



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

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

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Running Head: carbon limitation of heterotrophic respiration

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## Summary

1. Urban streams are degraded by a suite of factors, including burial beneath urban infrastructure, such as roads or parking lots, which eliminates light and reduces direct organic matter inputs to streams from riparian zones. These changes to stream metabolism and terrestrial carbon contribution will likely have consequences for organic matter metabolism by microbes and carbon limitation in streams. Respiration by heterotrophic biofilms drives other nutrient cycles, but we lack a clear understanding of how stream burial and seasonality affect microbial carbon use.

2. We studied seasonal changes (autumn, spring, summer) in organic matter metabolism by microbial communities in open and buried reaches of three urban streams in Cincinnati, Ohio. We characterized organic matter quality using fluorescence spectroscopy, extracellular enzyme profiles, 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 autumn and in buried reaches.

3. Several fluorescence metrics showed higher quality DOM in spring than autumn, but only the metric of recalcitrant humic compounds varied by reach, with more humic DOM in open compared to buried reaches. This likely reflected open reaches as an avenue for direct terrestrial inputs from the riparian zone.

4. Extracellular enzyme assays showed that microbes in buried reaches allocated more effort to degrade recalcitrant carbon sources, consistent with a lack of labile carbon compounds due to limited photosynthesis. Nitrogen acquisition enzymes were highest in autumn coincident with riparian leaf inputs to the streams. Buried and open reaches were both more carbon-limited in autumn when terrestrial leaf inputs dominated compared to the spring when vernal algal blooms were pronounced.

5. 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. Different carbon quality and use patterns coupled with widespread carbon limitation suggest that these urban streams likely export recalcitrant carbon to downstream water bodies, and that the cycling of nitrogen and/or phosphorus could decrease if heterotrophic metabolism is limited by labile carbon availability. Management actions that increase light to buried streams could help alleviate carbon limitation by allowing more autochthony in urban streams.

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1 Introduction

2 As suburban sprawl converts farmland and forests to urban infrastructure, and as the global trend of  
3 urbanization continues (Grimm *et al.*, 2008), the biological function of urban streams and its role in  
4 water quality has received increased attention (Kaushal *et al.*, 2015). Relatively small increases in  
5 impervious surface cover through urbanization can lead to a “flashy” hydrologic regime that reinforces  
6 entrenchment and channel incision in streams that are often already channelized to promote storm  
7 water drainage (Dunne and Leopold, 1978). These channelized streams are less retentive of particulate  
8 organic carbon (Paul and Meyer, 2001) which, in combination with greater nutrient loads (Carpenter *et al.*,  
9 1998) and reduced riparian canopies (Griffiths *et al.*, 2013), can alter the contribution of  
10 heterotrophic and autotrophic processes to stream metabolism (Kaushal *et al.*, 2014). For example,  
11 canopy opening and nutrient enrichment can increase autotrophy (Bernot *et al.*, 2010; Griffiths *et al.*,  
12 2013; Alberts, Beaulieu & Buffam, 2016), but stream burial can increase the importance of heterotrophy  
13 relative to autotrophy (Beaulieu *et al.*, 2014; Pennino *et al.*, 2014). Depending on management, changes  
14 in organic matter processing by headwater streams may have an influence on the quantity and quality of  
15 organic matter subsidies further downstream along the urban watershed continuum (e.g., Kaushal &  
16 Belt, 2012; Pennino *et al.*, 2014; Kaushal *et al.*, 2014)

17 Urban infrastructure expansion frequently results in low order streams being contained in buried pipes  
18 (Elmore & Kaushal, 2008). Further, open and buried stream reaches often alternate in an urban  
19 hydrological network so that stream metabolism can be vastly different in alternating stream reaches.  
20 For example, the severe reduction or absence of photosynthetically active radiation (PAR)  
21 fundamentally alters a stream ecosystem by eliminating the contribution of primary production to the  
22 food web. Although metabolism in buried streams shifts to net heterotrophic conditions, buried  
23 streams support a lower overall rate of ecosystem respiration compared to open reaches (Beaulieu *et al.*,  
24 2014; Pennino *et al.*, 2014). Because buried stream reaches are often optimized to convey water  
25 quickly and efficiently for drainage purposes, they have increased water velocity which, in conjunction  
26 with net reduction in overall biological demand for nutrients (Beaulieu *et al.*, 2014; Pennino *et al.*, 2014),  
27 promotes nutrient export to downstream reaches and ecosystems (Beaulieu *et al.*, 2015). Burial also  
28 severely affects standing stocks of organic matter in streams, and buried reaches have lower overall  
29 coarse and fine benthic organic matter, periphyton, and chlorophyll a standing stocks compared to open  
30 reaches (Beaulieu *et al.*, 2014). Organic matter standing stocks in buried reaches also have little  
31 seasonality, except for higher coarse benthic organic matter (CBOM) in the fall whereas organic matter  
32 standing stock exhibit pronounced seasonal patterns in open streams (Beaulieu *et al.*, 2014). Although  
33 the effect of stream burial on particulate organic matter standing stocks has been investigated, how this  
34 effect propagates through the microbial community to determine the abundance and quality of  
35 dissolved organic matter (DOM) is unknown.

