RESISTANCE AND RECOVERY OF RIVER BIOFILMS RECEIVING SHORT PULSES OF TRICLOSAN AND DIURON

L. Proia [1], Morin S.[1/2], Peipoch M.[1/3], Romaní A.M.[1], S. Sabater [1/4]

[1] Institute of Aquatic Ecology, University of Girona, E-17071 Girona (Spain)

[2] Cemagref, UR REBX, F-33612 Cestas Cedex (France)

[3] Centre d'Estudis Avançats de Blanes, Consejo Superior de Investigaciones Científicas, E-17300 Blanes (Spain)

[4] Catalan Institute for Water Research (ICRA), E-17003 Girona (Spain)

***Research Highlights**

>We studied the effects of Diuron and Triclosan pulses on river biofilms structure and function. > Diuron affected directly autotrophs and indirectly bacteria. > Triclosan affected directly bacteria, indirectly diatoms and reduces phosphorus uptake capacity. > Direct effects recovered rapidly and indirect ones later on. > Late recovery of phosphorus uptake capacity by biofilm highlights risk for ecosystem service.

ABSTRACT

The effects of the herbicide Diuron (DIU) and the bactericide Triclosan (TCS) were assessed on laboratory-grown stream biofilms. Four week-old biofilms were exposed in mesocosms to 48-hours of short pulses of either DIU or TCS. The direct and indirect effects of each toxicant on the biofilms, and the subsequent recovery of the biofilms, were evaluated according to structural and functional biomarkers. These parameters were analyzed immediately before exposure, immediately after exposure, and 9 and 16 days post-exposure. DIU caused an increase in diatom mortality (+79%), which persisted until the end of the experiment. TCS also affected diatom mortality (+41%), although the effect did not appear until one week post-exposure. TCS caused an increase in bacterial mortality (+45%); however, this parameter returned to normal values 1 week post-exposure. TCS compromised the cellular integrity of the green alga *Spirogyra* sp., whereas DIU did not. TCS also strongly inhibited phosphate uptake (-71%), which did not return to normal values until 2 weeks post-exposure. DIU directly affected algae, but barely affected the heterotrophs, whereas TCS seriously impaired bacteria (direct effect) as well as autotrophs (indirect effect). However, the biofilms recovered their normal structure and function within only a few days to a few weeks. These findings demonstrate the capacity of biofilms to cope with periodic inputs of toxicants, but also the risks associated to repeated exposure or multi-contamination in aquatic ecosystems.

Keywords:

Biofilms, Resistance, Recovery, Triclosan, Diuron, Pulses.

1. INTRODUCTION

Pollutants from agricultural, industrial and domestic activities enter watercourses either continuously (producing potentially chronic effects) or in pulses (causing potentially acute effects), in function of flow episodes, crop treatments and/or industrial release (Ellis, 2006). These chronic and periodic inputs are likely to have unexpected effects on the organisms living in aquatic environments. River ecosystems feature various ecological services (*e.g.* self-depuration and organic matter mineralization) directly related to processes driven by complex microbial communities (Mathuriau and Chauvet, 2002; Findlay et al., 1993; Sabater et al., 2007). These communities include benthic biofilms, which play a fundamental role in the trophic web and in the geochemical cycles within aquatic ecosystems (Battin et al., 2003; Lock, 1993). As interfaces between the water column and the substrata, biofilms are the first communities to suffer the consequences of pollutants (Sabater et al., 2007). Thus, understanding the resistance and resilience of biofilm communities to pollutants is crucial for ecological risk assessment of priority and emerging compounds.

This study analyzes the effects of two compounds on stream biofilms: the herbicide Diuron (DIU; 3-(3,4-dichlorophenyl)-1,1-dimethylurea), and the broad-spectrum bactericide Triclosan (TCS; 5-chloro-2-(2,4-dichlorophenoxy)phenol), which operate by different modes of action.

DIU is a photosynthesis inhibitor included on the list of priority pollutants of the EU Water Framework Directive (European Commission, 2000). As herbicide, DIU is active against phototrophic microorganisms and higher plants by blocking the chloroplast electron transport chain in Photosystem-II (Moreland, 1967). It has been used to control various annual and perennial broadleaf and grassy weeds, and is applied for vineyard protection. It has been also used on non-crop areas such as roads, garden paths and railway lines, and on many agricultural crops such as fruit, cotton, sugar cane, alfalfa and wheat (Giacomazzi and Cochet, 2004). Several

studies have reported the presence of DIU in surface waters (Azevedo et al., 2000; Blanchoud et al. 2004; Rodriguez-Mozaz et al., 2004).

TCS is active against both gram-positive and gram-negative bacteria. It is an inhibitor of the enzyme enoyl–acyl carrier protein reductase (ENR), which is involved in bacterial lipid biosynthesis (Adolfsson-Erici et al., 2002). For over 30 years TCS has been used in products such as anti-bacterial hand soaps, deodorants, household cleaners, dental hygiene products, and textiles (Singer et al., 2002). This emerging compound has been reported in sewage wastewater and sludge at significant concentrations (Halden and Paull, 2005; Samsøe-Petersen et al., 2003). Although wastewater treatment plants (WWTPs) are rather effective at removing TCS (Samsøe-Petersen et al., 2003; McAvoy et al., 2002), this compound still reaches freshwater systems, and has been reported in various aquatic habitats, including rivers, streams (Ellis, 2006; Kuster et al., 2008; Morral et al., 2004), lakes (Loos et al., 2007; Singer et al., 2002) and the sea (Xie et al., 2008). Both DIU and TCS have been widely tested for toxicity to myriad cultured aquatic organisms (Canesi et al., 2007; Capdevielle et al., 2008; De Lorenzo et al., 2007; Farré et al., 2008; Flaherty and Dodson, 2005; Giacomazzi and Cochet, 2004; Orvos et al., 2002; Wilson et al., 2003); however, they have not been extensively studied for toxicity to natural complex communities (Franz et al., 2008; Lawrence et al., 2009; Morin et al., 2010a; Pesce et al., 2006, 2008; Ricart et al., 2009).

DIU reaches running waters primarily via pulses from diffuse source, whereas TCS enters them periodically from fixed sources (chiefly, WWTPs). DIU pulses of up to 134.0 μg L^{-1} have been described during flooding events in vineyard catchments. These chronically affected environments show baseline concentrations of about 1 μg DIU L^{-1} between flood events (Rabiet et al., 2010). In contrast, TCS enters running waters chronically at low concentrations via urban sewage effluents, and its removal during wastewater treatment is variable (Ellis, 2006; Ricart et al., 2010). Measured TCS concentrations can be reduced to 80% on average trough waste water

85 treatment plants (Kantiani et al., 2008), but still can reach river waters, where observed
86 concentration range between 0.027 and 2.7μg L^{-1} (Ricart et al., 2009). In spite of these low
87 concentrations, brief spikes of compounds such as TCS could occur during dry periods, and
88 might result in transient perturbations of river ecosystems, with unknown long-term implications.
89 These inputs may have specific or non-specific effects on both target and non-target organisms.
90 Studying how biological communities are affected from these events is an ecological priority.
91 Studying how communities recover from transient perturbations is important for assessing the
92 risks associated with chronic contaminations

93 This study gauged the ability of biofilms to cope with short pulses of either DIU or TCS,
94 assessing their initial responses and their subsequent recoveries. Considering the intrinsic
95 complexity of biofilms, it was hypothesized that in addition to the specific effects of DIU on
96 autotrophs, and of TCS on bacteria, these toxicants could provoke indirect effects deriving from
97 ecological interactions at the microbial scale. The direct effects were expected to occur
98 immediately upon toxicant exposure, whereas the indirect ones were expected to appear later on.
99 Moreover, it was predicted that the time required for the biofilms to recover from the pollutant
100 pulse could be correlated to the presence of target organisms (direct effects) and non-target
101 organisms (indirect effects) in the biofilms. Given that indirect effects can involve much more
102 complex mechanisms (Ricart et al., 2009), they can imply longer recovery times. Thus, the core
103 hypothesis in this work was that early recovery of biofilm endpoints could be related to direct
104 effects of pollutants, whereas late recovery could be related to indirect ones. In order to verify our
105 hypothesis toxicants concentrations were selected after considering ecotoxicological data
106 available (i.e. EC50, NOEC) both on single cultured species and on natural biofilm communities.
107 It has been demonstrated how complex biofilm communities can result more resistant to toxicants
108 than single species composing it (Franz et al., 2008). Several studies demonstrated the protective
109 function of extracellular polymeric substances produced by biofilm organisms (Admiraal et al.,

1999; Samrakandi et al., 1997). These evidences were considered for the selection of toxicants concentrations as well as for consequent comparison of results with other studies.

