Dynamics of bacterial populations during bench-scale bioremediation of oily seawater and desert soil bioaugmented with coastal microbial mats

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Summary
This study describes a bench-scale attempt to bioremediate Kuwaiti, oily water and soil samples through bioaugmentation with coastal microbial mats rich in hydrocarbonoclastic bacterioflora. Seawater and desert soil samples were artificially polluted with 1% weathered oil, and bioaugmented with microbial mat suspensions. Oil removal and microbial community dynamics were monitored. In batch cultures, oil removal was more effective in soil than in seawater. Hydrocarbonoclastic bacteria associated with mat samples colonized soil more readily than seawater. The predominant oil degrading bacterium in seawater batches was the autochthonous seawater species *Marinobacter hydrocarbonoclasticus*. The main oil degraders in the inoculated soil samples, on the other hand, were a mixture of the autochthonous mat and desert soil bacteria; *Xanthobacter tagetidis*, *Pseudomonas geniculata*, *Olivibacter ginsengisoli* and others. More bacterial diversity prevailed in seawater during continuous than batch bioremediation. Out of seven hydrocarbonoclastic bacterial species isolated from those cultures, only one, *Mycobacterium chlorophenolicum*, was of mat origin. This result too confirms that most of the autochthonous mat bacteria failed to colonize seawater. Also culture-independent analysis of seawater from continuous cultures revealed high-bacterial diversity. Many of the bacteria belonged to the *Alphaproteobacteria*, *Flavobacteria* and *Gammaproteobacteria*, and were hydrocarbonoclastic. Optimal biostimulation practices for continuous culture bioremediation of seawater via mat bioaugmentation were adding the highest possible oil concentration as one lot in the beginning of bioremediation, addition of vitamins, and slowing down the seawater flow rate.

Introduction
Remediation of sites contaminated with xenobiotic compounds is achieved by physical and chemical methods, e.g. land filling and incineration (Kuiper et al., 2004). However, the physical removal of pollutants from all contaminated sites on earth is obviously very costly (Rosenberg, 1993). In addition, incineration is associated with air pollution, and land filling frequently leads to leachates in the form of gases and liquids which can pollute the ground water (Kuiper et al., 2004). The much more cost-effective and more environmentally friendly technology of bioremediation implies the use of microbial activities in pollutant biodegradation (Atlas and Pramer, 1990). It comprises two major practices. Bioaugmentation (inoculation or seeding), which implies the introduction of suitable oil-degrading microorganisms into the contaminated site. The second practice is biostimulation, whose objective is to enhance the activities of indigenous (autochthonous) pollutant-degrading microorganisms via environmental management, e.g. the addition of nutrients and other growth-limiting factors, especially nitrogen and phosphorus (Atlas and Bartha, 1998; Radwan, 2009). Bioremediation commonly is recommended as an alternative technology to the use of chemicals and other toxic materials for removing hydrocarbon contaminants (Piskonen and Itävaara, 2004).

As already mentioned, bioaugmentation implies the inoculation of the contaminated sites with laboratory grown, hydrocarbon-degrading microorganisms (Al-Awadhi et al., 1996; Van Limbergen et al., 1998; Kuiper et al., 2004). This leads to the introduction of additional gene pools complementary to the already existing ones, with the purpose of enhancing degradation of contaminants (Domde et al., 2007). In a study on the effect of bioaugmentation with a consortium of bacteria on the remediation of hydrocarbon contaminated waste water, the water chemical oxygen demand, which reflects the organic substance content, dramatically decreased (Domde et al., 2007). Obviously, the proper consortia of microorganisms should be used in order to complete the
degradation process (Kapley and Purohit, 2001; Moharikar et al., 2003; Domde et al., 2007). In literature reports, exogenous pure cultures as well as unidentified mixtures of microorganisms have been used for bioaugmentation (Atlas and Bartha, 1998). Based on their ability to degrade a wide range of organic compounds, species of Pseudomonas have been frequently selected (Atlas and Bartha, 1998). Evidently, the bioaugmented organisms should be adapted to physicochemical parameters of the contaminated site. Imported Arthrobacter strains, in contrast to locally isolated ones, failed to colonize local oil-polluted soils due to their inability to compete with the already existing strains (Radwan et al., 1997). Although proper microorganisms may be inoculated, they may fail to remove the pollutant (El Fantroussi and Agathos, 2005). Reportedly, this could be due to the absence of a single bacterium that possesses the entire set of enzymes needed to biodegrade the pollutant. Another five reasons have been suggested (Goldstein et al., 1985): the contaminant concentration is too low to support bacterial growth, presence of inhibitors that suppress microbial growth and/or activity, reduction of bacterial numbers due to protozoan grazing, presence of better utilizable sources of carbon and inability of the microbial cells to spread and reach the pollutant.

