Carbon limitation of heterotrophic respiration is linked to dissolved organic matter quality in urban streams

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Abstract

Urban streams are degraded by a suite of factors, including burial beneath urban infrastructure (i.e. roads, parking lots) that eliminates light and reduces direct organic matter inputs to streams, with likely consequences for the quality and quantity of dissolved organic carbon in stream water, the primary energy source for microbial communities.We studied seasonal changes in microbial carbon use patterns in open and buried reaches of three urban streams in Cincinnati, Ohio. We characterized organic matter quality using fluorescence, microbial carbon use, and carbon limitation patterns. We hypothesized: 1) that algal production would lead to higher quality dissolved organic matter (DOM) in spring compared to other seasons and in open compared to buried reaches, 2) lower reliance of microbial respiration on recalcitrant carbon sources in spring and in open reaches, and 3) that microbial respiration would be more carbon limited in the fall and in buried reaches. DOM quality was generally higher in Spring than Fall, but the only DOM quality metric that varied by reach was an indicator of recalcitrant humic compounds, which showed more humic DOM in open reaches compared to buried. This likely reflected open reaches as an avenue for direct terrestrial inputs from the riparian zone. Extracellular enzyme assays showed thatmicrobes in buried reaches consistently allocated more effort to degrade recalcitrant carbon sources, consistent with a lack of labile carbon compounds due to limited photosynthesis. Finally, buried and open reaches were both more carbon limited in Fall when terrestrial leaf inputs dominated organic matter inputs compared to the Spring when vernal algal blooms were pronounced. Taken together, our data show that stream burial affects the quality of DOM pool with consequences for how microbes use those carbon sources, and that buried and open stream reaches were limited by labile carbon in all seasons. The alteration of carbon quality and use patterns coupled with widespread carbon limitation suggests that these urban streams likely export recalcitrant carbon to downstream water bodies, and that the cycling of nitrogen and/or phosphorus could decrease if labile carbon sources limit heterotrophic metabolism.

Introduction

As suburban sprawl converts farmland and forests to urban infrastructure, and as the global trend of urbanization continues (reference, e.g. Grimm etal 2008 Science), the biological function of urban streams and their role in water quality has received increased attention (Kaushal et al 2015). Relatively small increases in impervious surface cover through urbanization can lead to a “flashy” hydrologic regime that reinforces entrenchment and channel incision in streams that are often already channelized to promote storm water drainage (Dunne and Leopold 1979). These channelized streams are less retentive of particulate organic carbon (Paul and Meyer 2001) which, in combination with greater nutrient loads (Carpenter et al. 1998) and reduced riparian canopies (Griffiths et al. 2013), can alter the contribution of heterotrophic and autotrophic processes to stream metabolism. For example, canopy opening and nutrient enrichment can increase autotrophy (Bernot et al. 2010; Griffiths et al. 2013; Alberts et al. in press), but stream burial can increase the importance of heterotrophy relative to autotrophy (Beaulieu et al. 2014; Pennino et al. 2014).

The expansion of urban infrastructure to facilitate development frequently results in low order streams being contained in buried pipes (Elmore and Kaushal 2008). Further, open and buried stream reaches often alternate in an urban hydrological network so that stream metabolism can be vastly different in alternating stream reaches. For example, the severe reduction or absence of photosynthetically active radiation (PAR) fundamentally alters a stream ecosystem by eliminating the contribution of primary production to the food web. Although metabolism in buried streams shifts to net heterotrophic conditions, buried streams support a lower overall rate of ecosystem respiration compared to open reaches (Beaulieu et al. 2014; Pennino et al. 2014). Because buried stream reaches are often optimized to convey water quickly and efficiently for drainage purposes, they have increased water velocity which, in conjunction with net reduction in overall biological demand for nutrients (Beaulieu et al. 2014; Pennino et al. 2014), promotes nutrient export to downstream reaches and ecosystems (Beaulieu et al. 2015). Burial also severely affects standing stocks of organic matter in streams. Buried reaches have lower overall coarse and fine benthic organic matter, periphyton, and chlorophyll a standing stocks compared to open reaches (Beaulieu et al. 2014). Organic matter standing stocks in buried reaches also have little seasonality, except for higher coarse benthic organic matter (CBOM) in the fall (Beaulieu et al. 2014), whereas organic matter standing stocks vary exhibit pronounced seasonal patterns in open streams. Although the effect of stream burial on particulate organic matter standing stocks has been investigated, how this effect propagates through the microbial loop (Meyer 1994) to determine the abundance and quality of dissolved organic matter (DOM) is unknown.

Dissolved organic matter is an important energy source for ecosystem respiration (Meyer and Edwards 1990), and is processed in a microbial loop that transfers this energy from dissolved sources to higher trophic levels (Meyer 1994). Streams depend on allochthonous organic carbon inputs from the terrestrial landscape including direct inputs from the riparian zone and DOM exported from soil by groundwater, as well as autochthonous sources from in-stream production of algae and/or macrophytes. These sources of organic matter partly determine the quality of the DOM pool available to stream microbial communities. Allochthonous inputs are generally more recalcitrant than autochthonous sources (McKnight et al. 2001) due to the presence of structurally complex carbon compounds such as lignin and tannin. In contrast, autochthonous carbon sources have fewer complex structural compounds and relatively more polysaccharides like cellulose and hemicellulose, and are generally considered a higher quality energy source for microbial metabolism. Therefore, the lability of the DOM pool is likely to vary seasonally in conjunction with autumn leaf inputs and vernal algal blooms. Moreover, urban infrastructure likely also affects the composition of the DOM pool with open reaches having more labile DOM than buried reaches due to greater light availability and associated higher levels of primary production. These seasonal and reach-scale differences in organic matter dynamics in urban streams are likely to influence the quality of the organic carbon pool and associated rates of microbial carbon processing.

