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Title: Seasonal resource limitation of heterotrophic biofilms in boreal streams

Running head: Resource limitation in boreal streams

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Abstract

Unraveling the potentially shifting controls over microbial activity among habitats and across seasonal transitions is critical for understanding how freshwater networks influence broader elemental cycles, and how these ecosystems may respond to global changes. We used nutrient-diffusing substrates to investigate seasonal patterns and constraints on microbial activity of biofilms in streams draining distinct landscape features of the boreal biome (forests, mires, and lakes). Microbial respiration (MR) largely mirrored spatial and temporal variation in water temperature. However, limitation by labile carbon (C) was a constraint to microbial activity during ice-covered periods, when MR of control nutrient-diffusing substrates fell below rates predicted from stream temperature alone. Variation in C limitation

among the study streams was reflective of putative organic C availability, with C limitation of biofilms weakest in the dissolved organic C (DOC)-rich, mire-outlet stream and greatest in the relatively DOC-poor, forest stream. Incidences of nutrient limitation were only observed during warmer months. Our study illustrates how variation in processes mediated by heterotrophic biofilms and seasonal shifts in resource limitation can emerge in a stream network draining a heterogeneous landscape. In addition, our results show that, for a large portion of the year, heterotrophic processes in boreal streams can be strongly limited by the availability of labile C, despite high DOC concentrations. Metabolic constraints to dissolved organic matter processing at near-freezing temperatures, coupled with hydrological controls over the delivery of more labile organic resources to streams (e.g., soil freezing and flooding), have potentially strong influences on the productivity of boreal streams.

Key words: Microbial processing, resource limitation, metabolism, boreal forest, wetlands, dissolved organic carbon.

Introduction

There is currently much interest in understanding the functional role(s) of freshwater ecosystems within the landscapes and regions they drain (Raymond et al. 2013). Streams and rivers are the primary recipients of terrestrial resources and support microbial processes that can influence the carbon (C) balance of landscapes (Hotchkiss et al. 2015) and alter the downstream fate of inorganic nutrients (Mulholland et al. 2008). The rates and significance of these processes are strongly influenced by dynamic changes in resource supply and habitat properties, which are in turn linked to seasonal climate conditions and the nature of land-water connections (Roberts et al. 2007). Resolving these interactions has thus emerged as an important challenge to understanding how microbial organisms in river networks influence broader elemental cycles and how these ecosystems may respond to global climate change

(Davis et al. 2013). Meeting this challenge is particularly important for high latitude regions, where terrestrial ecosystems and hydrological processes are notably sensitive to ongoing climate changes (Tetzlaff et al. 2013).

Heterotrophic biofilms, comprising bacteria and fungi that exist alongside other microbial groups attached to benthic and other substrates (Lock 1993), are a key element of stream food webs (Hall and Meyer 1998). The activities of these organisms constitute key linkages in the processing of organic matter (OM; Fisher and Likens, 1973) and the immobilization and transformation of nutrients (Peterson et al. 2001). Biofilm communities and rates of activity are influenced by many physical and chemical factors and stressors that are themselves spatially and temporally variable and interactive (Cross et al. 2015). For instance, while metabolic rates in streams are thought to be strongly influenced by water temperature (Acuña et al. 2008; Yvon-Durocher et al. 2012), pulsed inputs of organic resources (Valett et al. 2008), the seasonal depletion of autochthonous energy stores (Huryn et al. 2014), and variance in the quality of terrestrial OM stored in channel sediments (Jankowski et al. 2014) can all cause deviation from predictions based strictly on metabolic theory. Taken together, these results suggest that patterns of ecosystem functioning mediated by stream biofilms emerge from interactions between thermal regimes and energy supply (as organic matter or light) that can be highly sensitive to the specifics of land-water connections.

It is well established that the organic energy balance of headwater streams is closely linked to the vegetation, soils, and geomorphic structures of surrounding catchments (Fisher and Likens 1973), and the nature of these relationships differ across major biomes (Dodds et al. 2015). In northern boreal landscapes, variation in physical and chemical properties among streams is strongly influenced by differences in the relative cover of coniferous forests, wetlands (mires), and lakes within drainage networks (Laudon et al. 2011). For instance,

concentrations of dissolved nutrients and organic carbon (DOC) are generally greater in mire-dominated compared to forest-dominated catchments (Dillon and Molot 1997). Yet, the DOC pool from forested catchments is thought to be more bioavailable due to greater lateral inputs of fresh exudates from roots and mycorrhizal fungi (Berggren et al. 2010), as well as freshly leached organic acids from leaf litter and decaying OM (Ågren et al. 2008). In addition to these differences in resource pools, water temperature in boreal stream networks is spatially variable and strongly influenced by abundant lakes that can elevate summer temperature in receiving streams by 6-11 °C (Mellina et al. 2002). While heterogeneity in physical and chemical properties associated with these different landscape units are well-documented, how these attributes interact with seasonal thermal regimes to govern biofilm activity within aquatic networks remains poorly understood.

Seasonal variability in factors such as light and water temperature are also well known regulators of microbial processes in streams (e.g., Roberts et al. 2007), often eclipsing more subtle variation that arises across individual reaches within a drainage system (Malcolm et al. 2004). Such seasonality is likely to be particularly important in boreal regions that experience extreme changes in temperature, incident light, and hydrology throughout the year. Despite this likelihood, most research in boreal streams has focused on single seasons; for example, on microbial responses to resource export during the spring flood (e.g., Ågren et al. 2008), or on dynamics during the autumn when rates of biological activity are thought to be elevated (Burrows et al. 2015). By contrast, very little is known about the rates and controls of the activities of microbial biofilms, or even of freshwater ecosystems more generally, during the long and severe winters that characterize high latitude regions (Hampton et al., 2015; but see Huryn et al., 2014). Given the length of the winter season, biogeochemical processes in ice-covered streams likely have important implications for the overall productivity of these

ecosystems and for the broader role(s) they play as processors of terrestrially-derived resources. Moreover, processes during this period may be particularly vulnerable to the anticipated changes in winter climate that include a reduction in the duration snow and ice cover in the region (IPCC 2014).

