STUDY OF WETLAND RESTORATION STRUCTURES ON MICROBIAL FUNCTIONALITY

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Abstract. Wetland restoration is a topic gaining traction as understanding of wetland ecosystem services advances. Here we assessed the success of habitat heterogeneity, aimed to increase microbial functionality, in a restored freshwater tidal marsh. The study site, Gays Point, integrates alcoves and rootwads (side-pool excavations and woody debris mounds, respectively) and was compared to Hallenbeck Creek, a natural wetland of the same type. We took 52 sediment samples with 2-3 replicates per sample over the course of 2 sampling periods from the study site. We also took 24 sediment samples with 2 replicates per sample from the reference site. We measured sediment organic matter (SOM), rate of oxygen respiration, and Extracellular Enzymatic Activities (EEAs). We hypothesized that rootwads and alcoves would score higher within all three variables than the mid-channel. Within the study site Alcoves had higher SOM than other locations ($p < 0.015$), and there was not a significant difference in SOM between rootwads and mid-channel, ($p = 0.56$). There was not a significant correlation with SOM and respiration, contrary to expectations. Mean EEA was greater in Gays Point than Hallenbeck Creek ($p < 0.0001$). Alcove mean EEA was equal to that of Hallenbeck and less than other locations ($p < 0.001$). We conclude Alcoves were successful in management goals, and are likely microbial hotspots. Several other factors not accounted for in this study, such as hydrology, land cover, SOM characterization, and microbial community structure, would enhance the scope of this study and help better assess microbial functionality. Implications from this study's findings could potentially aid in progressing wetland restoration practices and wetland management.

INTRODUCTION

Wetland restoration is an increasingly prominent and pressing topic, and wetland management practices will be the dictator of restoration success. Here we examine a specific wetland restoration case and explore the idea that increased habitat heterogeneity increases secondary production and biodiversity (Strayer and Findlay 2010). We compare microbial metabolism and function, sediment organic matter, and sediment respiration between restored and reference sites and within microsites at each of the two systems. Conclusions from these investigations will allow us to assess the success and resilience of this wetland, and will potentially aid in creating future restoration plans with clearer foreseeable ecosystem functions. This study will add to the collection of knowledge concerning wetland management and restoration practices. The environmental benefits and ecosystem services provided by wetlands coupled with the historical degradation of Hudson wetlands, exemplifies the importance of successful wetland restoration to future ecosystem and human health.

The Hudson River, north of Kingston, NY was originally a series of shallow interconnected channels. The native landscape was primarily composed of shallow and intertidal wetland habitat, with other wetland habitats including shoreline (or fringe) and tributary streams also present. In the 1820s construction plans began to create a mainstream, navigable channel for ship access to Albany. Through dikes and dredging secondary channels were converted to backwaters and the mean bottom depth increased from >1m in 1818 to >9.7m since 1972. The 152 mile stretch of estuary lining the Hudson River was filled with >3,300 acres of dredge spoils (Miller, Daniel E. 2013). In the process islands decreased in area by approximately 65%-75%, secondary channels decreased in area by 70%, and contiguous backwaters increased in area by over 1000%. The increase in backwater area represents a direct consequence of dredge spoils partially filling

side channels. The resultant lower flow velocity in backwaters creates conditions favoring increased temperatures and decreased dissolved oxygen. Such huge losses of the Hudson's wetland habitat had detrimental effects on wetland inhabitants. Migrating fish were cut off from upstream or downstream routes, native submerged vegetation were unable to survive in the deeper, high velocity main channel (Collins and Miller 2011), and several native animals were listed as threatened or endangered (Miller, Daniel E. 2013). In addition to providing habitat for a multitude of species, wetlands stabilize sediments, sequester heavy metals and improve water quality, provide storm and wave protection, and store more carbon per unit area than any other terrestrial habitat, whilst comprising just 4% of terrestrial area (Hossler and Bouchard 2010).

Thankfully, in 1989 President Bush signed an executive policy of "no-net wetland loss," which stated that all efforts must be made to avoid wetland destruction, and if destruction is necessary a wetland of equal area to the one destroyed must be created or restored (US EPA 2015). In 1990, plans to restore the Hudson estuary followed suit and the remaining unaltered wetlands provided models for restoration of degraded wetlands. In the Hudson Estuary Restoration Plan four "Priority Hudson Habitats" are defined: tributary streams, shorelines, shallow water, and intertidal land. Secondary channel restoration includes all but the first mentioned habitat. Successful side channel restoration will increase ecosystem functions such as a dynamic trophic food web, sediment stabilization, primary production, bird habitat, overwintering refuge, fish nurseries, and over time these functions will sum to improved water quality and stocks of fish (Miller, Daniel E. 2013). This study will look at the restored side channel habitat of Gay's Point, which is north of Hallenbeck Creek, the reference wetland (Wawiernia 2016). The use of a natural wetland as a model in restoration plans is critical in setting restoration goals. The reference site provides functionality reference data for setting goals, however there is no assurance that created wetlands will meet their functionality goals. Sediment organic matter (SOM) accumulation is a common restoration goal, but increased flow velocity as a result of hydrologic alterations creates scour and works against SOM build up (Findlay et al. 2002). Created wetlands have been shown to have lower sediment organic carbon (SOC) than natural wetlands. Likewise created wetlands have lower microbial activity, lower transformation rates of SOC to CO_2, and lower biodiversity (Hossler and Bouchard 2010). The reality is that people don't yet fully understand the intricate bio-physical-chemical connections that sustain wetland ecosystem integrity, so design plans can be quite experimental. A caveat in wetland restoration is unaccounted differences from the reference wetland to the study site. Hallenbeck for example has more submerged aquatic vegetation than Gay's Point, which presents an extra variable influencing SOM and microbial function that is difficult to account for. Flow velocity, invertebrate species, proximate urban areas, and many other factors can also cause ecosystem function differences in the restored site relative to the reference site.

A variety of microhabitat types within a habitat potentially increases niche availability and subsequently increases biodiversity. Strayer and Findlay 2010 found that habitat heterogeneity positively correlates with high biodiversity, and a diverse shoreline that accumulates fine sediment particles is ideal for assemblage of microbial communities (Strayer and Findlay 2010). Microhabitats collectively compose dynamic metaecosystem habitats with consistent feedback from the components. Increased connectivity between the microhabitats of a metaecosystem creates more opportunities for diverse plant and animal communities. Many species facultatively use several microhabitats through life stages, and transport chemicals and other organisms between habitats (Schofield et al. 2018). Gay's Point managers attempted to increase habitat heterogeneity with the inclusion of rootwad and alcove structures, meant to accumulate SOM and stimulate microbial hotspots. Rootwads are partially buried tree stems with intact roots which are placed strategically in clusters and partially in the flow of water to maximize OM accumulation. Alcoves are pools fringing the secondary channel excavated to match the secondary channels prevailing bottom elevation. Tide-induced bidirectional flow increases connectivity and will expectedly amplify the effects of these structures in the whole system. Highly productive microsites provide downstream areas with nutrient loads. Periods of pulsing flow have high productivity, and biodiversity is greatest in the high marsh compared to the low marsh and subtidal regions of a freshwater tidal marsh, so alcoves are designed to drain with the tide to prevent standing water. In this study microsites that accumulate organic matter, in relation to average OM

loading in the system, and thus provide ideal and preferential habitat for microbes, are referred to as "microbial hotspots".

