MICROBIAL COLONIZATION OF DIFFERENT MICROPLASTIC TYPES AND BIOTRANSFORMATION OF SORBED PCBs BY A MARINE ANAEROBIC BACTERIAL COMMUNITY

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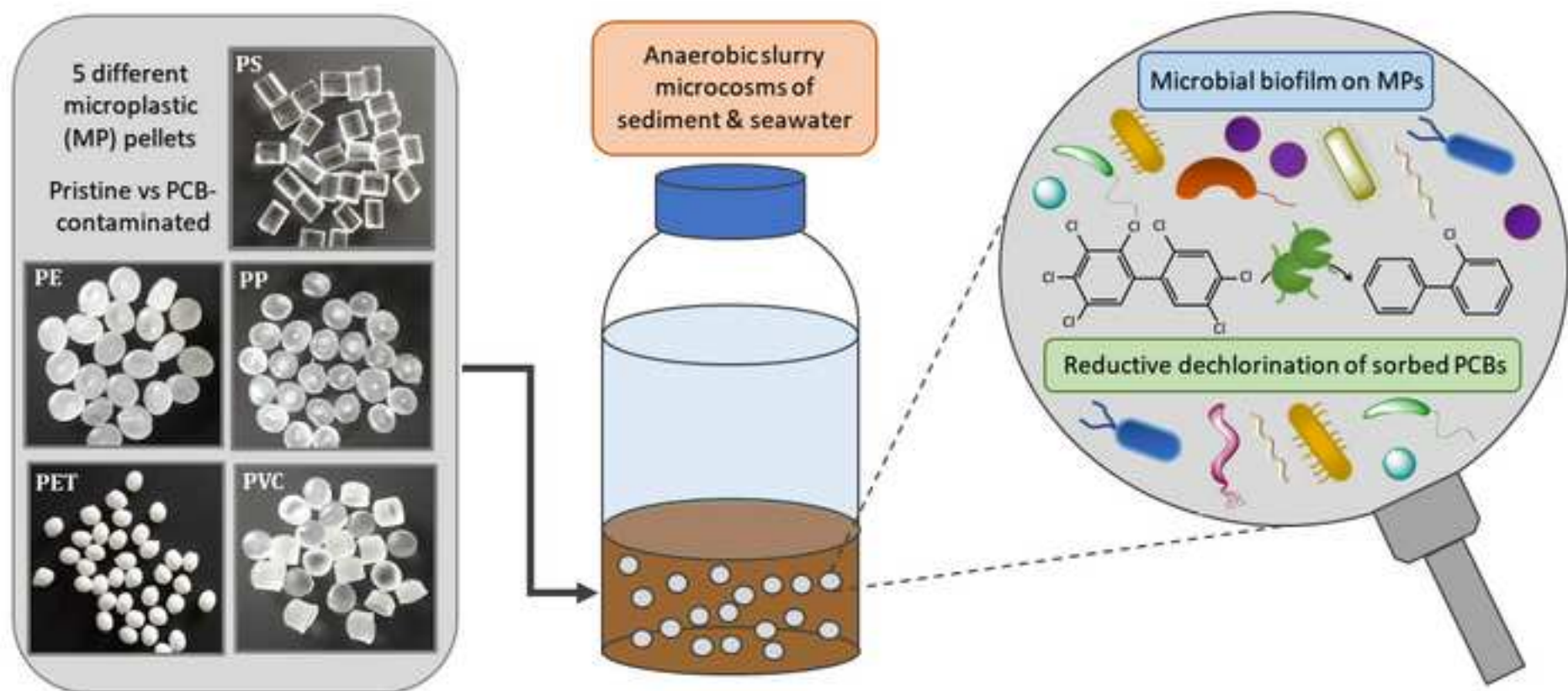
Highlights

- Marine bacterial biofilm composition changes on different MP types.

- MP-associated bacterial communities differ from the surrounding sediment ones.

- MP-sorbed PCBs do not significantly affect the MP-biofilm composition.

- PCBs sorbed on MPs can be rapidly dehalogenated by anaerobic marine biofilms.

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1   **Abstract**

2   We investigated the colonization dynamics of different microplastic (MP) pellets, namely,

3   polyethylene (PE), polyethylene terephthalate (PET), polystyrene (PS), polypropylene (PP)

4   and polyvinyl chloride (PVC), either pristine or contaminated with polychlorinated biphenyls

5   (PCBs), by an organohalide respiring marine microbial community and its biotransformation

6   activity towards PCBs sorbed on MPs, in anaerobic laboratory microcosms of a marine

7   sediment.

8   All MPs were rapidly colonized by the microbial community within 2 weeks of incubation,

9   when approximately 10^{10} 16S rRNA gene copies cm^{-2} were detected on PVC, 10^9 copies cm^{-2}

10   on PE, and 10^8 copies cm^{-2} on PET, PP and PS. A greater biofilm growth on PVC pellets than

11   other MPs was confirmed by quantification of the reducing sugars of the EPS and biofilm

12   staining with crystal violet. Illumina sequencing of the 16S rRNA genes and Principal

13   Coordinate Analysis (PCoA) revealed that the biofilm community on MPs significantly differed

14   from the sediment community, being enriched of chemoorganotrophic fermenting species,

15   and was significantly affected by the type of polymer. The presence of sorbed PCBs did not

16   significantly affect the overall community composition, and mainly resulted in the enrichment

17   of Dehalococcoidia, i.e., of the organohalide respiring members of the community.

18   Reductive dechlorination of PCBs sorbed to MPs was observed after two weeks of incubation,

19   when the average number of chlorines per biphenyl molecule was reduced from 5.2 to 4.8 -

20   4.3, and was faster (35.2 ± 1.9 to 61.2 ± 5.8 µmoles of Cl removed kg_{MP}^{-1} $week^{-1}$) than that of

21   sediment-sorbed ones (33.9 ± 9.1 µmoles of Cl removed $kg_{sediment}^{-1}$ $week^{-1}$), which started only

22   after 10 weeks of incubation. These data suggest that microbial colonization of contaminated

23   MPs might change the composition of sorbed PCB mixtures and therefore the toxicity

24   associated to PCB-polluted MPs.

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1   Introduction

Plastics use and production has enormously increased since 1950, reaching around 335 million tonnes in 2016, with 60 million tonnes generated in Europe alone (PlasticsEurope, 2018). The most common polymers are high-density and low-density polyethylene (PE), polyethylene terephthalate (PET), polystyrene (PS), polypropylene (PP) and polyvinyl chloride (PVC), which together represent approximately 90% of the global plastic production (Andrady and Neal, 2009). A huge amount of plastic materials ends up in the marine environment, becoming an ever-increasing problem due to the toxicity, persistence and universal presence of such debris. The estimation of plastic released in the oceans varies from 4.8 to 12.7 million tons annually (Haward, 2018), with size ranging from meters to micrometers (Ryan et al., 2009). The term microplastics (MPs) was introduced within the last decade to describe small particles of plastic, commonly defined as < 5mm in diameter (Frias and Nash, 2019). Once discharged into the marine environment, plastic litter undergoes different processes, such as weathering, fragmentation and fouling (Cole et al., 2011). In particular, biomass accumulation on MPs due to bio(fouling) can lead to an increase of their density and thus their sinking (Morét-Ferguson et al., 2010; Chubarenko et al, 2016; Miao et al., 2019a). The anaerobic sediment has been indicated as a possible long-term sink for MPs (Andrady, 2011; Van Cauwenberghe et al., 2015). Microplastic contamination in marine sediments has been reported by several authors (Thompson et al., 2004; McDermid and McMullen, 2004; Claessens et al., 2011), with some of the highest concentrations of MPs found in sediments located in Nova Scotia and Arctic Ocean (up to 8000 and 6595 MPs/kg of sediment, respectively) (Mathalon and Hill, 2014; Bergmann et al., 2017).

The first evidence of microbial colonization of plastic fragments dates back to the early 70s (Carpenter et al., 1972). Recent studies reported that MPs are readily colonized by environmental microbial communities in few hours or days of incubation in seawater

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(Ogonowski et al., 2018) and coastal marine sediments (Harrison et al., 2014). The bacterial assemblages colonizing MPs in freshwater and marine environments have been reported to significantly differ in taxonomic composition and structure from those present the in the surrounding water and/or sediment (Miao et al., 2019b; Frère et al., 2018; Ogonowski et al., 2018; Rummel et al., 2017; De Tender et al., 2015). Microplastic-associated microbial communities were also reported to have a lower alpha diversity (richness, evenness, and diversity) than those associated to natural substrates, indicating a remarkable differentiation between microbial communities and a substrate-type-coupled species sorting (Miao et al., 2019b; Ogonowski et al., 2018). MPs can thus be considered a distinct ecological habitat for diverse microbial communities, the "plastisphere" (Zettler et al., 2013), potentially characterized by distinct microbial and ecological functions (Miao et al., 2019b; Arias-Andres et al., 2018). Less conclusive information has been reported on the selective colonization of MPs of different materials, possibly as the consequence of the potential confounding temporal and environmental variability associated to the dynamic exposure conditions typically occurring in situ (Ogonowski et al., 2018). Recently, Li et al. (2019) reported that environmental factors, such as the salinity and the nutrients (total nitrogen and total phosphorus), affected the growth rate of biofilms on five types of plastics debris (polyvinyl chloride, polypropylene, polyethylene, polystyrene, and polyurethane) in the Haihe Estuary. The same authors also showed that some genera in the bacterial communities exhibit selectivity for the different polymer types (Li et al., 2019).

