

1 Microcosm study reveals the microbial
2 and environmental effects on tributyltin
3 degradation in an estuarine sediment

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9 **Abstract**

10 Tributyltin (TBT) is one of the most harmful contaminants ever released into the aquatic environment.
11 Despite being banned, it is still present at many locations throughout the world. Its degradation in
12 sediment mainly occurs through microbial biodegradation, a process that remains unclear. This study
13 therefore aimed at better understanding TBT biodegradation in estuarine sediment and the microbial
14 community associated with it. Microcosm experiments were set up, embracing a range of
15 environmental control parameters. Major community shifts were recorded, mainly attributed to the
16 change in oxygen status. The highest percentage of degradation (36,8%) occurred at 4°C in anaerobic
17 conditions. These results are encouraging for the *in-situ* bioremediation of TBT contaminated muddy
18 sediment in temperate ports worldwide. However, with TBT able to persist in the coastal environment

19 for decades when undisturbed in anoxic sediment, further research is needed to fully understand the
20 mechanisms that triggered this biodegradation observed in the microcosms.

21 **Keywords**

22 Tributyltin – Biodegradation – Microbial communities – Sediment Resuspension - 16S rRNA.

23 **Introduction**

24 Tributyltin (TBT) is an organotin compound that has been used as a biocide in antifouling paint for
25 years before being globally banned in 2008 due its high toxicity (Sonak et al., 2009). TBT affects the
26 development, reproduction and survival of many marine species, notably through its endocrine
27 disruption properties. Its most notorious effect is to trigger imposex, the masculinisation of female
28 organisms in gastropods (Shi et al., 2005) thus preventing reproduction. Despite its global ban, TBT is
29 still found in sediment around the world (Castro et al., 2018; Egardt et al., 2017), due to its continued
30 use in some countries that did not take part in the ban, illegal manufacturing and use in other countries
31 (Uc-Peraza et al., 2022) and also due to its very high persistence in sediment. In fact, half-lives range
32 from 3 months in the water column to decades in sediment (Langston et al., 2015). TBT amounts
33 recently recorded in sediment vary widely but can be as high as 90 µg TBT/g in Norway (Beyer et al.,
34 2022), 50 µg TBT/g in the Montenegrin Coast (Mikac et al., 2022) or 8 µg TBT/g in the Southern Baltic
35 coastal zone (Filipkowska and Kowalewska, 2019).

36 In the absence of light, biodegradation is supposed to be the major pathway for TBT elimination from
37 the environment, and therefore the main mechanism for the degradation of TBT in sediment (Cruz et
38 al., 2015). Research has focused on better understanding the mechanisms of TBT biodegradation as
39 well as the microbial actors of this degradation. Numerous studies have targeted the isolation of TBT-
40 resistant and TBT-degrading microorganisms (Cruz et al., 2015). Some studies have investigated the
41 factors controlling this biodegradation using microcosm experiments (Beolchini et al., 2014; Cruz et al.,

42 2014; Heroult et al., 2008; Sakultantimetha et al., 2011; Stasinakis et al., 2005; Suehiro et al., 2006) but
43 rarely using the native microbial community of sediment.

44 The vast majority of the studies have concentrated on aerobic biodegradation of TBT as anaerobic
45 degradation is generally considered less efficient. Very long half-lives of TBT in sediment are usually
46 interpreted as a limited or a lack of TBT biodegradation in anoxic conditions (sediment being
47 notoriously anoxic below the first few millimetres). However, some studies recorded TBT degradation
48 in anoxic conditions with reasonably short half-lives and sometimes at a higher rate than in aerobic
49 conditions (Bridou et al., 2018; Sakultantimetha et al., 2010; Shizhong et al., 1989). One of these
50 studies looked at the degradation of TBT by a sediment culture that was previously enriched in aerobic
51 conditions, which therefore selects for aerobic bacteria (Sakultantimetha et al., 2010). They observed
52 the same degradation rate in anaerobic conditions than in unenhanced aerobic conditions, the
53 degradation rate was however higher in enhanced aerobic conditions (Sakultantimetha et al., 2010).
54 Only two studies reported very slow degradation rate of TBT in anoxic sediment compared to aerobic
55 sediment (Brandsch et al., 2001; Furdek et al., 2016). Many variables have to be taken into account to
56 explain the contrasting rates of degradation. First, the matrix changes from study to study (sediment,
57 sludge, culture medium, soil), but also the anaerobic conditions tested are not always controlled and
58 identified (sulphate reducing, nitrate reducing, fermentative or methane producing). Finally, countless
59 other parameters can be considered such as temperature, organic and inorganic nutrient levels,
60 texture of soil and sediment, etc. These parameters can, for example, influence TBT adsorption to
61 sediment (Fang et al., 2017), which will likely modify its bioavailability. Considering the complexity
62 involved, many more studies are needed to clarify the factors influencing TBT biodegradation,
63 especially in sediment.

64 In aerobic conditions, some studies have focused on the influence of diverse factors like pH,
65 temperature and nutrient addition (Beolchini et al., 2014; Cruz et al., 2014; Sakultantimetha et al.,
66 2011, 2010). However, the number of studies remain limited compared to quantity of factors involved.

67 No studies have investigated the microbial taxa associated with TBT-contaminated sediment and only
68 one study to date has investigated the shifts in the composition of microbial communities along TBT-
69 biodegradation experiments over time (Cruz et al., 2014).

70 The present study aims to address some of these gaps and therefore investigate the factors controlling
71 TBT biodegradation by the native microbial community of estuarine sediment within a microcosm
72 experiment. The capacity of degradation of the native community of Liverpool Docks sediment was
73 investigated as well as the influence of nutrient addition on this capacity. Temperature in accordance
74 with environmental temperature were chosen for the experiment. In addition, anaerobic degradation
75 could be studied due to the pause of the experiment during the national lockdown imposed by Covid-
76 19 pandemic. Insights into the microbial community evolution throughout the TBT-degradation
77 experiments was obtained through 16S rRNA gene sequencing.

