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Effects of temperature on enzyme activity of aquatic litter-associated fungi

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Abstract

The decomposition of leaf litter in aquatic ecosystems is carried out mostly by microorganisms, including bacteria and fungi. Fungi, known as aquatic hyphomycetes, dominate microbial communities in the decomposition of leaf litter. These fungi produce extracellular enzymes that aid in the sequestration of carbon and nutrients and lead to the breakdown of complex plant polymers. We evaluated the effects of temperature on extracellular enzyme activity within the framework of the Metabolic Theory of Ecology (MTE). The activity of β-1,4-glucosidase and β-1,4-xylosidase was estimated fluorometrically using artificial substrate analogs. Phenol oxidase activity was estimated spectrophotometrically from oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine). We found greater temperature sensitivity of oxidative enzymes (phenol oxidase) involved in degradation of recalcitrant substrates compared to hydrolytic enzymes (β-glucosidase and β-xylosidase). In addition, we found that the activity of microbial enzymes involved in carbon sequestration does not follow simple monotonous response across experimental temperatures (4-20°C) predicted by the MTE. Instead, we observed greater temperature sensitivity (higher apparent activation energy) of hydrolytic enzymes at colder temperatures. These findings may have important implications for stream ecosystems under climate change scenarios since both peak leaf litter availability and microbial activity occur during the coldest seasons in autumn-winter.

Introduction

  The functioning of forested headwater stream ecosystems is affected by a multitude of factors including temperature, biodiversity, community structure, and nutrient abundance (Ferreira et al. 2012). In particular, temperature affects the activity of microorganisms in these ecosystems, where they form the foundation of trophic webs, drive global carbon (C) and nutrient cycles, and affect composition of the atmosphere (Sinsabaugh and Follstad Shah 2012). Headwater forest streams are capable of significant terrestrial C sequestration, processing, and transport, which demonstrates their important role in the global C cycle (Fisher and Likens 1973; Cole et al. 2007). The catabolism of organic matter in aquatic ecosystems by means of extracellular enzymes is considered a rate controlling step in the global C cycle (Cole et al. 2007; Sinsabaugh and Follstad Shah 2012). For this reason, several studies have focused on the effects of potential changes in climate on aquatic ecosystems (Davidson and Jannsens 2006; Ferreira and Chauvet 2011; Sinsabaugh and Follstad Shah 2012). Predictions by climate researchers suggest a 1-4°C increase in global average surface temperature by the end of the century (IPCC 2014). This may have important implications for stream ecosystem processes under climate change since increases in the rate of microbially mediated litter decomposition might lead to food shortage for higher trophic levels in aquatic ecosystems (Ferreira and Chauvet 2011, Ferreira et al. 2012).

  The photosynthesis and, hence, primary production in headwater forest streams are limited by light availability due to shading. The organisms found in these systems are mainly heterotrophic, meaning that they rely on organic sources of carbon (Brown et al. 2004; Gessner et al. 2007). As a result of natural senescence and abscission, forest streams receive large allochthonous inputs of organic matter from the riparian zone, which include leaves and twigs of

riparian trees and shrubs (Fisher and Likens 1973, Gulis 2001). These inputs fuel microbial metabolism of litter-associated decomposers taking place in stream ecosystems. Thus, litter-associated microorganisms are responsible for the mediation of energy and nutrient transfer to higher trophic levels in these ecosystems (Gessner et al. 2007).

Decomposition of organic matter in freshwater streams is carried out mainly by fungi and bacteria (Gulis et al. 2019). Increasing evidence suggests fungi, known as aquatic hyphomycetes, dominate microbial communities in decomposing plant litter under aerobic conditions (Gulis and Suberkropp 2003; Gessner et al. 2007; Gulis and Bärlocher 2017; Gulis et al. 2019). The decomposition of this organic matter is at the base of aquatic food webs, where it fuels aerobic metabolism, while litter C is incorporated into fungal biomass and lost as CO_2 via microbial respiration (Gessner et al. 2003; Ferreira et al. 2012). The process of fungal decomposition enhances litter palatability for aquatic invertebrate shredders generating fine particulate organic matter (FPOM) to be used downstream by filter-feeders and collectors (Ferreira et al. 2012; Gulis and Bärlocher 2017). Aquatic hyphomycetes are therefore important intermediates between decomposing organic matter, secondary production (accumulation of microbial biomass), and higher trophic levels in forest stream ecosystems.

In an effort to quantitatively describe how ecological processes may respond to changes in temperature, James H. Brown formulated the Metabolic Theory of Ecology (MTE) (Brown et al. 2004). MTE is a quantitative theory predicting how metabolic rates will vary with body size and temperature within normal biological temperature range (0-40°C) (Brown et al. 2004). Brown establishes that metabolism is an exclusively biological process, which must abide by the laws of mass, energy and thermodynamics (Brown et al. 2004). Brown reasons that metabolic rate is the fundamental biological rate, as it describes the rates of energy uptake, transformation,

and allocation across living organisms (Brown et al. 2004). In general, metabolic reactions of litter-associated decomposers involve acquisition and incorporation of C and other nutrients (e.g. N and P) into microbial biomass (secondary production) and release of CO_2 as a result of aerobic respiration (Gulis and Bärlocher 2017).

According to the MTE, rates of individual biochemical reactions, metabolic rates, and nearly all other rates of biological activity scale exponentially with temperature (Brown et al. 2004). In litter-associated aquatic microorganisms, the metabolic rate is related to the rate of respiration, since they obtain energy through oxidation of organic compounds. This relationship can be described in conjunction with the Van't Hoff-Arrhenius expression:

$$R \sim e^{-E/kT}$$

where R is respiration, E is activation energy, k is the Boltzmann constant, and T is temperature (Arrhenius 1889; Brown et al. 2004; Sierra 2012). Although temperature sensitivity may vary by metabolic process, the temperature dependence of the respiratory complex has been represented by an apparent activation energy (E_a) of ~0.65 eV based on multiple experimental estimates (Gillooly et al. 2001; Yvon-Durocher et al. 2012). The apparent activation energy of a metabolic process, which is determined by the structure and function of involved enzymes, can also be used to characterize kinetics of microbial enzymes involved in C sequestration (Sierra 2012; Sinsabaugh and Follstad Shah 2012). However, experiments are needed to better understand how the extracellular enzymatic activity of aquatic litter-associated microorganisms is affected by changes in temperature and if the responses follow simple MTE prediction of monotonous (exponential) increase across wide temperature intervals.

Communities of aquatic hyphomycetes in streams are well equipped with lignocellulolytic enzymes used to break down major plant polymers found in detritus (Gulis et al.

