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MANGANESE-REDUCING BACTERIAL COMMUNITIES FROM A HYPERSALINE ESTUARY IN SOUTH TEXAS UNDER CONDITIONS OF CHANGING SALINITY

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Abstract.–Manganese reducing bacteria were quantified and enriched from sediments at two sites (ABC and LMT050) in the Laguna Madre of South Texas. Bacteria were tested for Mn(IV) reduction under different salinities. Denaturing gradient gel electrophoresis (DGGE) was used to profile bacterial communities from Mn(IV) saline enrichments. Results showed that the density of manganese reducing bacteria at ABC was 30X greater than LMT050. The ABC bacteria were able to reduce Mn(IV) at similar rates at different salinities; however, at LMT050, Mn(IV) reduction rates decreased at higher salinities. Three population genotypes were observed by DGGE. All were present and similarly abundant in LMT050 cultures at all salinities. In site ABC, one population was below detection at salt concentrations < 60.0 ppt but observed at higher salt concentrations. We conclude that site ABC consists of a large manganese-reducing community that alters in structure when salinity changes, whereas site LMT050 contains a smaller but somewhat more adapted community.

In marine water columns and sediments, the redox potentials of inorganic nutrients change resulting in stratified layers where reduction of O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2 are favored depending on redox potential and availability (Myers & Nealson 1988; Koretsky et al. 2005). Microorganisms within marine sediments are important in biogeochemistry, bioremediation, and biotechnology. The ability of bacteria to mediate metal (e.g., manganese) transformations between oxidation states is important in these processes (Lovley 1991).

Manganese can exist in several oxidation states; the $^+2$, $^+3$, and $^+4$ forms are common in nature (Ehrlich 2002). Manganese is a trace nutrient required in microbial, plant and animal nutrition. For example, Mn(II) is required in oxygenic photosynthesis in plants and green algae (Ehrlich 2002) and serves as a cofactor for several important enzymes (Madigan & Martinko 2005). At high levels, manganese can be detrimental to the health of aquatic plants and

animals, and humans (Shukla & Singhal 1984; Whelan et al. 2005). Contaminant levels of manganese are the result of industrial effluents and smelter emissions (Agency for Toxic Substances and Disease registry [ATSDR] 1997).

Manganese biogeochemistry includes Mn(II), or the manganous form, as a soluble metal in water columns. Under aerobic conditions, the Mn(II) ion is stable at pH less that 5.5; however, at pH 8.0 Mn(II) ions can spontaneously oxidize to Mn(IV) (Atlas & Bartha 1993; Van Cappellen et al. 1998). The Mn(IV) ion is insoluble in water, cannot be taken up by aquatic vegetation, and may be seen as manganese nodules in some aquatic ecosystems (Nealson 1997). Sediment metal-reducing bacteria, such as manganese-reducing bacteria, couple metal reduction to the oxidation of organic compounds to generate ATP necessary for cellular activities (Myers & Nealson 1988; 1992). Mn(IV) reduction converts insoluble Mn(IV) to soluble Mn(II) and yields -18.5 kcal of energy. This may allow manganese to be more easily taken up by aquatic plants and animals where it may bioaccumulate (Whelan et al. 2005). To transfer electrons to insoluble Mn(IV) minerals, microorganisms must make contact with the surface of the mineral directly or transfer electrons by way of extracellular electron shuttles such as humic acids, quinones, or *c*- type cytochromes found within the outer membrane (Myers & Nealson 2001; DiChristina et al. 2002). Several groups of Mn(IV)-reducing bacteria are known, including members of the genera *Shewanella*, *Geobacter, Bacillus* and others (Lovley & Phillips 1988; Lovley 1991). *Shewanella* species are gram-negative, facultative anaerobes that use a wide array of potential terminal electron acceptors for respiration (Myers & Nealson 1990; DiChristina et al. 2002). *Geobacter* species are gram-negative obligate anaerobes (Lovley 1991; Madigan & Martinko 2005). Manganese-reducing *Bacillus* are gram-positive facultative bacteria found in soils and sediments (Boone et al. 1995). *Deferribacter thermophilus*, a bacterium isolated from a petroleum reservoir, is a gram-negative thermophile capable of Mn(IV) reduction (Greene et al. 1997).

Salinity changes may be one of several environmental factors that influence the types of bacterial communities residing within an aquatic environment. This, in turn, may affect the rate of anaerobic processes such as metal reduction by sediment bacteria. High salt concentrations affect bacteria by dehydrating cells or disrupting the tertiary structure of proteins (Atlas & Bartha 1993; Campbell et al. 1999). As the salinity in an aquatic ecosystem increases, the biological diversity in that ecosystem often decreases (Gerday & Glansdorff 2007) and the activities of microorganisms may decrease as well.

