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1 Influence of bacteria on the response of microalgae to contaminant mixtures

2

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10

11

12 **Abstract**13 When microalgae are exposed to contaminants, the role of associated bacteria within
14 the phycosphere, the microenvironment surrounding algal cells, remains largely unknown.15 The present study investigated the importance of algae-associated bacteria on the responses of
16 microalgae growth to metallic and organic toxicant exposure. The effects of a polluted
17 sediment elutriate, and of metal or pesticide mixtures at environmentally relevant18 concentrations ($<10 \mu\text{g L}^{-1}$) were assessed on the growth of two microalgae strains: *Isochrysis*
19 *galbana*, a prymnesiophyte, and *Thalassiosira delicatula*, a centric diatom. Both cultures20 were maintained as axenic or bacterized under similar conditions in batch cultures. In axenic
21 conditions, the metal mixture addition at low concentrations alleviated limitation of growth by22 metals for *T. delicatula* relative to control, but inhibited *I. galbana* growth at highest
23 concentration. In similar axenic conditions, both *T. delicatula* and *I. galbana* growth were24 negatively inhibited by pesticide mixture at concentrations as low as 10 ng L^{-1} . The bacterial
25 diversities associated with the two microalgae strains were significantly different (Bray–

26 Curtis dissimilarity greater than 0.9) but their impact on microalgae growth was similar. The
27 presence of bacteria reduced algal growth rate by *ca.* 50% compared to axenic cultures,
28 whereas no significant effect of sediment elutriate, metal or pesticide mixtures was noticed on
29 non-axenic algal growth rates. These results show that bacteria may have a negative effect on
30 algal growth but can reduce pesticide toxicity or metal availability to algae.

31

32 *Keywords: microbial interactions; metallic and pesticide contaminants; sediments*

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33 **1. Introduction**

34

35 Microalgae as primary oxygen producers in aquatic ecosystems are of prime
36 ecological importance, and represent the first trophic level in the aquatic food web (Azam and
37 Malfatti, 2007; Field et al., 1998). The region surrounding individual algal cells, named the
38 phycosphere, enriched in exuded organic molecules, is considered as an aquatic analogue of
39 the rhizosphere where microorganisms interact with plants in the terrestrial ecosystem
40 (Seymour et al., 2017). Within the phycosphere, microalgae interact with bacteria within a
41 large range from symbiosis to parasitism, conferring advantages or disadvantages to both
42 partners (Bell and Mitchell, 1972). The mechanisms of interactions between bacteria and
43 phytoplankton are diverse and involve specific cellular processes and fine communication (e.g.
44 quorum sensing) (Amin et al., 2012). Such mechanisms may result in antibacterial or
45 algaecide activities (Mu et al., 2007; Ribalet et al., 2008) or substrate competition as
46 experimentally observed between manipulated consortium of microalgae and bacteria (Le
47 Chevanton et al., 2013). On the other hand, the presence of bacteria could offer to microalgae
48 a capacity for tolerance and adaptation to stressful conditions, such as chemical exposure.
49 Indeed, the heterotrophic metabolism of highly diverse bacterial communities in the field and
50 their ability to degrade, metabolize and immobilize a large number of organic and inorganic
51 compounds (Bouwer and Zehnder, 1993; Bruins et al., 2000), make it possible to assign them
52 an ecological role of protecting microalgae, particularly in polluted environments. It can also
53 be hypothesized that microalgal growth may be further improved when the latter are
54 associated with bacteria subjected to chronic contaminants that could develop greater
55 tolerance capacities than naive bacteria and therefore allow microalgae to benefit from these
56 bacterial capacities to cope with pollutants (Bauer et al., 2010).

