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Water browning controls tolerance acquisition and associated trade-offs in phytoplankton stressed by chemical pollution

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Abstract

Acquisition of tolerance to an environmental stressor can cause trade-offs and result in organisms with slower growth. While this is theoretically grounded, assessments of the nature of this trade-off, environmental controls, and implications for organisms' fitness, are insufficient. Here, we report the effects of water browning on the toxic responses, tolerance acquisition and associated trade-offs in a population of microalgae exposed to sub-lethal concentrations of organic micropollutants over multiple generations. Our results show that dissolved organic matter (DOM) reduces toxic responses and modulates tolerance acquisition by the algae, possibly by complexing micropollutants. Microalgae that acquire tolerance allocate resources in fitness at the cost of a reduced cell size. They yield higher productivity than non-adapted ones when grown in presence of micropollutants, but lower in their absence. This growth efficiency trade-off is positive, indicating that - despite the costs of adaptation - tolerant organisms will have higher productivity and fitness in recurrently stressed environments.

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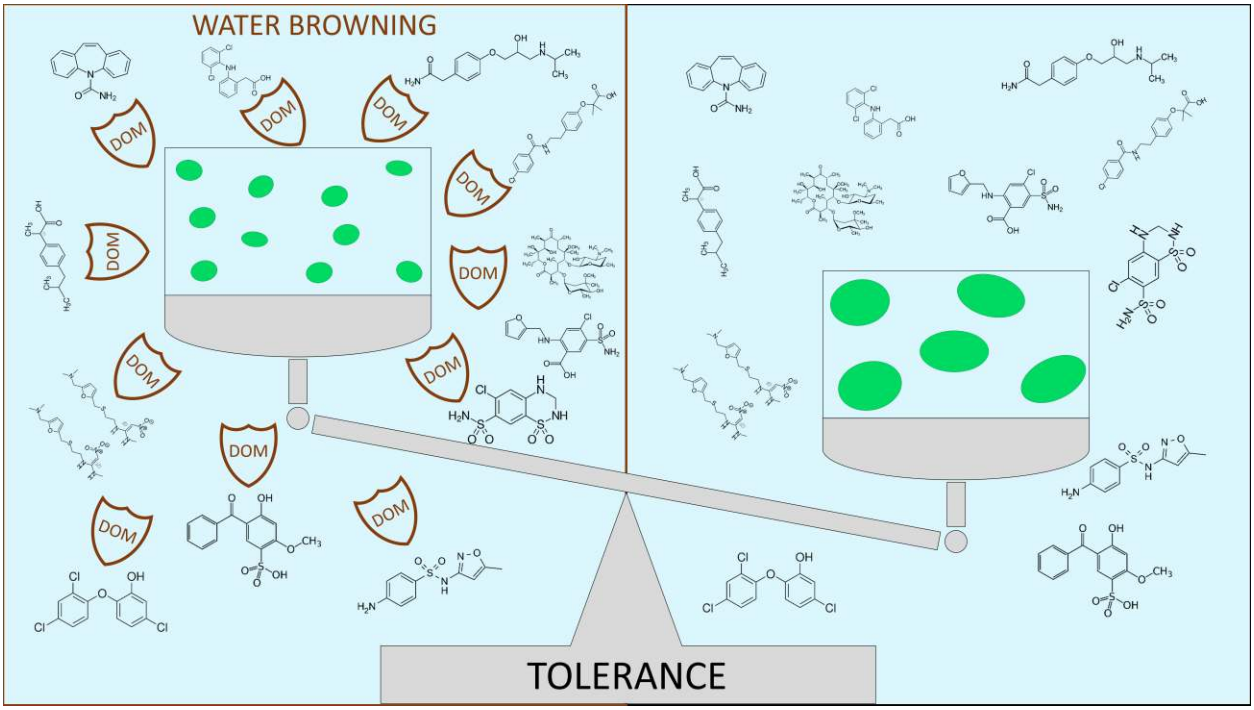

1. Introduction

Populations that have been exposed over multiple generations to the selective pressure of a recurrent stressor may acquire tolerance through physiological and evolutionary adaptation [1]. Although these processes usually occur at different time scales [2], evolutionary adaptation can also be rapid, arising a few generations after the stress onset [3]. Populations that acquire tolerance towards a specific stressor often show lower growth in another context, such as in the absence of the stressor [4–7]. Existence of these trade-offs is a fundamental postulate of resource-based allocation theory [6,7]. Drawing predictions of the net positive effect of tolerance acquisition on the functioning of a population requires accounting for these antagonistic processes, and is therefore complex. In addition, the magnitude of the stress can be modulated by other environmental factors. This is the case for example, for water pollutants, the availability and/or toxic action of which can

55 be affected by interaction with natural dissolved organic matter (DOM) or water pH [8,9]. How the
56 interaction of chemical stressors with environmental factors influences tolerance acquisition and
57 associated costs is mostly uncharted.

58 Chemical pollution acts as an important selective pressure on aquatic biota [10]. Among the range of
59 widespread freshwater chemical pollutants, pharmaceutical and personal care products (PPCPs)
60 are concerning as they are continuously discharged from wastewater effluents, and are biologically
61 active at low concentrations [11]. PPCPs can interfere with fundamental metabolic pathways related
62 to chlorophyll-a and lipids synthesis [12,13], which increases their likelihood to adversely impact
63 phytoplankton [14–17]. Evidence that microalgae can adapt to diffuse anthropogenic contaminants is
64 available [1,18,19], but documentation on the environmental controls on tolerance acquisition and on
65 the occurrence and nature of trade-offs is scant [20].

66 During the last decades, climate and land-use change and recovery from past acidification have
67 caused water browning [21] which haven a diffuse increase of natural DOM and changed pH in many
68 ecosystems [21–23]. DOM (commonly analyzed as the concentration of dissolved organic carbon –
69 DOC) can adsorb, bind and/or transform PPCPs by forming less bioavailable and toxic complexes
70 [24,25]. This process can be pH dependent since many fresh water contaminants, including many
71 PPCPs, exist simultaneously as ionic and neutral forms in the aqueous phase at environmental
72 conditions [26,27]. Neutral species dominate at water pH lower than the compound's acid dissociation
73 constant (pKa) and tend to be more toxic, possibly because the organisms' lipid membranes are
74 often more permeable to non-polar molecules [28]. Neutrality in the molecular charge can in turn
75 increase the likelihood of hydrophobic interactions with DOM [25], possibly resulting in lower

bioavailability and toxicity. The influence of these environmental factors on the form, availability and toxicity of PPCPs has been the subject of research [24,25,29], however the potential implications for driving adaptation and related trade-off are currently unexplored. Given the current widespread browning and the wide range of DOM concentrations in natural surface waters, a better understanding of this factor's role as a modulator of toxic responses and the development of tolerant strains, is needed.

In order to address these gaps, we designed a two-phase experiment assessing the role of DOM on the toxic outcomes and acquisition of tolerance and associated fitness trade-offs in a microalgae population exposed to a mixture of PPCPs. First, we postulated that:

i) DOM inhibits the insurgence of negative effects induced by PPCPs on algal growth [30];

ii) prolonged exposure to sub-lethal concentrations of PPCPs induces tolerance in microalgae.

Then, after testing these premises, we hypothesized that:

i) acquisition of tolerance to PPCPs trades-off with growth efficiency in the absence of the pollutants;

ii) DOM during the adaptation period controls both acquisition of tolerance and emergence of fitness trade-offs.

The experiment was designed as follows:

- in phase I we assessed microalgae growth and cell size response to PPCPs under different conditions of DOM and pH;

- then we subjected the microalgae to a two-month adaptation period, where they were exposed to sub-lethal PPCP levels and different levels of DOM, under the pH conditions that in phase I yielded highest growth inhibition;

- finally, in phase II the growth and cell size of non-adapted and adapted populations to PPCPs under different levels of DOM were compared in the presence and absence of PPCPs.

Addressing the implications of the two-way interaction between environment and environmental stressors on biota growth and fitness represents a challenge of considerable complexity. This multiple stressor – multiple interaction situation prevails in nature and it is important to understand and quantitatively balance synergistic/antagonistic effects, inform realistic extrapolations of results to real environmental conditions, and ultimately address the broader ecological implications of these interactions.

2. Materials and Methods

2.1. Experimental Design

112 The experiment consisted of two-phases (Figure 1), interposed by an adaptation period. An
113 acclimation phase preceded the first phase of the experiment, where the cultures were acclimated
114 for five days to combinations of DOM (0, 5 and 15 mg L^{-1} DOC) and pH (6.5 and 8). During phase
115 I, the growth response of the microalgae population to the mix of PPCPs was tested for
116 combinations of three DOM levels (0, 5, 15 mg L^{-1} DOC) and pH (6.5, 8) in a factorial design
117 (Figure 1). Then, the algae were allowed to adapt for 2 months under the same experimental
118 conditions of PPCPs and DOM (Figure 1) at pH 8 only (following results from phase I). In phase
119 II, subsamples from the cultures taken from the experimental adaptation period were exposed to
120 the mix of PPCPs only (at the same concentration used in phase I and during the adaptation period,
121 but in the absence of DOM (Figure 1), to assess acquisition of tolerance, growth performance and
122 ultimately trade-offs in growth efficiency.

