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1 EFFECTS OF A BACTERICIDE ON THE STRUCTURE AND SURVIVAL OF BENTHIC  
2 DIATOM COMMUNITIES

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5

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10

11 Running title: Effects of a bactericide on diatom communities

12

13 ABSTRACT

14 We studied the adverse effects of triclosan, a widely used biocide commonly reported in  
15 surface waters, on the structure and function of benthic diatom communities. Laboratory-  
16 grown biofilms were exposed (i) to chronic contamination by increasing concentrations of  
17 triclosan and (ii) to a short-pulse of sublethal triclosan concentrations followed by a 2-week  
18 recuperation period.

19 The first experiment was performed using 6 nominal concentrations ranging from 0.05 to 500  
20 µg/L triclosan to obtain the concentration – effect relationships for benthic diatom  
21 communities. Here effects at the highest triclosan concentration in the diatom community  
22 consisted of a 63% increase in diatom mortality, with respect to control conditions. The  
23 second experiment aimed at determining the long-term effects of the toxicant and biofilm  
24 recuperation after addition of 60 µg/L triclosan for 48h exposure. After two weeks the  
25 sublethal pulse had caused a decrease in diatom growth rates and a significant delay in the

26 exponential phase of growth. The triclosan pulse provoked a decrease in diatom species  
27 richness and diversity. The diatom communities were dominated by 6 species, with  
28 *Achnantheidium minutissimum* being highly preponderant, and variations were not large  
29 enough to provide information about sensitivities / tolerance to triclosan in different species.

30

## 31 KEYWORDS

32 Bacillariophyceae; benthic communities; triclosan; dose-response; bioassays; live and dead  
33 cells; recovery

34

## 35 INTRODUCTION

36 Residues of pharmaceutical and personal care products (PPCPs) have been detected in  
37 freshwater bodies for decades, but assessing their impact on the ecosystem and on the  
38 biological communities inhabiting these ecosystems (especially non-target organisms) is  
39 relatively new (Fent *et al.* 2006). The use of triclosan (TCS), a broad-spectrum bactericide,  
40 disinfectant and fungicide, in PPCPs is increasing and the compound is, together with its  
41 transformation products, frequently found in surface waters after biological degradation. The  
42 degradation products include methyltriclosan (Balmer *et al.* 2004; Singer *et al.* 2002; Wind *et al.*  
43 *et al.* 2004) and others, originating from photolytic and abiotic degradation, such as 2,8-  
44 dichlorodibenzo-p-dioxin (Aranami & Readman 2007; Buth *et al.* 2009) and chlorophenols  
45 (Canosa *et al.* 2005). Further degradation compounds, still not completely identified, are  
46 produced by direct photolysis (Wong-Wah-Chung *et al.* 2007) and their effects are still  
47 completely unknown.

48 Triclosan has several modes of action against bacteria such as the inhibition of lipid synthesis  
49 (Sivaraman *et al.* 2004) and modifications of the cell membrane permeability (Villalaín *et al.*  
50 2001). Though triclosan is intended for use as a bactericide, it has also been shown to have

51 deleterious effects on various other aquatic organisms. Recent reviews (Capdevielle *et al.*  
52 2008; Sanderson & Thomsen 2009) reported a high sensitivity of algae to TCS, with growth  
53 inhibition EC<sub>50</sub> values less than 5 µg/L. However, significant differences in sensitivity were  
54 observed among algal groups (Orvos *et al.* 2002) and algal life forms (Franz *et al.* 2008).  
55 Quick recovery of algal cultures following triclosan contamination events has also been  
56 reported (Orvos *et al.* 2002).  
57 Though evidence exists that triclosan may affect algal growth, the potential sensitivity of  
58 fluvial (benthic) diatom assemblages is unknown. The arrival of this pharmaceutical into  
59 stream systems through sewage waters may cause relevant effects in the diversity, sensitivity  
60 and recovery capacity of diatom communities (Ricciardi *et al.* 2009). Here, we propose to use  
61 a dose-response test to determine the sensitivity of fluvial diatom assemblages to triclosan.  
62 Further, using a sublethal concentration, the recuperation potential of benthic diatom  
63 communities to this toxicant was assessed through functional and structural descriptors, i.e.  
64 chlorophyll-a *in vivo* fluorescence, live / dead diatom cell ratio and taxonomic composition.

65

## 66 MATERIALS AND METHODS

### 67 1. Experimental design

#### 68 *Experiment 1: Dose – response relationships*

69 Benthic algal communities were collected from rocks in a small stream and inoculated in  
70 aerated incubators with flow-like water circulation to allow settlement of biofilms on glass  
71 slides (1 cm<sup>2</sup> each). After 3 weeks, the colonised slides were deposited in eight artificial  
72 channels supplied with micro-filtered water to which nutrients (N and P) were added  
73 continuously. The channels received a constant flow of about 1.2 L/min and a light/dark cycle  
74 of 12/12h (as described in Ricart *et al.* submitted). The biofilms were acclimated for three  
75 days to the new conditions before being exposed to an increasing range of triclosan

76 concentrations for 48 hours. The stock triclosan solution was prepared by dissolving triclosan  
77 powder (Irgasan DP-300) in 100% methanol and was stored in the dark to avoid  
78 photodegradation. From this, working standard solutions were prepared in the range of 0.05-  
79 500 µgTCS /L, in a solution containing 25% methanol. One of the eight artificial channels  
80 was used as a “control” *sensu stricto* (no additions to the initial culture medium), and a  
81 second one was used as the “control solvent” (culture medium + 0.05% methanol). The other  
82 six channels received triclosan at one of the nominal concentrations: 0.05, 5, 25, 125, 250 and  
83 500 µgTCS/L. At the end of the 48-hour exposure period, 4 glass slides per channel were  
84 collected at random for chlorophyll-a *in vivo* fluorescence measurements and diatom  
85 enumerations.

