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Increment in Anaerobic Hydrocarbon Degradation Activity of Halic Bay Sediments via Nutrient Amendment

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Abstract In this study, hydrocarbon (HC) degradation activity of a HC-rich marine sediment was assessed in anaerobic microcosms during a 224 days incubation period. Natural TOC/N/P ratio of the sediment porewater (1,000/5/1) was gradually decreased to 1,000/40/6 which resulted in approximately ninefold increase in gas production (CH_4+CO_2) and HC removal. Addition of external HCs to the microcosms was also resulted in approximately twofold higher gas production and HC removal. A high proportion (92%) of aromatic HCs and all *n*-alkanes were removed from the microcosms under unlimited nutrient supply conditions without external HC addition. The microorganisms of the sediment degraded a wide range of aliphatic (*n*-$C_{9–31}$ alkanes and acyclic isoprenoids) and aromatic (18 different one- to five-ring aromatics) HCs. Monitoring functional gene and transcript abundances revealed that methanogenesis and dissimilatory sulfate reduction took place simultaneously during the first 126 days, afterwards, only the syntrophic methanogenic consortium was active. Genes and transcripts related to initial activation of HCs were highly abundant throughout the incubation period showing that fumarate addition was the main pathway of anaerobic HC degradation. In conclusion, biostimulation of highly polluted anoxic marine sediments via nutrient amendment is effective and may constitute a suitable and cost-effective field-scale bioremediation strategy.

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Introduction

Petroleum hydrocarbons (HCs) are one of the most important organic pollutants in marine environments [19, 21]. Although anaerobic HC degradation is widespread and has been reported under nitrate-, iron-, manganese- and sulfate-reducing and methanogenic conditions [14, 18, 62], natural attenuation of oil in anoxic marine sediments is slow and oil deposits persist for many years [14, 19, 26, 35]. Biological HC removal from the sediments can be accelerated through addition of limiting nutrients (biostimulation) or HC-degrading microbes (bioaugmentation) [38].

Bioaugmentation with bacteria capable of anaerobic degradation is untested. This is because added microbes are at a disadvantage in competition with the indigenous microbiota and, even under aerobic conditions, successful bioaugmentation trials are sparse [14].

Since the type and concentration of terminal electron acceptors (TEAs) have a major effect on the outcome of bioremediation, TEA addition is the most studied way of accelerating HC biodegradation in anoxic environments. Addition of sulfate [2] and nitrate [49] was shown to enhance in situ degradation of mono aromatic HCs in petroleum-contaminated aquifers. Three independent studies reported that amending with both nitrate and sulfate rather than the

just one was advantageous due to existence of a heterogeneous microbial population: (1) Cunningham et al. [11] accelerated the natural attenuation of petroleum-contaminated groundwater; (2) in situ phenanthrene biodegradation was enhanced threefold in a marine sediment [55]; (3) the rate of diesel fuel degradation was the highest under mixed electron acceptor conditions in marine sediment microcosms [5]. Overall outcomes of these studies pointed out that a response to TAE amendment depends on indigenous microbiota and redox conditions, and the contaminated environment must be well characterized to apply a site-specific biostimulation strategy.

HC-polluted environments usually experience complex mixtures of contaminants that can influence the success of TAE amendment. In a survey of various aquatic sediments incubated under four different redox conditions, Phelps and Young [41] found that degradation of a mixture of benzene, toluene, ethylbenzene, and *m*+*p*+*o*-xylene (BTEX) compounds in cultures amended with aliphatic HCs was slower and incomplete. This negative effect contrasts with the stimulatory effect of aliphatic HCs on aromatic HC degradation in microcosms under methanogenic and sulfate-reducing conditions [44]. These conflicting observations emphasize the unpredictability of site-specific responses to mixed substrates.

The stimulatory effect of providing nutrients, such as fixed nitrogen and/or phosphate, has not been as thoroughly studied under anaerobic conditions as under aerobic conditions [14, 38]. Two cases show the benefit of fertilizing nutrient-poor anaerobic environments contaminated with diesel fuel: Cross et al. [10] observed enhanced anaerobic degradation when contaminated groundwater microcosms were amended with ammonium, nitrate, and phosphate, and Powell et al. [43] noted the stimulatory effect of nitrate, ammonium, phosphate and calcium on denitrifying HC degraders in nutrient-poor Antarctic soils. The fertilization to increase anaerobic HC degradation activity of marine microbenthos has not been tested yet.

Massive C loads from oil spills or chronic HC pollutions can be removed from marine sediments by microorganisms as long as (1) dissolution and hydrolysis of particulate nutrient forms are not rate limiting for microbial growth and (2) dissolved C/N/P ratio of the porewater is close to that of marine bacterioplankton (100/20/3) [60]. Although excessive N–P inputs from anthropogenic pollution sources change the nutrient balance toward low C ratios in the sediments, C/N/P ratio of the porewaters might still be much higher than 100/20/3 due to very slow dissolution/hydrolysis of particulate N–P forms. This has been the case for Marmara Sea, which has very high C/N/P ratios in the porewaters (~100/0.5/0.1) despite the low C ratios in the sediments (~100/50/10) [24]. N and P amendment is an option to increase microbial HC removal in this ecosystem.

Marmara Sea connects Black Sea to Aegean Sea via Bosphorus Strait, covering an area of 214,000 km^2. It has been over polluted with petroleum HCs [29]. The pollution has been originated from: highly polluted Black Sea, spills from oil tankers (~35 accidents/year), discharges during marine transportation (~50,000 tankers/year), municipal wastes (~20 million people), industrial wastes (40% of the Turkish Industry), atmospheric deposition, and urban surface and river runoff [40, 59]. The extreme pollution has led to approximately fivefold increase in anoxic areas during the last 30 years (Turkish Water Agency, personal communication).

Halic Bay is located in Istanbul, at a junction between Bospshorus Strait and Marmara Sea. The 8-km long bay has a water surface area of 2.6 million m^2. Its depth changes between 2 and 60 m depending on the regional waste input level and the deep water flow rate. Aromatic and aliphatic hydrocarbon levels in the sediments were in ranges of 4,000–6,000 and 1,500–3,000 ppm, respectively, which were similar to that of the extremely hydrocarbon-contaminated marine environments [1, 57]. The high and chronic pollution has resulted in formation of a highly anaerobic deep sludge over the years [23].

Water and Sewerage Administration of Istanbul (ISKI) and Istanbul Metropolitan Municipality (IBB) started "Halic Cleaning Project" in the late 1980s (http://www.iski.gov.tr/Web/statik.aspx?KID=1000470); ~5-million-m^3 sludge was collected by physical screening, and then the water body was aerated. The water quality was improved significantly in a very short time period (15 months); on the other hand, the remediation cost was remarkably high. Halic Bay ecosystem slowly returned to its over polluted status after the completion of the project (during the last 20 years).

