Effects of a bactericide on the structure and survival of benthic diatom communities

Soizic Morin, L. Proia, M. Ricart, C. Bonnineau, A. Geiszinger, F. Ricciardi, H. Guasch, A.M. Romani, Sergi Sabater

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

Soizic Morin[1,2], Lorenzo Proia[2], Marta Ricart[2], Chloé Bonnineau[2], Anita Geiszinger[2], Francesco Ricciardi[2], Helena Guasch[2], Anna Romaní[2], Sergi Sabater[2,3]

[1] Cemagref, UR REBX, 50 avenue de Verdun, 33612 Cestas cedex, France

[2] Institut d'Ecologia Aquàtica, Campus Montilivi, 17073 Girona, Spain

[3] Catalan Institute for Water Research (ICRA), Scientific and Technologic Park of the University of Girona, 17003, Girona, Spain

Running title: Effects of a bactericide on diatom communities

ABSTRACT

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

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

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

KEYWORDS

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

INTRODUCTION

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

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

deleterious effects on various other aquatic organisms. Recent reviews (Capdevielle *et al.* 2008; Sanderson & Thomsen 2009) reported a high sensitivity of algae to TCS, with growth inhibition EC_{50} values less than 5 µg/L. However, significant differences in sensitivity were observed among algal groups (Orvos *et al.* 2002) and algal life forms (Franz *et al.* 2008). Quick recovery of algal cultures following triclosan contamination events has also been reported (Orvos *et al.* 2002).

Though evidence exists that triclosan may affect algal growth, the potential sensitivity of fluvial (benthic) diatom assemblages is unknown. The arrival of this pharmaceutical into stream systems through sewage waters may cause relevant effects in the diversity, sensitivity and recovery capacity of diatom communities (Ricciardi *et al.* 2009). Here, we propose to use a dose-response test to determine the sensitivity of fluvial diatom assemblages to triclosan. Further, using a sublethal concentration, the recuperation potential of benthic diatom communities to this toxicant was assessed through functional and structural descriptors, i.e. chlorophyll-a *in vivo* fluorescence, live / dead diatom cell ratio and taxonomic composition.

MATERIALS AND METHODS

1. Experimental design

Experiment 1: Dose – response relationships

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

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

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

Eight independent incubators equipped with bubbling systems were used to grow biofilms in as described in Experiment 1. Colonisation lasted for 4 weeks, with a 12/12h light/dark cycle. The incubators were filled with liquid medium (1.5L each, detailed composition in Table 1). The liquid was replaced in each incubator twice per week, in order to avoid nutrient depletion. Then, in 4 of the 8 incubators triclosan was added, to reach the nominal concentration of 60 µgTCS/L. In order to maintain the contamination level, water was renewed every 3 hours during illuminated periods. After the 48-hour exposure with triclosan, all the incubators were re-filled with regularly replaced unpolluted liquid medium (as described above), and the biofilms incubated for 2 weeks.

Biofilm samples were taken just before triclosan addition (called T0d), at the end of the 48-hour exposure (T2d), and then after 7 days (T9d) and 13 days (T15d) in uncontaminated medium. For each sampling, 4 glass slides were randomly taken from each incubator and

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

2. Determination of the physicochemical characteristics

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

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

The HPLC system consisted of a Waters 717 autosampler and a Waters 1525 binary pump. HPLC separation was achieved on a 5-μm, 150 × 4 mm i.d. C18 reversed-phase column (SunFire). The injection volume was set at 50 μL, and the flow rate was 1 mL/min of 90% methanol - 10% water with isocratic flow. Detection was carried out using a UV−vis detector (Waters 2489) at 280 nm.

3. Diatom analyses

In vivo fluorescence measurements

An estimation of diatom-specific chlorophyll concentration was obtained by measuring the specific F0 (minimum fluorescence yield of dark-adapted cells) by means of a PhytoPAM fluorometer (Heinz Walz, Effeltrich, Germany) at 520 nm (Schmitt-Jansen & Altenburger 2007). The F0 was determined after applying a weak measuring light to the periphyton samples (directly on the colonised slides), after a 20-minute incubation in the dark to ensure that all the reaction centres were open. The photosynthetic capacity of the diatom community (Ymax) was also determined on dark-adapted samples. After being kept in the light for 20 minutes, the samples were exposed to actinic light for the determination of their photosynthetic efficiency (Yeff). PhytoPAM measurements for each parameter were repeated 5 times per slide and averaged.

