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Drought episode modulates the response of river biofilms to triclosan

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ABSTRACT

The consequences of global change on rivers include altered flow regime, and entrance of compounds that may be toxic to biota. When water is scarce, a reduced dilution capacity may amplify the effects of chemical pollution. Therefore, studying the response of natural communities to compromised water flow and to toxicants is critical for assessing how global change may affect river ecosystems. This work aims to investigate how an episode of drought might influence the response of river biofilms to pulses of triclosan (TCS). The objectives were to assess the separate and combined effects of simulated drought (achieved through drastic flow alteration) and of TCS exposure on biofilms growing in artificial channels. Thus, three-week-old biofilms were studied under four conditions: Control (normal water flow); Simulated Drought (1 week reduced flow + 2 days interrupted flow); TCS only (normal water flow plus a 48-h pulse of TCS); and Simulated Drought + TCS. All channels were then left for 2 weeks under steady flow conditions, and their responses and recovery were studied. Several descriptors of biofilms were analyzed before and after each step. Flow reduction and subsequent interruption were found to provoke an increase in extracellular phosphatase activity, bacterial mortality and green algae biomass. The TCS pulses severely affected biofilms: they drastically reduced photosynthetic efficiency, the viability of bacteria and diatoms, and phosphate uptake. Latent consequences evidenced significant combined effects caused by the two stressors. The biofilms exposed only to TCS recovered far better than those subjected to both altered flow and subsequent TCS exposure: the latter suffered more persistent consequences, indicating that simulated drought amplified the toxicity of this compound. This finding has implications for river ecosystems, as it suggests that the toxicity of pollutants to biofilms may be exacerbated following a drought.

1. Introduction

The consequences of global change on rivers include altered flow regime, and entrance of compounds that may be toxic to biota. Based on climate change scenarios, researchers have predicted substantial increases in both the frequency and magnitude of fluctuations in many ecosystems (Acuña, 2010). Alterations in flow regime, resulting from fewer precipitation days and more heavy rain events (Hirabayashi et al., 2008; Sillman and Roeckner, 2008), are expected to severely affect certain regions (e.g. the Mediterranean), chiefly through more frequent and more intense floods and droughts. Although flooding may strongly affect biogeochemical processes, drought implies more persistent consequences (Sabater and Tockner, 2010), e.g. by disrupting hydrological connectivity and consequently favoring the extension of lentic habitats. During drought periods, shallow sections disappear and the stream becomes a series of fragmented, short-lived pools (Lake, 2003). Flow cessation triggers a cascade of effects on community structure and ecosystem function (Lake, 2003; Sabater and Tockner, 2010). Specifically, the benthic microbial community may respond to changes in water quality during drought (e.g. increased temperature, or altered quality of organic matter; Ylla et al., 2010), since it serves as an interface between the water column and the substrata (Sabater et al., 2007). Benthic biofilms are complex microbial communities adhered to solid surfaces (Mathuriau and Chauvet, 2002; Findlay et al., 1993; Sabater et al., 2007). They are fundamental in the trophic web and in the geochemical cycles within aquatic ecosystems (Battin et al., 2003; Lock, 1993). Field studies have shown the consequences of drought and intermittency on biofilm primary production and algal recolonization (Robson and Matthews, 2004; Robson et al., 2008; Stanley et al., 2004; Ryder, 2004). Some benthic autotrophic groups and species are better adapted to drought than others, owing to structural traits (e.g. a higher content of extracellular polymeric substances) and/or

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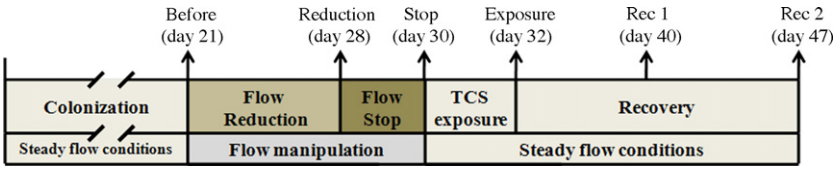

Fig. 1. Schematic figure of the experimental design.

to physiological properties (Ledger et al., 2008). The effects of drought on benthic heterotrophic microbial communities have been described mainly for sediment. They include changes in bacterial diversity, microbial activity and biomass due to water stress (Amalfitano et al., 2008; Fierer et al., 2003).

In watersheds strongly influenced by human activity, the principal consequences of drastic flow reduction or interruption during drought periods include compromised dilution capacity, which may exacerbate chemical pollution (Guasch et al., 2010). Climate change and pollution pressures may simultaneously affect systems that receive wastewaters from industrial, agricultural and urban areas and that are located in regions affected by increasing water scarcity. Pollutants of agricultural, industrial or domestic origin enter watercourses either continuously (producing potentially chronic effects) or in pulses (causing potentially acute effects). These inputs occur in function of flow episodes, crop treatments and/or industrial release (Ellis, 2006). Both chronic and periodic inputs may affect stream biota more severely during drought periods than under normal flow conditions.

In this study, we investigated the combined effects of drought and pollution on river biofilms by following the respective impacts of drastic flow alteration (as a simulation of drought), and of exposure to the antibacterial agent triclosan (TCS), on biofilm community structure and function using a multi-biomarker approach.

The broad-spectrum bactericide TCS (5-chloro-2-(2,4-dichlorophenoxy)phenol) 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 hand soaps, deodorants, household cleaners, dental hygiene products and textiles (Singer et al., 2002). Triclosan can reach freshwaters after the incomplete removal by wastewater treatment plants (Ellis, 2006; Kantiani et al., 2008; McAvoy et al., 2002). In consequence, it has been reported in rivers and streams (Kuster et al., 2008; Morrall et al., 2004), lakes (Loos et al., 2007; Singer et al., 2002) and the sea (Xie et al., 2008). Dry periods may cause drastic flow reduction and may entail brief spikes of compounds from sewage effluents, where TCS is commonly found. These spikes can provoke transient perturbation of river ecosystems with unknown long-term implications. The toxicity of TCS on aquatic organisms has been assessed by laboratory experiments (Capdevielle et al., 2008). Exposure to TCS may have significant consequences for biofilm structure and function. These effects may reach the autotrophic compartment, as reflected in biofilm responses to increasing concentrations (Franz et al., 2008; Ricart et al., 2010) or to short-term pulses (Morin et al., 2010; Proia et al., 2011) of TCS.

