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Impacts of copper and lead exposure on prokaryotic communities from contaminated contrasted coastal seawaters: the influence of previous metal exposure

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Running title: Impacts of copper on prokaryotic communities depends on metal exposure history

ABSTRACT

Our understanding of environmental factors controlling prokaryotic community is largely hampered by the large environmental variability across spatial scales (e.g. trace metal contamination, nutrient enrichment, physico-chemical variations) and the broad diversity of bacterial pre-exposure to environmental factors. In this article, we investigated the specific influence of copper (Cu) and lead (Pb) on prokaryotic communities from the uncontaminated site, using mesocosm experiments. In addition, we studied how pre-exposure (i.e. life history) affects communities, with reference to previous metal exposure on the response of three prokaryotic communities to similar Cu exposition. This study showed a stronger influence of Cu contamination than Pb contamination on prokaryotic diversity and structure. We identified 12, and 34 bacterial families and genera, respectively, contributing to the significant differences observed in community structure between control and spiked conditions. Taken altogether, our results point towards a combination of direct negative responses to Cu contamination and indirect responses mediated by interaction with phytoplankton. These identified responses were largely conditioned by the previous exposure of community to contaminants.

Keywords: prokaryotic communities; copper contamination; metal exposure history; coastal seawater; mesocosm experiment; high throughput sequencing

INTRODUCTION

Marine coastal environments are especially vulnerable to anthropogenic pressures such as chemical contamination (Halpern *et al.* 2008; The Mermex Group *et al.* 2011). For example, copper (Cu) contamination in coastal environments is mainly caused by urban and industrial wastes (Levin *et al.* 2001; Oursel *et al.* 2013), or antifouling coatings close to harbors (Turner 2010). Lead (Pb) contamination is mainly associated with historical short-term events (Xu *et al.* 2014; Dang *et al.* 2015a, 2015b) or historical fuel consumption for nautical traffic (Callender 2003). Cu contamination is mainly encountered in seawaters of harbor areas while Pb contamination are found in sediments, probably due to past events (Dang *et al.* 2015b). In Toulon Bay, Cu and Pb concentrations can be enriched up to more than 100-fold above geochemical background levels depending on the compartment (e.g. sediment or water column) (Casas *et al.* 2008; Tessier *et al.* 2011; Jean *et al.* 2012; Cossa *et al.* 2014; Dang *et al.* 2015b; Twining *et al.* 2015; Coclet *et al.* 2018, 2019). Thus, the unique feature of Toulon Bay relates to a gradient of multiple trace metal elements (TME) with very low concentrations in open parts comparable to levels measured in open Mediterranean Sea (Morley *et al.* 1997). By comparison, most enclosed and anthropized areas exhibit very high TME concentrations (Dang *et al.* 2015a; Coclet *et al.* 2018, 2019). Therefore, Toulon bay is a model ecosystem for the study of ecological consequences of TME contamination in the marine coastal environment.

In Toulon Bay where TME gradients are wider than classical physico-chemical gradients, TME contamination have been proposed to influence directly the

bacterioplankton diversity and structure (Coclet *et al.* 2019). Several field studies clearly suggested TMEs as structuring factors of abundance, diversity and composition of bacterioplanktonic communities by selecting metal resistant taxa (Sheeba *et al.* 2017; Coclet *et al.* 2018, 2019; Goni-Urriza *et al.* 2018). A microcosm-based study revealed that artificial mixture of contaminants (nutrients, metals, and herbicides) induced a strong inhibition of growth, production, respiration and significant modifications of the functional patterns of bacterioplankton community (Pringault *et al.* 2016). Furthermore, both industrial effluent discharge and the resuspension of metal-contaminated marine sediments have been shown to affect microbial diversity in a resuspension experiment (Zouch *et al.* 2018). TME contamination typically found in coastal environments have also been observed to affect phytoplanktonic communities (Mackey *et al.* 2012; Lafabrie *et al.* 2013; Coclet *et al*. 2018), which can have indirect consequences for bacterioplankton community (Goni-Urriza *et al.* 2018; Coclet *et al.* 2019).

Life history of a microbial community can be defined as its past exposure to a multitude of environmental changes, like top-down (e.g. grazing pressure, exposition to toxic substances) as well as bottom-up (e.g. phytoplanktonic production) control. The pre-exposure of communities to harmful contaminants like TME can affect the microbial ecology of the marine system and therefore is of major concern (Hughes-Martiny *et al.* 2006; Bissett *et al.* 2010; Bell *et al.* 2013; Sjöstedt *et al.* 2018; Ward *et al.* 2019). However, most studies exploring patterns in microbial community diversity and structure in response to environmental change have focused on direct effects, without consideration of life history. Previous TME exposure can lead to an increase

of metal tolerance in microbial communities by the elimination of the most sensitive individuals and the replacement of sensitive species by tolerant ones (Gustavson *et al.* 1999). Serra *et al.* (2009) found that copper pulse exposure may be toxic to periphyton communities while continuous copper exposure may lead to community adaptation, which is often related to changes in species composition. All of these studies are in agreement with the pollution-induced community tolerance (PICT) model (Blanck, Wangberg and Molander 1988; Blanck 2002). However, while numerous studies developed the PICT approach based on photosynthetic efficiency, extracellular enzyme activities or respiration activity (Carman, Fleeger and Pomarico 2000; Soldo *et al.* 2005; Serra, Corcoll and Guasch 2009; Tlili 2013), little is known about how metal exposure impacts diversity and composition of marine prokaryotic communities previously exposed to similar contamination.