57 Therefore, the main hypothesis tested in this study proposes that the presence of
58 bacteria with degrading or immobilizing ability would reduce the sensitivity of microalgae to
59 organic or metal contaminants, counterbalancing any potential bacterial algacide activity.
60

61 In order to test this hypothesis, the present study focused on the effect of a sediment
62 elutriate issued from the resuspension of polluted sediments on the growth of two microalgae
63 strains commonly found in marine environments: *Isochrysis galbana*, a small prymnesiophyte,
64 and *Thalassiosira delicatula*, a centric diatom. *Isochrysis galbana* is a well-known
65 phytoplankton species, traditionally used in aquaculture and biotechnology due its capacity to
66 produce large biomass (Williams and Laurens, 2010) whereas *Thalassiosira delicatula*
67 represents a model for diatom study, belonging to a genus widely distributed throughout the
68 world's oceans (Armbrurst et al., 2004). Both strains were growing either in axenic or non-
69 axenic condition, i.e. associated with bacteria naturally selected during culture selection and
70 maintenance processes. The growth of xenic and axenic strains were compared when exposed
71 to the total (including native bacteria) or dissolved fraction of the resuspended sediment, or
72 artificial mixtures containing either the main metallic or organic contaminants found in these
73 sediments.

74

75

76 **2. Materials and Methods**

77

78 *2.1. Elutriate and contaminated artificial mixtures*

79

80 *2.1.1. Elutriate and filtered elutriate preparation*

81 The elutriate was obtained by mixing seawater (3/4 by volume) and sediment (1/4)
82 sampled in February 2015 from the Bizerte Lagoon, for 12 hours, followed by decantation for
83 12 hours. The elutriate thus represented the supernatant obtained after decantation. It still
84 contained unsettled particulate matter, resident bacteria and water-soluble contaminants. The
85 filtered elutriate was obtained after filtration of the elutriate on a 0.2 μm membrane, leading
86 to a sterile mixture with only the dissolved fraction of chemical compounds. More details on
87 sediment location and sampling can be found in (Pringault et al., 2016).

88

89 *2.1.2 Artificial mixture of contaminants*

90 Two types of artificial mixtures of contaminants were produced in the laboratory,
91 using actual concentrations found after chemical analyses in the elutriate (HydroSciences
92 Montpellier laboratory (HSM) for metals and by the Ecole des Mines d'Alès for pesticides,
93 see (Pringault et al., 2016) for analytical set-up). One of these mixtures contained the organic
94 contaminants, mostly pesticides resulting from the agricultural activity of the Bizerte area,
95 whereas the other mixture contained the metallic fraction resulting from the industrial and
96 harbour activities of the city.

97 The artificial metal mixture was prepared in Milli-Q water, containing 0.026 $\mu\text{g L}^{-1}$ of
98 cadmium Cd, 0.068 $\mu\text{g L}^{-1}$ of copper Cu, 0.208 $\mu\text{g L}^{-1}$ of nickel Ni, 0.13 $\mu\text{g L}^{-1}$ of lead Pb,
99 1.615 $\mu\text{g L}^{-1}$ of zinc Zn and 4.896 $\mu\text{g L}^{-1}$ of arsenic As.

100 The artificial pesticide mixture was prepared in Milli-Q water with a mixture of the
101 pesticides measured in the elutriate and containing 7.5 ng L^{-1} of DIA (deisopropylatrazine, an
102 atrazine metabolite), 7.7 ng L^{-1} of DCPU (N-3,4 dichlorophenylurea, a diuron degradation
103 product), 8.8 ng L^{-1} of diuron, 10.1 ng L^{-1} of simazine and 12.7 ng L^{-1} of alachlor. All the
104 pesticides measured in sediment elutriate were herbicides or their degradation products. A

105 concentrated stock solution of both mixtures was performed in order to test a range of mixture
106 concentrations.

107

108 *2.2 Microalgae cultures*

109 The microalgal strains studied were *Isochrysis Galbana* (CCAP 927) isolated from a
110 marine fish pond in the British Isles and *Thalassiosira delicatula* (RCC 2560) isolated from
111 the English Channel. They were grown in a F/2 + Si medium and subjected to light exposure
112 according to a 16h/8h day/night cycle in an incubator maintained at 20 °C under neon lighting
113 (Vossloh Schwabe ELXe 218.526), providing an average intensity of 100 $\mu\text{E m}^2 \text{s}^{-1}$
114 (measured with a LI-COR®, Li-1400 equipped with a Walz US-SQS/L spherical micro
115 sensor). The microalgae cultures were inoculated in fresh medium every 2 weeks to maintain
116 active growth before the experiments.

