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Higher oil biodegradation potential at the Arabian Gulf coast than in the water body

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Abstract Littoral materials collected from the intertidal zone along the coast of Kuwait City were associated with much higher numbers of oil-utilizing microorganisms than inshore and offshore water samples. Animate materials viz. epilithic biomass, cyanobacterial mats and roots of higher plants were richer in such microorganisms than inanimate materials, e.g. littoral sand, rock pieces, shells and others. Those numbers remained highest during the autumn, winter and spring and decreased dramatically during the hot summer. By far, the predominant indigenous oil-utilizing bacterium in the marine environment of Kuwait was *Acinetobacter calcoaceticus*. Less dominant organisms included *Micrococcus* sp., nocardioforms and others. Coast-immobilized strains of *A. calcoaceticus* and *Micrococcus* sp. had a higher hydrocarbon degradation potential than planktonic strains of the same organisms. It was concluded that marine coasts have a much higher potential for oil biodegradation than the water body.

Introduction

The management of oil pollution in the sea is technically difficult. Being open, this environment is much less controllable than the terrestrial environment. Therefore, bioremediation approaches such as seeding with hydrocarbon-degrading microorganisms and fertilization with microbial growth-enhancing nutrients may be infeasible in the open sea (Applied Biotreatment Association 1990; US Congress, Office of Technology Assessment 1991).

In contrast, indigenous oil-utilizing microorganisms immobilized on coastal materials may prove more promising than planktonic microorganisms for hydrocarbon attenuation in the marine environment. This may be expected particularly for the Arabian Gulf, in which oil pollutants released naturally or during transport normally drift along the coasts with water currents and tidal movement.

After the 1990/91 Iraq–Kuwait conflict, massive growth of cyanobacterial mats was observed on the top of oil sediments, currently present in the intertidal zone of the western coasts of the Gulf (Sorkhoh et al. 1992). Such mats immobilize rich populations of organotrophic bacteria which, together with the cyanobacteria, contribute to oil biodegradation (Al-Hasan et al. 1994, 1998; Sorkhoh et al. 1995). There is an information gap regarding the frequencies and identities of hydrocarbon degraders that may be associated with other coastal materials, such as epilithic macroalgal biomass, shells and sand.

The main objectives of this work were to count and identify oil-utilizing microorganisms associated with animate and inanimate materials near Kuwait City coasts and to compare them with planktonic microorganisms in adjacent waters. We also compared the potential of individual isolates from coastal materials and waters for hydrocarbon consumption. This information should be useful in the management of marine oil pollution.

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Materials and methods

Sampling

Samples were collected from three coastal localities near Kuwait City, two at both distal ends of the City, Al-Shuwaikh and Al-Messilah coasts, and one in the middle; Al-Salmiyah coast. Sampling was done during the four seasons of the year. The samples

included inanimate and animate littoral materials, as well as adjacent inshore (1 m from the coast) and offshore (2 km) waters. The inanimate materials included littoral rock, wood, metal bars, cloth, shells (empty), plastic and littoral sand (surface 2 cm). The animate materials were: epilithic biomass (rich in macroalgae), cyanobacterial mats (mainly *Phormidium* sp. and *Microcoleus* sp.) and roots of the halophile *Suaeda aegyptiaca* (Chenopodiaceae). The samples were collected in sterile plastic bags. Water samples at different depths of the water column were collected in sterile vials. Processing of all samples began within 3 to 5 h after collection.

