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Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill

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ABSTRACT

A significant portion of oil from the recent Deepwater Horizon (DH) oil spill in the Gulf of Mexico was transported to the shoreline, where it may have severe ecological and economic consequences. The objectives of this study were to: 1) identify and characterize predominant oil-degrading taxa that may be employed as model hydrocarbon-degraders or as microbial indicators of contamination, and 2) characterize the *in situ* response of indigenous bacterial communities to oil contamination in beach ecosystems. This study was conducted at municipal Pensacola Beach, Florida, where chemical analysis revealed weathered oil petroleum hydrocarbon (C8-C40) concentrations ranging from 3.1 to 4500 mg kg^{-1} in beach sands. Twenty-four bacterial strains from 14 genera were isolated from oiled beach sands and confirmed as oil-degrading microorganisms. Isolated bacterial strains were primarily *Gammaproteobacteria*, including representatives of genera with known oil-degraders (*Alcanivorax, Marinobacter, Pseudomonas, Acinetobacter*). Sequence libraries generated from oiled sands revealed phylotypes that showed high sequence identity (up to 99%) to rRNA gene sequences from the oil-degrading bacterial isolates. The abundance of bacterial SSU rRNA gene sequences was approximately 10 times higher in oiled (0.44 – 10.2 x 10^7 copies g^{-1}) vs. clean (.024 – 1.4 x 10^7 copies g^{-1}) sand. Community analysis revealed a distinct response to oil contamination, and SSU rRNA gene abundance derived from the genus *Alcanivorax* showed the largest increase in relative abundance in contaminated samples. We conclude that oil contamination from the DH spill has a profound impact on the abundance and community composition of indigenous bacteria in Gulf beach sands, and our evidence points to members of the *Gammaproteobacteria* (*Alcanivorax*, *Marinobacter*) and *Alphaproteobacteria* (*Rhodobacteraceae*) as key players in oil degradation there.

56 **INTRODUCTION**

57 The blowout of the Deepwater Horizon (DH) drilling rig resulted in the world's largest
58 accidental release of oil into the ocean in recorded history. The equivalent volume of
59 approximately 4.9 million barrels of light crude oil were discharged into the Gulf of Mexico
60 from April to July, 2010 (OSAT, NOAA report, (56); Oil Budget Calculator, (43)), and the total
61 hydrocarbon discharge was 40 % higher if gaseous hydrocarbons are included (34). A large
62 amount of the discharged oil was transported to the surface and reached the shoreline. Although
63 cleanup efforts have remained aggressive, a substantial portion of the oil remains trapped in
64 coastal ecosystems, especially in benthic areas.

65 Permeable sandy sediments cover large areas of the seafloor in the Gulf of Mexico,
66 including beach ecosystems. Marine sands act as efficient biocatalytic filters that play an
67 important role in the biogeochemical cycles of carbon and nutrients in shallow Gulf waters (11,
68 19, 20). Marine sands in the Gulf are covered with biofilms of highly diverse microbial
69 communities (30, 53), and bacterial abundance in sands exceeds that of the overlying seawater
70 by orders of magnitude (7, 35, 38). Enhanced porewater exchange in highly permeable marine
71 sands stimulates microbial metabolism through the delivery of growth substrates and the removal
72 of waste products (7, 15, 28, 29).

73 Similar to the microbially-mediated breakdown of natural organic matter, biodegradation
74 mediated by indigenous microbial communities is the ultimate fate of the majority of oil
75 hydrocarbon that enters the marine environment (4, 42, 59). Hydrocarbon-degrading
76 microorganisms are ubiquitous in the marine environment (27, 76), and biodegradation was
77 shown to be successful in naturally remediating oil contamination associated with several spills
78 that impacted shorelines predominated by permeable marine sediments (8, 46, 66). Though

79 bioremediation field trials were often carried out, there is a paucity of information on the
80 indigenous microbial communities that catalyze oil degradation under *in situ* conditions at spill
81 sites (2). Even less information is available on which members of the microbial community are
82 active in degrading hydrocarbons, and the impacts of various environmental parameters in
83 controlling the activities of indigenous hydrocarbon-degrading microorganisms have not been
84 specifically addressed. Thus, the theoretical basis to understand and predict the dynamics of
85 hydrocarbon-degrading microorganisms *in situ* is lacking (27).

86 Contamination of beach ecosystems by oil has the potential to cause severe
87 environmental and economic consequences in the Gulf region. The risk of accidental oil
88 discharge to the marine environment will remain high for the foreseeable future as increased
89 economic pressure to access new oil reserves in deep marine waters will require less tested
90 technologies (32). Although technologies for oil drilling have advanced rapidly in recent
91 decades, strategies to respond to oil spills and to assess environmental impacts of oil
92 contamination have lagged behind (33). An understanding of the impacts of oil on indigenous
93 microbial communities and identification of oil-degrading microbial groups are prerequisite for
94 directing the management and cleanup of oil-contaminated beach ecosystems. Thus, the
95 objectives of this study were to: 1) identify and interrogate the ecophysiology of predominant
96 oil-degrading taxa that may serve as model organisms and microbial indicators of contamination,
97 and 2) characterize the *in situ* response of indigenous bacterial communities to oil contamination
98 in Gulf beach sands.

99 **MATERIALS AND METHODS**

100 **Site and sample description.** This study focused on beach sands collected from Pensacola
101 Beach, FL, in the area of the municipal beach (30°19.57N, 087°10.47W) which was exposed to

102 heavy oil contamination from the Deepwater Horizon (DH) oil spill beginning in the first week
103 of June, 2010. Sands in the supratidal zone of the beach where thick buried oil layers were found
104 was dry with no extractable pore water and no dissolved nutrients. Nutrients in the pore water of
105 shallow submerged sand in the region range from 2 to 10 µmol L^{-1} for dissolved inorganic
106 nitrogen, and from 0.1 to 1 µmol L^{-1} for dissolved inorganic phosphate and typically are about
107 one order of magnitude higher than the respective nutrient concentrations in the overlying water
108 column (Huettel, unpublished).

109 Samples were collected on 07/02/2010, 07/30/2010, and 09/01/2010. Sample
110 designations were assigned based on collection order, with OS55 through OS320 samples
111 represented in this study. During each trip, sediment cores of 50 cm in depth were taken in
112 duplicate from the intertidal and supratidal zones. Cores were aseptically sectioned into 10 cm
113 intervals on site, homogenized, and sub-sampled into sterile 50 ml conical tubes (7/02/2010
114 cores were labeled OS55-OS77; 7/30/2010 cores were labeled OS82-OS91; 9/01/2010 cores
115 were labeled OS291-OS320). During the latter two sampling trips, a 10 m x 1m x 1m trench was
116 excavated perpendicular to the beach face. Samples were taken along the visible oil layer
117 spanning the length of the trench, and vertically through the oil layer (7/30/2010 samples were
118 labeled OS200-OS244; 9/01/2010 samples were labeled OS248-OS290). All samples for nucleic
119 acid extraction were immediately frozen on dry ice and stored at -80 ^{o}C. For cultivation, samples
120 were immediately transported to the lab on ice, stored at 4 ^{o}C, and processed within a few days.
121 A second control site, unaffected by oil contamination, was sampled at St. George Island FL,
122 (29°44'88.1"N, 084°42'58.6"W) prior to the DH blowout in April of 2010. Cores were sampled
123 in triplicate and the surface 0-5 cm interval was archived as above for further analysis. Source oil

124 was sampled from the DH wellhead and used for chemical analysis and cultivation. This oil was
125 collected on 5/20/10 aboard the drillship Discoverer Enterprise.
126 **Analysis of oil hydrocarbon composition.** Oil contamination was assessed by visual
127 examination in the field according to the following scale: 0 for clean or no visible contamination,
128 1 for low, 2 for medium, and 3 for high levels of oil contamination. A subset of core samples was
129 ultrasonically extracted and subjected to chemical analysis using established, EPA approved, gas
130 chromatography-mass spectrometry (GC-MS) techniques at TestAmerica Laboratories,
131 Tallahassee, FL. Total petroleum hydrocarbons were determined using the Florida Residual
132 Petroleum Organic Method (FL-PRO) in core samples as well as in source oil from the DH
133 wellhead. In addition, more detailed analysis of oil hydrocarbon composition was conducted for
134 the source oil and oiled beach sands visually assessed as containing medium (2) or high (3)
135 levels of oil contamination. Semivolatile organic compounds (polycyclic aromatic
136 hydrocarbons, aromatics, aliphatics) were determined by GC-MS method SW846 8270C LL.
137 **Enumeration, enrichment and isolation of oil degrading bacteria.** Oil-degrading bacterial
138 populations were enumerated by the three-tube most-probable-number (MPN) assay using 10-
139 fold serial dilutions of beach sand in growth medium. A minimal, artificial seawater medium was
140 prepared and dispensed according to Widdel and Bak (74). Source oil was sterilized according to
141 Widdel (73) and added as the sole electron donor and carbon source to a final concentration of
142 0.2 % (w/v). Under anaerobic conditions, the medium was purged with a 80% N_2/20% CO_2 gas
143 mixture, sealed into Hungate tubes, and nitrate was provided as the sole electron acceptor to 3
144 mM. For enumeration under aerobic conditions, the medium was prepared aerobically and
145 dispensed into 16 x 150 mm tubes that were loosely capped and continuously shaken at 150 rpm
146 on a shaker table. Tubes were incubated at room temperature for one month and bacterial growth

147 was monitored by culture turbidity and depletion of added oil at regular intervals in comparison
148 to autoclaved controls. The MPN index was determined from statistical tables published by the
149 American Public Health Association (3).