2019). The degradation of plant polymers by fungi may also make simpler carbohydrates available for bacteria with enzymatic deficiencies (Gulis and Suberkropp 2003; Romani et al. 2006). Aquatic hyphomycetes secrete enzymes that break down hemicellulose, cellulose, and lignin-like or phenolic plant polymers (Hendel and Marxsen 2005; Hendel et al. 2005). However, the activity of ligninolytic enzymes used in the degradation of lignin can be limited but does occur in some species and not others (Gulis et al. 2019). Decomposition of plant polymers driven by microbial extracellular enzymes is important in contributing to the flow of C towards fungal respiration and secondary production, and eventually to stream detrital food webs (Gulis and Bärlocher 2017).

β-1,4-xylosidase catalyzes the hydrolysis of β-1,4-linkages found in hemicellulose xylooligosaccharides, a ubiquitous component of plant cell walls (Sinsabaugh and Follstad Shah 2012). β-1,4-glucosidase catalyzes the terminal reactions in the hydrolysis of cellulose β-1,4-linkages found in the cell wall and fibers of decomposing plant litter (Hendel and Marxsen 2005; Sinsabaugh and Follstad Shah 2012). Fluorogenic substrate analogs can be used for precise quantification of β-1,4-xylosidase and β-1,4-glucosidase activity (Hoppe 1983) in decomposing leaf litter samples. These methods, having been used in practice for decades, have shown to be reliable in determining the activity of hydrolytic enzymes present in biological samples (Hoppe 1983; Hendel and Marxsen 2005; Sinsabaugh and Follstad Shah 2012).

One of the most abundant compounds found in nature, lignin, is a principal constituent of vascular plants (Hendel et al. 2005). Due to its association with cellulose fibers in plants, lignin is significant in contributing to the flow of C in detrital food webs of aquatic ecosystems (Davidson and Jannsens 2006; Gessner et al. 2007). Degradation of lignin is an oxidative process carried out by fungi and bacteria equipped with ligninolytic enzymes that include phenol oxidase

(a monooxygenase) and peroxidase (Hendel et al. 2005). Assays of oxidative enzyme activity most commonly involve L-3,4- dihydroxyphenylalanine (L-DOPA) as the electron-donating substrate for the detection of phenol oxidase activity. Due to its water solubility and electron donating ability, L-DOPA is the preferred substrate to be used with environmental samples in spectrophotometric determination of phenol oxidase activity (Hendel et al. 2005). DOPA oxidation results in a red tint which is quantified by measuring absorbance at a wavelength of 460 nm (Hendel et al. 2005). L-DOPA can also be administered with a small concentration of H_2O_2 for the determination of peroxidase activity (Hendel et al. 2005).

In an effort to explore the temperature response of enzymatic activity in litter-associated aquatic decomposers, an experiment in laboratory microcosms simulating stream conditions was carried out. It was hypothesized that (1) there are differences in temperature sensitivity of microbial hydrolytic and oxidative enzymes, (2) the responses of microbial enzymes to temperature follow predictions of the MTE, and (3) temperature sensitivity of microbial enzymes is consistent across temperatures commonly found in streams (0-20°C).

Materials and Methods

Laboratory Microcosms

Experiments were conducted in laboratory microcosms to simulate stream conditions of aquatic litter-associated fungi. Microcosm leaf disks were pre-colonized by natural microbial assemblages in a headwater stream draining watershed 5a at the Coweeta Hydrologic Laboratory in Macon County, North Carolina. Pre-colonization took place in November 2017 during peak autumn abscission. Pre-weighed, dried *Liriodendron tulipifera* leaf disks were placed in litter bags in groups of 45, sterilized by autoclaving (dry, 15 minutes) and submerged in the stream for

four days. After colonization by stream microorganisms, leaf disks were placed into laboratory microcosms. Microcosms were 250-mL tissue culture flasks with membrane-filter caps filled with 100 mL of sterile nutrient solution (Figure 1). Nutrient solution was composed of 0.25 g L^{-1} of 3-(N-morpholino)-propanesulfonic acid (MOPS) (pH adjusted to 6.5), as well as inorganic nitrogen and phosphorus added as sterile stocks of $NaNO_3$ and KH_2PO_4 at final concentrations of 2.0 mg L^{-1} (NO_3-N) and 0.275 mg L^{-1} (PO_4-P) for a molar ratio of 16:1 (N:P). Nutrient solutions were replaced every two days via aseptic evacuation and refilled with fresh sterile nutrient solution to ensure that nutrient availability did not limit microbial activity. Microcosms were incubated on shakers at 5 different temperatures (4, 8, 12, 16, and 20°C) in environmental chambers for 28 days.

Sample Collection

Microcosm flasks were harvested on days 0, 6, 12, 18, 24, and 28 after the start of laboratory incubations, and sets of 5-15 leaf disks were distributed into containers for the measurement of dry leaf mass (DM), fungal biomass, microbial enzymatic activity and other microbial parameters that were measured by collaborating students (Pates 2019). A set of 5 leaf disks for enzymatic assays were immediately frozen at -20°C until analyses.

Enzymatic Assays

In this study, enzymatic assays were conducted to estimate activity of extracellular enzymes that aid in microbial decomposition of leaf material and carbon sequestration. The activity of extracellular enzymes β-xylosidase and β-glucosidase was estimated fluorometrically using artificial fluorogenic substrates that release 4-methylumbelliferone (MUF) upon attack by corresponding hydrolytic enzymes (Hendel and Marxsen 2005; Romani et al. 2006; Chen et al. 2011). Substrate analogs 4-methylumbelliferyl-β-D-glucopyranoside (MUF-GL, substrate for β-

glucosidase, E.C. 3.2.1.21) and 4-methylumbelliferyl-β-D-xylopyranoside (MUF-X, substrate for β-xylosidase, E.C. 3.2.1.37) were used to estimate enzymatic activity. Also, phenol oxidase activity was estimated spectrophotometrically using electron-donating substrate analog L-3,4-dihydroxyphenylalanine (L-DOPA) (Hendel et al. 2005; Chen et al. 2011).

Fluorometric Assays

For each replicate (four) of each temperature treatment (4, 8, 12, 16, 20°C) on each harvesting day (d. 0, 6, 12, 18, 24, 28), five leaf disks were placed into sterile 2 mL screw-cap tube containing four 3.2-mm diameter stainless steel beads. Then, 1.3 mL of sterile 5.0 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (pH 6.5) was pipetted into each tube. Tubes were homogenized at 3500 rpm in a bead-beating homogenizer for three minutes. Cut pipette tips were then used to transfer 0.1 mL aliquots of plant tissue homogenate from each sample into three amber microcentrifuge tubes to determine activity of glucosidase, xylosidase as well as quenching for each sample due to color present in leaf homogenates. Each of three tubes received 1.0 mL of corresponding sterile substrate analog stock solution (MUF-GL and MUF-X at 0.55 mM stock concentration) or quench solution (Q, prepared to a stock concentration of 0.5 mM MUF). Two additional tubes received sterile 5.0 mM MOPS buffer instead of plant tissue homogenate to serve as controls for fluorescence due to abiotic degradation of substrates and impurities. These additional tubes each received either 1.0 mL of MUF-GL or MUF-X substrate.

