4.5 h and allowed to cool to room temperature (20–25 °C). Each filter was then divided (pieces were no larger than 10 x 10 mm) using acid-washed surgical scissors and placed into a pre-cleaned 20 mL glass vial. After addition of HCl (5 mL, 0.5 M), vials were placed in a sonic-bath for 60 minutes then centrifuged at 3000 rpm for 30 minutes. The supernatants were analysed for P by inductively coupled plasma optical emission spectrometry at a wavelength of 177.495 nm. A certified reference material (CRM; NIST Apple leaf) was used as the analytical control to measure recovery of P from the sample filters. Samples containing CRM were prepared according to the Hawaii Ocean Time Series protocol (http://hahana.soest.hawaii.edu/hot/protocols/chap11.html), with some adaptations. The CRM was initially freeze-dried (48 h), then weighed into glass vials to give a range of P concentrations in 10 mL HPW (0, 5, 10, 15 and 20 µM). The added CRM was suspended in solution using a vortex mixer and aliquots pipetted on to an acid-washed and combusted GF/F filter paper. Filter papers were then oven-dried at 40 °C and prepared for analysis as described for the sample filters.

EXTRACTION AND DETECTION OF ORGANIC MOLECULES

SOLID PHASE EXTRACTION

Water was sampled from cultures (1 L samples) at harvesting after gentle mixing, and gravity-filtered through GF/F filters (0.7 µm) then amended with formic acid (FA) to a final concentration of 0.1 % FA v/v. Solid phase extraction (SPE) was then performed using Strata-X 33µ Polymeric Reversed Phase 500 mg/12 mL Giga Tubes, using a method adapted from Curtis-Jackson *et al.* (2009). The tubes were conditioned with 12 mL of a methanol (MeOH) and water mixture (50 : 50 v/v), followed by equilibration with a 12 mL mixture of MeOH and water at 1 : 99 v/v. Once the FA-amended sample had been passed through the cartridge, a single wash step was performed with a further 3 mL of the MeOH and water mixture (1 : 99 v/v). The cartridge was eluted with 3 volumes (2 mL, 2 mL, 1 mL) of a MeOH : FA mixture (99 : 1 v/v) into a glass vial. Eluted samples were further pre-concentrated by removal of the elution solvent under a gentle flow of N_2 gas then reconstituted in 100 µL HPLC-grade water for analysis by liquid chromatography tandem mass spectrometry LC-MS/MS.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

The aqueous samples were analysed by LC-MS/MS, on an Ultimate3000^{TM} system (Dionex, Odense, Denmark) connected to the LTQ Orbitrap Discovery instrument (Thermo Fisher Scientific, Bremen, Germany), operating in collision induced dissociation (CID) or higher energy collisional (HCD) mode. Standard mass spectrometric conditions for all experiments were: spray voltage, 4.5 kV; capillary voltage 47, sheath gas flow 20 ; heated capillary temperature 200 °C; predictive automatic gain control (AGC) enabled. Structures were manually deduced from the resulting fragment ion spectra and compared with the spectral library for the instrument.

A 5 μL aliquot of sample was separated on a 100mm analytical column (2.1mm inner diameter) packed with 3.6 μm C_{18} beads (Aeris Peptide, Phenomenex). A gradient comprising 0.1% acetic acid in water (A) and acetonitrile (B) was applied over a total run time of 30 minutes. The following proportions of solvent B were used for elution: 0–15 min, 0–50 % ; 15–18 min, 50-100 % ; 18–23 min, 100 % ; and 23–30 min, 0 %, with a flow rate of 0.25 mL min^{-1}. The analytes were detected at two wavelengths (206 and 280 nm).

Effluent from the analytical column was directly electrosprayed into the mass spectrometer. The linear trap quadrupole (LTQ) Orbitrap instrument was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.5.5 and Xcalibur 2.1. For the low-resolution collision induced dissociation method (CID-MS/MS top5), full scan MS spectra (from *m/z* 50 to 2000) were acquired in the Orbitrap analyzer after accumulation to a target ion count value of 5.10 E^5. The 5 most intense ions with charge states ≥ 2 were sequentially isolated to a target value of 30,000 and fragmented in the linear ion trap by CID with normalized collision energy of 15 %, and wideband-activation enabled. The ion selection threshold was 1.10 E^5 for MS/MS, and the maximum allowed ion accumulation times were 500 ms for full scans in the orbitrap, and

200 ms for CID-MS/MS measurements in the LTQ. An activation of $q = 0.25$ and activation time of 30 ms were used.

For the high-resolution HCD-MS/MS top3 method, full scan MS spectra (from *m/z* 50 to 1000) were acquired in the orbitrap with resolution $r = 30,000$. The three most intense ions with charge states ≥ 2 were sequentially isolated to a target value of 3 E^6 and fragmented in the HCD collision cell with normalized collision energy of 35 %. The resulting fragments were detected in the orbitrap with resolution $r = 7500$. The ion selection threshold was 1.10 E^5 for HCD, and the maximum allowed ion accumulation times were 500 ms for full scans and 200 ms for HCD.

