USE OF NEXT GENERATION SEQUENCING TO STUDY THE FEASIBILTY TO APPLY MONITORED NATURAL RECOVERY IN FRESHWATER SEDIMENTS AFFECTED BY ANCIENT HUMAN ACTIVITY

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Introduction

Aquatic environments in industrialized countries were frequently exposed to numerous pollutants generated by various discharges from municipal, hospital and industrial wastewaters, agricultural effluents and nonpoint source pollution (Rabodonirina et al., 2015). Aquatic sediments are repositories of this debris and act as sources and sinks for a wide variety of organic and inorganic pollutants (Perelo, 2010). Therefore, even if water quality improves, sediment contamination will remain a 'legacy of the past' (Förstner et al., 2004).

Hydrocarbons may enter the aquatic ecosystem either directly, by effluents or spills, or indirectly by terrestrial runoff or atmospheric deposition. Their persistence in the environment depends mainly on their chemical and physical characteristics (Perelo, 2010). Whereas polycyclic aromatic hydrocarbons (PAHs) are considered to be the most toxic component of oils, recalcitrant aliphatics and other high–molecular weight petroleum hydrocarbons are the primary hydrocarbon fractions found in sediment (Pettigrove and Hoffmann, 2005); although the latter might not be directly toxic to aquatic organisms, they can alter the physical properties of sediments, coat and smother organisms, and contribute to organic enrichment of sediments (Anson et al., 2008).

The assessment and management of contaminated sediments is inherently more complex than managing many relatively small and simple contaminated soil and groundwater sites (Reible, 2013). The three main objectives of sediment management strategies are to minimize contaminant risk to human health and the environment, to minimize associated with the remediation technique itself, like habitat destruction and/or modification and to minimize cost (Perelo, 2010). Three sediment management options are currently used, dredging, capping or monitored natural recovery (MNR), and due to the complexity and expensive of sediment remediation process, high technology solutions are not forthcoming.

Environmental dredging creates challenges including identification of disposal facilities or the design and construction of confined disposal facilities, controlling resuspension, and minimizing post dredging residual contamination. Installation of sand caps has its own set of challenges. This technology if not

designed and placed correctly can cause advective flow that might result in rapid breakthrough of contaminants depending on geo-chemistry and characteristics of the sediments.

The monitored natural recovery (MNR) is a remedial approach that relies on natural physical, chemical, and biological processes to reduce ecological and human health risks. MNR is a non-invasive technology which, carefully planned, allows remediation of sediment sites and can result in risk reduction in comparison with dredging and capping.

The aim of this work was to evaluate the application of MNR from a freshwater course affected by human activity using sediment biological parameters and next generation sequencing methods (NGS) as potential indicators of biological processes.

Materials and Methods

Sampling sites

The studied sediments come from a freshwater course with history of involvement by oil pollution, and whose recovery began projecting over 20 years ago. The sampling area corresponds to a length of 2205 m, 30 sediments cores distributed in 5 transects separated by approximately 400m were extracted by Laboratory of Soundings of Continental and Marine Environments (UBA-CONICET, Argentina) using a Hammer Piston tool. Water samples corresponding to the each core site were collected using 1 L pre-cleaned amber glass bottles that were immediately capped with Teflon-lined lids.

Physicochemical analysis

Physicochemical analysis were performed by INDUSER S.A. Total Petroleum (TNRCC 1005), Aliphatic and Aromatic Hydrocarbons (TNRCC 1006), pH (EPA 9045 D), total sulfide (EPA 9030 B/ 9034), total nitrogen (SM 4500-Norg C/ NH3 C) and phosphate (SM 2580 or Acid digestion/ SM 4500-P C/E) were determined in the water samples and at the different horizons defined in each core.

Humidity (SM 2540 G) and depth were also determined in the sediment samples, and O_2 dissolved in water samples (SM 4500-O G).

Biological analysis

Dehydrogenase (Thalman, 1968), lipase (Margesin et al., 2002), urease (Kandeler and Gerber, 1988) aril-sulfatase (Whalen and Warman, 1996), protease and phosphatase (Verchot and Borelli, 2005) activity were determined in the horizons from each core.

