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Molecular Characterization of Bacterial Phylogenetic and Functional Groups at Terrebonne Bay along the Coastline of the Gulf of Mexico

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Abstract

The detection and quantification of bacterial phylogenetic and functional groups as well as community diversity at the site of the Deepwater Horizon oil spill in Terrebonne Bay along the Gulf of Mexico were carried out using nucleic acid staining, Fluorescence *in situ* Hybridization (FISH) and 16S rRNA gene cloning and sequencing approaches. Results from the 16S rRNA gene clone library analysis revealed high occurrences of bacterial members belonging to the *Cyanobacteria* (28%), *β- Proteobacteria* (21%), *Bacteroidetes* (17%), *Actinobacteria* (12%) and the *α- Proteobacteria* (10%). Particularly, bacterial members identified within the clone library as belonging to the *β- Proteobacteria* subclass were mostly hydrocarbon degraders, including *Methylibium petroleiphilum, Burkholderia cepacia, Hydrogenophaga taeniospiralis* and *Methylobacillus flagellates*. Simultaneous analyses of both planktonic and benthic bacterial communities by FISH revealed the numerical dominance of members of the type I Methanotrophic Bacteria (MB) over the type II populations. The results from the study clearly reveal a shift in the bacterial community structure and composition in response to the tragic methane and crude oil discharges from the Deepwater Horizon rig along the Gulf of Mexico.

Keywords: 16S rRNA gene; Fluorescence *in situ* hybridization; Pollution; Coastal marine

Introduction

The diversity and global distributions of bacterial populations within indigenous microbial assemblages in marine environments have been well documented because of their significant ecological importance within various milieus [1-8]. For instance, there is currently ample and incontrovertible evidence that bacterial assemblages within coastal marine milieus do not only rapidly respond to oil spills, but also contribute their wide arrays of hydrocarbon degrading capabilities to the effective bioremediation of oil residues in contaminated environments [9-11]. Given the presence of diverse degradative genes needed for *in situ* clean up of complex hydrocarbon pollutants, accurate delineation of *in situ* microbial assemblages is therefore paramount in order to effectively understand the overall dynamics of microbial response and biodegradation process in oil polluted sites. Even more so that it is common knowledge that microbial assemblages are influenced by various controlling factors, including pollutant type and bioavailability, nutrient dynamics as well as continuous fluxes in site-specific hydrodynamic conditions within marine environments [12-15].

This study elucidated *in situ* microbial compositions in response to the tragic crude oil and methane discharges that resulted due to the severance of the Deep water Horizon rig from its well offshore on the Gulf of Mexico (GOM) in 2010. This tragic pollution event ultimately released approximately 1.3 X 10^{10} moles of methane and 205 million gallons (i.e.780,000 m^3) of crude oil into surrounding environments of affected GOM sites [14]. Shortly after the incidence, the widespread dispersal of hydrocarbon plume was linked to the stimulation of indigenous bacterial populations, especially the γ- *Proteobacteria* members known to be closely associated with petroleum degradation [15,16]. Furthermore, these studies also found strong correlations between the occurrences of several hydrocarbon-degrading genes and various components of the hydrocarbon plume at the GOM sites examined.

Therefore, combinations of 16S ribosomal RNA gene sequencing, nucleic acid staining and fluorescence *in situ* hybridization (FISH) analyses were employed to qualitatively and quantitatively examine the phylogenetic composition and community diversity within the bacterioplankton assemblages at one of the most contaminated coastal location along the Gulf of Mexico. Quantitative analysis by FISH was particularly employed to target bacterial phyla with hydrocarbon-utilizing capabilities, including two subclasses (i.e. α- and γ-) of *Proteobacteria* and members of the methanotrophs (i.e. type I and II), since methane was documented as the most abundant hydrocarbon released into the GOM sites during the spill [15]. Generally, the methanotrophs are bacterial groups capable of both aerobic and anaerobic methane oxidation and belong mainly to either the α-*Proteobacteria* (type II methanotrophs) or the γ- *Proteobacteria* (type I methanotrophs) as well as some acidophilic members of the *Verrucomicrobiae* [17,18]. However, several other recent studies have also documented widespread anaerobic methane oxidation among diverse groups and consortia of marine microbial populations [18-22]. In this study, Terrebonne Bay in southern Louisiana was selected as the study site, based on the extent of the Deepwater Horizon oil spill at this particular milieu, combined with several past ecological antecedents and the direct connection of this coastal region to the Mississippi River [23].