In this work, 4-week old biofilms were subjected to 48-hours of short pulses of either DIU or TCS. Their responses to these compounds were measured just after exposure, and then once weekly for 2 weeks post-exposure. Each toxicant's effects on the biomass and survival of algae and bacteria were used to gauge their respective effects on biofilm structure. Furthermore, their effects on extracellular enzymatic activity and phosphate uptake were used to assess the ecological implications of their entry into running water—namely, in the context of nutrient retention and river self-depuration.

2. MATERIAL AND METHODS

2.1 Experimental design

Biofilms were scraped from rocks of the Fuirosos Stream, a third order pristine stream located in the Natural Park of Montnegre-Corredor, (50 km N of Barcelona, NE Spain), and then inoculated and colonized in twelve independent mesocosms. They were colonized on glass slides (1 cm^2 each) placed at the bottom of each mesocosm (35-40 slides per mesocosm).The mesocosms comprised sterile glass jars (19 cm in diameter, 9 cm high), filled with 1.5 L of artificial stream water which was recirculated using a submersible pump (Hydor, Pico 300, 230 V 50 Hz, 4.5W). Artificial stream water was produced adding pure salts to MilliQ water (Millipore) as described in Ylla et al. (2009). To avoid nutrient depletion the water in each mesocosm was changed twice weekly. All mesocosms were maintained in an incubator (Radiber AGP-570) under controlled temperature (17.5 ± 1.1 °C) and light irradiance (160-180 µmol photons m^{-2} s^{-1}; darkness/light cycle of 12 h/12 h). After 4 weeks colonization, four mesocosms were treated with TCS (IRGASAN, Sigma Aldrich, >97% , CAS: 3380-34-5) up to nominal concentrations of 60 µg/L, and another four, with DIU (Sigma Aldrich, minimum 98%, CAS: 330-54-1) up to nominal

concentrations of 15 µg/L. The last four mesocosms were left untreated and used as control. To minimize photodegradation of the toxicants and ionization of TCS (pKa = 8.1), water and toxicants were renewed every 3 h (during the light cycle) for 48 hours. The pH was monitored between water changes during toxicant exposure. After the exposure period, the mesocosms were refilled with unpolluted artificial river water (as described above) that was changed twice per week during the following 2 weeks. The biofilm was sampled four times: before contamination (day 0), after the 48-hour exposure (day 2), and 1 and 2 weeks post-exposure (days 9 and 16).

Glass tiles from each mesocosm were randomly sampled. Extracellular enzyme activities, photosynthetic parameters (F_0, Y_{eff} and Y_{max}), and bacterial densities were immediately measured after collection. Diatom samples for enumeration and taxonomical identification were preserved in formalin before being processed. Samples for chlorophyll determination were frozen (-20 °C) until analysis. Samples for Scanning Electron Microscopy (SEM) observation were collected at day 16. The phosphorus uptake (P-uptake) of the biofilm in each mesocosm was experimentally determined on each sampling day.

2.2 Water analysis

Concentrations of TCS, methyl-Triclosan (Me-TCS) and DIU were determined using high performance liquid chromatography (HPLC). Stock solutions (1 mg/mL) were prepared by dissolving pure standards of the highest purity available (HPLC grade, Sigma Aldrich) in methanol. An external calibration curve was then built for each compound by injecting different concentrations of individual standards prepared by different dilutions of the stock solution. Water samples were collected once from each mesocosm. Samples were filtered through 0.45µm nylon membrane filters (Whatman) and immediately loaded onto C18 SPE cartridges (Sep-Pak® Vac 3 cc tC18, Waters, Ireland) previously conditioned with 5 mL of HPLC water and methanol at a flow rate of 1 mL min^{-1}. Samples (500 mL) were loaded at a flow rate of 5 mL min^{-1}. After pre-concentration, the cartridges were completely dried *in vacuo* for 20 minutes

160 to avoid hydrolysis and kept frozen until analysis. Thereafter, cartridges were eluted with 4 mL
161 of methanol. Eluted samples were partially evaporated under a gentle nitrogen stream and
162 reconstituted in a final volume of 1 mL methanol. Samples were then analyzed by liquid
163 chromatography. The HPLC system comprised a binary HPLC Pump (Waters 1525), an auto
164 sampler (Waters 717 Plus) and a UV-detector (Waters 2487 Dual λ Abs. Detector). The HPLC
165 separation entailed use of a µm C_{18} reverse-phase column (Sunfire 4.6x150 mm). For DIU
166 analysis, the injection volume was set at 20 µL and separation was performed using an isocratic
167 gradient of 45% methanol/55% water at 0.8 mL min^{-1}. The DIU peak was detected at 251 nm.
168 For TCS and Me-TCS analysis the injection volume was set at 50 µL and the flow rate was 1
169 mL min^{-1} of 90% methanol with isocratic flow. The TCS and Me-TCS peaks were detected at
170 280 nm.

171 **2.3 Biofilm structure and function**

172 Several biofilm endpoints were measured in order to describe structural and functional responses
173 of autotrophic and heterotrophic biofilm compartments of biofilms to toxicants pulses. In
174 particular the structure of the autotrophic community was investigated by measuring the
175 Chlorophyll-*a* density (as surrogate of autotrophic biomass) as well as the growth rate, mortality
176 and composition of diatom community. The structural response of heterotrophs was investigated
177 by counting the live and dead bacterial cells. This counting allowed the calculation of live/dead
178 ratio and therefore provides information about bacterial mortality. Scanning Electron Microscopy
179 (SEM) observations were useful to compare the structure of treated and non-treated biofilm at the
180 end of the experiment.

181 The *in vivo* fluorescence measurements described the functional response of phototrophic
182 organisms (green algae, diatoms and cyanobacteria). The extracellular enzymatic activities
183 described the heterotrophic capacity to degrade organic matter. Finally, the phosphorus uptake

184 rate measurement was used to describe the capacity of the whole biofilm community to remove
185 phosphate from the water column.
186
187
188 **2.3.1 Chlorophyll-a density.** Glass tiles for chlorophyll analysis were collected from the
189 mesocosms and stored in the dark at -20 °C until analysis. Chlorophyll-a in the biofilms was
190 quantified after extraction from the glass substrata in 90% acetone in the dark at 4 °C for 12 h.
191 Concentration was determined spectrophotometrically after filtration (GF/F Whatman) of the
192 extract, following the procedure of Jeffrey and Humphrey (1975).
193 **2.3.2 Diatom community structure and live/dead ratio.** Glass slides were scraped using
194 polyethylene cell lifters (Corning Inc., NY), preserved with a drop of formalin solution, and then
195 diluted with artificial stream water to a final volume of 5 mL. Samples were ultrasonicated for 7
196 minutes (sonication bath Bransonic 220, Technofix) to separate the aggregated cells. A 125 μL
197 aliquot of each sample were then pipetted onto a Nageotte counting chamber. The cells were
198 counted in ten fields (1.25 μL each, 0.5 mm depth) by light microscopy (Nikon Eclipse 80i,
199 Nikon Co., Tokyo, Japan) at 10× magnification. Data were recorded as the number of cells per
200 unit area of sampled substrate (number of cells per cm^2), and the cells were classified as either
201 live (if they contained chloroplasts) or dead (if they were empty) (Morin at al., 2010b). Growth
202 rates (expressed as number of cell divisions per day) of the diatom community were calculated
203 from live diatom counts (Guillard, 1973). Diatom community composition was determined based
204 on permanent slides following ANSP protocols (Charles et al. 2002). Approximately 500
205 frustules were counted per slide at 1,000× magnification; they were identified using European
206 taxonomic literature (Krammer and Lange-Bertalot, 1986-1991) and recent nomenclature
207 updates.

