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## Advances and limits of two model species for ecotoxicological assessment of carbamazepine, two by-products and their mixture at environmental level in freshwater

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### ► To cite this version:

Fanny Desbiolles, Xavier Moreau, Laetitia de Jong, Laure Malleret, Quentin Grandet-Marchant, et al.. Advances and limits of two model species for ecotoxicological assessment of carbamazepine, two by-products and their mixture at environmental level in freshwater. *Water Research*, 2020, 169, pp.115267. 10.1016/j.watres.2019.115267 . hal-03587360

**HAL Id: hal-03587360**

**<https://hal.science/hal-03587360v1>**

Submitted on 24 Feb 2022

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1 **Advances and limits of two model species for ecotoxicological assessment of**  
2 **carbamazepine, two by-products and their mixture at environmental level in**  
3 **freshwater**

4

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20

21 **Abstract**

22 In order to evaluate the environmental impacts associated with the presence of low-  
23 level of pharmaceuticals in waters, chronic ecotoxicity of carbamazepine (CBZ),  
24 oxcarbazepine (OxCBZ) and acridine 9-carboxylic acid (9-CAA) and their mixture was  
25 investigated using two species from different trophic levels. Innovative approaches were  
26 developed by monitoring: (i) phytometabolites in the duckweed *Lemna minor* L. and, (ii)  
27 alterations at the population, cellular and molecular levels on the cnidarian *Hydra*  
28 *circumcincta* Schulze 1914, to assess the effects of 14-day chronic exposure. On *H.*  
29 *circumcincta*, the approach was conducted at 19.5°C, the optimal growth temperature, and  
30 at 23°C, to simulate global warming impact.

31 In *L. minor*, results showed alterations of the nitrogen balance and the chlorophyll  
32 indices for OxCBZ and 9-CAA separately at environmental concentrations and for the mixture  
33 of the three studied products. However, phenolic compound index deeply varied depending on  
34 pharmaceuticals and time of exposure with no specific trend. In *H. circumcincta*, effects on  
35 morphology, reproduction and lipid peroxidation were observed at low level of CBZ. OxCBZ  
36 impacted the total antioxidant capacity of *H. circumcincta*, whereas exposure to 9-CAA  
37 affected all parameters, except the reproduction rate. Finally, 14-day exposure to the mixture  
38 of the three products also slowed the reproduction rate and altered the morphology of this  
39 cnidarian. Higher damages were detected when experiments were conducted at a temperature  
40 3.5°C above the optimum for *Hydra* growth, suggesting the relevance of taking into account  
41 possible ecotoxicological consequences of global warming. Our results confirmed the  
42 necessity of selecting sensitive organisms at different trophic levels to better assess ecotoxicity  
43 of pharmaceuticals and their mixture, especially at environmental levels and facing global  
44 changes.

45

46 **Keywords (6):** chronic exposure, sensitive aquatic organisms, low concentrations, cocktail  
47 effect, pharmaceuticals, biochemical responses

48

## 49 **1. Introduction**

50 Aquatic biota is chronically exposed to multiple exogenous substances. Many of these  
51 compounds are suspected to impact living organisms and human health, and their  
52 combination in complex mixtures could exacerbate their harmful effects (Delfosse et al.,  
53 2015). Among them, pharmaceuticals have recently emerged as pollutants of high concern  
54 in the aquatic environment, where they are commonly introduced as complex mixtures via  
55 wastewater treatment plant (WWTP) effluents (Desbiolles et al., 2018; Gros et al., 2007).  
56 Many of the past studies on aquatic organisms have focused on toxicity assessment of single  
57 pharmaceuticals (for review see Blaise et al., 2006; Crane et al., 2006; Fent et al., 2006;  
58 Godoy et al., 2015). Most of the bioassays currently used investigate short time exposure (72  
59 h or 96 h) at concentrations in the mg/L range, i.e. well above those found in the aquatic  
60 environment (Desbiolles et al., 2018). Since the concentrations of each pharmaceutical in  
61 receiving waters and effluents are low, their potential toxic effects on aquatic organisms and  
62 even on humans have been often neglected until recent years. Nonetheless, it has been  
63 demonstrated that combined low concentrations of pharmaceuticals having similar biological  
64 activities could lead to additive or synergistic effects, that could be ecotoxicologically  
65 relevant (Brain et al., 2004; Gros et al., 2007; Kolpin et al., 2002). However, data about  
66 chronic ecotoxicity of mixture of compounds at low concentrations are still poorly  
67 illustrated.

68 Pharmaceuticals are degraded with highly variable efficiencies at the outlet of WWTP  
69 ranging from upper than 80% abatement for ibuprofen to less than 20 % for carbamazepine  
70 (Miège et al., 2009). Owing to its low abatement in conventional WWTP, the present study

71 focuses on the anticonvulsant carbamazepine (CBZ) together with two of its by-products,  
72 namely oxcarbazepine (OxCBZ) and acridine 9-carboxylic acid (9-CAA). Typical  
73 concentrations of CBZ, OxCBZ and 9-CAA range from tens to several hundreds of ng/L in  
74 receiving natural waters (Ferrari et al., 2003; Gros et al., 2012; Kaiser et al., 2014).

75 The ecotoxicity of CBZ is poorly documented, especially its possible effects on  
76 aquatic macrophytes such as *Lemna minor* (Cleuvers, 2003), while some interesting data are  
77 available on the freshwater cnidarian, *Hydra attenuata*, a synonym species of *H.*  
78 *circumcincta* (Quinn et al., 2004, 2008a, 2008b, 2009). In this latter species, little effect was  
79 observed after short exposure (96 h) to a mixture of 11 pharmaceuticals, including CBZ,  
80 present at concentrations levels found in a treated municipal effluent.

81 As commonly admitted in ecotoxicological researches, a battery of several  
82 representative aquatic organisms belonging to different biological groups and to different  
83 trophic levels is recommended, referred as base-set (Arkhipchuk et al., 2000; Côté et al., 1998;  
84 Desbiolles et al., 2018; Vacchi et al., 2013). Thus, in the present study, we have selected a  
85 primary producer, the freshwater fast-growing macrophyte *Lemna minor* L. 1753 (Ziegler et  
86 al., 2016) and the freshwater carnivorous cnidarian *Hydra circumcincta* Schulze, 1914  
87 (Quinn et al., 2008a).

