

Project Title: Collaborative Research: Exploration of the mechanistic basis and biogeochemical implications of differential nutrient limitation among trophic levels (Award Number: 0816593)

Final Report
January 2013

(A collaborative study with Duke University, Baruch Marine Field Laboratory and South Dakota School of Mines and Technology)

Introduction, Hypothesis and Objectives

The structure and function of ecosystems is governed by the patterns of nutrient limitation of the primary producers (e.g., plants) and heterotrophs (e.g., soil microbes). Often, these groups of organisms are limited by the same nutrient. However, an increasing body of evidence indicates that different nutrients can limit primary producers and heterotrophs in some ecosystems; this is known as differential nutrient limitation (DNL). This study funded in the fall of 2008 examined why DNL occurs in some ecosystems (but not others), and what the consequences of DNL are with respect to the utilization vs. storage of carbon. Our project addressed these questions in four wetlands ranging from Georgia to Rhode Island, including both freshwater and saline systems, which were projected to have either N or P limitations based on traditional N: P ratio methods.

The key hypotheses we are testing in our DNL project are focused on the effects of DNL on carbon flux within a range of N and P limited wetland ecosystems along the eastern U.S. coast.

a) First, we hypothesize that DNL in an ecosystem is a function of P turnover rates in the sediment chemical environment such that DNL will occur in ecosystems with greater net P mineralization rates.

b) Second, we hypothesize that a greater proportion of gross primary production (GPP) will be stored in the ecosystem when primary producers and free-living microbial heterotrophs are limited by the same nutrient. Alternately, because the loss of fixed C is inversely related to C storage, the hypothesis can be restated as: the ratio of ecosystem respiration (ER) / GPP will be greater when differential nutrient limitation exists within the ecosystem. The nature of nutrient limitation will be altered through our selective fertilization (no fertilization, +N, +P, and +N+P) in twelve experimental plots (Figure 1) at each of four wetland sites. As detailed below, a suite of measurements were made to address our hypotheses and the following specific objectives:

Mechanistic basis for DNL:

1. Evaluate the relative nutrient limitations of plants and free-living soil microbial heterotrophs by measuring primary production and bacterial production under different fertilization regimes.

2. Quantify the availability of P and N in the experimental plots across the network of study sites.
3. Measure the net P mineralization rate in the experimental plots across the network of study sites.
4. Evaluate the abiotic sink strength for P in these ecosystems. For example, the P sorption capacity of each wetland.

Implications of DNL for carbon throughput:

1. Relate rates of C throughput to the relative soil heterotrophs by measuring rates of community respiration (CR) and gross ecosystem production (GEP).
2. Assess rates of photosynthesis, dark respiration and soil respiration as influenced by fertilization.
3. Measure biomass and plant carbon storage as well as indicators of decomposition rates.

Experimental Design, Research Sites and Methods

Early in 2009 we surveyed a number of potential sites and established our research sites in NC (pocosin freshwater bogs), GA (tidal marsh), SC (salt marsh), and RI (salt marsh). We collected baseline data on nutrients (C, N and P) in soils, and porewater as well as establish field-sampling protocols. In early spring of Year 2 of this project we fully instrumented each of our sites and developed our quarterly fertilization design as shown on Figure 1.

Wetland study sites in Georgia (tidal freshwater marsh), South Carolina (tidal salt marsh with mineral soil), North Carolina (pocosin shrub bog), and Rhode Island (tidal salt marsh with organic soil) received quarterly fertilizations of N, P and N +P, with dosages scaled to the nutrient sorption characteristics of the soils at each site. A full set of plant, soil and water measurements at these sites have been completed (See Figure 2) and work on data analysis of measurements has commenced to assess vegetation responses (photosynthesis, respiration, biomass and nutrient), GEP measurements as well as soil respiration, soil and water nutrient status, phosphatase and enzymatic changes as well as trophic analysis of microbial and plant responses to quantify DNL nutrient limitations. A serious marsh dieback event in Rhode Island in 2010 impacted about half of our experimental plots and has complicated efforts to understand the effects of fertilization at that site. Although visits to the site in July and October 2011 indicated that the site is slowly recovering, our analysis efforts have focused on the sites in Georgia, South Carolina, and North Carolina where dieback events did not occur.

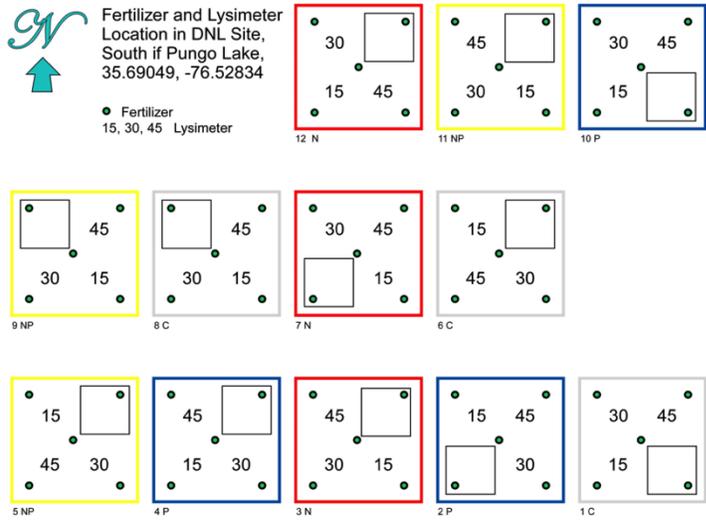


Figure 1. Sample fertilization design used for our treatment plots for our wetlands. The example shown is for the Pocosin site in NC. Shown are the replicated 2 x 2 m plots with fertilizer treatments locations and GEP sampling frames used to estimate ecosystem gas responses. Lysimeters for pore water analyses are placed at 15 cm, 30 cm and 45 cm in each plot; due to a low water table in NC, water samples are also collected from 60 cm (the lysimeters not shown on figure).



Figure 2. View of team collecting vegetation, water and soil data in July 2011 in the salt marsh in Prudence Island Rhode Island DNL site. Note the lysimeter pore water sampling tubes placed in each fertilizer treatment and the NEE chambers setup. *Spartina patens* is the dominant species shown.

Quarterly during 2010 and 2011, researchers from Duke University, the University of South Carolina, and the South Dakota School of Mines and Technology took soils samples, biomass samples and a suite of pore water samples to complete baseline chemistry for all the sites. In addition GEP, ER, and NEE (gross primary production, ecosystem respiration, and net ecosystem exchange) were measured under each fertilizer treatment (See Figure 3). In addition we collected our full complement of samples to test our hypotheses.



Figure 3. Measurement of ecosystem respiration (ER) in the Pocosins of North Carolina in July 2011.

Results

Data collection for this project did not finish until the late fall of 2012. Therefore results are still being analyzed with the final DNL analysis awaiting some key analytical analysis of soils and enzymes in the lab. The final QA/QC on all data and full exchange of data, and statistical analysis by the PI's across the three universities will refine our final analysis so that we can fully test our hypotheses and complete three manuscripts that are in draft. The results shown below are a first cut at some very interesting trends that have appeared with our first analysis of an enormous and complex dataset.

Background P sorption soil chemistry and N:P and C:P stoichiometry

Each site was initially tested for soil sorption capacity to determine the strength of P soil sorption pool (figure 4). The GA and SC sites have the highest P sorption capacity followed by RI salt marshes and Pocosin fresh water bogs in NC. These data showed an enormous difference among the sites for P retention and availability. This allowed us to test DNL across a wide range of sites. Soil chemical values for C, N and P for each site as well as extractable nutrients were assessed to determine if fertilizer treatments altered the major limiting nutrient in each system,

providing a basis for DNL testing (Figure 5). Importantly, the analysis for total C, N and P concentrations and extractable ions in the top 5 cm of the soils at each site reveal significant changes in site nutrient chemistry and fertilization effects during the last summer of the experiment. Earlier years showed similar trends but the major differences in fertilization effects did not appear until the second year of treatment. However, the SC site always had the lowest total P, N and C content in the soil. The highest NO_3 and Mehlich exchangeable P values were found at RI. Control plots in the upper 5 cm at NC, GA, SC and RI have a wide range of C:N, and N:P ratios. The highest C:N and N:P ratios were found at the NC pocosin site, the site with the higher organic mater content. The responses to fertilization varied by nutrient with most sites showing significant differences with the N, NP or P treatments compared to controls. The highest extractable P was always found at the P treated plots and the highest NO_3 and NH_4 at the N or NP plots. The specific differences among the treatments are shown with statistical cross comparisons for each treatment (Figure 5). These data clearly show the vast range in the amount of N and P in the initial sites before treatment and that N:P ratios decline at each site with increased P fertilization, indicating a possible shift in soil nutrient limitations, with NC having the highest P soil limitation. The recent N and P fertilizations were designed to shift these limitations and allow us to test for DNL in heterotrophs and autotrophs as discussed in the the following sections.

