CONTROLS ON MICROBIAL EXTRACELLULAR ENZYMES IN NORTHEAST PENNSYLVANIA AND EASTERN TENNESSEE FRESH WATERS

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

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August 2017

ACKNOWLEDGEMENTS

Thank you to Drew Steen and to my committee, Ed Perfect and Mike Mckinney, for all their knowledge and guidance.

ABSTRACT

A longstanding goal of organic biogeochemistry is to open the “black box” of microbial community metabolic capacity in order to understand how the vast complexity of organic chemical structures and microbial metabolisms give rise to observed patterns of organic matter reactivity. Microbial communities use diverse suites of extracellular enzymes to access complex organic compounds, which constitute the majority of bioavailable organic matter. Peptidase enzymes are responsible for degradation of protein, there are both endopeptidase and exopeptidase enzymes which cleave proteins from the end or the middle, respectively. Other than the exopeptidase enzyme Leucyl Aminopeptidase, constraints on peptidase activity are unknown. The purpose of this research is to determine what, if any, environmental variables control the production peptidase enzymes. Here we investigate the extent to which the freshness of organic matter and other water quality parameters control pathways of protein degradation as indicated by the ratio of exopeptidases to endopeptidases. Potential endo- and exopeptidase activities were measured in 32 freshwater samples from eastern TN and northeastern PA. We compared enzyme activities to organic matter concentrations and character, cell abundance, nutrient concentrations, and environmental parameters. There is evidence indicating that the ratio of endo- to exopeptidase activities are influenced by dissolved organic carbon (DOC) concentration and freshness. There is a similar relationship between endopeptidase to exopeptidase activity and cell densities, despite there

being no correlation between DOC concentration and cell density. Fluorescent dissolved organic matter (FDOM) data indicates that terrestrial DOC dominates systems when DOC > 275 µmol [micromoles] L^{-1} (per liter) whereas DOC < 275 µmol [micromoles] L^{-1} there is a mixture of carbon derived from microorganisms and terrestrial sources. These results indicate that the metabolic capacities of freshwater microbial communities influence the reactivity of specific classes of molecules within the protein-like fraction of dissolved organic matter (DOM) and are the characteristics of organic matter. Additionally, relationships exist between the ratio of endopeptidase to exopeptidase activity and temperature, pH, dissolved oxygen, and cell abundance. Despite the influence organic carbon (OC) freshness and cell density have on the production of endopeptidases and exopeptidases this is a very dynamic system which is influenced by several environmental factors.

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CHAPTER 1: INTRODUCTION

Biogeochemical cycles, both terrestrial and oceanic, are regulated and shaped by microorganisms (Burgin et al., 2011). Microorganisms, such as bacteria and fungi, are differentiated from multicellular organisms by the presence of one cell. They also have short generation times, large absolute population sizes and higher rates of dispersal than multicellular organisms (Dolan, 2005). Ecosystems are dependent on microorganisms as they perform the critical task of cycling nutrients, decomposing organic matter and forming a base for the food web (Adams et al., 2010).

Heterotrophic microorganisms cannot directly mineralize organic molecules larger than 600 - 800 Da because anything larger cannot cross cell membranes and requires the assistance of extracellular enzymes to aid in decomposition (Weiss et al., 1991). Extracellular enzymes are responsible for introducing detritus into the food web by breaking down macromolecules into smaller molecules (Arnosti et al., 2014). Microbes are then able to consume the products and assimilate them for metabolism and growth (Allison, 2005).

Microbial communities are capable of shifting enzyme production based on substrate availability or nutrient limitations (Montuelle & Volat, 1998). The degree to which the microbial communities shift production of enzymes is not well understood. However, enzymatic production is material- and energy-intensive, not only for the individual but for the community as a whole (Sinsabaugh et al., 2014). There are known organisms which do not produce

specific extracellular enzymes, but benefit from other organisms' extracellular enzymes. They are considered "opportunists" (Moorhead & Sinsabaugh, 2006) or "cheaters" (Allison, 2005) because they obtain organic matter that is broken down as a result of another microorganisms deploying extracellular enzymes.

Enzyme production rates depend on nutrient availability, specifically nitrogen and phosphorous, carbon allocation, and growth efficiency of the microorganism and are poorly understood because of the variability (Münster & De Haan, 1998). Studying enzymatic activity on an ecological scale can give insight to nutrient cycling within the ecosystem, specifically organic matter cycling (Allison, 2005).

Natural organic matter (NOM) is a large carbon source in aquatic ecosystems and acts as an important source of energy in the detritus food web. It is estimated roughly 1.9 Pg C y^{-1} are inputted into freshwater systems, from terrestrial sources (Cole et al., 2007). Dissolved organic matter (DOM) can be defined as any organic compound that passes through a 0.2 μm filter (Hansell & Carlson, 2014) and is made up of several thousand organic compounds, typically a conglomeration of amino acids, carbohydrates, and humic substances (Evans et al., 2005). Natural waters export DOC derived from decomposition of plants as well as microbial derived organic matter, that which ^{14}C-ages ranging from modern to >1,000 years old (Butman et al., 2015). DOM is either allochthonous, a derivative of soil organic matter and vegetation, or autochthonous, a product of phytoplankton production (Arvola & Tulonen, 1998). Allochthonous sources are a

result of catchment drainage and are, on average, more recalcitrant (less bioavailable) than autochthonous sources, while labile carbon is easier to obtain leading to faster cycling times and less accumulation (Sachse et al., 2005) freshwater ecosystems that have high concentrations of organic carbon maybe a result of accumulation of recalcitrant DOC (Evans et al., 2005).

Large quantities (250×10^{12} kg C/yr) of DOM are exported from terrestrial ecosystems to costal marine, estuarine, and inland bodies of water annually (Spencer et al., 2008). Between 0.4 and 0.9×10^{15} g yr^{-1} of organic carbon are transported into the ocean annually (Ran et al., 2013). The chemistry of natural water bodies strongly reflects the chemistry of the respective catchments, in turn controlling the export of DOC and the source and structure (Evans et al., 2005). DOC concentrations, in natural waters, range from 1-50 mg L^{-1} (Thurman, 1985) where highest concentrations are observed in peatlands, wetlands, and soil porewater and clear water lakes and rivers, and groundwater tend to have the lowest concentrations (Evans et al., 2005).

In freshwater ecosystems, such as streams, rivers, ponds, and lakes, DOM is primarily utilized by bacteria and fungi and provides energy to phagotrophic organisms (Münster & De Haan, 1998). Microbial processes, including bacterial respiration and bacterial productivity, are driven by DOM and the ability of organisms to efficiently utilize it, via extracellular enzymes, fuels food webs (Lennon & Pfaff, 2005).

The characteristics and composition of DOM pools are influenced by DOM

source as well as the microbial community responsible for degradation. These communities not only remineralize OM but they degrade it in a way that can determine its turnover and long term storage capabilities (del Giorgio & Davis, 2003). Organic carbon not only provides energy but can influence temperature, pH, light, and mobility of heavy metals (Stanley et al., 2012). We should study the breakdown of organic matter, in terms of extracellular enzyme degradation, in order to better understand the impacts DOM have on aquatic ecosystems.

There are several enzyme classes responsible for breaking down the major components of organic matter. Peptidases are responsible for breaking down proteins, glycosyl hydrolases and polysaccharide hydrolases break down polysaccharides and alkaline phosphatase break down phosphate esters (Chróst, 1990). Within the peptidase enzyme class, there are exo- and endo-acting enzymes. Endopeptidases cleave polymers in the middle and exopeptidase enzymes cleave polymers at the end. With the exception of Obayashi and Suzuki (2005, 2008) and Steen (2015), the only peptidase enzyme studied is leucine aminopeptidase, an exopeptidase. Limiting knowledge of peptidases by studying only exo- acting enzymes could be detrimental in our understanding of the role peptidases play in organic matter degradation (Obayashi & Suzuki, 2008). Research done by Mullen et al. (in prep) suggests that in some areas, that have similar environmental characteristics, the relative activity of endo- and exo- peptidases in freshwaters vary, indicating there is variability in the pathways of protein degradation and the presence of

endopeptidases.

To date, the controls on the production of endopeptidases in freshwater have not been constrained, however the variability maybe able to be explained through community composition or DOM composition. In freshwater, there are several gradients which are thought to naturally control community composition (Crump et al., 2004) including: spatial variability of microbial communities, which can range from kilometers to millimeters (Fortunato & Crump, 2011; Hanson et al., 2012) salinity, temperature, pH, depth (Fortunato & Crump, 2011), nutrient concentration, and OM composition (Judd et al., 2006).

Temperature can control rates of ecosystem functions as well as modify systems including bacterial processes. The metabolic rates of bacteria increase or decrease as a function of temperature (Adams et al., 2010). For example, bacteria are able to break down organic matter at a faster rate when temperature is elevated, as a result secondary production increases. Temperature shifts occur in many ecosystems on a yearly basis allowing for bacteria with different optimal living conditions to shift as temperature and substrate availability changes. These rates likely have an impact on community composition.

The purpose of this work is to identify what environmental factors, specifically the freshness of organic matter and cell densities, control specific ratios of endopeptidase:exopeptidase in freshwater. The stability of potential hydrolysis rates, within the first hour(s) to days of sample collection, is not well understood. The first objective was to understand how samples should be stored

to obtain hydrolysis rates similar to those at time of collection. The second objective was to determine controls on extracellular enzymes in freshwater and what environmental parameters, if any, control the ratio of endopeptidase to exopeptidase activity. The last objective was to experimentally test the hypothesis that OM lability/availability/quality affects enzyme activity.

CHAPTER 2:MATERIALS AND METHODS

2.1 Determination of the Stability of Enzymes During Storage

The purpose of this experiment was to determine the best way to store a sample in order to obtain relative fluorescent units (RFU) values similar to those initially taken at the time of sampling. Freshwater samples were collected from two locations at three time points and stored either in the refrigerator or in a dark box at room temperature. Enzyme assays were conducted several times over a 50-hour time span.

2.1.1 Sampling and Process for Storage Experiment

We collected 1 L samples of water from Volunteer Landing Dock and Second Creek, in Knoxville, TN (Table 1). We then filtered 500 mL of each sample at 3 μm, separated that sample into two 250 mL sections and stored one 250 mL bottle in the refrigerator and one in a dark box at room temperature. The remaining, unfiltered, 500 mL of each sample were divided into two 250 mL as well and one was stored in the refrigerator and one in a dark box at 20.5 ºC.

2.1.2 Enzyme Assays for Storage Experiment

We measured the potential hydrolysis rates of Leucine-AMC, Glycine-Glycine-Arginine-AMC, MUB-β-D-glucopyrinoside, and MUB-Phosphate Fluorimeter (Table 2) using a Promega Glomax Jr Fluorimeter in the UV setting (λ_{ex}=365 nm, λ_{em}=410-450 nm). Triplicates of each sample, whole and filtered, as well as a killed control were created by adding 860 μl of sample, 100 μl of $NaPO_4$ buffer, and 40 μl of substrate to a 2 ml cuvette. Bo 250 m L of each sample to

Table 1 Site names and GPS coordinates in both Tennessee and Pennsylvania.

site	latitude	longitude
Front Pond	41.1707	-74.9148
West Prong	35.6430	-83.7156
Briscoe Swamp	41.1777	-74.9192
Tyson Park	35.9538	-83.9425
Basket Ball Court 1	41.1714	-74.9095
Sequoyah Hills	35.9282	-83.9582
Basket Ball Court 2	41.1714	-74.9102
Fish Pond	35.9559	-83.9236
Pickerel Pond	41.1676	-74.9180
Meads Quarry	35.9528	-83.8657
Toll Creek	35.9522	-83.8650
Wempler Kieth Park	35.7971	-84.2642
Telico Lake	35.7784	-84.2499
Ten Mile	35.9249	-84.0729
Victor Ashe	35.9823	-83.9971
Sinking Creek	35.9249	-84.0765
Third Creek	35.9827	-84.0046
Clinton City Park	36.0947	-84.1329
Clinch River	35.9961	-84.1929
Cove Lake	36.3079	-84.2170
Norris Dam	36.2259	-84.0968
Volunteer Landing	35.9611	-83.9132
Second Creek	35.9582	-83.9237
Anchor Down	35.9687	-83.5198
Douglas Dam	35.9585	-83.5471
Chilowee Park	35.9994	-83.8844
Holston Hills Park	35.9779	-83.8595
Fort Dickerson Quarry	35.9428	-83.9174
UT Medical Pond	35.9384	-83.9447
Cherokee Reservoir	36.1304	-83.5064
Mossy Spring	36.1227	-83.4807

obtain a killed control. Boiling denatures the enzymes and distinguishes hydrolysis rates that are enzymatic versus abiotic hydrolysis. Each cuvette was capped, inverted, and incubated for 10 minutes. Readings were taken several times over a 2-3 hour period. This procedure was carried out for time points of 0 hr, 4 hrs, 24 hrs, 36 hrs, and 200 hrs after the start of the experiment, each time making new cuvettes. The starting time point, zero, was taken approximately 45 minutes after collecting the sample.

Calibration curves for all four sample types were created at each time point using AMC standard and MUB standard. The AMC-standard calibration curve consisted of 5 cuvettes with standard volumes of 0 µl, 10 µl, 20 µl, 30 µl, and 40 µl and MUB-standard calibration curve had standard volumes of 0 µl, 10 µl, 30 µl, 50 µl, and 70 µl. Final standard concentrations equated to 0 µmol/L, 100 µmol/L, 200 µmol/L, 300 µmol/L, 400 µmol/L and 0 µmol/L, 100 µmol/L, 300 µmol/L, 500 µmol/L, 700 µmol/L for AMC and MUB, respectively.

The data were analyzed using R, software for statistical computing (R Core Team, 2016). The slope of the RFU values was calculated at each time point and divided by the slope of the calibration data, this resulting in v_0. R was then used to perform data analysis and statistical testing.

2.2 Enzyme activity in of east Tennessee and north east Pennsylvania

2.2.1 Sample Plan

In order to test the hypothesis that organic matter (OM) quality or quantity influences the nature of extracellular enzymes expressed by the community, a total of 32 freshwater samples were collected using a 1 L Nalgene bottle from 27 sources in East Tennessee and 5 near Dingmans Ferry, PA. Assessing the quantity and quality of OM ahead of time is not possible due to natural fluctuations in OM input therefore locations were selected based on (a) practical accessibility, and (b) coverage of a range of OM quality/quantity based on prior assumptions. The DOM and enzyme activity in freshwater systems vary, as we see in the storage data as well as data collected by Mullen et al. (in prep), therefore doing preliminary measurements of DOM are not realistic. Sites were selected using a visual assessment of trophic status, anthropogenic disturbance, urbanization, and site type. Sample locations included 4 lakes, 9 ponds, 7 low order rivers, 2 quarries, and 8 creeks. Sites in Dingmans Ferry, PA (n = 5) had indistinguishable environmental parameters, by performing a one way ANOVA, to those in TN (n = 27), such as pH ($p > 0.615$), temperature ($p > 0.855$), DOC ($p > 0.369$), and SPC ($p > 0.615$), cell density ($p > 0.268$), and were used to maximize the range of OM quantity and freshness.

All samples were obtained ~2-3 inches below the surface and at least one foot from the bank. Geochemical parameters, including temperature, pH, dissolved oxygen, and specific conductivity were recorded at the time of

sampling. Samples were transported back to lab in a cooler and stored in the refrigerator (<1 hr) until used.

2.2.2 Potential Hydrolysis Rates

The hydrolysis rates of seven fluorogenic substrates, five of which targeted peptidases enzymes, one polysaccharide hydrolase, and one alkaline phosphatase (Table 2), were measured in all 32 samples. 400 μM of each substrate was used because this concentration is larger than observed K_m, so measured v_0 is close to V_{max}. Measurements were taken of both unfiltered sample and filtered sample (3 μm). 860 μl of sample, unfiltered or filtered, were added to a 1 mL methacrylate cuvette. In order to maintain a similar in situ pH, we added 100 μL of buffer. For all substrates excluding MUB-phosphate, we used 0.2 M dibasic potassium phosphate, pH = 7.29, and for MUB-phosphate, we used 0.2 M borate buffer, pH = 7.4. We used borate buffer for MUB-Phosphate as a result of dibasic potassium phosphate interfering with phosphatase activity. 40 μL of 10 mM substrates were added to each cuvette, cuvettes were then capped, mixed by inverting, and left to incubate in the dark for 15-20 minutes before the first measurements were taken.

We measured fluorescence using the same procedure in section 2.2.1. Enzyme assays were completed no later than 4 hours after the sample was

Table 2 Targeted Enzymes and the corresponding substrates used

Enzyme	Class	Substrate	Abbreviation	Concentration (µm)
Arginyl Aminopeptidase	Exopeptidase	Arginine-7-amino-4-methylcoumain	Arg-AMC	400
Glycine Aminopeptidase	Exopeptidase	Glycine-AMC	Gly-AMC	400
Leucine Aminopeptidase	Exopeptidase	Leucine-AMC	Leu-AMC	400
Pyroglutamate Aminopeptidase	Exopeptidase	Pyroglutamic-Acid-AMC	Pyr-AMC	400
Trypsin	Endopeptidase	Glycine-Glycine-Arginine- AMC	GGR-AMC	400
glucosidase	Polysaccharide hydrolase	MUB--D-glucopyranoside	MUB-gluc	400
Alkaline Phosphatase	Phosphatase	MUB-Phosphate	MUB-phos	400

collected, per determination from methods development, to ensure measured enzyme activity reflected in situ activity. Average hydrolysis (v_0) rates were calculated by taking the mean of the three replicates, per substrate, and subtracting the activity of the killed control. The total hydrolysis rates were then determined by taking the sum of v_0 from all 7 substrates. The ratio of endopeptidase

Eq. 1
$$\sum v0_{Exo} = (v0_{s1}) + (v0_{s2}) + (v0_{s3}) + (v0_{s4})$$

$$EI = \frac{v0_{Endo}}{\sum v0_{Exo}}$$

Equation 1- The activity (v_0) of endoppetidase (GGR-AMC) was divided by the sum of all 4 exopeptidases (Leu-AMC, Arg-AMC, Gly-AMC, Pyr-AMC) to obtain the ratio of endopeptidase to exopeptidase activity (EI).

to exopeptidase (EI) was then calculated using (Eq. 1).

2.2.3 Nutrient Concentrations

Concentrations of PO_4, SO_4, NH_3, and NO_2, were measured after collecting the sample (< 2 hours) using a HACH Colorimeter DR 900 (HACH, Loveland, CO). The manufacturers protocols were followed for each respective nutrient and can be found in the appendix.

2.2.4 Cell Counts

Cells were enumerated via direct microscopic counts using DAPI (4',6-diamidino-2-phenylindole, dihydrichloride) stained cells. 3.6 ml of unfiltered sample was preserved at the time of sampling with 400 µL of 0.2 µm-filtered glutaraldehyde and stored in a 5 mL cryovial at -20 °C. At the time of analysis, samples were defrosted to room temperature. 1 mg ml^{-1}DAPI stock was diluted with filtered deionized water, to 0.0082 µg ml^{-1}. A 0.45 µm Milipore absorbent pad topped with 0.2 µm Milipore membrane filter were dampened with 0.2 µm filtered deionized water to aid in even cell distribution. 2 mL of sample and 200 µL of 0.0082 µg ml^{-1} DAPI stain were pipetted into a filtration system and left to sit in the dark for 10-12 minutes (Porter and Fieg 1980). The sample was then filtered and set to dry in the dark for 15 minutes. Filters were mounted onto microslides using one drop of vectashield hardest (Vector Laboratories, Burlingame, CA), to secure the filter, slide and cover slip. Slides were stored in the freezer, for a maximum of two weeks, until the cell counts were done. Slides were viewed using a Zeiss AXIO Imager.M2 at 100x magnification with a DAPI

filter. Roughly 20-30 representative images were captured on a Axiocam MRm and total cell concentrations were estimated using the images.

2.2.5 Fluorescence Spectroscopy

I filtered 5 ml of sample into a combusted glass vial using a LuerLock, 0.2 µm polypropylene RESTEK syringe filter, and sterile syringe. The sample was stored at -20 °C until the time of processing. We thawed the samples to room temperature at the time of measurement then ~3 mL of sample was poured (to reduce contamination) into a semi-macro quartz cuvette. Absorbance was measured using an Evolution 201 UV-Vis Spectrophotometer and fluorescence was measured using a Horiba FluoroMax-4 Spectrofluorometer. Blank samples were measured using HPLC water as a control. Prior to fluorescence scans, water-Raman scans were completed. The fluorescence intensity was measured at excitation wavelengths ranging from 240- 450 nm and emissions wavelengths 300-560 nm with 2.5 nm increments at 0.1 s intervals per recommendation of (Mcknight et al., 2001). Data was exported and later analyzed using MATLAB.

2.2.6 Dissolved Organic Carbon and Total Nitrogen

We collected measurements of non-purgeable organic carbon (NPOC) as well as total nitrogen in all samples using a Shimadzu TOC-L. We filtered 12 mL of sample through a 0.2 µm polyethylene syringe filter into a combusted glass vial and stored at -20 °C at the time the sample was collected. At the time of measurement samples were defrosted at room temperature in the dark. We poured 6 ml of sample into 9 mL glass vials. Calibration curves were created for

inorganic carbon (IC), total carbon(TC) and total nitrogen (TN) using sodium bicarbonate, potassium nitrate, and potassium hydrogen phthalate respectively all with a concentration of 100 mg/L. NPOC is measured by acidifying a sample and sparging it with purified air to create carbon dioxide and remove the inorganic carbon from the sample. All carbon that is not purged from the sample as NPOC is assured to be dissolved organic carbon.

2.2.7 Particulate Organic Carbon

20 ml of sample was filtered through a pre-combusted Whatman 0.22 µm GF/F. The filters were placed on combusted foil, folded in half (per recommendation of the USGS (Ward 2000) and stored at 4 °C until time of measurements. Before analysis was done filters were fumigated in an acidic environment to remove inorganic carbon (IC). 60 mL of 1 M HCl were placed in the bottom of a glass desiccator, beneath a sample tray, containing filtered samples (removed from tin foil). The desiccator was vacuum sealed and left to sit for 6 hours. After fumigation filters were removed and dried on a hot plate at 50 °C for 3.5 hours. Each filter was then ground up in an agate mortar and pestle and placed in a tin boat to be used for measurements. Carbon and nitrogen analysis was done using an elemental analyzer.

2.2.8 Geochemical and Environmental Parameters

Temperature, pH, specific conductivity and dissolved oxygen were measured, at the time of sampling, using a YSI Pro-DSS Sonde. The sonde was lowered into the water slowly, at the time of sampling, ensuring no sediment was

disturbed that may disrupt the natural conditions. The sonde was left in the water until the temperature and pH stabilized and the measurements were then read.

2.2.9 Statistical Analysis

Analysis of variance was performed in R and SAS to look at the variance between hydrolysis rate and site type as well as EI and site type. The data were log and rank transformed to meet assumptions of ANOVA (residuals are normally distributed and homoscedastic) however, transformations did not produce different results in terms of the residuals and homoscedasticity. Therefore, the untransformed data was used for analysis. ANOVA tests were also performed to measure the variance between nutrients and geochemical parameters as a function of the state the sample was collected in. Lastly a stepwise multiple regression model was implemented in SAS to look at the relationship between all environmental parameters (elevation, pH, DO, SPC, temperature, total nitrogen, POC, DOC, and EI.

2.3 Effect of organic carbon amendments on enzyme activity

The purpose of this experiment was to observe the effects of adding, different types of organic carbon on enzyme activity. We added 2 labile organic protein sources, bovine serum albumin and acetate, and 1 recalcitrant organic carbon source, Suwannee River natural organic matter, plus nitrogen in order to see if these additions effect enzyme activity.

2.3.1 Experiment setup and treatment addition

18, 1 L samples were collected from Chilhowee Park in polypropylene Nalgene bottles, following the same sampling technique described in section 2.2.1. Six treatments were set up using three liters of water per treatment for biological replicates to test variability within samples and eliminate contamination error. Each treatment, with the exception of the control and killed, were amended with equivalent concentrations of nitrogen and different types of organic carbon, all equaling 100 µM C and 26.6 µM N. The treatments consisted of:

Treatment 1: Bovine serum albumin (BSA) which is commonly used as a labile protein source. BSA is a single polypeptide chain and is the major protein found in blood plasma.

Treatment 2: Acetate and ammonium hydroxide

Treatment 3: Suwannee River natural organic matter (SR-NOM) and ammonium hydroxide

Treatment 4: ammonium hydroxide

Treatment 5: No Addition- whole/unfiltered sample

Treatment 6: Kill- Boiled sample to denature enzymes

Enzyme assays were performed all on six treatments, and the respective biological replicates, using the substrates Arginine-AMC, Leu-AMC, and Gly-Gly-Arg-AMC. Each cuvette contained 860 µl sample, 100 µl buffer, dabasic sodium phosphate pH = 7.14, and 20 µl substrate.

Three biological replicates were capped and inverted several times and incubated for 10-15 minutes. Each cuvette was read a minimum of 4 times over a 2-3 hour period. Assays were completed over a 27 day period with reads on days 1-4 and 7, 9, 14, 16, 23, 27. At the time of sampling DOC measurements were taken from each treatment in a Shimadzu 500 TOC-L, using method previously explained ensuring samples were filtered at 0.2 μm before the time of measurement. Additionally, oxygen consumption rates were measured at the time of enzyme assay for each treatment using a ProSens O_2 microsenser. Samples were stored on a Thermofisher Scientfic Benchtop Orbital Shaker, at 65 rpm, at room temperature.