We used a nutrient diffusing substratum (NDS) approach coupled with extracellular enzyme activity (EEA) assays and characterized DOM via fluorescence to understand how organic carbon demand varies seasonally in buried and open stream reaches of urban streams. EEA assays have been used to characterize how microbes allocate energy to acquire different compounds (e.g., labile or recalcitrant carbon, nitrogen, etc.). These assays illuminate resource demand for, and environmental availability of, substrates required for microbial metabolism (Sinsabaugh and Follstad Shah 2012), and they have been used to infer microbial organic nutrient limitation patterns in soils and sediments (e.g, Sinsabaugh et al. 2009) and within river networks (Hill et al. 2012). Using fluorescence to characterize the bulk DOM pool allows the definition of various fractions of DOM as more or less labile, allochthonous or autochthonous, etc. This technique has been used to investigate seasonal (Catalan et al. 2013) and landscape (Williams et al. 2016) in the composition of the DOM pool in surface waters. We defined three hypotheses based on anticipated seasonal and spatial patterns of organic matter and light availability in urban streams containing open and buried reaches. We hypothesized that spring would have higher quality DOM than other seasons due to higher algal production prior to leaf-out of riparian trees and warming stream temperatures, and that open reaches would have higher quality DOM than buried reaches due to more algal production in open reaches versus buried reaches. Consequently, we hypothesized that microbes in spring would produce lower extracellular enzyme indicators associated with recalcitrant carbon acquisition, and that microbes in open reaches would exhibit less effort to acquire recalcitrant carbon compared to buried reaches. Finally, we hypothesized that microbial respiration would be more carbon limited in the fall due to the pulse of low quality terrestrial organic matter from the riparian zone, and that buried reaches would be more carbon limited than open reaches due to lower primary production and allochthonous carbon inputs. Regardless of reach or season, we predicted that microbial respiration would respond more strongly to the higher quality carbon compared to lower quality carbon.

Methods

Study Sites and Experimental Design

We studied three urban streams in or near Cincinnati, Ohio (USA) consisting of paired buried and open study reaches separated by a 30-100 m buffer reach. Two buried reaches flowed through corrugate pipe and one through a concrete tunnel, and buried stream widths ranged from 0.5-4.5 m. Open reaches were generally incised with restricted riparian zones, contained mobile sandy sediments, and ranged in width from 2.1-3.9 m. A more detailed site description can be found in Beaulieu et al. (2014).

We collected water samples to characterize dissolved organic matter quality in summer and autumn 2011 and in spring 2012. Concurrently, we collected biofilms from unglazed clay tiles that were deployed in the streams for a minimum of 6 weeks for extracellular enzyme activity analysis. Microbial arbon limitation patterns were measured using nutrient diffusing substrata. This design allowed us to compare how carbon quality, microbial enzyme activity, and the biofilm response to added carbon varied in space (buried versus open stream reaches) and time (summer, autumn, and spring). We also collected a suite of other environmental data including water chemistry, hydrologic parameters, organic matter standing stocks, and whole stream metabolism and nitrate (NO3-) uptake to understand how those factors relate to the microbial response to variations in DOM quality. Nitrate uptake and hydrologic parameters (i.e. transient storage) were measured using whole stream 15N-NO3- and bromide (Br-) releases. Methods that describe the processing of isotope samples, calculating NO3- uptake rate, and modeling one- and two-station whole-stream metabolism are beyond the scope of this paper, but are detailed in Beaulieu et al. (2014).

DOM Characterization

Dissolved organic matter quality was characterized using fluorescence excitation-emission matrices (EEMs; Coble et al. 1990, Coble 1996, Cory et al. 2010) measured on a Fluoromax-4 spectrofluorometer (Horiba Instruments, Kyoto, Japan). This technique allows the differentiation of humic-like, fulvic-like, and protein-like fractions of the bulk DOM pool, which in turn are generally related to the lability or recalcitrance of the DOM pool. EEMs were measured using excitation wavelength at 10 nm intervals between 240-450 nm and wavelengths at 2 nm intervals from from 290-600 nm. Three-dimensional EEMs were then instrument corrected, blank subtracted, and normalized by the water Raman signal (Cory et al. 2010) using Matlab software, but we did not measure absorbance for each sample, so we could not perform the standard inner-filter correction on the EEMs. Therefore these results will be most useful for relative differences across sites and time rather than for comparison to literature values.

The EEMs were used to calculate indices of DOM quality, including the humification index (HIX; Zsolnay et al. 1999; Huguet et al. 2009), the biological freshness index (BIX; Huguet et al. 2009), the fluorescence index (FI; McKnight et al. 2001), and the protein-to-humic ratio (P/H; Coble 1996; Stolpe et al. 2010). HIX characterizes the humic or autochthonous fractions of DOM (Zsolnay et al. 1999; Ohno 2002), and it is calculated as the ratio of integrated fluorescence emission intensity between 300-345 nm and between 435-480 nm at 254 nm excitation. Higher HIX values indicate DOM with humic character whereas lower values indicate either less humic or more autochthonous DOM. BIX was calculated from the ratio of emission at 380 and 430 nm at excitation of 310 nm (Huguet et al. 2009). BIX values <0.7 are associated with allochthonous DOM, values 0.8-1.0 are associated with autochthonous DOM, and values >1.0 are associated with aquatic bacterial sources; higher values indicate greater lability than lower values. FI is calculated from the ratio of the fluorescence intensity at 450 nm and 400 nm at excitation of 370 nm. FI values of about 1.9 indicate fulvic acids from microbes and values of about 1.4 indicate terrestrial fulvic acids. Finally, P/H was calculated from the EEMs whereby excitation at 275 nm and emission at 340 nm is associated with protein-like organic matter and excitation at 350 and emission at 480 is associated with humic-like organic matter (Coble 1996; Stolpe et al. 2010). We did not perform inner filter corrections on the data so HIX cannot be compared to literature values or among seasons, but the P/H ratio will allow us to make inferences about relative abundance of labile and recalcitrant DOM among streams and seasons.

Extracellular enzyme activities (EEA)

Periphyton collected from tiles deployed in buried and open reaches was analyzed for extracellular enzyme activities (EEA). Microbial assemblages produce extracellular enzymes to degrade organic matter and to acquire nutrients from their environment, and the activity of those enzymes serves as an index of environmental resource availability (Sinsabaugh and Foreman 2001). Acquisition of labile carbon compounds was measured as -D-glucosidase activity, and acquisition of recalcitrant carbon compounds was measured as polyphenol oxidase (POX) and peroxidase activity. The ratio of recalcitrant carbon acquisition to total carbon acquisition (as -D-glucosidase + polyphenol oxidase) characterizes the overall quality of the DOM pool (LCI) whereby values greater than 0.5 indicate greater effort to acquire recalcitrant carbon and values less than 0.5 indicate greater effort to acquire labile carbon (Sinsabaugh and Follstad Shah 2011). An alternate metric of recalcitrant carbon acquisition was measured as the activity of L-3,4-dihydroxyphenylalanine (DOPA) + H2O2 as a substrate. Nitrogen acquisition was measured as the activity of -N-acetylglucosaminidase (NACE: EC 3.2.1.50).

