

2020-09

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<http://hdl.handle.net/10026.1/15994>

10.1016/j.jembe.2020.151434

Journal of Experimental Marine Biology and Ecology

Elsevier BV

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1 Post-print version
2 DOI: 10.1016/j.jembe.2020.151434
3 Accepted: 2nd July 2020
4 Published: 10th July 2020
5 Embargo period: 24 months
6

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

9

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30

31 **Abstract**

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

53

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

56

57 **Introduction**

58 As limiting nutrients for algal growth, phosphorus (P) and nitrogen (N) play an essential
59 role in the biological productivity of aquatic ecosystems (Redfield 1958; Hecky & Kilham
60 1988). Most nutrient cycling studies have focussed on dissolved inorganic N and P (DIN and
61 DIP, respectively). However, recent studies show that the dissolved organic pools (DON and
62 DOP, respectively) also merit consideration. For example, DON frequently comprises the
63 largest part (60–69 %) of total dissolved N in rivers, estuaries and surface ocean waters (Bronk
64 2002), while DOP was shown to account for at least 40 % of the total dissolved phosphorus
65 pool in an estuary (McKelvie 2005; Monbet *et al.* 2009), and 70–90 % in oligotrophic waters
66 (Ruttenberg & Dyhrman 2012). Although bacteria are primarily responsible for the processing
67 of DON in aquatic environments (Berman & Bronk 2003), a variety of phytoplankton species
68 have been shown to utilise DON to meet their N needs (Antia *et al.* 1991; Moschonas *et al.*
69 2017). Studies of DON uptake by phytoplankton (Gobler & Boneillo 2003; Mulholland & Lee
70 2009) demonstrated that both external hydrolysis and direct assimilation occurred, depending
71 on molecular size, with highest rates measured in the size fraction containing the dominant
72 phytoplankter.

73 In marine waters, the supply of P to phytoplankton to meet their cellular demands is
74 thought to be mainly in the form of orthophosphate (Cembella *et al.* 1984; Nicholson *et al.*
75 2006; Mahaffey *et al.* 2014) rather than DOP. During times of DIP depletion, relative to other
76 nutrients, microbial activity and phytoplankton growth are often considered to be P-limited
77 (Karl *et al.* 1995; Shaked *et al.* 2006), even though the concentration of marine DOP can be 5–
78 10 times higher than DIP (Mather *et al.* 2008). However, when DIP is depleted, a number of
79 marine organisms, including dinoflagellates (Dyhrman & Palenik 1999; Lin *et al.* 2012),
80 coccolithophores (Dyhrman & Palenik 2003), diatoms (Dyhrman & Ruttenberg 2006) and
81 bacteria (Huang & Hong 1999) are known to synthesize hydrolytic enzymes in order to access

82 the DOP pool to derive their P requirement (Monaghan & Ruttenberg 1999; Ruttenberg &
83 Dyhrman 2005) via expression of the alkaline phosphatase (AP) enzyme (Perry 1972;
84 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
86 DIP, or elevated ratios of dissolved inorganic N to DIP (DIN : DIP). Studies from a range of
87 marine environments indicate variable inorganic phosphate concentration thresholds, below
88 which alkaline phosphatase activity (APA) is induced; specifically, below 10 nM in the
89 Sargasso Sea (Lomas *et al.* 2010), ~20 nM in the subtropical Pacific (Suzumura *et al.* 2012)
90 and ~100 nM in the northwest African upwelling region (Sebastian *et al.* 2004). As such, APA
91 has been used to determine phytoplankton community P status (Sebastián *et al.*, 2004;
92 Nicholson *et al.* 2006; Suzumura *et al.* 2012; Mahaffey *et al.* 2014). However, several studies
93 have shown that P from DOP can be taken up by bacteria or phytoplankton, even in the presence
94 of DIP, via enzymatic hydrolysis, depending on their competing strengths, substrate
95 concentrations, saturation, storage capacity and the availability of other nutrients such as
96 organic carbon (Cotner *et al.* 1997; Labry *et al.* 2005; Luo *et al.* 2011). The combination of
97 inducible and constitutive behaviour of AP means that its relationship with phosphate may be
98 complex when considered across a spectrum of marine environments.

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

113

114 **Materials and methods**

115 CLEANING PROCEDURE

116 Glass- and plasticware were first degreased (2% Nutracon solution, 24 h), then acid-washed
117 (10 % HCl, 24 h) and thoroughly rinsed with high purity water (HPW; Millipore, 18.2 M Ω
118 cm). Cleaned items were stored in resealable plastic bags. Glass fibre filters (GF/F) were
119 cleaned by combustion in a muffle furnace (450 °C, 6 h). Clean techniques were used
120 throughout the study and critical handling steps were performed in a laminar flow cabinet.