Given their small size, MPs may be ingested and accumulated by a wide range of marine organisms, causing direct effects such as physical damage in their intestinal tract, or in other tissues or organs (Van Cauwenberghe et al., 2015). However, the uptake of MPs might also have indirect impacts, since they can absorb and concentrate persistent organic pollutant (POPs) by partition and then transfer such contaminants to the marine food web through

79   ingestion (Andrady, 2011; Wang et al., 2018). Polychlorinated biphenyls (PCBs), polycyclic

80   aromatic hydrocarbons (PAHs), dichloro-diphenyl-trichloroethane (DDTs), polybrominated

81   diphenyl ethers (PBDEs), alkylphenols and bisphenol A (BPA), are the main contaminants that

82   have been found on plastics debris in the marine environment, at concentrations ranging from

83   0.001 to 10 mg/kg (Hirai et al., 2011).

84   Despite their production ban in the 1980s, PCBs are still common contaminants in marine

85   sediments and, as reported recently, on marine plastic pellets, where they have been detected

86   at concentrations up to 7.5 mg/kg (Taniguchi et al., 2016). Due to their high lipophilicity, PCBs

87   are accumulated through the food chain up to humans, where they act as endocrine disrupters

88   and possible carcinogens. Besseling et al. (2013) showed that an exposure of lugworm

89   (*Arenicola marina*) to low concentrations of polystyrene MPs mixed with PCB-contaminated

90   sediment enhanced the bioaccumulation of PCBs. Similar results were obtained by Koelmans

91   et al. (2013) in their model analysis with *A. marina*, although their experiments showed that

92   the role of plastic in the bioaccumulation of POPs may not be considered a significant hazard.

93   Therefore, the release of MPs in the marine environment can have contrasting effects on the

94   PCBs bioaccumulation in marine organisms, which can depend on different causes, such as the

95   experimental conditions or the type of PCB congeners, and further investigations in this

96   regard are necessary (Ziccardi et al., 2016). The toxicity of PCBs varies with the position and

97   number of chlorine atoms on the biphenyl ring, being the coplanar congeners more toxic

98   (Tanabe et al., 1987; Hashmi et al., 2017). Under anoxic conditions, PCBs can undergo

99   reductive dechlorination by organohalide respiring microorganisms occurring in marine

100 sediments, typically belonging to the Dehalococcoidia class of the Phylum Chloroflexi

101 (Zanaroli et al., 2015; Nuzzo et al., 2017). This process replaces chlorine atoms mainly in the

102 *meta* and *para* positions with hydrogen, thus converting highly chlorinated congeners into

103 less chlorinated products that are often less toxic, less prone to bioaccumulation (less

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104 hydrophobic) and more amenable to degradation by indigenous aerobic bacteria (Fava and

105 Agathos, 2006). If taking place on MP-sorbed PCBs, this microbial process might change the

106 composition of PCBs, and thus the toxicity and bioavailability of the sorbed PCB mixture for

107 consumers ingesting MPs. The effect of microbial colonization of plastics debris on the

108 environmental fate and the biodegradation/biotransformation of the pollutants absorbed on

109 the MPs surface has not been adequately evaluated yet. Microbial colonization of low-density

110 PE pellets incubated in marine and river sediments under aerobic conditions has been shown

111 to increase the biotransformation of MP-sorbed DDTs and PAHs, but not of PCBs (Wu et al.,

112 2017), suggesting that it might affect the toxicity associated with polluted microplastics.

113 However, to the best of our knowledge, no studies investigated the influence of microbial

114 colonization of MPs on MP-sorbed pollutants under anoxic conditions typically present in

115 sediments.

116 The aim of this study was to investigate the colonization dynamics on different types of MPs

117 (low-density PE, PET, PS, PP and PVC) by an anaerobic marine bacterial community

118 containing organohalide respiring bacteria, and the effect of the microbial biofilm on the

119 biotransformation of sorbed PCBs. The process was studied in anaerobic slurry microcosms

120 consisting of marine sediment suspended in seawater, i.e. under biogeochemical conditions

121 mimicking those occurring in situ and controlled exposure conditions.

122

123 **2 Materials and Methods**

124 *2.1 Microplastics and their contamination*

125 Five types of MPs were selected for this study: low density polyethylene pristine pellets (PE),

126 crystalline poly(ethylene terephthalate) pristine pellets (PET), general purpose polystyrene

127 pristine pellets (PS), homo-polypropylene pristine pellets (PP), and poly(vinyl chloride) soft

128 pristine pellets (PVC). The size of the plastic particles ranged from approximately 2.5 to 3 mm.

All MPs were sterilized in 70% ethanol for 15 min under shaking, followed by rinsing 3 times with sterilized deionized water, before their use. MPs were contaminated in the laboratory with a commercial mixture of PCBs (Aroclor 1254, UltraScientific, Bologna, Italy) at the final concentration of 30 mg_{PCBs}/kg_{MPs}. This concentration, which is approximately the same order of magnitude of the highest PCBs concentration reported on marine plastic debris (7.5 mg/kg; Taniguchi et al., 2016), was selected in order to obtain final concentrations of single PCB congeners (or co-eluting congeners) of the mixture in the range 20 µg/kgMPs - 4.8 mg/kgMPs, and thus to better assess the microbial dechlorination processes of the spiked PCB mixture. The contamination protocol was adapted from Beckingham and Ghosh (2017); synthetic marine water (1.5 L) was spiked with 0.6 mL of Aroclor 1254 stock solution (20000 mg/L in acetone) under shaking (acetone:water 0.04%), 400 g of MPs were added immediately after and incubated under shaking (180 rpm) for 10 days. MPs were recovered by sieving and dried at room temperature under sterile conditions. The actual amount of PCBs sorbed on MPs was indirectly estimated by measuring the residual mass of PCBs in the water phase via solid phase extraction with polydimethylsiloxane (PDMS) fibers. Five-cm PDMS fibers (outer diameter 558.8 µm, inner diameter 486 µm, thickness annulus 35.4 µm, fiber volume 0.597 µL/cm) were incubated in the water phase under mixing (150 rpm) at room temperature for 40 days. PCBs were then eluted for 16 h in 0.1 mL hexane and analysed as described in section 2.3. The water concentration of PCBs was calculated from their concentration in the PDMS fiber using the fiber-water coefficient (K_{PDMS-W}). The latter was calculated, for each PCB congener, from the octanol–water partition coefficient (K_{ow}) using the equation $logK_{PDMS-W} = 0.725\ logK_{ow} + 0.479$ (Thomas et al., 2014). Octanol–water partition coefficients for all PCB congeners were obtained from Hawker and Connell (1988). An almost negligible mass of PCBs was detected in the water phase, corresponding to 0.71%, 0.15%, 0.16%, 0.01% and 0.01%

153 (in case of PET, PP, PS, PE and PVC, respectively) of the total mass of PCBs initially added for

154 MPs contamination. The residual mass of PCBs was assumed to be sorbed on MPs.

155

156 *2.2   Microcosms set up, incubation and sampling*

157 Sacrificial anaerobic slurry microcosms (200 mL total volume) consisting of sediment (20%

158 w/v) and seawater collected from Piallassa Baiona (Ravenna, Italy) were set up according the

159 procedure described in Nuzzo et al. (2017). Microcosms were autoclave-sterilized for 1 h on

160 three consecutive days, inoculated at 5% (v/v) with a PCB-dechlorinating microbial culture

161 enriched previously from marine sediments (Nuzzo et al., 2017) and supplemented with 5 g of

162 MPs, corresponding to a number of MPs ranging from approximately 175 to approximately

163 300, depending on the density and size of the MP type. This resulted in a final concentration

164 ranging from approximately 4375 to approximately 7500 MPs/kg of sediment, which is

165 comparable with the concentrations of microplastics reported in the most MP-impacted

166 marine sediments (Bergmann et al., 2017; Mathalon and Hill, 2014). For each type of MPs, the

167 following microcosms sets were prepared: i) supplemented with MPs contaminated by PCBs;

168 ii) supplemented with pristine MPs (not contaminated). A sterile control set was set up for

169 each MP type by supplementing sterile, not inoculated slurry microcosms with MPs

170 contaminated by PCBs. In addition, a MP-free biologically active control was set up by spiking

171 Aroclor 1254 PCBs in the sediment and inoculating the same marine culture.

172 The microcosms were incubated at 20°C in the dark under static conditions for 28 weeks.

173 After 2, 5, 10, 14, 19 and 28 weeks of incubation, 3 microcosms for each MP type were

174 sacrificed and MPs were recovered through sieving on sterilized 0.5 mm sieves for the

175 analysis of biofilm microbial communities (except from sterile controls) and of the sorbed

176 PCBs (where spiked).

177

2.3   PCB extraction and analysis

179   PCBs were batch extracted from 1 g of MPs (i.e., approximately 35 to 60 MPs, depending on

180   the MP type) with 4 mL of hexane overnight (30°C, mixing at 150 rpm) and sonication (Hong

181   et al., 2017). PCBs in the organic extracts were analyzed with a 6890N gas-chromatograph

182   equipped with a 63Ni electron capture detector (μECD) and a 6890 series-automatic sampler

183   (Agilent Technologies) using a 30m×0.25mm HP-5 capillary column (Agilent Technologies)

184   under the conditions described elsewhere (Fava et al., 2003). Calibration curves were

185   obtained and verified monthly using standard mixtures of Aroclor 1254 and Aroclor 1242 (0.5

186   to 30 mg/L concentration range).

