Microb Ecol
DOI 10.1007/s00248-015-0651-2

MICROBIOLOGY OF AQUATIC SYSTEMS

Microbial Community Composition and Extracellular Enzyme Activities Associated with *Juncus roemerianus* and *Spartina alterniflora* Vegetated Sediments in Louisiana Saltmarshes

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Received: 7 May 2015 / Accepted: 14 July 2015

Abstract Saltmarshes are typically dominated by perennial grasses with large underground rhizome systems that can change local sediment conditions and be important in shaping the sediment microbial community. Factors such as salinity that control plant zonation in saltmarshes are also likely to influence the microbial community, but little is known as to whether microbial communities share distribution patterns with plants in these systems. To determine the extent to which microbial assemblages are influenced by saltmarsh plant communities, as well as to examine patterns in microbial community structure at local and regional scales, we sampled sediments at three saltmarshes in Louisiana, USA. All three systems exhibit a patchy distribution of *Juncus roemerianus* stands within a *Spartina alterniflora* marsh. Sediment samples were collected from the interior of several *J. roemerianus* stands as well as from the *S. alterniflora* matrix. Samples were assayed for extracellular enzyme activity and DNA extracted to determine microbial community composition. Denaturing gradient gel electrophoresis of rRNA gene fragments was used to determine regional patterns in bacterial, archaeal, and fungal assemblages, while Illumina sequencing was used to examine local, vegetation-driven, patterns in community structure at one site. Both enzyme activity and microbial community structure were primarily influenced by regional site. Within individual saltmarshes, bacterial and archaeal communities differed between *J. roemerianus* and *S. alterniflora* vegetated sediments, while fungal communities did not. These results highlight the importance of the plant community in shaping the sediment microbial community in saltmarshes but also demonstrate that regional scale factors are at least as important.

Keywords Microbial diversity · Extracellular enzyme activity · Wetland microbial communities · Saltmarshes

Introduction

Saltmarshes are highly productive ecosystems dominated by perennial grasses with large underground rhizome systems [1, 2]. These rhizomes can change local sediment conditions through root exudates, radial oxygen loss, and soil temperature effects [3–5]. Each of these factors can be important in shaping the sediment microbial community, which in turn drives vegetation development and composition through biogeochemical transformations [6]. Plant communities in coastal saltmarshes exhibit clear patterns of zonation driven by edaphic conditions, competition, and elevation [7, 8]. Several abiotic factors are thought to control this zonation, primarily salinity, sulfide concentration, redox potential, water movement, iron input, and pH [9, 10]. These same factors are also likely to influence the sediment microbial community, but little is known as to whether microbial communities share similar patterns of distribution to plants in these systems.

Evidence from studies in upland ecosystems suggests that microbial communities demonstrate a high degree of plant specificity, as well as being influenced by soil type [11].

Electronic supplementary material The online version of this article (doi:10.1007/s00248-015-0651-2) contains supplementary material, which is available to authorized users.

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Published online: 14 August 2015

However, studies of microbial diversity in wetland ecosystems have often tended to be narrower in scope and generally have focused on specific microbial populations responsible for biogeochemical processes (e.g., methanogenesis, nitrogen fixation) rather than whole community analysis. This is especially true in saltmarsh systems, where a significant body of research exists on sulfate-reducing bacteria [12–14] and diazotroph (nitrogen-fixing) communities [15–17]. Studies relating overall sediment microbial community structure to the plant community in such wetlands are much scarcer. Those studies that have related wetland sediment microbial assemblages to plant species often find some degree of plant specificity, although this can be confounded by geographical differences. For example, phospholipid fatty acid analysis (PLFA)-based comparisons of microbial communities associated with *Phragmites australis* and *Spartina alterniflora* in two brackish wetlands showed PLFA profiles that were significantly different between the two species at one site but not at the other [18]. Furthermore, microbial communities were markedly different between the two sites, leading to the conclusion that those microbial communities were primarily affected by the nature of the site [18]. Similarly, local-scale environmental heterogeneity has been suggested as the major factor structuring saltmarsh bacterial communities rather than nutrient inputs [19].

While studies relating the general microbial ecology of saltmarshes to plant zonation are limited, various studies describe the diversity and ecology of saltmarsh microorganisms [20–22], as well as their biogeography [23, 24]. These studies show that saltmarsh ecosystems harbor diverse microbial assemblages which respond to a wide variety of biotic and abiotic factors, and suggest that at a broad spatial scale, microbial community composition may be correlated with latitude [23]. Geographic distance has been shown to have a strong effect on microbial community similarity within a saltmarsh but has reduced importance at larger scales [25].

We sought to determine whether microbial communities in saltmarshes are structured by the type of vegetation present or by broader environmental conditions as dictated by the site. To examine these patterns in microbial community structure, we sampled sediments at three saltmarsh sites in south Louisiana, USA. All three sites are primarily *S. alterniflora* marsh, with smaller zones of *Juncus roemerianus* embedded within the *S. alterniflora* matrix. *S. alterniflora* is common to the Atlantic and Gulf coastal regions of the USA and is widespread along the coast of Louisiana. Typically, *S. alterniflora* dominates lower in the saltmarsh, while the plant community in the high marsh shifts to species with a lower salinity tolerance, such as *J. roemerianus*. However, in many areas of coastal Louisiana, *J. roemerianus* exists in distinctive patches within large monospecific stands of *S. alterniflora* without any obvious change in elevation. These *J. roemerianus* patches have been reported in the literature [26, 27] and are stable from year to year [28]. While these patches are prevalent in many coastal saltmarshes, the only prior analysis of their microbial assemblages we found analyzes only the composition of diazotroph assemblages [29]. These patches may represent excellent natural systems to test the influence of the plant community on sediment microbial community structure. As part of our sampling effort, we characterized microbial (bacterial, archaeal, and fungal) community structure using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified rRNA gene fragments and next-generation (paired-end Illumina) sequencing of bacterial and archaeal 16S rRNA gene fragments and fungal internal transcribed spacer (ITS) gene fragments. We also assayed patterns in community function through the activity of a suite of six extracellular enzymes related to organic matter processing and nutrient cycling.

Methods

Study Area and Sample Collection

Sediment samples were collected on June 25 and 27 and July 2, 2013 from three separate intertidal wetlands in south Louisiana, USA, with each site containing multiple (>20) large patches of *J. roemerianus* embedded within a matrix of *S. alterniflora*, as well as more smaller patches. The first site was located along the lower portion of Bayou Dularge near Theriot, LA (29° 17′ 12.39 N, 90° 53′ 17.80 W), the second site was near the Louisiana Universities Marine Consortium (LUMCON) facility in Cocodrie, LA (29° 15′ 20.94 N, 90° 39′ 38.46 W), and the third site at Four League Bay, adjacent to Atchafalaya Bay, south of Morgan City, LA (29° 13′ 56.69 N, 91° 8′ 33.88 W).

At each site, four *J. roemerianus* patches, each with a diameter of at least 12 m and at least 10 m apart, were selected for sampling. At each patch, three sediment samples were taken along a transect ranging from the interior of the *J. roemerianus* patch out into the *S. alterniflora* matrix. Samples were taken 6 m inside the interior of the *J. roemerianus*, at the edge of the *J. roemerianus*/*S. alterniflora* interface, and 6 m outside the patch in the *S. alterniflora* matrix, directly beneath the vegetation in the rhizosphere. At each sampling point, one sediment sample (approximately 50 g) was taken for microbial analysis and a second sample (approximately 50 g) taken for sediment chemical analysis. Water depth was also determined, as was pH, salinity, and temperature with a YSI meter (YSI Inc, Yellow Springs, OH). Samples were collected by filling sterile 50-mL centrifuge tubes directly with sediment from a 5–10-cm depth. This depth was visibly within the root zone of the plant species sampled and was chosen to be representative of the rhizosphere community. Samples were immediately placed on ice and then refrigerated (4 °C) once returned to the laboratory. The

following day, samples for microbial analysis were shipped chilled (frozen cold packs) overnight to the University of Mississippi for further analysis. Samples for sediment chemical analysis were refrigerated until all sites had been sampled, at which point the samples were taken to the Louisiana State University AgCenter Soil Testing and Plant Analysis Laboratory (STPAL). Sediment moisture content was determined by gravimetric loss after drying (75 °C, 48 h), and dried samples were subsequently combusted (500 °C, 2 h) to determine organic content as ash free dry weight (AFDW). Carbon and nitrogen content was determined by dry combustion on a Leco CN Elemental Analyzer, and concentrations of calcium, copper, magnesium, phosphorus, potassium, sodium, sulfur, and zinc were determined via inductively coupled plasma mass spectrometry.