Assuming that the life history of its planktonic communities in Toulon Bay would mainly reflect TME exposure history due to the strong and chronic Cu and Pb contamination gradients of the area (Layglon *et al*., under revision), in this study we aimed to answer two main questions: (1) to what extent do Cu and Pb contamination affect prokaryotic communities from uncontaminated site at relevant concentrations; and (2) does prokaryotic TME exposure history play a role in shaping prokaryotic diversity after a Cu exposure?

MATERIALS AND METHODS

Study area, seawater collection, processing, and storage

106 The study was conducted in Toulon Bay, located in the northwestern part of the
107 Mediterranean Sea (Figure 1 and Supplementary Material). Seawater used for the
108 first mesocosm experiment was collected at 1-m depth from site 41p (low
109 contaminated site) (Figure 1) on May 10th 2016 and seawater used for the second
110 mesocosm experiment was collected at 1-m depth from both sites LAZ (intermediate
111 contaminated site) and 6ext (highly contaminated site) on May 2nd 2017 (Figure 1).
112 The 3 sampling seawaters used for mesocosm experiments were geochemically
113 contrasted. They were representative of the North-West to South-East decreasing
114 gradient of trace metal contamination (mainly Cu and Pb) previously observed in
115 Toulon Bay (Figure 1). Minimal concentrations were recorded in the open-sea
116 sampling site (41p), while maximal concentrations were recorded in the northern and
117 enclosed part of the little bay (6ext) where the TME contamination is chronic (Coclet
118 et al., 2019, 2017; Layglon et al., under revision). Details on sample's information are
119 given in the supplementary Table 1. Seawater was collected using an air-
120 compressed Teflon pump (AstiPure™ II, Saint-Gobin) connected with Teflon tubing. It
121 was filtered on site through a 5-10 µm cellulose Polycap filter (Whatman) connected
122 to the Teflon tubing after the pump in order to remove the largest grazers. Filtered
123 seawater was collected in 50-L low-density polyethylene (LDPE) tanks (Nalgene®).
124 Back to the lab, each mesocosm was filled with 20-L of filtered seawater. The
125 remaining seawater was filtered through 0.2 µm Teflon Polycap filters (Whatman) to
126 eliminate all organisms, then stored at 4°C and subsequently used to re-fill
127 mesocosms during the experiment. All the sampling material, bottles, tanks, and
128 filters were pre-cleaned following rigorous protocols, detailed in supplementary

129 information and in Coclet *et al.* (2018), in order to keep metal concentrations as close
130 as possible to their initial levels.
131
132 **Mesocosm setup, and experimental design**
133 For both mesocosm studies, twelve 20-L LDPE tanks (Nalgene®) were enclosed in a
134 large box covered of plastics in order to avoid contamination in TMEs from ambient
135 particles. Fresh air was continuously provided inside the box by a filtered-equipped
136 fan in order to avoid humidity accumulation and to equilibrate temperature with the
137 surrounding room. The tanks were filled with 5-10 µm filtered water, and thus
138 included a natural marine microbial community mainly composed of free-living
139 organisms. Seawater in each tank was continuously mixed with a Teflon magnetic stir
140 bar used at low speed and oxygenized with 0.2 µm-filtered and humidified ambient
141 air. The 12 tanks were submitted to a daily light/dark cycle of 14/10h, using full solar
142 spectrum aquariophilly neon tubes and a surface light irradiance of 50 µmol.m^2.s^1 of
143 white light. In the first year, the temperature of the room and of the mesocosms' box
144 were kept constant at 19 ± 1.6°C, and 23 ± 1.5°C, respectively. In the second year,
145 the room temperature and the mesocosm interior were kept constant at 19 ± 2.2°C
146 and, 22 ± 2.0°C, respectively. To maintain resource availability during the
147 experiments, each tank had a continuous opened water circulation system, using a
148 peristaltic pump (Minipuls Evolution®, Gilson), to renew 50% of the total water volume
149 in a week by 0.2 µm filtered seawater sampled at the same site.
150 The first mesocosm experiment was performed from May 10th to June 17th
151 2016 over a 6-week period. Twelve pre-cleaned 20-L LDPE (Nalgene®) tanks were
152 filled with 5-10 µm filtered water from site 41p (Figure 1). A 2-week acclimation period