86

87

#### 88 *Experiment 2: Long-term effects of a 48h-pulse of triclosan*

89 Eight independent incubators equipped with bubbling systems were used to grow biofilms in  
90 as described in Experiment 1. Colonisation lasted for 4 weeks, with a 12/12h light/dark cycle.  
91 The incubators were filled with liquid medium (1.5L each, detailed composition in Table 1).  
92 The liquid was replaced in each incubator twice per week, in order to avoid nutrient depletion.  
93 Then, in 4 of the 8 incubators triclosan was added, to reach the nominal concentration of 60  
94 µgTCS/L. In order to maintain the contamination level, water was renewed every 3 hours  
95 during illuminated periods. After the 48-hour exposure with triclosan, all the incubators were  
96 re-filled with regularly replaced unpolluted liquid medium (as described above), and the  
97 biofilms incubated for 2 weeks.  
98 Biofilm samples were taken just before triclosan addition (called T0d), at the end of the 48-  
99 hour exposure (T2d), and then after 7 days (T9d) and 13 days (T15d) in uncontaminated  
100 medium. For each sampling, 4 glass slides were randomly taken from each incubator and

101 analysed for *in vivo* fluorescence, the diatoms were enumerated and live and dead cells  
102 distinguished. Then, 4 replicate slides for each incubator were pooled for taxonomic purposes,  
103 to give one integrated sample per incubator.

104

## 105 2. Determination of the physicochemical characteristics

106 Temperature, pH, conductivity and dissolved oxygen in the channels and incubators were  
107 measured using a multiparametric probe (WTW, Weilheim, Germany). Water samples were  
108 collected and filtered (nylon membrane filters 0.2 µm, Whatman International Ltd,  
109 Maidstone, England) prior to analysis. Nutrient concentrations were determined using ion  
110 chromatography (761 Compact IC, Metrohm Ltd., Herisau, Switzerland). Soluble reactive  
111 phosphate was measured following Murphy and Riley (1962).

112 Triclosan and methyl-triclosan standards were purchased from Sigma–Aldrich (St. Louis,  
113 MO, USA). Standard solutions were prepared in pure methanol. Water samples (500 mL)  
114 were filtered through Millipore 0.45 µm membrane filters and pre-concentrated immediately  
115 at a flow rate of 5 mL/min on Waters C18 6-mL solid phase extraction cartridges (Oasis HLB,  
116 Waters, Milford, MA), previously activated and conditioned with 5 mL of acetone, methanol  
117 water (1 mL/min). Finally, the SPEs were eluted with 4 mL of methanol (1 mL/min) and the  
118 sample was directly injected.

119 The HPLC system consisted of a Waters 717 autosampler and a Waters 1525 binary pump.

120 HPLC separation was achieved on a 5-µm, 150 × 4 mm i.d. C18 reversed-phase column

121 (SunFire). The injection volume was set at 50 µL, and the flow rate was 1 mL/min of 90%

122 methanol - 10% water with isocratic flow. Detection was carried out using a UV–vis detector

123 (Waters 2489) at 280 nm.

124

125           3. Diatom analyses

126    *In vivo fluorescence measurements*

127    An estimation of diatom-specific chlorophyll concentration was obtained by measuring the  
128    specific F<sub>0</sub> (minimum fluorescence yield of dark-adapted cells) by means of a PhytoPAM  
129    fluorometer (Heinz Walz, Effeltrich, Germany) at 520 nm (Schmitt-Jansen & Altenburger  
130    2007). The F<sub>0</sub> was determined after applying a weak measuring light to the periphyton  
131    samples (directly on the colonised slides), after a 20-minute incubation in the dark to ensure  
132    that all the reaction centres were open. The photosynthetic capacity of the diatom community  
133    (Y<sub>max</sub>) was also determined on dark-adapted samples. After being kept in the light for 20  
134    minutes, the samples were exposed to actinic light for the determination of their  
135    photosynthetic efficiency (Y<sub>eff</sub>). PhytoPAM measurements for each parameter were repeated  
136    5 times per slide and averaged.

137    *Quantitative estimates of diatom community structure and live / dead ratio*

138    After fluorescence measurements, the glass slides were scraped using polyethylene cell lifters  
139    (Corning Inc., NY, USA), and cells preserved with a drop of formalin solution and diluted to  
140    a final volume of 5 mL. Samples were ultrasonicated for 7 minutes to separate the aggregated  
141    cells without destroying the frustules. 125 µL of each sample were then pipetted onto a  
142    Nageotte counting chamber to count the total number of diatom cells in 10 microscope fields  
143    (1.25 µL each, 0.5 mm depth) selected at random, using light microscopy at a 10×  
144    magnification (photomicroscope Nikon Eclipse 80i, Nikon Co., Tokyo, Japan). Data were  
145    recorded as cells per unit area of sampled substrate (number of cells/cm<sup>2</sup>). Countings were  
146    separated into 2 types: empty cells that were considered as ‘dead’, and cells occupied by  
147    chloroplasts that, whatever their color (from pale yellow to green or brown), shape and  
148    number, were considered as ‘alive’ (Cox 1996). From live diatom counts, growth rates (as

149 expressed in cell divisions/day) of the diatom community were calculated according to  
150 Guillard (1973).

151 The optimal use of the live / dead cell indicator (L/D ratio) required:

152 1) The observations / countings to be carried out within 1 month after sampling. Chlorophyll  
153 content has been shown to decrease very quickly in formalin-preserved samples (Dell'Anno *et*  
154 *al.* 1999), and a decrease in pigment content would make it harder to distinguish between live  
155 and dead cells. Storing the formalin-fixed samples in the dark will however delay chlorophyll  
156 degradation (M.-J. Chrétiennot-Dinet, pers. comm.). Additionally, we used the same dilution  
157 per unit substratum surface area in a single study, in order to maximize comparability  
158 between data. Moreover, as the difference between live and dead cells may be subjective in  
159 some cases (e.g. very slight coloration of the cell content), all observations were performed by  
160 a single operator;

161 2) The proportion of live vs. dead diatoms was based on counting entire cells, *i.e.* those that  
162 exhibit an entire frustule. This required the ultrasonication to be limited to a maximum of 7  
163 minutes, in order to minimise frustule damage.