In this study, we ask how spatial and temporal variation in resource availability interacts with seasonal temperature regimes to shape patterns of microbial activity in headwater streams. We answer this question using repeated incubations of nutrient-diffusing substrata (NDS) amended with inorganic nutrients and labile OM to quantify seasonal patterns in the microbial respiration of heterotrophic biofilms that develop in streams draining distinct headwater units within the same drainage system. A key objective was to determine how differences in physical conditions and resources drive variation in rates of biofilm activity and the nature and magnitude of resource limitation among strongly contrasting stream types all occurring within the same network. In addition, while a previous survey of biofilm productivity in this region suggested that N limitation is a key constraint during the autumn (Burrows et al. 2015), we sought to determine the extent to which inorganic nutrients and/or energy limitation changes across a strong seasonal gradient in water temperature. To this end, we also included assays of biofilm activity during the coldest times of year, and thereby shed new light on the rates and controls of metabolic processes in ice-covered streams in northern latitudes.

Materials and methods

Study region and streams

The study was conducted in the Krycklan catchment, a long-term research site in northern Sweden (Laudon et al. 2013). The region has a sub-arctic climate, with an annual precipitation of 614 mm and a mean annual temperature of 1.8°C (30-year average; Laudon

et al. 2013). Four catchments were chosen as study sites (Fig. 1), each with a 90° v-notch weir located in an ice-free house at the downstream end of each catchment. The catchments have characteristics typical of the broader boreal zone (Laudon et al. 2013), and were selected because each differs in the relative proportion of distinct boreal landscape features (i.e., forests, mires, and lakes), and thus variation in the source and input pathways of stream resources. The ‘forest stream’ (Västrabäcken or C2) drains a 12-ha catchment with 100% forest cover. DOC in the forest stream contains comparatively greater amounts of low-molecular-weight compounds (Ågren et al. 2008) that are flushed into streams when soil flow paths become activated during high flow events (Bishop et al. 2004; Laudon et al. 2011). The ‘mire stream’ (Kallkälsmyren or C4) drains an 18-ha catchment strongly influenced by a minerogenic mire (or wetland). DOC concentrations are generally highest in the mire stream, compared to the other study streams, because flow is reliant on deep, preferential flowpaths that percolate through DOC-rich, peat for much of the year (Peralta-Tapia et al. 2014; Schiff et al. 1998). The ‘lake stream’ (Stortjärnen outlet stream or C5) drains a 65-ha catchment with a lake covering 6.4% of its area. Finally, the ‘mixed stream’ (Svartberget or C7) drains a 47-ha catchment, encompassing the confluence of forest and mire stream. The weir at the mire and lake streams are located approximately 50 m downstream of the mire and lake outlets, respectively.

Nutrient-diffusing substrata

Nutrient-diffusing substrata (NDS) were constructed following Tank et al. (2006) and deployed on nine occasions in 2014 (every five to six weeks, starting 8th January). This intensive temporal sampling regime was necessary to encompass seasonal changes in water temperature, flow, and terrestrial inputs for each stream that may influence the dynamics of heterotrophic biofilms. Thirty mL plastic cups (United States Plastics Corporation, Ohio,

USA) were filled with 2% agar solution enriched with 0.5 M NaNO_3 (nitrogen; N treatment), 0.5 M KH_2PO_4 (phosphorus; P treatment), 0.5 M $\text{C}_2\text{H}_3\text{NaO}_2$ (C treatment), N and P (NP treatment), all nutrients (CNP treatment), or unamended agar control (A treatment). Acetate was chosen as the C treatment because acetic acid is the dominant low-molecular weight organic C fraction in these streams (Berggren et al. 2010). NDS were capped with a 30-mm diameter cellulose-sponge surface (Freudenberg Household Products, Weinheim, Germany). Five replicates of each treatment (30 NDS cups per stream) were randomly attached with cable ties to a stainless steel bar. NDS were placed underwater in the flume located in each weir house. Throughout the entire study period light was excluded from the weir houses to inhibit autotrophic growth. After 19 days, NDS were removed from the flume and cellulose sponges placed individually in 50-mL Falcon centrifuge tubes (Corning Incorporated, New York, USA) filled with unfiltered stream water. Samples were stored on ice until transfer to the laboratory, where they were kept at 4°C until respiration measurements.

Microbial respiration (MR) incubations of cellulose sponges were conducted within 24 hours of retrieval from each stream. Respiration was measured using the modified dark-bottle method (Johnson et al. 2009) at room temperature following Burrows et al. (2015). MR was calculated as the difference in O_2 between start and finish of a three-hour incubation, correcting for background O_2 consumed from bacterioplankton, and represented per area of sponge surface (as $\mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ consumed).

Although organic surfaces favour the development of heterotrophic-dominated biofilms (Johnson et al. 2009), it is possible that autotrophic microbes adapted to very low light conditions colonised our NDS sponges, and this may have influenced final rates of heterotrophic MR. Furthermore, we did not assess the linearity of respiration during the incubation period, which could be non-linear in dark-chamber incubations. Lastly, previous

research has indicated that microbial diversity of biofilm communities that have developed on NDS can be lower than natural organic substrates such as leaves (Hoellein et al. 2010). This may reflect 1) the heterogeneous nature of C compounds present in leaves which potentially support a more diverse microbial community compared to cellulose, or 2) the short colonisation period for NDS experiments relative to leaf litter in streams (Hoellein et al. 2010).

