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Ph.D. Thesis

Microbial diversity of tropical wetlands in relation to nutrient content

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Annotation:

Research was conducted to understand the role of heterotrophic microorganisms in carbon, nitrogen and phosphorous transformation processes in sediments of tropical wetlands (Belize, Central America) that are being endangered by external P loading. Special emphasis was given to nitrogen fixation ability and functional and structural diversity of microorganisms. The study was performed at selected marshes varied in water salinity, within each wetland having sites with different levels of eutrophication and plant cover due to experimental phosphorus loading.

I hereby declare that Ph.D. thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

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Barbora Pivničková

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List of papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Pivničková B, Elhottová D, Rejmánková E, Šantrůčková H. Microbial community composition and activity potential in tropical marsh sediment profile influenced by P enrichment. *Manuscript*

II Černá B, Rejmánková E, Snyder JM, Šantrůčková H (2008) Heterotrophic nitrogen fixation in oligotrophic tropical marshes: changes after phosphorus addition. Hydrobiologia 627:55-65

III Pivničková B, Rejmánková E, Snyder JM and Šantrůčková H (2010) Heterotrophic microbial activities and nutritional status of microbial communities in tropical marsh sediments of different salinities: the effects of phosphorus addition and plant species. Plant and Soil; accepted 13th May 2010, DOI 10.1007/s11104-010-0439-6

IV Šantrůčková H, Rejmánková E, Pivničková B, Snyder JM (2010) Nutrient enrichment in tropical wetlands: shifts from autotrophic to heterotrophic nitrogen fixation. Biogeochemistry, accepted 25th May 2010, DOI 10.1007/s10533-010-9479-5

Co-author agreement

We hereby declare that Barbora Pivničková had a major contribution to Paper I, II and III.

Hana Šantůčková	Eliška Rejmánková	Jenise Maria Snyder	Dana Elhottová

SOUHRN

Tato práce byla součástí dlouhodobého americko-českého výzkumného projektu. Cílem projektu bylo navrhnout vhodný management ochrany mokřadů (travinný typ mokřadů) v Belize, Střední Amerika, které jsou ohroženy přísunem fosforu (P, živina) ze zemědělských hnojiv. Mokřady, nejen v této oblasti, plní mnoho významných funkcí - jsou domovem mnoha druhů organismů a zvyšují biodiverzitu krajiny, dále mají funkci v toku energie, vody, látek, jsou uložištěm uhlíku atd.. Současné zintenzivňování zemědělské činnosti v této oblasti vede ke zvyšování přísunu živin, zejména P a dusíku (N), z použitých hnojiv do mokřadů a mokřadních půd (sedimentů). Zvýšená koncentrace těchto, jinak limitujících, živin má za následek nahrazení původního sinicového porostu porostem vegetace vyšších rostlin a to bahničky (*Eleocharis* spp.) a orobince (*Typha domingensis*). Změny vegetačního pokryvu jsou následovány změnami diverzity sedimentárních mikrobiálních společenstev, jejich funkcí a změnami v koloběžích prvků v ekosystému mokřadů. Na rozdíl od původních sinicových porostů nově vzniklý porost orobince slouží jako úkryt malarického komára rodu *Anopheles vestitipennis*. Znehodnocení původního charakteru belizských mokřadů vede k vyššímu výskytu malárie v této oblasti.

Pro sledování dlouhodobých změn vegetačního pokryvu po přídavku P byly již v roce 2001, v pěti (z patnácti) vybraných mokřadech, založeny pokusné plochy (10x10 m), do kterých byl třikrát přidán P (rok 2001, 2002, 2005; 10 g m^{-2} y^{-1}). Původní vegetace byla přídavkem P nahrazena hustým porostem bahničky (experimentální plocha 5x10 m). Na části ploch byla vysázena 1 rostlina orobince, která se rozrostla a v podmínkách s odstraněnou limitací fosforem vytvořila husté porosty (experimentální plocha 5x10 m).

Tato dizertační práce je zaměřena na studium struktury a funkcí mikrobiálního společenstva v sedimentech mokřadů subtropické oblasti, Belize. Mikrobiální společenstva mokřadních ekosystémů jsou ovlivněna mnoha faktory, jakými jsou množství a kvalita zdroje energie (C) a živin (N, P), a dále pak míra anaerobie prostředí, která je závislá na míře prokořenění. Externím přísunem živin se mění rovnováha mezi poutáním organické hmoty v sedimentu mokřadů a jejím obratem (přeměna živin), což vede k znehodnocení těchto ojedinělých ekosystémů.

N základě předchozích experimentálních studií v těchto mokřadech jsme předpokládaly, že funkční a strukturní diverzita mikrooganismů bude ovlivněna zvýšeným vstupem P do sedimentů, což následně zvýší růst makrofyt na úkor mikrofyt (sinic). Následně se zvýší přísun uhlíku z vegetačního opadu a z kořenů (rhizodepozice) do sedimentu, čímž se změní rychlosti procesů přeměn prvků prováděných především heterotrofními mikroorganizmy.

Hlavním cílem práce bylo (a) porovnat mikrobiální procesy (procesy přeměn C, N, P) a strukturu společenstev v sedimentech oligotrofních mokřadů s mokřady s přídavkem limitujícího prvku (P). Dalšími cíli pak bylo zjistit jak jsou mikrobiální společenstva a mikrobiální aktivity ovlivněny (b) přídavkem P v profilu sedimetu (minerální vs. organická vrstva); (c) typem vegetačního pokryvu (*Eleocharis* vs. *Typha*); (d) salinitou vody; a podrobněji byl sledován proces fixace dusíku do ekosystému (e) (autotrofní vs. heterotrofní fixace N).

Odběry vzorků byly provedeny dvakrát (rok 2005, 2007) a to z experimentálních ploch bez i s přídavkem P a na gradientu salinity mokřadů. Vzorky byly analyzovány v laboratořích v Orange Walk (Belize), na Přírodovědecké fakultě PřF JCU, České Budějovice (Česká republika) a na University of California, Davis (USA). Mikrobiální procesy přeměn prvků C, N a P a biomasa byly zjištěny použitím standardních laboratorních inkubačních technik. Mikrobiální společenstva byla popsána analýzou fosfolipidických mastných kyselin (PLFA) - extrakcí fosfolipidických mastných kyselin z cytoplazmatických membrán veškerého mikrobiálního společenstva sedimentů mokřadů a jejich následnou detekcí plynovou

chromatografií. Metoda PLFA umožňuje porovnat složení (fingerprint) a popsat strukturu (významné mikrobiální skupiny) mikroorganismů, a kvantifikovat jejich biomasu.

Výsledky šestiletého polního experimentu:

(a) Přídavek P do mokřadů zvýšil mikrobní biomasu sedimentu a především pak zabudování P do mikrobní biomasy. Dále urychlil mikrobiální aktivitu, zejména pak procesy mineralizace (C, N) a heterotrofní fixace N. Složení mikrobiálního společenstva nebylo přídavkem P významně ovlivněno; výjimkou byl pouze nárůst relativního množství mononenasycených mastných kyselin (MUFA) ve společenstvu, jež je spojováno s dobrou dostupností C substrátu.

(b) Přísun P významně zvýšil mikrobiální aktivity a biomasu v obou vrstvách sedimentu. V minerální vrtsvě (10-30 cm) byl nárůst mikrobiálních aktivit a biomasy vyšší ve srovnání s vrchní organickou vrstvou sedimentu (1-10 cm); výjimkou bylo navýšení zabudování P do mikrobiální biomasy, které bylo významnější v organickém horizontu. Struktura mikrobiálního společenstva se mezi vrstvami lišila a přídavek P ji významně neolivnil.

(c) Typ vegetace ovlivnil především zásobení mikroorganismů fosforem. Do mikrobiální biomasy ovlivněné porostem bahničky bylo zabudováno více P, přestože orobinec uvolňoval více P do sedimentu ve srovnání s bahničkou; v sedimentu ovlivněným porostem bahničky byla zjištěna vyšší heterotrofní fixace N. Vliv typu vegetace se na struktuře společenstva mikroorganismů významně neprojevil.

(d) Salinita mokřadních vod (konduktivita 7 mS) byla stresujícím faktorem působícím na mikroorganizmy, což se projevilo snížením efektivity využití C a snížením rychlosti procesů přeměn C, N, P. Zatímco mikrobiální biomasa byla salinitou snížena, množství N v biomase a zastoupení koncově větvených (iso-/anteiso-) mastných kyselin (TBFA) v cytoplasmatických membránách mikroorganismů rapidně vzrostlo.

(e) Přídavkem P se snížila autotrofní fixace sinicemi, čímž se N stal limitujícím prvkem v ekosystému. Následkem toho byla autotrofní fixace N nahrazena heterotrofní fixací N v sedimentu a to především síran redukujícími bakteriemi. Nejvyšší fixace N byla změřena v rhizosféře, ale vzhledem k tomu, že kořeny zaujímají jen malou část půdy, kořeny nepřispěly významně k celkové fixaci N v ekosystému.

Výsledky ukázaly, že zvýšené množství P (v mikrobiální biomase i v sedimentu) a dostupného uhlíku, dále přechod od autotrofní k heterotrofní fixaci N a nárůst mononenasycených mastných kyselin (ku nasyceným) v mikrobiálním společenstvu jsou dobrými indikátory významných negativních ekosystémových změn mokřadů v Belize způsobených nadměrným přísunem P.

Naše výsledky významně přispěly k porozumění fungování mokřadního ekosytému, který je ohrožen vnějším přísunem P z hnojiv. Výsledky byly zveřejněny ve třech publikacích a jednom manuskriptu (Paper I – IV)

Součástí práce bylo zavedení a otestování PLFA metody v laboratořích PřF JCU. Metoda (*Simple PLFA extraction Method)* se na pracovišti nadále využívá ke stanovení složení mikrobiálního společenstva a jejího množství.

SUMMARY

This work was a part of a long-term Czech-USA research project aimed to propose suitable management for protection of native wetlands (grass-like type, marsh) in Belize, Central America, endangered by phosphorus (P; nutrient) loading from agriculture fertilizers. Wetlands around the world are valued ecosystems for their rich species diversity and important ecological functions in the landscape, such as wildlife habitats, flows of energy, water and nutrients, and storage of carbon (C). Recent intensification of agriculture in Belize has lead to an increase in nutrients loading, mainly P and nitrogen (N), from fertilizers into the wetlands and underneath soils (sediments). Higher concentrations of these otherwise limiting nutrients result in substitution of native cyanobacterial cover with plant vegetation formed by *Eleocharis* spp. and *Typha domingensis*. These changes in the vegetation cover are subsequently followed with changes in diversity and functioning of belowground microbial communities and elemental nutrient cycling in the ecosystem. Unlike the native cyanobacterial mats, the new dense *Typha* vegetation may serve as a shelter for a malaria disease vector, the mosquito *Anopheles vestitipennis,* and thus the degradation of native Belize wetlands directly leads to higher occurrence of malaria disease in the region.

Experimental plots (10 x 10 m) have been designed in Belize wetlands since 2001. Five out of total 15 selected marshes were enriched with 10 g m^{-2} y^{-1} of P in 2001, 2002 and 2005 in order to monitor long-term changes in the vegetation cover after P addition. In the P-enriched plots, the native cyanobacterial mats were naturally superseded by dense *Eleocharis* spp. overgrowth. These P-rich plots were also highly sensitive to invasion from *Typha*, which after plantation of a single individual in the plot, widely spread and formed a dense vegetation cover.

This thesis is focused on the study of structure and functioning of microbial communities in sediments of subtropical wetlands located in Belize. Microbial assemblages in these marsh ecosystems are influenced by many factors, such as the quantity and quality of an energy source (C) and nutrients (N, P) and to a lesser degree by the extent of anaerobiosis (affected by density of roots). Under inadequate management of the wetlands, the balance between bounding of organic matter into the sediment and its turnover by transformation processes of nutrients could be altered, resulting in degradation of these unique ecosystems.

Based on previous experimental studies in the region, we hypothesized that the functional and structural diversity of microorganisms will be affected by the increased P input into the associated sediments, which will consequently increase the growth of macrophytes (*Eleocharis* spp., *Typha domingensis*) over microphytes (cyanobacteria) in the wetlands. Additionally, this should lead to higher influx of C from the vegetation litter-fall and from roots (rhizodeposition) into sediments and to changes in cycling of elemental nutrients.

The main objective of this work was to compare microbial activities (rates of C, N and P transformation processes) and structure of microbial communities in sediments of the native oligotrophic marshes with those experimentally enriched by phosphorus, P (a). Furthermore, we investigated how the microbial communities and activities are affected by: b) differences in soil vertical profile (mineral vs. organic layer); c) the different vegetation cover (*Eleocharis* vs. *Typha*); d) changes in wetland water salinity; and e) changes in nitrogen availability (autotrophic vs. heterotrophic N-fixation).

Samples of sediments underneath the marshes from native and P-enriched plots (both *Eleocharis* and *Typha* cover), located in gradient of salinity, were collected in 2005 and 2007. Samples were analyzed in laboratories in Orange Walk (Belize), University of South Bohemia, České Budějovice (Czech Republic) and University of California, Davis (USA). Standard laboratory incubation techniques were used for biomass quantification and analyses of C, N and P transformation rates. Microbial communities were described by analysis of phospholipids fatty acids (PLFA) – by the extraction of phospholipids fatty acids from cytoplasmatic membrane of all microorganisms in the sediment, and its subsequent

detection on gas chromatograph. This method enables comparison of the composition (fingerprint), to describe microbial community structure (notable microbial groups) and to quantify its biomass.

Results after 6 years long P enrichment field experiment:

(a) Addition of P into the wetland increased amount of available carbon, the overall microbial biomass in the sediment and incorporation of P into this biomass but did not significantly change the microbial community composition; the only exception was monounsaturated fatty acids, indicator of good C availability that significantly increased. Microbial activities, mainly mineralization (C, N) and N-fixation, increased.

(b) Microbial activities and biomass increased after P addition more significantly in the deeper mineral sediment layer (10-30 cm) compared to upper organic layer (1-10 cm). On the contrary the incorporation of P into the biomass was significantly higher in the organic layer. Microbial community structure originally differed between the two soil layers, which did not change after the P addition.

(c) In spite of the fact that *Typha* released more P into the sediments compared to *Eleocharis* we measured an order of magnitude higher P incorporation and higher heterotrophic N-fixation in the *Eleocharis* overgrowth compared to *Typha*. There were no significant differences in microbial community structure between these two vegetation cover types.

(d) Salinity of wetland water negatively affected microbial activity. Higher salinity decreased effectiveness of C usage and C, N, P transformation rates. While microbial biomass decreased with salinity, the amount of nitrogen in biomass rapidly increased with salinity, as did the amount of terminally branched fatty acids (TBFA's) in microbial community structure.

(e) Since the P addition into the experimental plots resulted in decline of N-fixing cyanobacteria, N became the limiting nutrient in the ecosystem. As a consequence, cyanoabacterial autotrophic N-fixation was substituted in the sediments by heterotrophic N-fixation, primarily by sulfur reducing bacteria (SRBs). Highest rates of N-fixation where measured in the rhizosphere but because the root areas cover only a small fraction of the soils, they didn't contribute significantly to the total ecosystem N-fixation.

Our data showed that increased amount of P (both in microbial biomass and sediment), amount of available C, switch from autotrophic to heterotrophic N-fixation and higher ratios of monounsaturated to saturated fatty acids in the microbial communities are good microbial indicators of significant negative ecosystem changes in Belize wetlands caused by overloading of excessive P.

Our results significantly contributed to the understanding of wetland ecosystem functioning endangered by external P loading from fertilizes. These results were published as three articles in peer-reviewed journals and as one manuscript (Paper I-IV).

As a part of this project we tested and standardized protocols to implement the PLFA method into the PřF JCU laboratories. This method has been now used at PřF JCU for the determination of microbial community composition and biomass.

