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Consumption of terrestrial dissolved organic carbon by stream microorganisms

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ABSTRACT: Terrestrial dissolved organic carbon (DOC) is the largest organic C pool in lotic systems, yet its role in supporting these ecosystems remains challenging to quantify. To assess the reliance of stream heterotrophic microorganisms on terrestrial DOC, this study utilized a ^{13}C-labeled leaf leachate as a proxy for stream-water DOC of terrestrial origin. This leachate was introduced into dark, stream-water-fed bioreactors for 41 d and a suite of analytical techniques was used to evaluate community responses. Metabolic responses were quantified through measurements of DOC uptake, oxygen consumption, and bacterial production and abundance. Microbial community structure was assessed using phospholipid fatty acid (PLFA) analysis and metabolically active microbes were identified through compound specific isotope analysis. Leachate additions increased stream-water DOC concentration on average by 6% and changed its $\delta^{13}C$ from −28 to +1021‰. Leachate additions did not change microbial community structure or bacterial production and growth efficiency, but affected DOC and O_2 consumption. Prokaryotes comprised 80% of the bioreactor microbial biomass, with aerobic and facultative anaerobic bacteria dominating, and heterotrophic microeukaryotes comprising the remaining 20%. All PLFAs were enriched in ^{13}C, indicating that the leachate moved through the bacterial community and across trophic levels. The trophic transfer of leachate ^{13}C to heterotrophic microeukaryotes was 32%. These findings indicate that labile terrestrial DOC plays an important role in stream metabolism, as well as supporting higher trophic levels in the microbial loop.

KEY WORDS: Allochthonous · Bacteria · Streams · DOC · PLFA · Stable isotopes

INTRODUCTION

Heterotrophic benthic microbes play critical ecological roles in lotic ecosystems, including the processing of detrital organic carbon (C) (Bott et al. 1984, Fischer et al. 2002). These organisms often dominate community respiration in streams and rivers (Edwards et al. 1990) and support higher trophic levels through the microbial loop (Hall & Meyer 1998). Thus, the organic molecules fueling heterotrophic benthic microbial metabolism drive lotic ecosystem processes and support secondary production of consumers.

The proximate source of C sustaining benthic heterotrophic microbial metabolism in lotic systems is dissolved organic C (DOC). DOC is the largest organic C pool in rivers, with 170–360 Tg of terrestrial DOC entering the global ocean annually through riverine transport (reviewed in Dai et al. 2012). While terrestrial DOC has long been considered recalcitrant (Mantoura & Woodward 1983), the fact that twice as much terrestrial DOC is delivered

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to inland waters than to the global ocean suggests that it is more biologically reactive in freshwater systems than previously thought (Cole et al. 2007). This inference is supported by direct measurements showing that a portion of terrestrial DOC is bioavailable (Volk et al. 1997, Frazier et al. 2003, 2005, Kim et al. 2006) and respired in rivers (Cole & Caraco 2001, Mayorga et al. 2005). However, the importance of terrestrial DOC in supporting stream and riverine ecosystem metabolism, as well as secondary production, is not well known. Whole-stream stable isotope additions and natural abundance studies have begun to shed light on the metabolic role and trophic transfer of terrestrial DOC in lotic systems (Hall 1995, Hall & Meyer 1998, McCallister et al. 2004, Augspurger et al. 2008, Kaplan et al. 2008). They have shown that terrestrial DOC can account for 49–85% of C assimilated and respired by benthic and pelagic riverine bacteria, and 9–77% of C assimilated by higher trophic levels, including protozoans and invertebrates.

Despite these advances underscoring the importance of the terrestrial DOC flux through lotic ecosystems, little is known about which microbes drive detrital C fluxes in stream ecosystems (Foreman & Covert 2003, Hullar et al. 2006). Several approaches have been used to determine which microbes participate in DOC uptake, degradation, and mineralization across aquatic environments, but few employed the use of natural substrates. Terminal restriction length polymorphism analysis of 16S rRNA gene amplicons has been used to identify estuarine bacteria growing on low- and high-molecular weight DOC fractions (Covert & Moran 2001), while taxonomic analysis of mRNA transcripts have identified bacteria synthesizing proteins for DOC transport (Poretsky et al. 2010). Microradiographic fluorescence *in situ* hybridization using fluorescent rRNA-targeted oligonucleotide probes has been used to phylogenetically classify bacteria that have consumed tritiated DOC molecules (i.e. ^{3}H-glucose, -amino acids, -chitin) (Cottrell & Kirchman 2000). These approaches provide important insight into detailed classification of bacteria and their activity relevant to DOC uptake. However, they do not provide a means to assess actual DOC flow through whole microbial communities, including the transfer of DOC from bacteria to their consumers. Further, these approaches do not enable estimates of the relative differences in DOC use among different microbial groups.

Stable isotope probing (SIP), however, does provide a means for tracking C flow through microbial communities and assessing the relative difference in C use by different groups within those communities (Dumont & Murrell 2005). This approach has been used to identify metabolically active microbes across environments by tracking the incorporation of labeled substrates into either microbial lipid biomarkers (reviewed in Boschker & Middelburg 2002) or DNA that is then sequenced for phylogenetic identification (Nelson & Carlson 2012). Application of SIP has been used to follow the flow of benthic algal C (Lyon & Ziegler 2009, Ziegler et al. 2009, Ziegler & Lyon 2010, Risse-Buhl et al. 2012) and sucrose (Augspurger et al. 2008) in stream biofilms. To our knowledge, however, this approach has not yet been used to identify which heterotrophic microbes in streams are responsible for terrestrial DOC consumption. Identifying these microbes will help elucidate the linkage between microbial community structure and DOC metabolism, a key focus in microbial ecology.

