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EFFECTS OF A CHRONIC LOWER RANGE OF TRICLOSAN EXPOSURE ON A STREAM MESOCOSM COMMUNITY

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Abstract: Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is an antimicrobial found in consumer soaps and toothpaste. It is in treated wastewater effluents at low parts-per-billion concentrations, representing a potentially chronic exposure condition for biota inhabiting receiving streams. For the present study, a naturally colonized benthos was created using flow-through indoor mesocosms; then, the benthic communities were dosed to achieve different in-stream triclosan concentrations (control, 0.1 μg/L, 0.5 μg/L, 1.0 μg/L, 5.0 μg/L, and 10 μg/L) for 56 d. Water quality parameters and endpoints from bacteria to macroinvertebrates, as well as interacting abiotic components, were measured. Effects of triclosan on specific microbial endpoints were observed at all doses, including an effect on litter decomposition dynamics at doses of 1.0 μg/L and higher. Resistance of periphytic bacteria to triclosan significantly increased at doses of 0.5 μg/L and above. By the end of dosing, the antimicrobial appeared to stimulate the stream periphyton at the 3 lowest doses, while the 2 highest doses exhibited decreased stocks of periphyton, including significantly lower bacteria cell densities and cyanobacteria abundance compared with the control. Other than an effect on benthic ostracods, the changes that occurred in the periphyton did not translate to significant change in the colonizing nematodes, the macroinvertebrate community as a whole, or other measurements of stream function. The results shed light on the role a low, chronic exposure to triclosan may play in effluent-dominated streams. *Environ Toxicol Chem* 2013;32:2874–2887. © 2013 SETAC

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INTRODUCTION

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol; CAS number 3380-34-5) is an antimicrobial agent used in many consumer products such as toothpastes, soaps, and antiseptic ointments. Triclosan has been used as a biocide in the healthcare industry since the early 1970s [1]. It acts at biocidal concentrations by several mechanisms related to destruction of the cytoplasmic membrane in bacteria and fungi [1]. At lower, bacteriostatic concentrations, it prevents lipid synthesis [2] as it mimics the natural substrate *fabI*, the reduced nicotinamide adenine dinucleotide–dependent trans-2-enoyl-acyl carrier protein reductase, which is critical for the final step in fatty acid synthesis in bacteria [3]. Fatty acid metabolism in plants utilizes the same biochemical pathway as bacteria, but takes place within the endosymbiotically derived plastid, therefore imparting demonstrated sensitivity in some aquatic flora [4]. The mode of action for triclosan toxicity shown in other organisms is not entirely clear [5].

More than 96% of the triclosan that is manufactured is used in consumer products that ultimately make their way to sewers [6], resulting in influent triclosan concentrations to wastewater treatment plants ranging from less than 1 μg/L to more than 100 μg/L [7–10]. Triclosan that does not biodegrade in the activated sludge process or sorb to settled solids is discharged in effluent at concentrations at levels of detection (0.0002 μg/L [10]) to approximately 10 μg/L [7,8,10]. This results in detection frequencies of approximately 60% in effluent-receiving waters and concentrations that average approximately 0.1 μg/L in studies focusing on surface waters internationally [11] and in studies focusing on Canada [12], England [13], and the United States [10,14]. However, concentrations in the 1-μg/L to 10-μg/L range have been reported in receiving waters [10,14,15] and may be related to poor treatment processes, combined or sanitary sewer overflows, or failing septic systems. Triclosan will deprotonate to its negatively ionic phenolate form at $pH > 8.1$. The ionized form of triclosan is much more susceptible to photodegradation than the fully protonated form [16,17], and when ionized, the toxicity of triclosan decreases [5,18].

Triclosan concentrations equivalent to or above the means reported for surface waters have been shown to have significant effects on aquatic organisms [5]. Microbes are most affected, and bacteria genera are the most sensitive among organisms tested using traditional toxicological approaches. Some algal species are sensitive to acute affects in the single μg/L range [12]. For example, cell growth was inhibited at triclosan concentrations greater than 0.6 μg/L for *Scenedesmus subspicatus* over a 72-h period [18]. Aquatic macrophytes, such as *Lemna gibba* and wetland plant seedlings may be susceptible in the low μg/L range under certain conditions [18–20]. Protozoans may also be sensitive to observed low levels [21,22], but benthic macroinvertebrates [23] and zooplankton [18,24] do not appear to be directly impacted by triclosan concentrations below those that are 5 times to 10 times the level that is environmentally relevant.

Triclosan sensitivities observed for bacteria and algae are potentially important because the effect concentrations

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commonly occur in effluent-impacted systems. Organisms comprising these groups sit at the base of stream food webs and are responsible for driving a significant fraction of the carbon and nutrient cycling in stream systems [25]. The hypothesis follows that triclosan may be impacting whole ecosystems from ecological cascades, if the basal producers and osmotrophs of streams are sensitive. Several studies have assessed potential effects of triclosan at higher levels of ecological organization, focusing on understanding whether interacting structural and functional changes occur with exposure in river biofilms (also termed periphyton) [22,26–31]. Generally, it can be concluded from these studies that triclosan at relatively low concentrations ($\leq$10 μg/L) could significantly alter periphyton structure and activity. These basal impacts may be important to other components of stream function, such as the composition of the reach-scale consumer community or carbon and nutrient source or sink strength, but no studies geared toward this level of assessment could be found to date.

Furthermore, spread of triclosan resistance is a growing concern as an increased risk to human and environmental health [32,33]. Some bacteria are intrinsically resistant to triclosan. This is imparted via permeability barriers or efflux pumps that occur in genera ubiquitous in the environment [34], including inhabitants of aquatic biofilms [35]. Mutations in the genes encoding for *fabI* could also result in resistance to triclosan [3]. With its role in fatty acid synthesis, *fabI* is a cellular target of many antibiotics and is, therefore, a critical pathway for drug development [36]. Recently, Ciusa et al. [36] described an additional *sh-fabI* allele that imparted resistance mechanisms in clinical isolates, and they had a high potential for horizontal gene transfer. Selection for, acclimation to, or adaption to low levels of triclosan persistent in the environment may contribute to spread of resistance to antibiotics generally, which are important for staving off pathogenic infections in higher aquatic organisms.

The potential system-level consequences and risk of spreading resistance prompted the need for the present study. It was designed to focus on the potential of triclosan exposure to impart interacting structural and functional effects on a whole stream benthos, including naturally and continuously colonized microbes through macroinvertebrates. Flow-through mesocosms were used to simulate stream riffle or run habitat and its associated ecological compartments. After a 28-d colonization, 5 nominal triclosan concentrations—0.1 μg/L, 0.5 μg/L, 1 μg/L, 5 μg/L, and 10 μg/L, with controls—were dosed continuously to the mesocosms for 56 d. The ecological effects observed under these conditions can be crucial for the interpretation of the role triclosan may be playing in real exposure scenarios.

MATERIALS AND METHODS

Mesocosm set-up and experimental design

The US Environmental Protection Agency (USEPA) Experimental Stream Facility was used to examine the impacts of triclosan exposure on a stream benthos from July to September 2008. A detailed description of the Experimental Stream Facility is provided elsewhere [37,38]. The benthic biology, including bacterial and fungal osmotrophs, algal primary producers, and micro- and macroinvertebrate consumers colonized mesocosm substrates naturally and continuously in a semicontrolled environment (i.e., hydrology, slope, substrate, flow, and irradiance were tightly controlled). The intent was to simulate conditions within the riffle–run habitat features of natural streams, for which there is a continuous source of upstream organisms for colonization. Source water was supplied from the East Fork of the Little Miami River, Clermont County, OH, USA. Seven Experimental Stream Facility mesocosms (each 12 m total length) were divided into tile (0.3 m width) and gravel (0.6 m width) substrate sections, both 4.25 m long. Flow was uniform over the study period with a constant river water inflow rate of 34.1 L/min, and recirculation rates were set to 61 L/min. The recirculation decouples the mesocosms from the water quality dynamics in the source water so that community-level carbon and nutrient processing can be assessed. Total flow through the mesocosm substrate sections was 95 L/min.

Unglazed terra-cotta ceramic tiles and solid gravel-filled trays were used as streambed-sampling units before and during exposure. Each mesocosm contained 144 tiles and 45 trays, arranged in columns of 3. The gravel trays were positioned in acrylic holders that isolated each one to surface water exchange only (i.e., no intergravel flow could occur parallel or perpendicular to surface flow.) Three tiles were sampled weekly, and the gravel ($n = 2$–4) was sampled less frequently (Table 1). Each was sampled randomly without replacement. New tiles and gravel-filled trays were added after retrieval, but they were not resampled. Over the study, 25% and 62% of the original tile and gravel substrate was sampled, respectively.