Sediment Extracellular Enzyme Activity

The activity of six microbial extracellular enzymes was assayed in each sediment sample. Potential activities of β-glucosidase (EC 3.2.1.21), phosphatase (EC 3.1.3.2), *N*-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.52), cellobiohydrolase (CBH; EC 3.2.1.91), phenol oxidase (EC 1.10.3.2), and peroxidase (EC 1.11.1.7) were determined using the protocols demonstrated by Jackson et al. [30] that have previously been used for wetland sediments [31, 32]. For each sample, a 10-g subsample was homogenized in pH 5.5 50 mM acetate buffer to make a sediment/buffer slurry, and 150-μL aliquots of the slurry were used in individual reactions. Five millimolar 3,4-dihydroxyl-L-phenylalanine (L-DOPA) was used as the substrate for the phenol oxidase and peroxidase assays, and peroxidase assays also received 0.015 % H_2O_2. Five millimolar *p*-nitrophenyl (*p*NP)-linked substrates were used for the β-glucosidase and phosphatase assays, while 2 mM *p*NP-linked substrates were used to determine NAGase and CBH activity [30]. All substrates were dissolved in pH 5.5 50 mM acetate buffer. Assay incubation times were 0.5–1 h for phosphatase and β-glucosidase, 2 h for phenol oxidase and peroxidase, and 3 h for NAGase and CBH. Analytical procedures included three replicate assays of each sample for each substrate used, two replicate control assays of the sample with no substrate, and two control assays for abiotic hydrolysis/oxidation of each substrate in the presence of buffer but no sample.

After incubation, assays were centrifuged (4,000×*g*, 10 min) and 100 μL supernatant transferred to reading microplates containing 200 μL 0.067 M NaOH (*p*NP-linked substrates) or 200 μL H_2O (L-DOPA substrates). Absorbance was read at 410 nm for *p*NP-linked substrates and 460 nm for L-DOPA substrates using a Synergy HT microplate spectrophotometer (BioTek, Winooski, VT). Enzyme activity was calculated after accounting for control absorbance and expressed as micromoles substrate consumed per hour per gram dry weight of sediment. Differences in the activity of each enzyme between samples were analyzed using ANOVA followed by a Tukey's honest significance test using the open-source software R.

DNA Extraction, rRNA Gene Amplification, and Denaturing Gradient Gel Electrophoresis

DNA was extracted from 0.2-g subsamples of each sediment sample using a Power Soil DNA kit (MO BIO, Carlsbad, CA), followed by additional purification using a Power Clean DNA Clean-Up Kit (MO BIO, Carlsbad, CA). Samples were then amplified specifically for bacterial, archaeal, and fungal DNA prior to DGGE analysis. For bacterial community analysis, a 322-bp region of the bacterial 16S rRNA gene was amplified using a nested protocol involving the primers Bac8f and Univ1492r [33] followed by Bac1070f and Univ1392GC [33, 34]. For Archaea, a 461-bp section of the archaeal 16S rRNA gene was amplified with primers Arc2f and Univ1492r [33] followed by primers Arc931f and Univ1392GC [32, 33]. Reaction conditions for the bacterial and archaeal amplifications have been described previously [33]. For analysis of fungal communities, the 5′ end of the fungal 18S rRNA gene was amplified with primers NS1 and GCfung [35, 36] under conditions described by Nikolcheva et al. [37].

PCR products from the three sets of amplifications were analyzed by DGGE, following established procedures [32, 38]. Briefly, the bacterial and archaeal amplicons were electrophoresed separately along a 40–70 % urea–formamide gradient in 8 % acrylamide gels at 75 V and 60 °C for 24 h (Bacteria) or 19 h (Archaea). Fungal amplicons were electrophoresed through a 20–55 % urea–formamide gradient at 50 V for 20 h. All samples were loaded at 500–800 ng PCR product per lane. DGGE gels were stained using SYBR Green I and imaged using Molecular Imaging Software (Eastman Kodak, Rochester, NY). DGGE banding patterns were converted into binary data (indicating presence or absence of a specific band), and community profiles compared using the Jaccard similarity index followed by non-metric multidimensional scaling (NMDS) [39, 40] using the bioinformatics software Mothur. Distribution of points in NMDS plots were analyzed using analysis of molecular variance (AMOVA) and analysis of similarity (ANOSIM) to examine differences in microbial communities between samples taken from *J. roemerianus*, edge, and *S. alterniflora* locations, as well as between the three saltmarshes sampled. The Bonferroni correction was used to counteract any type I error for multiple pairwise comparisons.

Illumina Sequencing of Bacterial, Archaeal, and Fungal Communities

Based on outcomes of the amplifications and DGGE analysis, one site (Bayou Dularge) was chosen for more in-depth community analysis by paired-end indexed Illumina MiSeq sequencing of the bacterial, fungal, and archaeal community in

each sample. The Bayou Dularge site was chosen because it had the highest rate of amplification success for Bacteria, Fungi, and Archaea (100, 91.7, and 83.3 % success, respectively). Sequencing was carried out by a commercial facility (Molecular Research DNA, Shallowater, TX), essentially following standard Illumina protocols. For Bacteria, the V4 variable region of the 16S gene was targeted using the primer sets bac515F and bac806R [41]; for Fungi, the ITS1 region of the 18S gene was targeted using the primers ITS1F and ITS4R [42]; and for Archaea, a region of the archaeal 16S gene was targeted using the primers Arch 349F and Arch 806R [43]. Sequences were screened, aligned, and classified using the bioinformatics software Mothur [44] following general procedures recommended for Illumina data [45]. The SILVA database was used to align and classify bacterial and archaeal sequences. Number of operational taxonomic units (OTUs) analyzed was normalized by subsampling the lowest number of OTUs found in any one sample. A reliable fungal database has yet to be completed, so fungal sequences were clustered into OTUs at 97 % similarity using CD-HIT [46], and major OTUs were subsequently identified by BLAST searching the GenBank database for 100 randomly selected fungal sequences from each sample.

Results

Physicochemical Characteristics

Environmental variables did not differ significantly between sediments taken from stands of *J. roemerianus* and *S. alterniflora*. This was true when the data from all samples were analyzed together or when stands from each wetland were analyzed individually (Table 1). Rather, physicochemical characteristics were more dependent on the specific site sampled, with a number of parameters showing significant differences between the three saltmarshes (Table 1). The Four League Bay site was the most unlike the other two wetlands, having deeper waters, higher salinity, and lower pH than either the Bayou Dularge or Cocodrie sites. Physiochemical characteristics at Cocodrie were more intermediate, showing similarity to both of the other two sites in regards to water depth, temperature, and pH. The Bayou Dularge saltmarsh was more nutrient rich (higher %C, %N, P, and organic content) than either Four League Bay or Cocodrie and also had lower salinity. Across all sites, phosphorous content in the sediment was positively correlated with both percent carbon and percent nitrogen (R=0.72 and 0.79, respectively), and percent carbon and percent nitrogen were also highly correlated to each other (R=0.96). Organic content derived from AFDW was positively correlated with percent carbon, as would be expected, and with percent nitrogen (R=0.79 and 0.72, respectively) and was negatively correlated with water temperature (R=−0.73). Water pH was negatively correlated to sulfur content, water depth, and salinity (R=−0.71, −0.83, and −0.79, respectively), while water depth was correlated with water temperature and salinity (R=0.85 and 0.82, respectively). In regards to micronutrients, sediment magnesium content was positively correlated with the concentration of potassium, sodium, and sulfur (R=0.79, 0.82, and 0.71, respectively), and sodium content was also positively correlated with the amount of potassium (R=0.70) and sulfur (R=0.86).