The goal of our study was to identify and determine the relative importance of heterotrophic microbial groups metabolizing terrestrial DOC. We accomplished this by labeling lipids of heterotrophic microbes in dark, stream-water-fed, plug-flow bioreactors. Dark bioreactors colonized with stream-water microbes are laboratory tools that have been successfully used in stream ecology to chemically characterize bioavailable DOC (BDOC) (Volk et al. 1997) and quantify BDOC lability classes (Kaplan et al. 2008). Microbes were labeled using trace additions of a ^{13}C-labeled tree tissue leachate, a proxy for terrestrial DOC (Wiegner et al. 2005a). Previous studies linking stream-water DOC and stream microbes have used low-molecular weight, labile C compounds (sucrose, acetate) that typically comprise less than 0.1% of total DOC in stream water (Hall 1995, Hall & Meyer 1998, Simon et al. 2003, Augspurger et al. 2008, Foulquier et al. 2010). As leaf leachate can account for 30% of daily DOC export from small, forested streams (Meyer et al. 1998), our terrestrial DOC tracer is the most representative tracer of allochthonous DOC inputs to streams to be used to date (Kaplan et al. 2008). Phospholipid fatty acid (PLFA) analysis was used to determine microbial community structure, and metabolically active microbes were identified through compound specific stable isotope analysis of PLFA following exposure to the ^{13}C-labeled leachate. Metabolic responses of the microbial community to the leachate additions were examined through measurements of bacterial production, respiration, and DOC uptake.

MATERIALS AND METHODS

Study site

Water used in this experiment was collected from East Branch White Clay Creek (WCC), a third-order stream located in southeastern Pennsylvania, USA (39° 53′ N, 75° 47′ W). WCC drains an agricultural watershed and has elevated concentrations of dissolved nitrogen (3–4 mg NO_3^--N l^{-1}) and phosphorus (10–60 µg PO_4^{3-}-P l^{-1}).

Bioreactors

Five once-through plug-flow bioreactors with an empty bed contact time of 150 min were used. They were constructed out of glass chromatographic columns (Kontes) filled with sintered glass beads (Schott Glassworks, Siran) with 10-µm bed supports at both ends of the column. The bioreactors were incubated in the dark, and continuously fed filtered WCC stream water at 4 ml min^{-1} in an up-flow mode (Kaplan & Newbold 1995). Stream water was filtered using a 3-stage (75, 25, 0.3 µm) Balston glass fiber cartridge filter system, which produced a filtrate that contained greater than 90% of the bacteria suspended in stream water (L. A. Kaplan unpubl. data). Thus, the filtered stream water provided a continuous source of microbes and energy for bioreactor colonization and growth. Two of the bioreactors used in this study were in continuous operation for 4 years (established 1999; referred to as K4-1 and K4-2) and 3 were in continuous operation for 11 yr (established 1992; referred to as K2-1, K2-2, and K2-3). All bioreactors exhibited similar rates of stream water DOC uptake (L. A. Kaplan unpubl. data) prior to exposure to ^{13}C-labeled leachate. Additionally, previous analyses have shown that WCC streambed sediments and bioreactor Siran support similar microbial communities, except that the dark bioreactors do not support phototrophic microeukaryotes (R. H. Findlay unpubl. data).

Bioreactors K2-1, K2-2, and K2-3 were continuously fed stream water amended with ^{13}C-labeled tree tissue leachate for 41 d (03 July to 14 August 2003) and served as replicate 'treatment' bioreactors. The 2 remaining bioreactors (K4-1 and K4-2) served as controls and were fed unamended stream water. ^{13}C-labeled tree tissue leachate was generated following the methods of Wiegner et al. (2005a) using ^{13}C-labeled *Liriodendron tulipifera* L. (tulip poplar) tissue. The leachate had a δ^{13}C value of +4843‰, substantial polymeric character composed of humic substances (25% of C) and polysaccharides (8% of C), and 2 distinct DOC lability classes (readily and intermediately labile) (Wiegner et al. 2005a, Wiegner et al. 2005b, Kaplan et al. 2008). Weekly, the leachate was diluted approximately 10-fold with C-free deionized water, sterile filtered (0.1-µm Spiral Capsule Supor Membrane) into a pre-combusted 5-l glass container, and Tyndallized to prevent any microbial growth. Tyndallization is a low temperature sterilization procedure involving heating the water to 60°C for 0.5 h, allowing the water to sit at room temperature for 24 h, and then repeating the process; this process has been shown to not affect DOC bioavailability (L. A. Kaplan unpubl. data). The leachate was pumped from its reservoir into a static mixing tee (Upchurch Scientific) at 100 µl min^{-1} with a peristaltic pump, where it mixed with filtered stream water and fed into the bioreactors at 4 ml min^{-1} (a bioreactor diagram can be found in Kaplan & Newbold 1995). The treatment bioreactors were all fed from the same leachate reservoir, but each bioreactor had its own static mixing tee (IDEX Health & Science) and filtered stream water feed line. Sterile technique was used when connecting the leachate reservoir to the bioreactors.

Influent and effluent waters from the bioreactors were sampled weekly for DOC, ^{13}C-DOC, and dissolved oxygen concentrations. Effluent samples for DOC and ^{13}C-DOC were not re-filtered prior to analysis as the bioreactor beads and bed supports act as a tortuous path filter, and repeated tests of bioreactor effluents showed no difference in the DOC concentrations between filtered and unfiltered effluent samples (L.A. Kaplan unpubl.). BDOC concentrations were calculated from the difference between the DOC concentrations in the influent and effluent waters from the bioreactors. O_2 consumption was calculated similarly to BDOC.

At the end of the experiment (14 August 2003), all 5 bioreactors were destructively sampled for microbial biomass, community structure, δ^{13}C PLFA analysis, and bacterial abundance and production. Siran was removed from ca. 12 cm from the inlet and outlet of each bioreactor, homogenized, and sub-sampled by wet weight. Lipid samples (microbial biomass, community structure, and δ^{13}C PLFA analysis) were stored at −20°C until lyophilized (Federle & White 1982) as recommended for complex samples such as soils (Liu et al. 2009). Bacterial abundance samples were preserved in 10% formalin until analysis.

Microbial biomass and community structure analysis

Siran from the bioreactors was lyophilized and lipids were extracted through a modified (dichloromethane–methanol–water) Bligh-Dyer lipid extraction (Findlay et al. 1989). Total microbial biomass was quantified from phospholipid phosphate (PLP) concentrations, which were determined from the persulfate oxidation of the lipids and colorimetric analysis of the resulting orthophosphate (Findlay et al. 1989). Phospholipid fatty acids (PLFAs) were recovered from the remaining lipid fraction and were analyzed as their fatty acid methyl esters (FAMEs) (Findlay 2004) on a gas chromatograph with a flame ionization detector (GC-FID). FAMEs were identified based on relative retention times, co-elution with known standards, and mass spectrometer (MS) analysis.