Environmental variables

Water samples for physico-chemical characteristics were collected at each site with a sampling frequency of two to four weeks, increasing to two to three times per week during the spring snowmelt. We collected samples for analysis by ultraviolet (UV) spectroscopy and to measure ambient concentrations of DOC, dissolved organic N (DON), nitrate (NO_3^-), ammonium (NH_4^+), total dissolved N (TDN), soluble reactive phosphorus (SRP), specific electrical conductivity, and pH. Samples were filtered in the field (0.45 μm nylon membrane filters; Sarstedt, Nümbrecht, Germany) and transferred to the laboratory refrigerator (4 °C) or freezer (for inorganic nutrients) within 6 hours.

Dissolved organic carbon (DOC) and TDN was analyzed using the combustion catalytic oxidation method on a Shimadzu TOC-V_{CPH} analyzer (Shimadzu, Duisburg, Germany). NO_3^- (Method G-384-08 Rev. 2), NH_4^+ (Method G-171-96 Rev. 12) and SRP (Method G-297-03 Rev. 1) were analyzed using a SEAL Analytical AutoAnalyzer 3 (SEAL Analytical, Wisconsin, USA). DON was calculated by subtracting DIN from TDN. Absorbance spectra were measured in 1-cm quartz cuvettes with a Hewlett Packard 8452A diode array spectrophotometer (Palo Alto, California, USA). The absorbance spectra were used to calculate the specific UV absorbance and the absorbance ratio. The specific UV absorbance of DOC (SUVA_{254}) is calculated by dividing the UV absorbance measured at $\lambda = 254 \text{ nm}$ by

DOC concentration and is positively correlated to aromatic C content (Weishaar et al. 2003). The absorbance ratio is calculated as the ratio between absorbance at 254 nm and 365 nm and is negatively correlated with the molecular weight of DOC (Dahlén et al. 1996). Stage-height ($\pm 1\%$) and water-temperature ($\pm 0.3^\circ\text{C}$) data was recorded using WT-HR capacitance water-level probes (Intech Instruments Ltd, Christchurch, New Zealand). Rating curves were developed for each weir from manual measurements of discharge measured at the weir v-notch during regular sampling.

Data treatment and statistical analyses

Principal component analysis (PCA) was performed to visualize the key physico-chemical parameters that drive differences among streams and dates. The values used were the average or median (for flow) of parameters collected over each NDS incubation period (i.e., 19 days) at each stream. TN was excluded as it was highly correlated with DOC ($r=0.87$), as were NO_3^- ($r=0.66$) and NH_4^+ ($r=0.80$) due to their strong correlation with DIN.

The spatial and temporal dynamics of nutrient treatment effects on MR was explored using nutrient response ratios; these allow the comparison of trends within and among sites after normalizing treatment effects to spatial and temporal variation in control rates. Response ratios were calculated by dividing the mean treatment (N, P, C, NP, and CNP) response in MR to that of the average unamended agar control (A). Response ratios above 1 (or 0 when transformed by natural logarithm) indicate that the mean response of MR to the respective treatment is greater than for control NDS.

Temporal trends in the ambient MR (for each site) and treatment response ratio (for N, P, NP, C, and CNP) data were examined using orthogonal polynomial contrasts, which test for linear and polynomial trends whilst preserving the chronological sequence of time (Quinn and

Keough 2002). Quadratic (1 change in direction), cubic (2 changes in direction), quartic (3 changes in direction), and quintic (4 changes in direction) polynomial terms were investigated. Time was represented as day of year (chosen to be the 10th day of each NDS incubation period) and the contrast coefficients accounted for unequal spacing between factor levels.

We assessed overall differences between control and treatment MR using linear mixed-effects models (LMMs) incorporating the nested covariance structure associated with sample date. In a separate LMM for each site, NDS treatments were treated as fixed-factors nested within time of NDS incubation period (random variable), allowing for unconstrained intercepts and slopes at each time.

We investigated the temperature dependence of MR (as $\mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ consumed) using Arrhenius plots, which display $\ln \text{MR}$ against reciprocal water temperature (kT^{-1}), where k is the Boltzmann constant ($8.616 \times 10^{-5} \text{ eV}^\circ \text{ K}^{-1}$) and T is temperature in degrees Kelvin. When biological processes are rate-limited and thermally-activated, the Arrhenius plot gives a straight line, and the slope of this line describes its temperature dependence or activation energy (E). The E of heterotrophic respiration often ranges between 0.6 and 0.7 electronvolts (eV) with an average of 0.65eV (Allen et al. 2006). The temperature dependence of metabolism, along with body size, lies at the core of the metabolic theory of ecology, and has been shown to govern a broad range of biological and ecological processes, from the physiology of individuals, to the composition of communities, and structure and function of ecosystems (Brown et al. 2004, Wang et al. 2009). In this study, E was determined by plotting average laboratory measurements of MR (i.e., respiration of biofilms that developed on our artificial substrates) against the average in situ water temperature recorded over each NDS incubation period. Importantly, this assessment of ‘temperature dependence’ differs

from studies where the sensitivity of reaction rates are directly assessed across a range of temperatures (e.g., Huryn et al. 2014; Jankowski et al. 2014). Instead, using MR as a proxy, we are exploring how biofilm development scales as a function of the water temperature experienced during each incubation period. The use of this proxy is supported by the correlation between similar short-term MR measurements and total PLFA biomass on NDS surfaces from streams in this same area (ln/ln relationship, $r=0.78$, $p<0.001$, $n=80$; data from Burrows et al. (2015)). Due to the limited number of data points for each site (nine data points for the lake stream, ten for the other sites), temperature dependence was assessed using data pooled from all four study sites.

We assessed if rates of MR were influenced by C amendment (C and CNP treatments) by conducting a linear model on the mean MR data for each site and date combination. We explored relationships between explanatory variables and MR on unamended agar NDS (i.e., a measure of ambient MR), and the nutrient response ratios using Spearman's rank correlation (r_s). We considered results significant if $p\leq 0.05$. LMMs were performed using the 'lme4' R package and was, along with all other analyses, conducted in R (R Development Core Team 2011).