121

122 ALGAL CULTURING

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

138 under a light microscope, and sub-culturing was performed every 3-7 days prior to inoculation
139 of larger volumes of culture media. Culture volumes of 2 L were maintained over 21 days;
140 these cultures were maintained through addition of fresh medium (25 % addition by volume)
141 weekly over this period. Larger volume (6 L) cultures of *E. huxleyi* were prepared from sub-
142 cultures of established 2 L volume cultures, to provide adequate volume for the extraction and
143 detection of organic molecules via solid phase extraction and analyses of dissolved nitrate and
144 phosphate. The 6 L cultures were not refreshed over the experimental period.

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

151

152 DISSOLVED INORGANIC NUTRIENTS

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

160

161 ALKALINE PHOSPHATASE ACTIVITY

162 Kinetic assays of APA were performed using a sensitive fluorometric protocol similar to Perry
163 (1972). The APA in culture samples was assayed as a change in fluorescence through
164 enzymatic hydrolysis of the artificial P substrate, 4-methylumbelliferyl phosphate (MUF-P)
165 releasing the fluorescent product methylumbelliferone (MUF). A 4 mL aliquot of unfiltered
166 culture water was distributed into triplicate series (blank control plus 5 concentrations between
167 12.5 and 200 nM MUF-P in 10 nM Tris buffer) in 12 mL glass tubes. These samples were
168 incubated at 15 °C in the dark for 1 hour and their fluorescence determined (Turner Designs
169 Laboratory Trilogy Fluorometer) at excitation and emission wavelengths of 365 and 455 nm,
170 respectively. The procedure was calibrated on each occasion against six MUF standards
171 (concentration range 5-40 nM), measured in triplicate, which was sufficient to account for all
172 samples. Kinetic data were estimated using the Lineweaver-Burk transformation of the
173 Michaelis-Menten equation.

174

175 TOTAL PHOSPHATE

176 Frozen GF/F filters were thawed, wrapped in a double-layer of aluminium foil then combusted
177 at 450 °C for 4.5 h and allowed to cool to room temperature (20–25 °C). Each filter was then
178 divided (pieces were no larger than 10 x 10 mm) using acid-washed surgical scissors and placed
179 into a pre-cleaned 20 mL glass vial. After addition of HCl (5 mL, 0.5 M), vials were placed in
180 a sonic-bath for 60 minutes then centrifuged at 3000 rpm for 30 minutes. The supernatants
181 were analysed for P by inductively coupled plasma optical emission spectrometry at a
182 wavelength of 177.495 nm. A certified reference material (CRM; NIST Apple leaf) was used
183 as the analytical control to measure recovery of P from the sample filters. Samples containing
184 CRM were prepared according to the Hawaii Ocean Time Series protocol

185 (<http://hahana.soest.hawaii.edu/hot/protocols/chap11.html>), with some adaptations. The CRM
186 was initially freeze-dried (48 h), then weighed into glass vials to give a range of P
187 concentrations in 10 mL HPW (0, 5, 10, 15 and 20 μ M). The added CRM was suspended in
188 solution using a vortex mixer and aliquots pipetted on to an acid-washed and combusted GF/F
189 filter paper. Filter papers were then oven-dried at 40 °C and prepared for analysis as described
190 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 gravity-
195 filtered through GF/F filters (0.7 μ m) then amended with formic acid (FA) to a final
196 concentration of 0.1 % FA v/v. Solid phase extraction (SPE) was then performed using Strata-
197 X 33 μ Polymeric Reversed Phase 500 mg/12 mL Giga Tubes, using a method adapted from
198 Curtis-Jackson *et al.* (2009). The tubes were conditioned with 12 mL of a methanol (MeOH)
199 and water mixture (50 : 50 v/v), followed by equilibration with a 12 mL mixture of MeOH and
200 water at 1 : 99 v/v. Once the FA-amended sample had been passed through the cartridge, a
201 single wash step was performed with a further 3 mL of the MeOH and water mixture (1 : 99
202 v/v). The cartridge was eluted with 3 volumes (2 mL, 2 mL, 1 mL) of a MeOH : FA mixture
203 (99 : 1 v/v) into a glass vial. Eluted samples were further pre-concentrated by removal of the
204 elution solvent under a gentle flow of N₂ gas then reconstituted in 100 μ L HPLC-grade water
205 for analysis by liquid chromatography tandem mass spectrometry LC-MS/MS.

206

207

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

217 A 5 µL aliquot of sample was separated on a 100mm analytical column (2.1mm inner
218 diameter) packed with 3.6 µm C₁₈ beads (Aeris Peptide, Phenomenex). A gradient comprising
219 0.1% acetic acid in water (A) and acetonitrile (B) was applied over a total run time of 30
220 minutes. The following proportions of solvent B were used for elution: 0–15 min, 0–50 % ;
221 15–18 min, 50-100 % ; 18–23 min, 100 % ; and 23–30 min, 0 % , with a flow rate of 0.25 mL
222 min⁻¹. The analytes were detected at two wavelengths (206 and 280 nm).

