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ORIGINAL PAPER

In Situ Visualization of Natural Microbial Communities in Black Sea Coastal Shelf Sediments

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Abstract

A comparison of bacterial community structure in Black Sea coastal shelf sediments in two sites was conducted through cultivation-independent molecular techniques. Analysis of sediment samples with fluorescence in situ hybridization (FISH) showed that 75% of DAPI-stained cells hybridized with the general bacterial probe EUB338. Cytophaga-Flavobacterium cluster, along with the Gammaproteobacteria were the most abundant groups. We expected to find a higher percentage of Gammaproteobacteria in Agigea area, many microorganisms affiliated to this group are typically involved in biodegradation processes, but we found that it represents only 26.6% of the total cells counted comparing with Cytophaga-Flavobacterium group that represents 30.8%. On the contrary, in Northern Constanta area, we found a much higher percentage of Gammaproteobacteria subclass 47%, compared to Cytophaga-Flavobacterium cluster that represents 12.6% of the total cells counted.

Keywords**:** microbial communities, fluorescent *in situ* hybridization, Black Sea, marine sediments

1. Introduction

A method for the rapid and specific identification of individual microbial cells within their natural environments has been long awaited [1,3,11,20,22]. Traditional cultivation methods are time-consuming and frequently work only for a minority of the bacterial species present in a sample [1,2,3,8]. In recent years, molecular biological methods have extended our view to those microorganisms that have proved very hard or even impossible to be cultivated, therefore there was a need for a microscopy technique similar to that of the famous Gram-staining method [2,3]. The test needed to be as sensitive as the well-established immunofluorescence techniques [3], but instead of targeting antigens they based it on nucleic acids sequences. Most notably are the ribosomal RNA (rRNA) molecules and the genes encoding them, molecules that over the past 25 years profoundly changed our view of microbial systematics [1,2,16]. Applications of fluorescently labeled, rDNA-targeted oligonucleotide probes have become a common tool for the direct, cultivation-independent identification of individual bacterial cells. Fluorescence *in situ* hybridization (FISH) with rDNA-targeted oligonucleotide probes has been developed in studies on the structure and dynamics of various natural microbial communities. An important first step towards understanding the roles of various bacteria in the marine ecosystems is determining the

numbers and relative abundances of different bacterial groups [7,15,16]. Culture independent studies are essential for determining how many different types of bacteria are present in microbial communities, because only 1% of bacteria in nature can be cultivated with currently available methods [3,7,16].

Sediments play an important role in the remineralization of deposited organic matter in highly productive continental shelf areas [3,10]. The aim of this study was to compare microbial communities from two different areas of the Black Sea coastal self sediments using fluorescent *in situ* hybridization. It is important to know which phylogenetic groups of bacteria dominate marine bacterioplankton communities because abundant groups may be proportionally more influential in carbon cycling and other biogeochemical processes [6,8,10,19,21]. Furthermore, understanding why particular bacteria dominate microbial communities is a fundamental ecological question, especially by comparing unpolluted and highly polluted ecosystems.

2.Experimental Section

2.1. Sites description and sampling

This study was conducted in April 2005, on the Romanian Black Sea coast shelf sediments in two stations, Agigea and Northern Constanta (Fig. 1). Main industrial sites and harbors on Romanian Black Sea coast are located around Constanta. We took sediment samples from two areas: in north of Constanta ("Northern Constanta Area") and, respectively, south of Constanta ("Agigea Area"). As in spring time, marine currents are active from north to south in Black Sea (as indicated on the map below), the northern Constanta area is much less polluted than the Agigea Area.