Results

ALGAL CULTURING

Culture media were prepared to ensure that concentrations of DIN and DIP were adequate to support algal species relying on these forms of N and P throughout the experimental period. Concentrations of dissolved nitrate and phosphate remained replete throughout the experimental period and were never exhausted at the time of harvesting (Figure 1). Cell counts were made for each of the three cultures (Figure 2) reflecting the successful growth of each species though some differences were apparent. In the *E. huxleyi* cultures, cell numbers were highest in the k/2 medium at the time of final harvest, while those for the k/2 DON + DOP medium were highest at final harvest for *P. minimum*, although the maximum cell counts during the experimental period were measured in the k/2 medium on day 20. A change in cell density was measured for *Chaetoceros sp*, but the strain did not appear to flourish in any of the culture media, with low cell numbers generally measured throughout the culturing period.

Interestingly, at the time of harvesting the cell count of *E. huxleyi* in 6 L cultures was higher in the k/2 DON+DOP medium compared with k/2 (Day 7), while the k/2 medium cell count was higher than for the k/2 DON medium samples when the latter was harvested on Day 11.

ALKALINE PHOSPHATASE ACTIVITY

Rates of APA were measured in all cultures. With respect to those species grown in the k/2 media which had V_{max} rates for APA of 0.03, 1.78 and 4.66 fmolP cell^{-1}h^{-1} for *E. huxleyi*, *P. minimum and Chaetoceros sp.*, respectively, there was an apparent increase in APA for each species in cultures containing DON, with V_{max} rates of APA of 0.07, 39.7 and 80.5 fmolP cell^{-1}h^{-1} for *E. huxleyi*, *P. minimum and Chaetoceros sp.* respectively. (Table 2). The response in cultures containing DON+DOP varied for each group; APA for *E. huxleyi* reduced by ~ 67 % whilst *P. minimum and Chaetoceros sp.* both increased from 1.78 to 10.8 and from 4.66 to 106 fmolP cell^{-1}h^{-1}, respectively. Whilst these cultures were not grown axenically, they were all treated using identical clean handling procedures and, whilst a contribution to APA from bacteria is possible, the variable response between the different algal species and treatments gives confidence that these observations are dominated by the differential response to treatments by the algal cells.

PARTICULATE PHOSPHORUS

Particulate P was measured in cultures and used to calculate the concentration of P per algal cell in each medium according to Equation 1:

$$\text{P cell}^{-1} = (\text{P}_f /100)/\text{C} \qquad (1)$$

Where P_f is the molar P concentration measured on the filter after filtration of 100 mL of medium and C is the cell count per mL. The theoretical concentration based on uptake of PO_4^{3-} only was calculated according to Equation 2:

$$\text{P cell}^{-1}\text{ (theoretical)} = ([\text{P}_0\text{-P}]/1000)/\text{C} \qquad (2)$$

Where P_0 is the initial dissolved PO_4^{3-}concentration in the culture medium and P is the measured dissolved PO_4^{3-} concentration, in moles L^{-1} at the time of harvesting; this allowed comparison of the amount of P in cells that could be accounted for if only DIP had been taken up to cells. A value below equal to, or below, the theoretical amount indicated that cell uptake of P could be accounted for by DIP only, consistent with induced uptake of DOP. The data in Table 3 for *E. huxleyi,* shows that the measured decrease in PO_4^{3-} concentration could account for the particulate cellular P concentrations in the k/2 and k/2 DON samples, while the P cell^{-1}

concentration in the k/2 DON+DOP samples exceeded the theoretical value (27.6 versus 22.2 fmol P cell^{-1}) at the time of harvesting.

MOLECULAR UPTAKE OF DOP

Samples collected from 6 L cultures of *E. huxleyi* in the 3 media were pre-concentrated by SPE before chromatographic separation and detection by mass spectrometry. Mass spectra were examined for evidence of direct utilisation of DOP (ATP and ADP). A peak at *m/z* 268.1028 (Figure 4a) was prominent in the mass spectrum of k/2 DON+DOP samples, with an ion count of 7.24 x 10^{7}. The same peak in the k/2 DON samples was much weaker (ion count of 1.77 x 10^{3}) and absent in k/2 samples (Table 4). The spectral library identified this ion as adenosine and its further fragmentation by MS/MS confirmed the structure. The MS/MS mass spectrum contained a base peak at *m/z* 136.0609, consistent with the *m*/*z* for protonated 1H-Imidazo[4,5-d]pyridazin-4-amine after loss of 1,4-Anhydropentitol (Figure 4b)

