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Use of two spatially separated plant species alters microbial community function in horizontal subsurface flow constructed wetlands

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ABSTRACT

Both the presence and diversity of plants are integral to the development and functional abilities of microbial communities within constructed wetlands (CWs). The aim of this study was to assess the impacts of different individual and paired plant species combinations on microbial community function in HSSF mesocosm CWs. Experimental systems were quadruplicated and operated as two mesocosms in series planted with *Phragmites australis* (X) or *Phalaris arundinacea* (O), giving four possible sequential combinations (XX, XO, OX, OO). Wastewater was loaded each day into position 1 mesocosm with the outflow entering position 2 mesocosm, and the corresponding position 2 mesocosm outflow representing an overall experimental system effluent. The metabolic function of the interstitial-based microbial communities within each of the 16 mesocosm pairs was assessed at a single time point in the spring (May) based on community-level physiological profiles (CLPPs) gathered using Biolog® EcoPlates. Microbial activity and metabolic richness (number of carbon sources utilised) were found to be higher in position 1 mesocosms compared to position two. Microbial community carbon source utilisation patterns (CSUPs), overall activity and metabolic richness were similar between all mesocosms from position 1—irrespective of plant species. When assessing microbial communities in position 2 of each pairing a greater variety of CSUPs, activities and metabolic richness' could be found suggesting the sequence and choice of plant can alter the microbial community function in the HSSF mesocosms. Of particular interest were the mesocosm/plant species combinations containing *Phalaris* in the position 2 which led to higher overall microbial activity and richness, distinct carbon source utilisation patterns (CSUPs) and an increased utilisation of specific carbon sources further from the inlet. More specifically the XO pairings (*Phragmites-Phalaris*) seemed to offer the most promising overall microbial function throughout both positions 1 and 2 in series suggesting plant diversity may help enhance microbial community function, and therefore microbial based water treatment capacity. The findings also suggest that the microbial communities associated with each plant species respond differently to factors such as nutrient availability, and although not yet clearly defined, further highlights the potential for improved or tailored water treatment in CWs through selection of specific plant species and combinations. Broader microbial community function or greater activity did not correlate with improved water treatment efficiency in this study. This may have been due to the relatively low contaminant loads utilised, or the limited amount of data collected. Further study across several seasons or with higher contaminant loads is recommended.

1. Introduction

Constructed Wetlands (CWs) are an increasingly popular method for wastewater treatment due to their low cost, ease of use, provision of wildlife habitat and success in treating a wide range of water pollutants from a vast array of contaminated water sources (Faulwetter et al., 2009). One of the most widely employed designs is the Horizontal Sub-Surface Flow (HSSF) CW, typically consisting of a rectangular bed lined with an impermeable membrane and commonly planted with *Phragmites australis* or *Phalaris arundinacea* (Vymazal, 2005). The overall efficacy of CWs for wastewater treatment is determined by a combination of wetland design, vegetation type and microbial processes. Plants play a vital role within CWs mainly as a promoter of microbial community development by

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providing structural support for microbial community attachment to root systems, oxygen transfer from aerial tissues into the rhizoshpere and through the secretion of root exudates containing a diverse assortment of enzymes and carbon-containing metabolites (Bais et al., 2006; Weber and Gagnon, 2014). These root exudates may be beneficially utilised by microorganisms for both growth and the development of a biofilm consisting of a microbial community bound together in a matrix of extracellular polymeric substances (EPS). The biofilm is where the majority of contaminant biodegradation takes place within CWs (Faulwetter et al., 2009; Iasur-Kruh et al., 2010; Weber and Gagnon, 2014) and provides a suitable habitat for the development of plant growth promoting rhizobacteria (PGPR) which enhance plant growth via the provision of inorganic nutrients and growth promoting substances (Iasur-Kruh et al., 2010). In this way plants and microbial communities in CWs are inherently interdependent with changes in one affecting the other. Until recently CW research dealt primarily with aspects of technological design with the main issue of concern being inlet and outlet loads and the internal active zones of microbial community activity viewed as a 'black box' (Stottmeister et al., 2003). However with recent advancements in our understanding of the central role of microorganisms to the biogeochemical processes in CWs it is now generally accepted that to improve the performance of constructed wetlands it is essential to have an understanding of the functional diversity and metabolic properties of the intrinsic microbial community (Deng et al., 2011).

Plant species differ in terms of growth rates, root morphology, production of root exudates, and oxygen transfer, opening the possibility of microbial community characteristics being specific to plant species. For example, in a study on bacterial dynamics in an HSSF CWs at Nucice, Czech Republic, Vymazal et al. (2001) reported significantly more bacteria on the roots of *Phragmites* by comparison to *Phalaris*. A more recent study on the influence macrophyte species on microbial density and activity in constructed wetlands found conversely that significantly elevated levels were associated with *Phalaris* in comparison to *Typha* and *Phragmites* (Gagnon et al., 2007). Distinct microbial communities were also correlated to *A. donax* and *Sarcocornia* sp. in an HSSF CWs used to treat high salinity industrial wastewater (Calheiros et al., 2010). Increased species (plant, animal, microbial) richness is known to lead to greater functional diversity and improved ecological stability (Hooper et al., 2005; Peterson et al., 1998; Tilman et al., 1996) providing for a more resilient and healthy ecosystem. In the context of treatment wetlands it is hypothesized that through capitalizing on the complimentary nature of different plant species, greater plant diversity can lead to greater microbial functional diversity, and possibly an enhancement in the microbial community function in terms of water treatment abilities. This theory has not yet been demonstrated conclusively in CWs and the few studies that have addressed the issue of plant diversity have produced mixed results with the recommendation that the effect of plant species diversity on microbial community functional diversity be studied further. Zhang et al. (2010) looked at the effects of plant diversity on microbial biomass and metabolic profiles, and found that increased plant species richness was correlated to increased microbial biomass but not diversity. A later study by the same authors reported that plant functional group richness, as opposed to plant species richness, exerted little effect on most microbial communities in the substrate of the studied VFCW with the exception of fungal abundance but did significantly impact several microbial enzyme activities (Zhang et al., 2011). When looking at four different free-floating plant species in mono, bi, and quadri-cultures Bissegger et al. (2014) found that increasing the number of plant species did not promote the development of microbial communities with a more active and diverse metabolic capability, but did find that plant/plant interactions were important on defining the carbon source utilisation patterns CSUPs as collected with Biolog® EcoPlates.