All EEA assays used microplate protocols (Sinsabaugh et al. 1997; Sinsabaugh and Foreman 2011) modified by Hill et al. (2010). Microplate arrays were run with quadruplicate assays for each tested enzyme and reference standard, which were prepared in sterile deionized water. Fluorescence quenching, or the decrease of emissions caused by interaction between target enzyme substrates and non-reactant chemicals, was measured by comparing fluorescence of standard solutions mixed with sample to that of standard solution mixed with buffer. We measured fluorescence (Model FLX800T, BioTek Instruments, Winooski, VT, USA) at excitation wavelength of 350 nm and emission wavelength of 450 nm.

Nutrient diffusing substrata (NDS)

NDS arrays were deployed throughout the open reaches, and at the upstream and downstream end of the buried reaches. Each NDS array had one of four 0.5 M carbon amendments (glucose, arabinose, cellobiose, or a no-carbon control (n=8 each)) to represent differences in carbon bioavailability. The NDS were supplemented with 0.5 M N as NH4Cl and 0.5 M P as KH2PO4 to alleviate any potential nutrient limitation that could confound interpretation of the heterotrophic response to added carbon, and we used porous glass disks rather than cellulose sponges to eliminate the heterotrophic response to the sponge as a particulate carbon source. NDS arrays were installed within open-ended lengths of PVC pipe for shade and to reduce the potential for autotrophic biofilms to colonize the glass disks. NDS arrays were collected after a two week deployment and shipped overnight on ice for laboratory analysis within 24 h.

Laboratory analysis for biofilm respiration consisted of submerging the NDS disks in site water, incubating the disks in the dark for 3.5 h, and recording net oxygen change from the start to the end of the incubation. The glass disks were saved for calculation of biomass after weighing oven-dried (60 °C) samples before and after combustion in a muffle furnace (500 °C). The respiration response was scaled by disk area (g O2 cm-2 h-1) and by biomass (mg O2 gAFDM-1 h-1), and in order to compare the respiration response among streams and seasons, we calculated the nutrient response ratio (NRR) as respiration response for an individual NDS cup divided by the mean control response for that particular deployment.

Water chemistry and hydrologic parameters

We collected filtered (0.45 m) water samples in the field and stored them on ice for transport to the laboratory where they were acidified or frozen depending on the analyte. We used standard colorimetric methods to measure nitrate + nitrite (hereafter, NO3-), dissolved reactive phosphorus (DRP), ammonium (NH4+), and bromide (Br-) on a flow injection analyzer (Lachat Instruments, Loveland, CO USA). Dissolved organic carbon (DOC) concentration was measured using high-temperature Pt-catalyzed combustion and NDIR detection (Shimadzu TOC-VCPH, Columbia, MD, USA).

The breakthrough curve of Br- released in conjunction with the 15N-NO3- release was used in OTIS-P (Runkel, 1998), a one-dimensional advection, dispersion and transient storage model, to estimate solute hyporheic exchange parameters such as the cross-sectional area of the transient storage zone (As) and the storage zone exchange coefficient (). From these parameters, we calculated the storage zone residence time (Tsto)

Tsto = As/ \* A

where A is the cross-sectional area of the stream channel calculated from the bromide breakthrough curve and channel measurements. We calculated the storage exchange flux (qs)

qs = \*A

which represents the average water flux through the storage zone per unit length. We also calculated fraction of the median travel time due to transient storage, F200med (Runkel, 2002).

Organic matter standing stocks

We collected 10-20 samples of organic matter from different habitat units in a stratified-random sampling design. Samples for coarse (>1 mm), fine (<1 mm), and attached (i.e., periphyton) organic matter were collected from a 0.052 m2 area isolated by an open-ended plastic cylinder placed no more than 5 cm into the sediment. Coarse benthic organic matter (CBOM) was removed by hand, and the sediments were agitated before taking a fine benthic organic matter (FBOM) sample. We collected periphyton by scraping a known area (0.006-0.04 m2) of a rock with a wire brush. We calculated sample dry mass and ash-free dry mass of samples by weighing oven-dried (60 °C) samples before and after combustion in a muffle furnace (500 °C). We used a subsample of periphyton to measure chlorophyll a using the trichromatic method (APHA 2005) following hot ethanol extraction (Sartory and Grobbelaar 1984).

We deployed unglazed clay tiles for six weeks at all sites to provide a standardized surface for algae and bacteria to colonize. Periphyton on tiles was removed with a toothbrush and razor blade, rinsed into a bottle with in situ water, and stored on ice until analysis. Subsets of tiles were analyzed for algal abundance using a Palmer-Maloney counting cell (Charles et al. 2002), total bacterial counts using qPCR, and extracellular enzyme activity assays. Detailed methods for these analyses are described in Beaulieu et al. (2014).

Statistical Analysis

We used multivariate generalized least squares linear models (GLS) to test how DOM quality (HIX, BIX, FI, P/H) differed among seasons (summer, autumn, spring), between reaches (buried, open), and among streams. We also used GLS to test for differences in extracellular enzyme activity (POX, DOPA-H2O2, LCI, NACE) and carbon limitation patterns among seasons, between reaches, and among streams. We used linear modeling to test relationships between carbon limitation patterns and water chemistry, hydrologic parameters, organic matter standing stocks, and whole stream metabolism and nitrate (NO3-) uptake. We used permutational multivariate analysis of variance using distance matrices (adonis in the vegan package for R; Oksanen et al. 2016) to test detect a relationship between the aggregated response of microbial respiration to glucose, arabinose, and cellobiose additions and CBOM and FBOM standing stocks. . All statistical analyses were done using R (R Core Team 2016).

Results

Patterns in DOM Variability

We examined differences in dissolved organic matter quality among seasons (summer, autumn, spring) and between reaches (buried, open). HIX, the humification index derived from EEMs, differed by season (GLS, p=0.0005), with autumn having higher HIX than spring or summer, which were not different from each other, and also differed by reach (GLS, p=0.021) with open reaches having higher HIX than buried reaches (Figure 1). Because we did not perform the standard inner-filter corrections on these samples, these values cannot be compared to literature values, and using these data alone, we cannot determine whether our sites or seasons have more allochthonous or autochthonous organic content. Rather, they can be used to show relative difference between reaches and among seasons whereas other metrics from the EEMs can clarify allochthonous/autochthonous DOM content.

