Environmental Toxicology and Chemistry—Volume 38, Number 11—pp. 2414–2425, 2019
Received: 11 April 2019 | Revised: 29 May 2019 | Accepted: 26 July 2019

Environmental Toxicology

N,N-Diethyl-m-Toluamide Exposure at an Environmentally Relevant Concentration Influences River Microbial Community Development

John R. Lawrence,[a,*] Marley J. Waiser,[a] George D.W. Swerhone,[a] Julie L. Roy,[a] Armelle Paule,[b] and Darren R. Korber[c]

[a]Environment and Climate Change Canada, Saskatoon, Saskatchewan, Canada
[b]Global Institute for Water Security, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
[c]Department of Food and Bioproducts Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Abstract: Studies of the South Saskatchewan River confirmed that *N,N*-diethyl-m-toluamide (DEET) is ubiquitous at 10 to 20 ng/L, whereas in effluent-dominated Wascana Creek, levels of 100 to 450 ng/L were observed. Effects of DEET exposure were assessed in microbial communities using a wide variety of measures. Communities developed in rotating annular reactors with either 100 or 500 ng/L DEET, verified using gas chromatography-mass spectrometry analyses. Microscale analyses indicated that both DEET concentrations resulted in significant ($p < 0.05$) declines in photosynthetic biomass, whereas bacterial biomass was unaffected. There was no detectable effect of DEET on the levels of chlorophyll a. However, pigment analyses indicated substantial shifts in algal–cyanobacterial community structure, with reductions of green algae and some cyanobacterial groups at 500 ng/L DEET. Protozoan/micrometazoan grazers increased in communities exposed to 500 ng/L, but not 100 ng/L, DEET. Based on thymidine incorporation or utilization of carbon sources, DEET had no significant effects on metabolic activities. Fluorescent lectin-binding analyses showed significant ($p < 0.05$) changes in glycoconjugate composition at both DEET concentrations, consistent with altered community structure. Principal component cluster analyses of denaturing gradient gel electrophoresis indicated that DEET exposure at either concentration significantly changed the bacterial community ($p < 0.05$). Analyses based on 16S ribosomal RNA of community composition confirmed changes with DEET exposure, increasing detectable beta-proteobacteria, whereas actinobacteria and acidimicrobia became undetectable. Further, cyanobacteria in the subclass Oscillatoriophycideae were similarly not detected. Thus, DEET can alter microbial community structure and function, supporting the need for further evaluation of its effects in aquatic habitats. *Environ Toxicol Chem* 2019;38:2414–2425. © 2019 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Biofilm; DEET; Microbial community; Pigment; Protozoa; Sequencing

INTRODUCTION

N,N-Diethyl-m-toluamide (DEET) is the active ingredient in most commercial insect repellents used around the world. It has been registered for general use since 1957 and is found in over 200 registered veterinary and human use products, with production of up to 1.8 million kg/yr in the United States (US Environmental Protection Agency 1998). In general, DEET is not rated as persistent, bioaccumulative, or toxic according to European Union criteria (KemI 2010; Weeks et al. 2012). However, as a consequence of extensive use, DEET has been detected in many water surveys, including surface waters (Kolpin et al. 2002; Sandstrom et al. 2005), groundwaters and sewage effluents (Glassmeyer et al. 2005), drinking water (Cordy et al. 2004; Stackelberg et al. 2004), and ocean waters (Weigel et al. 2002, 2004). Indeed, DEET was one of the contaminants most frequently detected in US streams in 74% of cases at concentrations up to 1.1 μg L^{-1} (Kolpin et al. 2002) and is clearly entering the aquatic environment and persisting at low levels. Concentrations detected in the environment have ranged from 40 to 3000 ng L^{-1} (Costanzo et al. 2007). Reports from sewage influents have indicated that DEET may reach levels of 0.6 to 1.2 μg/L (Sui et al. 2010). However, reports on the efficiency of removal of DEET by sewage treatment indicate that its concentrations may be decreased by more than 80%

* Address correspondence to john.lawrence2@canada.ca
Published online 31 July 2019 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.4550

during biological treatment (Okuda et al. 2009; Sui et al. 2010). It is thought to enter the aquatic environment mainly through sewage effluents as a result of the washing off of skin-applied DEET and limited excretion from humans. The possibility of localized introductions attributable to the presence of high-use recreational areas or use in horse facilities may also contribute to environmental loading (Costanzo et al. 2007). However, although atmospheric introductions have been suggested, they are considered minor (Weigel et al. 2002).

Information on the ecological toxicity of DEET, however, is sparse. Despite this deficiency, it has been suggested that the risk to aquatic biota at observed environmental concentrations is minimal (Aronson et al. 2012; Weeks et al. 2012). Limited studies show it to be slightly toxic to freshwater fish (75 mg/L, rainbow trout) and invertebrates (75 mg/L, *Daphnia magna*; US Environmental Protection Agency 1998; Seo et al. 2005). Recent metabolomics studies have suggested that the in situ presence of DEET has a strong relationship to changes in endogenous metabolites in fathead minnows (*Pimephales promelas*; Davis et al. 2016), indicating that ambient concentrations of DEET (0.019–0.902 μg/L) elicited a biological response. A 30-d chronic toxicity value for fish of 10.8 mg/L, a 96-h value of 6.5 mg/L for green algae, and a 16-d value for *D. magna* of 5.0 mg/L have been estimated based on its classification as a neutral organic using the program ECOSAR (Aronson et al. 2012). A toxicological summary for DEET was prepared for the National Institutes of Health in 1999 (Tice and Brevard 1999) and points to a variety of impacts in standard toxicity testing and examinations of animal and human exposures. Thus, there appear to be significant gaps in our understanding of the environmental chemodynamics and potential effects of DEET in aquatic environments. Costanzo et al. (2007), reiterated by Brausch and Rand (2011), suggested that DEET is not likely to produce biological effects at environmentally relevant concentrations. Indeed, a hazard quotient analysis for DEET in Wascana Creek (Waiser et al. 2011) suggested that there was not a significant risk from DEET exposures in this ecosystem, although levels in the range 100 to 450 ng/L were observed. However, there is an acknowledged lack of data regarding microorganisms other than algae and an absence of complex community exposures. Further, there is a lack of information on DEET in the area of chronic toxicity to aquatic organisms.

Microbial communities are considered sensitive, relevant indicators of ecotoxicity with high ecological realism and reliability for assessing ecological effects of contaminants (Porsbring et al. 2007; Sabater et al. 2007; Proia et al. 2012; Lawrence et al. 2016a). Although in situ studies provide greater realism, the use of various micro- and mesocosm designs provides the necessary control and replication for toxicological studies (Lawrence et al. 2016a). The approach allows simultaneous assessment of effects on numerous organisms as well as their interactions. Further, community-level testing has been shown to be sensitive and to incorporate organisms and processes not assessed in standardized single-species testing (Lawrence et al. 2005, 2016a, 2016b). In addition, it may suggest areas of concern for additional study (Lawrence et al. 2016a). A number of studies have demonstrated that the application of a multimetric approach including traditional, molecular, and genomic techniques can effectively determine the responses of complex microbial communities to a range of stresses (Lawrence et al. 2004, 2005, 2007, 2008, 2012, 2015, 2016a, 2016b; Sabater et al. 2007; Porsbring et al. 2007; Proia et al. 2012).

In the present study, river-derived microbial communities were cultivated in rotating annular reactors with and without exposure to 100 or 500 ng/L DEET. A variety of methods, including confocal laser microscopy–based microscale analyses, pigment analyses, thymidine incorporation/carbon utilization, and polymerase chain reaction (PCR)-based molecular and genomic methods, including denaturing gradient gel electrophoresis (DGGE) and 16S ribosomal RNA (rRNA) gene sequence analyses, were used to assess the impacts of DEET exposure.

MATERIALS AND METHODS

Microcosm operation

The experimental setup and reactor design for biofilm development have been described in detail (Lawrence et al. 2000, 2004). Natural river water (South Saskatchewan River, Saskatoon, SK, Canada) was used as inoculum and as a source of carbon and nutrients. Two separate experiments were performed in summer (July–September) and winter (December–January). Gas chromatography-mass spectrometric analyses confirmed DEET levels in the river water and treatment concentrations in Wascana Creek (100–450 ng/L), South Saskatchewan River (10–20 ng/L), and treatment reactors of 100 or 500 ng/L. Control reactors that received river water alone were included in the experimental design. Replicated biofilms (3× reactors) were grown under treatment and control conditions in bioreactors for a period of 8 wk, at which time coupons were removed for immediate analysis (confocal laser scanning microscopy [CLSM], microscopic; Biolog), frozen at –80 °C, and stored for subsequent DNA extraction and analyses.

CLSM and image analysis

Examination of all stained and control materials was carried out with an MRC 1024 CLSM attached to a Microphot SA microscope (Nikon) or a Nikon C2 confocal laser microscope mounted on a Nikon Eclipse 80i standard microscope along with a ×40, NA 0.80 water-immersible lens.