Community-level physiological profiling (CLPP) has proven to be a useful tool for the assessment of microbial community function and functional diversity in both terrestrial and aquatic environments (Frac et al., 2012; Weber and Legge, 2013; Zhang et al., 2010). The method utilizes Biolog® EcoPlates containing 31 sole carbon sources plus a blank well (no C source) in triplicate. Carbon sources contained on the EcoPlate (Table 1) are designed to be ecologically relevant and diverse with many commonly found in plant root exudates (Campbell et al., 1997). An overall community-level physiological profile based on pattern and rate of utilisation of the 31 carbon sources is generated giving an indication of the community functional abilities. One of the strengths in using CLPP is the large amount of information which is obtained. Authors frequently discount the majority of information which is gained, focusing on the differentiation of microbial communities based on the CSUPs. Wastewater composition itself varies depending on origin but is most often a complex mixture of compounds, with many of those compounds containing carbon. The diverse set of carbon sources found on the BIOLOG® EcoPlate have varying compositions and can be separated into carbohydrates, polymers, carboxylic acids, amins/amides, and amino acids. Although not exhaustive the 31 different carbon sources on the BIOLOG® EcoPlate represents a fairly large range of compounds. By evaluating the degree with which specific compounds are utilised on the plate, a relative understanding of the water treatment potential of the microbial community being evaluated can be gained (Button et al., 2015b). In this way the CLPP method can be used to more closely evaluate and represent the water treatment potential of a specific microbial community (originating from a treatment wetland or other water treatment technology) for a range of compounds.

The objective of the present study was to assess the impacts of different individual and paired plant species combinations on microbial community function in HSSF CWs. Microbial community function, rather than structure, was chosen for study in order to more closely connect findings to CW water treatment potential. Mesocosm systems planted with *P. australis* (X) and/or *P. arundinacea* (O) were operated under four possible sequential combinations (i.e. XX, OO, OX, XO), and the function of the intrinsic microbial communities assessed via CLPP.

2. Methods

2.1. Experimental set-up and sampling

The experiment was carried out in a controlled greenhouse at the Montreal Botanical Garden, Québec (Canada). Sixteen mesocosm scale experimental constructed wetlands were set-up and operated for 12 months (Jul. 2012-Jul. 2013). The temperature of the greenhouse ranged from 35 °C in summer, 15 °C in autumn and spring, and 5 °C in winter. Each experimental unit consisted of two mesocosms connected in series (L 70 cm W 51 cm H 36 cm) filled with granitic river gravel (Ø = 10–15 mm) giving an interstitial/free water volume of 24L. To facilitate water distribution, each experimental unit had a 0.1 m section at the inlet with granitic coarse gravel (Ø = 30–40 mm). The mesocosms were planted according to each of the following four treatments: monocultures of *Phragmites* (XX) and *Phalaris* (OO) and the combination of the two plant species, *Phragmites* followed by *Phalaris* (XO), as well as *Phalaris* followed by *Phragmites* (OX). Each treatment was replicated four times following a randomized block design (Fig. 1). Following plant establishment (from spring 2009 to spring 2012), the mesocosms were fed from Apr. 2012 to Aug. 2013 with 15 L d^{-1} of reconstituted wastewater from diluted fish farm sludge, urea (20.2 mg L^{-1})

Table 1
Utilisation of the 31 individual carbon sources, grouped according to guild type (adapted from Weber and Legge, 2009), on the Biolog® EcoPlate. √ = carbon source utilised, X = carbon source not utilised. P1 and P2 = position 1 and position 2 of the mesocosms. %< refers to the decrease in utilisation from position 1 to position 2. Brackets denote well number. Well (C0) is a blank. [a] = identified root exudate (Campbell et al., 1997; Dell'mour, 2010). A carbon source was considered utilised when a blank-corrected absorbance value was above 0.25. na = not utilised in either position and therefore no change reported.

Guild group	Plant sequence											
(Well number) Carbon source	XX			OO			OX			XO		
Carbohydrates	P1	P2	%<	P1	P2	%<	P1	P2	%<	P1	P2	%<
(C1)Pyruvic acid methyl ester	√	√	−4	√	√	11	√	√	13	√	√	13
(C6)D-cellobiose	√	√	9	√	√	18	√	√	48	√	√	9
(C7)Alpha-D-lactose	√	√	78	√	√	79	√	√	83	√	√	58
(C8)Beta-methyl-D-glucoside	√	√	42	√	√	38	√	√	30	√	√	15
(C9)D-xylose[a]	√	√	55	√	√	20	√	√	38	√	√	31
(C10)I-erythritol	√	X	92	√	√	20	√	X	90	√	X	93
(C11)D-mannitol[a]	√	√	38	√	√	20	√	√	30	√	√	29
(C12)*N*-acetyl-D-glucosamine[a]	√	√	41	√	√	13	√	√	10	√	√	19
(C14)Glucose-1- phosphate[a]	√	√	32	√	√	63	√	√	57	√	√	35
(C15)D,L-alpha-glycerolphosphate	√	X	60	√	X	59	√	√	47	√	√	32
Carbohydrates Average			44			34			45			33
Carboxylic/acetic acids												
(C13)D-glucosaminic acid	√	√	32	√	√	13	√	√	57	√	√	33
(C16)D-galactonic acid-gamma lactone	√	√	30	√	√	33	√	√	42	√	√	36
(C17)D-galacturonic acid	√	√	41	√	√	29	√	√	38	√	√	44
(C18)2-Hydroxy Benzoic acid	√	X	83	√	X	100	√	X	100	√	X	37
(C19)4-Hydroxy Benzoic acid	√	√	45	√	√	14	√	√	27	√	√	18
(C20)Gamma-hydroxybutyric acid	√	X	57	X	X	na	X	X	na	X	X	na
(C21)Itaconic acid	√	√	66	√	√	45	√	X	82	√	√	51
(C22)Alpha-Ketobutyric acid	X	X	na	X	X	na	X	X	na	X	X	na
(C23)D-malic acid[a]	√	√	−26	√	√	22	√	√	−7	√	√	8.9
Carboxylic/acetic acid average			41			37			48			33
Polymers												
(C2)Tween 40	√	√	47	√	√	20	√	√	40	√	√	43
(C3)Tween 80	√	√	35	√	√	23	√	√	10	√	√	42
(C4)Alpha-cyclodextrin	√	X	91	√	√	67	√	√	71	√	√	6.1
(C5)Glycogen	√	√	40	√	√	7	√	√	15	√	√	19
Polymer average			53			29			34			28
Amines/amides												
(C30)Phenylethylamine	√	√	35	√	√	31	√	√	40	√	√	27
(C31)Putrescine[a]	√	√	16	√	√	29	√	√	26	√	√	5.7
Amine/amide average			26			30			33			16
Amino acids												
(C24)L-arginine	√	√	20	√	√	62	√	√	24	√	√	39
(C25)L-asparagine	√	√	29	√	√	32	√	√	12	√	√	18
(C26)L-phenylalanine[a]	√	X	67	√	X	92	√	X	87	√	X	94
(C27)L-serine[a]	√	√	49	√	√	48	√	√	58	√	√	32
(C28)L-threonine[a]	X	X	na	X	X	na	X	X	na	X	X	na
(C29)Glycyl-L-glutamic acid	√	X	71	√	X	57	√	X	61	√	√	14
Amino acid average			47			58			48			39
Total utilised	29	22		28	24		28	23		28	25	