223 Effluent from the analytical column was directly electrosprayed into the mass
224 spectrometer. The linear trap quadrupole (LTQ) Orbitrap instrument was operated in data-
225 dependent mode to automatically switch between full scan MS and MS/MS acquisition.
226 Instrument control was through Tune 2.5.5 and Xcalibur 2.1. For the low-resolution collision
227 induced dissociation method (CID-MS/MS top5), full scan MS spectra (from *m/z* 50 to 2000)
228 were acquired in the Orbitrap analyzer after accumulation to a target ion count value of 5.10
229 E⁵. The 5 most intense ions with charge states ≥ 2 were sequentially isolated to a target value
230 of 30,000 and fragmented in the linear ion trap by CID with normalized collision energy of 15
231 %, and wideband-activation enabled. The ion selection threshold was 1.10 E⁵ for MS/MS, and
232 the maximum allowed ion accumulation times were 500 ms for full scans in the orbitrap, and

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.

235 For the high-resolution HCD-MS/MS top3 method, full scan MS spectra (from m/z 50
236 to 1000) were acquired in the orbitrap with resolution $r = 30,000$. The three most intense ions
237 with charge states ≥ 2 were sequentially isolated to a target value of 3×10^6 and fragmented in the
238 HCD collision cell with normalized collision energy of 35 %. The resulting fragments were
239 detected in the orbitrap with resolution $r = 7500$. The ion selection threshold was 1.10×10^5 for
240 HCD, and the maximum allowed ion accumulation times were 500 ms for full scans and 200
241 ms for HCD.

242

243 **Results**

244 ALGAL CULTURING

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

256 Interestingly, at the time of harvesting the cell count of *E. huxleyi* in 6 L cultures was
257 higher in the k/2 DON+DOP medium compared with k/2 (Day 7), while the k/2 medium cell
258 count was higher than for the k/2 DON medium samples when the latter was harvested on Day
259 11.

260

261 ALKALINE PHOSPHATASE ACTIVITY

262 Rates of APA were measured in all cultures. With respect to those species grown in the k/2
263 media which had V_{\max} rates for APA of 0.03, 1.78 and 4.66 fmolP cell⁻¹h⁻¹ for *E. huxleyi*, *P.*
264 *minimum* and *Chaetoceros sp.*, respectively, there was an apparent increase in APA for each
265 species in cultures containing DON, with V_{\max} rates of APA of 0.07, 39.7 and 80.5 fmolP cell⁻¹
266 h⁻¹ for *E. huxleyi*, *P. minimum* and *Chaetoceros sp.* respectively. (Table 2). The response in

267 cultures containing DON+DOP varied for each group; APA for *E. huxleyi* reduced by ~ 67 %
268 whilst *P. minimum* and *Chaetoceros sp.* both increased from 1.78 to 10.8 and from 4.66 to 106
269 fmolP cell⁻¹h⁻¹, respectively. Whilst these cultures were not grown axenically, they were all
270 treated using identical clean handling procedures and, whilst a contribution to APA from
271 bacteria is possible, the variable response between the different algal species and treatments
272 gives confidence that these observations are dominated by the differential response to
273 treatments by the algal cells.

274

275 PARTICULATE PHOSPHORUS

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

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

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

$$282 \quad P \text{ cell}^{-1} \text{ (theoretical)} = ([P_0-P]/1000)/C \quad (2)$$

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

290 concentration in the k/2 DON+DOP samples exceeded the theoretical value (27.6 versus 22.2
291 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
295 before chromatographic separation and detection by mass spectrometry. Mass spectra were
296 examined for evidence of direct utilisation of DOP (ATP and ADP). A peak at m/z 268.1028
297 (Figure 4a) was prominent in the mass spectrum of k/2 DON+DOP samples, with an ion count
298 of 7.24×10^7 . The same peak in the k/2 DON samples was much weaker (ion count of $1.77 \times$
299 10^3) and absent in k/2 samples (Table 4). The spectral library identified this ion as adenosine
300 and its further fragmentation by MS/MS confirmed the structure. The MS/MS mass spectrum
301 contained a base peak at m/z 136.0609, consistent with the m/z for protonated 1H-Imidazo[4,5-
302 d]pyridazin-4-amine after loss of 1,4-Anhydropentitol (Figure 4b)

