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Enzyme activity responses to nutrient loading in subtropical wetlands

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Abstract Eutrophication caused by anthropogenic nutrient inputs is one of the greatest threats to the integrity of freshwater wetlands. The resultant changes in organic carbon cycling and nutrient mineralization may be expressed through increased decomposition rates, which are ultimately dependent on the metabolism of the resident microbial community. Specifically, microbial nutrient acquisition is controlled through the activity of enzymes, which are in turn influenced by local biogeochemical conditions. This study examines enzyme activities along distinct North-South P gradients within four distinct hydrologic units of the Florida Everglades. The results indicate that nutrient enriched sites exhibit lower N and P limitations on microbially constrained C mineralization, in addition to enhanced cellulose decomposition rates. Nutrient loading resulted in decreased microbial mobilization of resources for P mineralization, resulting in greater energetic allocation for C mineralization. Additionally, N appears to become less limiting to C mineralization in the enriched sites within Everglades National Park, the least P enriched area within the Everglades. A simple two component model, incorporating total P and the relationship between the enzymes involved in C and P mineralization accounted for between 46 and 92% of the variability in measured cellulose decomposition rates and thus demonstrates the significant influence that P loading plays in these systems. These results also suggest there is an environmental threshold TP concentration below which changes in enzyme-based resource allocation will not occur.

Keywords Decomposition · Enzyme · Everglades · Microbial · Nitrogen · Phosphorus

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Introduction

Most organic C in aquatic and marsh systems is processed and recycled entirely by the microbial community without entering the higher order food webs (Wetzel 1984). Alterations in microbial decomposition associated with nutrient loading

may drastically affect the higher trophic levels of the system.

Enzyme activities have the potential to reflect changes in nutrient cycling as a result of changes in water and soil quality (Wetzel 1991). For example, in a phosphorus (P)-limited system the activities of phosphatases may be a dominant regulatory mechanism controlling microbial productivity. Phosphatase regenerates inorganic P through the hydrolysis of organic P to inorganic P, is repressed by P enrichment and dissolved reactive phosphorus (DRP) (Jansson et al. 1988; Chróst 1991; Newman et al. 2003), and has been recommended as a parameter to assess P impact in P limited systems (Newman et al. 2003). Phosphatase is one example of a suite of enzymes involved in organic matter degradation and nutrient cycling that can be affected by nutrient loading (Wetzel 1991; Newman and Reddy 1993; Marx et al. 2001). For example, phosphatase and β-glucosidase activities are positively related to microbial biomass and decomposition (Frankenberger and Dick 1983; Güsewell and Freeman 2003). The measurement of multiple enzymes that tie together different aspects of C, N and P cycling has significantly enhanced our mechanistic understanding of decomposition. One strategy utilizes a resource allocation model (MARCIE) for exposing linkages between individual enzymes (Sinsabaugh and Moorhead 1994). Components of this model have been linked to bacterial productivity, microbial biomass, and particulate organic carbon turnover times in several systems (Sinsabaugh and Findlay 1995; Sinsabaugh et al. 1997). As mediators of soil microbial decomposition, enzyme activities are intrinsically linked to environmental conditions and the resident microbial communities (Frankenberger and Dick 1983) and thus provide a means to assess changes in nutrient cycling in response to anthropogenic enrichment.

The Everglades ecosystem encompasses over 10,000 km^2 ranging from a softwater peatland ecosystem in the north, to a calcareous marsh in the south. Historically a P-limited ecosystem, over 40 years of nutrient loading from the Everglades Agricultural Area (EAA) has caused the establishment of distinct nutrient gradients throughout the remnant Everglades. Consequently, shifts in macrophyte species composition (Davis 1943, 1991), increases in net primary production (NPP) (Davis 1991) and peat accumulation (Reddy et al. 1993), loss or taxonomic shifts of native periphyton assemblages (McCormick and O'Dell 1996), and increases in microbial activity and biomass (White and Reddy 2000) have been observed in Everglades areas adjacent to discharge points (McCormick et al. 1996). The majority of our understanding of nutrient enrichment effects on the Everglades is based on the numerous studies conducted in the northern section of the ecosystem (WCA2A), however, nutrient gradients are prevalent to some degree throughout the entire landscape. The objectives of this study were to investigate the specific microbial mechanisms that have been impacted by nutrient loading throughout the entire ecosystem by (1) using a suite of enzyme activities reflecting C, N and P cycling to assess the effects of nutrient loading across the entire Everglades region, (2) investigating differences in microbial responses to nutrient impacts across different hydrologic units within the region, and (3) establishing a potential decomposition model relating enzyme activity to a standard substrate to assess in-situ decomposition.

Methods

Site description

The Florida Everglades is an oligotrophic system with background (reference) surface water total phosphorus (TP) levels averaging less than 10 $\mu g\ l^{-1}$ throughout the interior of the marsh (McCormick and O'Dell 1996; McCormick et al. 2000). Nutrient enriched sites are generally characterized by robust stands of *Typha domingensis* (Pers) (cattail) and a large quantity of particulate organic matter composed of plant litter in various stages of decay. Reference sites generally consist of *Eleocharis* spp. (spike rush), *Nymphaea odorata* (water lily), *Utricularia spp.* (bladderwort) and benthic and floating periphyton mats surrounded by stands of *Cladium jamaicense* Crantz (sawgrass), with little suspended organic matter.

Field study sites were situated along nutrient gradients in four distinct hydrologic units of the Everglades (Fig. 1): Arthur R. Marshall Loxahatchee National Wildlife Refuge (LNWR), Water Conservation Area 2A (WCA-2A), Water Conservation Area 3A (WCA-3A), and Taylor Slough within Everglades National Park (ENP-TS). Two sites were selected within each area, representing nutrient enriched and reference conditions.

The Loxahatchee National Wildlife Refuge is the northernmost of the hydrologic units within the Everglades, completely impounded by levees and canals, and encompasses 566 km^2. The P gradient in this area exhibits a steep decline with surface water and soil TP levels decreasing to reference levels within 2.2 km of the L-7 canal (SFWMD 2003). LNWR hydrology is primarily rainfall driven (54%) and is, unlike the rest of the Everglades, a slightly acidic softwater system. Water Conservation Area 2A is a 442 km^2 area immediately south of LNWR and is completely enclosed by canals and levees. The primary source of water and nutrient loading to the area are the S-10 structures that transfer water from agricultural areas and LNWR via the Hillsboro Canal. Nitrogen and P gradients exist in the water column and periphyton tissue (McCormick and O'Dell 1996) and soil P concentrations ranged from 400 to 1,600 mg kg^{-1} in the reference and enriched areas, respectively (Reddy et al. 1993; DeBusk et al. 1994). Water Conservation Area 3A encompasses 2,012 km^2 and is predominantly a vast sawgrass marsh interspersed with sloughs, tree islands, and wet prairies. It is the only area not completely enclosed by levees. The extent of

