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Microbial and vegetative changes associated with development of a constructed wetland

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ABSTRACT

Wetlands may be constructed to provide several ecosystem functions. A constructed wetland receiving agricultural runoff water was observed prior to, and for more than two years after, establishment. The excavated portion of this wetland was compared to an undisturbed, upland area and to an adjacent, natural, depressional, reference wetland. After construction the excavated cell was rapidly colonized by wetland plant species, including some exotic invasive weeds. Flourescein diacetate (FDA) hydrolysis and triphenyl tetrazolium chloride (TTC) dehydrogenase assays indicated that the soil microbial community was more active in the excavated wetland cell than either of the two reference areas. In 2003 and 2004 TTC activity was greater than twice as high in the constructed wetland than the reference wetland. FDA was greatly stimulated in the constructed wetland immediately after construction, but by 2004 the three systems were not significantly different. The soil microbial community of the constructed wetland rapidly decreased in the abundance of fungi and gram-negative bacteria and increased in gram-positive bacteria and overall bacteria, as measured by fatty acid methyl esters. These shifts in the microbial community were consistent with the differences noted between the communities of the upland system and the reference wetland. During the time of observation the constructed wetland did not sequester carbon relative to the upland system ($P = 0.11$) and did not significantly increase in carbon content ($P = 0.41$).
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1. Introduction

Agricultural non-point source pollution is the primary cause of impaired surface waters in the U.S. (EPA, 2002). Runoff from fields often contains nutrients and suspended sediments that can diminish water quality through eutrophication and turbidity. The decline in the value of the oxbow lakes of Mississippi Delta for boating and fishing as a consequence of this runoff has long been noted (Coleman, 1969). In-field agronomic practices such as reduced tillage, targeted application of fertilizer and pesticides, and the selection of pesticides have potential to reduce the negative impact of agricultural surface runoff water (reviewed in Locke et al., 2002). In some systems agricultural non-point source pollution is intercepted by wetlands, which may further reduce the movement of agricultural contaminants to surface water bodies by sequestering and/or processing pollutants. In addition to nutrient and pesticide mitigation, wetlands have high net primary productivity, mitigate floods and provide wildlife habitats. Consequently, these areas are valuable and are protected by U.S. law and by various conservation programs (e.g., Ducks Unlimited, the 'Swampbuster' provision of the Food Security Act of 1985 and subsequent US federal farm bills, the Wetland Reserve Program).

With these well recognized ecological services provided by wetlands, there is an effort to protect these systems and, in some locations, to duplicate them in constructed wetlands. Just as there are many reasons for the construction of wetlands, there are several means to evaluate their performance. Other studies have detailed the macro-flora (Kadlec, 2008; Bastviken et al., 2009) and fauna of constructed wetlands (Knutson et al., 2004; Fairchild et al., 2000); the effectiveness of constructed wetlands to process animal (Lin et al., 2002; Muñoz et al., 2006) or municipal wastewater (Coleman et al., 2001); retain sediment (reviewed in Cooper and Moore, 2003) and sequester and/or degrade insecticides (reviewed in Schultz, 2004; Cooper and Moore, 2003; Moore et al., 2009) and herbicides (Locke et al., 2011; Weaver et al., 2004a,b). These constructed wetlands, at least initially, often lack some key biotic characteristics of natural wetlands. For example the constructed wetlands may

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have lower plant species richness and higher prevalence of invasive species (Mitsch and Wilson, 1996; Balcombe et al., 2005; Spieles, 2005 and references therein).

The Beasley Lake watershed is in the Mississippi Alluvial Delta, an area of intense row-crop agricultural production, large agrichemical inputs, and a prevalence of impacted water bodies. This region has a subtropical climate with an average annual rainfall of 128 cm. Beasley Lake is a part of the multi-agency Mississippi Delta Management Systems Evaluation Project (MSEA) and Conservation Effects Assessment Project (CEAP) (Locke, 2004; Locke et al., 2008). We describe here the establishment of a surface-flow, constructed wetland at one inlet of Beasley Lake. The changes in the plant community and the microbial community structure and activity were monitored for two years after construction and these characteristics compared to a naturally occurring adjacent reference wetland.

2. Methods

2.1. Study sites

Located ca. 6 km south of Indianola, MS (Lat: 33.40417 Long: −90.66808) Beasley Lake is an agriculturally impacted, sediment stressed water body receiving runoff water from a watershed of ca. 850 ha. The Beasley watershed is part of the Mississippi Delta Management Systems Evaluation Areas (MD-MSEA) program (Locke, 2004; Locke et al., 2008; Zablotowicz et al., 2010). Meteorological conditions are monitored and are available from the Mississippi State Experiment Station (http://ext.msstate.edu/anr/drec/stations.cgi). Northeast of this lake is a <10 ha sub-watershed that drains into Beasley lake via a metal flume and a long, shallow natural inlet.

After a preliminary survey of the topology and hydrology of this vegetated waterway in September 2001, seven points were selected along the center of the channel and marked with flags for subsequent sampling. At each sample point in the channel, two points midway up the adjacent banks were identified and flagged. Each of these 21 points was geo-referenced using global positioning satellites (GPS).

