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Comparative molecular analysis of bacterial communities inhabiting pristine and polluted with polycyclic aromatic hydrocarbons Black Sea coastal sediments

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ABSTRACT

Molecular analysis was applied to characterize bacterial community structure in sediment samples collected from pristine site and oil-polluted Black Sea harbor. Amplified Ribosomal DNA Restriction Analysis (ARDRA) revealed a high similarity in the restriction patterns of both samples thus not demonstrating the effect of the pollutant on the structure of the bacterial communities. Constructed 16S rRNA gene libraries gave more detailed assessment of members. Results showed that α- and γ-*Proteobacteria* were dominant in the oil polluted site, whereas the pristine site was characterized by prevalence of *Actinobacteria*. The biodegradative potential of the adapted bacterial community in the oil-polluted sediments was demonstrated by the presence of the aromatic ring hydroxylating dioxygenase genes.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are among the most widespread anthropogenic pollutants of free water column and sediments in the coastal zones (Choiseul et al., 1998; Dean-Ross et al., 2002). Because of their high resistance to degradation crude oil fractions and oil derivatives can persist in the environment over months and years (Whittle et al., 1982; Law et al., 1997; Awata et al., 1998; Baumard et al., 1998). PAH are among the priority pollutants in the Water Framework Directive, 2000 of European Commission (2000/60/EC) due to their toxic and carcinogenic effect on marine biota and attempts are made to develop appropriate strategies for sediment monitoring. Global legislation as the Marine Directive (2008/56/EC) as well as regional ones as the Strategic Action Plan for the Environmental Protection and Rehabilitation of the Black Sea 2009 outlined the increased risk of oil pollution as a priority problem of the coasts, especially emphasizing the risk caused by localized chronic pollutions with weak but frequent oil discharges. Despite the positive tendency showing improvement of the ecological situation alongside the Bulgarian part of the Black Sea coast (National strategy about regional development in Bulgaria, 2005–2015), benthic habitats are still affected by chronic oil pollution. As a result of continuous entering of oil, chronic input is hard to be traced or eliminated and repeated pollution hinders restoration (IFAW, 2006, Status report; Stricherz, 2006). The problem is severe near harbors, where re-release of heavy weight oil fractions (including PAH) from sand beaches further enhances their chronic effect on coastal environment.

On the other hand analytical assessment of PAH concentrations gives little information about their effect on the biota, raising the need of appropriate bioindicators. Contamination of the coast with oil results in pollution, and in particular poses a chronic problem to commercial fisheries and recreational resources. As only microorganisms are capable of oil degradation, their role in selfrecovery of impacted habitats is significant (Röling et al., 2002; Verrhiest et al., 2002; Sathishkumar et al., 2008). The processes of self-purification are actively passing in coastal marine ecosystems, where bioremediation is identified as the main natural process by which non-volatile hydrocarbons are removed from the environment (Swannell and Head, 1994; Kennish, 1998; Röling et al., 2004; Alonso-Gutiérrez et al., 2009). Many bacterial species are capable of degrading directly or indirectly oil hydrocarbons (van der Meer et al., 1992; Widada et al., 2002; Habe and Omori, 2003; Head et al., 2006). The quick response of bacteria to even relatively minor pollutant concentrations occurs before other components of the coastal ecosystem show a noticeable effect (Leahy and Colwell, 1990). Therefore the reaction of microbial populations to environmental variations makes them a promising pollutant indicator

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(Kawahara et al., 2009) although chronic oil pollution may affect their structure in a quite unpredictable manner (Röling et al., 2004; Head et al., 2006).

As natural bacteria are difficult to be cultured, the main source of information about their diversity is the environmental distribution of gene sequences (Lozupone and Knight, 2007). Genetic fingerprinting generates specific profiles of microbial communities based on direct analysis of PCR products amplified from environmental DNA (Muyzer, 1999). Some of the mainly used techniques include DGGE/TTGE (Denaturing- or Temperature-Gradient Gel Electrophoresis), ARDRA (Amplified Ribosomal DNA Restriction enzyme Analysis), T-RFLP (Terminal Restriction Fragment Length Polymorphism). The resulting community fingerprints are based on either sequence or length polymorphism. As they are rapid and allow simultaneous analyses of multiple samples, fingerprinting approaches have been used to demonstrate differences between microbial communities (Rastogi and Sani, 2011).

Metagenomic analysis (Venter et al., 2004; DeLong et al., 2006) and ribosomal tag pyrosequencing strategies (Huber et al., 2007) have previously identified unexpectedly high *Bacteria* and *Archaea* phylogenetic and functional diversity. Next-generation sequencing (NGS) methods have greatly increased sequencing throughput via the use of massive parallel sequencing (Sogin et al., 2006) of small but highly variable regions of the 16S rDNA (Staley et al. 2013). However all current high-throughput technologies have serious limitations due to the average error rate that is considerably higher than the typical observed for high-quality Sanger sequences (Kircher and Kelso, 2010). This feature together with the generation of longer sequence reads for phylogenetic affiliation still make Sanger 16S rRNA gene sequencing the more widely used strategy for getting information from environmental microbial assamblages and used in our further analysis.