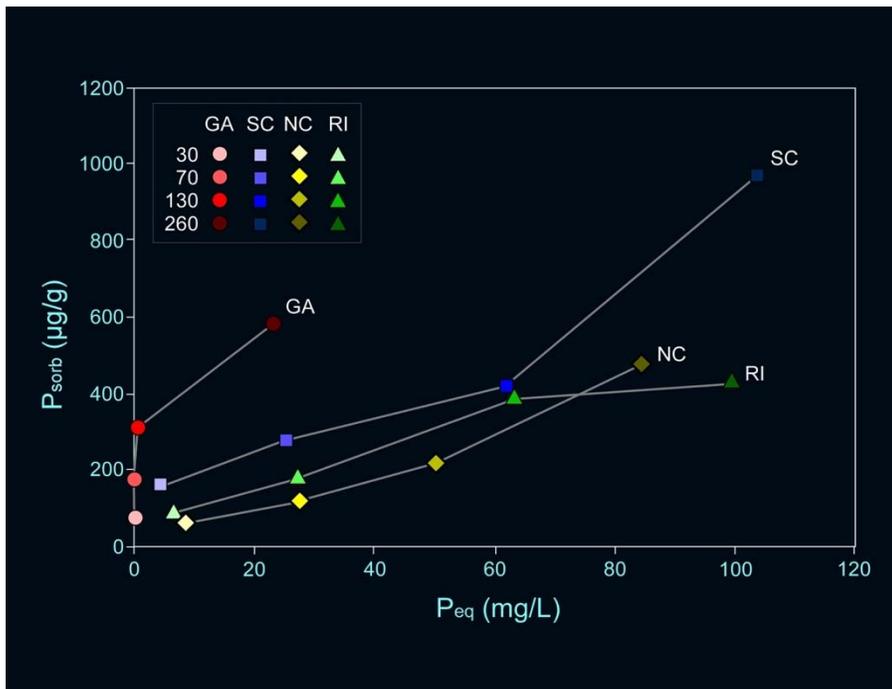
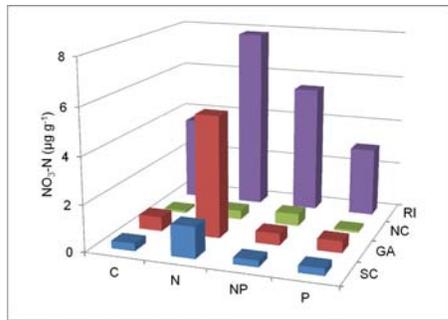
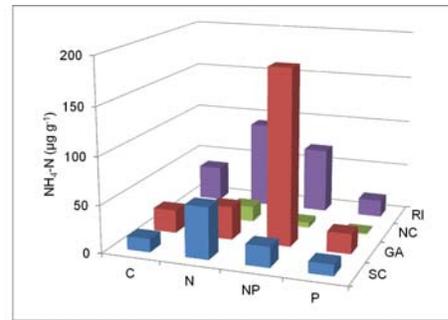


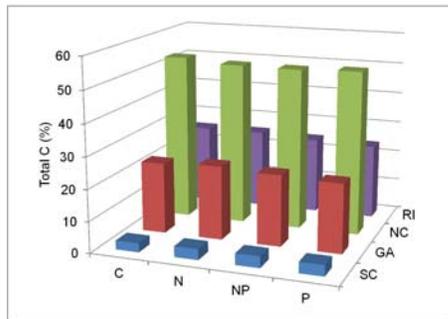
Figure 4. Initial soil P sorption curves for wetland site in NC (North Carolina Pocosins), SC (South Carolina salt marsh), GA (Georgia tidal marsh), and RI (salt marsh) used to determine fertilization levels and P sorption potential.



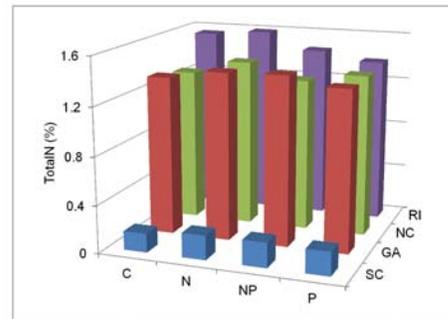
GA- C v N: 0.0427 // N v NP: 0.0390
 NC- C v NP: 0.0097 // N, NP v no N: 0.0038 // NP v P: 0.0085



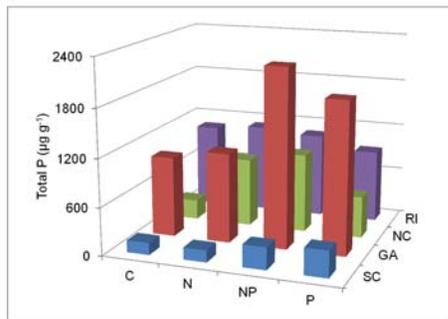
GA- C v NP: 0.0210 // N, NP v no P: 0.0584 // NP v P: 0.0191
 RI- N, NP v no N: 0.0564
 SC- C v N: 0.0442



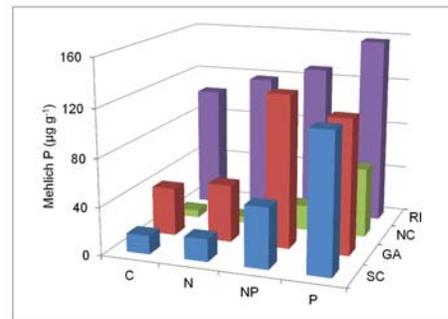
SC- C v others: 0.0484 // C v NP: 0.0543



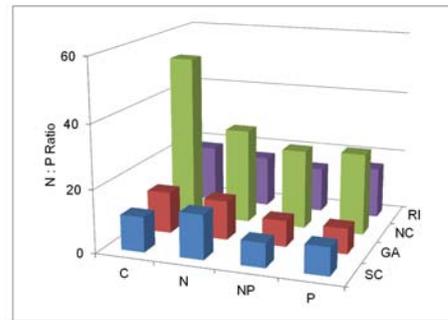
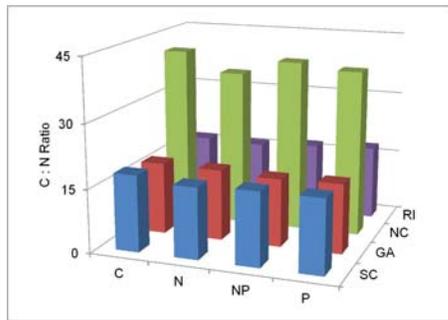
RI- NP, P v no P: 0.0300
 SC- C v others: 0.0182 // C v N: 0.0430 // C v NP: 0.0279



SC- NP, P v no P: 0.0402



NC- C v P: 0.0590
 SC- C v P: 0.0116 // NP, P v no P: 0.0162



GA- NP, P v no P: 0.0278
 NC- C v others: 0.0295 // C v NP: 0.0488 // C v P: 0.0537

Figure 5. A comparison of soil chemistry variable responses in July 2011, after 2.5 years of fertilizer treatments. Measurements are for only the top 0-5cm. Wetland sites locations are in Georgia (GA), South Carolina (SC), North Carolina (NC) and Rhode Island (RI). Significant differences among treatment are shown below figures.