#### 164 *Relative abundances of the diatom species*

165 Diatom identifications were performed only in experiment 2, after having prepared permanent  
166 slides following European standard NF EN 13946. About 500 frustules were counted per slide  
167 at 1,000× magnification and diatoms were identified to the lowest taxonomic level possible  
168 using standard references and recent nomenclature updates. References consisted of:

169 Krammer & Lange-Bertalot (1986 - 1991), Sala et al. (1993), Round & Bukhtiyarova (1996),  
170 Krammer (1997), Compère (2001), Bukhtiyarova (2006) and Potapova (2006).

171 Diatom life forms were separated following the guilds as defined by Passy (2007). Species  
172 with solitary life forms were divided into 3 groups: motile, erected and prostrate, according to



173 the growth forms described by Cox (1996), Hudon & Legendre (1987), Katoh (1992), and  
174 Kelly et al. (2005).

175

#### 176 4. Processing the data

177 The values of chlorophyll-a *in vivo* fluorescence and L/D ratios with increasing triclosan  
178 concentrations (Experiment 1) were analysed for significant differences using 1-way ANOVA  
179 performed with Statistica v5.1 (StatSoft Inc. Tulsa, Oklahoma). The EC<sub>50</sub> of the L/D ratio was  
180 estimated from the dose / response curve, using the 3 replicates of the 2 controls and 6  
181 measured triclosan concentrations.

182 The variation of the same parameters with treatment and duration of the experiment  
183 (Experiment 2) were explored by means of a linear mixed-effect model (LME) for repeated  
184 measurements. For this purpose, the treatments and sampling dates were considered as fixed  
185 effects and the replicate samples (*i.e.* the samples taken from replicate incubators) as random  
186 effects. These statistical analyses were computed with the package nlme in the statistical  
187 modelling environment R (Ihaka & Gentleman 1996).

188 Calculating the ratio between live and dead cells was quite independent of the number of cells  
189 counted. Indeed, relative counts allowed minimization of the influence of microhabitat factors  
190 (as shown by the reduced error in the intra-treatment L/D values compared to absolute cell  
191 density data), especially in the case of small-sized substrates as used in these experiments,  
192 where algal development is highly variable.

193

## 194 RESULTS

### 195 *Experiment 1: Dose – response relationships*

196 Apart from the TCS additions, all channels were fed with the same water; its characteristics  
197 are given in Table 1. TCS concentrations measured in the channels (Table 2) differed slightly

198 from the nominal concentrations. The highest concentration was 461.29  $\mu\text{g/L}$ . Concentrations  
199 of methyltriclosan, a degradation product of triclosan, always remained below the detection  
200 limit.

201 After a 48-hour exposure, chlorophyll-a *in vivo* fluorescence parameters did not display any  
202 significant trend related to triclosan addition, with mean values of  $F_0 = 100.2 \pm 3.0$  ( $n = 36$ ),  
203  $Y_{\text{max}} = 0.43 \pm 0.01$  ( $n = 36$ ) and  $Y_{\text{eff}} = 0.30 \pm 0.01$  ( $n = 36$ ).

204 Live diatom densities tended to decrease with increasing TCS concentrations but did not  
205 express statistically significant differences ( $p = 0.09$ ). Up to  $130,000 \pm 24,000$  cells/cm<sup>2</sup>  
206 occurred in the controls, while  $91,000 \pm 9,000$  cells/cm<sup>2</sup> were counted in the maximum TCS  
207 concentration, representing a decrease of about 30% (Figure 1). The highest diatom densities  
208 were observed in the channel exposed to 5.41  $\mu\text{gTCS/L}$ , that reached ca.  $170,000 \pm 37,000$   
209 cells/cm<sup>2</sup>.

210 The ratio between live and dead diatoms ranged from  $6.2 \pm 0.2$  in the controls down to  $3.5 \pm$   
211  $0.3$  in the most contaminated channel. Diatom L/D ratio expressed as a percentage of the  
212 mean control values fitted the sigmoidal curve reported in Figure 2. The dose-response curve  
213 was used to evaluate the  $EC_{50}$  of TCS for the diatom community. An  $EC_{50}$  value of 560  $\mu\text{g/L}$   
214 was derived from the regression equation of the curve (confidence interval: 534-584  $\mu\text{g/L}$ ).  
215 This value was taken as a criterion to use a 10-fold lower concentration (60  $\mu\text{g/L}$ ) to perform  
216 Experiment 2.

217

#### 218 *Experiment 2: Long term response after a 48h-pulse of triclosan*

219 Among the *in vivo* parameters, the linear mixed-effect model did not show any statistical  
220 differences in  $F_0$  and  $Y_{\text{eff}}$  values between treatments, duration of exposure, nor combined  
221 effects. Indeed, T0d values were  $F_0 = 138.4 \pm 26.0$  ( $n = 40$ ) and  $Y_{\text{eff}} = 0.37 \pm 0.03$  ( $n = 40$ ),  
222 with maximum variations of 29% of the control (T15d, where a slight date x treatment effect

