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ORIGINAL PAPER

Elucidating stream bacteria utilizing terrestrial dissolved organic matter

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Abstract

Terrestrial dissolved organic matter (tDOM) is susceptible to microbiological and photolytic oxidations and contributes significantly to the energy flow in aquatic ecosystems. However, bacterial species actively utilizing this tDOM are still not determined. We here elucidated the microbial groups actively utilizing tDOM. We characterized sediment microbial biomass and community structure using phospholipid phosphate and phospholipid fatty acids (PLFAs) analysis, respectively, and identified metabolically active members using PLFA stable-isotope-probing. Prokaryotes comprised 61% of the streambed microbial community consisting of aerobic, facultative anaerobic and anaerobic bacteria while microeukaryotes comprised the remaining 39%. Sediments were incubated in re-circulating mesocosm chambers amended with leachate from composted ^{13}C-labelled tulip poplar (*Liriodendron tulipifera L.*) tree-tissues and examined for ^{13}C incorporation into microbial PLFAs. The structure of stream sediment microbial communities prior to and after mesocosm incubation, in both the presence and absence of ^{13}C-labeled DOM, showed no significant differences and indicated our mesocosm-based experimental design as sufficiently robust to investigate the utilization of ^{13}C-DOM by sediment microbial communities. After 48 h of incubation, bacterial fatty acids i15:0, a15:0, 16:0, 16:1ω9, 18:1ω9c, 18:1ω7c, 10me16 and cy19:0 showed increased abundance of ^{13}C. This identified the aerobic, facultative anaerobic and anaerobic bacteria as actively utilizing the ^{13}C-labeled DOM. A single dark 48 h incubation showed incorporation into both bacterial and microeukaryotic fatty acids (20:4ω6, 20:5ω3) suggesting that microeukaryotic predators consumed bacteria that utilized ^{13}C-labeled DOM. Hence, our data support the hypothesis that streamwater tDOM is utilized by stream bacteria, and substantially contributes to the energy flow in aquatic ecosystems.

Keywords Dissolved organic matter · Mesocosm · Fatty acids · Bacteria · Microeukaryotic · Stable isotope

Introduction

Dissolved organic matter (DOM) plays a significant metabolic role in aquatic ecosystems as carbon and energy sources for the microbial food web (Peduzzi et al. 2008; Wiegner et al. 2009; Wong and Williams 2010; Wu et al. 2019). It influences the availability of dissolved nutrients and metals, and modifies the optical properties of aquatic ecosystems (Findlay and Sinsabaugh 1999; Sulzberger and Durisch-Kaiser 2009). In addition, DOM is now seen as an important driver of ecosystem functions in freshwater environments and a major component in global carbon cycling and climate change (Amon and Benner 1996; Battin et al. 2008; Besemer et al. 2009; Wu et al. 2019). DOM as a complex mixture is present in all natural waters and is continuously supplied to aquatic ecosystems from both allochthonous (terrestrial) and autochthonous (aquatic) sources (Peduzzi et al. 2008; Wagner et al. 2015). Amon and Benner (1996) reported that humic substances of terrestrial origin are the major constituents of the DOM pool in stream ecosystems and comprise up to 88% of the DOM in the high molecular weight fraction in the Amazon River water. These findings agree with other studies, which found that, in most cases, terrestrial DOM (tDOM) comprised a large portion of DOM in streams and rivers (Benner and Hedges 1993; Hedges et al. 1994; Peduzzi et al. 2008; Kaplan and Cory 2016). Conventionally, tDOM has been

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considered recalcitrant to bacterial biodegradation and to move conservatively through aquatic ecosystems due to the apparent biochemical refractory nature of humic substances (Mantoura and Woodward 1983; Thurman 1986; Rosenstock et al. 2005). However, Volk et al. (1997) found that humic substances account for 75% of the biodegradable fraction of DOM in White Clay Creek. Similar studies confirm the susceptibility of tDOM to microbiological and photolytic oxidations, as well as their value as microbial substrates and their significant contribution to energy flow in aquatic ecosystems (Amon and Benner 1996; Bano et al. 1997; Carlsson et al. 1999; Frazier et al. 2005; Kaplan et al. 2008; Battin et al. 2008; Fagerberg et al. 2009; Lipczynska-Kochany 2018; Bowen et al. 2019).

Heterotrophic benthic bacteria are important organisms in lotic ecosystems and are responsible for several biogeochemical transformations, including DOM uptake, degradation, and mineralization (Kaplan and Newbold 1993; Pusch et al. 1998**;** Fischer and Pusch 2001; Tank et al. 2010). While it is clearly established that bacteria provide an important trophic linkage between DOM and many stream fauna (Hall and Meyer 1998; Kaplan and Cory 2016), their relative importance in overall stream carbon processing remains relatively understudied (Tank et al. 2010). In particular, little is known about which heterotrophic benthic bacteria drive these ecosystem dynamics. Moreover, research efforts to understand DOM utilization through microbial processes have been complicated by the chemical heterogeneity of the DOM pool, since the relationship between both is complicated and bidirectional (Mosher et al. 2010) and a lack of methods for measuring in situ microbial activities (Kaplan et al. 2008; Bourguet et al. 2009). Few studies have attempted to identify substrates that would be representative of natural molecules providing realistic data regarding DOM utilization. Such attempts include $NaH^{13}CO_3$ additions in lakes (Kritzberg et al. 2004; Pace et al. 2004) and ^{13}C-enriched sodium acetate additions in streams (Hall and Meyer 1998; Johnson and Tank 2009). However, being chemically much simpler than terrestrially derived DOM, these tracers are not reflective of natural streamwater DOM. This major drawback has been addressed by the production of a tDOM tracer; ^{13}C-labeled tree tissue leachate from tulip poplar tree leaves, small twigs and roots with polymeric and monomeric constituents and lability fractions approximating those of streamwater DOM (Wiegner et al. 2005a). Using this ^{13}C-DOM tracer, Wiegner et al. (2005b) reported that labile DOM is taken up quickly at or near its point of entry to the stream, whereas intermediately labile /humic DOM is consumed more slowly and a substantial proportion is exported to downstream reaches, thus humic DOM serves as an important energy link between upstream and downstream systems (Kaplan et al. 2008; Bowen et al. 2019). These results indicated that ^{13}C-labeled tree tissues leachate is the most representative tracer of tDOM inputs to streams that has been used to date and is well suited to investigate the utilization of DOM by heterotrophic benthic microbes.