Enzymatic reactions were timed at the moment of dispensing fluorogenic substrates into microcentrifuge tubes. Samples were incubated at corresponding temperatures (4-20°C) in environmental chambers on orbital shakers at 80 rpm. Following incubation, 0.1 mL of 0.2 M Na_2CO_3 stop buffer was dispensed into each reaction tube including controls for a total reaction time of 1 hour. Addition of carbonate stop buffer stopped enzymatic reactions by raising pH to

~10.5 which is also ideal for fluorescence of MUF. Tubes were then centrifuged for one minute at 16,000·g. Serial dilutions ranging from 10^{-1} to 10^{-2} as needed to measure fluorescence were then prepared using combined buffer solution (carbonate buffer: MOPS buffer, 1:10 ratio). Fluorescence was recorded using a Promega QuantiFluor Portable Fluorometer with a modified cuvette insert for 0.5 mL PCR tubes.

A set of 6 working standards were prepared daily by diluting 0.2 mM MUF stock with MOPS buffer to generate a standard curve with MUF concentrations of 0.4, 1, 2, 5, 10, 20 µM. Standards were treated consistently with homogenized samples by adding 0.1 mL of carbonate stop buffer, elevating pH to ~10.5 to achieve ideal MUF fluorescence. Final standard concentrations considered addition of carbonate stop buffer when calculating the standard curve. All buffers and solutions were stored in a refrigerator at 4°C while being protected from exposure to light.

Spectrophotometric Assays

After sample homogenization as described above for fluorometric assays, cut pipette tips were then used to transfer 0.1 mL aliquots of plant tissue homogenate from each sample into four microcentrifuge tubes to estimate phenol oxidase (Ph) and peroxidase (P) activity as well as to adjust absorbance in each assay for the presence of yellowish coloration from leaf homogenate (Ph-C and P-C samples). Two additional tubes received 0.1 mL of MOPS buffer instead of homogenate to serve as controls for absorbance due to abiotic degradation or contamination of L-DOPA (Hendel et al. 2005).

Enzymatic reactions were started by the addition of 1.0 mL of sterile 3.25 mM L-DOPA in MOPS solution to Ph and P samples. At the same time, 1.0 mL of sterile 5.0 mM MOPS buffer was added to Ph-C and P-C samples. Peroxidase reactions also received 65 µL of 3% H_2O_2

solution. Control tubes also received 1.0 mL of L-DOPA in MOPS solution and were treated consistently with processed samples. All tubes were incubated for 4-24 hours at corresponding temperatures (4-20°C) in environmental chambers on orbital shakers at 80 rpm. Tubes were centrifuged for one minute at 16,000·g. Absorbance was immediately recorded at 460nm with a Beckman DU520 UV/VIS spectrophotometer by transferring 1.0 mL of supernatant to 1.0 cm cuvettes. In general, no peroxidase activity was detected in most samples (i.e. comparable absorbance for phenol oxidase and peroxidase assays), thus, only phenol oxidase activity was considered for data analysis in this study.

Statistical Analyses

Enzymatic activities expressed per gram of litter dry mass and per gram of fungal biomass were used for statistical analyses. Data for litter dry mass and litter-associated fungal biomass was provided by graduate student Hunter Pates who worked on a closely associated project (Pates 2019). For analysis of variance (ANOVA), enzymatic activity data was ln-transformed to achieve normality. Main effects ANOVA with time and temperature as categorical variables was performed to determine if responses of enzymatic activity to changes in temperature were significant. Also, linear regressions of log-transformed data on enzymatic activity against inverse temperature parameter (1/kT) from the MTE were used to estimate apparent activation energy for each type of enzymatic activity across different temperature intervals. Statistical analyses were performed with SPSS 26.0.

Results

In general, enzymatic activity increased with increases in temperature. β-xylosidase activity per unit of litter dry mass increased throughout the duration of the experiment (d. 0-28)

(Figure 2A). ANOVA confirmed the effect of temperature on β-xylosidase activity per unit of litter dry mass to be statistically significant ($F_{4, 103}$ = 139.64, p < 0.0001) (Figure 2A). The effect of temperature on β-xylosidase activity per unit of litter-associated fungal biomass was also statistically significant (main effects ANOVA, $F_{4, 103}$ = 39.89, p < 0.0001) (Figure 2B). On day 0, temperature sensitivity of β-xylosidase activity per unit of litter-associated fungal biomass was comparable to that of respiration (E_a of respiration ~0.65 eV as predicted by the MTE). A linear regression of day 0 β-xylosidase activity for 4-16°C interval showed an activation energy (E_a) of 0.72 eV (p = 0.0039) (Figure 3) with no further increases in activity above 16°C. On day 12, β-xylosidase activity per unit of litter-associated fungal biomass showed somewhat lower temperature sensitivity with estimate of E_a for 4-12°C interval of 0.48 eV (p = 0.0029) (Figure 3). A linear regression for 12-20°C interval showed an E_a of 0.26 (p = 0.042) suggesting lower temperature sensitivity of β-xylosidase at higher temperatures (Figure 3).

β-glucosidase activity per unit of litter dry mass generally increased throughout the duration of the experiment (d. 0-28) (Figure 4A). β-glucosidase activity in this case also generally increased with increases in temperature. ANOVA showed that the effect of temperature on β-glucosidase activity per unit of litter dry mass was significant ($F_{4, 104}$ = 52.0, p < 0.0001) (Figure 4A). The effect of temperature on β-glucosidase activity per unit of litter-associated fungal biomass was also statistically significant (main effects ANOVA, $F_{4, 104}$ = 12.64, p < 0.0001) (Figure 4B). On days 0 and 12, β-glucosidase activity per unit of litter-associated fungal biomass appeared to show higher temperature sensitivity at lower temperatures (Figure 5). Although not significant, a linear regression of day 0 data for 4-20°C interval showed an E_a of 0.30 eV (p = 0.0745) (Figure 5). A linear regression of day 12 β-glucosidase activity for 4-12°C

interval gave an E_a of 0.37 eV (p = 0.0594) with no further increases in activity above 12°C (Figure 5).

