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Response of Archaeal Communities in Beach Sediments to Spilled Oil and Bioremediation

Wilfred F. M. Röling,[1]† Ivana R. Couto de Brito,[1] Richard P. J. Swannell,[2] and Ian M. Head[1]*

School of Civil Engineering and Geosciences and Centre for Molecular Ecology, University of Newcastle, Newcastle upon Tyne NE1 7RU,[1] and AEA Technology, Didcot, Oxfordshire OX11 OQJ,[2] United Kingdom

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While the contribution of *Bacteria* to bioremediation of oil-contaminated shorelines is well established, the response of *Archaea* to spilled oil and bioremediation treatments is unknown. The relationship between archaeal community structure and oil spill bioremediation was examined in laboratory microcosms and in a bioremediation field trial. 16S rRNA gene-based PCR and denaturing gradient gel analysis revealed that the archaeal community in oil-free laboratory microcosms was stable for 26 days. In contrast, in oil-polluted microcosms a dramatic decrease in the ability to detect *Archaea* was observed, and it was not possible to amplify fragments of archaeal 16S rRNA genes from samples taken from microcosms treated with oil. This was the case irrespective of whether a bioremediation treatment (addition of inorganic nutrients) was applied. Since rapid oil biodegradation occurred in nutrient-treated microcosms, we concluded that *Archaea* are unlikely to play a role in oil degradation in beach ecosystems. A clear-cut relationship between the presence of oil and the absence of *Archaea* was not apparent in the field experiment. This may have been related to continuous inoculation of beach sediments in the field with *Archaea* from seawater or invertebrates and shows that the reestablishment of *Archaea* following bioremediation cannot be used as a determinant of ecosystem recovery following bioremediation. Comparative 16S rRNA sequence analysis showed that the majority of the *Archaea* detected (94%) belonged to a novel, distinct cluster of group II uncultured *Euryarchaeota*, which exhibited less than 87% identity to previously described sequences. A minor contribution of group I uncultured *Crenarchaeota* was observed.

Biodegradation of oil spilled on shorelines is in general strongly enhanced by treatment with inorganic compounds, especially nitrogen and phosphorus (3, 33). Bacteria are considered to be the predominant agents of hydrocarbon degradation in this environment (19). Bacterial community structure changes in response to oil spills and subsequent bioremediation treatments, and members of the alkane-degrading genus *Alcanivorax* become dominant (17, 18, 20, 26, 27).

The response of the archaeal community to oil spilled on beaches and to bioremediation treatments has not been investigated. Until a few years ago, the domain *Archaea* was thought to consist only of methanogens living under strictly anaerobic conditions or extremophiles inhabiting inhospitable environments. Culture-independent molecular analysis has revealed that *Archaea* are also present in many nonextreme, aerobic ecosystems (6), and *Archaea* are now known to inhabit many marine environments, including coastal and open-ocean waters and sediments and marine animals (5, 7, 11, 12, 22, 35, 36). *Archaea* usually account for at least a few percent of the total cell count and often more in these environments. Their widespread distribution at a relatively high level suggests that these organisms also have ecological significance in many low-temperature, oxic environments.

* Corresponding author. Mailing address: Civil Engineering and Geosciences, University of Newcastle, Newcastle upon Tyne NE1 7RU, United Kingdom. Phone: 44 191 2226605. Fax: 44 191 2225431. E-mail: i.m.head@ncl.ac.uk.

† Present address: Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit, 1081HV Amsterdam, The Netherlands.

Archaea have been detected in several oil-containing environments, such as petroleum reservoirs (21), underground crude oil storage cavities (37), and hydrocarbon-polluted aquifers (8). However, the conditions in these environments were either extreme (high temperature) or strictly anaerobic. To our knowledge, the only study of the effect of pollution on the archaeal community in a nonextreme, aerobic ecosystem was a study of heavy-metal-contaminated soils (29). This study revealed that there was a decrease in archaeal numbers with increasing heavy-metal contamination, as well as differences in the structures of the communities.

In order to gain a better understanding of the ecological significance of *Archaea* in relation to oil spills on marine shorelines, we examined the composition of archaeal communities in beach sediment and their response to oil pollution and subsequent bioremediation treatments. Culture-independent molecular tools were applied to DNA extracts obtained from beach microcosm and field experiments for which the relationship between bacterial community dynamics and oil degradation has been described elsewhere (26, 27).