Percent contributions of eukaryotes and prokaryotes to the total microbial community biomass were determined using the approach of Findlay & Dobbs (1993), where it was assumed that 50% of the PLFAs from eukaryotes were polyenoic (Findlay et al. 1986, Wood 1988) and eukaryotic biomass was twice the sum of all polyenoic PLFAs. Prokaryotic biomass was calculated as the difference between the total microbial biomass and eukaryotic biomass. The biomass of eukaryotes and prokaryotes, in terms of C, was calculated using conversion factors of 0.02 g C µmol^{-1} P for eukaryotes and 0.01 g C µmol^{-1} P for prokaryotes (Findlay & Dobbs 1993). Prokaryotic biomass was converted into bacteria cell abundances from PLP concentrations using a conversion factor of 1 nmol PLP · 4×10^7 cell^{-1} (Balkwill et al. 1988).

Microbial community utilization of the ^{13}C-labeled tree tissue leachate

δ^{13}C values of individual PLFAs were determined by analyzing FAMEs on a GC (Agilent 6890) equipped with a 50-m 70% cyanopropyl polysilphenylene-siloxane capillary column (BPX-70, SGE) that was interfaced with a GC/CIII (GC-C [combustion]; ThermoFinnigan) to an isotope ratio MS (IRMS; ThermoFinnigan Delta$^+$). A correction for the addition of the methyl C from the BF_3-methanol derivatization was calculated for each fatty acid by mass balance from the analysis of nonadecanoate with a known δ^{13}C composition, as commonly done for GC-C-IRMS of derivatized compounds (Silfer et al. 1991, Abrajano et al. 1994). The correction was determined by calculating the difference between the known nonadecanoate and the measured value of its methyl ester within each sample. Stable isotopic ratios were measured relative to high-purity reference gas expressed relative to the international standard Vienna PeeDee Belemnite (VPDB), as:

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

where R is ^{13}C/^{12}C. A standard mixture containing phosphatidylcholine tetradecanoyl, phosphatidylcholine heptadecanoyl, phosphatidylcholine nonadecanoyl, and phosphatidylcholine dodecanoyl was saponified and methylated along with each set of samples to assess the precision of the GC-C-IRMS analysis, which was below 0.5‰. Error associated with the analyses of highly ^{13}C-enriched samples is expected to be higher than that of natural abundance samples because of the IRMS configuration used to detect ^{13}C and ^{12}C equally (Brenna et al. 1997). For the 2 ^{13}C-enriched standards used (glucose and vanillin), the error for our instrumentation was 15‰ and 26‰, respectively.

Wet chemistry methods

DOC was measured by Pt-catalyzed persulfate oxidation using either an OI 700 or an OI 1010 (O. I. Corp.) C analyzer. Dissolved oxygen was determined using the Winkler method (Oudot et al. 1988; Mettler-Toledo DL50 titrator). DOC samples for isotope analyses were prepared according to Gandhi et al. (2004) using a Eurovector elemental analyzer interfaced to a PRIME (Micromass) IRMS. Stable C isotope ratios were expressed as δ^{13}C (Eq. 1). The standard was VPDB and the reproducibility of these measurements at natural abundance levels was ≤0.2‰, and <0.03 atom% at enriched levels (Gandhi et al. 2004, Wiegner et al. 2005a).

^{13}C-labeled tree tissue leachate uptake in bioreactors

Because of the high level of ^{13}C enrichment within the tree tissue leachate and the desire to follow the ^{13}C from the leachate into the DOC and microbial C pools throughout the experiment, fractional abundance (F) notation was used (Fry 2006). Fractional abundance in all DOC samples (stream water with and without ^{13}C-labeled tree tissue leachate) was calculated using measured δ^{13}C-DOC values as follows:

$$F = R_{sample}/(R_{sample} + 1) = {}^{13}C/({}^{12}C + {}^{13}C) \quad (2)$$

These data were then used in a mixing model to provide estimates of the fraction of DOC in the bioreactors' influent and effluent waters originating from the leachate (Wiegner et al. 2005b):

$$f_{leachate} = (F_{mix} - F_{stream})/(F_{leachate} - F_{stream}) \quad (3)$$

where F_{mix} is the measured fractional abundance of ^{13}C in the DOC of the bioreactors' influent or effluent waters, F_{stream} is the measured fractional abundance of ^{13}C in the stream water DOC prior to the leachate addition, and $F_{leachate}$ is the measured fractional abundance of ^{13}C in the leachate. The concentration of ^{13}C from the leachate in the bioreactors' influent and effluent waters ($^{13}C_{leachate}$) was calculated as:

$$^{13}C_{leachate} = f_{leachate} \times C_{mix} \times F_{leachate} \quad (4)$$

where C_{mix} is the measured bioreactors' influent or effluent DOC concentrations. Percent of total PLFA ^{13}C originating from the leachate (PLFA-$^{13}C_{leachate}$) at the end of the experiment was calculated as:

$$\%\ \text{PLFA-}{}^{13}C_{leachate} = [(F_{treatment\ bioreactor} - F_{control\ bioreactor})/(F_{leachate} - F_{control\ bioreactor})] \times 100 \quad (5)$$

where $F_{treatment\ bioreactor}$ and $F_{control\ bioreactor}$ are the measured fractional abundance of ^{13}C in each PLFA (quantified as their FAME) from the treatment and control bioreactors, respectively.

Bacterial abundance

Bacterial abundance on Siran from the bioreactors was determined from epifluorescence microscopic counts (EMCs) of formalin-fixed cells that were detached and separated from the Siran by sonication and centrifugation in a glycerol gradient, filtered onto Nuclepore membranes, and stained with propidium iodide (Sigma; Bott & Kaplan 1993, Battin et al. 2001). Bacterial cells were enumerated in 20 fields on a Zeiss Universal microscope equipped with epifluorescence illumination. Three filters were counted for each Siran sample.

