Influence of macrophyte species on microbial density and activity in constructed wetlands

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Abstract It is often assumed that planted wastewater treatment systems outperform unplanted ones, mainly because plants stimulate belowground microbial population. Yet, fundamental interactions between plants and associated microorganisms remain only partly understood. The aim of our project was to evaluate microbial density and activity associated to the rhizosphere of three plant species. Experimental set-up, in six replicates, consisted of four 1.8-L microcosms respectively planted in monoculture of *Typha angustifolia*, *Phragmites australis*, *Phalaris arundinacea* and unplanted control. Plants were grown for two months with 25 L m^{-2} d^{-1} of secondary effluent (in g m^{-2} d^{-1}: 1.3 TSS, 7.5 COD, 1.0 TKN). Sampling of substrate, roots and interstitial water was made according to depth (0–10, 10–20 cm). Biofilm was extracted with 500 mL of a buffer solution. Microbial density was directly estimated by flow cytometry and indirectly by protein measurements. Biological activity was determined using respirometry assays, dehydrogenase and enzymatic activity measurements.

Our results show that microbial density and activity are higher in the presence of plants, with significantly higher values associated with *Phalaris arundinacea*. Greater density of aerobic or facultative bacteria was present in planted microcosm, particularly on root surface, suggesting root oxygen release. Microbes were present on substrate and roots as an attached biofilm and abundance was correlated to root surface throughout depth. Plant species root morphology and development seem to be a key factor influencing microbial–plant interaction.

Keywords Constructed wetlands; microbial activity; microbial density; plant species; rhizosphere

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Introduction

It is generally assumed that planted wetlands outperform unplanted controls, mainly because plant rhizosphere stimulates microbial communities. Yet, fundamental interactions between plants and associated microorganisms remain poorly understood. It has been suggested that plant rhizosphere enhances microbial density and activity by providing root surface for microbial growth, a source of carbon compound through root exudates and a micro aerobic environment via root oxygen release (Gersberg *et al.*, 1986; Brix, 1997). Higher microbial densities in planted systems were reported in full scale constructed wetlands, with 2.7×10^6 CFU/g of gravel evaluated by culture method (Hatano *et al.*, 1993) and in planted microcosms with 3.2×10^9/g of gravel measured by fluorescence microscopy (Münch *et al.*, 2005). Collins *et al.* (2004) showed that, while microbial populations will grow on any surface, plants influence microbial composition and abundance. Microbial density can also differ according to plant species, with greater densities observed with *Phragmites australis* compared to *Phalaris arundinacea* (Vymazal *et al.*, 2001) and *Typha angustifolia* (Hatano *et al.*, 1993). Plant species specific morphology can also influence microbial density as suggested by Kyambadde *et al.* (2004), who measured higher root surface and microbial density in a constructed wetlands planted with *Cyperus papyrus* compared to *Miscanthidium violaceum*. Plant

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species differ in the amount of root exudates they produce (Zhu and Sikora, 1995) and by root oxygen release (0.02–12 g O_2 m^{-2} day^{-1}) as reviewed by Brix (1997), which may in turn influence microbial communities. Several microbial parameters have been measured in constructed wetlands, including biofilm components (protein, phospholipids, exopolysaccharide), enzymatic activities (protease, phosphatase, urease) and respiration (dehydrogenase) (Hatano *et al.*, 1993; Shackle *et al.*, 2000; Liang *et al.*, 2003; Larsen and Greenway, 2004; Ragusa *et al.*, 2004). However, most studies generally evaluate only one or two microbial parameters and rarely associate those findings to plant presence or species.

Our project aimed at evaluating the influence of the presence of plants and possible differential effects of plant species on microbial density and activity in a well replicated experiment using microcosms. We also assessed microbial spatial distribution according to depth and localisation (on root, sand or free living).

The presence of plants significantly enhanced microbial density and activity with significantly higher values associated with *Phalaris arundinacea* than with *Typha angustifolia* and *Phragmites australis*. Plant species morphology and development seem to be a key factor influencing microbes in microcosm.

