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Bacterial Community Dynamics and Hydrocarbon Degradation during a Field-Scale Evaluation of Bioremediation on a Mudflat Beach Contaminated with Buried Oil

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A field-scale experiment with a complete randomized block design was performed to study the degradation of buried oil on a shoreline over a period of almost 1 year. The following four treatments were examined in three replicate blocks: two levels of fertilizer treatment of oil-treated plots, one receiving a weekly application of liquid fertilizer and the other treated with a slow-release fertilizer; and two controls, one not treated with oil and the other treated with oil but not with fertilizer. Oil degradation was monitored by measuring carbon dioxide evolution and by chemical analysis of the oil. Buried oil was degraded to a significantly greater extent in fertilized plots, but no differences in oil chemistry were observed between the two different fertilizer treatments, although carbon dioxide production was significantly higher in the oil-treated plots that were treated with slow-release fertilizer during the first 14 days of the experiment. Bacterial communities present in the beach sediments were profiled by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA gene fragments and 16S rRNA amplified by reverse transcriptase PCR. Similarities between the DGGE profiles were calculated, and similarity matrices were subjected to statistical analysis. These analyses showed that although significant hydrocarbon degradation occurred both in plots treated with oil alone and in the plots treated with oil and liquid fertilizer, the bacterial community structure in these plots was, in general, not significantly different from that in the control plots that were not treated with oil and did not change over time. In contrast, the bacterial community structure in the plots treated with oil and slow-release fertilizer changed rapidly, and there were significant differences over time, as well as between blocks and even within plots. The differences were probably related to the higher concentrations of nutrients measured in interstitial water from the plots treated with slow-release fertilizer. Bacteria with 16S rRNA sequences closely related (>99.7% identity) to *Alcanivorax borkumensis* and *Pseudomonas stutzeri* sequences dominated during the initial phase of oil degradation in the plots treated with slow-release fertilizer. Field data were compared to the results of previous laboratory microcosm experiments, which revealed significant differences.

The ability to degrade hydrocarbon components of crude oil is widespread among marine bacteria (10), and bioremediation has proven to be an effective method for cleaning up residual oil in a variety of coastal environments, such as rocky shorelines (6) and pebble (29) and coarse sand (33) beaches. Due to the high carbon content of oil and the low levels of other nutrients essential for microbial growth, treatment of beached oil with phosphorus and nitrogen is generally required to enhance the growth of hydrocarbon-degrading bacteria and to stimulate oil degradation (3, 6, 27).

Most oil spill bioremediation studies have focused on surface contamination of relatively coarse shoreline sediments (6, 28, 29, 33), and much less attention has been paid to fine sediments, such as those found in the upper reaches of mudflats around the coast of the United Kingdom. Oil deposited on these beaches is readily buried under clean sediment by tidal action and is therefore difficult to clean by conventional methods. Suitable nutrient amendment levels required for optimal hydrocarbon biodegradation are often determined by laboratory incubations. Laboratory microcosm studies performed with sediment collected from a beach used in the present field study demonstrated that a wide range of nutrient additions selected for different bacterial communities, but oil was degraded to similar extents irrespective of the structure of the predominant bacterial communities present (21).

However, laboratory incubations do not necessarily accurately reflect field conditions, and field trials must be conducted to corroborate findings of laboratory experiments (28). In experiments to assess hydrocarbon degradation on contaminated shorelines, careful attention must be paid to experimental design (33). Unknown and uncontrollable factors, like longshore currents, spatially distinct underground flows, and winds, may have different effects on different parts of the same experimental area. These effects can be accounted for by using a completely randomized block design, in which treatments are replicated and randomly assigned to replicate blocks. A completely randomized block design allows assignment of statisti-

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cally significant differences to treatments. In recent years such a design has been used to determine the effects of bioremediation treatments on oil degradation on polluted beaches (17, 29, 33). Microbial community structure has been studied in detail in only one experiment in which a completely randomized block design was used (17). However, in that study, samples from the early stages of the bioremediation were not analyzed, and the effects of treatment and block position on microbial community structure were not statistically assessed.

In order to fill gaps in our understanding of the potential of bioremediation of buried oil on mudflat beaches and the effects of treatment and plot allocation on bacterial community structure, we performed a field experiment with a completely randomized block design. Four different treatments were randomly assigned to three replicate blocks prepared on a fine-sediment beach, Stert Flats in the United Kingdom. The treatments consisted of two controls, one not treated with oil and the other treated with oil, and two bioremediated plots, one treated with oil and liquid fertilizer and the other treated with oil and slow-release fertilizer. Degradation of buried oil was monitored by measuring carbon dioxide production in the field and by analysis of oil composition. Bacterial community structure was determined by cultivation-independent analysis of PCR-amplified 16S rRNA gene fragments and 16S rRNA by denaturing gradient gel electrophoresis (DGGE) and analysis of 16S rRNA gene clone libraries (18). Numerical analysis of the DGGE profiles (21) was used to assess changes in bacterial community structure and to statistically evaluate the effect of bioremediation treatments and plot location on bacterial community structure.

MATERIALS AND METHODS

Field site. Stert Flats in Somerset, United Kingdom (51°12.3′N, 03°03.9′W), is a mudflat with fine sand (mud content, 3.2%; 80% of the particles with diameters ranging from 125 to 180 μm) deposited on the upper part of the intertidal zone. Details of the sediment properties have been described previously (21). The fine sand is highly mobile and moves between 7 and 13 cm during a single tide. The site has a history of oil contamination, including previous oil spill field experiments (30). The beach has a shallow gradient (on average, 4.2% in the sandy area) and is generally subjected to low wave energy. The amount of time that the experimental plots were covered by the tide depended on the height of the tide and the lunar cycle; however, observations made during the experiment showed that the minimum coverage time was approximately 1 h for each tidal cycle.

Experimental design. A completely randomized block design was used, with three blocks each containing four randomly assigned treatment plots receiving different treatments. The treatments and their designations were as follows: UC, control that was not treated with oil (no oil, no bioremediation treatment); OC, oil-treated control (treated with oil, no bioremediation treatment); SR, treated with oil and with slow-release fertilizer; and LF, treated with oil and with liquid inorganic fertilizer added regularly. The plots were marked out on an approximately 90-m stretch of the upper intertidal zone by using stainless steel poles on 28 June 1999. Nitex nylon mesh enclosures (pore size, 200 μm; length, 63 cm; width, 96 cm; depth, 7 cm) were filled with oil-treated (OC, LF, and SR plots) or untreated (UC plots) beach material and closed with a Nitex nylon mesh lid by using safety clips. Within each enclosure a smaller Nitex nylon bag (pore size, 200 μm; length, 30 cm; width, 30 cm; depth, 2.5 cm) was placed. This bag was used to measure CO_2 production. The enclosures were anchored to the steel poles and were buried in the beach at a depth of 10 cm 4 m apart to avoid cross contamination.