The Laguna Madre of South Texas is a subtropical, hypersaline estuary divided into upper and lower regions. Although an estuary, salinity in the Laguna Madre is typically 30 ppt or slightly higher, and salt concentrations as high as 80 ppt have been observed (Tunnell & Judd 2002; Whelan et al. 2005). High temperatures play an important role in concentrating salts in the lagoon. During hot summers, increased evaporation rates can result in the salinity nearly tripling, causing massive fish kills and ecological changes in the ecosystem (Tunnell & Judd 2002). Precipitation input into the Laguna Madre averages approximately 73 cm/yr, but the rate of evaporation can exceed precipitation by two or three times (Tunnell & Judd 2002). Freshwater into the Laguna Madre comes primarily from the Arroyo Colorado, a man-made waterway used for agricultural drainage. The Arroyo Colorado's water volume varies seasonally because of droughts and floods and thus its contribution of freshwater into the Laguna Madre can fluctuate.

The Laguna Madre provides habitat to many rare, endangered and migratory animals. The macroecology of the ecosystem has been the focus of numerous studies (Bates et al. 2009; Cebrian et al. 2009; McMahan 1970; Quammen & Onuf 1993). A recent study (Berlanga et al. 2009) showed that bacteria present in Laguna Madre sediment may be involved in the cycling of anthropogenic compounds in the ecosystem. However, little is known about metal-reducing microbial communities that inhabit the Laguna Madre and the effects of

changing salinity on these organisms. In this study, we examined the effects of changing salinity on manganese reduction rates by microorganisms enriched from Laguna Madre sediments and used molecular profiles to assess changes in the manganese reducing bacteria (MnRB) microbial community under different salt concentrations.

METHODS AND MATERIALS

Sediment sample collection.–Two sites were selected in the lower Laguna Madre of South Texas. One site was designated ABC (26°10'09.7"N, 97°11'05.3"W). The second collection site in the Laguna Madre was south of the ABC site and designated LMT050 (26°08'17.4"N, 97°10'41.0"W). These sites were chosen based on a pilot study performed in 2005 of several sites in the lower Laguna Madre to estimate the abundance of sediment manganese reducing bacterial communities. Samples for this study were collected in September 2006. Three sediment cores were taken from each site using PVC pipe fitted with a rubber plunger at one end to aid in pulling sediment into the pipe. The PVC pipe was approximately 40 cm in length with an inside diameter of approximately 5 cm. The cores were collected and brought back to the lab on ice. The upper 10 cm of the sediment core was removed, homogenized and used for standard plate counts and enrichment cultures (described below). Water salinity levels for the collection sites were determined using a handheld refractometer (Mannix Testing & Measurement, New York, NY) and converted to parts per thousand (ppt). Water pH was determined by using a handheld portable pH meter (Oakton Instruments, Vernon Hills IL). Water temperature was measured using a manual thermometer.

Culture medium.–Growth medium contained the following ingredients per liter: 15 mM sodium acetate, 9.0 mM $(NH_4)_2SO_4$, 5.7 mM K_2HPO_4, 3.3 mM KH_2PO_4, 2.0 mM $NaHCO_3$, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$, 70.0 μM disodium ethylenediaminetetraacetic acid (EDTA), 56.6 μM H_3BO_3, 10.0 μM NaCl, 6.0 μM $FeSO_4$, 5.0 μM

$CoCl_2$, 5.0 μM $Ni(NH_4)_2(SO_4)_2$, 4.0 μM Na_2MoO_4, 1.5 μM Na_2SeO_4, 1.3 μM $MnSO_4$, 1.0 μM $ZnSO_4$, 0.2 μM $CuSO_4$, L-arginine hydrochloride (10 mg/L), L-glutamine (20 mg/L), L-glutamic acid (10 mg/L), vitamin B_1 (0.5 mg/ml), and L-serine (10 mg/L). Two mM manganese oxide (MnO_4) was added after sterilization as the only electron acceptor to enrich for growth of MnRB and no other organisms. The media was used in both a liquid and solid (1.5% agar) form. The media was supplemented with NaCl in order to achieve salinities of 0, 15, 30, 40, 60 or 80 ppt for experiments to simulate changing salinity (see below).

Community Density.–Sediment samples of 1 g were diluted (10^{-1}, 10^{-2}, and 10^{-3}) using phosphate buffered saline (PBS) at pH = 8 (Sambrook & Russell 2001). 100-μL aliquots of each dilution were plated onto Mn media agar plates supplemented with NaCl to a salinity of 30 ppt. The media plates were placed in an anaerobic chamber (Coy Laboratory Products, Grass Lake MI) and incubated at 27°C for 30 d. The gas composition of the anaerobic chamber was 85% N_2 - 10% CO_2 - 5% H_2. After 30 d, MnRB were observed and colony forming units per gram (cfu/g) wet sediment were estimated by visual counting and multiplying by the dilution factor.