Biostimulation, the second bioremediation, practice implies, among others, the addition of nutrients, usually nitrogen, phosphorous and trace elements (Korda et al., 1997). Enhancing effects of biostimulation on hydrocarbon biodegradation have been documented (Bossert and Bartha, 1984; Leahy and Colwell, 1990; Atlas, 1991; Margesin and Schinner, 1998, Namkoong et al., 2002, Jimenez et al., 2007; For review see Nikolopoulou and Kalogerakis, 2009). On the other hand, a few investigators found that the rate of hydrocarbon degradation was not affected following the addition of nutrients (Seklemova et al., 2001). It has been reported that the percentage of oil degraded was inversely proportional to the concentration of the contaminating oil (Rahman et al., 2002). Bioremediation in the field is unpredictable because of the lack of knowledge of the persisting microorganisms in the site (Head, 1998).

Ideally, biostimulation should be coupled with bioaugmentation (Odokuma and Dickson, 2003; Coppotelli et al., 2008; Nikolopoulou et al., 2013a,b). When the efficiency of bioaugmentation and biostimulation in Long Beach soils and Hong Kong soils was compared, it was found that biostimulation achieved more hydrocarbon removal (Bento et al., 2005). However, there is no feasible technology for enhancing nutrient availability in the open seas (Rosenberg, 2006). Evidently, autochthonous microorganisms are to be chosen for bioaugmentation (Hosakawa et al., 2009). In view of the fact that biostimulation enhances autochthonous microorganism (DiGregorio et al., 2015), the two practices (autochthonous bioaugmentation and biostimulation) could be regarded as two faces of one coin. Autochthonous microorganisms of a habitat are the natural inhabitants, contributing to biochemical activities therein. Their counterparts, the allochthonous microorganisms are foreign survivals which do not contribute significantly to activities in the habitat.

The following snap shots summarize the history of the ‘autochthonous bioaugmentation (ABA)’ concept, and contribute to highlighting the objectives of this study. About two and half decades back, one of our group (Radwan, 1991) warned from using imported microbial cocktails, instead of depending on indigenous microorganisms for combating the greatest man-made oil spill in the history of mankind (the spill associated with the 1990–1991 occupation of Kuwait by the Iraqi forces). Experimental studies supported the validity of this concept (Vecchioli et al., 1990; Weber and Corseuil, 1994). However, it was Ueno and colleagues (2007) who coined the term ‘autochthonous bioaugmentation (ABA)’, which necessitates the use of natural microbial inhabitants of an environment for its bioremediation. With this background in mind, the major objective of this paper was to study, in bench-scale experiments, the feasibility of using local microbial mats from Kuwaiti coasts, instead of laboratory-grown microbial cocktails, as bioaugmentation materials for bioremediation of local oil-contaminated seawater and desert soil samples. We selected microbial mats on the basis of our earlier report (Sorkhoh et al., 1992) that they were the primary colonizers of coastal oil sediments, and consequently the first sign of self-cleaning of the dead coasts that had been heavily polluted during the Iraqi occupation of Kuwait. Reportedly, such coastal mats were rich in hydrocarbonoclastic bacteria, well adapted to the Kuwaiti conditions. There are still only a very few studies worldwide on the ABA strategy (Hosakawa et al., 2009), and almost none on the contaminated Kuwaiti habitats. These facts highlight the need for the current study.

Results

Oil removal in batch culture

The Kuwait map in Fig. 1 shows where the environmental samples have been taken.

The results in Fig. 2 show that about 60% of the oil in the seawater batches were consumed after the first month of incubation. The consumption values did not increase thereafter. There were also no marked differences between the consumption values obtained from the sterilized and unsterilized seawater samples. It will be shown soon that the typical seawater bacterium Alcanivorax hydrocarbonoclasticus was the active organism in both
Fig. 1. Kuwait map showing the sampling sites of coastal mats, seawater and desert soil samples.

Fig. 2. Oil consumption and numbers of cultivable hydrocarbonoclastic bacteria during bioremediation of seawater and desert soil samples in batch cultures using microbial mats for bioaugmentation. Solid lines, oil consumption; broken lines, bacterial numbers; closed symbols, sterile samples; open symbols, fresh samples.

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samples. It reached the sterilized seawater with the bioaugmented mat which was suspended in seawater.

In the desert soil batches, oil consumption increased from time zero, reaching maximum values between the third and sixth months. Higher oil removal values were commonly measured in soil batches than in seawater batches.

**Numbers of hydrocarbonoclastic bacteria in batch cultures**

With the exception of the unsterilized seawater batches from the Kuwait Towers, whose bacterial numbers increased reaching a maximum in month 3, the numbers of bacteria in the seawater samples were highest after the first month, and decreased with prolonged incubation (Fig. 2). In several batches, the bacteria died after 4 months, as indicated by the stinky, anaerobic smell. In the soil batches, the numbers of the hydrocarbonoclastic bacteria kept increasing from time zero till the end of the 6-month bioremediation period.