153 was carried out to establish relatively stable conditions in each tank. After the
154 acclimation period, seawater from the 12 tanks was pooled, homogenized and then
155 tanks were re-filled. Following the acclimation period, seawater in tanks from the first
156 mesocosm study were assigned to four treatments: (1) site control (41p CTRL, i.e. no
157 TME added to 41p seawater), (2) Cu addition (150 nM) (41p Cu), Pb addition (7 nM)
158 (41p Pb), and (4) Cu and Pb addition (150 nM + 7 nM, respectively) (41p Cu+Pb)
159 (Supplementary Figure 1). The Cu treatments were spiked with a stock solution of Cu
160 nitrate (10^{-3} M) and the Pb treatments were spiked with a stock solution of Pb nitrate
161 (10^{-3} M). According to the initial Cu and Pb concentrations measured in the 41p
162 seawater used to fill the tanks, final concentrations of 150 nM and 7 nM for Cu and
163 Pb respectively, were targeted. The water supplies were simultaneously spiked with
164 similar concentrations of Cu, Pb, or both depending of treatment to maintain metal
165 concentrations in tanks.

166 The second mesocosm was performed from May 2nd to June 12th 2017 over a
167 6-week period. Six 20-L LDPE (Nalgene®) tanks were filled with 5-10 µm filtered
168 water from each sampling site (LAZ and 6ext sites) (Figure 1). The treatments in the
169 second mesocosm experiment were (1) site control (LAZ CTRL, i.e. no TME added to
170 LAZ seawater), (2) Cu addition to LAZ seawater (150 nM) (LAZ Cu), (3) site control
171 (6ext CTRL, i.e. no TME added to 6ext seawater), and (4) Cu addition to 6ext
172 seawater (150 nM) (6ext Cu) (Supplementary Figure 2). The Cu treatments were
173 spiked with a stock solution of Cu nitrate (10^{-3} M). According to the initial Cu
174 concentrations measured in LAZ and 6ext seawaters used to fill the tanks, final
175 concentration of 150 nM for Cu were targeted. The water supplies were simultaneous
176 spiked with Cu depending of treatment to maintain metal concentrations in tanks.

177

178 **Experimental time plan and sampling**

179 After the acclimation period, seawater was sampled from the 12 tanks using a
180 peristaltic pump connected with Teflon tubing at different times over the course of the
181 first mesocosm experiment (0, 3, 7, 10, 14, 17, 21, and 25 days) after the Cu/Pb
182 spikes, and the second mesocosm experiment (0, 3, 7, 10, 14, 17, 21, 25 and 28
183 days) after the Cu spikes. At each sampling time, 333 mL of seawater was sampled
184 in each replicate of a given experimental condition and pooled in 1L FEP bottles
185 (Nalgene®) to obtain composite samples for each condition. It was then immediately
186 resampled for future trace metals (TM), dissolved organic carbon (DOC), and total
187 nitrogen (TN) quantifications as previously described (Omanović *et al.* 2006; Cindrić
188 *et al.* 2015), and fully explained in Supplementary Material. Additionally, seawater
189 (10-mL) for flow cytometry analysis were directly collected in 15-mL centrifuge tubes
190 (Falcon) from each of 12 tanks, fixed with 0.25% (final concentration) glutaraldehyde,
191 and stored at -80°C until analysis. Finally, at 0, 7, 14, 21, and 25 days, 1-L of
192 seawater from each of 12 tanks was collected in 1-L HDPE bottles and filtered
193 through 0.2 µm polycarbonate membranes (Millipore). Filters were stored at -80°C
194 until DNA extraction.

195

196 **Seawater physico-chemical measurements and analyses**

197 Temperature (°C), pH, dissolved oxygen concentration ($mg.L^{-1}$) and saturation (%)
198 were directly monitored daily into tanks, over the course of both mesocosm
199 experiments, using LDO10105 (Hach) multiparameter probe. Differential pulse
200 anodic stripping voltammetry (DPASV) was used to measure Cd, Cu, Pb, and Zn

dissolved concentrations. Details about instruments, analysis conditions, and data treatment are provided in Supplementary material and elsewhere (Oursel *et al.* 2013; Cindrić *et al.* 2015). DOC and TN concentrations were determined using a TOC-V_{CSH} analyzer (Shimadzu) (further details in Dang *et al.*, 2014; Coclet *et al.*, 2018, 2019).

Flow cytometry analysis

Autotrophic prokaryotes (*Synechococcus*-like, SYN), photosynthetic picoeukaryotes (PPE) and photosynthetic nanoeukaryotes (PNE) populations were characterized and enumerated using a BD Accury™ C6 (BD Biosciences) flow cytometer, as previously described (Coclet *et al.* 2018, 2019). Heterotrophic prokaryotes were enumerated after staining with SYBR Green as previously described (Cabrol, Quéméneur and Misson 2017).

DNA extraction, PCR amplification, and sequencing of 16S rRNA gene amplicons

DNA was extracted from the polycarbonate filters by a combination of enzymatic cell lysis (Ghiglione, Conan and Pujo-Pay 2009) and AllPrep DNA/RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Prokaryotic community structure was assessed by targeting the V4-V5 region of the 16S rRNA gene (Parada, Needham and Fuhrman 2016) and using Illumina Miseq 2 x 250pb paired-end sequencing (Genoscreen, France). The protocol for the DNA extraction and the library preparation is fully described in Coclet *et al.* (2019).