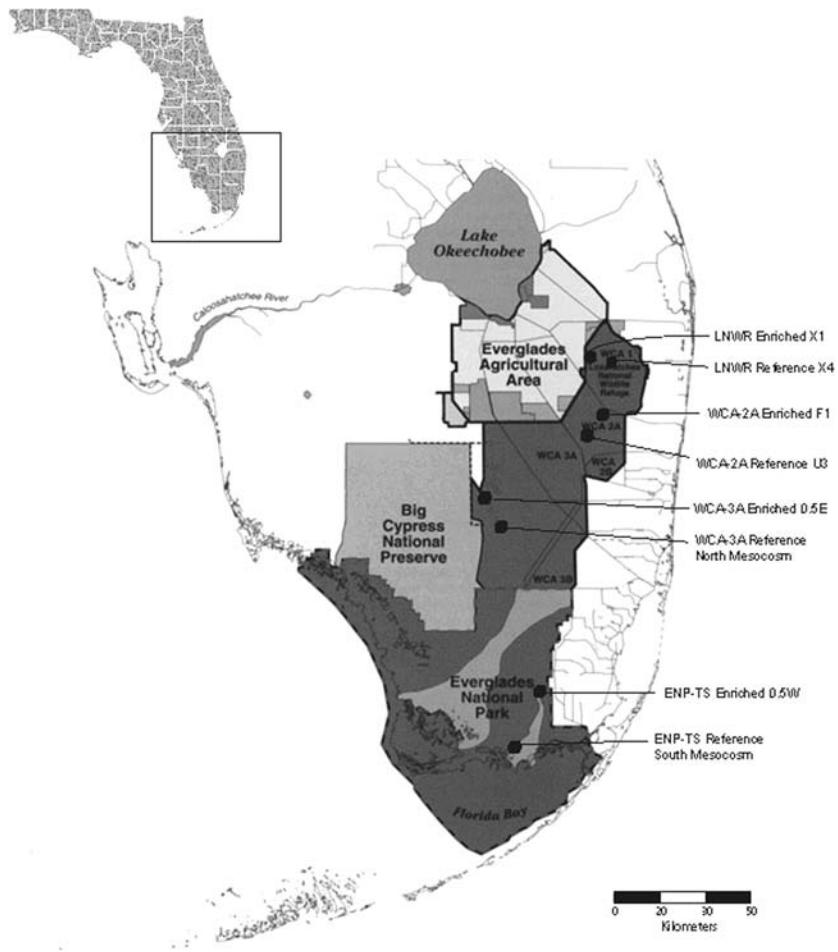

Fig. 1 Sampling sites within four hydrologic units of the Everglades. Site designations refer to South Florida Water Management District station IDs

downstream enrichment is similar to that of LNWR and extends approximately 3 km from the inflow (Newman et al. 2002). Everglades National Park is a 5,569 km^2 wetland consisting primarily of marl forming wet prairies, sawgrass stands, sloughs, and mangrove stands at the southern periphery. The overlying water column at the enriched site contains less suspended organic matter and lower net primary productivity. Vegetative changes relating to nutrient input occur in a relatively short distance from the canal inflow. Surface water flow into Taylor Slough originates at the S-332 structure at the southeastern side of ENP.

Soil sampling and preparation

Intact soil cores were obtained from each site to a depth of approximately 30 cm in triplicate using a 10 cm diameter thin-walled stainless steel corer with butyrate inserts on 12/14/2001, 5/18/2002, and 10/14/2002. The benthic matter, defined as the unconsolidated or pourable core fraction, was separated from the 0 to –10 cm soil layer and both layers were stored separately on ice for transport to the laboratory. Sample analysis began within 24 h of field collection. Each layer, corresponding to the 0 to –10 cm soil and benthic layers, was processed separately. Dry mass (DM) and ash free dry mass (AFDM) were determined from homogenized samples. The slurry was diluted 10^{-3} with deionized H_2O and homogenized an additional 5 min. To minimize cell lysis due to freezing, the suspension was refrigerated until use (Sinsabaugh et al. 1991). Enzyme analysis began within 6 h of sample preparation.

Enzyme analysis

Hydrolytic enzyme activity was determined using methylumbelliferyl (MUF) and amidomethylcoumarin (AMC) substrates. Substrate concentrations were optimized at saturating conditions. The activities of β-glucosidase (BGL) (EC 3.2.1.21), phosphatase (PHO) (EC 3.1.3.1), leucine aminopeptidase (LEU) (E.C. 3.4.11.1), phenol oxidase (PHE) (EC 1.14.18.1) and peroxidase (PER) (EC 1.11.1.7) were assayed using MUF-β-D-glucoside (Sigma M3633), MUF-phosphate (Sigma M8168), L-Leucine amidomethylcoumarin (Sigma L2145), L-3,4-dihydroxyphenylalanine (DOPA), and DOPA + H_2O_2 as substrates, respectively.

MUF and AMC substrate conversion was measured using a Cytofluor 600^{TM} (PerSeptive Biosystems, Inc., Framingham, MA) automated spectrofluorimeter with Kineticalc^{TM} software at 360 nm excitation and 460 nm emission wavelengths at 20°C. Assays were performed in quadruplicate per sample (twelve per site) using Corning® 48-well culture plates in which 400 µl sample, 360 µl 10 mM Tris–HCl pH 8.5 and 40 µl substrate was added. Stock substrate concentrations were 2000 µM MUF-β-D glucoside, 1000 µM MUF-phosphate, and 6000 µM L-Leucine aminomethylcoumarin resulting in concentrations of 100, 50, and 300 µM, respectively. Initial, final, and 5 min interval fluorescence measurements were taken during the 1 h incubation. Graphs produced from interval readings were analyzed to ensure linear kinetics were being observed.

Quenching effects on MUF and AMC substrates were determined to account for fluorescence blocking or absorption effects caused by coloring, particle suspension, humic matter or self-quenching. Final and initial fluoresences were converted based on the standard based quench percentage. Phenol oxidase and peroxidase activities were determined per Sinsabaugh and Linkins (1990) with quench corrections. Final activities for both hydrolytic and oxidative enzymes were calculated as µmoles substrate released g^{-1} AFDM h^{-1}.

Sample nutrient analyses were performed by DB Labs, Rockledge, FL. Total phosphorus (TP) (EPA 365.2), total nitrogen (TN) (MVP), total organic carbon (TOC) (MVP), and lignin (AOAC 973.18) analyses were performed using standard methods on homogenized samples (U.S. EPA 1983, 1986; Horwitz 2000). Nutrient ratios were expressed in terms of mass-mass relationships.