MATERIALS AND METHODS

Microcosm and field experiments. The same DNA extracts that were analyzed previously to examine the dynamics of bacterial communities in laboratory microcosms (27) and during a field trial of oil spill bioremediation (26) were used in this study. Full details concerning the field site and experimental setup have been published previously (27) and are briefly summarized here. Sediment for microcosm experiments was obtained from the upper part of the intertidal zone of Stert Flats in Somerset, United Kingdom (51°12.3′N, 03°03.9′W). This site was also used for the field trial. Each laboratory microcosm contained 2.0 kg of sediment and was kept at 20 ± 3°C. Fresh synthetic seawater was used to provide the microcosms with two tidal cycles each day. A weathered, water-in-oil emul-

TABLE 1. Characteristics of the laboratory beach microcosms examined for the presence of *Archaea* by PCR with *Archaea*-specific primers[a]

Treatment[b]	Amt of oil added (kg/m^2)	Amt of $NaNO_3^-$ added (% N [wt/wt])	Amt of $KH_2PO_4^-$ added (% P [wt/wt])	% of biodegraded oil after 26 days	Amt of CO_2 produced in 26 days (mmol)	No. of *Archaea*-positive PCRs/ no. of samples analyzed		
						Zero time	6 days	26 days
FO	0	2	0.2		26.0	3/3	3/3	3/3
0%N	3.7	0	0	5.2	25.7	3/3	0/3	1/3
1%N	3.7	1	0.1	52.8	290.0	3/3	0/3	0/3

[a] Oil degradation data are from reference 27. Archaeal presence was determined by PCR with specific primers by using samples taken at zero time and 6 and 26 days after the start of the experiment.
[b] FO, fertilizer only; 0%N, no N added; 1%N, 1% (wt/wt) N added (based on the amount of oil added).

sion of crude oil was added to the sediment surface in the microcosms at a level of 3.7 kg/m^2. Bioremediation treatments consisted of addition of an inorganic nutrient solution (sodium nitrate and potassium dihydrogen phosphate) 24 h and 7, 14, and 21 days after oil addition. Characteristics of oil degradation (27) in the three microcosms investigated in this study are summarized in Table 1. At each sampling point three independent samples were taken from a single microcosm and subjected to DNA extraction (27). The field experiment is described in the accompanying paper (26). DNA extracts from block 2 of the field experiment were used in this study.

PCR and DGGE analysis. A nested PCR approach was used to amplify archaeal 16S rRNA gene sequences from the DNA extracts. PCR was performed in a 50-μl (total volume) mixture containing 0.2 μM forward primer, 0.2 μM reverse primer, 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 DNA template. In the first round primers ARCH46f (24) and ARCH1017r (4) were used to amplify a 0.97-kb fragment, and in the second round primers pARCH344f-GC (25) and UNIV522r (2) were used with a 1:1,000 dilution of the first-round PCR product, which resulted in a PCR fragment that was 0.22 kb long. Amplification was performed with a Hybaid Omnigene thermocycler as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 0.5 min, 55°C (first round) or 53°C (second round) for 1 min, and 72°C for 1 min and a final elongation step consisting of 72°C for 10 min. Denaturing gradient gel electrophoresis (DGGE) analysis and subsequent statistical analysis were performed as described in the accompanying paper (26), except that the PCR product was electrophoresed on gels containing a linear 30 to 70% denaturant gradient (100% denaturant was 7 M urea and 40% [vol/vol] formamide).

Cloning, sequencing, and phylogenetic analysis of 16S rRNA gene fragments. 16S rRNA gene fragments (ca. 1 kb) were amplified by using primers ARCH46f and ARCH1017r, as described above. PCR products were cloned with a TOPO TA cloning kit (Invitrogen Ltd., Paisley, United Kingdom), and the 16S rRNA gene libraries were screened by amplified ribosomal DNA restriction analysis (ARDRA). *Escherichia coli* clones were initially categorized into different ARDRA types based on the patterns obtained after simultaneous digestion (3 h, 37°C) with restriction enzymes RsaI and HaeIII (5 U each). To determine the banding positions of the clones in DGGE gels, vector inserts that were the correct size were reamplified with primers pARCH344f-GC and UNIV522r, and the products were subjected to DGGE next to 16S rRNA gene fragments amplified from the beach sediment DNA used to construct the clone library. At least one representative of every ARDRA type was completely sequenced, and for ARDRA types that appeared more than once in the library at least two representatives were sequenced. Phylogenetic analysis was performed as previously described (1, 9, 16, 26, 28, 34).

