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# Heterotrophic microbial activities and nutritional status of microbial communities in tropical marsh sediments of different salinities: the effects of phosphorus addition and plant species

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**Abstract** Oligotrophic, phosphorus (P) limited herbaceous wetlands of northern Belize are being impacted by P loading from fertilizer runoff. P enrichment causes a shift in autotroph communities from a microphyte (cyanobacterial mats, CBM) to macrophyte (*Eleocharis* spp., *Typha domingensis*) dominated system. To document potential effects of P, salinity, and macrophyte species on the heterotrophic microbial community nutritional status (represented especially by specific phospholipid fatty acids and specific respiration rate), biomass and activities, we took soil samples from established P enrichment plots in replicated marshes of two salinity levels. P addition increased microbial biomass carbon (C), nitrogen (N) and P, as well as soil nutrient transformation rates (nitrogenase activity, N mineralization and immobilization, methanogenesis). The effect of plant species (*Eleocharis* vs *Typha* sites) was gener-

ally lower than the effect of P addition (CBM vs *Eleocharis* sites) and was most evident at the low salinity sites, where *Eleocharis* dominated plots had enhanced nitrogenase activity and P microbial immobilization. Salinity reduced the overall rates of microbial processes; it also weakened the positive effect of both P addition and plant species on microbial activities. Lastly, the amount of N stored in microbial cells, likely in form of osmoprotective compounds, was enhanced by salinity.

**Keywords** Phosphorus loading · *Eleocharis* · *Typha* · Salinity · Microbial biomass · Nitrogen

## Abbreviations

CBM	Cyanobacterial mats
NA	Nitrogenase activity
DEA	Denitrification enzyme activity
PLFA <sub>tot</sub>	Amount of phospholipid fatty acids
TBFA	Terminally branched fatty acid
SRB	Sulfate reducing bacteria
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
MBP	Microbial biomass phosphorus
P <sub>ox</sub>	Oxalate extractable sediment P
C <sub>avail</sub>	Available carbon
Q <sub>CO2</sub>	Specific respiration rate
MUFA/ STFA	Monounsaturated/saturated fatty acids ratio

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## Introduction

In aquatic and wetland systems, microorganisms are the most abundant and important biological component involved in organic matter turnover (Pomeroy and Wiegert 1981). Organic matter is used by microbes as a growth substrate and nutrient supply; it is the location, quantity, and quality of organic matter that determine microbial abundance and production (Cunha et al. 2000; Boschker et al. 1999).

In marsh sediments, microbial production is generally high and strongly influenced by organic matter released from primary producers' exudates and/or decomposing litter (Moriarty and Pollard 1981). Carbon from the organic matter is assimilated into the microbial biomass or rapidly transformed to CO<sub>2</sub> (Créach et al. 1999). The size, composition, and activity of the heterotrophic microbial pool are all a reflection of the dominant plant species' partitioning of resources and growth (Semenov et al. 1999). Among the possible mechanisms, plants regulate nutrient availability by competing with microorganisms for nutrient resources and control sediment aeration by consuming or releasing oxygen to the rhizosphere.

Plant production is determined by a wide range of environmental factors, among which, nutrient availability often plays a major role, especially in oligotrophic wetlands. While macrophyte community changes following nutrient loading have been reported for various types of wetlands (Childers et al. 2003; Wolin and Mackeigan 2005), it is not clear how the changes in macrophytes, in turn, affect the sediment microbial communities (Ravit et al. 2006; Francoeur et al. 2006). The effect of macrophytes on heterotrophic microbial activity, as well as on rhizospheric bacterial composition may be species specific (Bagwell et al. 2002; Burke et al. 2002). The presence of plant species with distinct growth patterns and resource allocation can lead to differences in the proportion of modified bulk soil, and, thus, result in different populations and/or degrees of soil microbial activities (Semenov et al. 1999).

Wetlands can vary widely in salinity level, with high salinity posing a potential stress to plants as well as microbial growth. In saline environments, bacteria have to cope with ionic stress, which is balanced by intracellular osmoprotective compounds, many of which are sugars (Welsh and Herbert 1999) or contain

N (amino acids and their derivatives; Csonka 1989; Galinski and Trüper 1994). Even under moderate increases in salinity, bacteria may modify the chemical composition of their cell membranes, synthesizing and altering the patterns of proteins, lipids, fatty acids and polysaccharides (Zahran 1997).

This study focuses on a wetland site in northern Belize that is well suited to assess the impact of different plant species on sediment microbial communities. The site includes marshes of a wide range of salinities with a well established system of nutrient enriched plots, which are dominated by only a few macrophyte species (Rejmánková et al. 2008). Under natural un-impacted conditions, all of these wetlands are dominated by benthic cyanobacterial mats with scattered macrophytes, mainly *Eleocharis cellulosa* and *E. interstincta*, and are strongly P limited. The region is experiencing increasing nutrient inputs from fertilizer runoff due to the expansion of sugar cane cultivation and its impact, specifically the expansion of *Typha domingensis*, has been documented in some of the marshes (Johnson and Rejmánková 2005). In 2001, we initiated a long term manipulative experiment using these wetlands as a model system, to obtain a mechanistic explanation for an ecosystem level response to increased nutrient input across a salinity gradient. We have already confirmed that P addition leads to almost total elimination of cyanobacterial mats due to the expansion of *Eleocharis cellulosa*, and, eventually, the replacement of *Eleocharis* by *Typha domingensis* (Rejmánková et al. 2008).

Both *Eleocharis* and *Typha* are rhizomatous perennials, but while *Eleocharis* is a good example of a stress tolerator adapted to P limitation stress, *Typha* behaves as a competitor (*sensu* Grime 2001) and is able to outcompete *Eleocharis* once the P-limitation has been removed (Macek and Rejmánková 2007). We expected that due to their different biomass production, nutrient uptake, and resorption (Rejmánková 2005), these two macrophytes will provide organic material of contrasting amounts and quality to the sediments and, consequently, they will differentially impact the microbial activities in the respective sediments.