117 Axenization of cultures was carried out according to the protocol of The Culture
118 Collection of Algae and Protozoa (CCAP) C.N. Campbell
119 (<https://www.ccap.ac.uk/knowledgebase.htm>) using a mixture of antibiotics (Cefotaxime 500
120 mg L⁻¹, Carbenicillin 50 mg L⁻¹, Kanamycin 200 mg L⁻¹, Augmentin 200 mg L⁻¹) at
121 concentrations of 0 to 10% and with contact times of 24 to 80 hours. The verification of the
122 axenization was carried out by adding 4 ', 6'- β -diamidino- α -2- α -phenylindole (DAPI),
123 filtration and observation under epifluorescence microscopy with UV excitation at 360 nm ;
124 spreading in a Petri dish on Marine Broth medium (15% agar) was also performed. The axeny
125 of microalgae cultures was checked regularly, especially prior to the beginning of the toxicity
126 experiments.

127

128 *2.3 Bacterial diversity analysis*

129 Bacterial 16S rDNA was extracted from 10mL of sample filtered on a 0.2 μ m
130 membrane (PALL Supor® 200 PES), using the DNeasy PowerWater Kit (Qiagen) according
131 to the manufacturer's instructions.

132 The V4-V5 region of the 16S rRNA gene was amplified over 30 amplification cycles at an
133 annealing temperature of 65 °C, with the forward primer and the reverse primer (Table 1)
134 with their respective linkers. The resulting products were purified and loaded onto the
135 Illumina MiSeq cartridge for sequencing of paired 300 bp reads following manufacturer's
136 instructions (v3 chemistry). Sequencing and library preparation were performed at the
137 Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France
138 (get.genotoul.fr).

139 A modified version of the standard operation procedure for MiSeq data (Kozich et al.,
140 2013) in Mothur version 1.35.0 (Schloss et al., 2009) was used for alignment and as a
141 taxonomic outline. Using Mothur, representative sequences of bacterial operational
142 taxonomic units (OTUs) were identified at the 3% level.

143 An R script was used to perform a hierarchical clustering using R command hclust
144 with the Bray–Curtis index

146 2.4. *Experimental design and statistical analysis*

147 An experimental design has been carried out to study the growth of microalgae in the
148 absence of bacteria (axenic microalgal strains) or in the presence of bacteria (xenic strains
149 with culture associated bacteria) under i) a range of concentrations of a mixture of metals at
150 concentrations found in elutriate x1, and at greater concentrations: x3,x10, x31; and ii) a
151 range of concentrations of a mixture of pesticides at concentrations found in elutriate x1, and
152 at greater concentrations: x10, x31, x97, x310; iii) the sediment elutriate containing bacteria;

153 iv) the filtered (0.2 μm) sediment elutriate; v) culture medium without contaminants (=
154 positive control).

155 All the tests were carried out in 96-well microplates, 6 replicates were made for
156 artificial mixture treatments, and 12 replicates for controls, elutriate and filtered elutriate
157 treatments. Each well (300 μL) contained 10% of microalgae inoculum, 10% of artificial
158 mixtures or elutriate, and 80% of culture media. Therefore, the concentrations of elutriate and
159 all the artificial mixtures were diluted to 1/10. The microplates were covered with a Breathe-
160 Easy® membrane allowing gas exchange and ensuring an absence of contamination
161 throughout the follow-up of the growth which lasted ten days. This test was carried out for
162 each of the strains (*Isochrysis galbana* and *Thalassiosira delicatula*) in axenic and non-axenic
163 conditions. The growth of algal strains was monitored by measuring the optical density at 650
164 nm every 24 h until reaching the plateau (i.e. stationary phase) on a Chameleon (Hidex,
165 Finland) microplate reader. The wavelength of 650 nm was chosen according to previous
166 studies (Ben Othman et al., 2012) corresponding to the absorption peak of chlorophyll
167 pigments with a minimal contribution of bacterial cells to light attenuation. The maximum
168 growth rates were obtained by fitting Verhulst growth curve (see (Fouilland et al., 2014)) to
169 experimental data. An analysis of variance was used to determine the significance of the
170 difference in growth rates between treatments and with or without axenization, followed by a
171 Bonferroni post hoc test using SYSTAT 11 version. Significance threshold was set at $p < 0.05$.