Our main objective in this study was to determine the structural and functional responses of fluvial biofilms to the separate and the combined effects of simulated drought (altered water flow) and of TCS exposure. Although several studies have revealed the independent effects of drought or TCS on river biofilm structure and function, the toxicity of TCS to biofilms experiencing multiple stressors (i.e. altered temperature, pH, oxygen levels, etc.) has not been assessed to date. Studying the combined effects of drought and of TCS exposure on river biological communities will facilitate risk assessment for this compound in freshwater ecosystems under stress. Thus, our specific objectives were, firstly, to assess the individual effects of altered low and of TCS pulses on biofilm structure and function, secondly, to determine any combined action between these two stressors and, lastly, to evaluate the recovery potential of biofilms after these perturbations. We hypothesized that: (i) altered water flow, as a simulation of drought, would affect the structure and function of biofilms, consequently making them more sensitive to short-term TCS pulses; (ii) TCS would have direct and indirect effects on autotrophs; and (iii) recovery after short-term perturbations would differ according to whether the observed consequences were direct or indirect, and would be influenced by any combined effects from the two stressors.

2. Material and methods

2.1. Experimental setup

The experiment was conducted using twelve recirculating Perspex channels (170 cm long × 10 cm wide). Each channel unit was affixed with a Perspex piece to keep the water column height at 1.5 cm. Water input at the head of the first channel unit was provided by a 10 L carboy using a pump connected with silicone tubes (Pico Evolution1000, Hydor). The system was supplied with dechlorinated tap water filtered through an active carbon filter. All the carboys were placed in a water bath for water temperature control (21 °C). To avoid nutrient depletion, the water in the carboys was renewed twice weekly. Phosphate supply was provided after each water change to reach a concentration of 30 $\mu g\,L^{-1}$. Light-emitting diodes (LEDs; Lightech, Spain) were used to provide natural light between 450 and 660 nm, at an intensity of 136.7 ± 8.0 μmol photons $m^{-2}\,s^{-1}$ ($n = 72$), following a 12 h/12 h light and dark cycle. The bottom of each channel was covered with a sandblasted glass substratum (1 × 1 cm and 8.5 × 2 cm). Biofilm colonization was achieved by introducing aliquots of a natural microbenthic community obtained from the Llémena stream (NE Spain; Serra et al., 2009) weekly during the first 2 weeks. The biofilms colonized the artificial substrata under controlled flow conditions (3.5 L min^{-1}) for 21 days (Fig. 1).

After colonization, four different conditions were assessed for their respective effects on biofilm structure and function: Control (normal water flow); Simulated Drought (1 week reduced flow + 2 days interrupted flow); TCS only (normal water flow plus a 48-h pulse of TCS); and Simulated Drought + TCS (Fig. 1). First, six channels were maintained under Control, and the other six were subjected to Simulated Drought, whereby the flow was gradually decreased to one-third of the control value, over the course of 1 week (days 21–28), and then ultimately stopped for 2 days (days 28–30). The physico-chemical parameters (conductivity, dissolved Oxygen, pH and temperature) of all the channels were measured twice daily during the zero-flow period. The flow conditions were restored overnight in the Simulated Drought channels. Six channels (three Control and three Simulated Drought) were then treated with TCS (IRGASAN, Sigma–Aldrich, >97%, CAS: 3380-34-5) for 48 h (nominal concentration of 100 $\mu g\,TCS\,L^{-1}$), and the remaining six channels (three Control and three Simulated Drought) were left untreated. Thus, each of the four established conditions (Control, Simulated Drought, TCS, and Simulated Drought + TCS) was tested in triplicate (three independent channels per treatment) (Fig. 1).

Triclosan can be photo-degraded to dioxins, especially at high pH values (Mezcua et al., 2004). Therefore, to maintain the TCS concentration levels, the water in the system was renewed every 3 h during the light period of the light/dark cycle during TCS exposure. The water was sampled twice daily during TCS exposure, and to avoid TCS ionization (TCS pK_a = 8.1, Tixier et al., 2002), the pH was monitored, and adjusted with diluted hydrochloric acid drops (as needed), to maintain values below 8.0. The biofilms were sampled after establishment of each condition to ascertain their structural and functional responses to each one as well as to any possible combined effects. Thus, the biofilms were sampled six times: before any manipulation (day 21, Before); after 1 week of flow reduction (day 28, Reduction); after 48 h of interrupted flow (day 30, Stop); after 48 h of TCS exposure (day 32, Exposure); 1 week after the end of TCS exposure (day 40, Recovery 1); and 2 weeks after the end of TCS exposure (day 47, Recovery 2) (Fig. 1). On each sampling day, tiles of the colonized glass substrata were randomly collected from each channel and analyzed for extracellular-enzyme activity (EEA); photosynthetic parameters (F_0, Y_{eff} and Y_{opt}); phosphate uptake capacity (U); ratio of live-to-dead bacteria; ratio of live-to-dead diatoms; and biofilm biomass (ash-free dry mass [AFDM]).

2.2. Water analysis

The TCS concentrations were determined by a magnetic particle-based immunoassay using the TCS IA kit (Abraxis LLC, Warminster, PA, USA). The sensitivity expressed as the limit of detection (LOD) was calculated as the mean of 20 replicate blanks plus three standard deviations from ultra-pure water (Kantiani et al., 2008). All contaminated channels were sampled twice daily after TCS addition and before water changes (n = 48). Furthermore, one contaminated channel was intensively sampled (n = 10) during the second day of exposure to determine the decay kinetics of the dissolved TCS. For this assay, the first five samples were collected every 5 min within the first 20 min (starting at time 0) after TCS addition; the sixth sample was taken 10 min later (30 min after addition), and the remaining samples, every 30 min until the next water change (3 h after addition). Finally, samples were collected once a day in the Control channels (n = 12). The samples were immediately filtered through 0.2 μm nylon membrane filters (Whatman) and stored at −20 °C in the dark until analysis.

2.3. Biofilm sampling

Different sampling strategies were used depending on the endpoint. Biofilm functional measurements were performed on the day of sampling. One tile of the colonized glass substratum (1 cm^2 per activity) was collected from each channel for EEA measurements (Leucine-aminopeptidase, alkaline phosphatase and β-D-1,4-glucosidase). To measure the phosphate uptake capacity of the biofilms, five large tiles of the glass substratum (8.5 × 2 cm) were collected per channel. For the photosynthetic parameters analyzed by Phyto-PAM, three tiles of colonized glass substrata (17 cm^2) were used. Sample staining and preparation for microscopy (to count the live and dead bacteria) were also immediately performed after collection of one tile of glass substratum per channel.