Discussion

It is important to acknowledge that this study was not performed axenically and that there was potential for some bacterial cycling of DON and DOP within the culture media. The experimental matrix of algal species and inorganic : organic nutrient ratios was therefore designed to enable comparative interpretation of nutrient use by utilising analytical procedures to independently follow enzymatic activity, dissolved and cellular nutrient content and molecular changes in media composition. In this study, for each phytoplankton species, rates of APA in cultures amended with DON were higher than in cultures grown in unamended k/2 media, even though the absolute N : P ratio was kept constant. Whilst some bacterial remineralisation of DON to DIN was likely, there was no evidence that the original DIN pool was enriched in the media where DON was added, neither was DIN significantly depleted in those cultures not receiving DON (Figure 1). Butler *et al.* (1979) described the seasonal balance of inorganic to organic N and P in waters of the English Channel and the succession between inorganic to organic dominance during the transition from spring to summer, so that phytoplankton were likely to rely on the DON and DOP fractions during the summertime. A summertime survey of these waters by Davies & Smith (1988) confirmed this, where all phytoplankton communities displayed APA. They proposed that DOP could have an important role supporting phytoplankton and bacterial productivity during periods of P-stress. Rees et al. (2009) found that intense periods of summertime rainfall altered the inherent nutrient stoichiometry of coastal waters of the Western English Channel from an N-limited condition to one where microbial communities were P-stressed, invoking AP expression. Interestingly, Butler *et al.* (1979) measured a relatively constant total N : P ratio throughout the year (17-24, with a ratio of about 20 reflecting the overall chemical-biological balance in the system), while the NO_3^- : PO_4^{3-} ratio varied from 3-13 and for DON : DOP the ratio was 25-42. They suggested that the yearly succession of phytoplankton species occurring in these waters may be partly explained by the hypothesis that when NO_3^- is exhausted there may be a change in the phytoplankton population such that species capable of utilizing DON became dominant.

Constitutive uptake of DOP occurs in phosphorus-replete systems (Sebastian *et al.* 2004; Dyhrman & Ruttenberg 2006; Sato *et al.* 2013); however, upregulation of AP through the presence of DON is a new finding and the data from this study suggest that expression of AP may be sensitive to the form of N available, rather than a focussed response to changes in inorganic N : P ratios. A recent study found that some dinoflagellate species maintained AP even when DIP was supplied in excess, further suggesting that APA is not necessarily an absolute indicator of phosphorus stress nor tightly controlled by ambient DIP level. It seems likely that APA activity in these species could indicate selective use of DOP, or a metabolic response to changes in P forms (Martinez Soto *et al*. 2015). A recent study assessed the relative lability of model P compounds representing the major bond classes of marine DOP in diatom cultures of the genus *Thalassiosira*, as well as coastal field sites of the western North Atlantic (Diaz *et al.* 2018). They found that ATP degradation rates were always suppressed under P-replete culture conditions but the effect of P availability on DOP uptake was inconsistent among diatom strains.

When cultures of *E. huxleyi* were harvested for SPE processing, the cell count for k/2 DON+DOP samples was also significantly higher than k/2 samples compared on the same day ($P < 0.001$; Figure 3), though this was not apparent when k/2 DON samples were compared with k/2; this might be indicative of a requirement for organically-derived P to support organic N uptake. Direct uptake of DON by phytoplankton, including diatoms and dinoflagellates, has been reported in estuarine and coastal waters (Jauffrais *et al.* 2016; Moschonas *et al.* 2017; Mulholland *et al.* 2009; Zhang *et al.* 2015). Low DIN concentrations appeared to be a factor in some cases (Mulholland *et al.* 2009) but requirements may vary within the phytoplankton population. For example, in the Scottish fjord of Loch Creran, Moschonas *et al.* (2017)

observed that N sources correlating with the multivariate pattern in phytoplankton community composition and abundance were, in order of statistical importance: urea, dissolved free amino acids (DFAA), total DON, and DIN. The measured drawdown of DON during the spring bloom was calculated to have contributed up to 37 % of the total measured dissolved N drawdown compared to 63 % from NO_3^-, clearly showing the importance of DON for phytoplankton N nutrition. Indeed, in the smaller phytoplankton size fraction (< 10 µm), NO_3^- contributed only 28 % during spring and summer but generally much less, while NH_4^+ (up to 55 %), urea (up to 59 %), and DFAA (up to 38 %) contributions were considerable during spring and summer when regenerated N uptake rates were highest. These studies measured N in isolation and our study indicates that DON uptake could also be linked to the presence of DOP.

Particulate P concentrations in *E. huxleyi* provided evidence for direct uptake of DOP by phytoplankton cells. While the k/2 and k/2 DON samples measured had particulate P concentrations consistent with DIP loss from the media, the P concentrations in the k/2 DON + DOP samples exceeded the amount that could be accounted for by DIP uptake alone (Table 3). Mass spectra confirmed utilisation of DOP by *E. huxleyi*, as adenosine, a fragment of ADP and ATP, which was present in the k/2 DON+DOP culture medium (Figure 4), could only have been produced through hydrolysis of the triphosphate chain on these molecules. Casey *et al.* (2009) used ATP to represent labile DOP in the oligotrophic North Atlantic Ocean, which was taken up directly by phytoplankton. Interestingly, while uptake of DIP increased in that study, in line with its abundance, ambient DOP concentrations had no apparent effect on whole seawater utilization of either DIP or ATP. Interestingly, although ADP and ATP are N-rich molecules (containing 5 nitrogen atoms) the presence of adenosine in the medium suggested that cells took up the P content after external hydrolysis of ADP and ATP. Direct uptake of DON by marine phytoplankton has been reported (Hu *et al.* 2012, Mulholland & Lee 2009), though a molecular mass limit has not been established.