Bioinformatic analysis

225 Sequences were demultiplexed and assigned to corresponding samples using
226 CASAVA (Illumina). Bioinformatic analysis were performed on sequences from two
227 sequencing MiSeq runs in the same sequencing company were used for our two
228 mesocosm experiments. The analysis of the raw sequences was done by following
229 the standard pipeline of DADA2 (Callahan *et al.* 2016) in RStudio (R Core Team
230 2017) with the following parameters: maxN= 0, maxEE= c(2,2), truncQ= 2. Briefly,
231 the package includes the following steps: filtering, dereplication, sample inference,
232 chimera identification, and merging of paired-end reads. DADA2 infers exact
233 amplicon sequence variants (ASVs) from sequencing data, instead of building
234 operational taxonomic units from sequence similarity. The taxonomy assignments
235 were done with the SILVA v.128 database (Pruesse *et al.* 2007; Quast *et al.* 2013)
236 and the “assignTaxonomy” function in DADA2. For some ASVs, in order to obtain a
237 finer taxonomical resolution, DADA2 package implements a method to make species
238 level assignments based on exact matching between ASVs and sequenced
239 reference strains in the SILVA v.128 database, using “addSpecies” function.
240 Sequences classified as mitochondria or chloroplast were removed from the ASV
241 table. The datasets from the samples were normalized by random subsampling to
242 include an equal number of reads (n = 2078 reads). Samples with lower number of
243 sequences than the number of reads chose to the normalization were discarded
244 (Supplementary Table 1).
245
246 **Statistical analysis**
247 All plots and statistical analyses were performed with RStudio (R Core Team 2017).
248 Alpha diversity and error estimates were performed using QIIME script

core_diversity_analyses.py (Caporaso *et al.* 2010), including Chao1, equitability, observed richness, Shannon and Simpson's diversity. Two-way analysis of variance (ANOVA) was applied to investigate the influences of Cu and Pb addition, experimental time, and their interaction on α-diversity metrics and the abundance heterotrophic prokaryotes' community. Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distance was applied to evaluate the overall differences in prokaryotic community structure, using the *vegan* package in R (Oksanen *et al.* 2016). Global and pairwise PERMANOVA tests, using *vegan* and *RVAideMemoire* packages, respectively were applied to test significant differences between treatments and sampling time. To find representative phylotypes associated with the treatments, similarity percentage (SIMPER) analyses were first applied to screen OTUs primarily responsible for the overall dissimilarity (i.e. contributing to more than 1% of total dissimilarity) in the prokaryotic communities between treatments at each time point using *vegan* package.

Results

Trace metal elements and additional physicochemical parameters

Initial seawater samples (Ti) used for the first (i.e. 41p) and the second (i.e. LAZ and 6ext) experiments were geochemically contrasted and presented variations in trace metal concentrations as expected (Supplementary Table 2 and Supplementary Table 3). Total Cu, Pb, and Zn concentrations for 41p were 2.44 nM, 0.13 nM, and 5.34 nM, respectively while the concentrations were 2.5-, 3-, and 3.6-fold higher than in LAZ respectively, representing 15 nM, 0.79 nM, and 40 nM. For initial 6ext seawater, total

Cu, Pb, and Zn concentrations were 15-, 18-, and 26-fold higher than in 41p respectively, representing 36 nM, 2.32 nM, and 139 nM.

At the beginning of both mesocosm experiments (T0), trace metal concentrations measured in control conditions were close to the ones encountered in initial seawater samples (Ti) from 41p, LAZ, 6ext. Thus, this confirms that no adsorption or contamination in metals occurred in our experiments. In both mesocosms, metal concentrations added were close to the targeted concentrations (150 nM of Cu and 7 nM of Pb) (Supplementary Table 2 and Supplementary Table 3). Finally, both Cu and Pb concentrations at the beginning (T0) were not different to those measured at the end (TF) of each experiment, confirming no metal loss in treatments where metals were added (Supplementary Table 2 and 3).

The DOC and TN concentrations were not significantly different between the 3 initial seawaters (Supplementary Table 2 and Supplementary Table 3). For both mesocosm experiments, water temperature, pH, DO, and DOC did not exhibit significant differences among conditions but all of these parameters experienced temporal significant changes during the entire course of experiments (Kruskal-Wallis, $p < 0.05$). Globally, for both mesocosm experiments, pH and water temperature increased with time while DO saturation and DOC concentration decreased with time (Supplementary Table 2 and Supplementary Table 3).