The goal of our study was to elucidate the heterotrophic benthic microbes within stream sediments that actively utilize tDOM, thereby controlling carbon flux to higher trophic levels and to downstream reaches. We examined incorporation of tDOM into microbial biomass by incubating stream sediment in recirculating mesocosms with natural streamwater to which tracer-levels of ^{13}C-labeled tree tissues leachate were added. We used phospholipid fatty acid (PLFA) analysis to characterize the benthic microbial biomass and community structure (Findlay 2004) and PLFA stable isotope probing (SIP) to determine the metabolically active community members (Boschker et al. 1998; Boschker 2004). After microbial cell death, fatty acids are quickly degraded ensuring that only viable microbes are investigated. Thus, the carbon or energy transfer within the microbial food web can be investigated by PLFA. We have extended the specificity of functional assignments of PLFA-SIP by utilizing clone libraries produced from White Clay Creek (WCC) sediments by Hullar et al. (2006) and published phenotypic descriptions of identified species (e.g., Hahn et al. 2010; Jin et al. 2012).

Materials and methods

Study site

Streamwater and sediments were collected from 3rd order WCC adjacent to the Stroud Water Research Center in Avondale, Pennsylvania. The WCC watershed is agriculturally dominated with upstream riparian forests and is within the Piedmont Province of southeastern Pennsylvania and northern Delaware (39°53'N, 75°47'W), joining the Christina River near the Christina's discharge to the Delaware Bay. WCC drains 725 ha of approximately 52% of agricultural, 22% of tilled/hayed and 23% of wooded lands (Newbold et al. 1997; Wiegner et al. 2005b). The immediate area surrounding the study site is forested and the local drainage is a patchwork of pasturelands grazed by horses and cattle. The dominant tree species reported are tulip poplar (*Liriodendron tulipefera*), beech (*Fagus grandifolia*), red oak (*Quercus rubra),* and black oak (*Quercus velutina*) (Wiegner et al. 2005b). Stream flow and streamwater chemistry have been monitored at regular intervals since the 1970s with mean annual stream flow, streamwater temperature, and local precipitation of 115 L/s, 10.6 °C, and 105 cm y^{-1}, respectively (Newbold et al. 1997). Streambed sediments consist of clay-, silt-, and sand-sized particles in pools and runs, with gneiss- and schist-derived gravel and cobble in riffles (Kaplan et al. 1980).

Synthesis of ^{13}C-labeled DOM

Wiegner et al. (2005a) describes the generation of ^{13}C-labeled stream DOM. Briefly, thirty-two 1-y-old tulip poplar seedlings (*Liriodendron tulipefera L.*) were grown with $^{13}CO_2$ at the National Phytotron located at Duke University, Durham, North Carolina, USA. ^{13}C-tree tissue leachate was generated by leaching approximately 4 g of dried, ground tulip poplar seedlings tissues (60.9% leaves, 24.6% stems, 14.5% roots; % weight of new tissues) in 4 L of sterile-filtered (0.2-µm, Gelman Supor) C-free de-ionized cold water in the dark at 4 °C for 24 h. The mixture of all tree tissue types (leaf, stem, and root) was used to generate a leachate representative of fresh tree litter inputs (organic matter inputs) from trees to streams. Tree tissue leachate was Tyndallized in a 70 °C water bath for 0.5 h twice, separated by 24 h at room temperature to ensure biological stability, and stored in 2-L sterile plastic containers in the dark at 4 °C (Wiegner et al. 2005a) until the experiment began. The leachate had a δ^{13}C value of +4843‰, comprising of humic substances (25% of C) and polysaccharides (8% of C), and two distinct DOM lability classes (readily and intermediately labile) (Wiegner et al. 2005a).

Mesocosms

Two 15-L recirculating plug-flow bioreactors with Venturi flume inserts and an empty bed contact time of 150 min were used to elucidate the microbes responsible for utilization of DOM in streams. Bioreactors accepted a 0.014 m^2 galvanized box used to sample stream sediments such that the stream sediments were level with the bioreactor bed with their top surfaces contiguous with the front ramps of the flumes (Fig. 1). The Venturi flume improved control of current velocity over the sediments, and sondes with dissolved O_2 and temperature/conductivity probes (YSI Model 600 XL, Yellow Springs, Inc., Yellow Springs, Ohio) were inserted into the recirculation line of each mesocosm (Wiegner et al. 2005b). Bioreactors could be operated in either open or recirculating mode (i.e. water was recirculated through the system) and were contained within a 1000-L flowing streamwater tank to maintain ambient stream temperature. These tanks also served as the source of streamwater during open mode operation. Mesocosms were set up in the experimental greenhouse facility of Stroud Water Research Centre under natural photoperiod except for experiment 1 when mesocosms were covered with black plastic to simulate night condition (see below).

Sediment collection and the mesocosm experiments

Four sediment cores were collected from WCC twice within a four-week period in October and November 2009 to reduce seasonal differences (Table 1). To collect stream sediments with a minimum of disturbance, a galvanized box (surface area 0.014 m^2) perforated by 0.32-cm diameter holes (bottom only) that allowed streamwater to escape as the box was inserted into the sediments until the bottom just touched the sediment surface. Plexiglas plates were slipped under and over the box trapping the sediments and allowed them to be lifted from the stream intact. A second box, just large enough to accommodate the first, was place over the first box, the core inverted and the inner (first) box removed. This process yielded a rectangular core or sample of intact stream sediment that was placed into a bioreactor in the proper vertical orientation. Two samples were processed immediately and served as a reference for sediment and microbial community characteristics before the ^{13}C-DOM uptake experiment (stream control; referred to as T0). The other two boxes were

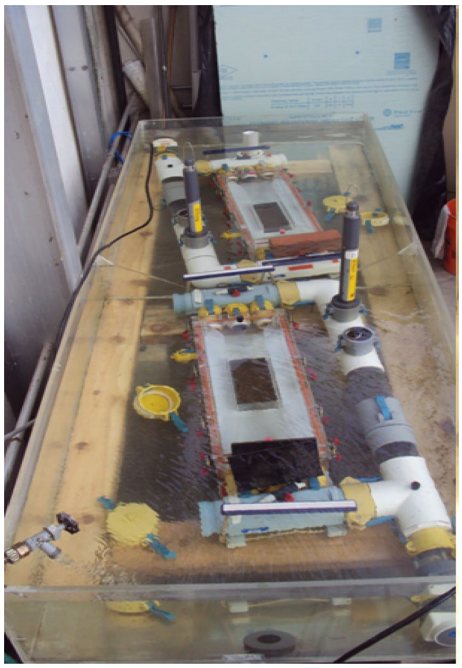

Fig. 1 Mesocosm setup for ^{13}C leachate uptake measurement, including streamwater-fed bioreactors/ mesocosm chambers with Venturi flume inserts containing sediments and water jackets. One chamber without ^{13}C leachate amendment served as experimental control