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Materials and Methods

Description of study sites and sample collection

Water and sediment samples were collected in triplicates at three separate locations along the coastline of the Gulf of Mexico on July 19th, 2011, specifically at Terrebonne Bay (29.14134º, -90.56258º) in southern Louisiana (USA) as previously described [7]. Subsamples were later removed from each sample and preserved in 8% (w/v) paraformaldehyde and 1X phosphate-buffered saline solution for nucleic acid (4', 6'-diamidino-2-phenylindole {DAPI} staining and FISH analyses. During sampling, various water characteristics including temperature, pH, conductivity, oxidation-reduction potential were measured using the YSI model 556 MPS multi-probe system (YSI Incorporated, USA).

DNA extraction, PCR and clone sequencing

Approximately 500 mL of water samples were filtered through sterile, 47 mm, 0.2 um pore-size filters before storing at -80°C until processed. Total DNA was later extracted from the preserved filters using FastDNA SPIN Extraction kit (MP Biomedicals, Solon, OH, USA) and eluted in 50 uL of sterile deionized water. PCR amplification was then carried out on the extracted and pooled DNA from the replicate samples by targeting the almost full-length 16S rRNA gene with the universal bacterial primer pair 8F (5' AGA GTT GTA TCC TGG CTC AG 3') and 1492R (5'GGT TAC CTT GTT ACG ACT T3') as previously described [7]. The amplified PCR products were then confirmed on agarose gel and purified with a QIAquick PCR purification kit (QIAGEN, Valencia, CA) before subsequently utilized for cloning and sequencing. Clone libraries were constructed using the TOPO TA cloning kit (Invitrogen, Carslbad, CA). Colony PCR was used in screening transformed cells with vector-specific primers [24] and size of products verified by agarose gel electrophoresis.

Phylogenetic and diversity analysis

A total of 83 clones were successfully sequenced and analyzed using the Sequencher program (version 4.5; Gene Codes Co., Ann Arbor, MI). They were then compared with previously published GenBank sequences using the BLAST system [25] in order to determine their close relatives. Alignment and other manual editing were carried out with ClustalW [26]. The clones were then classified into 66 Operational Taxonomic Units (OTUs) using sequence identity values ranging from ≥ 98% to 100%. The OTUs were analyzed for species richness, Shannon Index, Simpson's (Reciprocal) Index of diversity, species evenness and Chao-1 richness indicator [27-29]. Rarefaction analysis was also performed to determine the diversity of the clone libraries using the freeware program Analystical Rarefaction version 1.3 (S. M. Holland: www.uga.edu/strata/software/Software.html).

Bacterial enumeration

Direct Counts (DAPI Staining): Total bacterial numbers in the preserved samples were determined by concentrating onto 0.2 µm pore-size black polycarbonate filters (Poretics, Livermore, CA) and staining with DAPI solution for 5 minutes. Filters were rinsed with sterile water and then mounted onto glass slides with Type FF immersion oil [30]. Bacterial cells in 10 separate fields were then counted using an epifluorescence microscope.

Fluorescence In Situ Hybridization: FISH analyses were used to determine the abundance of different bacterial phylogenetic groups as described in Lomans et al. [31] and Mills et al. [32]. Briefly, bacterial cells in the preserved water samples were concentrated onto 0.2 µm pore-size anodisc or polycarbonate filters (Whatman, Maidstone, UK), and then rinsed with deionized water, treated with 1 mL 0.1% Nonidet P-40 (Sigma Aldrich, St. Louis, MO). 40 µL of Texas red-labeled probe (Sigma Genosys, The Woodlands, TX; [5 ng/µL final concentration]) dissolved in hybridization buffer (6X standard saline citrate {SSC}, 0.02 M TRIZMA base at pH7.0, 0.1% sodium dodecyl sulfate {SDS}, 0.01% polyadenylic acid, and 30% formamide) were then added to the filters before incubating for 4 h at the appropriate temperature (Table 1). After the incubation, filters were washed twice with 400 µL of wash buffer (0.9 M NaCl, 0.02 M Tris-pH7, 0.1% SDS) and incubated with 80 µL of wash buffer for 10 minutes at the hybridization temperature. The filters were then rinsed twice with 400 µL sterile deionized water before they were mounted on glass slides with immersion oil. Cells that hybridized to each probe were enumerated using the epifluorescence microscopy by counting at least 300 fields on triplicate slides.