Results

Stream characteristics

Clear differences in environmental characteristics were evident among streams (Fig. 2), with 55% of the total variance accounted for by the first two axes of the PCA ordination plot. The first axis (34%) separates the majority of the forest stream sampling dates from the other streams and is strongly weighted by DIN (-0.49), molar DIN:SRP (-0.44), the absorbance ratio (0.42), DOC (-0.42), and pH (0.40). The second axis (21%) separates the majority of the mire stream sampling dates from the lake stream sampling dates, and is strongly weighted by

SUVA₂₅₄ (-0.54), median flow (-0.53), and DOC (0.39). DOC concentrations were greater, and pH lower, in the mire stream for most sampling dates (Fig. 2; Table S1, see Supporting Information). The third axis (17%, not shown) was strongly weighted by mean water temperature (0.70) and separates sampling dates along a logical winter-summer gradient. Water temperature in the lake stream was consistently greater than other streams (Fig. 2; Fig. 3a), with 2336 degree days over the study period compared to 1457 for the mire stream, 1366 for the forest stream, and 1325 for the mixed stream.

Spatial and temporal patterns in microbial respiration

Microbial respiration (MR) at all sites displayed similar seasonal trends, with the fastest rates during summer, and lowest rates during winter (Fig. 3b). This seasonal variation in MR was strongly related to water temperature (All sites, $r_s=0.82$; Table 1). However, there were differences among sites in the magnitude and timing of seasonal peaks in MR, as described by the linear polynomial contrasts. In the lake, mire, and mixed streams, MR peaked during late summer, with a quadratic model best explaining seasonal variation for the mire stream (Mire, $F_{5,44}=42.6$, $R^2=0.82$, $p<0.001$), whereas a cubic model was best for the lake and mixed streams (Lake, $F_{5,37}=69.9$, $R^2=0.90$, $p<0.001$; Mixed, $F_{5,44}=44.5$, $R^2=0.84$, $p<0.001$). In contrast, MR in the forest stream peaked after the spring snowmelt, followed by a decrease in MR with increasing water temperature as summer progressed, and a second peak during autumn when mean water temperature was substantially lower (Fig. 3b). Accordingly, a quintic polynomial model (i.e. 4 changes in direction) best described seasonal trends for the forest stream ($F_{5,44}=24.2$, $R^2=0.73$, $p<0.001$). MR in the forest stream during the summer period was negatively correlated with water temperature ($r=-0.94$) and DOC concentration ($r=-0.99$), but was positively correlated with median flow ($r=0.93$, logarithmic trend).

Microbial respiration response to C and nutrient amendment

Contrasting seasonal trends were evident in the response of MR to C and nutrient amendment, pooling all sites (Fig. 4). The response ratio of NDS amended with C displayed positive quadratic trends (C: $F_{4,30}=2.89$, $p=0.006$; CNP: $F_{4,30}=3.12$, $p=0.03$), with the greatest response in winter (Fig. 4). During the winter period (for season cut-off dates see Table S1, Supporting information), C-amended rates of MR were 1.2 to 8.4 times greater (mean=3.3), and CNP-amended rates were 1.2 to 35 times greater (mean=7.1), than unamended controls, pooling all sites. In summer, however, the response of MR to C amendment was much lower, with C-amended rates 0.7 to 4.0 (mean=1.7) greater and CNP-amended rates 0.8 to 5.1 (mean=2.7) greater than unamended controls. In contrast to NDS amended with C, the response of MR to amendment with only nutrients displayed either a negative quadratic (P: $F_{4,28}=6.24$, $p=0.001$; NP: $F_{4,32}=6.06$, $p=0.001$) or negative cubic trend (N: $F_{4,32}=33.4$, $p<0.0001$), with positive response ratios of greatest magnitude occurring during summer (Fig. 4). The response of MR to nutrient amendment was, however, lower than unamended controls during winter, with responses ratios varying from 0.82 to -0.64 (mean=0.34) for N-amended, 2.88 to -0.18 (mean=0.4) for NP-amended, and 0.75 to -0.39 (mean=0.25) for P-amended NDS.

Although all streams displayed similar seasonal trends in C and nutrient limitation, there were site-specific differences in the overall magnitude of MR response to C and nutrient amendment (Table 2). MR in the mire stream showed the most muted response to C amendment, and in contrast to all other streams, its overall response was not significantly greater than ambient MR (LMM, $p=0.1$, Table 2). In contrast, rates of MR showed the greatest response to C and CNP amendment in the Forest stream (Table 2). In all streams, MR was significantly greater in response to CNP amendment and significantly lower in

response to P amendment, but the mean response of MR to N and NP amendment was not different than ambient MR (LMM, Table 2). The random effect term in all LMMs, incorporating the nested covariance structure associated with sampling time, was highly significant ($p < 0.0001$), indicating that treatment effects varied through time.

Temperature dependence

Visual inspection of the Arrhenius plot for the unamended, control sponges showed an abrupt change in the relationship between $\ln$ MR and kT^{-1} (Fig. 5), indicating a point at which rates for MR were lower than predicted by theoretical temperature dependence. A piecewise regression, conducted using the methods outlined in Crawley (2007), identified a temperature breakpoint of 0.8°C . Microbial respiration of biofilms on unamended sponges was very close to the theoretical temperature dependence of heterotrophic respiration at water temperatures greater than this breakpoint (Fig. 5), with an activation energy of -0.65 eV (linear regression, pooling all sites; $F_{1,18}=25.5$, $R^2=0.59$, $p < 0.001$). Below 0.8°C , there was a breakdown in theoretical temperature dependence, with MR lower than that predicted for these temperatures (Fig. 5). The average rate of MR below 0.8°C (all observations from a stream-water temperature range of -0.08 to 0.8°C) was $1.40 \mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$, less than half of the theorised rates for this temperature range (3.29 to $3.53 \mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$). Average rates of MR for C- ($3.64 \mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$) and CNP-amended ($8.41 \mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$) biofilms at water temperatures below 0.8°C were, however, significantly ($p < 0.008$) elevated compared to the unamended controls (see Supporting Information Fig. S1). The number of days during 2014 that the average-daily streamwater temperature was below 0.8°C was 136 for the mire stream, 151 for the mixed stream, 157 for the lake stream, and 161 for the forest stream.