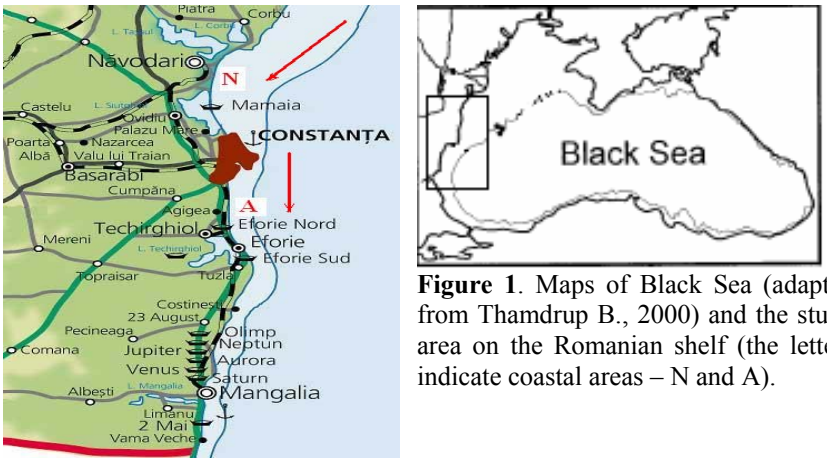

Figure 1. Maps of Black Sea (adapted from Thamdrup B., 2000) and the study area on the Romanian shelf (the letters indicate coastal areas – N and A).

At the stations, the sediment samples were prelevated from the oxic layer, 1-2cm from the surface, in sterile 250ml bottles. Sediment for molecular analyses was subsampled and processed immediately after retrieval of the bottles. At both stations, the sediment was covered by an 1-cm-thick layer of small shells filled in with fine-grained sediment. Samples were paraformaldehyde fixed, and after several dilutions, cells were aliquoted in PBS (phosphate-buffered saline: 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4, pH 7.2) / Ethanol 1/1(vol/vol), and preserved for long term at -20^{o}C.

2.2. Fluorescent in situ hybridization

The marine sediment samples from the two sites were fixed at 4°C for 16 h in 4% paraformaldehyde–PBS. After fixation, the samples were washed in PBS three times and stored in ethanol-PBS (1:1 [vol/vol]) at -20°C. The aliquoted samples were vortexed for 1 min, diluted in PBS, and sonicated 30 seconds with a 1-second pulse using a Sonoplus HD70, Bandelin, Berlin, Germany. Hybridizations were carried out on 0.22 mm-pore-size polycarbonate filters (Millipore) with a 2.5 cm diameter after filtration at a pressure of 100 mbarrs of the diluted marine sediment in a total volume of 20ml, for obtaining an improved dispersion of the cells on the filters. After air drying, the filters can be hybridized or conserved at -20^{o}C for at least one year. Oligonucleotide probes were synthesized with Cy3 fluorochrome at the 5' end by Interactive Biotechnology GmbH, Ulm, Germany. The probes used in this study are presented in Tab.1. To enhance single-mismatch discrimination during hybridization with oligonucleotides probe GAM42a, BET42a and Pla886a, equal concentrations of the appropriate competitor probe were included in the hybridization buffer. The hybridization buffer contained 0.9 M NaCl, a percentage (vol/vol) of formamide as shown in Tab.1, 20 mM Tris-HCl (pH 7.4), and 0.01% (wt/vol) SDS(Sodium Dodecyl Sulfate). Hybridization was performed for 3h at 46°C, in a hybridization chamber in a horizontal position, using a 5 ng/ml concentration probe. Filters were washed at 48°C for 15 min in a washing buffer containing 20 mM Tris-HCl (pH 7.4), 0.01% (wt/vol) SDS, and a recommended concentration of NaCl determinate by formamide concentration from hybridization buffer. For counterstaining, filters were covered for 3 minutes with DAPI (4',6'-diamino-2-phenylindole) 1 μg/ml, in dark, washed in distillated water for several seconds and than in 80% ethanol for another few seconds, to remove unspecific staining followed by rinsing in distilled water. After air drying in the dark on chromatografic paper, the filter were mounted on slides with Citifluor/Vecta Shield 1/3 (vol/vol).

Table 1.. Oligonucleotide probes used in this study.

