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Tidal Freshwater Marshes Harbor Phylogenetically Unique Clades of Sulfate Reducers That Are Resistant to Climate-Change-Induced Salinity Intrusion

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Abstract Rates of sea level rise associated with climate change are predicted to increase in the future, potentially altering ecosystems at all ecological levels. Sea level rise can increase the extent of brackish water intrusion into freshwater ecosystems, which in turn can affect the structure and function of resident microbial communities. In this study, we performed a year-long mesocosm experiment using intact tidal freshwater marsh sediment cores to examine the effect of a 5-part per thousand (ppt) salinity increase on the diversity and community composition of sulfate-reducing prokaryotes. We used a clone library approach to examine the *dsrA* gene, which encodes an important catalytic enzyme in sulfate reduction. Our results indicate that tidal freshwater marshes contain extremely diverse communities of sulfate-reducing bacteria. Members of these communities were, on average, only 71 % similar to known cultured sulfate reducers and 81 % similar to previously sequenced environmental clones. Salinity and associated increases in sulfate availability did not significantly affect the diversity or community composition of sulfate-reducing prokaryotes. However, carbon quality and quantity, which correlated with depth, were found to be the strongest drivers of sulfate-reducing community structure. Our study demonstrates that the sulfate-reducing community in tidal freshwater marsh sediments appears resistant to increased salinity in the face of sea level rise. Additionally, the microorganisms that comprise this sulfate-reducing community appear to be unique to tidal freshwater marsh sediments and may represent novel lineages of previously undescribed sulfate reducers.

Keywords Sulfate reduction · *dsrA* · Tidal freshwater marsh · Sea level rise

Introduction

Tidal freshwater marshes (TFMs) are highly productive ecosystems (Odum 1988) that serve as habitats for a wide variety of organisms (Mitsch and Gosselink 2007). In addition to their importance to food webs, TFMs are effective at both reducing the effect of storm surges and at removing anthropogenic nutrients derived from the surrounding land (Yang 1998; Gribsholt et al. 2005; Neubauer et al. 2005; Barbier et al. 2008). Marshes rely on accretion of sediment and macrophyte biomass for vertical growth, which allow them to keep pace with sea level rise (Reed 1995; Morris et al. 2002). Accelerated sea level rise due to anthropogenic forcings (Nakada and Inoue 2005; Church and White 2006), however, is predicted to exceed the accretion rates of many marshes, causing increased inundation and eventual marsh loss (Craft et al. 2008; Kirwan et al. 2010). Increased inundation in TFMs may be accompanied by increases in salinity as sea levels rise and freshwater

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inputs are altered due to changing climate and land use (Hamilton 1990; Knowles 2002; Weston et al. 2014). Inundation of freshwater sediments with brackish water containing elevated concentrations of sulfate appears to switch the primary anaerobic microbial decomposition mechanism from methanogenesis to sulfate reduction (Rysgaard et al. 1999; Canavan et al. 2006; Weston et al. 2006; Craft 2007). Sulfate reduction is a more energetically favorable form of anaerobic respiration than methanogenesis, and increased SO_4^{2-} concentrations may result in higher rates of carbon mineralization and, hence, a loss of marsh carbon storage capacity (Weston et al. 2011). Since sulfate reduction forms an important link between the sulfur and carbon cycles, as sea level increases, it is important to understand the effect of salinity changes and inundation on rates of sulfate reduction and the microbial communities that facilitate this process.

In freshwater systems, which generally have low sulfate concentrations, terminal carbon mineralization is typically carried out by methanogenic archaea (Ward and Winfrey 1985). This is in contrast to marine systems where a substantial portion of anaerobic decomposition is carried out via sulfate reduction (Capone and Kiene 1988). Sulfate reduction can, however, play an important role in anaerobic decomposition in freshwater systems (Ingvorsen et al. 1981; Bak and Pfennig 1991; Vile et al. 2003a). Sulfate-reducing prokaryotes may compete with methanogens for carbon (CO_2, acetate, or HCO_3^-) and hydrogen (Ward and Winfrey 1985; Holmer and Kristensen 1994), but may also form intricate syntrophic relationships (Stams 1994). While measured sulfate levels are generally low in freshwater systems, recycling of sulfur compounds via the "thiosulfate shunt" (Jørgensen 1990) or reoxidation of reduced sulfur species (Wieder and Lang 1988; Muyzer and Stams 2008) may drive sulfate reduction in otherwise sulfate-depleted freshwater environments. Further, global atmospheric sulfur pollution and deposition via acid rain can provide excess sulfate to freshwater ecosystems (Vile et al. 2003b; Gauci et al. 2004); however, globally, this source is decreasing annually (Waldner et al. 2014).

