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1 **Legacy and dispersant influence microbial community**
2 **dynamics in cold seawater contaminated by crude oil**
3 **water accommodated fractions**

4
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20 **Abstract**

21 Dispersants, used for combating oil spills, increase hydrocarbon bioavailability
22 promoting their biodegradation. Oil weathering process introduces harmful soluble
23 hydrocarbons, such as polycyclic aromatic hydrocarbons (PAHs), into the water
24 column, resulting in water-accommodated fraction (WAF). The presence of dispersants
25 can influence the weathering process by increasing PAHs solubility, toxicity and
26 biodegradability. However, little is known on how dispersants affect microbial
27 communities and their degradation capacities, especially in cold environment where low
28 temperature decreases microbial activity and thus hydrocarbon degradation. Here, we
29 investigated the microbial community dynamics in cold water contaminated by WAF
30 prepared from crude oil with or without a commercial dispersant (Finasol OSR52).

31 The WAFs, prepared with Naphthenic North Atlantic crude oil, were used to
32 contaminate seawater from Norwegian cold sites, one oil-contaminated and the other
33 pristine. The WAF-contaminated seawaters were maintained in microcosms at 4°C for
34 21 days. The content of PAHs and microbial compositions (16S rRNA gene
35 sequencing) were determined at days 0, 7, 14 and 21. In addition, the 96 hours toxicity
36 assay with adult *Acartia tonsa* revealed WAFs toxicity at days 0 and 21.

37 The toxicity of WAF mixtures, with and without dispersant, against *Acartia tonsa* was
38 reduced during the experiment, but PAHs removal was not increased. The water from
39 the oil-contaminated site showed the highest PAHs removal revealing legacy effect
40 (presence of microorganisms adapted to PAHs). Additionally, our results reveal: i)
41 microbial community plasticity allowing the adaptation to the presence of PAHs and
42 dispersant, ii) specific bacteria taxa probably involved in PAHs degradation, and iii)
43 dispersants shape the microbial communities dynamics by stimulating potential

44 dispersant-degrading taxa, such as *Fusibacter*. Thus, our results provide valuable
45 insights on the role of microbial community in determining the fate of water-solubilized
46 hydrocarbon in cold environment while questioning the role of dispersant used for
47 fighting oil spill.

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49 Keywords: Water Accommodated Fractions, dispersant, Cold water, Microbial
50 communities, Bioremediation, Crude oil.

51

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62 **1. Introduction**

63 Cold areas, such as the sub arctic area, are increasingly threatened by human activities
64 due to climate change effects opening novel sea lines (Farrington, 2014). Particularly,
65 the Arctic seas are exposed to oil-related activities presenting a high risk of oil spill
66 accidents (Vergeynst et al., 2019). Among hydrocarbon compounds, polycyclic
67 aromatic hydrocarbons (PAHs) are the most toxic, being of global concern due to their
68 persistence in the environment, and their toxicity towards a wide range of organisms
69 (Patel et al., 2020). In addition, the fate of PAHs in marine ecosystems is largely
70 dependent on microbial activities and abiotic weathering processes (Duran and Cravo-
71 Laureau, 2016). The abiotic weathering processes, including mechanical actions (e.g.
72 wave movements) and chemical reactions (e.g. photo-oxidation), favor PAHs solubility,
73 especially low molecular weight PAHs (LMW-PAHs), increase the hydrocarbon
74 bioavailability and thus toxicity (Duran and Cravo-Laureau, 2016; Neff et al., 2005). In
75 order to simulate weathering processes, scientists are using water-accommodated
76 fractions (WAF), which contain harmful aromatic hydrocarbons and aliphatic
77 compounds, PAHs being the most toxic compounds (Jiang et al., 2012). WAF is
78 considered as useful model for studying oil spills dynamics, determining the fate of
79 solubilized hydrocarbons, their toxicity and degradation by microorganisms (Faksness
80 et al., 2020).

81 In response to oil spill accidents, several intervention approaches have been developed
82 for clean up processes, including bioremediation, the use of dispersants, and mechanical
83 removal (Hazaimah and Ahmed, 2021). The cost of oil spill clean up is difficult to
84 evaluate because it depends on several factors including the type of the oil, the
85 environmental conditions, the nation, and the incident site (Prendergast and Gschwend,
86 2014). The clean up cost has been evaluated for the Exxon Valdez, the Prestige, and the
87 Deepwater Horizon oil spills was around 3, 12, and 63 billion dollars respectively, both

88 combining several strategies including the use of dispersant, bioremediation and
89 mechanical recovery (Dave and Ghaly, 2011). It has been shown that the clean up
90 strategies can be ranked in increasing order of cost as follow: bioremediation (including
91 natural attenuation, bio-stimulation by fertilizer addition, and bio-augmentation by
92 addition of microorganisms), in-situ burning, use of dispersants, and mechanical and
93 manual recovery (Etkin 2000; Prendergast and Gschwend, 2014). Thus, bioremediation,
94 involving microbial activities, is considered as a less costly, highly effective, and
95 environmental-friendly approach in comparison to the other remediation procedures
96 (Ali et al., 2020; Hazaimh and Ahmed, 2021). It has been estimated that cleaning costs
97 can be reduced by about 30 to 50% by bioremediation approaches (Lü et al., 2011). In
98 addition, the application of chemical and physical treatments after oil spills are difficult
99 to implement under certain situations, such as large areas covered by spilled oil, and bad
100 weather conditions (Kumar and Yadav, 2019; Ventikos et al., 2004). Therefore,
101 bioremediation is then the most suitable strategy (Alvernia et al., 2021).

102 The use of dispersants is a strategy implemented to stimulate the microbial degradation
103 by increasing hydrocarbon solubility and thus bioavailability (George-Ares et al., 2000;
104 Yamada et al., 2003), but unfortunately increasing also toxicity (Baca et al., 2006).
105 Indeed, several studies have shown that dispersant have adverse toxic effects on marine
106 organisms (Lee et al., 2013; Cohen and Nugegoda, 2000; Mitchell and Holdway, 2000),
107 including benthic organisms (Cuny et al., 2015) and microbial communities (Brakstad et
108 al., 2015; McFarlin et al., 2018; Garneau et al., 2016; Kleindienst et al., 2015).
109 However, only few studies have investigated the effect of dispersant on PAHs microbial
110 degradation at low temperature (Pan et al., 2017). In cold environments, the low
111 temperature and the seasonal variation of nutrient availability limit PAHs
112 biodegradation by microorganisms (Vergeynst et al., 2019; Brakstad et al., 2008).

113 Therefore, further knowledge on microbial hydrocarbon degradation in cold conditions
114 is required in order to develop adapted microbial management strategies for fighting oil
115 spills. In addition, industrialization and urbanization introduce PAHs into coastal areas
116 (Duran et al., 2015), resulting in chronic PAHs pollution threatening the human health
117 (Duran and Cravo-Laureau, 2016; Lv et al., 2020). Such chronic PAHs pollution allows
118 the adaptation of microbial communities exhibiting PAHs degradation capabilities
119 (Bordenave et al., 2004; Louati et al., 2013; Stauffert et al., 2014), which constitute the
120 legacy of the microbial communities.

121 We hypothesize that microbial communities have their own PAHs degradation capacity
122 at low temperature, which probably depend whether or not they have been adapted to
123 the presence of PAHs and to the presence of dispersant. In order to verify the
124 hypothesis, we investigated the effect of WAF, prepared in presence of dispersant or
125 not, on water from two cold environments, one oil-polluted and the other pristine, in
126 microcosm experiment during 21 days, focusing on PAHs because they are the most
127 toxic compounds in WAF. The study focused on three main objectives: i) determine the
128 PAHs degradation capacities of the bacterial communities assessed by GC-MS analysis,
129 and how the addition of dispersant affects PAHs degradation capabilities ii) evaluate the
130 resulting toxic effect of the introduction of WAF and WAF+dispersant by toxicity
131 assays on the copepod *Acartia tonsa*, and iii) characterize microbial community changes
132 in the water column identifying microbial taxa linked with PAHs degradation by 16S
133 rRNA gene Illumina sequencing. The comparison of microbial communities' behavior
134 from oil-contaminated site to that from pristine site allowed the characterization of the
135 microbial legacy effect, which was more important than the presence of dispersant in
136 shaping the organization of microbial community. Our study provides useful

137 information for the development of strategies for the management of microbial
138 communities in response to an oil-spill.

139 **2. Materials and methods**

140 *2.1 Sampling sites*

141 Sediment and water samples were collected from two sites, a pristine and an oil-
142 contaminated site. The oil-contaminated site was located in a harbor in the middle of
143 Tromso, Norway (69°39'08.7"N 18°57'50.2"E) characterized with busy boat activity.
144 Whereas the pristine site located in a sound in Tisnes, Norway (69°36'24.4"N
145 18°49'56.3"E) with little anthropogenic influences nearby. Samples were collected from
146 the coast at a shallow depth, stored in polypropylene containers at 4 °C. The containers
147 were then shipped to Pau, France at constant temperature of 4 °C.