Discussion

Boreal rivers are fed by headwater streams that drain a patchwork of forest, lake, and mire ecosystems, each having distinct physical and chemical properties (Laudon et al. 2011).

While these different stream types were associated with heterogeneity in the rates, temporal dynamics, and resource limitation of stream biofilm activity on our experimental substrates, the clearest biological patterns were linked to seasonal changes in water temperature. The strong effect of temperature on biofilm activity is not surprising in the boreal zone, where the seasonal amplitude of climate conditions is notably dramatic (e.g., Mahli et al. 1999).

Superimposed on this overarching, temperature-driven signal, seasonal and spatial shifts in resource availability also constrained biofilm activity. Most notably, MR of biofilms was energy limited during winter, when C-amended rates were up to 8.4 times greater than unamended controls. By contrast, limitation by inorganic nutrients (primarily N) was observed during summer and autumn months. Overall, our results highlight how variation in seasonal habitat conditions can interact with the availability of multiple resources to shape patterns of in-stream biological activity.

The strength of C limitation of biofilm development in the winter is surprising given that boreal streams typically have high DOC concentrations linked to widespread peat formation (Aitkenhead and McDowell 2000). Nevertheless, further inspection of our data using Arrhenius plots illustrates the significance of winter C limitation. Specifically, to the extent that laboratory measures of MR serve as proxies for the accrual of microbes during field incubations, the activation energy for this development on unamended samples was consistent with predictions from metabolic theory for much of the year (when water temperatures were $> 0.80^{\circ}\text{C}$). This value was well within the range of those observed for multiple variables representing different levels of biological and ecological organization

(Brown et al. 2004; Gillooly et al. 2001; Yvon-Durocher et al. 2012). However, at water temperatures $<0.8^{\circ}\text{C}$, which in 2014 represented between 37 and 44% of the year in our study streams, rates of biofilm MR were lower than predicted from theoretical temperature dependence, unless experimentally supplied with a low-molecular-weight C source (see Fig. S1, Supporting Information). Given the length of the winter season in northern boreal regions, energy limitation during this period may have widespread implications for the overall productivity of these ecosystems.

Deviations from theoretical temperature dependence can occur due to changes in resource supply which can both enhance (Welter et al. 2014) and inhibit (Huryn et al. 2014) metabolic processes, and these resource controls are often exemplified when comparing distinct landscape patches and seasons (Valett et al. 2008). A breakdown in the temperature dependence of ecosystem respiration was observed during the winter in a spring-fed stream in the Alaskan arctic (Huryn et al. 2014). These authors did not experimentally test for C limitation but suggested the non-linearity in temperature dependence was due to the exhaustion of photosynthetic C that had accumulated during the summer. Similarly, in a review of the controls of oceanic bacterial production, greater temperature dependencies were found in polar regions (the Ross Sea and Western Arctic) compared to warmer, lower-latitude regions with the most parsimonious explanation being lower C availability along with reduced microbial activity at low temperatures (Kirchman et al. 2009). Although we can only speculate as to what mechanisms lead to C limitation of biofilms in our humic study streams, we know that it was largely alleviated by amendment with acetate at all sites, indicating that labile-C substrate availability (chemical quality and/or supply) during winter was a key constraint.

The mechanisms underlying the winter C limitation of biofilm MR in our study streams are likely a combined effect of the high activation energy required at low temperatures to assimilate complex C substrates and a reduction in the delivery of more bioavailable C resources from land. The temperature dependence (and activation energy) of biotic reactions increases with greater molecular complexity (Davidson and Janssens 2006), and DOC in boreal streams is generally humic with a high-molecular-weight (Thurman 1985). Moreover, a reduction in the supply of freshly-leached C resources was coupled with low temperatures during winter, when soil frost isolates riparian and upland hydrological pathways and the relative contribution of deep, older groundwater increases (Ågren et al. 2007; Peralta-Tapia et al. 2015). Reduced insolation for ice-covered streams may also eliminate the production of labile C from both photochemical degradation (Köhler et al. 2002) and exudation from aquatic autotrophs (e.g., Baines and Pace, 1991) during winter months. This winter C limitation may be exacerbated in northern boreal headwaters, where coniferous trees dominate riparian zones in mature forests and autumn pulses of particulate C (as deciduous litter) are relatively low and patchy. At lower latitudes, deciduous litter inputs are known to be critical for stream ecosystems during autumn (Tank et al. 2010) and may continue as important resources throughout the winter (Sinsabaugh and Shah 2010).

Variation among streams in the magnitude of MR response to C amendment is broadly consistent with our understanding of the origin, delivery, and bioavailability of C resources in boreal landscapes (Laudon et al. 2011). For instance, the relatively muted response of MR to C amendment in the mire stream corresponds with high average DOC concentrations (Table S1, Supporting information). Although stream DOC originating from wetlands is generally less bioavailable (by percentage) when compared to forested catchments (Berggren et al. 2007), our results suggest that the overall size of the C pool exported from mires contains

sufficient bioavailable DOC to at least partially satisfy the energetic needs of heterotrophic microbes. In contrast, DOC in the forest stream contains comparatively greater amounts of low-molecular-weight compounds thought to be a higher quality C source for stream microbes (Berggren et al. 2007). For much of the boreal winter, however, plant photosynthesis is low (Hari and Mäkelä 2003), surface and soil flowpaths are inactive, and DOM inputs to streams are derived from deeper groundwater sources (Ågren et al. 2007; Peralta-Tapia et al. 2014). Additionally, in the absence of precipitation events that flush resources to streams, this labile, forest soil-derived DOC pool is thought to be rapidly exhausted (days to weeks) by microbial consumption (Berggren et al. 2007). Subsequently, during winter base flow there is likely to be a strong reduction in the availability of labile C substrate, which may be the reason the greatest response to C amendment was observed in the forest stream.