78 **Material and methods**

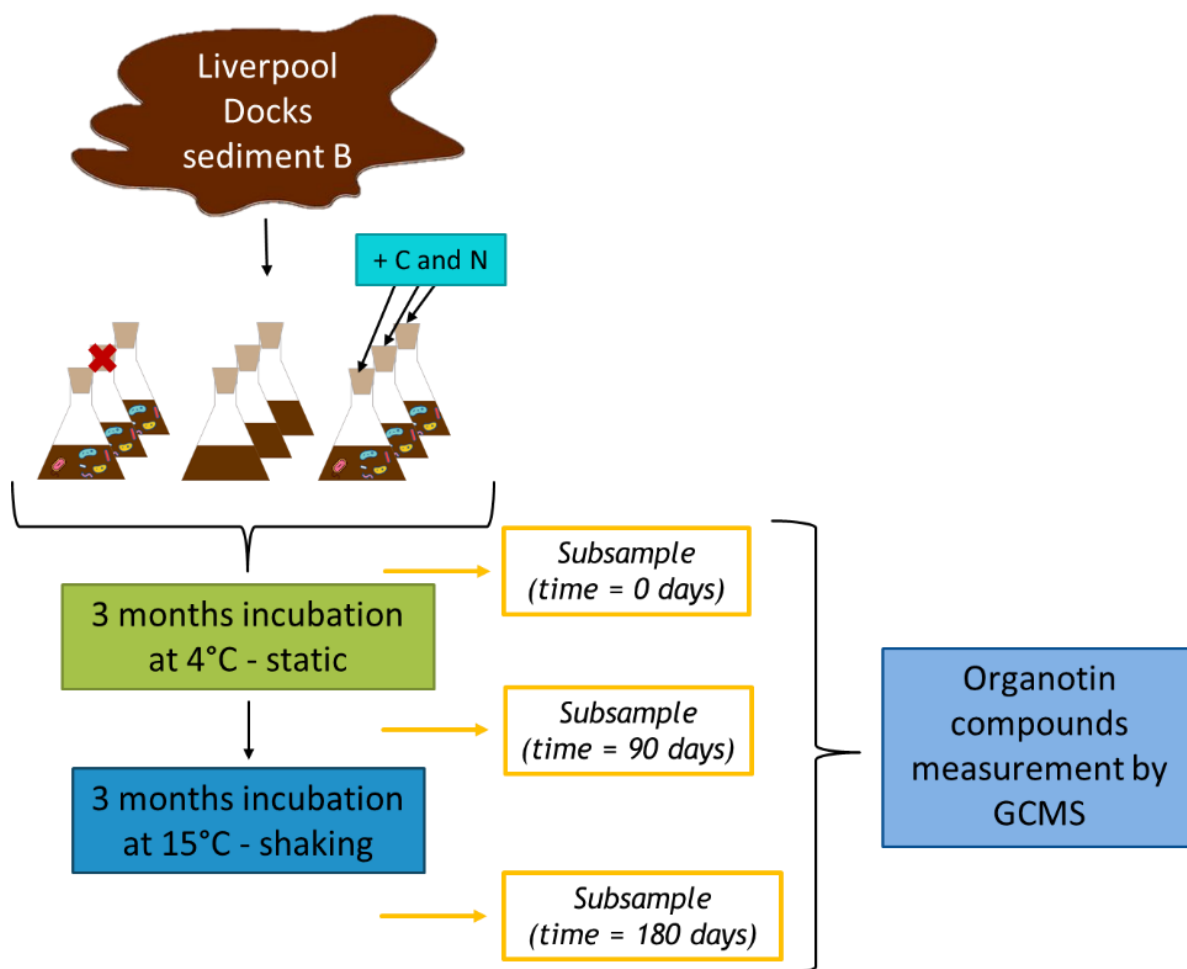
79 *Microcosm study*

80 Sediment samples were taken at Sandon Docks in Liverpool in October 2019. The first 20 centimetres
81 of the sediment layer were taken with a grab sampler (Supplementary figure S1) and transported via
82 plastic buckets to the laboratory for storage at 4°C the same day as sampling. At each sampling
83 location, 2 L of seawater was sampled as well. Sediment was characterised as 'slightly sandy mud'
84 (Flemming, 2000) and its physicochemical characteristics are described in Supplementary table S1. The
85 temperature of sediment at the time of sampling was 8°C. Sampling locations in the docks were chosen
86 according to TBT contamination data from 2010 (data provided by Peel Ports). Analyses revealed that
87 the organotin compounds levels were below detection limits at the time of sampling, which could be
88 explained by the fact that the previous contamination was present in localised hotspots that were
89 missed during our sampling campaign or suggest a capacity of the indigenous microbial community to
90 degrade TBT. This latter hypothesis was also supported by the isolation of TBT-degrading bacteria from
91 the same sediment, as described in a previous study (Polrot et al., 2022).

