

## Horizontal and vertical complexity of attached and free-living bacteria of the eastern Mediterranean Sea, determined by 16S rDNA and 16S rRNA fingerprints

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### Abstract

The community structure of attached and free-living bacteria in the Aegean Sea (eastern Mediterranean Sea) was analyzed with use of terminal-restriction fragment length polymorphism (T-RFLP) fingerprinting. Since the Aegean Sea is characterized by rather small temperature fluctuations between surface and deep-water layers, it represents an ideal study site to determine the variations in the community structure of bacteria with depth, since environmental factors other than temperature are likely to determine depth zonation of bacteria. The analysis of 132 T-RFLP electropherograms indicated pronounced differences among the attached and free-living bacterial communities defined as operational taxonomic units (OTUs). Distinct vertical differences of attached and free-living OTUs were found between mesopelagic waters (>200 m depth) and the upper mixed water column (~10–200 m). Attached and free-living OTUs differed considerably throughout the water column, with only ~35% for the South Aegean and ~24% for the North Aegean of all OTUs in both free-living and attached OTUs. Approximately 50% of attached and free-living OTUs were present throughout the water column. Fingerprinting analysis using 16S rDNA indicated that only ~14% of the attached and ~33% of the free-living OTUs were identical to the 16S rDNA fingerprints. The distribution of free-living versus attached bacteria as obtained in this study suggests that even in the absence of temperature as a major selective factor, a distinct deep-water bacterial community exists (particularly in the free-living mode). The deep-water free-living bacterial community appears to be as compositionally complex as the surface water free-living bacterial community.

The eastern part of the Mediterranean Sea is a highly oligotrophic, phosphorus limited system with corresponding low phytoplankton biomass and production (Salihoglu et al. 1990; Krom et al. 1991; Robarts et al. 1996). In oligotrophic seas, bacterioplankton biomass often exceeds phytoplankton biomass even in the euphotic zone, and >50% of the primary production can be channeled through bacterioplankton (Azam et al. 1983; Cho and Azam 1990). This bacterioplankton community has been shown to be phylogenetically diverse, but most bacteria fall within only a few major clusters (Giovannoni et al. 1990). The comparison of surface waters from different regions of the oligotrophic Atlantic and Pacific showed that many gene lineages were similar (Mullins et al. 1995) and that gene clusters related to specific depths in the water column were present (Giovannoni et al. 1996). Large differences between surface and deep-water bacterial communities were also found by DNA-DNA hybridization (Lee and Fuhrman 1991). These differences in community composition between surface and deep-water

bacteria have been interpreted to reflect differences in the physicochemical conditions between these layers.

Sequence comparison of attached and free-living bacteria from the upper mixed water column indicated distinct phylogenetic differences between these two communities (DeLong et al. 1993; Acinas et al. 1999; Crump et al. 1999). Acinas et al. (1997) found a generally lower diversity of the attached community when compared with the free-living community. Bidle and Fletcher (1995) concluded that the composition of particle-associated bacteria is rather stable, whereas the free-living community rapidly changes along a transect in an estuary. The studies mentioned above are based on the analysis of 16S rDNA, which is a valuable indicator of the standing stock but does not necessarily resolve the composition of metabolically active bacteria (Giovannoni et al. 1990). RNA, particularly the 16S rRNA, has been suggested as an indicator for active bacteria (Poulsen et al. 1993). In the present study, we used terminal-restriction fragment length polymorphism analysis (T-RFLP; Moeseneder et al. 1999) to characterize complex marine bacterial communities. We compared the community structure of attached and free-living bacteria in an extremely oligotrophic environment (the South Aegean Sea) with an oligo- to mesotrophic environment (North Aegean Sea) using T-RFLP fragments of amplified 16S rDNA and, additionally, 16S rRNA. T-RFLP fingerprinting has proved sensitive to detect even bacteria present in low abundance (Moeseneder et al. 1999), which nevertheless could be a highly active fraction of the bacterial community.

An important, albeit still unsolved, question is the resource partitioning between different taxa of bacterioplankton in a seemingly rather homogenous environment, such as a mixed water column. Recently, Blackburn et al. (1998)

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showed that motile bacterioplankton are able to cluster around nutrient-rich micropatches. This might be a mechanism to facilitate resource partitioning among different bacterioplankton species by establishing concentration gradients of nutrients in the water column. These nutrient-rich patches or particles might be present at different scales, ranging from micrometer-sized detrital and colloidal matter to marine snow of up to 1 cm in diameter. Colonization of suspended particles by bacterioplankton has been suggested as a strategy to escape nutrient-depleted conditions in the ambient water, since particles harbor nutrients in concentrations at least one order of magnitude higher than that in the surrounding water (Goldman 1984; Azam and Cho 1987). Higher ectoenzymatic activity and biomass production have been frequently reported for particle-associated bacteria; however, this is not a universal feature, and it depends to a large extent on the nutritional quality of these particles (Karner and Herndl 1992; Smith et al. 1992). Most studies have been performed in coastal and estuarine environments, in which the particle load is higher and qualitatively different from open sea conditions, in which almost all the particles are autochthonously produced. The overall nutritive quality of autochthonously produced particles in the ocean is largely independent of the overall trophic condition of the surrounding water—only their abundance differs (Herndl 1992).

Thus, we expected a similar community composition of the particle-associated bacteria in the North and South Aegean Sea, whereas larger differences were expected for the free-living bacterial community between the two sites. We also addressed the question whether the composition of the attached bacterial community is more stable throughout the water column than that of the free-living bacterioplankton. Sedimentation of particles might rapidly transfer the particle-associated bacterial community to greater depth, which would ultimately lead to a more uniform distribution of the particle-associated community over the water column. On the contrary, free-living, nonsinking bacteria might develop distinct communities in different water layers, reflecting possible differences in the physicochemical conditions in different depth layers.

Furthermore, we hypothesized that the attached and the free-living bacterial communities experience a higher degree of similarity in the surface waters, where freshly produced particles might be colonized by bacterioplankton than in deep-water layers. The Mediterranean Sea is an ideal site to address all these aspects related to the vertical distribution of bacterioplankton and particle-associated bacteria, since there are no pronounced temperature gradients between surface and deep waters.

## Materials and methods

**Sample collection**—Raw seawater (RSW) was collected at four stations in the eastern Mediterranean Sea (Fig. 1) during a cruise on R/V *Aegaeo* in March 1998. Several depth layers were sampled with 10-liter Niskin bottles attached to a Sea-Bird CTD profiler. Fifty-milliliter subsamples of RSW were fixed with 0.2- $\mu\text{m}$ -filtered formaldehyde (2% v/v final concentration) and stored at 4°C for later bacterial enumer-