Randomly selected slides from among the 12 slides (polycarbonate 1 cm wide × 10 cm long) in each of the replicate reactors were cut into 1-cm^2 pieces and mounted in small Petri dishes using Dow Corning #3140 acid-free silicone (WPI) and then stained and analyzed according to the following procedures. For observation, the following water-immersible lenses were used: ×63, 0.9 NA (Zeiss); ×40, 0.55 NA; and ×10, 0.35 NA (Nikon). Biofilms were observed using a double-labeling procedure and 3-channel recording: bacteria were stained with the fluorescent nucleic acid stain SYTO 9 (excitation wavelength, 488 nm; emission wavelength, 522–532 nm); in this assay the lectin (*Triticum vulgaris*–tetramethyl rhodamine isothiocyanate [TRITC]; excitation, 568 nm; emission, 605–632) was used to visualize exopolymer, and

autofluorescence (excitation, 647; emission, 680–632) was used to detect algal and cyanobacterial cells (Neu et al. 2004). Digital image analysis of the CLSM optical thin sections in each of the 3 channels determined such parameters as biofilm depth, bacterial cell area (biomass), exopolymer biomass, cyanobacterial biomass, and total photosynthetic biomass at various depths. Image analyses were performed using the National Institutes of Health's Image, Ver 1.61, with macros written for semiautomated quantification as described in Manz et al. (1999). In addition, 3-color projections (bacterial cells stained green, polymer stained red, and algal autofluorescence stained blue) of the biofilms were computed for visual comparisons of biofilm structure.

Exopolymer analyses

Lectins labeled with either fluorescein isothiocyanate or TRITC (Sigma) or cyanine 5 (Research Organics) were applied for exopolymer analyses. In this assay the following lectins were used in separate applications for in situ analyses of polymer composition: *T. vulgaris* (target residues β[1,4]*N*-acetylglucosamine, *N*-acetylneuraminic acid), *Arachis hypogaea* (terminal β-galactose, *N*-acetylgalactosamine [associated with algal–cyanobacterial polymers]), *Canavalia ensiformis* (α-linked mannose or glucose residues), and *Ulex europaeus* (α-L-fucose [associated with bacterial polymers]). Staining, imaging, image analyses, and calculations of lectin binding volumes were carried out using the equations of Neu et al. (2001).

Protozoan and micrometazoan enumeration

Protozoa and micrometazoa (rotifers, nematodes) were enumerated weekly by counting manually on triplicate 2-cm^2 subsamples using phase contrast on an Olympus BH-2 microscope with an MS plan ×10, NA 0.03, objective lens. The entire 2-cm^2 subsample was examined at each time period.

Carbon utilization spectra

Carbon utilization spectra were determined for biofilm samples using commercial Eco-plates (Biolog), as described (Lawrence et al. 2004, 2008).

Thymidine incorporation

Thymidine incorporation was determined using tritiated thymidine following the standard protocol of Robarts and Wicks (1989). All negative controls were killed with formaldehyde at 0.4% (v/v) final concentration.

Chlorophyll a and pigment analyses

Biofilm strips (10 cm^2) were scraped using a sterile silicone rubber spatula to remove the biofilm, after which chlorophyll a was extracted using 90% boiling ethanol (Nusch 1980) and analyzed fluorometrically using a Turner Designs Model 10-AU digital fluorometer (Waiser and Robarts 1997). Detailed analyses of pigments were carried out using the high performance liquid chromatography methods as described by Leavitt and Hodgson (2001) and Lawrence et al. (2015).

Chemical analyses

Reference materials, as well as influent and effluent from the reactors, were collected, placed on ice, and submitted to the Alberta Innovates Analytical Facility for extraction and determination of DEET levels. A liquid–liquid (dichloromethane) extraction method was used, and extracts were analyzed on an Ion Trap, Varian GC-MS.

Molecular analyses

Total community DNA extraction. For each replicate treatment bioreactor (3×), a frozen (–80 °C) polycarbonate strip was aseptically cut (2 cm^2) and transferred to a 50-mL polypropylene tube (Falcon, Becton Dickinson). Microbial cells from the frozen biofilm samples were removed from the polycarbonate strip with a sterile metal scraper, and total DNA was extracted using the FastDNA spin kit for soil (Bio101 Systems, Qbiogene) according to the manufacturer's instructions.

PCR amplification. The bacterial 16S rRNA gene was amplified using "universal" primers to perform DGGE. The primer consensus sequence was, forward, 5′-CCT ACG GGA GGC AGC AG-3′ (preceded by a GC clamp for DGGE [not for sequencing] = CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC G [40 nt]) and, reverse, 5′-ccg tca attcmt ttg agt tt-3′ position (length) 341 to 357 (17 nt) and 907 to 926 (20 nt), respectively; and the PCR amplicon size was 586 bp (Muyzer et al. 1993; Muyzer and Ramsing 1995). Amplification by PCR was conducted in a 25-μL reaction volume containing 1 μL of DNA template, 10 pmol of each appropriate primer as described by Muyzer et al. (1993; Muyzer and Ramsing 1995), 1.25 U Taq DNA polymerase (New England Biolabs) 1 × PCR buffer, 2.5 mM $MgCl_2$, and 200 μM deoxynucleoside triphosphate. A touchdown PCR program using the PTC-200 thermocycler (MJ Research) consisted of an initial denaturation step of 94 °C for 5 min, followed by 10 cycles of denaturation at 94 °C for 1 min, annealing at 66 °C (decreasing in each cycle by 1 °C) for 1 min, and an elongation step of 72 °C for 1 min. Following these steps, another 20 cycles of 95 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min, with a final elongation step of 72 °C for 7 min, were performed. The correctly sized PCR product was verified by electrophoresis on a 1.5% w/v agarose gel in 1.0 × Tris–acetate–ethylenediamine tetraacetic acid (EDTA) buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) for 1.0 h at 100 V. Gels were stained using ethidium bromide and documented using the AlphaImager 3300 gel documentation and image analysis system (Alpha Innotech).

DGGE. After the specificity and size of the amplified products were checked on agarose gels, the PCR product was separated

by DGGE (Muyzer et al. 1993; Muyzer and Ramsing 1995) using an Ingeny phorU2 system. Aliquots (20 uL) of PCR product were mixed with 4 µL of loading dye buffer and resolved on a 6% (w/v) polyacrylamide gel in 1.0 × Tris–acetate–EDTA buffer, using denaturing gradients from 45 to 65% (100% denaturant contains 7 M urea and 40% deionized formamide). The DGGE procedure was carried out at 40 V for 10 min and then 100 V for 18 h at 60 °C. After electrophoresis, the gel was stained with SYBR Green I (1:10 000 dilution; Molecular Probes) for 15 min with gentle agitation and photographed using the AlphaImager 3300 gel documentation and image analysis system.

MiSeq sequencing analyses. The DNA was extracted from frozen biofilm communities (–80 °C) using the MoBio Powersoil Kit (Qiagen Sciences). Barcoded amplicons (560 bp) of bacterial 16S rRNA genes, covering the V3 to V4 region, were generated. The DNA library was prepared using the Illumina Nextera XT DNA kit and sequenced on a MiSeq platform (Illumina), as explained in the following section.

Bacterial 16S rRNA gene library preparation and high-throughput amplicon sequencing. Sequence libraries were prepared by PCR amplification of the V3 to V4 conserved regions of the bacterial 16S rRNA gene using the primers Bakt_341F and Bakt_805R (Herlemann et al. 2011) that included 50-bp overhangs complementary to priming sequences for Nextera XT-indexed adapters. The reaction was comprised of a 5-mL template, 12.5 mL 2xKAPAHiFi HotStart ReadyMix (Kapa Biosystems), 1.0 mmoL reverse and forward primers, 0.5 mL 25 mmol/L $MgCl_2$, with a final volume adjusted to 25 mL using molecular-grade water. Thermal cycling involved initial denaturation at 96 °C (4 min), followed by 30 cycles of denaturation at 96 °C (30 s), primer annealing at 56 °C (30 s), and primer extension at 72 °C (30 s), followed by extension at 72 °C for 5 min.

Amplicons were visualized by gel electrophoresis to verify size (~425 bp), purified by the Agencourt AMPure XP system (Beckman Coulter), eluted in PCRapace elution buffer (STRATEC Molecular), and quantified using a Qubit dsDNA HS kit (Invitrogen).

Amplicons were barcoded with sequencing adapters using the Nextera XT index kit. Obtained libraries were normalized to 4 nM and pooled prior to sequencing and further processing. Libraries (6 pM final concentration) were sequenced using a MiSeq Reagent Kit V3 (600 cycles) on the MiSeq System.