A cost-effective remediation strategy that can be sustained for a long time with a minimal human intervention is needed to overcome the chronic HC pollution in Halic Bay. The best candidate for this purpose is bioremediation under anaerobic/anoxic conditions as long as oil-degrading anaerobes are abundant and active in the sediments, and there is a way to increase activity of this population [21]. In order to assess the anaerobic bioremediation feasibility of Halic Bay, a Turkish Academy of Sciences (TUBITAK) project was carried out [24]. Monitoring the physicochemical and microbiological sediment characteristics revealed that (1) the total petroleum HC levels (the sum of aliphatics, aromatics, asphaltenes, and resenes; 11,000–18,000 ppm) were similar to those from extremely polluted marine environments [30]; (2) the microbial cell contents were very high ($9–12\times10^{10}$ cells/ml) compared with the other marine environments [29]; (3) the sediments were dominated by methanogens and anaerobic HC degraders, and these microbes were active [30]; and (4) N and P were limited in the porewaters for biological activity [30]. These indications raised the question that anaerobic HC degrada-

tion activity of Halic Bay sediments can be increased by N–P amendment under methanogenic conditions. This paper describes assessment of this hypothesis through anaerobic HC degradation microcosms.

Methods

Sampling and Characterization of the Sediment

Sediment samples were taken from Halic Bay (41°33.66′ N and 28°56.64′ E), in the northwest of Marmara Sea, at a water depth of 2 m. The samples were taken using a Van Veen grab with a volume of 3.5 L and a penetration depth of 15 cm on the day of microcosm set up (December 2008). The samples were taken in three replicates and then subdivided for molecular and chemical analyses and stored at −20°C.

The samples had a grayish-black color and a fine-grained nature being rich in mud (>90%) and poor in sand. The porewater was brackish (12±0.8 psu). The bottom water temperature, pH, and redox potential were 19°C, 7.8±0.3, and −230±18 mV, respectively. The porewater NO_3^- concentration (300±21 μM) was considerably higher than that of typical seawater (10–50 μM) while the SO_4^{2-} level (13±1.2 mM) was lower than the typical levels (26–32 mM) [34]. No oxygen was detected in the porewater.

TOC/N/P ratio of the sediment (100/35/8) was very low compared with Redfield ratio (106/16/1) indicating pollution from anthropogenic sources. N and P were limited in the porewaters for biological activity which was evident from much lower TOC/N/P ratio of the porewater (862/5/1) compared with that of the exponentially growing marine bacterioplankton (100/20/3) [60]. Total aromatic and aliphatic HC level in the sediment (8,120±450 ppm) was similar to those from highly polluted marine environments [1, 57].

Microcosm Setup

An anaerobic cabinet (Coy Laboratory Products) fitted with an oxygen sensor and with a regulated atmosphere of nitrogen (100%) was used in the preparation and incubation of the anaerobic microcosms. The microcosms were set up in glass 120-ml serum bottles sealed with butyl rubber stoppers and aluminum crimps (Aldrich). The total volume of liquid was 100 ml with 20 ml of headspace volume. Each microcosm comprised a carbonate-buffered nutrient medium containing sources of nitrogen and phosphorus, vitamins and trace minerals, made up in deionized water, according to the brackish medium of Widdel and Bak [61]. Methanogenic conditions were established by the exclusion of the exogenous electron acceptors.

Microcosms were seeded with 10 g of the Halic Bay sediment and amended with 200 mg of the HC mix (HC composition on day 0 in Figs. 6 and 7) except the controls, which were used to determine the extent of methanogenesis on natural HC content of the sediments. Composition of the HC mix was defined based on the detected HC types in the sediments during the 2-year monitoring study [30].

The overall TOC/N/P ratio of Halic Bay sediments (~1,000/5/1) was chosen as a nutrient limited condition. The unlimited nutrient condition was calculated as 1,000/40/6 (C/N/P) based on the following assumptions: (1) molecular formula of the HC mix was $C_{5n}H_{8n}$ (derived from the HC composition); (2) the maximum biomass yield was as high as 0.2 gcell/g HC mix [16]; and (3) C/N/P ratio of the marine microbes was 100/20/3 [60]. Hence, the nutrient amendment was done by gradually decreasing TOC/N/P ratio from 1,000/5/1 to 1,000/40/6.

Microcosms were prepared in triplicate. In addition to the control microcosms without external HC addition, two control microcosms were included: (1) Na_2MoO_4 was added to inhibit dissimilatory sulfate reduction; (2) *NaN_3 (1 g/L) treatment* was applied *to* suppress microbial activity.

The experimental conditions and controls, and abbreviations of the sample names were summarized in Table 1. Five sets of each condition were prepared for destructive sampling. Total of 360 microcosms were set up.

Gas productions in the microcosms were monitored periodically (2 weeks) and the incubations were done till the all gas productions stopped (224 days). The destructive samplings were carried out on days 0, 84, 126, 168, and 224. The samples on days 84, 126, and 168 were taken because of the discernable increases in gas production (Figs. 1 and 2).

Genomic DNA and Total RNA Extraction, and cDNA Synthesis

Genomic DNA was extracted using the FastDNA Spin Kit for Soil (Qbiogene, UK), and total RNA was extracted using the ChargeSwitch® Total RNA Cell Kit (Invitrogen, Germany) by following the manufacturer's instructions. To test for a DNA contamination, the RNA extracts were used in Q-RT-PCR (quantitative real-time PCR combined with reverse transcription**)** as a negative control. The first-strand cDNA was synthesized from the total RNA using random hexamers and SuperScript® First-Strand Synthesis System for RT-PCR according to the kit's manual (Invitrogen, Germany).

Quantitative Real-Time PCR

The primer sets and their targets were given in Table 2; $10^{3–7}$ copies of the standard sequences were used to obtain the

Table 1 Summary of the experimental conditions and controls, and abbreviations of the sample names

TOC/N/P→	1,000/40/6	1,000/5/6	1,000/20/6	1,000/40/1	1,000/40/3	1,000/5/1
External HC[a] added→	HC(+)-UL	HC(+)-NL1	HC(+)-NL2	HC(+)-PL1	HC(+)-PL2	HC(+)-L
Control↓						
Without external HC	HC(−)-UL	HC(−)-NL1	HC(−)-NL2	HC(−)-PL1	HC(−)-PL2	HC(−)-L
Inhibited	HC(+)-I-UL	HC(+)-I-NL1	HC(+)-I-NL2	HC(+)-I-PL1	HC(+)-I-PL2	HC(+)-I-L
Sterilized	HC(+)-S-UL	HC(+)-S-NL1	HC(+)-S-NL2	HC(+)-S-PL1	HC(+)-S-PL2	HC(+)-S-L

[a] The added HC mixture contained *n*-$C_{9–31}$ alkanes, acyclic isoprenoids, and 18 different one- to five-ring aromatic HCs

calibration curves. Roche LightCycler DNA Master SYBR Green I kit and Roche LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim, Germany) were utilized for all reactions. Reaction mixes contained 25 ng template DNA, 0.5 μM of each primer and 2.5 μM $MgCl_2$. The following thermocycling program was applied: 95°C for 10 min, 45 cycles of 10 s at 95°C, 5–10 s at primer-dependent annealing temperature, 15 s at 72°C. A melt-curve analysis was performed from 55°C to 95°C to determine if only one amplified product was generated during quantitative real-time PCR (Q-PCR). Q-PCR runs were analyzed using Roche LightCycler Software 4.05. The efficiencies were between 1.8 and 2.0, and the correlation factors (r^2) were not lower than 0.97 in all reactions. To convert gene abundances into cell numbers, averages of 3.6 and 1.6 copies of 16S rRNA gene were estimated for *bacteria* and *archaea*, respectively [27]. It was assumed that a single copy of the target functional genes exists in a prokaryotic genome [4, 12, 42, 63], and functional gene abundances were divided by total cell abundances to calculate the gene percentage.