Data for phenol oxidase activity was generally below detection limit across treatment temperatures (4-20°C) on days 0 and 6. In general, phenol oxidase activity increased from days 12 to 28 during the experiment (Figure 6). Phenol oxidase activity per unit of litter dry mass generally increased with increases in temperature (Figure 6A). ANOVA showed the effect of temperature on phenol oxidase activity per unit of litter dry mass was statistically significant ($F_{4, 69}$ = 142.3, p < 0.0001) (Figure 6A). The effect of temperature on phenol oxidase activity per unit of litter-associated fungal biomass was also significant (main effects ANOVA, $F_{4, 69}$ = 116.54, p < 0.0001) (Figure 6B). On day 12, phenol oxidase activity per unit of litter-associated fungal biomass showed higher temperature sensitivity than that of respiration according to the MTE predictions (Figure 7). A linear regression of day 12 phenol oxidase activity for 4-20°C interval gave an E_a of 0.92 eV (p < 0.0001) (Figure 7). On day 28, phenol oxidase activity per unit of litter-associated fungal biomass also showed very high temperature sensitivity with an E_a estimate of 1.25 eV (p < 0.0001) for 4-20°C interval (Figure 7).

Discussion

Overall, the results of this experiment suggest that the responses of microbial extracellular enzymatic activity associated with submerged plant litter to changes in temperature are likely more complex than predicted by the Metabolic Theory of Ecology. With the possible exception of phenol oxidase, enzymatic activity did not follow simple monotonous exponential response predicted by the MTE in the 4-20 °C interval that was examined. Instead, for β-xylosidase and β-glucosidase, greater temperature sensitivity of enzymatic activity was observed

at lower temperatures. Linear regressions of day 12 β-xylosidase activity per unit of fungal biomass for 4-12°C and 12-20°C intervals showed estimates for E_a of 0.48 eV and 0.26 eV respectively, while no further increases in β-xylosidase activity was observed at temperatures above 12°C (Figures 3 and 5). Estmates of E_a for β-xylosidase and β-glucosidase activity varied depending on decomposition stage and temperature interval (0.26-0.72 eV, Figures 3 and 5) and were generally lower or comparable to E_a of repiration (0.65 eV) as predicted by the MTE (Brown et al. 2004). In contrast, estimates of E_a for phenol oxidase activity (0.92-1.25 eV, Figure 7) were considerably higher than that of respiration. These findings indicate that temperature responses of litter-associated extracellular enzymes cannot be easily predicted by the MTE, since temperature sensitivity depended on examined temperature interval (i.e. monotonous exponential response was not detected in all cases). In addition, the temperature sensitivity of respiration may not be a reliable predictor of temperature responses of extracellular enzymatic activity of aquatic litter-associated microorganisms, since we observed clear differences in temperature sensitivity (E_a) between hydrolytic and oxidative enzymes involved in plant litter decomposition and C sequestration.

As expected, the results of this experiment demonstrated that that enzymatic activity generally increased in response to increases in temperature. This agrees with findings from previous publications as the rates of enzymatic reactions are known to increase with temperature (Hendel and Marxsen 2005; Sinsabaugh and Follstad Shah 2012; Fenoy et al. 2016). A closer look at the responses to changes in temperature for each kind of activity measured in this experiment (β-xylosidase, β-glucosidase, and phenol oxidase) revealed some peculiarities. For example, activity of β-xylosidase and β-glucosidase per unit of fungal biomass decreased from day 0 to day 6 of the experiment (Figures 2B and 4B). The decrease in activity per unit of fungal

biomass was observed due to a very large increase in fungal biomass (Pates 2019) coupled with smaller increases in enzymatic activity across temperature treatments. This is what would be expected, since fungal biomass was very low and resource (C, N, P) availability was high at the very beginning of the experiment (Sinsabaugh and Follstad Shah 2012).

Phenol oxidase activity was very low (below detection limit) across treatment temperatures (4-20°C) in the beginning of the experiment on days 0 and 6 (Figure 6). This can be explained by the fact that the production and release of ligninolytic enzymes involved in the degradation of recalcitrant substrates requires substantial resource investment from fungi; thus, early in decomposition, microorganisms will be relying mostly on more easily digestible plant polymers that can be mined with hydrolytic enzymes (e.g. β-xylosidase and β-glucosidase in this experiment). At later stages of decomposition when less recalcitrant plant polymers become exhausted and fungal biomass accumulates to high levels, microbial investment in ligninolytic enzymatic machinery will occur (Sinsabaugh et al. 2014; Fenoy et al. 2016; Gulis et al. 2019).

The temperature sensitivity of phenol oxidase activity per unit of fungal biomass was higher than what was observed for β-xylosidase and β-glucosidase activity in this experiment. This finding is supported by previously mentioned higher estimates of apparent activation energy (E_a) of phenol oxidase (0.92-1.26 eV) than those of β-xylosidase and β-glucosidase (0.26-0.72 eV). Thus, in this experiment, temperature sensitivity was found to be consistently higher for oxidative ligninolytic enzymes than hydrolitic enzymes involved in degradation of hemicellulose and cellulose. The higher temperature sensitivity of these enzymes involved in the degradation of recalcitrant substrates has been reported in previous works. In terms of temperature sensitivity of decomposition and respiration rates, it has been reported that recalcitrant substrates are more sensitive to changes in temperature than labile substrates processed mostly by hydrolytic

enzymes (Craine et al. 2010; Sierra 2012; Wang et al. 2012). Thus, the findings from this experiment generally agree with previously published data.

Conclusions

In this study, it was hypothesized that (1) there are differences in temperature sensitivity of microbial hydrolytic and oxidative enzymes, (2) the responses of microbial enzymes to temperature follow predictions of the MTE, and (3) temperature sensitivity of microbial enzymes is consistent across temperatures commonly found in streams (0-20°C). First, it was observed that enzymatic activity was temperature dependent and generally increased with temperature; however, the temperature sensitivity of oxidative enzymes (phenol oxidase) involved in degradation of recalcitrant substrates was much greater than that of hydrolytic enzymes (β-xylosidase and β-glucosidase), thus, supporting our first hypothesis. In addition, we found that the activity of microbial enzymes involved in carbon sequestration did not follow simple monotonous response across experimental temperatures (4-20°C) predicted by the MTE. Instead, we observed greater temperature sensitivity (higher apparent activation energy) of hydrolytic enzymes at colder temperatures. Thus, our second and third hypotheses were rejected. These findings may have important implications for stream ecosystems under climate change scenarios, since peaks in leaf litter availability and microbial activity both occur during the coldest season in autumn and winter months.