Emulsified, weathered Forties crude oil was applied at a rate of 70 g of oil per kg of beach sediment in metal trays, and the preparations were thoroughly mixed before they were placed in the mesh enclosures in the OC, LF, and SR plots. The oil was weathered by agitation with air at room temperature until a constant weight was obtained. This process removed 20% of the oil by volume. The oil was then emulsified with artificial seawater (Instant Ocean) by using a mechanical mixer (Silverson, Bucks, Chesham, United Kingdom) to form a 25% water–oil emulsion. The weathering and emulsification were used to simulate oil spilled at sea and washed ashore. The LF and SR plots were treated with fertilizer 1 week after oil application (6 July 1999); 4.17 g of $NaNO_3$ per kg of sediment and 0.30 g of KH_2PO_4 per kg of sediment were applied in solution to LF plots once a week for 15 weeks (the last application was on 15 October 1999). Fertilizer was added by adding nitrogen N and phosphorus P at levels that were 1 and 0.1% of the mass of oil, respectively. The SR plots received the same total amount of N as the LF plots received over a 15-week period, in the form of a single addition of 5.5 kg of Osmocote 14-14-14 (Scotts, Ipswich, United Kingdom), an organic resin-coated granular fertilizer containing 14% N, 14% P, and 14% K with controlled release over 4 months; 5.2 kg of Osmocote was placed in a mesh bag that was anchored into the beach sediment within each mesh enclosure containing oil-treated sediment. The smaller nylon enclosure used for carbon dioxide measurements contained a mesh bag with 0.3 kg of Osmocote.

Carbon dioxide measurements. During the first 2 weeks, microbial activity was determined on a daily basis by monitoring CO_2 evolution by an in situ respirometry method described previously (27). For measurement of CO_2 production the small mesh bags were removed from the plots and placed on a clean plastic bag on the beach surface nearby. A flux box was placed over the bag and was gently pushed into the beach surface around the bag to obtain a good seal. The air within the flux box was circulated through the cell of an infrared gas analyzer (Servomex, Crowborough, United Kingdom) in order to determine CO_2 production.

Nutrient and dissolved oxygen analysis. The beach sediments were highly water saturated, and a syringe was used to remove interstitial water that remained immediately following removal of sediment samples. Samples (10 ml) of pore water from within the plots were filtered with a 0.2-μm-pore-size filter, transferred into acid-washed tubes, and kept frozen at −20°C until analysis. Inorganic nitrogen and phosphorus concentrations in the seawater were determined by using a Technicon autoanalyzer system (16). A Horiba water quality analyzer equipped with an oxygen electrode was used to determine dissolved oxygen concentrations in the field. A fresh sediment sample was removed, and the probe of the water analyzer was used to measure dissolved oxygen in the pool of water that remained when the sediment sample was taken.

Oil chemistry. During every sampling event, three sampling locations per plot were randomly selected. Sediment samples (50 g) were excavated, transferred into steel containers, and kept frozen at −20°C for oil chemistry analysis. Hydrocarbons in oil-treated sediments (10 g), spiked with squalane and 1,1-binaphthyl standards to determine the extraction efficiency, were extracted, analyzed by gas chromatography with flame ionization detection and mass spectrometric detection, and quantified as described previously (27). On average, the recovery efficiency of the added standards was 83%. Replicate analyses showed that the variability of measured values was always less than 10%. To distinguish between physical removal of oil and biodegradation, the levels of total petroleum hydrocarbons, total gas chromatography-resolvable hydrocarbons, n-C_{11} to n-C_{35} alkanes, and polycyclic aromatic hydrocarbons were expressed relative to the amount of 17α(H),25β(H)-hopane, a degradation-resistant compound present in crude oil (6).

Statistical analysis of chemical data. Statistical analysis (parametric two-way analysis of variance) was performed by using Systat 7.0 (SPSS Inc.).

Nucleic acid extraction. During every sampling event, three samples (20 g) were randomly collected from each plot by using a grid and kept frozen at −20°C until extraction and analysis. After thawing and homogenization, DNA and RNA were extracted from 0.6-g samples, which were mixed with 0.6 ml of 0.12 M sodium phosphate buffer (pH 8.0), 80 μl of 10% sodium dodecyl sulfate, and 0.6 ml of phenol (pH 8.0) in Ribolyzer tubes (bacterial matrix; Hybaid, Ashford, United Kingdom). The tubes were agitated in a Hybaid Ribolyzer for 15 s at 5.5 m/s, incubated at 65°C for 30 min, and agitated again for 15 s at 5.5 m/s. After centrifugation, the supernatant was extracted once with 0.5 ml of phenol and once with 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1; pH 8.0). Nucleic acids were precipitated by adding 0.1 volume of 3 M sodum acetate (pH 5.5) and 0.6 volume of isopropanol to the supernatant. The mixture was incubated on ice for 1 h and centrifuged (10 min, 14,000 × g) to recover the precipitated nucleic acids. The pellet was washed with 70% ethanol at −20°C and, after air drying, was dissolved in 100 μl of 10 mM Tris-HCl–1 mM EDTA (pH 8.0). For rRNA isolation, 50 μl of the nucleic acid extract was cleaned with an RNeasy column (Promega, Madison, Wis.) used according to the manufacturer's instructions. Residual DNA was removed by DNase treatment (20 U of DNase I [Boehringer-Roche, Mannheim, Germany]) for 2 h at 37°C. After DNA digestion, the extract was again cleaned with an RNeasy column. PCR without the reverse transcriptase step was performed with the RNA extract, and extracts not giving rise to a PCR product were subsequently used for reverse transcriptase reactions. All

solutions were prepared with sterile diethyl pyrocarbonate-treated water. Bottles and plastic ware were baked for at least 4 h at 180°C.

DGGE analysis. RNA was converted to cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega) as recommended by the manufacturer and primer 3 (18), which corresponded to positions 534 to 517 in *Escherichia coli* 16S rRNA. PCR was performed in a 50-μl (total volume) mixture containing 0.2 μM primer 2 (18) (corresponding to positions 341 to 358 in *E. coli* 16S rRNA), 0.2 μM primer 3, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 U of BioTaq enzyme, buffer supplied with the enzyme (Bioline, London, United Kingdom), and 1 μl of cDNA template. Amplification was performed with a Hybaid Omnigene thermocycler as follows: 95°C for 3 min, followed by 30 cycles of 94°C for 0.5 min, 55°C for 1 min, and 72°C for 1 min and a final elongation consisting of 72°C for 10 min. DGGE was performed with the Bio-Rad DCode system. The PCR product was loaded on 1-mm-thick 8% (wt/vol) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels containing a linear 30 to 55% denaturant gradient; 100% denaturant was 7 M urea and 40% (vol/vol) formamide. The gels were electrophoresed in 1× TAE buffer (40 mM Tris-acetate, 1 mM Na-EDTA; pH 8.0) at 60°C and 200 V for 4 h. The gels were stained in 1× TAE buffer containing SYBR Green I (Sigma, St. Louis, Mo.) and were examined with UV transillumination by using a Fluor-S multiimager (Bio-Rad, Hercules, Calif.). Images were analyzed by using the Quantity One 4.1 software (Bio-Rad), and data were exported to Excel and used for numerical analysis with Systat 7.0 (SPSS Inc.). Similarities between tracks were calculated by using the band-based Dice coefficient (S_D) ($S_D = 2n_{AB}/[n_A + n_B]$, where n_{AB} is the number of bands present in both track A and track B and n_A and n_B are the numbers of bands in tracks A and B, respectively) and band-independent, whole-densitometric-curve-based Pearson product-moment correlation coefficients (r) (22). Statistically significant differences between sets of samples were determined as follows. First, the 95% limits of confidence for similarity values were determined from a set of similarity values for 13 replicate samples (replicate DNA extraction, PCR, and DGGE procedures for the same sample; the samples were obtained from a control plot that was not treated with oil and fertilizer in order to allow statistical discrimination of the bacterial community profiles from the treated plots relative to the untreated control). These similarity values were arcsine transformed to obtain normal distribution of the data (11). Next, the mean and standard deviation were calculated. To calculate the 95% lower limit of confidence ($\alpha = 0.05$) (4), the critical value at $\alpha = 0.05$ was multiplied by the standard deviation and subtracted from the mean; then this value was back-transformed to give the lower 95% confidence limit. As three samples per plot were analyzed, the within-plot variation was determined from three similarity values, and the between-plot variation was determined from nine similarity comparisons. Based on binomial distributions (4), with data from three similarity measurements no values below the confidence limit are acceptable, while for comparison of nine similarity values at most one value below the 95% lower limit of confidence is acceptable in order to conclude with 95% certainty that no significant differences in community structure are present between plots.