CHAPTER 3: RESULTS

3.1 Storage

3.1.1 Enzyme Assay

The storage experiment indicates that enzyme activity varies and in many cases after the initial day (Figure A1), hydrolysis rates are much different than when measured immediately after collection. In order to quantify the change in hydrolysis rates at each time point the mean hydrolysis rate, across all 4 time points, was calculated at the initial time point (t =0), after four hours (t =4), 24 hours (t =24), and 50 hours (t =50) (Figure A2). We then performed an ANOVA (Table 3) test to determine if there was statistical significance in hydrolysis rates at each time point. The ANOVA showed that there is a significant difference ($p < 0.01$) between the means of each time point within each substrate and sample type. The data were also transformed, log and rank, to try and satisfy all assumptions needed in ANOVA testing and both transformations confirmed significant differences at $p < 0.05$ and $p < 0.05$, respectively.

Table 3 Storage data ANOVA table. Statistically significant difference occur between time points and substrates.

Source	DF	Mean Square	F Value	Pr > F
Time	3	12024.84	6.40	0.0009
Substrate	3	111178.50	59.22	0.0001
Sample Type	3	451.1976	0.24	0.8678

3.2 Controls on Freshwater microbial extracellular enzymes in east TN and north east PA

3.2.1 Hydrolysis Rates

An ANOVA indicated that total hydrolysis rates groups by site type (Figure A4) where ponds have different mean hydrolysis rates than any other site type ($p < 0.001$) and do not relate to rivers, creeks, lakes, or quarries. The ANOVA table also indicated that when observing each site by EI (Figure A6), the same patterns do not exist and site types overlap ($p > 0.05$). Creeks and lakes generally group close to one another but ponds, lakes, and quarries are all very different, indicating that there is variability in the way microorganisms are metabolizing the organic matter present. ANOVA tables were done on all other environmental parameters to determine if the addition of Pennsylvania data effects the outcome of the analysis. No significant differences were seen between TN versus PA data (Table 4).

3.2.2 Dissolved Organic Carbon

The relationship between dissolved organic carbon (DOC) and EI describes an "L" shape (Figure A5). The majority of samples had less than 275 μmol C L^{-1} with corresponding EI between 1 and 4. At concentrations over 275 μmol C L^{-1}, the EI is consistently less than or equal to one indicating there is higher exopeptidase activity in areas that have higher concentrations of DOC. Likewise when concentrations of DOC are below 275 μmol C L^{-1} there is higher endopeptidase activity. Although, there is no statistically significant relationship ($p > 0.05$)

Table 4 ANOVA on environmental parameters and the impacts on states samples were obtained from.

Environmental Parameter	p
Temperature	0.855
Cell Densities	0.268
pH	0.615
Specific Conductivity	0.615
Dissolved Organic Carbon	0.369
Total Nitrogen	0.595

between EI and site type, determined by performing an analysis of variance, it is obvious when looking at the data visually. This is not surprising, because bodies of water with similar geochemical/ environmental properties would expect to have similar concentrations of DOC. V_0 of each substrate, other than Pyr-AMC (Figure A11), is highest in samples that have concentrations of DOC over 275 µmol C L^{-1} (Figures 7-10).

3.2.3 Particulate Organic Carbon

Particulate organic carbon measurements follow very similar, but less pronounced, “L” shape pattern to that of DOC (Appendix B1). The majority of POC concentrations are less than 50 µmol C with corresponding EI less than or

equal to 1. Again, indicating higher exopeptidase activity in higher C concentrations and higher endopeptidase activity in lower C concentrations.

3.2.4 Fluorescently Dissolved Organic Matter

We used the resulting excitation-emission matrices to calculate several indices. We calculated the fluorescence index using the ratio of emission intensity at 400-500 nm (Mcknight et al., 2001). FI values of ~1.4 indicate terrestrially derived organic matter and FI values ~1.9 indicate microbial derived OM (Mcknight et al., 2001). FI values that range from 1.4-1.85 indicate there is a range of terrestrially derived and microbial derived organic matter. The fluorescence index (Appendix B2) indicates that the majority of the DOM in these systems is influenced by both terrestrial and microbial derived organic matter. However, there are some locations that have primarily terrestrial derived organic matter (FI~1.4) and a few that are microbial dominated (F1~1.9).

Specific ultraviolet absorbance at 254 nm ($SUVA_{254}$) is calculated by dividing the absorbance at 254 nm by DOC concentrations. The majority of ($SUVA_{254}$) values range between 0-6 with one value of 32 (Appendix B3). Biological Index (BIX) is a relatively new index (Huguet et al., 2009) used to determine the presence of the β fluorophore (Em_{max} = 430-450), which indicates autochthonous biological activity in water samples. The β component is composed of protein like fluorophores and is representative of humic substances (Parlanti et al., 2000). It is measured by dividing the emission at 380 nm by emission at 430 nm when excitation wavelength is 310 nm. BIX displays the

same “L” shape trend where ponds have more terrestrial derived organic carbon and other bodies have microbial derived OC (Appendix B4). The humification index (HIX) is calculated by taking ratio of the intensity at emission wavelengths 300 nm - 345 nm and dividing it by the intensity at emission wavelengths 435 nm - 480 nm. HIX values did not give consistent results with the other indices and all values were less than 1, indicating microbial derived carbon (Appendix B5). The freshness index, defined as β:α, where β has been linked to plankton productivity and is measured by dividing the relative fluorescence at Ex/Em 300–310 by Ex/Em 380–420 nm (Wilson & Xenopoulos, 2008) representing fresher organic matter. α indicates humic-like OM, is measured at Ex/Em 310–330/420–430 nm and represents the presence of older organic matter. The freshness index shows that ponds have lower freshness values, meaning older OM, and water bodies with higher freshness values are a mix of sample sites (Appendix B6).

3.2.5 Cell Count

At lower concentrations of cells EI is variable and at higher concentrations EI is low (Appendix B7). There is a very clear divide at cell concentrations of 5×10^{21} cells mL^{-1}. Corresponding ratios less than 5×10^{21} cells mL^{-1} range from 0-4.5 where corresponding ratios are greater than 5×10^{21} cells $mL^{-1}$are all less than or equal to 1. Despite the fact that the relationships between DOC and EI and cell count and EI both form an “L” shape, no clear relationship between DOC concentration and cell densities (Appendix B8).

3.2.6 Nutrient Concentrations

Ammonia concentrations ranged from 0-7 μmol L^{-1} and corresponding EI of 0-4.5 (Appendix B9). There is a no significant correlation ($R = 0.04$, $p > 0.05$) between the ratio and concentration and no obvious grouping by site type. There is no significant relationship between the concentration of nitrate and EI activity ($R = 0.0028$, $p > 0.05$; Appendix B10). There is some evidence suggesting a possible optimal NO_3 level (1-4 μmol L^{-1}), where the ratio is low and with increase or decrease of nitrate the ratio increases. However, with more data this pattern may not hold true. These data also indicate creeks and rivers have higher nitrate levels than ponds, lakes, and quarries. The ratio of EI decreases as phosphate concentration increases (Appendix B11). The majority of the locations with high levels of phosphate are ponds and in some cases lakes.

3.2.7 pH

The relationship between EI and pH is one of the few that is not “L” shaped. At pH values between 7-8 the mean EI is much greater than that of values at $pH < 7$ and $pH > 8$. Where $pH < 7$ and $pH > 8$ the mean EI is equal or less than 0.5 (Appendix B12). However, when $7< pH < 8$ the corresponding ratios increase and peak around $pH = 7.5$.

3.2.8 Temperature

There is no significant relationship between temperature and EI ($R = 0.012$) (Appendix B13). However, although there is no justification removing the two outlying points (EI >3) may increase the strength of the relationship. Where T<

21 ºC endopeptidase production is more prevalent, where T > 21 ºC exopeptidase production is higher.

3.2.9 Dissolved Oxygen

Dissolved oxygen ranged from 0-12.5 mg L^{-1}, rivers and creeks had the highest concentrations (Appendix B14). The data showed no significant relationship (R = 0.12, p > 0.05) between dissolved oxygen and EI, but higher endopeptidase activity generally occurs with higher DO concentrations.

3.2.10 Specific Conductivity (SPC)

Specific conductivity ranged from 7.6 – 521.00 µs cm^{-1}. SPC appears lower in ponds and rivers and higher in creeks, however, there is no correlation (R = 0.008) between EI and SPC (Appendix B15).

3.2.11 Statistical Analysis

The stepwise multiple regression model (Table 5) that elevation (R = 0.3494, p = 0.0048), dissolved oxygen (R = 0.2525, p = 0.0033), and pH (R = 0.09, p = 0.0328) significantly influence the model. Removing elevation from the model results in total nitrogen being the only significant parameter influencing the model (R = 1. 00, p < 0.001).

Table 5 Stepwise multiple regression of the impacts of environmental controls on EI.

Variable	Partial R-Squared	Model R-Squared	F Value	Pr > F
Elevation	0.3494	0.3494	10.21	0.0048
Dissolved Oxygen	0.2525	0.6019	21.6808	0.0033
pH	0.0959	0.6979	14.84	0.0328

3.3 Effect of carbon addition on enzymes in freshwater

The activity of leucine aminopeptidase, is highest within the first 150 hours of the experiment (Figure A12). The activity of GGR-AMC remains lower than Leu-AMC and Gly-AMC in all samples until approximately 120-150 hours into the experiment. The treatments consisting of SR-NOM and acetate with ammonia both follow very similar patterns with similar V_0 values between 0-80 μmol L^{-1} hr^{-1}. V_0 of trypsin is roughly 62 times higher in the BSA treatment than other treatments after 300 hours of incubation (Figure A12). A repeated measures ANOVA was performed and shows that the hydrolysis rates are effected by both treatment and experimental elapsed time as well as the interaction between treatment and experimental elapsed time (Table 6). Therefore, we know that there are statistical differences between the additions of nutrients and time points.

Table 6 Repeated measures ANOVA on carbon addition experiment.

Effect	DF	F	p
Treatment	4	6.9632	.000049
Experimental Elapsed Time	1	5.0444	.0267
Treatment: Experimental Elapsed Time	4	6.1039	.000178

CHAPTER 4: DISCUSSION

By conducting the storage experiment, we determined that after the initial read (t = 0) v_0 changes and after four hours of storing a sample the mean v_0 is statistically different than at t=0. Enzyme production is most variable after the initial read and the data show that measuring enzyme activity as soon as possible was critical to obtain the most accurate v_0 values and collect activities that are most similar to in situ measurements. Data show that although there is variation, storing in a cool dark place and measuring as soon as possible after collection will yield the most consistent results. When comparing multiple sites it would be ideal to keep the same sample/analysis plan to minimize error and aid in stability. We were able to use these data to create a proper sample plan to determine the controls on extracellular enzymes in east Tennessee and Northeastern Pennsylvania.

The data collected in this study provide evidence that DOC concentration and cell density exhibit similar relationships with EI. Both relationships follow nonlinear “L” shape patterns, where high concentrations of DOC and cells show low EI and low concentrations have varying EI. However, they do not show a correlation between DOC concentration and cell density. Water bodies characterized by low cell densities and low DOC concentrations vary EI, whereas water bodies characterized by high cell densities and DOC concentrations have relatively low EI. We see a relationship between endopeptidase and exopeptidase production and cell density in the storage data. The data collected

in the storage experiment, on March 23, 2016 at Second Creek, show the v_0 of exopeptidase (leucyl aminopeptidase), in room temperature samples, was almost 2 times as high as the v_0 of samples stored in the refrigerator. The opposite pattern occurred in endopeptidase (GGR-AMC) where the rate of decrease was faster in unfiltered room temperature water than sample that was stored in the refrigerator. We may be able to infer from these data that with cell growth there is increased exopeptidase activity. It is also possible that where there are more cells there is a higher chance the community would be more diverse, producing a wider range of enzymes or producing exopeptidase enzymes has a faster return rate than endopeptidase due to the nature of the enzyme (Arnosti et al., 2014). Alternatively, where there is more carbon there are more cells being produced and as a result the enzymatic production is much higher.

Several factors have been shown to structure community composition and abundance including, temperature (Furhman et al., 2008), light availability (Wagner et al., 2015), and salinity (Dupont et al., 2014; Fortunato et al., 2011; Crump et al., 2011). Although, it is unlikely there is a single control on community composition and abundance, one idea is DOC concentration is a driving force on microbial community abundance, as OC is critical for life and growth. Several studies (Bianchi et al., 2015; Crump et al., 2004; Fasching et al., 2016; Judd et al., 2006; Catalán et al., 2013) illustrate situations where organic matter is the dominating factor controlling community composition and abundance in freshwater.

Freshwater bodies do not just act as passive pipes for DOC transport, but are critical in transformation and storage of carbon (Cole et al., 2007; Evans & Thomas, 2016) through microbial processes such as respiration. Chemical diversity of DOC decreases as stream order increases (Creed et al., 2003). Often, low stream orders contain DOC which is characteristic of the catchment in which it originates and higher stream orders (12) have more heterogeneous DOC concentrations, resulting in more terrestrial derived DOC in small creeks and streams and a mixture in ponds, lakes, and large rivers (Creed et al., 2003). Allochthonous, terrestrial derived, organic carbon is generally less bioavailable making it harder to breakdown resulting in accumulation.

DOM is extremely complex in terms of composition and molecular structure and as a result it is difficult to characterize DOM (Carlson & Hansell 2002). One way to study OM is by looking at the fluorescence properties of dissolved organic matter. Although FDOM refers to the small portion of colored organic matter (CDOM) that fluoresces it fairly accurately and relatively fast measurements that can be used to identify organic carbon source (Chen, 1999). This is a longstanding method and has been used to identify sources of organic matter (Cabaniss & Shuman, 1987). Over the years several indices have been proposed to try and classify and understand the optical properties of DOM. These indices look at certain sections of the excitation/emission spectra and provide information on aromaticity and OM origin. A few common indices, derived from excitation-emission spectra, used are fluorescence index (FI)

(Mcknight et al., 2001), humification index (HIX) (Zsolnay et al., 1999), biological index (BIX) (Huguet et al., 2009), and freshness index (Wilson & Xenopoulos, 2008). The specific ultraviolet absorbance at 254 ($SUVA_{254}$) (Miller & McKnight, 2010) also provides insightful information from UV-Vis data. It is important to look at several different indices to avoid interference error from other dissolved constituents. For example, $SUVA_{254}$ can be altered by iron and nitrate, which absorb across the region including 254 nm. This specific error occurs more frequently when DOC concentrations are low, as iron and nitrate greater in abundance (Miller & McKnight, 2010). We calculated several indices in order to understand how the source and freshness of DOM influence enzyme activity.

I used fluorescence index to obtain a better idea of what type of organic carbon was present in our samples. The FI values in ponds was lower, providing evidence that higher concentrations of recalcitrant organic carbon are a result of terrestrial inputs (Appendix B2). The highest FI values were in creeks, ranging from 1.7-1.85, suggesting more microbial influence. A mix of terrestrial and microbial sourced DOM was seen in creeks, lakes, and quarries and was indicated by FI values between 1.5 and 1.65. A mix of terrestrial and microbial derived carbon is expected as the lakes and rivers that were sampled are more of a transitional fresh water body. These data support the hypothesis that EI activity is controlled by the freshness of organic matter. There is higher exopeptidase activity in samples that have low FI values, and higher FI values where endopeptidase production is more prevalent (Appendix B2).

$SUVA_{254}$ is used as a surrogate for DOC aromaticity and can be a good indication of humic substances by measuring absorption by fulvic acids (Miller & McKnight, 2010). Fulvic acids that are microbial derived are composed of roughly 12-17% aromatic carbon where as the aromatic portion of terrestrial derived fulvic acids is closer to 25-30% total carbon. As a result of lower aromaticity microbial derived fulvic acids absorb less visible and ultra violet light than terrestrial derived fulvic acids (Mcknight et al., 2001). Therefore we use UV absorbance data to provide structural information about the DOC and to understand the origin of the fulvic acids, which make up a large portion of humic substances (Mcknight et al., 2001). $SUVA_{254}$ is used as a proxy for aromaticity, as it is an average absorptivity for all DOC molecules. We see that in the majority of ponds (higher DOC concentrations) there are higher values of $SUVA_{254}$ and in bodies of water with low concentrations of DOC the $SUVA_{254}$ are lower.

The humification index (HIX) was introduced by Zsolnay et al. (1999) and is used to identify the source of DOM, ranging from humic to microbial derived. Humic material fluoresces to a greater degree than extracellular material (Zsolnay et al., 1999). HIX indicates aromaticity and is therefore used as proxy for the source of OM (allochthonous vs autochthonous). HIX values (<4) indicate autocthonous OM sources and high (10-12) indicate allochthonous. These data do not follow the same patterns that the other indices measured show (Appendix B5). There are values ranging from 0.4-0.9 with EI ratios varying throughout. Although we see ponds with HIX values greater than 0.6 and creeks

being less than 0.65, in this system HIX does not reflect the patterns observed using other indices (Appendix B5). The other indices measured indicate there are inputs of both terrestrial and microbial derived organic carbon and the HIX values suggests all of the OM is microbial derived.

Increasing BIX is an indication of an increase in the concentration of β fluorophore. Autochthonous OM is indicated by a high BIX value (>1) and allochthonous OM is represented by low BIX values (0.6-0.7) (Huguet et al., 2009). Ponds have lower BIX values indicating they are more allocthonous in source while the other site types (for the majority) are greater than 7, meaning more autochthonous sources of carbon. Although most ponds sampled had visible algal growth, there was also significant decomposition of vegetation, including leaves, branches, and other plants.

Higher values of the freshness index, β:α, represent a system that has more autochthonous or fresh organic matter. Systems that have low β:α have higher values, indicating that the system may have larger abundance of old organic matter, or allochthonous (Wilson & Xenopoulos, 2008). The freshness index shows that in this system overall ponds have lower β: α, indicating the presence of older organic matter. Many of the samples fall between the highest and lowest values, signifying a variation and range in age of organic carbon. At the highest values present (>0.8) the majority of samples are creeks, representative of fresher organic matter and higher EI.

Bodies of water with higher concentrations of terrestrial DOC, show higher

exopeptidase activity, implying that exopeptidase production is more prevalent when recalcitrant organic carbon is present and less prevalent in areas with labile organic carbon. Likewise, it is a possibility that endopeptidase enzymes are produced in areas that have higher biological growth rates and more microbial activity and exopeptidase enzymes are produced in abundance when there is a lack of easily accessible carbon. The same trends are seen in particulate organic carbon.

We would expect that when ammonia concentrations increase there would be an increase in overall peptidase activity because ammonia is being created through decomposition. If reduction in ammonia leads to increase in nitrate (Wetzel, 2001) there would be a direct effect on the hydrolysis rates of protein degrading enzyme production. Increased nitrate levels could also be an indication of high OM mineralization rates. We can see that in (Appendix B9) as NH_3 concentration decreases, there is an decrease in exopeptidase production.

Environmental parameters consisting of pH, temperature, salinity, and dissolved oxygen all influence and are influenced by both community composition and DOC concentration (Bianchi et al., 2015; Crump et al., 2004; Fasching et al., 2016; Judd et al., 2006; Catalán et al., 2013). Extracellular enzyme activity is temperature dependent, often having slower reaction rates in colder temperatures and higher rates in warmer temperatures (Arnosti et al., 1998). As a result, in marine environments, enzyme activity and organic matter degradation occur at higher rates in the summer months (Arnosti et al., 1998).

The reaction is not only slowed down, but growth rates and membrane permeability is altered with temperature change (Arnosti et al., 1998). Enzymes have temperature optima, below temperature optima the reaction is kinetically controlled (Scopes, 2002). These reactions can increase 4-6% per degree. However, when the temperature exceeds the optima the enzymes are denatured and the activity is greatly reduced (Scopes, 2002). These data suggest that in T < 21 ºC there is greater production of endopeptidase and when T > 21 ºC exopeptidase is more prevalent (Appendix B13). It is hard to determine the driving factor for this trend, as temperature not only effects microorganisms but can be affected by several other environmental parameters that may be more prominent such as pH.

The same pattern is not observed between pH and EI (Appendix B12), as temperature and EI. Although assays were carried out at a defined neutral pH, these data show a definite peak in EI suggesting an optimal pH (7-8) where endopeptidase production occurs and in conditions below pH = 7 and above pH = 8 exopeptidase production occurs. It also appears that the majority of the sites within this “optimal range” are creeks with varying DOC concentrations.

Dissolved oxygen is essential for aquatic ecosystems. It both fuels respiratory metabolisms and drives ecosystem productivity (Wetzel, 2001). These data show that when DO > 7 endopeptidase activity is more prominent and when DO <7 exopeptidase activity is more prevalent. Low concentrations of DO could indicate an impulse or high DOM concentrations, which is likely the

case as ponds had the highest DOC concentrations and have the lowest DO (Wetzel, 2001). Aquatic animals must alter breathing patterns when there is a significant reduction in dissolved oxygen (Cox, 2003). Dissolved oxygen, in a temperate circulating lake at sea level, is normally at or near saturation, 12-13 mg mL^{-1}, as it equilibrates with atmospheric oxygen in the spring when temperatures are near 4 ºC (Wetzel, 2001). However, concentrations decrease in summer months with increased temperature. DO can also be affected by organic matter input and longer residence times. Large input of organic matter greatly increases the demand for oxygen, as it is needed for microbial degradation. Loading of organic matter can occur during times of excess leaf fall, anthropogenic input, and large storm events.

Specific conductivity (SPC) was the last environmental parameter analyzed showing no relationship between EI and SPC (Appendix B15). SPC shows some segregation by site type but no statistically significant relationship ($p < 0.05$). Four major ions (calcium, magnesium, sodium, and potassium) comprise salinity in freshwater and averages 125 mg L^{-1} in surface waters (Wetzel, 2001). Salts can come from several sources including weathering of rocks and soil, anthropogenic inputs, and atmospheric precipitation, which is most common source of saline in fresh water sources (Wetzel, 2001). However, east Tennessee is underlain by karst topography, composed of limestone ($CaCO_3$) and dolostone ($CaMg(CO_3)_2$). When precipitation levels are high some of the karsts fill with water then drain into streams, bringing salts, and eventually

increased salts may be introduced in to the river network (Maclay, 1962). Microorganisms in freshwater bodies can be affected by an increase in salt concentrations, mainly as a function of anthropogenic input (Nielsen et al., 2003). Although there are natural high and low periods, these fluctuations do not normally affect the threshold which microorganism can tolerate (Nielsen et al., 2003). Increased saline concentrations can lead to alteration of light and nutrient mixing, in turn affecting photosynthesis by altering light penetration. Freshwater organisms are influenced equally by pH and dissolved ion concentration, making saline an important environmental parameter (Nielsen et al., 2003). However, according to Nielsen et al. (2003), freshwater microbial communities can generally be unaffected by concentrations up to 1000 mg L^{-1} which was not seen in these samples.

The multiple regression model indicated that dissolved oxygen and pH were the most influential on EI. However, these models cannot be fully accepted because the assumptions were not met. The data was transformed several ways including log and rank transformation and neither case satisfied the assumptions of the residuals being normally distributed and homoscedastic. The validation of the model was further disregarded when removing elevation resulted in total nitrogen being the only significant parameter. It wouldn't make sense that pH and DO would heavily influence the model only when elevation was included.

We can also look at the effects, on enzyme activity, of adding known carbon sources. Preliminary data suggests that in all treatments except for the

BSA addition, Lue- AMC, GGR-AMC and Arg- AMC all follow similar patterns. The average hydrolysis rates vary between treatment but we see the same trends, where Leu-AMC is highest in the first week and then drastically decreases, Arg-AMC and GGR-AMC both start off fairly low ($v_0 < 20$) and increase after the first week ($v_0 > 20$). In the treatment with the addition of BSA, GGR-AMC increased almost 60 times that of other treatments. This indicates that peptidase production it is not dependent on the type of carbon that is added but rather the composition of the carbon source. Both BSA and acetate are forms of labile organic carbon, however BSA is a protein, derived from cow blood, and acetate is not a viable protein source. We know that microbial communities can shift enzyme production based on substrate availability, and that can happen in a matter of minutes, however these data indicate that it can take longer ($t > 200$ hrs) for the community to start utilizing those enzymes. These data also suggest we may be able to infer what the composition of the organic matter is based on the types of enzymes that are present. In areas that have “fresh”-protein rich organic matter there is much higher endopeptidase activity and areas that do not have not protein enrichment exopeptidase activity was higher.

CHAPTER 5: CONCLUSION

This study aimed to provide insight on what environmental parameters control the production of endopeptidase and exopeptidase enzymes. We can conclude that, despite DOC and cell densities not correlating, they both have a role in determining which type of peptidase enzyme is produced. There were higher levels of exopeptidase activity in areas with high concentrations of recalcitrant organic carbon and in areas with low concentrations of labile organic carbon there is higher endopeptidase activity. These ratios imply that protein degradation pathways are variable in freshwater systems and the mechanisms used to break down organic matter vary based on the freshness of OM and the cell density.

We also learned that trypsin can be used as a proxy for bioavailable protein. When adding fresh intact protein (BSA) to a freshwater system we saw a positive response in terms of trypsin activity. This indicates that the community was able to shift enzyme production to go after that bioavailable protein. This is the first time, we are aware of, that a proxy for bioavailable protein has been found.

Understanding what controls extracellular enzyme activity, specifically the effect of carbon freshness, can lead to a better understanding of big picture carbon cycling and implications of anthropogenic impacts. If specific extracellular enzyme production is a function of carbon freshness and cell density, with a

changing environment we will see significant impacts on degradation and utilization of organic carbon.