For example, the biological freshness index (BIX) varied by season (GLS, p<<0.0001) but did not differ between buried and open reaches (Figure 2A). Although BIX did not differ between spring and summer, both were significantly higher compared to autumn. Typically BIX values between 0.6-0.7 are associated with DOM having low autochthonous content whereas BIX >0.9 are associated with high autochthonous content. The fluorescence index (FI) characterized whether fulvic acids in the DOM pool are microbially derived (~1.9) or terrestrially derived (~1.4), and FI was higher in summer and spring compared to autumn (GLS, p<<0.0001) but did not differ between buried and open reaches (Figure 2B). Summer and spring did not differ from each other.

A fourth index, the protein to humic ratio (P/H), compares autochthonous and labile tryptophan-like and protein-like content (more autochthonous and labile) to more terrestrial and recalcitrant humic-like content, and it was generally less than 1 indicating high humics. This ratio varied by season (GLS, p<<0.001), with spring and summer having a higher ratio (more protein) compared to fall (Figure 3), and also by reach (GLS, p<<0.0002) with open reaches having lower ratio (more humic-like) than buried reaches when all seasons were combined.

Patterns in extracellular enzyme activity

Extracellular enzyme activity characterizes microbial energy allocation to acquire nutrients and carbon from the environment. Extracellular enzymes that degrade L-3,4-dihydroxyphenylalanine (DOPA) + H2O2 (DOPAH2) correlate to lignin degradation and is a metric of recalcitrant carbon use. While we found no significant differences in DOPAH2 among seasons, buried reaches had higher DOPAH2 than open reaches (GLS, p=0.024) when we expressed DOPAH2 per unit dry mass (Figure 4a) or per unit carbon (data not shown). We found a similar pattern in the polyphenol oxidase (POX) extracellular enzyme activity. POX is an alternate metric of recalcitrant carbon use, and we found higher POX in buried reaches compared to open reaches (GLS, p=0.0043) (Figure 4b).

We found no evidence of spatio-temporal differences in extracellular enzyme activity (EEA) associated with labile carbon use. However, when we composited metrics of carbon use together to calculate the LCI, an index of carbon lability that compares recalcitrant carbon use to total carbon use, we found that buried reaches had higher use of recalcitrant carbon (GLS, p=0.014), and microbes in summer had greater use of recalcitrant carbon than in autumn (GLS, p=0.027). There were no differences in carbon use between spring and autumn (Figure 5). The LCI was also correlated to the CQI, an alternate carbon quality index derived from EEA on different substrates (data not shown).

Because carbon uptake and use is often linked to the acquisition of nitrogen from the environment, we also analyzed differences in N uptake as activity of -N-acetylglucosaminidase. We measured highest values in the autumn, intermediate values in summer, and lowest values in spring with all seasons significantly different from each other (GLS, p<<0.0001), but there were no differences between reaches (Figure 6).

Carbon limitation

We deployed NDS amended with different carbon sources (glucose, arabinose, cellobiose, and a no-carbon control) to see if patterns in carbon limitation differed between buried and open stream reaches or among seasons. Unfortunately, the NDS we deployed during summer were washed away by thunderstorms that produced very flashy runoff in these urban streams.. Therefore, we focus our analysis on autumn and spring to contrast the carbon limitation response to a time when leaf inputs dominate compared to when vernal algae blooms dominate.

When respiration on NDS disks was scaled by biomass (mg O2 gAFDM-1 h-1), we found no differences among carbon amendments including the no carbon control. However, when the respiration response was scaled by disk area (g O2 m-2 h-1), all NDS carbon amendments were significantly different than the control in all streams, seasons, and reaches (GLS, p<<0.001), and we found no instances where the respiration response differed among the three carbon amendments during any deployment (GLS, p>0.05). Therefore, we analyzed the nutrient response ratio (NRR) of all carbon types together to detect differences between seasonal and/or reach-scale responses. Furthermore, although we deployed NDS arrays at the top and bottom of the buried reaches, there was no difference in the response, so we categorized them all as “buried.” Generally, NRR was higher in fall compared to spring in both reaches (LME, p<<0.0001; Figure 7). We found a significant interaction (GLS, p=0.0009) between season (autumn versus spring) and reach (buried versus daylight) whereby the respiration response to added carbon was stronger for open compared to buried reaches in autumn, but it was stronger for buried compared to open reaches in spring (Figure 7). Overall, these results indicate carbon limitation in all streams and season.

To see what factors might predict the areal NRR response to added carbon among streams and between seasons and reaches, we analyzed a suite of reach-scale variables including standing stocks (e.g., chl a, periphyton biomass, bacterial cell counts, FBOM, CBOM etc.), water chemistry (e.g., NH4+, NO3-, SRP, DOC, etc.), hydrologic variables (e.g., Q, As/A, travel time, etc.), ecosystem-scale functional attributes (e.g., NO3- uptake, whole-system metabolism), metrics of microbial effort to acquire nutrients using EEA assays, and metrics of dissolved organic matter quality derived from excitation-emission matrices. We found no relationships between the NRR response and water chemistry, hydrology, or ecosystem-scale functional attributes. Although EEA and DOM quality metrics often differed between seasons and reaches, there was no direct relationship between NRR and those metrics. Further, most standing stock metrics were also unrelated to the NRR response, but we did find weak positive relationships between reach-scale standing stocks of CBOM (adonis, p=0.036) and FBOM (adonis, p=0.053) with glucose, arabinose, and cellobiose.

Discussion

Seasonal patterns of DOM characteristics

These urban streams had higher CBOM biomass in autumn compared to other seasons and higher chlorophyll a biomass in spring than in other seasons (see Beaulieu et al. 2014 Fig 3). Because terrestrial carbon sources are typically of lower quality than aquatic autochthonous DOM sources (McKnight et al. 2001), these changes in particulate organic matter stocks should be reflected in changes to the quality of the DOM pool, with lower quality DOM dominating in fall and higher quality DOM dominating in the spring. As we hypothesized, BIX and FI, metrics of labile DOM, show a clear pattern of less labile carbon during autumn and more labile carbon during spring, likely due to riparian leaf fall producing a large influx of recalcitrant terrestrial DOM in the fall and vernal algal blooms producing a large influx of labile autochthonous DOM in the spring. HIX, which measures the recalcitrant humic fraction of DOM, is similar to BIX and FI with autumn having higher humic character than spring or summer. This pattern is also seen in the P/H (protein/humic) ratio, which shows more humic-like components in the autumn compared to the spring whereas summer was not distinctly different. Collectively, these patterns reflect the reach-scale standing stock data collected during this study. This seasonal pattern is seen in temperate forested mountain streams (Villanueva et al. 2016), ephemeral Mediterranean streams that flow during the autumn-spring wet season (Catalan et al. 2013), and in other urbanized streams (Hosen et al. 2014). Therefore, temperate zone seasonality of autumn riparian leaf inputs and spring algal blooms imparts the dominant seasonal signature to the DOM pool of these temperate urban streams even though they have limited riparian zones due to channelization.