**16S rRNA gene sequencing for hydrocarbonoclastic bacterial isolates from batch cultures**

Table 1 presents the results of 16S ribosomal (r)RNA gene sequencing of the autochthonous, hydrocarbonoclastic bacteria isolated by the culture-dependent method from coastal mat, desert soil and seawater samples. Most isolates showed 99–100% similarities in their sequences to their closest relatives in the GenBank database. Many of the autochthonous mat inhabitants were affiliated with the class of *Alphaproteobacteria*, with fewer members affiliated with the *Sphingobacteridae*, *Gammaproteobacteria* and *Actinobacteridae*. Autochthonous desert soil inhabitants and seawater inhabitants belonged predominantly to the *Actinobacteridae* and *Proteobacteria* respectively. In other words, autochthonous bacteria in the three habitats belong to diverse systematic taxa. The phylogenetic tree in Fig. 3 illustrates phylogenetic relationships among those hydrocarbonoclastic isolates. Figure 4 shows that the individual autochthonous bacterial isolates from the three habitats consumed in batch cultures within 14 d between about one fifth and one third of the crude oil.

**Population dynamics of hydrocarbonoclastic bacteria during bioremediation in batch cultures**

The three oil-contaminated seawater samples that had been bioaugmented with coastal mat were colonized by *Marinobacter hydrocarbonoclasticus*. In comparison, the

| Isolates | Total bases | Subdivision | Nearest GenBank match | Similarity % | Bases compared | Accession numbers |
|----------|-------------|-------------|-----------------------|--------------|----------------|------------------|
| **Microbial mats** | | | | | | |
| M1 | 468 | Alphaproteobacteria | Xanthobacter tagetidis strain TagT2C | 99 | 475/478 | KP276687 |
| M2 | 514 | Gammaproteobacteria | *Pseudomonas* cnicalata strain KNJ2C110 | 100 | 514/514 | KP276688 |
| M3 | 469 | Gammaproteobacteria | Phenyllobacterium koreense strain SBR9 | 99 | 480/485 | KP276689 |
| M4 | 514 | Gammaproteobacteria | *Pseudomonas* pachastrellae strain mjp2-PW8-OH9 | 99 | 517/518 | KP276690 |
| M5 | 484 | Actinobacteria | Dietzia maris strain W13107 | 100 | 484/484 | KP276691 |
| M6 | 510 | Actinobacteria | Agrobacterium agile | 100 | 510/510 | KP276692 |
| M7 | 490 | Actinobacteria | Mycobacterium chlorophenolicum isolate 42C8 | 100 | 490/490 | KP276693 |
| M8 | 494 | Actinobacteria | Rhodococcus ruber strain Z17-3 | 100 | 494/494 | KP276694 |
| M10 | 458 | Sphingobacteria | Olivibacter ginsengisoli strain Gsoil 060 | 96 | 494/513 | KP276695 |
| M11 | 498 | Gammaproteobacteria | *Pseudomonas* alcaligens strain SM-26 | 99 | 507/511 | KP276696 |
| M12 | 491 | Sphingobacteria | Olivibacter jilunii strain 14.2A | 99 | 500/504 | KP276697 |
| M14 | 487 | Actinobacteria | Prasereilla muralis strain 05-Be-005 | 99 | 489/490 | KP276698 |
| **Desert soil** | | | | | | |
| S17 | 481 | Actinobacteria | Dietzia maris strain DSM 43672 | 100 | 481/481 | KP223302 |
| S18 | 383 | Betaproteobacteria | Cupriavidus taiwanensis | 100 | 383/383 | KP223303 |
| S19 | 335 | Actinobacteria | Nocardioides rubidaea strain S1 | 98 | 352/360 | KP223304 |
| S20 | 513 | Gammaproteobacteria | *Pseudomonas* stutzeri strain ECP10 | 100 | 513/513 | KP223305 |
| S21 | 508 | Gammaproteobacteria | *Pseudomonas* psychrotolerans strain ZAP069 | 99 | 515/512 | KP223306 |
| S22 | 503 | Betaproteobacteria | Massilia timoneae strain WK-79s | 99 | 508/510 | KP223307 |
| S23 | 496 | Betaproteobacteria | Massilia varians strain E26 6-3 | 99 | 506/510 | KP223308 |
| S24 | 457 | Alphaproteobacteria | Brevundimonas diminuta strain 2P06AC | 98 | 478/487 | KP223309 |
| S25 | 507 | Betaproteobacteria | Oxalobacteraceae strain NR185 | 99 | 510/511 | KP223310 |
| **Seawater** | | | | | | |
| W26 | 489 | Gammaproteobacteria | Alcanivorax venustus strain 5P57-5 | 99 | 498/503 | KP223311 |
| W27 | 499 | Gammaproteobacteria | Strain Alcanivorax balaenica strain G06.163s VO | 100 | 499/499 | KP223312 |
| W28 | 514 | Gammaproteobacteria | Marinobacter hydrocarbonoclasticus strain SBU2 | 100 | 514/514 | KP223313 |
| W29 | 478 | Alphaproteobacteria | Thalassospira profundimarina strain S8-2 | 98 | 500/510 | KP223314 |
| W30 | 459 | Alphaproteobacteria | *Amorphous orientalis* strain YIM D10 | 99 | 471/477 | KP223315 |
| W31 | 500 | Betaproteobacteria | Aquabacterium citratiphilum strain B4 | 100 | 500/500 | KP223316 |
| W32 | 487 | Actinobacteria | Gordonia terrae strain DSM 43249 | 99 | 489/490 | KP223317 |

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Fig. 3. 16S rRNA gene phylogeny of 28 hydrocarbonoclastic bacterial isolates from mat, soil and seawater. Values shown in each node of the tree are bootstrap values; 2000 bootstrap replicates were performed. M, microbial mat; S, desert soil; W, sea water.