Table 1 Experimental design of ^{13}C-DOM microbial uptake experiments of White Clay Creek sediments

Experiment/ Mesocosm condition	Sampling Date/^{13}C DOM injected (µg/L)	Experiment duration (h)
1. Dark	12–15 Oct., 2009 / 17.47	48
2. Natural photoperiod	20–23 Oct., 2009 / 17.47	50
3. Natural photoperiod	3–6 Nov., 2009 / 17.47	50
4. Natural photoperiod	9–12 Nov., 2009 / 8.73	50

placed into 15-L recirculating bioreactors; one per bioreactor (Fig. 1), which were operated for 24 h in open mode with a flow of 0.06 cm/s (equivalent to measured stream velocities prior to the experiment). The depth of the water directly above the sediments was 0.04 m, equivalent to WCC water depth. The chambers were switched to recirculate mode and one of the chambers (experimental; referred to as T^{13}C) received ^{13}C DOM while the other chamber (mesocosm control; referred to as TM) received no ^{13}C DOM.

Addition of the tracer increased total DOM by approximately 1–5%. Sediments were incubated in recirculation mode for 48 h or 50 h, and the ^{13}C exposure period was based upon the effective depth of the chambers (ratio of chamber volume to sediment tray area) and the mass transfer coefficient for the ^{13}C tracer (estimated from a prior whole-stream injection). At the end of the incubation period, the core was removed from the bioreactor, the upper 2 mm of sediments were scraped off with a clean spatula, placed in an aluminum weigh boat, well mixed, and subsampled for total microbial biomass, microbial community structure, δ^{13}C of PLFAs, organic matter contents and particle-size analyses. In total four mesocosm experiments were conducted over a month period. Experiment 1 was run in the dark for 48 h; the mesocosm chambers were covered with 2.2 cm Styrofoam sheets and black plastic sheeting to exclude light (Table 1). In experiment 2 sediments were incubated uncovered (exposed to natural sunlight at natural photoperiod) for 50 h. Experiments 3 and 4 were replicates of experiment 2. The concentrations of the ^{13}C- tree tissue leachate used and exposure times are shown in Table 1.

Phospholipid fatty acids analysis

Samples for lipid analysis were stored at −80 °C until lyophilization. Microbial biomass and the community structure of freeze-dried sediments (approximately 10 g dry weight) were determined using phospholipid analyses following the methods of Findlay (2004). Briefly, stream and mesocosm sediments were extracted in the dark at 4 °C in 50 ml screw-cap glass tubes with 27 ml of a 1:2:0.6 (*v*/v/v) dichloromethane-methanol-50 mM phosphate buffer (pH 7.4) solution. The solution was partitioned into organic and aqueous phases with 7.5 ml dichloromethane and 7.5 ml deionized water, after which the organic phase (containing total lipid) was collected through a dry 2 V filter (Whatman, Schleicher & Schuell) into 15 ml test tubes and the solvent dried under nitrogen at 37 °C. The dried lipid was dissolved in 2 ml chloroform and two 100 µl subsamples were oxidized with potassium persulfate at 100 °C overnight in sealed ampoules to release orthophosphate. Phosphate content was determined spectrophotometrically (610 nm) using a dye-coupled reaction between ammonium molybdate and malachite green. The remainder of the lipid was fractionated into neutral, glyco-, and phospholipid with silica gel solid phase extraction chromatography. Phospholipid fatty acids were converted into their respective methyl esters by base methanolysis and purified by octadecyl bonded silica gel (C18) reverse-phase column chromatography. Purified fatty acid methyl esters (FAMEs) were identified and quantified using an Agilent gas chromatograph equipped with an automatic sampler, a 60 m × 0.25 mm non-polar DB-1 column and a flame ionization detector. Hydrogen was used as the carrier gas at a flow rate of 2.3 ml/min. The initial chromatograph oven temperature was 80 °C followed by a temperature rise of 4 °C/min to 250 °C which was then held at this temperature for 10 min. FAME identification was based on relative retention times, coelution with standards, and mass spectral analysis. Standard nomenclature was used to refer to the fatty acids: the total number of carbon atoms is followed by a colon, and the number of double bonds. The position of the first double bond is indicated by ω and the number of carbon atoms from the aliphatic end. For example, the fatty acid 16:1ω7, is 16 carbons long, and has one double bond that occurs at the seventh carbon from the *omega* end of the molecule. All double bonds are *cis*, unless designated as *trans* configuration using a suffix of *t*. Methyl branching at the *iso* and *anteiso* positions and at the 10th carbon atom from the carboxyl end is designated by the prefixes *i*, *a,* and 10Me, respectively. The prefix *cy* denotes cyclopropane fatty acids. Individual fatty acids were analyzed for both absolute and relative abundance. Relative abundance or weight percent data (gram individual fatty acids x gram^{-1} total fatty acids × 100) was used to determine community structure (Findlay and Dobbs 1993). In addition to the standard combination of functional group and marker fatty acid assignments (Findlay 2004), we increased the specificity of PLFA taxonomic assignments using a previously published 16S rRNA gene library constructed from WCC sediments (Hullar et al. 2006) and the taxonomic descriptions of these operational taxonomic units (OTUs) or closely related species.

Microbial community utilization of ^{13}C DOM

The δ^{13}C was determined by gas chromatography/combustion/isotope ratio mass spectrometry using an Isoprime isotope ratio mass spectrometer (IRMS; Elementar, UK) coupled to a 7890-gas chromatograph (GC; Agilent Technologies, USA) via a combustion and reduction furnace. Analyses were conducted on two different gas chromatographic columns (DB-1 and DB-23; 60 m × 0.25 mm, 0.25 µm film thickness) to allow the analysis of a greater number of resolved FAMEs. For FAMEs that were not resolved by either column during GC-IRMS analysis, we report the δ^{13}C of the summed feature. Stable carbon isotope ratios were expressed as:

$$\delta^{13}C = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000 \quad (1)$$

where R is $^{13}C/^{12}C$ in the samples and standard. Data were reported relative to Vienna PeeDee-.