The ability of *E. huxleyi* to adjust to changes in composition of the N and P pool has recently been reported (McKew *et al.* 2015, Rokitta *et al.* 2016). McKew *et al.* (2015) found that acclimation of *E. huxleyi* to nutrient limitation led to marked increases in the abundance of proteins involved in inorganic nutrient transport and both the scavenging and internal remobilization of organic N and P, including AP. However, this was a highly targeted reorganization of the proteome towards scavenging of DON and DOP under N and P limitation, with proteins that were upregulated under these conditions accounting for only 1.7 and 5.7 % of the total spectral counts, respectively. Rokitta *et al.* (2016) observed that *E. huxleyi's* outstanding endurance under nutrient deficiency related to its versatile high-affinity uptake systems and an efficient, NAD-independent malate oxidation that was absent from most other taxa. However, the metabolic adjustments made during senescence involved conserved and ancient pathways, such as proline oxidation or the glycolytic bypass, that prolong survival but give rise to toxic messengers (e.g. reactive oxygen species or methylglyoxal) so that continued senescence promoted various processes that eventually lead to cell death. The data from our study is particularly novel as it shows that a recognised indicator of P-stress, APA, was also upregulated by a change in the DIN : DON ratio, rather than low DIP concentrations or the presence of DOP. We acknowledge that the DON and DOP pools are varied and complex, such that proxies of these fractions may not represent the cycling of both labile and refractory components. For example, the DOP pool ranges from relatively labile compounds like phosphomonoesters to more refractory molecules like phosphonates (Kolowith *et al*., 2001). However, ATP has been used as a proxy for the labile DOP fraction and, as phosphoesters, ADP and ATP contain a functional groups shared by the class of compounds comprising the majority of the DOP pool (Young & Ingall, 2010). Dissolved free amino acids are also labile molecules within the DON pool, but their varied functionality, acidity and solubility has facilitated their application as proxies to study DON cycling in aquatic environments (Hedges *et al*., 1994; Tappin *et al*. 2010). Uptake of DON by phytoplankton in the upper water column is widely recognized (reviewed in Mulholland & Lomas 2008), and marine phytoplankton, including *P. minimum*, can take up dipeptides directly as well as dissolved free amino acids (Mulholland & Lee, 2009).

There was potential for bacterial contribution to this study, though our consideration of all measurements made would indicate that this had a minor impact on our findings. Bacterial remineralisation could have proved problematic if cultures were grown on organic nutrients only. As all experiments were permanently replete in DIN and DIP any bacterial generation of inorganic nutrient did not likely contribute significantly to: 1) the enhanced algal growth observed and 2) elevated cellular P content of algal cells observed following DON and DOP amendments. Additionally the stoichiometric balance of inorganic nutrients was maintained in favour of P, so that elevations of APA observed following addition of DON were not a result of P stress from algal or bacterial communities according to canonical understanding. This study confirms the contention offered by several other authors that organic nutrients are, at times, of significance to the growth and function of several algal groups. Additionally, we argue that the expression of AP or the absence of DIP do not necessarily indicate a phosphorus-stressed community, but that there are occasions when the uptake of DOP is in preference to DIP and may be enhanced by the presence of DON. These data do not allow us to indicate the mechanism by which this happens but provide three lines of evidence of this process occurring: APA, P cleaved from ATP/ADP, and elevated particulate P. Environmental conditions of coastal waters and open ocean regions are projected to change over the next few decades. These changes include increased storminess and hence turbulence, altered freshwater delivery, elevated seawater temperatures which might lead to enhanced stratification restricting nutrient input to surface waters from depth (Rost & Riebesell 2004; Steinacher *et al.* 2010; Doney *et al.* 2012). Altered wind systems may strengthen eastern boundary upwelling, and thus enhance primary productivity (Bakun *et al.* 2010). Such alterations to abundances of macronutrients like N and P are expected to affect phytoplankton community composition, ecosystem functioning and, ultimately, biogeochemical cycles.

While our finding that the *E. huxleyi* cultured in media amended with DON and DOP grew faster during the early stages of culturing than in cultures containing only DIN and DIP, this does not necessarily mean that the difference endured over the lifetime of the culture (e.g. Table 2). However, in a marine environment where P is less replete, the ability to access DOP earlier than competing species might enable *E. huxleyi* to better adapt to DIP limitation. Changes in algal metabolism, such as uptake of DOP, may occur as a result of more nuanced changes in the balance of the macronutrient pool rather than under conditions of N- or P-stress. As phytoplankton form the basis of the marine food web and drive the biogeochemical cycles of elements in the oceans (Field *et al.* 1998), understanding their functioning is a prerequisite for modelling behaviour to simulate their reactions to a changing environment.