Microbiological analysis

Microorganisms capable of utilizing crude oil were counted using the standard plate method. We used a solid inorganic medium supplemented with 1% (w/v) weathered crude oil as a sole source of carbon and energy (Sorkhoh et al. 1990). The inorganic medium had the following composition (l^{-1}): 30 g NaCl, 0.85 g $NaNO_3$, 0.56 g KH_2PO4, 0.86 g Na_2HPO_4, 0.17 g K_2SO_4, 0.37 g $MgSO_4{\cdot}7H_2O$, 0.007 g $CaCl_2{\cdot}H_2O$, 0.004 g Fe (III) EDTA; and trace element solution (0.25 ml) consisted (l^{-1}) of: 2.32 g $ZnSO_4{\cdot}7H_2O$, 1.78 g $MnSO_4{\cdot}4H_2O$, 0.56 g KBO_3, 1.0 g $CuSO_4{\cdot}5H_2O$, 0.39 g $Na_2MoO_4{\cdot}2H_2O$, 0.66 g KI, 1.0 g EDTA, 0.4 g $FeSO_4{\cdot}7H_2O$, 0.004 g $NiCl_2{\cdot}6H_2O$, 15.0 g agar; pH 7. Cultures were incubated at 30 °C. After counting the total and individual organisms, representative strains were isolated on the same medium, purified and identified by consulting pertinent keys, and comparing them with previously identified strains in our collection.

Hydrocarbon utilization potential

To estimate qualitatively the potential of isolated strains for utilizing individual alkanes and aromatic hydrocarbons, a loopful of the liquid culture was streaked on solid inorganic basal medium aliquots containing 0.5% (w/v) of individual hydrocarbons spread on their surfaces. The cultures were incubated at 30 °C for 7 d, and the growth visually estimated and compared with that on conventional nutrient agar after the same incubation period.

The potential of the strains to consume hydrocarbons was determined quantitatively. Each 0.5 g fresh cells of the test strain (48 h cultures) was suspended in 25 ml inorganic medium provided with 10 mg of the individual test hydrocarbons, *n*-octadecane or phenanthene. These compounds were selected because they are natural constituents of crude oil. A control sample was prepared by following the same procedure but adding autoclaved biomass. The culture bottles were sealed and incubated in a shaking water bath, 170 rpm at 30 °C for 12 h. Bacterial cell samples were removed by centrifugation and quickly washed with boiling inorganic medium to recover adhering hydrocarbons. Both the supernatant and the washing aliquots were used to extract residual hydrocarbons by three 5 ml aliquots of diethylether. After volatilizing the solvent, the residual hydrocarbons were dissolved in 3.0 ml hexane, and 1 µl aliquots were analyzed by gas liquid chromatography (GLC) using a Chrompack CP-9000 instrument equipped with a flame ionization detector, a WCOT fused silica capillary column, and a temperature program of 150 to 310 °C, raising the temperature 20 °C min^{-1} starting from 182 °C. The peak areas for the hydrocarbons were measured, and the percent decreases based on the areas of the control peaks calculated; these values were taken as a quantitative measure of hydrocarbon consumption.

Results

Table 1 shows the numbers of oil-utilizing microorganisms attached to littoral materials and free in water, as determined by the standard plate method. The data indicate that the littoral materials were much richer in oil-utilizing microorganisms than the water samples. The animate materials, viz. epilithic macroalgae, cyanobacterial mats and roots of coastal plants, were associated with much higher numbers of these microorganisms than the inanimate materials. The inshore water samples (maximum depth about 100 cm) as well as the offshore surface water samples were much poorer than the coastal materials in oil-utilizing microorganisms.

Table 1 shows further that the highest numbers of oil-utilizing microorganisms associated with all materials studied were in the autumn, winter and spring. In summer the microbial numbers in all littoral samples exhibited a dramatic drop. Even the planktonic oil-utilizing microorganisms decreased in the summer, albeit relatively to a much lesser extent.

The results in Table 2 show that *Acinetobacter calcoaceticus* was by far the most dominant oil-utilizing microorganism in the marine environment of Kuwait City. This was true both for littoral animate and inanimate materials as well as water samples throughout the year. Characteristic of this aerobic, Gram-negative, nonmotile, short rod-like species was its ability to utilize ethanol and acetate as sole sources of carbon and energy. In some samples during certain seasons *A. calcoaceticus* strains were the only oil-utilizing microorganisms that grew on the counting plates. Less dominant, yet usually predominant over the rest of the oil-utilizing microorganisms were various strains of the genus *Micrococcus*. Minor oil-utilizing microorganisms included various strains of nocardioforms, actinomycetes and yeasts.