Gas Measurements

Headspace gas (10 ml) was removed, periodically (2 weeks), throughout the course of the experiment from all microcosms and injected into evacuated gas tubes. The gas removed was replaced by 10 ml of 100% N_2. Gas samples were analyzed for CH_4 using a Hewlett-Packard (HP) 5972 II gas chromatograph–mass spectrometer (GC–MS). The samples were analyzed using a HP Plot Q column (30 m×530 μm, 1 μm) with nitrogen as carrier gas. The column temperature was programmed from 60°C to a final temperature of 120°C at a rate of 8°C/min. Peak areas were calibrated using the CH_4 and CO_2 gas standards and the reproducibility (n=4) of replicate standard analyses were typically less than 1% relative standard deviation.

HC Analysis

The analytical procedure for extraction of HCs derived from a modified UNEP's protocol [58]. The samples were Soxhlet-extracted with chloroform (1:2 *m*/*v*) for a period of 1 h at 50°C and concentrated on a rotary evaporator. The total HC content of extracts was quantified by infrared spectroscopy.

The extracts were fractionated into aliphatic and aromatic HCs by adsorption liquid chromatography using a column of alumina and silica gel, and gradient solvents as eluent/ *n*-hexane and 2:1 *n*-hexane/chloroform for aliphatic and aromatic fractions, respectively. The extracts for aromatic HC analyses were evaporated under gentle steam of nitrogen after addition of 50 μl dimethylformamide as keeper and diluted with acetonitrile for high-performance liquid chromatography (HPLC) analysis. The resulting solution were analyzed by a Hewlett-Packard 1046A HPLC with a programmed fluorescence detector. The

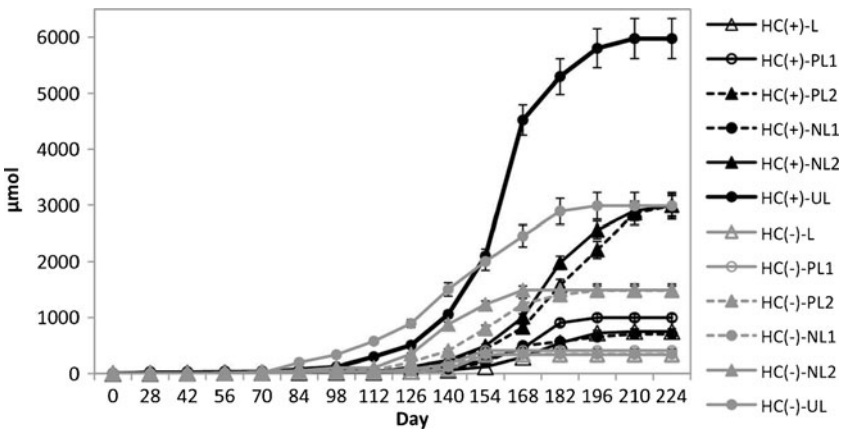

Figure 1 Cumulative CO_2 production in the microcosms

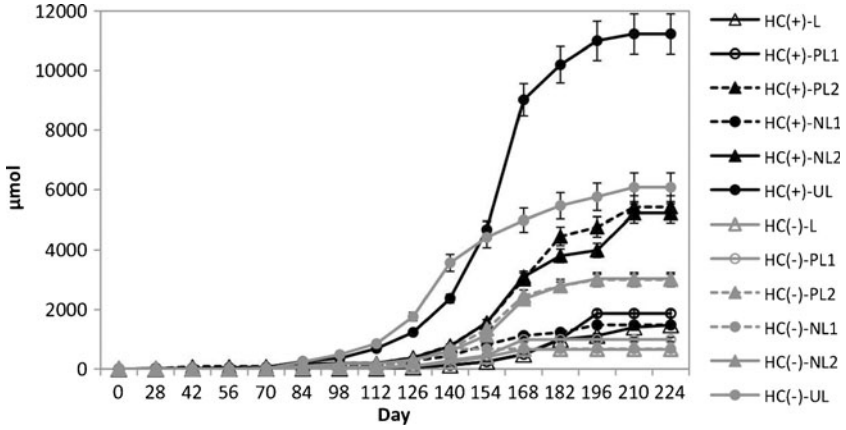

Figure 2 Cumulative CH_4 production in the microcosms

column (MZ-polyaromatic HCs (PAHs) 250×3 mm, 5 µm, from MZ-Analysentechnik, Mainz, Germany) was maintained at 35°C and the flow of mobile phase was 0.5 ml/min. A linear gradient was applied from 52% acetonitrile in water to 100% acetonitrile and held constant for 10 min. Fourteen different PAHs and BTEX were analyzed in the samples. Certified reference materials (CRM 535) were used to assess the accuracy (> 85%) of the measurements. Another CRM (NIST-1647) was also used for recovery test and analyzed three times.

Aliphatic HC analyses were conducted on a HP 5972 II GC–MS. The samples were analyzed using a fused silica capillary column (25 m×0.32 mm, 0.52 µm) with nitrogen as carrier gas. The column temperature was programmed from 80°C to a final temperature of 280°C at a rate of 8°C/min. The MS operating conditions were: electron ionization of 50 eV and linear scanning over the mass range 35–500 Da were used. The samples were analyzed in the splitless mode. Compound identification was based on individual mass spectra and GC retention times in comparison to the literature,

Table 2 Q-PCR primer sets and their targets

Primer	Target gene	Target organism/metabolism	Reference
Bac519f Bac907r	16S rRNA	Bacteria Bacteria	[48]
Arc349f Arc806r		Archaea Archaea	[54]
mcrA1f mcrA1r	Methyl-coenzyme M reductase (mcrA)	Methanogens Methanogens	[9]
DSRp2060F DSR4R	Dissimilatory sulfite reductase (dsrB)	Dissimilatory sulfate reducers Dissimilatory sulfate reducers	[17]
BCR697f BCR1178r	Benzoyl-coenzyme A reductase (bcrA)	Degraders of aromatics Degraders of aromatics	[52]
bssA_715f[a] bssA_1107r[b]	Benzylsuccinate synthase (bssA)	Toluene–xylene degraders Toluene–xylene degraders	[30]
assA_1578f[c]	Alkylsuccinate synthase (assA)	Aliphatic HC degraders	
assA_1967r[d]		Aliphatic HC degraders	
nosZf nosZr	Nitrous oxide reductase (nosZ)	Denitrifiers Denitrifiers	[22]
nrfAf nrfAr	Periplasmic nitrite reductase (nrfA)	Dissimilatory nitrate reducers to NH_4^+ Dissimilatory nitrate reducers to NH_4^+	[51]

[a] 5′-WGGATCGMCAAGATCGAYRA-3′

[b] 5′-RTGYTCKGARACYTTSAGNC-3′

[c] 5′-KGAYTTTGAGSASCTTTTCS-3′

[d] 5′-TCGTCCACRTARTCGTCGTC-3′

library data, and authentic standards. Standards were injected and analyzed under the same conditions as the samples. Quantification was made owing to internal standards such as n-C_{18}, n-C_{20}, n-C_{22}, and n-C_{24}. Blank analyses were carried out, and all values were corrected for these blank concentrations.