and mono potassium phosphate (10.1 mg L^{-1}) at a hydraulic loading rate of 42 L m^2 d^{-1}. Average influent concentration (in g m^2 d^{-1}) was TSS 11; COD 21; TP 1.5; TN 5.8. Wastewater was fed on a daily basis from a large mixed chamber which fed all experimental units (mesocosm pairs). The daily loading of 15 L of wastewater was loaded into the inlet section of mesocosm 1 and flowed horizontally towards the outlet, pouring the overflow by gravity into mesocosms 2. The subsequent overflow from mesocosms 2 was collected in plastic buckets by gravity at the outlet representing an overall experiment system effluent. This experimental system thus mimics a horizontal flow subsurface constructed wetland with batch loading. This simplified design allowed some replication and the environmental conditions were controlled in a way that the influence of plant diversity on microbial community functional diversity could be assessed. Dissolved oxygen readings were generally between 1.5 and 2 mg L^{-1}, with REDOX between −130 and 50 mV.

Sampling was completed using a perforated 1 inch PVC pipe placed at the centre of each mesocosm, which spanned the entire vertical profile. Plant root development in these systems also spanned the entire (or close to the entire) vertical profile (36 cm) and therefore there was little or no opportunity to differentiate between the rhizospheric and bulk media zones in this study. Additionally this microbial community focused study was part of a larger investigation looking at the pollutant removal capabilities of these same experimental units (Rodriguez and Brisson, 2016). Therefore no opportunity for destructive gravel, biofilm, or root sampling was available. In May, 2013 aqueous based microbial community samples were drawn, placed in sterile 50 mL C-tubes, conserved on ice, and shipped to the Royal Military College of Canada, Kingston, Ontario for microbial community analysis. The microbial community samples gained were observed to originate from both the interstitial space and biofilm. A relatively vigorous extraction technique was employed where a large 50 mL pipette was used to quickly extract water from the vertical PVC pipe. This allowed for some sheering of biofilm for sample collection, thus each microbial community sample did not represent either the interstitial or biofilm communities alone, but rather a mixture of both.

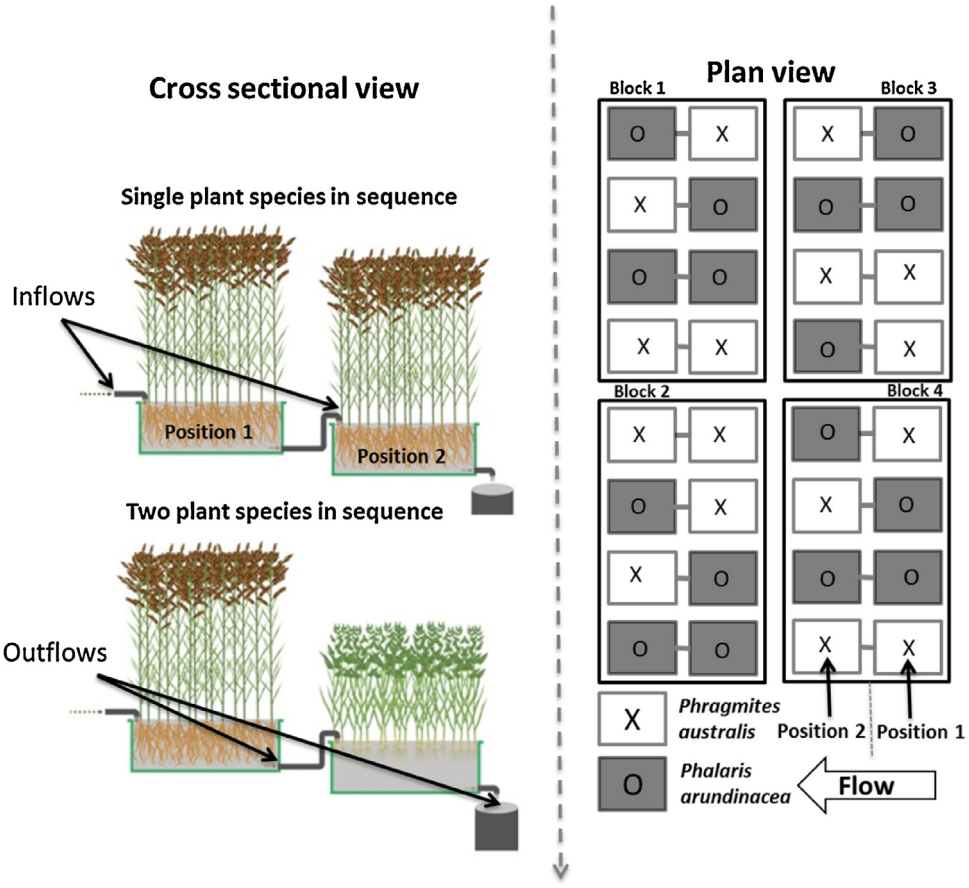

Fig. 1. Illustration of the experimental set up showing a cross-sectional view depicting mesocosm design and sequencing with inflow into position 1 and outflows from position 1 into position 2 and out of position 2. The plan view shows the entire experimental set-up with the 4 different plant combinations of X, *Phragmites australis* and O, *Phalaris arundinacea* (XX, XO, OO, OX) quadruplicated giving a total of 16 mesocosms and 32 individual positions.

2.2. Community level physiological profiling

CLPP analysis of microbial community samples was performed within 48 h of sample collection. CLPP followed the methods described by Weber and Legge (2010). Briefly, all laboratory work was performed in an aseptic environment and equipment involved in the procedure was sterilised using 70% ethanol. Samples were homogenised by gentle end over end shaking before 100 μL was added to each well of a Biolog® EcoPlates (Biolog Inc., Hayward CA., USA). The plates were incubated in the dark at room temperature on an orbital shaker (VWR® Mini Shaker) at low speed (100 rpm). Plates were then read using a microplate absorbance reader (Bio Rad IMark™) at an absorbance of 590 nm at 0, 18, 24, 42, 48, 66, 72, 90 and 96 h.