Sulfate-reducing prokaryotes form a polyphyletic group, with members found in both bacterial and archaeal domains. Sulfate reduction is partially catalyzed by the enzyme encoded by the dissimilatory (bi) sulfite reductase (*dsrAB*) gene cluster, which has been widely used as a molecular marker for sulfate-reducing microorganisms. While *dsrAB* phylogeny is generally congruent with 16S rDNA phylogeny, several studies have also shown lateral transfer of *dsrAB* between the major lineages of sulfate reducers (Klein et al. 2001; Mussmann et al. 2005; Zverlov et al. 2005). Sulfate reducers occupy a wide range of habitats including salt marshes (Bahr et al. 2005), acidic fens and bogs (Vile et al. 2003a; Schmalenberger et al. 2007), hypersaline microbial mats (Roychoudhury et al. 2013), hot springs (Fishbain et al. 2003), hydrothermal vents (Cottrell and Cary 1999; Frank et al. 2013), acid mine drainage sites (Moreau et al. 2010; Quillet et al. 2012), and marine and freshwater sediments (Leloup et al. 2007; Miletto et al. 2008). Their widespread distribution may be attributed to their tolerance of fluctuating environments (Zhou et al. 2011) and their ability to use a number of metabolic pathways (Plugge et al. 2011). Additionally, sulfate-reducing microorganisms have been shown to enter intricate syntrophic relationships with several clades of microorganisms, whereby they share carbon sources and electron acceptors (Ward and Winfrey 1985; Kuivila et al. 1990; Muyzer and Stams 2008). Their ability to enter syntrophy may also contribute to their widespread distribution, allowing them to proliferate in unfavorable environments.

Edmonds et al. (2009) demonstrated that the sulfate-reducing community in freshwater riverine sediments did not shift in response to saltwater intrusion over a 35-day experiment. The results of Edmonds et al. (2009) suggest the sulfate-reducing community demonstrates short-term resistance (no response to perturbation). To build on the results of Edmonds et al. (2009), we undertook a mesocosm experiment lasting 1 year and using intact TFM sediment cores and a simulated tidal cycle to determine the effect of saltwater intrusion on the sulfate-reducing community. We tested two hypotheses: (1) Over longer time frames, saltwater intrusion will shift the TFM sulfate-reducing community towards a community representative of more saline marshes and (2) changes in the community structure of sulfate reducers will vary with sediment depth due to differential carbon sources and quantities. We demonstrate that after a full year sulfate-reducing communities in TFMs remain resistant to saltwater intrusion as they demonstrated no change in community composition or diversity during the experiment. Instead, carbon quality and quantity, which varied with depth, were the primary drivers of sulfate-reducer community structure. In addition, our results suggest that TFMs harbor novel clades of sulfate-reducing prokaryotes with substantial divergence from both cultured representatives and previously sequenced environmental clones.

Methods

Experimental Design

We evaluated the effect of saltwater intrusion on the sulfate-reducing microbial community composition during a 1-year laboratory experiment using TFM soil cores collected from the Delaware River estuary. Long-term increases in salinity have been observed due to sea level rise in the oligohaline region of the Delaware River estuary (Ross et al. 2015), indicating saltwater will intrude into previously freshwater marshes in the lower region of the freshwater tidal zone. In this study, we collected intact soil cores (10 cm i.d. to a depth of 25 cm) from the tidal freshwater Woodbury Marsh

(39.8594 N, 75.1729 W) in April of 2006 prior to the germination of plants. Cores were incubated in the laboratory in two sets of tanks with a simulated diel tidal cycle (6 h of flooding followed by 6 h of exposure) at 20 °C in the dark for 1 year. After a 14-day pre-equilibration period, the salinity in one set of tidal tanks was amended to 5 parts per thousand (ppt) to simulate saltwater intrusion while the other set of tanks was maintained as tidal freshwater controls. Five parts per thousand represents a moderate increase in salinity that would follow a storm surge or prolong sea level rise. Salinity was amended using a mix of salts (NaCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, and KCl) to achieve concentrations of major ions representative of 5-ppt dilute seawater. Both the salinity-amended and freshwater control tanks received $NaHCO_3$ (669 μM) and nutrients ($NaNH_4$, KPO_4, and KNO_3 at 28, 11, and 99 μM, respectively) to achieve concentrations similar to those found in the Delaware River estuary. More details on this experimental design can be found in Weston et al. (2011).

Duplicate soil cores were removed from both the freshwater control and salinity-amended treatments on days −14 (immediately following core collection), 0 (just prior to salinity amendment), 27, 82, 160, and 364 and sectioned into 2-cm sections. Porewater and soil biogeochemistry were measured on soil sections, as were rates of sulfate reduction (Weston et al. 2011). Briefly, porewater was obtained following centrifugation of soil, and concentrations of dissolved organic carbon (DOC; high-temperature oxidation on Shimadzu TOC-V_{CSH}), dissolved inorganic carbon (DIC; acidification on Shimadzu-V_{CSH}), low molecular weight fatty acids (LMW-FA; derivatization followed by high-pressure liquid chromatography on Agilent 1200; Albert and Martens 1997), nitrate (NO_3^-; flow injection analyzer following cadmium reduction), ammonium (NH_4^+; phenohypochlorite method; Solorzano 1969), phosphate (PO_4^{3-}; molybdenum blue method; Murphy and Riley 1962), Cl^-, and SO_4^{2-} (ion chromatography on Dionex DX-500) were measured. A portion of the soil was dried for water content analysis and measurement of soil carbon and nitrogen content (Leco TruSpec CN). Rates of microbial sulfate reduction were determined immediately following sectioning on subsamples of soil incubated after addition of approximately 0.2 μCi $Na^{35}SO_4$, followed by cold distillation (Kallmeyer et al. 2004) and liquid scintillation counting of the reduced sulfur pool. The total reduced sulfur (TRS) pool was measured by colorimetry (Cline 1969) following distillation. Subsamples of soil from each section were immediately frozen (−80 °C) for analysis of microbial community composition.

