

PERIPHYTIC CHLOROPHYLL-A RESPONSE TO TRICLOSAN EXPOSURE: APPLICATION OF A PASSIVE DIFFUSION PERIPHYTOMETER

K. L. White, B. E. Haggard, M. D. Matlock, J.-W. Kim

ABSTRACT. *In situ* assessments of chemical toxicity in streams may be accomplished using natural periphytic communities when allowed to colonize an artificial substrate. The proliferation of Triclosan (TCS) in consumer products has resulted in its presence in wastewater influent, effluent, and subsequently in streams. In this study, the two objectives were: 1) assess the utility of a passive-diffusion periphytometer in toxicity tests, and 2) evaluate the growth-inhibiting effects of TCS on periphytic algae at the White River, Northwest Arkansas. The periphytometer was deployed for one week with seven replicates of nine treatments, including control (deionized H₂O), methanol, low TCS (50 µg L⁻¹), medium TCS (100 µg L⁻¹), high TCS (500 µg L⁻¹), nutrients (2000 µg PO₄-P L⁻¹ and 20000 µg NO₃-N L⁻¹), low TCS with nutrients, medium TCS with nutrients, and high TCS with nutrients. Relatively low stream nutrient concentrations were observed; maximum nitrate-nitrogen (NO₃-N), total N, ammonium-nitrogen (NH₄N-N), total organic carbon (TOC), and soluble reactive phosphorus (SRP) concentrations during the deployment were 230, 603, <50, 12500, and 15 µg L⁻¹, respectively. The Student-Newman-Kuels test ($\alpha = 0.05$) identified three significantly different groups within the treatments. The nutrients and low TCS with nutrients treatments had chlorophyll-a means of 10.9 and 5.8 mg m⁻², respectively, which were significantly different from each other and all other treatments. Chlorophyll-a content means of the remaining treatments ranged from 1.8 to 3.5 mg m⁻² and were not significantly different from each other. Exponential regression of chlorophyll-a contents in nutrient and TCS with nutrient treatments against TCS concentration produced a significant decreasing trend; however, no trend in chlorophyll-a content was observed in treatments without nutrients. The observed inhibition of periphytic algal growth in the treatments with additional NO₃ and SRP suggests that the mechanism of TCS may be more complex than postulated. Periphytic response to TCS exposure was quantified using an innovative, *in situ* approach that warrants additional investigation.

Keywords. Antimicrobial agents, Chlorophyll-a, Triclosan, Periphytometer.

Chemical toxicity in aquatic systems is often tested using laboratory techniques following appropriate toxicological methods. Target organisms used in toxicological assessments should represent populations in target aquatic systems; this is often difficult to achieve without use of model aquatic systems, such as periphyton from natural substrate in artificial streams (Rodgers et al., 1996). Use of natural periphytic communities in streams for toxicity tests provides insights into shifts in community structure and growth rate responses to chemical or physical stimuli. A passive diffusion periphytometer (Matlock et al., 1998) was developed for quantifying *in situ* periphytic growth response to nutrients. It is possible to evaluate *in situ* chemical toxicity in streams or other aquatic systems

using this passive diffusion periphytometer. We used this passive diffusion periphytometer to evaluate growth-limiting effects of a widely used antimicrobial agent, Triclosan, on periphytic algae colonizing an artificial substrate.

Triclosan (TCS) [also known as Irgasan DP 300, Irgacare MP, and 5-chloro-2-(2,4-dichlorophenoxy)phenol] is an antimicrobial agent commonly used in deodorants, soaps, plastics, and many other products (Tixier et al., 2002). The value of TCS as a disinfectant is its acute toxicity; the postulated mechanism of toxicity is apoptosis (Zuckerbraun et al., 1998). This compound has been widely used because of intended effects on bacteria on (adult) human skin and oral cavities.

Widespread consumer use of products containing TCS has resulted in the introduction of it to municipal wastewater treatment plant (WWTP) influents (Lindstrom et al., 2002), WWTP effluents (Tixier et al., 2002), and streams of the continental United States (Kolpin et al., 2002). A daily TCS load of 5 g per 10,000 human units is estimated to be in WWTP influent (derived from Swiss wastewater influent analyses; Lindstrom et al., 2002). As TCS travels through typical WWTP processes, approximately 85% is removed via biodegradation and biotransformation (Federle et al., 2001; McAvoy et al., 2002; Tixier et al., 2002). High TCS concentrations and loads may reduce efficiencies of WWTP removal of target constituents (Federle et al., 2001), increasing concentrations discharged to streams or other receiving water bodies.