2.3. Data analysis

Analysis of the CLPP data was performed as previously described (Weber and Legge, 2010; Weber et al., 2007). For all samples, absorbance readings at 66 h were identified as the time point for further CLPP data analysis. One plate was used per sample for a total of 32 plates representing individual positions for each of the 16 2-position systems (Fig. 1). Each plate contained 31 carbon sources and a blank well in triplicate (96 wells in total) providing 3 replicated carbon source utilisation patterns (CSUPs) per mesocosm. This equated to 3072 data points for analysis. The carbon sources contained in the Biolog® EcoPlate are outlined and classified into five broader 'guild' groups as described in Table 1.

Two different metrics were calculated from the CSUPs for further analysis: (1) The average well colour development (AWCD), and (2) the number of carbon sources utilised (Richness). The AWCD represents the average metabolic activity over all wells and per Weber and Legge (2010) is calculated as:

$$AWCD = \frac{1}{31}\sum_{i=1}^{31}(A_i - A_0)$$

Where A_i represents the absorbance reading of well i and A_0 is the absorbance reading of the blank well (inoculated, but without a carbon source). Where there is very little response in a well, negative values of standardized absorbance may occur, in which case they are coded as zeroes for further analysis.

The number of carbon sources which a microbial community is able to utilize on any one plate provides a representation of the metabolic potential of a particular community. This can also be referred to as metabolic richness which is calculated as the number of wells with a corrected absorbance (A_i–A_0) greater than 0.25 (Weber and Legge, 2010). The two metrics thereby provide complimentary information with AWCD representing overall metabolic activity and richness demonstrating them etabolic or 'functional' potential of the microbial community.

The statistical significance ($p < 0.05$) of any differences in the AWCD or richness data between mesocosm systems was assessed using 1-way analysis of variance (ANOVA) for initial identification of differences followed by post-hoc Tukey HSD to look for specific differences between each system.

Principle component analysis (PCA) was performed using the covariance (n-1) matrix of AWCD normalized CSUP data (Weber and Legge, 2010) to further assess for differences between mesocosms systems. Datasets were subjected to LN transformation based on assessment of normality, homoscedasticity and linear correlations following the recommendations of Weber et al. (2007). Statistical and PCA analysis was completed using XLSTAT 2015 (Addinsoft New York, NY). The significance of differences between observed groupings in PCA plots was assessed using One-way Permutational Analysis of Variance (PERMANOVA) with Bray-Curtis distance. PERMANOVA analysis was completed using the free Paleontological statistics software package (PAST) (Hammer et al., 2001).

3. Results

3.1. Microbial community metabolic activity and richness

Fig. 2 shows the microbial community metabolic activity and richness for mesocosms in position 1 with plant species X and O (X, *Phragmites*; O, *Phalaris*), and mesocosms in position 2 for plant species from the sequences XX, OO, OX, XO. Overall, microbial activity and richness was higher in position 1 compared to position 2. The activity and richness of the microbial communities associated with both plant species were not significantly different ($p < 0.05$) in position 1 with activities (AWCD) of 1.32 and 1.34 and richness values (no. of carbon sources utilised) of 26.5 and 27.3 for *Phragmites* and *Phalaris* respectively. Some variation was observed in position 2 based on plant species sequence with the highest activities and richness values observed where *Phalaris* (O) was present as the second plant species. Plant sequences OO and XO gave rise to microbial activities of 0.91 and 0.92 and richness values of 21.8 and 23.1 respectively compared to the plant sequences with *Phragmites* in the position 2 (XX and OX) which had lower associated microbial activities of 0.66 and 0.75 and richness values of 19.1 and 21.1 respectively.

3.2. Microbial community metabolic profiles

The Carbon Source Utilisation Patterns (CSUPs) of microbial communities associated with each single (position 1) and mixed plant sequence (position 2) were visualised using principal component analysis (PCA) to assess for differences in the CSUPs (Figs. 3 and 4). When all 32 individual positions of the 16 systems were ordinated in a single PCA (Fig. 3a) objects representing microbial CSUPs formed two clear groups. One group containing samples from position 1 and another representing the samples from position 2. These two groups were significantly different ($p < 0.05$). Additionally, it could be seen that samples from position 1 grouped more closely than those from position 2. Looking in more detail at the grouping of samples from position 1 (Fig. 3b) revealed no clear differences between plant species. When subjected to PCA in isolation, samples from the position 2 showed a different trend (Fig. 4a) whereby distinct groups were evident according to plant species present in position 2. These groupings, representing *Phragmites* or *Phalaris*, were significantly different from one another ($p < 0.05$). Within these two groups it could also be seen that single plant species combinations (O-O and X-X) showed less variation than mixed plant species (X-O and O-X). The contribution of each of the carbon sources available on the EcoPlate to the groupings is visualised in the distance biplot (Fig. 4b). Several of the carbon sources contributing most (shown by length of the variable vector) to the upper group (*Phragmites* in position 2) were root exudates (see Table 1).

Principal component analysis of each of the four plant species combinations (XX, OO, OX, XO) (Fig. 5) showed district groupings for the plant species in each position for all the combinations except XO (*Phragmites* in position 1 followed by *Phalaris* in position 2) suggesting less variation in the CSUP between positions 1 and 2 for this particular combination of plant species. It should also be again noted that this particular combination showed the highest activity and richness in position 2.

3.3. Utilisation of specific carbon source guilds

Potential differences in the functional profiles of microbial communities associated with each of the plant sequences was further investigated by looking at the rate of utilisation of the five key guild types (Table 1) contained on the EcoPlate. The average guild utilisation rates for position 1 and 2 are displayed for each of the four plant sequence combinations (Fig. 5). Utilisation of the five guild types (carbohydrates, carboxylic acids, polymers, amines/amides and amino acids) was relatively uniform for all plant sequence combinations with a general trend of slightly increased utilisation of carbohydrates, polymers and amines/amides compared to carboxylic and amino acids. The utilisation of all five carbon source guilds decreased between positions 1 and 2 in all instances. This decrease between positions was statistically significant for all plant species combinations for carbohydrates with the exception of the *Phrgamites* followed by *Phalaris* combination (XO).

The decrease in utilisation of the carbon guilds from position 1 to 2 in each mesocosm sequence was investigated further by analysing the change in utilisation from position 1 to 2 for each specific carbon source on the EcoPlate (Table 1). A greater number of carbon sources were utilised in position 1 than in position 2 for all plant species combinations. All amines/amides and the majority of carbohydrates were utilised in both positions for all systems whilst fewer amino acids and carboxylic/acetic acids were utilised in position 2. System specific patterns were observed. The biggest drop in utilised carbon sources occurred in the Phragmites–Phragmites systems (XX) with utilisation of 29 carbon sources in position 1 but only 20 in position 2. The Phalaris–Phalaris (OO) system showed more similarity in the utilisation of carbon sources between positions 1 and 2 at 28 and 24 respectively. The plant combination of Phragmites followed by Phalaris (XO) showed the lowest percentage decrease in utilisation between positions 1 and 2 for all five of the guild groups. This trend was most notable for the carbohydrates alpha-D-lactose, beta-methyl-D-glucoside, the carboxylic acid 2-hydroxy benzoic acid and the amine putrescine. The utilisation of the carbohydrate pyruvic acid methyl ester and the polymer D-malic acid were the only carbon sources that were utilised at a greater rate in position 2, specifically when Phragmites was in position 2.