DNA Extraction, PCR Amplification, and Functional Gene Sequencing

DNA was extracted from the surface (0–6 cm) and at depth (8–14 cm) from each soil core ($n = 12$ freshwater cores, $n = 8$ salinity-amended cores), to evaluate the composition of the sulfate-reducing microbial community using the *dsrAB* functional gene. Surface samples consisted of soil homogenized from equal subsamples (~0.25 g) from each of three surface sections (0–2, 2–4, and 4–6 cm) and deep soils from subsamples of sections at depth (8–10, 10–12, and 12–14 cm). DNA was extracted using MOBIO PowerSoil® DNA Extraction Kits (Carlsbad, CA, USA) following the manufacturer's protocol. The *dsrAB* functional gene was PCR amplified after combining 3 μL of extracted DNA template, 45 μL of Invitrogen Platinum® PCR SuperMix (Invitrogen, Grand Island, NY, USA), and 2 μL of PCR primers. The degenerate DSR1F (ACBCAYTGGAARCAYG) and DSR1FB (GGCCACTGGAAGCACG) forward primers and DSR4R (GTGTARCAGTTDCCRCA) reverse primer (Zverlov et al. 2005 and references therein) were synthesized by Invitrogen™ and added to the PCR at final concentrations of 200 nM each. After initial denaturation at 95 °C for 5 m, *dsrAB* was amplified for 30 cycles consisting of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C and a final extension for 5 min at 72 °C.

We loaded amplified PCR products (50 μL) and Invitrogen™ low DNA Mass Ladder onto a 1 % agarose gel and visualized the gel with ethidium bromide to confirm amplification of the ~1.9-kb product. We then excised the bands from the gel and purified them using QIAGEN QIAquick® gel extraction kits (Qiagen, Valencia, CA, USA). The PCR amplification products were then cloned into plasmid vectors and transformed into chemically competent *Escherichia coli* cells using Invitrogen™ TOPO TA cloning kits with TOP10 cells following the manufacturer's instructions. Glycerol (20 %) was added to the transformations, which were then frozen (−80 °C) for shipping to the Genome Sequencing Center at Washington University in St. Louis. Transformations were plated, and 192 colonies from each sample were robotically picked for sequencing using the DSR1F forward primer on an Applied Biosystems® 3730 DNA Analyzer.

Sequence and Statistical Analyses

We checked sequence quality in MEGA 5.0 (Tamura et al. 2011), and all reads were aligned and trimmed to 712 bp. Reads with excessive ambiguous bases and sequences with low-quality scores were discarded. Sequences were then checked for chimeras in Mothur (Schloss et al. 2009) using UCHIME (Edgar et al. 2011) and for frameshifts using BLAST. Sequences were clustered at 90 % sequence identity (Liu et al. 2009) in Mothur, and BLASTn was used to find the closest cultured and environmental sequence. Amino acid alignments were generated with ClustalW, and a maximum likelihood tree was constructed in RAxML (Stamatakis 2014). The tree was visualized using the Interactive Tree of

Life (Letunic and Bork 2007). Sequences were deposited in GenBank under accession numbers KM379151–KM381954.

We used Mothur to calculate the abundance-based coverage estimator (ACE), Shannon diversity index, and Chao1 richness estimator for our dataset. To assess difference in diversity metrics between treatments (fresh versus salt) and depths (shallow versus deep), we used one-way ANOVA implemented in R (R Core Team 2012). To assess community similarity, we used a non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity values calculated in Mothur and used analysis of similarity (ANOSIM) on a normalized operational taxonomic unit (OTU) table with 5000 permutations to assess the degree of similarity between treatments and depths.

To assess which taxa were driving differences between shallow and deep samples, we used Kruskal-Wallis test (non-parametric ANOVA) implemented in R. We assessed significance of any OTU that was differentially abundant and that was present at least five times in the dataset with a Bonferroni-corrected p value <0.001. Finally, we used weighted UniFrac (Lozupone et al. 2006), a metric that uses the phylogenetic relatedness of the microbial taxa present to assess community similarity, to test for the effect of treatment and depth on similarity of the sulfate-reducing communities.

Due to the observed differences between shallow and deep sulfate-reducing communities, we used linear regression of significantly different geochemical variables (one-way ANOVA, $p<0.05$) between shallow and deep samples and the primary principal coordinates axis from the Bray-Curtis analysis in R to determine which environmental drivers were correlated with community structure. We assessed the significance of the correlation with an F test.

Results

Geochemical Data

Measurements of geochemical parameters displayed (Fig. 1; Table S2) a strong difference not only between fresh- and saltwater cores but also between shallow and deep samples within the cores. Saltwater cores had significantly more sulfate in shallow sediments; however, it did not appear to significantly change sulfate concentrations in deep samples in saltwater treatments (Fig. 1a). Additionally, higher levels of sulfate in saltwater cores likely drove higher rates of sulfate reduction (Fig. 1b); however no significant difference in sulfate reduction rates between shallow and deep samples in saltwater cores was noted. Surface sediments had a significantly higher total reduced sulfur (Fig. 1c; ANOVA; $p<0.001$, $F=14.21$) and percent nitrogen (Fig. 1f; $p<0.05$, $F=24.24$) than deep samples; however, they did not vary as a

