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Carbon speciation and composition of natural microbial communities in polluted and pristine sediments of the Eastern Mediterranean Sea

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Abstract

Sediment samples collected from polluted (Augusta Bay) and pristine regions of the Eastern Mediterranean Sea (South Ionian Sea, Thracian Sea) were analyzed for black carbon, aliphatic hydrocarbons and phospholipid ester-linked fatty acids (PLFA). The aim of the study was to investigate the anthropogenic and biogenic inputs into the Eastern Mediterranean Sea and to evaluate the effects of refractory organic matter (e.g. black carbon) and the level of hydrocarbon contamination on benthic microbial community composition. Black carbon, generally considered to be chemically and biologically inert, comprised a significant but highly variable fraction of the sedimentary carbon pool in the analyzed sediments with a ratio to total organic carbon ranging from 0.02 to 0.66. Principal component analysis of the chemical characteristics of the sediments (organic carbon content, black carbon, bioavailable organic carbon, chlorophyll *a*, phaeopigments, aliphatic hydrocarbons) revealed clustering of samples along a gradient from the most productive and contaminated region of Augusta Bay to the carbon-poor and pristine sediments of the Thracian Sea. PLFA analysis revealed that gram-negative bacteria and microeukaryotes were most abundant in Augusta Bay and in the most impacted station of the Thracian Sea. The high levels of branched and odd-chain fatty acids recorded for these stations is probably linked to the elevated amounts of hydrocarbons at these stations; e.g. microbial communities may have developed the ability to degrade either naturally occurring aliphatic hydrocarbons or hydrocarbons derived from oil contamination.

Keywords: Black carbon; Carbon bioavailability; Eastern Mediterranean Sea; Sediments; Microbial communities; Phospholipids

1. Introduction

The input of various types of organic matter to the sediment surface is mainly governed by the origin of the sedimenting material and the diagenetic processes taking place in the water column and in the surface sediments (Teece et al., 1998). Black carbon has recently received attention as it represents a form of refractory organic matter that may preferentially be preserved in sediments (Middelburg et al., 1999; Gélinas et al., 2001; Dickens et al., 2004). Black carbon (BC) is produced from incomplete combustion of a variety of fossil fuels and biomass materials (Middelburg et al., 1999; Elmquist et al., 2004). It is mainly formed on land and introduced to the marine environment by riverine transport or aerosols (Masiello and Druffel, 1998; Dickens et al., 2004).

In order to assess the balance between terrestrial, marine or anthropogenic organic matter inputs to the ocean, tracers such as aliphatic hydrocarbons have been evaluated. Many of these compounds are derived from allochthonous

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sources such as higher plants, whereas others are derived from autochthonous sources such as phytoplankton and bacteria (Wakeham, 1996). Bacteria play a key role in the ecosystem as degraders and scavengers of the different fractions of organic matter (Boschker et al., 2001). A number of culture-independent techniques have recently been developed, offering insights into the composition of the largely uncultivated communities that inhabit natural ecosystems (Head et al., 1998; Macnaughton et al., 1999). For example, fatty acid profiling is widely used to describe microbial strains or communities or to differentiate among environmental samples by their fatty acid "fingerprint" (White et al., 1979).

Studies that link the composition of sediment microbial communities in the Eastern Mediterranean Sea to environmental variables (e.g. black carbon and oil contamination) are scarce and hence urgently needed (Polymenakou et al., 2005a,b). The Eastern Mediterranean Sea is among the most oligotrophic regions on Earth (Ignatiades, 1969; Krom et al., 1991) and includes areas differentially impacted by anthropogenic activities (Polymenakou et al., 2005a). Here we report a survey of black carbon, aliphatic hydrocarbons and phospholipid-linked fatty acids in polluted and pristine areas of the Eastern Mediterranean Sea. The main goal was to evaluate the anthropogenic and biogenic inputs into the Eastern Mediterranean Sea and identify the major environmental features controlling the composition of benthic microbial community structure.

2. Materials and methods

2.1. Samples collection and sediment characteristics

Sediment samples were collected from three different regions of the Eastern Mediterranean Sea (Fig. 1). A detailed description of the sediment samples collected from the South Ionian Sea close to hypersaline anoxic basins of Bannock, L'Atalante, Discovery and Urania has been presented elsewhere (Polymenakou, 2005). Sediment samples (0–1 and 8–10 cm) from the Thracian Sea and Augusta Bay have been collected using a Bowers and Connelly Multiple-corer (eight cores, i.d. 9.0 cm) (Barnett et al., 1984) in September 2003 and November 2003, respectively. All sampling was carried out onboard the R/V's *Aegaeo* and *Urania*. Subsamples for analysis of the different chemical parameters were sealed in aluminum foil and stored at −20 °C until further analysis took place. Chlorophyll *a* (Chl-*a*) and phaeopigment (Phaeop.) concentrations were