Cotton strips

Cotton strips (Shirley Institute, Manchester, England) were used to determine a measure of cotton rottenness rate (CRR) as a standardized in-situ decomposition linkage to enzyme

activities. Standardized substrates are often used across ecosystems with differing litter types in order to isolate changes that occur due to nutrient loading. Cotton strips were selected as a standardized substrate as CRR was previously shown to be a sensitive indicator of P enrichment in the Everglades (Newman et al. 2001). Strips were prepared by cutting 12 cm wide strips that were secured in duplicate to 6 mm thick stainless steel wire frames. Two frames were deployed within 10 meters of the soil coring sites to a depth of at least 15 cm below the soil surface for a period of 2 weeks at all sampling sites, corresponding within a week to soil coring events. Control cotton strips were placed within the soil, promptly removed and rinsed with in-situ water. The remnant tensile strength was tested using a tensiometer (Chatillon TCD-200) with a digital force gauge (DFIS 200, Chatillon, Greensboro, North Carolina, USA). Because the loss of tensile strength is analogous to first-order decay, the calculation of the rate constant requires linearization of the curve. CRR was calculated as (Hill et al. 1985):

$$\mathrm{CRR} = \left(((y_o - y)/y)^{1/3} / \#\,\text{of days deployed}\right)^{*} 365$$

where y_o is the tensile strength of the control strips and y is the tensile strength of the test strips at each 2 cm increment. Two of the three sampling dates were included in the analysis due to weather issues.

Resource allocation and impact index

Extracellular enzymes were divided into four categories: Ec (BGL), En (LEU), Ep (PHO), and Eox (mean(PHE and PER)), reflecting those involved in C, N, and P mineralization and lignin degradation, respectively. Enzyme activities were normalized on a scale of 0–1 to eliminate the weighting effects of the more active enzymes and were formulated to reflect the premise of resource allocation derived from the MARCIE (Microbial Allocation of Resources Among Community Indicator Enzymes) model (Sinsabaugh and Moorhead 1994, 1996; Sinsabaugh and Findlay 1995; Sinsabaugh et al. 1997, 2002). Three additional ratios were formulated: Ec/En, Ec/Ep and Ec/Eox, which reflect apparent N, P, and lignin influences on C mineralization, respectively. Variants of Ec/En and Ec/Ep have also been used in another decomposition study (Güsewell and Freeman 2003).

A qualitative impact index was constructed to compare the varying parameters on the same scale and to allow the comparisons between the enriched and reference sites within each area (Reddy et al. 1999). The equation for each parameter is:

$$\text{Impact Index} = \log((\text{Enriched site value})/(\text{Reference site value}))$$

In general, impact index values above 0.5 reflect severe changes in a specific parameter between the enriched and reference sites. Positive values reflect increased values at the enriched site while negative values reflect higher values at the reference site.

Statistics

Data were analyzed with SAS v.8© statistical software (SAS 1999) using mixed model repeated measures to determine significant differences ($P < 0.05$) between enriched and reference nutrient sites within each hydrologic unit at each time period. There was a significant area*site*sampling period interaction, therefore all site and area comparisons were conducted separately for each time period. Bonferroni corrections were used for multiple contrasts. Pearson correlations were performed on the entire data set. Data were log transformed which resulted in datasets that were normally distributed with equal variance. The only exception was phosphatase, however the analysis of this parameter was based primarily on the relationship with Ec as Ec/Ep, of which all assumptions were met. Regressions for the enzyme model of cellulose decomposition were performed using SYSTAT® 10.2 (SYSTAT 2002) for individual time periods. Mean values of all sampling dates were combined for tables and charts. Significant differences and correlation coefficients are significant at the $P < 0.05$ level, unless otherwise noted.

Results

Benthic Nutrient and Enzyme Data

Results from this study demonstrate greater microbial activity in the benthic layers compared to the soil layers. Other Everglades studies have shown increased microbial biomass (White and Reddy 2000) as well as faster decomposition in this layer (DeBusk and Reddy 2005), versus the soil layer. Additionally, the soil layer exhibited few significant differences in enzyme activity between enriched and reference sites (Appendix 1), presumably due to lower activities driven by anaerobiosis and increasing recalcitrance in the deeper sections. Therefore, the results and analyses for this study were focused on the more active benthic layers.

Significantly higher TP concentrations were present at the enriched sites in all four areas (Fig. 2), with the greatest concentrations present in the enriched LNWR and WCA-3A sites. In contrast, TN and TOC concentrations did not significantly differ between sites or areas. However, ENP-TS TN and TOC concentrations were as much as 60% lower than the other areas. TOC was most correlated with TP ($r = 0.68$) and TN ($r = 0.98$). C:P ratios were significantly lower at the enriched sites in all four study areas as a consequence of P loading, with the largest difference in ENP-TS (Fig. 3). However, C:N ratios were significantly higher at the enriched sites in only 50% of the comparisons. Lower C:N:P ratios at the enriched sites also reflected P loading in each hydrologic unit. Because lignin and cellulose were only measured on one field replicate from the 2nd and 3rd sampling events, we averaged these values to look for trends. With the exception of WCA3A, lignin was higher in enriched than reference sites, while cellulose was higher in enriched sites in WCA3A and ENP-TS (Fig. 4). The lignocellulose index (LCI) calculated as (Lignin/(Lignin + Cellulose) (Melillo et al. 1989) ranged from 0.46 in ENP-TS to values generally greater than 0.7 in peat based regions of the Everglades.

Though generally not significant, BGL activities were consistently higher at the enriched sites within WCA2A, WCA3A, and ENP-TS.