Nucleotide sequence accession numbers

Nucleotide gene sequences obtained were already submitted to DDBJ/GenBank/EMBL under accession numbers AB691143 to AB691225.

Results

Environmental variables at study site

Mean values for the various water characteristics that were measured in triplicates at the study sites are: temperature (30.74°C), pH (7.85), dissolved oxygen (109.8%), conductivity (14.27mS/cm) and oxidation-reduction potential (10.87 mV).

Clone library composition and community diversity assessment

Analysis of the 83 16S ribosomal RNA gene sequences revealed eight distinct phylogenetic groups, including bacterial members belonging to the *Cyanobacteria,* four subclasses (i.e. α-, β-, γ- δ-) of the *Proteobacteria, Bacteroidetes, Actinobacteria,* and the *Firmicutes* (Figure 1). Overall, members of the *Cyanobacteria,* accounted for the highest clone representation with 28%, followed by the β-*Proteobacteria* (20.5%), *Bacteroidetes* (17%) and *Actinobacteria* (12%). The percent representations by the different OTUs detected within the clone library constructed after comparing to their closest relatives in the NCBI database are presented in Table 2.

Probe	Taxa	Sequence (5'-3')	Hybridizing Temp (°C)	Reference
EUB338	Domain *Bacteria*	GCTGCCTCCCGTAGGAGT	48	Amann et al. [46]
ALF1b	*α-proteobacteria*	CGTTCG (C/T)TCTGAGCCAG	54	Amann et al. [47]
GAM42a	*γ-proteobacteria*	GCCTTCCCACATCGTTT	57	Manz et al. [48]
SRB385	Sulfate-Reducing-Bacteria	CGGCGTCGCTGCGTCAGG	53	Amann et al. [46]
β -AO233	Ammonia-oxidizing-Bacteria	AGCTAATCAGRCATCGG	44	Stephen et al. [49]
M-450	Type I Methanotrophs	ATCCAGGTACCGTCATTATC	46	Eller et al. [4]
M-84	Type II Methanotrophs	CCACTCGTCAGCGCCCGA	46	Eller et al. [4]

Table 1: Oligonucleotide sequences, target and hybridization conditions for probes used in this study.

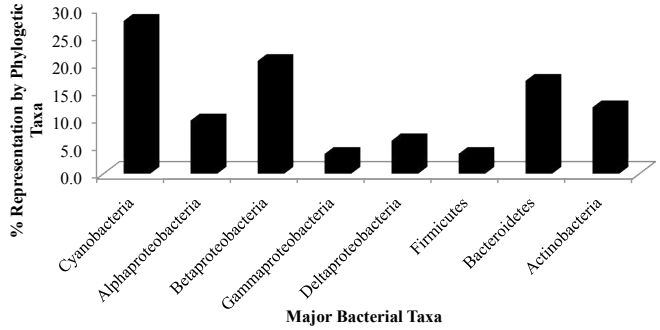

Figure 1: Percent distribution of major bacterial phylogenetic groups based on 16S rRNA gene sequences from the coastal bacterioplankton assemblages in Terrebonne Bay along the Gulf of Mexico.

Among the *Proteobacteria*, members of the β-*Proteobacteria* subclass dominated especially including several hydrocarbon-utilizing bacterial species as *Methylibium petroleiphilum, Burkholderia cepacia* and *Methylophilus methylotrophus.* Conversely, bacterial members of the α-*Proteobacterial* subclass accounted for only 9.6% of the total clone library composition including species *of Oceanicola pacificus* and *Shinella zoogloeoides* belonging to the pyrene and pyridine-degrading consortia. Clones belonging to the γ- and δ- *Proteobacteria* accounted for only 3.6% and 6.0% of total populations, respectively, including species such as *Hydrocarboniphaga effuse* and *Desulfofaba fastidiosa.*

Results from the various diversity measures analyzed revealed high bacterial diversity within the bacterioplankton assemblage at the bay site examined. Specifically, the Simpson's (Reciprocal) index of diversity was 46.2, while the species evenness was 0.969. Equally high were the numbers calculated to be 217.3 and 4.06 for the Chao 1 estimate of species richness and the Shannon Weiner Index, respectively. Rarefaction analysis revealed that the amounts of clones sequenced and screened are probably not sufficient for the estimation of the bacterial diversity within the clone libraries (Figure 2).