Table 3 shows the estimated growth qualities of bacterial isolates on solid media containing individual hydrocarbons as sole sources of carbon and energy. The results demonstrate the rather wide range of pure hydrocarbons that the predominant isolates could utilize. Medium- and long-chain *n*-alkanes, with up to C40-chains, as well as various aromatic hydrocarbons could serve as sole carbon and energy sources for the predominant microorganisms, to varying extents.

Table 4 presents quantitive data of hydrocarbon consumption by predominant isolates, as determined by GLC analysis. The results show that strains of *Acinetobacter calcoaceticus* and *Micrococcus* sp. immobilized on epilithic macroalgae had a higher potential for *n*-octadecane and phenanthrene consumption than planktonic strains of the same organisms isolated from inshore water.

Discussion

It is quite interesting that the numbers of oil-utilizing microorganisms immobilized on littoral materials were by far much higher than those of planktonic microorganisms suspended in water (Table 1). These microorganisms belong predominantly to the genera *Acinetobacter* and *Micrococcus* (Table 2) and could grow on a wide range

Table 1 Numbers ($\times 10^5$) of oil-utilizing microorganisms immobilized on littoral materials and free in the water body. Each value is the mean of three parallel determinations, the standard deviation values did not exceed 6.5% of the mean values (*ND* not determined)

Materials (No. of samples)	Autumn	Winter	Spring	Summer
Inanimate materials				
Rock pieces (11)[a]	36.7–347.0	24.0–50.0	1.8–55.0	0.01–0.3
Wood piece (1)[a]	180.4	98.0	178.3	0.01
Iron bar (1)[a]	44.0	46.1	52.7	< 0.01
Cloth piece (1)[a]	51.7	42.0	56.1	< 0.01
Shells (13)[a]	11.1–43.8	0.2–6.0	0.1–1.3	0.01
Synthetic material (plastic) (1)[a]	27.5	22.1	0.3	< 0.01
Sand (10)[b]	170–375	291–415	98–152	< 0.01
Animate materials				
Epilithic biomass (8)[b]	ND	27800–59800	3.6–10.7	< 0.1
Cyanobacterial mats (9)[b]	980–1460	560–1320	2300–4123	5.0–25.0
Plant roots (3)[a]	3400–6600	2100–4300	ND	ND
Inshore water[c]				
Surface (10)	0.2–0.3	0.01–0.02	0.01–0.02	< 0.01
5 cm deep (10)	0.1–0.2	0.01–0.02	0.02–0.03	< 0.01
10 cm deep (10)	0.02–0.03	0.01–0.02	0.02–0.03	< 0.01
100 cm deep (10)	0.03–0.05	0.02–0.03	0.02–0.03	< 0.01
Offshore water[c]				
Surface (5)	0.1–0.3	0.01–0.02	0.01–0.02	< 0.01

[a] Number cm^{-2} of the material surface
[b] Number g^{-1} of the material
[c] Number ml^{-1} of water

Table 2 Composition of oil-utilizing microorganisms immobilized on littoral materials and free in the water body. Data are percent values of the total microorganisms (*ND* not determined)