A naturally occurring, depressional wetland, located on the North side of Beasley Lake, approximately 600 m west of the constructed site was also under observation as a reference site.

2.2. Wetland construction and field observations

In April of 2002, this site was converted to a constructed wetland. Three berms were placed across the waterway, with two upstream berms breached by two 40-cm pipes and the drainage through the final berm via 10-cm corrugated field tile and two 40-cm overflow pipes. The placement of the berms resulted in the creation of three cells: a low volume sediment trap and two additional cells each approximately 90-m long (Fig. 1). The final, downstream cell was excavated as follows: the surface 10 cm was removed and stockpiled while approx 1000 m^3 of additional soil was removed before replacing the surface soil, resulting in a nearly level, excavated cell of approximately 90-m long and 25-m wide. For analysis in this study, only the two downstream cells are examined and are referred to as the upland and constructed wetland systems.

Prior to the construction of the wetland, the site experienced variable, seasonal hydrology. For example, in the early months of 2002 all the sampling points were submerged, but during most of the warmer months there was no standing water. After construction the excavated cell remained continuously submerged for the duration of the study. The upland cell also retained a small amount

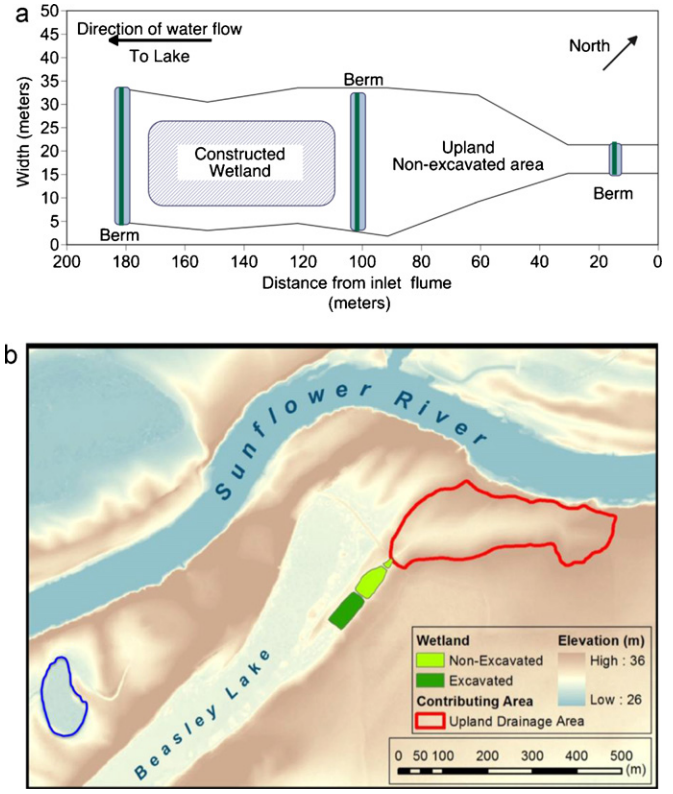

Fig. 1. (a) Schematic of the constructed wetland and (b) an aerial view of the wetland (light and dark green) and adjacent drainage area (outlined in red). Beasley Lake is located southwest of the constructed wetland. The reference wetland, outlined in blue, is between the Sunflower river and Beasley Lake. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of water periodically after rainfall events, but would experience drydown in the summer.

On October 9, 2001 (pre-construction) and on September 16, 2003 the flora of the site was surveyed and the abundance and distribution of plant species was recorded. The hydrophytic status of each species was assigned according to Reed (1988). Dominant plants were determined by the "50/20" rule for wetland delineation (Environmental Laboratory, 1987).

Soil samples (ca. 500 g) were collected from the upper 2.5 cm on September 26, 2001; January 4, 2002; March 10, 2002; April 15, 2002; June 4, 2002; October 8, 2002; January 6, 2003; April 12, 2003; July 10, 2003; October 20, 2003; and December 19, 2003. Samples were immediately placed on ice for transport to the laboratory where sub-samples were stored at −80 °C for fatty acid methyl ester (FAME) analysis or 4 °C for all other analysis. Textural analysis before construction indicated that the lower elevation points in the system were clay soils and the higher elevation points were silty clay soils. Soil surveys reported the site as a Dowling clay (very-fine smectitic, thermic Typic Endo-aqualfs; hydric, clayey alluvium of recent Holocene age) (Soil Survey Staff, 1959). Degradation of the herbicides atrazine and fluometuron in moist or submerged soil from this site has been observed and reported (Weaver et al., 2004a,b).

The upland and constructed wetland systems were compared with a naturally occurring reference wetland located on the North side of Beasley Lake, approximately 600 m west of the constructed site. Samples were collected from six geo-referenced, submerged points in reference wetland on the same dates as in the constructed wetland and subject to the same analyses.

Table 1
Plant species and their hydrophytic adaption observed in the reference, upland and constructed wetland systems before construction and nineteen months following establishment in Beasley Lake watershed.