208 **2.3. 3 Bacterial density**. Live and dead bacteria were counted with epifluorescence microscopy
209 using the LIVE/DEAD® Bacteria Viability Kit L7012 (*Bac*Light™, Molecular Probes,
210 Invitrogen). Biofilm samples were sonicated (< 1 min, sonication bath at 40 W and 40 kHz,
211 Selecta) and scraped (Nunc sterile silicone cell scraper) to obtain a biofilm suspension. Samples
212 were then diluted with pre-filtered sterilized water from the mesocosms, and 2 mL subsamples
213 were stained with 3 µL of a 1:1 mixture of SYTO 9 and propidium iodide for 15-30 minutes in
214 the dark. After incubation, samples were filtered through a 0.2 µm black polycarbonate filters
215 (Nuclepore, Whatman). Filters were then dried, placed on a slide with mounting oil (Molecular
216 Probes) and counted by epifluorescence microscopy (Nikon E600, 100× in immersion oil). Green
217 (live) and red (dead) bacterial cells were counted in 20 random fields per filter.
218 **2.3.4 SEM observation.** One glass tile from each mesocosm was collected at day 16 for SEM
219 observation. Samples were fixed immediately with 2.5% glutaraldehyde in 0.1 M cacodylate
220 buffer, pH 7.2–7.4. Samples were dehydrated in graded ethanol (65–100%) and dried at the
221 critical point of CO_2. Finally, samples were sputter coated with gold, and then observed by SEM
222 (Zeiss DSM 960).
223 **2.3.5 In vivo fluorescence measurements.** Fluorescence emission from chlorophyll was
224 measured with a PhytoPAM (Pulse Amplitude Modulated) fluorometer (Heinz Walz GmbH),
225 which uses a set of LEDs at four wavelengths (470, 520, 645, and 665 nm). Five analytical
226 replicates (1 cm^{-2} glass tiles) for each experimental replicate (mesocosm) were analyzed and
227 averaged (Serra et al., 2009). The photosynthetic efficiency (Y_{eff}) of photosystem II (PSII) and
228 the photosynthetic capacity (Y_{max}) of PSII were also estimated (Ricart et al., 2010).
229 **2.3.6 Extracellular enzymatic activity.** Leucine-aminopeptidase (EC3.4.11.1) and alkaline
230 phosphatase (EC 3.1.3.1-2) in the biofilms were quantified spectrofluorometrically using the
231 fluorescent-linked substrates L-leucine-4-methyl-7-coumarinylamide (Leu-AMC, Sigma-Aldrich)
232 and MUF-phosphate (MUF-P, Sigma-Aldrich), respectively. Colonized glass substrata were

collected from the mesocosms and placed in vials filled with 4 mL of pre-filtered water (0.2 μm Nylon Membrane filters, Whatman). Samples were immediately incubated at saturating conditions (0.3 mM, Romaní and Sabater, 1999) for 1 h in the dark in a shaking bath. Blanks and control samples were used to correct non-enzymatic hydrolysis of the substrate and of fluorescent substances in the solution, respectively. Blanks, control samples, and AMC and MUF standards (0–100 μM) were also incubated in the shaking bath. Following incubation, 4 mL of glycine buffer (pH 10.4) solution was added (1:1, v/v), and fluorescence was measured at 364 and 445 nm (excitation & emission) for AMC and at 365 and 455 nm (excitation & emission) for MUF (Kontron, SFM25). The calculated AMC and MUF concentrations were then standardized for glass surface and incubation time; therefore, the activity values are expressed as nanomoles (of AMC or MUF) per cm^2 of biofilm surface area per hour. To determine the specific activity per live cell, the activities per unit area were further normalized per the number of live bacteria (Ricart et al., 2009).

2.3.7 Phosphorus uptake. Soluble reactive phosphorus (SRP) uptake (P-uptake) of the biofilms was calculated by measuring the decay of SRP following a spike. Background SRP concentrations were analyzed at each sampling date. SRP concentration was increased by adding a spike of 10 mM Na_2PO_4, which increased basal concentrations by roughly four to eight times. Samples for SRP concentration (10 mL) were taken five times between 1 and 20 minutes post-spike in each replicate. The P-uptake was calculated as the mass of phosphorus removed from the water column per unit area per time (μg P cm^{-2} h^{-1}). Abiotic controls showed that SRP had not decayed during the experiment.

2.4 Statistical tests

Differences in the biofilm endpoints were tested daily using one-way analysis of variance (ANOVA), in which treatment (DIU or TCS) was set as the fixed factor. Effects were analyzed

post hoc with Tukey's b test. Statistical significance was set at p = 0.05. Analysis was performed using SPSS Version 15.0. Growth rates were calculated from the slope of the linear portion of a curve showing the log of the cell number as a function of time, as described by Morin et al. (2008), and tested with ANOVA.

3. RESULTS

3.1 Physical and chemical conditions in the mesocosms

Conductivity, pH and dissolved oxygen in the mesocosms remained steady during the experiment: their mean values were 143.4 (± 34.1) µS cm^{-1}, 7.9 (± 0.5), and 9.2 (± 0.2) mg L^{-1} (n= 48), respectively. SRP concentration ranged from 16.9 (± 2.1) to 4.5 (±1.9) µg L^{-1} between water replacements. pH was monitored between water changes and ranged from 7.45 (± 0.02) to 7.62 (±0.04) (n = 8), never reaching the TCS pKa value of 8.1.

During the 48 hours of toxicant exposure, the DIU-contaminated mesocosms had 13.4 ± 1.3 µg DIU L^{-1} (n = 4), and the TCS-contaminated mesocosms, 60.8 ± 30.1 µg TCS L^{-1} (n = 4). The level of Me-TCS in the TCS-contaminated mesocosms was consistently below the detection limit. The water of control mesocoms resulted in no trace of both DIU and TCS (n = 8).

3.2 Biofilm structure: microbial biomass, diatom community composition, and SEM observations

Before toxicant exposure, the biofilms had chlorophyll-a concentration of 3.04 ± 0.72 µg cm^{-2} and a mean bacterial density of 3.06 ± 1.14 x 10^7 cells cm^{-2}. The diatom community was dominated by *Achnanthidium minutissimum* (Kützing), and also contained *Achnanthidium biasolettianum* (Grunow), *Ulnaria ulna* (Nitzsch) and *Gomphonema*. Live diatom density was 11.3 ± 3.7 x 10^4 cells cm^{-2}, and the diatom live/dead ratio was 10.9 ± 0.9. The bacteria live/dead ratio was 1.48 ± 0.58.

Chlorophyll-a density increased in all the treatments from day 0 to day 16 (Fig. 1a); however, that of the DIU-treated biofilms was not significantly different than that of the control

biofilms. Chlorophyll-a density in TCS-treated biofilms decreased relative to that of the control by day 2, and subsequently increased significantly ($p = 0.026$ and $p = 0.007$, for days 9 and 16, respectively) (Fig. 1a).

Live diatom density increased exponentially (Fig. 2) from day 0 to day 16 in all treatments. Diatoms growth rates (Fig. 2) were higher (0.14 divisions day^{-1}) in DIU-treated biofilms than in either control or TCS-treated biofilms (Fig. 2; $p = 0.001$). Diatom composition did not significantly change in either DIU or control biofilms, but *Achnanthidium minutissimum* became dominant in the TCS-treated biofilms. The diatom live/dead ratio significantly decreased in DIU-treated biofilms starting from day 2 and remained lower than that of the control biofilms until the end of experiment (Fig. 1b). Diatoms of TCS-treated biofilms responded late to exposure (Fig. 1b): the live/dead ratio significantly decreased by day 9 (7.4 ± 0.7; 69% of the control value; $p = 0.001$), recovering moderately by day 16 (Fig. 1b).

The SEM showed that the filamentous green alga *Spirogyra* sp. thrived with unharmed filaments both in control and DIU-contaminated mesocosms (Fig. 3, day 16). However, the *Spirogyra* filaments were less abundant and visually damaged, hosting abundant epibionts (*Achnanthidium* species in Fig. 3b and c) in TCS-treated mesocosms. The green algae contribution to the total chlorophyll *a* content of the TCS-treated samples was less than 0.5% (data derived from fluorescence, not shown).

DIU exposure did not affect bacterial mortality. However, TCS did cause a significant but temporary surge in bacterial mortality (145% of the control value; $p < 0.001$; Fig. 1c).

3.3 Biofilm function: photosynthesis, extracellular enzyme activities, and phosphorus uptake

The results from photosynthetic capacity are included in Figure 4 while results from extracellularar enzymes are reported in this section. The extracellular enzymatic activity and the

307 physiological parameters of the biofilms were similar among mesocosms before toxicant
308 exposure. Leucine-aminopeptidase activity was 419 ± 78 nmol AMC cm^{-2} h^{-1} and alkaline
309 phosphatase activity was 136 ± 21 nmol MUF cm^{-2} h^{-1}. Photosynthetic capacity was 0.47 ± 0.06
310 photon yield and the photosynthetic efficiency was 0.4 ± 0.05 photon yield. The P-uptake before
311 exposure was 2.1 ± 0.3 µg P cm^{-2} h^{-1}.