Microbes are important secondary producers. In the detritus-based food chain fungi begin breaking down plant matter, and bacterial microbes finish the process. In the absence of primary producers, microbes occupy the base of the food chain. Microbial nutrient transformations create plant-available element forms and facilitate in sequestering toxins. Microbes transform SOC into CO_2, sediment organic nitrogen into NO_3, and sediment organic phosphorus into PO_4. Microbial metabolism drives global cycles of C, N and P (Sinsabaugh et al. 2014). Both the study site and its reference site are intertidal freshwater marsh secondary channel habitats which are generally characterized by having salinity ≤0.5ppt, receiving influx of P from upland tributaries, decay of organic matter, or rock weathering, and an influx of N and SOM with high tide. In freshwater tidal marshes microbes inhabit the sediment and an oxidized surface layer creates a chemical gradient that maintains movement of molecules (Levinton and Waldman 2006). Organic C facilitates microbial transformation and digestion of N and P. Carbon acquisition is common in studies of wetland microbes because of its importance in the acquisition of other elements, and because of the historical difficulties in having sufficient SOC for ecosystem health in created wetlands (Hossler and Bouchard 2010). Gays Point and Hallenbeck Creek are both connected to the main channel and experience twice daily high and low tides. N is thought to be the most limiting nutrient in saline waters while N and P are co-limiting in fresh waters such as the study site. Sources of P and N loading would suggest P to be more abundant at low tide and more consistent through the tide cycle than N. P is conceivably representative of autochthonous nutrient cycling, and N of allochthonous nutrient cycling (Levinton and Waldman 2006). Studies cannot count on retention in the system for consistent levels of N, as N loads peak at high tide and sampling is usually done over several days with different tide cycles. Additionally, nutrient budget generalizations are not always true, so organic matter composition should be characterized on a site by site basis to best manage any wetland.

Microbes metabolize organic matter externally by releasing enzymes specific to the organic molecule being lysed. Extracellular enzymatic activities (EEAs) can be characterized to determine the function of a microbial community (Findlay and Sinsabaugh 2006). EEAs have been shown to be positively correlated with SOM, so where OM accumulates, one would expect to see high EEA and thus microbial metabolism. Likewise, respiration rates often positively correlate with SOM (Sinsabaugh et al. 2014). In addition to quantity of SOM, the quality of SOM is also important for microbes. Microbes are able to occupy habitats such as redox gradients where macro fauna could not survive Baker et al., 1999; Kemp and Dodds, 2001 (as cited in Findlay 2016), and are able to regulate community composition to maximize occupancy of niches in the sediment (Freimann et al. 2015). Microbes exhibit plasticity in detrital stoichiometry, whereas macro fauna are intolerant of such energy source variations (Findlay 2016). Heterotrophic bacteria alter enzymatic expression to accommodate changes in carbon, nitrogen, and phosphorus supply Godwin and Cotner, 2015 (as cited in Logue et al., 2015). However, microbes are sensitive to changes in quantity of SOM, content of SOM, pH (Findlay 2016), and mechanical "washing" by high velocity flow. Also, microbial tolerance to varying detrital stoichiometry is limited, Sinsabaugh et al. 2014 demonstrated that EEA, and thus microbial function (Freimann et al. 2015), is limited by OM availability. Studies showed that the concentration of sediment elements correlates with, but does not equal, the concentration of sediment elements available for microbial metabolism. Microbial metabolism is limited by nutrient availability, or quantity of available binding sites for an enzyme. Microbes make tradeoffs within enzymatic expression in response to nutrient availability, to optimize substrate to product reactions without expending unnecessary energy. Extracellular enzymatic reactions are the first step in microbial nutrient acquisition and enzymatic assays can reveal nutrient limitations in a system by showing where the community allocates enzymatic expression. Sediment C: P: N ratios, from enzymatic digestion, have been shown to be about 1:1:1 and represent the bacterial community's homeostasis, not the sediments bulk elemental composition or SOM composition (Sinsabaugh et al., 2014).

Microbial communities comprise many kinds of microscopic biota including archaea, eukaryotes, bacteria, and fungi (Logue et al. 2015). Current studies on sediment microbes involve identifying key-stone species within the microbial community. By sequencing microbial DNA in company with characterizing EEAs, the specific species most involved in a reaction can be identified. Furthermore, researchers are interested in finding the nutrient limitations of these specific microbial individuals. An important question given our global state is how microbial communities will respond to climate change (Findlay, 2016). Factors such as rising sea levels, increased salinity, rising temperatures, and shifts in carbon supplies can potentially affect microbial respiration, production, activity of enzymes, or carbon transformation and sequestration (Logue et al. 2015). To predict such responses would be useful in managing wetlands for resilience, but a complete understanding of parameters around microbial assemblage will be crucial to such predictions.

The main question facing wetland restoration, and facing the restoration of Gay's Point, is will the system meet restoration goals and be comparable in ecosystem services to natural wetlands? This study will attempt to answer whether the rootwad and alcove structures included in Gay's Point accumulate organic matter and stimulate microbial hotpots. Specifically this study asks, does mass of SOM differ between rootwads, alcoves, and the secondary channel, does EEA C, N, and P linked enzymes differ between rootwads, alcoves, and the secondary channel, and does microbial metabolism differ between rootwads, alcoves, and the secondary channel?

There are a few possible outcomes. The null hypothesis is that neither alcoves nor rootwads will accumulate organic matter and stimulate microbial hotspots, i.e. have higher EEA compared to the secondary channel. Secondly, it is possible either alcoves or rootwads will function to accumulate organic matter and stimulate microbial hotspots. In this case there could be a hierarchy of OM accumulation and microbial activity, with respect to each structure and followed by the secondary channel. Or, either rootwads or alcoves will function as intended and the other structure will be equal with the secondary channel in OM accumulation and microbial activity. Third and where this study leans, both rootwads and alcoves will function in accumulating organic matter and will have increased EEA compared to the secondary channel, but EEA will have negligible variation between rootwads and alcoves. Answers to these questions will primarily allow managers and contributors of Gay's Point to assess if alcoves and rootwads are working. If they function as intended, Gay's Point could become a reference for other restoration projects. Structures to accumulate OM could become commonplace in created wetlands. If the structures do not function, then adjustments should be made to the structures or another plan to accumulate OM should be developed. This study will shed light on the idea that variation in hydrology and topography, or habitat heterogeneity, creates more functionality. This study will also fit amongst others investigating sediment stoichiometry, enzymatic elasticity, and EEAs.

MATERIALS AND METHODS

Description of Field Site

Gays Point is approximately 2,000,000 ft^2 in area with a 1000ft long channel of average bottom width 65ft and a 650ft intertidal designed to be inundated at high tide and above mean water level at low tide (Wawiernia 2016). Core samples were taken at each of the 3 alcove locations, each of the 4 rootwad locations, and at 4 mid-channel locations without alcoves or rootwads (Fig 1.).

Sampling for Gays Point was done on two different dates. 3 sediment core samples were taken at each location for a total of 33 sediment core samples in the first trip. 3 sediment core samples were taken at each alcove, rootwads 1 and 2, and midchannels 1 and 2. Due to unexpected thunder sampling time was limited and thus 2 sediment core samples were taken from rootwads 3 and 4, and none from midchannel 3, for a total of 28 sediment core samples in the second trip. Core samples were roughly 5-10 cm depth, collected

using a polycarbonate corer of inner diameter 8cm (Tritthart et al., 2011). Sediment samples were taken at mid-channel and edge locations of Hallenbeck Creek for comparison to Gays Point (Fig 2).

Organic Matter

Total organic matter content of sediment samples was obtained as mass (mg) of non-ash dry weight by loss-on ignition at 450°C (Findlay 2017). Water samples were taken at each mid channel location and later measured for nitrate concentrations, as compared to a standard curve, in order to account for nitrate and phosphate removal by the system (data not shown). Sediment samples were taken at mid-channel and edge locations of Hallenbeck to Gays Point.

Microbial Function

For the acquisition of C, enzymatic activities of esterase, β-glucosidase, α-glucosidase, and β-xylosidase were measured using 4-MUB linked acetate, β-D-glucopyranoside, α-D-glucoside, and β-D-xylopyranoside substrate analogs, respectively. For the acquisition of N, enzymatic activities of Leu-aminopeptidase, β-N-acetylglucosaminidase, and endopeptidase were measured using L-Leucine 6-amido-4-methyl-coumarin substrate analog and 4-MUB linked β-N-acetyl-β-glucosaminide and P-guanadinobenzoate substrate analogs, respectively. For the acquisition of P, enzymatic activities of Phosphatase were measured using 4-MUB-phosphate as the substrate analog. Substrate analogs of 1mM were prepared according to Findlay 2018.