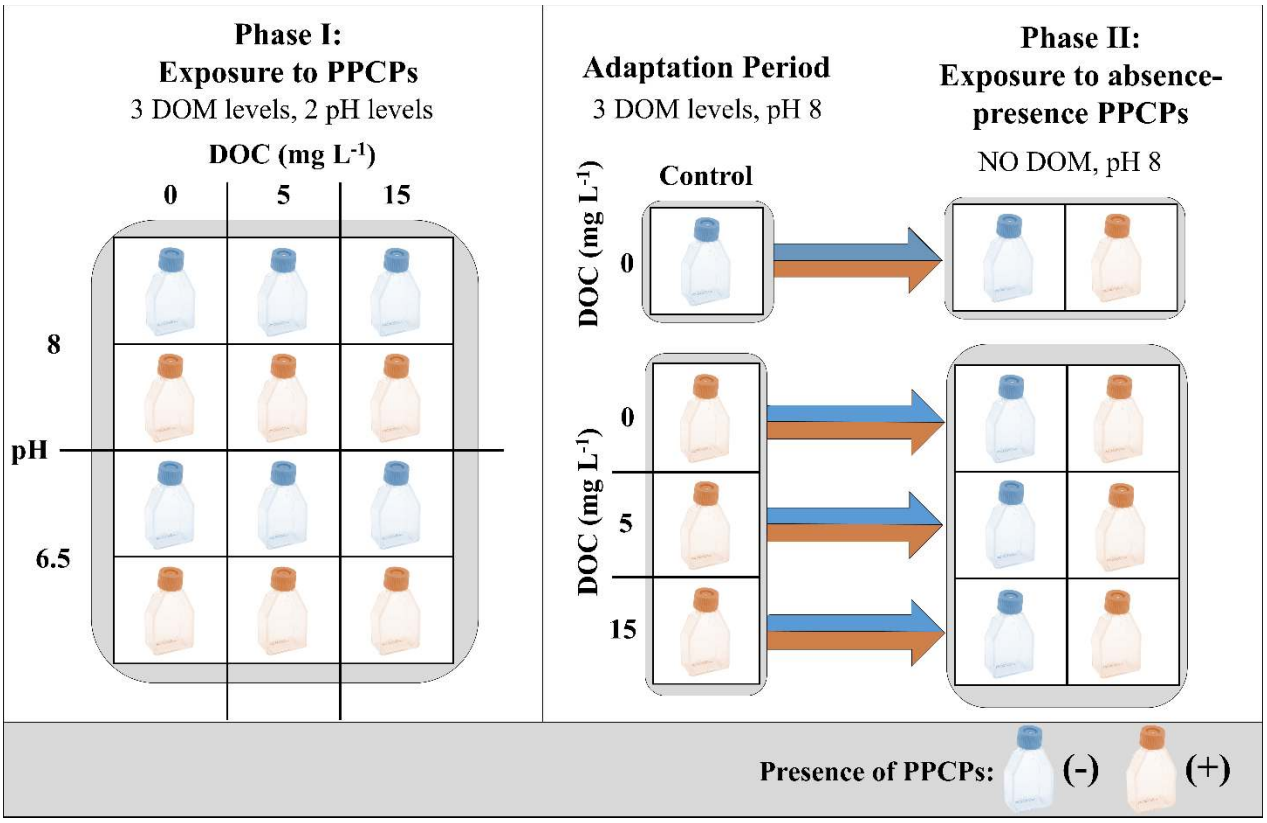

Figure 1. Factorial experimental design. Phase I; exposure of the algal population to the absence (-) and the presence (+) of a mix of 12 PPCPs under different DOM and pH levels. Adaptation period; multi-generational exposure of the algal population to the presence (+) of PPCPs under different levels of DOM (0, 5, 15 mg L^{-1} DOC) at pH 8. Phase II; exposure of the algal population previously adapted to the presence of PPCPs under different levels of DOM, and of the control population which never experienced the contaminants and/or the DOM during the adaptation period, to the absence (-) and the presence (+) of PPCPs.

2.2. Selection of algal culture

The chlorophyte *Chlamydomonas reinhardtii* (strain CC-1690 21 gr mt+) was used in laboratory growth experiments. This is a widely used model organism for toxicological and evolution studies [31].

2.3. Selection of DOM and pH

DOM originated from the Hellerudmyra tarn (Norway) and was previously isolated through reverse osmosis [32]. All the physical-chemical properties of this DOM are reported by Gjessing et al. [32]. The levels of DOM and pH applied represent the range typically found in Northern European lakes [33,34]. The nutrient concentrations (mesotrophic lakes, P= 30 μg L^{-1}) minimized the effect induced by the algal photosynthesis on the sequestration of carbon dioxide increasing the level of hydroxide and therefore pH of the cultures. The increase in pH for the algal cultures exposed to the effect of PCPPs was very modest (not shown).

2.4. Selection of chemical contaminants

A mixture of 12 PPCPs was taken as chemical stressor model (Table S2), according to a number of previous studies [35–38] and reflecting most commonly detected substances in European wastewater and surface water (Table S1). PPCPs analytical standards were purchased from Sigma-Aldrich (USA), mixed and diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to create a stock solution. The exposure level used in this experiment to induce toxic effects from PPCPs in phase I and II and during the adaptation period (Table S2) was chosen as the concentration that yielded a 30% decrease in growth rate in a pilot toxicity test (Table S3), following the OCED guidelines [39]. The concentration of individual PPCPs was determined at the end of both experimental phases (Table S4) as described in Text S2.

2.5. Algal culturing and biomass measurements

155 The algae were grown as batch cultures in 60 mL non-treated polystyrene cell culture flasks (Nunc,
156 Thermo-scientific, US), using WC medium [40] with P concentration of 30 µg L^{-1}. Cultures were
157 incubated at 16 °C in a temperature controlled room under constant white light (100 µmoles of
158 photons m^{-2} s^{-1}; this resulted in no light limitation, based on earlier experiments with *C. reinhardtii*
159 [41]). Each treatment was replicated four times (total number of experimental units was 48 in both
160 phases).

161 The relative biomass development was monitored in both phases as the chlorophyll a *in vivo*
162 fluorescence (excitation at 460 nm and emission at 680 nm, Figure S1, S2), using a plate reader
163 equipped with a spectrophotometer (BioTek Synergy MX; Winosky, VT, USA). Triplicates from
164 each experimental unit were loaded on clear flat bottom 96 well black microplates (300 µL in each
165 well) (Corning, USA). Biomass assessments were further constrained through cell number and
166 size distribution determination, measured by a coulter counter (Multisizer 3, Beckman Coulter Life
167 Sciences, USA). For phase I, samples of 1 mL were collected from each experimental unit at the
168 end of the exponential growth phase (on day 5, as judged from the chlorophyll *in vivo* fluorescence,
169 Figure S1). For phase II, samples for cell counting were taken daily.

170 **2.6. Phase I**

171 DOM enriched medium was prepared by spiking MQ-diluted DOM in two bulk solutions of
172 modified WC medium (see experimental design paragraph) to reach concentrations of 5 and 15
173 mg DOC/L, respectively. A third batch (control) with no added DOM was also prepared. The
174 volume of the three bulk solutions was split into two separate sets, the pH of which was adjusted

by titration with HCl or NaOH to 6.5 and 8, respectively. Finally, 20 µL of PPCPs stock solution was added to half of the units, to reach the concentrations shown in Table S3. 40 mL of each of the 12 different media (3 DOM-levels x 2 pH levels x 2 PPCPs levels) were added to four replicate culture flasks and inoculated with 100 µL of algal stock culture. This resulted in a starting concentration of ca. 1000 cells per mL (measured in a coulter counter). Phase I was run for 7 days under the light and temperature conditions described earlier.

2.7. Experimental adaptation period

Following phase I, the algal cultures from the pH=8 set were grown for 2 months in the presence of PPCPs, under the same experimental conditions as in phase I. Only the higher pH conditions was chosen because these conditions only induced growth inhibition by PPCPs in phase I. Such a prolonged sub-lethal exposure was aimed at inducing selection of resistant traits and promote adaptations that could affect population dynamics and result in the postulated fitness trade-offs. Exposure was conducted under three DOM levels (0, 5 and 15 mg L^{-1} DOC), to account on the influence of DOC on the emergence of tolerance and growth trade-offs. A control culture was grown at the same level of pH, in the absence of PPCPs and DOM. The cultures were transferred to new growth medium every week (0.5 mL culture to 40 mL of fresh medium) during the adaptation period.

2.8. Phase II

193 Following the adaptation phase, subsamples (100 µL) from each cultures were inoculated in two
194 separate sets of four replicate culture flasks and diluted with 40 mL of DOM-free growth medium.
195 One set was spiked with 20 µL of the PPCP solution (at the same concentrations used in phase I),
196 while the other one was spiked with 20 µL of the carrier solvent (DMSO) only (excluding the
197 contaminants). Phase II was run for 7 days during which cultures were grown exponentially under
198 the same light, nutrient and temperature conditions used in phase I.

199 **2.9. Data treatment, response parameters and statistical analysis**

200 All the analyses were conducted using R (version 3.5.1) statistical software (R Core Development
201 Team 2015). Growth rate was calculated using the total algal biovolume as determined from the
202 cell counter. Total algal biovolume (BV_t) was calculated based on the number of cells (N) and their
203 radius (r), assuming a spherical shape of the cells:

204 $$BV_t = \sum_{i=1}^{n} \frac{4}{3} \pi {r_i}^3 \, N_i$$

205 Specific growth rate µ (d^{-1}) of each experimental unit was calculated as the slope of a linear
206 regression of log-transformed biovolume against time, using data from the exponential growth
207 phase (Figure S3). For the comparison of cell size between treatments, we calculated peak cell
208 diameter (µm; here called "cell size") as the mode of cell size distribution. Growth rate based on
209 the cell count (here called "recruitment rate") was also calculated to disentangle the growth of the
210 microalgae from the variation of the cell size caused by the treatments.

In phase I, the toxic responses of the algal population to PPCPs under different combinations of DOM and pH was evaluated by a two-step procedure. As response variables we used total algal biovolume and cell size. First, we used a three-way ANOVA to test the significance of the treatment factors and their interactions. Secondly, we used linear modelling (with all predictor variables coded as factors) to test for significant differences in toxic responses between groups of interest (e.g. whether the response of total algal biovolume or cell size to contaminants differed significantly between different DOC levels at a given pH).

In phase II, we first tested whether the adaptation period had caused algae to develop tolerance to PPCPs, and whether an eventual adaptation led the trade-off (i.e. reduced growth rate and/or cell size when grown without contaminants). We did this by modelling specific growth rate, cell size and recruitment rate as a function of contaminants exposure (factor variable with two levels; yes/no) and whether they were allowed to adapt to PPCPs in the adaptation period (factor variable with two levels; yes/no). We tested for main effects and interactions between the two treatment factors. For the populations that underwent the adaptation phase under different levels of DOM, we tested how specific growth rate and cell size responded to contaminant exposure in phase II in the absence of DOM. This was done by modelling specific growth rate and cell size as a function of two factors: the presence/absence of PPCPs and DOM-level during the adaptation period (factor variable with three levels; 0, 5 and 15 mg L^{-1} DOC). We tested for main effects and interactions between two treatment factors.

3. Results

3.1. Phase I

3.1.1. Effects of DOM and PPCPs on biomass

The mix of PPCPs had a highly significant effect on the total algal biovolume yield ($F = 97.025$, $p<0.001$; Table 1 and Figure 2A). This effect was strongly dependent on pH and DOM, as shown by the significant interactions between PPCPs and pH ($F =20.807$, $p<0.001$) and PPCPs and DOM ($F = 5.684$, $p<0.05$). While exposure to PPCPs generally reduced the total biovolume yield ($t=-6.07$, $p<0.05$), the toxic effect was significantly stronger at pH 8 than at pH 6.5 ($t=-4.55$, $p<0.001$). Low concentrations of DOM (5 mg L^{-1} DOC) at pH 8 decreased the negative effect of contaminants exposure on the total biovolume yield, relative to the control without DOM ($t=2.272$, $p<0.05$). A similar positive effect was not observed at the higher level of DOM (15 mg L^{-1} DOC), where the total biovolume did not differ from the control with no DOM ($t=0.56$, $p = 0.586$). At pH 6.5, the detrimental effect of PPCPs was not influenced by the DOM ($F= 0.1865$, $df= 2.9$, $p = 0.83$).

