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Evidence for key enzymatic controls on metabolism of Arctic river organic matter

Running title: Organic matter metabolism in the Kolyma River

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Abstract

Permafrost thaw in the Arctic driven by climate change is mobilizing ancient terrigenous organic carbon (OC) into fluvial networks. Understanding the controls on metabolism of this OC is imperative for assessing its role with respect to climate feedbacks. In this study we examined the effect of inorganic nutrient supply and dissolved organic matter (DOM) composition on aquatic extracellular enzyme activities (EEAs) in waters draining the Kolyma River Basin (Siberia), including permafrost derived OC. Reducing the phenolic content of the DOM pool resulted in dramatic increases in hydrolase EEAs (e.g. phosphatase activity increased > 28 fold) supporting the idea that high concentrations of polyphenolic compounds in DOM (e.g. plant structural tissues) inhibit enzyme synthesis or activity, limiting OC degradation. EEAs were significantly more responsive to inorganic nutrient additions only after phenolic inhibition was experimentally removed. In controlled mixtures of modern OC and thawed permafrost endmember OC sources, respiration rates per unit dissolved OC were 1.3 – 1.6 times higher in waters containing ancient carbon, suggesting that permafrost derived OC was more available for microbial mineralization. In addition, waters containing ancient permafrost derived OC supported elevated phosphatase and glucosidase activities. Based on these combined results, we propose that both composition and nutrient availability regulates DOM metabolism in Arctic aquatic ecosystems. Our empirical findings are incorporated into a mechanistic conceptual model

highlighting two key enzymatic processes in the mineralization of riverine OM: 1) the role of phenol oxidase activity in reducing inhibitory phenolic compounds; and 2) the role of phosphatase in mobilizing organic P. Permafrost derived DOM degradation was less constrained by this initial "phenolic-OM" inhibition; thus, informing reports of high biological availability of ancient, permafrost derived DOM with clear ramifications for its metabolism in fluvial networks and feedbacks to climate.

Introduction

Arctic climatic warming is occurring faster than the global average, with air temperature increases of 4 – 7 °C expected for the coming century (ACIA 2004; Trenberth *et al.* 2007). Rapid warming results in the thaw and mobilization of extensive amounts of OC currently locked up in frozen permafrost soils, releasing it into the contemporary carbon cycle (Schuur *et al.* 2008; Schaefer *et al.* 2011). Permafrost soils are estimated to contain on the order of 1672 Pg C, of which approximately 25 % is stored as frozen yedoma soils in the Siberian Arctic (Zimov *et al.* 2006; Tarnocai *et al.* 2009). Permafrost soils thus contain carbon stocks approximately twice the size of the entire current atmospheric carbon pool and almost five times estimated live forest biomass (Tarnocai *et al.* 2009; Pan *et al.* 2011). Examining the reactivity and fate of permafrost derived OC is imperative in understanding northern high-latitude watershed and Arctic Ocean carbon dynamics as well as potential climate feedbacks.

River networks efficiently mineralize terrigenous OC, acting as important sites for heterotrophic metabolism and greenhouse gas release to the atmosphere (Cole *et al.* 2007; Battin *et al.* 2008; Aufdenkampe *et al.* 2011). Thawing permafrost and thermokarst processes significantly affect Arctic hydrology and geomorphology (Walvoord *et al.* 2012) resulting in the

direct and indirect release of ancient OC into aquatic ecosystems. Studies have highlighted permafrost derived organic matter to not be protected from microbial degradation by physical protection mechanisms (Fan *et al.* 2008) and to display geochemical and optical properties indicative of less decomposed OC fractions (Hugelius *et al.* 2012). Evidence suggests permafrost OC may be readily utilized by modern microbial communities (Dutta *et al.* 2006; Uhlirova *et al.* 2007; Waldrop *et al.* 2010; Vonk *et al.* 2013a). Furthermore, changing hydrologic conditions resulting from permafrost degradation may result in shifts in nutrient availability as well as DOM composition (Frey & McClelland 2009). Both inorganic nutrient concentrations and DOM quality have been linked to aquatic OC mineralization in Arctic River systems (Holmes *et al.* 2008; Wickland *et al.* 2012; Mann *et al.* 2012). Yet, mechanisms regulating the degradation of DOM including permafrost derived OC remain unclear.

Induction of extracellular enzymes (EEAs) allows heterotrophic bacteria to degrade complex organic compounds into smaller molecules for assimilation, and may be an initial rate limiting step for decomposition and nutrient mineralization (Asmar *et al.* 1994; Sinsabaugh 1994). Hydrolase enzymes catalyze the hydrolysis of covalently bonded compounds (e.g. peptides/phosphate), whereas oxidative enzymes are capable of cleaving the aromatic nuclei of phenolic compounds, and thus can be produced to degrade polyphenolic compounds often associated with terrestrially derived DOM (Sinsabaugh 2010). Step-wise degradation of DOM therefore appears to involve a plethora of enzymes, with their activities often used as indicators of freshwater nutrient status or the presence of complex substrates (Sinsabaugh & Moorhead 1994; Allison & Vitousek 2005). Environmental constraints that act to minimize the activities of specific enzymes have been posited to be responsible for inhibiting peat decomposition, resulting

in what has been considered an enzymatic "latch" upon global carbon stores (Freeman *et al.* 2001a).