Manganese reduction rate experiments.–Two types of manganese reduction rate experiments were performed. In the first type, designated Enrichment Experiments, 1 g of homogenized sediment was inoculated into 15 ml Hungate tubes (Hungate & Macy 1973) containing 9 ml of liquid Mn minimal media. The media salinity was adjusted to 0, 15, 30, 40, 60 or 80 ppt with NaCl to enrich for MnRB that could grow at the indicated salinity. Tubes were purged with anaerobic gas mix (85% N_2 -10% CO_2 - 5% H_2), plugged with rubber stoppers, capped and incubated for 14 d in the anaerobic chamber. As the enrichment cultures incubated, the sediment settled to the bottom of the tube and cell growth was observed in the media above the sediment. After 14 d, cells were collected from the media portion, centrifuged, and re-inoculated into tubes containing fresh media. Cells were re-inoculated into the same salinity for which they had

been enriched. That is, cells enriched at 0 ppt salinity were re-inoculated into media with 0 ppt salinity; cells that had been enriched at 15 ppt were re-inoculated into media adjusted to 15 ppt salinity and so on. The number of cells was normalized by diluting or concentrating the culture in minimal media equal to the turbidity of a 0.5 McFarland Standard, approximately 1.5×10^8 cells/ml (bioMérieux, Inc.; Durham, NC).

Five tubes in triplicate (total of 15 tubes) were set up for the salinities tested. Tubes were labeled 1 through 5 to indicate the growth day. Tubes were purged with anaerobic gas, plugged and capped, and incubated for 5 d. Tubes were sacrificed daily in triplicate and tested using benzidine to estimate the concentration of Mn(IV). A 'time zero' measurement of Mn(IV) was also performed. The rate of manganese reduction was determined by plotting [Mn(IV] vs. time and calculating the slope of a regression line.

In the second type of manganese reduction rate experiments designated Consortium Experiments, 1 g of homogenized sediment was inoculated into 15 ml Hungate tubes containing 9 ml of Mn minimal media. Salinity was adjusted to 30 ppt with NaCl, which was similar to the salinity measured at the time of sample collection (Table 1). Tubes were purged with anaerobic gas mix, capped and incubated in the anaerobic chamber. After 14 d, cells were collected, centrifuged, and re-inoculated into tubes containing fresh media. Cells were re-inoculated into media with 0, 15, 30, 40, 60 or 80 ppt salinity to simulate increases and decreases in salinity, such as has been observed in the Laguna Madre. The number of cells was normalized by diluting or concentrating the culture to 1.5×10^8 cells/ml.

Five tubes in triplicate (total of 15 tubes) were set up for the salinities tested. Tubes were labeled 1 through 5 to indicate the growth day. Tubes were purged with anaerobic gas, plugged and capped, and incubated for 5 d. Tubes were sacrificed daily in triplicate and tested using benzidine to estimate the concentration of

Mn(IV). A 'time zero' measurement of Mn(IV) was also performed. The rate of manganese reduction was determined by plotting [Mn(IV] vs. time and calculating the slope of a regression line.

Benzidine Assay.–Benzidine (benzidine dihydrochloride; Sigma Aldrich, St. Louis MO) was used to estimate the concentration of Mn(IV) ions in the Hungate tubes (Stratton et al. 1982; Burnes et al. 1998). Benzidine complexes with Mn(IV) ions to produce a blue color that can be measured with a spectrophotometer. Tubes were vortexed for 30 seconds to create a homogeneous mixture; 1 ml was removed from the tube using a sterile syringe and transferred to a fresh tube. Twice the volume (2 ml) of 2 mM Benzidine solution was then added to each tube. After 3 minutes, 1 ml was extracted from the mixture, transferred to a cuvette and read at 424 nm in a spectrophotometer. The concentration of Mn(IV) was estimated from standard curves constructed using known concentrations of MnO_4.

Polymerase Chain Reaction.–Bacterial DNA was extracted from Time 0 and Day 5 cultures grown during the Consortium Experiments using the QIAamp DNA Mini Kit (QIAGEN Sciences, Germantown, MD) according to the manufacturer's instructions. Extracted DNA was verified by electrophoresis using 1% agarose gels and quantified with a spectrophotometer at 260 nm.

The Polymerase Chain Reaction (PCR) was performed to amplify the 16S rRNA genes of the bacterial community. PCR was conducted using PCR Master Mix (Promega, Madison WI), 250 ng of template DNA, sterile water and 50 pmol of the forward and reverse primers that corresponded to a conserved 200 bp region of the bacterial 16S rRNA genes. The sequences of the primers were as follows: forward primer 5'- CCT ACG GGA GGC AGC AG-3' and the reverse primer 5' – ATT ACC GCG GCT GCT GG – 3' (Muyzer et al. 1993). The primer set corresponds to regions 341 to 534 in *Escherichia coli*. The PCR mixture was placed into a MyCycler PCR thermocycler (Bio Rad, Hercules, CA) and heated to 95°C for 6 min in order to initially denature the template DNA. Then 40 cycles were run with the

following conditions: denaturing at 95°C for 30 sec, annealing at 52°C for 30 sec, and primer extension at 72°C for 30 sec. A final extension at 72°C for 1 min was done and the PCR products were held at 4°C. A DNA-free negative control was included in the PCR experiments but did not yield a product (data not shown). PCR products were verified by electrophoresis in 1.5% agarose gels. Gels were exposed to an electric field of 41 mAmps for 1.5 h and visualized with a UV illuminator.

