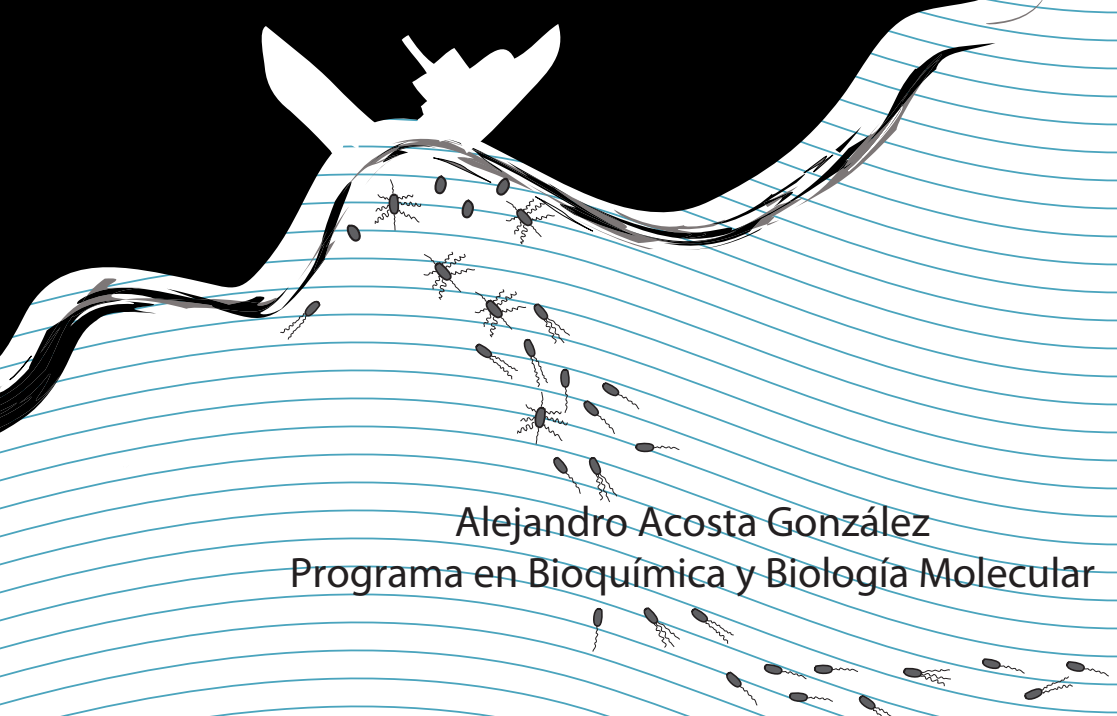
Universidad de Granada

COMUNIDADES MICROBIANAS EN SEDIMENTOS MARINOS CONTAMINADOS POR EL VERTIDO DEL *PRESTIGE*

TESIS DOCTORAL
2013



Alejandro Acosta González
Programa en Bioquímica y Biología Molecular

UNIVERSIDAD DE GRANADA

Comunidades microbianas en sedimentos marinos contaminados por el vertido del *Prestige*

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Comunidades microbianas en sedimentos marinos contaminados por el vertido del *Prestige*

Memoria presentada por el licenciado en Biología, Luis Alejandro Acosta González, para optar al título de Doctor,

Programa de Doctorado:
Bioquímica y Biología Molecular

Fdo. **Luís Alejandro Acosta González**

VºBº de la Directora de Tesis

Dra. **Silvia Marqués Martín**
Doctora en Biología
Investigador Científico CSIC

VºBº del Director de Tesis

Dr. **Ramon Rosselló-Móra**
Doctor en Biología
Investigador Científico CSIC,

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Dra. Silvia Marqués Martín
Director de la tesis

Dr. Ramon Rosselló-Móra
Director de la tesis

Alejandro Acosta González
Doctorando

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List of abbreviations

Contig: Contiguous sequence
FISH: Fluorescent *in situ* hybridization
IRB: Iron reducing bacteria
NGS: Next generation sequencing
NRB: Nitrate reducing bacteria
mbsf: meters below seafloor
MPN: Most probable number
MRB: Manganase reducing bacteria
ppm: parts per million
ppb: parts per billion
$OTU_{0.03}$: Operational taxonomic unit at 3% of sequence dissimilarity.
POP: persistent organic pollutant
SMM: seawater minimal medium
SMTZ: Sulfate-methane transition zone
SRB: Sulfate reducing bacteria
SIP: Stable Isotope Probing

Resumen

Los ecosistemas costeros son ambientes vulnerables a la contaminación por vertidos de petróleo cuyos efectos ocasionan problemas ecológicos y socioeconómicos que perduran por mucho tiempo. El vertido accidental del *Prestige* ocurrido en 2002 contaminó zonas costeras desde Galicia hasta el País Vasco, alcanzando incluso algunas zonas de Francia. Las labores manuales de limpieza se llevaran a cabo principalmente en las zonas litorales, de fácil acceso, mientras que en los sedimentos sub-litorales (sumergidos) afectados por la contaminación las labores de recuperación fueron más complicadas. Comparada con suelos o acuíferos contaminados, la ecología microbiana de estos ambientes ha sido poco estudiada. Para entender la respuesta de la microbiota autóctona ante la presencia del vertido del *Prestige*, decidimos analizar muestras de un área contaminada a los 18 y 53 meses de ocurrida la catástrofe. Tres profundidades del sedimento fueron analizadas: la zona superficial con más influencia del oxígeno; la zona de transición (condiciones oxidantes/reducidas) y la zona anaerobia. Además, realizamos experimentos en microcosmos para simular y comparar eventos de contaminación accidental. Las comunidades microbianas se caracterizaron mediante metodologías clásicas de microbiología y biología molecular: el recuento de degradadores aerobios y anaerobios mediante la técnica del número más probable; el análisis de la diversidad microbiana mediante microscopia de fluorescencia (FISH), librerías de ARNr 16S y piro-secuenciación del mismo gen; la detección de genes de degradación anaerobia de hidrocarburos, y finalmente el análisis funcional mediante metagenómica.

Aunque los metabolismos aerobio y desnitrificante asociados a la degradación de hidrocarburos fueron importantes en los sedimentos analizados comparados con resultados en estudios similares, el metabolismo dominante fue la sulfato-reducción, como era de esperar en este tipo de ambientes anóxicos. Los resultados de altos recuentos de oxidadores de hidrocarburos aerobios y anaerobios pusieron en evidencia el potencial degradador de las comunidades que habitan estos sedimentos. Paralelamente, la presencia de genes específicos de degradación anaerobia de compuestos aromáticos (tolueno y metil-naftaleno) y alifáticos (alcanos) corroboró el potencial degradador y además permitió correlacionar la presencia de dichos genes con los tipos de contaminantes más comunes en las muestras. La toxicidad de los hidrocarburos produjo una disminución en el número de células y resultó especialmente aguda en el caso del microcosmos contaminado con naftaleno. A pesar de ello, el porcentaje de la fracción cultivable de organismos oxidadores de hidrocarburos fue alta y aumentó en los casos de mayor contaminación.

Especies de *Proteobacteria*, especialmente *Deltaproteobacteria* y *Gammaproteobacteria*, dominaron las librerías de ARNr 16S. Dentro de *Deltaproteobacteria*, el orden *Desulfabacterales* fue el más abundante y por primera vez se describió la presencia

de mixobacterias en sedimentos marinos contaminados. La piro-secuenciación del gen ARNr 16S permitió confirmar la dominancia de los grupos descritos anteriormente. Por otra parte, la diversidad de las comunidades microbianas de los microcosmos cambió drásticamente en comparación al resultado obtenido de la caracterización *in situ*, especialmente en el tratamiento con naftaleno, donde más del 60% de las secuencias correspondían a un grupo no cultivable, OD1/*Parculobacteria*, poco descrito en sedimentos marinos aunque sí en ambientes anóxicos como aguas profundas de lagunas o biodigestores.

Los datos de la piro-secuenciación se utilizaron para hacer una comparación global de la diversidad presente en sedimentos costeros contaminados y no contaminados. No se detectó un patrón de diversidad determinante, pero de manera general se pudo observar que dentro de los grupos de organismos que son abundantes hay mayores similitudes entre sí, que entre los grupos de organismos de ambientes no contaminados. La importancia de *Proteobacteria* en las muestras contaminadas no fue reveladora, pero el análisis detallado a niveles taxónomicos inferiores sí permitió establecer la importancia de ciertos grupos en estos ambientes dentro de *Proteobacteria* (*Desulfobacterales* en *Deltaproteobacteria*, y *Alteromonadales* y *Chromatiales* en *Gammaproteobacteria*) o dentro de *Bacteroidetes* (*Flavobacteriales*).

Por último , el análisis de los metagenomas produjo resultados poco esperados. Por una parte, el tratamiento con petróleo no produjo muchos cambios a nivel funcional respecto al control sin contaminar, a pesar de que la diversidad microbiana cambió significativamente. Esto implica que las comunidades están pre-adaptadas a la contaminación. Por otra parte, y como se mencionó anteriormente, el tratamiento de naftaleno ejerció una presión selectiva mayor sobre las comunidades dominantes. Curiosamente, la mayoría de genes asociados a metabolismo de degradación de aromáticos se encontraron en este metagenoma. Nuestros resultados no permiten determinar si el grupo OD1/*Parculobacteria* juega un papel en la degradación de compuestos tóxicos.

De manera general, se observó que el historial de contaminación de estos sedimentos es el determinante de la capacidad de degradación de las comunidades microbianas. Los resultados descritos anteriormente permiten predecir una respuesta potencialmente activa de la microbiota frente a la contaminación.

I. General Introduction

1. Characteristics of coastal sediments.

Unlike other planets in the solar system, the Earth contains an immense mass of water available in the liquid form. Oceans represent approximately 70.8% of the Earth's surface with an average depth of 3682 m (Charette and Smith, 2010), where 97.2% of all the water is on or near the surface (Trujillo and Thurman, 2011). One-half of the global net primary production (NPP) is estimated to be produced in Oceans (Field *et al.*, 1998). After the ocean's water column, marine sediments constitute the largest habitat in the world.

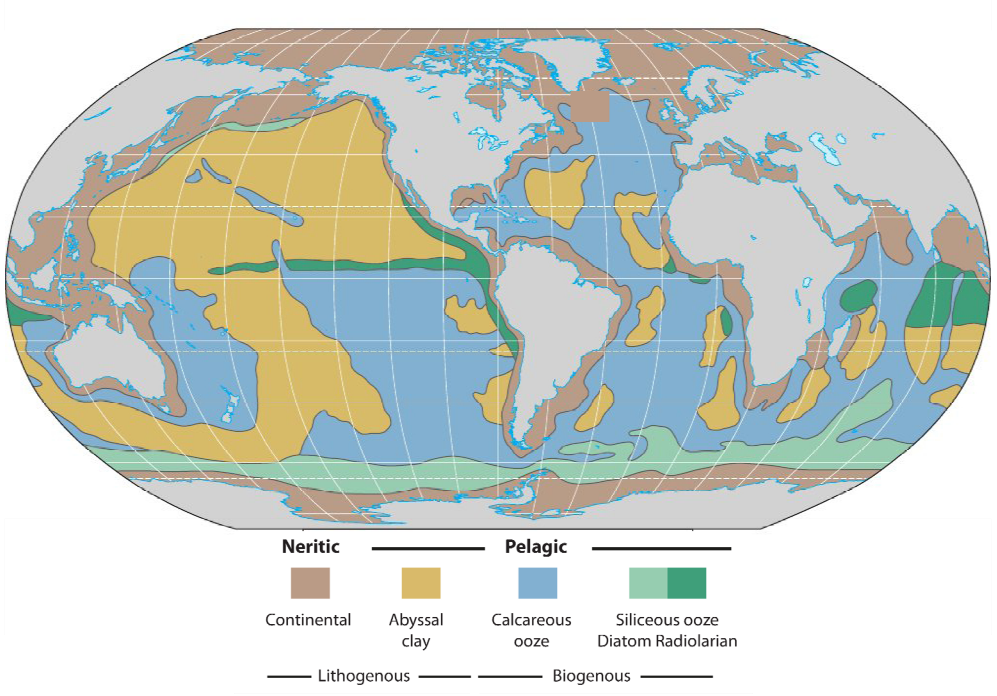

Figure 1. Distribution of neritic and pelagic sediments. The main sources of sediment deposits are indicated in the legend. Note that neritic sediments are dominated by lithogenous sources while at pelagic zones various types of biogenous and lithogenous deposits are present. Taken from Trujillo & Thurman, 2011.

The complexity and diversity of the sediment characteristics hampers the elaboration of a satisfactory classification scheme. An oceanographic division in neritic (continental) and pelagic (open ocean) sediments is the simplest classification (Figure 1), but generally more complex categories are used. According to the origin of the deposition sources four divisions have been established: biogenous, hydrogenous, cosmogenous and lithogenous (Trujillo and Thurman, 2011). Continental sediments are composed primarily by lithogenic deposits that include shelf sediments and intertidal mud flats of the coastal shoreline (Llobet-Brossa *et al.*, 2002). Lithogenous sediments (also known as terrigenous sediments) are composed of material eroded from continents or islands that are mainly transported by

rivers to the continental margin, or by winds or volcanic eruptions to the open ocean. Additionally to the near-shore shallow-water deposits of coastal sediments, lithogenic deposits are found in the river delta foreset beds of continental margins, slump deposits at continental rises produced by gravity transport, and the terrigenous-detrital shelf sediments transported into the deep sea (mixed sediments) by the activity of debris flows and turbidity currents (Fütterer, 2006). Grain size is another parameter used to classify sediments. The development of terrigenous sediments is evidenced basically by the grain-size distribution and the resulting sediment characteristics. According to the Udden-Wentworth scale (Wentworth, 1922), sedimentary particles comprise four size ranges (Table A1, appendix): granular and pebble gravel (> 2 mm), sand (2 - 0.0625 mm), silt (0.0625 - 0.0039 mm) and clay (< 0.0039 mm).

Continental shelves (coastal sediments) constitute 9% of the ocean's area and represents 15% of the total volume of marine sediments (Garrison, 2010). Currently, about 40% of the world's population inhabits areas within 100 kilometers of the coastline, with a consequent important influence on the sediment ecosystem (CIESIN, 2000). Two zones can be ecologically differentiated in coastal sediments: intertidal and subtidal zones. The intertidal zone comprises the area covered by the highest tides and exposed during the lowest tide. This includes diverse habitats, such as abrupt rocky cliffs, sandy beaches, or wetlands. The biogeochemical activities in this zone will depend of the tide fluctuations and availability of carbon matter (Taillefert *et al.*, 2007). On the contrary, the subtidal zones are submerged most of the time, only exposed at extreme low tides. Sandy sediments cover >50% of coastal and shelf areas worldwide and display a relative high-permeability due to their grain size, sorting and bulk sediment compaction (Wilson *et al.*, 2008). These properties allow a considerable sub-seafloor pore water advection (Santos *et al.*, 2012) and facilitate exchange of solutes, particles, microorganisms, dissolved substances or degradation products to and/or from the sediment. Unfortunately, most studies of sediment microbial ecology have focused muddy sediments because of their high organic content (OM), at least 10 fold higher than OM in sandy sediments, and to the higher cell density inherent to the surface of the sediment smallest particles (Musat *et al.*, 2006).

Oceans act as a natural depository of products of continental weathering. Marine sediments are the most important sites for carbon burial where microbes regulate the efficiency burial rates (Archer and Maier-Reimer, 1994). Fluxes of organic and inorganic material between the water column and the sediment bed highly influence the sediment ecology. In general, deposits occurs through processes like gravity sedimentation, benthic filtration activities, and sediment-water interface aggregation (Huettel *et al.*, 1996). Across most of the ocean realm, the material sinking from the surface is decomposed before reaching the sediments. On average, only 1% of the organic matter from the water column reaches the sediment surface, but just 3% of it remains buried (Berger *et al.*, 1989).

Continental shelf sediments constitute an exception because of the shallow depth. Delivery, reaction and burial patterns of continentally derived organic carbon are not uniform (Blair and Aller, 2012). Variances in deposit and burial rates between open ocean and coastal sediment beds could be up to 30 times in many sites (Zabel and Hensen, 2006). The organic fraction or particulate organic matter (POM) in the water column is mostly composed of detritus, except in phytoplankton blooms, where living organic matter prevails. A fraction of this material is mineralized in the sediment and many decomposition products that become nutrient sources return to the water column, where they are recycled (Nedwell *et al.*, 1993). The remaining material could move down from the upper sediment's layers to the bottom where they are used (Huettel *et al.*, 1996). In the continental shelf, carbon mineralization could correspond to up to 50% of the primary production that is deposited from the water column (Jorgensen *et al.*, 1990). As already mentioned, the sediment inputs of coastal sediments have a lithogenous origin, mainly attributed to the rivers efflux that constitutes 95% of the contributions incoming to the ocean (Syvitski *et al.*, 2003), with discharges estimates of 12 to 20 x 10^9 tons/year of sediment (Milliman and Mei-e, 1995; Syvitski *et al.*, 2005). The rivers from southeast Asia and Oceania transport more than two-third of the global sediment discharge to the oceans (Fütterer, 2006). Anthropogenic activities increase the quantity of sediment loads but the global contribution of this process is limited in Europe and North America because of the retention of approximately 20% of the material transported by rivers within artificial reservoirs (Syvitski *et al.*, 2005; Fütterer, 2006). In contrast, in areas with low or insignificant physical retention by dams, such as tropical zones, transport of eroded soil increases muddiness in coastal sediments (Wolanski and Spagnol, 2000). In both cases the ecological impact on benthic communities is significant (Wolanski and Spagnol, 2000). These effluxes could also potentially transport contaminants from continental anthropogenic sources to marine environments (Walling, 2006).

2. Microbial diversity in coastal sediments

Microbial biodiversity, defined as *the total, specific, taxonomic or genetic richness contained in nature or in any local or taxonomic part of it* (Pedrós-Alió, 2006), has been gradually to extensively studied since the first microbe was discovered more than three centuries ago (Kluyver and van Niel, 1956). Microbes include microorganisms from both prokaryotic and eukaryotic domains, as well as virus. Marine microbes occur in massive numbers in marine habitats and increase in higher productivity areas. Biochemical cycles crucial for the functioning of the ecosystem are mainly driven by microorganism. Bacteria and Archaea encompass the majority of the genetic diversity (Glöckner *et al.*, 2012).