Soil and pore water enzyme activity

We measured soil and pore water enzyme activities in all our experimental plots across the four wetlands. However, soil and pore water enzyme activities for the pocosin site in NC presented methodological challenges due high amount of colored organic matter in soils and inconsistent availability of sample in pore water sampling wells from the rooting zone (15 and 30 60 cm wells). Since colored organic matter interfere with spectrophotometric and fluorometric assays, we are presently testing an HPLC method that will allow us to potentially fractionate the substrate-analog hydrolysis products and high-molecular weight dissolved colored organic compounds (e.g. phenolics). At other sites, soil enzyme activities were measured using established protocols but at sample pH. We measured the activities of the following enzymes in marsh soils α and β -1, 4-glucosidase (BG), α and β – galactosidase, β -1, 4-N-acetylglucosaminidase (NAG), Leucine amino peptidase (LAP), and Phosphatase (PA).

Pore water samples were assayed for LAP and PA activities to determine microbial activity as it relates to N, P and C metabolism in the ecosystem. BG is an enzyme that hydrolyzes sugar as a source of energy, and high activity reflects greater demand for carbon. Higher NAGase activity reflects greater N-and C acquisition. NAGase activity is also correlated with fungal biomass indices such as ergosterol content. LAP is another key enzyme involved in N-regeneration and acquisition, while phosphatases are inducible enzymes that regenerate inorganic phosphates from monoesters. Generally, during the summer (July 2010) sampling event LAP activity was highest in organic rich RI salt marsh, followed by the GA freshwater marsh, with least activity per unit dry weight of wetland soil in the mineral RI salt marsh. BG activity was highest in the freshwater marsh, followed by the organic rich salt marsh and then the mineral salt marsh. Soil phosphatase activity was highest in the freshwater marsh and similar for the two salt marshes. During the October sampling event the enzyme activities were significantly lower with the organic rich RI salt marsh showing the highest activities with the exception of soil PA, which was highest in the freshwater marsh. We also measured NAGase activity in the month of October, which was highest in the organic rich salt marsh, followed by the freshwater marsh and then mineral salt marsh in South Carolina.

In the month of April 2011, Soil phosphatase activity was higher in SC mineral salt marsh N treated plot suggesting P limitation as a result of N fertilization. Also, there was higher Phosphatase and BG activity in mineral salt marsh's N treated plots. There was no significant difference in Soil NAGase activity. In the month of July 2011, P demand was higher in mineral salt marsh's NP treated soil followed by high N demand in NP and P treated plots. Also soil NAGase activity was higher in P and NP treated plot but no difference in BG activities. This suggests, the SC mineral salt marsh is primary limited by phosphorus and secondary limited by nitrogen. In the month of October there was no phosphatase activity in NP treated soil in this marsh. Also difference in LAP activity was not significant. In the GA freshwater marsh and organic rich RI salt marsh, we didn't observe any significant difference in soil enzyme activities over the period of 2011 as a result of fertilization.

We also looked at α -glucosidase, α -galactosidase, and β -galactosidase enzyme activities in the control plots of our study sites. Higher activity of these different types of enzyme suggests demand for different types of carbon. For e.g., high β -galactosidase activities in GA in April and

October suggests demand for more simpler form of carbon in GA freshwater marsh but higher demand in SC mineral salt marsh in July. Also, α -glucosidase activity was higher in mineral salt marsh control plots than in GA freshwater marsh and organic rich RI salt marsh throughout the 2011 sampling season. Overall activity of α -glucosidase was two times higher in July suggesting more demand for the type of carbon hydrolyzed by α -glucosidase enzyme activities in the summer. GA had high α -galactosidase activity in October and April but the SC mineral salt marsh had higher activity in July suggesting higher demand of this type of glucose in summer time in the mineral salt marsh.

Since the relative activities of these ectoenzymes reflect microbial nutrient demands, we examined ratio of $\ln(\text{BG}):\ln(\text{LAP+NAG})$, $\ln(\text{BG}):\ln(\text{PA})$, and $\ln(\text{LAP+NAG}):\ln(\text{PA})$ to assess the relative demands for C, N and P in these wetlands, respectively (Figures 6ab). Our results indicate that despite differences in organic matter content, the $\ln(\text{BG}):\ln(\text{PA})$ was very similar across the wetlands (1.097; 1.086; 1.047 for freshwater, organic salt marsh, mineral salt marsh, respectively). This suggests that the demand for C relative to P (C:P ratio) was very similar across sites. In contrast, the ratio of $\ln(\text{BG}):\ln(\text{LAP+NAG})$ was less than 1 (0.61; 0.60; 0.55 for freshwater, organic salt marsh, mineral salt marsh, respectively) indicating a greater demand for nitrogen in these soils. Similarly, $\ln(\text{LAP+NAG}):\ln(\text{PA})$ was greater than 1 indicating relatively greater demand for N than P. When compared to the global averages of these ratios, our sites supported higher-level of activity but were well within the spread of the global data. Changes in nutrient content of the soil altered these activities. Since, our sites varied in organic matter content, we also examined the trends in these activities after normalizing them with the site-specific carbon content in the soil. When expressed on a 'per unit carbon' basis, the values increased dramatically especially for the mineral salt marsh in South Carolina. Our study suggests that extracellular enzyme activities reflect microbial nutrient acquisition and can provide insights into the biogeochemical processing of C, N and P in coastal wetlands.

Our enzyme activity assays also reveal that the nutrient demand for soil microbes varies seasonally and may be structured by changes in competition for limiting resources with plants. Remarkably, the stoichiometry of ectoenzyme activity at our sites appeared to follow the trajectory of recently published global trends for rivers, soils and wetlands. Seasonal changes and nutrient availability affects were manifest as shifts along this trend line. A 1:1 ratio of activity of BG (C-generating enzyme) versus (LAP and NAGase – N – generating enzymes) suggests a balanced demand for C and N by soil microbes. Similarly, a 1:1 ratio of BG vs. AP would signify a balanced demand for C and P. Interestingly, we have observed that these ratios were constrained across late fall and early spring at the GA (freshwater) and SC (saltmarsh) sites (Figure 6ab). Nutrient amendments result in deviation from this trend. For example, in the freshwater marsh in GA, where plants are primarily limited by N availability, N additions stimulate C demand and N+P amendments increases C demand further. In contrast, P additions alone stimulate N demand for soil microbes at the freshwater marsh in GA (Figure 6a). Similar changes are also observed for activities of organic C and organic P acquisition (Figure 6b).