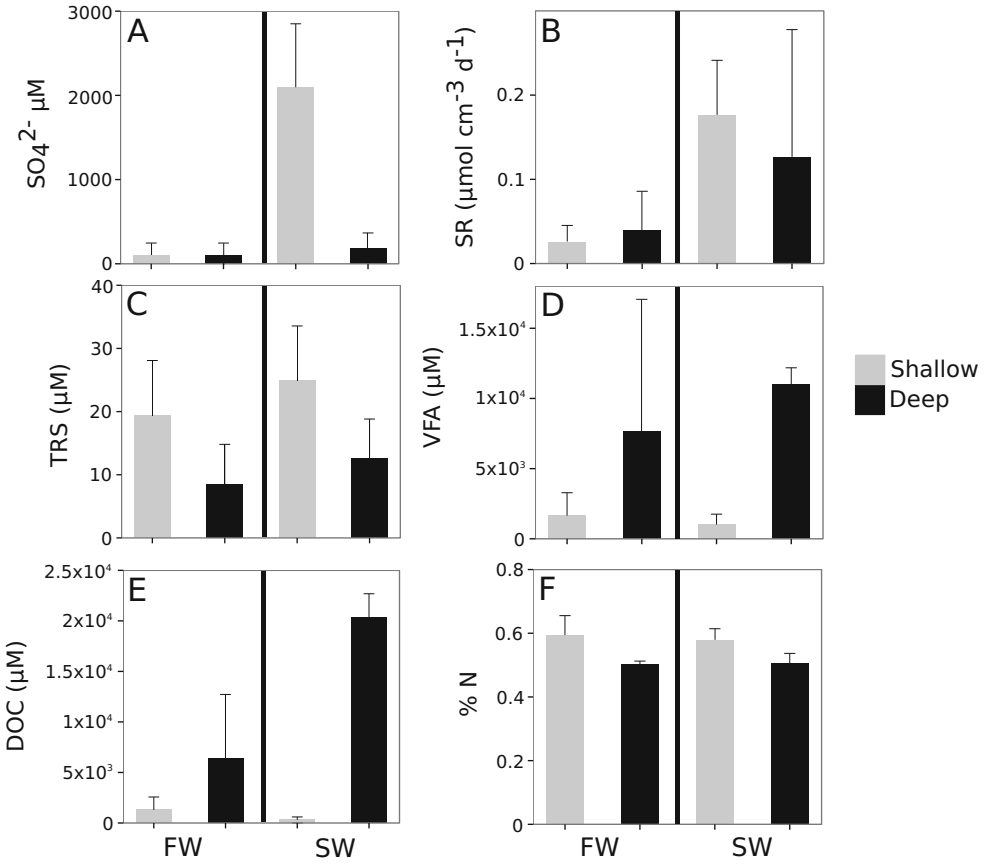

Fig. 1 Bar charts of the significantly different (ANOVA $p<0.05$) geochemical data measured in the study. *A* Sulfate (µM), *B* sulfate reduction (SR; µmol cm^{-3} day^{-1}), *C* total reduced sulfur (TRS; µM), *D* volatile fatty acids (VFA; µM), *E* dissolved organic carbon (DOC; µM), and *F* percent nitrogen

function of treatment. Deep samples, however, had significantly higher volatile fatty acids (Fig. 1c; $p < 0.01$, $F = 31.45$) and dissolved organic carbon (Fig. 1e; $p < 0.05$, $F = 9.90$).

Diversity

We sequenced a total of 3139 clones from 32 samples. After quality filtering, we retained 2803 partial sequences of the alpha subunit of the dissimilatory bisulfite reductase gene (*dsrA*). Clustering of the sequences at 90 % sequence identity resulted in the identification of 1079 unique OTUs. We chose 90 % sequence identity as it has been previously used for the *dsrA* gene (Liu et al. 2009); however, we also ran all analyses at sequence identities ranging from 80 to 95 % and the ecological patterns were consistent with what we present at the 90 % clustering threshold. We used multiple metrics to assess differences in alpha diversity in our experiment (Table 1). Comparing saltwater cores to freshwater cores, we found no significant changes in Shannon diversity, OTUs observed, or estimated taxonomic richness. Comparisons among sulfate reducers in surface and deep sediments also resulted in no significant changes to Shannon diversity, taxonomic richness, or the number of OTUs observed.

Table 1 OTUs observed and calculated Shannon diversity, Chao1 richness, and abundance-based coverage estimator (ACE) for each of the cores in the study. The 95 % confidence interval ranges are indicated next to each value