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Triclosan is subject to additional removal or degradation in natural waters because TCS ionizes when the pH is greater than the pKa of 8.1 (Tixier et al., 2002). Orvos et al. (2002) showed that TCS ionizes into a readily photodegradable form in high pH streams and becomes less toxic to aquatic communities. However, Latch et al. (2003) reported that direct photolysis of TCS under normal stream conditions can produce a form of dioxin, one of the most active carcinogenic chemicals known to exist. In spite of the potential for additional degradation in natural waters after passing through WWTPs, TCS has been found in streams across the United States with concentrations as great as $2.3 \mu\text{g L}^{-1}$ and a median of $0.14 \mu\text{g L}^{-1}$ (Kolpin et al., 2002). The magnitude of these concentrations may not be of immediate concern because the effect of TCS on algae is most often observed when concentrations are greater than the lowest observed-effect concentration (LOEC) of $1.2 \mu\text{g L}^{-1}$ (Orvos et al., 2002). The EC₅₀ (effective concentration) of TCS in laboratory algal assays has been reported to be $1.5 \mu\text{g L}^{-1}$ (Ciba, 1998). Several streams in the recent nationwide assessment (Kolpin et al., 2002) have TCS concentrations exceeding the LOEC and EC₅₀, and the potential impact on algal communities may need to be evaluated in these streams. Furthermore, TCS concentrations of $2.7 \mu\text{g L}^{-1}$ have been observed in WWTP influent (McAvoy et al., 2002).

Naturally high numbers of species, rapid response times, ease of sampling and analysis, and sensitivity to environmental changes make natural stream assemblages of periphyton ideal for toxicity testing (Rodgers et al., 1979). Because periphyton are a commonly used biological indicator for testing toxicity and algae are affected by TCS, we used a passive diffusion periphytometer to evaluate the periphytic chlorophyll-a response to TCS exposure at the West Fork of the White River, Northwest Arkansas. The objectives of this study were two-fold: 1) assess the use of a passive diffusion periphytometer in toxicity tests in streams, and 2) evaluate the effect of TCS on periphytic growth on an artificial substrate *in situ*. Historically, periphytometers had been used to assess nutrient limitation (Matlock et al., 1998; 1999), and this study was an initial deployment of a periphytometer in a toxicity test.

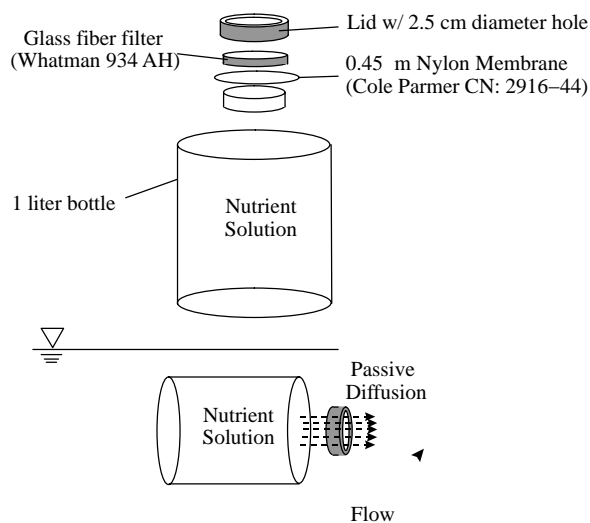
STUDY SITE AND METHODS

The overall objective of this study was to assess the use of a passive diffusion periphytometer in a toxicity test of TCS in an Ozark stream. A passive diffusion periphytometer (or Matlock Periphytometer) was deployed in a northwest Arkansas stream not heavily impacted by WWTP effluent discharge, hence not likely containing measurable amounts of TCS. The stream selected was the West Fork of the White River (fig. 1A) which has a catchment land cover characterized mostly by forests and pastures; however, there is an increasing trend in urbanization (King, 2001). The stream is characterized by riffle-pool morphology typical of Ozark streams in the region where the periphytometer was secured in an unshaded pool, slightly upstream of a riffle in October 2002.