148 *2.2 Water accommodated fractions preparation*

149 Two types of water accommodated fractions were prepared for the microcosm
150 experiments: 1) crude oil WAF, and 2) dispersant added to WAF (WAF+dispersant).
151 The soluble fractions were made from an arctic crude oil provided by Norwegian
152 university of science and technology (NTNU). Naphthenic North Atlantic (NNA) is a
153 naphthenic based light crude oil with a low aromatic hydrocarbons content, low wax
154 content, low viscosity index (299 cP at 2 °C) and density of (0.900 g mL⁻¹) (Hafez et al.,
155 2021). The crude oil is characterized by high amount of polycyclic aromatic
156 hydrocarbons (Katsumiti et al., 2019). Preparation of WAF was similar to the protocol
157 described by Singer et al., (2000) but with slight modifications. Briefly, in 8 L glass
158 bottles, crude oil was added to artificial seawater of salinity 30 ppt at a ratio of 5 g of oil
159 per 1 L water. The oil water mixture was stirred for 42 h at 10 °C and under dark

160 conditions using a magnetic stirrer without creating vortex. For WAF+dispersant, the
161 dispersant Finasol OSR 52 (Total energies company, France) was added first to the oil
162 at a ratio of 1 g of dispersant per 10 g of crude oil, gently mixed, then the
163 WAF+dispersant was produced following the same procedure than for WAF.

164 ***2.3 Microcosm set up***

165 For each microcosm, 200 g of sediment, 500 mL of collected water and 500 mL of
166 WAF were added to a 2 L sterile glass flasks covered with filter rubber mesh tap to
167 allow gas exchange. Control, WAF and WAF+dispersant microcosms were prepared in
168 triplicate with water and sediments collected from either the pristine or the
169 contaminated sites. Thus a total of (3 control + 3 WAF + 3 WAF+dispersant) x 2 sites =
170 18 microcosms were prepared. All microcosms were incubated at 4 °C for 3 days before
171 the addition of either WAF or WAF+dispersant to allow acclimatization. After addition
172 of WAFs, the microcosms were incubated for 21 days at 4 °C in the dark to prevent
173 photosynthesis and photoreactions. Every day, each microcosm was gently mixed to
174 allow the resuspension of particles and oxygenation. Water samples were collected after
175 0, 7, 14, and 21 days for microbial and PAHs analyses. In addition, samples at day 0
176 and day 21 were collected for copepods toxicity assays. The samples for microbial
177 analysis were collected by filtration (100 mL water) on 0.22 µm membrane under
178 vacuum. The filters were stored at -80 °C until analysis.

179 ***2.4 Chemical analysis of the PAHs content***

180 The PAHs composition and concentrations of WAF and WAF+dispersant were
181 determined by GC/MS analysis. A mix standard solution of 16 PAHs was used
182 [Norwegian Standard (NS 9815: S-4008-100-T): phenanthrene (Phe), anthracene (An),
183 fluoranthene (Fluo), pyrene (Pyr), 11 H-benzo[a]fluorene (11H-B[a]F), 11 H-

184 benzo[b]fluorene (11H-B[b]F), benz[a]anthracene (B[a]A), chrysene (Chry),
185 benzo[b]fluoranthene (B[b]F), benzo[k]fluoranthene (B[k]F), benzo[a]pyrene (B[a]P),
186 benzo[e]pyrene (B[e]P), indeno[1,2,3-cd]pyrene (Ind), dibenz[a,h]anthracene (D[a,h]A),
187 benzo[g,h,i]perylene (B[g,h,i]P) and dibenzo[a,e]pyrene (D[a,e]P). Stir bars (poly
188 dimethylsiloxane polymer coated twisters, supplied by Gerstel GmbH, Germany) with
189 10 mm length and 0.5 mm film thick were used. The twisters were introduced in
190 aqueous samples (35 mL) and stirred during 315 minutes. Then, twisters were cleaned
191 with Milli-Q water, in order to eliminate seawater, and dried with paper tissue. Twisters
192 were desorbed using a commercial thermal desorption TDS-2 unit (Gerstel GmbH)
193 connected to a CIS-4 injector (Gerstel GmbH) with the following conditions: desorption
194 time (10 min), desorption temperature (300 °C), desorption flow (23 mL min⁻¹), cryo-
195 focusing temperature (-50 °C) and vent pressure (7 psi). This desorption unit was
196 coupled in an Agilent 6890 gas chromatograph also coupled to an Agilent 5975 mass
197 spectrometer system (Agilent Technologies, USA). The Mass spectrometer (MS) was
198 operated in selected ion monitoring (SIM) for quantification of target compounds. An
199 Agilent DB-5MS+DG column with the following dimensions 30 m x 0.25 mm and 0.25
200 µm was used. Analytes were separated using the following conditions: carrier gas,
201 helium (1.3 mL min⁻¹); transfer line, ion source and quadrupole analyser temperatures
202 were maintained at 300, 230 and 150 °C, respectively. The following temperature
203 program was used for target PAHs and lineal hydrocarbons: 170 °C for 5 min; ramp at
204 30 °C min⁻¹ to 260 °C; ramp at 8 °C min⁻¹ to 300 °C and hold 15 min.

205 ***2.5 DNA extraction and 16S rRNA gene PCR amplification***

206 The membrane filters were cut into small pieces then homogenized using metal beads.
207 The total DNA was extracted using power soil total DNA isolation kit (Qiagen,
208 Germany) following the extraction protocol recommended by manufacturer. DNA

209 quantity was determined using Quant it dsDNA HS kit (Theremofisher scientific, USA).
210 The 16S rRNA gene was amplified using the universal prokaryotic primers U515-532-
211 GTGYCAGCMGCCGCGGTA and U909-928-CCCCGYCAATTCMTTTRAGT
212 targeting the V4-V5 region of the 16S rRNA gene (Wang and Qian 2009). The reaction
213 medium (25 μ L) consisted of 12.5 μ L AmpliTaq Gold 360 Master Mix (Invitrogen,
214 USA), 1 μ L of forward and reverse primer at concentration of (20 μ M), 5.5 μ L water
215 and 5 μ L DNA template (10 ng). The polymerase chain reaction was performed using
216 thermocycler (Applied biosystems, USA) with the following cycling conditions: 10 min
217 at 95 $^{\circ}$ C followed by 30 cycles of: 30 s at 95 $^{\circ}$ C, 30 s at 60 $^{\circ}$ C and 40 s at 72 $^{\circ}$ C,
218 followed by a final step of 10 min at 72 $^{\circ}$ C. Successful amplification of the PCR was
219 checked on 2% agarose gel electrophoresis, stained with ethidium bromide. PCRs were
220 performed in triplicates and then pooled for further sequencing.

221 ***2.6 Amplicon sequencing and bioinformatics analysis***

222 The 16S rRNA gene barcoding was performed using NGS Illumina MiSeq at the
223 GenoToul platform (Toulouse, France). Demultiplexed sequences obtained were treated
224 with Qiime2 software (Quantitative insights into microbial ecology; Caporaso et al.,
225 2010) according to Quince et al. (2009). Briefly, sequences were trimmed and then
226 denoised using DADA2 pipeline integrated in the Qiime2 software to remove chimeric
227 sequences. The total number of sequences for each sample obtained after denoising is
228 presented in Supplementary Materials (Table S1). Denoised sequences were assigned
229 with taxonomic affiliation using RNA gene reference database SILVA version 132
230 (Quast et al., 2013). In addition, singletons were filtered. Finally, sequences were
231 rarefied according to the lowest number of total sequences observed in samples.
232 Rarefaction curves are presented in Supplementary Materials (Figure S1). An
233 abundance table with the total available amplicon sequence variant (ASVs) was

234 produced removing those with less than 250 counts (representing about 1% of total
235 counts per sample). The complete dataset was deposited in the NCBI Sequence Read
236 Archive (SRA) database (SUB11018949) under the Bioproject (PRJNA802112).

237 ***2.7 Copepod mortality assays and gene transcription assays***

238 Total of 30 adults of the copepod *Acartia tonsa* were selected under a dissection
239 microscope. Adult copepods were placed in 75 mL water samples collected from the
240 microcosms for 96 hours. Copepods were not fed during exposure and no gender
241 preference was considered when collecting the copepods for the experiments. Everyday,
242 copepod mortality was checked and dead copepods were removed. A copepod was
243 considered dead when is not moving after a gentle stimulation with a pipette. After 96
244 hours, the surviving copepods were collected and placed in cryotubes filled with RNA
245 later (Invitrogen, USA). The pooled copepods were homogenized using beads tissue
246 homogenizer (Precellys 24 tissue homogenizer, France) with the following program: 45
247 secs at 6500 rpm, then 2 min on ice, then another 6500 rpm. RNA was extracted using
248 RNA easy minikit (Qiagen, Germany) following the manufacture protocol. The
249 extracted RNA quantity and quality were observed using 2100 bioanalyzer (Agilent
250 technologies, USA). RNA from the extracted samples showed to have minimum Rin
251 score of 7, indicating intact extracted RNA. In addition, RNA purity was determined by
252 measuring samples' absorbance ratios of 260/280 nm using a spectrophotometer
253 (Epoch, Biotek, USA). All samples had acceptable ratios between 1.8 and 2.3. cDNA
254 was synthesized from purified RNA using the AffinityScript Multiple Temperature
255 cDNA synthesis kit (Agilent Technologies, USA) with random primers and following
256 the manufacturer's protocol. In addition, cDNA concentration was quantified using
257 Quant-it Oil green stain (Invitrogen, USA).