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APPENDICES

Appendix A

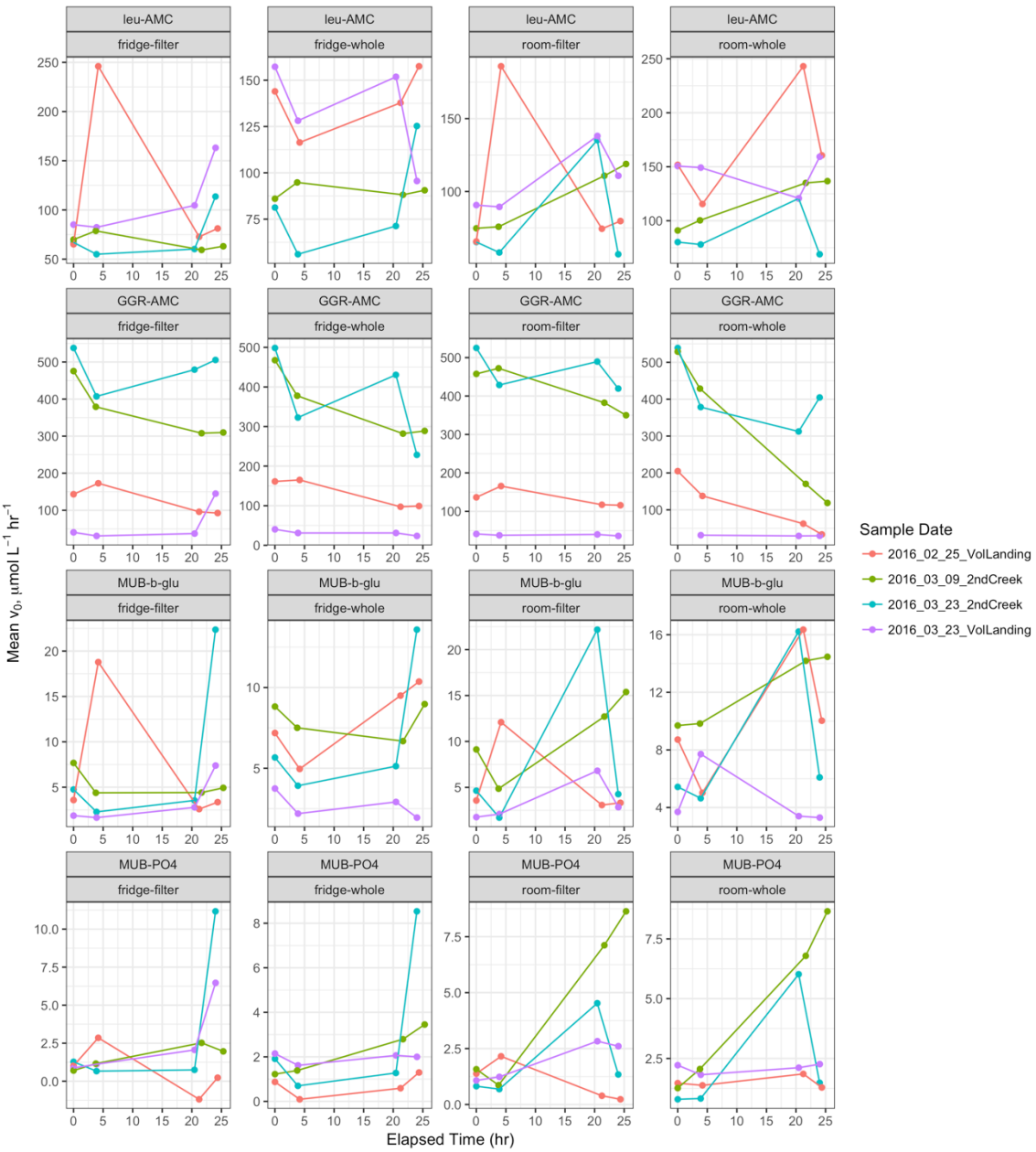


Figure A1 Enzyme activity at Second Creek March 9, 2016 and March 23, 2016 and Volunteer Landing February 25, 2016 and March 23, 2017.

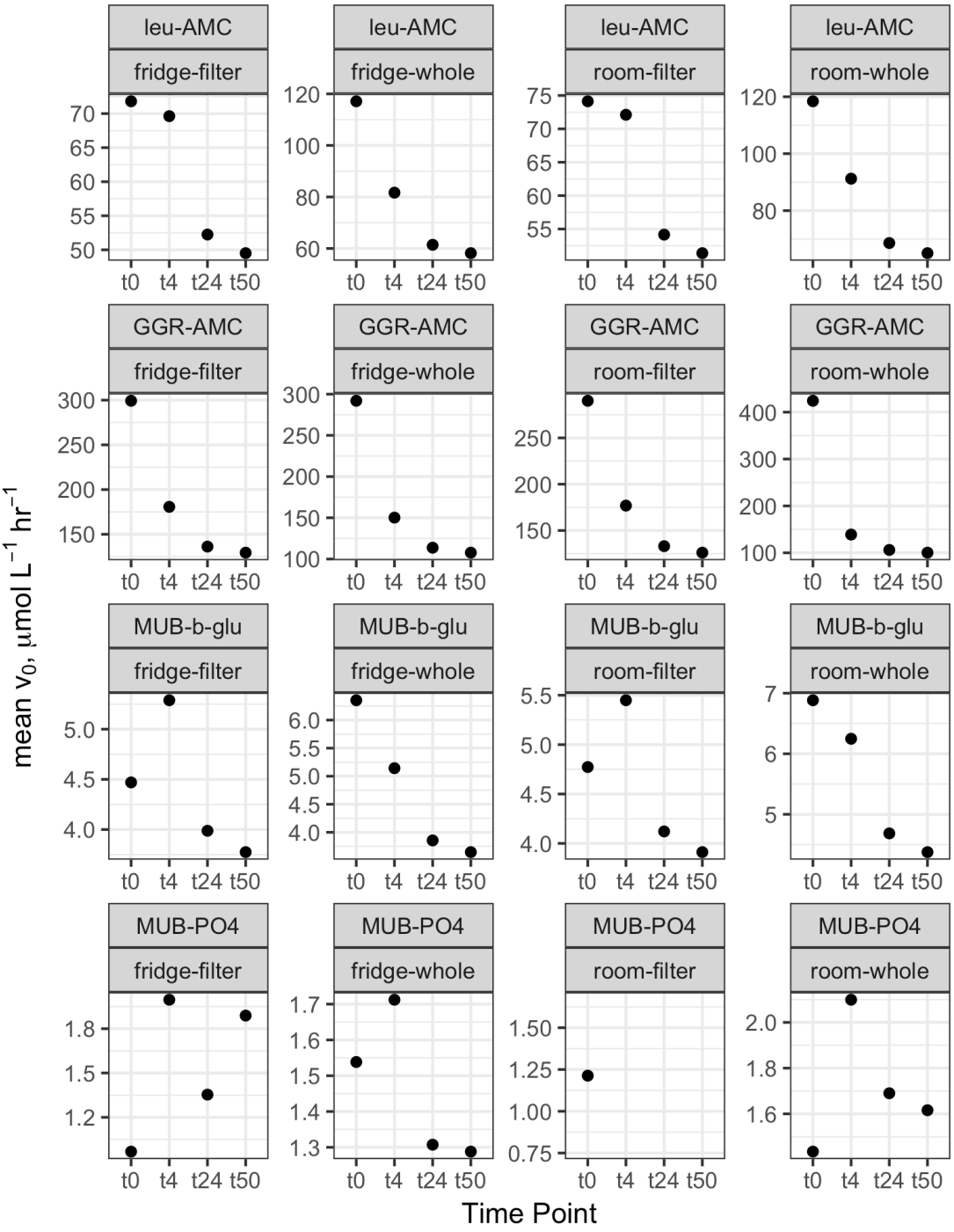


Figure A2 Average hydrolysis rate, of all four samples, at each time point.

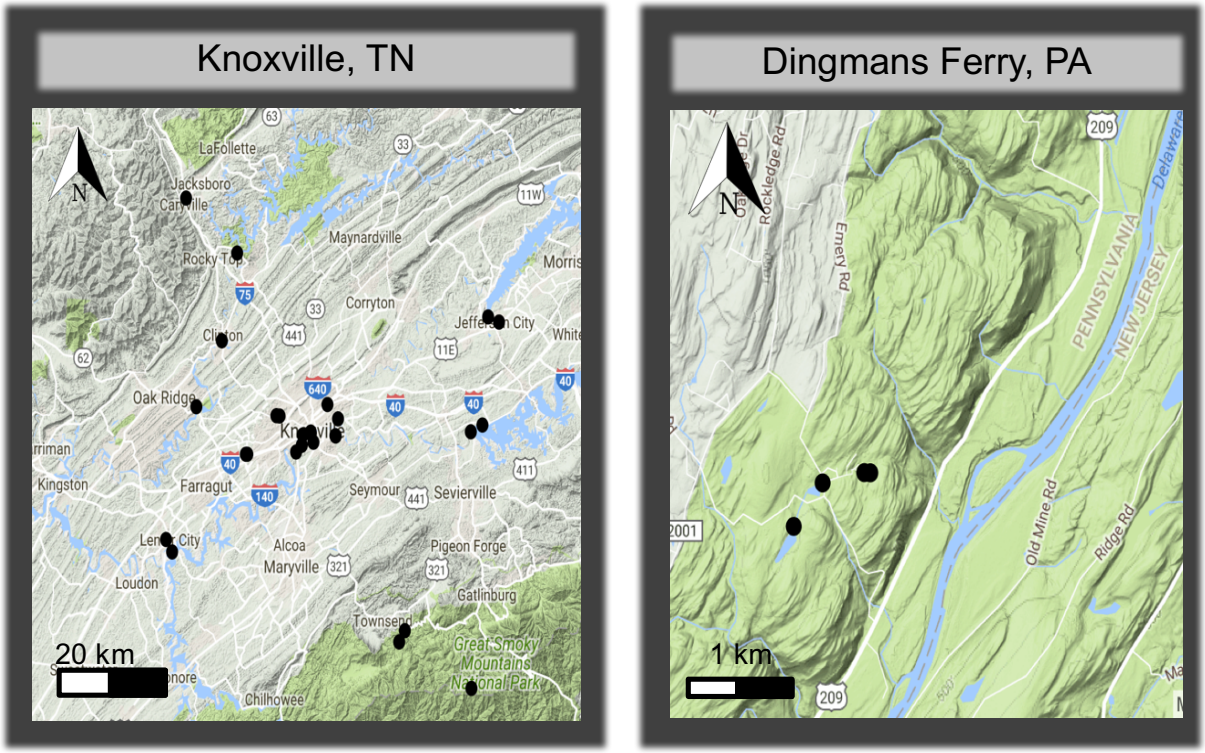


Figure A3- Sample locations in the Knoxville, TN area and Dingmans Ferry, PA

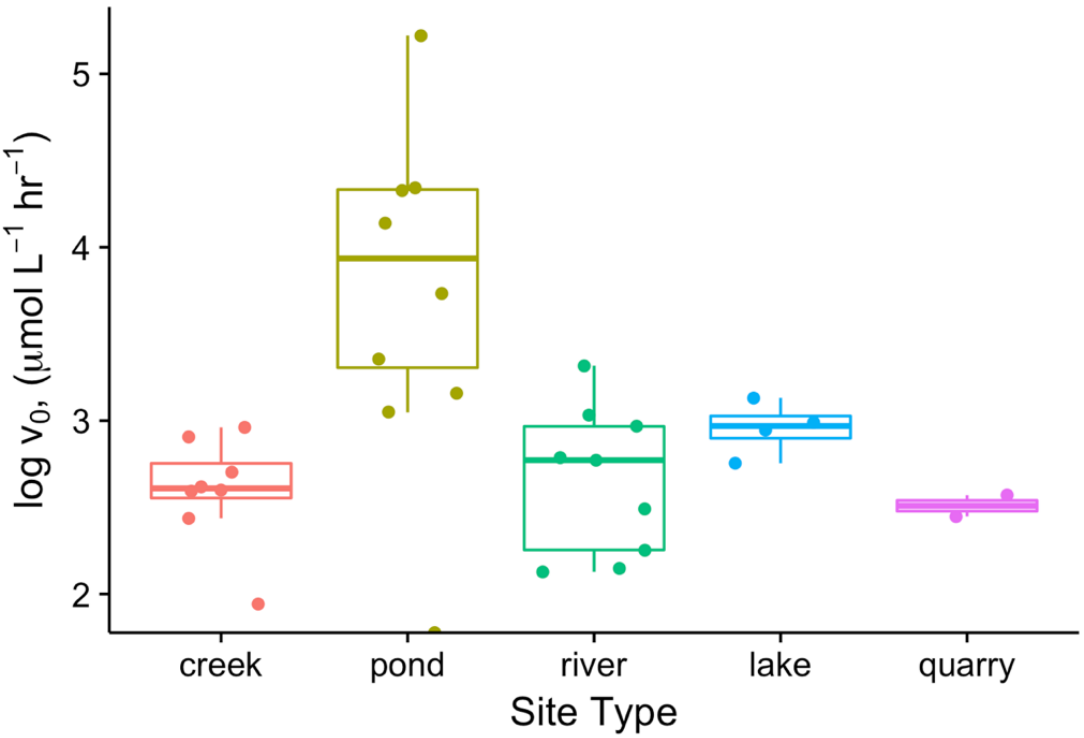


Figure A4 Total Hydrolysis Rate per Site, ordered by decreasing hydrolysis rate. Sites group by site type, where ponds have highest hydrolysis rates and creeks and quarries at the lower end. Rivers and lakes are dispersed within the other site types.

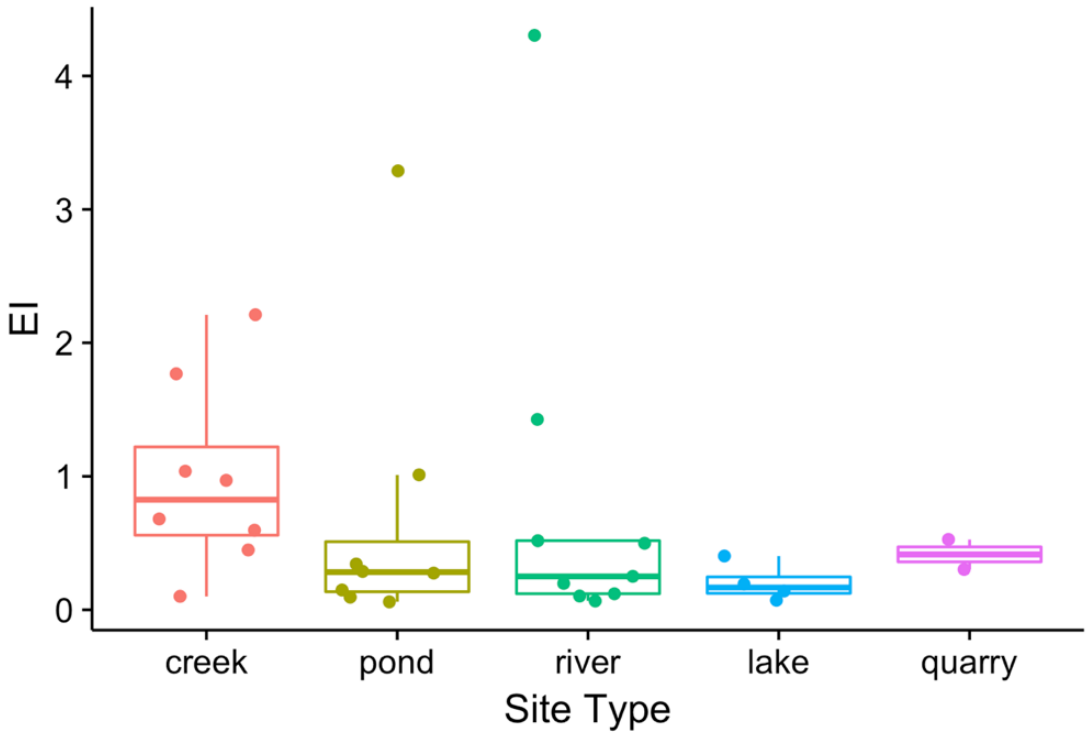


Figure A5 Endopeptidase to Exopeptidase Activity per Site. The same patterns do not exist when looking at the ratio of degradation mechanisms.

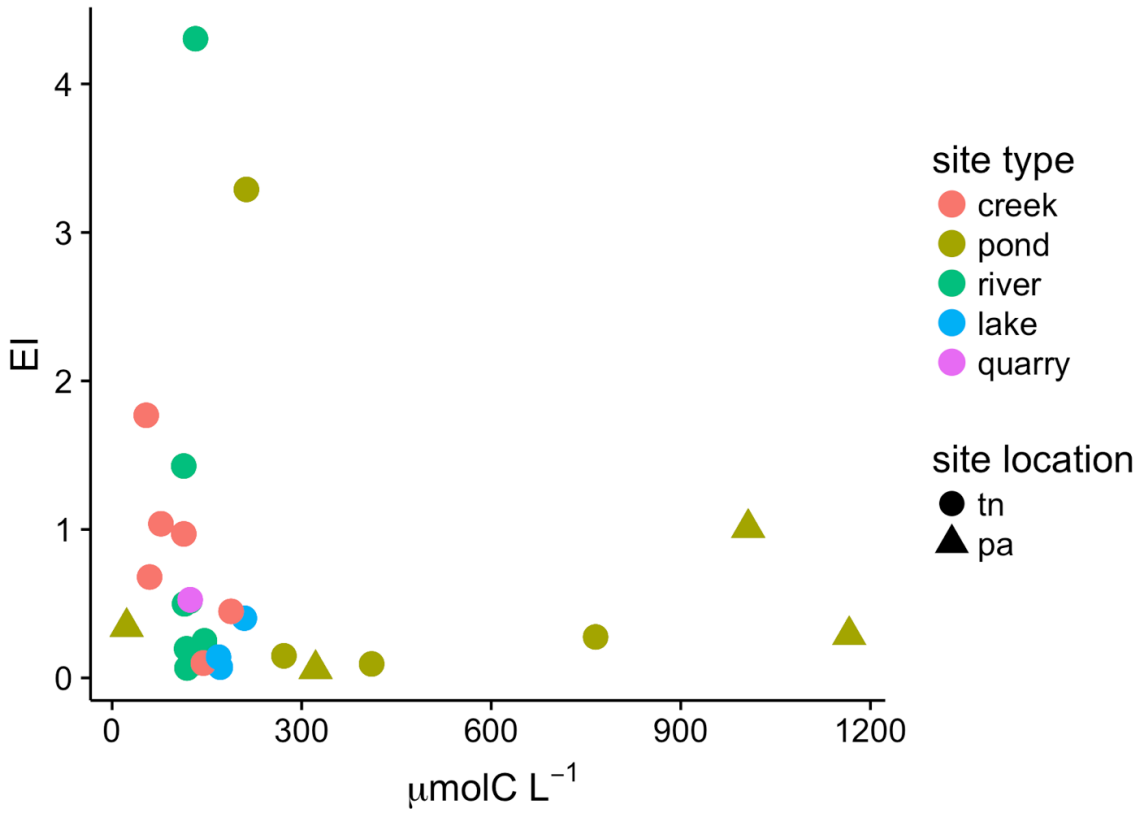


Figure A6 EI as a function of DOC concentrations.