High-throughput sequence data analysis

Paired-end MiSeq reads from the samples examined were aligned and transformed to contigs (2 × 300 bp), yielding a total of 65 511 (control), 70 593 (DEET 100), and 65 234 (DEET 500) reads. Reads passing quality filtering were control, 55 916 (85.33%); DEET 100, 60 349 (85.37%); and DEET 500, 55 964 (85.73%). In addition, rarefaction analyses confirmed sufficient sequencing depth and that the diversity was captured by these analyses. Contig sequences were further subjected to error elimination, chimera checking, and contaminant removal using Mothur, Ver. 1.32.2, with SILVA database, Ver. 123, as a bacterial taxonomy reference (Schloss et al. 2009; Kozich et al. 2013). SILVA bacterial reference sequence data sets were normalized to the size of the smallest library of filtered reads per sample for community data analysis to ensure consistent sequencing depth. Operational taxonomic units were generated using average-neighbor clustering with a 3% cutoff using algorithms implemented in Mothur, Ver. 1.31.2.

Experimental design and statistical analyses

The experimental design consisted of an untreated control and DEET at 100 or 500 ng/L, reflective of levels observed in Wascana Creek. Experiments were run in both summer and winter periods for 8 wk. Each treatment had 3 identical replicate reactors randomly assigned to it on the reactor bench (replications). Furthermore, each analysis was done on subsamples of randomly selected biofilm coupons from among the 12 identical coupons in each replicate reactor. Analysis of variance was used to detect significant differences among sample means at $p < 0.05$ (MiniTab). Band detecting, matching, and processing of DGGE gels were performed using GelCompare II software, Ver 4.6 (Applied Maths).

Fingerprint data were processed by generating a band-matching table (Boon et al. 2002). The binary data were exported and compared by principal component analysis (PCA) with PRIMER, Ver 6, software (PrimerE). Statistical analyses of PCA scores generated from the first 2 axes were run using an analysis of similarity (ANOSIM) algorithm with PRIMERr, Ver 6, software (Clarke 1993). The inclusion of DGGE ladders allowed GelCompare II to normalize the position of bands in all of the lanes under examination.

RESULTS AND DISCUSSION

Our studies of municipal waste water effluents from the city of Saskatoon, Saskatchewan, Canada, and its receiving environment (South Saskatchewan River) have confirmed that DEET was ubiquitous in the river at 10 to 20 ng/L. The South Saskatchewan River thus made an ideal water source for exposure experiments with low background levels of DEET versus Wascana Creek (Saskatchewan), an effluent-dominated system (Waiser et al. 2011), where levels of 100 to 450 ng/L have been reported. Although DEET is commonly detected, relatively little is known regarding its degradation and metabolites. A few studies have recorded degradation of DEET, including by a fungus (Seo et al. 2005) with 2 metabolites, *N,N*-diethyl-m-toluamide-*N*-oxide and *N*-ethyl-m-toluamide, and aerobic degradation of DEET by *Pseudomonas putida* DTB (Rivera-Cancel et al. 2007). In the latter case, the bacteria used DEET as a sole carbon source and produced 2 metabolites: 3-methylbenzoate and 3-methylcatechol (Rivera-Cancel et al. 2007). A study of wastewater-treatment plants in Germany indicated that limited degradation of DEET occurred (Knepper 2004). Clearly, biological degradation of DEET occurs; however, at this time there is no explanation for its environmental

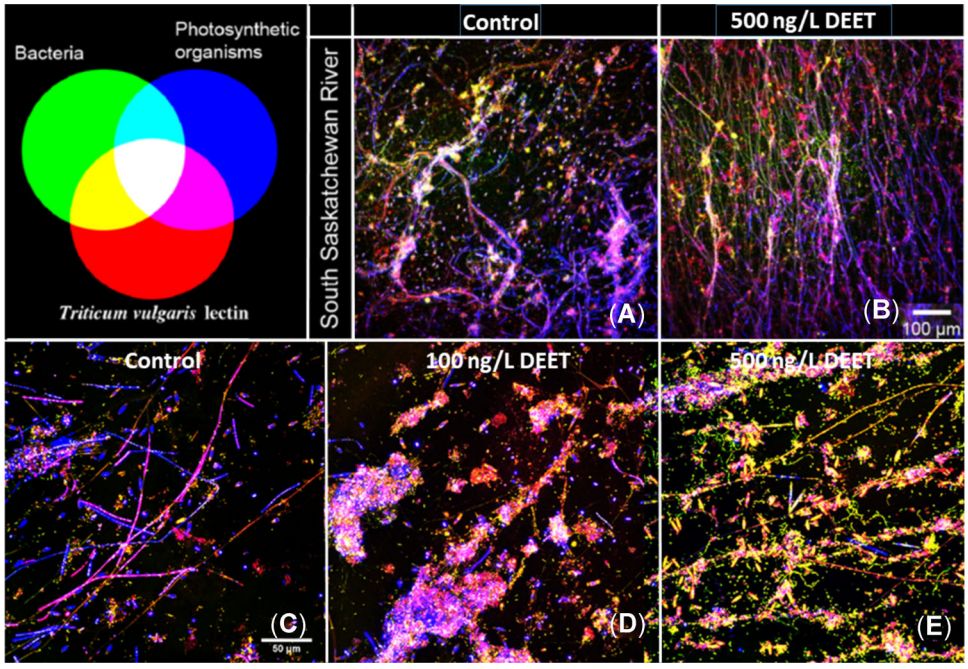

FIGURE 1: Representative confocal laser scanning microscopy photomicrographs of control and *N,N*-diethyl-m-toluamide-exposed river biofilm communities. Color wheel indicates bacteria (green), *Triticum vulgaris*-tetramethyl rhodamine isothiocyanate lectin binding polymer (red), and photosynthetic biomass (blue/magenta). (**A**, **B**) Summer; (**C–E**) winter. DEET = *N,N*-diethyl-m-toluamide.

persistence. Based on these observations and the range of DEET concentrations reported in the literature (40–3000 ng/L [Costanzo et al. 2007]; 13-660 ng/L [Brausch and Rand 2011]), we selected exposure levels of 100 and 500 ng/L.

Algae–Cyanobacteria–Bacteria

We performed a wide range of analyses on replicate microbial biofilm communities, derived from South Saskatchewan River waters, developed in rotating annular reactors (Lawrence et al. 2000) during continuous exposure to 100 or 500 ng/L DEET. Detailed microscopic analyses suggested that the microbial communities were affected by DEET exposure, as indicated by a shift to a community dominated by filamentous microbial morphotypes during summer exposures (Figure 1A, B). Similar, although different, responses were observed during winter, as shown in Figure 1C, D, and E. These observations substantiate both the clear change in biofilm architecture and membership as well as a differential seasonal response. We noted previously that when using either in situ or derived microbial communities, there are seasonal effects on the outcome (Chénier et al. 2003) consistent with changes in the nature of the community available for recruitment during biofilm formation.

Digital image analyses of replicate confocal image stacks showed that DEET exposure resulted in a significant decline in photosynthetic biomass ($p < 0.05$) in South Saskatchewan River biofilm communities. In contrast, bacterial biomass was not significantly affected by either DEET concentration (Figure 2). Determination of chlorophyll a levels in these biofilms indicated no significant change in total chlorophyll in any of the communities ($p < 0.05$; Table 1). However, pigment analyses (Figure 3) indicated that there were significant shifts in community composition, particularly the disappearance of green algae with pheophytin-b in South Saskatchewan River communities exposed to DEET (Figure 3). Consistent reductions in diatoms were also observed (Figure 3). There were increases in pigments of 2 groups of cyanobacteria (Figure 3). There are reports of impacts of DEET on algal photosynthesis (Brausch and Rand 2011) wherein *Chlorella protothecoides* was affected, although at a very high concentration of 388 mg/L.

We also exposed pure cultures of algae and cyanobacteria to concentrations of DEET between 0.5 and 100 µg/L. However, we detected no effects on the growth of cultures relative

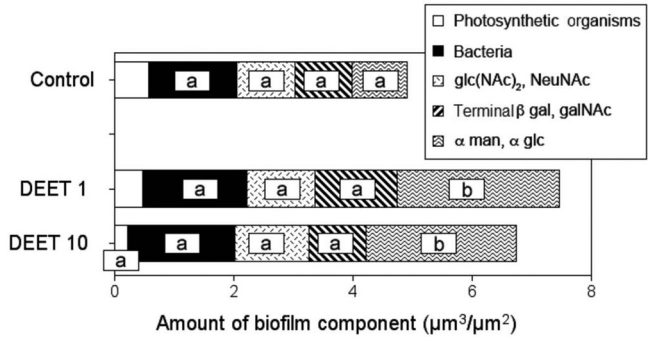

FIGURE 2: Results of image analyses of confocal laser micrographs illustrating the effect of the *N,N*-diethyl-m-toluamide treatment on the relative abundance of photosynthetic biomass and bacterial biomass as well as composition of exopolymeric substances using lectin binding analyses (see also Figure 6) in the winter river biofilms by treatment. Parameters indicated by different letters are significantly different from the control at $p < 0.05$. DEET = *N,N*-diethyl-m-toluamide; β-gal = β-galactose; galNAc = *N*-acetylgalactosamine; glc = glucosamine; glcNAc = *N*-acetylglucosamine; man = mannose; NeuNAc = *N*-acetylneuraminic acid.