Abundances of bacterial phylogenetic and functional groups

Numbers of total bacteria within the bacterioplankton and benthic assemblages averaged about 3.5 x 10^7/mL and 1.5 x 10^8/g, respectively. While, Domain Bacteria occurrence accounted for between 11% and 20% of total bacterial counts in the water and sediment at average abundance of 4.0 x 10^6/mL and 3.0 x 10^7/g respectively (Figure 3a and 3b). When two subclasses (*i.e.* α-, and γ-) of the *Proteobacteria* were enumerated, their numbers were comparable between both the bacterioplankton and benthic assemblages, although members of α-*Proteobacteria* were found to be numerically more dominant within both habitats at the bay site (Figure 4a and 4b).

The occurrences of both type 1 and II methanotrophic functional bacterial populations followed the same trend and on average were at least one order of magnitude higher within the sediment than in the bacterioplankton communities (Figure 4a and 4b). Comparatively, the type 1 group were more numerically dominant than the type II, in both water and sediment samples examined. In contrast, the abundances of the other two functional groups examined i.e., the sulfate-reducing and the ammonia-oxidizing bacterial populations differed in their pattern of occurrences within both the bacterioplankton and benthic assemblages. Specifically, numbers of AO233-hybridized cells were found to be higher than those detected with the SRB385 probe in the sediment; conversely, the entire opposite in occurrence was the case for both populations enumerated in the water samples (Figure 4a and 4b).

Discussion

In this study, by applying combinations of several culture-independent (i.e. nucleic acid staining, fluorescence *in situ* hybridization

	Closest Phylogenetic Taxa from NCBI	GenBank Acc. Number	Abundance (n/%)a
A	**Cyanobacteria**		
1	*Uncultured Cyanobacterium*	HQ242211	1/1.20
2	*Uncultured Cyanobacterium*	JF966676	1/1.20
3	*Uncultured Cyanobacterium*	EU930687	1/1.20
4	*Uncultured Cyanobacterium*	AM690936	6/7.23
5	*Uncultured Cyanobacterium*	AB491631	2/2.41
6	*Uncultured Cyanobacterium*	FJ352328	1/1.20
7	*Uncultured Cyanobacterium*	GQ349130	1/1.20
8	*Uncultured Cyanobacterium*	FM995186	1/1.20
9	*Uncultured Cyanobacterium*	KC545747	1/1.20
10	*Uncultured Cyanobacterium*	FJ763779	1/1.20
11	*Uncultured Cyanobacterium*	EU780238	1/1.20
12	*Uncultured Cyanobacterium*	JF966674	1/1.20
13	*Uncultured Cyanobacterium*	EU800916	1/1.20
14	*Uncultured Cyanobacterium*	HM057705	1/1.20
15	*Uncultured Cyanobacterium*	AM259752	1/1.20
16	*Uncultured Cyanobacterium*	HQ914635	1/1.20
17	*Uncultured Cyanobacterium*	GU074287	1/1.20
B	**Alphaproteobacteria**		
18	*Rhodobacter veldkampii*	NR043405	1/1.20
19	*Shinella zoogloeoides*	NR041341	1/1.20
20	*Shinella zoogloeoides*	NR041342	1/1.20
21	*Rhodoplanes serenus*	NR040936	1/1.20
22	*Anderseniella baltica*	NR042626	2/2.41
23	*Skermanella aerolata*	NR043929	1/1.20
24	*Oceanicola pacificus*	NR043915	1/1.20
C	**Betaproteobacteria**		
25	*Methylibium petroleiphilum*	NR041768	1/1.20
26	*Burkholderia ginsengisoli*	NR041288	1/1.20
27	*Massilia lutea*	NR043310	2/2.41
28	*Burkholderia cepacia*	NR041719	1/1.20
29	*Methylophilus methylotrophus*	NR041257	1/1.20
30	*Denitratisoma oestradiolicum*	NR043249	2/2.41
31	*Burkholderia endofungorum*	NR042584	1/1.20
32	*Massilia dura*	NR043307	1/1.20
33	*Methylobacillus flagellatus*	NR043691	1/1.20
34	*Hydrogenophaga pseudoflava*	NR028717	1/1.20
35	*Azoarcus buckelii*	NR027190	1/1.20
36	*Methylobacillus flagellatus*	NR043691	1/1.20
37	*Hydrogenophaga taeniospiralis*	NR028716	1/1.20
38	*Burkholderia cepacia*	NR041719	1/1.20
39	*Methylobacillus flagellatus*	NR043691	1/1.20
D	**Gammaproteobacteria**		
40	*Thioalkalivibrio denitrificans*	NR028745	1/1.20
41	*Singularimonas varicoloris*	NR042175	1/1.20
42	*Hydrocarboniphaga effusa*	NR029102	1/1.20
E	**Deltaproteobacteria**		
43	*Desulfofaba fastidiosa*	NR025746	1/1.20
44	*Desulfuromusa bakii*	NR026175	1/1.20
45	*Geobacter thiogenes*	NR028775	1/1.20
46	*Desulfuromonas alkaliphilus*	NR043709	1/1.20
47	*Desulfuromusa bakii*	NR026175	1/1.20