Patterns in Extracellular Enzyme Activity

As with the physicochemical conditions, sediment extracellular enzyme activity was largely influenced by site rather than location on the *J. roemerianus* to *S. alterniflora* transects with few exceptions at within individual sites (Table 2). Generally, activities of β-glucosidase, phosphatase, NAGase, and CBH were all significantly higher ($p \leq 0.05$, ANOVA) in the Bayou Dularge saltmarsh than the other two sites. The Cocodrie and Four League Bay wetlands showed similar activity profiles for these enzymes, while sediments from all three saltmarshes showed similar phenol oxidase and peroxidase activity (Table 2). These oxidative enzymes were generally the least active of the six measured, with phenol oxidase, in particular, approaching zero activity in some samples. Activities of the four hydrolytic enzymes (phosphatase, β-glucosidase, NAGase, CBH) were positively correlated with each other (R=0.82–0.96 for pairwise comparisons). None of the six enzyme assayed show significant differences in activity between *J. roemerianus* or *S. alterniflora* stands (Table 2), although there was a suggestion that edge sediments showed slightly higher activity than *J. roemerianus* or *S. alterniflora* sediments in the Bayou Dularge and Cocodrie wetlands. However, these differences were not significant.

Relating enzyme activity to environmental physicochemical conditions revealed that β-glucosidase activity was generally higher in sediments with higher organic content, percent carbon, and percent nitrogen, being positively correlated with those factors (R=0.87, 0.74, and 0.70, respectively). Activity of this enzyme was negatively correlated to water temperature (R=−0.72), although all of these correlations reflect the site effect of Bayou Dularge, which has both a different physicochemical profile and a different enzymatic profile than the other two sites (Tables 1 and 2). NAGase and CBH activity showed similar correlations to environmental parameters as β-glucosidase, being positively correlated to percent carbon, percent nitrogen, and organic content (NAGase R=0.68, 0.61, and 0.82, respectively; CBH R=0.75, 0.68, and 0.87, respectively) and negatively correlated to water temperature (NAGase R=−0.71, CBH R=−0.73). Phosphatase activity showed the same trends, being positively correlated to organic content, % C and % N (R=0.63, 0.69, and 0.77, respectively), but was also positively correlated with sediment phosphorus content (R=0.63).

Table 1 Physicochemical conditions in sediments taken from three southeast Louisiana, USA, saltmarshes (Bayou Dularge, Four League Bay, and Cocodrie) that were sampled for microbial activity and community structure

Site	Bayou Dularge			Four League Bay			Cocodrie		
Vegetation type	*J. roem.*	Edge	*S. altern.*	*J. roem.*	Edge	*S. altern.*	*J. roem.*	Edge	*S. altern.*
Water depth (cm)	7.4±0.7a	7.0±0.6a	7.6±0.5a	16.0±0.9b	15.2±1.0bc	16.4±1.2b	9.7±1.3a	9.9±1.2a	10.9±1.5ac
Water temp. (°C)	28.5±0.1a	28.6±0.6a	29.1±0.6ab	30.9±0.3bc	30.9±0.5bc	31.3±0.3c	29.9±0.3abc	30.1±0.3abc	30.4±0.3abc
Salinity (ppm)	1.4±0.1a	1.4±0.1a	1.4±0.1a	9.5±0.3b	9.3±0.3b	9.3±0.3b	0.9±0.8a	2.9±1.7a	1.9±1.8a
pH (water)	7.3±0.1a	7.3±0.1a	7.3±0.1a	6.3±0.1b	6.0±0.1b	6.0±0.1bc	6.6±0.2cd	7.2±0.1ad	7.3±0.2a
Sediment moisture (%)	85±2a	86±2a	86±1a	71±1b	76±1bc	75±2bc	78±1abc	79±2ac	75±2bc
Sediment carbon (%)	19.1±1.9a	14.9±2.9abd	16.2±1.1ab	9.2±0.4cd	9.7±0.2cd	10.6±0.6bcd	13.3±1.4abcd	9.1±0.1cd	8.2±0.5c
Sediment nitrogen (%)	1.0±0.1ab	0.8±0.2abc	1.0±0.1a	0.6±0.0c	0.6±0.0bc	0.7±0.0abc	0.7±0.1abc	0.5±0.0c	0.5±0.0c
Organic content (%)	40.0±4.2ab	40.8±5.8b	35.0±2.6abc	21.0±1.5cd	25.0±1.1acd	22.5±1.6cd	25.0±2.2acd	28.0±4.5abcd	19.0±1.9d
Calcium (ppm)	2,603±613a	1,670±116ab	1,971±217ab	1,765±55ab	1,561±90ab	1,504±110b	1,682±38ab	1,628±84ab	1,568±22ab
Copper (ppm)	2.5±0.1ab	2.5±0.5ab	2.1±0.2ab	2.1±0.2ab	1.8±0.3a	1.9±0.3a	2.6±0.3abc	3.8±0.1c	3.3±0.4bc
Magnesium (ppm)	3,581±205a	3,514±394a	3,570±245a	4,440±79a	3,802±403a	3,735±327a	4,354±216a	3,620±72a	3,549±254a
Phosphorus (ppm)	55.6±4.8ad	61.1±11.5b	91.9±7.4c	35.4±2.4ad	32.2±2.0ad	26.3±2.3a	53.5±9.3ab	40.2±5.8ab	31.0±2.7a
Potassium (ppm)	1,337±85a	1,326±113a	1,225±105a	1,606±47ab	1,474±82ab	1,427±107ab	1,757±106b	1,510±34ab	1,337±71a
Sodium (ppm)	2,990±1,824a	5,316±2,964ac	2,684±1,318a	18,897±1,151b	12,624±3,976abc	9,668±2,386abc	14,426±3,383bc	5,003±1,463ac	2,042±640a
Sulfur (ppm)	858±156ab	894±235abd	707±142a	1,763±70c	1,519±161cd	1,465±94bcd	1,325±101abcd	993±108abd	760±79a
Zinc (ppm)	3.6±0.3a	3.5±0.4a	4.3±1.0a	6.6±0.7a	5.7±0.5a	5.8±0.6a	7.9±0.5a	12.3±5.2a	7.5±1.1a

Sediments were vegetated with *Juncus roemerianus*, *Spartina alterniflora*, or taken from the edge between those zones. Values are means ±standard error (n=4). Lowercase letters indicate a sample that was significantly (p<0.05, ANOVA) different from the other samples for that variable

J. roem. Juncus roemerianus, *S. altern. Spartina alterniflora*, *Edge* edge between zones

Table 2 Potential extracellular enzyme activity (μmoles h^{-1} g DW^{-1}) in sediments taken from three southeast Louisiana, USA saltmarshes (Bayou Dularge, Four League Bay, and Cocodrie) that were sampled for microbial activity and community structure

Site/vegetation	Bayou Dularge			Four League Bay			Cocodrie		
Enzyme	*J. roem.*	Edge	*S. altern.*	*J. roem.*	Edge	*S. altern.*	*J. roem.*	Edge	*S. altern.*
Phosphatase	2.14±0.51ab	3.68±1.00a	3.06±0.53ab	0.93±0.12b	1.32±0.24b	1.44±0.11b	0.92±0.18b	1.07±0.38b	1.13±0.28b
β-glucosidase	2.04±0.38ac	2.26±0.39a	1.71±0.40ab	0.61±0.09b	0.76±0.08b	0.70±0.08b	0.73±0.12b	0.86±0.3bc	0.75±0.1b
NAGase	0.98±0.18ab	1.30±0.41a	0.65±0.12ab	0.28±0.04b	0.42±0.05b	0.47±0.05b	0.45±0.08b	0.48±0.14b	0.48±0.05b
CBH	0.46±0.09a	0.45±0.1a	0.32±0.06ab	0.11±0.02b	0.16±0.02b	0.16±0.02b	0.16±0.04b	0.19±0.06ab	0.17±0.03b
Peroxidase	0.16±0.02a	0.21±0.08a	0.15±0.03a	0.16±0.05a	0.22±0.05a	0.23±0.03a	0.10±0.05a	0.13±0.04a	0.13±0.01a
Phenol oxidase	0.06±0.03a	0.09±0.02a	0.05±0.01a	0.03±0.01a	0.02±0.01a	0.02±0.01a	0.07±0.04a	0.03±0.01a	0.04±0.02a