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in the GenBank database under accession numbers AY396000 to AY396007.

RESULTS

Response of archaeal communities to oil and nutrient addition in laboratory microcosms. Microcosms that were treated with oil but no nutrients (no N) or were treated with oil and a solution containing inorganic nutrients (1% N) and control microcosms that received nutrients only and no oil were sampled at zero time and 6 and 26 days after nutrient treatment (27). DNA extracts were subjected to a nested PCR with primers specific for *Archaea*. *Archaea* were detected in all microcosms immediately after oil addition and in all DNA extracts from the microcosm that was not treated with oil (n = 15) (Table 1). However, exposure to oil had a negative effect on the ability to amplify archaeal 16S rRNA gene fragments. *Archaea* were detected in only 1 of 12 samples taken on days 6 and 26 from the oil-treated microcosms. This reflected a real reduction in the archaeal community and not inhibition of PCR due to oil in the samples or bound to archaeal DNA. This was apparent because it was possible to amplify archaeal 16S rRNA gene fragments from zero-time samples which contained oil, as well as from the bioremediated microcosm on day 26, and bacterial 16S rRNA gene fragments could be readily amplified from the same DNA extracts (27). Archaeal 16S rRNA genes have also readily been amplified from other oil-containing environments (8, 37). DGGE profiling and subsequent cluster analysis of the DGGE data (based on the whole-track densitometric curves; Pearson correlation) revealed differences in archaeal community structure between the different microcosms at the start of the experiment (zero time) (Fig. 1, top). The archaeal communities of the bioremediated microcosms were clearly different from the communities in unfertilized, oil-treated microcosms and the control that was not treated with oil, clustering only at Pearson product-moment correlation coefficients (r) of >0.61. Triplicate samples taken at the same time from a microcosm sometimes clustered differently when the whole-track densitometric curve information was taken into account (compare the 0%-N zero-time samples in Fig. 1, top, and the fertilizer-only day 26 samples in Fig. 1, bottom). However, when only the data for band presence or absence were compared, these triplicate samples clustered together (data not shown), showing that the differences were due to variation in the intensity of the bands rather than differences in the composition of the predominant *Archaea*. For the microcosms that were not treated with oil no clear-cut changes in community structure over time were observed, both when clustering was based on the whole-track densitometric curve (Fig. 1, bottom) and when clustering was based on band positions only (data not shown). Despite the differences in clustering of the DGGE profiles, the most intense bands were found in all samples.

Phylogenetic analysis of archaeal communities in microcosms. Phylogenetic analysis of cloned 16S rRNA sequences was performed in order to obtain specific insight into which types of *Archaea* were present on unpolluted beaches and were potentially adversely affected by oil pollution. As the banding patterns in all the samples were similar in terms of band positions (Fig. 1, bottom) (see above), sequences were cloned