F	Firmicutes		
48	*Anaeroarcus burkinensis*	NR025298	1/1.20
49	*Parasporobacterium paucivorans*	NR025390	1/1.20
50	*Thermincola carboxydiphila*	NR043010	1/1.20
G	**Bacteroidetes**		
51	*Flavisolibacter ginsengisoli*	NR041500	2/2.41
52	*Owenweeksia hongkongensis*	NR040990	5/6.02
53	*Flavobacterium sp*	NR040990	1/1.20
54	*Perexilibacter aurantiacus*	NR041534	2/2.41
55	*Robiginitalea myxolifaciens*	NR041514	1/1.20
56	*Lishizhenia caseinilytica*	NR041043	1/1.20
57	*Fluviicola taffensis*	NR041911	1/1.20
58	*Haliscomenobacter hydrossis*	NR042316	1/1.20
H	**Actinobacteria**		
59	*Streptomyces hebeiensis*	NR029091	1/1.20
60	*Ferrimicrobium acidiphilum*	NR041768	1/1.20
61	*Ilumatobacter fluminis*	NR041633	2/2.41
62	*Ferrimicrobium acidiphilum*	NR041798	2/2.41
63	*Streptomyces hebeiensis*	NR029091	1/1.20
64	*Ilumatobacter fluminis*	NR041633	1/1.20
65	*Kitasatospora saccharophila*	NR041538	1/1.20
66	*Patulibacter minatonensis*	NR041254	1/1.20

a = total of 83 clones

Table 2: Percent occurrences of bacterial phylogenetic groups in the bacterioplankton assemblage at Terrebonne Bay along the Gulf of Mexico.

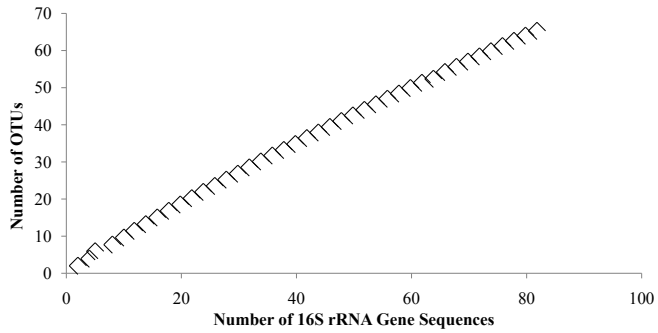

Figure 2: Percent abundance of different OTUs presented by genus from Terrebone Bay along the Gulf of Mexico.