General water quality and nutrient chemistry

Background water quality conditions were continuously measured (at 5-min intervals) using process sensors (Hach) positioned in the tail tank of each mesocosm, including 1 for dissolved oxygen (DO), pH, specific conductivity, water temperature, and turbidity. These water quality data were acquired along with output from flow meters on the inflow and recirculation lines, light sensors, and indoor and outdoor climate sensors on the same time step. Surface water grab samples of the inflow were collected and analyzed for nutrient species daily. Nutrients were analyzed using a flow injection autoanalyzer (QuickChem 8000, Lachat Instruments) and the manufacturer's methods for total nitrite plus nitrate [39], total ammonium nitrogen [40], total nitrogen [41,42], total phosphorus, and total reactive phosphorus [43]. Total organic carbon was analyzed via USEPA methods 415.1 and 9060 by wet oxidation ultraviolet/persulfate using a Phoenix 8000 analyzer (Tekmar-Dohrmann). All nutrient species were analyzed with no preservative added within 24 h of collection or were immediately frozen and held for a maximum of 1 wk until analysis.

Triclosan dosing and measurement

After the colonization period, triclosan, purchased as Irgasan (assay >97%, Fluka, from Sigma-Aldrich) was dosed from stock solutions, renewed every other day, and pumped continuously at 0.110 L/min. Five mesocosms received 1 triclosan dose (0.1 μg/L, 0.5 μg/L, 1 μg/L, 5 μg/L, or 10 μg/L) and 2 more were used as controls, receiving a null dose of deionized water and carrier used to prepare the triclosan stocks (see the Supplemental Data for more details on dosing logistics). A background river water sample and samples collected from the inflow and overflow of each mesocosm were analyzed weekly using a magnetic particle enzyme-linked immunoassay (ELISA) kit supplied by Abraxis and following the manufacturers' protocol precisely. The ELISA had a reported method detection limit of 0.02 μg/L. In addition, sample splits were taken 5 times and measured for triclosan with a high-performance liquid chromatograph (HPLC) equipped with a diode array detector (Varian [now Agilent]). Although it was expected that this method would be more sensitive, the calculated method detection limit was 0.1 μg/L. The ELISA method, however, is

Table 1. Variables for the triclosan study with number of measures taken at each sampling event

Variable (unit)	Method ref.	No. of measures per mesocosm per event (dosing day)								
		0	7	14	21	28	35	42	49	56
From prepared periphyton slurry										
Periphyton dry weight (mg/cm^2)[a]	[41]	5	5	5	5	5	3	6	3	6
AFDM (mg/cm^2)[a]	[41]	5	5	5	5	5	3	6	3	6
C, N, P (mg/cm^2)[b]	See text	2	2	2	2	2		3		3
C:N, N:P (ratio)[b]	Derived	2	2	2	2	2		3		3
Chla (ug/cm^2)[a]	[73]	5	5	5	5	5	3	6	3	6
Cyanobacteria (% of total algae)[c]	Derived	2	2	2	2	2	2	2	2	2
Urease activity (mg/cm^2.hr, mg/mg.hr)[c]	[49]	2		2		2		2		2
Bacteria cell density ($cell/cm^2$)[c]	[74]	2	2	2	2	2	2	2	2	2
Triclosan resistance (%)[c]	[44]	2	2	2	2	2	2	2	2	2
Diatom taxa (relative sbundance)[c]	[41]					2				2
Algal taxa (relative abundance)[c]	[41]	2	2	2	2	2	2	2	2	2
On leaf litter										
Organic matter, C, N, P decay (d^{-1})	[41]	4				4				4
Ergosterol (ug/gAFDM)	[46]	3				3				3
C:N, N:P (ratio)	Derived	4				4				4
From gravel trays										
Macroinvertebrate taxa, biomass (no., μg/tray)	See text	4		2		4		2		4
Nematode count, biomass (no., ng/tray)	[45]	2	2	2	2	2		2		2
<250 μm sediment dry wt (mg/cm^2)	[41]	2	2	2	2	2		2		2
<250 μm sediment organic matter (mg/cm^2)	[41]	2	2	2	2	2		2		2
<250 μm sediment C, N, P (mg/cm^2)	See text	2	2	2	2	2		2		2
Intergravel water										
TN, TNO_{2-3}, TNH_4, TP, TRP, TOC (μg/L)	See text	5		3		5		3		5
Inorganic N:P, ratio)	Derived	5		3		5		3		5
Over whole mesocosm										
Cyanobacteria cover (%)	See text									23
Net O_2 metabolism	[48]	7	7	7	7	7	7	7	7	7

[a] Included 3 tiles and gravel taken from 2 trays at each sampling event except on dosing days 35 and 49, when only tile was sampled, and on dosing days 42 and 56, when gravel from 3 trays was sampled.
[b] Measured on gravel-collected periphyton only.
[c] Measured on tile-collected periphyton only.
AFDM = ash-free dry mass; Chla = chlorophyll *a*; TN = total nitrogen; TNO_{2-3} = total nitrite plus nitrate; TNH_4 = total ammonium nitrogen; TP = total phosphorous; TRP = total reactive phosphorous; TOC = total organic carbon.

probably more influenced by cross-reactivity compared with the HPLC method used, so the results of each are provided (Table 2). Mean percentage recoveries of quality control samples ($n = 10$) were 91.6 ± 5.7 (standard deviation [SD]) and 90 ± 9.2 (SD) for the ELISA and HPLC methods, respectively.

Table 2. Triclosan concentrations (μg/L) measured in mesocosm inflow and overflow[a]

Nominal dose	Location	ELISA method	HPLC method
	EFLMR supply	0.03 ± 0.02	$<0.1 \pm$ —
Controls	Inflow	0.04 ± 0.02	$<0.1 \pm$ —
	Overflow	0.03 ± 0.02	$<0.1 \pm$ —
0.1	Inflow	0.17 ± 0.06	$<0.1 \pm$ —
	Overflow	0.15 ± 0.04	$<0.1 \pm$ —
0.5	Inflow	0.80 ± 0.27	0.4 ± 0.27
	Overflow	0.77 ± 0.25	$0.33^* \pm 0.23$
1.0	Inflow	1.41 ± 0.37	0.9 ± 0.12
	Overflow	1.25 ± 0.28	$0.7^* \pm 0.12$
5.0	Inflow	5.84 ± 1.52	4.9 ± 0.53
	Overflow	5.59 ± 1.21	$3.3^* \pm 0.63$
10.0	Inflow	10.64 ± 1.21	10.6 ± 1.6
	Overflow	10.32 ± 10.93	7.6 ± 3.03

[a] Data are dosing period means ± 1 standard deviation. For ELISA, $n = 7$ and for HPLC, $n = 5$ at each location sampled.
[*] Significantly different from inflow at $p < 0.05$.
ELISA = enzyme-linked immunosorbent assay; HPLC = high-performance liquid chromatography; EFLMR = East Fork of the Little Miami River.

Periphyton methods

Periphyton-specific variables were assessed from slurries produced by scraping and scrubbing the accumulated material on tiles and individual pieces of surficial gravel collected from the trays into a clean plastic container at each sampling event. Each slurry was rinsed into a graduated cylinder, and then deionized water was added to bring it to a consistent volume. It was then homogenized with a hand mixer and subsampled for bulk dry weight, ash-free dry mass (AFDM), total carbon, nitrogen, and phosphorous content, chlorophyll *a*, urease activity, bacteria cell density, triclosan-resistant bacteria, and algal taxonomy (Table 1). Bulk periphyton variables estimated from the gravel were normalized by calculating the area equivalent (cm^2) of half the weight of tin foil it took to wrap the individually scraped gravel pieces. For sample dates that included measurements for both tile and gravel periphyton, these data were first tested for no difference (via *t* test within mesocosm) and then merged before additional transformation and statistics were performed.

Microbial resistance to triclosan was determined using dilution plating according to methods described by McArthur and Tuckfield [44]. Briefly, bacteria from serially diluted periphyton slurries were placed on control plates with half-strength nutrient agar ($n = 2$) and triclosan-enriched plates (100 μg/mL; $n = 2$). To control fungal overgrowth, both sets of plates contained 100 μg/mL of cycloheximide. All plates were

incubated at room temperature (~23 °C) in darkness for 6 d. The relative frequency of triclosan-resistant bacteria was calculated as the ratio of triclosan-resistant colonies counted to control counts.

Subsamples for the identification and enumeration of soft algae and diatoms were preserved with a final concentration of 1% gluteraldehyde. All algae were identified to the lowest possible taxonomic resolution using a Palmer counting cell at a magnification of 400× and processing no fewer than 300 cells or algal colonial units for each sample. Algal community structure was assessed at the end of the colonization period and at every week of the dosing period. Diatoms were taken to species-level taxonomy on 2 dates: day 28 and day 56 of dosing. Diatom specimens were acid-rinsed, mounted, and identified at 1000× to assess relative species abundances. At the end of the dosing period a 5-cm by 5-cm gridded quadrat, 45 cm long by 30 cm length, was used to estimate the percentage of coverage of cyanobacteria mats in each mesocosm. The entire mesocosm was surveyed with the whole quadrat sampled and moved 23 times. The cyanobacteria mat coverage per quadrat was estimated as a percentage.