36 DOM is an important energy source for ecosystem respiration (Meyer & Edwards, 1990), and microbial  
37 assimilation transfers this energy from dissolved sources to higher trophic levels (Meyer, 1994). Streams  
38 depend on allochthonous organic carbon inputs from the terrestrial landscape including leaf litter inputs  
39 from the riparian zone and DOM exported from soil by groundwater, as well as autochthonous sources  
40 from in-stream production of algae and/or macrophytes. These organic matter sources partly  
41 determine the quality of the DOM pool available to stream microbial communities. Allochthonous  
42 inputs are generally more recalcitrant (i.e., lower quality) than autochthonous sources (McKnight *et al.*,  
43 2001) due to the presence of more structurally complex carbon compounds (e.g., lignin, tannin). In

contrast, autochthonous carbon sources have fewer complex structural compounds and relatively more polysaccharides (e.g., cellulose, hemicellulose), so these carbon sources are generally considered more labile (i.e., higher quality). 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 DOM pool composition with open reaches having more labile DOM than buried reaches due to greater light availability and associated higher levels of primary production (Kaushal *et al.*, 2014), and greater hyporheic exchange and higher respiration rates in open reaches (Beaulieu *et al.*, 2014) could influence microbial use and transformation of DOM. 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 characterize how microbes allocate energy to acquire different compounds (e.g., labile or recalcitrant carbon, nitrogen, etc.). These assays quantify the microbial demand for and environmental availability of substrates (Sinsabaugh & Follstad Shah, 2012), and they have been used to infer microbial organic nutrient limitation patterns in soils and sediments (e.g., Sinsabaugh, Hill & Follstad Shah, 2009) and within river networks (Hill *et al.*, 2012). DOM fluorescence properties can characterize various fractions of DOM as more or less labile, and allochthonous or autochthonous. This technique has been used to investigate seasonal (Catalan *et al.*, 2013) and landscape (Williams *et al.*, 2016) differences 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, warming stream temperatures, higher sun angle, and longer day length, and that open reaches would have higher quality DOM than buried reaches due to more algal production in open 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 those in buried reaches. Finally, we hypothesized that microbial respiration would be more carbon-limited in autumn 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 lower inputs and less retention of allochthonous carbon inputs. Regardless of reach or season, we predicted that microbial respiration would respond more strongly to higher quality carbon amendments in the NDS compared to lower quality carbon amendments.

## 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 corrugated 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. In two of the three streams, the buried reach was upstream of the open reach. A more detailed site description can be found in Beaulieu *et al.* (2014).

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3 86 We collected water samples to characterize dissolved organic matter quality in summer and autumn  
4 87 2011 and in spring 2012. Concurrently, we collected biofilms from unglazed clay tiles that were  
5 88 deployed in the streams for a minimum of 6 weeks for extracellular enzyme activity analysis. Microbial  
6 89 carbon limitation patterns were measured using nutrient diffusing substrata. This design allowed us to  
7 90 compare how carbon quality, microbial enzyme activity, and the biofilm response to added carbon  
8 91 varied in space (buried versus open stream reaches) and time (summer, autumn, and spring). We also  
9 92 collected a suite of other environmental data including water chemistry, hydrologic parameters, organic  
10 93 matter standing stocks, and whole stream metabolism and nitrate ( $\text{NO}_3^-$ ) uptake to understand how  
11 94 those factors related to the microbial response to variations in DOM quality. Nitrate uptake and  
12 95 hydrologic parameters (i.e., transient storage) were measured using whole-stream  $^{15}\text{N}\text{-NO}_3^-$  and  
13 96 bromide ( $\text{Br}^-$ ) releases. Methods that describe the processing of isotope samples, calculating  $\text{NO}_3^-$   
14 97 uptake rate, and modeling transient storage parameters and one- and two-station whole-stream  
15 98 metabolism are beyond the scope of this paper, but are detailed in Beaulieu *et al.* (2014).  
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20 99 DOM Characterization  
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22 100 Dissolved organic matter quality was characterized using fluorescence excitation-emission matrices  
23 101 (EEMs; Coble *et al.*, 1990; Coble 1996; Cory *et al.*, 2010) measured on a Fluoromax-4 spectrofluorometer  
24 102 (Horiba Instruments, Kyoto, Japan). This technique quantifies humic-like, fulvic-like, and protein-like  
25 103 fractions within the bulk DOM pool, which in turn are generally related to the lability or recalcitrance of  
26 104 DOM pool. EEMs were measured using excitation wavelength at 10 nm intervals between 240-450 nm  
27 105 at and emission wavelengths at 2 nm intervals from 290-600 nm. Three-dimensional EEMs were then  
28 106 instrument corrected, blank subtracted, and normalized by the water Raman signal (Cory *et al.*, 2010)  
29 107 using Matlab software, but we did not measure absorbance for each sample, so we could not perform  
30 108 the standard inner-filter correction on the EEMs. Therefore these results will be most useful for relative  
31 109 differences across sites and time rather than for comparison to literature values.  
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35 110 The EEMs were used to calculate several DOM quality indices, including the humification index (HIX;  
36 111 Zsolnay *et al.*, 1999; Huguet *et al.*, 2009), the biological freshness index (BIX; Huguet *et al.*, 2009), the  
37 112 fluorescence index (FI; McKnight *et al.*, 2001), and the protein-to-humic ratio (P/H; Coble, 1996; Stolpe  
38 113 *et al.*, 2010). HIX characterizes the humic or autochthonous fractions of DOM (Zsolnay *et al.*, 1999;  
39 114 Ohno, 2002), and it is calculated as the ratio of integrated fluorescence emission intensity between 300-  
40 115 345 nm and between 435-480 nm at 254 nm excitation. Higher HIX values indicate DOM with humic  
41 116 character whereas lower values indicate either less humic or more autochthonous DOM. BIX was  
42 117 calculated from the ratio of emission at 380 and 430 nm at excitation of 310 nm (Huguet *et al.*, 2009).  
43 118 BIX values <0.7 are associated with allochthonous DOM, values 0.8-1.0 are associated with  
44 119 autochthonous DOM, and values >1.0 are associated with aquatic bacterial sources; higher values  
45 120 indicate greater lability than lower values. FI is calculated from the ratio of the fluorescence intensity at  
46 121 450 nm and 400 nm at excitation of 370 nm. FI values of about 1.9 indicate fulvic acids from microbes  
47 122 and values of about 1.4 indicate terrestrial-origin fulvic acids. Finally, P/H was calculated from the EEMs  
48 123 whereby excitation at 275 nm and emission at 340 nm is associated with protein-like organic matter and  
49 124 excitation at 350 and emission at 480 is associated with humic-like organic matter (Coble, 1996; Stolpe  
50 125 *et al.*, 2010).  
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55 126 Extracellular enzyme activities (EEA)  
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Biofilm collected from tiles 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 & Foreman, 2001). Acquisition of labile carbon compounds was measured as  $\beta$ -D-glucosidase activity, and acquisition of recalcitrant carbon compounds was measured as polyphenol oxidase (POX) and peroxidase activity. An alternate metric of recalcitrant carbon acquisition was measured as the activity of L-3,4-dihydroxyphenylalanine (DOPA) +  $H_2O_2$  as a substrate, and this metric correlates with lignin degradation. The ratio of recalcitrant carbon acquisition to total carbon acquisition (as  $\beta$ -D-glucosidase + polyphenol oxidase) characterizes the overall quality of the DOM pool (equivalent to lignocellulose index or 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 & Follstad Shah, 2011). We also used CQI, the ratio of labile to recalcitrant carbon acquisition enzymes whereby larger values indicate greater effort to acquire labile carbon, as alternate metric of overall carbon quality. Nitrogen acquisition was measured as the activity of  $\beta$ -N-acetylglucosaminidase (NACE: EC 3.2.1.50).