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Table 1. Details of the N and P components of the k/2 and f/2 media used in this study. The DON fraction comprised 20 proteinogenic amino acids and urea. Individual amino acids were added to a final concentration of 3.5 µM in the media, equivalent to 101 µmol-N L^{-1}; urea was added to a final concentration of 43 µmol-N L^{-1}. The DOP fraction comprised ADP and ATP at a combined concentration of 9 µmol-P L^{-1}. As ADP and ATP each contain 5 atoms of N, in the form of aromatic and amino N, the DON-fraction in these media was amended to remove the equivalent amount of structurally-similar DON; specifically, histidine, tryptophan and proline were not added to media containing DOP

Medium	**NO_3^- (µM)**	**PO_4^{3-} (µM)**	**DON (µM)**	**DOP (µM)**
k/2	288	18	0	0
k/2 (DON)	144	18	144	0
k/2 (DON+DOP)	144	9	144	9
f/2	288	18	0	0
f/2 (DON)	144	18	144	0
f/2 (DON+DOP)	144	9	144	9

Table 2. Cell counts, DIN : DIP ratio and V_{max} (the maximum activity rate achieved by the system, at saturating substrate concentration) for alkaline phosphatase, measured in cultures of algal species studied. The measurements were performed on sub-samples (n = 3) from cultures and V_{max} was normalised to cell count. The DIN : DIP ratios were based on measured concentrations of NO_3^- and PO_4^{3-}; starting ratios are given in brackets of the same column.

E. huxleyi	**Cell count (mL^{-1})**	**DIN : DIP**	***V**max* ***(nM h^{-1})***	***V*max (fmol $cell^{-1}$ h^{-1})**
k/2	208581	18 (16)	5.5	0.03
k/2 (DON)	244148	13 (8)	17.4	0.07
k/2 (DON+DOP)	297310	14 (16)	4.0	0.01
P. minimum				
k/2	621	24 (16)	1.1	1.78
k/2 (DON)	307	10 (8)	12.2	39.7
k/2 (DON+DOP)	443	8 (16)	4.8	10.8
Chaetoceros sp.				
k/2	536	20 (16)	2.5	4.66
k/2 (DON)	657	11 (8)	52.9	80.5
k/2 (DON+DOP)	943	11 (16)	99.7	106.0

Table 3. Particulate phosphorus per cell in the different culture media used to grow *Emiliania huxleyi* at the time of harvesting. The theoretical concentration per cell is based on uptake of DIP alone.

Culture medium	**fmol P cell^{-1} (theoretical)**	**fmol P cell^{-1} (measured)**
k/2	97.7	93.5
k/2 (DON)	40.0	37.0
k/2 (DON + DOP)	22.2	27.6

Table 4. Mean ion current for peak occurring at retention time window 4.71-5.19 minutes in media sampled from *Emiliania huxleyi* cultures (n = 3). A full MS scan (*m/z* 50.00 – 2000.00) revealed a base peak at m/z 268.1028, corresponding to adenosine.

Culture medium	**Ion current**
k/2	0
k/2 (DON)	1773
k/2 (DON+DOP)	27395000

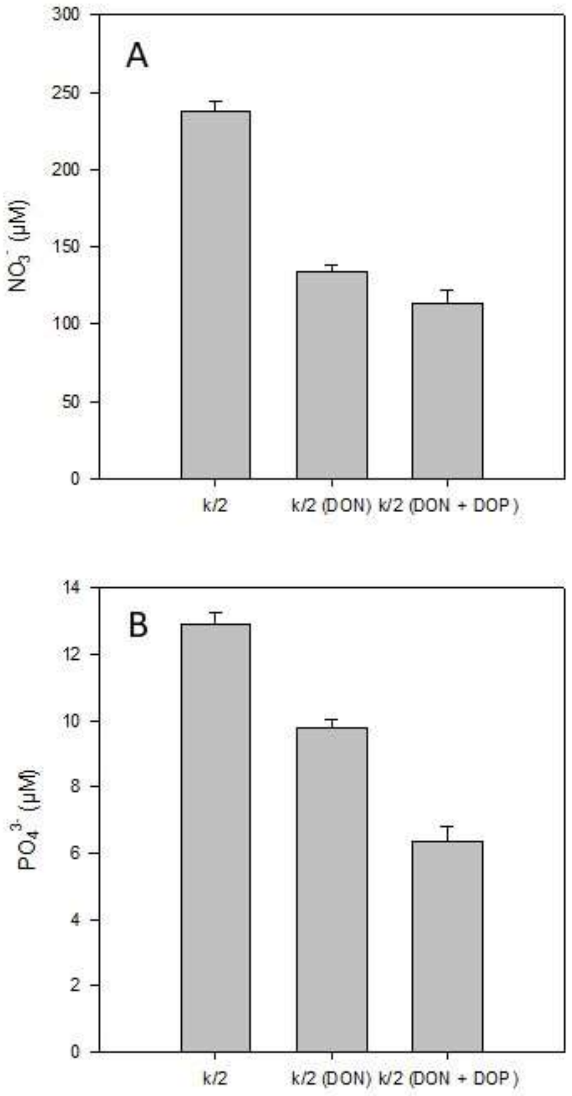

Figure 1. Concentrations of A, dissolved nitrate; B, dissolved phosphate in 6 L cultures of *Emiliania huxleyi* at the time of harvesting.