4. Discussion

4.1. Activity and richness

P. australis and *P. arundinacea* are two commonly employed plant species in HSSF CWs (Vymazal, 2005). In a field-scale design comparable to mesocosms set-up employed here, Vymazal et al. (2001) studied an HSSF CW planted with alternate stripes of these two plant species perpendicular to the flow direction and found significantly more bacteria on the roots of *Phragmites* compared to *Phalaris* and a steep decrease in bacterial numbers within the first few metres of the bed. Gagnon et al. (2007) found significantly increased bacterial density and activity associated with rhizospheric biofilms in monocultures of *Phalaris* compared to both *Phragmites* and *Typha*. In contrast, here we did not observe any

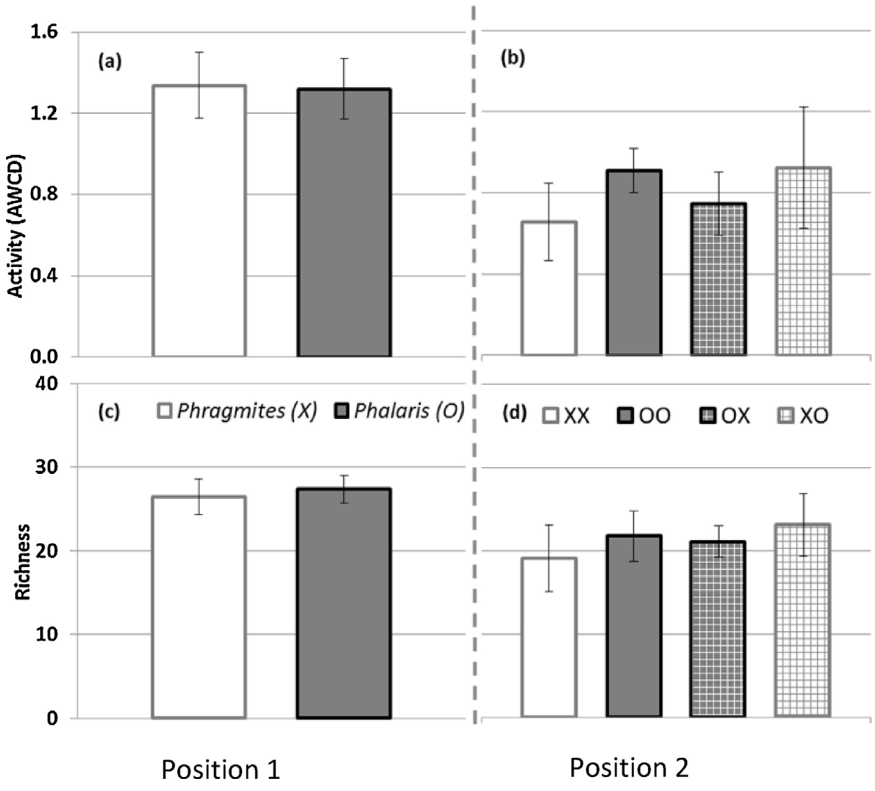

Fig. 2. Microbial community metabolic activity (AWCD) and richness (number of carbon sources utilised in the Biolog® EcoPlate) in position 1 (a and c) and position 2 (b and d) respectively. Error bars represent one standard deviation where n = 8 in position 1 and n = 4 in position 2.

significant differences in microbial community activity and richness between *Phragmites* and *Phalaris* (Fig. 2a and c) in position 1. In the present study microbial communities were sampled in a way to represent the greater biofilm/rhizospheric areas of each system whilst in the previous studies (Gagnon et al., 2007; Vymazal et al., 2001) microbial communities were sampled from the root surface and enumerated (rather than profiled for overall function via carbon source utilisation).

Microbial community activity and richness was lower in position 2 regardless of the plant species combination which is expected as the distance is increasing from the inlet between position 1 to 2 and hence the source of energy and nutrients contained in the wastewater is less in position 2. This observation is in agreement with Button et al. (2015a) where the activity and richness of HSSF microbial communities, representative of mixed rhizospheric and biofilm origin, decreased in the direction of flow away from the inlet in pilot scale systems in Langenreichenbach, Germany. The study of Button et al. (2015a) was comprehensive in the variety of designs (intensified vs non-intensified, gravel depth 25 cm or 50 cm, planted vs. unplanted) where the same observations (decreasing activity and richness along the direction of flow away

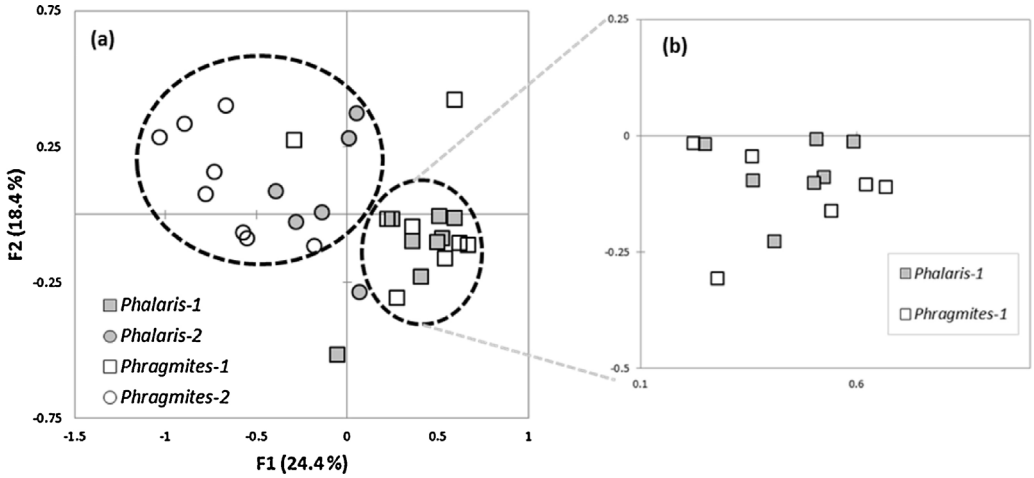

Fig. 3. Principal component analysis: (a) based on LN transformed carbon source utilisation patterns (CSUPs) of microbial communities from individual positions of the mesocosms. Dashed circles are used to indicate significant differences between groupings ($p < 0.05$, PERMANOVA). *Phalaris*-1/*Phragmites*-1 are samples from mesocosms in position 1 with the respective plants species, *Phalaris*-2/*Phragmites*-2 are samples from mesocosms in position 2 with the respective plants species. (b) Expanded view of grouping with mesocosms from position 1.