172

173 **3. Results and Discussion**

174

175 *3.1. Differential sensitivity of axenic microalgal strains to contaminants*

176 Axenisation of both algal strains was successfully maintained during the present study,
177 as no bacterial cells were observed using epifluorescence microscope and culture techniques
178 performed just before the experiments. A significant reduction in the growth rates of both
179 axenic strains was observed when supplemented with the total elutriate, but not with the
180 filtered elutriate (Fig. 1A). A reduction of light availability due to the presence of large
181 particles in the total elutriate is unlikely as no difference in light absorbance (DO) was
182 observed between control (culture media only) and after total elutriate addition (10% of total
183 well volume) at the beginning of the experiment. This observation suggests that only the
184 particulate fraction of the elutriate containing the bacterial community can affect the growth
185 of both algal strains. The potential negative effect of bacteria presence in the elutriate on the
186 algal growth is supported by the results obtained with xenic microalgae as discussed below.

187 When comparing the responses of the two axenic microalgae strains tested in the
188 present study, *Thalassiosira delicatula* showed a higher sensitivity to metal and pesticide
189 additions compared to *Isochrysis galbana*. Growth rates of *T. delicatula* increased by 40%
190 when low concentrations of metals were added to their media corresponding to x1 and x3 of
191 the concentrations measured in the elutriate (Fig. 1A). A reduction of 60% of *T. delicatula*
192 growth rates was observed for all the pesticide concentrations tested except the one
193 corresponding to the pesticide concentration measured in the elutriate (x1). The growth rates
194 of *I. galbana* were more slightly modified and only at higher concentrations of the metal or
195 pesticide mixture (Fig. 1A). These results suggest that the additions of the metal mixture at
196 low concentrations relieved a growth limitation by metal ions for *T. delicatula* in culture
197 rather than a growth inhibition, while the growth of *I. galbana* was significantly inhibited at

198 the highest concentration of the metal mixture tested (x31). Arsenic, lead, nickel and
199 cadmium are not normally present in the F/2 culture media, amongst them only cadmium at
200 low concentrations was found in the literature to induce growth enhancement in diatoms (Lee
201 et al., 1995; Masmoudi et al., 2013). On the other hand, *T. delicatula* was negatively affected
202 by the pesticide mixture in a magnitude greater than observed for *I. galbana* (Fig. 1A).
203 Previous studies highlighted the interspecific difference in toxicity of various pesticides on
204 marine algal growth rates (Staley et al., 2015; Walsh, 1972) at mg L⁻¹ scale, but no conclusive
205 evidences were generally provided regarding the phylogenetic importance in the tolerance or
206 sensitivity of microalgae species. Our results suggest that the diatom *T. delicatula* and the
207 prymnesiophyte *I. galbana* can be negatively inhibited by exposure to a mixture of pesticides
208 at concentrations as low as 10 ng L⁻¹, the diatom being even more sensitive than the
209 prymnesiophyte, confirming what has been observed in oceanic and coastal phytoplankton
210 strains (Huertas et al., 2010).

211 Therefore, the lack of effect, or the positive effect, of metal and pesticide mixtures on
212 microalgal growth at concentrations found in elutriate (x1) may explain why microalgae
213 growth was not affected when supplemented with filtered elutriate containing both
214 contaminants. This is also consistent with the absence of any toxic effect observed during a
215 previous experiment performed using a filtered sediment elutriate from Bizerte Lagoon and
216 added to a natural phytoplankton community (Ben Othman et al., 2017).