Sample slurries were made with ~2 g wet mass of sediment and filled to ~45 mL with DI in a 45mL tubes. Slurries were homogenized by vortexing and sediment weight and volume was measured for each slurry. 150μL of slurry was added to plate wells, with 2 replicates per well and 100μL of each substrate analog were added subsequently. 250μL of each sample and substrate analog were added to wells and run concurrently as blanks (Findlay 2017). The mixture was placed on a shaker table and incubated in the dark (Findlay and Sinsabaugh 2006). Fluorometric data were collected at roughly 1, 2, 3, 8, and 20 hours after beginning incubation using a microplate reader set at 365nm excitation and 450nm emission wavelengths (Findlay 2017). The rate of enzymatic activity was calculated as the linear increase of fluorescence over time, and converted to μmol MUB/mg SOM hr-1. Quenching and any pH effects on fluorescence detection was accounted for by running plates with 8 replicates of 150μL for each sample concurrently with 8 replicate wells of 250μL methylumbelliferone of the same molarity. Variation from just methylumbelliferone was calculated for each sample, for later correction of sample EEAs (Findlay and Sinsabaugh 2006).

Respiration is one of the major pathways of metabolism and was measured as rate of oxygen consumption (DO saturation, min-1). 60-mL BOD bottles were filled with sediment and sealed. Initial DO was measured, as well as DO approximately 3 hours, 6 hours, and 9 hours later using YSI 5905 BOD probe. Control blanks were run in parallel (Findlay and Sinsabaugh 2006).

Statistical Analysis

Two-tailed t-tests were used to compare factors between gays Point and Hallenbeck Creek, as well as to compare low marsh vs edge locations within Hallenbeck. Anova multi way tests were used to find variances by locations, sample group, microsites, and by enzyme for EEAs. Regression slopes were used to relate respiration and SOM, EEAs and SOM, and EEA and respiration.

RESULTS

"Sites" refers to Gays Point (GP) and Hallenbeck (HB) as wholes. Microsites are abbreviated as: Alcoves (AC), Rootwads (RW), and Midchannels (MC). Specific microsites are abbreviated as: Alcove 1 (AC 1), Alcove 2 (AC 2), Alcove 3 (AC 3), Rootwad 1 (RW 1), Rootwad 2 (RW2), Rootwad 3 (RW 3), Rootwad 4 (RW 4), Midchannel 1 (MC 1), Midchannel 2 (MC 2), Midchannel 3 (MC 3), Midchannel 4 (MC 4), Low marsh, and Edge.

Organic Matter

SOM ranged from 12.1 % dry mass (mg) in AC 1 to 0.6 % dry mass (mg) in RW 3. Organic matter in sediment was not significantly different between sites (*two-tailed t test;* df = 1, p = 0.54). Within Hallenbeck Creek there was also no significant variation in SOM by microsite, (*two-tailed t test;* df = 1, p = 0.14), so for assessment of Gays Point microsite types, comparisons were to Hallenbeck SOM summed over all samples (n = 24, see Table 1 for values). Specific microsites within Gays Point were compared to Hallenbeck microsites (Table 2). SOM varied by microsite type (*Anova;* df = 3, F = 5.78, p = 0.001), and varied within microsites (*Anova;* df = 12, F = 6.704, p < 0.001). Posthoc results of SOM revealed ACs > MCs, ACs > RWs, ACs > HB (p = 0.001, p = 0.007, p = 0.014, respectively), and RW=HB, RW=MC, MC=HB (p > 0.94, p = 0.813, respectively).

Gays Point microsites were additionally analyzed individually, without Hallenbeck data, for clearer variance within microsite type (Fig. 3). For alcoves, (*Anova;* df = 2 , F=34.471, p < 0.001), post hoc results were AC 1 > AC 2, AC 3 > AC2 (p < 0.001). For rootwads, (*Anova;* df = 3, F = 7.853, p = 0.002), post hoc results RW 2 > RW 4, RW 1 > RW 3, and RW 2 > RW 3 (p = 0.025, p = 0.029, p = 0.001, respectively). For midchannels, (*Anova;* df = 3, F = 7.577, p = 0.002), post hoc results MC 2 > MC 1, MC 3 > MC 1, MC 2 > MC 4, and MC 3 > MC 4 (p = 0.008, p = 0.047, p = 0.009, p = 0.049, respectively).

SOM in Hallenbeck Creek correlated positively with sample group (*Anova;* Fig. 3, p < 0.001), with groups 5 and 6 being equal to each other and less than all other sample groups (p < 0.015, *Post-hoc*). In light of this result sample group numbers were given to Gays Point in relation to distance from start, (see Fig. 1 for reference; AC 1, MC 1, RW 1= "1", AC 2, RW 2, MC 2 = "2", RW 3 = "3", MC 3 = "4", RW 4 = "5", MC 4, AC 3 = "6"). Anova results indicated no significance in SOM by this variable (p=0.06).

Respiration

Within Hallenbeck Creek there was also no significant difference in respiration by microsite, (*Anova;* df = 1, p = 0.54), so as with SOM, respiration rates of Gays Point microsite types were compared to Hallenbeck respiration rate of the whole site (n=24, see Table 1 for values). Individual microsites in Gays Point were compared to Hallenbeck microsites. Respiration rate was greater in Hallenbeck than in Gays Point (*two-tailed t-test;* t = 2.39, p = 0.015). Respiration in Hallenbeck was not correlated with sample number. Respiration did not significantly differ by microsite types or within microsites (p = 0.088 and p = 0.688 respectively, Anova), nor did it differ when microsite types were analyzed for each site individually (p > 0.5, Anova). Respiration was not positively correlated with SOM in any measure.

Extracellular Enzymatic Activity

Nutrient limitations based on stoichiometry of enzymatic activities, by the ratios of C:N:P linked activities, have been found to reflect nutrient limitations in sediment. Ratios diverging form 1:1:1 indicate a nutrient limitation of the microbial community (Sinsabaugh et al. 2014), so EEAs can be used as proxies for nutrient limitations of systems. The ratios of C linked EEA to P linked EEA for Gays Point and Hallenbeck sites were 1.01 + 0.094 SE and 1.19 + 8.44 SE, respectively. The ratios of C to N linked EEA for Gays Point and Hallenbeck sites were 1.22 + 0.56 SE, and 0.99 + 0.95 SE, respectively. The ratios of N to P linked EEA for Gays Point and Hallenbeck sites were 0.828 + 0.08 SE and1.19 + 8.72 SE, respectively. Ratios

were not statistically different from each other, nor did they differ by site (Anova; $p = 0.1939$, $p = 0.8472$, $p = 0.9776$, respectively), indicating there is not a large difference in nutrient limitations by site. However, within Gays Point ratios were statistically different ($df=3$, $F = 15.303$, $p = 0.00112$), with P:N > N:P, and C:P > C:N ($p = 0.0021$, $p = 0.0019$), suggesting that within Gays Point there is a greater effort by the microbial community for N acquisition over P acquisition. C:N and C:P were not compared to N:P or P:N.

As with EEA nutrient ratios, the relative activities of C, N, and P linked enzymes have been found to reflect relative nutrient acquisition effort, or nutrient availability, of microbial assemblages in sediment. EEA was separated into C linked EEAs, N linked EEAs, and P linked EEAs and analyzed by microsite, microsite type, and site to assess spatial variation in nutrient acquisition. C linked EEAs were greater in Gays Point (*two-tailed t-test;* $t=3.109$, $p = 0.002$), and differed by microsite type ($p = 0.0012$, *Anova*) where RWs > Hallenbeck and MCs > Hallenbeck (*Anova*; $p = 0.037$, $p = 0.003$). P linked EEAs were greater in Gays Point (*two-tailed t-test;* $t=2.517$, $p = 0.015$), and differed by microsite type ($p = 0.0422$, *Anova*) where RWs > HB ($p = 0.0399$, *post-hoc*). N linked EEAs did not differ by site ($p = 0.084$, *two-tailed t-test*). Results indicate there may be less available sources of C and P for microbial degradation within Gays Point, while sources of N may be equitable in sites and locations. Given that rootwads were the main cause of variation by location for C and P linked EEAs, it is likely the full scope of causes to spatial variation of nutrient acquisition is underestimated in this study.