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three oil-contaminated desert soil samples that had been bioaugmented with coastal mat exhibited much more diversity, as far as their hydrocarbonoclastic bacterial population was concerned (Table 2). Throughout the incubation period, this population consisted of a mixture of autochthonous mat and desert soil bacterial inhabitants. *Xanthobacter tagetidis*, *Pseudomonas geniculata* and albeit in much fewer numbers, *Olivibacter ginsengisoli* were found in all the soil samples throughout the incubation period. *Phenylobacterium koreense* formed considerable proportions of the total bacteria at time zero and after 1 month, but decreased in months 2 and 3. However, the population re-increased in months 4 and 5. The proportions of *Agrobacterium agile*, *Mycobacterium chlorophenolicum* and *Pseudomonas alcaligenes* showed sharp fluctuation during the bioremediation process (Table 2). In addition, some of the soil samples contained one or more of the following hydrocarbonoclastic species in the proportions specified in Table 2 notes: *Pseudomonas pachastrellae*, *Dietzia maris*, *Rhodococcus ruber*, *Olivibacter jilunii* and *Prauserella muralis*. Evidently, the autochthonous mat bacterioflora colonized the soil batches more readily than the seawater batches. All the above species are hydrocarbonoclastic, as judged by their ability to grow on the mineral medium with oil vapor as a sole source of carbon and energy. As already men-

tioned, quantitative determinations revealed that those organisms consumed considerable proportions of the available oil (see Fig. 4).

**Oil consumption and numbers of hydrocarbonoclastic bacteria in continuous cultures**

In culture vessels of the six chemostat-like units, the crude oil remained as separate phases for 2 to 3 d, after which it dispersed in the water, probably via extracellular biosurfactants. A small proportion remained as droplets adhering to the vessel walls and connection tubes. However, all the residual oil in the individual units was completely recovered and analysed, as described in the experimental part.

The results in Fig. 5 show that starting the bioremediation process by adding the whole 3% oil as one lot at time zero resulted in the maximum oil removal (70%, unit IV). As described in the experimental part, the 3% oil had been added in all other chemostat-like units as six 0.5% aliquots at 2-week intervals. Addition of yeast extract as a vitamin source (unit V), was associated with 65% oil removal. The lowest oil consumption value of 39% was obtained when the seawater flow rate (unit VI) was five times quicker (30 ml h$^{-1}$) than in all the other five chemostat-like units. Neither the addition of

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*Fig. 4.* Oil consumption values by autochthonous bacterial isolates from three habitats. Values are means of three replicates.

1. *Xanthobacter tagetidis*, 2. *Pseudomonas geniculata*, 3. *Phenylobacterium koreense*, 4. *P. pachastrellae*, 5. *D. maris*, 6. *Agrobacterium agile*, 7. *M. chlorophenolicum*, 8. *R. ruber*, 9. *Olivibacter ginsengisoli*, 10. *P. alcaligenes*, 11. *O. jilunii*, 12. *P. muralis*, 13. *Dietzia maris*, 14. *Cupriavidus taiwanensis*, 15. *Nocardia fluminea*, 16. *Pseudomonas stutzeri*, 17. *P. psychrotolerans*, 18. *Massilia timonaee*, 19. *Massilia varians*, 20. *Brevundimonas diminuta*, 21. *Oxalobacteraceae bacterium*, 22. *Alcanivorax venustensis*, 23. *Alcanivorax balearicus*, 24. *Marinobacter hydrocarbonoclasticus*, 25. *Thalassospira profundimaris*, 26. *Amorphus orientalis*, 27. *Aquabacterium citratiphilum*, 28. *Gordonia terrae*. 

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the reducing substance thioglycollic acid (unit III), nor the deletion of NH₄NO₃ (unit II) were associated with any dramatic reduction of oil removal, which amounted to about 50%. Oil removal was also quite effective (57%) in the dark-incubated culture vessel (unit I). All the seawater samples in the six chemostat-like units at the end of bioremediation were rich in hydrocarbonoclastic bacteria. The numbers of the cfu ml⁻¹ were in the magnitudes of 10⁹ and 10¹⁰, compared with only 10⁵ at time zero. The highest bacterial numbers were associated with the highest oil removal values (units IV and V).

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16S rRNA gene sequencing for hydrocarbonoclastic bacteria from continuous cultures

Table 3 presents the results of 16S rRNA gene sequencing of hydrocarbonoclastic bacteria isolated by plating from the six chemostat-like units after incubation for 12 weeks. The seven bacterial isolates exhibited sequence similarities of 99% and 100% to the closest GenBank relatives; they were affiliated with the Actinobacteridae and Gammaproteobacteria.

