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# Environmental Pollution

## Transient effect of bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP) on the cosmopolitan marine diatom *Chaetoceros decipiens-lorenzianus* --Manuscript Draft--

<b>Manuscript Number:</b>	ENVPOL-D-21-00859R2
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<b>Abstract:</b>	Incubation under controlled laboratory conditions were performed to assess the toxic effects of two plastic derived chemicals, bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP), on the growth, photosynthetic efficiency and photosynthetic activity of the cosmopolitan diatom <i>Chaetoceros decipiens-lorenzianus</i> . Non-axenic diatom cells were exposed to concentrations of BPA and DEHP (separately and in mixture), mimicking concentrations observed in contaminated marine ecosystems, for seven days. Upon short-term exposure (i.e., during the first 48 h), BPA and DEHP induced a slight but significant stimulation of biomass and photosynthetic activity relative to the control, whereas, no significant impact was observed on the photosynthetic efficiency. Nevertheless, this pattern was transient. The stimulation was followed by a return to control conditions for all treatments at the end of incubation. These results showed that the cosmopolitan diatom <i>Chaetoceros</i> was not impacted by representative in situ concentrations of plastic derivatives, thus confirming its ability to thrive in coastal anthropogenic environments.
<b>Suggested Reviewers:</b>	Marta Staniszewska, PhD Uniwersytet Gdanski marta.staniszewska@ug.edu.pl Dr. Staniszewska is working on marine pollution and environmental pollutants such the plastic derived chemicals.  Wan Jun Kit University of Kuala Lumpur: Universiti Kuala Lumpur wanjunkit@gmail.com Dr. Jun Kit is working on the impacts of hazardous compounds on microalgae.  Jie Wei Dalian Ocean University weijie@dlo.u.edu.cn One of the main interest of Prof. Wei is the ecotoxicological answer of phytoplankton to plastic additives: phthalate esters.  Hyeon Ho Shin, PhD Korea Institute of Ocean Science and Technology shh961121@kiost.ac.kr Dr. Shin is a marine biologist. He is also expert on environmental changes, pollutants and marine microorganisms.

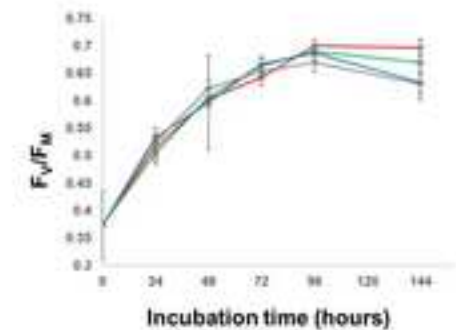
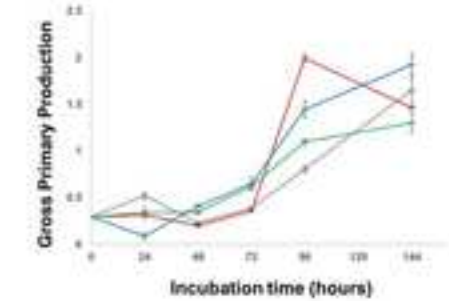
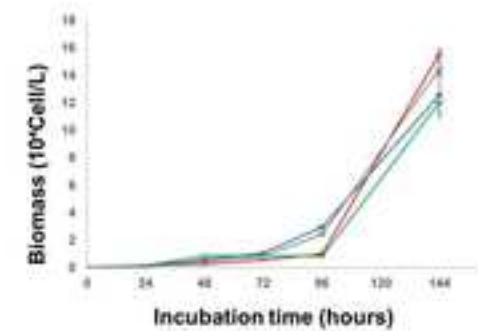
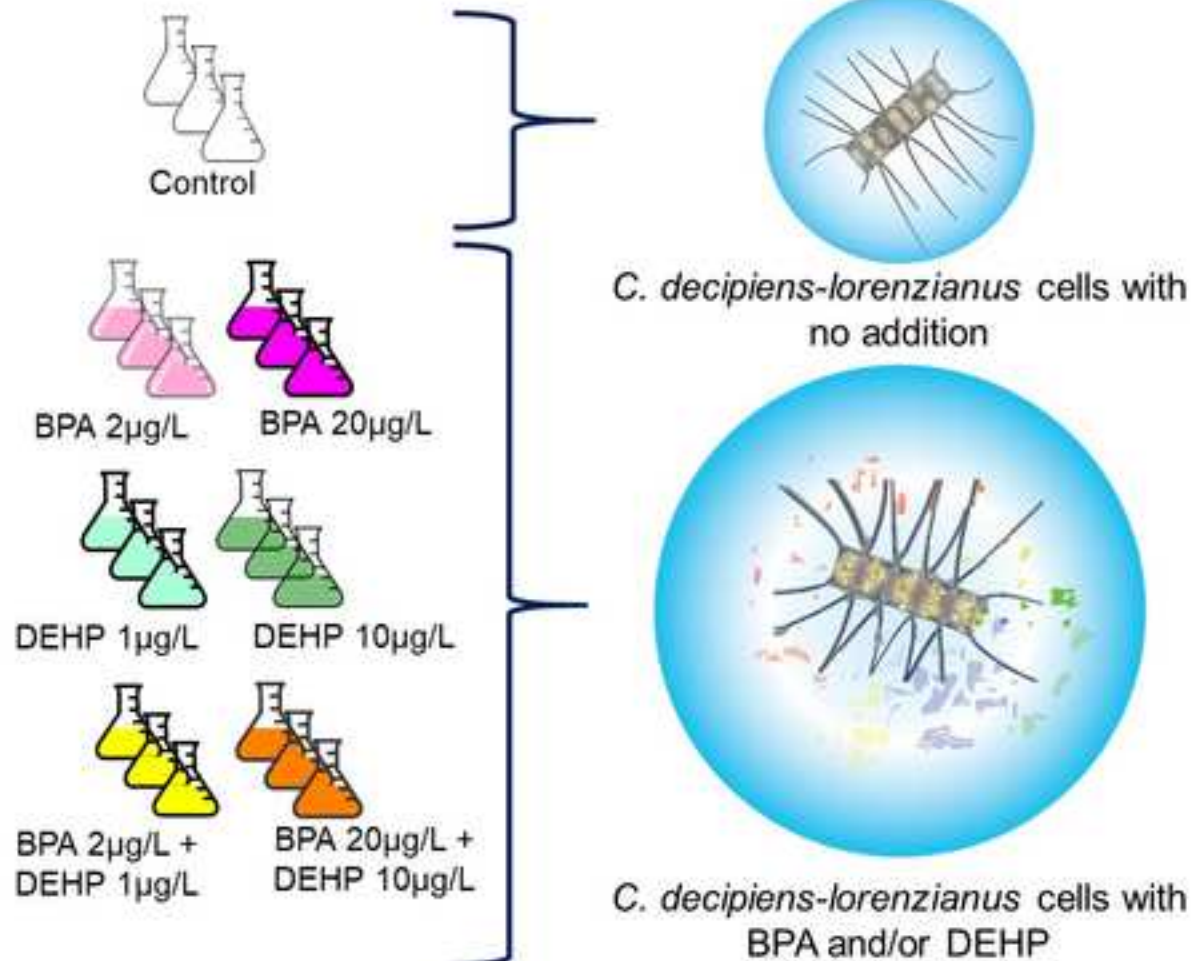
	Suk Min Yun, PhD Sangmyung University horriwar@nnibr.re.kr Dr. Yun is a marine ecologist. He is expert in phytoplankton, particularly diatoms.
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<b>Response to Reviewers:</b>	
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**Highlights:**

- BPA and DEHP contamination do not significantly impact *C. decipiens-lorenzianus*.
- Slight impacts were observed on the biomass and photosynthetic activity over 48 h.
- *C. decipiens-lorenzianus* might be identified as a tolerant species to BPA and DEHP.
- This diatom might be tolerant to BPA and DEHP at environmental concentrations.

## Incubation under controlled laboratory conditions



1 **Transient effect of bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP)**  
2 **on the cosmopolitan marine diatom *Chaetoceros decipiens-lorenzianus***