217

218 3.2. Bacteria affect algal growth rates and algal sensitivity to contaminants

219 For both microalgae strains studied here, the presence of bacteria did significantly
220 reduce their growth rate by *ca.* 50%, from 0.8 to 0.4 d⁻¹ for *Isochrysis galbana* and from 0.9
221 to 0.5 d⁻¹ for *Thalassiosira delicatula* (Table 1) in control conditions. This suggests that
222 bacteria i) may act as competitors for nutrient resources and/or ii) may release toxic

223 compounds partially inhibiting, algal growth rates (Mayali and Azam, 2004). This would
224 explain the reduction in the growth rate for axenic strains in the presence of total elutriate
225 containing bacteria. Competition for nutrients is unlikely in the present study, as both bacteria
226 and microalgae were growing in high macro- and micronutrient culture media and the
227 maximal growth rates of microalgae are calculated using the first 2–3 days of the growing
228 phase. The algal growth inhibition by the presence of bacteria seems not to be related to a
229 specific bacterial diversity associated with the microalgae, as the bacterial community was
230 rather different between the two strains (Bray–Curtis dissimilarity greater than 0.5), being
231 dominated by the class of Flavobacteria for *Thalassiosira delicatula*, and the class of Alpha-
232 and Betaproteobacteria for *Isochrysis galbana* (Fig. 2). This is further supported by the
233 growth reduction of both axenic microalgae strains when supplemented with the elutriate
234 containing a diverse bacterial community (Fig. 2). The additional presence of bacteria from
235 elutriate in the xenic culture of both strains did not lead to an extra reduction in their growth
236 (Fig. 1B) relative to the controls. We therefore suggest that bacteria growing with microalgae
237 strains are probably opportunistic bacteria that benefit from the microalgae phycosphere but
238 to the detriment of algal growth. This is supported by a recent study showing that all the
239 bacteria isolated from a marine algal culture collection had a negative effect on the growth
240 rate of *Dunaliella* sp. when experimentally associated with the axenic microalgae, even when
241 these bacteria were initially isolated from *Dunaliella* cultures (Le Chevanton et al., 2013).

242 Interestingly, in contrast to the observations made under axenic algal growth
243 conditions, in the presence of bacteria, neither microalgal strain was significantly affected by
244 either the metal or pesticide mixture (Fig. 2B). This suggests that even if bacteria may have an
245 algaecide effect, they can also reduce toxicity or metal availability to algae (e.g. pesticide
246 degradation, metal immobilization). For example, *T. delicatula* under pesticide mixtures
247 showed growth rates as low as 0.3 d⁻¹ without bacteria but always greater than 0.5 d⁻¹ with

248 bacteria (Table 2). It seems that the reduction of *ca.* 50% of algal growth rate with the
249 presence of bacteria allowed microalgae to better resist pesticide contamination. A reduction
250 in toxicity by bacteria may be suggested, although further investigation is required to clearly
251 evidence their ability to degrade or immobilize these toxic compounds within the 2–3 days of
252 the microalgae growing phase. The herbicides and degradation products included in the
253 mixture assayed during this work are considered as being resistance to rapid biodegradation,
254 and half-lives are often comprised of between a week and a few months. For example,
255 atrazine and degradation products lasted more than a month in marine sediments (Smalling
256 and Aelion, 2006), whereas simazine degradation was only observed with selected bacterial
257 strain supplemented with carbon sources (Liu et al., 2018). Diuron and its metabolites
258 exhibited half-lives of longer than five days in selected bacterial pure cultures (Villaverde et
259 al., 2017), and it took more than 100 days to degrade alachlor in artificial wetlands (Elsayed
260 et al., 2015). As a consequence, it appears unlikely that significant degradation of the
261 pesticide mixture occurred within the duration of the present experiment.

262 We also suggest that the presence of bacteria would induce microalgae to dedicate
263 parts of their resources to defense rather than growth, such as the release of molecules with a
264 high adsorption capability allowing them to cope with all other stressing factors, including
265 toxic compounds. Such a strategy can be seen as a growth–defense trade-off, similar to the
266 phenomenon that was first observed in forestry studies of plant–insect interactions. This
267 trade-off strategy is based on the assumption that plants possess a limited pool of resources
268 that can be invested either in growth or in defense (Coley et al., 1985). A similar strategy was
269 evidenced for microalgae associated with inducible defenses against predators (Zhu et al.,
270 2016) but never suggested when in interaction with bacteria, as far as we know.