88 To address the issue of toxic effects of chemicals at low levels in mixtures, we aimed  
89 at testing effects of chronic (exposure time higher than 7 days) exposures to CBZ, OxCBZ,  
90 9-CAA and their mixture at environmentally relevant concentrations on various biological  
91 parameters of the two selected model organisms. Photosynthetic pigments and flavonoids  
92 are good indicators of plant health (Croft and Chen, 2018). However, sampling for  
93 phytometabolites bioassays are usually done at the end of exposure experiments with  
94 destructive techniques. We hypothesized that a daily-monitoring of those metabolites will  
95 be more accurate for early-detection of effect of pharmaceutical exposure using a non-

96 destructive analytical equipment. Moreover, since in animal models, pharmaceutical  
97 exposure may act on physiological and behavioral features, an integrative analysis of  
98 pharmaceutical exposure may need to include the study of many endpoints from biochemical  
99 to populational levels. Bearing in mind to fit tightly to environmental realistic conditions,  
100 our work aimed to investigate how far a panel of tests covering molecular to macroscopic  
101 level using two biological models could improve the capability to detect a potential effect of  
102 selected pharmaceuticals. Furthermore, crossed-effects between chemical pollutions and  
103 climate change were noticed on community structure and ecosystem functioning (Dossena  
104 et al., 2012). Thus, to question potential cross-effects of pollution and climate change  
105 experiments have been carried out at the classic breeding temperature (19.5°C) and at a  
106 higher temperature of 23°C. Indeed, it could be hypothesized that temperature rising affects  
107 the sensitiveness of organisms to pollutants and reversely chemical exposure may decrease  
108 the thermal tolerance of organisms. Therefore, the crossed effects of these stressors must be  
109 investigated as it could entrain a decreased in the fitness of wild populations.

110

## 111 **2. Materials and methods**

### 112 **2.1. Pharmaceuticals**

113 Carbamazepine (CBZ) and acridine 9-carboxylic acid (9-CAA) were provided by  
114 Sigma-Aldrich (Saint Quentin-Fallavier, France) and oxcarbazepine (OxCBZ), by VWR-  
115 International-France (Fontenay-sous-Bois, France) and were available as powders (purity >  
116 97 %). The stock solutions of each compound were prepared in ultrapure water (Direct-Q  
117 5UV, Millipore®) at concentrations of 100, 40 and 20 mg/L for CBZ, OxCBZ and 9-CAA,  
118 respectively, after stirring 2 day-long in the dark and at 25 °C. The solutions were stored in  
119 the dark at 4 °C for 2 months, thus enabling the ecotoxicological tests on chronic exposure.  
120 The stability of the compounds under these conditions was checked by HPLC monitoring.

121 Test solutions of 600, 27 and 900 ng/L were prepared for CBZ, OxCBZ and 9-CAA  
122 respectively and a mixture of them at the same latter concentrations was also prepared. This  
123 range of concentrations was chosen based on concentrations found in treated municipal  
124 effluents (Ferrari et al., 2003; Gros et al., 2012; Kaiser et al., 2014).

125

## 126 **2.2. Plant bioassay using phytometabolites**

127 Duckweeds (*Lemna minor* L.) were collected from a freshwater basin of the botanic  
128 garden in Saint-Jérôme campus (Aix Marseille University, France). The population was  
129 cultivated in a modified 10 % Hoagland solution (Sigma-Aldrich) in glass beakers. Beakers  
130 with duckweeds at 100 % confluence were then put in a phytotron (MLR-351H, Sanyo) with  
131 a 11-13 h day-night cycle with 60 % of humidity and light intensity of 20  $\mu\text{mol}/\text{m}^2/\text{s}$  at 15 °C  
132 to lower algal proliferation for 17 days. Each condition of exposure to pharmaceuticals and  
133 their mixture was done in 5 replicates in 40 mL-glass dishes and compared with a control  
134 containing only the culture solution. Duckweeds were maintained in the same solution during  
135 all the experiment time. Phytometabolites were monitored with a non-destructive  
136 measurement equipment (Multiplex®, FORCE-A, Orsay, France) using fluorescence  
137 technology with multiple excitations to measure constitutive and induced flavonols,  
138 anthocyanins, chlorophylls and a chlorophyll-to-flavonoid ratio referred to as nitrogen  
139 balance index (NBI), as previously described by Ellili et al. (2017). For each condition, the  
140 duckweed fronds were in contact with the measurement cell of the multiplex to ensure that  
141 the focal distance was the same for all samples and measurements were done in triplicate per  
142 40 mL-glass dishes.

143

## 144 **2.3. Animal bioassays**

145 The clonal population of *Hydra circumcincta* arises from a polyp given by the  
146 “Laboratoire Interdisciplinaire des Environnements Continentaux” (Lorraine University, Metz,  
147 France). Culture maintenance procedure was adapted from that developed by Trottier et al.  
148 (1997). Briefly, TES buffer (pH 7) (N-[Tris(hydroxymethyl)-methyl]-2-aminoEthaneSulfonic  
149 acid, Merck Chimie SAS, Fontenay-sous-Bois, France) was used as culture media and the  
150 polyps were fed every three days with 24 h hatched *Artemia sp.* nauplii. The polyp culture was  
151 raised at a constant temperature of  $19.5 \pm 0.1^\circ\text{C}$  with a 12-12 h light-dark cycle.

152

### 153 **2.3.1. Chronic toxicity test**

154 All pharmaceutical solutions were diluted in TES buffer. Experiments were carried out  
155 on six sterile multi-well polystyrene microplates. Healthy non-budding *H. circumcincta* polyps  
156 of similar size were randomly collected among a population of more than 1000 polyps under a  
157 stereomicroscope. For each condition, 12 polyps per well were filled with 8 mL of test solutions  
158 and tests were done in triplicate. The multi-well microplates were covered to prevent  
159 evaporation and placed into a thermo-regulated incubator at  $19.5 \pm 0.1^\circ\text{C}$  or  $23 \pm 0.1^\circ\text{C}$  under  
160 a 12-12 h light-dark cycle for two weeks. Animals were fed the day before the beginning of the  
161 experiment and then each three days (day 3, 6, 9 and 12) with about 10 *Artemia sp.* larvae per  
162 polyp. Capacity to catch and ingest prey was recorded. For each well, the media was renewed  
163 after a 4-hours feeding period. Daily, morphology was monitored, and the number of buds and  
164 new polyps was counted to determine the asexual reproduction. At the end of the two-week  
165 exposure period, the media was carefully removed, and the living polyps were rapidly frozen  
166 and kept at  $-80^\circ\text{C}$  until oxidative stress measurements. Criteria used to classify the intensity of  
167 toxic effects based on experimental endpoints (i.e. morphological changes, budding and  
168 reproductive *Hydra* rates) were the following: (1) non-toxic: 0-20 % effect, (2) moderately



169 toxic: 21-40 % effect, (3) toxic: 41-60 % effect, (4) highly toxic: 61-80 % effect, and (5)  
170 extremely toxic: 81-100 % (Arkhipchuk et al., 2006; Wang, 1990).

171

### 172 **2.3.2. Asexual reproduction: evaluation of budding and reproductive hydra** 173 **rates**

174 The budding rate ( $K_{tot}$ ) considering the number of attached buds and new polyps (i.e.  
175 separate buds) was calculated according to the following formula:

$$176 K_{tot} = (\ln(n_{tot}) - \ln(n)) / d$$

177 in which  $n$  is the number of polyps at the beginning of the experiment and  $n_{tot}$  the sum of the  
178 number of attached buds and the number of polyps (detached buds and initial polyps) after  $d$   
179 days of exposure (according to de Jong et al. (2016)).

180 The reproductive *Hydra* rate (RHR) during the two-week exposure to pharmaceuticals  
181 was calculated according to the following formula:

$$182 RHR = ((N - n) / n) \times 100$$

183 with  $N$  number of *Hydra* polyps after  $d$  days of exposure and  $n$  number of *Hydra* polyps at the  
184 beginning of the experiment (according to Arkhipchuk et al. (2006)).

