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1 Colimitation assessment of phytoplankton growth using a resource use efficiency approach in
2 the Bay of Seine (French-English Channel).

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30

31 Abstract

32

33 Eutrophication and dystrophy are two of the main problems affecting coastal ecosystems. In the
34 Bay of Seine, phosphorus (P) inputs from the Seine estuary have been largely reduced in the
35 last decade, in contrast to nitrogen (N), which leads to high N/P ratio inputs. To study the effect
36 of dystrophy, an enrichment bioassay using water sampled from the Bay of Seine was repeated
37 19 times over a period of 18 months with six different enrichments. After a few days,
38 chlorophyll *a* (chl *a*), alkaline phosphatase activity (APA), transparent exopolymeric particles
39 (TEPs), cytometric size structure, and maximum quantum yield of photosystem II were
40 measured. The data provide strong evidence for an N & P colimitation system in the vast
41 majority of the incubations, as only the N+P and N+P+Si enrichments supported phytoplankton
42 growth, and Si only appeared to play a secondary role in our incubations. A N/P ratio of 16
43 equal to the Redfield ratio was identified as the optimum for balanced growth, as chl *a* was the
44 highest and TEP and APA production was the lowest at this ratio. To fit the requirements of the
45 colimited system, a new resource use efficiency (RUE_{NP}) calculation was developed to account
46 for N and P colimitation instead of only one nutrient, as is usually the case. This calculation
47 allows better representation of RUE in dystrophic conditions, as found in many highly
48 anthropized ecosystems. The relationships between RUE_{NP} and the size structure of the
49 phytoplankton community were explored, and a significant positive correlation between RUE_{NP}
50 and larger cells ($> 2 \mu\text{m}$) and a negative correlation with smaller cells ($< 2 \mu\text{m}$) were noted,
51 showing a better use of nutrients by larger cells. This study highlights an increase of RUE_{NP}
52 with the phytoplankton cell size in a colimited system.

53

54 Keywords: Eutrophication; N/P ratio; Alkaline phosphatase activity; Transparent
55 exopolymeric particles; Flow cytometry, ecophysiological parameters

1. Introduction

56
57
58 Coastal ecosystems provide important economic services, and their decline, due to multiple
59 human pressures, could have long-term impacts (Barbier et al., 2011). The main problem facing
60 coastal ecosystems is eutrophication (Rabalais et al., 2009), caused by excessive inputs of
61 nutrients, usually nitrogen (N) and phosphorus (P), into the system associated with a dystrophic
62 ratio. Phytoplankton uptake is mainly described by the Redfield ratio ($N/P = 16$), which
63 regulates the nutrient system (Falkowski, 2000), but in dystrophic conditions, deviation from
64 this paradigm can be observed at multiple scales from cellular to environmental requirements
65 (Fraga, 2001; Geider and La Roche, 2002; Glibert and Burkholder, 2011; Ptacnik et al., 2010).
66 However, unbalanced nutrient inputs affect phytoplankton community composition (Leruste et
67 al., 2019; Shen et al., 2019) and growth rate (Nwankwegu et al., 2020). Thus, knowing which
68 nutrients limit phytoplankton growth is crucial. Recently, multiple coastal ecosystems have
69 been described as colimited systems (Chorus and Spijkerman, 2020; Conley et al., 2009;
70 Harpole et al., 2011) with N and P as independent nutrients (Saito et al., 2008). The Bay of
71 Seine (France) is a typical eutrophic system, and nutrients are mainly supplied by the Seine
72 River (Aminot et al., 1998) and at a smaller scale by local rivers (Lemesle et al., 2015). In recent
73 decades, nutrient management programmes in the Seine River have successfully reduced
74 nutrient inputs, particularly phosphorus inputs (Aissa-Grouz et al., 2018) by improvement of
75 domestic wastewater treatment, but N levels have nevertheless remained high (Garnier et al.,
76 2019). This causes an abrupt change in nutrient stoichiometry with high N/P ratios measured in
77 both the Seine estuary and the Seine River (Garnier et al., 2019; Meybeck et al., 2018).
78 One way to better assess the link between the phytoplankton community and nutrients is the
79 resource use efficiency (RUE) parameter. RUE measures the supplied resources converted into
80 biomass (Hodapp et al., 2019) and is useful to characterize the use of nutrients by phytoplankton
81 populations. To our knowledge, only a few authors, e.g., Han et al. (2016), have broached the

82 multiple limitations of RUE, one being that RUE usually focuses on one nutrient at a time (e.g.,
83 RUE_N , RUE_P). Today, given the growing interest in colimitation systems, new insights are
84 needed to describe these nutrient regimes more precisely.

85 Enrichment bioassays are an effective way to investigate the effects of nutrient inputs on the
86 phytoplankton community (Rahav et al., 2018; Reed et al., 2016; Song et al., 2019; Van
87 Meerssche and Pinckney, 2019). Varying the composition, stoichiometry and quantity of
88 nutrients added pinpoint limitation patterns more easily (Tamminen and Andersen, 2007).
89 Repeating the bioassays throughout the year enables the identification of time-dependent
90 interactions (Xu et al., 2010).