All EEA assays used microplate protocols (Sinsabaugh *et al.*, 1997; Sinsabaugh & Foreman, 2001) 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 where light was extinguished at the upstream and downstream ends of the buried reaches. We initially predicted increased carbon limitation at the downstream end of each buried reach due to microbial processing of DOM through the buried reach, but we found no difference in carbon-limitation or EEM metrics between the upstream and downstream ends of the buried reaches. Therefore upstream and downstream NDS arrays were both considered "buried" in the statistical analysis. 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 increasing recalcitrance. The NDS were supplemented with 0.5 M N as  $NH_4Cl$  and 0.5 M P as  $KH_2PO_4$  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 PVC for shade 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 ( $\mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ ) and by biomass ( $\text{mg O}_2 \text{ gAFDM}^{-1} \text{ h}^{-1}$ ), 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 ( $0.45 \mu\text{m}$ ) 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 (APHA, 2005) to measure nitrate + nitrite (hereafter,  $\text{NO}_3^-$ ), dissolved reactive phosphorus (DRP), ammonium ( $\text{NH}_4^+$ ), and bromide ( $\text{Br}^-$ ) 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  $\text{Br}^-$  released in conjunction with the  $^{15}\text{N-NO}_3^-$  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 ( $A_s$ ), the storage zone exchange coefficient ( $\alpha$ ), the storage zone residence time ( $T_{\text{sto}}$ ), and the storage exchange flux ( $q_s$ ) fraction of the median travel time due to transient storage,  $F_{\text{med}}^{200}$  (Runkel, 2002). These methods are described in detail in Beaulieu et al. (2014).

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 ( $>1 \text{ mm}$ ), fine ( $<1 \text{ mm}$ ), and attached (i.e., periphyton) organic matter were collected from  $0.052 \text{ m}^2$  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) subsample. We collected periphyton by scraping a known area ( $0.006\text{-}0.04 \text{ m}^2$ ) of a rock with a wire brush. We calculated sample dry mass and ash-free dry mass of samples by weighing oven-dried ( $60^\circ\text{C}$ ) samples before and after combustion in a muffle furnace ( $500^\circ\text{C}$ ). We used a subsample of periphyton to measure chlorophyll a using the trichromatic method (APHA, 2005) following hot ethanol extraction (Sartory & Grobbelaar, 1984).

We deployed unglazed clay tiles for six weeks at all sites to provide a standardized surface for algae and bacteria to colonize. Biofilm on tiles was removed with a toothbrush and razor blade, rinsed into a bottle with site water, and stored on ice until analysis. Subsets were analyzed for algal abundance using a Palmer-Maloney counting cell (Charles, Knowles & Davis, 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) with alternate variance structures and model optimization (Zuur et al., 2009) to test how DOM quality (HIX, BIX, FI, P/H) differed among seasons (summer, autumn, spring) and between reaches (buried, open). We also used GLS to test for differences in extracellular enzyme activity (POX, DOPA- $\text{H}_2\text{O}_2$ , LCI, NACE) and carbon limitation patterns among seasons and between reaches. We examined the relationship between CQI and LCI using



Spearman's rank correlation. 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  $\text{NO}_3^-$  uptake. We used permutational multivariate analysis of variance using distance matrices (adonis in the vegan package for R; Oksanen *et al.*, 2016) to detect a relationship between the aggregated response of microbial respiration to glucose, arabinose, and cellobiose NDS additions and CBOM and FBOM standing stocks. Other parameters were not significantly related to the NDS data. All statistical analyses were done using R (R Core Team, 2016)

## Results

### Patterns in DOM Variability

DOM quality differed among seasons (summer, autumn, spring) and between reaches (buried, open). HIX, the humification index, differed by season (GLS,  $p=0.0005$ ), with autumn having higher HIX than spring or summer, which were not different from each other. HIX also differed by reach (GLS,  $p=0.021$ ) with open reaches having higher HIX than buried reaches when compared across all seasons (Figure 1). Because we did not perform the standard inner-filter corrections on these samples, these values show relative differences between reaches and among seasons.

BIX, the biological freshness index, and FI, the fluorescence index, varied by season (GLS,  $p<<0.0001$ ) but did not differ between buried and open reaches (Figure 2A and 2B, respectively). Although BIX and FI did not differ between spring and summer, both indices had significantly lower values in autumn compared to spring and summer. The BIX and FI values we measured indicate low autochthonous content and terrestrially-derived fulvic acids respectively in all seasons and reaches.