In this study we undertook a series of controlled experiments that examined microbial-EEA controls on the utilization of aquatic DOM, including permafrost derived DOM in the Kolyma watershed, Siberia. We examined: 1) the effect of inorganic nutrient supply and DOM composition on OC metabolism, and; 2) EEAs in waters containing modern carbon or thawed permafrost endmember OC sources, recognizing potential microbial inhibitors and accelerators. Here we report on and synthesize several controlled laboratory experiments in conjunction with in-situ field observations collected over a two-year period (2011 – 2012) that provide the empirical basis for our model of Arctic riverine DOM degradation.

Materials and Methods

Site description & sampling

The Kolyma River Basin covers 652,924 km^2 of North-East Siberia, and represents the largest watershed on Earth underlain by continuous permafrost (Fig 1). Sampling was focused in the lower basin region and was conducted during the summer months of June/July 2011 and 2012. The sample region is highly diverse containing mixed forest and tundra vegetation and mountain to floodplain landscapes (Fig 1). The area receives relatively low annual precipitation rates (~185 mm/yr; Welp *et al.* 2005), but experiences large mean monthly air temperature variations, ranging from ~ 12 °C in summer to ~ -35 °C during winter. Waters from four diverse endmembers were selected and collected for manipulation and incubation experiments (Table 1; Fig 1). The Kolyma River mainstem was sampled at two different locations (Sites 1 & 5, Fig 1). Site 1 waters were collected from the Kolyma mainstem approximately 2 km upstream from the

city of Cherskiy. The second Kolyma River mainstem site (Site 5; Fig 1) was collected approximately 120 km upstream from the first (Fig 1). Both Kolyma River sites represented an integrated signature of waters draining a large proportion of the Kolyma watershed. Site 2 was a series of first-order streams draining a yedoma exposure known as Duvanni Yar (DY), which was sampled in both years 2010 and 2011 and hereafter referred to in the text as DY-A and DY-B respectively. This exposure has previously been extensively studied and is known to contain soil deposits ranging from between 13,000 – 45,000 years in age (Vasil'chuk & Vasil'chuk 1997). The small streams are fed by ice-wedge meltwaters draining a cut permafrost bank and thus represent an excellent yedoma permafrost endmember with waters containing OC aged between 20,000 – 30,000 years bp (Vonk *et al.* 2013a). The remaining two sites (site 3 - FPS & site 4 - Y4) were small tributary streams draining into the Pantaleikha River; a major tributary of the Kolyma River (Fig 1). FPS is a small lowland stream draining predominantly floodplain sediments, whereas Y4 represents an upland stream draining largely yedoma soils (Site 3 & 4; Fig 1). In addition to these four endmember sites, samples were also collected from 42 different waters during 66 different sampling events across the lower basin region (streams, rivers & lakes) over 2011 and 2012, to assess ecosystem scale patterns in EEAs and respiration rates (Fig 1; Table S1).

Samples for DOC and nutrient analyses were filtered immediately after collection (precombusted 450 °C 0.7 µm, GF/F), and then acidified with HCl (pH < 2). Filtered, unacidified waters were collected for optical analyses and biological oxygen demand (BOD) assays. Enzyme activity assays were also measured upon GF/F filtered waters during the laboratory-based and incubation experiments. EEAs in survey samples were measured on unfiltered surface waters,

reducing preparation and assay measurement times. Optical analyses were always conducted within 48 hrs of collection. Enzyme and BOD assays were started immediately after filtration. All samples were stored cool (4 °C) and in the dark until processing began. All measurements, except nutrient analyses, were conducted on-site at the North-East Science Station, Siberia.

Dissolved organic carbon, total nitrogen and optical analyses

Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured on a Shimadzu TOC-V analyzer equipped with an inline chemiluminescence detection unit (TNM-1) using established protocols (Mann *et al.* 2012). Chromophoric dissolved organic matter (CDOM) measurements were made using a dual-beam spectrophotometer (Shimadzu UV-1800) and referenced against blank nanopure water spectra. UV-visible scans between 200 – 800 nm were collected at room temperature in 10 mm quartz cells. Specific UV absorbance ($SUVA_{254}$) was calculated as the decadal absorption coefficient at 254 nm divided by the DOC concentration (L mgC^{-1} m^{-1}). $SUVA_{254}$ has been shown to be a sensitive indicator of DOM aromaticity (Weishaar *et al.* 2003; Spencer *et al.* 2012).