Ultraphytoplanktonic and heterotrophic prokaryotes' abundances

During the first mesocosm experiment, total ultraphytoplanktonic abundances were determined by flow cytometry. Abundances were comparable between sampled

seawater (Ti) from 41p and both conditions 41p CTRL and 41p Pb (1.1 ± 0.1 x 10^4 cell.mL^{-1} on average), with low temporal variability during the entire course of experiment (Supplementary Figure 3A). Total ultraphytoplanktonic abundances in conditions 41p Cu and 41p Cu+Pb, started to decrease 3 days after metal addition, leading to significantly lower abundances than in conditions 41p CTRL and 41p Pb until the end of the experiment (ANOVA, $P < 0.001$). At the beginning of the experiment (T0), heterotrophic prokaryotes' abundance measured in all conditions (11 ± 0.3 x 10^4 cell.mL^{-1} on average) was significantly lower than in sampled seawater (Ti) from the 41p (2.5 x 10^5 cell.mL^{-1}) (ANOVA, $P < 0.001$) (Supplementary Figure 3B). From three days after metal addition to the end of the experiment, heterotrophic prokaryotic abundance was significantly higher in conditions 41p Cu and 41p Cu+Pb than in conditions 41p CTRL and 41p Pb (ANOVA, $P < 0.001$).

During the second mesocosm experiment, total ultraphytoplanktonic abundance in LAZ (1.9 x 10^4 cell.mL^{-1}) was significantly higher than in 6ext seawater (7.9 x 10^3 cell.mL^{-1}) (ANOVA, $P < 0.001$). After the acclimation period (T0), total ultraphytoplanktonic abundance followed a similar pattern in all conditions, decreasing 3 days after metal addition (Supplementary Figure 4A). From 3 days after metal addition to the end of the experiment, total ultraphytoplanktonic abundance was significantly lower in Cu-treated conditions than in LAZ and 6ext control conditions (ANOVA, $P < 0.001$). Heterotrophic prokaryotic abundance was not significantly different between LAZ (5.8 x 10^5 cell.mL^{-1}) and 6ext (5.0 x 10^5 cell.mL^{-1}) initial seawaters (Supplementary Figure 4B). At the beginning of the experiment (T0), this abundance was similar in all conditions (41 ± 1.0 x 10^4 cell.mL^{-1}), while from 7 days after metal addition to the day 17, it was higher in Cu-treated conditions than in

control conditions (ANOVA, $P < 0.001$). From 21 days after metal addition to the end of the experiment, heterotrophic prokaryotic abundance was similar in 6ext CTRL and 6ext Cu. Heterotrophic prokaryotic abundance from LAZ Cu was higher than in LAZ CTRL.

Prokaryotic alpha-diversity

For the first mesocosm experiment, all the α-diversity indices except equitability varied significantly over time (Table 1A and Supplementary Figure 5). Globally, prokaryotic α-diversity indices significantly decreased over time in all conditions (ANOVA, $P < 0.05$). After the metal addition (T0), the Chao1 index was significantly lower in Cu-treated conditions (i.e., 41p Cu and 41p Cu+Pb) relative to both initial seawaters from 41p and condition 41p CTRL (ANOVA, $P < 0.001$). Simpson's diversity was significantly higher in metal-treated conditions (i.e., 41p Cu, 41p Pb, and 41p Cu+Pb) relatively to condition 41p CTRL (ANOVA, $P < 0.001$). No significant difference was observed in equitability, observed richness, and Shannon indices between conditions.

For the second mesocosm experiment, all the α-diversity indices except equitability varied significantly over time (Table 1B and Supplementary Figure 6). All prokaryotic α-diversity indices significantly decreased over time in all conditions, especially after the 7th day of the experiment (ANOVA, $P < 0.05$). Average Chao1, observed OTUs, and Shannon indices varied across conditions, and they were lower in condition LAZ Cu relative to LAZ CTRL (ANOVA, $P < 0.05$). No significant difference was observed in all α-diversity indices between conditions 6ext CTRL and

342 6ext Cu. Finally, all α-diversity indices measured in the second mesocosm
343 experiment were lower compared to those of the first mesocosm experiment.
344
345 **Prokaryotic community is strongly shaped by Cu in contrast to Pb**
346 In the first mesocosm, the most abundant dominant phyla/classes (> 1% of all
347 sequences across all samples) were *Alphaproteobacteria*, *Gammaproteobacteria*,
348 *Flavobacteriia* and *Cyanobacteria,* representing 96% of the overall relative
349 abundance. Archaea represented less than 1% of the prokaryotic community of all
350 samples and thus were grouped with all the “other” rare taxa (< 1% of all sequences
351 across all samples) in subsequent analysis.
352 Among the abundant families, 10 representative families, determined by
353 SIMPER analysis, significantly differed treatments over time (Supplementary Table
354 5). Their relative abundances are shown in Figure 2. *Rhodobacteraceae*, *Family I*
355 (*Cyanobacteria*), *Alteromonadaceae*, and *SAR11 Surface 1 clade* decreased in Cu-
356 treated conditions compared to 41p CTRL or 41p Pb. These families contributed to
357 20 ± 1.3%, 16 ± 4.6%, 6.7 ± 2.5%, and 3.0 ± 0.76% of the overall dissimilarity
358 between these two conditions, respectively. Conversely, the relative abundance of
359 *Hyphomonadaceae*, *Flavobacteriaceae*, and *Halieaceae* increased in Cu-treated
360 conditions compared to 41p CTRL or 41p Pb and contributed to 34 ± 8.1%, 17 ±
361 4.4%, and 4.8 ± 1.6% respectively of the overall dissimilarity between these two
362 groups of conditions.
363 Moreover, 19 genera showed overall significant differences between
364 conditions at the end of the experiment (TF) (Supplementary Table 6). Among these
365 genera, only 4 were responsible for the structural change in Pb-treated condition.