Biofilm biomass (AFDM) was measured in samples (one glass tile per channel) that were collected and immediately stored in vials at 50 °C. Since Phyto-PAM is a non-destructive, the three tiles of colonized glass substrata (17 cm^2) used for the fluorescence measurements were later fixed and used to determine the ratio of live to dead diatoms. For the latter task, the biofilms were scraped from the glass substrata using polyethylene cell lifters (Corning Inc., NY, USA), preserved with a drop of formalin solution, and diluted to a final volume of 5 mL.

2.3.1. Activities of extracellular enzymes

The activities of the extracellular enzymes leucine-aminopeptidase (EC 3.4.11.1), alkaline phosphatase (EC 3.1.3.1-2) and β-D-1,4-glucosidase (EC 3.2.1.21) in the biofilms were measured spectrofluorometrically using the fluorescent-linked substrates L-leucine-4-methyl-7-coumarinylamide (Leu-AMC, Sigma–Aldrich), 4-methylumbelliferyl-phosphate (MUF-P, Sigma–Aldrich) and 4-methylumbelliferyl β-D-glucopyranoside (4-MUF β-glucoside, Sigma–Aldrich), respectively. Colonized glass tiles 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 under saturating conditions (0.3 mM; Romaní and Sabater, 1999) for 1 h in the dark in a shaking bath. Blank, control, AMC and MUF standards (0–100 μM) were also incubated in the shaking bath. The blank and control samples were used to adjust for the non-enzymatic hydrolysis of the substrate and for the fluorescent substances in the aqueous solution, respectively. At the end of the incubation, 4 mL of glycine buffer (pH 10.4) were added (1/1, v/v), and the fluorescence was measured at 364/445 nm (excitation/emission) for AMC and at 365/455 nm (excitation/emission) for MUF (Kontron, SFM25). The values obtained for the AMC and MUF concentrations were then standardized for the glass surface and incubation time; therefore, the activity values are expressed as nmol (AMC or MUF) h^{-1} cm^{-2} biofilm.

2.3.2. Phosphorus uptake rate

Phosphorus (soluble reactive phosphorus, SRP) uptake rates (U) were calculated by measuring the temporal decay of SRP after spiking. From each of the twelve channels, five randomly selected tiles of colonized substrata (8.5 × 2 cm) were removed and then transferred into one of twelve sterile glass jars (19 cm in diameter, 9 cm high; each jar corresponded to one channel) filled with 1.5 L of dechlorinated tap water recirculated using a submersible pump (Hydor, Pico 300, 230 V 50 Hz, 4.5 W). The starting SRP concentration in each channel was analyzed. The biofilms were then incubated for 45 min (Radiber AGP-570) under controlled temperature (21 °C) and light (130–150 μmol photons m^{-2} s^{-1}) conditions. Subsequently, to quadruple the background concentration of phosphorus, each jar was spiked with Na_2PO_4 (10 mM). Aliquots (10 mL) for SRP concentration measurements were then taken at 1, 5, 10, 15, 30 and 45 min after spiking, and immediately filtered through 0.2 μm nylon membrane filters (Whatman). The phosphate uptake rate (U) was calculated as the mass of phosphorous removed from the water column per unit area per unit time (μg P cm^{-2} h^{-1}), and the phosphate uptake rate coefficient (K, min^{-1}) was calculated as the coefficient of the negative exponential model represented by the decay of SRP concentration over time. Abiotic controls were performed under the same experimental conditions and for the same time: they did not exhibit any SRP decay.

2.3.3. In vivo fluorescence measurements

Three colonized glass substrata were randomly removed at each sampling date from each channel. These samples were used for fluorescence parameters measurements, taken on a Phyto-PAM fluorometer (version EDF, Walz, Effeltrich, Germany).

Biofilm samples were first incubated for 20 min in the dark to ensure that all the photosynthetic reaction centers were opened. A weak measuring light was then applied to the samples (directly on the colonized substrata) to measure the photosynthetic capacity of the community (Y_{opt}) and the minimum fluorescence yield of the dark-adapted cells (F_0). The F_0 values were also used to estimate the chlorophyll concentration, based on the method described by Schreiber (1998). After 20 min of light adaptation, the samples were exposed to actinic light to determine their photosynthetic efficiency (Y_{eff}). Using both dark and light fluorescence measurements,

the non-photochemical quenching (NPQ) values also were calculated. Minimal fluorescence attributed to cyanobacteria, (F_0[Bl]), green algae (F_0[Gr]) and diatoms (F_0[Br]) were used for evaluating the contribution of each class to the biofilm community.

2.3.4. Bacterial density and viability (live-to-dead ratio)

Live and dead bacteria were counted with epifluorescence microscopy using the LIVE/DEAD® Bacteria Viability Kit L7012 (BacLight™, Molecular Probes, Invitrogen). Colonized glass substrata were sonicated (<60 s, sonication bath at 40 W and 40 kHz, Selecta) and scraped (sterile silicone cell scraper, Nunc) to obtain a biofilm suspension. The samples were then diluted with prefiltered sterilized water from microcosms, and 2 mL subsamples were incubated with 3 μL of a 1:1 mixture of SYTO 9 and propidium iodide, for 15–30 min in dark. After incubation, the samples were filtered through 0.2 μm black polycarbonate filters (Nuclepore, Whatman). The filters were then dried, placed on a slide with mounting oil (Molecular Probes), and counted by epifluorescence microscopy (Nikon E600, 1000× in immersion oil). Green and red (live and dead, respectively) bacterial cells were counted in 20 random fields per filter.

2.3.5. Quantitative estimates of diatom community structure and live-to-dead ratio

To separate any aggregated cells, the samples were sonicated (7 min), and then 125 μL of each one were pipetted onto a Nageotte counting chamber for counting of the total number of diatom cells. Ten fields (1.25 μL each, 0.5 mm depth) were selected at random, and then observed by light microscopy at 10× (Nikon Eclipse 80i, Nikon Co., Tokyo, Japan). The data were recorded as cells per unit area (cells cm^{-2}), whereby the empty cells were considered dead, and the cells containing chloroplasts, as alive, regardless of their color (pale yellow, or green or brown), shape or number (Cox, 1996).