Statistical Analysis

Bivariate correlation analyses were performed using the softwares MINITAB 15 (Minitab Ltd., England) and SPSS 17.0 (SPSS Inc., USA). Correlations were evaluated using Pearson's method. Statistical significance was taken as $p<0.05$.

Results

Correlation Analysis

Statistically significant correlations were given in Table 3. All of the measured parameters except sulfate-reducing bacteria (SRB) abundance and activity were related to the gas production. The comparative evaluation of gas production data from the limiting and the unlimited nutrient microcosms reflected the relative changes in the other parameters. This is why only the results from unlimited nutrient conditions were given for the other parameters.

CO_2 and CH_4 Production

The cumulative gas production data were shown in Figs. 1 and 2. CO_2 and CH_4 production in sterilized and inhibited control microcosms were lower than 21 μmol; their gas production data were not shown. The much higher CO_2 and CH_4 production in HC(+) and HC(−) microcosms compared with the sterile controls indicated that the gas production was of microbial origin.

Addition of the dissimilatory sulfate reduction (DSR) inhibitor inhibited both the DSR and methanogenic activity; the origin of CO_2 production (dissimilatory sulfate, Fe(III) and Mn(IV) reduction, fermentation, and methanogenesis) was discussed based on the transcript abundance of functional genes (Fig. 10) and the previous data on microbial composition of Halic Bay sediment [24]. The initial SO_4 content (120–127 μmol) was completely removed before day 126 (the data were not shown). DSR activity (dsrB transcript production) was not detected after the day 84 (Fig. 10). Methanogenesis activity (mcrA transcript production) was very high during the all incubation period. Dissimilatory nitrate reduction activity (nosZ and nrfA transcript production) was not observed in the microcosms. Dissimilatory Fe(III) and Mn(IV) reducing bacteria were not monitored in the microcosms because frequencies of 16S rRNA genes from Fe(III)- and Mn(IV)-reducing bacteria in the previously constructed clone libraries from Halic Bay sediments were very low (~6% and ~2%, respectively) [24].

Table 3 Statistically significant ($p<0.05$ and $n=24$) Pearson's product-moment correlation coefficients (r) between the measured parameters and the methane production

	HC(+) CH_4 production	HC(−) CH_4 production
Removal		
TOC	0.98	0.99
HC	0.97	0.96
N	0.95	0.96
P	0.95	0.95
Production		
CO_2	1	1
Gene		
assA	0.84	0.82
bssA	0.87	0.85
bcrA	0.85	0.84
Archaea-rRNA	0.9	0.89
Bacteria-rRNA	0.91	0.89
mcrA	0.9	0.87
Transcript		
assA	0.87	0.88
bssA	0.9	0.86
bcrA	0.89	0.86
Archaea-rRNA	0.94	0.89
Bacteria-rRNA	0.94	0.92
mcrA	0.93	0.87
Aliphatic HC%	−0.64	−0.83
Aromatic HC%	0.64	0.83

Since the microbial activity stopped at an uncertain time between days 196 and 224, the data from the destructive sampling set on day 224 were omitted in the correlation analysis

As seen in Figs. 1 and 2, the higher initial amount of N and P was resulted in higher amount of total gas produced. Relative total gas production in HC(+) and HC(−) L/PL1/PL2/NL1/NL2/UL microcosms were ~1/1.3/3.7/1/3.5/7.6 and ~1/1.5/4.5/1/4.6/9.2, respectively. Addition of external HCs to the microcosms was resulted in approximately twofold higher gas production for UL microcosms. This indicated that the added HCs were biologically more available compared to the natural C sources in the sediments for the microbial growth.

C Balance

C balance in the microcosms was shown in Fig. 3 and Eq. 1 (after 224 days of incubation); 51% and 57% of the initial

TOC were removed from HC(+)-UL and HC(−)-UL microcosms, respectively. These correspond to 56% and 97% HC removal in HC(+)-UL and HC(−)-UL microcosms, respectively.

	TOC(from HCs)	+ TOC(from the other C sources)	→ Cell − C	+ CH_4	+ CO_2	
HC(+)	77%	23%	10%	57%	30%	(1)
HC(−)	55%	45%	10%	58%	27%	

N and P Removal

Changes in TOC, N, and P content of HC(+)-UL and HC(−)-UL microcosms were shown in Fig. 4. Initial TOC/N/P ratios of both microcosms were adjusted to be 1,000/40/6. Interestingly, addition of external HCs resulted in higher N and P consumption rate for HC(+)-UL microcosms. TOC/N/P removal ratio (1,000/78/12) was lower than the initially adjusted ratio (1,000/40/6) in HC(+)-UL microcosms, which resulted in incomplete C removal. Although TOC/N/P removed from HC(−)-UL microcosms at the expected ratio (1,000/47/7), C removal stopped before the all N and P depleted. This was probably due to depletion of the biodegradable fraction of TOC.

Changes in HC Composition

Changes in aromatic and aliphatic HC levels of HC(+)-UL and HC(−)-UL microcosms were shown in Fig. 5; 55% and 57% of aromatic and aliphatic HCs in HC(+)-UL microcosms were removed, respectively. High proportion (92%) of aromatic HCs and all aliphatic HCs were removed from HC(−)-UL microcosms; 2.4× and 2.1× higher amounts of aliphatic HCs were consumed compared with those of aromatic HCs in HC(+)-UL and HC(−)-UL microcosms, respectively.

Changes in aromatic and aliphatic HC fractions were shown in Figs. 6 and 7. As seen, the short-chain HCs were degraded faster than the long-chain HCs. Aromatic HCs were only degraded after significant removal of *n*-alkanes and alteration of acyclic isoprenoids pristane and phytane.

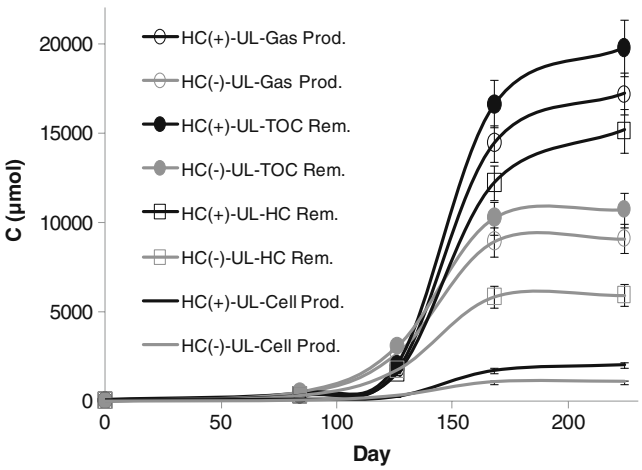

Figure 3 C balance in the HC(+)-UL and HC(−)-UL microcosms: TOC removal, HC removal, gas production, and cell production. In order to convert the removed mg HC into µmol C, molecular formula of the HC mix was calculated based on the composition of aliphatic and aromatic HCs (Figs. 6 and 7). Cell numbers were converted into µmol C, based on the assumptions that molecular formula and average weight of a bacterial cell are $C_5H_7NO_2$ and 1 pg [32, 39]

Aromatic HC Fractions

Changes in aromatic HC fractions were shown in Fig. 6. Complete removal of 1–3 ring aromatic HCs was achieved in both HC(+)-UL and HC(−)-UL microcosms. Only antracene was partially (40%) removed in HC(+)-UL microcosms. Four- to five-ring aromatic HCs were not degraded in HC(+)-UL microcosms whereas those in HC(−)-UL microcosms were completely consumed except benzo(g,h,i) perylene.