2.1. Prokaryotes

Marine sediments harbor different types of microbes, but prokaryotes constitute the bulk of the biomass and chemical activity (Nealson, 1997). *Archaea* and *Bacteria* share common features that differentiate them from other microorganisms (Stanier and Niel, 1962). However, they show an enormous range of distinct metabolic capacities whereas both groups are similar in appearance (Glöckner *et al.*, 2012). The term prokaryote emerged from the necessity to distinguish both groups (the bacteria *sensu lato*) from the rest of organisms (*Eucarya*). Despite controversial, the term is usually employed and still valid in microbiology (Martin and Koonin, 2006; Pace, 2006; Pace, 2009; Whitman, 2009). During this thesis, we will use the term prokaryote when refer to Bacteria and Archaea together. Prokaryotes are essential omnipresent inhabitants of most environments that dominate the biosphere and represent the second most abundant biological group after virus (Breitbart, 2012). There is no clear definition of what "species" means in the prokaryotes. Besides the problem of defining and understanding what a prokaryotic species is (further discussed in part 4), the prokaryotic species richness is imagined to range from thousands to millions, and an accurate estimation is imprecise and problematic, and probably over- or underestimated (Rossello-Mora and Amann, 2001). The most accepted contribution of prokaryotes to the total biological diversity has been established in 3.2% (Claridge *et al.*, 1997), although this value is relative because the total number of bacterial species on Earth still remains unresolved, and the estimations are based on classified taxa that are composed by pure cultures in the laboratory.

Prokaryotes inhabiting marine sediments are involved in the main geochemical cycles of elements like sulphur, nitrogen and iron and are metabolically very versatile (Nealson, 1997). Geochemical conditions governing the sediments (electron acceptor availability, organic matter quantity and quality) determine the structure and dynamics of the microbial communities. *Proteobacteria*, as the largest phylum of *Bacteria*, include species with enormous morphological, phenotypic and metabolic differences (Kersters *et al.*, 2007). *Gammaproteobacteria* and *Deltaproteobacteria* (included in *Proteobacteria)* are the dominant groups of bacteria in intertidal and subtidal sediments; classes from the phyla *Bacteroidetes, Acidobacteria* and *Planctomycetes* are common; and *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Verrucomicrobia*, *Nitrospirae*, *Spirochaeta*, *Beta-proteobacteria* and *Lentisphaerae* are detected at a lower scale (Musat *et al.*, 2006; Li *et al.*, 2009; Polymenakou *et al.*, 2009; Köchling *et al.*, 2011; Kolukirik *et al.*, 2011; Gobet *et al.*, 2012; Zinger *et al.*, 2012; Acosta-González *et al.*, 2013). These major groups are common to other environments, with differences. For example, the class *Bacteroidetes* (within the same phyla) is almost absent from soils (Janssen, 2006). *Betaproteobacteria* and *Epsilonproteobacteria* are not always detected (Mills *et al.*, 2008). *Archaeal* communities, both *Euryarchaeota* and *Crenarchaeota*

phyla, are also present in coastal marine sediments and are associated to processes such as methanogenesis or anaerobic oxidation of methane.

2.2. Virus

Due to the abundance of prokaryotic organisms, the majority of the viruses are phages commonly named bacteriophages". As a general rule, the smaller the organism, the more abundant it is, and viruses are by far the most abundant biological entities in the planet (Kirchman, 2012). The virosphere probably prevails in every environment on Earth, present primarily in the Oceans where 94% of nucleic acid containing particles are virioplankton (planktonic virus from aquatic systems) that constitutes only 5% of the total biomass of the marine environment (Suttle, 2007). Studies on viriobenthic (virus inhabiting sediments) diversity and community structure are at a pioneering stage (Danovaro *et al.*, 2008).

The viriobenthos is highly diverse and active in shore-line coastal sediments where 10^9 to 10^{13} viruses and at least 10^4 viral types per kilogram of sediment are found (Breitbart *et al.*, 2004; Filippini and Middelboe, 2007; Danovaro *et al.*, 2008). Although viruses are more abundant in the sediment, the viral lysis rates are higher in the water column (Glud and Middelboe, 2004). Identification of virus based on culturing phage (restricted by the bacterial isolates available) and measuring physical parameters of the free virion limits the measuring of phage diversity (Breitbart *et al.*, 2004). Molecular data (metagenomic) for a genomic-based taxonomy system is increasing our knowledge of this diversity (Rohwer and Edwards, 2002; Suttle, 2007).

It has been proposed that virions control host-specific microbial populations, structure communities and also influence geochemical cycles (Fuhrman, 1999; Suttle, 2007; Breitbart, 2012). Virus impact the global carbon cycle in marine environments by releasing bacterial content via cellular lysis (Riemann and Middelboe, 2002; Siem-Jørgensen *et al.*, 2008). The DNA released can be easily metabolized by prokaryotes. Phages can be responsible for 10-60% of bacterial mortality in deep anoxic sediments and 15-35% in coastal anoxic sediments (Corinaldesi *et al.*, 2007), contrasting with freshwater sediments where bacterial infection and mortality rates are low (Filippini *et al.*, 2006).

2.3. Eukaryotes

The marine realm represents ~99.83% of the habitable volume on this planet but eukaryotic richness is 3-4 orders of magnitude lower than that of terrestrial and freshwater habitats (Dawson, 2012). Studies of eukaryotic communities in anoxic environments have been restricted by the general assumption that oxygen depletion and sulphide's

occurrence (resulting from sulfate respiration) limit the existence of nearly any eukaryote in these habitats (Dawson and Hagen, 2009). However, species of meiobenthic animals and mainly of protists (flagellates and ciliates) are common inhabitants of anoxic environments because they can tolerate these adverse conditions and survive in the absence of oxygen (Levin, 2003). Both aerobic and anaerobic species are equally distributed across groups of eukaryotes, therefore respiratory metabolism was not a guiding force of phylogenetic divergence (Müller *et al.*, 2012). Anaerobic capacities are confined to unicellular organisms among eukaryotes but recently the first animal species able to live in permanently anoxic conditions was reported (Danovaro *et al.*, 2010). Protists derive energy through fermentation rather than aerobic respiration in marine sediments (Dawson and Hagen, 2009) although there are reports of unicellular eukaryotes like benthic foraminifera or planktonic diatoms that store intracellular nitrate to support their growth by denitrification in anoxic conditions (Risgaard-Petersen *et al.*, 2006; Kamp *et al.*, 2011).

The ecological role of eukaryotes is to graze on *Bacteria* and *Archaea* present in the sediments (Kemp, 1998; Radajewski *et al.*, 2000). The lower protists densities in anoxic habitats are attributed to the low growth efficiencies inherent to fermentation (Kirchman, 2012). Consequently, grazing bacterial rates mediated by protists are higher in oxic than anoxic environments, contrasting with the major relevance of bacterial mortality induced by virus in the anoxic marine sediments (Epstein, 1997; Wilhelm and Matteson, 2008). Another important ecological role of eukaryotes in marine sediments is bioturbation. The term refers to the biological reworking of soils and sediments by benthic animals (Meysman *et al.*, 2006). This process plays a key role in the structure and functioning of the subsurface because it influences food caching and prey excavation, redistributes food resources (organic matter, viruses, bacteria, resting stages and eggs) and supports the infilling of abandoned burrow structures (Meysman *et al.*, 2006), among others. The re-mineralization of organic matter is promoted by bioturbation when redox conditions (for example through oxygen inflows) are temporally altered (Aller, 1994). As a consequence, patterns of diversity and structure of microbial communities are altered (Laverock *et al.*, 2010). Bioturbation could also help to increase the sulfate seawater concentration, demonstrating its evolutionary relevance (Canfield and Farquhar, 2009).

2.4. Patterns of microbial diversity

The ubiquity of prokaryotes is favored by their widespread dispersal, metabolic versatility and flexibility, as well as by their capacity to resist extreme and hostile conditions (Schlegel and Jannasch, 2006). The most accepted estimation of the number of prokaryotic cells in the earth is 4-6 x 10^{30} and represents at least the half of the "living protoplasm" (protoplastic biomass other than extracellular components like cell walls or structural

polymers) on earth, and is equivalent to 60-100 % of the total carbon content estimated in plants (Kluyver and van Niel, 1956; Whitman *et al.*, 1998). Marine sediments (both continental and pelagic) contain approximately 85% (3.55 $x10^{30}$ cells) of the estimated number of total prokaryotic cells, becoming the most inhabited environments on earth (Table 1) (Whitman *et al.*, 1998). Virus exceeds the number of bacteria by at least an order of magnitude in the biosphere (Wommack and Colwell, 2000). Viral abundance in the coastal sediment can surpass 10 to 100 times the abundances observed in the overlying water column, correlating with the increase in bacterial abundance in the sediments with respect to the water column (Maranger and Bird, 1996; Glud and Middelboe, 2004). Environments where bacteria beat phage abundance are Mediterranean deep sediments and non-cold seep sediments from the Gulf of Mexico (Danovaro *et al.*, 2002; Kellogg, 2010), but these seem to be exceptional cases.

Table 1. Estimated number of prokaryotes in different habitats[1].

Habitat	Volume (m^3)	Cells/ml $x10^5$	Total number of cells ($x10^{26}$)
Marine water			
Continental shelf	2.03 x 10^{14}	5	1.0
Open ocean			
water, upper 200m	3.0-7.2 x 10^{16}	5	360
water, below 200m	1.3 x 10^{18}	0.5	650
sediment (top 10 cm)	3.6 x 10^{13}	4600	170
Total marine water			**1180**
Freshwater			
Lakes	1.25 x 10^{14}	10	1.3
Rivers	1.20 x 10^{12}	10	0.012
Total freshwater			**1.312**
Saline lakes	1.04 x 10^{14}	10	**1.0**
Subsurface sediments			
Coastal plains (terrestrial)	9.8 x 10^{16}		2530
Continental shelf and slope			7990
Deep ocean			27510
Total subsurface sediments			**38030**
Others			
Soil*	9.14 x 10^{14}	21000	2556
Atmosphere (lower 3km)			0.0000001
Whole human population**			0.007-0.07
Grand total			**41767**

[1] Table adapted from Whitman et al., 1998. The subsurface was limited between 10 cm and 8 m for terrestrial habitats, and to 4 km for marine sediments. *Soil refers to the first 8 m depth analysed in soil biomes (tropical rain and seasonal forest, temperate evergreen and deciduous forest, boreal forest, woodland and shrubland, savanna, temperate grassland, desert scrub, cultivated land, tundra and alpine, and swamps and marsh). ** data obtained from Ursell *et al.*, 2012.

All microbial communities are governed by only a few species (known as resident populations) that support the major biogeochemical cycles, but the role of the remaining fraction of minority communities, known as the "rare biosphere", is still unknown (Sogin *et*

al., 2006; Dawson and Hagen, 2009; Gobet *et al.*, 2012; Pedrós-Alió, 2012). As a generalization, ecosystems with rough geochemical conditions usually harbor high cells numbers that represent a few species. In contrast, an ecosystem with mild environmental conditions, as it is the case for oceans and sediments, harbor a high number of species present in low cell numbers (Schlegel and Jannasch, 2006).

Different attempts to give a comprehensive picture of the microbial diversity and community structure in marine sediments have been made. Both sediment and soil environments seem to harbor a higher prokaryotic diversity that differ many orders of magnitude from the diversity in aquatic environments (Curtis *et al.*, 2002; Torsvik *et al.*, 2002; Zinger *et al.*, 2011). Although soil has been reported as an environment with the highest diversity at the species level, sediment types such as coastal sediments or hydrothermal vents are the most phylogenetically diverse habitats (Lozupone and Knight, 2007). Differences in community composition have been mainly attributed to environmental conditions (mainly salinity) rather than the own environmental type (Lozupone and Knight, 2007). However, comparison of the functional potential between water and solid substrates indicates that substrate type (over salinity, microbial abundance or nutrient status) is the crucial factor that determines the prevailing microbial metabolism (Jeffries *et al.*, 2011). In coastal sediments, differences in diversity between microbial communities from pore water and sand are observed (Gobet *et al.*, 2012). On the other hand, pyrosequencing of marine samples from global pelagic (water column) and benthic (sediment) realms established that composition and distribution of microbial communities displayed an horizontal and vertical large-scale patterns determined by variables like productivity or spatial distribution (Zinger *et al.*, 2011). Sediment communities showed a higher dissimilarity with increasing distance as compared to pelagic communities (Zinger *et al.*, 2011), suggesting that local physical mixing of sediments may contribute to changes in the distribution patterns of marine bacteria. A small fraction of the bacterial species is shared by pelagic and benthic communities (Lozupone and Knight, 2007; Zinger *et al.*, 2011). Only a few investigations have compared the cultivated and non-cultivated fraction of microorganisms obtained from the same sediment samples, and surprisingly, isolates from different sediment environments were phylogenetically closer between them than with the uncultured counterparts found in the same environment (Lozupone and Knight, 2005; Lozupone and Knight, 2007). Seasonal abundance of viriobenthos is correlated with the fluctuations of bacterial communities because bacterial activities can regulate the viriobenthic production (Glud and Middelboe, 2004; Siem-Jørgensen *et al.*, 2008). Although virus could be highly diverse on a local scale, at global scale the diversity is relatively limited because they can move between environments (Breitbart and Rohwer, 2005). The phylogenetic diversity of eukaryotes in marine sediments is considered generally low, but access to the undetected diversity by pyrosequencing approaches has demonstrated that

the previously determined richness was underestimated (Stoeck *et al.*, 2009). In the marine realm, there are more eukaryotic phyla that in the terrestrial realm, although the species density is lower (Dawson, 2012).

3. Microbial metabolism in coastal marine sediments

Before the appearance of oxygenic photosynthesis 2.5 billion years ago, anaerobic metabolism dominated the biosphere (Lozupone and Knight, 2007). Nowadays, many habitats such as marine sediments are under permanent anoxic conditions (Rockne and Strand, 1998; Brune *et al.*, 2000). The high oxygen demand for aerobic degradation processes in initially oxic sites causes a rapid oxygen depletion resulting in the development of anoxic conditions. Additionally, hypoxia processes are extending over marine ecosystems as consequence of coastal eutrophication and global warming, and unfortunately, considerations about its effects on marine life and geochemical processes have been underestimated (Vaquer-Sunyer and Duarte, 2008; Conley *et al.*, 2011).

As already mentioned, coastal sediments are characterized by an important input of organic matter, obtained through the sedimentation of material from the continental or upwelling areas. In the open ocean, only a small fraction of the complex organic matter is accumulated on the seafloor as a result of the mineralization process carried out through the water column depth. In contrast, a limited processing of the organic material in shallow waters settles in the seafloor sediment higher amounts of organic matter to be further degraded (Engelen and Cypionka, 2009). Within the sediment, the mineralization processes reduce the organic matter quality with depth (Julies *et al.*, 2010; Moxley and Schmidt, 2012). As a consequence, there is a general decrease of bacteria cell numbers through depth due to the organic matter depletion, although in many cases the subsurface of the superficial layers also display a high degree of microbial activity (Parkes *et al.*, 2000). Chemoorganotrophic bacteria are the main players responsible of the oxidation of organic matter in the sediments by reducing different available electron acceptors (Llobet-Brossa *et al.*, 2002). The most abundant terminal electron acceptor (TEA) in marine sediments is sulfate from seawater (with an average concentration of 28 mM). Other common electron acceptors in sediments are oxygen, iron and manganese oxides, nitrate and carbon dioxide. In a typical sediment profile, a succession of electron acceptors is observed. First, microorganisms consume the oxygen that penetrates the first millimeters/centimeters of the sediments (Braker *et al.*, 2001). Once the oxygen consumption exceeds its supply, anoxic conditions are established. Below the zone of oxygen-influence, anoxic processes are stimulated if biodegradable organic matter is available. Nitrate, manganese, iron and sulfate are used for anaerobic respiration if present (Sørensen, 1978; Canfield *et al.*, 1993b; Koike and Sorensen, 1998). Subsequently, different redox zones are formed in the coastal

sediments that can be visualized by simple eye inspection (Figure 2): i) an upper oxidized brown layer that can range between a few millimeters to several centimeters; ii) an anoxic but oxidized transition zone where nitrate, manganese and/or iron oxides are reduced; and iii) finally a dark grey reduced sulfidic zone, in which sulfate reduction predominates (Moxley and Schmidt, 2012). Methanogenesis develops in the sediment depending on the interaction between methanogens and sulfate reducers, generally described as mutually exclusive (Lovley and Phillips, 1987). Methanogenic *Archaea* would be expected to succeed in the deeper sulfate-depleted layers of the sediment (Plugge *et al.*, 2011). Other important types of chemoorganotrophic metabolisms present in the sediment include sulfur reducers, syntrophs, acetogens and fermenters (Nealson, 1997). On the other hand, both biotic (chemolithotrophs) and abiotic processes can oxidize the compounds reduced by chemoorganotrophs, thus recycling elements in the sediments. The order of use of the available electron acceptors in the sediment is explained by the tendency of these compounds to accept electrons (see the electron tower in Figure 2): oxygen is firstly depleted and carbon dioxide is latest. The theoretical yield of energy obtained from the oxidation of organic matter using each one of the electron acceptors is the driving force for microbes to use a specific metabolism when oxygen is absent.

3.1. Aerobic respiration

In the continental margin sediments, the penetration of oxygen from the water column is limited to a depth ranging from millimeters to less than 10 cm (Cai and Sayles, 1996). In the sediment surface, the microbial communities consume oxygen at rates that exceeds natural oxygen supply by molecular diffusion from seawater and production in the diffusive boundary layer, resulting in fast oxygen depletion (Fenchel and Finlay, 2008). The extent of the oxidized horizon depends on the input and quality of the degradable organic matter and the depth of oxygen penetration. Thus, a steep decreasing oxygen gradient is formed. If total sediment metabolism is considered, oxygen respiration represents a relatively small percentage of organic carbon oxidation (Canfield *et al.*, 1993b). However, the microbial activity (photosynthesis and respiration) of the upper millimeters/centimeters of the sediment can be 100-1000 times higher than the activity of the entire water column (Glud, 2008).