2.4. Data analysis

To assess temporal variations and then determine the effects of drought and of TCS exposure on biofilms, the means of each biological variable measured per each channel at each sampling date were used as independent replicates. This dataset comprised the values for extracellular enzymatic activities (phosphatase, peptidase and β-glucosidase), photosynthetic parameters (Y_{eff}, Y_{opt}, non-photochemical quenching [NPQ], total F_0, and F_0[Br], F_0[Gr], F_0[Bl]), biofilm phosphate uptake capacity (U and $|K|$), the ratio of live-to-dead bacteria, the ratio of live-to-dead diatoms and the biomass (AFDM). All variables were log-transformed. Firstly, a principal component analysis (PCA) was performed to study the temporal variation of biofilm structure and function. Then, the effect of time was removed from the two sets of variables by performing a within PCA on each matrix, whereby, for each variable, the mean value for the samples is subtracted from the value for each sample in the group. All the group centers are, therefore, at the origin of the factorial map and samples are represented with the maximal variance around this origin. This enables comparison of variation patterns. The percentage of variance explained by the within PCA, *the intra-group variance*, corresponds to the variance due to factors other than time; it was calculated as the ratio between the sum of the Eigenvalues of the within PCA and the sum of the Eigenvalues of the simple PCA (Dray and Dufour, 2007). Statistical analysis was performed on the program R (R Development Core Team, 2008; Ihaka and Gentleman, 1996) and on the software packages ade4 (Dray and Dufour, 2007) and Hmisc (Harrell, 2007). Statistical significance was set at $p < 0.05$.

The effect of the simulated drought episode on each parameter was also tested daily (sampling after flow reduction and after flow interruption) by one-way analysis of variance (ANOVA) in which Simulated Drought (F) was set as the fixed factor. The responses of the biofilms after TCS exposure were also analyzed daily (Exposure, Recovery 1 and Recovery 2 samplings) by two-way analysis of variance (ANOVA) to test for the effect of Simulated Drought (F), TCS treatment (T) and any combined effects of the two factors ($F+T$). Statistical significance was set at $p < 0.05$. Both analyses were performed using SPSS software, v.15.0.

3. Results

3.1. Experimental conditions

No significant differences in the physico-chemical parameter values among the channels were observed during the colonization period ($p > 0.05$). The conditions were set as follows: water flow, 3.5 ± 0.2 L min^{-1}; temperature, 21.7 ± 0.2 °C; pH 7.7 ± 0.3; conductivity, 407.8 ± 6 μS cm^{-1}; dissolved oxygen, 8.5 ± 0.3 mg L^{-1} ($n = 72$); and phosphorus concentration, 33.7 ± 3.8 μg P L^{-1} ($n = 18$). During the flow reduction period, the water flow of the manipulated channels was 1.2 ± 0.6 L min^{-1}, significantly lower than in the control channels ($p < 0.001$). The remaining parameter values during this period did not differ significantly between the Control channels and the Simulated Drought channels ($p > 0.05$). When the flow was stopped, the water temperature, conductivity and dissolved oxygen all increased significantly ($n = 26$): the temperature rose to 25.6 ± 0.8 °C (2.4 °C higher than in the control; $p < 0.001$), conductivity to 432.8 ± 20.4 μS cm^{-1} ($p = 0.001$) and dissolved oxygen to 10.7 mg L^{-1} ($p = 0.031$). However, after the flow was restored, the values of the physico-chemical variables no longer differed significantly among the channels ($p > 0.05$).

After TCS addition, the measured TCS concentration (LOD = 14 ng L^{-1}) in the treated channels was 87.2 ± 7.8 μg TCS L^{-1} ($n = 24$), 12.2% below the nominal concentration. The TCS concentration followed exponential decay kinetics, with a rate (K) of 0.018 min^{-1} ($r = 0.90$, $p < 0.05$). By 3 h after addition, the concentration had dropped to 4.6 ± 0.3 μg TCS L^{-1} ($n = 24$) (5.2% of the initial concentration). The TCS concentration in the Control channels was always below the detection limit.

3.2. Biofilm responses to drought and to TCS exposure

3.2.1. Temporal variation

The biofilm changes over throughout the course of the experiment are described by the PCA (Fig. 2). The first two axes of the PCA explain 53.5% of the variance. Axis 1 clearly arranges samples by their sampling date (40.7%, Fig. 2). The live-to-dead ratios of bacteria and of diatoms, the phosphatase activity, and the photosynthetic efficiency and capacity all decreased over time. On the contrary, the activity of β-glucosidase and of peptidase, the AFDM, the algal biomass (F_0), the fluorescence of cyanobacteria (F_0[Bl]) and of green algae (F_0[Gr]), and the phosphate uptake rate (U) all increased during the experiment (Fig. 2, Table A.1).

Supplementary material related to this article found, in the online version, at doi:10.1016/j.aquatox.2012.01.006.

3.2.2. Effects of drought and of TCS exposure

The within PCA showed that 47.8% of the variance (intra-group variance) was explained by factors other than time. Both flow alteration and exposure to triclosan affected the different biofilm structural and functional parameters, especially on days 30, 32 and 40 (Fig. 3). The first two axes of the within PCA explain 40.7% of variance (wPCA1: 25.0%, wPCA2: 15.8%) and are linked to flow-alteration effects and to TCS effects, respectively. The specific effects of flow alteration and of TCS exposure on the measured

Table 1
Results of F and p values of the one-way ANOVA and two-ways ANOVA test performed. The most responsive endpoints are reported. p values below 0.01 are represented in bold.