271

272 The present study clearly shows that interactions between bacteria and phytoplankton
273 can influence the sensitivity of microalgae to toxic compounds and to metal availability.
274 These interactions did not seem to be species dependent, as they were not related to the
275 bacterial community composition or phytoplankton species. This suggests that the influence
276 of bacteria on algal sensitivity to contaminants could be generalized to various species of
277 microalgae and bacteria. Such results can be applied for bioremediation of toxic contaminants
278 in heavily polluted environments or as wastewater final treatment. These purposes will benefit
279 from innovative approaches such as the design and the use of artificially optimised microbial
280 consortia to remediate toxic chemicals. Microalgae-bacteria consortia can favour the presence
281 of cometabolism which is recognized as a successful bioremediation approach to biodegrade
282 recalcitrant molecules (Hazen, 2010). But further studies are required to assess the
283 degradation and/or immobilization of pesticide and metal compounds by bacteria
284 communities usually observed within the phycosphere and to evidence any change in the
285 metabolism and physiology of microalgae (e.g. release of scavenging molecules) when in the
286 presence of bacteria. This study was performed under nutrient replete conditions but
287 additional investigations would be necessary to explore any change in the bacteria-microalgae
288 relationships when exposed to nutrient limitation in addition to contaminants. Such limitation
289 may affect algal growth in a stronger extent than toxic compounds, further limiting the
290 potential protective effect of bacteria.

291

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299

300

301

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406 Table 1. 16S rRNA gene sequencing primers

forward primer	5'-CTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA-3'
reverse primer	5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCGYCAATTCMTTTRAGT-3'

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409 Table 2. Growth rates (mean and standard error – SE) of axenic and xenic algal strains measured with or without (control) the addition of total
 410 elutriate, filtered elutriate, mixture of metals and pesticides at concentrations from those found in elutriate (x1) to x31 for metals and x310 for
 411 pesticides. Greyed values represent significant differences with Control ($p < 0.05$)