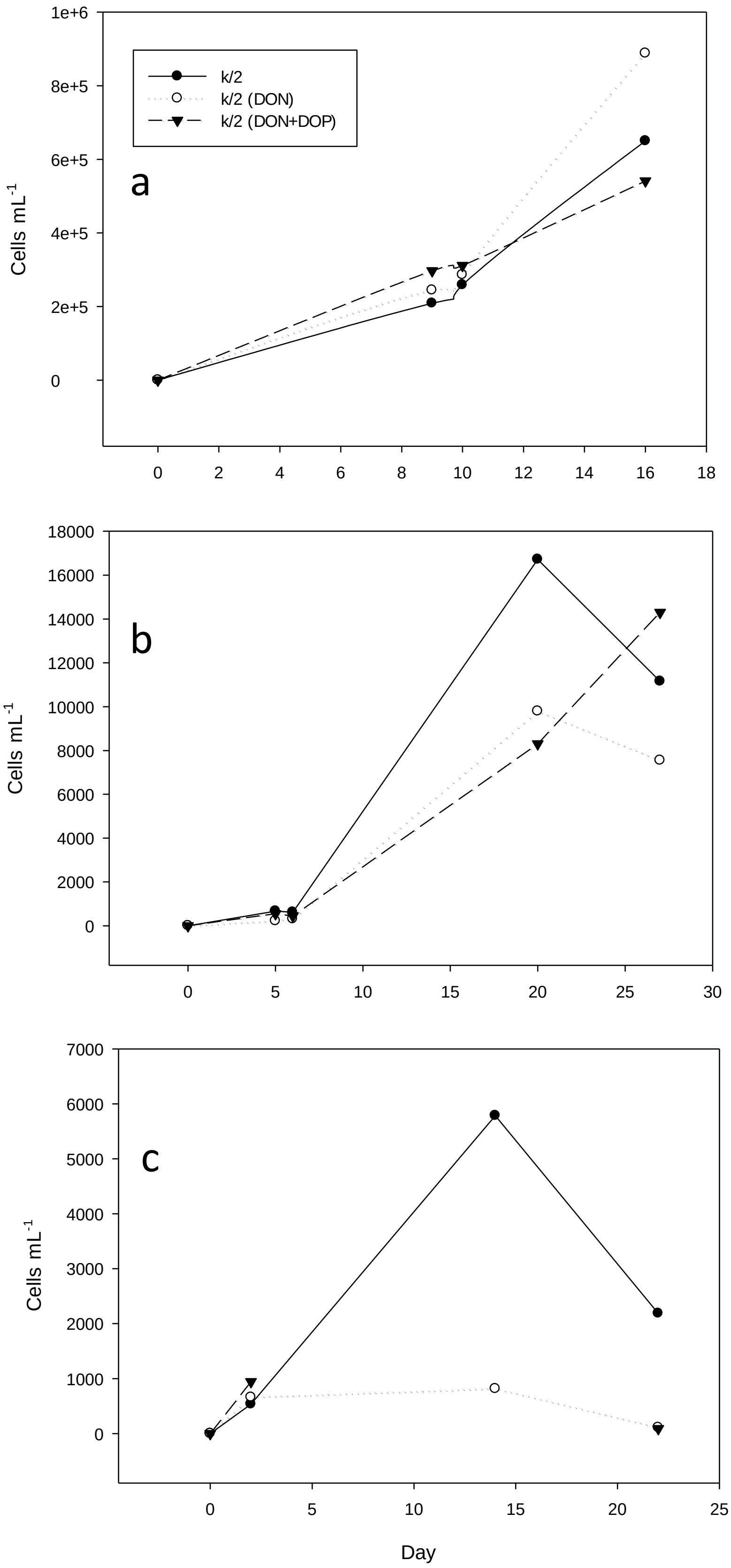

Figure 2. Measured cell counts for *Emiliania huxleyi* (a); *Prorocentrum minimum* (b); *Chaetoceros sp*. (c). The culture volume was 2 L and the culture vessels amended with fresh medium (0.5 L) at intervals during the culturing period.