Despite the strong and consistent seasonal differences seen across multiple DOM optical properties, the low absolute values of BIX and FI show that the DOM pool in all seasons has a weak autochthonous component and a strong signature of terrestrially-derived fulvic acids, despite higher productivity and higher algal standing stocks in the spring. The dominance of terrestrial or humic derived carbon in the DOM pool may be a general pattern in streams draining urbanized basins. For example, microbial humic-like DOM compounds were associated with higher population density and greater proportion of developed land across nearly 200 catchments in southeast Canada (Williams et al. 2016). In contrast to the higher microbial humic signature in Williams et al. (2016), we found more terrestrial humic-like DOM compounds instead, suggesting a terrestrial DOM source. Terrestrial DOM sources include upwelling ground water, leaking stormwater infrastructure (Kaushal and Belt 2012), and runoff from impervious surfaces (Hope et al. 2014). DOM derived from these sources may overwhelm any *in situ* aquatic signature. Alternatively, the year-round stronger terrestrial/recalcitrant characteristics could indicate that heterotrophic biofilms, which are typical in urban streams (Johnson et al. 2009), rapidly remove high quality DOM from the water column. For example, Franke et al. (2013) found that labile carbon, such as that from autochthonous sources, stimulated water column carbon use for energy metabolism and/or assimilation, and algal biofilms enhanced the EEA of heterotrophic biofilms, suggesting the rapid use of labile DOM in the presence of autochthonous production (Rier et al. 2014). Rapid use of high quality DOM would be consistent with systemic carbon limitation, which we found in all reaches and seasons.

Spatial patterns of DOM characteristics

Our hypothesis that open reaches would have more labile carbon than buried reaches was not supported by the optical properties of the DOM pool. Although reach-scale standing stock data showed lower overall chlorophyll and CBOM in buried compared to open reaches, reach was not a significant predictor of BIX or FI, metrics that indicate labile DOM. One explanation for the lack of a burial effect on BIX and FI is that these optical properties of the DOM pool are determined by processes at the larger stream segment or catchment scale, rather than the reach scale. For example, in a previous study at the stream network scale and across a range of discharges in urbanized catchments, BIX never had a strongly autochthonous character despite many instances of net ecosystem productivity in the spring across 30 months of continuous sampling (Smith and Kaushal 2015). Further, a cross-system study found that catchment scale land use was a good predictor of DOM composition (Williams et al. 2016), which implies that catchment urbanization could have overwhelmed reach-scale differences in organic matter dynamics at our highly urbanized streams (16-34% impervious surface cover; Beaulieu et al. 2014).

In contrast to BIX and FI, which were not affected by burial, HIX (an indicator of humic DOM) was higher in open reaches compared to buried reaches, which was contrary to our hypothesis that buried reaches would have lower quality DOM. This pattern was also reflected in the P/H (protein-to-humic) ratio, which was likely driven by the relative abundance of humic-like compounds (denominator of the ratio) rather than patterns in aquatic production that affected low molecular weight fractions of the DOM pool (numerator of the ratio), consistent with the year-round humic nature of DOM in these urban streams. The pattern of higher HIX in open reaches was largely driven by high HIX in autumn (Figure 1) when open reaches received and retained more leaf litter that could leach recalcitrant terrestrial DOM. In contrast, buried reaches neither received direct inputs of riparian leaf litter nor retained litter exported from upstream due to higher velocities and fewer retention structures (Beaulieu et al. 2014). Alternatively, several abiotic mechanisms could account for lower HIX in buried reaches including dilution of the DOM pool by lower HIX sewage sources that leak into the buried reaches (Smith and Kaushal 2015) or by sorption of humic compounds during transport through the buried reaches (Ohno 2002; Zsolnay et al. 1999). We collected water for EEM metrics from the top and bottom of the buried reaches, and there was no significant difference in HIX collected at either end of a buried reach (data not shown). Therefore, although the EEA data indicated greater use of recalcitrant carbon in buried reaches compared to open reaches (see below), microbial processing was not enough to change the humic character of the DOM as water flows through the buried reach. Therefore microbial processing of humic compounds was not likely a dominant enough process to reduce the HIX of the DOM pool. Higher HIX in the open reach in spring is counter-intuitive given the presence of large algal standing stocks and high GPP, which would be expected to produce labile DOM. It is possible that the high HIX values resulted from DOM leached from greenfall inputs during leaf out and/or flower or seed production (Lewis and Likens 2007). However, in the overall context of this study, the median spring HIX values are lower than autumn in open and buried reaches, which is still consistent with an overarching seasonal affect driven by terrestrial sources in the autumn and aquatic sources in the spring.

Despite spatio-temporal differences in the DOM composition driven by seasonal differences in CBOM and algae, the DOM data show that these urban streams are dominated by terrestrial humic sources, likely from constant seepage of DOM from soils to streams throughout the watershed. These data also show secondary control over DOM quality due to spatial differences in organic matter inputs that alter the characteristics of the DOM pool in buried versus open reaches. Therefore urban infrastructure can influence the characteristics of the DOM pool in the urban stream network.

Patterns in Carbon Use – Extracellular Enzyme Activities (EEA)

Extracellular enzyme activity (EEA) reflects the composition of the DOM pool, as perceived by the microbial community. Although P/H and HIX indicated more humic and recalcitrant DOM in open reaches, buried reaches had higher DOPAH2 and POX (indicators of recalcitrant carbon) than open reaches. This supports our hypothesis that the microbial community in buried reaches would allocate more energy toward acquiring recalcitrant carbon sources than in the open reach, regardless of season. This pattern is consistent with experiments showing greater POX in low light conditions (Wagner et al. 2015). Lower values of DOPAH2 and POX in the open reach indicate less effort to acquire recalcitrant carbon, likely because DOM leached from primary producers supplies an alternative, high quality carbon source. Conversely, the greater effort to acquire recalcitrant carbon in buried reaches is consistent with low chlorophyll, limited periphyton cover, and an extremely low reach scale GPP estimates (also observed in Beaulieu et al. 2014 and Pennino et al. 2014) that suggest more recalcitrant carbon is available/used/what?. This implies rapid use of high quality carbon produced in the open reaches and little export to downstream buried reaches, and is consistent with generally greater EEA in the presence of algal biofilms (Rier et al. 2014).