Extracellular enzyme assays (EEA's) and biological oxygen demand

The potential enzyme activities of up to six extracellular enzymes, involved in catalyzing the cleavage of a range of organic matter compounds, were investigated (Table 2). Assays were conducted on freshly collected samples using *p*-nitrophenol (pnp) linked substrates (Sigma Aldrich) and measured on a 96-well plate reader (Biotek Powerwave XS2). Phenol oxidase was assayed using L-3,4-dihydroxyphenylalanine (L-DOPA) and leucine aminopeptidase using L-leucine p-nitroanilide (Table 2). Substrate concentrations of 5 mM were used for all enzymes,

except LAP and NAG due to their solubility (2 mM). At least four replicate wells were averaged across each sample and triplicate sub-samples used for each sample treatment. The variation across wells was typically < 5% (max. 10%) and linear activity kinetics were assured by measurements at 4+ timepoints per assay. Each well received 200 µL of sample waters with 50 µL of substrate and enzyme. Substrate and sample blank controls were always run on the same plate and were subtracted from sample values. Plates were incubated at 20 ºC and measured periodically over 1-8 hours. Potential activities were expressed as the rate of pnp accumulation per hour (nmol L^{-1} h^{-1}). Protocols for pnp-linked assays are available from the Enzymes in the Environment RCN webpage (enzymes.nrel.colostate.edu). We acknowledge that by using these fixed substrate concentrations we did not measure in-situ activity rates per se, instead we assessed potential activity rates that are comparable across sites or treatments and influenced only by DOM composition, which was our stated aim. We avoided directly comparing EEAs measured in experimental waters with those measured in survey waters due to the differences in sample preparation.

Sensitive oxygen demand assays were conducted on filtered water samples over 5 day incubation periods (BOD_{5d}). Total oxygen loss over the 5 d period was used as an indicator of relative DOM bioavailability. Oxygen loss was converted into estimates of stream and river respiration, assuming a respiratory quotient of 1. Oxygen concentrations were measured using self-stirring BOD probes (YSI, ProOBOD, ±0.1 mg/L) in triplicate glass BOD bottles (300 mL). Assays were run at room temperature (~ 20 ºC) and kept dark in-between measurements.

DOM composition and inorganic nutrient experiments

To examine the effect of DOM quality and inorganic nutrient concentrations upon EEA expression in our four endmember waters, the response of a suite of enzymes was followed after amendments to examine dissolved phenolic content and additions of inorganic nitrogen (N), phosphorus (P) or combined N & P. The dissolved phenolic content of waters were reduced by selectively removing them using polyvinylpyrrolidone (PVP; ≥ 95%, molecular weight 40 KD, Fisher Scientific) at a final concentration of 0.01 g/mL (Carpenter *et al.* 1976; Freeman *et al.* 2001a). PVP-phenolic complexes were not extracted, but blanks containing PVP alone showed no effect upon enzyme activity rates. Potassium phosphate and potassium nitrate were used as a source of P and N respectively, and added to yield a final concentration approximately ten times mean natural concentrations in the Kolyma River Basin as determined using data from 2010 (http://www.thepolarisproject.org/data/). Waters were well mixed and split into two treatments, with one left unamended and the other treated with PVP to reduce dissolved phenolic content. Sub-samples of both unamended and PVP waters were then amended by additions of inorganic N, P, N & P, or none to act as a control. All treatment waters were incubated in the dark, at a fixed temperature of 20 °C, for 24 hours before EEA measurements. Using this approach, we were able to assess the independent and combined effects of phenolic content and inorganic nutrient content upon EEA expression.

To investigate the direct response of dissolved phenolics upon freshwater respiration rates, we conducted a separate BOD_{5d} assay on Kolyma River waters (Site 1; Fig 1) containing different dissolved phenolic concentrations. Respiration rates were measured in three independent treatments; an unamended control and two identical waters amended with different final PVP concentrations (treatment A: 0.010 g/mL & treatment B: 0.005 g/mL).

Incubation experiments

To assess OC loss and EEA expression during degradation of Kolyma waters mixed with ancient OC (DY-A waters) we conducted a controlled incubation experiment. Waters were collected from the Kolyma River mainstem (Site 5; Fig 1) and four small tributary streams draining the DY exposure (Site 2; Fig 1; Vonk *et al.* 2013a). Samples were filtered (precombusted 450 °C 0.7 µm, GF/F), and a composite sample (DY-A) produced by mixing the filtrate of the four smaller streams. The composite DY-A water contained a Δ^{14}C-DOC signature of -933 representing an OC pool aged 21,700 ±85 y before present (Vonk *et al.* 2013a). Studies of major Arctic Rivers including the Kolyma River, by contrast, report generally modern Δ^{14}C-DOC ages (Neff *et al.* 2006; Raymond *et al.* 2007). Three treatments containing 0%, 1.0% and 10% dilutions of the DY-A composite with Kolyma River water were then prepared and incubated in triplicate in BOD glass bottles (20 °C in the dark).