CHAPTER 1

GENERAL INTRODUCTION

1 Wetlands

Wetlands belong to most biologically productive ecosystems on the earth (Tiner 1999). Productivity of wetlands is comparable to rainforests and coral reefs. Integral part of a wetland ecosystem is an immense variety of species of microorganisms, plants, insects, amphibians, reptiles, birds, fish and mammals. Basically all concepts of wetlands imply the existence of characteristic vegetation, which serves as a criterion for classifying a habitat as a wetland (EPA 1993). Functions of wetlands are the physical, chemical and biological processes that characterize wetlands ecosystems; the major functions are water storage and groundwater recharge, flood control, moderating climate and community structure, biodiversity and wildlife support. Wetlands are regarded as a complex habitat type, in their full range of development from oligotrophic to mesothropic, eutrophic and dystrophic, calcerous fens to acidic bogs, lowland swamps to marsh etc. While marsh is transition zone between water and land principally inhabited by partially submerged herbaceous vegetation, swamp is seasonally flooded bottomland with more woody plants than a marsh and better drainage than a bog.

Marshes of northern Belize in Central America (Fig. 1) were chosen as a unique opportunity to compare ecosystem processes in systems under similar climatic and hydrologic conditions, dominated by the same vegetation but differing substantially in their sediment and water chemistry. Yucatan Peninsula is an uplifted marine platform composed of a 2–3 km thick sequence of cretaceous and Tertiary limestone, dolomite, and gypsum (Weidie 1985). Hydrology of these marshes is closely linked to the ground water system and water levels are controlled primarily by regional precipitation patterns. Porous limestone aquifer allows intrusion of seawater inland and, as a result, mixing of fresh and marine waters can occur. The conductivity of the inland wetlands varies by an order of magnitude (0.2 mS to 7 mS) and chemical analyses of ion content revealed large differences in sulfate, bicarbonate and chloride; marshes with limestone marls bedrock having higher salinity while others underlain by alluvial sands, usually formed by peaty clays, are poor in calcium with regular freshwater salinity (Rejmánková and Post 1996). The climate of the Yucatan peninsula 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. Main primary producers in these systems are several species of emergent macrophytes (*Eleocharis cellulosa, E. interstincta, Cladium jamaicense and Typha domingensis*) and speciose communities of microphytes represented mostly by cyanobacteria (Rejmánková et al. 2004). Both macro- and microphytes in these wetlands are generally P limited as has been experimentally confirmed in cyanobacterial mats and *Eleocharis* dominated marshes (Rejmánková and Komárková 2000; Rejmánková 2001). No nitrogen limitation has been detected in any of the reported studies. The region is experiencing an increasing nutrient input 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).

Fig. 1 Localization of study site – Orange Walk, Belize, Central America.
Picture from worldatlas.com

2 Microbial metabolic activity

Most of soil microorganisms are heterotrophs using organic substrates, as carbon, as energy source and are, therefore, largely responsible for soil organic matter (OM) transformation. Metabolic activities of microorganisms as well as their growth efficiencies are influenced by environmental conditions. Soil temperature and soil water content belong to the most important factors. While soil temperature directly affects enzyme activities and changes their reaction kinetics, the effects of soil water content on microbial activity are much more complicated. Unicellular soil microorganisms, such as bacteria and protozoa, some algae, and fungal zoospores, require an aquatic environment to perform their life processes, and are largely dependent on existence of water films in soil (Van Gestel et al. 1991). Soil moisture determines mobility of soil microorganisms and their possibility to reach new substrates. Moreover, soil water content is closely associated with nutrient and substrate diffusion in the soil, as well as with soil aeration status. In affects nutrient availability for microorganisms and shifts between aerobic and anaerobic conditions in soil; all these factors influence the soil microbial community in combination and cannot be separated from each other (Griffin 1981).

O_2 stratification in the sediment profile creates relatively well-defined habitats for the different groups of gas metabolizing microorganisms. The different redox zones, that are not sharply separated (Conrad 1996; Fig. 2), are characterized by the dominance of the electron acceptors O_2, NO_3^-, Mn_4^+, Fe_3^+, SO_4^{2-}, and CO_2 (Zehnder and Stumm 1988). Most wetland soils are devoid of O_2 (below 2 to 3 mm deep) and also contain no other electron acceptors (below a few centimeters deep) other than CO_2 and H_2, therefore this zone is dominated by fermentation and methanogenesis. Methane is an end product of the microbial metabolism in this zone (Kiene 1991). The sulfate reduction zone can also be quite extensive in wetland soils, especially in marshes that are influenced by seawater. The NO_3^- reduction zone is the zone where production and consumption of NO and N_2O occur by the action of denitrifiers and DNRA bacteria (Keller 1994). Results (Rysgåård et al. 1994) show that nitrification of ammonium is an important source of nitrate and that denitrification is often tightly coupled to this process. The oxic zone is dominated by consumption of trace gases, i.e., oxidation of

CH_4, H_2, CO, and H_2S with O_2 as the electron acceptor. Although the zonation in wetland soil is usually relatively well defined, activity overlaps may occur. For example, although CH_4 production is usually inhibited by the presence of SO_4^{2-}, Fe^{3+} or NO_3^-, mainly since sulfate reducers, iron reducers, and nitrate reducers outcompete methanogens for common electron donors (Ward and Winfrey 1985), the simultaneous operation is possible if electron donors are not limiting, e.g., in organic-rich soils and sediments (Westermann and Ahring 1987; Achtnich et al. 1995). However, more complex interactions on the community level have also been discussed (Achtnich et al. 1995).

The same redox zonation as in the vertical dimension theoretically also occurs radially around the roots of O_2-transporting aquatic plants. Releases of O_2 by aquatic plants and increased redox potentials around the roots have been documented (Armstrong 1979; Christensen et al. 1994). The availability of O_2 allows the operation of chemical and microbial oxidation reactions in the rhizosphere. The roots of aquatic plants are also sources of organic material, which is either actively excreted or derived from sloughed-off cells or decomposing dead roots (Lin and You 1989). Also, the amount of microbial available organic substrates should influence the extent of the redox layers around the root, since these substrates fuel the reduction of O_2, NO^{3-}, Fe^{3+}, SO_4^{2-}, and CO_2. Nothing is known about the actual composition of the microbial aggregate communities in anoxic soil and sediment. Soils and sediments probably can contain only small microbial aggregates because of spatial limitations. The aerobes allow the operation of CH_4 production even in an oxic environment. Investigation of both function and community structure will be necessary to elucidate these kinds of microbial interactions in soil and other environments. There are many others abiotic and biotic environmental factors, which affect microbial activity in soils. Soil texture and structure, pH or redox potential belongs to important abiotic factors, determining character of soil microbial community. Besides these factors, human influence, land use (management), markedly affect soil microbial processes directly or indirectly.

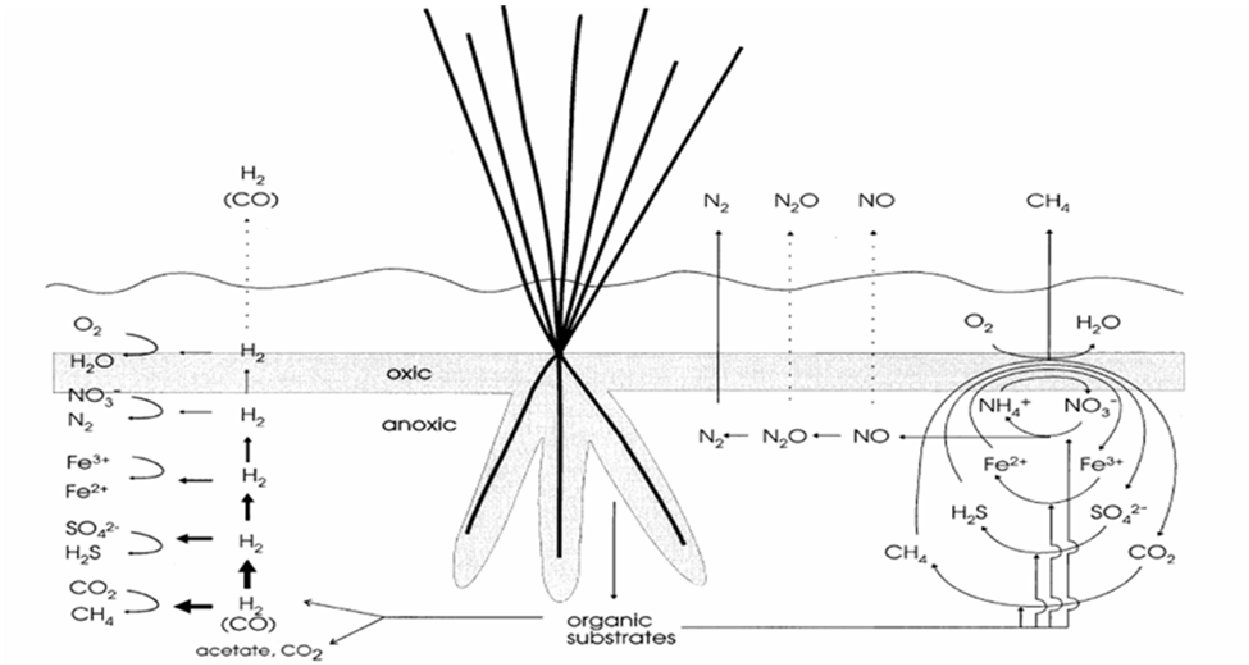

Fig. 2 Conceptual scheme of the vertical distribution of different redox reactions that influence the flux of H_2 and other trace gases (CO, CH_4, NO, and N_2O) from submerged vegetated soil and of the reoxidation of reduced inorganic electron acceptors by O_2 in the oxic layers at the soil-water interface and the rhizosphere of aquatic plants. Taken over from Conrad (1996)

3 Carbon in wetlands

Wetlands are major carbon sinks (IPCC 2001) and thus its destruction ultimately releases carbon to the atmosphere. Although wetlands occupy only 4-6% of the earth´s land area, they store a substantial amount of carbon. Gorham (1995) estimated that wetlands contain 20-25% of the world´s organic soil carbon. Wetland soils accumulate carbon at rates of 10 to 100 g/m^2/yr, whereas rates for upland areas range between 1 to 5 g/m^2/yr. In comparison to other biomes, wetlands cover a smaller area but with relatively high carbon storage in it (240 Gt; Mitra et al. 2005). The net-sink of wetlands is attributed to low decomposition rates in anaerobic soils. High variability in soil types and ecosystem types are among the many factors that give rise to disparate estimates of carbon quantities stored in peat (Mitra et al. 2005). Many wetlands trap large quantities of sediment from natural and anthropogenic watershed sources, adding to the carbon accumulation. Carbon flow in soil is represented by processes of decomposition and mineralization of organic matter and formation of soil humus (humification), with salt marshes having primary productivity matching tropical forests. Various factors, as groundwater level, temperature, substrate availability, nutrient level, and microbial population, affect the decomposition rate and hence carbon sequestration. Though wetlands are globally a major sink for carbon, releases of carbon dioxide may exceed photosynthesis in some circumstances; moreover wetlands emit large amounts of methane. Natural wetlands are the largest natural source of methane release to the atmosphere (Fung et al. 1991). Larger amount of methane are produced from the lower levels of peat (catotelm), while the upper levels (acrotelm) produce carbon dioxide and at least partially oxidize methane released from the lower levels. Wetlands may affect the atmospheric carbon cycle in four ways (Adhikari et al. 2009): (1) tropical peatlands are highly labile carbon reservoirs, carbon is released under conditions of water level decrease or of land management practices that result in oxidation of soils; (2) by sequestration of carbon from the atmosphere through photosynthesis by wetland plants and subsequent carbon accumulation in the soil; (3) by horizontal carbon transport pathways among different ecosystems; (4) by producing the greenhouse gas methane as a result of the anoxic conditions occurring in their flooded soils and their high rates of primary production (Barlett and Harris 1993) which is regularly emitted to the atmosphere even in the absence of climate change.

3.1. Carbon and microorganisms

Carbon is main element of all living components in soil. One of the major pathways by which C from the atmosphere enters the soil C cycle is through primary production of plants, and specifically via **senescent litter and rhizodeposition** of exudates into soil (Marschner et al. 2004). The rhizodeposits have diverse functions in plant nutrition and soil ecology; some of the compounds are able to improve nutrient (Fe, P, micronutrients) availability, other can be signalling substances for the establishment of symbiotic relations between plant roots and microorganisms (Hutsch et al. 2002). A large percentage (60-85%) of C released via roots serves as an important carbon and energy source and is rapidly respired by rhizosphere soil microorganisms (Cheng 1996; Hutsch et al. 2002). The remaining C is incorporated into soil microorganisms and **soil organic matter** (Kuzyakov et al. 2001). Root and shoot remains contribute to the accumulation of SOM due to **humification** after plant death. SOM **decomposition** and **mineralization** rates are general parameters of microbial activity in soil; mineralization rates correspond to CO_2 evolution from soil, soil respiration; decomposition rate can be measured as a loss of a particular substrate added to soil. **Process of mineralization** occurs much faster in oxygenated zones (Kadlec et al. 2000). The rates of mineralization are dependent on temperature, pH, the C:N ratio of the residue, available nutrients in the system, and soil conditions such as texture and structure. Carbon transformation is quite difficult process mediate by range of microorganisms. Soil organic

carbon was found to be a powerful predictor of heterotrophic activity, linearly increasing **CO_2 flux, methane production** and potential denitrification. The two main processes that operate with carbon transformation is process of **methane oxidizing** and methane formation. The nonsporulating, obligately anaerobic **methanogens** are able to survive in soil under adverse conditions largely than was previously believed possible.

4 Nitrogen and microorganisms

Nitrogen (N) is a key element in wetland biogeochemical cycles. **Organic N** is present in wetlands in the form of amino acids, urea, uric acid, amines, purine, and pyrimidines (Stevenson 1986). Biological and physical processes such as plant uptake, sediment/peat accumulation, adsorption of ammonium on to the organic sediments/peat, and nitrification denitrification processes can transform N between these different forms (Mitsch and Gosselink 2000). The major and more permanent **removal** mechanism of organic nitrogen in wetlands is the sequential processes of **ammonification, nitrification** and **denitrification**. As part of the nitrogen cycle, the various forms of N are converted into gaseous components that are expelled into the atmosphere as nitrogen gas (N_2) or nitrous oxide (N_2O). Soil moisture is considered a key determinant of N trace gas production through stimulation of microbial activity, delivery of electron donors (NH_4^+, dissolved organic carbon) and acceptors (O_2, NO_3^-), and the diffusion of N trace gases from soils (Stark and Firestone 1995). Processes involved in production (nitrification) and in consumption (denitrification) are regulated differently (Conrad 1996) - one particular process can be composited of different bacterial species that may be phylogenetically distant from each other and express different types of enzymes. The available information on the regulation of enzyme synthesis suggests that O_2 partial pressure and concentrations of nitrogen substrates are major regulators (Tiedje 1988). The real situation in soil probably involves more types of microbial metabolism, such as autotrophic and heterotrophic nitrification, nitrate respiration, and DNRA, which produce and consume NO and N_2O (Conrad 1996).

(1) Nitrification occurs in aerobic regions of the sediment profile, soil-water interface, and root zone (Reddy and D´Angelo 1997). The oxygen required for the nitrification process is supplied by diffusion from the atmosphere and leakage from macrophyte roots (Sorrell and Armstrong 1994). The rhizosphere of oxygen-releasing wetland plants provides a niche for oxygen-consuming microorganisms such as chemolithotrophic ammonia-oxidizing bacteria and facultative chemolithotrophs that oxidizing nitrite to nitrate. These bacteria are adapted to oxygen limitation with respect to their affinity for oxygen, ability to survive periods of anoxia, and immediate response to the appearance of oxygen. Although nitrification is widely believed to be an oxic process, investigations have shown that at least ammonia oxidizers are able to oxidize ammonia under anoxic conditions (Schmidt and Bock 1997). Various heterotrophic and lithotrophic microorganisms, including bacteria (actinomycetes), algae and fungi have also been reported to have nitrifying activity (Focht and Verstraete 1977; Jetten et al. 2001; Stevens et al. 2002). Autotrophic nitrification usually occurs at higher rates than heterotrophic nitrification.