150 Strains of oil-degrading bacteria were isolated from the highest positive dilutions of the
151 MPN enrichments as well as from parallel enrichments conducted in larger volumes of the
152 artificial seawater medium or with filter-sterilized seawater as the enrichment medium. When
153 filtered seawater was used, it was supplemented with vitamin and trace element solutions
154 according to Widdel and Bak (74). In oxic seawater enrichments, the medium was buffered with
155 10 mM HEPES (pH 7.0), dispensed into Erlenmeyer flasks, and flasks were shaken at 150 rpm
156 on a shaker table. Anaerobic seawater enrichments were prepared in serum bottles as described
157 above and sealed with butyl rubber stoppers. Flasks and serum bottles both were inoculated to 10
158 % (w/v) with oiled sands and 0.2 % (w/v) of sterile source oil served as the sole source carbon
159 and electron donor. Bacterial growth was monitored by measuring the optical density at 600 nm
160 (OD_{600}). After four successive transfers to fresh medium, strains were isolated onto Zobell
161 marine agar (HiMedia, India), or artificial seawater medium supplemented with 5 % peptone + 1
162 % yeast extract, 10 mM HEPES, and molecular grade agar (Sigma, Aldrich USA) as a
163 solidifying agent. Denitrifying bacteria were isolated on agar plates of the artificial seawater
164 medium equilibrated inside a Coy anaerobic chamber with 20 mM nitrate as the sole electron
165 acceptor (22). Repeated streaking on solid media purified isolated strains, representative pure
166 cultures were again screened in liquid media for oil degradation potential, and frozen stocks were
167 prepared at -80 ^{o}C in 20% glycerol.

168 **Phenotypic characterization of pure cultures.** Representative isolates were further screened
169 for oil degradation capability under aerobic conditions by quantifying the residual oil in liquid

170 cultures using gravimetric and spectrophotometric methods (see supplementary material for
171 details).

172 Initial physiological characterization of representative oil-degrading isolates was
173 conducted using Phenotypic MicroArray™ (PM) analysis. PM tests were performed by Biolog,
174 Inc. (Hayward, CA) according to methods described elsewhere (6, 44) (see supplementary
175 material for further details).

176 **Nucleic acid extraction, DNA fingerprinting, and analysis of SSU rRNA sequences.**
177 Total genomic DNA was extracted using a MoBio Powersoil DNA extraction kit (Mo Bio
178 Laboratories, Carlsbad, CA) following the manufacturer's protocol. Bacterial community
179 structure was initially assessed in DNA extracts by community fingerprinting using the
180 automated ribosomal intergenic spacer analysis (ARISA) method (61). This technique allowed
181 for the rapid comparison of a large number of samples and aided in identifying critical samples
182 for pyrotag sequencing (see supplementary material for a complete description of the ARISA
183 methods). Total RNA was extracted from sand samples according to Chin et al. (10).
184 Contaminating DNA was removed using DNase I (Ambion, Austin, TX, USA). Reverse
185 transcription reactions for SSU rRNA were performed using GoScript reverse transcriptase
186 (Promega, Madison, WI, USA) and the general bacterial 1492R reverse primer at 0.2 µM (72).
187 Reactions were conducted following the manufacturer's instructions.

188 For pyrotag sequencing, approximately 100 ng of extracted DNA or cDNA was sent to
189 the Research and Testing Laboratory (Lubbock, TX) for bacterial ribosomal RNA (rRNA) gene
190 tag-encoded FLX amplicon pyrosequencing. PCR amplification was performed using primers
191 Gray28F and Gray519r 5' (Table 1; (31)) and sequencing reactions utilized a Roche 454 FLX
192 instrument (Roche, Indianapolis, IN) with Titanium reagents, titanium procedures, a one-step

193 PCR, mixture of Hot Start and HotStar high fidelity *Taq* polymerases. Following sequencing, all
194 failed sequence reads, low quality sequence ends and tags were removed and sequences were
195 depleted of any non-bacterial ribosome sequences and chimeras using custom software (21) as
196 has been described previously (1). Pyrosequences were trimmed to 300 bases, and sequences
197 shorter than 300 bases were removed from further analysis. Sequence data were submitted to the
198 European Nucleotide Archive (ENA) Sequence Read Archive (SRA) under the study accession
199 number ERP000807.

200 **Analysis of bacterial community composition.** Sequence analysis was accomplished through
201 the software packages QIIME (9) and Primer6 (Primer-E, Lutton, IvyBridge, UK). Sequences
202 were clustered into operational taxonomic units by 97% sequence identity using UCLUST (17)
203 and reference sequences were picked using scripts in QIIME. OTU were classified in QIIME
204 using the RDP classification algorithm set at a 50% confidence rating. Representative sequences
205 were aligned using the PyNAST algorithm and the alignment was filtered to remove common
206 gaps. A phylogenetic tree was built de novo using FastTree (58).

207 Jackknifed beta diversity was assessed by iteratively rarefying samples to 1600 sequences
208 in order to remove the effect of sampling effort, in QIIME. Bray-Curtis distance was calculated
209 for each rarefied OTU abundance table, and an average pairwise distance and standard deviation
210 was calculated for each pair of samples. The resulting distance matrix was imported into Primer6
211 and visualized using a non-parametric multidimensional scaling plot (NMDS). The same method
212 was used to generate an NMDS plot of an averaged weighted UniFrac distance matrix and is
213 included in the supplementary material. An analysis of similarity test (ANOSIM) was performed
214 on the Bray-Curtis distance matrix to determine if grouping samples by oil presence was
215 significant. Indicator species were identified using a similarity of percentages (SIMPER) test in

216 Primer6. Samples were grouped into 5 types by location (Pensacola, St. George Island), oil
217 presence and nucleic acid type.

218 **Quantitative molecular analyses for tracking total Bacteria and *Alcanivorax***

219 Quantification of bacterial small subunit rRNA genes (SSU rRNA) was performed
220 according to Nadkarni et al. 2002 (Table 1; (55)). Primers and probes were ordered from
221 Integrated DNA Technologies (IDT; Coralville, IA), and all had working concentrations of 100
222 nM in the PCR mastermix. The probe was labeled with a 6 - Carboxyfluorescein (6-FAM)
223 fluorophore, and contained an Iowa Black™ fluorescent quencher (IBFQ), and an internal ZEN
224 quencher (IDT) located nine bases from the 5' end. All reactions were performed in triplicate,
225 and analyzed using the ABI 7900HT Fast RealTime PCR system. A new primer set was
226 developed to target SSU rRNA genes from bacteria of the genus *Alcanivorax* (Table 1; see
227 supplementary material for further details). Briefly, primers were designed using the 'probe
228 design' feature of the phylogenetic analysis program package ARB (48), and checked for
229 specificity within ARB, and by basic local alignment search tool (BLAST) analyses. These
230 primers were used with the probe developed for domain-level bacterial qPCR analyses, under
231 similar PCR conditions. Primers were designed to have similar annealing properties to the
232 general primers described by Nadkarni et al. (55), and the qPCR was performed as described
233 above. For both Bacterial and *Alcanivorax*-specific qPCR assays, absolute quantification was
234 performed using a standard curve derived from PCR products of the *Alcanivorax dieselolei*
235 isolate from this study generated by near-full gene amplification of SSU rRNA genes using the
236 general bacterial primer set 27F and 1492R (41, 72). Standard curves were linear across a six-
237 order of magnitude range (from 2.79×10^8 to 2.79×10^2 copies/reaction), with similar
238 efficiencies (92-94%). qPCR data were analyzed using principal components analysis, as

239 implemented within the software package Primer6. Gene abundance values were fourth-root
240 transformed to meet assumptions of normality.

241 **RESULTS**

242 **Changes in hydrocarbon composition.** Representative samples were selected for hydrocarbon
243 analysis in order to quantify the extent of contamination, to verify our visual observations of oil
244 contamination as well as to determine which fractions of oil hydrocarbons in beach sands were
245 depleted in comparison to the source oil collected from the Deepwater Horizon (DH) wellhead.
246 Chemical analysis revealed petroleum hydrocarbon (C8-C40) concentrations ranging from 3.1 to
247 4500 mg kg^{-1} in Pensacola Beach sands. All beach sand samples analyzed contained detectable
248 oil hydrocarbon concentrations. At the lower range of contamination detected by chemical
249 analysis (< 10 mg kg^{-1}), oil was not observed visually (Table 2).