The aim of this paper is to document how microbial biomass and activities (nitrogen and carbon mineralization, denitrification enzyme activity and nitrogenase activity) in wetland sediments are impact-

ed by the addition of a limiting nutrient (P) and the accompanied changes in macrophyte species and production. Based on previous results on macrophyte growth and plant tissue composition in P enriched wetlands, we expect that the functioning of sediment microflora will be positively impacted by a greater input of organic carbon through plant litter and root exudates and by more favorable C/P ratio of the plant litter (Fig. 1). Using wetlands of two contrasting salinity levels allows us to interpret the data in the salinity context.

We hypothesize that:

1. The nutrient content (N and P stoichiometry) in microbial biomass will be enhanced either directly by the removal of P limitation, or indirectly by increased available C from macrophyte exudation and a litter.
2. Plant species will differ in quality (C, N, P favorable stoichiometric ratio) and quantity of a litter thus affecting microbial biomass and activity.
3. An enhanced microbial N demand will be reflected in faster N transformations.
4. The rate and direction of the changes in microbial activities and biomass will be salinity dependent.

## Materials and methods

### Study site

Our study area is located in the lowlands of northern Belize, Central America within a 50 km radius of 18°9' 58" N and 88°31'28". A detailed description has been provided elsewhere (Černá et al. 2009; Rejmánková et al. 2008). Briefly, the limestone geology and occasional intrusion of seawater result in diverse range of water conductivities (0.2–7 mS) with large differences in sulfate, bicarbonate and chloride. The climate of the region is tropical wet–dry. The majority of wetlands in the study area remain flooded or water saturated year round, although the total flooded area may vary as water levels rise and fall.

The main primary producers in these systems are several species of emergent macrophytes (*Eleocharis cellulosa*, *E. interstincta*, *Cladium jamaicense* and *Typha domingensis*) and species rich communities of microphytes represented mostly by cyanobacteria (Rejmánková et al. 2004). Both the macro- and

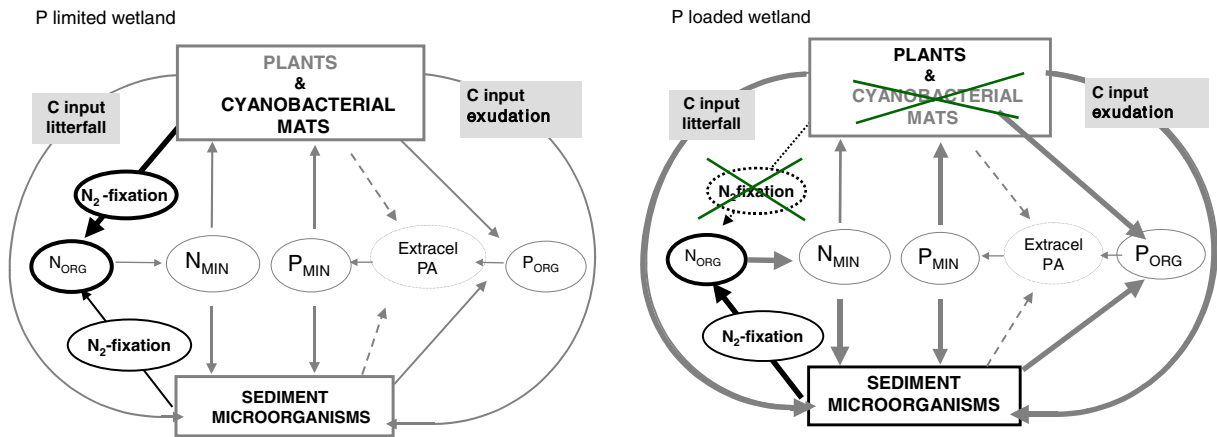
microphytes in these wetlands are generally P limited (Rejmánková 2001; Rejmánková and Komárková 2000). No nitrogen limitation has been detected in any of the reported studies.

### Treatment plot history

Fifteen marshes of diverse salinities, all dominated by sparse macrophytes (*Eleocharis* spp.) and cyanobacterial mats (CBM), have been studied as a part of a project aimed at assessing the ecosystem response to nutrient addition along a salinity gradient (Rejmánková et al. 2008). In August of 2001, four 10×10 m plots were established in each marsh, with one representing a control, and the remaining three receiving N, P and N & P additions in August 2001, August 2002 and March 2005. N was added as ammonium nitrate and P as triple super phosphate in amounts corresponding to 20 and 10 g m<sup>-2</sup> y<sup>-1</sup>, respectively. In March 2003, one individual of *Typha domingensis* was planted in each plot. While the *Typha* plant did not survive in the majority of controls and N addition plots, it grew and spread vigorously in the P enriched plots, and out-competed *Eleocharis*. In January of 2005 to current, the P addition plots in six of the 15 marshes have been manipulated and maintained to be half dominated by *Eleocharis* and half dominated by *Typha* (Fig. 2). In March 2007, four of these six marshes, two from the low and two from the high salinity categories, were sampled to dually measure the impact of P addition and macrophyte dominance on the microbial activities and community composition. For the general characteristics of these marshes see Table 1.

### Soil sampling

During the time of sampling, all plots were flooded and the water depth ranged from 25 cm to 70 cm. At each marsh, samples were collected from (1) control plots with sparse growth of *Eleocharis* (low-phosphorus *Eleocharis*, LP/E), (2) P-addition plots dominated by *Eleocharis cellulosa* (high-phosphorus *Eleocharis*, HP/E) and (3) P-addition plots dominated with *Typha domingensis* (high-phosphorus *Typha*, HP/T); for experimental design and sampling strategy see Fig. 2. Recently deposited, readily distinguishable plant detritus on the soil surface was gently removed before sampling. Eight randomly located sediment samples were collected with a 5.5 cm diameter sharp



**Fig. 1** Conceptual diagram of microbial processes in (a) P limited and (b) P loaded wetlands. P enhances the role of sediment microorganisms (indicated by extended arrows) in nutrient transformation processes due to an increase of organic

