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The impact of long-term hydrocarbon exposure on the structure, activity, and biogeochemical functioning of microbial mats

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ABSTRACT

Photosynthetic microbial mats are metabolically structured systems driven by solar light. They are ubiquitous and can grow in hydrocarbon-polluted sites. Our aim is to determine the impact of chronic hydrocarbon contamination on the structure, activity, and functioning of a microbial mat. We compared it to an uncontaminated mat harboring similar geochemical characteristics. The mats were sampled in spring and fall for 2 years. Seasonal variations were observed for the reference mat: sulfur cycle-related bacteria dominated spring samples, while *Cyanobacteria* dominated in autumn. The contaminated mat showed minor seasonal variation; a progressive increase of *Cyanobacteria* was noticed, indicating a perturbation of the classical seasonal behavior. Hydrocarbon content was the main factor explaining the differences in the microbial community structure; however, hydrocarbonoclastic bacteria were among rare or transient Operational Taxonomic Units (OTUs) in the contaminated mat. We suggest that in long-term contaminated systems, hydrocarbonoclastic bacteria cannot be considered a sentinel of contamination.

1. Introduction

Microbial mats dominated by *Cyanobacteria* are considered to be the oldest structured ecosystem on Earth (Margulis et al., 1980). They are nearly auto-sufficient systems in terms of nutrient cycling, containing high taxonomic and metabolic diversity within few-millimeter scales (Bolhuis and Stal, 2011). Microbial populations within a mat are stratified by steep light, oxygen, sulfide, and pH gradients; in turn, their metabolisms participate in maintaining these gradients. *Cyanobacteria* play key roles within a photosynthetic mat. They are precursors of microbial mats, since they fix dinitrogen and carbon dioxide, supporting the community growth (Severin and Stal, 2010). Other functional groups in photosynthetic microbial mats are aerobic/anaerobic heterotrophs, fermenters, sulfide oxidizers, and methanogens (van Gemerden, 1993). The structure of the mats changes depending on season, as purple sulfur bacteria increase at the end of winter and spring and cyanobacteria at the end of summer and fall (Pinckney et al., 1996). Microbial mats can be found in a diverse range of environments around the world, including but not limited to hot springs (Coman et al., 2013; Roeselers et al., 2007), polar ponds (Vincent and Whitton, 2002), and hypersaline waters (Allen et al., 2009; Dillon et al., 2009; Schneider et al., 2013). They also have been observed in petroleum-contaminated areas (Barth, 2003; Paissé et al., 2008).

Previous studies on hydrocarbon-contaminated microbial mats showed a reduction of microbial diversity in the short term associated with the disappearance of certain groups of microorganisms and a strong selection for specialist hydrocarbon-degrading marine bacteria (*i.e.*, *Alcanivorax* and *Marinobacter*) (Abed et al., 2007; Bordenave et al., 2007). *Alcanivorax* belong to the obligate hydrocarbonoclastic bacteria, which are known to feed exclusively on hydrocarbons (Yakimov et al., 2007). Other obligate hydrocarbonoclastic bacteria are *Cycloclasticus* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolituus* spp., and some members of the genus *Planomicrobium*. *Alcanivorax* and related alkane degraders have been detected rapidly after an oil input (Head

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et al., 2006). *Cycloclasticus* spp., an aromatic hydrocarbon degrader, was shown to appear at later stages, when the alkanes have been degraded (Head et al., 2006). Microbial diversity in chronically or long-term hydrocarbon-polluted microbial mats is less documented. High diversity has been observed in chronically hydrocarbon-polluted microbial mats even though 16S rRNA gene sequences related to well-known hydrocarbon degraders (*i.e.*, *Alcanivorax* and *Cycloclasticus*) were not (or rarely) detected (Paissé et al., 2008). Finally, microbial mats show a high resilience capacity after petroleum contamination (Bordenave et al., 2007), suggesting that the petroleum impact is only transient and that the mat structure and functioning are robust enough to recover.

The archaeal community has been tackled rarely in hydrocarbon-polluted environments. In oiled sediments, the *Euryarchaeota* dominated, with particular contributions of *Methanococcoides*, *Methanosarcina*, and *Methanolobus* sequences (Miralles et al., 2010). The *Euryarchaeota* have been also found to be dominant in an uncontaminated microbial mat developing on the Dutch barrier island Schiermonnikoog, mainly by the *Methanobacteria* and the *Methanomicrobia* (Bolhuis and Stal, 2011). As far as we know, the archaeal community never has been investigated in hydrocarbon-polluted microbial mats.

The goal of this study was to determine the impact of chronic hydrocarbon contamination on the biogeochemical functioning, structure, and activity of the prokaryotic community in natural microbial mats. In order to determine to which extent hydrocarbon contamination is able to drive the structure and activity of microbial mats, we compared a highly hydrocarbon-contaminated mat, whose *in vitro* hydrocarbon degradation capacity has been proven (Paissé et al., 2010), with a reference mat. The second mat is located in close proximity to the same lagoon and shows similar physical and chemical parameters but without a hydrocarbon contamination history. We performed an extensive biogeochemical characterization of both mats and deep phylogenetic and transcriptomic analyses using high-throughput sequencing of the bacterial and archaeal community 16S rRNA genes and transcripts. Hydrocarbonoclastic bacteria were specifically targeted, since previously they were poorly observed in the contaminated mat (Paissé et al., 2008). Because microbial mat functioning is influenced by seasonal variation, the analyses were performed in spring and autumn during 2 consecutive years in order to assess the seasonal variation of mat structure and activity and to determine the impact of hydrocarbon contamination.