For DGGE experiments, 16S rDNA PCR products were re-amplified using the same primers; however, the forward primer contained an additional 40-nucleotide GC rich sequence (GC Clamp) (Muyzer et al. 1993). A designed "touchdown" PCR protocol was used. The PCR protocol was as follows. First, 1 cycle of initial denaturing was performed at 94°C for 5 min to separate the DNA. Then 20 cycles of PCR were conducted using the steps: denaturing at 94°C (1 min), followed by annealing at 65°C - 55°C (1 min) and extension at 72°C (3 min). The annealing step was reduced by 0.5°C each cycle until reaching the touchdown temperature of 55°C. Once the touchdown temperature of 55°C was reached, an additional 10 cycles were performed. A final cycle held the product at 4°C.

Denaturing Gradient Gel Electrophoresis (DGGE).–DGGE was conducted following the protocol of Muyzer et al. (1993). Commercially-purchased solutions (BioRad, Hercules CA) were used to produce denaturing gradients. Initially, a broad gradient of 20 – 80% denaturant (urea) was used in DGGE assays of the cultures; however, DNA bands in these initial DGGE assays were only observed in the higher denaturing concentrations (greater than 60%). Therefore, the gradient range was significantly reduced to 60 – 70% denaturant and the DGGE assays repeated. A DNA-free negative control was included but did not yield any bands in the DGGE assay (data not shown). Gels were run in the DCODE apparatus tank (BioRad, Hercules CA) in 1X Tris-acetate-EDTA (TAE) buffer heated to 60°C. Samples of 30 μL (20 μL of PCR sample and 10 μL of loading dye) were added to the gel. The gel was run at 200 volts

at 60°C for 2 h. The gel was gently removed from the glass and submerged into 1X TAE and 30 μL of ethidium bromide for 15 min to stain the gel. The gel was photographed using a UV light and a digital camera.

Statistical analysis.–A two-tailed student t-test was used to determine whether manganese reduction rates from the enrichment and consortium experiments were correlated to increased or decreased salinity, and to compare mean manganese reduction rates between sites. The two-tailed student t-test was measured using the Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL) computer software at a confidence interval of 0.05.

RESULTS

Site characteristics and manganese-reducing bacteria density.– Salinity, pH, water temperature, and MnRB density of the Laguna Madre sites are given in Table 1. Site ABC had a slightly higher salinity and pH compared to site LMT050; the water temperature was the same in both sites.

Manganese reduction rates of enrichment experiments.–Sediment samples collected from sites ABC and LMT050 were used to culture MnRB to be used in manganese reduction rate experiments. Enrichment experiments consisted of sediment that was inoculated into manganese media adjusted to 0, 15, 30, 40, 60, or 80 ppt salinity, grown anaerobically for 14 d to enrich for MnRB at that salinity, then re-inoculated into media with the same salinity to determine the manganese reduction rate over a 5 d time frame. Manganese reduction rates were measured in μM manganese reduced per day (μM/d). Manganese reduction rates from site ABC enrichment experiments confirmed that MnRB communities in the sediments consisted of organisms that could grow and reduce manganese at a range of salinities as we were able to enrich for those organisms at different salinities (Fig. 1A). Manganese reduction rates ranged from approximately 10 μM/d at 0 ppt salinity to 41 μM/d at both 60 and

Table 1. Environmental conditions and density of manganese-reducing bacteria (MnRB) from the Laguna Madre, September 2006.

Site	Salinity (ppt)	pH	Temperature (°C)	Cfu/g
ABC	32.4	8.27	27	$2.38 \pm 0.885 \times 10^6$
LMT050	31.2	8.17	27	$7.77 \pm 2.98 \times 10^4$

80 ppt salinity. A significant trend was observed showing that manganese reduction rates increased as salinity increased ($p < 0.05$). Manganese reduction rates from LMT050 enrichment experiments were tested at all salinities; however, the reduction rates at 0 ppt and 60 ppt were below detection (Fig. 1C). Unlike what was observed in ABC enrichment experiments, the reduction rates of LMT050 enrichments were not correlated to increasing salinity ($p = 0.056$). The highest manganese reduction rate was measured at 80 ppt salinity and was approximately 37 μM/d.