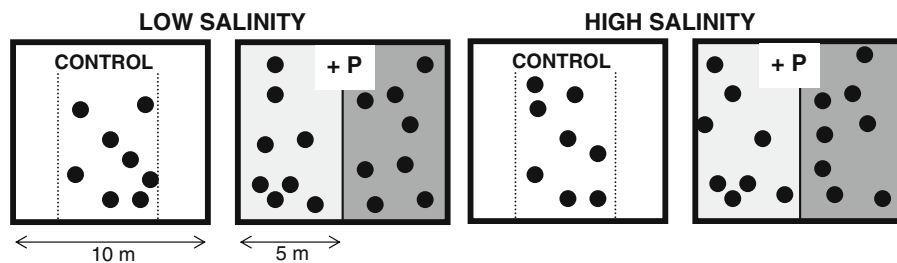
C input through plant litter and root exudates and by the elimination of cyanobacterial mats. Extracel PA = extracellular phosphatase activity

edge PVC corer to a depth of approximately 30 cm. The upper section (1–10 cm) representing mostly the rhizosphere, was used for the analyses. Samples were placed in ziplock bags and transported on ice. Large plant debris, roots and shells were removed and samples were homogenized. Nitrogenase activity and N-NH<sub>4</sub> and N-NO<sub>3</sub> contents in sediment were measured immediately. Samples for the remaining analyses were divided into two parts. One part was stored in sealed bags for a maximum of 1 month at 4°C and the other was oven dried at 105°C to a constant weight for determination of the gravimetric water content and elemental composition (C, N, reactive P) of the soil. All the subsequent analyses were conducted in triplicate.

#### Plant biomass and tissue analyses

For *Eleocharis*, plant height was measured and shoots were counted in ten randomly selected 20×20 cm

subplots in each plot. Ten mature stems from each plot were collected and their length measured before drying (80°C) and weighing to calculate the specific stem weight (g cm<sup>-1</sup>). The biomass was expressed as a product of the average number of stems × average height × specific stem weight. The same dried plant material was ground on a Wiley mill and used for nutrient analysis. To assess the nutrient content of litter, representative samples of recently senescent leaf/stem tissue were collected, dried, ground and analyzed. For *Typha*, the number of leaves and average leaf length were measured on 8–10 randomly selected individuals in each plot and the number of individuals was counted in 8–10 random square m. The biomass was expressed using the correlation between total leaf length and dry mass. The annual primary production was expressed as biomass multiplied by the turnover rate (2.6 and 3 for *Eleocharis* and *Typha*, respectively; calculated from plant longevity, see Rejmánková et al. 2008). The annual



**Fig. 2** Schematics of the experimental design and sampling strategy. Eight soil cores were randomly collected (black dots) from control plots (white) and P-enriched plots dominated by

*Eleocharis* (light gray) and *Typha* (dark gray). To avoid the discrepancy in plot size; only 5×10 m area (indicated by dotted line) was sampled in controls

**Table 1** Characteristics of the selected marshes from August 2001, before the beginning of the nutrient addition experiment. Values represent means of four 10×10 m plots in each marsh for water conductivity, and soil total P and N. Standard deviations are given within the parentheses

Marsh #	Area ha	Conductivity mS cm <sup>-1</sup>	Sediment type	Soil P mg cm <sup>-3</sup>	Soil N mg cm <sup>-3</sup>
Low salinity					
F10	4.7	0.231 (0.068)	Peaty clay	0.09 (0.01)	3.88 (0.68)
F12	11.3	0.658 (0.164)	Marly clay	0.09 (0.01)	3.31 (1.01)
High salinity					
F6	63.4	6.671 (1.479)	Marl	0.10 (0.03)	2.16 (0.50)
F7	18.2	5.667 (1.328)	Marl	0.10 (0.01)	1.54 (0.03)

primary production and litter nutrient content from 2006 were used to characterize the impact of macrophytes on the sediment microorganisms.

Root primary production was assessed in two marshes (high and low salinity) using mesh in-growth cores (Steingrobe et al. 2000). Each in-growth core (4.3 cm diameter, 20 cm length) was constructed from 2×3 mm flexible mesh screening and filled with root-free soil taken from each plot to match the mean bulk density of plot soils ( $N=3$ ). In each plot, three in-growth cores were inserted into cored holes and were left to incubate for 3 months. After this time, the cores were carefully removed and rinsed with marsh water. Roots protruding from the exterior were cut and the contents of each mesh core were sequentially washed through a 1 mm and 0.5 mm sieve. Living and dead roots were then separated from each sample using a two-phase decanting technique in Ludox TM-50, originally developed for mangrove roots (Robertson and Dixon 1993). As with the aboveground plant material, the separated roots were dried, weighed and ground. Due to the relatively small size of each sample, the ground roots were pooled by plots and then analyzed for nutrient content. From the remaining two marshes, roots were also non-quantitatively collected and analyzed for nutrient content. To gain annual root production rates, this process was repeated every three months throughout 2007.

#### Chemical analyses

Soil and plant total organic carbon (TOC) and total nitrogen (TN) were analyzed on a Carlo-Erba series 5,000 CHN-S analyzer. Soil samples were pretreated with 0.1 M HCl to remove carbonates. Available C was measured as organic C in 0.5 M K<sub>2</sub>SO<sub>4</sub> extract (Ettema et al. 1999) using an organic C analyzer

(Shimadzu total organic carbon analyzer TOC-5050A). Total phosphorus (soil P) was analyzed using ascorbic acid reduction of phosphomolybdate complex after acid digestion (McNamara and Hill 2000). Oxalate extractable sediment P ( $P_{ox}$ ) was analyzed using ascorbic acid reduction of phosphomolybdenate complex in oxalate extracts (Owens et al. 1977). The N-NH<sub>4</sub> and N-NO<sub>3</sub> concentrations in 0.5 M KCl extract were analyzed by flow injection analyzer (FIAstar 5012, Foss Tecator, Sweden).

#### Nutrients in microbial biomass

We used two fumigation extraction procedures to determine microbial biomass carbon (MBC), nitrogen (MBN) and phosphorus (MBP) within 10-g of the refrigerated soil subsamples. MBC and MBN were calculated after subsequent 0.5 M K<sub>2</sub>SO<sub>4</sub> extractions (Vance et al. 1987) as the difference in K<sub>2</sub>SO<sub>4</sub>-extractable C and N between the fumigated and unfumigated samples (Shimadzu total organic carbon analyzer TOC-5050A) and corrected by extraction efficiency factors of  $k_{EC}=0.37$  and  $k_{EN}=0.54$ , respectively. The MBP was calculated after subsequent NaHCO<sub>3</sub> extraction and quantification of P by the molybdophosphate complex method (Olsen and Sommers 1982). All biomass values were expressed per gram soil dry weight.

