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ORIGINAL PAPER

Application of T-RFLP analysis to the study of the coastal Black Sea bacterioplankton

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Abstract

Marine bacterioplankton play important roles in biogeochemical cycles in coastal regions of the sea. Despite its ecological importance, the composition of bacterioplankton community of coastal Black Sea, a unique marine basin in the World, is poorly characterized. Thus, the aim of this study was to analyze the bacterial communities in the Black Sea surface waters by terminal-restriction fragment length polymorphism (T-RFLP) analysis, a relatively new, fast and easy molecular method for assessing microbial diversity and community structure. Bacterioplankton assemblages were sampled in shelf surface waters in Constanta Bay between May and August 2004. T-RFLP patterns derived from enzyme digestion with HhaI were analyzed in order to provide a preliminary picture of the relative diversity of this complex microbial community. Members of both prokaryote domains, Archaea and Bacteria, have been successfully identified using T-RFLP analysis of amplified total community 16S rDNA from surface seawaters and were related to the environmental conditions. A minimum of 20 operational taxonomic units (OTU) were identified with T-RFLP. This study contributes to the evaluation of the Black Sea bacterioplankton diversity and it was used for the first time in Romania.

Keywords: bacterioplankton, Bacteria, Archaea, diversity, genetic fingerprinting method, T-RFLP, Black Sea

Abbreviations: T-RFLP: Terminal-restriction fragment length polymorphism, RFLP: Restriction fragment length polymorphism, OTU: operational taxonomic unit, T-RF: Terminal-restriction fragment, RFU: Relative Fluorescence Units, PCR: Polymerase Chain Reaction, psu: practical salinity units

Introduction

Understanding of bacterioplankton diversity (species richness and evenness) is important for marine microbiology due to its potential correlation to marine ecosystem function and stability (AZAM & al. [1], FUHRMAN, 2002 [2]).However, until recently our knowledge of diversity of natural marine bacterial communities have been limited by classical culture-dependent methods. More than 99.9% of the natural bacterioplankton community in seawater could not be cultured on standard culture media (FERGUSON & al. [3], CURTIS & al. [4]).

As the limitations of culture methods became clear, many different molecular approaches for evaluating microbial communities have been developed over the last decade. Most of them use the Polymerase Chain Reaction (PCR) to amplify genes of interest directly

from environmental samples, without the need to culture the microorganisms. The application of such techniques has resulted in detection of forms never before isolated. Among all available molecular techniques allow the estimation of marine bacterial species richness and community composition in a rapid and accurate way (AMANN R & al. [5], HUGENHOLTZ & al. [6]).

Terminal restriction fragment length polymorphism (T-RFLP) analysis is one of the newest community fingerprinting techniques for evaluating complex bacterial communities from the marine environments based on variation in the 16S rRNA gene (16S rDNA). It offers rapid characterization of bacterial community structure and dynamics populations in natural habitats, but may also be used to identify bacteria within the community without the need for any genomic sequence information (GRANT & al. [7], OSBORN & al. [8])

T-RFLP method is a significant advance compared with the initial designed restriction fragment length polymorphism (RFLP) analysis (DENMAN, & al. [9]). In a generic sense, T-RFLP refers to the use of fluorescently labelled primers combined with restriction digests to visualize sequence variation in either single- or mixed-species DNA samples. The initial step involves the extraction of total DNA from the microbial community and PCR-amplification of fragment of the 16S rDNA with fluorescently-labelled universal primers. The amplified DNA fragments (amplicons) are then immediately digested using a restriction enzyme that recognizes a particular genetic sequence that occurs repeatedly. This step generates fluorescently-labeled terminal restriction fragments (T-RFs). Next, the digested amplicons are separated and detected on either a polyacrylamide gel or a capillary gel electrophoresis apparatus, usually a DNA sequencer with a fluorescence detector. The output will be a series of peaks (fragments) of various sizes and heights that represents the distinct profile (T-RFs pattern or fingerprint) dependent on the species composition of the communities of the sample. The data are then either analyzed based on the number of peaks and the similarity of peak profiles across samples or individual species are identified and analysis focuses on these identified species (LIU & al. [10], DUNBAR & al. [11])