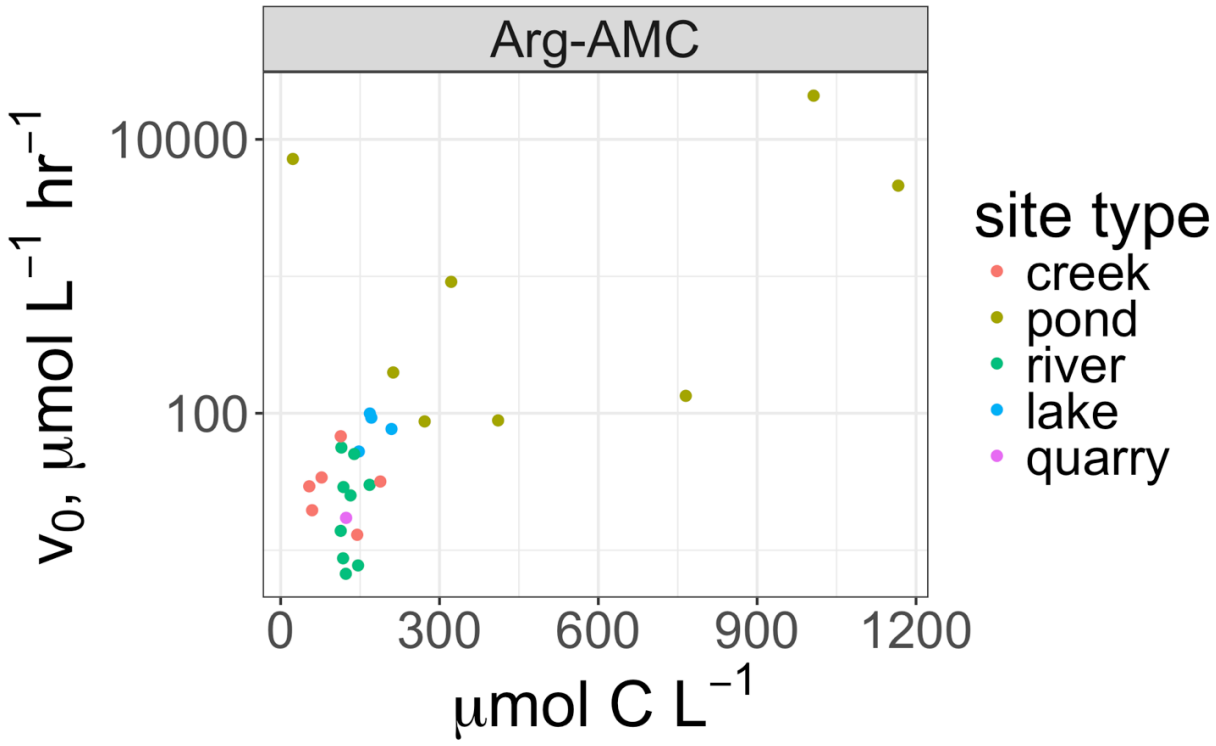


Figure A7 v_0 of Arg-AMC as a function of DOC

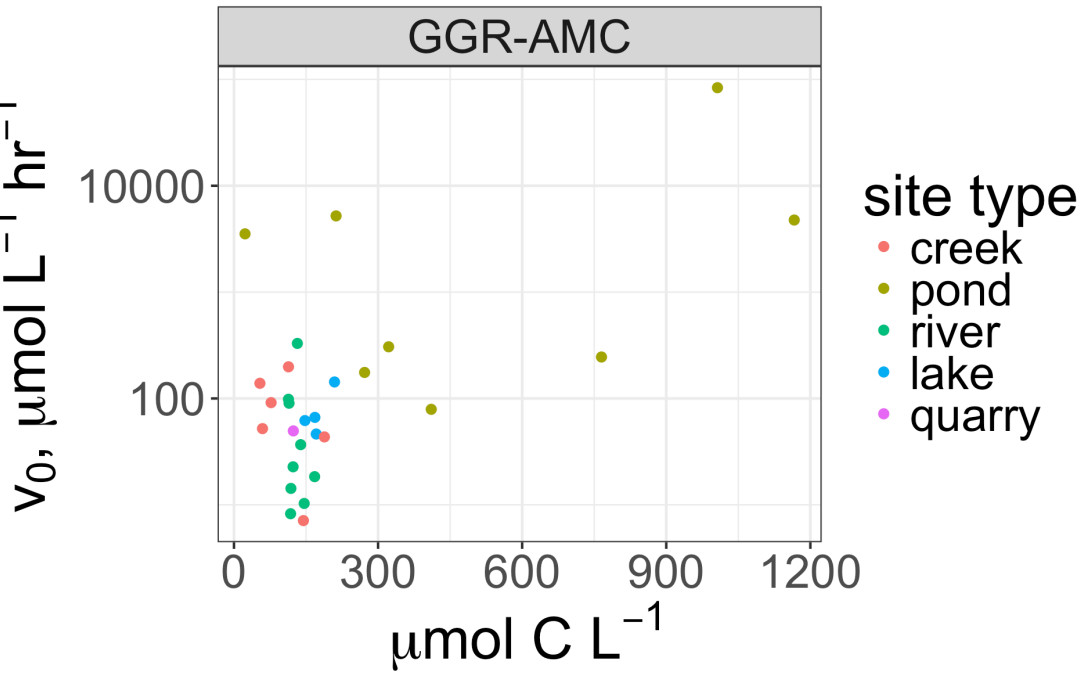


Figure A8 v_0 GGR-AMC as a function of DOC

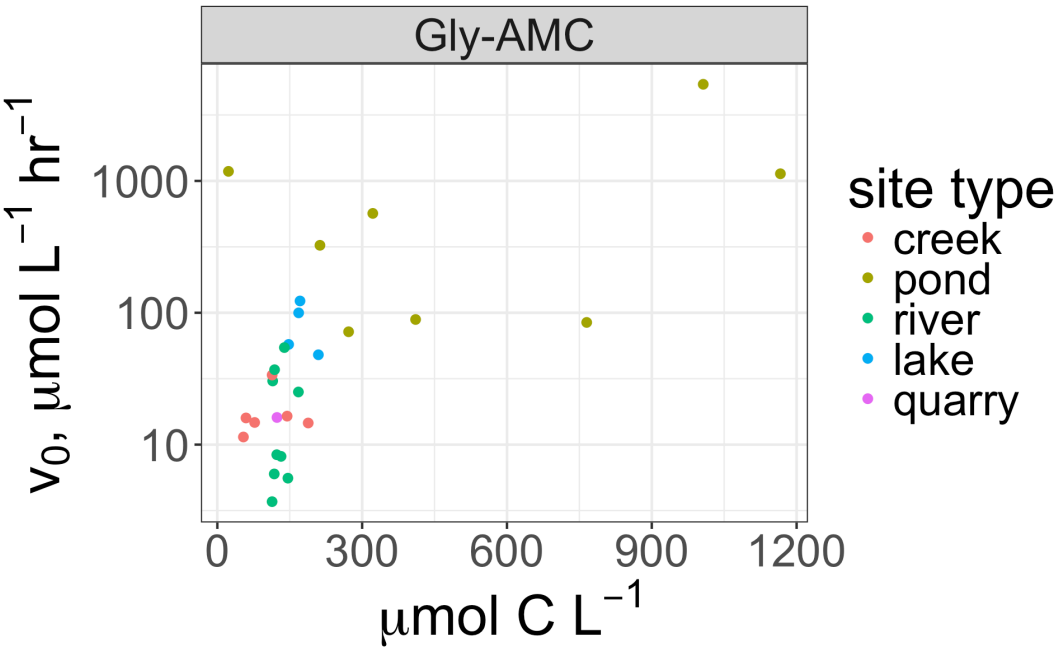


Figure A9 v_0 Gly-AMC as a function of DOC

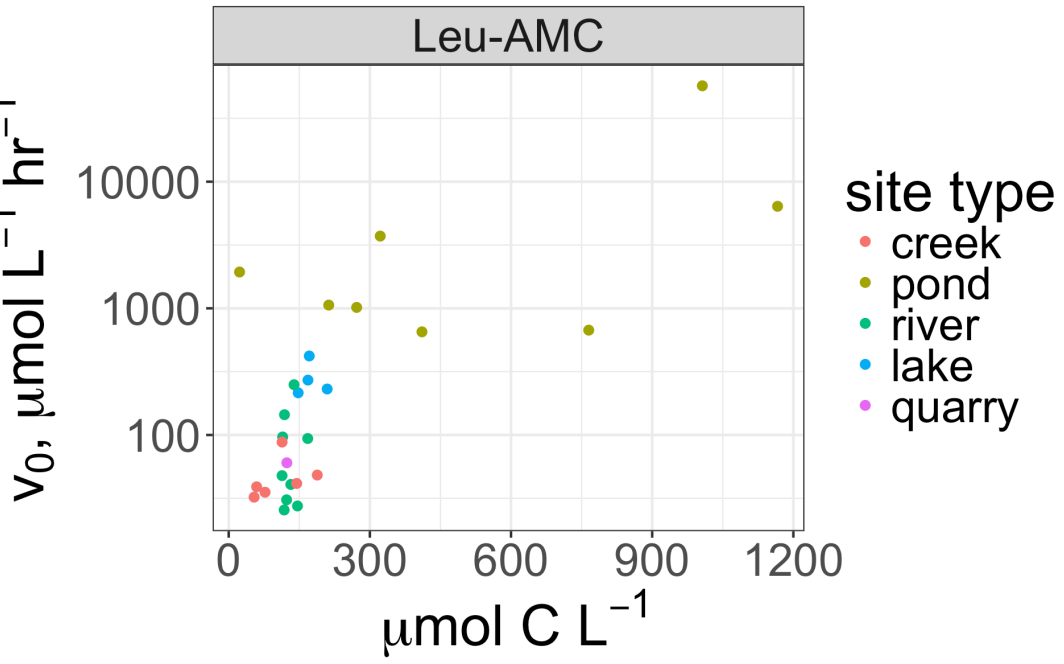


Figure A10 v_0 of Leu-AMC as a function of DOC

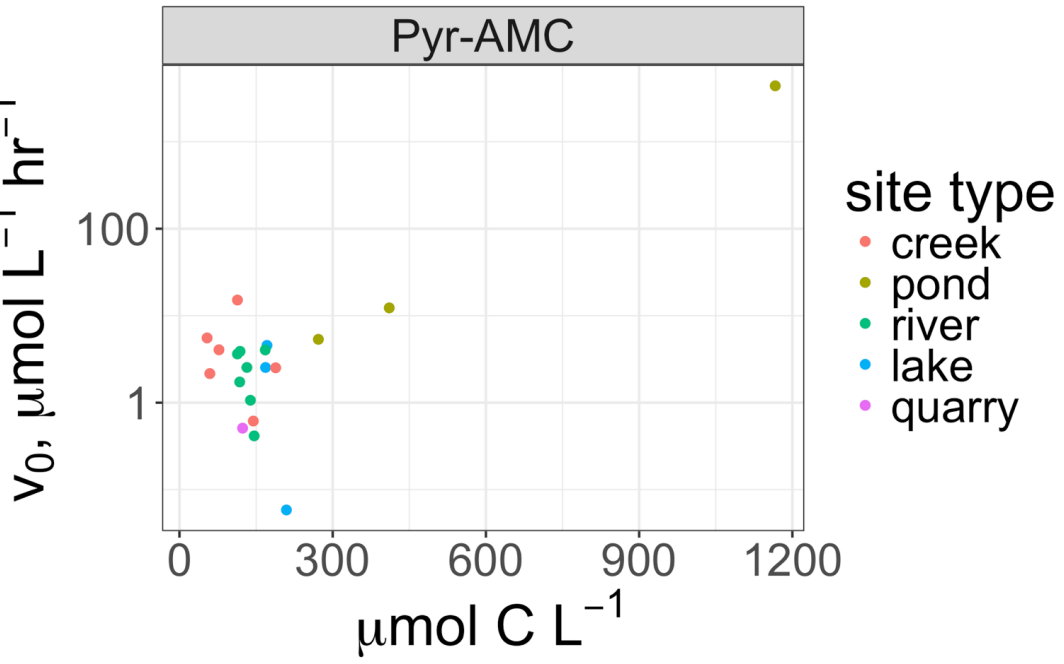


Figure A11 v0 of Pyr-AMC as a function of DOC

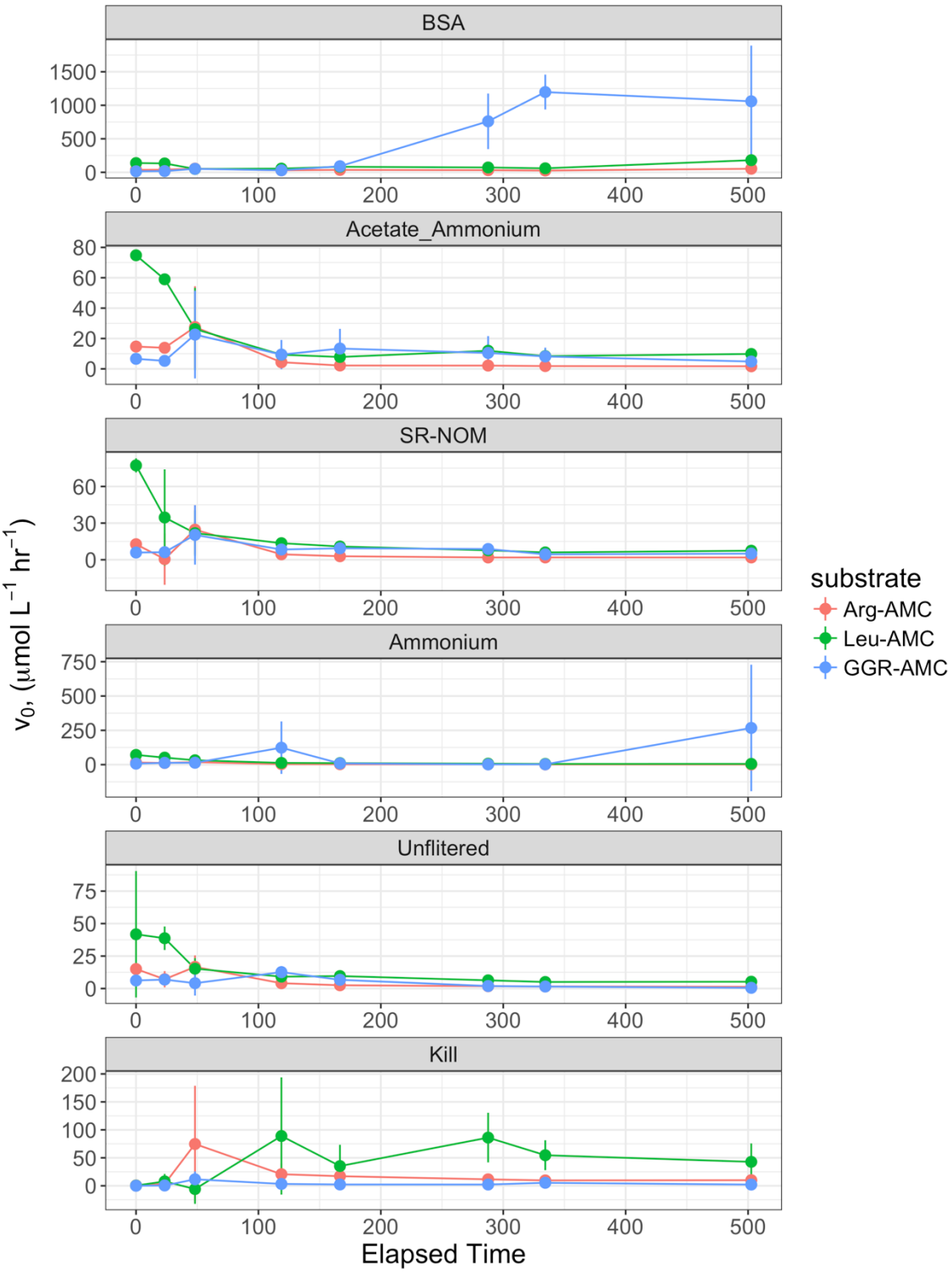


Figure A12 Effects of carbon addition on El. Treatment A-BSA B- Acetate & Ammonium C-SR-NOM D- Ammonium E-Unfiltered Sample F-Killed Control

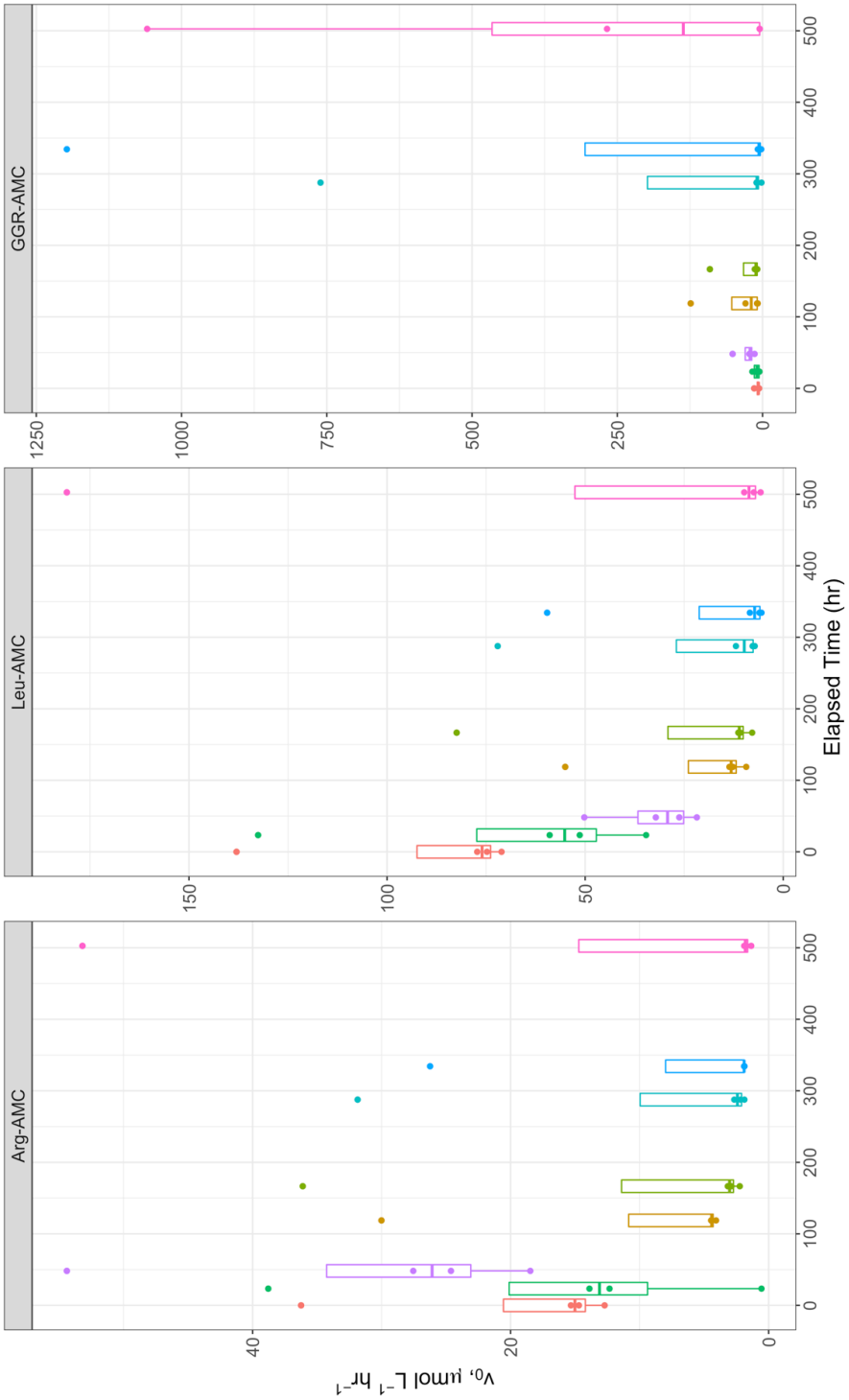
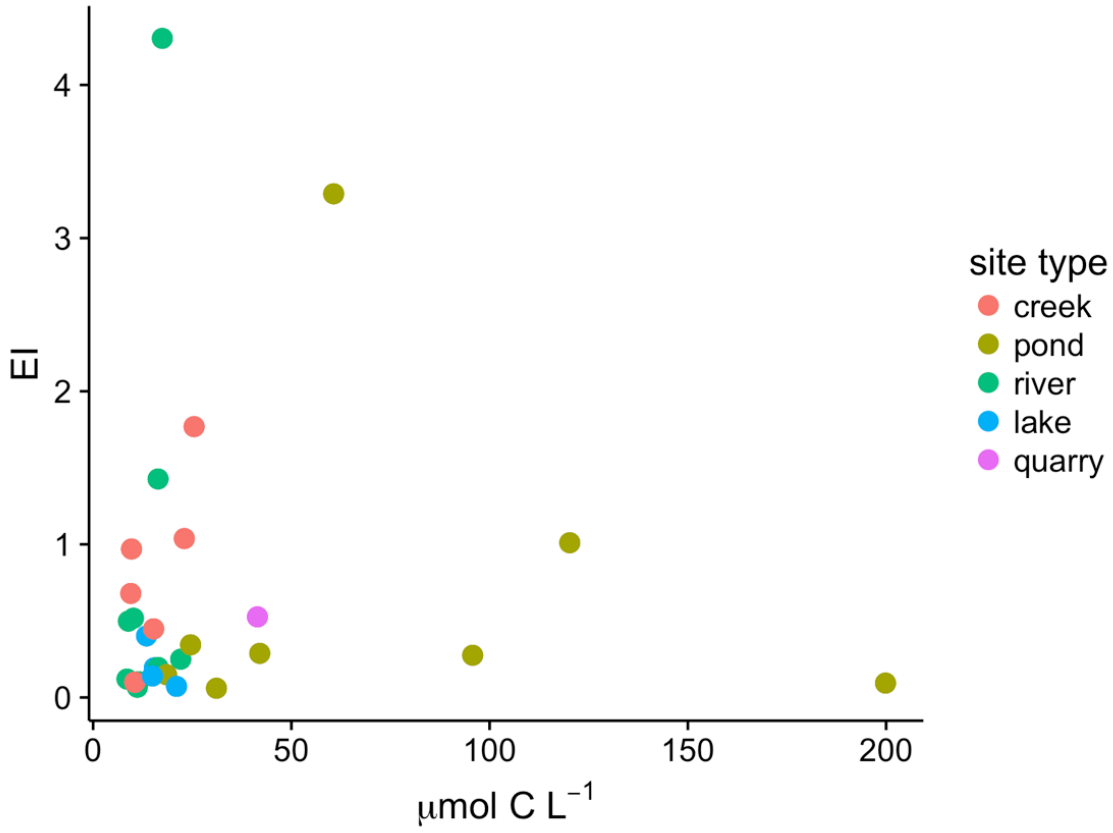
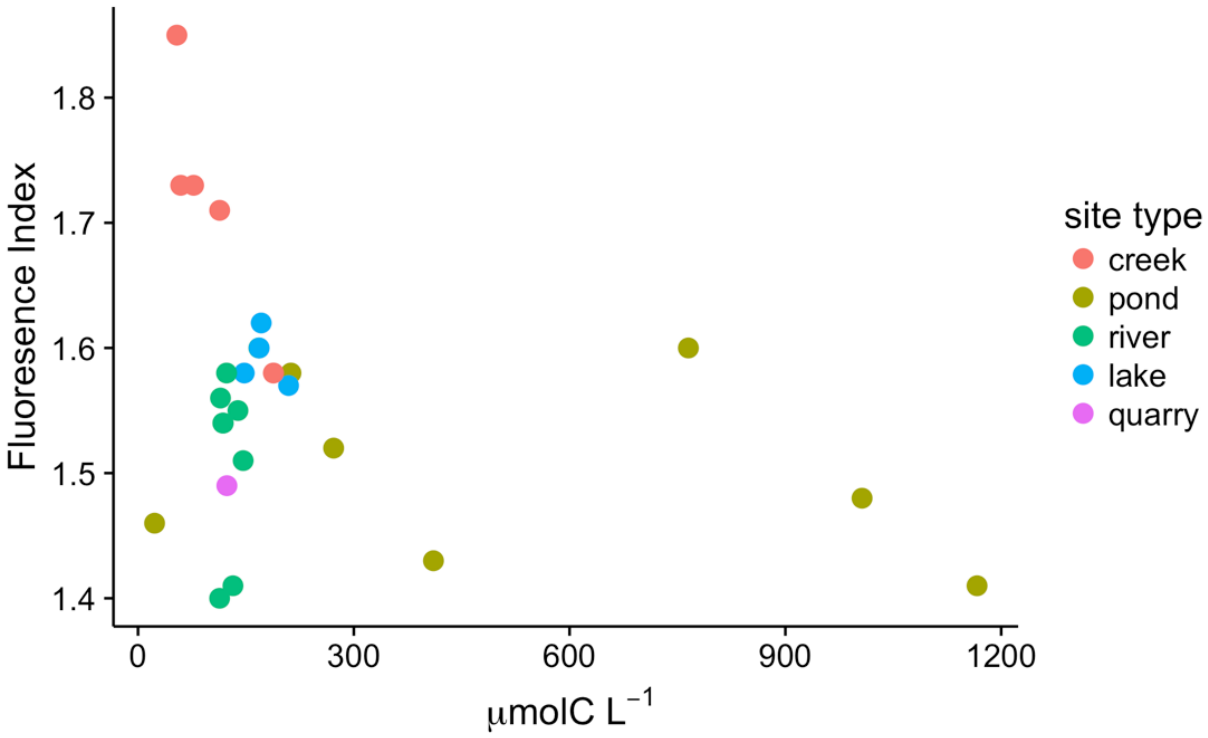


Figure A13 Hydrolysis rates, for each substrate, per time point. Repeated measures ANOVA indicates there is statistical differences between treatments in each substrate. Colors represent measured time point.

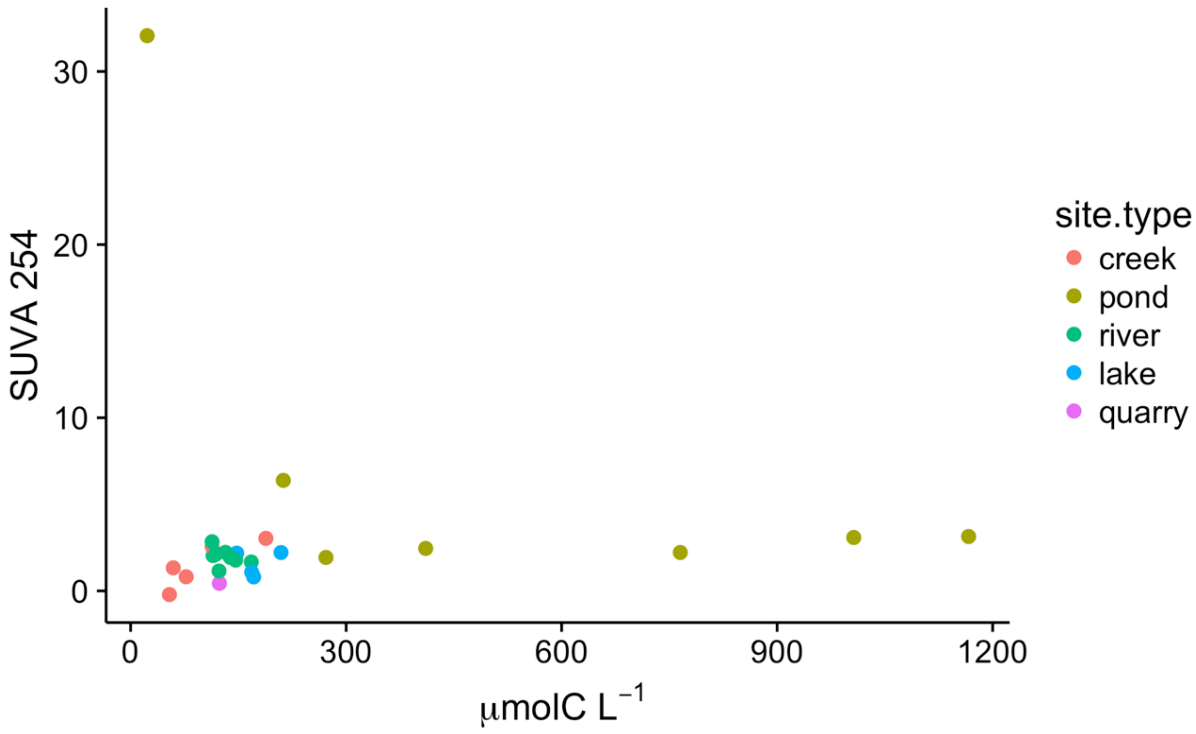
Appendix B



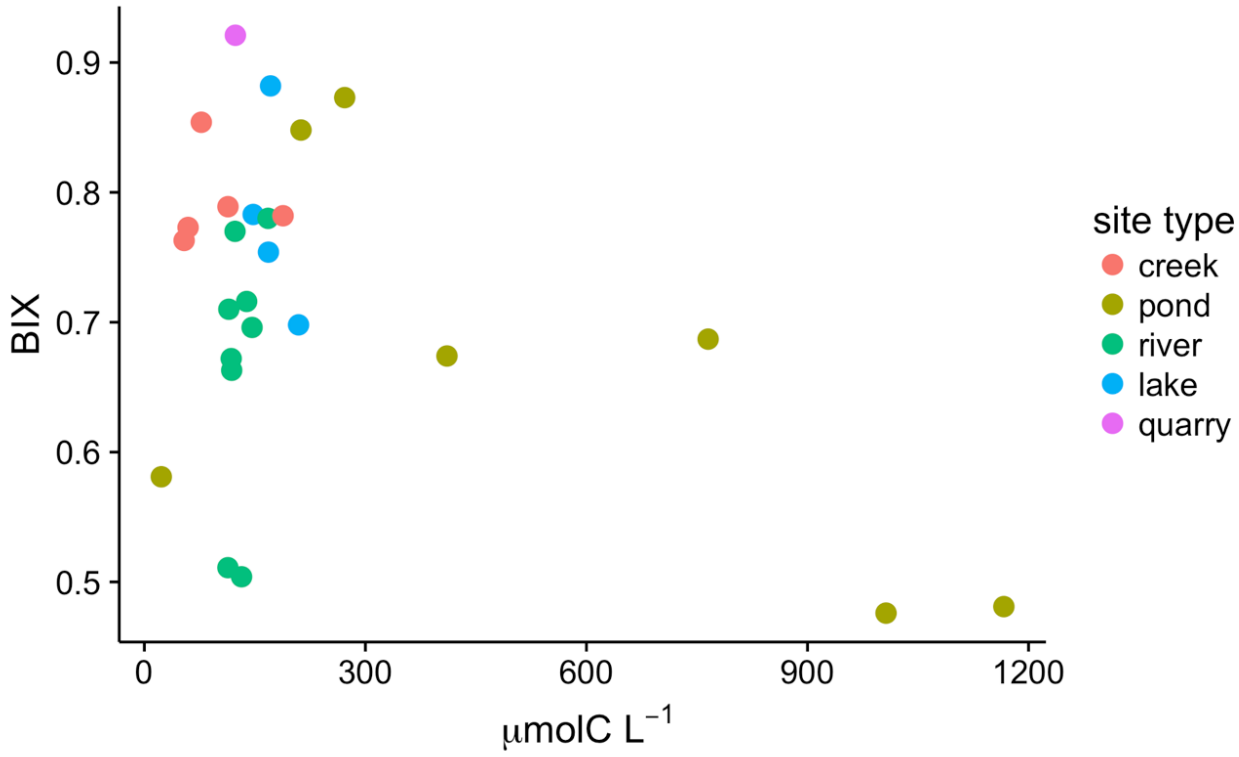
B1- EI as a function of POC



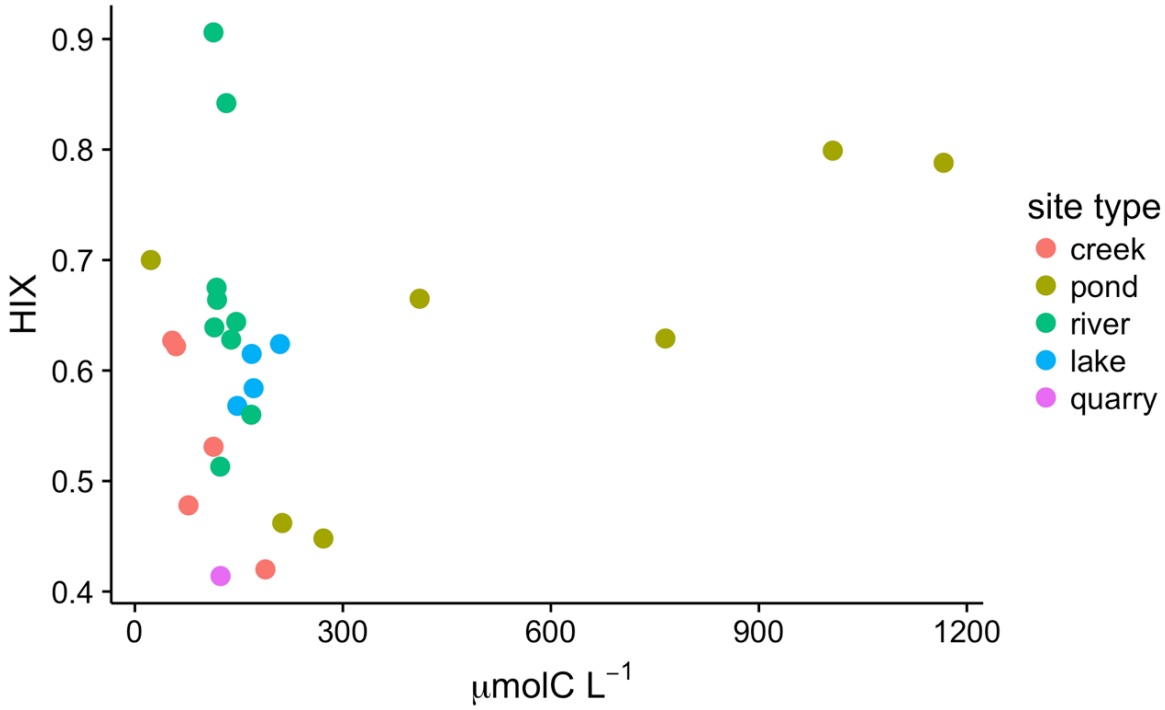
B2- Fluorescence Index as a function of DOC concentration. FI closer to 1.4 = terrestrial derived OC and FI closer to 1.9 = microbial derived OC