(2) Denitrification is a stepwise enzymatic anoxic reduction process in which nitrite and nitrate are reduced to molecular nitrogen or nitrogen gases by chemoorganotrophic, lithoautotrophic, and phototrophic bacteria (Kadlec et al. 2000). The denitrifying organisms to support respiration use the free energy conserved as ATP (Kadlec et al. 2000). Although denitrification takes place preferably under anoxic conditions, there is accumulating evidence however, that some bacteria also denitrify aerobically (Plessis et al. 1998). The ability to denitrify has also been found in some Archaea, in the halophilic and hyperthermophilic branches, and in mitochondria of certain fungi (Zumft 1997).

(3) Nitrogen fixation is a process by which molecular nitrogen is reduced to form ammonia. Nitrogen-fixing bacteria (diazotrophs) carry out this complex process. Since the triple bond of

atmospheric N_2 is extremely stable, the reduction of N_2 to ammonia during its biological fixation is an energetically expensive process, therefore an incidence and activity of diazotrophs are highly dependent on the availability of energy rich carbon (C) source.

Diazotrophs comprise a taxonomically diverse group that includes aerobes, microaerophiles, facultative and strict anaerobes (Capone and Kiene 1988). Those diazotrophs associated with the roots of non-crop plant species are extraordinarily diverse, and apart from a few select groups such as cyanobacteria and rhizobia, are particularly understudied (Lovell et al. 2000). *Enterobacter, Klebsiella, Vibrio, Desulfobacter, Desulfovibrio* and *Clostridium* represent anaerobes of particular importance in wetland and water ecosystems. Since the late 1950's, it has been accepted that sulfate-reducing bacteria (SRB) can fix N.

Across ecosystems, N fixation is controlled by a variety of abiotic (bottom-up) and biotic (top-down) factors (Vitousek et al. 2002). In wetlands, these factors include light, redox potential, salinity, carbon substrate availability, inorganic N and P concentrations, Fe and Mo availability and grazers (Howarth et al. 1999).

5 Phosphorus and microorganisms

Phosphorus in soils and sediments originates from the weathering of residual minerals and from phosphorus additions in the form of fertilizer, plant residues and agricultural wastes. Phosphorus tends to accumulate in sediments in organic and inorganic forms. The phosphorus binding capacity, a function of sediment surface area, cat ion exchange capacity and mineral composition, is sufficient to explain phosphorus limitation in freshwater wetlands and SRP (soluble reactive phosphorus) surplus in salt marshes. **Inorganic P** is usually associated with compounds of varying solubility (Al, Fe, Ca, Mg) and availability to plants, affording long-term storage of P. In calcareous soils inorganic P is associated with **Ca** (Gale et al. 1994). In anaerobic and chemically reduced soils, oxides dissolve and release the bound P. **Organic P** consists of undecomposed residues, microbes, and organic matter in the soil. A large fraction of soil organic P consists of phytin; phosphatases cannot decompose phytin, but microbial phytases can, thus enabling plants to use also P from phytin. Phosphorus-solubilising and associative N_2-fixing bacteria enhance nutrient uptake by plant.

In most soils, the P content of surface horizons is greater than that of the subsoil because of higher adsorption rates of added P, greater biological activity, cycling of P from roots to aboveground plant biomass, and organic material accumulation in surface layers. Wetland vegetation and microbes help to remove some of the dissolved P by incorporating it into their own biomass. Immobilization of *P* by plants is less significant than immobilization of N because biological requirements for P are lower. A number of wetland studies have shown that soil/litter compartment is the major (>95%) long-term storage pool for P (Dunfield and Knowles 1995). The long-term application of P on soils can decrease the adsorption capacity of soils since soils have a finite capacity to retain P through adsorption and precipitation reactions (Rhue and Harris 1999). Many studies have shown that plants contain only a small proportion of the total P that occurs in wetlands indicating that the uptake of macrophytes in wetlands is limited (Reddy and D´Angelo 1997).

6 Soil microbial community and its describing method

Microbial communities play critical roles in the processing of matter and energy in wetland ecosystems. The composition, structure, and function of these complex assemblages are thought to be controlled either by top-down mechanisms (grazing), or by bottom-up effects (nutrient availability, temperature, and salinity). Wetland conditions support anaerobic bacteria such as methanogens, denitrifiers, sulfate reducers, fermenters, and acetogens (Conrad 1996). Aerobic organisms such as nitrifiers and methanotrophs may play an important role in wetland ecosystem functioning. It has now been demonstrated that

mycorrhizal fungi are often abundant in wetlands and may play a significant role. Actinomycetes, phylogenetically defined as a number of taxa within the gram-positive phylum (Embley and Stackebrandt 1994), are involved in important processes in a wide range of habitats (Williams et al. 1984); they are active in the decomposition of organic materials in soil, including lignin and other recalcitrant polymers. With increasing soil depth and as nutrient addition effect different carbon sources for the two main groups of soil bacteria could be determined. Gram-negative bacteria were found to use preferably recent plant-derived carbon and Gram-positive bacteria to use older OM-derived carbon. In the sediment profile, specific functional microbial communities become established dependent on aeration status and the availability of inorganic electron acceptors (Reddy and D´Angelo 1994).

General community structure (fingerprints) has been described using biochemical techniques as phospholipids fatty acid analysis (PLFA). PLFA analysis has been used to report differences in community composition in different wetlands wetlands (Borga et al. 1994; Sundh et al. 1997). PLFA profiles offer sensitive reproducible measurements for characterizing the numerically dominant portion of soil microbial communities without cultivating the organisms. The technique gives estimates of both microbial community composition, structure and biomass size, and results represent *in situ* conditions in the soil. Fatty acids are the key component of cellular membrane of all living cells. These lipids form in the membrane a bilayer with hydrophilic ends towards the outer surface of the membrane and hydrophobic ends buried in the interior. PLFA can be classified into ester-linked phospholipids fatty acids (60–90% of the total) and non-ester linked phospholipids fatty acids (10–40% of the total). Ester-linked are further subdivided into several groups depending on the number of double bonds and number and character of substitutions. The concentration of total PLFA provides quantitative insight into the soil viable/active microbial biomass because the phospholipids are rapidly degraded after cell death and are not found in the storage products. They are useful biomarkers or signatures for fingerprinting the soil microbial community because of relative abundance of certain PLFAs, which differ considerably among the specific group of microorganisms (Zelles et al. 1999). Fatty acid extraction techniques that have been used so far for analysing the microbial community structure are: cellular fatty acid analysis by microbial identification system (MIDI), simple PLFA extraction method and extended PLFA extraction method (for review see Kaur et al. 2005). *Simple PLFA extraction method* was used to analyse all samples. The most widely used extraction and separation method to obtain fatty acids derived from phospholipids is that proposed by Bligh and Dyer (1959) and modified by Frostegård et al. (1993). Briefly, the soil sample is extracted with single-phase mixture of chloroform: methanol: buffer solution (1: 2: 0.8 v/v/v) for lipid extraction. After extraction, the lipids are separated into neutral, glyco and phospholipids on a silicic acid column. Phospholipids are methylated and resultant PL-FAME (phospholipid fatty acid methylester) is separated and quantified by GC (Fig. 3). This method is simple, rapid and has been used for a wide range of soil types for microbial community analysis (Frostegård et al. 1993) as well as the total microbial biomass determination. However, through this method, only ester-linked PLFA can be analysed and not the nonester-linked PLFAs, which are key biomarkers of certain anaerobic bacteria (Zelles 1999). Gram-positive bacteria have sometimes been considered stress tolerators that grow slowly and tend to be able to metabolize complex carbon substrates more readily than Gram-negative bacteria (Waldrop et al. 2005). Fungi and actinomycetes are commonly considered producers of oxidative enzymes. Thus, subsets of the microbial community identified by PLFA biomarker analysis can be related to function.

Main disadvantages of PLFA method (*Simply extraction method*) is that there are no specific PLFA biomarkers for bacteria involved in nitrogen metabolism. The only known exception so far are Terminally branched fatty acids (iso-, anteiso-; TBFA) that were found to have amino acids as primers and so are expected to be found in higher portion in presence of N (Kaneda 1991). Archaea, such as methanogens, cannot be monitored directly with phospholipids fatty acids analysis (PLFA) methodology because Archaea contain only ether-linked lipids but PLFA analysis measures only ester-linked lipids. It may be possible to

monitor changes caused by methane production in soils, however, by using a biomarker for methane-consuming bacteria.

When studying microbial diversity, replicates of 1 to 5 g of soil are often used to measure diversity and then conclusions about the community are made (Kirk et al 2004). Problem is the innate heterogeneity of soil and thus of spatial distribution of the microorganisms (Trevors 1998). Microbial communities exist on such a small scale, that possibly 1 to 5 g of soil could bias results and favour detection of dominant populations (Grundmann and Gourbiere 1999) thus homogenized samples from a few samplings are recommended and were used in our survey. Plants also influence the spatial distribution of soil bacteria and fungi, as shown by an approximately two-fold increase in bacterial numbers in the rhizosphere over bulk soil (Smalla et al. 2001).

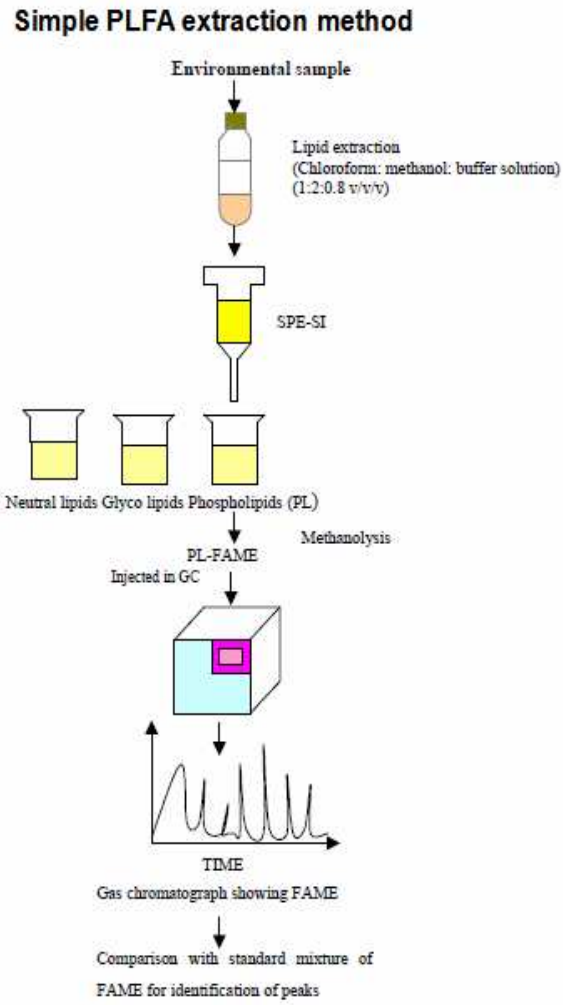

Fig. 3 Flow chart for extraction and detection of phopsholipid fatty acids using Simple PLFA extraction method. SPE-SI, Solid-phase-extraction silicic acid bonded phase column. *Adopted from Kaur et al. 2005.*

7 Plants

There is limited information on the significance of individual **plant species effects** at the ecosystem scale, although they are presumably instrumental in determining patterns of soil nutrient turnover (Chen and Stark 2000) and are likely to lead to strong positive and negative feedbacks that act as structuring forces within plant communities (Bever et al. 1997). Plant

species have been shown to have a major selective influence on microbial communities in their rhizospheres (Smalla et al. 2001). It has been postulated that this is due to variation in plant rhizodeposits (Grayston and Campbell 1996), more readily utilizable carbon is released into the rhizosphere result in greater stimulation and carbon utilization by bacterial communities. Nitrogen is required for plant growth, and plants do remove some N (both in NH_4-N and NO_3-N forms) for use in biomass production. The removal of N by plant uptake into wetland plant biomass is called immobilization. *As* long as the plant survives and continues to grow, it acts as a sink for N. However, much of this N is released back into the sediment when plants die and are decomposed by microbes in a process called mineralization. Although the net removal of N by wetland plants may not be great, plant roots can help promote some soil microbial processes that do remove N. The release of organic substances by plant roots has an interesting ecological aspect, since it influences the nutrient availability in the rhizosphere and indirectly acts on the soil microorganisms that in turn influence plant growth (Armstrong 1979). Microbes that are closely associated with roots are likely to be primarily influenced by patterns of root exudation that are known to vary both quantitatively and qualitatively between plant species (Gransee and Wittenmayer 2000). Plant specific differences in microbial communities were explained largely in terms of variations in exudation patterns and plant nutrient acquisition strategies, rather than by differences in productivity of individual plants (Bardgett and Shine 1999). Plants in nutrient poor environment are often characterized by high nutrient resorption resulting in poor litter quality and consequently slow decomposition. Cyanobacterial (microphyte) mats are dynamic contributors to ecosystem primary production and elemental cycling. Inorganic carbon was found (Megonigal et al. 2004) to be fixed in microbial mats primarily by phototrophs, 75% of which employ oxygenic photosynthesis, 12% of CO_2 is fixed by chemolithotrophs. Bacterial numbers and diversity are thought to be directly proportional to the quantity and diversity of carbon-containing compounds released by roots. Several studies have indicated that the structural and functional diversity of rhizosphere populations is affected by the plant species due to differences in root exudation and rhizodeposition in different root zones (Bardgett and Shine 1999). Rhizosphere microorganisms exert strong effects on plant growth and health by nutrient solubilisation, N_2 fixation, or the production of plant hormones (Asakawa and Hyano 1995).

8 Nutrient enrichment of wetlands

Organic matter decomposition is an important process controlling internal nutrient cycling and soil accumulation/loss. Nutrients are considered primary regulator of this complex process. Tropical ecosystems are often characterized by (1) low availability of inorganic nutrients; (2) phosphorus (P) limitation, and (3) species-rich communities and landscapes with a diversity of community types. The oligotrophic freshwater wetlands of the Caribbean basin display all of these tropical characteristics (Jones and Amador 1992; Rejmánková et al. 1995, 1996; Daoust and Childers 1998). These wetlands are an excellent venue for studying the effects of anthropogenic nutrient enrichment, particularly low-level eutrophication, on nutrient poor ecosystems. The loss of P in agricultural runoff increases the risk of surface water and wetland eutrophication and is affected by a number of factors including hydrology, soil type, soil P content and amount and placement of P added as fertilizer or manure (Carpenter et al. 1999). Accelerated land-use change in the tropics increases nutrient loading to aquatic and wetland ecosystems (Downing et al. 1999). Field experiments are essential for assessment of ecosystem responses to these impacts. Nutrient limitation in tropical wetland ecosystems has been little studied so far.

Since oligotrophic systems are vulnerable to even low levels of nutrient addition, questions of how macronutrient availability controls ecosystem structure and function in nutrient poor systems are important (Vounatsou and Karydis 1991). Nutrient enrichment alters ecological structure in oligotrophic systems in a variety of ways—from shifts in species composition and production of primary producers (Vymazal and Richardson 1995; McCormick et al. 2001) to shifts in trophic dynamics (Polis and Strong 1996; Elser and Urabe

1999). Cultural eutrophication is altering ecosystems (Boesch 2002) and its impacts have been addressed in a number of ecological systems, including rivers (Hart and Robinson 1990), seagrass meadows (Burkholder et al. 1992), coastal saltmarshes (Boyer and Zedler 1996), and prairie grasslands (Tilman 1984). In general, nutrient enrichment in the soil or water tends to increase decomposition rates; however, several studies have shown that increased nutrient input may cause either no change in decomposition, or a variable response (Rybczyk et al. 1996). As limitation by nitrogen is more common in the temperate terrestrial and wetland ecosystems, more attention has been paid to nitrogen as a limiting nutrient (Bedford et al. 1999). Contrary to the temperate ecosystems, tropical and arctic ecosystems are often P limited (Rejmánková 2001). In P-limited systems, P enrichment has resulted in increased decomposition as described by Qualls and Richardson (2000). Plant growth rate generally increases with increasing availability of the resource currently limiting growth (Jones and Hartley 1999). In some cases, removal of nutrient limitation can lead to a species replacement by a stronger competitor. In wetlands, this has been documented repeatedly with strong competitors such as *Typha* spp. replacing species adapted to low nutrient conditions (Wisheu and Keddy 1992). Also in the subtropical Florida Everglades wetlands change from *Eleocharis* spp. and periphyton dominated 'sloughs' to a dense productive *Typha domingensis* monoculture following the P input was documented (Craft and Richardson 1997). Decomposition correlates with a wide variety of chemical characteristics of the initial litter, and litter decomposition rate is affected by soil fertility (Morris and Bradley 1999). Higher nutrient concentrations in live tissue result in higher nutrient concentrations in litter and, consequently, faster decomposition (Vitousek 1998). The nutrient availability to microorganisms and plants can be strongly influenced by the sediment type. In carbonate rich environments (in the sediment interstitial water), dissolved inorganic phosphate concentrations decrease by adsorption onto $CaCO_3$ aragonite and calcite crystals; under Ca rich conditions, phosphates occluded in inorganic soil pools are less soluble (De Kanel and Morse 1978).