Methods

The experiment was conducted under greenhouse conditions in microcosms (16 × 4 × 28 cm) filled with 2 mm silica sand (porosity: 0.33). The experimental set-up, in six replicates, consisted of four microcosms respectively planted in monoculture of *Typha angustifolia*, *Phragmites australis*, *Phalaris arundinacea* and unplanted control. Four platelets (produced by *in vitro* technique) were planted per microcosm. Microcosms were fertilised for the first 2 months to insure appropriate plant establishment. During the following 2 months, the microcosms were fed with 25 L m^{-2} d^{-1} of reconstituted fish farm effluent (in g m^{-2} d^{-1}: 1.3 TSS, 7.5 COD, 1.0 TKN). Then, microcosms were entirely sampled and analysed. After plant harvesting, interstitial water was slowly pumped (200 mL), from depths between 0–10 cm and 10–20 cm, then sand and roots were collected from each depth (0–10 and 10–20 cm). Biofilm was extracted by washing the samples (sand and roots) with 500 mL phosphate buffer (K_2HPO_4 9.3 g/L; KH_2PO_4 1.8 g/L) for 5 min. Extracted biofilm (500 mL) and interstitial water (200 mL) were combined and filtered (Ø: 112 μm) to eliminate rootlets. For one replicate, biofilm on sand and roots was extracted and analysed separately in order to assess spatial pattern of microbial density and activity in the microcosms.

Protein contents were determined using bicinchoninic acid (BCA, Sigma-Aldrich) and the method provided by Smith *et al.* (1985). Bacteria were counted by flow cytometry (FACScan, Becton and Dickinson, San Jose, USA) on pre-fix extracted solution (1% glutaraldehyde) stored at 4 °C. Pre-fix extracted solution (50 μL) was diluted 20 times, and stained with 5 μM final concentration of fluorescent dye (SYTO-13, Molecular Probes) for 10 min. A known concentration of 1 μm florescent bead (FluoSpheres, Invitrogen) was added as standard to the sample. The concentration of bacteria was determined by comparing the amount of fluorescent beads to the number of bacteria counted (adapted from Del Giorgio *et al.*, 1996). Potential aerobic respiration was measured with a respirometer (AER-200, Challenge Environmental Systems) on 500 mL of extracted solution, under continuous agitation for 5 hours. Enzyme activity was measured using fluorescein diacetate (FDA, Sigma-Aldrich) with a protocol adapted from Adam and Duncan (2001): 20 μL of FDA (2 mg/mL) was added to 1 mL of extracted solution and incubated for 30 min at 30 °C under agitation. Reaction was stopped with 0.5 mL of chloroform/methanol (2:1 v/v) and aqueous phase was measured by absorbance (490 nm) and

compared to standards. Dehydrogenase activity was measured by the reduction rate of p-iodonitro-tetrazolium violet (INT, Molecular Probes) to INT-Formazan (INT-F). Dehydrogenase was assessed using 250 µL of INT (4 mM) and 1 mL of extracted biofilm solution, incubated during 1 hour at room temperature. Reaction was stopped with 250 µL of formaldehyde (37%) and the Formazan precipitate was extracted using methanol for 30 min. Supernatant absorbance (480 nm) was measured and INT-F concentration was calculated using Beer-Lambert's law ($\epsilon = 15{,}500\,M^{-1}\ cm^{-1}$). Root surface was measured using WinRHIZO 4.1c software (Regent Instruments, Canada) on representative root samples of known dried weight. The entire root surface was estimated using the proportion surface/weight of root samples extrapolated to the whole root mass present in the microcosm.

Results and discussion

Influence of the presence of plants on microbial density and activity

The presence of plants enhanced microbial density and activity in the microcosms (Table 1). Our results showed a bacterial density ratio of 10.3 between planted and unplanted microcosms. This is in the range (7–13) found in other studies conducted in experimental microcosm (Münch *et al.*, 2005) and in full scale constructed wetlands (Hatano *et al.*, 1993).