Cloning, sequencing, and phylogenetic analysis of 16S rRNA gene fragments. Almost full-length 16S rRNA gene fragments were amplified by using primers pA and pH′ (8). Except for the primers, the PCR conditions were the same as those described above. PCR products were cloned with an AdvanTAge kit (Clontech, Palo Alto, Calif.), and the 16S rRNA gene libraries were screened by amplified ribosomal DNA restriction analysis (ARDRA). *E. coli* clones were categorized into different ARDRA types based on the patterns obtained after simultaneous digestion with restriction enzymes RsaI and HaeIII. Representatives of ARDRA types that occurred more than once in a library were subjected to DGGE, and clones corresponding to dominant bands in the DGGE fingerprint of the same sample from which the clone library was constructed were completely sequenced (8). Sequence data were obtained from DGGE bands by excising the bands from the DGGE gels and reamplifying them with primers 2 and 3 (18), followed by another round of DGGE analysis and excision of the appropriate bands. 16S rRNA gene fragments from the DGGE analysis were sequenced by using primers pC and pD′ (8). If unambiguous sequences could not be obtained, the excised bands were cloned, and transformants were screened by using DGGE. Three clones with mobility in DGGE gels similar to that of the excised band were sequenced. Sequences were compared to sequences deposited in the GenBank DNA database by using the BLAST algorithm (1). Nearly full-length 16S rRNA sequences were aligned manually with representative 16S rRNA sequences from the Ribosomal Database Project. Only unambiguously aligned base positions were used in the analysis. Distance analysis with the Jukes-Cantor correction (13) and bootstrap resampling (100 times) were done with the TREECON package (32), and the distance matrix was used to construct a tree by neighbor joining (24). Parsimony analysis was done with DNAPARS from the PHYLIP package (9). Data analysis and manipulation were performed by using the Genetic Data Environment software running on a SPARC 10 workstation (Sun Microsystems).

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in the GenBank database under accession numbers AF548761 to AF548767.

RESULTS

Inorganic nutrient levels in experimental treatments. Measurements of nitrogen and phosphorus concentrations in interstitial water sampled daily between days 5 and 9 and on day 80 after fertilization showed that the slow-release fertilizer treatment led to nitrogen and phosphorus concentrations that were significantly higher (21.2 ± 30.8 mg of N/liter and 18.7 ± 27.9 mg P/liter; $P < 0.001$) than the concentrations in the plots with no fertilizer treatment or in the plots treated with liquid fertilizer (0.8 ± 0.7 mg of N/liter and 0.9 ± 0.8 mg of P/liter). The nitrogen concentrations in the plots treated with liquid fertilizer were higher than the concentrations in the unfertilized oil-treated plots only immediately after the weekly fertilization. No effect of block position on nutrient concentration was observed.

Effects of bioremediation on oil degradation. Oil degradation was assessed by measuring carbon dioxide evolution and determining changes in oil composition. Carbon dioxide evolution was monitored intensively during the first 14 days of the experiment. One day after fertilizer treatment, high rates of carbon dioxide production were observed for the plots treated with slow-release fertilizer, as well as for the liquid fertilizer-treated plot in block 3 (Fig. 1A). Large fluctuations in carbon dioxide evolution occurred over time (Fig. 1A); these appeared to be related to lunar cycle-induced differences in tidal height and plot-covering times (data not shown). The observed fluctuations resulted in large standard deviations for the average daily carbon dioxide evolution rates (Fig. 1B). None of the other liquid fertilizer-treated plots and none of the oil-treated and non-oil-treated control plots showed elevated carbon dioxide production. Two-way analysis of variance of data that were log transformed (in order to obtain normal distribution of data) revealed significant effects of both treatment and block ($P < 0.001$) on carbon dioxide production. CO_2 production was significantly greater in block 3 than in block 1 ($P < 0.001$) (Fig. 1B). Bioremediation treatment had positive effects on CO_2 production (Fig. 1); CO_2 production was greater with the slow-release fertilizer treatment than with the other three treatments ($P < 0.001$). Also, the CO_2 production in the plots treated with liquid fertilizer was greater than that in the oil-treated and non-oil-treated control plots ($P < 0.001$). However, the carbon dioxide production in the oil-treated control was not significantly greater than that in the non-oil-treated control ($P = 0.463$). Dissolved oxygen measurements showed that the sediment remained oxygenated (data not shown).

Oil composition was determined at zero time and 80 and 315 days after fertilization. Non-oil-treated plots were found to contain some oil, but the amounts were negligible compared to the amount applied to the oil-treated plots (<1% of the amount added to the oil-treated plots). Significant biodegradation ($P < 0.001$) of total petroleum hydrocarbons (Fig. 2), total resolvable hydrocarbons, n-C_{11} to n-C_{31} alkanes, and substituted and unsubstituted naphthalenes was observed for all

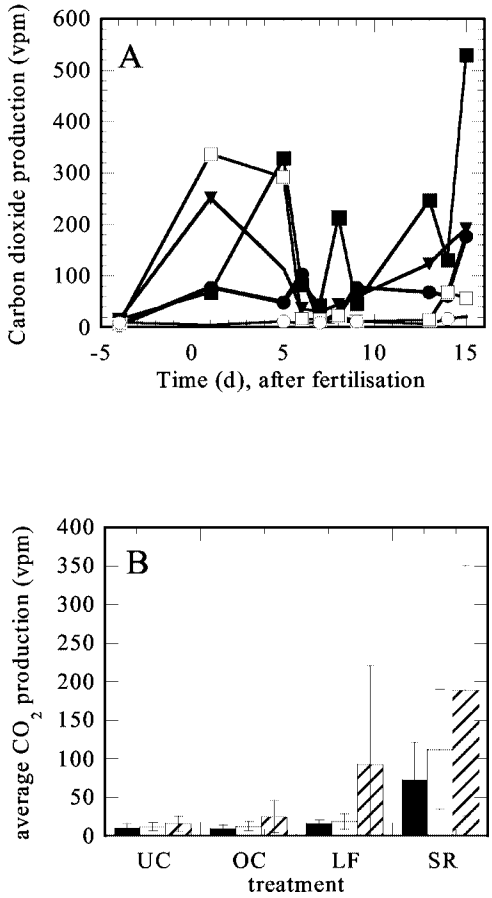

FIG. 1. Carbon dioxide production during the first 14 days of the field experiment at Stert Flats, United Kingdom. (A) Fluctuations in production in the plots in which the largest amounts of carbon dioxide were produced. Symbols: ●, SR plot in block 1; ▼, SR plot in block 2; ■, SR plot in block 3; □, LF plot in block 3; ○, UC plot in block 1, representing a treatment with low carbon dioxide production. Note that oil was added to the plots on day −7 and fertilizer was added at zero time. (B) Average carbon dioxide production in plots subjected to different treatments and located in different blocks. Solid bars, block 1; open bars, block 2; striped bars, block 3. The error bars indicate standard deviations (n = 10). Vpm, parts per million by volume.