Oxygen respiration is carried out by heterotrophic microorganisms with strict aerobic metabolism, but also by facultative bacteria (in particular nitrate-, iron- and manganese-reducing bacteria and fermentative bacteria) can participate in the process. *Gammaproteobacteria* dominates the first centimeters of most coastal (at inter- and sub-tidal zones) but also pelagic sediments (Li *et al.*, 2009; Liao *et al.*, 2009; Orcutt *et al.*, 2011). Populations shallower than 10 cm below surface were temporally variable yet uniform

between sites, while below this depth, populations were more site-specific (Reese *et al.*, 2013). *Cyanobacteria* are abundant in microbial mats established in the tidal-free zone (Bolhuis and Stal, 2011).

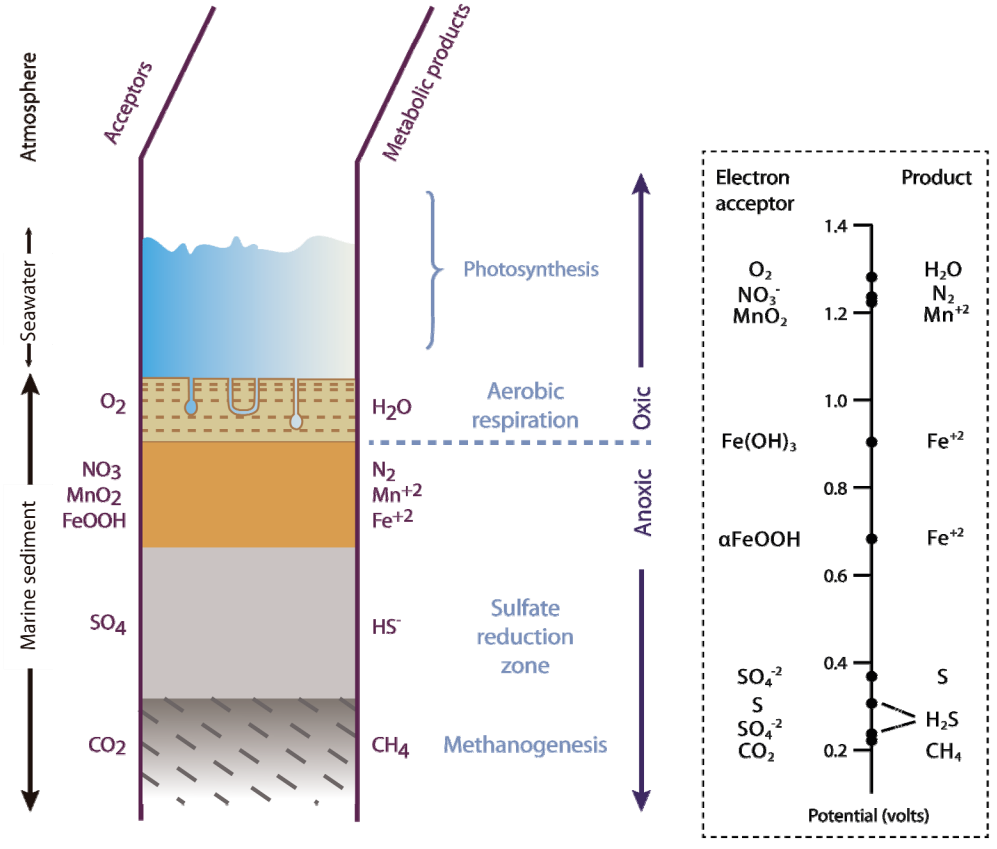

Figure 2. Geochemical processes in coastal sediments. The vertical profile shows a gradient from an oxidized to a reduced zone, determined by the electron acceptor availability and the quality of electron donors (left). The geochemical zones from the scheme are not scaled for better visualization, but represent a maximum of 1 meter of the sediment profile. The electron tower (right) shows the potential for some half reactions involving TEA used by microbes in the sediments (Adapted from Kirchman, 2012).

3.2. Denitrification

Denitrification occurring in coastal sediments is considered to be responsible for less than 10% of organic carbon oxidation in marine sediments (Canfield *et al.*, 1993b), where denitrification seems to be limited by the availability of nitrate (Sørensen *et al.*, 1979). Oceans are acidified by increasing inputs of anthropogenic CO_2, which results in an increase of nitrogen fixation by *Cyanobacteria*. It has been suggested that ocean acidification would reduce ammonia availability and therefore the nitrification processes

(ammonia is considered the preferential substrate of nitrifiers like *Nitrosomonas*). Consequently one important source of oxidized nitrogen forms of denitrifiers would also be reduced (Ward, 2008; Hutchins *et al*., 2009). However, the potential for denitrification may be important in zones exposed to a large and continuous input of nitrogen, as it is the case in the coastal and shelf areas (Galloway, 2003). Depending of the oxygen concentration, oxygen and nitrate reduction can take place simultaneously. The latest steps of denitrification are especially sensitive to high oxygen concentrations (Bonin and Raymond, 1990; Gao *et al*., 2009).

Nitrate reducers (NR) are widespread among prokaryotes and eukaryotes *(Devol, 2008)*. The most studied group of NRB is *Proteobacteria* (*Alpha*, *Beta*, *Gamma* and *Epsilon* classes) (Kraft *et al*., 2011), although NRB belonging to other phylogenetically distinct bacterial groups like *Firmicutes*, *Bacteroidetes*, *Actinobacteria* were also identified (Heylen *et al*., 2006; Verbaendert *et al*., 2011; Falcão Salles *et al*., 2012). The majority of denitrifiers are chemoorganotrophs, although chemolithotrophic denitrifiers growing autotrophically are also common in freshwater and marine sediments (Straub *et al*., 1996; Jones, 2007). The analysis of denitrifying genes distribution indicates that denitrifier communities change through time, and that a vertical decrease of diversity is associated to observed decreases of nitrate and oxygen concentrations (Scala and Kerkhof, 2000; Tiquia *et al*., 2006). In coastal sediments, *Alphaproteobacteria* have been reported to be the largest fraction of denitrifying communities (Mills *et al*., 2008). Species from filamentous *Beggiatoa sp*. can store nitrate and grow anaerobically by coupling sulphide oxidation to the reduction of nitrate (Mußmann *et al*., 2003). Among *Archaea*, both *Crenarchaeota* and *Euryarchaeota* included NR organisms (Cabello *et al*., 2004).

3.3. Iron and Manganese reduction

Iron content is the fourth most abundant element on Earth and the sixth in the Universe. Continental dust and shallow and coastal sediments are the main iron sources to the open ocean (Raiswell *et al*., 2008; Boyd and Ellwood, 2010). It was shown that natural iron fertilization induced a phytoplankton bloom in the ocean, demonstrating the importance and influence of iron in the carbon cycle (Blain *et al*., 2007). Besides its importance as nutritional requirement, its abundance supports the energy metabolism of both chemoorganotrophic (anaerobic iron reduction) and chemolithotrophic (both aerobic and anaerobic iron oxidation) microbial populations in soil and sediment systems (Weber *et al*., 2006). Manganese is the tenth most abundant element in the earth's crust, and the presence of large deposits in the deep seafloor reflects the migration of this element (Glasby, 2006). Although manganese oxides are ten times less abundant that iron oxides, the proportion of bioavailable oxides for respiration is higher for manganese than for iron

(Lovley, 1991). The importance of iron and manganese reduction in coastal sediments was recognized many years ago (Sørensen and Jeørgensen, 1987; Hines *et al*., 1991).This process in coastal sediments is confined to the first superficial centimeters (Canfield *et al*., 1993b; Canfield *et al*., 1993a), between the nitrate and sulfate-reduction zones (Nealson and Saffarini, 1994). Iron reduction is inhibited by denitrification and only when nitrate and nitrite have been depleted, iron respiration increases, presumably by facultative anaerobic denitrifying bacteria (Sørensen, 1982). Some studies suggested that metal reduction rates have been underestimated in some near shore sediments, and that these processes are favored by active bioturbation processes that mix the manganese oxides and the organic matter through the sediment sub-oxic/anoxic zones (Aller, 1990; Canfield, 1993). In marine ecosystems sulfide tends to remove Iron (+2) as iron sulfide (pyrite) as consequence of an active sulfur cycle (Donald E, 1989; Nixon *et al*., 2012). .

3.4. Sulfate reduction

Beneath the superficial layers, the sediment becomes reduced, and in this zone sulfate reduction (SR) predominates. The final product of sulfate respiration, hydrogen sulfide, precipitates as iron sulfide that gives the typical dark-grey zonation of the reduced sediment. In marine coastal sediments, about 50% of the deposited material is mineralized via SR (Jorgensen, 1982), converting this process in the most relevant respiratory metabolism of this habitat. The electron donors commonly used by SRB primarily include hydrogen and acetate, so that marine sediments are dominated by acetate/hydrogen oxidizing SRB (Jørgensen and Bak, 1991). In addition, SRB can also oxidize methanol, ethanol, lactate, propionate, butyrate, sugar and molasses (Liamleam and Annachhatre, 2007). If sufficient concentrations of electron acceptors like iron (Lovley and Phillips, 1987) or manganese oxides (Canfield *et al*., 1993a) are present in the sediment, the predominant SR is inhibited (Myers and Nealson, 1988; Thamdrup *et al*., 1994). Sulfate respiration is mediated by a diversity of phylogenetically and physiologically different groups of microorganisms including in four bacterial phyla: *Desufotomaculum*, *Desulfosporomusa*, *Desulfosporosinus* and *Thermodesulfobium narugense* (*Clostridiales*) within *Firmicutes,* species of *Deltaproteobacteria*, species of *Thermodesulfovibrio* (*Nitrospira*), *Thermodesulfobacterium* and *Thermodesulfatator* (*Thermodesulfobacteria)*; and two archaeal divisions: *Archaeoglobus* in *Euryarchaeota* and *Thermocladium* and *Caldivirga* in Crenarchaeota. More than 220 species included in 60 genera of SRB described to date (Barton and Fauque, 2009). Sulfate reducing *Deltaproteobacteria* govern the anoxic reduced zone of the sediment (Jorgensen, 1977; Skyring, 1987; Leloup *et al*., 2009; Enning *et al*., 2012) although this group could inhabit the sediment surface too (Bühring *et al*., 2005). Sulfate reduction is considered a strictly anaerobic process. However, evidences of

tolerance or even oxygen respiration when exposed to air during short periods of time have been reported in some *Desulfovibrio* species (Cypionka, 2000). Additionally, SRB species use strategies like aggregate formation, aerotaxis or enzymatic systems to reduce and detoxified oxygen reactive species as a defense mechanism (Cypionka, 2000; Dolla *et al.*, 2006).

3.5. Methanogenesis

Once the sulfate is depleted as consequence of a high organic matter oxidation activity, methanogenesis becomes a dominant process. The distribution of methanogenic and sulfate reducing communities is thought to be reciprocally exclusive due to competition for hydrogen and acetate, the two major substrates for which these communities show the greatest affinity (Hines and Buck, 1982; Ferry and Lessner, 2008). The main terminal acceptors in methanogenesis are carbon dioxide and acetate. Other small carbon compounds (C1 compounds) that are not used by SRB can serve as substrate for methanogens. As a consequence, in some cases both sulfate reduction and methanogenesis can co-exist in a biogeochemical zone named sulfate-methane transition zone (SMTZ) (Oremland and Polcin, 1982; Holmer and Kristensen, 1994). This zone is generally observed at shallow depths of 1-30 mbsf (meters below seafloor) in methane-rich marine sediments (Malinverno and Pohlman, 2011). Below this zone, methane is produced; above, methane could be oxidized by both aerobic bacteria and anaerobic *Archaea*. Two processes reduce sulfate at and above the SMTZ, anaerobic oxidation of methane (AOM) and classical organoclastic sulfate reduction (OSR).

4. Oil pollution in marine sediments

The oil pollution inputs to marine habitats include both anthropogenic and natural sources with a contribution of 53% and 47%, respectively (Kvenvolden and Cooper, 2003). After delivery *via* marine transportation, oil spills are the second cause of crude oil coastal contamination, and the immediate visual impact caused by tanker's accidents generate higher social concern (Neuparth *et al.*, 2012). Other relevant anthropogenic sources are oil production and industrial and urban emissions (Charriau *et al.*, 2009). Natural sources of hydrocarbons range from terrestrial plant waxes and marine phytoplankton to volcanic eruptions, biomass combustion and natural oil seeps (Gonul and Kucuksezgin, 2012).

4. 1. Petroleum characteristics

Petroleum oils consist of many thousands of different compounds. Hydrocarbons and heterocycles are the main constituents of petroleum and consequently are extremely abundant in geological systems. Besides their petrol origin, hydrocarbons occur in a great structural diversity as consequence of sub-products biosynthesis by living organisms or as abiotic transformation products of biogenic organic matter (Wilkes and Schwarzbauer, 2010). Petroleum composition depends on the source material depositions and the biogeological maturation processes (persistence, microbial degradation, pressure, temperature and catalytic processes) (Readman, 2010). The density and solubility in seawater regulate the mobility, distribution and toxicity of hydrocarbons within the marine environment (Rogowska and Namieśnik, 2010). Hydrocarbons *sensu stricto* contain by definition exclusively the elements carbon and hydrogen, and they include three main classes of compounds namely saturated, unsaturated, and aromatic hydrocarbons.

4.1.1. Saturated hydrocarbons

These are the hydrocarbons where all the valences of carbon atoms are fully utilized by single covalent bonds. Saturated hydrocarbons comprise three structurally different compound classes:, *n*-alkanes, branched alkanes and cyclic alkanes.

The term *n*-alkane (commonly called paraffin) or *normal*-alkanes (molecule without branches) refers to linear hydrocarbons with the general formula C_nH_{2n+2} that are bonded exclusively by single bonds, representing a straight chain of carbon atoms. As the majority of the *n*-alkanes of the oil reservoirs are substantially biodegraded, their presence is an indication of undegraded crude oil. Biogenic alkanes characteristically have a major odd-over-even C number predominance because they are synthesized from fatty aldehydes with even-numbered carbon chains (Cheesbrough and Kolattukudy, 1984). Methane is the simplest *n*-alkane, the smallest hydrocarbon compound and the most abundant low-molecular-weight organic compound in the biosphere, formed through both biological and geological processes. More than one constitutional isomer its possible when at least 4 carbons form an *n*-alkane. These structural isomers that do not possess straight chains of carbon atoms are termed *branched* alkanes. Biodegradability is limited by increasing branching complexion and after biodegradation of crude oil a relative accumulation of these compounds is observed. The low natural abundance of conformational isomers has not favored the evolution of appropriate biodegradation pathways for these compounds (Wilkes and Schwarzbauer, 2010).

Finally cycloalkanes are represented by the general formula $C_nH_{2(n+1-r)}$, where n is the number of carbon atoms and r the number of rings in the molecule. This structure is

generated by the removal of two hydrogen atoms from two different carbon atoms in *n*-alkanes or branched alkanes.

4.1.2. Unsaturated hydrocarbons

Unsaturated hydrocarbons are molecules that contain at least one C–C double bond (alkenes or olefins) or one C–C triple bond (alkynes). Unsaturated hydrocarbons can be represented by the general formula $C_nH_{2(n+1-r-d-2t)}$ where n is the number of carbon atoms, r the number of rings, d the number of C–C double bonds and t the number of C–C triple bonds in the molecule. The simplest alkene is ethylene and the simplest alkyne is acetylene. Alkenes (and cycloalkenes) of great structural diversity are present in some organisms as natural products: muscalure, the sex pheromone of housefly; α-farnesene, the coating of apples and other fruits; γ-terpinene from various plant sources (Wilkes and Schwarzbauer, 2010). Alkenes quantities in crude oil are insignificant. These compounds are industrially produced by cracking (thermally breaking down certain fractions from crude oil in the presence of a catalyst) and converted into compounds like plastics, ethylene glycol, alcohols and gasses like ethylene or mustard gas.

4.1.3. Aromatic hydrocarbons

Aromatic compounds consist of conjugated planar ring systems with delocalised pi electron clouds instead of discrete alternating single and double bonds. These compounds (mainly found as lignin components, flavonoids, quinones, aromatic amino acids or constituents of fossil fuels) are the most abundant class of organic compounds in nature, after carbohydrates. In fact, lignin is the second most abundant polymer in nature, after cellulose (Boerjan *et al.*, 2003). The basic example of an aromatic hydrocarbon is benzene, the second chemical structure most distributed in nature (about 25% of the Earth's biomass is composed of compounds that have a benzene ring as the main structural constituent) (Gibson and Harwood, 2002). Aromatic compounds are more stable due to resonance stabilization behind the conjugation of the double bonds (Wilkes and Schwarzbauer, 2010); in fact, resonance energy of the ring confers a high thermodynamic stability that increases the persistence of aromatics as major environmental pollutants in nature (Krygowski *et al.*, 2000). Many xenobiotic compounds belong to this class of compounds. Polycyclic aromatic hydrocarbons (PAHs) consist of two or more benzene rings in linear, angular, or cluster structural arrangements. The structurally simplest representative of this class of compounds is naphthalene. Additional to petroleum refining and fossil fuel combustion, PAHs anthropogenic emissions include domestic, industrial, power plant, traffic, and coke incomplete combustion (Inomata *et al.*, 2012). Their low aqueous solubility and

hydrophobicity determines hydrocarbons tendency to associate with suspended particulate material, which is finally deposited and accumulated in the sediment (Tolosa *et al.*, 2004; Lu *et al.*, 2011). PAHs are ubiquitous in natural environments, including, water, soil and sediments (Wilkes and Schwarzbauer, 2010).

4.2. Oil spills

Oil spills is an important source of marine pollution that causes enormous damages to organisms and their environment (Goldstein *et al.*, 2011). In fact, human impact on continental shelf and coral reefs is higher than on any other marine environment (Halpern *et al.*, 2008). Unfortunately, a conscious society waked up just few decades ago and still today much effort in prevention and monitoring of spills has to be done (Peperzak *et al.*, 2010; Rogowska and Namieśnik, 2010; Neuparth *et al.*, 2012).

Oil spills range from minor discharges and leaks (less than 1 tons) to immense releases by tanker accidents (300000 tons) (Peperzak *et al.*, 2010). If intentional tanker large discharges are considered (as what occurred in the 90's Gulf war), release size increases to 1.8 millions of tons. Despite the progressive decrease of oil spill accidents in the marine environment (figure 3), the European Atlantic waters have been considered as a "hotspot" because until the last decade the highest number of oil spills worldwide occurred here (Vieites *et al.*, 2004; Neuparth *et al.*, 2012).