185

### 186 **2.3.3. Morphological changes reflecting increasing toxicity**

187 The hereby morphological stage nomenclature used was previously described (de Jong  
188 et al., 2016; Pachura-Bouchet et al., 2006). Classically, five morphological stages are reported:  
189 *a stage*, normal, *Hydra sp.* polyps exhibit an extended body and tentacles; *b stage*, first sign of  
190 intoxication, appearance of bulbs at tips of tentacles; *c stage*, second sign of intoxication,  
191 shortened tentacles and body (early phase); *d stage*, shortened tentacles and body (late phase);  
192 *e stage* or tulip stage, tentacles and body drastically shortened. This last intoxication stage leads

193 to the animal death by disintegration (*f* stage). Stages *b* to *d* are reversible and are considered  
194 as sub-lethal endpoints, while *e* and *f* stages are irreversible and considered as lethal endpoints.

195

#### 196 **2.3.4. Biomarkers of oxidative stress**

197 The disturbance of the oxidative balance equilibrium was evaluated in *H. circumcincta*  
198 polyps exposed to chronic (14 days) pharmaceutical concentrations by measuring the total  
199 antioxidant capacity (AOC kit, Cayman Chemical, Bertin Pharma, France) and the lipid  
200 peroxidation by means of the thiobarbituric acid reactive substance assay (TBARS assay kit,  
201 Cayman Chemical, Bertin Pharma). The AOC with the pharmaceuticals was compared with that  
202 of Trolox, a water-soluble tocopherol analogue, and was quantified as molar Trolox equivalents.  
203 In the TBARS assay, the quantification of the malondialdehyde (MDA) was performed by  
204 colorimetry following its controlled reaction with thiobarbituric acid.

205

#### 206 **2.4. Data analysis**

207 Data from phytometabolite monitoring were expressed as ratios. All values of each  
208 group on *H. circumcincta* were expressed as mean  $\pm$  S.E.M (standard error of the mean). At the  
209 end of the exposures, the time, pharmaceutical, and temperature interactions for the hydra  
210 bioassays were analyzed by the three-way ANOVA followed by Tukey test for paired  
211 comparisons. When the use of parametric tests was not possible, the comparisons between  
212 conditions for the duckweed and hydra bioassays were done with the Kruskal-Wallis test  
213 followed, if needed, by the *post-hoc* Dunn test.

214

### 215 **3. Results**

#### 216 **3.1. Plant bioassay using phytometabolites**

217 Three of the monitored indices were considered: one for chlorophylls i.e. part of the  
218 primary metabolites, one for phenolic compounds i.e. part of the secondary metabolites and  
219 finally, one corresponding to a nitrogen balance index (NBI), proxy of the allocation of N  
220 and C resources in the plant. Each index was monitored on *L. minor* for 17 days of exposure  
221 to the pharmaceuticals.

222

### 223 **3.1.1. Index of chlorophylls**

224 Variations of chlorophyll indexes during the exposure to each pharmaceutical  
225 compound tested separately at environmental concentration as well as to the mixture are  
226 presented in figure 1A. No significant effects of CBZ or 9-CAA were observed on  
227 chlorophyll contents in *L. minor* compared to control. However, duckweeds exposed to  
228 OxCBZ at 27 ng/L had a chlorophyll index constantly significantly higher than the control  
229 along the experiment. Finally, no significant effects were detected on the chlorophyll index  
230 in *L. minor* exposed to the mixture as compared to control.

231

### 232 **3.1.2. Index of phenolic compounds**

233 No significant differences were detected for the index of phenolic compounds of  
234 duckweeds when exposed to each pharmaceutical (CBZ, OxCBZ, 9-CAA) separately and to  
235 the mixture as compared to control ones (figure 1B), but considerable data fluctuations were  
236 noticed.

237

### 238 **3.1.3. NBI ratio**

239 No significant effects on the NBI ratio were observed over time at the environmental  
240 concentration of CBZ (figure 1C). Exposure to OxCBZ significantly increased the NBI ratios  
241 as compared to control ones along the exposure period except at days 11 and 17 (figure 1C).

242 These results showed an increase in the pool of nitrogen compounds compared to the pool  
243 of carbon compounds when *L. minor* was exposed to OxCBZ. For 9-CAA, the NBI ratios  
244 were significantly higher compared to the control at days 2, 5, 9, 11, 14 and 17. For *L. minor*  
245 exposed to the pharmaceutical mixture, the NBI ratios were always significantly higher than  
246 control ones from the first day of experiment (figure 1C). Consequently, the ecotoxicity on  
247 NBI ratio was expressed continuously when the 3 compounds were combined.

248

## 249 **3.2 Animal bioassay using multi-scale analysis**

### 250 **3.2.1 Asexual reproduction and feeding behavior**

251 A significant decrease of the budding rate ( $K_{tot}$ ) was recorded at 19.5 °C in polyps  
252 exposed to CBZ at day 6 and in those exposed to the mixture at days 6 and 9 (figure 2A). At  
253 23 °C,  $K_{tot}$  was significantly lower from day 9 in polyps exposed to CBZ and OxCBZ. A  
254 significantly lower  $K_{tot}$  was also observed in polyps exposed to the mixture at days 7, 12  
255 and 14 (figure 2B). Moreover, a significant effect of the temperature on the  $K_{tot}$  values was  
256 observed at day 3 ( $F_{9,59} = 9.839$ ,  $P < 0.0001$ ): the  $K_{tot}$  values of each group of exposed polyps  
257 at 23 °C were significantly increased as compared with those obtained at 19.5 °C (at 19.5 °C,  
258  $K_{tot}$  mean is 0.006 and, at 23 °C,  $K_{tot}$  mean is 0.066) (figure 2A and B). At the end of the  
259 experiment, on day 14, a significant effect of the temperature on the budding rate was  
260 observed: the  $K_{tot}$  values obtained at 23 °C were lower than those obtained at 19.5 °C ( $F_{1,1} =$   
261  $77.002$ ,  $P < 0.0001$ ), and a significant effect of the exposure to pharmaceuticals was also  
262 noticed ( $F_{1,4} = 9.415$ ,  $P < 0.0001$ ). However, statistical analyses did not show any interactions  
263 between the effects of temperature and pharmaceuticals on  $K_{tot}$  ( $F_{1,4} = 1.623$ ,  $P = 0.169$ ).

264

265 At 19.5 °C, the reproductive rate (RHR) was significantly lower in polyps exposed to  
266 CBZ than controls from day 9 to day 14. Moreover, a significantly lower RHR was also

267 observed in polyps exposed to the mixture from day 12 (figure 2C). At 23 °C, a significantly  
268 lower RHR was observed in polyps exposed to CBZ, OxCBZ and the mixture from day 12  
269 to the end of the exposure (figure 2D). Significant interactions were detected between  
270 temperature and pharmaceutical on the effects on RHR ( $F_{1,4} = 6,946$ ,  $P = 0,0001$ ).