Quantitative estimates of diatom community structure and live / dead ratio

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

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

4. Processing the data

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

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

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

RESULTS

Experiment 1: Dose – response relationships

Apart from the TCS additions, all channels were fed with the same water; its characteristics 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 µg/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 F0 = 100.2 ± 3.0 (n = 36),
203 Ymax = 0.43 ± 0.01 (n = 36) and Yeff = 0.30 ± 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 ± 24,000 cells/cm^2
206 occurred in the controls, while 91,000 ± 9,000 cells/cm^2 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 µgTCS/L, that reached ca. 170,000 ± 37,000
209 cells/cm^2.
210 The ratio between live and dead diatoms ranged from 6.2 ± 0.2 in the controls down to 3.5 ±
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 µg/L
214 was derived from the regression equation of the curve (confidence interval: 534-584 µg/L).
215 This value was taken as a criterion to use a 10-fold lower concentration (60 µg/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 F0 and Yeff values between treatments, duration of exposure, nor combined
221 effects. Indeed, T0d values were F0 = 138.4 ± 26.0 (n = 40) and Yeff = 0.37 ± 0.03 (n = 40),
222 with maximum variations of 29% of the control (T15d, where a slight date x treatment effect

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

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

A total of 32 diatom species were identified during this study, but only 6 of them accounted for more than 1% in at least one of the samples (Figure 4). In all treatments, diatom communities were dominated by *Achnanthidium minutissimum* Kützing (87.3 ± 1.4 %), followed by *Gomphonema angustatum* (Kützing) Rabenhorst (5.5 ± 0.9 %), *Achnanthidium pyrenaicum* (Hustedt) Kobayasi (1.9 ± 0.5 %), *Gomphonema minutum* (Agardh) Agardh (1.0 ± 0.2 %), *Ulnaria ulna* (Nitzsch) Compère (0.8 ± 0.2 %) and *Fragilaria capucina* Desmazières var. *capucina* (0.6 ± 0.3). Relative abundances of these main species remained 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 ± 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 ± 0.8
259 taxa), whereas it was constant through time in the controls (16.7 ± 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_{50} 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 Ymax 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 ± 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

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

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

Acknowledgements

This study was partially funded by the European projects Modelkey (511237, GOCE) and Keybioeffects (MRTN-CT-2006-035695). The authors are grateful to M. Laviale and S. Boutry for their help in analysing the data.

346

347 References

348 Aranami K & Readman JW 2007. Photolytic degradation of triclosan in freshwater and
349 seawater. *Chemosphere* 66(6):1052-1056

350 Balmer ME, Poiger T, Droz C, Romanin K, Bergqvist P-A, Muller MD & Buser H-R 2004.
351 Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish
352 from various lakes in Switzerland. *Environ. Sci. Technol.* 38(2):390-395

353 Bérard A & Pelte T 1999. Les herbicides inhibiteurs du photosystème II, effets sur les
354 communautés algales et leur dynamique. *Rev. Sci. Eau* 12(2):333-361

355 Berney M, Hammes F, Bosshard F, Weilenmann H-U & Egli T 2007. Assessment and
356 interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination
357 with flow cytometry. *Appl. Environ. Microbiol.* 73(10):3283-3290

358 Blanck H, Wängberg SA & Molander S 1988. Pollution-Induced Community Tolerance - A
359 new ecotoxicological tool. *In* Functional Testing of Aquatic Biota for estimating Hazards of
360 Chemicals, Cairns J. Jr, Pratt J.R. (Eds), Philadelphia, 219-230 p.

361 Bukhtiyarova LN 2006. Additional data on the diatom genus *Karayevia* and a proposal to
362 reject the genus *Kolbesia*. *Nova Hedw. Beih.* 130:85-96

363 Buth JM, Grandbois M, Vikesland PJ, McNeill K & Arnold WA 2009. Aquatic
364 photochemistry of chlorinated triclosan derivatives: Potential source of polychlorodibenzo-*p*-
365 dioxins. *Environ. Toxicol. Chem.* 28(12):2555-2563

366 Calabrese EJ 2005. Paradigm lost, paradigm found: The re-emergence of hormesis as a
367 fundamental dose response model in the toxicological sciences. *Environ. Pollut.* 138(3):378-
368 411