(FISH), gene cloning and sequencing approaches, the impact of the Deepwater Horizon oil spill on indigenous microbial assemblages was assessed qualitatively and quantitatively at the study site in Terrebonne Bay. Sequence diversity analyses revealed the presence of eight distinct bacterial phyla comprising mostly of various hydrocarbon-utilizing phylotypes that strongly suggests that such a shift was stimulated by the oil disaster at the GOM. This result corroborates earlier documentation that also showed strong stimulation of indigenous bacterial populations, especially the γ- *Proteobacteria* members closely associated with petroleum degradation at the same polluted GOM sites [33,15]. Interestingly, results documented in some earlier studies that were conducted along the same coastlines of the GOM, prior to the oil spillage [1,32,7] appeared to show different compositions of the microbiota compared to those reported in this current study, therefore indicating that a shift in the dominant microbial groups must have occurred. Particularly, Olapade [7] observed majority of sequences (*i.e.* between 30% and 60% of cloned libraries) within the bacterioplankton communities in three GOM sites to be closely related to gene sequences belonging to unknown bacteria. This shift in the major microbial phylogenetic groups probably further validate the suggestion that the phyla with hydrocarbon-degrading abilities were relatively more stimulated and supported by the oil plume at these GOM locations [14,33,15].

Generally, variations of sequences belonging to globally distributed bacterial taxa are typically found within microbial communities in coastal marine environments [34,2,5]. However, in this study the relatively high occurrence of β-*Proteobacteria* found is quite suggestive of the oil plume influence, especially given the relatively rare occurrence of this particular taxa in marine milieus as compared to freshwater environments [28,7,8]. Moreover, the bacterial members identified as belonging to the *β- Proteobacteria* subclass within the clone library were mostly hydrocarbon (e.g., methanol) degraders, including *Methylibium petroleiphilum, Burkholderia cepacia, Hydrogenophaga taeniospiralis* and *Methylobacillus flagellates* species [35-37].

Furthermore, bacterial members of the δ- *Proteobacteria* and the *Firmicutes* that represented about 6% and 3% of total clone populations comprised of several species such as *Desulfofaba fastidiosa, Desulfuromusa bakii* and *Parasporobacterium paucivorans* which are capable of utilizing various crude-oil derived compounds including methyloxylated aromatics, propionate and sulfur [38-40]. Typically, majority of bacterial species belonging to these two taxa are reportedly associated with soil and sediment in coastal marine environments especially during tidal events [1,7]. Therefore, the presence of high numbers of hydrocarbon-utilizing bacterial phyllotypes in this study within the bacterioplankton communities strongly suggest a possible change in the physiological and metabolic profiles of some of the taxa in response to the available hydrocarbon substrates.

The relatively high representations by the α-*Proteobacteria* (10%) and the *Bacteroidetes* (17%) were not at all surprising and further

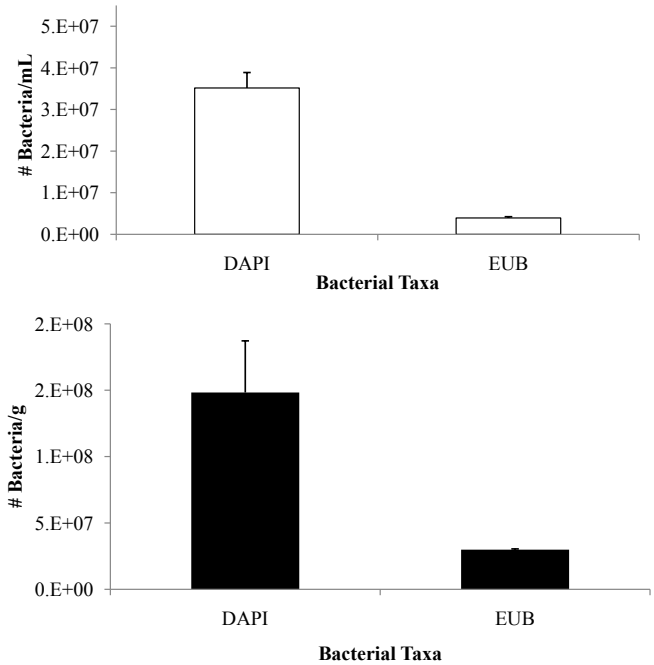

Figure 3: Numbers of total bacteria (as determined by DAPI staining) and domain bacteria (determined by FISH) in the coastal bacterioplankton [A] and benthos [B] assemblages in Terrebonne Bay along the Gulf of Mexico. Values represent mean ± 1 SE (n = 3).