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16 **Abstract**

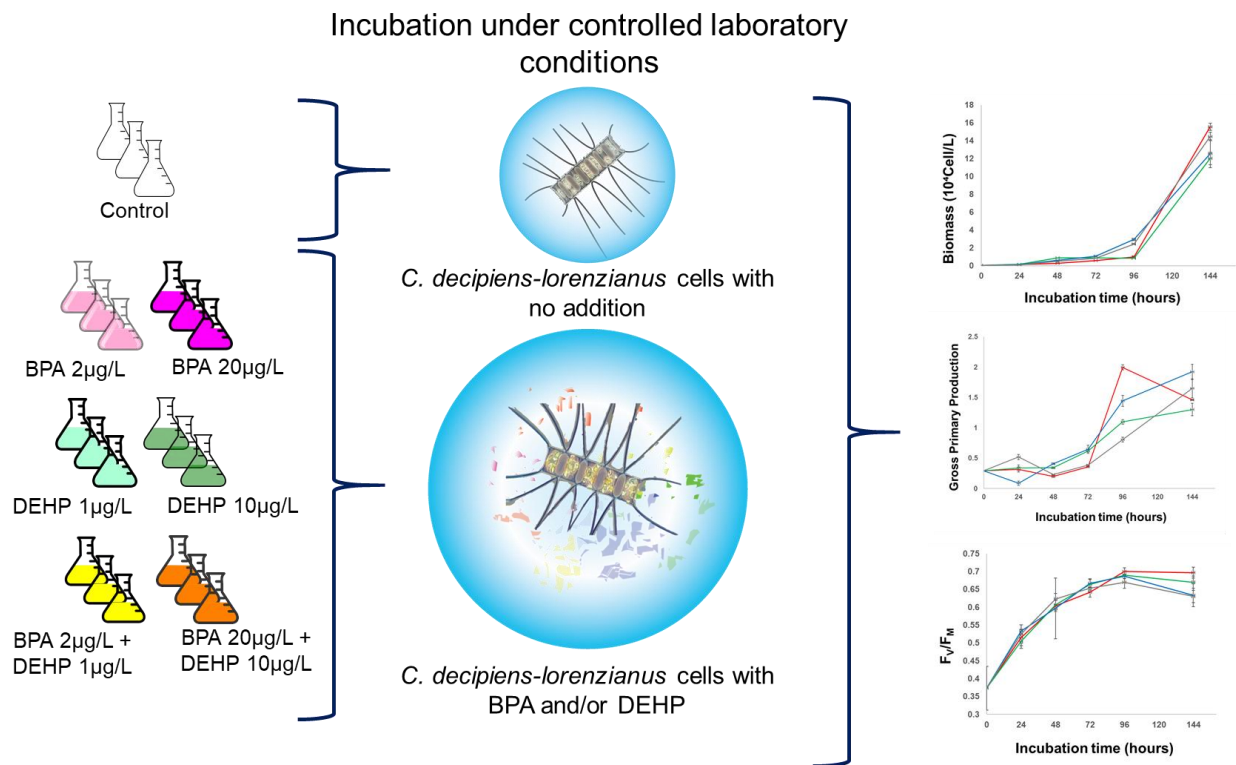
17 Incubation under controlled laboratory conditions were performed to assess the toxic  
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25 significant impact was observed on the photosynthetic efficiency. Nevertheless, this pattern  
26 was transient. The stimulation was followed by a return to control conditions for all  
27 treatments at the end of incubation. These results showed that the cosmopolitan diatom  
28 *Chaetoceros* was not impacted by representative *in situ* concentrations of plastic  
29 derivatives, thus confirming its ability to thrive in coastal anthropogenic environments.

30 **Keywords:** *Chaetoceros decipiens-lorenzianus*, bisphenol A, di-(2-ethylhexyl) phthalate,  
31 biomass, photosynthetic efficiency and activity

32 **Capsule:**

33 The marine cosmopolitan diatom *C. decipiens-lorenzianus* seemed to be tolerant to BPA and  
34 DEHP contamination, at in situ concentrations.

35 **Graphical abstract:**



37 **Highlights:**

- 38
- BPA and DEHP contamination do not significantly impact *C. decipiens-lorenzianus*.
  - Slight impacts were observed on the biomass and photosynthetic activity over 48 h.
  - *C. decipiens-lorenzianus* might be identified as a tolerant species to BPA and DEHP.
  - This diatom might be tolerant to BPA and DEHP at environmental concentrations.
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- 40
- 41

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## 43 **1. Introduction**

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44 As marine plastic debris becomes a topic of emerging concern (Galgani et al., 2019;  
45 Hahladakis, 2020), scientific interest on the environmental impact of this debris increased  
46 remarkably. The number of scientific publications dealing with this topic reached over 37,200 in  
47 the last decade (Abalansa et al., 2020; Schmid et al., 2021). In aquatic environments, this plastic  
48 debris is accumulated and degraded by various means (e.g., biodegradation, hydrolysis), forming  
49 smaller particles called microplastics (Booth et al., 2017; Sharma and Chatterjee, 2017).  
50 Furthermore, microplastic fragments release harmful compounds (Hermabessiere et al., 2017),  
51 associated with plastic derived compounds or plastic additives, such as phthalic esters, bisphenol,  
52 lindane, atrazine and polybrominated diphenyl ethers (Hong et al., 2018; Lithner et al., 2011;  
53 Sharma and Chatterjee, 2017).

54 Among these plastic additives, bisphenol A (BPA) and di-(2-ethylhexyl) phthalate (DEHP)  
55 are used extensively on a global basis as plasticizers (Erythropel et al., 2014; Warner and Flaws,  
56 2018). These two man-made compounds are known to act as endocrine disruptor chemicals  
57 (Brossa et al., 2005; Oehlmann et al., 2009). Moreover, in the environment, another potential  
58 danger is known as the 'cocktail effect' due to the occurrence of pollutants in the mixture  
59 (Backhaus et al., 2003; Lloyd-Smith and Immig, 2018; Svingen and Vinggaard, 2016). Several  
60 studies reported the occurrence of these compounds in the marine environment at concentrations  
61 between 0.1-10 µg/L in estuaries (Careghini et al., 2014; Liu et al., 2010b; Zhang et al., 2018),  
62 < 0.1 µg/L in lagoons (Pojana et al., 2007), ≤ 0.1 - 6 µg/L in coastal seawaters (M'Rabet et al.,  
63 2019; Paluselli et al., 2017; Sánchez-Avila et al., 2012) and < 0.1 µg/L in ocean (Corrales et al.,  
64 2015; Zhang et al., 2019).

65 As they are associated with Endocrine Disruptor Chemicals (EDCs), studies have  
66 highlighted their hazardous impacts on marine organisms (Pojana et al., 2007; Rehman et al., 2017;  
67 Windsor et al., 2018). BPA and DEHP potentially affect the physiology, growth, metabolism, and  
68 reproduction of fishes (Kim et al., 2011; Saili et al., 2012), crustaceans (Cole et al., 2011; Liu et  
69 al., 2009), invertebrates (Canesi et al., 2007; Fromme et al., 2002; Porte et al., 2006), seabirds  
70 (Coffin et al., 2019) and marine mammals (Hart et al., 2018; Mathieu-Denoncourt et al., 2015; Net  
71 et al., 2015).



72 Studies on their toxic effect were more focused on aquatic organisms possessing an  
73 endocrine system while less attention has been given to those lacking this system, such as primary  
74 producers. Most studies focused on the ability of microalgae to bioaccumulate, biodegrade, and  
75 transfer these molecules to higher trophic levels with little attention being given to their  
76 ecotoxicological impact (Crain et al., 2007; Liu et al., 2010a; Xu et al., 2015). Nevertheless, recent  
77 studies highlighted the significant negative impact of these EDCs on the growth and metabolic  
78 activity of some phytoplankton species. For example, contamination with DEHP at 10 mg/L,  
79 which is much higher than environmental concentrations, showed growth inhibition and  
80 physiological disruption associated with oxidative stress in Chlorophyceae *Chlorella vulgaris*  
81 (Shen et al., 2019). Similarly, a significant biomass decrease was reported with the dinoflagellate  
82 *Cochlodinium polykrikoides*, renamed *Margalefidinium polykrikoides* (Gómez et al., 2017), upon  
83 72 h of exposure to BPA at 68.5 mg/L (Ebenezer and Ki, 2012). Although the ecotoxicological  
84 response of phytoplankton to organic pollutants has been considerably investigated (Echeveste et  
85 al., 2016; Filimonova et al., 2016; Menezes-sousa et al., 2018), nowadays, and to our knowledge,  
86 few studies have assessed the impact of EDCs at realistic environmental concentrations.  
87 Interestingly, relevant environmental concentrations of EDCs, resulted in a biomass decrease as  
88 well as physiological inhibition, for the potentially toxic dinoflagellate *Alexandrium pacificum* (C.  
89 M'Rabet et al., 2018) and diatom *Nitzschia palea* (Debenest et al., 2011).

90 Among marine phytoplankton, diatoms are a highly productive and diverse group (Norton et al.,  
91 1996; Jensen and Moestrup, 1998). Diatoms contribute to ocean biodiversity with 136 genera, with  
92 a considerable number of species without precise identification (20,000 - 200,000) (Guiry, 2012;  
93 Mann and Vanormelingen, 2013). They are cosmopolitan organisms, occurring in coastal  
94 ecosystems, open waters, temperate environments, and polar regions (Deng et al., 2017; Gogorev  
95 et al., 2016; Malviya et al., 2016). Diatoms are one of the principal sources of oxygen production  
96 in marine environments accounting for up to 40% of total production (Hildebrand, 2008). They  
97 are also involved in different biogeochemical cycles, ensuring approximately 20% of CO<sub>2</sub> fixation  
98 (Falkowski et al., 1998; Field et al., 1998; Goldman, 1993). Due to their characteristics  
99 (cosmopolitan, short generation time and efficient proxy for environmental changes), diatoms are  
100 commonly used in ecotoxicological studies (Fourtanier and Kociolek, 1999; Puspitasari et al.,  
101 2018; Stevenson and Pan, 2010). They have been classified as bioindicators of aquatic  
102 environmental perturbation (Hourmant et al., 2009; Pandey et al., 2017).

103 Within the diatom group, the *Chaetoceros* genus is considered as one of the most abundant diatoms  
104 in marine phytoplankton (De Luca et al., 2019), with an abundance that can exceed  $8 \cdot 10^5$  during  
105 phytoplankton bloom Cells/L (Leblanc et al., 2012; Shevchenko et al., 2006). *Chaetoceros*  
106 *decipiens* is a cosmopolitan species, commonly found in coastal waters as well as in the open sea  
107 (Balzano et al., 2017; Batistić et al., 2017; Li et al., 2017). In the Mediterranean sea, *C. decipiens*  
108 is one of the dominant phytoplankton species (Tas and Hernández-Becerril, 2017; Yahia-Kéfi et  
109 al., 2005). For example, in the northeastern Adriatic Sea, *C. decipiens* occurred among the  
110 dominant phytoplankton species with  $9 \cdot 10^4$  Cells/L (Viličić et al., 2009). Notably, that several  
111 studies reported remarkable similarities between *C. decipiens* and *C. lorenzianus*, suggesting the  
112 existence of an intermediate form between these two species, the *C. decipiens-lorenzianus*  
113 complex (Bosak et al., 2016; Kownacka et al., 2013; Sunesen et al., 2008).