Gravel benthos methods

The counts and biomass densities of macroinvertebrates and nematodes (as primary members of inhabiting microinvertebrates) were estimated from retrieved gravel trays. Tray contents were gently emptied into a 2.0-mm sieve, atop a 250-μm sieve stack, which was placed over a tared 20-L bucket. Material retained on the 250-μm sieve was split using a Folsom plankton splitter. One-half of the split was passed through a 500-μm sieve, elutriated, preserved with 75% ethanol, and sorted for macroinvertebrates. Macroinvertebrates were identified mostly to family-level (some to tribe and genus), counted in-total for each gravel tray sampled, and measured with an ocular micrometer to estimate biomass density using allometric relationships obtained from the literature.

Nematodes were sampled from the contents retained in the tared bucket. This sample was collected under mixing and then filtered through a 63-μm sieve [45]. Microinvertebrates retained on the sieve were preserved with phloxine B:formalin reagent equal to the sample volume, then weighed. Nematodes were counted using a Sedgewick Rafter Counting Chamber at 100× magnification for each sample (minimum of 100 nematodes per sample).

The other half of the 250-μm fraction underwent the same gravimetric analysis as the coarse sediment fraction, as did a subsample obtained under mixing of the <250 μm material collected from the tared bucket (the fine sediment fraction). Bulk dry weight, organic content, and C, N, and P contents were measured on both fractions. Given that the <250 μm fraction constituted more than 96% of the accumulating sediments, results are provided for dosing effects on this fraction only. However, the final dry weight estimates of each of the 2 fractions were added to determine the total benthic sediment accumulation.

Leaf litter decomposition assay

Platanus occidentalis leaves had been collected in suspended catchers the fall prior to the experiment. Discs (2.5 cm diameter), cut from air-dried leaves, were added to nylon mesh bags, And the bags were buried in the gravel trays. Six bags were retrieved from each mesocosm at day zero, day 28, and day 56 of dosing. On retrieval, the remaining leaf material was removed from the bag and gently rinsed of adhering fine particulates. Two bags were used for an ergosterol analysis, an indicator of fungal biomass, with HPLC following Gessner [46]. Material from the remaining bags was dried to constant weight at 50 °C, pulverized, and subsampled for organic content, and then total P analysis [47] and C and N content via an elemental analyzer (Exeter). Decay constants were obtained from a first-order model fit to the original and ash-corrected organic C, N, and P contents, and C:N ratios were calculated for use as endpoints to determine whether triclosan affected stream detritus decomposition.

Functional indicators

In addition to the litter bag assay being an indicator of detritus processing effects and being able to quantify changes in carbon and nutrient stocks in the periphyton and gravel, net oxygen (O_2) metabolism, intergravel nutrients, and periphyton urease activity were also assessed to determine whether triclosan was affecting aspects of streambed function.

Net O_2 metabolism is an indicator of the relative balance between carbon production and mineralization. It was estimated as net daily DO consumption following the single-station procedures outlined in Bott [48]. Briefly, changes in DO due to stream photosynthesis and respiration recorded by the sensor placed near the outflow of each mesocosm were integrated over light and dark periods, separately. The dark period integral represented community respiration (CR). The average dark respiration rate was applied to the changes in DO occurring during the light period to estimate gross primary production (GPP). Water column reaeration rate, measured during preliminary gas tracer tests, was accounted for in the estimates [48]. The difference between GPP and CR was the net daily community metabolism. Negative values represented net DO consumption or net heterotrophy, and positive values represented production or net autotrophy. Daily net O_2 metabolism was averaged across each week during the dosing period and tested along with the other univariates at each time point of sampling.

Intergravel water was obtained and sampled for the same nutrient species as were measured on surface water using diffusion-based solute equilibration by burying within each gravel tray a glass vial filled with deionized water and covered with 63-μm mesh. Preliminary salt tracer tests showed that these vials equilibrated with matrix water in a matter of hours. Differences in nutrient concentration, or the relative proportion of specific nutrient species, or both served as indicators of potential dosing effects on streambed biogeochemistry. For example, an increase in the relative proportion of the ammonia to nitrite-nitrate (NH_4:NO_{2-3}) is indicative of different bed redox conditions due to changed condition of the periphyton affecting water exchange. Also, absolute differences in bed nutrient concentrations are indicative of changes in biotic driven mineralization or uptake. Furthermore, the relative contents of carbon and nutrients assessed on the periphyton and deposited fine sediment can also be indicative of potential for changes in nutrient source/sink relationships with respect to surface water.

Finally, a urease activity assay was conducted on an aliquot taken from the periphyton slurry. After a known addition of urea, ammonia was analyzed after 24 h, along with controls receiving no urea. Urease activity was inferred from ammonium production in excess of the assay control. Urease is ubiquitous in the environment and is an important regulator of nitrogen uptake [49]. Relative differences in the specific urease activity (i.e., per mg AFDM) or aerial wide activity (i.e., per cm^2 periphyton) were used as indicators of changing periphytic function with respect to nutrient cycling. Table 1 shows the endpoints, methods, and numbers of samples collected for the present study.

Statistical analysis

The general linear modeling (GLM) procedure in SAS version 2.0 (SAS Institute) was used to model effects of triclosan on all endpoints assessed except for the observed algae and macroinvertebrate taxonomy data. These were analyzed with multivariate ordination techniques (see below). Data from samples collected at weekly to monthly intervals after dosing had begun were used (Table 1). Dosing day (defined as the day of sampling after dosing began) and triclosan dose were included in the GLM as categorical fixed factors. A dosing day–by–dose interaction factor was also included.

With only a small number of mesocosms available, and a main objective of providing a community-level context for triclosan risk assessment, the regression approach was adopted to assess the chronic dose-response [50]. Dose is therefore not replicated at the mesoscale. Instead, we consider it replicated at the microhabitat scale of individual tiles and gravel trays. In analysis of variance (ANOVA), these experimental units would be considered pseudoreplicates if a strict interpretation of independence was applied, because tiles and gravel trays sampled over the dosing period are not equally likely to receive a given dose through time. We rationalize the 2-factor least squares modeling approach as appropriate if samples taken from all mesocosms are similar before dosing begins, and given that the setup is tightly controlled to minimize the chance for divergence among units by something other than the manipulated main dosing factor. To further account for this potential, however, the design included 2 mesocosms treated as control, unbalancing the design. Also to help address suspected autocorrelation with time, least squared means, homoscedasticity tests of residuals, and variance homogeneity tests were conducted. A repeated measures approach was not taken because mesocosm, or subject, is confounded with dose, and tiles and trays collected at each dosing day differ. Box–Cox models were run on each response variable using the TRANSREG software procedure to determine the best transformation to achieve normality prior to modeling the responses. The transformed data were tested using the Shapiro–Wilk statistic in the UNIVARIATE procedure of the SAS software, to confirm normality.

A 2-sided Dunnett's test was used post hoc to compare the different doses with the control when the dose effect was significant and the dosing day $\times$ dose interaction was not. If the dosing day $\times$ dose interaction was significant, then specific contrasts comparing each dose with the control for each sampling event were made, and a Scheffe adjustment was applied to the returned p values as the most conservative option. Student residuals for each GLM were tested for homoscedasticity, again via the Shapiro–Wilk normality test. Both Bartlett and Levene tests for variance homogeneity were also performed on all response models. For any modeled variable, if the tests of normality, homogeneity of variance, or homoscedasticity failed, then the variable was analyzed using the nonparametric Wilcoxon exact test according to Newman [51] for dosing effects at the final sampling time during the dosing period.

For the sampling event at the end of the colonization period, a one-way ANOVA using GLM was used to test for differences in response variables among mesocosms before dosing began. This did not prove to be the case for any variable results reported subsequently, except for O_2 metabolism in the mesocosm scheduled to receive the 5.0-μg/L dose; it was determined that this was due to a DO sensor offset problem that was not corrected until midway through the dosing period. This dose was eliminated from further testing. The statistics comparing doses with control allowed for the reporting of the lowest-observed-effect level (LOEL), which was strictly evaluated at $p < 0.05$ and is given for significant effects in Table 3.

Multivariate approaches were applied to the macroinvertebrate relative abundance and biomass variables as well as the algal taxa count data and all periphyton-related response variables, in an overall analysis [52]. Two ordination techniques were used: nonmetric multidimensional scaling (NMS) [53] and principle response curve (PRC)-redundancy analysis [54]. The latter has been recommended for inferring community-level effects from mesocosm studies [55]. The PRC analysis was conducted using the CANOCO 4.5 software program (Microcomputer Power) following the guidelines presented in ter Braak and Smilauer [56] and Leps and Smilauer [57]. The NMS techniques were implemented on the algal and macroinvertebrate community data following McCune and Mefford [58] and Peck [59] using PCORD 6.0 software (MjM Software).