91 To investigate the effect of nutrient enrichment on phytoplankton in the Bay of Seine, we
92 conducted repeated bioassays. After a few days of incubation, the community structure of
93 natural populations of phytoplankton was measured to assess the nutrient regime of the
94 phytoplankton community. A new approach was proposed to calculate a RUE constrained by
95 two colimiting nutrients.

96 The specific objectives of our study were to investigate the ratio of N/P consumed by the
97 phytoplankton community under unbalanced nutrient input conditions and characterize
98 physiological status and population structure responses to sudden nutrient enrichments. This
99 approach should allow us to identify the limitation system in a dynamic anthropized macrotidal
100 bay at a temperate latitude.

101

102 **2. Materials and Methods**

103

104 **2.1 Measurements in the Bay of Seine**

105 High-frequency data were monitored *in situ* over a two-year period using the SMILE
106 (System of Measurement Integrated for Littoral and Environment) buoy. It is a moored buoy
107 localized in the Bay of Seine (0°19'41.00"O 49°21'14.00"N) equipped with physico-chemical
108 and biological sensors working in continuous and autonomous conditions (data available
109 at Claquin et al., 2018).

110 *In vivo* fluorescence (Cyclops-6K, Turner Design, USA), fluorescein fluorescence units (FFU),
111 temperature (WTW™, TetraCon, Germany) and turbidity (Seapoint Turbidity Meter, Seapoint
112 Sensors, USA) were measured together in a multiparameter probe NKE instrument (MP7, NKE
113 Instrumentation ®). Photosynthetically active radiation (PAR) was measured with a Satlantic
114 sensor (Satlantics, Italy). NO₃⁻ was measured with an OPUS optical sensor (TriOS Mess- &
115 Datentechnik GmbH Germany). All these parameters were measured at 20-min intervals at a
116 depth of one metre since 2016.

117 PO₄³⁻ data and additional NO₃⁻ data come from a bimonthly sampling program (French Coastal
118 Observation Service, <https://www.somlit.fr/>, Cocquempot et al., 2019) where water has been
119 sampled near the SMILE buoy at a depth of one metre since 2013 and analysed as described in
120 Part 2.2.1.

121

122 **2.2 Enrichment experiment**

123 Bioassays were designed to assess the impacts of enrichment on the phytoplankton
124 assemblages and on their physiological responses. The design was modified from Ly et al.
125 (2014) to fit our specific requirements as described in Serre-Fredj et al. (2021). The
126 experiment was repeated 19 times over a period of 2 years on different dates (see

127 supplementary table for starting date, and Supplementary figure 2). Each incubation will be
128 referred to by its number corresponding to chronological order. Seawater was sampled at the
129 SMILE buoy site. To remove large grazers, the seawater was filtered through a 100- μm mesh
130 immediately after sampling. For the bioassays, enrichments were added to 500-ml subsamples
131 in polycarbonate bottles one hour after sampling and placed in an incubator. All bioassays
132 were incubated in a water bath incubator for 4 to 5 days (see supplementary table for specific
133 duration) under natural sunlight in a greenhouse. The water bath incubator was fuelled
134 continuously with seawater pumped directly from the sea maintaining the incubator to the
135 temperature of the bay. Seawater temperature and PAR were recorded at 5-min intervals using
136 an RBRsolo T logger and an RBR solo³ PAR logger connected to a Li-COR LI-192
137 Underwater Quantum Sensor, respectively. Up to six types of enrichment treatments, each
138 with five replicates, were performed in the incubator for each incubation experiment: Control
139 with no addition of nutrients (C), +P (P), +N (N), +N+Si (NSI), +N+P (NP), or +N+P+Si
140 (PNSI). The enrichments applied in the bioassays were defined by the maximum value of N
141 ($50 \mu\text{mol.l}^{-1}$), P ($3 \mu\text{mol.l}^{-1}$) and Si ($50 \mu\text{mol.l}^{-1}$) measured in 2018 in the Bay of Seine by the
142 French Coastal Observation Service (SOMLIT, <https://www.somlit.fr/>, Cocquempot et al.,
143 2019). Two enrichments were added during the experiment: NSI was added after the 4th
144 incubation experiment, and NP was added after the 13th incubation experiment.

145
146 After 4 or 5 days, 25 ml was sampled in all bioassay bottles and homogenized before measuring
147 photosynthetic, flow cytometer and alkaline phosphatase activity (APA). TEP, nutrient and
148 chlorophyll *a* (chl *a*) concentrations were also measured at day 4 or 5. APA was included after
149 the 7th incubation and TEPs after the 9th incubation.