366 The relative abundance of *Alteromonas, Glaciecola*, and unknown *SAR11 Surface 2*
367 *clade* significantly increased while *Erythrobacter* nearly disappeared when only Pb
368 was added. *Synechococcus*, *Litorimicrobium*, and to a lesser extent, *CHAB XI 27*
369 decreased in 41p Cu compared to 41p CTRL. Conversely, *Parvularcula*, *NS5 marine*
370 *group* and *Hyphomonas* increased in 41p Cu compared to 41p CTRL (Figure 3).
371 Samples from the first mesocosm study were mainly clustered by conditions
372 (PERMANOVA, R^2 = 0.41; P < 0.001) and in a lesser extent by sampling times
373 (PERMANOVA, R^2 = 0.34; P < 0.001) or interaction (PERMANOVA, R^2 = 0.17; P <
374 0.001) (Figure 4 and Supplementary Table 4A). At the beginning of the experiment
375 (T0), prokaryotic community structures were similar between all conditions, and
376 significantly different from the initial seawater at 41p (Supplementary Table 4B). After
377 the 7th day, Cu-treated conditions (41p Cu and 41p Cu+Pb) shown significantly
378 different prokaryotic community structures from the other conditions (41p, 41p CTRL,
379 and 41p Pb) at each sampling time, especially at the end of the experiment (TF).
380 Finally, there was no significant difference in communities between 41p CTRL and
381 41p Pb, as well as between 41p Cu and 41p Cu+Pb at each sampling time
382 (Supplementary Table 4).
383
384 **Influence of TME exposure history on copper effects**
385 *Prokaryotic community composition and structure*
386 Concerning prokaryotic communities sampled from 3 geochemically contrasted sites
387 (a low contaminated site (41p), an intermediately contaminated site (LAZ), and a
388 highly contaminated site (6ext)), the most abundant phyla/classes (> 1% of all
389 sequences across all samples) were *Alphaproteobacteria*, *Gammaproteobacteria*,

390 *Flavobacteriia* and *Cyanobacteria*, representing 94% of the overall relative
391 abundance. At the family taxonomic rank, *Rhodobacteraceae* (25 ± 1.5%),
392 *Flavobacteriaceae* (16 ± 1.1%), *Hyphomonadaceae* (11 ± 1.3%), *SAR11 Surface 1*
393 *clade* (6.3 ± 0.89%), and *Halieaceae* (6.0 ± 0.59%) included the majority of the total
394 sequences (Figure 5). As for the first mesocosm experiment, Archaea represented
395 less than 1% of the prokaryotic community of all samples, and they were considered
396 in the group "other" corresponding to the rare taxa in further analyses.
397 NMDS plot and PERMANOVA tests showed significant clusters corresponding
398 to both sampling times and conditions (Figure 6 and Supplementary Table 7A). At all
399 taxonomic rank, Cu addition (PERMANOVA, $R^2 = 0.58$; $P < 0.001$) showed a greater
400 influence on community variations than time (PERMANOVA, $R^2 = 0.14$; $P < 0.001$) or
401 interaction between time and experimental condition (PERMANOVA, $R^2 = 0.20$; $P <$
402 0.001) (Supplementary Table 7A). Prokaryotic community structures were
403 significantly similar between initial seawaters from LAZ and 6ext and significantly
404 different to the initial seawater from 41p (Supplementary Table 7B). At the beginning
405 of the experiment (T0), similarly to what was observed for 41p in the first experiment,
406 the prokaryotic community structures in each control conditions were not significantly
407 dissimilar from related Cu-treated ones (LAZ CTRL vs LAZ Cu, and 6ext CTRL vs
408 6ext Cu). After the 7th day, and until the end of the experiment, significant differences
409 were clearly observed between Cu-treated and non-Cu-treated communities for 41p
410 and LAZ. Conversely, pairwise comparisons showed no significant difference
411 between 6ext CTRL and 6ext Cu, whatever the sampling time.
412

Representative taxa responsible for structural changes between control and Cu-treated conditions

SIMPER analyses revealed that 12 representative families significantly differed initial seawaters, but also control and Cu-treated conditions throughout experiment duration (Figure 5 and Supplementary Table 8). *SAR11 Surface 1* and *Surface 2 clades*, *Hyphomonadaceae*, and *Family I* (*Cyanobacteria*) were higher in 41p than in LAZ and 6ext, while *Flavobacteriaceae* and *Rhodobacteraceae* were more abundant in LAZ and 6ext than in 41p (Supplementary Table 8). Finally, major differences between LAZ CTRL and LAZ Cu were explained by the variation of relative abundances of *Hyphomonadaceae*, *Rhodobiaceae*, *Cryomorphaceae* and *Alteromonadaceae*.