TABLE 1: Effects of *N,N*-diethyl-m-toluamide on bacterial production and chlorophyll a levels in South Saskatchewan River microbial communities[a]

Treatment	Bacterial production (pmole/L/h)	Chlorophyll a (μg/mm^2)
SSR control	53 ± 5.8	3.6 ± 0.47
SSR DEET	49 ± 4.4	3.8 ± 0.30

[a]No significant ($p < 0.05$) effects were detected, $n = 3$.
DEET = *N,N*-diethyl-m-toluamide; SSR = South Saskatchewan River.

to controls (data not presented). Similar observations were obtained previously for other chemicals, where significant effects of chlorhexidine, triclosan, and triclocarban were observed on the natural community with no detectable impacts on selected pure cultures (Lawrence et al. 2008, 2009, 2015). It has been suggested in a number of studies (Lawrence et al. 2008, 2009; Wilson et al. 2003) that natural assemblages and communities provide greater insight into potential impacts than studies using selected pure cultures. Community-level testing can be a very useful tool in ecotoxicology because of its relatively high ecological realism and reliability when assessing the ecological effects of a contaminant or a mixture (Porsbring et al. 2007). The studies of Lawrence et al. (2004, 2005, 2007, 2009, 2015) and Chénier et al. (2003, 2006) have demonstrated that the application of an array of traditional and molecular techniques can effectively determine the responses of complex microbial communities to a range of stresses.

Changes in photosynthetic and bacterial biomass were also reflected in the amount of exopolymer detected using lectin binding analyses (Figure 2). Altered production of exopolymeric substances (EPS) is frequently consistent with increased availability of nutrients, particularly in the oligotrophic South Saskatchewan River (Lawrence et al. 2004, 2005) or the observed increase in protozoan and micrometazoan grazers. However, the significant decline in photosynthetic biomass in the oligotrophic South Saskatchewan River may be more consistent with a toxic effect of DEET. The potential for mixed toxicity and nutrient effects of pharmaceuticals and personal care products has been noted in studies of microbial community response (Lawrence et al. 2005, 2012). However, the levels of DEET detected by gas chromatography-mass spectrometry in the reactors were consistent with the exposure level, suggesting a lack of degradation.

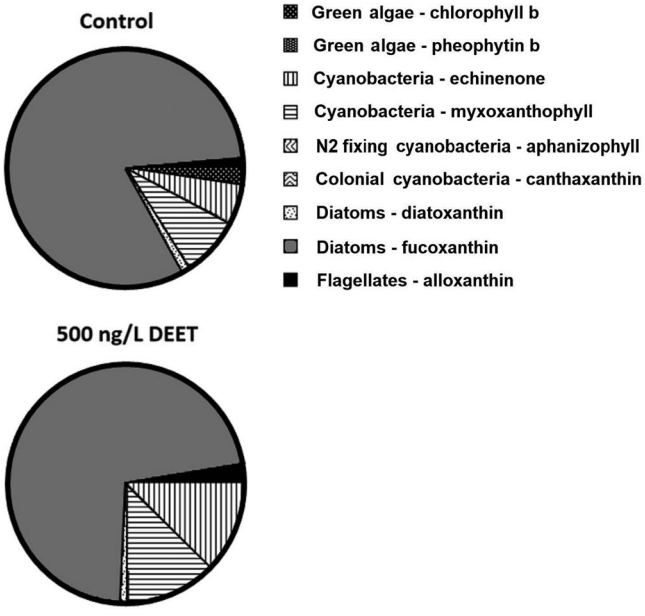

FIGURE 3: Results of detailed pigment analyses indicating that there were significant shifts in the composition of the photosynthetic community makeup, predominantly in pigments associated with cyanobacteria and green algae. DEET = *N,N*-diethyl-m-toluamide.

Community metabolism

Assessment of carbon utilization spectra indicated that DEET had few significant effects (Figure 4A,B). Principal component analyses of average well color development by substrate family (i.e., amino acids, amines, carbohydrates, carboxylic acids, polymers, phosphates, and esters) indicated no significant effects in South Saskatchewan River communities (Figure 4A). However, there were significant ($p < 0.05$) changes in the use of specific substrates (Figure 4B); South Saskatchewan River communities decreased use of D-galactonic acid/D-glacturonic acid/L-serine/L-arginine at 100 ng/L DEET, whereas at 500 ng/L increased utilization of α-D-lactose and decreased use of D-mannitol and λ-hydroxybutyric acid were observed. The changes in carbon substrate usage were minor and restricted to a narrow range of compounds, more in keeping with the effects of relatively inert contaminants such as carbon nanomaterials (Lawrence et al. 2016b) rather than generally suppressive as with metals (Lawrence et al. 2004) or stimulatory as observed with degradable substrates such as pharmaceuticals (Lawrence et al. 2005, 2015). These observations were also consistent with the lack of evidence for degradation of DEET during these exposures. Thymidine incorporation assays indicated no significant effects of any treatment ($p < 0.05$) relative to control values (Table 1). These results, and those of several previous studies, on the effects of antimicrobials and personal care products have tended to suggest that thymidine incorporation appears relatively insensitive as an indicator of contaminant effects at environmentally relevant concentrations (Lawrence et al. 2008, 2009, 2012).

Protozoa and micrometazoa

Direct counts of protozoans and micrometazoans showed a trend for a delay in the onset of maximum populations of protozoa (Figure 5A–C). However, the final populations were larger in 500 ng/L DEET-treated communities in the oligotrophic South Saskatchewan River. A similar pattern was observed for micrometazoans (rotifers; Figure 5B). This pattern was repeated for protozoans in the winter experiment at 500 ng/L DEET but was not observed at 100 ng/L DEET exposure (Figure 5C). This difference may suggest a lack of impact on protozoa at the lower concentration. Protozoa play a key role in nutrient cycling and

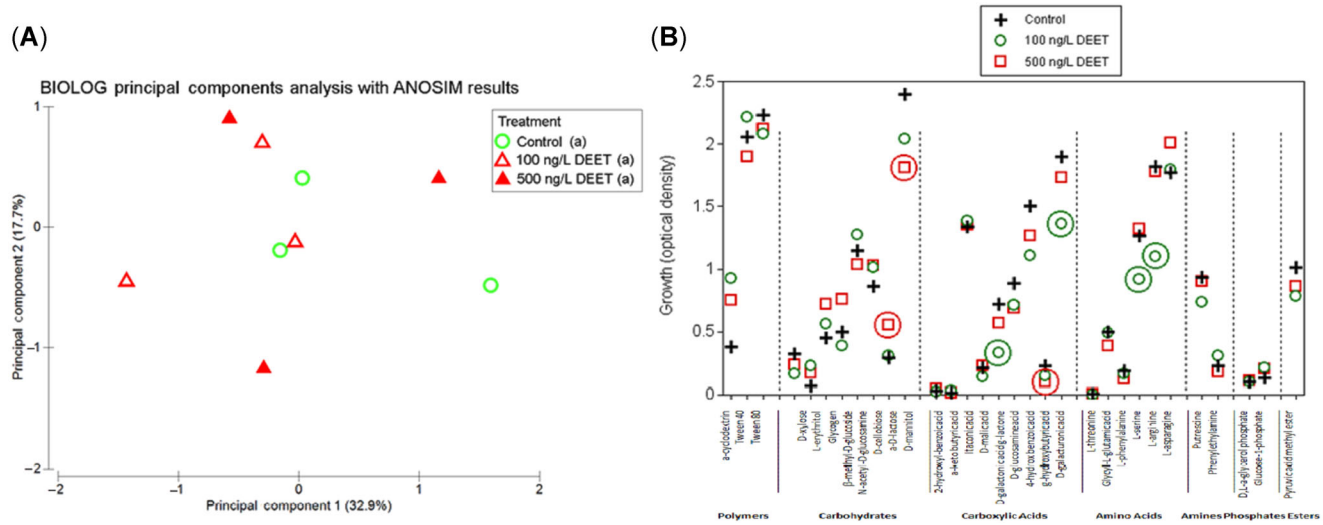

FIGURE 4: **(A)** Principal component analyses of carbon utilization spectra for South Saskatchewan River microbial biofilm communities. Letter (a) indicates no significant difference ($p < 0.05$). (**B**) Differential display of the carbon utilization assays of control biofilms and those growing with *N,N*-diethyl-m-toluamide in the South Saskatchewan River symbols, with circles indicating results that are significantly different from the control ($p < 0.05$). ANOSIM = analysis of similarity; DEET = *N,N*-diethyl-m-toluamide; SSR = South Saskatchewan River.