150 The analyses, duration, and enrichments performed are summarized in the additional table.

151

152 **2.2.1 Measurements of inorganic nutrients (NO_3^- , PO_4^{3-} , Si(OH)_4)**

153 Water samples were collected and filtered through a ClearLine, CA, 33 mm, 0.45 μm cellulose
154 acetate filter and immediately frozen ($-20\text{ }^\circ\text{C}$), with the exception of Si(OH)_4 , which was stored
155 at $4\text{ }^\circ\text{C}$. Analyses were conducted using a SEAL Analytical AA3 system (Aminot and K erouel,
156 2007). The quantification limits were $0.02\text{ }\mu\text{mol.l}^{-1}$ for PO_4^{3-} and $0.05\text{ }\mu\text{mol.l}^{-1}$ for NO_3^- , NO_2^- ,
157 Si(OH)_4 . The N/P ratio was calculated as $\text{NO}_3^- / \text{PO}_4^{3-}$. The $\text{N/P}_{\text{consumed}}$ was calculated as $(\text{N}_{\text{start}} -$
158 $\text{N}_{\text{end}}) / (\text{P}_{\text{start}} - \text{P}_{\text{end}})$, where N_{start} and P_{start} represent the concentrations of N and P at the beginning
159 of the incubation (i.e., the stock of nutrients in the sampled water plus specific enrichment) and
160 N_{end} and P_{end} the concentrations of N and P at the end of the incubation.

161 **2.2.2 Chlorophyll-*a* measurements**

162 Water samples (250 ml) were filtered through a Whatman GF/F 47 mm, 0.7 μm glass-fibre filter
163 and immediately frozen ($-20\text{ }^\circ\text{C}$) until analysis. Ten millilitres of 90% acetone (v/v) was added
164 to extract the pigment, and the samples were then left in the dark at $4\text{ }^\circ\text{C}$ for 12 h. After being
165 centrifuged for 5 min at 1,700 g twice, the concentration of chl *a* in the extracts was measured
166 using a Trilogy fluorometer (Turner Designs, Sunnyvale, USA) according to the method of
167 (Strickland and Parsons, 1972). The $\Delta\text{chl } a$ is calculated as $\text{chl } a_{\text{end}} - \text{chl } a_{\text{start}}$.

168 **2.2.3 Transparent exopolymeric particles (TEP)**

169 Water samples (150-200 ml) were filtered through Millipore, 0.4 μm polycarbonate
170 membrane filters and immediately frozen ($-20\text{ }^\circ\text{C}$) until analysis. Following Claquin et al.
171 (2008) adapted from Passow and Alldredge (1996), the filters were stained with a solution of
172 0.02% Alcian Blue solution (Sigma) with 0.06% acetic acid (pH: 2.5). Excess dye was removed
173 by adding water before centrifugation at 3,000 g at $19\text{ }^\circ\text{C}$ for 15 min. This washing cycle was
174 repeated twice, after which 6 ml of 80% H_2SO_4 was added. After 2 h, measurements were taken
175 using a Pharmacia Biotech Ultrospec 1000 Spectrophotometer at 787 nm. Calibration was
176 performed using xanthan gum (10-700 μg) as a standard, as described in Claquin et al., (2008).

177 After being divided by the chl *a* concentration, TEP concentrations are expressed in $\mu\text{g Xanthan}$
178 $\text{eq.}\mu\text{g chl } a^{-1}$.

179 **2.2.4 Alkaline phosphatase activity (APA)**

180 The potential maximum APA per chlorophyll unit was measured according to Hrustic et
181 al. (2017). Samples (3,920 μl) were placed in a UV cuvette, and 80 μl of 500 μM 4-
182 methylumbelliferyl phosphatase (MUF-P) substrate was added. While incubating at room
183 temperature, the samples were measured at hourly intervals over a total period of 7 h.
184 Measurements were made with an RF-6000 spectrofluorophotometer (Shimadzu, Japan). APA
185 was calculated as the slope of the linear regression. Using an MUF standard curve, the results
186 are expressed in concentration units per hour divided by the concentration in chl *a* to normalize
187 by the biomass, thus obtaining $\text{APA}_{\text{chl } a}$ ($\text{nM}\cdot\text{h}^{-1}\cdot\mu\text{g chl } a^{-1}\cdot\text{l}^{-1}$).

188

189 **2.2.5 Fluorometry to assess photosynthetic parameters**

190 To measure the maximum quantum efficiency of photosystem II photochemistry (F_v/F_m), three
191 fluorimeters were used during the experiment: a Water PAM (Walz, Germany), a FRRf-ACT2
192 (Chelsea Technologies, UK) and a LabSTAF (Chelsea Technologies, UK). Oxidation of
193 quinone A (Q_A) in the samples was analysed after a 5-min period of incubation in the dark.

194 For the water-PAM measurements, the sample was excited by weak blue light (1 μmol
195 $\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 470 nm, frequency 0.6 kHz) to record the minimum fluorescence (F_0). The
196 maximum fluorescence (F_m) was obtained during a multiturnover (MT)-saturating light pulse
197 (0.6 s, 1700 $\mu\text{mol photons}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, 470 nm), which enabled reduction of the quinone A (QA)
198 pool.

199 For the ACT2-FRRF measurements, a single turnover (ST) saturation phase was delivered with
200 one hundred 1- μs flashlets at 2- μs intervals to measure F_0 and maximum fluorescence (F_m) (452
201 nm) using the biophysical model of Kolber et al. (1998).

202 For the LabSTAF measurements, a single turnover (ST) saturation phase was delivered with a
203 solid 100- μ s flash (450 nm) to measure minimum and maximum fluorescence, as described in
204 Boatman et al. (2019).