Species* Common name[a]	Hydrophytic status[b]	Percent abundance				
		Oct. 2001			Sept. 2003	
		Beasley lake inlet	Reference wetland	Non-excavated cell	Excavated Cell	Reference wetland
Alternanthera philoxeroides (Mart.) Griseb. Alligatorweed	OBL	1	70	10	70	70
Andropogon virginicus L. Broomsedge	FAC-	13	0	2	0	0
Brachiaria platyphylla (Griseb.) Nash Broadleaf signalgrass	FAC+	10	0	5	2	0
Cephalanthus occidentalis L. Buttonbush	OBL	1	0	2	0	0
Cyperus iria L. Rice flatsedge	FACW	3	0	2	2	0
Digitaria ischaemum (Schreber) Muhl. Small crabgrass	UPL	13	0	0	0	0
Diodia virginiana L. Virginia buttonweed	FACW	1	0	5	2	0
Echinochloa colonum (L.) Link Junglerice	FACW	7	0	5	2	0
E. crus-galli (L.) Beauv. Barnyardgrass	FAC-	3	0	5	2	0
Eleocharis obtusa (Willd.) Schultes Blunt spikerush	OBL	0	0	2	5	0
Leptochloa filiformis (Lam.) Beauv. Red sprangletop	FACW	3	0	0	0	0
L. panicoides (Presl) Hitchc. Amazon sprangletop	FACW	3	0	0	0	0
Ludwigia repens Forst. Floating primrose	OBL	1	30	2	10	30
Panicum dichotomiflorum Michaux Fall panicum	FACW	4	0	15	2	0
Polygonum lapathifolium L. Pale smartweed	FACW	3	0	2	2	0
Polygonum pennsylvanicum L. Pennsylvania smartweed	FACW	3	0	0	2	0
Sesbania exaltata (Raf.) A.W. Hill Hemp sesbania	FACW-	2	0	2	2	0
Setaria parviflora (Poir.) Kerguelen Knotroot foxtail	FAC	7	0	2	2	0
Sida spinosa L. Prickly sida	FACU	7	0	0	2	0
Sorghum halepense (L.) Pers. Johnsongrass	FACU	13	0	5	0	0

Invasive species are denoted with an asterisk. Percent abundance based on visual estimate.

[a] Common names, where applicable, are from Weed Science Society of America Composite List of Weeds Jan 2010.pdf; http://www.wssa.net/weeds/ID/WeedNames/namessearch.php, accessed September 21, 2010.

[b] Species with an obligate (OBL), facultative wetland (FACW), facultative (FAC), facultative upland (FACU) and upland (UPL) classifications from Reed (1988). OBL and FAC ratings meet the definition of "typically adapted for life in anaerobic soil conditions" by the Army Corps of Engineering manual for wetland delineation (Environmental Laboratory, 1987).

2.3. Soil enzyme assays

Flourescein diacetate (FDA) hydrolysis enzyme assays (modified from Schnürer and Rosswall, 1982) were conducted as a general indicator of soil microbial hydrolytic activity (esterase, lipase and protease). Assays were conducted using 4 g of soil (fresh weight) in 20 ml of 50 mM phosphate buffer (pH 7.6) with 0.5 mg of FDA incubated for 1 h in a shaking incubator (28 °C, and 150 rpm). Assays were terminated by addition of 12 ml of acetone. Dehydrogenase was measured using triphenyl tetrazolium chloride (TTC) as substrate and yeast extract as a carbon source (Casida, 1977). Soil (4 g fresh weight) was placed in 25-ml corex tubes with 8 ml aqueous TTC (3%) and yeast extract (0.1%) and briefly sonicated to enhance equilibria with substrate and incubated statically for 22 h at 37 °C. Dehydrogenase assays were terminated with 12 ml of acetone, vortexed and centrifuged (10 min 10,000 × *g*). The absorbance of the supernatant analyzed using a spectrophotometer at 490 nm and 485 nm for products of the FDA and TTC assays (fluorescein and triphenyltetrazolium formazan), respectively. All enzyme assays were analyzed in triplicate using one control without substrate in order to correct for background. Activity is reported on a soil dry weight basis.

2.4. FAME extraction and analysis

A protocol was developed that is similar to the Ester-Linked method of Schutter and Dick (2000) and others (Gagliardi et al., 2001; Franzluebbers et al., 1999; Weaver et al., 2007). Methyl-ester linked fatty acids were released from two grams of soil (fresh weight) during incubation with 10 ml of 0.2 N methanolic KOH at 37 °C in Teflon-capped glass tubes. After incubation, 3 ml of 1 M acetic acid was added to neutralize the suspension and the FAMEs were extracted twice into 5 ml of hexane. The organic phases were combined, concentrated, but not dried, under N2 and dissolved in ca. 1 ml of 1:1, hexane:methyl-tert butyl ether. Fatty acid methyl esters were then separated, identified, and quantified using an Agilent 6890 gas chromatograph and the MIDI EUKARY protocol