Nine treatments with seven replicates were established in a split plot, randomized block design (table 1). Triclosan was dissolved in a methanol and H₂O solution, and less than 5 mL of the TCS solution in a methanol and H₂O matrix was added



(A)



(B)



(C)

Figure 1. (A) Study site (Lat. $36^{\circ}03'00''$, Long. $94^{\circ}04'42''$) where the passive diffusion periphytometer was deployed at the West Fork of the White River, Northwest Arkansas. [The study site is near U.S. Geological Survey stream gage no. 07048550 West Fork White River East of Fayetteville, Ark.] (B) Schematic diagram of the passive diffusion periphytometer bottles. (C) Picture of the periphytometer treatment array during deployment at the West Fork of the White River in October 2002.

to each bottle that was filled with either deionized H₂O or a nutrient solution of 2000- μg phosphate-phosphorus (PO₄-P)

Table 1. Treatments for evaluating an *in situ* toxicity test of Triclosan on periphytic algae using a passive diffusion periphytometer at the West Fork of the White River, Northwest Arkansas, during October 2002.

Treatment	Symbol	Bottle Solutions
Control	C	Deionized H ₂ O
Methanol	M	<2% methanol in deionized H ₂ O
Low TCS	LT	50 µg TCS L ⁻¹
Medium TCS	MT	100 µg TCS L ⁻¹
High TCS	HT	500 µg TCS L ⁻¹
Nutrients	NU	2000 µg PO ₄ -P L ⁻¹ and 20000 µg NO ₃ -N L ⁻¹
Low TCS plus nutrients	LTN	LT plus NU
Medium TCS plus nutrients	MTN	MT plus NU
High TCS plus nutrients	HTN	HT plus NU

L⁻¹ and 20000 µg nitrate–nitrogen (NO₃-N) L⁻¹ (~300 mL). A methanol treatment was included in the experimental design to determine if the H₂O and methanol matrix had any effect on periphytic algal growth. Nutrients were also included in the experimental design to insure accelerated periphytic growth rate by removing any potential nutrient limitation of ambient stream concentrations. The periphytometer uses the principles of diffusion to expose periphytic algae colonizing the artificial substrate to additional nutrients, and in this application TCS. This creates a non-uniform exposure concentration over time, with the greatest exposure concentrations occurring early in the deployment period and reduced exposure over time. Historical deployments of this type of periphytometer had measurable amounts of nutrients remaining in the bottles after a 14-d deployment (Matlock et al., 1998).

Periphytometer bottles (Nalgene HDPE 250 mL) were filled with respective treatment solutions of control (deionized H₂O, C), methanol and deionized H₂O (M), low TCS (50 µg TCS L⁻¹, LT), medium TCS (100 µg TCS L⁻¹, MT), high TCS (500 µg TCS L⁻¹, HT), nutrients (2000 µg PO₄-P L⁻¹ and 20000 µg NO₃-N L⁻¹, NU), low TCS with nutrients (LTN), medium TCS with nutrients (MTN), and high TCS with nutrients (HTN) (see table 1). A 0.45-µm nylon membrane (47-mm diameter) was placed over the bottle opening, and a glass fiber filter was placed on top of the nylon membrane to provide the growth surface (artificial substrate) for natural stream periphyton (fig. 1B). A bottle cap with a 25-mm diameter hole was screwed on the bottle to hold the membrane and glass fiber filter in position. The bottles were attached with zip ties to a floating platform constructed with a PVC frame and 18-gauge wire utility paneling. The platform provided a stable structure for treatment bottles (fig. 1C). Potential grazing by fish and macroinvertebrates was prevented by 10-mesh Al screen secured loosely over the bottlenecks with a zip tie. Additional details on periphytometers and deployment may be found in Matlock et al. (1998, 1999).