Manganese reduction rates of consortia experiments.–Consortia experiments were performed in which sediment was inoculated into Mn media adjusted to the salinity similar to conditions at the time of collection (Table 1). Cultures were incubated anaerobically for 14 d then re-inoculated into media with 0, 15, 30, 40, 60 or 80 ppt salinity to determine the manganese reduction rate over a 5 d time frame. The ABC consortium was able to reduce manganese better at salinities below or above the measured salinity at the time of collection (Fig. 1B). The highest reduction rate was 43.4 μM/d in the 0 ppt salinity sample. The manganese reduction rate was lowest at media adjusted to 15 ppt salinity. The manganese reduction rates amongst consortia were correlated to increasing salinity ($p < 0.05$).

The consortium experiment for LMT050 showed a slightly different trend. MnRB from LMT050 were able to reduce manganese at salt concentrations at or lower than 40 ppt; however, above 40 ppt, the organisms' ability to reduce manganese declined (Fig. 1D). As was observed in ABC consortium experiments, the highest manganese reduction rate was seen at 0 ppt and then decreased at 15 ppt. The lowest value was obtained in experiments with cultures

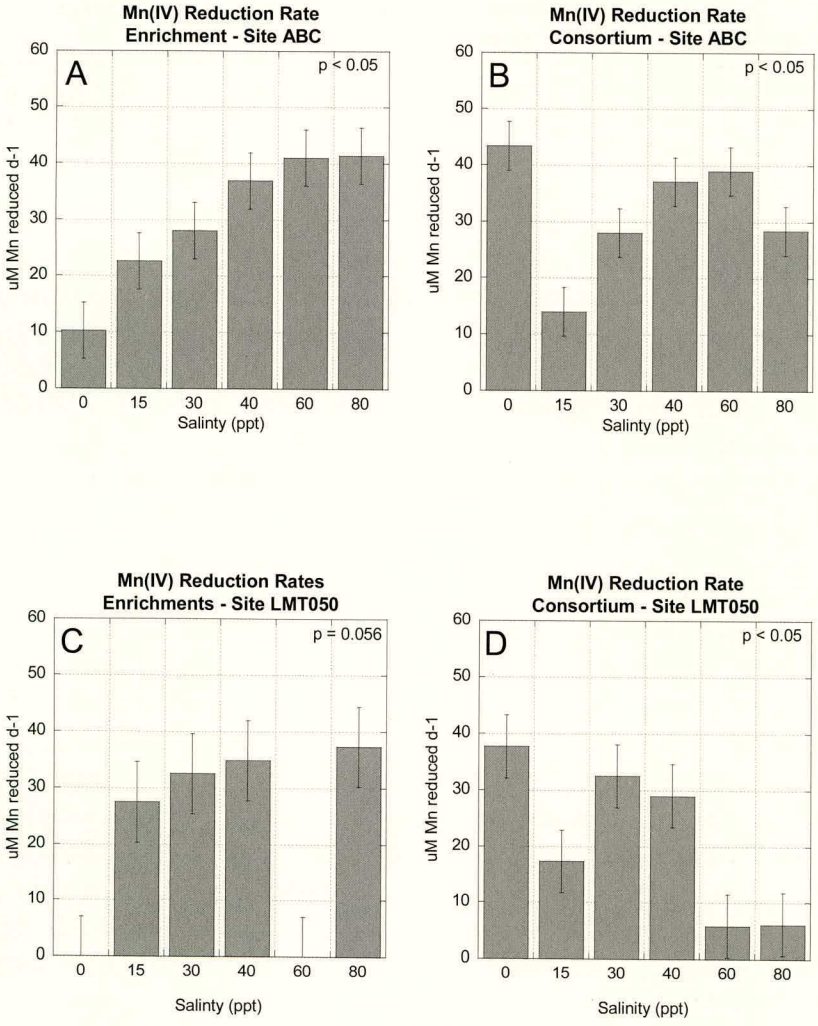

Figure 1. Mn(IV) reduction rates by MnRB from the Laguna Madre. For Enrichment Experiments (Panels A & C), sediment MnRB were enriched in Mn media at salinities of 0, 15, 30, 40, 60, and 80 ppt and tested for Mn(IV) reduction anaerobically for 5 d. For Consortium Experiments (Panels B & D), MnRB were grown in Mn media with 30 ppt NaCl then transferred to media with salinities of 0, 15, 30, 40, 60, and 80 ppt and tested for Mn(IV) reduction anaerobically for 5 d. Panel A: site ABC enrichment experiments Mn(IV) reduction rates; Panel B: site ABC consortium experiment Mn(IV) reduction rates; Panel C: site LMT050 enrichment experiments Mn(IV) reduction rates; Panel D: site LMT050 consortium experiment Mn(IV) reduction rates. Rates (μM Mn per day) are the mean of triplicate experiments. Error bars represent the standard error of the mean.

tested a salinity of 60 ppt. There was a significant trend that manganese reduction rates decreased as salinity increased ($p < 0.05$). The consortia experiments suggest that growing the organisms at 30 ppt salinity, then quickly changing the environmental salt conditions, affected anaerobic manganese reduction by microorganisms from both sites.