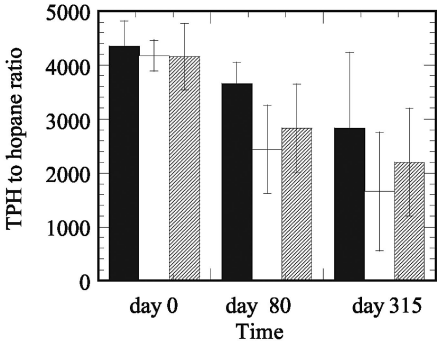

FIG. 2. Effects of bioremediation treatments on biodegradation of total petroleum hydrocarbons (TPH), expressed as the ratio of total petroleum hydrocarbons to hopane. Solid bars, oil-treated control; open bars, plots treated with liquid fertilizer; striped bars, plots treated with slow-release fertilizer. The error bars indicate standard deviations.

oil-treated plots. Substituted and unsubstituted phenanthrenes and benzothiophenes were not significantly degraded ($P > 0.05$) in any of the nine oil-treated plots for almost 1 year. No effect of block position on oil degradation was evident. Significant differences were observed between the different treatments after 80 days, but the differences were no longer significant after 315 days, when the plots had not received fertilizer treatment for 215 days (Fig. 2). After 80 days, the hydrocarbon degradation in the plots treated with liquid fertilizer was significantly greater than that in the oil-treated control plots ($P < 0.01$). Surprisingly, in view of the CO_2 evolution during the first 14 days (Fig. 1B), only a marginal difference in hydrocarbon degradation was observed between the slow-release fertilizer treatment and the oil-treated control ($P = 0.056$) (Fig. 2). This appeared to be related to the slow-release fertilizer plot in block 3, in which the degradation was not obviously greater than that in the oil-treated, unfertilized control. When this plot was not included in the statistical analysis, a significant difference between the plot that received the slow-release fertilizer and oil-treated control plots was observed.

Bacterial community structure in relation to treatment and block position. The plots were sampled in triplicate for bacterial community analyses on the day that the plots were set up and oil was added (day −7; 7 days before fertilizer addition), 5 and 2 days before first fertilizer addition (zero time), and 1, 5, 11, 80, 101, and 315 days after fertilizer treatment started. Bacterial community profiles were determined by DGGE analysis of 16S rRNA gene fragments amplified from nucleic acid extracts. The reproducibility of DNA extraction and PCR-DGGE was extensively tested with 13 pairwise comparisons of replicate samples from control plots that received no oil or fertilizer treatment; samples from different time points and blocks were examined. Similarities between replicate DGGE fingerprints were calculated by using the following two coefficients: S_D, which indicated similarities based on the absence or presence of bands; and r, which compared the whole-track densitometric curve information for the DGGE fingerprints and was independent of band assignment. These two coefficients were complementary and resulted in optimal separation between DGGE fingerprints (22). The levels of reproducibility were 0.983 ± 0.016 (S_D) and 0.929 ± 0.025 (r). The lower 95% limits of confidence correspond to similarity values of 0.933 and 0.872, respectively (see Materials and Methods for the method of calculation).

Matrices containing similarity coefficients for 16S rRNA gene-based DGGE profiles for a particular sampling event were statistically evaluated (see Materials and Methods) in order to determine significant variation within a plot, between plots that received the same treatment, or between different treatments on a particular day, depending on which samples were compared. The data from these statistical analyses are summarized in Table 1. The within-plot variation was in most cases not significant; the exceptions were plots treated with slow-release fertilizer and the liquid fertilizer-treated plot in block 3 on several sampling occasions. An example of significantly different DGGE profiles (r = 0.777 ± 0.012), corresponding to three samples taken independently from the same

TABLE 1. Statistical comparison of microbial community structure over time during a field experiment to examine the degradation of buried oil, in which several bioremediation treatments were used[a]

Time (days)[b]	Similarity coefficient	UC	OC	LF, block 1	LF, block 2	LF, block 3	SR, block 1	SR, block 2	SR, block 3
−5	Dice	a	ND[d]	ND	ND	ND	a	a	a
	Pearson	a	ND	ND	ND	ND	a	a	a
−3	Dice	a	ND	ND	ND	ND	a	a	a
	Pearson	a	ND	ND	ND	ND	a	a	a
1	Dice	a	a	a	a	b[e]	c	d	d[e]
	Pearson	a	a	a[e]	a	b[e]	c	bc[e]	d[e]
5	Dice	a	a	a	a	b[e]	c	d[e]	e
	Pearson	a	a	b	b	c/d/e[f]	f[e]	g	f
11	Dice	a	a	a	a	a[e]	a	b	a
	Pearson	a	a	a	a	a	a[e]	b	a
80	Dice	a	a	a	a	a	b/c/d[f]	e[e]	f/g/h[f]
	Pearson	a	a	a	a	a	b	b[e]	b/c/d[f]
315[c]	Dice	a	a	a	a	a	a	b	c
	Pearson	a	a	a	a	a	a	b	c

[a] Similarities between DGGE tracks were calculated by using either band presence-absence data (Dice coefficient) or whole-track densitometric information (Pearson coefficient), followed by statistical analysis of the similarity matrices. Different characters in a row indicate significant differences in community structure ($P < 0.05$) between treatments and/or blocks on the day of sampling. Significant between-block differences were never observed for the OC and UC plots, and data from all blocks are grouped into a single column for these treatments.

[b] Time after fertilizer treatment.

[c] Only a single sample was analyzed for each plot.

[d] ND, not determined.

[e] There was significant within-plot variation; one sample was significantly different from the other two. The different sample was not used when we compared different treatments or plots with a similar treatment in another block.

[f] There was significant within-plot variation, and all three samples were significantly different from each other. All samples were separately compared to other plots, and the results are separated by slashes.

plot, is shown in lanes 1a to 1c of Fig. 3A, while lanes 2a to 2c contained samples from another plot that did not show significant differences ($r = 0.959 \pm 0.023$).

