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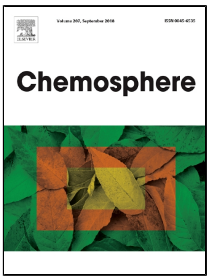

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Influence of bacteria on the response of microalgae to contaminant mixtures

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Abstract

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

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

Keywords: microbial interactions; metallic and pesticide contaminants; sediments

1. Introduction

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

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

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

2. Materials and Methods

2.1. Elutriate and contaminated artificial mixtures

2.1.1. Elutriate and filtered elutriate preparation

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

2.1.2 Artificial mixture of contaminants

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

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

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

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

2.2 Microalgae cultures

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

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

2.3 Bacterial diversity analysis

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

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

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

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

2.4. Experimental design and statistical analysis

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

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

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

3. Results and Discussion

3.1. Differential sensitivity of axenic microalgal strains to contaminants

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

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

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

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

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

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

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

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

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

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

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

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406 Table 1. 16S rRNA gene sequencing primers

forward primer	5'-CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA-3'
reverse primer	5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTRAGT-3'

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

		Isochrysis galbana				*Thalassiosira delicatula*			
		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
metal mixture	x1	0.98	0.13	0.38	0.04	1.28	0.05	0.71	0.02
	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
pesticide mixture	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
	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

