Carbon limitation patterns are linked to spatio-temporal changes in dissolved organic matter quality in an urban stream

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Abstract

Introduction

Methods

Study Sites and Experimental Design

We studied three urban streams in or near Cincinnati, Ohio (USA), and each stream consisted 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 concrete, 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 samples to characterize dissolved organic matter quality in summer and autumn 2011 and in spring 2012. Concurrently, we deployed tiles to measure extracellular enzyme activity and nutrient diffusing substrata to measure carbon limitation patterns. 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 was measured with 15N-NO3- release in conjunction with bromide (Br-) as a conservative tracer to calculate and model hydrologic parameters. 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 they 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 MODEL INFORMATION. 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 available to microbial consumers in the stream. To produce the indices to distinguish among these fractions of DOM, water samples were analyzed on a spectrofluorometer that measured excitation between 240-450 nm at 10 nm intervals and emission from 290-600 nm at 2 nm intervals. The three-dimensional EEMs were then instrument corrected, blank substracted, and normalized by the water Raman signal (Cory et al. 2010), but we did not measure absorbance so we did perform the standard inner-filter correction. Therefore these results will be most useful for relative differences across sites and time rather than for comparison to literature values.

The EEMs we produced allowed us to calculate a variety of indices to characterize the quality of the DOM pool, 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). 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 to make inferences about relative abundance of labile and recalcitrant DOM among streams and seasons.

Extracellular enzyme activities (EEA)

Periphyton cultured on tiles that we deployed in the 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, acquisition of recalcitrant carbon compounds was measured as polyphenol oxidase (POX) and peroxidase activity. The ratio of recalcitrant carbon acquisition total carbon acquisition (as -D-glucosidase + polyphenol oxidase) characterizes the overall quality of the DOM pool (LCI) whereby larger values represent more recalcitrant carbon (Sinsabaugh and 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 3-N-acetylglucosaminidase (NACE: EC 3.2.1.50).

All EEA assays used microplate protocols developed by Sinsabaugh and colleagues (Sinsabaugh et al. 1997; Sinsabaugh and Foreman 2011) and subsequently modified by Hill et al. (2010). Microplate arrays were run with quadruplicate assays for each tested enzyme and reference standard, where 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 in 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 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 in PVC to shade them and reduce the potential for autotrophic biofilms to colonize the glass disks, and they were deployed for two weeks. Upon collection, the samples were sent overnight on ice for laboratory analysis within 24 h.

Laboratory analysis consisted of submerging the 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 ice for transport to the laboratory where they were acidified or frozen depending on the analyte. We used standard colorimetric methods to measure nitrate+nitrate (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 with a total organic C analyzer with 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 in order to minimize any potential among site differences. Tiles were collected with the rest of the samples, and periphyton was removed with a toothbrush and razor blade, rinsed into a bottle with site water, and held on ice until arrival at the laboratory. A subset of tiles was analyzed for algal abundance using a Palmer-Maloney counting cell (Charles et al. 2002), a subset of tiles was analyzed for total bacterial counts using qPCR, and a subset of tiles was used for laboratory 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 simultaneously how CBOM and FBOM standing stocks affect the response to glucose, arabinose, and cellobiose. 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 (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 reach 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. 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. This ratio varied by season (GLS, p<<0.001), with spring and summer having a higher ratio (more protein) compared to fall, and also by reach (GLS, p<<0.0002) with open reaches having lower ratio (more humic-like) than buried reaches.

Patterns in extracellular enzyme activity

We deployed standard tiles for microbes to colonize for 6 weeks prior to collecting all our samples, and we measured extracellular enzyme activity to characterize microbial effort to acquire nutrients and use different carbon sources available in the environment. Extracellular enzymes that degrade L-3,4-dihydroxyphenylalanine (DOPA) + H2O2 (DOPAH2) as a substrate correlate to lignin degradation, so it is a metric of recalcitrant carbon use. While we found no significant differences in DOPAH2 among seasons, we did find that 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 we also found that summer had greater use of recalcitrant carbon than autumn (GLS, p=0.027). There were no differences 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 dominated by impervious surface cover. 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.” 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 in autumn compared to spring, but open reaches had the strongest response in the fall and buried reaches had strongest response in the spring (Figure 7).

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).