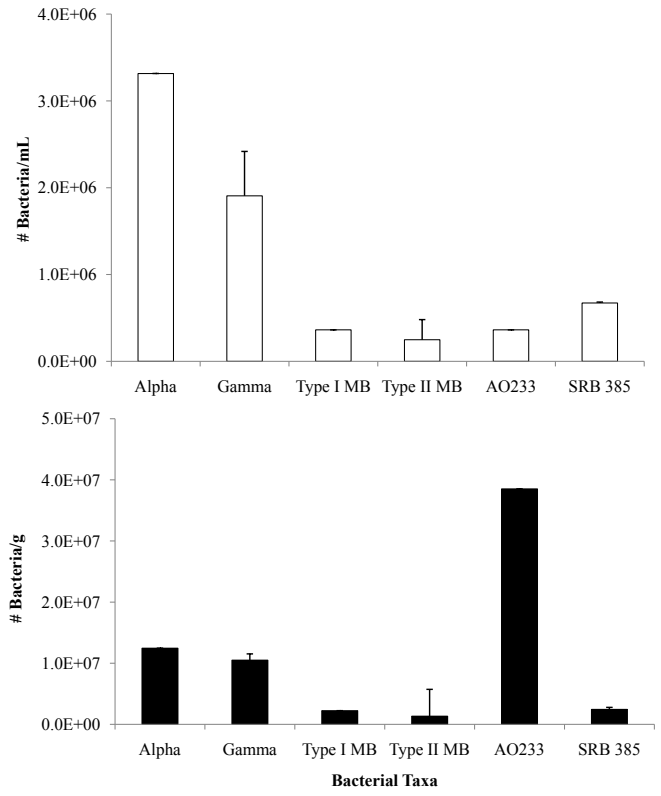

Figure 4: Numbers of α- and γ- *Proteobacteria*; Type I and Type II methanotrophs and Ammonia-oxidizing and sulfate-reducing bacterial populations (determined by FISH) in the coastal bacterioplankton [A] and benthos [B] assemblages in Terrebonne Bay along the Gulf of Mexico. Values represent mean ± 1 SE (n = 3).

corroborate earlier documentations of the dominance of the two bacterial taxa in coastal marine systems [5]. Species such as *Shinella zoogloeoides* that are capable of pyridine degradation [41] dominated α-*Proteobacteria* clones, while members of the *Bacteroidetes* that typically play significant roles in the decomposition of high-molecular-weight organic compounds in marine environments [42], were mostly represented by *Owenweeksia hongkongensis,* within the bacterioplankton communities.

The numerical dominance of both types of the methanotrophs examined within the sediment matrixes as compared to populations in the bacterioplankton is further indicative of their typical rapid response towards the source of methane release [43], which in this case was close to the ocean sub-seafloor [28]. The close proximity to methane source may also partly explain the higher presence of bacterial members belonging to the ammonia-oxidizing and sulfate-reducing bacterial populations, despite the frequent methane oxidization in both aerobic and anaerobic environments [22,44]. The disparity observed here regarding the occurrence of the AOB populations strongly agrees with an earlier similar study from the Northwestern Mediterranean Sea which indicated that members of this functional group might probably occupy distinct environmental niches in marine systems, because of their lower representations in planktonic libraries as compared to those that were particle-associated [45]. Similar contrasting patterns have also been observed especially in the diversity of aerobic methanotrophs between planktonic and sediment- associated assemblages in marine environments [17]. In this study conducted in two seep systems, they reported that while the sediment associated methanotrophs were less diverged and dominated by single taxa, in contrast the diversity within the planktonic community were found to be limited to a small number of moderately diverged clades within the same *Methylococcaceae* genus.

In conclusion, the results from both FISH and 16S rRNA gene clone sequences clearly reflects the subsequent shift in the bacterial community structure and composition at Terrebonne Bay in southern Louisiana in response to the tragic methane and crude oil discharges from the Deepwater Horizon rig along the Gulf of Mexico. This obvious shift in bacterial community diversity to mostly hydrocarbon-degrading phylotypes at the GOM site examined, further highlights both the ecological importance as well as various degradative potentials of autochthonous bacterial assemblages within contaminated coastal marine milieus.

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