205 Following Genty et al. (1989), the maximum quantum efficiency of photosystem II
206 photochemistry (F_v/F_m) was calculated in Equation 1:

207

$$208 \quad \frac{F_v}{F_m} = \frac{(F_m - F_0)}{F_m} \quad (1)$$

209

210 A discrepancy in the F_v/F_m measurement is known between the different methods of variable
211 fluorometry used (Kromkamp and Forster, 2003). Therefore, data normalization per incubation
212 (between 0 and 1) allowed us to compare the whole set of incubations.

213

214 **2.2.6 Flow cytometry**

215 Unfixed samples were analysed within one hour of sampling with a CytoSense
216 (Cytobuoy b.v., Netherland) equipped with a blue laser (488 nm, 50 mW) and a green laser (552
217 nm, 50 mW). This produces pulse shapes based on the inherent optical properties of the particle
218 when they cross the laser: sideward angle scatter (SWS), forwards scatter (FWS), red (FLR,
219 668-734 nm), orange (FLO, 601-668 nm) and yellow fluorescence (FLY, 536-601 nm). The
220 threshold was set at 16 mV to reduce data acquisition concerning nonphotosynthetic particles
221 triggered on FLR; for each sample (5 per enrichment), 380 μ l was analysed with a speed of 2.0
222 μ l.s⁻¹. CytoSense can analyse chains, cells, or colonies between 1 and 800 μ m in diameter.
223 Microspheres of 1 μ m (yellow–green fluorescent, FluoSpheres®), 1.6 μ m (nonfluorescent,
224 provided by Cytobuoy) and 2 μ m, 6 μ m, 10 μ m, and 20 μ m (Fluoresbrite® YG microspheres,
225 Polyscience) were used to calibrate size recordings (daily use). To distinguish the phytoplankton

226 (Supplementary Fig. 1), five clusters were determined using the cells' optical properties and
227 attributed to *Synechococcus* spp., picoeukaryotes, nanoeukaryotes and microphytoplankton and
228 cryptophytes. The *Synechococcus* spp. cluster has the smallest FWS signal and a high orange
229 fluorescence (FLO) signal, which matches very small cells with a high concentration of
230 phycoerythrin.

231 Picoeukaryote cells are small cells ($< 2 \mu\text{m}$) and produce low FLR and FWS signals.
232 Nanoeukaryotic (2–20 μm) and microphytoplankton ($> 20 \mu\text{m}$) cells were differentiated from
233 picoeukaryotic cells using the amplitude of the FLR signal and the bead signal. Cryptophytes
234 clusters have higher FLO than *Synechococcus* due to the high concentrations of phycoerythrin
235 in their cells and an FWS equivalent to the nanoeukaryotic and microphytoplankton cells (Olson
236 et al., 1989; Thyssen et al., 2014).

237

238 **2.3 RUE calculation**

239 RUE is the amount of biomass produced per unit of supplied resource (Hodapp et al., 2019).
240 The equation proposed by Ptacnik et al. (2008) to calculate RUE (chl *a*/total P) is quite simple
241 and is the one most frequently reported in the literature (Chai et al., 2020; Filstrup et al., 2019;
242 Lehtinen et al., 2017; Yang et al., 2021) with total P as the limiting nutrient. This equation can
243 also be used with other limiting nutrients, including N (Amorim and Moura, 2021; Frank et al.,
244 2020; Olli et al., 2015; Otero et al., 2020). The dissolved organic fraction (DIN or DIP) can also
245 be used instead of the total elemental concentration (Amorim and Moura, 2021; Otero et al.,
246 2020). The need to consider stoichiometry constraints for RUE calculation has already been
247 highlighted in Frank et al. (2020).

248 Thus, to fit the colimitation hypothesis (Arrigo, 2005; Saito et al., 2008), we considered more
249 than one nutrient. The concept of colimitation and/or alternation between N and P limitation
250 according to Davidson and Howarth (2007) and Elser et al. (2007) describes how, depending

251 on the balance, N and P could incrementally limit growth one after another until one is too low
 252 for uptake. The nutrient present in the smallest proportion will be the last limiting nutrient in
 253 the sequence. To directly compare the concentration of both nutrients (N & P), a scale of
 254 limitation has to be applied. The simplest approach largely admitted is to consider the Redfield
 255 ratio as the balanced N/P ratio (Falkowski, 2000; Lips and Lips, 2008), even if it can be
 256 discussed primarily in the context of coastal anthropized ecosystems (Arrigo, 2005; Chorus and
 257 Spijkerman, 2020; Glibert and Burkholder, 2011). Then, N/P ratios lower than 16 are considered
 258 N limited, and ratios higher than 16 are considered P limited.

259 Based on the equation of Ptacnik et al. (2008), a RUE_{NP} equation using the N/P ratio was
 260 applied:

$$261 \quad RUE_{NP} = \frac{chl\ a_{end}}{\min\{\frac{N}{N/P_{balanced\ ratio}}, P\}} \quad (2)$$

262 where $chl\ a_{end}$ is the concentration at the end of the incubation, N and P concentrations
 263 correspond to values of N_{start} and P_{start} of NO_3^- and PO_4^{2-} , respectively, at the beginning of
 264 each incubation (i.e., the stock of nutrients in the sampled water plus specific enrichment).
 265 Here, the $N/P_{balanced\ ratio}$ accounts for the 16/1 Redfield ratio (Redfield, 1958) (Equation 2).
 266 The minimum function (min) makes it possible to account for the last limiting nutrient in the
 267 alternating nutrient limitation system.