187

188   *2.4   Quantification of the bacterial biofilm*

189   Prior to each analysis, the MPs were separated from the sediment by sieving (mesh 0.5 mm),

190   rinsed three times with sterile water, in order to remove not-attached cells, and air dried

191   under sterile conditions. Three different approaches were used for the quantification of the

192   biofilm growth on MPs: i) metagenomic DNA extraction followed by qPCR of bacterial 16S

193   rRNA genes; ii) cell/biofilm staining with crystal violet; iii) quantification of the reducing

194   sugars after alkaline hydrolysis of the EPS polysaccharides (Costerton et al., 1995).

195   Metagenomic DNA was extracted from a mixture of replicate samples (0.5 g of MPs, i.e.,

196   approximately 18-30 MPs, depending on the MP type) with the UltraClean Soil DNA kit

197   (MoBio Laboratories, Carlsbad, CA, USA) following the procedure described by the provider,

198   with an additional enzymatic cell lysis step before mechanical cell lysis. In particular, MPs

199   were incubated in the bead solution (provided with the kit) in the presence of Lysozyme (11.9

200   μL of a 100 mg mL^{-1} solution) at 37 °C on a rotary shaker for 30 min, and then of Proteinase K

201   (3 μL of a 20 mg mL^{-1} solution) at 37 °C on a rotary shaker for 45 min, prior to addition of

202   SDS and mechanical cell lysis (bead beating on vortex at maximum speed for 10 min). Total

203   DNA was quantified using Qubit® dsDNA HS Assay Kit with a Qubit 3.0 fluorimeter, following

204   the manufacturer's specifications. The 16S rRNA genes were quantified via qPCR with primers

205   905f (5'-AAACTCAAAGGAATTGACGG-3') and 1044r (5'-GACARCCATGCASCACCTG-3') using

206   the reactions conditions described in Nuzzo et al. (2017). 7-point standard curves were

207   included in each plate using *E. coli* 16S rRNA gene. Samples and standards were analyzed in

208   triplicate reactions and 16S rRNA gene copy numbers per cm^2 of MPs were finally calculated.

209   A colorimetric biofilm quantification protocol using crystal violet was adapted from Burton et

210   al. (2007) and Zanaroli et al. (2011). MPs (0.5 g, i.e., approximately 18-30 MPs, depending on

211   the MP type) were stained 20 minutes with 2 mL of crystal violet solution (0.05% w/v). MPs

212   were then rinsed with sterile distilled water and air-dried for 15 min. The crystal violet on

213   MP-associated biofilm was solubilized by adding 2 mL of 96% (v/v) ethanol and the

214   absorbance of the destaining solution was spectrophotometrically measured at 570 nm

215   versus a blank solution obtained with the same procedure using ethanol-sterilized MPs (see

216   above).

217   Reducing sugars occurring in the EPS polysaccharides of the biofilm were measured via the

218   colorimetric method described by Bailey (1988), using 3,5 dinitrosalicylic acid, potassium

219   tartrate and NaOH. MPs (0.5 g, i.e., approximately 18-30 MPs, depending on the MP type) were

220   incubated in a boiling water bath for 15 minutes. After cooling in ice, the absorbance of the

221   supernatant was measured at 540 nm versus a blank solution obtained with the same

222   procedure using ethanol-sterilized MPs (see above).

223   To identify statistically significant differences between MP types, multiple pair-wise

224   comparisons were performed with the Tukey test ($P < 0.05$).

225

2.5   Analysis of the biofilm community via PCR-DGGE of the 16S rRNA genes

PCR-DGGE analysis of 16S bacterial rRNA genes was used to investigate changes in richness (Rr) and community structure (Co) of the bacterial biofilm on different MPs over time. The V3-V5 variable regions of the bacterial 16S rRNA genes were PCR amplified with primers GC-357f, containing a 40 bp GC-clamp (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C CC TAC GGG AGG CAG CAG-3') and 907r (5'-CCG TCA ATT CCT TTG AGT TT-3') (Sass et al., 2001). The PCR program consisted of an initial denaturation at 95 °C for 5 min, 30 cycles of repeated denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min. The PCR reaction (50 µL) consisted of mixtures containing 1× colorless GoTaq® Flexi buffer (Promega Corporation, Italy), 1.5 mM MgCl$_2$, 0.2 mM each dNTP, 0.4 µM each primer, 1 U of GoTaq® G2 Flexi DNA Polymerase (Promega Corporation, Italy), and of 4 µL template DNA.

The amplicons were resolved with a D-Code Universal Mutation Detection System (Bio-Rad, Milan, Italy) on a 7% (w/v) polyacrylamide gel (acrylamide-N,N'-methylenebisacrylamide, 37:1) containing a denaturing gradient from 40% (top) to 60% (bottom) denaturant (100% denaturant: 7M urea, 40% v/v formamide) as described in Nuzzo et al. (2017). DGGE image analysis was used to calculate the community richness (Rr) and community organization (Co) indexes (Nuzzo et al., 2017).

2.6   Analysis of the biofilm community via Illumina sequencing of 16S rRNA genes and
    statistical analysis

DNA extracted from biofilms of selected samples was subjected to sequencing in order to investigate the bacterial community composition. The selection of samples was based on the observed colonization dynamics in terms of i) amount of bacterial biofilm formed and, ii) changes of the community richness and organization over time, detected via PCR-DGGE

11

251 analysis (section 3.1), as well as on the observed reductive dechlorination of MP-sorbed PCBs

252 (section 3.3). V1-V3 hypervariable regions of 16S rRNA were PCR amplified and sequenced on

253 Illumina Miseq with 300 bp paired-end approach (BMR genomics, Padova, Italy).

254 The 16S rRNA gene raw sequences were processed using the open source software pipeline

255 Quantitative Insights Into Microbial Ecology 2 (QIIME 2) version 2017.12 (http://qiime2.org).

256 SILVA was used as reference database to classify the representative sequences from our

257 dataset, and Jaccard distance matrices as beta-diversity measures. The Principal Coordinate

258 Analysis (PCoA) plot was created in R (version 3.5.1) through community ecology vegan

259 package. The significance of separation among study groups was determined by permutation

260 test with pseudo-F ratios using the function adonis of vegan package. Significant differences in

261 the relative abundance of Dehalococcoidia between MPs groups – pristine and PCB-

262 contaminated – were evaluated with the Wilcoxon test.

263

264 **3   Results**

265 *3.1   Colonization dynamics of different MPs*

266 The colonization dynamics was evaluated during a 28-weeks experiment using different

267 approaches: i) quantification of the Bacterial 16S rRNA genes via qPCR, as an approximation

268 of cell density on the MPs surface (16S rRNA gene copies per cm^2 of MPs), ii) cell/biofilm

269 staining based on crystal violet, which targets both the bacterial cells and the extracellular

270 polymeric substances (EPS) they produce when growing attached to surfaces, and iii)

271 quantification of reducing sugars occurring in the polysaccharide fraction of EPS.

272 The qPCR analysis revealed that all MPs were rapidly colonized by the bacterial community

273 within the first 2 weeks of incubation, without any remarkable difference between the PCB-

274 contaminated MPs and the pristine (not-contaminated) MPs (Figure 1A,D). The highest cell

275 density was found on PVC pellets, which reached approximately 10^{10} 16S rRNA gene copies

276   per cm^2 of MPs, followed by PE (approximately 10^9 16S rRNA copies cm^{-2}), and then PET, PP

277   and PS (approximately 10^8 16S rRNA copies cm^{-2}). Since the surface area of MPs was

278   estimated using the geometry of each plastic pellet, i.e., not considering the irregularities of

279   the shapes and the porosities, the calculated superficial cell density may be an

280   underestimation of the actual microbial colonization. No remarkable cell density changes

281   were observed along the rest of the incubation, indicating that cells attachment and growth

282   were not taking place and/or that were balanced by cells death and detachment from the

283   surface (McDougald et al., 2012).

284   Staining with crystal violet (Figure 1B,E) and quantification of the reducing sugars (Figure

285   1C,F) revealed a biofilm increase up to weeks 10-15 on PE, PS, PP and PET, and up to the end

286   of incubation (week 28) on PVC, without remarkable differences in the presence and absence

287   of the sorbed pollutants. The biofilm increase over time may indicate the occurrence of a

288   biofilm maturation phase, taking place after a rapid cells adhesion and growth. The biofilm

289   amount then changed less markedly, showing slight decreases followed by small increases

290   that may indicate the succession of detachment and regrowth events involving portions of the

291   biofilm, which are typical of these dynamic structures (McDougald et al., 2012). The greatest

292   biomass concentration, approximately one order of magnitude higher than on the other MP

293   types, was detected on PVC MPs also according to crystal violet and quantification of reducing

294   sugars and was significantly higher than that on other MP types (P<0.05, Tukey test)

295   according to all measured parameters.

296   A preliminary investigation of the evolution of the biofilm community established on the

297   different MPs was carried out through PCR-DGGE analysis of the 16S rRNA genes. PCR-DGGE

298   profiles (Figure S1) were mainly used to evaluate if major changes were taking place over

299   time in the community developed on each MP type, rather than for an in-depth assessment of

300   the community diversity and structure or to compare the biofilm communities on the

301 different MP types. The following parameters were considered: i) community richness (Rr),

302 i.e., the number of species, and ii) community organization (Co), which indicates if the

303 individuals of the population are homogeneously distributed between the species (even

304 community) or if individuals belonging to few species are predominant over those belonging

305 to all others species (uneven community).

