Resistance and recovery of river biofilms receiving short pulses of Triclosan and Diuron

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

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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

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

62 TCS is active against both gram-positive and gram-negative bacteria. It is an inhibitor of
63 the enzyme enoyl–acyl carrier protein reductase (ENR), which is involved in bacterial lipid
64 biosynthesis (Adolfsson-Erici *et al.*, 2002). For over 30 years TCS has been used in products
65 such as anti-bacterial hand soaps, deodorants, household cleaners, dental hygiene products, and
66 textiles (Singer *et al.*, 2002). This emerging compound has been reported in sewage wastewater
67 and sludge at significant concentrations (Halden and Paull, 2005; Samsøe-Petersen *et al.*, 2003).
68 Although wastewater treatment plants (WWTPs) are rather effective at removing TCS (Samsøe-
69 Petersen *et al.*, 2003; McAvoy *et al.*, 2002), this compound still reaches freshwater systems, and
70 has been reported in various aquatic habitats, including rivers, streams (Ellis, 2006;Kuster *et al.*,
71 2008; Morral *et al.*, 2004), lakes (Loos *et al.*, 2007; Singer *et al.*, 2002) and the sea (Xie *et al.*,
72 2008). Both DIU and TCS have been widely tested for toxicity to myriad cultured aquatic
73 organisms (Canesi *et al.*, 2007; Capdevielle *et al.*, 2008; De Lorenzo *et al.*, 2007; Farré *et al.*,
74 2008; Flaherty and Dodson, 2005; Giacomazzi and Cochet, 2004; Orvos *et al.*, 2002; Wilson *et*
75 *al.*, 2003); however, they have not been extensively studied for toxicity to natural complex
76 communities (Franz *et al.*, 2008; Lawrence *et al.*, 2009; Morin *et al.*, 2010a; Pesce *et al.*, 2006,
77 2008; Ricart *et al.*, 2009).

78 DIU reaches running waters primarily via pulses from diffuse source, whereas TCS enters
79 them periodically from fixed sources (chiefly, WWTPs). DIU pulses of up to 134.0 μg L^{-1} have
80 been described during flooding events in vineyard catchments. These chronically affected
81 environments show baseline concentrations of 1 μg DIU L^{-1} between flood events (Rabiet *et al.*,
82 2010). In contrast, TCS enters running waters chronically at low concentrations via urban sewage
83 effluents, and its removal during wastewater treatment is variable (Ellis, 2006; Ricart *et al.*,
84 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 provide information about bacterial mortality. Scanning Electron Microscopy
179 (SEM) observations were useful to compare the structure of treated and non-treated bniofilm at
180 the end of the experiment. The *in vivo* fluorescence measurements described the functional
181 response of phototrophic organisms (green algae, diatoms and cyanobacteria). The extracellular
182 enzymatic activities described the heterotrophic capacity to degrade organic matter. Finally, the
183 phosphorus uptake rate measurement was used to describe the capacity of the whole biofilm
184 community to remove phosphate from the water column.

2.3.1 Chlorophyll-a density. Glass tiles for chlorophyll analysis were collected from the mesocosms and stored in the dark at -20 °C until analysis. Chlorophyll-a in the biofilms was quantified after extraction from the glass substrata in 90% acetone in the dark at 4 °C for 12 h. Concentration was determined spectrophotometrically after filtration (GF/F Whatman) of the extract, following the procedure of Jeffrey & Humphrey (1975).

2.3.2Diatom community structure and live/dead ratio. Glass slides were scraped using polyethylene cell lifters (Corning Inc., NY), preserved with a drop of formalin solution, and then diluted with artificial stream water to a final volume of 5 mL. Samples were ultrasonicated for 7 minutes (sonication bath Bransonic 220, Technofix) to separate the aggregated cells. A 125 μL aliquot of each sample were then pipetted onto a Nageotte counting chamber. The cells were counted in ten fields (1.25 μL each, 0.5 mm depth) by light microscopy (Nikon Eclipse 80i, Nikon Co., Tokyo, Japan) at 10× magnification. Data were recorded as the number of cells per unit area of sampled substrate (number of cells per cm^2), and the cells were classified as either live (if they contained chloroplasts) or dead (if they were empty) (Morin at al., 2010b). Growth rates (expressed as number of cell divisions per day) of the diatom community were calculated from live diatom counts (Guillard, 1973). Diatom community composition was determined based on permanent slides following ANSP protocols (Charles *et al.* 2002). Approximately 500 frustules were counted per slide at 1,000× magnification; they were identified using European taxonomic literature (Krammer and Lange-Bertalot, 1986-1991) and recent nomenclature updates.