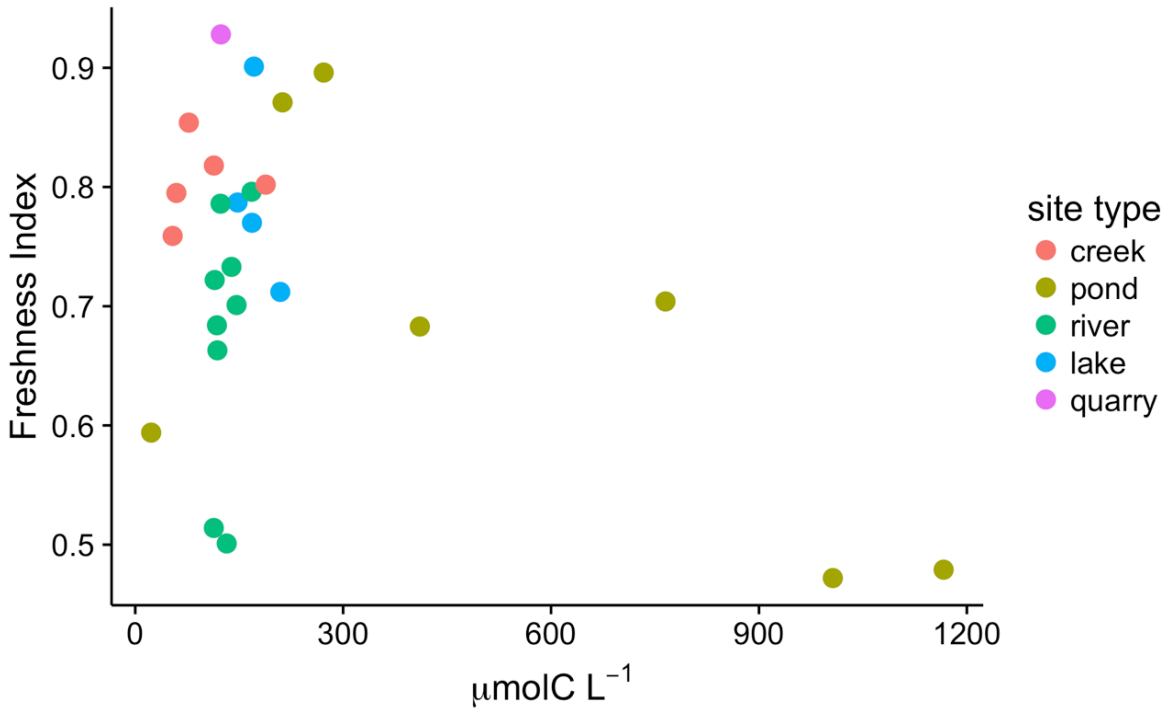
B3- Specific ultraviolet absorbance at 254 nm as a function of OC



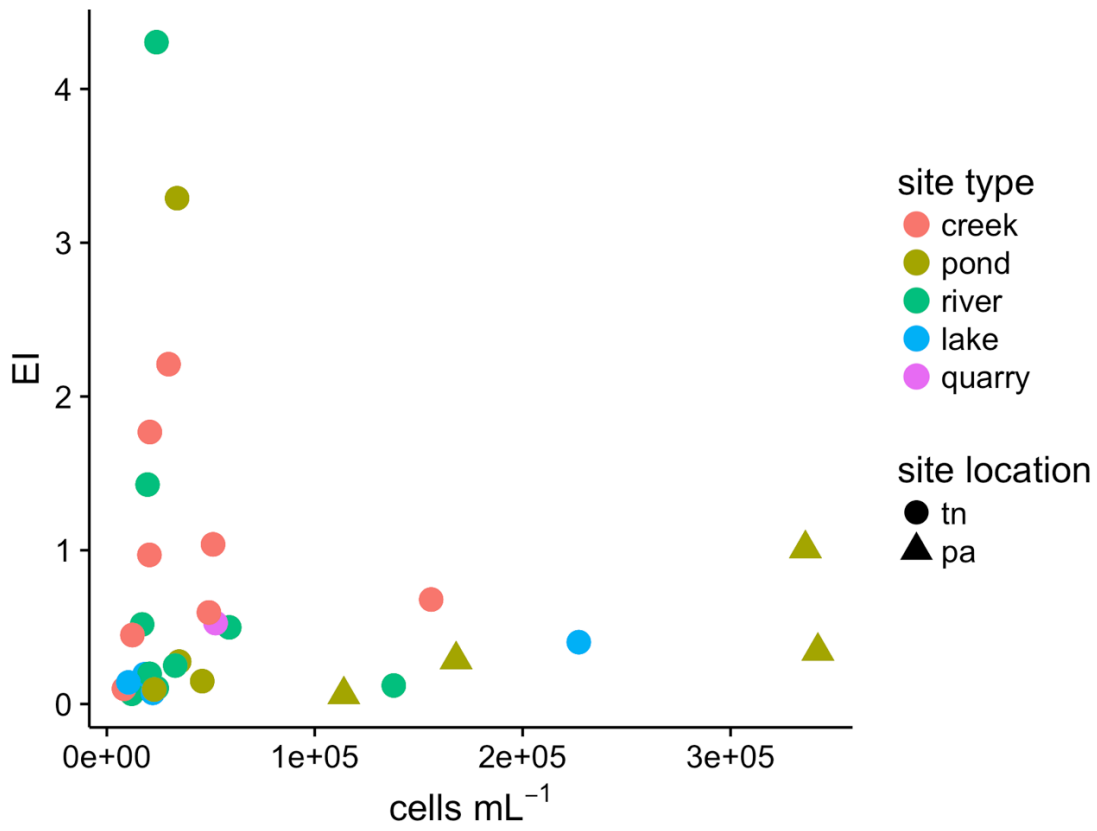
B4- Biological Index as a function of OC



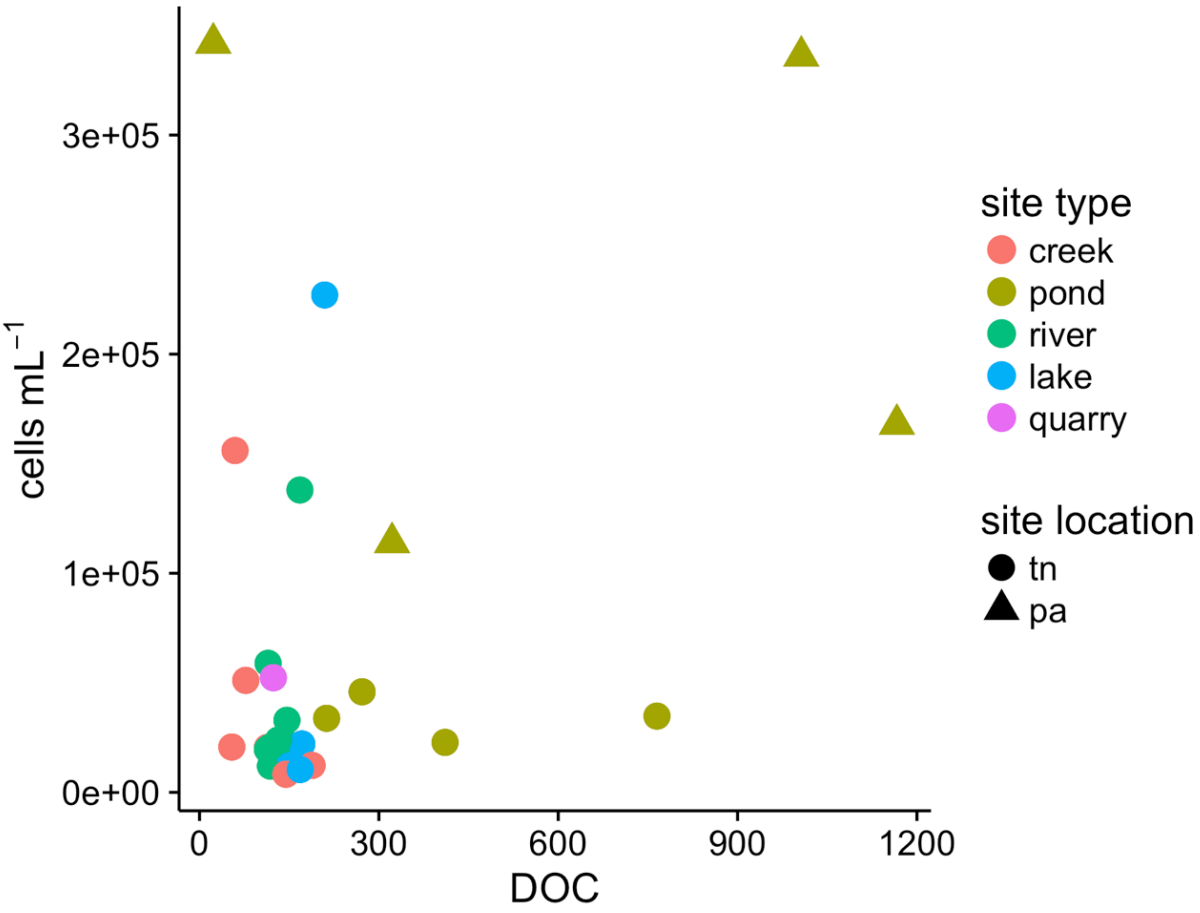
B5- Humification index as a function of OC



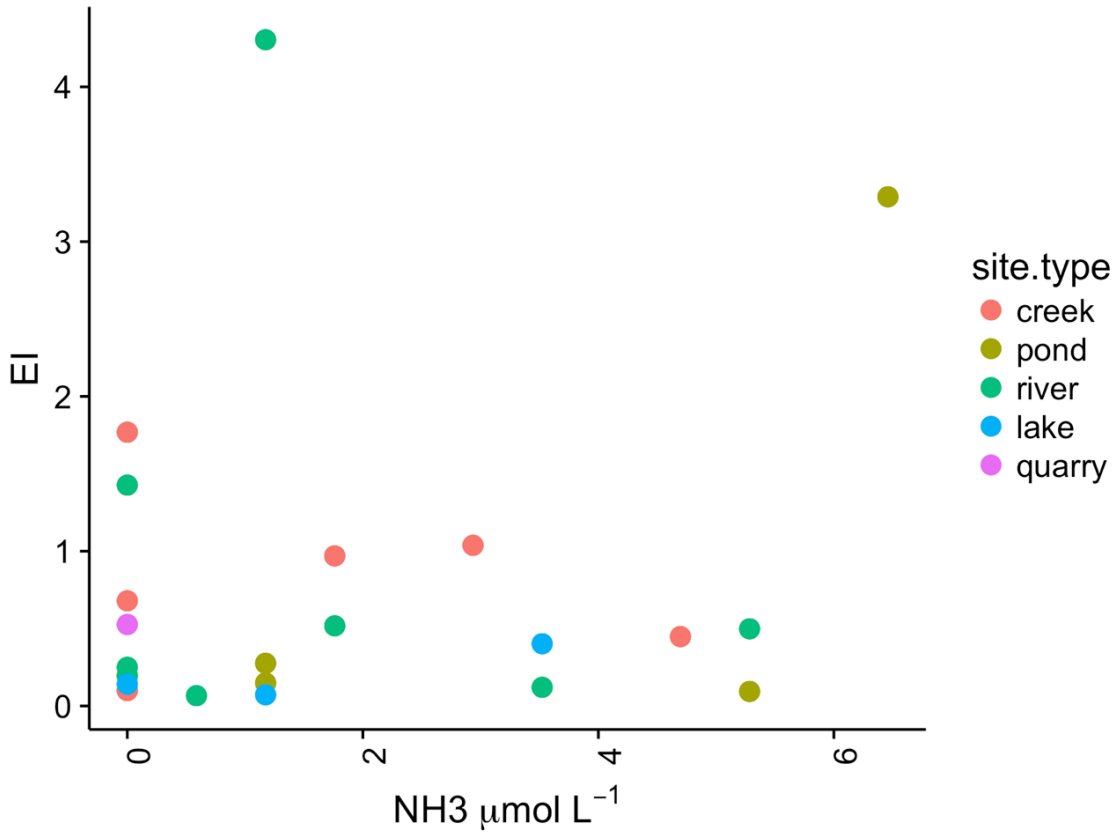
B6- Freshness index as a function of organic carbon



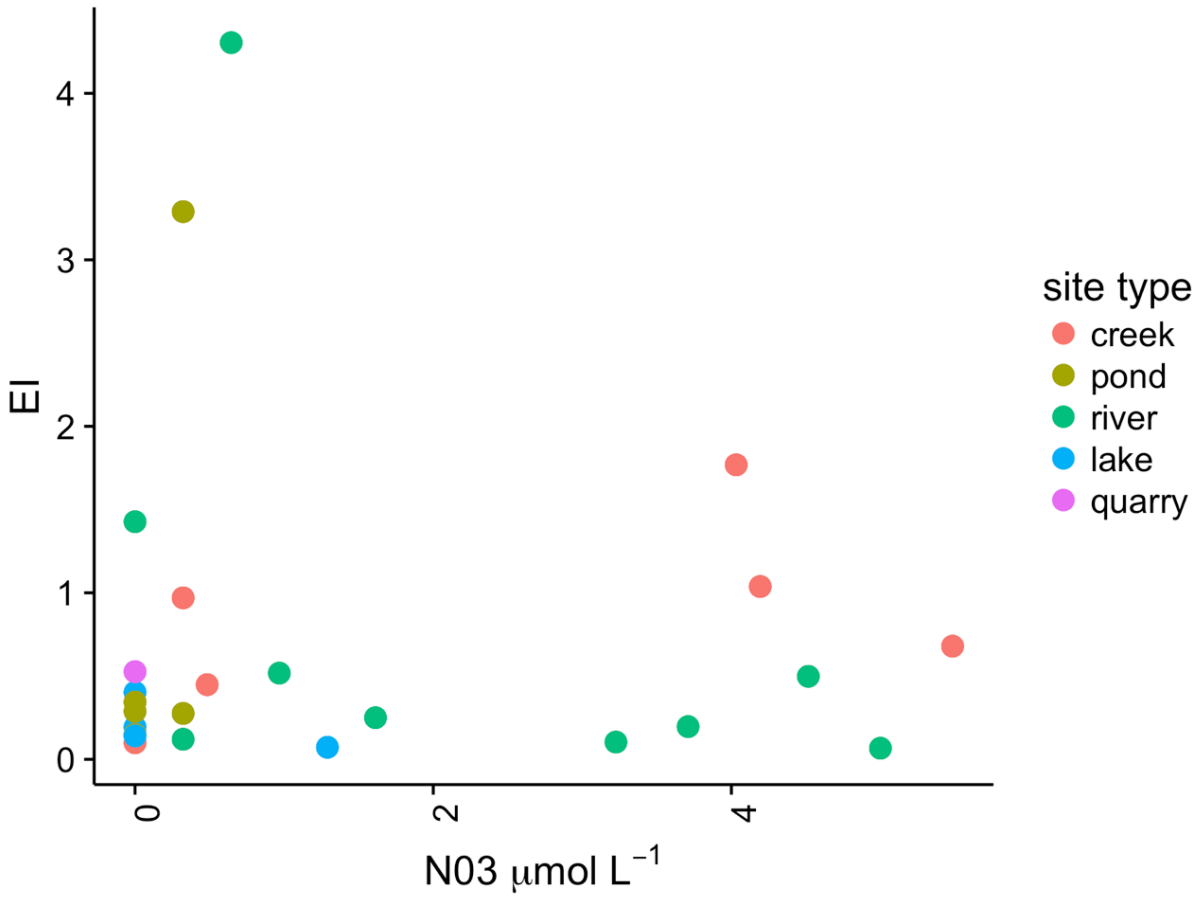
B7- EI as a function of cell densities



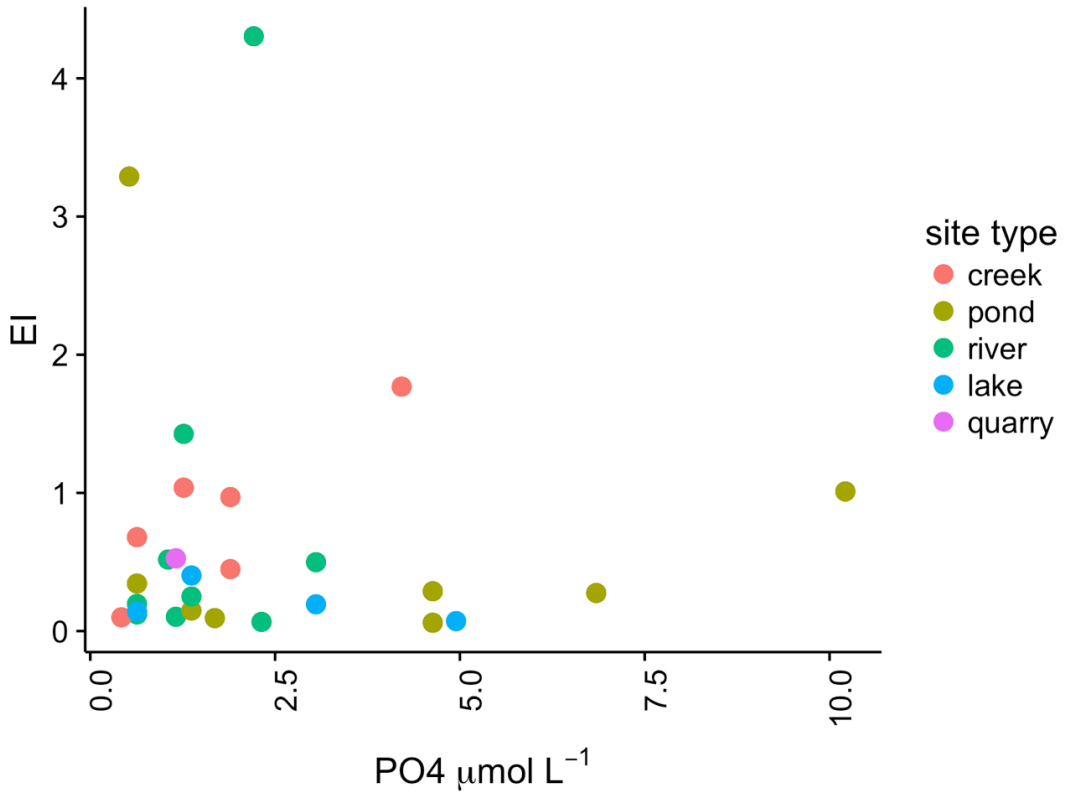
B8- Cell densities as a function of DOC concentration



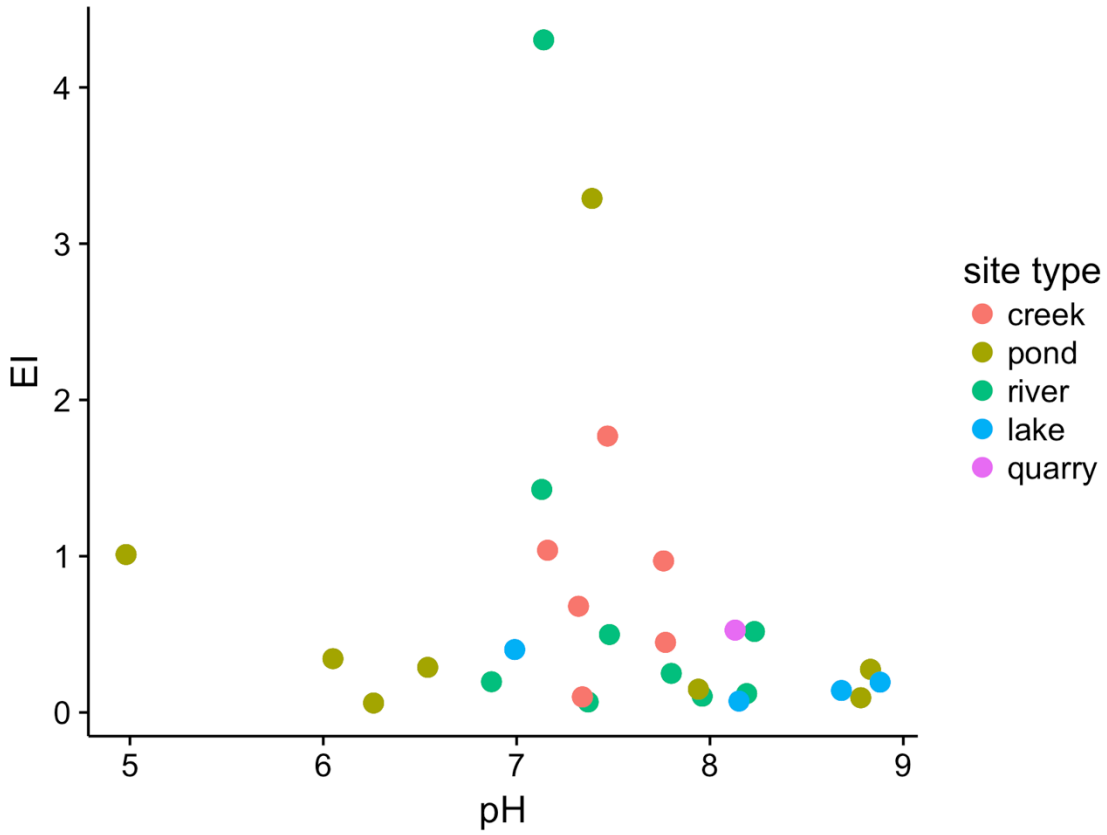
B9- EI as a function of ammonia concentration



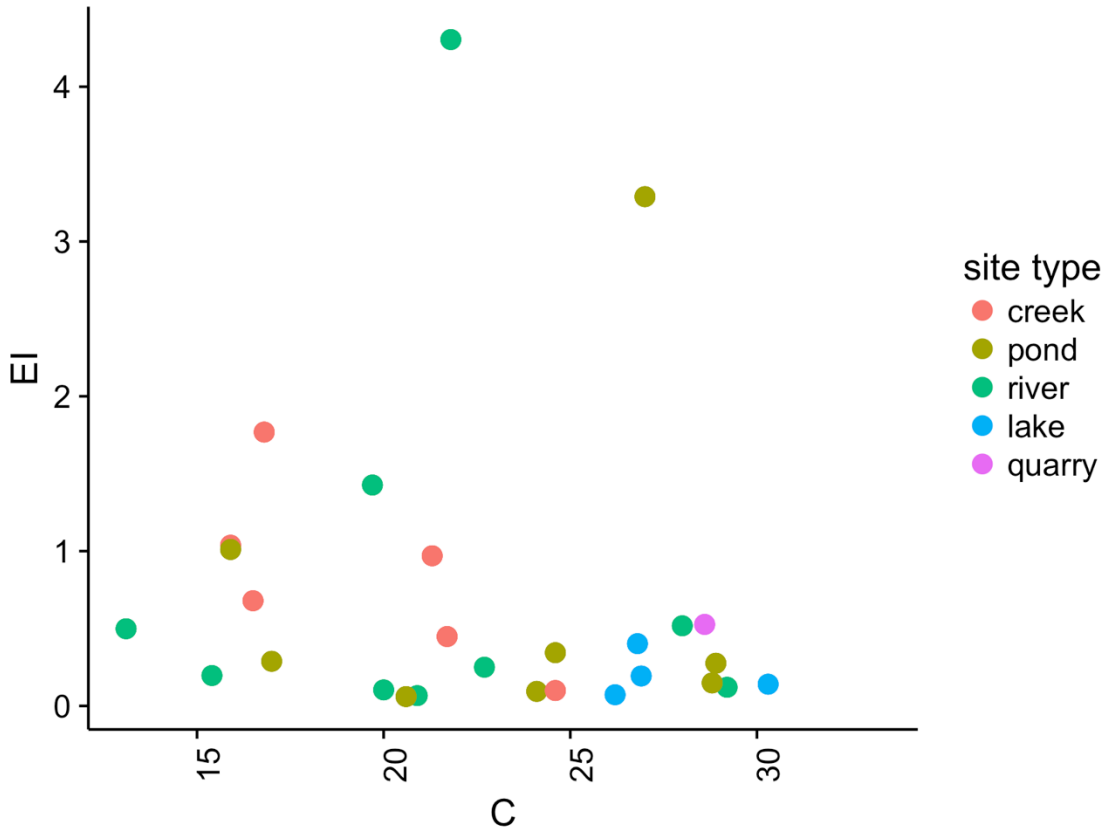
B10- EI as a function of nitrate concentration