306 The Rr and Co varied markedly between the different type of MPs, but not between the MPs

307 contaminated by PCBs and those not contaminated (Figure S2). In particular, the biofilm

308 communities on PET, PS and PP did not change remarkably in Rr and Co over time, and were

309 characterized by an average Rr of approximately 25 and 18, for the pristine and PCB-

310 contaminated MPs, respectively. The Co resulted to be approximately 25 or lower for all this

311 plastic samples (PET, PS and PP, contaminated and pristine), which indicates a high degree of

312 community evenness. In contrast, a remarkable change of the biofilm community appeared to

313 occur on PE during the first 10 weeks of incubation, where a noteworthy reduction of Rr and a

314 substantial increase of Co where observed. This indicates that a fraction of the biofilm

315 microbial community on the PE surface was able to become dominant over time, causing a

316 loss of biodiversity. Conversely, an increase of Rr and limited fluctuations of the Co were

317 observed on PVC over time. This indicates that an increase of biodiversity took place without

318 affecting the functional organization of the community.

319

320 *3.2   Composition of the biofilm community on different MPs*

321 The composition of the biofilm communities was then investigated more in detail through

322 Illumina sequencing of the 16S rRNA genes. Samples were selected based on the observed

323 colonization dynamics in terms of i) amount of bacterial biofilm formed and, ii) changes of the

324 community richness and organization over time (section 3.1), as well as on the observed

325 reductive dechlorination of MP-sorbed PCBs (section 3.3). In particular, the biofilm

community composition was investigated for all samples after 2 weeks of incubation, since at this sampling point all MP types were colonized by the maximum cells concentration, which then remained almost constant (Figure 1A,D), and a remarkable reductive dechlorination of sorbed PCBs was observed on all MP types (section 3.3, Figure 5); in addition, the final sampling point (28 weeks of incubation) was analysed only for PE and PVC pellets, since substantial changes in Rr and Co were observed between 2 and 28 weeks of incubation only on these materials (Figure S2). The PCB-dechlorinating marine culture inoculated in the microcosms was also analysed to investigate if adhesion and biofilm formation on MPs involves specific members of the community or the whole population. In addition, the sediment present in the microcosms supplemented with PCB-contaminated MPs was analysed to evaluate any differences between the microbial communities adhering to the MPs and those associated to the sediment particles.

At the phylum level (Figure 2), the microbial inoculum mainly consisted of Proteobacteria (58%), Chloroflexi (30%) and, at lower extent, Firmicutes (6%). After 2 weeks of incubation, Firmicutes became dominant in the biofilm communities on all MP types, having a relative abundance that ranged from 88% (pristine PS) to 49% (PCB-contaminated PVC). Conversely, Proteobacteria represented a minor fraction of the biofilm communities, ranging from 1 to 27%, and the fraction of Chloroflexi decreased on all plastic pellets (0.3-13%,), except for PVC contaminated by PCBs (45%). Plastic biofilm communities also included, at quite high relative abundances, the phyla Lentisphaerae (0-26%), Deinococcus-Thermus (0-7%), Actinobacteria (0-13%) and Acidobacteria (0-9%), which were not detected in the inoculum or represented less than 2% of the original microbial community.

Relevant changes in composition were detected after 28 weeks of incubation on PE and PVC, when Chloroflexi became more abundant and Firmicutes decreased.

The bacterial communities associated to the sediment surrounding the different PCB-contaminated MPs were very similar to each other and mainly consisted of bacteria belonging to the phyla Firmicutes (30-43%), Chloroflexi (20-28%), Proteobacteria (12-22%), and to less extent, Acidobacteria (6-9%), Synergistetes (4-8%), Lentisphaerae (3-7%), and Actinobacteria (2-3%). These bacterial communities therefore remarkably differed from the microbial inoculum and those colonizing the MPs.

At lower taxonomic levels (Table S1), the original marine microbial community was dominated by bacteria of the genera *Sulfurovum* (20.4%) and *Sulfurimonas* (15.5%), which are both typically sulphur oxidizing, nitrate-reducing bacteria, *Dethiosulfatibacter* (5.3%), a sulphur and thiosulfate-reducing genus, *Magnetovibrio* (3.6%), which includes members capable of anaerobic thiosulfate oxidation using nitrous oxide, *Celeribacter* and another member (unidentified genus) of the *Rhodobacteraceae* family (12.0%) and *Dehalobium* (17.7%), an organohalide respiring Dehalococcoidia, along with other non-dehalorespiring members of the Chloroflexi phylum (unidentified genus, *Anaerolineacea* family, 12.8%). Most of these genera were not detected in the biofilm communities grown on pristine MPs, or they were at much lower abundances. After 2 weeks of incubation, the biofilm present on PS and PVC was dominated by a Firmicutes bacterium of the *Peptostreptococcaceae* family (unidentified genus), which represented 42.4% and 37.6% of the community, respectively. This bacterium was also present at high relative abundance on PE (19.4%) and, at lower abundances, on PET (9.4%) and PP (8.5%) after 2 weeks of incubation. The microbial community on PE and PP after 2 weeks of incubation was mainly dominated by a different Firmicutes bacterium belonging to the SRB2 family of *Thermoanaerobacterales* (unidentified genus), which represented approximately 29% of the biofilm community on both MPs types. The same bacterium was also detected at high abundance on PET (17.6%) and at much lower abundance on PS (5.6%), while was not detected on PVC. A member of the *Clostridiaceae 1*

16

375 family (unidentified genus) was also dominant or present at relatively high abundances on

376 PET (20.5%), PP (21.0%) and PS (16.4%), whereas the community on PVC included at high

377 abundances a member of Clostridiales (22.5%), *Celeribacter* (15.0%) and a member of

378 Dehalocccoidia (10.0%). Other community members that enriched on pristine MPs after 2

379 weeks of incubation were an uncultured *Coriabacteriaceae* (unidentified genus) in PVC

380 (12.5%), PE (6.6%), PET (5.3%) and PP (4.3%), a member of Deinococci in PP (6.8%), PE

381 (5.9%) and PS (5.3%), *Sedimentibacter* in PE (10.2%), PET (7.3%), PP (6.3%) and PS (3.1%),

382 and *Clostridium senso strictu 1* only in PET (7.9%) and PS (5.4%).

383 After 28 weeks of incubation, the SRB2 family of *Thermoanaerobacterales* and

384 *Sedimentibacter* were lost by the biofilm community on PE, where *Peptostreptococcaceae*

385 further enriched (40.3%) along with *Anaeroplasma* (6.5%) and *Anarolinaceae* (17.9%). A

386 remarkable change was also observed in the biofilm composition of PVC after 28 weeks of

387 incubation, when the uncultured *Coriobacteriaceae*, Dehalococcoidia, Clostridiales and

388 *Celeribacter* drastically reduced, and uncultured *Anaerolinaceae* remarkably enriched

389 (35.4%).

390 The presence of sorbed PCBs had very limited effects on the overall composition of the biofilm

391 on MPs, resulting mainly in the enrichment of the class Dehalococcoidia, i.e., the organohalide

392 respiring Chloroflexi members of the community (Figure 3). This behavior appears even

393 more marked in the case of PVC, where the increase of Dehalococcoidia in the biofilm

394 community in the presence of PCBs reaches the statistics significance (P-value = 0.05,

395 Wilcoxon test).

396 The PCoA analysis based on Jaccard similarity index was then used to assess the overall OTU-

397 level compositional differences in the microbial communities adhering to the different plastic

398 types (PE, PET, PS, PP, PVC) and sediment. The data show a clear separation of microbial

399 community profiles by MPs type and between MPs and the surrounding sediment (P-value <

400   0.03, permutation test with pseudo-*F* ratio) (Figure 4A). Permutation test with pseudo-*F* ratio

401   of the significance of pairwise separation between each MP type and MPs and the sediment is

402   reported in Table S2. The PCoA analysis also demonstrated that the communities on PCB-

403   contaminated and pristine MPs did not significantly differ between each other (P-value = 0.4)

404   (Figure 4B). The sorption of PCBs on MPs therefore did not significantly change the taxonomic

405   composition of the overall microbial community adhering to them.

406

407   *3.3   PCB dechlorination*

408   No dechlorination of MP-sorbed PCBs was observed in sterile controls during the 28 weeks of

409   incubation. On the contrary, a significant reductive dechlorination of PCBs associated to all

410   different types of MPs was observed in the microcosms inoculated with the PCB-

411   dechlorinating culture after only two weeks of incubation, when the average number of

412   chlorines per biphenyl molecule was reduced from 5.2 to 4.8 on PP, 4.7 on PE, 4.6 on PET, 4.4

413   on PS and 4.3 on PVC (Figure 5), corresponding to dechlorination rates of 35.2 ± 1.9 (PP), 44.6

414   ± 2.6 (PE), 56.8 ± 6.2 (PET), 57.8 ± 5.4 (PS) and 61.2 ± 5.8 (PVC) µmoles of Cl removed kg_{MP}^{-1}

415    $week^{-1}$. The reductive dechlorination of sorbed PCBs then proceeded more slowly, leading to

416   an average number of chlorines per biphenyl molecules after 28 weeks of incubation in the

417   range 4.4-3.9. Remarkably, the biotrasformation of PCBs sorbed on MPs was faster than that

418   of PCBs sorbed on sediment, which started only after 10 weeks of incubation and proceeded

419   with a dechlorination rate of 33.9 ± 9.1 µmoles of Cl removed $kg_{sediment}^{-1}$ $week^{-1}$ until week 28,

420   when the average number of chlorines per biphenyl molecule was reduced to 4.0 (Figure 5).