Treatment	Day	Depth	Duplicate	No. of clones	OTUs observed	Chao	ACE	Shannon
FW	−14	Surface	1	68	53	631 (248–1729)	579 (212–1746)	3.7 (3.4–3.9)
FW	−14	Deep	1	119	90	641 (258–1739)	589 (222–1756)	4.3 (4.1–4.5)
FW	0	Surface	2	74	65	242 (137–486)	275 (154–550)	3.9 (3.8–4.2)
FW	0	Surface	2	90	77	402 (222–807)	430 (237–856)	4.2 (4.1–4.4)
FW	0	Deep	2	102	88	413 (242–771)	417 (247–767)	4.4 (4.2–4.5)
FW	28	Surface	1	140	109	760 (411–1513)	2389 (1883–3038)	4.5 (4.3–4.6)
FW	28	Deep	1	41	40	410 (165–1135)	820 (58–32,928)	3.6 (3.4–3.8)
FW	28	Surface	2	56	51	223 (121–474)	285 (87–1578)	3.9 (3.7–4.1)
FW	28	Deep	2	90	78	413 (227–828)	408 (229–800)	4.2 (4.1–4.4)
FW	83	Surface	2	69	60	335 (210–590)	666 (485–930)	4.1 (3.8–4.2)
FW	83	Deep	1	147	116	289 (155–610)	307 (162–656)	4.6 (4.4–4.7)
FW	83	Surface	2	77	62	601 (358–1085)	926 (682–1275)	4.1 (3.8–4.1)
FW	83	Deep	2	101	87	339 (210–600)	358 (219–642)	4.4 (4.2–4.5)
FW	161	Deep	1	41	40	410 (165–1135)	820 (58–928)	3.6 (3.4–3.8)
FW	161	Deep	2	90	79	604 (299–1332)	1984 (1332–2975)	4.2 (4.1–4.4)
FW	161	Deep	2	126	102	547 (317–1019)	1673 (1255–2243)	4.4 (4.3–4.6)
FW	365	Surface	1	105	84	264 (173–446)	341 (203–637)	4.3 (4.1–4.4)
FW	365	Deep	1	158	102	232 (170–351)	367 (276–506)	4.4 (4.3–4.5)
FW	365	Surface	2	78	61	290 (156–611)	1364 (1006–1858)	3.9 (3.7–4.1)
FW	365	Deep	2	94	80	314 (191–571)	362 (212–684)	4.3 (4.1–4.4)
SW	27	Surface	1	86	61	181 (113–335)	194 (124–344)	3.9 (3.7–4.1)
SW	27	Deep	1	139	105	399 (257–675)	1254 (969–1634)	4.4 (4.3–4.6)
SW	27	Surface	2	118	83	227 (154–373)	246 (164–409)	4.2 (4.1–4.4)
SW	27	Deep	2	73	64	212 (132–387)	259 (120–737)	4.1 (3.9–4.3)
SW	82	Deep	1	75	62	291 (157–612)	264 (151–520)	4.1 (3.8–4.2)
SW	82	Deep	1	33	26	57 (36–122)	66 (42–124)	3.2 (2.9–3.4)
SW	82	Deep	2	126	94	545 (299–1086)	1432 (1097–1879)	4.3 (4.1–4.5)
SW	82	Deep	2	93	78	423 (232–848)	2001 (1448–2778)	4.2 (4.1–4.4)
SW	160	Surface	1	52	44	229 (112–548)	268 (125–663)	3.7 (3.5–3.9)
SW	160	Deep	1	92	82	467 (255–938)	495 (266–1006)	4.3 (4.2–4.5)
SW	364	Deep	1	63	51	108 (75–181)	133 (92–217)	3.8 (3.7–4.1)
SW	364	Surface	2	78	58	233 (132–469)	301 (155–667)	3.7 (3.5–4.1)
SW	364	Deep	2	114	86	208 (148–326)	222 (157–348)	4.3 (4.2–4.4)

FW freshwater, *SW* saltwater

Community Composition and the Effect of Salinity

To assess community similarity, we used Bray-Curtis dissimilarity as a metric of total community dissimilarity between each pair of samples in our experiment. The NMDS plot, which was used to visualize the community dissimilarity, indicated a clear separation in communities between shallow and deep samples (Fig. 2, compare black to grey symbols). This separation was statistically significant, as assessed with ANOSIM ($p < 0.0005$, $r = 0.76$). However, no significant effect of salinity intrusion was evident in our analysis (Fig. 2, compare triangles to circles). Our results indicate that despite a year of incubation in saltwater (~5 ppt), the sulfate-reducing microbial community remained unaffected by the added SO_4^{2-}. Depth, however, was a clear driver of sulfate-reducing microbial community structure.

Environmental Drivers of Community Structure

Linear regression between carbon content (DOC, VFA, and acetate) and the primary axis coordinates from our NMDS plots (Fig. 2) indicated a significant ($p < 0.01$) relationship between deep community composition and carbon concentrations (Fig. 3). Our results indicate that higher concentrations of carbon may be driving the observed changes in community composition between shallow and deep samples. The coefficient of determination values for the regressions between carbon content and the NMSD primary axis indicate that both acetate and DOC explain the largest proportion of the variance in the relationship ($r^2 = 0.28$ for both), but the coefficient of determination for all three carbon types was low.

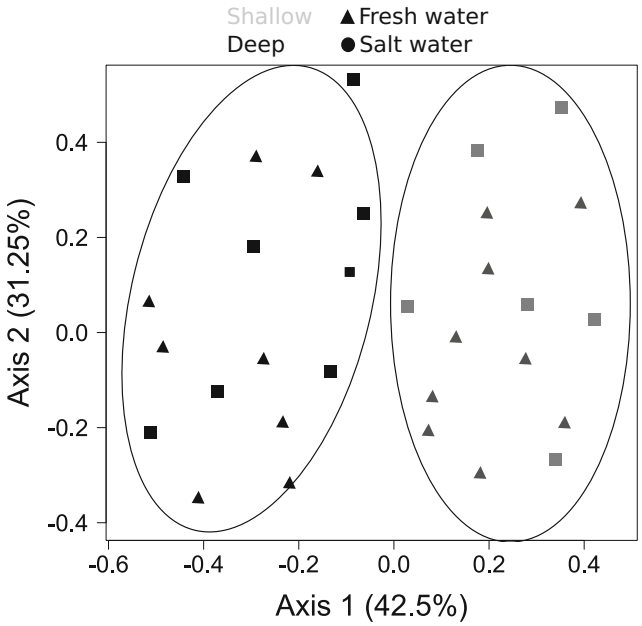

Fig. 2 Non-metric multidimensional scaling plot of Bray-Curtis similarity values. *Grey symbols* are from shallow (0–6 cm) samples and *black symbols* are from deep (8–14 cm) samples. Treatment is denoted by *triangles* for the control treatment and *circles* for the saltwater addition treatment

Taxonomic Drivers of Community Composition

A total of 32 OTUs, present at least five times, were significantly different (Kruskal-Wallis test, $p < 0.001$) between shallow and deep samples (Fig. 4). The bacterial types most abundant in surface sediments were Deltaproteobacteria from the order *Syntrophobacterales*, while deep samples contained more Deltaproteobacteria outside the order *Syntrophobacterales*. There was a significant difference (UniFrac significance test, $p < 0.0005$) in the phylogenetic grouping of communities between shallow (0–6 cm) and deep (8–14 cm) samples for both fresh- and saltwater cores. Our results indicate changes in depth resulted in changes in the phylogenetic composition of the sulfate-reducing community due to different types of Deltaproteobacteria dominating different depths.