While the results of several molecular investigations of the bacterial assemblages of the central part of the Black Sea (oxic/anoxic chemocline) have been published in the last decade, relatively few refer to the coastal area of this basin (BIRD & al. [12], VETRIANI & al. [13], MORGAN & al. [14], WAKEHAM & al. [15]). Moreover, little is known about the identity and function of the planktonic bacteria (both Archaea and Bacteria) that inhabit particular shallow waters of the NW Black Sea even if these organisms are involved in most significant biogeochemical processes (BECQUEVORT & al. [16]). Therefore, in this study, T-RFLP fingerprinting technique was applied to explore and characterize the composition of complex marine bacterial community samples from surface water collected at one coastal site of the Black Sea.

Materials and methods

Study site and sample preparation

The investigation was conducted in a shallow coastal station located in Constanta Bay (44°10’N, 28°41’E, Romanian sector of the Black Sea, Fig. 1). Constanta Bay has features common to many shallow coastal ecosystems that are influenced by natural and anthropogenic sources of nutrients (COCIASU & al. [17]). Surface seawater samples (0.5 m depth) were taken twice a month from sampling site between May to August 2004. Between 300-500 ml seawater were filtered through a 0.2-μm-pore size polycarbonate filter (Millipore GTTP). All filters were stored at –20°C until processing. Water temperature, salinity and

inorganic nutrients (ammonium, nitrite, nitrate, and phosphate) were also determined by using standard methods (PARSONS & al. [18]).

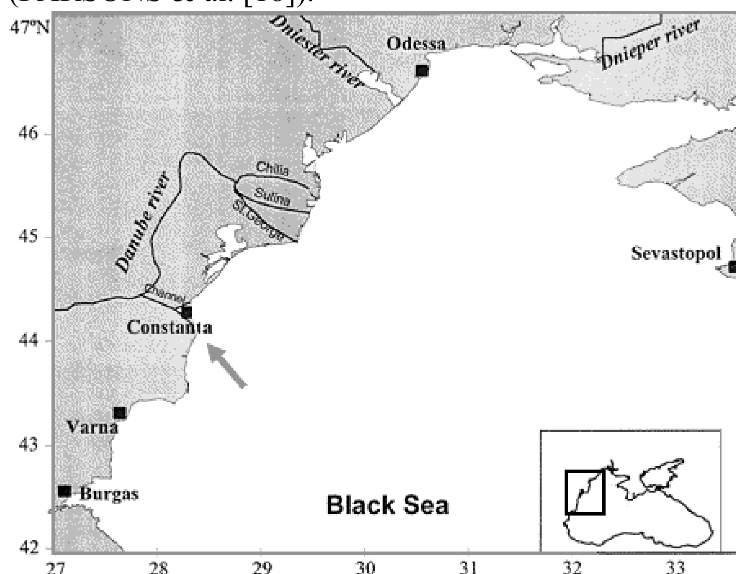

Figure 1. Location of the sampling site (Constanta Bay, Romanian Black Sea sector)

Terminal restriction fragment length polymorphism (T-RFLP)

In the present study, bacterioplankton communities were analyzed following the major steps of T-RFLP analysis on capillary electrophoresis (CE) systems as previously described (MOESENEDER & al. [19]).

DNA extraction and purification. Total DNA of bacterial community was extracted and purified from GTTP filters using the UltraClean™ Soil DNA Isolation Kit following the directions of the manufacturer (MoBio, Carlsbad, CA, USA). Extracted nucleic acids were quantified and sized by agarose gel electrophoresis, ethidium-bromide staining and visualized with UV transilluminator.