Significant differences in community structure were never detected between non-oil-treated and oil-treated control plots (Table 1), and on most occasions (88% of the cases [$n = 15$] based on S_D and 73% of the cases based on r) differences were not apparent between oil-treated control plots and liquid fertilizer-treated plots (Table 1). Principal-component analysis also did not result in separation of these samples into different groups (data not shown). Only for samples taken 5 days after fertilizer treatment was a small but significant difference observed in the bacterial community profiles between oil-treated control plots and the liquid fertilizer-treated oil-treated plots in blocks 1 and 2 based on a comparison of whole densitometric curve data ($r = 0.834 \pm 0.072$). However, when these samples were analyzed based solely on the presence or absence of bands, no significant differences were observed ($S_D = 0.966 \pm 0.029$). Also on day 5, very clear differences in community structure were observed between the liquid fertilizer-treated replicates and the control plots in block 3 ($r = 0.400 \pm 0.126$). This is clear from a comparison of lanes 1a to 1c (samples from liquid fertilizer-treated plot in block 3) with lanes 2a to 2c (samples from oil-treated control plot in block 3) in Fig. 3A. The bacterial community structure in this plot was also significantly different from that in the control plot 1 day after fertilization ($r = 0.654 \pm 0.111$). It is interesting that only the liquid fertilizer-treated plot in block 3 showed obvious differences in bacterial community structure compared to the untreated and oil-treated controls (Table 1). This is consistent with the observation that liquid fertilizer treatment stimulated carbon dioxide production only in block 3 (Fig. 1).

In contrast to treatment with oil alone and treatment with oil plus liquid fertilizer, rapid changes in community structure were observed following treatment with slow-release fertilizer, and the changes were long lasting (Table 1). For the plots treated with slow-release fertilizer it was only in samples from block 1 that the bacterial community structure was similar to that in non-oil-treated plots by day 315. Remarkably, 11 days after treatment with slow-release fertilizer the community structure in blocks 1 and 3 was temporarily not significantly different from that in the non-oil-treated and oil-treated control plots. In general there were significant (Table 1) and large differences in the bacterial community structure among the three blocks, as shown clearly in Fig. 3B.

Comparison of 16S rRNA- and 16S ribosomal DNA-based DGGE. Oil addition alone and treatment with liquid fertilizer appeared to have little effect on the bacterial community structure. We might have expected inhibition of the activity and growth of some community members by oil and stimulation of the activity and growth of other community members. These effects could have been obscured by the DGGE approach in which rRNA gene fragments were amplified, since rRNA gene-based analysis also detects inactive microbes. Reverse transcription of rRNA, followed by PCR and DGGE, was expected to give a more representative view of the active bacterial community. rRNA was isolated from 12 samples taken from the four different treatment plots in block 3, 1, 5 and 11 days after the addition of fertilizer. Samples from this block were used as the highest activities (highest carbon dioxide production rates following addition of fertilizer) (Fig. 1) were observed in this block. The rRNA-based PCR products were analyzed by DGGE next to rRNA gene-derived fragments. The rRNA-based DGGE profiles always resembled the corresponding rRNA gene-derived profiles from the same treatment to some extent (Fig. 4). Although the rRNA- and rRNA gene-derived profiles were similar, they could be distinguished statistically ($S_D = 0.827 \pm 0.063$; $r = 0.823 \pm 0.094$). Nevertheless, a between-treatment comparison of rRNA-based DGGE profiles showed that the degree of similarity was not significantly different from that for a comparison of rRNA gene-derived profiles ($P > 0.05$). This was the case even when samples from plots showing the highest metabolic activity based on carbon dioxide production were analyzed (data not shown). Thus, it was not possible to detect any treatment-associated differences in the bacterial community profiles by 16S rRNA-based analyses that could not be determined from the results of 16S rRNA gene-based DGGE analyses.

Temporal dynamics and phylogenetic analysis of bacterial communities. Time series were analyzed for block 2 in order to detect dynamics in bacterial communities in response to oil pollution and bioremediation treatments. Community fingerprints of the non-oil-treated plots, the oil-treated plots that received no fertilizer, and the oil-treated plots treated with liquid fertilizer showed that there were no major changes over time (Fig. 5A and 6). In contrast, clear changes over time were

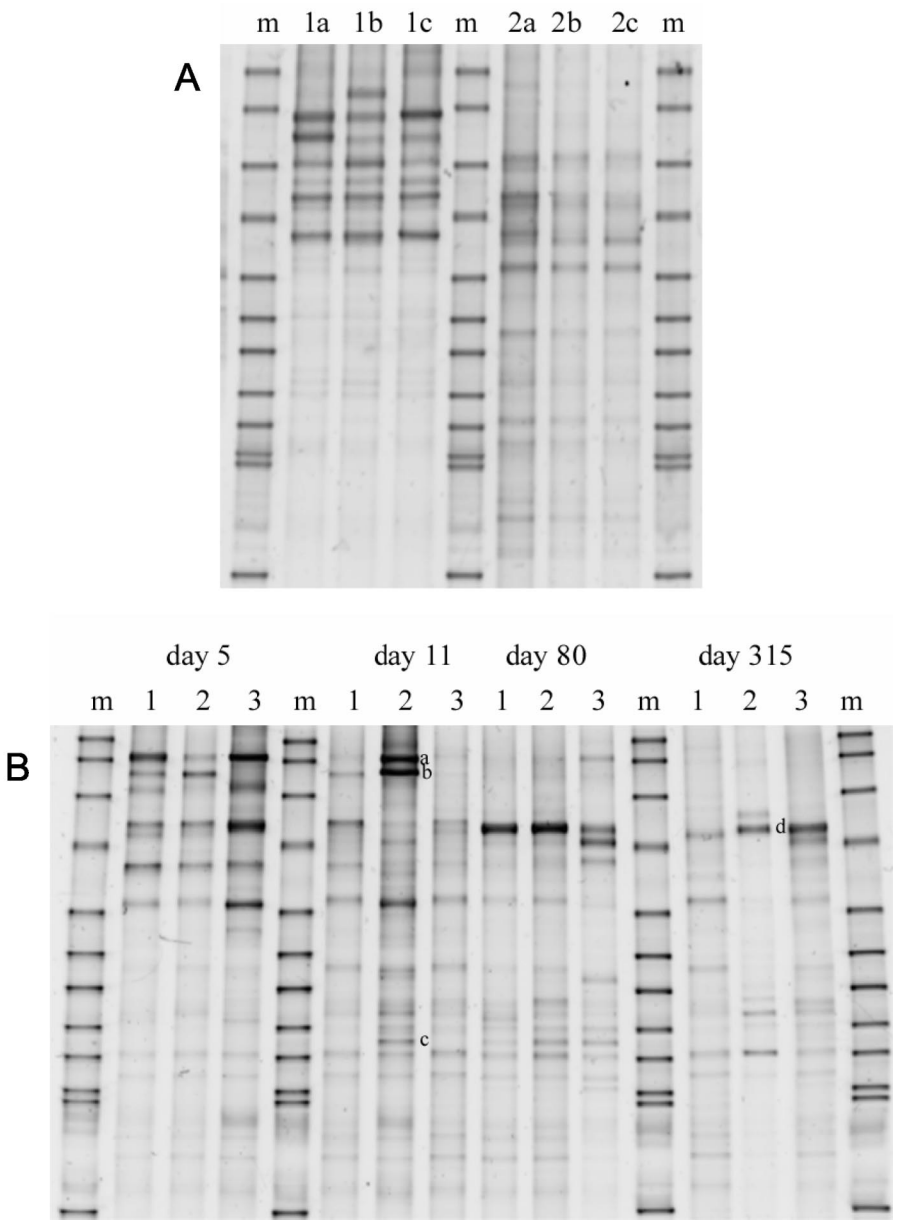

FIG. 3. Within-plot and between-block heterogeneity of microbial communities as revealed by rRNA gene-based DGGE profiling. (A) Within-plot heterogeneity of the microbial communities of the plot treated with liquid fertilizer in block 3 on day 5 after fertilization (lanes 1a to 1c). Lanes 2a to 2c show the results for a plot without obvious heterogeneity (the unfertilized, oil-treated plot in block 3 on day 5). (B) Between-block heterogeneity of the microbial communities in the oil-treated plots treated with slow-release fertilizer. The numbers above the lanes indicate the blocks, and the days indicate the numbers of days after fertilizer treatment. The letters indicate DGGE bands for which sequence data were obtained (see text). Lanes m contained markers.

observed for the bacterial community in the oil-treated plots that were treated with slow-release fertilizer (Fig. 3B and 5B). Nondimensional metric scaling (Fig. 6) revealed a nonsystematic pattern of changes over time, and no clear trend was observed. This may in part have reflected the length of time between sampling events since laboratory microcosm experiments with sediments obtained from the beach studied here and sampled more intensively showed clear successional changes in the bacterial communities (21); also, the observed spatial heterogeneity in the blocks and plots treated with slow-release fertilizer may have contributed to the findings (Table 1).