Further support for the ‘flowpath control’ over bioavailable C resources to streams is found when evaluating the temperature dependence of biofilm MR in the forested stream during the snow-free period. In contrast to other streams, MR in the forest stream peaked in late spring, declined through summer as temperature increased, and then displayed a second peak during autumn when temperature was already declining (Fig. 3b). This initial late-spring peak likely corresponds to the flush of labile C that has previously been documented for this catchment (Ågren et al. 2008; Berggren et al. 2007). Indeed, during this ice-free period, MR was negatively correlated with stream water temperature but positively correlated with median flow ($r=0.93$, logarithmic trend). We hypothesize that the declining trend in MR during the spring-summer transition reflects the reduction in labile-DOC transported to streams during periods of low flow. Supporting this notion is the positive response of MR to C amendment over the same period and the increase in ambient MR as flow increased again during autumn.

Elsewhere, allochthonous pulses of resources delivered via snowmelt or storm-related events influence patterns of respiration at episodic and seasonal timescales (Baker et al. 2000; Roberts et al. 2007). In our study streams, and perhaps throughout the boreal zone, it is likely that the turning on (via snow melt and rainfall events) and off (via soil frost and drought) of flow paths is the key factor driving rates of MR, at least for heterotrophic biofilms.

A positive response to nutrient amendment was only evident during the warmer part of the year when C limitation was weakened, and this was generally greatest in the nutrient-poor forest stream and lowest in the comparatively nutrient-rich mire stream (Fig. 4; Table 2).

These findings support previous research in the same region that revealed nutrient (primarily N) limitation of biofilm MR during autumn, with the degree of limitation negatively correlated with stream nutrient concentrations (Burrows et al. 2015). Despite the short duration of nutrient limitation throughout the year, particularly in comparison to the period during which C limitation was paramount, we emphasize that its occurrence corresponds with the greatest rates of biofilm activity. For instance, average rates of MR among our study sites were 3 to 10 times greater during summer than winter (Fig. 3). Consequently, stream N enrichment during this time of year, whether natural or anthropogenic, may increase rates of biofilm respiration in boreal streams, and this should be most evident in forest-dominated catchments. In contrast, owing to the overwhelming C limitation, our results suggest that the boreal study streams we studied have a very limited capacity to retain inorganic nutrients during the winter season, a period when terrestrial N losses are likely to be elevated (Sponseller et al. 2014).

A phenomenon common to all streams was nutrient inhibition of biofilm MR that was strongest during the winter months. Nutrient inhibition has previously been observed in NDS research, mainly associated with P amendment (Atkinson et al. 2013; Tank and Dodds 2003),

but as Tank and Dodds (2003) assert, it is probably reported less often than occurs. Our study, nonetheless, found equally strong inhibition for N amendments, and strongest inhibition for combined N and P amendment (Fig. 4). Some possible explanations include the production of growth inhibitors during agar preparation that has been observed in laboratory growth media (Tanaka et al. 2014) and the toxicity of high nutrient concentrations to microbes, both of which may be more pronounced when microbial biomass and resource demand are low during the winter season. Inhibition by N and P, however, was not observed when combined with C amendments, which yielded the greatest rates of MR consistently in excess of those recorded for control biofilms. Regardless, the interactive effects of very low temperatures and elevated nutrient supply on microbial biofilm dynamics warrants further investigation.

Our research uncovered clear seasonal trends in resource limitation of heterotrophic biofilms, with the magnitude varying in accordance with our understanding of the origin, delivery, and bioavailability of resources among stream types representative of dominant boreal landscape units. The strong seasonal imprint revealed for biofilm functioning in our study streams indicates that changes to the extent of winter, due to climate change, may have implications for the overall heterotrophic activity of these boreal streams and potentially alter the rates of many biogeochemical transformations that these microbes mediate. Research is needed to unravel the concurrent seasonal patterns and resource limitation of autotrophic biofilms in order to better understand implications for the overall productivity of boreal streams.

Warming conditions are predicted to reduce the period of snow and ice cover in northern latitudes and increase the likelihood of rainfall and rain-on-snow melt events (IPCC 2014). These conditions would increase the period that surface and sub-surface flowpaths are activated in boreal streams (Spence et al. 2015) and potentially lead to greater quantities of

in-stream C resources, whose availability during winter currently constrains microbial activity.

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Figure legends

Fig. 1. The location and land-cover characteristics of the four study sites. C2 = Forest stream, C4 = Mire stream, C5 = Lake stream, C7 = Mixed stream.

Fig. 2. Plot of the first two principal component axes from a principal component analysis of the physico-chemical parameters measured at each stream and date (represented as day of year). DOC, dissolved organic carbon; DIN, dissolved organic nitrogen; SRP, soluble reactive phosphorus; a_{254}/a_{365} , absorbance ratio.

Fig. 3. Seasonal patterns for each of the four study streams of a) stream-water temperature recorded every hour (values less than 0.8°C are colored red) and b) mean microbial respiration (MR; $\pm$ standard deviation of five replicates) of heterotrophic microbial biofilms that colonized the artificial cellulose substrates during each incubation period; a polynomial line of best fit for the mean MR data is overlaid with 95% confidence intervals (shaded grey).

Fig. 4. Seasonal patterns in the mean ($n=5$) response ratio (treatment response relative to control) for microbial respiration on heterotrophic microbial biofilms that developed on

artificial cellulose substrates. Response ratio is displayed with natural logarithmic spacing. A polynomial line of best fit is overlaid with 95% confidence intervals (shaded grey).