RESULTS

Boundary conditions

Apart from the specific triclosan dose, water quality conditions were similar across all mesocosms, with turbidity, water temperature, specific conductivity, pH, DO, and total sediment accumulation not differing significantly among them (Figure 1). Of 22 outside precipitation events that can affect the quality of the source water to the mesocosms, 8 produced significant increases in turbidity, and all of these occurred in the colonization period (Figure 1A). Over the entire study, water temperature ranged from 20 °C to 29 °C, specific conductance averaged 400 μS/cm, and pH underwent diel fluctuations between 7.0 and 8.0 (Figure 1B, C, and D, respectively). The pH conditions would have set the neutral form of triclosan at approximately 77% of the nominal concentration [5]. Because of uncertainty associated with microscale pH conditions in the benthos, however, to be conservative this fact was not used to adjust the doses beyond that targeted. The DO did not supersaturate or fall below 5 mg/L at night (Figure 1E). Significant shifts in surface water nutrient content (Figure 1G) and N:P ratios (Figure 1H) occurred as a result of seasonally changing hydrological conditions in the natural stream supplying the mesocosms.

Triclosan dosing targets

Background concentrations of triclosan in the source water to the mesocosms were generally at the detection limits of the analytics used, as was the case for the 2 control mesocosms (Table 2). Both methods suggested some loss of triclosan while resident in the dosed mesocosms, observed by the difference between inflow and overflow for each dose; over the dosing period, the difference was significant (via t test) for the results returned by the HPLC method, but not for those returned by ELISA (Table 2). The ELISA method indicated at most 13% loss, while the HPLC results indicated greater loss, approximately 30% for the 2 highest doses. Generally, ELISA suggested an average loss of 6% and the HPLC method 23% at doses equal and above 0.5 μg/L triclosan. Overall, though, these results showed dosing targets to be well met, and given uncertainty about microscale exposure concentrations, we did not feel it rational to cite subsequent dosing effects different from the nominal concentrations targeted.

Table 3. Significance of results for fixed factors and adjusted nominal dose to control comparisons

Variable	Fixed factors *f*-test *p* values[a]			Dose w/ Dunnett test*	Scheffe adjusted contrast or Wilcoxon significances*			LOEL @ $p < 0.5$ (μg/L)
	Dosing day	Dose	Day × dose[b]		42 d[c]	49 d	56 d	
Periphyton								
Dry weight	<.0001	0.0007	0.7192	5 L	—	—	—	5.0
AFDM	<.0001	0.0001	0.3179	5 L	—	—	—	5.0
Carbon	<.0001	<.0001	0.3466	5 L	—	—	—	5.0
Nitrogen	<.0001	0.0443	0.8329	5 L	—	—	—	5.0
Phosphorus	<.0001	<.0001	0.3195	5 L	—	—	—	5.0
Chla	<.0001	<.0001	<.2993	0.1 H; 5, 10 (L)	—	—	—	0.1
Urease activity (specific)	<.0001	0.6368	0.0601	—	—	—	—	—
Urease activity/cm^2	<.0001	0.0125	0.0082	—	5 L	—	—	5.0
Cyanobacteria mats[d]	na	na	na	—	—	—	0.5, 1 (H); 5, 10 (L)	0.5
Cyanobacteria RA	0.8172	0.0331	0.0824	10 L	—	—	—	10.0
Bacteria cells	<.0001	<.0001	<.0001	—	5L	0.1, 0.5, 1 (H); 5 L	0.1, 0.5, 1 (H); 5 L	0.1
Triclosan resistance	0.0075	<.0001	0.2533	0.5 1, 5, 10 (H)	—	—	—	0.5
Leaf litter								
Organic decay	<.0001	0.0294	0.2619	0.5 H	nd	nd	—	0.5
Carbon decay	<.0001	0.0151	0.1792	0.5 H	nd	nd	—	0.5
Nitrogen decay	0.1477	0.0108	0.3694	5 L	nd	nd	—	5.0
Phosphorus decay	0.4327	0.0565	0.3052	—	nd	nd	—	—
Ergosterol	<.0001	0.2685	0.7068	—	nd	nd	—	—
C:N	<.0001	0.0002	0.0304	—	nd	nd	1, 5, 10 (H)	1.0
N:P	<.0001	<.0001	0.0172	—	nd	nd	1,10 (L)	1.0
Intergravel water								
Organic carbon	<.0001	0.0232	0.0495	—	—	—	—	—
Total nitrogen	<.0001	00433	0.9524	—	—	—	—	—
Ammonium	0.0083	0.0062	0.3068	0.5 L	—	—	—	0.5
Nitrite—nitrate[d]	na	na	na	na	na	na	—	—
Total phosphorus[d]	na	na	na	na	na	na	—	—
Reactive phosphorous[d]	na	na	na	na	na	na	—	—
Inorganic N:P[d]	na	na	na	na	na	na	—	—
NH_4:$NO_{2\text{-}3}$	0.3255	0.0008	0.4012	0.5 L	—	—	—	0.5
<250 μm deposited sediment								
Dry wt	<.0001	0.0006	0.3421	1H	—	—	—	1.0
Organic matter	<.0001	<.0001	0.2506	0.5, 1 (H)	—	—	—	0.5
Carbon	<.0001	0.0006	0.1222	1 H	—	—	—	1.0
Nitrogen	0.1578	0.7501	0.9999	—	—	—	—	—
Phosphorus	<.0001	0.0039	0.0679	1 H	—	—	—	1.0
Net O_2 metabolism	<.0001	<.0001	<.0001	—	1 H	1 H	10* L	1.0
Invertebrates								
Macroinvertebrate count	<.0001	0.1872	0.4728	—	—	—	—	—
Ostracoda count	<.0001	<.0001	0.1675	0.5, 1, 5, 10 (L)				0.5
Nematode count	0.0013	0.1485	0.2764	—	—	—	—	—

[a] Fixed factor *f*-test probabilities are given for type III sum of squares.
[b] If the interaction term was not significant, then Dunnett's results are given; otherwise Scheffe or Wilcoxon adjusted differences at specific sample times are given.
[c] No doses were significantly different from control for sample events < 42 d when dosing day × dose interaction was significant.
[d] Variable did not meet requirements of general linear modeling. Instead, a nonparametric Wilcoxon test was performed at the end of the dosing period.
* If $p \leq 0.05$ dose level significantly different from control is given with direction of response.
LOEL = lowest-observed-effect level; AFDM = ash-free dry mass; Chla = chlorophyll *a*; RA = relative abundance; L = lower than control; H = higher than control; (H) = all proceeding doses are higher than the control; (L) = all proceeding doses are lower than the control; nd = no data collected; na = not applicable.

Stock periphyton dynamics

Throughout the colonization period, the dry weight of periphyton covering the mesocosm tile and gravel substrates was similar across units. Between sampling on days 7 and 14 of dosing, the standing stock of periphyton began to rise, and this increasing trend continued across all mesocosms, irrespective of dose, until the sampling event occurring on day 35 of dosing. The temporal trend among mesocosms is exemplified in Figure 2A, which shows the experimental time series for the indicator of periphytic algal biomass, chlorophyll *a*. While differences among doses were not apparent until later in the experiment, the dosing day × dose interaction factor was not significant. The post hoc Dunnett's test suggested that the 0.1-μg/L dose was higher in chlorophyll *a* relative to the control, while the 2 highest doses, 5.0 μg/L and 10.0 μg/L, were significantly lower than the control (Table 3). A similar response pattern among doses, as shown in Figure 2B for chlorophyll *a*, was observed for total periphytic dry weight, AFDM, and periphytic nutrient contents, with the 0.1-μg/L and intermediate doses trending higher than the control and the 5.0-μg/L and 10-μg/L doses trending lower; but the only significantly different comparison was between the control and the 5.0-μg/L dose for these variables (Table 3).