A phylogenetic survey was conducted for the plots treated with slow-release fertilizer. A clone library was constructed from a DNA extract from a block 2 sample taken 11 days after the addition of slow-release fertilizer. This sample was chosen as in the corresponding DGGE track two intense bands were apparent (bands a and b in Fig. 5B). Corresponding bands were also observed in samples taken at other times and/or in other plots treated with slow-release fertilizer (Fig. 3B and 5B)

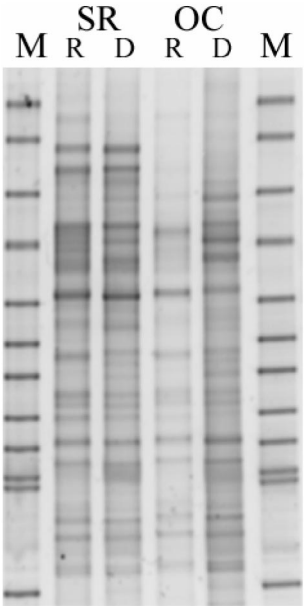

FIG. 4. Example of rRNA- and rRNA gene-based DGGE analysis of samples from the Stert Flats field trial. Samples were obtained from block 3, 11 days after fertilization. Lanes R, rRNA based; lanes D, rRNA gene based; lanes M, marker.

and, at much lower intensities, in oil-treated plots treated with liquid fertilizer and plots treated with oil only (Fig. 3A and data not shown). The clones (n = 40) were screened by ARDRA, which revealed that five restriction patterns occurred more than once. DGGE analysis of representatives of these five ARDRA types showed that two of these representatives comigrated with bands a and b in Fig. 5B. These ARDRA types represented 12.5 and 37.5% of the clones in the library, respectively. Sequencing of the cloned, nearly complete 16S rRNA genes indicated that the ARDRA type that comigrated with band a was very similar to *Pseudomonas stutzeri* 16S rRNA (99.7%), while the ARDRA type that comigrated with band b was almost identical to *Alcanivorax borkumensis* 16S rRNA (99.9%) (Fig. 7). Sequencing of bands a and b excised from a DGGE gel yielded high-quality sequences containing 160 nucleotides, which were identical to the corresponding sequences of the full-length 16S rRNA gene clones with similar DGGE migration characteristics. This provided stronger evidence that there was selection for bacteria related to *P. stutzeri* and *A. borkumensis* in the plots that showed the most effective hydrocarbon degradation. In the day 11 DGGE profile for the plot in block 2 treated with slow-release fertilizer a third band (band c in Fig. 3B and 5B) stood out as it was not observed in DGGE profiles for the other treatments and was still detectable 101 days after treatment with the slow-release fertilizer. DGGE screening of clones from the 16S rRNA gene clone library did not reveal a match with this band. Therefore, band c was excised from the DGGE gel and sequenced. The sequence recovered exhibited 96% identity with the 16S rRNA from the gamma-proteobacterium *Idiomarina loihiensis* (accession no. AF288370). After 80 days, a dominant band was observed in most of the DGGE profiles from the plots treated with slow-release fertilizer (band d in Fig. 3B and 5B); this

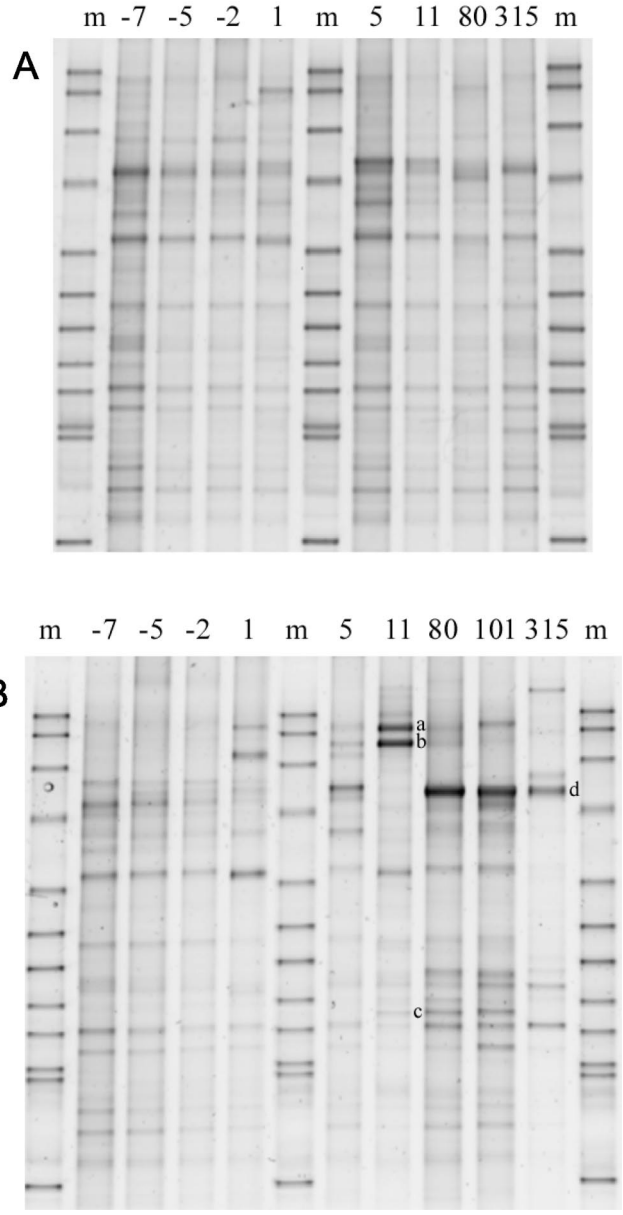

FIG. 5. Changes over time in rRNA gene-based DGGE profiles for plots treated with oil but not with fertilizer (A) and plots treated with slow-release fertilizer (B) in block 2 of the field experiment at Stert Flats. The numbers above the lanes indicate the numbers of days after fertilizer was added to the treated plots. Oil was added on day −7, and fertilizer was added at zero time. The letters indicate DGGE bands for which sequence data were obtained (see text). Lanes m contained markers.

band was detectable until the end of the experiment. The sequence of this 16S rRNA gene fragment exhibited 90.2% identity with the sequences of the gamma-proteobacteria *Microbulber hydrolyticus* (accession no. U58338) and *Serratia plymythica* (AJ233433).