Fig. 5. Arrhenius plot for the temperature dependence of microbial respiration (MR; plotted as O₂ consumption) for heterotrophic biofilms that colonized cellulose sponges on agar (control) nutrient-diffusing substrata. The ln-transformed MR is plotted against the mean reciprocal water temperature during three-week incubations ($1/kT$; temperature decreases from left to right of x-axis). The dotted vertical line is the temperature breakpoint (0.8°C) identified for this relationship on control substrata using piecewise regression. The line of best fit (slope=-0.65) is for data with water temperature >0.8°C.

Tables

Table 1. Spearman's rank correlation coefficient (r_s) describing the association between microbial respiration (ambient) and physico-chemical variables for each stream across seasons. Bold numbers indicate that the r_s statistic was significantly different than zero ($\alpha=0.05$; two-sided test).

Date	Forest	Mire	Lake	Mixed	All
Streamwater temperature (°C)	0.68	0.88	0.83	0.95	0.82
Q (L s ⁻¹)	0.05	0.33	0.52	0.01	0.07
pH	-0.12	-0.45	0.52	-0.36	-0.05
DOC (mg C L ⁻¹)	0.42	0.37	-0.58	0.81	<i>0.31</i>
TN (mg N L ⁻¹)	0.37	0.37	-0.42	<i>0.60</i>	0.21
DIN (µg N L ⁻¹)	0.13	-0.02	-0.77	-0.59	-0.15
SRP (µg P L ⁻¹)	-0.67	-0.30	-0.83	0.47	-0.22
Molar DIN:SRP	0.73	0.55	0.55	-0.49	0.19
Absorbance ratio	-0.03	-0.45	0.6	-0.13	-0.09
SUVA ₂₅₄	0.32	0.81	0.57	0.76	0.33

Table 2. The output (untransformed mean coefficient $\pm$ standard error and P value) from linear mixed-effects models assessing the response of microbial respiration (MR; $\mu\text{g O}_2 \text{ cm}^{-2} \text{ h}^{-1}$ consumed) to nutrient and carbon amendment for each study stream. NDS treatments were treated as fixed-factors nested within time of incubation period (random variable), allowing for unconstrained treatment intercepts and slopes for each period. A=agar, C=carbon, N=nitrogen, P=phosphorus. MR of A is assessed compared to a value of 0, and each treatment MR is compared to A.

	Forest Coefficient ($\pm$ SE)	P	Mire Coefficient ($\pm$ SE)	P	Lake Coefficient ($\pm$ SE)	P	Mixed Coefficient ($\pm$ SE)	P
A	3.35 (0.93)	0.0003	4.39 (0.87)	<0.0001	3.60 (1.25)	<0.001	3.70 (1.04)	0.02
C	7.45 (1.27)	0.01	5.99 (0.80)	0.07	6.91 (0.89)	0.003	6.91 (0.79)	0.003
CNP	13.4 (2.20)	0.001	9.95 (1.64)	0.003	11.2 (2.24)	0.001	12.9 (1.92)	0.001
N	5.19 (1.17)	0.8	4.29 (0.62)	0.2	4.84 (0.98)	0.9	4.69 (1.00)	0.09
NP	4.54 (1.16)	0.6	4.09 (0.84)	0.1	5.50 (1.45)	0.6	5.04 (1.06)	0.1
P	2.09 (0.54)	0.003	3.06 (0.40)	0.002	2.56 (0.42)	0.0006	1.72 (0.59)	0.002

Figure 1. The location and land-cover characteristics of the four study sites. C2 = Forest stream, C4 = Mire stream, C5 = Lake stream, C7 = Mixed stream.

191x160mm (300 x 300 DPI)

Acce

Figure 2. Plot of the first two principal component axes from a principal component analysis of the physico-chemical parameters measured at each stream and date (represented as day of year). DOC, dissolved organic carbon; DIN, dissolved organic nitrogen; SRP, soluble reactive phosphorus; a254/a365, absorbance ratio.

158x132mm (300 x 300 DPI)

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Figure 3. Seasonal patterns for each of the four study streams of a) stream-water temperature recorded every hour (values less than 0.8°C are colored red) and b) mean microbial respiration (MR; $\pm$ standard deviation of five replicates) of heterotrophic microbial biofilms that colonized the artificial cellulose substrates during each incubation period; a polynomial line of best fit for the mean MR data is overlaid with 95% confidence intervals (shaded grey).

165x200mm (300 x 300 DPI)

Figure 4. Seasonal patterns in the mean ($n=5$) response ratio (treatment response relative to control) for microbial respiration on heterotrophic microbial biofilms that developed on artificial cellulose substrates. Response ratio is displayed with natural logarithmic spacing. A polynomial line of best fit is overlaid with 95% confidence intervals (shaded grey).

254x190mm (300 x 300 DPI)

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Arrhenius plot for the temperature dependence of microbial respiration (MR; plotted as O_2 consumption) for heterotrophic biofilms that colonized cellulose sponges on agar (control) nutrient-diffusing substrata. The ln-transformed MR is plotted against the mean reciprocal water temperature during three-week incubations ($1/kT$; temperature decreases from left to right of x-axis). The dotted vertical line is the temperature breakpoint (0.8°C) identified for this relationship on control substrata using piecewise regression. The line of best fit (slope= -0.65) is for data with water temperature $>0.8^\circ\text{C}$.

Figure 5
147x190mm (300 x 300 DPI)

Supporting information

Table S1. The seasonal mean ($\pm$ standard deviation) of physico-chemical variables recorded

at each stream during the seasons. Seasons were determined according to the official

meteorological season starting dates for the Svartberget field station in 2014: Spring - 6th

March, Summer - 17th May, Autumn - 20th September, Winter – 4th November. DOC,

dissolved organic carbon; TN, total nitrogen; DIN, dissolved inorganic nitrogen; SRP, soluble

reactive phosphorus; SUVA₂₅₄, specific ultraviolet absorbance at 254 nm; a₂₅₄/a₃₆₅,

absorbance ratio; Q , discharge.