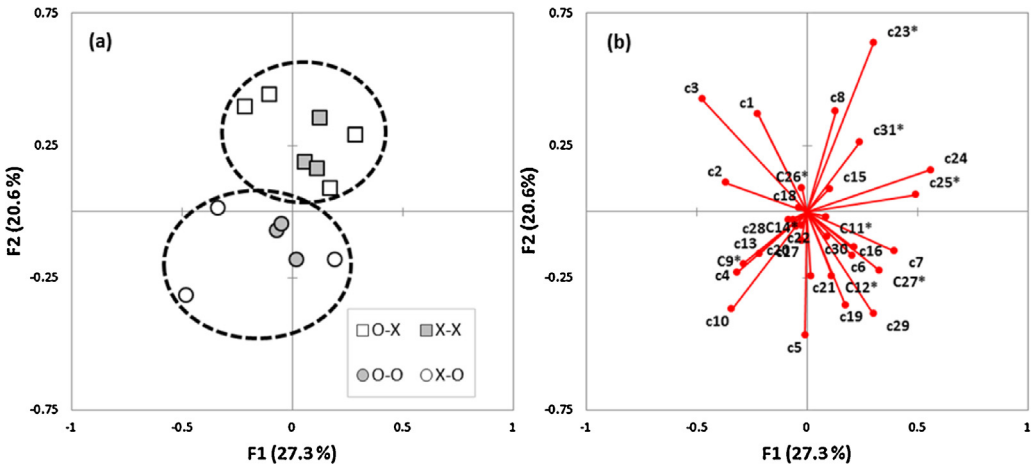

Fig. 4. Principal component analysis (a) based on LN transformed carbon source utilisation patterns (CSUPs) of microbial communities from the position 2 of each mesocosm. Dashed circles are used to indicate significant differences between groupings ($p < 0.05$, PERMANOVA). Distance biplot (b) represents the contribution of each variable (31 carbon sources on the Ecoplate) to the ordination of observation in the initial PCA. The length of a variable vector in the representation space is indicative of the variable's level of contribution. * = carbon source is a known root exudate. Three outlier data points (plate reading errors) were excluded from the analysis.

from the inlet) were seen in all systems, therefore agreement with this laboratory based study is not unexpected.

Plant species combinations with *Phalaris* (O) in position 2 gave rise to higher, although not statistically significant, microbial activity and richness than when *Phragmites* (X) was in the position 2 (Fig. 2b and d). This effect was enhanced further still for microbial community richness when *Phragmites* was followed by *Phalaris*. This may suggest the existence of potential mechanisms by which *Phalaris* is better able to support microbial communities in situations of lower nutrient availability. *Phalaris* roots are known to penetrate only half as deeply into the bed material compared with *Phragmites* (Vymazal and Krőpfelová, 2005). Due to the shallow depth of the mesocosms used here both plant species penetrated the entire depth of the mesocosm (or very near) however *Phalaris* was observed to be more dense with finer branching which may have provided an improved habitat for biofilm development as the specific area for attachment is increased.

When evaluating the effect of plant diversity on the water treatment capabilities of CWs different observations have been collected. Coleman et al. (2001), Fraser et al. (2004) and Picard et al. (2005) did not find polyculture CWs to be more efficient than monocultures for nutrient removal, while Zhu et al. (2010), Zhang et al. (2010), and Ge et al. (2015) found a positive correlation between nitrogen removal and number of plant species in CWs. Rodriguez and Brisson (2016) describes a study completed in parallel to the subject investigation, using the same experimental systems, and found that when combining plant species overall treatment performance was enhanced in some cases. When using *Phalaris* followed by *Phragmites* (OX) COD, total phosphorus, and ammonium removal were partially enhanced. When using *Phragmites* followed by *Phalaris* (XO) nitrate removal was enhanced. Although Rodriguez and Brisson (2016) observed the above mentioned trends, the majority of those were not statistically significant. Similar to this study, the findings of Rodriguez and Brisson (2016) suggest that combining plant species can be beneficial depending on removal goals, however, did not find overwhelming evidence supporting the idea that plant diversity positively influences treatment ability of subsurface flow CWs. The present investigation underlines the useful type of microbial community function data that can be generated and highlights the potential enhancements available through plant selection and combinations. Rodriguez and Brisson (2016) describe the removal efficiencies for COD, total phosphorus, ammonium and nitrate to be on the order of 85–95%. As shown by Ge et al. (2015) increasing plant number had a positive influence on nitrogen removal, under a high ammonia loading scenario. Future work should include investigating whether microbial activity, and overall treatment performance (COD, phosphorus, nitrogen species) differs depending on plant species number or plant type in CWs under varying loading scenarios, especially where loading is higher and removal efficiencies are lower than 85–95%. It would also be useful to perform the same type of study over an extended temporal period to capture potential seasonal dynamics to help fully understand the effects of plant selection and combinations on large scale systems operated year-round.

4.2. Metabolic profiles

In addition to overall activity and richness, assessment of the microbial community function can be gained by using data obtained from the Biolog® EcoPlates to perform multivariate analysis. The CSUP data for each sample takes into account both the rate and number of carbon sources utilised allowing for a more detailed comparison of functional profiles. The CSUPs were clearly different for position 1 and 2 and this is to be expected as a result of nutrient availability decreasing with distance from the inlet. Both plant species led to comparable microbial community CSUPs in position 1. A greater spread (dissimilarity) between data points was observed for *Phragmites* (X) suggesting that microbial communities associated with *Phalaris* (O) are more reproducible between systems i.e. the 8 individual systems in the experimental set up (Fig. 1). Significant differences have been reported in the functional profiles of microbial communities between planted and unplanted systems both when profiled at one point in time (Truu et al., 2009; Zhang et al., 2010) and when investigated temporally (Weber and Legge, 2011). Differences between microbial community functional profiles between plant species is however less clear. Zhang et al. (2010) looked at the effects of plant diversity on microbial biomass and community metabolic profiles reporting that plant species richness was positively correlated to the size of microbial communities yet changes in the microbial function were found to be dependent only on the presence or absence of plants and not plant species richness. Bissegger et al. (2014) also found that increasing the number of plant species did not promote greater microbial community functional richness or diversity, but rather specific plant species had more or less influence based on overall root mass. Unlike Zhang et al. (2010) and Bissegger et al. (2014) this study looked at mesocosm systems setup in series. Physically separating the plants between systems created a nutrient availability gradient between