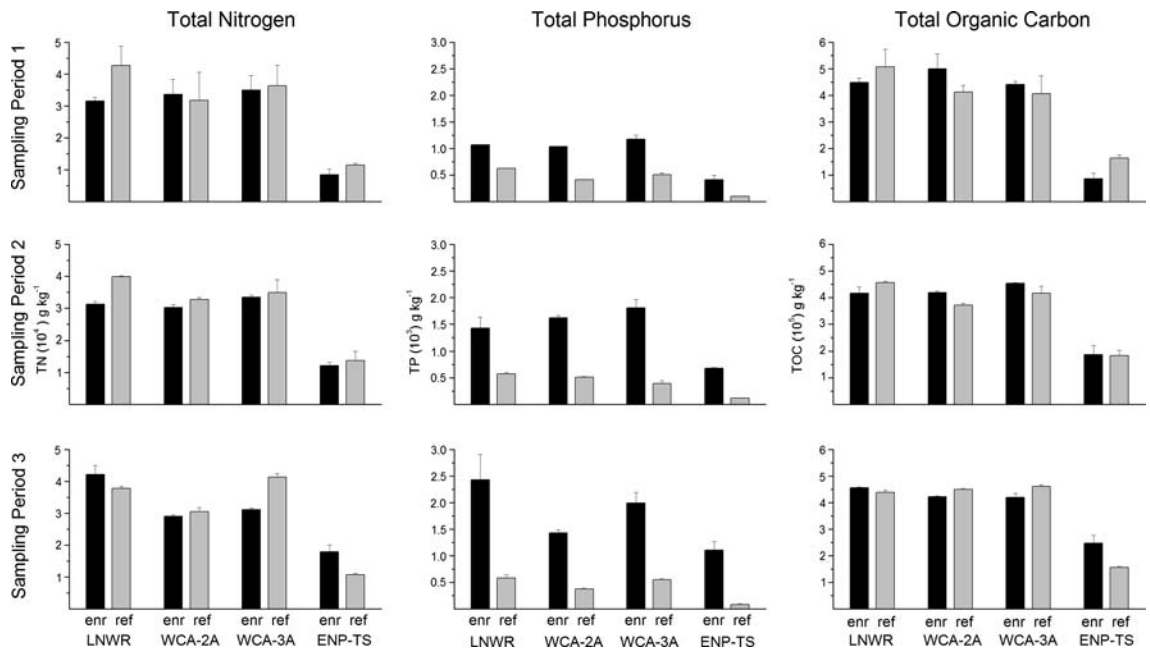

Fig. 2 TN, TP, and TOC concentrations (g/kg) per sampling period with standard error. enr = enriched site, ref = reference site, L = Loxahatchee National Wildlife Refuge, 2A = Water Conservation Area 2A, 3A = Water Conservation Area 3A, TS = Taylor Slough, Everglades National Park

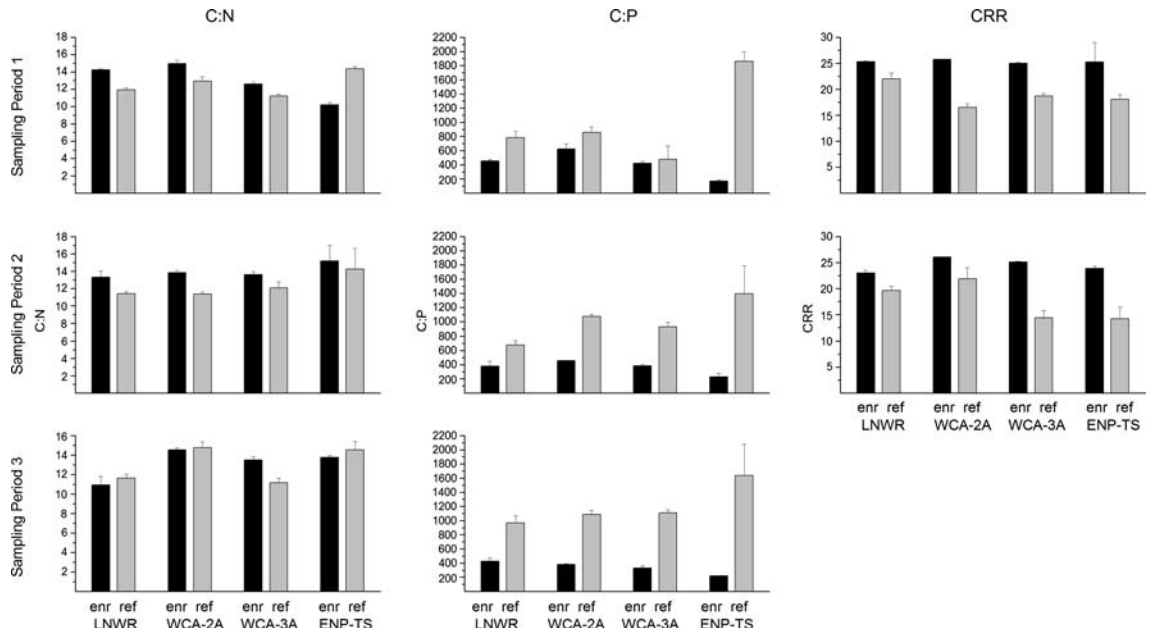

Fig. 3 C:N (mass–mass), C:P (mass–mass), and CRR values per sampling period with standard error. enr = enriched site, ref = reference site, L = Loxahatchee National Wildlife Refuge, 2A = Water Conservation Area 2A, 3A = Water Conservation Area 3A, ENP-TS = Taylor Slough, Everglades National Park

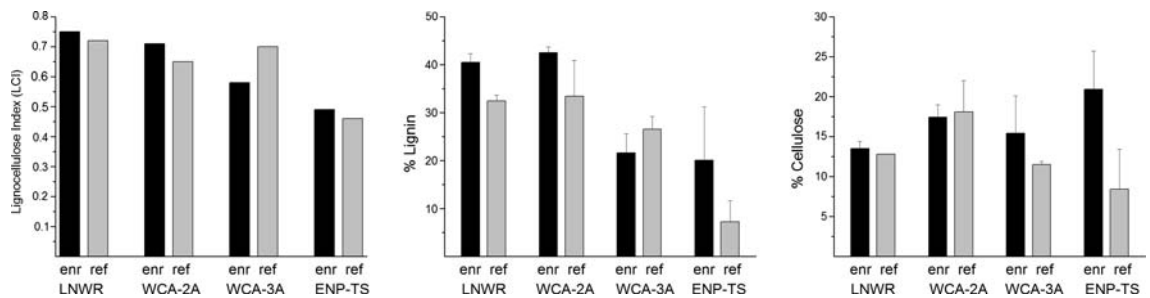

Fig. 4 LCI, lignin, and cellulose values per sampling period with standard error. enr = enriched site, ref = reference site, L = Loxahatchee National Wildlife Refuge, 2A = Water Conservation Area 2A, 3A = Water Conservation Area 3A, ENP-TS = Taylor Slough, Everglades National Park. Values are averaged over all samples with standard error

Conversely, LEU was higher in LNWR and ENP-TS reference sites in the first sampling period but higher at enriched sites in all areas during the second sampling event (Fig. 5). In correlating BGL and LEU enzyme activities with nutrient parameters, only BGL and TP were significantly correlated ($r = 0.39$). Significantly lower PHO activity was found in the enriched sites in the majority of comparisons and was correlated to TP ($r = –0.66$), TN ($r = –0.48$), and TOC ($r = –0.48$). The highest PHO activities were associated with the ENP-TS sites, which mirrored low benthic TP values. There were no significant patterns apparent in the PHE activities between enriched and reference sites.