369 Canosa P, Morales S, Rodríguez I, Rubí E, Cela R & Gómez M 2005. Aquatic degradation of
370 triclosan and formation of toxic chlorophenols in presence of low concentrations of free
371 chlorine. *Anal. Bioanal. Chem.* 383(7):1119-1126
372 Capdevielle M, Van Egmond R, Whelan M, Versteeg D, Hofmann-Kamensky M, Inauen J,
373 Cunningham V & Woltering D 2008. Consideration of exposure and species sensitivity of
374 triclosan in the freshwater environment. *Integr. Environ. Assess. Manag.* 4(1):15-23
375 Compère P 2001. *Ulnaria* (Kützing) Compère, a new genus name for *Fragilaria* subgen.
376 *Alterasynedra* Lange-Bertalot with comments on the typification of *Synedra* Ehrenberg. *In*
377 Lange-Bertalot-Festschrift: Studies on Diatoms, Jahn R, Kociolek JP, Witkowski A, Compère
378 P. ARG Gantner Verlag, Ruggell, 97-102 p.
379 Coogan MA, Edziyie RE, La Point TW & Venables BJ 2007. Algal bioaccumulation of
380 triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater treatment plant
381 receiving stream. *Chemosphere* 67(10):1911-1918
382 Cox EJ 1996. Identification of freshwater diatoms from live material. Chapman & Hall
383 London.
384 Dell'Anno A, Fabiano M, Bompadre S, Armeni M, Leone L & Danovaro R 1999.
385 Phytopigment and DNA determinations in long-time formalin-preserved trap samples. *Mar.*
386 *Ecol. Prog. Ser.* 19171-77
387 Escalada MG, Russell AD, Maillard JY & Ochs D 2005. Triclosan-bacteria interactions:
388 single or multiple target sites? *Lett. Appl. Microbiol.* 41(6):476-481
389 Fent K, Weston AA & Caminada D 2006. Ecotoxicology of human pharmaceuticals. *Aquat.*
390 *Toxicol.* 76(2):122-159
391 Franz S, Altenburger R, Heilmeier H & Schmitt-Jansen M 2008. What contributes to the
392 sensitivity of microalgae to triclosan? *Aquat. Toxicol.* 90(2):102-108

393 Grover JP 2000. Resource competition and community structure in aquatic micro-organisms:
394 experimental studies of algae and bacteria along a gradient of organic carbon to inorganic
395 phosphorus supply. *J. Plankton Res.* 22(8):1591-1610
396 Guillard RRL 1973. Division rates. *In* Culture methods and growth measurements. Handbook
397 of phycological methods, Stein JR ed, Cambridge University Press, Cambridge, 289-311 p.
398 Hudon C & Legendre P 1987. The ecological implications of growth forms in epibenthic
399 diatoms. *J. Phycol.* 23(3):434-441
400 Ihaka R & Gentleman R 1996. R: A language for data analysis and graphics. *J. Comput.*
401 *Graph. Statist.* 5299-314
402 Katoh K 1992. Correlation between cell density and dominant growth form of epilithic diatom
403 assemblages. *Diat. Res.* 777-86
404 Kelly MG, Bennion H, Cox EJ, Goldsmith B, Jamieson J, Juggins S, Mann DG & Telford RJ.
405 2005. Common freshwater diatoms of Britain and Ireland: an interactive key. *In* Environment
406 Agency, Bristol
407 Krammer K 1997. Die cymbelloiden Diatomeen. Teil 2: Encyonema part., Encyonopsis und
408 Cymbellopsis. Bibliotheca Diatomologica Band 37. J. Cramer, Stuttgart
409 Krammer K & Lange-Bertalot H 1986 - 1991. Bacillariophyceae 1. Teil: Naviculaceae; 2.
410 Teil: Bacillariaceae, Epithemiaceae, Surirellaceae; 3. Teil: Centrales, Fragilariaceae,
411 Eunotiaceae; 4. Teil: Achnanthaceae. Kritische Ergänzungen zu *Navicula* (Lineolatae) und
412 *Gomphonema*. G. Fischer Verlag., Stuttgart
413 Lawrence JR, Zhu B, Swerhone GDW, Roy J, Wassenaar LI, Topp E & Korber DR 2009.
414 Comparative microscale analysis of the effects of triclosan and triclocarban on the structure
415 and function of river biofilm communities. *Sci. Total Environ.* 407(10):3307-3316
416 Levy CW, Roujeinikova A, Sedelnikova S, Baker PJ, Stuitje AR, Slabas AR, Rice DW &
417 Rafferty JB 1999. Molecular basis of triclosan activity. *Nature* 398(6726):383-384