Bacterial production

Bacterial production was measured with ^{3}H-leucine using a method modified from Kirchman (1993). Siran from the control and treatment bioreactors was placed into sterile tubes with sterile-filtered stream water amended with ^{3}H-leucine, incubated in the dark at 20°C for 1 h on a shaker table, and killed by adding trichloroacetic acid (TCA) to a final concentration of 5%. Samples were sonicated and centrifuged, and supernatants were sterile filtered through polycarbonate filters (Poretics). The pellet and filter were then rinsed separately with 5% TCA, 40 mmol l^{-1} leucine, 80% ethanol, and C-free deionized water. The pellets and filters were pooled and an extractant (0.5 mol l^{-1} NaOH, 25 mmol l^{-1} ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate) was added to the tubes, which were then autoclaved and centrifuged. A subsample of the supernatant was placed into Ecolite scintillation cocktail and radioassayed using a Beckman 3801 Liquid Scintillation Counter.

Bacterial growth efficiency

Bacterial growth efficiency (BGE) was calculated for both the control and treatment bioreactors using BDOC and O_2 consumption measurements. These measurements were used to calculate a BGE that was integrative over the entire bioreactor. Direct bacterial production measurements were not used because they were limited to the first and last 60 cm^3 of the bioreactors, and they were significantly different between these 2 locations in the bioreactors. For our BGE calculations, measurements of O_2 consumption were converted into units of C using a respiratory quotient of 1 in molar units, and we assumed that changes in BDOC concentrations were equivalent to the sum of bacterial production and respiration (del Giorgio & Cole 1998). Bacterial production was calculated from the difference between BDOC and O_2 consumption concentrations with both parameters in units of C, and BGE was calculated as bacterial production divided by BDOC concentration.

Statistical methods

Differences in microbial parameters calculated from PLFA analysis (PLP concentration, total microbial biomass, microbial community composition [% eukaryotes versus prokaryotes], and bacterial cell abundance) between the inlets of the control (n = 2) and treatment bioreactors (n = 3) were examined with 2-sample *t*-tests. Comparisons of bacterial abundance and production between inlet and outlet regions of the bioreactors were examined using a 2-way ANOVA with leachate exposure

(control versus treatment bioreactors) and location within the bioreactor (inlet versus outlet) as main effects. Other measures of microbial activity measurements (% BDOC,% O_2 consumption, and BGE) were analyzed using a mixed between- and within-subjects ANOVA, with time serving as the within-subjects factor and leachate exposure serving as the between-subjects factor. Levene's test was used to test the homogeneity of variance for the mixed model analyses. A one-way ANOVA examined differences in % PLFA-$^{13}C_{leachate}$ among microbial functional groups. Log and rank transformations were performed on data that did not meet the normality and equality of variance requirements for parametric analyses (Potvin & Roff 1993). These analyses were run using either Systat 11 or SPSS version 21, with an α-level of 0.05. The exposure effect of the ^{13}C-labeled tree tissue leachate on microbial community structure was examined using principal component analysis (PCA) of PLFA profiles after log transformation [ln(x + 1)] of weight percent fatty acid data. PCA was conducted using SPSS version 7.5; PLFA profiles were interpreted using a functional group and biomarker approach (Findlay 2004).

Table 1. Mean (±1 SD) dissolved organic carbon (DOC) concentrations and $\delta^{13}C$ values at the inlets and outlets of the control and treatment (^{13}C-labeled) bioreactors. DOC was measured weekly from 13 July 2003 to 14 August 2003; $\delta^{13}C$-DOC was measured 28 July and 04 August 2003

Bioreactor	DOC (µg l^{-1})	$\delta^{13}C$-DOC (‰)
Control		
Inlet		
K4-1	2537 (1552)	−26.83 (1.37)
K4-2	2537 (1552)	−26.83 (1.37)
Mean	2537 (1499)	−26.10 (1.37)
Outlet		
K4-1	1869 (1212)	−28.49 (0.028)
K4-2	1848 (1183)	−29.26 (1.44)
Mean	1859 (1157)	−28.37 (0.19)
Treatment		
Inlet		
K2-1	2310 (1650)	1061.81 (1026.72)
K2-2	2658 (1436)	1046.17 (1053.14)
K2-3	2730 (1602)	954.34 (854.79)
Mean	2662 (1449)	1021.78 (762.55)
Outlet		
K2-1	1432 (1059)	1.01 (2.59)
K2-2	1687 (1040)	22.87 (6.60)
K2-3	1629 (972)	301.37 (404.43)
Mean	1642 (953)	108.42 (234.86)

RESULTS

Effects of ^{13}C-labeled tree tissue leachate additions on stream water DOC

^{13}C-labeled tree tissue leachate additions increased the DOC concentration in the influent stream water by 6 ± 9% (mean ± 1 SD) and changed its $\delta^{13}C$-DOC signature from −28 ± 2‰ to +1021 ± 763‰ (Table 1). Natural variability in stream-water DOC concentrations (1553 to 6083 µg l^{-1}) resulted in large SD values during the experiment; daily SDs among the influent water of the treatment bioreactors ranged from 27 to 275 µg l^{-1}. Additionally, on the 2 days when $\delta^{13}C$-DOC for the influent waters to the treatment bioreactors was measured, the values were +1712 ± 133‰ (28 July 2003) and +349 ± 25‰ (04 August 2003).

Effects of ^{13}C-labeled tree tissue leachate additions on bioreactor microbial biomass and community structure

Total microbial (as PLP, p = 0.054, t = −3.727, df = 2) and prokaryotic biomasses (as C, p = 0.039, t = −4.182, df = 2) at the inlet of the treatment bioreactors were 1.6-fold higher than those in the control bioreactors (Table 2). Total microbial biomass could not be biochemically determined at the outlets of the bioreactors due to insufficient biomass. Prokaryotes comprised ~80% of the total microbial biomass in the control and treatment bioreactors (p = 0.63, t = −0.619, df = 1); the remainder comprised heterotrophic microeukaryotes (Table 2). Microbial community structure, as determined by a comparison of individual PLFA weight percent values, was similar for control and treatment bioreactors (Table 3). PCA analysis of PLFA profiles also indicated that communities from control and treatment bioreactors were similar (data not shown).