Molecular analysis

The different horizons of one of the core of each transect and additionaly two cores that showed high hydrocarbons concentration were analyzed. The microbial genomic DNA was extracted from the samples utilizing an E.Z.N.A Soil DNAkit (OMEGA, USA) following the manufacturer's protocol and stored at 80°C until analysis. The bacterial and archaea diversity was determined by NGS (Illumina). 16S rDNA

fragments flanking the V3 region were amplified using universal primers 341F and 518R for Eubacteria (Bartram et al., 2011) and 349F and 806R for Archaea (Li et al., 2016). Barcode on the forward primer was used in a 30 cycle PCR (5 cycle used on PCR products) using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations. Pooled samples are purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare DNA library by following Illumina TruSeq DNA library preparation protocol. Sequencing was performed at Molecular Research Laboratory (MR DNA; Shallowater, TX) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov, DeSantis et al 2006, http://rdp.cme.msu.edu). Good's coverage and Shannon index at genus level were were calculated using EstimateS (Version 9) (Colwell, 2013). Canonical correspondence analysis (CCA) between biodiversity and physicochemical and biological parameters were performed using the software Canoco version 4.5. Parameters with significant correlations were examined by a Monte Carlo permutation.

Result and Discussion

In the water samples pH ranged from 6.2 to 7.5, total nitrogen from <1.0 to 2 $mg.l^{-1}$, whereas phosphate and total hydrocarbons concentrations were below de detection limit (3 mg.l-1 for hydrocarbons) and the concentration of oxygen dissolved was higher than 6.4 mg.l-1 in all the samples. The physicochemical parameters suggest a low level of contamination that would not affect the functioning and survival of biological communities.

In all studied sediments cores could be differentiated a surface horizon, called H0, that showed the lowest or not detectable hydrocarbons concentrations (Fig. 1). Hydrocarbons concentration increased in deep horizons, showing a heterogeneous distribution. The concentrations of ΣPAH in the H0 ranged from 0.01 to 3.9 mg/Kg^{-1}, whereas in the deepest horizons the higher concentration found was 55.2 mg/Kg^{-1}.

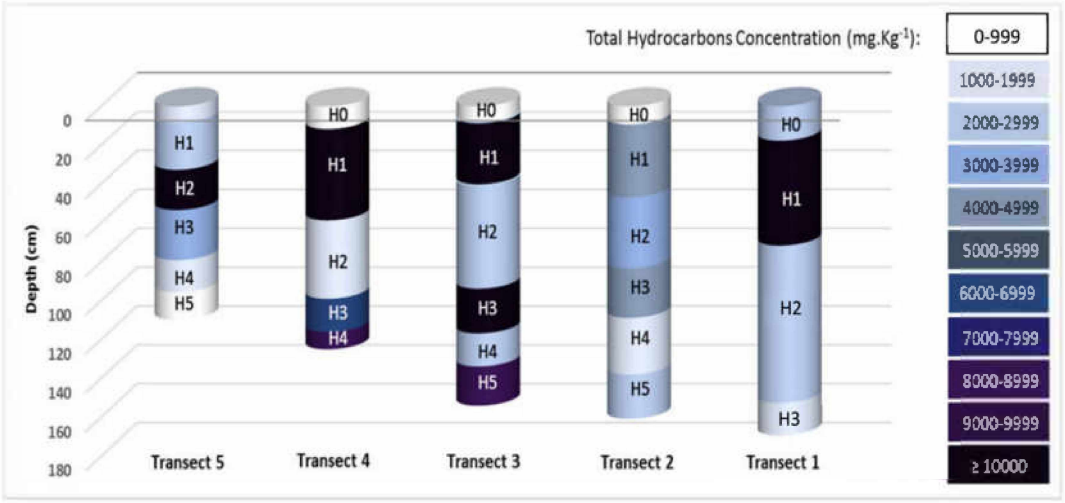

Figure 1. Total hydrocarbons concentration ($mg.kg^{-1}$) in each horizons found in the different transects (means of 3 cores per transect).

Molecular analysis was performed on cores 39 and 38 (transect 1), 36 (transect 2), 34 and 33 (transect 3), 31 (transect 4) and 26 (transect 5). Sequencing generated an average of 98,829 (range 39,744-168,863) and 121,032 (range not detected-196,678) quality-filtered sequence reads per sample for bacteria and archaea domains respectively. High Good's coverage (>0.98) indicated that the OTUs of archaea and bacteria of each sediment samples was accurately captured.