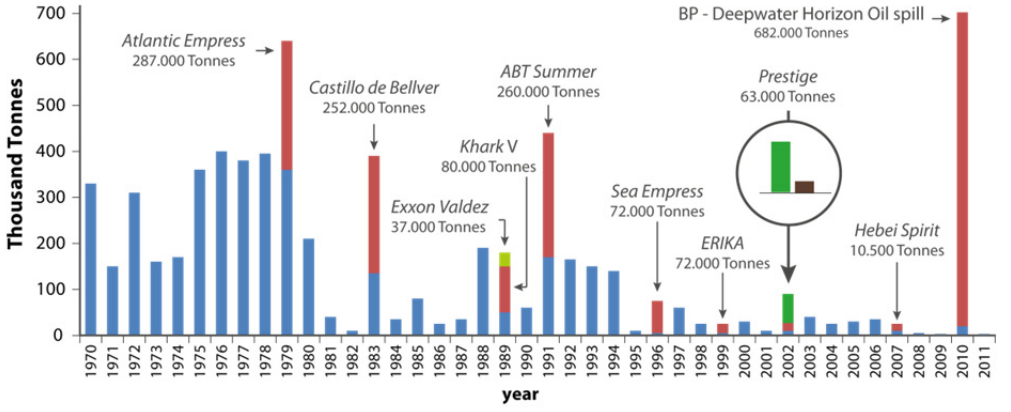

Figure 3. Annual oils spills since 1970. The biggest tanker accidents are shown as red bars and the size of the spill is indicated. The Exxon Valdez oil spill is highlighted in light-green as reference. For the *Prestige* spill estimative, both official (recognized by Spanish authorities) and alternative estimates (data from http://www.disasterscharter.org) are shown in brown and green, respectively. Figure and data adapted from ITOPF (2011) and NRT (2011) documents.

The amount of oil remaining in the sea after a spill can decrease through different simultaneous mechanisms: evaporation, dissolution, biodegradation and sedimentation,

while winds and marine currents tend to disperse the contaminant in the marine environment (Peperzak *et al.*, 2010). As a result of oil depositions on the shoreline, extensive and long-term deterioration of coastal ecosystems is evidenced.

4.3. The *Prestige* oil spill

In November 2002, the sinking of the oil tanker *Prestige* in the northern Spanish coast (3500 meters of water off) released an estimated 77,000 tons of heavy fuel oil into the sea (Figure 3). The *Prestige* tanker was 26-year-old and contained approximately 78,000 tons of heavy fuel oil at the moment of the accident. The ship had a single-hulled vessel that was not adequate to retain the load after tank non-severe collisions (Höfer, 2003). Several hundred kilometers of coastline were covered by an oil slick, made up of a complex mixture of saturated (23%) and aromatic (53%) hydrocarbons, asphaltenes and resins (24%). The aromatic fraction was mainly composed of naphthalene, phenanthrene and alkyl derivatives, while the saturated fraction consisted of lineal and cyclic hydrocarbons of variable length (Barrett *et al.*, 2011). During the first week, 2,000 tons reached the Spanish coast. A steady flow of oil from the wreck out of cracks, gauging and outlets was evidenced (approximately 100 tons per day) (Höfer, 2003).

The Atlantic coast of Galicia is rich in fish species and an important sector of the local population depends on fishery activities. Galicia coastline harbors one of the largest aquaculture of mussels and barnacles, important activities for the fish industry. The toxic effects produced by the spill on the ecosystem have been studied. Loss of species richness of invertebrates (de la Huz *et al.*, 2005; Veiga *et al.*, 2010), plankton (Varela *et al.*, 2006), fishes (Morales-Caselles *et al.*, 2006) and microalgae (Lobon *et al.*, 2008; Diez *et al.*, 2009) have been determined. The analysis of sediment contamination revealed that after four years, the toxicity had decreased, although the biota surrounding polluted sediments still suffered the toxic effects of pollutants (Morales-Caselles *et al.*, 2008). Also, consequences for human health like prolonged respiratory symptoms in spill clean-up workers were verified (Zock *et al.*, 2007). The increase of microbial activity as response against the presence of pollutants was detected in the water column (Martín-Gil *et al.*, 2004; Medina-Bellver *et al.*, 2005; Bode *et al.*, 2006), intertidal (Alonso-Gutiérrez *et al.*, 2008; Alonso-Gutiérrez *et al.*, 2009) and subtidal sediments (Acosta-González *et al.*, 2013).

5. Biodegradation of hydrocarbons

The presence of hydrocarbon in the biosphere since the primitive earth explains the evolution of microbial metabolic pathways to utilize these compounds as energy and carbon sources (Wilkes and Schwarzbauer, 2010). The ability of bacteria to utilize

hydrocarbons as sole carbon and energy source have been documented since the beginning of the past century (Zobell, 1946). The versatility of the catabolic enzymes allows microbes to degrade even xenobiotic compounds that share similar structures with naturally occurring aromatic compounds (Díaz, 2004). Bacteria have specialized to obtain energy from numerous compounds coupled to aerobic and anaerobic respirations and to fermentation (Sleat and Robinson, 1984). Hydrocarbon oxidation by aerobic bacteria has been extensively studied for decades (Atlas, 1981; Leahy and Colwell, 1990; Korda *et al.*, 1997; Van Hamme *et al.*, 2003; Cao *et al.*, 2009) but it is only in the past 25 years that the anaerobic hydrocarbon metabolism has attracted the general interest (Das and Chandran, 2011). The rate of anaerobic oxidation of hydrocarbons was initially considered to be very low and its ecological importance non-significant in most environments (Bailey *et al.*, 1973; Ward and Brock, 1978; Atlas, 1981). However, substantial hydrocarbon degradation in marine sediments or oil wells turned the attention to bacterial anaerobic metabolism (Rueter *et al.*, 1994; Coates *et al.*, 1997; Caldwell *et al.*, 1998). In oxygen depleted environments, anaerobes metabolize organic matter that otherwise would persist in nature and would constitute up to one-quarter of the global estimated biomass (Fuchs *et al.*, 2011).

5.1. Biodegradation of aromatic compounds

The metabolisms associated to aromatic degradation in nature are quite diverse. The degradative capacities are not restricted to a specific metabolism. Toluene, for example, can be metabolized aerobically (Zylstra *et al.*, 1988) or anaerobically by nitrate reducers (Schocher *et al.*, 1991), sulfate reducers (Rabus *et al.*, 1993), methanogens consortia (Grbic-Galic and Vogel, 1987; Winderl *et al.*, 2010) or phototrophic bacteria (Zengler *et al.*, 1999). Some species can metabolize aromatic compounds both aerobically and anaerobically (Harwood and Gibson, 1988).

Prokaryotes are considered as the only organisms in nature able to oxidize aromatic compounds through an anaerobic metabolism, and the process seems to be predominantly carry out by bacteria (Harwood and Gibson, 1997). In the early 20th century, *Bacillus hexacarbovorum* was the first aromatic oxidizing bacteria isolated in the laboratory on minimal mineral media, with toluene and xylene as sole carbon source and oxygen as electron acceptor (Störmer, 1908). Although the first report of complete benzoate degradation under strictly anoxic conditions dates back to the 1930s, detailed studies did not appear until the late 1970s (Williams and Evans, 1975; Evans, 1977). The first isolates of anaerobic aromatic degraders were sulfate reducers (Schnell *et al.*, 1989; Beller *et al.*, 1996) and denitrifying bacteria (Rabus and Widdel, 1995; Ball *et al.*, 1996; Coates *et al.*, 1996), although methanogenic consortia were reported earlier (Tarvin and Buswell, 1934; Clark

and Fina, 1952; Kuhn *et al.*, 1988). The first *Archaea* able to degrade an aromatic compound was isolated aerobically just a decade ago (Fu and Oriel, 1999). To date, *Ferroglobus placidus* is the only isolated *Archaea* capable of anaerobically oxidizing aromatic compounds (Tor and Lovley, 2001). Even though only limited species of *Archaea* able to oxidize aromatic compounds have been isolated, it is generally accepted that the predominant metabolism used by *Archaea* to oxidize these compounds is dependent on the presence of oxygen (Fuchs *et al.*, 2011).

The enzymatic pathways for the aerobic degradation of a wide variety of aromatic compounds have been elucidated, the genes characterized, their regulation, distribution and diversity have been analysed, so that the current cumulative knowledge allows a thorough and deep view of the processes involved (Harayama *et al.*, 1992; Gibson and Parales, 2000; Díaz, 2004). The enzymatic mechanism is much conserved and invariably involves mono- or di-oxygenases for the initial activation of the aromatic ring, converting the aromatic pollutants to dihydroxy aromatic intermediates. These are the substrate of intradiol or extradiol dioxygenases that cleave the aromatic ring using molecular oxygen (Harayama *et al.*, 1992). The first oxygenases were described for the aerobic oxidation of catechol and dimethyl-phenol, respectively (Hayaishi *et al.*, 1955; Mason *et al.*, 1955) and since then many new oxygenases have been found in eukaryotes and prokaryotes (Hayaishi and Nozaki, 1969; Mallick *et al.*, 2011).

As compared to aerobic degradation, a relatively higher diversity of the initial steps used by anaerobic microorganisms to degrade aromatic compounds has been reported. The anaerobic pathways always involve a preliminary activation step of the benzene ring, followed by the corresponding enzymatic cleavage. Anaerobic activation of a broad range of aromatic compounds converges into a few major central metabolites, which are further dearomatized and channeled to the central cell metabolism (Heider and Fuchs, 1997a; Schink *et al.*, 2000). The anaerobic activation reactions are rather slow as compared to the aerobic ones (Widdel *et al.*, 2006). The better studied mechanism is fumarate addition to a methyl group which takes place e.g. in the activation of toluene. The reaction is catalyzed by the glycyl radical enzyme benzylsuccinate synthase (Bss), encoded by the *bssDCA* genes, which mediates the addition of the methyl group of toluene to a fumarate co-substrate to produce (R)-benzylsuccinate (Seyfried *et al.*, 1994; Biegert *et al.*, 1996). The activation generates a stable protein-bound glycyl radical that is extremely unstable under oxic conditions (Selmer *et al.*, 2005). The reaction product is further converted in several steps to the final key metabolite benzoyl-CoA (Altenschmidt and Fuchs, 1991; Harwood *et al.*, 1998; Heider, 2007). This mechanism of activation is a general strategy for anaerobic aromatic activation of alkyl-aromatics like BTEX (Beller and Spormann, 1997; Krieger *et al.*, 1999; Annweiler *et al.*, 2000; Kniemeyer *et al.*, 2003), *m*- and *p*-cresols (Müller *et al.*, 1999; Müller *et al.*, 2001) or 2-methylnaphthalene compounds (Annweiler *et al.*, 2000; Musat *et al.*, 2008).

On the other hand, benzoate is directly transformed to benzoyl-CoA via a benzoate-CoA ligase encoded by the *bzdA* (*badA*) gene. The central metabolite benzoyl-CoA is dearomatized by the benzoyl-CoA reductase, followed by further reduction an opening of the ring through a series of reactions similar to β-oxidation (Heider and Fuchs, 1997b). The reactions involved in the complete degradation of aromatics through these pathways have been described with all kind of anaerobic respiratory metabolisms, and the gene clusters for the pathways have been identified. The *bzd* and *bad* clusters of *Azoarcus* sp. strain CIB and *Rhodopseudomonas palustris* respectively are the best studied genetic systems for benzoate degradation, while *Aromatoleum aromaticum* EbN1 *bss* cluster can be considered the model system for toluene degradation (Barragán *et al.*, 2004; Larimer *et al.*, 2004; Rabus, 2005). The regulatory mechanisms controlling expression of the pathways have also been elucidated in several model organisms (Barragán *et al.*, 2005; Peres and Harwood, 2006). The regulators controlling the anaerobic pathways described so far belong to the major regulatory protein families known in prokaryotes, such as NtrC, LysR, MarR, FNR and the two component regulatory systems, among others. Most of them exert and effector specific regulation where their activating or repressing activity is modulated by the presence of a substrate or and intermediate in the degradation pathway (Carmona *et al.*, 2009). Anaerobic enzymes are sensitive to O_2 and therefore not functional when oxic conditions are present.

A second activation mechanism, initially reported for polycyclic aromatic hydrocarbons (PAHs), was the direct carboxylation of naphthalene and phenanthrene (Zhang and Young, 1997). This mechanism was recently confirmed in SRB and sulfate reducing consortia and, as in the case of benzoate, it is followed by the direct ligation of a CoA thioester to the carboxyl group (DiDonato Jr. *et al.*, 2010). A third mechanism involves the oxygen-independent hydroxylation of C1-methylated carbon of ethylbenzene and *n*-propylbenzene (Johnson *et al.*, 2001; Kniemeyer and Heider, 2001). Finally, in NRB, some di-hydroxylated aromatics undergo oxygen-independent oxidation as main activation of the aromatic ring (Schink *et al.*, 2000).

5.2. Biodegradation of saturated hydrocarbons

The anaerobic degradation of aliphatic compounds was demonstrated earlier than the anaerobic oxidation of aromatics under anaerobic (methanogenic) conditions (Novelli and Zobell, 1944). The sulphide production in oil deposits and production plants led to the discovery of the direct oxidation of *n*-alkanes in crude oil (Rueter *et al.*, 1994). The anaerobic oxidation of methane (AOM) in marine sediments is carry out by closest relatives of methanogenic *Archaea* belonging to three cluster of *Euryarchaeota* (ANME-1 ,-2 and -3) (Knittel and Boetius, 2009). Linear, branched and cyclic alkanes have been shown to be

biodegradable by several sulfate reducing and nitrate reducing bacteria (Mbadinga *et al.*, 2011). The initial reaction of the anaerobic degradation pathway of *n*-alkanes is the addition to fumarate via a radical mechanism, in an analogue way to the anaerobic toluene activation step, and, as the homolytic cleavage of a terminal C–H bond in alkanes requires more energy than the homolytic cleavage of a subterminal C–H bond, the anaerobic activation mechanism takes place at the subterminal carbon where the addition offumarate is done (So and Young, 1999; Rabus *et al.*, 2001). Generally, *n*-alkanes degraders are not able to utilize aromatic compounds, and *vice versa*, although the co-metabolism of *n*-alkanes and toluene has been described recently (Rabus *et al.*, 2011).

6. Tools for studying marine polluted sediment ecology

Microbial ecology examines the diversity and activity of microbes in Earth's biosphere. Microbial ecology has become an integrated discipline where basic microbiology techniques, modern molecular biology approaches ("omics") and integrated geochemistry analysis converge to expand our view on the unknown microbial world (Konopka, 2009).

6.1. Culture dependent approaches

Although the isolation of bacteria gave the first perceptions of the diversity of microbial communities in the environment, conventional culturing methods have limited the isolation of many microorganisms, especially from nutrient-poor environments, reducing the inventory of microbial diversity to a few thousand of species. In oligotrophic (oceans waters, deep oceanic sediments and subsurface soil) or mesotrophic (coastal sediments and lakes) environments, less than 1% of the total viable bacterial could be enumerated or isolated by different culturing methods (Staley and Konopka, 1985). On the other hand, the efficiency of bacterial recovery could increase up to twofold in eutrophic environments (Staley *et al.*, 1982). This phenomena, called the "great plate count anomaly" (Staley and Konopka, 1985), has restricted studies on microbial ecology and underestimated in orders of magnitude the bacterial diversity (Amann *et al.*, 1995; Pedrós-Alió, 2006).

The cultivation technique dramatically influences the isolation of prokaryotes, resulting in the isolation of groups of bacteria specific to each method (Schloter *et al.*, 2000; Kopke *et al.*, 2005). General cultivation methods fail to reproduce fundamental characteristics of the isolation source (Vartoukian *et al.*, 2010). Even factors such as the agar type used in the isolation media could inhibit the growth of bacterial strains (Evans *et al.*, 1991; Tamaki *et al.*, 2009). A renaissance in culturing techniques led to the isolation of abundant phenotypes that are successively studied in the laboratory. Recovery of cells has

been enhanced up to one order of magnitude by using dilution culture methods (Button *et al.*, 1993). This technique allowed the isolation of *Candidatus Pelagibacter ubique* (Rappe *et al.*, 2002), the first cultivated representative from the SAR11 clade, the most abundant microbial group in seawater, a novel lineage of *Alphaproteobacteria* previously discovered using 16S rRNA molecular approach (Morris *et al.*, 2002). Non-conventional techniques based on natural simulation of the environment with diffusion chambers (Kaeberlein *et al.*, 2002; Bollmann *et al.*, 2010), stimulated growth by co-culturing other bacteria (Tanaka *et al.*, 2004) or single cell encapsulation (Zengler *et al.*, 2002) increased considerably the recovery and diversity of isolated microbes (Stewart, 2012).

The cultivation of anaerobic prokaryotes requires special procedures like oxygen exclusion, use of reducing agents or cultivation in anaerobic chambers (Widdel and Bak, 1992). Defined media and guidelines for cultivation of hydrocarbon degraders are well established (Widdel *et al.*, 2006; Widdel, 2010). Serial agar or liquid dilutions are the recommended methods for the isolation of pure cultures if the hydrocarbon is sufficiently soluble. In general it is more demanding to cultivate anaerobic than aerobic bacteria in the laboratory. In addition, cultivation of anaerobic hydrocarbon oxidizers is more arduous than cultivation of conventional anaerobes (Widdel, 2010). Special care should be taken with the bioavailability of the tested compound for microbial degradation or with a possible growth inhibition due to toxicity (Wick and Holliger, 2010). Most probable number (MPN) statistical estimates allow the quantification of viable physiological groups of interest through the combination of different carbon compounds with different electron acceptors. Oxidizer populations able to degrade different hydrocarbon compounds in marine sediments have been usually enumerated via MPN technique (Brakstad and Lødeng, 2005; Medina-Bellver *et al.*, 2005; Suárez-Suárez *et al.*, 2011; Acosta-González *et al.*, 2013).

6.2. Analysis of microbial diversity based on ribosomal markers

Despite all the efforts, the cultivation of microbes is still limited and less than 1% of marine bacteria can be isolated (Button *et al.*, 1993; Schut *et al.*, 1993; Amann *et al.*, 1995). Uncultivated microbial taxa dominate most natural ecosystems and play critical roles in element cycles (Pace, 1997; Stewart, 2012). In the course of the past two decades, the implementation and application of novel and powerful molecular biology techniques allowed the deep analysis of the microbial diversity and the description of the dynamics among communities directly in their environment.