McMurry LM, Oethinger M & Levy SB 1998. Overexpression of *marA*, *soxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiol. Lett.* 166(2):305-309

Murphy J & Riley JP 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* 2731-36

Orvos DR, Versteeg DJ, Inauen J, Capdevielle M, Rothenstein A & Cunningham V 2002. Aquatic toxicity of triclosan. *Environ. Toxicol. Chem.* 21(7):1338-1349

Potapova M 2006. *Achnanthidium zhakovschikovii* sp. nov. (Bacillariophyta) and related species from rivers of Northwestern Russia. *Nova Hedw.* 82(3-4):399-408

Ricart M, Barceló D, Geiszinger A, Guasch H, López de Alda M, Romaní AM, Vidal G, Villagrasa M, Sabater S 2009. Effects of low concentrations of the phenylurea herbicide diuron on biofilm algae and bacteria. *Chemosphere* 76(10):1392-1401

Ricart M, Guasch H, Alberch M, Bonnineau C, Farré M, Ferrer J, Geiszinger A, Morin S, Proia L, Ricciardi F, Romaní AM, Sala Ll, Sureda D & Sabater S. Effects of short term exposure of triclosan on biofilm communities (submitted)

Ricciardi F, Bonnineau C, Faggiano L, Geiszinger A, Guasch H, Lopez J, Muñoz I, Proia L, Ricart M, Romaní AM, Sabater S 2009. Is the chemical contamination linked to the diversity of biological communities in rivers? *Trends Analyt. Chem.* 28(5):592-602

Round FE & Bukhtiyarova L 1996. Four new genera based on *Achnanthes* (*Achnanthidium*) together with a re-definition of *Achnanthidium*. *Diat. Res.* 11:345-361

Sabater S, Guasch H, Ricart M, Romaní AM, Vidal G, Klünder C, Schmitt-Jansen M 2007. Monitoring the effects of chemicals on biological communities. The biofilm as an interface. *Anal. Bioanal. Chem.* 387(4):1425-1434

Sala SE, Guerrero JM, Ferrario ME 1993. Redefinition of *Reimeria sinuata* (Gregory) Kociolek & Stoermer and recognition of *Reimeria uniseriata* nov. spec. *Diat. Res.* 8:439-446

443 Sanderson H & Thomsen M 2009. Comparative analysis of pharmaceuticals versus industrial
444 chemicals acute aquatic toxicity classification according to the United Nations classification
445 system for chemicals. Assessment of the (Q)SAR predictability of pharmaceuticals acute
446 aquatic toxicity and their predominant acute toxic mode-of-action. *Toxicol. Lett.* 187(2):84-93
447 Schmitt-Jansen M & Altenburger R 2007. The use of pulse-amplitude modulated (PAM)
448 fluorescence-based methods to evaluate effects of herbicides in microalgal systems of
449 different complexity. *Toxicol. Environ. Chem.* 89(4):665-681
450 Serra A, Corcoll N & Guasch H 2009. Copper accumulation and toxicity in fluvial
451 periphyton: The influence of exposure history. *Chemosphere* 74(5):633-641
452 Singer H, Muller S, Tixier C & Pillonel L 2002. Triclosan: Occurrence and fate of a widely
453 used biocide in the aquatic environment: Field measurements in wastewater treatment plants,
454 surface waters, and lake sediments. *Environ. Sci. Technol.* 36(23):4998-5004
455 Sivaraman S, Sullivan TJ, Johnson F, Novichenok P, Cui G, Simmerling C & Tonge PJ 2004.
456 Inhibition of the bacterial enoyl reductase FabI by triclosan: A structure-reactivity analysis of
457 FabI inhibition by triclosan analogues. *J. Med. Chem.* 47(3):509-518
458 Villalaín J, Mateo CR, Aranda FJ, Shapiro S & Micol V 2001. Membranotropic effects of the
459 antibacterial agent triclosan. *Arch. Biochem. Biophys.* 390(1):128-136
460 Wilson BA, Smith VH, deNoyelles F & Larive CK 2003. Effects of three Pharmaceutical and
461 Personal Care Products on natural freshwater algal assemblages. *Environ. Sci. Technol.*
462 37(9):1713-1719
463 Wind T, Werner U, Jacob M & Hauk A 2004. Environmental concentrations of boron, LAS,
464 EDTA, NTA and Triclosan simulated with GREAT-ER in the river Itter. *Chemosphere*
465 54(8):1145-1154