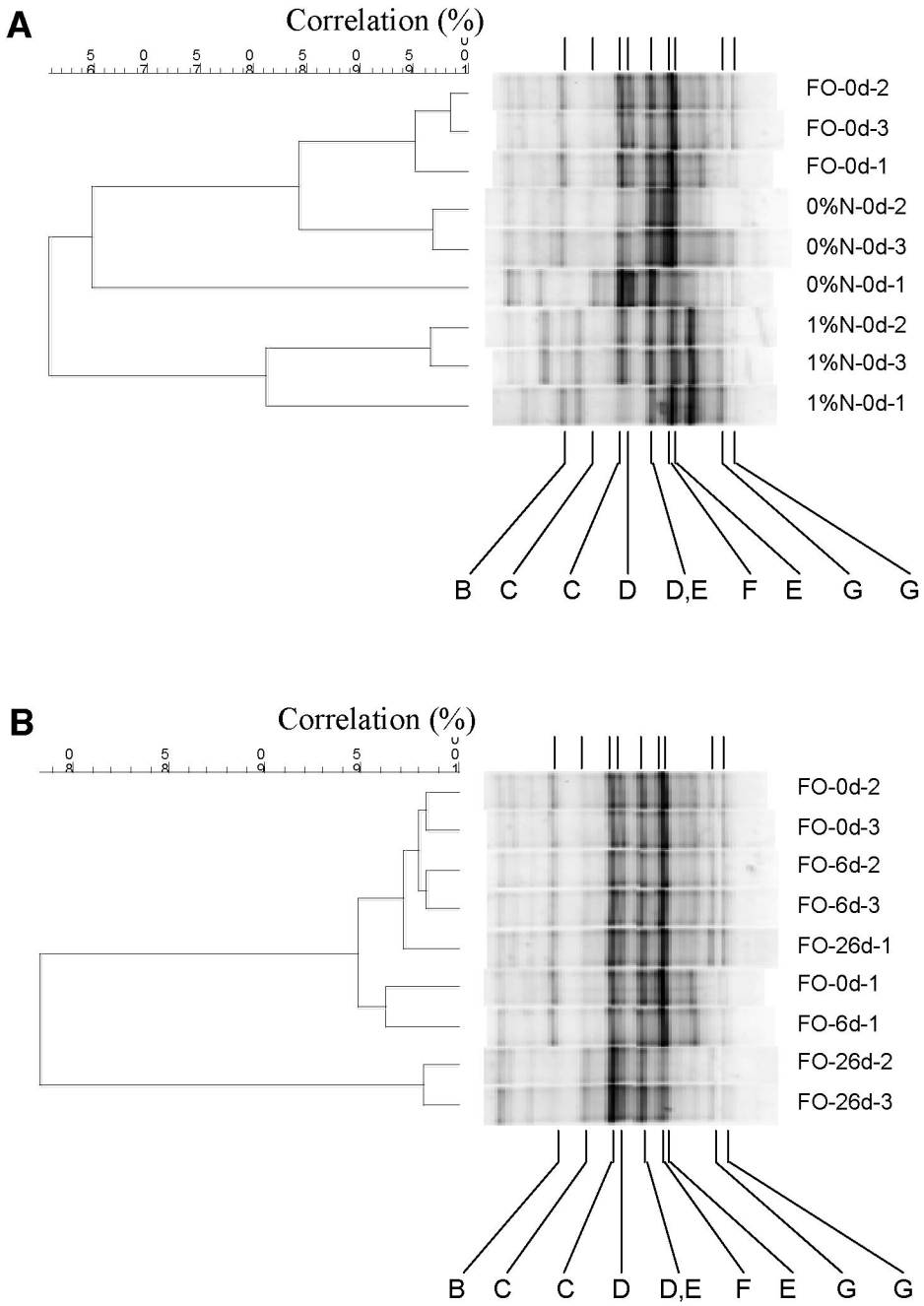

FIG. 1. Unweighted pair group method with arithmetic mean clustering of DGGE profiles of archaeal 16S rRNA gene fragments (30 to 70% denaturant gradient) from laboratory microcosms after Pearson correlation of whole-track densitometric curves of the profiles. Samples are indicated according to the treatments applied to the microcosms from which the profiles were derived (FO, fertilized only; 0%N, oil treated only; 1%N, oil treated and fertilized) and the time of sampling (at the start of the experiment [0d] and 6 and 26 days after the start [6d and 26d, respectively]). At each sampling time three samples were independently taken from a microcosm, and nucleic acids were extracted and subjected to PCR-DGGE analysis. Each replicate sample from a single microcosm was labeled 1, 2, or 3. (A) Clustering of the community profiles in the microcosms at the start of the experiment. (B) Clustering of the community profiles in the fertilized microcosm that was not treated with oil at different times. Matching of cloned archaeal sequences to bands in the environmental DGGE patterns is indicated by letters. The identities of the coded bands are shown in Table 2. Note that a number of clones gave multiple bands due to the use of degenerate PCR primers. Also note that clone A is not included as it did not correspond to a visible band in the DGGE patterns.

from the day 26 sample from the microcosm that was not treated with oil (Fig. 1, bottom). ARDRA of 31 clones with inserts that were the correct size revealed eight unique restriction patterns. For each clone type at least one representative was sequenced and subjected to phylogenetic analysis. When more than one representative of a clone type was sequenced, the sequences proved to be identical (data not shown). The majority of the clones (94%) belonged to the uncultured ma-

TABLE 2. Identities of clones related to bands in Fig. 2, as determined by sequencing of cloned 0.97-kb 16S rRNA gene fragments