	One-way ANOVA	Two-way ANOVA									
	Day 28 (low Q)	Day 30 (Stop)	Day 32 (Effect)			Day 40 (Rec 1)			Day 47 (Rec 2)		
	Flow (F)	Flow (F)	Flow (F)	Triclosan (T)	Interaction ($F \times T$)	Flow (F)	Triclosan (T)	Interaction ($F \times T$)	Flow (F)	Triclosan (T)	Interaction ($F \times T$)
Phosphate uptake rate (U)	n.s.	n.s.	n.s.	0.013 $F = 10.2$	n.s.	n.s.	**0.008** $F = \mathbf{12.2}$	0.036 $F = 6.3$	n.s.	0.019 $F = 8.5$	n.s.
Alkaline phosphatase activity	n.s.	**<0.001** $F = \mathbf{61.6}$	n.s.	0.029 $F = 7.0$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
L/D bacteria	0.024 $F = 7.1$	**<0.001** $F = \mathbf{31.3}$	n.s.	**0.009** $F = \mathbf{11.8}$	n.s.	n.s.	**0.001** $F = \mathbf{23.4}$	n.s.	n.s.	0.012 $F = 10.6$	n.s.
Y_{eff}	n.s.	n.s.	0.011 $F = 10.9$	**0.002** $F = \mathbf{19.6}$	n.s.	0.014 $F = 9.7$	n.s.	n.s.	n.s.	0.014 $F = 9.9$	0.047 $F = 5.5$
F_0(Gr)	n.s.	**0.003** $F = \mathbf{14.8}$	**0.002** $F = \mathbf{18.8}$	n.s.	n.s.	0.044 $F = 5.7$	n.s.	0.023 $F = 7.9$	n.s.	n.s.	n.s.
F_0(Br)	n.s.	**0.009** $F = \mathbf{10.2}$	**0.001** $F = \mathbf{26.5}$	n.s.	n.s.	**0.004** $F = \mathbf{16.3}$	n.s.	0.030 $F = 6.9$	n.s.	n.s.	n.s.
L/D diatoms	n.s.	n.s.	n.s.	n.s.	**0.001** $F = \mathbf{28.8}$	0.041 $F = 5.9$	n.s.	n.s.	**<0.001** $F = \mathbf{128.9}$	**<0.001** $F = \mathbf{25.4}$	0.044 $F = 5.7$

Fig. 2. (a) Factorial map of the sample ordination by the Principal Component Analysis (PCA). The samples are grouped by sampling day (indicated by the label) to highlight temporal variation. The solid black dots correspond to Control samples, and the outlined dots correspond to TCS-exposed samples. (b) Normalized coefficients of the different variables on the first two axes of the PCA. F_{diato} = Fluorescence of Diatoms; F_{green} = Fluorescence of Green algae; F_{cyano} = Fluorescence of Cyanobacteria; F_0 = fluorescence of whole community; Ph_{Eff} = Photosynthetic efficiency; Ph_{Cap} = Photosynthetic capacity; NP_Q = non-photochemical quenching; U = Phosphorus uptake rate; K = phosphorus uptake rate coefficient; AFDM = Ash free dry mass; Peptidase = Leucine-aminopeptidase, Phosphatase = Alkaline phosphatase; BGlucosidase = β-D-1,4-glucosidase; BacteriaLD = live-to-dead bacteria ratio; DiatomsLD = live-to-dead diatoms ratio.

biofilm variables were further evidenced by the ANOVA analyses (Table 1).

3.2.2.1. Effects of drought. At 2 days after flow interruption, the Control biofilms (C) and the Simulated Drought (F) biofilms are clearly separated along axis 1 of the within PCA (Fig. 3c). Although the gap between them decreases during the recovery period, they remain separated along axis 1 until the end of the experiment (Fig. 3). Flow reduction and further flow interruption caused a significant decrease in the number of live bacteria and of live diatoms (Fig. 4a and b, respectively, and Table 1), in F_0(Br) (Fig. 6 and Table 1) and in photosynthetic capacity (Fig. 5b and Table 1), but it caused a significant increase in phosphatase activity (Fig. 5a and Table 1) and in F_0(Gr) (Fig. 6 and Table A.1). The differences in the proportion of algal groups remained until the end of the experiment (Fig. 6). A lower proportion of live diatoms (i.e. a lower live-to-dead ratio) was still observed at day 47 in the Simulated Drought samples, relative to the Control samples (Fig. 4b, Table 1).

3.2.2.2. Effects of TCS. At 48 h after TCS exposure (day 32), the exposed biofilms appear separate from the control biofilms along axis 2 of the within PCA (Fig. 3d). These effects persisted for 1 week after the end of exposure (day 40, Fig. 3e). However, 2 weeks after the end of exposure (day 47), exposed and non-exposed biofilms appear together (Fig. 3f); nonetheless, some parameter values of the