Phylogeny

BLASTn was used to determine the closest cultured and environmental representative to the observed OTUs. The 1079 *dsrA* OTUs, on average, showed only ~72 % identity to known cultured sulfate-reducing prokaryotes and ~81 % identity to previously published environmental sequences (SI Table 1). Based on *dsrA* sequencing, OTUs were most closely related to three major sulfate-reducer clades: Firmicutes, Euryarchaeota, and Deltaproteobacteria (SI Table 1). Of the Deltaproteobacteria-like OTUs, they most closely resembled the orders *Desulfarculales*, *Desulfovibrionales*, and *Syntrophobacterales*. Maximum likelihood analysis revealed the presence of 12 distinct clusters consisting of only the OTUs from this study (Fig. 5). As with the BLASTn results, phylogenetic analysis indicated all OTUs grouped within the Firmicutes, Euryarchaeota, and Deltaproteobacteria but typically with a great deal of divergence from previously cultured sulfate reducers. Most sequences, however, were affiliated with the Deltaproteobacteria, with 994 OTUs grouping with members of this class. Of the remaining 85 OTUs, 36 OTUs grouped into a single cluster with the archaeon *Archaeoglobus veneficus* and 4 distinct clusters ($n = 49$) closely clustered within the Firmicutes clade.

Discussion

Sea level rise, resulting both from thermal expansion of warming seawater and melting of continental ice, is predicted to accelerate as the planet becomes warmer (Meehl et al. 2005; Rignot et al. 2011). Local changes in salinity due to sea level rise will result in changes in marsh plant communities (Bertness

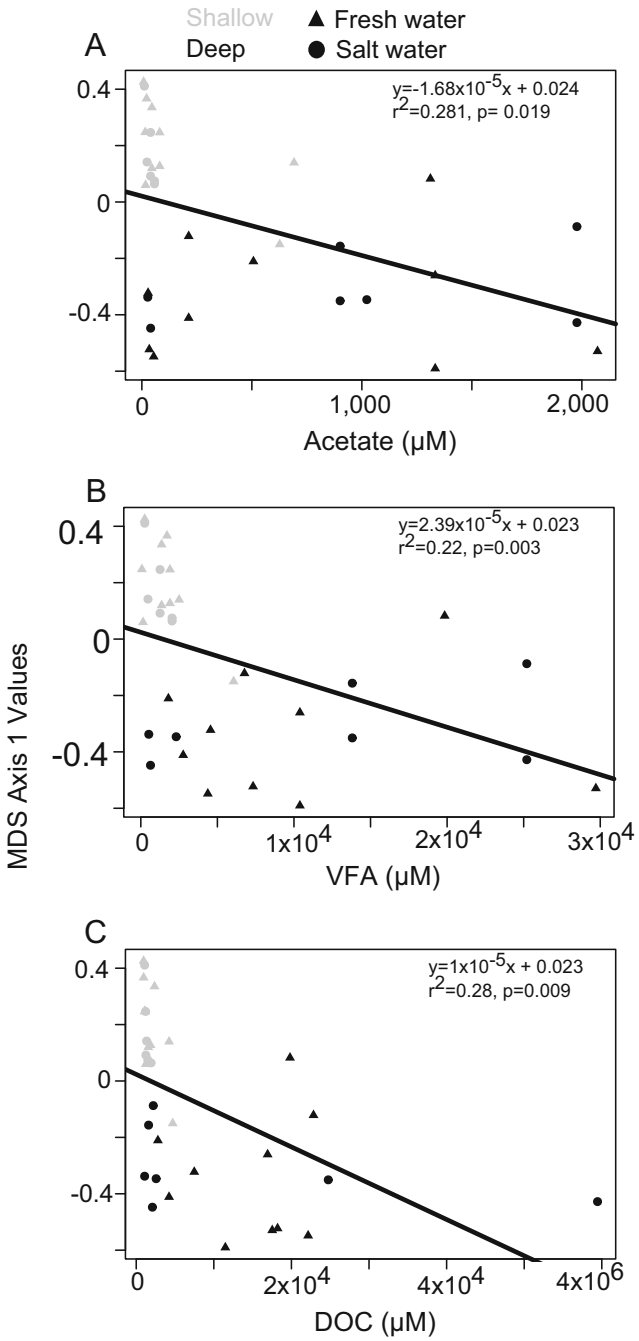

Fig. 3 Biplot of the non-metric multidimensional scaling plot (NMDS) coordinates (axis 1 from Fig. 2) as a function of three significantly different carbon types: **a** acetate, **b** volatile fatty acids (VFAs), and **c** dissolved organic carbon (DOC)