312 After 48 hours treatment, DIU significantly affected both photosynthetic efficiency ($p <$
313 0.001) and capacity ($p = 0.004$), whereas TSC did not significantly affect either one (Fig. 4).
314 However, in the DIU-treated biofilms, both photosynthetic parameters returned to normal levels
315 by 1 week post-exposure.

316 Extracellular enzyme activity did not differ among the different biofilms and generally
317 increased with time. Phosphatase activity increased up to 266 ± 28 nmol MUF cm^{-2} h^{-1} (day 9),
318 and leucine-aminopeptidase increased up to 544 ± 58 nmol MUF cm^{-2} h^{-1} (day 9). These
319 temporary changes were not associated to the effects of DIU or TCS. In contrast, DIU had
320 enhanced specific extracellular enzyme activity per cell by day 2: the DIU-treated biofilms
321 exhibited an increase in specific alkaline phosphatase and leucine aminopeptidase activity per
322 cell, up to 250 % of the values in the control biofilms (data not shown). Both specific activities
323 returned to normal levels by 1 week post-exposure (day 9).

324 P-uptake was not affected in the DIU-treated biofilms, but decreased significantly in the
325 TCS-treated biofilms (Fig. 5): at day 2 its value was 1.1 ± 0.3 µg P cm^{-2} h^{-1} (29.3 % of the control
326 value; $p < 0.001$). The TCS-induced effects persisted until 1 week post-exposure: at day 9 P-
327 uptake was 2.1 ± 0.1 µg P cm^{-2} h^{-1} (77.4 % of the control value; $p = 0.001$), although by day 16, it
328 had returned to normal levels (Fig. 5).

329

330 **4. DISCUSSION**

Several studies have investigated the effects of DIU (Pesce et al., 2006; Ricart et al., 2009; Tlili et al., 2008) and TCS on river biofilms (Franz et al., 2008; Lawrence et al., 2009; Ricart et al., 2010). Nevertheless, these studies mainly used dose-response designs, and exposure of biofilms to the toxicants, to describe the effects of chronic contamination. The results reported in the present work show that short pulses of either compound also affect natural biofilm communities, and that post-pulse behavior depends on the toxicant used and on the endpoint considered. Due to the different target of the two toxicants and the interactions between autotrophs and heterotrophs within the biofilm, direct and indirect effects are highlighted. Moreover, the study of the post-pulse behavior shows the timing of these effects (either rapid or delayed) as well as potential recovery (return to values not significantly different respect to the control). The multi-biomarker approach (Boninneau et al., 2010) employed in this study enabled description of direct and indirect effects, as well as their recovery, associated with short pulses of either toxicant. Short-term pulses can be considered as transitory perturbations, which can generate responses in the structure and function of fluvial biofilms. Whether the responses after these short-term perturbations are immediate or delayed in time, responses depend on the organisms directly or indirectly targeted by the stressor, as well as on the mechanism associated to the measured parameter. Once a significant response occurred (i.e. increase or decrease of some activity; increase or decrease of mortality; shift in community composition etc.), the effects can persist in time, or instead recover to the original status. We considered that parameters recovered when values after the disturbance were close to values in the control after the disturbance. However, the significance of perturbations and the recovery at the ecosystem scale is a more complex subject that the one being dealt in the paper at a mesocosms scale, and therefore cannot be directly extrapolated to real systems..

The short pulses of DIU inhibited photosynthetic efficiency and capacity and increased diatom mortality. These effects are related to its inhibition of photosynthesis via blockage of electron

356 transport in photosystem II (Van Rensen, 1989). Several studies have confirmed this effect on
357 natural epipelic and epilithic biofilm communities (Legrand et al., 2006; López-Doval et al.,
358 2010). However, the short-term pulses had transient functional effects on the autotrophs and the
359 photosynthetic parameters had rapidly recovered (returned to control values) by 1 week post-
360 exposure. On other hand, recovery of functional parameters may hide specific effects on
361 community (i.e. species replacement, composition shift). Ricart et al. (2009) evidenced how
362 chronic DIU exposure induced shift in diatoms community composition and decrease in diatoms
363 biovolume. Nevertheless the same study also evidenced that photosynthetic parameters did not
364 recover despite the shift of community composition. The recovery of photosynthetic parameters
365 in our study occurred despite the low resistance of diatoms and their slow recovery after 48 hours
366 of short pulses (10 μg L^{-1}) of DIU. The significant increase in diatom growth rate (Fig. 2) and the
367 absence of shift in community composition after the DIU pulses might indicate that the diatoms
368 recovery was occurring at the end of the experiment. These results could be explained by the
369 short time of exposure leading to transient direct effects on photosynthesis and on diatoms
370 viability. A relevant side-effect was that algal biomass (chlorophyll-*a*) was moderately enhanced
371 by DIU exposure. Other authors have also observed this increase in chlorophyll density and have
372 related it to the interruption of electron flow in PSII provoked by DIU (Ricart et al., 2009; Tlili et
373 al., 2008), as well as to the induction of shade-type chloroplasts with a higher concentration of
374 photosynthetic pigments (Chesworth et al., 2004). On the other hand any significant effect of
375 DIU on bacterial viability has been observed. In other studies, DIU did indirectly affect bacteria
376 mortality and extracellular enzyme activity, but these studies involved long-term exposure (Ricart
377 et al., 2009). Moreover, chronic exposure has been demonstrated to induce shift in bacterial
378 community composition of biofilms in the case of DIU (Pesce et al., 2006; Tlili et al., 2008)

379 The moderate effects on periphyton structure and function after the DIU pulses showed in this
380 study are consistent with the three-stage model proposed by Mølander and Blanck (1992). This

model joins various effects of DIU on periphyton structure and function. In the first stage no long-term effects can be detected in spite of short-term effects, such as inhibition of photosynthesis. The second stage would be characterized by slight long-term effects such as the increase of chlorophyll-*a*. The final stage would occur when the sensitive species would be eliminated resulting in restructured community and increased community tolerance. Achieving this last stage should imply that the diuron stress would be sufficiently severe to cause cell mortality. The recovery of photosynthetic parameters and the increase of chlorophyll-*a* density evidenced in this study suggest that 48 hours exposure to 13µg L^{-1} of DIU can be considered a threshold between first and second stage impact (Mølander and Blanck, 1992) in biofilm communities.

In contrast to DIU, the mode of action of the bactericide TCS leads to a strong direct effect on bacterial viability in the biofilm. TCS might be inhibiting fatty acid synthesis and bacterial growth (Escalada et al., 2005). However, one week after the end of exposure, live/dead bacteria ratio values were similar than controls indicating a recovery of the bacterial community. Nevertheless, considering the biology and the short life cycle of bacteria, selection of resistant species and consequent shift in community composition could occur, although this would be most probably in response to longer exposure time. However, to our knowledge, no experimental data are available about chronic contamination effects of TCS on biofilm bacteria.

Although TCS has chiefly been described as a bactericide, it also significantly affected autotrophs (non-target organisms). Diatom mortality increased for 1 week post-exposure (day 9), and TCS exposure retarded development of the diatom community relative to the control. These results may reflect a delayed direct effect of TCS on diatoms or an indirect effect of bacteria mortality as a result of the tight interaction between these two biofilms components. The difficulty of growing axenic cultures of benthic diatoms (Bruckner and Kroth, 2009) demonstrates that diatoms require bacteria for proper development (*e.g.* bacteria vitamin

406 production for algae; Croft et al., 2005). The delayed effect on diatoms (day 9) could have been a
407 late indirect response to the increase in bacterial mortality that had occurred on day 2. This
408 scenario is corroborated by the fact that the diatoms recovered within 1 week after the bacteria
409 had recovered. Chlorophyll concentration had decreased after 48 hours of TCS exposure, but
410 eventually returned to normal levels. The negative effect of TCS on chlorophyll-a, described
411 elsewhere (White et al., 2005), has been associated to modifications of biofilm architecture
412 (Lawrence et al., 2009). In the present work, SEM images (Fig. 3) showed that TCS had damaged
413 *Spirogyra* sp. filaments and reduced chlorophyll density. The effects of TCS on the cell walls of
414 *Spirogyra* sp. can be related to its blocking of fatty acid synthesis. This has been described in
415 bacteria (McMurry et al., 1998), and has been reported to compromise permeability-barrier
416 functions (Phan and Marquis, 2006) and to destabilize cell membranes (Villalaín et al., 2001).
417 Although no specific mode-of-action for TCS has yet been established for algae, in some aspects
418 this contaminant could affect algae similarly to the way it affects bacteria (Lawrence et al., 2009;
419 Ricart et al., 2010; Morin et al., 2010b). Moreover, some studies have described that algae are
420 more sensitive to TCS than are bacteria (Tatarakazo et al., 2004).