114 In this study, we aimed to understand the impact of two commonly used plastic additives,  
115 BPA and DEHP on the cosmopolitan diatom *C. decipiens-lorenzianus*. Since plastic derived  
116 chemicals can have a negative impact on phytoplankton activities, we hypothesized that a  
117 representative EDC would provoke growth inhibition and damage of the photosynthetic apparatus  
118 expressed through the reduction of photosynthetic efficiency and activity.

## 119 **2. Material and methods**

### 120 **2.1- Diatom culture**

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121 A strain of the *C. decipiens-lorenzianus* complex, an autotrophic diatom species, was  
122 isolated from Bizerte Channel (South-Western Mediterranean Sea 37.269°N, 9.877°E) in  
123 December 2016. This diatom strain was grown in F/2 medium (34.5‰ salinity) enriched with  
124 silicates (Guillard and Kilham, 1976). The species was maintained under controlled laboratory  
125 condition at 22°C and illuminated at an irradiance level of approximately 110  $\mu\text{mol photons /m}^2 \cdot \text{s}^{-1}$   
126 under a 12:12 light: dark cycle.

### 127 **2.2. Taxonomic identification of the strain**

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128 Taxonomic identification was performed using PCR amplification of a partial large subunit  
129 of ribosomal DNA (LSU rDNA, 28S). A polymerase chain reaction (PCR) was carried out using  
130 1  $\mu\text{l}$  of concentrated culture resuspended in distilled water. According to Taq Polymerase  
131 (Promega) instructions, amplification of the region D1/D2 was obtained using primers D1R and

132 D3B (Nézan et al., 2012). Sequencing products were run on an ABI PRISM 3130 Genetic Analyzer  
133 (Applied Biosystems). The obtained sequence was aligned with 145 other sequences of different  
134 putative *Chaetoceros* spp. and two outgroup sequences used by Chen et al (2018) using BioEdit  
135 v. 7.0.9.0 software (Hall 1999). Alignment was produced using the MUSCLE v. 3.7 algorithm  
136 (Edgar, 2004). The resulting matrix comprised 148 OTU and 650 characters. The evolutionary  
137 model and parameters were selected after running Smart Model Selection in PhyML (Lefort et al.,  
138 2017). For the present dataset, SMS selected the general time reversible (GTR) model with a  
139 gamma correction (G) for among-site rate variation and invariants sites. Maximum likelihood  
140 analyses were performed using PhyML version 3.0. (Guindon et al., 2010) and Bayesian analyses  
141 were run using Mr Bayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Bootstrap analysis  
142 (1000 replicates) was used for the maximum likelihood (ML) to assess the relative robustness of  
143 branches (bootstrap values, bv). Initial Bayesian analyses were run with a GTR model (nst = 6)  
144 with rates set to invgamma and nucleotide frequencies set to equal. Each analysis was performed  
145 using four Markov chains (MCMC) with two millions cycles for each chain. Trees were saved to  
146 a file every 100 cycles and the first 2000 trees were discarded. Therefore, a majority-rule consensus  
147 tree was created from the remaining 18000 trees to examine the posterior probabilities (pp) of each  
148 clade. The consensus trees were edited using TreeView version 1.6.6. The best ML phylograms  
149 were shown with their robustness values for each node (ML, bv / BI, pp).

### 150 **2.3. Experimental design**

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151 Toxicity experiments were performed with non-axenic batch cultures of *C. decipiens-*  
152 *lorenzianus* exposed to BPA and DEHP in 250 mL Erlenmeyer flasks following the protocols of  
153 M'Rabet et al (2018). Inoculum of 300 cells/mL were used to start the culture experiments.  
154 Different nominal concentrations of BPA and DEHP were added separately to the cultures and in  
155 mixture (BPA and DEHP), as follow: BPA at 2 µg/L and 20 µg/L, DEHP at 1 µg/L and 10 µg/L,  
156 and mixtures with BPA 2 µg/L + DEHP 1 µg/L and BPA 20 µg/L+ DEHP 10 µg/L. Incubations  
157 were run in triplicate, with respect to the control conditions (no BPA and DEHP addition), for  
158 144h.

### 159 **2.4. Chemical analyses**

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160 Analytical analyses are described in more detail in the supplementary material. BPA and  
161 DEHP analysis were performed at the beginning of the experiment ( $T_0$ ) to validate the nominal  
162 concentration added, using LC/MS and SPME coupled with GC/MS (M'Rabet et al., 2019).

## 163 **2.5. Growth measurement**

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164 A 1 mL aliquot of each treatment was fixed with acidic lugol to enumerate cell abundances  
165 at 0 (incubation-start), 24, 48, 72, 96 and 144 h (incubation-end), to assess the impact on the short,  
166 mid-, and long-term exposure to pollutants. Like all phytoplankton species, *C. decipiens-*  
167 *lorenzianus* has an exponential growth (lag phase, exponential phase, stationary phase and decline  
168 phase). In this study, the cellular concentration was checked by inverted microscope counting  
169 (Leica 521,234) during the incubation period. For each treatment, the growth rate (GR) was  
170 calculated after logarithmic transformation of the cell density. The following equation was applied:  
171  $GR = (\ln N_i - \ln N_{i-1}) / (T_i - T_{i-1})$ , where  $N_i$  and  $N_{i-1}$  represent the cell density (cells/mL) at the  
172 beginning ( $T_{i-1}$ ) and end ( $T_i$ ) of the exponential phase of the incubation period, respectively  
173 (Guillard et al., 1973).

## 174 **2.6. Photosystem II (PSII) efficiency**

### 175 **2.6.1. Quantum yield of the PSII: $F_V/F_M$ measurements**

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176 To assess the effect of BPA and DEHP on photosynthetic performance, in vivo chlorophyll  
177 fluorescence was measured using a portable Pulse Amplitude Modulation fluorometer PSI-AP-  
178 100 (AquaPen- Photon Systems Instruments). The OJIP-transient protocol was applied to calculate  
179  $F_V/F_M$  (Strasser and Srivastava, 2000). Before measurement, a subsample of 3 mL of each  
180 treatment was dark-adapted for 30 min to inactivate the photosynthetic activity according to the  
181 manufacturer's operating manual. The quantum yield of PSII was calculated according to the  
182 following equation  $F_V/F_M = (F_M - F_0) / F_M$ , where  $F_0$  is the minimum fluorescence in the dark-  
183 adapted state,  $F_M$  is the maximum fluorescence in the dark-adapted state, and  $F_V$  is the maximum  
184 variable fluorescence yield.

### 185 **2.6.2. Light curves and photosynthetic parameters ( $\alpha$ , $P_{MAX}$ , and $E_K$ )**

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186 To determine the relative electron transport rate versus irradiance (rETR versus E) curves,  
187 the LC3 protocol of the Pulse Amplitude Modulation fluorometer PSI-AP-100 (AquaPen- Photon

188 Systems Instruments) was used. A 3 mL subsample of the phytoplankton culture (from each  
189 treatment) were submitted to seven actinic light levels from 10 to 1000  $\mu\text{mol}/\text{m}^2\cdot\text{s}^1$  (10, 20, 50,  
190 100, 300, 500, and 1000  $\mu\text{mol}/\text{m}^2\cdot\text{s}^1$ ). The hyperbolic tangent model fitted to rETR versus E  
191 curves were obtained as indicated by  $r\text{ETR} = \alpha * E_K * \tanh(E * E_K^{-1})$  (Jassby and Platt, 1976; Silsbe  
192 and Kromkamp, 2012). This model was used to estimate the light curve parameters:  $\alpha$  (the  
193 coefficient of maximal utilization of energy) and  $E_K$  (the photon flux density from which the  
194 quantum efficiency of photosynthetic activity does not increase proportionally to the light  
195 intensity). Then, light saturated  $P_{\text{MAX}}$  was deduced as  $P_{\text{MAX}} = \alpha * E_K$ .

## 196 **2.7. Oxygen metabolism**

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197 To evaluate the impact of BPA and DEHP on primary production (GPP) and respiration  
198 (R), dissolved oxygen evolution was determined using an oxygen microelectrode (Unisense,  
199 Denmark) and the light-dark method (Pringault et al., 2007). A 1 mL vial filled with subsamples  
200 from each treatment were used to measure net production (NP) during the light phase and  
201 respiration (R) during the dark phase. All vials were exposed to controlled light-dark and  
202 temperature conditions. GPP was then calculated as  $\text{GPP} = \text{NP} + |\text{R}|$ .

## 203 **2.8. Statistical analyses**

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204 Difference between treatments were analyzed by a two-way analysis of variance  
205 (ANOVA), considering the incubation time and concentrations of the plastic derived chemicals as  
206 independent variables. Data normality and homoscedasticity were checked before the ANOVA  
207 test. The growth rate was analyzed by a one-way ANOVA and the Tukey's post hoc test.  
208 Differences were considered as significant at  $p \leq 0.05$ . All statistical analyses were performed  
209 using STATGRAPHICS Centurion software (STATGRAPHICS 12.0).

## 210 **3. Results**

### 211 **3.1. Strain identification**

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212 The molecular identification dataset was comprised of 148 LSU and 650 characters. The  
213 sequences obtained were aligned with sequences of *Chaetoceros* spp. from GenBank  
214 using MUSCLE (EMBL-EBI) software. The ML phylogenetic tree inferred from the LSU rDNA  
215 (28S) dataset revealed that the sequence obtained for the Bizerte strain (MW111279) formed a

216 well-supported group, together with accession number KX065227 and EF423436 of *C. decipiens*  
217 and *C. lorenzianus*, respectively (ML bootstrap 90). This confirmed that the identified species  
218 belonged to the *C. decipiens-lorenzianus* complex (Figure 1).