258 **2.8 Primer design and Quantitative Real-time PCR (qRT-PCR)**

259 In order to evaluate the effects of PAHs exposure in *Acartia tonsa*, the gene encoding
260 the multifunctional enzyme glutathione S-transferase (*gst*) has been selected as a
261 biomarker because its expression has been shown to be responsive to PAHs exposure in
262 *Acartia tonsa* (Zhou et al., 2020). The primers for the amplification of *gst* genes were
263 designed from conserved regions observed by aligning *Acartia tonsa* genome
264 (PRJEB20069) with *Tortanus forcipatus* (KT755427.1) and *Acartia pacifica*
265 (KT754520.1) using Clustal omega (Mадiera et al., 2019). Hybridization Taqman
266 Probes were designed according to the sequenced amplicons obtained from the *gst*
267 primers. For the quantitative PCR, the reaction mixture (25 μ L) consisted of 12.5 μ L
268 Taqman gene expression master mix (Applied Biosystems, USA), 0.3 μ L (800 nM)
269 forward and reverse primers, 0.25 μ L hybridization probe, 8.2 μ L water and 3 μ L
270 cDNA template, undiluted. Quantitative PCR was performed in 384 optical well plates
271 (Applied Biosystems, USA) using ViiA 7 Real-Time PCR system (Applied Biosystems,
272 USA). The amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed
273 by 40 cycles of 95 °C for 15 s and then 1 min. of annealing at 60 °C.

274 **2.9 Statistical analysis**

275 Analysis of the bacterial community dynamics such as alpha diversity indices, bacterial
276 abundances at the phylum level, were conducted on R software using RAM package
277 (Chen et al., 2018). Correspondence analysis (CA), canonical correspondence analysis
278 (CCA), permutational multivariate analysis of variance (PERMANOVA), and similarity
279 percentages analysis (SIMPER) were performed using PAST statistical software version
280 4.03 (Hammer et al., 2001).

281 Linear discriminant analysis effect size (LEfSe, Segata et al., 2011) was performed with
282 galaxy (<http://huttenhower.sph.harvard.edu/LEfSe/>). Briefly, non-parametric Kluskal
283 Wallis was used to detect taxa with significant abundances ($p < 0.05$). Biological
284 consistency was conducted using pair wise Wilcoxon test. Finally, LDA with threshold
285 score of 4.0 and 1000 bootstraps was applied.

286 Network analyses of bacterial communities overtime were constructed using Molecular
287 Ecological Network Analysis (MENA) pipeline (<http://129.15.40.240/mena/>; Deng et
288 al., 2012). Briefly, a pair wise similarity matrix was constructed from log transformed
289 ASV abundance table using spearman correlation. A similarity threshold cut off value
290 was automatically generated for the similarity matrix. Network topological features,
291 such as global network properties, individual nodes' centrality, module separation and
292 modularity, were performed. Mantel test was performed using Pearson correlation to
293 investigate correlation between Bacterial taxa connectivity and the concentrations of
294 total PAHs. Finally, the network modules were generated with “greedy modularity
295 optimization mode” and the contaminated and pristine networks were visualized using
296 *cytoscape* (Shannon et al., 2003). Finally, *Acartia tonsa* gene transcript levels were
297 normalized with the amount of input cDNA using formula adapted from the $\Delta\Delta CT$
298 normalization method (Rojo-Bartolome et al., 2016).

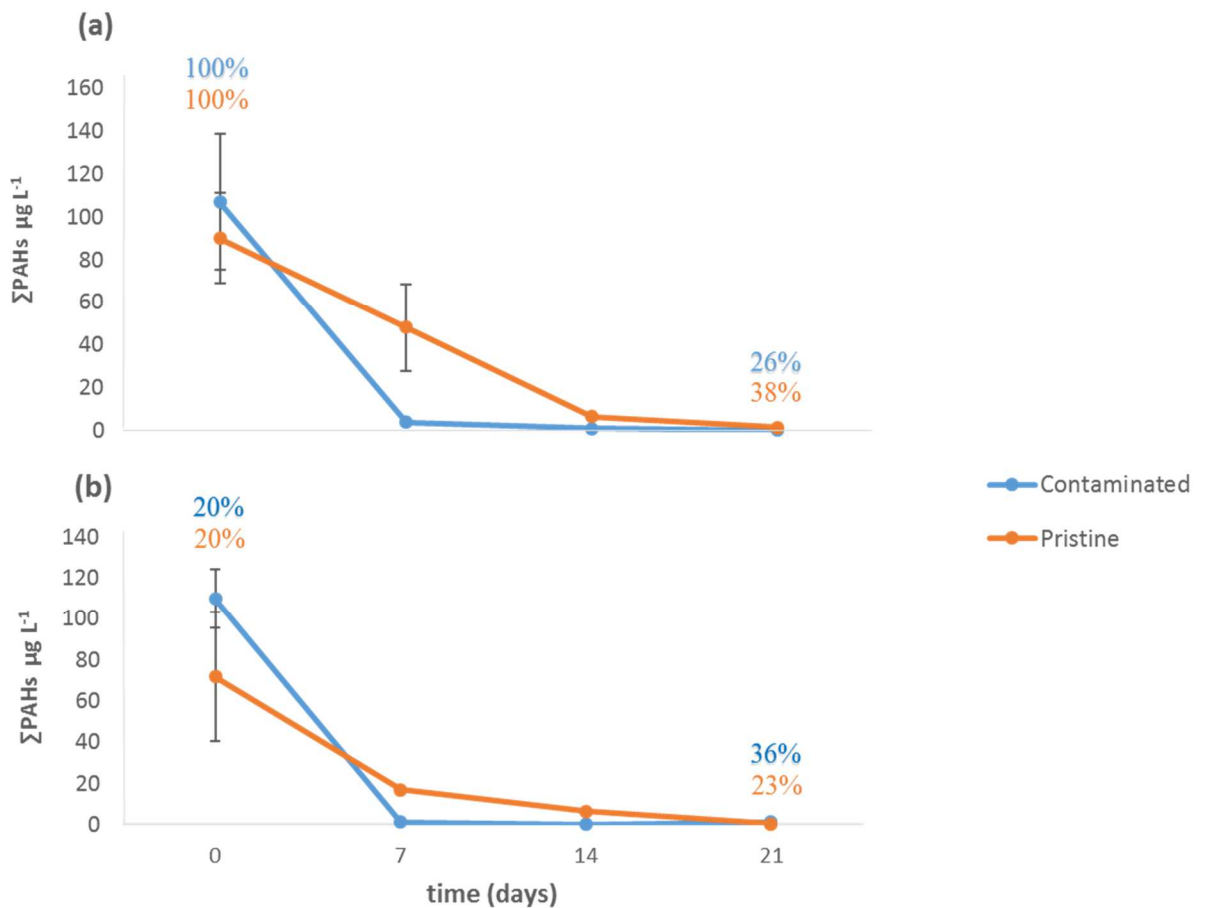
299 **3. Results and Discussion**

300 ***3.1 PAHs degradation and toxicity***

301 The initial water accommodated fractions (WAF) contained similar total PAHs
302 concentration whether they were prepared with or without dispersant, and irrespective
303 of the water origin. They exhibited similar PAHs composition (unpaired t test $p > 0.05$)
304 containing mainly naphthalene and derivatives (acinaaphthalene and acenaphthlene, > 50

305 $\mu\text{g L}^{-1}$), while the content of high molecular weight PAHs (> 3 rings) was below $1 \mu\text{g L}^{-1}$. For both contaminated and pristine control microcosms the mortality at time 0 and
306 1 . For both contaminated and pristine control microcosms the mortality at time 0 and
307 time 21 days was below 15 %. However, the WAF+dispersant showed higher toxicity
308 with 100% copepod (*Acartia tonsa*) mortality whereas the WAF exhibited 20% copepod
309 mortality (Figure 1), showing the toxic effect of the WAF+dispersant. It has been shown
310 that WAF+dispersant has high toxicity toward marine organisms compared to WAF
311 (Lee et al., 2013), especially toward *Acartia tonsa* (Rattes de Almeida et al., 2014). In
312 addition, dispersed oil droplets have been shown to be more toxic than WAF due to the
313 bioavailability of low soluble and high molecular weight PAHs (Berrojalbiz et al.,
314 2009).

