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Dissolved organic phosphorus uptake by marine phytoplankton is enhanced by the presence of dissolved organic nitrogen

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7	Dissolved Organic Phosphorus Uptake by Marine Phytoplankton is
8	enhanced by the presence of Dissolved Organic Nitrogen
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31 Abstract

Organic nutrients can constitute the major fractions (up to 70 %) of aquatic nitrogen (N) and 32 33 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 34 expression of alkaline phosphatase (AP), which hydrolyses orthophosphate from organic 35 molecules, and is thought to occur either at low concentrations of dissolved inorganic P (DIP), 36 37 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 38 39 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 40 the rate of DOP uptake by each algal species. The addition of DON and DOP enhanced the 41 42 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 43 and DIN, an increase in alkaline phosphatase activity (APA) was measured. This suggested 44 that the presence of DON triggered the selective uptake of DOP. The uptake of organic P was 45 confirmed by detection of adenosine in DOP-amended culture media, indicating that P had 46 been cleaved from ADP and ATP added to the media as DOP, and cellular P concentration in 47 these cultures exceeded the calculated concentration based on uptake of DIP only. Our data 48 demonstrates that organic nutrients can enhance and sustain marine algal productivity. The 49 50 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 51 the inorganic to organic balance of the nutrient pools. 52

53

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

57 Introduction

As limiting nutrients for algal growth, phosphorus (P) and nitrogen (N) play an essential 58 role in the biological productivity of aquatic ecosystems (Redfield 1958; Hecky & Kilham 59 1988). Most nutrient cycling studies have focussed on dissolved inorganic N and P (DIN and 60 DIP, respectively). However, recent studies show that the dissolved organic pools (DON and 61 DOP, respectively) also merit consideration. For example, DON frequently comprises the 62 largest part (60–69 %) of total dissolved N in rivers, estuaries and surface ocean waters (Bronk 63 64 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 65 (Ruttenberg & Dyhrman 2012). Although bacteria are primarily responsible for the processing 66 of DON in aquatic environments (Berman & Bronk 2003), a variety of phytoplankton species 67 have been shown to utilise DON to meet their N needs (Antia et al. 1991; Moschonas et al. 68 69 2017). Studies of DON uptake by phytoplankton (Gobler & Boneillo 2003; Mulholland & Lee 2009) demonstrated that both external hydrolysis and direct assimilation occurred, depending 70 71 on molecular size, with highest rates measured in the size fraction containing the dominant 72 phytoplankter.

In marine waters, the supply of P to phytoplankton to meet their cellular demands is 73 thought to be mainly in the form of orthophosphate (Cembella et al. 1984; Nicholson et al. 74 75 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 76 77 (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 78 marine organisms, including dinoflagellates (Dyhrman & Palenik 1999; Lin et al. 2012), 79 coccolithophores (Dyhrman & Palenik 2003), diatoms (Dyhrman & Ruttenberg 2006) and 80 bacteria (Huang & Hong 1999) are known to synthesize hydrolytic enzymes in order to access 81

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.

85 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 86 marine environments indicate variable inorganic phosphate concentration thresholds, below 87 88 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) 89 90 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; 91 Nicholson et al. 2006; Suzumura et al. 2012; Mahaffey et al. 2014). However, several studies 92 have shown that P from DOP can be taken up by bacteria or phytoplankton, even in the presence 93 of DIP, via enzymatic hydrolysis, depending on their competing strengths, substrate 94 95 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 96 inducible and constitutive behaviour of AP means that its relationship with phosphate may be 97 complex when considered across a spectrum of marine environments. 98

Climate change scenarios predict both episodic conditions of elevated rainfall and 99 extended periods of dry conditions (Stocker et al. 2013), leading to variable riverine inputs to 100 101 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 102 (Bronk 2002; McKelvie 2005; Monbet et al. 2009), but their cycling is still poorly understood 103 104 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 105 106 P pools could impact on marine ecosystem function and health.