In the absence of PPCPs, the total biovolume yield was significantly lower at the higher level of DOM (15 mg L^{-1} DOC) compared to the control without DOM ($t=-3.45$, $p = 0.0027$) at both pH levels. Total biovolume tended to be more sensitive to high DOM levels at pH 6.5 than at pH 8 (Figure 2A), but the difference was border-line significant ($p = 0.08$).

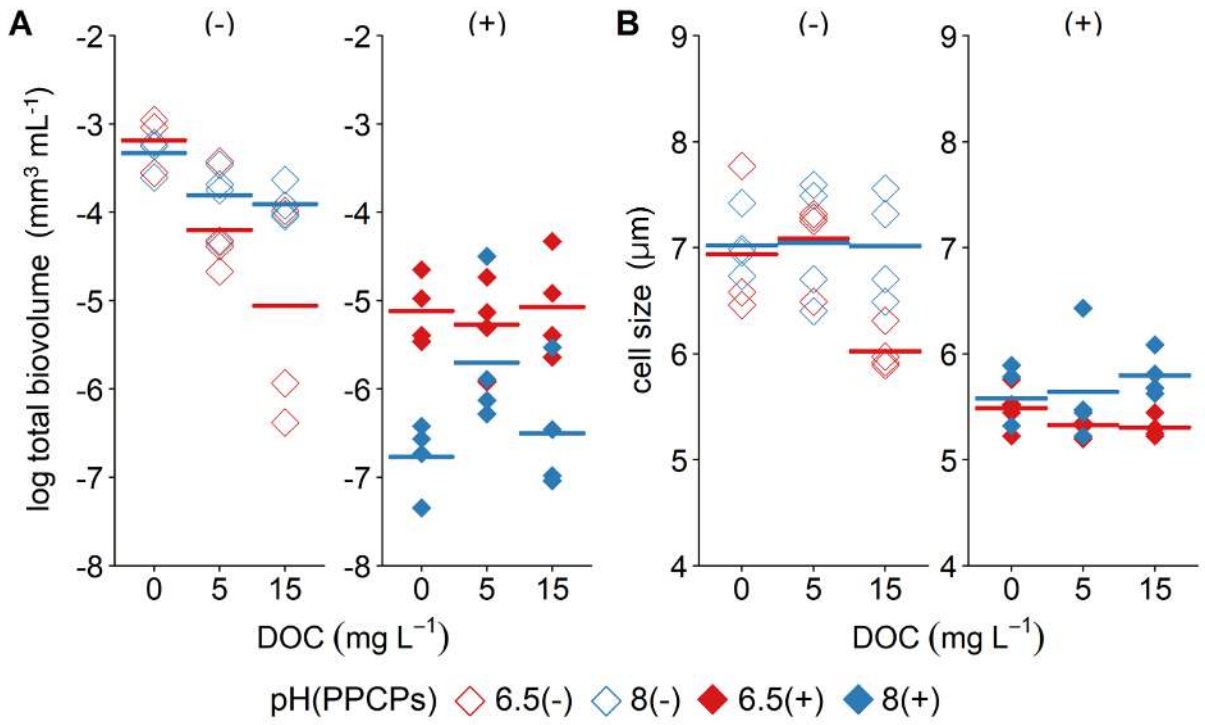

Figure 2. Phase I results. (A) Log total biovolume yield (mm^3/mL) and (B) mean cell size (µm) of *C. reinhardtii* as a function of DOM (0, 5, 15 mg L^{-1} DOC) and pH (6.5, 8) in the absence (-) and the presence (+) of the mix of PPCPs in phase I. Short horizontal bars represent the each group.

Table 1. ANOVA table of phase I results. Main outcome from a three-way ANOVA which tested the effects of PPCPs (the absence/presence), DOM (0, 5, 15, mg L^{-1} DOC) and pH (6.5, 8) on log(total algal biovolume yield) and mean cell size. The three-way interactions were not significant and are not shown in the table. df; degree of freedom. SS; Sum of square means. F; F value. Significant values are reported in bold.

Variables	Factors and interactions	df	SS	F	p
log total biovolume (mm^3/mL)	**PPCPs**	**1**	**37.77**	**97.02**	**< 0.001**
	DOM	2	2.15	2.76	0.08
	pH	1	1.33	3.42	0.07
	PPCPs : DOM	**2**	**4.43**	**5.68**	**0.01**
	PPCPs : pH	**1**	**8.1**	**20.21**	**< 0.001**
	DOM : pH	2	1.7	2.18	0.13
	residuals	*37*	*14.40*		
cell size (µm)	**PPCPs**	**1**	**20.77**	**141.20**	**< 0.001**
	DOM	2	0.55	1.92	0.16
	pH	**1**	**1.29**	**8.80**	**0.005**
	PPCPs : DOM	**2**	**0.81**	**2.75**	**0.05**
	PPCPs : pH	1	0.01	0.07	0.79

	DOM : pH	**2**	**1.047**	**3.56**	**0.04**
	residuals	*35*	*5.15*		

256

257 ### 3.1.2. DOM and PPCPs effects on cell size

258 The mix of PPCPs consistently decreased the mean cell size of the population ($F= 141.20$, $p<0.001$,
259 Table 1 and Figure 2B). This effect was also modified by the presence of DOM, as shown by the
260 significant interaction term ($F= 2.75$, $p<0.05$), while the interaction with pH was not significant.
261 The negative effect of PPCPs on cell size ($t= -7.323$, $p<0.001$) was lower ($t= 2.579$, $p<0.05$) at pH
262 8 than at pH 6.5. In the absence of contaminants, the higher level of DOM (15mg L^{-1} DOC)
263 negatively affected the cell size only in the treatment with pH 6.5 ($t= -3.20$, $p<0.05$).

264 ## 3.2. Phase II

265 ### 3.2.1. Trade-offs of tolerance acquisition in the absence of DOM

266 Exposure to PPCPs in phase II in absence of DOM decreased algal growth rates (defined as the
267 increase in total algal biovolume over time) in all cultures, regardless of previous adaptation ($F=$
268 43.68, $p<0.001$; Figure 3A and Table 2). The growth inhibition effect was, however, significantly
269 lower for the adapted cultures ($df = 12$, estimated mean difference = 0.51 μ (d^{-1}), $p<0.05$). At the
270 same time, when grown in the absence of PPCPs in phase II, adapted cultures had a significant
271 slower growth than not-adapted ones ($df=12$, estimated mean difference =-0.27 μ (d^{-1}), $p<0.05$,
272 Figure 3A and B, Table S7), indicating that acquisition of tolerance trades-off with growth in
273 absence of contaminants.

274

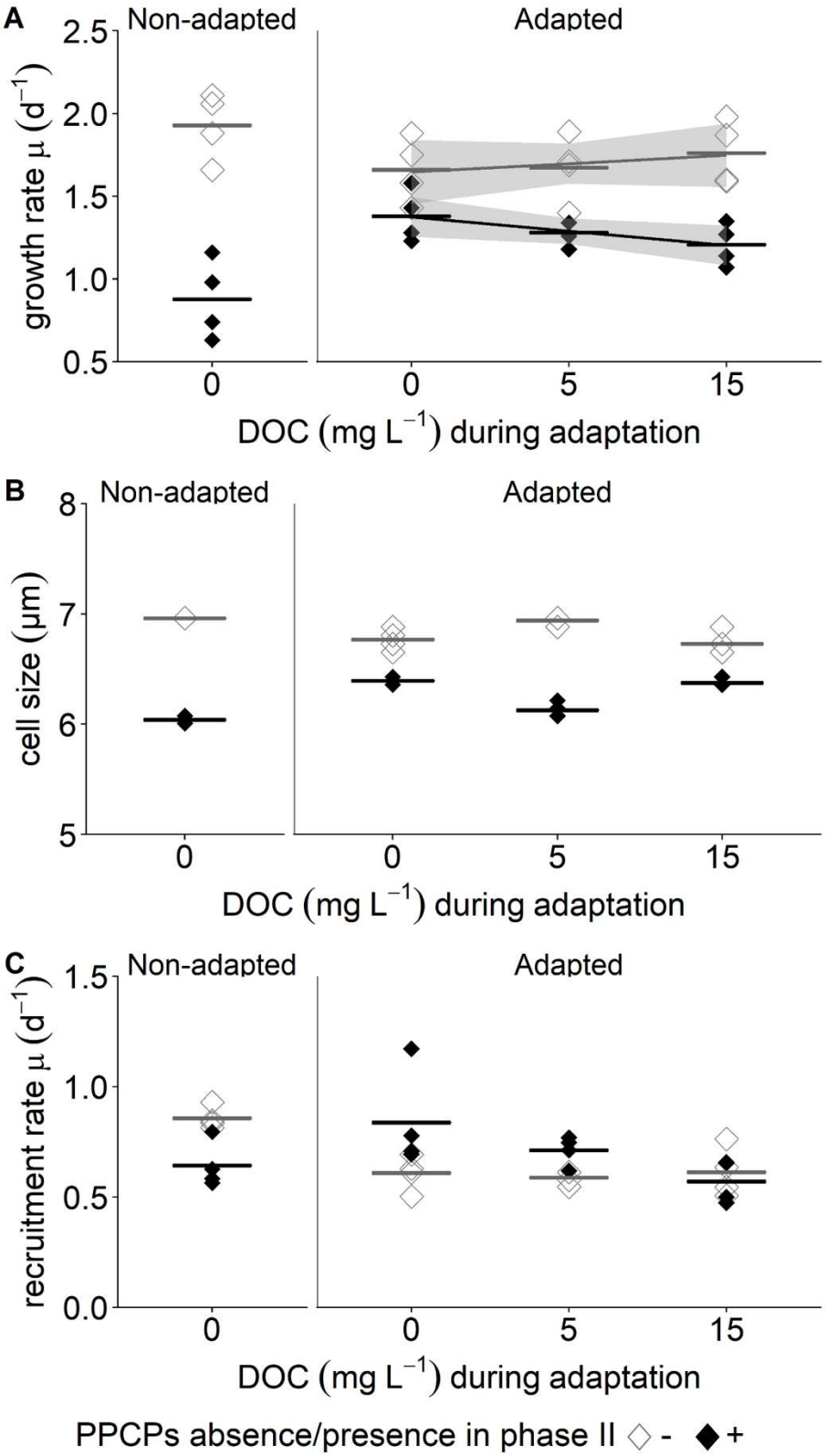

Figure 3. Phase II results, growth rate and cell size. Growth rate (A), mean cell size (B) and recruitment rate (C) of the population which did not experimented PPCPs and DOM during the adaptation period (non-adapted), and the population cultivated with PPCPs and DOM levels during

the adaptation period (adapted), in response to the absence (-) and the presence (+) of the mix of PPCPs in phase II. Short bar report the mean values.