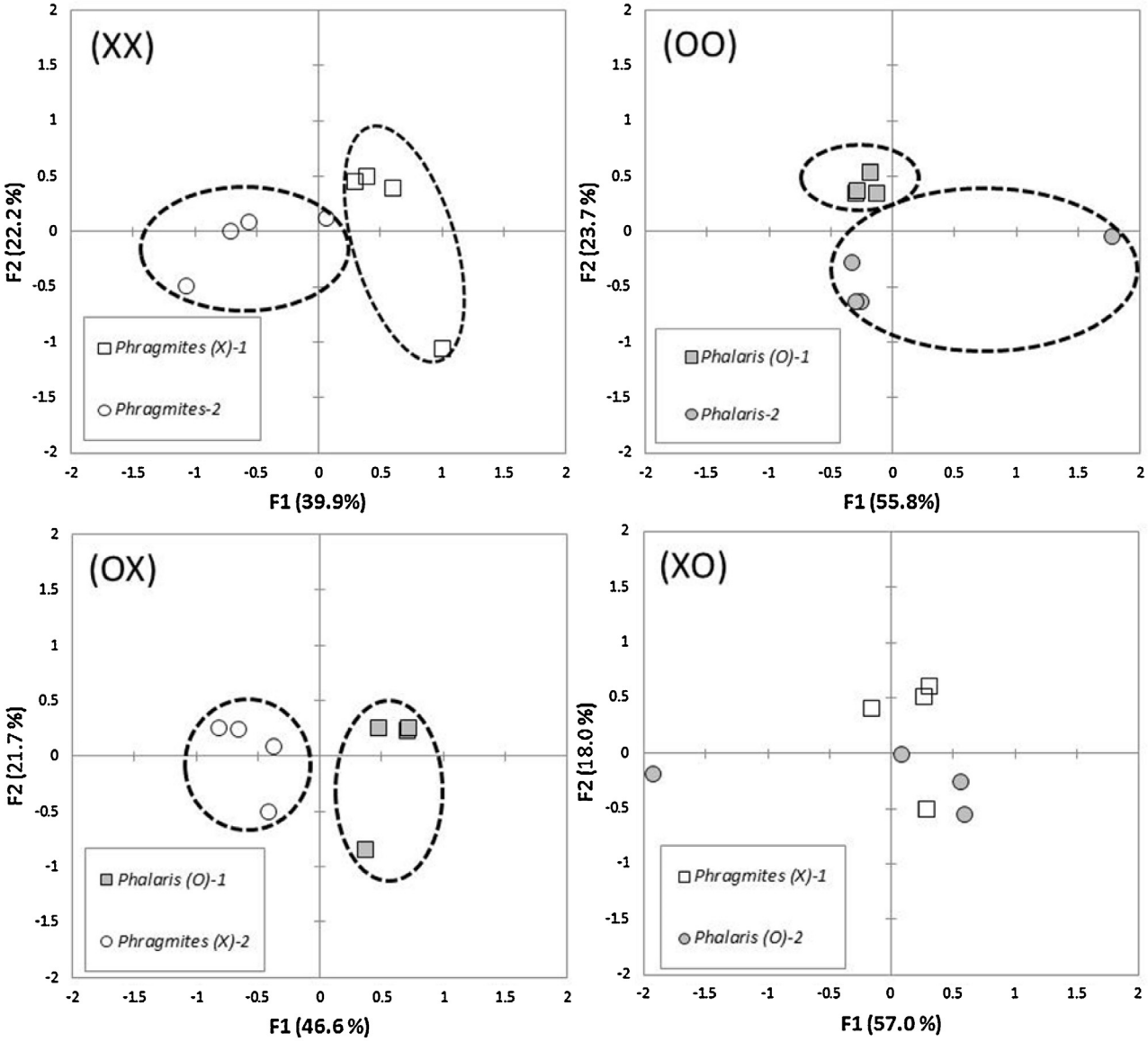

Fig. 5. Principal component analysis based on LN transformed carbon source utilisation patterns (CSUPs) of microbial communities associated with the four plant species combinations tested, XX: Phragmites position 1 and 2, OO: Phalaris in position 1 and 2, OX: Phalaris in position 1 followed by Phragmites in position 2, XO: Phragmites in position 1 followed by Phalaris in position 2. Dashed circles are used to indicate significant differences between groupings ($p < 0.05$, PERMANOVA).

position 1 and 2 and here we did see differences based on plant sequence. Position 1 in general had a more active and metabolically diverse microbial community than position 2, regardless of the species. The exception was the XO combination, *Phragmites* followed by *Phalaris,* which maintained a similar functional profile in both positions 1 and 2. This result may suggest that this combination of species in HSSF CWs may offer increased microbial based water treatment capabilities. This finding is of course from a lab-scale study and testing at the pilot or field scale would be needed for confirmation.

Here it was also observed that the microbial community functional profiles (CSUPs) from systems planted with multiple species in series did not group as closely as systems planted with the same species in series (Fig. 4a), particularly systems consisting of *Phalaris* alone (e.g. OO). This suggests that microbial communities associated with mixed plant species systems are not as clearly defined and may be more susceptible to fluctuations in functional profiles between replicated systems.

4.3. Guild analysis

All of the plant species combinations (XX, OO, OX, XO) led to similar patterns in the utilisation of the five key guild groupings contained on the Biolog® EcoPlate (Fig. 5). Polymers were the most utilised and amino acids the least, and in general the difference in utilisation between guild groups for plant species combinations was not noteworthy. It is interesting that *Phalaris* in position 2 led to higher utilisation of polymers compared to other guilds and that the differences were most pronounced for the single plant species combination with *Phalaris* (OO). Some of the differences between plant combinations observed here point to the potential for optimisation of water treatment with specific planting regimes. For example, wastewaters high in carbohydrates may be better treated by planting *Phalaris* further from the inlet, since we found no significant decrease in the utilisation of carbohydrates for the *Phragmites* followed by *Phalaris* combination. The biggest drops in utilisation between positions 1 and 2 occurred with *Phragmites* in position 2 both in terms of overall utilisation (Fig. 6) and the number of specific