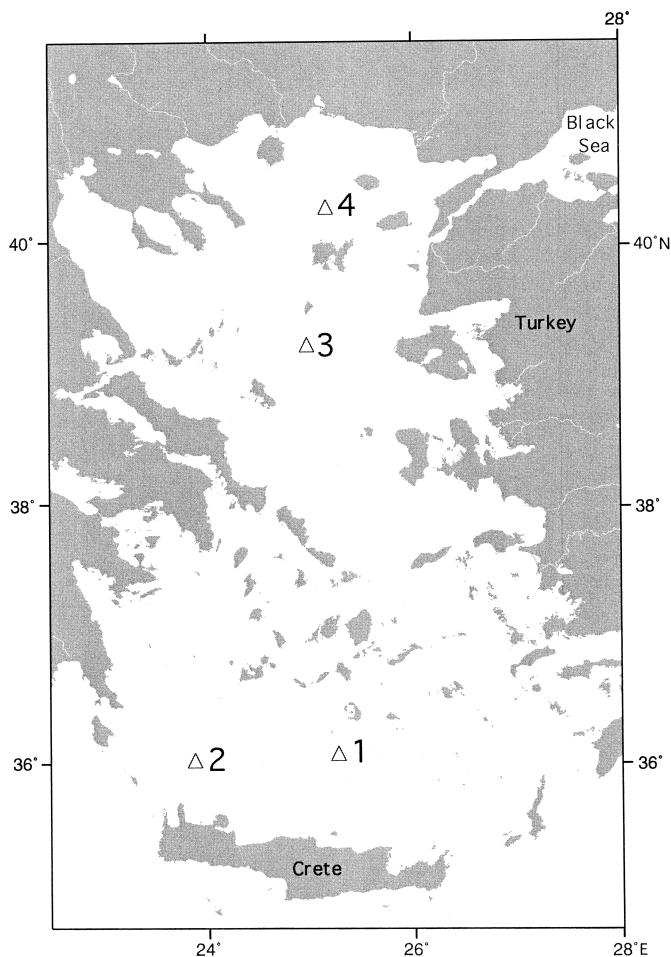


Fig. 1. Sampling sites (indicated by triangles) in the South (Sta. 1 and 2) and North (Sta. 3 and 4) Aegean Sea during the MATER cruise in March 1998. Sta. 1 represents MATER station msb1, Sta. 2 represents MATER station msb3, Sta. 3 represents MATER station mnb3, and Sta. 4 represents MATER station mnb1.

ation. For chlorophyll *a* determinations, 1 liter of RSW was filtered onto a Whatman GF/F filter and stored at  $-20^{\circ}\text{C}$  until analysis. For total organic carbon (TOC) measurements, 10 ml of RSW was filled in combusted ( $450^{\circ}\text{C}$ , 4 h) glass ampoules, acidified with 6N HCl to a pH  $<2$  and stored at  $-20^{\circ}\text{C}$ . For molecular characterization of the bacterial communities, 3–5 liters of RSW were filtered through Whatman GF/C filter to collect “particle-associated” bacteria. These filters were stored in sterile tubes (Greiner). The fraction of the bacterial community passing the Whatman GF/C filters was considered to be the “free-living” bacteria. These free-living bacteria were collected onto 0.2- $\mu\text{m}$  Sterivex cartridges (Millipore) applying a pressure  $<100$  mbar. All filters for molecular characterization of the attached and the free-living bacteria were stored at  $-80^{\circ}\text{C}$ .

**Bacterial abundance, Chl *a*, and TOC determinations**—Bacterial abundance was determined by acridine orange counting (Hobbie et al. 1977) and Chl *a* and pheopigments according to Yentsch and Menzel (1963). TOC concentrations were determined by the high temperature combustion

method using a Shimadzu TOC-5000 analyzer with a platinum catalyst on quartz (Benner and Strom 1993). Standards were prepared with potassium hydrogen phthalate (Kanto Chemical Co. Inc.). Both the water blank (i.e., Milli-Q water redistilled with 10 mM potassium peroxide sulfate and 20 mM *o*-phosphoric acid [Sigma]) and instrument blank were assessed before and after sample analysis. Blanks typically ranged between 3.3 and 4.2  $\mu\text{M C}$  (water) and between 1.6 and 2.5  $\mu\text{M C}$  (instrument), respectively. The overall analytical precision was always better than 3%. To determine the contribution of particulate organic carbon to the TOC pool, the organic carbon concentration in unfiltered versus filtered water samples was compared in a set of samples. TOC concentrations in the unfiltered samples were, on average, only slightly higher than those in the filtered DOC samples, with occasionally higher carbon concentrations in the filtered water samples. Thus, the reported TOC also roughly represents the DOC concentrations.

*Nucleic acid extraction and purification*—Each 15-ml tube containing a Whatman GF/C filter or a Sterivex cartridge was filled with 2 ml of lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA, and 50 mM Tris-HCl; pH 9.0) and incubated with lysozyme (final concentration 1 mg ml<sup>-1</sup>, Sigma) at 37°C for 30 min. Proteinase K (final concentration 100  $\mu\text{g ml}^{-1}$ , Fluka) and sodium dodecyl sulfate (final concentration 1% [wt/v], Sigma) were added and incubated at 55°C for 2 h. The lysate (checked under the microscope for complete lysis) was extracted with 2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) after a precipitation at -20°C overnight in 0.1× the volume of 3 M sodium acetate (pH 5.2, Sigma) and 3× the volume of 100% (v/v) ethanol. Nucleic acid pellets were resuspended in 200  $\mu\text{l}$  diethyl pyrocarbonate-treated water and stored at -80°C. RNA was removed from DNA in 40  $\mu\text{l}$  nucleic acid subsamples with DNase-free RNase (final concentration 100  $\mu\text{g ml}^{-1}$ , Pharmacia Biotech) at 55°C for 30 min. DNA was further purified by use of the Qiaex II Extraction Kit (Qiagen), following the manufacturer's instructions. For RNA analysis, DNA was removed from 40  $\mu\text{l}$  nucleic acid subsamples with 20 U RNase-free DNase (Pharmacia Biotech) at 37°C for 30 min. RNA was extracted and precipitated as described for DNA. The efficiency of the DNA removal from RNA was checked by amplifying 1  $\mu\text{l}$  of each RNA preparation in 50  $\mu\text{l}$  polymerase chain reaction (PCR) reactions (conditions as described below). PCR reactions that did not give a product (i.e., DNA was removed efficiently, and RNA is not amplified by the *Taq* polymerase) were used for cDNA synthesis. Five microliters of these PCR reactions were analyzed in a 1% agarose gel (SeaKem LE, FMC) run in 1× TBE at 75 V for 50 min. Gels were poststained in an ethidium bromide solution (final concentration 0.5  $\mu\text{g ml}^{-1}$ ) for 20 min and acquired with the Fluoro-S MultiImager (BioRad) by exposing the gels for 30 s.

*cDNA synthesis for reverse transcription-PCR (RT-PCR)*—For eight samples (10 m and greatest depth from every station), a transcription of 16S rRNA into cDNA was performed with "first-strand-reaction-mix beads" (Pharma-

cia Biotech), according to the manufacturer's protocol, by use of 1  $\mu\text{l}$  pd(N)6-primer (0.2  $\mu\text{g ml}^{-1}$ , Pharmacia Biotech). The cDNA was purified with Qiaquick PCR columns (Qiagen), and 1  $\mu\text{l}$  cDNA was used per 50  $\mu\text{l}$  PCR reaction. The reliability of this cDNA approach for subsequent reactions was first tested in experiments by mixing equal amounts of pure RNA from three marine bacterial strains for cDNA synthesis (Moeseneder et al. in prep.). The 16S rDNA sequences obtained from these strains were submitted to GenBank (Benson et al. 1998) and are available under the accession numbers AF237975, AF237976, and AF237977. The RT-PCR product (primers as described below but without fluorescent label) resulting from this mixture was cloned by use of the pGEM-T cloning kit (Promega) and the whole inserts (>1,460 bp) of 35 randomly picked clones were sequenced. In this small clone library, all three strains were detected again, and none of the 35 clones indicated a chimeric structure when analyzed with the program CHECK\_CHIMERA from the Ribosomal Database Project II (Maidak et al. 1999). On the basis of these results, we concluded that our approach did not lead to a preferential production of chimeric structures, which would result in artificial differences in the T-RFLP pattern between the 16S rDNA and 16S rRNA approach.