PCR for T-RFLP. The PCR conditions and chemicals were applied as described by MOESENEDER & al. [19]. Briefly, 1 to 2 μl of the cleaned nucleic acid extract was used as a template in a 50μl PCR mixture. The *Bacteria*-specific primer EUBAC27F-FAM (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R-JOE (LANE, 1991 [20]) were used to amplify a ~1,480-bp fragment of the bacterial 16S rRNA gene. The *Archaea*-specific primer ARCH21F-FAM (5'-TCCGGTTGATCCYCCGG-3') and the universal primer 958R-JOE (MOESENEDER & al. [21]) were used to amplify a ~ 920-bp fragment of the archaeal 16S rRNA gene. Both forward primers (EUBAC27F-FAM and ARCH21F-FAM) were 5'end-labeled with phosphoramidite fluorochrome 5-carboxy-fluorescein (5' 6-FAM) obtained by Eurogentec (Searing, Belgium). Several PCR reactions have been performed to obtain sufficient archaeal and bacterial PCR products (200-300 ng of DNA) for further steps. After PCR amplification, the FAM-labeled PCR products were analyzed by electrophoresis in a 1% (wt/vol) agarose gels (Gibco BRL). Then, the PCR products were visualized with a UV transilluminator, and bands were excised and purified with the QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.). With this protocol, the fluorescently labelled primers were efficiently removed from the PCR products.

Restriction digestion. PCR-amplified 16S rRNA gene fragments were digested with the *Hha*I restriction enzyme (Amersham Pharmacia) for at least 6 h at 37ºC and desalted by isopropanol precipitation (MOESENEDER & al. [21]).

Separation and detection of restriction fragments (T-RFs). The digested fragments were separated on an ABI Prism 310 automated capillary sequencer (Perkin-Elmer Applied Biosystems). Samples were detected with laser-induced fluorescence detection using the virtual filter set A of the 310 acquisition software.
Numerical analysis of T-RFLP patterns. For each sample, the sizes and numbers of the unique fluorescently labelled terminal restriction fragments were determined by comparison with the internal TAMRA-2500 size standard (ABI Biosystems) using GeneScan 3.1 software (ABI). The term operational taxonomic unit (OTU) was used to refer to individual restriction fragments in T-RFLP patterns, with recognition that each OTU may comprise more than one distinct bacterial ribotype (KENT & al. [22]). Peaks over a threshold of 50 units above background fluorescence were analyzed by manually aligning fragments to the size standard. Only those peaks with a peak area > 1% of the total peak area of the electropherograms were counted (LUKOW & al. [23]). T-RFLPs patterns were compared by calculating the relative abundances of individual T-RFs within samples. Relative abundance was estimated for each T-RF by dividing the peak area by the total peak area and multiplying by 100, and the results were displayed as histograms (BRAKER & al. [24]).

Results

Physicochemical properties of the sampling site

Generally, the temperature changed significantly between months, with the lowest temperatures (13.6°C) at the beginning of the sampling in May and an average maximum temperature in August (23.6°C). Salinity ranged from a minimum of 11.59 psu to a maximum of 17.86 psu which is the typical full strength marine water value for coastal marine Black Sea water. Monthly averages for inorganic nutrients concentrations varied over the investigation period with higher ammonium (6.07 μM), nitrate (8.03 μM) and phosphate (4.10 μM) concentrations level recorded in surface waters during the final phase of a sporadic upwelling event (July-August).

Diversity of 16S rDNA OTUs in coastal Black Sea waters

The composition and dynamics of bacterioplankton communities were investigated in one coastal Black Sea site over a period of four months (May-August). Replicate samples were taken fortnightly in each month, corresponding to the two seasons (late spring and summer), and analyzed by T-RFLP. The presence of different T-RFLP peaks defined as operational taxonomic units (OTUs) and their relative abundances was determined in each seawater sample.

T-RFLP measurements yielded a total of 74 different OTUs with an average of 23 to 36 OTUs in each sample. Fifty-nine OTUs were recorded for Bacteria community, whereas only 15 OTUs were obtained for Archaea community. The distribution of all T-RFLP fragments analyzed showed that 88% of bacterial OTUs and 65% of archaeal OTUs were smaller than 586 bp (Fig. 2 and Fig.3). Including larger fragments to this data set, 92% of bacterial OTUs and 81% of archaeal OTUs were smaller than 812 bp. The biggest fragments analyzed were approximately 1000 bp.