92 Sediment was first sieved at 2mm. To standardize its density at 1.18 g.cm^{-3} , it was resuspended with
93 excess water and left for 24 hours after which the water remaining above the sediment layer was
94 removed. The sediment was then spiked with tributyltin chloride (Sigma-Aldrich) at $10 \mu\text{g}$ per gram of
95 dry sediment ($\mu\text{g/g}$ sed dw), physically mixed by hands in the sampling container and allowed to
96 equilibrate for four weeks at 4°C . Long equilibration steps are recommended for the stabilization of
97 binding and partitioning of spiked compounds (Northcott and Jones, 2000). After TBT equilibration, a
98 part of the mud was spiked with 5 g/kg dw of glucose (Sigma-Aldrich) and 5 g/kg dw of ammonium
99 nitrate (Sigma-Aldrich). Glucose and ammonium nitrate were added as a readily available source of
100 carbon and nitrogen in an attempt to boost microbial activities. On the first day of incubation, triplicate
101 250 mL conical flasks were filled up with 150 mL of amended and non-amended mud. For the sterile
102 microcosms used as a control (stated as “abiotic” in the rest of the manuscript), triplicate flasks were
103 filled up a few days before the experiment and three consecutive autoclave cycles at 121°C for 40
104 minutes were done, the flasks were kept in the cold room (4°C) until the start of the experiment.

105 On the first day of the experiment, subsamples of 15 g were taken, and the flasks were incubated for
106 3 months in the cold store at 4°C , then another set of subsamples were taken before the flasks were
107 put in a shaking incubator for 3 months at 15°C . A last set of subsamples were taken at the end of this
108 second incubation period. At each subsampling event, for the abiotic flasks, a control of the microbial
109 contamination was also performed by plate count on tryptic soy agar medium; no contamination was
110 observed throughout the experiment. All the subsamples were freeze-dried immediately and kept in
111 the freezer at -20°C before further processing. A summary of the microcosms set-up can be seen in
112 Figure 1.

113



114

115 **Figure 1: Microcosm experiment set-up using sediment from Liverpool Docks.** The sediment was spiked with 10 µg TBT/g
116 sed dw prior to the experiment. Organotin compounds were analysed in all the subsamples and microbial communities were
117 analysed for the subsamples coming from the amended flasks and from the native sediment used to set-up these microcosms.
118 The red cross represents the loss of a flask after the 3 months incubation at 4°C, for these flasks the only analyses performed
119 were the organotin measurements.

120

121 Organotin measurement:

122 Organotin compounds were measured using a method inspired from (Okoro et al., 2012) and fully
123 described in Polrot (2022). A known amount of approximately 1 g of freeze-dried sediment, weighed
124 on an analytical scale, was placed in a fresh 50 mL Duran bottle and 20 mL of acetic acid in methanol

125 (3:1) was added. The mixture was sonicated for 20 minutes, then left 5 minutes to stand. 1 mL of
126 supernatant (sediment extract) was transferred to clean 50 mL Duran bottles. To this 1 mL of sediment
127 extract or to 1 mL organotin standards in acetic acid:methanol (3:1), 20 mL of 1 M acetate buffer pH
128 5.4, 200 μ L of 10 μ g/L internal standard (triphenyltin chloride), 200 μ L of 0.2% NaBet4 and 1.5 mL of
129 trimethylpentane were added. The bottles were then shaken for 30 minutes on a mechanical shaker.
130 The trimethylpentane layer laying on the top of the aqueous phase and containing the analytes of
131 interest (TBT and any associated degradation products) was then recovered in vials for further analyses
132 by gas chromatography mass spectrometry (GCMS) using the parameters detailed in the
133 supplementary material (Supplementary table S2 and S3). Technical duplicates of the samples were
134 measured, and each analysis was performed with a mix of tributyltin (TBT), dibutyltin (DBT) and
135 monobutyltin (MBT) standards for calibration purposes (0.05 to 0.5 μ g/ml). The analysis was retained
136 when the regression coefficient R^2 of the standard curve was greater than 0.995. Analysis was also
137 performed with an in-house reference sediment (4 μ g/g dw sediment) in triplicate along with the
138 samples in order to calculate recovery percentages which were used to correct the samples values.
139 Concentrations are expressed as μ gTBT/g; μ gDBT/g and μ gMBT/g. Average sample recoveries
140 obtained for TBT, DBT and MBT were 97.1%, 98.6% and 84.5% respectively. Detection limits were
141 calculated at 0.02 μ g TBT / g sed dw, 0.01 μ g DBT/g sed dw and 0.02 μ g MBT/g sed dw.

142 16S rRNA gene amplicon sequencing:

143 DNA was extracted from sediment coming from the sampling container directly after sampling and
144 from the amended microcosm subsamples at the different steps of incubations (after equilibration,
145 after the incubation at 4°C and after the incubation at 15°C) using a known amount of approximately
146 250 mg of sediment, weighed on an analytical scale, and the DNeasy Power Soil Kit (Qiagen) following
147 the manufacturer's instructions. DNA concentration was measured using the Qubit High Sensitivity
148 (Thermofisher). DNA was normalized at 1 ng/ μ l prior to PCR amplification. 16S rRNA genes were
149 amplified using primers 515F (Parada et al., 2016) matched to 806R (Aprill et al., 2015) followed by

150 library preparation and sequencing on an Illumina MiSeq platform with MiSeq Reagent Kit V2 as
151 previously described (Haendiges et al., 2021) The sequencing data were analysed using the ASV-based
152 pipeline through the DADA2 tool (v.1.1.6) (Callahan et al., 2016) with R (v.3.5.1). Briefly, primer
153 sequences and low quality-reads were trimmed using filterAndTrim function trimLeft=c(19,20),
154 truncLen=c(240,160). After removing the reads with expected errors using learnErrors function, the
155 reads were merged using mergePairs function. Chimera sequences were removed using
156 removeBimeraDenovo function. Taxonomic assignment of representative amplicon sequence variants
157 (ASVs) was then carried out using assignTaxonomy function with SILVA REF database (v.138).