Composition of the bacterial populations in the six chemostat-like units

Table 4 shows that out of the seven different hydrocarbonoclastic bacterial species identified, only three to five were found in the individual reaction vessels that had been subjected to the studied cultural variables. *Vibrio parahaemolyticus*, *V. diabolicus*, *Alcanivorax dieselolei* and *Mycobacterium chlorophenolicum* shared the predominance in the dark-incubated unit I. The 2 *Vibrio* spp. and *M. chlorophenolicum* in addition to *Gordonia bronchialis* shared the predominance in the NH₄NO₃-deprived seawater samples (unit II). *Vibrio parahaemolyticus* and *D. maris* contributed more than 50% of the total bacterial species in the thioglycolic-acid-amended vessel (unit III). In unit IV, which had received the whole 3% oil lot at time zero, *M. chlorophenolicum*, *V. parahaemolyticus*, *D. maris* and *G. terrae* shared the predominance. In the yeast extract-amended seawater (unit V), *D. maris* formed >60%, and *M. chlorophenolicum* was about 24% of the total. In unit VI with the quick water flow rate, *G. bronchialis*, *D. maris* and *G. terrae* shared the predominance.

Culture-independent analysis of the total bacteria in the six chemostat-like units

The typical denaturing gradient gel electrophoresis (DGGE) profiles in Fig. 6 show that 29 16S rDNA-
amplicon bands were recognized in the seawater samples of the six chemostat-like units. However, sequencing was successful with 14 bands only. Sequences of the residual bands were of low quality, probably due to failure of clean multiple band separation. Table 5 shows that none of the hydrocarbonoclastic bacteria that had been isolated by the culture-dependent method (Table 3) showed up in the list of taxa captured by the culture-independent analysis. As will be discussed soon, such technical problems faced earlier investigators; their bases need to be studied. Even the bacterial classes in both lists were quite different. The culture-independent approach captured among others, two phototrophic organisms, *Lyngbya aestuarii* and an uncultured *Chloroflexi bacterium*, which apparently had their origin in the microbial mat inoculum. Four Alphaproteobacteria; *Paracoccus pantotrophus*, *Maricaulis maris*, *Tistrella mobilis* and *Mesorhizobium thiogeangeticum* as well as two Flavobacteriales; two Gammaproteobacteria and 1 each of the Bacteroidetes; Microgenomates, Tenericutes and Actinobacteria were also detected. Several of those taxa had been recorded in the literature as hydrocarbonoclastic (see below).

**Discussion**

In the beginning, a few relevant facts need to be addressed. Organisms indigenous to a habitat should be able to colonize it easily. Furthermore, they should contribute significantly to activities in this habitat. Organisms not doing so should be regarded as allochthonous. A strain autochthonous in one environment may be allochthonous in another.

To recall, the objective of this contribution was to investigate the feasibility of bioaugmenting local oily desert soil and seawater samples with local microbial mats, for the purpose of soil and water bioremediation. Therefore, we monitored oil removal in bioaugmented batch and continuous cultures, and correlated it with the dynamics of the hydrocarbonoclastic bacterial populations in the studied cultures. Confirming earlier reports on bioaugmentation and biostimulation (*Jimenez et al.*, 2007; Nikolopoulou and Kalogerakis, 2009), our results indicated that both practices were effective in bioremediating hydrocarbons, albeit to varying extents. The fact that individual autochthonous isolates consumed between one fifth and one third of oil implies that the total microbial consortia must be quite effective in cleaning suitable oily habitats. The results generally indicate that the microbial mats are more suitable bioaugmentation materials for bioremediation of hydrocarbon-contaminated soil than seawater samples.

In batch cultures, bioaugmentation-mediated oil removal was more effective in soil than in seawater samples. This is apparently due to the fact that soil contains more nutrients for microorganisms than seawater. Oil removal in seawater ceased 1 month after bioaugmentation, obviously due to the rather quick depletion of the limited nutrients and oxygen. The fact that the bacterial numbers decreased after an initial phase of increase coordinates with the typical growth curve in batch cultures. Judged by the culture stinky smell, anaerobiosis prevailed quite early in seawater, but not in soil cultures. In other words, bioremediation in seawater started under aerobic conditions, which turned anaerobic with time. In soil, on the other hand, aerobic conditions prevailed through the total incubation period. The results imply that batch culture bioremediation for seawater should not exceed 1 month. On the other hand, soil bioremediation should extend for several months. Interestingly, seawater batches, unlike soil batches, did not ‘welcome’ the autochthonous bacterial species of the mat inocula. Instead, the seawater batches were enriched with *M. hydrocarbonoclasticus*, a typical seawater autochthonous bacterium. In other words, the physicochemical parameters in soil, but not in seawater, were suitable for microorganisms indigenous to the mat habitat. This is surprising, since the mats *in situ* are frequently submerged in seawater during high tide. However, the natural resistance of such bacteria to being washed out by tidal movement coordinates with their failure to colonize seawater batches following mat bioaugmentation.