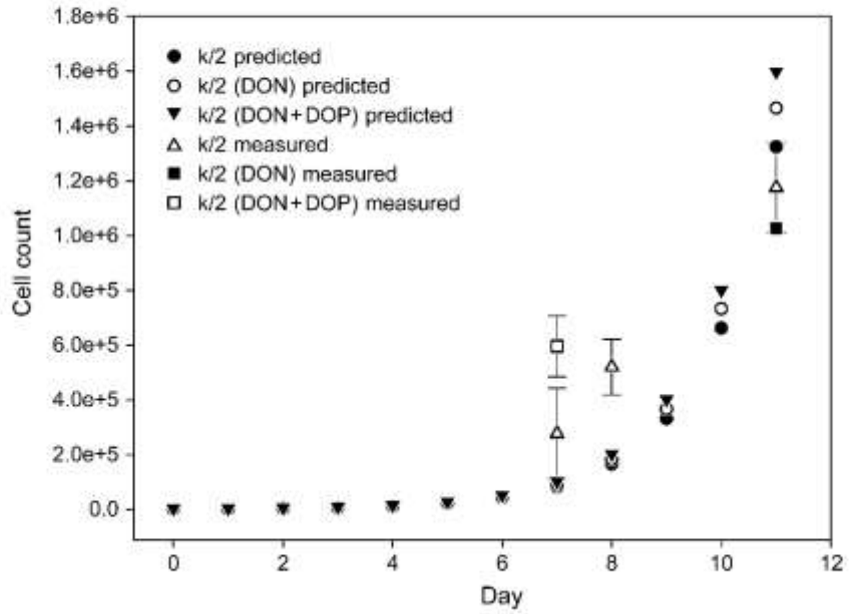

Figure 3. Measured cell counts for *Emiliania huxleyi* cultured in 6 L volumes without replenishment in: k/2 culture medium (Δ), k/2 medium containing DON (■) and k/2 medium containing DON+DOP (□). Predicted growth rates for cultures were based on cell counts at the time of sub-culturing to 6 L and assumed one cell division per day (● = k/2$_{\text{predicted}}$, ○ = k/2 DON$_{\text{predicted}}$, ▼ = k/2 DON+DOP$_{\text{predicted}}$)

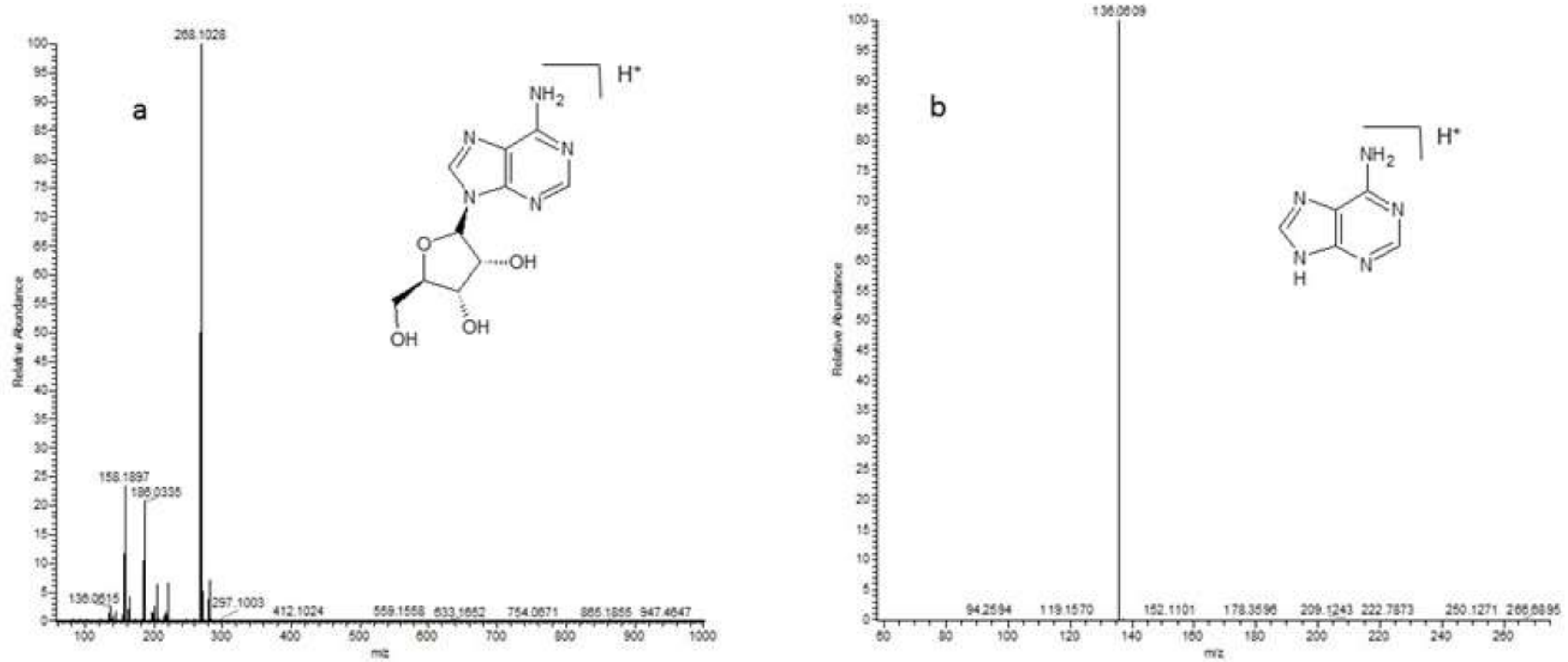

Figure 4. Mass spectra for a) adenosine and b) 1H-Imidazo[4,5-d]pyridazin-4-amine detected in cultures of *Emiliania huxleyi* at the time of harvesting.

