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## **Influence of grazing on triclosan toxicity to stream periphyton**

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## Abstract

1. Interactions between emerging contaminants and other drivers of community structure and function are poorly known. We used laboratory microcosms to investigate the single and combined effects of grazing and triclosan toxicity on the structure and function of stream periphyton.
2. Grazing alone strongly reduced algal biomass, but also reduced oxidative stress and increased periphyton productivity and phosphorus (P) uptake capacity. This suggests that grazed algal communities can compensate for reduced biomass by enhanced growth after being released from grazing.
3. Triclosan exposure ( $11.6 \pm 1.1 \mu\text{g L}^{-1}$ ) reduced the detoxification capacity and P-uptake capacity of periphyton and altered diatom taxonomic composition. This indicates that triclosan at environmentally relevant concentrations affects the capacity of periphyton to remove dissolved nutrients and to cope with toxicant mixtures commonly occurring in streams.
4. Triclosan exposure and grazing pressure had negative synergistic effects on algal size-class distribution and diatom mortality, since the effects of triclosan were higher than expected when periphyton was subject to grazing.
5. Periphyton exposed to toxic substances such as triclosan had a lower capacity to cope with grazing than unexposed communities, because toxicity can limit algal regrowth after release from grazing and promote the loss of less abundant species. This synergism may have important implications because grazing pressure will magnify the negative effects of toxicants on community structure and ecosystem functions such as primary production and nutrient cycling.

Keywords: algae, ecotoxicology, herbivores, periphyton, triclosan toxicity, trophic interaction

## **1. Introduction**

In aquatic ecosystems, communities are commonly exposed to multiple stresses, including chemical contamination (Piggott et al., 2015) and biotic interactions (Liess & Hillebrand, 2004). Those multiple stressors can individually structure communities and influence their function. In addition, the presence of one stressor can also modify the community response or sensibility to another one. Thus, the combination of two stressors can often induce a synergistic effect stronger than the sum of the individual effects. For instance, natural biotic stressors such as food limitation or parasitism can modify or amplify the negative effects of chemicals on organisms (Heugens et al., 2001; Holmstrup et al., 2010; Laskowski et al., 2010; De Coninck et al., 2013). Although both contamination and biotic interactions occur simultaneously in stream ecosystems, the effects of toxicants on biotic interactions are not well understood (Barranguet et al., 2003; Real et al., 2003; Evans-White & Lamberti, 2009).

To explore the interaction between chemical contamination and biotic interactions, this study focused on periphyton, an association of autotrophic and heterotrophic micro-organisms embedded in a polymer matrix and attached to submerged surfaces of aquatic ecosystems. Periphyton is an important energy source for aquatic consumers, an active site of nutrient cycling and an important place for the adsorption and accumulation of chemicals (Guasch et al., 2010). Since changes in periphyton community structure and biotic interactions caused by chemicals can reveal indirect effects that would not be detected by standard toxicity tests with single species, periphyton is considered an ecologically relevant model in community ecotoxicology (Ricart et al., 2009).

Periphyton community structure is influenced by various abiotic and biotic factors, one of which is grazing. Grazing effects may differ, however, depending on periphyton productivity (Liess et al., 2009). High productivity reduces the probability of species to disappear, since populations can compensate for grazing losses, whereas any factor that limits biomass accrual (e.g. nutrient limitation or low light availability) increases the probability of species loss. In this situation, synergistic interactions between grazing and a growth-limiting factor might be expected. Similarly, grazing may exacerbate the negative effects on periphyton exerted by contaminants, which limit the ability of periphyton to recover from grazing pressure (Munoz et al., 2001).

The antimicrobial agent triclosan, 5-chloro-2-(2,4-dichlorophenyl)-phenol, is a broad-spectrum antimicrobial agent that is extremely widespread in medical, household and personal care products such as shampoos, toothpaste, deodorants, medical skin creams and detergents (Bhargava & Leonard, 1996; Daughton & Ternes, 1999; Schweizer, 2001). Most of the triclosan used in this vast array of products ends up in wastewater treatment plants (Singer et al., 2002; Boyd et al., 2003). However, triclosan is not degraded during conventional wastewater treatment (Adolfsson-Erici, Pettersson & Parkkonen Sturve, 2002), resulting in discharges to surface waters, where concentrations of up to  $2.3 \mu\text{g L}^{-1}$  are reached (Chalew & Halden, 2009; Dann & Hontela, 2011). Triclosan is highly toxic to periphytic bacteria (no-effect concentration of  $0.21 \mu\text{g L}^{-1}$ ; Ricart et al., 2010), periphytic algae (Franz et al., 2008; Ricart et al., 2010), periphyton architecture (Lawrence et al., 2009) and grazers feeding on periphyton (Canesi et al., 2007; Binelli et al., 2009a). As a bactericide, triclosan inhibits the biosynthesis of fatty acids at the level of the enoyl-acyl carrier protein reductase (FabI) (Heath et al., 1999).

Our aim in this study was to assess effects of grazing pressure and triclosan on periphyton community structure and ecosystem functions such as primary production and nutrient cycling. We experimentally determined the effects of these two factors on a suite of functional and structural endpoints relating to diatoms, all autotrophic periphyton and the entire periphyton community, to test for both the individual and combined effects of

triclosan and grazing and to determine whether any interactions are synergistic or antagonistic.