Wong-Wah-Chung P, Rafqah S, Voyard G & Sarakha M 2007. Photochemical behaviour of triclosan in aqueous solutions: Kinetic and analytical studies. *J. Photochem. Photobiol. A Chem.* 191(2-3):201-208

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

	Experiment 1	Experiment 2
pH	7.7 ± 0.0 *(n=8)*	7.9 ± 0.5 *(n=11)*
Temperature (°C)	15.8 ± 0.0 *(n=8)*	17.5 ± 1.1 *(n=11)*
Conductivity (µS/cm)	289.9 ± 0.2 *(n=8)*	143.4 ± 34.1 *(n=11)*
Dissolved oxygen (mg/L)	9.0 ± 0.1 *(n=8)*	9.2 ± 0.2 *(n=11)*
Oxygen saturation (%)	94.3 ± 0.4 *(n=8)*	7.9 ± 0.5 *(n=11)*
NO_3 (mg/L)	1.85 ± 0.17 *(n=16)*	0.75 *(n.c.)*
PO_4 (µg/L)	77.3 ± 8.6 *(n=16)*	16.9 ± 2.1 *(n=12)*

474 Table 2. Nominal and effective concentrations of triclosan (μg/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

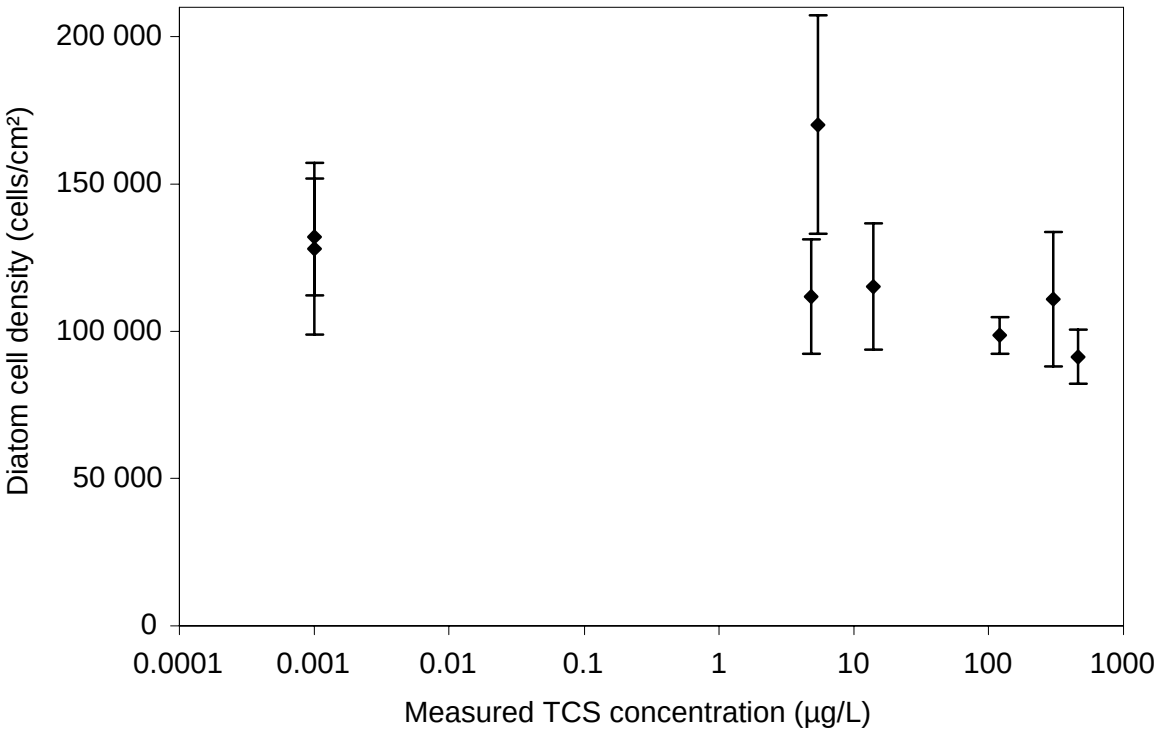
477

Figure captions

Figure 1

Densities of live diatom cells (number of cells/cm²) after a 48h-exposure, for the different concentrations of triclosan measured in the channels.