Community structure of metabolically active microbes

Only samples collected at the bioreactor inlets had sufficient biomass to be analyzed for the incorporation of the ^{13}C-labeled tree tissue leachate into microbial phospholipids. Control bioreactor PLFA $\delta^{13}C$ ranged from −44 to −25‰, with the most depleted values found in the microeukaryotic biomarkers 20:4ω6 and 20:5ω3, and the most enriched values in the sulfate-reducing bacteria biomarker 10Me16:0

Table 2. Microbial biomass, community structure, and bacterial cell abundance determined from phospholipid fatty acid (PLFA) analysis and epifluorescence microscopy counts at the inlets and outlets of the control and treatment (^{13}C-labeled) bioreactors. There was not enough microbial biomass for PLFA analysis at the outlet of the bioreactors. % Eukaryotic and % prokaryotic are the percent contributions of eukaryotes and prokaryotes to the total microbial community biomass, respectively. PLP: phospholipid phosphate; DW: dry weight

Bioreactor	Inlet						Outlet
	PLP (nmol g^{-1} DW)	% Eukaryotic	% Prokaryotic	Prokaryotic biomass (mg C g^{-1} DW)[a]	Bacterial abundance (cells g^{-1} DW)[a]	Bacterial abundance (cells g^{-1} DW)[b]	Bacterial abundance (cells g^{-1} DW)[b]
Control							
K4-1	219.9	23	77	2.71	6.74×10^9	5.30×10^9 (0.77×10^9)	0.17×10^9 (0.03×10^9)
K4-2	282.3	18	82	3.34	9.24×10^9	9.80×10^9 (0.07×10^9)	0.19×10^9 (0.05×10^9)
Mean (±1 SD)	251.1 (44.12)	21 (4)	79 (4)	3.02 (0.44)	7.99×10^9 (1.77×10^9)	7.55×10^9 (0.49×10^9)	0.18×10^9 (0.01×10^9)
Treatment							
K2-1	442.3	17	83	5.19	14.6×10^9	12.0×10^9 (1.90×10^9)	0.27×10^9 (0.03×10^9)
K2-1	355.4	20	80	4.26	11.4×10^9	8.30×10^9 (0.83×10^9)	0.35×10^9 (0.04×10^9)
K2-3	403.5	20	80	4.84	12.9×10^9	9.30×10^9 (0.41×10^9)	0.40×10^9 (0.05×10^9)
Mean (±1 SD)	400.4 (43.53)	19 (2)	81 (2)	4.76 (0.47)	13.0×10^9 (1.63×10^9)	9.87×10^9 (0.77×10^9)	0.34×10^9 (0.07×10^9)

[a]Determined from PLP concentrations. See Materials and methods section for calculations
[b]Determined from epifluorescence microscopy counts. Mean (±1 SD), n = 3

(Table 3). Variability between the 2 replicate control bioreactor samples was less than 0.6‰ and within analytical precision of the method. The δ^{13}C values of PLFAs from the treatment bioreactors ranged from +246 to +1096‰, and were similar in isotopic composition to the influent δ^{13}C-DOC values (+1021‰), but more depleted than the original ^{13}C-labeled tree tissue leachate (+4843‰; Table 1). All PLFAs were enriched in ^{13}C and the variability in the δ^{13}C of PLFAs from the 3 replicate treatment bioreactors ranged from +76 to +292‰, and as such, exceeded the analytical precision of the PLFA method for δ^{13}C > +250 ± 6‰ (n = 15). This variability is expected with highly ^{13}C-enriched samples (see 'Materials and methods'). The δ^{13}C of the microeukaryotic biomarkers 20:4ω6 and 20:5ω3 exhibited the least variability, while cy19:0 exhibited the most. The most enriched PLFAs were cy19:0 and cy17:0, while the least enriched were a17:0, 18:1ω5, and the co-eluted 16:1ω9 and 16:1ω13t. Variation among δ^{13}C values of PLFAs indicated that different functional groups within the community varied in the percentage of their C obtained from the leachate (% PLFA-$^{13}C_{leachate}$) ranging from 8.5% (18:1ω5) to 19% (cy19:0; Fig. 1), but this difference was not statistically significant (p = 0.089, F = 3.103, df = 2).

Metabolic response of microbes to ^{13}C-labeled tree tissue leachate additions

The percentage of stream-water DOC that was biodegradable (percent BDOC) significantly increased (p = 0.006, F = 47.55, df = 1,3, η^2 = 0.94) from 28 ± 2% in the control to 39 ± 6% in the treatment bioreactors (Fig. 2A). Ninety-three percent (±7%) of the ^{13}C-labled tree tissue leachate was consumed within the bioreactors and δ^{13}C declined approximately 10-fold from the inlet to the outlet of the bioreactors (Table 1). Percent O_2 consumption significantly increased (p = 0.012, F = 29.394, df = 1,3, η^2 = 0.907) from 20 ± 5% in the control bioreactors to 33 ± 9% in the treatment bioreactors (Fig. 2B). Bacterial cell abundance was 1.6-fold higher in the treatment bioreactors than the controls (p = 0.001, F = 113.413, df = 1), and was more than an order of magnitude higher at the inlets than at the outlets (p < 0.001, F = 67.440, df = 1; Table 2). In contrast, bacterial production was similar (p = 0.680, F = 0.174, df = 1) between the control and treatment bioreactors (Fig. 2C). In both the control and treatment bioreactors, bacterial production was 40- to 50-fold higher at the inlets than at the outlets (p < 0.0001, F = 126.420, df = 1; outlet data not shown). BGE was slightly higher in

Table 3. Weight percent—(ng $FAME_i$ / ng total FAME) ×100; FAME: fatty acid methyl ester—and stable carbon isotopic composition ($\delta^{13}C$, ‰) of individual phospholipid fatty acids (PLFAs) resolved from the inlet of control and treatment (^{13}C-labeled) bioreactors at the end of the labeling experiment. Values for individual bioreactors and means (±1 SD) of the 2 treatments (control versus ^{13}C-labeled) are shown. Dashes indicate where PLFAs were unable to be resolved or detected