The first classifications of prokaryotes were constructed based on phenotypic characteristics of isolated microbes. The use of the 16S rRNA SSU (ribosomal small subunit) gene sequence as phylogenetic molecular marker provided scientist an accessible tool to

classify prokaryotes that revolutionized microbial systematics (Fox *et al.*, 1977). In the early 90's, an accurate classification of organisms proposed three domains: *Archaea*, *Bacteria* (both constitute the "Prokaryotes") and *Eukarya* (Woese *et al.*, 1990). Furthermore, the 16S rRNA approach has the additional advantage of accessing the presumable microbial uncultivable fraction from different habitat, defining novel microbial lineages to conclude that microbial diversity was far more extensive than had ever been considered (Pace, 1997). In fact, only 29 (31 if "candidatus status" is considered) of the 59 proposed phyla have at least one cultivated representative strain according to the Formerly *List of Prokaryotic names with Standing in Nomenclature* – LPSN (http://www.bacterio.net/). It is estimated that 88% of all microbial isolates belong to only four bacterial phyla, the *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Rinke *et al.*, 2013). Until now, the isolated strains of prokaryotes have been classified in 10.954 (May 2013) distinct species. The contrasting number of classified prokaryotic species versus the total 16S rRNA entries deposited in the databases is displayed in Figure 4.

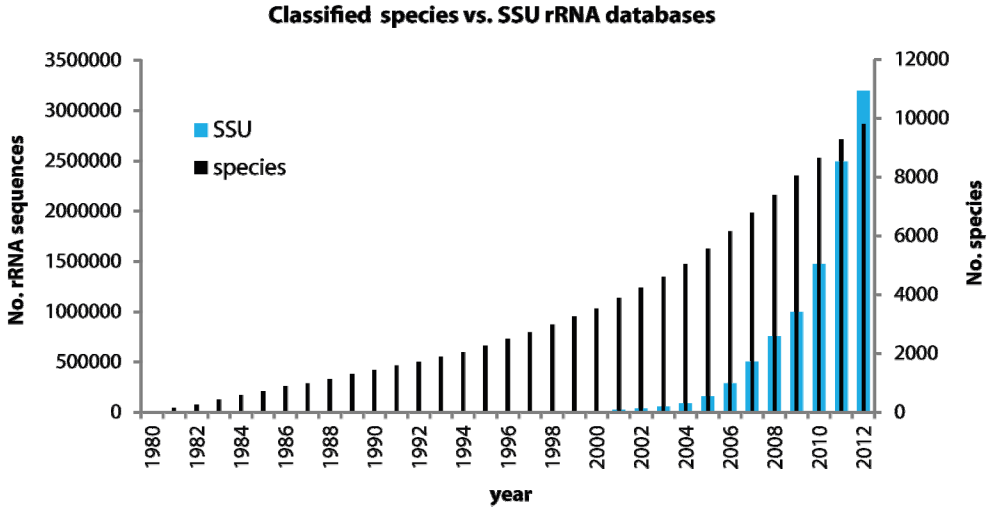

Figure 4. Number of recognized prokaryotic species versus SSU rRNA (16S small unit) entries found in repositories. Environmental 16S rRNA databases have grown enormously in the last years, especially if compared with the number of isolated and classified species during the same period of time. Data of recognized and classified prokaryotic species are taken from LPSN (http://www.bacterio.cict.fr/number.html). SSU rRNA data correspond to sequence entries to databases from uncultured organisms. For years 1986 to 2005, data were retrieve from the NCBI searching the keywords "16S AND uncultured". For 2006 to date, data correspond to SILVA "Parc" releases (http://www.arb-silva.de/projects/living-tree/). Figure modified from (Yarza *et al.*, 2008).

One of the limitations to describe prokaryotic diversity is to define the basic unit to measure biodiversity: the species. *There is probably no other concept in biology that has remained so consistently controversial as the species concept* (Mayr, 1982). Species concepts used in classical ecology for animals and other macro-organisms are meaningless for

prokaryotes. As a consequence different efforts to describe a pragmatic and useful species concept for prokaryotes have been proposed (Rossello-Mora and Amann, 2001). One has to distinguish between concept and definition. Concept is the idea of what a species may be, whereas the definition is the way to embrace units based on the available methods of observation (Rosselló-Móra, 2012). The concept of species explains that it is a group of organisms that are monophyletic and share enough genomic and phenotypic coherence to be included in the same unit. However, the definition of species for prokaryotes is based on measurable genetic and phenotypic parameters, and just applies to pure cultures. Currently, the modern species concept recommended for a prokaryotic entity is defined as a group of strains that share ≥98.5% similarity of their 16S rRNA gene and have a DNA reassociation value of at least 70% (Stackebrandt and Ebers, 2006) or an Average Nucleotide Identity (ANI) between genomes above 94% (Richter and Rosselló-Móra, 2009). However, a 97% of similarity level to the corresponding type species is the most used cut-off for a species circumscription (Stackebrandt and Goebel, 1994). With the advances in genomics and ultra-sequencing approaches the concept is being re-evaluated (Lan and Reeves, 2000; Gevers *et al.*, 2005). It has been formulated that due to its artificial nature, the species entity probably does not exist or cannot be recognized (Stackebrandt and Goebel, 1994; Torsvik *et al.*, 2002). However, metagenomic data reveal that detectable populations of bacteria hold the attributes expected for the species definition (Caro-Quintero and Konstantinidis, 2012). Different terms (OPU, OTU, ecotype) to describe uncultivable diversity have been used (Rosselló-Mora and López-López, 2008). Operational Taxonomic Unit (OTU) usually defined as "the number of distinct 16S ribosomal RNA sequences at a certain cut-off level of sequence diversity" (Achtman and Wagner, 2008) is a neutral term frequently used because it is well defined and thus useful for comparison between diversity studies (Hughes *et al.*, 2001). It should be taken into account that the number of copies of the 16S rRNA genes varies significantly among bacterial genomes. The relative abundance of 16S in environmental samples is therefore a subjective estimation that needs to be corrected (Kembel *et al.*, 2012).

The new high throughput sequencing technologies are accelerating the data generation at extraordinary scale with a relevant decrease of cost and accession to researchers (Caporaso *et al.*, 2010; Caporaso *et al.*, 2012). The Next Generation Sequencing (NGS) technologies could generate such quantity of information that just in a few years the produced data exceeded the total data compiled in three decades of Sanger sequencing application. For example, a recent extensive project (509 marine samples) generated by 454 pyrosequencing almost 9.6 million of reads, which clustered in approximately 120,000 $OTU_{0.03}$ (Zinger *et al.*, 2011). In addition, NGS has allowed the description of the rare microbial diversity (Sogin *et al.*, 2006). Thanks to NGS data, we will probably start to see in

the next years the linearization of the rarefaction curves and the estimated total number of prokaryotes will become more realistic.

6.3. Fluorescent *in situ* hybridization (FISH)

Fluorescent *in situ* hybridization allows the detection of individual cells by hybridization with fluorescently labeled nucleic acid probes (DeLong *et al.*, 1989; Amann *et al.*, 1990). This technique has been applied to directly identify cells in the original environmental samples by designing group-specific probes. The identification of phylogenetic groups could be determined at any taxonomic level if sequence information for the design of the suitable probe is available. The description of community structures and population dynamics is less biased by FISH techniques as compared to PCR based methods. The quantification of rRNA gene amplicons from certain populations in PCR-based cloning libraries is not precise due to the existence of multiple copies of rRNA operons in some prokaryotic genomes (Amann *et al.*, 1995; Tourova, 2003). Intrinsic PCR artifacts like selective sequence priming, chimeric products formation or clone efficiencies are avoided with FISH (Amann *et al.*, 1995). The truthful probe design guarantees that a specific hybridization over the target microbial communities is precise. Despite an accurate probe design and the use of optimized hybridization conditions, mismatches (Pernthaler *et al.*, 2002; Yeates *et al.*, 2003) and deficient probe coverage (Daims *et al.*, 1999) have been reported. The classical FISH focuses on 16S rRNA as target gene, but the technique is continuously improved through integration with other methods. For example, the MAR-FISH (Micro Auto Radiography - FISH), the Raman-FISH (Raman microspectroscopy - FISH) and the NanoSIMS (FISH coupled to nanometer-scale secondary ion mass spectrometry) simultaneously determines identity of microorganisms and up-take activity of a specific substrate (radioactive in the first case or a stable isotope in the last two (Wagner *et al.*, 2006; Huang *et al.*, 2007; Wagner, 2009); in addition, FISH combined with microelectrode measurements can better define microenvironments where activity and identity of particular microbial guilds among stratified microbial communities are determined (Kofoed *et al.*, 2012). The use of FISH has also been considered in the last years for the detection of functional genes (Moraru *et al.*, 2010; Wagner and Haider, 2012).

6.4. Functional gene analysis

Catabolic genes from anaerobic degradation pathways are used as biomarkers of hydrocarbon metabolisms in polluted sites. Pathways from anaerobic degraders of BTEX, *n*-alkanes and 2-methylnapthalene have been described and the detection of these genes has been possible due to the design of degenerated probes based on the homology of the

respective amino acid sequences. The most useful biomarker genes are those involved the activation step of the particular pathway. Probes that amplified partial sequences of benzylsuccinate synthase (*bssA*) have been extensively tested (Botton *et al.*, 2007; Washer and Edwards, 2007; Winderl *et al.*, 2007). The alkylsuccinate synthase gene (*assA*) involved in the activation of *n*-alkanes was concurrently described in the NRB *Azoarcus* sp. HxN1 (Grundmann *et al.*, 2008) and the SRB *Desulfatibacillum alkenivorans* AK-01 (Callaghan *et al.*, 2008). Sequences of naphthyl-methyl succinate synthase (*nmsA*), a homologous of BssA in the 2-methylnapthalene degradation pathway, were detected in the genomes of the SRB strains *naphS2*, *naphS3* and *naphS6* and in the SR enrichment N47 (DiDonato Jr. *et al.*, 2010; Selesi *et al.*, 2010). Recently, *assA* (Callaghan *et al.*, 2010) and *nmsA* (von Netzer *et al.*, 2012) probes detected the presence of these genes in polluted environments. Genes like *bamA* from pathways of central intermediates are also used as indicators of the metabolic potential for anaerobic hydrocarbon degradation (Li *et al.*, 2012). A number of aerobic hydrocarbon degradation genes have also been used for the assessment of alkanes, naphthalene and other aromatic hydrocarbon pathways in contaminated sites, including superficial and intertidal marine sediments (Andreoni and Gianfreda, 2007; Kuhn *et al.*, 2009; Marcos *et al.*, 2009; Wasmund *et al.*, 2009; Mulet *et al.*, 2011). Functional genes from dominant respiratory pathways have been used to describe the relevance of each particular metabolism in the sediments. Denitrifying bacteria have been monitored by amplification of the genes for nitrite reductase (*nirS*, *nirK*) and nitrous oxide reductase (*nosZ*) (Throbäck *et al.*, 2004; Tiquia *et al.*, 2006; Mills *et al.*, 2008), while SRB were targeted using genes for the sulfate reduction pathway, such as APS reductase (*Apr*) and dissimilatory and sulfite reductase (*Dsr*) (Meyer and Kuever, 2007; Basen *et al.*, 2011).

6.5. Metagenomic analysis

The implementation of NGS approaches opens an opportunity to explore the unrevealed microbial world from many diverse environments by ultrasequencing of metagenomic DNA. The metagenome represents the collection of genomes belonging to the entire microbiota found in every environment. Metagenomic analysis focuses on the potential functionality of the microbial communities (genome structure and gene content) rather than on their identity (diversity and community composition), allowing the inference of the features relevant for the functioning of each particular environment (Tringe *et al.*, 2005). Initially, large fragments of DNA recovered from natural environments were cloned to access the metagenomes belonging to the uncultured subset of organisms (Handelsman *et al.*, 1998; Rondon *et al.*, 2000), but now the advances of ultrasequencing facilities unlock the opportunity to directly pyrosequence the metagenomic DNA. At present, the majority of metagenomic surveys are focused in describe microbial diversity, in part due to the

complication that represents the analysis of the derived data from whole metagenomic sequencing (Fuhrman, 2012; Teeling and Glöckner, 2012). Online platforms for the deposit, management, comparison and analysis of metagenomic data are available and the most popular include MG-RAST (Meyer *et al.*, 2008), IMG/M (Markowitz *et al.*, 2012) and CAMERA (Sun *et al.*, 2011).

Since pyrosequencing based technologies development (Margulies *et al.*, 2005), the platform has vastly employed and use for analysis of genetic variation associated with whole genome sequencing and multilocus sequence typing, quantitative analysis of copy number variation, allele frequency and loss of heterozygosity, identification of heterozygous mutations and genotyping and large-scale analysis of bacterial populations (Novais and Thorstenson, 2011). Insights on the mechanisms displayed by microorganism in biodegradation and bioremediation of environmental pollutants are potentially gained by "omics" application (Desai *et al.*, 2010). Novel organization of aromatic degradation pathway genes or diversity, abundance and prevalence of genes and pathways has been described using metagenomic surveys (Brennerova *et al.*, 2009; Suenaga *et al.*, 2009; Fang *et al.*, 2012). Studies of marine metagenomes from polluted sites just started to be analyzed recently (Andreote *et al.*, 2012; He *et al.*, 2013)

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II. Objectives

The subtidal sediments from the north-west coast of Spain heavily affected by the *Prestige* oil spill had previously been poorly characterized. The goal of this work was to determine the potential response of autochthonous microbiota (anaerobic bacterial communities) against the presence of high levels of hydrocarbon pollution in marine sediments from the Cíes Island Natural Park (Galicia, Spain). We have applied culture-dependent and molecular culture-independent techniques to characterize the bacterial diversity by classical and massive sequencing approaches, with the following specific objectives:

i) To characterize the *in situ* response of indigenous bacterial communities to oil contamination in coastal sediments affected by the *Prestige* oil spill.

ii) To determine if microbial diversity patterns along coastal polluted sediments could be inferred by comparison of available pyrosequencing data.

iii) To determine the diversity of functional genes associated to anaerobic degradation pathways of hydrocarbon compounds in polluted sediments.

iv) To compare the metagenomes from sediment microcosms subjected to different contamination treatments in the laboratory.

III. Experimental Procedures

1. Sampling site

Two neighboring beaches in the eastern shore of the Cíes Island (Atlantic Isle National Park, Galicia, Spain) were chosen for sediment sampling: Rodas beach (42°13'31N, 8°53'59W) and Figueiras beach (42°13'55N, 8°53'50W) (Figure 1.1, Chapter I). In both sites, sediment cores were collected by scuba-divers at nine meters depth with one meter-long cores. The samples were kept at 4ºC until processed. All analyses further described were carried out independently for each collected core as indicated in Table 1.

Table 1. Description of samples used during this study.

	Year	Location	Sample	name	Core depth *	notes	Chapter
First	2004	Rodas beach	RI	RI	1-20		III
		Figueiras beach	FI	FI- OX	2-5[1]		II, III
				FI-TR	11-14[2]		II, III
				FI-AN	32-35[3]		II, III
				FI-PET	1-15[4]		II, III
Second	2007	Rodas beach	RII	RII-OX	2-5[1]		II
				RII-TR	9-13[2]		II
				RII-AN	23-27[4]		II
		Figueiras beach	FII	FII-OX	2-5[1]		II
				FII-TR	12-15[2]		II
				FII-AN	32-35[3]		II
			FII	FII-0		Time zero	IV
				FI-mCON	4-16[2]	Microcosm, 8 months (18ºC)	
				FII-mNAP			
				FII-mOIL			
		Mallorca	M	M-CON	2-16[2]	Microcosm, 4 months (22ºC).	III
				M-NAP			
				M-OIL			

* Depth in centimeters

[1] Nitrogenous zone (probably oxygen influenced), white sand; [2] Transition zone (nitrogenous-sulfidic zone), mixed white-grey sand; [3] Sulfidic zone, grey sand with sulphide odor; [4] Gray sand mixed with residuary petroleum.

1.1. First sampling campaign. The first sampling campaign was carried out on May 25th, 2004. The selected site at Figueiras beach was heavily contaminated and still presented traces of crude oil over the seafloor during the sampling campaign time. Triplicate cores collected at each site (cores FI) were analyzed in each case. An additional 20 cm core was collected in a petroleum spot visualized by the diving team in this site (FI-PET). An apparent pristine beach close to Figueiras was sampled for comparison (RI). Each core was fractionated every three centimeters and used for immediate analysis (MPN anf FISH), or stored at -80°C for future analysis (DNA extraction).

1.2. Second sampling. On June 27th 2007 a similar campaign was carried out (cores FII), and was again extended to Rodas beach (cores RII), where at this time a heavily contaminated spot was detected in the deepest part of the core. This site was protected by a pear and resulted less contaminated during the spill in 2002, and was immediately cleaned by diving teams.

1.3. Microcosms. Microcosm experiments from Mallorca for *bss*A analysis (Chapter III), were set up in June 2007 with sediments collected at Alcúdia Bay (Mallorca, Spain) within a pristine seabed. Additionally to FII samples, three cores collected at Figueiras were transported to the laboratory to set-up experiments used in the metagenomic analysis (Chapter IV). *Microcosms setup:* The cores were artificially contaminated with naphthalene (M-NAPH and FII-mNAP) or crude oil (M-OIL and FII-mOIL) as follows: naphthalene was provided by inserting a perforated 10 ml propylene pipette filled with naphthalene crystals in the originally collected core; four lanes of 1 mm holes distributed equidistantly along the pipette to allow for diffusion. *Prestige*'s crude oil was introduced to another core after impregnation onto a nylon stick.