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

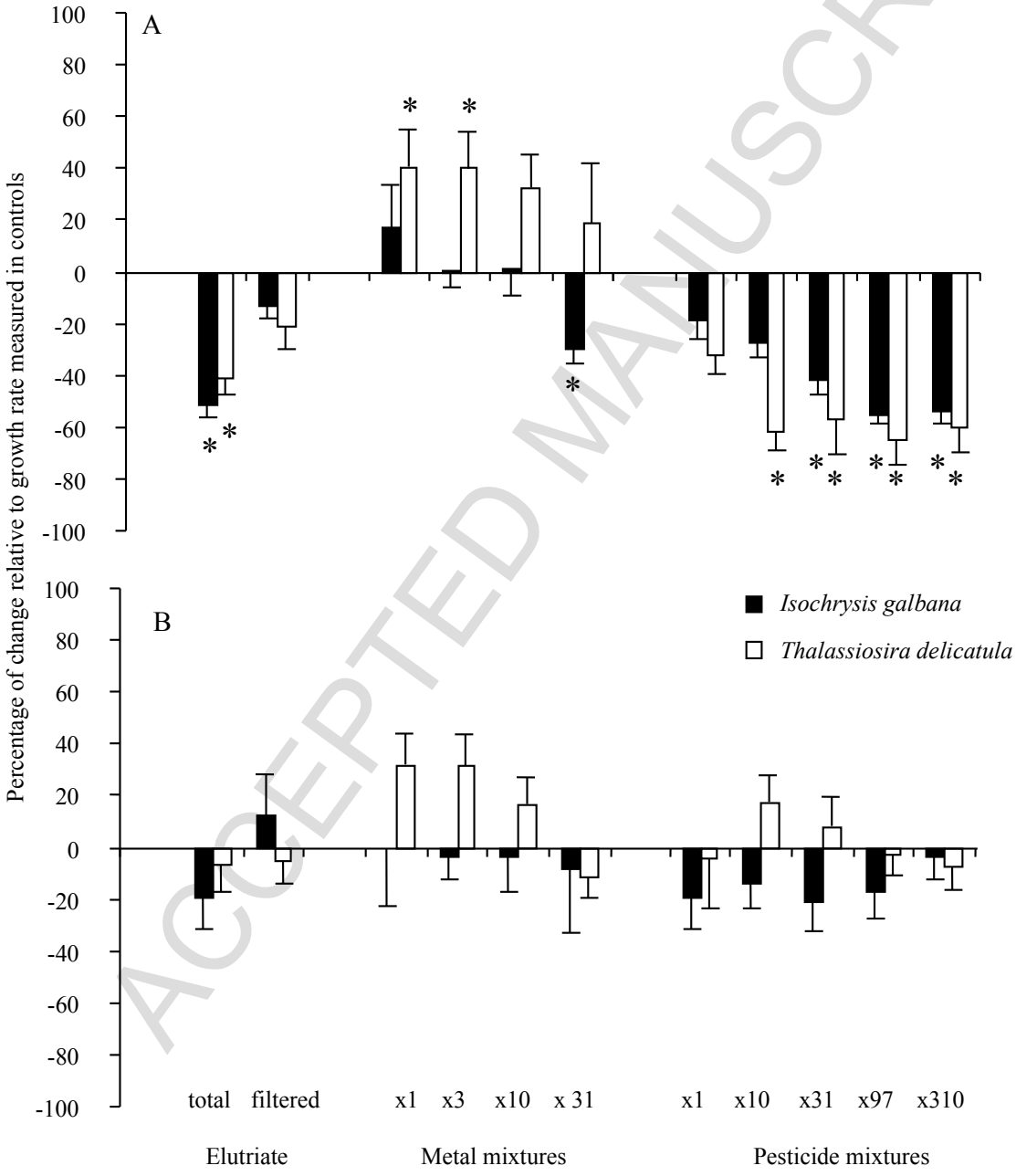
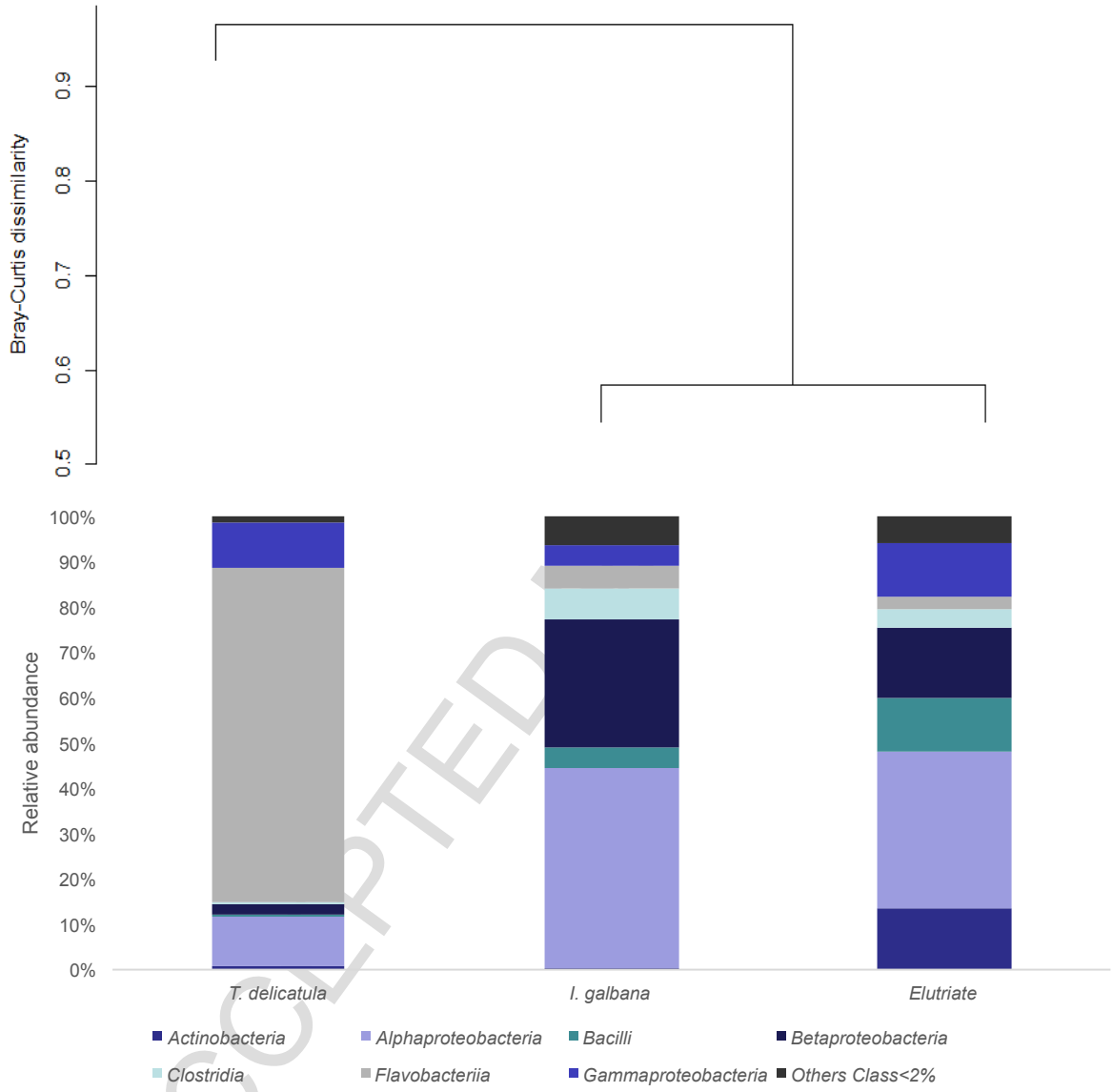


Figure 2. Bacterial diversity observed in xenic cultures of *Thalassiosira delicatula* and *Isochrysis galbana* and in the sediment elutriate: the dendrogram shows the clustering of bacterial communities found in cultures, based on Bray–Curtis similarities calculated at the OTU level and bar plots show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



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