PLFA	Control bioreactors						Treatment bioreactors					
	PLFA weight percent[a]			$\delta^{13}C$-PLFA			PLFA weight percent[a]			$\delta^{13}C$-PLFA		
	K4-1	K4-2	Mean	K4-1	K4-2	Mean	K2-1	K2-2	K2-3	Mean	K2-1	K2-2	K2-3	Mean
i14:0	–	0.07	0.04 (0.05)	–29.5	–28.7	–29.1 (0.59)	–	0.06	0.08	0.04 (0.05)	440	784	717	647 (182)
14:0	0.12	0.03	0.08 (0.06)	–30.1	–29.7	–29.9 (0.29)	0.29	0.06	0.13	0.16 (0.12)	312	546	466	441 (119)
i15:0	1.17	0.59	0.88 (0.41)	–28.9	–29.4	–29.1 (0.37)	2.18	0.88	1.42	1.49 (0.66)	448	895	649	664 (224)
a15:0	1.20	0.47	0.84 (0.52)	–28.1	–28.3	–28.2 (0.09)	1.66	0.67	1.27	1.20 (0.50)	387	828	613	609 (220)
15:0	0.23	0.12	0.17 (0.08)	–30.2	–30.3	–30.3 (0.05)	0.27	0.13	0.22	0.21 (0.07)	314	514	462	430 (104)
i16:0	1.32	0.97	1.14 (0.25)	–27.4	–26.9	–27.1 (0.33)	1.51	1.03	1.27	1.27 (0.24)	260	514	452	409 (132)
16:0	11.44	9.13	10.28 (1.63)	–28.3	–27.8	–28.0 (0.32)	9.88	8.36	9.92	9.39 (0.89)	331	607	514	484 (141)
16:1[b]	1.84	1.35	1.59 (0.32)	–27.1	–27.4	–27.3 (0.22)	1.95	1.50	1.90	1.78 (0.24)	248	422	445	372 (108)
16:1ω7ct	8.86	6.04	7.44 (1.97)	–31.0	–30.8	–30.9 (0.17)	8.54	6.30	8.12	7.66 (1.17)	387	753	612	584 (185)
16:1ω5c	5.77	4.65	5.21 (0.80)	–27.7	–28.1	–27.9 (0.28)	5.33	5.33	6.55	5.49 (0.99)	287	587	433	436 (150)
a17:0	2.07	2.09	2.08 (0.01)	–30.1	–30.7	–30.4 (0.44)	2.83	2.52	2.31	2.55 (0.26)	246	520	412	393 (138)
10Me16:0	3.79	5.69	4.74 (1.35)	–25.2	–25.2	–25.2 (0.00)	5.81	6.87	5.08	5.92 (0.90)	464	693	783	647 (165)
cy17:0	1.66	2.01	1.84 (0.25)	–31.8	–31.7	–31.7 (0.05)	1.47	1.38	1.89	1.58 (0.27)	543	931	803	759 (197)
18:0	4.64	5.92	5.28 (0.90)	–27.9	–28.1	–28.0 (0.16)	4.26	5.12	4.52	4.63 (0.44)	446	821	684	650 (190)
18:1ω9	5.61	6.74	6.18 (0.80)	–28.8	–28.5	–28.7 (0.20)	5.95	6.38	5.63	5.99 (0.38)	276	615	460	450 (170)
18:1ω7ct	16.81	19.59	18.20 (1.85)	–30.9	–31.3	–31.1 (0.33)	16.87	19.28	16.37	17.50 (1.51)	442	806	708	652 (188)
18:1ω5	4.73	4.46	4.60 (0.19)	–28.0	–28.6	–28.3 (0.42)	3.79	5.27	4.92	4.66 (0.77)	262	504	340	369 (123)
cy19:0	4.68	7.53	6.11 (2.02)	–32.9	–33.4	–33.1 (0.35)	5.75	6.65	5.17	5.86 (0.75)	534	1096	949	860 (292)
20:4ω6	5.05	3.36	4.21 (1.20)	–38.7	–38.2	–38.4 (0.34)	3.83	4.65	4.83	4.44 (0.53)	450	637	507	531 (96)
20:5ω3	2.99	1.92	2.46 (0.76)	–44.1	–43.5	–43.8 (0.38)	1.63	1.81	1.67	1.71 (0.10)	388	536	436	453 (76)

[a]PLFA concentration determined by GC-FID; however, values are reported only for PLFA detected by IRMS analysis
[b]Co-eluted value for 16:1ω9 and 16:1ω13t, which comprised 96 ± 0.5% and 4 ± 0.5% of the PLFA concentration, respectively

the controls (0.20 ± 0.37) compared to the treatment bioreactors (0.13 ± 0.45; Fig. 2D); however, this difference was not significant ($p = 0.216$, $F = 2.440$, df = 1, 3, $\eta^2 = 0.440$).

DISCUSSION

Metabolically active members of the microbial community

All PLFAs recovered from the bioreactor microbial community showed significant incorporation of the ^{13}C-labeled tree tissue leachate, with 8.5 to 19% of the microbial ^{13}C originating from the leachate (Fig. 1). This indicates that the entire heterotrophic microbial community utilized this terrestrial DOC source, either directly or indirectly, as a source of C. In addition, individual PLFAs were not all enriched in ^{13}C to the same extent, and the most prevalent PLFAs as determined by weight percent were not necessarily the most enriched (Table 3). The most abundant fatty acid (18:1ω7; by weight %) was highly enriched in ^{13}C ($\delta^{13}C$ = 652 ± 188‰), as were several of the less abundant PLFAs (e.g. i15:0; $\delta^{13}C$ = 649 ± 224‰), suggesting that both abundant and some of the more rare microbes were metabolically active. This has also been observed in other freshwater and coastal ecosystems (Jones & Lennon 2010, Campbell et al. 2011), and suggests that microbial abundance is not necessarily indicative of microbial DOC uptake and utilization. Lastly, the distribution of tree tissue leachate ^{13}C among the PLFAs is indicative of 3 or possibly 4 functional groups within the bioreactor microbial community that either were actively metabolizing the leachate or were predators of those bacteria; these functional groups are based solely on the degree of ^{13}C labeling.