Band clone	Closest relative in GenBank database (accession no.)	% Identity	Phylogenetic group	No. of microcosm profiles with corresponding band visible/total no. of profiles
A	Uncultured clone 74A4 (AF393466)	98.9	*Crenarcheota*	0/15
B	Uncultured clone 74A4 (AF393466)	98.0	*Crenarcheota*	12/15
C	Uncultured clone CCA47 (AY179969)	85.9	*Euryarcheota*	15/15
D	Uncultured archaeal symbiont PA203 (AB062320)	84.3	*Euryarcheota*	12/15
E	Uncultured clone ARCP1-30 (AF523939)	84.7	*Euryarcheota*	15/15
F	Uncultured clone M2 (AB034186)	86.9	*Euryarcheota*	15/15
G[a]	Uncultured clone WCHD3-16 (AF050618)	84.1	*Euryarcheota*	14/15
G′	Uncultured clone WCHD3-16 (AF050618)	84.2	*Euryarcheota*	14/15

[a] Two different ARDRA types had similar banding positions in DGGE, and phylogenetic information was obtained for both ARDRA types. These types are indicated by G and G′. The in silico restriction map agreed with the observed ARDRA patterns.

rine group II *Euryarchaeota*, and all these clones clustered together but were distinct (less than 87% identity) from previously described sequences from the marine group II *Euryarchaeota* (Table 2). Two clones (6%) belonged to group I of the *Crenarchaeota* and showed 98% identity to previously encountered sequences (Table 2).

DGGE analysis of cloned sequences alongside the 16S rRNA gene fragments amplified directly from the beach sediment DNA showed that sequences corresponding to the majority of the bands in the DGGE profile were recovered in the clone library. (Fig. 1), and most clones were related to bands present not only in the DGGE profiles from the sample that was used to generate the clone library but also in DGGE profiles from the other samples of beach sediment (Table 2 and Fig. 1). Thus, it appears that despite the low number of clones screened ($n = 31$), the clone library covered a considerable part of the archaeal diversity in the beach sediments detectable by DGGE. This was corroborated by calculation of the clone distributions by using 97% identity in 16S rRNA sequences to group sequences (31). A coverage value (13) of 87% was calculated, while the Chao estimator of richness (15) indicated the presence of 9.4 ± 3.4 operational taxonomic units. In one instance two clone types with different ARDRA profiles migrated to the same position in DGGE gels. Despite having distinct ARDRA profiles, the sequences of these clones exhibited >99.5% identity (band clones G and G′ in Table 2).

Dynamics of archaeal communities during an oil spill bioremediation field trial. Oil had an adverse effect on archaeal communities in laboratory microcosms. If this is also true under field conditions, the recovery of archaeal communities might be a useful parameter for determining the ecological end point of bioremediation. This hypothesis was tested by using beach sediment samples from a field trial of oil spill bioremediation that were previously analyzed in relation to the dynamics of bacterial communities (26). *Archaea*-specific PCRs were performed with DNA extracts obtained from plots that were not treated with oil, from oil-treated plots that were not treated with nutrients, and from bioremediated plots treated with oil and a liquid nutrient solution or with oil and slow-release fertilizer. In contrast to the microcosm experiment, detection of *Archaea* was somewhat variable, and no clear effect of oil on the ability to detect *Archaea* was observed (Table 3). DGGE analysis was performed to determine whether in the oil-treated plots the *Archaea* community structure differed from that in plots that were not treated with oil, thus indicating a response of *Archaea* to oil and bioremediation. DGGE profiling and subsequent cluster analysis based on a comparison of the whole-track densitometric curves (Pearson correlation) showed neither a clear trend over time nor clustering of samples with respect to treatment (Fig. 2). Nevertheless the DGGE profiles from the field samples clustered at a high level of similarity ($r > 0.8$) and were distinct from the DGGE profiles obtained from microcosm samples (Fig. 2 and data not shown). The high degree of similarity is related to the fact that the most intense bands were present in all DGGE profiles from the field samples. When cluster analysis was performed solely for band positions (Jaccard coefficient), more variation was observed (all samples clustered at a low similarity, ca. 28%) due to the variable presence of bands with low intensities (Fig. 2); however, still no clear trend over time or clustering with respect to treatment was evident. The dominant bands in the DGGE profiles from the field experiment corresponded to some of the dominant bands in the DGGE profiles of the microcosm samples (Fig. 2), namely, bands B and E (Table 2).