### 219 **3.2. Plastic derived chemicals measurement**

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220 To confirm the nominal concentrations added to the *Chaetoceros* cultures, dissolved BPA  
221 and DEHP were measured at the beginning of incubation. For all treatments, the measured  
222 concentrations of dissolved BPA and DEHP were close to the nominal one (Table S1), regardless  
223 of the conditions (single compounds or mixture).

### 224 **3.3. Effect of BPA and DEHP on the growth of *C. decipiens-lorenzianus***

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225 Cell density in the control showed a regular increase from the beginning (an average of  
226 315 Cells/L) until the end of the incubation period (7 days,  $1.56 \cdot 10^5$  Cells/L), associated with a  
227 growth rate of  $0.82 \pm 0.05 \text{ day}^{-1}$ . Upon short-term exposure (i.e., during the first 72h), BPA and  
228 DEHP contamination induced a slight but significant increase ( $p < 0.05$ ) in the diatom cell number  
229 relative to the control (Figure 2). Simultaneously, growth rates were significantly enhanced ( $p <$   
230  $0.05$ ), particularly with the presence of DEHP (Table 1). The most pronounced density increase  
231 was observed with BPA and DEHP mixture, both at low and high concentrations, with a growth  
232 rate of  $1.17 \pm 0.07 \text{ day}^{-1}$  and  $1.19 \pm 0.02 \text{ day}^{-1}$ , respectively, relative to  $0.82 \pm 0.05 \text{ day}^{-1}$  in the  
233 control. Short-term cell stimulation was observed, followed by a return to control levels at the end  
234 of the incubation period for all treatments (Figure 2).

### 235 **3.4. Impact of BPA and DEHP on the photosynthetic efficiency**

#### 236 **3.4.1. Impact of BPA and DEHP on the quantum yield of PSII**

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237 The quantum yield of PSII ( $F_v/F_M$ ) in *C. decipiens-lorenzianus* from the control treatment  
238 showed a continual increase from 0.3 to 0.71 during incubation (Figure 3).  $F_v/F_M$  in the  
239 contaminated treatments exhibited the same trend as the control conditions. An increase was  
240 observed for all treatments from 0.3 to about 0.69 (Figure 3), with no significant differences  
241 relative to the control conditions ( $p > 0.05$ ).

### 242 **3.4.2. Impact of BPA and DEHP on the photosynthetic performances:** 243 **Light-response-curves (LR-curves) and photosynthetic efficiency** 244 **parameters**

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245 The initial measurement of the Light Response-Curve (LR-curve) of *C. decipiens-*  
246 *lorenzianus* showed an increase and saturation between 300 to 500  $\mu\text{mol photon/ m}^2 \cdot \text{s}^{-1}$ , followed  
247 by clear photo-inhibition above 500  $\mu\text{mol photon/ m}^2 \cdot \text{s}^{-1}$ . Using the photosynthetic model of Jassby  
248 and Platt (1976), characteristic LR-Curve parameters were calculated. The efficiency ( $\alpha$ ) with  
249 which *C. decipiens-lorenzianus* cells harvested the light was about 0.5 ( $\mu\text{mol O}_2/ \text{mg}^1 \text{ Chl } a \cdot \text{h}^{-1}/$   
250 ( $\mu\text{mol photons/ m}^2 \cdot \text{s}^{-1}$ ) in control conditions. The maximum photosynthetic production ( $P_{\text{MAX}}$ ) was  
251 about 236.41 ( $\text{mg C/ mg Chl } a \cdot \text{h}^{-1}$ ), at an irradiance ( $E_K$ ) of 117.47 ( $\mu\text{mol photon/ m}^2 \cdot \text{s}^{-1}$ ). As  
252 observed previously with  $F_v/F_M$ , the same pattern was obtained with photosynthetic performance.  
253 No changes were observed on the LR-curves for all treatments, with no significant differences  
254 between contaminated treatments and controls (Figure S1). Concomitantly, no significant  
255 differences were observed on the parameters of the photosynthetic efficiency ( $\alpha$ ,  $E_K$ ,  $P_{\text{MAX}}$ ) (Table  
256 S3). Alpha values increased with incubation time to an average of 0.71 ( $\mu\text{mol O}_2/ \text{mg}^1 \text{ Chl}$   
257  $a \cdot \text{h}^{-1}/(\mu\text{mol photons/ m}^2 \cdot \text{s}^{-1})$ , independent of the contamination conditions (Table S3). This  
258 increase of  $\alpha$  was followed by an increase of  $E_K$  and a decrease of  $P_{\text{MAX}}$  for all treatments (Table  
259 S3).

### 260 **3.5. Impact of BPA and DEHP on O<sub>2</sub> metabolism**

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261 O<sub>2</sub> metabolism was evaluated through the measurement of gross primary production (GPP)  
262 and respiration (R). In the control treatment, the photosynthetic activity varied between  $\text{GPP}_{0\text{h}} =$   
263  $0.3 \pm 0.1 \mu\text{mol O}_2/ \text{L} \cdot \text{min}$  ( $\text{R}_{0\text{h}} = 0.1 \pm 0.001 \mu\text{mol O}_2/ \text{L} \cdot \text{min}$ ) and  $\text{GPP}_{14\text{h}} = 1.46 \pm 0.01 \mu\text{mol}$

264  $\text{O}_2/\text{L} \cdot \text{min}$  ( $R_{144\text{h}} = 0.38 \pm 0.02 \mu\text{mol O}_2/\text{L} \cdot \text{min}$ ). As observed previously with the biomass of *C.*  
265 *decipiens-lorenzianus*, GPP in the contaminated treatments showed a slight, but significant ( $p <$   
266  $0.05$ ) stimulation (Figure 4A-C) within the first 72 h of incubation relative to the control  
267 conditions, particularly with BPA at a high concentration ( $0.6 \pm 0.1 \mu\text{mol O}_2/\text{L} \cdot \text{min}$ ) relative to  
268 the control ( $0.4 \pm 0.04 \mu\text{mol O}_2/\text{L} \cdot \text{min}$ , Figure 4A). Similarly, GPP stimulation was concomitant  
269 to slight but significant ( $p < 0.05$ ) respiration stimulation within the first hours of incubation  
270 (Figure 4D-F). Short-term  $\text{O}_2$  metabolism stimulation was followed by a return to control levels in  
271 all treatments. Overall, no significant differences were observed in the ANOVA test ( $p > 0.05$ )  
272 when the whole incubation time was considered. From GPP values and cell densities, the specific  
273 phytoplankton activity was calculated (specific GPP = GPP / Cell density). Considering the total  
274 experimental period (7 days), the specific GPP of all treatments did not vary significantly relative  
275 to the control (Figure 5).

## 276 **4. Discussion**

277 Molecular analyses performed on the strain isolated from the Bizerte lagoon (MW111279)  
278 formed a common sub-group between *C. decipiens* and *C. lorenzianus* (Figure 1). This signature  
279 led us to confirm that the identified strain belongs to the *C. decipiens-lorenzianus* complex.  
280 Previous studies reported close similarities between these two diatom species in different areas of  
281 the world including the north-eastern Adriatic Sea, the southern Baltic Sea, and the coast of Buenos  
282 Aires Province, Argentina (Bosak et al., 2016; Kownacka et al., 2013; Sunesen et al., 2008),  
283 suggesting the existence of an intermediate form of both *C. decipiens* and *C. lorenzianus*.

284 Despite the high accumulation of plastic debris in the marine environments, BPA and  
285 DEHP occur at low concentrations, in the range of  $6 \mu\text{g/L}$  (M'RABET et al., 2019; Paluselli et al.,  
286 2017; Sánchez-Avila et al., 2012), close to those used in the present study (Table S1). To assess



287 their toxicity on a representative of marine primary producers, we selected the cosmopolitan  
288 diatom *C. decipiens-lorenzianus*, considering its large marine occurrence. This species is  
289 commonly observed in coastal ecosystems, as well as in open waters (Bosak et al., 2016; Gogorev  
290 et al., 2016). Species belonging to the genus *Chaetoceros* contribute considerably to ocean primary  
291 production (Booth et al., 2002). In this study, we isolated strains of *C. decipiens-lorenzianus*, that  
292 were grown under non-axenic conditions, as microscopic observation revealed the presence of  
293 bacteria associated with the diatom. Moreover, the experimental conditions were chosen to mimic  
294 *in situ* conditions as much as possible, where the diatoms are naturally associated to bacteria (Amin  
295 et al., 2012; Vincent and Bowler, 2020).

#### 296 **Transient stimulation of *C. decipiens-lorenzianus*: first hypothesis**

297 At realistic environmental concentration of EDCs, the cosmopolitan diatom *C. decipiens-*  
298 *lorenzianus* showed a relative tolerance to BPA and DEHP after 7 days of incubation. In all  
299 treatments, a transient slight but significant stimulation of biomass and photosynthetic activity was  
300 observed, especially upon DEHP exposure. Two hypotheses can be proposed to explain this  
301 significant transient stimulation.

302 First, the stimulation of biomass and oxygen gross production might be explained by the  
303 direct impact of the environmental concentration of EDCs on the photosynthetic apparatus, as  
304 recently observed in microalgae cultures. A recent work highlighted that BPA released from small  
305 fragments of expanded polystyrene (EPS) at a concentration of 1.5 ng/L provoked a significant  
306 stimulation of the photosynthetic efficiency of Chlorophyceae species (Chae et al., 2020). They  
307 showed that BPA exposure significantly stimulated the quantum yield of PSII ( $F_V/F_M$ ), which  
308 consequently induced a growth stimulation of several phytoplankton strains (*Dunaliella salina*,  
309 *Scenedesmus rubescens*, *Chlorella saccharophila*, and *Stichococcus bacillaris*) (Chae et al., 2020).