2. Physico-chemical features

2.1. Geochemical parameters. All determinations were carried out in duplicate for each sample, as follows: **i)** sediment density (ρ_{bulk}) was calculated by weighting a known volume of each fresh sample (20 ml); **ii)** water content was determined after drying sediment at 100°C during 24 hours and calculating weight difference; **iii)** sediment grain size was determined from dried sand from each sample that was sieved through sieves of 2000, 1000, 710, 630, 500, 400, 315, 250, 177, 100 and 63 µm opening. Each sieve's fraction was weighted to determine the portion of grain size composition over total and the software GRADISTAT was used to calculate the sorting coefficient (Blott and Pye, 2001); **iv)** porosity was calculated with the formula: $Ø=1 - (\rho_{bulk}/\rho_{particle})$ assuming grain density ($\rho_{particle}$) to be approximately 2.69 g cm^{-1} (Libes, 2009); **v)** Organic matter was determined by a Loss-on-ignition method according to (Erftemeijer and Koch, 2001) with some modifications: 10 ml of sediment were dried at 105°C for 16 hours, combusted at 500°C for 5 hours and, once cooled in a desiccator, weighted to determine the loss. All glass material was previously treated at 450°C in a muffle oven to eliminate organic contaminants; **vi)** Iron was determined in duplicate samples using inductively coupled plasma-optical emission spectrometry with a Varian ICP-OES 720-ES. Approximately 0.5 g of sediment was extracted with 12 ml of extraction solution (7.6 M HCl/2.7 M HNO_3). Yttrium was used as internal standard; **vii)** For the analysis of nitrate and sulfate, interstitial water was extracted from 5 grams of sediment with two 5 ml aliquots of deionized water and quantification of nitrate,

nitrite and sulfate was done by ion chromatography (IC) using a Metrohm 761 Compact Ion Chromatograph with a Metrosep A sup 4-250 column with chemical suppression (50 mM H_2SO_4). Eluent solution was composed of 1.7 mM $NaHCO_3$ and 1.8 mM $NaCO_3$. The detection limit for ions was 0.5 ppm and the associated error was 5%.

2.2. Hydrocarbon determination. Triplicate frozen sediment samples from each core were used to determine aliphatic and aromatic hydrocarbon compounds. Before the extraction, a volume of 20 µl Deuterated Mix 37 (manufactured by Dr. Ehrenstorfer) and 10 µl of 1000 ppm solution of 5a-cholestane (Aldrich) were added as internal standards to quantify hydrocarbons. Aliquots of 2 ml of sediment were dissolved in 4 ml of hexane/acetone mixture (2:1), the mixture was shacked vigorously for 1 minute and sonicated for 3 minutes. An equal volume of salt saturated solution (40% NaCl in water) was added to each tube. Once the phases separated, the supernatant was percolated through a BOND ELUT TPH column (Varian) that included both anhydrous Na_2SO_4 and Si-CN-U matrix previously hydrated with 2 ml of hexane. The aliphatic fraction was first eluted by gravity with 4 ml of pure hexane. Then, positive pressure was applied to completely remove the solvent, and the aromatic fraction was eluted with 6 ml of dichloromethane. Both fractions were concentrated by gentle solvent evaporation under a nitrogen flow until dryness, resuspended in 0.5 ml of the corresponding solvent and kept at -20°C. The analysis was carried out with a Varian 450-GC attached to a 240-IT Mass Spectrometer, equipped with a programmed temperature vaporization/split-splitless injector and a CTC-GCpal auto-sampler. Injection mode was: 1µl splitless (splitless time 1 min), carrier gas He at 1ml/min, injector temperature 300°C, ramp 50°C, 5 min >>150 (15°C/min) >> 325°C (6°C/min), 12 min, capillary column FactorFour VF5ms 30 m-0.25 mm-0.25 µm, transfer line temperature 300°C, MS in Full Scan mode 50-490m/z, EI ionization, 4 min of solvent delay, Software MS Work Station. Identification was performed using DRH-008S-R2 alkane mix (C8-C40, pristane and phytane) standard from AccuStandard Inc.

2.3. BTEX analysis. A static headspace gas chromatographer mass spectrometer (Varian 450GC 240MS with CTC CombiPal autosampler) was used for the determination of BTEX in contaminated sediments (Shin, 2012). Duplicate samples of 2 g of defrosted sediment were placed in 10 ml vials. Fluorobenzene (50 ng) was added to each vial as internal standard. The vial was incubated in a heating block at 70°C for 10 min. A volume of 1 ml was injected at 250°C in split mode (1:20) at a flow rate of 1 ml/min. The temperature was held at 50°C for 1min, then increased to 95°C at a rate of 20°C/min, held for 2 min, then increased to 150°C at a rate of 25°C/min and held for 0.6 min. The MS acquisition was as follows: 0.5 min delay, scan range of 45m/z to 200m/z until 3.4 minutes and 80m/z to 200m/z until 8 minutes. Identification and quantification was performed in single ion

monitoring (SIM) mode using the most abundant ions. The detection limit of the assays was 1-5 ppb for the standard analytes.

2.4. Metabolic biomarkers. Triplicate frozen sediment samples were used to determine metabolic biomarkers from characterized anaerobic degradation pathways of BTEX (Beller *et al.*, 1995), alkanes and naphthalene (Gieg and Suflita, 2002; Oka *et al.*, 2011). Four ml of ethyl acetate were added to 2 g of sediment, mixed by vortexing and once the phases separated, the supernatant was collected and completely dried. Then, the extract was derivatized by adding one volume of ethyl acetate and one volume of BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) to inject in the GC-MS. A volume of 0.5 ml was injected in split mode and the MS acquisition was between 100 and 300 m/z.

Gas chromatography–mass spectrometry (GC-MS), ion chromatography (IC), and Inductively Coupled Plasma (ICP) analysis were done at the Scientific Instrumentation Unit in the Estación Experimental del Zaidín (CSIC) and Instituto de Ciencias de la Tierra (CSIC).

3. Most probable number (MPN) enumeration of bacteria.

For the cultivation of bacteria we used an artificial seawater minimal medium (SMM) described previously (Widdel and Bak, 1992) that was supplemented with different electron acceptors for sulfate-reducing bacteria (SRB), nitrate reducing bacteria (NRB) and iron-reducing bacteria (IRB) at final concentrations of 10 mM ($NaSO_4$), 5 mM ($NaNO_3$) and 20 mM (ferrihydrite), respectively, prepared as previously described (Lovley and Phillips, 1986). After autoclaving, the medium was cooled under an atmosphere of N_2/CO_2 (90:10, vol/vol) and supplemented with 1 ml of a vitamin solution (Widdel and Bak, 1992), 0.5 ml of trace element solution SL10 (Pfenning and Trüper, 1992) and 30 ml of a 0.5 M CO_2-saturated sodium bicarbonate solution per liter of media. Table 2 indicates the composition of SMM medium, SL 10 and vitamin solution. The SRB media was supplemented with sodium sulphide (0.5 M $Na_2S_2{\cdot}6H_2O$) at a final concentration of 1 mM. The pH of the medium was adjusted to 7.2-7.4 by addition of sterile 2 N HCl (if necessary). Oxygen-free 12 ml vials (LABCO LIMITED, UK) were filled with 9 ml of each media. An oxidized iron nail washed in sterile 5% HCl and rinsed with sterile milli-Q water was autoclaved and added to each tube of MPN media for SRB in order to detect the growth of bacteria as the formation of a black FeS precipitate (Cifuentes *et al.*, 2003). Growth of NRB was determined by measuring the nitrite produced from nitrate respiration according to the colorimetric method (Snell and Snell, 1951). Growth of IRB was considered positive when ferrihydrite turned from brown to black, as indication of iron reduction. This usually required more than one-year incubation. Microbial growth was tested in the following carbon sources at a final concentration of 2 mM: benzene, toluene, lactate and acetate, which were added directly in the medium

during the flow of N_2/CO_2; for media with naphthalene and anthracene, the carbon source was previously distributed in the tubes from stock solutions dissolved in pure hexane, which was evaporated before delivering the media.

Table 2. SMM, SL10 and Vitamins components.

	Compound	Quantity (per liter)	Molarity (final)
SMM	KH_2PO_4	0.2 g	1.47 mM
	NH_4Cl	0.25 g	4.7 mM
	NaCl	20 g	342 mM
	$MgCl_2 \cdot 6H_2O$	3 g	14.7 mM
	KCl	0.5 g	6.7 mM
	$CaCl_2 \cdot 2H_2O$	0.15 g	1 mM
SL10	HCl (25%, 7.7N)	10 ml	
	EDTA-di-Na	-	
	FeCl2·4H2O	1.5 g	
	ZnCl2	70 mg	
	MnCl2·4H2O	100 mg	
	H3BO3	6 mg	
	CoCl2·6H2O	190 mg	
	CuCl2·2H2O	2 mg	
	NiCl2·6H2O	24 mg	
	Na2MoO4·2H2O	36 mg	
Vitamins	4-Aminobezoic acid	40 mg	
	D (+)-Biotin	10 mg	
	Nicotinic acid	100 mg	
	Ca- D(+)-pantothenate	50 mg	
	Pyridoxamine dihydrochloride	150 mg	
	Thiaminium dichloride	100 mg	
	Cyanocobalamine	50 mg	

SL 10 and vitamins are filter sterilized.

For aerobe MPN, hydrocarbon sources dissolved in *n*-hexane were added to polypropylene microtitre 96-well sterile plates as described previously (Johnsen *et al*., 2002). Each well was filled with 20µl of 5 mg ml^{-1} stock solutions of naphthalene, phenanthrene, pyrene or undecanel; for anthracene, 80 µl of saturated 5 mg ml^{-1} stock solution (also dissolved in *n*-hexane) was used. Plates contained only *n*-hexane were used as negative control. The solvent was evaporated at room temperature and plates were kept at 4°C until use. Each well was filled with 270 µl of autoclave-sterilized artificial seawater medium (40g SIGMA Sea Salts, 250 mg $(NH_4)_2SO_4$, 25 mg $FeSO_4 \cdot 7H_2O$ per liter) supplemented with filter-sterilized K_2HPO_4 pH 6.8 at a final concentration of 10 mM. Microtitre plates were covered with gas-permeable sealing membrane (Breathe-Easy™, Diversified Biotech) and covered with autoclaved filter paper moistened with sterile milli-Q water to minimize evaporation and edge effects under the plastic lid.

Bottles with 90 ml of artificial seawater minimal medium were used to dilute 10 cm^3 of each selected fresh sediment sample keeping anaerobic conditions through constant

N_2/CO_2 flow. For each sample, triplicates (anaerobes) and quintuplicates (aerobes) tubes were inoculated with 1 ml and 30 µl, respectively, of the corresponding dilution in tenfold (anaerobes) and fivefold (aerobes) serial dilutions for MPN calculation. Both tubes and plates were incubated at 16°C in the dark and growth was checked during one year. Counts are the result of combining counts from three replicate cores per sample within the 95% confidence interval.

4. Molecular Biology approaches

4.1. DAPI counts and fluorescent in situ hybridization (FISH). Approximately 20 cm^3 of sediment from each sample (each specific horizon of each core) were fixed for 6 hours at 4°C with one volume of 4% formaldehyde solution (wt/vol) in 4 x phosphate buffered saline (PBS). Samples were treated by mild ultrasonication in a water bath for 10 minutes and different supernatant volumes were filtered on white 0.2 µm polycarbonate membranes (Ø47 mm) by applying reduced pressure (<150 mm Hg). An additional wash was done with 1 x PBS, membranes were air dried and stored at -20°C.

Cy3 labeled probe mix EUB338 targeting Bacteria (Amann *et al*., 1990; Daims *et al*., 1999) and probes GAM42a targeting *Gammaproteobacteria*, DSS658 targeting *Desulfobacteraceae*, DSV698 targeting *Desulfovibrionaceae* (Manz *et al*., 1998), DSB985 targeting *Desulfobacter*, CF339a targeting the phylum *Bacteroidetes* (Manz et al., 1996) and ARCH915 targeting *Archaea* were used for the analysis at concentrations of 5 ng/ml. Each filter was cut into several sections, placed on glass slides and covered with 16 µl of prewarmed (46°C) hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS), 35% of formamide (except DSS658, which needed 60%) and 2 µl of the respective probe. *In situ* hybridizations were incubated at 46°C for 2 hours. Hybridization mixture was removed, and the slides were rinsed in prewarmed (48°C) washing buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 70 mM NaCl (4 mM for DSS658) and 0.01% SDS. Incubation was performed at 48°C for 15 min. After drying, filter sections were stained with 20 µl of DAPI (10 µg ml^{-1}) for 10 minutes in the darkness, at room temperature. The slides were manually enumerated under an epifluorescence microscope (Zeiss) under red light for detection of CY3-labeled eubacteria and under UV light for DAPI-stained cells at 1250 x magnification.

4.2. Nucleic acids extraction

4.2.1. KIT extraction. Frozen sediment samples from each core horizon (2 grams each) were used for direct extraction of DNA with the UltraClean Soil DNA Kit (MoBio Laboratories, USA) following procedures described by the manufacturer except in the last step, where the elution with solution S6 was repeated twice. The integrity and yield of

extracted nucleic acids were checked by standard agarose gel electrophoresis and ethidium bromide staining. Nucleic acids were quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). Equal amounts of DNA from triplicate cores were mixed and used in PCR reactions.

4.2.2. SDS-based extraction. An SDS-based DNA extraction method commonly described in literature was use with some modifications: approximately 5 g of sediment were mixed with 12.5 ml of high-salt extraction buffer (1.5 M NaCl, 100 mM Tris-HCl , 100 mM sodium EDTA , 100 mM sodium phosphate (Aguilo-Ferretjans *et al.*) and 1% CTAB) containing 125 µl of proteinase K (12.5 mg/ml) and lysozyme (100 mg/ml) and incubated for 30 min at 37°C with constant horizontal shaking at 200 rpm. Then, 1.5 ml of SDS (20% w/v) was added and tubes were incubated for 2 hours at 65°C. Extracts were centrifuged at 6000 g for 20 minutes and supernatants were transferred to a new tube. Extraction was repeated once using 7.5 ml of extraction buffer with 75 µl of proteinase K and lysozyme prepared as above. Supernatants of both extractions were combined and treated with one volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v), then vortexed and centrifuged at 6000 g for 10 min. The supernatant was extracted again with chloroform-isoamylalcohol (24:1, v/v). Nucleic acids were precipitated from the aqueous phase after addition of NaCl to a final concentration of 0.2 M and isopropanol to 0.7 volumes of. The pellet was washed with 70% ethanol and resuspended in 100 µl of milli-Q water.

4.2.3. Extraction of metagenomic DNA. The metagenomic DNA was extracted with the same SDS-based protocol described above using 10 g of sediment. Additional purifications steps were performed to improve the pyrosequencing run. Once the DNA was resuspended in water, columns for DNA elution from the UltraClean Soil DNA Kit were used, as recommended by the manufacturer: 2 volumes of S3 solution (200 µl) were added and vortexed for 5 seconds. Then, the entire volume was loaded in a spin filter and centrifuged at 10,000 xg for 1 minute. Filter was cleaned with 300 µl of S4 solution and centrifuged two times as describe before. The DNA was eluted from the filter with 30 µl of DNA-free PCR grade water and stored at -20ºC.

4.3. 16S analysis by PCR

4.3.1 PCR amplification and gene libraries constructions. PCR amplification of the complete 16S rRNA gene was performed with the bacterial universal primers GM3F and GM4R (Muyzer *et al.*, 1995). Approximately 10-20 ng of DNA (combined triplicates) were used as template for PCR in 50 µl reactions containing 1 x PCR Buffer (Sigma) supplemented with 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 mM of each primer (synthetized by Genosys, Sigma)

and 1U of Tag DNA Polymerase (Sigma). To determine optimal PCR cycles and annealing temperatures for the primers, gradient PCR was performed with annealing temperatures between 42°C and 52°C (T-Gradient, BIOMETRA). PCR conditions comprised initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 46°C for 20 s, and extension at 72°C for 1.5 min, and a final extension step at 72°C for 5 min. The PCR fragments were eluted from standard agarose gels after electrophoresis (1% agarose, 100V, 1 h) and purified using the Gel Extraction Kit (Qiagen). Amplicons were cloned in pGEM-T (Promega) according to the manufacturer instructions. For each sample, 146 positive clones were selected for sequence analysis. *Big Dye* Terminator Cycle *Sequencing* was performed at SECUGEN (Madrid).

4.3.2 Pyrosequencing of 16S rRNA gene. Duplicate samples from independent DNA extractions were sequenced by pyrosequencing. Primers were based on the 6F and 532R oligonucleotide sequences targeting V1-V3 region of the 16S rRNA gene were used. To distinguish among samples, primers carried a 10-bp barcoded (Table 3). Approximately 25 ng of DNA from each replicate were used as template for PCR. Several reactions of 50 µl containing 1 x PCR Buffer (Sigma) supplemented with 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 mM of each primer (Roche) and 1U of High Fidelity Tag DNA Polymerase (Fisher) were run in parallel and mixed before the analysis. PCR conditions comprised initial denaturation at 94°C for 5 min, 28 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 1.5 min, and a final extension step at 72°C for 5 min. The PCR fragments were purified using the Gel Extraction Kit (Qiagen). The samples were run in one quarter PicoTiterPlate by unidirectional sequencing (from Adaptor A side) on the Roche GS FLX (Titanium Sequencing Kit XLR70) at CITIUS (Sevilla, Spain).

4.4. Amplification of benzylsuccinate synthase alpha-subunit gene (*bssA*) for functional gene analysis. Primers described previously (Winderl *et al.*, 2007) were used to amplify the alpha-subunit of benzylsuccinate synthase gene (*bssA*). A gradient PCR was performed with annealing temperatures ranging from 45°C to 60°C for all primer combinations. After 32 cycles, primers 7772f (5′ GACATGACCGACGCSATYCT 3′) and 8542r (5′ TCGTCGTCRTTGCCCCAYTT 3′) successfully amplified partial *bssA* genes of expected size and were chosen for the gene library preparations**.** DNA from *Thauera aromatica* K172 was used as positive control of the PCR. As unspecific amplicons were also obtained with these primers, the amplified fragment with the expected size was separated onto a 15 cm-long agarose gel (1.5% w/v) over 2 hours and extracted with the QIAGEN Gel extraction Kit according to the manufacturer instructions. Purified fragments were cloned in pGEM-T vector (Promega) and 70 positive clones were selected for sequencing.

Table 3. Segments constituting the oligonucleotides used for pyrosequencing analysis.