421 Neither DIU nor TCS affected the extracellular activity of either phosphatase or leucine-
422 aminopeptidase in any of the biofilms. This indicates that the toxicant pulses did not compromise
423 the ability of the biofilms to process organic matter (proteins) or organic phosphorus, despite the
424 increased microbial mortality. Thus, one could infer that the biofilm maintained certain major
425 functions even when its constituent organisms were directly or indirectly affected. This could be
426 explained by either a change in the bacterial community or by a relatively higher specific activity
427 per live cell (Francoeur and Wetzel, 2003). In fact, normalization of extracellular activities per
428 live bacteria cell revealed that each toxicant had a distinct effect. In the TCS-treated biofilms,
429 specific activity per cell was not affected, suggesting that an alternative mechanism dictates
430 extracellular enzymatic activity in the biofilms. In contrast, the DIU-treated biofilms exhibited

significant increases in both specific phosphatase and specific peptidase activity per live cell by day 2. Ricart et al. (2009) reported a similar indirect effect of DIU on the metabolism of live bacteria in the long-term. They concluded that the increase in extracellular leucine-aminopeptidase activity per live cell was a response to the release of proteinaceous material from DIU-induced lysis of algae cells.

DIU did not affect P-uptake, whereas TCS did. The bacterial death caused by TCS, together with its delayed indirect effect on diatoms and its toxicity to *Spirogyra* sp., could have caused the reduction in P-uptake. The fact that damage to heterotrophs and autotrophs generally had negative consequences for P-uptake highlights the utility of this endpoint as a descriptor of total biofilm function. Given that biofilms are the most important compartment in the biotic removal of inorganic dissolved nutrients from water columns (Sabater et al., 2007), TCS-induced loss in P-uptake is a clear threat to this ecological service of river ecosystems, which purifies water.

The results obtained with the large set of biomarkers confirm the central hypothesis of this work: that direct effects on target organisms would occur earlier and are recovered in a short period (especially if only physiological mechanisms were affected). The strong resistance of bacteria to DIU, and the rapid recovery of photosynthetic parameters following exposure to DIU, are consistent with the toxicant's specific mode-of-action, and support the aforementioned hypothesis. Nevertheless, slow recovery of diatoms suggests that a combination of direct and indirect effects could be at play. The hypothesis on delayed effects from indirect interactions was confirmed by the results from the TCS exposure experiments. These effects on diatom mortality appeared one week post-exposure and delayed biofilm recovery. Biofilms were more resistant and resilient after the DIU pulses than after the TCS pulses. The fact that DIU did not affect P-uptake, and that TCS did, agrees with the more complex behavior of direct and indirect effects associated to this community function.

To conclude, the present study has confirmed the existence of direct effects of DIU and of TCS on specific biofilm components, as well as indirect effects of each toxicant due to ecological interactions within biofilms, as consequences of short-term pulses. Biofilms have shown their recovery capacity (by 2 weeks post-exposure they had recovered from nearly all the effects), but also that even very short pulses of toxicants can have relevant consequences for biofilm structure and function. It is reasonable to establish that short pulses can be seen as initial phases for effects on biofilms, that longer toxicant pulses could imply more persistent effects and finally that chronic concentrations of these toxicants could represent the most severe threat to biofilm diversity and function.

Acknowledgements

This study was supported by the European Commission projects MODELKEY (Project 511237-2 GOCE) and KEYBIOEFFECTS (MRTN-CT-2006-035695). Additional funds were provided by the SCARCE project (Consolider-Ingenio 2010, CSD2009-00065) of the Spanish Ministry of Science and Innovation.

5. REFERENCES

Admiraal W, Blanck H, Buckert-De Jong M, Guasch H, Ivorra N, Lehmann V, Nyström, Paulsson M, Sabater S. Short-term toxicity of Zinc to microbenthic algae and bacteria in a metal polluted stream. Wat Res 1999; 3:1989-1996.

Adolfsson-Erici MM, Pettersson J, Parkkonen Sturve J. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere 2002; 46: 1485–1489.

Azevedo, D, Lacorte S, Vinhas T, Viana P, Barceló D. Monitoring of priority pesticides and other organic pollutants in river water from Portugal by gas chromatography–mass spectrometry and liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. J Chromatogr A 2000; 879: 13–26.

Battin TJ, Kaplan LA, Newbold JD, Hansen, CME. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. Nature 2003; 426 (6965): 439–442.

Blanchoud H, Farrugia F, Mouchel JM. Pesticide uses and transfers in urbanized catchments. Chemosphere 2004; 55: 905–913.

Bonnineau C, Guasch H, Proia L, Ricart M, Geiszinger A, Romaní A.M, Sabater S. Fluvial biofilms: A pertinent tool to assess β-blockers toxicity. Aquatic Toxicology 2010; 96: 225–233.

Bruckner CG, Kroth PG. Protocols for the removal of bacteria from freshwater benthic diatom cultures. Journal of Phycology 2009; 45: 981–986.

Canesi L, Ciacci C, Lorusso LC, Betti M, Gallo G, Pojana G, Marcomini A. Effects of Triclosan on Mytilus galloprovincialis hemocyte function and digestive gland enzyme activities: Possible modes of action on non target organisms. Comparative Biochemistry and Physiology 2007; 145(C): 464–472.

Capdevielle M, Van Egmond R, Whelan M, Versteeg D, Hofmann-Kamensky M, Inauen J, Cunningham V, Woltering D. Consideration of Exposure and Species Sensitivity of Triclosan in the Freshwater Environment. Integrated Environmental Assessment and Management 2008; 4: 15–23.

Charles DF, Knowles C, Davies RS. Protocols for the analysis of algal samples collected as part of the U.S. Geological Survey National Water-Quality Assessment Program. Patrick Center for Environmental Research, The Academy of Natural Sciences; 2002, Philadelphia, PA.

504 Chesworth JC, Donkin ME, Brown MT. The interactive effects of the antifouling herbicides
505 Irgarol 1051 and Diuron on the seagrass Zostera marina (L.). Aquatic Toxicology 2004;
506 66: 293–305.
507 Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG. Algae acquire vitamin B12
508 through a symbiotic relationship with bacteria. Nature 2005; 438:90–3.
509 De Lorenzo ME, Keller JM, Arthur CD, Finnegan MC, Harper HE, Winder VL, Zdankiewicz
510 DL. Toxicity of the Antimicrobial Compound Triclosan and Formation of the Metabolite
511 Methyl-Triclosan in Estuarine Systems. Environmental Toxicology 2007; 23: 224–232.
512 Ellis JB. Pharmaceutical and personal care products (PPCPs) in urban receiving waters.
513 Environmental Pollution 2007; 144: 184-189.
514 European Commission. Directive 2000/60/EC of the European Parliament and of the Council –
515 Establishing a Framework for Community. Action in the Field of Water Policy 2000;
516 European Commission, Belgium.
517 Escalada M, Russell AD, Maillard JY, Ochs D. Triclosan–bacteria interactions: single or multiple
518 target sites? Letters in Applied Microbiology 2005; 41: 476–481.
519 Farré M, Asperger D, Kantiani L, González S, Petrovic M, Barceló D. Assessment of the acute
520 toxicity of triclosan and methyl triclosan in wastewater based on the bioluminescence
521 inhibition of Vibrio fischeri. Anal Bioanal Chem. 2008; 390:1999–2007.
522 Findlay S, Strayer D, Goumbala C, Gould K. Metabolism of streamwater dissolved organic
523 carbon in the shallow hyporheic zone. Limnology and Oceanography 1993; 38: 1493-1499.
524 Flaherty CM, Dodson SI. Effects of pharmaceuticals on Daphnia survival, growth, and
525 reproduction. Chemosphere 2005; 61: 200–207.
526 Francoeur SN, Wetzel RG. Regulation of periphytic leucine-aminopeptidase activity. Aquatic
527 Microbial Ecology 2003; 31: 249-258.