Sediments were vegetated with *Juncus roemerianus*, *Spartina alterniflora*, or taken from the edge between those zones. Values are means ±standard error (n=4). Lowercase letters indicate significant differences in activity (p<0.05, ANOVA) from other samples for that enzyme

J. roem. Juncus roemerianus, *S. altern. Spartina alterniflora*, *Edge* edge between zones

Molecular Analysis of Microbial Community Structure

Bacterial 16S rRNA fragments were successfully amplified from all 36 sediment samples. DGGE revealed 48 total bands across all samples with a mean of 15 (±1 SE) bands per sample. When all of the samples were analyzed together, NMDS of presence–absence data obtained from DGGE banding patterns showed no consistent separation of bacterial communities by vegetation type. However, there was a site effect with differences in communities apparent among the three saltmarshes sampled (Jaccard-based AMOVA, p<0.001, Fig. 1a). Thus, subsequent analyses focused on determining the influence of vegetation on the sediment microbial community within each saltmarsh individually. For the Bayou Dularge and Cocodrie wetlands, there were differences in bacterial community structure between *J. roemerianus* and *S. alterniflora* sediments (Jaccard-based AMOVA, p=0.026 and 0.031, respectively), with the sediments sampled from the

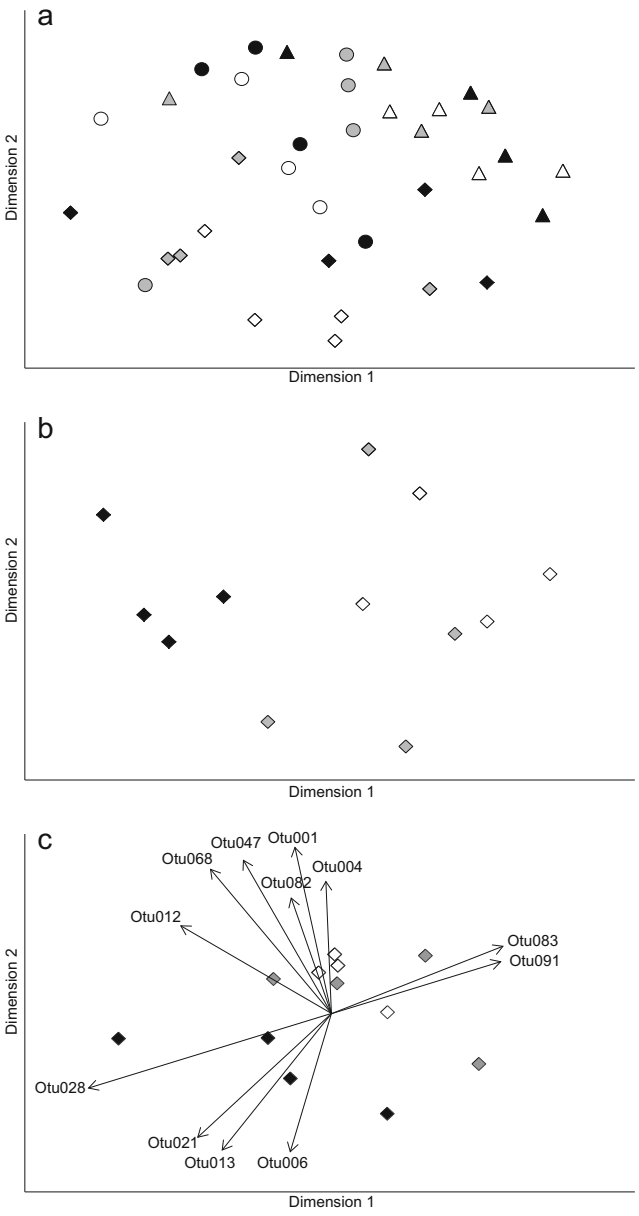

Fig. 1 The first two dimensions from three-dimensional NMDS ordinations of bacterial community structure in sediments from three saltmarshes (Cocodrie (*circles*), Four League Bay (*triangles*), Bayou Dularge (*diamonds*)) in southeast Louisiana, USA that were taken from areas vegetated with *Juncus roemerianus* (*black symbols*), *Spartina alterniflora* (*white symbols*), or the edge between these vegetation types (*gray symbols*). Ordinations were based on Jaccard similarity scores of DGGE profiles of amplified 16S rRNA gene fragments from all sites (**a**; stress=0.21), DGGE profiles of amplified 16S rRNA gene fragments from the Bayou Dularge site only (**b**; stress=0.15), or theta similarity scores of each community following Illumina sequencing of 16S rRNA gene fragments from the Bayou Dularge site only (**c**; stress=0.13). Panel **c** also shows important taxa driving the separation between *J. roemerianus* and *S. alterniflora* sediment samples at this site, which represent members of the Chromatiales (Gammaproteobacteria; OTU1), Sphingobacteriales (Bacteroidetes; OTU4, OTU68), Desulfobacterales (Deltaproteobacteria; OTU6, OTU47), Alteromonadales (Gammaproteobacteria; OTU12), Thiohalophilus (Gammaproteobacteria; OTU13), Syntrophobacterales (Deltaproteobacteria; OTU21), Hydrogenophilales (Betaproteobacteria; OTU28), Spirochaetales (Spirochaetes; OTU82), Methylococcales (Gammaproteobacteria; OTU83), and Caldilineales (Chloroflexi; OTU91)

edge of these two zones grouping predominantly with those found in *S. alterniflora* sediments (e.g., Fig. 1b). The Four League Bay site did not suggest differentiation between bacterial communities in *J. roemerianus* and *S. alterniflora* stands (Jaccard-based AMOVA, $p=0.35$).

Focusing in greater detail on the bacterial communities at Bayou Dularge using Illumina high-throughput sequencing confirmed the effect of vegetation on bacterial community structure. Valid bacterial sequences (838,302) were obtained across the 12 Bayou Dularge samples, which were classified into 77,445 OTUs. Sample coverage averaged 86 %. Bacterial communities from sediments in *J. roemerianus* and *S. alterniflora* stands were distinct from each other when compared using theta [47] similarity scores (AMOVA, $p=0.028$) and, as with the DGGE analysis, edge bacterial communities tended to be more similar to those associated with *S. alterniflora*. NMDS ordination confirmed these groupings (Fig. 1c). Several OTUs drove these community differences as determined from correlations of OTU representation with NMDS axes scores. OTU1, identified as a member of the Chromatiaceae (Gammaproteobacteria), accounted for 7.2 % of the sequences derived from *S. alterniflora* sediments, 5.9 % from edge sediments, and just 3.1 % from *J. roemerianus* sediments. In contrast, OTU6, a member of the Desulfobacteraceae (Deltaproteobacteria), was found in greater relative abundance in *J. roemerianus* and edge sediments than in *S. alterniflora* sediments (accounting for 2.6, 1.4, and 0.9 % of the *J. roemerianus*, edge, and *S. alterniflora* sequences, respectively). Other significant OTUs driving community differences included OTU83, a representative of *Methylococcus* (Gammaproteobacteria), that accounted for 0.2 % of the bacterial community in *S. alterniflora* sediments but just 0.04 % in *J. roemerianus* sediments and OTU91, a member of the genus *Caldilinea* (*Chloroflexi*), that accounted for 0.15 % of the community in *S. alterniflora* sediments as opposed to 0.07 % for *J. roemerianus*.

In terms of major bacterial phyla, *S. alterniflora* sediment communities had greater proportions of bacterial sequences classified as members of the Bacteroidetes and Lentisphaerae compared to *J. roemerianus* sediments (Fig. 2), though this was not statistically significant (ANOVA, $p=0.279$ and 0.605 for each taxon, respectively), while *J. roemerianus* sediments contained a higher proportion of sequences classified as Alphaproteobacteria (ANOVA, $p=0.049$) and Betaproteobacteria (ANOVA, $p=0.036$; Fig. 2). Edge sediments contained a greater relative abundance of sequences classified as members of the *Chlorobi* (ANOVA, $p=0.048$) compared to *S. alterniflora* sediments. For all sediment types, the most dominant bacterial lineages present, as based on the proportion of sequences obtained and classified, were the Deltaproteobacteria and Gammaproteobacteria (Fig. 2).