107 This study was undertaken to: 1) investigate algal growth rates using culture media 108 containing mixtures of N and P components; 2) examine the effect of culture media 109 macronutrient compositions on alkaline phosphatase activity; 3) Monitor uptake of P by algal 110 species. The experimental conditions were designed to facilitate a comparison of the growth of 111 algal species in media containing both inorganic and organic forms of N and P so that uptake 112 was not governed by the lack of alternative forms of each macronutrient.

114 Materials and methods

115 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 M Ω cm). Cleaned items were stored in resealable plastic bags. Glass fibre filters (GF/F) were cleaned by combustion in a muffle furnace (450 °C, 6 h). Clean techniques were used throughout the study and critical handling steps were performed in a laminar flow cabinet.

121

122 ALGAL CULTURING

Three species of algae isolated from the English Channel were obtained from the Roscoff 123 Culture Collection; a coccolithophore, Emiliania huxleyi (BIO 8), a dinoflagellate, 124 Prorocentrum minimum (RCC 2563), and a diatom, Chaetoceros sp. (RCC 2565). Stock 125 cultures of Emiliania huxleyi and Prorocentrum minimum were maintained in k/2 medium, 126 whilst Chaetoceros sp., a diatom requiring Si, was grown in k/2 media with added Si; full 127 details of the culture media are provided as supporting information. The N and P component 128 of the media was adjusted to include media containing DON and DOP. The DON component 129 130 comprised protein-forming amino acids and urea, while DOP was prepared using adenosine diand tri-phosphate (ADP and ATP, respectively). ATP is a labile form of DOP, in the low 131 molecular weight fraction (< 10 kDa); algal uptake of ATP has been studied in marine systems 132 and it was reported to be the preferred DOP source, after DIP, in recent comparison studies 133 (Diaz et al., 2018, Nausch et al., 2018). Details of the N and P components of the culture media 134 are shown in Table 1. All cultures were maintained at 15 °C under a 16 : 8 h light : dark cycle. 135 For the first inoculation, 100 mL of culture medium was transferred to a 125 mL sterile culture 136 flask and 5 mL of stock culture added. Cell growth was subsequently monitored visually and 137

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 subcultures 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 AccuriTM C6 Cytometer on *in vivo* samples, with a typical analysis time of 1 minute at a flow rate of 35 μ L 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.

151

152 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 precleaned 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.

161 ALKALINE PHOSPHATASE ACTIVITY

162 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 163 enzymatic hydrolysis of the artificial P substrate, 4-methylumbelliferyl phosphate (MUF-P) 164 releasing the fluorescent product methylumbelliferone (MUF). A 4 mL aliquot of unfiltered 165 culture water was distributed into triplicate series (blank control plus 5 concentrations between 166 167 12.5 and 200 nM MUF-P in 10 nM Tris buffer) in 12 mL glass tubes. These samples were incubated at 15 °C in the dark for 1 hour and their fluorescence determined (Turner Designs 168 Laboratory Trilogy Fluorometer) at excitation and emission wavelengths of 365 and 455 nm, 169 170 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 171 samples. Kinetic data were estimated using the Lineweaver-Burk transformation of the 172 173 Michaelis-Menten equation.

174

175 TOTAL PHOSPHATE

Frozen GF/F filters were thawed, wrapped in a double-layer of aluminium foil then combusted 176 at 450 °C for 4.5 h and allowed to cool to room temperature (20–25 °C). Each filter was then 177 divided (pieces were no larger than 10 x 10 mm) using acid-washed surgical scissors and placed 178 into a pre-cleaned 20 mL glass vial. After addition of HCl (5 mL, 0.5 M), vials were placed in 179 a sonic-bath for 60 minutes then centrifuged at 3000 rpm for 30 minutes. The supernatants 180 181 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 182 as the analytical control to measure recovery of P from the sample filters. Samples containing 183 prepared according to the Hawaii Ocean Time Series 184 CRM were 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 μ M). 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.