223 was observed,  $p \sim 0.05$ ) and 4 % (T2d) respectively. Conversely, the photosynthetic capacity  
224 ( $Y_{max}$ ) expressed differences both due to sampling date, treatment ( $p < 0.0001$ ) and date x  
225 treatment ( $p < 0.01$ ).  $Y_{max}$  increased with time (from  $0.47 \pm 0.04$  at T0d), with values  
226 slightly higher in the TCS-treated samples ( $0.52 \pm 0.02$ ) than in the controls ( $0.48 \pm 0.02$ ).  
227 Diatom communities in the controls showed exponential growth during the 15 days of the  
228 experiment ( $R^2 = 0.93$ ;  $p < 0.001$ , see Figure 3), from  $117,000 \pm 12,000$  cells/cm<sup>2</sup> (T0d) to  
229  $690,000 \pm 88,000$  cells/cm<sup>2</sup> (T15d). In contrast, diatom densities in the incubators receiving  
230 triclosan were quite stable throughout the 9 first days following the beginning of exposure  
231 ( $106,000 \pm 22,000$  cells/cm<sup>2</sup>) and then reached  $447,000 \pm 55,000$  cells/cm<sup>2</sup> at T15d. Whatever  
232 the treatment, the increase in cell densities from T0d to T15d was highly significant ( $p <$   
233  $0.0001$ ), and a date x treatment effect was also observed at the last dates of the experiment ( $p$   
234  $< 0.05$ ).

235 Before TCS exposure, the ratio between live and dead diatoms averaged  $10.9 \pm 0.4$  in the  
236 incubators. This ratio decreased down to  $7.4 \pm 0.7$  (more than 30% decrease) in the TCS-  
237 exposed systems in comparison to the control values (treatment x date effect:  $p < 0.0001$  at  
238 T9d) and then increased at T15d but remained significantly lower than the controls (-16.6%,  $p$   
239  $< 0.05$ ).

240 A total of 32 diatom species were identified during this study, but only 6 of them accounted  
241 for more than 1% in at least one of the samples (Figure 4). In all treatments, diatom  
242 communities were dominated by *Achnantheidium minutissimum* Kützing ( $87.3 \pm 1.4$  %),  
243 followed by *Gomphonema angustatum* (Kützing) Rabenhorst ( $5.5 \pm 0.9$  %), *Achnantheidium*  
244 *pyrenaicum* (Hustedt) Kobayasi ( $1.9 \pm 0.5$  %), *Gomphonema minutum* (Agardh) Agardh ( $1.0$   
245  $\pm 0.2$  %), *Ulnaria ulna* (Nitzsch) Compère ( $0.8 \pm 0.2$  %) and *Fragilaria capucina*  
246 Desmazières var. *capucina* ( $0.6 \pm 0.3$ ). Relative abundances of these main species remained  
247 quite stable in the control incubators during the course of the experiment, and slight changes

248 occurred in the TCS incubators after the 2-day exposure. Indeed, relative abundances of *A.*  
249 *minutissimum* decreased by about 6%, whereas increased percentages of *G. angustatum*  
250 (almost reaching 10 %) and *F. capucina* (from 0.2 to 2.7 %) were observed. Twisted valves of  
251 *F. capucina*, *G. angustatum* and *U. ulna* were also recorded in the TCS-exposed communities  
252 but cumulative abundances of abnormal cells did not exceed 2 % (at T2d). At the end of the  
253 experiment, diatom community structure was comparable between treatments in terms of  
254 dominant species ( $p = 0.095$ ).

255 Diatom species richness and Shannon diversity displayed differences between controls and  
256 TCS-incubators. At T0d, Shannon index was of  $0.70 \pm 0.10$ ; it slightly decreased in the  
257 controls down to about 0.61 at T15d and was much lower (0.33) in the TCS-treated samples.  
258 Species richness also suffered a significant decrease in the treated incubators ( $13.7 \pm 0.8$   
259 taxa), whereas it was constant through time in the controls ( $16.7 \pm 0.5$  taxa).

260

## 261 Discussion

### 262 *TCS dose – response relationships*

263 As a bactericide, triclosan inhibits the enzyme enoyl-acyl carrier protein reductase (ENR),  
264 which is involved in the bacterial lipid biosynthesis (Levy *et al.* 1999; McMurry *et al.* 1998).  
265 However, it is also known as a broad-spectrum antimicrobial agent and as such it could  
266 generate effects on non-target components of the fluvial community (Wilson *et al.* 2003). The  
267 results obtained in this study reinforced this hypothesis and showed effects on non-target  
268 organisms within the biofilm community. Chlorophyll-a *in vivo* fluorescence parameters were  
269 expected to show significant variations among the concentrations tested (Franz *et al.* 2008),  
270 that were not observed in our study. However, diatom countings provided information about  
271 some possible unexpected effects of TCS towards algae. The total number of live cells  
272 enumerated per unit surface area seemed to indicate that, at quite low concentrations, diatoms

273 could be favoured (higher cell densities), probably reflecting a decrease in competition  
274 between bacteria and diatoms (Grover 2000) due to the bactericidal effects of TCS (Escalada  
275 *et al.* 2005). Indeed, bacterial mortality was significantly higher in the TCS-treated channels  
276 compared to the controls ( $p < 0.001$ , data not shown). Toxicant effects on non-target  
277 organisms are common in biofilms, where algae and bacteria interact in a small space (Ricart  
278 *et al.* 2009). The L/D ratio (Figure 2) confirmed the adverse effects of TCS on diatom  
279 communities, inhibiting diatom community growth and / or increasing mortality at the highest  
280 concentrations tested.