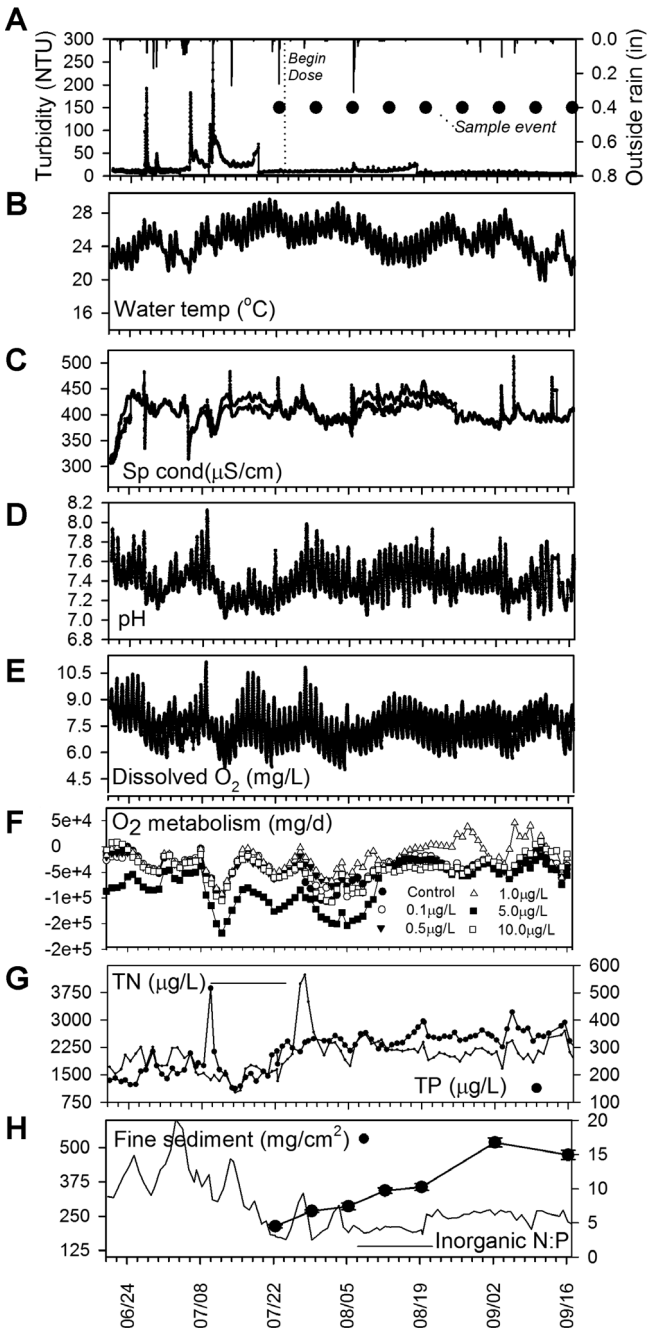

Figure 1. Experimental conditions during the triclosan study, including 15-min trends for turbidity, water temperature, specific conductance (Sp cond), pH, and dissolved oxygen (**A–E**, respectively), as well as net O_2 metabolism (**F**). Nutrients and inorganic N:P ratios are shown in (**G**) and (**H**) from the water supply to all mesocosms. Fine sediment data shown in (**H**) are grand means with 1 standard deviation across doses after pooling gravel tray units within each mesocosm. TN = total nitrogen; TP = total phosphorus.

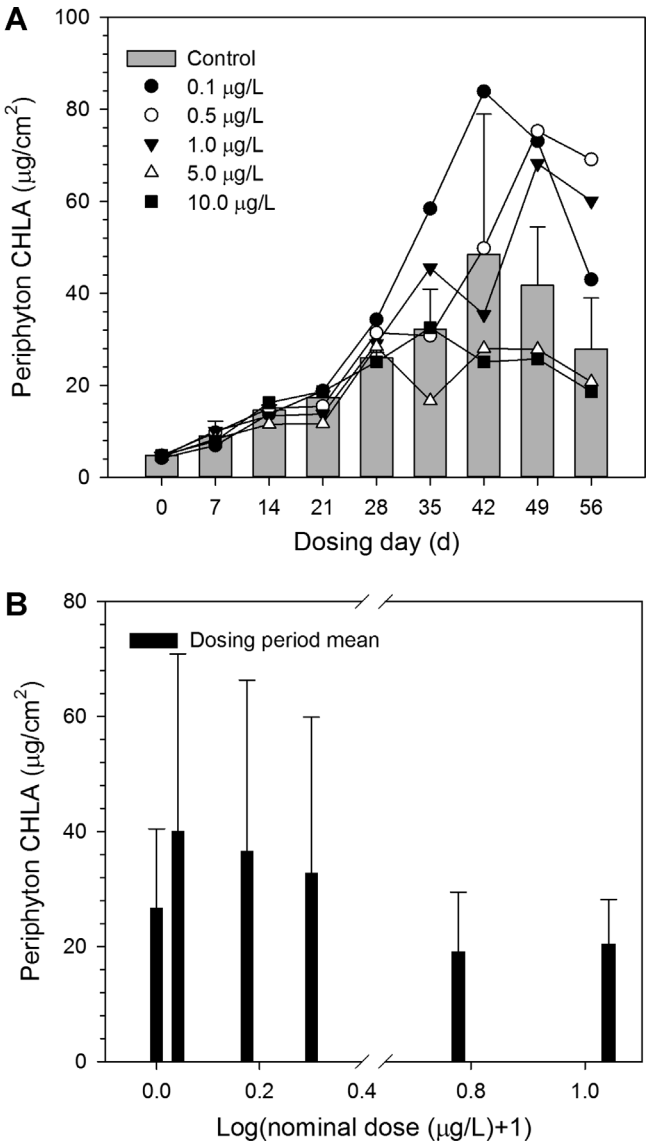

Figure 2. Experimental trends for periphyton chlorophyll *a* (CHLA). (**A**) Trends among doses for sampling events across the dosing period. Data for the controls are mean ± 1 standard deviation. (**B**) Comparison among doses, averaged across the dosing period. For sample sizes and statistical significance, see Tables 1 and 3, respectively.

Periphytic bacteria (including cyanobacteria)

There was an overall increase in bacteria cell densities over the first half of the dosing period in all mesocosms, in accordance with the stock periphyton dynamics noted above. This increase was significantly suppressed at the 2 highest triclosan doses (Figure 3 and Table 3). The suppression in bacteria cell density began after 14 d of dosing. By the time of sampling on day 28, a negative dose–response relationship was observed across doses (Figure 3B); however, none of the doses were significantly different from the control at this point. Effects of dosing became significant for the sampling event on the 42nd d of dosing. By the end of the dosing period, the 3 lowest doses were exhibiting significantly higher bacteria cell densities relative to the control, whereas the 2 highest doses were lower (Figure 3B, Table 3).

Observed cyanobacteria taxa in the periphyton included *Microcystis, Lyngbya, Merismopedia, Oscillatoria*, an unidentified mat-forming species, *Anabaena, Chamaesiphon*, and *Spirulina* (Table 4). The relative abundance of cyanobacteria among all periphytic algae was significantly lower in the highest triclosan dose compared with the control (Table 3), and their tendency to form mats noticeable to the naked eye in the mesocosm beds also differed in a trend reflective of the one for bacteria cell density by the end of the dosing period. Based on the survey of cyanobacteria mat coverage at the end of the dosing period, the 0.5-µg/L and 1.0-µg/L doses had significantly higher coverage, and the 2 highest doses had significantly lower coverage than the control (Table 3). The counts of *Lyngbya,*

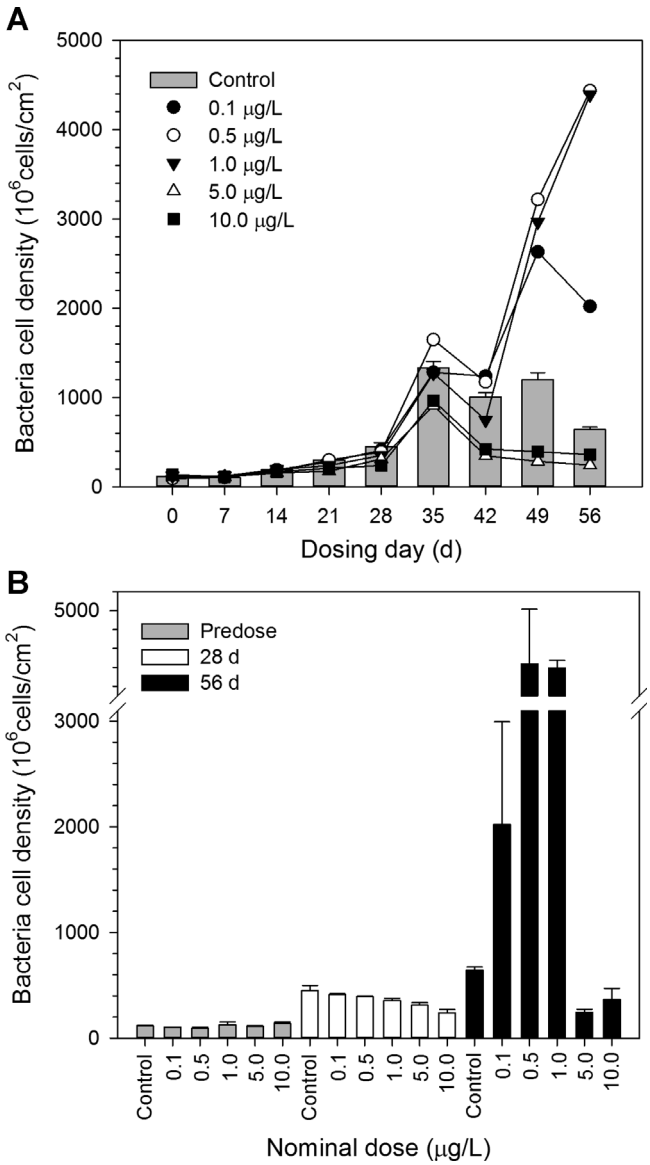

Figure 3. Experimental trends for periphyton bacteria cell density. (**A**) Trends among doses for sampling events across the dosing period. Data for the controls are mean ± 1 standard deviation. (**B**) Comparison among doses, for dosing day 0 (predose), day 28, and day 56, as the dose × dosing day interaction was significant. For sample sizes and statistical significance among doses, see Tables 1 and 3, respectively.