191

192 EXTRACTION AND DETECTION OF ORGANIC MOLECULES

193 SOLID PHASE EXTRACTION

194 Water was sampled from cultures (1 L samples) at harvesting after gentle mixing, and gravityfiltered through GF/F filters (0.7 µm) then amended with formic acid (FA) to a final 195 concentration of 0.1 % FA v/v. Solid phase extraction (SPE) was then performed using Strata-196 197 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) 198 and water mixture (50: 50 v/v), followed by equilibration with a 12 mL mixture of MeOH and 199 water at 1 : 99 v/v. Once the FA-amended sample had been passed through the cartridge, a 200 single wash step was performed with a further 3 mL of the MeOH and water mixture (1:99 201 v/v). The cartridge was eluted with 3 volumes (2 mL, 2 mL, 1 mL) of a MeOH : FA mixture 202 (99:1 v/v) into a glass vial. Eluted samples were further pre-concentrated by removal of the 203 elution solvent under a gentle flow of N₂ gas then reconstituted in 100 µL HPLC-grade water 204 205 for analysis by liquid chromatography tandem mass spectrometry LC-MS/MS.

206

208 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

The aqueous samples were analysed by LC-MS/MS, on an Ultimate3000TM system 209 (Dionex, Odense, Denmark) connected to the LTQ Orbitrap Discovery instrument (Thermo 210 Fisher Scientific, Bremen, Germany), operating in collision induced dissociation (CID) or 211 higher energy collisional (HCD) mode. Standard mass spectrometric conditions for all 212 experiments were: spray voltage, 4.5 kV; capillary voltage 47, sheath gas flow 20; heated 213 capillary temperature 200 °C; predictive automatic gain control (AGC) enabled. Structures 214 were manually deduced from the resulting fragment ion spectra and compared with the spectral 215 library for the instrument. 216

A 5 μ L aliquot of sample was separated on a 100mm analytical column (2.1mm inner diameter) packed with 3.6 μ m C₁₈ 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 223 spectrometer. The linear trap quadrupole (LTQ) Orbitrap instrument was operated in data-224 dependent mode to automatically switch between full scan MS and MS/MS acquisition. 225 Instrument control was through Tune 2.5.5 and Xcalibur 2.1. For the low-resolution collision 226 227 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 5.10 228 E^5 . The 5 most intense ions with charge states ≥ 2 were sequentially isolated to a target value 229 230 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 231 the maximum allowed ion accumulation times were 500 ms for full scans in the orbitrap, and 232

233 200 ms for CID-MS/MS measurements in the LTQ. An activation of q = 0.25 and activation 234 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 E⁶ 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 E⁵ for HCD, and the maximum allowed ion accumulation times were 500 ms for full scans and 200 ms for HCD.

243 **Results**

244 ALGAL CULTURING

245 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. 246 Concentrations of dissolved nitrate and phosphate remained replete throughout the 247 248 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 249 species though some differences were apparent. In the *E. huxlevi* cultures, cell numbers were 250 highest in the k/2 medium at the time of final harvest, while those for the k/2 DON + DOP 251 medium were highest at final harvest for *P. minimum*, although the maximum cell counts 252 during the experimental period were measured in the k/2 medium on day 20. A change in cell 253 density was measured for *Chaetoceros sp*, but the strain did not appear to flourish in any of the 254 culture media, with low cell numbers generally measured throughout the culturing period. 255

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.

260

261 ALKALINE PHOSPHATASE ACTIVITY

Rates of APA were measured in all cultures. With respect to those species grown in the k/2media which had V_{max} rates for APA of 0.03, 1.78 and 4.66 fmolP cell⁻¹h⁻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⁻¹h⁻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⁻¹h⁻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.

274

275 PARTICULATE PHOSPHORUS

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

278
$$P \operatorname{cell}^{-1} = (P_f / 100)/C$$
 (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:

(2)

282 P cell⁻¹ (theoretical) = $([P_0-P]/1000)/C$

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.