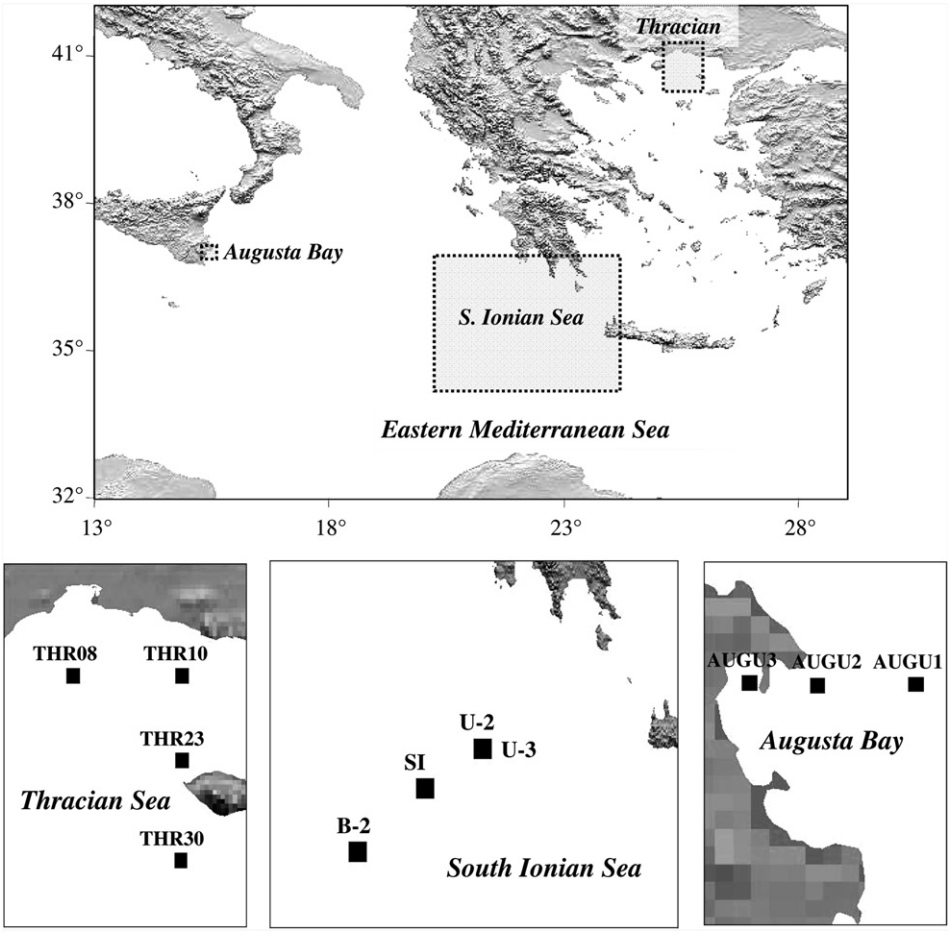

Fig. 1. Map of the Eastern Mediterranean Sea showing the sampling stations in Thracian Sea, South Ionian Sea and Augusta Bay.

determined according to the fluorometric method of Yentsch and Menzel (1963) and Lorenzen and Jeffrey (1980) using a Turner TD-700 fluorometer. Chloroplastic pigment equivalents (CPE) are considered as the sum of chlorophyll *a* and phaeopigments. Total organic carbon measurements were carried out according to the method of Hedges and Stern (1984), using a Perkin Elmer CHN 2400 analyser. Black carbon was estimated using the method described by Gustafsson and coworkers (1997) where samples were oxidized at 375 °C for 24 h in the presence of excess oxygen (air) and further analyzed in the Perkin Elmer 2400 CHN analyzer. Bioavailable organic carbon (BOC) was estimated by subtracting the amount of black carbon from the total organic carbon content. All solvents used were purchased from Merck (Suprasolv, Darmstadt, Germany). Silica gel 60 (0.063–200 mm) was also from Merck. Alkane standards were purchased from PolyScience (Analytical Standards, Merrimac Avenue, Niles, IL). Identification of fatty acids was based on comparison of retention times to purchased standards (FAME Mix, Supelco).

2.2. Aliphatic hydrocarbons and phospholipids analyses

For aliphatic hydrocarbons, 1 g of pre-weighed freeze-dried sediment material was Soxhlet extracted in *n*-hexane:dichloromethane (1:1 v/v). 1-Chlorohexadecane was added prior to extraction as an internal standard. Subsequently, the solvent was evaporated to 1 ml and applied on top of a glass column (0.5 cm i.d.) containing 1.5 g of silica gel, activated at 150 °C for 3 h. Aliphatic hydrocarbons were eluted using 15 ml *n*-hexane in gravity flow. Sulfur was removed by treating the extract with activated copper. Samples were processed following published procedures for lipid extraction and phospholipid linked fatty acids (PLFA) recovery (Polymenakou et al., 2005a). Total lipids were extracted from pre-weighed freeze-dried sediment material (3 g) using a modified Bligh and Dyer (1959) method (for details see Polymenakou et al., 2005a).

2.3. GC/MS analysis

A gas chromatograph equipped with a Mass Spectrometer was used to identify and quantify aliphatic hydrocarbons and fatty acid methyl esters (FAME). A Hewlett Packard Model 6890 GC equipped with a split–splitless injector was directly coupled with the fused silica capillary column (HP-5 MS with 0.25 μm film thickness, 30 m × 0.25 mm i.d.) to the ion source. Helium was used as a carrier gas. The chromatographic conditions were the following (temperature program for *n*-alkanes): injector temperature 270 °C; temperature program, 70 °C (1 min), 70–150 °C (10 °C/min), 150–290 °C (5 °C/min), and 290 °C (30 min). Temperature program for FAME analysis: injector temperature 270 °C; temperature program, 70 °C (1 min), 70–140 °C (10 °C/min), 140–240 °C (4 °C/min), and 240 °C (30 min). For both methods, 1 μl of each sample was injected in the splitless mode. Identification of *n*-alkanes and fatty acid methyl esters was based on comparisons of retention times to purchased standards (Polyscience and Supelco FAME mix, respectively). Quantification of *n*-alkanes and PLFA were carried out using the internal standards 1-chlorohexadecane and nonadecanoic acid methyl ester, respectively. The classical fatty acid terminology was used: total number of carbon atoms:number of double bonds, followed by the position (ω) of the double bond from the methyl end of the molecule. Methyl branching at the iso and anteiso positions is designed by the prefixes i- and a-. Prefix br- indicates methyl branched at unidentified position. All results are presented as mean ± standard deviation ($n = 3$).

2.4. Statistical analysis

In order to detect differences in chemical characteristics (organic carbon content, black carbon, bioavailable organic carbon, chlorophyll *a*, phaeopigments, aliphatic hydrocarbons) between the sediment samples, principal component analysis (PCA) was applied to the data set. Prior to analysis, the raw data was transformed using a $\mathrm{Log}_{10}(n + 1)$ transformation and extracted into a correlation matrix. The correlation matrix was evaluated and eigenvalues were determined (Kleikemper et al., 2002). PCA was performed using the PRIMER 5.2.2 software package (Plymouth Routines on Multivariate Ecological Research).

In order to compare stations based on PLFA data, the following indices were determined: Shannon diversity index (H', calculated on ln; Magurran, 1988) and Pielou index (J' evenness; Pielou, 1975). In all calculations we assumed that each of the fatty acids represents a different species and that their concentration represents their relative abundance. Calculations were performed using the PRIMER software.

3. Results and discussion

3.1. Sediment characteristics

Water column depth ranged from 52 m in the Thracian Sea to 3320 m in the South Ionian Sea. The organic carbon content, ranging from 0.25 ± 0.08% to 1.73 ± 0.06% (Table 1) was comparable to values found in other areas of the Eastern Mediterranean Sea (0.38–1.47%; Polymenakou et al., 2005a; 0.30–0.82%; Gogou et al., 2000; 0.23–0.99%; Bianchi et al., 2003) as well as the Western Mediterranean Sea (0.38–1.47%; Bouloubassi et al., 1997).