P/H, the protein to humic ratio was generally  $< 1$  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

Although differences in DOPAH2 activity among seasons were not detected, DOPAH2 activity was higher in biofilm from buried reaches than in biofilm from open reaches (GLS,  $p=0.024$ ) when we expressed DOPAH2 per unit dry mass (Figure 4a) or per unit carbon (data not shown). Polyphenol oxidase (POX) extracellular enzyme activity within biofilm was higher 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, biofilm LCI values from buried reaches reflected higher use of recalcitrant carbon than open reaches (GLS,  $p=0.014$ ), and summer biofilm had greater use of recalcitrant carbon than autumn biofilm (GLS,  $p=0.027$ ). There were no differences between spring and autumn (Figure 5). The LCI was also correlated to the CQI ( $r_s=-0.98$ ,  $p<<0.0001$ , data not shown).

Because carbon uptake and use is often linked to the acquisition of N from the environment, we also analyzed differences in N uptake as activity of  $\beta$ -N-acetylglucosaminidase. We measured highest values in the autumn, intermediate values in summer, and lowest values in spring with all seasons significantly

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3 248 different from each other (GLS,  $p < 0.0001$ ) (Figure 6), but there were no differences between open and  
4 249 buried reaches.  
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6 250 Carbon limitation  
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8 251 We deployed NDS amended with different carbon sources (glucose, arabinose, cellobiose, and a no-  
9 252 carbon control) to see if patterns in carbon limitation differed between buried and open stream reaches  
10 253 or among seasons. The NDS we deployed during summer were washed away by stormflows. Therefore,  
11 254 we focus our analysis on autumn and spring to contrast the carbon limitation response to a time when  
12 255 leaf inputs dominate compared to when vernal algae blooms dominate.  
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15 256 Respiration rates on NDS disks were not different among carbon source treatments when the data were  
16 257 scaled by biomass ( $\text{mg O}_2 \text{ gAFDM}^{-1} \text{ h}^{-1}$ ). However, when the respiration response was scaled by disk  
17 258 area ( $\mu\text{g O}_2 \text{ m}^{-2} \text{ h}^{-1}$ ), all NDS carbon amendments were significantly different than the control in all  
18 259 streams, seasons, and reaches (GLS,  $p < 0.001$ ). Respiration response were not detectably different  
19 260 among the three carbon amendments during any deployment (GLS,  $p > 0.05$ ). Generally, fall had higher  
20 261 NRR (ratio of the treatment response to the control) compared to spring in both reaches (LME,  
21 262  $p < 0.0001$ ; Figure 7). We found a significant interaction (GLS,  $p = 0.0009$ ) between season (autumn  
22 263 versus spring) and reach (buried versus daylight) whereby the respiration response to added carbon was  
23 264 stronger for open compared to buried reaches in autumn, but it was stronger for buried compared to  
24 265 open reaches in spring (Figure 7). Further, the difference between the seasonal responses was less  
25 266 pronounced in buried reaches than in open reaches. Overall, these results indicate carbon limitation in  
26 267 all streams and season.  
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29 268 No relationships between NRR and water chemistry, hydrology, or ecosystem-scale functional attributes  
30 269 were detected. Although EEA and DOM quality metrics often differed between seasons and reaches,  
31 270 there was no direct linear relationship between NRR and those metrics. Although most standing stock  
32 271 metrics were also unrelated to NRR, there were weak positive relationships between reach-scale  
33 272 standing stocks of CBOM (adonis,  $p = 0.036$ ) and FBOM (adonis,  $p = 0.053$ ) with glucose, arabinose, and  
34 273 cellobiose NRR.  
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40 275 Discussion  
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42 276 Seasonal patterns of DOM characteristics  
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44 277 These urban streams had higher CBOM biomass in autumn compared to other seasons and higher  
45 278 chlorophyll a biomass in spring than in other seasons (Figure 8). Because terrestrial carbon sources are  
46 279 typically of lower quality than aquatic autochthonous DOM sources (McKnight *et al.*, 2001), these  
47 280 changes in CBOM and chlorophyll biomass should result in lower quality DOM dominating in autumn  
48 281 and higher quality DOM dominating in the spring. As we hypothesized, BIX and FI, metrics of labile  
49 282 DOM, show a clear pattern of less labile carbon during autumn and more labile carbon during spring,  
50 283 likely due to riparian leaf fall producing a large influx of recalcitrant terrestrial DOM in the fall and vernal  
51 284 algal blooms producing a large influx of labile autochthonous DOM in the spring. HIX, which measures  
52 285 the recalcitrant humic fraction of DOM, is similar to BIX and FI with autumn having higher humic  
53 286 character than spring or summer. This pattern is also seen in the P/H (protein/humic) ratio, which  
54 287 shows more humic-like components in the autumn compared to the spring whereas summer was not  
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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, Navarro & Albarino, 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 measured across multiple DOM optical properties and higher primary production and algal standing stocks in the spring, the low absolute values of BIX and FI show that the DOM pool in the seasons we studied has a weak autochthonous component and a strong signature of terrestrially-derived fulvic acids. The dominance of terrestrial or humic derived carbon in the DOM pool may be a general pattern in streams draining urbanized basins. For example, terrestrial DOM sources include upwelling ground water, leaking stormwater infrastructure (Kaushal & Belt, 2012), and runoff from impervious surfaces (Hope *et al.*, 2004). DOM derived from these sources may overwhelm any autochthonous signature in streams. Alternatively, the year-round stronger, more recalcitrant terrestrial characteristics could indicate that heterotrophic biofilms, which are typical in urban streams (Johnson, Tank & Dodds, 2009), rapidly remove high quality DOM from the water column. For example, labile autochthonous carbon stimulates water column carbon use for energy metabolism and/or assimilation (Franke, Bonnell & Ziegler, 2013). Furthermore, the presence of algal biofilms enhanced the EEA of heterotrophic biofilms, suggesting the rapid use of labile DOM in the presence of autochthonous production (Rier, Shirvinski & Kinek, 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 there were lower overall chlorophyll and CBOM biomass in buried compared to open reaches, reach type 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 & 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 in 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