303

304

305 **Discussion**

306 It is important to acknowledge that this study was not performed axenically and that there was
307 potential for some bacterial cycling of DON and DOP within the culture media. The
308 experimental matrix of algal species and inorganic : organic nutrient ratios was therefore
309 designed to enable comparative interpretation of nutrient use by utilising analytical procedures
310 to independently follow enzymatic activity, dissolved and cellular nutrient content and
311 molecular changes in media composition. In this study, for each phytoplankton species, rates
312 of APA in cultures amended with DON were higher than in cultures grown in unamended k/2
313 media, even though the absolute N : P ratio was kept constant. Whilst some bacterial
314 remineralisation of DON to DIN was likely, there was no evidence that the original DIN pool
315 was enriched in the media where DON was added, neither was DIN significantly depleted in
316 those cultures not receiving DON (Figure 1). Butler *et al.* (1979) described the seasonal balance
317 of inorganic to organic N and P in waters of the English Channel and the succession between
318 inorganic to organic dominance during the transition from spring to summer, so that
319 phytoplankton were likely to rely on the DON and DOP fractions during the summertime. A
320 summertime survey of these waters by Davies & Smith (1988) confirmed this, where all
321 phytoplankton communities displayed APA. They proposed that DOP could have an important
322 role supporting phytoplankton and bacterial productivity during periods of P-stress. Rees *et al.*
323 (2009) found that intense periods of summertime rainfall altered the inherent nutrient
324 stoichiometry of coastal waters of the Western English Channel from an N-limited condition
325 to one where microbial communities were P-stressed, invoking AP expression. Interestingly,
326 Butler *et al.* (1979) measured a relatively constant total N : P ratio throughout the year (17-24,
327 with a ratio of about 20 reflecting the overall chemical-biological balance in the system), while
328 the $\text{NO}_3^- : \text{PO}_4^{3-}$ ratio varied from 3-13 and for DON : DOP the ratio was 25-42. They suggested
329 that the yearly succession of phytoplankton species occurring in these waters may be partly

330 explained by the hypothesis that when NO_3^- is exhausted there may be a change in the
331 phytoplankton population such that species capable of utilizing DON became dominant.

332 Constitutive uptake of DOP occurs in phosphorus-replete systems (Sebastian *et al.*
333 2004; Dyhrman & Ruttenberg 2006; Sato *et al.* 2013); however, upregulation of AP through
334 the presence of DON is a new finding and the data from this study suggest that expression of
335 AP may be sensitive to the form of N available, rather than a focussed response to changes in
336 inorganic N : P ratios. A recent study found that some dinoflagellate species maintained AP
337 even when DIP was supplied in excess, further suggesting that APA is not necessarily an
338 absolute indicator of phosphorus stress nor tightly controlled by ambient DIP level. It seems
339 likely that APA activity in these species could indicate selective use of DOP, or a metabolic
340 response to changes in P forms (Martinez Soto *et al.* 2015). A recent study assessed the relative
341 lability of model P compounds representing the major bond classes of marine DOP in diatom
342 cultures of the genus *Thalassiosira*, as well as coastal field sites of the western North Atlantic
343 (Diaz *et al.* 2018). They found that ATP degradation rates were always suppressed under P-
344 replete culture conditions but the effect of P availability on DOP uptake was inconsistent
345 among diatom strains.

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

355 observed that N sources correlating with the multivariate pattern in phytoplankton community
356 composition and abundance were, in order of statistical importance: urea, dissolved free amino
357 acids (DFAA), total DON, and DIN. The measured drawdown of DON during the spring bloom
358 was calculated to have contributed up to 37 % of the total measured dissolved N drawdown
359 compared to 63 % from NO_3^- , clearly showing the importance of DON for phytoplankton N
360 nutrition. Indeed, in the smaller phytoplankton size fraction ($< 10 \mu\text{m}$), NO_3^- contributed only
361 28 % during spring and summer but generally much less, while NH_4^+ (up to 55 %), urea (up
362 to 59 %), and DFAA (up to 38 %) contributions were considerable during spring and summer
363 when regenerated N uptake rates were highest. These studies measured N in isolation and our
364 study indicates that DON uptake could also be linked to the presence of DOP.

365 Particulate P concentrations in *E. huxleyi* provided evidence for direct uptake of DOP
366 by phytoplankton cells. While the k/2 and k/2 DON samples measured had particulate P
367 concentrations consistent with DIP loss from the media, the P concentrations in the k/2 DON
368 + DOP samples exceeded the amount that could be accounted for by DIP uptake alone (Table
369 3). Mass spectra confirmed utilisation of DOP by *E. huxleyi*, as adenosine, a fragment of ADP
370 and ATP, which was present in the k/2 DON+DOP culture medium (Figure 4), could only have
371 been produced through hydrolysis of the triphosphate chain on these molecules. Casey *et al.*
372 (2009) used ATP to represent labile DOP in the oligotrophic North Atlantic Ocean, which was
373 taken up directly by phytoplankton. Interestingly, while uptake of DIP increased in that study,
374 in line with its abundance, ambient DOP concentrations had no apparent effect on whole
375 seawater utilization of either DIP or ATP. Interestingly, although ADP and ATP are N-rich
376 molecules (containing 5 nitrogen atoms) the presence of adenosine in the medium suggested
377 that cells took up the P content after external hydrolysis of ADP and ATP. Direct uptake of
378 DON by marine phytoplankton has been reported (Hu *et al.* 2012, Mulholland & Lee 2009),
379 though a molecular mass limit has not been established.