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**Table 4.** Composition of the hydrocarbonoclastic bacterial populations in oily seawater at the end of continuous culture bioremediation as analysed by the culture-dependent method.

| Isolates | % of cfu of total hydrocarbonoclastic bacteria after bioremediation for 12 weeks |
|----------|---------------------------------------------------------------------------------|
|          | % Dark incubated | No NH₄NO₃ added + Thioglycollate | 3% Oil from time zero | + Yeast extract | Quick flow rate |
| Mycobacterium chlorophenolicum | 18.7 | 22.7 | 26.2 | 24.7 | 3% Oil from time zero |
| Vibrio parahaemolyticus | 26.8 | 26 | 30.4 | 26.4 | 14.8 | 3% Oil from time zero |
| Vibrio diabolicus | 24 | 29.1 | 6.5 | 6.4 | – | 3% Oil from time zero |
| Dietzia maris | 8.1 | – | 26.8 | 20.1 | 60.4 | 27.4 | 3% Oil from time zero |
| Alcanivorax dieselolei | 22.2 | – | 13.7 | – | 2.4 | 3% Oil from time zero |
| Gordonia bronchialis | – | 22.2 | 7.2 | – | 38.7 | 3% Oil from time zero |
| Gordonia terrae | – | – | – | 20.6 | – | 26.9 | 3% Oil from time zero |
The facts that oil removal values in soil were higher than in seawater batches and that many of the typical mat bacteria appeared in the oily soil samples reflect and confirm the ready colonization of soil but not seawater by autochthonous mat bacteria. However, oil removal in soil seems to have been due to the collective activity of indigenous mat and indigenous soil bacteria. The dynamic changes of the microbial communities as described in this study confirm that, and probably coped with the types of intermediates of oil biodegradation at the time of analysis.

The short-term continuous culture approach was adopted in this study for seawater remediation via mat bioaugmentation in an attempt to avoid the rather quick cell death in batch cultures. Meanwhile, it was proposed to couple bioaugmentation with biostimulation, as recommended by earlier workers (Odokuma and Dickson, 2003; El Fantroussi and Agathos, 2005). Although much more bacterial diversity in seawater was noted during continuous than batch culture, out of the seven identified hydrocarbonoclastic bacterial species, only *M. chlorophenolicum* seemed to have had its origin in the mat. Other species were indigenous seawater inhabitants. However, the culture-independent analysis revealed a different list of bacteria, many of which belonged

Fig. 6. Upper: Typical DGGE profiles of 16S rRNA amplicons of total genomic DNA samples extracted from seawater in the reaction vessels of the six chemostats. For band identities see Table 5. Lower: Cluster analysis using Euclidean distances.

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to the Alphaproteobacteria, Gammaproteobacteria and Flavobacteriales. Earlier researchers, too, found that Gammaproteobacteria (Alcanivorax, Marinobacter) and Alphaproteobacteria were ‘key players’ in oil degradation in contaminated Mexico beach sands (Kostka et al., 2011). The fact that culture-dependent and culture-independent approaches capture dissimilar bacterial taxa confirms and consolidates earlier reports from our (Al-Awadhi et al., 2013) and other laboratories (Polz and Cavanaugh, 1998; Sipos et al., 2007).

The aerobic continuous culture approach was effective in oil removal under certain conditions; the following relevant recommendations may be made. First, to start the continuous fermentation with the highest tolerable oil concentration, i.e. not to feed it as smaller aliquots during the course of bioremediation. Second, to provide the system with vitamin-containing natural products. In this context, vitamins have been reported earlier to enhance microbiological hydrocarbon biodegradation (Radwan and Al-Muteirie, 2001; Al-Mailem et al., 2013). Third, the water flow should be considered critically; too quick flow inhibits oil removal. No addition of nitrogenous compounds is needed, probably many of the inhabitants are diazotrophic, e.g. Dietzia, Gordonia and most of the hydrocarbonoclastic bacteria (Dashti et al., 2015). Reducing substances such as thioglycollate do not seem to inhibit the bioremediation process, even though molecular oxygen is involved in the initial step of microbial attack on the hydrocarbon substrate (Ratledge, 1978; Radwan, 2009). The mats seem to harbour adequate oxygenic phototrophic inhabitants, which keep the cultures well aerated.

In conclusion, although local, environmental samples must be used for bioaugmentation, the physicochemical parameters in the targeted, contaminated site must be suitable for the inoculated microbial taxa. Should this not be the case, the bioaugmented bacteria would not contribute significantly to bioremediation. Specifically in this study, bioaugmentation of desert soil with costal mat is a typical autochthonous bioaugmentation practice. The mat bacteria showed up and exhibited dynamic behaviour in the soil. On the other hand, bioaugmentation of seawater with the same material merits the designation ‘allochthonous bioaugmentation’. With the exception of taxa naturally inhabiting both materials (see Table 1), mat bacteria failed to show up in seawater, which obviously was bioremediated via the typical seawater bacteria only. From a practical viewpoint, allochthonous bioaugmentation is obviously useless as a bioremediation approach.