## 2. Methods

### *Experimental design*

Four treatments were applied to periphyton exposed to triclosan and grazing by snails in four replicate crystallising dishes per treatment. Control dishes received neither triclosan nor snails (Ctrl); grazing treatments received snails but no triclosan (S); triclosan treatments received triclosan but no snails (TCS); and triclosan plus grazing treatments received both triclosan and snails (TCS+S). The four treatments were randomly assigned to the dishes. The experiment was run for 48 h at 20 °C and under a light–dark cycle of 12:12 h at 130–150  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ .

### *Periphyton*

Periphyton was grown for 3 weeks on sandblasted glass substrata in 16 crystallising dishes. The dishes were filled with 1.5 L of artificial fresh water ( $\text{Na}_2\text{SO}_4$ ,  $\text{Na}_2\text{SiO}_3$ ,  $\text{CaCl}_2 \cdot 9 \text{ H}_2\text{O}$ ,  $\text{KCl}$ ,  $\text{MgSO}_4 \cdot 9 \text{ H}_2\text{O}$  and  $\text{NaHCO}_3$  in deionised water, neutral pH) resembling that of the River Llemena in northeastern Spain in terms of ionic composition (Table 1; Bonnineau et al., 2010). Once a week, an aliquot of a microbenthic community, scraped from three colonised cobbles from the River Llemena (small tributary of the Ter River, northeastern Spain) was resuspended in artificial fresh water and added to the dishes. The water was renewed three times per week and water samples were collected and filtered (nylon membrane filters, 0.2- $\mu\text{m}$  pore size, Whatman, Maidstone, UK) both before and after each water renewal. Soluble reactive phosphorus (SRP) was measured according to Murphy & Riley (1962) and  $\text{Cl}^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were analysed by ion chromatography (761 Compact IC, Metrohm Ltd., Herisau). Conductivity, pH and dissolved oxygen were measured with WTW probes (Weilheim).

### *Grazers*

Snails (*Radix ovata*, Lymnaeidae) were collected from the River Llemena and transported to the laboratory (<3 h travel time) in plastic containers filled with stream water. The plastic containers were placed in a bigger container with stream water and cooler packs to maintain temperature at around 15 °C. The snails were placed in glass aquaria filled with dechlorinated tap water and fed with TetraMin fish food during a 2-day acclimatisation period before starting the experiment. Forty-eight individuals were used in the experiment and 15 additional individuals were directly frozen in liquid  $\text{N}_2$ , later defrosted and the soft body removed from the shell, lyophilised and weighed to the nearest  $\mu\text{g}$ . Maximum shell height was measured with digital callipers. The mean shell length was 12.2 mm with a standard deviation (SD) of 0.6 mm, corresponding to a mean dry mass and SD of the soft body tissue of  $21.0 \pm 4.2 \text{ mg}$ .

### *Experimental set-up*

Each dish contained 40 colonised glass substrata. Triclosan was added from a stock solution in methanol. The nominal triclosan concentration was  $15 \mu\text{g L}^{-1}$  and the final methanol concentration was 0.03%. Control dishes received methanol only. The triclosan concentration was within the range reported from polluted effluents and high enough to affect periphyton in the short term. Water of all dishes was renewed every 3 h to maintain the triclosan concentrations at the target level. On both days of the experiment, physical and chemical variables were measured once as described in the Periphyton section above.

Six snails were added in the grazing treatments (Lopez-Doval et al., 2010). This density was intended to avoid

food limitation of and resource competition among the snails. Dead snails were counted and removed after 1 day and at the end of the experiment after 2 days. To ensure the same grazing pressure on periphyton in all crystallising dishes, the snails removed after the first day were replaced by individuals of the same size (shell length). These individuals were marked and excluded from the analysis of endpoints.

#### *Endpoints for periphyton and snails*

Colonised glass substrata from each dish were randomly collected and used to determine all endpoints at both the start and end of the experiment. *In vivo* fluorescence of chlorophyll a (chl a) was measured with a PhytoPAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany) in a dark chamber at room temperature after 30 min of acclimation to darkness (Corcoll et al., 2011). The samples were exposed to a saturating pulse of light to induce maximal fluorescence yield ( $F_m$ ). The maximum quantum yield (photosynthetic capacity),  $Y_{max}$ , was then calculated as  $(F_m - F_0)/F_m$ , where  $F_0$  is the minimum fluorescence yield of dark-adapted cells (Genty, Briantais & Baker, 1989). Photosynthetic capacity was used as an indicator of photosynthetic performance, and thus productivity, and  $F_0$  was used as a proxy for algal biomass (Corcoll et al., 2011).

Algal biomass was also estimated by extracting chl a with 90% acetone for 12 h in the dark at 4 °C. Samples were sonicated (40 W, 40 kHz; SELECTA, Abrera, Spain) for 2 min to improve extraction efficiency. Chl-a concentrations were subsequently determined on a Lambda UV/VIS spectrophotometer (Perkin-Elmer, Waltham) by measuring absorbance at 430 and 665 nm, without phaeophytin correction (Jeffrey & Humphrey, 1975).