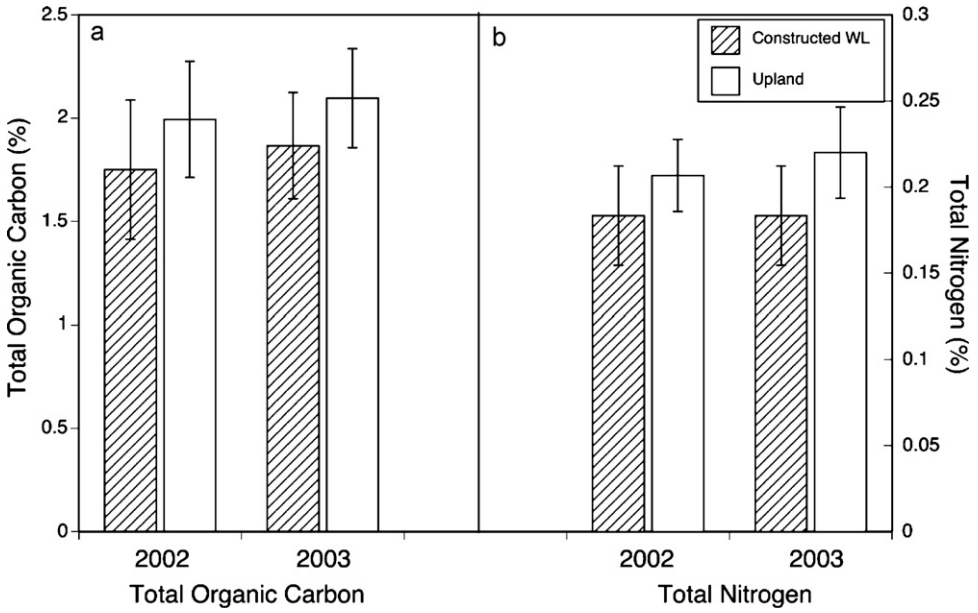

Fig. 2. Total organic carbon (a) and nitrogen (b) content of soil in the upland area and in the constructed wetland. Each bar depicts the mean and standard deviation. Year, ecosystem and year × ecosystem interactions were not significant for carbon ($P = 0.41$, 0.11 and 0.96, respectively). The upland system had higher nitrogen ($P = 0.04$) but no significant year or ecosystem × year interaction ($P = 0.47$ and 0.66, respectively).

and verified by using MIDI FAME standards (Microbial ID, Newark, NJ).

These FAMEs were grouped following by functional commonalities and according to D'Angelo et al. (2005): branched chain, gram-positive-associated FAMEs (iso and anti-iso); unsaturated, gram-negative-associated FAMEs; fungal FAMEs (16:1 cis 5 and 18:2 cis 9; 18:2 cis 6); hydroxy FAMEs; cyclo FAMEs; low molecular weight saturated FAMEs; and high molecular weight saturated FAMEs.

2.5. Statistics

The experiment was analyzed as a split–split-plot with six replications of each treatment. Year (2002 and 2003) was the whole plot, habitat (upland, constructed wetland, natural wetland) was the sub-plot, and season (summer, fall, winter) was the sub–sub-plot. Data were subjected to principle component analysis (PCA) and analysis of variance in Proc Mixed using SAS 9.2 (SAS, 2009), and least squared means were calculated ($P \leq 0.05$) using PDMIX800 (Saston, 1998).

3. Results and discussion

A summary of the vegetation observed at the two sampling dates is presented in Table 1. Prior to construction of the wetland a substantial number of the plant species present, including several of the dominant species, were adapted to inundation and/or saturation during the growing season. This vegetation was not