Belemnite (VPDB). Incorporation of ^{13}C into PLFAs was estimated using the equation (Abraham et al. 1998):

$$\delta^{13}C_{FA} = \left[\left(C_n + 1\right) * \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}\right]/C \quad (2)$$

where $\delta^{13}C_{FA}$ is the $\delta^{13}C$ of the fatty acid, C_n is the number of carbons in the fatty acid, $\delta^{13}C_{FAME}$ is the $\delta^{13}C$ of the fatty acid methyl ester, and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ of the methanol used for the methylation reaction, which was determined to be −43.23‰.

Statistical analysis

We used comparisons of total microbial biomass and community structure to assess the efficacy of our mesocosm approach with a comparison of stream sediments (T0) to the control mesocosm sediments (TM) used to assess mesocosm effects, and comparison of control mesocosm sediments to treatment mesocosm sediments ($T^{13}C$) used to check for unwanted stimulation due to tracer-level DOM additions. Potential differences in microbial biomass and the percent of eukaryotes vs. prokaryotes were examined with analysis of variance (ANOVA) with a α-level of 0.05. Fatty acid profiles for the bioreactors and WCC sediments were subjected to principal component analysis (PCA) after log transformation [ln (x + 1)] of weight percent fatty acid data and analyzed using SPSS 19. Changes in microbial community structure were examined by comparing principal component 1 scores as above (for microbial biomass). Increases in $\delta^{13}C$ values of individual fatty acids, or when necessary summed features, upon exposure to $\delta^{13}C$-DOM were detected using matched pairs t-tests of the difference in $\delta^{13}C$ values from control and treatment mesocosm sediments.

Results

Utilization of ^{13}C-labeled DOM by the sediment microbial community

The combined DB-1 and DB-23 columns allowed quantification of the $\delta^{13}C$ values of 15 features; these were either individual PLFAs, two co-eluting PLFAs or summed features (more than 2 co-eluting PLFAs). For PLFAs that were resolved by both columns, values presented are the mean of both analyses (Table 2). PLFA $\delta^{13}C$ values for stream and bioreactor control sediments (treatments T0 and TM) ranged from −37.26 to −28.53‰ and −35.92 to −28.70‰, respectively, with the most depleted values found in the microeukaryotic biomarkers 20:4ω6 and 20:5ω3, and the most enriched values found in cy17:0 (Table 2). No significant differences were observed between the $\delta^{13}C$ values of PLFAs from T0 and TM treatments. The $\delta^{13}C$ values of PLFAs from the ^{13}C-labeled bioreactor ($T^{13}C$) ranged from −34.75 to −24.69‰. Eight features (5 individual PLFAs, two co-eluting pairs and 1 summed feature) showed significant ^{13}C enrichment ($p < 0.05$); these were i15:0, a15:0, 16:0, 10me16:0, cy19:0, 16:0/16:1ω9, 18:1ω9/18:1ω7 and

Table 2 Microbial PLFA $\delta^{13}C$ values (‰; mean ± SD) from mesocosm experiments determined using DB-1 and DB-23 chromatographic columns

FAMEs	T0	TM	$T^{13}C$	Mean of d[a]	p[b]
i15:0	−29.01 ± 0.75	−28.69 ± 0.84	−24.69 ± 3.30	4.00 ± 3.01	0.02
a15:0	−29.34 ± 0.99	−29.12 ± 0.86	−25.58 ± 3.08	3.54 ± 2.82	0.02
16:0	−32.35 ± 2.12	−31.89 ± 1.51	−28.12 ± 3.16	3.77 ± 2.92	0.02
Summed feature 1*	−33.16 ± 0.86	−32.50 ± 1.23	−30.20 ± 4.23	2.30 ± 4.57	0.23
16:0, 16:1w9	−33.17 ± 1.81	−32.84 ± 1.49	−28.73 ± 2.81	4.11 ± 2.84	0.01
10me16:0	−30.60 ± 1.76	−29.88 ± 0.49	−28.80 ± 0.72	1.13 ± 0.64	0.02
cy17:0	−28.53 ± 2.18	−28.70 ± 2.44	−27.84 ± 2.40	0.86 ± 2.10	0.55
Summed feature 2*	−31.54 ± 1.52	−31.60 ± 1.40	−27.36 ± 3.36	4.24 ± 2.88	0.02
18:2w6	−33.07 ± 2.11	−32.83 ± 1.32	−32.09 ± 2.00	0.24 ± 1.85	0.50
18:1w9c, 18:1w7c	−30.35 ± 0.91	−30.56 ± 0.97	−24.70 ± 3.16	5.87 ± 3.03	< 0.01
cy19:0	−30.46 ± 0.80	−30.84 ± 0.23	−30.03 ± 0.50	0.82 ± 0.54	< 0.01
20:4w6, 20:5w3	−36.69 ± 1.52	−35.92 ± 1.95	−34.59 ± 1.62	1.33 ± 2.62	0.23
20:4w6, coelluter	−35.25 ± 1.71	−33.85 ± 1.75	−32.42 ± 4.29	0.40 ± 4.73	0.50
20:5w3, coelluter	−37.26 ± 1.44	−35.92 ± 2.01	−34.75 ± 1.38	1.17 ± 2.81	0.27
22:6w3	−33.85 ± 1.49	−33.40 ± 1.62	−33.29 ± 1.68	0.05 ± 2.85	0.92

T0 = natural sediment control, TM = experimental control, $T^{13}C$ = treatment sediment. * Summed feature 1 includes 16:1ω9, 16:1ω7c, 16:1ω5c, 16:1ω13t; summed feature 2 includes 18:2ω6, 18:3w3, 18:1ω9, 18:1ω7c, 18:1ω5, [a] = difference in $\delta^{13}C$ values of $T^{13}C$- TM, [b] = p of paired t-test ($T^{13}C$-TM)

summed feature 2 (18:2ω6, 18:3ω3, 18:1ω9, 18:1ω7c, 18:1ω5). The PLFA 18:2ω6 and 18:3ω3 were resolved by the DB-23 and did not show significant enrichments in $T^{13}C$, suggesting that significant enrichment in summed feature 2 detected using the DB-1 column was driven by enrichment of 18:1ω9/18:1ω7. Two polyenoic fatty acids, 20:5ω3 and 20:4ω6, indicative of microeukaryotes were significantly labeled in the dark-incubated bioreactor with 4.15‰ and 3.45‰ differences (TM vs $T^{13}C$), respectively, while labeling of these fatty acids was not detected when incubations were conducted using the natural photoperiod. In assigning microbial identity to the enriched PLFAs using a functional group approach (Findlay 2004), bacteria actively metabolizing streamwater DOM were aerobic Gram-negative bacteria (16:0/16:1ω9, 18:1ω9/18:1ω7), Gram-positive or facultative anaerobic Gram-negative bacteria (i15:0, a15:0), and anaerobic Gram-negative bacteria (10Me16:0, cy19:0).