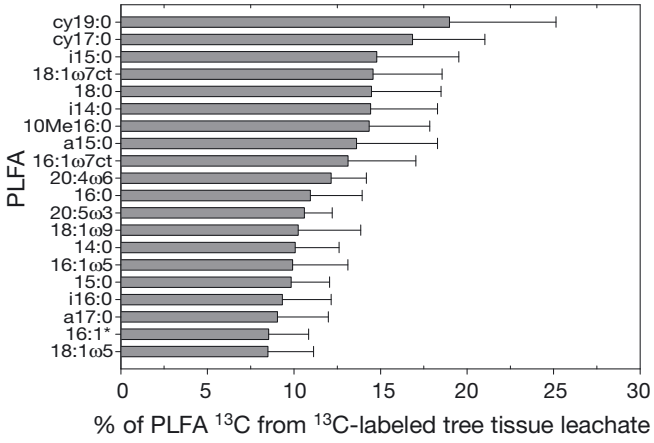

Fig. 1. Mean (±1 SD, n = 3) percentage of total phospholipid fatty acid (PLFA) ^{13}C in the treatment bioreactors derived from the ^{13}C-labeled tree tissue leachate amendments to stream water after a 41-d exposure. 16:1* is a co-eluted value for 16:1ω9 and 16:1ω13t, which comprised 96 ± 0.5% and 4 ± 0.5% of the PLFA concentration, respectively

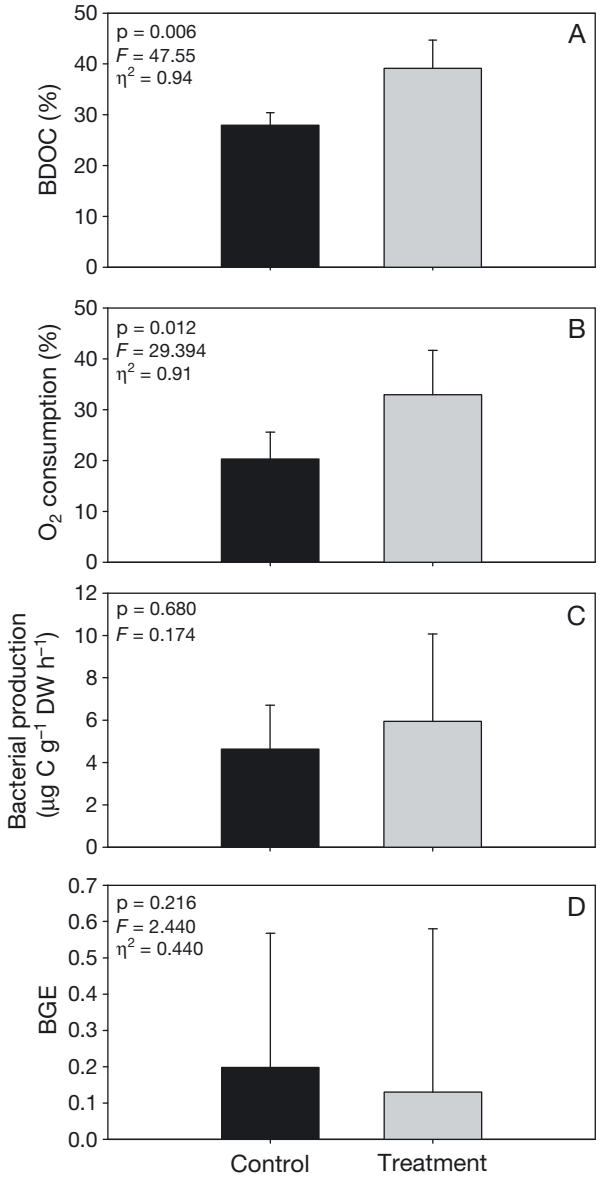

Fig. 2. Mean (±1 SD) (A) percent bioavailable dissolved organic carbon (BDOC), (B) percent O_2 consumption, (C) bacterial production (results from inlet of bioreactors shown), and (D) bacterial growth efficiency (BGE) in control and treatment (^{13}C-labeled) bioreactors. Data in A, B, and D were analyzed using a mixed between- and within-subjects model, while those in C were analyzed using a 2-way ANOVA (α = 0.05). Statistical results from the main factor (leachate exposure: treatment versus control bioreactors) are shown

The first functional group comprised bacteria and is represented by the fatty acids i15:0, 18:1ω7, 18:0, i14:0, 10Me16:0, a15:0, and 16:1ω7, with 13 to 15% of ^{13}C from these fatty acids originating from the ^{13}C-labeled tree tissue leachate. Of these, 18:1ω7 and 16:1ω7 are indicative of aerobic and facultative anaerobic Gram-negative bacteria (reviewed in Findlay 2004). The fatty acids i14:0, i15:0, and a15:0 can be found in both aerobic and facultative anaerobic Gram-negative bacteria, and Gram-positive bacteria (reviewed in Findlay 2004). i15:0 and i14:0 were 2 of the fatty acids with low PLFA weight percentages that had high ^{13}C enrichment, suggesting that some less abundant bacteria were metabolically active. The fatty acid 10Me16:0 is generally considered a marker fatty acid for sulfate-reducing bacteria, and 18:0 has a wide phylogenetic distribution among microorganisms (reviewed in Findlay 2004). The high level of PLFA ^{13}C originating from the leachate and the fact that these above fatty acids, along with cy19:0 and cy17:0, comprise ~45% of total PLFAs suggest that these organisms competed favorably for the labile DOC classes present in the leachate, and highlights their importance within the stream sediment community, both in overall biomass and in DOC processing.

The second functional group also comprised bacteria and is represented by the fatty acids 14:0, 16:1a, 15:0, i16:0, a17:0, 16:1ω5, and 18:1ω5, with 8.5 to 10% of the ^{13}C in the PLFAs originating from the ^{13}C-labeled tree tissue leachate. In general, these fatty acids are associated with Gram-positive and some anaerobic Gram-negative bacteria, although the 2 monoenoic fatty acids are generally associated with aerobic microorganisms (reviewed in Findlay 2004). The extent of leachate-derived C in these PLFAs suggests that these microbes are a metabolically active component of the community.