#### Nutritional status of microbial community

Phospholipid fatty acids (PLFA) were analyzed in 2 g of the refrigerated soil according to Frostegård et al. (1993). Phospholipids were separated, quantified and identified by gas chromatography (HP 6890 with flame ionization detector, FID). PLFA were identified by comparing the retention times of the sample with FAME standards (Supelco, Larodan Fine Chemicals



AB). The mol% of terminally iso- and anteiso-branched fatty acids was combined into terminally branched fatty acids group (TBFA; Haack et al. 1994) indicating the presence of amino acids in the environment as primers for TBFA formation (Kaneda 1991). The ratio of monounsaturated to saturated FAs (MUFA/STFA) was examined as it can change due to membrane changes in response to environmental stresses; where an increasing ratio signifies an increase in carbon (energy) availability (Kieft et al. 1997). Since the quantity of microbial PLFA is a function of microbial biomass (Zelles et al. 1992), we used the total amount of all bacterial PLFA identified in the samples as an index of live microbial biomass (PLFA<sub>tot</sub>). The specific respiration rate (CO<sub>2</sub> per unit microbial biomass; Q<sub>CO<sub>2</sub></sub>) was also calculated. Higher specific respiration rates indicate increasingly stressful environmental conditions to the microbial community (Anderson and Domsch 1990).

#### Microbial nutrient transformation

##### *Nitrogen mineralization*

Three week incubations of 10 g fresh soil subsamples in 100 mL flasks covered with perforated parafilm at 25°C in the dark were used for measuring net N mineralization rate (Ste-Marie and Paré 1999; Šantrůčková et al. 2001). Net mineralization rate was calculated as a difference between final (21 days) and beginning (7 days) concentrations of N–NH<sub>4</sub>, divided by the number of days.

##### *Carbon mineralization*

Soil subsamples (30 g) were slowly stirred with 20 mL of physiological salt solution (0.9% NaCl) in tightly closed 100 mL bottles incubated at 25°C in the dark. Incubations were conducted under both aerobic and anaerobic conditions. Aerobic respiration (aerobic CO<sub>2</sub>) was measured under an atmosphere of 21% O<sub>2</sub> after 24 h. Anaerobic conditions were achieved by bubbling the water phase of the samples with helium gas for 10 min. Samples for anaerobic respiration (anaerobic CO<sub>2</sub>) and CH<sub>4</sub> production (methanogenesis) were incubated for 14 days and gas production was measured using HP 5,890 gas chromatograph (Agilent, USA), with a thermal conductivity detector for CO<sub>2</sub>, and FID for CH<sub>4</sub>.

##### *Denitrification enzyme activity (DEA)*

Denitrification enzyme activity was measured using an acetylene-inhibition technique (Balderston et al. 1976). Ten grams of fresh weight soil were incubated in 100 mL flasks with 15 ml of nutrient solution containing excess nitrate (0.5 g l<sup>-1</sup> KNO<sub>3</sub>, 1 g l<sup>-1</sup> glucose). At the start of experiment, 10% of the gas phase of the samples was replaced with acetylene. The production of nitrous oxide was taken (0.2 ml) and measured from the headspace at time zero and after 30 and 60 min using a HP 6,890 gas chromatograph equipped with an electron capture detector.

##### *Nitrogenase activity (NA)*

To measure potential nitrogenase activity, we used a modification of the acetylene reduction assay method (Hardy et al. 1968). Glucose (0.184–0.696 mg C g<sup>-1</sup>) was mixed into the 50 g of fresh soil and sealed in 100 mL glass bottles, which were equilibrated to atmospheric pressure. From each bottle, 30 ml of the headspace was removed and subsequently replaced with 20 ml of N<sub>2</sub> gas to lower the partial pressure of oxygen. Ten mL of acetylene, freshly prepared from CaC<sub>2</sub>, were added to each bottle, which were then vigorously shaken for 1 min, and incubated under dark conditions at 28°C. Bottles were shaken again after 12 h and at the end of the incubation. After 24 h, approximately 15 ml of headspace were withdrawn and analyzed using a Shimadzu 14 gas chromatograph, equipped with FID. Controls run with samples without acetylene as well as blanks showed no endogenous ethylene production. Rates of acetylene reduction were expressed as nmol of acetylene reduced per gram dry mass of sample per day of incubation.

#### Data analysis

Two-way ANOVAs, with Neumann-Keuls Post Hoc tests, were used (STATISTICA version 7.0) to evaluate: (1) the effect of P addition and salinity comparing LP/E and HP/E plots at the high and low salinity sites; and (2) the effect of plant species and salinity comparing HP/E and HP/T at the high and low salinity sites on sediment characteristics and microbial communities and processes. We could not use a 3-way ANOVA due to the unbalanced design (there is no

combination of low P and *Typha*). Root production data were Box-Cox transformed to meet the assumptions of normality and homoscedasticity. Root tissue nutrients could not be statistically tested, as the samples were pooled for each plot, but data were correlated to shoot tissue.

## Results

### P addition and salinity effect

P addition significantly increased aboveground biomass production (NPPshoot; Table 2a&b). It also significantly increased P content and slightly increased N content of live tissue (data not shown, but see Rejmánková et al. 2008), and because live and dead tissue nutrient content is closely correlated, the same increase was observed in the senescent tissue (litter) (Table 2a&b). The increase of shoot P content was much higher than that of N leading to highly significant changes in shoot nutrient ratios, especially C/P and N/P, which decreased up to more than five-fold (Table 2a). There was no, or marginal effect of salinity on litter nutrients with the exception of C/P ratio which was lower at high salinity marshes (Table 2a&b). Neither P addition, nor salinity had a significant effect on root production (Table 2b). However, the ratios of roots to shoots dramatically decreased in response to P (Table 2a&b). Senescent root nutrient content, specifically P and C/P were closely correlated with senescent shoot nutrient contents ( $R^2=0.877$ ;  $P=0.02$ ;  $N=6$ , and  $R^2=0.848$ ;  $P=0.09$ ;  $N=6$  for tissue P and C/P, respectively; data not shown).

