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1 **Assessment of individual and mixture toxicity of bromoform, tribromoacetic-acid and 2,4,6**
2 **tribromophenol, on the embryo-larval development of *Paracentrotus lividus* sea urchin.**

3
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11
12 **Keywords:** Bromoform, Tribromoacetic acid, Tribromophenol, Disinfection by-products, Sea
13 urchin, *Paracentrotus lividus*, Ecotoxicology, Genotoxicity.

14
15 **Abstract**

16 Water chlorination is the most widely used technique to avoid microbial contamination and
17 biofouling. Adding chlorine in bromide-rich waters leads to the rapid oxidation of bromide ions and
18 leads to the formation of brominated disinfection by products (bromo-DBPs) that exert adverse
19 effects on various biological models. Bromo-DBPs are regularly encountered within industrialized
20 embayments, potentially impacting marine organisms. Of these, bromoform, tribromoacetic acid
21 and tribromophenol are among the most prevalent. In the present study, we tested the potential
22 toxicity and genotoxicity of these disinfection by-products, using sea urchin, *Paracentrotus lividus*,
23 embryos. We highlighted that tribromophenol showed higher toxicity compared to bromoform
24 and tribromoacetic acid. Furthermore, a synergistic effect was detected when tested in
25 combination. Pluteus cells exposed for 1h to mixtures of DPBs at several concentrations
26 demonstrated significant DNA damage. Finally, when compared to a non-exposed population, sea
27 urchins living in a bromo-DPB-polluted area produced more resistant progenies, as if they were
28 locally adapted. This hypothesis remains to be tested in order to better understand the obvious
29 impact of bromo-DBPs environments on marine wildlife.

30
31

32 Introduction

33 Chlorination has been used worldwide, for decades, and is one of the most effective treatment for
34 water disinfection in treatment plants for the production of tap water. Chlorination is also
35 employed in the management and upkeep of industrial installations, where water is often used for
36 cooling or heating purposes, as well as to prevent and control biofouling and corrosion of pipelines.
37 Hence, in some industrialized embayment areas, huge volumes of coastal seawater are
38 continuously being pumped and chlorinated before being re-released into the original embayment
39 (Allonier et al. 1999; Boudjellaba et al. 2016; Manasfi et al. 2018). As chlorine is very reactive in the
40 presence of natural organic matter, seawater chlorination generates a complex set of brominated
41 and chloro-brominated disinfection-by-products (DBPs). Finally, benthic organism, resident in the
42 embayment, are exposed to these DBPs (Singer 1999; Westerhoff et al. 2004; Halpern et al. 2008).
43 Although the diversity and occurrence of brominated DBPs in contaminated coastal seawater is
44 relatively undocumented (Richardson et al. 2007; Manasfi et al. 2017), bromoform (halomethane),
45 tribromoacetic acid (haloacetic acid) and tribromophenol (halophenol) are among the most
46 prevalent molecules, often measured at relatively high concentrations (i.e. $\mu\text{g/L}$) (Manasfi et al.
47 2018).

48 Most of the DBPs so far tested have been found to be toxic and genotoxic in diverse model systems,
49 such as bacteria, mammalian cells or zebrafish embryos. Furthermore, brominated DBPs have
50 generally been shown to be more toxic than their chlorinated analogues (Richardson et al. 2007,
51 2010; Yang and Zhang 2013; Hanigan et al. 2017). It is worth noting that at the single molecule
52 level, visible effects have always been following exposure to high concentrations of DBPs,
53 suggesting that toxic and genotoxic risks are limited (Teixidó et al. 2015). Nevertheless, recent
54 epidemiological research has suggested that lifetime exposure to DBPs, mainly through ingestion,
55 would significantly increase the risk of bladder cancer in humans (Villanueva et al. 2004, 2006).
56 Hence, there are great concerns about the level of human exposure, causing health and sanitary
57 surveillance agencies to enact maximum concentration limits (MCL). For instance, the US EPA
58 recommends MCLs for total halomethane and haloacetic acid of $80 \mu\text{M}$ and $60 \mu\text{M}$, respectively,
59 in safe drinking water. It is notable that these values are relatively consistent with the maximum

60 concentrations measured in the effluents of water treatment plants from the USA, Canada or
61 France (CAREX Canada 2009; Mouly et al. 2009).

62 There are currently no MCLs for environmental waters that harbor functional ecosystems, and
63 which are potentially chronically exposed to DBPs. Studies that assess the toxicity of DBPs in
64 aquatic animals remain scarce and to our knowledge, only two have tested brominated DBPs in
65 recent years, using a zebrafish embryo model (Teixidó et al. 2015; Hanigan et al. 2017). These two
66 studies tested a total of 11 brominated DBPs, including bromoform (BMF) and tribromoacetic acid
67 (TBAA). Most of the brominated DBPs that were compared, proved toxic for larval development,
68 though only at high concentration (i.e. >100 µM). Moreover, short-term exposure to chlorinated
69 wastewater did not have any significant toxic effect. Finally, a genotoxic effect was only detected
70 for chlorodibromomethane and sodium borate at their EC₅₀ levels, 160 µM and 0.049 µM,
71 respectively.