DISCUSSION

In this study, by using a randomized block design, we demonstrated that buried oil was degraded significantly in the field

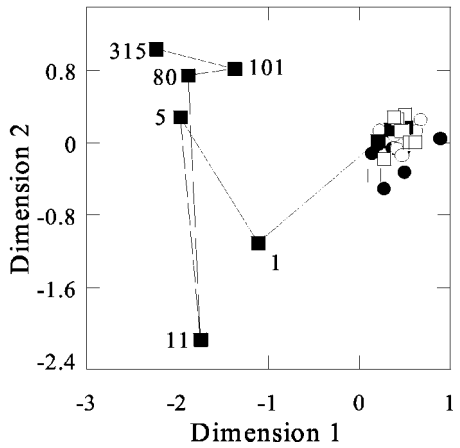

FIG. 6. Nonmetric multidimensional scaling map showing the changes in bacterial community structure during the field experiment at Stert Flats for the four different treatments. Symbols: ○, non-oil-treated control plot; ●, oil-treated plot; □, oil-treated plot treated with liquid fertilizer; ■, oil-treated plot treated with slow-release fertilizer. In order to avoid problems with interpretation of the map, the data points are connected with a line only for the plot treated with slow-release fertilizer; the numbers next to the data points indicate the time elapsed (in days) since the plots were fertilized. The numbers of days for the three data points obtained prior to fertilizer are not indicated, since they are close to the data points for the three other plots.

during the first 3 months of the experiment when plots were fertilized with nutrients. When fertilizer treatment was stopped, the extent of oil degradation (percent removed) in unfertilized plots equaled the extent of degradation in the fertilizer-treated plots. However, the time that it took to achieve the same level of hydrocarbon degradation was longer in plots that did not receive fertilizer treatment. The type of treatment did not influence the oil components that were degraded. Although during the first 2 weeks significant differences were observed between blocks (as determined from carbon dioxide production), differences were not evident from oil chemistry measurements after 3 months. Interestingly however, the absolute amount of oil was smaller with both fertilizer treatments (25). The results of this study of bioremediation of buried oil are consistent with the results of two previous experiments in which a randomized block design was used, which showed that bioremediation treatment significantly enhanced oil degradation on surface-contaminated shorelines (29, 33). To the best of our knowledge, our study uniquely integrated a robust randomized block design with a comprehensive statistical analysis of bacterial community dynamics. In addition, higher-resolution molecular analysis demonstrated the importance of and rapid proliferation of the oil-degrading organism *Alcanivorax* in oil spill bioremediation under field conditions, suggesting that *Alcanivorax* spp. are key for dissipation of hydrocarbon pollution on maritime beaches.

Previous laboratory experiments performed with beach sediment from the site of the field experiment reported here (Stert Flats, United Kingdom) had shown that addition of nutrients strongly stimulated oil degradation in this beach sediment (21). However, a comparison of the characteristics of oil degradation revealed differences between the laboratory and field experiments. Degradation in nutrient-amended laboratory experiments was faster, and *n*-alkanes were depleted within 1 month. Also, the extent of oil degradation was greater in the laboratory experiments. In laboratory microcosms, phenanthrenes and dibenzothiophenes were degraded, but this was not observed in the field experiment. Large changes in community structure occurred in the laboratory experiments in which the preparations were amended with liquid fertilizer, while addition of liquid fertilizer had only some short-term effects on community structure in the field. The differences probably are related to differences in the experimental conditions in the laboratory and field studies. In the laboratory experiment a temperature of 20°C was maintained. Although tidal cycles were simulated in the laboratory experiment, the amount of water added to the microcosm per cycle (1 liter per microcosm containing 1.3 kg of sediment) was relatively small compared to the field situation, and this was probably the cause of the relatively high residual concentrations of nutrients that were maintained in the microcosms, despite the use of water-soluble fertilizer. In the field experiment the nitrogen concentrations in the plots treated with liquid fertilizer were in general not significantly different from those in the unfertilized, oil-treated plots, and this may well explain the minor differences in bacterial communities noted in the majority of these plots. Nutrient levels are an important factor in structuring microbial communities, as discussed below. The obvious differences between the field and laboratory experiments indicate that great care should be taken when results of laboratory experiments are extrapolated to field situations, and they underline the need to support results obtained in the laboratory with field experiments (27).

Nevertheless, some similarities between field and laboratory experiments were observed. With the slow-release fertilizer treatment significantly higher nutrient concentrations were maintained in the pore water, similar to the high nutrient concentrations maintained in the laboratory experiments with liquid fertilizer (21). In both cases, a sustained change in bacterial community structure occurred. Furthermore, in both field and laboratory experiments selection for *Alcanivorax*-like bacteria was noted. *A. borkumensis* is capable of using only a few organic substrates, especially alkanes (36) and the alkyl groups of *n*-alkylbenzenes and *n*-alkylcycloalkanes (7). Alkanes are among the most easily degradable oil components (2). Our observation is the second observation that these bacteria are important components of an oil-degrading community in the field, indicating their significance in hydrocarbon degradation. Previously, the presence of *Alcanivorax* was noted in beach oil paste and seawater after an oil spill in the Japan Sea (14). Clear selection for *P. stutzeri* was also observed. *P. stutzeri* is capable of aerobic degradation of many pollutants (5, 12, 15, 23, 34), and a strain closely related to *P. stutzeri* (99.6% 16S rRNA sequence identity to clone SR11d28) has been reported to degrade naphthalene under denitrifying conditions (20). Interestingly, 16S rRNA gene sequences related to *P. stutzeri* were not detected in laboratory experiments in which sediments from the same beach used in this field study were used (515 clones were screened) (21).

Significantly greater hydrocarbon degradation during the first 80 days of the experiment occurred in the oil-treated plots treated with fertilizer than in the oil-treated plots that received no fertilizer. However, the oil-treated plots that received no