Post hoc tests of variance in P linked EEAs by microsite revealed RW3 > AC1, AC3, Edge, Low Marsh, MC 2, RW 1, and RW 2 ($p < 0.05$). Post hoc tests of variance in C linked EEAs by microsite revealed RW3 > AC1, AC2, AC 3, Edge, Low Marsh, MC 2, MC4, RW 1, RW 2, ($p < 0.001$), and RW 4 > AC 1 and Low Marsh ($p < 0.05$). Post hoc tests of variance in N linked EEAs by microsite revealed RW3 > AC1, AC2, AC 3, Edge, Low Marsh, MC 1, MC 2, MC4, RW 1, RW 2, ($p < 0.01$) and RW 4 > AC 1 and Low Marsh ($p < 0.05$). Due to the similarities in results of nutrient linked EEAs by microsite, with RW 3 and RW 4 again carrying highest significance, we concluded there was not a large difference in local constraints to nutrient acquisition effort by individual microsites, and subsequent analysis was done on EEAs separated by nutrient and grouped by site or microsite type.

We analyzed total EEA to assess spatial variation in overall microbial activity and overall plasticity of the systems. Total EEAs of both sites varied by site and microsite type ($p < 0.0001$, Anova) with Gays Point having greater mean EEA (two-tailed t-test; $t = 4.021$, $p < 0.0001$,), and RWs = MCs > ACs = HB (post-hoc; $p = 0.5327$, $p < 0.01$, $p = 0.9984$, respectively), suggesting Gays Point may have less readily degradable material. Within Gays Point total EEA differed by microsite type and within microsites (Anova; see Fig 4, $p = 0.0007$, $p < 0.0001$). Post hoc results revealed ACs < RWs and MCs ($p = 0.0005$, $p = 0.257$, respectively), suggesting ACs contain more degradable material in sediment than RWs or MCs. Within Hallenbeck total EEA did not differ by microsite but did differ by sample group, with group 2 > group 1, and group 2 < group 3, group 4, group 5, and group 6 (post-hoc; $p < 0.0001$, Fig 5), further indicating a cause of spatial variation outside of this studies parameters. Total EEA of both sites decreased with increasing organic matter ($t = -5.426$, $p < 0.0001$) and decreased with increasing respiration ($t = -3.543$, $p = 0.0004$), as was expected. However mean EEA in Gays Point decreased with increasing SOM ($t = -10.35$, $p < 0.0001$) and within Hallenbeck Creek total EEA did not correlate with SOM ($p = 0.769$). Neither site's mean EEA correlated with SOM (*Hallenbeck;* $p = 0.628$, *Gays Point;* $p = 0.085$), however EEA was normalized to SOM so given the lack of relationship between SOM and respiration, this result was expected. Additionally respiration and EEA data are from a single sampling effort per site, so it is possible increased sampling would yield different results and possibly show more consistency in trends of respiration to SOM and EEA.

Studies concerning wetland sediment microbial function often focus on the EEAs of β-glucosidase (BG), Phosphatase (AP), Leucine aminopeptidase (LU), and N-acetylglucosaminidase (NAG). BG degrades cellulose, a primary component of plant cell walls. AP degrades polysaccharides, nucleotides, and

phospholipids. LU hydrolyzes peptide bonds of leucine and alanine, the two most abundant protein amino acids (Freimann et al., 2015). NAG degrades chitin, the second most environmentally abundant polymer after cellulose, and a large reserve of sediment bound nitrogen. Therefor activity of NAG and LU are indicative of nitrogen cycling, BG is indicative of C cycling, and AP is indicative of P cycling (Kang et al. 2005).

Similarly, we found that of all eight enzymes in this study only BG, NAG, AP, and LU were those of significance by any parameter, supporting the assumption that these enzymes are particularly important in microbial nutrient acquisition. LU, BG, NAG, and AP activities differed by microsite (*Anova*; $p = 0.0262$, $p = 0.0002$, $p = 0.0024$, $p = 0.0422$). In all cases variation was due to RW 3 and RW 4 (Table 2), suggesting these enzymes may be sensitive to small factors and RW 3 and RW 4 likely from the rest of Gays Point. Across all sites BG activity correlated with that of LU, AP, and NAG ($t = 9.831$, $t = 10.286$, $t = 11.979$, respectively, $p < 0.0001$). NAG activity correlated with AP activity and LU ($t = 7.336$, $t = 14.74$, $p < 0.0001$). AP activity correlated with LU activity ($t = 9.755$, $p < 0.0001$). Within Hallenbeck only BG correlated with AP ($t = 5.372$, $p = 0.0058$). Within Gays Point, none of the enzymes which differed in EEA by microsite correlated with each other ($p > 0.07$). Results suggest that generally all enzymes are needed to break down detritus and uptake nutrients, and BG is manipulated for the uptake of other compounds. However it is likely the composition of detritus in Hallenbeck differs from Gays Point and contains a source composed majorly of cellulose and phosphomonoesters, implied by the positive correlation of BG and AP (Luo et al. 2017). Gays Point is likely more heterogeneous in sources of nutrients, so no particular enzyme correlates with another in the systems normal detrital breakdown.

DISCUSSION

Assessing the microsites within Gays Point fits into a larger picture of improving practices of wetland restoration. In this study we conclude Hallenbeck and Gays Point to be comparable in all measured parameters, although inclusion of other environmental factors would improve the picture of restoration success in this study. Alcoves finished as the most successful microsite type, and rootwads were not different than mid channel locations within Gays Point. In this study it appears implementing habitat heterogeneity does increase microbial function and SOM accumulation, and potentially culminates to allow the restored site to match its reference site in ecosystem function.

Organic Matter

Gays Point did not differ from Hallenbeck in accumulation of SOM, and alcoves as a group were more successful in SOM accumulation than rootwads, midchannels, and Hallenbeck Creek as a whole. Alcove 1 had the highest mean SOM accumulation amongst microsites. Hallenbeck did not differ in SOM by microsite (low marsh vs edge), although generally the low marsh (or edge) locations of a freshwater marsh have higher SOM accumulation, respiration, and microbial functionality than the low marsh locations. Alcove 1 was the only microsite to have a meaningful difference with edge microsites of Hallenbeck, and was greater in mean SOM accumulation suggesting that at the very least Alcove 1 is more successful than Hallenbeck in SOM accumulation. Further, it is possible alcoves are functioning to amend the repeated problem of low SOM accumulation in restored wetlands.

SOM did not vary by microsite in Hallenbeck but did so in Gays Point, which could indicate the natural wetland, Hallenbeck, has reached a greater state of maturity or equilibrium. On the other hand, it is possible the implemented structures in Gays Point are providing habitat heterogeneity so that there are a greater variety of niches, exhibited by variation in SOM buildup by microsite. The difference in SOM within microsites suggests future restoration plans planning on implementing similar structures should streamline factors such as depth and area, which differed within Gays Point alcoves (Wawiernia 2016). It is also possible the variation in microsite reflects an effect of proximity to the main channel. Alcoves 1 and 3

surpassed most other microsites in SOM build up and happen to be on the latitudinal endpoints of the side channel (Fig. 3), and SOM only varied within Hallenbeck by sample group, with the two northernmost groups having the least SOM (Fig. 3). This pattern could reflect an interaction with hydrology and connectivity to the main channel where in Hallenbeck the northern end is narrow enough to receive OM inputs from mainly downstream, and SOM settles as it enter resulting in higher accretion downstream, and in Gays Point the northern and southern points are broad enough to equally allow flow of high tide, so SOM accumulates in the alcoves closest to latitudinal endpoints.

Generally in marshes, the supply of organic matter is dependent on primary production and subsequent detrital input, as well as inputs from incoming tides. Therefore hydrology and land cover present factors capable of influencing SOM and should be quantified for a more complete picture of SOM accumulation in either Gays Point or Hallenbeck Creek.