Probes	Specificity	Sequence 5' →3'	% PA*	Competitor	Reference
ALF968a	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	35		[14]
BET42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	35	Gam42 a	[11]
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	35	Bet42a	[11]
Pla886	*Planctomycetales*	GCCTTGCGACCATACTCCC	35	cPla	[14]
CF319a	*Cytophaga/ Flavobacterium* cluster	TGGTCCGTGTCTCAGTAC	35		[12]
NonEUB 338	For detection unspecific bindings	ACTCCTACGGGAGGCAGC	0		[22]
EUB338	Almost all *Bacteria*, less *Planctomycetales*	GCTGCCTCCCGTAGGAGT	0		[1]
ARCH915	*Archeaea*	GTGCTCCCCCGCCAATTCCT	20		[20].
EUK513	Most *Eukarya*	ACCAGACTTGCCCTCC	0		[2]

*Values represent percent formamide in the hybridization buffer.

2.3. Microscopy:

The slides were examined with an Axioplan II microscope (Zeiss, Jena, Germany) with a 100 X 1.3 objective. The pictures were taken with an AxioCam MRC Zeiss photo

camera and vizualized on computer with AxioVision Zeiss 3.1. program. Hybridization and microscopy counts of hybridized and 4',6'-diamidino-2-phenylindole (DAPI)-stained cells were performed on the same microscopical field by changing the filters. Were calculated 10 to 20 randomly chosen fields on each filter section, corresponding to 800 to 1,000 DAPI stained cells. Counting results were always corrected by subtracting signals observed with the probe NON338.

3. Results and Discussions

Molecular ecology studies have recently showed that marine sediments are characterized by diverse microbial communities and culture-independent molecular methods are the most appropriate tools for studying microbial communities [3,13,20]. Knowledge of the diversity of microbial communities inhabiting polluted environments is useful since it provides clues about the type of bacteria able to adapt to such habitats. In this study we comparatively analyzed the microbial communities from two coastal sediment samples, one low-polluted site (Northern Constanta Area) and a highly polluted site (Agigea Area). The results, allowed us to identify bacteria groups which were presumably metabolically active and therefore responsible for the functionality of the bacterial community in both marine sediments.

In our experiments, total cell counts determined by DAPI staining of the oxic layer of marin coastal shelfes sediments were 1.3 x 10^8cell $ml^{-1}$for Agigea area and 3.7 x 10^8cell $ml^{-}1$ for Northern Constanta area, values which were in accordance with what has been reported previously [21]. As shown in other studies, quantitative molecular methods, such as fluorescence *in situ* hybridization, have shown that the abundance of microorganisms in sandy sediments is about one order of magnitude lower that in organic-rich sediments, of up to 10^8 cells ml^{-1} sediment [13]. The highest number of cells is usually recorded at depths of 1−2 cm, probably a consequence of the active mixing and washing of the sediment surface [8,9,13,16,17].

Only 75% of the total cells counted were hybridized with EUB338, the rest of 15% was represented by microscopic eukaryotic microorganisms and confirmed by counts obtained with EUK516, more than 10% represents unspecific bindings detected by NonEUB338 probe, and DAPI staining. Also, we have to note the absence of *Archaea* in Agigea sample, and just 0.6% in North Constanta sample, but this is in accordance with previous studies from other marine sediments, were made up only a minor part of the marine sediments community, no more then 3%[17,18,21].

Typical FISH detection yield of the CY3-monolabeled probe EUB338, which is specific for the domain *Bacteria* with almost 62% of DAPI-stained cells and the bright probe signals [4,8], indicated that the sediment microbial community, as a whole, was highly active at the time of sampling [3]. In general, only 50% of the bacterioplankton community can be detected with domain-specific probes [15,16], and much less (60%, i.e., sum of probes) of the bacterial fraction can usually be assigned to specific phylogenetic groups[6,13]. Detection rates as well as single-cell signal intensities with FISH using the general probe for bacteria EUB338 were higher in both sediments. Some trends in the marine environment are emerging. Glöckner et al. [8] reported 96% detectability of DAPI-stained cells with EUB338 and 87% assignability with phylogenetic group probes in a sample collected from North Sea.

Members of the *Cytophaga-Flavobacterium* group could be found in all marine and freshwater samples investigated and usually formed the largest bacterial group in marine water ecosystems [3,5,6,812]. In Black Sea costal shelf sediments, this group reached 26.6% in Agigea sample and 47% in North Constanta sediment of the total bacterial community,

which is between the range of 2% in Baltic Sea and 72% in Artic Ocean reported by other studies [4,5,17,21]. This cluster typically contains aerobic species, but recently some studies reported a significant cell number of both groups in marine sediments, even in oxic/anoxic layers [13, 21]. These findings indicate a strong input of organic substrates to anaerobic sediments and indicate a high potential ecological relevance of these bacteria as hydrolytic fermentative organisms[18].