528 Franz S, Altenburger R, Heilmeier H, Schmitt-Janse M. What contributes to the sensitivity of
529 microalgae to triclosan? Aquatic Toxicology 2008; 90: 102–108.
530 Giacomazzi S, Cochet N. Environmental impact of diuron transformation: a review.
531 Chemosphere 2004; 56: 1021–1032.
532 Guillard RRL. Division rates. In: Stein JR editor. Culture methods and growth measurements.
533 Handbook of phycological methods. Cambridge University Press, Cambridge; 1973. p.
534 289-311.

535 Halden R, Paull DH. Co-occurrence of triclocarban and triclosan in U.S. water resources. Environ
536 Sci Technol. 2005; 39:1420–1426.
537 Kantiani L, Farré M, Asperger D, Rubio F, González S, López de Alda M, Petrović M, Shelver
538 W, Barceló D. Triclosan ans methyl-triclosan study in the northeast of Spain using
539 magnetic particle enzyme immunoassay and confirmatory analysis by gas cromatography-
540 mass spectrometry. Journal of Hydrology 2008; 361:1-9.
541 Krammer K. Lange-Bertalot H. Bacillariophyceae 1. Teil: Naviculaceae. p. 876; 2. Teil:
542 Bacillariaceae, Epithemiaceae, Surirellaceae, p. 596; 3. Teil: Centrales, Fragilariaceae,
543 Eunotiaceae, p. 576; 4. Teil: Achnanthaceae. Kritische Ergänzungen zu Navicula
544 (Lineolatae) und Gomphonema. p. 437 G. Fischer Verlag., Stuttgart. 1986 – 1991.

545 Kuster M, López de Alda M, Hernando MD, Petrovic M, Martín-Alonso J, Barceló D. Analysis
546 and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in
547 sewage treatment plant effluents, river water and drinking water in the Llobregat river
548 basin (Barcelona, Spain). Journal of Hydrology 2008; 358: 1123-123.

549 Jeffrey S, Humphrey GF. New spectrophotometric equations for determining chlorophylls a, b,
550 c1 and c2 in higher plants, algae and natural phytoplankton. Biochemie und Physiologie
551 der Pflanzen 1975; 167: 191–194.

552 Lawrence JR, Zhu B, Swerhone GDW, Roy J, Wassenaar LI, Topp E, Korber DR. Comparative
553 microscale analysis of the effects of triclosan and triclocarban on the structure and
554 function of river biofilm communities. Science of the Total Environment 2009; 407:
555 3307–3316.
556 Legrand H, Herlory O, Guarini J, Blanchard GF, Richard P. Inhibition of microphytobenthic
557 photosynthesis by the herbicides atrazine and diuron. Cahiers de Biologie Marine 2006;
558 47: 39-45.
559 Lock MA. Attached microbial communities in rivers. In: Fored TE (editor) Aquatic
560 Microbiology: an Ecological Approach. Blackwell Scientific Publications, Oxford 1993;
561 p. 113–138.
562 Loos R, Wollgast J, Huber T, Hanke G. Polar herbicides, pharmaceutical products,
563 perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its
564 carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern
565 Italy. Anal Bioanal Chem. 2007; 387: 1469–1478.
566 López-Doval JC, Ricart M, Guasch H, Romaní AM, Sabater S, Muñoz I. Does Grazing Pressure
567 Modify Diuron Toxicity in a Biofilm Community? Archives of Environmental
568 Contamination and Toxicology 2010; 58: 955-962.
569 Mathuriau C, Chauvet E. Breakdown of litter in a neotropical stream. J. N. Am. Benthol. Soc.
570 2002; 21: 384-396.
571 McAvoy, DC, Schatowitz B, Jacob M, Hauk A, Eckhoff WS. Measurement of triclosan in
572 wastewater treatment systems. Environmental Toxicology and Chemistry 2002; 21: 1323–
573 1329.
574 McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. Nature 1998; 394: 531-
575 532.
576 Mølander S, Blanck H. Detection of pollution-induced community tolerance

577 (PICT) in marine periphyton communities established under diuron exposure. Aquatic
578 Toxicology 1992; 22: 129–144.
579 Morin S, Coste M, Delmas F. A comparison of specific growth rates of periphytic diatoms of
580 varying cell size under laboratory and field conditions. Hydrobiologia 2008; 614: 285–
581 297.
582 Morin S, Pesce S, Tlili A, Coste M, Montuelle B. Recovery potential of periphytic communities
583 in a river impacted by a vineyard watershed. Ecological Indicators 2010a; 10: 419–426.
584 Morin S, Proia L, Ricart M, Bonnineau C, Geiszinger A, Ricciardi F, Guasch H, Romani AM,
585 Sabater S. Effects of a bactericide on the structure and survival of benthic diatom
586 communities. Life and Environment 2010b; 60: 107-114.
587 Morrall D, McAvoy D, Schatowitz B, Inauen J, Jacob M, Hauk A, Eckhoff W. A field study of
588 triclosan loss rates in river water (Cibolo Creek, TX). Chemosphere 2004; 54: 653–660.
589 Orvos DR, Vergsteeg DJ, Inauen J, Capdeville M, Rothrnstein A, Cunnigham V. Aquatic toxicity
590 of Triclosan. Environmental Toxicology and Chemistry 2002; 21: 1338–1349.
591 Pesce S, Fajon C, Bardot C, Bonnemoy F, Portelli C, Bohatier J. Effects of the phenylurea
592 herbicide diuron on natural riverine microbial communities in an experimental study.
593 Aquatic Toxicology 2006; 78: 303–314.
594 Pesce S, Bardot C, Lehours A, Batisson I, Bohatier J, Fajon C. Effects of diuron in microcosms
595 on natural riverine bacterial community composition: new insight into phylogenetic
596 approaches using PCR-TTGE analysis. Aquatic Sciences 2008; 70: 410 – 418.
597 Phan T, Marquis RE. Triclosan inhibition of membrane enzymes and glycolysis of *Streptococcus*
598 *mutans* in suspension and biofilm. Canadian Journal of Microbiology 2006; 52: 977-983.
599 Rabiet M, Margoum C, Gouy V, Carluer N, Coquery M. Assessing pesticide concentrations and
600 fluxes in the stream of a small vineyard catchment – Effect of sampling frequency.
601 Environmental Pollution 2010; 158: 737–748.

602 Ricart M, Barceló D, Geiszinger A, Guasch H, López de Alda M, Romaní AM, Vidal G,
603 Villagrasa M, Sabater S. Effects of low concentrations of the phenylurea herbicide diuron
604 on biofilm algae and bacteria. Chemosphere 2009; 76: 1392–1401.
605 Ricart M, Guasch H, Alberch M, Barceló D, Bonnineau C, Geiszinger A, Farré M, Ferrer J,
606 Ricciardi F, Romaní A, Morin S, Proia L, Sala L, Sureda D, Sabater S. Triclosan
607 persistence trough wastewater treatment plants and its potential toxic effects on river
608 biofilms. Aquatic Toxicology 2010; 100: 346-353.
609 Rodriguez-Mozaz S, López de Alda MJ, Barceló D. Monitoring of estrogens, pesticides and
610 bisphenol A in natural waters and drinking water treatment plants by solid-phase
611 extraction–liquid chromatography–mass spectrometry. J. Chromatogr. 2004; 1045(A):
612 85–92.
613 Romaní AM, Sabater S. Effect of primary producers on the heterotrophic metabolism of a stream
614 biofilm. Freshwater Biology 1999; 41: 729-736.
615 Sabater S, Tockner K. Effects of hydrologic alterations on the ecological quality of river
616 ecosystems. In: Sabater S and Barceló D (editors). Water scarcity in the Mediterranean:
617 Perspectives Under Global Change. Springer Verlag. Hdb Env. Chem. 2010; 8: 15–39.
618 Sabater S, Guasch H, Ricart M, Romaní AM, Vidal G, Klünder C, Schmitt- Jansen M.
619 Monitoring the effect of chemicals on biological communities. The biofilm as an
620 interface. Anal. Bioanal. Chem. 2007; 387, 1425–1434.
621 Samrakandi MM, Roques C, Michel G. Influence of trophic conditions on exopolysaccharide
622 production: bacterial biofilm susceptibility to chlorine and monochloramine. Can J
623 Microbiol 1997; 3: 751-758.
624 Samsøe-Petersen L, Winther-Nielsen M, Madsen T. Fate and Effects of Triclosan. Environmental
625 Project No. 861 2003. Miljøprojekt. Danish Environmental Protection Agency. Danish
626 Ministry of The Environment, 2003.