268 In other words, this RUE_{NP} calculation is equal to RUE_P when $N/P > N/P_{balanced}$ and to RUE_N
 269 when $N/P < N/P_{balanced}$ (Figure 1). This prevents underestimation of the RUE value in
 270 dystrophic cases. To illustrate the case of an extremely high N/P (e.g., 300), the RUE_N would
 271 be reduced due to the excessive presence of N, even if the limiting nutrient here is P, thus
 272 making the RUE_P more appropriate. The RUE_{NP} allows to cover both dystrophic cases.

274

275 **2.4 Statistical analysis**

276 All analyses were conducted in R (R-project, CRAN) version 3.6.1. To better display
277 variations due to the enrichment data used for the heatmap (e.g., Figures 2, 3, 4 and 5) are all
278 normalized between 0 and 1 per incubation, such that the maximum value of the parameter in
279 each incubation is 1 and the minimum is 0. As the incubation data did not follow a normal
280 distribution, a Kruskal–Wallis test was performed using the “stats” package, and a Conover-
281 Iman pairwise test was performed using the “conover.test” package. Only significant tests
282 with a p value < 0.05 were accepted. PCA and hierarchical cluster analyses were performed
283 using the “FacoMineR” (Lê et al., 2008) and “factoextra” (Kassambara, 2017) packages. The
284 last six incubations were included in the multivariate analysis (13th to 19th incubations) to
285 include all types of enrichment. The number of clusters for the k-means clustering analysis
286 was chosen using silhouette analysis.

287

288 **3. Results and discussion**

289

290 3.1 Environmental parameter in the Bay

291

292 Figure 2 shows two years of high-frequency data in the Bay of Seine measured by the SMILE

293 buoy. The temperature ranged from 8.1 °C to 22.5 °C, and a maximum fluorescence value of

294 481 FFU was recorded on the date of the 19th sampling (12/10/2020). Fluorescence followed

295 standard patterns (Napoléon et al., 2012) with higher values in spring and summer, while late

296 blooms could occur in autumn, as shown by the 9th incubation event, which represented the

297 highest value in 2019 (170 FFU) (Fig. 2. A). The two inorganic nutrients (NO_3^- and PO_4^{3-})

298 displayed the opposite pattern of temperature, i.e., replenished in winter and depleted in

299 summer due to nutrient consumption. NO_3^- ranged from the limit of detection 0.01 to 69.27

300 $\mu\text{mol.l}^{-1}$ (Fig. 2. C) and PO_4^{3-} ranged from the limit of detection 0.02 to 1.01 $\mu\text{mol.l}^{-1}$ (Fig. 2.

301 D). A wide range of variation is depicted by the incubation that may affect phytoplankton

302 community and response even if a majority (60%) are gathered within the high production

303 zone from May to October. Out of this time period, light intensity may be more limiting than

304 nutrients, as displayed by replenishing nutrient levels (Napoléon et al., 2014, 2012).

305 Moreover, some extreme events are present, such as the 9th or 19th incubation, which both

306 account for massive blooms, as flagged by the fluorescence increase.

307

308 3.2 Phytoplankton growth link with nutrient balance

309 Figure 3 presents the chl a_{start} concentration measured at the beginning of the incubation and

310 final concentration for each enrichment for all bioassays and all enrichments. The results

311 showed that in 17 incubations out of 19, NPSi enrichment presented the highest chl a

312 concentration (Fig. 3. A). Both NP and PNSI enrichments showed high chl a concentrations

313 with averages of 20.9 $\mu\text{g chl } a^{-1}.\text{l}^{-1}$ and 24.5 $\mu\text{g chl } a^{-1}.\text{l}^{-1}$, respectively, and differed

314 significantly from other treatments (Kruskal–Wallis: $\chi^2 = 29.26$, $p_{\text{value}} = 5.4 \times 10^{-5}$; followed by
315 Conover test, $p_{\text{value}} = 3.6 \times 10^{-4}$, 8.7×10^{-4}). This result highlights evidence for colimitation of N
316 and P, as NP and PNSi enrichment displays maximum growth. The lack of a significant
317 difference between NP and NPSi indicates the secondary role of Si. Incubations 3, 5, 10, 11
318 and 12 display little to no growth regardless of the enrichment, indicating other limitations. As
319 underlined in Section 3.1, this could be due to suboptimal temperature and light conditions for
320 phytoplankton growth.

321 When compared to the log of the N/P_{consumed} ratio during the incubation, the chl *a* distribution
322 showed a peak. The weighted mean of the 9th percentile of the chl *a* distribution
323 corresponding to the N/P ratio peak value was 16.01, which corresponds to the Redfield ratio
324 (Fig. 3. B). This optimum growth of the phytoplankton community at a N/P Redfield is not
325 surprising (Klausmeier et al., 2004) and is in agreement with the results of a recent study by
326 Zheng et al. (2020), who showed a consumed N/P ratio of 16 in the overenriched Bohai Sea in
327 China. However, a discrepancy from this ratio could have been expected under dystrophic
328 input (Geider and La Roche, 2002; Glibert and Burkholder, 2011).