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3 330 open reaches received and retained more leaf litter that could leach recalcitrant terrestrial DOM (Figure  
4 331 8). In contrast, buried reaches neither received direct inputs of riparian leaf litter nor retained litter  
5 332 exported from upstream due to higher velocities and fewer retention structures (Beaulieu *et al.*, 2014).  
6 333 Alternatively, dilution of the DOM pool by lower HIX sewage sources that leak into the buried reaches  
7 334 (Smith & Kaushal, 2015) could reduce HIX in the buried reaches. Sorption of humic compounds has  
8 335 been observed in other studies (Ohno, 2002; Zsolnay *et al.*, 1999), but we collected water for EEM  
9 336 metrics from the top and bottom of the buried reaches, and there was no significant difference in HIX  
10 337 collected at either end of a buried reach (data not shown). Although the EEA data indicated greater use  
11 338 of recalcitrant carbon in buried reaches compared to open reaches (see below), the lack of change in HIX  
12 339 as water flows through the buried reaches implies that microbial processing of humic compounds was  
13 340 not enough to reduce the HIX of the DOM pool. Higher HIX in the open reach in spring is counter-  
14 341 intuitive given the presence of large algal standing stocks and high GPP, which would be expected to  
15 342 produce labile DOM. It is possible that the high HIX values resulted from DOM leached from greenfall  
16 343 inputs during leaf out and/or flower or seed production (Lewis & Likens, 2007), but we did not observe a  
17 344 CBOM peak in spring. However, in the overall context of this study, the median spring HIX values are  
18 345 lower than autumn in open and buried reaches, which is still consistent with an overarching seasonal  
19 346 affect driven by terrestrial sources in the autumn and aquatic sources in the spring.

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25 347 Despite spatio-temporal differences in the DOM composition driven by seasonal differences in CBOM  
26 348 and algae, the DOM data show that these urban streams are dominated by terrestrial humic sources,  
27 349 likely from constant seepage of DOM from soils to streams throughout the watershed. These data also  
28 350 show secondary control over DOM quality due to spatial differences in organic matter inputs that alter  
29 351 the characteristics of the DOM pool in buried versus open reaches. Therefore urban infrastructure can  
30 352 influence the characteristics of the DOM pool in the urban stream network.

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33 353 Patterns in Carbon Use – Extracellular Enzyme Activities (EEA)  
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35 354 Extracellular enzyme activity (EEA) reflects the composition of the DOM pool, as perceived by the  
36 355 microbial community. Although P/H and HIX indicated more humic and recalcitrant DOM in open  
37 356 reaches, buried reaches had higher DOPAH2 and POX activity (indicators of recalcitrant carbon) than  
38 357 open reaches. This supports our hypothesis that the microbial community in buried reaches would  
39 358 allocate more energy toward acquiring recalcitrant carbon sources than in the open reach, regardless of  
40 359 season. This pattern is consistent with experiments showing greater POX activity in low light conditions  
41 360 (Wagner *et al.*, 2015). Lower values of DOPAH2 and POX in the open reach indicate less effort to acquire  
42 361 recalcitrant carbon, likely because DOM leached from primary producers supplies an alternative, high  
43 362 quality carbon source. Conversely, the greater effort to acquire recalcitrant carbon in buried reaches is  
44 363 consistent with low chlorophyll, limited periphyton cover, and an extremely low reach-scale GPP  
45 364 (reported in Beaulieu *et al.*, 2014). This implies rapid use of high quality carbon produced in the open  
46 365 reaches and little export to downstream buried reaches, and is consistent with generally greater EEA in  
47 366 the presence of algal biofilms (Rier *et al.*, 2014).

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52 367 The LCI, which aggregates several EEA measures into a composite index of carbon use, also shows  
53 368 greater use of recalcitrant carbon in buried reaches. However, LCI shows a seasonal effect whereby  
54 369 summer has greater use of recalcitrant carbon than autumn, but that autumn and spring were not  
55 370 different. This pattern may be driven by low CBOM, low chlorophyll a, and high FBOM in open reaches  
56 371 during the summer, and it suggests that the autumn pulse of terrestrial CBOM leaches a labile fraction

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of DOM that microbes can use despite being dominated by low BIX and FI compounds. 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, EEA patterns 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 DOM pool that varies on a diurnal basis with primary production, 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 & 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  $\text{NO}_3^-$  or  $\text{NH}_4^+$  concentrations (reported in Beaulieu *et al.*, 2014), suggesting that higher quality spring DOM acted as a nitrogen source as well as a carbon source. Previous work has found seasonal changes in microbial demand for organic N in response to changes in C:N ratio and composition of organic matter (Kaushal & Lewis, 2005), and more work needs to be done to understand the role of organic matter as an energy source vs. a nitrogen source in some urban streams. 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

Biofilms in autumn were always more carbon-limited than in spring, which supported our hypothesis that terrestrial leaf fall would depress DOM quality in autumn. However, the pattern of carbon limitation by reach (i.e., buried or open) varied among seasons. Open reaches were more strongly carbon-limited than buried reaches in autumn, but were less carbon-limited than buried reaches in the spring. Stronger carbon limitation in open reaches during autumn may be a result of the pulse of recalcitrant DOM from terrestrial leaves that entered the open reaches during leaf-fall whereas lower carbon limitation in open reaches during spring may be a result of the pulse of labile DOM derived from



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3 414 algal sources. These explanations are corroborated by the DOM optical properties. DOC concentration  
4 415 did not vary between seasons (data not shown), suggesting that the pulse of autumn leaves and spring  
5 416 algae blooms changed DOM composition rather than quantity.