421   In general, highly similar dechlorination patterns were observed for PCBs sorbed on different

422   MP types, as indicated by the depletion of the same highly-chlorinated congeners and the

423   accumulation of the same low-chlorinated ones (Figure 6). The lower reduction of the highly-

424   chlorinated congeners and the lower accumulation of the low-chlorinated ones detected on

425 PE and PP pellets at the end of incubation was in accordance with the less extensive

426 dechlorination process occurred on these MPs. The dechlorination patterns of PCBs sorbed on

427 MPs was similar to that observed on PCBs sorbed on sediment (without MPs), with a main

428 difference: the accumulation in the sediment of the co-eluting hexa-/penta-chlorinated

429 congeners 234-35, 235-34 and 2356-24, which did not accumulate on the MPs, and the

430 concomitant accumulation of the tri-chlorinated congeners 25-3 and 24-3 on MPs and not in

431 the sediment. This indicates that a *meta* and *para* dechlorination of 235-34 and 234-35 to 25-

432 3 and 24-3, respectively, occurred only on MPs.

433

434 **4   Discussion**

435 Microbial colonization of MPs in the marine environment may have several implications on

436 their fate and sedimentation behavior, the fate of MP-sorbed pollutants and their toxicity and

437 bioavailability to marine organisms (Cole et al., 2011; Wang et al., 2018; Mohamed Nor and

438 Koelmans, 2019). While the colonization of plastic debris by marine microorganisms have

439 been recently reported in seawater (Dussud et al., 2018; Frère et al., 2018; Xu et al., 2019),

440 very limited information is available on plastic colonization in marine sediments (Harrison et

441 al., 2014), which are the ultimate sink, as well as an entry point in the food chain through

442 benthic organisms, of plastic debris and hydrophobic pollutants in the marine environment

443 (Kaiser et al., 2017). Very limited information is available also on the fate of pollutants sorbed

444 on plastics and, in particular, on the potential role of microbial biofilms in the

445 biodegradation/biotransformation of plastic-sorbed pollutants (Wu et al., 2017). In this study,

446 the colonization dynamics of microplastic (MP) pellets of different materials, namely, PE, PET,

447 PS, PP and PVC, either pristine or contaminated with polychlorinated biphenyls (PCBs), was

448 investigated in laboratory microcosms of an anoxic marine sediment and seawater collected

449 from the same site, i.e., under laboratory biogeochemical conditions mimicking those

450 occurring in situ and controlled exposure conditions. The sediment was inoculated with a

451 marine anaerobic microbial community previously selected for its ability to dehalogenate

452 PCBs (Nuzzo et al., 2017), in order to better assess the potential role of microbial reductive

453 dehalogenation processes on the fate of MP-sorbed PCBs. The use of a well-defined source

454 community was also made to better evaluate the possible effects of the MP material and of

455 MP-sorbed pollutants on the surface colonization dynamics and on structure and composition

456 of the biofilm (Ogonowski et al. 2018).

457 All MPs were rapidly colonized during the first 2 weeks of incubation, up to a cell density

458 range of 1.0×10^8 to 1.0×10^{10} cells/cm^2. Harrison et al. (2014) reported a comparable, rapid

459 colonization of low-density PE by bacteria of coastal marine sediments incubated in Petri

460 dishes, that reached a density of 1.0×10^6 to 1.0×10^9 16S rRNA genes DNA per mm^2 of PE

461 within 7 days. Our work confirms that MPs are a good anthropogenic substrate for

462 colonization by marine sediment microbial communities also under strictly anoxic conditions,

463 which are typically present in situ few millimeters below the sediment surface.

464 Remarkable differences were observed in terms of colonization of different MP types. In

465 particular, biofilm formation was remarkably higher on PVC pellets than on other MP types,

466 both in terms of cell density reached and amount of EPS produced. Such a higher biofilm

467 growth on PVC is likely the consequence of a higher availability of energy and carbon sources

468 that might have been released from the MPs. Many organic additives are commonly

469 supplemented to plastic materials in order to improve their proprieties, such as tensile

470 strength, flexibility and durability (Teuten et al., 2009). In particular, PVC is among the

471 plastics containing the highest amounts of additives, mainly plasticizers and stabilizers, that

472 may reach up to 50% w/w of the plastic material. Phthalates (alkyl/aryl esters of 1,2-

473 benzenedicarboxylic acid), as well as low molecular weight, easily biodegradable citrate,

474 adipate and hexanoate esters, are very commonly used plasticizers in PVC (Markarian, 2007;

Babinsky, 2006). The microbial degradation of phthalates to methane and carbon dioxide has been also reported under anaerobic conditions (Chang et al., 2005; Liang et al., 2008). All these additives can thus sustain the growth of anaerobic microbes/microbial communities and could have therefore favored a more extensive biofilm growth on PVC pellets.

All MP types selected specific fractions of the inoculated microbial community. The dominant members of the latter were mainly associated with sulphur cycling, a common process taking place in marine sediments (Wasmund et al., 2017), as well as with organohalide respiration, which was a specific feature of the marine microbial community inoculated in the microcosms (Nuzzo et al., 2017). Most of these members were not detected in any of the biofilm communities grown on MPs, which were dominated by taxa mainly including chemoorganotrophic, fermenting bacteria.

Remarkable differences were also observed in terms of composition between the microbial communities associated to the different MP types and the surrounding sediment. Recent studies have demonstrated that the bacterial assemblages that develop on the surface of plastic material in seawater or freshwater systems can significantly differ from those developing on non-plastic substrates, such as wood, cobblestone, cellulose and glass, as well as from the sediment and water bacterial communities (Miao et al., 2019b, Ogonowski et al., 2018; Frère et al., 2018; De Tender et al., 2015). Some of these studies have also reported that the microbial communities developed on PE and PP were quite similar to each other, and more distinct to those associated with PS pellets (Ogonowski et al., 2018; Frère et al., 2018). The differentiation of biofilm communities on different MP types occurred in this study may be attributed to many factors. For example, Ogonowski et al. (2018) recently reported a significant correlation between the substrate hydrophobicity and bacterial composition on different plastic and non-plastic (glass, cellulose) materials. Since the hydrophobicity of the materials tested in this study typically changes in a relatively narrow range (contact angle 80-

500   100; Ogonowski et al., 2018; Gotoh et al., 2011; McGinty and Brittain, 2008), the difference in

501   biofilm composition could be more probably related to other features, such as the presence of

502   different organic and inorganic additives and the different rates they are released from the

503   plastic material. Indeed, the additives released from different MPs may either promote the

504   growth of some microbial species able to use them as carbon and energy source, or limit the

505   growth and survival of some other species that are sensitive to these chemicals. However, the

506   very high variety of additives used in different plastics, as well as the use of different additives

507   for the same plastic type by different producers, make impossible to identify a typical

508   additives composition for each plastic and thus to relate the specific compositions of the

509   biofilm communities observed in this study to the release of specific chemicals. Interestingly,

510   Klaeger et al. (2019) recently reported that the release of biodegradable residual monomers

511   and oligomers from polymers can lead to a remarkable overestimation of the plastic materials

512   degradation, in particular during the first 19 days of incubation. Indeed, styrene and vinyl

513   chloride monomers have been widely reported to leach from PS and PVC pellets, respectively

514   (Pilevar et al., 2019; Fayad et al., 1997) and are known to be susceptible, as well as other

515   hydrocarbons, to anaerobic biodegradation (Varjani, 2017). This suggests that the leaching of

516   residual monomers and/or oligomers from the different plastic types may have also

517   contributed to the differentiation of biofilm compositions on the different MPs we observed

518   especially during the first 2 weeks of incubation. This is in agreement also with the selective

519   enrichment of Dehalococcoidia (i.e., of the organohalide respiring Chloroflexi members of the

520   inoculated community) in the biofilm promoted by the sorbed PCBs on all MPs, without any

521   significant change of the overall community.

522   The PCBs sorbed on the different MP types were rapidly converted into less chlorinated

523   congeners by the anaerobic marine biofilms. Indeed, the rate of PCB dechlorination we

524   observed may be much higher than that potentially taking place in situ, given the extremely

low concentration of organohalide respiring bacteria typically present in environmental samples and the specialized enriched community with marked organohalide-respiring activity we used in our study under controlled conditions. However, to our knowledge, this is the first report describing the potential occurrence of reductive dechlorination processes towards PCBs sorbed on MPs. Very few studies investigated the biotransformation/biodegradation of pollutants sorbed on MPs; Wu et al. (2017) reported for example that colonization of low-density PE pellets increased the biotransformation of dichloro-diphenyl-trichloroethanes (DDTs) and polycyclic aromatic hydrocarbons (PAHs) in marine and river sediments incubated aerobically, although had not significant effect on PCBs. This claims the need of further studies on the biotransformation of pollutants sorbed on MPs under different environmental conditions and by different microbial communities.