Respiration

Respiration proved to be the puzzling variable, as it did not correlate with SOM in any location or microsite, and did not follow patterns of variation seen with SOM or EEA. Respiration was greater in Hallenbeck, but within Hallenbeck respiration did not correlate with sample group as SOM did. Unlike SOM, respiration did not vary by microsite within Gays Point. Although respiration did not correlate with SOM as expected, this finding was shared amongst both sites, so Gays Point is at least behaving similarly to Hallenbeck in the relationship between respiration and SOM. It is likely there are other interacting factors accounting for this variation not measured in this study. Respiration was measured on samples from just one trip per site, so perhaps increasing sampling periods for measurement of respiration would yield different mean respiration rates.

Extracellular Enzymatic Activity

Microbial enzymatic expression at the community level, as is this studies concern, can be discussed as an example of allometry, where rather than a scaling of body function with body size, microbial function scales with community complexity. Assessments of EEA reveal what nutrients microbes are transforming, which relates to sediment nutrient concentrations, stoichiometry, and depends on the microbial species present. Both sites appear to be near equilibrium enzymatic ratios, as nutrient linked EEA ratios did not greatly diverge from unity, the greatest difference being 0.172 for Gays Point N:P EEAs. Enzymatic ratios reflect allocation of microbial extracellular enzymes in response to substrate availability. A ratio of one for C:N:P linked EEAs reflects community homeostasis and should correlate with sediment nutrient ratios close to one. Similarly, C:P, C:N, and N:P ratios of one indicate equal expression of nutrient linked enzymes, and thus no dominantly limiting nutrient. As sediment nutrient ratios differ from one, microbial communities must alter enzymatic expression to maintain homeostasis, and nutrient linked EEA ratios consequently diverge from one. EEA ratios of N:P linked enzymes in Hallenbeck were > 1 and suggest Hallenbeck to be somewhat P limited. N:P ratios in Gays Point were < 1 and suggest some N limitation in Gays Point. Nutrient limitations can have implications for the efficiency of nutrient removal from waterways, where an N limited system would better remove N and a P limited system would better remove P. However because ratios did not drastically differ from one we can assume neither site is drastically limited by P or N, although co-limitation is probable. N linked EEA was the only nutrient linked EEA not to differ by site and P and C linked EEAs were both greater in Gays Point. These results imply P and C are more available to microbes in Hallenbeck, complicating a conclusion of nutrient limitations in either site and exemplifying the need for characterization of fractions of N, P, and C within SOM.

Mean EEA followed a similar patter to SOM, although it was greater in Gays Point as a whole. Given that mean EEA decreased with respiration and SOM as expected, this study reinforces the use of EEAs as indicators for nutrient dynamics of a system. Within Gays Point mean EEA correlated with SOM and

alcoves were equal in mean EEA to Hallenbeck and less than other locations. Alcoves 1 and 3 occupied the lower end of mean EEA. Following the finding that these microsites exceeded others in SOM, it would appear that these microsites are in fact microbial hotspots. Although mean EEA did not correlate with SOM in Hallenbeck creek, mean EEA also seemed to follow a pattern of proximity, described by sample group, similar to that of SOM with the sample groups closest to the main channel having highest microbial function. Increased sampling should be done within Hallenbeck to clarify the existence of any spatial patterns, and inclusion of a measurement for hydrology would additionally provide for more concrete conclusions.

It is likely that the quality of organic matter differs within microsites, and thus accounted for some variation in EEA and EEA ratios by specific microsites. Sediment organic matter directly influences microbial functionality, as microbes regulate extracellular enzymatic expression to optimize nutrient availability, through carbon use and assimilation (Sinsabaugh et al. 2015). We did not characterize the composition of organic matter, so we cannot confirm a correlation with nutrient availability and microsites. Detritus is bound in a biopolymer matrix resistant to enzymatic bonding, and requires fungi to convert complex polymers into monomers (Reddy 2008), so perhaps microsites with high SOM but low microbial function accumulate coarse particles of SOM rather than fine particles required for extracellular enzymatic bonding. Without characterizing the composition of SOM, the fractions of organic N, P, and C, we cannot assess microbial availability and thus it is possible microsites differ in composition of organic matter.

Many studies include just AP, LU and BG (for P, N, and C acquisition respectively), in experiments concerning microbial nutrient acquisition. This study similarly found these enzymes to be of interest, exhibiting variance by microsite in EEA. We also found N-acetyl glucosaminide (NAG) to differ by microsite. Variation was almost completely due to rootwads 3 and 4, indicating some environmental interaction unique to these microsites, influencing microbial acquisition of cellulose, chitin, peptides, and phospholipids. EEAs of these enzymes all positively correlated with each other when both sites were grouped together, a result contrary to that expected. If the expression of one enzyme is a cost that reduces the ability to create and express enzymes for different targets, then EEAs for different targets should have a negative correlation. However it is possible different nutrient sources are bound together and nutrients acquired from within the detrital matrix are thus assimilated together. Given that EEA was greater in rootwads 3 and 4, it is also possible the positive correlation of EEAs for specific enzymes could be a reflection of variation from these specific microsites. Within Hallenbeck BG correlated with AP, implying carbon acquisition in correlation with P acquisition and supports the suggestion that Hallenbeck is more P limited than N limited. Increased sampling should be done within microsites, especially rootwads 3 and 4, to more accurately assess EEA relationships between specific enzymes.

CONCLUSION

In this study we wanted to investigate the success of integrating the theory of habitat heterogeneity in a restored wetland by the inclusion of Alcove and Rootwad structures. We conclude that Alcove structures were the highest functioning within Gays Point, and did serve as points of accumulation for SOM as well as microbial hotspots. Within Alcoves, Alcove 1 was the most successful microsite by these parameters. Gays Point, the restored site, does not dramatically differ in functionality from Hallenbeck, exhibited by statistically equal accumulation of SOM and nutrient ratios. Respiration was greater in Hallenbeck, and total EEA was greater in Gays Point, both of which could indicate a certain degree of higher microbial functionality, however given that SOM and mean EEA were statistically equal between sites, we conclude the sites are generally comparable.

We can at this point only make assumptions concerning nutrient limitations in Gays Point and Hallenbeck Creek. Plans to further this study include assessment of microbial community structure and characterization of sediment organic matter. This study adds to a growing amount of research concerning wetland restoration

and methods of assessing microbial functionality, and has potential to benefit future restoration plans and study questions.

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APPENDIX

TABLE 1. Mean Experimental Values. Values presented as 'Value' ± "Standard Deviation,' in respective units: SOM as % of dry mass (mg). Respiration as loss of dissolved Oxygen / minute (DO/min). EEA as μmol / MUB μg SOM hr^{-1}, and grouped by mean EEA of Carbon (C), Nitrogen (N), and Phosphorus (P) linked enzymes. Gays Point grouped by Alcoves (AC), Rootwads (RW), and Mid-channels (MC).

Locations		SOM	Respiration	EEA		
				C	N	P
Gays Point	AC Average	8.478 ± 2.323	0.0610 ± 0.0195	3682807 ± 3485380	2774485 ± 2425434	3810750 ± 2748793
	RW Average	5.065 ± 2.729	0.0491 ± 0.0174	6099833 ± 5692661	5267372 ± 5126929	6205920 ± 5036755
	MC Average	3.963 ± 3.030	0.0574 ± 0.0129	5533863 ± 4506935	4484551 ± 3378777	5134148 ± 2382735
	Average	5.284 ± 3.457	0.0556 ± 0.0175	5141013 ± 4763638	4214466 ± 3973118	5091546 ± 3658501
Hallenbeck	Average	5.284 ± 3.457	0.0785 ± 0.0406	3280797 ± 3689177	3277622 ± 3647157	2743037 ± 2955888

TABLE 2. Post Hoc Results of Variance. Organic Matter (SOM) as % of dry mass (mg). Respiration as loss of Dissolved Oxygen / minute (DO/min). EEA as μmol MUB, μg SOM-1, hr-1.