biomass removal; thus, impacts of DEET on grazers such as protozoa and micrometazoans may influence biomass as observed in the algal, cyanobacterial, and bacterial components of the communities. Although effects on invertebrates were apparent at much lower concentrations in the present study, DEET has been reported to have effects on a number of invertebrate species. Seo et al. (2005) reported that DEET and its metabolites may be chronically toxic, with DEET median lethal concentration (LC50) values of 108 mg/L with *D. magna* and for the metabolite *N*-ethyl-m-toluamide of >190 mg/L. Xue et al. (2000) reported that the DEET median lethal dose for *Cypricercus* sp. (Ostracoda), *Moina* sp. (Cladocera), and *Eucyclops agilis* (Copepoda) were 120, 240, and 140 mg/L, respectively. These reports and a 96-h LC50 of 75 mg/L for rainbow trout are consistent with the view that DEET is only slightly toxic to aquatic invertebrates and vertebrates (US Environmental Protection Agency 1998). However, in the oligotrophic South Saskatchewan River, we observed consistent, although not statistically significant ($p < 0.05$), increases in grazing populations with a pattern of delayed appearance that may be indicative of selection of

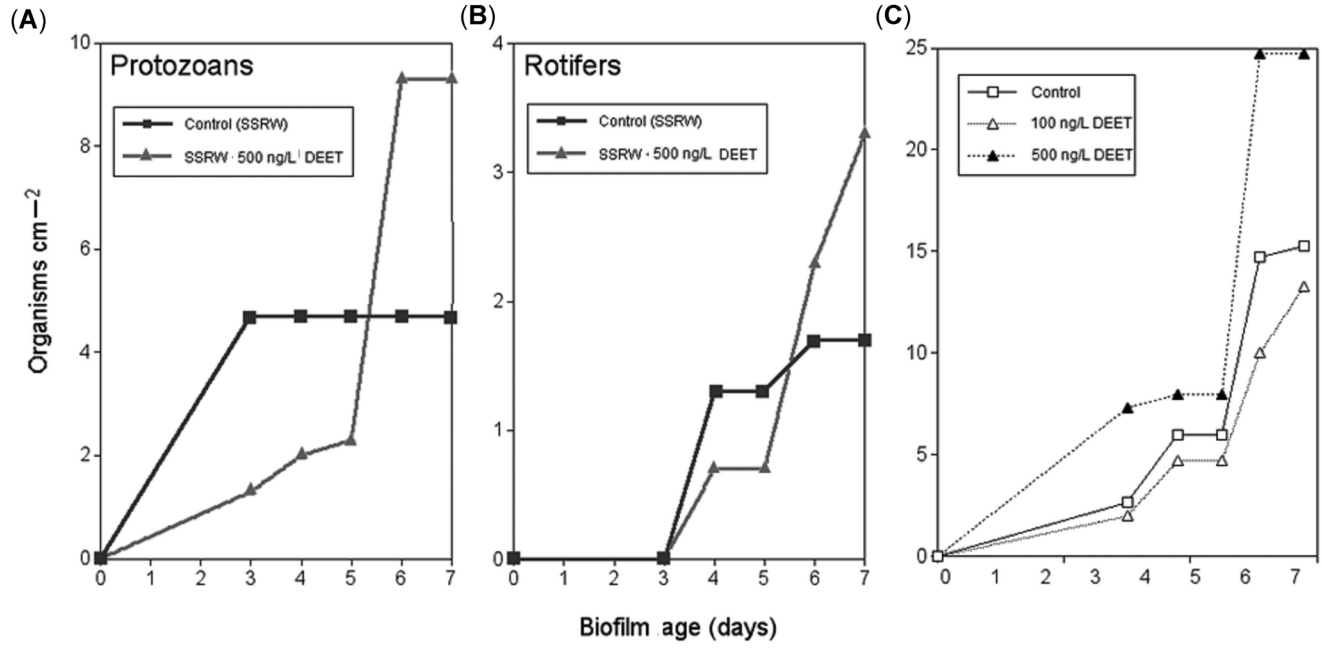

FIGURE 5: Enumeration of protozoa (ciliates, flagellates, amoeba) and micrometazoa (rotifers, nematodes) showing the effects of *N,N*-diethyl-m-toluamide on the cumulative grazer density within South Saskatchewan River summer (**A**, **B**) and winter (**C**) biofilm communities. DEET = *N,N*-diethyl-m-toluamide; SSRW = South Saskatchewan River water.

tolerant species. Certainly, additional studies examining direct and indirect effects on protozoa and other invertebrate grazers are suggested by these community-level observations.

Comparative analyses of microbial community composition

Lectin profiling of the communities in both the winter and summer microcosm studies (Figures 2 and 6) showed that there were significant changes in the composition of the exopolymer matrix with DEET exposure. Summer exposures (Figure 6) resulted in increased levels ($p < 0.05$) of *C. ensiformis* binding indicative of an increase in the presence of α-linked mannose residues and *A. hypogaea* (terminal β-galactose) residues in the biofilm communities. Winter exposure also resulted in significant ($p < 0.05$) increases in the binding of *C. ensiformis* lectin (Figure 2). In contrast, binding sites for *U. europaeus* lectin (α-L-fucose) were significantly reduced ($p < 0.05$) in summer exposures, whereas for winter exposures there were no other significant changes in lectin binding affinities in the exopolymeric matrix (Figure 2). We have shown previously for other chemicals (Chénier et al. 2003) that biofilms developed from river water collected at different times of the year will have different microbial activities and community compositions, with the net effect being that the response to stress varies with season as well.

These changes in EPS are all consistent with an altered community structure and changes in photosynthetic and bacterial community composition in response to DEET exposure. For example, *C. ensiformis* (α-linked mannose or glucose residues) are associated with binding to algal–cyanobacterial polymers, whereas *U. europaeus* (α-L-fucose) is associated with binding to bacterial polymers (Lawrence et al. 2015). Further, it parallels the observations regarding shifts in photosynthetic pigments (Figure 3). We have previously demonstrated a relationship between changes in bacterial and photosynthetic populations and shifts in lectin profiling of the EPS (Chénier et al. 2003; Lawrence et al. 2004, 2005; Neu et al. 2005). There appears to be strong general agreement based on biomass, algal–cyanobacterial pigment abundance, as well as lectin typing that DEET at 500 ng/L has significant effects on the

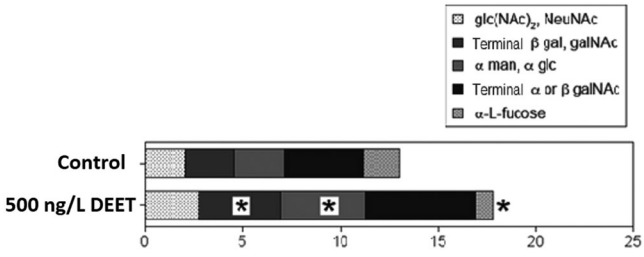

FIGURE 6: Effect of *N,N*-diethyl-m-toluamide on the composition of river biofilm exopolymer, as determined by in situ lectin binding analyses for communities cultivated in the summer. A panel of 5 lectins was used to assess the nature of the glycoconjugates present in the biofilms. *Significantly different at $p < 0.05$. DEET = *N,N*-diethyl-m-toluamide; β-gal = β-galactose; galNAc = *N*-acetylgalactosamine; glc = glucosamine; glcNAc = *N*-acetylglucosamine; man = mannose; NeuNAc = *N*-acetylneuraminic acid.

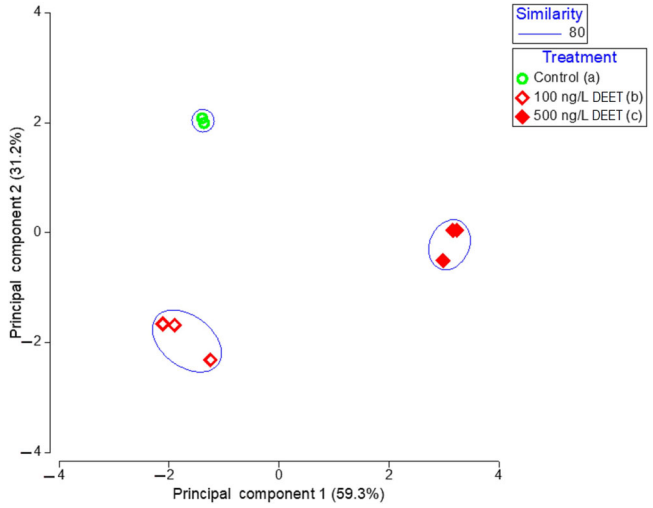

FIGURE 7: Results of principal component analyses on triplicate denaturing gradient gel electrophoresis analyses showing the differences between *N,N*-diethyl-m-toluamide-treated communities relative to the control in the South Saskatchewan River at both 100 and 500 ng/L. DEET = *N,N*-diethyl-m-toluamide. Treatments followed by a different letter (a), (b), (c) are significantly different from each other $p < 0.05$.

nature of the photosynthetic community in these riverine biofilms. The results of molecular analyses of the eubacterial populations using DGGE further substantiate these conclusions, indicating that significant differences occurred between the banding patterns observed in control and DEET-exposed communities for South Saskatchewan River. Pairwise ANOSIM of the PCA results showed that there were significant differences ($R = 1.0$, $p < 0.05$) in the structure of microbial communities between the control and both DEET treatments for South Saskatchewan River biofilm communities (Figure 7). In addition, significant differences were detected between the 2 levels of DEET exposure in South Saskatchewan River river biofilms (Figure 7).