Aliphatic HC Fractions

Changes in aliphatic HC fractions were shown in Fig. 7. *n*-$C_{9–31}$ alkanes and acyclic isoprenoids were depleted completely in HC(−)-UL microcosms. *n*-$C_{9–20}$ alkanes, pristane, and phytane were degraded, and *n*-$C_{21–31}$ alkanes remained unchanged in HC(+)-UL microcosms. *n*-$C_{9–18}$ and *n*-C_{20} alkanes were completely removed from HC(+)-UL microcosms.

Changes in Microbial Abundance and Activity

Initial cell and transcript abundances in HC(+)-UL and HC(−)-UL microcosms were shown in Fig. 8. The results were in accordance with the monitoring data from Halic sediments obtained between the years 2006 and 2008 [30]. Bacteria ($19\pm2\times10^9$ cells/ml) dominated over archaea ($7.1\pm0.6\times10^9$ cells/ml); archaeal community almost completely composed of methanogens. Methanogenic archaea (MA) ($6.5\pm0.6\times10^9$ cells/ml) was highly dominant over SRB ($2.7\pm0.1\times10^9$ cells/ml), denitrifying bacteria (DB; $1.1\pm0.1\times10^9$ cells/ml) and dissimilatory nitrate reduction

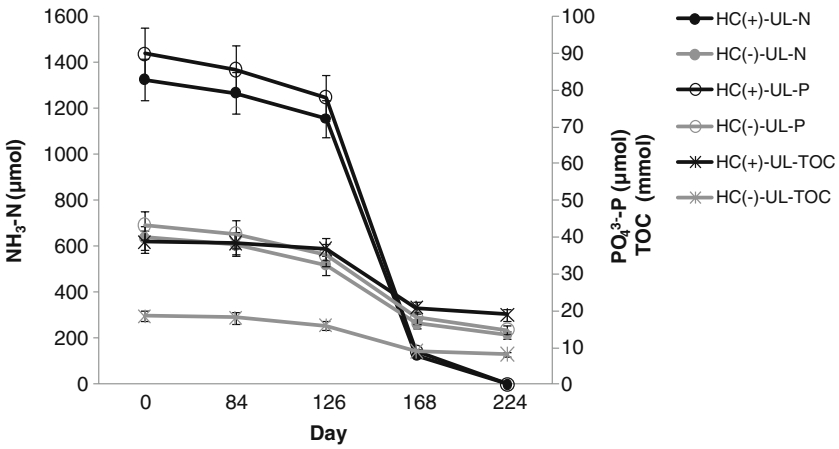

Figure 4 Changes in TOC, N and P content of HC(+)-UL and HC(−)-UL microcosms

to ammonia bacteria (DNRB; $2.2\pm0.2\times10^9$ cells/ml). Anaerobic aliphatic HC-degrading bacteria (AAHDB; $5.9\pm0.6\times10^9$ cells/ml), anaerobic aromatic HC-degrading bacteria (AArHDB; $6.4\pm0.5\times10^9$ cells/ml), and anaerobic aromatic-degrading bacteria (AArDB; $7.7\pm0.8\times10^9$ cells/ml) were as abundant as MA. All of the assessed microbial groups were active. The most active processes at the time 0 were methanogenesis ($7.2\pm0.6\times10^9$ mRNA/ml) and HC degradation ($3.5–6.9\times10^9$ mRNA/ml).

Bacteria, Archaea, MA and SRB Abundance, and Activity Changes

Differential changes in cell and RNA abundances compared to the levels on day 0 in HC(+)-UL and HC(−)-UL microcosms were shown in Figs. 9 and 10. The most considerable increase occurred in the abundance of SRB between the days 0 and 84 during which period sulfate concentration decreased from 1,310 to 660 and 170 μM in HC(+)-UL and HC(−)-UL microcosms, respectively. Sulfate was completely depleted in the both microcosms before the day 126 after which SRB abundance decreased and no SRB activity was observed. Initial nitrate concentration in the microcosms was very low (~30 μM); DNRB and DB activities and nitrate were not detected throughout the incubation period.

Activity and abundance of archaea, bacteria, and MA were very high and related to the C removal (Table 3) all through the incubation period. Increase in archaea abundance was ~1.5× higher than those of bacteria; 74% and 67% of the total C removal occurred between days 126 and 168 during which period archaeal, bacterial, and methanogenic activity levels increased to ×6–8. Overall microbiological results implied that C removal in this period could be attributable to the activities of syntrophic consortium of fermentative bacteria and MA.

Changes in Activity and Abundance of HC Degraders

Differential changes in gene and transcript abundance of enzymes related to anaerobic HC degradation were shown in Fig. 11. As given in Table 3, the changes were highly correlated to C and HC removal. Anaerobic HC-degrading bacteria (AHDB) were as abundant and active as MA in the

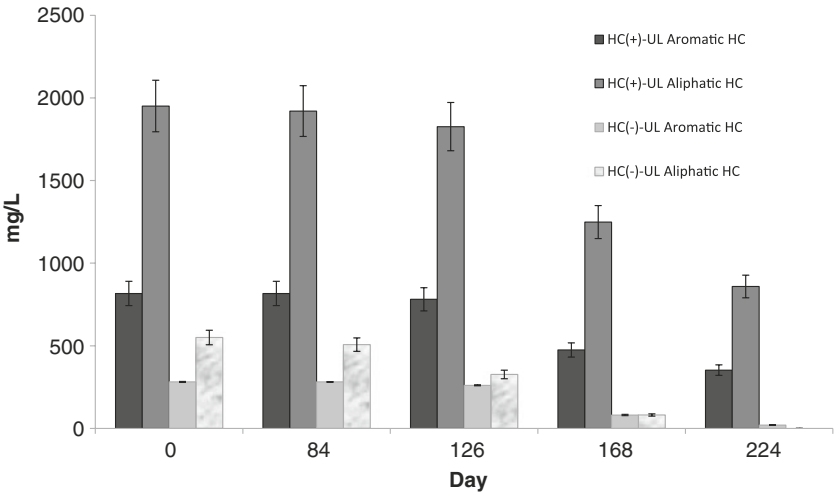

Figure 5 Changes in aromatic and aliphatic HC levels of HC (+)-UL and HC(−)-UL microcosms