310 Furthermore, this would suggest that BPA and DEHP contamination at environmental  
311 concentrations can stimulate the photosynthetic apparatus of phytoplanktonic cells, subsequently  
312 inducing the stimulation of biomass and photosynthetic activity. Nevertheless, in our study  
313 photosynthetic efficiency of PSII, measured through the Fv/Fm ratio, as well as specific activity  
314 (Figure 3 and 5), were not significantly impacted by the presence of BPA or DEHP. Consequently,  
315 a direct transient effect of EDCs on PSII of *C. decipiens-lorenzianus* cannot explain the short-term  
316 stimulation observed for growth and oxygen production.

### 317 **Transient stimulation of *C. decipiens-lorenzianus*: second hypothesis**

318 The second hypothesis to explain this transient significant stimulation relies on the  
319 presence of bacteria in the diatom culture. Recently, Romera-castillo et al (2018) demonstrated  
320 that bacterial activity was stimulated in response to phthalate exposure when released from  
321 polyethylene (PE) and polypropylene (PP). They observed that PE and PP particles in seawater  
322 promoted the release of dissolved organic carbon (DOC), which induced the stimulation of  
323 bacterial activity.

324 In the present study, although DOC was not measured, we speculated that BPA and DEHP  
325 promoted the metabolic activity of bacteria associated with *C. decipiens-lorenzianus*, which in turn  
326 induced biomass and photosynthetic stimulation. The microbiome associated with the non-axenic  
327 diatom might have benefited from diatom-bacterium interactions. In fact, this association can  
328 promote synergistic interactions between both compartments, through the availability of nutrients,  
329 such as vitamins (B<sub>1</sub> and B<sub>12</sub>), iron, DOC, and nitrogen (Amin et al., 2012; Seymour et al., 2017).  
330 We hypothesized that the short-term observed stimulation of *C. decipiens-lorenzianus* upon EDC  
331 exposure at environmental conditions could be the consequence of a cascading effect of BPA and  
332 DEHP on its phycosphere, which subsequently induced cell growth and photosynthetic activity

333 stimulation. Furthermore, phytoplankton possess a unique microbiome (Behringer et al., 2018)  
334 that could act as a protective barrier (Fouilland et al., 2018; Ramanan et al., 2016) against toxic  
335 contaminant effects, ensuring an optimal phytoplankton production. The association between the  
336 cosmopolitan *C. decipiens-lorenzianus* and its phycosphere could form an effective consortium  
337 able to cope with the toxic effects of BPA and DEHP at realistic environmental concentrations. In  
338 addition, the large size of *C. decipiens-lorenzianus* might represent a defense mechanism, relative  
339 to other smaller phytoplankton cells. The low cell volume ratio of diatoms reduces the diffusion  
340 of organic pollutants through the cell relative to smaller phytoplankton cells, favoring their  
341 tolerance to chemical contamination (Ben Othman et al., 2012; Staniszewska et al., 2015).

#### 342 **Situation of the ecotoxicological answer of *C. decipiens-lorenzianus* compared to other species**

343 Previous works studying the ecotoxicological impacts on microalgae have often used  
344 higher concentrations of EDCs than those employed in the present study (Table S1). Significant  
345 toxic effects of EDCs on phytoplankton were reported for concentrations far above the observed  
346 *in situ* concentrations. For example, growth and activity inhibition were observed with diatom  
347 species at high BPA concentration of > 0.04 mg/L with *Ditylum brightwelli* and *Navicula incerta*  
348 (Lee et al., 2014; Liu et al., 2010a). Similarly, a high EC<sub>50</sub> was measured for the Chlorophyceae  
349 *Chorella vulgaris* (6.02 mg/L) with DEHP (Shen et al., 2019) and for the diatom *Chaetoceros*  
350 *muelleri* (EC<sub>50</sub> at 96 h: 194 mg/L) with diethyl phthalate (Chi et al., 2019). Recent ecotoxicological  
351 works studying the impact of EDCs at environmental realistic concentrations have observed  
352 contrasting effects depending on the phytoplankton species studied. Exposure to EDCs drastically  
353 inhibited the biomass and physiological activity of the toxic dinoflagellate *Alexandrium pacificum*  
354 at concentrations of 2 and 20 µg/L BPA and 1 and 10 µg/L DEHP, similar to those used in the  
355 present study M'Rabet et al (2018). Similarly, Cunha et al (2019) observed significant inhibition

356 of cell abundance after exposure of a Chlorophyceae *Scenedesmus* sp. to dibutyl phthalate (DBP).  
357 They reported an EC<sub>50</sub> after 48 h of DBP exposure of 41.88 µg/L. In contrast, Chae et al (2020)  
358 showed that BPA at 0.0019 µg/L enhanced the photosynthetic activity, as well as the growth of  
359 Chlorophyceae species. Thus, these observations indicate that the ecotoxicological impact of  
360 EDCs on phytoplankton at relevant environmental concentrations is very likely species dependent.

## 361 **5. Conclusions**

362 Overall, results of the present study highlighted that in simulating environmental  
363 conditions, the cosmopolitan diatom *C. decipiens-lorenzianus* was not significantly impacted by  
364 BPA and DEHP contamination, the two most observed EDCs in contaminated marine ecosystems.  
365 This apparent tolerance might rely on the heterotrophic compartment associated with the diatom  
366 *Chaetoceros*. This laboratory ecotoxicological study confirms what was observed at the  
367 community scale, where environmentally relevant EDC contamination promoted the dominance  
368 of the genus *Chaetoceros* relative to other phytoplankton species (M'Rabet et al., 2019). Future  
369 ecotoxicological studies are required to better understand the possible role of the phycosphere  
370 associated with *Chaetoceros* to face EDC contamination.

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## 379 **Conflict of interest**

380 The authors declare that they have no known competing financial interests or personal  
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382

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## Tables and figures

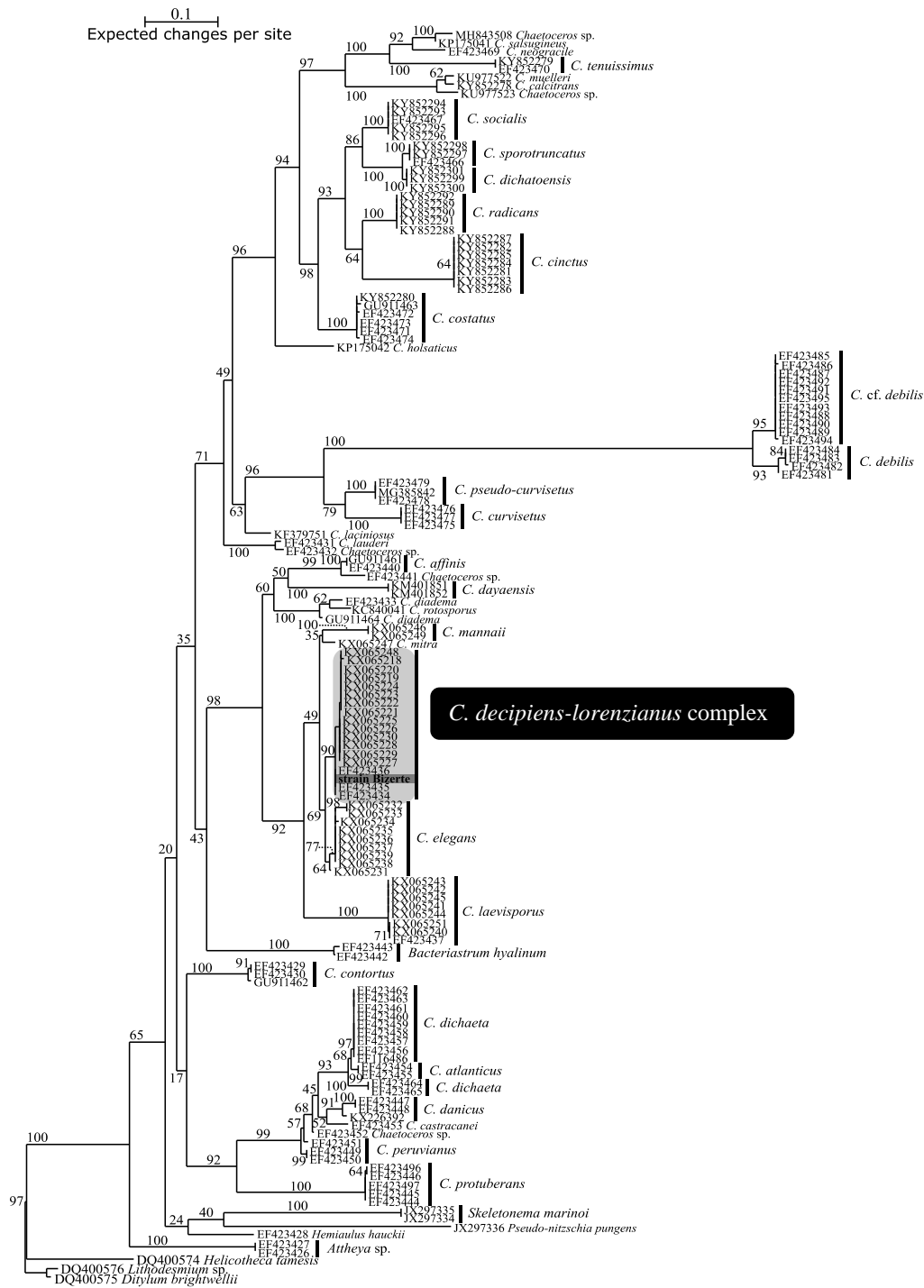
702 **Table 1** : Growth rate (/day) of *Chaetoceros decipiens-lorenzianus* during the exposure to BPA and/or  
703 DEHP mean of three replicate  $\pm$  standard deviation (SD), the letters (a–d) indicate homogeneity of different  
704 treatments ( $P < 0.05$ ).