Discussion

Seasonal patterns of DOM characteristics

BIX and FI show a clear pattern of more recalcitrant carbon during autumn and more labile carbon during spring, possibly due to a large influx of terrestrial DOM in the fall, which is typically lower quality than aquatic autochthonous DOM sources (McKnight et al. 2001), and a greater contribution of autochthonous DOM in the spring. HIX is similar to BIX and FI with autumn having higher humic character than spring or summer, and this pattern is also seen in the P/H ratio, which shows more humic-like components in the autumn compared to the spring whereas summer was not distinctly different. This is consistent in the reach standing stock data showing higher CBOM in autumn compared to other seasons and higher chlorophyll a in spring than in other seasons (get Beaulieu et al. 2014 Fig 3). This seasonal pattern is also seen in temperate forested mountain streams (Villanueva et al. 2016) and ephemeral Mediterranean streams that flow during the autumn-spring wet season (Catalan et al. 2013), as well as 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 with 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 in these streams is dominated by recalcitrant characteristics, presumably from terrestrial sources, despite higher productivity and higher algal standing stocks in the spring. Urban streams could exhibit generally stronger terrestrial or humic DOM signature than streams with other land use cover. 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 stronger terrestrial/recalcitrant characteristics year-round could indicate that heterotrophic biofilms rapidly removed 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).

Spatial patterns of DOM characteristics

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 correlate to biological “freshness” and labile DOM. Because buried reaches have no avenue for direct riparian inputs, chlorophyll a is nearly absent, and primary production is undetectable (Beaulieu et al. 2014), this implies that organic matter inputs to open reaches impart the BIX and FI characteristics to the DOM pool in the buried reaches. Biological activity in buried reaches, which is lower than in the open reaches (Beaulieu et al. 2014), likely does little to alter those seasonal aspects of the DOM signature due to the absence of DOM production via photosynthesis and limited DOM consumption via ecosystem respiration (Beaulieu et al. 2014). Alternatively, BIX and FI could be determined by processes at the larger stream segment or catchment scale, rather than the reach scale. For example, 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 that included streams found that catchment scale land use was a 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 was higher (more humic) in open reaches compared to buried reaches. This pattern was reflected in the P/H ratio, which was driven more by the relative abundance of humic-like compounds rather than patterns in aquatic production that affect the low molecular weight protein fractions of the DOM pool, consistent with the year-round humic nature of DOM in these urban streams. . However, it is unclear if the pattern in HIX is driven by differences in the supply of organic matter to the reaches, use of DOM in the reaches, or both. More humic DOM in the open reaches during autumn is consistent with open reaches receiving and retaining more leaf inputs than the buried reaches, making the humic character stronger in the open reaches. Assuming the HIX value at the top of the buried reach is identical to that of the upstream open reach, a lower HIX value further down the buried reach implies that the DOM character has changed as water flows through the buried reach. Several abiotic mechanisms could account for this including dilution of the DOM pool by lower HIX groundwater or 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). Alternatively, lower HIX in the buried reach could imply consumption of high HIX compounds. Higher HIX in the open reach in spring could be from terrestrial organic matter leached from greenfall inputs during leaf out and/or flower or seed production, and greenfall can be an important source of carbon in forested streams (Lewis and Likens 2007). However, in the overall context of this study, the median spring values are lower than autumn values on a reach-by-reach basis, which is still consistent with an overarching seasonal affect driven by terrestrial sources in the autumn and aquatic sources in the spring. (Pennino -- Could this also suggest that the P/H ratio is more sensitive to biological processing of the DOM pool in different reaches given that there is more effort to degrade recalcitrant carbon sources in the buried reaches, which would drive this ratio toward protein in the buried reach?).

Taken together, the DOM data show that these urban streams are dominated by humic sources despite spatio-temporal differences in the DOM composition driven by seasonal differences in CBOM and algae. These data also show secondary control over DOM quality due to spatial differences in organic matter inputs (buried versus open) that alter the characteristics of the DOM pool.

Patterns in Carbon Use – Extracellular Enzyme Activities (EEA)

Extracellular enzyme activity (EEA) reflects the composition of the DOM pool, as perceived by microbial activity. Although BIX and FI indicated no effect of burial on DOM composition and HIX indicated more humic (e.g. more recalcitrant) DOM in open reaches, buried reaches had higher DOPAH2 and POX than open reaches. This indicates that the microbial community in buried reaches allocated more energy toward acquiring recalcitrant carbon sources than in the open reach, regardless of season. This pattern is consistent with differential biological processing of DOM in buried versus open reaches similar to what has been seen in experiments showing greater POX activity 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 in parallel with higher levels of chlorophyll a in autumn, winter, and spring that suggest more labile carbon. 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 that suggest more recalcitrant carbon. This suggests rapid use of high quality carbon produced in the open reaches and little export to downstream buried reaches, and it is consistent with generally greater EEA in the presence of algal biofilms (Rier et al. 2014).