271

272         Whatever the conditions, no alteration of the feeding behavior was observed during  
273 the 2-week exposure as all living polyps were still able to catch and ingest their prey.

274

### 275         **3.2.2 Morphological changes reflecting intoxication**

276         At 19.5 °C, significant morphological changes appeared at the very first day of  
277 mixture exposure: only 34.7 % of exposed polyps showed normal morphology (*a* stage)  
278 while *b* and *c* stages represented 48.6 % and 16.7 % respectively (figure 3A). Significant  
279 morphological changes were still observable at days 2, 3 and 4 in polyps exposed to the  
280 mixture. At day 4, *d* stages appeared in the population exposed to the mixture (4.6 %) and in  
281 polyps exposed to CBZ (2.8 %), while *a* stages represented 46.2 % and 56.3 %, respectively.  
282 From day 5 to 8, all the polyps exhibited normal morphology. Then, at day 9, some polyps  
283 exhibited *b* stages, but the percentage of injured polyps were significantly higher only in  
284 those exposed to OxCBZ, where *b* stages represented 17.6 %. Finally, from day 10 to the  
285 end of the two-week exposure, none of the exposed polyps exhibited signs of impaired  
286 morphology.

287

288         At 23 °C, significant morphological changes were recorded only from day 12, where  
289 the percentage of *b* stages was significantly higher than in controls for all exposure  
290 conditions and represented 10.8 % to 15.9 % of the exposed population (figure 3B). At day  
291 14, *c* stages appeared in OxCBZ and mixture exposed polyps and their percentage was

292 significantly higher than in controls; *b* stages were still present for all exposure conditions  
293 but as *b* stages also appeared in control group, no significant morphological changes were  
294 lasting compared to controls.

295

### 296 **3.2.3. Biomarkers of oxidative stress**

297 At 19.5 °C, an overall significant difference ( $F_{9,29}= 87.04$ ,  $p<0.0001$ , two-way  
298 ANOVA) in the total antioxidant capacity (AOC) was observed: a 2-week exposure to  
299 OxCBZ induced a significant two-fold increase in the AOC compared to controls  
300 (figure 4A). Concerning the lipid peroxidation (LPO), an overall significant difference  
301 ( $F_{9,29}= 68.70$ ,  $P<0.0001$ , two-way ANOVA) was detected at 19.5 °C. Indeed, compared to  
302 controls, the LPO was significantly higher in polyps exposed to CBZ and to 9-CAA, and  
303 significantly lower in those exposed to the mixture, whereas no significant differences were  
304 observed in those exposed to OxCBZ (figure 4B).

305 At 23 °C, there were no significant differences in the AOC values between control  
306 and exposed groups (figure 4A). However, a significant increase in LPO level was observed  
307 in polyps exposed to CBZ and 9-CAA and a significant decrease in polyps exposed to OxCBZ  
308 compared with controls (figure 4B). The data showed a significant effect of the temperature  
309 since differences were observed between control groups ( $F_{1,4}= 540.55$ ,  $P<0.0001$  for AOC  
310 and  $F_{1,4}= 124.55$ ,  $P<0.0001$  for LPO). Indeed, in control groups, it was observed a significant  
311 lower AOC levels at 23 °C than 19.5 °C, while the LPO level was higher at 23 °C than at  
312 19.5 °C. Same significant variations according to temperature were observed in exposed  
313 polyps: AOC values were lower at 23 °C than at 19.5 °C while, except for OxCBZ, LPO  
314 values were higher at 23 °C than at 19.5 °C. (figures 4A and 4B). Moreover, three-way  
315 ANOVA results have shown an interaction between the effects of both temperature and

316 pharmaceuticals on AOC ( $F_{1,4} = 31.445$ ,  $P < 0.0001$ ) and on LPO level ( $F_{1,4} = 7.147$ ,  $P =$   
317 0.001).

318

#### 319 **4. Discussion**

320 The aim of this study was to determine the potential ecotoxicological effects of CBZ,  
321 OxCBZ, 9-CAA and their mixture at environmental levels and for exposure times above 7  
322 days as required by chronic experiments. No general trends in model organism responses to  
323 pharmaceutical exposure were identified, since biochemical responses were diverse.  
324 However, mixture effect was mostly different than the effects of pharmaceuticals taken  
325 individually, thus confirming research needs on mixture of pharmaceuticals and not only on  
326 single pharmaceutical exposure, which is far away from environmental conditions  
327 (Desbiolles et al., 2018). Moreover, time of exposure is a key element in the detection of  
328 chronic ecotoxicological effect. In this study thanks to the model organisms used, to the  
329 multi-parameters monitored and to the non-destructive approach, experimenting long time  
330 of exposure was feasible. In this attempt, reduced acute toxicity but significant chronic  
331 effects were evidenced for exposure to pharmaceuticals added at environmental  
332 concentrations levels.

333

##### 334 **4.1. Chronic effects of CBZ, OxCBZ and 9-CAA on *Lemna minor***

335 A stimulating effect on the chlorophyll index of duckweeds exposed to OxCBZ at  
336 27 ng/L was observed with constantly significantly higher values than the control ones.  
337 Large variations of the index of phenolic compounds in duckweeds exposed to each  
338 pharmaceutical were obtained and precluded any general conclusion. By comparing the  
339 exposure to the mixture and to each pharmaceutical taken separately, the effects resulting  
340 from the mixture on the NBI ratio were lower than the sum of the effects generated by each

341 compound. Indeed, the mixture exposure resulted in an infra-additive effect or an  
342 antagonism. Therefore, the NBI ratio was the most sensitive indicator in duckweeds to  
343 mixture exposure showing a cocktail effect of the pharmaceuticals.

344 Literature about ecotoxicity of CBZ on *L. minor* is scarce. However, an EC<sub>50</sub> of  
345 25.5 mg/L for CBZ was already determined by Cleuvers (2003) after 7 days. In 2015, the  
346 concentration of chlorophylls in *L. minor* exposed to various synthetic chemical compounds,  
347 including some pharmaceutical products, have been monitored (Fekete-Kertész et al., 2015).  
348 This approach was destructive by harvesting duckweeds after 7 days of exposure and  
349 performing spectrophotometric assays from the extracts. The authors demonstrated that  
350 duckweeds are less sensitive to a pharmaceutical i.e. diclofenac than to pesticides. In our  
351 study, the use of a non-destructive measuring equipment to determine indices of  
352 phytometabolites allowed to put forward early effects on the metabolism of *L. minor* from  
353 the lowest concentrations used. However, the great variability of responses between  
354 replicates and over time did not allow to identify a particular trend in the plant response to  
355 pharmaceutical exposure.