Antioxidant Enzyme Activities (AEAs) were determined to provide information on the stress status and the ability of periphyton to cope with oxidative stress (Bonnineau et al., 2013). We measured the activity of catalase (CAT) and ascorbate peroxidase (APX), two antioxidant enzymes directly involved in the scavenging of ROS, as well as the activity of glutathione-S-transferase (GST), a class II detoxification enzyme. By catalysing the conjugation of electrophilic chemicals (such as oxidative toxicants) with glutathione, GST inactivates oxidative toxicants and facilitates their excretion. CAT activity was determined according to Aebi (1984), APX activity according to Nakano & Asada (1981) and GST activity according to Grant, Bender & Hammock (1989). Periphyton was removed from the glass substrata using polyethylene cell lifters (Corning Inc.) and collected into an Eppendorf tube. Samples were centrifuged for 5 min at 10 °C and 5000 g. The pellets were immediately frozen in liquid nitrogen and stored at -80 °C for later protein analysis following Bonnineau et al. (2011). Briefly, proteins were extracted in a bead beater after the addition of extraction buffer (100 mM  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.4, 100 mM KCl, 1 mM EDTA) and quantified with the Bradford (1976) method using bovine serum albumin as a standard. The assays were performed in microplates and absorbance was measured in a microtiter plate reader Synergy4 (BioTek, Winooski).

The capacity of periphyton to take up phosphorus was determined by measuring the rate of disappearance of soluble reactive phosphorus (SRP) added to the crystallising dishes with a known area of periphyton on the glass slides (Proia et al., 2013). The dishes were spiked with 12  $\mu\text{L}$  of  $1\text{Na}_2\text{PO}_4$  ( $1.41\text{ g L}^{-1}$ ) to quadruple the background concentration of SRP ( $3.7 \pm 1.9\text{ }\mu\text{g L}^{-1}$ ) and incubated for 120 min in an illuminated incubator under controlled temperature (21 °C) and light ( $130\text{--}150\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ) conditions (Radiber AGP-570).

Suspensions of periphyton scraped off the slides were preserved with a drop of formalin and diluted with water (4%) from the respective crystallising dish (5 mL final volume). Diatoms were subsequently identified and counted and their live–dead ratio (Morin et al., 2010) determined in a Nageotte chamber at a magnification of 109. The presence of chloroplasts was used as a criterion to distinguished live and dead cells. Samples were



digested in oxygen peroxide to prepare permanent slides for diatom identification. A minimum of 400 individuals was counted per sample (Morin et al., 2010). Diatom taxonomy was used to assess treatment effects on the structure of the community (in terms of species composition and traits) and the ratio between live and dead diatom cells to assess effects on diatom viability. In addition, four different growth forms of the periphyton were distinguished: solitary unattached, solitary stalked and heap-forming cells as well as linear colonies. The size distribution of diatom cells was also determined, based on theoretical biovolumes (<http://hydrobio-dce.irstea.fr/telecharger/diatomees-ibd>).

Snail mortality was recorded after 24 and 48 h. Oxygen consumption by the snails was measured at the start and at the end of the experiment with an Oxygen Measurement Controller (Model 10, Rank Brothers, Cambridge). A total of three (beginning) or four (end) snails were used for each treatment. Only one snail from each replicate was used. The measuring chamber of the oxygen meter was filled with 15 mL of air-saturated bubble-free water at a constant temperature ( $20 \pm 1$  °C) and a snail placed inside for 10 min. The difference between the initial and final concentration was assumed to represent the oxygen consumption by the snail. Rates were normalised by snail dry mass determined at the end of the experiment. Snails were marked to recognise them at the end of the experiment and calculate their individual dry mass.

### *Triclosan analysis*

Triclosan concentrations were measured with the magnetic particle-based immunoassay using the triclosan IA kit developed and supplied by Abraxis LLC (Warminster) as described by Kantiani et al. (2008). One water sample was collected from all replicate crystallising dishes 30 min after triclosan addition. In addition, one triclosan-treated dish was sampled every 20 min between two consecutive water exchanges. Water samples were adjusted with methanol to a concentration of 25% (v/v) prior to analysis and diluted to fall within the calibration range. Suspended antibody-coupled paramagnetic particles supplied with the kit were mixed in a glass test tube and incubated at room temperature for 30 min. Next, 250 µL of HRP-4-[3-bromo-4-(2,4-dibromophenoxy) phenoxy] butyrate was added and the mixture was incubated for another 30 min at room temperature. A magnetic field was applied to hold the paramagnetic particles and bound reagents in the tube. Unbound reagent was removed before washing the particles with water. A chromogenic substance (3,3',5,5'-tetramethylbenzidine; TMB) and hydrogen peroxide (500 µL) were then added to the particles and the mixture was incubated for 20 min at room temperature. The colour reaction was stopped by adding 500 µL of 2% sulphuric acid and absorbance at 450 nm was read within 15 min. Standard curves were based on eight triclosan concentrations between 20 and 1000 ng L<sup>-1</sup>. The standard curves were obtained by nonlinear curve fitting to a four-parameter equation (Kantiani et al., 2008).