*PCR and RT-PCR for T-RFLP and restriction digests*—The primers used for PCR and RT-PCR were the *Bacteria*-specific primer 27F-FAM 5'-AGA GTT TGA TCC TGG CTC AG-3' and the universal primer 1492R 5'-GGT TAC CTT GTT ACG ACT T-3' (Lane 1991). 27F-FAM was 5' end-labeled with phosphoramidite fluorochrome 5-carboxy-fluorescein (5' 6-FAM) by Eurogentec (Searing, Belgium). Each 50- $\mu\text{l}$  PCR or RT-PCR reaction consisted of 0.2  $\mu\text{M}$  of primers, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  of each dNTP, and 2.5 U of *Taq* polymerase (both from Pharmacia Biotech). Samples were amplified by use of an initial denaturation step at 94°C (3 min), followed by 30 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), and an extension at 72°C (1 min). Cycling was completed by a final extension at 72°C (7 min). The FAM-labeled PCR products were purified with Qiaquick PCR columns (Qiagen). With this protocol, we removed >99% of the fluorescent primer from the PCR product. The PCR and RT-PCR products were checked for purity under the same conditions as those applied for T-RFLP separation and detection, and the remaining primer peak was excluded from subsequent T-RFLP analyses. PCR and RT-PCR products always gave a PCR product in the expected size range (~1500 bp, data not shown). FAM-labeled PCR products were digested at 37°C for 6 h. Each digest contained 70 ng cleaned PCR product, 10U *HhaI* or *RsaI* (Pharmacia Biotech), and the recommended buffer (final reaction volume 100  $\mu\text{l}$ ). Previous results with identical amounts of PCR products and the same reaction conditions indicated a complete digestion of the PCR products after an incubation time of 3 h (data not shown). Restriction digests were desalted with the Qiaquick Nucleotide Removal Kit (Qiagen).

*T-RFLP separation and detection*—FAM-labeled fragments were separated and detected with an ABI Prism 310

capillary sequencer (Perkin Elmer) run under GeneScan mode. Separation was performed in a 47-cm noncoated capillary (inner diameter 75  $\mu\text{m}$ , Perkin Elmer) by use of POP4 polymer and sequencing buffer (both from Perkin Elmer). According to the manufacturer, fragments of up to 500 bp can be sized with an accuracy of 1 bp with POP4. Our results indicate that the resolution is  $\sim 2$  bp for fragments up to 700 bp and  $\sim 5$  bp for fragments up to 1,000 bp (determination with size standards, data not shown). For T-RFLP analysis, 7- $\mu\text{l}$  restriction digest was denatured in the presence of 7.5- $\mu\text{l}$  deionized formamide at 94°C for 3 min. Additionally, each sample contained 0.5  $\mu\text{l}$  TAMRA 2500 marker (Perkin Elmer) for size determination of FAM-labeled fragments. Injection was performed electrokinetically at 15 kV for 10 s, and the runs at 15 kV were completed within 45 min. Samples were detected with laser-induced fluorescence detection using the virtual filter set (a) of the 310 acquisition software. In the present study, we separated the fragments under denaturing conditions using an automated sequencer.

**Reproducibility of T-RFLP fingerprinting from complex bacterial communities**—Initial evaluations on the reproducibility of the T-RFLP approach were performed on a duplicate 1:10 dilution culture of a complex North Sea bacterioplankton community, incubated at 20°C in the dark for 48 h (C. Winter, M. M. Moeseneder, and G. J. Herndl pers. comm.). The cultures were filtered and extracted as described above.

**T-RFLP pattern and unweighted pair group with mathematical averages (UPGMA) analysis**—The T-RFLP fingerprints generated with each of the two enzymes were pooled and used for UPGMA analysis (Liu et al. 1997). The size of the FAM-labeled fragments (representative for different operational taxonomic units [OTUs]) was determined by comparison with the internal TAMRA 2500 size standards with use of the local southern size-calling method of the GeneScan 3.1 software (Perkin Elmer). The detection threshold was set to 50 RFU (relative fluorescent units) for the GeneScan software. In our hands, peaks larger than 50 RFU could be clearly separated from background noise of the electropherogram and were therefore included in the analysis. Additionally, all peaks were checked manually for correct size and shape. These checks corrected for two kinds of artifacts in T-RFLP electropherograms. First, on rare occasions, smaller secondary peaks ( $\sim 100$  RFU) were detected by the program, usually associated with a larger peak and only  $\sim 0.5$  nucleotides apart, probably representing partial degradation of the restriction fragment. These secondary peaks were excluded from the analyses. Second, some runs indicated differences in size for fragments larger than 536 bp, since the GeneScan software is using either the 827- or the 1,115-bp size standard peak for sizing larger fragments. For a size standard peak to be considered a match, the size standard peak had to be within  $\pm 400$  scans of its expected position (as defined by the corresponding size standard definition peak). Peak drifts for larger size standard fragments can cause artifacts which resulted in a  $\sim 20$ -bp shift in size determination. Whenever necessary, we corrected for these differences by standardizing all larger peaks ( $> 536$  bp) to

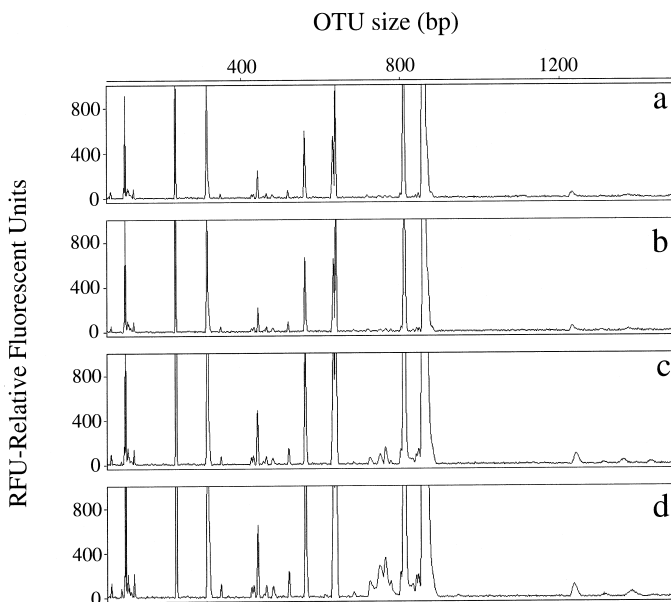


Fig. 2. Reproducibility test for the T-RFLP approach. A complex bacterial community was grown in duplicate dilution cultures for 48 h, and nucleic acids were extracted separately. Independent analysis of the T-RFLP patterns of this duplicate culture indicates identical results for the experiments where 16S rDNA (a, b) and 16S rRNA (c, d) were used as template for PCR. However, differences in the 700–800 bp range were found between the DNA and RNA approach.

the 827-, 1,181-, and 2,162-bp size standard peaks, to allow comparison of larger fragments. However, most peaks were clearly discernable, and the accurate size determination was independent of the peak intensity, as shown elsewhere (Moeseneder et al. 1999). Representative T-RFLP electropherograms are shown in Fig. 3. Figure 3b contains two major peaks in the size range  $> 800$  bp; nevertheless, the GeneScan software determined four additional peaks, which were confirmed manually when the y scale was set to an upper limit of 6000 RFU (data not shown). Figure 3c shows differences in size determination for fragments larger than 536 bp when compared with Fig. 3d. These differences were corrected manually, as described above. A matrix was established according to the presence (+) or absence (–) of a peak (i.e., 1 OTU,  $\pm 1$  bp for fragments up to 536 bp and  $\pm 5$  bp for fragments up to 1,000 bp). This matrix was further analyzed by use of the beta version of PAUP 4b2 (Sinauer Associates). UPGMA was applied with the restriction site distance matrix method described by Nei and Li (1979), to determine similarities between T-RFLP fingerprints.