250 Two oiled beach sand samples were subjected to detailed chemical analysis (Table 3). In
251 these sand samples considered to be moderately or heavily contaminated by visual observations,
252 total petroleum hydrocarbon concentrations were 1900 and 4500 mg kg^{-1}, respectively.
253 Comparison to the source oil from the DH wellhead indicated substantial weathering in that the
254 majority of detectable hydrocarbon compounds that remained resided in the higher molecular
255 weight aliphatic (>C16) and aromatic (>C35) fractions (Table 3). When these samples were
256 collected on 1 September, approximately 18 weeks after the onset of the DH spill, detectable
257 polycyclic aromatic hydrocarbons and lighter aliphatics (C6 to C16) and aromatics (C8 to C21)
258 were reduced to near the detection limit. Heavier compounds such as larger alkanes (> C21) have
259 been shown to be degraded more slowly than corresponding lighter alkanes (< C21). Thus, the
260 ratio of lighter alkanes (C6 to C16) to heavier alkanes (C16 to C35) was used as a chemical
261 proxy for the biodegradation process (59, 62). The ratio observed in oiled beach sands (0.025 to

262 0.16) was reduced by 1 to 2 orders of magnitude in comparison to the ratio observed in the DH
263 source oil (2.79; see Table 3).

264 **Enumeration of oil-degrading bacteria.** In the same sand samples for which detailed chemical
265 analysis was performed (Table 3), MPN counts of cultivatable hydrocarbon-degrading bacteria in
266 oil-contaminated sands (2.4 x 10^{10} cells ml^{-1}) exceeded those from "clean" sands sampled in
267 parallel by 3 to 4 orders of magnitude (2.4 – 9.3 x 10^{6} cells ml^{-1}).

268 Enumeration of bacteria with quantitative molecular-based approaches corroborated
269 cultivation-based evidence. Ribosomal RNA gene abundances were determined for samples
270 taken from all sampling trips, resulting in a dataset of 468 sand samples. Counts of cultivatable
271 hydrocarbon-degraders paralleled the molecular-based quantification of bacterial rRNA gene
272 abundance. In oiled sands that were analyzed for hydrocarbon content, bacterial rRNA gene
273 abundance was approximately 10 times higher in oiled (0.44 – 14.2 x 10^{7} copies g^{-1}) vs. clean
274 (0.024 – 1.57 x 10^{7} copies g^{-1}) sand. *Alcanivorax* spp. were not detected with qPCR methods in
275 the clean sands used for MPN enumeration, but were detected in Pensacola Beach sands without
276 visible oil contamination. *Alcanivorax* were more abundant in sand samples with visible oil
277 contamination (0.27 – 2.77 10^{7} copies g^{-1}) than in sands without visible oil contamination (0.12 –
278 8.9 x 10^{5} copies g^{-1}). Differences in total bacterial and *Alcanivorax* gene abundances between
279 oiled and non-oiled sands are both statistically significant, as assessed by a t-test on log-
280 transformed data ($P<0.008$).

281 Principal components analysis (PCA) was performed to investigate the effect of visible
282 oil contamination and sampling date on bacterial abundances for the larger qPCR dataset of 468
283 samples (Figure 1). Total bacterial SSU rRNA gene abundance and *Alcanivorax* specific SSU
284 rRNA gene abundance co-varied and increased with respect to visible oil contamination (Figure

285 1). The percent abundance of *Alcanivorax* did not co-vary with *Alcanivorax* SSU rRNA gene
286 abundance. The percent abundance of *Alcanivorax* was strongly associated with oil presence in
287 the earliest (July 2nd) sampling trip, and weakly associated with oil contamination in the later
288 trips. The first sampling trip was also characterized by a relatively small increase in total
289 bacterial SSU rRNA gene abundance associated with oil contamination compared to later
290 sampling trips. Linear regression analysis of the abundance of overall bacteria in comparison to
291 the abundance of *Alcanivorax* spp. for each sampling trip supported observations from the
292 principal components analysis (Figure 2). During the July sampling trip, *Alcanivorax* spp. were
293 most abundant and were shown to co-vary with the abundance of overall bacteria. In subsequent
294 sampling trips, the abundance of *Alcanivorax* declined and did not strongly co-vary with overall
295 bacterial abundance. Initially, the average percent composition of *Alcanivorax* gene copies
296 comprised approximately 10% of the total bacterial gene copies, which fell to less than 1% in
297 sampling trips after September 2010.
298 **Enrichment and isolation of oil-degrading bacteria.** Bacteria capable of using source oil as the
299 sole carbon and energy source were isolated from oiled beach sands under aerobic and anaerobic
300 conditions (Table 4). Analysis of bacterial SSU rRNA gene sequences revealed that the
301 characterized isolates belong to 14 genera from 7 orders and 4 classes within 3 phyla
302 (*Proteobacteria*, *Firmicutes*, and *Actinobacteria*). The majority of isolates were identified as
303 members of four orders (*Oceanospiralles*, *Alteromonadales*, *Vibrionales*, and *Pseudomonadales*)
304 within the class *Gammproteobacteria*. A single isolate was identified from the
305 *Alphaproteobacteria*. Two Gram positive organisms were isolated from the phyla *Firmicutes*
306 and *Actinobacteria*. The SSU rRNA gene sequences of all isolates were highly similar to those
307 retrieved from oiled sands of Pensacola Beach (Figure 3; Table 5). In particular, SSU rRNA gene

308 sequences from isolates of the genera *Alcanivorax*, *Marinobacter*, and *Pseudoalteromonas*, and
309 *Pseudomonas* shared a high sequence identity (> 97 %) with recovered sequences from beach
310 sands.

311 **Phenotypic characterization of oil-degrading bacteria.** Pure cultures from five of the 14
312 genera (SSU rRNA gene sequences show high sequence identity to those from *Alcanivorax*
313 *dieselolei, Acinetobacter sp., Pseudidiomarina maritima, Marinobacter hydrocarbonoclasticus,*
314 and *Vibrio hepatarius*) were tested for oil consumption under aerobic conditions. Oil degradation
315 capability was confirmed from the quantification of residual oil and concomitant growth as
316 optical density and cell protein (see supplementary material). Little to no oil was consumed by
317 the *Vibrio* strain in comparison to killed control cultures and minimal growth was observed. In
318 contrast, all of the remaining strains were capable of rapid aerobic growth with source oil as the
319 sole carbon substrate and electron donor. Gravimetric analysis indicated that strains most similar
320 to *A. dieselolei* and by *Acinetobacter sp.* showed highest oil degradation potential, with 93 and
321 90 % of the chloroform-extractable portion of amended crude oil degraded, respectively, in
322 comparison to autoclaved and uninoculated control cultures (see Figure S1). Strains most similar
323 to *M. hydrocarbonoclasticus* and *P. maritima* showed moderate amounts of oil degradation, with
324 36 % and 12 % of the chloroform-extractable oil fraction removed, respectively. Results from
325 culture experiments in which oil consumption was quantified by spectrophotometry confirmed
326 the abovementioned results from gravimetric analysis (see Figure S2).

327 Initial physiological characterization was conducted for five of our isolates (SSU rRNA
328 gene sequences most similar to those from *A. dieselolei*, *Acinetobacter* sp., *M.*
329 *hydrocarbonclasticus*, *P. maritima*, and *V. hepatarius*) using Phenotypic MicroArray^{TM} (PM)
330 analysis. PM testing included salt tolerance, a wide variety of carbon sources (alcohols, amines,

331 amino acids, carbohydrates, carboxylic acids, esters, fatty acids, and polymers) as well as
332 inorganic and organic forms of major nutrients (N, P, and S) (Table S2). Consistent with their
333 marine habitat, all strains metabolized in the presence of NaCl concentrations up to 10%. In
334 addition, Tween (polyoxyethylene sorbate) compounds, which contain long-chain alkyl moieties,
335 were metabolized by all strains tested. Utilization of Tween compounds is considered diagnostic
336 of the substrate specificity of marine bacteria toward the use of hydrocarbons as a source of
337 carbon and energy (76). However, contrasts were observed in phenotype between oil-degrading
338 strains that indicate niche specialization in carbon and major nutrient metabolism (see
339 supplementary material for details). *Vibrio*, *Acinetobacter*, and *Marinobacter* strains all utilized a
340 fairly broad range of carbon substrates. In contrast, the *Alcanivorax* strain utilized relatively few
341 carbon sources among those tested.

342 **Cultivation-independent analysis of bacterial community structure in beach sands.**
343 Bacterial community structure was initially assessed in DNA extracts of 178 samples from 3
344 field trips by community fingerprinting using the automated ribosomal intergenic spacer analysis
345 (ARISA) method (see supplementary material for details). Twenty six samples were chosen for
346 pyrotag sequencing of SSU rRNA amplicons to represent substantial shifts in bacterial
347 community structure. Pyrosequence data were analyzed using a non-metric multidimensional
348 scaling plot (NMDS) of an averaged Bray-Curtis distance matrix (Figure 4). Distances reveal a
349 pronounced, uniform response to oil contamination in the total and active bacterial communities.
350 The pattern of sample grouping in ordination space reveals community response to oil presence,
351 with the most heavily oiled samples (dark shading) distinct from clean samples (light shading).
352 The pristine or "clean" samples group together and are most distant to the oiled samples. RNA-

353 based pyrosequence libraries cluster with the corresponding DNA-based library, in most cases,
354 and are also spatially oriented with respect to oil presence.