### *Data analysis*

Treatment effects on periphyton endpoints determined at the end of the experiment were tested by analysis of variance (ANOVA) with two factors, triclosan exposure and grazing by snails. All endpoints were log<sub>10</sub>-transformed prior to statistical analyses to meet assumptions of normality and homogeneity of variance. Statistically significant differences in the two main factors were interpreted as a positive or negative effect, depending on the sign of changes (see De Coninck et al., 2013). Statistically significant triclosan 9 grazing interactions are interpreted as statistically significant deviations from the Independent Action model of joint stressor effects (Jonker et al., 2004; De Coninck et al., 2013). The interaction was synergistic when the effect observed in the combined treatment was higher than the sum of the effects of the individual factors and antagonistic when the effect observed in the combined treatment was smaller than the sum of the individual effects. Treatment effects on snail mortality and oxygen consumption were tested by one-way ANOVA.

Differences between the start and end of the experiment were also assessed by one-way ANOVA to test for changes during the experiment independent of the treatment applied. All ANOVAs were performed using SPSS v.17.0.

Non-metric multi-dimensional scaling (nMDS) was performed using the PRIMER 6 program to visualise the similarity/dissimilarity between diatom communities exposed to different treatments. The analysis was based on a Bray-Curtis distance matrix created from square-root-transformed relative abundance data.

### 3. Results

Physical and chemical variables varied relatively little during the 3-week colonisation period (Table 1) and the 2-day experiment. SRP concentrations decreased over time because P was taken up by periphyton between water exchanges, but it was never depleted during the colonisation period. Water temperature during the experiment was  $20.8 \pm 0.3$  °C, pH was  $7.9 \pm 0.1$ , conductivity was  $150 \pm 3$   $\mu\text{S cm}^{-1}$  and the dissolved oxygen concentration was  $8.8 \pm 0.3$   $\text{mg L}^{-1}$  (mean  $\pm$  SE,  $n = 16$  in all cases). Triclosan concentration in the triclosan-exposed dishes was  $11.6 \pm 1.1$   $\text{lg L}^{-1}$  ( $n = 8$ ).

No significant differences in any of the endpoints were detected between treatments at the beginning of the experiment (data not shown). However, oxygen consumption by snails increased from  $0.35 \pm 0.04$   $\text{g O}_2 \text{ g}^{-1}$  snail dry mass (mean  $\pm$  SD) at the start of the experiment to  $0.64 \pm 0.18$  and  $0.59 \pm 0.18$   $\text{g O}_2 \text{ g}^{-1}$  snail dry mass at the end in the absence and presence of triclosan respectively ( $F = 6.78$ ,  $P = 0.03$ ).

#### Grazing effects

Several effects of grazing on periphyton were observed at the end of the experiment (Fig. 1, Table 2). Algal biomass (chl a) was three times lower in the presence of snails, photosynthetic capacity and phosphorus uptake capacity increased and activities of the antioxidant enzymes CAT and APX decreased (Fig. 1, Table 2). Diatom abundance was also strongly reduced (Fig. 1) and the size-class distribution was changed (Fig. 2, Table 2). Specifically, grazing led to a decrease in the relative abundance of small diatoms and an increase in medium-sized cells (Fig. 2). The live/dead ratio of diatoms also increased (Fig. 1, Table 2), whereas the number of diatom taxa and diatom community composition were unaffected (Figs 1 & 3).

#### Triclosan effects

Triclosan affected both snails and algae. Snail mortality was higher in triclosan treatments ( $F = 8.93$ ,  $P = 0.03$ ). The mean number of dead individuals in controls ( $1.3 \pm 0.58$  individuals) was 2.3 times lower than in dishes exposed to TCS ( $3.0 \pm 0.8$  dead individuals). GST activity of periphyton and P-uptake capacity were also reduced (Fig. 1, Table 2). A lower live/dead ratio of diatoms indicated increased diatom mortality, and diatom richness also decreased to some extent (Table 2, Fig. 2). nMDS separated diatom communities exposed to triclosan from those that were not exposed (Fig. 3), indicating that triclosan was the main factor determining diatom community composition. The relative abundance of some species was affected by triclosan (Table 2); *Achnanthes pyrenaicum* increased from 6% to 10% and *Nitzschia palea* from 0.4% to more than 1%.

The distribution of biovolume classes and types of growth form were also affected (Fig. 2). Large species like *Ulnaria ulna*, *Gomphonema truncatum* and *Gyrosigma attenuatum* decreased in abundance more than twofold when periphyton was exposed to triclosan, leading to a lower proportion of large diatom cells (Fig. 2, Table 2). Similarly, linear colonies, associated particularly with *Fragilariaceae* and *Encyonema* species, were the diatom growth form affected most (Fig. 2b, Table 2).



### *Interactive grazer and triclosan effects*

Three of the measured endpoints indicated statistically significant interactions of grazing pressure and triclosan exposure (Fig. 1, Table 2). Two of these were synergistic (live/dead ratio of diatoms, abundance of medium-sized diatoms) and one was antagonistic (GST).