Optical parameters, EEA's and nutrient concentrations (NH_4^+& PO_4^-) were measured at the start, and after 5-days of incubation. Oxygen concentrations were measured frequently over the incubation period and continued for a total of 9 days. Ammonium concentrations were measured immediately on-site using the fluorometric method developed by Holmes *et al.* (1999) and modified by Taylor *et al*. (2007). Phosphate concentrations were measured immediately using the molybdate colorimetric method (U.S. Environmental Protection Agency 1984). No flocculation was observed in optical or visual inspections in any of the treatments over the incubation period.

Statistical analyses

Paired sample t-tests were used to assess pre- and post-treatment differences in control waters with and without PVP treatments, and EEAs pre- and post-incubation. Effects of inorganic nutrient additions on EEAs were assessed using one-way analysis of variance (ANOVA) to test for significant differences among treatments versus the control within each experiment. Significant responses were investigated using pairwise comparisons between group means after Bonferroni correction (p <0.05). Data were normalized using log transformations where non-normal distributions were observed before analyses. Linear regressions were used to assess correlations between parameters and the coefficient of determination (R^2) calculated. All statistical analyses were conducted using SPSS 20 (IBM, NY).

Results

Endmember and incubation water properties

The initial geochemical properties of each of the four endmember waters studied varied considerably (Table 1). DOC concentrations were lowest in Kolyma mainstem waters (3.8 mgC L^{-1}), intermediate in FPS and Y4 waters (10.7, 23.3 mgC L^{-1}, respectively), and highest in the DY-B waters draining yedoma deposits (67.1 mgC L^{-1}) (Table 1). TDN and inorganic nitrogen concentrations also ranged dramatically between samples, DY-B waters containing high concentrations of NO_3^- and NH_4^+ causing a low DOC:TDN ratio of 6.1 (Table 1). Highest DOC:TDN concentrations, indicating low inorganic N concentrations were observed in Y4 (28.8) and FPS waters (15.4). PO_4^-concentrations were generally low (2.5 – 8.7 µgP L^{-1}) in all of the sample waters (Table 1). DOM composition also varied between endmember waters (Table 1). DOM aromaticity and molecular weight, inferred from $SUVA_{254}$ values, was similar across

sites except for DY-B waters, which contained very low aromatic content waters (low $SUVA_{254}$; Table 1).

Initial incubation waters displayed a gradient in DOC content, nutrient concentration and DOM quality (Table 1). DOC concentrations increased from 5.3 mgC L^{-1} in Kolyma River waters to 6.6 and 19.6 mgC L^{-1} with 1% and 10% DY-A additions, respectively. Additions of DY-A waters resulted in an increase in inorganic N concentrations and associated reduction in DOC:TDN (Table 1). PO_4^- concentrations increased with DY-A additions, increasing from 2.7 µgP L^{-1} in Kolyma waters, to 5.0 µgP L^{-1} in the 10% DY-A treatment (Table 1). Successive DY-A water additions also led to a reduction in DOM molecular weight and total aromaticity (lower $SUVA_{254}$; Table 1).

Effect of phenolic removal in endmember waters on EEAs & respiration

After experimental manipulations to reduce phenolic content via PVP amendments, mean potential phenol oxidase (POx) activities declined significantly in each of the endmember waters (52 – 62% losses) except the Kolyma River (Table 3; Table S2). Kolyma River waters, by contrast, demonstrated a significant rise in POx expression with phenolic reduction resulting in a 210 % increase in activities (Table 3). Hydrolase activities displayed markedly different responses to phenolic removal in each of the endmember waters. Largest responses to phenolic removal were observed in APase activities, with increases of 28 and 48-fold in Kolyma River and FPS samples, respectively (Table 3). No response was observed in APase activity rates in DY-B waters after phenolic removal ($P = 0.24$). LAP activities approximately doubled in Kolyma waters after PVP amendments, but resulted in small reductions (50 % loss) or no significant response at each of the other three sites. Furthermore, substantial stimulation of β-

gluc activities (> 25-fold) was observed in Kolyma River waters with phenolic removal (Table 3).

The removal of dissolved phenolics led to dramatic increases in the observed mean respiration rate of Kolyma River mainstem waters (Fig 2). Unamended Kolyma mainstem waters displayed low respiration rates throughout the entire incubation period (0.78 $gC\text{-}O_2\ L^{-1}\ g^{-1}$ DOC after 5 d; Fig 2). PVP treated Kolyma waters demonstrated consistently higher respiration rates over 7-8 fold (treatment B) and 13-16 fold (treatment A) greater than measured in untreated waters (Fig 2). Significantly higher respiration rates persisted over the entire 5 d incubation period in both PVP treatments.

Effect of inorganic nutrient supply upon enzyme activities

Inorganic nutrient additions generally had little effect upon EEAs relative to control waters in unamended endmember waters ('Unamended' Table 4). Activities of APase were significantly lower in DY-B waters after combined N & P additions. The activity of LAP increased significantly, but relatively modestly (~ 1.5 fold), with combined N and P supplements in the Kolyma River sample (Table 4).