## Results

**Overall physical, chemical, and biological characteristics of the sampling sites**—Generally, the salinity and temperature profiles revealed rather uniform water masses in the South and North Aegean Sea. Pronounced vertical gradients in temperature and salinity were lacking (Table 1). TOC concentrations were highest in the surface layers and decreased slightly with depth, although, occasionally, small peaks in

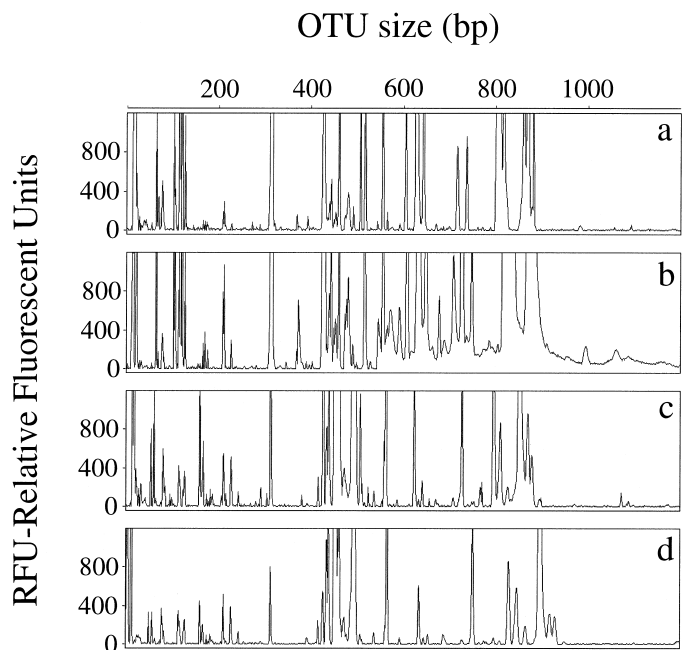


Fig. 3. T-RFLP fingerprints from free-living bacteria at Sta. 4 when the restriction enzyme *RsaI* was used; (a) at 10 m depth with 16S rDNA used as template for PCR; (b) at 10 m depth with 16S rRNA as template for PCR; (c) at 1,200 m depth with 16S rDNA as template for PCR; and (d) at 1,200 m depth with 16S rRNA as a template for PCR. For the description of the community structure at our four stations and six depths, a total of 132 T-RFLP fingerprints were used for UPGMA analysis, as shown in Fig. 5.

TOC were detected in specific depth layers (Table 1). At all stations except for Sta. 4, highest Chl *a* and pheopigment concentrations were detected at 50–100 m depth. The top 50 m layer at Sta. 4 was characterized by low salinity and temperature and, in comparison with the other stations, high concentrations in TOC, Chl *a*, pheopigments, and bacteria, reflecting the influence of Black Sea water (V. Zervakis, D. Georgopoulos, E. Papageorgiou, and V. Papadopoulos pers. comm.).

*Quantitative aspects of the horizontal and vertical distribution of attached and free-living bacteria*—The reproducibility test for the T-RFLP approach, using a complex community grown in duplicate dilution cultures, indicated identical patterns for both the 16S rDNA and the 16S rRNA approach, respectively (Fig. 2). Differences found between the 16S rDNA and the 16S rRNA approach in our samples therefore reflect actual differences between the bacterial community present and that metabolically active. Occasional migration shifts as for fragments larger than 536 bp (Fig. 3d) were corrected as described in the Methods section. The distributions of all T-RFLP fragments analyzed (i.e., attached and free-living OTUs for both enzymes) indicate that ~72% (range 66%–82%) of the 16S rDNA OTUs and ~76% (range 68%–82%) of the 16S rRNA OTUs were smaller than 536 bp. Including larger fragments to this data set, ~88% (range 84%–93%) of the 16S rDNA OTUs and ~87% (range 83%–

92%) of the 16S rRNA OTUs were smaller than 827 bp. The biggest fragments analyzed were ~1,000 bp.

In general, the two restriction enzymes used to determine the number of 16S rDNA OTUs of the attached and the free-living bacteria gave different results. Usually, the yield of OTUs was higher when the restriction enzyme *RsaI* was used, compared with *HhaI* (Fig. 4). Pooling the data of the two stations of the North and South Aegean Sea, respectively, revealed no significant differences in the number of OTUs of the attached bacteria between the South and North Aegean Sea (Fig. 4). When different depth strata were compared, the number of OTUs of the free-living bacteria, however, was significantly lower in the North Aegean Sea than in the South Aegean Sea (Wilcoxon,  $P < 0.001$  for the restriction enzymes combined,  $n = 20$ ). There was a clear decrease in the number of OTUs detectable with depth if the restriction enzyme *HhaI* was used (Fig. 4). When this restriction enzyme was used, depth explained between 66% and 88% and between 75% and 91% of the variation in the number of OTUs for free-living and attached bacteria, respectively. When the restriction enzyme *RsaI* was used, there was no clear tendency with depth detectable.

In the South Aegean Sea, the number of OTUs obtained for the free-living bacteria was significantly higher than the number of OTUs obtained from the attached bacteria (Wilcoxon,  $P = 0.003$  for *HhaI* and  $P = 0.001$  for *HhaI* and *RsaI* combined). No significant differences in the number of OTUs were found between the free-living and the attached bacteria in the North Aegean Sea.

*Qualitative aspects of the horizontal and vertical distribution of attached and free-living bacteria*—UPGMA analysis of the free-living and attached bacterial communities on the 16S rDNA level resulted in a clustering distinctly different from that based on the 16S rRNA (Fig. 5). This indicates that the active bacterial community comprises only a minor fraction of the bulk bacterial communities (active and dormant). Both the attached and the free-living bacteria formed distinct clusters. The composition of the free-living bacteria inhabiting the surface waters (<200 m) was similar at the two stations in the South, as well as at the two stations in the North Aegean Sea on both the 16S rDNA and the 16S rRNA level (Fig. 5). The free-living community in the surface waters was distinctly different from the community >200 m depth. UPGMA analysis indicated that the free-living community on the 16S rRNA level was more closely related to the free-living community on the 16S rDNA level than to the attached bacteria. The attached bacteria on the 16S rRNA level were distinctly different from all the other bacterial communities (Fig. 5).

Detailed analysis of the OTU pattern obtained for both restriction enzymes revealed that, on the 16S rDNA level, ~35% (range 24%–47%,  $n = 13$ ) of the OTUs of the attached bacteria were identical to the OTUs of the free-living bacteria in the South Aegean and ~24% (range 21%–28%,  $n = 12$ ) in the North Aegean Sea (Table 2). The percentage of identical OTUs of the attached and free-living bacteria was significantly higher in the South, compared with the North Aegean Sea (Mann-Whitney,  $P < 0.0001$ ,  $n = 13$  and 12 for the South and North Aegean Sea, respectively). On

Table 1. Selected physical, chemical, and biological parameters of the different sampling sites and depths. TOC = total organic carbon, Chl *a* = chlorophyll *a*, Pheo = pheopigments, BA = bacterial abundance.