Materials (No. of samples)	Winter				Spring			
	Acinetobacter calcoaceticus	*Micrococcus* sp.	No cardioforms[a]	Others[b]	*Acinetobacter calcoaceticus*	*Micrococcus* sp.	No cardioforms[a]	Others[b]
Inanimate materials								
Rock pieces (11)	70.5–100.0	0.0–19.1	0.0–9.3	0.0–1.1	95.3–100.0	0.0–4.3	0.0–0.4	0.0
Wood piece (1)	95.4	0.2	3.3	1.1	100.0	0.0	0.0	0.0
Iron bar (1)	84.7	6.0	8.1	1.2	96.6	3.3	0.1	0.0
Cloth piece (1)	85.8	11.4	2.1	0.7	56.6	42.1	0.3	0.0
Sea shells (13)	81.2–96.7	1.6–17.6	1.0–1.7	0.0–0.2	99.2–100	0.0–0.8	0.0	0.0
Plastic piece (1)	84.7	1.3	11.5	2.3	58.5	40.5	1.0	0.0
Sand (10)[b]	76.1–100.0	0.0–13.2	0.0–8.7	0.0–2.0	91.0–100	0.0–9.0	0.0	0.0
Animate materials								
Epilithic biomass (8)	95.3–100.0	0.0–3.7	0.0–0.8	0.0–0.2	98.1–100.0	0.0–1.7	0.0–0.2	0.0
Cyanobacteriol mats (9)	86.2–87.6	3.1–10.5	1.8–2.4	0.1–1.1	40.5–49.9	59.5–50.1	0.0	0.0
Plant roots (3)	32.5–66.6	20.9–32.2	0.0	1.2–46.5[c]	ND	ND	ND	ND
Inshore water								
Surface (10)	79.1–94.9	2.8–10.9	2.0–8.1	0.3–1.9	25.1–75.5	74.9–24.5	0.0	0.0
5 cm deep (10)	98.0–100.0	0.0–1.5	0.0–0.4	0.0–0.1	98.2–100.0	0.0–1.8	0.0	0.0
10 cm deep (10)	77.2–100.0	0.0–22.7	0.0–0.1	0.0	ND	ND	ND	ND
100 cm deep (10)	81.3–100.0	0.0–18.3	0.0–0.4	0.0	ND	ND	ND	ND
Offshore water								
Surface (5)	18.5–22.7	18.5–38.6	4.2–19.4	19.3–58.8[c]	28.2–35.6	71.8–64.4	0.0	0.0

[a] Mainly *Rhodococcus* strains
[b] Unless otherwise specified, mainly *Pseudomonas*, *Arthrobacter* and *Bacillus* strains
[c] Mainly actinomycetes tentatively identified as *Streptomyces* strains

of individual hydrocarbons (Table 3), demonstrating their potential for biodegrading oil constituents. Each gram of littoral epilithic biomass in the autumn or winter may be associated with more than one- to ten-million-fold more oil-utilizing microorganisms than 1 ml (1 g) of inshore water. The fact that also littoral sand was rather rich in such microorganisms underlines the higher oil degradation potential of coast-immobilized than planktonic microorganisms. Obviously, such organisms are spread through the whole intertidal zone in numbers much higher than in the water body. To our knowledge this significant result has not been reported

Table 3 Growth qualities of predominant isolates from littoral materials and inshore water on solid media containing individual hydrocarbons as sole sources of carbon and energy. Growth comparable with that on conventional nutrient agar was designated "excellent"; weaker growth was estimated as "good" or "weak". Benzene, phenanthrene, naphthalene, xylene, tolune, pyrene, biphenyl (*aromatics*) were tested individually (*KCC* Kuwaiti Culture Collection)