627 Serra A, Corcoll N, Guasch H. Copper accumulation and toxicity in fluvial periphyton: the
628 influence of exposure history. Chemosphere 2009; 74: 633–641.
629 Singer H, Muller S, Tixier C, Pillonel L. Triclosan: occurrence and fate of a widely used biocide
630 in the aquatic environment: field measurements in wastewater treatment plants, surface
631 waters, and lake sediments. Environ. Sci. Technol. 2002; 36: 4998–5004.
632 Tatarazako N, Hishibashi H, Teshima K, Kishi K, Arizono K. Effects of Triclosan on Various
633 Aquatic Organisms. Environmental Sciences 2004; 11: 133-140.
634 Tlili A, Dorigo U, Montuelle B, Margoum C, Carluer N, Gouy V, Bouchez A, Bérard A.
635 Responses of chronically contaminated biofilms to short pulses of diuron. An
636 experimental study simulating flooding events in a small river. Aquatic Toxicology 2008;
637 87: 252–263.
638 Van Rensen JJS. Herbicides interacting with photosystem II. In: Dodge, A.D. (Ed.), Herbicides
639 and Plant Metabolism. Cambridge University Press, Cambridge 1989; 21–36.
640 Villalaín J, Reyes Mateo C, Aranda FJ, Shapiro S, Micol V. Membranotropic Effects of the
641 Antibacterial Agent Triclosan. Archives of Biochemistry and Biophysics 2001; 390: 128–
642 136.
643 White KL, Haggard BE, Matlock MD, Kim MD. Periphytic chlorophyll-a response to triclosan
644 exposure: application of a passive diffusion periphytometer. Applied Engineering in
645 Agriculture 2005; 21 : 307−311.
646 Wilson BA, Smith V, Denoyelles F Jr, Larive CK. Effects of Three Pharmaceutical and Personal
647 Care Products on Natural Freshwater Algal Assemblages. Environmental Science &
648 Technology 2003; 37: 1713-1719.
649 Xie Z, Ebinghaus R, Flöser G, Caba A, Ruck W. Occurrence and distribution of triclosan in the
650 German Bight (North Sea). Environmental Pollution 2008; 156: 1190–1195.

651 Ylla I, Borrego C, Romaní AM, Sabater S. Availability of glucose and light modulates the
652 structure and function of a microbial biofilm. FEMS Microbiology Ecology 2009; 69: 27-
653 42.

654 **FIGURE CAPTIONS**

655 1) Changes of control (black) and treated (greys) biofilms in following endpoints: (a) Chla
656 density; (b) live/dead diatom ratio and (c) live/dead bacteria ratio in each sampling date.
657 Values are means and standard deviation (n = 4). Post-hoc Tukey-b analysis results are
658 showed when treatment effect resulted significant. Statistical signicance was set at $p \leq$
659 0.05 (one-way ANOVA).

660 2) Diatom community evolution during experiment. a) Increase of live cell density from
661 day 2 to day 16. Slope of linear curve is the grow rate (div day^{-1}) of diatoms

662 3) SEM pictures of Control (1), DIU-treated (2) and TCS-treated (3) biofilms at day 16.

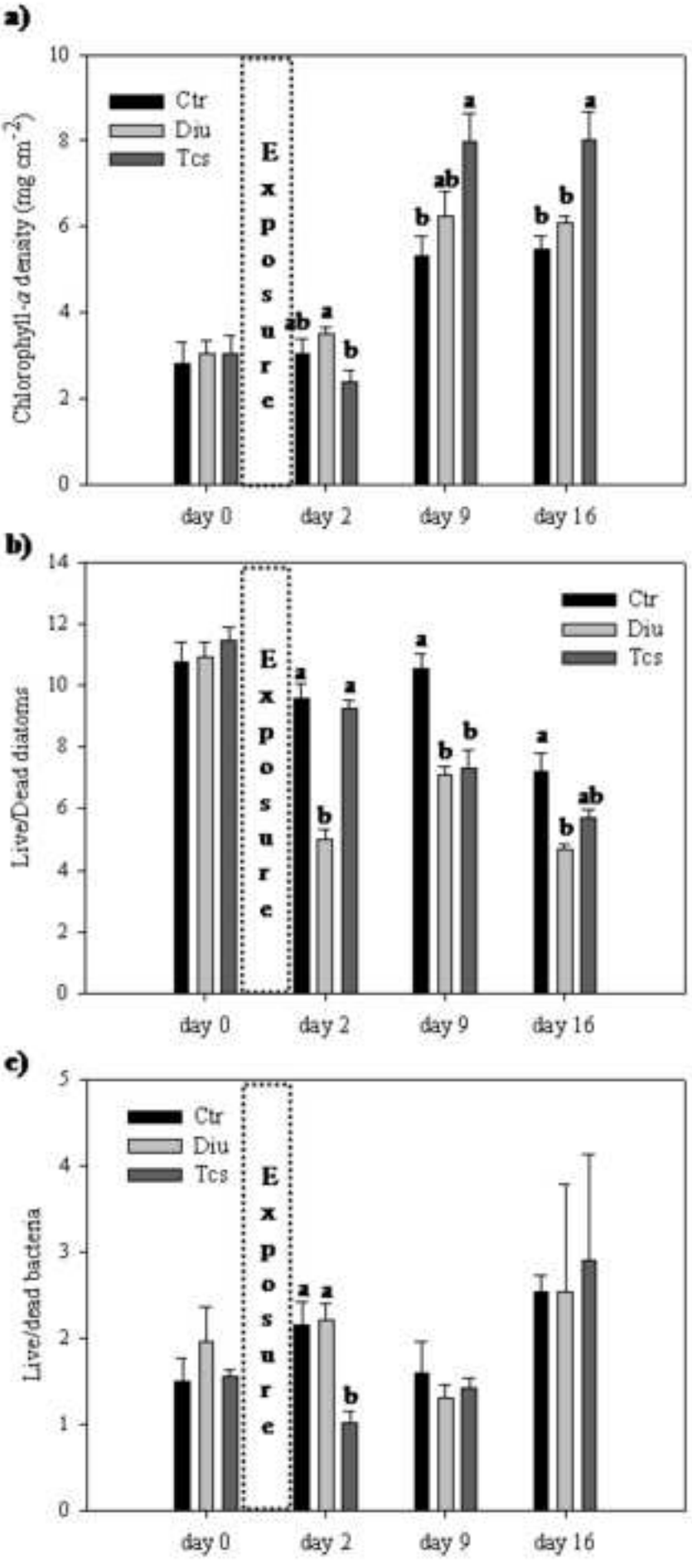
663 4) Changes of control (black) and treated (greys) biofilms in community photosynthetic
664 efficency; in each sampling date. Values are means and standard deviation (n = 4). Post-
665 hoc Tukey-b analysis results are showed when treatment effect resulted significant.
666 Statistical signicance was set at $p \leq 0.05$ (one-way ANOVA).

667 5) Changes in Phosphate Uptake rates (U) of control (black) and treated (greys) biofilms in
668 each sampling date. Values are means and standard deviation (n = 4). Post-hoc Tukey-b
669 analysis results are showed when treatment effect resulted significant. Statistical
670 signicance was set at $p \leq 0.05$ (one-way ANOVA).