7 417 Alternatively, differences in C limitation between reaches might be related to secondary reach-scale  
8 418 factors. For example, EEA assays confirm that biofilms in buried reaches always invested more effort to  
9 419 acquire recalcitrant carbon, so they might have been better able to utilize the autumn pulse of  
10 420 terrestrial DOM compared to the open reaches. In contrast, biofilms in open reaches always invested  
11 421 less in recalcitrant carbon acquisition which, when compounded by the fact that the pulse of autumn  
12 422 leaves was delivered directly to the open reaches, could have led to more intensified carbon limitation.  
13 423 Similarly, in the spring, open reaches responded less to the simple carbon sources in the NDS because  
14 424 the system had higher levels of high quality algal DOM, but P/H ratio shows that buried reaches appear  
15 425 to receive less of this higher quality DOM, so they responded more strongly to the NDS. Less high  
16 426 quality DOM exported to buried reaches is consistent with the potential for rapid use of algal DOM *in*  
17 427 *situ* by heterotrophic biofilms (Franke *et al.*, 2013; Rier *et al.*, 2014) and is reflected in the carbon  
18 428 acquisition effort devoted to recalcitrant carbon sources.

23 429 We found different results when we expressed carbon limitation by area or biomass (i.e., gAFDM<sup>-1</sup>).  
24 430 When expressed by area, the temporal and spatial patterns were highly significant, but no patterns were  
25 431 evident when expressed by biomass. Therefore, the biofilm response to added carbon is not to increase  
26 432 the per cell carbon use rate, but simply to accumulate greater biomass. Given the fact that we relieved  
27 433 N and P limitation to focus on the carbon amendment response, these results might be most applicable  
28 434 to agricultural and urban streams which tend to have chronically high background nutrient  
29 435 concentrations (Carpenter *et al.*, 1998). The rapid processing of added carbon could also be a function  
30 436 of generally high inorganic nutrient concentrations in these urban streams in combination with the  
31 437 nutrients added to the NDS (Rosemond *et al.*, 2015).

35 438 Although we hypothesized that responses to the different carbon types in the NDS arrays would vary,  
36 439 biofilms responded similarly to all carbon sources (glucose, arabinose, cellobiose). Although arabinose  
37 440 has been used as a less labile form of carbon in some studies (e.g., Kaplan *et al.*, 2006), our results show  
38 441 that it is just as bioavailable as glucose in this study system. Similarly, we used cellobiose as a  
39 442 breakdown product of cellulose that we predicted would be less bioavailable than glucose or arabinose,  
40 443 yet it was equally bioavailable to those more simple carbon sources. It is unclear if arabinose and  
41 444 cellobiose bioavailability is equally high as glucose in most streams or if it was high in these urban  
42 445 streams because of the systemic dominance of recalcitrant carbon and/or the presence of N and P in the  
43 446 NDS agars.

47 447 Overall, these results indicate spatio-temporal variation in biofilm carbon use patterns related primarily  
48 448 to seasonal changes in the DOM pool and secondarily to reach scale patterns, such as stream burial.  
49 449 We found that the pulse of labile autochthonous carbon in the spring might have acted as a nutrient  
50 450 source as well as an energy source, but more work is needed to resolve this conclusively. Additionally,  
51 451 we documented widespread carbon limitation in these urban streams which could have been induced  
52 452 by the dominance of recalcitrant DOM sources from the watershed, limited *in situ* production of labile  
53 453 DOM due to stream burial, high background nutrient concentration leading to rapid CBOM consumption  
54 454 (e.g., Rosemond *et al.*, 2015), or some combination of those factors. Together, differences in carbon use  
55 455 patterns among buried and open reaches likely have implications at the river network scale, particularly  
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in drainages dominated by urban infrastructure that alternate between buried and open stream reaches. Because the limited quantity of labile carbon is more likely to be used *in situ*, urban systems with buried reaches may export a higher proportion of recalcitrant carbon than unburied streams, possibly increasing C flux from streams to receiving water bodies, but reducing labile carbon subsidies. 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 along the urban stream continuum is likely to have consequences for biogeochemical cycling of other nutrients and for downstream export of DOM, nutrients, and inorganic carbon.

Our work also suggests important considerations for management and restoration of urban streams. Stream daylighting is an engineering approach to urban stream restoration whereby buried streams are redesigned to be open to light (Pinkham, 2000). Daylighting is increasingly seen as an effective management approach to improve stream water quality and ecosystem function with respect to nutrient cycling in urban ecosystems (Beaulieu *et al.*, 2014; Pennino *et al.* 2014). Our results show that an additional mechanism of improvement may be to increase high quality autochthonous labile organic carbon availability to microbes that can support nitrogen removal processes such as denitrification (Newcomer *et al.*, 2012). Few studies have examined the biogeochemical impacts of daylighting streams (Newcomer Johnson *et al.*, 2016) and how the ecosystem changes over time after daylighting. Future research on carbon limitation in buried streams should elucidate how daylighting affects stream ecosystem function and provisioning of ecosystem services like nutrient reduction through *in situ* labile carbon production, especially in the broader context dissolved carbon from watershed, riparian, and floodplain or groundwater sources.

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## Tables

Table 1. Coefficients from adonis (Oksanen et al. 2016), 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	<i>P</i> -value
CBOM	0.072	0.060	0.064	0.036
FBOM	0.014	0.011	0.01	0.053

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Figure Legends

Figure 1. Spatio-temporal variation in the humification index (HIX) values derived from excitation-emission matrices. Lines within boxes are medians, box ends are 1<sup>st</sup> and 3<sup>rd</sup> quartiles, whiskers are 1.5 times the interquartile range.

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

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

Figure 4. Reach-scale variation in (A) L-3,4-dihydroxyphenylalanine (DOPA) + H<sub>2</sub>O<sub>2</sub> (DOPAH<sub>2</sub>) and (B) polyphenol oxidase (POX) activities.