705

Treatment	Growth rate
<b>Control</b>	0.82 $\pm$ 0.05 <sup>a</sup>
<b>BPA 2<math>\mu</math>g/L</b>	0.85 $\pm$ 0.02 <sup>a</sup>
<b>BPA 20<math>\mu</math>g/L</b>	0.80 $\pm$ 0.05 <sup>a</sup>
<b>DEHP 1<math>\mu</math>g/L</b>	1.11 $\pm$ 0.03 <sup>c</sup>
<b>DEHP 10<math>\mu</math>g/L</b>	0.98 $\pm$ 0.02 <sup>b</sup>
<b>BPA 2<math>\mu</math>g/L &amp; DEHP 1<math>\mu</math>g/L</b>	1.17 $\pm$ 0.07 <sup>cd</sup>
<b>BPA 20<math>\mu</math>g/L &amp; DEHP 10<math>\mu</math>g/L</b>	1.19 $\pm$ 0.02 <sup>d</sup>

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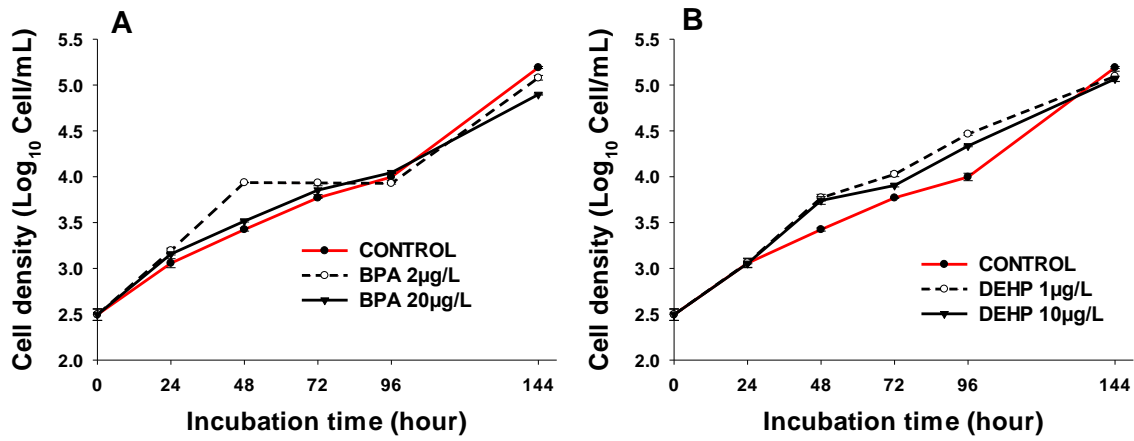
**Figure 1:** Maximum likelihood (ML) phylogenetic tree based on LSU rDNA sequences (148 sequences/650 characters). The tree was rooted using *Lithodesmium* / *Ditylum* as out-group

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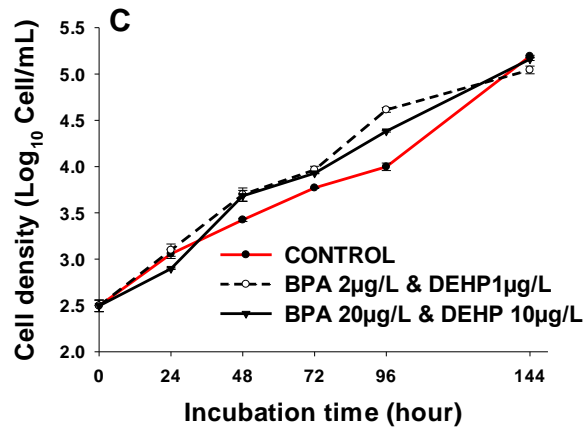
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716 **Figure 2:** Growth curve of *C. decipiens-lorenzianus* (mean of three replicates  $\pm$  standard deviation (SD)),  
717 expressed as Log<sub>10</sub> Cell/mL during the exposure to BPA at 2 µg/L and 20 µg/L (panel A),  
718 the exposure to DEHP at 1 µg/L and 10 µg/L (panel B), the exposure to the mixture of BPA 2µg/L + DEHP 1µg/L and  
719 BPA 20µg/L + DEHP 10µg/L (panel C).

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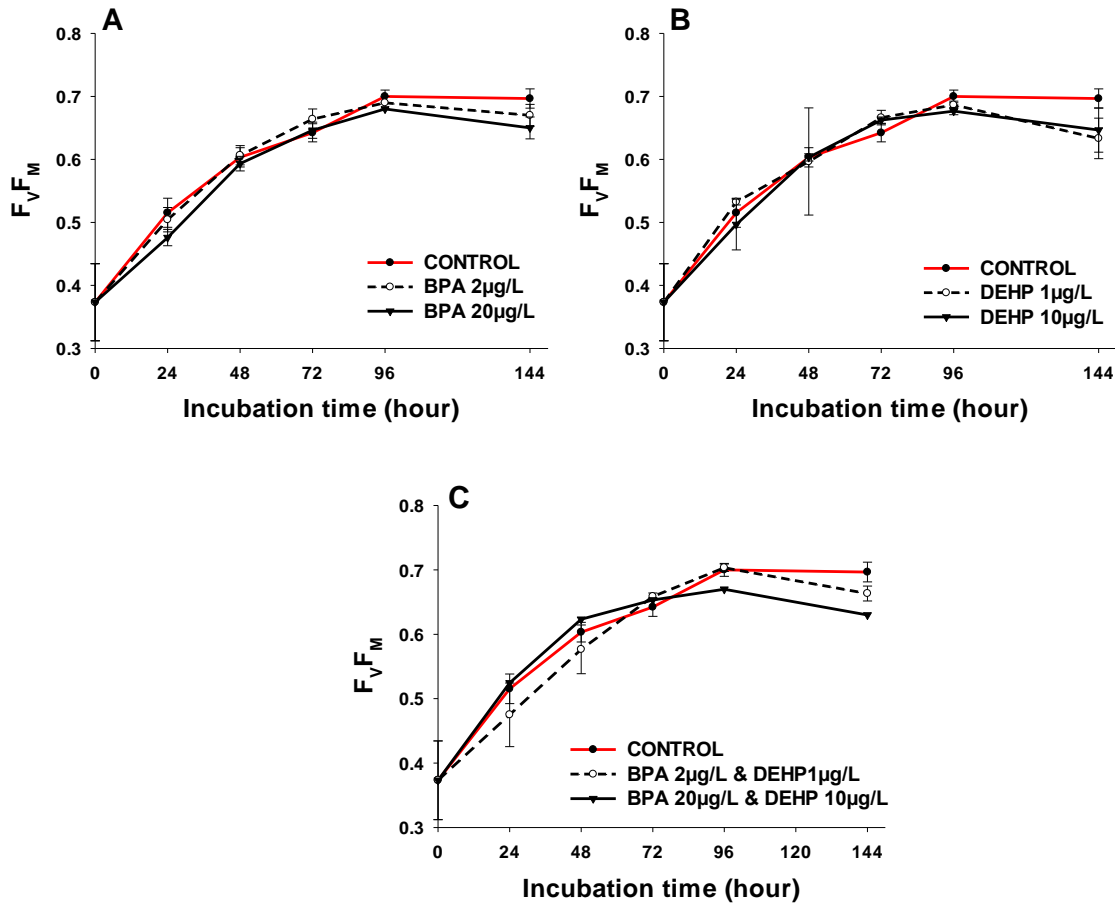
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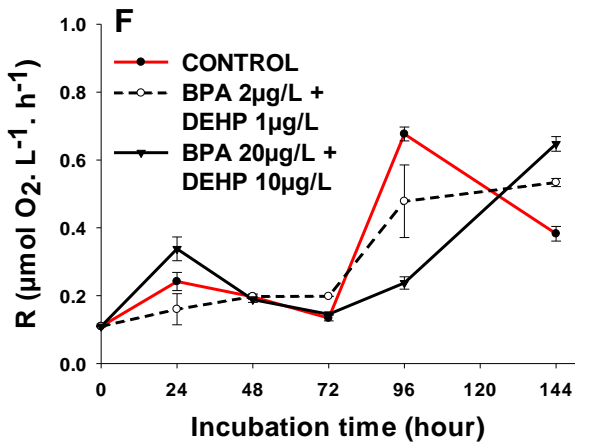
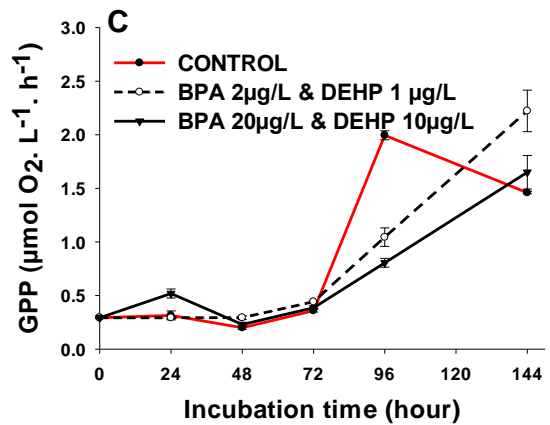
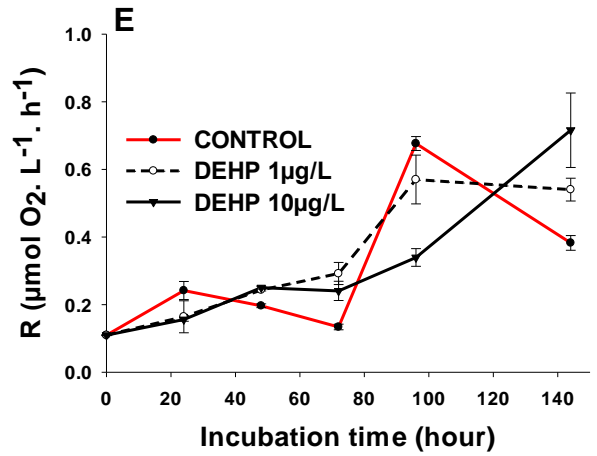
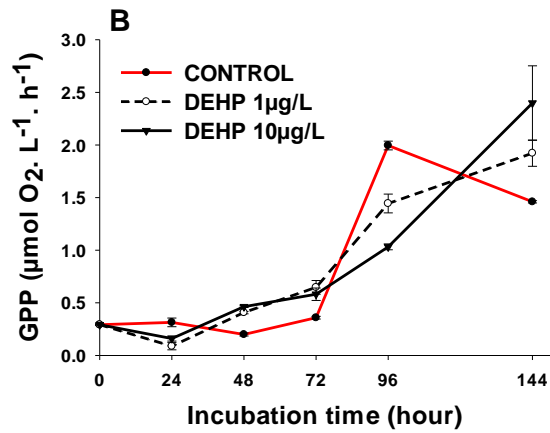
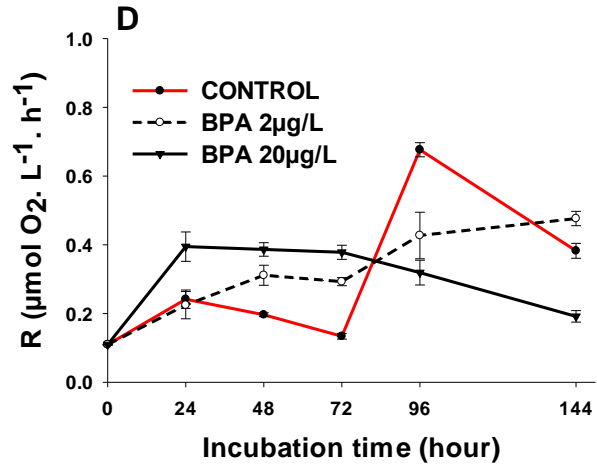
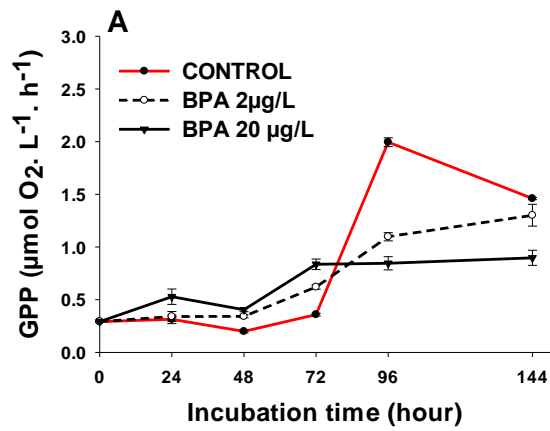
731 **Figure 3:** Variation of the maximum quantum yield of PSII ( $F_v/F_M$ ) during the time incubation (7 days) of  
732 *C. decipiens-lorenzianus* to BPA (A), to DEHP (B) and to the mixture of BPA and DEHP (C). The values  
733 are expressed of mean of three replicates  $\pm$  standard deviation (SD)).