Sampling site	Depth (m)	Temperature (°C)	Salinity	TOC (μM C)	Chl <i>a</i> (μg L <sup>-1</sup> )	Pheo (μg L <sup>-1</sup> )	BA (N × 10 <sup>5</sup> ml <sup>-1</sup> )
South Aegean							
1	10	16.017	39.149	57.6±3.0	0.199	0.166	6.4
1	50	16.028	39.148	78.6±2.9	0.260	0.220	7.6
1	100	16.002	39.142	71.1±2.2	0.265	0.173	6.3
1	200	15.213	39.070	61.5±3.6	0.097	0.088	4.5
1	500	14.495	38.999	69.3±1.7	0	0.008	2.4
1	1,000	14.240	39.030	47.3±1.8	0	0.001	1.5
1	1,600	14.192	39.072	46.3±2.2	0	0.002	1.4
2	10	15.649	39.014	72.9±0.7	0.241	0.179	5.3
2	50	15.656	39.070	64.0±1.7	0.213	0.175	7.3
2	100	15.618	39.064	55.3±1.1	0.311	0.239	6.3
2	200	15.411	39.033	71.8±3.1	0.025	0.047	3.8
2	500	14.532	38.985	59.1±1.8	0.001	0.013	1.6
2	1,000	14.315	39.020	78.7±1.5	0	0.002	2.2
North Aegean							
3	10	15.134	39.040	63.3±2.1	0.169	0.184	7.8
3	50	15.107	39.034	60.5±0.9	0.234	0.236	8.9
3	100	15.008	39.011	59.5±2.1	0.216	0.249	8.4
3	200	14.987	39.001	59.2±2.1	0.164	0.186	8.8
3	500	13.432	39.093	50.8±2.8	0.001	0.020	2.1
3	800	13.302	39.189	48.6±1.3	0	0.007	2.2
4	10	10.996	34.360	108.9±2.1	0.641	0.484	19.2
4	50	13.523	37.552	85.8±2.1	0.163	0.243	11.5
4	100	14.990	38.717	75.9±2.7	0.084	0.154	6.2
4	200	14.739	38.916	73.2±1.6	0.026	0.093	5.1
4	500	13.286	39.019	84.0±4.9	0.002	0.027	2.5
4	1,200	13.264	39.045	61.3±1.3	0.001	0.021	2.4

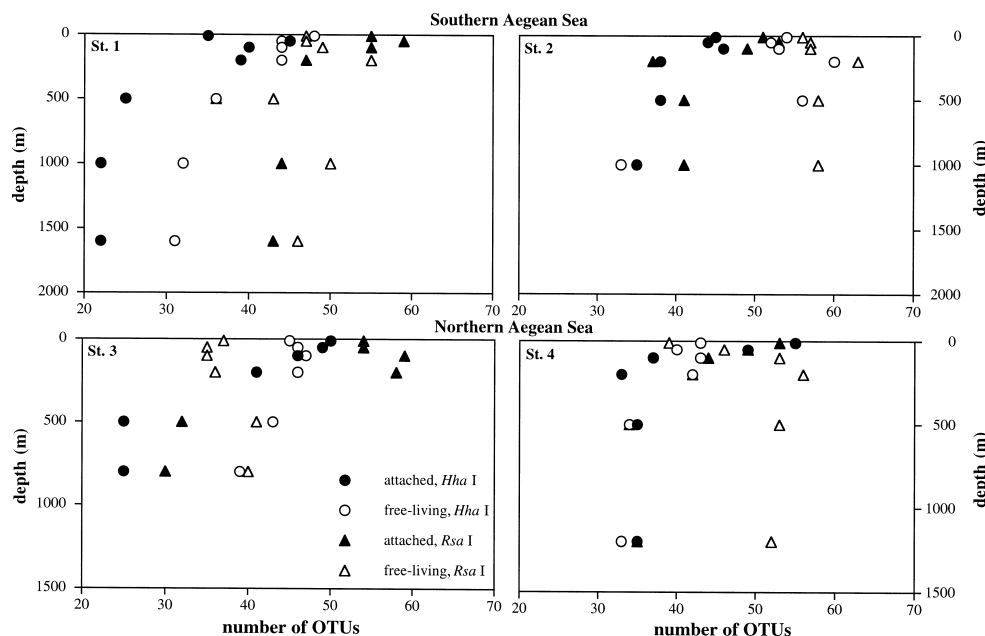


Fig. 4. Number of OTUs detected on the 16S rDNA level for the restriction enzyme *Hha*I and *Rsa*I at different depth horizons for the attached and the free-living bacteria at the four stations.

the 16S rRNA level, ~21% (range 16%–24%,  $n = 4$ ) and ~19% (range 17%–23%,  $n = 4$ ) of the OTUs were identical for attached and free-living bacteria in the South and North Aegean Sea, respectively (Table 2). When the OTUs detected for attached bacteria on the 16S rDNA and 16S rRNA level were compared, only ~16% (range 12%–17%,  $n = 4$ ) were found to be identical in the South Aegean Sea and ~12% (range 9%–16%,  $n = 4$ ) in the North Aegean Sea. For the free-living bacteria, however, ~38% (range 35%–42%,  $n = 4$ ) of the OTUs were found to be identical on the 16S rDNA and the 16S rRNA level in the South Aegean Sea and 29% (range 26%–39%,  $n = 4$ ) in the North Aegean Sea (Table 2). Thus, the percentage of identical OTUs on the 16S rDNA and 16S rRNA level was significantly higher for the free-living bacteria, compared with the attached bacteria (Mann Whitney,  $P = 0.0003$ ,  $n = 8$  for both, free-living and attached bacteria).

The distribution pattern of OTUs for attached bacteria at each individual station (Fig. 6) indicated a constant contribution of three categories of OTUs throughout the water column, consisting of OTUs ubiquitously present (at all stations and all depths), OTUs only found at one particular station, and OTUs only present either in the South or North Aegean Sea (Fig. 6). These three distribution categories of OTUs for attached bacteria made up ~50% of all OTUs detected in the surface waters and ~75% in the deeper water layers, because of the general decline in the number of OTUs with depth. The vast majority of the remaining 50%–25% of the OTUs for attached bacteria were specific for either surface waters (<200 m) or deep-water layers (>200 m depth), respectively. Usually, <5% of the OTUs present at a specific station appeared to be unique (only detected in a specific depth layer at a single station), but at Sta. 1 at 50

m depth, ~15% of unique OTUs in the attached bacterial fraction were detected.

Similar to the attached bacteria, these three distribution categories (i.e., ubiquitously occurring, station-specific, and specific for either the South or the North Aegean Sea) comprised ~50% of the total number of OTUs of the free-living bacteria found in the different depth layers (Fig. 7). In contrast to the attached bacteria, no increasing contribution of these three distribution categories toward greater depth was detectable when compared with the total number of OTUs. The number and the percentage of station-specific OTUs was significantly lower in the free-living than in the attached bacteria (Wilcoxon,  $P = 0.028$ ,  $n = 6$ ), whereas no general trend was detectable in the number and percentage of OTUs specific for either the South or the North Aegean Sea. A higher number and percentage of unique OTUs was found in the free-living bacteria and a more complex deep-water community than in attached bacteria (Figs. 6, 7).

## Discussion

In this study, we separated the free-living from the attached bacterial community using glass-fiber filters (Whatman GF/C) with a nominal pore size of ~1.2  $\mu\text{m}$ . In a comparable region of the Mediterranean Sea, it has been shown that ~90% of the free-living bacteria were found in the filtrate when GF/C filters were used (Gasol and Moran 1999). Generally, >95% of bacteria in the oligotrophic ocean are free living (Cho and Azam 1988). Because of the general scarcity of detrital particles in the oligotrophic Mediterranean Sea, the small sample size filtered (10 ml), and the fragile nature of marine snow, microscopic examinations during bacterial enumeration did not reveal distinct particles.