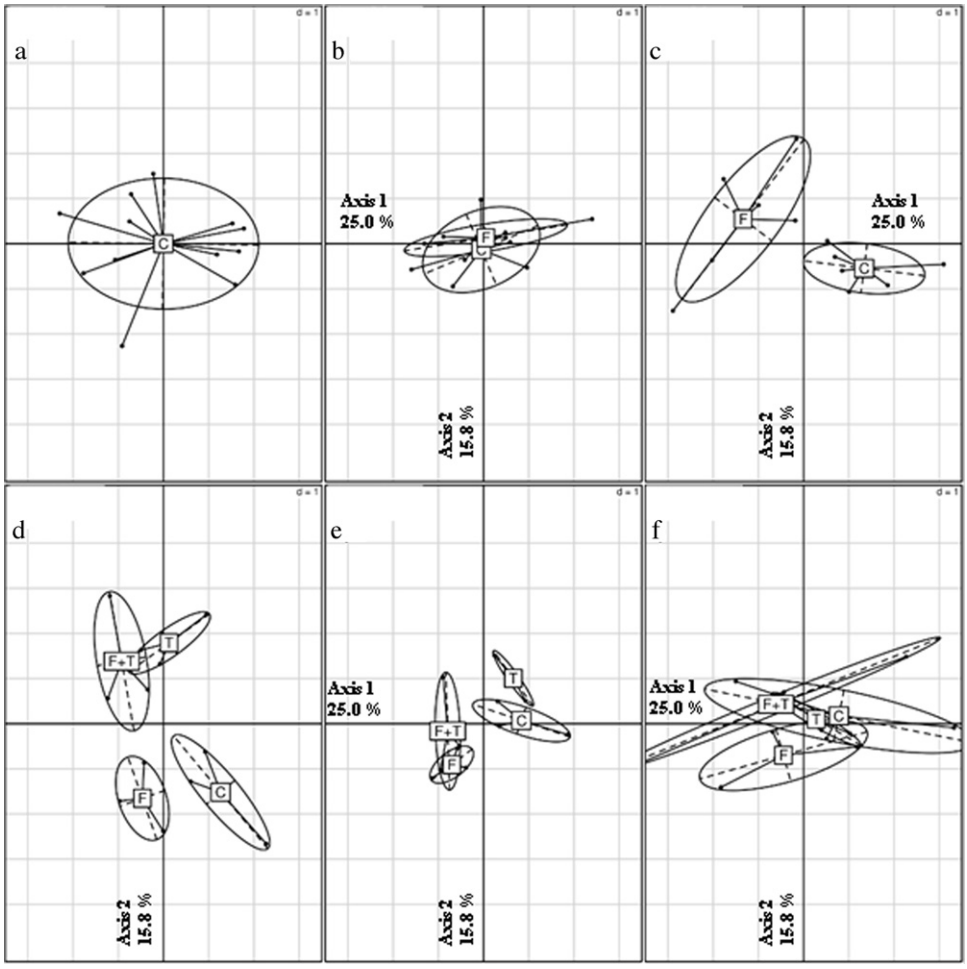

Fig. 3. Factorial map of the sample ordination by the within PCA, shown for each sampling day: (a) day 21, Before; (b) day 28, Reduction; (c) day 30, Stop; (d) day 32, Exposure; (e) day 40, Recovery 1; and (f) day 47, Recovery 2. Samples are grouped by treatments: *C* = Controls; *F* = Simulated Drought (altered flow); *T* = TCS exposure; and *F* + *T* = Simulated Drought + TCS exposure.

TCS-exposed biofilms (TCS, and Simulated Drought + TCS) were still significantly different from the non-exposed communities (Controls, and Simulated Drought) (Table 1).

In the TCS-treated biofilms, the peptidase activity and the F_0(Br) increased, whereas the F_0(Gr) and the AFDM decreased. The phosphatase activity in these biofilms increased significantly compared to controls on day 32, but recovered to control levels within 1 week after the end of exposure (Fig. 5a and Table 1). The photosynthetic parameters (Ph. capacity) were also significantly affected by TCS, but the effect was transient: the values had recovered by day 40 (Fig. 5b and Table 1). In contrast, the negative effects of TCS exposure on the ratio of live-to-dead bacteria (Fig. 4a) and on phosphate uptake rate (Fig. 5c) persisted until the end of the experiment (Table 1).

3.2.2.3. Combined effects of drought and TCS exposure. The within PCA indicates that the short-term effects of TCS were not influenced by flow variations (days 32 and 40). However, the biofilms exposed to TCS only (*T*) recovered more quickly (Fig. 3), and at day 47, the biofilms exposed to both Simulated Drought and TCS (*F* + *T*) appear separate from those exposed only to Simulated Drought (*F*) (Fig. 3f). The delayed combined effects of Simulated Drought + TCS were observed in several structural and functional descriptors of biofilms (Table 1). For example, F_0(Gr) decreased in the TCS biofilms, but was not affected in the Simulated Drought + TCS biofilms (Fig. 6). The ratio of live-to-dead bacteria and the phosphate uptake capacity remained lower in the TCS and Simulated Drought + TCS biofilms. However, the live-to-dead bacteria ratio of TCS communities recovered to normal values by day 47 (Fig. 4a). In the F and the Simulated Drought + TCS biofilms F_0(Br) decreased; the decline was more severe in the latter (Fig. 6 and Table A.1). In the TCS-treated biofilms, the ratio of live-to-dead diatoms decreased after TCS exposure, but ultimately recovered to values higher than those of the control biofilms. However, in the Simulated Drought + TCS biofilms, it never recovered (Fig. 4b).

4. Discussion

In this study, we investigated how simulated drought and TCS exposure can affect natural river biofilm communities, both separately and when combined. Although several studies have revealed the independent effects of drought or TCS on river biofilm structure and function, the toxicity of TCS to biofilms experiencing multiple stressors (i.e. altered temperature, pH, oxygen levels, etc.) has not been assessed to date. Studying the combined effects of drought and TCS exposure on natural biological communities will facilitate risk assessment for this compound in freshwater ecosystems under stress. Our results confirmed the hypothesis that altered water flow, as a simulation of drought, can modulate the responses of biofilms to chemical exposure, even long after the flow is re-established.

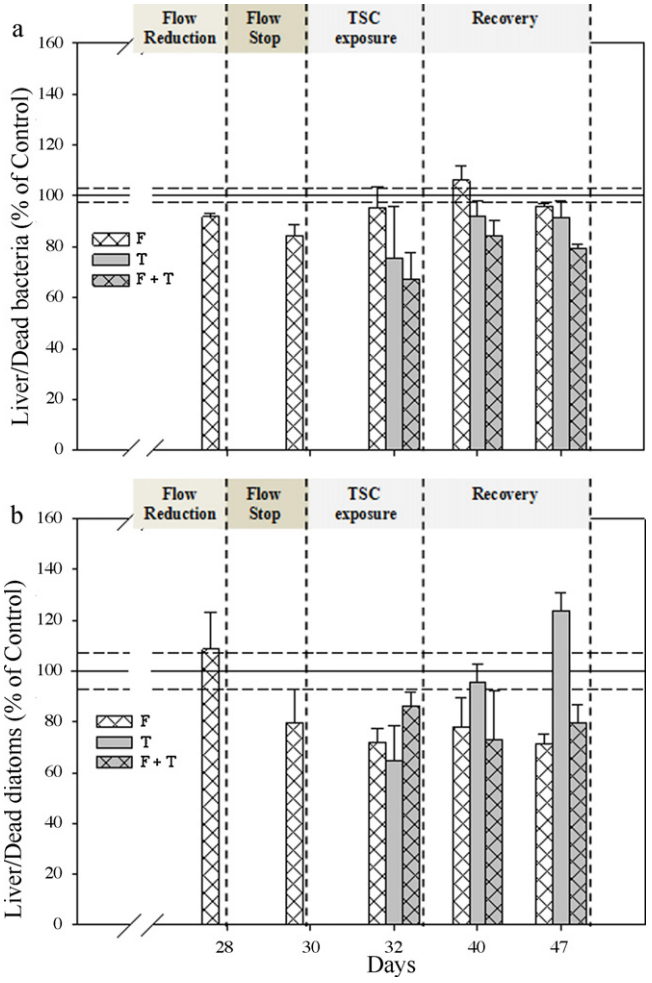

Fig. 4. Altered composition in treated biofilms. Changes in (a) the live-to-dead bacteria ratio and (b) the live-to-dead diatoms ratio on each sampling date. The values are means with standard errors ($n = 3$). The solid horizontal line represents controls at ±95% CI (dashed horizontal lines).