Using high-throughput sequencing, 16S rRNA gene amplicons from control and DEET-exposed communities were analyzed, confirming that there were changes in community composition with DEET exposure, resulting in shifts in the nature of the detectable taxa (Figures 8 and 9). The control and the 100- and 500-ng/L groups had 7 shared taxonomic groups: Synechococcophycideae, Chloroplasts, Phycisphaerae, Planctomycetia, Gammaproteobacteria, Betaproteobacteria, and Alphaproteobacteria. The overlap of taxonomic groups between treatments is illustrated in the Venn diagram shown in Figure 8. However, it is also evident that there are 4 taxonomic groups that occur exclusively in the control community—Actinobacteria, Acidimicrobiia, Oscillatoriophycideae, and "other"—and one group common to both the control and 500-ng/L DEET treatment groups (the Bacilli). However, there were no taxa in common with the 100-ng/L DEET treatment. Interestingly, both the 100- and 500-ng/L DEET treatments had detectable levels of Cytophagia, a taxon unique to these treatments (Figure 9). Bacteroidetes are widely distributed in aquatic and terrestrial habitats and are frequently degraders of polymeric organic

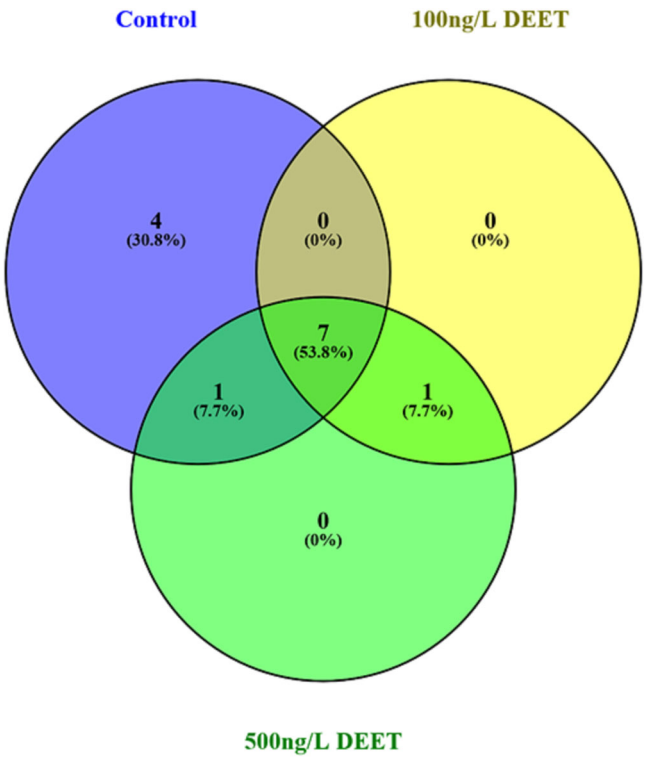

FIGURE 8: Venn diagram showing the differential detection of taxonomic groups in the control and the 100- and 500-ng/L *N,N*-diethyl-m-toluamide treatments. DEET = *N,N*-diethyl-m-toluamide.

matter and common in river biofilm communities (Anderson-Glenna et al. 2008). They have been reported as part of the core community in river plankton (Staley et al. 2013; Mukherjee et al. 2013). However, they are viewed as being most common in high-nutrient habitats (O'Sullivan et al. 2006), which is not consistent with the nutrient-limited status of the South Saskatchewan River. Shifts in abundance of Bacteroidetes and other members of the microbial community have been attributed to a variety of factors, for example, increased water residence time (Read et al. 2015) and decreasing inputs from soil and groundwater (Savio et al. 2015), whereas de Oliveira and Margis (2015) and Staley et al. (2015) indicated that physicochemical and hydrometeorological factors (including temperature, day length, and nutrient concentrations) were key. The results obtained with 16S rRNA gene amplicon analyses also indicate the loss of cyanobacteria belonging to the Oscillatoriophycideae in both DEET treatments. This is a diverse group which has been used as an indicator of contamination (Tanimu et al. 2011). They may indicate the presence of polycyclic aromatic hydrocarbons, heavy metals, high salinity, organic pollution, increased nitrogen and phosphorus, and increased fecal coliform concentration—in general, eutrophication (Johnson 2016).

However, in the controlled microcosm environment the essential factors are limited to the nature of the planktonic community available for recruitment and reproduction in the

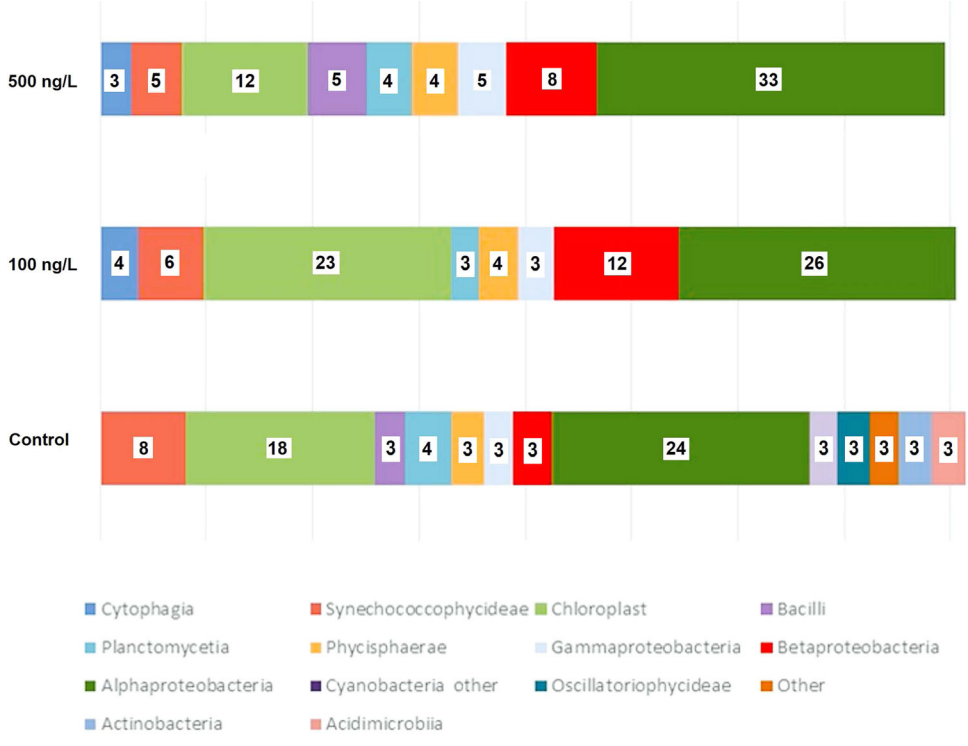

FIGURE 9: Class- and subclass-level taxonomic diversity and relative abundance of 16S ribosomal RNA gene fragments in the high-throughput library retrieved from riverine microbial biofilm communities cultivated in rotating annular reactors with and without *N,N*-diethyl-m-toluamide exposure.

biofilm community and one environmental variable, the addition of either 100 or 500 ng/L. Thus, the observed changes in community composition are mainly attributable to the direct or indirect treatment effects of DEET.

Other studies have shown that pharmaceutical and personal care compounds may be highly selective for specific groups of bacteria (Paje et al. 2002; Lawrence et al. 2005). For example, using fluorescent in situ hybridization, Lawrence et al. (2005) reported that ibuprofen increased the Alpha-proteobacteria, Beta-proteobacteria, Cytophaga-flavobacteria, and SRB385 probe-positive populations, whereas caffeine and carbamazepine additions resulted in significant increases in the high GC354c and low GC69a probe-positive cells. It is possible that persistent low-concentration contaminants such as DEET have significant subtle effects on the structure and function of the microbial community not detected by gross measures such as biofilm thickness, biomass, chlorphyll a, carbon utilization, or thymidine incorporation.