Figure 2 shows a comparison of the mean manganese reduction rates from all the enrichments and consortia experiments. Rates from site ABC enrichments were statistically higher than the mean rates from site LMT050 ($p = 0.492$) and rates from site ABC consortia were statistically higher than the mean rates from site LMT050 ($p = 0.594$). This was despite normalizing the cell numbers so that the same number of cells was used in the experiments. Therefore, on average, microorganisms from site ABC reduced manganese at higher rates than microorganisms from site LMT050. Combined with the greater density of MnRB observed at site ABC (Table 1), the higher reduction rates by ABC microbes suggests that this site has great potential for manganese cycling.

Denaturing gradient gel electrophoresis.–DGGE was used to construct profiles of the MnRB communities that grew during the course of the consortia experiments. DGGE allows for the separation of same-sized DNA fragments based on the guanine + cytosine (G + C) content of the DNA. The polyacrylamide gel used in DGGE contained a gradient of denaturing chemicals (60 – 70%) which caused double stranded DNA to unwind and halted the progress of the DNA in the gel. The hindered DNA was visualized as a band in the gel. DNA bands at different positions in the gel represented different G + C genotypes and different populations of organisms. In addition, the intensity (thickness) of the band was indicative of the relative amount of DNA with that sequence. For DGGE, DNA was extracted from consortium cultures at the onset and on the final day of the manganese reduction experiment. The 16S rDNA was amplified and subsequently re-amplified using primers specific for DGGE (e.g., the forward primer contained a 40 bp GC clamp). The

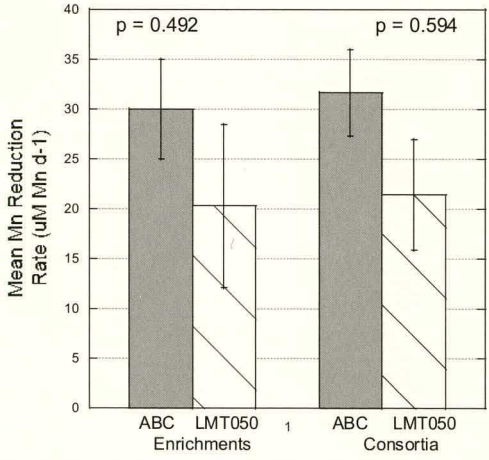

Figure 2. Comparison of mean Mn reduction rates from enrichment (left columns) and consortia (right columns) experiments. Rates were estimated as μM of Mn reduced per day (μM Mn d^{-1}). Error bars represent the standard error of the mean. Mean reduction rates from site ABC cultures, both enrichments and consortia, were statistically higher than rates from site LMT050.

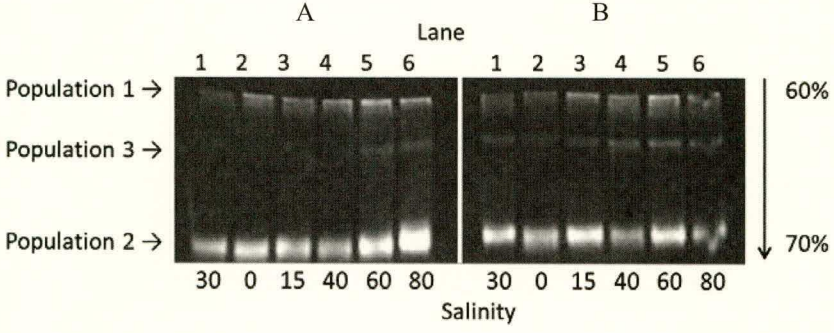

Figure 3. DGGE Profiles of Laguna Madre sites ABC and LMT050. PCR-amplified DNA from consortia experiments was exposed to a denaturant (urea) gradient of 60% to 70% in a polyacrylamide gel. Panel A, site ABC: Population 3 was below detection at salinities less than 60 ppt but became prevalent at higher salinities. Panel B, LMT050: Populations 1, 2, and 3 were present at all salinities.

initial 16S rDNA PCR products were all approximately 200 bp in length; DGGE allowed us to separate the fragments in different populations with different amounts of G + C. Figure 2 displays the DGGE profiles of sites ABC (Fig. 3A) and LMT050 (Fig. 3B).

The ABC consortium was first grown in medium adjusted to 30 ppt NaCl then re-inoculated into other salinities. The MnRB community profile (Fig. 3A, lane 1) contained 2 clearly visible populations at the beginning of the manganese reduction experiment. Population 1 formed a band near the top of the gel at the low denaturant region, and a second population, Population 2, formed a band near the bottom of the gel in the region of highest denaturant. Population 2 produced a distinctly darker band. This indicated that the second population contained a higher amount of G +C compared to the first population and was in greater abundance. When the consortium was re-inoculated into the different salinities, the DGGE profiles remained the same in the 0, 15, and 40 ppt communities (Fig. 3A, lanes 2-4). This suggests that the composition of the MnRB community did not change when the cultures were subjected to these salinities. However, at salinities of 60 and 80 ppt, a third band representing a third type, Population 3, became visible in the gel (Fig. 3A) and the density of the lowest band, Population 2, became more pronounced.