329

330 3.3 Limitation assessment by physiological parameter

331 3.3.1 APA - TEP

332 $TEP_{\text{chl } a}$ displayed a nonhomogenous distribution in the “high- $TEP_{\text{chl } a}$ concentration” groups,
333 with C, P, N, and NSi enrichments, and in the “low- $TEP_{\text{chl } a}$ concentration” group, with D₀, NP
334 and PNSi enrichments (Kruskal–Wallis: $\chi^2 = 36.5$, $p_{\text{value}} = 2.2 \times 10^{-6}$; followed by the Conover
335 test: mean p value = 5.9×10^{-4}) (Fig. 4. A). When $TEP_{\text{chl } a}$ concentrations were compared to
336 $\log(N/P)$, the minimum value of $TEP_{\text{chl } a}$ identified by the regression function corresponded to

337 an N/P ratio of 26.42, diverting from this minimum the function rise up at low and high N/P
338 values (Fig. 4. B).

339 The same approach was applied to the APA_{chl a} dataset. Data analysis of APA_{chl a} (Kruskal–
340 Wallis: $\chi^2 = 16.78$, $p_{\text{value}} = 1.0 \times 10^{-2}$; followed by the Conover test: mean $p_{\text{value}} = 3.0 \times 10^{-4}$)
341 highlighted two groups: “high APA_{chl a}” corresponding to C, N, NSi enrichments and “low
342 APA_{chl a}” corresponding to D0, P, NP and PNSi enrichments (Fig. 4. C). When compared to the
343 log(N/P ratio), the minimum value of APA_{chl a} of the regression function, which resembled a
344 parabola, corresponded to an N/P ratio of 16.26, diverting from this minimum, the function rose
345 faster at a high N/P ratio than at a low N/P ratio (Fig. 4. D).

346 As APA is produced by phytoplankton and prokaryotes to convert organic P into accessible
347 inorganic P (Falkowski and Raven, 1998; Lin et al., 2016), it is used as an indicator of P
348 limitation (Serre-Fredj et al., 2021; Tanaka et al., 2006). Enrichment without P addition (*e.g.*,
349 C, N and NSI) displays a higher APA value, which is in agreement with Elser and Kimmel
350 (1986). Surprisingly, no difference was found between the D0 value of APA and the APA
351 measured for P, NP and PNSI enrichments. This result may contradict a potential P limitation.
352 Even after sustaining P, APA levels may remain high due to internal storage of the alkaline
353 phosphatase enzyme (Litchman and Nguyen, 2008).

354 When compared to the N/P ratio values, the increase in APA at a low N/P (*i.e.*, N-limited) ratio
355 can be disconcerting but has already been reported in the literature (Kuenzler and Perras, 1965).
356 APA production can be triggered by N limitation (Kuenzler and Perras, 1965) or change as a
357 function of the community structure (Lin et al., 2015; Yuan et al., 2017). Our results show that
358 a high level of APA may reveal an unbalanced N/P ratio rather than being an absolute indicator
359 of P limitation.

360 TEPs are indicators of carbon excretion (Claquin et al., 2008; Klein et al., 2011) and could be
361 used as a metabolic overflow of carbon under nutrient limitation. Although Claquin et al. (2008)

362 showed that TEPs are also produced during balanced growth, in the literature, an increase in
363 TEP production has been reported to be associated with N limitation (Beauvais et al., 2003;
364 Corzo et al., 2000; Deng et al., 2016), with P limitation (Pandey and Pandey, 2015) and with
365 variations in the N/P ratio (Engel et al., 2015; Mari et al., 2005). Our results are consistent with
366 these hypotheses, suggesting that digressing from a balanced N/P ratio (i.e., N/P = 16) increases
367 TEP production. Only the addition of at least N and P appeared to reduce TEP production. All
368 other partial enrichments (C, N, P and NSi) increased TEP, which is consistent with the nutrient
369 limitations of the phytoplankton community we identified in this study.

370 Some exceptions to this general trend were observed. During the 10th (16/09/2019) incubation,
371 a high concentration of TEPs was associated with an N/P ratio near the Redfield ratio. High
372 TEP concentrations are frequently observed at the end of summer due to increased
373 phytoplankton biomass (Parinos et al., 2017; Serre-Fredj et al., 2021), and Claquin et al. (2008)
374 showed that under balanced growth, an increase in temperature was associated with an increase
375 in TEP production by diatoms. The high value of TEPs in the 10th incubation could be caused
376 by a high initial stock of TEPs in the water sample because of a bloom of *Lepidodinium*
377 *chlorophorum* (Serre-Fredj et al., 2021), a huge TEP producer (Claquin et al., 2008), which
378 occurred two weeks before (Serre-Fredj et al., 2021).

379

380 The physiological response of the phytoplankton community, illustrated here by APA and TEPs,
381 supports the hypothesis of the optimum balance of the N/P ratio of approximately 16, as the
382 addition of balanced nutrients reduces the values of both physiological indicators. In addition,
383 the dynamics of both indicators (APA and TEPs) confirmed the secondary role played by Si
384 inputs in the bioassays.