281 In the first experiment, performed in artificial channels, we observed 50% diatom mortality at  
282 concentrations far higher than those obtained by Sanderson and Thomsen (2009), who found  
283 values for 24h-LC<sub>50</sub> of 4.5 µgTCS/L for algal growth in laboratory cultures. However, our  
284 results were consistent with the concentration effects determined by Franz *et al.* (2008). This  
285 variation of results between experiments underlines the relevance of abiotic factors  
286 (experimental conditions, and in particular water flow regime modifying boundary layer  
287 effects, and thus TCS bioavailability) in the assessment of the hazards of toxicants. Besides,  
288 Franz *et al.* (2008) compared the tolerance of monospecific diatom suspensions and semi-  
289 naturally grown biofilms and reported different responses depending on biotic factors (e.g.  
290 single species tests *vs.* life forms and community structure). Increased resistance of a natural  
291 algal community (the object of our study) by comparison to laboratory culture could be linked  
292 to the intrinsic characteristics of the biofilms, in which the cells are embedded in a  
293 polysaccharide matrix acting as a protective barrier. The thickness of the matrix also leads to  
294 diffusion gradients within the biofilms, limiting the penetration of triclosan in the inner layers.  
295

296 *Long term effects of a 48h-pulse of triclosan*

297 The moderate increase of the *in vivo* fluorescence parameter  $Y_{max}$  in the exposed samples  
298 could be attributed to a hormetic response of the community (Calabrese 2005), *i.e.* to the  
299 capacity of the system to initiate damage-repair processes. This adaptative response could  
300 either be linked to enhanced photosynthetic capacity of the diatoms, or to structural changes  
301 in the community favouring species with higher efficiency (Sabater *et al.* 2007). Previous  
302 studies (Bérard & Pelte 1999; Ricart *et al.* 2009; Serra *et al.* 2009) have already described  
303 increases in chlorophyll concentration and photosynthetic capacity under sublethal pulses of  
304 toxicants. Here the persistent hormetic response suggested that the compensation process was  
305 not totally achieved, or that exposure durably selected for species with higher photosynthetic  
306 potential, able to modify community tolerance to further exposure (Blanck *et al.* 1988).

307 The two quantitative diatom estimates (cell densities, L/D ratios) indicated deleterious effects  
308 of a 60 µg/L triclosan concentration on diatoms, lasting in time. The exponential growth of  
309 diatoms (as seen in the controls) was significantly delayed by the 48-hour exposure to  
310 triclosan. Cell densities after 2 weeks were not as high in the TCS-treated incubators as they  
311 were in the controls, which could also indicate a persistence of toxicant exposure linked to  
312 TCS or methylTCS bioaccumulation within biofilms (Coogan *et al.* 2007). The L/D ratio also  
313 expressed an increased mortality of the communities exposed, with a  $6.1 \pm 0.5$  % decrease in  
314 the ratio after the 48-h exposure. This value fitted the curve of Experiment 1 ( $R^2=0.90$ ;  $p <$   
315  $0.001$ ), suggesting that the response of this endpoint was not significantly modified by the  
316 differences in flow conditions between Experiments 1 and 2. After 15 days, the communities  
317 recovered slightly, although they did not attain the L/D ratio of the controls. This was also  
318 seen in the diatom community composition, which was quite comparable between controls  
319 and treated incubators at the end of the experiment. However, when looking at the life forms  
320 (Figure 5), some differences were observed, the treated samples having a lower proportion of

321 species forming clumps (4%, like *Ulnaria ulna* or *Gomphonema* sp.) or filaments (0.6%, e.g.  
322 *Melosira varians* and *Fragilaria* sp.), than in the controls (7.5 and 1.5% resp.). Lawrence *et*  
323 *al.* (2009) observed that the biofilm architecture was affected by relatively low concentrations  
324 of TCS (10 µg/L), that caused a less tightly packed structure and the disappearance of  
325 filamentous species. In the present study, TCS exposure was also followed by a decrease in  
326 colony-forming diatom species. Moreover, the control glass slides were covered by other  
327 organisms with filamentous physiognomy, which were almost absent in the TCS-treated  
328 incubators, even after the 2-week recovery period (L. Proia, unpubl. data).

329

330 Data about TCS effects on non-target organisms such as algal communities are scarce, and  
331 apparently contradictory. However, the results of this study indicate that high concentrations  
332 of pharmaceuticals occurring in sewage water may have unexpected effects on the receiving  
333 aquatic ecosystems. Given the current lack of knowledge about sensitivities to PPCPs and the  
334 multiplicity of toxic modes of action, it cannot be expected at this stage that any index of  
335 toxicity based on diatom community structure can be immediately developed but these results  
336 confirm the high sensitivity of diatoms to various environmental stressors, including  
337 pharmaceutical inputs. Diatom mortality as inferred from the L/D ratio proved to be sensitive  
338 to TCS contamination, and could be a useful tool for future biomonitoring purposes.

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340

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469

470 Table 1. Mean values  $\pm$  standard error (number of samples analysed) of physicochemical  
471 parameters during the course of both experiments. n.c. nominal concentration.

472

	Experiment 1	Experiment 2
pH	$7.7 \pm 0.0$ ( $n=8$ )	$7.9 \pm 0.5$ ( $n=11$ )
Temperature ( $^{\circ}\text{C}$ )	$15.8 \pm 0.0$ ( $n=8$ )	$17.5 \pm 1.1$ ( $n=11$ )
Conductivity ( $\mu\text{S}/\text{cm}$ )	$289.9 \pm 0.2$ ( $n=8$ )	$143.4 \pm 34.1$ ( $n=11$ )
Dissolved oxygen (mg/L)	$9.0 \pm 0.1$ ( $n=8$ )	$9.2 \pm 0.2$ ( $n=11$ )
Oxygen saturation (%)	$94.3 \pm 0.4$ ( $n=8$ )	$7.9 \pm 0.5$ ( $n=11$ )
$\text{NO}_3$ (mg/L)	$1.85 \pm 0.17$ ( $n=16$ )	0.75 ( <i>n.c.</i> )
$\text{PO}_4$ ( $\mu\text{g}/\text{L}$ )	$77.3 \pm 8.6$ ( $n=16$ )	$16.9 \pm 2.1$ ( $n=12$ )

473

474 Table 2. Nominal and effective concentrations of triclosan ( $\mu\text{g/L}$ ) in the channels (Experiment  
475 1). d.l.: detection limit.