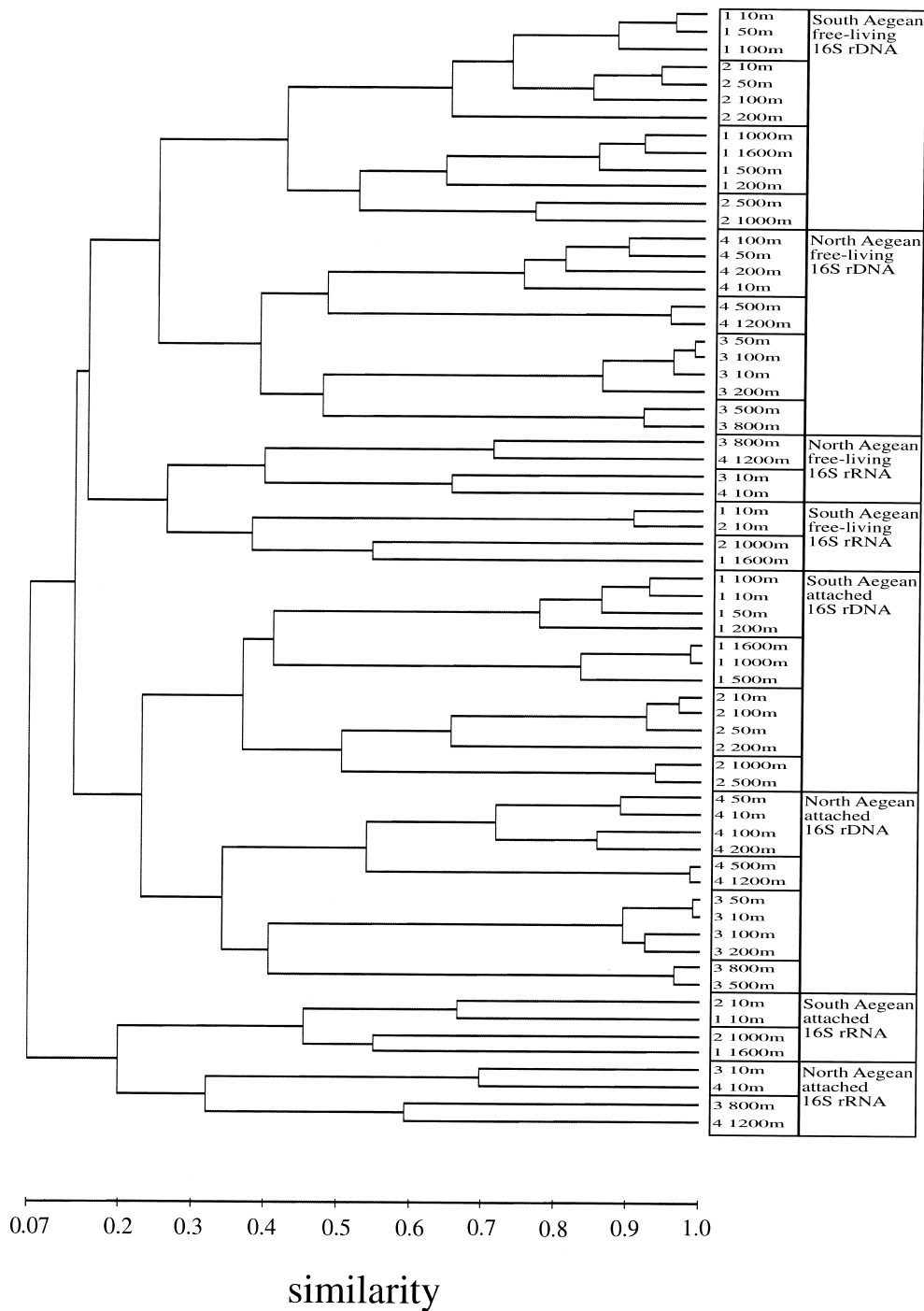


Fig. 5. UPGMA dendrogram of T-RFLP samples for attached and free-living bacteria from the South and North Aegean Sea. The information from two separate restriction digests (with the restriction enzyme *HhaI* and *RsaI*) were pooled and used for the construction of the matrix. Therefore the matrix consisted of 66 samples  $\times$  663 binary characters in total. 16S rDNA/rRNA indicates whether DNA or RNA was used as template for PCR.

Although we applied a low vacuum during the filtration procedures, it is possible that a fraction of the particle-associated bacteria, particularly the loosely associated ones, are included in the free-living fraction. As shown in Table 2, ~35% of the OTUs found in the attached bacterial fraction

were also found in the free-living bacteria in the South Aegean Sea and ~24% in the North Aegean Sea. These percentages represent the maximum possible error introduced to the OTU distribution of free-living and attached bacteria by our fractionation procedure. Our UPGMA analyses in-

Table 2. Total number of OTUs found for attached and free-living bacteria using T-RFLP analysis and restriction enzymes *HhaI* and *RsaI*. Either 16S rDNA or 16S rRNA reverse-transcribed into cDNA was used as a template for PCR.

Sampling site	Sampled at (m)	16S rDNA		16S rDNA		16S rDNA		16S rDNA/rRNA		16S rDNA/rRNA	
		OTUs total	OTUs attached	OTUs total	OTUs free-living	OTUs total	OTUs attached	OTUs total	OTUs attached†	OTUs total	OTUs free-living‡
South Aegean											
1	10	90	95	28.9	73	80	21.9	12.3	(%)	35.0	(%)
1	50	104	91	27.9	ND	ND	ND	ND	ND	ND	ND
1	100	95	93	31.6	ND	ND	ND	ND	ND	ND	ND
1	200	86	99	31.4	ND	ND	ND	ND	ND	ND	ND
1	500	61	79	34.4	ND	ND	ND	ND	ND	ND	ND
1	1,000	66	82	31.8	ND	ND	ND	ND	ND	ND	ND
1	1,600	65	77	33.8	58	60	15.5	17.2	17.2	36.7	36.7
2	10	96	110	43.8	92	80	20.7	17.4	17.4	38.8	38.8
2	50	97	109	45.4	ND	ND	ND	ND	ND	ND	ND
2	100	95	110	40.0	ND	ND	ND	ND	ND	ND	ND
2	200	75	123	46.7	ND	ND	ND	ND	ND	ND	ND
2	500	79	114	31.6	ND	ND	ND	ND	ND	ND	ND
2	1,000	76	91	23.7	66	62	24.2	16.7	16.7	41.9	41.9
North Aegean											
3	10	104	82	26.9	124	94	16.9	10.5	10.5	25.5	25.5
3	50	103	81	28.2	ND	ND	ND	ND	ND	ND	ND
3	100	105	82	27.6	ND	ND	ND	ND	ND	ND	ND
3	200	99	82	25.3	ND	ND	ND	ND	ND	ND	ND
3	500	57	84	24.6	ND	ND	ND	ND	ND	ND	ND
3	800	55	79	23.6	79	83	20.3	8.9	8.9	38.6	38.6
4	10	108	82	21.3	95	116	16.8	15.8	15.8	21.6	21.6
4	50	98	86	22.4	ND	ND	ND	ND	ND	ND	ND
4	100	81	96	21.0	ND	ND	ND	ND	ND	ND	ND
4	200	75	98	22.7	ND	ND	ND	ND	ND	ND	ND
4	500	69	87	21.7	ND	ND	ND	ND	ND	ND	ND
4	1,200	70	85	21.4	74	95	23.0	12.2	12.2	31.6	31.6

\* Percentage of OTUs in the attached fraction which were also found in the free-living fraction of the 16S rRNA fingerprints.