356 Otherwise, *L. minor* is recognized as a pollutant-tolerant plant species used in  
357 phytoremediation to metabolize some pharmaceuticals (Farrell, 2012; Matamoros et al.,  
358 2012; Reinhold et al., 2010). Consequently, more research is needed due to the limited  
359 number of studies on the effects of CBZ on this plant species and their discordant results.  
360 Matamoros and colleagues (2012) hypothesize that the difference in results between studies  
361 could be due to different initial concentrations of pharmaceuticals and in the density of plant  
362 biomass. *L. minor* may also have a defined photoperiodism dependent on its circadian clock  
363 as it has been reported for another duckweed species (Hillman, 1970). Some xenobiotics may  
364 also cause a phase shift in the circadian rhythm as demonstrated in *Lemna gibba* (Kondo,  
365 1989). Phenolic compound synthesis in plants is controlled by the circadian rhythm



366 (Lattanzio, 2013), which results in a regulatory effect of the biosynthetic pathway via its key  
367 enzymes. Among the many functions of phenolic compounds, they play a preponderant role  
368 in the response to plant stress (Cheynier et al., 2013). The variability of the metabolic  
369 responses in *L. minor* in our work may correspond to multiple responses at the  
370 phenylpropanoid pathway leading to the absence of a clear signal related to CBZ and by-  
371 product exposure. Given that these phytometabolites cannot be easily used as early  
372 biomarkers of the pharmaceutical effects, further studies including more complex mixtures  
373 containing chemicals having different usage purpose (pharmaceuticals, pesticides, industrial  
374 detergent...) should be explored before renouncing to this nondestructive approach for  
375 ecotoxicity assessment.

376

#### 377 **4.2. Chronic effects of CBZ, OxCBZ and 9-CAA on *Hydra circumcincta***

378 According to Maruyama and Sugiyama (1979), two factors determine the growth rate  
379 of a hydra population: how frequently mature polyps produce buds and how rapidly newly  
380 formed buds mature to start producing their own buds. The time for bud to form a fully  
381 developed new hydra is dependent of both feeding rate and temperature (Stiven, 1962). In  
382 our study, the feeding rate was constant (each three days), the polyps were equally fed and,  
383 whatever the exposure conditions in the present experiments, all polyps were still able to  
384 catch and ingest their prey. Therefore, significant differences in the budding rates between  
385 control group and exposed ones could only be attributed to exposure conditions. Hence, at  
386 day 3, the velocity at which polyps produced new buds at 23 °C, as compared to those  
387 maintained at 19.5 °C, can be attributed to the increase of temperature leading to a higher  
388 metabolism. At 19.5 °C, the lower budding rate observed at day 6 in polyps exposed to CBZ  
389 and at days 6 and 9 in polyps exposed to the mixture could explain in part the lower RHR  
390 observed at the following days for the same pharmaceutical exposure. However, as after day

391 9, no more differences in budding rate were observed, it could be hypothesized that the  
392 decrease of RHR could also be explained by the action of CBZ and pharmaceutical mixture  
393 on the highly molecular conserved signaling pathways involved in the development. For  
394 example, pharmacological approaches have demonstrated the importance of the FGFR  
395 (“*fibroblast growth factor receptor*”) /Notch (membrane receptor) signaling in bud  
396 development because when the FGFR or Notch pathways are inhibited, buds develop well  
397 but never detach from the parent (see Galliot, 2012, for review). Therefore, if CBZ and the  
398 pharmaceutical mixture acted on such pathways, it could retard the bud detachment (i.e.  
399 lower RHR, observed at days 12 and 14) but not necessarily slow the budding development  
400 ( $K_{tot}$ ). At 23 °C and from day 7, significant differences in budding rates of exposed polyps  
401 were more frequently observed than at 19.5 °C. Indeed, in polyps exposed to CBZ, OxCBZ  
402 but also the mixture, the budding rate ( $K_{tot}$ ) was lower. This latter result is consistent with  
403 a lower observed RHR from day 12 at this temperature. Thus, it seems that the increase of  
404 temperature affected the sensitiveness of exposed organisms to CBZ, OxCBZ and the  
405 mixture leading to decreases of both  $K_{tot}$  and RHR at the end of the exposure. Finally, maybe  
406 the decrease of reproductive rates (RHR) at both temperatures and after long term exposure  
407 to low concentration of pharmaceuticals, especially to the mixture, could lead to possible  
408 deleterious effects on the fitness of chronically exposed wild populations.

409         At 19.5 °C, from the first to the fourth day of mixture exposure, morphological  
410 changes revealed effects that can be ranged from moderately toxic to highly toxic according  
411 to the nomenclature of Wang (1990) and Arkhipchuk et al. (2006). Then, from day 5 to the  
412 end of exposure time (two weeks), a return to a normal morphology was observed. These  
413 observations suggest an early setting up of efficient defense mechanisms. At 23 °C, it seemed  
414 that the increase in metabolism activity, suggested by an elevated  $K_{tot}$  at day 3, may protect  
415 from intoxication at the beginning of exposure. Interestingly, at 23 °C, morphological signs

416 of intoxication appeared at day 12 and persisted at day 14, in polyps exposed to OxCBZ and  
417 the mixture. It could be hypothesized that the energetic allocation to survive to thermal stress  
418 may protect the polyp from intoxication for a while. However, the set-up defense  
419 mechanisms could not be maintained to preserve homeostasis for a long time. This  
420 hypothesis supports the apparition of *b* stages in controls and an increasing sensitiveness of  
421 polyps exposed to OxCBZ and the mixture at day 14.

422 At the end of the two-week exposure, morphological changes did not allow to classify  
423 the effects according a toxicity scale (Arkhipchuk et al., 2006; Wang, 1990), as less than  
424 20 % of the exposed population showed toxicity signs. Interestingly, at the end of the 2 weeks  
425 exposure at 23 °C, even if morphological alterations did not reach 20 % and did not allow to  
426 classify the effects according a toxicity scale (Arkhipchuk et al., 2006; Wang, 1990), they  
427 were greater than 10 % in polyps exposed to the low pharmaceutical concentrations used in  
428 this study. These results showed that *H. circumcincta* is a sensitive species that could be used  
429 for detection of early intoxication signs and therefore be a good candidate for utilization in  
430 regulatory framework for the determination of classical ecotoxicological endpoints (No  
431 Observable Effect Concentration= NOEC, Lowest Observed Effect Concentration= LOEC,  
432 Maximum Acceptable Threshold Concentration= MATC=  $(\text{NOEC} \times \text{LOEC})^{1/2}$ ) and  
433 especially EC<sub>10</sub>, an alternative endpoint recommended by several authors instead of the three  
434 other above mentioned (Warne and van Dam, 2008). The sensitiveness of *H. circumcincta*  
435 to pharmaceuticals was already supported by Quinn et al. (2009).