72 In this study, we were interested in broadening the understanding of the toxicological impact of
73 DBPs on coastal marine organisms, using the sea urchin, *Paracentrotus lividus*, as a proxy for the
74 echinoderm class. *Paracentrotus lividus* lives in the first few meters of coastal areas. The species is
75 widely distributed along the north-eastern Atlantic coast and on all Mediterranean coasts.
76 Furthermore, they can be encountered in numerous contrasted habitats that include differences
77 in temperature, salinity and chemical contamination (Bellas et al. 2008). While adults are benthic
78 and relatively sedentary, the larvae produced at each reproductive event are pelagic for up to 4
79 weeks, thus ensuring species dispersion. The sea urchin is a relevant model organism to assess the
80 effect of potentially harmful molecules. It is more closely related to human than the mussel (Tu et
81 al. 2006), with two species having been fully sequenced. Reproduction is easily performed in vitro,
82 resulting in high numbers of translucent larvae. Sea urchin larvae have been extensively used to
83 assess the embryo-toxicity and embryo genotoxicity of molecules (Hose 1985; McGibbon and
84 Moldan 1986; Gharred et al. 2016; Morroni et al. 2016; Trifuoggi et al., 2017; Neves et al., 2018;
85 Messinetti et al., 2018; Periera et al., 2018; Dorey et al., 2018).

86 In this study, our objective was to generate additional data in a model marine organism, of the
87 toxic influence of three major brominated contaminants found in coastal seawater that is
88 subjected to massive anthropogenic disturbance. With this aim in mind, we used sea urchin

89 embryos to assess the toxicity and genotoxic potential of bromoform (halomethane),
90 tribromoacetic acid (haloacetic acid) and tribromophenol (halophenol), tested either alone or in
91 combination, on two populations of *P. lividus*, either chronically exposed or unexposed.

92

93 **Materiel and methods**

94 Sea urchins

95 Adult *Paracentrotus lividus* sea urchins were harvested by scuba diving, on the morning of each
96 experiment, at a non-polluted site (NPS) near the Toulon (Var, France) embayment (Garonne Bay:
97 43.098503-6.018430) and at a polluted site (PS) where seawater is chronically chlorinated (Manasfi
98 et al., 2018). *P. lividus* were induced to spawn by gentle shaking.

99 Sperms and eggs were individually collected, respectively dried with a micropipette and kept on
100 ice and in 100mL of filtered seawater (FSW). Eggs were microscopically observed to verify maturity
101 before adding dry sperm.

102 Ten independent (i.e. unrelated) larval populations were produced by arbitrarily mixing one sperm
103 with one egg suspension, in 50mL of FSW at 20°C at a concentration of 500 eggs/mL, under
104 agitation for 45 min, then the fertilization rates were assessed.

105 Observed fertilization rates were 100% in every experiment.

106

107 Chemicals

108 Bromoform (CAS 75-25-2), tribromoacetic acid (CAS 75-96-7) and tribromophenol (CAS 118-79-16)
109 were purchased from SigmaAldrich. Stock solutions of bromoform and tribromoacetic acid were
110 prepared by direct dissolution in filtered sea water (FSW). Tribromophenol was first dissolved in
111 dimethylsulfoxide solvent (DMSO) to compensate for its very low water solubility, then it was
112 dissolved in FSW, with a final DMSO concentration of 0.1%.

113

114 Experimental design

115 *Toxicological assessments*

116 Ten unrelated larval populations were used and tested in triplicates, as follows: in 24-wells
117 microplates, suspensions of 500 fertilized eggs in 2 mL FSW were exposed for 48 hours to 7

118 concentrations of each chemical (Table 1). At the end of the exposure time, larvae were fixed by
119 adding ethanol at 15% final concentration and kept at 4°C until microscopic observations were
120 carried out. Normal and abnormal (i.e. delayed growth, developmental anomalies) Pluteus larvae
121 were recorded.

122 Percentages of normal Pluteus larvae were plotted against chemical concentrations. We then
123 determined the dose-response curves using R software and deduced the half maximal effective
124 concentrations, EC₅₀, here considered as the concentration of chemical at which we could observe
125 only 50% of normal and viable larvae among the pool. The no and lowest observed effective
126 concentrations (respectively NOEC and LOEC) were deduced from the chemical concentrations
127 used in the experiments.

128 We then assessed the toxicity of the chemicals combined in ten other independent experiments.
129 Specifically, we tested three mixtures named mix NOECs, mix LOECs and mix EC₅₀, that is each
130 chemical within a mixture is at its specific endpoints (NOEC, LOEC or EC₅₀). Depending on the
131 chemical tested, controls were either exposed to FSW alone or FSW containing 0.1% DMSO.

132

133 *Genotoxicity test*

134 The genotoxicity of the mix NOECs, mix LOECs and twice mix LOECs was assessed using the comet
135 assays, based on the Tice et al. (2000) procedure.