355 Nearly all of the groups represented in our culture collection of oil-degrading bacteria
356 were detected in pyrosequence libraries from oiled beach sands (Table 5). *Alcanivorax spp.*, in
357 particular, were shown to comprise the most abundant OTU in the pyrosequence libraries. Thus,
358 the relative abundance of *Alcanivorax* OTU is overlaid on the NMDS plot, demonstrating a
359 strong co-variation with oil presence (Figure 4).

360 As a proxy for the metabolically active bacteria present in beach sands, RNA-based
361 pyrosequence libraries were generated and compared to DNA-based libraries for four samples
362 collected on July 2th and selected to represent a range in visual oil contamination (Figure 5).
363 RNA- and DNA-based libraries revealed similar patterns in community composition grouped at
364 the class level, especially in the oiled samples. Members of the *Gamma*- and
365 *Alphaproteobacteria* were shown to dominate beach sand communities, regardless of oil
366 presence (Figure 5, Table 5). *Alphaproteobacteria* as well as *Actinobacteria* show a
367 proportionately higher representation in the RNA-based libraries compared to the DNA-based in
368 all samples. In the most heavily oiled sample, this increase in relative abundance of
369 *Alphaproteobacteria* is due primarily to a single OTU in the family *Rhodobacteraceae* (Table 5).

370 **DISCUSSION**

371 The overall goal of our research is to determine the environmental and ecological
372 controls of hydrocarbon biodegradation under *in situ* conditions in coastal benthic environments
373 impacted by the Deepwater Horizon (DH) oil spill in the Gulf of Mexico. The oil-degradation
374 capacity of microbial populations in marine sediments is likely limited by stressors such as
375 anoxia or nutrient starvation as well as ecological interactions such as mutualistic production and

376 exchange of biosurfactants between bacterial populations (27). Knowledge of bacterial
377 community structure and the response of key microbial players in oil-contaminated environments
378 provide a first glance at metabolic potential and the physiological mechanisms that might drive
379 hydrocarbon degradation.

380 **Response of indigenous bacterial communities to oil contamination in marine sands.**

381 Microorganisms with the capacity to degrade hydrocarbons are among the best-studied
382 microbial groups in applied and environmental microbiology. Indeed, more than 200 bacterial,
383 algal, and fungal genera, encompassing over 500 species, have been recognized as capable of
384 hydrocarbon degradation (see reviews by Head et al. (27), Yakimov et al. (76)). Much progress
385 has been made to determine the response of specific bacterial taxa to oil contamination in marine
386 environments impacted by oil spills, see reviews (4, 5, 23, 27, 42, 50). However, our ability to
387 understand and predict the dynamics of *in situ* bacterial communities responding to
388 environmental stimuli such as the presence of oil contamination remains in its infancy (27, 60).
389 The majority of previous work on hydrocarbon-degrading bacterial communities in the marine
390 environment has been conducted under enrichment conditions in laboratory microcosms (12, 13,
391 45, 52, 57, 69, 75). Among the fewer studies that have been conducted in the field, most all have
392 focused on biostimulation, for instance the application of nutrients to enhance oil bioremediation
393 (46, 49, 64, 68). Thus, a paucity of information exists on the response of indigenous bacterial
394 communities to oil contamination under *in situ* or natural attenuation conditions (2, 36, 51). The
395 DH oil spill disaster in the Gulf of Mexico has inspired a number of studies that address this
396 knowledge gap, with most of them to date centered on bacterioplankton communities of the deep
397 ocean (26, 37, 70).

398 In our study, cultivation-based and molecular-based enumeration of bacterial
399 communities showed a distinct impact of oil on overall bacterial numbers and specifically on the
400 abundance of known oil-degraders in Gulf beach sands. Not surprisingly, bacterial abundance
401 increased as oil provides a major source of carbon and electrons in an otherwise nutrient-starved
402 marine environment. Based on an extensive molecular dataset from 3 field campaigns, we show
403 that bacteria in Pensacola Beach sands were on average 2 to 4 orders of magnitude more
404 abundant in the presence of oil contamination (Figures 2 and 3); and high cultivatable counts as
405 well as RNA-based analyses support the premise that the majority of bacteria in oiled sands are
406 active. These results are consistent with past field research on oiled beaches (2, 46, 49, 63). We
407 further show that the abundance of the known hydrocarbonoclastic group of *Alcanivorax* spp.
408 increased in response to oil contamination, and a comparison of bacterial ribosome abundance in
409 RNA extracts indicated that *Alcanivorax* abundance was proportionately greater within the active
410 bacterial community (data not shown). Few previous studies have applied quantitative molecular
411 approaches to determine the abundance of hydrocarbon-degraders *in situ*. In a field
412 bioremediation experiment, Singh et al. (68) reported an elevated abundance of *Alcanivorax* spp.
413 in beach plots amended with oil plus fertilizer but not in plots treated with oil alone.
414 Oil began to come ashore at Pensacola Beach in early June and we observed a bloom of
415 *Alcanivorax spp.* and overall bacteria by early July, approximately 4 weeks after the initial oiling
416 event. Maximum abundance was observed in our aerobic bacterial cultures after 1 to 2 weeks of
417 incubation. Thus, our results indicate that the native microbial communities in beach sands
418 respond fairly quickly to oil contamination, metabolizing oil to support growth at a rate which is
419 within a factor of 2 to 4 in comparison to pure cultures. Coupled with our observation that oiled
420 sands were depleted in low molecular weight aliphatic and aromatic hydrocarbons, the elevated

421 abundance of hydrocarbon-degraders provides an early indication that natural attenuation is a
422 viable strategy for the mitigation of oil contamination in Pensacola Beach sands. The proportion
423 of *Alcanivorax* spp. to the total community was lower at Pensacola Beach in comparison to past
424 studies which employed nutrient amendments to stimulate biodegradation. Conditions in
425 subtropical sands (temperature, oxygen supply, nutrients; see below) appear to favor a broader
426 diversity of hydrocarbon-degraders that might render biostimulation unnecessary.

427 The *in situ* metabolism of oil-degrading bacteria is likely to be limited by a number of
428 environmental parameters including: temperature, the availability of oxygen and major nutrients,
429 oil hydrocarbon content, and weathering or dispersal of the oil (59). Temperatures in this
430 subtropical environment remain optimal for microbial growth throughout the summer, and
431 oxygen profiling indicates that the Pensacola Beach sands remained aerobic throughout the study
432 period with oxygen concentrations at all sampled depth >90% of sediment surface oxygen
433 concentration. The rapid *in situ* growth we observed after 4 weeks indicates that microbial cells
434 had access to the oil. The majority of our beach sand samples were collected in the supratidal
435 zone and the interstitial pore space of these sands was not saturated with seawater. Therefore, we
436 conclude that the growth of oil-degrading communities was likely limited by dessication and/or
437 nutrient depletion in the Pensacola Beach sands we studied. Dessication would exacerbate
438 carbon and nutrient depletion by shutting off the supply of dissolved substrates from inundating
439 tidal waters.

440 In corroboration of previous work on marine sands in the Gulf of Mexico (30, 53), we
441 observed that Pensacola Beach sands contain highly diverse bacterial communities that are
442 predominated by members of the *Gammaproteobacteria* and *Alphaproteobacteria* (Figure 5;
443 Table 5). Bacterial diversity was higher in beach sands in comparison to the bacterioplankton

444 communities that we sampled (see supplementary information). Shannon indices place the
445 bacterial communities of beach sands (see Table S1) as more diverse than bacterioplankton but
446 not as diverse as the bacterial communities of marine muds or soils (54).

447 Concomitant with changes to bacterial abundance, a pronounced shift in bacterial
448 community structure was observed in Pensacola beach sands in response to DH oil
449 contamination (Figure 4). These results emphasize that the embedded oil exerted a strong
450 selective pressure on the sand bacterial community, in concurrence with past research (see
451 reviews by Berthi-Corti and Nachtkamp (5); Greer (23)). The consensus of studies conducted in
452 oil-contaminated marine environments points to a succession of bacterial populations associated
453 with the early stages of contamination reflecting the initial utilization of various highly-
454 degradable hydrocarbon compound classes (aliphatics, aromatics, polyaromatics; (27)). In our
455 study, shifts in community composition in beach sands in response to oil presence were
456 manifested at the strain to family level (Table 5), but little to no change was observed at the class
457 level (Figure 5). This study along with past studies show that the *Gammaproteobacteria*, and to a
458 lesser extent the *Alphaproteobacteria*, predominate the bacterial communities of marine
459 sediment ecosystems following exposure to oil hydrocarbons (see reviews by Head et al. (27);
460 Yakimov et al. (76); Berthe-Corti and Nachtkamp (5); Greer (23)). Among the
461 *Gammaproteobacteria* in the oiled Pensacola Beach sands, members of the *Alcanivorax* genus
462 were by far the most abundant. The *Alcanivorax* genus has been associated with the early stages
463 of hydrocarbon degradation (weeks to months post spill) and has been shown to utilize saturated
464 hydrocarbons such as straight chain and branched alkanes (50, 76). Other hydrocarbon-degrading
465 members of the *Gammaproteobacteria* that were detected in this study (including *Acinetobacter*,
466 *Marinobacter*, and *Pseudomonas*), although they also tend to be less abundant at the onset of

467 hydrocarbon degradation, are more metabolically versatile than *Alcanivorax* and have been
468 shown to degrade PAHs as well as alkanes.