Moreover, our study shows that PCBs sorbed on MPs underwent reductive dechlorination much more rapidly than those sorbed on the sediment. While Mato et al. (2001) showed that sorption of organic compounds, such as PCBs and DDE, is approximately two orders of magnitude higher in PP pellets than in marine sediments, probably due to the plastic hydrophobic surfaces, our results suggest that PCBs are more bioavailable to the dechlorinating marine microbes when sorbed on MPs, or that the colonization of MPs might increase the bioavailability of MP-sorbed PCBs, e.g., through biosurfactants production, better than of the sediment-sorbed ones. PCB dechlorination was more extensive on PVC, PS and PET, followed by PE and PP, which might indicate a slight different degree of PCB bioavailability on different MP materials. The most abundant highly-chlorinated PCB congeners were extensively converted into less chlorinated products, that are commonly less toxic and less hydrophobic, thus potentially more bioavailable and less prone to bioaccumulation. The toxicity of contaminated MPs is potentially due to the combination of several factors, e.g., the intrinsic toxicity of the sorbed pollutants, their bioavailability and

550 capability to bioaccumulate; the observed change in congener composition of the PCB mixture

551 associated to MPs might thus have remarkable effects on the overall toxicity associated to

552 polluted MPs, that deserves more investigations.

553

554 **5 Conclusions**

555 The colonization dynamics of different MP pellet types (PE, PET, PS, PP, and PVC) by an

556 anaerobic marine microbial community was investigated for the first time in anoxic marine

557 sediments, which represent the main sink for MPs in the marine environment. This study

558 showed that microbial colonization took place very rapidly on all MPs and that the biofilm

559 composition differed significantly between the five plastic types and from the surrounding

560 sediment community. The colonization of MPs is thus a selective process that may depend on

561 several factors, such as the different properties of polymer surface and the presence of

562 different additives and/or different residual monomers/oligomers that may be released from

563 the plastics and promote or limit selectively the growth of distinct bacterial species. However,

564 the factors driving the enrichment of different biofilm communities on the MPs may be

565 multiple and diverse and need further investigation. This work also showed that PCBs sorbed

566 on different MP types can be dehalogeated much faster than those sorbed on sediments,

567 suggesting that they are highly bioavailable. In addition, their susceptibility to be

568 bioconverted to even more bioavailable, although less toxic, low-chlorinated PCBs indicate

569 that microbial biofilms growing on contaminated MPs, by changing the congener composition

570 of the sorbed PCB mixture, might remarkably affect the toxicity of contaminated MPs in the

571 marine environment, as well as the uptake and bioaccumulation of MP-associated PCBs by

572 marine organisms. Further investigation should be carried out in order to assess ecotoxicity

573 changes associated to the biotransformation of PCBs sorbed to MPs particles.

574

583

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Figures

Figure 1. Colonization dynamics of PCB-contaminated (A-C) and pristine (D-F) MPs determined via qPCR of 16S rRNA genes (A, D), reducing sugars in the EPS (B, E), and biofilm staining with crystal violet (C, F). Values are the mean of triplicate cultures ± standard deviation.

Figure 2. Composition at the Phylum level of the inoculated microbial community, the biofilm grown on all PCB-contaminated and pristine MPs after 2 weeks of incubation, the biofilm grown on PCB-contaminated and pristine PE and PVC after 28 weeks of incubation and the microbial community associated to the sediment particles surrounding the PCB-contaminated MPs after 2 weeks of incubation.

Figure 3. Boxplots showing the relative abundance (%) of Dehalococcoidia in pristine and PCB-contaminated MPs. *: P-value = 0.05; Wilcoxon test.

Figure 4. PCoA plots of bacterial communities in the inoculum, in the sediment and associated to MPs (A) and of bacterial communities associated to PCB-contaminated and to pristine MPs (B).

Figure 5. Reductive dechlorination of PCBs sorbed on the sediment (biotic control microcosms) and on the different MP types (biologically active microcosms and sterile controls). Values (average number of chlorines/biphenyl molecule) are the mean of triplicate cultures ± standard deviation.

Figure 6. PCB congeners and their concentrations on MPs (A) and in the sediment (B) at the beginning and the end of incubation (28 weeks). Value are the mean of triplicate cultures ± standard deviation.

797   **Supplementary information**

798   **Table S1.** Composition at the genus level of the inoculated microbial community, of the

799   biofilm grown on all MP types after 2 weeks of incubation and after 28 weeks of incubation on

800   PE and PVC MPs, and of the microbial community associated to the sediment particles.

801   **Table S2.** Results of permutation test with pseudo-F ratio statistics applied to ordination

802   analysis based on Jaccard similarity index (related to Figure 4A).

803   **Figure S1.** PCR-DGGE gels of the 16S rRNA genes of the inoculated marine microbial culture

804   (A) and the biofilm microbial community grown on pristine MPs (A) and on PCB-

805   contaminated-MPs (B).

806   **Figure S2.** Evolution of the biofilm community richness (Rr) and organization (Co) over time

807   on PCB-contaminated (A) and pristine (B) MPs, based on PCR-DGGE analysis of 16S rRNA

808   genes (Figure S1).