Metabolically active members of the sedimentary microbial community

Hullar et al. (2006) produced bacterial 16S rRNA gene clone libraries from WWC sediment (same site as used in this study). Due to the scarce knowledge of direct integration of microbial biomarkers and microbial roles in stream biogeochemistry, using the highest available taxonomic resolution (species, genius, or phylum) and published phenotypic descriptions, we matched bacterial taxa known to inhabit WWC to the PLFAs showing ^{13}C enrichment during incubation with ^{13}C-labeled DOM (Table 3). The

Table 3 Phylogenetic affiliation of bacterial fatty acids functional groups extracted from White Clay Creek sediment*

Phylum or Subphylum	Species #	Fatty acids	Morphology	Metabolism	Citation
Betaproteobacteria	*Rhodoferax ferrireducens*	**16:0,** 16:1w7, **18:1w7**	Gram −ve	Aerobic, facultative anaerobic	Hahn et al. (2010)
	Variovorax paradoxus	**16:0,** 16:1w7, cy17:0, **18:1w7**	Gram −ve	Aerobic, facultative anaerobic	Jin et al. (2012)
	Herbaspirillum rubrisubalbicans	16:1w7, **16:0, 18:1w7**	Gram −ve	Aerobic, facultative anaerobic	Jung et al. (2007)
	Burkholderia cepacia	**16:0,** 16:1w7, **cy19:0**	Gram −ve	Strict aerobe	Stead (1992)
Gammaproteobacteria	*Lysobacter antibioticus*	**i15:0,** i17:1w9	Gram −ve	Aerobic	Srinivasan et al. (2010)
	Nevskia ramosa	16:1w7, **16:0, 18:1w7**	Gram −ve	Aerobic	Losey et al. (2013)
Deltaproteobacteria	"*Polyangium vitellum*" [*Kofleria flava*] (*Haliangium ochraceum*)	i16:0,**16:0**	Gram −ve	Strict aerobe	Fudou et al. (2002)
Alphaproteobacteria	"*Pedomicrobium fusiforme*" [*Filomicrobium fusiforme*]	**18:1w7, 16:0, cy19:0**	Gram –ve	Aerobe	Wu et al. (2009)
Bacteroidetes	*Flavobacterium aquatile*	**i15:0, a15:0,** 15:1w6	Gram −ve	Strict aerobic	Lee et al. (2012)
	Dysgonomonas gadei	i14:0, **i15:0, 16:0**	Gram −ve	Aerobe, facultative anaerobic	Hofstad et al. (2000)
	Runella slithyformis	**a15:0, i15:0,** 16:1w5	Gram −ve	Strict aerobe	Copeland et al. (2012)
Firmicutes	*Bacillus niacini*	**a15:0, i15:0, 16:0,** 16:1w11, 18:0	Gram +ve	Facultative anaerobe	Hong et al. (2012)
	Bacillus silvestris	**i15:0,** i16:1	Gram +ve	Aerobic	Reddy et al. (2008)
	Dendrosporobacter quercicolus	15:1, 17:1	Gram −ve	Anaerobic	Strömpl et al. (2000)
Acidobacteria	*Acidobacterium capsulatum*	**i15:0, 18:1w9**	Gram −ve	Facultative anaerobic	Kulichevskaya et al. (2012)
Planctomycetes	*Pirellula (Blastopirellula) marina*	**16:0, 18:1w9**	Gram −ve	Strict aerobe	Schlesner et al. (2004)
Nitrospirae	*Nitrospina moscoviensis*	**16:1w9, 16:0** (11Me16:0)	Gram −ve	Aerobe	Lipski et al. (2001) and Spieck et al. (2006)
Gemmatimonadetes	*Gemmatimonas aurantiaca*	**i15:0,** 16:1, 14:0	Gram −ve	Aerobe	Zhang et al. (2003)
Actinobacteria	*Kutzneria kofuensis*	i16:0 (10Me17:0)	Gram +ve	Aerobe	Stackebrandt et al. (1994) and Suriyachadkun et al. (2013)

*Fatty acids that showed ^{13}C enrichment in this study are in bold type, # Hullar et al. (2006) bacterial 16S rRNA gene clone libraries

fatty acid i15:0 is a major fatty acid in several described bacteria closely related to those from the WWC sediment clone libraries. These include: *Lysobacter antibioticus*, (*Gammaproteobacteria*, aerobic, Gram-negative), *Bacillus niacina*, *B. silvestris* (*Firmicutes*, aerobic, Gram-positive), *Acidobacterium capsulatum* (*Acidobacteria*, facultative anaerobic, Gram-negative), *Flavobacterium aquatile, Dysgonomonas gadei, Runella slithyformis* (*Bacteroidetes*, aerobic, Gram-negative), and *Gemmatimonas aurantiaca* (*Gemmatimonadetes*, aerobic, Gram-negative). The fatty acid a15:0 is a major fatty acid in two bacteria closely related to those from the WWC sediment clone libraries - *Bacillus niacina* and *B. silvestris* (*Firmicutes*, aerobic, Gram-positive). The fatty acid 16:0 is widely distributed and found in many of the described bacteria that are closely related to those from the WWC sediment clone libraries. The fatty acid 10me16:0 is not a major fatty acid of any described bacteria that are closely related to those identified from the WWC sediment clone libraries. The fatty acid 16:1ω9 is a major fatty acid in one bacterium [*Nitrospira* cf. *moscoviensis* (*Nitrospirae*, aeorobic, Gram-negative)] that is closely related to those identified from the WWC sediment clone libraries. The fatty acid 18:1ω9 is a major fatty acid in two bacteria (*Acidobacterium capsulatum, Acidobacteria*, facultative anaerobic, Gram-negative; *Blastopirellula marina, Planctomycetes*, facultative anareobic, Gram-negative) that is closely related to those identified from the WWC sediment clone libraries. The fatty acid 18:1ω7 is a major fatty acid in several described bacteria closely related to those from the WWC sediment clone libraries. These include *Burkholderia cepacia*, *Herbaspirillum rubrisubalbicans, Rhodoferax ferrireducens, Variovorax paradoxus* (*Betaproteobacteria*, aerobic, Gram-negative), *Nevskia ramosa* (*Gammaproteiobacteria*, aerobic, Gram-negative) and *Filomicrobium fusiforme* (*Alphaproterobacteria*, areobic, Gram-negative). The fatty acid cy19:0 is a major fatty acid in two described bacteria (*Burkholderia cepacia* and *Filomicrobium fusiforme*) closely related to those from the WWC sediment clone libraries. Assigning microbial identity to the enriched PLFAs using this extended approach, bacteria actively metabolizing streamwater DOM were aerobic Gram-negative bacteria including species related to *Burkholderia cepacia*, *Nevskia ramosa, Lysobacter antibioticus, Kofleria flava, Filomicrobium fusiforme, Flavobacterium aquatile, Runella slithyformis, Blastoperillula marina Gemmatimonas aurantiacca* and *Nitrospira* cf. *moscoviensis;* Gram-positive or facultative anaerobic Gram-negative bacteria including species related to *Bacillus niacini* and *B. silvestris, Rhodoferax ferrireducens, Variovorax paradoxus, Herbaspirillum rubrisubalbicans*, *Dysgonomonas gadei,* and *Acidobacterium capsulatum*.