469 Evidence from this study also points to other microbial groups as key players in oil
470 degradation. RNA-based pyrosequence libraries supported the DNA-based results as members of
471 the *Rhodobacteraceae* family of the *Alphaproteobacteria* were among the most abundant
472 phylotypes detected in the presence of oil. A shift toward the *Rhodobacteraceae* and Gram
473 positive groups may indicate that a succession has begun in Pensacola Beach oiled sands toward
474 microbial groups involved in the degradation of more recalcitrant oil hydrocarbons. In field
475 studies of weathered marine sediments which were depleted in alkanes, members of the
476 *Alphaproteobacteria* and Gram positives were the prevailing groups detected (2, 36), and
477 members of these groups represented in our culture collection (*Labrenzia*, *Bacillus*,
478 *Microbacterium*) were shown to degrade PAHs in pure culture (40, 67). OTUs that showed a
479 high sequence identity to *Sulfitobacter* were the most abundant members of the
480 *Rhodobacteraceae* that we could assign at the genus level in our pyrosequence libraries.
481 *Sulfitobacter* is a sulfite-oxidizing bacterium that has been isolated from a variety of marine
482 environments. Thus, the capabilities of the *Alphaproteobacteria* genera *Labrenzia* and
483 *Sulfitobacter* to degrade specific oil compounds should be further explored.

484 **Isolation, identification, and characterization of model hydrocarbon-degrading bacteria.**
485 Evidence from cultivation-based approaches corroborated results from our cultivation-
486 independent molecular-based analyses. We isolated organisms from several well known oil-
487 degrading bacterial genera (*Alcanivorax*, *Marinobacter*, *Pseudomonas*, *Acinetobacter*, *Bacillus,*
488 *Microbulbifer*) that were detected in abundance in oiled Pensacola Beach sands. All 24 of our
489 isolates were screened initially in minimal media with oil as the sole carbon and electron source,

490 thereby assessing their potential to degrade oil. The majority of strains from oiled Pensacola
491 Beach sands showed high SSU rRNA gene sequence identity to isolates previously cultured from
492 marine or saline habitats that were contaminated with oil hydrocarbons (14, 40, 47, 65, 71). All
493 of these organisms were demonstrated to degrade oil hydrocarbons in pure culture in this study
494 or by others (14, 40, 47, 65, 71, 76) or were detected in oil-contaminated marine environments
495 (27) (Figure 3; Table 5). Bacteria from the genus *Alcanivorax* and other members of the
496 *Gammaproteobacteria* isolated in this study have been demonstrated to degrade alkanes in pure
497 culture (76). Representatives from at least 6 of the species we isolated (*Acinetobacter* sp.,
498 *Bacillus* sp., *Labrenzia* sp., *Microbacterium* sp., *M. hydrocarbonoclasticus*, *P. pachastrellae*, and
499 *P. stutzeri*) have been linked to PAH degradation in pure culture (27, 40, 67). Although the
500 *Vibrio* species represented in this study have not yet been shown to degrade oil in pure culture,
501 some vibrios have been found to metabolize hydrocarbons including PAHs (24).
502 Our results are in general agreement with previous cultivation studies of these
503 hydrocarbonoclastic taxa. Amongst the OTUs that could be classified to the genus level,
504 *Alcanivorax* and *Marinobacter* groups showed the highest relative abundance amongst known
505 hydrocarbon-degraders in our pyrosequence libraries (Table 5) and were well represented
506 amongst our isolates (Table 4). *Marinobacter* is a metabolically versatile hydrocarbon-degrading
507 taxon, capable of utilizing a broad range of carbon substrates including aliphatics and PAHs (18,
508 25). In contrast, the 15 described species of the *Alcanivorax* group are thought to be highly
509 specialized (50), with *A. dieselolei* listed as an "obligate hydrocarbonoclastic bacterium
510 (OHCB)" that is capable of utilizing a very narrow range of carbon substrates (76). Our isolate
511 with 100% sequence identity (across 850 bases) with *A. dieselolei* (AB453732) did show the
512 narrowest range of carbon substrate utilization amongst the strains we tested (see results and

513 supplementary material). The results of this study and others indicate that the OHCB designation
514 may need to be reconsidered, however, and genome sequencing will be essential to verify the
515 absence of metabolic pathways. Two different phylotypes of *Alcanivorax* spp. were abundant in
516 our pyrosequence libraries and previous work has shown that the physiology of *Alcanivorax* is
517 likely to be strain-specific (27). As with other functional guilds of bacteria, the taxonomy of
518 hydrocarbon-degraders is rapidly evolving. Many species have not been validly described. Of
519 those that have been formally described, the phenotype of most strains has been largely
520 characterized using BIOLOG testing. BIOLOG tests are effective for range finding and for
521 testing many strains at once, as we have done here. However, these should be considered as
522 preliminary, and they require verification with traditional physiological screening in pure culture.
523 A further understanding of the ecophysiology of hydrocarbon-degraders will be crucial to
524 uncovering the *in situ* controls of oil degradation and to the development of improved mitigation
525 strategies for oil spills. Through the isolation of model organisms, physiological testing of
526 isolates, and genome sequencing, the activity, physiological potential and environmental
527 distribution of hydrocarbon-degraders can be confirmed and understood.
528 **Conclusions.** The quantitative increase in bacterial gene sequences, the increased relative
529 abundance of known oil-degrading taxa, and the isolation of oil-degrading pure cultures from
530 many of the same taxa confirmed the strong selective response of indigenous bacterial
531 communities in Pensacola Beach sands to the presence of oil from the DH oil spill. We
532 hypothesize that *Alcanivorax* spp. of the *Gammaproteobacteria* are effective microbial indicators
533 or sentinels of the early stages of oil hydrocarbon degradation in Gulf beach sands when more
534 reactive components such as n-alkanes abound, whereas members of the *Alphaproteobacteria*
535 (*Labrenzia*, *Rhodobacteraceae*) and Gram positive groups (*Bacillus*, *Microbacterium*) may be

536 used as sentinels for the later stages of degradation when more recalcitrant oil hydrocarbon
537 compounds such as PAHs predominate. In addition, we provide a number of pure cultures from
538 these groups that may be used as model organisms to investigate the physiological ecology of
539 hydrocarbon-degraders in benthic habitats of the Gulf of Mexico. Further study is needed to
540 delineate the specific role for each of these groups in hydrocarbon degradation on contaminated
541 beaches.

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793 **FIGURE LEGENDS**

794

795 Figure 1. Principal components analysis of bacterial SSU rRNA gene abundance and days since
796 oil arrival. Dark bubbles represent visibly oil contaminated samples, while light ones indicate
797 visibly clean samples. Bubble size is relative to the number of bacterial SSU rRNA gene copies
798 per gram of sand. Vectors represent variables used to generate Euclidean distance. SSU rRNA
799 abundance data was initially fourth root transformed to meet assumptions of normality.

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801 Figure 2. Linear regression analysis of the abundance of overall bacteria in comparison to the
802 abundance of *Alcanivorax* spp. for each sampling trip, with the abundance determined as SSU
803 rRNA gene copies per gram of sand.

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805 Figure 3. Phylogenetic comparison of SSU rRNA gene sequences from oil-degrading bacterial
806 isolates (★) and sequences retrieved from oiled Pensacola Beach sands (bolded). Only
807 *Gammaproteobacteria* lineages are included in the analysis. The most similar sequences
808 identified by BLAST are indicated by GenBank accession number. The bootstrapped neighbor-
809 joining phylogenetic tree was generated in MEGA using the maximum composite likelihood
810 model with Gamma-distributed rates and pairwise deletion (39). SSU rRNA sequences from oil-
811 degrading isolates, similar environmental OTU from the pyrosequencing dataset, and top isolated
812 BLAST hits were aligned using the GreenGenes NAST aligner (16). Nodes with >70%
813 bootstrapping support, out of 500 replications, are shown.

814

815 Figure 4. Multidimensional scaling plot of SSU rRNA pyrosequence libraries derived from oiled
816 Pensacola beach sands (black), clean Pensacola sands (gray), and pristine sands (gray) from St.
817 George Island. Samples above the black line were obtained from gDNA extracts, while samples
818 below the line were derived from total RNA extracts reverse transcribed using a bacterial SSU
819 rRNA gene primer. Lowercase letters (a – e) indicate pyrosequence libraries derived from the
820 same sand sample. Averaged Bray-Curtis distance is shown. Bubble size is relative to percent
821 abundance of *Alcanivorax*-like OTU as assigned by RDP classifier.
822
823 Figure 5. Phylum- and class- level phylogenetic analysis of RNA and DNA-based bacterial SSU
824 rRNA pyrosequence libraries for Pensacola Beach sand samples collected on July 2nd, 2010.
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Table 1. PCR primers used in this study.