Relationship	**p value**
SOM	
AC 1 > RW 3	p = 0.00001
AC 1 > MC 1	p = 0.00003
AC 1 > MC 4	p = 0.00003
AC 1 > Low Marsh	p = 0.0002
AC 3 > RW 3	p = 0.0006
AC 1 > RW 4	p = 0.0011
AC 3 > MC 1	p = 0.0019
AC 3 > MC 4	p = 0.0021
RW 2 > RW 3	p = 0.0082
AC 3 > Low Marsh	p = 0.0153
AC 1 > AC 2	p = 0.0235
RW 2 > MC 1	p = 0.0236
RW 2 > MC 4	p = 0.0257
AC 3 > RW 4	p = 0.0335
MC 2 > RW 3	p = 0.0411
Edge > RW 3	p = 0.0467
AC 1 > Edge	p=0.0553
EEAS	
Beta Glucosidase	
RW 3 > AC 3	0.0024
MC 1 > Low Marsh	0.0025
RW 3 > RW 2	0.0025
RW 3 > RW 1	0.0025
RW 4 > Low Marsh	0.0063
MC > Edge	0.0109
RW 3 > MC 2	0.0187
RW 4 > Edge	0.0210
RW 3 > MC 4	0.0302
AC 2 > Low Marsh	0.0528
Leucine	
RW 3 > AC 1	> 0.0001
RW 4 > AC 1	0.0007
RW 3 > AC 2	0.0002
RW 3 > AC 3	> 0.0001
RW 4 > AC 3	0.0049
RW > Edge	> 0.0001
RW 4 > Edge	0.0251

Table 2 - continued	
RW 3 > Low Marsh	> 0.0001
RW 4 > Low Marsh	0.0005
RW 3 > MC 1	0.0013
RW 3 > MC 2	> 0.0001
RW 3 > MC 4	> 0.0001
RW 3 > RW 1	> 0.0001
RW 4 > RW 1	0.0013
RW 3 > RW 2	> 0.0001
RW 4 > RW 2	0.0057
Phosphatase	
RW 3 > Edge	0.0006
RW 3 > AC 1	0.0034
RW 3 > RW 1	0.0077
RW 3 > RW 2	0.0109
RW 3 > AC 3	0.0143
RW 3 > MC 2	0.0371
RW 4 > Edge	0.0481
NAG	
RW 3 > Low Marsh	0.0001
RW 3 > Edge	0.0011
RW 3 > AC 1	0.0027
RW 3 > AC 3	0.0050
RW 3 > RW 1	0.0130
RW 3 > RW 2	0.0414
RW 4 > Low Marsh	0.0420

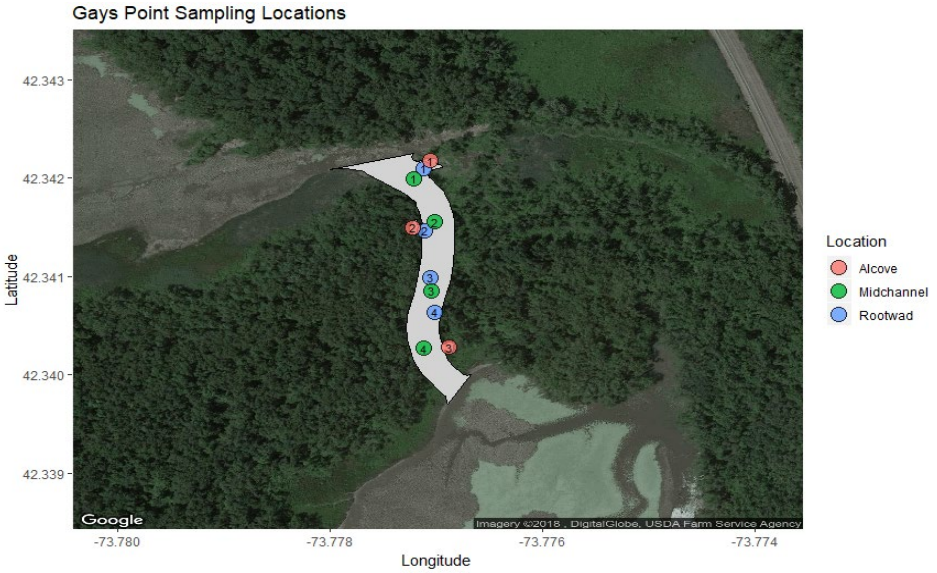

FIGURE 1. Map of Gays Point. Numbers indicate microsites.

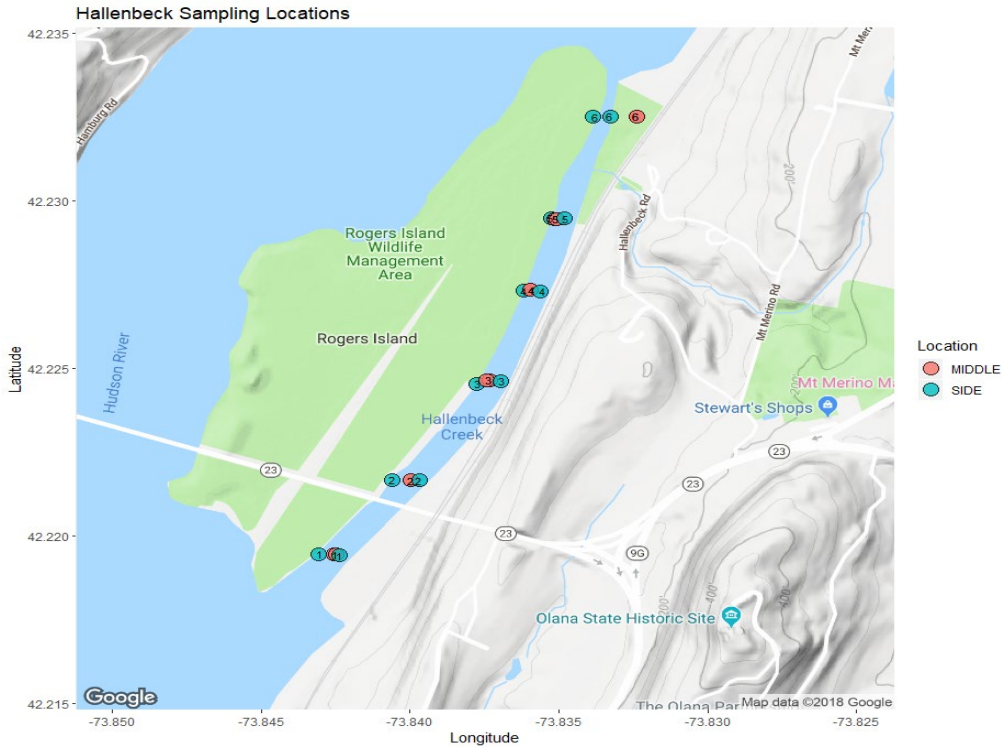

FIGURE 2. Map of Hallenbeck. Numbers indicate sampling group.