Benthic Ec/Ep values were significantly lower at the reference sites (Fig. 6). The lowest Ec/Ep values occurred within ENP-TS, reflecting higher PHO activity in relation to BGL. Ec/Ep was positively correlated with the nutrient parameters TP ($r = 0.76$), TN ($r = 0.41$), TOC ($r = 0.44$), and negatively correlated with C:P

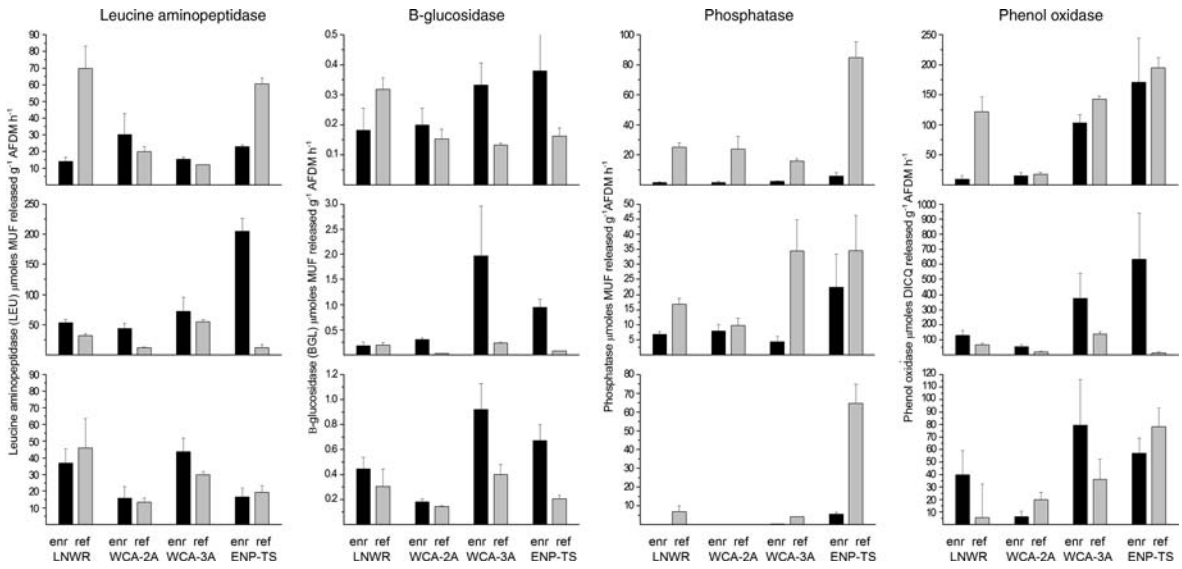

Fig. 5 Leucine aminopeptidase (LEU), β-glucosidase (BGL), Phosphatase (PHO), and Phenol oxidase (PHE) activities per sampling period with standard error. enr = enriched site, ref = reference site, L = Loxahatchee National Wildlife Refuge, 2A = Water Conservation Area 2A, 3A = Water Conservation Area 3A, ENP-TS = Taylor Slough, Everglades National Park

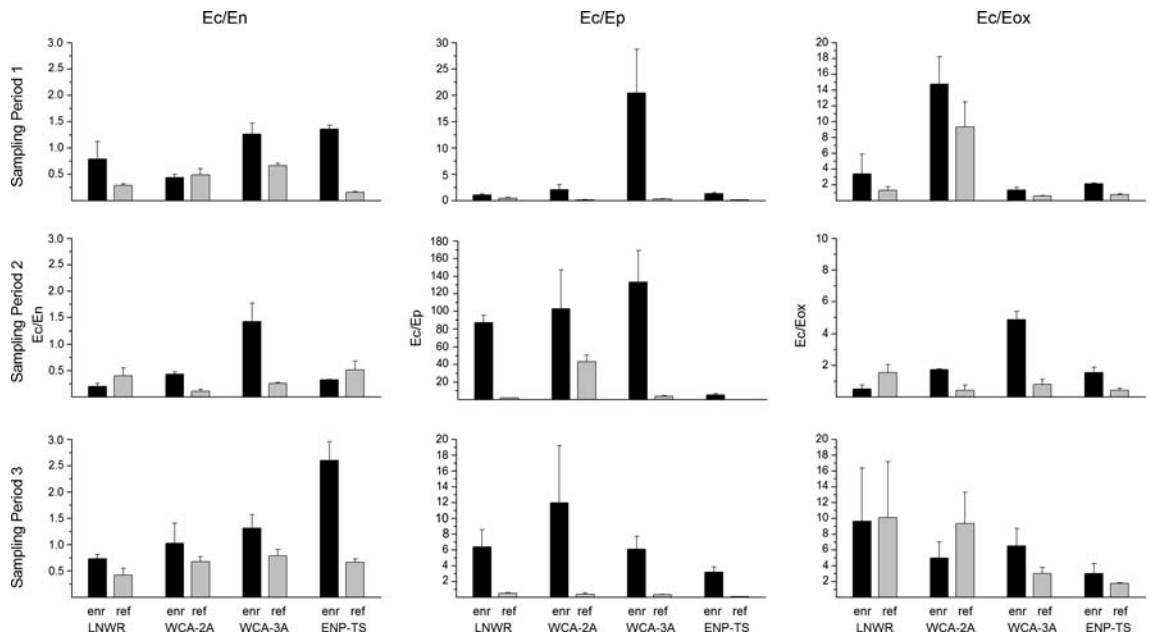

Fig. 6 Ec/En, Ec/Ep, and Ec/Eox values per sampling period with standard error. enr = enriched site, ref = reference site, L = Loxahatchee National Wildlife Refuge, 2A = Water Conservation Area 2A, 3A = Water Conservation Area 3A, ENP-TS = Taylor Slough, Everglades National Park

(r = –0.63). Benthic Ec/En significantly increased with P loading in 5 of 12 comparisons with the largest range found in ENP-TS. Due to large variabilities, Ec/Eox values were significantly higher at the enriched sites in only 50% of the comparisons.

Table 1 Impact index (log(impacted/reference)) for nutrient and enzymic data in the benthic layers for LNWR, WCA-2A, WCA-3A, and ENP-TS for averages calculated over the three sampling periods. Bolded items reflect parameters that are 0.4 or greater, indicating potentially large variations across the nutrient gradient

	LNWR	WCA-2A	WCA-3A	ENP-TS
TP	**0.42**	**0.49**	**0.53**	**0.83**
TN	–0.06	–0.01	–0.05	0.04
TOC	–0.03	0.04	0.01	-0.02
C:P	**–0.42**	**–0.46**	**–0.47**	**–0.88**
C:N	0.04	0.05	0.06	-0.05
Lignin %	0.10	0.12	-0.09	**0.47**
Cellulose %	0.02	-0.01	0.10	**0.48**
Glucosidase	–0.04	**0.45**	**0.56**	**0.66**
Leucine amin.	–0.19	0.27	0.13	0.24
Phosphatase	**–1.06**	**–0.49**	**–0.95**	**–0.81**
Phenol oxidase	–0.29	–0.04	0.21	**0.51**
Peroxidase	0.21	0.03	0.13	-0.03
Ec/Ep	**1.03**	**1.04**	**1.54**	**1.42**
Ec/En	0.13	0.24	**0.41**	**0.44**
Ec/Eox	–0.09	0.28	**0.50**	**0.40**

CRR

Mean CRR decreased at the reference sites in all areas (Fig. 3, Appendix 1) with the largest change between the enriched and reference sites in ENP-TS and WCA-3A. A model was created using enzyme data to test whether enzyme activities could predict CRR. The ratio Ec/Ep ($r = 0.62$) and TP ($r = 0.70$) were found to be most strongly correlated with CRR and were therefore chosen for the resulting benthic model:

$$\text{Predicted CRR} = (0.04(\text{Ln}^{\text{Ec:Ep}}) + 1.4) + (0.35(\text{Ln}^{\text{TP}}) + 1)$$

This model most strongly predicted CRR in sampling period 1 ($r^2 = 0.92$) with the weakest in sampling period 3 ($r^2 = 0.46$) and predicted significantly greater cellulose decomposition at the enriched sites in all four areas at all time periods ($P < 0.0001$).