136 Five thousand, 48-h, Pluteus were exposed as described above for 1 h to the mixtures. After
137 exposure, larvae were collected by centrifugation at 1500g for 10 min at 4°C. The pellet was
138 resuspended in 1mL of FSW, then gently mixed with 1 mL of glycine 1M and incubated on ice for 5
139 min to allow for complete dissociation of cells. Fifty thousand cells were then collected and placed
140 in 1 mL of phosphate buffer saline (PBS) 1× then centrifuged at 3000g for 5 min at 4°C. Pelleted
141 cells were then collected and gently mixed with 50 µL of low melting point agarose at 37°C, then
142 plated onto a pre-coated laboratory microscope slide (Tice et al. 2000).

143 Once prepared, slides were immersed in a lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 2H₂O, 10
144 mM Tris pH10, 10 g N-Lauroylsarcosine, 10% DMSO and 1% Triton X100) for 90 min at 4°C. Slides
145 were then placed in an electrophoresis tank containing an alkaline solution (200 mM Na₂EDTA
146 2H₂O, 10 N NaOH) for 20 min at room temperature, to allow DNA to denature. This was followed

147 by electrophoresis which was conducted for 20 min at 25V and 350mA. Thereafter, slides were
148 plunged in a neutralization buffer (0.4 M Tris-HCl pH 7.5) for 20 min. Finally, slides were fixed with
149 100% methanol and dried at room temperature overnight.

150 DNA was stained with a solution of SyberGreen (Sigma-Aldrich) deposited onto each slide and left
151 to stand for 20 min in the dark. Slides were then read using an epifluorescence microscope
152 equipped with a digital camera and dedicated software. Whole cells and comets observed within
153 several randomly selected microscopic fields were captured, irrespective of the length and the
154 shape of the comet (Gyori et al. 2014). Pictures were then analysed using Open Comet v1.3.1
155 (cometbio.org) implemented in ImageJ (SciJava) program (Gyori et al. 2014).

156 DNA damage was assessed considering the olive tail moment (OTM) that consider both the length
157 and the distribution of DNA within the comet's tail (Olive et al. 1991).

158 The control experiment consisted in larval suspensions exposed to FSW containing 700µM of H₂O₂.
159 This concentration has been chosen after a preliminary experiment in which we tested the
160 genotoxicity of H₂O₂ for a range of concentrations ($R^2 = 0.9967$), as previously described (Nahon et
161 al. 2008).

162 DNA damage was assessed via the olive tail moment (OTM), which takes into consideration both
163 the length and the distribution of DNA within the comet's tail (Olive et al., 1991).

164 The positive control experiment consisted of larval suspensions to FSW containing 500 µM of H₂O₂.
165 This concentration was chosen after a preliminary experiment in which we tested the genotoxicity
166 of H₂O₂ for a range of concentrations ($R=0.9983$), as previously performed (Nahon et al., 2008).

167

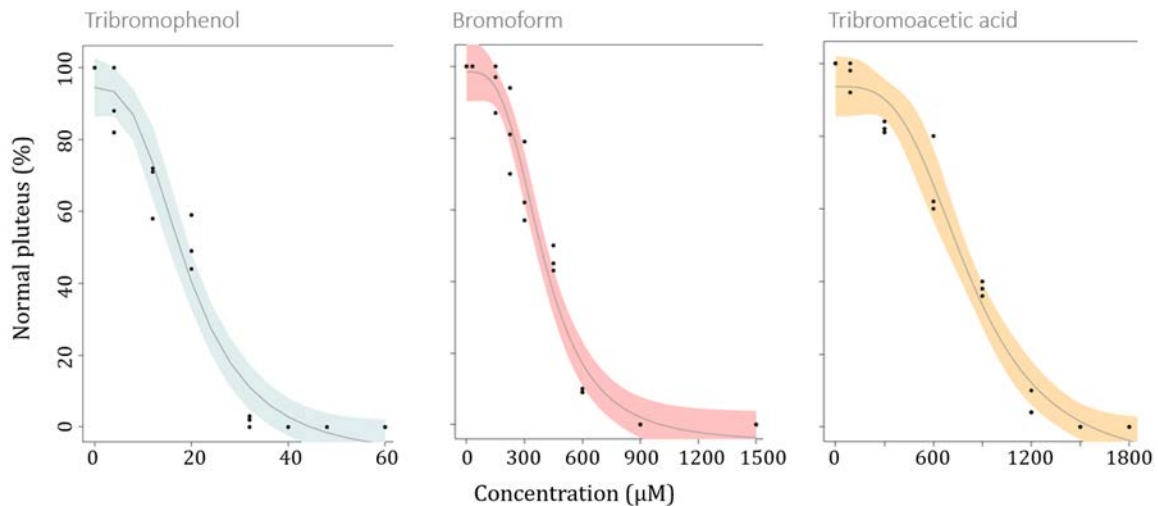
168 Statistical analysis

169 Statistics were performed with Rstudio (version 1.1.442) and XLSTAT (Addinsoft, 2017). Dose-
170 response curves were produced using regression models. Effective concentrations (i.e. EC₁₀, EC₅₀
171 and EC₉₀) values were generated with the “estimating effective doses” (ED.drc) from R package.
172 Prior to Kruskal-Wallis test, ecotoxicological and comet assay data were tested for normality. The
173 Mann-Whitney U test was proceeded to compare data from non-polluted site (NPS) to polluted
174 site (PS). Differences between each condition were considered significant when p -value < 0.05,
175 data were expressed as mean ±S.E.M (standard error of the mean).