Table 2. Effect of the presence of PPCPs and DOM during the adaptation period. ANOVA-table showing the main outcome from the two-way ANOVA which tested the effects of the presence of PPCPs during the adaptation period on the growth rate and cell size of the algal populations exposed to the absence/presence of PPCPs in phase II (non-adapted vs. adapted), and the effects induced by the presence of DOM during the adaptation period with PPCPs on the growth rate and cell size of the algal population exposed to the absence/presence of PPCPs in phase II (adapted with no DOM vs. adapted with DOM). df; degree of freedom. SS; Sum of square means. F; F value. Significant values are reported in bold.

Contrast	Variables	Factors and interactions	df	SS	F	p
Non-adapted vs. Adapted with no DOM	growth rate μ (d^{-1})	PPCPs during adaptation	1	0.05	1.36	0.26
		PPCPs in phase II	**1**	**1.77**	**43.68**	**<0.001**
		PPCPs during adaptation : PPCPs in phase II	**1**	**0.59**	**14.64**	**<0.01**
		residuals	*12*	*0.49*		
	cell size (µm)	**PPCPs during adaptation**	**1**	**0.02**	**7.64**	**<0.05**
		PPCPs in phase II	**1**	**1.67**	**509.56**	**<0.001**
		PPCPs during adaptation : PPCPs in phase II	**1**	**0.3**	**90.97**	**<0.001**
		residuals	*12*	*0.04*		
	recruitment rate μ (d^{-1})	PPCPs during adaptation	1	0.002	0.15	0.7
		PPCPs in phase II	1	0.001	0.01	0.9
		PPCPs during adaptation : PPCPs in phase II	**1**	**0.2**	**11.9**	**<0.05**
		residuals	12			
Adapted with no DOM vs. Adapted with DOM	growth rate μ (d^{-1})	**PPCs in phase II**	**1**	**1**	**36.43**	**<0.001**
		DOM during adaptation with PPCPs	2	0.009	0.16	0.85
		PPCPs in phase II : DOM during adaptation with PPCPs	2	0.07	1.37	0.28
		residuals	*18*	*0.49*		
	cell size (µm)	**PPCPs in phase II**	**1**	**1.58**	**309.84**	**<0.001**
		DOM during adaptation with PPCPs	2	0.008	0.84	0.45
		PPCPs in phase II : DOM during adaptation with PPCPs	**2**	**0.27**	**26.52**	**<0.001**
		residuals	*18*	*0.092*		
	recruitment rate μ (d^{-1})	**PPCPs in phase II**	**1**	**0.06**	**4.58**	**<0.05**
		DOM during adaptation with PPCPs	1	0.07	2.46	0.11
		PPCPs in phase II : DOM during adaptation with PPCPs	1	0.07	2.61	0.11
		residuals	*12*			

289 Cell size response was similar to that of growth rate. Exposure to PPCPs in phase II yielded smaller
290 cells in all treatments ($F= 509.56$, $p<0.001$; Figure 3B and Table 2). The magnitude of the effect,
291 however, was strongly dependent on the adaptation ($F= 90.97$, $p<0.001$). In phase II experiments,
292 the mean cell size of cultures exposed to PPCPs during the adaptation period was significantly
293 larger in the presence of the contaminants than that of non-adapted cultures (df= 12, estimated
294 mean difference= 0.352 µm, $p<0.001$, Table S7). Concurrently, their cell size was smaller than the
295 non-adapted ones when grown in phase II in absence of PPCPs (df= 12, estimated mean
296 difference= -0.194 µm, $p<0.001$, Table S7). This further reinforces confidence on the existence of
297 a trade-off between tolerance acquisition and reduced cell size.

298 PPCP exposure during phase II decreased recruitment rates (taken as a proxy of fitness and
299 measured here simply as the increase of cell number over time) of the non-adapted population ($F=$
300 4.58, $p<0.05$). The adapted population, on the contrary, yielded higher recruitment rates when
301 algae were exposed in phase II to the PPCPs (df= 12, estimated mean difference= -0.229 µ (d^{-1}),
302 $p<0.05$). The effect of adaption on the recruitment rates mirrored observed growth rate and cell
303 size patterns (Figure 3C, Table S7). For instance the adapted population yielded a higher
304 recruitment rate when exposed to the PPCPs in phase II (df= 12, estimated mean difference= 0.194
305 µ (d^{-1}), $p<0.001$, Table S7), but lower in the absence of the contaminants (df=12, estimated mean
306 difference= -0.25 µ (d^{-1}), $p<0.05$, Table S7), relative to the non-adapted population. This indicates
307 that beyond the negative relation with cell size, acquisition of tolerance also trades-off with
308 recruitment rates, and therefore with the population fitness.

309 **3.2.2. Effects of DOM on tolerance acquisition and trade-offs**

Similar to the response of adapted algae in absence of DOM, the exposure to PPCPs in phase II significantly affected growth rates, cell size and recruitment rates of algae adapted in presence of DOM (Figure 3, Table 2). Growth rates and recruitment rates of adapted algae exposed to PPCPs declined along the DOM gradient applied during the adaptation period. The recruitment rate of algae that acquired adaptation in presence of the highest level of DOM was significantly lower relative to that of algae adapted in its absence ($t = -2.27$, $p<0.05$). The DOM gradient during the adaptation period did not significantly affect growth rates, cell size and recruitment rates of the adapted algae in absence of contaminants (Table 2, Figure 3), despite those were, altogether, lower than that of non-adapted algae (Table S7).

4. Discussion

We assessed the effects of the interaction of micropollutants and DOM on growth, cell size and fitness (through the use of recruitment rate as a proxy) of a freshwater microalgae population. We focused in particular on the emergence of trade-offs associated to adaptation acquisition (i.e. whether tolerance acquisition to chemical stress [20] influences these variables when algae grow in the absence of the stressor) as well as the role of an important environmental factor (namely, DOM) on the development of tolerance acquisition and related costs. Our results show that algae responses depend on PPCPs, DOM and their interaction during the adaptation period. In particular we observe:

i) a mitigating effect induced by the combination of DOM and pH on the toxic effect of the PPCPs (Figure 2, Table 1);

ii) Emergence of tolerant populations upon the adaptation period;

iii) tolerance acquisition and emergence of related trade-off are influenced by DOM levels during the adaptation period (Figure 3, Tables 2, S5-S6);

whereby, points i) and ii) verify the study's postulates, and point iii) supports our main hypothesis. The following sections discuss these findings and their implications in detail.

4.1. Phase I - Effects of DOM and pH on algal population responses to PPCPs

PPCPs negatively affected growth rate (Figure 2A) and cell size (Figure 2B) of the tested population during phase I. Previous studies have shown that PPCPs can affect growth of microalgae [14]. Our findings shows that the interaction between DOM, pH and PPCPs has a significant effect on the algal growth rate (Table 1). This translates into a positive effect of the interaction of DOM, pH and PPCPs on algal growth that is observed in particular at the lower DOM concentration (5 mg L^{-1} DOC) and pH 8. Under these conditions observed growth hindrance effects by PPCPs are minimal. This verify the first of our postulates. pH can vary the speciation/form of both contaminants and DOM, and modify contaminants' ionic configuration. These, in turn, can affect both their toxicological properties and/or their complexation with DOM, and thereby their availability. The majority of the compounds (7 out of 12) within the mix of PPCPs used in the present study (Table S2), are in their associated form, moderately to highly hydrophobic (log_{kow} ranging from 2.03 to 4.76), while the remaining are highly hydrophilic (log_{kow} ranging from -0.07 to 0.89, Table S2). Hydrophobic compounds have a significant interaction with DOM [24] that likely influenced our results. In addition, higher pH (8) forms neutral species also for some of the more hydrophilic compounds, promoting their toxicity and their complexation. Among the PPCPs in the mixture, carbamazepine, clarithromycin and triclosan have pKa between 7.9 and

354 13.9 (Table S2). This explains the dependency of the toxicity results on pH. Our findings are in
355 line with previous studies [25,42–44].

356 At higher DOM concentration (15 mg L^{-1} DOC) instead, such a toxicity inhibition effect vanished
357 (Figure 3B). We argue that this is caused by direct, negative impacts of DOM on the algae. For
358 instance, DOM can actually directly stress algae [30] in various ways (an effect that is found in our
359 experiment to be more pronounced where algae are grown in absence of PPCPs at lower pH)
360 (Figure 2A). In particular, DOM can i) reduce growth by reducing light availability [30]; ii) in
361 nutrient-limited environments, affect algal growth by adding organically bound nutrients (e.g. P
362 [30]), hinder it by complexing or adsorbing key elements (e.g. Fe [30]), or promote the growth of
363 heterotrophic bacteria with higher affinity for limiting nutrients (e.g. P [30]); iii) produce of harmful
364 free radicals and reactive oxygen species from photoactivation stressing the algae [45]; and iv) affect
365 directly the photosynthetic machinery [46]. In the experimental conditions, lack of short-wave
366 irradiation and nutrient saturated conditions exclude negative impacts due to formation of reactive
367 species and nutrient limitations. Direct negative effects of high DOM levels on algae are more
368 plausible mechanisms. This explanation is consistent with the observed interactive effect between
369 pH and DOM (Table 2) on growth inhibition in absence of PPCPs.

370 **4.2. Phase II – Tolerance acquisition and trade-offs**

371 During the adaptation period the algae were exposed over multiple generations to the mix of
372 PCPPs. This results in acquisition of tolerance as demonstrated by the higher growth rate of the
373 adapted population in phase II (Figure 3) compared to non-adapted ones under PPCPs exposure.
374 Considering the time frame of the adaptation period (> 2 months) [1], PPCPs may have favored the
375 emergence of tolerant strains through selective filtering. While this can be the result of rapid

evolution, a physiological component of this response cannot be excluded, in principle. To disentangle the nature of the adaptation process is notoriously difficult and is outside the scope of this study. However, the rapid changes in mean cell size observed in experimental phase II as response to PPCP especially in the non-adapted population (Figure 3B) points at fast physiological responses that can affect resource allocation. Similar findings indicating tolerance acquisition triggered by rapid adaptation to chemical stress are also reported by others [20], including attempts to isolate physiological, ecological and evolutionary processes [47].