CONCLUSIONS

Microscale analyses using confocal laser microscopy and selected probes revealed a visible impact of DEET on the biofilm community architecture, morphology of cell types, and community composition. Digital image analyses of the confocal microscopic images indicated significant reductions in photosynthetic biomass ($p < 0.05$). Toxicity to phototrophs may have significant impacts on the phototrophic–heterotrophic bacterial linkages in the community, impacting productivity, energy flow, and carbon cycling (Haak and McFeters 1982a, 1982b) as well as biogeochemical processes such as nitrogen cycling (Lawrence et al. 2004; Dokianakis et al. 2004). Given that microbial biofilms also represent the base of the food web (Battin et al. 2009) and a significant food source for a wide variety of invertebrate and vertebrate grazers in aquatic habitats, reductions in the quantity (biomass) and quality (i.e., community composition and nature) of the exopolysaccharides present may have significant impacts for these higher trophic levels. Indeed, effects of contaminants on "biofilm" quality have been shown to influence the development and growth of aquatic invertebrates (Lowell et al. 1995). Biodiversity analyses using DGGE assessed by PCA substantiated that the presence or absence of specific community members resulted in strong separation of DEET-exposed and control communities. Gene sequence analyses confirmed significant changes in community composition, indicating effects on bacterial taxa including cyanobacteria. Impacts of DEET on invertebrates and protozoa can significantly alter their critical role as ecosystem engineers (Jones et al. 1994), with far-reaching impacts on community activity. These community-level studies suggest greater biological relevance for this contaminant and a need for further toxicological assessments of the effects of DEET on protozoa, bacteria, and, in particular, phototrophic organisms.

Acknowledgment—The present study was supported by funding from Environment and Climate Change Canada, the University of Saskatchewan, and Health Canada.

Disclaimer—The authors declare no conflict of interest.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (john.lawrence2@canada.ca).

REFERENCES

Anderson-Glenna MJ, Bakkestuen V, Clipson NJW. 2008. Spatial and temporal variability in epilithic biofilm bacterial communities along an upland river gradient. *FEMS Microbiol Ecol* 64:407–418.

Aronson D, Weeks J, Meylan B, Guiney PD, Howard PH. 2012. Environmental release, environmental concentrations, and ecological risk of N,N-diethyl-m-toluamide (DEET). *Integr Environ Assess Manag* 8:135–166.

Battin TJ, Kammer FVD, Weilhartner A, Ottofuelling S, Hofmann T. 2009. Nanostructured TiO_2: Transport behavior and effects on aquatic microbial communities under environmental conditions. *Environ Sci Technol* 43:8098–8104.

Boon N, Windt WD, Verstraete W, Top EM. 2002. Evaluation of nested PCR DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol Ecol* 39:101–112.

Brausch JM, Rand GM. 2011. A review of personal care products in the aquatic environment: Environmental concentrations and toxicity. *Chemosphere* 82:1518–1532.

Chénier MR, Beaumier D, Fortin N, Roy R, Driscoll BT, Lawrence JR, Greer CW. 2006. Influence of nutrient inputs, hexadecane and temporal variations on denitrification and community composition of river biofilms. *Appl Environ Microbiol* 72:575–584.

Chénier MR, Beaumier D, Roy R, Driscoll BT, Lawrence JR, Greer CW. 2003. Impact of seasonal variations and nutrient inputs on the cycling of nitrogen and the degradation of hexadecane by replicated river biofilms. *Appl Environ Microbiol* 69:5170–5177.

Clarke KR. 1993. Non-parametric multivariate analyses of changes in community structure. *Austral Ecol* 18:117–143.

Cordy G, Duran N, Bower H, Rice R, Furlong ET, Zaugg SD, Meyer MT, Barber LB, Kolpin DW. 2004. Do pharmaceuticals, pathogens, and other organic wastewater compounds persist when wastewater is used for recharge? *Ground Water Monit Remediat* 24:58–69.

Costanzo SD, Watkinson AJ, Murby EJ, Kolpin DW, Sandstrom MW. 2007. Is there a risk associated with the insect repellent DEET (*N,N*-diethyl-m-toluamide) commonly found in aquatic environments? *Sci Total Environ* 384:214–220.

Davis J, Ekman D, Teng Q, Ankley G, Berninger J, Cavallin J, Jensen K, Kahl M, Schroeder A, Villeneuve D, Jorgenson Z, Lee K, Collette T. 2016. Linking field-based metabolomics and chemical analyses to prioritize contaminants of emerging concern in the Great Lakes basin. *Environ Toxicol Chem* 35:2493–2502.

de Oliveira LFV, Margis R. 2015. The source of the river as a nursery for microbial diversity. *PLoS One* 10:e120608.

Dokianakis SN, Kornaros ME, Lyberatos G. 2004. On the effect of pharmaceuticals on bacterial nitrite oxidation. *Water Sci Technol* 50:341–346.

Glassmeyer ST, Furlong ET, Kolpin DW, Cahill JD, Zaugg SD, Werner SL, Meyer MT, Kryak DD. 2005. Transport of chemical and microbial compounds from known wastewater discharges: Potential for use as indicators of human fecal contamination. *Environ Sci Technol* 39:5157–5169.

Haak SK, McFeters GA. 1982a. Microbial dynamics of an epilithic mat community in a high alpine stream. *Appl Environ Microbiol* 43:702–707.

Haak SK, McFeters GA. 1982b. Nutritional relationships among microorganisms in an epilithic biofilm community. *Microb Ecol* 8:115–126.

Herlemann DP, Labrenz M, Jurgens K, Bertilsson S, Waniek JJ, Andersson AF. 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J* 5:1571–1579.

Johnson WH. 2016. Stream microbial communities as potential indicators of river and landscape disturbance in north-central Arkansas. MS thesis, University of Arkansas, Fayettevill, AK, USA. [cited 2019 March 18]. Available from: http://scholarworks.uark.edu/etd/1624

Jones CG, Lawton JH, Shackak M. 1994. Organisms as ecosystem engineers. *Oikos* 69:373–386.

KemI. 2010. Assessment report, N,N-diethyl-meta-toluamide (DEET), Product-type 19 (repellents and attractants). KemI (Swedish Chemicals Agency), Sundbyberg, Sweden. [cited 2011 May 25]. Available from: http://www.kemi.se/default____550.aspx

Knepper TP. 2004. Analysis and fate of insect repellents. *Water Sci Technol* 50:301–308.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. *Environ Sci Technol* 36:1202–1211.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 79:5112–5120.

Lawrence JR, Chénier M, Roy R, Beaumier D, Fortin N, Swerhone GDW, Neu TR, Greer CW. 2004. Microscale and molecular assessment of the impacts of nickel, nutrients and oxygen level on river biofilm communities. *Appl Environ Microbiol* 70:4326–4339.

Lawrence JR, Neu TR, Paule A, Korber DR, Wolfaardt GW. 2016a. Aquatic biofilms: Development, cultivation, analyses, and applications. In Yates M, Nakatsu C, Miller R, Pillai S, eds, *Manual of Environmental Microbiology*, 4th ed. ASM, Washington, DC, pp 4.2.3-1–4.2.3-33.

Lawrence JR, Swerhone GDW, Neu TR. 2000. Design and evaluation of a simple rotating annular reactor for replicated biofilm studies. *J Microb Methods* 42:215–224.

Lawrence JR, Swerhone GDW, Topp E, Korber DR, Neu TR, Wassenaar LI. 2007. Structural and functional responses of river biofilm communities to the non-steroidal anti-inflammatory diclofenac. *Environ Toxicol Chem* 26:236–245.

Lawrence JR, Swerhone GDW, Wassenaar LI, Neu TR. 2005. Effects of selected pharmaceuticals on riverine biofilm communities. *Can J Microbiol* 51:655–669.

Lawrence JR, Topp E, Waiser MJ, Tumber V, Roy J, Swerhone GDW, Leavitt P, Paule A, Korber DR. 2015. Resilience and recovery: The effect of triclosan exposure timing during development, on the structure and function of river biofilm communities. *Aquat Toxicol* 161:253–266.

Lawrence JR, Waiser MJ, Swerhone GDW, Tumber V, Roy J, Paule A, Hitchcock AP, Dynes JJ, Korber DR. 2016b. Effects of fullerene (C60), multi-wall carbon nanotubes (MWCNT), single wall carbon nanotubes (SWCNT) and hydroxyl and carboxyl modified single wall carbon nanotubes on riverine microbial communities. *Environ Sci Pollut Res* 23:10090–10102.

Lawrence JR, Zhu B, Swerhone GDW, Roy J, Tumber V, Waiser MJ, Topp E, Korber DR. 2012. Molecular and microscopic assessment of the effects of caffeine, acetaminophen, diclofenac and their mixtures on river biofilm communities. *Environ Toxicol Chem* 31:508–517.

Lawrence JR, Zhu B, Swerhone GDW, Topp E, Roy J, Wassenaar LI, Rema T, Korber DR. 2008. Effects of the broad spectrum antimicrobial chlorhexidine on river microbial biofilm development, community composition and activity. *Appl Environ Microbiol* 74:3541–3550.