176 **Results and discussion**

177 Assessment of BMF, TBAA and TBP toxicity on early embryo development

178 In this study, embryos were exposed to a range of bromoform (BMF), tribromoacetic acid (TBAA)
179 and tribromophenol (TBP) concentrations for 48 h (Table 1), from fertilization to the Pluteus stage.
180 The control with 0.1% DMSO was not toxic for larvae, and we found typical dose-response curves,
181 from which EC₅₀ and EC₉₀ were extrapolated (Figure 1 and Table 2).



182
183 **Figure 1: Effect of tribromophenol, bromoform and tribromoacetic acid singly, on the percentage**
184 **of normal pluteus growth after 48h**
185

186 TBP (halophenol) was by far the more toxic molecule compared to BMF (halomethane) and TBAA
187 (haloacetic acid) (Table 2). BMF was found more toxic than TBAA. This order of toxicity has regularly
188 been reported in several aquatic organisms living in fresh or marine water (Yoshioka et al. 1985;
189 Yang and Zhang 2013; Liu and Zhang 2014; Teixidó et al. 2015; Hanigan et al. 2017).

190 **Table 1: Different endpoints (µM) for three DBPs, tribromophenol, bromoform and tribromoacetic**
191 **acid obtain on pluteus larva after 48 hours of exposure**

	Tribromophenol	Bromoform	Tribromoacetic acid
LOEC µM	3,02E+00	1,98E+02	3,37E+02
EC ₅₀ µM	1,45E+01	5,26E+02	9,23E+02
EC ₉₀ µM	3,14E+01	1,05E+03	1,78E+03

192

193 Like other aquatic organisms (Yoshioka et al. 1985; Delacroix et al. 2013; Liu and Zhang 2014;
 194 Teixidó et al. 2015; Hanigan et al. 2017), *P. lividus* embryos were sensitive to relatively high
 195 concentrations of the tested molecules. The EC₅₀ was 14.5 μM for TBP, 526.31 μM for BMF and
 196 923.5 μM for TBAA, and the lowest LOEC was 3 μM with TBP (Table 2). Except for bromoform, *P.*
 197 *lividus* appears to be more sensitive to TBP and TBAA, than the embryos of the marine polychaete,
 198 *Platynereis dumerilii* (Yang and Zhang 2013), or the phytoplanktonic green algae, *Tetraselmis*
 199 *marina* (Liu and Zhang 2014). Indeed, according to the three previous studies, the EC₅₀ for
 200 bromoform in *P.lividus* is situated between the EC₅₀ in zebrafish (200 μM (Teixidó et al. 2015)) and
 201 the EC₅₀ in polychaete embryos (730 μM (Yang and Zhang 2013)). With regard to TBP and TBAA,
 202 our model seems 4 to 14 times more sensitive to TBP and 4 to 6 times more sensitive to TBAA,
 203 when compared with other, different, biological systems (Yang and Zhang 2013; Liu and Zhang
 204 2014; Teixidó et al. 2015).

205 The first observable effect (LOEC) of the three chemicals in our model was the abnormal
 206 development of a fraction of Plutei. At higher concentrations, we observed both abnormal and
 207 delayed development, mostly between the morula and gastrula stages, although in different
 208 relative proportion, depending upon the molecule and the concentration. Of the developmental
 209 anomalies triggered by all three chemicals, we mainly observed crossed spicules, which are among
 210 the most regularly reported malformations in sea urchin larvae (Gharred et al. 2016). Interestingly,
 211 TBAA-induced mortality was detected early, since we observed a black-coloured dead eggs
 212 (*unpublished data*).

213 **Table 2: Percentage of normal pluteus larva growth when exposed to NOEC and LOEC of**
 214 **bromoform, tribromophenol and tribromoacetic acid singly and combined**

	% pluteus larva	
	NOEC	LOEC
Bromoform (bmf)	99 ± 1	94 ± 5
Tribromophenol (tbp)	96 ± 1	90 ± 8
Tribromoacetic acid (tbaa)	94 ± 1	80 ± 4
Cocktail	63 ± 3 ↓ -33%	18 ± 6 ↓ -70%