Our results show that acquiring tolerance introduces a cost. This is evident when the adapted population grows in absence of PPCPs (Table 2), yielding a lower growth rate (compared with the non-adapted one (Figure 3). Physiological and evolutionary trade-offs are broadly treated and described in biological literature, and different theoretical bodies provide explanation or acknowledge their existence as a postulate [7,48]. Trade-offs between growth and cell size can reflect the need to balance investment in tolerance at expense of energy expenditure on other fundamental processes. Trade-offs can theoretically originate both from physiological, ecological or evolutionary adaptations. Their effects on population demographic rates emerge when individuals capable of expressing metabolic paths or molecular arrangements conferring stress tolerance (at the expense of other fundamental functions) increase their frequency in the population. Here we show that a two-month continuous sub-lethal exposure to PPCPs set a new environmental optimum selecting tolerant organisms with a significantly different morphology (i.e. cell size) and higher fitness (i.e. higher recruitment rate). This results in a stress tolerant population with growth dynamics that are different from the wild type both in the presence and in the absence of the stressors (Figure 3). Similar findings indicating emergence of trade-offs in rapidly adapted phytoplankton ae also reported elsewhere [49] Our study complements and expand these results

showing that the selectivity of the environment is significantly controlled by ambient DOM levels (Figure 3).

The co-variance between growth rates, recruitment rates and cell size indicates a tight interconnection between stress response of these variables and the acquisition of tolerance (Figure 3). Cell size results basically mirrored the patterns observed for growth rate (Figure 3B). Similarly to growth rates, tolerance acquisition reduces negative effects of PPCPs on cell size (Figure 3B). At the same time, the occurrence of trade-off results in a smaller cell size of the adapted population in the absence of the contaminants, relative to the non-adapted population. Recruitment rates respond similarly but in this case the benefits of tolerance acquisition appear more clearly. Adapted microalgae growing in the presence of the contaminants yield recruitment rates comparable to those of the wild type growing in absence of stress (Figure 3C).

Recruitment rates are taken here as a proxy of fitness, whereby fitness is fundamentally defined as the probability of producing off-springs and is measured through the increase in the population cell number over time) [50]. As we used culture batches in microcosms, recruitment depends only on the generation of off-springs and dispersal is absent. Recruitment rate results demonstrate that tolerance acquisition fundamentally concerns allocation of resources toward maximizing fitness in the selective environment (i.e. in the presence of PPCPs) at the cost of a smaller cell size. Cell size changes accounted in fact for a considerable fraction of the biovolume-derived growth rate response. Reduced cell volume explains in fact almost 100% of the observed growth rate inhibition in phase II of the population adapted in the absence of DOM (not shown). Instead, the relative contribution of cell size change in the growth rate loss in the presence of PPCPs ranges 20-50% (not shown). Disentangling the influence of recruitment rate and cell size on the growth rate allows to reveal another interesting effect related to tolerance acquisition. When grown in presence of

PPCPs, the adapted population yields a higher recruitment of larger-sized cells, compared with the non-adapted population (Figure 3B-C). This is especially visible for the treatment with no DOM addition during the adaptation period.

Whether the acquisition of tolerance implies a net advantage when balanced against its costs is a question deserving attention. To address it, we formulated a rigorous definition of trade-off. First, we defined the benefit of the adaptation (B_{adp}, t^{-1}) as the gain in growth rate the adapted population displays when growing in the presence of PPCPs. This was calculated as:

$$B_{adp} = gr_{A,+} + gr_{nonA,+} \qquad 1)$$

where $gr_{A,+}$ and $gr_{nonA,+}$ represents the growth rates of the adapted population and non-adapted population in the presence of PPCPs in phase II (Figure 3B, Table 2).

Similarly, we defined the costs of adaptation (D_{adp}, t^{-1}) as the reduction in growth rate the adapted population displays when growing in the absence of PPCPs in phase II, calculated as:

$$D_{adp} = gr_{A,-} + gr_{nonA,-} \qquad 2)$$

Note that, based on the experimental results B_{adp} and D_{adp} are positive and negative, respectively. Their net trade-off is therefore their sum.

$$Net\ trade-off = B_{adp} + D_{adp} \qquad 3)$$

Figure 4 shows that the net trade-off tends to be positive, suggesting that the acquisition of tolerance generally results in a net benefit for the population. This has implications on how adapted populations will behave in variable environments in which phases of stress periodically follow phases of non-stress (i.e. a lake receiving contaminated waters intermittently). In such an

environment (assuming stress periods are equivalent to periods of non-stress) the adapted population can theoretically have a two-fold competitive advantage: first, by yielding higher biomass over time that the non-adapted one. Second, by having a net fitness advantage (Figure 3C). This indicates that PPCPs potentially represent an important selective force in impacted ecosystems, and that chemical pollution should be included more frequently in the study of multi-stressor ecosystem responses.

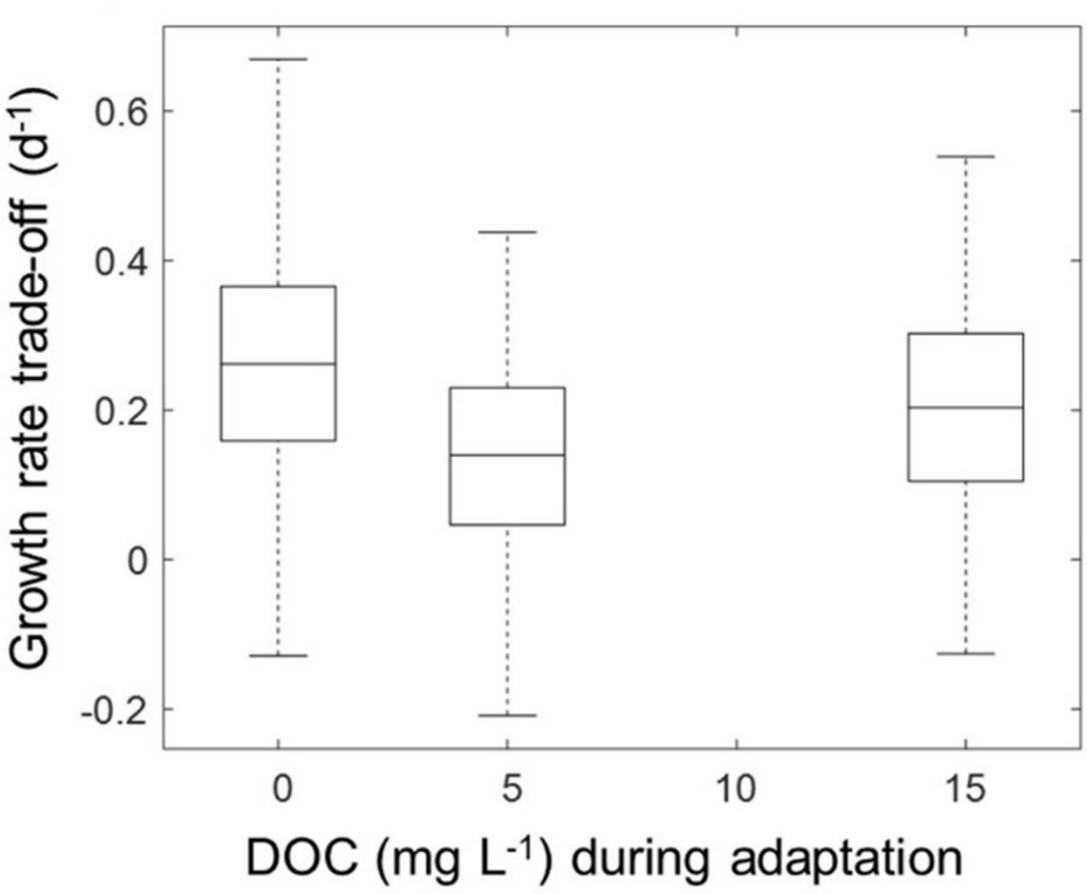

Figure 4. The net trade-off from tolerance acquisition. These variables were calculated after bootstrapping estimated growth rate values from gaussian distributions fitted to the experimental growth rate data. Data variability and uncertainties were tracked down to the final values of gap and trade-off using a Montecarlo frame ($N=10^5$).

4.3. Phase II - Effects of DOM on tolerance acquisition and trade-off

The presence of DOM during the adaptation period reduces tolerance acquisition (both in terms of growth and recruitment rates) and resulted in both lower B_{adp} and D_{adp} (Figure 3 and 4, Table S5), in line with our hypothesis. Based on the results of phase I (Figure 2A, B), DOM and high pH mitigate the selective pressure hindering tolerance acquisition by the stressed algae. Similar findings suggesting a proportional response of tolerance acquisition in relation to stress intensity are reported elsewhere [51,52]. In our case, stress mitigation depends upon an environmental factor (DOM) of great relevance for freshwater ecosystems and under fundamental biogeochemical control [21,23]. While growth rate and recruitment rates are dependent on DOM levels during the adaptation period (Figure 3), the net trade-off is not (Figure 4). This is obviously because both B_{adp} and D_{adp} grows in their absolute value at increasing level of DOM during the adaptation period, compensating for their off-set. As discussed above, despite a positive net trade-off of adaptation that is apparently independent from DOM, the increasingly large gap in the growth response of adapted algae in the presence and the absence of PPCPs has interesting implications. It suggests, in fact, that the population that gained tolerance in the absence of DOM, developed faster response dynamics to changes in stress levels. As a result of a similar net trade-off, this population is expected to experience more rapid biomass losses at the onset of the stressor, and to recover faster at the stress release, compared to the populations that partially acquired tolerance in the presence of DOM. In contrast, this population is expected to respond to changes in stress levels smoothing biomass loss and gains. These different behaviors, embodied in the different growth dynamics and trade-offs, represent two alternative strategies to stress-response. In the broader ecological context, the co-existence of adapted and non-adapted populations in a community can have implication on community structuring, functioning and ultimately ecosystem resilience [48].

4.4. Environmental significance

Through the use of sub-lethal concentrations of a mixture of PPCPs as stressor model and DOM as model of environmental control, we showed that the interaction of stressors and the environment modulates adaptation processes and the unfolding of associated functional trade-offs. We add here more empirical evidence for the key role of DOM and pH in mediating toxic responses to PPCPs [24,25], showing that both the direct effect of DOM, as well as its interaction with chemical pollutants on algae growth is highly dependent on pH. Furthermore, our results complement the findings of other recent studies showing acquisition of tolerance to chemical stress triggered by multi-generational exposure to the same stressor [1,20]. At the same time, we report new empirical evidence of the costs and net trade-offs associated to tolerance acquisition. DOM can counteract the process of tolerance acquisition when algae are exposed to sub-lethal levels of chemical stressors for multiple generations. This, in turn, has implications also for costs associated to tolerance acquisition. Adapted algae have relatively higher growth rates when growing in the presence of the stressor compared to non-adapted ones, and, on the contrary, have lower growth rate in pristine conditions. While DOM affects these rates, their net trade-off is positive and DOM-independent, suggesting that acquiring tolerance is generally advantageous for the algae, and can represent a significant selective pressure in impacted ecosystems.

Tolerant microalgae display higher recruitment rates and smaller cell size when grown in the presence of PPCPs, indicating tolerance acquisition coincided with allocation in fitness at the cost of a smaller cell size. This strategy allowed tolerant microalgae to compensate a considerable part of the growth rate loss due to PPCPs.