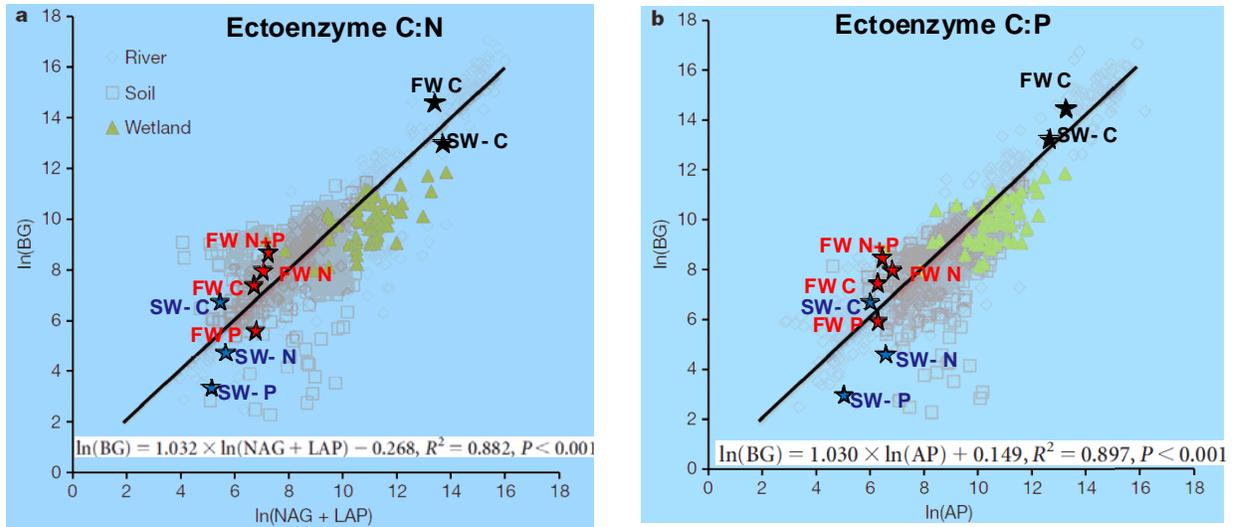


Figure 6ab: Relative acquisition activity of organic carbon (C) and organic nitrogen (N) by soil microbes in freshwater (FW) site in GA and mineral salt marsh (SW) site in SC. Letters N, P and C indicate treatments and controls, respectively. Although the activity of enzymes is typically greater in the freshwater GA marsh than the SC site, the ratio of the activity of these enzymes is similar. Points that lie above the line reflect greater C demand relative to N, while those below the line reflect relatively greater N demand. Enzyme activity at FW and change across seasons, but the ratio appears to be conserved. Late fall (October 2010 - Black) ratios though high cluster around the 1:1 trend line, but shift downward along this line during early spring (April 2011 – Red / Blue). Generally, at FW there is greater demand for C than N (expect under P enrichment), while at the SW site there appears to be shift from greater demand for N relative to C during the late fall to greater C demand during early spring.

It will be interesting to see if strong competition for nutrients and greater primary production during the summer months skews the trend towards nutrient limitation by either N or P, or towards energy limitation manifested in the activity of BG. Theoretically these trends should reflect the microbial nutrient limitation as discerned from bacterial production assays. By comparing pore water and bulk soil enzymes over our full data set we may be also able to tease out the responses of plants versus soil microbes.

When we looked simply at soil enzyme activities at our sites we didn't find significant difference in activities between treatments, as soil enzyme activity is a combination of both microbes and plant root activities. The insignificant difference in activities between treatments may be a result of this combined enzyme activity so we looked in more detail at soil enzyme activities and porewater enzyme activities in soil and porewater of SC mineral marsh, GA freshwater marsh, and organic rich RI salt marsh using $\ln(\text{LAP}):\ln(\text{PA})$ for relative N:P demand on these marshes. Pore water enzyme activities represent microorganism's only enzyme activity. Activities in the porewater were lower compared to soil because of reduced microbial activity in the porewater as seen in Figure 7 abc. In SC mineral salt marsh scattered plots shows demand that deviates from the linear line suggesting variation in nutrient demand; there is a higher demand for N in P treated plot. Also, porewater $\ln(\text{LAP}):\ln(\text{PA})$ is higher than 1 in porewater P for SC salt marsh site suggesting N limitation. When we looked at relative nutrient demand in GA freshwater marsh, they tend to show linearity suggesting there is equal amount of demand for N and P on this marsh. The porewater P treated plots and control plots shows higher demand for N in GA marsh suggesting N limitation. When we looked at RI porewater and soil enzyme

for relative nutrient demand, it followed similar trend that of GA marsh. Control plots and phosphorous treated plots in both porewater and soil enzyme showed relatively higher demand of nitrogen compared to phosphorus.

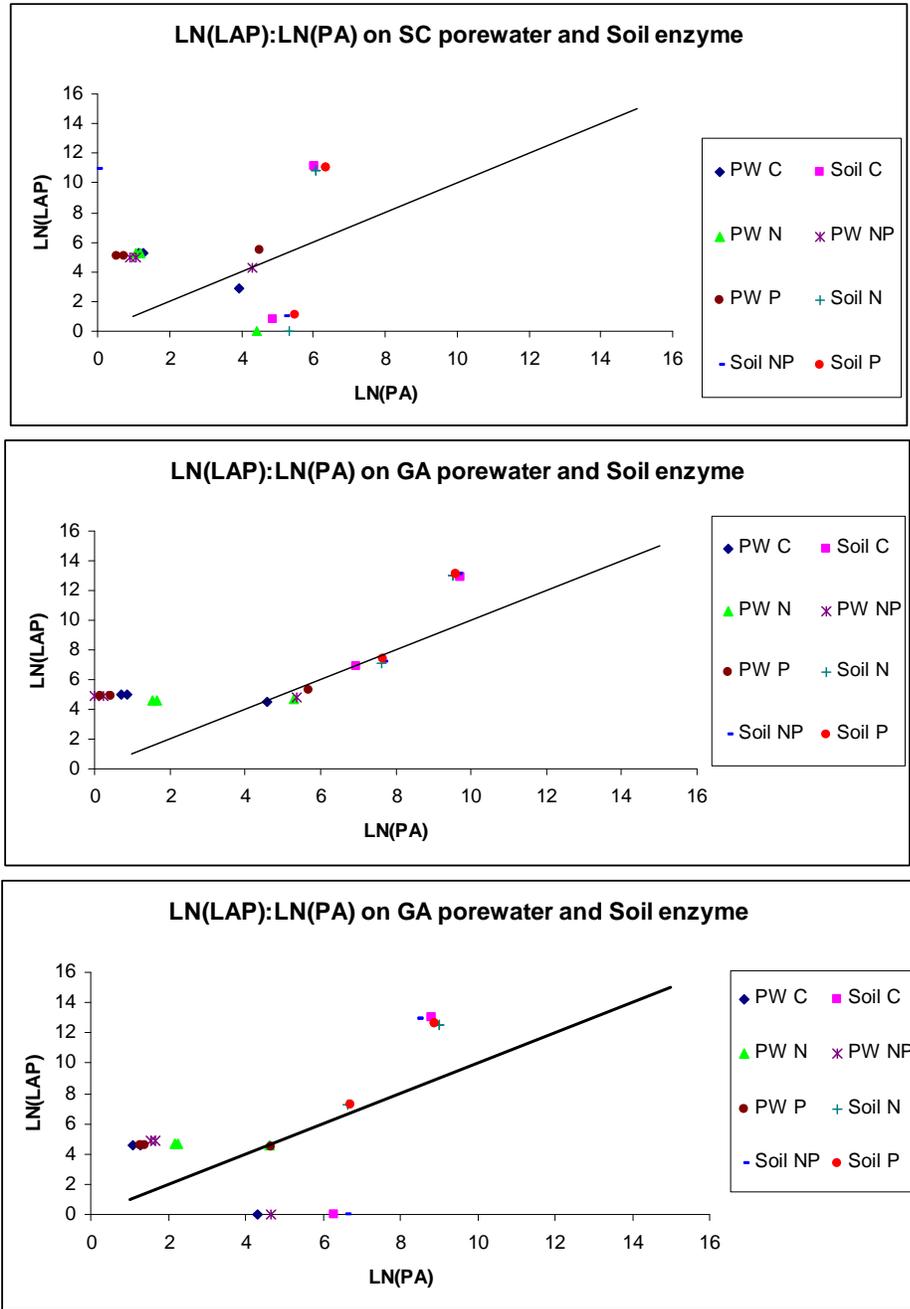


Figure 7 abc showing relative demand of N:P in soil and porewater. Activities are higher in soil due to presence of plant roots and soil microbes.

Soil microbial productivity

Microbial productivity is measured in soils (5-10 cm depth) by tracing the incorporation of ³H-labelled leucine into protein, using the methods of Buessing and Gessner (2003). Briefly, soil samples are incubated with ³H-leucine, the incubation stopped with TCA, and proteins extracted using a hot NaOH-SDS-EDTA extraction following several cleanup steps designed to remove unincorporated ³H-leucine. During 2010, our focus was on measuring rates of microbial productivity within each plot but interpretation of the 2010 data is equivocal due to high ³H activity in killed samples (most likely due to abiotic sorption to soil). In 2011, our focus has shifted to identifying the limiting nutrient(s) at each site. We have been collecting soil in the vicinity of (but outside) our experimental plots. In laboratory incubations, the soils from all sites are amended with all combinations of N, P, and C (final concentration of 600 μM NH₄NO₃, 60 μM triple superphosphate, and/or 10 mM glucose). The 12-h incubations at ambient soil temperatures are long enough to allow soil microbes to respond to the added nutrient(s) such that there are significant differences in ³H-leucine incorporation between treatment and control samples. Importantly, the abiotic ³H sorption to soil does not change significantly over time so the ³H activity in killed samples is proportionally low in 12-h incubations compared to the shorter (1-2 h) incubations that were performed in 2011. Analysis of this phase is being reanalyzed due to method problems.