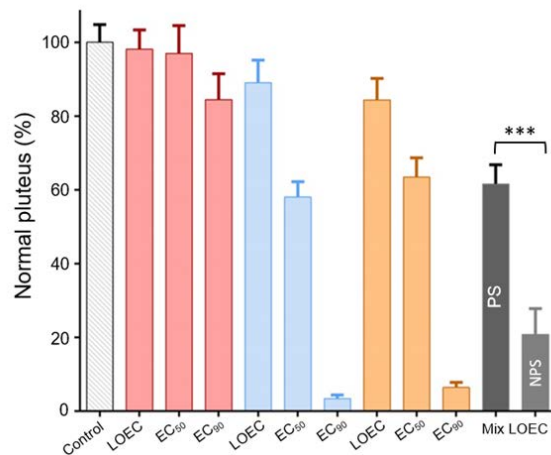
215
 216 Exposure for 48h to mix NOECs and mix LOECs critically reduced the proportion of normal Pluteus,
 217 to 63% ± 2 and 18% ± 1, respectively, while none survived exposure to mix EC₅₀ condition,
 218 demonstrating the combined effect of these chemicals (Table 2). The results obtained with a

219 mixture of chemicals were entirely expected. However, in zebrafish embryos, the influence of
 220 chlorinated water had no detectable phenotypic impact after 5 days of exposure, suggesting that
 221 a complex water environment, containing a wide diversity of DBPs at much lower concentrations
 222 than those tested, may not be deleterious for the population (Hanigan et al. 2017). Nevertheless,
 223 chronic exposure of adult fish to 0.9 nM of TBP could potentially have a significant effect over a
 224 number of generations, on survival, larval development and the malformation rate (Deng et al.
 225 2010).

226 **Table 2: Percentage of normal pluteus larva growth when exposed to NOEC and LOEC of**
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Cocktail	63 ± 3 ↓ -33%	18 ± 6 ↓ -70%

228
 229
 230 We further tested the impact of embryo exposure at polluted site (PS), with embryos generated
 231 by *P. lividus* adults chronically exposed to chlorinated sea water. These PS embryos were exposed
 232 to TBP, BMF and TBAA at their respective LOEC, EC₅₀ and EC₉₀, and to mix LOECs condition. They
 233 were found slightly less sensitive to TBP and TBAA than NPS embryos, while very resistant to BMF,
 234 which is the most prevalent molecule found in seawater where genitors were harvested (Figure 2)
 235 (Manasfi et al. 2018). Moreover, exposure to mix LOECs reduced the proportion of normal Pluteus
 236 by 40% instead of 80% for the NPS embryos. This increased PS embryo resistance could be
 237 explained by either natural selection of larva, with higher fitness for the polluted site, or a parental
 238 effect (Ross et al., 2016).

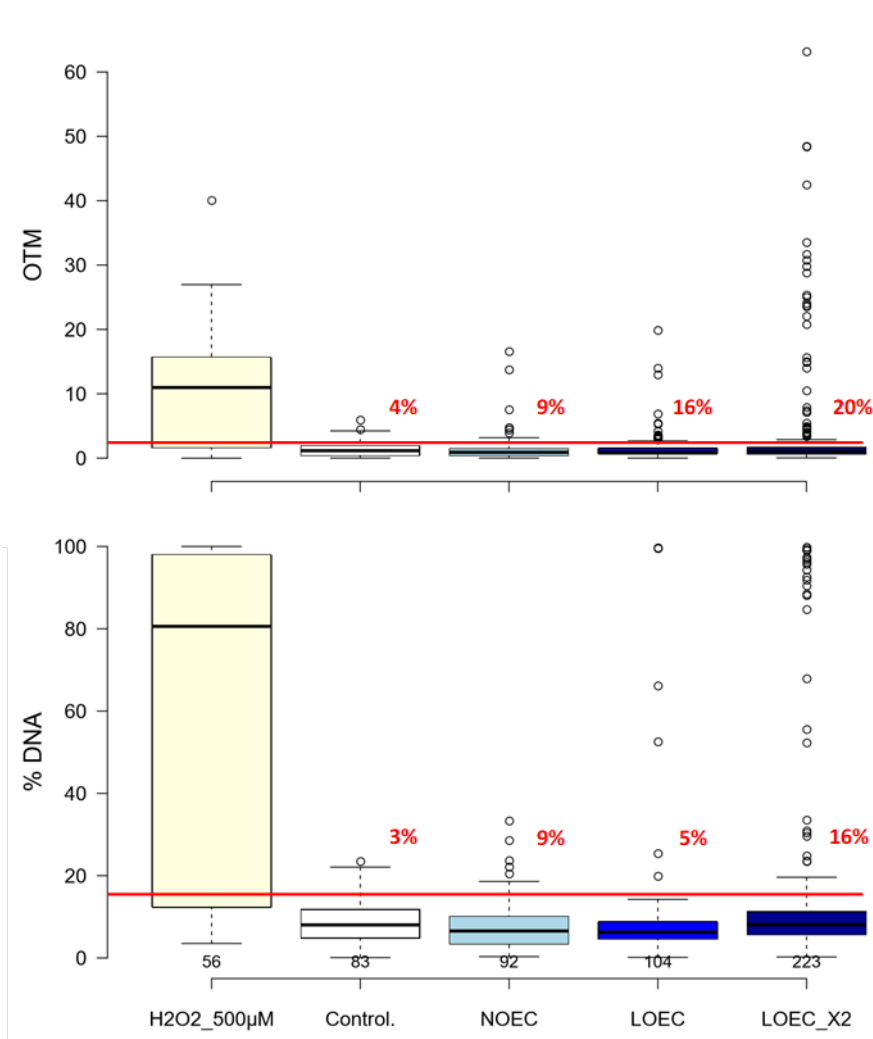


239
 240 Figure 2: Effect of bromoform (pink), tribromophenol (blue), tribromoacetic acid (orange), singly
 241 and in combination (grey), on the percentage of normal pluteus larva growth from a polluted site,
 242 after 48h of exposure. *** represent statistically significant differences at $p < 0.05$ from Dunnet's
 243 test, between the combine molecules toxicity on larva from a non-polluted site and from a DBPs
 244 polluted site.
 245