158 Statistical analyses:

159 Statistical analyses were performed using the vegan package in R (Oksanen et al., 2015). Alpha diversity
160 indices including Shannon, Simpson and richness were calculated using the diversity and specnumber
161 function, respectively. Prokaryotic community structure of each sample was analysed with Principal
162 Coordinates Analysis (PCoA) using Bray-Curtis dissimilarities calculated using the metaMDS function
163 with ASV relative abundance table. Permutational multivariate analyses of variance (PERMANOVA)
164 were performed on Bray-Curtis dissimilarities with the adonis function. A heatmap of the relative
165 abundance of ASVs at phylum level was generated with the microbiomeutilities R package.

166 **Results**

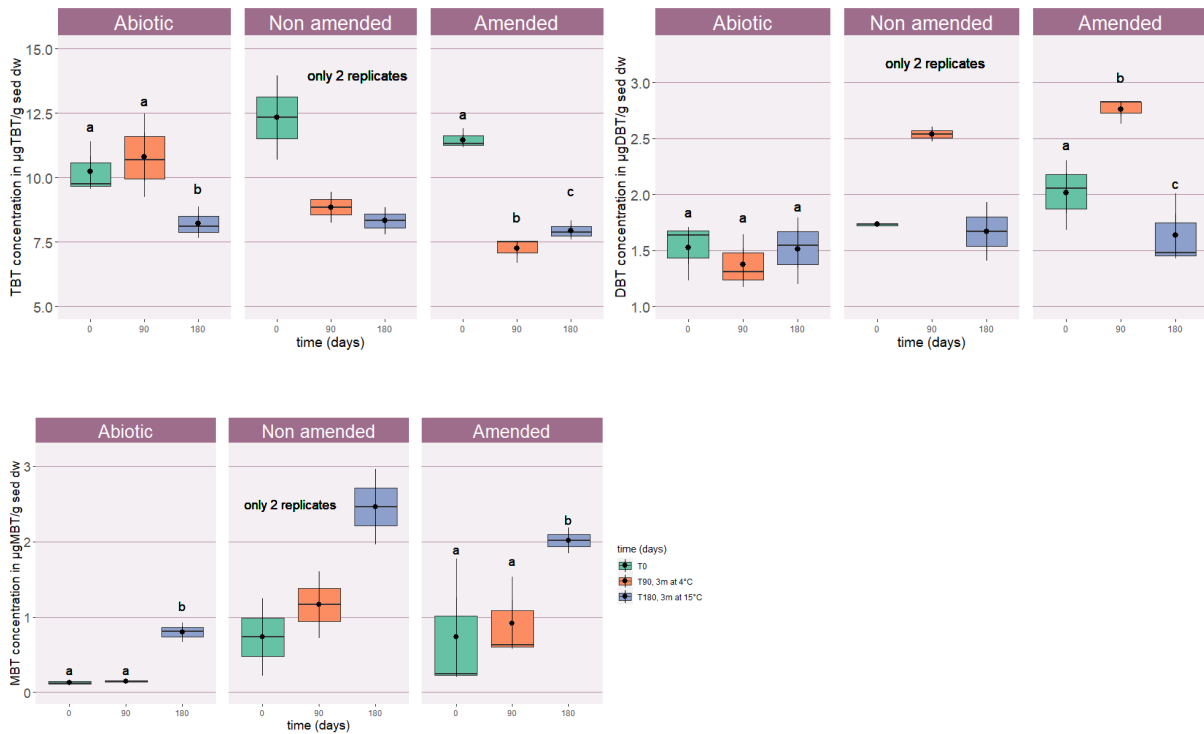
167 Temporal variation in organotin compounds in the microcosms:

168 Tributyltin (TBT) concentration significantly decreased by $36.8 \pm 4.0\%$ in the amended septic flasks and
169 by $27.6 \pm 6.6\%$ in the non-amended septic flasks incubated for 3 months at 4°C compared to no
170 degradation in the sterile flasks incubated in the same condition (Figure 2). This biodegradation was
171 matched by an increase of dibutyltin (DBT) and not monobutyltin (MBT).

172 During the following 3 months of incubation at 15°C a slight but statistically significant increase of TBT
173 ($9.7 \pm 4.5\%$) was recorded in the amended flasks but a decrease of DBT matched with an increase of

174 MBT. In the non-amended flasks, this increase was not recorded and a decrease of $33.9 \pm 10.3\%$ of DBT
 175 occurred, also matched with an increase of MBT. At this temperature, a decrease of TBT by $20.1 \pm 3.5\%$
 176 was observed in the sterile flasks, accounting for abiotic degradation. This decrease was matched with
 177 an increase of MBT.