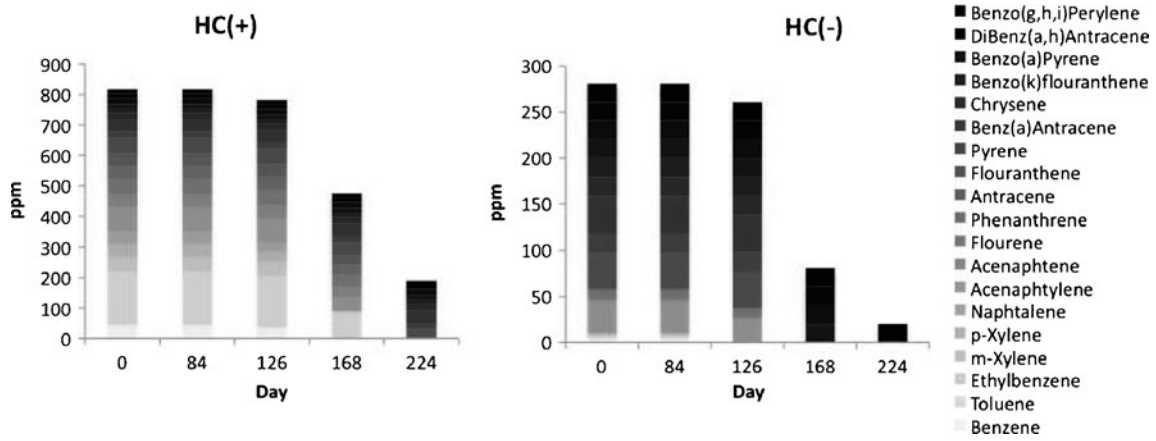

Figure 6 Changes in one- to five-ring aromatic HCs

whole incubation period. AHDB activity increased to ×9–12 and ×4–5 in HC(+)-UL and HC(−)-UL microcosms, respectively, between days 126 and 168 during which period ~70% of the HC removal took place.

Discussion

Interactions Between the Functional Microbial Groups

Shallow marine sediments are characterized by intense and diverse microbial activities, which generate steep chemical gradients [50]. As the products of O_2, NO_3^-, Mn(IV), Fe(III), and SO_4^{2-} reduction enter consecutively deeper zones of the sediments, vertical cascades of electron-accepting processes are sustained. Methanogenesis occurs after electron acceptors that yield higher standard-free energies have been depleted.

Methanogens in Halic Bay sediments were highly abundant and active along with nitrate and sulfate-reducing bacteria in 15 cmbsf (penetration depth of the grab sampler.) This was an expected result since NO_3^- and SO_4^2 levels in the sediments were very low compared to the exceptionally high electron donor (TOC and TPH) contents (please check "sampling and characterization of the sediment" part in "Methods"). Most of the Fe(III) and Mn(IV) in sediments form insoluble oxides, colloids, and organic complexes [31]; the low abundance of dissimilatory Fe(III) and Mn(IV) reducers was an indicator of bio-available Fe(III) and Mn(IV) limitation in Halic Bay sediments [24]. It can be speculated that limited amount of electron acceptors were quickly depleted in a very short distance below the sediment surfaces which resulted in dominancy of methanogens [50]. This was also a case for the microcosms in which no external e^--acceptor was added. The low NO_3^- and SO_4^2 contents of the sediments were completely depleted during the initial stages of microcosm incubation, afterwards methanogenic activity and abundance increased substantially. Dissimilatory sulfate reduction was dominant over methanogenesis till the sulfate

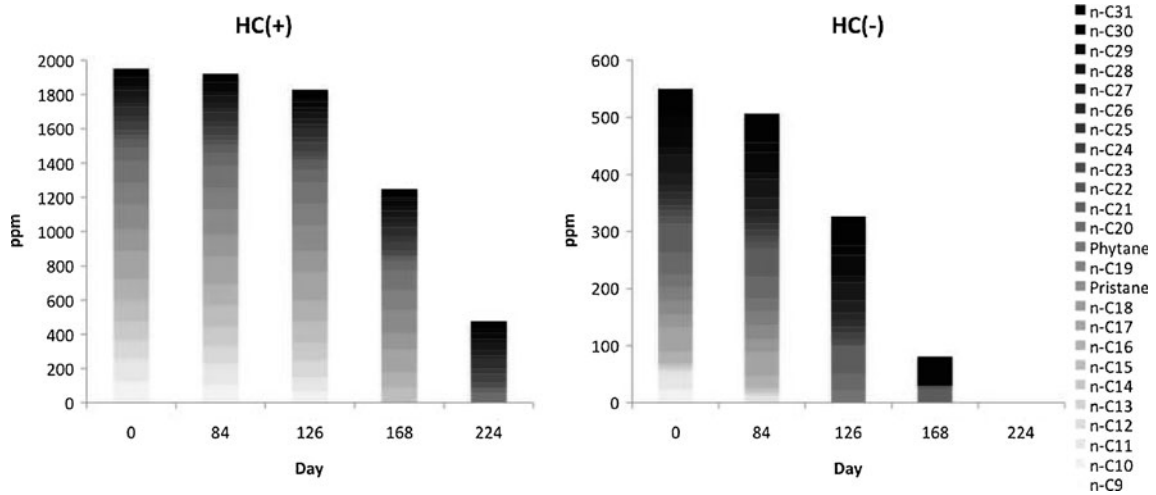

Figure 7 Changes in n-C_{9-31}-alkanes

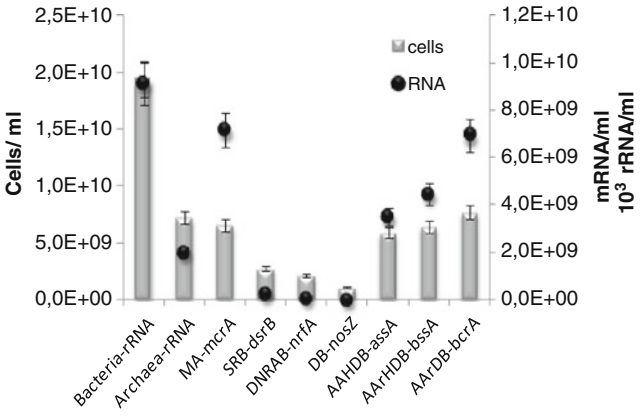

Figure 8 Bacteria, archaea, methanogenic archaea (*MA*), sulfate-reducing bacteria (*SRB*), denitrifying bacteria (*DB*), dissimilatory nitrate reduction to ammonia bacteria (*DNRAB*), anaerobic aliphatic HC-degrading bacteria (*AAHDB*), anaerobic aromatic HC-degrading bacteria (*AArHDB*), and anaerobic aromatic-degrading bacteria (*AArDB*) abundances and transcription levels of the related genes in HC(+)-UL and HC(−)-UL microcosms on day 0

was depleted, which was apparently due to higher standard-free energy yield of sulfate reduction [50]. The overall results showed that gas production after the day 126 was originated from activities of syntrophic consortium of fermentative bacteria and methanogenic archaea.