In contrast to unamended samples, waters containing lower concentrations of dissolved phenolic compounds ('PVP treated' Table 4) showed strong responses in EEAs to inorganic nutrient additions. Enzymes associated with N (LAP) and P (APase) cycling were most responsive to nutrient amendments. LAP activity was consistently higher in all endmember waters after combined N and P additions and in all, except for DY-B, in response to P additions

alone. The activity of LAP decreased significantly, with only N fertilization in the DY-B sample (Table 4).

The effect of inorganic nutrients on APase activities was often considerable, but differed between the four endmember waters. APase activities in Kolyma River water increased by ~ 3.5 fold with P and combined N & P fertilization. Similar patterns were observed in Y4 waters, alongside additional increases of APase activities with N amendments alone (~ 2.5 fold). APase activities also increased in FPS waters with N additions alone, but declined significantly with P and combined additions (Table 4). DY-B waters showed no significant change in APase activities in response to nutrient additions after PVP amendment.

Activities of β-gluc significantly increased in response to both P and combined additions in Y4 and FPS waters post PVP amendment. In contrast, β-gluc activities were suppressed in the Kolyma River sample with P amendments (P, N+P). Activities of POx also varied in response to both P and N & P amendments, causing a decrease in activities in the Kolyma River sample and significant increases in FPS waters after PVP treatment (Table 4).

Incubation experiments of ancient organic carbon

Respiration rates and EEAs measured within the 0%, 1.0% and 10% DY-A permafrost treatments differed dramatically over the incubation period (Fig 3; Table S2). Respiration rates were higher in both treatments amended with DY-A waters (1.0 & 10%), relative to Kolyma River mainstem waters, after only one-day of incubation (Fig 3a; $P < 0.01$). Normalized respiration rates ($gC\text{-}O_2\ L^{-1}\ g^{-1}$ DOC), accounting for differences in initial DOC concentrations, were also consistently higher in DY-A supplemented waters than Kolyma waters after 76 h

onwards (P <0.01; Fig 4). However, no significant differences in respiratory losses were observed between the 1% and 10% DY-A treatments when C-normalized over the entire incubation period (Fig 4).

Enzyme activity potentials of LAP and APase measured after 5 d of incubation, significantly increased across all treatments relative to pre-incubation rates ($P < 0.05$; Fig 3b, c, d). LAP and APase activities increased approximately 3 to 4 fold over the 5 d period in both the Kolyma River and 1% DY-A treatments. The 10% DY-A sample, by contrast, supported far higher enzyme synthesis with LAP activities increasing over 6 fold and APase activities by 15 fold during the 5 d period. Enzyme activities associated with glucose and chitin degradation (α-gluc, β-gluc & NAGase) were either undetectable or showed no significant increase in Kolyma River waters. Kolyma River water containing 1.0% DY-A, by contrast, showed significant differences in α-gluc activities with increases of 16 fold over the incubation (Fig 3c). Kolyma River with 10% DY-A waters also displayed significant differences in α-gluc activities (4 fold increase), as well as significant increases in the activity potentials of β-gluc and NAGase (18 & 4 fold increases respectively, Fig 3d). In contrast to the hydrolase enzymes, POx activities declined consistently and significantly over the incubation period in all of the treatments ($P < 0.05$). Final NH_4^+ concentrations, after the 5 day incubations were very similar across each treatment (9.7, 8.7 and 10 μg L^{-1} respectively). Final PO_4^- concentrations were extremely low in each of the treatments ranging from 2 μg L^{-1} (1% treatment) to below the detection limit (~ 0.5 μg L^{-1}) in Kolyma and 10% samples.

Ecosystem scale patterns in DOM bioavailability

Enzyme activities varied greatly across diverse waters (streams, rivers and lakes) within the lower Kolyma Basin watershed (Table S1). Activities of POx, APase and β-gluc in surface waters were all significantly correlated to respiration rates as determined by biological oxygen demand assays, but only POx explained a large proportion of the variance (R^2 = 0.41, 0.15, 0.06, P <0.05, n = 65 respectively) (Fig 5a). DOC and TDN concentrations, as well as DOM compositional indices ($SUVA_{254}$) also correlated with respiration rates but showed low explanatory power in explaining variance in rates (R^2 = 0.21, 0.16, 0.10, P <0.05, n = 63 respectively). Inorganic PO_4^- concentrations were highly correlated to respiration rates (R^2 = 0.53, P <0.01, n = 65; Fig 5b). Nitrate and ammonium concentrations showed no significant relationship with respiration (P > 0.05; Table S1).

Discussion

The bioavailability of permafrost derived OC will prove critical in determining the fate and response of northern high-latitude ecosystems to climate induced hydrologic and biogeochemical change. As important mediators of nutrient cycling and decomposition pathways, extracellular enzymes can provide useful information on the constraints limiting the continued breakdown of OC in aquatic ecosystems.