178



179

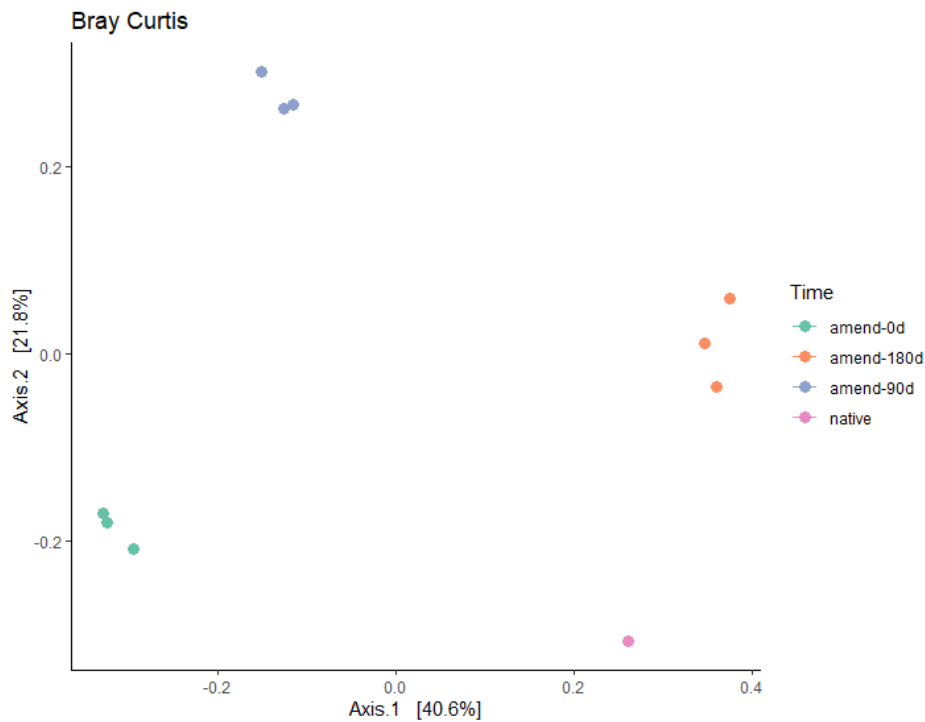
180

181 **Figure 2: Tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) concentration along the microcosm experiments.**

182 Statistical significance under the Student's t-test is indicated by letters. Differing letters indicate a statistically significant
 183 difference in mean between the samples of the same condition ($P < 0.05$), the same letters indicate that there is no statistical
 184 difference in mean between the samples of the same condition.

185 Microbial community analyses:

186 Principal coordinate analysis (PCoA) of prokaryotic community composition shows significant
 187 correlation between the different times of the microcosms (Figure 3), with microbial communities
 188 appearing as three distinct clusters.



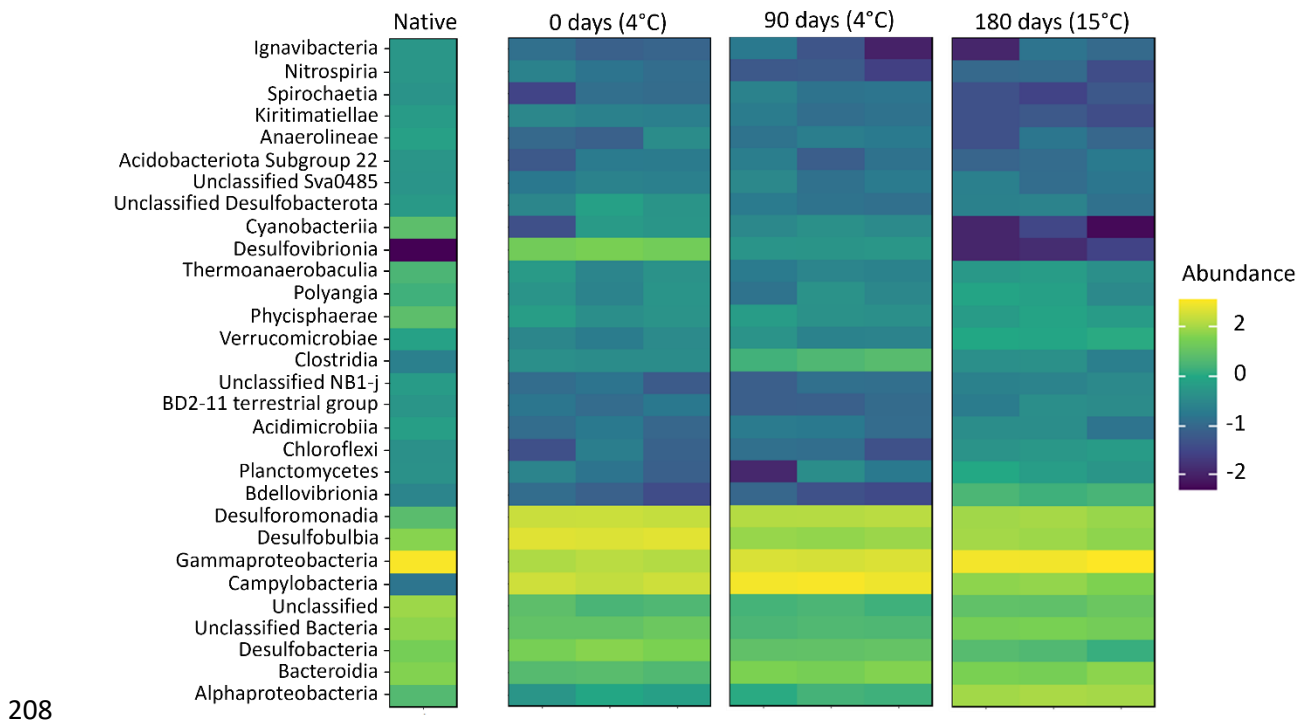
190

191 **Figure 3: Principal coordinate analysis (PCoA) of prokaryotic community structure based on Bray-Curtis dissimilarities**
 192 **derived from the relative abundance of amplicon sequence variants.** Each colour represents a group of subsamples from
 193 amended microcosms as described in Figure 1 (triplicates subsamples were taken at day 0, 90 and 180 of the experiment),
 194 the sediment at the time of sampling is also represented as "native".

195

196 A change in community composition was observed between the different treatments of the microcosm
 197 experiment (Figure 4). The abundance of *Campylobacteria*, *Desulfobulbia* and *Desulfomonadia*
 198 increased respectively by a factor of 116, 3.4 and 6.64 after TBT spiking and equilibration for one month
 199 at 4°C. During this step, *Desulfovibrionia* also became detectable in the flasks. On the contrary the
 200 abundance of many other taxa decreased, such as *Cyanobacteria*, *Bacteroidia*, *Alphaproteobacteria*,
 201 *Nitrospira*, *Acidimicrobiia*, *Phycisphaera*, which decreased by a factor of 7, 2.9, 2.9, 3.2, 6, 5.3 and 5.4
 202 respectively. After carbon and nitrogen amendment and an incubation at 4°C for 3 months without
 203 shaking, the abundance of *Clostridia*, *Bacteroidia*, *Bacilli* increased respectively by a factor of 5.3, 2.7,
 204 and 30.7 whereas the abundance of *Desulfovibrionia* decreased by a factor of 10.4. Finally, after 3

205 months of incubation at 15°C with shaking, the abundance of *Alphaproteobacteria*, *Bdellovibrionia*,
 206 *Chloroflexi* increased by a factor of 7.5, 32.5 and 5.8 whereas the abundance of *Campylobacteria*,
 207 *Desulfovibrionia*, *Clostridia*, *Bacilli* decreased by a factor of 4.3, 47.3, 5.7 and 21 respectively.