8.1. Nutrient addition experiment in Belize

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 the assessment of ecosystem response to nutrient addition along a salinity gradient (Rejmánková et al. 2008). Four 10 x 10m plots were established in each marsh in August of 2001, one represents a control (Fig. 4), and the remaining three received N, P and N & P addition in August 2001, August 2002 and March 2005 (Fig. 5). N was added as ammonium nitrate and P as triple super phosphate in amounts corresponding to 20 and 10 g m^{-2} y^{-1}, respectively. In March 2003, one individual of *Typha domingensis* was planted in each plot. In majority of controls and N addition plots *Typha* plants did not survive, while in P enriched plots they have been growing vigorously and outcompeting *Eleocharis.*

In January of 2005, P addition plots in 6 marshes were manipulated to contain one half dominated by *Eleocharis* and the second half dominated by *Typha*. Four of these 6 marshes, two from the low and two from the high salinity categories, were selected to measure the impact of P addition and macrophyte dominance on the microbial activities and community composition and were sampled in August 2005 and March 2007 (Fig. 6).

Responses to the fertilizer treatment in marshes of Belize were documented in many publications (Rejmánková 2001; Rejmánková and Houdová 2006; Macek and Rejmánková 2007; Rejmánková and Sirová 2007; Rejmánková et al. 2008; Rejmánková and Snyder 2008; Rejmánková and Macek 2008) as changes in: plant height, plant density, plant biomass, net primary production, nutrient resorption, decomposition, plant and soil nutrient concentrations, percent cover of cyanobacterial mats, and potential colonization by *Typha*. Decomposition of litter and cellulose assays was significantly faster in fertilized plots. Increased plant density led to elimination of a key component of these ecosystems, the nitrogen fixing cyanobacterial mats (Rejmánková and Komárková 2000). Loss of the calcareous periphyton mat and replacement by green algae has occurred in most other

Everglades P enrichment experiments as well as along eutrophication gradients in northern parts of the system (Chiang et al. 2000; McCormick et al. 2001; Gaiser et al. 2005).

Fig. 4 Oligotrophic marsh dominated by sparse *Eleocharis* spp. and Cyanobacterial mats (in detail picture no. 1). Sample of sediment core - upper organic layer and lower mineral layer are shown in detail (picture no.2). Photos – B.Pivničková.

9 Management of wetlands

Eutrophication, alongside with increasing temperatures, changes in precipitation and sea level rise, is the main aspects that affect distribution and function of wetlands (Wright 2009). It has become necessary to consider how landuse change and climate change may affect the role of wetlands, mainly carbon cycle and its biodiversity. The adaptation ability of wetland ecosystems to these variabilities will undoubtedly depend on the rate and extent of these changes. Lack of accurate knowledge on the location, area, distribution and condition of wetlands makes it more difficult to standardize a management plan or policy or to set management priorities. While many anthropogenic activities, such as city development, agriculture, use of pesticides and pollution can potentially affect soil microbial diversity, it is unknown how changes in microbial diversity can influence belowground and aboveground ecosystems. Before we can address how changes in microbial community structure influences ecosystem functions, there is the need for reliable and accurate mechanisms of studying soil microorganisms. An understanding of the relationships between community composition, nutrient status, and biogeochemical cycling may yield sensitive indicators of environmental impacts. Bacteria respond to environmental change much more rapidly than do higher organisms such as plants, and characterization of shifts in bacterial assemblage composition as a response to changes in nutrient concentrations may provide very sensitive early warning indicators of ecosystem change. These indicators could be useful in identifying ecologically sensitive concentrations of nutrients, and conversely, may be used to determine appropriate restoration endpoints.

Fig. 5 P addition procedure, here in *Typha* plot (August 2005). Plastic barier was installed for the period of 14 days to prevent P outflow from the defined plot.

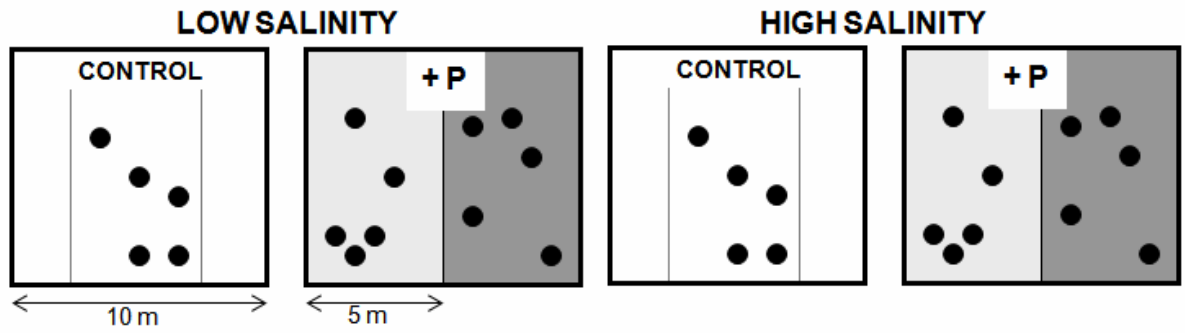

Fig. 6 Schematic of the experimental design and sampling strategy in marshes of low and high salinity. Soil cores (black dots; see also fig. 4a) were randomly collected from control plots (white) and P-enriched plots dominated by *Eleocharis* (light gray) and *Typha (dark gray)*.

11 Hypotheses and Aims

Hypotheses:

Using wetlands in northern Belize district as a model system, we have established a field manipulative experiment to obtain a mechanistic explanation for an ecosystem level response to increased nutrient input along a water salinity gradient. As previously confirmed P addition promotes the growth of macrophytes *(Eleocharis* spp. and *Typha domingensis*), which rapidly reduces cyanobacterial mats. We hypothezise that:

HY 1 Elimination of cyanobacterial mats in P-enriched plots shifts the microbial activity from autotrophs to heterotrophs and increases the heterotrophic microbial activity in the sediment; P addition will enhance the nutrient content (N and P stoichiometry) in microbial biomass either directly by removing P limitation or indirectly by increased available C from macrophyte exudation and litter

HY 2 Based on previous results (Rejmánková et al. 2008) that showed different plant biomass production, nutrient uptake and resorption from senescing tissues of Typha and Eleocharis, we expect different quality (C, N, P stoichiometry) and quantity of their litter to affect heterotrophic microbial biomass and activity

HY 3 P enrichment will affect microbial activities and community composition in both sediment layers of the profile (organic and mineral)

HY 4 Rate and direction of the changes in microbial activities, biomass and community composition will be salinity dependent. Water salinity will cause stress to microorganisms, more energy will be invested to osmotic regulation that will constrain heterotrophic activities, microbial biomass

HY 5 The replacement of cyanobacterial mats by macrophytes following P addition limits N-fixation by cyanobacteria (Rejmánková and Komárková 2000). We expect that, due to processing plant litter with high C/N ratio, soil microorganisms will require extra N to produce new microbial biomass. Whether this will eventually lead to competition between plants and microorganisms for N will depend on the availability of organic matter, and, consequently, heterotrophic N-fixation potential. Rate of heterotrophic N-fixation will be plant species and salinity dependent

a) Heterotrophic N-fixation will be higher in rhizosphere than in sediment and will be enhanced by P addition
b) The input of N_2 from autotrophic N-fixation can be replaced by heterotrophic N-fixation after P addition
c) In Typha dominated plots, the large quantity of litter and larger amount of C-rich exudates will result in higher demand by sediment microorganisms for N and will lead to higher heterotrophic N-fixation than in Eleocharis dominated plots
d) Heterotrophic N-fixation, as high energy demand activity, will be constrained by salinity

HY 6 Physiological status of heterotrophic microbial community (represented especially by microbial community composition, specific phospholipids fatty acids and specific respiration rate) will be affected by effects of P addition, plant species cover, sediment layer and water salinity

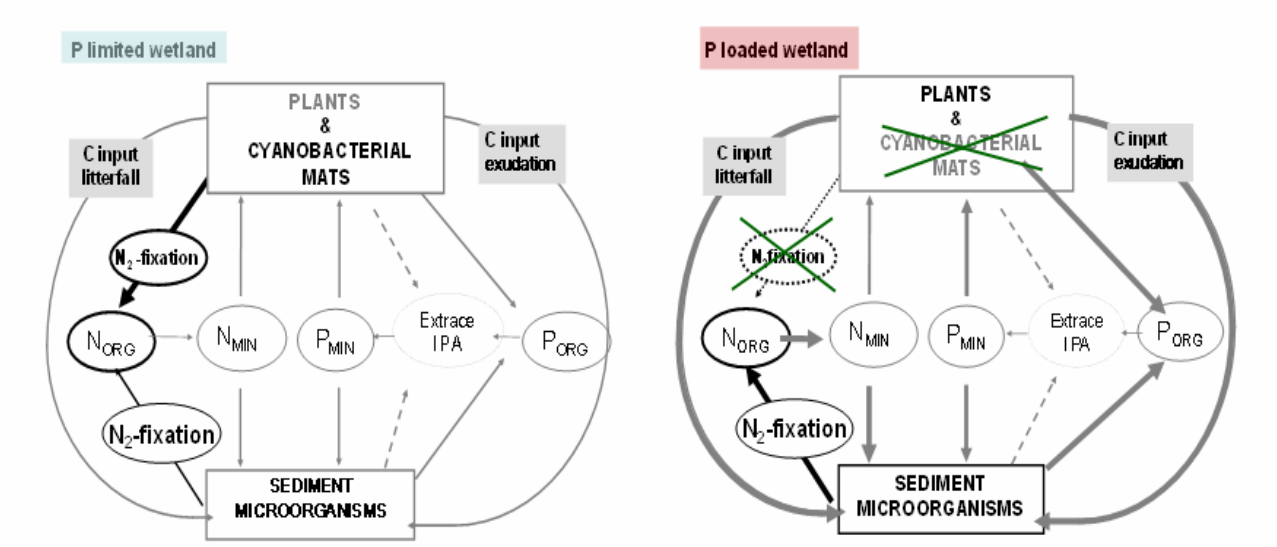

Fig. 7 Description of ecosystem functioning, based on above mentioned hypotheses, in P limited and P loaded wetland ecosystem. In P limited system nutrient turnovers are managed mainly via cyanobacterial mats with autotrophic nitrogen fixation being important process. In P loaded wetlands cyanobacterial mats are eliminated and functioning of sediment microorganisms is positively impacted by higher input of organic carbon through enhanced plant litter and root exudates and by more favorable C/P ratio of the plant litter (highlighted by bold arrows). Autotrophic N fixation is replaced by heterotrophic N fixation, amount of sediment P is increased. *Picture was kindly provided by Hana Šantrůčková.*

Aims:

- To document how wetland sediment heterotrophic microbial activities and microbial community physiological status are impacted by
  - the addition of a limiting nutrient, phosphorus
  - the effect of macrophyte species cover as an organic matter provider to the sediment
  - the effect of water salinity

  (see Paper III)

  and
  - to which depth (1-10 cm or even 10-30 cm) of the sediment profile are microorganisms sensitive to P loading **(see Paper I)**

- Report the differences in the sediment heterotrophic N-fixation between
  - controls (dominated by cyanobacteria mats and sparse *Eleocharis*) and P enriched plots (dominated by dense *Eleocharis* spp.)
  - plots dominated by *Eleocharis* spp. and by *Typha domingensis* (plant species effect)

  and attempt to explain these differences in the context of abiotic by either
  - vertical profile layer effect
  - water salinity effect

  (see Paper II and Paper IV)

- To master technique of phospholipids fatty acids analysis (PLFA); test and standardized protocol to implement PLFA method into the PřF JCU laboratories

11 Outline of the thesis

This research was conducted to understand the role of microorganisms in carbon, nitrogen and phosphorous transformation processes in wetland sediments, with special emphasis on nitrogen fixation ability and the functional and structural diversity of microorganisms. The study was performed at four wetlands varied in water salinity (two of high and at two of low salinity), in relation to environmental conditions – within each of four wetlands having three plots with different levels of eutrophication and plant cover due to experimental phosphorus loading.

Chapter 1 contains a review of carbon, nitrogen and phosphorus cycles and role of microbial community in sediment organic matter transformation. Known effects of nutrient enrichment on sediment microbial community are summarized here and study site is introduced. Finally, hypotheses and aims of the thesis are formulated.

Chapter 2 displays P enrichment effect on heterotrophic microorganisms in sediment vertical profile - organic (1-10 cm) and mineral (10-30 cm) layers of the sediment. The microbial community and its metabolic diversity were compared in P enriched and pristine plots of the wetlands. The microbial community composition and structure was determined by biochemical technique targeting the phospholipids fatty acids (PLFA). Laboratory incubation and enriched techniques using aerobic and anaerobic analyses of homogenized sample were used to assess C, N, P transformation processes in these types of freshwater/saline marshes. Both layers of sediment profile were found to be impacted by P enrichment; **see Paper I**

Chapter 3 focuses on the determination of N-fixation (potential nitrogenase activity) in P enriched, *Eleocharis* and *Typha* dominated plots, and in pristine plots (caynobacterial cover) of low and high salinity marshes. Data sets (environmental and microbial characteristics accompanied with root biomass measurements) for organic and mineral layer in two years (2005, 2007) were analyzed. P addition was found to control heterotrophic N-fixation mainly via root development, although litter input quality also appeared to be important. *Eleocharis* litter supported N-fixation more than *Typha litter*, while effect of salinity was not conclusive; **see Paper II**

Chapter 4 reveals microbial C, N, P transformation processes in sediment organic layer with its link to physiological status of microbial community, additionally to Chapter 2 also its link to plant species and water salinity. Acceleration of C and nutrients turnover rates and microbial biomass in P enriched sediments and its lowering by salinity are described here. Balanced nutrients turnover was concluded to be more dependent on P addition in *Typha* compared to *Eleocharis* plots. Water salinity was found to increase an amount of N in microbial biomass and to change chemical composition of microbial membranes; **see Paper III**

In **Chapter 5**, the effect of water salinity and the contribution of sulfur reducing bacteria (SRB) to the overall N fixation of collected marsh sediments, roots, and cyanobacterial mats was measured in laboratory incubation experiments (acetylene reduction assay calibrated by $^{15}N_2$ reduction assay) with and without the addition of sodium molybdate (SRB inhibitor). PLFA technique was used to assess the dynamic of SRB assemblages. Simple N budget to determine if N demands are met following P addition is introduced in this chapter; **see Paper IV**

Finally, the most important results of this research are summarized in **Chapter 6**, and the concluding remarks are defined. It is anticipated that the results of this study will contribute to a greater understanding of the factors controlling C and nutrients turnover with respect to plant species cover and water salinity status in tropical wetland sediments in Belize.