MID#	adaptor	key	MID	primer	sample
86	cgtatcgcctccctcgcgcca	tcag	cgagtcatcgt	tcagagtttgatcctggctcag	FII-mCON1
	ctatgcgccttgccagcccgc	tcag	cgagtcatcgt	caccgcggckgctggcac	
87	cgtatcgcctccctcgcgcca	tcag	cgatcgtatat	tcagagtttgatcctggctcag	FII-mCON2
	ctatgcgccttgccagcccgc	tcag	cgatcgtatat	caccgcggckgctggcac	
90	cgtatcgcctccctcgcgcca	tcag	cgcgctatact	tcagagtttgatcctggctcag	FII-mNAP1
	ctatgcgccttgccagcccgc	tcag	cgcgctatact	caccgcggckgctggcac	
91	cgtatcgcctccctcgcgcca	tcag	cgtacagatat	tcagagtttgatcctggctcag	FII-mNAP2
	ctatgcgccttgccagcccgc	tcag	cgtacagatat	caccgcggckgctggcac	
92	cgtatcgcctccctcgcgcca	tcag	cgtagctctct	tcagagtttgatcctggctcag	FII-mOIL1
	ctatgcgccttgccagcccgc	tcag	cgtagctctct	caccgcggckgctggcac	
93	cgtatcgcctccctcgcgcca	tcag	cgtatagtgct	tcagagtttgatcctggctcag	FII-mOIL2
	ctatgcgccttgccagcccgc	tcag	cgtatagtgct	caccgcggckgctggcac	
128	cgtatcgcctccctcgcgcca	tcag	cactcgcacg	tcagagtttgatcctggctcag	FI-PET1
	ctatgcgccttgccagcccgc	tcag	cactcgcacg	caccgcggckgctggcac	
129	cgtatcgcctccctcgcgcca	tcag	cagacgtctg	tcagagtttgatcctggctcag	FI-PET2
	ctatgcgccttgccagcccgc	tcag	cagacgtctg	caccgcggckgctggcac	
132	cgtatcgcctccctcgcgcca	tcag	cgatctgtcg	tcagagtttgatcctggctcag	FI-TR1
	ctatgcgccttgccagcccgc	tcag	cgatctgtcg	caccgcggckgctggcac	
133	cgtatcgcctccctcgcgcca	tcag	cgcgtgctag	tcagagtttgatcctggctcag	FI-TR2
	ctatgcgccttgccagcccgc	tcag	cgcgtgctag	caccgcggckgctggcac	
134	cgtatcgcctccctcgcgcca	tcag	cgctcgagtg	tcagagtttgatcctggctcag	FI-AN1
	ctatgcgccttgccagcccgc	tcag	cgctcgagtg	caccgcggckgctggcac	
1	cgtatcgcctccctcgcgcca	tcag	acgagtgcgt	tcagagtttgatcctggctcag	FI-AN2
	ctatgcgccttgccagcccgc	tcag	acgagtgcgt	caccgcggckgctggcac	
2	cgtatcgcctccctcgcgcca	tcag	acgctcgaca	tcagagtttgatcctggctcag	RI1
	ctatgcgccttgccagcccgc	tcag	acgctcgaca	caccgcggckgctggcac	
3	cgtatcgcctccctcgcgcca	tcag	agacgcactc	tcagagtttgatcctggctcag	RI2
	ctatgcgccttgccagcccgc	tcag	agacgcactc	caccgcggckgctggcac	

4.5. Pyrosequencing of metagenomic DNA. Three standard genomic libraries for single read sequencing (fragmentation of DNA and adapter ligation) were constructed at GATC biotech (Germany). The samples were run in one half PicoTiterPlate by unidirectional sequencing (from Adaptor A side) on the Roche GS FLX (Titanium Sequencing Kit XLR70):

4.6. Accession numbers. Sequences from 16S rRNA (Chapter I) were deposited with accession numbers JF343979 to JF344708 and JQ579650 to JQ580525. Sequences from *bssA* (Chapter III) were deposited with accession numbers KC463912 to KC464320.

5. Bioinformatics analysis

5.1 Phylogenetic and statistical analyses of 16S rRNA full-length gene. Chromatograms of sequences were checked and edited with the programs Chromas (Technelysium) and DNA Baser (Heracle Biosoft). Gene libraries were screened for chimeras with Mallard and Pintail software (Ashelford *et al.*, 2006). Phylogenetic reconstructions

based on 16S rRNA gene sequences were done with the ARB package (Ludwig *et al.*, 2004) using the online SINA alignment service and Silva database version SSU Ref 108. OTU's assignation, rarefaction curves, Venn diagrams and diversity index were calculated with MOTHUR version 1.23 (Schloss *et al.*, 2009). OTUs were classified at ≥97% sequence similarity level. We performed PCA hierarchical clustering analyses with the UniFrac web interface (Lozupone *et al.*, 2007) using a NJ tree generated with ARB software to determine dissimilarities among communities form different samples. X-Fig and Adobe Illustrator CS5.5 were used to edit image files when necessary.

5.2. Bioinformatics analysis of 16S rRNA amplicons. We searched in the databases for the available data from microbial diversity studies of polluted and unpolluted subtidal coastal sediments. As our analysis was limited by the need to compare the same pyro-sequenced region (V1-V3), only a few datasets were found as compared to datasets available for other regions (V4-V6). SRA accession numbers are: subtidal sediments (9 meters depth) from Fundy Bay (Atlantic Canada), SRA065197 to SRA065205; King Island, Antartic Ocean, SRA061199; affected sediments from DWH (deep water horizon spill) in Gulf of Mexico (USA), SRA056124. We used SRA Toolkit (May 9 2013, version 2.3.2-5 release for Ubuntu, NCBI) to extract the .sff or .fastq files from the selected .sra archives. Mothur v. 1.31 (Schloss *et al.*, 2009) was used to convert .sff or .fastq files to .fasta and .qual files, as required to perform the analysis with Qiime (Caporaso *et al.*, 2010). Qiime version 1.7 was used in a Virtualbox (version 4.2.16) for Ubuntu. Briefly, parameters commands to run the program were:

split_libraries.py (split sequences):
split_libraries.py –m map_file.txt -f file1_name.fasta,file2_name.fasta -q file1_name.qual, file2_name.qual -o split_library_subtidal.output -L 600 -l 150 -w 50 -e 0 -k -b variable_length
Parameters: -m=map file; -f=fasta file, -q=qual file, -o=output folder, -L=maximum sequence length, -l=minimum sequence length; -b=barcode length variable; -k=keep primer

pick_de_novo_otus.py (build an OTU table):
pick_de_novo_otus.py -i split_library_subtidal.output/seqs.fna -o otus
Parameters: -i=imput file, -o=output folder

summarize_taxa_through_plots.py (summarize OTUs by taxonomic categories):
summarize_taxa_through_plots.py -i otus/otu_table.biom -o taxa_summary -m map_file.txt -s
Parameters: -i=imput file, -o=output folder, -m=map file, -s=sort

beta_diversity_through_plots.py (perform beta diversity and principal coordinate analysis):
beta_diversity_through_plots.py: -i otus/otu_table.biom -m map_file.txt -o bdiv/ --color_by_all_fields -t otus/rep_set.tre
Parameters: -i=imput file, -m=map file, -o=output folder, -t=path to phylogenetic tree file

make_3d_plots.py (PCoA with taxonomy plots):
make_3d_plots.py -i bdiv/unweighted_unifrac_pc.txt -m map_file.txt -t taxa_summary/otu_table_sorted_L3.txt --n_taxa_keep 15 -o biplot/unweighted_L3
Parameters: -i=imput, -m=map file, -t=taxa level to be plotted (L3=order), --n=number of taxa to display, -o=output folder

Note: The map_file.txt contains all of the information about the samples necessary to perform the data analysis. At a minimum, the mapping file should contain the name of each sample, the barcode sequence used for each sample, the linker/primer sequence used to amplify the sample, and a Description column.

5.3. Sequence analysis of *bss*A genes. DNA sequences obtained in this work were translated to perform a protein blast (blastp) search against NCBI databases (nr; refseq_protein; swissprot; pat; pdb; env_nr) and IMG 4 databases (Markowitz *et al.*, 2012) in order to select the closest matches. Some matching sequences found in the blastp search included sequences not annotated as BssA but showing at least 60% amino acid similarity with the query were included in subsequent analysis. Sequences including less than 75% of the protein region analyzed in this work (region amplified with primers 7772f 8542r) were discarded. To compile all possible BssA-like sequences, we first retrieved all published BssA, NmsA and AssA sequences described in the literature (Table S3.1, Chapter III). In addition, we searched all NCBI databases for the keyword "benzylsuccinate synthase", "naphthylmethylsuccinate synthase" or "alkylsuccinate synthase". Mega 5 software was used to align the sequences and construct phylogenetic trees. FastUniFrac was used for PCoA analyses (Hamady *et al.*, 2009). Amino acid sequences were compiled in OTUs (operational taxonomic units) at 5% ($OTU_{0.05}$) and 2% ($OTU_{0.02}$) sequence dissimilarity level for tree construction and PCoA analysis, respectively. SPEER software (specificity prediction using amino acids' properties, entropy and evolution rate) available in the SPEER-SERVER web was applied to elucidate the specific conservation pattern of the amino acid residues by using the automated subgrouping tool SCI-PHY, allowing a gap of 20% per column and a weight of 1.0 for relative entropy and physicochemical PC property distance. This method can define protein subfamilies and predicts residues that are relevant for family protein functions by quantifying patterns of conserved sites, based on their physico-chemical properties and the heterogeneity of evolutionary changes between and within the protein subfamilies (Chakrabarti *et al.*, 2007; Chakraborty *et al.*, 2012). This method identifies Type I sites (conserved in one subgroup and variable in the remaining groups) and Type II sites, where different types of amino acids are conserved across different subfamilies. WebLogo software (Crooks *et al.*, 2004) was used to build the logo of each sequence cluster.

Exportable documents were edited with Illustrator 5.0 to compare the different profiles. A computational model of the three-dimensional structure of the translated *bssA* amplified fragment was constructed with the Phyre2 Server (Protein Homology/analogY Recognition Engine V 2.0, http://www.sbg.bio.ic.ac.uk/phyre2/) (Kelley and Sternberg, 2009). The structures were displayed as ribbon using Pymol software.

5.4. Metagenomic annotation. Sequences obtained from each metagenome as well as amplicons of 16S rRNA gene were uploaded to MG-RAST platform (sff files) and submitted for annotation using default parameters. For comparison of GO categories, a maximum e-value threshold of 10^{-3} was used. The heat map was made with Euclidean distance to determine the similarity/dissimilarity among samples.

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IV. Results

Chapter I

Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the Prestige oil spill

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1.1. Introduction

Accidental oil spills are an important source of marine pollution that causes huge damages to the environment, resulting in extensive and long-term deterioration of coastal and ocean ecosystems. The sinking of the oil tanker *Prestige* in November 2002 in 3500 meters of water off the northern Spanish coast released an estimated 17,000 tons of heavy fuel oil into the sea. Several hundred kilometers of coastline were covered by an oil slick, made up of a complex mixture of saturated (23%) and aromatic (53%) hydrocarbons, asphaltenes and resins (24%). The aromatic fraction was mainly composed of naphthalene, phenanthrene and alkyl derivatives, while the saturated fraction consisted of lineal and cyclic hydrocarbons of variable length (Alzaga *et al*., 2004). Despite extensive hand- and mechanically-assisted removal techniques used during the initial clean-up of the shore, the heavy nature of the spill and the strong tidal activity in the region allowed large oil deposits to accumulate in the sediments, interspersed with the sand (Bernabeu *et al*., 2006; Alonso-Gutiérrez *et al*., 2008). Further removal of these heavy oil layers became impracticable.

Stable isotope tracing of seawater dissolved inorganic carbon carried out early after the spill revealed active biodegradation processes occurring on the spilled oil *in situ* (Medina-Bellver *et al*., 2005). Microbiological analysis of intertidal water and of sand and rock environments affected by the spill detected bacterial activities towards the hydrocarbon contaminant, and aerobic bacterial strains able to degrade aromatic and aliphatic hydrocarbons could be isolated, including the hydrocarbonoclastic aerobe *Alcanivorax* (Alonso-Gutiérrez *et al*., 2008; Alonso-Gutiérrez *et al*., 2009). Microbial biodegradation mediated by indigenous microbial communities is known to be a relevant natural process in the decontamination of oil-polluted environments. Prokaryotes use a huge metabolic repertoire to degrade a wide range of organic contaminants, and in many cases this degradation produces water and CO_2 as end products. The use of autochthonous bacteria to restore contaminated sites is an innovative and environment friendly technology, widely used in the remediation of aerobic sites (Harayama *et al*., 2004). However, this technology is hardly applicable to marine sediments, where oxygen concentration is limited or nil. Anaerobic biodegradation of petroleum components has been repeatedly observed in nature, and hydrocarbon-degrading microorganisms with all types of respiratory metabolisms have been isolated and described (Widdel and Rabus, 2001). While biochemical and molecular determinants of aerobic hydrocarbon biodegradation processes are well established (Parales and Haddock, 2004), current knowledge on the molecular basis of anaerobic degradation processes is limited (Widdel and Rabus, 2001).

The implementation of powerful molecular ecology techniques during the past decade has enabled the scrutiny of microbiological processes directly in their environment.

The analysis of different contaminated habitats shows that the presence of a contaminant produces changes in the bacterial community, both in structure and function. In long-term contaminated sites, the bacterial population tends to be dominated by strains able to use the hydrocarbons as carbon source (Harayama *et al*., 2004). Most bioremediation projects have studied soil, fresh water, shorelines and surface water contamination. The characterization of the bacterial response to an oil spill of this magnitude has generally focused on aerobic populations in aquatic and superficial sediments (Lindstrom *et al*., 1991; Kasai *et al*., 2001; Alonso-Gutiérrez *et al*., 2009; Kostka *et al*., 2011), but has generally not analyzed subtidal coastal sediments, basically characterized by sand movement interruption that creates a stable anoxic environment.

Sediments are among the most phylogenetically diverse environments (Lozupone and Knight, 2007). Coastal sediments are characterized by an important input of organic matter, obtained through sedimentation of material from the land or in upwelling areas. The limited processing of this organic material in the relatively shallow waters results in high quality organic matter (with respect to degradability) at the top of the sediments (Engelen and Cypionka, 2009). The microbial processes degrading sunk organic matter promote a vertical zonation determined by the distinct availability of electron acceptors for respiration, resulting in a reduction of the redox potential with depth (Fenchel and Finlay, 1995), together with a reduction of the quality of degradable organic matter. The vertical sequence of electron acceptors roughly follows the order of decreasing free energy yield. Redox reactions first consume oxygen, followed by nitrate and manganese, iron and sulfate (Braker *et al*., 2001). Therefore, marine sediments are typically characterized by a thin oxic surface layer that can range between few millimetres to several centimetres, an anoxic but oxidized zone, in which nitrate, manganese and iron oxides are the main electron acceptors, and a reduced sulfidic zone, in which sulfate reduction predominates (Fenchel and Finlay, 1995). In deeper zones methanogenesis may dominate, although the coexistence of both sulfate reduction and methanogenesis has been reported (Holmer and Kristensen, 1994). Normally, the oxidized nature of the surface shore sediments can be observed as a brown or light-grey surface layer. The extent of this oxidized horizon depends on the input and quality of degradable organic matter, on the rate of oxygen diffusion into the sediment, which relies on temperature, grain size, water flow, and other sediment characteristics (Gray and Elliot, 2009), and on the availability of the different electron acceptors (Fenchel and Finlay, 1995). Denitrification occurring in this zone is considered to be responsible for less than 10% of organic carbon oxidation in marine sediments (Canfield *et al*., 1993). However, the potential for denitrification may be important in zones exposed to a large and continuous input of nitrogen, as it is the case in the coastal and shelf regions (Galloway, 2003). Beneath this layer, the sediment becomes reduced, and in this zone sulfate reduction predominates. The final products of sulfate

respiration are iron sulfides that give the typical dark-grey zonation of reduced sediments. In coastal marine sediments, about 50% of the deposited material is mineralized via sulfate reduction (Jorgensen, 1982), which is the most relevant sediment surface respiratory metabolism.

Our study focuses on two beaches in the Cíes Islands, located at the mouth of the Ría de Vigo, in the NW of Spain (Figure 1.1). The sediments in this area consist mainly of siliciclastic material, made up of well-rounded quartzes, and biogenic carbonate sands and gravels (Costas *et al*., 2006). The coastline is protected from the open ocean, and is dominated by seasonal winds and mesotidal activity, with a mean tidal range of 3 m. The region is under the influence of the Gulf Stream, and is regularly subjected to an upwelling phenomenon in spring. When this occurs, nutrient rich deep waters reach surface supplying additional nutritional elements to the area. Although a number of studies analyzing the impact of the *Prestige* slick on coastal environments have been carried out, none of them analyzed the effect on the microbial communities in the subtidal sediments.

The goal of this work was to provide a detailed characterization of the bacterial communities in sediments heavily affected by the *Prestige* oil spill in an environment that had previously been poorly characterized. The response to the presence of high levels of aromatic contaminants, and the natural microbiota potential to respond to the impact has been analyzed. We have applied culture-dependent and molecular, culture-independent, techniques to characterize the bacterial populations at two stages after the spill. The data obtained provide an overview of the population behavior in response to a toxic spill, and of the main factors involved in population dynamics under these conditions.

1.2. Experimental procedures

Sampling. Two neighboring beaches in the eastern shore of the Cíes Island (Atlantic Isle National Park, Galicia, Spain) were chosen for sediment sampling. One of them (Figueiras beach) had been heavily contaminated and still presented traces of crude oil over the seafloor in the first sampling campaign, while the other one (Rodas beach), was protected by a pear and resulted less contaminated during the spill, and was immediately cleaned. Sediment cores were collected by scuba-divers at nine meters depth with one meter-long cores and kept anaerobic at 4°C until processed. The first sampling campaign (cores FI) was carried out on May 25th, 2004 in Figueiras beach (FI; 42°13'55N, 8°53'50W), where an additional 20 cm core was collected in a petroleum spot visualized by the diving team in this site (FI-PET). On June 27th 2007 a similar campaign was carried out (cores FII), which was extended to Rodas beach (cores RII; 42°13'31N, 8°53'59W), where at this time a heavily contaminated spot was detected (Figure 1.1). Triplicate cores collected at each site were analyzed in each case, except for FI-PET. Each core was fractionated every three

centimeters and used for immediate analysis, or stored at -80°C for future analysis. A description of the sediment samples used for the analysis is shown in Table 1.1 and Figure 1.1. All determinations in the following analyses were carried out independently for each collected core.