385

386 3.3.2 Maximum quantum efficiency of photosystem II (Fv/Fm)

387 The Fv/Fm in the bioassays ranged from 0.19 to 0.62. These values display intraincubation
388 patterns that distinguish the two groups. In 9 out of 13 incubations (70%), complete enrichment
389 showed the highest values among incubation, resulting in PNSI being a separate group
390 (Kruskal–Wallis: $\chi^2 = 20.6$, $p_{\text{value}} = 3.8 \times 10^{-4}$, Conover: mean = 2.2×10^{-3}). In all other cases, D0
391 displayed the maximum value but was not significantly different from the other enrichments
392 (Fig. 5. A). In addition to influencing the biomass or physiology of the bulk community,
393 enrichment with a different stoichiometry affects the structure (Piehler et al., 2004), growth
394 (Watanabe et al., 2017) and photosynthesis (Song et al., 2019) of the phytoplankton community
395 (Serre-Fredj et al., 2021). A decrease in the maximum quantum yield of photosystem II (Fv/Fm)
396 has been associated with many factors, including temperature (Zhang et al., 2012), parasite
397 attacks (Park et al., 2002) and particularly nutrient limitation (Behrenfeld et al., 2004; Claquin
398 et al., 2010). Here, the addition of both N and P increased this physiological indicator status in
399 most cases, which is in accordance with the APA and TEP dynamics.

400

401 3.5 Phytoplankton community

402 The concentration of *Synechococcus* dropped in most incubations regardless of the enrichment.
403 NP enrichment was still identified as a separate group (Kruskal–Wallis: $\chi^2 = 16.8$, $p_{\text{value}} =$
404 7.8×10^{-3} , Conover: mean = 4.4×10^{-3}) due to the high concentration of *Synechococcus* measured
405 in the final (from the 15th -16/06/2020 to the 19th – 12/10/2020) incubations, with a maximum
406 of 8.9×10^4 cells.cm⁻³ in the 19th incubation (Fig. 5. B).

407 The concentration of picoeukaryotes appeared to be affected over time, and PNSI enrichment
408 was identified as a separate cluster due to the high concentration of this population (Kruskal–
409 Wallis: $\chi^2 = 22.3$, $p_{\text{value}} = 4.0 \times 10^{-4}$, Conover: mean = 5.2×10^{-4}), as the maximum value was
410 1.7×10^5 cells.cm⁻³ in the 4th incubation, PNSI enrichment (Fig. 5. C). Nanoeukaryotes followed
411 exactly the same pattern as picoeukaryotes, with PNSI enrichment identified as a separate

412 population (Kruskal–Wallis: $\chi^2 = 25.2$, $p_{\text{value}} = 1.3 \times 10^{-4}$, Conover: mean = 3.6×10^{-3}). The pattern
413 of cryptophytes was less certain, depending on the incubation, the maximum value could be
414 reached by all enrichments, nonetheless, NP was highlighted as a separate group (Kruskal–
415 Wallis: $\chi^2 = 20.0$, $p_{\text{value}} = 9.3 \times 10^{-4}$, Conover: mean NP= 4.4×10^{-3} , mean PNSI= 2.3×10^{-3}), as the
416 *Synechococcus*-normalized value of cryptophytes was high in the final incubations (15th to 19th)
417 (Fig. 5. C).

418 The concentration of microphytoplankton followed a pattern similar to that of the
419 nanoeukaryotes, and PNSI was again identified as a separate group (Kruskal–Wallis: $\chi^2 =$
420 25.0 , $p_{\text{value}} = 1.1 \times 10^{-4}$, Conover: mean = 5.0×10^{-4}) (Fig. 5. D). The size structure of the
421 phytoplankton population must be taken into account, as PNSi enrichment allowed maximum
422 growth of the picoeukaryotes, nanoeukaryotes and microphytoplankton in most of the
423 incubations. With the exception of the 15th and 16th incubations, *Synechococcus* seemed
424 unable to grow in these conditions. This result could be a bias caused by the closed system,
425 which could increase predatory pressure associated with a higher death rate of this taxon
426 (Agawin et al., 2000). Another possible hypothesis is that larger cells, such as diatoms,
427 compete more successfully with high enrichment pulses than *Synechococcus* and cryptophytes
428 (Van Meerssche and Pinckney, 2019).

429

430 3.6 Multivariate analysis and overall trend

431 Figure 6 shows the multivariate results of the last six incubations (see Section 2.4 for
432 precision on the choice). The two dimensions of the PCA explained up to 66.3% of the total
433 variance and are summarized by a few statements (Fig. 6. A): The chl *a* concentration was
434 positively correlated with the concentration of picoeukaryotes, nanoeukaryotes and
435 microphytoplankton (i). APA, TEPs, RUE and the proportion of picoeukaryotes were
436 positively intercorrelated and negatively correlated with chl *a* (ii). The proportions of

437 microphytoplankton and nanoeukaryotes were opposite but not correlated with any of the
438 other parameters (iii). This pinpoints a change in the phytoplankton community linked with
439 nutrient enrichment and the N/P ratio, as limitations seem to favour populations with higher
440 proportions of picoeukaryotes, while replete and balanced conditions favour nanoeukaryote
441 and microphytoplankton populations.