From measured chemical parameters of the sediment, P addition positively affected mainly  $P_{ox}$ , whose concentration increased by about an order of magnitude over LP, and also TOC and TN contents (Table 3a&b). C avail increased with P addition, but only in the low salinity plots (Table 3a&b). The nitrate content was significantly lower in the high salinity than in the low plots (Table 3a&b). The nitrate content increased in the P-added plots only for the high salinity sites while it decreased in the low salinity sites (Table 3a). Nutrient and C contents were, in general, lower at high salinity (Table 3a). Ammonium content was not significantly affected by P or salinity (Table 3a, b).

Microbes responded to the altered nutrient availability in the sediment of the P-added plots with a significant increase of MBC, MBN, MBP, while microbial biomass, indicated by  $PLFA_{tot}$ , did not significantly change (Table 4a&b). The increase in MBC and MBP was salinity dependent with significantly higher values at the low salinity sites (Table 4a&b). Similar to plant C/P ratios, microbial MBC/MBP significantly decreased at the HP plots, and the ratio was lower at high salinity (Table 4a&b). Similar to plant C/N, MBC/MBN was not affected by P addition (Table 4a&b). MBC/MBN was significantly lower, however, in high salinity marshes. Both MBN and MBC were lower at high salinities, while  $PLFA_{tot}$  was higher at high salinity (Table 4a&b).

The effect of P addition on microbial community nutritional status was indicated by an increase of MUFA/STFA ratio and by a decrease of  $PLFA_{tot}/MBC$  (Table 5a&b). While the response of  $PLFA_{tot}/MBC$  to P was salinity dependent, the response of MUFA/STFA and  $Q_{CO_2}$  were not. Salinity per se negatively affected MBC/TOC and enhanced all other parameters related to the nutritional status of microbial community ( $PLFA_{tot}/MBC$ ,  $Q_{CO_2}$ , and TBFA).

Nitrogen mineralization, denitrification enzyme activity, nitrogenase activity and methanogenesis were enhanced by P addition, while aerobic and anaerobic respiration were not significantly affected (Table 6a&b). Salinity decreased N mineralization, denitrification enzyme activity and methanogenesis and weakened the positive effect of P addition on these processes (Table 6a&b).

### Plant species and salinity effects

*Eleocharis* and *Typha* had similar shoot and root production (NPPshoot, NPProot, respectively) at the high salinity sites, but *Eleocharis* had a higher production at the low salinity sites (Table 2a&c). Shoot litter of *Typha*, as compared to *Eleocharis*, had slightly but significantly higher C content and similar P content. Shoot litter N content was slightly greater at high salinity sites, but there were no significant differences in C and P of litter. Both species had similar root N content that was comparable with shoot N, but *Typha* had always almost two times higher root P content than *Eleocharis* (data not shown). *Eleocharis* also had higher ratios of root to shoot biomass compared to *Typha* (Table 2a).



**Table 2** Aboveground primary production ( $NPP_{shoot}$ , data from 2006), root production ( $NPP_{root}$ ) and litter carbon (C), nitrogen (N) and phosphorus (P) contents, for low and high salinity marshes and three different treatments: P unenriched sites dominated by *Eleocharis cellulosa* (LP/E) and P enriched sites dominated either by *E. cellulosa* (HP/E) or *Typha domingensis* (HP/T). NPP values are averages of  $N=4$  (two marshes and two

sampling dates, February and August 2006) except for root production, which was measured only in two marshes, from high and low salinity each. Nutrient data are replicates from two marshes. Section 2a refers to treatment means; section 2b refers to  $p$ -values of P addition and salinity effect; section 2c refers to  $p$ -values of plant species and salinity effect; “ns” means  $P>0.05$  (ANOVA)

	Salinity	$NPP_{root}$ g m <sup>-2</sup> y <sup>-1</sup>	$NPP_{shoot}$ g m <sup>-2</sup> y <sup>-1</sup>	Root/Shoot	Litter nutrients (% dry mass)					
					C	N	P	C/N	C/P	N/P
2a Treatment means										
LP/E	Low	260	120.1	2.8	43.3	0.46	0.007	95.2	6,404.5	68.1
HP/E	Low	516	481.3	0.7	44.4	0.56	0.033	86.6	1,865.5	19.9
HP/T	Low	148	217.9	0.5	47.4	0.47	0.031	103.1	1,554.8	15
LP/E	High	251	88.4	2.2	43.7	0.55	0.012	80.6	3,644.9	46.7
HP/E	High	167	237.5	1.1	43.3	1.05	0.053	42.3	819.1	20.2
HP/T	High	176	377.8	0.7	46.1	0.59	0.05	75.5	950.2	13.7
2b $p$ -values—effect of P addition and salinity										
P effect		ns	0.006		ns	ns	0.018	ns	0.004	0.015
Salinity effect		ns	0.043		ns	ns	ns	ns	0.04	ns
P Salinity		ns	ns		ns	ns	ns	ns	ns	ns
2c $p$ -values—effect of plant species and salinity										
Plant effect		ns	ns		0.006	ns	ns	ns	ns	ns
Salinity effect		ns	ns		ns	ns	ns	ns	ns	ns
Plant × salinity		ns	ns		ns	ns	ns	ns	ns	ns

The effect of plant species on chemical and microbial characteristics of the sediment was generally lower than the effect of P addition, which was mainly evident at the low salinity sites (Tables 3b&c, 4b&c, and 5b&c). As compared to *Eleocharis* plots, *Typha* plots were significantly higher in  $P_{ox}$  content at both salinity levels and in TOC at only low salinity (Table 3a&c). Other sediment nutrients were unaffected by plant species (Table 3c). The contents of TOC, TN and  $P_{ox}$  were generally lower at high salinity sites (Table 3a).

In *Typha* plots, MBN and microbial biomass, indicated by  $PLFA_{tot}$ , were higher, but MBC/MBN ratio was lower than in *Eleocharis* plots (Table 4a). Conversely, phosphorus immobilization in microbial cells, indicated by higher MBP and lower MBC/MBP, was enhanced in *Eleocharis* plots. All the above differences were more distinctive at the low salinity sites (Table 4a). High salinity weakened plant species effect in all cases but  $PLFA_{tot}$ .

Microbial community nutritional status was not affected by plant species (Table 5c). It was negatively

influenced by high salinity, as indicated by lower MBC/TOC ratio and higher  $PLFA_{tot}/MBC$ ,  $Q_{CO2}$  and proportion of TBFA groups (Table 5a&c).