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Figure 1. Laboratory microcosms (tissue culture flasks) containing naturally colonized tulip poplar leaf disks with nutrient solution that were incubated at 5 temperature treatments (4-20°C) for 28 days.
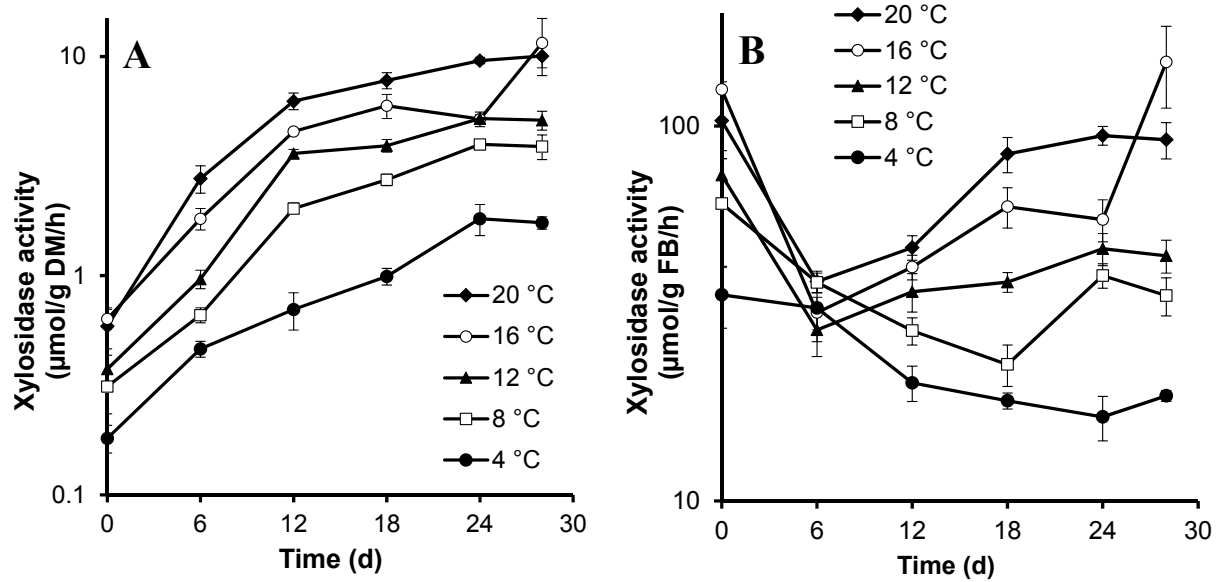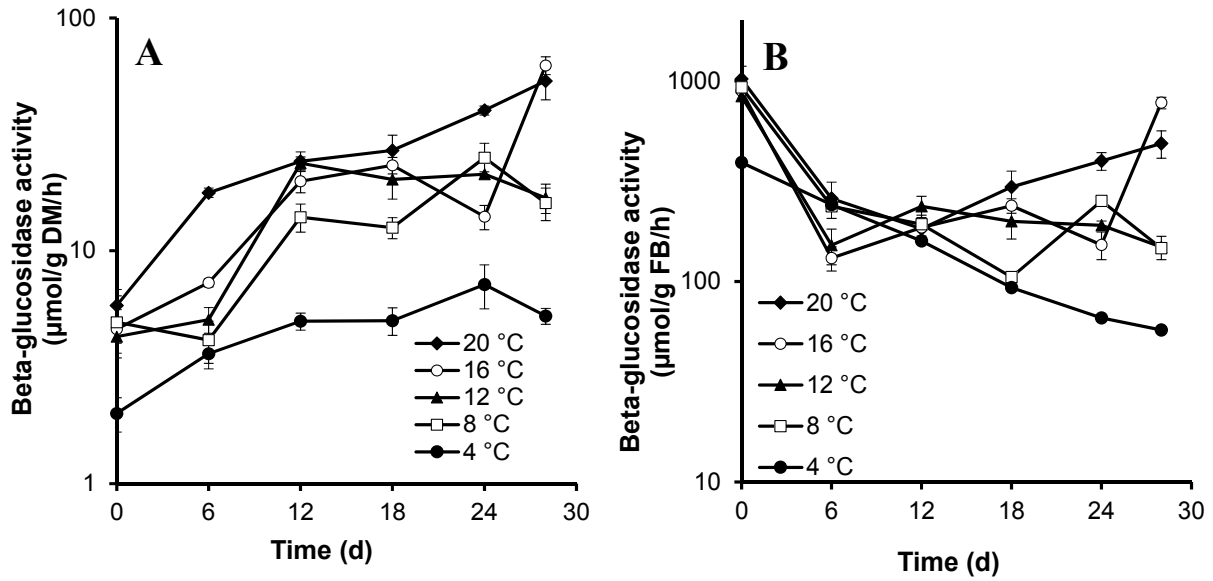
Figure 2. A. Dynamics of β-xylosidase activity per unit of litter dry mass over the course of the experiment (days 0-28) for each temperature treatment (4-20°C). B. β-xylosidase activity per unit of litter-associated fungal biomass over the course of the experiment (days 0-28) for each treatment temperature (4-20°C). Error bars represent standard errors (SE) of means.
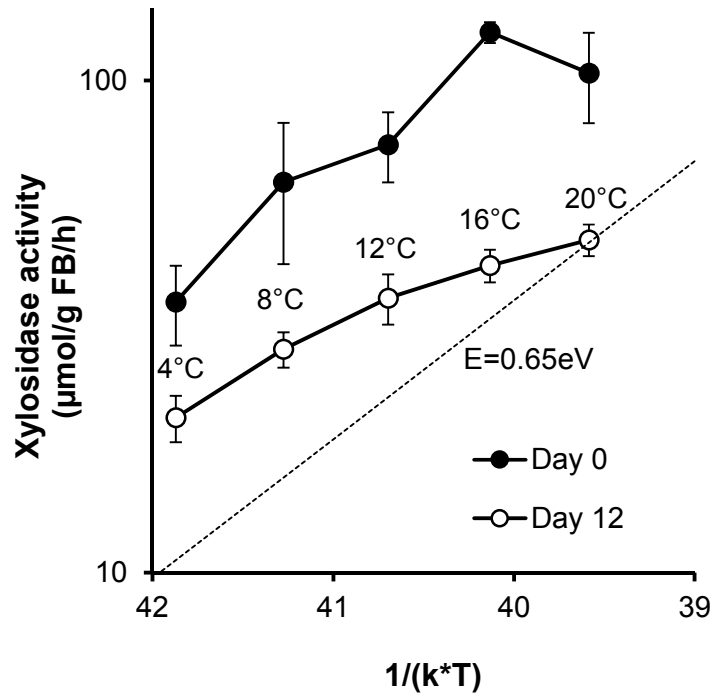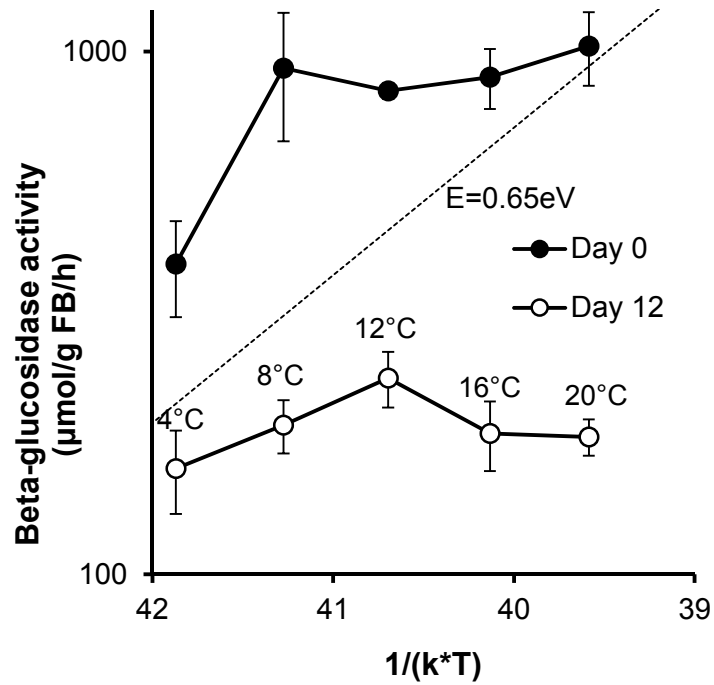
Figure 3. Temperature sensitivity of β-xylosidase activity per unit of litter-associated fungal