292

293 MOLECULAR UPTAKE OF DOP

294 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 295 examined for evidence of direct utilisation of DOP (ATP and ADP). A peak at m/z 268.1028 296 (Figure 4a) was prominent in the mass spectrum of k/2 DON+DOP samples, with an ion count 297 of 7.24 x 10^7 . The same peak in the k/2 DON samples was much weaker (ion count of 1.77 x 298 10^3) and absent in k/2 samples (Table 4). The spectral library identified this ion as adenosine 299 and its further fragmentation by MS/MS confirmed the structure. The MS/MS mass spectrum 300 301 contained a base peak at m/z 136.0609, consistent with the m/z for protonated 1H-Imidazo[4,5d]pyridazin-4-amine after loss of 1,4-Anhydropentitol (Figure 4b) 302

303

305 Discussion

It is important to acknowledge that this study was not performed axenically and that there was 306 potential for some bacterial cycling of DON and DOP within the culture media. The 307 experimental matrix of algal species and inorganic : organic nutrient ratios was therefore 308 designed to enable comparative interpretation of nutrient use by utilising analytical procedures 309 to independently follow enzymatic activity, dissolved and cellular nutrient content and 310 molecular changes in media composition. In this study, for each phytoplankton species, rates 311 312 of APA in cultures amended with DON were higher than in cultures grown in unamended k/2media, even though the absolute N : P ratio was kept constant. Whilst some bacterial 313 remineralisation of DON to DIN was likely, there was no evidence that the original DIN pool 314 315 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 316 of inorganic to organic N and P in waters of the English Channel and the succession between 317 inorganic to organic dominance during the transition from spring to summer, so that 318 phytoplankton were likely to rely on the DON and DOP fractions during the summertime. A 319 summertime survey of these waters by Davies & Smith (1988) confirmed this, where all 320 phytoplankton communities displayed APA. They proposed that DOP could have an important 321 role supporting phytoplankton and bacterial productivity during periods of P-stress. Rees et al. 322 (2009) found that intense periods of summertime rainfall altered the inherent nutrient 323 stoichiometry of coastal waters of the Western English Channel from an N-limited condition 324 to one where microbial communities were P-stressed, invoking AP expression. Interestingly, 325 Butler et al. (1979) measured a relatively constant total N : P ratio throughout the year (17-24, 326 with a ratio of about 20 reflecting the overall chemical-biological balance in the system), while 327 the NO_3^- : PO_4^{3-} ratio varied from 3-13 and for DON : DOP the ratio was 25-42. They suggested 328 that the yearly succession of phytoplankton species occurring in these waters may be partly 329

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. 332 2004; Dyhrman & Ruttenberg 2006; Sato et al. 2013); however, upregulation of AP through 333 the presence of DON is a new finding and the data from this study suggest that expression of 334 AP may be sensitive to the form of N available, rather than a focussed response to changes in 335 inorganic N : P ratios. A recent study found that some dinoflagellate species maintained AP 336 337 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 338 likely that APA activity in these species could indicate selective use of DOP, or a metabolic 339 340 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 341 cultures of the genus Thalassiosira, as well as coastal field sites of the western North Atlantic 342 (Diaz et al. 2018). They found that ATP degradation rates were always suppressed under P-343 replete culture conditions but the effect of P availability on DOP uptake was inconsistent 344 345 among diatom strains.

When cultures of *E. huxleyi* were harvested for SPE processing, the cell count for k/2 346 DON+DOP samples was also significantly higher than k/2 samples compared on the same day 347 (P < 0.001; Figure 3), though this was not apparent when k/2 DON samples were compared 348 with k/2; this might be indicative of a requirement for organically-derived P to support organic 349 N uptake. Direct uptake of DON by phytoplankton, including diatoms and dinoflagellates, has 350 351 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 352 some cases (Mulholland et al. 2009) but requirements may vary within the phytoplankton 353 population. For example, in the Scottish fjord of Loch Creran, Moschonas et al. (2017) 354