Our results also add new insights to the impacts of water browning. Since browning is caused by increasing levels of DOM [21], our findings suggest that while this process may mitigate the detrimental effects caused by ubiquitous organic contaminants, at the same time antagonistic effects on the tolerance acquisition of stressed populations should be considered as one of its potential implications. Hence, results presented here can be useful to guide future assessments on the ecological and evolutionary consequences induced by the process of browning in freshwater ecosystems that are also recipient of wastewater discharges, and might be beneficial to inform environmental management in a multi-stressor context.

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Supplementary Materials for:

Water browning controls tolerance acquisition and associated trade-offs in phytoplankton stressed by chemical pollution

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Figure S3: Log daily biovolume development during phase II.

Figure S4: *In vivo* fluorescence per unit of biomass data of the microalgae population during phase I.

References

Table S1. Summary data on the occurrence and concentration (ng/L) of PPCPs used in this study found in European freshwaters (lakes and rivers). The data was obtained from the Norman database. Norman is the Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances (www.Norman-network.net). This table was modified from the paper published by Pomati et al. (*60*) and Baho et al. (*59, 62*).

Chemical	Time analyzed	Times detected	Percentage detection (%)	Min conc. (ng/L)	Max conc. (ng/L)	Mean conc. (ng/L)	standard deviation (ng/L)	Q1 conc. (ng/L)	Median conc. (ng/L)	Q3 conc. (ng/L)
Atenolol	977	723	74	0.1	900	26.3	70.7	6	11	19
Bezafibrate	1384	764	55.2	0.3	21200	108.5	1162.7	8	13	28
Carbamazepine	22270	19361	86.9	0.8	7600	158.3	295.8	33	70	160
Clarithromycin	945	730	77.2	0.9	1100	21	44.7	10	13	21
Diclofenac	6320	4439	70.2	0.2	110000	785	5977.4	23	57	130
Furosemide	507	84	16.6	0.5	283000	9253.7	44732.1	12.25	35	76
Hydrochlorothiazole	484	235	48.6	4	389000	4425	36594.8	22	41	85.5
Ibuprofen	5154	3668	71.2	1.2	303000	214.5	5167.9	15	32	70
Ranitidine	50	29	58	1.3	200	33.4	55.1	2.3	5.4	40
Sulfamethoxazole	2616	2133	81.5	0.7	700	33.3	46	12	20	40
Benzophenone-4	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Triclosan	11565	9053	78.3	1	3060	20.4	56.9	8	12	20

Table S2. Chemical properties (acid dissociation constant – pKa, and octanol/water partition coefficient – log K_{ow}), spiked concentrations (µg/L), and reported effective concentrations (µg/L) inhibiting 50% of growth (EC50) in phytoplankton species for the 12 studied chemical compounds. Toxicity values were obtained from the U.S. Environmental Protection Agency ECOTOXicology Database System (2015, Version 4.0, www.epa.gov/ecotox/). This table was modified from the papers published by Pomati et al. (*60*) and Baho et al. (*59, 62*).

Chemical	CAS ID	mm (g/mol)	pKa	Log K_{ow}	Spiked conc. (µg/L)	Mean EC50 (µg/L)	SD (µg/L)	Num. studies
Atenolol	29122-68-7	266.34	9	0.16	22	$3.18E^{+05}$	$2.63E^{+05}$	3
Bezafibrate	42859-67-0	361.822	3.83	4.25	2.2	$3.50E^{+04}$	$2.63E^{+03}$	3
Carbamazepine	298-46-4	236.274	13.9	2.45	22	$1.37E^{+05}$	$2.83E^{+05}$	24
Clarithromycin	81103-11-9	747.964	8.99	3.16	22	$1.97E^{+01}$	$2.33E^{+01}$	3
Diclofenac	15307-86-5	296.147	4.15	4.51	22	$6.27E^{+04}$	$6.73E^{+04}$	6
Furosemide	54-31-9	330.739	4.25	2.03	2.22	> $7.000E^{+04}$	NA	1
Hydrochlorothiazole	58-93-5	297.728	7.9	-0.07	22	NA	NA	NA
Ibuprofen	15867-27-1	206.285	4.91	3.97	22	$3.29E^{+05}$	$1.92E^{+04}$	2
Ranitidine	66357-35-5	314.404	7.8	0.08	2.2	$2.70E^{+04}$	$4.87E^{+04}$	
Sulfamethoxazole	723-46-6	253.276	1.6/5.7	0.89	2.2	$2.15E^{+03}$	$3.10E^{+03}$	7
Benzophenone-4	4065-45-6	308.304	7.6	0.37	22	$1.00E^{+04}$	NA	1
Triclosan	3380-34-5	289.536	7.9	4.76	2.2	$5.86E^{+02}$	$7.82E^{+02}$	24

Table S3. Growth inhibition test of *Chlamydomonas reinhardtii* exposed to the mix of PPCPs. The exposure levels used in our study were based on a preliminary test conducted on *C. reinhardtii* following the OECD guidelines (*63*). Eight levels of exposure were applied following a factorial increase (0, 1, 3, 10, 30, 100, 300, 1000). The concentrations used in this study were the one from level 5 (in bold), causing 28.6% growth inhibition.

	Concentrations (µg/L)							
Chemical	**Ctrl**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**
Atenolol	0	0.22	0.66	2.2	6.6	**22**	66	220
Bezafibrate	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Carbamazepine	0	0.22	0.66	2.2	6.6	**22**	66	220
Clarithromycin	0	0.22	0.66	2.2	6.6	**22**	66	220
Diclofenac	0	0.22	0.66	2.2	6.6	**22**	66	220
Furosemide	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Hydrochlorothiazole	0	0.22	0.66	2.2	6.6	**22**	66	220
Ibuprofen	0	0.22	0.66	2.2	6.6	**22**	66	220
Ranitidine	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Sulfamethoxazole	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
Benzophenone-3	0	0.22	0.66	2.2	6.6	**22**	66	220
Triclosan	0	0.022	0.066	0.22	0.66	**2.2**	6.6	22
n	6	3	3	3	3	**3**	3	3
mean growth rate µ (d^{-1})	1.62	1.79	1.61	1.64	1.66	**1.16**	0.90	0.55
SD	0.05	0.05	0.05	0.09	0.02	**0.01**	0.06	0.01
% growth inhibition		-10.7	0.8	-1.6	-2.8	**28.6**	44.4	65.7

Text S2. PPCPs stability test

In order to check for degradation of the mix of PPCPs, the experimental units exposed to the contaminants were sampled during both phases of the experiment as follows. 1 mL samples were collected in triplicates, stored in 2 mL GC amber glass vials at -20°C in the dark. The compounds were extracted through SPE extraction using HLB cartridges (Oasis) in 5 mL of MeOH. The extract was blown down to dryness with a gentle N^2 flow, reconstituted in 1 mL MeOH, and filtered through 0.2 µm PP syringes filters (Pall, UK) into a 2 mL GC vial. The samples were analysed by HPLC-MS (Shimadzu, 8040), using an XBridge BEH C18 column (2.1 mm x 100 mm, 3.5 µm) to separate the compounds. The mobile phases were A, 0.2% Ammonium hydroxide in MQ water, and B, 50% Methanol and Acetonitrile. The gradient procedure was optimized at: 0-1 min 20% B, then increased to 100% within 8 min, held at 100% for 5 min, after that decreased to the initial conditions (20% B) within 1 min. Finally, 6 minutes of post-run ensured re-equilibration of the column before the next injection. The injection volume was 15 µL and the column and the tray temperature were set to 35°C. The quantification of the compounds was based on internal standard method (Atenolol d7 and Ibuprofen d3, Sigma Aldrich), and the instrument detection limit was 3.87 ng/mL.

Table S4. Percentage of recovery (± standard deviation) of the mix of PPCPs at different levels of DOM and pH at the end of phase I and phase II.

	Chemical	Spiked conc. (ng/L)	Recovery DOC 0 (% ± sd)	Recovery DOC 5 (% ± sd)	Recovery DOC 15 (% ± sd)	
phase I	Atenolol	22	100 ± 0.3	94.3 ± 6.3	99.4 ± 0.1	pH 6.5
	Bezafibrate	2.2	99.3 ± 2.2	100.3 ± 1.0	97.6 ± 1.0	
	Carbamazepine	22	102.9 ± 1.2	101.2 ± 2.3	104.3 ± 3.3	
	Clarithromycin	22	98.2 ± 1.9	104.3 ± 2.3	105.2 ± 4.2	
	Diclofenac	22	99.4 ± 2.1	102.2 ± 2.0	100.3 ± 1.1	
	Furosemide	2.22	96.4 ± 4.1	99.3 ± 2.4	98.2 ± 2.4	
	Hydrochlorothiazide	22	103.7 ± 1.3	98.4 ± 2.5	98.8± 6.7	
	Ibuprofen	22	100.3 ± 0.3	99.4 ± 4.1	96.8 ± 7.4	
	Ranitidine	2.2	99.4 ± 2.8	98.7 ± 4.4	102.3 ± 2.3	
	Sulfamethoxazole	2.2	97.3 ± 2.1	95.6 ± 6.3	104.2 ± 4.0	
	Benzophenone-4	22	104.4 ± 3.4	99.2 ± 4.4	105.3 ± 4.0	
	Triclosan	2.2	99.2 ± 2.1	104.3 ± 5.4	99.7 ± 1.0	
	Atenolol	22	99.3 ± 0.9	100.4 ± 0.8	100.9 ± 1.0	pH 8
	Bezafibrate	2.2	102.4 ± 1.0	102.9 ± 3.2	101.7 ± 0.4	
	Carbamazepine	22	100.2 ± 2.0	98.2 ± 2.2	100.3 ± 0.8	
	Clarithromycin	22	103.3 ± 0.4	99.2 ± 4.2	105.3 ± 5.7	
	Diclofenac	22	98.4 ± 2.4	101.0 ± 1.2	104.5 ± 0.4	
	Furosemide	2.22	99.7 ± 2.4	104.2 ± 5.0	101.5 ± 6.3	
	Hydrochlorothiazide	22	95.4 ± 5.2	100.4 ± 1.0	98.5 ± 3.7	
	Ibuprofen	22	100.9 ± 1.3	99.8 ± 1.4	100.0 ± 1.2	
	Ranitidine	2.2	102.5 ± 3.3	98.9 ± 0.2	100.2 ± 3.2	
	Sulfamethoxazole	2.2	101.0 ± 3.0	96.2 ± 5.0	104.7 ± 7.0	
	Benzophenone-4	22	97.6 ± 2.2	102.8 ± 2.0	98.5 ± 0.3	
	Triclosan	2.2	96.6 ± 4.4	101.2 ± 0.2	99.3 ± 3.3	
phase II	Atenolol	22	99.7 ± 2.8	104.3 ± 6.4	100.0 ± 1.0	pH 8
	Bezafibrate	2.2	104.2 ± 1.5	102.3 ± 2.6	100.2 ± 1.0	
	Carbamazepine	22	100.3 ± 2.2	103.2 ± 3.7	102.0 ± 2.4	
	Clarithromycin	22	97.8 ± 2.5	99.8 ± 1.1	102.4 ± 1.0	
	Diclofenac	22	98.3 ± 1.1	98.8 ± 2.0	101.3 ± 0.2	
	Furosemide	2.22	99.1 ± 1.4	97.3 ± 4.0	99.2 ± 0.2	
	Hydrochlorothiazide	22	102.2 ± 2.7	98.6 ± 2.1	100.8 ± 1.0	
	Ibuprofen	22	101.3 ± 3.5	100.2 ± 2.4	101.2 ± 1.0	
	Ranitidine	2.2	98.8 ± 4.4	101.2 ± 2.3	99.6 ± 2.0	
	Sulfamethoxazole	2.2	102.4 ± 0.3	101.0 ± 2.1	99.3 ± 3.2	
	Benzophenone-4	22	101.0 ± 1.1	95.6 ± 4.2	96.6 ± 3.1	
	Triclosan	2.2	97.1 ± 3.0	99.0 ± 1.5	100.2 ± 2.0	

Table S5. Pairwise comparison post-hoc Tukey test on the gap between the growth rate in the absence/presence of the PPCPs in phase II in the non-adapted population, and in the population adapted to PPCPs at different levels of DOM. Significant values are reported in bold.