biomass shown for days 0 and 12 of the experiment. Error bars represent standard errors (SE) of

means. The dotted line, shown for comparison, is the temperature sensitivity of respiration (the

slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE).

Figure 4. A. Dynamics of β-glucosidase activity per unit of litter dry mass over the course of the experiment (days 0-28) for each temperature treatment (4-20°C). B. β-glucosidase activity per unit of litter-associated fungal biomass over the course of the experiment (days 0-28) for each treatment temperature (4-20°C). Error bars represent standard errors (SE) of means.

Figure 5. Temperature sensitivity of β-glucosidase activity per unit of litter-associated fungal biomass shown for days 0 and 12 of the experiment. Error bars represent standard errors (SE) of means. The dotted line, shown for comparison, is the temperature sensitivity of respiration (the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE).
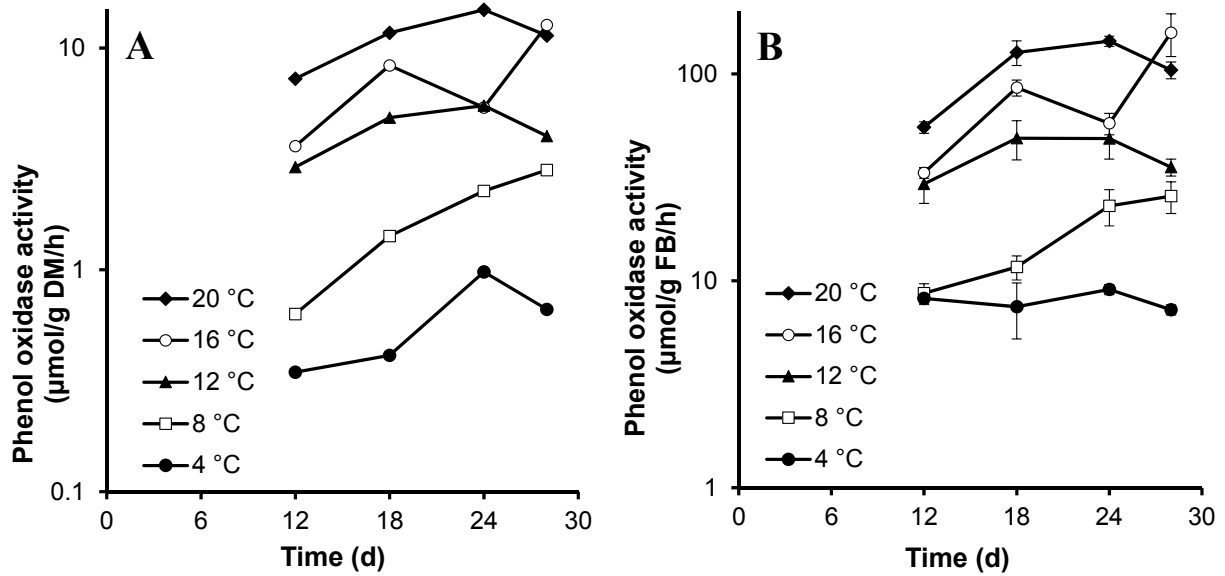
Figure 6. A. Dynamics of phenol oxidase activity per unit of litter dry mass over the course of the experiment (days 12-28) for each temperature treatment (4-20°C). B. Phenol oxidase activity per unit of litter-associated fungal biomass over the course of the experiment (days 12-28) for each treatment temperature (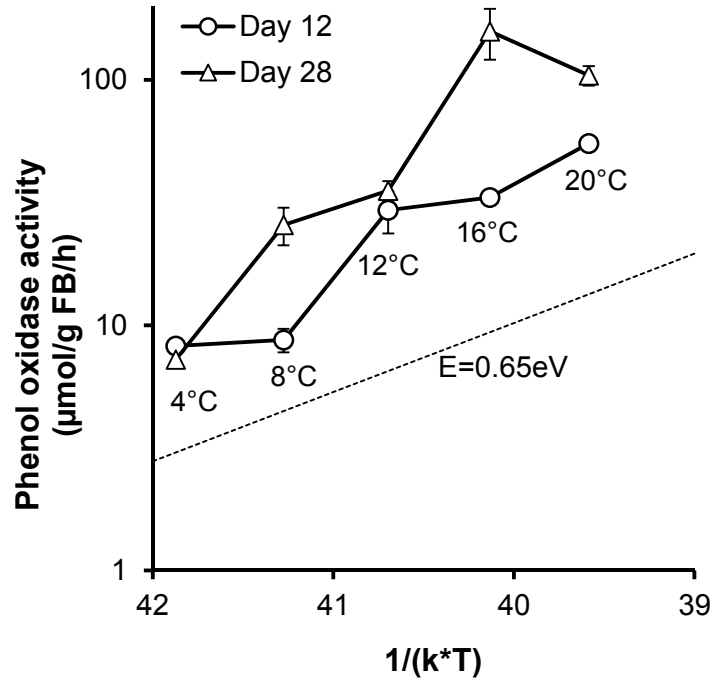4-20°C). Error bars represent standard errors (SE) of means. Activity of phenol oxidase was generally below the detection limit on days 0-6.

Figure 7. Temperature sensitivity of phenol oxidase activity per unit of litter-associated fungal biomass shown for days 12 and 28 of the experiment. Error bars represent standard errors (SE) of means. The dotted line, shown for comparison, is the temperature sensitivity of respiration (the slope corresponding to the activation energy of respiration (0.65 eV) according to the MTE).