355 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 356 acids (DFAA), total DON, and DIN. The measured drawdown of DON during the spring bloom 357 was calculated to have contributed up to 37 % of the total measured dissolved N drawdown 358 compared to 63 % from NO₃⁻, clearly showing the importance of DON for phytoplankton N 359 nutrition. Indeed, in the smaller phytoplankton size fraction ($< 10 \,\mu$ m), NO₃⁻ contributed only 360 28 % during spring and summer but generally much less, while NH_4^+ (up to 55 %), urea (up 361 to 59 %), and DFAA (up to 38 %) contributions were considerable during spring and summer 362 363 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. 364

Particulate P concentrations in E. huxleyi provided evidence for direct uptake of DOP 365 366 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 367 + DOP samples exceeded the amount that could be accounted for by DIP uptake alone (Table 368 3). Mass spectra confirmed utilisation of DOP by E. huxleyi, as adenosine, a fragment of ADP 369 and ATP, which was present in the k/2 DON+DOP culture medium (Figure 4), could only have 370 been produced through hydrolysis of the triphosphate chain on these molecules. Casey et al. 371 (2009) used ATP to represent labile DOP in the oligotrophic North Atlantic Ocean, which was 372 taken up directly by phytoplankton. Interestingly, while uptake of DIP increased in that study, 373 374 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 375 molecules (containing 5 nitrogen atoms) the presence of adenosine in the medium suggested 376 377 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), 378 though a molecular mass limit has not been established. 379

380 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 381 that acclimation of E. huxleyi to nutrient limitation led to marked increases in the abundance 382 383 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 384 reorganization of the proteome towards scavenging of DON and DOP under N and P 385 limitation, with proteins that were upregulated under these conditions accounting for only 1.7 386 and 5.7 % of the total spectral counts, respectively. Rokitta et al. (2016) observed that E. 387 388 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 389 most other taxa. However, the metabolic adjustments made during senescence involved 390 391 conserved and ancient pathways, such as proline oxidation or the glycolytic bypass, that 392 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 393 394 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 395 than low DIP concentrations or the presence of DOP. We acknowledge that the DON and 396 DOP pools are varied and complex, such that proxies of these fractions may not represent the 397 cycling of both labile and refractory components. For example, the DOP pool ranges from 398 399 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 400 DOP fraction and, as phosphoesters, ADP and ATP contain a functional groups shared by the 401 402 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 403 functionality, acidity and solubility has facilitated their application as proxies to study DON 404

405 cycling in aquatic environments (Hedges *et al.*, 1994; Tappin *et al.* 2010). Uptake of DON by
406 phytoplankton in the upper water column is widely recognized (reviewed in Mulholland &
407 Lomas 2008), and marine phytoplankton, including *P. minimum*, can take up dipeptides
408 directly as well as dissolved free amino acids (Mulholland & Lee, 2009).

There was potential for bacterial contribution to this study, though our consideration of 409 all measurements made would indicate that this had a minor impact on our findings. Bacterial 410 411 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 412 413 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 414 amendments. Additionally the stoichiometric balance of inorganic nutrients was maintained in 415 416 favour of P, so that elevations of APA observed following addition of DON were not a result 417 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 418 419 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-420 421 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 422 mechanism by which this happens but provide three lines of evidence of this process occurring: 423 424 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 425 changes include increased storminess and hence turbulence, altered freshwater delivery, 426 427 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 428 al. 2012). Altered wind systems may strengthen eastern boundary upwelling, and thus enhance 429

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.