Analyzing the distribution of the bacterial OTUs during the entire four-month period, we found that 18 bacterial OTUs occurred in all samples, 15 were unique for a specific month (3 for May, 1 for June, 5 for July and 6 for August) and 16 were shared between the months (Fig. 4). Three fragments of the 15 archaeal OTUs obtained after cleavage with *Hha*I (331 bp, 586 bp, 812 bp and 915 bp) were common within all samples and had a similar relative

abundance (less than 20%). The other 10 archaeal OTUs were found only in July (69 bp and 509 bp) or in August (100 bp, 120 bp, 124 bp, 190 bp, 193 bp, 202 bp, 509 bp, and 577 bp) and represented 25% of the archaeal population. Only one archaeal OTU (234 bp fragment) was found to be shared between all four investigated months (Fig. 4).

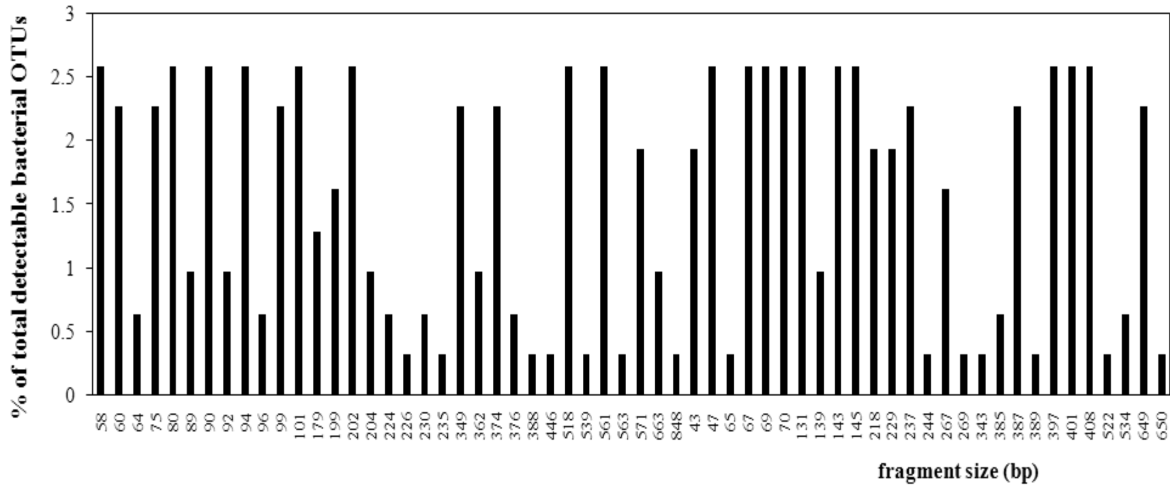

Figure 2. Size heterogeneity of T-RFLP OTUs found in bacterial community of the Black Sea