Protocol for Determining Extracellular Enzymatic Activity of Microorganisms in Plant Litter (01/12/2018, VG) (beta-glucosidase, xylosidase, phenol oxidase, peroxidase)

Prior to Analyses
1. Place 3.2mm diam. stainless steel beads into a beaker and cover the surface of the beads with diH_2O. Cover beaker with foil **tightly**. Autoclave.
2. Place 4 stainless steel beads in each 2-mL screw-cap tubes. **Cap loosely**. Autoclave.
3. Autoclave some 1.5-mL regular and black microcentrifuge tubes.
4. Cut approximately 5 mm off of the tapered end of the 1 mL pipette tips for each expected sample. Autoclave.
5. Prepare 5 mM MOPS buffer solution (see below). Autoclave.
6. Prepare 0.2 M carbonate stop buffer (see below). Autoclave.

Buffers
5.0 mM MOPS buffer (Mr=209.26; 1.046 g per 1 L)
Add 1.046 g of MOPS to a 1L beaker. Fill container with 1 L diH_2O using a graduated cylinder. Adjust pH to 6.5 by adding 1N NaOH solution dropwise.
0.2 M Carbonate stop buffer (Na_2CO_3 monohydrate, Mr=124; 24.8 g per 1 L).
Add 12.4 g of Na_2CO_3 x·$1H_2O$ to a 1L bottle. Fill container with 500 mL diH_2O. pH should be about 11.2 (no adjustment needed).
Combined buffer (pH 10.5)
Mix 40 ml of MOPS buffer with 4 mL of carbonate buffer (44 mL total).
L-DOPA for spectrophotometric assays
Primary (and only) stock of L-DOPA is prepared by dissolving the appropriate amount in 5 mM MOPS buffer (see Table below). All stocks should be aliquoted (in 50-mL tubes) and frozen at -20C. Protect from light – wrap tubes in foil.
Fluorogenic substrate solutions
Primary (and only) stocks of MUF-GL and MUF-X are prepared as 0.55 mM solutions. Prepare stocks by dissolving the appropriate amount of substrate (see Table below) in 5 mM MOPS buffer. Before adding buffer to the powder, add cellosolve to help in dissolution. MUF-GL, MUF-X are difficult to dissolve, use sonication and gentle heating (to ca. 35 C). Dissolution takes a couple of hours. Aliquot into 15-mL or 50-mL tubes for daily use and store in the freezer at -20 C. Protect from light (black tubes or wrap in foil).
MUF Standards
Before adding buffer to the powder, add cellosolve to help in dissolution. MUF is difficult to dissolve, use sonication and gentle heating (to ca. 35 C). Dissolution takes a couple of hours. Stocks of MUF standards are prepared as 0.2 mM concentrations. First dissolve powder in 100 mL of 5.0 mm MOPS buffer (see table below) to get 0.5mM solutions. Then dilute to 0.2mM with MOPS (e.g. 12mL of 0.5 mM solution + 18 mL of MOPS). Filter through 0.22 um membrane filter into a couple of sterile 15-mL tubes. Wrap in foil. Store in refrigerator at 4C.

Spectrophotometric assays

Stock	Molecular Weight	Concentration (mM)	Substrate (mg)	MOPS buffer (mL)	Cellosolve (mL)
L-DOPA	197.19	3.25	320.43	500.0	----

Fluorometric assays

Stock	Molecular Weight	Concentration (mM)	Substrate (mg)	MOPS buffer (mL)	Cellosolve (mL)
MUF-GL	388.32	0.55	85.52	400.0	0.4
MUF-X	326.28	0.55	44.91	250.0	0.25
MUF	176.17	0.5 (final 0.2)	8.81	100.0	0.1

Daily routine
1. In the morning, take one 50-mL tube of L-DOPA from the freezer. Take two 15-mL tubes for each fluorogenic substrate (MUF-GL, MUF-X) from the freezer and thaw. These will be used as substrate solutions. No further dilution is necessary. Shake vigorously. Sonicate/heat to 35 C if necessary (up to 20 min) to aid in thawing/dissolving.
2. Label each 2-mL screw-cap tube that contains steel beads with sample ID.
3. Pipet 1.3 mL of cold MOPS buffer into each of those tubes.
4. Add 5 leaf disks to each screw-cap tube. **Cap tightly.**
5. Homogenize tubes for 3 minutes at 3,500 rpm for maple leaf disks (3 samples at a time). (Adjust time if different type of material is used, i.e. 2 minutes for tulip poplar, longer for rhododendron leaves and wood).

In general, we have 5 temperature treatments (4, 8, 12, 16, 20C), 4 replicates, 6 sampling dates, (20, 20, 20, 20, 48 and 48 samples per date), i.e. at least 176 samples.

Spectrophotometric assays

6. Prepare 0.3 % hydrogen peroxide solution by mixing 0.1 mL of 30% stock H_2O_2 and 9.9 mL of sterile diH_2O in 15-mL tube.
7. Transfer 0.2 mL of homogenate using a cut pipettor tip (invert tube to mix, and remove subsamples from the middle of the suspension) from a screw-cap tube into 4 labeled transparent microcentrifuge tubes for spectrophotometric assays (phenol oxidase, peroxidase, phenol oxidase color, peroxidase color; i.e. Ph, P and Ph-C, P-C, respectively). I.e. take out 0.2, 0.2, 0.2 and 0.2 mL of homogenate. Repeat this process for each homogenized sample.
8. Place the remaining homogenates (screw-cap tubes with beads) temporarily in the fridge.
9. Each day, label just 2 additional tubes (Ph1 and P1) but do not add the homogenate. Instead, add 0.2 and 0.2 mL of MOPS buffer. These will be used as controls to check background absorbance of L-DOPA, i.e. these tubes will not receive any biological material.
10. Pipet into groups of 4 microcentrifuge tubes:
    Tube "Sample_ID_Ph": 1.0 mL of 3.25 mM L-DOPA (final concentration of substrate 2.5 mM) (phenoloxidase substrate)
    Tube "Sample_ID_P": 1.0 mL of 3.25 mM L-DOPA *and 65 µL of 0.3% hydrogen peroxide solution*
    Tube "Sample_ID_Ph-C": 1.0 mL of MOPS buffer (this sample will serve as control for colored substances leaching from leaf material; do not add L-DOPA or peroxide)
    Tube "Sample_ID_P-C": 1.0 mL of MOPS buffer and *65 µL of 0.3% hydrogen peroxide solution* (this sample will serve as control for colored substances leaching from leaf material and decolorization due to peroxide; do not add L-DOPA)
    Record start time for each group of 4 tubes separately.
11. In addition, just 2 tubes labeled Ph1 and P1 (see above) should receive 1.0 mL of L-DOPA or 1.0 mL of L-DOPA *plus 65 µL of 0.3% hydrogen peroxide*, respectively, and will serve as controls for absorbance due to abiotic degradation of substrate or impurities.
12. Incubate microcentrifuge tubes **for about 4-6 hours (spectrophotometric assay)** at appropriate temperature (i.e. 4, 8, 12, 16 or 20C) in the environmental chamber with shaking.
13. About **30 min before measuring samples**, turn on the spectrophotometer and set up the program (460 nm). Blank the instrument using 1 mL of MOPS buffer in the cuvette.
14. **After about 4-6 hours**, centrifuge the first group of 4 microcentrifuge tubes for 1 min at 16,000 g (rcf). **Record time (to a minute) when the incubation was stopped** for each set of 4 tubes separately. Consider the end of centrifugation as stop time.
15. Carefully transfer (remove subsample from the middle of solution without disturbing the pellet) 1.0 mL from each of those 4 microcentrifuge tubes into cuvette.
16. Measure absorbance immediately at 460 nm, record results.
17. Repeat for other groups of 4 samples. **Record stop time.**
18. Measure absorbance of samples Ph1 and P2 (just 2 of them) in the same way. Record absorbance and stop time.