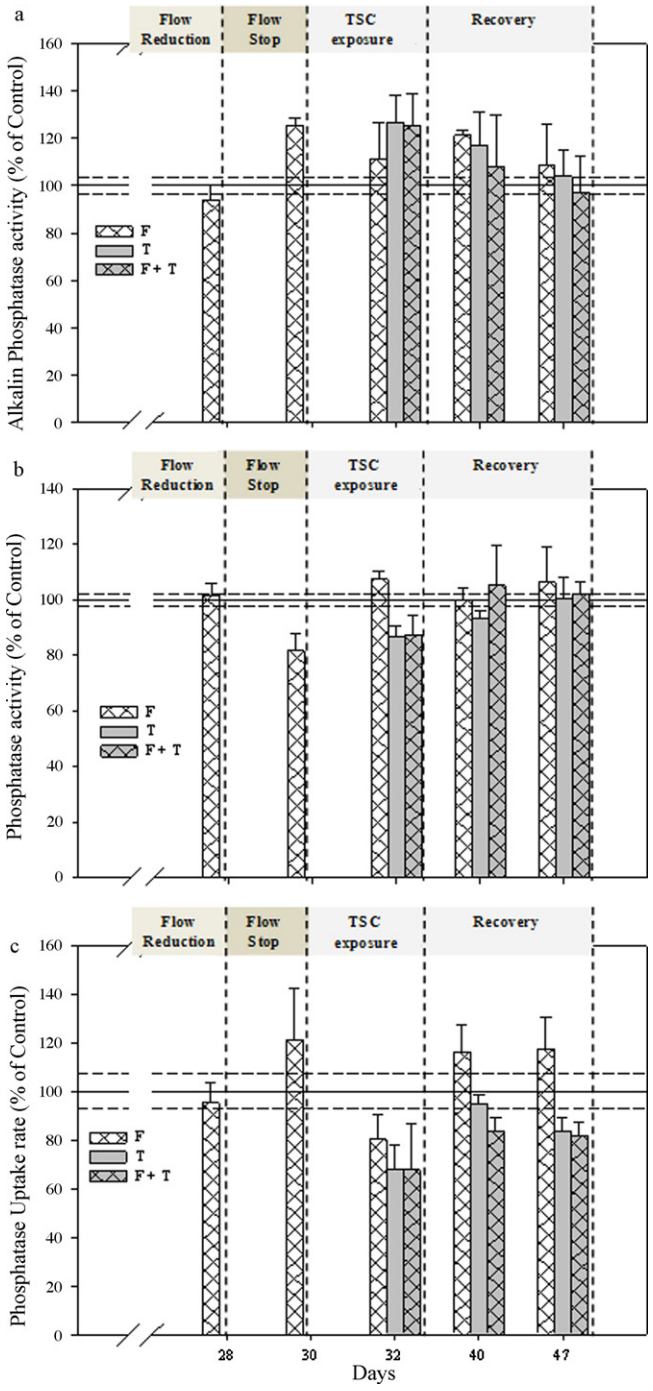

Fig. 5. Altered biological activity in treated biofilms. Changes in (a) alkaline phosphatase activity, (b) photosynthetic capacity, and (c) phosphate uptake rate on each sampling date. The values are means with standard errors ($n = 3$). The solid horizontal line represents controls at ±95% CI (dashed horizontal lines).

4.1. Effects of drought

Our study revealed that the simulated drought episode (1 week of drastically reduced flow, followed by 2 days of totally blocked flow) was enough to cause structural and functional changes in biofilm communities. It induced changes in the structure and photosynthetic capacity of the autotrophic compartment, and provoked increased bacterial mortality and extracellular phosphatase activity.

Drought episodes in running waters produce drastic flow reduction and the subsequent disruption of longitudinal hydrological connectivity, with altered physico-chemical conditions and specific biotic responses (Lake, 2003; Sabater and Tockner, 2010). After two days of interrupted flow, we observed increases in temperature, conductivity and dissolved oxygen, all which have been described in disconnected systems (Stanley et al., 1997; Caruso, 2002). Researchers have shown that in unshaded pools, the higher temperatures and the photosynthesis-activating radiation can trigger algal blooms (Freeman et al., 1994). Indeed, in our lentic channels, the temperature increase associated with the steady photosynthetic radiation fostered an initial phase of such a bloom. Green algal biomass—particularly of filamentous forms—increased significantly, which could be associated to the decrease in inorganic phosphorus (Dahm et al., 2003). The subsequent increase in the activity of extracellular alkaline phosphatase that we observed after the 2 days of flow interruption could be associated to this apparent algal growth. Indeed, alkaline phosphatase catalyzes the hydrolysis of phosphate esters, liberating inorganic phosphorus available for microbial uptake, particularly when inorganic phosphate is limiting (Berman, 1970; Siuda and Chrost, 1987; Allison and Vitousek, 2005; Romaní et al., 2004). The reduced flow was also detrimental to diatoms, either because of direct effects, or because the diatoms had to compete with autotrophic groups that are more prone to lentic conditions (e.g. green algae). The observed changes in the algal compartment (increase in green algae and decrease in diatoms) associated to a slight decrease in biofilm photosynthetic capacity, but not to any changes in photosynthetic efficiency, indicating that

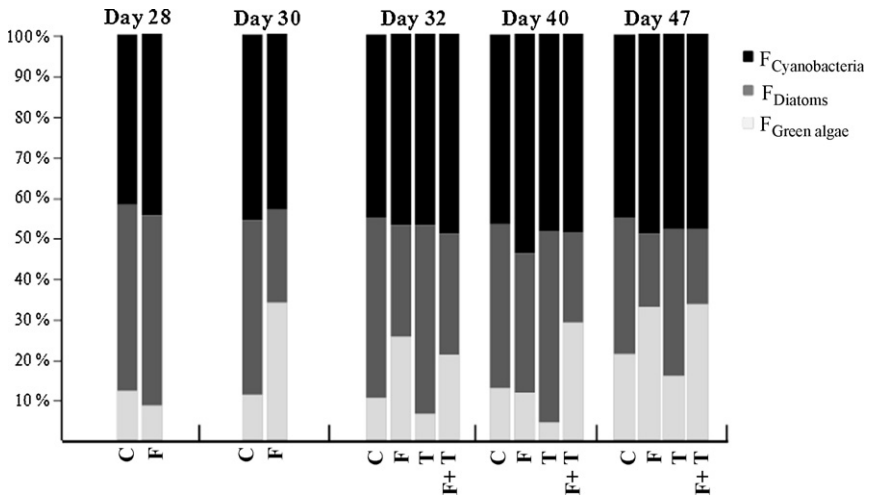

Fig. 6. Altered fluorescence in treated biofilms. Changes in the relative percentage of the fluorescence signal corresponding to each of the three autotrophic groups on each sampling date. The values are means ($n = 3$). Samples are grouped by treatments: C = Controls; F = Simulated Drought (altered flow); T = TCS exposure; and $F+T$ = Simulated Drought + TCS exposure.