476

Nominal	0 (Control)	0 (Control solvent)	0.05	5	25	125	250	500
Measured	< d.l.	< d.l.	4.8	5.4	13.9	120.7	300.0	461.3

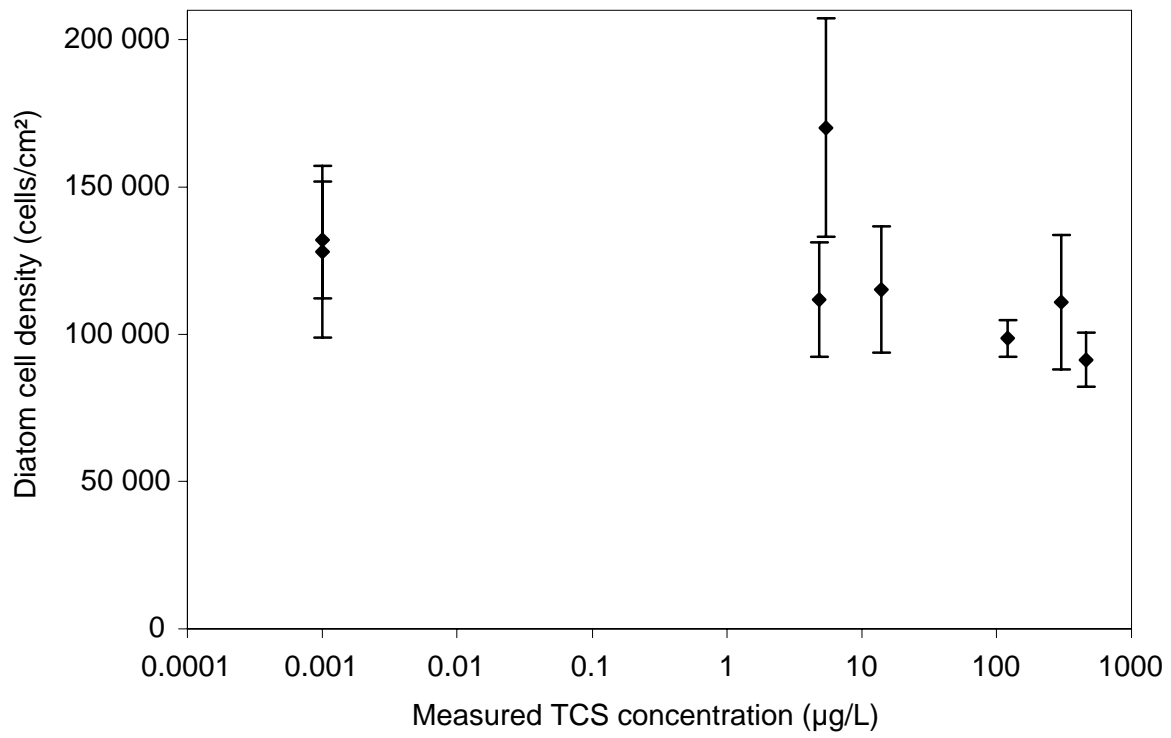
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478 Figure captions

479 Figure 1

480 Densities of live diatom cells (number of cells/cm<sup>2</sup>) after a 48h-exposure, for the different  
481 concentrations of triclosan measured in the channels.

482 Note the logarithmic scale on the X-axis





483 Figure 2

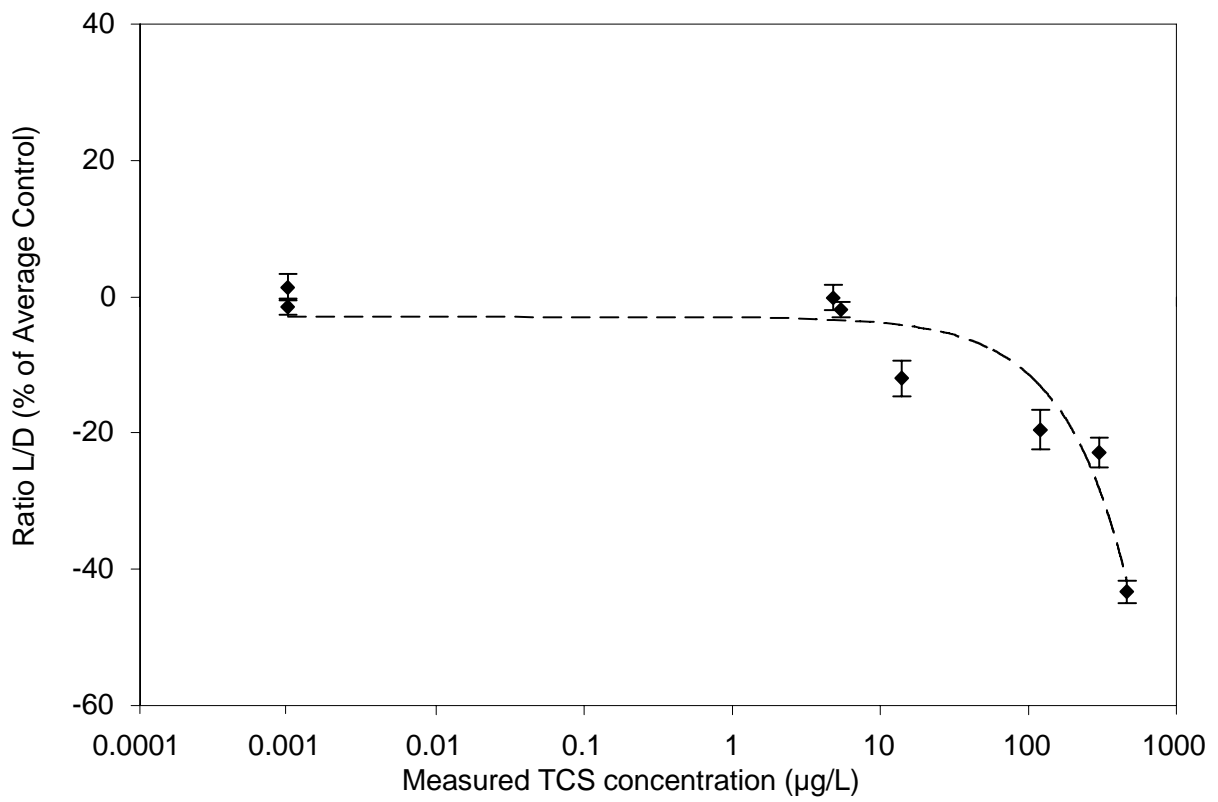
484 Triclosan dose-response curve for diatom live / dead ratio (in percentage of average control)