To reduce the influence of OTUs present in minor numbers from over-emphasizing community differences, rare OTUs were removed from the dataset at varying cutoff points and the data analyzed when just keeping sequences affiliated with OTUs that accounted for >0.001, >0.01, or >0.1 %, of the total sequences. Removing OTUs that comprised less than 0.001 or 0.01 % of the total reads left 7,313 OTUs or 1,315 OTUs, respectively, and in each case still yielded significant differences in bacterial community structure between *J. roemerianus* and *S. alterniflora* samples (Jaccard-based AMOVA, $p<0.05$). Removing a greater portion of "rare" OTUs (those accounting for less than 0.1 % of the total community) resulted in the loss of this significance and no difference between the two vegetation types, although this analysis was now just based on 123 remaining OTUs.

Archaeal 16S rRNA was successfully amplified from 15 of the 36 sediment samples (ten from Bayou Dularge (three from *J. roemerianus* sediments, four from *S. alterniflora* sediments, and three from edge sediments), three from Cocodrie (one from *J. roemerianus*, one from *S. alterniflora*, and one from edge sediments), and two from Four League Bay (one from *S. alterniflora*, and one from edge sediments)). DGGE of archaeal amplicons yielded 59 total bands across all sample types, with each sample having a mean of 21 (±1) DGGE bands. Differences in archaeal composition between the three sites could not be thoroughly tested because of the limited number of samples that amplified from the Cocodrie and Four League Bay sites, although there was a suggestion that samples taken from *J. roemerianus* sediments differed from those under *S. alterniflora* (Fig. 3a). Further analysis of the archaeal community focused on samples taken from the Bayou Dularge site, where NMDS of DGGE profiles further suggested differences in archaeal communities based on vegetation type (Fig. 3b), although these difference were not significant (Jaccard-based AMOVA, $p=0.063$).

Illumina sequencing of the sediment archaeal communities from Bayou Dularge yielded 278,840 valid archaeal sequences, representing 24,208 OTUs. Sample coverage of these assemblages averaged 90 %. The archaeal communities associated with sediments vegetated with *J. roemerianus* or *S. alterniflora* were not significantly different upon analysis of the full dataset (theta-based AMOVA, $p=0.301$); however, differences became apparent following the removal of rare OTUs. Removing OTUs that contributed to <0.001 or <0.01 % of the total community removed over 90 % of the OTUs, leaving 2,283 or 472 OTUs for analysis, respectively, although this did not change the outcome, and archaeal communities in sediments from *J. roemerianus* or *S. alterniflora* were still not significantly different (Jaccard-based AMOVA, $p=0.189$ and 0.298). However, removing OTUs that accounted for <0.1 % of the community (those represented by 278 reads or less) did indicate a significant difference between archaeal communities in *J. roemerianus* and *S. alterniflora* sediments (Jaccard-based AMOVA, $p=0.029$), although this was now just based on the 107 most

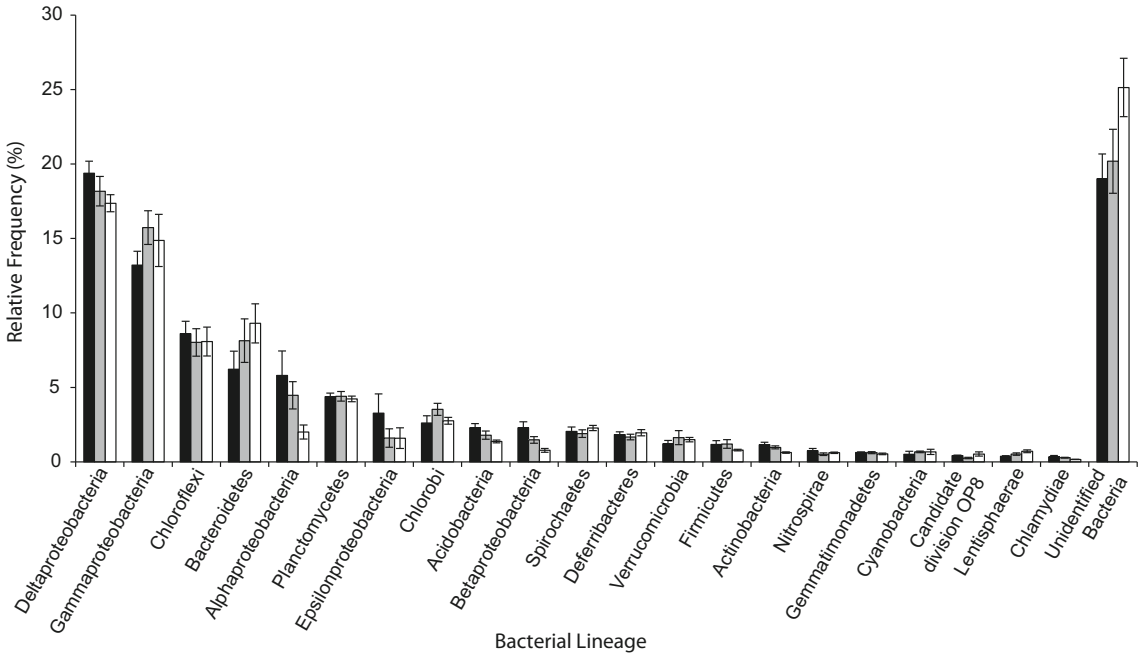

Fig. 2 Relative proportions of major bacterial lineages in sediments taken from a southeast Louisiana saltmarsh (Bayou Dularge) as determined from Illumina 16S rRNA gene sequencing. Sediments were vegetated with either *Juncus roemerianus* (*black bars*), *Spartina alterniflora* (*white bars*), or taken from the edge between these vegetation types (*gray bars*). Each *bar* represents the mean (±SE) composition based on four samples, with a mean of 69,858 sequence reads per sample

abundant archaeal OTUs. Ordination by NMDS clearly showed a separation of *J. roemerianus* and *S. alterniflora* samples using this criterion (Fig. 3c). Correlations of OTU representation with NMDS axes scores revealed that archaeal OTUs contributing to these differences included OTU1, identified as a member of the candidate order *pGrfC26*, which accounted for 76 % of the archaeal community in *S. alterniflora* sediments and 46 % in *J. roemerianus* sediments. OTU18 classified as a member of the same candidate order and pulled the NMDS ordination in a similar direction. Other OTUs that were important in separating the sample types included OTU141 (a member of the E2 group in the Euryarchaeota) which accounted for 8.7 % of the sequences from *J. roemerianus* sediments and just 1.7 % of those from *S. alterniflora* sediments. The majority of influential OTUs acted to separate the *S. alterniflora* community from the rest of the samples on the ordination, while only OTU141 was more prevalent in the *J. roemerianus* sediment archaeal community. At a broader taxonomic level, just over half of the archaeal sequences detected in Bayou Dularge saltmarsh sediments classified as members of the Euryarchaeota, which could be further divided into thermophilic, methanogenic, and miscellaneous Euryarchaeota (Fig. 4). Sequences identified as members of the Crenarchaeota were also abundant, typically accounting for 40–50 % of the dataset (Fig. 4).

Fungal 18S rRNA gene fragments could be amplified from 27 of the 36 samples: 11 from Bayou Dularge (3 from *J. roemerianus* sediments, 4 from *S. alterniflora*, 4 from edge sediments), 6 from Cocodrie (2 from *J. roemerianus* sediments, 2 from *S. alterniflora*, 2 from edge sediments), and 10 from Four League Bay (4 from *J. roemerianus* sediments, 3 from *S. alterniflora*, 3 from edge sediments). A total of 88 different bands across all 27 samples were observed following DGGE, with each sample having a mean of 16±1 bands. NMDS ordination of DGGE banding profiles suggested that there were no differences in sediment fungal communities between the different vegetation types; rather, fungal communities differed between each of the three saltmarshes sampled (Jaccard-based AMOVA, $p<0.001$, Fig. 5a). Fungal assemblages at all three sites were dissimilar from each other in individual pairwise analyses, with Bayou Dularge and Four League Bay being the most different (Jaccard-based AMOVA, $p<0.001$), followed by Four League Bay and Cocodrie (Jaccard-based AMOVA, $p=0.004$), and Cocodrie and Bayou Dularge (Jaccard-based AMOVA, $p=0.018$). The latter two sites were not significantly different because of the Bonferroni correction required for multiple pairwise comparisons (critical $p=0.0167$) but were highly suggestive of being dissimilar. Taking the Bayou Dularge site as an example, even within each individual wetland, there was no apparent effect of *J. roemerianus* and *S. alterniflora* on the sediment fungal community as NMDS ordination showed no separation of points by vegetation type (Fig. 5b).