Impact index

The most variable nutrient parameters to the P gradient were the P-related biogeochemical indicators TP, C:P, PHO and Ec/Ep in the benthic layer (Table 1). The majority of large impact indices in the benthic layer were within ENP-TS, despite a less pronounced P gradient in this area. Ec/En index values were higher in the southern areas, corresponding with the North-South flow from the EAA to Florida Bay. Higher index values were associated with the benthic layer, indicating that this layer is more responsive to changes in biogeochemical conditions.

Discussion

Phosphorus gradients

This study examined microbial responses at sites with differing nutrient loads and hydrologic regimes throughout the Everglades. Decreased nutrient loading at the reference sites are reflected in higher C:P values, which have been correlated with suppressed microbial respiration (Amador and Jones, 1993). Overall, C:N:P ratios were within the range reported in WCA-2A (Koch and Reddy 1992) and indicate low quality substrates that may exert stoichiometric control over microbial colonization and decomposition (Cross et al. 2005). Furthermore, high N:P ratios which are generally within the range found in periphyton at the reference sites (Vyzamal and Richardson 1995), in concert with increasing phosphatase activities, support the P limitation of decomposition rates (Güsewell and Freeman 2005). While C:N ratios have been specifically

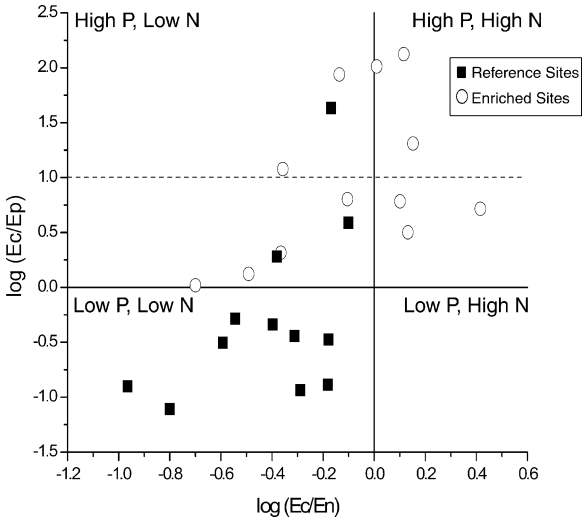

Fig. 7 Log plot of Ec/En vs. Ec/Ep for enriched and reference samples. Each point represents the mean from one sampling period. Ec/Ep values greater than 1 indicate high P availability

used as indicators of potential decomposition in other systems, it appears that C:P ratios may be more useful in this historically P-limited system, particularly considering the lack of significant enzyme or CRR correlations with C:N. The LCI index and total P are widely used indicators of substrate quality in various systems (Melillo et al. 1982) and have been found to account for 91% of the variability in aerobic C mineralization of plant litter along the WCA-2A gradient (DeBusk and Reddy 1998). However, once LCI values reach 0.7, the litter is described as decomposed to a state in which substrate quality is no longer controlling decay and environmental conditions alone drive the decomposition process (Melillo et al. 1982). With the exception of 3 sites, LCI values approximated 0.7. This demonstrates that P availability, not LCI, is controlling enzyme activity in this study.

The MARCIE model relates changes in resource allocation over the decomposition process, as substrate quality changes (Sinsabaugh and Moorhead 1994). We adopted the concept of enzyme ratios from the MARCIE model to investigate resource allocation changes between the enriched and reference sites in each area. Higher Ec/En and Ec/Ep values at all the enriched sites (Fig. 7) are concurrent with decreases in nutrient limitations that have been documented (McCormick *et al.* 1996), as well as lower relative P mineralization rates in enriched areas of the Everglades (Newman et al. 2003; White and Reddy 2000). Specifically, greater Ec/Ep values at the enriched sites appear to reflect a decrease in apparent P control on C mineralization which was as much as 20 times higher than those reported in an aquatic study (Sinsabaugh et al. 1997) and are a reflection of lower C:P values. Higher Ec/En values at the enriched sites appear to be due to generally elevated BGL activities, which have been correlated with microbial production in nutrient enriched mesocosms (Chróst and Rai 1993). Since algae generally contain a higher protein percentage than macrophytes (Boschker and Cappenberg 1998), the lower Ec/En values at the reference sites may also be attributed to the higher algal inputs due to the prevalence of periphyton as compared to the primarily macrophytic inputs at the enriched sites. Interestingly, there was no significant correlation between PHO and LEU which have been shown to influence each others activity in marine systems (Nausch 2000).

The small increase in Ec/Eox values at the enriched sites of LNWR, WCA-3A and ENP-TS, although not generally significant, indicate slightly decreased apparent lignin control on C mineralization. The higher Ec/En values at the enriched sites in these areas are a response to increased N availability which is not reflected in the TN content of the soils, suggesting that TN is not a well suited measure for judging microbial N availability. Additionally, a large portion of endogenous N may be shielded by refractory compounds within the litter (Sinsabaugh and Moorhead 1994). As exogenous N loading has been shown to repress lignin degradation (Eriksson et al. 1990; Sinsabaugh et al. 1993; Carreiro et al. 2000) by the repression of phenol oxidase, the higher Ec/Eox values at the enriched sites may be linked to the higher Ec/En values, which are reflections of greater N concentrations. This high N may be repressing phenol oxidase activity, supported by a positive correlation between LEU and PHE. Lastly, while algal productivity is higher in enriched areas, biomass is much lower

(McCormick and Laing 2003). This is coupled with increased O_2 consumption due to higher respiration. Therefore, the enriched areas tend to have lower dissolved oxygen which could also reduce phenol oxidase activity (Freeman et al. 2001).

Nutrient loading to the four areas of the Everglades appear to have resulted in overall decreased N and P constraints to benthic C mineralization. This is supported by consistently higher CRR at the enriched sites that were within the range of values reported in a mesocosm P loading experiment in LNWR (Newman et al. 2001) and concurrent with elevated decomposition at these sites (DeBusk and Reddy 2005). Phosphatase activity alone exhibited the greatest number of correlations with nutrients, illustrating the possible role of PHO as an important enzyme in the Everglades regulating microbial responses. The CRR decomposition model, utilizing TP and Ec/Ep as parameters, also predicts the strong influences that P loading alone has on cellulose decomposition rates. Additionally, the inclusion of Ec/Ep in the model indicates the relative strength of BGL and PHO in predicting cellulose decomposition, comparable to the correlation between BGL and PHO with bacterial production in nutrient enriched and un-enriched mesocosms, respectively (Chróst and Rai 1993).