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

Figure 6. Seasonal variation in β-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 (μg O<sub>2</sub> cm<sup>-2</sup> h<sup>-1</sup>).

Figure 8. (A) Coarse benthic organic matter (CBOM) standing stocks, (B) fine benthic organic matter (FBOM) standing stocks, (C) benthic chlorophyll *a*, and (D) periphyton standing stocks in the buried and open reaches during each sample season. All error bars are standard errors of the mean. Figure originally in Beaulieu et al. 2014.

## Figures

Figure 1.

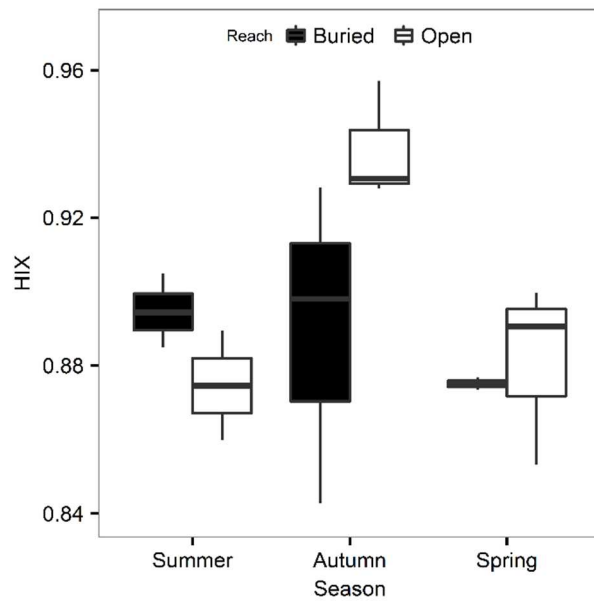


Figure 2.

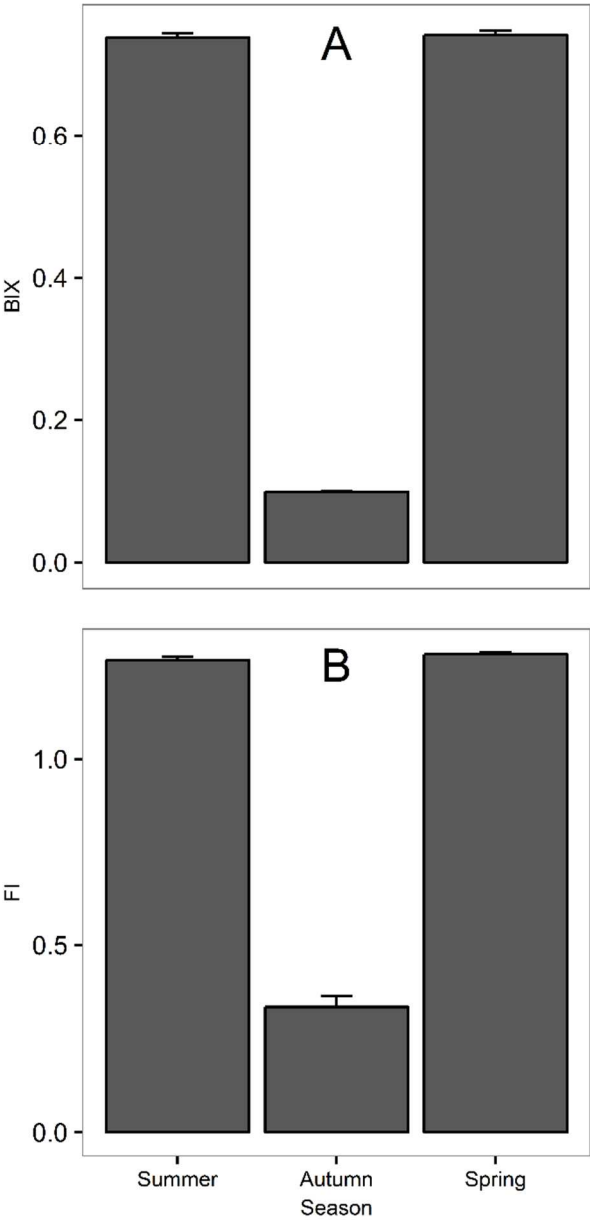


Figure 3.

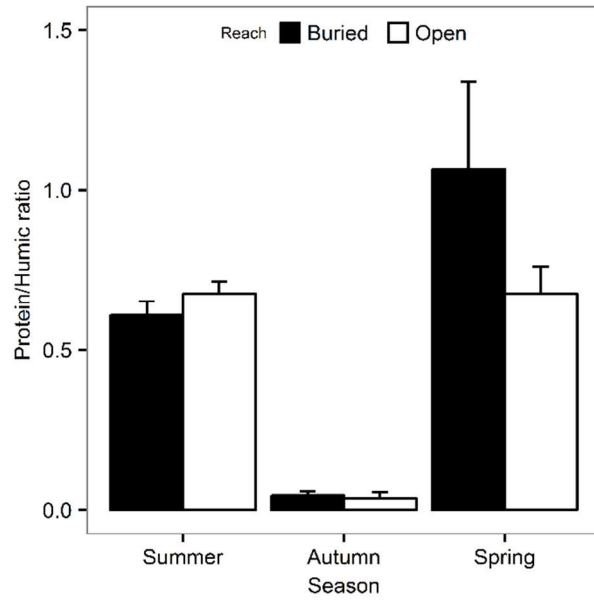


Figure 4.