† Percentage of OTUs in the attached fraction from the 16S rDNA fingerprints which were identical to the ones in the attached fraction from the 16S rRNA fingerprints.

‡ Percentage of OTUs in the free-living fraction from the 16S rDNA fingerprints identical to the ones in the free-living fraction from the 16S rRNA fingerprints. ND = not determined.

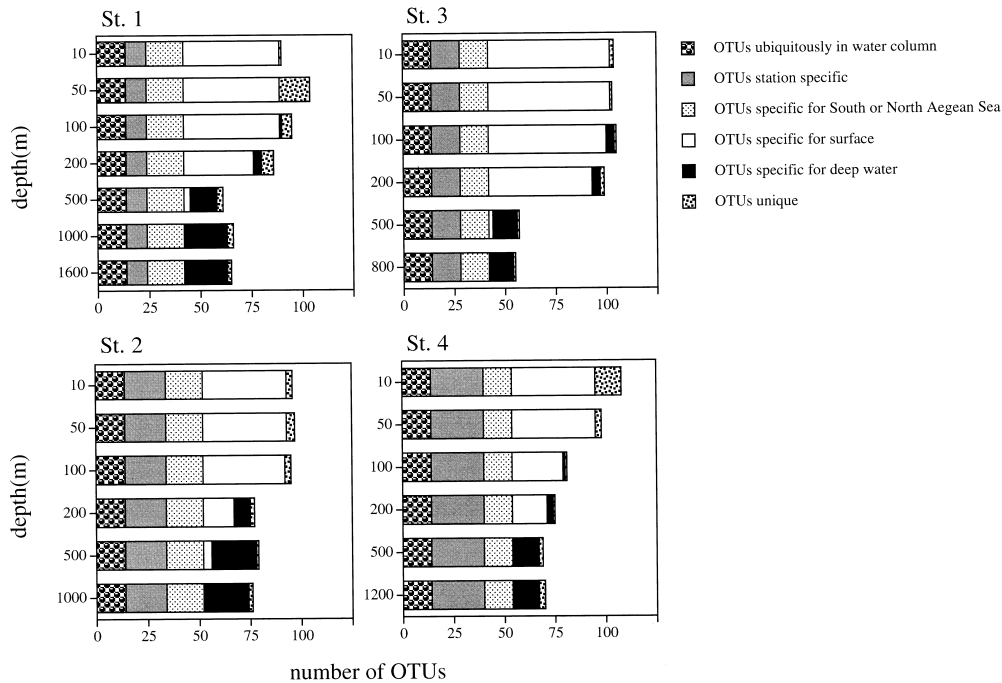


Fig. 6. Analysis of the spatial distribution of OTUs for attached bacteria by use of 16S rDNA and T-RFLP. The number of OTUs obtained from the 16S rDNA is grouped into six different categories of occurrence: OTUs found at every station at every depth (ubiquitously occurring); OTUs found only at a single station but throughout the water column (station specific); OTUs found at either in the South or North Aegean Sea but throughout the water column (specific for South or North Aegean Sea); surface-specific OTUs found only at one station; deep-water-specific OTUs found only at one station; and OTUs unique for a specific depth layer.

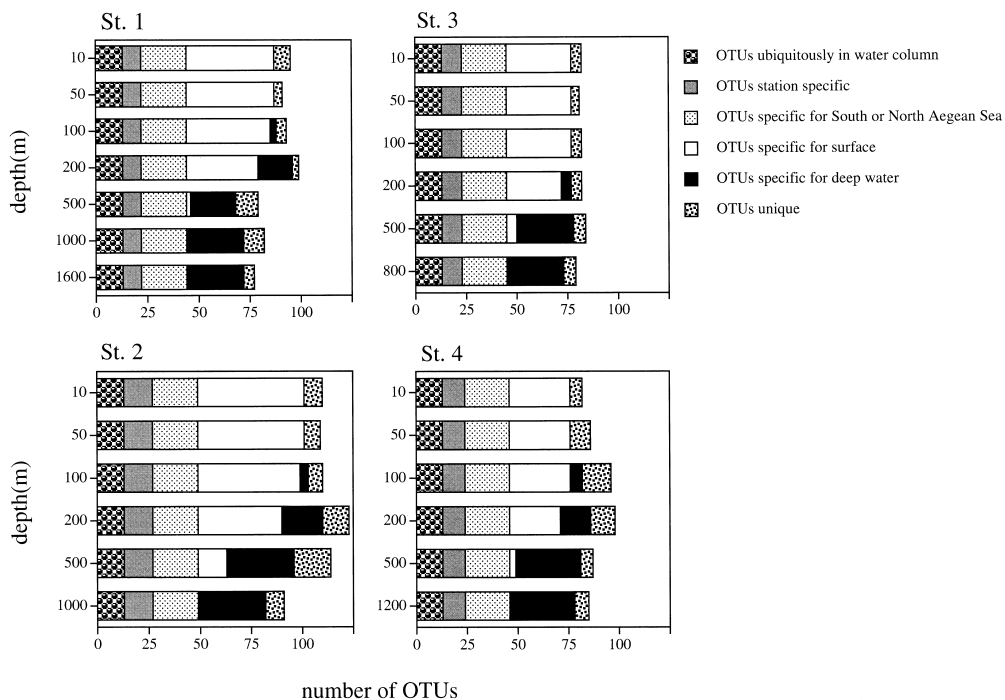


Fig. 7. Analysis of the spatial distribution of OTUs for free-living bacteria by use of 16S rDNA and T-RFLP. Grouping into categories of occurrence was performed as described in Fig. 6.

dicates that attached and free-living bacteria differed considerably. Distinctly different OTUs were found throughout the water column (Table 2), resulting in clusters of attached bacteria considerably different from free-living bacteria (Fig. 5).

A potential bias inherent to PCR is the possible amplification of templates such as plastids from phytoplankton. Although we cannot exclude that some of the peaks might be of phytoplankton origin, we consider this bias in our T-RFLP fingerprints rather small. The low concentration of phytoplankton in near-surface waters ( $<0.64 \mu\text{g L}^{-1}$ , Table 1) and their absence at greater depths contrasts with the high bacterial abundance throughout the water column (Table 1) and suggests that this kind of bias is probably negligible. Additionally, preliminary results of clone libraries from surface waters of the Aegean Sea do not indicate inserts of phytoplankton origin (M. M. Moeseneder unpubl. data).