The H0 showed the highest biodiversity indexes (Table 1), mainly represented by *Deltaproteobacteria*, *Actinobacteria*, *Anaerolinea* and *Methanobacteria* classes (Fig. 2 and 3). A wide spectrum of genera was found at relatively high abundances in the surface horizons, many of them contain known oil-degrading species, both aerobic and anaerobic.

Bacteria and archaea diversity decreased in deeper horizons (Table 1) characterized by a remarked dominance of *Gammaproteobacteria* and *Bacilli* classes (Fig. 2 and 3). Within the *Gammaproteobacteria* class, *Acinetobacter* was the most abundant genus (over 30% of reads). The species of the genus *Acinetobacter* are "famous" hydrocarbon degraders (Jung y Park, 2015). In addition, the *Bacillus* genus that was the most represented within the *Bacilli* class (over 10% of reads) had been frequently reported as alkane degrader (Uzoigwe y col., 2015).

Shannon Index

	T1 Core 39	T1 Core 38	T2 Core 36	T3 Core 34	T3 Core 33	T4 Core 31	T5 Core 26
H0	6.0	6.3	6.3	5.6	5.2	5.3	5.5
H1	3.6	4.0	4.5	1.4	0.9	3.0	4.5
H2	3.2	3.6	3.9	1.4	1.8	1.1	2.4
H3	1.3	1.3	3.4	2.4	1.6	1.0	--
H4	--	--	3.4	1.5	--	1.0	--
H5	--	--	3.3	1.5	--	--	--

Table 1. Shannon diversity index at genus level for the different sediment samples obtained by analysis of Illumina sequencing data.

CCA axes between bacterial community composition and physicochemical variables (Fig. 2) or enzymatic activities (Fig. 3), explained respectively 42.6% and 46.3% of the total variance in community composition and 72.1% and 73.6% of the cumulative variance of the species–environment relationship.

Spatial distribution of the communities correlated significantly ($P<0.05$) with depth ($F=8.10$,), total phosphate ($F=3.27$), protease ($F=5.19$), and lipase activities ($F=4.94$) but not for hydrocarbons concentration ($P>0.05$, $F=1.25$).

The bacterial community of H0 showed a positive correlation with total nitrogen, humidity and lipase, protease, urease and alkaline phosphatase activities (Fig. 2 and 3), suggesting that these horizons were characterized by their activity in recycling nutrients.