At a lower taxonomic rank, among the 176 genera recorded, 34 significantly differed control and Cu-treated conditions at the end of the experiment (TF) (Figure 3 and Supplementary Table 9). In addition, the genera *Salinihabitans* and *Rhodobium* discriminating 41p CTRL from 41p Cu, decreased in LAZ Cu compared to LAZ CTRL while *Jannaschia*, unknown *Cryomorphaceae*, and *Hirschia* increased in LAZ Cu compared to LAZ CTRL. *Ruegeria* decreased in 6ext Cu compared to 6ext CTRL while *Formosa*, OCS116 clade, and *Methylotenera* increased in 6ext Cu compare to 6ext CTRL. Finally, representative genera responsible for structural changes between control conditions and Cu-treated conditions for 41p and LAZ were mainly abundant genera while for 6ext, representative genera were mainly rare genera (< 1% of all sequences across all samples).

436 ## Discussion

437 **Copper exposure induced changes in prokaryotic community**

438 In both mesocosm experiments, temporal variations in prokaryotic community
439 structure were observed regardless of metal additions. However, metal addition led
440 to stronger changes of community structure than the experimental setup or the
441 experiment time. Copper exposure killed more than 99% of the ultraphytoplanktonic
442 community. The selective pressure of metals on phytoplankton and more precisely
443 on ultraphytoplankton is now well documented (Echeveste, Agustí and Tovar-
444 Sánchez 2012; Lafabrie *et al.* 2013; Coclet *et al.* 2018). In parallel to the
445 phytoplankton mortality, we observed an increase in heterotrophic prokaryotes'
446 abundance and a decrease in their α-diversity if they originated from the lower and
447 intermediately contaminated sites. This abundance increases thus seemed to relate
448 the development of a limited number of opportunistic *r*-strategists that efficiently use
449 the organic resources released by the phytoplanktonic decay (Odum, 1969; Pianka,
450 1970). This behavior has already been observed with the highly reactive marine
451 microbial community (Buchan *et al.* 2014; El-Swais *et al.* 2015). Taken altogether,
452 these observations highlight the strong interdependence between prokaryotic and
453 phytoplanktonic communities, which could partly explain Cu-responses of microbial
454 communities through indirect trophic relationships.

455 When considering the widely reported bacterial resistance to metals (Nies
456 1999; Baker-Austin *et al.* 2006), the response of the prokaryotic community to Cu
457 addition might also be due to the persistence or proliferation of resistant taxa, which
458 can be higher than the extinction rate of sensitive taxa (Wang *et al.* 2015; Qian *et al.*
459 2017).

Significant differences in community structure between the control and the Cu-added conditions were found at all sampling time. Particularly, *Hirschia* (*Hyphomonadaceae*), *NS4 and NS5 marine groups*, *Formosa* and *Tenacibaculum (Flavobacteriaceae)* increased in Cu-treated conditions for both mesocosm experiments, indicating that they could be more resistant or take advantage from copper addition and phytoplankton decay (Pinhassi *et al.* 2004). *Hyphomonadaceae* were known to be abundant in plastic-attached biofilm communities (Zettler, Mincer and Amaral-zettler 2013; Bryant *et al.* 2016; De Tender *et al.* 2017; Oberbeckmann *et al.* 2018), especially in disturbed environments (Briand *et al.*, 2017; Coclet, *et al.*, submitted). However, the results in this paper demonstrate for the first time the significant presence of this group in the prokaryotic community of metal contaminated seawater. *Flavobacteriacea* was also a major component of the microbial community in strongly disturbed and metal-contaminated environments (Allen *et al.* 2013; Wang *et al.* 2015; Zouch *et al.* 2018; Coclet *et al.* 2019). Finally, the previous observation that *Tenacibaculum* and *Formosa* genera are positively selected in Cu-amended 6ext water as abundant and representative taxa from the most contaminated site (6ext) during a one-month-long survey, is in agreement with the idea of a similar selective pressure *in situ* as that observed in our previous field study experiment (Coclet *et al.* 2019). Conversely, members of *Rhodobacteraceae*, *Family I* (*Cyanobacteria*, mainly *Synechococcus*), Alteromonadaceae, and *SAR11 Surface 1 clade* families have been proposed to be sensitive *in situ* to high trace metal levels (Cassier-Chauvat and Chauvat 2014; Coclet *et al.* 2018, 2019; Corcoll *et al.* 2019) or characteristic of marine zones away from anthropogenic influence (Gilbert *et al.* 2009, 2012; Fuhrman, Cram and Needham 2015).

Thus, our study reveals the sensitivity of a part of the prokaryotic community to Cu contamination representative from a harbor, by a combination of direct (i.e. toxicity) or indirect (i.e. related to phytoplankton decay) processes, as suggested recently in a field study (Coclet *et al.* 2019).