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

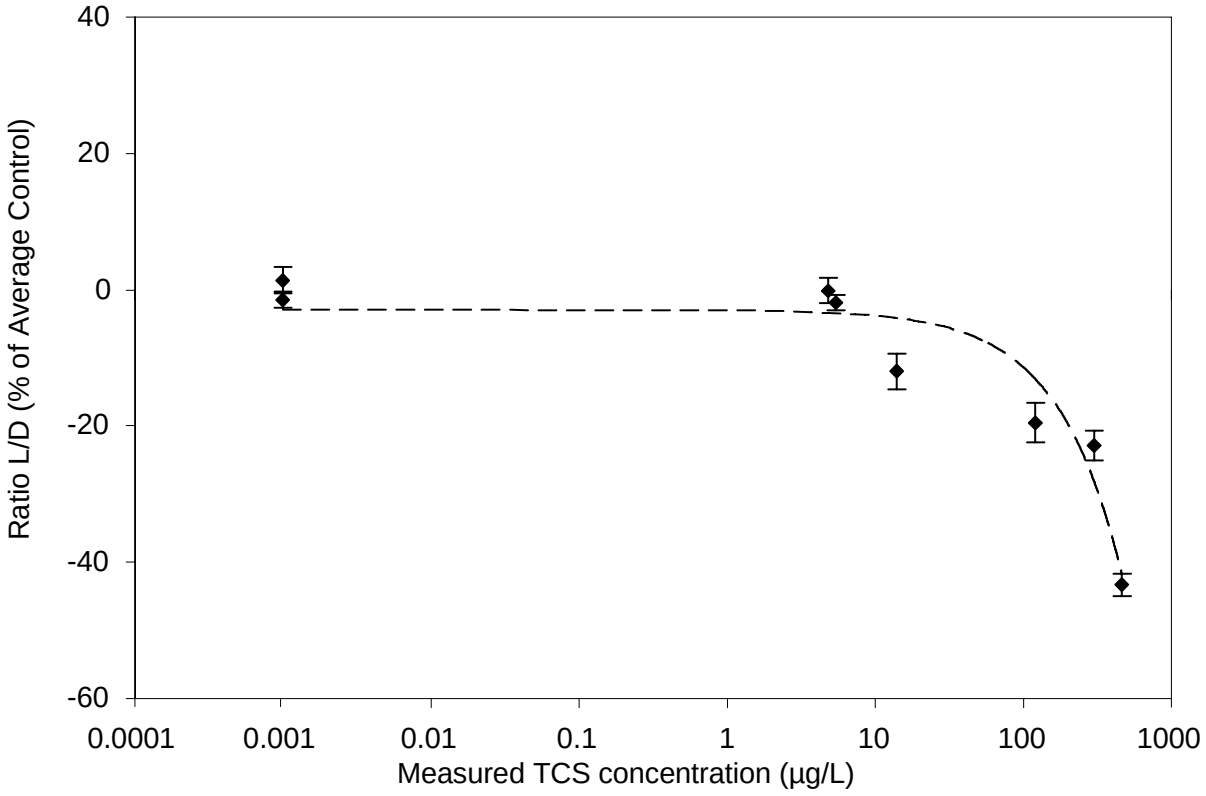


Figure 3

Diatom cell densities (in ln cells/cm^2) in the control (○) and treated (▲) incubators during the course of Experiment 2.