the treatment affected the structure of the photosynthetic apparatuses without comprising their photosynthetic performance. The photosynthetic efficiency increased after day 32, supporting the existence of a selection pressure that favors the growth of green algae (Corcoll et al., 2012). The significant decrease in the ratio of live-to-dead bacteria observed after the 2 days of flow interruption could be also associated to a described coupling between diatoms and bacteria (Croft et al., 2005). Indeed, indirect effects on bacteria have been induced by direct effects on autotrophs (López-Doval et al., 2010; Ricart et al., 2009; Pesce et al., 2006). Nevertheless, bacterial mortality was the only parameter that significantly increased after 1 week of flow reduction (day 22; Tables 1 and A.1, and Fig. 4a), confirming the high sensitivity of bacteria to water flow and suggesting that other mechanisms (direct and/or indirect) could have affected the biofilm heterotrophs during the first phases of the simulated drought episode. In particular, flow reduction may provoke changes in the internal water (and consequently, in the nutrients) that cycle into biofilms (Battin et al., 2003), which can directly affect bacteria in the first phases of a drought episode. Subsequent effects of interrupted flow on autotrophic community composition, as evidenced in our study, may indirectly contribute to maintaining and even reinforcing this negative direct effect.

4.2. TCS toxicity

Our results confirm that TCS may directly and/or indirectly affect fluvial biofilm structure and function as indicated in several studies (Franz et al., 2008; Lawrence et al., 2009; Morin et al., 2010; Proia et al., 2011; Ricart et al., 2010). The TCS pulses in our experiments provoked increased mortality among bacteria and diatoms, and led to reduced phosphate uptake, photosynthetic capacity and green algae biomass.

The TCS concentrations in water after the pulse exposure followed exponential decay kinetics: within 3 h after addition, nearly 94% of the TCS disappeared from the water during the illuminated period. Photochemical transformation has been described as a main degradation process of dissolved TCS (Aranami and Readman, 2007; Mezcua et al., 2004; Tixier et al., 2002). Photochemical degradation rapidly occurs in water (Latch et al., 2003), and our observations agree with others that specifically investigated the kinetics of TCS photodegradation (Lores et al., 2005; Sanchez-Prado et al., 2006). Nevertheless, organic compounds (such as TCS) can be degraded to several bioactive, and therefore potentially toxic, sub-products that can be adsorbed and trapped in biofilms by both extracellular and intracellular pathways. The formation of the 2,8-dichlorodibenzo-p-dioxin from TCS as a consequence of photoreaction in aqueous solution has been confirmed by several studies (i.e. Latch et al., 2003; Lores et al., 2005).

The TCS exposure markedly influenced the biofilm communities, affecting both target (bacteria) and non-target (autotrophs) organisms. The mechanisms of TCS toxicity to bacteria are well documented: these include blocking of fatty acid synthesis (McMurry et al., 1998), compromising permeability-barrier functions (Phan and Marquis, 2006) and destabilizing cell membranes (Villalaín et al., 2001). Although no specific mode-of-action of TCS in algae has yet been determined, this agent could affect algae similarly as it does on bacteria (Lawrence et al., 2009; Ricart et al., 2010; Morin et al., 2010). The negative impact of TCS on green algal biomass, diatom viability and photosynthetic parameters reinforced our hypothesis that this bactericide would have direct and indirect effects on autotrophs. Similar negative effects of TCS on autotrophs in biofilms have been found in several studies (Franz et al., 2008; Morin et al., 2010; Proia et al., 2011; Ricart et al., 2010). Some researchers have described algae to be even more sensitive to TCS than bacteria (Tatarazako et al., 2004), whereas others, having evaluating axenic algal cultures, have confirmed the existence of some direct—albeit unexplainable—effect of TCS on algae (Capdevielle et al., 2008). The significant reduction in biofilm phosphate uptake induced by TCS could derive from its negative effects on algae and bacteria (Proia et al., 2011).

4.3. Influence of flow reduction history on TCS toxicity

The persistent effects of TCS on biofilm structure and function differed in relation to their water flow history (at day 32, the four treatment groups appear at distinct locations along the two axes of the within PCA analysis). The Simulated Drought + TCS biofilms were more sensitive to TCS exposure than were the TCS only biofilms: the former suffered more persistent effects and exhibited lower recovery potential than did the latter. Among the biofilms subjected to both Simulated Drought and TCS, the persistently higher mortality of bacteria and of diatoms (until the end of the experiment) correlated to the reduced phosphorus uptake, thereby confirming the aforementioned effects. Since biofilms are the most important biotic compartment in rivers, in terms of retaining inorganic nutrients from the water column (Sabater et al., 2007), any reduction in their phosphate uptake induced by organic compounds such as TCS, and worsened by previous

drought episodes, can severely compromise the health of river ecosystems.

The present study investigated the acute response and recovery potential of biofilm communities after transient perturbations (simulated short-term drought followed by a highly-concentrated pulse of TCS). Proia et al. (2012) has theorized that in rivers that have experienced a sequence of perturbations, the recovery process will dependent on the duration and magnitude of disturbances (short-term and/or long-term), the observed effects (direct and/or indirect) and the resilience potential of the affected organisms (target and/or non-target). Biofilms that experienced water flow reduction and interruption were more sensitive to subsequent TCS pulses than those submitted to constant flow. Our findings indicate that the flow history of rivers may alter the response of biofilms to pollutant exposure, confirming our assumption that drought episode would modulate the response of biofilms to chemical exposure. The evidence that the toxicity of pollutants to biofilms may be augmented following a drought period should be further investigated for compound mixtures in field conditions, especially in regions affected by water scarcity, more prone to be affected by global change.

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