Table 4. Periphyton algae encountered in the 300 taxa counts and correlations with nonmetric multidimensional scaling (NMS) ordination axes

Taxa[a]	Group	Pearson's r[b] axis 1
Melosira	Diatom	0.935
Navicula	Diatom	−0.867
Pleurosira laevis	Diatom	0.677
Cocconeis	Diatom	−0.910
Nitzschia	Diatom	−0.801
Synedra	Diatom	0.701
Cyclotella	Diatom	−0.875
Microcystis	Cyanobacteria	0.159
Thalassiosira weissflogii	Diatom	−0.761
Scenedesmus	Green	0.340
Lyngbya	Cyanobacteria	0.323
Merismopedia	Cyanobacteria	0.215
Gyrosigma	Diatom	−0.198
Oscillatoria/Phormidium	Cyanobacteria	0.465
Cyanobacteria mat sp.	Cyanobacteria	−0.236
Surirella	Diatom	−0.130
Caloneis	Diatom	−0.218
Bacillaria paradoxa	Diatom	0.201
Fragilaria	Diatom	0.126
Gomphonema	Diatom	0.334
Crucigenia quadrata	Green	0.133
Cosmarium	Green	−0.081
Achnanthes	Diatom	−0.149
Cymbella affinis	Diatom	0.093
Anabaena	Cyanobacteria	0.097
Amphora	Diatom	−0.061
Cymatopleura solea	Diatom	0.420
Pediastrum bi	Green	−0.065
Phacus	Green	0.147
Rhoicosphenia curvata	Diatom	0.334
Ankistrodesmus	Green	0.339
Meridion	Diatom	0.169
Chamaesiphon incrustans	Cyanobacteria	−0.521
Chlorella	Green	−0.307
Closterium	Green	−0.050
Euglena	Green	0.028
Pinnularia	Diatom	0.254
Diatoma	Diatom	−0.050
Spirulina	Cyanobacteria	0.278
Eutreptia	Green	0.224
Cladophora	Green	0.134
Amphipleura pellucida	Diatom	0.163
Micrasterias	Green	0.090
Spirogyra	Green	0.068

[a] The taxa list is ordered in terms of rank abundance.
[b] Pearson's r correlation coefficients for taxa-specific ordination and primary NMS axis.

Oscillatoria, and the mat-forming species were responsible for the increase in the intermediate doses of 0.5 µg/L and 1.0 µg/L.

Bacterial resistance to triclosan exposure was evident by the sampling event on day 14 of dosing (Figure 4A), and generally increased with dose and length of exposure. Overall, the interaction term was not significant, and triclosan resistance was significantly higher compared with the control in all doses 0.5 µg/L and above (Table 3, Figure 4B).

Other periphytic autotrophs

The relative abundance of 44 unique algal taxa, including cyanobacteria, was identified as part of the periphyton community assessment (Table 4). Diatom cells accounted for 95% of the periphytic algae counted. The most prevalent genera were *Melosira* (51%), *Navicula* (12%), and *Pleurosira* (9%) (Table 4). Green algae accounted for only 1% of the community, and cyanobacteria comprised 4% of total cells counted.

A PRC analysis used to model the periphytic algal community response in relation to the dosing day by triclosan dose interaction did not identify a significant response. The NMS method was also used to determine whether triclosan dose was influencing algal community structure. A 2-dimensional solution was specified after arcsine square root transformation (final stress = 8.8, distance measure = Sorensen [Bray–Curtis]) with the first axis explaining 92% of the variation in the ordination and highly correlated with dosing day ($r^2 = 0.73$). The 2nd axis only explained an additional 0.05% of the variation and was poorly correlated with dose ($r^2 = 0.003$). Pearson's correlations among taxa and axis 1 (dosing day) given in Table 4 suggested that *Melosira, Pleurosira*, and *Synedra* abundances increased with dosing day, while *Navicula*, *Cocconeis*, *Nitzchia*, *Cyclotella*, and *Thalassiosira* abundances decreased over the dosing period.

Because diatom taxa dominated the algal community, they were identified separately in samples to the lowest possible

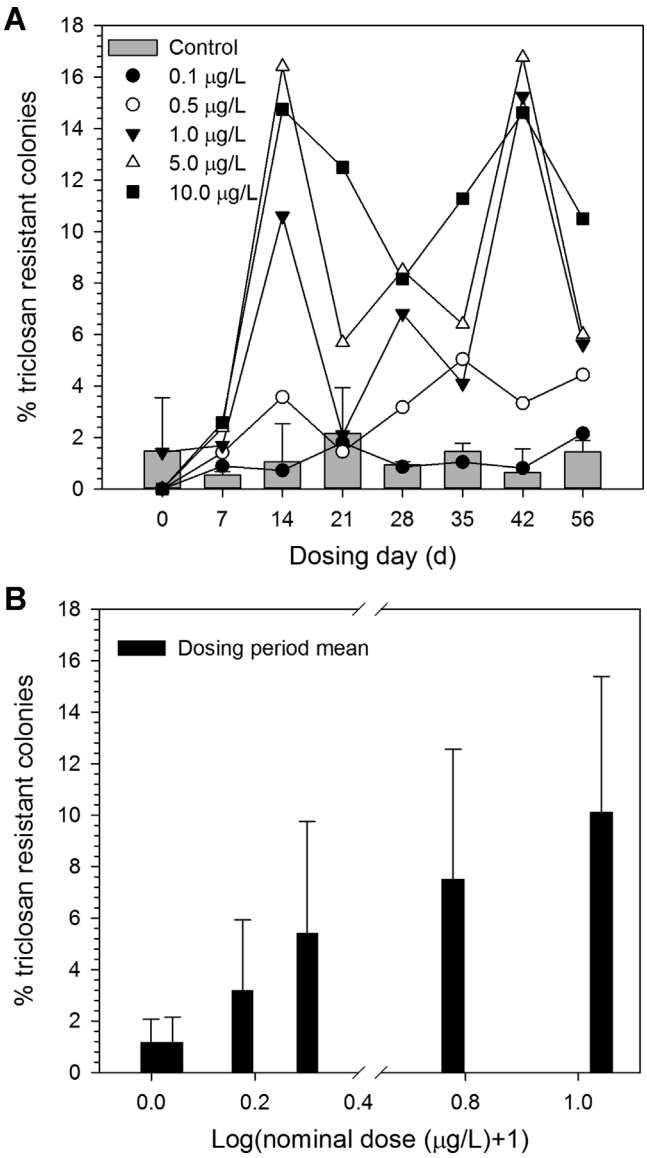

Figure 4. Experimental trends for triclosan resistance in periphyton bacteria. (**A**) Trends among doses for sampling events across the dosing period. Data for the controls are mean ± 1 standard deviation. (**B**) Comparison among doses, averaged across the dosing period. For sample sizes and statistical significance among doses, see Tables 1 and 3, respectively.

taxonomic resolution after 28 d and 56 d of dosing. (The Supplemental Data Table S1 contains the diatom taxa list and correlations with the main NMS axes.) The diatom relative abundance data were analyzed with NMS after applying an arcsine square root transformation. A 2-dimensional solution was specified (final stress = 8.75, distance measure = Sorensen [Bray–Curtis]), and time correlated well ($r^2 = 0.87$) with the first axis, whereas dose correlated moderately with the second axis ($r^2 = 0.55$). The second axis explained 35% of the variation in the ordination. In all, 109 unique taxa (mostly identified to species level) were observed. *Melosira varians*, a chain-forming species, was more abundant in the higher triclosan doses by the end of the dosing period (Pearson's $r = -0.876$). *Navicula capitatoradiata* and *Tryblionella levidensis*, other community dominants, had lower relative abundance at the higher doses (Pearson's $r = 0.901$ and 0.920, respectively), and these are unicellular species (Supplemental Data Table S1.).

Time by dose response of periphyton

The stock periphyton variables were merged with bacterial cell density and the 300 count-algal taxonomy data, and the PRC analysis was rerun. In this case, the total periphyton response in relation to the dosing day × dose interactions indicated a significant response in the first canonical axis of the redundancy analysis (RDA)-based ordination (eigenvalue = 0.066, f ratio = 14.135, $p = 0.0020$). The PRC for the ordination results indicated a significant departure from the position of the controls in the later part of the dosing period, being driven by change in stock periphyton endpoints, bacterial cell density, and *Lyngbya* and *Synedra* relative abundances at the intermediate doses, 0.5 μg/L and 1.0 μg/L (Figure 5). The relative abundances of *Nitzchia*, *Navicula*, and *Pleurosira* diatoms were driving the ordination of the 2 highest doses relative to the controls. Based on this PRC, the LOEL for the periphyton community would be 0.5 μg/L.

Consumers

Nematodes followed the same general pattern (data not shown) among doses as did the periphytic AFDM, chlorophyll *a*, and bacteria cell density, with slightly higher densities found in the mid-doses and lower ones in the 2 higher doses relative to the controls, but the dose effect was not significant (Table 3). There were 33 unique macroinvertebrate taxa observed (Table 5).). The most abundant in terms of biomass were Ferrisid snails, small *Corbicula* clams, and naidid worms (20%, 17%, and 26% of taxa counts, respectively). Insect taxa comprised 20% of the macroinvertebrates and were mostly dominated by chironomids (midges). Ephemeroptera, Plecoptera, and Trichoptera taxa only accounted for 6% of the mesocosm insects. The NMS-based ordinations for both macroinvertebrate counts and biomass converged on 2-dimensional solutions. Dosing day was a significant correlate with the primary NMS axis ($r^2 = 0.96$ and 0.92 for count and biomass measurements, respectively). Dose was not a strong correlate with the second NMS axis in either case of the taxa count ($r^2 = 0.13$) or biomass ($r^2 = 0.19$) ordinations. Similar nonsignificance was the result of the PRC analysis of the macroinvertebrate community, as well as the GLM applied to the total macroinvertebrates counted (Table 3). Ostracoda was the only macroinvertebrate taxa well correlated with the second NMS axis derived from the count data ($r^2 = -0.773$; Table 5), and the post hoc Dunnett's test for this taxa's density suggested that all doses of 0.5 μg/L and above had significantly lower densities compared with the control.