209 **Figure 4: Heatmap of the relative abundance of the 30 taxa that varied the most along the microcosm experiment in the**
 210 **amended microcosms.**

211 Alpha diversity as shown by both Shannon and Simpson indices (Table 1) displayed a decrease after
 212 the equilibration and tributyltin spiking step. It then remained stable during the incubation at 4°C but
 213 increased during the incubation at the 15°C stage.

214

215 **Table 1: Alpha diversity indices including Shannon-Weiner's (Shannon) and Simpson's diversity indices in the amended**
 216 **microcosms**

	SHANNON	SIMPSON
NATIVE	6,082	0,993
0 DAYS	4,404 ± 0,110	0,956 ± 0,004

90 DAYS	4,503 ± 0,033	0,945 ± 0,009
180 DAYS	5,359 ± 0,064	0,988 ± 0,006

217

218

219 **Discussion**

220 *Biodegradation activity:*

221 Several studies have explored the impact of different factors on Tributyltin (TBT) biodegradation but
 222 the degradation at low temperature and the influence of oxygen status on biodegrading activity by
 223 native sediment communities in sediment microcosms has never been investigated. In a study of TBT
 224 in water microcosms Sakultantimetha et al. (2011) found an increase in biodegradation activity with
 225 temperature, until a plateau was reached at 28°C. In the present study, the effect of temperature
 226 contrasted with the expectations. As anticipated abiotic degradation, which was observed in the
 227 abiotic flasks and most likely resulted from chemical cleavage, seemed to be positively influenced by
 228 higher temperatures. However, this study is the first to report a biodegradation of TBT at 4°C. TBT
 229 degradation appears to be supported by psychrophilic microorganisms in the sediment used. A
 230 temperature of 4°C is close to the ambient winter temperature of sediment in Liverpool and reveals
 231 an intrinsic biodegradation potential of the natural community present in the Docks. Studies on TBT
 232 adsorption to sediment suggest that it increases for marine and estuarine sediment with increasing
 233 temperature in the range of 20°C to 40°C (Bangkedphol et al., 2009), meaning that its bioavailability
 234 would decrease with increased temperature. This phenomenon could explain the observations of TBT
 235 degradation in the present study but cannot explain the contrast with other studies.

236 The majority of the studies used room temperature or above (25-28°C) as a standard for their TBT
 237 biodegradation microcosm experiments. For some of them, this temperature is relevant as they used
 238 sediment from warm locations where the sea bottom temperature spans around 20-35°C all year

239 round (Suehiro et al., 2006; Yonezawa et al., 1994). But for others, the sediment originated from
240 Scotland, Portugal or Italy, where the sea bottom temperature can be below 10 °C in the winter
241 (Beolchini et al., 2014; Cruz et al., 2014; Sakultantimetha et al., 2011). These new results are
242 encouraging as they demonstrate TBT degradation at a temperature closer to natural conditions in
243 temperate climates. However, these results also demonstrate that factors other than temperature
244 have a significant influence on TBT biodegradation rates.

245 The explanation for this contrast with the other studies most likely lies in the oxygen status of the
246 microcosms. Preliminary experiments showed a rapid decrease in dissolved oxygen in the microcosms
247 in the absence of agitation (Supplementary table S4). Sediment in the environment is notoriously
248 anoxic after a few millimetres of depth. Little is known about TBT biodegradation under anaerobic
249 conditions and the process is generally considered inefficient and slow. The vast majority of microcosm
250 studies therefore focus on aerated conditions. The few studies which focused on TBT anaerobic
251 biodegradation reported contrasting results. One precursor study observed TBT degradation in sludge
252 fermenters at 20°C, biodegradation was faster in anaerobic conditions compared to aerobic conditions
253 with respective half-lives of 3 and 5 days (Shizhong et al., 1989). Another study described TBT
254 biodegradation in nitrate reducing conditions but did not make a comparison with aerobic
255 biodegradation (Yonezawa et al., 1994). Other studies reported nearly inexistant degradation in
256 anaerobic condition such as the study from Voulvoulis et al. (2006) or another study that reported a
257 rate of TBT biodegradation so slow that it was impossible to calculate half-lives after 12 months
258 (Brandsch et al., 2001). In 2010, Sakultantimetha *et al.* experimented on TBT degradation in water
259 microcosms, using a microbial community enriched from sediment under aerobic conditions, and
260 performed the microcosms in both anaerobic, unenhanced aerobic and enhanced aerobic condition.
261 The authors recorded a faster degradation in enhanced aerobic conditions (half-life of 3.2 days), but
262 still recorded a similar degradation rate for anaerobic compared to unenhanced aerobic conditions
263 (half-lives of 8.16 and 9.5 days respectively), despite selecting for aerobic bacteria during the
264 enrichment of the sediment culture (Sakultantimetha et al., 2010). Finally, a more recent study

265 evaluated the capacity of different strains to biodegrade TBT in different oxygenation conditions and
266 recorded significant degradation rates in fermentative and sulphate reducing conditions by a
267 *Desulfobulbus propionicus* strain (respective half-lives of 85.5 and 16 days) and in nitrate reducing
268 conditions by a *Pseudomonas sp.* Strain (half-life of 18.6 days) at 30°C (Bridou et al., 2018). This
269 literature and the present study demonstrate not only that TBT biodegradation occurs in anaerobic
270 conditions, but that the resulting half-lives can approach those of aerobic biodegradation. The many
271 other environmental factors involved (pH, organic matter content, temperature, texture, etc.) can
272 explain the contrasting biodegradation rates between studies at the same oxygen status. Therefore,
273 future work should pay close attention to anaerobic biodegradation of TBT and its coproducts.