Illumina sequencing of fungal ITS fragments from the Bayou Dularge wetland sediments returned 572,387 valid sequences. As with the DGGE data, fungal communities from

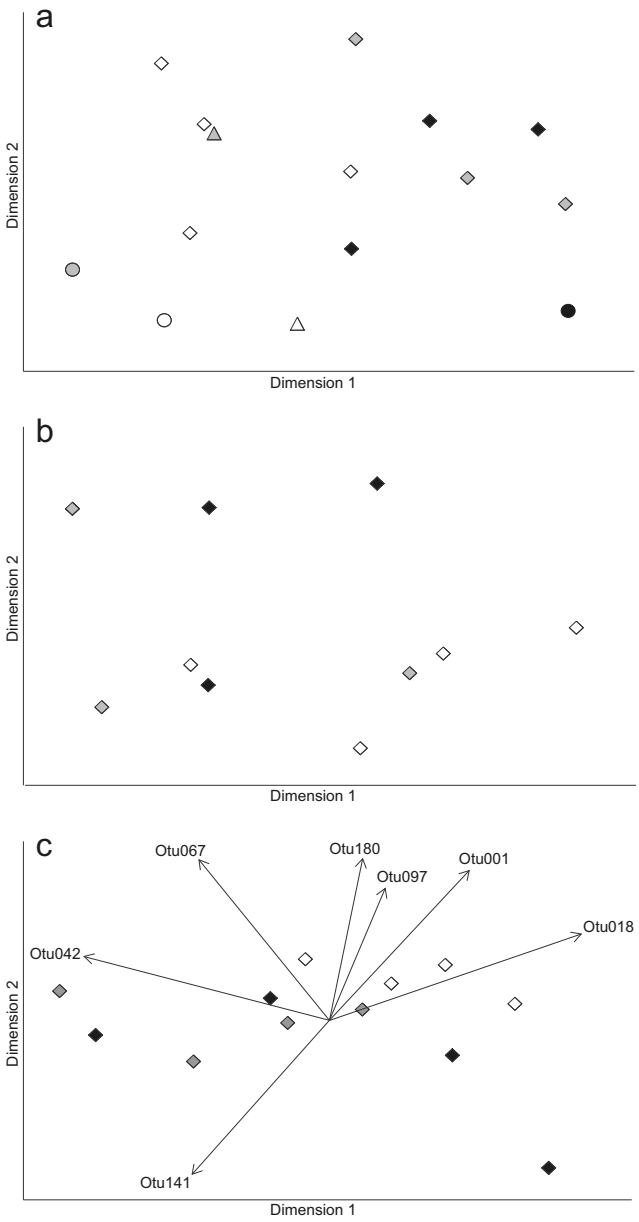

Fig. 3 The first two dimensions from three-dimensional NMDS ordinations of archaeal community structure in sediments from three saltmarshes (Cocodrie (*circles*), Four League Bay (*triangles*), Bayou Dularge (*diamonds*)) in southeast Louisiana, USA that were taken from areas vegetated with *Juncus roemerianus* (*black symbols*), *S. alterniflora* (*white symbols*), or the edge between these vegetation types (*gray symbols*). Ordinations were based on Jaccard similarity scores of DGGE profiles of amplified 16S rRNA gene fragments from all sites (**a**; stress=0.19), DGGE profiles of amplified 16S rRNA gene fragments from the Bayou Dularge site only (**b**; stress=0.15), or theta similarity scores of each community following Illumina sequencing of 16S rRNA gene fragments from the Bayou Dularge site only with 0.1 % of rare sequences removed (**c**; stress=0.19). Panel **c** also shows important taxa driving the separation between *J. roemerianus* and *S. alterniflora* sediment samples, which represent members of *pGrfC26* (*Crenarchaeota*; OTU1, OTU18), *E2* (*thermophilic Euryarchaeota*; OTU42, OTU97), *pMC2A36* (*thermophilic Euryarchaeota*; OTU67), unclassified Crenarchaeota (OTU141), unclassified *DHVE3* (*miscellaneous Euryarchaeota*; OTU180)

J. roemerianus and *S. alterniflora* sediments were not significantly different (theta-based AMOVA, p=0.405, Fig. 5c). The removal of rare sequence types accounting for <0.001, 0.01, or 0.1 % of the community did not change these findings (Jaccard-based AMOVA, p=0.286, 0.290, or 0.590, respectively). Fungal assemblages in all three types of sediment (*J. roemerianus*, *S. alterniflora*, or edge) were dominated by members of Pezizomycotina (Ascomycota), which accounted for 60.7 % of the fungal sequences obtained (Fig. 6). Fungal community structure was similar across all sediment samples in regards to relative abundances of sequences from major fungal taxa, the one exception being sequences related to order Lobulomycetales (Chytridiomycota), which accounted for 2 % of the fungal sequences obtained from *S. alterniflora* sediments, but were absent from the *J. roemerianus* dataset, although this difference was not quite statistically significant (ANOVA, p=0.069). Many of the fungal sequences obtained could not accurately be identified beyond the phylum level, and 8 % could not be classified further than being recognized as Fungi (Fig. 6).

Correlations of environmental variables to NMDS axes scores for each microbial group (Bacteria, Archaea, Fungi) indicated calcium content to be a significant driver of differences in bacterial community structure between *J. roemerianus* and *S. alterniflora* sediments (Spearman ranked correlation, p=0.026). Fungal communities were influenced by water pH and percent sediment moisture (p= 0.025 and 0.024, respectively). There were no significant correlations between enzyme activity and microbial community composition as expressed as NMDS axes scores.

Discussion

We examined the effects of vegetation type on microbial community composition and function in saltmarsh ecosystems to determine whether microbial communities were structured primarily by vegetation or by specific site conditions. The data suggest that saltmarsh bacterial communities can be related to vegetation type, but that site specific characteristics may be of equal or greater importance for overall community composition. Our data does not show an effect of vegetation on sediment fungal communities in these systems. For archaeal communities, our data suggest an effect of vegetation type; however, the lower sample recovery of archaeal 16S rRNA gene fragments (compared to bacterial fragments) limited the statistical power of our analyses. Relatively low levels of sediment Archaea have been noted in other saltmarshes [48], likely because they are outcompeted by sulfate-reducing bacteria in typically sulfate-rich saltmarsh sediments.

Site effects have been noted by other studies investigating microbial communities in salt and brackish marshes and contradict studies of upland microbial communities that suggest