Detritus processing

Potential effects of triclosan on stream detritus processing were inferred from relative differences in the decay rates of carbon and nutrient species from leaf litter (Table 3, Figure 6). Comparison among doses with respect to organic matter and carbon decay were not reflective of a negative dose response (Figure 6A). The trend in ergosterol content (Figure 6B), the indicator of fungal biomass, ostensibly degrading the litter, tended toward a negative dose response with a lower measurement after 28 d and 56 d of dosing relative to the controls. However, dose was not a significant effect in the GLM (Table 3). By day 56 of dosing, the ergosterol content of the litter had nearly doubled compared with that sampled previously. Evidence of a potential triclosan effect on detritus decomposition was provided by the significant increase in litter C:N ratio in doses of 1.0 μg/L and above after 56 d of dosing (Figure 6C, Table 3).

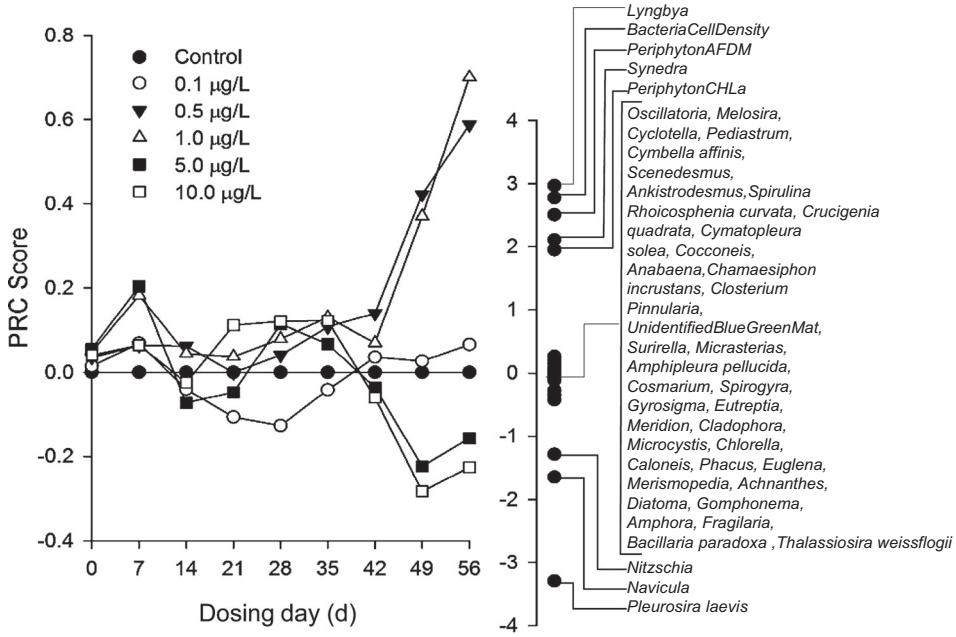

Figure 5. Principle response curve (PRC) for the redundancy analysis (RDA) ordination of dosing day by dose covariates for all periphyton variables. See text for RDA statistics.

Table 5. Macroinvertebrates inhabiting gravel trays and correlations with nonmetric multidimensional scaling (NMS) ordination axes

Taxa[a]	Group	Pearson's r[b] axis 1	Pearson's r axis 2
Ferrissia	Gastropod	0.457	−0.162
Corbiculidae/Sphaeriidae	Bivalve	0.821	−0.2
Naididae/Tubificidae	Oligochaete	−0.337	−0.458
Tanytarsini	Insect	−0.782	−0.294
Ostracoda	Crustacean	0.121	−0.773
Prostoma	Nemertean	0.847	−0.29
Elmidae	Insect	0.071	−0.607
Chironomidae	Insect	−0.95	−0.005
Nematoda	Nematode	0.14	−0.467
Orthocladiinae	Insect	−0.944	0.11
Planorbidae	Gastropod	0.479	−0.602
Copepoda	Crustacean	−0.472	−0.291
Hydroptila	Insect	−0.191	−0.677
Turbellaria	Worm	0.804	−0.333
Heptageniidae	Insect	−0.839	0.131
Tanypodinae	Insect	−0.8	0.13
Tricorythodes	Insect	−0.584	−0.302
Caenidae	Insect	−0.644	−0.119
Elmidae, adult	Insect	0.116	−0.505
Stenonema	Insect	−0.555	−0.151
Chimarra	Insect	−0.63	−0.282
Baetidae	Insect	−0.607	−0.128
Ephemeroptera	Insect	−0.338	−0.043
Coenagrionidae	Insect	−0.028	−0.116
Ceratopogonidae	Insect	0.027	−0.068
Cheumatopsyche	Insect	−0.536	0.226
Pleuroceridae	Gastropod	0.462	0.627
Simuliidae	Insect	−0.535	0.045
Hydropsychidae	Insect	0.101	−0.517
Polycentropodidae	Insect	0.335	−0.214
Lymnaeidae	Gastropod	−0.028	−0.268
Perlidae	Insect	−0.334	−0.119
Leptoceridae	Insect	−0.274	−0.045

[a] The taxa list is ordered in terms of rank abundance.
[b] Pearson's r correlation coefficients for taxa-specific ordination and primary and secondary NMS axes.

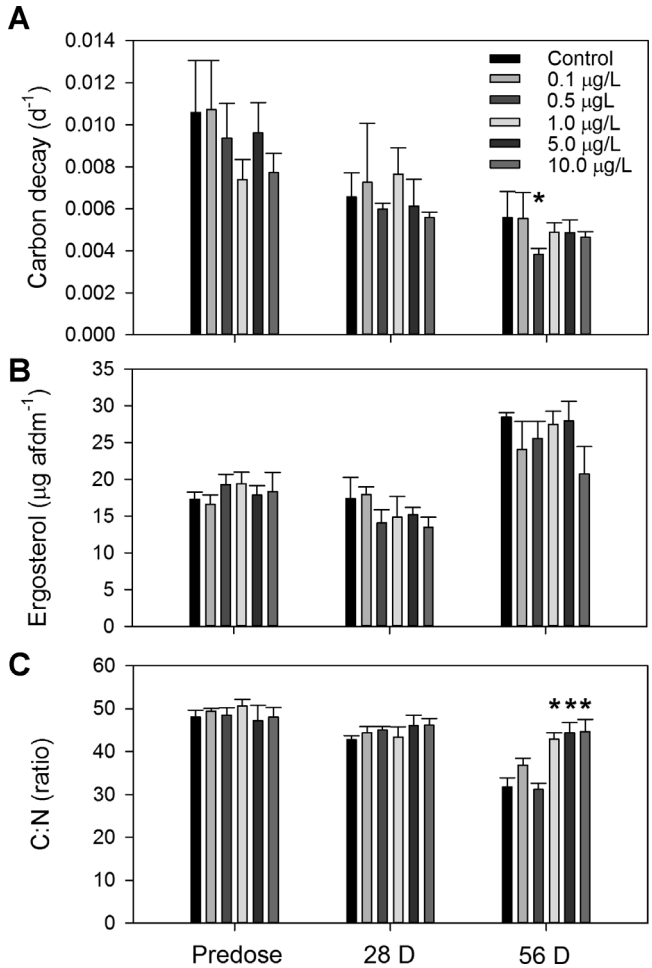

Figure 6. Leaf litter bag results. (**A**) Litter carbon decay rate trends among doses at each sampling event. (**B**) Ergosterol trends. (**C**) C:N ratios for decayed leaf litter. Data are means + 1 standard deviaition. * $p < 0.05$ (see Table 3).

Net metabolism and nutrient processing indicators

The mesocosms were net heterotrophic overall, with net daily O_2 metabolism only rarely being positive (Figure 1F). The O_2 metabolism rates separated slightly during the first half of the dosing period, but then converged again in the later half. The 1.0-μg/L dose was more autotrophic than the rest for most of this period (Figure 1f, Table 3). There was not a clear linkage between the general periphyton trend with the dose noted above and the trends in net O_2 metabolism, although the 10-μg/L dose was exhibiting greater heterotrophy than the control by the end of dosing, and the significant relative autotrophy of the 1.0-μg/L dose coincided with relative differences in periphytic algal biomass between these doses (Table 3).

The mesocosm beds with the most periphyton (0.5-μg/L and 1.0-μg/L doses) also tended to have higher contents of carbon or organic matter and phosphorus in deposited sediment (Table 3). However, any difference in embeddedness, presumably caused by differences in periphytic stock, did not translate to significant difference in intergravel nutrient availability. Only in the 0.5-μg/L dose was a significant difference observed in the concentrations and relative distribution of ammonia and nitrite-nitrate species (Table 3). The effect of triclosan on urease activity was only significant for the 5-μg/L dose sampled on day 42 of dosing. Triclosan did not affect periphyton-specific urease activity.