246 Evaluation of mixtures' genotoxicity

247 The alkaline comet assay has been widely used to test potential genotoxic effects of environmental
 248 pollutants, in several model organisms such as animal embryos and plant roots, as it is an efficient
 249 method for detecting single- and double-strand DNA breaks (Yıldız et al. 2009; Liman et al. 2011).

250 The results obtained from the comet assay are summarized in Figure 3.



251

252 Figure 3: Distribution of OTM (A) and % of DNA within the comet tail (B) according to the level of
 253 exposure of *Pluteus* larvae to the chemicals' mixture. Boxplots encompass the 1st and the 3rd
 254 quartile. The black line within the boxplot represents the median. Tukey's whisker extents are
 255 presented (defined as 1.5 times the 1st and 3rd quartiles). The red horizontal line indicates the
 256 95% percentile of the control condition used as a reference, and the numbers in red indicate the
 257 proportion of comets presenting a higher value than the reference
 258

259 Here we tested whether 1h exposure to mixtures of TBP, BMF and TBAA, at their respective NOEC,
 260 LOEC and twice their LOEC concentrations, could induce DNA strand breakages in *Pluteus* larvae.
 261 It is likely that given the short-term exposures to the mixtures, we observed the obvious potential
 262 of our mixtures to induce DNA strand breakages, prior to any activation of DNA repair mechanisms
 263 that would counteract the induced DNA damage. The level of genotoxicity was assessed in positive
 264 and negative controls and exposed groups according to the OTM and the percentage of DNA

265 (%DNA) measured in the comet tail. Furthermore, we only assessed the proportion of cells within
266 each group that exceeded a threshold value, corresponding to the 95th percentile of the control
267 group for the OTM and %DNA. Under controlled conditions, the 95th percentile for OTM and %DNA
268 were 2.6 and 16.5%, respectively.

269 For the two parameters considered, we found normal data distributions within the negative
270 control groups (Shapiro-Wilk, $p=0.1$) but not in the tested groups or in the positive control (Shapiro-
271 Wilk, $p<0.0001$). The non-parametric test did not reveal any influence of exposure level based on
272 the OTM but demonstrated that the group exposed to the mixture at twice the LOEC concentration
273 (mix LOEC_ X 2), had significantly more DNA within the comet tail, compared to all other groups
274 (Kruskal-Wallis, $p<0.033$). This result would suggest that this last condition only had a genotoxic
275 effect. However, the discrepancy in the results between controls and tested groups for normality
276 of data distribution indicates exposure had an influence on a fraction of the whole cells within each
277 group, as previously reported (Taban et al. 2004). Indeed, the proportion of cells with an OTM
278 higher than 2.6 regularly increases with the concentration and gives higher OTM values than
279 observed in the negative control group (Figure 3, A). This increase of the proportion of cells has
280 also been observed for the %DNA parameter, although it is clearly noticeable at the mix LOEC_X2
281 condition (Figure 3, B). Hence, overall, our results suggest a dose response when taking into
282 consideration both the effect on DNA and the occurrence of responding cells. Similar results have
283 already been reported in short-term exposure experiments, such as those performed on CHO
284 (Chinese hamster ovary) cells exposed to TBA for 4h (Plewa et al. 2008) and occurrence (Taban et
285 al. 2004).

286 It is interesting to note that at the mix LOEC_X2 condition, we found that 7% of the cells were
287 distributed with more than 90 % of their DNA within the comet tail. Among these, a few cells also
288 displayed a high OTM value, which relates to the length of the comet. A similar result was obtained
289 with some cells from the positive control group (Figure 3, B). This could be indicative of ongoing
290 apoptosis (Ríos et al. 2003; Collins et al., 2004). Evidence for this has already been demonstrated
291 in other studies. It has been shown that hydrogen peroxide can induce apoptosis, causing cell death
292 in less than 2 h in 293T cells exposed to more than 400 μM of H_2O_2 (Xiang et al. 2016). A recent
293 study has also suggested that halogenated DBPs might trigger apoptosis at high concentration (Ríos

294 et al. 2003; Collins, 2004). Thus, in our study, the mixtures might have the potential to also trigger
295 apoptosis in a fraction of the larval cells.