DISCUSSION

This study indicated that *Archaea* are present on shorelines but that their abundance is negatively affected by oil spills in laboratory experiments. The samples analyzed here to determine the composition of archaeal communities were used previously to investigate the relationship between bacterial community structure and biodegradation (27). Since in the previous study rapid degradation of oil in the microcosms

TABLE 3. Relationship between the presence of oil and PCR-detectable *Archaea* during an oil spill bioremediation field trial with various treatments applied to plots

Treatment	PCR results at the following times (days) after first fertilization of the plots[a]						
	−7	−3	1	5	11	80	315
No oil	−	−	−	−	+	+	+
Oil	−	−	+	+	+	+	−
Oil + liquid fertilizer	+	ND	+	+	+	−	−
Oil + slow-release fertilizer	+	ND	ND	+	+	+	+

[a] Fertilization occurred at zero time, which was 7 days after oil was added. −, negative; +, positive; ND, not determined.

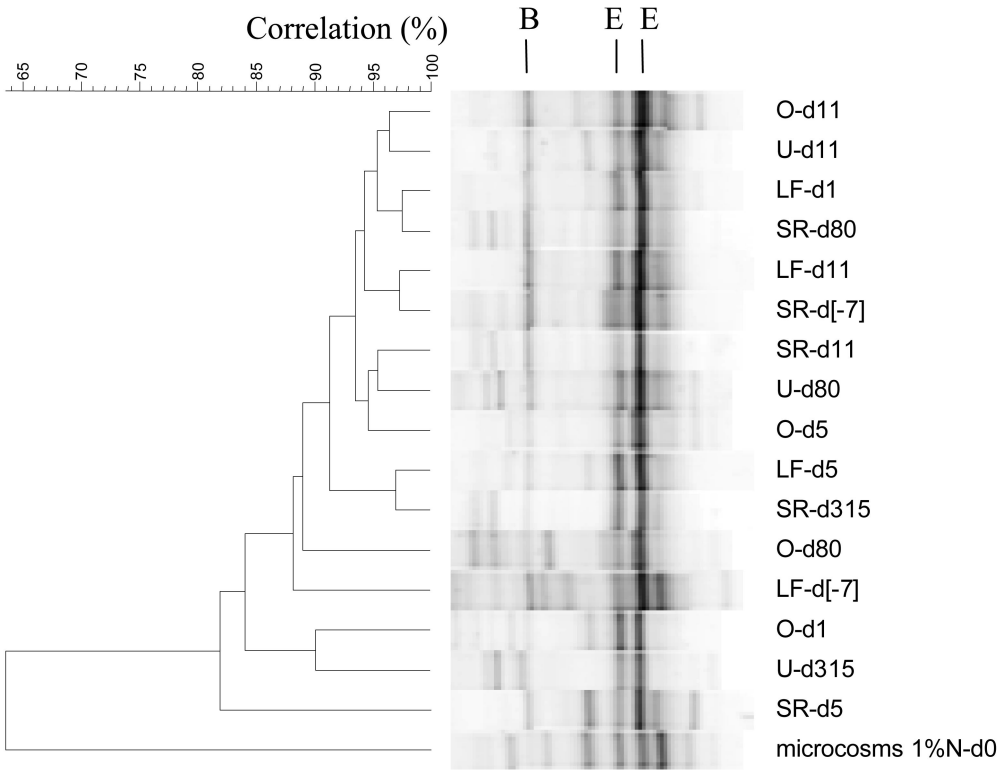

FIG. 2. Unweighted pair group method with arithmetic mean clustering of DGGE profiles of archaeal 16S rRNA gene fragments (30 to 70% denaturant gradient) in beach sediments from an oil spill bioremediation field trial containing plots which received various treatments, after Pearson correlation. The profiles are labeled according to the treatments used for the plots (U, not treated with oil; O, treated with oil only; LF, treated with oil and liquid fertilizer; SR, treated with oil and solid slow-release fertilizer) and time before or after the first addition of fertilizer (d[−7], day −7; d1, day 1; d5, day 5; d11, day 11; d80, day 80; d315, day 315). Oil was added 7 days before the addition of fertilizer. Dominant bands that comigrated with 16S rRNA gene fragments from microcosm experiments (Table 2) are indicated.