Primer Set	Individual Primers	Sequence (5' → 3')	Primer Location	Annealing Temp. (°C)	Intended Target	Source
PCR, near-full length SSU rRNA, Bacteria	27F	AGA GTT TGA TCM TGG CTC AG	8 - 27[a]	55	Bacteria	Lane, 1991 (41)
	1492R	GGT TAC CTT GTT ACG ACT T	1510 - 1492[a]			
Pyrosequencing, SSU rRNA, Bacteria	Gray28F	GAG TTT GAT CNT GGC TCA G	9 - 27[a]	60	Bacteria	*e.g.* Ishak et al. 2010 (31)
	Gray519r	GTN TTA CNG CGG CKG CTG	536 - 519[a]			
Quantitative PCR, SSU rRNA, Bacteria	Primer 331F	TCC TAC GGG AGG CAG CAG T	340 - 358[a]	60	Bacteria	Nadkarni et al. 2002 (55)
	Primer 772R	GGA CTA CCA GGG TAT CTA ATC CTG TT	806 - 781[a]			
	Probe 515R	CGT ATT ACC GCG GCT GCT GGC AC	537 – 515[a]			
Quantitative PCR, SSU rRNA, *Alcanivorax*	Primer Alcvx-464F	GAG TAC TTG ACG TTA CCT ACA G	464 - 485[b]	60	*Alcanivorax*	This study
	Primer Alcvx-675R	ACC GGA AAT TCC ACC TC	675 - 659[b]			This study
	Probe 515R	CGT ATT ACC GCG GCT GCT GGC AC	528 - 506[b]			Nadkarni et al. 2002 (55)
ARISA, SSU rRNA, Bacteria	S-D-Bact-1522-b-S-20 [1522F]	TGC GGC TGG ATC CCC TCC TT	1522 - 1541[a]	50	Bacteria	Ranjard et al. 2000 (61)
	L-D-Bact-132-a-A-18 [LSU132R]	CCG GGT TTC CCC ATT CGG	132 - 115[c]			

[a]According to *rrS* gene of *Escherichia coli* S88 (NC_011742)
[b]According to *rrS* gene of *Alcanivorax dieselolei (*AY683537) [offset from *E. coli* numbering by 7 bases at the start of the rRNA gene]
[c]According to *rrL* gene of *Escherichia coli* S88 (NC_011742)

Table 2. Summary comparison of the chemical analysis of total petroleum hydrocarbons, qualitative determination of oil presence, and the molecular-based quantification of bacterial abundance in Pensacola Beach sands collected from 2 July to 2 September, 2010.

Sample ID	**Total Hydrocarbons mg kg^{-1}**	**Qualitative Oil Assessment**	**Total Bacterial SSU rRNA gene copies g^{-1}**	***Alcanivorax* SSU rRNA gene copies g^{-1}**	**% *Alcanivorax***
OS-71b	3600	3	4.44E+06	3.10E+06	69.9%
OS-67	3.8	0	2.42E+05	nd[a]	0.0%
OS-69	4.2	0	2.45E+06	1.69E+04	0.7%
OS-82	130	1	1.42E+08	2.77E+07	19.4%
OS-88a*	680	2	3.38E+07	4.04E+06	12.0%
OS-88b*		2	4.45E+07	6.23E+06	14.0%
OS-89a*	320	1	3.99E+07	3.82E+06	9.6%
OS-89b*		1	5.34E+07	7.05E+06	13.2%
OS-90a*	7.7	0	1.40E+07	6.30E+05	4.5%
OS-90b*		0	1.57E+07	8.90E+05	5.7%
OS-306	3.1	0	8.21E+05	1.20E+04	1.5%
OS-307	55	0	2.75E+05	3.06E+04	11.1%
OS-311	3600	3	1.02E+08	1.36E+07	13.3%
OS-312	480	1	1.84E+07	2.68E+06	14.5%

* Denotes replicate beach sand samples collected at the same depth and time. Only single replicates were analyzed for total petroleum hydrocarbons.
[a] nd = None detected.

Table 3. Detailed chemical analysis of hydrocarbon compounds from two oiled sand samples collected on September 1st,2010 from Pensacola Beach in comparison to source oil sampled on May 20th, 2010, from MC Block 252 after the Deepwater Horizon blowout.

Analyte	MODERATELY OILED	HEAVILY OILED	SOURCE OIL
Polycyclic aromatic hydrocarbons	[mg compound / kg sand]	[mg compound / kg sand]	[mg compound / kg oil]
1-Methylnaphthalene	-	-	1000
2-Methylnaphthalene	-	-	1600
Anthracene	0.029	0.024	-
Benzo[a]anthracene	0.037	-	30
Benzo[a]pyrene	0.052	-	23
Benzo[b]fluoranthene	0.075	0.11	26
Benzo[g,h,i]perylene	0.032	-	18
Benzo[k]fluoranthene	0.050	0.071	20
Chrysene	0.260	0.62	77
Dibenz(a,h)anthracene	0.040	-	18
Fluoranthene	0.042	-	25
Fluorene	0.014	0.011	140
Indeno[1,2,3-cd]pyrene	0.024	-	16
Naphthalene	-	-	910
Phenanthrene	0.027	0.022	320
Pyrene	0.047	-	32
Aliphatic hydrocarbons			
C5-C6 aliphatics	-	-	5370
C6-C8 aliphatics	35	24	62 400
C8-C10 aliphatics	13	10	57 800
C12-C16 aliphatics	9.5	14	72 500
C16-C35 aliphatics	370	1900	69 100
Aromatic hydrocarbons			
C5-C7 aromatics	-	-	2590
C7-C8 aromatics	-	-	7010
C8-C10 aromatics	73	81	20 200
C10-C12 aromatics	-	-	12 800
C12-C16 aromatics	31	6.8	25 100
C16-C21 aromatics	72	92	25 600
C21-C35 aromatics	280	550	64 200
Total Petroleum Hydrocarbons (C8-C40)	1900	4500	500 000
Ratio of Lighter (C6-C16) to Heavier (C16-C35) Aliphatics	0.16	0.025	2.79

Table 4. Genotypic and phenotypic characterization of oil-degrading bacteria isolated from beach sands impacted by the Deepwater Horizon oil spill. Results based on comparison of SSU rRNA gene sequences of the isolates to the sequence that shows the highest sequence identity to the isolate. All strains were isolated in a minimal artificial seawater medium with DH source oil as the sole carbon and electron source. Oil degradation activity was quantified for representative isolates using the gravimetric assay described in the methods section. Anoxic enrichment conditions indicate denitrifying enrichments.

Closest BLAST Match	**% Similarity**	**Genbank Accession #**	**Enrichment Condition**	**Quantitative Test for Oil Degradation**
Pseudomonas pachastrellae	100	EU603457	Anoxic	
Pseudidiomarina maritima	99.9	EU600203	Anoxic	Yes
Marinobacter hydrocarbonoclasticus	99.6	DQ768638	Anoxic	
Shewanella algae	99.2	GQ372877	Anoxic	
Vibrio alginolyticus	97.7	GQ455008	Anoxic	
Pseudomonas stutzeri	99.7	GU396288	Anoxic	Yes
Alcanivorax dieselolei	100	AB453732	Anoxic	
Vibrio hepatarius	98.6	EU834019	Anoxic	Yes
Marinobacter vinifirmus	99.2	FJ161339	Anoxic	
Marinobacter vinifirmus	99.1	FJ161339	Anoxic	
Vibrio sp.	99.9	HM640395	Oxic	
Acinetobacter sp.	100	FJ876296	Oxic	Yes
Pseudoalteromonas sp.	99.8	AY394863	Oxic	
Acinetobacter venetianus	99.8	DQ912805	Oxic	
Bacillus sp.	99.2	HQ588864	Anoxic	
Halomonas shengliensis	99.7	EF121853	Anoxic	
Vibrio hepatarius	99	HM584097	Anoxic	
Vibrio alginolyticus	99.3	GQ455008	Anoxic	
Marinobacter hydrocarbonoclasticus	100	DQ768638	Anoxic	Yes
Labrenzia sp.	99.3	EU440961	Oxic MPN	
Alcanivorax sp.	99.9	AB435642	Oxic MPN	
Microbulbifer sp.	98.7	GQ334398	Oxic MPN	
Microbacterium schleiferi	99.9	EU440992	Oxic MPN	
Marinobacter hydrocarbonoclasticus	99.4	DQ768638	Oxic MPN	

Table 5. Summary of taxa detected in the highest relative abundance in DNA-derived and RNA-derived pyrosequence libraries from beach sands in this study. Notable OTU abundances are bolded.