The sediment content of black carbon ranged from 0.01% to 0.63% (BC; Table 1) and was correlated with total organic carbon ($R^2 = 0.69$; $n = 15$). Ratios of black carbon (BC) to total organic carbon (BC/TOC; Table 1) varied from 0.02 to 0.66 demonstrating that BC may constitute a significant pool of sedimentary organic carbon in the Eastern Mediterranean. Deep-sea samples did not contain higher values of BC/TOC ratios (0.16–0.50; Table 1) compared to shallow sampling sites as was previously observed in various deep-sea sediments in the Atlantic Ocean, the

Table 1
Water column depth (m), total organic carbon (TOC), black carbon (BC), proportion of BC to TOC (BC/TOC), bioavailable organic carbon (BOC), chlorophyll *a* (Chl-*a*), phaeopigments (Phaeop.), and chloroplastic pigment equivalents (CPE) of the sediment samples are presented

Station	Sediment depth (cm)	Area	Water column depth (m)	TOC (%)		BC (%)		BC/TOC	BOC (%)	Chl-*a*		Pheaop.		CPE	
				Mean	sd	Mean	sd			Mean	sd	Mean	sd	Mean	sd
SI	0–1	South Ionian	2840	1.30	0.69	0.21	0.03	0.16	1.09	0.06	0.00	0.35	0.06	0.40	0.06
B-2	0–1	South Ionian	3179	0.58	0.03	0.28	0.04	0.49	0.30	0.03	0.01	0.12	0.02	0.15	0.03
U-3	0–1	South Ionian	3310	0.76	0.09	0.35	0.08	0.46	0.41	0.05	0.02	0.30	0.04	0.35	0.06
U-2	0–1	South Ionian	3320	0.67	0.09	0.33	0.07	0.50	0.33	0.04	0.00	0.27	0.01	0.30	0.01
AUGU1	0–1	Augusta Bay	1700	0.55	0.02	0.27	0.02	0.48	0.29	0.48	0.07	2.95	0.05	3.43	0.02
AUGU2	0–1	Augusta Bay	600	1.73	0.06	0.60	0.10	0.35	1.13	1.72	0.41	6.62	0.47	8.34	0.05
AUGU3	0–1	Augusta Bay	100	1.37	0.07	0.63	0.10	0.46	0.74	1.11	0.00	4.78	0.18	5.90	0.17
THR08	0–1	Thracian Sea	52.1	0.38	0.10	0.08	0.00	0.21	0.30	0.05	0.00	1.01	0.11	1.06	0.10
THR08	8–10	Thracian Sea	52.1	0.55	0.08	0.13	0.01	0.23	0.43	0.02	0.00	0.43	0.12	0.45	0.12
THR10	0–1	Thracian Sea	62.5	0.34	0.09	0.01	0.00	0.02	0.33	0.70	0.18	2.01	0.21	2.70	0.39
THR10	8–10	Thracian Sea	62.5	0.25	0.08	0.01	0.00	0.04	0.24	0.03	0.00	0.69	0.01	0.72	0.02
THR23	0–1	Thracian Sea	81	0.42	0.06	0.05	0.05	0.13	0.36	0.66	0.26	2.12	0.05	2.78	0.21
THR23	8–10	Thracian Sea	81	0.33	0.04	0.08	0.01	0.24	0.25	0.04	0.00	0.51	0.22	0.54	0.22
THR30	0–1	Thracian Sea	223	0.45	0.11	0.15	0.00	0.34	0.29	2.30	0.15	2.87	0.21	5.17	0.06
THR30	8–10	Thracian Sea	223	0.33	0.03	0.22	0.01	0.66	0.11	0.08	0.03	0.37	0.11	0.46	0.14

sd indicate standard deviation among replicate samples ($n = 3$).

Madeira Abyssal Plain and the Eastern Mediterranean (0.16–0.40; Middelburg et al., 1999).

Although microbiological breakdown during laboratory experiments has been reported, black carbon in the marine environment is generally considered to be chemically and biologically inert (Middelburg et al., 1999; Dickens et al., 2004). In addition, Dickens and coworkers (2004) suggested that the recycling of BC in sediments may be separated from the biologically mediated carbon cycle, most likely as the result of the slow rates of formation and destruction.

In the present study, we considered BC content as the most refractory form of carbon and estimated the bioavailable organic carbon (BOC) by subtracting the amount of black carbon from the total organic carbon. BOC content in the sediments varied from 0.11% to 1.13% C with a maximum recorded value occurring at station AUGU2 of Augusta Bay (1.13; Table 1).

Chlorophyll *a* and phaeopigment concentrations varied significantly between the sampling stations with maximum values at the shallow stations of Augusta Bay (Table 1) and at station THR30 (0–1) of the Thracian Sea (Table 1). The latter station is strongly influenced by the less saline and nutrient rich Black Sea water masses flowing into the North Aegean Sea through the Dardanelles Strait (Poulos et al., 1997). As a result, high amounts of chloroplastic pigments (e.g. chlorophyll *a*, phaeopigments; Table 1) tend to accumulate in the region.

3.2. Anthropogenic and biogenic inputs

In the present study, aliphatic hydrocarbon distributions were examined in order to evaluate the anthropogenic, terrigenous and marine organic carbon inputs in different areas of the Eastern Mediterranean Sea (Fig. 1 and Table 2). Most chromatograms from analyses of aliphatic hydrocarbons (AHC) were dominated by resolved *n*-alkanes (NA) and an unresolved complex mixture (UCM) (Table 2). However, the aliphatic fraction at station AUGU2 was dominated by UCM and specific αβ-hopanes (Fig. 2a and Table 3). The presence of the UCM together with the detection of hopanes indicated the presence of petroleum-related hydrocarbon inputs (Wang et al., 1999; Frysinger et al., 2003).

Absolute (AHC; in μg g^{-1} of dry sediment) and organic carbon normalized concentrations (AHC/OC; in μg g^{-1} of organic carbon) of aliphatic hydrocarbons showed large variation between the sampling sites; i.e. 1.2–462.8 μg g^{-1} and 371–26,698 μg g^{-1}, respectively (Table 2). For sediments, total hydrocarbon concentrations exceeding 500 μg g^{-1} are indicative of significant pollution, whereas values below 10 μg g^{-1} are considered to represent unpolluted sites (Tolosa et al., 2004). Maximum levels were observed within the Augusta Bay stations, indicating that strong anthopogenic influences dominated this region and were comparable to chronically oil-contaminated sediments in Hong Kong (e.g. up to 646 μg g^{-1}; Hong et al., 1995) and the Caspian Sea (up to 1820 μg g^{-1}; Tolosa et al., 2004). In contrast, AHC in the Thracian Sea as well as in the offshore station of Augusta Bay and the characteristic offshore site of the South Ionian Sea exhibited concentration levels typical of non-polluted regions.

n-Alkanes displayed a wide distribution ranging from *n*-C_{14} to *n*-C_{39}. Short chain homologues ($\sum(C_{14}–C_{20})$; Table 2), mainly derived from phytoplanktonic sources (Gogou et al., 2000), as well as long-chain homologues ($\sum(C_{21}–C_{36})$; Table 2), characteristic of terrestrial higher plant waxes (Gogou et al., 2000), were prominent at the shallow station of Augusta Bay (Table 2) and at the deep stations close to hypersaline anoxic basins of the South Ionian Sea (Polymenakou, 2005).