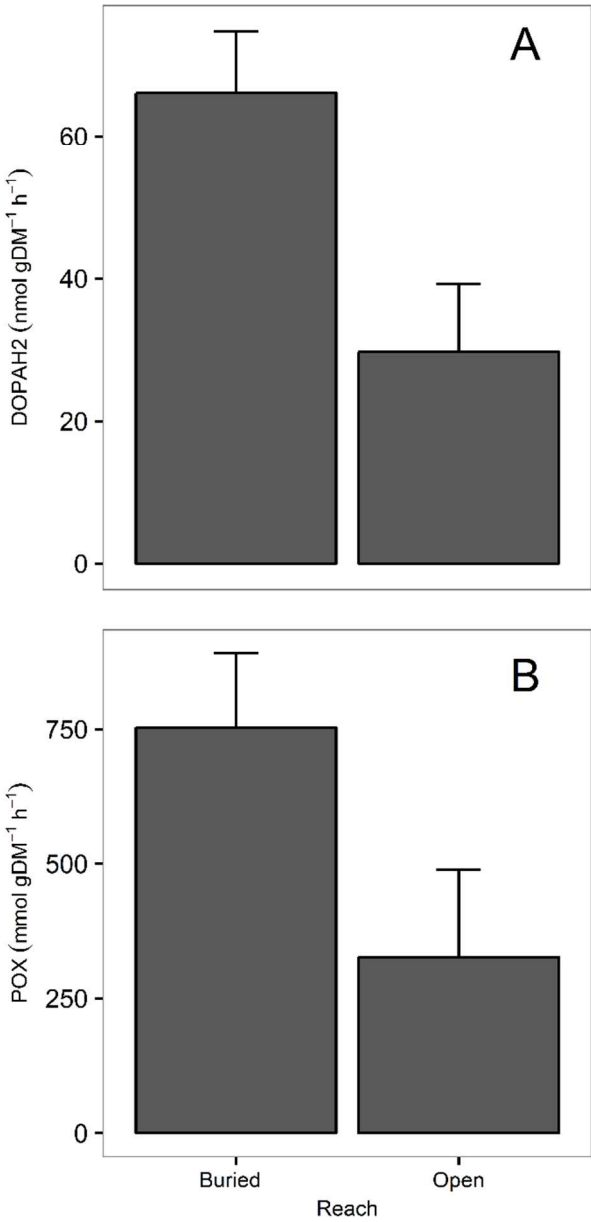




Figure 5.

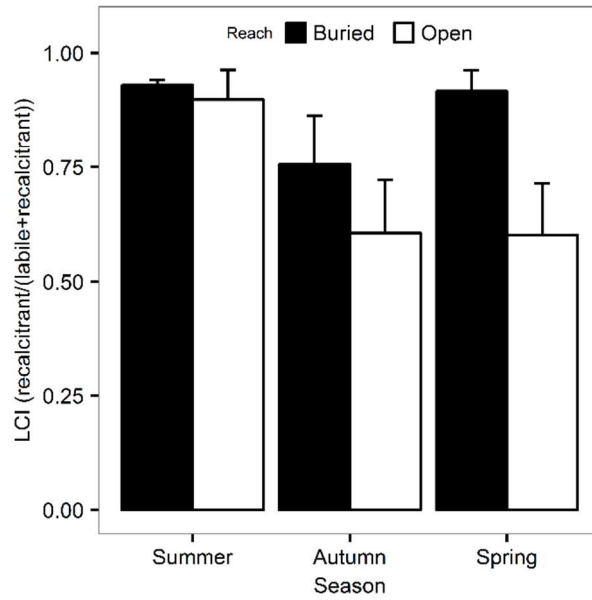


Figure 6.

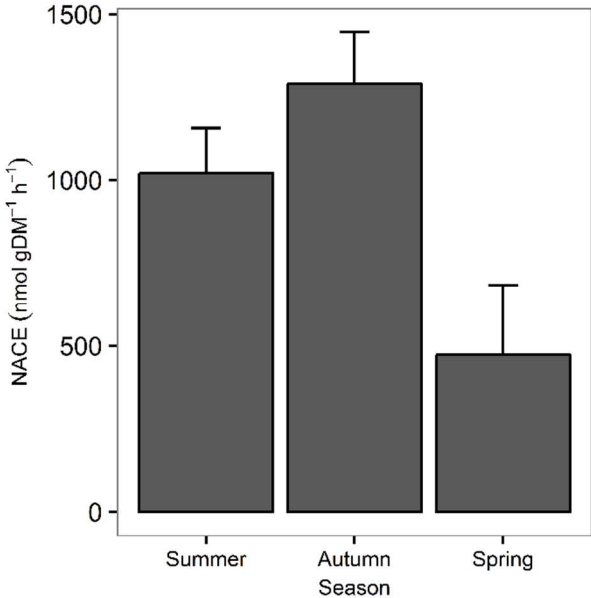


Figure 7.

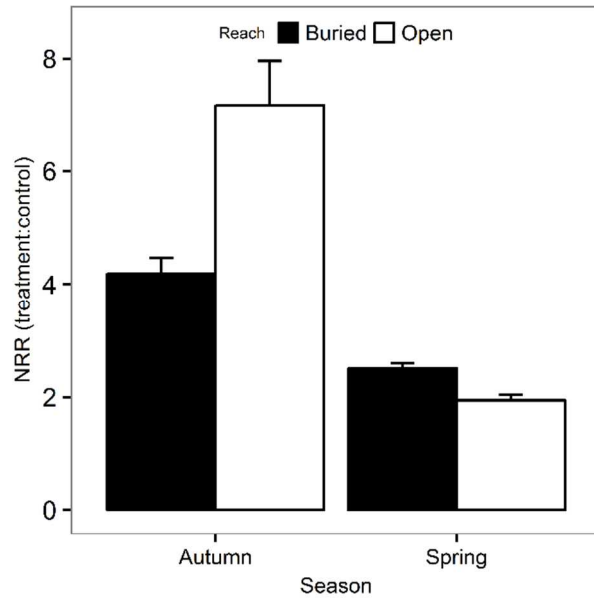


Figure 8.