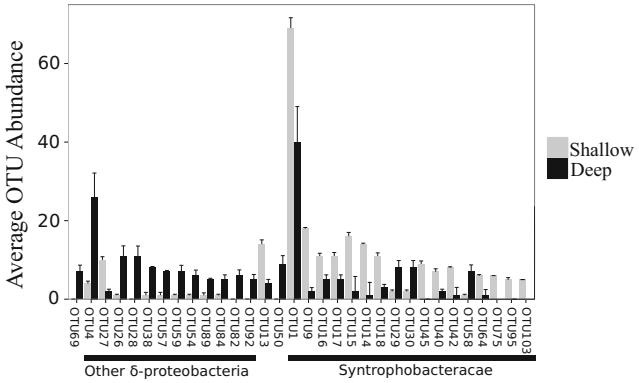

Fig. 4 Plot of significantly different OTUs between shallow (*grey*) and deep (*black*) samples as assessed by a Kruskal-Wallis test ($p < 0.001$) for OTUs that were present at least five times in the data. OTU 69 has been classified as an archaeon while OTUs 13 and 50 are classified as members of the Firmicutes phylum

and Ellison 1987; Crain et al. 2004), which could lead to changes in ecosystem function. In addition to being a driver of change in structuring macroscopic communities, salinity has been shown to be a strong driver of both total microbial community composition (Crump et al. 2004; Lozupone and Knight 2007) and sulfate-reducing microbial community composition (Miletto et al. 2008; Steger et al. 2011; Fan et al. 2012).

Our results demonstrate that the sulfate-reducing community structure is resistant to low levels of saltwater inundation (Fig. 2). Despite the increase in rates of sulfate reduction in saltwater-amended cores (Fig. 1b; SI Table 2), the sulfate-reducing community composition did not significantly change in response to saltwater intrusion and increased SO_4^{2-} concentrations (Fig. 1a) over a year-long incubation. Our results are surprising given the often rapid response of microbial communities to saltwater inundation (e.g., Reed and Martiny 2013) and suggests resistance within the sulfate-reducing community, which may be due to adaptation resulting from the occasional saltwater inundation TFMs receive during storm surges or to increased salinity exposure during periods of drought (Odum 1988). Further, rates of sulfate reduction between shallow and deep samples did not significantly differ, despite significantly ($p < 0.01$) higher amounts of carbon and less nitrogen in deep samples (Fig. 1c–e). The lack of significant difference in sulfate reduction rates between shallow and deep samples indicates that the sulfate-reducing communities, despite being different (Fig. 2), contain a large degree of functional redundancy enabling them to perform a similar set of ecosystem services within TFM sediments despite changes in environmental conditions.

Although the total sulfate-reducing community did not appear to respond to a 5-ppt increase in salinity, our data show a distinct difference in microbial community structure between shallow and deep sediments (Figs. 2 and 4). Depth is considered a strong driver of bacterial (Blümel et al. 2007; Treusch et al. 2009) and sulfate-reducing community structure (Schmalenberger et al. 2007) and can even overwhelm other strong drivers such as seasonality (Fortunato et al. 2012). In our study, both changes in carbon sources (acetate, VFAs, DOC) and the quantity of carbon differentiated deep sediment samples from surface sediment samples (Fig. 3). Further, a