	OTU Classification	Control	DNA PB Oiled	RNA PB Oiled	DNA PB Clean	RNA PB Clean
Gammaproteobacteria		28.4%	41.5%	31.6%	32.9%	21.9%
Oceanospirillales		0.0%	19.2%	15.2%	3.1%	3.6%
	Alcanivorax Group I	0.0%	**8.9%**	**6.4%**	1.7%	2.6%
	Alcanivorax Group II	0.0%	**4.4%**	**5.1%**	0.6%	0.3%
	Oceanospirillaceae	0.0%	**4.3%**	**2.0%**	0.0%	0.1%
	Alteromonadaceae Group I	0.0%	0.5%	0.8%	0.1%	0.0%
	Alteromonadaceae Group II	0.0%	0.3%	0.1%	**4.1%**	**9.4%**
	Marinobacter	0.0%	**1.6%**	**2.9%**	0.1%	0.1%
Xanthomonadales		0.0%	2.9%	0.3%	0.5%	0.0%
	Sinobacteraceae	0.0%	2.6%	0.3%	0.2%	0.0%
Chromatiales		3.5%	4.5%	2.2%	9.8%	3.2%
	Ectothiorhodospiraceae Group I	0.8%	1.2%	0.7%	3.3%	1.2%
	Ectothiorhodospiraceae Group II	1.6%	0.7%	0.1%	5.2%	1.6%
	Ectothiorhodospiraceae Group III	0.0%	0.1%	0.0%	0.5%	0.1%
	Unclassified	0.0%	2.3%	1.4%	0.6%	0.0%
	Unclassified	1.1%	0.0%	0.0%	0.2%	0.2%
Pseudomonadales		0.3%	0.2%	4.7%	0.2%	0.0%
	Pseudomonas	0.2%	0.0%	**4.2%**	0.1%	0.0%
Alphaproteobacteria		6.7%	14.5%	30.0%	3.0%	12.1%
Rhodobacterales		2.9%	5.3%	25.6%	0.7%	7.4%
	Sulfitobacter	0.2%	0.4%	**2.9%**	0.1%	1.4%
	Hyphomonadaceae	0.0%	1.1%	0.1%	0.0%	0.0%
	Rhodobacteraceae Group I	0.0%	0.9%	1.0%	0.0%	0.0%
	Rhodobacteraceae Group II	0.0%	0.2%	1.3%	0.1%	0.3%
	Rhodobacteraceae Group III	0.0%	0.1%	**9.7%**	0.0%	0.0%
	Rhodobacteraceae	0.1%	0.0%	1.4%	0.0%	0.4%

	Group IV					
Bacteroidetes		10.4%	18.1%	2.3%	14.2%	3.5%
	Muricauda Group I	0.0%	**3.0%**	0.0%	0.0%	0.0%
	Muricauda Group II	0.0%	**2.4%**	0.0%	0.0%	0.0%
	Robiginitalea	0.1%	0.6%	0.0%	2.1%	0.3%
Cyanobacteria		2.5%	0.0%	0.2%	0.1%	4.6%
	Synechoccocus	1.7%	0.0%	0.0%	0.1%	0.4%
Firmicutes		0.1%	0.3%	2.3%	0.3%	0.1%
	Bacillus	0.0%	0.0%	1.3%	0.0%	0.0%

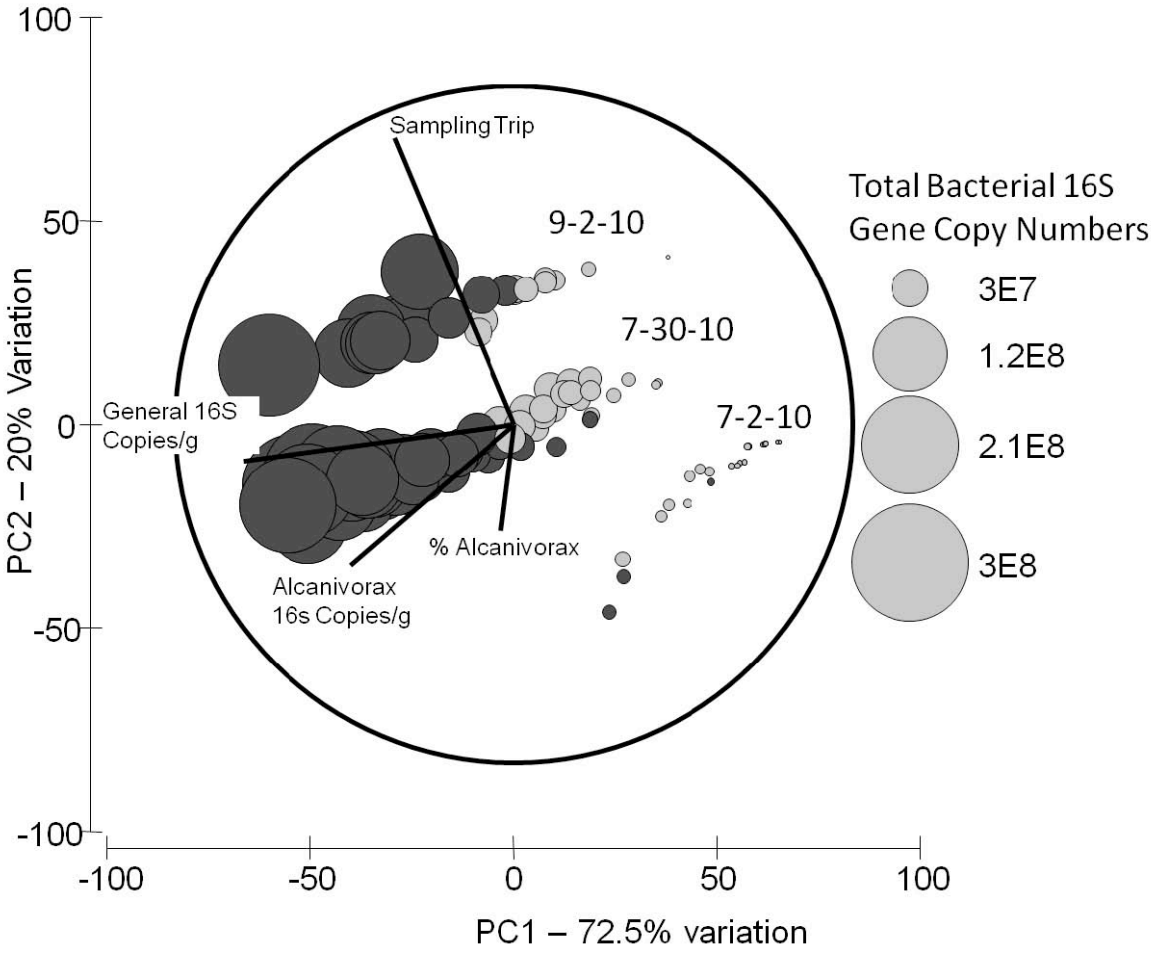

Figure 1. Principal components analysis of bacterial SSU rRNA gene copy numbers and days since oil arrival. Dark bubbles represent visibly oil contaminated samples, while light ones indicate visibly clean samples. Bubble size is relative to the number of bacterial SSU rRNA gene copies per gram of sand. Vectors represent variables used to generate Euclidean distance. SSU rRNA copy number data was initially fourth root transformed to meet assumptions of normality.

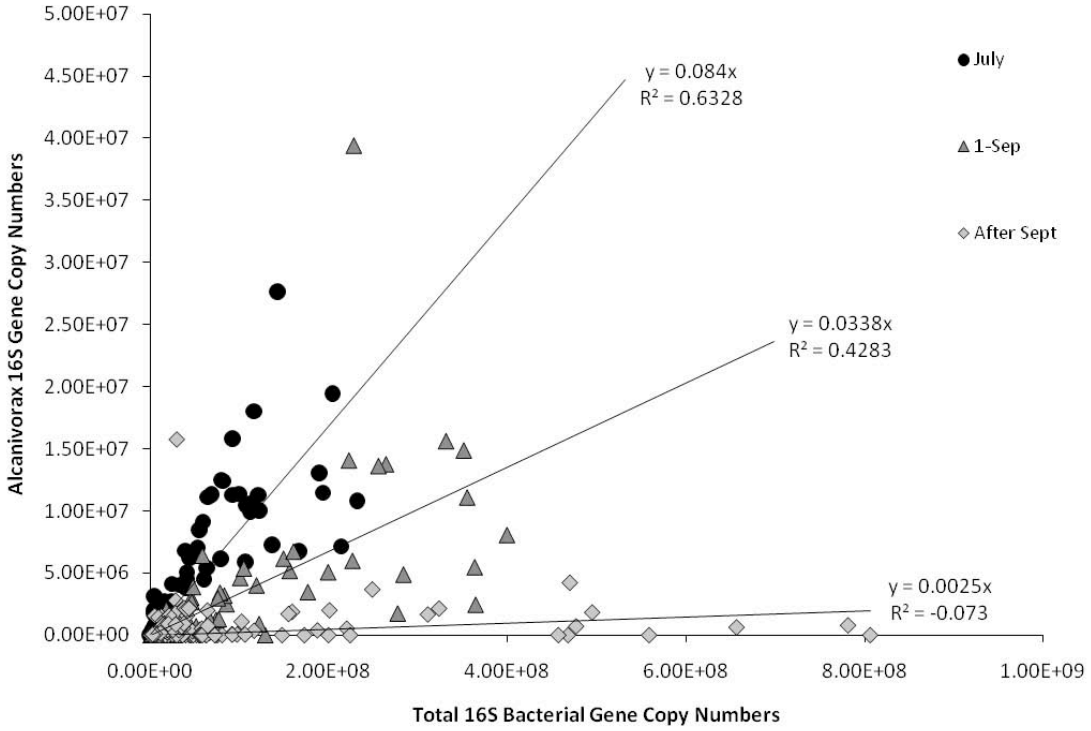

Figure 2. Linear regression analysis of the abundance of overall bacteria in comparison to the abundance of *Alcanivorax* spp. for each sampling trip, with the abundance determined as SSU rRNA gene copy numbers per gram of sand.