436 Regarding the biomarkers of the oxidative stress in *H. attenuata*, it has been  
437 previously shown that after a 48 h exposure to CBZ at 20 °C, the inducible threshold  
438 concentrations (MATC) for heme oxidase and glutathione S-transferase activities are  
439 0.03 µM and 0.2 µM CBZ respectively (equivalent to 7 and 47 mg/L respectively) (Quinn et  
440 al., 2004). However, these concentrations are considerably higher than the environmental

441 levels of tens to hundreds ng/L tested here separately and in mixture. Our results indicated  
442 that, at 19.5 °C, the exposition at realistic pharmaceuticals environmental concentrations of  
443 CBZ and 9-CAA did not induce the antioxidant defense systems which in turns lead to a  
444 membrane lipid peroxidation. In contrast, as the antioxidant defense systems were induced  
445 in polyyps exposed to OxCBZ, no lipid peroxidation was measured in those polyyps. This can  
446 be explained by the fact that low doses of CBZ and 9-CAA induce membrane lipid  
447 peroxidation that is not counteracted by the antioxidative systems. In polyyps exposed to  
448 OxCBZ, the induction of antioxidative system seemed to have prevented lipid peroxidation.  
449 The exposure to mixture seemed to have no impact on the oxidative balance parameters (no  
450 induced oxidative defense mechanisms and low lipid peroxidation). It can be hypothesized  
451 an unknown (agonist/antagonist/synergistic) effect of the combination of the three molecules  
452 on the oxidative balance. These results indicated that *Hydra* species is a sensitive model to  
453 detect the effects of low concentration of pharmaceuticals on cellular membrane lipid  
454 peroxidation. At higher concentrations, it has been reported that hydra may reduce the toxic  
455 effect of pharmaceuticals mixture by metabolizing the toxicants (Quinn et al., 2009). Hence,  
456 hydra were found to possess glutathione S-transferase activity (Stenersen et al., 1987), an  
457 enzyme that plays an important role during phase II biotransformation in the detoxification  
458 and metabolism of xenobiotics and endogen compounds (Hoarau et al., 2004). The fact that  
459 the increase of temperature led to significantly lower levels of AOC in all groups of *H.*  
460 *circumcincta* is consistent with the observations of Dash and Phillips (2012), which have  
461 reported that *H. vulgaris* responds to thermal stress by regulating its catalase mRNA  
462 transcription, another enzyme involved in the regulation of the oxidative balance. In their  
463 study, these authors have investigated the effects of acute thermal stresses (heat shocks) on  
464 levels of HvCatalase mRNA. They have observed a decrease in HvCatalase mRNA level in  
465 hydra groups exposed to 30 °C or 37 °C for 6 h, as compared to control groups maintained

466 at 18 °C. Our thermal exposure condition (+ 3.5 °C for 14 days) led to similar results, as we  
467 observed a lower level of AOC in controls and groups exposed to pharmaceuticals.  
468 Moreover, the ANOVA results, showing significant interactions between pharmaceutical  
469 and temperature, argue in favor of an increased hydra sensitiveness to pharmaceuticals when  
470 temperature rises. Thus, it could be hypothesized that *Hydra* species are able to regulate  
471 mRNA antioxidative enzyme levels according to surrounding temperature. As proposed by  
472 Dash and Phillips (2012), it would be expected that exposure to increased temperatures for  
473 long duration would impair the transcriptional machinery of the cell thereby negatively  
474 affecting transcription of mRNA of antioxidative enzymes, and thus, decreasing the total  
475 antioxidative capacity. This decrease in AOC could, in turns, lead to an increase in cellular  
476 membrane lipid peroxidation.

477 In summary, at the end of the two-week pharmaceutical exposure, none of the  
478 biological endpoints allowed to classify the effects according to a toxicity scale (Arkhipchuk  
479 et al., 2006; Wang, 1990), as less than 20 % of the exposed population showed toxicity signs.  
480 However, altogether, the significant variations of biological parameters studied led to the  
481 same conclusions: (1) long term exposure to low pharmaceuticals in mixture had deleterious  
482 effects on *H. circumcincta* polyps, and especially decreased reproductive rates suggesting  
483 possible harmful effects on the fitness of wild populations chronically exposed; (2)  
484 temperature rising also seemed to have harmful effects on the studied biological endpoints.  
485 When the temperature rising was combined with exposure to very low doses of  
486 pharmaceuticals in mixture, the effects seemed to be more pronounced on some of the  
487 biological endpoints (i.e. altered morphology). In a context of global warming climate  
488 change, it is possible that the toxic effects of mixtures in non-target aquatic organisms will  
489 be higher.

490

491 **5. Conclusion**

492 Our results confirmed the necessity of identifying sensitive organisms belonging to  
493 different trophic levels, to better assess ecotoxicity of pharmaceuticals and their mixture  
494 especially at environmental levels and facing global changes.

495 Although some variations of phytometabolite composition were noticed under  
496 exposure with the selected pharmaceuticals, no specific trend could be identified. The  
497 involvement of complex interactions between the pharmaceuticals and the plant metabolic  
498 pathways may induce time-dependent effects, which may also be connected with the  
499 circadian cycle of the duckweed. Further investigations are needed, especially considering  
500 the activities of key-enzymes, which may give more contrasting results. *Hydra circumcincta*  
501 appears as an appropriate sensitive model for ecotoxicological studies aiming at  
502 investigating long term effect of complex environmental mixtures. Future steps could  
503 involve a screening of other biological endpoints and the use of experimental designs,  
504 allowing to study more complex environmental mixtures and crossed effects between  
505 chemical pollutions and climate change.

506

507 **Acknowledgements**

508 The authors thank the Provence-Alpes-Côte d'Azur regional council and the company  
509 Seakalia (Marseille, France) for funding this research through a PhD grant (DEB  
510 n°141198/2014). The ECCOREV federation is also acknowledged for funding the project  
511 entitled POPMETABOTOX. We also thank the students who participated at several  
512 preliminary experiments with *Hydra circumcincta*: L. Bosco, J. Dujardin, M. Caceres and F.  
513 Tournier. Many thanks to Labex OT-MED scientific network for inspiring our reflection  
514 about warming climate change potential impact.