Soil microbial community responses

Microbial community populations in response to fertilization were analyzed from 0-10 cm soil cores at the Georgia (GA), North Carolina (NC) and South Carolina (SC) sites. Soil DNA extraction was done via a MO BIO Ultra Clean DNA extraction kit and 16S DNA sequencing was used for bacterial analysis. Informatics was done using QIIME, RDP and also MG-RAST. There were statistical differences in the bacterial communities across sites as expected and fertilization treatments significantly altered the microbial communities at both the GA and SC sites but not NC (Table 1). An analysis of the relationship of the microbial communities and soil variables indicates that bacterial communities are primarily linked with some components of phosphorus (total P, or extractable P) at each site but not nitrogen components, which included exchangeable NH₄ and NO₃, (Table 2). The C:P ratios and N:P ratios of soils at the NC site also was correlated with bacterial communities.

Table1. Statistical comparison of bacterial communities by non-parametric bootstrapped distance-based ANOVA.

Site	all	0.911	0.001
Treat site	GA	0.352	0.037
	NC	0.090	0.246
	SC	0.494	0.005

Table 2. Relationships between bacterial communities and environmental soil variables across wetland sites using a Mantel test.

Soil Var.	<u>ALL SITES</u>		<u>Partial by site</u>		
	Partial	Pure Partial	GA	NC	SC
C	0.60	0.49			
N	0.43	0.46			
P	0.27	0.22		0.32	0.44
Ext. NH ₄	0.14				
Ext. NO ₃	0.16				
Ext. P			0.39		
pH	0.64	0.13			
% Moisture	0.34	0.15			
C:N	0.58	0.20			
C:P	0.62	0.33		0.32	
N:P	0.50	0.27		0.54	
Ext N:P					

Aboveground biomass

Aboveground biomass at all sites has been measured for all dominant species using allometric equations based on parameters including height, leaf size, and basal diameter. Measurements have been made within the gas flux collars so that aboveground biomass can be correlated with ecosystem-scale C fixation. In Georgia, we have developed allometric equations for five species that were common across the study plots at the beginning of the study; *Bidens* sp. (beggarticks), *Eleocharis obtusa* (blunt spikerush), *Polygonum hydropiperoides* (swamp smartweed), *Pontederia cordata* (pickerelweed), and *Zizaniopsis miliacea* (giant cutgrass). Our site in South Carolina contains only *Spartina alterniflora* (smooth cordgrass) and we have found a strong relationship between the height of this plant and its aboveground biomass. Allometric equations for the two species dominating the NC Pocosin sites (*Lyonia lucida*) and (*Ilex glabra*) and RI salt marshes (*Spartina patens*) were completed in 2009.

In Georgia, N additions (alone or with P) resulted in 70-96% (Jul 2010) and 95-122% (Oct 2010) increases in total aboveground biomass (where “total aboveground biomass” is defined as the aboveground biomass of the five species for which we have allometric relationships (Figure 8a). In contrast, P had no effect on standing stocks of aboveground biomass in Georgia, suggesting that phosphorus is neither a primary nor a secondary limiting nutrient for plant production at this site. However, the fertilization responses that we observed during 2010 did not

repeat in 2011. Instead, there was a “crash” in the populations of *Z. miliacea* in most of the N and N+P fertilized plots, resulting in a decrease in a ~10% decrease in stem density for this species from 2010 to 2011 (e.g., 37 vs. 33 plants m⁻² for Jul 2010 and 2011). Additionally, the mean height of *Z. miliacea* was significantly lower in 2011 than 2010 (e.g., 152 vs. 135 cm for Jul 2010 and 2011). The fact that this crash in plant productivity was centered around one species and happened only in the N and N+P plots suggests that the initially high growth response to the added nutrients in 2010 may have created a secondary nutrient limitation (perhaps Si). When that nutrient demand could not be met in 2011, the plants were unable to support high biomass, such that the nitrogen fertilization effect that was seen in 2010 was not repeated in 2011. In both years (although the trend was less pronounced in 2011), aboveground biomass peaked in mid- summer (July) and stayed high through October. Although aboveground biomass of the total plant community responded to nutrients, it is difficult to determine the nutrient status of individual plant species due to interspecific competition. *Z. miliacea*, which accounted for 50-80% of total biomass, responded in the same manner as the entire plant community. The other species, however, showed either no response to fertilization or inconsistent responses across seasons. It is difficult to assess if those plants are limited by something other than N and/or P (e.g., perhaps light is the limiting resource for the understory species) or if the dominant *Z. miliacea* is outcompeting the other species for limiting nutrients. The fact that the nitrogen concentration (%) of aboveground biomass increased in N and N+P plots for *Z. miliacea* and in the N-only plots for *Bidens* sp. and *P. cordata* suggests that nitrogen is the limiting nutrient for each of these species (data not shown). In contrast, the leaf N concentration of *P. hydropiperoides* did not vary across the fertilization treatments.

In South Carolina, there was a significant increase in *S. alterniflora* biomass with N additions across both years of the project (Figure 8b). The addition of both N and P significantly increased aboveground biomass beyond that in the plots receiving nitrogen additions. P, by itself, had no effect on standing stocks of aboveground biomass. These data suggest the production of *Spartina alterniflora* biomass is primarily limited by N, with a secondary P limitation that occurs once the plant’s N requirements have been met. Aboveground biomass increased throughout the year, reaching a maximum in October. Nitrogen additions (alone or with added P) significantly increased leaf N concentrations (data not shown), providing additional evidence of N limitation of this species.