The significant increase in hydrolase enzyme activities we report across diverse waters after reductions in dissolved phenolic content highlights the inhibitory effect polyphenolic compounds can play upon enzyme expression and thus OC decomposition pathways (Fig 6, phenolic inhibition). Additionally, the marked and dynamic response of hydrolase enzyme

activities to inorganic nutrient supply, after reductions in the dissolved phenolic content of waters, demonstrates that the microbial community responds to variability in exogenous nutrient availability only after initial composition constraints have been removed (Fig 6, nutrient availability). This may have been caused by a shift in the microbial community or an upregulation of enzyme production by the initial microbial population. The immobilization of extracellular enzyme activities has previously been reported in soils as a result of mineral stabilization and the formation of polyphenolic-enzyme complexes (Ladd 1985). Furthermore, a phenolic inhibition (or "latch") mechanism has also been proposed to be critical in preserving carbon stocks in low oxygen terrestrial peatland systems (Freeman *et al.* 2001a; Fenner & Freeman 2011), suggesting that this preservation mechanism could be globally pervasive. A number of natural pathways may act to reverse the formation of polyphenolic-enzyme complexes in freshwaters. These include the auto-digestion of phenolic compounds via alternate enzymes (e.g. POx), dilution processes or ultraviolet photolytic decomposition (Boavida & Wetzel 1998; Spencer *et al*., 2009; Mann *et al*., 2012; Fig 6).

Extracellular POx enzymes have been shown to be crucial in enabling the degradation of lignin and other polyphenols, acting as one of the few enzymes capable of removing inhibitory phenolic compounds (McLatchey & Reddy 1998). Previous studies in temperate soils have reported that POx activities were limited by oxygen availability, and suggested that soil OC degradation rates were therefore limited by low environmental oxygen concentrations (Freeman *et al.* 2001a; Fenner & Freeman 2011). Ecosystem scale relationships observed here between freshwater POx activities and respiration rates in waters across the Kolyma Basin (Fig 5a), suggest that the activity of POx may play a crucial role in regulating the biological reactivity of

DOM across aquatic ecosystem types. Relationships between DOM phenolic content and bioavailability have also been shown across a permafrost gradient in the Yenisei River (Siberia), with lower phenolic concentrations and higher bioavailability in tributaries with greater active layer depths (Kawahigashi *et al.* 2004). If initial POx activities are largely a function of soil enzyme concentrations, changes in vegetation type, dominant flow paths or soil enzyme production may dramatically influence aquatic OC bioavailability. POx activities may also be expected to increase in warmer environments; experiments in peatland soils have demonstrated increased POx activities in warmer soils accompanying increased DOC release with higher phenolic compound content (Freeman et al. 2001b). Recent findings from Arctic inland surface waters report >40 % increases in the susceptibility of dissolved ancient OC to microbial degradation when exposed to UV light, as compared to dark controls (Cory *et al.* 2013). These results provide evidence that photochemical alteration can result in increased bioavailability, likely as a result of phenolic removal (Spencer *et al.*, 2009; Stubbins *et al.*, 2010) and easing of biochemical constraints upon degradation (e.g. extracellular enzyme production).

After compositional constraints are removed, nutrient availability became the dominant factor determining continued aquatic OC losses. Across our study sites, APase activities were closely associated with the availability of phosphate (after phenolic removal; Table 4; Fig 5b). APase activities generally increased when inorganic N availability increased and were suppressed when inorganic PO_4^- was in ample supply. These results agree with experiments in soils (Clarholm 1993), and suggest APase activities are a sensitive indicator of the P status of freshwaters across the Kolyma watershed. Contrastingly, LAP and β-gluc activities responded little to N and P addition alone, but increased when both N and P were abundant. These enzymes

were therefore likely produced in response to higher carbon processing rates, or a reduction in carbon availability. The rapid synthesis of APase enzymes in our endmember waters and observed during incubations of ancient OC, in association with the strong relationships between PO_4^- concentration and respiration rates (Table 3; Fig 5b) indicate that phosphate availability plays a dominant role in influencing long term OC bioavailability rates. The release of organic P via APase activities will therefore become the limiting factor once exogenous inorganic P has been depleted (Fig 6, nutrient availability), yet APase activities will continue to be depressed and unresponsive until phenolic constraints have been removed (Tables 3, 4, Fig 6, phenolic inhibition).

A major concern of warming air temperatures across Arctic regions is that it may lead to enhanced permafrost thaw, resulting in the transfer of previously sequestered ancient OC to the atmosphere via respiration in soils, or via increased mobilization to inland waters and metabolism in the aquatic environment. Yedoma derived OC (DY) displayed higher respiration rates per unit DOC than OC containing largely modern carbon, indicating that ancient OC was inherently more bioavailable over short timescales. These results are in good agreement with a recent study that traced dissolved OC loss, rather than O_2 removal, at similar sites (Vonk *et al.* 2013a). These findings suggest that ancient permafrost carbon inputs to the stream network may be extensively processed within Arctic stream and river networks, resulting in a flux of aged CO_2 to the atmosphere, where it may serve to accelerate future climate warming. Despite the largely modern ^{14}C-DOC signatures observed in many northern river systems during non-baseflow conditions (e.g. Evans *et al.* 2004; Raymond *et al.* 2007), selective in stream degradation of DOC may therefore result in the loss of an ancient DOC fraction that is masked by larger continued modern DOC inputs.