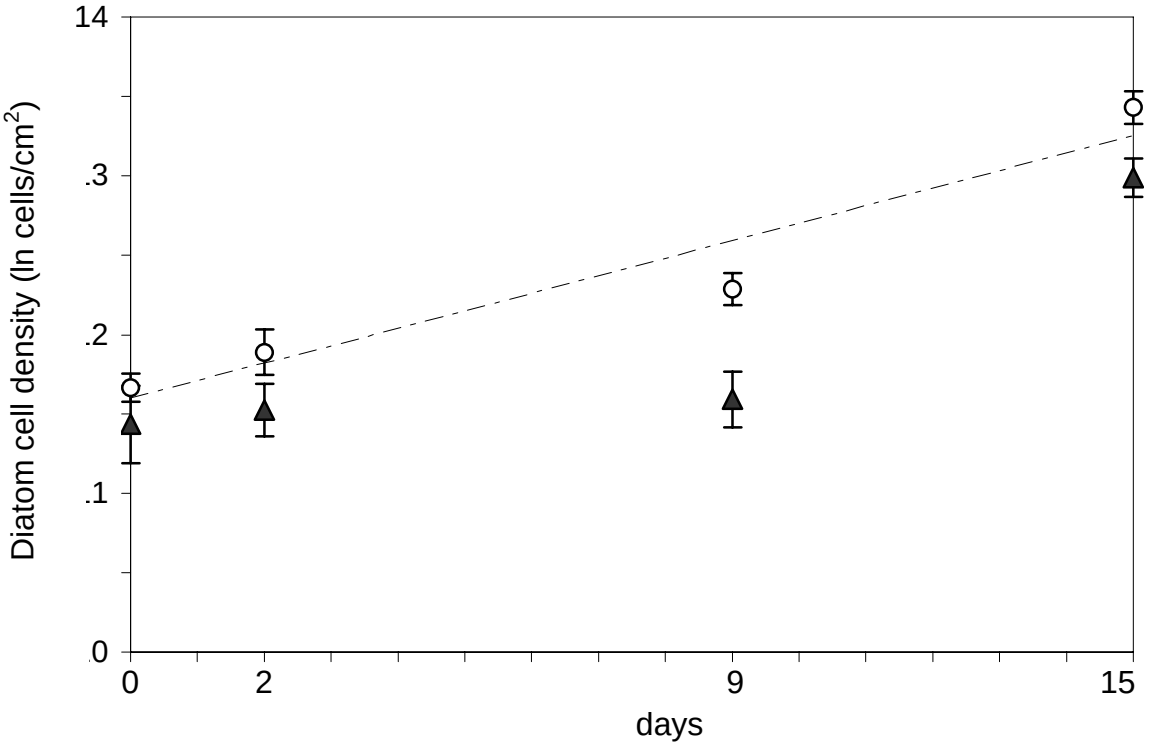
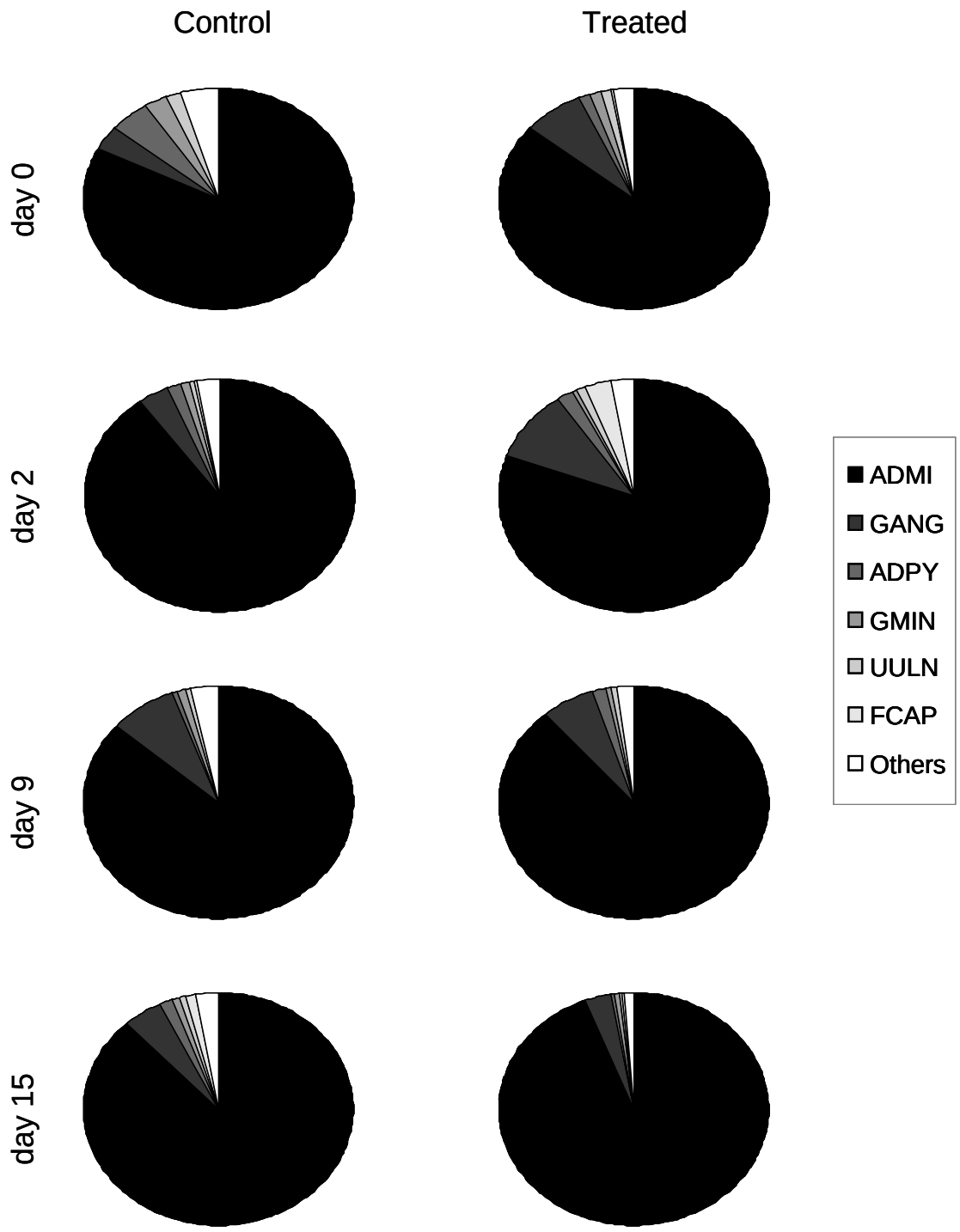