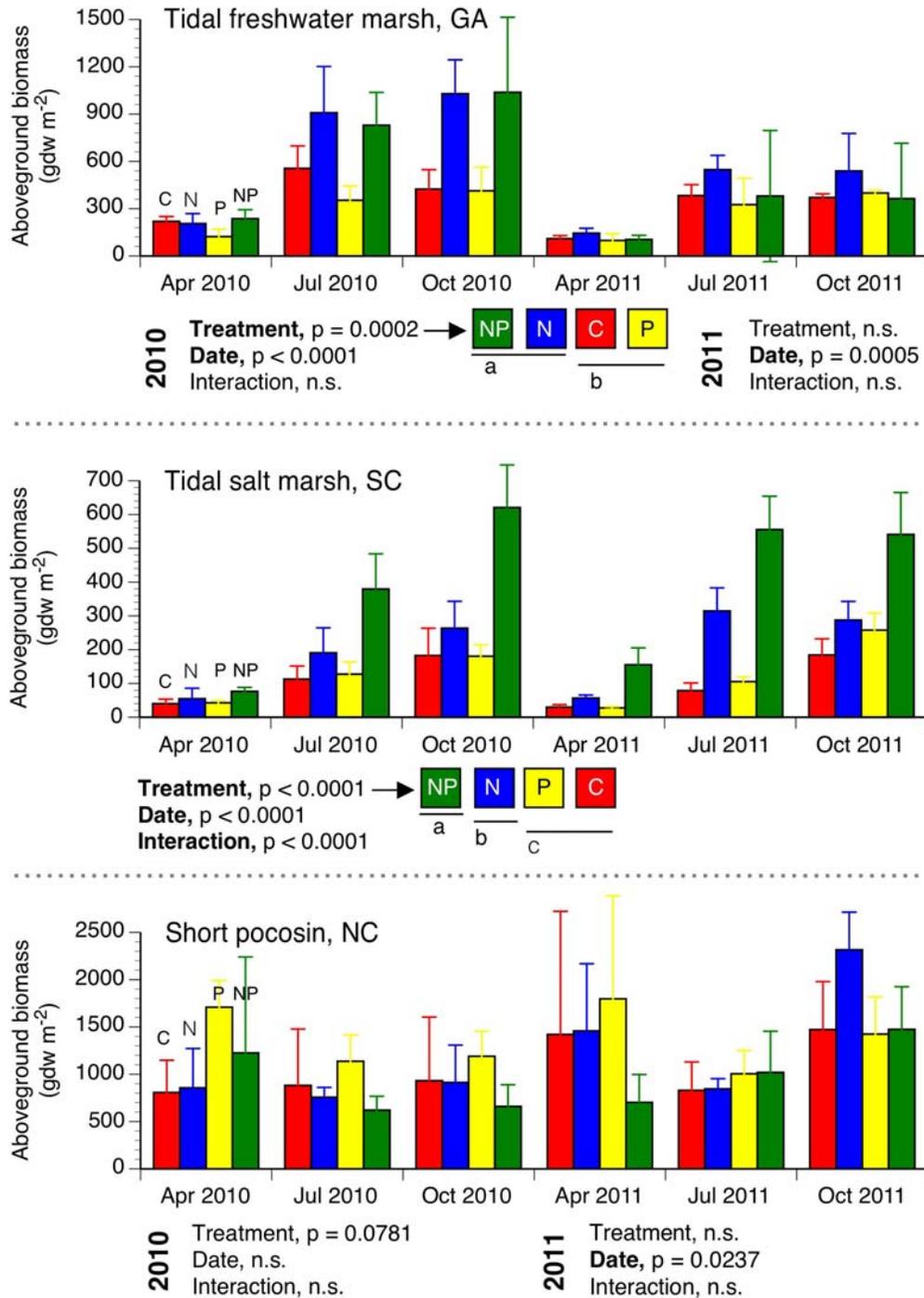


Figure 8abc. Aboveground biomass in experimental plots in Georgia, South Carolina, and North Carolina, as determined using allometric relationships for the dominant species at each site. Total aboveground biomass in Georgia is defined as the sum biomass for four species for which allometric relationships were developed; there are roughly a dozen species within the experimental plots. In South Carolina, *S. alterniflora* was the only plant species present. In North Carolina, the dominant plants were *Lyonia lucida* and *Ilex glabra*, although scattered individuals from several other species were periodically observed in the plots. Each bar represents the average (\pm standard deviation) of three replicate plots. Colored boxes with lettered lines indicate statistical similarities and differences between treatments within a site. “n.s.” indicates not significant, i.e., $p > 0.05$.

The vegetation biomass response in NC did not show a fertilizer effect for either N, P, or N+P during the study period (Figure 8c). This may be due to the excessively dry conditions the sites experienced in both 2010 and 2011, the slow growth rates of the pocosin vegetation, or a combination of both. Visits to the site during 2012 provided evidence of P limitation of the plants, although we did not quantify aboveground biomass during those visits. Our ability to discern treatment effects on aboveground plant response in RI was compromised due to an unexpected marsh die off, which affected some of our experimental plots; in general, data from the Rhode Island site are not presented in this report.

Ecosystem gas exchanges measurements

Rates of gross primary production, ecosystem respiration (as CO₂ and CH₄ emissions), and net ecosystem exchange (balance between GPP and ER) were measured quarterly within each fertilization plot using gas exchange chambers (Figure 3). Each field plot contained an 81 x 81 cm collar that was permanently installed in the plot and that served as a base for the gas exchange chamber during measurements. During 20-40 min intervals (depending on observed flux rates), CO₂ and CH₄ fluxes were measured at multiple light levels (full sun, multiple intermediate levels, and dark). Chamber CO₂ concentrations are measured in the field using a LI-COR LI-7000 infrared gas analyzer. Gas samples for CH₄ were analyzed in the lab using a gas chromatograph with flame ionization detector (GC-FID). Photosynthesis was measured at full sunlight with a LI-COR 6400XT IRGA.

Net Photosynthesis

Net photosynthesis (P_N) was measured on the dominant species at each site (Figure 9). Highest rates for photosynthesis were found, for species at the tidal marsh in GA, followed by *Spartina alterniflora*, a C₄ species, which dominated the SC marshes. Lowest rates were found for *S. patens* in RI. This may be due to the dieback and stressed conditions of the plots mentioned earlier. Pocosin shrub species had moderate rates of P_N and showed no treatment effect. All species had low respiration rates except *S. alterniflora* in SC (data not shown). The only sites, which looked to have a trend related to fertilization, were RI, SC, and GA where NP and P treatments increased P_N for the dominant species. Additional comparisons of P_N and R are being made for each season to further relate plant responses to fertilizations and assess DNL. These data are being used in conjunction with NEE and GEP data to assess C flux across treatments.

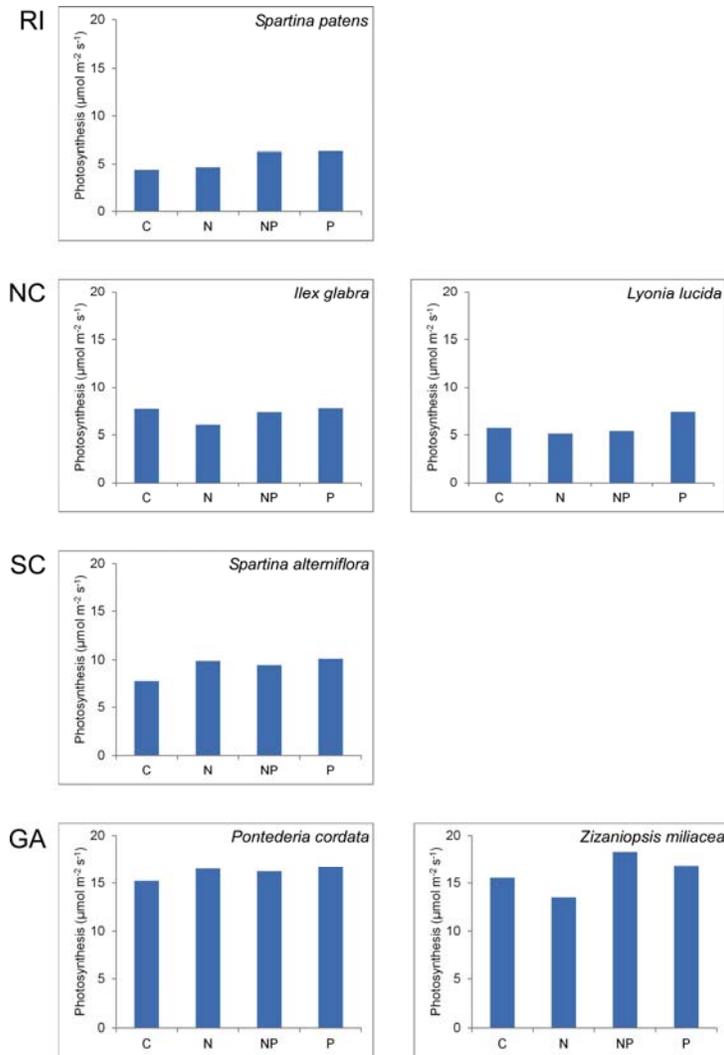


Figure 9. Net plant photosynthesis (P_N) measured on the dominant species in RI, SC, GA and NC wetlands in the summer of 2011. Measurements for P_N were conducted at irradiation level close to full sunlight.

Ecosystem carbon flows

One of our key metrics for determining the biogeochemical effects of differential nutrient limitation is through the measurement of gross ecosystem production and respiration, with an assessment of the ER/GEP ratio. There have been significant effects of fertilization on ecosystem carbon flows at some sites, but not others (Figures 10 and 11). During our first sampling in April 2010, rates of GEP ranged from $0.3 \text{ mg C m}^{-2} \text{ min}^{-1}$ in Rhode Island (control and N+P plots) to $16 \text{ mg C m}^{-2} \text{ min}^{-1}$ in Georgia (N+P plots). This 50-fold range in gross ecosystem production rates represents inherent variations in the plant community between the different sites (the GA site was dominated by 1+ m tall *Zizaniopsis miliacea* whereas the salt marshes in SC and RI were vegetated with *Spartina alterniflora* (SC) or *S. patens* (RI), with total heights of 10-30 cm), nutrient availability, and differences in temperature/phenology across the 10° latitude range of our four sites. There were no visible differences in the aboveground plant

canopy between experimental treatments at any sites in April 2010 (and this is reflected in the aboveground biomass data for April 2010 – Figure 8abc).