274 In the present study however, dibutyltin (DBT) biodegradation displayed a different trend, with more
275 degradation (biotic and abiotic) at 15°C under agitation compared to static at 4°C. This has to be taken
276 into account in a remediation context as DBT is also recognised to be toxic (Ferreira et al., 2013; Zhang
277 et al., 2018). Whilst the toxicity seems to reduce significantly for MBT (Ferreira et al., 2013; Marin et
278 al., 2000), the two compounds have been less subjected to toxicity studies than TBT. The accumulation
279 of MBT in both scenarios isn't surprising as MBT accumulation during TBT degradation experiment is
280 often reported in the literature and this last step of debutylation seems to display a considerably higher
281 kinetic constant (Beolchini et al., 2014).

282 The evidence, both in this and other studies demonstrating non-negligible TBT biodegradation in
283 anaerobic conditions contrasts with the records of high levels of TBT persistence in sediment around
284 the world. However, the varying conditions between environmental sediment are numerous and could
285 explain some of the disparities. The type of anaerobic condition has also to be taken into account, as
286 shown by Bridou *et al.* (2018) and Yonezawa *et al.* (1994). TBT biodegradation rate can differ between
287 fermentative, nitrate-reducing, methane producing and sulphate reducing conditions. In addition,
288 many other environmental factors have been shown to influence TBT partitioning behaviours in
289 sediment and therefore affect its bioavailability (Fang et al., 2017). For example, Furdeck (2016)

290 investigated organotin persistence in contaminated marine sediment and concluded that the limiting
291 step for TBT biodegradation was desorption into the porewater (rather than microbial activity). The
292 study also showed that TBT persistence increases for sediment rich in organic matter (Furdek et al.,
293 2016). The implications are that for locations where TBT persists, the native community may as well
294 display an intrinsic capacity to degrade TBT at the environmental level of oxygen and temperature and
295 the key for bioremediation would be to increase TBT bioavailability. For the case of high persistence
296 due to the adsorption on the high amounts of organic matter, promoting organic matter degradation
297 could boost TBT biodegradation at the same time for example.

298 The only effect of carbon and nitrogen amendment that could be observed in this study is the slight
299 increase in TBT in the amended flasks after the incubation at 15°C compared to no change in the non-
300 amended flasks. This increase can only be explained by the release of glass-adsorbed TBT in the
301 amended flasks at 15°C. The fact that it does not occur in the non-amended flasks could mean that TBT
302 degradation was faster in these latter, masking this increase. Further targeted research is necessary to
303 investigate this potential phenomenon.

304 Microbial communities:

305 The decrease of alpha diversity after TBT spiking is not surprising as it will have killed a consequent
306 part of the population (Table 1). In addition, storage under static conditions after spiking will have
307 triggered a drop in dissolved oxygen which is also associated with a decrease in diversity (Broman et
308 al., 2017). This diversity only increased significantly during the incubation at a warmer incubation and
309 under agitation, which suggests that these parameters are more favourable to community resilience.

310 The significant difference in community structure between microcosm steps (Figure 3) highlights the
311 influence of TBT-spiking, shaking and temperature on microbial community structure. It is not
312 surprising that spiking high concentrations of TBT and storage at 4°C for one month induces a
313 community change. The same can be said for supplementing with carbon and nitrogen and
314 resuspending the sediment, as well as raising the temperature of incubation to 15°C under shaking and

315 therefore oxygenation as all of these parameters have previously been reported to influence microbial
316 community structure (Broman et al., 2017; Li et al., 2019; Pala et al., 2018; Sinkko et al., 2019).

317 The taxa that increased during the equilibration step were all composed of either microaerophilic (e.g.
318 *Campylobacter*) or anaerobic bacteria (e.g. *Desulfobulbia*, *Desulfomonadia*, *Desulfovibrionia*) (Figure
319 4), which are likely to be favoured by the decrease in dissolved oxygen that should occur in static
320 sediment during the equilibration step. This dissolved oxygen drop also explains the decrease in the
321 abundance of many other taxa that include aerobic bacteria, but the addition of TBT is likely to be
322 responsible for most of the observations as it will have killed a major part of the microbial population.
323 The decrease in *Cyanobacteria* could also be attributed to the absence of light as they are
324 photosynthetic.

325 After the amendment with carbon and nitrogen and incubation of the microcosms for 3 months at 4°C
326 without shaking, which corresponds to the step at which TBT biodegradation was recorded, the
327 increase in abundance of *Clostridia*, *Bacteroidia*, *Bacilli* and *Alphaproteobacteria* (Figure 4) could have
328 a different explanation. A part of the community may slowly adapt to the presence of high
329 concentrations of TBT (and may be helped by its degradation at the microscale) and start displaying
330 some resilience after the strong perturbation that represented the addition of TBT. Nutrient addition
331 could also have boosted the growth of these taxa. This may be the case for *Clostridia* for example as
332 they are strict anaerobes and the addition of nutrients will have caused a rapid decrease in dissolved
333 oxygen concentration in the absence of flask shaking (to initiate aeration) and may have favoured their
334 development.