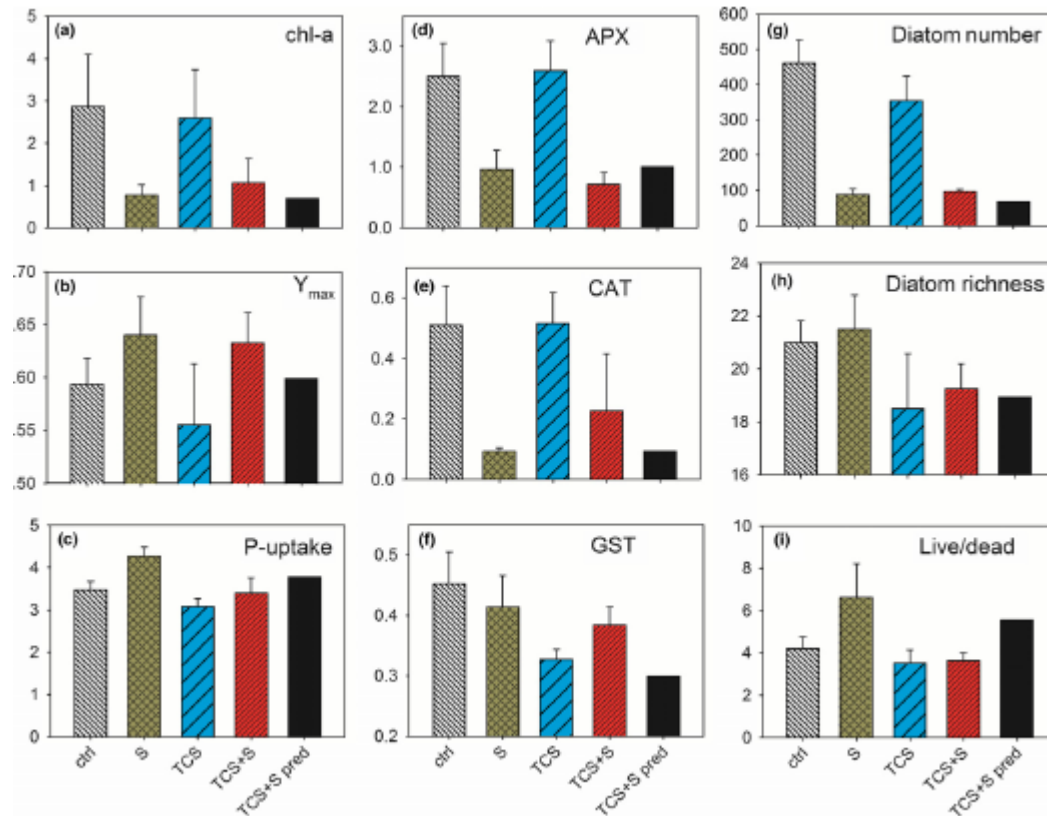
**Table 1** Physical and chemical conditions during the periphyton colonisation period. Mean and standard error (in parentheses) are given (n=21).

Variable	Before water exchange	After water exchange
pH	8.3 (0.1)	7.6 (0.1)
Conductivity ( $\mu\text{S cm}^{-1}$ )	189 (6)	136 (5)
Oxygen ( $\text{mg L}^{-1}$ )	9.3 (0.1)	8.73 (0.1)
Temperature ( $^{\circ}\text{C}$ )	19.9 (0.2)	21.9 (0.4)
$\text{Cl}^{-}$ ( $\text{mg L}^{-1}$ )	28.7 (2.1)	24.1 (1.7)
$\text{NO}_3^{-}$ ( $\text{mg L}^{-1}$ )	1.0 (0.2)	1.9 (0.1)
$\text{SO}_4^{2+}$ ( $\text{mg L}^{-1}$ )	9.8 (0.9)	9.0 (0.8)
$\text{Na}^{+}$ ( $\text{mg L}^{-1}$ )	12.6 (3.0)	10.8 (2.4)
$\text{K}^{+}$ ( $\text{mg L}^{-1}$ )	1.1 (0.4)	0.9 (0.3)
$\text{Ca}^{2+}$ ( $\text{mg L}^{-1}$ )	20.7 (6.0)	13.4 (4.8)
$\text{Mg}^{2+}$ ( $\text{mg L}^{-1}$ )	0.4 (0.1)	0.3 (0.1)
SRP ( $\mu\text{g L}^{-1}$ )	7.1 (0.8)	14.9 (3.6)