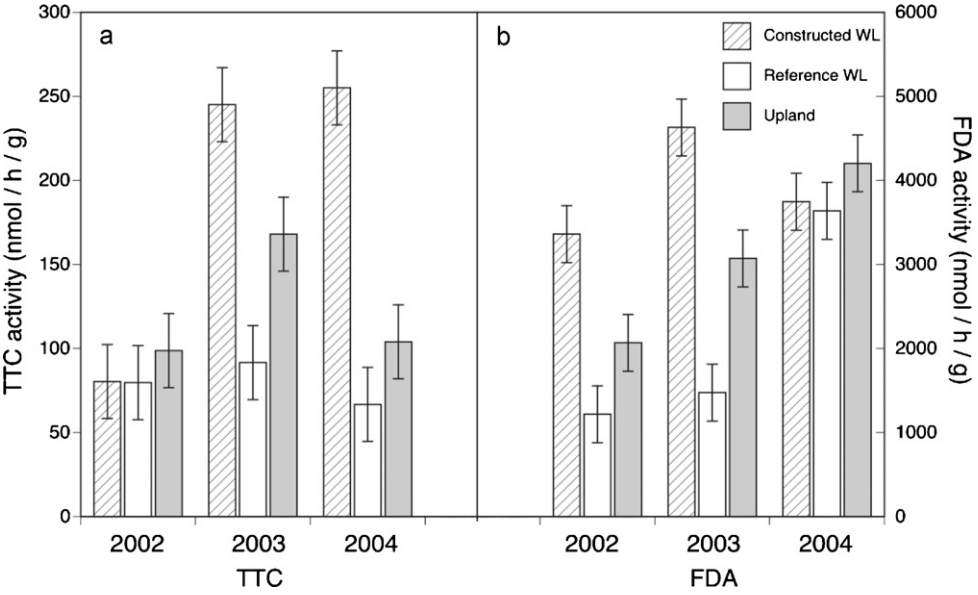

Fig. 3. Soil enzyme activity, triphenyl tetrazolium chloride (TTC) dehydrogenase and fluorescein diacetate (FDA) hydrolytic activity observed for the three ecotones averaged among all yearly samples (LSD for TTC = 62, LSD for FDA = 775). Each bar depicts the mean and standard deviation. Year by ecosystem effects significant for TTC ($P = 0.007$) and FDA ($P = 0.02$).

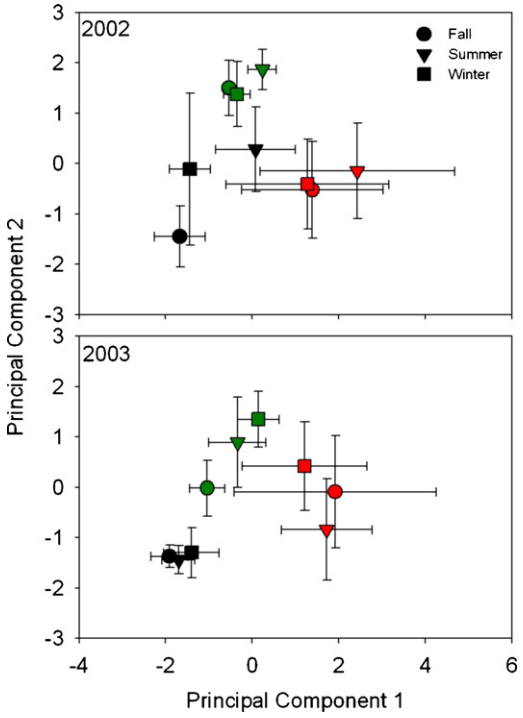

Fig. 4. Mean and standard deviation of principal component ordinates for the three ecotones for a given season and year based on principal component analysis of soil microbial fatty acid methyl esters (FAMEs). Upland observations are in red, reference wetland in green and constructed wetland in black. Error bars are 1 standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

uniformly distributed, and appeared to correspond to the elevation of a specific point. For example, *Polygonum lapathifolium*, an obligate wetland species, was a dominant species along the base of the channel, but was completely absent along the banks. At the time of the initial plant inventory, two of the four dominant species were wetland-adapted species (according to Reed, 1988), as were all of the plants in the reference wetland.

Numerous studies on vegetated filter strips (Krutz et al., 2003, 2004, 2005), ditches (Moore et al., 2001; Crum et al., 1998) and wetlands (Kao et al., 2001; Matheson et al., 2002; Dierberg et al., 2002; Hoagland et al., 2001) have highlighted the importance of plants in the capture of sediments, binding of pesticides and uptake and sequestration of nutrients. Others have noted that ditches have potential as weed reservoirs. For example Boutin et al. (2003) reported that 10 of the 97 species they inventoried in Eastern Canadian riparian buffer strips were introduced species and 30% of the vegetation was "noxious weeds." The observations of the ditch in the present study are somewhat worse: nine of the 50 species found were invasive, and these invasive species represented two of the four most abundant species. Of particular concern were *Sesbania exaltata*, *Sorghum halepense* and *Polygonum pennsylvanicum*, which are significant agronomic pests. Also noted during the 2001 survey were 8 invasive species, including two dominant species. For comparison, the submerged portion of the natural wetland was dominated by the invasive species *Alternanthera philoxeroides*.

Within a week after construction there was sufficient rainfall to inundate the excavated cell and within six weeks there was a dense, uniform stand of *Leptochloa panicoides*, a native, wetland-adapted species. As that annual grass matured and senesced, other plant species dominated the excavated cell, particularly *A. philoxeroides* and *Ludwigia repens*, two obligate wetland species. By fall of 2003, only four species were found in the constructed wetland that were not obligate (OBL) or facultative wetland adapted (FACW) (Reed, 1988), each comprising 2% or less of the total vegetation. The three most dominant species were all OBL wetland species, *A. philoxeroides*, *L. repens* and *Eleocharis obtuse*. At the same time, *A. philoxeroides* and *L. repens* comprised 100% of the vegetation in the reference wetland. In contrast, OBL or FACW vegetation accounted for 41% in the upland area.

Given the regional and local prevalence of *A. philoxeroides* (Geo-Reseources Institute), it should not be a surprise that it efficiently colonized the excavated cell. Others have noted the rapid colonization of wetlands by aquatic species; these wetland plant communities, however, often lack richness and are colonized by exotic invasive species (Mulhouse and Galatowitsch, 2003). Consequently, constructed wetlands tend to take on the characteristics of "degraded" wetlands due to these invasions (Matthews and

Table 2
Pearson coefficients of functional fatty acid methyl ester groups contributing to microbial community structure based on principal component analysis and the relationship between year, ecosystem, season and interaction of these fixed effects.