Population	DOC (mg L^{-1})	contrast PPCPs	estimate	df	t ratio	p
non-adapted	0	(-) vs (+)	**1.05**	**12**	**7.38**	**< 0.001**
adapted	0		**0.28**	**18**	**2.39**	**0.03**
	5		**0.39**	**18**	**3.35**	**0.04**
	15		**0.55**	**18**	**4.71**	**< 0.001**

Table S6. Pairwise comparison post-hoc Tukey test between the populations adapted in presence of PPCPs at different levels of DOC, in the absence/presence of PPCPs in phase II. In the table are reported the growth rate, mean cell size and recruitment rate. Significant values are reported in bold.

Variable	PPCPs	contrast (DOC levels)	estimate	df	t ratio	p
growth rate μ (d^{-1})	(-)	0-5	-0.0125	18	-0.107	0.994
		0-15	-0.1	18	-0.853	0.675
	(+)	0-5	0.1	18	0.853	0.675
		0-15	0.172	18	1.472	0.327
cell size (μm)	(-)	**0-5**	**-0.174**	**18**	**-3.451**	**< 0.05**
		0-15	0.038	18	0.755	0.735
	(+)	**0-5**	**0.266**	**18**	**5.264**	**< 0.001**
		0-15	0.018	18	0.359	0.932
recruitment rate μ (d^{-1})	(-)	0-5	0.022	18	0.257	0.964
		0-15	-0.003	18	-0.04	0.999
	(+)	0-5	0.126	18	1.496	0.316
		0-15	**0.267**	**18**	**3.168**	**0.014**

Table S7. Pairwise comparison post-hoc Tukey test between the populations adapted in presence of PPCPs at different levels of DOC and the non-adapted population, in the absence/presence of PPCPs in phase II. In the table are reported growth rate, cell size and recruitment rate. Significant values are reported in bold.

			0 mg L^{-1} DOC				**5 mg L^{-1} DOC**				**15 mg L^{-1} DOC**			
Variable	**Contrast**	**PPCPS**	**df**	**estimated mean difference**	**t ratio**	**p**	**df**	**estimated mean difference**	**t ratio**	**p**	**df**	**estimated mean difference**	**t ratio**	**p**
growth rate μ (d^{-1})	adapted at different DOM levels vs. non-adapted	(+)	**12**	**0.51**	**3.53**	**<0.05**	**18**	**0.4**	**2.99**	**<0.05**	**18**	**0.33**	**2.39**	**<0.05**
		(-)	**12**	**-0.27**	**-1.88**	**<0.05**	18	-0.25	-1.89	0.08	18	-0.17	-1.21	0.24
cell size (μm)		(+)	**12**	**0.35**	**8.7**	**<0.001**	**18**	**0.09**	**0.69**	**<0.01**	**18**	**0.33**	**7.81**	**<0.001**
		(-)	**12**	**-0.19**	**-4.79**	**<0.001**	18	-0.02	-2.81	0.5	**18**	**0.23**	**-5.42**	**<0.001**
recruitment rate μ (d^{-1})		(+)	**12**	**0.19**	**2.08**	**<0.05**	18	0.06	1.43	0.18	18	-0.07	1.06	0.3
		(-)	**12**	**-0.25**	**-2.63**	**<0.05**	**18**	**-0.27**	**-5.52**	**<0.001**	**18**	**-0.24**	**-3.62**	**<0.05**

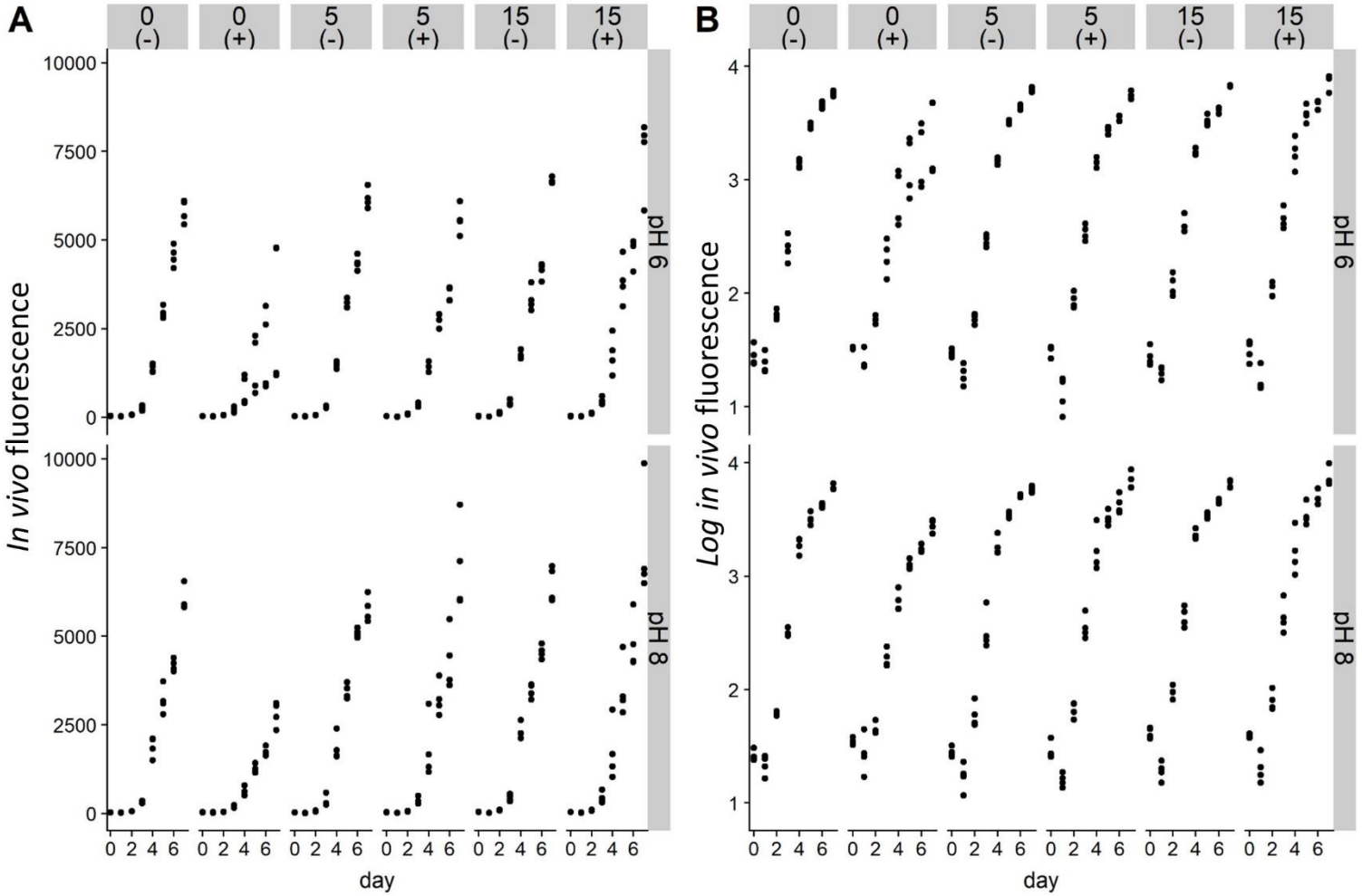

Figure S1. (A) Daily biomass development measured as the *in vivo* fluorescence and (B) log *in vivo* fluorescence data of the phytoplankton population under different DOM (DOC 0, 5, 15 mg L^{-1}) and pH levels (6.5, 8), in the absence (-) and the presence (+) of PPCPs, during phase I.