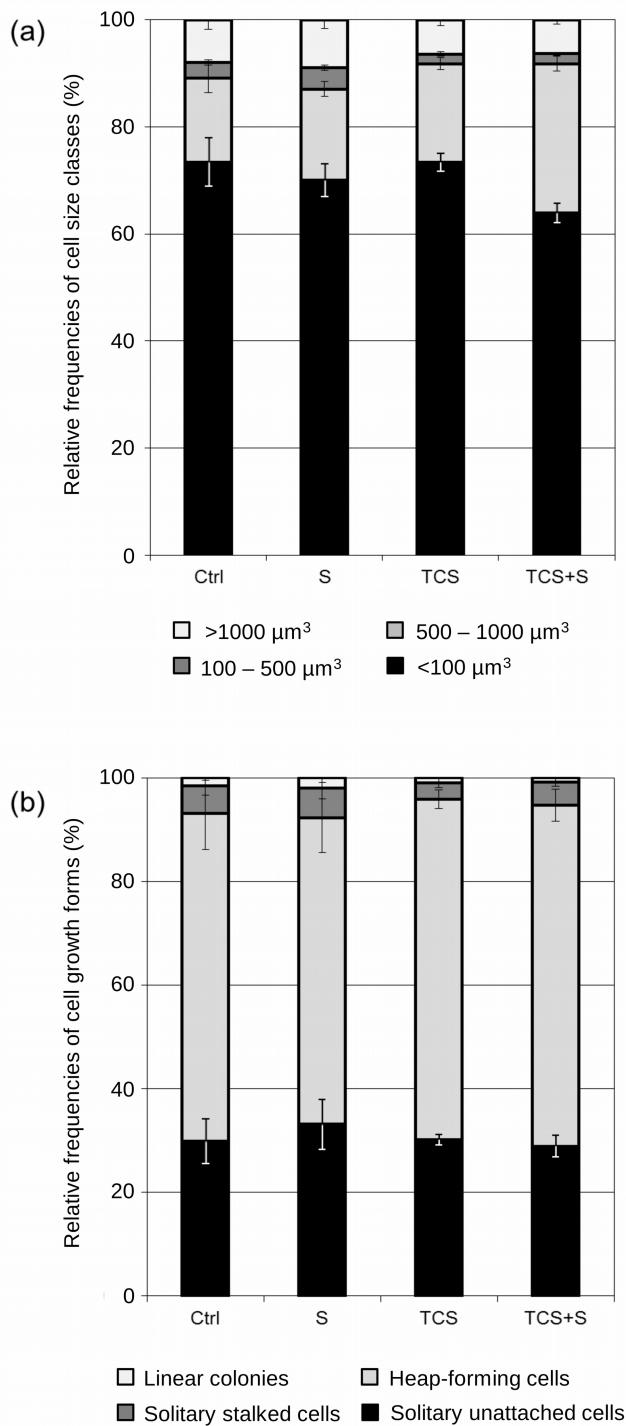
**Table 2** Two-way ANOVA results of periphyton endpoints: phosphorus uptake capacity, antioxidant enzyme activities of ascorbate peroxidase (APX), catalase (CAT) and glutathione-S-transferase (GST), photosynthetic capacity ( $Y_{\max}$ ), chl-a concentration, diatom relative abundance (%), diatom richness (number of taxa), relative abundance (%) of *Achnanthes pyrenaicum* and *Nitzschia palea*, ratio between live and dead diatoms (live–dead ratio), diatom biovolume class and growth forms.

Endpoint	Grazing effect	TCS effect	TCS × S effect
<b>Whole periphyton</b>			
Phosphorus uptake	F = 25.0, P < 0.01	F = 19.1, P < 0.01	n.s.
CAT	F = 5.9, P < 0.05	n.s.	n.s.
APX	F = 65.5, P < 0.01	n.s.	n.s.
GST	n.s.	F = 15.5, P < 0.01	F = 5.2, P < 0.05
<b>Autotrophs</b>			
$Y_{\max}$	F = 8.0, P < 0.05	n.s.	n.s.
Chl a	F = 18.0, P < 0.01	n.s.	n.s.
<b>Diatoms</b>			
Abundance	F = 17.5, P < 0.01		n.s.
Richness	n.s.	F = 4.6, P = 0.05	n.s.
<i>Achnanthes pyrenaicum</i> (r.a.)	n.s.	F = 62.3, P < 0.01	n.s.
<i>Nitzschia palea</i> (r.a.)	n.s.	F = 15.0, P < 0.01	n.s.
Live–dead ratio	F = 7.9, P < 0.05	F = 16.0, P < 0.01	F = 4.4, P = 0.05
<b>Biovolume class</b>			
Small: < 100 $\mu\text{m}^3$	F = 4.4, P = 0.05	n.s.	n.s.
Medium: 100–500 $\mu\text{m}^3$	F = 9.5, P < 0.01	n.s.	F = 5.2, P < 0.05
Large: 500–1000 $\mu\text{m}^3$	n.s.	F = 15.0, P < 0.01	n.s.
Very large: >1000 $\mu\text{m}^3$	n.s.	n.s.	n.s.
<b>Growth form and posture</b>			
Solitary unattached	n.s.	n.s.	n.s.
Solitary stalked	n.s.	n.s.	n.s.
Heap-forming	n.s.	n.s.	n.s.
Linear colonies	n.s.	F = 8.9, P < 0.05	n.s.