Principal component 1[a] Eigen value PC1 = 0.42	Principal component 2 Eigen value PC2 = 0.23
Fungal 0.951 $P < 0.0001$	High molecular weight saturated 0.662 $P < 0.0001$
Unsaturated, Gram-negative 0.913 $P < 0.0001$	Cyclo 0.654 $P < 0.0001$
Branched, Gram-positive −0.8351 $P < 0.0001$	Low molecular weight saturated 0.620 $P = 0.038$
Hydroxy −0.661 $P < 0.0001$	Hydroxy −0.573 $P < 0.0001$
Low molecular weight saturated −0.1997 $P = 0.038$	Unsaturated, Gram-negative −0.225 $P = 0.019$

	Test of fixed effects[b]	
	Principal component 1[a]	Principal component 2
Year	$P = 0.04$	$P = 0.512$
Ecosystem	$P < 0.0001$	$P = 0.298$
Year by ecosystem	$P = 0.006$	$P = 0.006$
Season	$P = 0.0019$	$P = 0.458$
Ecosystem by season	$P = 0.005$	$P = 0.007$
Year by ecosystem by season	$P = 0.019$	$P = 0.119$

[a] Positive values indicate a positive association with this FAME group and this PCA axis and the absolute value is the magnitude the group effects the position on the axis. Numbers in parentheses represent the statistical significance.
[b] Fixed effects tests the significance of each variable on the given principal component.

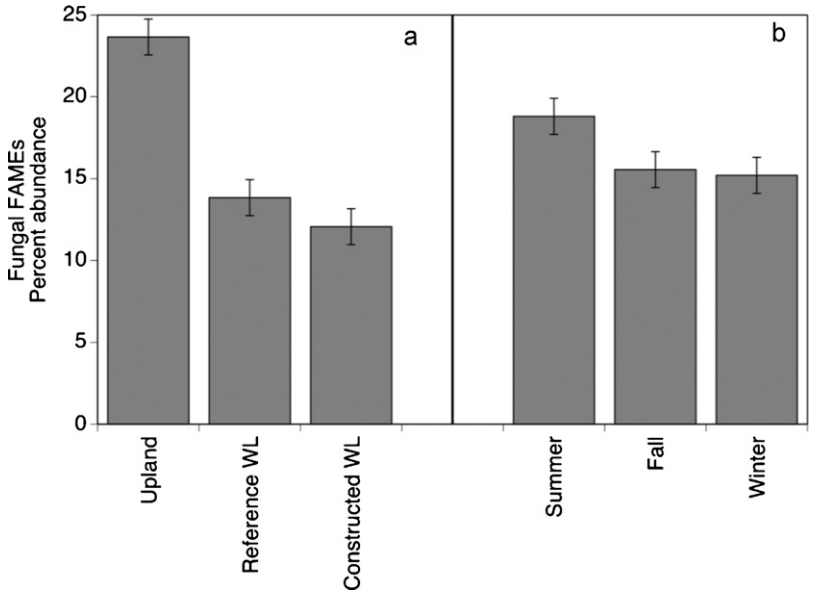

Fig. 5. Recovery of fungal fatty acid methyl esters (FAMEs) from wetland soil in the upland, reference and constructed wetlands. Effect of ecotone (a) LSD = 2.2 and season (b) LSD = 2.2. *P* value for ecosystem effect <0.0001; *P* value for season = 0.075. Each bar depicts the mean and standard deviation.

Spyreas, 2010). The success of the invasive weed in the present system underscores the need for proactive revegetation of these disturbed sites. Given the observed presence these invasive species prior to wetland construction, their later dominance might have been anticipated. Introduction of desirable native wetland species or supplementation with propagules and soil from a high quality wetland might have delayed or prevented the establishment of the invasive species. Alternatively, we cannot reject the hypothesis that the "weedy" invasive species have an ecological function. For example, we have documented that this wetland system, with it's "degraded" plant population, improves water quality through the processing or sequestering of herbicides and insecticides in agricultural runoff (Locke et al., 2011; Moore et al., 2009; Weaver et al., 2004a,b).

Wetlands are sometimes constructed with the intent to process or sequester dissolved or particulate-associated carbon. The extent to which constructed wetlands are a sink for carbon or a source of methane emissions is debatable (e.g., Hossler and Bouchard, 2010; Brantley et al., 2008; Bridgham et al., 2006), and C sequestration may be a trait that develops in more mature systems, but in the present constructed wetland, over the time period evaluated, there was no evidence of C sequestration; there were no sampling dates that revealed significantly elevated C content in the constructed wetland relative to the upland area and there was no measurable increase in organic carbon over time ($P = 0.41$) or ecosystem by time ($P = 0.96$) (Fig. 2a). There was no time effect for soil N ($P = 0.47$) or ecosystem by time effect ($P = 0.951$) during the study period (Fig. 2b), but, overall, the denitrification rate in the excavated cell is maintaining the soil N content below that of the upland system ($P = 0.04$).