671

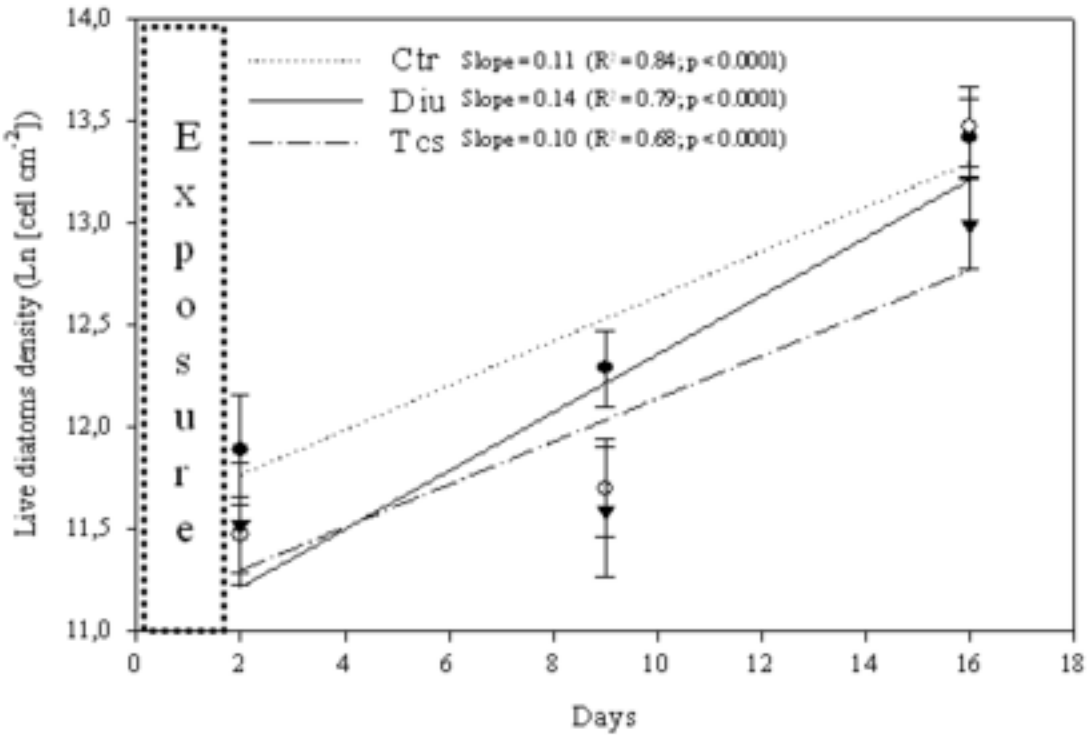
Figure

Click here to download high resolution image



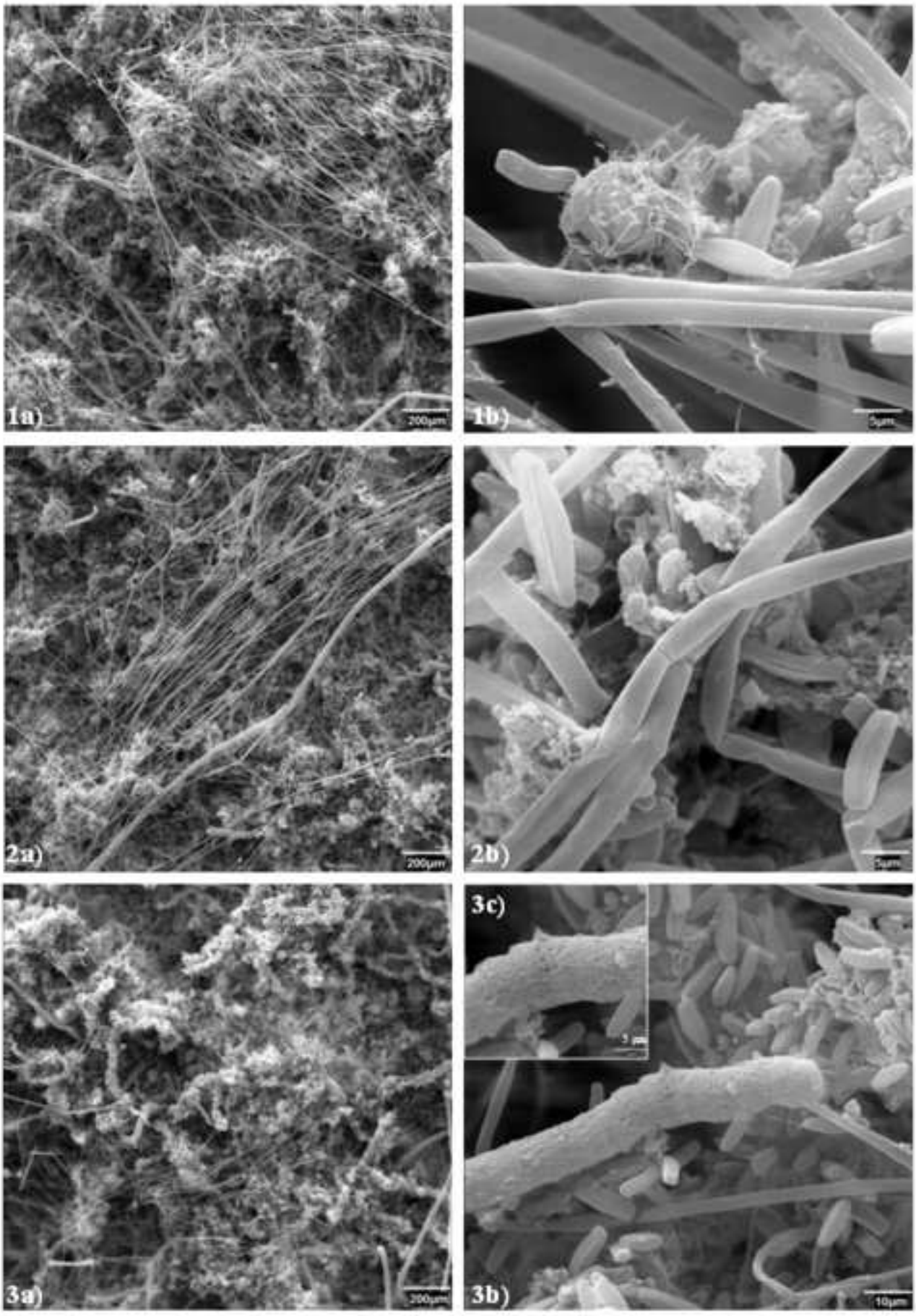
Figure

Click here to download high resolution image



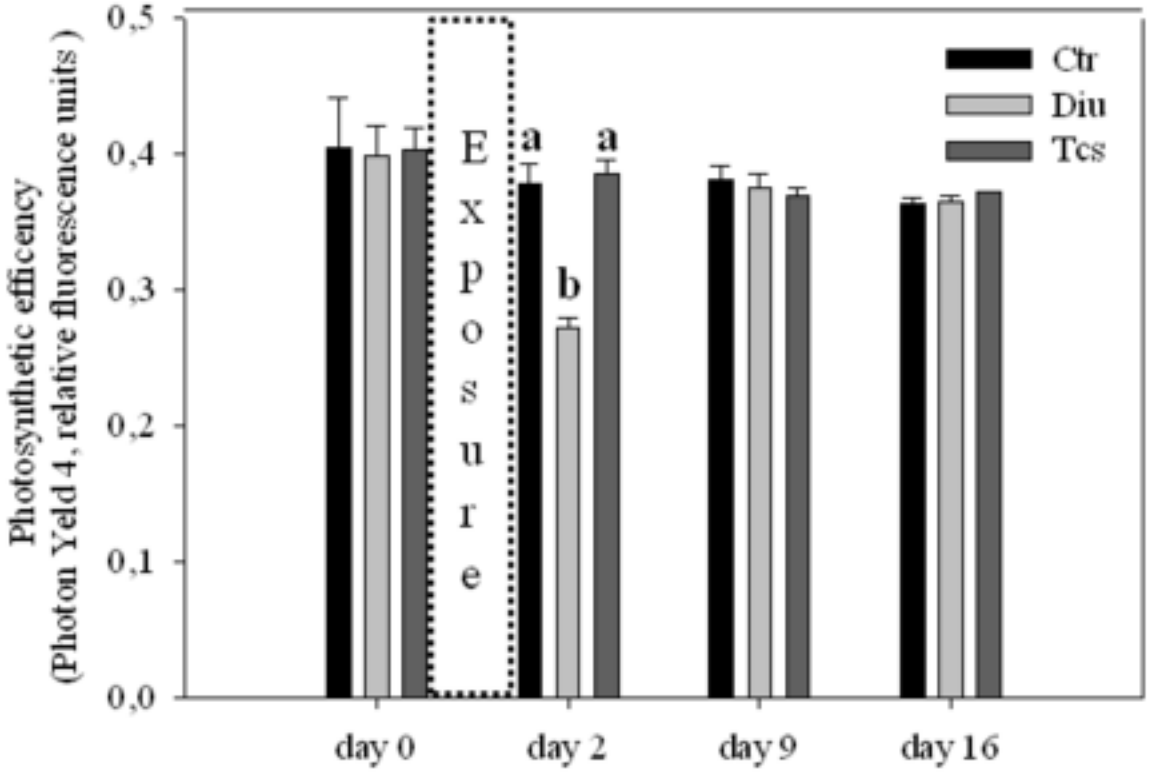
Figure

Click here to download high resolution image



Figure

Click here to download high resolution image



Figure

Click here to download high resolution image