Differences among hydrologic units

Because the majority of the knowledge concerning nutrient inputs to the Everglades is based on data gathered in WCA-2A, differences among the hydrologic units of the Everglades are important to address. While the general influences of phosphorus loading throughout the four hydrologic units examined in this study are fairly consistent, there are certain questions that arise from unit specific results. The greatest Ec/Ep shift and Ec/Ep impact index in WCA-3A both suggest that the largest P impact on the microbial community occurs within this area. The smallest shift in Ec/Ep occurs in ENP-TS, although the impact index does not support this. The highest TP impact index (0.83) is actually found in ENP-TS, driven by the very low TP value at the reference site. This lower intensity of P loading within ENP-TS does not appear to induce resource allocation shifts and, as such, the magnitude of the TP shift does not solely influence the magnitude of the microbial community response between the enriched and reference sites. Rather, it appears that enzyme-based resource allocation significantly responds to changes only above a certain TP threshold value that drives higher order changes in the microbial community structure and function and thus the impact index does not appear to be a robust measure in this hydrologic unit.

While most attention has been focused on the role of P in the Everglades ecosystem, it appears that there are some trends within the Ec/En data that are worthy of exploration. Apparent N limitation on the microbial community appears to become less important in the southern regions along the canals, according to a North-South gradient of increasing Ec/En ratios at the enriched sites: LNWR < WCA-2A < WCA-3A < ENP-TS. This may be due to changes in substrate composition. For example, compared to the northern Everglades, significantly lower C:N ratios of the DOC/DON fraction of surface and shallow pore water were found within the southern Everglades (Qualls and Richardson 2003), which mirrors the higher Ec/En values. Several hypotheses concerning the North-South trend of decreasing DOC/DON ratios are presented by Qualls and Richardson (2003) and include increased rates of biodegradation, greater plant production of soluble organic matter, and greater production of soluble organic matter by-products where peat is being decomposed faster. Although the ENP-TS enriched site is exhibiting higher LEU activity, the relationship with the amount of C mineralization that appears to be occurring suggests that, energetically, this community is expending a smaller proportion of its resources on the acquisition of N from organic sources. This may be explained by greater algal biomass in the southern Everglades which constitute highly cohesive N-fixing periphyton mats that may supplement N availability to the heterotrophic community. In addition to the highest Ec/En values at the enriched site, ENP-TS exhibits the largest

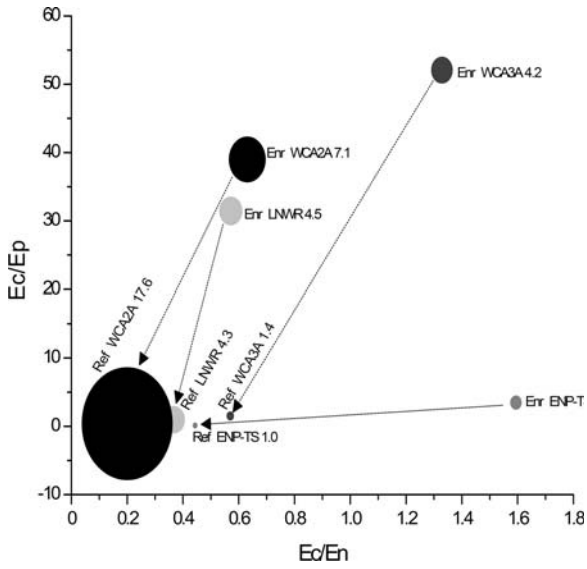

Fig. 8 Plot of Ec/Ep vs. Ec/En with Ec/Eox represented by the numerically identified bubble size with the site designation. Arrows denote shifts from the enriched (enr) to the corresponding reference (ref) sites in each area

change in apparent N dynamics between enriched and reference sites, as compared to the relatively large shifts in P dynamics in the other areas (Fig. 8). Lastly, the lowest overall Ec/Eox at the ENP-TS enriched site also points to increased lignin control on C mineralization. This indicates that lignin content is playing a potentially important role in the C mineralization process, with increased combined oxidative enzyme activities, although the lignin content is lowest at this site.

In contrast, the overall lowest Ec/Ep, TP, TN, TOC, lignin, and cellulose and the highest C:N:P ratio occurs at the ENP-TS reference site. Specifically, the high N:P ratio at this site is within the high range found in periphyton (Vyzamal and Richardson 1995), further supporting the primarily periphytic composition of this site and P limitation on decomposition. Phosphatase is therefore the primary driving force, based on resource allocation strategies, that leads to the decreased production of the C and N acquiring enzymes at this site as well as the possible role of N fixation and other interactions by the extensive consolidated periphyton mat. For example, BGL activity may also be tied to the resident periphyton community such that photosynthetically produced extracellular organic carbon (EOC) from the periphyton community may supply substantial amounts of labile carbon that is sufficiently degraded for direct microbial uptake (Espeland et al. 2001). If the majority of DOC released is of sufficiently low molecular weight there may be no need for microorganisms to acquire it through enzymatic action (Chr&oacgr;st and Rai 1993). The possible nutritional supplementation with EOC, resulting in lower BGL activities, may also be exhibited among the suite of cellulase enzymes, which coincides with the lowest overall benthic CRR at the ENP-TS reference site.

Conclusions

Previous studies examining mechanisms driving decomposition have primarily been based in the northern Everglades. This is the first study to investigate nutrient loading effects to the Florida Everglades simultaneously in all four hydrologic units using enzyme activities to derive relationships with measured *in-situ* decomposition rates. Nutrient loading consistently decreased P and N limitations on C mineralization, which results in increased decomposition rates at the enriched sites near the canal inflows. While the four hydrologic units exhibited similar patterns of enzyme activities with nutrient loading, there were marked differences between the units. Water Conservation Area 3A was found to currently exhibit the greatest nutrient loading impact, reflected by the greatest change in Ec/Ep and highest impact index between sites. This contradicts the currently accepted evidence that WCA-2A is the most impacted area. Conversely, the current nutrient loading regime to ENP-TS appears to have not surpassed an available P threshold that induces resource allocation shifts within the microbial community. While there was evidence of differences between the enriched and reference sites at ENP-TS, the extent of the change was relatively small compared to other areas. Increasing Ec/En in a North-South pattern supports previous evidence of decreasing C:N ratios. This suggests a larger N reservoir in

the Southern Everglades and a potential source of N efflux into Florida Bay which warrants further investigation.

Various methods have been used to derive relationships between enzyme activities and nutrients with measured decomposition rates. For system-wide comparisons, the generally high LCI values indicate that standardized substrates such as cotton strips are applicable because decomposition is influenced more strongly by environmental factors than substrate quality. An impact index appears to be a valid method for quickly identifying the largest change across a landscape gradient. However, this simple measure can easily be skewed when reference sites vary dramatically among areas. Enzyme ratios appear to be the most robust method to provide information regarding the relative influences of P and N on C mineralization. In this study Ec/Ep was the strongest enzymatic predictor of decomposition, reflecting the resource allocation shifts that occur when the availability of the most limiting nutrient changes. Other systems have shown that N or lignin mineralization limits decomposition, illustrating the need to validate the components of an enzyme derived decomposition model on a system-specific basis.