DISCUSSION

The results from the present study demonstrate the effects of triclosan on several properties of stream ecosystems that until now have largely been speculative and based on effects of the biocide on single organisms or single-system compartments (e.g., attached biofilm). Of prime speculation has been the potential for cascading ecological consequences for stream communities, given demonstrated biofilm-specific effects [22,27,29,31]. Our results equally demonstrated changes in the biofilm community, in terms of bacterial cell density, algal biomass, and algal structure (decreased cyanobacteria densities, potential change in diatom species). However, the responses were not linear over the course of the experiment or across the triclosan dosing gradient. Neither the significant increase nor decrease in periphyton-specific endpoints observed appeared great enough to cause similar responses in the indicators of community-scale effects or among the interacting components of stream ecosystems that were simultaneously considered. That is, although nematode densities trended with the periphytic stocks, the only discernible effect in the benthic invertebrate community was the negative dose-response of Ostracoda densities. The macroinvertebrate community as a whole was weakly and nonsignificantly driven by dose. Hence, the concern about an ecological cascade resulting from the effects of triclosan on periphytic structure as a primary resource supporting macroinvertebrates appears unwarranted.

For the nematodes, their density trended, though not significantly, with the periphyton stock, which they rely on for both food and habitat [60]. Lawrence et al. [22] reported a direct effect of triclosan as a LOEC of 0.5 μg/L for *Rotifera* species exposed in microtiter plate culture; they did not detect a response in this or other members of the microinvertebrates inhabiting their flow-through reactor biofilm communities that were dosed with 10 μg/L triclosan. Nor do we think the trend in nematode density was directly related to triclosan, but rather to resource availability.

Ostracods also rely on the periphyton for sustenance, but their abundance appeared to be significantly and directly affected by triclosan dose. These crustaceans are related to others of their kind that have been tested using traditional toxicological approaches, such as *Hyallela azteca*, *Ceriodaphnia dubia*, and *Daphnia magna*, all of which have published no-observed-effect concentrations (NOECs) well above those tested in the present study [18,61,62]. The lowest NOEC reported for an invertebrate crustacean previously was 4 μg/L [5]. The LOEL for the ostracod density was 0.5 μg/L, and if directly related to triclosan toxicity, then the mode of action is unknown and uncharacteristic of sensitivities observed from standard toxicological tests.

Extremes in periphyton stock did appear to mildly translate to potential effects on net O_2 metabolism, but in-common effects on intergravel carbon and nutrient chemistry were not consistent across the dose gradient. Hence, the nutrient chemistry results suggested that the observed changes in periphyton stock and structure were not extensive enough to cause differences in streambed nutrient chemistries, which can be mediated by periphyton through regulation of surface water–streambed water and solute exchange.

Furthermore, a dramatic change in detritus processing was not observed based on results from the litter bags. We can infer, however, that the microbial community controlling litter carbon turnover was affected at triclosan doses of 1 μg/L and higher. Fungal biomass increased and C:N ratios decreased at the lower doses between 28 d and 56 d of dosing, which is consistent with the notion that a litter leached of nitrogen needs to immobilize a fraction as microbial biomass builds [25,63]. Collectively, the endpoints assessed in the litter decomposition assay point to a potential negative effect on osmotrophic biomass and a decrease in litter nutritional quality with higher net nitrogen losses at the higher triclosan doses (1.0 μg/L, 5.0 μg/L, and 10 μg/L). This suggests that triclosan could affect the quality of a primary consumer food resource in stream communities at a moderate level of chronic dosing. A decrease in detrital quality in streams could also have significant consequences for food webs and nutrient processing.

The effects observed in the periphytic algal community were not reflective of an assumed negative monotonic response among its members with varying degrees of sensitivity as is suggested by triclosan risk assessments [5,12]. The cyanobacteria component of periphyton, along with unicellular diatoms, accounts for most of the autotrophy in small streams with closed canopies [60]. Cyanobacteria were significantly affected by the dosed triclosan, stimulated in the intermediate doses tested, while being mostly absent at the 2 highest doses. This contrasts to the individual species sensitivities showing that the same genera of cyanobacteria (i.e., *Lyngbya* and *Oscillatoria*) found in abundance in the mesocosms are negatively impacted at 1.0 μg/L [5]. Studies suggesting that other periphytic autotrophs can be quite sensitive to triclosan [28,30,31] were also not reflected here. Moderate correlation between the ordination of diatom species and the triclosan dose gradient was observed, but we suggest that this was related more to the loss of cyanobacteria at the higher doses rather than a direct toxic affect. Finally, although certain green algae in the present study appear quite sensitive to triclosan—even more so than some cyanobacteria [5], green algae represented such a small fraction of the total algal community that triclosan affects could not be inferred. A low abundance of green algae is typical for small receiving streams with intact riparian zones. The closed canopies create light-limiting conditions favoring diatoms over green algae [64].

Triclosan significantly altered the structure and density of the periphytic autotrophs, and this was largely the result of a direct influence on the relative abundance of cyanobacteria. Triclosan species sensitivity distributions (SSDs) only speak to negative effects, mostly on single species [5,12]. We note stimulation at a concentration lower than the cyanobacteria-species threshold of 1.0 μg/L in the SSD compiled by Lyndall et al. [5].

The widespread use of triclosan as a biocidal agent likely imparts a selective pressure in microbial communities favoring mechanisms of resistance that are in common with antibiotics. The demonstration of horizontal gene transfer and mobile genetic elements makes the risk to human and environmental health a concern [32]. McBain et al. [65] used sink drain microcosms to show that triclosan, through innate resistance or insusceptibility, altered community composition but did not decrease the susceptibility of the community to antibiotics. Svenningsen et al. [66], using soil drain-field models, similarly noted a presumed innate community shift among soil bacteria to more resistant varieties that were less effective at degrading xenobiotics from a simulated sanitary sewage. The conditions created in the present study represent a more distant endmember in a continuum of triclosan-exposed microbial communities from the point of domestic water use through wastewater treatment systems and post discharge to the receiving water environment.

In the present model, receiving stream environment resistance to triclosan significantly increased in periphyton communities exposed to all doses of 0.5 μg/L and higher (Figure 4). Resistance generally increased with increasing level of triclosan and length of exposure, suggesting an overall adaptive response. Within a bacterial biofilm, triclosan induced an upregulation of a gene encoding for the main efflux pump in gram-negative bacteria, the major regulator of a multidrug resistance controlling genetic cascade, and cellulose synthesis [67]. This translates to altered membrane permeability, increased efflux, and more exopolysaccharide production. The change last mentioned could increase the standing stock of periphyton in the system.

The alterations in cell membranes and signaling for exopolysaccharide production triggered by low levels of triclosan are similar to the notion that low concentrations of antibiotic-like compounds in the environment act as chemical cues for biofilm development [68]. In the clinical setting, typically planktonic bacteria have long been known to respond to antimicrobial agents by inducing biofilm growth [69]. This signaling in response to the sub-biocidal concentrations may help to explain why the periphyton, as a whole, exhibited a stimulatory response to the lowest doses of triclosan. Cyanobacteria are gram-negative bacteria similar to medically relevant human pathogens and other groups known to have innate resistant responses to sub-biocidal exposure conditions. Despite a variable and fluctuating growth and development pattern typical of the transient stream environment, triclosan resistance established quickly in the mesocosms, was largely maintained, and trended upward with the period of exposure, suggesting the potential for a consistent reservoir of genes encoding for resistance to antibiotics. Some of these may be transferred to microbial pathogens of aquatic vertebrates (such as fish species) that rely on antimicrobial agents in secretory material to maintain health [70–72].

CONCLUSIONS

Triclosan caused change in at least 1 measurable structural aspect of a complete stream periphyton community at all doses tested. In contrast to species-specific sensitivities, the responses at 1 μg/L (nominal) and lower of those doses targeted appeared to be generally stimulatory for periphytic endpoints in this chronic exposure condition. At the higher concentrations tested, 5 μg/L and 10 μg/L. the effect on periphyton was negative. The stimulatory response at environmentally relevant doses of triclosan probably occurs thru innate, but also potentially acquired, mechanisms of resistance responding to sub-biocidal concentrations. The measured changes in the periphyton were not reflected in the same manner in the macroinvertebrate community, the net O_2 metabolism, or the nutrient source or sink strengths and species distributions in the streambed matrices. However, a potential affect on detritus food quality that has not been considered previously was observed. We suggest that this, coupled with the selective pressure for resistance that triclosan may place on the stream microbial community, should be considered in risk assessments that traditionally focus on direct toxicological sensitivity. The effects measured for triclosan in this community-level context are within the range reported for streams currently permitted to receive treated wastewater effluents.

SUPPLEMENTAL DATA

Supplemental Methods, Supplemental Results, Supplemental Discussion, and Supplemental References.

Table S1. (42 KB DOCX).

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