Across the three sampling dates in 2010, there were significant effects of fertilization on Gross Ecosystem Production (GEP) in the Georgia site (Figure 11). In Georgia during 2010, N additions (alone or with P) resulted in 70-75% (July) and 172-180% (Nov) increases in gross ecosystem production whereas P had no effect on the photosynthesis trends in GA. In 2011, there were no treatment effects on plot-scale gross ecosystem production. Across both years, the gas flux data parallel the patterns in aboveground biomass that were observed at this site (Figure 8a). In fact, the seasonal variations in GEP in Georgia can be explained as a function of plant biomass and rates of leaf photosynthesis (Figures 8a and 9). In South Carolina, N additions increased GEP during 2010 by 56-90% (recall that biomass in SC did not significantly respond to N only during 2010). The N+P additions further increased GEP by 70-94% (versus rates in N only plots). P, by itself, had no effect on GEP. These treatment patterns persisted in 2011. The South Carolina data indicate that GEP is a function of both plant biomass and leaf-level photosynthesis (as in Georgia) although the slope of this relationship varies between July and October (Figure 10ab), the two photosynthesis data dates that are available and have been analyzed. In North Carolina, gross ecosystem production in 2011 was significantly higher in the N+P plots than in the controls (by ~55%) but there were no differences between any of the other treatments in 2011, or at all in 2010.

There were large differences in rates of ecosystem respiration ($\text{CO}_2 + \text{CH}_4$ emissions) across the gradient of our four sites, with rates ranging from < 1 (RI, control plots, Oct) to $> 10 \text{ mg C m}^{-2} \text{ min}^{-1}$ (GA, N+P plots, July; Figure 11). It should be noted that this is total system respiration and includes gaseous CO_2 and CH_4 emissions from both plant and soil sources. In the final analysis separate soil respiration rates measured by the LICOR 6400 soil chamber system will be included. In GA, rates of $\text{CO}_2 + \text{CH}_4$ emissions were greater in the N and N+P plots relative to the control and P plots (in 2010 but not 2011) and paralleled the treatment-related patterns in gross ecosystem production and biomass. In SC, respiration rates were always higher in the N+P plots than the N plots, which themselves had higher respiration rates than the control plots. In 2010, there was no difference in total $\text{CO}_2 + \text{CH}_4$ emissions between the +N and +P plots, although $\text{CO}_2 + \text{CH}_4$ emissions were higher from the +N plots than the +P plots in 2011. In North Carolina, ecosystem $\text{CO}_2 + \text{CH}_4$ emissions in 2010 were significantly higher in the N+P plots than in the controls (by ~35%), possibly reflecting nutrient limitation, but there were no differences between any of the other treatments in 2010, or in 2011. However, there were no differences in CO_2 emissions toward the end of the growing season or at all in 2011. CH_4 emissions tended to be highest in GA and lower in the NC pocosin and SC and RI salt marshes, but there have been no treatment-related effects on plot-scale CH_4 emissions. Our next step in the analysis will be to relate ecosystem respiration (ER; $\text{CO}_2 + \text{CH}_4$ emissions) to gross ecosystem production (GEP) as an indicator of carbon throughput as a final test of our key hypothesis.

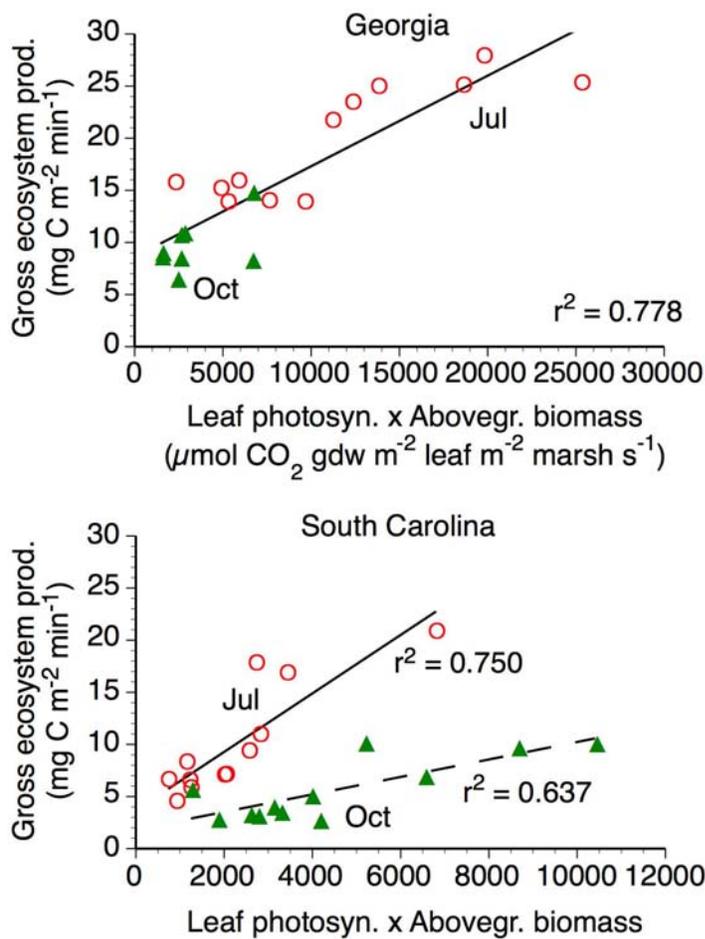


Figure 10ab. Plot-scale gross ecosystem production (GEP) is a function of aboveground biomass (g m^{-2}) and rates of leaf photosynthesis ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ leaf s}^{-1}$). In Georgia, there is a common pattern across months but the relation appears to differ by month in South Carolina. Each data point represents measurements within a single experimental plot; all treatments are shown on the graphs.