296 This distribution pattern is difficult to explain at this stage, and it would be interesting to assess
297 whether a certain cell type would be more sensitive to mixtures than others, or if the external cells
298 are more impacted than those embedded within the larval body, for instance. This has already
299 been reported in zebrafish exposed for a much longer time (i.e. 72 h) to BMF (Teixidó et al. 2015).

300 Finally, we have highlighted with our experimental conditions that a significant genotoxic effect,
301 observed with high doses of a cocktail of DBPs, are generally consistent with previous findings
302 (Teixidó et al. 2015; Hanigan et al. 2017).

303

304 **Conclusion**

305 This study has furthered our knowledge of the impact of bromoform, tribromophenol and
306 tribromoacetic acid, tested alone and in combination, on sea urchin, *Paracentrotus lividus*,
307 development. We have revealed that all three molecules could impair embryo development with
308 the order of toxicity TBP>BMF>TBAA. TBP is 10 to 30 times more toxic compared to the two others
309 molecules. The mixture of the three DBPs also significantly impaired development and were proved
310 weakly genotoxic at low doses (i.e. mix NOECs). Increasing the concentration of the mixture to mix
311 LOECs significantly increases cytotoxicity, as well as increasing the level of DNA damage and the
312 proportion of cells affected.

313 In this study, we have highlighted a potential genome by environment interaction, particularly
314 regarding BMF. Further experiments will be required in order to assess the relative influence of a
315 potential parental effect, as well as local genetic adaptation in the observed resistance.

316

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321

322 **References**

323 Allonier, A.-S. et al. 1999. Determination of dihaloacetonitriles and halophenols in chlorinated sea
324 water. *Talanta*

325 Bellas, J. et al. 2008. Integrative assessment of coastal pollution in a Ría coastal system (Galicia,
326 NW Spain): Correspondence between sediment chemistry and toxicity. *Chemosphere* 72(5), pp.
327 826–835. doi: 10.1016/j.chemosphere.2008.02.039.

328 Boudjellaba, D. et al. 2016. Chlorination by-product concentration levels in seawater and fish of an
329 industrialised bay (Gulf of Fos, France) exposed to multiple chlorinated effluents. *Science of The*
330 *Total Environment* 541, pp. 391–399. doi: 10.1016/j.scitotenv.2015.09.046.

331 CAREX Canada 2009. *Les sous-produits de chloration*. School of Environmental Health University of
332 British Columbia. Available at: <https://www.carexcanada.ca/fr/chloration.pdf> [Accessed: 12
333 December 2018].

334 Delacroix, S. et al. 2013. Disinfection by-products and ecotoxicity of ballast water after oxidative
335 treatment – Results and experiences from seven years of full-scale testing of ballast water
336 management systems. *Marine Pollution Bulletin* 73(1), pp. 24–36. doi:
337 10.1016/j.marpolbul.2013.06.014.

338 Deng, J. et al. 2010. Chronic exposure to environmental levels of tribromophenol impairs zebrafish
339 reproduction. *Toxicology and Applied Pharmacology* 243(1), pp. 87–95. doi:
340 10.1016/j.taap.2009.11.016.

341 Gharred, T. et al. 2016. Assessment of the individual and mixture toxicity of cadmium, copper and
342 oxytetracycline, on the embryo-larval development of the sea urchin *Paracentrotus lividus*.
343 *Environmental Science and Pollution Research* 23(18), pp. 18064–18072. doi: 10.1007/s11356-016-
344 6988-3.

345 Gyori, B.M. et al. 2014. OpenComet: An automated tool for comet assay image analysis. *Redox*
346 *Biology* 2, pp. 457–465. doi: 10.1016/j.redox.2013.12.020.

347 Halpern, B. et al. 2008. A Global Map of Human Impact on Marine Ecosystems. *Science* 319, pp.
348 948–952. doi: 10.1126/science.1149345.

349 Hanigan, D. et al. 2017. Zebrafish embryo toxicity of 15 chlorinated, brominated, and iodinated
350 disinfection by-products. *Journal of Environmental Sciences* 58, pp. 302–310. doi:
351 10.1016/j.jes.2017.05.008.