The periphytometer was deployed approximately 7 d in the West Fork of the White River, from 2 to 9 October 2002 (fig. 1A and C). Water samples were collected at deployment (2 October) and harvest (9 October) and analyzed for NO₃-N, ammonium-N (NH₄-N), total N (TN), total organic carbon

(TOC), and soluble reactive phosphorus (SRP), physicochemical measures of temperature, electrical conductivity, and pH were also made. At periphytometer harvest, the glass fiber filters were removed from the bottles, preserved with aqueous acetone, and stored at 4°C prior to chlorophyll-a analysis via pigment extraction and spectrophotometer determination (APHA, 1995). Pheophytin correction was used in conjunction with chlorophyll-a determination; the mass of chlorophyll-a was measured per unit area (mg m⁻²). Statistical comparisons ($\alpha = 0.05$) between mean chlorophyll-a content in each treatment were conducted using the Student–Newman–Keuls (SNK) Test. In addition, regression analysis was used to determine the relation between reduced chlorophyll-a response and initial TCS concentration in each treatment.

RESULTS

Ambient nutrient concentrations were relatively low and potentially limited periphytic algal growth rates at the West Fork of the White River (table 2). Physicochemical properties were within the range of natural occurrence for streams in this region; however, the pH was slightly greater than the pKa of TCS at deployment. Ambient conditions were representative of base flow or low flow conditions over the weeklong deployment because no surface runoff occurred during the study period.

Statistical analysis identified three significantly different groups between the nine treatments (fig. 2). NU and LTN treatments had greater chlorophyll-a means that were significantly different from each other and all other treatments. The nutrient enrichment treatment (NU) treatment had the greatest chlorophyll-a mean (10.9 mg m⁻²) suggesting nutrients supply (particularly NO₃-N and SRP) limited algal growth (NU>>C) in the stream in the absence of TCS.

Analyzing TCS within each treatment block (with and without nutrients in the treatment solutions) showed no significant differences between treatment means without nutrient enrichment; that is, TCS elicited no detrimental response (i.e., reduced chlorophyll-a content relative to the control treatment). Chlorophyll-a means within the nutrient enrichment block exhibited a significant exponential decline in values with an increase in initial TCS concentration (fig. 3). This is described by the following regression equation:

$$\text{chl}a = 3.15 + 7.85e^{-0.2554x} \quad (1)$$

where chl a = chlorophyll-a concentration in mg m⁻² and x = Triclosan concentration in µg L⁻¹ (R² = 0.76, F = 35.74, and P < 0.0001). Thus, the addition of nutrients stimulated periphytic algal growth but increasing initial concentrations of TCS inhibited algal growth.

Table 2. Ambient nutrient concentrations and physicochemical properties at deployment and harvest of a passive diffusion periphytometer at the West Fork of the White River, Northwest Arkansas, during October 2002.

Date	Temp. (°C)	Cond. (µS cm ⁻¹)	pH	SRP (µg L ⁻¹)	NH ₄ -N (µg L ⁻¹)	NO ₃ -N (µg L ⁻¹)	TN (µg L ⁻¹)	TOC (µg L ⁻¹)
Deployment: 2 October 2002	21	295	8.2	5	<50	230	600	10,900
Harvest: 9 October 2002	17	303	7.2	15	<50	140	530	12,500

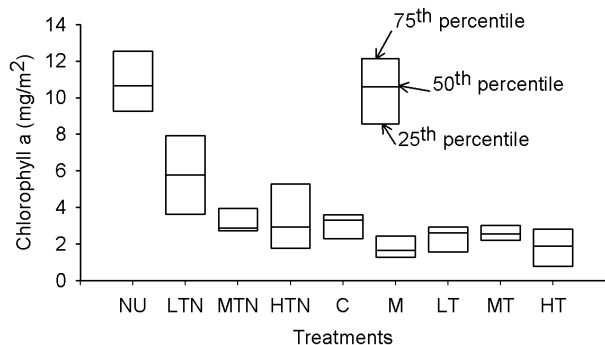


Figure 2. Box plots depicting chlorophyll-a 25th, 50th, and 75th percentile values for all treatments in the passive diffusion periphytometer deployment during October 2002.

DISCUSSION

Mean values of chlorophyll-a content for non-nutrient treatments (C, M, LT, MT, and HT) were not significantly different; thus, periphyton in these treatments showed no response to increasing initial TCS concentrations. In these treatments, TCS was not able to elicit a toxic response because algal growth was limited by ambient nutrient supply. However, when periphytic growth was stimulated by additional nutrients, TCS inhibited algal growth in all but the lowest exposure concentrations; this inhibition of growth represents an explicit toxic response. This toxic response was not lethal (i.e., decreased algal biomass), but inhibited algal growth or chlorophyll-a production over the 7-d deployment (i.e., chronic response).