There were more aerobic or facultative bacteria in planted microcosms, as measured by microbial densities and aerobic respiration rate (Table 1). This supports the assumed role of plants in generating micro aerobic environment in the rhizosphere (Brix, 1997; Wießner *et al.*, 2002). Greater microbial densities and activities in planted microcosms can also be explained by high carbon availability in the rhizosphere resulting of root exudates (Andrews and Harris, 2000; Karjalainen *et al.*, 2001). Global enzymatic activities were significantly higher with plant presence (Figure 1(d)). While this may suggest greater microbial activity, direct plant release of enzymes may also have contributed: plant roots can exude a variety of other compounds, including a small quantity of enzymes (e.g. amylase, phenolase, phosphatase, protease) (reviewed in Uren, 2001). Further investigations must be made to evaluate the possible production of enzymes by macrophyte species used in constructed wetlands and their interaction with pollutants. It has also been suggested that plants enhanced microbial density by providing a surface for microbial growth (Gersberg *et al.*, 1986). Within planted microcosm (all species confounded), root surface was correlated to bacterial density ($r = 0.91$, $P < 0.001$), microbial aerobic respiration ($r = 0.78$, $P < 0.001$), dehydrogenase activity ($r = 0.65$, $P < 0.01$), protein content ($r = 0.58$, $P < 0.05$) and enzyme activities ($r = 0.57$, $P < 0.05$). In all cases, microbial density and activity were poorly correlated to root biomass ($r = 0.02$–0.13, $P = 0.31$–0.48). It must be noted, however, that root surface is also highly correlated to the relative abundance of young roots and rootlets. Thus, the correlation between root surface and microorganisms may in fact indirectly be related to

Table 1 Total microbial density and activity in planted (mean of all species) and unplanted microcosms

	Bacterial density 10^{11} bacteria microcosm^{-1}	Protein content mg protein microcosm^{-1}	Dehydrogenase activity 10^{-5} mol INT-F. h^{-1} microcosm^{-1}	Enzymatic activity mg fluo. h^{-1} microcosm^{-1}	Aerobic respiration rate mg O_2 h^{-1} microcosm^{-1}
Planted	2.0	29.7	3.0	155.5	9.1
Unplanted	0.2	10.7	0.9	59.5	2.2
Ratio	10.3	2.8	3.5	2.6	4.1

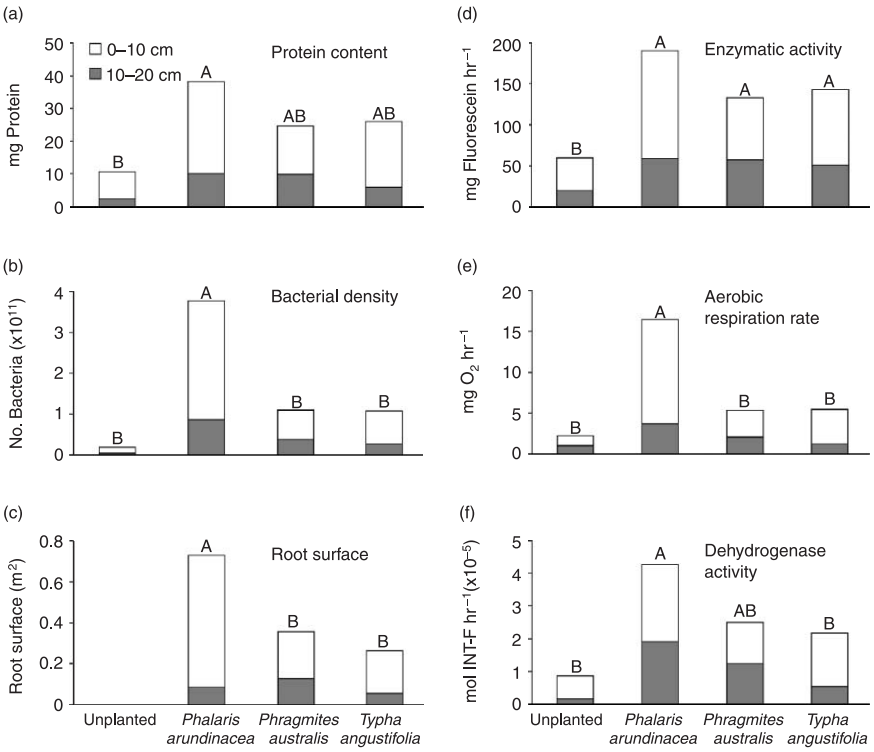

Figure 1 Microbial densities, activities and root surface per microcosm depending on depth (0–10, 10–20 cm) (A,B significant value $P < 5\%$) (mass of sand per depth zone 0–10, 10–20 cm = 700 g)

root oxygen release and exudates coming from young roots and rootlets (Armstrong and Armstrong, 1988; Andrews and Harris, 2000).