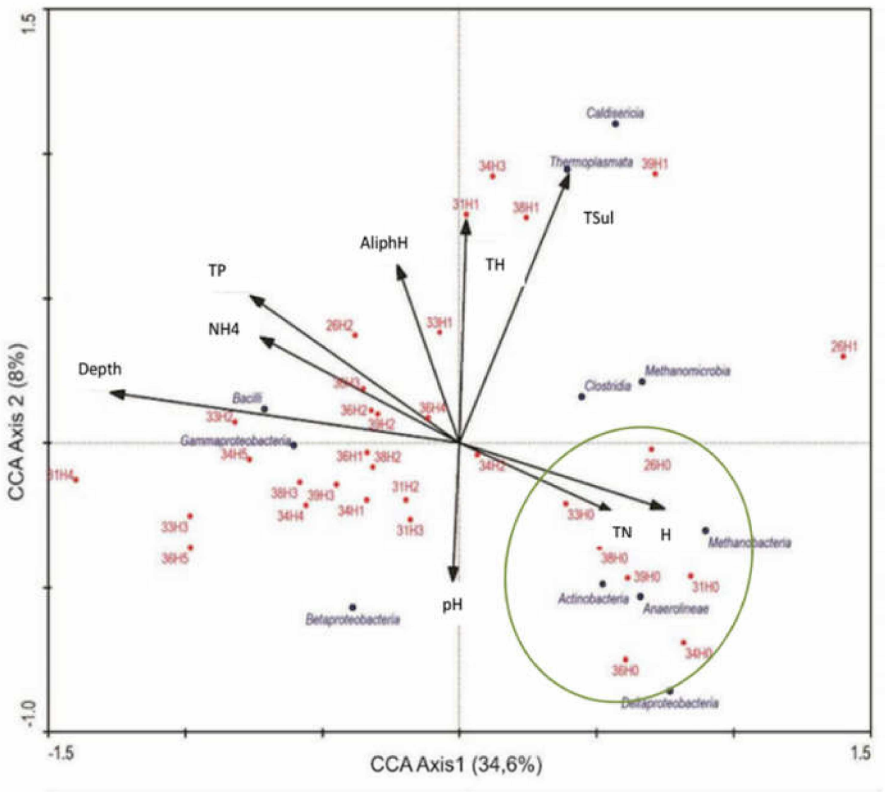

Figure 2. CCA of 16S gene data and physicochemical parameters. Physicochemical parameters indicated in black text include Depth, NH_4, TP (total phosphorus), AliphH (aliphatic hydrocarbons), TH (total hydrocarbons), TS (total sulfide), H (humidity), TN (total nitrogen) and pH. Arrows indicate the direction and magnitude of physicochemical parameters associated with bacterial community structures. The red dots represent the different bacterial community structures from the different samples core. The green circle indicates the position of bacterial community of all H0.

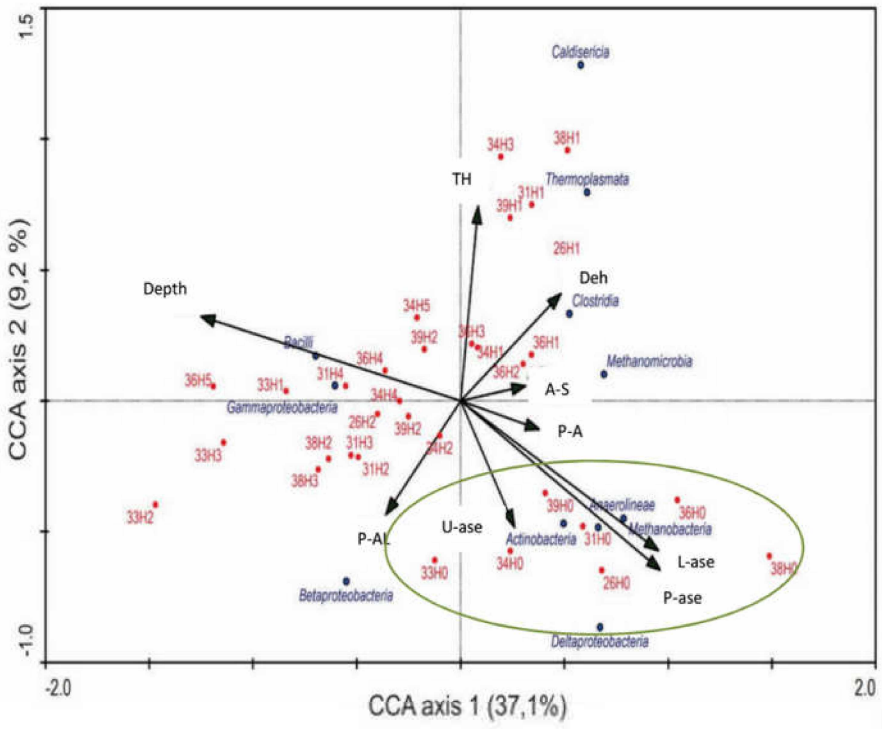

Figure 3. CCA of 16S gene data and biological parameters. Biological parameters indicated in black text include enzymatic activity of Deh (dehydrogenase), L-ase (lipase), U-ase (urease), A-S (aril-sulfatase), P-ase (protease), P-A (acid phosphatase) and P-AL (alkaline phosphatase). Depth, TH (total hydrocarbons) were also included. Arrows indicate the direction and magnitude of physicochemical parameters associated with bacterial community structures. The red dots represent the different bacterial community structures from the different samples core. The green circle indicates the position of bacterial community of all H0.

The great bacterial diversity and enzymatic activities detected in the H0, in correspondence with the low level of contaminants found in water samples, allow us to infer that it works as natural "biofilter", which could be able to process the organic matter, contributing to the pollutant degradation, and to the confinement of the contaminants to the deepest horizons and preventing the hydrocarbons reentry to the watercourse. Furthermore the deep horizons exhibited a high abundance of the genera *Acinetobacter* and

Bacillus, suggesting that they may be functionally important hydrocarbons degraders. These results indicate that MNR would be a sustainable option for the recovery of this site.
Moreover, our study illustrates how the application of emerging molecular techniques can contribute substantially to the advancement of our knowledge about biodegradation capacities of indigenous microbial communities and to monitor the effectiveness of in-situ biodegradation processes.

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