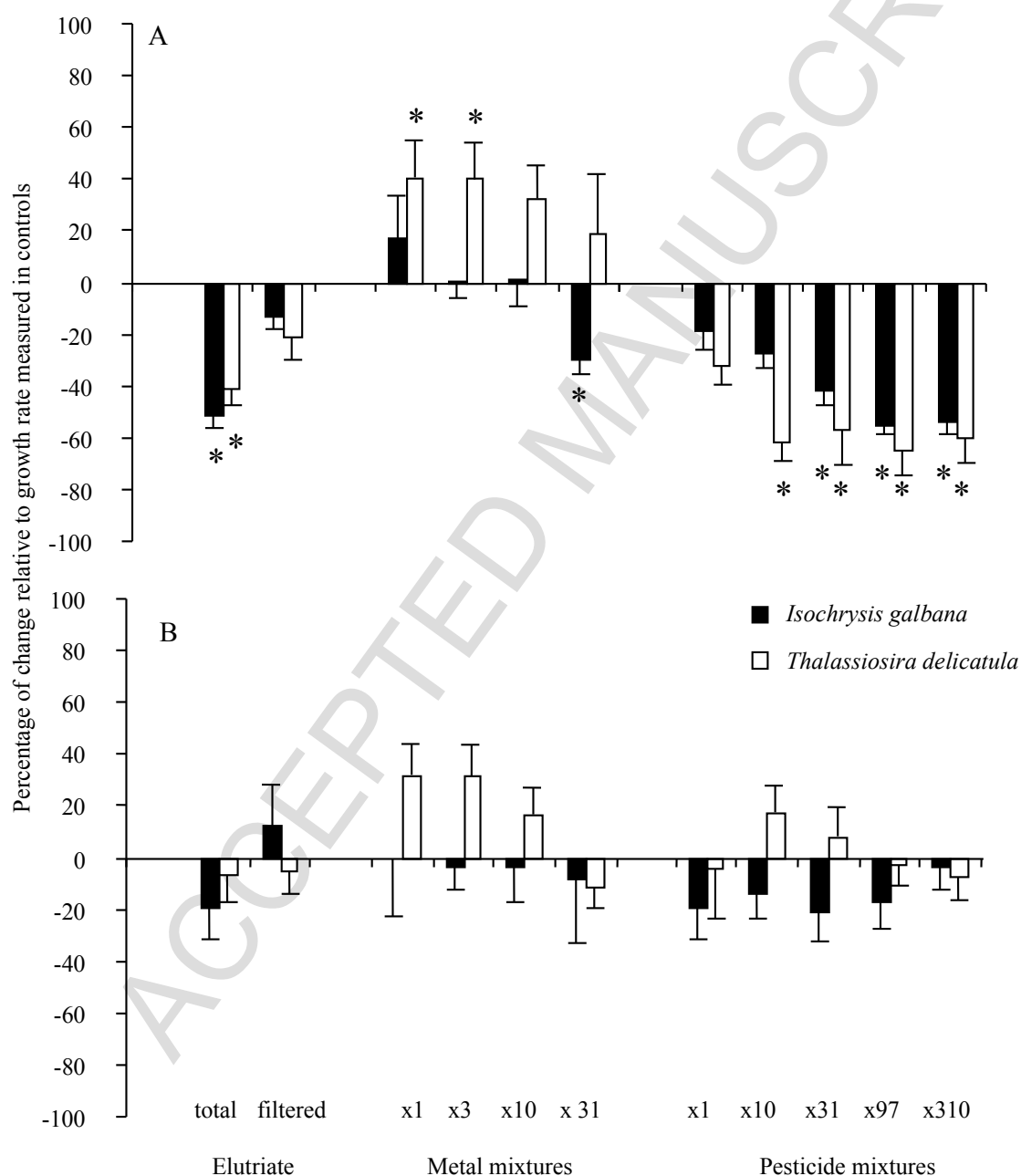
		<i>Isochrysis galbana</i>				<i>Thalassiosira delicatula</i>			
		axenic		xenic		axenic		xenic	
		mean	SE	mean	SE	mean	SE	mean	SE
	Control	0.83	0.04	0.38	0.03	0.92	0.09	0.54	0.05
	total elutriate	0.40	0.03	0.31	0.02	0.54	0.02	0.51	0.04
	filtered elutriate	0.72	0.03	0.43	0.02	0.72	0.03	0.51	0.02
	x1	0.98	0.13	0.38	0.04	1.28	0.05	0.71	0.02
metal mixture	x3	0.84	0.04	0.37	0.01	1.28	0.06	0.71	0.02
	x10	0.84	0.07	0.37	0.02	1.21	0.03	0.63	0.01
	x31	0.58	0.04	0.35	0.04	1.09	0.18	0.48	0.02
	x1	0.68	0.05	0.31	0.02	0.62	0.02	0.52	0.10
	x10	0.60	0.04	0.33	0.01	0.35	0.06	0.63	0.02
pesticide mixture	x31	0.49	0.05	0.30	0.02	0.39	0.12	0.58	0.03
	x97	0.37	0.03	0.32	0.02	0.32	0.08	0.53	0.01
	x310	0.38	0.03	0.37	0.01	0.36	0.08	0.50	0.02