515

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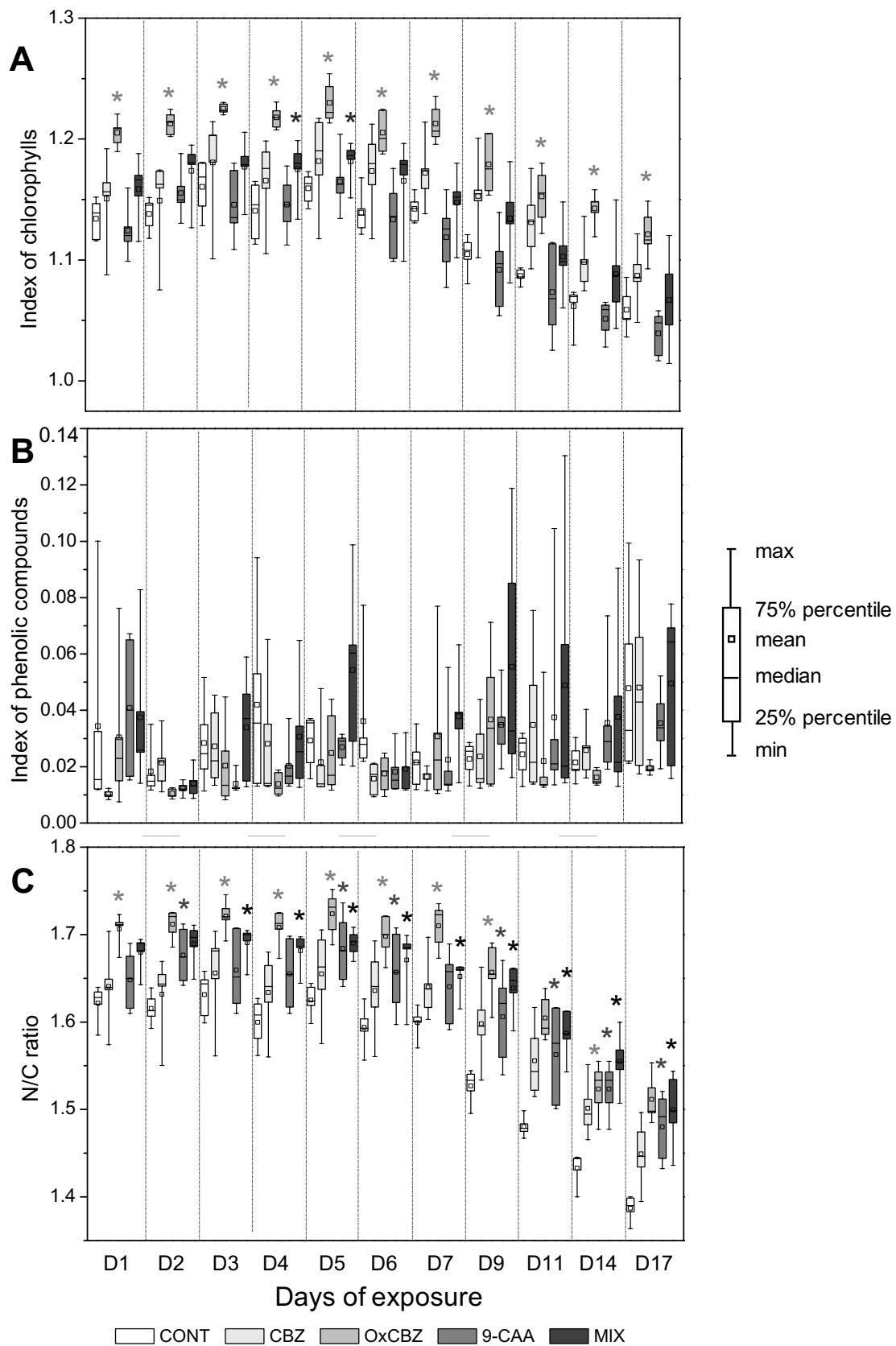
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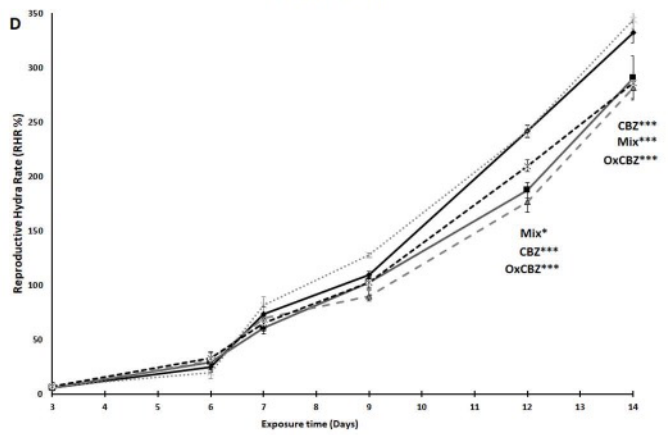
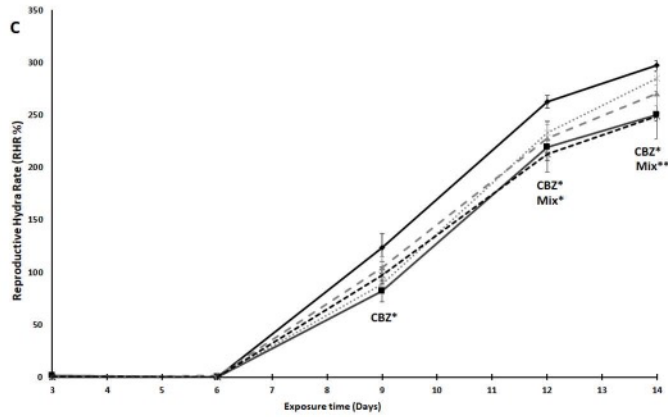
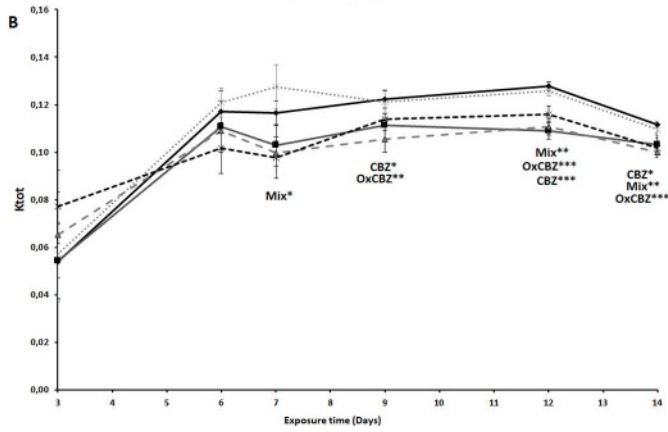
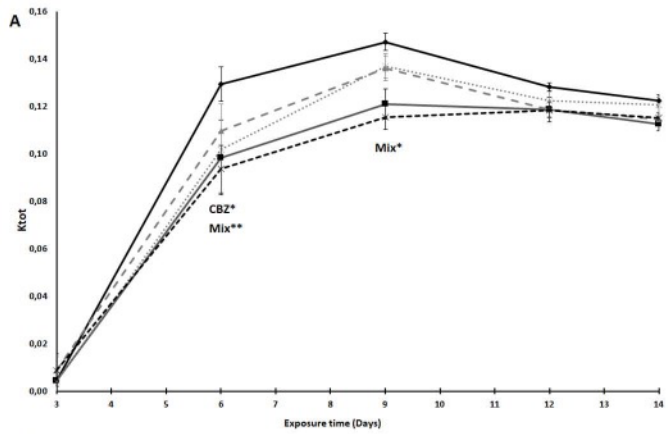
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666 Figure 1: Boxplots of chlorophyll index **(A)**, phenolic compound index **(B)**, and nitrogen  
667 balance index (NBI) **(C)** measured during 17 days in *Lemna minor* after exposure to single  
668 pharmaceuticals and the low-dose mixture (CONT: control; CBZ: Carbamazepine (600 ng/L);  
669 OxCBZ: Oxcarbazepine (27 ng/L); 9-CAA: acridine 9-carboxylic acid (900 ng/L), MIX:  
670 mixture of CBZ, OxCBZ and 9-CAA at respectively 600, 27 and 900 ng/L. Boxplots,  
671 considered per day of measure, those marked with asterisks \* are significantly different from  
672 controls, at  $p \leq 0.05$ .