380 The ability of *E. huxleyi* to adjust to changes in composition of the N and P pool has
381 recently been reported (McKew *et al.* 2015, Rokitta *et al.* 2016). McKew *et al.* (2015) found
382 that acclimation of *E. huxleyi* to nutrient limitation led to marked increases in the abundance
383 of proteins involved in inorganic nutrient transport and both the scavenging and internal
384 remobilization of organic N and P, including AP. However, this was a highly targeted
385 reorganization of the proteome towards scavenging of DON and DOP under N and P
386 limitation, with proteins that were upregulated under these conditions accounting for only 1.7
387 and 5.7 % of the total spectral counts, respectively. Rokitta *et al.* (2016) observed that *E.*
388 *huxleyi*'s outstanding endurance under nutrient deficiency related to its versatile high-affinity
389 uptake systems and an efficient, NAD-independent malate oxidation that was absent from
390 most other taxa. However, the metabolic adjustments made during senescence involved
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
393 methylglyoxal) so that continued senescence promoted various processes that eventually lead
394 to cell death. The data from our study is particularly novel as it shows that a recognised
395 indicator of P-stress, APA, was also upregulated by a change in the DIN : DON ratio, rather
396 than low DIP concentrations or the presence of DOP. We acknowledge that the DON and
397 DOP pools are varied and complex, such that proxies of these fractions may not represent the
398 cycling of both labile and refractory components. For example, the DOP pool ranges from
399 relatively labile compounds like phosphomonoesters to more refractory molecules like
400 phosphonates (Kolowitz *et al.*, 2001). However, ATP has been used as a proxy for the labile
401 DOP fraction and, as phosphoesters, ADP and ATP contain a functional groups shared by the
402 class of compounds comprising the majority of the DOP pool (Young & Ingall, 2010).
403 Dissolved free amino acids are also labile molecules within the DON pool, but their varied
404 functionality, acidity and solubility has facilitated their application as proxies to study DON

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).

409 There was potential for bacterial contribution to this study, though our consideration of
410 all measurements made would indicate that this had a minor impact on our findings. Bacterial
411 remineralisation could have proved problematic if cultures were grown on organic nutrients
412 only. As all experiments were permanently replete in DIN and DIP any bacterial generation of
413 inorganic nutrient did not likely contribute significantly to: 1) the enhanced algal growth
414 observed and 2) elevated cellular P content of algal cells observed following DON and DOP
415 amendments. Additionally the stoichiometric balance of inorganic nutrients was maintained in
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
418 study confirms the contention offered by several other authors that organic nutrients are, at
419 times, of significance to the growth and function of several algal groups. Additionally, we argue
420 that the expression of AP or the absence of DIP do not necessarily indicate a phosphorus-
421 stressed community, but that there are occasions when the uptake of DOP is in preference to
422 DIP and may be enhanced by the presence of DON. These data do not allow us to indicate the
423 mechanism by which this happens but provide three lines of evidence of this process occurring:
424 APA, P cleaved from ATP/ADP, and elevated particulate P. Environmental conditions of
425 coastal waters and open ocean regions are projected to change over the next few decades. These
426 changes include increased storminess and hence turbulence, altered freshwater delivery,
427 elevated seawater temperatures which might lead to enhanced stratification restricting nutrient
428 input to surface waters from depth (Rost & Riebesell 2004; Steinacher *et al.* 2010; Doney *et*
429 *al.* 2012). Altered wind systems may strengthen eastern boundary upwelling, and thus enhance

430 primary productivity (Bakun *et al.* 2010). Such alterations to abundances of macronutrients
431 like N and P are expected to affect phytoplankton community composition, ecosystem
432 functioning and, ultimately, biogeochemical cycles.

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

443

444 **Acknowledgements**

445 The comments from reviewers were much appreciated and helped to strengthen the manuscript.
446 We are grateful for the help of Dr Kate Schofield, University of Plymouth (particulate P
447 measurements), Dr Thierry Cariou, Station Biologique de Roscoff (dissolved nutrient
448 measurements) and the SOMLIT sampling team (seawater for algal culturing). The study was
449 supported through awards to APR and MFF from the ASSEMBLE FP7 research infrastructure
450 initiative (<https://www.assemblemarine.org/>), while MFF is grateful for an additional award
451 from the Marine Institute, University of Plymouth.