Heterotrophic microeukaryotes comprised the third functional group (fatty acids 20:4ω6, 16:0, 20:5ω3, and 18:1ω9), and ~10 to 12% of the ^{13}C in these PLFAs originated from the ^{13}C-labeled tree tissue leachate. The polyenoic fatty acids are only synthesized (with rare exception) by eukaryotes, and the greater weight percent of 20:4ω6 versus 20:5ω3 indicates the presence of heterotrophic, rather than phototrophic, eukaryotes (White et al. 1997). The incorporation of ^{13}C from the leachate indicates that C from this source not only served as a food resource for stream bacteria, but also that the C and energy consumed by these bacteria entered into the food web via predation by heterotrophic microeukaryotes. Our results also suggest that these predators fed on both groups of bacteria defined in this study. We calculate that ~32% of the incorporated ^{13}C from the leachate reached these protozoan grazers; this was calculated by dividing the percent of ^{13}C from the leachate in the heterotrophic microeukaryotes by the percent incorporated by the bacteria, including what was incorporated into the heterotrophic microeukaryotes. Our percentage is slightly higher than the transfer of sucrose (13–23%; Augspurger et al. 2008) and algal-derived C (10–29%; Risse-Buhl et al. 2012) measured in biofilms from 2 German streams. Our result suggests that labile classes of terrestrial DOC may have as great a potential for supporting higher trophic levels in the microbial loop as algal sources. The importance of terrestrial DOC in supporting higher trophic levels has also been documented in lake systems (e.g. Karlsson et al. 2003, Jansson et. al 2007, Caraco et al. 2010, Cole et al. 2011, Berggren et al. 2014).

The 2 fatty acids cy17:0 and cy19:0 may represent a fourth functional group. These fatty acids are widely distributed among bacteria (Grogan & Cronan 1997) and they are formed through post-synthesis modification of 16:1ω7 and 18:1ω7 within intact phospholipids by an enzyme that adds a methyl group to the PLFAs (Londry et al. 2004, Zhang & Rock 2008). Given the potential for enhanced ^{13}C incorporation via the methyl incorporation pathway and the high percentage of ^{13}C from the leachate in cy17:0 and cy19:0 (16.2 and 18.3%, respectively), it is prudent to interpret these 2 fatty acids as originating from the functional group of bacteria (aerobic Gram-negative bacteria), as indicated by their precursor fatty acids (16:1ω7 and 18:1ω7, respectively). The synthesis of these cyclopropane fatty acids has been linked to the physiological status of the bacteria, as they are thought to enhance viability of slow-growing or quiescent cells in hostile environments such as those experiencing starvation or exposed to low O_2 concentrations (Guckert et al. 1986, reviewed in Langworthy et al. 2002, Zhang & Rock 2008). Ratios of cyclopropyl PLFAs to their precursors (cy19:0/18:1ω7c and cy17:0/16:1ω7) have been used to assess the physiological status of prokaryotes in stream sediments (Langworthy et al. 2002, Sutton & Findlay 2003). Unstressed sedimentary microbial communities have cy/cis ratios less than 0.05, and stressed communities have ratios greater than 0.1 (White et al. 1997). Riverine sediment cy19:0/18:1ω7c and cy17:0/16:1ω7 ratios range from 0.17 to 0.25 (Langworthy et al. 2002, Sutton & Findlay 2003), and are generally lower than those observed in these bioreactors (0.18 to 0.40). The ratios in the bioreactors were greater than 0.1, suggesting that microbes were physiologically stressed, most likely from locally reduced O_2 concentrations, or that a significant proportion of the community was in stationary phase. Both conditions may result from low water flow rates and thick biofilms that together generate a diffusion barrier for O_2 between the overlying water and the actively metabolizing cells within the biofilm (Sutton & Findlay 2003).

Effects of ^{13}C-labeled tree tissue leachate on microbial community structure and function

Trace-level additions of ^{13}C-labeled tree tissue leachate derived from tulip poplar tissues to the bioreactors did not affect microbial community structure. This is not surprising, because tulip poplar is a common tree species in the WCC watershed and not a novel C source. However, the lower $\delta^{13}C$-DOC in the bioreactor outlet compared to the inlet suggests a preferential utilization of the leachate DOC. The leachate was highly enriched in labile DOC, as 93% of the additions were metabolized in the bioreactors compared to the roughly 30% BDOC content of stream water (Fig. 2A). Approximately 5% of the C present in the leachate was monosaccharide-C and an additional 8% was polysaccharide-C (Wiegner et al. 2005a). This contrasts to a 0.06–0.33% monosaccharide-C content in stream-water DOC under baseflow conditions (Gremm & Kaplan 1997). Even though the monosaccharides were present in μg C l^{-1} amounts (6 to 21 μg C l^{-1}) in the leachate, they represented as much as a 2- to 15-fold increase in the concentrations of glucose present in stream water, along with lesser amounts of fructose and mannitol (Gremm & Kaplan 1997, Cheng & Kaplan 2001).

The labeling of 2 bacterial functional groups determined from *a posteriori* analysis indicates that a community of microbes, and not a single population, was involved in degradation and consumption of the terrestrial-derived DOC. Further, the percentage of PLFA ^{13}C derived from the leachate was similar between the 2 bacterial functional groups, suggesting that the labile components of the leachate were equally bioavailable to both groups; experiments examining degradation of lake DOC under oxic and anoxic conditions support this finding (Bastviken et al. 2004). In contrast, it has been suggested that aerobic bacteria capable of producing oxygenases are responsible for consumption of more recalcitrant DOC (Bastviken et al. 2004). Our results neither support nor refute this finding, as both functionally defined groups contain fatty acids that are associated with anaerobic bacteria. Therefore, we surmise that labile DOC decomposers were composed of both aerobic and anaerobic bacteria.

Summary

Increasing the $\delta^{13}C$-DOC signature in the stream water from −28‰ to +1021‰ with only a 6% increase in DOC concentration was sufficient for labeling microbial lipids within bioreactors. From our labeling experiment, we were able to identify microbial groups that actively consumed the ^{13}C-labeled tree tissue leachate. They were aerobic prokaryotes and eukaryotes, as well as facultative anaerobic and anaerobic bacteria. The leachate ^{13}C incorporated into the heterotrophic bacteria was transferred to heterotrophic microeukaryotes with 32% efficiency, higher than any efficiency reported for algal-derived DOC to date. While the leachate additions did not appear to change the microbial community structure, bacterial production, or BGE in the bioreactors, they did increase DOC and O_2 consumption. These findings indicate that labile terrestrial DOC plays an important role in stream metabolism, and underscores its importance in supporting higher trophic levels in the microbial loop.

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