Evaluation of the mesocosm approach

Total sediment microbial biomass measured as phospholipid phosphate (nmol PLP gdw^{-1}) was increased by removal from WCC and incubation within the mesocosms; however, this increase was not significant ($F=0.456$, $p=0.654$) (Fig. 2a). The sediment microbial community structure was unchanged by incubation within the mesocosms as neither the percentage that prokaryotes comprised of the total community ($F=2.739$, $p=0.173$) nor the PC1 score from a PCA analysis of PLFA profiles ($F=4.745$, $p=0.095$) were significantly different for stream and mesocosm control sediments (Fig. 2b, c). Thus, the addition of ^{13}C-DOM to the natural

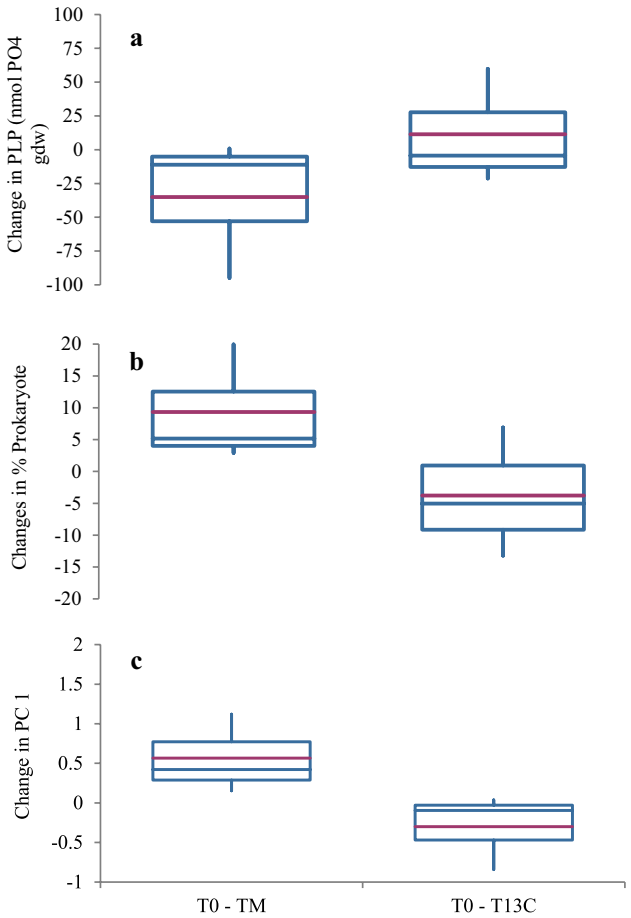

Fig. 2 Box plots of changes in (**a**) microbial biomass, measured as phospholipid phosphate (nmol PLP gdw^{-1}) ($F=0.456$, $p=0.654$); (**b**) percent prokaryotes ($F=2.739$, $p=0.173$); and (**c**) community structure summarized by Principle Component Analysis (PC1), ($F=4.745$, $p=0.095$) among treatments and sampling dates for all experiments (with the exception of dark experiment), $n=3$. T0-TM = Differences attributed to mesocosm effect, TM-T^{13}C = Differences attributed to the effects of ^{13}C-labeled DOM. The red line inside the rectangle indicate the mean of the sample distribution. The upper and lower boundaries of each rectangle indicate the upper quartile and lower quartile respectively

streamwater DOM or mesocosm control versus treatment sediments did not affect total microbial biomass, the percentage that prokaryotes comprised of the total community and microbial community structure (the PC1 score from a PCA analysis of PLFA profiles).

Discussion

Current studies of DOM metabolism within freshwater streams acknowledge that the structure of sediment microbial communities may modulate the degradation and use of this important carbon and energy resource (e.g. Mineau et al. 2013). However, few, if any, studies directly examine the bacteria that are responsible for the utilization of DOM in streams. In this study, the ^{13}C incorporated into the microbial phospholipid fatty acids revealed that many, but not all, heterotrophic microorganisms present in WCC sediments assimilated components from an allochthonous detrital source. The tracer used was designed to mimic, to a far greater extent than any previously used DOM tracer (Wiegner et al. 2005a). It contains humic and polysaccharide components (Wiegner et al. 2005a), both labile and semi-labile fractions (Wiegner et al. 2005b) and has been used for direct measurement of stream dissolved organic carbon (DOC) uptake rates coefficients (Kaplan et al. 2008). Kaplan et al. (2008) determined that uptake rate coefficients for the labile and semi-labile fractions were 4.20 and 0.22 km^{-1}, respectively, and calculated that labile tracer uptake was 272 mg C m^{-2} d^{-1} and semi-labile tracer uptake was 40 mg C m^{-2} d^{-1}. These rates were sufficient such that all labile tracer DOC and ~ 25% of the semi-labile fraction were taken up during the mesocosm incubations. PLFA-SIP revealed that aerobic Gram-negative bacteria, Gram-positive and/or facultative anaerobic Gram-negative bacteria, and anaerobic Gram-negative bacteria utilized streamwater DOM.