One way to describe the origin of sedimentary organic matter is the carbon preference index (CPI) following the equation: $CPI = \sum(C_{21}–C_{35})/\sum(C_{22}–C_{36})$ (Simoneit, 1999). CPI values corresponding to petroleum contamination or input of degraded planktonic material are approximately

Table 2
Characteristic parameters of aliphatic hydrocarbons estimated in the sediment samples

Sampling sites	AUGU1	AUGU2	AUGU3	THR08		THR10		THR23		THR30	
	0–1	0–1	0–1	0–1	8–10	0–1	8–10	0–1	8–10	0–1	8–10
Parameters											
AHC ($\mu g\ g^{-1}$)	3.4	462.8	163.3	14.4	8.4	6.9	7.4	1.9	9.8	13.8	1.2
AHC/OC ($\mu g\ g^{-1}$)	611.7	26,698.4	11,911.8	3829.1	1530.4	2041.0	2955.3	445.9	3030.5	3098.0	370.8
NA ($ng\ g^{-1}$)	1829.8	5127.9	4941.2	1899.4	2444.7	1750.9	532.7	1775.8	2293.0	1839.3	812.5
NA/OC ($\mu g\ g^{-1}$)	330.6	295.8	360.5	503.4	443.6	515.3	212.9	424.2	705.5	411.4	246.5
UCM ($\mu g\ g^{-1}$)	1.3	447.4	155.8	12.4	6.0	5.2	6.9	0.1	7.4	11.9	0.4
$\sum(C_{14}–C_{20})$ ($\mu g\ g^{-1}$)	0.3	0.7	1.0	0.5	0.6	0.6	0.1	0.5	0.5	0.4	0.2
$\sum(C_{21}–C_{36})$ ($\mu g\ g^{-1}$)	1.5	4.4	3.9	1.4	1.8	1.1	0.4	1.3	1.8	1.5	0.6
Mar ($ng\ g^{-1}$)	128.7	342.8	494.9	156.6	220.1	268.4	61.7	222.2	206.3	118.2	87.5
Ter ($ng\ g^{-1}$)	572.3	1767.0	1171.0	442.2	704.1	386.0	145.5	473.6	600.8	425.9	226.4
% Mar to NA	7.0	6.7	10.0	8.2	9.0	15.3	11.6	12.5	9.0	6.4	10.8
% Ter to NA	31.3	34.5	23.7	23.3	28.8	22.0	27.3	26.7	26.2	23.2	27.9
CPI ($C_{21}–C_{36}$)	1.6	1.4	1.9	1.6	1.8	1.8	1.5	1.7	1.5	1.5	1.8
Pr ($ng\ g^{-1}$)	86.4	84.0	122.3	93.6	18.1	102.6	59.3	54.4	119.1	39.1	30.2
Ph ($ng\ g^{-1}$)	57.0	75.2	108.7	75.8	8.7	43.4	39.1	44.9	41.7	31.8	17.5

AHC: aliphatic hydrocarbon total concentration; OC: organic carbon; NA: *n*-alkane concentration; UCM: unresolved complex mixture; Pr: pristane; Ph: phytane; $\sum(C_{14}–C_{20})$: sum of concentrations of NA from C_{14} to C_{20}; $\sum(C_{21}–C_{36})$: sum of concentrations of NA from C_{21} to C_{36}; Mar: sum of concentrations of marine NA C_{15}, C_{17} and C_{19}; Ter: sum of concentrations of terrestrial NA C_{27}, C_{29} and C_{31}; CPI($C_{21}–C_{36}$): carbon preference index estimated for NA from C_{21} to C_{36}.

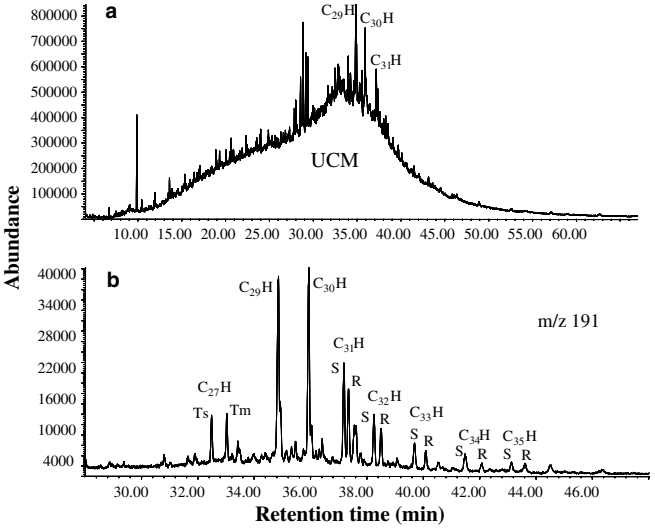

Fig. 2. (a) Representative gas chromatographic pattern of aliphatic hydrocarbons in the highly polluted station AUGU2 of Augusta Bay. UCM: unresolved complex mixture. (b) Example of ion chromatogram (*m*/*z* 191) of hopanes determined in station AUGU2. Names of hopane abbreviations are presented in Table 3.

1, whereas odd to even predominance (CPI > 1) suggests inputs of plant wax *n*-alkanes (Simoneit, 1999). The relative high CPI values for the sediments studied here (1.4–2.2; Table 2), combined with the predominance of long chain homologues compared to short chain compounds (Table 2), and the higher concentrations of the three most abundant terrestrial NA, C_{27}, C_{29} and C_{31} (Ter: 146–1767 $ng\ g^{-1}$) versus the main marine homologues, C_{15}, C_{17} and C_{19} (Mar: 61.7–268.4 $ng\ g^{-1}$) indicate a predominantly land-derived organic matter contribution to marine sediments (Gogou et al., 2000). The above results imply that atmospheric transport was the major mechanism by which hydrocarbons enter the Eastern Mediterranean Sea (Santos et al., 1994) at both shallow (Augusta Bay, Thracian Sea) and deep-sea (South Ionian Sea, Augusta Bay) sampling sites.