The LCI, which aggregates several EEA measures into a composite index of carbon use, also shows greater use of recalcitrant carbon in buried reaches. However, LCI shows a seasonal effect whereby summer has greater use of recalcitrant carbon than autumn, but that autumn and spring were not different. This pattern may be driven by low CBOM, low chlorophyll a, and high FBOM in open reaches during the summer. The lack of chlorophyll and reliance on FBOM, a highly processed particulate carbon source, may explain the high use of recalcitrant carbon sources in summer. Furthermore, the lack of difference in enzyme activity between spring and autumn, despite the major differences in CBOM and chlorophyll, may reflect the overall terrestrial signature of the DOM pool, which is dominated by terrestrial sources even in the spring.

While the spatial patterns in EEA are consistent with our hypotheses, they do not match patterns in the optical properties of the DOM pool. This discrepancy may be due to differences in the composition of the DOM pool in the water column, where the EEM samples were collected, and at the sediment-water boundary layer where the microbial community was sampled for EEA. For example, labile carbon produced in the benthos of the open reaches may be rapidly and selectively processed by microbes with little being transported to the water column. Alternatively, the discrepancy between the optical and microbial indicators may be due to a temporal mismatch. The microbial EEA indicators likely reflect the integrated response of the microbial community to a temporally variable DOM pool, whereas the optical indices reflect the composition of the DOM pool at the moment the grab sample was collected. The mismatch between EEA and EEMs might also be related to specific substrates used in the EEA assays not corresponding to the compounds that determine the optical properties of DOM.

Although some EEA metrics did not conform to the DOM characteristics, others did. In our streams, N-acquiring enzymes had the lowest abundance in the spring, coincident with higher quality algal DOM, and highest values in summer and autumn, when overall chlorophyll is low and the system is dominated by lower quality FBOM and CBOM standing stocks respectively. Greater N acquiring activity is associated with increasing C recalcitrance (Sinsabaugh and Follstad Shah 2012), so this finding is consistent with a more labile pool of carbon in spring and a more recalcitrant pool in other seasons. For example, organic matter C:N ratio was lower during spring in forested Mediterranean streams (Villanueva et al. 2016), and higher quality spring DOM in temperate rainforest streams was likely used as a source of labile C and N (Fellman et al. 2009). We found no significant seasonal differences between NO3- or NH4+ concentrations (data not shown), suggesting that higher quality spring DOM acted as a nitrogen source as well as a carbon source. The combined approach of using EEA and EEMs provides complementary information about the characteristics of, and microbial use of, the DOM pool, and the combined approach confirms that spatio-temporal differences in the DOM pool, driven in part by urban infrastructure, translate to spatial differences in how microbes use carbon sources in the urban stream network.

Patterns in Carbon Use – NDS

We assessed overall carbon limitation in autumn and spring and found an interaction between season and reach when expressed on an areal basis. Biofilms in autumn were always more carbon limited than in spring, which supported our hypothesis that terrestrial leaf fall would depress DOM quality in the fall. However, the pattern of carbon limitation by reach (i.e. buried vs open) varied among seasons. Open reaches were more strongly limited by carbon than buried reaches in the autumn, but were less limited by carbon than the buried reaches in the spring. Stronger carbon limitation in open reaches during the fall may be a result of the pulse of recalcitrant DOM from terrestrial leaves that entered the open reaches during leaf-fall, lower carbon limitation in open reaches during the spring may be a result of the pulse of labile DOM derived from the vernal algal bloom; explanations which are corroborated by the DOM optical properties. Total DOC concentration did not vary between seasons (data not shown), suggesting that the pulse of autumn leaves and spring algae blooms changed DOM composition rather than quantity.

Alternatively, differences in C limitation between reaches might be related to secondary reach-scale factors. For example, EEA assays confirm that biofilms in buried reaches always invested more effort to acquire recalcitrant carbon, so they might have been better able to utilize the autumn pulse of terrestrial DOM compared to the open reaches. In contrast, biofilms in open reaches always invested less in recalcitrant carbon acquisition which, when compounded by the fact that the pulse of autumn leaves was delivered directly to the open reaches, could have led to more intensified carbon limitation. Similarly, in the spring, open reaches responded less to the simple carbon sources in the NDS because the system had higher levels of high quality algal DOM, but P/H ratio shows that buried reaches appear to recieve less of this higher quality DOM, so they responded more strongly to the NDS. Less high-quality DOM exported to buried reaches is consistent with the potential for rapid use of algal DOM *in situ* by heterotrophic biofilms (Franke et al. 2013; Rier et al. 2014) and is reflected in the carbon acquisition effort devoted to recalcitrant carbon sources.

We found different results when we expressed carbon limitation by area or biomass (i.e., gAFDM-1). When expressed by area, the temporal and spatial patterns were highly significant, but no patterns were evident when expressed by biomass. Therefore, the biofilm response to added carbon is not to increase the per cell carbon use rate, but simply to accumulate greater biomass. Given the fact that we relieved N and P limitation to focus on the response to added carbon, these results might not apply to carbon additions in low nutrient streams, but they may be applicable to agricultural and urban streams which tend to have chronically high background nutrient concentrations (Carpenter et al. 1998). The rapid processing of added carbon could also be a function of generally high inorganic nutrient concentrations in these urban streams in combination with the nutrients added to the NDS (Rosemond et al. 2015).

Although we hypothesized that responses to the different carbon types in the NDS arrays would vary, biofilms responded similarly to all carbon sources (glucose, arabinose, cellobiose). Although arabinose has been used as a less labile form of carbon in some studies (e.g., Newbold et al. 2006), our results show that it is just as bioavailable as glucose in this study system. Similarly, we used cellobiose as a breakdown product of cellulose that we predicted would be less bioavailable than glucose or arabinose, yet it was equally bioavailable to those more simple carbon sources. It is unclear if arabinose and cellobiose bioavailability is equally high as glucose in most streams or if it was high in these urban streams because of the systemic dominance of recalcitrant carbon and/or the presence of N and P in the NDS agars.