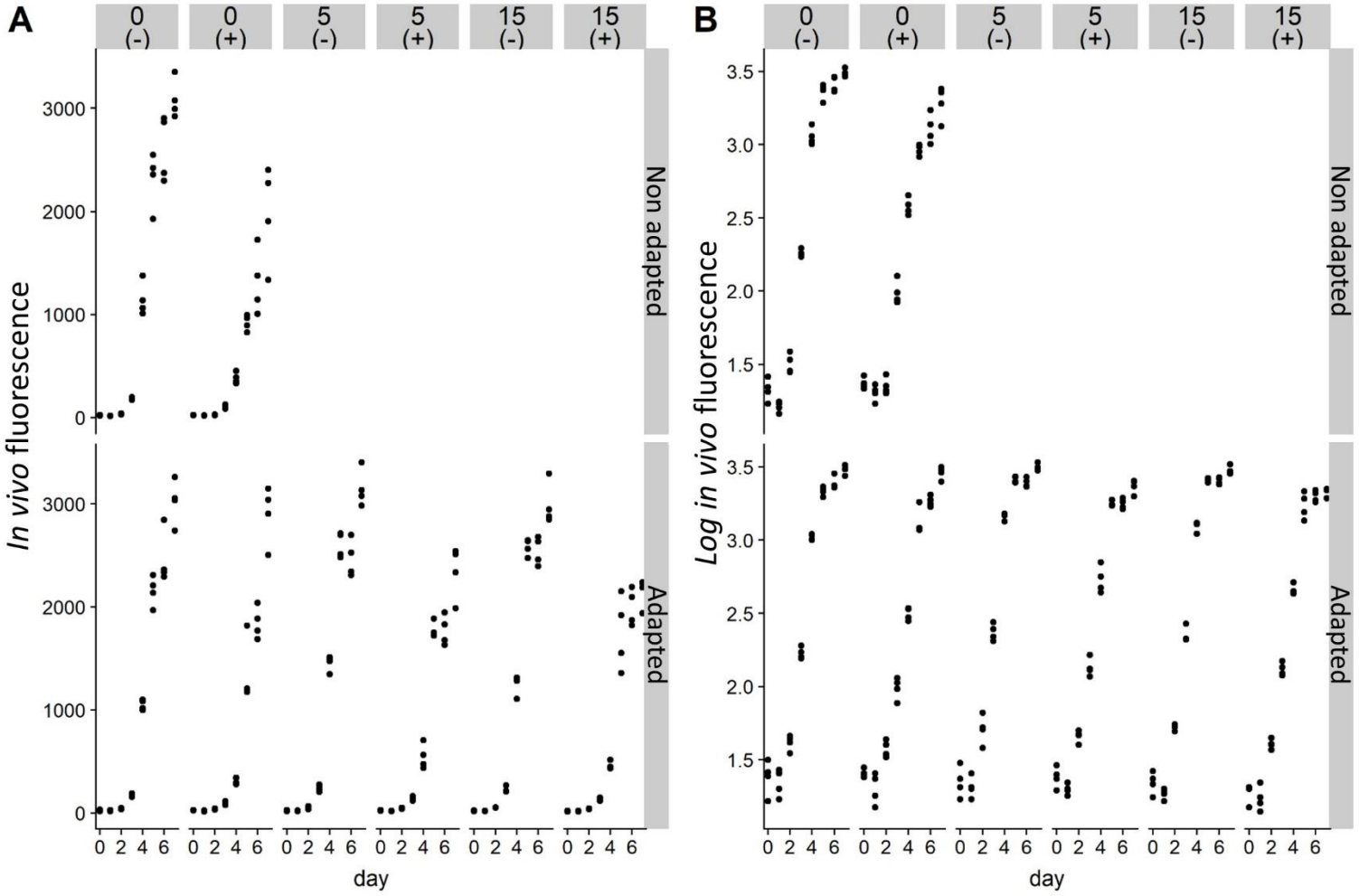

Figure S2. (A) Daily biomass development measured as the i*n vivo* fluorescence and (B) log *in vivo* fluorescence data of the phytoplankton populations under different DOM levels (DOC 0, 5, 15 mg L^{-1}), in the absence (-) and the presence (+) of PPCPs, in the non-adapted and adapted populations during phase II.

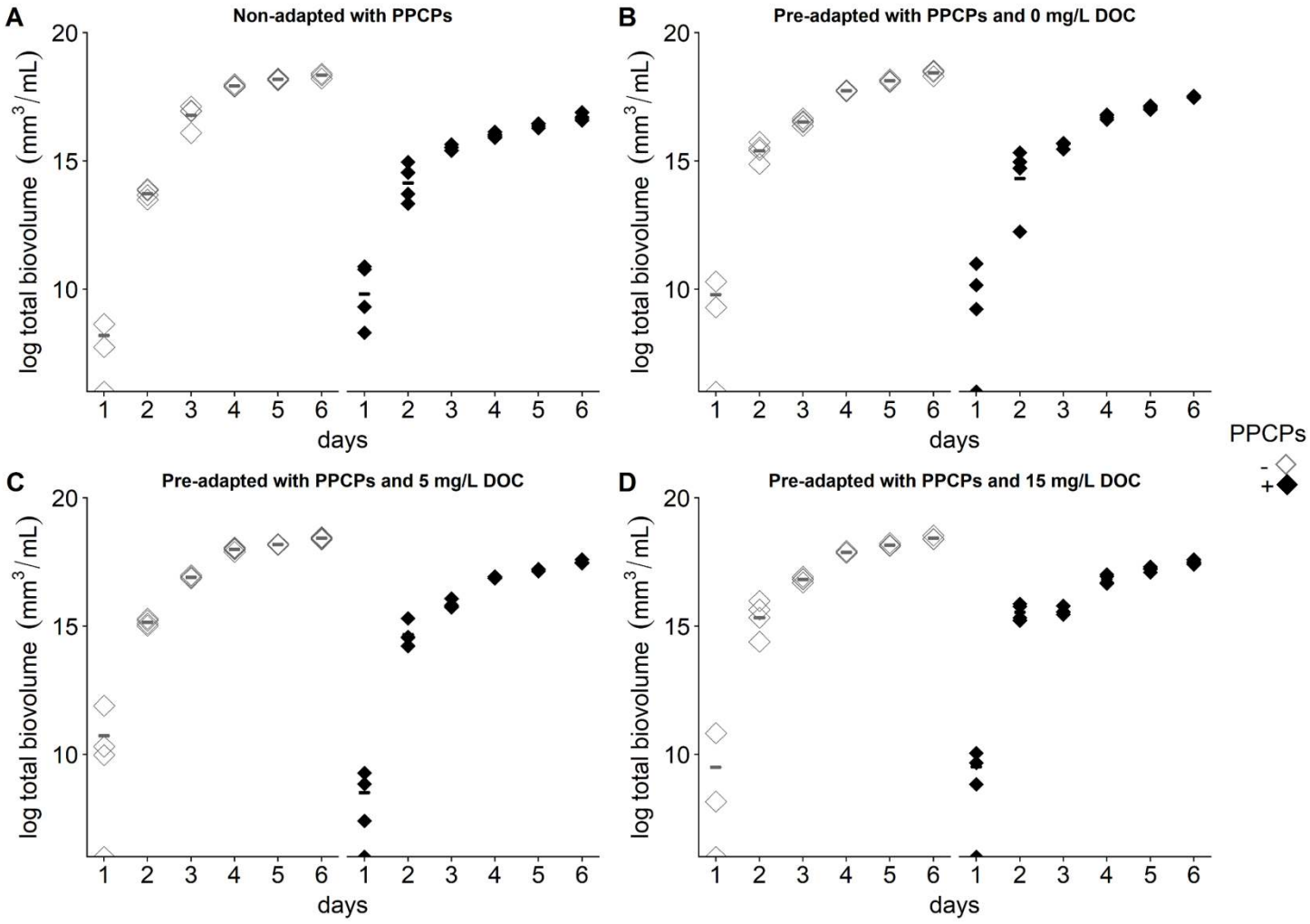

Figure S3. Log daily biovolume development (mm^3/mL) of *C. reinhardtii* in the non-adapted (A), and adapted to PPCPs at 0 mg L^{-1} DOC (B), 5 mg L^{-1} DOC (C) and 15 mg L^{-1} DOC (D), in the absence (-) and the presence (+) of PPCPs during phase II. Short horizontal bars represent the mean of each group.

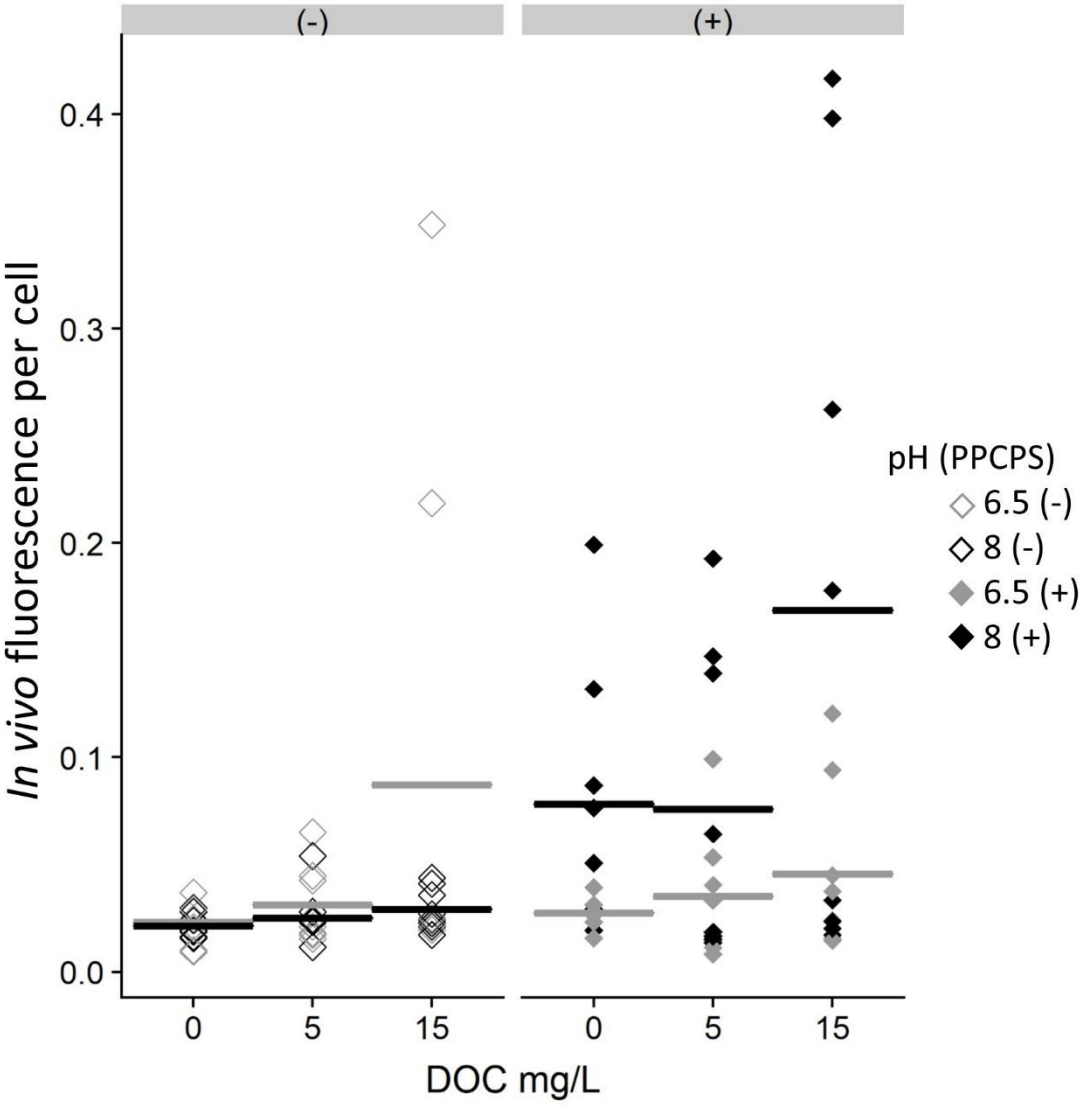

Figure S4. *In vivo* fluorescence per unit of biomass data of *C. reinhardtii* under different DOM (0, 5, 15 mg L^{-1} DOC) and pH (6.5, 8) levels, in the absence (-) and the presence (+) of PPCPs, during phase I.