Besides *n*-alkanes, UCM was found to be a significant component of the aliphatic hydrocarbon fraction at the sampled stations (Table 2). A recent study has shown that UCM comprises a mixture of branched alkanes, cycloalkanes, monoaromatics, naphthalenes and multi-ring polyaromatic hydrocarbons (Frysinger et al., 2003) and are linked to degraded or weathered petroleum residues (Tolosa et al., 2004). UCM concentrations were higher in the sediments of Augusta Bay (up to 447.4 $\mu g\ g^{-1}$) whereas the lowest values were recorded in the deeper sediment layers of stations THR23 (0.1 $\mu g\ g^{-1}$; Table 2) and THR30 (0.4 $\mu g\ g^{-1}$; Table 2) of the Thracian Sea.

Additional evidence for the presence of petroleum residues in the sediment samples was also provided by the detection of the isoprenoid hydrocarbons pristane (Pr) and phytane (Ph) (Table 2; Volkman and Maxwell, 1986). The occurrence of oil pollution was confirmed by GC/MS monitoring of characteristic fragment ions of pentacyclic terpenoids (Wang et al., 1999; Gogou et al., 2000; Tolosa et al., 2004). Thus, we identified a series of C_{27}–C_{35} pentacyclic terpanes (hopanes) characteristic of oil-derived inputs in most of the stations (Philp, 1985) (Table 3). Homologues with number of carbon atoms $\geqslant C_{29}H$ exhibited the thermodynamically more stable 17α(*H*),21β(*H*) configuration with a peak occurring at the $C_{30}H$ homologue in most of the stations ($C_{29}H/C_{30}H$ ratios varied from 0.6 to 1.1) (Fig. 2b). The hopane series of C_{31}–C_{35} occurred as pairs of the C-22 diastereoisomers (22*S* and 22*R* epimers) (Table 3). The values of the ratio $22S/(22S + 22R)$ varied from 0.51 to 0.67 and were close to the equilibrium value of 0.6 for mature petroleum (Tolosa et al., 2004).

Table 3
Concentration of hopanes (ng g^{-1}) determined in the sediment samples

	Sampling sites	SI 0–1	U-2 0–1	U-3 0–1	AUGU1 0–1	AUGU2 0–1	AUGU3 0–1	THR08 0–1	THR23 8–10	THR30 0–1
Hopanes (ng g^{-1})	Name									
$C_{27}H$ Ts	18α(*H*)-22,29,30-Trisnorneohopane	–	239.5	50.0	–	410.9	103.0	–	–	–
$C_{27}H$ Tm	17α(*H*)-22,29,30-Trisnorhopane	–	–	–	–	423.4	106.5	–	16.8	–
$C_{29}H$	17α(*H*),21β(*H*)-29-Norhopane	51.1	211.5	157.4	64.7	2726.6	531.7	33.3	24.7	26.5
$C_{30}H$	17α(*H*),21β(*H*)-Hopane	55.9	285.2	205.4	84.8	2535.2	537.3	50.5	43.4	33.2
$C_{29}H/C_{30}H$		0.9	0.7	0.8	0.8	1.1	1.0	0.7	0.6	0.8
$C_{31}H$ (22*S*)	17α(*H*),21β(*H*)-Homohopane	41.6	224.9	167.0	31.3	1165.7	291.4	18.0	14.1	18.9
$C_{31}H$ (22*R*)	17α(*H*),21β(*H*)-Homohopane	27.2	130.0	111.9	20.8	783.4	211.6	17.3	11.2	13.4
$C_{32}H$ (22*S*)	17α(*H*),21β(*H*)-Bishomohopane	29.6	180.5	116.3	14.6	625.2	174.1	–	–	12.9
$C_{32}H$ (22*R*)	17α(*H*),21β(*H*)-Bishomohopane	18.1	90.7	80.4	–	438.9	130.1	–	–	8.0
$C_{33}H$ (22*S*)	17α(*H*),21β(*H*)-Trishomohopane	21.0	115.1	83.6	–	367.4	98.6	–	–	–
$C_{33}H$ (22*R*)	17α(*H*),21β(*H*)-Trishomohopane	17.7	47.1	43.2	–	192.8	63.7	–	–	–
$C_{34}H$ (22*S*)	17α(*H*),21β(*H*)-Tetrakishomohopane	17.7	37.4	74.8	–	267.9	96.7	–	–	–
$C_{34}H$ (22*R*)	17α(*H*),21β(*H*)-Tetrakishomohopane	11.6	–	33.2	–	99.7	36.5	–	–	–
$C_{35}H$ (22*S*)	17α(*H*),21β(*H*)-Pentakishomohopane	16.1	–	54.0	–	126.7	43.7	–	–	–
$C_{35}H$ (22*R*)	17α(*H*),21β(*H*)-Pentakishomohopane	8.6	–	42.5	–	113.7	45.7	–	–	–

Missing values denote levels below the detection limit.

3.3. Statistical analysis of geochemical data

Principal component analysis (PCA) of the data set (e.g. total organic carbon, black carbon, bioavailable organic carbon, chlorophyll *a*, phaeopigments, aliphatic hydrocarbon parameters) was carried out in order to get further insights into the geochemical features among sampling stations (Fig. 3). Data from a recent study in the South Ionian Sea were also included in the multi-variate analysis (Polymenakou, 2005). The results indicated that 78.4% of the total variance in environmental parameters could be represented by two principal components (PC) where PC1 and PC2 explained respectively 53.9% and 24.5% (Fig. 3).

The first component (PC1) in the score plot discriminates the most contaminated stations of Augusta Bay and South Ionian Sea which appear in the left half of the plot, with the more pristine sampling stations in the right half (Fig. 3). Both the deeper station of Augusta Bay (AUGU1) and the pristine station of the South Ionian Sea (SI) are grouped together with the stations from the Thracian Sea. The dominant projection of this component is mostly associated with the general concentrations of the different organic constituents and pigments. Thus, the distribution of samples from left to right reflects a gradient of decreasing primary productivity and organic matter content. The second principal component (PC2) discriminates the samples of the South Ionian Sea from Augusta Bay according to the levels of the different organic carbon forms (e.g. BC and BOC; Tables 1 and 2, and Fig. 3).