452

453

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639 **Table 1.** Details of the N and P components of the k/2 and f/2 media used in this study. The
 640 DON fraction comprised 20 proteinogenic amino acids and urea. Individual amino acids were
 641 added to a final concentration of 3.5 μM in the media, equivalent to 101 $\mu\text{mol-N L}^{-1}$; urea was
 642 added to a final concentration of 43 $\mu\text{mol-N L}^{-1}$. The DOP fraction comprised ADP and ATP
 643 at a combined concentration of 9 $\mu\text{mol-P L}^{-1}$. As ADP and ATP each contain 5 atoms of N, in
 644 the form of aromatic and amino N, the DON-fraction in these media was amended to remove
 645 the equivalent amount of structurally-similar DON; specifically, histidine, tryptophan and
 646 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

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

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<i>E. huxleyi</i>	Cell count (mL^{-1})	DIN : DIP	V_{\max} (nM h^{-1})	V_{\max} ($\text{fmol cell}^{-1} \text{h}^{-1}$)
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
<i>P. minimum</i>				
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
<i>Chaetoceros sp.</i>				
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

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

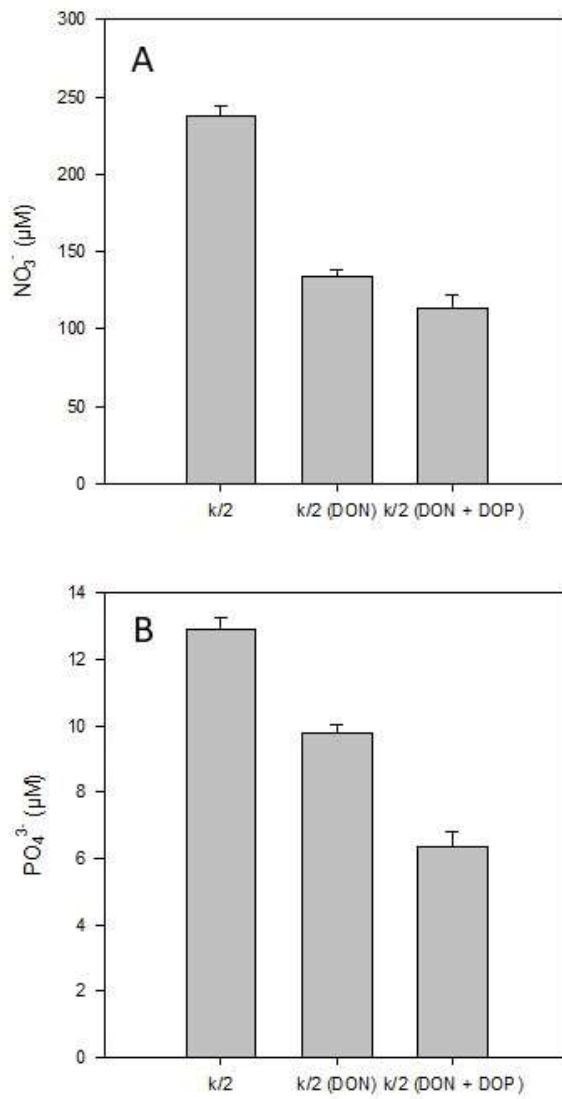
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685 **Table 4.** Mean ion current for peak occurring at retention time window 4.71-5.19 minutes in
686 media sampled from *Emiliania huxleyi* cultures (n = 3). A full MS scan (*m/z* 50.00 – 2000.00)
687 revealed a base peak at *m/z* 268.1028, corresponding to adenosine.
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Culture medium	Ion current
k/2	0
k/2 (DON)	1773
k/2 (DON+DOP)	27395000

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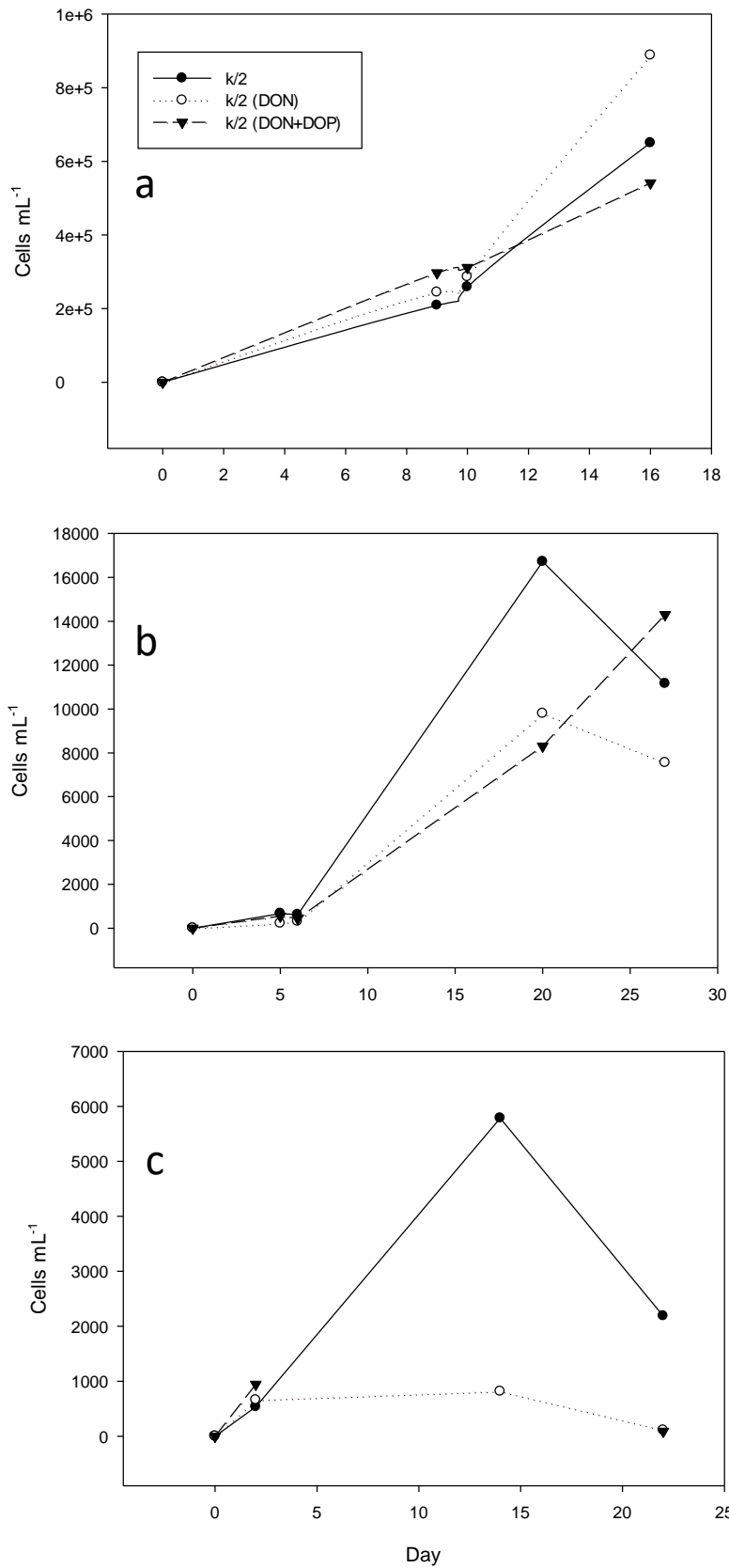