The DGGE profile for LMT050 MnRB contrasted with that observed in site ABC. The initial consortium contained all three genotypes observed in ABC (Fig. 3B, lane 1); however, the 3 population bands were consistently visible and had similar density at all salinities tested. The DGGE profile, and therefore the composition of the MnRB community, remained the same despite the lower or higher concentrations of salt present in the media (Fig. 3B, lanes 2-5).

DISCUSSION

The microbial ecology of the Laguna Madre has been largely ignored; therefore, this study represents a novel examination of the manganese-associated microbial processes in this ecosystem. Sites ABC and LMT050 were chosen for this study based on preliminary bacterial population estimates obtained in January 2005, June 2005, and October 2005 that showed the presence of MnRB in the Laguna Madre. In addition, geochemical analysis of Laguna Madre sediments showed an average Mn concentration of 243 μg/g dry weight sediment (Whelan et al. 2005). In 2005, the population density of MnRB was estimated from four different locations in the Laguna Madre, including site LMT050, and displayed high variability. The MnRB ranged from $0 - 5.6 \times 10^5$ cfu/g wet sediment in the 2005 samples (data not shown). In the current study, site ABC had approximately 30X greater density of MnRB compared to LMT050 despite the similarity in environmental conditions and proximity of the two sites. This demonstrates that the presence and abundance of MnRB can vary greatly in Laguna Madre sediments.

Thamdrup et al. (2000) quantified manganese-reducing bacteria in Black Sea sediments. They estimated that the Black Sea sediments contained approximately 10^5 cells/cm^3 which is comparable to the densities we obtained for Laguna Madre sediments. Furthermore, the authors concluded that these were true manganese reducing bacteria and not facultative metal reducing bacteria (i.e., capable of reducing either iron or manganese) because microorganisms capable of iron reduction were very low. Whelan et al. (2005) performed chemical analyses from Laguna Madre sediments a year prior to our study. They reported that iron was the most abundant metal in the sediment followed by manganese. Copper, arsenic, zinc and lead were also detected. Of these, iron, manganese, and arsenic can serve as potential electron acceptor for anaerobic bacteria. Several manganese-reducing bacteria have been shown to also reduce ferric iron, and members of the genus *Shewanella* can reduce ferric iron, oxidized manganese and arsenate. However, the Mn(IV) enrichment

media used in this study did not contain arsenic or ferric iron (a small amount of ferrous iron was present but this cannot be used as an electron acceptor) and thus grew manganese-reducing bacteria. It is possible that some of the enriched organisms would also be able to reduce iron and/or arsenate, but we did not test for that. In addition, previous attempts by our lab yielded very low numbers of ferric iron-reducing bacteria from Laguna Madre sediments (data not shown).

There have been several recent studies of manganese-reducing bacteria in other marine environments, including salt lakes and coastal environments. For example, Sorokin & Muyzer (2010) examined manganese reduction in hypersaline lakes in Russia. In that study, colloidal manganese was rapidly reduced and a pure culture was obtained that could reduce manganese at both high salinity and high pH. However, that study is not comparable to ours for several reasons. First, the environmental conditions in the Russian salt lakes were very different from ours (e.g., pH 10 and higher salinity) and the authors did not characterize the manganese reducing microbial community using DGGE or other methods. DGGE has been used in recent studies of marine bacterial communities symbiotically associated with other organisms, such as marine sponges (Anderson et al. 2010), marine algae (Lachnit et al. 2011) and marine copepods (Brandt et al. 2010) and to describe microbial communities in different sediments impacted by anthropogenic pollutants (Cetecioğlu et al. 2009; Song et al. 2002; Zanaroli et al. 2010).

We used a combination of traditional community counts, enrichments cultures and DGGE to quantify and characterize the manganese reducing bacteria in the Laguna Madre. Köpke et al. (2005) examined microbial diversity in North Sea coastal sediments using a similar approach. Communities cultivated with manganese as an electron acceptor were approximately 10^7 cells/g. Enrichment cultures were prepared and enriched cultures were examined using DGGE. A narrow DGGE gradient (50-70%), which was similar to the gradient we employed, was used to profile the enriched community. A total of nine bands were observed in the gels;

however, the DGGE phenotypes in the North Sea study were not manganese reducing bacteria. The DGGE profiles were based on enrichments using organic substrates rather than manganese.

Salinity in the Laguna Madre is usually slightly above 30 ppt but has been reported as high as 80 ppt. In analysis of the Laguna Madre sites, both sites contained sediment MnRB communities that reduced Mn(IV) at salt concentrations higher than 30 ppt and lower than 30 ppt salinity. This data suggests that Laguna Madre MnRB are broadly halotolerant organisms capable of growth and Mn(IV) reduction under different osmotic conditions and are not obligate halophiles.