16S rRNA was reversely transcribed into cDNA and used as a template for the characterization of active members of the attached and free-living bacterial community. Initial tests on this cDNA approach indicated that we did not produce chimeric structures when using random hexamer primers (see the Methods section). Some differences between the 16S rRNA and 16S rDNA fingerprints might be attributable to differences in the methods (like random priming and cDNA synthesis), although it is unlikely that these differences in the methods are responsible for the discrepancy between the 16S rRNA and 16S rDNA fingerprints. Numbers of attached and free-living OTUs based on 16S rRNA showed a vertical decrease of complexity similar (Table 2) to that obtained by the 16S rDNA approach (Fig. 4). However, 16S rRNA fingerprints indicate the presence of a large number of bacteria containing 16S rRNA, which were not detected in the 16S rDNA fingerprints, and only a small number of 16S rRNA OTUs are identical to the 16S rDNA OTUs (Table 2). These differences between the 16S rDNA and the 16S rRNA fingerprints might be explained by the numbers of 16S rDNA and 16S rRNA templates in bacterial cells. Analyses of bacterial strains indicated that bacteria can have 1–13 rRNA operons, possibly reflecting different strategies for growth (Schmidt 1997). Recent studies show that marine bacteria capable of growing under low nutrient concentrations tend to have a single rRNA operon (Button et al. 1998; Fegatella et al. 1998). One explanation could be that keeping the cell volume, the genome, and the number of rRNA operons of the bacterium small might allow higher metabolic rates and faster reproduction in an oligotrophic environment (Button et al. 1998; Fegatella et al. 1998). On a per cell base, ribosomes are much more abundant than rRNA operons. Depending on the growth rate of the bacterium, between 6,800 and 72,000 ribosomes  $\text{cell}^{-1}$  have been found for *Escherichia coli* (Bremer and Dennis 1996) and between 200 and 2,000 for an oligotrophic ultramicrobacterium (Fegatella et al. 1998). Because of the lower number of rRNA operons per cell, it is reasonable to assume that the successful detection of OTUs based on 16S rDNA fingerprints might therefore be mainly determined by the abundance of the organisms in the environment. In contrary, bacteria present in low abundance but with a high ribosome content might be still detectable on the basis of 16S rRNA fingerprints. The observed mismatch between the 16S rRNA

and 16S rDNA OTUs suggests that many bacteria may be low in abundance but metabolically highly active, as shown by their 16S rRNA fingerprints.

Because of these variations in 16S rDNA and 16S rRNA template copy number within the bacterial cell and the possibility of preferential amplification of certain templates during PCR, the relative contribution (peak area) of each OTU was not considered for T-RFLP analysis. Interpretation of peak areas might be possible if template concentrations and PCR biases are known. In our study, we described complex bacterial communities qualitatively. Thus, only the peaks (OTUs) above a threshold of 50 RFU were included in the analysis of presence or absence of the T-RFLP electropherograms.

Generally, the number of 16S rDNA OTUs declined with depth, particularly with the restriction enzyme *HhaI* (Fig. 4). This decline in the number of OTUs was more pronounced in the attached bacteria, which decreased by ~25%–50% from the surface to the deep-water layers ( $>200$  m depth, Fig. 4). A similar decline was observed for free-living bacteria but only at the stations in the South Aegean Sea (Fig. 4). The number of OTUs detected for free-living bacteria was significantly higher in the South than in the North Aegean Sea, whereas no differences in the number of OTUs were detectable for the attached bacteria between these two areas (Table 2). Although the South Aegean Sea is extremely oligotrophic and limited in phosphorus (Becacos-Kontos 1977), the North Aegean Sea is characterized by the inflow of mesotrophic Black Sea waters (V. Zervakis, D. Georgopoulos, E. Papageorgiou, and V. Papadopoulos pers. comm., see also Table 1), and thus the differences in OTU diversity may stem from differences in the nutrient status of the two areas. Attached bacteria living on particles create their own microenvironment and are therefore probably more independent from the trophic situation of the surrounding water. However, as particles sediment through the water column, the metabolic activity of attached bacteria might lead to a depletion of labile particle compounds, resulting in more refractory particles, which, in turn, might cause a decline in the complexity of the attached bacterial community with depth.

UPGMA analyses showed a distinct clustering of bacteria according to geographic origin and life mode (attached versus free-living), as well as differences based on 16S rRNA and rDNA templates (Fig. 5). When 16S rRNA templates were used, these differences were less pronounced for the free-living than for the attached bacteria, indicating that a smaller subset of the community on particles is metabolically active. The free-living, as well as the attached, bacteria clustered according to the adjacent depth at the specific stations on the 16S rDNA level, but nearest neighbors on the 16S rRNA level were usually at similar depth layers at the two stations in the North or South Aegean Sea, respectively. Overall, this UPGMA analysis indicates relatively little similarity between the free-living and the attached bacteria, suggesting limited exchange between the two communities. These results agree with previous studies showing distinctly different attached and free-living bacterial communities in the coastal marine environment (DeLong et al. 1993; Crump et al. 1999).

Contrary to our initial hypothesis, attached and free-living bacteria did not exhibit a higher degree of similarity in the surface layers, as might be expected if attached bacteria primarily originate from colonization of newly formed particles by free-living bacteria. The number of identical OTUs between the free-living and the attached bacteria was more or less constant throughout the water column (Table 2). However, shifts in the composition of the attached, as well as of the free-living, bacteria were detectable from the surface layers to the deep waters (>200 m) (Figs. 6, 7). The pronounced decrease in the number of OTUs with depth in attached bacteria (Figs. 4, 6) is largely due to the decline in the number of OTUs specific for surface water particles. At depth >200 m, the OTUs characteristic for surface waters are virtually absent (Fig. 6). This remarkable shift in the attached bacterial community composition was detectable within 300–400 m, suggesting that sedimentation of particles was slow. The attached bacteria consisted of ~20%–25% of OTUs ubiquitously present on particles at all stations and depths and ~15%–25% of station-specific OTUs with a generally higher fraction (up to 35%) of the latter category at Sta. 4, probably indicating the influence of Back Sea waters. OTUs specific for either the attached bacteria of the South or North Aegean Sea made up another 15%–25% of the total number of OTUs found in attached bacteria (Fig. 6). Thus, these three categories together made up ~50% in the surface waters and up to 75% in the deep waters. Generally, the contribution of deep-water-specific OTUs in attached bacteria was lower (~24%) than that in the free-living bacterial community (~35%). Overall, the distribution pattern of attached bacteria suggests that deep-water particles are colonized by a limited number of bacteria, probably adapted to exploit these poor habitats.

The percentage of the number of ubiquitously occurring OTUs in the free-living bacteria was lower than that for the attached bacteria. However, the percentage of OTUs specific for either the North or the South Aegean Sea was significantly higher in the free-living than in the attached bacterial community (Fig. 7). The number of unique OTUs specific for a distinct depth at a specific station was also higher in the free-living community. In contrast to the attached bacteria, a complex free-living bacterial community was found in the deep water (Fig. 7). The larger number of OTUs unique for free-living as opposed to attached bacteria found in distinct depth layers indicates slower vertical transfer of free-living than attached bacteria. However, similar to the attached bacterial community, ~50% of the OTUs detected were present throughout the water column, suggesting that a considerable fraction of the free-living and attached bacterial community is capable of at least surviving the expected changes in substrate supply with depth. For the free-living bacteria, depth-specific OTUs (surface and deep water) were the two categories contributing most to the total number of OTUs, again indicating vertical exchange of free-living bacteria lower than that of attached bacteria (Fig. 7).

In conclusion, we have shown that there are remarkable differences between the community detectable on the 16S rDNA versus the 16S rRNA level, indicating that the community present might considerably deviate from the active bacterial community. Contrary to our initial hypothesis that

the composition of the attached bacterial community should be similar to the free-living community in the surface layers, colonization of particles appears to be largely mediated by bacteria specialized in exploiting the specific microenvironment such particles offer. Generally, the exchange between the attached and free-living bacterial community seems to be rather limited. The free-living and attached bacteria showed a similar percentage of ubiquitously occurring OTUs, whereas deep-water-specific OTUs were more prominent in the free-living bacteria. Our data on the distribution of free-living versus attached bacteria when T-RFLP was used on the 16S rDNA and 16S rRNA level suggest that, even in the absence of temperature as a major selecting factor, there is a distinct deep-water community present, particularly in the free-living mode. The composition of the free-living deep-water bacterial community appears to be as complex as the surface water bacterial community.

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