Figure 1. Legend: PCB-contaminated PE, PCB-contaminated PET, PCB-contaminated PS, PCB-contaminated PP, PCB-contaminated PVC, Pristine PE, Pristine PET, Pristine PS, Pristine PP, Pristine PVC
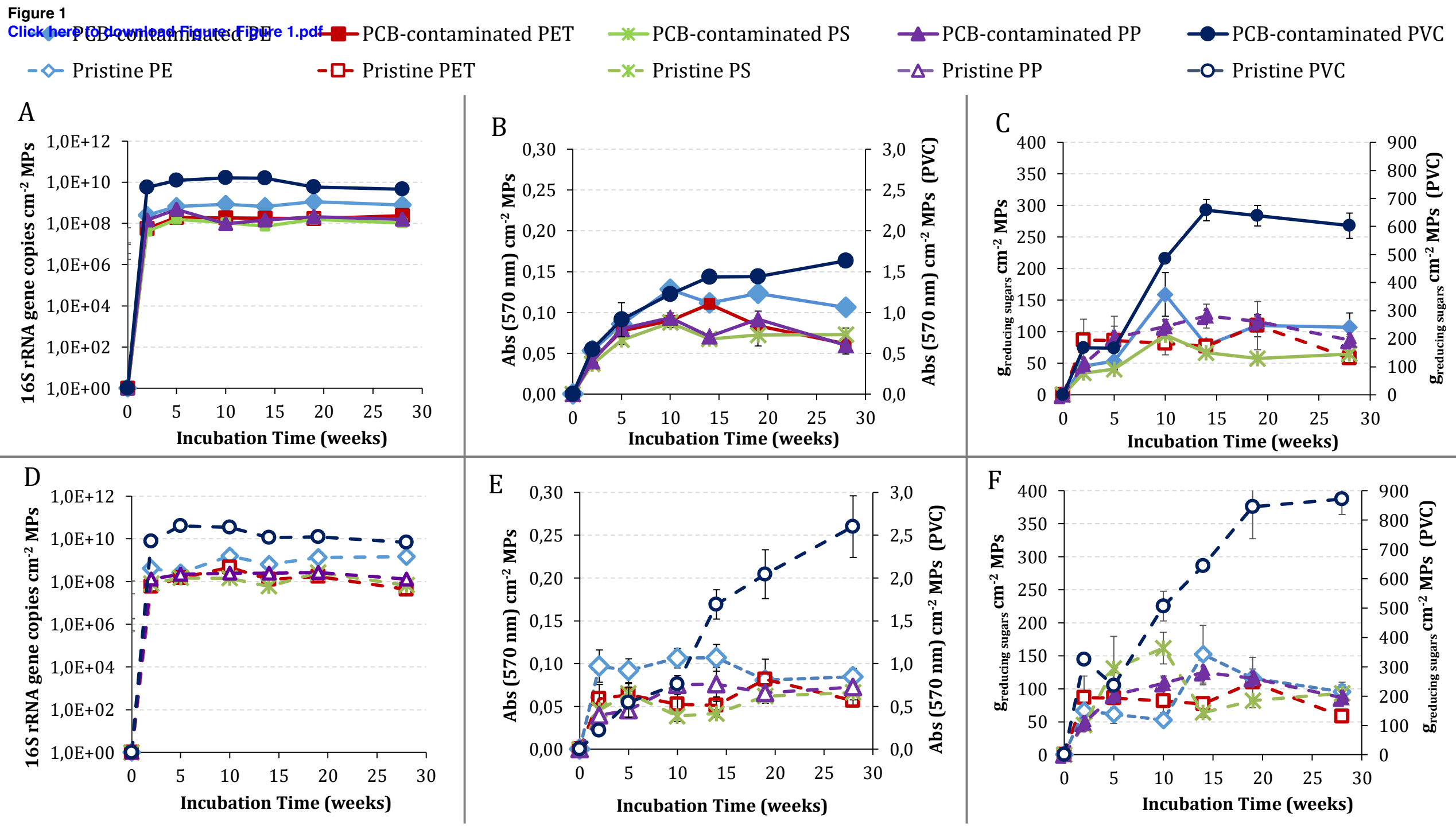
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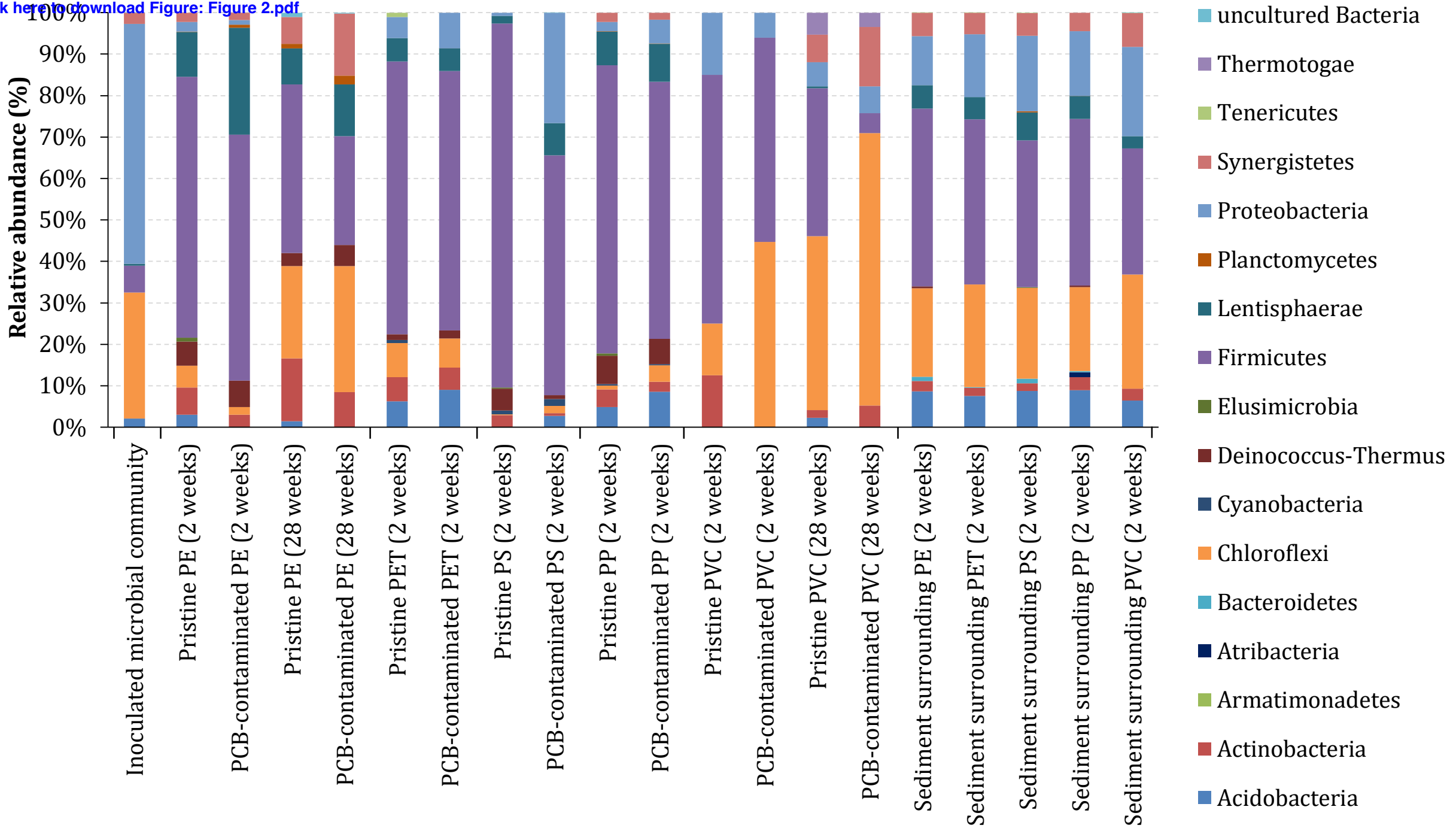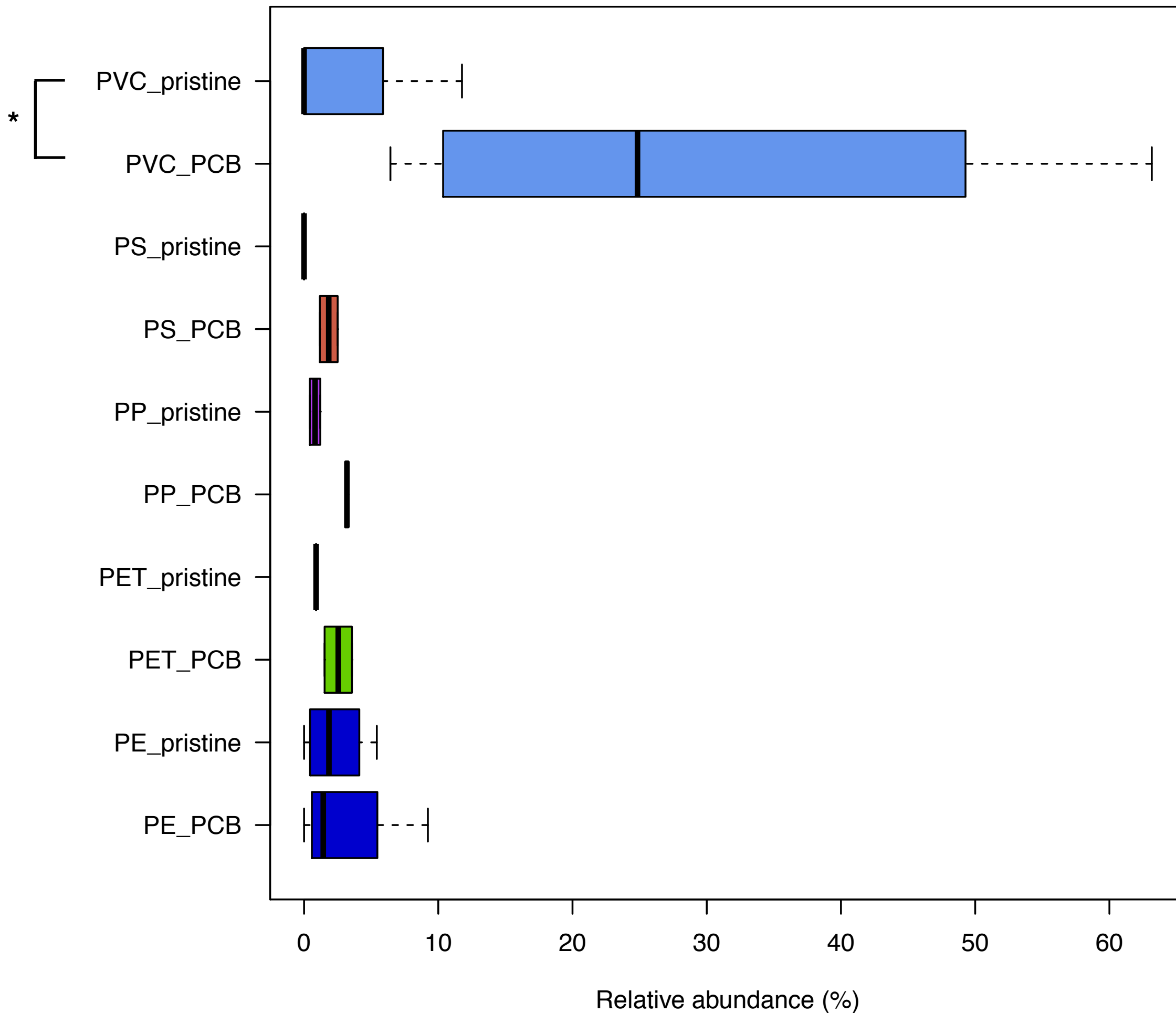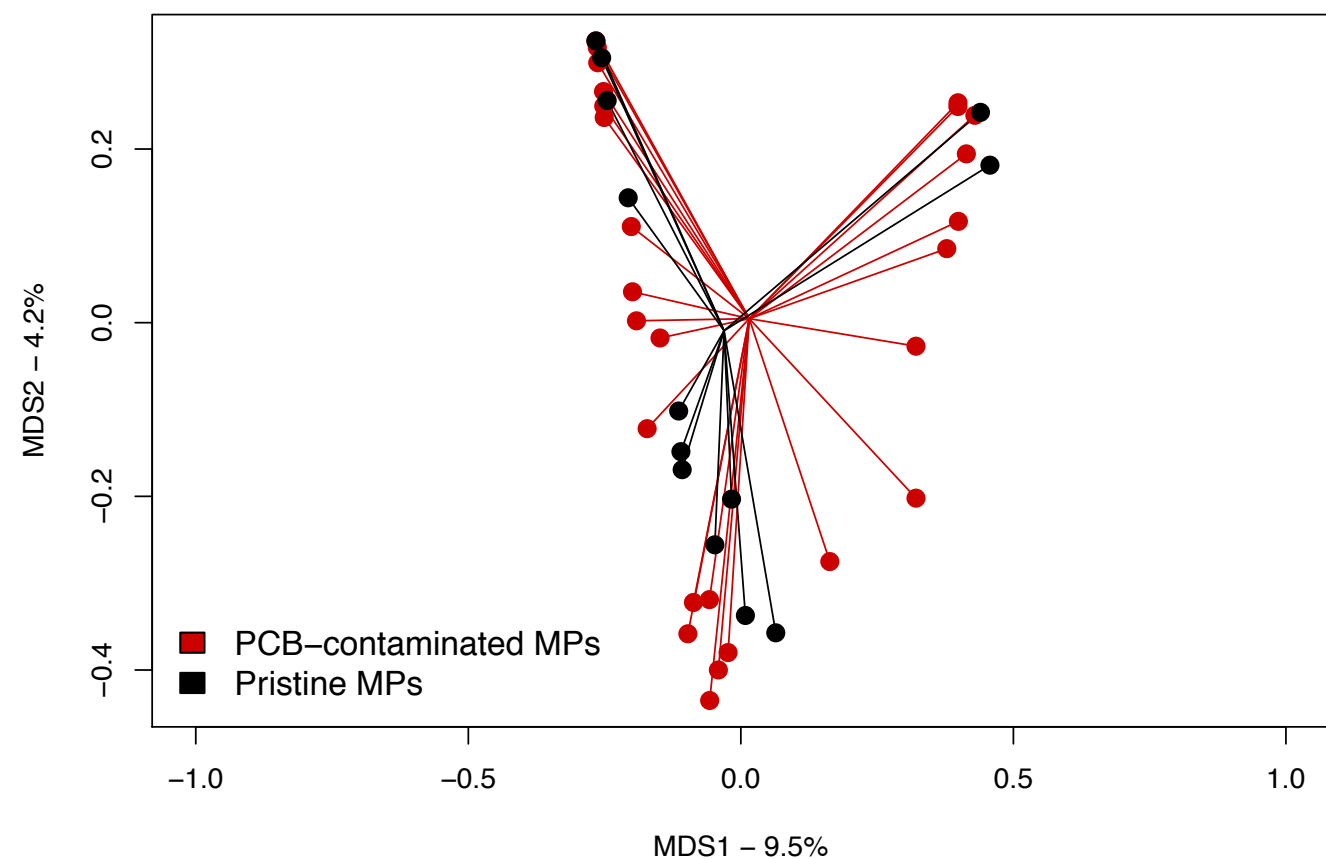