2. Materials and methods

2.1. Sampling sites and procedure

The Berre lagoon is located on the French coast of the Mediterranean Sea near Marseille. Microbial mat samples were collected from two sites within the lagoon at a distance of 4.9 km: a contaminated site located in a retention basin receiving hydrocarbon wastewaters from a petrochemical industry (EDB1) and a non-hydrocarbon-contaminated site located within the “Les salins du Lion” bird reserve (SL) considered as a reference. The EDB1 retention basin had been collecting hydrocarbon-charged waters from the petrochemical industry for decades, and it currently is continuously supplied with hydrocarbon-contaminated rainfall waters (Paissé et al., 2008). The waters flow from industrial facilities to a sedimentation tank and then to the retention basin by an overflow system. In September 2009, an accidental oil spill occurred due to the overflow of a hydrocarbon-polluted water retention pond (Beau-Monvoisin, 2009). Four sampling campaigns were carried out: two in spring (April 2011 and 2012) and two in autumn (September 2011 and 2012), named Apr11 and Apr12 for April sampling and Sept11 and Sept12 for September sampling, respectively. The first 2 cm of sediment were collected in triplicate for molecular analysis and stored at −80 °C until analyses. Sediments for hydrocarbon analyses were sampled with a glass container and, for metal and other chemical analyses, with a polypropylene container.

2.2. Physical and chemical analyses

Complete physical and chemical characterization was performed for Sept11, Apr12, and Sept12 campaigns. Metals and transition metals (Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Sn, and Zn) were quantified in sediments *via* inductively coupled plasma mass spectrometry by Ultra Trace Analyses Aquitaine (Pau, France). Sediments were freeze-dried and passed through a 2-mm sieve. Total sediment mineralization was performed in a microwave oven using 0.2 g of sediment, 2 mL of concentrated HNO_3, and 2 mL of concentrated HF. The solution was then diluted with MilliQ water. Metal concentrations were measured with an internal standard of rhodium (Rh). Water samples were quantified without pre-treatment with an Rh internal standard. Hydrocarbon analyses (alkanes and polyaromatic hydrocarbons [PAH]) in sediments were performed by Cedre (Brest, France) using gas chromatography coupled to mass spectrometry, as described by Stauffert et al. (2013). Sulfates and sulfides were quantified in water with the method described by Kolmert et al. (2000) and with the methylene blue method (Cline, 1969), respectively. Total carbon (TC) and total sulfur (TS) contents were measured in freeze-dried sediments by infrared spectroscopy using a LECO C-S 125. Organic carbon content also was measured in freeze-dried material after removal of carbonates with 2 M HCl from 50 mg of powdered sample (Etcheber et al., 1999; Pastor et al., 2011). Total nitrogen (TN) content in the sediment was measured in freeze-dried material by combustion using an automatic Thermo Finnigan EA 1112 Series Flash Elemental Analyzer. The C/N ratios were expressed as atomic ratios. Redox potential, pH, salinity, and dissolved oxygen were measured *in situ* (*via* a WTW pH/mV 340 meter, an Atago S10 refractometer, and a CellOx 325 electrode, respectively). Net photosynthesis, areal rates of gross photosynthesis, and dark and light respiration were measured in microbial mats as described by Pringault et al. (2015). The pore water concentration of NH_4^+, NO_2^-, and NO_3^-, the total denitrification, and the nitrogen fixation were measured as described by Bonin and Michotey (2006). The sulfate reduction rates were determined using the single-step chromium reduction method (Fossing and Jørgensen, 1989) by adding 1 mL of $35SO_4^{2-}$ (37 kBeq) in 5 mL of homogenized sediment and incubated for 6 h at 21 °C. Specific activities of $35S^{2-}$ and $35SO_4^{2-}$ were determined with a Beckman LS6500 liquid scintillation counter.

2.3. DNA/RNA extraction and cDNA synthesis

DNA and RNA co-extractions were performed using an RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories, Inc.) coupled with an AllprepDNA/RNA Mini Kit and an RNase-Free DNase set (QIAGEN). RNA qualities were verified using an RNA nanochip on a Bioanalyzer 2100 (Agilent); only RNA extracts with RNA quality higher than 7 RIN (RNA Integrity Numbers) were used for following experiments. Absence of DNA contamination within RNA samples was determined by PCR amplification of RNA without reverse transcription. cDNA synthesis was achieved on RNA using M-MLV RT and RNase OUT (Invitrogen) with 1 μL of RNA. Reverse transcription was performed with random primers (1 μL at 5 U/mL) according to manufacturer's instructions.

2.4. Quantification of genes and transcripts

16S rRNA genes and transcripts of archaea and bacteria were quantified using the DyNAmo Flash SYBR Green qPCR Kit (ThermoFisher Scientific) in an Mx 3005PTM (Stratagene) as described by Paissé et al. (2012) using bac1055YF and bac1392R primers (Ritalahti et al., 2006) for bacteria and arch349F and arch806R primers (Takai and Horikoshi, 2000) for archaea. PCR products were cloned in the pCR2.1 Topo TA Cloning Kit (Invitrogen) to generate the standard gene count curve. The standard curve for the transcript quantification was performed with the cDNA obtained after *in vitro* transcription. The cloned PCR