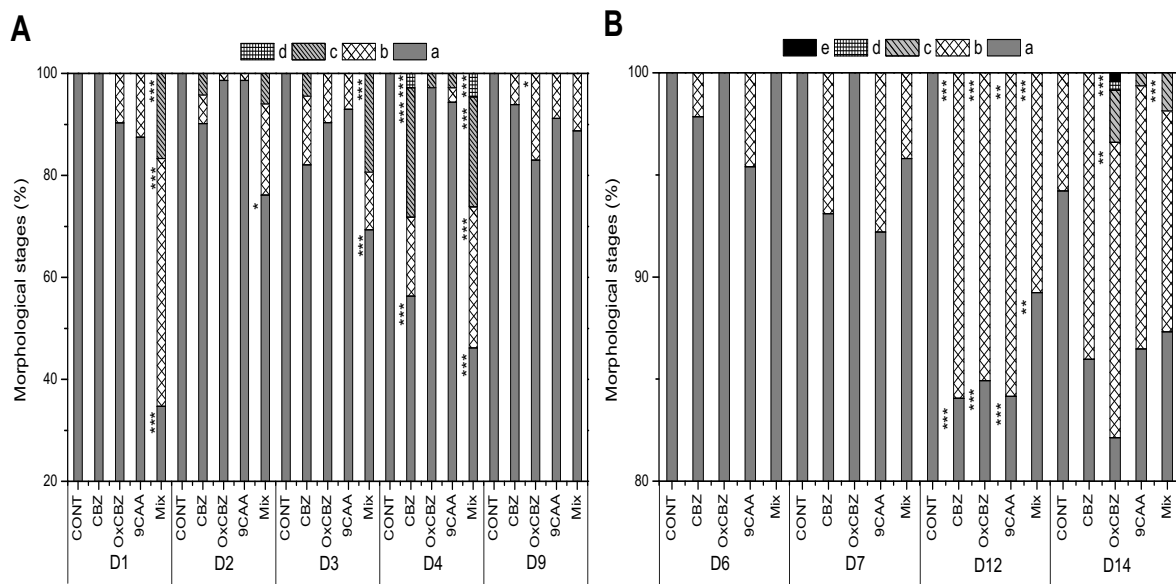


Control    CBZ    OxCBZ    \*\*\* 9CAA    \* Mixture

674 **Figure 2:** Asexual reproduction expressed as mean budding rate (Ktot) at 19.5 °C (A) and at  
 675 23 °C (B) in *Hydra circumcincta* exposed 14 days to pharmaceuticals. Asexual reproduction  
 676 expressed as mean reproductive *Hydra* rate (RHR) at 19.5 °C (C) and at 23 °C (D) in *H.*  
 677 *circumcincta* exposed 14 days to pharmaceuticals.

678 CONT: control; CBZ: Carbamazepine (600 ng/L); OxCBZ: Oxcarbazepine (27 ng/L); 9-  
 679 CAA: acridine 9-carboxylic acid (900 ng/L); Mix: Mixture of CBZ, OxCBZ and 9-CAA at  
 680 600, 27 and 900 ng/L respectively.

681 \*: P ≤ 0.05, \*\*: P ≤ 0.01, \*\*\*: P ≤ 0.001 significantly different from controls at the corresponding  
 682 day.

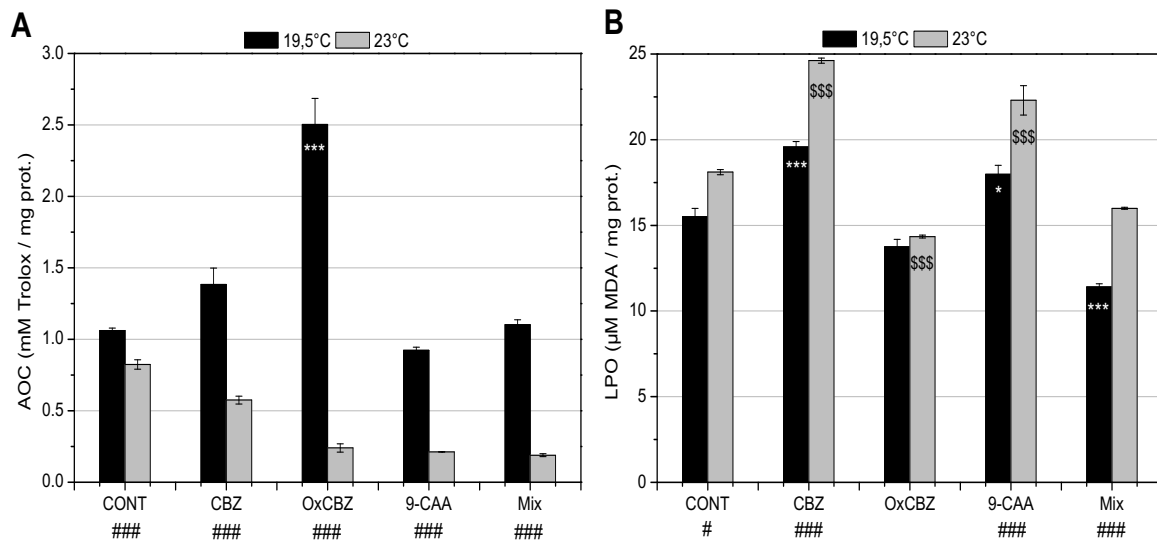


683

684 **Figure 3:** Cumulative histograms of morphological stage occurrence (in percent) in *Hydra*  
 685 *circumcincta* reflecting intoxication according to time (from day 0 to day 14) during  
 686 pharmaceutical exposures at 19.5 °C (A) and at 23 °C (B). For better readability, standard  
 687 errors and days where morphological changes were not observed (98-100 % of a stages) are  
 688 omitted.



689 CONT: Control; CBZ: Carbamazepine (600 ng/L); OxCBZ: Oxcarbazepine (27 ng/L); 9-  
 690 CAA: acridine 9-carboxylic acid (900 ng/L); Mix: Mixture of CBZ, OxCBZ and 9-CAA at  
 691 600, 27 and 900 ng/L, respectively. For stage description see 2.3.3. section. \*:  $P \leq 0.05$ , \*\*:   
 692  $P \leq 0.01$ , \*\*\*:  $P \leq 0.001$  significantly different from controls at the corresponding day.



693  
 694 **Figure 4:** Effects of 14-day exposure to pharmaceuticals on biomarkers of oxidative stress  
 695 in *Hydra circumcincta* at two different temperatures. **(A)** Antioxidant capacity (AOC)  
 696 expressed as mM Trolox/mg proteins and, **(B)** Lipid peroxidation (LPO) measured by means  
 697 of the TBARS assay (expressed in malondialdehyde-MDA- concentration in μmole/mg  
 698 proteins).

699 CONT: Controls; CBZ: Carbamazepine (600 ng/L); OxCBZ: Oxcarbazepine (27 ng/L); 9-  
 700 CAA: acridine 9-carboxylic acid (900 ng/L); Mix: Mixture of CBZ, OxCBZ and 9-CAA at  
 701 respectively 600, 27 and 900 ng/L. \*:  $P \leq 0.05$ , \*\*\*:  $P < 0.001$ , significantly different from  
 702 corresponding control at 19.5 °C; \$\$\$:  $P \leq 0.001$ , significantly different from corresponding  
 703 control at 23 °C; #:  $P \leq 0.05$ , ####:  $P \leq 0.001$ , significantly different between 19.5 °C and 23 °C.

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