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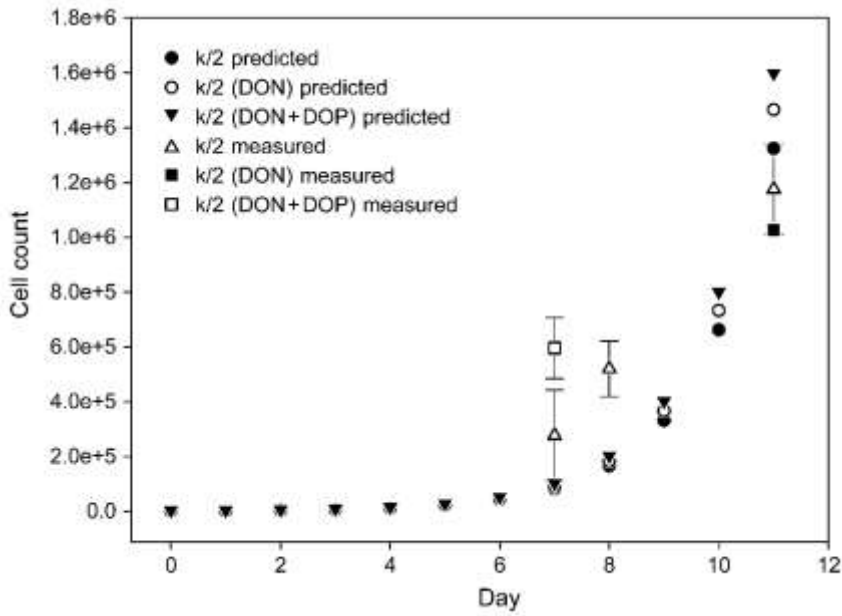
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Figure 1. Concentrations of A, dissolved nitrate; B, dissolved phosphate in 6 L cultures of *Emiliana huxleyi* at the time of harvesting.