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CHAPTER 2

Paper I

Microbial community composition and activity potential in tropical marsh sediment profile influenced by P enrichment

manuscript

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Abstract

Phosphorous loading is gradually changing oligotrophic P limited marshes in northern Belize; this ecosystem change is coupled with malaria disease transmission. Understanding of sediment microorganisms, governing nutrients turnovers, are of highly need for successful management of these endangered marshes. How microbial communities naturally differ in profile layers (UL, 1-10 cm; LL, 10-30 cm) and to which depth of sediment profile P loading influenced heterotrophic microbial composition/structure and its activity potentials were tasks of the study. Four marshes of two salinities levels were used for the experiment. Sediment samples from P enrichment plots (dominated with dense *Eleocharis* spp. overgrowth) were compared with samples from native marsh sites (influenced by cyanobacteria mats debris and sparse *Eleocharis* spp.). Microbial biomass, community structure as well as composition were estimated by phospholipids fatty acids (PLFA) analysis. Microbial biomass (MBC) and nutrients (N, P) turnover potential (heterotrophic nitrogenase activity, NA; potential denitrification enzyme activity) were found to be higher in UL while portion of active microorganisms (PLFA-to-MBC) increased with depth. Organic matter content, immobilization of P into the microbial biomass and NA was enhanced by P addition more in UL than in LL. P addition enhanced microbial biomass and activities in both layers thus decreased differences between layers.

Microbial community composition did not differ between layers (P = 0.49), salinity effect seemed to contra-indicated the layer effect. P addition did not significantly affect community composition in UL (P = 0.378) neither in LL (P = 0.845). Using PLFAs as microbial community biomarkers we showed that portion of monounsaturated FAs (MUFA) and terminally branched FAs decreased with depth, the portion of MUFA only increased after

P addition. The distribution of these biomarkers we attribute to carbon availability – its decline with soil depth and its increase with P addition. Sulphate reducing bacteria were more abundant in LL and its portion in community was not changed by P addition. Both layers partly changed its microbial community (biomass, activity, structure) after P addition thus having a potential for response to P loading.
Key words: sediment profile, P loading, PLFA community composition, microbial activity

Introduction

Knowledge of microbial characteristics in soil profile is mostly limited to agriculture, forest and marine environments (Blume et al. 2002; Fierer et al. 2003; Kramer and Gleixner 2008), and less frequently available is the information on microbial community composition and activity in profile of wetland sediment (Bossio et al. 2006). Microbial communities in wetlands function mostly under restricted aeration conditions that result in increasing anaerobiosis with depth. This not only slows rates of organic matter (OM) decomposition, but also may significantly affect the amount and types of decomposition products. The majority of OM in wetland ecosystems originates from wetland macrophytes, its aboveground and belowground litter as well as root carbon exudates (Moriarty and Pollard, 1981). OM degradation controls internal nutrient cycling and sediment accumulation/loss. Biochemical processes in deeper layers (>10 cm) of wetlands were found to be not as responsive to changes in the overlaying water chemistry, driven by a more stable OM and possibly reflecting historical levels of nutrients (White and Reddy 2001; DeBusk and Reddy 1998; Corstanje et al. 2007). The decline in total soil nutrients with increasing soil depth has been previously reported for various soils and wetland sediments (White and Reddy 2001). In addition, different microbial carbon sources in different soil depth intervals might be found due to changes in microbial community structure that also depends on soil depth (Fierer et al. 2003).

Nutrient enrichment to previously oligotrophic systems alters species composition and production of primary producers and shifts trophic dynamics (Vymazal and Richardson 1995; Polis and Strong 1996; McCormick et al. 2001). Macrophyte community changes following increase of limited nutrient input have been reported from various types of wetlands (Childers et al. 2003; Wollin et al. 2005) and quality and quantity of OM provided by macrophytes is therefore changed, mainly increased. Generally, an addition of readily hydrolysable sources and increase of nutrient availability resulted in increased microbial growth (Anderson and Domsch 1985), faster turnover of organic matter (DeBusk and Reddy 2003), faster mineralization and release of nutrients back to the environment as observed in the Everglades wetland system (Davis 1991; DeBusk and Reddy 1998). Results of Kramer and Gleixner (2008) showed that with increasing soil depth more carbon derived from

decades old OM and less recent carbon derived from plants is used as carbon source by all microorganisms. In the sediment profile, specific functional microbial communities become established dependent on aeration status and the availability of inorganic electron acceptors (Reddy and D´Angelo 1994). Wetland conditions support anaerobic bacteria such as denitrifiers, methanogens, sulfate reducers, fermenters, and acetogens (Conrad 1996), but also aerobic organisms, present preferably in the vicity of roots, such as nitrifiers and methanotrophs play an important role in wetland ecosystem. To identify soil microbial community structure and its viable portion biochemical method, based on analysis of phospholipids ester-linked fatty acids (PLFA) is recommended (White et al. 1979; Borga et al. 1994; Ibekwe and Kennedy 1998). Certain PLFAs isolated from prokaryotic and eukaryotic cell membrane can serve as unique signatures for certain functional groups of microorganisms (specific biomarker fatty acids; reviewed by Findlay and Dobbs 1993). Signature lipid biomarker analysis cannot detect every species of microorganisms in soil samples as many share overlapping PLFA patterns. Nevertheless, comparison of total community PLFA profiles accurately mirrors shifts in community composition and provides a way to correlate community composition to specific microbial activities and environmental conditions (White et al. 1996).

Our wetland study site in northern Belize is well suited to assess the impact of phosphorus (P) input on sediment microbial communities. Benthic cyanobacterial mats with scattered macrophytes, mainly *Eleocharis cellulosa* and *E. interstincta*, dominate the wetlands that 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 the oligotrophic, P limited wetlands as a model system, to obtain a mechanistic explanation for an ecosystem level response to increased nutrient input. 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* (Macek and Rejmánková 2007). In tropical wetlands of Central America, expansion of *Typha*, which provides an excellent habitat for an efficient malaria vector, *Anopheles vestitipennis*, can have serious consequences for disease transmission (Rejmánková et al. 2006).

The aim of this paper is to document sediment profile layer (UL 1-10 cm; LL 10-30 cm) dependent impact of a limiting nutrient addition, P, on the microbial community composition, growth and nutrient status, and activity potential. We expect that composition and functioning of sediment microflora will be impacted by OM content and organic carbon input both increased through *Eleocharis* enhanced growth (debris, root biomass,

rhizodeposition) in P enriched sites compared to control sites. We hypothesize that microbial biomass and activities will be enhanced by higher amount of OM and available carbon in upper layer of the sediment compared to lower layer, and will be enhanced by P addition. Rate and directions of microbial biomass and activities changes will be layer dependent but P addition will decreased differences between layers. Microbial community composition will differ between layers and will be changed by P addition. P addition will improve in both layers nutrient and growth status and will decrease stress.

Methods

Study site

Our study area is located in lowlands of northern Belize, Central America within a 50 km radius of 18_9°58°° N and 88_31°28°°. This part of the Yucatan Peninsula is an uplifted marine platform composed of a 2–3 km thick sequence of cretaceous and Tertiary limestone, dolomite, and gypsum (Weidie, 1985). Marsh hydrology is closely linked to the ground water system, and water levels are controlled primarily by regional precipitation patterns. 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 ''fresh'' ground waters are nearly saturated with carbonate and sulfate, derived from dissolution of the platform rocks. The net result of these factors is the conductivity of the inland wetlands varying by an order of magnitude (from 0.2 mS to 7 mS low to high salinity respectively). Sediments in low conductivity marshes are usually formed by peaty clays while high conductivity marshes have predominantly marl sediments (table 1). Main primary producers in these systems are several species of emergent macrophytes (*Eleocharis cellulosa, E. interstincta, Cladium jamaicense* and *Typha domingensis*) and specious communities of microphytes represented mostly by cyanobacteria (Rejmánková et al. 2004). Both macro- and microphytes in these wetlands are generally P limited as experimentally confirmed in cyanobacterial mats and *Eleocharis* dominated marshes (Rejmánková, 2001; Rejmánková and Komárková 2000). No nitrogen limitation has been detected in any of the reported studies.

Soil sampling

Four marshes, two of low and two of high water salinity, dominated by cyanobacterial mats (CBM) and sparse macrophytes (*Eleocharis* spp.) were chosen for the experiment (table 1, fig. 1). Plots of diameter 10 x 10m were established in each marsh: one represents a control and second received P additions (P as triple super phosphate in amounts corresponding to 20 and 10 g m^{-2} y^{-1}) in August 2001, August 2002 and March 2005. All plots were flooded with water depth ranging from 25 to 70 cm during the time of sampling. Samples were collected in March 2007 from (1) control plots and from (2) P-addition plots dominated by *Eleocharis* spp.. Recently deposited, readily distinguishable plant detritus on the soil surface (app. 1 cm) was gently removed before sampling. Eight randomly located sediment samples were collected with a 5.5 cm diameter sharp edge PVC corer to a depth of approximately 30 cm. The upper section (1-10 cm) represented mostly the rhizosphere, and the lower section (10-30 cm) represented mostly the mineral part of sediment, were 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 to one sample; analyses were conducted in triplicates. From various analyses only nitrogenase activity was measured immediately, others were done after delivery of samples to Czech Republic (max. 1 month after sampling). Samples for the remaining analyses were divided into two parts. One part was stored fresh in ziplock bags for a maximum of 1 month at 4℃ and the other was oven

dried at 105℃ to a constant weight for determinati on of gravimetric water content and elemental composition (C, N, reactive P) of soil.

Fig. 1 Schematic of the experimental design and sampling strategy. Samples were sampled at 4 marshes (two defined as high water saline marshes and two of low salinity; see table 1). In each plot eight cores eight cores (black dots) were randomly collected from control plots and P-enriched plots. To avoid discrepancy in plot size of controls, samples were collected in only a 5 x 10 m area (indicated by line). Cores were separated in two parts according to the color and texture (upper, UL, and lower, LL, layer) and homogenized. From homogenized sample, three subsamples were used as pseudoreplicates. Data of respective salinity sites were analyzed together thus having for one parameter 12 replicates.

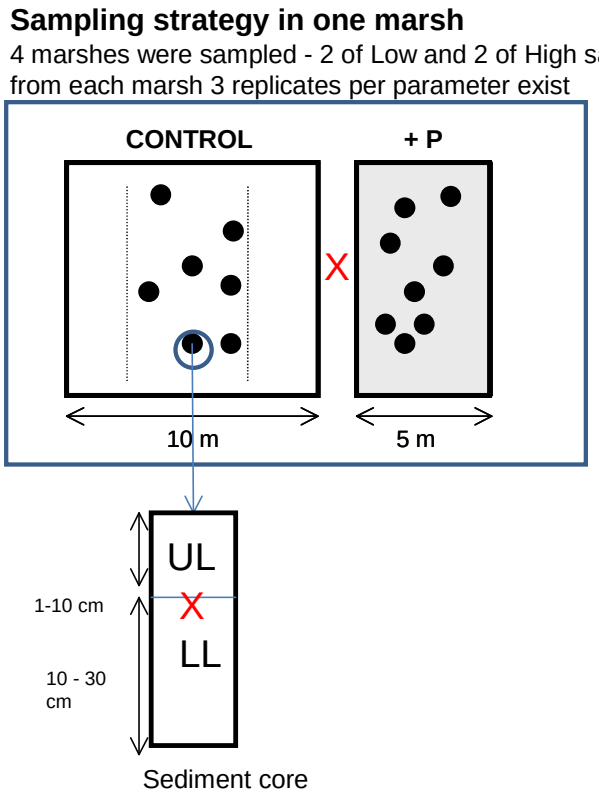


Table 1 Characteristics of four marshes selected for the experiment. Values represent means and standard deviations (in paretheses) for water conductivity and bulk density (BD, year 2007 only). Data from August 2001, before the beginning of the nutrient addition experiment. NPP is the aboveground net primary production of *Eleocharis* in Control and P enriched plot (year 2007).

Marsh #	**Area**	**Conductivity**	**Sediment**	**BD**	**NPP**
				UL / LL	Control/P
	ha	ms cm^{-1}	Type	g cm^{-3}	g m^{-2} y^{-1}
LOW SALINITY					
F10	4.7	0.231 (0.068)	Peaty Clay	0.52/ 0.55	180/440
F12	11.3	0.658 (0.164)	Marly Clay	0.42/ 0.50	126 /481
HIGH SALINITY					
F6	63.4	6.671 (1.479)	Marl	0.23/ 0.49	219 /315
F7	18.2	5.667 (1.328)	Marl	0.25/ 0.45	205 /218

Environmental characteristics

Organic matter content was gained by lost on ignition method using Muffle´s oven (15 hours, 450℃). Total soil organic carbon (TOC) was analyze d in soil samples pretreated with 0.1M

HCl (carbonate removal) on a Carlo-Erba series 5000 CHN-S analyzer. Available C (Cav) was measured as organic C in 0.5 M K_2SO_4 extract (Ettema et al., 1999) using organic C analyser (Shimadzu total organic carbon analyzer TOC-5050A). The oxalate extractable P (P_{ox}) was analyzed using ascorbic acid reduction of phosphomolybdenate complex in oxalate extracts (Owens 1977).

Nutrients in microbial biomass

Microbial biomass carbon (MBC) and phosphorus (MBP) were determined by fumigation extraction procedures of 10-g subsamples. The MBC was calculated after a subsequent 0.5 M K_2SO_4 extraction (Vance et al. 1987) as the difference in the dissolved organic C (Shimadzu total organic carbon analyzer TOC-5050A) from fumigated and unfumigated sample, corrected by an extraction efficiency factor k_{EC} = 0.37 for C. The MBP was calculated after subsequent $NaHCO_3$ extraction in molybdophosphate complex (Olsen and Sommer 1982). Biomass values were expressed per gram soil dry weight.

Physiological status of microbial community

Microbial community composition was determined based on a phospholipids fatty acid (PLFA) pattern (Frostegård et al. 1993) in 2 g of fresh soil (4℃). Phospholipids were separated, quantified and identified by gas chromatography (HP 6890, FID, capillary column HP5, 30m, 0.33 mm, 025 µm). The identification of PLFA was based on comparison of retention time with retention times of FAME standards (Supelco, Larodan Fine Chemicals AB). All together, 50 PLFAs were identified from samples, only 35 FAs were involved for statistical analyses (PLFAs with distribution less than 0.1 mol% were removed from PLFA profile - 8:0; 10:0; 2OH 10:0; 3OH 10:0; 13:0; i15:1; 20:3ω3; 20:1ω9; 22:5ω3; 22:1ω9; 3OH 12:0; 23:0; 20:3ω6; 19cy 9,10; 2OH 16:0). The mol% of certain fatty acids was combined into groups representing different portions of the microbial community (Table 2). The total PLFA content was used for active microbial biomass estimation (Frostegård et al., 1993) and numbers of different PLFA for the community richness (Maire et al. 1999).

Fatty acid nomenclature used is as follows: total number of C atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule; cis and trans geometry are indicated by the suffixes *c* and *t*. The prefixes *a* and *i* refer to anteiso- and iso-branching; 10Me indicates a methyl group on the tenth C atom from the carboxyl end of the molecule; position of the hydroxy (OH) groups are noted; and *cy* indicates cyclopropane fatty acids.

Specific respiration rate (CO_2 per unit microbial biomass) was used as an indicator of catabolic activity; the higher the catabolic activity the higher the stress effect of environmental conditions on microbial community (Anderson and Domsch 1990). **MBC-to-TOC ratio** was used as an indicator of C availability and ability of microbes to utilize it; the lower the ratio, the lower the availability of C or ability to use it (Anderson and Domsch 1989). **PLFAtot-to-MBC** ratio was used to determine the active portion of microbial community. Increasing **MUFA/STFA** ratio was used as indicator of C (energy) improvement availability (Bossio and Scow 1998; Zelles et al. 1992).

Microbial activity analyses

Carbon mineralization

Soil (30g fresh weight) was incubated with 20 mL of physiological salt solution (0.9% NaCl) in tightly closed 100 mL bottles under anaerobic conditions with slow stirring at 25℃ in the dark. Aerobic respiration was measured under an atmosphere of 21% O_2 after 24 hours. Samples were incubated for 24 hours. Carbon dioxide production was detected using thermal conductivity detector (HP 5890 gas chromatograph (Agilent, USA).