Figure 4

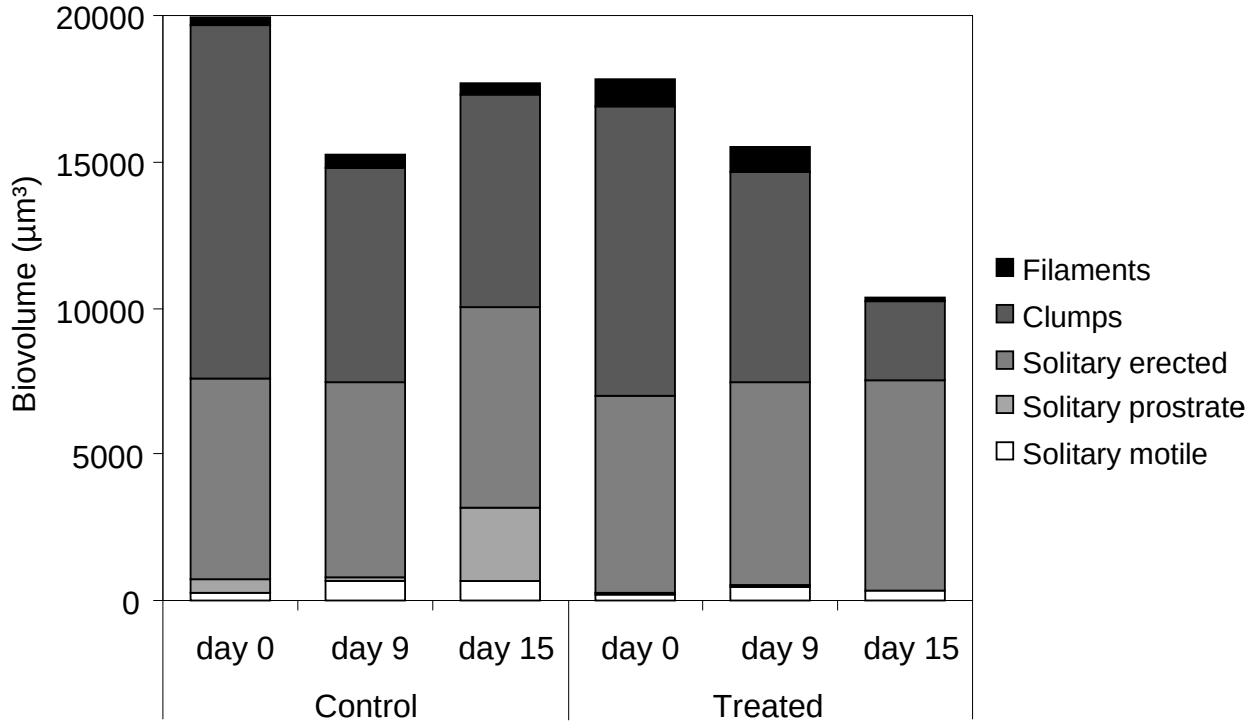
Changes in species relative abundances during the long-term response experiment. Species abbreviations: ADMI: *Achnanthidium minutissimum*, ADPY: *A. pyrenaicum*, FCAP: *Fragilaria capucina* var. *capucina*, GANG: *Gomphonema angustatum*, GMIN: *G. minutum*, UULN: *Ulnaria ulna*.



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