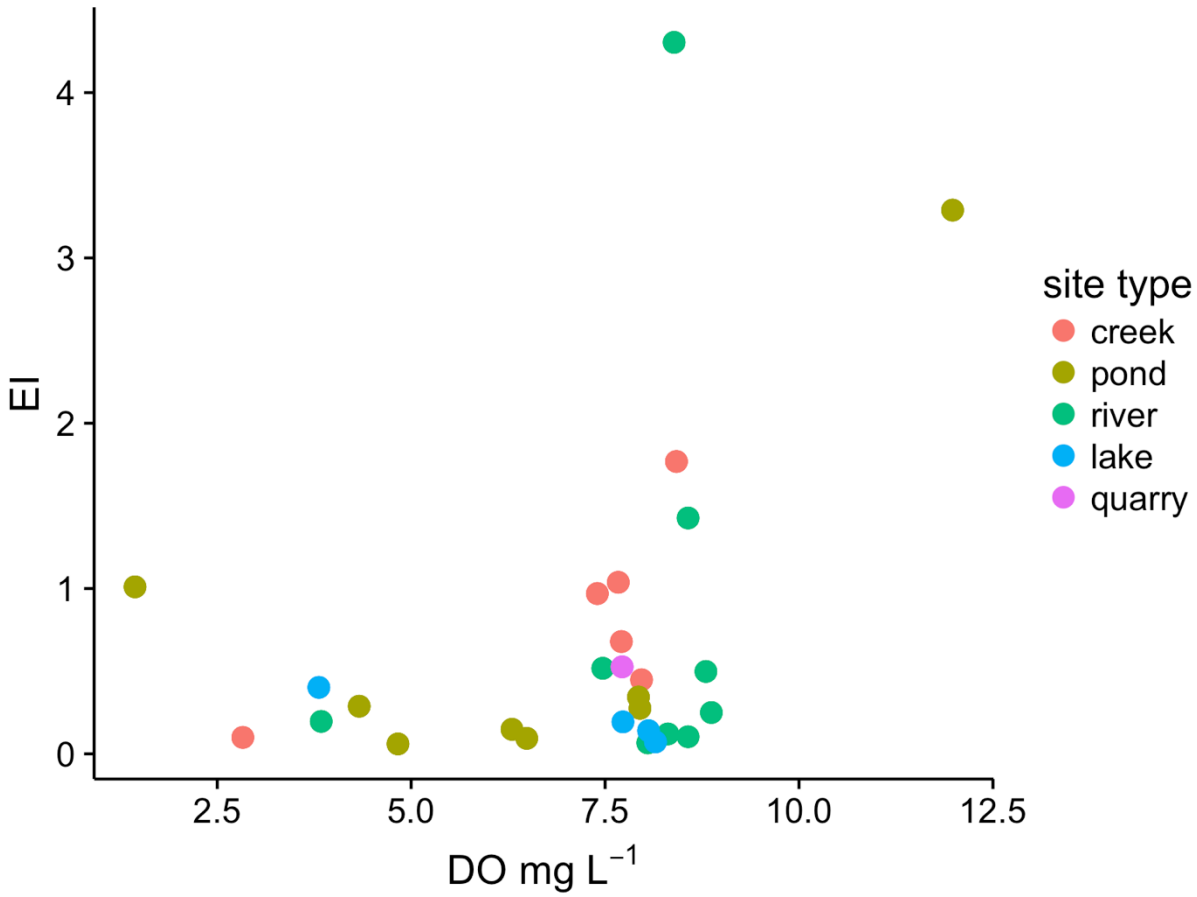
B11- EI as a function of Phosphate Concentration



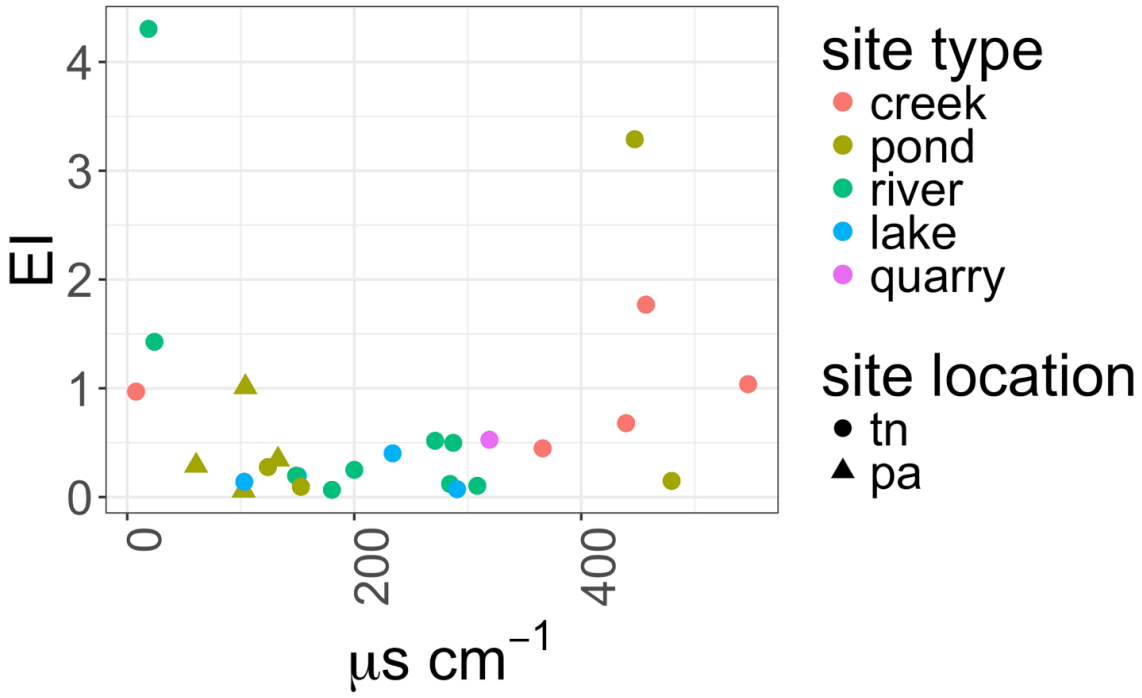
B12- EI as a function of pH



B13- EI as a function of Temperature (ºC)

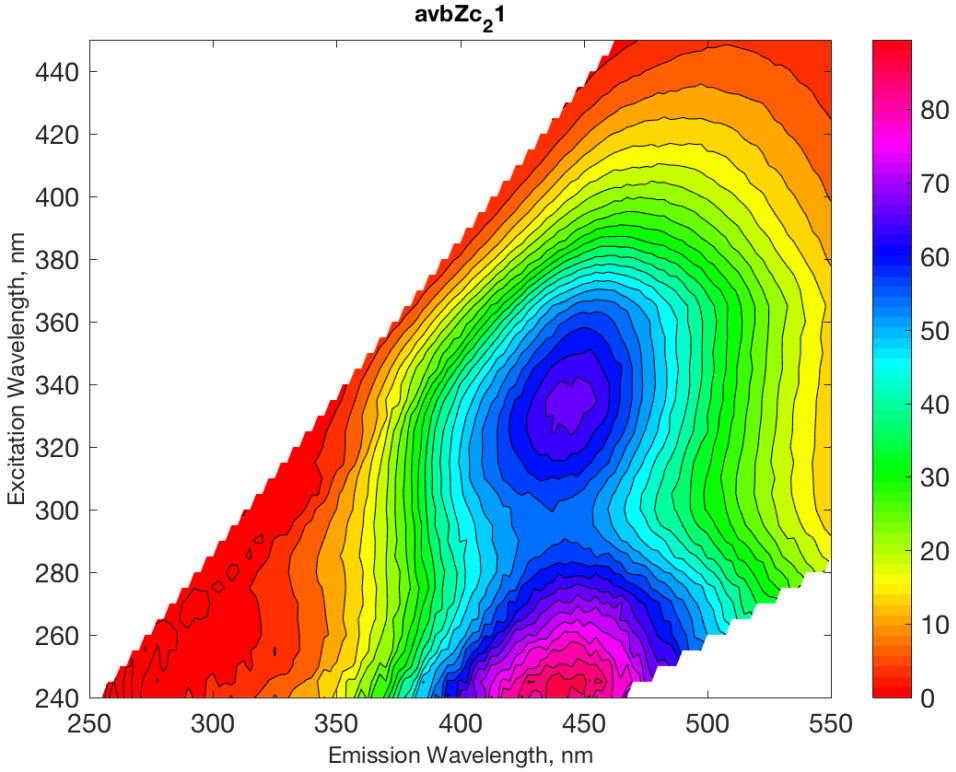


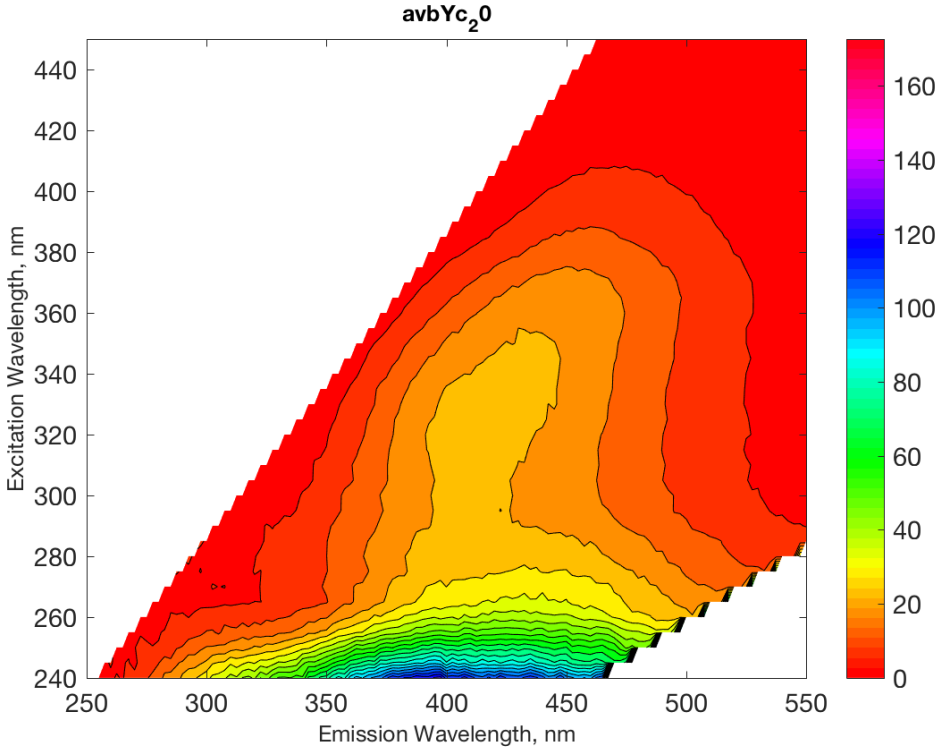
B14- EI as a function of dissolved oxygen



B15- EI as a function of specific conductivity.

B16-Emission excitation spectra for all sites.

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Emission Wavelength, nm
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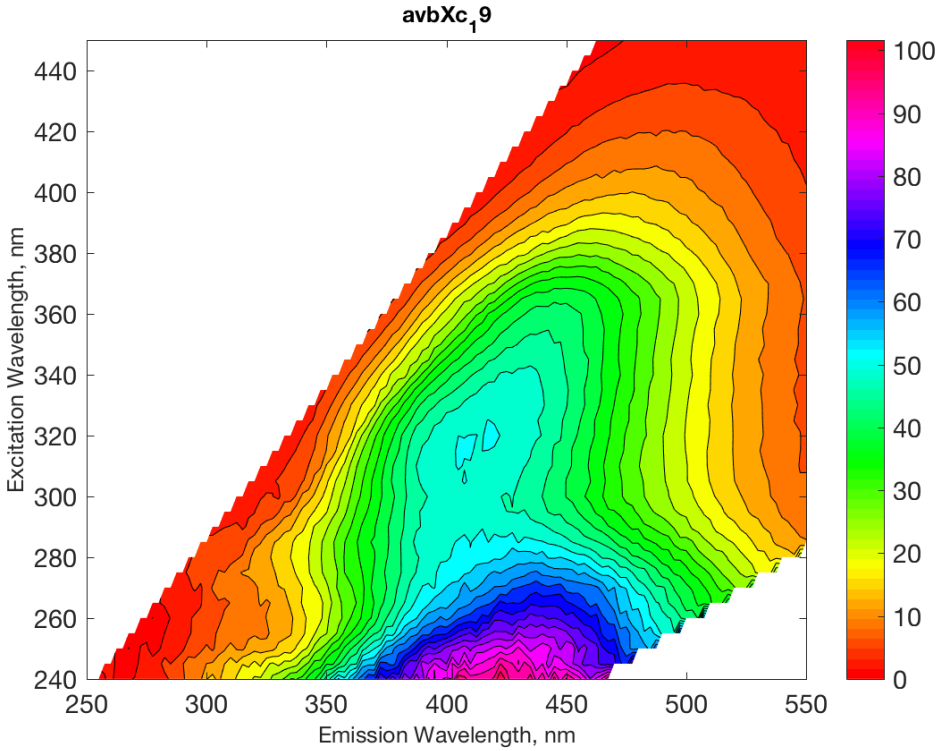
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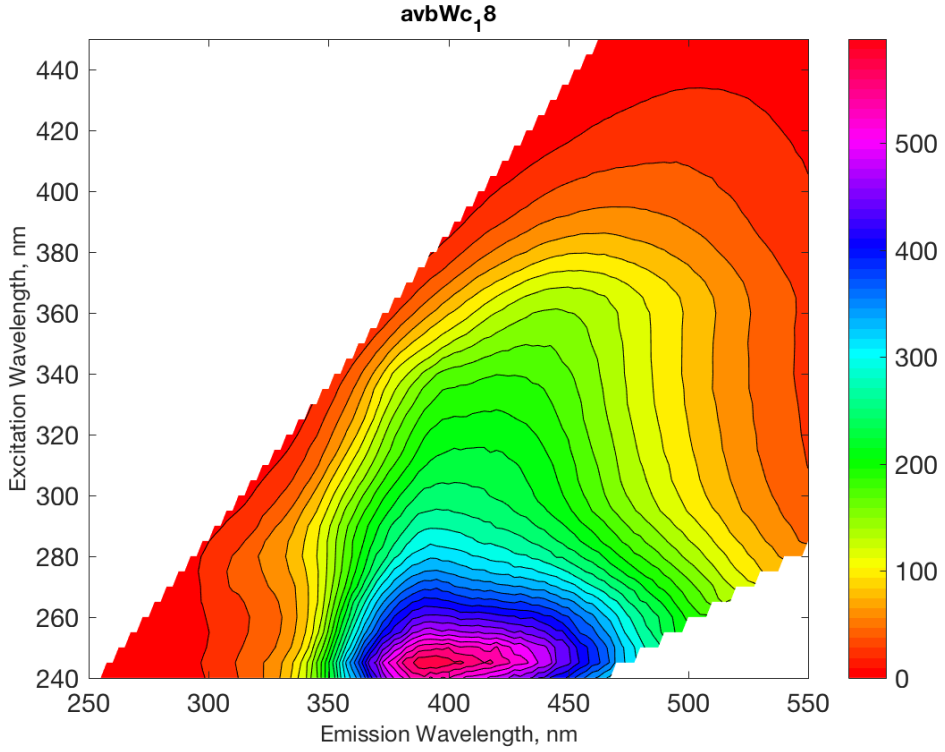
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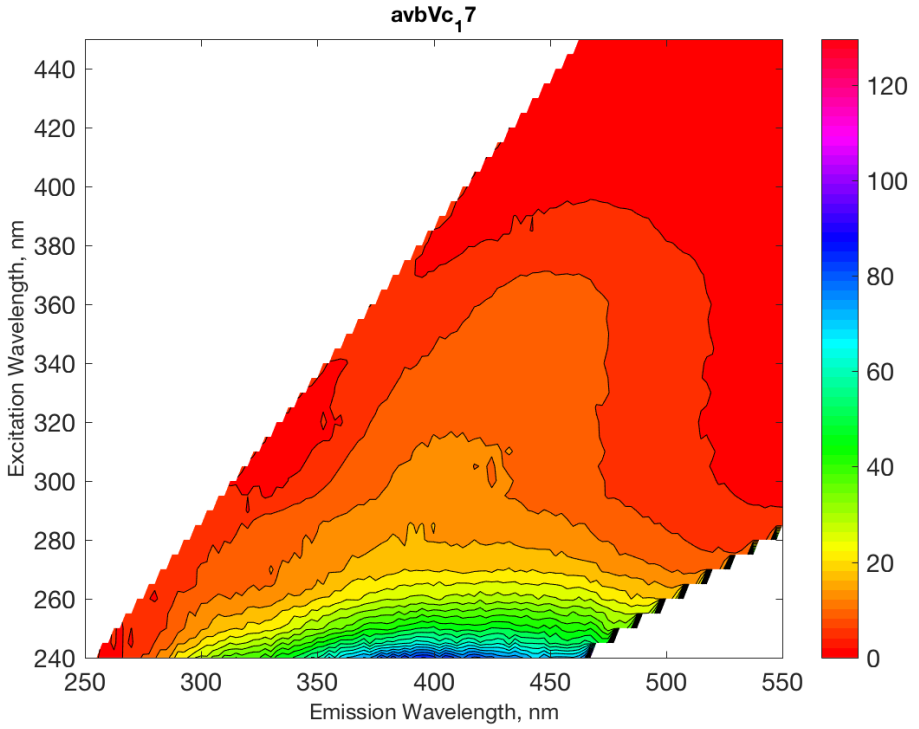
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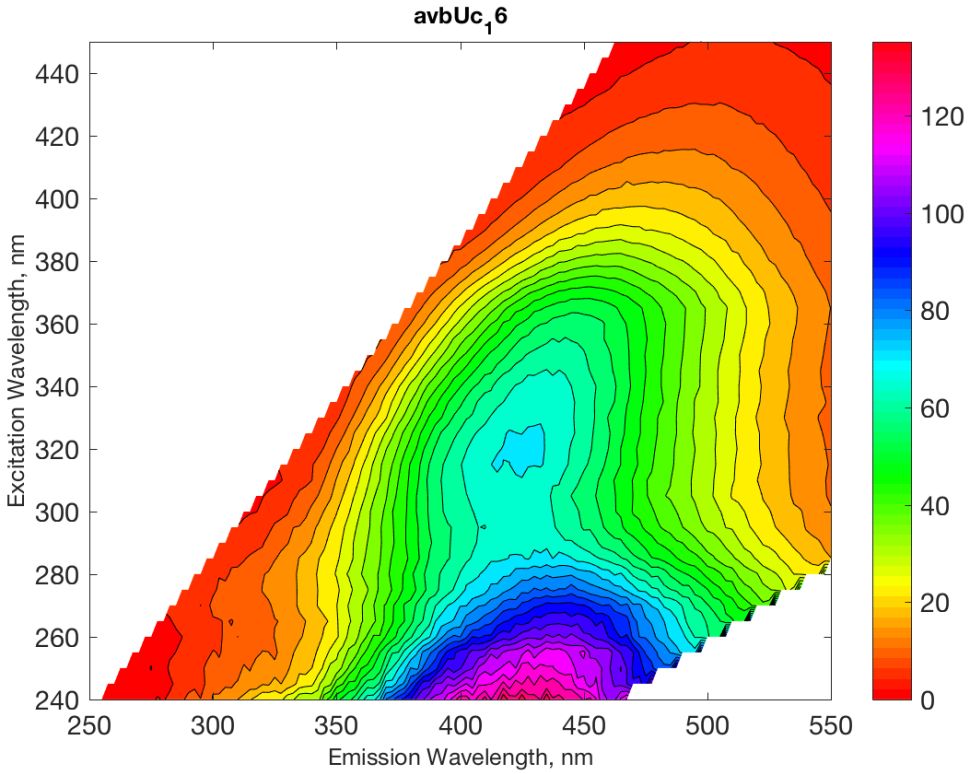
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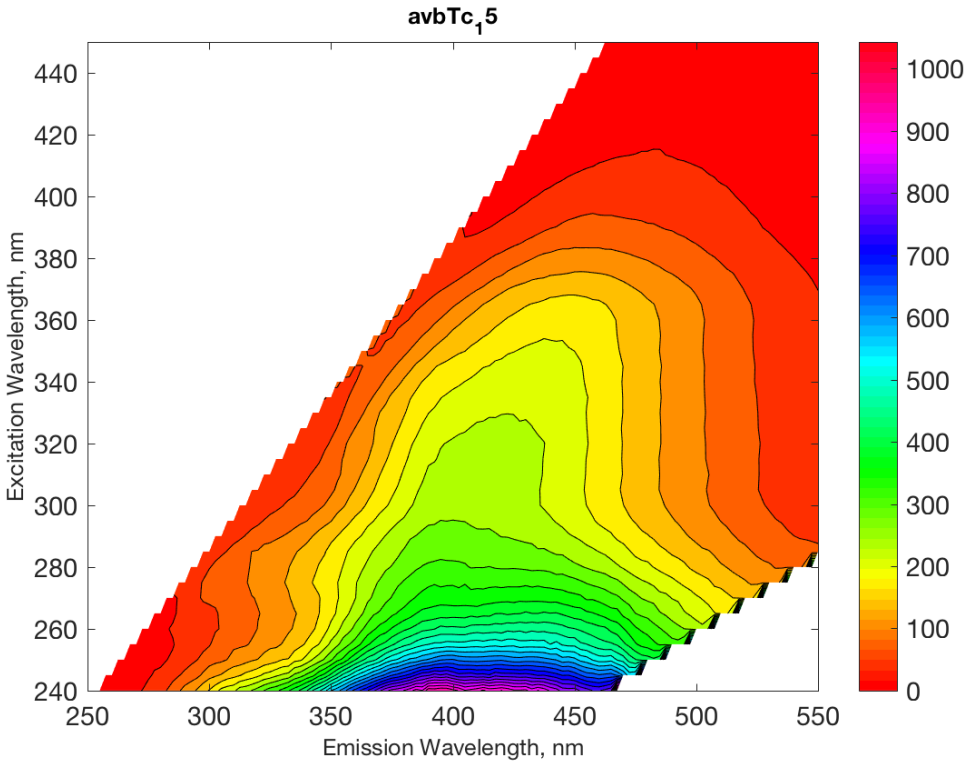
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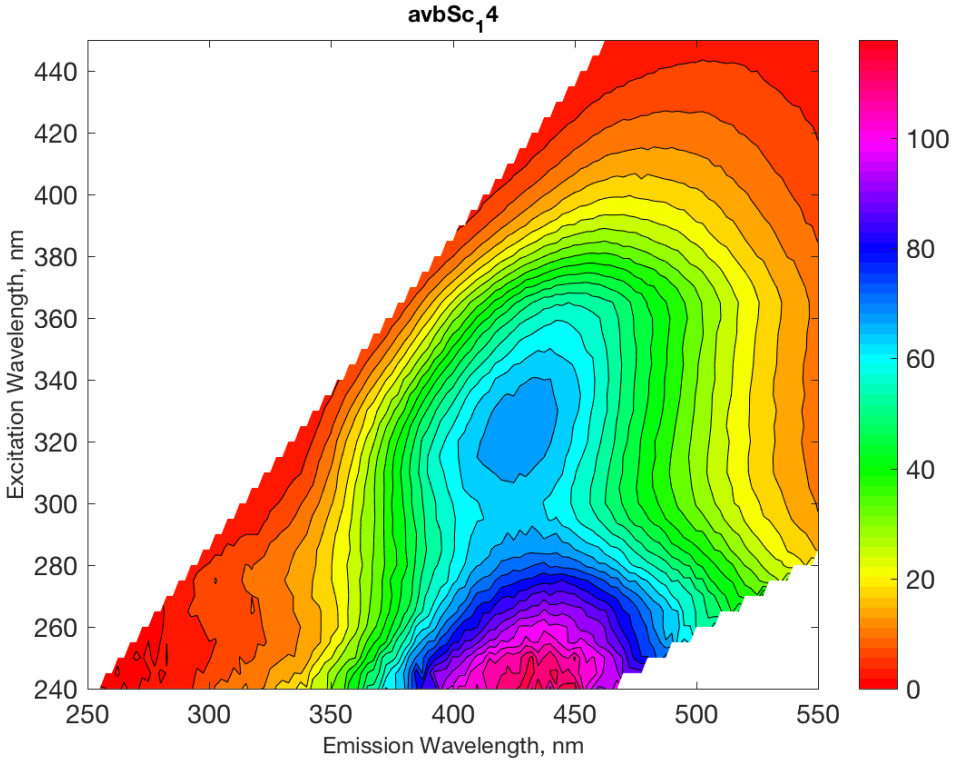
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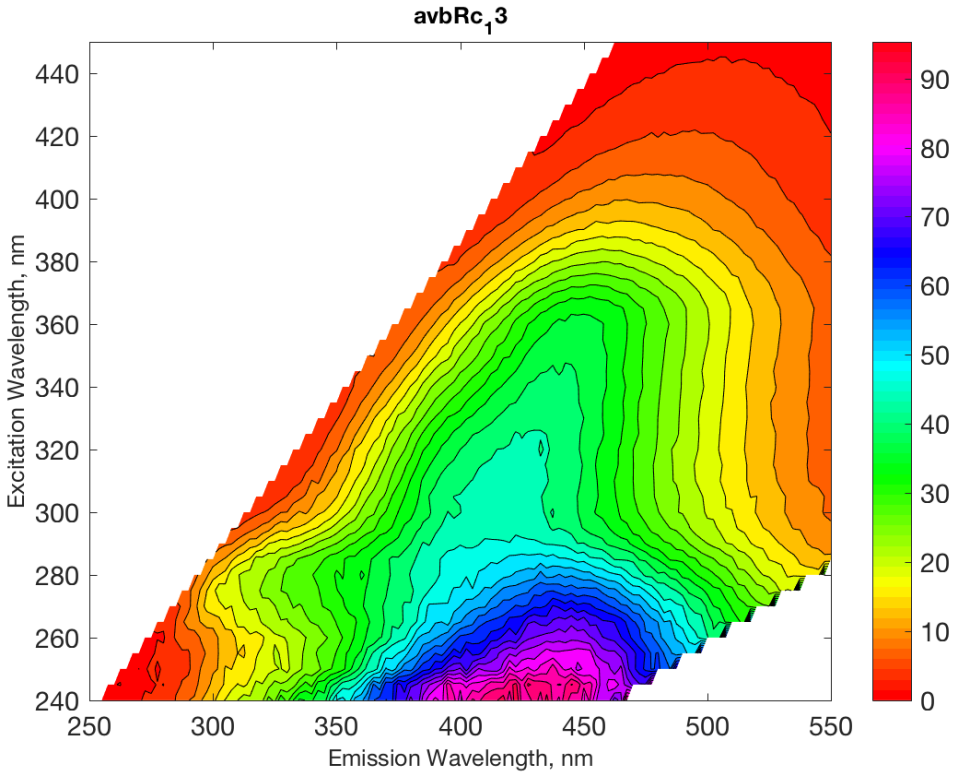
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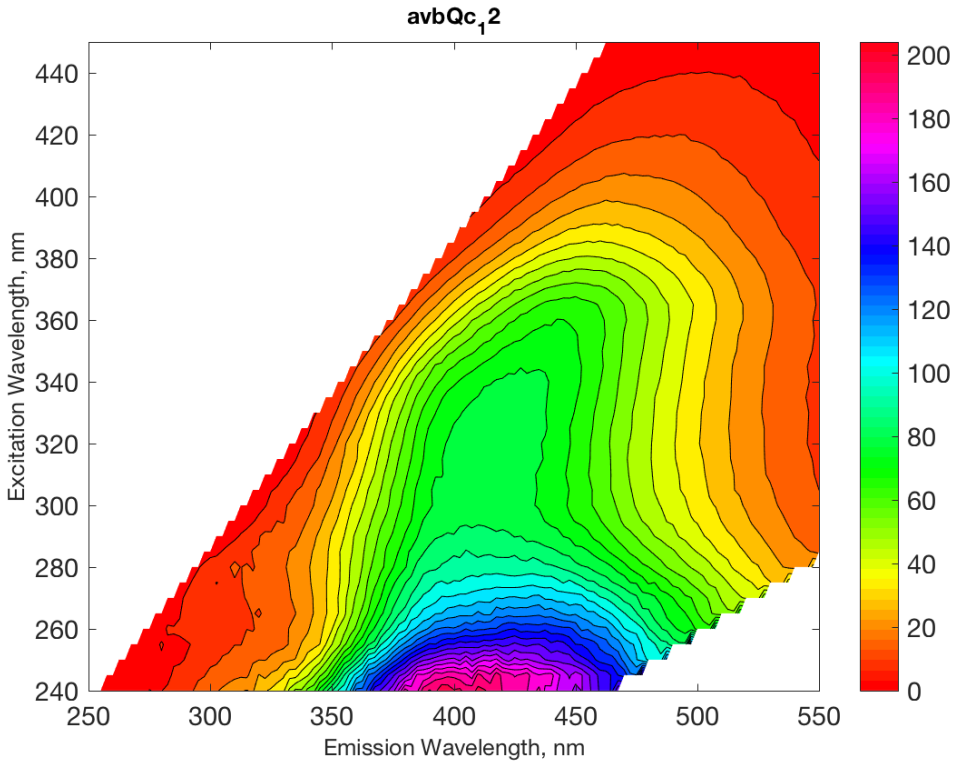
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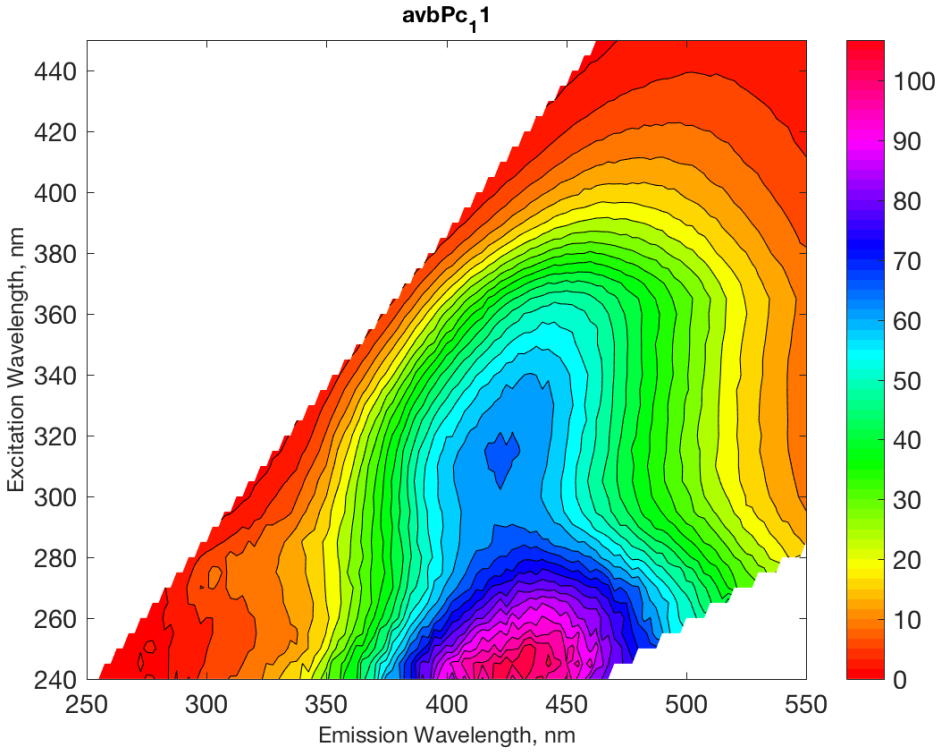
avbPc$_1$1
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Emission Wavelength, nm
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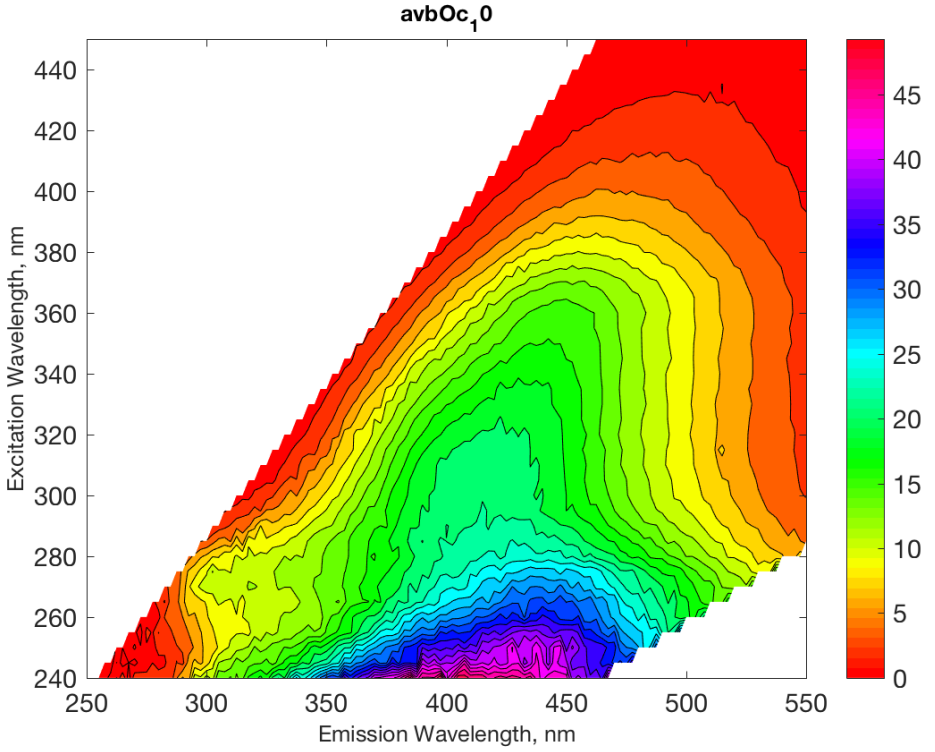
90
100