Overall, these results indicate spatio-temporal variation in biofilm carbon use patterns related primarily to seasonal changes in the DOM pool and secondarily to reach scale patterns, such as stream burial, that can alter the microbial effort to acquire different carbon sources. Additionally , we documented widespread carbon limitation in these urban streams which could have been induced by the dominance of recalcitrant terrestrial sources from the watershed, limited production of labile DOM due to burial, high background nutrient concentration and rapid consumption of particulate carbon source (e.g., Rosemond et al. 2015), or some combination of those factors. Together, differences in carbon use patterns within the stream system likely have implications at the stream network scale, particularly in streams systems dominated by urban infrastructure that alternate between buried and open stream reaches. Because labile carbon is more likely to be used *in situ*, recalcitrant carbon is likely to be exported to downstream ecosystems, possibly increasing C flux from streams to receiving water bodies. Further, when DOM sources are dominated by recalcitrant carbon, uptake and use of nitrogen and phosphorus could decrease, further loading downstream ecosystems with nutrients. Therefore, differential carbon use in an urban stream continuum is likely to have consequences for biogeochemical cycling of other nutrients and for downstream export of DOM, nutrients, and inorganic carbon.Management implications - Stream daylighting is an engineering approach to urban stream restoration whereby buried streams are redesigned to be open to light (refs). Daylighting may represent an effective management approach to improving stream water quality and ecosystem function in urban ecosystems (Beaulieu et al. 2015). Our results show that the mechanism of improvement may be dependent upon increasing high quality labile organic carbon available to microbes by management of vegetation in the riparian zone (Newcomer et al. 2012). Future research on carbon limitation in buried streams should be directed at elucidating the effects of daylighting on stream function, as well as the influence of natural carbon sources, soil type in the watershed, hydrology and floodplain connection, etc etc

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Citations

Alberts, J.M, Beaulieu, J.J., Buffam I. In Press. Watershed Land-use and Seasonal Variation Constrain the Influence of Riparian Canopy Cover on Stream Ecosystem Metabolism. Ecosystems.

APHA (2005) Standard methods for the examination of water and wastewater. American Public Health Association, Washington DC

Beaulieu, J. J., P. M. Mayer, S. S. Kaushal, M. J. Pennino, C. P. Arango, D. A. Balz, T. J. Canfield, C. M. Elonen, K. M. Fritz, B. H. Hill, H. Ryu, and J. W. Santo Domingo. 2014. Effects of urban stream burial on organic matter dynamics and reach scale nitrate retention. Biogeochemistry 121:107-126.

Beaulieu JJ, HE Golden, CD Knightes, PM Mayer, SS Kaushal, MJ Pennino, CP Arango, DA Balz, CM Elonen, KM Fritz, and BH Hill. 2015. Urban stream burial increases watershed-scale nitrate export. PLOS One DOI:10.1371/journal.pone.0132256

Booth DB, Jackson CR. 1997. Urbanization of aquatic systems: degradation thresholds, stormwater detection, and the limits of mitigation. J. Am.Water Resour. Assoc. 33:1077–90

Catalan N, Obrador B, Alomar C, and Pretus JL. 2013. Seasonal and landscape factors drive dissolved organic matter properties in Mediterranean ephemeral washes. Biogeochemistry 112:261-274.

Charles DF, Knowles C, Davis RC (2002) Protocols for the analysis of algal samples collected as part of the U.S. Geological Survey National Water-Quality Assessment Program. In: Report No. 02-06. Patrick Center for Environmental Research, The Academy of Natural Sciences, Philadelphia, p 124

Coble. P. G. 1996. Characterization of marine and terrestrial DOM in seawater using excitation emission matrix spectroscopy. Marine Chemistry 51(4):325–346

Coble P. G., S. A. Green, N.V. Blough, and R. B. Gagosian. 1990. Characterization of dissolved organic-matter in the black-sea by fluorescence spectroscopy. Nature 348(6300):432–435

Cory RM, Miller MP, McKnight DM, Guerard JJ, Miller PL. 2010. Effect of instrument-specific response on the analysis of fulvic acid fluorescence spectra. Limnology and Oceanography Methods 8:67–78

Dunne T, Leopold LB. 1978.Water in Environmental Planning. New York: Freeman. 818 pp.

Elmore AJ and SS Kaushal. 2008. Disappearing headwaters: patterns of stream burial due to urbanization. Frontiers in Ecology and Environment 2008 6:308-312

Fellman JB, Hood E, D’Amore DV, Edwards RT, White D. 2009. Seasonal changes in the chemical quality and biodegradability of dissolved organic matter exported from soils to streams in coastal temperate rainforest watersheds. Biogeochemistry 95:277-293

Franke D, Bonnell EJ, and Ziegler SE. 2013. Mineralisation of dissolved organic matter by heterotrophic stream biofilm communities in a large boreal catchment. Freshwater Biology 58:2007-2026.

Griffiths NA, JL Tank, TV Royer, SS Roley, EJ Rosi-Marshall, MR Whiles, JJ Beaulieu, and LT Johnson. 2013. Agricultural land use alters the seasonality and magnitude of stream metabolism. Limnology and Oceanography 58:1513-1529.

Hansen AM, Kraus TEC, Pellerin BA, Fleck JA, Downing BD, Bergamaschi BA. 2016. Optical properties of dissolved organic matter (DOM): effects of biological and phytolytic degradation. Limnology and Oceanography 61:1015-1032.

Hill BH, McCormick FH, Harvey BC, Johnson SL, Warren ML, Elonen CM (2010) Microbial enzyme activity, nutrient uptake and nutrient limitation in forested streams. Freshw Biol 55(5):1005–1019

Hill BH, CM Elonen, LR Seifert, AA May, and Ellen Tarquinio. 2012. Microbial enzyme stoichiometry and nutrient limitation in US streams and rivers. Ecological Indicators 18:540-551

Hope, D, Naegeli MW, Chan AH, Grimm NB. (2004). Nutrients on asphalt parking surfaces in an urban environment. Water, Air, and Soil Pollution 4:371-390.

Hosen JD, McDonough OT, Febria CM, Palmer MA. 2014. Dissolved organic matter quality and bioavailability changes across an urbanization gradient in headwater streams. Environmental Science and Technology 48:7817-7824.

Huguet A, Vacher L, Relexans S, Saubusse S, Froidefond JM, Parlanti E (2009) Properties of fluorescent dissolved organic matter in the Gironde Estuary. Organic Geochemistry 40(6):706–719

Johnson LT, JL Tank, and WK Dodds. 2009. The influence of land use on stream biofilm nutrient limitation across eight North American ecoregions. Canadian Journal of Fisheries and Aquatic Sciences 66:1081-1094

Kaushal, SS and KT Belt. 2012. The urban watershed continuum: evolving spatial and temporal dimensions. Urban Ecosystems 15:409-435.