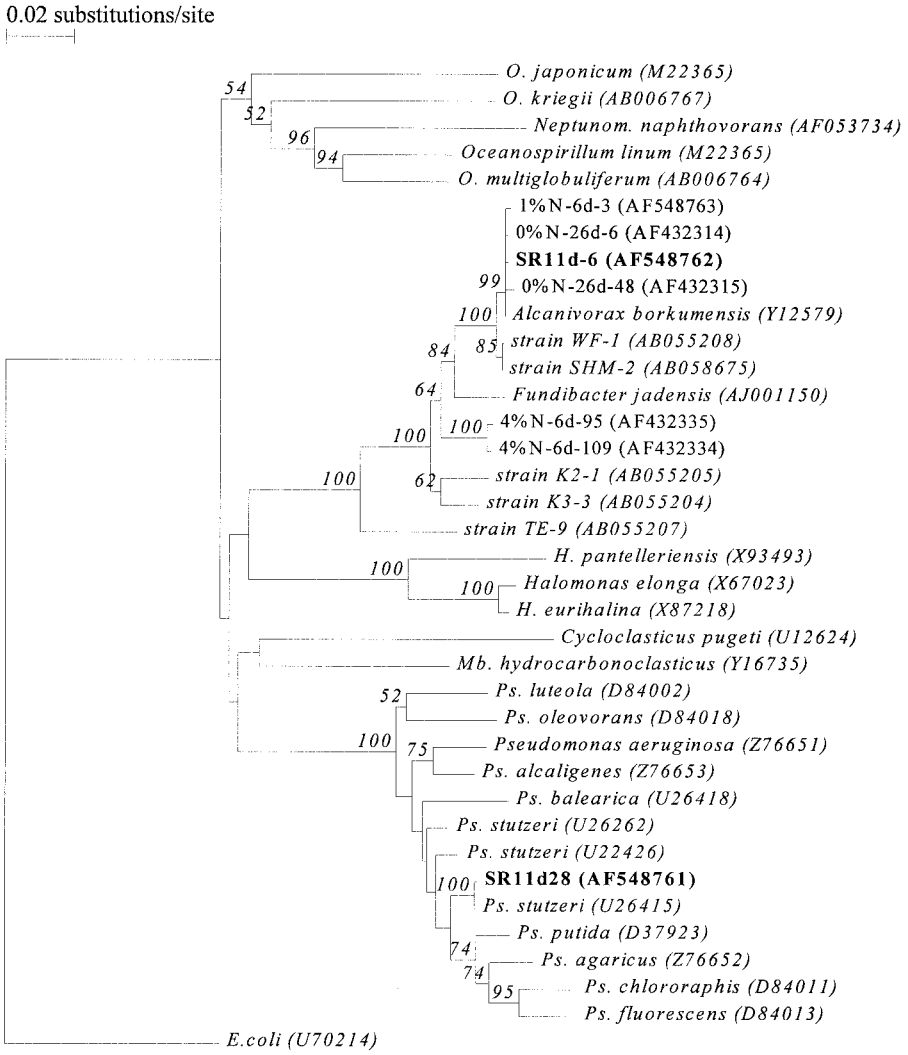

FIG. 7. Phylogenetic tree based on almost complete 16S rRNA sequences for two dominantly occurring sequences (clones 6 and 28, indicated by boldface type) in a clone library constructed from a sample taken from the plot treated with slow-release fertilizer (SR) in block 2 of the Stert Flats field trial, 11 days (11d) after fertilization. Related sequences from a previous microcosm study of oil spill bioremediation in which sediment from the same beach was used (21) are also shown. These are indicated by "a % N-bd-number," where a is the amount of fertilizer added and b is the number of days after fertilizer addition. A neighbor-joining analysis with Jukes-Cantor correction was performed. Only bootstrap values greater than 50% are indicated at the nodes. *O.*, *Oceanospirillum*; *Neptunom.*, *Neptunomonas*; *H.*, *Halomonas*; *Mb.*, *Marinobacter*; *Ps.*, *Pseudomonas*.

fertilizer did show significant degradation over time. Remarkably, only a few, very-short-lived significant differences in bacterial community structure were observed between the oil-treated plots that received no fertilizer and the oil-treated plots treated with liquid fertilizer, while no significant differences were observed between the oil-treated plots that received no fertilizer and the non-oil-treated control plots. Interestingly, in laboratory experiments Wikström et al. (35) noted changes in the microbial community of groundwater contaminated with nitroaromatic compounds following amendment with hydrocarbons to which the microbial community had not been previously exposed, but not after amendment with excessive amounts of hydrocarbons to which the microbial community had been exposed previously. The research site used in the present study has a history of oil pollution due to accidental spills and previous oil spill experiments (30). Therefore, an oil-degrading community may already have been established and active at Stert Flats, and no changes in community structure may have been required in order to express the degradation potential. It is also possible that changes in community structure as the result of oil pollution and bioremediation treatment were too small to be detected by our PCR-DGGE approach and statistical analysis.

The occurrence of significant, lasting changes in bacterial community composition in plots treated with slow-release fertilizer appears to contradict the idea that the Stert Flats sediments harbored an already established bacterial community in the oil-treated plots that received no fertilizer or were treated with liquid fertilizer, since one might expect that the same communities would be responsible for hydrocarbon degrada-

tion in the plots treated with slow-release fertilizer. However, in the plots treated with slow-release fertilizer the nutrient concentrations were greater than the concentrations in the other plots. Degradation of beached oil is in general limited by the supply of N and P (3, 28). The observed stimulation of oil degradation by nutrient amendment in this study, as well as in previous laboratory experiments (21), indicates that this is also the case for our field site. Resource ratio theory predicts that different communities are selected as the result of competition for limiting resources (31). The outcome of competition is determined by differences in species-specific substrate affinity, the maximum growth rate, and the mortality rate. Therefore, the sustained higher nutrient concentrations present in the plots containing slow-release fertilizer granules may have resulted in selection of a bacterial community that differed from that in the other oil-treated plots. It is unlikely that the differences in community structure between the plots treated with slow-release fertilizer and the other plots were due to growth of bacteria on the oleophilic coating of the slow-release fertilizer granules. The slow-release fertilizer was added to the oil-treated plots in mesh bags and thus was physically separated from the oil-treated sediment that was sampled for analysis. Also, the hulls of the granules remained in the mesh bags after the nutrients had been lost from the granules. Furthermore, a population of bacteria closely related to *A. borkumensis* (99.9% similarity) developed quickly in the plots treated with slow-release fertilizer, and significant carbon dioxide production occurred in these plots, but the type strain of *A. borkumensis* (DSM 11573) was unable to grow with the fertilizer granules as a sole source of carbon (data not shown). The statistical analysis performed in our study showed that there were clear differences in bacterial community structure in the plots treated with slow-release fertilizer, both in time and between different blocks. Sometimes even within plots significant variation was observed. The reasons for these differences are not understood, since the plots were treated with the same amount of slow-release fertilizer. However, they may be related to heterogeneity in the beach sediments; similar observations were made in a randomized block bioremediation experiment on the shoreline of Delaware (17), although in that study community patterns were not analyzed in a rigorous statistical manner. Also, the addition of the slow-release fertilizer to the plots in single mesh bags may have resulted in an uneven release of nutrients and a heterogeneous distribution in the oil-contaminated beach sediment. This factor probably contributed to heterogeneity in bacterial communities between blocks and within plots. However, in replicate microcosms containing homogenized Stert Flat beach sediment and treated with the same amount of liquid fertilizer, clear variation in community structure was observed (21). Therefore, besides heterogeneity in nutrient levels and sediment, other unknown factors may have contributed to heterogeneity in bacterial communities in plots treated with slow-release fertilizer. Our results clearly indicate that it may not be sufficient to sample a single location when the bacterial community structure of oil-contaminated shorelines is studied (19).

It has been suggested that restoration of the bacterial community structure to a state similar to that present prior to pollution could be used as a parameter for determination of the ecological end point of bioremediation (26). For this study, such a measure may have been of little value; in the plot treated with slow-release fertilizer in block 1, the bacterial community structure after 315 days was comparable to the prepollution community structure, while considerable amounts of oil, especially polycyclic aromatic hydrocarbons, remained. Also, the community structure in the oil-treated plots that received no fertilizer or were treated with liquid fertilizer could in general not be distinguished from that in non-oil-treated plots, despite the presence of oil and the occurrence of significant oil degradation.

In conclusion, the randomized block experiment showed that biodegradation of buried oil is stimulated by addition of nutrients, either in a liquid form or in a solid form. The relationship between community structure and degradation appears to be complex since communities with similar structures showed different rates of degradation, while communities with different structures showed similar degrees of degradation.

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