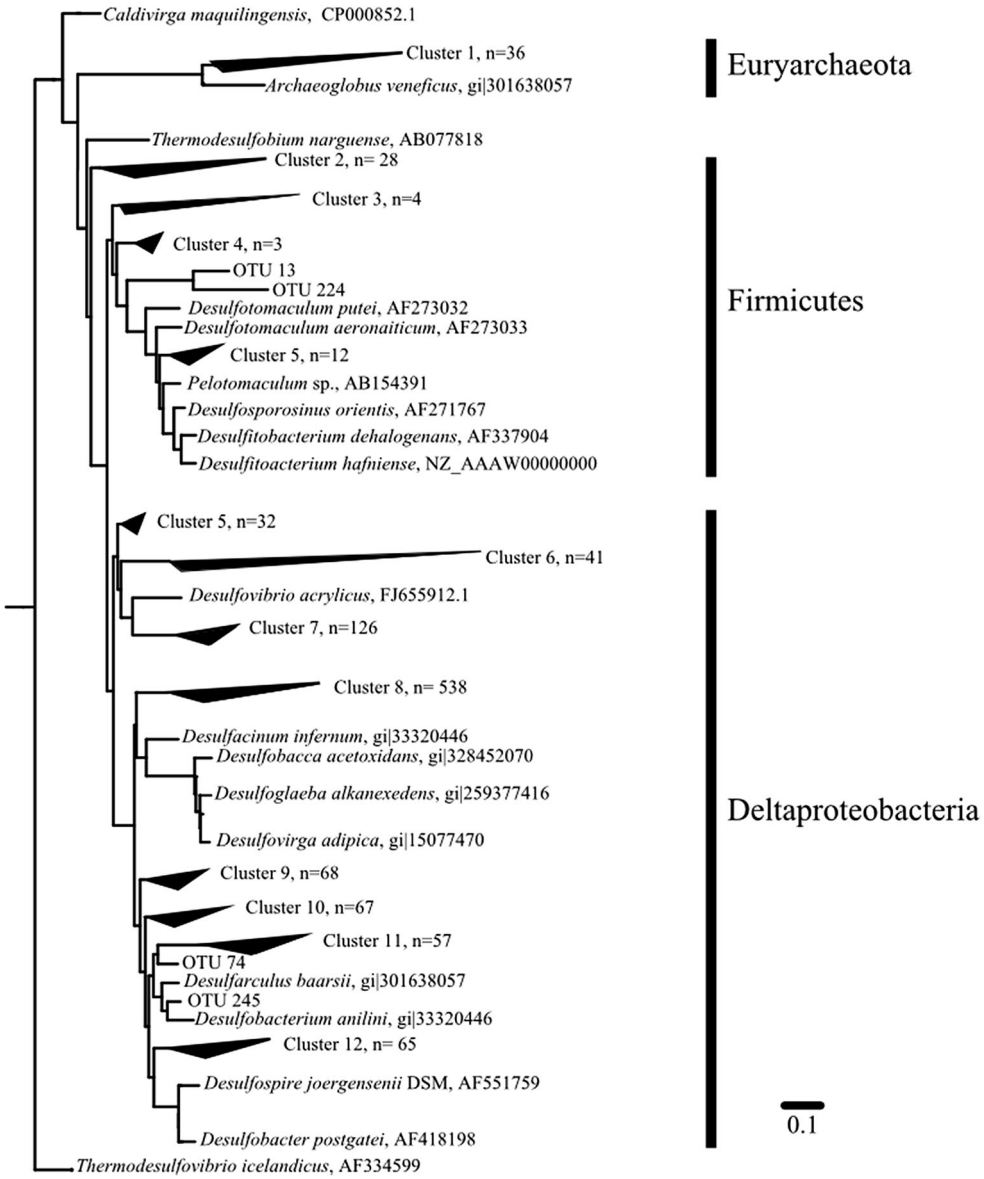

Fig. 5 Phylogenetic tree based on maximum likelihood for all observed OTUs in the study. Clades of OTUs were collapsed, and the *number* of OTUs present in each cluster is indicated next to each cluster. Sequences from cultured sulfate reducers were added to show phylogenetic affiliation, and *GenBank accession numbers* are given next to the name of the cultured representative. The *scale bar* indicates 10 % sequence divergence

weighted UniFrac test and a Kruskal-Wallis test indicated that phylogenetically different groups of sulfate reducers inhabited shallow and deep samples. Shallow samples had a higher presence of the members from the order *Syntrphobacterales* while deep samples were depleted in this order, but had a higher presence of Deltaproteobacteria from other orders. This is likely a result of the varying abilities of sulfate-reducing lineages to compete for and process different carbon substrates (Plugge et al. 2011 and references within), allowing them to be better adapted to the different habitats present in shallow and deep environments.

In freshwater systems, sulfate availability is typically low and methanogenesis is generally the dominant form of anaerobic decomposition (Capone and Kiene 1988; Kelley et al. 1990). While our study demonstrates novel groups of *dsrA*-containing prokaryotes, we are unable to assess if all the observed diversity is functional or a remnant of past ability to reduce sulfate. For example, Imachi et al. (2006) demonstrated that bacteria closely related to *Desulfotomaculum* genus lost the ability to reduce sulfate despite possessing the *dsrAB* gene cluster. Both sulfate reduction and methanogenesis are active processes in our experiment (Fig. 1b, SI Table 1), and many of the observed sulfate reducers may be in syntrophic relationships with methanogens due to the low levels of sulfate in their natural environment. The geochemical role of the taxa discovered in this study remains unclear, and future work is needed to determine the metabolic capabilities and role of the specific sulfate reducers found in TFMs.

Sulfate reducers are a taxonomically diverse group of microorganisms, belonging to five distinct bacterial and two archaeal phyla. Previous studies, however, have demonstrated novel groups of sulfate reducers that have yet to be described

by cultivation (Castro et al. 2002; Dhillon et al. 2003; Loy et al. 2004; Bahr et al. 2005). The OTUs identified in our study also demonstrate a high degree of divergence from cultured representatives (~71 % identity at the nucleic acid level) and previously sequenced environmental clones (~81 % at the nucleic acid level). Given the high degree of divergence, it is difficult to assign taxonomy to the sequences generated in this study, but they most closely resemble Deltaproteobacteria, Firmicutes, and Euryarchaeaota (Fig. 1, SI Table 1). Our inability to definitively link *dsrA* fragments to known sulfate reducers may be due to the ability of *dsrAB* to be horizontally transferred (Bahr et al. 2005; Mussmann et al. 2005; Zverlov et al. 2005). Consistent with our findings, recent work by Quillet et al. (2012) and Steger et al. (2011) have both demonstrated deeply branching and highly divergent clades of sulfate reducers as core members of the sulfate-reducing community in salt marshes and in low-salinity peatlands. The divergence of the sulfate-reducing community found in our TFMs from previously sequenced environmental clones indicates they have yet to be identified in other systems, supporting the notion that TFMs contain novel lineages that may be endemic to these habitats.

In conclusion, our study has identified a very diverse community of sulfate reducers within TFM sediments and they display considerable divergence from previously sequenced *dsrA* fragments. Our results are indicative of new lineages of sulfate-reducing prokaryotes that may be endemic to TFM sediments. Carbon quality and availability, which were linked to sample depth, were the strongest drivers of community composition, while increased salinity and associated SO_4^{2-} availability had little effect on the communities sampled. As sea levels continue to rise, TFMs will be faced with increased inundation with brackish water and, as a result, increased levels of sulfate. Because of their carbon storage capacity, understanding sulfate-reducing prokaryotic communities as well as other carbon degradation pathways (e.g., denitrification) in TFM sediments will provide much-needed information regarding the response of TFMs to future sea level rise.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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