315 During microcosm incubation, the PAHs content decreased drastically, faster in
316 microcosms prepared with water from the contaminated site, with removal of almost all
317 total PAHs (96%) within 7 days while with pristine site water the complete PAHs
318 removal (98%) was obtained after 14 days (Figure 1). Such discrepancy on PAHs
319 content decrease according to the origin of water suggests that biodegradation
320 participate to PAHs removal, consistent with the fact that abiotic removal is limited at
321 low temperature as the volatility of low molecular weight PAHs decreases increasing
322 their viscosity (Atlas, 1975). Such observation is further supported by studies showing
323 hydrocarbon biodegradation at low temperature (Gomes et al., 2022; Ribicic et al.,
324 2018). Noteworthy, the PAHs degradation rate was not increased by the presence of
325 dispersant (Figure 1a), probably due to the bioavailability of solubilized PAHs in WAF
326 (Liu and Kujawinski, 2015; Berrojalbiz et al., 2009). Accordingly with PAHs removal,
327 the toxicity at the end of the experiment (21 days), assessed by *Acartia tonsa* mortality,
328 was similar in all conditions (unpaired t test $p > 0.05$), close to that observed at the
329 beginning of the experiment in WAF (Figure 1b) without dispersant.



330

331 **Figure 1:** PAHs removal in a) WAF+dispersant and b) WAF microcosms. Numbers above points denote
 332 percentage of mortality of *Acartia tonsa* after 96 hours exposure at microcosm water samples obtained at
 333 day 0 and day 21. Colors of numbers represent the origin of water. N=3 Error bars = ±1 Standard error

334

335 **3.2 Microbial communities characterization and composition**

336 After trimming, the retained 296,386 sequences were rarefied to 3,224 sequences per
 337 samples, corresponding to the lowest sequences number in a sample. The retained
 338 sequences were then dispatched within 4,024 ASVs (Supplementary Materials, Table
 339 S1). The rarefaction curves for the different conditions indicated that the sequencing
 340 depth was sufficient to reveal the microbial diversity (Supplementary Materials, Figure
 341 S1). Nevertheless, despite several attempts, it was not possible to characterize the
 342 microbial communities from contaminated water at day 14 under the WAF+dispersant
 343 condition, and at days 14 and 21 under the WAF condition, probably because the

344 microbial abundance was low. We hypothesize that the depletion of hydrocarbon
345 (Figure 1) in water caused the collapse of microbial communities inhabiting the
346 contaminated water at day 14 in WAF and WAF+dispersant. Hydrocarbons available in
347 WAF and in WAF+dispersant probably represent the main carbon source for the growth
348 of oil degraders (Xu et al., 2018). However, in the case of WAF+dispersant, the added
349 hydrocarbons and dispersant represent an easily degradable carbon source for
350 prokaryotes rapidly consumed (after 7 days, Figure 1) corresponding to a priming effect
351 usually described after addition of fresh bioavailable carbon source (Pascault et al.,
352 2013). Then after adaptation (around 21 days, Figure 1), microbial community use less
353 bioavailable carbon source present on the water.

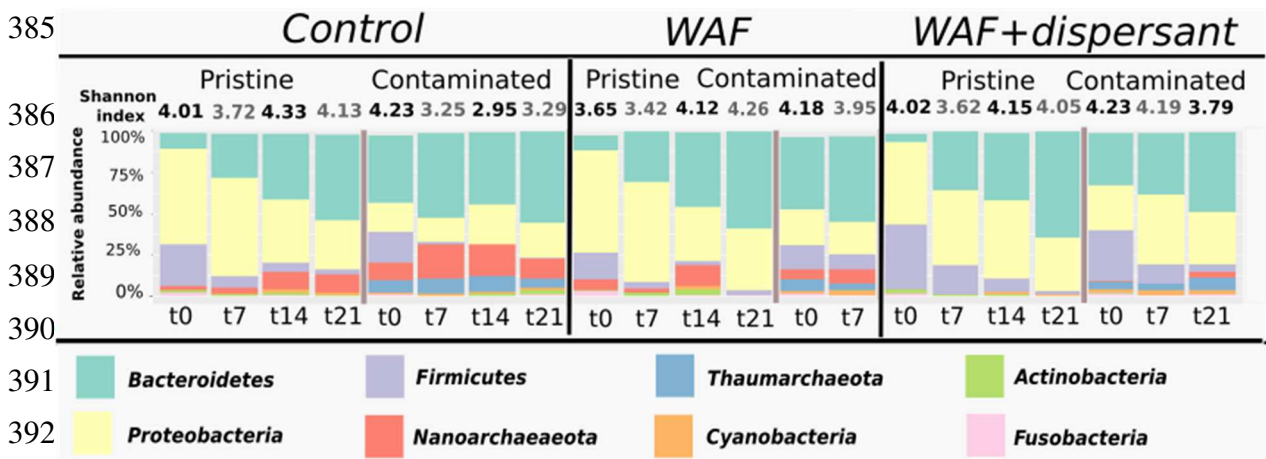
354 The Shannon index was between 2.95 and 4.26, increasing in pristine water during the
355 experiment while decreasing in polluted water (Figure 2, and Supplementary Materials,
356 Table S1). However, according to one way ANOVA, no significant differences were
357 observed among Shannon indices during the experiment in all conditions.

358 The microbial communities were dominated by *Proteobacteria* (65% - 25%),
359 *Bacteroidetes* (60% - 10%), *Firmicutes* (40% - 5%) and *Nanoarchaeaota* (20% - 1%)
360 phyla, while *Cyanobacteria*, *Actinobacteria*, *Fusobacteria*, *Thaumarchaeota*, phyla
361 were present at lower abundance, less than 10% (Figure 2). Noteworthy, changes in
362 microbial community composition were observable during the incubation period
363 characterized by increasing abundance of *Bacteroidetes* with concomitant decreasing of
364 *Proteobacteria* and *Firmicutes* abundances irrespective of the condition and the origin
365 of water (Figure 2). However, it can be noticed that at the beginning of the experiment,
366 the *Bacteroidetes* abundance was lower in the pristine water but reached similar
367 abundance to that observed in contaminated water at the end of the experiment. Such
368 observation suggested that members of the phyla *Proteobacteria*, *Bacteroidetes* and

369 *Firmicutes* play crucial roles in PAHs degradation within the microbial community.
 370 These phyla have been described to be dominant in hydrocarbon-enriched environments
 371 (Kwon et al., 2019). For example, during the Deepwater Horizon oil spill accident,
 372 members of *Bacteroidetes* became the major phyla in the water column (Redmond and
 373 Valentine, 2012), contributing to 3% of the total hydrocarbon degradation activity
 374 (Kwon et al., 2019). Crisafi et al. (2016) demonstrated that at 4 °C for 90 days, oil
 375 enriched samples favored several hydrocarbon degrader strains belonging to
 376 *Gammaproteobacteria* and *Alphaproteobacteria*, being capable to degrade up to 80% of
 377 the available hydrocarbons. In addition, *Firmicutes* showed to be important components
 378 in the bacterial communities composition from samples from western Siberia oil fields
 379 (Frank et al., 2016). In fact, several members of the phyla *Firmicutes* and
 380 *Proteobacteria* are considered to be opportunistic heterotroph (Nguyen-Lopez Lozano
 381 et al., 2013) that probably explains the increase abundance of such phyla after the
 382 addition of WAF and WAF+dispersant.

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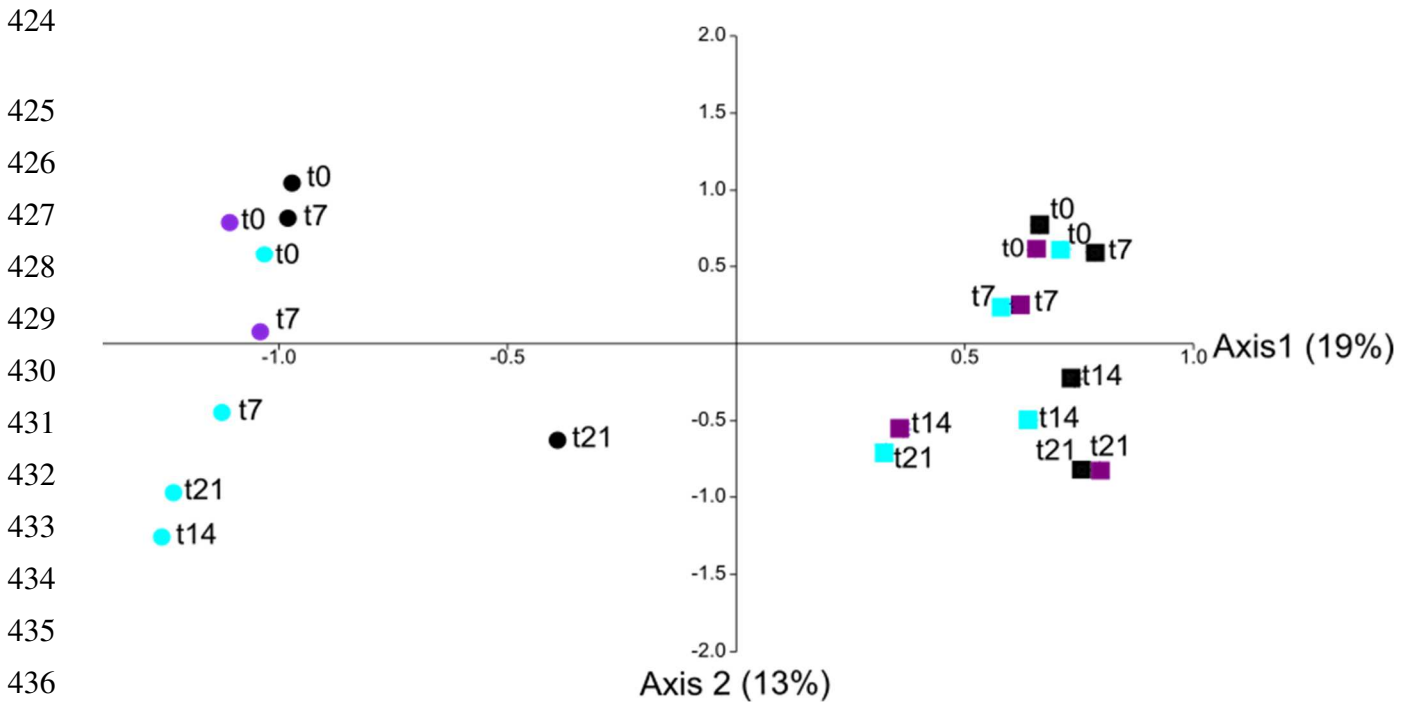