Impact of plant species on microbial processes was not consistent and was largely insignificant (Table 6c). The exception was NA and SRB; NA was significantly higher while the mol% of the SRB biomarker was lower in the soil from the *Eleocharis* plots (Table 6a). *Typha* supported denitrification enzyme activity and respiration (both aerobic and anaerobic), and reduced methanogenesis at the low salinity sites, while its influence was opposite at the high salinity sites (Table 6a&c). High salinity mostly reduced the rate of the microbial processes (Table 6a).

## Discussion

### P addition effect

In the studied marshes, P addition significantly increased aboveground production and ratio of shoot

**Table 3** Chemical parameters in the sediment (1–10 cm) for low and high salinity marshes and three different treatments: P unenriched sites dominated by *Eleocharis cellulosa* (LP/E) and P enriched sites dominated either by *E. cellulosa* (HP/E) or *Typha domingensis* (HP/T). Values are averages of  $N=6$  (three replicates from two marshes). Section 3a refers to treatment means; section 3b refers to  $p$ -values of P addition and salinity effect; section 3c refers to  $p$ -values of plant species and salinity effect; “ns” means  $P>0.05$  (ANOVA)

	Salinity	TOC	TN	C avail	$P_{ox}$	$NH_4-N$	$NO_3-N$
3a Treatment means							
LP/E	Low	110.20	9.16	43.0	0.76	2.75	1.88
HP/E	Low	195.0	11.51	238.6	7.30	3.25	0.70
HP/T	Low	219.9	12.98	250.3	16.11	1.50	0.45
LP/E	High	145.50	5.08	185.3	0.93	2.47	0.03
HP/E	High	165.4	6.87	172.2	6.17	2.84	0.20
HP/T	High	163.8	7.26	111.0	9.03	2.06	0.16
3b $p$ -values—effect of P addition and salinity							
Phosphorus effect		0.0001	0.016	0.0001	0.0001	ns	0.027
Salinity effect		ns	0.0001	ns	ns	ns	0.0001
Phosphorus $\times$ Salinity		0.0001	ns	0.0001	0.029	ns	0.006
3c $p$ -values—effect of plant species and salinity							
Plant effect		0.032	ns	ns	0.0001	ns	ns
Salinity effect		0.0001	0.0001	0.0001	0.0001	ns	ns
Plant $\times$ salinity		0.017	ns	ns	0.0001	ns	ns

TOC, TN total organic carbon and total nitrogen in  $mg\ g^{-1}$ ; C avail available carbon,  $P_{ox}$  oxalate extractable phosphorus,  $NH_4-N$ ,  $NO_3-N$  ammonium and nitrate nitrogen, all in  $\mu g\ g^{-1}$

**Table 4** Microbial biomass and microbial biomass nutrients in low and high salinity marshes and three different treatments: P unenriched sites dominated by *Eleocharis cellulosa* (LP/E) and P enriched sites dominated either by *E. cellulosa* (HP/E) or *Typha domingensis* (HP/T). Values are averages of  $N=6$  (three replicates from two marshes). Section 4a refers to treatment means; section 4b refers to  $p$ -values of P addition and salinity effect; section 4c refers to  $p$ -values of plant species and salinity effect; “ns” means  $P>0.05$  (ANOVA)

	Salinity	MBC	MBN	MBP	MBC/MBN	MBC/MBP	PLFA <sub>tot</sub>
4a Treatment means							
LP/E	Low	2,601	81.8	2.71	31.8	959.8	94.4
HP/E	Low	4,583	146	32.60	31.4	140.6	131.2
HP/T	Low	5,390	240	7.70	22.5	700.0	224.3
LP/E	High	657.0	56.0	2.39	11.7	274.9	218.5
HP/E	High	1,117	121	18.80	9.2	59.4	212.0
HP/T	High	1,202	153	4.65	7.9	258.5	221.7
4b $p$ -values—effect of P addition and salinity							
Phosphorus effect		0.0001	0.0001	0.0001	ns	0.0001	ns
Salinity effect		ns	0.012	0.027	0.0001	0.0001	0.0001
Phosphorus $\times$ Salinity		0.0001	ns	0.034	ns	0.0001	ns
4c $p$ -values—effect of plant species and salinity							
Plant effect		ns	0.0003	0.0001	0.028	0.0001	0.0083
Salinity effect		0.0001	0.0001	0.0107	0.0012	0.0001	0.037
Plant $\times$ Salinity		ns	0.0413	0.087	ns	0.0029	0.0275

MBC, MBN, and MBP microbial carbon, nitrogen, and phosphorus, respectively, in  $\mu g\ g^{-1}$ ; PLFA<sub>tot</sub> total amount of phospholipids fatty acids in  $nmol_{PLFA}\ g^{-1}$

**Table 5** Parameters related to the status of microbial community for low and high salinity marshes and three different treatments: P unenriched sites dominated by *Eleocharis cellulosa* (LP/E) and P enriched sites dominated either by *E. cellulosa* (HP/E) or *Typha domingensis* (HP/T). Values are averages of  $N=6$  (three replicates from two marshes). Section 5a refers to treatment means; section 5b refers to  $p$ -values of P addition and salinity effect; section 5c refers to  $p$ -values of plant species and salinity effect; “ns” means  $P>0.05$  (ANOVA)

	Salinity	MBC/TC	PLFA <sub>tot</sub> /MBC	$Q_{CO_2}$	MUFA/STFA	TBFA
5A Treatment means						
LP/E	Low	0.023	0.036	0.060	0.605	16.01
HP/E	Low	0.023	0.029	0.037	0.759	17.26
HP/T	Low	0.024	0.042	0.059	0.866	17.13
LP/E	High	0.004	0.333	0.231	0.657	22.80
HP/E	High	0.006	0.189	0.211	0.847	21.61
HP/T	High	0.007	0.184	0.112	0.802	22.30
5b $p$ -values—effect of P addition and salinity						
Phosphorus effect		ns	0.0001	ns	0.0002	ns
Salinity effect		0.0001	0.0001	0.0001	0.033	0.001
Phosphorus $\times$ salinity		ns	0.0001	ns	ns	ns
5c $p$ -values—effect of plant species and salinity						
Plant effect		ns	ns	ns	ns	ns
Salinity effect		0.0001	0.0001	0.0001	ns	0.0014
Plant $\times$ salinity		ns	ns	0.0049	ns	ns