- 352 Liman, R. et al. 2011. Determination of genotoxicity of Fenaminosulf by Allium and Comet tests.
353 *Pesticide Biochemistry and Physiology* 99(1), pp. 61–64. doi: 10.1016/j.pestbp.2010.10.006.
- 354 Liu, J. and Zhang, X. 2014. Comparative toxicity of new halophenolic DBPs in chlorinated saline
355 wastewater effluents against a marine alga: Halophenolic DBPs are generally more toxic than
356 haloaliphatic ones. *Water Research* 65, pp. 64–72. doi: 10.1016/j.watres.2014.07.024.
- 357 Manasfi, T. et al. 2017. Assessing the genotoxicity of two commonly occurring byproducts of water
358 disinfection: Chloral hydrate and bromal hydrate. *Mutation Research/Genetic Toxicology and
359 Environmental Mutagenesis* 813, pp. 37–44. doi: 10.1016/j.mrgentox.2016.11.009.
- 360 Manasfi, T. et al. 2018. Characterization of chlorination byproducts in marine waters and sediments
361 in a semi-enclosed bay exposed to multiple industrial chlorinated effluents.
- 362 Mouly, D. et al. 2009. Les sous-produits de chloration dans l'eau destinée à la consommation
363 humaine en France., p. 76.
- 364 Nahon, S. et al. 2008. Improved Comet assay for the assessment of UV genotoxicity in
365 Mediterranean sea urchin eggs. *Environmental and Molecular Mutagenesis* 49(5), pp. 351–359.
366 doi: 10.1002/em.20391.
- 367 Plewa, M.J. et al. 2008. Comparative Mammalian Cell Toxicity of N-DBPs and C-DBPs. In: Karanfil,
368 T. et al. eds. *Disinfection By-Products in Drinking Water*. Washington, DC: American Chemical
369 Society, pp. 36–50. Available at: <http://pubs.acs.org/doi/abs/10.1021/bk-2008-0995.ch003>
370 [Accessed: 7 June 2018].
- 371 Richardson, S. et al. 2007. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging
372 disinfection by-products in drinking water: A review and roadmap for research. *Mutation
373 Research/Reviews in Mutation Research* 636(1–3), pp. 178–242. doi:
374 10.1016/j.mrrev.2007.09.001.
- 375 Richardson, S.D. et al. 2010. What's in the Pool? A Comprehensive Identification of Disinfection By-
376 products and Assessment of Mutagenicity of Chlorinated and Brominated Swimming Pool Water.
377 *Environmental Health Perspectives* 118(11), pp. 1523–1530. doi: 10.1289/ehp.1001965.
- 378 Ríos, J.C. et al. 2003. Tribromophenol induces the differentiation of SH-SY5Y human neuroblastoma
379 cells in vitro. *Toxicology in Vitro* 17(5–6), pp. 635–641. doi: 10.1016/S0887-2333(03)00110-3.
- 380 Singer, P.. 1999. Humic substances as precursors for potentially harmful disinfection by-products.
381 *Water Science and Technology* 40(9). Available at: <http://wst.iwaponline.com/content/40/9/25>
382 [Accessed: 6 June 2018].
- 383 *Some drinking-water disinfectants and contaminants, including arsenic*. 2004. Lyon: IARC.

- 384 Taban, I.C. et al. 2004. Detection of DNA damage in mussels and sea urchins exposed to crude oil
385 using comet assay. *Marine Environmental Research* 58(2), pp. 701–705. doi:
386 10.1016/j.marenvres.2004.03.018.
- 387 Teixidó, E. et al. 2015. Developmental effects and genotoxicity of 10 water disinfection by-products
388 in zebrafish. *Journal of Water and Health* 13(1), pp. 54–66. doi: 10.2166/wh.2014.006.
- 389 Tice, R.R. et al. 2000. Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic
390 toxicology testing. *Environmental and Molecular Mutagenesis* 35(3), pp. 206–221. doi:
391 10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J.
- 392 Tu, Q. et al. 2006. Sea urchin Forkhead gene family: Phylogeny and embryonic expression.
393 *Developmental Biology* 300(1), pp. 49–62. doi: 10.1016/j.ydbio.2006.09.031.
- 394 Villanueva, C.M. et al. 2004. Disinfection Byproducts and Bladder Cancer: A Pooled Analysis.
395 *Epidemiology* 15(3), pp. 357–367.
- 396 Villanueva, C.M. et al. 2006. Bladder Cancer and Exposure to Water Disinfection By-Products
397 through Ingestion, Bathing, Showering, and Swimming in Pools. *American Journal of Epidemiology*
398 165(2), pp. 148–156. doi: 10.1093/aje/kwj364.
- 399 Westerhoff, P. et al. 2004. Reactivity of natural organic matter with aqueous chlorine and bromine.
400 *Water Research* 38(6), pp. 1502–1513. doi: 10.1016/j.watres.2003.12.014.
- 401 Xiang, J. et al. 2016. Is Hydrogen Peroxide a Suitable Apoptosis Inducer for All Cell Types? *BioMed*
402 *Research International* 2016, pp. 1–6. doi: 10.1155/2016/7343965.
- 403 Yang, M. and Zhang, X. 2013. Comparative Developmental Toxicity of New Aromatic Halogenated
404 DBPs in a Chlorinated Saline Sewage Effluent to the Marine Polychaete *Platynereis dumerilii*.
405 *Environmental Science & Technology* 47(19), pp. 10868–10876. doi: 10.1021/es401841t.
- 406 Yıldız, M. et al. 2009. Determination of genotoxic effects of copper sulphate and cobalt chloride in
407 *Allium cepa* root cells by chromosome aberration and comet assays. *Chemosphere* 75(7), pp. 934–
408 938. doi: 10.1016/j.chemosphere.2009.01.023.
- 409 Yoshioka, Y. et al. 1985. Testing for the toxicity of chemicals with *Tetrahymena pyriformis*. *Science*
410 *of The Total Environment* 43(1–2), pp. 149–157. doi: 10.1016/0048-9697(85)90037-3.