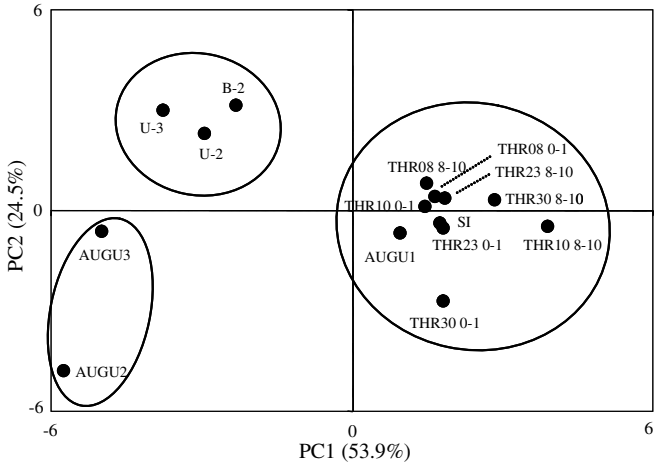

Fig. 3. Principal components analysis of the sediment characteristics (total organic carbon, black carbon, bioavailable organic carbon, chlorophyll *a*, phaeopigments, chloroplastic pigment equivalents, aliphatic hydrocarbons, *n*-alkanes, unresolved complex mixture, sum of short chain *n*-alkanes and sum of long chain *n*-alkanes). PC1 and PC2 explained respectively 53.9% and 24.5% of the total variance.

3.4. Microbial community composition

A useful approach to estimate the microbial biomass and community composition is to measure chemical components that are specific for the different microbial groups. For example, certain groups of microorganisms synthesize different arrays of PLFAs (White et al., 1979; Rajendran et al., 1993; Guezennec and Fiala-Medioni, 1996). PLFA analysis represents the current living community, since they are rapidly turned over following cell death (White et al., 1979). This makes PLFAs effective taxonomic markers, useful for characterizing the bulk composition of viable microbial communities. However, as there is an overlap in the PLFA composition between many species, it is not possible to assess the presence of individual species by PLFA analysis. Due to these limitations, a series of molecular based techniques have been developed using rRNA sequencing and gene coding for rRNA (rDNA). These latter methods have greatly enhanced our insights into the phylogeny and taxonomy of bacterial populations (Pace et al., 1986; Woese, 1987).

The fatty acids detected in the sediments included saturated, monounsaturated and polyunsaturated as well as branched-chain fatty acids ranging from $C_{14:0}$ to $C_{20:0}$ (Table 4). The concentration of total PLFA varied significantly between sampling stations with values ranging between 274 ± 97 and $10{,}656 \pm 959$ ng g^{-1} of sediment dry weight with the lower levels recorded in the deeper sediment layers (8–10 cm) of the Thracian Sea, likely indicating that phospholipids are not efficiently preserved in sediments. The levels of PLFA measured in the sediments from the Thracian Sea were comparable to the reported PLFA concentration in sediments from Thermaikos Gulf (North Aegean Sea) (219–2714 ng g^{-1}; Polymenakou et al., 2005a) and the Cretan Sea (South Aegean Sea) (524–1238 ng g^{-1}; Polymenakou et al., 2005a). In contrast, although PLFA concentrations from Augusta Bay were much higher they were still lower than those reported from eutrophic systems such as the Wadden Sea (36–71 μg g^{-1}; Langezaal et al., 2003), and a brackish lagoon in the German Baltic Sea (>40 μg ml^{-1} of sediment; Boschker et al., 2001).

The main marine microbial fatty acids found were the saturated $C_{16:0}$, $C_{18:0}$ and the monounsaturated $C_{16:1}$ and $C_{18:1}$ (Table 4). The saturated fatty acids, ranging from $33.4 \pm 2.1\%$ in the offshore station of Augusta Bay to $58.7 \pm 16.8\%$ in the shallower station THR08 (0–1) of the Thracian Sea, are generally ubiquitous in organisms from a wide range of genera (Rajendran et al., 1993). The high proportions of saturated fatty acids infer that a large fraction of the viable microbial communities inhabiting the sediments cannot be attributed to a certain functional group.

Monounsaturated PLFAs are typically found in gram-negative cell membranes (Ringelberg et al., 1997; Macnaughton et al., 1999). Previous studies have shown that both $C_{16:1}$ and $C_{18:1}$ fatty acids are characteristic indicators of bacterial origin (Oliver and Colwell, 1973; Langezaal et al., 2003) originating from methanotrophs (e.g. Virtue et al., 1996) or from thio-oxidizing bacteria (e.g. Guezennec and Fiala-Medioni, 1996). In the present study, the contribution of monounsaturated fatty acids to total fatty acids varied significantly between the stations of Augusta Bay and the Thracian Sea (Table 4). In Augusta Bay, gram-negative bacteria dominated microbial communities with concentrations ranging from $40.3 \pm 3.1\%$ to $41.8 \pm 0.4\%$ of the total fatty acids. In the Thracian Sea, with the exception of station THR10 (0–1), saturated fatty acids were much more abundant (Table 4). Moreover, the deeper layers (8–10 cm) of the Thracian Sea displayed the largest difference between saturated and monounsaturated fatty acids (Table 4). There was also a weak tendency for both saturated and monounsaturated fatty acids to correlate with the levels of organic carbon and CPE (Tables 1 and 4), possibly indicating that the abundance of functional groups inferred from fatty acid profiles, are associated with the different trophic states of the ecosystem.

White and coworkers (1996) reported that polyunsaturated PLFAs are found almost exclusively in eukaryotes whereas DeLong and Yayanos (1985) suggested that the polyunsaturated PLFAs can also be used as an indicator for the presence of barophilic bacteria since this group of lipids can play an important role in maintaining optimal membrane fluidity and hence making the organisms better adapted to the deep-sea environment (DeLong and Yayanos, 1985). The contribution of polyunsaturated to total fatty acids ranged between $0.2 \pm 0.3\%$ and $11.5 \pm 0.7\%$ with the minimum values recorded in the deeper sediment layers of the Thracian Sea (Table 2). The low abundances of polyunsaturated fatty acids strongly indicated that microeukaryotes or barophilic bacteria represent a minor fraction of the total benthic microbial community structure (Guezennec and Fiala-Medioni, 1996).

Branched saturated fatty acids are generally considered to indicate the presence of gram-positive bacteria (Zelles, 1999). The contribution of PLFAs associated with gram-positive bacteria, in relation to the total community, was similar for all stations with concentrations ranging from $12.4 \pm 4.4\%$ and $22.6 \pm 0.6\%$ (Table 4). Although branched-chain fatty acids are major components of gram-positive bacteria, they can be also found to the membranes of gram-negative bacteria. Indeed, recent studies using 16S rRNA gene clone library analysis in oxic sediments of the Eastern Mediterranean Sea revealed the absence of gram-positive bacteria (Polymenakou et al., 2005b). Thereby, we associated the presence of branched-chain fatty acids in the sediment samples to the presence of gram-positive and/or negative bacteria.