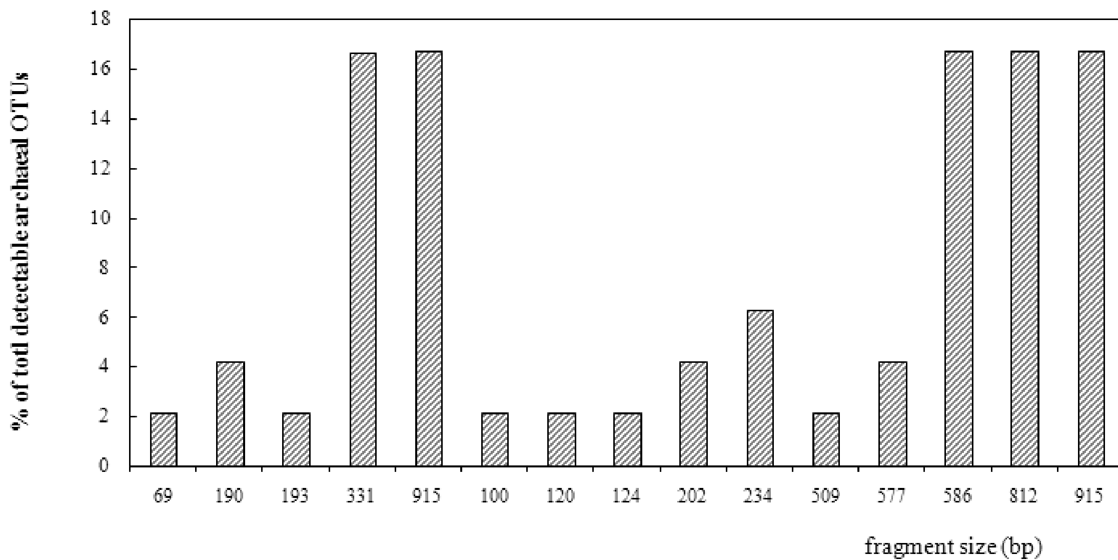

Figure 3. Size heterogeneity of T-RFLP OTUs found in archaeal community of the Black Sea waters (Constanta Bay). Each bar represents an OTU, and its length is in base pairs (bp).

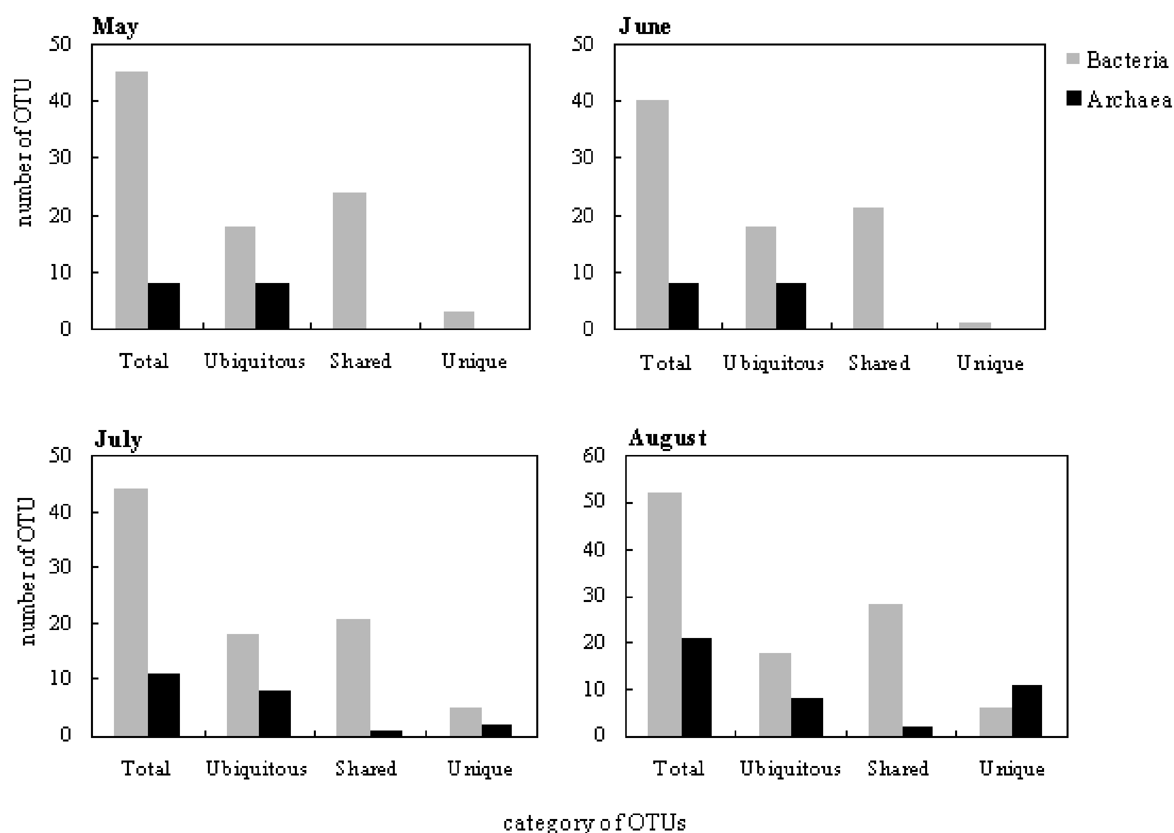

Figure 4. Analysis of temporal dynamics of bacterial and archaeal community composition in the Black Sea surface waters (Constanta Bay) between May to August 2004 by use 16S rDNA and T-RFLP. The number of OTUs is grouped into four categories of occurrence: OTUs found for the entire month analyzed (Total); OTUs found in every month (Ubiquitous); OTUS common for two or three months of analyzed period (Shared); and OTUs unique for a specific month (Unique).