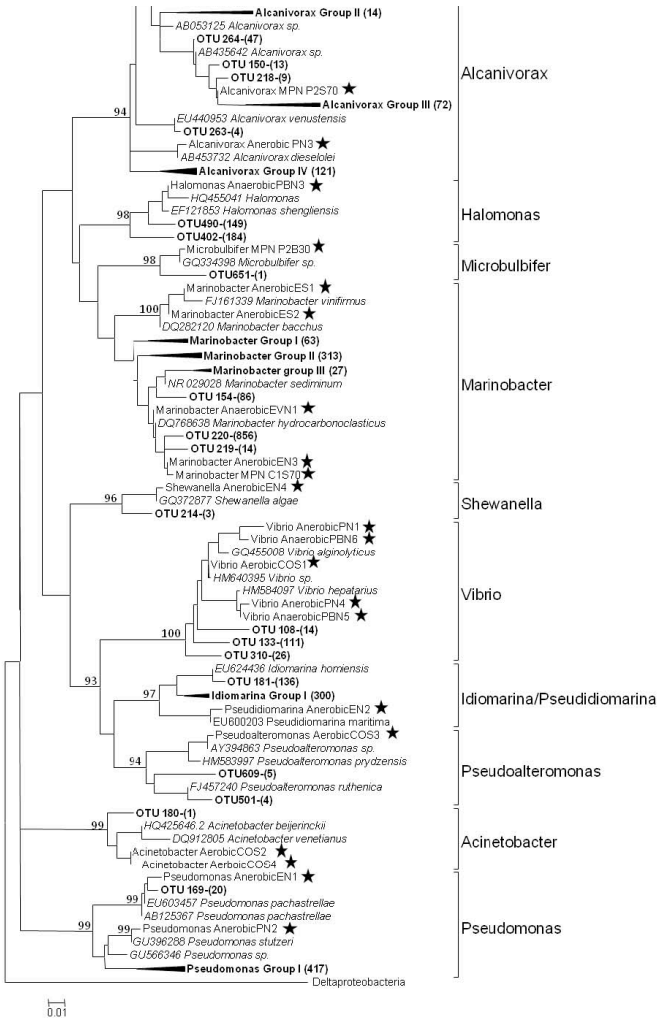

Figure 3. Phylogenetic comparison of SSU rRNA gene sequences from oil-degrading bacterial isolates (★) and sequences retrieved from oiled Pensacola Beach sands (bolded). Only *Gammaproteobacteria* lineages are included in the analysis. The most similar sequences identified by BLAST are indicated by GenBank accession number. The bootstrapped neighbor-joining phylogenetic tree was generated in MEGA using the maximum composite likelihood model with Gamma-distributed rates and pairwise deletion (39). SSU rRNA sequences from oil-degrading isolates, similar environmental OTU from the pyrosequencing dataset, and top isolated BLAST hits were aligned using the GreenGenes NAST aligner (16). Nodes with >70% bootstrapping support, out of 500 replications, are shown.

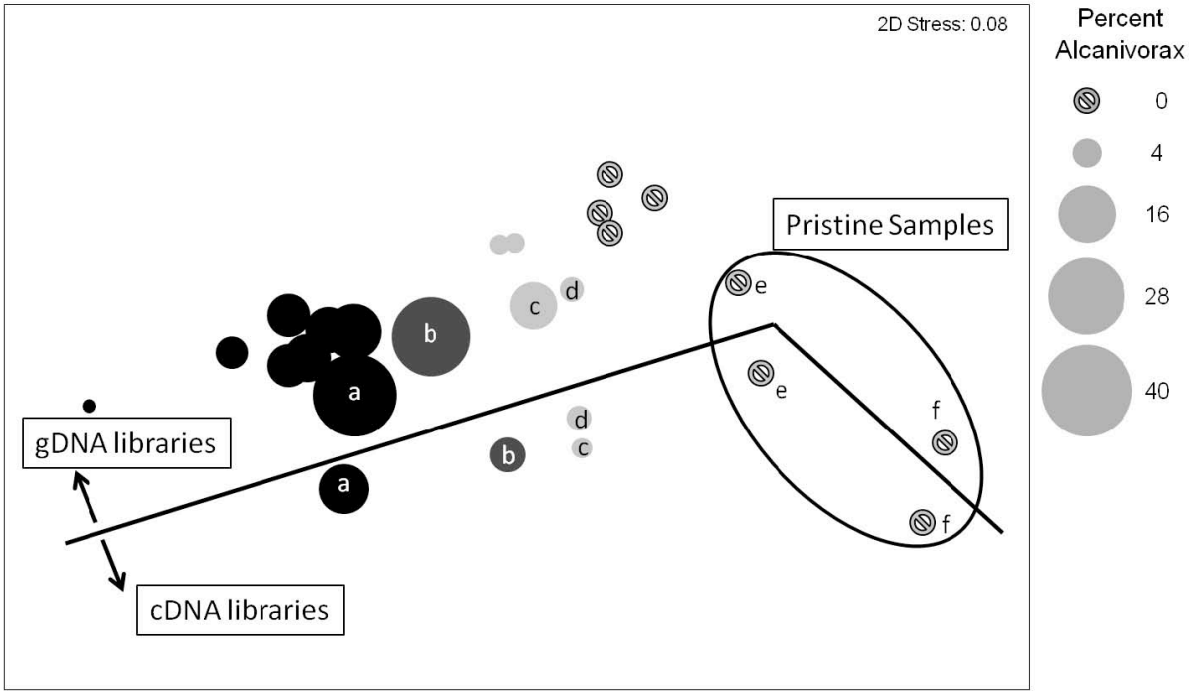

Figure 4. Multidimensional scaling plot of SSU rRNA pyrosequence libraries derived from oiled Pensacola beach sands (black), clean Pensacola sands (gray), and pristine sands (gray) from St. George Island. Samples above the black line were obtained from gDNA extracts, while samples below the line were derived from total RNA extracts reverse transcribed using a bacterial SSU rRNA gene primer. Lowercase letters (a – e) indicate pyrosequence libraries derived from the same sand sample. Averaged Bray-Curtis distance is shown. Bubble size is relative to percent abundance of *Alcanivorax*-like OTU as assigned by RDP classifier.

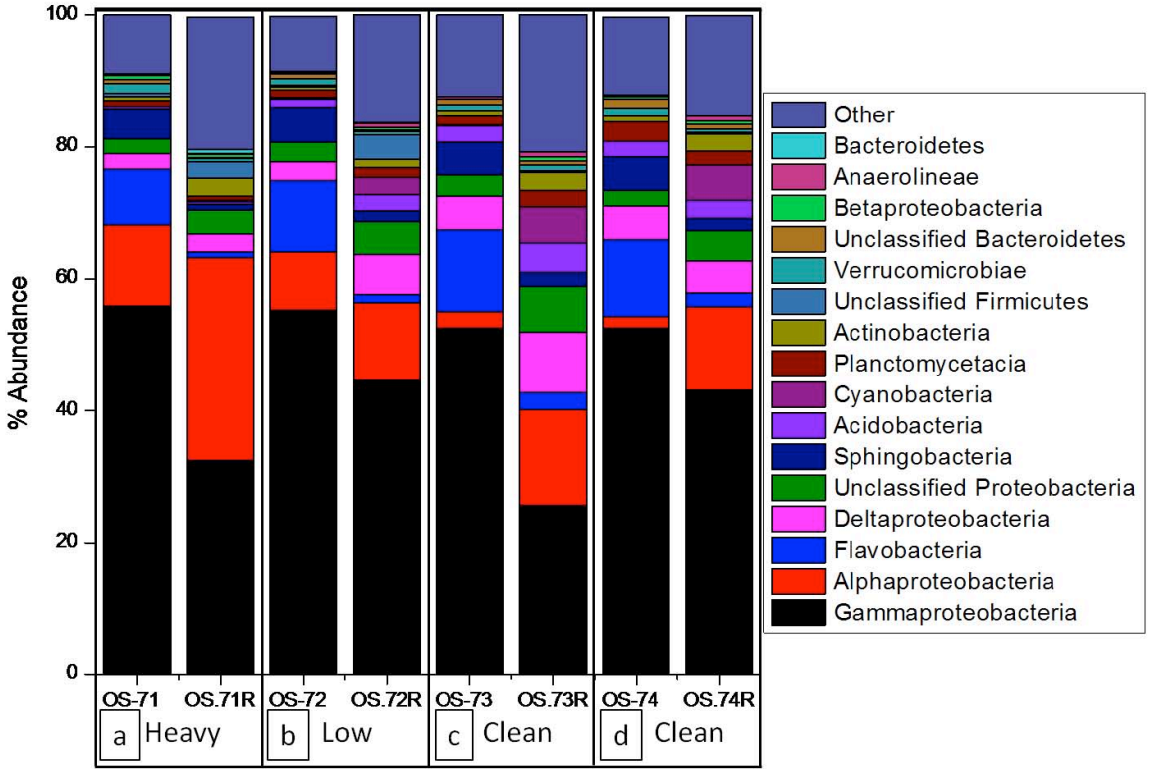

Figure 5. Phylum-and class-level phylogenetic analysis of RNA and DNA-based bacterial SSU rRNA pyrosequence libraries for Pensacola Beach sand samples collected on July 2nd, 2010.