2.3. 3 Bacterial density. Live and dead bacteria were counted with epifluorescence microscopy using the LIVE/DEAD® Bacteria Viability Kit L7012 (*Bac*Light™, Molecular Probes, Invitrogen). Biofilm samples were sonicated (< 1 min, sonication bath at 40 W and 40 kHz,

Selecta) and scraped (Nunc sterile silicone cell scraper) to obtain a biofilm suspension. Samples were then diluted with pre-filtered sterilized water from the mesocosms, and 2 mL subsamples were stained with 3 µL of a 1:1 mixture of SYTO 9 and propidium iodide for 15-30 minutes in the dark. After incubation, samples were filtered through a 0.2 µm black polycarbonate filters (Nuclepore, Whatman). Filters were then dried, placed on a slide with mounting oil (Molecular Probes) and counted by epifluorescence microscopy (Nikon E600, 100× in immersion oil). Green (live) and red (dead) bacterial cells were counted in 20 random fields per filter.

2.3.4 SEM observation. One glass tile from each mesocosm was collected at day 16 for SEM observation. Samples were fixed immediately with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4. Samples were dehydrated in graded ethanol (65–100%) and dried at the critical point of CO_2. Finally, samples were sputter coated with gold, and then observed by SEM (Zeiss DSM 960).

2.3.5 In vivo fluorescence measurements. Fluorescence emission from chlorophyll was measured with a PhytoPAM (Pulse Amplitude Modulated) fluorometer (Heinz Walz GmbH), which uses a set of LEDs at four wavelengths (470, 520, 645, and 665 nm). Five analytical replicates (1 cm^{-2} glass tiles) for each experimental replicate (mesocosm) were analyzed and averaged (Serra *et al.*, 2009). The photosynthetic efficiency (Y_{eff}) of photosystem II (PSII) and the photosynthetic capacity (Y_{max}) of PSII were also estimated (Ricart *et al.*, 2010).

2.3.6 Extracellular enzymatic activity. Leucine-aminopeptidase (EC3.4.11.1) and alkaline phosphatase (EC 3.1.3.1-2) in the biofilms were quantified spectrofluorometrically using the fluorescent-linked substrates L-leucine-4-methyl-7-coumarinylamide (Leu-AMC, Sigma-Aldrich) 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 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

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

309 Extracellular enzyme activity did not differ among the different biofilms and generally
310 increased with time. Phosphatase activity increased up to 266 ± 28 nmol MUF cm^{-2} h^{-1} (day 9),
311 and leucine-aminopeptidase increased up to 544 ± 58 nmol MUF cm^{-2} h^{-1} (day 9). These
312 temporary changes were not associated to the effects of DIU or TCS. In contrast, DIU had
313 enhanced specific extracellular enzyme activity per cell by day 2: the DIU-treated biofilms
314 exhibited an increase in specific alkaline phosphatase and leucine aminopeptidase activity per
315 cell, up to 250 % of the values in the control biofilms (data not shown). Both specific activities
316 returned to normal levels by 1 week post-exposure (day 9).
317 After 48 hours treatment, DIU significantly affected both photosynthetic efficiency ($p < 0.001$)
318 and capacity ($p = 0.004$), whereas TSC did not significantly affect either one (Fig. 4).
319 However, in the DIU-treated biofilms, both photosynthetic parameters returned to normal levels
320 by 1 week post-exposure.

321 P-uptake was not affected in the DIU-treated biofilms, but decreased significantly in the
322 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
323 value; $p < 0.001$). The TCS-induced effects persisted until 1 week post-exposure: at day 9 P-
324 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
325 had returned to normal levels (Fig. 5).

326

327 ## 4. DISCUSSION

328 Several studies have investigated the effects of DIU (Pesce *et al.*, 2006; Ricart *et al.*, 2009;
329 Tlili *et al.*, 2008) and TCS on river biofilms (Franz et al, 2008; Lawrence *et al.*, 2009; Ricart *et*
330 *al.*, 2010). Nevertheless, these studies mainly used dose-response designs, and exposure of
331 biofilms to the toxicants, to describe the effects of chronic contamination. The results reported in
332 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 transport in photosystem II (Van Rensen, 1989). Several studies have confirmed this effect on natural epipelic and epilithic biofilm communities (Legrand *et al.*, 2006; López-Doval *et al.*, 2010). However, the short-term pulses had transient functional effects on the autotrophs and the photosynthetic parameters had rapidly recovered (returned to control values) by 1 week post-exposure. On other hand, recovery of functional parameters may hide specific effects on community (i.e. species replacement, composition shift).