The relationship between soil enzyme activities and microbial processes is not always clear. Aon et al. (2001) related TTC dehydrogenase to microbial biomass and FDA hydrolysis to nutrient cycling, while most others (Silva et al., 2003; Sena et al., 2002) relate TTC dehydrogenase to microbial activity, specifically activity in the electron transport chain (Trevors, 1983). TTC has also been used as an indicator of the health of the soil microbial community (Guerra et al., 2002). In the present study there was a year by habitat interaction for both TTC ($P = 0.007$) and FDA ($P = 0.02$) activity (Fig. 3). TTC responded significantly to all main effects and two- and three-way interactions. Four of the five highest observed FDA values occurred in the constructed wetland. The lowest TTC levels were reported in all three systems during summer of 2002, but after this period of low activity, they all increased significantly by winter 2002. Over this time period the reference wetland increased TTC activity 3.3 fold, the unexcavated cell increased 4.5 fold and the excavated cell increased 6 fold. After the first year there was not a clear trend over time, but the four highest TTC values recorded in this study were all from the excavated cell. We have noted dramatic increases in TTC activity in rice fields during over-winter flooding (Patterson et al., 2005). The rice fields in that study were disturbed flooded agricultural sites with a large input of fresh organic matter from the decomposing rice crop. Similarly, the constructed wetland cell in this study had a major disturbance event followed by flooding and flush of high primary productivity, which enriched the surface sediment with fresh organic matter. Together, these events may have facilitated a stimulation of the electron transport chain of the soil microbial community. The reference wetland, in contrast, had generally depressed TTC and FDA activities, with four of the eight lowest and five of the eight lowest values, respectively. This low soil microbial enzyme activity, coupled with the low plant diver-

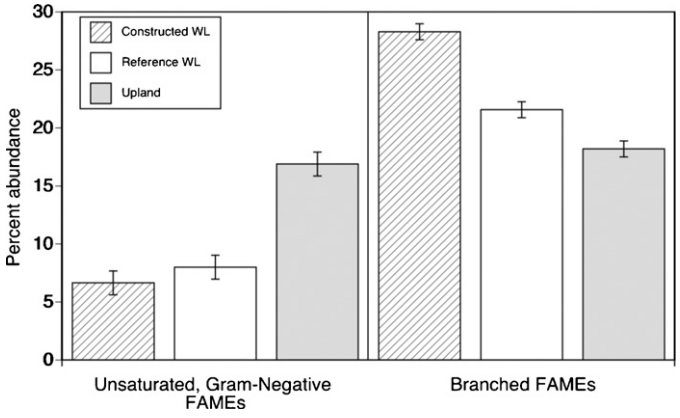

Fig. 6. Ecotone effect on abundance of unsaturated and branched fatty acid methyl esters (FAMEs) associated with Gram-negative (LSD = 2.1) and Gram-positive bacteria (LSD = 1.6), respectively. Each bar depicts the mean and standard deviation. $P < 0.001$ for unsaturated and for branched FAMES.

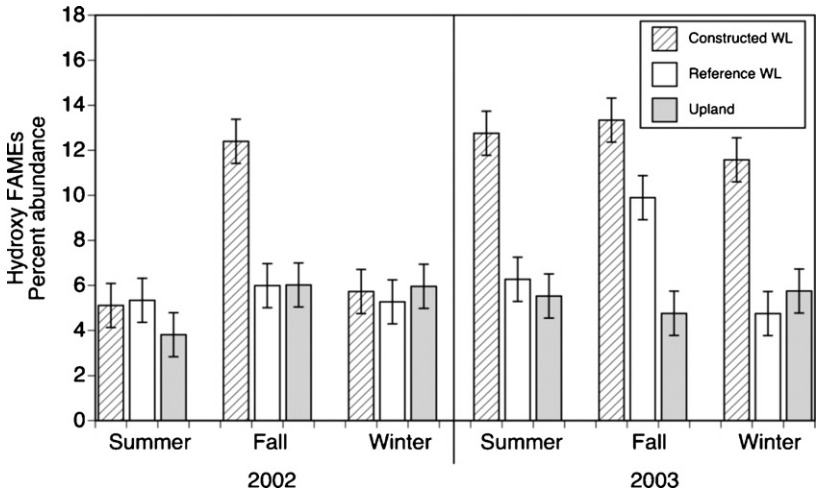

Fig. 7. Recovery of hydroxy fatty acid methyl esters (FAMEs) as affected by three-way ecotone by year by season interactions ($P = 0.004$; LSD 0.05 = 2.70). Each bar depicts the mean and standard deviation.

sity and the dominance of exotic invasive weeds indicate that the reference wetland is a low-quality system, likely with diminished ecosystem function.