The relatively low ambient $\text{NO}_3\text{-N}$ ($140\text{--}230\ \mu\text{g L}^{-1}$) and SRP ($\leq 15\ \mu\text{g L}^{-1}$) concentrations, and the N to P supply ratio of 43 suggested that P may have been the limiting nutrient in this reach of the West Fork of the White River during deployment. Periphyton in Ozark streams is generally not limited by $\text{NO}_3\text{-N}$ when concentrations exceeded $100\ \mu\text{g L}^{-1}$ (Lohman et al., 1991). The Lotic Ecosystem Trophic State

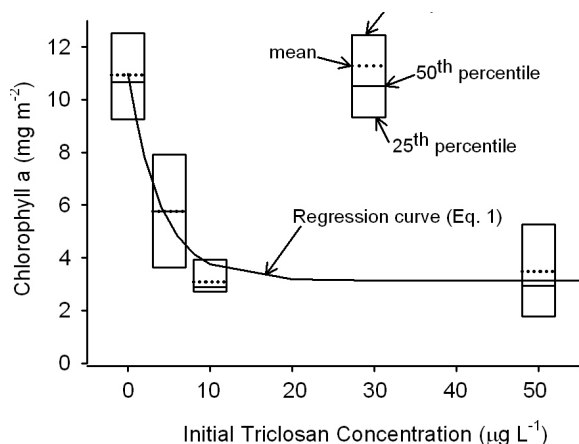


Figure 3. Box plots depicting chlorophyll-a 25th, 50th, and 75th percentile and mean values for nutrient enriched treatments (NU, LTN, MTN, and HTN) in the passive diffusion periphytometer deployment during October 2002. The regression curve (eq. 1) depicts the dose-response curve relating chlorophyll-a content in the NU, LTN, MTN, and HTN treatments with initial Triclosan concentration.

Index (LETSI sensu Matlock et al., 1999) measured in this experiment was least of LETSI values observed in several regional Ozark streams (Haggard, 2003), further supporting the conclusion that this system is nutrient limited with respect to algal growth during deployment. Observations that periphytic growth on the control treatment (C) did not differ from the TCS treatments absent additional nutrients suggested that TCS was not being delivered to the algae in lethal amounts.

Post-deployment analysis of TCS in the periphytometer bottles indicated that very little TCS ($<10\ \mu\text{g L}^{-1}$) remained at the end of the experiment in all the bottles (data not shown). Thus, most of the TCS diffused through the nylon membrane and across the artificial growth substrate (glass fiber filter) into the periphytic biofilm, and TCS toxicity was evident in nutrient-enriched treatments. The significant relation between periphytic algal growth, measured as chlorophyll-a content, and initial TCS concentrations suggested a toxic exposure-response relationship (fig. 3). Furthermore, a passive diffusion periphytometer may be useful to make *in situ* toxicity assessments in streams.

Using the exposure-response curve in figure 3, we estimated a periphytic biofilm EC_{50} for TCS of approximately $50\ \mu\text{g L}^{-1}$; this EC_{50} was based on initial TCS concentrations. In this study, exposure concentrations are functions of the diffusion rates across the nylon membrane and glass fiber filter to the periphytic biofilm. Quantifying the diffusion rate provides an exposure rate, but converting it to concentration at very low exposure levels makes it more difficult to ascertain an EC_{50} than traditional laboratory bioassays or the use of model stream systems. Previously reported EC_{50} in algal laboratory assays (Ciba, 1998; Orvos et al., 2002) were an order of magnitude less than that observed in this study (i.e., exposure-response curve in fig. 3), a reasonable comparison given the exponential rate of diffusion at deployment.

In spite of the inability to determine exposure concentrations, application of a passive diffusion periphytometer provided an alternative approach to *in situ* assessments of chemical toxicity in streams. Future investigations should focus on quantifying exposure concentrations by measuring diffusion rates of the chemicals used in toxicity testing. The use of this methodology may provide a useful precursor to toxicity testing in model stream systems and a simple *in situ* approach to be used in combination with traditional laboratory bioassays.

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