The limited response of DY waters to phenolic removal and the high initial enzyme activities we report suggest that yedoma influenced waters contain lower initial dissolved polyphenolic concentrations, or contain such elevated initial enzyme concentrations that they were unable to be fully complexed by phenolics within the DOM pool (Wetzel 1992). Alternatively, DY waters may contain high concentrations of intrinsic bacteria, limiting the need for an initial growth phase before EEA production (Rivkina *et al.* 1998). Successive DY additions to Kolyma mainstem waters, mimicking changing contributions that may be expected with increasing permafrost thaw, resulted in a shift in the overall OC composition toward less aromatic, low molecular weight DOM, as inferred from lower $SUVA_{254}$ (Table 1). These reductions in DOM aromaticity and molecular weight led to the observed increase in respiration rates (Fig 3 & 4).

Recent studies in the Yukon River Basin have highlighted that an increase in the contribution of organics from deeper soil horizons and groundwaters in permafrost regions is expected to result in the export of OC with lower aromaticity ($SUVA_{254}$), lower lignin carbon-normalized yields and with higher contributions of hydrophilic relative to hydrophobic acids (Striegl *et al.* 2007; Spencer *et al.*, 2008; O'Donnell *et al.* 2010; 2012). Ice wedge complexes, constituting up to 50% of the total volume of yedoma permafrost, have also been shown to contain low molecular weight, low aromaticity DOM which is readily available to microbial degradation (Vonk *et al.* 2013b). Furthermore, a linear relationship between Δ^{14}C-DOC and $SUVA_{254}$ values has also been reported in the Kolyma Basin, suggesting older OC pools typically display lower DOM aromaticity (Neff *et al.* 2006). In the context of the results presented here it seems apparent that yedoma derived OC is highly biologically labile in stream networks in comparison to modern

terrestrial OC inputs, primarily as a result of its composition (Fig 6, phenolic inhibition). As continued warming in the Arctic will lead to more extensive permafrost thaw it seems likely to result in a shift toward more readily available OC for metabolism, leading to increased mineralization of ancient organic carbon in downstream receiving aquatic ecosystems. Under this scenario it is apparent that the aquatic microbial processing of permafrost derived OC may play an important role in permafrost carbon and climate feedbacks with respect to global climate change.

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Supporting Information Legends

Supplementary Table 1. EEAs and sample characteristics for surface waters measured throughout the Lower Kolyma Basin (Fig 1).

Supplementary Table 2. EEAs for DOM composition and inorganic nutrient addition experiments (Table 3 & 4) and ancient organic carbon incubations (Fig 3).

Table 1. Initial chemical and optical measurements of four endmember waters used in PVP and nutrient experiments (1 -4) and Kolyma incubation waters mixed with permafrost waters (5). Site locations are displayed on Figure 1. Site DY was sampled on two different occasions in 2010 (DY-A) and 2011 (DY-B). *NH_4^+ and PO_4^- concentrations were measured using OPA and molybdate colorimetric methods respectively.

Exp.	Site no. & description	DOC (mgC L^{-1})	DOC:TDN (molar)	NO_3^- (µgN L^{-1})	NH_4^+ (µgN L^{-1})	PO_4^- (µgP L^{-1})	$SUVA_{254}$ (L mgC^{-1} m^{-1})
Endmem	(1) Kolyma River mainstem at Cherskiy	3.8	10.9	9.9	27.7	2.9	3.2

ber waters (PVP & nutrient)	(2) Duvanni Yar (DY-B) (permafrost waters)	67.1	6.1	2462	1007	3.1	1.1
	(3) Floodplain stream (FPS)	10.7	15.4	2.0	1.5	2.5	3.1
	(4) Yedoma stream (Y4)	23.3	28.8	1.8	0.4	8.7	3.0
Incubation waters*	(5) Kolyma River upstream of Duvanni Yar	5.3	22.9	-	1.2	2.7	2.2
	(Mixture of 5 & DY-A) Kolyma + 1% DY-A	6.6	19.0	-	2.0	3.6	2.2
	(Mixture of 5 & DY-A) Kolyma + 10% DY-A	19.6	16.9	-	51.4	5.0	1.6

Table 2. Enzyme assays measured, their function and the substrates used. α-Gluc and NAGase activities were only measured during laboratory incubation experiments.