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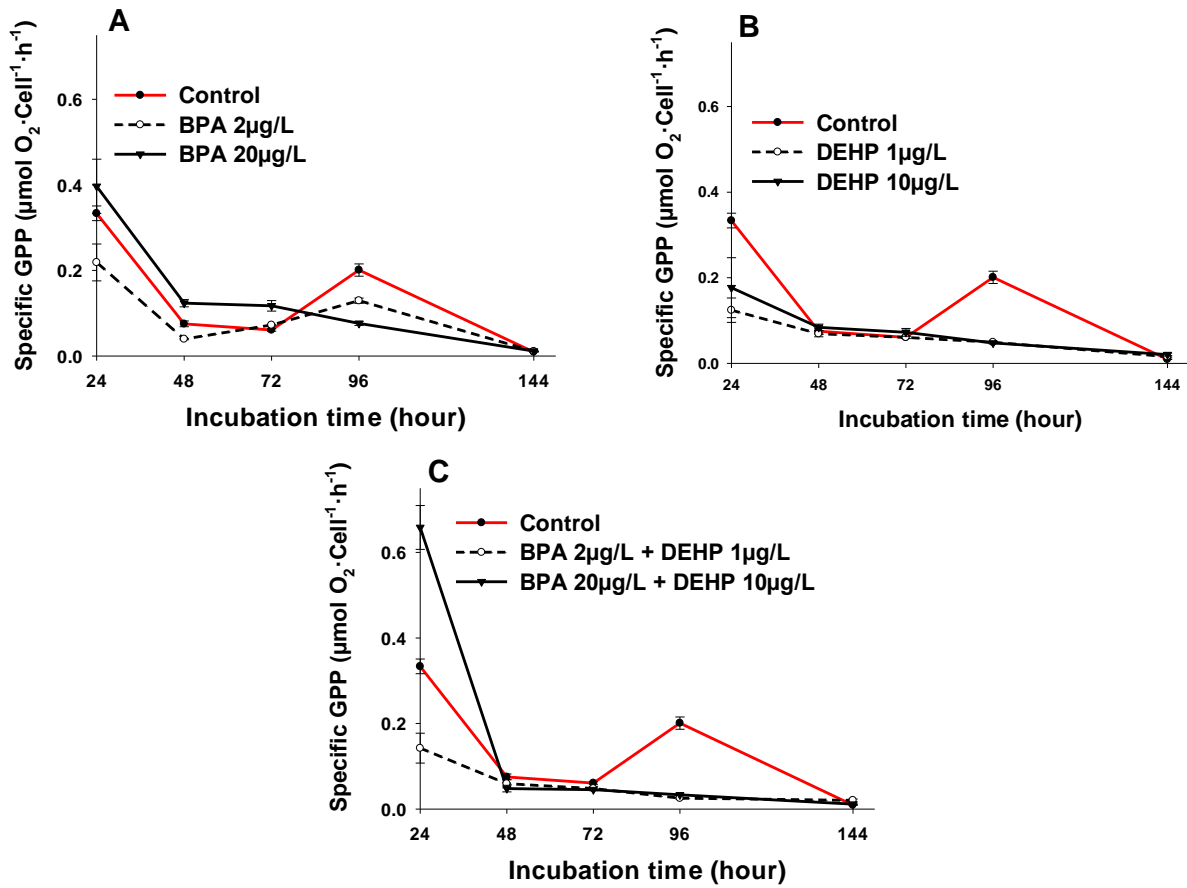
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741 **Figure 4:** Photosynthetic activity expressed as GPP (Gross Primary Production) and respiration activity  
 742 expressed as R, during the time exposure of *C. decipiens-lorenzianus* to BPA (A and D), DEHP (B and E)  
 743 and the mixture of BPA + DEHP (C and F) in the course of the time incubation (7 days) and with respect  
 744 to the control (red curve).

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748 **Figure 5:** Specific phytoplankton activity (specific GPP), expressed in  $\mu\text{mol O}_2 \cdot \text{Cell}^{-1} \cdot \text{h}^{-1}$  (mean  $\pm$  S.D) in  
 749 BPA (A), DEHP (B) and the mixture of BPA and DEHP (C) treatments, over time incubation and with  
 750 respect to the control (red curve).

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752

## Supplementary material

753 **Table S1:** Nominal and measured concentrations of BPA and DEHP, expressed in  $\mu\text{g/L}$  at the beginning of  
754 the experiment

Treatment	BPA ( $\mu\text{g/L}$ )		Treatment	DEHP ( $\mu\text{g/L}$ )	
	Nominal concentration	Measured concentration		Nominal concentration	Measured concentration
Control	0	< 0.3	Control	0	$0.2 \pm 0.03$
BPA 2 $\mu\text{g/L}$	2	$3.3 \pm 1.06$	DEHP 1 $\mu\text{g/L}$	1	$1.2 \pm 0.35$
BPA 20 $\mu\text{g/L}$	20	$20.1 \pm 2.53$	DEHP 10 $\mu\text{g/L}$	10	$10.1 \pm 1.35$

755

### 756 BPA and DEHP chemical analysis

757 DEHP was analyzed using Solid-Phase Micro-Extraction (SPME) coupled with Gas  
758 Chromatography/Mass Spectrometry (GC/MS). For the extraction of water samples, 100  $\mu\text{m}$  fiber  
759 SPME-PFMS (Supelco) was used. The quality parameters of the SPME were: i) an immersion of  
760 the fiber in liquid phase (15 mL of the sample), ii) an incubation temperature of 65  $^{\circ}\text{C}$ , iii) an  
761 incubation time of 5 min, iv) agitation 250 rpm and v) 30 min for extraction and 3 min for  
762 desorption. DEHP analysis was performed with GC/MS working in electro-ionization impact  
763 mode (GC-7890 A; MSD-5975C, Agilent Technologies). An HP5MS-UI column (5% phenyl  
764 methyl siloxane, 30 m  $\times$  0.25 mm ID  $\times$  0.25  $\mu\text{m}$  phase thickness, Agilent Technologies) was used.  
765 BPA analysis was performed using Liquid Chromatography/tandem Mass Spectrometry  
766 (LC/MSMS) in a negative ionization mode (UPLC Acquity; MSMS-Quattro Premier XE, Waters).  
767 The cartridge used was an Acquity UPLC BEH C18 (50 mm  $\times$  2.1 mm ID  $\times$  1.7  $\mu\text{m}$  granulometry,  
768 Waters). Direct injection volume was set at 40  $\mu\text{L}$ . The quality parameters of chromatography were  
769 as follows: i) solvent tank A: milliQ-water with 0.5 mM ammonium acetate, ii) solvent tank B:  
770 methanol, iii) a mobile phase flow rate of 0.5 mL min<sup>-1</sup>. BPA and DEHP were analyzed following



771 the analytical protocols of (Dévier et al., 2013) for DEHP and of (Gagnaire et al., 2009) for BPA  
772 (information about the validation parameters in Table S2).