### Experimental procedures

#### Coastal microbial mats

Information on the microbial composition of such mats and their relation to the self-cleaning of the Gulf is available in one of our earlier reports (Sorkhoh et al., 1992). The mats consist of phototrophic microorganisms, predominantly the filamentous cyanobacterium *Microcoleus* sp., which harbour in its filament sheaths millions of cells of hydrocarbonoclastic bacteria per gram fresh mat. Mat samples used in this study were freshly collected from the Soq Sharq coast of Kuwait City (see Kuwait map in Fig. 1). The samples were transported in sterile conical flasks to the laboratory to be processed in the same day. Culture-dependent counting (see below) revealed that each gram of fresh mat harbour 2.8 × 10^8 hydrocarbonoclastic bacterial cells.

#### Seawater and desert soil samples

Seawater samples from the Arabian Gulf and desert soil samples from stations at the north, middle and south of Kuwait (see the map in Fig. 1) were used for this bench scale
bioremediation study. Seawater samples were collected from Subbyah, Kuwait Towers and Khiran areas, about 5 m off-shore. Desert soil samples were collected from Kadma, Shuaybah and Wafra areas. The samples were transported to the laboratory and started to be processed in the same day. Three samples, 10 m apart, were collected from each site, pooled, mixed thoroughly and used in the bioremediation experiments, as described below. The water-holding capacity of the soil samples ranged between 57.3 and 57.7%, w/w.

Oil bioremediation in batch cultures

All experiments were done in triplicates. Fifty milliliter aliquots of pooled seawater were dispensed in 250 ml conical flasks. Pooled soil samples, 50 g, were also dispensed in 250 ml conical flasks and suspended in 50 ml aliquots of sterile tap water. Each flask received in addition 1%, w/v, weathered Kuwait light crude oil. To compare the behaviour of the inoculated microorganisms in the absence and presence of the inhabitant microorganisms, the flasks of one set were sterilized by autoclaving to kill the already existing microorganisms (designated sterile), and the flasks of another set were left unautoclaved (designated fresh). Each flask was inoculated with 1 ml (= 2 g mat) cell suspension prepared by homogenizing 200 g mat in 100 ml seawater. The flasks were sealed to avoid oil loss by volatilization and incubated at 30°C. At time zero and at monthly intervals, flasks were taken for residual oil recovery and quantitative determination, as well as for microbiological analysis. The mean values of the readings from the three replicates were determined and the standard deviations were calculated.

Oil bioremediation in continuous cultures

This approach was used for bioremediating oily seawater samples only. For this, six identical chemostat-like units were constructed, each consisting of a seawater vessel and a receiver vessel. The seawater vessel contained the freshly collected seawater to be continuously fed (by gravity effect) into the culture vessel. If not otherwise specified, the water flow rate was adjusted at 6 ml h\(^{-1}\). This rate was determined in preliminary experiments. Each culture vessel received at time zero 200 ml seawater which was inoculated only once with 1 ml mat suspension as bioaugmentation material. Five of the culture vessels received at time zero and every 2 weeks, 0.5 g aliquots (totally 3 g per vessel) of weathered Kuwaiti light crude oil. The sixth vessel received the whole 3 g oil in one lot at time zero. The six chemostat-like units were set up to compare oil bioremediation as affected by six different biostimulation treatments:

I – To study the effect of light; the culture vessel of unit I was dark incubated by wrapping it in three successive layers of aluminum foil. The remaining five culture vessels were left exposed to day (about 13 h)–night (about 11 h) cycles.

II – To study the effect of added nitrogen fertilizers, culture vessel II was set up without added NH\(_4\)NO\(_3\), unlike other culture vessels that contained 0.5%, w/v, NH\(_4\)NO\(_3\).

III – To study the effect of the redox potential, one of the day–night exposed, NH\(_4\)NO\(_3\)-containing culture vessels (unit III) was provided with 0.025%, w/v, thioglycollic acid. The remaining vessels did not receive this reducing substance.

IV – To study the feasibility of starting bioremediating using high-oil concentration, instead of adding it in aliquots during incubation, one culture vessel (unit IV) received at time zero the whole 3 g crude oil in one lot, as described above.

V – To study the effect of vitamins, only one (unit V) of the day–night-exposed, NH\(_4\)NO\(_3\)-containing culture vessels received 0.2%, w/v, yeast extract.

VI – To study the effect of the seawater flow rate, the rate in unit VI was adjusted at 30 ml h\(^{-1}\); in the other five it was kept at 6 ml h\(^{-1}\). All the chemostat-like units were incubated under room conditions for 12 weeks.