In our study *Cytophaga-Flavobacterium* cluster, along with the *Gammaproteobacteria* were the most abundant groups and it is presumable that are playing the major role in the Black Sea coastal shelf sediments. We expected to find a higher percentage of *Gammaproteobacteria* in Agigea station, as many microorganisms affiliated to this group are typically involved in biodegradation processes, instead we found that it represents only 26,6% of the total cells count comparing with

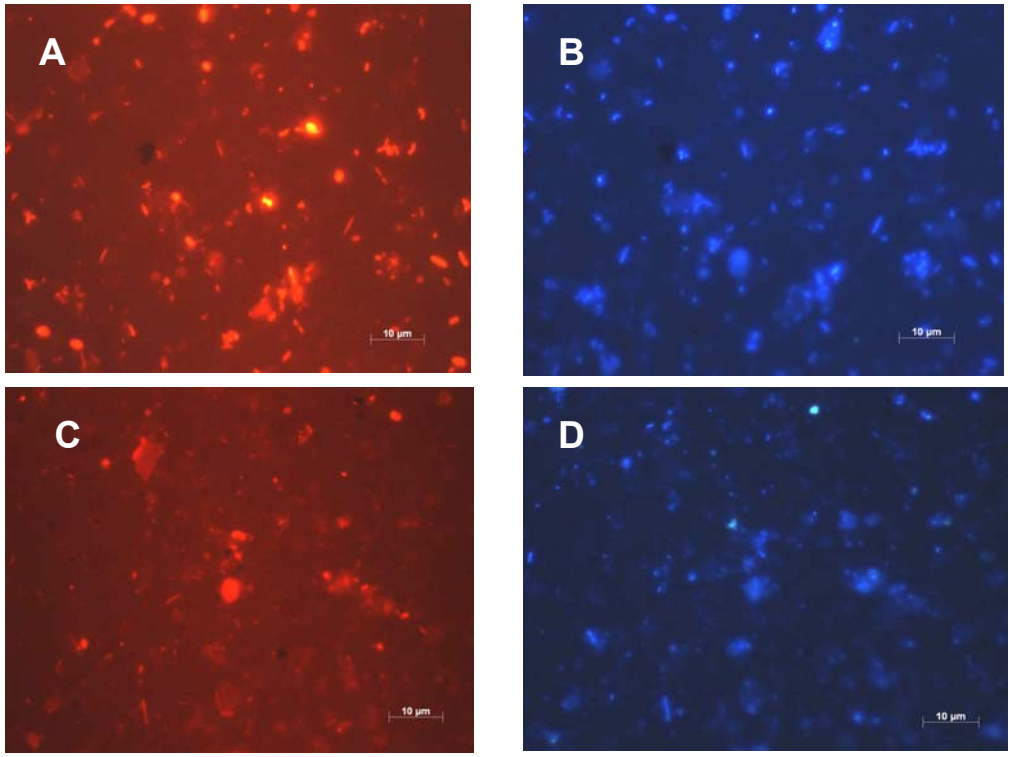

Figure 2. FISH on marin sediments from Black Sea. (A) Hybridization with probe EUB338 and (B) Identical microscopic field for DAPI staining for Agigea Area; (C and D) Identical microscopic fields showing results of hybridization with the same probe (C) and of staining with DAPI (D) for Northern Constanta Area

Cytophaga-Flavobacterium group that represents 30,8%. On the contrary, in Northern Constanta sediments (very low polluted area), we found a much higher percentage of *Gammaproteobacteria* subclass 47%, comparing with *Cytophaga-Flavobacterium* cluster that represents 12.6% of the total cells counted. These findings are not in accordance to our expectations but are not in total discrepancy with other studies because members of the *Cytophaga-Flavobacterium* cluster are mainly aerobic, gram-negative bacteria which are specialized for the degradation of complex macromolecules [9]. However, considering the brightness of the hybridization the cells detected by probes CF319a and also with GAM42a [2,12,19], seem to be intact and metabolically highly active.