MnRB growth and activity at 80 ppt salinity is very important during the long, dry and hot summers of South Texas. Summer temperatures in South Texas can reach 30°C or higher by mid-morning and low temperatures of 27°C are not unusual at night. In addition, the Laguna Madre experiences periods of drought and high rates of evaporation. As water levels decreases due to high temperatures, evaporation and other factors, salts in the lagoon become more concentrated.

The enrichment and consortia Mn(IV) reduction rate experiments support the notion that salinity has an effect on MnRB activity in aquatic sediments; however, rather than inhibiting manganese reduction, modest increases in salinity may actually enhance manganese reduction, as was observed in site ABC enrichment experiments. Rainfall, including rainfall events from seasonal tropical storms, and other freshwater inputs into the Laguna Madre will result in decreased salinity, which does not seem to inhibit manganese reduction rates by enriched MnRB communities from the Laguna Madre. We measured manganese reduction rates using the benzidine-blue method. Benzidine does react slightly with some inorganic and organic compounds (Miller & Kraemer 1952) but the amount of non-specific reaction is low. Furthermore, we added 2 mM MnO_4 to our media which would greatly mask any non-specific reactions.

The medium used to enrich for MnRB contained approximately 0.010 mM concentrations of sulfate; therefore, it was possible that sulfate reduction, as an alternative anaerobic respiratory pathway, might be occurring in the cultures. However, the amount of sulfate present was less than the typical amounts of used to culture Sulfate Reducing Bacteria (SRB), such as Postgate's B medium which contains 0.017 M sulfate (Ghazy et al. 2011). Media to cultivate SRB contains one or more reducing agents, such as thioglycollate and/or ascorbic acid. Reducing agents were not added to the media in this study making it unlikely that it would be conducive to growing SRB. Furthermore, the reduction of sulfate produces hydrogen sulfide and sulfate reduction can be qualitatively assessed by either the production of a black precipitate in the culture, due to the reaction of hydrogen sulfide with iron present in the media, or by the presence of a "rotten eggs" odor. Neither the precipitate nor the hydrogen sulfide odor were observed or detected in the cultures; therefore, we were confident that sulfate reduction, if occurring, was not prevalent in the MnRB cultures.

Changing salinity may lead to different microbial community profiles as some species of MnRB may be better at growing and reducing manganese at certain salinities or within a range of salinities. DGGE revealed genotypic differences in MnRB between sites ABC and LMT050 and changes in community structure within ABC at increased salinity. MnRB from LMT050 were more broadly halotolerant and were able to adapt to changing salinities without altering the community structure. Overall, the DGGE profiles of both ABC and LMT050 enrichment displayed similarity in the MnRB populations present. This can be seen by the presence of Population 2 near the bottom of the gel at the high denaturing concentration. Since these manganese-reducing bacteria were from enrichment cultures, it is likely that we enriched for similar organisms which would account for the low diversity of the MnRB in the DGGE. That these MnRB's 16S rDNA denatured at high concentrations of urea (>60%) suggests that Laguna Madre MnRB have relatively high G +

C content in their DNA. Furthermore, Population 3 was present but non-detectable at low salinity but visible when grown at higher salinities. Population 3 had a higher G + C content compared to Populations 1 and 2 since the 16S rDNA was able to migrate further in the gel. This suggests that at high salinities organisms with high G + C content could be better at growing under higher salt environmental conditions and become more active, prevalent members of the MnRB community. A high G + C content may be a mechanism to withstand the hypersaline or changing seasonal salt conditions in the Laguna Madre and protect the cells under conditions of salt stress. However, this is purely speculative. There is no information in the scientific literature that definitively links a high G + C content with halotolerance or halophily.

Last, there appears to be no obvious correlation between community density, community structure and manganese reduction rates. Site ABC had a greater density of MnRB and displayed higher average manganese reduction rates compared to LMT050. Site LMT050 showed a consistent community structure when viewed with DGGE; however, the DGGE profile of site ABC changed at higher salinity but the manganese reduction rates did not greatly change. Thus, site ABC would appear to have a large, plastic and highly active community of manganese-reducing bacteria whereas LMT050 has a smaller, less active but more stable MnRB community. Whether this is due to differences in available manganese or other nutrients or due to environmental factors is not yet known.

Laguna Madre MnRB communities are present in different densities in lagoon sediments and may possess different strategies to cope with changing salinities. MnRB communities may alter the community profile (ABC) with certain genotypes more prevalent at high or low salinity, or sediment communities may consist of MnRB that are salt-adaptable (LMT050) yet still maintain consistent rates of Mn(IV) reduction. Future experiments will be to identify the MnRB present in the Laguna Madre and determine the percent of G

+ C in the organisms' DNA. The density, activity, and structure of MnRB communities are critical to the cycling and availability of this metal in marine sediments.

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