394 **Figure 2:** Prokaryotic composition (relative abundance) at the phylum level in pristine and contaminated
 395 waters for the three treatments (control, WAF, WAF+dispersant) during the incubation. Number above
 396 each bar corresponds to the Shannon index.

397 **3.3 Comparison of microbial communities**

398 The correspondence analysis (CA) separated the microbial communities of pristine
399 water apart from those of contaminated water into two main clusters along the axis 1
400 that explained 19% of the data distribution (Figure 3), indicating that the microbial
401 community structures were different according to the site. Noteworthy, the microbial
402 communities of both sites were separated, according to time within each main cluster,
403 along the axis 2 explaining 13% of the data distribution despite the missing samples of
404 polluted water at days 14 and 21 (Figure 3). Such observation further confirmed the
405 modifications of microbial communities during the experiment, which corresponds to
406 the ecological succession relating the microbial plasticity for the adaptation to the
407 environmental conditions (Bordenave et al., 2004). The ecological succession has been
408 reported during PAHs-bioremediation processes (Cravo-Laureau et al., 2011) as well as
409 in environments with the presence of various pollutants, including metals (Giloteaux et
410 al., 2013), nanoparticles (Concha-Guerrero et al., 2014), pesticides (Vercaene- Eairmal
411 et al., 2010), and even during niche colonization in volcano (Medrano-Santillana et al.,
412 2017). Additionally, similarity percentage analysis (SIMPER) revealed differences
413 between the microbial community composition according to the conditions (control,
414 WAF, and WAF+dispersant) with dissimilarity between 38% and 54% at the beginning
415 of the experiment, and above 55% for the rest of the experiment. The dissimilarity was
416 higher between control and WAF+dispersant (60% < SIMPER > 80%) than between
417 control and WAF (32% < SIMPER > 62%), revealing the effect of both WAF and
418 WAF+dispersant on the microbial communities. These observations were supported by
419 permutation analysis of variance (PERMANOVA) indicating that the origin of water
420 was the main factor explaining data distribution followed by time and then the
421 conditions (Supplementary Materials, Table S2). The initial composition of the

422 microbial communities plays important roles in shaping the responses toward oil
423 contamination as observed (Ortmann et al., 2015; Al Jawasim et al., 2015).



437

438 **Figure 3:** Correspondence analysis (CA) comparing microbial communities from pristine (square) and
439 oil-polluted waters (dots) in the three treatments (controls, WAF, WAF+dispersant), at different
440 incubation time. Light blue, Control; Purple, WAF; Black, WAF+dispersant. Numbers indicate the
441 incubation time (days).

442

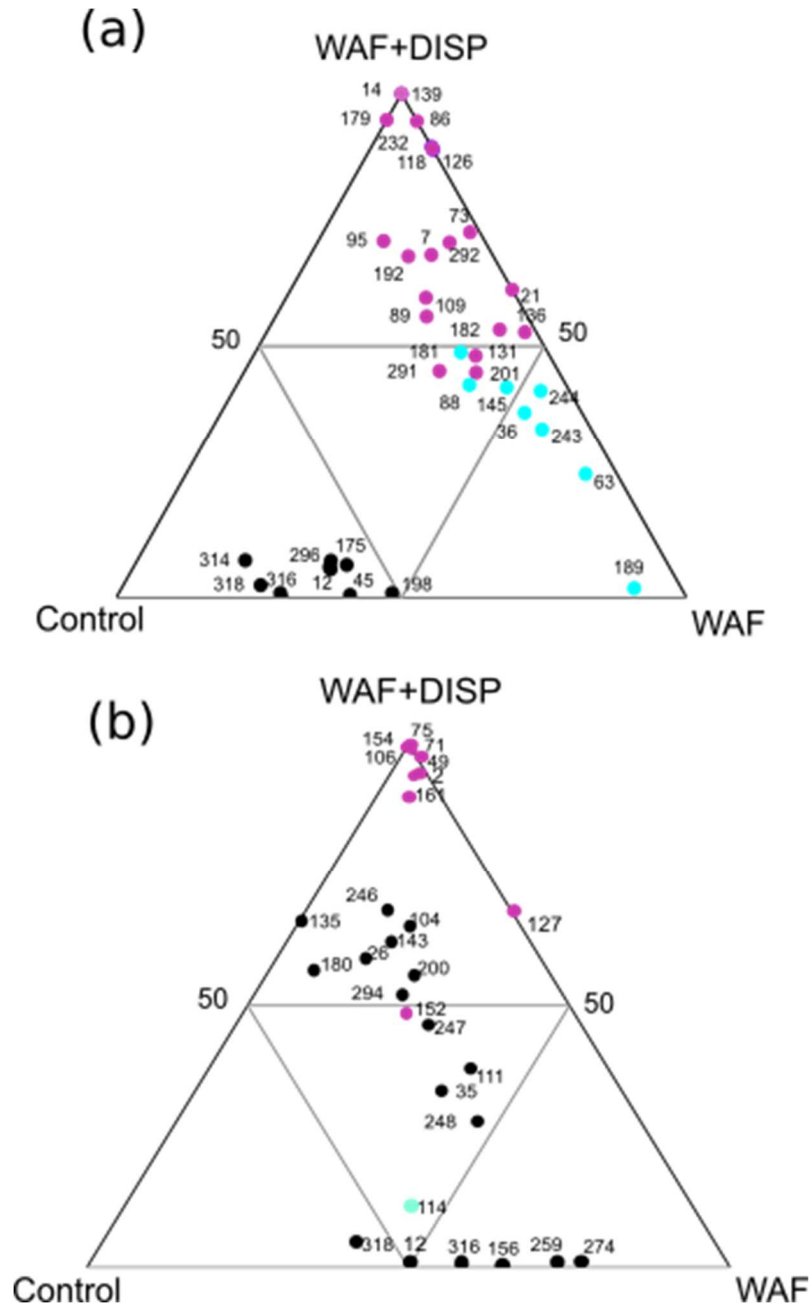
443 Linear discriminant analysis (LDA) effect size (LEfSe) revealed taxa significantly more
444 abundant according to the condition for both pristine and contaminated waters (Figure 4
445 and Supplementary Material, Figure S2) explaining the observed differences between
446 the microbial communities. Taxa found significantly more abundant in a condition have
447 been proposed as bioindicator (Segata et al., 2011). More bioindicators for
448 contamination were observed for contaminated water than for pristine water while, in
449 contrast, more bioindicators were detected for the control in the pristine water (Figure
450 4). Such observation indicates that due to legacy effects, the contaminated water
451 microbial community was adapted to the presence of hydrocarbon. Such legacy effect

452 has been described in temperate aquatic environments (Bourhane et al., 2022; Ben
453 Salem et al., 2019; Bordenave et al., 2008; Duran et al., 2015; Misson et al., 2016) but
454 not yet in cold environments.

455 The abundance distribution of the identified bioindicators by LEfSe according to the
456 treatment (control, WAF, and WAF+dispersant) further revealed the specificity of taxa
457 to the treatment (Figure 4a). For the pristine water, seven bioindicators were exclusive
458 for the WAF+dispersant (Figure 4a) while no exclusive ASVs were found for the other
459 conditions. It is likely that most of taxa associated to the bioindicators for pristine water
460 were able to cope with the presence of contaminant as they are equally distributed
461 within control and contaminated treatments, but only the WAF+dispersant treatment
462 provides favorable conditions for the development of specific microorganisms able to
463 use hydrocarbon and/or dispersant as carbon source. The seven bioindicators exclusive
464 for the WAF+dispersant include ASVs belonging to *Fusibacter* (ASV 75, ASV 2, and
465 ASV 154), *Flavobacterium* (ASV 49), *Psychrobacter* (ASV 71), *Psychromonas* (ASV
466 106), and *Shewanella* (ASV 161).