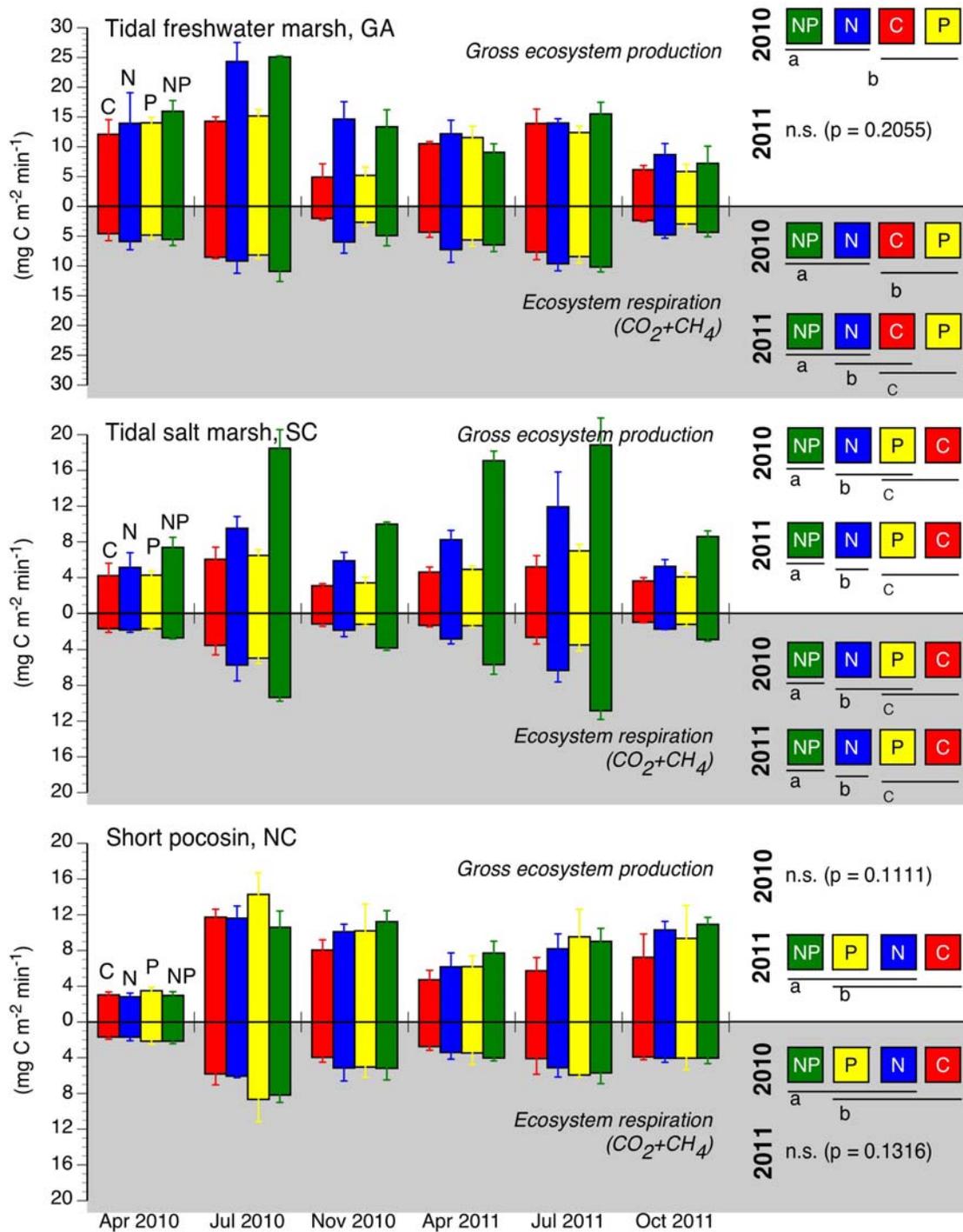


Figure 11. Gross ecosystem production and ecosystem CO₂+CH₄ emissions from GA, SC, and NC sites in 2010 and 2011. Rates were measured using gas exchange chambers that enclosed an 81x81 cm area, such that measured fluxes represent integrated plant+soil fluxes. Gross production rates shown here are for the maximum light level within each plot although rates have been measured across at least 3 light levels in each plot. Each bar represents the average (\pm standard deviation) of three replicate plots. In 2010, the “Oct 2010” sampling at the GA site actually took place in November; it had to be postponed at that site due to equipment failure. Colored boxes with lettered lines indicate statistical similarities and differences between treatments within a site. “n.s.” indicates not significant, i.e., $p > 0.05$ for the main treatment effect.

Soil nutrient controls on carbon flows across sites

To assess the overall effects of our treatments on key features of carbon cycling at our research sites we compared CO₂ flux, NEE, DOC and microbial biomass (MBC). Soil phosphorus was the primary control on the C cycling components we tested when compared across sites for the major microbial controlled aspects of the C cycle such as soil respiration, and DOC, a byproduct of microbial activity (Figure 12). NEE only showed a weak response to soil P, a possible artifact of several data points being below the other values. While soil CO₂ respiration did not respond to C or N content in the soil across sites was significantly relate to soil P content, as was microbial biomass (MBC). These features of C cycling were not related to other aspects (data not shown) such as soil C:N:P ratios or extractable N and P or porewater N and P. Overall these early analysis indicate a significant influence of P on microbial biomass across sites. A further analysis of the relationship of carbon flow and microbial communities is now underway.

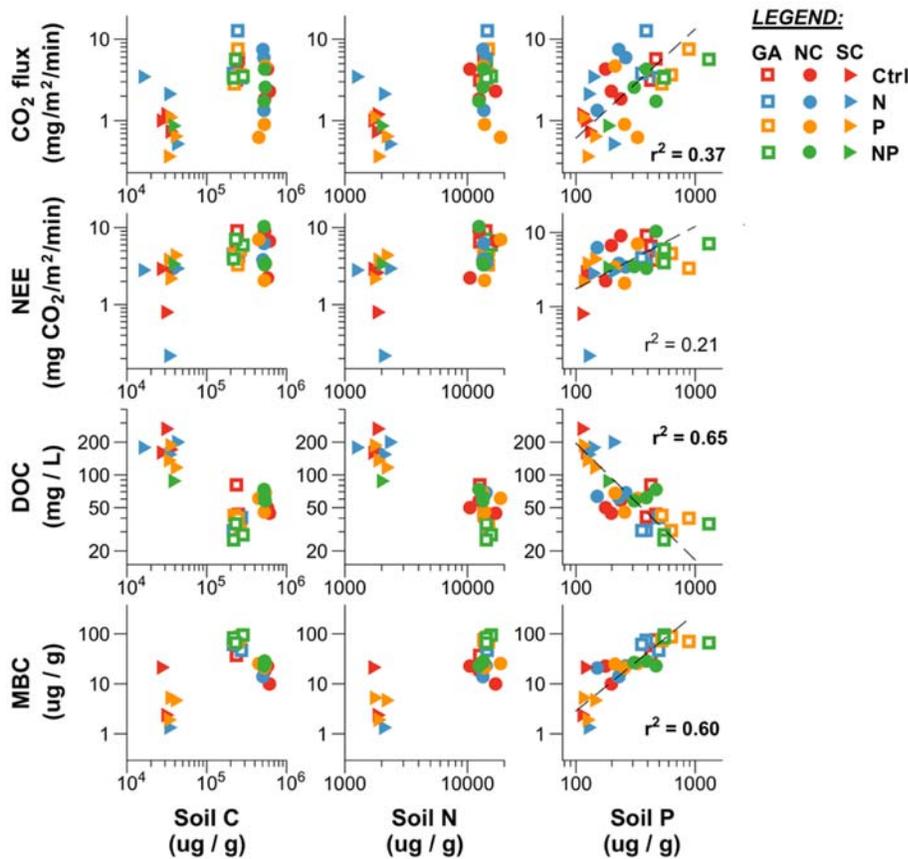


Figure 12. The relationship of total soil nutrients and microbial biomass (MBC), DOC, NEE and soil respiration of CO₂. The comparisons are from the tidal freshwater marsh in Georgia (GA), pocosin site in NC and a salt marsh in SC only due to dieback at the RI site.

Summary

The structure and function of ecosystems is governed by the patterns of nutrient limitation of the primary producers (e.g., plants) and heterotrophs (e.g., soil microbes). Often, these groups of organisms are limited by the same nutrient. However, an increasing body of evidence indicates that different nutrients can limit primary producers and heterotrophs in some ecosystems; this is known as differential nutrient limitation (DNL). The key hypotheses tested in our DNL project are focused on the effects of DNL on carbon flux within a range of N and P limited wetland ecosystems along the eastern U.S. coast. Hypothesis one poses that DNL in an ecosystem is a function of P turnover rates in the sediment chemical environment such that DNL will occur in ecosystems with greater net P mineralization rates. This was supported by increased phosphatase activity at our some of our sites. Second, it is hypothesized that a greater proportion of gross primary production (GPP) will be stored in the ecosystem when primary producers and free-living microbial heterotrophs are limited by the same nutrient. This was the case at the pocosin bog site according to analysis to-date. Alternately, because the loss of fixed C is inversely related to C storage, the hypothesis can be restated as: the ratio of ecosystem respiration (ER) / GPP will be greater when differential nutrient limitation exists within the ecosystem. This aspect is to be tested when all our data are available for comparisons. Results from a three year fertilizer experiment in four wetland types: evergreen shrub bog in North Carolina, tidal freshwater wetland in Georgia, salt marsh in South Carolina and a northern salt marsh in Rhode Island do suggest that different nutrients can limit primary producers and heterotrophs in some of these ecosystems, especially at the SC salt marsh site. Data from the final analysis of all sites in our project may provide key evidence of where and why DNL may occur and its effects on carbon flow in ecosystems. If DNL is a feature of some ecosystems, this will have significant ramifications for our understanding on how systems respond to nutrients as well as the effects of DNL on carbon flux and storage, key features of assessing the role of soils and plants on global climate change.