The archaeal and bacterial 16S rDNA-targeted T-RFLP electropherograms generated using *Hha*I are shown in Fig. 5 and Fig. 6. The number of OTUs in Fig. 5 was threefold higher than that in Fig. 6.

Discussion

In this study, we assess the diversity and temporal variability of coastal Black Sea bacterioplankton communities by T-RFLP analysis. This molecular approach was used for its ability to rapidly detect and identify microorganisms in the complex microbial communities (CLEMENT, & al. [25])

The T-RFLP analysis resulted in very complex and highly reproducible bacterioplankton community fingerprints patterns and showed a clear difference between the bacterial and archaeal diversity in the surface coastal waters of the Black Sea (Fig. 2 and Fig. 3). While comparing the 16S rDNA-based T-RFLP community fingerprints patterns between two major bacterioplankton communities (Bacteria and Archaea), it was observed that bacterial T-RFLP patterns had more number of relatively high OTUs with higher peak area. The highest number of distinct OTUs in all samples analyzed was detected from amplified bacterial 16S rDNAs (Fig.2 and Fig. 3). These results indicated that bacterial community was

much more diverse than the archaeal community. However, the composition of archaeal OTUs clearly showed the permanent presence of archaea in the Black Sea coastal samples (Fig. 4), which confirmed the common suggestion that Archaea, previously thought to inhabit only extreme environments, represent a stable and specific component in many marine habitats (DELONG, 1992 [26]).