Enzyme	Function	Substrate
Phenol Oxidase (POx)	Degradation of lignin	L-dihydroxyphenylalanine
Alkaline Phosphatase (APase)	Releases ester-bound phosphate	4-nitrophenyl phosphate disodium
Leucine aminopeptidase (LAP)	Degrades protein into amino acids	L-leucine p-nitroanilide
β-glucosidase (β-gluc)	Release glucose from cellulose decomposition	pnp- β -D- glucopyranoside
α-Glucosidase (α-gluc)	Releases glucose from soluble saccharides	pnp-α-D-glucopyranoside
Glucosaminidase (NAGase)	Degradation of chitin	pnp-N-acetyl glucosaminidase

Table 3. Effect of phenolic removal on freshwater enzyme activities (% change). Arrows represent direction of significant % change in EEAs ($P < 0.05$). *NS* represents non-significant response.

	POx	APase	LAP	β-gluc
Kolyma	↑ **210**	↑ **2800**	↑ **120**	↑ **2400**
Duvanni Yar	↓ **52**	*NS*	*NS*	↑ **77**
FPS	↓ **62**	↑ **4800**	↓ **58**	↓ **100**
Y4	↓ **55**	↑ **370**	*NS*	*NS*

Table 4. Effect of inorganic nitrogen (N), phosphorus (P) and combined additions (N+P) upon potential enzyme activities in unamended (no PVP) and treated waters (PVP added). Arrow represents direction of significant changes (P = * < 0.05, ** < 0.01 after Bonferroni correction). *NS* equals non-significant response ($P > 0.05$).

Enzyme	Site	Unamended			PVP treated		
		N	P	N&P	N	P	N&P
POx	Kolyma	*NS*	*NS*	*NS*	*NS*	↓**	↓**
	Duvanni Yar	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	FPS	*NS*	*NS*	*NS*	*NS*	↑*	↑**
	Y4	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
APase	Kolyma	*NS*	*NS*	*NS*	*NS*	↑**	↑**
	Duvanni Yar	*NS*	*NS*	↓**	*NS*	*NS*	*NS*
	FPS	*NS*	*NS*	*NS*	↑**	↓*	↓**
	Y4	*NS*	*NS*	*NS*	↑**	↑*	↑*
LAP	Kolyma	*NS*	*NS*	↑**	*NS*	↑*	↑**
	Duvanni Yar	*NS*	*NS*	*NS*	↓**	*NS*	↑**
	FPS	*NS*	*NS*	*NS*	*NS*	↑**	↑**
	Y4	*NS*	*NS*	*NS*	*NS*	↑*	↑*
β-gluc	Kolyma	*NS*	*NS*	*NS*	*NS*	↓**	↓**
	Duvanni Yar	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	FPS	*NS*	*NS*	*NS*	*NS*	↑**	↑**
	Y4	*NS*	*NS*	*NS*	*NS*	↑*	↑*

Figure Legends

Figure 1. Study site and sampling locations. Main study sites are represented by red circles with site numbers and names as Table 1. Yellow dots represent sampling locations for waters used to assess ecosystem scale patterns in EEAs (Table S1).

Figure 2. Cumulative microbial respiration from identical Kolyma waters amended to reduce dissolved phenolic content. Unamended waters (black diamond), PVP treatments A = 0.010 g/mL (open triangle) & B = 0.005 g/mL (open square). Respiration values were normalized to

DOC concentration to account for differences in sample OC concentrations. Values are means ± 1 standard error (n = 3). High specific respiration rates per unit DOC indicate a greater proportion of labile OC.

Figure 3. a) Microbial respiration in Kolyma, 1% and 10% DY-A treatments in laboratory incubations over 9 d. Values are means ± 1 standard error (n = 3). EEAs measured at time 0 and 5d (arrow). Enzyme activity potentials measured pre- and post 5 d incubation in b) Kolyma waters c) Kolyma and 1% DY-A waters, and d) Kolyma and 10% DY-A waters. Note different y-axis scales. Significant differences between pre- and post-incubation activities are indicated by * ($P < 0.05$).

Figure 4. Cumulative microbial respiration from Kolyma, 1% DY-A and 10% DY-A waters during incubations. Respiration values were normalized to DOC concentration to account for differences in initial sample OC concentrations. Values are means ± 1 standard error (n = 3).

Figure 5. Ecosystem scale patterns in microbial respiration measured over 2011 and 2012 plotted against a) phenol oxidase (POx) activity ($R^2 = 0.41$, $P < 0.05$, $n = 65$) and, b) inorganic phosphate (PO_4^-) concentrations ($R^2 = 0.53$, $P < 0.01$, $n = 65$).

Figure 6. Conceptual diagram of key controls upon metabolism of Arctic river organic matter (red circles). 'Phenolic inhibition' refers to the compositional constraints caused by inhibitory phenolic compounds. Inhibition from phenolics can be removed via enzymatic processes (stars) or photolysis (dotted line). Secondary constraints upon metabolism relate to limited 'nutrient availability' after exogenous nutrient supply has been exhausted, restricting continued organic carbon degradation. Hydrolase enzyme activity can release organic nutrients promoting continued degradation (stars) but only after initial phenolic constraints have been removed. Inset photographs courtesy of Chris Linder (http://www.chrislinder.com/).