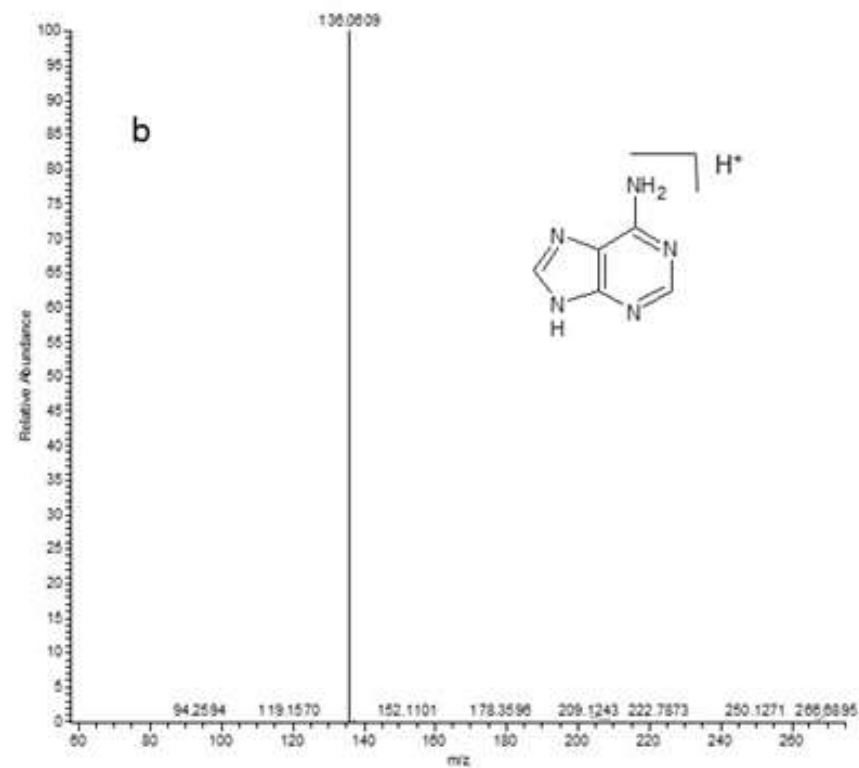
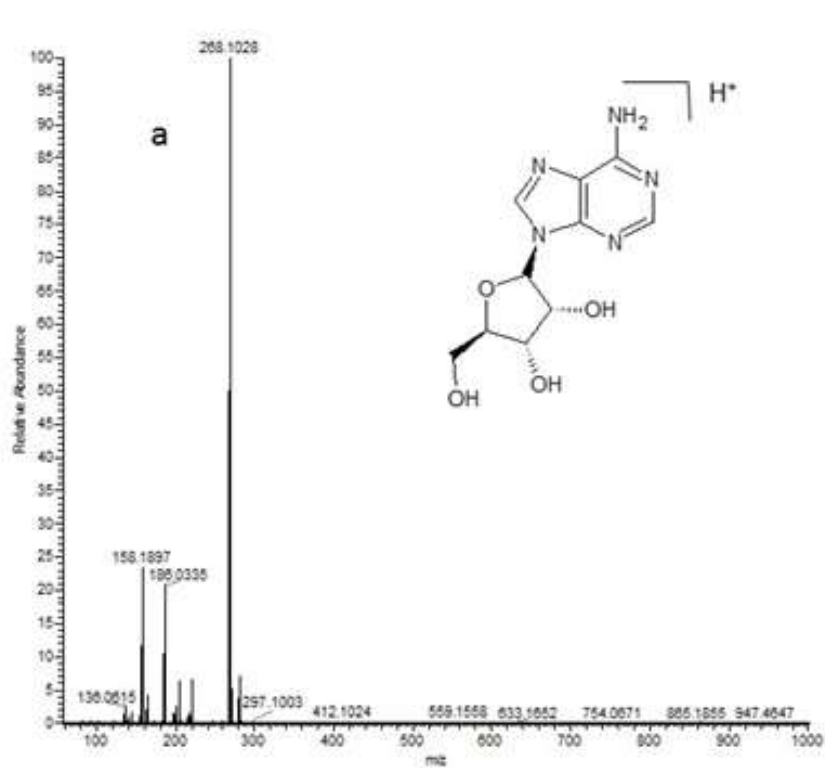
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Figure 2. Measured cell counts for *Emiliana huxleyi* (a); *Prorocentrum minimum* (b); *Chaetoceros sp.* (c). The culture volume was 2 L and the culture vessels amended with fresh medium (0.5 L) at intervals during the culturing period.



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700 **Figure 3.** Measured cell counts for *Emiliana huxleyi* cultured in 6 L volumes without
 701 replenishment in: k/2 culture medium (Δ), k/2 medium containing DON (\blacksquare) and k/2 medium
 702 containing DON+DOP (\square). Predicted growth rates for cultures were based on cell counts at the
 703 time of sub-culturing to 6 L and assumed one cell division per day ($\bullet = k/2_{\text{predicted}}$, $\circ = k/2$
 704 $\text{DON}_{\text{predicted}}$, $\blacktriangledown = k/2 \text{ DON+DOP}_{\text{predicted}}$)



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

708 *Fitzsimons et al. (2019)*

709

710 Supplementary information ; preparation of culture media

711

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

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

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 PO ₄	4.8992 g L ⁻¹ H ₂ O	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)

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

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

721

Trace metal solution

722 To 950 mL distilled H₂O 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 g L ⁻¹ H ₂ 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	CoSO ₄ .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 ₂ SeO ₃	1.29 g L ⁻¹ H ₂ O	0.005 μM

1.0 mL	NiCl ₂ ·6H ₂ O	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 H₂O 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 mg L ⁻¹ H ₂ O	2.0 nM
100.0 mg	Thiamine HCl	-	0.3 μM

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)**

729 For K-ET, add 10-30 mL marine soil extract (ET)

730

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

733

734 **DON solution**

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

737

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

738

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

741

742 **DOP Solution**

743

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

746

P species	g/100 mL
ADP	0.135
ATP	0.055

747