Fitzsimons et al. (2019)

Supplementary information ; preparation of culture media

K/2 culture medium with f/2 adaptations for *Chaetoceros sp.*

To 992.5 mL of seawater (optional: Heat seawater to 80°C for 2 hours and leave to cool – this should kill most organisms but should not chemically modify the medium too much) add:

Quantity	**Compound**	**Stock solution** (sterile)	**Final conc. in K medium**
0.25 mL	$NaNO_3$	48.9542 g L^{-1} H_2O	144 µM
1.0 mL	DON (urea + 20 amino acids)	(see recipe on page 2)	144.5 µM
0.25 mL	KH_2PO4	4.8992 g L^{-1} H_2O	9 µM
1.0 mL	DOP (ADP + ATP)	(see recipe on page 3)	9 µM
0.5 mL	FeEDTA solution	(see recipe below)	(see below)
0.5 mL	Trace metal solution	(see recipe below)	(see below)
1.0 mL	f/2 vitamin solution	(see recipe below)	(see below)

* optional

FeEDTA solution

To 950 mL distilled H_2O add:

Quantity	**Compound**	**Stock solution**	**Final conc. in K medium**
4.3 g	(Na)FeEDTA	-	5.85 µM

Make up to 1 L with high purity water (18.2 MΩ cm resistivity) , sterilize (filter 0.22 µm) and store in fridge.

Trace metal solution

To 950 mL distilled H_2O add:

Quantity	**Compound**	**Stock solution**	**Final conc. in K medium**
37.22g	$Na_2EDTA.2H_2O$	-	50 µM
1.0 mL	$CuSO_4.5H_2O$	2.497 g L^{-1} H_2O	0.005 µM
1.0 mL	$Na_2MoO_4.2H_2O$	7.2585 g L^{-1} H_2O	0.015 µM
1.0 mL	$ZnSO_4.7H_2O$	23.0 g L^{-1} H_2O	0.004 µM
1.0 mL	$CoSO4.7H_2O$	14.055 g L^{-1} H_2O	0.025 µM
1.0 mL	$MnCl_2.4H_2O$	178.11 g L^{-1} H_2O	0.45 µM
1.0 mL	H_2SeO_3	1.29 g L^{-1} H_2O	0.005 µM

1.0 mL	NiCl2.6H20	1.49 g L^{-1} H_2O	0.00314 µM

Make up to 1 L with high purity water, sterilize (filter 0.22µm) and store in fridge.

f/2 Vitamin solution

To 950 mL distilled H_2O add:

Quantity	**Compound**	**Stock solution**	**Final conc. in K medium**
1.0 mL	Vit. B_{12} (cyanocobalamin)	0.5 g L^{-1} H_2O	0.37 nM
1.0 mL	Biotin	5.0 mg L^{-1} H_2O	2.0 nM
100.0 mg	Thiamine HCl	-	0.3 µM

Make up to 1 litre with high purity water, filter sterilize into plastic vials and store in freezer.

After addition of supplements, adjust pH of medium to 8.2 (with 0.2 M solution of NaOH)
For K-ET, add 10-30 mL marine soil extract (ET)

Sterilization of medium : Filter sterilize through 0.22 µm filters (e.g. Millipore Steritop units) into sterile (autoclaved) polycarbonate bottles.

DON solution

Prepare the DON solution by adding AAs and urea in the quantities shown below, making up to 500 mL

Amino acid	**g/500 mL (3.5 mM)**	**N (mM)**
GLY	0.131	3.5
ALA	0.156	3.5
VAL	0.205	3.5
LEU	0.230	3.5
ILE	0.230	3.5
SER	0.184	3.5
THR	0.208	3.5
ASP	0.233	3.5
GLU	0.257	3.5
ASN	0.231	7

GLN	0.256	7
LYS	0.256	7
HIS*	0.272	10.5
ARG	0.305	14
PHE	0.289	3.5
TYR	0.317	3.5
TRP*	0.357	7
CYS	0.212	3.5
MET	0.261	3.5
PRO*	0.201	3.5
		80.5
Urea	0.131	43
	Total	123.5

* Not included in DON + DOP recipe due to 20 µM aromatic N added to medium from ADP and ATP.

DOP Solution

Prepare the DOP solution by adding ADP and ATP in the quantities shown below, making up to 100 mL with high purity water (3 mM ADP and 1 mM ATP).

P species	**g/100 mL**
ADP	0.135
ATP	0.055