Fluorometric assays. Proceed in the subdued light.

19. Transfer 0.1 mL of homogenate (from screw-cap tubes with beads) using a cut pipettor tip (invert tube to mix, and remove subsamples from the middle of the suspension) from a screw-cap tube into 3 labeled <u>black microcentrifuge</u> tubes for fluorescent assays (MUF-GL, MUF-X assays and quench control, Q).). I.e. take out 0.1, 0.1 and 0.1 mL of homogenate. Repeat this process for each homogenized sample.

20. Each day, label 2 additional tubes (GL1 and X1) but do not add the homogenate. Instead, add 0.1 and 0.1 mL of MOPS buffer. These will be used as controls to check for fluorescence in solutions of fluorogenic substrates; i.e. these tubes will not receive any biological material.

21. Prepare quench solution by diluting 0.2 mM (=200 µM) MUF stock from the refrigerator 100-fold. To do that, add 0.13 mL of this 0.2 mM stock to 12.87 mL of MOPS buffer in foil-wrapped 15-mL tube, mix. (This would be enough for ~12 samples, it actually equals to 2 µM std solution).

22. Pipet the following substrates into black microcentrifuge tubes in 20 second intervals.
    Tube "Sample_ID_GL": 1.0 mL of MUF-β-D-glucopyranoside (β-glucosidase substrate) (final concentration 0.5 mM)
    Tube "Sample_ID_X": 1.0 mL of MUF-β-D-xylopyranoside (xylosidase substrate) (final concentration 0.5 mM).
    Tube "Sample_ID_Q": 1.0 mL of quench solution.
    Tubes GL1 and X1 (see above) should receive 1.0 mL of appropriate substrate, respectively, and will serve as controls for fluorescence due to abiotic degradation of substrates or fluorescing impurities.

23. Incubate black microcentrifuge tubes for **exactly 1 hour (fluorometric assay)** at appropriate temperature (i.e. 4, 8, 12, 16 or 20C) in the environmental chamber with shaking.

24. In the meantime, **prepare a set of 6 working standards** and blank (1 mL each) of MUF from primary standard (0.2 mM stock) stored in the dark in the refrigerator (4 C). Protect working standards from light (i.e. prepare in black microcentrifuge tubes or keep them in the drawer). The final concentrations for these standards are given in the table below.
    a. To prepare these from 0.2 mM (=200 µM) stock, first prepare 10^{-1} dilution (i.e. 20 µM secondary stock). Pipette 300 µL of original stock to 2.7 mL of MOPS buffer in 15-mL tube (foil wrapped). That's 20 µM secondary stock.
    b. From this 20 µM secondary stock, prepare 6 working standards (see table below) in black microcentrifuge tubes. Use appropriate pipettors, i.e. for 20-200, 100-1000 µL.

Final standard concentration, µM	Volume of 20 µM secondary stock, µL	Volume of MOPS buffer, µL
blank	0	1000
0.4	20	980
1	50	950
2	100	900
5	250	750
10	500	500
20	1000	0

25. **After exactly 1 hour**, stop incubation of black centrifuge tubes from step 23 by pipetting 0.1 mL of 0.2 M carbonate stop buffer in 20-second intervals to the black microcentrifuge tubes in the same order/sequence as before to stop the enzymatic reaction. Invert/mix. **Record time (to a minute) if there is a deviation from 1-hour incubation time.**

26. Prepare a set of 6 "activated" MUF standards plus activated blank. Add 0.1 mL of carbonate stop buffer to microcentrifuge tubes with standards and blank before measuring fluorescence. (Total volume will become 1.1 mL; this change in concentration will be accounted for in the spreadsheet).

27. Pipette 200 µL of each "activated" standard and blank into 0.5-mL PCR tube (7 tubes). Store in the dark (drawer).

    Different dilutions may be needed to measure fluorescence for different substrates and biological samples. I.e. 10^{-1} would be likely the most suitable dilution for GL and X samples, however, sometimes undiluted samples or 10^{-2} dilution may be needed.

28. Centrifuge black microcentrifuge tubes of GL, X and Q series for 1 min at 16,000 g (rcf). Treat GL1 and X1 control in exactly the same way.

29. Carefully transfer (remove subsamples from the top or middle of the solution without disturbing the pellet) 0.1 mL from each black microcentrifuge tube of GL, X and Q series into corresponding transparent microcentrifuge tubes with 0.9 mL of combined buffer to get 10^{-1} dilution and maintain pH 10.5. Mix.

30. Transfer 200 µL of 10^{-1} dilution of GL, X and Q series to 0.5-mL PCR tube. Avoid bubbles or droplets on the walls. Keep these in the dark (drawer).

31. Turn on the fluorometer. Measure and record fluorescence readings for blank and 6 "activated" MUF standards.

32. Measure and record fluorescence readings for each sample of GL, X and Q series at least twice.

33. Remeasure all standards and blank.

34. Prepare additional dilutions (e.g. 10^{-2}) if needed for some samples or use undiluted samples, if fluorescence is low. Ideally, fluorescence should be in the 0.5 to 10 µM range (below 0.5 µM the sensitivity is questionable, above 10 µM the relationship between fluorescence and concentration becomes non-linear, also color may be serious issue in undiluted samples). If the fluorescence is too high (>10 µM), prepare appropriate dilutions. If the fluoresecence is too low (< 0.5 µM), move to the undiluted sample or prepare two-fold dilution by mixing 100 µL of undiluted sample from a black tube and 100 µL of combined buffer directly in the 0.5-mL PCR tube. Mix and remeasure fluorescence. Prepare other dilutions using a combined buffer if needed (final volume in the PCR tube should be 200 µL, protect from light, keep in the drawer).
    In general,
    a. 0.5 to 10 µM range is ideal
    b. 10^{-1} dilution is better than undiluted sample, since color (due to leaching) may be a problem in those samples.
    c. **If measuring any further dilutions or undiluted samples, it's absolutely necessary to also measure corresponding Q sample at the same dilution to account for quenching.**