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Appendix Soil layer mean nutrient, enzyme, and ratio parameters in P enriched and reference sites in LNWR, WCA-2A, WCA-3A and ENP-TS with standard errors. ß-glucosidase, Leucine aminopeptidase, phosphatase, phenol oxidase, and peroxidase activities expressed as µmoles substrate released g^{-1} AFDM h^{-1}. CRR = cotton rottenness rate, LCI = lignocellulose index. Asterisks reflect differences at the $P < 0.05$ level per sampling period between the sites.

	LNWR	LNWR	$P < .05$			WCA-2A	WCA-2A	$P < .05$			WCA-3A	WCA-3A	$P < .05$			ENP-TS	ENP-TS	$P < .05$		
	Enriched $n = 9$	Reference $n = 9$	1	2	3	Enriched $n = 9$	Reference $n = 9$	1	2	3	Enriched $n = 9$	Reference $n = 9$	1	2	3	Enriched $n = 9$	Reference $n = 9$	1	2	3
Total P g/kg	0.91 ± 0.14	0.37 ± 0.09	*			0.72 ± 0.13	0.33 ± 0.04	*	*		0.95 ± 0.42	0.31 ± 0.04	*	*	*	0.37 ± 0.11	0.23 ± 0.08	*		
Total N g/kg	32.0 ± 1.7	35.6 ± 3.2	*			29.1 ± 2.8	33.6 ± 1.2	*			25.4 ± 9.1	37.2 ± 1.1		*		7.7 ± 1.6	12.7 ± 2.1	*		*
Total Organic C g/kg	471.1 ± 3.7	462.1 ± 28.2	*			443.0 ± 17.2	464.7 ± 8.2				343.1 ± 129.2	473.4 ± 5.0		*		86.4 ± 25.8	153.5 ± 24.1	*		*
Calcium g/kg	30.3 ± 3.1	12.6 ± 0.2				34.8 ± 6.8	28.6 ± 2.7				87.9 ± 73.0	19.1 ± 0.9				183.44 ± 75.8	219.9 ± 30.9			
C:P (mass)	711.1 ± 122.3	1.96 ± 0.08	*			3.6 ± 0.1	3.2 ± 0.21				489.3 ± 142.7	1601.9 ± 220.8	*	*	*	239.3 ± 6.4	775.9 ± 60.0	*		*
C:N (mass)	14.9 ± 0.9	13.2 ± 0.6	*	*		16.4 ± 1.3	13.9 ± 0.7	*			13.1 ± 0.6	12.5 ± 0.3	*	*	*	10.9 ± 1.1	12.0 ± 0.1			
Lignin %	52.1 ± 3.1	45.7 ± 2.1				50.1 ± 2.8	46.5 ± 3.6				20.5 ± 11.5	45.1 ± 1.8				19.0 ± 12.2	10.9 ± 1.8			
Cellulose %	17.3 ± 0.2	18.8 ± 0.1				18.2 ± 1.7	19.7 ± 0.7				18.0 ± 1.6	18.7 ± 1.6				27.4 ± 8.4	13.3 ± 0.1			
Glucosidase (BGL)	0.24 ± 0.06	0.14 ± 0.06	*			0.08 ± 0.00	0.05 ± 0.01				0.39 ± 0.02	0.21 ± 0.02				0.28 ± 0.07	0.06 ± 0.01	*		*

Appendix Continued.

	LNWR Enriched $n = 9$	LNWR Reference $n = 9$	$P < .05$ 1 2 3	WCA-2A Enriched $n = 9$	WCA-2A Reference $n = 9$	$P < .05$ 1 2 3	WCA-3A Enriched $n = 9$	WCA-3A Reference $n = 9$	$P < .05$ 1 2 3	ENP-TS Enriched $n = 9$	ENP-TS Reference $n = 9$	$P < .05$ 1 2 3
Leucine amin. (LEU)	1.92 ± 0.13	2.69 ± 0.05	*	1.95 ± 0.03	2.16 ± 0.07	*	2.87 ± 0.08	2.31 ± 0.02	*	3.48 ± 0.04	2.72 ± 0.17	*
Phosphatase (PHO)	1.89 ± 0.34	2.46 ± 0.07		1.35 ± 0.05	1.14 ± 0.06	*	0.80 ± 0.14	2.18 ± 0.23		3.87 ± 0.54	10.84 ± 0.20	
Phenol oxidase (PHE)	110.5 ± 8.33	223.0 ± 45.9		78.6 ± 4.0	142.8 ± 23.4		253.0 ± 32.0	222.1 ± 34.0		577.9 ± 76.0	252.4 ± 18.0	*
Peroxidase (PER)	24.3 ± 0.8	36.1 ± 5.3		44.5 ± 9.1	41.1 ± 2.2		19.2 ± 3.6	32.6 ± 13.6		62.0 ± 14.0	24.9 ± 13.7	
Ec/Ep	18.47 ± 8.40	8.52 ± 3.25		13.08 ± 5.47	0.84 ± 0.99	* *	90.19 ± 9.15	9.37 ± 3.13	* *	2.74 ± 0.71	0.32 ± 0.10	* * *
Ec/En	0.60 ± 0.20	0.32 ± 0.15	*	0.27 ± 0.04	0.10 ± 0.03	*	1.28 ± 0.27	0.57 ± 0.11		0.63 ± 0.23	0.21 ± 0.07	* *
Ec/Eox	0.88 ± 0.32	1.44 ± 1.11		0.24 ± 0.06	2.29 ± 0.06		4.97 ± 3.19	1.29 ± 0.47		1.86 ± 1.20	0.34 ± 0.16	*
CRR	24.7 ± 0.5	19.4 ± 0.3	* * *	25.4 ± 0.2	18.7 ± 1.1	* * *	25.1 ± 0.1	18.7 ± 1.1	* * *	22.2 ± 0.2	19.6 ± 0.6	* * *
C:N:P ratio (mass)	517:35:1	1248:96:1		615:40:1	1408:102:1		361:27:1	1527:120:1		234:21:1	667:55:1	
LCI	.75	.71		.73	.70		.53	.71		.41	.45	

[1] Units for BGL, LEU, PHO are μmoles AMC or MUF released g^{-1} AFDM h^{-1}

[2] Units for PHE and PER are μmoles DICQ released g^{-1} AFDM h^{-1}

[3] C:P = Total organic carbon / total phosphorus, C:N = Total organic carbon / total nitrogen, CRR = Cotton rottenness rate, Leu = leucine aminopeptidase, LCI = lignocellulose index

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