The analysis of microbial diversity indices indicates that highest values are reported in the surface sediment layer (0–1 cm; H': 2.45–2.64; Table 4) compared to the deeper one (8–10 cm; H': 2.16–2.33; Table 4). The high values of biodiversity (as H') indicated that both polluted and pristine sediments of the Eastern Mediterranean Sea harbour highly diverse microbial assemblages. Our values were comparable with reported values from the literature where molecular fingerprinting techniques were applied to estimate bacterial diversity in Mediterranean sediments (H' for surface sediments: 2.1–2.4; H' for 8–10 cm sediment layer: 1.8–2.6; Luna et al., 2004).

3.5. Oil contamination and microbial communities

The abundance of PLFAs characteristic for different functional groups of microorganisms (e.g. gram-negative, gram-positive, microeukaryotes or barophilic) was also compared between the sampling stations (Table 4) in an attempt to assess major shifts in microbial community composition along the trophic gradient.

All marker lipids were most abundant in the Augusta Bay station and in the most impacted station of the Thracian Sea (THR30 0–1; Table 4). However, lower abundances of all marker PLFAs as well as diversity indices were recorded in the deeper sediment layers, most likely due to the lower amounts of available organic carbon sources (e.g. organic carbon and chloroplastic pigment content). Microeukaryotes were much more abundant in the surface sediment layer

Table 4
Mean percentages (mean) and standard deviation (sd) of PLFAs determined in the sediment samples

PLFA (%)	AUGU1		AUGU2		AUGU3		THR08 (0–1)		THR08 (8–10)		THR10 (0–1)		THR10 (8–10)		THR23 (0–1)		THR23 (8–10)		THR30 (0–1)		THR30 (8–10)	
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
SFA (bacteria)																						
$C_{14:0}$	4.5	0.6	6.1	0.1	5.9	0.3	6.8	0.7	6.3	0.9	5.0	0.4	4.4	1.1	2.6	1.8	3.8	0.0	5.4	0.5	4.6	0.7
$C_{15:0}$	1.3	0.1	2.0	0.1	1.7	0.5	2.0	0.3	3.7	1.5	1.4	0.0	1.6	0.4	1.8	0.0	2.1	0.7	1.3	0.1	2.1	0.1
$C_{16:0}$	21.1	1.8	24.2	1.4	25.2	0.6	28.3	3.7	29.5	7.4	24.3	0.4	28.1	3.6	25.6	1.8	28.4	0.8	27.4	6.5	29.1	0.1
$C_{17:0}$	0.9	0.1	1.1	0.1	1.1	0.1	0.9	1.3	1.4	0.4	1.1	0.1	1.5	0.3	1.6	0.5	1.4	0.3	1.2	0.5	1.6	0.3
$C_{18:0}$	4.4	0.7	4.6	0.3	5.3	0.2	9.1	0.1	14.9	8.5	6.1	0.3	14.8	4.5	7.9	2.5	16.9	8.7	6.4	3.4	15.8	1.3
$C_{20:0}$	1.2	0.0	0.8	0.1	0.8	0.1	1.2	0.2	2.9	1.8	0.8	0.0	–	–	1.0	0.2	2.0	0.3	0.6	0.1	2.9	0.1
MUFA (gram-negative)																						
cis-$C_{16:1\omega7}$	1.7	0.2	0.9	0.0	0.9	0.2	0.7	0.9	0.8	1.2	1.2	0.1	1.0	0.3	0.7	1.0	–	–	0.7	0.3	–	–
cis-$C_{16:1\omega9}$ + *trans*-$C_{16:1\omega7}$	9.4	0.8	10.6	0.7	10.4	1.2	5.3	0.2	2.9	1.3	12.7	0.2	4.6	1.7	9.2	5.3	6.5	2.2	12.7	3.9	4.9	2.7
trans-$C_{16:1\omega9}$	3.5	0.6	2.0	0.1	2.0	0.3	1.9	0.2	0.3	0.5	2.0	0.0	1.1	0.2	1.8	0.5	1.1	0.2	1.1	0.4	0.0	0.0
$C_{17:1}$	1.6	0.1	1.0	0.3	0.8	0.2	0.5	0.7	–	–	1.2	0.1	1.2	0.2	1.0	0.1	0.7	0.0	0.6	0.1	–	–
cis-$C_{18:1\omega7}$	7.0	0.4	6.0	0.4	6.5	0.4	8.0	1.6	6.5	0.4	6.7	0.3	8.0	1.4	7.5	0.4	7.7	1.9	5.9	1.6	6.5	0.6
cis-$C_{18:1\omega9}$	17.1	1.7	16.7	0.4	17.1	0.7	16.2	0.4	6.9	7.9	13.8	0.3	15.2	4.0	15.7	1.3	11.4	2.6	9.6	1.1	6.6	4.2
trans-$C_{18:1\omega9}$	–	–	4.6	1.0	2.9	0.1	1.0	0.6	–	–	4.4	0.2	1.3	1.8	0.8	0.3	0.5	0.7	3.1	3.6	1.7	2.4
Br-FA (gram-positive and/or gram-negative)																						
i-$C_{15:0}$	5.2	0.7	4.9	0.0	3.8	0.1	4.2	0.9	1.1	0.4	3.8	0.4	2.8	0.3	3.4	0.4	2.8	0.5	2.1	1.0	1.0	1.4
a-$C_{15:0}$	5.6	0.7	4.9	0.3	4.2	0.2	3.1	1.4	2.1	0.9	3.3	0.3	5.2	0.1	2.8	0.8	6.2	0.1	1.8	0.7	3.4	1.1
br-$C_{15:0}$	0.4	0.2	–	–	–	–	0.5	0.1	0.7	0.9	0.3	0.1	0.6	0.1	0.3	0.1	–	–	0.4	0.1	1.1	0.1
br-$C_{15:0}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.2	1.7	0.3	0.2	–	–
i-$C_{16:0}$	1.9	0.1	2.3	0.1	1.6	0.0	1.7	0.2	0.2	0.3	3.7	0.1	1.1	0.8	2.7	1.2	1.3	0.3	4.3	2.2	–	–
br-$C_{16:0}$	4.4	0.0	2.3	0.0	1.9	0.2	1.4	1.9	2.1	0.7	2.6	0.1	3.3	0.3	2.4	0.6	3.2	0.5	1.3	0.6	–	–
i-$C_{17:0}$	2.8	2.3	1.2	0.1	1.3	0.1	1.2	1.7	10.8	3.5	1.2	0.1	0.9	0.2	1.9	1.0	0.6	0.3	1.5	0.4	–	–
a-$C_{17:0}$	1.5	0.5	1.1	0.2	1.3	0.4	0.8	1.1	–	–	1.0	0.0	2.6	0.3	1.4	0.3	1.9	0.2	0.8	0.0	17.2	3.2
PUFA (Microeuk./barophilic)																						
$C_{18:3}$	0.5	0.2	0.4	0.2	0.4	0.1	0.2	0.3	0.9	0.1	0.9	0.6	0.1	0.1	0.3	0.3	–	–	0.5	0.2	–	–
$C_{18:2}$	1.3	0.3	0.6	0.2	0.9	0.1	1.2	0.2	2.4	1.2	0.7	0.3	0.5	0.7	1.3	0.1	0.2	0.3	1.0	0.3	1.1	1.6
$C_{20:4}$	0.7	0.1	0.7	0.1	0.9	0.1	1.0	0.3	0.6	0.3	1.1	0.1	–	–	1.5	0.4	–	–	1.6	0.2	0.5	0.7
$C_{20:3}$	0.9	0.1	–	–	1.5	0.1	–	–	3.0	1.9	–	–	–	–	2.4	1.1	–	–	5.7	0.2	–	–
$C_{20:3}$	–	–	–	–	–	–	0.8	0.6	–	–	0.1	0.0	–	–	0.3	0.1	–	–	0.4	0.3	–	–
$C_{20:2}$	0.8	0.4	0.2	0.3	–	–	1.3	0.5	–	–	0.5	0.0	–	–	1.2	0.8	–	–	0.6	0.4	–	–
$C_{20:1} + C_{22:6}$	0.2	0.4	0.7	0.4	1.5	0.6	0.8	0.3	–	–	0.3	0.4	–	–	0.8	0.2	–	–	1.7	1.0	–	–
Total PLFA (ng g^{-1})	4458	103	10656	959	7086	712	1372	348	726	272	2455	552	516	49	2529	753	488	263	5296	2492	274	97
H' (diversity index)	2.64	0.10	2.54	0.06	2.55	0.06	2.45	0.21	2.32	0.26	2.59	0.03	2.33	0.08	2.59	0.02	2.32	0.05	2.58	0.11	2.16	0.00
J' (eveness)	0.82	0.02	0.80	0.01	0.80	0.02	0.79	0.01	0.79	0.07	0.80	0.00	0.79	0.04	0.79	0.00	0.80	0.03	0.77	0.03	0.82	0.03