avbOc10
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
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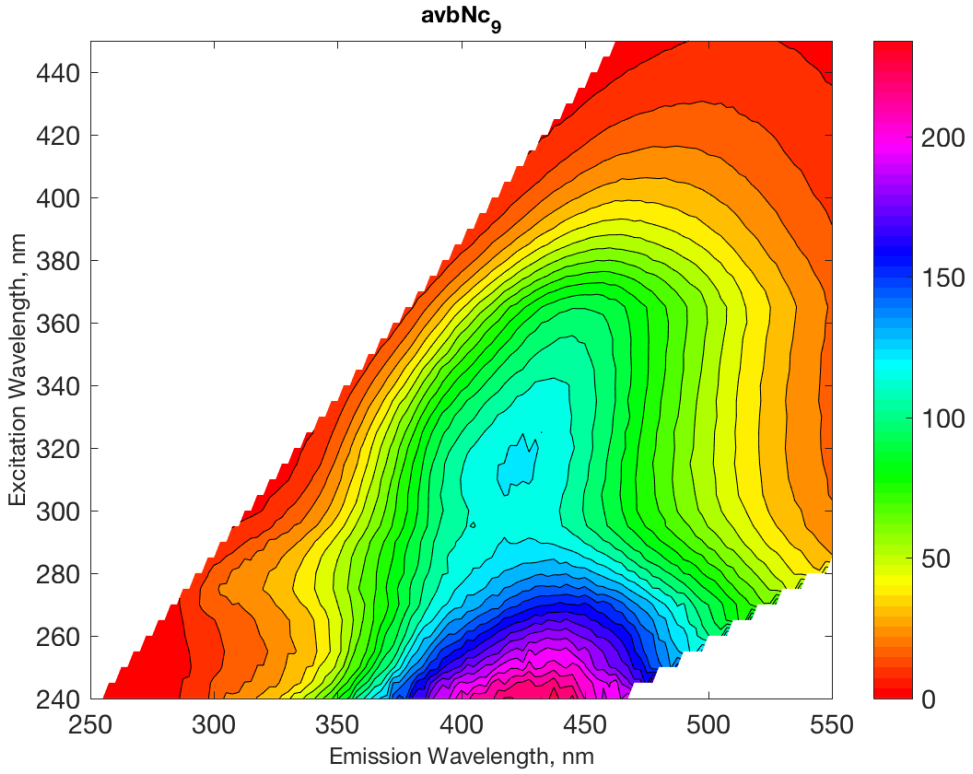
250
300
350
400
450
500
550
0
5
10
15
20
25
30
35
40
45


avbNc9
Excitation Wavelength, nm
Emission Wavelength, nm
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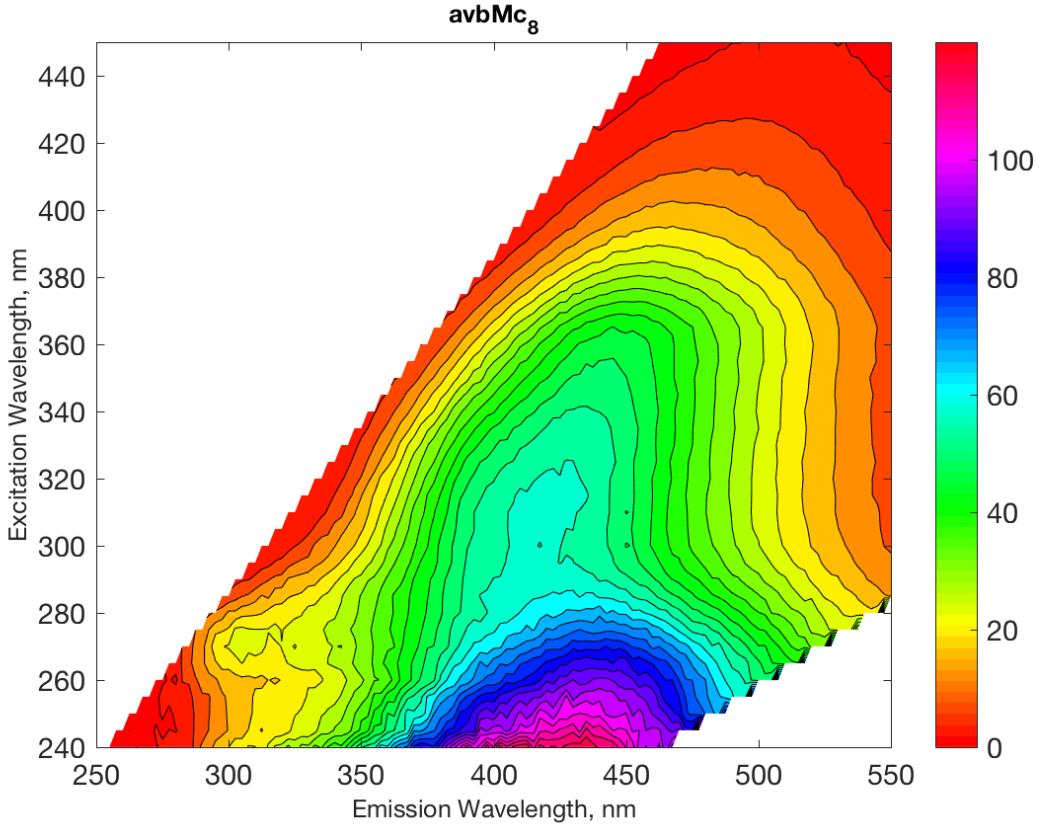
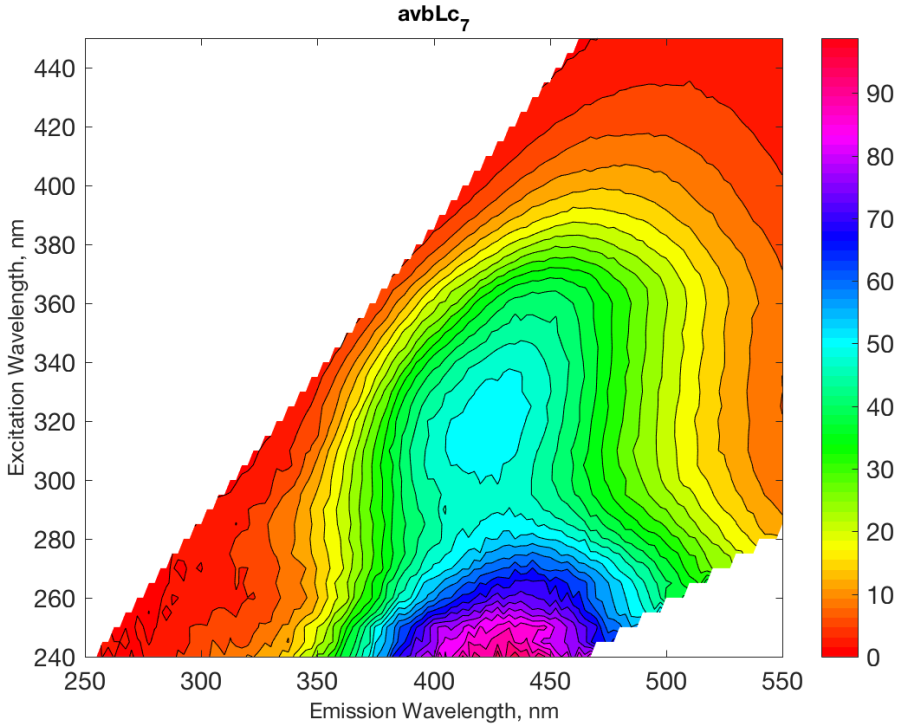
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
50
100
150
200


avbMc8
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
20
40
60
80
100


avbLc7
Excitation Wavelength, nm
Emission Wavelength, nm
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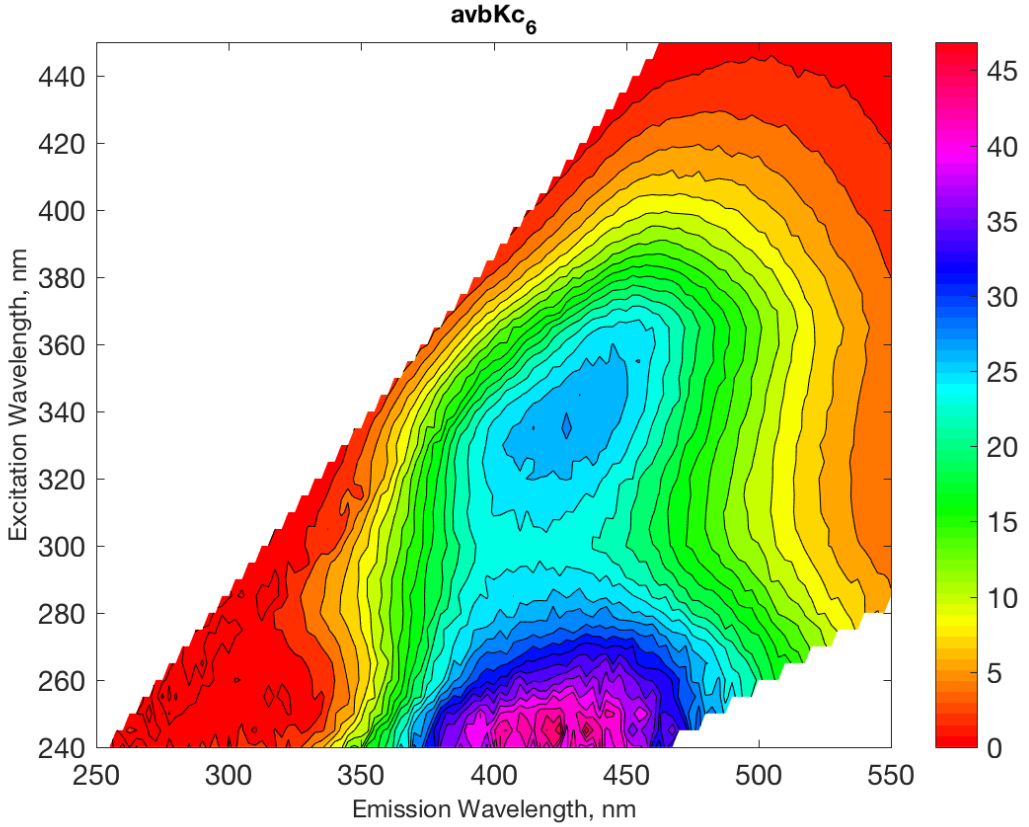
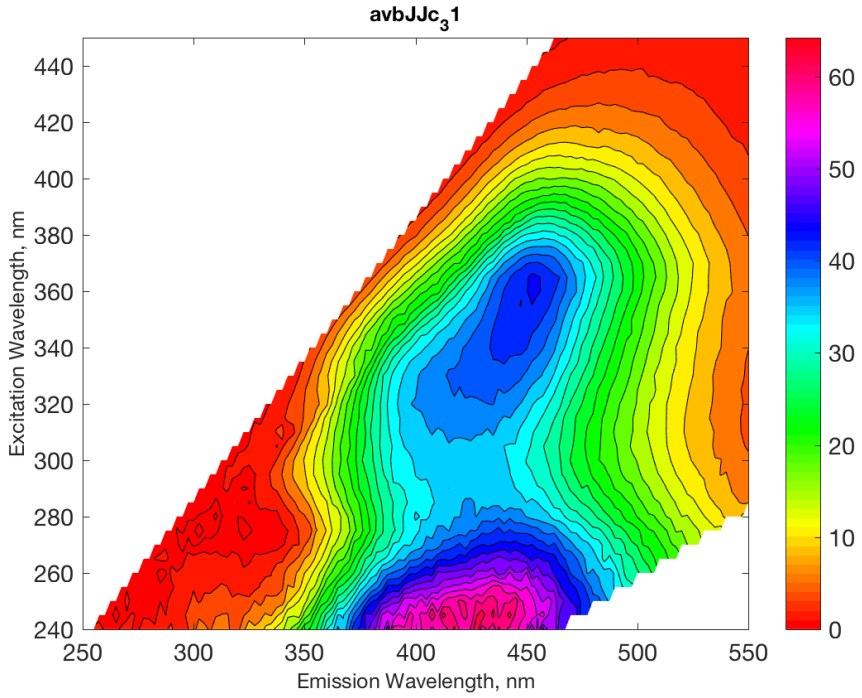
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
10
20
30
40
50
60
70
80
90


avbKc6
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
5
10
15
20
25
30
35
40
45


avbJJc31
Excitation Wavelength, nm
Emission Wavelength, nm
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260
280
300
320
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360
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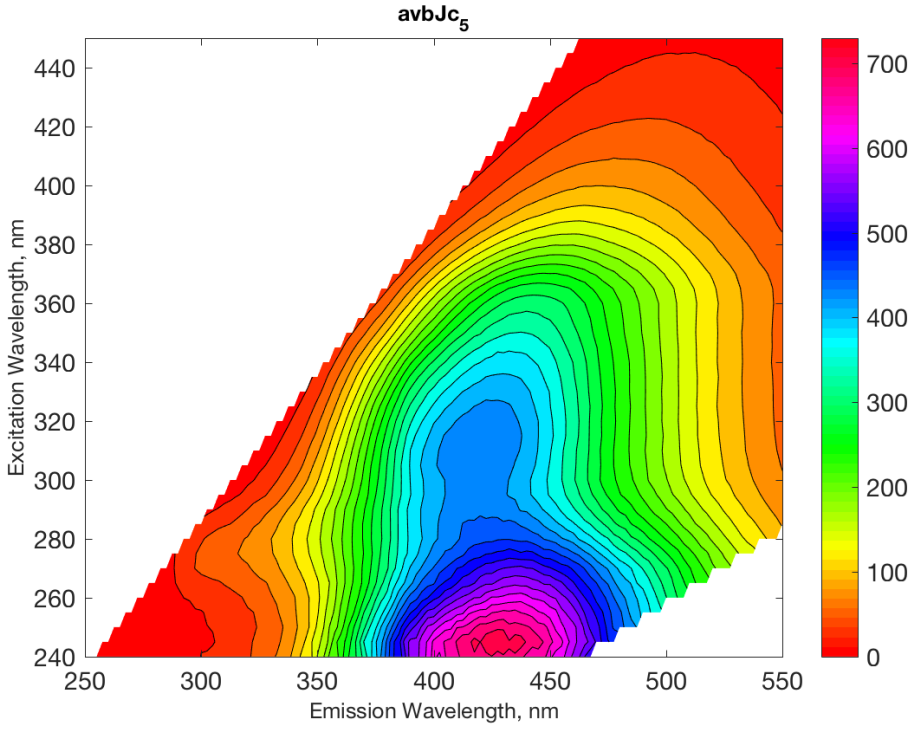
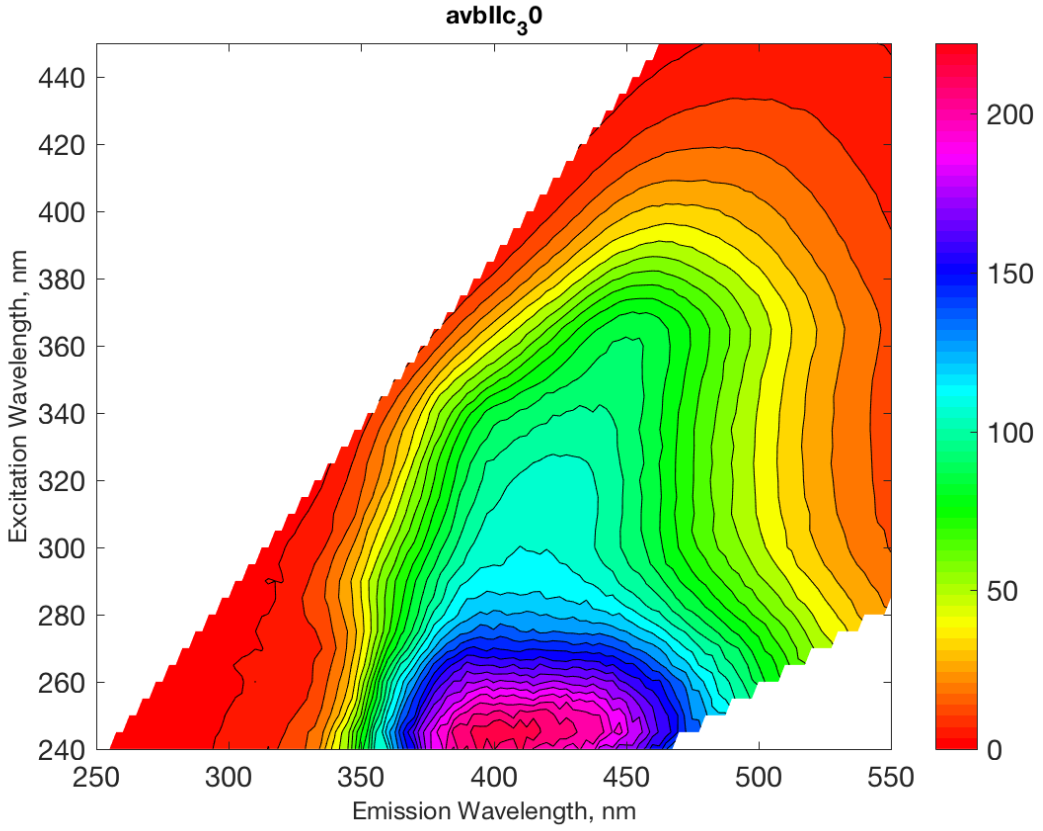
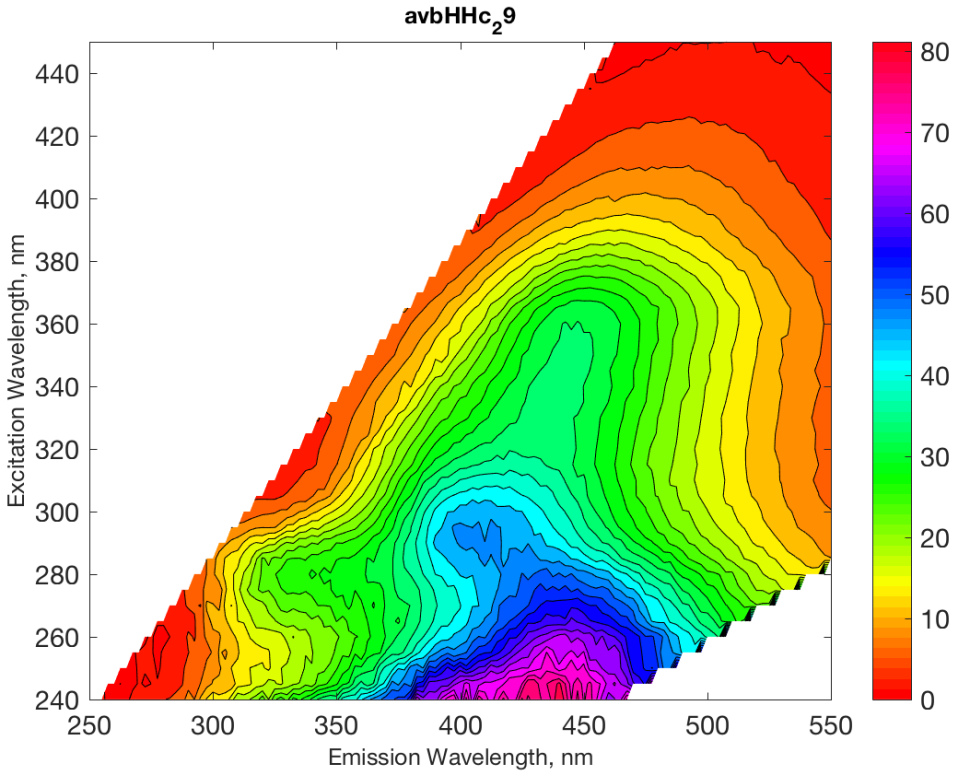
400
420
440
250
300
350
400
450
500
550
0
10
20
30
40
50
60