Figure 2

Dehalococcoidia

Figure 4
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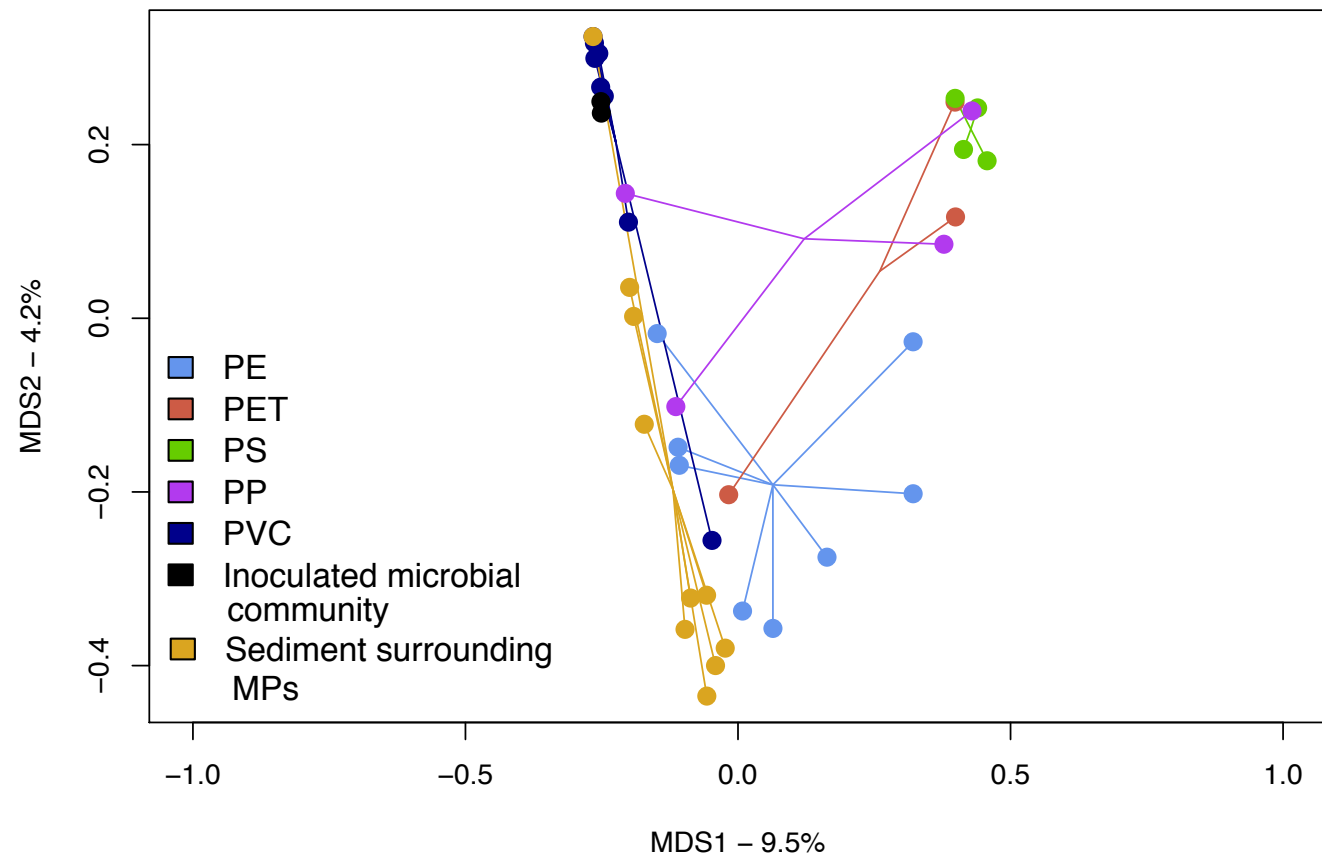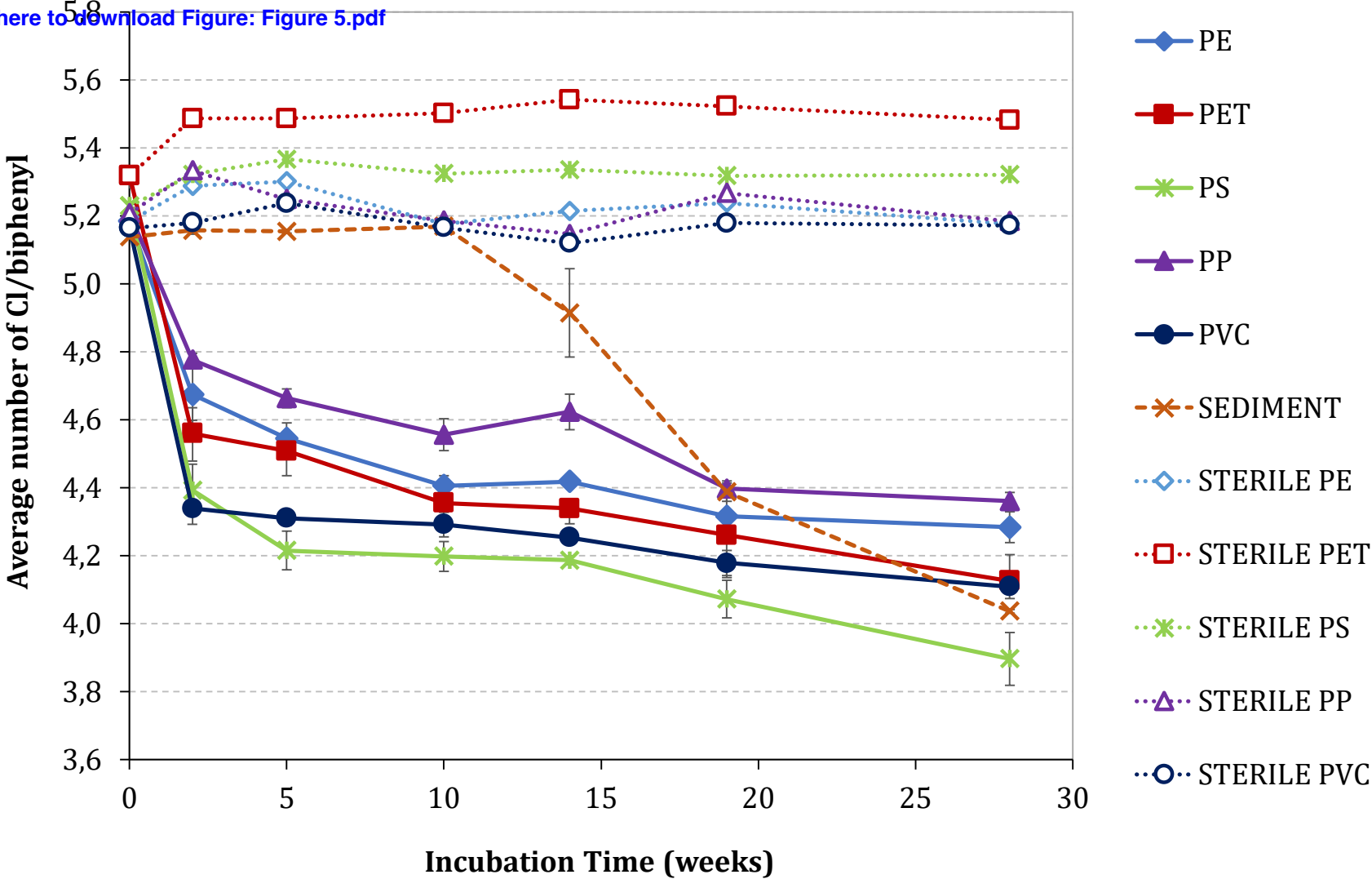
Figure 4

Figure 6
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Figure 6

Figure S1

Figure S2

Table S1

Table S2