Denitrification enzyme activity (DEA)
The potential denitrification enzyme activity was measured using acetylene-inhibition technique (Balderston et al. 1976). 10 grams of fresh weight soil were incubated in 100 mL flasks with 15 ml of nutrient solution containing excess nitrate (0.5 g l^{-1} KNO_3, 1g l^{-1} glucose). At the start of experiment, 10% of the gas phase of the samples was replaced with acetylene. The production of nitrous oxide was measured taken (0.2 ml) from the headspace at zero time and after 30 min and 60 min using Hewlett Packard 6890 Gas Chromatograph equipped with an electron capture detector.

Nitrogenase activity (NA)
We used modification of the acetylene reduction assay method (Hardy et al. 1968) for the measurement of potential nitrogenase activity. Glucose in the amount of 0.16 mg C cm^{-3} was mixed into the 50 g of fresh soil and sealed in 100 mL glass bottles. The bottles were equilibrated to the atmospheric pressure, 30 ml of the headspace were removed and subsequently replaced with 20 ml of N_2 gas to lower the partial pressure of oxygen. Ten mL of acetylene, freshly prepared from CaC_2, were added to each bottle, the bottles were vigorously shaken for 1 min and were incubated under dark conditions at 28^0C. Bottles were shaken again after 12 h and at the end of incubation. After 24 h, several ml of headspace were withdrawn and analyzed by gas chromatograph, Shimadzu 14 GC equipped with FID. Controls run with samples without acetylene addition 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.

Table 2 PLFA interpretive tools including: structural and functional biomarkers.

Indicator	Interpretation	References
Structural biomarkers		
Saturated FA (STFA)	all genera	Zelles 1999
Monounsaturated FA (MUFA)	mostly Gram-negative, aerobic bacteria	Ponder and Tadros 2002; Zelles 1999
Terminally branched FA (TBFA, *a-/i-branched*)	Gram-positive bacteria, aminoacid precursor is required	Haack et al. 1994; Kaneda 1991
Hydroxy FA (OH)	Gram-negative, anaerobic bacteria	O´Leary and Wilkinson 1988
Cyclopropyl FA (cy17:0, cy19:0)	mostly Gram-negative, anaerobic bacteria	Guckert et al. 1985; Vestal and White 1989
10Me17:0, 10Me18:0	Actinomycetes	Kroppenstedt 1985
18:2ω6 Polyunsaturated FA (PUFA)	Fungi	Frostegård and Bååth 1996
20:4ω6 Polyunsaturated FA (PUFA)	aerobic Protozoa	Erwin 1973
Functional biomarker		
10Me16:0	mostly Gram-positive, Sulphate-reducing bacteria (SRB), *Desulfobacter,* anaerobic bacteria	O´Leary and Wilkinson 1988; Dowling et al. 1986

Data analysis

The one-way ANOVAs, with level of probability $P<0.05$, were used (STATISTICA version 7.0) to evaluate: (1) sediment layer effect in Control treatments and (2) P addition effect separately for UL and for LL on selected biological and chemical variables and PLFA biomarkers. The principal-component analysis (PCA) was used to elucidate a major variation in PLFA patterns of samples (35 PLFAs, expressed as $\log_{10}$ moles percent, were used) (Canoco, Wageningen, Netherlands). PCA score values of individual samples were

compared by the same way as the other characteristics to show sediment layer effect and P addition effect in UL and in LL.

Results

Sediment layer effect (Control treatment)

The profile layer affected preponderance of the studied environmental and microbial parameters significantly, see Table 3A&C. No significant effect was observed only in case of Pox. Other variables were significantly higher in UL compared to LL. Focused on environmental parameters the biggest difference between layers was found in root biomass that was about 4-fold higher in UL compared to LL. OM content and C availability decreased 2-fold with increasing depth. Statistically significant profile layer effect on the growth and nutrient status of microbial community was confirmed; see Table 4A&C. Upper layer samples showed higher amount of microbial biomass C (23-fold), as well as higher (4-fold) amount of microbial PLFA that was accompanied by lower PLFA/MBC ratio compared to LL. The amount of microbial biomass created from total organic carbon (MBC/TOC) significantly increased with increasing depth (4-fold) as well as specific respiration activity (Q_{CO2}, 5-fold). Immobilization of P into the microbial biomass (MBC/MBP) was found to be increased (4-fold) in lower layer samples. Upper layer samples were found to have significantly higher denitrification potential (DEA, 19-fold) and nitrogenase activity (NA, 2-fold) compared to lower layer samples. Decreased activity in LL was followed by a decline in the MU/STFA ratio showing nutrient limitation (Table 4A&C).

Community composition, based on PCA scores comparison, did not differ between layers (P = 0.496, F = 0.48; Fig. 2). However, upper and lower layer samples, included high (F6, F7) and low salinity (F10, F12) category, were clearly separated into two groups according to PCA axes (Fig.2); variability of samples given by salinity was likely higher than variability given by profile layer. Microbial community structure was found to be dominated by G- bacteria with a co-dominance of G+ bacteria in both layers. Microbial community was dominated by STFA, followed by MUFA, TBFA, CYFA, SRB, OHFA biomarkers (Table 5A, Fig. 3). Actinomycetes, Fungi and Protozoa in both layers were present in relative distribution less than 2 mol%. Layers significantly differed in portion of STFA, MUFA, TBFA, OHFA and SRB, see Table 5C. Upper layer samples had significantly higher relative distribution of MUFA, TBFA, CYFA, and Protozoa while lower layer samples dominated by STFA, OHFA and SRB compared to UL (Table 5C). The number of detected fatty acids (PLFA richness) decreased from 35 in upper layer samples to 28 in lower layer samples; following fatty acids were not present in lower layer samples: 12:0, 2OH13:0, i17:1ω9c, 17:1ω8c, 20:5ω6, 20:4ω6, 22:6wω3.

P addition effect in sediment layers

The P addition effect was tested by comparing Control with P treatments in respective layers (UL, LL). Results (Table 3B&4B) showed that P addition enhanced most of tested variables in both layers. Increase of variables was found to be layer dependent. While all variables except of OM were affected in lower layer (Table 3D&4D), in upper layer P addition did not significantly changed C availability, root biomass, PLFAtot, PLFAtot/MBC and Q_{CO2} (Table 3D&4D). Root biomass increased in both layers similarly (2-fold). OM increased in upper layer samples (1.5-fold), while in lower layer samples remained unchanged. C availability increased in lower layer samples more (3-fold) than in upper layer samples (2-fold) thus having similar values in both layers. Microbial biomass C increased in both layers, in upper layer samples less (2-fold) than in lower layer samples (4-fold) but still having one order of magnitude higher values in upper layer samples. Amount of microbial PLFA increased 2-fold only in lower layer samples but active portion of microbial biomass (PLFAtot/MBC) decreased similarly (2-fold) in both layers. P_{ox} increased significantly in both layers (5-fold in upper layer samples and 2-fold in lower layer samples) but only in upper layer sample P immobilization into the biomass increased (6-fold) while in lower layer samples decreased (3-fold). Microbial activities were also enhanced (Table 4B&D); DEA increased more in lower layer samples (9-fold) compared to upper layer samples (2-fold), opposite trend was in NA where in upper layer samples increased 800-fold and in lower layer samples only 26-fold. Nutrient limitation given by MU/STFA ratio decreased in both layers similarly, upper layer samples still having higher value than lower layer samples. Specific respiration rate (Q_{CO2}) increased only in lower layer samples (3-fold) while in upper layer was not changed. Microbial community composition was changed by P addition neither in upper layer ($P = 0.378$, $F = 0.81$, Fig. 4) nor in lower layer ($P = 0.845$, $F = 0.04$, Fig. 5). In upper layer, four groups of samples were separated according to marshes (F6, F7, F10, F12); control and P enriched sites still relatively close to each other (Fig. 4). In lower layer three groups were separated by PCA; the one group of Control and P enriched samples of high salinity sites (F6 and F7 marshes), the other two groups were of low salinity samples, separately for F12 and F10 marshes. P addition did not affect the PLFA richness in upper layer samples while in lower layer samples PLFA richness increased from 28 to 30 FAs; following FAs appeared: 10Me17:0 and 22:6w3.

Table 3 Selected biological and chemical characteristics in upper and lower sediment layer in Control plots (3A) and P enriched plots (3B). Layer effect in Control treatments (3C) and P effect in both layers (3D) were tested by ANOVA. Values are averages of N=12 (high and low salinity involved)

	OM	P_{ox}	Cavail	RootB	MBC	MBC/MBP	PLFAtot
Treatment means							
3A Control							
upper layer	17.72	0.84	114.2	4.31	1632	640.0	156.5
lower layer	10.75	1.37	60.0	0.863	70.2	146.3	47.9
3B P effect							
upper layer	26.57	6.74	205.4	10.88	2850	110.9	174.7
lower layer	11.94	2.14	196.1	1.43	286.1	408.7	99.10
p-value, ANOVA							
3C Layer effect in Control		0.031	ns	0.001	0.001	0.001	0.001
3D P effect							
upper layer	0.081	0.001	ns	ns	0.059	0.009	ns
lower layer	ns	0.022	0.035	0.009	0.038	0.01	0.045

OM = organic matter, in %; C avail = available carbon, in µg g^{-1}, P_{ox} = oxalate extractable phosphorus, in µg g^{-1}; RootB – root biomass, in mg g^{-1}; MBC and MBP = microbial carbon, and phosphorus, respectively, in µg g^{-1}.

Table 4 Indicators of physiological status of microbial community and microbial activities tested in Control (4A) and P enriched plots (4B). Values are averages of N=12 (high and low salinity involved). Layer effect in Control treatments (4C) and P effect in both layers (4D) were tested by ANOVA.

	MBC/TOC	PLFAtot/MBC	MU/STFA	Q_{CO2}	DEA	NA
Treatment means						
4A Control						
upper layer	0.013	0.21	0.65	0.15	741.9	1.15
lower layer	0.003	0.97	0.53	0.74	40.3	0.52
4B P effect						
upper layer	0.016	0.14	0.80	0.13	1296	960.0
lower layer	0.004	0.40	0.61	0.24	363.5	12.81
p-value, ANOVA						
4C Layer effect in Control	0.008	0.009	0.045	0.001	0.01	0.001
4D P effect						
upper layer	0.001	ns	0.009	ns	0.001	0.001
lower layer	0.042	0.01	0.001	0.01	0.001	0.044

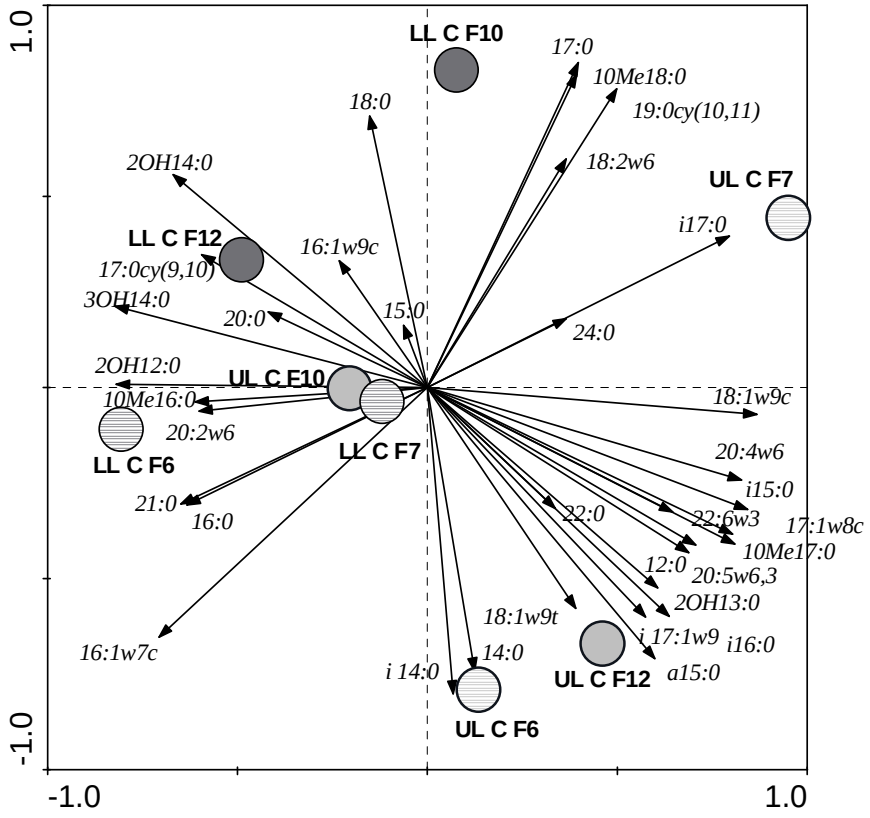
MBC = microbial carbon, µg g^{-1}; TOC = total organic sediment carbon, in µg g^{-1}; PLFAtot/MBC = active/total biomass, $nmol_{PLFA}$ μg^{-1} C; MUFA/STFA = ratio of Monounsaturated and Saturated fatty acids; Q_{CO2} = specific respiration rate, d^{-1}; DEA = denitrification enzymes activity, ng N_2O g^{-1} d^{-1}; NA = nitrogenase activity, nmol C_2H_4 $g^{-1}d^{-1}$

Table 5 PLFA structural and functional biomarkers (mol%) tested in Control (5A) and P enriched plots (5B). Values are averages of N=12 (high and low salinity involved). Layer effect in Control treatments (5C) and P effect in both layers (5D) were tested by ANOVA.

	STFA	MUFA	TBFA	CYFA	OHFA	SRB	Act	Fungi	Protozoa
Treatment means									
5A Control									
upper layer	32.21	21.15	19.41	14.17	2.15	10.64	1.70	0.55	0.20
lower layer	35.50	18.48	13.21	12.98	3.88	12.66	1.57	0.64	0
5B P effect									
upper layer	29.39	22.98	19.43	13.52	2.36	8.96	1.59	0.99	0.21
lower layer	35.25	20.68	13.82	15.08	3.22	11.88	1.78	1.00	0
p-value, ANOVA									
5C Layer effect in Control	0.028	0.035	0.001	ns	0.008	0.041	ns	ns	ns
5D P effect									
upper layer	0.045	0.041	ns	ns	ns	ns	ns	0.048	ns
lower layer	ns	0.048	ns	ns	ns	ns	ns	0.009	ns

Microbial community structure was only slightly changed by P addition. Relative distribution of MUFA and Fungi was enhanced in both layer samples (Table 5) and distribution of SRB was decreased but not significantly. Distribution of Fungi increased in both layers significantly thus having same value. Relative portion of TBFA, CYFA, OHFA, SRB, Actinomycetes and Protozoa was not changed by P effect. STFA decreased and MUFA increased in upper layer samples while in lower layer samples both were not changed.

Fig. 2 Sediment layer effect: Principal component analysis of PLFA fingerprints from Control treatment. Samples from upper (gray colour) and lower layer (dark colour) of the sediment profile are visualized. Amount of variability explained by PCA1 38.5%, by PCA2 30.6%. PCA scores revealed not significant differences between layers (P = 0.496, F = 0.48). Effect of water salinity, represented by High salinity marshes F6, F7 (lines, gray symbol) and Low salinity marshes F10, F12 (lines, dark symbol), likely contra-indicative effect of layer. One point is an average of three replicates.