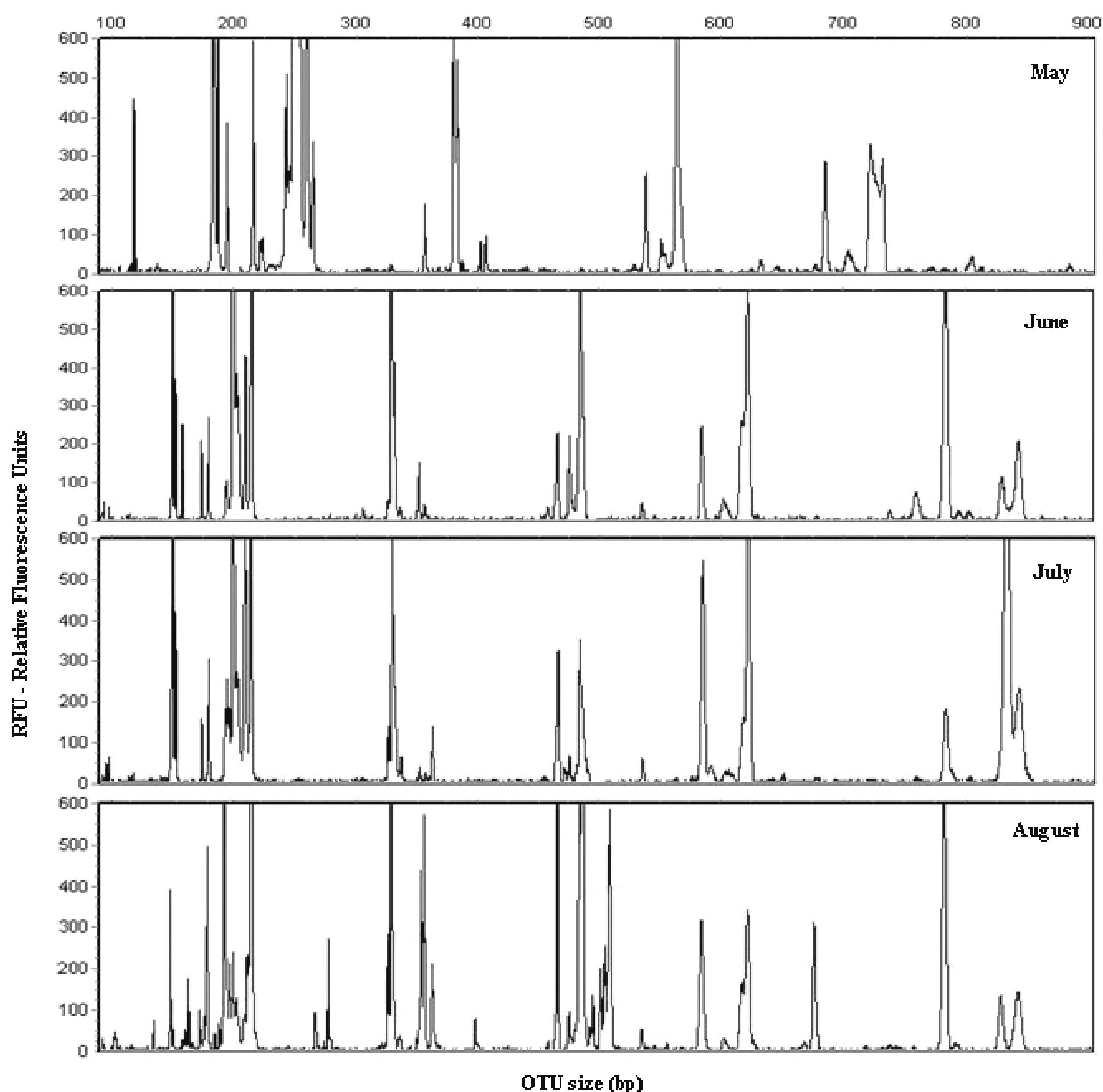

Figure 5.T-RFLP patterns from Constanta Bay generated by the enzyme *Hha*I and bacterial reverse primer (1492R-JOE). Each peak represents a different operational taxonomic unit (OTU) from the sample. The T-RFLP patterns are centered on the region of the major OTUs in the sample collected in May, June, July and August 2004

The richness and abundance of archaeal and bacterial communities as measured by T-RFLP, varied over the entire four-month period. The Bacteria community (70-91% of total detectable OTUs) was much more variable than Archaea community (10-12% of total detectable OTUs) in all seawater samples investigated (Fig. 4 and Fig. 5). The relative abundance bacterial OTUs increased slightly towards summer (late July and August).

However, the most differences between the mean values of bacterial OTUs were not significant. The dominant Archaea population was rather constant from late spring (May) through mid summer, but then shifted to a completely different community in July and maintained this community through the end of August (Fig. 4 and Fig. 6). Four (331 bp, 586 bp, 812 bp and 915 bp) of the 15 archaeal OTUs dominated the first period, and 11 OTUs the later period. Moreover, within 15 total archaeal OTUs detected in the Black Sea samples, 6 OTUs were found exclusively in samples collected in August.