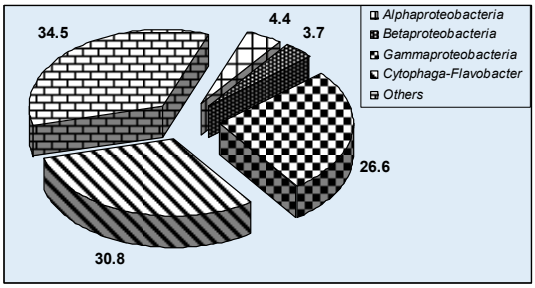

Figure 3. Bacterial community structure in Agigea coastal shelfs sediments, reaveled by FISH

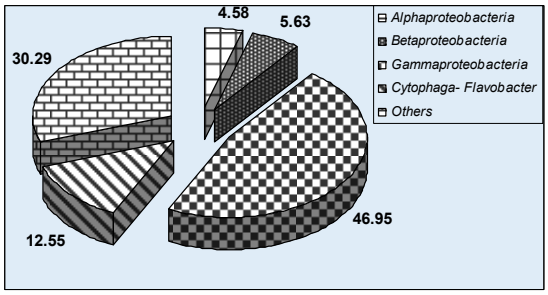

Figure 4. Bacterial community structure in Northern Constanta coastal shelfs sediments, reaveled by FISH

The relative abundance of *Alphaproteobacteria* subclass detected with the probe ALF968 was similar in both sediments, 4.4% in the Agigea sample and 4.6 % in the Northern Constanta sample, that is also between the range of relative abundance of *Alphaproteobacteria*, for examples in the North Sea 1% and 14% in the Baltic Sea [8].

Betaproteobacteria is less abundant in marine sediments than other bacterial groups, a fraction of 4.5% of the total cells was visualized, in both samples, with probe BET42a, that was in range of other studies [19]. Typically, are found in high numbers in fresh water habitats [8,18] and only in low abundances in marine bacterioplankton [9,13]. Their presence in sandy sediments is probably accidental and results from the washing and mixing of the sediment correlated with the sediment property to act as filter, capturing different particles that enter from the water column. This hypothesis is also sustained by their low abundances and presence only in the top layers (1−2 cm) of the sediment [9,13].

Conclusions

A comparison of studies based on FISH on marine sediments bacterial communities in high/low polluted environments is relevant even if this method has some drawbacks. Despite this, our results indicate that structure of the bacterial community in marine sediments differ significantly. However, there should be a direct interaction between the water phase and the sediment, which has implications for the community development in the upper layers of the sediments.

Overall, we can imagine a natural scenario in which an ecologically unique microbial population becomes predominant for a short period of time but that can easily be outcompeted by a close relative better adapted to newly established environmental conditions. These bacteria could represent a tremendous potential for rapid changes in community composition, since they could grow quickly and multiply over several orders of magnitude under appropriate conditions, such as in the presence of pollutant compounds. Thus, the function carried out by the microbes in the environment persists, but the identity of the microbial populations may change with time as an adaptative response to environmental pressures and to fluctuating conditions. This would provide an explanation as to why so many related isolates can be obtained from a natural sample or why these isolates do not match sequences of predominant organisms at a certain time [5].

In this respect, we think that one of the most interesting findings in our work is the unexpected high abundance of members of the *Cytophaga-Flavobacterium* cluster in Agigea site. Other studies have also reported similar results, indicating that the sedimentation of microbial aggregates from the water column plays an essential role in the formation of the microbial communities in marine sediments. Our results support this hypothesis, but there is

a question as to whether a high abundance of *Cytophaga-Flavobacterium* cluster and also *Gammaproteobacteria* subclass are common in polluted marine sediments, or not. Determination of the significance of the observed similarities and differences between the FISH results in the two samples will require more-comprehensive studies involving statistical analysis, determinations of seasonal fluctuations, monitoring pollution sources will also give relevant information.

Acknowledgments

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