Isolate	Source	Hydrocarbons		Growth
Acinetobacter calcoaceticus KCC 101	Epilithic biomass	*n*-Alkanes:	C_9–C_{11}, C_{24}–C_{30}	Good
			C_{12}–C_{23}	Excellent
			C_{32}, C_{36}, C_{40}	None
		Aromatics		Good
Acinetobacter calcoaceticus KCC 102	Water	*n*-Alkanes:	C_9–C_{15}, C_{19}, C_{30}	Weak
			C_{16}–C_{18}	Excellent
			C_{32}, C_{36}, C_{40}	None
		Aromatics		Good
Micrococcus sp. KCC 108	Epilithic biomass	*n*-Alkanes:	C_9–C_{11}, C_{24}–C_{30}	Weak
			C_{12}–C_{23}	Good
			C_{32}, C_{36}, C_{40}	Weak/None
		Aromatics		Excellent
Micrococcus sp. KCC 109	Water	*n*-Alkanes:	C_9–C_{15}, C_{19}, C_{30}	Weak
			C_{16}–C_{18}	Good
			C_{32}, C_{36}, C_{40}	Weak/None
		Aromatics		Good
Nocardioforms	Epilithic biomass	*n*-Alkanes:	C_9	Good
			C_{10}, C_{30}, C_{32}	
			C_{36}, C_{40}	Excellent
		Aromatics		Excellent
Nocardioforms	Water	*n*-Alkanes:	C_9–C_{12}	Good
			C_{13}–C_{30}, C_{32}	
			C_{36}, C_{40}	Excellent
		Aromatics		Good

Table 4 Consumption of pure hydrocarbons by predominant isolates from littoral materials and inshore water as determined by GLC. Data are means ± SD of three determinations (*KCC* Kuwaiti Culture Collection)

Isolates	Source	*n*-Octadecane consumed (%)	Phenanthrene consumed (%)
Acinetobacter calcoaceticus KCC 101	Epilithic biomass	73.9 ± 2.1	62.8 ± 1.9
Acinetobacter calcoaceticus KCC 102	Water	21.2 ± 0.4	32.1 ± 0.7
Micrococcus sp. KCC 108	Epilithic biomass	18.2 ± 0.5	47.3 ± 0.9
Micrococcus sp. KCC 109	Water	13.5 ± 0.8	13.2 ± 0.7

before for marine ecosystems. The fact that animate materials were associated with higher microbial numbers than inanimate materials is probably due, in part, to the former providing the microorganisms with better aerated and nutrient-rich microenvironments. Epilithic macroalgae and cyanobacteria produce oxygen via photosynthesis, and plant roots are known to pump oxygen from the aerial plant organs into the rhizosphere. It has been reported that oxygen concentration is a factor limiting hydrocarbon biodegradation in aquatic and terrestrial environments (Jamison et al. 1975; von Wedel et al. 1988). In this context, the initial step of hydrocarbon biodegradation involves substrate oxidation by oxygenases that need molecular oxygen (Cerniglia 1984; Perry 1984; Singer and Finnerty 1984). Animate materials may also provide microorganisms with specific needs such as vitamins.

The high oil degradation potential of coast-immobilized microorganisms is obviously limited to the autumn, winter and spring. In the summer this potential is dramatically lowered. Littoral epilithic biomass was associated in the winter with over one-million-fold more oil-utilizing microorganisms than in the summer (Table 1). The decrease in microbial numbers started in May, and the low numbers were maintained till October. Planktonic oil-utilizing microorganisms did not exhibit such dramatic seasonal fluctuation in numbers. It appears that water protects the microorganisms from the stressful temperatures (80 °C and higher) normally prevailing on the Arabian Gulf coast surfaces during the summer.

The higher hydrocarbon degradation potential of coast-immobilized microorganisms than that of planktonic microflora is not only due to variation in microbial numbers but also to differences in strain activity. Strains of coastal origin have a higher hydrocarbon consumption potential than planktonic strains belonging to the same genus and species (Table 4). This difference may be due to adaptive responses of coastal strains that are permanently exposed to higher levels of oil than planktonic strains. As mentioned above, oil is continuously carried by tidal movement from the open water to the coast.

In conclusion, marine coasts have a much higher potential for oil biodegradation than inshore and offshore waters. This result should be of value when

considering bioremediation technologies for oil-polluted marine habitats. If this finding proves valid globally, bioremediation strategies should be refocused on attenuating oil pollutants along the coast rather than in the water body. The coastal environment obviously provides the further advantage that it can be more easily managed and controlled than the open sea.

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