485 after a 48h-exposure ( $R^2 = 0.90$ ;  $p < 0.001$ ).

486 Note the logarithmic scale on the X-axis

487

488



489 Figure 3

490 Diatom cell densities (in ln cells/cm<sup>2</sup>) in the control (○) and treated (▲) incubators during the

491 course of Experiment 2.

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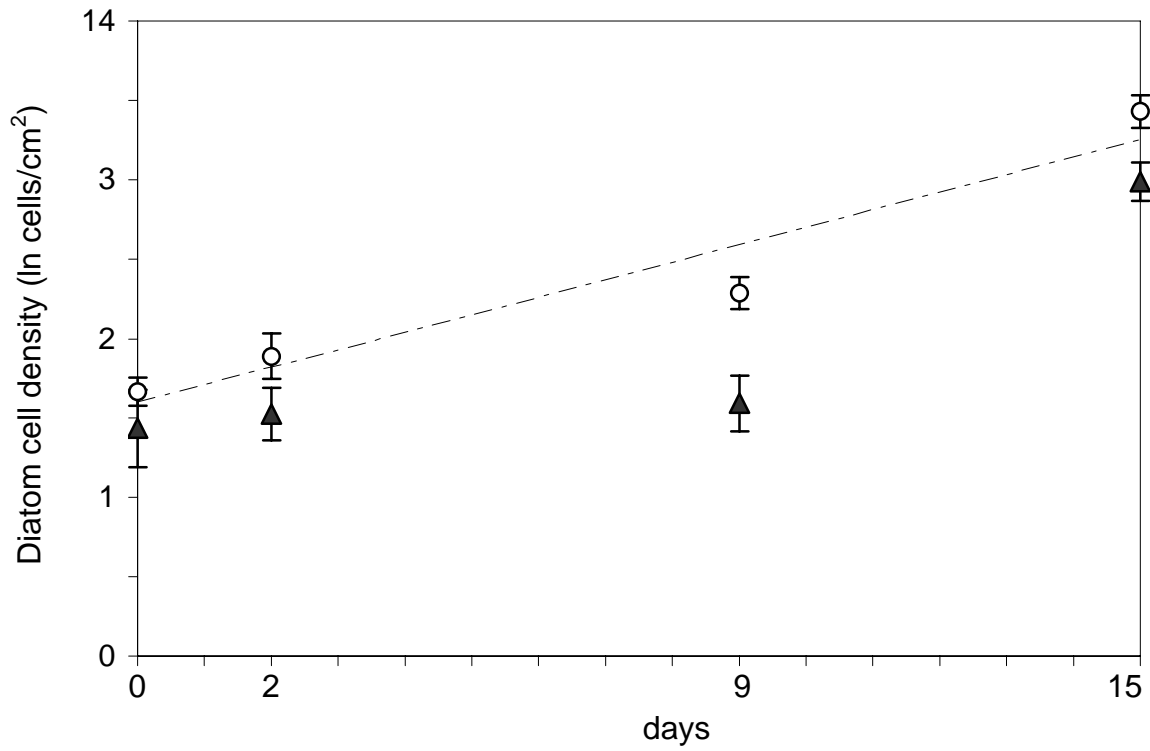
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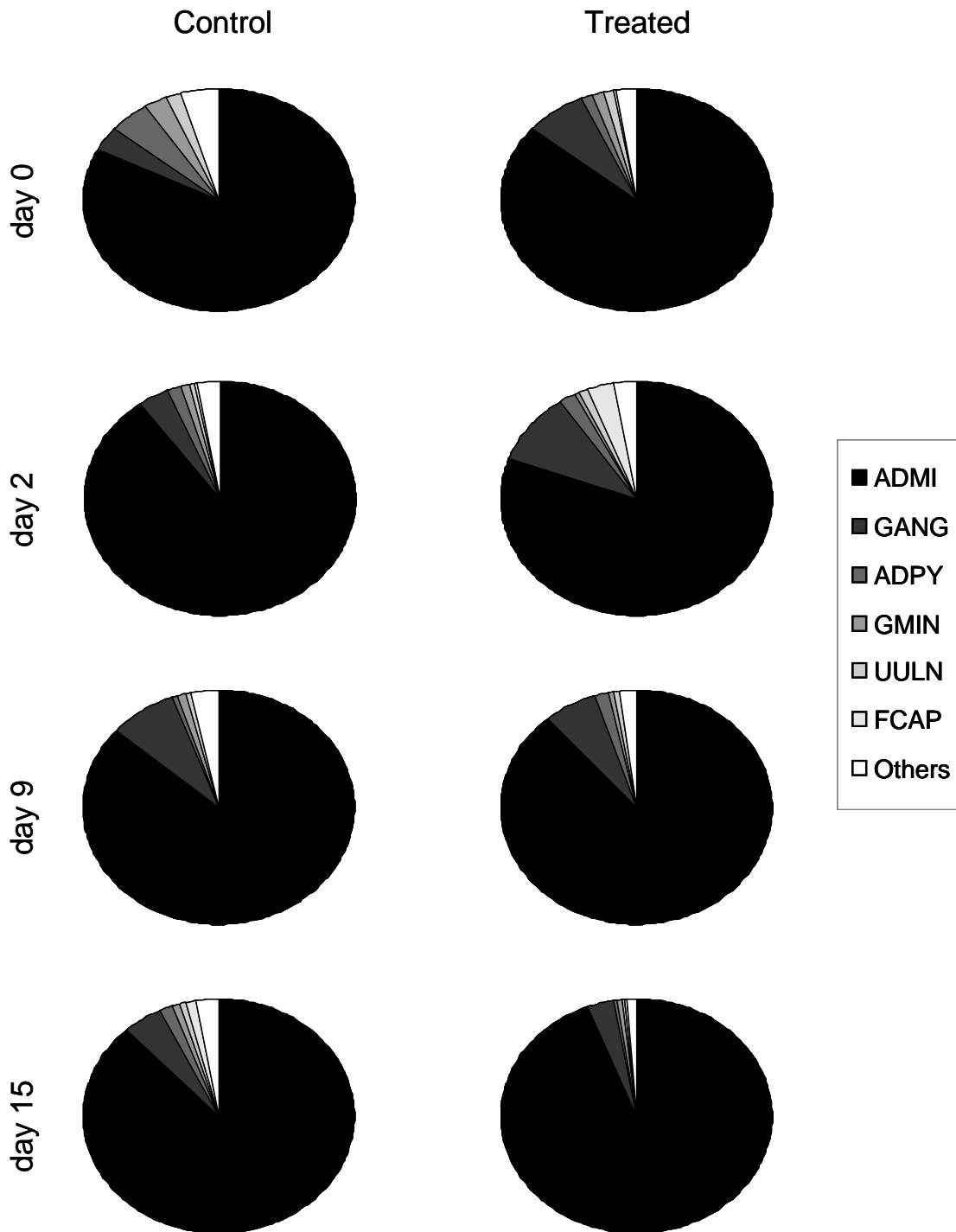
503 Figure 4