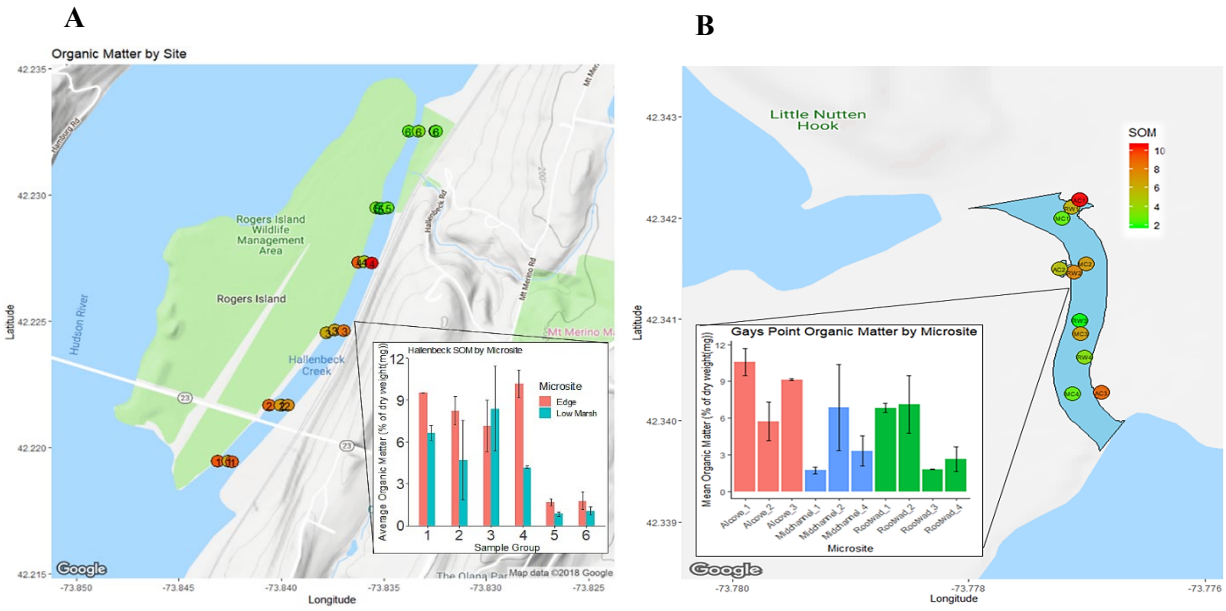

FIGURE 3. Site maps with SOM scaled to color. **A.** Map of Hallenbeck Creek with bar chart indicating variation by sample group. **B.** Map of Gays Point showing SOM with bar chart indicating variation within location types.

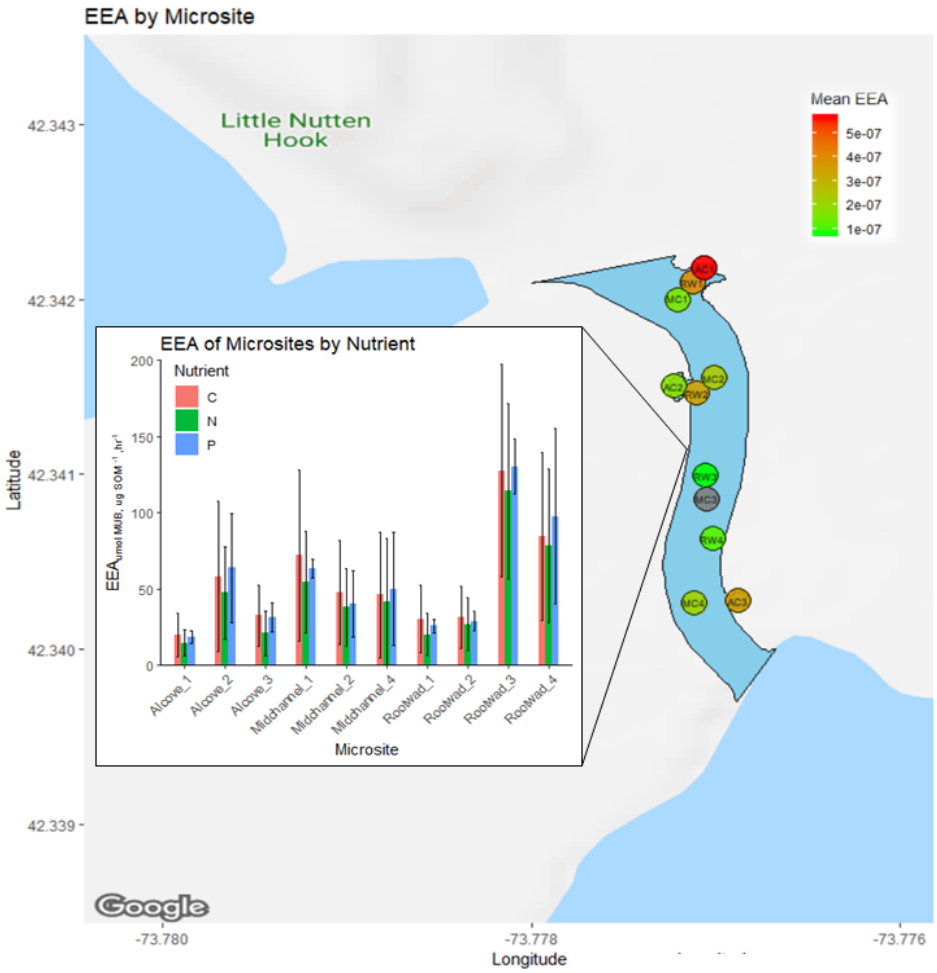

FIGURE 4. Map of Gays Point with mean EEA scaled to colors. EEA was transformed as 1 / mean (EEA) to equate color scale to figure 3. Bar chart indicates EEAs of microsites as grouped by linked nutrient.

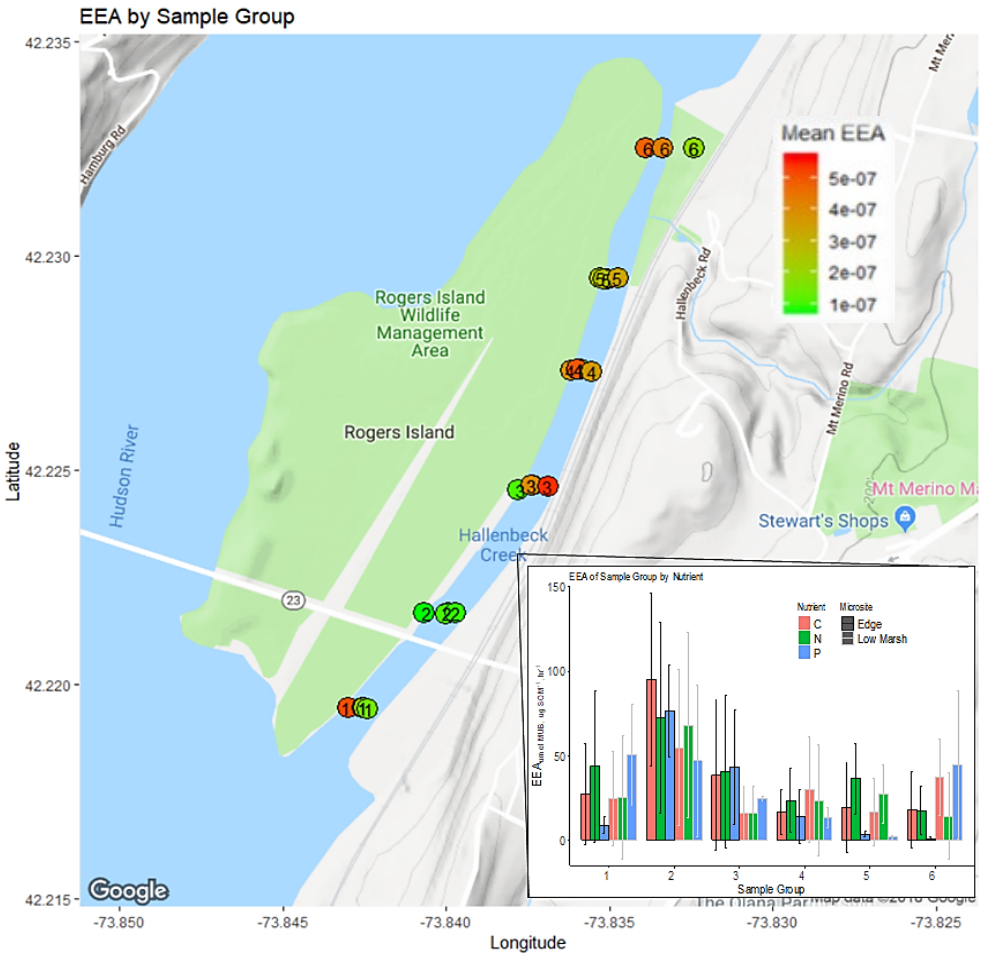

FIGURE 5. Map of Hallenbeck Creek with mean EEA scaled to colors. EEA was transformed as 1 / mean (EEA) to equate color scale to figure 3. Bar chart indicates EEAs of sample groups as grouped by linked nutrient and microsite.