The dominancy of DNRA over denitrification (Fig. 8) was not surprising in organic carbon rich Halic Bay sediments. Although the conditions promoting DNRA and denitrification are similar, DNRA is thought to be favored in nitrate-limited environments rich in organic carbon, while denitrification would be favored under carbon-limited conditions [6]. Tiedje [56] argued that high-labile carbon availability would favor organisms that used electron acceptors most efficiently; DNRA transfers eight electrons per mole of nitrate reduced, whereas denitrification only transfers five.

Biodegradability of the Added HC Types

The degraded HCs by Halic Bay sediments were shown in Figs. 6 and 7. Only benzo(g,h,i)perylene was recalcitrant to biodegradation and remained intact during the incubation period. The HC removal in microcosms was linked to biotic processes as attested by: (1) the much higher gas production in HC(+) and HC(−) microcosms compared with the sterile controls, (2) the increasing abundance and continuous transcription of the genes related to anaerobic HC degradation, and (3) the high correlations between C removal and microbial activity.

$C_{9–31}$ *n*-alkanes and acyclic isoprenoid alkanes (pristane and phytane) have previously been reported to be biodegradable under nitrate- and sulfate-reducing and methanogenic conditions ([18, 62] and references therein). Studies using marine sediments as inoculum produced much of the biodegradability reports on $C_{20–31}$ *n*-alkanes [25, 35, 36]. We confirmed that marine benthic microbiota is able to degrade long chain *n*-alkanes.

BTEX components are the best-studied substrates of anaerobic biodegradation since they are the most water soluble of the aromatic HCs. As expected, Halic Bay sediments degraded BTEX faster than the PAHs. Literature on PAH degradation by anoxic marine sediments was mainly obtained under sulfate-reducing conditions because of overwhelming abundance of sulfate in seawater ([14, 62] and references therein). Our study is the first that reports three- to five-ring PAHs were degraded by marine sediments under methanogenic conditions. Besides, this is the first indication of acenaphtylene biodegradation.

Our findings on biodegradation hierarchy of different HC types were coincided with the previous reports ([14, 18] and references therein): short-chain HCs were degraded faster than long chain HCs; aromatic HCs were only degraded after significant removal of *n*-alkanes and alteration of acyclic isoprenoids pristane and phytane.

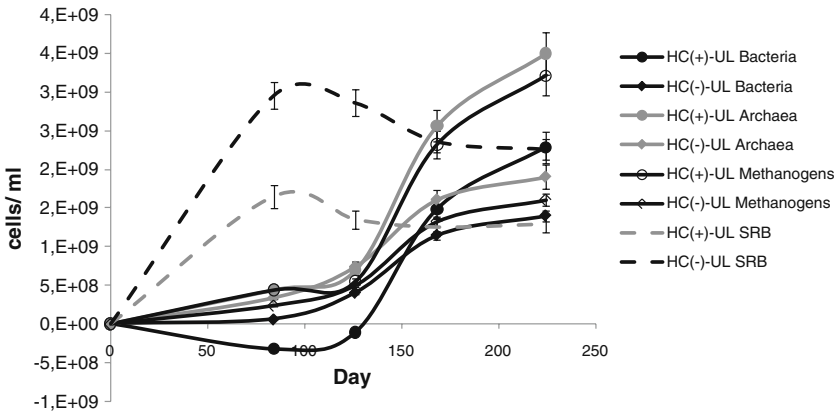

Figure 9 Differential changes in cell abundances compared with the levels on day 0 in HC (+)-UL and HC(−)-UL microcosms

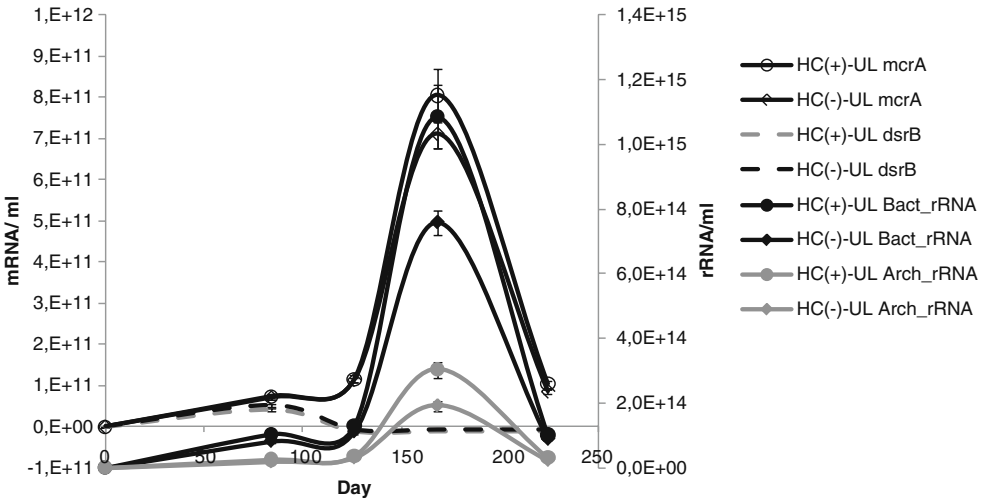

Figure 10 Differential changes in RNA abundances compared to the levels on day 0 in HC(+)-UL and HC(−)-UL microcosms

The most biodegradable HCs under anaerobic conditions are straight-chain *n*-alkanes, followed by more resistant branched acyclic and monocyclic hydrocarbons, the most resistant polycyclic steroidal and triterpenoidal hydrocarbons, and aromatic hydrocarbons ([14, 18] and references therein). In this study, the sequence of removal of different HC types coincided with the previous findings.

The Pathway of Anaerobic HC Degradation in Halic Bay Sediments

Overall microbiological results obtained in this study implied that assA, bssA, and bcrA genes were carried by syntrophic methanogenic consortium, and fermentative bacteria most probably carried out the initial attack on HCs during the exponential growth phase. Initial activation of aromatic HCs is crucial for anaerobic biodegradation, and four general enzymatic reactions are recognized: (1) addition of fumarate, catalyzed by a glycyl radical enzyme such as bss; (2) methylation of unsubstituted aromatics; and (3) hydroxylation of an alkyl substituent ([15] and references therein). The other proposed pathways represent a combination of these reactions. These activation reactions feed into pathways that result in production of central metabolites such as benzoyl-coenzyme A, which are eventually incorporated into biomass or completely oxidized.

Bss has been the only identified enzyme, which specifically attacks on aromatic HCs (toluene and xylene) [14]; bcr catalyzes dearomatization of the central metabolite benzoyl-coenzyme A. This is why we chose bcr and bss genes and their transcripts as indicators of anaerobic aromatic HC degradation. Abundance of these genes gradually increased during the incubation period and their transcription levels were very high even after depletion of toluene and xylene. These implied that fumarate addition was the main route of initial activation of the other aromatic HCs. Fumarate addition has previously been proposed to be included in degradation pathways of the other monoaromtic HCs (benzene, alkylbenzenes, and ethylbenzene) and PAHs (naphtalene and phenanthrene) ([14] and references therein).