504 Changes in species relative abundances during the long-term response experiment. Species

505 abbreviations: ADMI: *Achnantheidium minutissimum*, ADPY: *A. pyrenaicum*, FCAP:

506 *Fragilaria capucina* var. *capucina*, GANG: *Gomphonema angustatum*, GMIN: *G. minutum*,

507 UULN: *Ulnaria ulna*.

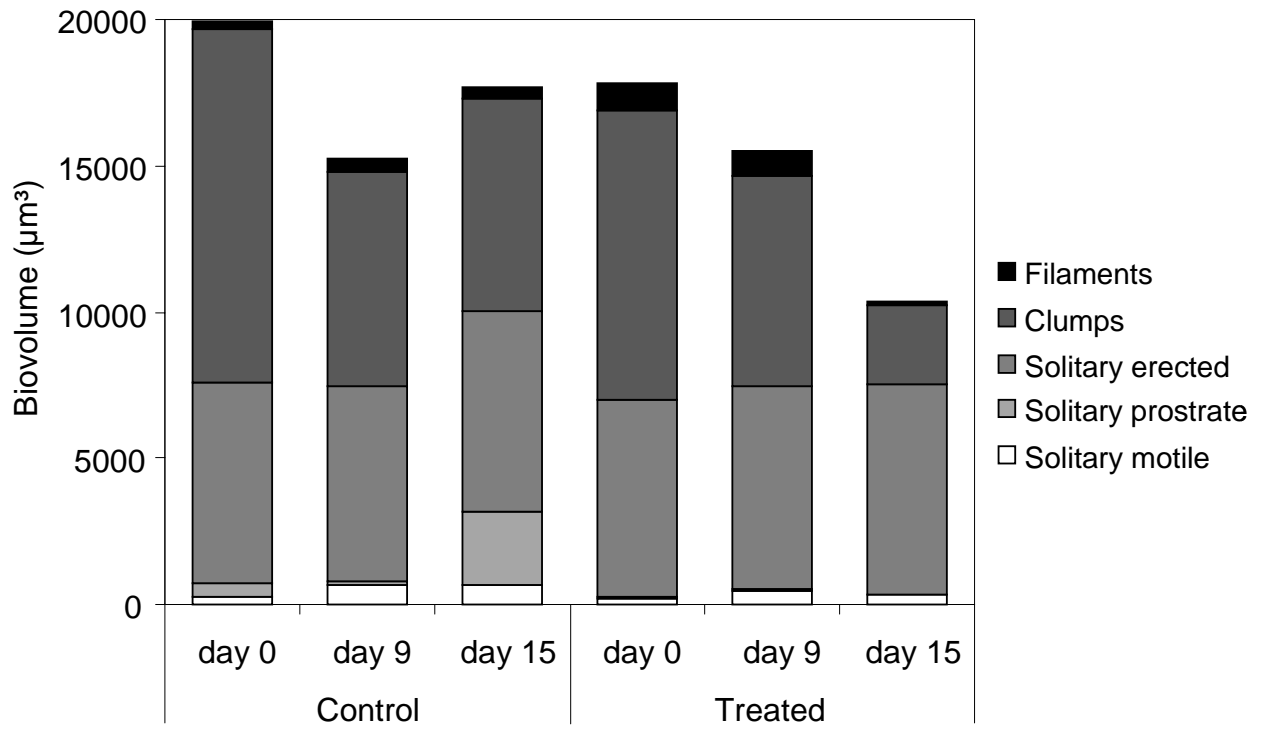


508

509 Figure 5

510 Distribution of diatom life-forms with respect to taxon abundances and specific cell

511 biovolume during the course of Experiment 2.



512