Discussion

Sediment layer effect

Our results brought clear information about higher microbial biomass production in upper (1-10 cm) tropical marsh sediment in comparison to the lower sediment layer (10-30 cm). Organic matter content, available carbon, soil P and microbial biomass declined with increasing soil depth. Our results confirmed the findings in Everglades and other wetlands (Qualls and Richardson 1995; White and Reddy 2001; Gathumbi et al. 2005) declaring the decline in total soil nutrients with increasing soil depth. Explanation states that biochemical processes in deeper layers of sediment profile (>10 cm) are not as responsive to changes in the overlaying water chemistry and processes are driven by a more stable organic matter that possibly reflecting historical levels of nutrients (Corstanje et al. 2007). In the studied marshes, OM content significantly differed between the layers (Table 3A&C). Accumulating of OM in the form of poorly decomposed plant remains (White et al. 2004) could be an explanation for lowering OM content with increasing depth. Key role of OM is in providing not only C but also of other nutrients (P and N) and micronutrients thus creating more convenient environment for microorganisms. Upper layer was confirmed to be the place better supplied with available C (Cav) more likely via roots that were 4-fold higher in this part (Table 3A). High availability of carbon in upper layer was verified by increased MUFA/STFA ratio as available energy and aeration conditions are required for biochemical transformation of saturated fatty acids (STFA) on monounsaturated fatty acids (MUFA) (Fulco et al. 1964). Better conditions for microorganisms in upper layer were also confirmed by an increase of microbial biomass to total organic carbon ratio (MBC/TOC) too; the higher the ratio, the higher the availability of C or ability to use it (Anderson and Domsch 1989). Smaller part of microbial community was active in high nutrient conditions, according to PLFAtot/MBC ratio. We consider microorganisms to be less stressed by the lack of nutrients in the nutrient rich environment thus enabling less active portion of its biomass. Five-fold higher specific respiration rate (Q_{CO2}) in lower layer indicated higher catabolic activity of microbial community and higher energy demand to keep microbial cell alived (Anderson and Domsch 1990). Uhlířová et al. (2005) found higher specific respiration activity with increasing moisture and decreasing availability of C, comparable with lower layer in our survey. P was immobilized into the biomass rather than used for biomass formation in lower layer. This result supports our theory about less favorable conditions with increasing depth, more likely driven by insufficient amount of C. Additionally less extensive, energetically highly demanding processes denitrification and nitrogenase activity in lower layer are more likely results of limited microbial growth and availability of available carbon. All these results support our hypothesis stating upper, more organic, layer is microbiologically more active

with higher turnover of organic matter and higher release of nutrients back to the environment.

Contrary to our hypothesis, results of the present study generally did not indicate significant shifts in microbial community composition, based on PLFA patterns, between layers (Fig. 3). We consider salinity effect to contra-indicate layer effect on microbial community composition. Focused on microbial structure, using biomarker methodology, successfully employed in a wide range of environments to study the regional variation in microbial communities (Guckert et al. 1985; Findlay et al. 1990; Frostegård et al. 1993; Rajendran et al. 1994, 1997), significant differences between layers were found. PLFA structural and functional biomarkers revealed that community of our sediments were found to be dominated by STFA, MUFA, TBFA, Cy and OH. The presence of the characteristic polyunsaturated fatty acids (PUFA), indicating presence of protozoa (20:4ω6) and fungi (18:2ω6), revealed that microeukaryotes were always present in minor proportions and prokaryotes were in predominance. Corresponding phospholipids fatty acids are being associated with distinct groups of microbes as Gram-positive bacteria are generally characterized by iso/anteiso (TBFA) but also mid branched PLFA. Similarly, monounsaturated (MUFA), cyclopropyl-substituted (CyFA) and hydroxyl-substituted (OHFA) PLFA are signature fatty acids for Gram-negative bacteria (Frostegård and Bååth 1996; Zelles 1999). In both layers microbial community was dominated by Gram-negative bacteria (MUFA, CYFA, OHFA). However, with increasing soil depth different carbon sources for the two main groups of soil bacteria could be determined. Gram-negative bacteria were found to use preferably recent plant-derived carbon and Gram-positive bacteria to use older OM-derived carbon.

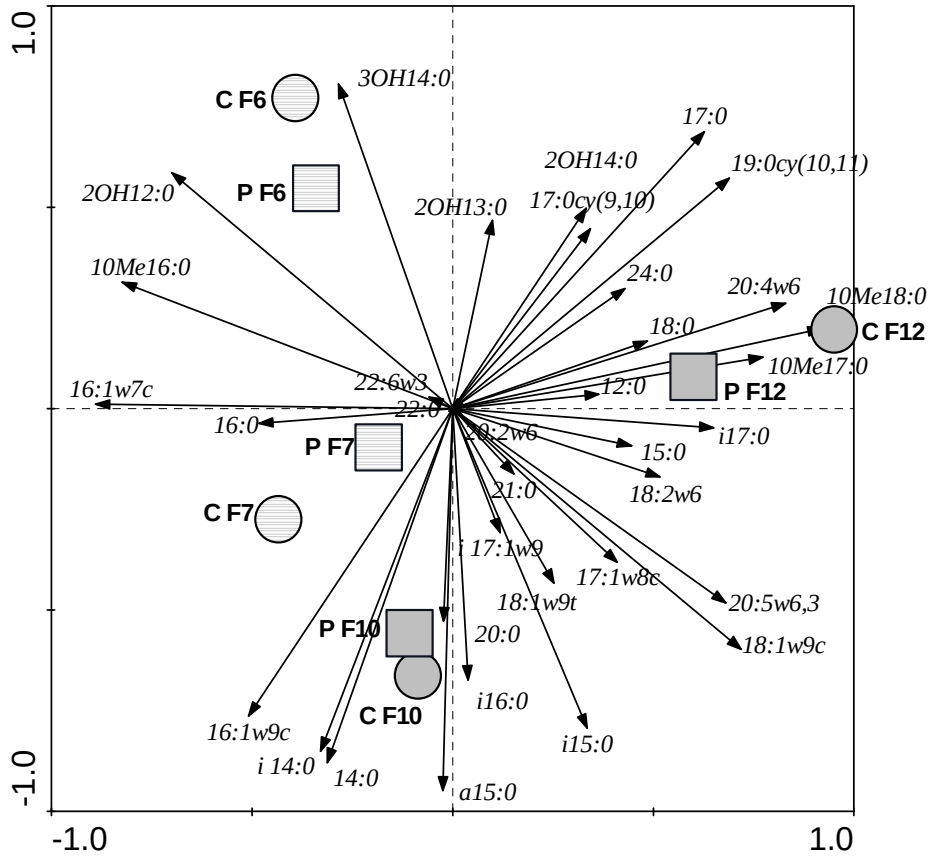
An increased portion of TBFA in upper layer was found to comply with significantly higher amount of root biomass and N processes rates (DEA, NA) that support our theory about the link of TBFA occurance and amount of N in root surroundings. Increased plant biomass was found to take up more inorganic N and support rhizospheric microorganisms by production of aminoacids (Weigelt et al. 2005). Aminoacids were found to be essentially needed primers for building up TBFA (Kaneda 1991). An increasing portion of hydroxyl-branched FA (OHFA) with increasing depth was found in our survey. The presence of hydroxyl-substituted FAs is related to anaerobic environment (O´Leary and Wilkinson 1988) that complied with our finding. Contrary to it cyclopropyl FAs that are also being connected with anaerobic conditions (Guckert et al. 1985) were not found to increase with increasing depth (Table 5). Sulfate reducing bacteria, indicated by 10Me16:0 biomarker (Dowling et al. 1986), contained in both layers more than 10 mol% but more abundant this group was in lower layer of the profile (12.66%). The increased portion of SRB in deeper layers (Table 5) can be attributed to low redox potential conditions, sulfate reduction process, in the

sediments that is in accord with results found elsewhere (Drake et al. 1996; Nielsen et al. 2001; White and Reddy 2001). The wetlands in Belize generally have high sulfate due to a significant proportion of gypsum in the underlying rock; and high rates of H_2S production have been documented (Rejmánková and Post 1996). Actinomycetes and both representatives of eukaryota (fungi and protozoa) created only minor part of microbial community (less than 2 mol%). From these only Protozoa were confirmed to be specifically present in upper layer, ie. more oxygenated part of the profile (by roots).

P addition effect

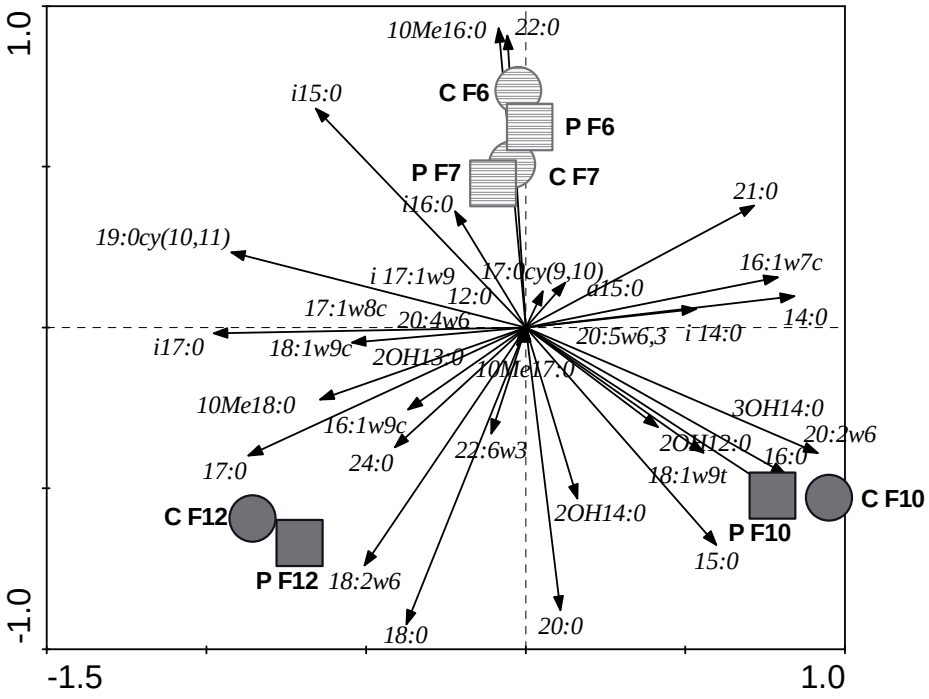
Our results brought clear information about significant stimulating P-effect on the microbial biomass and activity in this type of ecosystem. Based on above mentioned differences between layers following P addition it could be concluded that P addition decreased differences between layers (namely in C availability, microbial biomass C, microbial PLFA, DEA and specific respiration) by an enhancement of these variables more in lower layer samples compared to upper layer samples that conforms to our hypotheses.

Fig. 4 P addition effect in upper layer: Principal component analysis of PLFA fingerprints of upper layer samples from Control treatment (circle symbol) and P treatment sites (square symbol). Amount of variability explained by PCA1 39.2% and PCA2 27.7%. PCA scores revealed not significant differences between layers (P = 0.378, F = 0.81). High salinity marshes F6, F7 (lined symbol) and Low salinity marshes F10, F12 are depicted. One point is an average of three replicates.



Increased P availability in marsh system, measured as oxalate extractable P (P_{ox}), changed the overgrowth from originally cyanobacteria and sparse *Eleocharis* in P unenriched sites (Table 1) with net primary production (NPP) 180-219 g $m^{-2}y^{-1}$ to dense overgrowth of *Eleocharis* spp. in P enriched areas (218 - 481 g $m^{-2}y^{-1}$). This microphyte/macrophyte change gradually led to an increase of OM content in upper layer of the sediment profile and of root biomass quantity (2-fold) in both layers (Table 3; Černá et al. 2009). The change in plant production after P addition was in our survey followed by an increase of microbial biomass, both C and total PLFA, as well as microbial biomass P that is in agreement with observations in Everglades wetland system (DeBusk and Reddy 1998; White and Reddy 2001;). Lower layer reacted on P addition by higher enhancement of microbial biomass C (4-fold) and total PLFA (2-fold) compared to upper layer where microbial biomass increased 2-fold and total PLFA less than 2-fold. Immobilization of P, opposite to microbial biomass C, increased after P addition more in upper layer (6-fold) compared to lower layer (3-fold). Enhancement of microbial activities, denitrification and nitrogen fixation potentials after P addition confirmed the findings of others (DeBusk and Reddy 1998; Ingersoll and Baker 1998; Qualls and Richardson 2000; Bastviken et al. 2005) that addition of P, as a primary limiting factor, increased biochemical processes. Both activities measured, are energy demanding processes. N transformations indicated an importance of an indirect effect of nutrient supply via plant growth and litter quantity and likely also quality. We consider an increase of microbial activities to be more likely ruled by amount of available carbon. Lowering oxygen concentrations by supplying with available organic carbon favors activity of mainly denitrifiers (Reddy and Patrick 1984; D´Angelo and Reddy 1999). Increase of denitrification was higher in lower layer (9-fold) compared to upper layer (2-fold). As previously confirmed (Černá et al. 2009) P addition controls nitrogenase activity mainly via root development that explains more intensive process in upper layer (800-fold compared to 26-fold in lower layer). Nutrient, growth and stress status increased by P addition, as hypothesized. Independently on profile layer P addition increased C availability, as documented by both Cav and MU/STFA indicators. Zelles et al. (1992) identified a relationship between MUFAs and high C availability in agriculture soil and by Bossio and Scow (1998) in wetland sediments. P addition mediated increase of microbial biomass-to-organic C ratio (MBC/TOC) refers to improved conditions for microorganisms; as mentioned above the higher the ratio, the higher the availability of C or ability to use it (Anderson and Domsch 1989). It is evident from the presented results that amount of viable biomass portion decreased (PLFAtot/MBC) 2-fold in both layers with P nutrient supply. Decrease of stress condition in P enrichment sites was determined by lowering energy demand to keep microbial cell alive (Q_{CO2}) but only in lower layer.

Fig. 5 P addition effect in lower layer samples: Principal component analysis of PLFA fingerprints of lower layer samples from Control treatment (circle symbol) and P treatment sites (square symbol). Amount of variability explained by PCA1 40.5% and PCA2 24.5% PCA scores revealed not significant differences between layers (P = 0.845, F = 0.04). High salinity marshes F6, F7 (lines symbol) and Low salinity marshes F10, F12 are depicted. One point is an average of three replicates.



However, less stress conditions was not confirmed neither by a change of trans/cis configuration ratio of 18:1ω9 fatty acid nor by ratio of cyclopropane fatty acid 17:0cy(9,10) to its precursor 16:1ω7 (data not shown). Trans/cis ratio was found to be 2.5 for both layers and according to Kimura et al. (2001) the value of more than 1 indicates stress. Cy17/precursor was found to be 0.30 and values around 0.05 indicates well-being conditions and also shift from aerobic to anaerobic conditions value; value higher than 2.5 indicates stress (Navarrete et al. 2000; Uhlířová et al. 2005).

Our results did not reveal significant shifts in microbial community composition after P addition in respective layers. Focused on microbial structure, P addition significantly increased relative distribution of MUFA and Fungi in both layers. In contrast to results of Drake et al. (1996) sulfate reduction potential (the distribution of SRB) was not enhanced by P addition.

CONCLUSION

We confirmed upper sediment layer to be more active part of marsh sediment. P loading was proven to enhanced microbial biomass and activities to depth of 30 cm; amount of microbial biomass and denitrification enzyme activity were increased and stress to microorganisms was decreased more in lower layer compared to upper layer. Six years after the initiation of

long-term field P enrichment experiment organic matter content and P immobilization into microbial biomass increased only in upper layer of the profile.

Microbial community indicators of amount of C and N in the environment (MUFA and TBFAs) decreased with increasing depth, while the portion of MUFA only increased with P loading. Sulphate-reducing bacteria were more abundant in lower layer and P addition did not affect its distribution. Microbial community composition was markedly altered neither by layer location nor by P enrichment.

We assume the water salinity play important role in the community composition besides to sediment layer and P addition effect; additional studies are needed to separate the influence of the salinity to confirm our findings.