Measurements of oil consumption

Residual hydrocarbons in the contents of individual chemostat-like units were recovered at the end of the 12-week incubation by extraction with three successive 20 ml portions of pentane. Extraction involved oil still adhering to the vessel walls and connecting tubes. The combined extract was raised to 60 ml using pure pentane, and 1 μl was analysed by gas liquid chromatography (GLC) using a Varian 3900 (USA) instrument equipped with an Flame Ionization Detector (FID), a Wall coated Open Tubular (WCOT)-fused silica CP-Sil 5 CB capillary column (Varian, USA), and a temperature program 45–310°C with temperature rising 10°C min\(^{-1}\), using N\(_2\) as a carrier gas. The detector temperature was 300°C and injector temperature 270°C. The percentage of oil consumption was calculated as the percentage reduction of total hydrocarbon peak areas in the GLC profiles based on the total areas of peaks in the GLC profiles at time zero. A similar method was used to determine crude oil consumption by individual bacterial isolates in batch cultures. A mineral medium (Sorkhoh et al., 1990, see below) which 1 g l\(^{-1}\) oil as a sole source of carbon and energy was used. Each flask was inoculated with 1 ml of bacterial suspension (one loopful in 5 ml water). Triplicates were prepared throughout. The flasks were incubated under room conditions for 14 days.

Culture-dependant analysis of hydrocarbonoclastic bacteria

The conventional dilution plating method was used for counting hydrocarbonoclastic bacteria in the cultures. A solid mineral medium (Sorkhoh et al., 1990) with oil vapour as the sole source of carbon and energy was used. The medium consisted of (g l\(^{-1}\)): 30.0 NaCl, 5.0 NaNO\(_3\), 0.56 KH\(_2\)PO\(_4\), 0.86 Na\(_2\)HPO\(_4\), 0.17 K\(_2\)SO\(_4\), 0.37 MgSO\(_4\).7H\(_2\)O, 0.7 CaCl\(_2\)2H\(_2\)O, 2.5 ml of trace element mixture (g l\(^{-1}\)): 2.3 ZnSO\(_4\), 1.8 MnSO\(_4\), 0.6 H\(_3\)BO\(_3\), 1.0 CuSO\(_4\), 0.4 NaMoO\(_4\), 0.4 CoCl\(_2\), 0.7 KI, 1.0 EDTA, 0.4 FeSO\(_4\), 0.004 NiCl\(_2\), pH 7.0. Agar, 20 g l\(^{-1}\) was added for medium solidification. Each plate lid was provided with a filter paper impregnated with 2 ml crude oil, and the covered plates were sealed after inoculation with 0.25 ml of each inoculum. The volatile oil vapour was the sole source of carbon and energy available to the developing colonies. The plates were incubated at 30°C for 10 days. The total
colony-forming units (cfu) were counted, and the mean values ± standard deviation values calculated per millilitre seawater or gram soil. Parallel plates were pooled and colonies with identical morphologies were counted and their percentage of the total calculated. For characterization of individual bacterial isolates, their 16S rRNA gene sequences were compared with the closest sequences in the GenBank database. The PrepMan Ultra Kit (Applied Biosystems, Foster City, CA, USA) was used to extract genomic DNA from pure isolates, and the 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the GM5F (5′-CCTACGGAGAACGACG-3′) and 907R (5′-CCCCGTCGAATCTM TTGTAGGG-3′) primers (Santegoeds et al., 1998). The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) in order to remove the Taq polymerase, primers and deoxy nucleotide triphosphates (dNTPs). Partial sequencing of the 16S rRNA genes was performed using the BigDye version Terminator Kit (Applied Biosystems, Warrington, UK). Pure template DNA samples were processed in the 3130 × 1 genetic analyser (Applied Biosystems, Foster City, CA, USA). Sequencing analysis version 5.2 software (Applied Biosystems, Foster City, CA, USA) was used to analyse the results. Sequences were subjected to basic local alignment search tool analysis with the National Center for Biotechnology Information (Bethesda, MD, USA) GenBank database (Altschul et al., 1997). A phylogenetic tree was constructed by neighbour-joining including bootstrap analysis using PAUP* v.4 (Swafford). Bootstrap proportions were used on 2000 replicates.

Culture-independent analysis of total bacteria

To analyse the total bacterioplankton in various cultures, the total genomic DNA was extracted using the Rapid Water DNA Isolation Kit (MO-BIO, Carlsbad, CA) for (media) and the Fast Nucleic Acid Res Soil Kit (MP Biomedicals, LIC., France). The 16S rRNA genes in the genomic DNA samples were partially amplified using the universal primer pair GMSF (with a G (guanine) C (cytosine) clamp) and 907R (Schäfer and Muyzer, 2001). The resulting amplicons were resolved by parallel DGGE using the DCode Universal Mutation Detection System (Bio-Rad, California, USA). The denaturant concentrations were 45–60%. Electrophoresis was run under constant voltage of 50 V at 60°C for 16 h. Gels were stained with SYBR Green (Invitrogen, USA) in 1xTAE buffer (1:100,000) for 30 min and inspected using a Dark Reader transilluminator (Clare Chemical Research, CO, USA). The bands were transformed into binary matrix; the presence of bands was given the weight of ‘1’ and their absence ‘0’. The binary matrix produced was analysed using cluster analysis, and a dendogram was plotted. Gel bands carrying 16S rRNA fractions were excised and stored overnight in 50 μl molecular water (Sigma, UK) at 4°C to elute the DNA. One microliter of the eluted DNA was amplified using the above primer pair, sequenced, and the sequences were compared with those in the GenBank database.

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Conflict of interest

The authors declare that they have no conflict of interests.

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