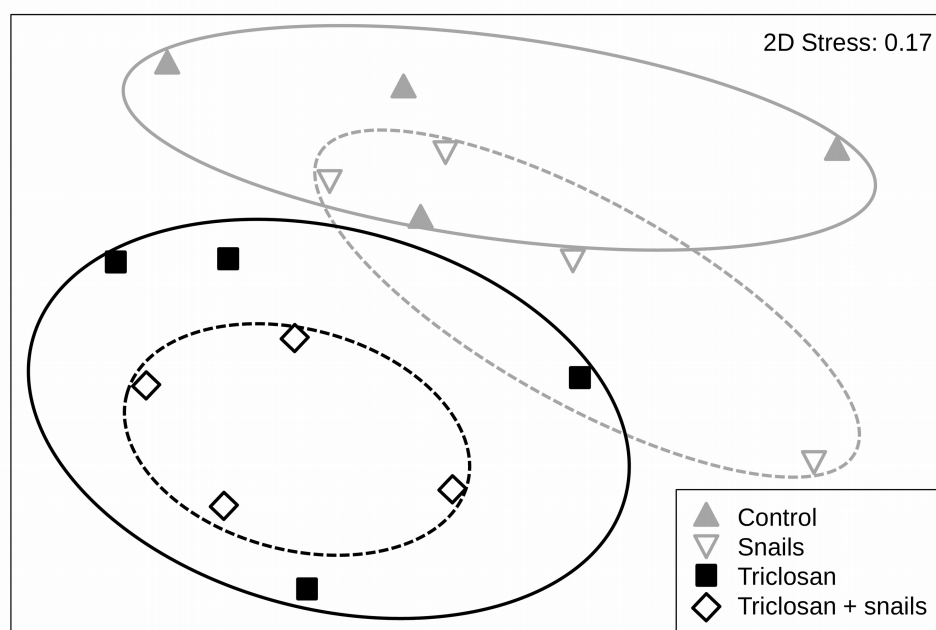
**Fig. 1** Mean and standard error ( $n = 4$ ) of periphyton endpoints measured after 48 h of exposure to triclosan (TCS), grazers (S) or both. Endpoints specific to autotrophic periphyton: (a) Chl-a concentration ( $\mu\text{g cm}^{-2}$ ) and (b) photosynthetic capacity (relative fluorescence); endpoints of periphyton as a whole: (c) phosphorus uptake capacity ( $\mu\text{g P cm}^{-2} \text{ h}^{-1}$ ), antioxidant enzyme activities (d) APX ( $\text{nmol Na ascorbate min}^{-1} \mu\text{g}^{-1} \text{ protein}$ ), (e) CAT ( $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \mu\text{g}^{-1} \text{ protein}$ ) and (f) the enzyme GST ( $\mu\text{mol CDNB conjugate min}^{-1} \mu\text{g}^{-1} \text{ protein}$ ) and diatom endpoints: (g) diatom abundance (number of cells  $\text{mm}^{-2}$ ), (h) diatom taxon richness and (i) diatom viability assessed as the ratio between live and dead cells. ANOVA results are presented in Table 2.



**Fig. 2** Mean and standard error (n = 4) of diatom biovolume classes (a) and growth forms (b) in periphyton communities after 48 h of exposure to triclosan (TCS) and grazers (S). ANOVA results are presented in Table 2.



**Fig. 3** Nonmetric multi-dimensional scaling (nMDS) ordination of diatom communities after 48 h of triclosan exposure, based on square-root-transformed relative abundances and Bray-Curtis similarities.



## 4. Discussion

Bactericides like triclosan can influence the phosphate uptake capacity, antioxidant activities and community structure of periphyton, and these effects are aggravated by grazing.

### *Grazing effects on periphyton*

Grazing was very intense, reducing algal biomass and shifting algal community structure to early stages of succession, as has been previously reported (DeNicola et al., 1990; Underwood & Thomas, 1990; Steinman, 1996; Sabater et al., 2002). Specifically, exposure to grazers reduced the abundance of diatoms as a whole, as generally observed for periphytic algae (Munoz et al., 2001). The proportion of small diatoms (biovolume < 100  $\mu\text{m}^3$ ) also decreased under grazing pressure, whereas the proportion of medium-sized cells (biovolumes between 100 and 500  $\mu\text{m}^3$ ) increased. Photosynthetic capacity and the viability of diatoms were also higher in grazed periphyton than in controls. These observations together with the observed reduction in CAT and APX activities in grazed periphyton might be due to a rejuvenating effect of grazing, since increases in these AEAs have been previously attributed to community ageing during periphyton succession (Bonnineau et al., 2013). The stimulation of P-uptake capacity of periphyton by grazing is also consistent with positive effects of herbivores on nutrient cycling related to nutrient remineralisation, which may decrease nutrient spiralling length in streams (Mulholland, 1996). Thus, although greatly reducing algal biomass, grazing clearly had a positive effect on periphyton activity.

### *Triclosan effects*

Triclosan affected both the snails and periphyton, but the impact was greater on the diatom community than on



the grazers. Indeed, our nMDS analysis showed that although described as a bactericide, triclosan clearly affected the structure and function of periphytic algae as non-target organisms. Increased diatom mortality (Ricart et al., 2010; Proia et al., 2011) as observed in this study, a decline in diatom richness (Wilson et al., 2003) and changes in periphyton architecture as a result of shifts in the relative abundance of species and growth forms (Lawrence et al., 2009) have also been found in previous investigations. In our study, the relative decrease in large cells following triclosan exposure could be related to structural damage of the cell membrane, whose surface area increases with cell size. This interpretation is consistent with a marked effect of triclosan on filamentous green algae observed in periphyton (Proia et al., 2013). However, it is unclear whether the TCS-induced decline in the P-uptake capacity of periphyton in our study was effectively caused by toxicity to algae, because similar effects have been observed in studies focusing on the bacterial component of periphyton (Proia et al., 2011, 2013).