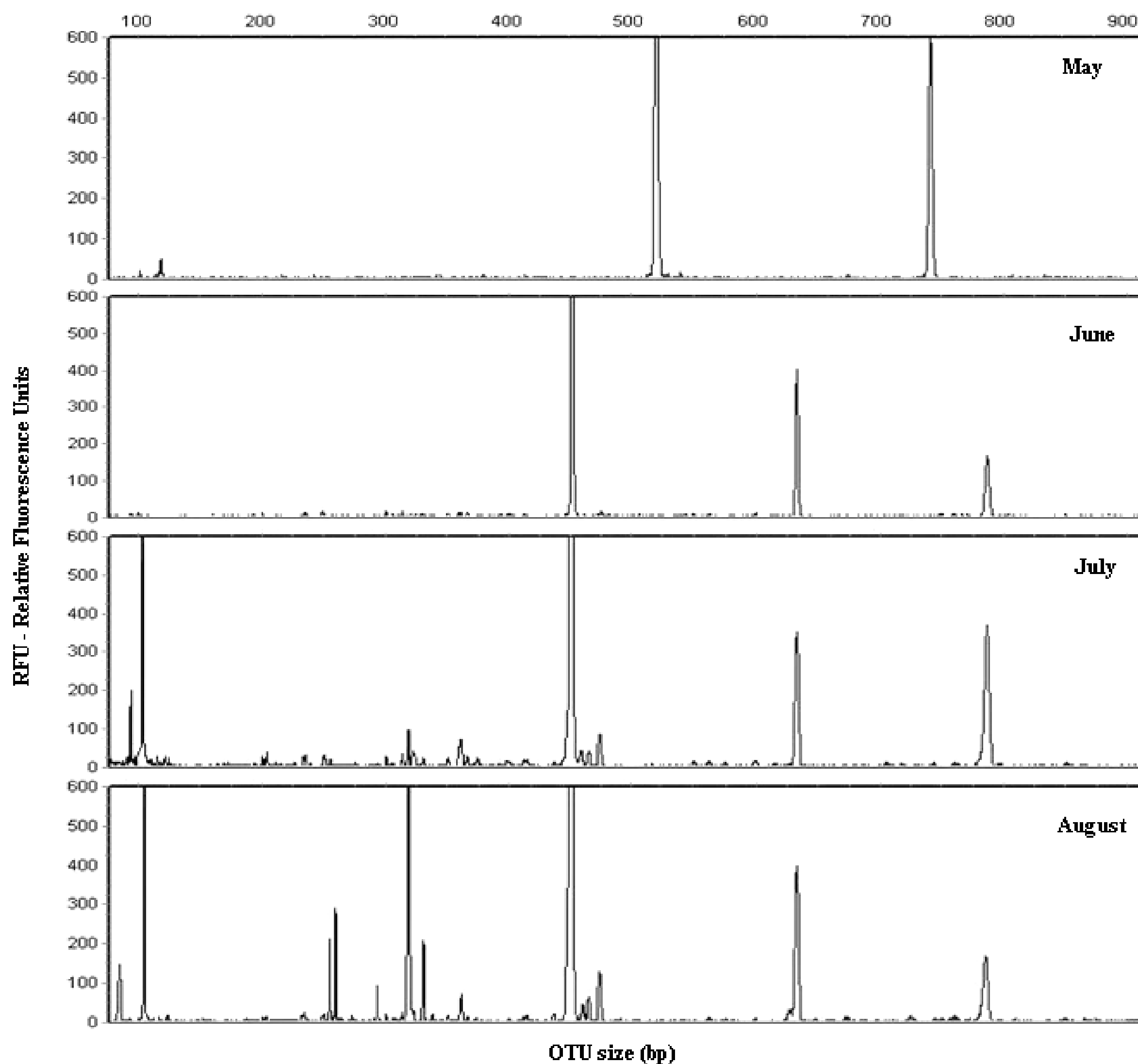

Figure 6. T-RFLP patterns from Constanta Bay generated by the enzyme *Hha*I and archaeal reverse primer (958R-JOE). Each peak represents a different operational taxonomic unit (OTU) from the sample. The T-RFLP patterns are centered on the region of the major OTUs in the sample collected in May, June, July and August 2004.

Although the differences seen were small and variable over time at the level of archaeal and bacterial communities, it is obviously that individual OTUs of *Archaea* and *Bacteria* were developed under the selection of differing environmental conditions. The soluble inorganic nutrients concentrations were relatively higher and variable in the near-shore waters of Constanta Bay throughout the investigation period. The increase in archaeal community richness (number of distinct OTUs) and abundance in the late summer, coincided with the

change in the environmental conditions (increase in nutrients concentrations and salinity concomitant with decreasing temperatures) determined by an episodic upwelling event found between July-August. Assuming a relationship between bacterioplankton taxonomic composition and nutrients availability (PINHASSI, & al. [27], FISHER, & al. [28]) this might lead to community variation along the four-month period investigation.

Overall, in our study bacterioplankton (Archaea and Bacteria) communities in Constanta Bay of the coastal Black Sea have been successfully explored using T-RFLP analysis. These results demonstrated, to our knowledge for the first time, that the coastal Black Sea harbour diverse and dynamic archaeal and bacterial communities that interact with differing environmental factors as previously observed in other temperate marine systems (POMEROY, & al. [29], BOUVIER, & al. [30]). Knowledge of the bacterioplankton composition, and how that composition varies over space and time, seems to be of major importance for understanding the role of bacteria in the biogeochemistry of the coastal NW Black Sea. Therefore we suggest that further detailed molecular studies of bacterioplankton would be worthwhile in this ecosystem of the Black Sea.

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