433 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, 434 this does not necessarily mean that the difference endured over the lifetime of the culture (e.g. 435 Table 2). However, in a marine environment where P is less replete, the ability to access DOP 436 earlier than competing species might enable E. huxleyi to better adapt to DIP limitation. 437 Changes in algal metabolism, such as uptake of DOP, may occur as a result of more nuanced 438 changes in the balance of the macronutrient pool rather than under conditions of N- or P-stress. 439 As phytoplankton form the basis of the marine food web and drive the biogeochemical cycles 440 441 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. 442

443

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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 639

- DON fraction comprised 20 proteinogenic amino acids and urea. Individual amino acids were 640
- added to a final concentration of 3.5 μ M in the media, equivalent to 101 μ mol-N L⁻¹; urea was 641
- added to a final concentration of 43 µmol-N L⁻¹. The DOP fraction comprised ADP and ATP 642 at a combined concentration of 9 µmol-P L⁻¹. As ADP and ATP each contain 5 atoms of N, in
- 643
- 644 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
- 645 proline were not added to media containing DOP 646

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

647

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 NO₃⁻ and PO₄³⁻; starting ratios are given in brackets of the same column.

E. huxleyi	Cell count	DIN :	Vmax	Vmax
		DII	$(nM h^{-1})$	(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

686 media sampled from *Emiliania huxleyi* cultures (n = 3). A full MS scan (m/z 50.00 – 2000.00) 687 revealed a base peak at m/z 268.1028, corresponding to adenosine.

688

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); 695 Chaetoceros sp. (c). The culture volume was 2 L and the culture vessels amended with 696 fresh medium (0.5 L) at intervals during the culturing period. 697





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 (**I**) and k/2 medium containing DON+DOP (\Box). 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_{predicted}, **•** = k/2 DON_{predicted}, **•** = k/2 DON+DOP_{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.

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709

Supplementary information ; preparation of culture media 710

711

712 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 713
- kill most organisms but should not chemically modify the medium too much) add: 714

715

Quantity	Compound	Stock solution (sterile)	Final conc. in K medium
0.25 mL	NaNO ₃	48.9542 g L ⁻¹ H ₂ O	144 μM
1.0 mL	DON (urea + 20 amino acids)	(see recipe on page 2)	144.5 μM
0.25 mL	KH ₂ PO4	4.8992 g L ⁻¹ H ₂ O	9 μΜ
1.0 mL	DOP (ADP + ATP)	(see recipe on page 3)	9 μΜ
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)

716 * optional

717

FeEDTA solution

718 To 950 mL distilled H₂O 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 M Ω cm resistivity), sterilize (filter 0.22 μ m) and store 719 720 in fridge.

721 722

Trace metal solution

<u>To 950 mL di</u>	stilled H ₂ O add:		
Quantity	Compound	Stock solution	Final conc. in K medium
37.22g	Na ₂ EDTA.2H ₂ O	-	50 µM
1.0 mL	CuSO ₄ .5H ₂ O	2.497 g L ⁻¹ H ₂ O	0.005 µM
1.0 mL	Na ₂ MoO ₄ .2H ₂ O	$7.2585 \text{ g } \text{L}^{-1} \text{ H}_2\text{O}$	0.015 µM
1.0 mL	ZnSO ₄ .7H ₂ O	23.0 g L ⁻¹ H ₂ O	0.004 µM
1.0 mL	CoSO4.7H ₂ O	14.055 g L ⁻¹ H ₂ O	0.025 μM
1.0 mL	MnCl ₂ .4H ₂ O	178.11 g L ⁻¹ H ₂ O	0.45 µM
1.0 mL	H_2SeO_3	1.29 g L ⁻¹ H ₂ O	0.005 µM

	1.0 mL	NiCl2.6H ₂ 0	1.49 g L ⁻¹ H ₂ O	0.00314 μM
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723 Make up to 1 L with high purity water, sterilize (filter 0.22µm) and store in fridge.

724

f/2 Vitamin solution

725 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 ₂ O	0.37 nM
1.0 mL	Biotin	$5.0 \text{ mg } \text{L}^{-1} \text{ H}_2\text{O}$	2.0 nM
100.0 mg	Thiamine HCl	-	0.3 μΜ

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

727

728 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)

730

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

733

734 DON solution

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

	g/500 mL	
Amino acid	(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

739 * Not included in DON + DOP recipe due to 20 μ M aromatic N added to medium from ADP and 740 ATP.

DOP Solution

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

P species	g/100 mL
ADP	0.135
ATP	0.055