Exposure to triclosan also affected the antioxidant defence response of periphyton. Given that CAT and APX, two enzymes involved in scavenging hydrogen peroxide, were unaffected by triclosan exposure, the 30% decrease of GST in our study suggests that the mechanism of triclosan toxicity did not involve the generation of free radicals. By catalysing the conjugation of glutathione to electrophilic compounds, such as some xenobiotics and cell constituents damaged by ROS, GST can reduce toxicity and facilitate the excretion of toxicants from cells (Hoque et al., 2007). Thus, the reduced GST activity in our study suggests that the detoxification capacity of periphyton was reduced by triclosan exposure. This observation contrasts with data on mussels (Canesi et al., 2007) and zebra fish larvae (Oliveira et al., 2009), which showed an increase in GST activity after exposure to triclosan for 24 and 96 h respectively. In animals, triclosan may be degraded by phase II enzymes (such as GST) and thus be detoxified (Wang, Falany & James, 2004). In algae or bacteria, however, triclosan sulphonation or glucuronation has not yet been demonstrated and different degradation mechanisms have been suggested (e.g. oxidation by laccase in green algae; Otto et al., 2015). These differences in degradation pathways might account for the opposite responses of periphyton and animals. Nevertheless, our study indicates that triclosan decreases the detoxification capacity of periphyton. This is likely to have repercussions on the capacity of periphyton to purify water and to cope with toxicant mixtures that commonly occur in streams (Sabater et al., 2002).

Triclosan also significantly increased snail mortality up to 50%. However, since snails also died in dishes without TCS (20%), this result needs to be interpreted with caution. Indeed, mortality of freshwater snails at low triclosan concentrations has not been previously detected, and the few studies reporting triclosan effects on other molluscs (Canesi et al., 2007; Coogan & Point, 2008; Binelli et al., 2009b) did not assess mortality. TCS killed the fish *Pimephales promelas* (Cyprinidae) at a concentration of 260  $\mu\text{g L}^{-1}$  after 4 days of exposure (Orvos et al., 2002) and also produced a dose-dependent toxic effect between 29 and 87  $\mu\text{g L}^{-1}$  on zebra mussels (*Dreissenidae*, Binelli et al., 2009a). Furthermore, *in vitro* experiments with tissue extracts of different organs of the mussel *Mytilus galloprovincialis* (Mytilidae) found effects on kinase-mediated cell signalling, lysosomal membranes and redox balance at 289  $\mu\text{g}$  triclosan  $\text{L}^{-1}$  (Canesi et al., 2007). However, genotoxicity in mollusc cells exposed to TCS for 24 h was found even at a concentration of 0.29  $\mu\text{g L}^{-1}$  (Binelli et al., 2009b). These results suggest that snails in our study might have suffered sublethal physiological damage that was undetectable by the endpoints used in our study. However, although oxygen consumption is considered to be a sensitive endpoint for toxicity screening (e.g. Brodeur, Dixon & McKinley, 2001; Khangarot & Rathore, 2003), no difference in oxygen consumption was observed between treatment and control conditions, suggesting that any physiological stress of triclosan on snails was limited in our experiment.

### *Interactive effects of triclosan and grazing*

As initially hypothesised, triclosan effects on periphyton resembled those expected in response to other factors limiting algal growth, such as nutrient or light supply. The combined effects of triclosan and grazing on diatom viability were synergistic, since the live/dead ratio in the TCS+S treatment was lower than predicted based on triclosan or snail effects alone, indicating that triclosan toxicity prevented the recovery of periphyton subject to grazing (Burns & Ryder, 2001). The capacity for P uptake showed a similar response pattern, possibly indicating an interactive effect of triclosan and grazing on this ecosystem function, although the interaction was not statistically significant.

A synergistic interaction was also apparent for medium-sized diatom cells. Their relative increase in the presence of both snails and triclosan was higher than predicted based on responses of periphyton to either snail or triclosan exposure. This finding, together with the synergistic interaction on diatom viability described above, indicates that triclosan not only affects the ability of periphyton to recover from grazing pressure but also exerts a selective grazing pressure on particular species. In contrast to these synergistic interactions, the detoxification capacity of periphyton exposed to triclosan and grazing simultaneously was lower than predicted when assessed as GST activity, indicating an antagonistic response. This could imply mitigation of toxicity by grazing, although firm conclusions to this effect are premature as long as the mechanistic basis of how the glutathione cycle in periphyton responds to toxicants remains poorly understood.

Overall, our results indicate complex responses of periphyton simultaneously exposed to grazing and triclosan. In particular, triclosan exposure suppressed the rejuvenating effect of grazing on periphyton by decreasing the capacity to recover when periphyton was released from grazing. Both synergistic and antagonistic interactions were observed, although insufficiently understood, suggesting that detailed mechanistic studies of toxicant effects on species interactions are required to assess the risks of toxicant release into natural ecosystems.

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