Limited effects of Pb contamination on prokaryotic community

In our study, the effect of Pb exposure on prokaryotic communities was far less pronounced than the effect of Cu exposure. Pb addition using nitrate salts appeared to have limited impact on prokaryotic diversity and structure, with no detectable effect on ultraphytoplankton. Moreover, it did not prove to have a synergistic interaction with Cu. The weak toxicity of Pb on prokaryotic communities could be explained by its resistance and detoxification mechanisms, which are very efficient in microbial organisms (Trevors, 1989; Nies, 1999; Nayar *et al.*, 2004; Naik and Dubey, 2013). Additionally, the dichotomy between Cu and Pb effects could be due to the difference in their concentrations added in our mesocosms as well as their bioavailability. Indeed, to mimic the contamination levels observed in Toulon Bay, Pb concentrations were 20-fold lower than Cu concentrations, thus reducing the dose at which the cells were exposed. No data is available in the literature concerning tolerance threshold of marine bacterial communities against Pb contamination. Pb dissolved concentration was constant throughout the experiment, indicating no Pb adsorption on mesocosm materials or on microbial cells. Thus, the surface reactive behavior of Pb might not have induced a reduced bioavailability by reducing its dissolved concentration. Therefore, our study does not allow us to explain precisely this difference of toxicity between Cu and Pb, but the non-essential nature of Pb and potential different ways

to enter the cells than Cu represent hypotheses that should be studied in future research (Han *et al.* 2014).

The relative abundance of *Glaciecola* taxon significantly increased while *Erythrobacter* became undetectable when only Pb was added. *Glaciecola* has been described to be an opportunistic member of prokaryotic communities, its abundance increasing rapidly following an increase in DOC concentration (Sipler *et al.* 2017; von Scheibner, Sommer and Jürgens 2017). Since DOC concentrations in our experiment were similar in all conditions, the ability of *Glaciecola* to take advantage compared to other taxa in a Pb contaminated sea water remains unexplained. The genus *Erythrobacter* was found to be important taxa in surface-attached communities (Zettler, Mincer and Amaral-zettler 2013; Dussud *et al.* 2018; Oberbeckmann *et al.* 2018), and notably in contaminated ecosystems (Catão *et al. submitted*; Briand *et al.*, 2017; Pollet *et al.*, 2018; Coclet, *et al.*, submitted). Thus, the strong decrease of *Erythrobacter* in the water after Pb addition could be explained by the ability of this genus to form biofilms in the mesocosms. Indeed, biofilms are one of the most widely distributed and successful modes of life in water environment (Stoodley *et al.* 2002; Flemming *et al.* 2016) and a strategy that microorganisms might use to protect them from toxic metals (Harrison, Ceri and Turner 2007).

TME exposure history determines Cu effects on prokaryotic communities

Trace metal contamination gradients in Toulon bay have been already described by punctual mappings (Jean *et al.* 2012; Dang *et al.* 2015b; Coclet *et al.* 2018) or during short (Coclet *et al.* 2019) and long-term surveys (Layglon *et al.*, under revision). These studies demonstrated that TME contamination gradients persists at a pluri-

annual scale in Toulon Bay. This led us to assume that prokaryotic communities sampled along this contamination gradient could present different metals exposure histories. Cu addition in both low (41p) and in intermediately (LAZ) contaminated seawaters induced important changes in prokaryotic community structure, while Cu addition in seawater originating from a chronically contaminated site (6ext) only weakly impacted the prokaryotic community structure. Based on the PICT theory (Gustavson *et al.* 1999; Blanck 2002), this observation validates our assumption and demonstrates the adaptive potential of prokaryotic communities to withstand elevated Cu concentrations. This adaptation must have developed rapidly since the water residence time in Toulon Bay has been estimated to a few days (Dufresne, Duffa and Rey 2014), even if this estimation is probably not representative to what happens in very enclosed docks such as the one where we sampled contaminated water (6ext). This adaptive potential could be the result of the presence of a higher proportion of tolerant species in the most contaminated seawater, representative of a strong and chronic metal-exposure history of these communities (Carman, Fleeger and Pomarico 2000). This hypothesis is in agreement with the very small variation of structure after Cu addition in the community sampled from the most contaminated site. Indeed, for this community, we mainly observed changes in the relative abundance of rare taxa.

We hypothesize that mechanisms of toxicity and resistance to Cu co-occur in prokaryotic communities from low and intermediately contaminated sites. The observed dissimilarity in prokaryotic structures between these communities after Cu exposure could be the result of differences in initial community composition in 41p and LAZ seawaters. To the best of our knowledge, our study provides the first

evidence that TME exposure history may play a critical role in marine prokaryotic community response to re-current metal contamination.

Conclusion

From our mesocosm experiences, we can conclude that harbor-relevant Cu exposure affects abundance, diversity and structure of prokaryotic communities from both low and intermediately TME contaminated seawaters. The effect of Cu addition is visible through both the disappearance of sensitive taxa and the selection of adapted ones. We also demonstrated that TME exposure history has a strong influence on the response of prokaryotic communities to newer-current contamination, as prokaryotic communities from contaminated site were less impacted by Cu addition. Conversely, we showed limited impacts of Pb exposure compared to Cu contamination, probably due to the low toxic potential against prokaryotes and the efficiency of its resistance and detoxification mechanisms. Finally, our observations suggest both direct (toxic) and indirect (trophic) effects of TME contaminations, highlighting the need for studies of the complexity of both biotic and abiotic interactions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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