335 When the microcosms were put at 15°C under agitation, another community shift could be recorded
336 (Figure 4), most likely attributed to the change from anaerobic to aerobic condition imposed by shaking
337 but also to the increase in temperature.

338 The studies linking microbial community and TBT biodegradation are scarce and even rarer for
339 anaerobic biodegradation. One of the few studies investigating TBT biodegradation in anaerobic

340 conditions notably demonstrated the ability of a bacteria belonging to *Desulfobulbia* to degrade TBT
341 under sulphate reducing conditions (Bridou et al., 2018). It is therefore possible that members of this
342 class, which was enriched after the equilibration step of the microcosms, were responsible for TBT
343 biodegradation in the present study. In the same study the other important strain used for TBT
344 degradation was a *Pseudomonas* member. Different *Pseudomonas* strains were repeatedly isolated in
345 TBT degradation studies (Cruz et al., 2015) and were also isolated from the microcosms of the present
346 study (Polrot et al., 2022). In the microbial community analysis, the class *Gammaproteobacteria* which
347 holds the *Pseudomonas* genus are relatively abundant throughout the entire experiment. They could
348 also account for a part of the degradation recorded at 4°C but were apparently inactive at 15°C in
349 aerobic conditions.

350 *Campylobacteria* were found to increase considerably during the experiment, this group mostly consist
351 of microaerophilic sulphur oxidising bacteria which are often found to increase following the
352 oxygenation of anaerobic sediment (Broman et al., 2017; Ihara et al., 2017). In the present study,
353 oxygenation of the anaerobic sediment will have occurred at the beginning of each phase, which could
354 explain the significant raise in *Campylobacteria*. In the environment, such a proliferation is usually
355 regarded as beneficial as SOB's are able to eliminate the toxic hydrogen sulphide (H₂S) produced by
356 sulphate reducers (Broman et al., 2017). *Campylobacteria* are not however particularly associated with
357 biodegradation activities. Their role in eliminating H₂S is nonetheless interesting as some of the other
358 taxa that increased during the static incubation steps, the steps at which TBT degradation was mostly
359 observed, are sulphate reducers (e.g. *Desulfobulbia*, *Desulfomonadia*, *Desulfovibrionia*). *Bacilli* is also
360 a taxa that increases substantially during the static incubation at 4°C, and although they were not
361 necessarily noticed in TBT degradation studies, members of this class are known to degrade other
362 sediment pollutants, in particular petroleum hydrocarbons which is biodegraded in anaerobic
363 conditions by *Bacillus spp* (Kaida et al., 2018).

364 *Potential for in-situ TBT bioremediation*

365 The level of TBT biodegradation recorded in this study is encouraging for the *in-situ* bioremediation of
366 TBT-contaminated sediment as the majority of today's contaminations do not exceed these amounts
367 or are even well below (Abreu et al., 2020; Kuprijanov et al., 2021; Metelkova et al., 2022). For other
368 locations with high TBT contamination however, the observed rate of degradation may not be
369 sufficient to achieve the degradation in an acceptable timescale. A study of the kinetics of degradation
370 would also be interesting here to determine whether the degradation reached a threshold or not. In
371 fact, a part of the TBT could be in a non-bioavailable form and therefore persist over time. This
372 phenomenon could also explain TBT's high persistence in anoxic sediment despite the existence of
373 anaerobic biodegradation.

374 In theory, TBT bioremediation would be useful for many locations around the world as TBT is still
375 recorded in most locations where intense maritime traffic occurs, with for example some of the
376 strongest contaminations ($> 2 \mu\text{g TBT/g sed dw}$) observed in Norway (Beyer et al., 2022), the
377 Montenegro coast (Mikac et al., 2022), or in Korea (Lam et al., 2017). TBT biodegradation at low
378 temperature is especially relevant for the sediment of temperate and cold regions but further research
379 could show that this anaerobic degradation is also efficient at warmer temperature, which was not
380 tested in the present study.

381 TBT contamination is often found in environments holding a cocktail of other contaminants like
382 petroleum hydrocarbons, polycyclic biphenyls or heavy metals. These other contaminants are also
383 subjected to much research in term of biodegradation and some of them have been studied for
384 anaerobic degradation in sediment at low temperature. A review discussing the use of extremophile
385 microorganisms in bioremediation of metal and organic pollutant highlights studies about polycyclic
386 aromatics hydrocarbons (PAHs) or petroleum hydrocarbon degradation by psychrophilic organisms but
387 notes the small number of studies on PAHs anaerobic degradation (Giovanella et al., 2020). Another
388 review discussing the recent advances in bioremediation strategies for oil-contaminated soil and
389 sediment in cold-environments also highlight the importance of recent findings on the use of

390 psychrophilic microorganisms and encourages more research in this field (Chaudhary and Kim, 2019).
391 This same review also underlines the importance in advancing knowledge on anaerobic degradation
392 of pollutants for the application of bioremediation in cold regions as aerobic degradation usually lacks
393 efficiency in such locations because of the limited availability of oxygen.

394 **Conclusion**

395 This study presents the first analysis of the native sediment microbial community during anaerobic
396 degradation of TBT in microcosms. Various bacteria could be responsible for TBT biodegradation in the
397 microcosms such as *Desulfobulbus* or *Pseudomonas* members. It is also the first study to record TBT
398 degradation by the native microbial community of estuarine sediment in microcosms at 4°C. In these
399 conditions however, DBT accumulated and was not further degraded to MBT which is the least toxic
400 form of TBT's degradation products. TBT biodegradation at 4°C is encouraging for the *in-situ*
401 remediation of contaminated sediment as it is the temperature of water for several months each year,
402 but the situation with DBT should also be taken into account. Other studies would be necessary to
403 draw further conclusion regarding the fluctuations in abundance of certain taxa.

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567 **CRedit author statement:**

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