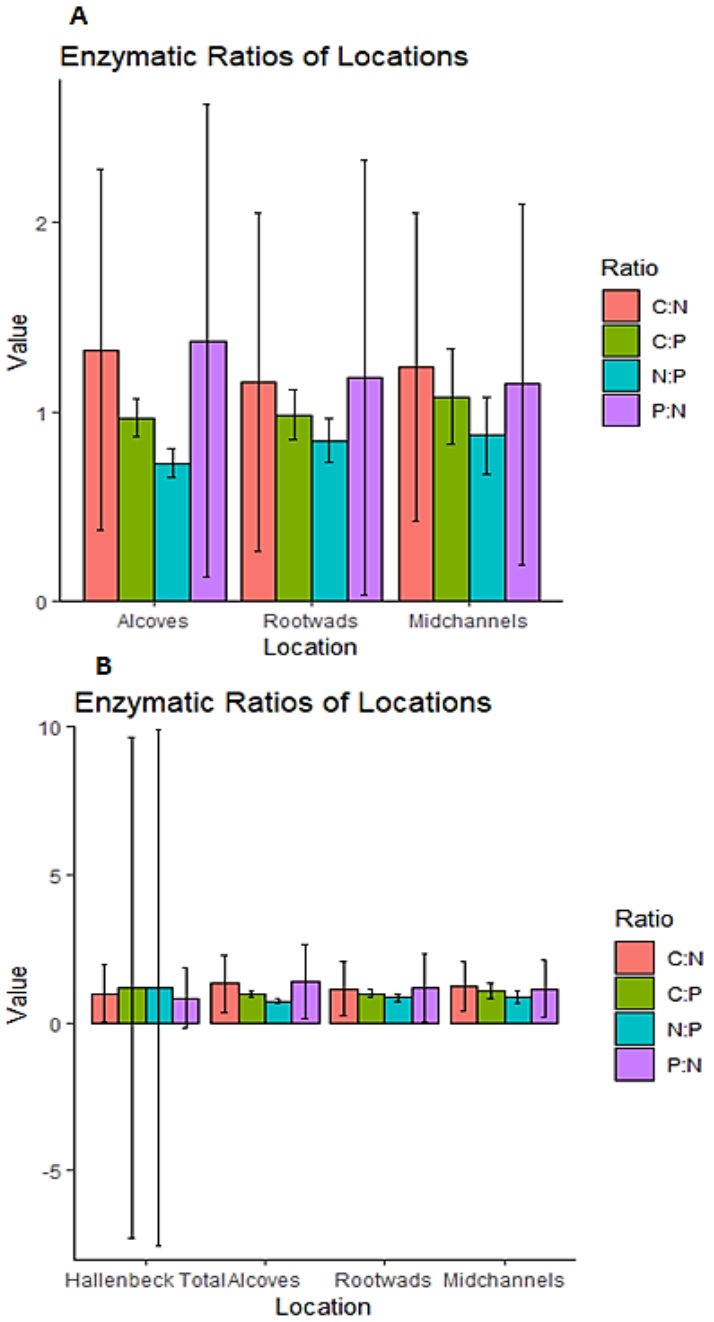

FIGURE 6. Charts of Nutrients linked EEA ratios, by locations. **A.** Only Gays Point microsite types. **B.** Gays Point Microsite types with Hallenbeck as a whole for comparison.

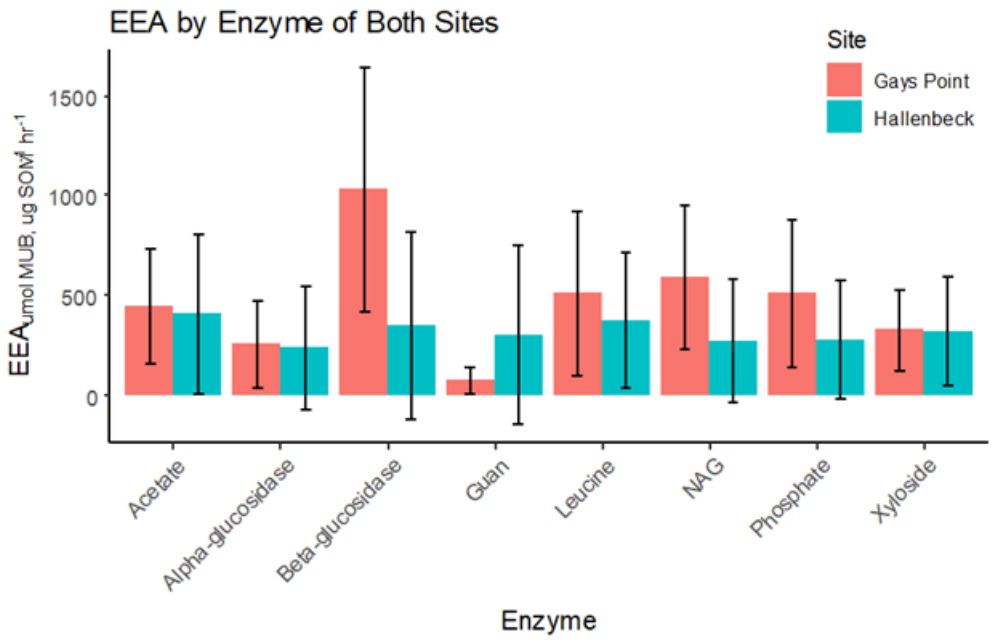

FIGURE 7. EEAs by specific enzymes, with colors indicating site.

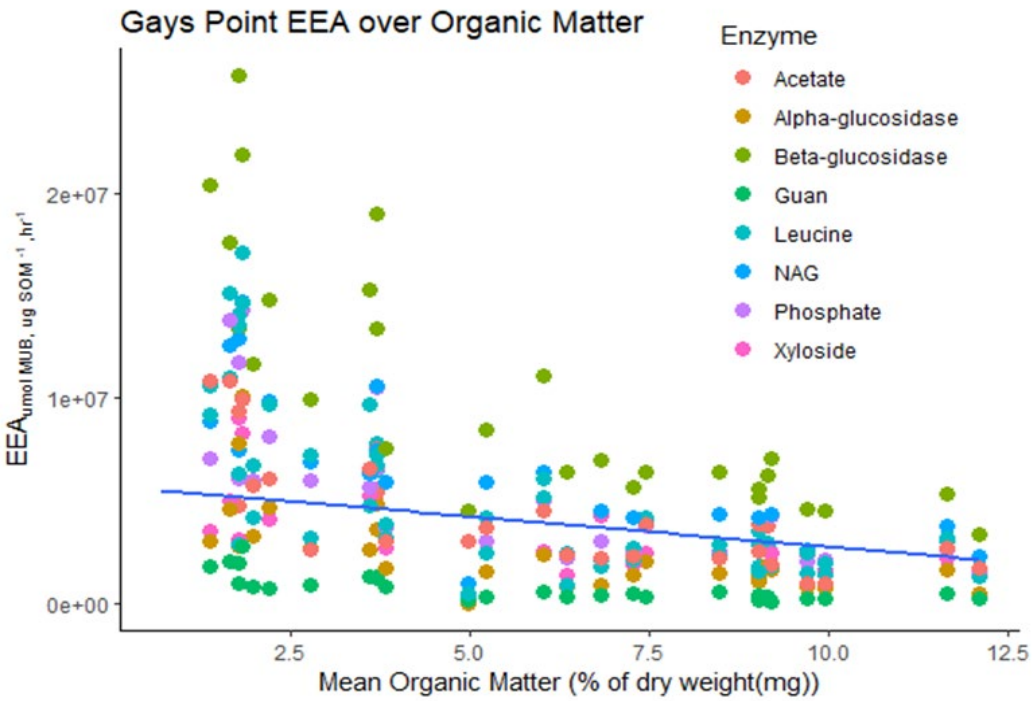

FIGURE 8. Scatter plot of Gays point EEAs over SOM, grouped by enzyme.