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9 Table S1.

Period	Stream	DOC (mg C L ⁻¹)	TN (mg N L ⁻¹)	DIN (µg N L ⁻¹)	SRP (µg P L ⁻¹)	pH	SUVA ₂₅₄	a ₂₅₄ /a ₃₆₅	Q (L s ⁻¹)	Water temperature (°C)
Spring	Forest	16.3 (2.91)	0.28 (0.04)	12.6 (1.03)	2.90 (0.95)	4.88 (0.11)	4.60 (0.17)	4.35 (0.09)	1.64 (1.46)	0.14 (0.49)
	Lake	25.1 (4.63)	0.46 (0.14)	50.1 (46.9)	2.16 (0.89)	4.65 (0.21)	5.06 (0.25)	3.95 (0.09)	18.4 (19.8)	1.77 (2.22)
	Mire	20.5 (6.50)	0.30 (0.10)	25.9 (10.6)	2.29 (1.26)	4.33 (0.09)	4.43 (0.24)	4.17 (0.17)	2.47 (2.76)	0.44 (0.79)
	Mixed	18.6 (2.05)	0.33 (0.05)	35.5 (5.07)	4.63 (1.35)	4.70 (0.13)	4.69 (0.18)	4.13 (0.25)	7.85 (6.76)	0.44 (0.42)
Summer	Forest	25.0 (5.75)	0.79 (1.01)	13.0 (5.32)	3.56 (1.87)	5.07 (0.42)	4.61 (0.38)	4.36 (0.15)	0.67 (1.00)	8.68 (2.33)
	Lake	20.1 (2.97)	0.39 (0.06)	35.4 (19.0)	5.35 (9.70)	5.05 (0.23)	5.30 (0.27)	3.89 (0.15)	8.09 (8.52)	15.5 (3.29)
	Mire	36.9 (6.91)	0.66 (0.30)	65.6 (39.7)	6.21 (4.84)	4.50 (0.51)	4.64 (0.25)	3.93 (0.21)	1.03 (1.58)	8.74 (2.10)
	Mixed	27.5 (8.84)	0.46 (0.14)	33.7 (8.34)	10.8 (7.06)	4.85 (0.40)	4.99 (0.43)	4.00 (0.26)	3.73 (4.84)	8.28 (2.46)
Autumn	Forest	23.0 (2.50)	0.40 (0.03)	13.4 (4.76)	6.31 (2.23)	4.98 (0.41)	4.30 (0.28)	4.39 (0.09)	0.79 (1.16)	4.48 (1.67)
	Lake	21.1 (1.11)	0.37 (0.02)	40.5 (8.95)	3.44 (0.61)	5.03 (0.25)	5.04 (0.19)	3.96 (0.02)	9.53 (9.68)	4.71 (2.24)
	Mire	39.3 (3.19)	0.53 (0.08)	67.6 (56.5)	30.2 (43.1)	4.37 (0.58)	4.22 (0.29)	4.16 (0.16)	1.41 (1.95)	5.13 (1.34)
	Mixed	28.0 (8.41)	0.41 (0.10)	26.1 (6.64)	11.9 (4.95)	4.86 (0.69)	4.48 (0.47)	4.12 (0.13)	4.66 (6.01)	4.27 (1.49)
Winter	Forest	16.2 (3.80)	0.29 (0.07)	13.6 (3.08)	7.52 (4.14)	5.07 (0.30)	4.47 (1.71)	4.40 (0.19)	0.46 (0.52)	0.38 (0.58)
	Lake	24.4 (0.99)	0.45 (0.02)	57.2 (8.33)	4.75 (1.37)	4.77 (0.08)	4.72 (0.50)	3.98 (0.08)	4.88 (3.72)	0.29 (0.29)
	Mire	30.6 (1.38)	0.42 (0.02)	54.8 (4.03)	8.84 (2.03)	4.35 (0.13)	4.01 (0.61)	4.12 (0.15)	1.03 (0.61)	0.80 (0.89)
	Mixed	19.7 (1.60)	0.35 (0.05)	49.6 (17.2)	10.5 (3.97)	5.01 (0.16)	4.46 (0.26)	4.07 (0.08)	2.43 (1.58)	0.41 (0.65)
Whole study	Forest	20.3 (5.88)	0.49 (0.63)	13.1 (3.80)	4.68 (3.05)	5.01 (0.33)	4.53 (0.88)	4.37 (0.14)	0.76 (1.03)	3.59 (4.12)
	Lake	22.4 (3.59)	0.42 (0.08)	44.6 (27.1)	4.14 (6.05)	4.89 (0.26)	5.07 (0.38)	3.94 (0.11)	8.58 (11.1)	6.22 (7.15)
	Mire	31.9 (8.82)	0.50 (0.24)	53.2 (34.5)	9.01 (16.3)	4.41 (0.37)	4.38 (0.43)	4.06 (0.20)	1.32 (1.68)	3.88 (3.97)
	Mixed	23.6 (7.42)	0.39 (0.11)	36.6 (12.1)	9.29 (5.64)	4.85 (0.35)	4.72 (0.40)	4.07 (0.21)	3.95 (4.78)	3.50 (3.92)

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Figure S1. Arrhenius plots of the temperature dependence of microbial respiration (MR; plotted as O_2 consumption) for heterotrophic biofilms that colonized the artificial cellulose substrates on agar (A, control), C, and CNP nutrient-diffusing substrata. The ln-transformed MR is plotted against the mean reciprocal water temperature during three-week incubations ($1/kT$; temperature decreases from left to right of x-axis). Dotted vertical lines are the temperature breakpoint (0.8°C) identified for this relationship on control substrata using piecewise regression. The line of best fit (slope=-0.65) of the A treatment is for data with water temperature $>0.8^\circ\text{C}$.

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