442 Cluster analysis identified three groups (Fig. 6. B & C): The first group was only composed of
443 C, N, P and NSi enrichments, the second group was composed of NP and PNSi enrichments,
444 with the exception of P enrichment in the 13th incubation, and the last group was composed of
445 all the enrichments in the 19th incubation already highlighted as an extreme event of massive
446 bloom.

447

448 3.7 Link between RUE and the phytoplankton community

449 RUE is usually calculated as chl *a* or the primary production divided by N or P (Filstrup et al.,
450 2014; Olli et al., 2015; Ptacnik et al., 2008), but our result highlights the fact that N and P
451 colimitation and the stoichiometry constraint summarized in the N/P ratio are important
452 regarding the RUE (Frank et al., 2020). Moreover, both the increase in chl *a* and phytoplankton
453 physiological parameters (TEPs and APA) suggest that the Redfield ratio is optimum for
454 community uptake. In our case, only accounting for nutrients would have involved loss of
455 information and an important skew due to a colimited system. The equation of RUE_{NP} we use
456 accounts for both the quantity of nutrients—by directly using the concentration—and the
457 balance of nutrients—by prioritizing the limiting nutrient. This kind of model, which resembles
458 multiple resource use efficiency (*mRUE*), could be more effective and help understand both
459 resource use efficiency and ecosystem production (Han et al., 2016).

460 The relation between RUE_{NP} and the cytometric size structure of the phytoplankton population
461 (Figure 7) pointed out two trends: the proportion of picoeucaryotes was negatively correlated

462 ($r^2 = 0.13$, $F = 13.7$, $p_{\text{value}} = 3.8 \times 10^{-4}$) with RUE_{NP} (Fig. 7. A), while both proportions of the
463 largest phytoplankton classes, nanoeukaryotes ($r^2 = 0.13$, $F = 11.3$, $p_{\text{value}} = 1.2 \times 10^{-3}$) and
464 microphytoplankton ($r^2 = 0.27$, $F = 29.2$, $p_{\text{value}} = 6.8 \times 10^{-7}$), were positively correlated with
465 RUE_{NP} (Fig. 7. B & C).

466 Concerning the RUE, we provide evidence for a relationship between size-structure groups and
467 RUE_{NP} , where smaller classes of phytoplankton ($< 2 \mu\text{m}$) are negatively correlated and higher
468 classes ($> 2 \mu\text{m}$) are positively correlated. RUE has been positively linked with diversity (Chai
469 et al., 2020; Otero et al., 2020; Paczkowska et al., 2019), but to our knowledge, no information
470 linking size structure with RUE has been reported to date. The use of size class instead of classic
471 diversity may be more powerful, as cell size largely controls the efficiency of the nutrient uptake
472 rate (Zaoli et al., 2019).

473 Furthermore, the coastal environment with rich inputs found in the Bay of Seine creates a
474 dynamic nutrient system with nutrient pulses (e.g., inputs from local rivers). Larger cells can
475 have 200,000 and 20,000 times more biovolume and carbon/cell volume, respectively, than
476 smaller cells (Harrison et al., 2015), and larger cells may grow better and especially have
477 more storage capacity, which helps them maintain a highly dynamic system (Malerba et al.,
478 2018). Their storage capacity and luxury uptake of carbon may also render night-time nutrient
479 uptake possible (Gardner-Dale et al., 2017), and larger cells may have even better carbon
480 acquisition (Malerba et al., 2021). These characteristics balance the lower nutrient uptake of
481 picoeukaryotes (Hein et al., 1995; Probyn and Painting, 1985). In this study, colimitation was
482 observed in the incubations. The balance of a Redfield $\text{N}/\text{P}_{\text{consumed}}$ ratio has been highlighted
483 as optimum for increased biomass of the phytoplankton population, and both APA and TEPs
484 pointed to the same ratio needed to reduce nutrient stress. No other single resource use
485 efficiency can fulfil these needs compared to RUE_{NP} . This study provides evidence that using
486 stoichiometry (Frank et al., 2020) and multiple resources in resource use efficiency ($m\text{RUE}$)

487 (Han et al., 2016) can advance our understanding of resource use efficiency and ecosystem
488 production. Furthermore, the relationship highlighted between RUE and size community
489 structure should help to understand and establish ecosystem trajectories as a function of
490 management programs to reduce nutrient inputs in coastal ecosystems.

491 **4. Conclusion**

492
493 This study investigated the effect of nutrient composition on the growth, population structure,
494 and physiological status of phytoplankton using bioassays. The N/P ratio was shown to be the
495 main driver of the phytoplankton community composition and physiology in the Bay of Seine,
496 even if both a balanced (e.g., N/P = 16) and a nutrient stock are needed to maintain
497 phytoplankton growth. The nutrient balance of the inputs affects both the physiological and
498 population structure of phytoplankton. To fulfil our requirements, we proposed a new RUE_{NP}
499 approach, which showed a correlation with the size structure of the phytoplankton
500 community.

501

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510

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