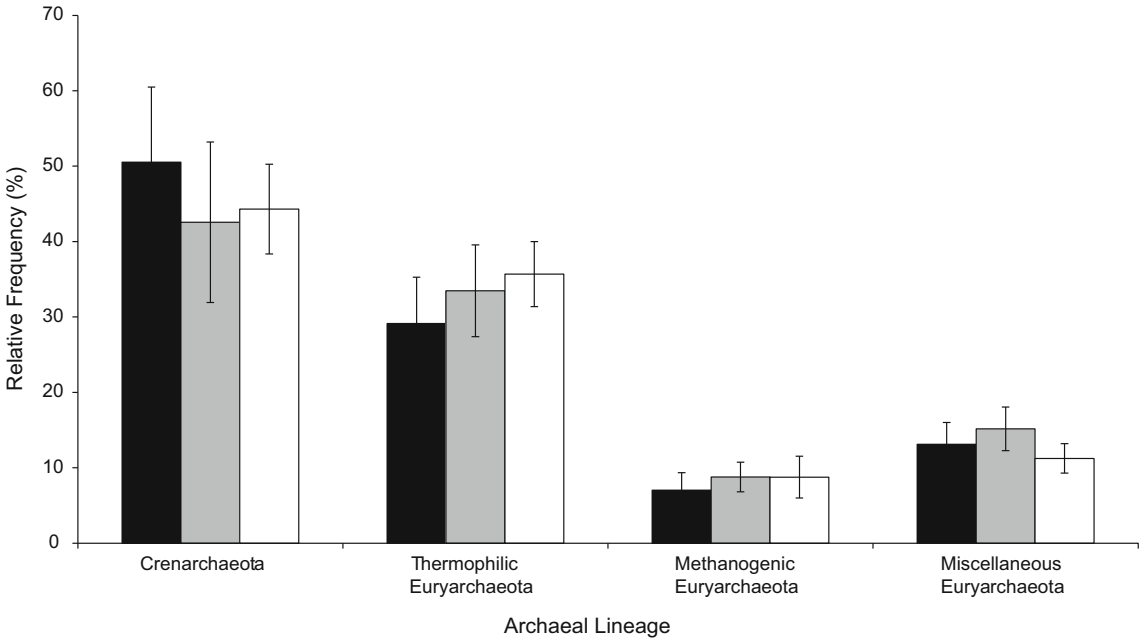

Fig. 4 Relative proportions of major archaeal lineages in sediments taken from a southeast Louisiana saltmarsh (Bayou Dularge) as determined from Illumina 16S rRNA gene sequencing. Sediments were vegetated with *Juncus roemerianus* (*black bars*), *Spartina alterniflora* (*white bars*), or taken from the edge between these vegetation types (*gray bars*). Each *bar* represents the mean (±SE) composition based on four samples, with a mean of 23,236 sequence reads per sample. The group Crenarchaeota consists of all reads that were identified as being related to the classes *C2*, and *Thermoprotei*, as well as the deep sea vent clone *pMC2A15*. *Thermophilic Euryarchaeota* contains the class Thermoplasmata and the hydrothermal vent clones *pMC2A209* and *SdNA*. *Methanogenic Euryarchaeota* consists of the classes Methanomicrobia, Methanococci, and Methanobacteria and the candidate division *MSBL1* which are involved in methanogenesis, *miscellaneous Euryarchaeota* contains all other Euryarchaeota identified which includes the Deep Sea Euryarchaeota Group (DSEG) members *BC07-2A-27* and *DHVE3*, the surface water clone *NO27FW*, the methanotrophic order *ANME-1*, Halobacteria, and miscellaneous/unidentified *WCHD3-30* and *pMC2A384*. The phylum Thaumarchaeota was also detected in three of the four *J. roemerianus* sediments but accounted for <0.5 % of the community in each sample

plant species composition is more important in shaping microbial communities than site. Studies ranging from forests and grasslands [49, 50] to agricultural systems [51] show a clear effect of vegetation on microbial community structure. The same, however, cannot be said for wetlands, where a comparatively smaller number of studies suggest that site characteristics may be more important [18, 52]. However, those previous studies used phospholipid fatty acid (PLFA) analysis for community identification rather than the finer scale next-generation sequencing methods reported here. Our finding that saltmarsh bacterial communities do appear to be influenced by vegetation type suggests the need to use more targeted methods for specific components of the overall microbial community in order to detect vegetation effects in wetlands.

Bacterial communities in *J. roemerianus* and *S. alterniflora* sediments differed using both DGGE and Illumina sequencing. OTUs that distinguished *S. alterniflora* from *J. roemerianus* included one of the most numerous in our sequence libraries, OTU01, identified as a member of the Chromatiaceae or purple sulfur bacteria. These anaerobic phototrophs oxidize sulfide to sulfate and represented a greater proportion of the bacterial community in *S. alterniflora* sediments. In contrast, OTU06, a member of the Desulfobacteraceae (a family of anaerobic sulfate-reducing bacteria), was found in greater relative abundance in *J. roemerianus* sediments. The sulfate requirement for members of the Desulfobacteraceae is likely to come directly from tidal water, whereas the Chromatiaceae require reduced sulfur (sulfide), which would presumably be generated from sulfate reduction in anaerobic pore water. Differences in sulfur chemistry could be accounting for some of the vegetation-linked patterns in bacterial community structure, perhaps through vegetation effects of the dominant sulfur species [53, 54]. Total sulfur content was not significantly different between the vegetation types as a whole, but within individual saltmarshes, *S. alterniflora* vegetated sediments tended to have lower total sulfur than the *J. roemerianus* sediments, suggesting that plant-related patterns in sulfur chemistry could potentially explain some bacterial community patterns. Calcium was found to be related to the distribution of bacterial communities in NMDS ordinations, and while its direct influence on bacterial communities may be difficult to determine, calcium can form complexes with organic acids and impact the availability of other nutrients [55, 56]. Regardless, it is important to keep in mind that saltmarshes often experience substantial temporal variability in aquatic and sediment

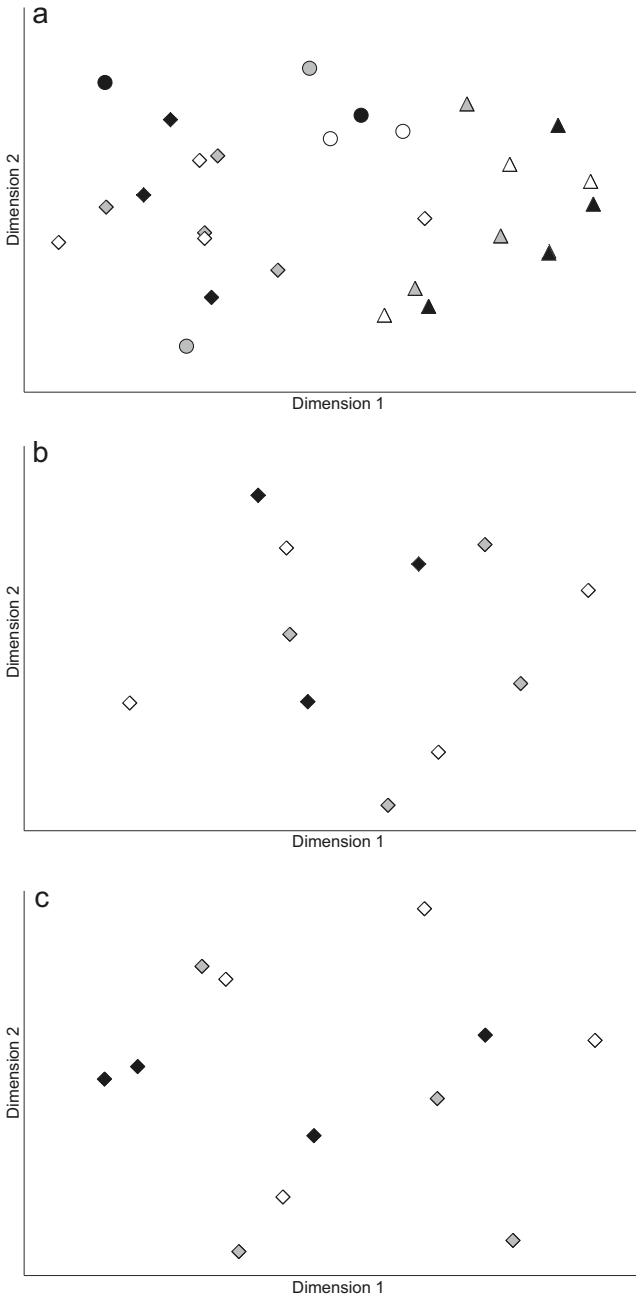

Fig. 5 The first two dimensions from three-dimensional NMDS ordinations of fungal community structure in sediments from three saltmarshes (Cocodrie (*circles*), Four League Bay (*triangles*), Bayou Dularge (*diamonds*)) in southeast Louisiana, USA that were taken from areas vegetated with *Juncus roemerianus* (*black symbols*), *Spartina alterniflora* (*white symbols*), or the edge between these vegetation types (*gray symbols*). Ordinations were based on Jaccard similarity scores of DGGE profiles of amplified 18S rRNA gene fragments from all sites (**a**; stress=0.25), DGGE profiles of amplified 18S rRNA gene fragments from the Bayou Dularge site only (**b**; stress=0.18), or theta similarity scores of each community following Illumina sequencing of ITS fragments from the Bayou Dularge site only (**c**; stress=0.18)

chemistry because of tidal influences, so whether differences in sediment chemistry between plant species could ultimately influence the microbial community is debatable.