MBC microbial carbon; TOC total sediment carbon; PLFA<sub>tot</sub>/MBC active/total biomass in  $\text{nmol}_{\text{PLFA}} \mu\text{g}^{-1} \text{C}$ ;  $Q_{CO_2}$  specific respiration rate in  $\text{d}^{-1}$ ; MUFA/STFA ratio of Monounsaturated and Saturated fatty acids; TBFA terminally branched fatty acids in mol%

**Table 6** Parameters related to nutrient mineralization and transformation processes for low and high salinity marshes and three different treatments: P unenriched sites dominated by *Eleocharis cellulosa* (LP/E) and P enriched sites dominated either by *E. cellulosa* (HP/E) or *Typha domingensis* (HP/T). Values are averages of  $N=6$  (three replicates from two marshes). Section 6a refers to treatment means; section 6b refers to  $p$ -values of P addition and salinity effect; section 6c refers to  $p$ -values of plant species and salinity effect; “ns” means  $P>0.05$  (ANOVA)

	Salinity	Nmin	DEA	NA	AnaerobCO <sub>2</sub>	AerobCO <sub>2</sub>	CH <sub>4</sub>	SRB
6a Treatment means								
LP/E	Low	-441.3	36.70	1.12	12.10	193.90	0.055	10.5
HP/E	Low	75.60	68.70	1,158.0	15.90	160.60	2.70	8.13
HP/T	Low	76.40	87.30	154.30	20.10	281.90	1.84	13.1
LP/E	High	-225.3	25.20	1.18	14.40	150.30	0.034	9.39
HP/E	High	61.90	39.20	816.0	15.70	230.10	0.073	9.78
HP/T	High	73.70	23.80	73.0	12.40	132.90	0.195	10.7
6b $p$ -values—effect of P addition and salinity								
Phosphorus effect		0.0001	0.0001	0.0001	ns	ns	0.0002	ns
Salinity effect		0.0001	0.0005	ns	ns	ns	0.0002	ns
Phosphorus $\times$ salinity		0.003	0.012	ns	ns	ns	0.0002	ns
6c $p$ -values—effect of plant species and salinity								
Plant effect		ns	ns	0.0001	ns	ns	ns	0.034
Salinity effect		ns	0.0001	0.0001	0.043	ns	0.0001	ns
Plant $\times$ salinity		ns	0.046	ns	ns	0.0005	ns	ns

Nmin nitrogen mineralization rate,  $\text{ng N-NH}_4 \text{g}^{-1} \text{d}^{-1}$ ; DEA denitrification enzyme activity,  $\text{ng N}_2\text{O g}^{-1} \text{d}^{-1}$ ; NA nitrogenase activity,  $\text{nmol C}_2\text{H}_4 \text{g}^{-1} \text{d}^{-1}$ ; AnaerobCO<sub>2</sub> anaerobic respiration rate and AerobCO<sub>2</sub> aerobic respiration rate in  $\mu\text{g CO}_2 \text{g}^{-1} \text{d}^{-1}$ ; CH<sub>4</sub> methanogenesis,  $\mu\text{g CH}_4 \text{g}^{-1} \text{d}^{-1}$ ; SRB sulfate reducing bacteria, mol%

to root; the increased shading led to the elimination of cyanobacteria (see also Rejmánková et al. 2008). This switch from microphyte to macrophyte dominated autotrophic production gradually led to an increase of total C and N contents and availability in soil, and microbial nutrient contents. The same trend was also observed in similar wetlands such as the Florida Everglades (Davis 1991; DeBusk and Reddy 1998).

The addition of readily hydrolysable nutrients and the increase of nutrient availability has been reported to augment microbial growth (Anderson and Domsch 1985), increase organic matter turnover (Reddy et al. 1999), and accelerate the mineralization and release of nutrients back to the environment. Plants are an essential source of the organic carbon in soil. Any increase in plant production increases the C supply for microorganisms thus enhancing the development and activity of the microbial community. The rate at which this occurs further depends on plant material quality and nutrient availability in soil. Accordingly, we found that where plant production increased, there was an increase in microbial biomass. The level of microbial biomass C and N (MBC and MBN) was similar to what has been reported from the Everglades (Wright and Reddy 2001; Corstanje et al. 2007). In accordance with our primary hypothesis, microbial biomass C, N and P and microbial activities were positively affected by P addition supporting the assumption that P is the limiting nutrient to the microbial biomass in unimpacted Belize marsh sediments. P limitation is further confirmed by decrease of C/P ratio in microbial biomass (MBC/MBP) in P enriched soils. Although P immobilization into microbial biomass increased after P addition, MBP was still an order of magnitude lower than what has been found in the Everglades (Corstanje et al. 2007). This discrepancy perhaps can be linked to the amount of time P has been augmented within the systems. While our experimental plots have been enriched for only several years, the Everglades has been enriched by P for several decades.

In the studied P enriched plots, N mineralization, nitrogenase activity and denitrification enzyme activity were accelerated, which confirms our secondary hypothesis. These processes were most likely accelerated by plants, which contributed higher quantity and better quality biomass in P enriched plots. The enhancement of heterotrophic microbial activity by P additions or in high P soils has been well documented

(Bridgham and Richardson 1992; DeBusk and Reddy 1998; Bastviken et al. 2005). The increased input of easily decomposable plant material and rhizodeposition (Kozub and Liehr 1999) and resulting lower oxygen concentrations (Nielsen 1990) may have been the main drivers for the recorded changes in N cycle processes.

We also detected an increase in methanogenesis within the P enriched plots. Methanogenesis and most likely sulfate reduction are the major anaerobic processes governing carbon cycling in wetland ecosystems. Traditionally in highly reduced freshwater wetlands with low sulfate input, methanogenesis is considered the dominant carbon mineralization process, while in marine and salt marshes with a higher sulfate input, sulfate reduction is the dominant process (Ward and Winfrey 1985). Sulfate reduction was not measured in our study, but its occurrence was suggested by the presence of sulfate reducing bacteria (SRB, 10Me16:0 biomarker, Table 6a). The wetlands in Belize generally have high sulfate due to a significant proportion of gypsum in the underlying rock; high rates of H<sub>2</sub>S production have been documented at these sites (Rejmánková and Post 1996). In contrast to results of Drake et al. (1996) sulfate reduction potential (the distribution of SRB) was not enhanced by P addition.