467 For the contaminated water, the bioindicator ASVs were specifically distributed
468 according to the conditions (Figure 4b), showing abundance above 50% in their
469 respective condition. The ASVs specifically identified for the WAF+dispersant
470 treatment were members of the *Proteobacteria* phylum, belonging to *Colwellia* (ASV
471 14, ASV 232, and ASV 7), *Marinomonas* (ASV 86, ASV 126, and ASV 73), *Moritella*
472 (ASV 95), *Sulfitobacter* (ASV 139), *Paraperlucidibaca* (ASV 118, ASV 179, and ASV
473 292) genera. The exclusive bioindicators for the WAF treatment included ASVs
474 belonging to *Bacteroidales* (ASV 189), *Cyanobacteria* (ASV 243), *Arcobacter* (ASV
475 63), *Sulfurovum* (ASV 244), *Moritella* (ASV 36), *Marinifilium* (ASV 145), and
476 *Fusibacter* (ASV 181) genera. Interestingly, some bioindicator ASVs were equally

477 distributed between WAF and WAF+dispersant treatments suggesting that they respond
 478 to the presence of hydrocarbons. These bioindicator ASVs belong to *Caedibacteraceae*
 479 (ASV 21) family, *Moritella* (ASV 109), *Olispera*, (ASV 89), *Fusibacter* (ASV 182,
 480 ASV 131, and ASV 291), and *Marinifilium* (ASV 201) genera.



481

482 **Figure 4:** Ternary plots showing the abundance of bioindicator taxa identified by LEfSe in microcosms
 483 prepared with water from (a) pristine and (b) contaminated sites. Numbers refer to ASV taxa.
 484 Bioindicator taxa identified for WAF+dispersant treatments are purple dots, for WAF are light blue dots,
 485 and for Control are black dots.

486

487 The most striking observation was that among the bioindicators taxa, *Colwellia* was
488 exclusively found in WAF+dispersant treatment for contaminated water. Such
489 observation was in agreement with previous studies showing that *Colwellia* was
490 detected in oil containing cold seawater (Garneau et al., 2016), dominating bacterial
491 communities after the Deepwater Horizon oil spill, and presenting the capacity to
492 biodegrade aromatic hydrocarbons (Mason et al., 2014). Additionally, *Colwellia* has
493 been shown to benefit from the presence of dispersant (Kliendiest et al., 2015).

494 Noteworthy, several ASVs belonging to *Fusibacter*, genus characterized by the ability
495 to degrade low molecular weight PAHs (Folwell et al., 2016), were identified as
496 contamination bioindicator (WAF and WAF+dispersant) in contaminated and pristine
497 waters (Figure 4b). Interestingly, it was demonstrated that *Fusibacter* dominated the
498 bacterial community after dispersant addition in oiled sediments, probably by using it as
499 carbon source (Calderon et al., 2018). Most of the ASVs revealed as contamination
500 bioindicators were related to taxa known as hydrocarbon degraders such as *Moritella*
501 (Bagi et al., 2014), *Paraperlucidibaca* (Brakstad et al., 2008). Similarly, the
502 psychrophilic *Shewanella* have shown pyrene degradation potential (Rathour et al.,
503 2018), some strains have even been isolated from Arctic Sea PAHs-rich sediment (Dong
504 et al., 2015). In addition, it is important to note that two ASVs belonging to the
505 Candidatus *Woesearchaeota* archaeal phylum (ASV 314 and ASV 318) were identified
506 as exclusive bioindicators for control contaminated water, suggesting that they were
507 sensitive to the addition of WAF and WAF+dispersant.

508 ***3.4 The relation between prokaryotic taxa and PAHs toxicity***

509 Canonical correspondence analysis (CCA) assisted in visualizing how microbial taxa
510 interacted with the PAHs toxicity through *Acartia tonsa* mortality and the
511 downregulation of *gst* transcription levels. For microcosms prepared with pristine site

512 water, the first two axes of the CCA explained 99% of the observed variance (axis 1:
513 72% and axis 2: 27%; Figure 5a). Axis 1 was positively explained by PAHs
514 concentration and *Acartia tonsa* mortality but negatively explained by the
515 downregulation levels of *gst* gene. Such observation indicated the correlation between
516 PAHs concentrations and toxicity. Microbial communities in microcosm prepared with
517 WAF+dispersant (at t0) were strongly correlated with the mortality and the
518 concentration of PAHs (Figure 5a). These microbial communities were dominated by
519 ASVs belonging mainly to the *Firmicutes Fusibacter* (ASV 58, ASV 97, ASV 42, ASV
520 46, ASV 96, ASV 93, ASV 91, ASV 22, ASV 28, ASV 19, and ASV 81),
521 *Proteobacteria, Burkholderiaceae* family (ASV 50, ASV 26, and ASV 38), and
522 *Actinobacteria, Microbacteriaceae* family (ASV 7). Stress levels in copepods,
523 measured by the transcription of *gst* gene, were positively correlated with
524 WAF+dispersant samples collected after 21 days, in which ASVs affiliated to
525 *Flavobacterium* (ASV 74), *Salinirepens* (ASV 4), and *Psychrobacter* (ASV 20) were
526 positively correlated with the *gst* gene expression levels (Figure 5a).

527 For microcosms prepared with water from contaminated site (Figure 5b), the CCA
528 explained 98% of the variance (axis 1: 87%, axis 2: 11%). The PAHs concentrations
529 and *Acartia tonsa* mortality were positively explained by the first axis while *gst* gene
530 downregulation levels were negatively explained by the second axis (Figure 5b). In
531 general, the communities of WAF and WAF+dispersant microcosms at t0 were
532 correlated with PAHs concentrations and with copepod mortality. However, the
533 communities at microcosm treatments at time 21 correlated with the *gst* gene down
534 transcription patterns.

535 The abundance of several ASVs showed strong positive correlation with copepod
536 mortality such as *Fusibacter* (ASV 73, ASV 58, ASV 93, ASV 67, ASV 26, ASV 39,

537 ASV 11, and ASV 74), an ASV of *Moritella* (ASV 33), and an ASV from the family
538 *Rhodobacteraceae* (ASV 42). In addition, the abundance of some ASVs was correlated
539 with the concentration of PAHs. These ASVs were affiliated to *Marinifilum* (ASV 83,
540 ASV 30, ASV 2, ASV 68, ASV 48, ASV 89, and ASV 9), *Rhodobacteraceae* family
541 (ASV 64), and *Colwellia* (ASV 88). In both pristine and contaminated microbial
542 communities, *Fusibacter*, showed to be an important taxa capable to survive the toxicity
543 of dispersant. Currently, *Fusibacter* is yet to be directly linked to PAHs biodegradation
544 (Kappel et al., 2014). However, we hypothesize that strains belonging to *Fusibacter*,
545 among the other correlated ASVs identified, played an important role in reducing the
546 toxicity of dispersant. The mineralization of dispersant (e.g. Corexit EC9500A) by a
547 consortium of marine microbes has been shown to be faster than that of crude oil at low
548 temperature (Lindstrom and Braddock, 2002).

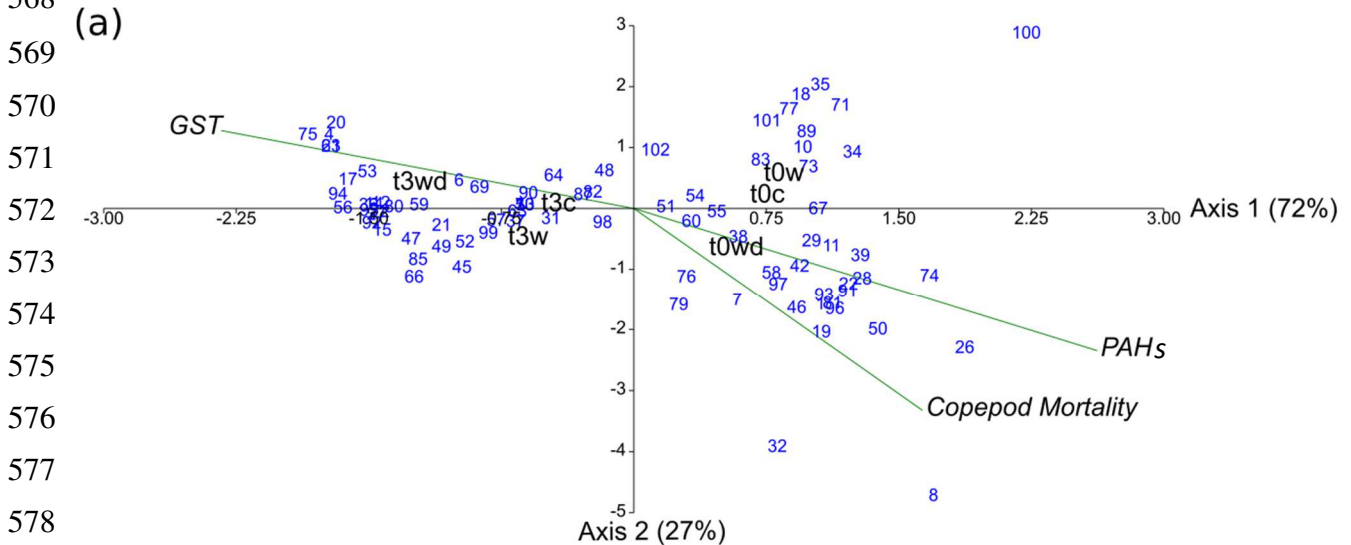
549 Several ASVs correlated with *gst* gene expression belonged to *Marinifilum*, *Lutibacter*,
550 *Salinirepens*, *Polaromonas*, *Colwellia*. These correlations probably result from the
551 reduction of dispersant toxicity, suggesting the sensitivity of these taxa toward the
552 toxicity of dispersant. It is likely that such taxa, stimulated after 21 days of microcosm
553 experiment, were initially out competed by the opportunistic taxa, which were rapidly
554 stimulated by the dispersant at the beginning. Then, later on the experiment, such
555 stimulated taxa were replaced upon the depletion of the carbon source provided from
556 dispersant. Dispersant have shown to be able to alter bacterial communities by
557 stimulating some taxa while inhibiting others (Yoshida et al., 2006).