treated with nutrients was observed and here we observed a detrimental effect of oil on archaeal communities irrespective of bioremediation treatment, it is apparent that *Archaea* are unlikely to play a significant role in oil spill bioremediation. It is difficult to speculate on the environmental role of the *Archaea* detected in beach sediment. Phylogenetic analysis revealed that the majority of the clones recovered (94%) belonged to the uncultured group II *Euryarchaeota*. The sequences showed only relatively low similarity (<87% identity) to previously encountered sequences and an even weaker relationship to cultured *Archaea*. A small percentage of the clones (6%) belonged to the uncultured group I *Crenarchaeota*, for which there are also no closely related cultured representatives. Therefore, the phylogenetic information cannot be used to infer with confidence any environmental role for the *Archaea* detected in these beach sediments. It is possible that the *Archaea* are not active. A comparable situation has been encountered in some oxic activated sludge plants in which a diverse community of *Archaea*, especially methanogens, was observed (14). Nevertheless, the sequences encountered in this study contribute to our knowledge of the diversity of *Archaea*.

At the outset of the experiment the archaeal communities of the bioremediated microcosms were clearly different from the communities in the unfertilized, oil-treated microcosm and the control that was not treated with oil, and the within-microcosm differences in archaeal communities in the oil-treated microcosms were much larger than those in the microcosm that was not treated with oil (Fig. 1). In contrast, the bacterial communities in these microcosms showed no clear-cut differences, and there were high degrees of similarity (>95%) between and within microcosms (27). A similar observation of apparent small-scale homogeneous communities of *Bacteria* and heterogeneous communities of *Archaea* was recently made for an agricultural soil, and the findings may have been related to the relatively low numbers of *Archaea* present (23). The differences in archaeal communities were probably also partially related to aspects of the experimental setup. The bioremediation microcosm experiments were performed at a later date than the other microcosm experiments; thus, although the beach sediment came from the same field location, the samples were collected on different days and may have contained slightly different archaeal communities (27). Also, weathered crude oil was added to the microcosms 24 h prior to addition of nutrients, and the time of nutrient addition was considered zero time. There was, therefore, 24 h, including two tidal cycles, during which changes in the community structure could have taken place prior to sampling. This may explain differences between the oil-treated samples (which received no N and 1% N) and the samples treated with fertilizer only. It is apparent that the oil-treated samples were much more variable than the untreated samples (which clustered at 95% similarity). It is therefore possible that the separation of the oil-

treated samples in the cluster analysis was influenced by the generally more heterogeneous nature of the oil-treated microcosms and the impact of the 24 h of exposure to oil.

Although archaeal communities are unlikely to have a significant role in oil spill bioremediation, the fact that microcosm experiments showed that oil contamination had a severe negative effect on the archaeal community offers the possibility that *Archaea* may be useful indicators of ecosystem recovery from a pollution incident. It has been suggested that restoration of the microbial community structure to a state similar to that prior to a pollution event could be used as a parameter for determining the ecological end point of bioremediation (32). Since in microcosm experiments a negative effect of oil on archaeal communities was observed, we hypothesized that the reestablishment of *Archaea* in contaminated environments might also be suitable for this purpose. However, analysis of samples from an oil spill bioremediation field experiment did not reveal a clear relationship between the presence of oil and the absence of *Archaea*. In contrast to the laboratory experiment, *Archaea* could be detected at a high frequency (11 of 15 samples analyzed) in samples in which oil was present. These contrasting results may be explained by the inability to completely reproduce environmental conditions in laboratory experiments. For example, in the field experiment *Archaea* from external sources may have continually reinoculated the beach sediments. Seawater contains *Archaea* (5, 11, 22), and the replacement of interstitial waters in the beach sediments by tidal cycles may maintain a detectable archaeal community even in the face of a local environmental stress, such as oil pollution. Furthermore, the intestines of invertebrates can harbor significant archaeal populations (10). Meio- and macrofauna were detected throughout the field trial (30) but were lost during the procedures used to prepare sediment for the beach microcosms. These sources of *Archaea* were therefore not present in the laboratory experiment, in which tidal cycles were simulated by using artificial seawater. Care should therefore be exercised when workers extrapolate the results of laboratory experiments to the field situation, and findings from laboratory experiments should be corroborated in the field.

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