Among the microbial PLFAs that showed significant ^{13}C enrichment, there were two trends - those that were enriched, on average, by 3.5‰ to 5.9‰ and those that were enriched, on average, by 0.8‰ to 1.1‰. The fatty acids showing the strongest enrichment were i15:0, a15:0, 16:1ω9, 16:0, 18:1ω7c and 18:1ω9. Coupling these PLFA-SIP findings with the results of previously constructed 16S rRNA gene sequence clone libraries via published phenotypic species descriptions indicated that several organisms closely related to several described species actively utilized ^{13}C-labeled leaf leachate (Table 3). Virtually all of these species are known for possessing versatile metabolisms. For example, the *Burkholderia cepacia* complex (Vandamme et al. 1997) is well known for its extraordinary degradative abilities, possessing broad substrate mono- and dioxygenases (Lessie et al. 1996). *Herbaspirillum rubrisubalbicans*, best known as a nitrogen-fixing, plant-growth-promoting rhizobacteria, is also a plant pathogen capable of penetrating plant cell walls (Monteiro et al. 2012). This genus contains a number of aquatic species (e.g. *H. aquaticum*) that can metabolize a wide variety of sugars and other low molecular weight compounds (Dobritsa et al. 2010). *Nevskia ramosa* is considered a neuston bacterium although related OTUs have been identified among the active bacteria present in drinking water biofilms (Keinanen-Toivola et al. 2006). *Nevskia ramosa* is capable of digesting complex organic polymers including starch and cellulose, as well as many low molecular weight compounds (Stürmeyer et al. 1998). Species of genus *Lysobacter* are typically found in soil and water habitats and *L. antibioticus* is capable of degrading a wide variety of complex substrates including carboxymethyl cellulose, chitin, gelatin, laminarin, protein, Tween-20, Tween-80 and yeast cell walls (Sullivan et al. 2003). ^{13}C DNA-SIP has shown utilization of 2,4,6-trinitrotoluene by an OTU related to *L. taiwanensis* in Norfolk Harbor sediments (Gallagher et al. 2010). *Acidobacteria* are one of the most common bacterial phyla in soil and can also be among the dominant taxa of aquatic sediments (Rawat et al. 2012; Spring et al. 2000). *Acidobacterium capsulatum* is known to degrade cellobiose, starch and xylan, and contains homologs to enzymes required for pectin degradation (Rawat et al. 2012). *Bacillus niacini* and *B. silvestris* are known to utilize a host of simple organic molecules, as well as degrade several complex organic molecules. *Flavobacterium aquatile* digests casein and aesculin, and exhibits cystine arylamidase esterase, esterase lipase, and α-glucosidase activity. *Runella slithyformis* is capable of growth on glycogen, D-arabitol, dulcitol, inositol, mannitol, sorbitol, ribose and sorbose and can hydrolyze starch (Copeland et al. 2012). *Blastopirellula marina* digests DNA, aesculin, gelatin and starch, exhibits lipase activity and growth on fructose, glycerol, glutamic acid and chondroitin sulfate (Schlesner et al. 2004). *Gemmatimonas aurantiaca* is capable of growth on yeast extract, polypeptone, succinate, acetate, gelatin, benzoate, glucose, sucrose, galactose, melibiose, maltose, formate and b-hydroxybutyrate (Zhang et al. 2003). Genomic sequencing of *Rhodoferax ferrireducens* indicates that this species possesses highly diverse metabolic capacities including utilization of sugars, acetate and aromatic compounds under both aerobic and anaerobic conditions (Risso et al. 2009). *Variovorax paradoxus* is capable of digesting a wide range of complex organic compounds including (but not limited to) amino acids, polychlorinated biphenyls, dimethylterephthalate, linuron, 2,4-dinitrotoluene, homovanillate, veratraldehyde, 2,4-dichlorophenoxyacetic acid, anthracene, poly(3-hydroxybutyrate), chitin, cellulose, and humic acids. *Dysgonomonas gadei* is known to utilize a wide range of sugars, to hydrolyze starch and aesculin, and to exhibit a wide range of derivative enzyme activities including N-acetyl-b-glucosaminidase, acid phosphatase and trypsin (Hofstad et al. 2000). Combined, the ^{13}C-enrichment of

many of the abundant fatty acids present in these species, the recovery of gene sequences closely related to these cultured species from clone libraries developed from WCC and their utilization of labile organic compounds strongly suggests that bacteria related to the species discussed above utilized the labile tracer DOM. The capacity of several species, notably *Rhodoferax ferrireducens, Variovorax paradoxus, Lysobacter antibioticus, Burkholderia cepacia* and *Nevskia ramosa* to digest complex organic compounds suggests that bacteria related to these species are responsible for the utilization of the semi- labile tracer DOM.

Among the cultures most closely related to recovered sequences from the sediment community only *Nitrospina moscoviensis* exhibits 16:1ω9 as a dominant fatty acid. *Nitrospina*-like bacteria are nitrite-oxidizing bacteria and members of the deep-branching bacterial phylum *Nitrospirae* with only one class Nitrospira (Bock and Wagner 2006). The enrichment of 16:1ω9 following sediment incubation with ^{13}C-labeled leaf leachate suggests that either species containing 16:1ω9 but not identified from the clone libraries also utilized components of the ^{13}C-labeled leaf leachate or that bacteria closely related to *Nitrospina moscoviensis* exhibit mixotrophic, rather than lithoautotrophic growth in WCC.

The PLFAs 10me16:0 and cy19:0 showed moderate ^{13}C enrichment following incubation with ^{13}C-labeled leaf leachate. The fatty acid 10me16:0 is viewed as a marker fatty acid for members of the genus *Desulfobacter* within the delta subclass of proteobacteria (Findlay 2004). Macalady et al. (2000) analyzed fatty acid profiles for 100 strains of bacteria including 12 genera of sulfate-reducing bacteria and several other anaerobic species. They concluded that *Desulfobacter* were the major sources of 10me16:0 in environmental samples. The Macalady et al. (2000) analysis also indicated that the PLFA cy19:0 was also strongly associated with *Desulfobacter.* This suggests that anaerobic sulfate-reducing bacteria, and in particular members of the genus *Desulfobacter,* utilized some component of ^{13}C-labeled leaf leachate, albeit at a lesser extent compared to those bacteria represented by the strongly labeled PLFAs.