558 Although the toxicity of the dispersant decreased during the experiment (Figure 1), the
559 WAF+dispersant microcosms still had deleterious sub-lethal effects towards *Acartia*
560 *tonsa* at day 21 as observed by the down expression of the *gst* gene. The *gst* genes are
561 known to be involved in antioxidant mechanisms capable to detoxify several

562 xenobiotics and protect the cell from oxidative stress (Lee et al., 2006). The low
 563 expression levels of *gst* gene observed in exposed *Acartia tonsa* suggests a decrease in
 564 the cellular antioxidant machinery resulting probably in the over accumulation of ROS
 565 in cells (Dvořák et al., 2021). However, further studies are needed to understand the
 566 significance of transcript downregulation of *gst* gene in *Acartia tonsa*.

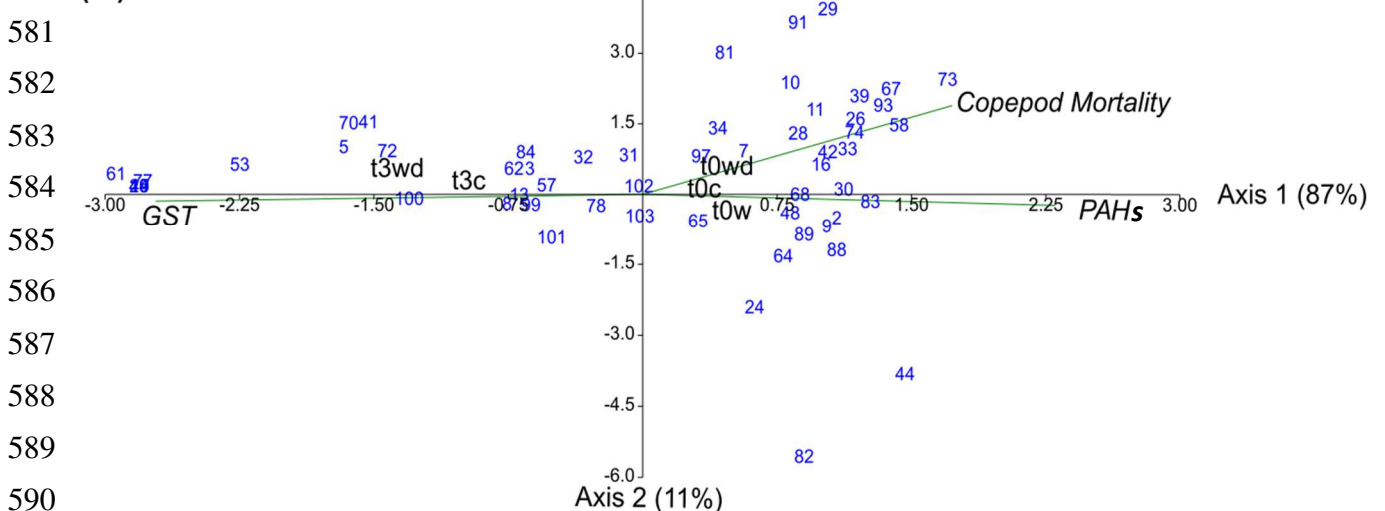
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580

581 (b)



591 **Figure 5:** Canonical correspondence analysis (CCA) showing correlation between the top 100 most
 592 abundant ASVs and the sum of concentration of PAHs, expression levels of *gst* gene in *Acartia tonsa*
 593 (*GST*) and the mortality exhibited by *Acartia tonsa* in the microcosm water (*Copepod mortality*). (a)
 594 Bacterial communities of microcosms prepared with pristine site water, (b) bacterial communities of
 595 microcosms prepared with contaminated water. Blue numbers denote the number of ASV, black names
 596 denote the microcosm communities: t(n): time (day) sample is collected from the microcosm, w, wd, and
 597 c: WAF, WAF+dispersant, and control respectively.

598

599 **3.5 Ecological association network**

600 In order to characterize the microbial interactions occurring in response to WAF or
601 WAF+dispersant exposures, a molecular ecological network analysis was performed.
602 Networks describing strong correlations (spearman correlation threshold at 0.910 and
603 0.950; p value < 0.05) were obtained (Supplementary Materials, Table S4). Pristine
604 communities' network showed more density by having 171 total nodes and 468 links,
605 while contaminated communities network showed 112 total nodes and 225 links.
606 However, R^2 of power law fitted better the pristine site network (0.68) compared to
607 contaminated site network (0.48), indicating that pristine site fits better as a scale free
608 network, where fewer taxa accounts for the majority of interactions. However, both
609 networks exhibited average path distance of 3.925 for contaminated and 4.296 for
610 pristine and similar average clustering coefficient (0.307 contaminated, 0.306 pristine).
611 Network indices from both networks were higher than the random network indices,
612 suggesting that both networks exhibited small world features as described for microbial
613 communities (Jeanbille et al., 2016 a,b). Such result indicated that the networks are
614 highly clustered and nodes are related (Watts and Strogatz, 1998). However, no
615 association was found between ASV and PAHs concentrations (Mantel test, for pristine
616 ($r = 0.007318$, $P = 0.375$) and contaminated ($r = -0.02143$, $P = 0.625$) networks).
617 Nevertheless, the networks, for both pristine and contaminated waters, were modular.
618 Contaminated site network yielded 17 modules with modularity value of 0.65 while
619 pristine site network yielded 14 modules with modularity value of 0.60. A plot was
620 constructed from the ASVs connectivity within the modules (Z_i) and ASVs connectivity
621 among other modules (P_i). According to Z_i and P_i values, the roles of ASVs in modules
622 were classified as: 1) Peripheral, 2) Connectors, 3) Module hubs, 4) Network Hubs

623 (Liang et al., 2016). The threshold for these classifications were selected at $Z_i = 2.5$ and
624 $P_i = 0.62$ as previously described by Guimera and Amaral (2005).

625 In general, most of the ASVs in both networks were peripherals, and therefore
626 considered as specialists (Figure 6a). In contaminated network, there are distinct
627 modules with little interconnection, evidenced by the ASVs with low P_i value. In
628 contrast, pristine network showed with more interconnected modules, evidenced by the
629 several ASVs with high P_i value (Figure 6a). In addition, several connector ASVs were
630 identified in pristine network, suggesting the complexity of the ecological interactions
631 within the network. Connectors are generalist species, which are capable to connect
632 different modules (Liang et al., 2016). The connector taxa identified belonged to
633 *Flavobacterium* (ASV 49) genus, 3 ASVs of uncultured bacteria from the order
634 *Rickettsiales* (ASV 259, ASV 224, and ASV 174), *Paraperlucidibacta* (ASV 227), ASV
635 from class *Nitrososphaeria* (ASV 321), an ASV from family *Cyanobiaceae* (ASV 153),
636 and 2 ASV from *Fusibacter* (ASV 37 and ASV 290) genus.

637 The networks, for microbial communities from microcosms of both pristine and
638 contaminated sites, showed a modularization according to time (Figure 6b & c). Indeed,
639 most interactions were observed at t14 and t21 days for the pristine network (Figure 6a),
640 and at t0 for the contaminated site network (Figure 6b). Such observation further
641 supports the ecological succession observed with the CCA (Figure 5). Interestingly, one
642 module in the pristine network clustered ASVs showing high bacterial abundances in
643 WAF+dispersant treatments, indicating that microbial community composition changes
644 in response to the presence of dispersant. Among the ASVs in this module, 7 ASVs
645 belonged to the genus *Fusibacter*, further showing the importance of such genus in the
646 microbial community dynamics, and four ASVs were connector nodes (ASV 321, ASV
647 277, ASV 153, and ASV 290).