Total amount of PLFA (ng g^{-1}), and the relative proportions of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and branched-chain fatty acids (Br-FA) are also presented. H': Shannon diversity index calculated on ln (Magurran, 1988); J': Pielou index for evenness (Pielou, 1975).

of station THR30 (Table 4) followed by the shallow sediments of Augusta Bay.

A previous study has shown that marine bacterial strains growing on petroleum products can generate high levels of odd saturated and monounsaturated fatty acids (Aries et al., 2000). Moreover, Pinturier-Geiss and coworkers (2002) suggested that the relative increase in phospholipid-linked branched and odd fatty acids is related to the development of hydrocarbonoclastic bacteria associated with the hydrocarbon-rich sediments. The relative increase in branched and odd fatty acids recorded in the shallow stations of Augusta Bay and the stations located close to the deep hypersaline anoxic basins of the South Ionian Sea (Polymenakou, 2005) could be associated with elevated amounts of hydrocarbons (Fig. 4).

Therefore, on the basis of these findings, we propose that the highly impacted sediments of the Eastern Mediterranean Sea represent an environment that has fostered microbial communities capable of degrading hydrocarbons in order to exploit all potentially available sources of carbon and energy. The question whether these microbial communities possess the ability to degrade natural hydrocarbons or hydrocarbons derived from oil contamination deserves further attention.

On the other hand no considerable correlation was recorded between total organic carbon ($R^2 = 50.3$, $n = 15$) or black carbon ($R^2 = 46.8$, $n = 15$) and PLFA content. Previous studies report that organic carbon is a poor indicator of bioavailable organic substrates since it contains an unknown refractory portion that is not available to benthic communities (Tselepides and Lampadariou, 2004). In contrast, chloroplastic pigments are considered as a fair indicator of labile organic substrates since they originate from primary production. In the present study, we estimated the refractory fraction of the total organic carbon and we found that the residual, presumably bioavailable organic matter, correlates well (despite their contamination levels) with the PLFA content only in the shallow sediments of Augusta Bay and Thracian Sea ($R^2 = 81.3$, $n = 10$). These results point to the key role of organic carbon partitioning between labile and refractory forms in regulating microbial community structure in the diverse habitats of the Eastern Mediterranean Sea. However, additional work is required before conclusive statements can be made about the importance of the refractory fraction of organic carbon for microbial community structure in differentially impacted sediments. With the use of molecular based techniques such as the 16S rRNA gene analysis we can get much more information about the effect of the refractory organic carbon on community composition at species level.

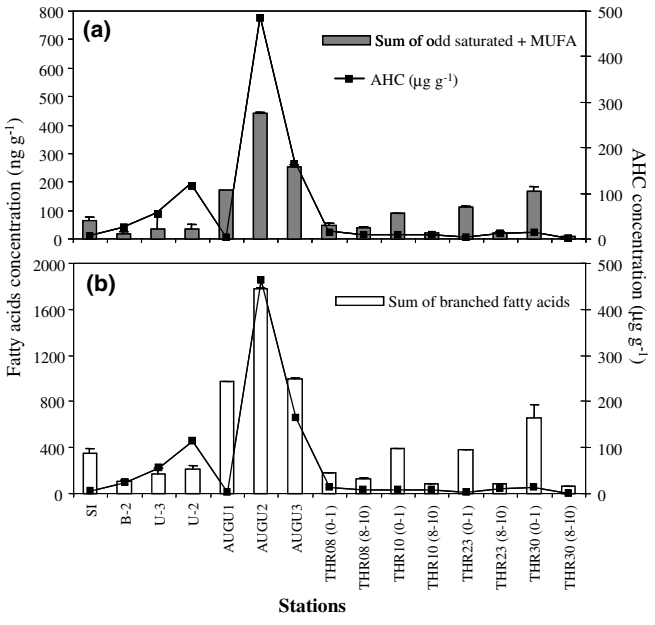

Fig. 4. Distribution pattern in the different sediment samples of (a) the sum of odd saturated and monounsaturated (MUFA) fatty acids and (b) the sum of branched fatty acids. Aliphatic hydrocarbons (AHC) are also plotted. Fatty acids and AHC concentrations are presented in ng g^{-1} and μg g^{-1}, respectively. Samples are listed in a random order (x-axis).

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