avbJc5
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
100
200
300
400
500
600
700


avbIIc30
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
50
100
150
200


avbHHc29
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
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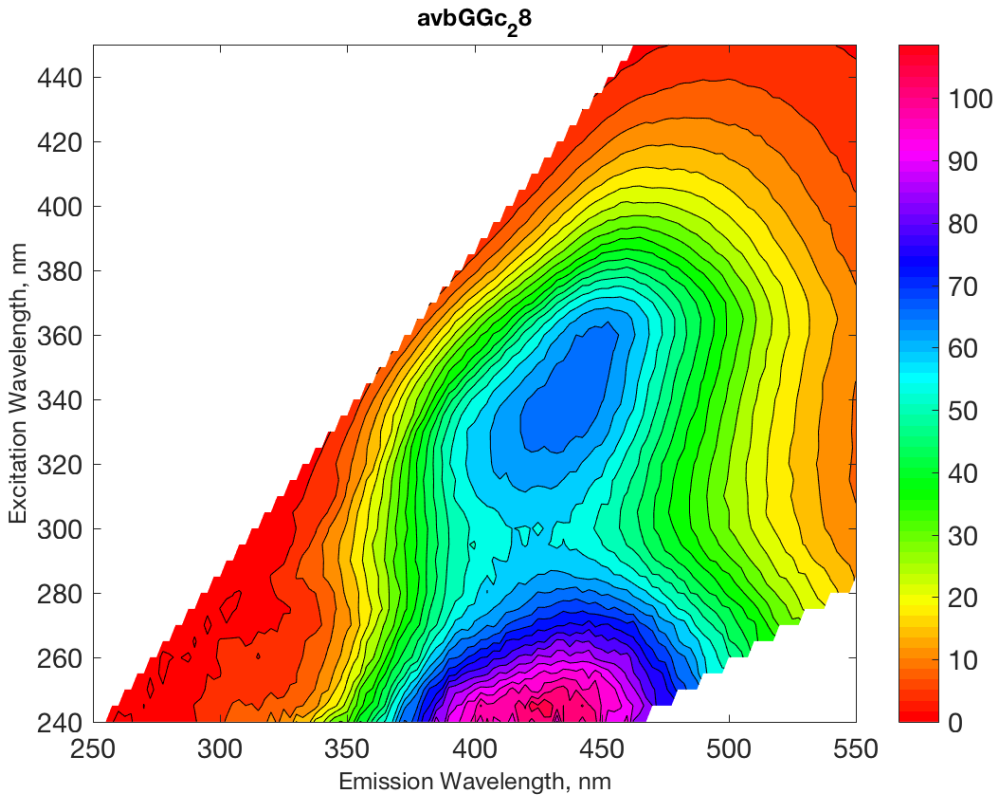
250
300
350
400
450
500
550
0
10
20
30
40
50
60
70
80


avbGGc28
Excitation Wavelength, nm
Emission Wavelength, nm
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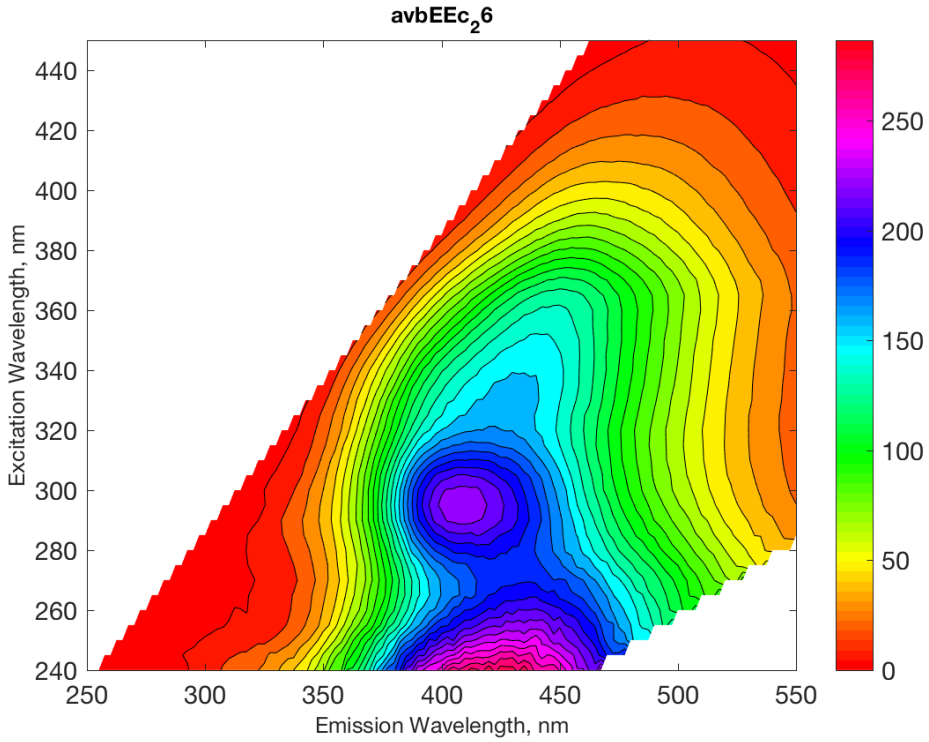
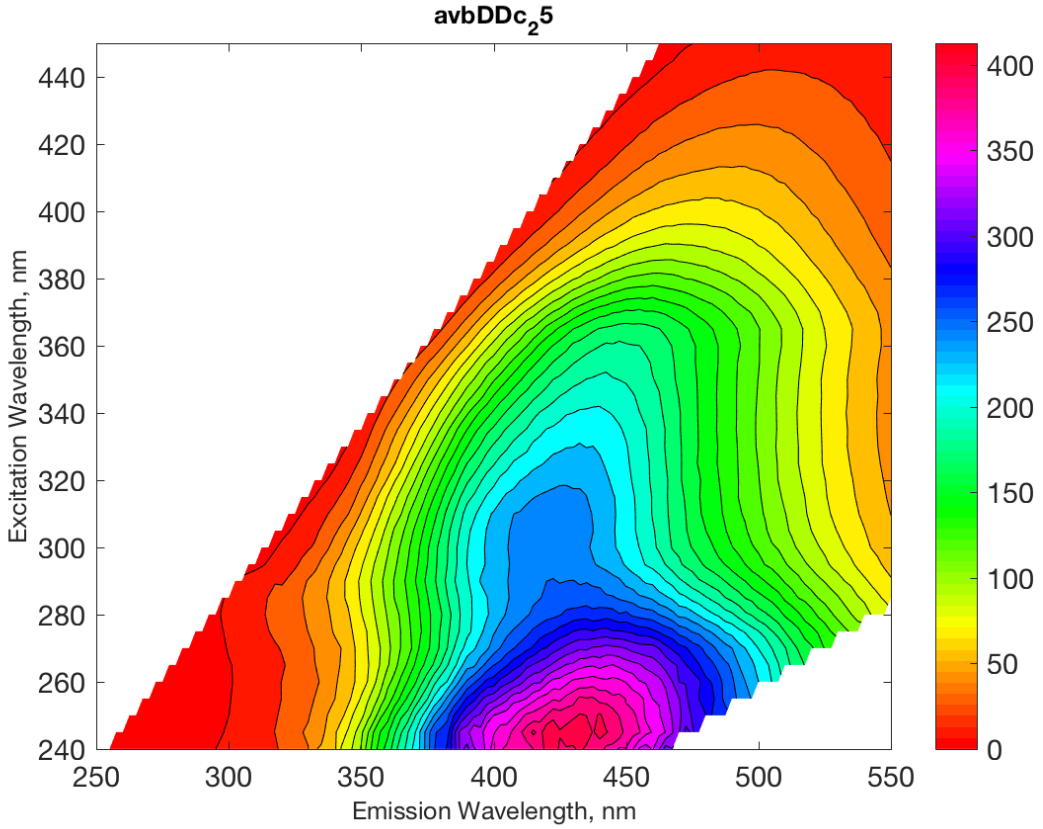
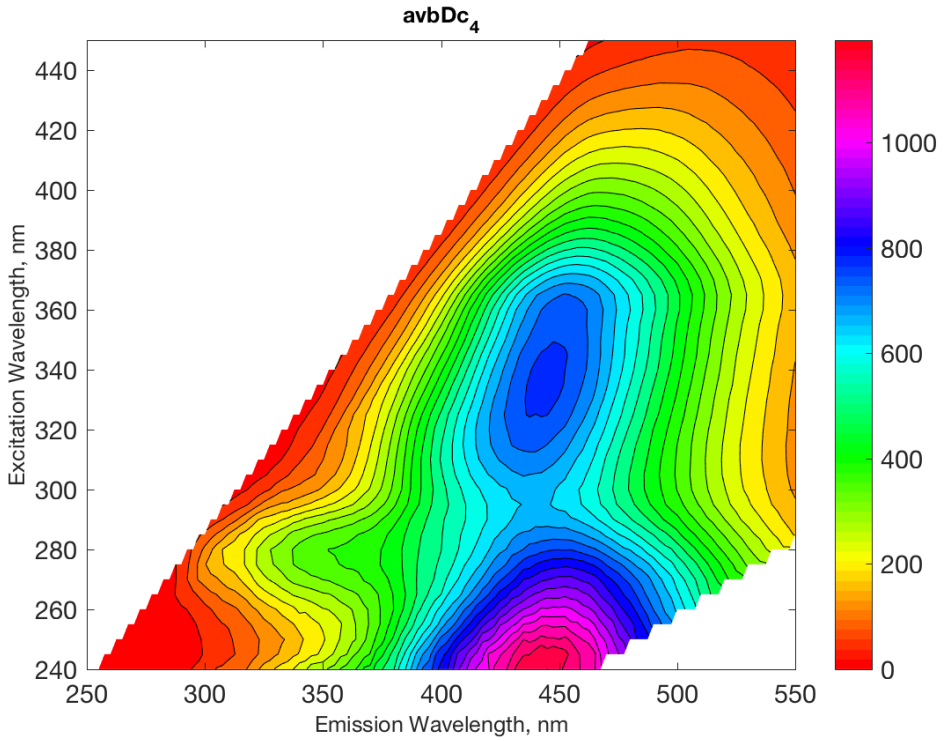
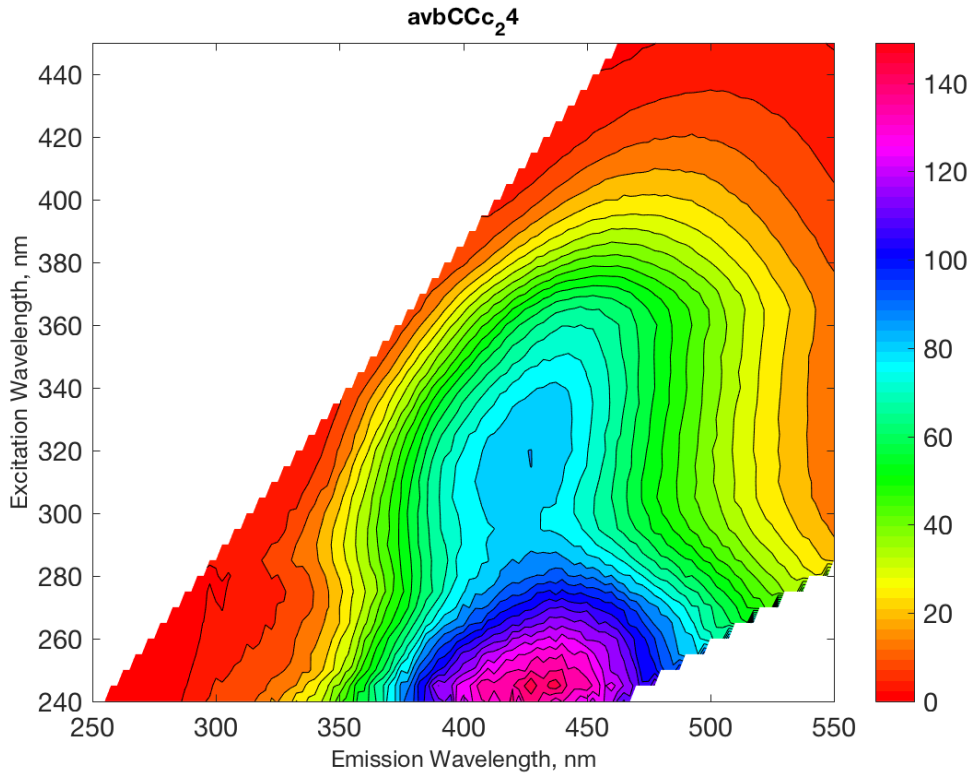
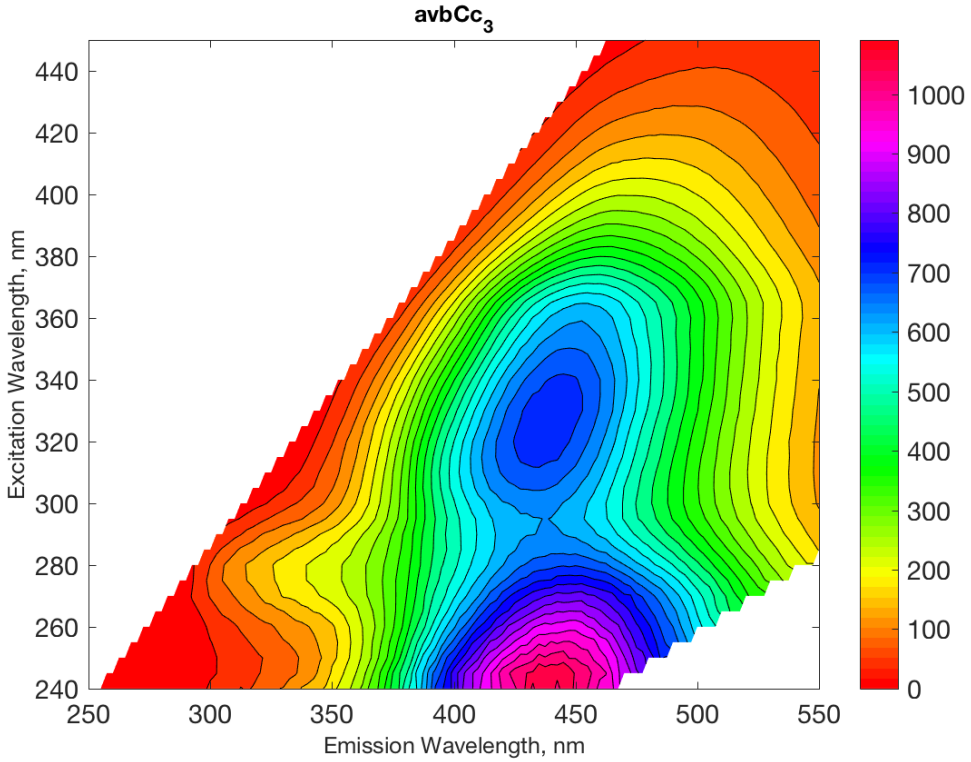
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
10
20
30
40
50
60
70
80
90
100

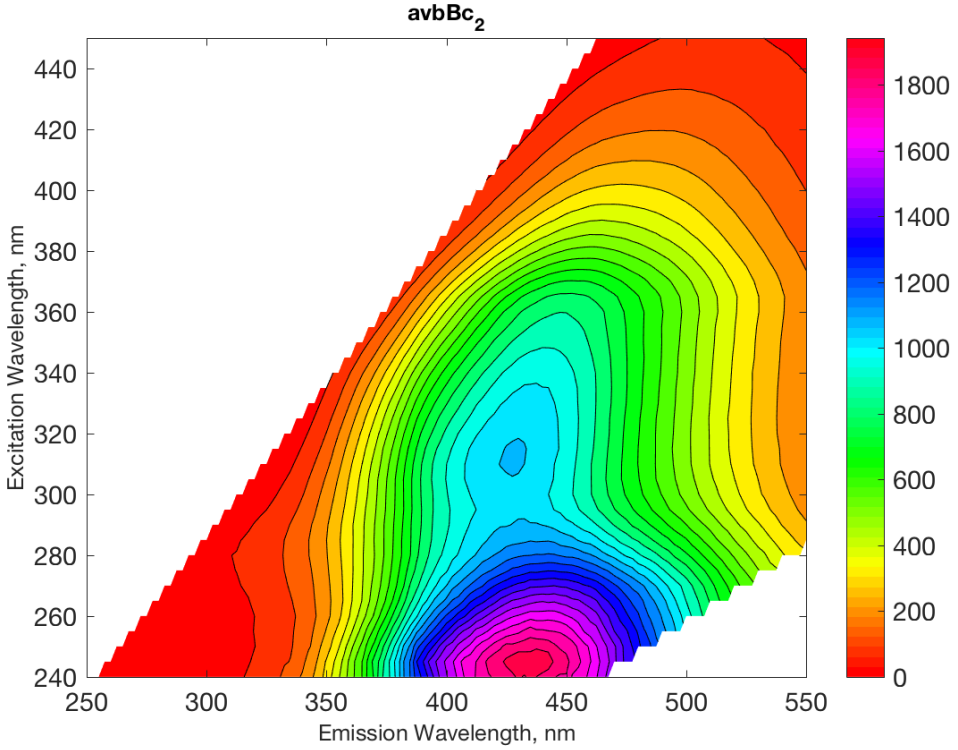

avbEEc2 6
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
50
100
150
200
250


avbDDc2 5
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
50
100
150
200
250
300
350
400


avbDc4
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
200
400
600
800
1000


avbCCc24
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
20
40
60
80
100
120
140


avbCc3
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
100
200
300
400
500
600
700
800
900
1000


avbBc2
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
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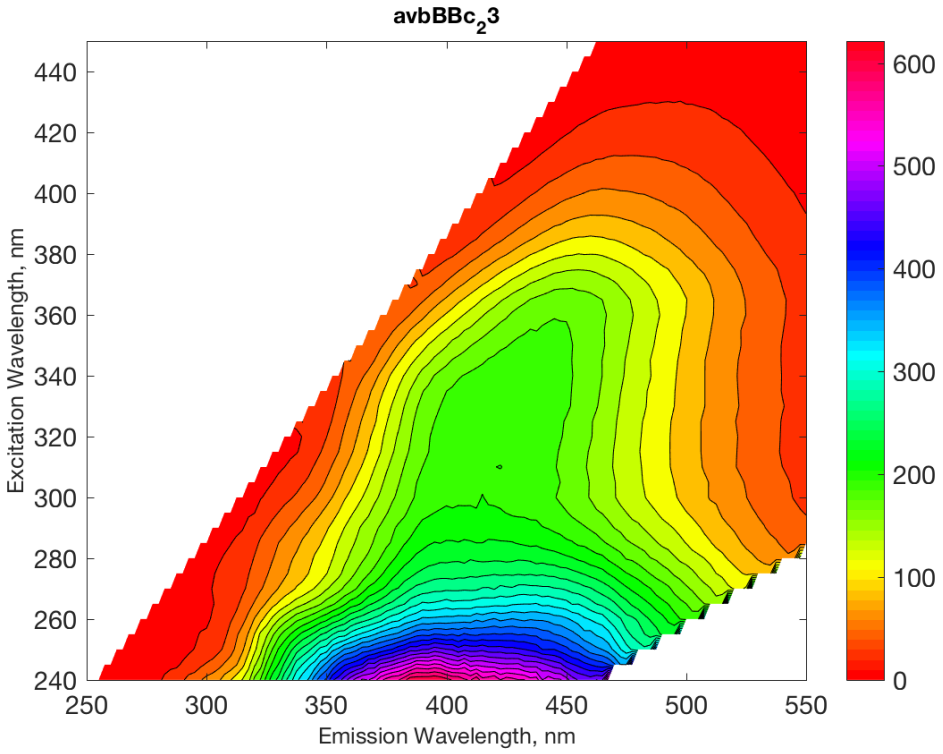
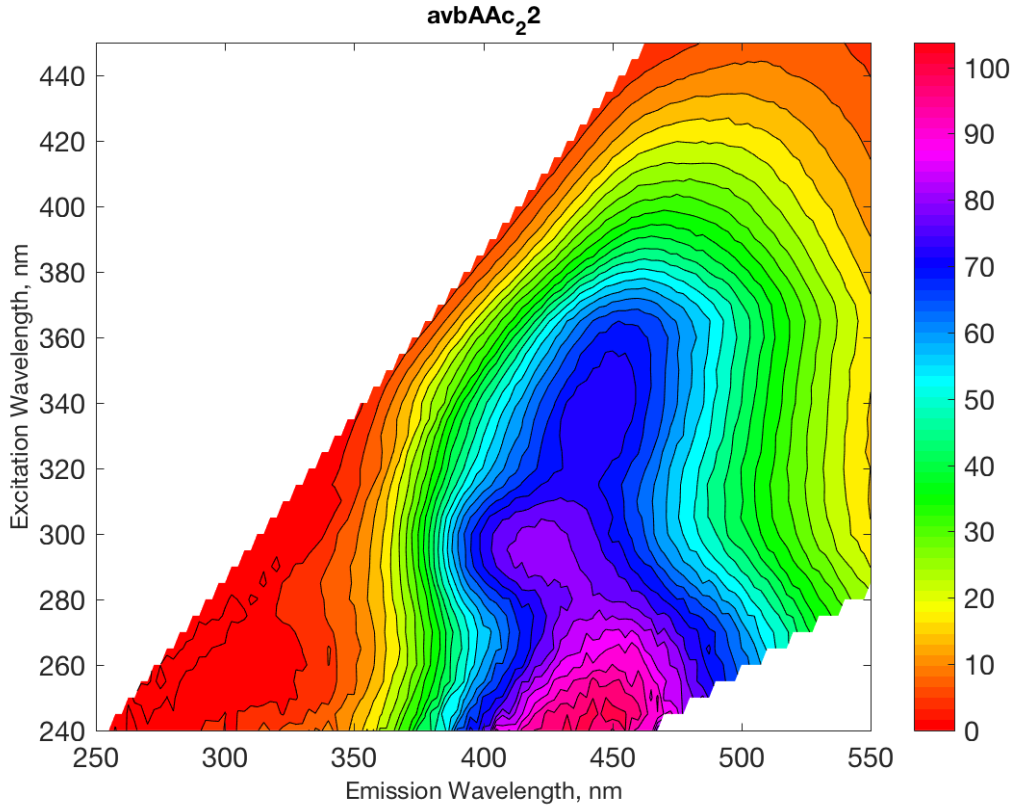
300
350
400
450
500
550
0
200
400
600
800
1000
1200
1400
1600
1800


avbBBc2 3
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
100
200
300
400
500
600


avbAAc2 2
Excitation Wavelength, nm
Emission Wavelength, nm
240
260
280
300
320
340
360
380
400
420
440
250
300
350
400
450
500
550
0
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20
30
40
50
60
70
80
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100

VITA

Abigail Barrett was born in Bridgeport, CT. She completed a BS in geology at the College of Charleston. She is now pursuing a MS in geology at the University of Tennessee Knoxville.