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779 **Table S2:** DEHP and BPA were quantified by isotopic dilution (Internal standard compound DEHP-d4 and BPA-d16, respectively), the validation  
 780 parameters for chemical analyses are mentioned in the table below:

Compound	Quantification transition (Collision energy) (m/z)	Quantification confirmation (Collision energy) (m/Z)	Retention time (min)	Dwell (ms)	Internal standard compound	Quantification transition (Collision energy) (m/z)	Rtention time (min)	Dwell (ms)
<b>DEHP</b>	149	167	23.98	100	DEHP-d4	153	23.97	100
<b>BPA</b>	227>212.1 (22)	227>132.9 (25)	1.1	120	BPA-d16	241.2>223.2 (20)	1.09	120

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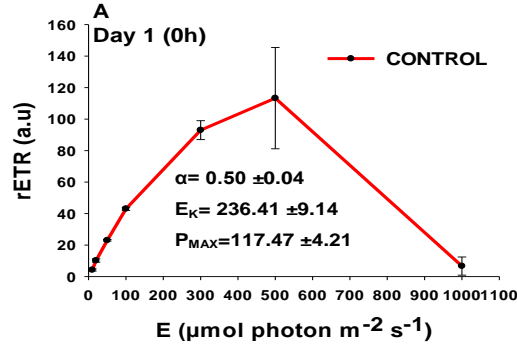
782 **Table S3:** Parameters ( $\alpha$ , light utilization coefficient;  $P_{MAX}$ , maximum gross photosynthesis;  $E_K$ , irradiance for the light saturation  
 783 of photosynthesis) of relative electron transport rate versus irradiance curves.  $\alpha$ : Arbitrary unit;  $P_{MAX}$ : Arbitrary unit;  $E_K$ :  $\mu\text{mol}$   
 784  $\text{photon. m}^{-2}.\text{s}^{-1}$ . The letters (a–c) indicate homogeneity of different treatments ( $P < 0.05$ )

photosynthetic performances		Control	BPA	BPA	DEHP	DEHP	BPA 2	BPA
			2 $\mu\text{g/L}$	20 $\mu\text{g/L}$	1 $\mu\text{g/L}$	10 $\mu\text{g/L}$	$\mu\text{g/L} +$ DEHP 1 $\mu\text{g/L}$	20 $\mu\text{g/L} +$ DEHP 10 $\mu\text{g/L}$
$\alpha$ (a.u)	4d	0.72	0.70	0.72	0.71	0.71	0.70	0.72
		$\pm 0.01^a$	$\pm 0.02^b$	$\pm 0.01^a$	$\pm 0.01^{ab}$	$\pm 0.01^{ab}$	$\pm 0.01^{ab}$	$\pm 0.01^a$
	7d	0.73	0.70	0.70	0.70	0.70	0.70	0.60
		$\pm 0.01^a$	$\pm 0.02^a$	$\pm 0.02^a$	$\pm 0.02^a$	$\pm 0.03^a$	$\pm 0.02^a$	$\pm 0.01^b$
E ( $\mu\text{mol photon m}^{-2}.\text{s}^{-1}$ )	4d	156	186	155	165	157	167	164
		$\pm 7.0^a$	$\pm 21.0^b$	$\pm 5.1^a$	$\pm 11.7^{ab}$	$\pm 11.7^a$	$\pm 21.1^{ab}$	$\pm 2.4^{ab}$
	7d	101	113	122	113	119	136	139
		$\pm 3.2^a$	$\pm 10.1^{ab}$	$\pm 21.4^{ab}$	$\pm 22.4^{ab}$	$\pm 12.4^{ab}$	$\pm 22.4^b$	$\pm 27.5^b$
$P_{MAX}$ (a.u)	4d	112	129	111	117	112	118	118
		$\pm 3.7^a$	$\pm 14.0^a$	$\pm 4.2^a$	$\pm 7.5^a$	$\pm 10.2^a$	$\pm 21.9^a$	$\pm 2.4^a$
	7d	74	79	83	74	81	93	91
		$\pm 3.34^a$	$\pm 9.4^a$	$\pm 16.4^a$	$\pm 14.03^a$	$\pm 9.2^a$	$\pm 13.0^a$	$\pm 19.1^a$

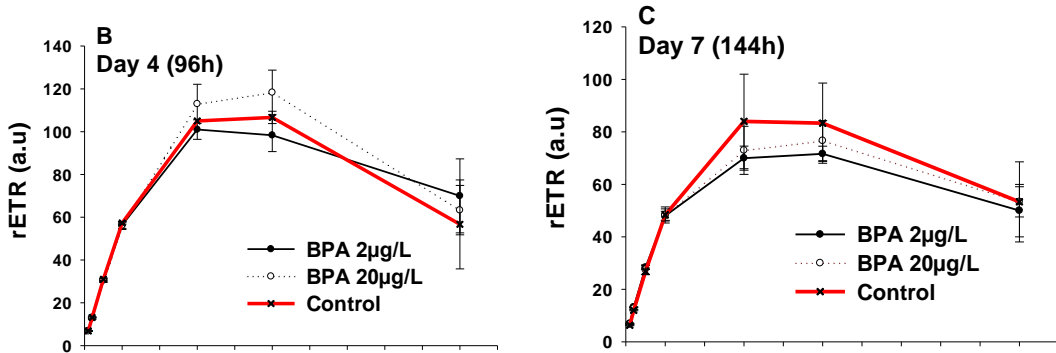
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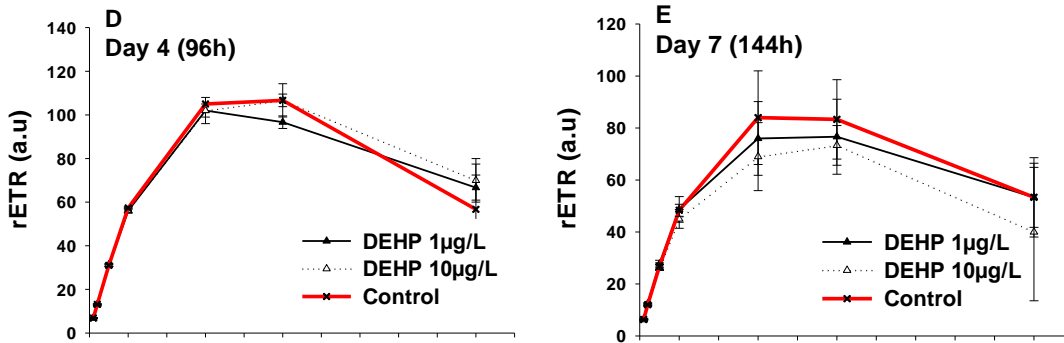
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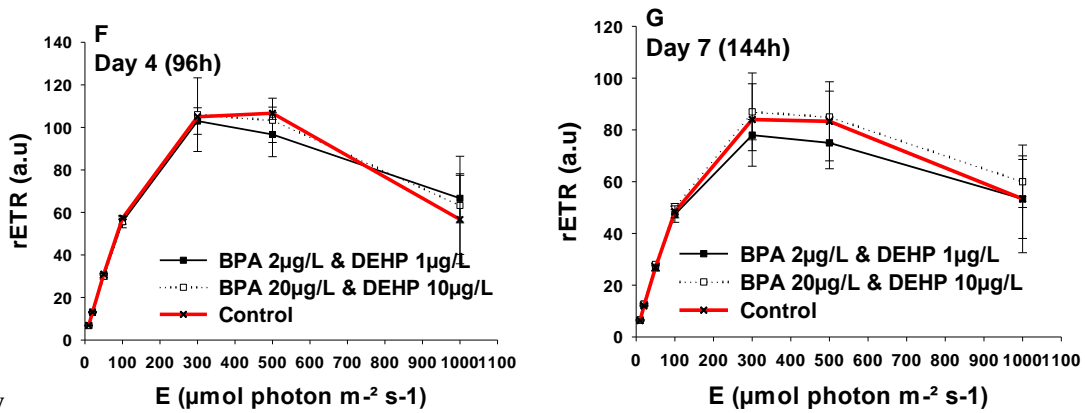
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791 **Figure S1:** Relative electron transport rate (rETR)-versus-Irradiance (E) curves for the diatom *C. decipiens-lorenzianus*: (A) at  
792 D1-0 h, which corresponds to the start of the experiment, (B) at D4-96 h which corresponds to the exposure of *C. decipiens-*

793 *lorenzianus* to BPA, (D) at D4-96 h which corresponds to the exposure of *C. decipiens-lorenzianus* to DEHP, (F) at D4-96 h which  
794 corresponds to the exposure of *C. decipiens-lorenzianus* to BPA and DEHP combined, (C) at D7-144 h which corresponds to the  
795 exposure of *C. decipiens-lorenzianus* to BPA, (E) at D7-144 h which corresponds to the exposure of *C. decipiens-lorenzianus* to  
796 DEHP, (G) at D7-144 h which corresponds to the exposure of *C. decipiens-lorenzianus* to BPA and DEHP combined.

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