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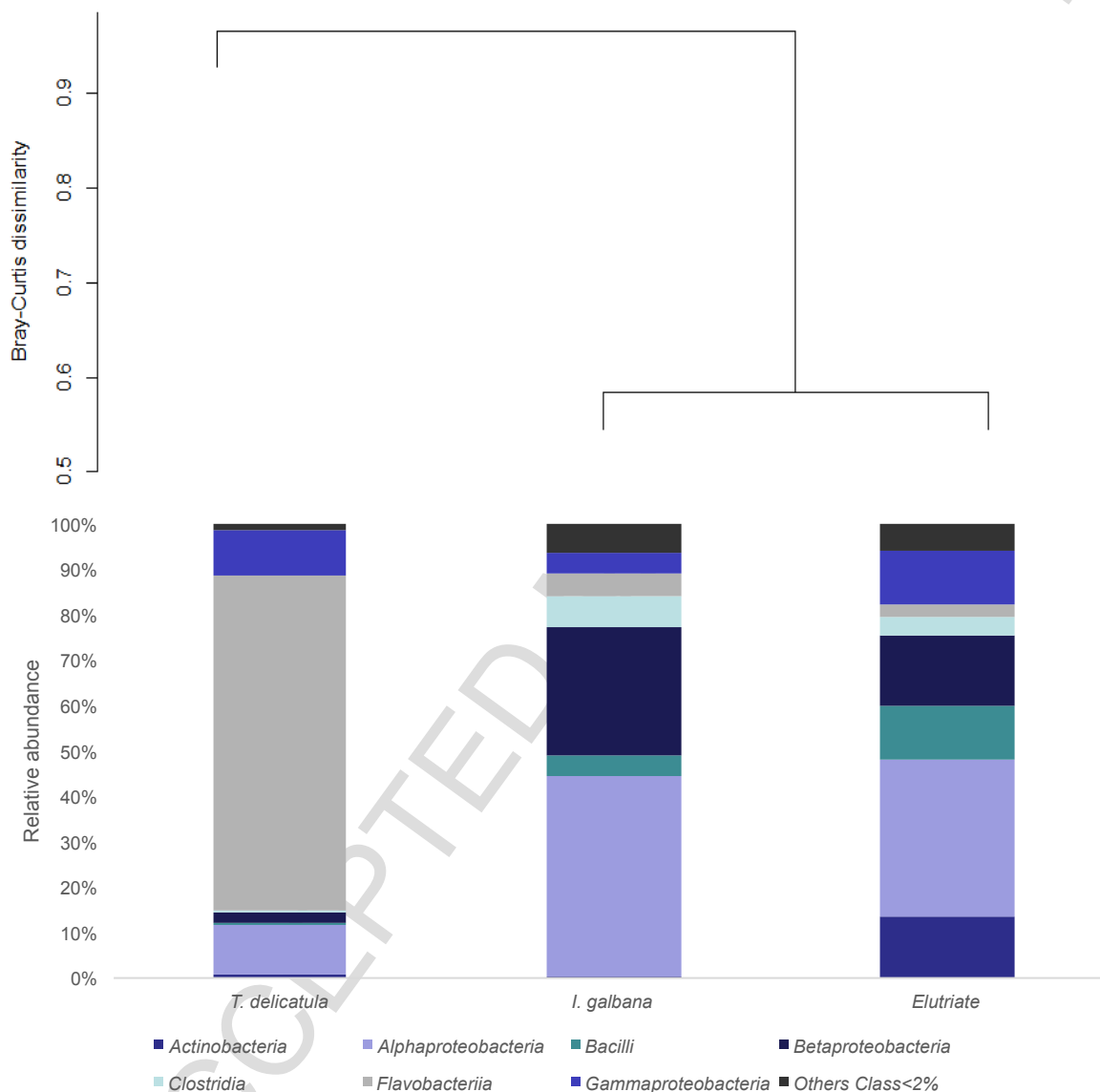
414 Figure 1. Change (mean and SE) in growth rates measured with elutriate, metal or pesticide
 415 mixture additions relative to control (no addition) and expressed in percentage for *Isochrysis*
 416 *galbana* and *Thalassiosira delicatula* under (A) axenic and (B) xenic culture conditions.
 417 Positive and negative values (%) correspond to, respectively, an increase and a decrease in
 418 growth rate relative to control. Asterisks denote a significant difference ($p < 0.05$) between
 419 treatments and control.



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422 Figure 2. Bacterial diversity observed in xenic cultures of *Thalassiosira delicatula* and
 423 *Isochrysis galbana* and in the sediment elutriate: the dendrogram shows the clustering of
 424 bacterial communities found in cultures, based on Bray–Curtis similarities calculated at the
 425 OTU level and bar plots show the relative abundance of the main bacterial classes observed.



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Highlights

- The presence of bacteria in the culture medium negatively affected microalgae growth
- The absence of bacteria resulted in short term microalgae response to metals and pesticides at low dose
- Metals and pesticides were not toxic to microalgae when growing with bacteria