The anaerobic respiration (anaerobic CO<sub>2</sub> production rate) in our study was about 10% of the aerobic respiration rate (Table 6a). DeBusk and Reddy (1998) found anaerobic CO<sub>2</sub> production rates to be 32% of the aerobic rates in the Everglades soils, while Benner et al. (1984) found values of 37%. Others have reported values of 34–63% (Bridgham and Richardson 1992) and 64% (Wright and Reddy 2001). The lower values of anaerobic respiration we found are likely due to the lower organic matter content (supply of electron donors) of Belizean soils and most likely also by lower C availability as compared to more peaty sediments studied elsewhere.

A great deal of energy is needed to support microbial growth and activities. The biochemical transformation of saturated fatty acids to monounsaturated fatty acids requires available energy and oxygen (Fulco and Bloch 1964), which justifies using the ratio of soil monounsaturated/saturated fatty acids (MUFA/STFA) to identify increasing substrate availability in the system. A relationship between MUFAs and high substrate availability was also identified by

Zelles et al. (1992) in agriculture soil, and Bossio and Scow (1998) found MUFA to increase with added carbon in wetland conditions. We found this ratio to increase in P enriched sites.

#### Plant species effect

Based on previous results (Rejmánková 2005; Rejmánková et al. 2008) that showed different biomass production, nutrient uptake and resorption from senescing tissues of *Typha* and *Eleocharis*, we expected different quality and quantity of their litter to affect microbial biomass and activity. This assumption has not been validated by this study. The differences in biomass production between the two species were not significant; largely because the *Typha* plots were not fully established (the biomass production of a well established *Typha* stand in the Everglades is around 2,500 g/m<sup>2</sup>/y; Weisner and Miao 2004). In terms of nutrients in senescent biomass, there were no differences between the species in N and P, but *Typha* litter contained consistently more C. The relatively weak plant species effect on microbial biomass and processes corresponded to small differences in plant litter stoichiometry.

Regardless of plant litter similarities, plant species significantly impacted the transformation of P, particularly SRP, MBP contents and MBC/MBP ratio in the sediment. This effect might be explained by the macrophytes different life strategies with respect to P use efficiency and allocation. *Typha* behaves as a competitor (sensu Grime 2001). Accordingly, compared to *Eleocharis*, *Typha*'s P use efficiency and P uptake was lower (more inorganic P left behind in sediment, see Table 3a). P resorption efficiency was also lower (unpublished data and Rejmánková 2005). Moreover, microbial P immobilization in *Typha* plots was lower, even though more reactive P was in the sediment. We also found that *Typha* released less extracellular phosphatases than *Eleocharis* (Rejmánková and Macek 2008). The higher P use and resorption efficiency of *Eleocharis* confirmed the status of this species as a stress tolerator.

The higher MBN in *Eleocharis* plots was most likely supported by the more N available due to an order of magnitude higher nitrogenase activity compared to *Typha* plots (Table 6a). The uptake of N by macrophytes was comparable (see Table 2a), therefore, it is likely that more available N within *Eleocharis*

plots was immobilized into microbial biomass. This further bolsters the role of *Eleocharis* as a stress tolerator, supporting microbial nutrient immobilization to keep microorganisms in fast turnover.

#### Salinity effect

The rate and direction of the changes in microbial activities and biomass were found, as hypothesized, to be salinity dependent. In low salinity marshes, we found a significant increase in microbial biomass and microbial processes within P added plots. This suggests that low salinity marshes are microbiologically more active, with a higher release of nutrients back to the environment (higher ratio of MBC/MBN, MBC/MBP). Lower activity at high salinity plots is significant for lower N mineralization, denitrification enzyme activity and methanogenesis. The other measured processes (nitrogenase activity, aerobic and anaerobic respiration) tended to be lower too, but the difference was not significant.

In the high salinity marshes, the microbial growth was limited, resulting in lower microbial biomass to total C ratio (Table 5a). This ratio is an indicator of C availability and the ability of microbes to utilize it; lower ratios imply lower C availability (Anderson and Domsch 1989). High salinity marshes also had distinctly higher PLFA<sub>tot</sub> to MBC ratios (Table 5). We suggest that high PLFA<sub>tot</sub> to MBC ratio indicates higher proportion of membrane phospholipids in total biomass due to lower bacterial size in high salinity soils. According to Zahran (1997), the morphology of the bacteria is usually modified with increasing salinity of the environment; cells usually shrink and change in cell and cytoplasm volume. Consequently, a close correlation between microbial biomass MBC and PLFA<sub>tot</sub> should not be expected (Calderon et al. 2001). Accordingly, we can speculate that even a lower amount of microbial cells of smaller size, with a lower C content, could have higher amount of phospholipids in the membranes (Table 5a).

The chemical composition of membranes may also be modified by salinity, as demonstrated by an increased portion of TBFA in samples from high salinity sites (Table 5a). To create TBFA, amino acid precursors are required (Kaneda 1991). At the same time, some amino acids are considered to be essential osmolytes for bacteria to adapt to saline environments (Imhoff 1986). From this, we speculate that micro-

organisms need more nitrogen to balance the salinity stress in high salinity marshes. This is supported by the elevated microbial storage of N. The higher recorded respiration per cell ( $Q_{CO_2}$ ) for the high salinity sites reflects, as discussed above, a higher cellular energy demand under salinity stress.

In conclusion, we found that nutrient in microbial biomass and C, N and P turnover rates in Belizian wetland sediments were impacted by P addition and the accompanied increase in macrophyte production. Microbial activities were affected differently based somewhat on plant species stoichiometry. Perhaps more important than litter stoichiometry are the distinctly different nutrient usage strategies employed by *Eleocharis* and *Typha* and the effect this has on microbial nutrient availability. As a result, *Eleocharis* supported a more balanced system with microorganisms kept in fast turnover, while microorganisms in *Typha* plots were more dependent on P addition. As hypothesized, microbial activities were salinity dependant. Salinity reduced the overall rates of microbial processes, weakened the positive effect of both P addition and plant species on microbial activities, and enhanced the amount of N stored in microbial cells.

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