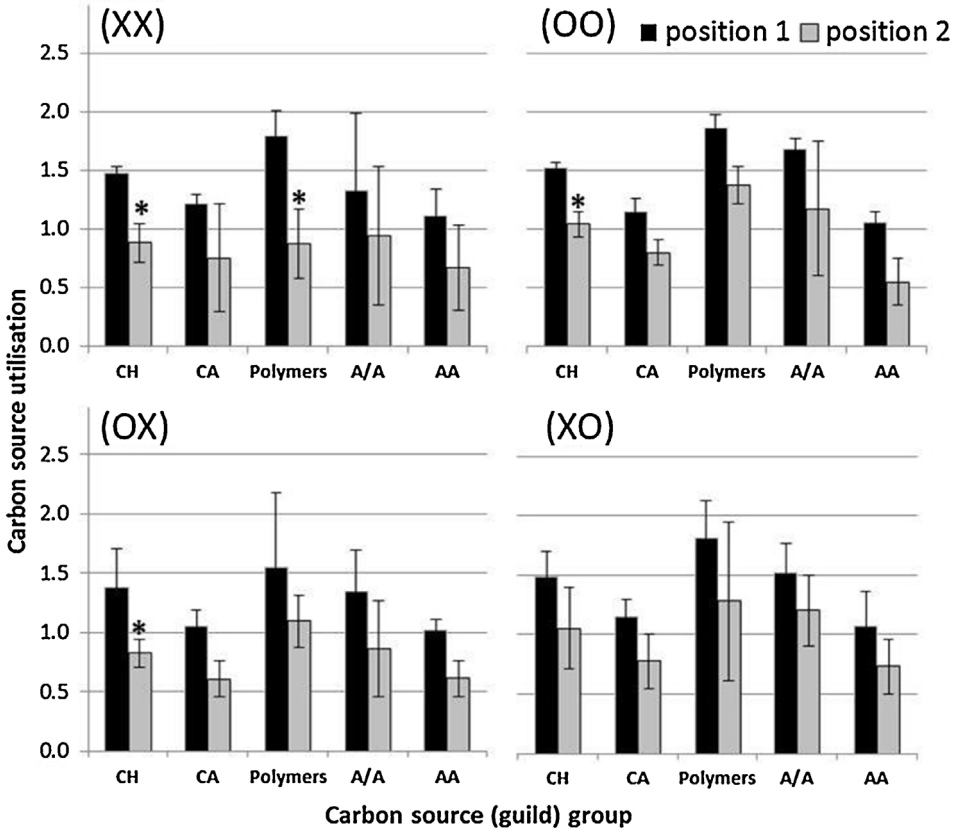

Fig. 6. Utilisation of the carbon source 'guild' groups in each position of the mesocosms for each plant species combination. Plant species and sequence are signified by X = *Phragmites* and O = *Phalaris*, e.g. XO = *Phragmites* in position 1 followed by *Phalaris* in position 2. Error bars represent 1 std. dev. of quadruplicated systems (n = 4). CH = carbohydrates, CA = carboxylic acids, A/A = Amines/amides and AA = amino acids. *Indicates a statistically significant difference between position 1 and 2 for corresponding guild group ($p < 0.05$, Tukey HSD).

carbon sources that the microbial community is capable of utilising (Table 1). Some of the findings here point to potential limitations of monocultures in HSSF constructed wetlands when compared to bi or poly culture alternatives. Zhang et al. (2010) report correlations between the utilisation of specific guilds and plant species richness with amino acid utilisation positively correlated to plant species richness whilst amine/amide utilisation decreased with increasing plant species richness. Similarly Liu et al. (2013) demonstrated increased utilisation of carbohydrates, polymers and amino acids with greater plant species richness and the presence of mono and dicot species. The identification of such trends in the utilisation of specific types of carbon sources depending on either plant species diversity or individual species characteristics presents potential for matching plants and their associated microbial communities to specific wastewater types. When examining the utilisation of carbon sources in position 2, *Phalaris* was able to maintain high glycogen utilisation rates in comparison to *Phragmites*. Similarly, higher rates of alpha-D-lactose utilisation were also maintained by the combination of *Phagmites/Phalaris*. With the increasing interest in the use of constructed wetlands for the treatment wastewaters such as dairy effluents (Healy et al., 2007) and challenging petrochemical effluents high in napthenic acids (Headley and McMartin, 2004), this type of very rigorous analysis of carbon source utilisation could be used to help select plants for optimal performance under specific circumstances. For example, maintaining high lactose utilisation rates in position 2, or further away from the inlet of a field scale HSSF, could be useful for treating dairy wastewaters.

Root exudate analysis of these two plants might also provide an insight into specific chemicals produced by each plant species that may selectively promote or support specific microorganisms. Both plant species and environmental conditions determine the quality and quantity carbon and nutrient sources secreted into the rhizosphere and the structure of microbial communities around roots (Bais et al., 2006). *Phalaris* with its shorter and more dense root systems, may for example exude more polymeric substances than *Phragmites* thereby promoting the development of communities that thrive on such substances.

Ecological theory suggests that greater plant diversity should lead to a greater microbial community functional diversity. Therefore, it is perhaps surprising that the influence of plant diversity on microbial community function was not more heavily observed. However, ecological theory is mostly developed in the context of the natural environment. CWs are most certainly an engineered environment. It is possible that the relentless and continuous feeding of wastewater to such an engineered ecosystem is an overwhelming adaptive driver thus forcing the microbial community function into a direction based largely on influent composition and loading rate alone. This could be one reason ecological theory does not perfectly describe such an extreme ecosystem case. Additionally, the employed sampling schedule and methodology did not allow for the evaluation of either spatial or temporal dynamics. It is possible that the single time point at which this study was performed does not represent the system conditions throughout the year. It is recommended that a similar study be completed over at least four seasons to evaluate for potential temporal dynamics. Additionally, when allowable, the same systems should be destructively sampled to allow for spatial analysis within the same systems. Future analysis could consider the use of microarray methods for the functional analysis of microbial communities. Although

not yet developed for CWs, the DNA based implied functional information may be a useful complement to CLPP.

5. Conclusions

The objective of this study was to assess the impacts of different individual and paired plant species combinations on microbial community function in HSSF CWs. The results presented show similar microbial community function for both *Phragmites* and *Phalaris* based on analysis of mixed microbial communities from CW mesocosms. However, when mesocosms are operated in sequence it appears that microbial community function can be altered by choice of plant species and spatial positioning along a nutrient gradient. Plant species combinations with *Phalaris* in position 2 (further from nutrient source) led to higher overall system microbial activity and richness and altered the utilisation of specific types of carbon sources. This finding provides an interesting incentive to investigate further the potential for improved or tailored water treatment in CWs with plant poly cultures, however at this stage the specific advantages are not proven. The current study assessed interstitial-based microbial communities at a single time point in the spring (May). Further insight would be gained through the comparative investigation of microbial communities associated with all regions of the CW (root, biofilm and interstitial water). The growing season of wetland plants is season specific (Picard et al., 2005) and as such it will also be important to assess the impacts of seasonality on any potential benefits to water treatment with the use of poly cultures.

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