Kaushal, Sujay, William H. McDowell, Wilfred M. Wollheim, Tamara A. Newcomer Johnson, Paul M. Mayer, Kenneth T. Belt, Michael J. Pennino. 2015. Urban Evolution: The Role of Water. Water 7:4063-4087; doi:10.3390/w7084063

Klein RD. 1979. Urbanization and stream quality impairment. Water Resour. Bull. 15:948–63

Lewis GP and Likens GE. 2007. Changes in stream chemistry associated with insect defoliation in a Pennsylvania hemlock-hardwoods forest. Forest Ecology and Mangement 238:199-211

McKnight DM, Boyer EW, Westerhoff PK, Doran PT, Kulbe T, Andersen DT (2001) Spectrofluorometric characterization of dissolved organic matter for indication of precursor organic material and aromaticity. Limnology and Oceanography 46(1):38–48

Meyer, JL. 1994. The microbial loop in flowing waters. Microbial Ecology 28:195-199.

Meyer, JL and RT Edwards. 1990. Ecosystem metabolism and turnover of organic carbon along a blackwater river continuum. Ecology 71:668-677

Newcomer, Tamara A., Sujay S. Kaushal, Paul M. Mayer, Amy R. Shields, Elizabeth A. Canuel, Peter M. Groffman, and Arthur J. Gold. 2012. Influence of natural & novel organic carbon sources on denitrification in forested, degraded-urban, & restored streams. Ecological Monographs 82:449–466

Ohno T (2002) Fluorescence inner-filtering correction for determining the humification index of dissolved organic matter. Environmental Science and Technology 36(4):742–746

Jari Oksanen, F. Guillaume Blanchet, Roeland Kindt, Pierre Legendre, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens and Helene Wagner (2016). vegan: Community Ecology Package. R package version 2.3-5. https://CRAN.R-project.org/package=vegan

Paul MJ and JL Meyer. 2001. Streams in the urban landscape. Annual Review of Ecology and Systematics 32:333-365

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Regnier P, Friedlingstein P, Ciais P et al. 2013. Anthropogenic perturbation of the carbon fluxes from land to ocean. Nature Geoscience 6:597-607.

Rier ST, Shirvinski JM, and Kinek KC. 2014. In situ light and phosphorus manipulations reveal potential role of biofilm algae in enhancing enzyme-mediated decomposition of organic matter in streams. Freshwater Biology 59:1039-1051.

Rosemond AD, JP Benstead, PM Bumpers, V Gulis, JS Kominoski, DWP Manning, K Suberkropp, and JB Wallace. 2015. Experimental nutrient additions accelerate terrestrial carbon loss from stream ecosystems. Science 347:1142-1145

Runkel R.L. (1998) One-Dimensional Transport with Inflow and Storage (OTIS): A Solute Transport Model for Streams and Rivers. U.S. Geological Society, Water Resources Investigations Report 98-4018.

Runkel R.L. (2002) A new metric for determining the importance of transient storage. Journal of the North American Benthological Society, 21, 529–543.

Sartory DP, Grobbelaar JU (1984) Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114(3):177–187

Sinsabaugh RL, Findlay S, Franchini P, Fisher D (1997) Enzymatic analysis of riverine bacterioplankton production. Limnol Oceanogr 42(1):29–38

Sinsabaugh RL, Foreman CM (2001) Activity profiles of bacterioplankton in a eutrophic river. Freshwater Biol 46(9): 1239–1249

Sinsabaugh RL, BH Hill, JJ Follstad Shah (2009) Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. Nature 462:795-798.

Sinsabaugh RL, Follstad Shah JJ (2011) Ecoenzymatic stoichiometry of recalcitrant organic matter decomposition: the growth rate hypothesis in reverse. Biogeochemistry 102(1–3):31–43

Sinsabaugh RL, Follstad Shah JJ (2011) Ecoenzymatic stoichiometry and ecological theory. Annual Review of Ecology, Evolution, and Systematics 43:313-343.

Smith RM and Kaushal SS. 2015. Carbon cycle of an urban watershed: exports, sources, and metabolism. Biogeochemistry 126:173-195.

Stolpe B, Guo L, Shiller AM, Hassellov M (2010) Size and composition of colloidal organic matter and trace elements in the Mississippi River, Pearl River and the northern Gulf of Mexico, as characterized by flow field-flow fractionation. Marine Chemistry 118(3–4):119–128

Villanueva VD, Navarro MB, Albarino R. 2016. Seasonal patterns of organic matter stoichiometry along a mountain catchment. Hydrobiologia 227-238.

Wagner K, Besemer K, Burns NR, Battin TJ, Bengtsson MM. 2015. Light availability affects stream biofilm bacterial community composition and function, but not diversity. Environmental Microbiology 17:5036-5047.

Williams CJ, Frost PC, Morales-Williams AM, Larson JH, Richardson WB, Chiandet AS, and Xenopoulos MA. 2016. Human activities cause distinct dissolved organic matter composition across freshwater ecosystems. Global Change Biology 22:613-626

Zsolnay A, Baigar E, Jimenez M, Steinweg B, Saccomandi F (1999) Differentiating with fluorescence spectroscopy the sources of dissolved organic matter in soils subjected to drying. Chemosphere 38(1):45–50

Tables (part of me wants to have another table with site descriptions, but then again we can just refer to your 2014 paper)

Table 1. Coefficients from adonis, a permutational multivariate analysis of variance using distance matrices, show weak relationships between nutrient response and particulate carbon standing stocks

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Glucose NRR | Arabinose NRR | Cellobiose NRR | P value |
| CBOM | 0.072 | 0.060 | 0.064 | 0.036 |
| FBOM | 0.014 | 0.011 | 0.01 | 0.053 |

Figure Captions

Figure 1. Spatio-temporal variation in the humification index (HIX) derived from excitation-emission matrices.

Figure 2. Seasonal variation in the (A) biological freshness index (BIX) and (B) fluorescence index (FI) derived from excitation-emission matrices.

Figure 3. Spatio-temporal variation in the protein-to-humic ratio (P/H) derived from excitation-emission matrices.

Figure 4. Reach-scale variation in ecoenzyme activity on (A) L-3,4-dihydroxyphenylalanine (DOPA) + H2O2 (DOPAH2) and (B) polyphenol oxidase (POX), both metrics of recalcitrant carbon use.

Figure 5. Spatio-temporal variation in the LCI, and index of carbon quality where larger values indicate more recalcitrant carbon in the dissolved organic matter pool.

Figure 6. Seasonal variation in -N-acetylglucosaminidase (NACE) activity of stream biofilms.

Figure 7. Spatio-temporal variation in the nutrient response ratio (NRR: respiration/mean control) to added carbon when measured on an areal basis (g O2 cm-2 h-1).

Figures

Figure 1.

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Figure 2.



Figure 3.

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Figure 4.



Figure 5.

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Figure 6.



Figure 7.

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