While the EEA results about recalcitrant carbon use are entirely consistent with expectations, they do not match the optical properties we measured in the DOM pool. The water column grab sample we took for EEMs might not reflect the DOM characteristics the biofilm is exposed to at the boundary layer, which could be different if labile carbon produced in the benthos of the open reaches was rapidly and selectively processed by microbes. Alternatively, the long incubation times of the tiles (5-6 weeks) to culture microbes for the EEA assays might integrate microbial exposure to a different DOM pool than was reflected in the single grab sample we used for EEMs at the end of the incubation period. 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. Regardless, the combined approach of using EEA and EEMs provides complementary information about the characteristics of, and microbial use of, the DOM pool.

The LCI index, which aggregates several measures of EEA 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 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.

Higher quality DOM typically has a lower C:N ratio. 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). 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 FBOM and CBOM standing stocks respectively. We found no significant seasonal differences between NO3- or NH4 concentrations (data not shown), suggesting that higher quality spring DOM acted as a N source as well as a carbon source.

Patterns in Carbon Use – NDS

We assessed overall carbon limitation in autumn and spring, and we found an interaction between season and reach when expressed on an areal basis. Biofilms in autumn were always more limited by carbon than biofilms in spring, but the pattern of which reach was more limited in a given season was reversed. Open reaches were more strongly limited by carbon than buried reaches in the autumn, but they were less limited by carbon than the buried reaches in the spring. The overall seasonal effect can be explained as autumn having a pulse of recalcitrant DOM from terrestrial leaves whereas spring has a pulse of labile DOM from algal sources. Total DOC concentration did not vary between seasons, suggesting that the pulse of autumn leaves and spring algae blooms changed the composition more than the quantity of DOM, a pattern that is confirmed by higher BIX, FI, and lower HIX in spring compared to autumn. The differential response might be related to secondary reach-scale factors. For example, biofilms in buried reaches always invested more effort to acquire recalcitrant carbon, so they might have been better able to use 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 buried reaches appear to get 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, results were highly significant, but when expressed by biomass, there were no differences. This implies that 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 reasonably applicable to agricultural and urban streams which tend to have higher background nutrient concentrations.

Biofilms responded the same 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 as glucose. It is unclear if arabinose and cellobiose bioavailability is equally high as glucose in most streams or if it was high in this stream because of the systemic dominance of recalcitrant carbon and/or the presence of N and P in the NDS agars.

Interestingly, although we measured distinct differences in the DOM pool and microbial use of different carbon and nitrogen sources, none of those metrics were directly related to the NDS response to added carbon. The only relationship we found was a positive response to CBOM and FBOM, and although this relationship is sensible, it is very weak and probably driven by the fall NDS response to significantly higher CBOM stocks. The lack of a relationship with nearly all of the environmental data collected, despite the NDS response being consistent with the spatio-temporal patterns of DOM quality and EEA, may reflect a significant difference in the carbon sources we used for the NDS. We used very simple carbon sources with respect to the sources in the overall bulk DOM pool, so the biofilm response to these highly labile sources could be different than the EEA response to the environmental DOM pool the biofilms were exposed to during incubation. The NDS could also have exerted high selection pressure for a particular subset of microbes that responded differently than the natural community as a whole. Alternatively, the fact that the NDS had added N and P that was unavailable to the environmental biofilms could have induced a different response to the carbon amendments. Despite not being able to link DOM characteristics or EEA metrics in a linear relationship with the NDS response, the NDS response was still consistent with those metrics suggesting this can be a good tool to explore patterns of carbon use in streams. Overall, these results suggest that spatio-temporal variation in biofilm carbon use patterns are related primarily to seasonal changes in the DOM pool and secondarily to reach scale patterns such as stream burial. Differential carbon use in an urban stream continuum can have consequences for biogeochemical cycling of other nutrients and for downstream export of DOM and nutrients.

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Citations

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.

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

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.

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

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

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

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

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

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/.

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.

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

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.

C:\Users\ArangoC\Documents\R Files\Cinn R dev\Cincy-Carbon-Limitation\output\figures\box.hixByReachSeason.tiff

Figure 2. (Make a 2 panel graphic eventually)



Figure 3.

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Figure 4.



Figure 5.

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Figure 6.



Figure 7.

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