Lawrence JR, Zhu B, Swerhone GDW, Roy J, Wassenaar LI, Topp E, Korber DR. 2009.Comparative microscale analysis of the effects of triclosan and triclocarban on the structure and function of river biofilm communities. *Sci Total Environ* 407:3307–3316.

Leavitt PR, Hodgson DA. 2001. Sedimentary pigments. In Smol JP, Birks HJB, Last WM, eds, *Tracking Environmental Change Using Lake Sediments: Terrestrial, Algal, and Siliceous Indicators, Vol 3—Developments in Peleoenvironmental Research*. Kluwer Academic, Dordrecht, The Netherlands, pp 2–21.

Lowell RB, Culp JC, Wrona FJ. 1995. Stimulation of increased short-term growth and development of mayflies by pulp mill effluent. *Environ Toxicol Chem* 14:1529–1541.

Manz W, Wendt-Potthoff K, Neu TR, Szewzyk U, Lawrence JR. 1999. Phylogenetic composition, spatial structure and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal scanning laser microscopy. *Microb Ecol* 37:225–237.

Mukherjee S, Kumar D, Nanda AK, Chakraborty R. 2013. 16S rRNA gene sequence analyses of the metagenome derived from waters of River Mahananda at Siliguri: An approach to understand bacterial diversity. *Indian J Biotechnol* 12:80–87.

Muyzer G, De Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700.

Muyzer G, Ramsing NB. 1995. Molecular methods to study the organization of microbial communities. *Wat Sci Technol* 32:1–9.

Neu TR, Swerhone GDW, Bockelmann U, Lawrence JR. 2005. Effect of carbon, nitrogen and phosphorus on the nature and development of lectin-specific glycoconjugates in lotic biofilms. *Aquat Microb Ecol* 38:283–294.

Neu TR, Swerhone GDW, Lawrence JR. 2001. Assessment of lectin-binding analysis for in situ detection of glycoconjugates in biofilm systems. *Microbiology* 147:299–313.

Neu TR, Wölfl S, Lawrence JR. 2004. Three-dimensional differentiation of phototrophic biofilm constituents by multi channel confocal and 2-photon laser scanning microscopy. *J Microbiol Methods* 56:161–172.

Nusch E. 1980. Comparison of different methods for chlorophyll and phaeopigment determination. *Archiv fur Hydrobiologie* 14:14–36.

Okuda T, Yamashita N, Tanaka H, Matsukawa H, Tanabe K. 2009. Development of extraction method of pharmaceuticals and their occurrences found in Japanese wastewater treatment plants. *Environ Int* 35:815–820.

O'Sullivan LA, Rinna J, Humphreys G, Weightman AJ, Fry JC. 2006. Culturable phylogenetic diversity of the phylum 'Bacteroidetes' from river epilithon and coastal water and description of novel members of the family *Flavobacteriaceae: Epilithonimonas tenax* gen. nov., sp. nov. and *Persicivirga xylanidelens* gen. nov., sp. nov. *Int J Syst Evol Microbiol* 56:169–180.

Paje MLF, Kuhlicke U, Winkler M, Neu TR. 2002. Inhibition of lotic biofilms by diclofenac. *Appl Microbiol Biotechnol* 59:488–494.

Porsbring T, Arrhenius A, Backhaus T, Kuylenenstierna M, Scholze M, Blanck H. 2007. The SWIFT periphyton test for high-capacity assessments of toxicant effects on microalgal community development. *J Exp Mar Biol Ecol* 349:299–312.

Proia L, Cassio F, Pascoal C, Tlili A, Romanı AM. 2012. The use of attached microbial communities to assess ecological risks of pollutants in river ecosystems: The role of heterotrophs In Guasch. H, Ginebreda A, Geiszinger A, eds, *Emerging and Priority Pollutants in Rivers: Bringing Science into River Management Plans, Vol 19—Handbook of Environmental Chemistry*. Springer Verlag, Berlin, Germany, pp 55–84.

Read DS, Gweon HS, Bowes MJ, Newbold LK, Field D, Bailey MJ, Griffiths RI. 2015. Catchment-scale biogeography of riverine bacterioplankton. *ISME J* 9:516–526.

Rivera-Cancel G, Bocioaga D, Hay AG. 2007. Bacterial degradation of *N,N*-diethyl-m-toluamide (DEET): Cloning and heterologous expression of DEET hydrolase. *Appl Environ Microbiol* 73:3105–3108.

Robarts RD, Wicks RJ. 1989. [Methyl-3H] thymidine macromolecular incorporation and lipid labelling: Their significance to DNA labeling during aquatic bacterial growth rate measurements. *Limnol Oceanogr* 34:213–222.

Sabater S, Guasch H, Ricart M, Romani A, Vidal G, Klunder C, Schmitt-Jansen M. 2007. Monitoring the effect of chemicals on biological communities. *The biofilm as an interface. Anal Bioanal Chem* 387:1425–1434.

Sandstrom MW, Kolpin DW, Thurman EM, Zaugg SD. 2005. Widespread detection of *N,N*-diethyl-m-toluamide in US streams: Comparison with concentrations of pesticides, personal care products, and other organic wastewater compounds. *Environ Toxicol Chem* 24:1029–1034.

Savio D, Sinclair L, Ijaz UZ, Parajka J, Reischer GH, Stadler P, Blaschke AP, Blöschl G, Mach RL, Kirschner AK. 2015. Bacterial diversity along a 2600 km river continuum. *Environ Microbiol* 17:4994–5007.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing Mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–7541.

Seo J, Lee YG, Kim SD, Cha CJ, Ahn JH, Hur HG. 2005. Biodegradation of the insecticide *N,N*-diethyl-m-toluamide by fungi: Identification and toxicity of metabolites. *Arch Environ Contam Toxicol* 48:323–328.

Stackelberg PE, Furlong ET, Meyer MT, Zaugg SD, Henderson AK, Reissman DB. 2004. Persistence of pharmaceutical compounds and other organic

wastewater contaminants in a conventional drinking water–treatment plant. *Sci Total Environ* 329:99–113.

Staley C, Gould TJ, Wang P, Phillips J, Cotner JB, Sadowsky MJ. 2015. Species sorting and seasonal dynamics primarily shape bacterial communities in the upper Mississippi River. *Sci Total Environ* 505:435–445.

Staley C, Unno T, Gould TJ, Jarvis B, Phillips J, Cotner JB, Sadowsky MJ. 2013. Application of Illumina next-generation sequencing to characterize the bacterial community of the upper Mississippi River. *J Appl Microbiol* 115:1147–1158.

Sui Q, Huang J, Deng S, Yu G, Fan Q. 2010. Occurrence and removal of pharmaceuticals, caffeine and DEET in wastewater treatment plants of Beijing, China. *Water Res* 44:417–426.

Tanimu Y, Bako SP, Adakole JA, Tanimu J. 2011. Phytoplankton as bioindicators of water quality in saminaka reservoir, Northern-Nigeria. *Proceedings*, International Symposium on Environmental Science and Technology, Dongguan, Guandong Province, China. Science Press, USA, pp 318–322.

Tice R, Brevard B. 1999. *N,N*-Diethyl-m-toluamide (DEET) [134-62-3]: Review of toxicological literature. National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. [cited 2007 April 22]. Available from: http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/DEET.pdf

US Environmental Protection Agency. 1998. Re-registration eligibility decision (RED) DEET. EPA 738-R-98-010. US Environmental Protection Agency, Washington, DC.

Waiser MJ, Humphries D, Tumber V, Holm J. 2011. Effluent-dominated streams. Part 2: Presence and possible effects of pharmaceuticals and personal care products in Wascana Creek, Saskatchewan, Canada. *Environ Toxicol Chem* 30:508–519.

Waiser M, Robarts RD. 1997. Impacts of a herbicide and fertilizers on the microbial community of a saline prairie lake. *Can J Fish Aquat Sci* 54:320–329.

Weeks J, Guiney P, Nikiforov A. 2012. Assessment of the environmental fate and ecotoxicity of *N,N*-diethyl-m-toluamide (DEET). *Integr Environ Assess Manag* 8:118–132.

Weigel S, Berger U, Jensen E, Kallenborn R, Thoresen H, Huhnerfuss H. 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromso/Norway with emphasis on ibuprofen and its metabolites. *Chemosphere* 56:583–592.

Weigel S, Kuhlmann J, Huhnerfuss H. 2002. Drugs and personal care products as ubiquitous pollutants: Occurrence and distribution of clofibric acid, caffeine and DEET in the North Sea. *Sci Total Environ* 295:131–141.

Wilson BA, Smith VH, deNoyelles F Jr, Larive CK. 2003. Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environ Sci Technol* 37:1713–1719.

Xue RD, Barnard DR, Ali A. 2000. Laboratory toxicity of three mosquito oviposition repellents to six nontarget aquatic invertebrates. *Environ Entomol* 29:437–441.