648 Interestingly, some taxa showed noticeable patterns in the networks, indicating their
649 ecological role on the microbial communities in either WAF or WAF+dispersant
650 microcosms. For example, in pristine network, four small modules exclusively belonged
651 to three taxa: *Fusibacter*, *Shewanella* and *Polarimonas*, and in contaminated water
652 network (Figure 6c), several small modules included *Fusibacter* at t0. These small
653 modules, which are lacking co-occurring interactions, suggest that such taxa could be
654 exhibiting other type of interactions with the other communities, such as competition.
655 At t0, ASVs affiliated to *Marinifilium* genus showed high node degree in the network of
656 contaminated water, suggesting that *Marinifilium* is an important key taxon.

657 The ASVs identified as bioindicators by LEfSe were found in specific modules within
658 the contaminated water network at time 7 days, further suggesting that these ASVs are
659 important for the organization of microbial assemblage. Such observation was in
660 agreement with a previous study showing that soil with a previous history of
661 contamination has fewer interactions than pristine soil (Sheng et al., 2021). It is likely
662 that the selection pressure exerted by the presence of contaminant reduces diversity, and
663 thus the interconnectivity among taxa.

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(a)

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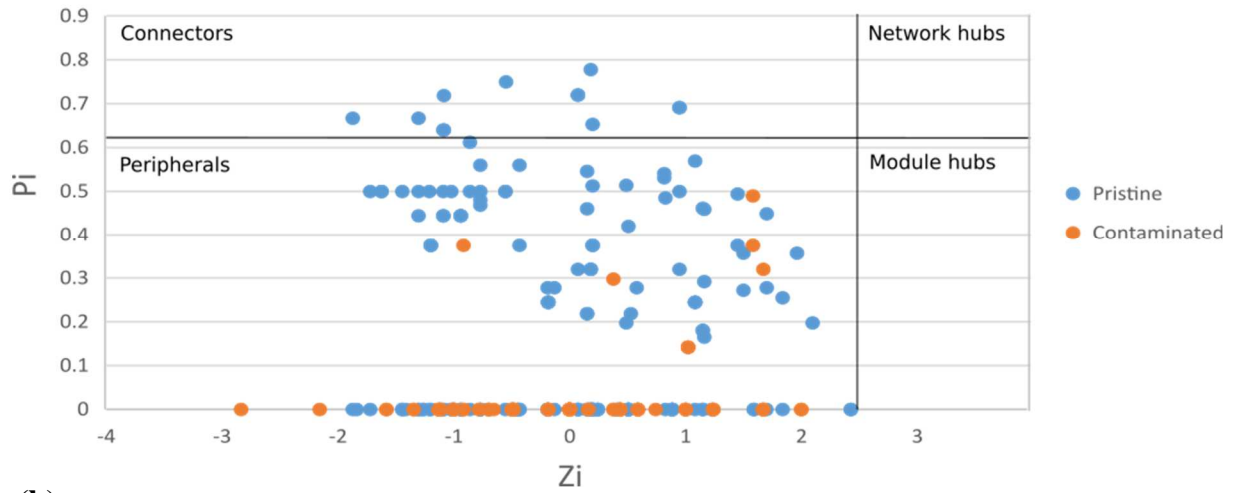
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(b)

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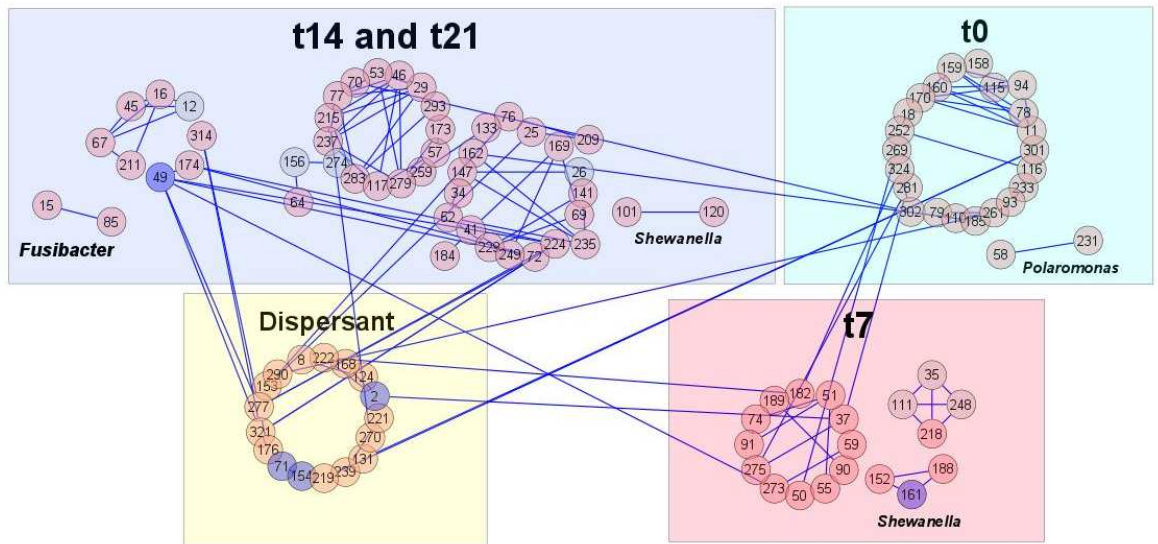
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(c)

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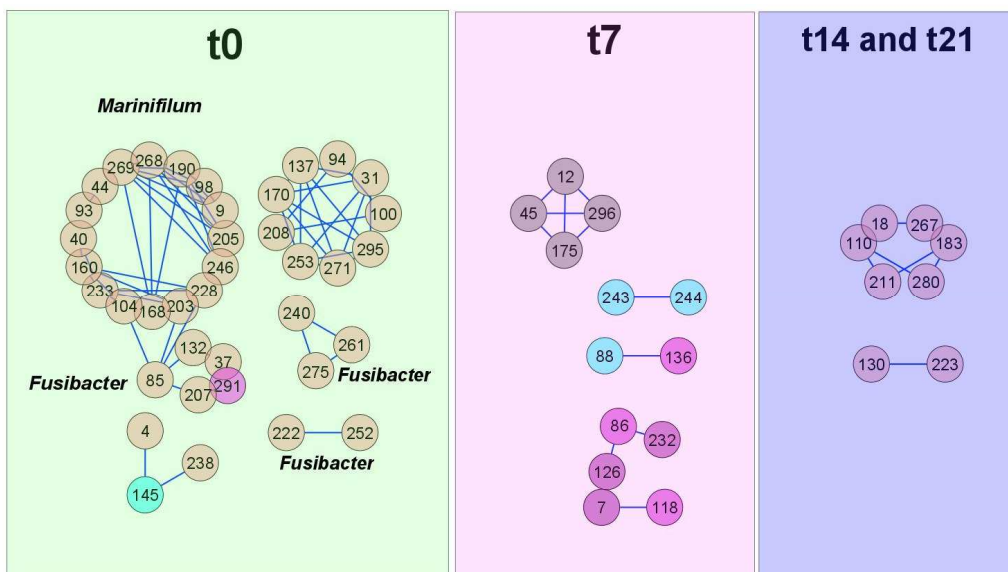
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686 **Figure 6: Molecular Ecological Network Analysis** of microbial communities interactions at t0,t7,t14
687 and t21 (days) of the microcosms from pristine and contaminated site. (a) ASV distribution according to
688 their topological features: within module connectivity (Z_i) and among module connectivity (P_i). (b)
689 Pristine microbial community and (c) contaminated microbial community networks. The networks were
690 constructed considering the microbial communities from all the samples collected during the microcosm
691 experiment from either pristine or contaminated sites. Blue lines are positive interactions (Co-
692 occurrence). Purple nodes are bioindicator taxa identified by LEfSe for WAF+dispersant treatment. Light
693 blue nodes are bioindicator taxa identified for WAF treatment. White and Grey nodes are bioindicator
694 taxa identified for control in pristine and contaminated networks respectively. Taxa names mentioned on
695 the networks denote taxa with observable effects on communities upon the introduction of WAF or
696 WAF+dispersant.

697

698 **4. Conclusions**

699 In cold environments, microbes are not dormant, active microbial communities were
700 observed in response to the addition of WAF and WAF+dispersant. Our results showed
701 that the legacy effect plays a vital role in shaping the microbial response toward WAF
702 and WAF+dispersant. Adapted microbial communities presented higher removal rates
703 allowing the identification of several WAF stimulated taxa, but the contribution of
704 dispersant to the PAHs removal is still questionable. Network analysis demonstrated
705 that the microbial interactions patterns were time dependent, but the presence of
706 dispersant also changed the microbial community structure, probably by stimulating
707 taxa with potential biodegradation capabilities. We demonstrated that WAF+dispersant
708 rapidly stimulated *Firmucutes* (*Fusibacter*), which consequently affected the microbial
709 communities' dynamics. The PAHs removal decreased the toxicity against marine
710 organisms (such as copepods). We assume that the toxicity decrease was due to PAHs
711 and dispersant removal by bacteria affiliated to specialized taxa, such as *Fusibacter* and
712 *Colwellia*. In conclusion, this study provides new insights on the behavior of water
713 microbial communities at 4 °C in response to WAF and WAF+dispersant simulating an
714 oil spill scenarios and a bioremediation efforts, respectively.

715

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