Much of the phylogenetic diversity in microbial communities comes from rare taxa, which can often comprise the majority of species at any given time [57–59]. Such rare taxa have also attributed to artifacts of PCR amplification or sampling [60, 61], although the consistent appearance of rare taxa in a temporal series of samples suggests that these populations are real and may be important contributors to microbial community dynamics [62]. Regardless of the origins of rare taxa in next-generation sequencing datasets, removing OTUs that compromised less than 0.001 or 0.01 % of our dataset still yielded significant differences in bacterial community structure between the two plant species, suggesting that these differences are true and did not just arise from methodological artifacts. It also supports previous findings that rare taxa may not necessarily be important for the comparison and analysis of overall bacterial community patterns [62, 63]. Archaeal communities responded differently to the removal of rare taxa and were only significantly different between the two plant species when OTUs comprising <0.1 % of the data set were removed. This effectively removed over 90 % of the archaeal OTUs and focused solely on the proportionally most abundant taxa. This suggests that while archaeal communities are similar between *J. roemerianus* and *S. alterniflora* when all taxa are considered, the most abundant archaeal taxa in the rhizosphere of these plants may be different. Increased proportions of archaeal OTUs 01 and 18 helped to distinguish the *S. alterniflora* sediment archaeal community from that of *J. roemerianus*, which had higher proportions of OTU141. However, both OTU01 and 18 could only be conclusively classified into the pGrfC26 order, part of the miscellaneous Crenarchaeota group [64], whereas OTU141 was classified as "unclassified Crenarchaeota." Thus, while there may be differences in the dominant archaeal taxa in sediments associated with *S. alterniflora* or *J. roemerianus*, the inconclusive state of archaeal taxonomy limits our understanding of these patterns.

DGGE indicated differences in the fungal communities between the sites, though not between vegetation types within individual sites using either DGGE or next-generation sequencing. One explanation for the similarity of the fungal community in sediment under *S. alterniflora* and *J. roemerianus* could be the large filamentous hyphal networks that many fungi develop and which can spread over large areas [65]. Our sediment samples were collected only 6 m apart, quite possibly resulting in the collection of fungi from the same hyphal network at each patch and even across patches at each site. This would account for the fairly homogenous fungal communities that our data suggests is present at each site. Many of our fungal OTUs were rare, and only present as one or two sequence reads, suggesting that some of that diversity could be an artifact of the OTU selection procedure used (97 % similarity of that ITS region). In any case, deficiencies in existing fungal databases prevented much taxonomic resolution for the fungal sequences, and many OTUs could only be classified to the phylum level.

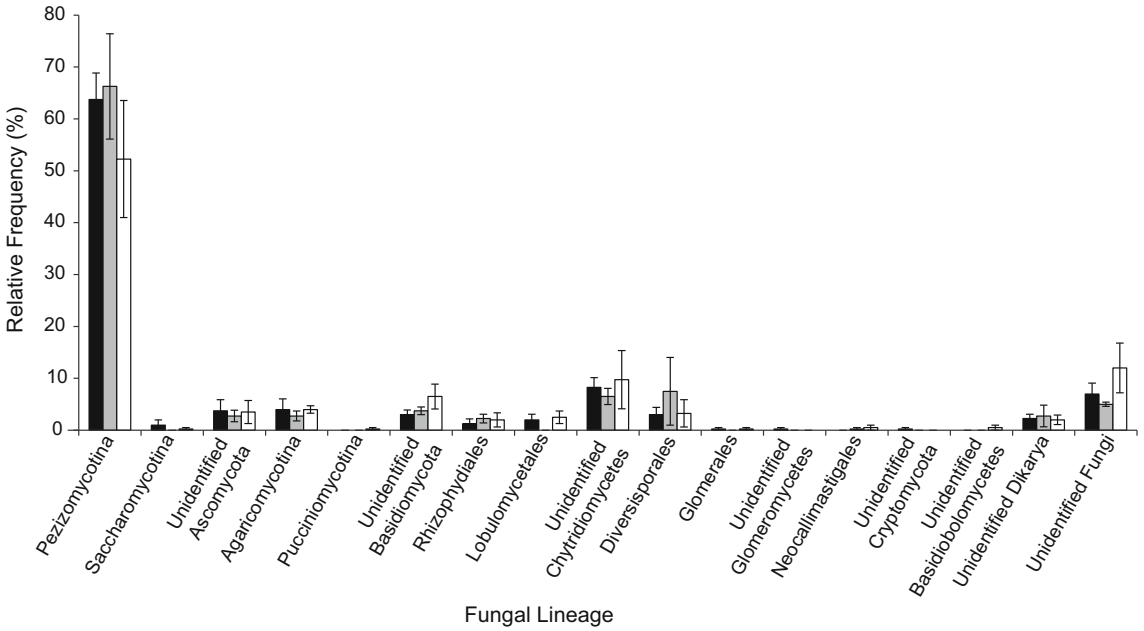

Fig. 6 Relative proportions of major fungal lineages in fungal communities in sediments taken from a southeast Louisiana saltmarsh (Bayou Dularge) as determined from Illumina ITS sequencing. Sediments were vegetated with *Juncus roemerianus* (*black bars*), *Spartina alterniflora* (*white bars*), or taken from the edge between those zones (*gray bars*). Proportions are derived from the results of BLAST searches of GenBank of 100 randomly selected reads from each sediment sample. *ss* represents the mean (±SE) composition based on four samples

In terms of functional characteristics of the sediment microbial community, extracellular enzyme activity was largely influenced by site, a pattern that has been found in other saltmarshes [18]. Despite the differences in bacterial communities, and the suggestion of differences in archaeal communities between *S. alterniflora* and *J. roemerianus* vegetated sediments, enzyme profiles in sediments under these plant species were not different. This suggests that while some of the specific bacterial and archaeal populations may differ between plant species, the function of these communities remains similar regardless of what phyla or families are most predominant. It is also possible that fungi accounted for the bulk of the extracellular enzyme activity that we detected, and fungal assemblages did not differ between the two plant species.

Based on nutrient levels and microbial analyses, the Bayou Dularge site sampled in our study differed from the other two sites. This site had significantly higher levels of C, N, and P and higher activities of β-glucosidase, phosphatase, CBH, and NAGase. Bayou Dularge runs from Houma, LA to the Gulf of Mexico and is bordered by many developments (seafood processing plants, recreational fishing camps) and small communities. Thus, various human factors maybe influencing the nutrient load to this area. This contrasts with the more pristine nature of the Four League Bay site which is only accessible by boat and has limited development around it. The Cocodrie site could also be receiving human impacts as there are numerous fishing camps in the area, although based on chemical analyses, this site was more similar to Four League Bay than to Bayou Dularge. These differences in land use around each site may be at least partially responsible for the site-level differences in community structure that we observed [66] and highlight the need for further study of human impacts on microbial communities in highly connected aquatic systems.

Our study shows that bacterial communities in saltmarsh habitats can be related to the type of vegetation present and that archaeal communities may also follow this pattern. While site-to-site differences may override this local-scale relationship, what is particularly interesting is that we could detect these vegetation-related patterns in stands of plants that were close together and within the same location in the marsh so presumably subject to the same tidal influences and other environmental perturbations. Identifying the specific environmental variables that drive these differences may be difficult, as exudates from plants themselves can also influence microbial community composition [11, 67], and vegetation effects on microbial community structure could arise from a combination of biotic and abiotic factors. The microbial ecology of wetlands, especially saltmarshes, is underrepresented in the literature compared to similar studies in upland terrestrial systems, and there is a need for further studies in these important systems. A better understanding of saltmarsh microbial ecology and the relationship between their plant and microbial communities could prove beneficial in future coastal restoration efforts which seek to maximize plant productivity in rapidly subsiding marsh.

Acknowledgments This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, McIntire Stennis project, under LAB94095. We would also like to thank Logan Boudreaux for help in the field and Bram Stone for help in the laboratory.

The nucleotide sequence data reported are available in the NCBI Sequence Reads Archive under accession numbers SRX1014006, SRX1014005, SRX1014004, SRX1013995, SRX1013994, SRX1013993, SRX1013992, SRX1013991, SRX1013990, SRX1013989, SRX1013955, and SRX1013921.

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