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CHAPTER 3

Paper II

Heterotrophic nitrogen fixation in oligotrophic tropical marshes: changes after phosphorus addition

Černá B, Rejmánková E, Snyder JM, Šantrůčková H

Hydrobiologia 627 (2008): 55-65

See file: **PhD_Thesis-Barbora_Pivnickova-Paper_II.pdf**

Abstract

In order to determine the impact of nutrient enrichment on phosphorus (P) limited wetlands, we established experimental P additions in marshes throughout northern Belize. P significantly increased macrophyte primary production, which led to the rapid elimination of cyanobacterial mats. The replacement of cyanobacterial mats by macrophytes constrained autotrophic nitrogen (N) fixation, increased the quantity, and changed the quality of organic matter input to the sediments. We predicted that the activity of sediment heterotrophic N fixers will be impacted by these alterations in carbon input. We used the acetylene reduction technique to measure potential (glucose amended) nitrogenase activity (NA) in sediments from controls and treatment plots that have been P enriched for four years and dominated either by *Eleocharis cellulosa*, or *Typha domingensis* for two years. NA in P-enriched plots was 2–3 orders of magnitude higher than NA in controls. NA was positively correlated with the soil reactive P, both total organic and microbial carbon, live root biomass, and total phospholipid fatty acids (PLFA) as an indicator of active microbial biomass. It was negatively correlated with the concentration of ammonium-N. Path analysis revealed that the indirect effect of P on NA through the root biomass was more important than the direct effect of P. NA of the upper sediment layer was consistently higher in *Eleocharis* than in *Typha* dominated plots, despite the higher litter input by *Typha*. We feel that the higher levels of lignin and phenolics occurring in *Typha* litter, relative to *Eleocharis*, constrained NA in *Typha* plots.

Abstrakt

Experimentálním pokusem přídavku fosforu (P) byl sledován vliv této živiny na mokřady v severní části Belize, které jsou P limitované. Přídavek P významně zvýšil primární produkci vyšších rostlin, což vedlo k rychlé eliminaci nárostů sinic. Nahrazení sinicových nárostů vyššími rostlinami omezilo autotrofní fixaci dusíku (N), zvýšilo množství a změnilo kvalitu organické hmoty vstupující do sedimentu. Předpokladem bylo, že aktivita heterotrofních sedimentárních fixátorů dusíku bude ovlivněna změnami ve zdroji vstupujícího uhlíku. Technika redukce acetylenu byla použita k měření potenciální (s přídavkem glukózy) nitrogenázové aktivity (NA) v sedimentech z kontrolních a v pokusných plochách, které byly obohaceny přídavkem P po dobu čtyř let. V pokusných plochách byl, po dobu dvou let, dominatním porostem bahnička (*Eleocharis celullosa*), nebo orobinec (*Typha domingensis*). NA byla na plochách s přídavkem P o 2-3 řády vyšší než v kontrolních plochách. NA byla pozitivně korelována s půdním reaktivním P, celkovým organickým i mikrobiálním uhlíkem, biomasou živých kořenů, a celkovým množstvím fosfolipidických mastných kyselin (PLFA) jako ukazatelem aktivní mikrobiální biomasy. NA byla negativně korelována s koncentrací amoniakálního dusíku. Path analýza ukázala, že nepřímý vliv přídavku P na NA přes biomasu kořenů byl důležitější než přímý vliv přídavku P. NA v horní vrstvě sedimentu byl soustavně vyšší v plochách s dominantou *Eleocharis* než s *Typha*, přestože *Typha* poskytovala větší množství opadu. Domníváme se, že vyšší množství ligninu a fenolů v opadu *Typha*, v porovnáná s *Eleocharis*, omezovala NA.

Podíl Barbory Pivničkové – 60%

CHAPTER 4

Paper III

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

Pivničková B, Rejmánková E, Snyder JM and Šantrůčková H

Plant and Soil; accepted 13th May 2010, DOI 10.1007/s11104-010-0439-6

See file: **PhD_Thesis-Barbora_Pivnickova-Paper_III.pdf**

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 phospholipids 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 generally 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.

Abstrakt

Oligotrofní, fosforem (P) limitující travinné typy mokřadů v severní Belize jsou ovlivněny vstupem P z hnojiv. Přídavek P způsobuje posun od autotrofních společenstev (sinicové nárosty, CBM) k systému s dominantou vyšších rostlin (bahnička, *Eleocharis* spp.; orobinec, *Typha domingensis*). Vzorky sedimentů z ploch s přídavkem P, z mokřadů mající 2 úrovně salinity vody, byly použity pro zjištění vlivu přídavku P, salinity vod mokřadů, a druhu vyšších rostlin na heterotrofní mikrobiální společenstva (popsaná především specifickými fosfolipidickými mastnými kyselinami a specifickou respirační rychlostí), biomasu a aktivity, byly použity. Přídavek P zvýšil uhlík (C), dusík (N) a P v mikrobiální biomase, stejně jako rychlosti přeměn živin v sedimentech (nitrogenázová aktivita, mineralizace N a zabudování N do mikrobiální biomasy, metanogeneze). Vliv druhu rostliny (plochy *Eleocharis* vs *Typha*) byl obecně nižší než vliv přídavku P (plochy CBM vs *Eleocharis*) a byl více průkazný v mokřadech s nízkou salinitou vod; plochy s dominantou *Eleocharis* měly vyšší nitrogenázovou aktivitu a větší zabudování P do mikrobiální biomasy. Salinita snížila rychlosti mikrobiálních procesů; snížilo pozitivní vliv přídavku P a vlivu druhu rostlin na mikrobiální aktivity. Množství N v mikrobiálních buňkách, pravděpodobně ve formě osmoprotektivních složek, bylo salinitou zvýšeno.

Podíl Barbory Pivničkové – 60%

CHAPTER 5

Paper IV

Nutrient enrichment in tropical wetlands: shifts from autotrophic to heterotrophic nitrogen fixation

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Abstract

We used established long-term experimental P additions plots in freshwater marshes of northern Belize to determine the impact of P addition on nitrogen (N) fixation. Marshes with different conductivities and sulfate concentrations were selected to elucidate the effect of salinity and the contribution of sulfur reducing bacteria to the overall N fixation. N fixation of collected marsh sediments, roots, and cyanobacterial mats were measured in laboratory incubation experiments (acetylene reduction assay calibrated by $^{15}N_2$ reduction assay) with and without the addition of sodium molybdate (sulfur reducing bacteria inhibitor). P has increased macrophyte primary production significantly, which led to the rapid elimination of cyanobacterial mats and the elimination of autotrophic N fixation. P addition enhanced heterotrophic N fixation in both the sediments and rhizosphere due primarily to increased C supply to the sediment. When expressed on a dry weight basis, root associated N fixation was higher than sediment N fixation, but the contribution of the root associated fixation to the total N fixation was small when expressed per square meter. Sulfur reducing bacteria were a important component of N fixation contributing from 20 to 53 % to the overall N fixation. A simple N budget was created to determine if N demands are met following P addition. The heterotrophic N fixation substituted in part for autotrophic cyanobacterial N fixation when P limitation was alleviated.

Abstrakt

Sladkovodní mokřadní plochy, s dlouhodobým experimentálním přídavkem fosforu (P) v severní Belize, byly použity pro sledování vlivu přídavku P na fixaci dusíku (N). Mokřady s rozlišnou vodivostí a koncentrací sulfátů byly vybrány k objasnění vlivu salinity a přispění sulfur redukujících bakterií k celkové fixaci N. Fixace N vybraných mokřadních sedimentů, kořenů a sinicových nárostů byla měřena laboratorními inkubačními experimenty (analýza redukce acetylenu kalibrovaná analýzou redukce 15N2) s přídavkem a bez přídavku molybdenanu sodného (inhibitor sulfur redukujících bakterií). P zvýšil primární produkci vyšších rostlin, což vedlo k rychlé eliminaci sinicových nárostů a k eliminaci autotrofní fixace N. Přídavek P zvýšil heterotrofní fixaci N v sedimentech i v rhizosféře a to především díky zvýšenému zásobení sedimentu uhlíkem. Fixace N kořeny byla vyšší než fixace N sedimentem při vyjádření na sušinu, ovšem při vyjádření na čtvereční metr příspěvek fixace N kořeny k celkové fixaci N byl nízký. Sulfur redukující bakterie byly důležitou složkou fixace N a přispívaly 20-53% k celkové fixaci N. Jednoduchá bilance N objasnila pokrytí požadavku N po přídavku P. Heterotrofní fixace N částečně nahrazuje autotrofní fixaci N prováděnou sinicemi v podmínkách se zmírněnou limitací P.

Podíl Barbory Pivničkové – 15%

CHAPTER 6

CONCLUDING REMARKS

Functional and structural diversity of heterotrophic microorganisms in oligotrophic tropical wetlands was evaluated in relation to P nutrient content (experimentally added).

Heterotrophic microbial activities (C, N, P transformation processes), biomass and community physiological status are shown to be somehow affected by the **effect of P addition** (cyanobacteria dominated plots vs. *Eleocharis* spp. dominated plots) as well as by **P addition effect within sediment profile layer** (mineral vs. organic), by **effect of plant species cover** (*Eleocharis* spp. vs. *Typha*) and by **water salinity effect** (conductivity of 0.2 – 7 mS).

Special interest was given to **heterotrophic nitrogen fixation process** (nitrogenase activity) in sediment as the crucial activity in N supply after elimination of autotrophic N_2-fixing process.

Finally some of the microbial and environmental parameters are recommended to be used as early warning indicators for ecosystem changes due to P loading.

Conclusions are presented as answers to given *hypotheses*.

HY 1 *Elimination of cyanobacterial mats in P-enriched plots shifts the microbial activity from autotrophs to heterotrophs and increases the heterotrophic microbial activity in the sediment; P addition will enhance the nutrient content (N and P stoichiometry) in microbial biomass either directly by removing P limitation or indirectly by increased available C from macrophyte exudation and litter*

CONCLUSION

- The addition of P to experimental plots significantly increased aboveground production and ratio of shoot to root, which led to elimination of both floating and benthic cyanobacterial mats; this switch from microphyte to macrophyte dominated autotrophic production gradually led to an increase of total soil C and N contents and its availability in sediment, and microbial nutrient contents.
- Elimination of cyanobacteria shifted the microbial activity from autotrophs to heterotrophs as documented by increased heterotrophic N-fixation
- Heterotrophic microbial activities (nitrogen mineralization, denitrification enzyme activity, nitrogenase activity and methanogenesis) were enhanced by P addition, while aerobic and anaerobic respiration were not significantly affected
- Microbial biomass C, N and P was positively affected by P addition supporting the assumption that P is the limiting nutrient to the microbial biomass; P limitation was confirmed by decrease of C/P ratio in microbial biomass in P enriched soils.

HY 2 *Based on previous results (Rejmánková et al. 2008) that showed different plant biomass production, nutrient uptake and resorption from senescing tissues of Typha and Eleocharis, we expect different quality (C, N, P stoichiometry) and quantity of their litter to affect heterotrophic microbial biomass and activity*

CONCLUSION

- P addition increased production of the shoot and root biomass of both *Eleocharis* and *Typha;* the differences in plant biomass production between the two species were not significant; *Eleocharis* had higher root to shoot biomass ratio compared to *Typha*
- Both plant species had similar N content in roots and shoots; *Typha* compared to *Eleocharis* had more C but less P in shoots, and two-times higher P content in roots.

- The relatively weak plant species effect on microbial biomass and processes corresponded to small differences in plant litter stoichiometry; plant species significantly impacted mainly the transformation of P: microbial P immobilization in *Typha* plots was lower eventhough *Typha*'s left more inorganic P behind in sediment *(Typha* behaved as a competitor, released less extracellular phosphatases). *Eleocharis* supported a more balanced system with microorganisms kept in fast turnover, while microorganisms in *Typha* plots were more dependent on P addition.
- *Eleocharis* debris affected mainly heterotrophic nitrogen fixation by providing higher amount of P to sediment

HY 3 *P enrichment will affect microbial activities and community composition in both sediment layers of the profile (organic and mineral)*

CONCLUSION

- upper (organic) layer compared to lower (mineral) sediment layer was confirmed to have higher organic matter content, higher microbial biomass C, N, P and N turnover processes (denitrification and nitrogenase activity potentials)
- P addition changed (at least partly) nutrients metabolism in both layers thus having a potential for response to P loading; upper layer was found to be more active but microbial activities and biomass increased after P addition more significantly in the deeper mineral sediment layer compared to upper layer
- P addition increased organic matter content and microbial biomass P only in upper layer of the sediment
- For effect of sediment layer on microbial community composition, see conclusions to HY 6

HY 4 *Rate and direction of the changes in microbial activities, biomass and community composition will be salinity dependent. Salinity will cause stress to microorganisms, more energy will be invested to osmotic regulation that will constrain heterotrophic activities, microbial biomass*

CONCLUSION

- salinity weakened P addition effect and plant species effect on microbial processes
- saline plots were found to be microbiologically less active, with lower N mineralization, denitrification and metanogenesis; effect of salinity on N-fixation was not conclusive
- salinity limited microbial biomass growth resulting in decreased effectiveness of C usage
- salinity decreased shoot and root C, C/P and C/N ratios and weakened positive effect of P addition on shoot biomass and C/P ratio
- salinity caused an increase of N amount in biomass (likely to form osmolytic compounds of N origin to solve the stress)
- For effect of salinity on microbial community composition, see conclusions to HY 6

HY 5 *The replacement of cyanobacterial mats by macrophytes following P addition limits N-fixation by cyanobacteria (Rejmánková and Komárková 2000). We expect that, due to processing plant litter with high C/N ratio, soil microorganisms will require extra N to produce new microbial biomass. Whether this will eventually lead to competition between plants and microorganisms for N will depend on the availability of organic matter, and, consequently, heterotrophic N-fixation potential. Rate of heterotrophic N-fixation will be plant species and salinity dependent*

e) Heterotrophic N-fixation will be higher in rhizosphere than in sediment and will be enhanced by P addition

f) The input of N_2 from autotrophic N-fixation can be replaced by heterotrophic N-fixation after P addition

g) *In Typha dominated plots, the large quantity of litter and larger amount of C-rich exudates will result in higher demand by sediment microorganisms for N and will lead to higher heterotrophic N-fixation than in Eleocharis dominated plots*
h) *Heterotrophic N-fixation, as high energy demand activity, will be constrained by salinity*

CONCLUSION

- Since the P addition into the experimental plots resulted in decline of N-fixing cyanobacteria, N became the limiting nutrient in the ecosystem. As a consequence, cyanoabacterial autotrophic N-fixation was substituted in the sediments by heterotrophic N-fixation. Highest rates of N-fixation where measured in the rhizosphere but because the root areas cover only a small fraction of the soils, they didn't contribute significantly to the total ecosystem N-fixation
- N-fixation was found to be strongly dependent on available C and root biomass
- heterotrophic N-fixation partially substituted for autotrophic cyanobacterial N-fixation when P limitation was alleviated
- *Eleocharis* dominated plots had consistently higher N-fixation compared to *Typha* dominated plots in organic layer of sediment; in the lower sediment layers the trend was opposite
- Effect of salinity on N-fixation was not conclusive

HY 6 *Physiological status of microbial community (represented especially by microbial community composition, specific phospholipids fatty acids and specific respiration rate) will be affected by effects of P addition, plant species cover, sediment layer and water salinity*

CONCLUSION

- Higher recorded respiration per cell (specific respiration rate; higher cellular energy demand) where found in saline sites and sites affected by increasing amount of organic matter
- Portion of active microorganisms (PLFAtot-to-MBC ratio) increased with salinity and with depth
- Microbial community composition (fingerprint) was not significantly changed by none of effects - P addition, sediment layer, plant species and salinity
- Relative distribution of microbial community structure biomarkers was partly changed by the above mentioned effects as follows:

a) Monounsaturated fatty acid-to-saturated fatty acids ratio, an indicator of C availability; increased after P addition and in upper layer
b) Terminally branched fatty acids (TBFA), an indicator of N presence, increased with salinity
c) PLFA biomarkers of diazotrophs (10Me16:0; anaerobic SRB) formed relatively constant part, 5%, of the microbial community and:
    - increased with depth and in *Typha* plots
    - contributed to the overall diazotrophic activity in sediment and rhizosphere (within upper layer) samples by about 50% and 20%, respectively.
    - Increased with an organic C input

Our data showed that increased amount of P (both in microbial biomass and sediment), amount of available carbon, switch from autotrophic to heterotrophic N-fixation, higher ratios of monounsaturated to saturated fatty acids in the microbial communities are good indicators of significant negative ecosystem changes in Belize wetlands caused by overloading of excessive P.