On one occasion, we incubated sediments in the dark and during this trial the δ^{13}C of the PLFAs 20:4ω6 and 20:5ω3 increased by 3.45‰ and 4.15‰, respectively, compared to an average increase of 1‰ when sediments were incubated under the natural photoperiod. It is important to note that we conducted but a single dark incubation and that 20:4ω6 and 20:5ω3 are found in both heterotrophic and autotrophic protists. Nevertheless, the presence of label in fatty acids 20:4ω6 and 20:5ω3 in the dark suggests that protozoan grazers used bacteria that are utilizing ^{13}C-DOM as food source and may contribute significantly to the transfer of allochthonous carbon via the microbial loop in stream ecosystems. Risse-Buhl et al. (2012) in a series of dark-incubated biofilms treated with ^{13}C labelled CO_2, showed that 29% of the labeled carbon reached protozoan grazers. As the system contains both heterotrophic and autotrophic protists, synthesis of 20:4ω6 and 20:5ω3 by algae during incubations under the natural photoperiod would produce a second, non-labeled source of these fatty acids, which would serve to isotopically dilute those produced by trophic interactions within the sediment microbial community.

Total sediment microbial biomass measured as phospholipid phosphate (nmol PLP gdw^{-1}) for the control and ^{13}C-labeled bioreactors fall within the range (2–280 nmol PLP g^{-1} dry wt) of PLP concentrations quantified in freshwater sediments of eastern deciduous forest and those previously published for WCC (Bott and Kaplan 1985; Sutton and Findlay 2003; Findlay et al. 2008). The increase in microbial biomass in the mesocosm sediments (Fig. 2a; comparison T0-TM), though not significant, is likely a response of stream microbial communities to placement within a mesocosm setting. While infrequently assessed and even less frequently discussed (but see Mortazavi et al. 2013, Suárez-Suárez et al. 2011), a 'mesocosm effect' appears to be an increase in sediment bacterial abundance. Sediments, in general and stream sediments in particular, are dynamic and sediment microbial communities experience frequent disturbances associated with storm flows, hydraulic turbulence, or macrofauna activities (Fisher et al. 1982; Schwendel et al. 2011) and are best viewed as rarely, if ever, at maximum biomass. The natural response of sediment microbial communities to disturbance is regrowth leading to increased biomass (Findlay et al. 1990; Traunspurger et al. 1997; Langworthy et al. 2002). In this study, sediments were obtained with the utmost care; however, obtaining a "disturbance free" microbial sediment sample is very difficult, if not impossible. In addition, once placed in the mesocosms sediments were protected from further in-stream disturbances which, combined with any disturbance during removal from the stream, likely led to the small observed increase in biomass (Riemann et al. 2000). Stream and mesocosm sediments (T0, TM, and T^{13}C) showed little change in ratios of prokaryotic to eukaryotic biomass (Fig. 2b). On average, in the bioreactors, ~60% of the microbial biomass was prokaryotic and the remaining 40% was eukaryotic, which is well within the range of estimates reported for stream sediments from several low-order forested streams (Sutton and Findlay 2003; Mosher and Findlay 2011) and is similar to previously reported ratios for WCC (Findlay et al. 2008). Thus, there was no change in community structure, or in microbial biomass in these mesocosms in response to the sampling procedure or mesocosm effect.

In this study, the additions of 8.73–17.47 µg/L of ^{13}C-DOM did not significantly alter the structure of the sediment microbial community during any of the mesocosm incubations. In a study where 50 µg C g^{-1} soil of universally ^{13}C

labeled glucose, glutamine, oxalate or phenol were added to samples of soil, no detectable changes in the soil PLFA profiles were found (Brant et al. 2006). Also, Griffiths et al. (1999) detected no changes in the soil PLFA profiles until rates of additions of a model root exudate exceeded 375 µg C $g^{-1}d^{-1}$ in a 14-d experiment. In other studies, the additions of 400 µg C g^{-1} soil of vanillin (Waldrop and Firestone 2004) and 726 µg C g^{-1} oxalate and glutamate to grassland sandy loam soil (Falchini et al. 2003) resulted in changes in microbial community composition or PLFA profile. The trend in these studies was that as substrate loading increased, the relative abundance of specific PLFAs increased leading to changes in total microbial community composition. Our tracer-level substrate addition was significantly smaller than additions used in studies where significant changes in biomass or community composition were observed; this was intentional and designed to avoid changes in biomass and PLFA profiles that could compromise our use of mesocosm-based experimental design.

Though mesocosm experiments have increased our understanding of community ecology, ecosystem dynamics and provided insight into global processes (Fraser and Keddy 1997; Jessup et al. 2004; Cardinale et al. 2006; Benton et al. 2007; Duffy 2009), mesocosms have been criticized as being unrealistic simplifications of natural systems with restricted utility (Carpenter 1996; Schindler 1998; Haag and Matschonat 2001). However, with appropriate scaling, accurate conclusions can be made (Spivak et al. 2011). Our analyses of microbial biomass and community structure indicate that our mesocosm-based experimental design, particularly the comparison of the TM mesocosm control to the $T^{13}C$ mesocosm treatment samples, was sufficiently robust to warrant examination of individual fatty acids for the incorporation of ^{13}C with the goal of determining the role of sediment microbes in processing streamwater DOM. Also, the significant ^{13}C-enrichment detected in microbial lipids in the $T^{13}C$ bioreactors clearly demonstrated the high sensitivity of stable isotope probing of PLFA as a technique to elucidate which microbial communities are responsible for the utilization of tDOM.

In conclusion, the present study provides direct experimental evidence that tDOM is readily utilized by a broad range of benthic heterotrophic aerobic, facultative anaerobic and anaerobic bacteria in forested headwater streams. We posit that terrestrially derived DOM exported from forested watersheds is not entirely lost to downstream systems but rather is assimilated and mineralized by a variety of heterotrophic bacteria which, in turn, are grazed by heterotrophic eukaryotes transferring allochthonous carbon and energy to higher trophic levels through the microbial loop (Meyer 1994). Thus, our data have important implications for protection of forested headwater streams where much of the DOM in transport is derived from the surrounding terrestrial ecosystem. As tDOM is an important source of carbon and energy for stream microbial communities, any human activity that disrupts or accelerates the delivery of tDOM to headwater streams may need regulation, since perturbation to ecological linkages between aquatic and terrestrial systems could have pronounced effects on microbial community structure and function. This is particularly true where the terrestrial and aquatic ecosystems are tightly linked by large internal fluxes of DOM in the forested landscape (McDowell and Likens 1988; Aitkenhead-Peterson et al. 2003).

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