Influence of plant species on microbial density and activity

Planted microcosms of each plant species always had higher microbial density and activities than unplanted controls, although the differences with *Phragmites* and *Typha* were not always statistically significant (Figure 1). There were also differences between plant species, with significantly higher values associated with *Phalaris* compared to *Typha* and *Phragmites*, at least for some measures (Figure 1). We hypothesised that plant species particular root and shoot morphology was a key factor influencing microbial density and activity. It has been shown that oxygen release rate by plants is strongly correlated to above ground biomass (Wießner *et al.*, 2002). In our study, *Phalaris* had the highest above ground biomass ($r = 0.93$, $P < 0.001$), the greatest number of stems per microcosm ($r = 0.96$, $P < 0.001$) (Table 2) and the highest root surface ($r = 0.91$, $P < 0.01$) (Figure 1). The difference in microbial aerobic respiration rates ($16\,\mathrm{mg\,O_2\,h^{-1}}$ microcosm^{-1} with *Phalaris* compared to about five for *Phragmites* and *Typha*) can also be due to the plant species differences in root oxygen release, as reported by Brix (1997).

Higher microbial density and activity associated with *Phalaris* was most likely caused by its rapid colonisation strategy, passing from 4 to 88 stems on average per microcosm during the first growth season (Table 2). In comparison, *Phragmites* and *Typha* did not reach maturity during the same period. In another study, *Phalaris* was shown to reach its maximum biomass faster (1–2 years) than *Phragmites* (3–4 years) (Vymazal and Krópfelová, 2005). Plant-microbe interaction will certainly change with time, depending

Table 2 Plant species biomass (below and aboveground) and shoot number after the first growth season

	Below ground biomass (g)	Above ground biomass (g)	Shoot number
Phalaris arundinacea	11.1 ± 3.1	57.3 ± 8.1	88 ± 24
Phragmites australis	5.7 ± 2.4	23.8 ± 10.6	32 ± 5
Typha angustifolia	19.7 ± 5.3	17.3 ± 4.8	6 ± 2

on plant species establishment period and other parameters such as organic matter accumulation or plant litter characteristic (Chazarenc and Merlin, 2005).

Plant species influence on microbial spatial distribution

Root spatial dispersion differs between plant species, resulting in specific microbial distribution. *Phalaris* and *Typha* had significantly ($P < 0.05$) higher root surface in the upper part of the microcosm (0–10 cm) and greater microbial density and activities in this region ($P < 0.01$) (Figure 1). In contrast, *Phragmites* root surface was similar throughout depth (0–20 cm), as were microbial density and activity. Unplanted control also had significantly higher density and activities in the upper part of the microcosm (0–10 cm), except for aerobic respiration rate, which was equally low at both depths. We believe that these higher values in the first 10 cm of the unplanted control are explained by alga proliferation.

Our results indicate that microbes were mainly located as an attached biofilm on substrate and root surface, while interstitial water had only a small amount of protein content and near zero microbial activities. However, a low enzymatic activity was detected in interstitial water, indicating the presence of free and active enzymes. Microbial aerobic respiration was higher on root surface than on substrate, suggesting an influence of root oxygen release on microbial population.

Conclusion

The presence of plants enhanced microbial density and activity with significantly higher values associated with *Phalaris*. Greater density of aerobic or facultative bacteria was present in planted microcosm, particularly on root surface, suggesting an influence of root oxygen release. Microbes were present as an attached biofilm on sand and roots and were proportional to root surface. The main limit of our investigation was the experimental duration which only reflects the microbial characteristic associated with a first growth season. Finally, knowledge of plant–microbial interaction resulting of this study lets us suggest the use of *Phalaris* as a start up plant due to its rapid colonisation and its high enhancement of microbial activity. Mixed culture of *Phalaris* and *Phragmites* can also be an option for a rapid start up, a good root dispersion with depth and possibly a long term efficiency.

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