Salinity is considered the major environmental determinant of microbial community composition (Lozupone and Knight, 2007). Based on the Black Sea unique environmental conditions (average salinity 17.5‰, Zaitsev et al., 2002) the development of specific bacterial consortia is expected. On the other hand, the south-western (SW) part of the Black Sea is characterized by a persistence of minor oil and oil derivatives concentrations due to the intensive maritime transport, summer recreational activities and commercial fishing. To date the Black Sea coastal zone has not been examined for the presence of PAH degrading bacteria. In our previous study we have explored controlled laboratory microcosm model systems where simulating pristine and oil-poluted coastal sediments. The physico-chemical characteristics of the control basin, such as oxygen concentrations, temperature, salinity, organic matter content were maintained as close as possible to the natural ones (Sozopol Bay, SW Black Sea coast), while chronic oil pollution was maintained by regular additions of mixture of crude oil and light fractions to final concentration in the overlying water of 0.5 mg l^{-1}. Applying ARDRA methodology that provides a rapid overview on the diversity in nonculturable microbial fractions (Junca and Pieper, 2003; Khetmalas et al., 2002; Porteous et al., 2002), we have detected a difference in bacteria profiles between pristine and oil polluted laboratory systems (Todorova et al., 2005). However, ARDRA of 16S rRNA genes gave only a rough estimate of the highly complex structure of the inhabiting bacterial communities.

In this study, by using 16S rRNA gene retrieval, ARDRA and gene sequencing, we provide comparative data on the diversity of bacterial communities in samples collected from pristine site and oil polluted harbor sediments, SW Black Sea coast. We made also an attempt to test the hypothesis of community shift over time proposed by Röling et al. (2002). To assess the putative oil biodegradative potential of the bacterial communities from the oil-polluted sediments, we targeted genes for the alpha subunit of the PAH-ring hydroxylating dioxygenases (PAH-RHDα), which are involved in the initial step of the aerobic PAH metabolism.

2. Methods

2.1. Site description

Natural pristine and PAH polluted sediment and water samples originated from the coastal zone near town of Sozopol (SW Black Sea coast). Triplicate samples were collected from SZ2, Sozopol harbor (42°25′N 27°41′E) and from SZ4, a pristine nearby site "Zlatna ribka" (42°24′N 27°40′E) in July 2006 (Fig. 1).

Sediments from both sampling stations were mainly sandy (sand > 95%), having similar sediment characteristics as sand porosity (22.85% SZ2, 25.15% SZ4) and percentage organic matter (dry weight 1.02% and 1.16%, respectively). The measured water salinity was 16.5‰, pH 8.41. The redox potential was consistently decreasing in depth, staying in oxidized condition (0–7 cm: 135–55 mV) with no abrupt changes (unpublished data).

Based on the harbor history, it is expected that oil derivatives are the main pollutants. To assess the degree of contamination, the 16 standard priority PAH in SZ2 and SZ4 were quantified by gas chromatography with flame ionization detector (GC-FID) as previously described (Todorova et al., 2008b). Due to the continuous flux of the pollutants between the different parts of the system the concentrations were measured in sediments and water samples simultaneously. The pristine sediment sample SZ4 showed lack or background PAH concentrations, while SZ2 contained 6.8 mg kg^{-1} total PAH_{16} (dry wt sediment). Standards for the permitted PAH concentrations in marine sediments do not exist, therefore to evaluate the pollution effect, local legislation classification for soils was used. According to it the permitted PAH concentration is less than 4 mg kg^{-1} PAH_{16} and thus the SZ2 sediment could be classified as "harmful to the environment".

2.2. Sampling

Undisturbed sediment cores (top oxygenated layer) were collected in triplicates via SCUBA diving. From the same stations 50 ml water samples were collected (no visible oil spots were detected on the water surface). Samples were preserved on ice and transferred to the laboratory in Sozopol. The sediment samples were processed immediately, while the water ones were first filtered through a 0.22 μm GV Millipore filter. Air dried filters were stored at − 20°C until further processing.

2.3. DNA extraction

DNA extracted from all the triplicates from each site was mixed in equal aliquots (forming SZ2 and SZ4 sample respectively) and used for further molecular analysis to assess the whole bacterial diversity in the aerobic zone. Nucleic acid extraction from sediments was done directly by a modified version of the method of Osborn et al., 2000. The protocol was optimized and tested in our previous study on laboratory microcosm system, which imitated chronic oil pollution (Todorova et al., 2005). Briefly, 1.5 cm^3 sediment samples were homogenized and incubated with 400 μl extraction buffer (100 mM Tris, 100 mM EDTA, 100 mM Na-phosphate buffer, pH 8.0), containing 500 μg lysozyme, for 1 h at 37 °C. After addition of 50 μg proteinase K, the temperature was gradually increased to 58 °C and the mixture was incubated for 30 min. Then, 150 μl 10% SDS, 220 μl 5 M NaCl and 80 μl CTAB were added and incubation was continued for 30 min at 58 °C, followed by repeated freeze–thaw cycles. The soluble phase was collected after 20 min centrifugation at 9000×g and subjected to one

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