Ricart et al (2009) evidenced how chronic DIU exposure induced shift in diatoms community composition and decrease in diatoms biovolume. Nevertheless the same study also evidenced that photosynthetic parameters did not recover despite the shift of community composition. The recovery of photosynthetic parameters in our study occurred despite the low resistance of diatoms and their slow recovery after 48 hours of short pulses (10 μg L^{-1}) of DIU. The significant increase in diatom growth rate (Fig. 2) and the absence of shift in community composition after the DIU pulses might indicate that the diatoms recovery was occurring at the end of the experiment. These results could be explained by the short time of exposure leading to transient direct effects on photosynthesis and on diatoms viability. A relevant side-effect was that algal biomass (chlorophyll-*a*) was moderately enhanced by DIU exposure Algal biomass (chlorophyll) was moderately enhanced by DIU exposure. Other authors have also observed this increase in chlorophyll density and have related it to the interruption of electron flow in PSII provoked by DIU (Ricart *et al.*, 2009; Tlili *et al.*, 2008), as well as to the induction of shade-type chloroplasts with a higher concentration of photosynthetic pigments (Chesworth *et al.*, 2004). On the other hand any significant effect of DIU on bacterial viability has been observed. In other studies, DIU did indirectly affect bacteria mortality and extracellular enzyme activity, but these studies involved long-term exposure (Ricart *et al.*, 2009). Moreover, chronic exposure has been demonstrated to induce shift in bacterial community composition of biofilms in the case of DIU (Pesce et al 2006; Tlili et al., 2008)

The moderate effects on periphyton structure and function after the DIU pulses showed in this 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

383 eliminated resulting in restructured community and increased community tolerance. Achieving
384 this last stage should imply that the diuron stress would be sufficiently severe to cause cell
385 mortality. The recovery of photosynthetic parameters and the increase of chlorophyll-*a* density
386 evidenced in this study suggest that 48 hours exposure to 13µg L^{-1} of DIU can be considered a
387 threshold between first and second stage impact (Mølander and Blanck, 1992) in biofilm
388 communities.

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

397 Although TCS has chiefly been described as a bactericide (and was like this in the present
398 study, where the ratio of live/dead bacteria indeed decreased), it also significantly affected
399 autotrophs (non-target organisms). Diatom mortality increased for 1 week post-exposure (day 9),
400 and TCS exposure retarded development of the diatom community relative to the control. These
401 results may reflect a delayed direct effect of TCS on diatoms or an indirect effect of bacteria
402 mortalityas a result of the tight interaction between these two biofilms components. The difficulty
403 of growing axenic cultures of benthic diatoms (Bruckner and Kroth, 2009) demonstrates that
404 diatoms require bacteria for proper development (*e.g.* bacteria vitamin production for algae; Croft
405 *et al.*, 2005). The delayed effect on diatoms (day 9) could have been a late indirect response to
406 the increase in bacterial mortality that had occurred on day 2. This scenario is corroborated by the
407 fact that the diatoms recovered within 1 week after the bacteria had recovered. Chlorophyll

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

420 Neither DIU nor TCS affected the extracellular activity of either phosphatase or leucine-
421 aminopeptidase in any of the biofilms. This indicates that the toxicant pulses did not compromise
422 the ability of the biofilms to process organic matter (proteins) or organic phosphorus, despite the
423 increased microbial mortality. Thus, one could infer that the biofilm maintained certain major
424 functions even when its constituent organisms were directly or indirectly affected. This could be
425 explained by either a change in the bacterial community or by a relatively higher specific activity
426 per live cell (Francoeur and Wetzel, 2003). In fact, normalization of extracellular activities per
427 live bacteria cell revealed that each toxicant had a distinct effect. In the TCS-treated biofilms,
428 specific activity per cell was not affected, suggesting that an alternative mechanism dictates
429 extracellular enzymatic activity in the biofilms. In contrast, the DIU-treated biofilms exhibited
430 significant increases in both specific phosphatase and specific peptidase activity per live cell by
431 day 2. Ricart *et al.* (2009) reported a similar indirect effect of DIU on the metabolism of live
432 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 1 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

457 recovery capacity (by 2 weeks post-exposure they had recovered from nearly all the effects), but
458 also that even very short pulses of toxicants can have relevant consequences for biofilm structure
459 and function. It is reasonable to establish that short pulses can be seen as initial phases for effects
460 on biofilms, that longer toxicant pulses could imply more persistent effects and finally that
461 chronic concentrations of these toxicants could represent the most severe threat to biofilm
462 diversity and function.

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466 **Acknowledgements**

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

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653 **FIGURE CAPTIONS**

654 1) Changes of control (black) and treated (greys) biofilms in following endpoints: (a) Chla
655 density; (b) live/dead diatom ratio and (c) live/dead bacteria ratio in each sampling date.
656 . Values are means and standard deviation (n = 4). Post-hoc Tukey-b analysis results are

showed when treatment effect resulted significant. Statistical signicance was set at $p \leq 0.05$ (one-way ANOVA).

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

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

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

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