Observations were collected on 42 individual FAMEs and on five functional/taxonomic groupings. Prior to construction, there was little significant difference between the area that would be excavated and the area that would not be excavated, with only 5 FAMEs statistically different between the two areas (data not shown). After construction, however, analysis of individual FAMEs indicated that soil microbial community within the constructed wetland differed from the upland area. To improve the resolution of principal component analysis and to understand the changes in specific groups of microorganisms, multiple FAMEs were combined into functional groups and subject to PCA (Fig. 4). PCA resolved the three ecosystems. The upland and reference wetland area exhibited little change over the period of observation, while the constructed wetland moved significantly along principle components 1 and 2 during 2002. In 2003, there was very low variance in the FAMEs within the constructed wetland. This low variance in the constructed wetland is consistent with the observed uniform elevation and uniform inundation. In 2003, the low variance also indicates a greater temporal stability than in 2002, suggesting that a large part of the rapid transition had already taken place. Part of the variance in the upland habitat is understandable given that it is a composite of sampling points with differing elevations and non-uniform hydrology and vegetation. Table 2 describes the correlation of the functional FAME groupings to principle components 1 and 2.

To more clearly discern the role of various FAME groups in describing the microbial community the FAME groups were examined individually by ANOVA. These results are summarized in Figs. 5–7. Fungal FAMEs were the greatest contributor to principal component 1. As revealed in Fig. 5a, these fatty acids responded strongly to the ecosystem ($P < 0.0001$). While these fatty acids accounted for about 24% of the overall FAMEs in the upland system, they only amounted to 13–14% in the two wetland systems. The small difference over season (Fig. 5b) may also be related to hydrology: while the wetland habitats were saturated year-round, the upland system experienced periodic winter flooding, but would dry during the growing season. The magnitude of response of the fungal FAMEs to the ecosystem relative to the response to season underscores the strong linkage between this indicator and the hydrology.

Unsaturated FAMEs associated with Gram-negative bacteria were also strong positive contributors to PC1 (Pearson coefficient 0.913, $P < 0.0001$) and negatively associated with PC2 (Pearson coefficient −0.225, $P = 0.019$). Unlike the fungal FAMEs however, the only significant factor linked with these FAMEs was ecosystem ($P < 0.001$). These FAMEs, overall, were about twice as abundant in the upland area as in either of the wetlands (Fig. 6). This strong association between ecosystem and unbranched Gram-negative FAMEs was quickly established, as there was no significant change after summer of 2003 (data not shown). The reverse pattern was seen for branched chain FAMEs (Fig. 6). This shift in abundance of branched FAMEs in response to the altered hydrology reflects the observations of Bossio and Scow (1998). Branched chain FAMEs are associated with Gram-positive bacteria, so, taken together, these observations are consistent with a shift in the bacterial community in the constructed wetland from Gram-negative species to Gram-positive species associated with the change in hydrology.

While Fungal FAMEs and the unsaturated, Gram-negative FAMEs distinguished the two wetlands from the upland system, the hydroxy FAMEs resolved the two wetlands. There were highly significant year, season and ecosystem effects (data not shown) and a significant ($P = 0.004$) three-way interaction (Fig. 7). While hydroxy FAMEs are common to many Gram-negative bacteria lipopolysaccharides, Zelles (1997) shown that these are also present in Gram-positive bacteria such as *Arthrobacter*. Thus the association is hardly specific and in the present system is contradictory with other Gram-negative markers (Fig. 6). The taxonomic significance of the hydroxy FAMEs may be unclear, but this marker clearly identified the unique microbial community in the present constructed wetland.

4. Conclusions

It is often noted that constructed wetlands may take years or even decades to mature (Balcombe et al., 2005; Spieles, 2005). Baclombe et al. cited a benchmark of 50 years and another analysis that concluded that the time to maturity would be affected by how different the initial conditions were. Balcombe et al. were in West Virginia and citing work from Oregon and The Netherlands, respectively. Given the much warmer climate in the present study; the documented prior adaptations to wetland biotic conditions, including occasional flooding and some wetland-adapted plant species; and the relatively high annual rainfall, there was reason to anticipate that this constructed wetland might develop on an accelerated timescale. In fact, by several indicators, the present wetland has undergone substantial transition during this period of observation.

The constructed wetland in the present study already provides several wetland services. It wetland intercepts agricultural runoff water and has an overall retention of ca. 4000 m^3. It has demonstrated a capacity for processing herbicides present in surface waters (Weaver et al., 2004a,b; Moore et al., 2009; Locke et al., 2011). Numerous wetland plant species now reside in the system. The examined microbial enzyme activities and FAME biomarkers indicate that the excavated cell rapidly assumed the community structure of the reference wetland, and is much more metabolically active than the undisturbed upland system or the reference wetland. There was less nitrogen present in the soil of the constructed wetland than the comparable upland ecosystem. However, if wetlands are constructed specifically to provide habitat for native wetland species, the present constructed wetland is a more equivocal success; there is more plant diversity in the constructed wetland than the reference wetland, but much of the plant biomass is represented by exotic invasive species. With regards to C sequestration, the constructed wetland has not measurably provided this ecosystem function in the time period evaluated. Perhaps carbon will accumulate as the wetland matures, but there is presently no evidence of the system acting as a net C sink. If wetlands are constructed to provide some ecosystem function, then this ecological service should be carefully defined. The observations over the first two years after constructed, presented here, indicate that in constructed wetlands provide many, but not all, wetland services.

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