The two main mechanisms of anaerobic *n*-alkane degradation involves (1) activation at the subterminal

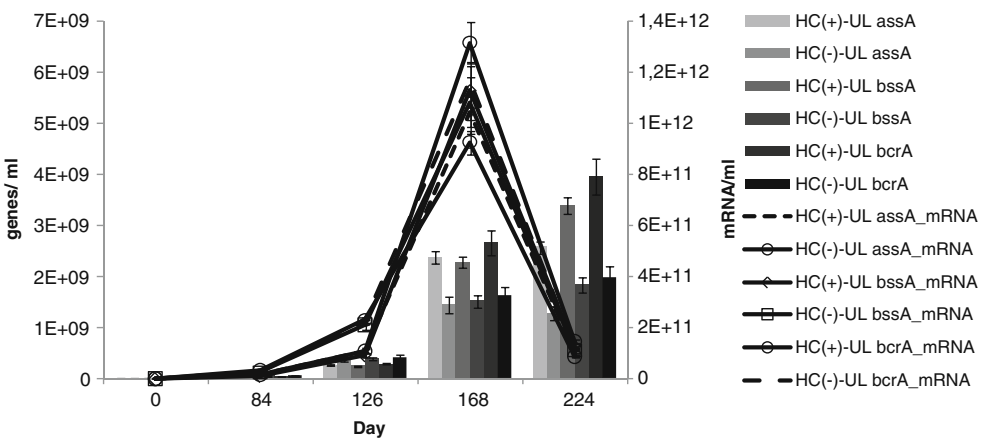

Figure 11 Differential changes in assA, bssA, and bcrA gene and transcript abundances compared with the levels on day 0 in HC(+)-UL and HC(−)-UL microcosms

carbon of the alkane and addition to a molecule of fumarate and (2) the alkane carboxylation at C-3 [18 and references therein]. Recently, Callaghan et al. [7] reported a glycyl radical type enzyme (assA) which involve in alkane activation through fumarate addition in the SRB strain AK-01. This was the first description of a gene specifically involved in anaerobic *n*-alkane metabolism. Our results imply that fumarate addition was the main mechanism of initial activation of *n*-alkanes since both abundance and transcription level of the ass gene was very high throughout the incubation period.

Anaerobic Bioremediation Feasibility of the Chronically Polluted Sediments

Anaerobic biodegradation processes are a significant component of natural attenuation owing to the abundance of anoxic electron acceptors relative to dissolved oxygen. Furthermore, clean-up systems based on anaerobic biodegradation require less human intervention [21]. Studies on the anaerobic/anoxic biodegradation of the HCs in natural habitats, microcosms and enrichment cultures were initiated to determine whether or not bioremediation processes are possible in petroleum/fuel-contaminated anoxic sediments, groundwaters and aquifers [62]. Although numerous reports have been published documenting natural attenuation of HCs in these environments [3, 14, 32, 33], bioremediation strategies based on anaerobic microbial processes are very limited because they proceed at much lower rates than the aerobic ones [19, 35, 36].

Recent reports on the anaerobic HC degradation rates of marine sediments or marine enrichment cultures were summarized in Table 4. As seen, none has achieved anaerobic HC degradation rates comparable to the aerobic ones. In this study, we obtained anaerobic HC degradation rates as fast as SRB enrichment cultures' rates via addition of the limiting nutrients to Halic Bay sediments which resulted in substantial microbial activity increases (approximately ninefold). Although the obtained rates were comparable to the aerobic ones, they are still much lower than the aerobic HC degradation rates. Moreover, aerobic microorganisms degrade a wider range of HC compounds than anaerobic microorganisms [8, 20]. Nevertheless, an aerobic bioremediation strategy is unfeasible for Halic Bay since oxygen penetration into the anoxic sediments is poor and oxygen mass transfer enhancement by mechanical means is inappropriate for the inaccessible sediments. Moreover, economical long-term solution for the chronic HC pollution by continuous aeration of the huge anoxic area in Halic Bay is out of the question. Under these conditions anaerobic HC degradation is the only alternative.

In summary, we have obtained three lines of evidence for demonstrating anaerobic bioremediation feasibility of petroleum HC pollution in Halic Bay sediments: (1) the anaerobic HC-degrading microorganisms were highly abundant in the sediments [30]; (2) anaerobic HC degradation was taking place in the sediments [30]; (3) the sediments were able to degrade wide range of HCs under anaerobic/anoxic conditions (this study); (4) high anaerobic HC degradation rates were achieved via biostimulation of the sediments through nutrient amendment (this study).

As given in Table 4, almost all of the studies on anaerobic hydrocarbon degradation in marine sediments were conducted ex situ. There were two studies reporting in situ anaerobic hydrocarbon degradation in marine sediments: (1) Miralles et al. [35, 36] subjected Mediterranean coastal sediments to massive crude oil inputs and observed alteration of *n*-alkanes by SRB; (2) Tang et al. [55]

Table 4 Literature on anoxic/anaerobic HC degradation rates obtained from marine sediments or SRB enrichment cultures obtained from the marine sediments

Inoculum	Place	Substrate	e^--Acceptor addition	Removal rate (μg/g sediment Lday	Reference
Sediment	In situ	^{14}C-Phenanthrene	Sulfate+nitrate	0.2	[55]
SRB enrichment	Ex situ	Short-chain alkanes	Sulfate	0,6	[28]
SRB enrichment	Ex situ	Phenanthrene	Sulfate	1.4	[13]
Sediment	In situ	Crude oil	None	7	[35, 36]
Sediment	Ex situ	Diesel fuel	Sulfate+nitrate	12.4	[5]
Sediment	Ex situ	2- to 5-ring PAH	Sulfate	48	[46]
Sediment	Ex situ	*n*-Alkanes	None	60	[25]
Sediment	Ex situ	PAH	None	228	[26]
SRB enrichment	Ex situ	Naphthalene	Sulfate	880	[37]
Sediment	Ex situ	*n*-Alkanes, PAH, and BTEX	none	700	This study
Sediment	Ex situ	Crude oil	Oxygen	1,970	[53]
Sediment	Ex situ	Crude oil	Oxygen	4,285	[47]

enhanced in situ anaerobic phenanthrene biodegradation rates by factors up to 2–3 in undisturbed marine sediments via controlled-release of nitrate and sulfate. The obtained in situ hydrocarbon degradation rates in these studies were very low compared to those from ex situ incubations (Table 4), which highlights the gap between microcosm results and reality of the field. Hence, we are planning to confirm our ex situ findings via a pilot-scale in situ biostimulation trial: Halic Bay sediments will be (1) sampled, (2) reworked by addition of slow release fertilizers which prolong duration of nutrient release up to 6 months [38], (3) placed in PVC cores, and (4) reinserted into sampling site. If a remarkable enhancement of natural attenuation is observed in the cores, the slow release fertilizers will be applied over a large area of Halic Bay subsurface to remove the accumulated HCs. The fertilizers will be supplied in the form of granules that were shown to adhere tightly to the oiled sediment material [45].

We are now making the preliminary preparations in conjunction with Maritime Undersecretariat of Turkish Republic, ISKI and IBB to scale-up our microcosm trials, the success of which will certainly lead to less human intervened and more economical field-scale bioremediation applications for highly polluted anoxic marine environments worldwide.

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