Geochemical parameters and hydrocarbon determination. Sediment density (ρ_{bulk}), porosity, water content, sediment grain size and total organic matter were calculated. Total iron was determined by inductively coupled plasma-optical emission spectrometry. Quantification of nitrate, nitrite and sulfate was done by ion chromatography (IC).

Aromatic and aliphatic compounds were extracted from the sediments with a mixture of hexane/acetone (2:1). The supernatant was percolated through a BOND ELUT TPH column (Varian). Recover fractions were concentrated by gentle solvent evaporation under a nitrogen flow until dryness and GC-MS was used to quantify hydrocarbon compounds.

Most probable number (MPN) enumeration of bacteria. For enumeration of anaerobic hydrocarbon oxidizers, we used an artificial seawater minimal medium described previously (Widdel and Bak, 1992) supplemented with different electron acceptors for sulfate-reducing bacteria (SRB), nitrate reducing bacteria (NRB) and iron-reducing bacteria (IRB). Microbial growth was tested in the following carbon sources at a final concentration of 2 mM: benzene, toluene, lactate and acetate; naphthalene and anthracene were dispensed in the MPN tubes from stock solutions dissolved in pure hexane, which was evaporated before delivering the media. For aerobe MPN determination, hydrocarbon sources (naphthalene, phenanthrene, pyrene, undecane anthracene) dissolved in *n*-hexane were added to polypropylene microtitre 96-well sterile plates and the solvent was evaporated at room temperature. Each well was filled with autoclave-sterilized artificial seawater medium (SIGMA Sea Salts) supplemented with filter-sterilized K_2HPO_4 pH 6.8. For each sample, tenfold (anaerobes) and fivefold (aerobes) serial dilutions were used for inoculate triplicates tubes (anaerobes) and quintuplicates (aerobes) wells, respectively. Both tubes and plates were incubated at 16°C in the dark and growth was checked during one year.

DAPI counts and fluorescent in situ hybridization (FISH). Probe mix EUB338 targeting Bacteria (Amann *et al*., 1990), GAM42a targeting *Gammaproteobacteria*, DSS658 targeting *Desulfobacteraceae*, DSV698 targeting *Desulfovibrionaceae* (Manz *et al*., 1998), DSB985 targeting *Desulfobacter*, CF339a targeting the phylum *Bacteroidetes* (Manz *et al*., 1996) and ARCH915 targeting *Archaea* were used for the analysis.

Nucleic acids extraction. Frozen sediment samples from each core horizon (2 grams each) were used for direct extraction of DNA with the UltraClean Soil DNA Kit (MoBio Laboratories, USA) following procedures described by the manufacturer except in the last step, where the elution was repeated twice. The integrity and yield of extracted nucleic acids were checked by standard agarose gel electrophoresis and ethidium bromide staining. Nucleic acids were quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). Equal amounts of DNA from triplicate cores were mixed and used in PCR reactions.

PCR amplification and 16S rRNA gene libraries constructions. PCR amplification of the complete 16S rRNA gene was performed with the bacterial universal primers GM3F and GM4R (Muyzer *et al*., 1995). The PCR fragments were eluted from standard agarose gels after electrophoresis (1% agarose, 100V, 1 h) and purified using the Gel Extraction Kit (Qiagen). Amplicons were cloned in pGEM-T (Promega) according to the manufacturer instructions and 146 positive clones from each sample were sequenced.

Phylogenetic and statistical analyses. For specific details, see section 5.1 (experimental procedures).

1.3. Results

Site description and sediment characterization.

The oil slick spilled by the *Prestige* reached the shore of the Cíes Islands the first days of December 2002, contaminating the entire shoreline. The oil was mechanically removed from the surface of most beaches during the following weeks, but a small area at Figueiras beach remained un-manipulated for monitoring purposes. All remaining areas were immediately cleaned and in our study were considered as not heavily affected by the spill. Sediments samples were collected from Figueiras in 2004 (FI) and 2007 (FII). Eye inspection of the samples revealed the presence of two different redox zones (Gray and Elliot, 2009): i) an upper brown-colored, oxidized zone and ii) a grey zone caused by ferrous sulfide precipitation resulting from sulfide formation (Figure 1.1).

We thus selected three zones along depth, which corresponded to an upper oxidized zone (OX), which included the upper 2 to 5 cm; an intermediate transition zone (TR) at the level where sediment colour changed from brown to grey (10 to 15 cm depth), and a reduced zone (AN) located at the bottom of the cores (25-35 cm) (Figure 1.1). During 2004 sampling, several crude oil patches (tar aggregates) intermingled in the sediment were detected, and were collected in 20 cm long cores (FI-PET). During the sampling process in

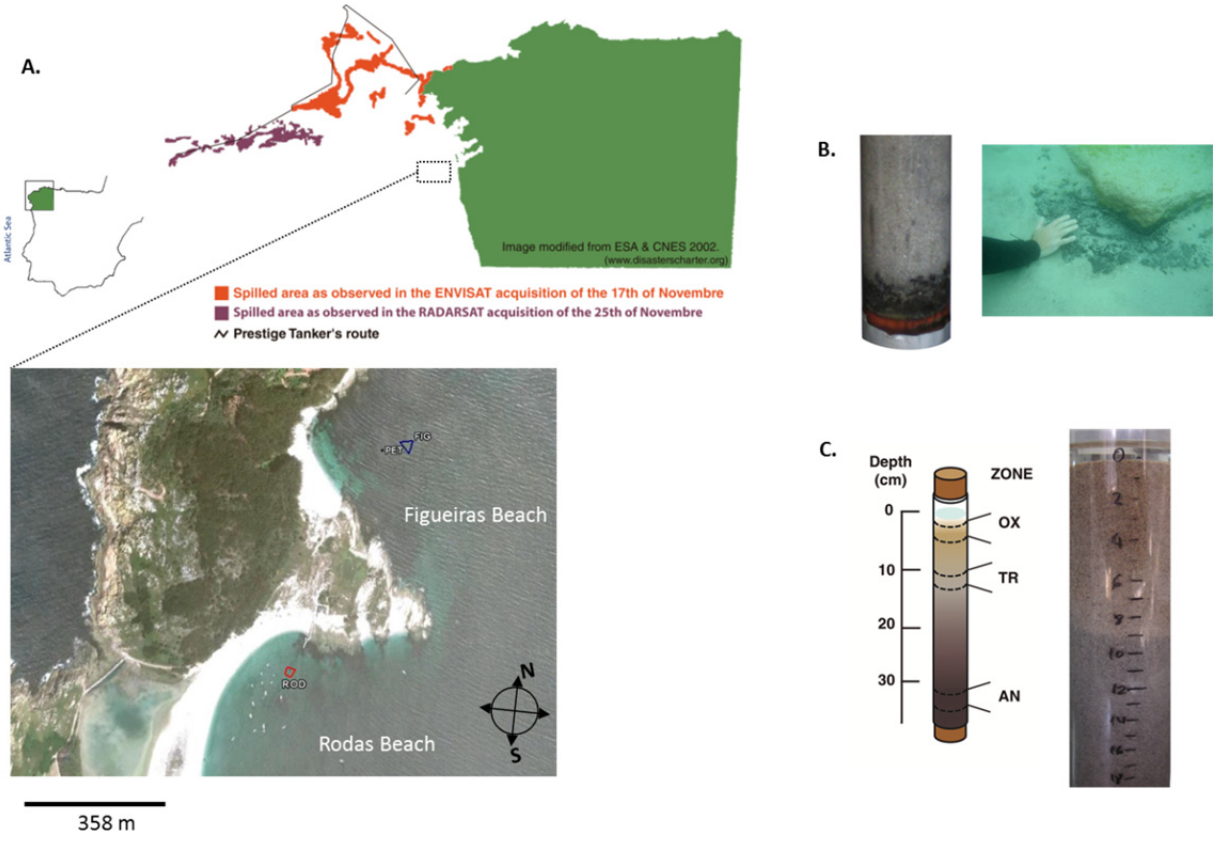


Figure 1.1. Sampling scheme. A) Sampling site. Picture from Figueiras and Rodas beaches in the Cies Island where sampling was carried out. Red and blue shapes delimit the sampling sites at each beach. B) Oil patch in the seafloor of Figueiras beach in 2004 (left) and an example of a petroleum patch found at the bottom of Rodas cores in 2007 (right). C) Scheme of the core depth zones analyzed in this work (left) and magnification of the oxic to anoxic transition zone typical of Figueiras sediment cores.

2007, several cores collected in a previously considered uncontaminated area (Rodas beach, Figure 1.1) showed a similar petroleum patch located in the reduced zone (RII-AN), and were selected for comparison. Table S1.1 summarizes some physicochemical characteristics of the samples. Sediments at the two sampling sites differed slightly in their bulk parameters. All samples were mainly composed of sand (>99%), but the grain size in Figueiras was finer than in Rodas and therefore the bulk density and water porosity varied accordingly (Table S1.1). Total sediment organic matter (SOM) content of samples containing visible oil residues (FI-PET and RII-AN) was between three to twenty times higher than the corresponding sand samples in the same site, which ranged from 0.26 to 1.2% of the dry weight (Table 1.1). Nitrate and total iron content were also determined. Nitrate concentration was highest in the oxidized zone, except in the FII cores, where higher values were obtained in the transition zone. Iron content was in all cases below 0.3% (Table 1.1).

Table 1.1. Sediment organic matter (SOM), total hydrocarbons and electron acceptors present in the samples.

Sample			Total organic matter		hydrocarbons (ppm)[b]		SO_4^{2-}		NO_3^-		Fe[a] (%)
			mg/ml	%[a]	Alk.	Arom.	mM[c]	mg/g	µM[c]	µg/g	
2004	FI	OX	12.8 ± 1.3	0.9	12.2	1.51	28.6 ± 0.6	0.85	67.8 ± 3.9	0.75	0.22
		TR	13.5 ± 2.3	1.0	8.1	0.91	24.3 ± 1.4	0.79	55.3 ± 15.2	0.70	0.15
		AN	11.8 ± 1.8	0.9	5.0	0.17	20.7 ± 1.6	0.73	26.5 ± 8.8	0.34	0.07
		PET	46.8 ± 14.4	3.1	261.0	87.18	17.9 ± 4.6	1.30	57.1 ± 12.0	0.59	n.d.
2007	FII	OX	4.1 ± 0.2	0.2	12.1	1.92	28.7 ± 1.0	0.89	50.2 ± 10.9	0.37	0.05
		TR	5.2 ± 0.9	0.3	13.5	2.70	25.7 ± 1.1	0.81	70.9 ± 11.5	0.41	0.09
		AN	6.5 ± 0.8	0.46	8.7	1.63	23.6 ± 0.6	0.80	46.4 ± 1.5	0.36	0.06
	RII	OX	9.1 ± 0.9	0.7	10.1	2.33	29.7 ± 0.8	0.91	104 ± 28.4	0.91	0.04
		TR	15.7 ± 3.3	1.2	53.9	3.76	20.5 ± 0.6	0.66	52.1 ± 3.3	0.51	0.07
		AN	258 ± 53.8	17.2	4557.8	421.33	20.8 ± 6.4	0.69	5.9 ± 0.6	0.06	n.d.

a. % of dry weight of sediment
b. Sum of total hydrocarbons detected in the analysis (see tables S1.2-S1.3)
c. Molarity in interstitial water
n.d: not determined due to the high hydrocarbon content

The hydrocarbon composition was determined in all the samples (Tables S1.2-S1.3). In Figueiras, the aliphatic hydrocarbon distribution of the sediment samples taken in 2004 and 2007 showed little variations, and C_{11}-C_{19} *n*-alkanes with a slight odd-even carbon number preference predominated, while naphthalene and derivatives were the main aromatic detected. In 2004 FI-PET tar patch, the aliphatic and polycyclic hydrocarbon profile resembled the previously described profile of *Prestige's* affected samples (Table S1.2-S1.3, Figure 1.2) [CEDRE (Centre de documentation, de recherche et d'expérimentations sur

les pollutions accidentelles des eaux), 2002].The (nC_{12}+nC_{13})/(C_{25}+nC_{26}) indicator ratio suggested a low degree of evaporation, in accordance with the buried location of the patch, while the low nC_{18}/phytane ratio indicated a substantial degree of biodegradation (Díez *et al*., 2005). In contrast, the oil patch found in Rodas beach in 2007 (RII-AN) was mainly composed of higher molecular weight *n*-alkanes (C_{24}-C_{38}), suggesting a high intensity of weathering processes (Figure 1.2). Naphthalene and alkylnaphthalenes with one to four substitutions represented the largest group of polycyclic hydrocarbons (PAHs) detected in samples FI-PET and RII-AN (Table S1.3). In the *Prestige* oil, naphthalenes represented the largest fraction (41.5%), and consequently naphthalene and derivatives predominated in our tar aggregate samples. In addition, negligible amounts of BTEX compounds were detected in most of the samples.

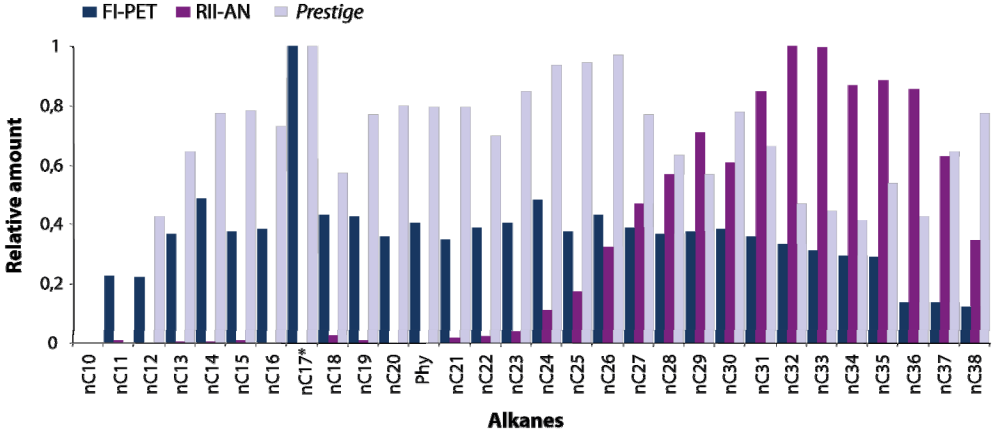


Figure 1.2. Relative distribution of alkanes in most polluted samples. Data are compared with the Prestige oil fingerprinting described in Alzaga *et al*., 2004. * sum of nC17 and pristane.

Bacterial community analysis and biodegradation potential in 2004 samples

Oxygen penetration in the sediments is limited to the upper few centimeters. Below this upper layer, sediments are under permanent anoxic conditions (Engelen and Cypionka, 2009). We therefore mainly investigated the presence in the sediment of bacterial populations able to degrade PAHs and simple aromatic compounds under anoxic conditions. Most probable numbers (MPN) of sulfate reducing (SRB), iron reducing (IRB) and nitrate reducing (NRB) bacteria with different aromatic compounds as electron donors were determined in the three different sections of the cores taken at Figueiras (Figure 1.3a). Growth was tested with benzene, toluene, naphthalene and anthracene. Acetate, a major electron donor for SRB in sediments (Sørensen *et al*., 1981), was also used as an internal reference. In addition, the MPN of aerobes growing on several PAHs, and n-hexane and undecane as simple alkanes was also determined.

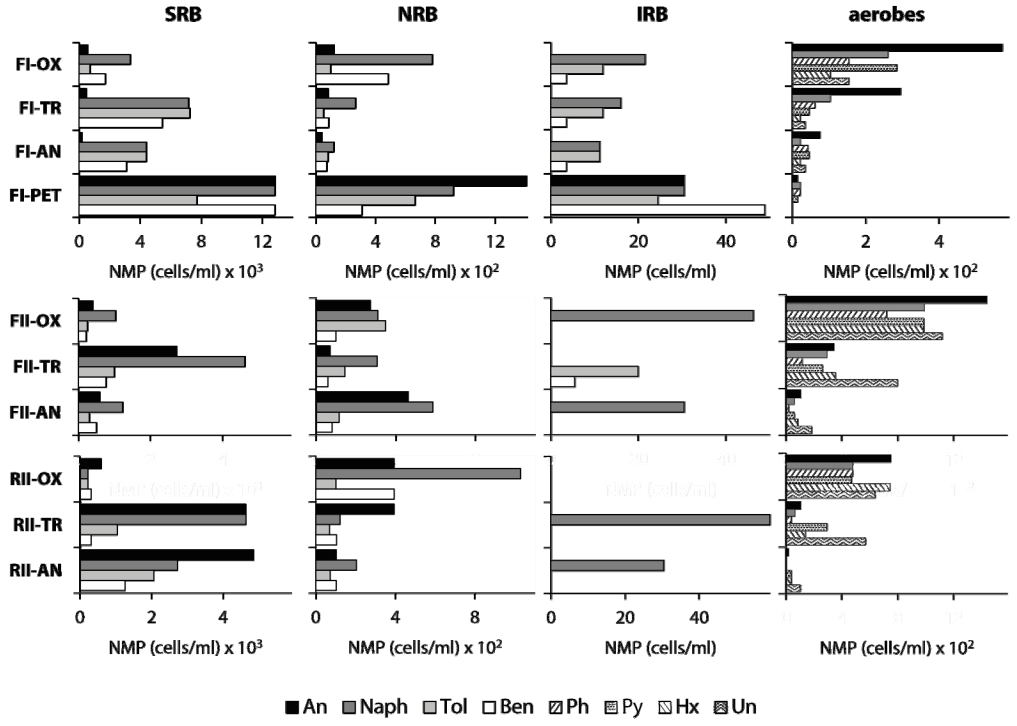


Figure 1.3. Most probable number of SRB, NRB, IRB and aerobes able to grow on different oil components at different depths. A) Samples collected in 2004 at Figueiras beach (FI); B) samples collected in 2007 at Figueiras (FII) and Rodas (RII) beaches. PET, tar aggregate; OX, TR and AN refer to the oxidized, transition and reduced zones, respectively, as described in the text (see Figure 1.1). An, anthracene; Naph, naphthalene; Tol, toluene; Ben, benzene; Ph, phenanthrene; Py, pyrene; Hx, hexane; Un, undecane. Counts were made in triplicate. Numerical values (95% C.I.) can be found in Supplementary Table S1.4a and S4b, respectively.

Growth in all tested media with aromatics was obtained with all the samples, especially with naphthalene, toluene and benzene as carbon sources. Following the vertical electron acceptor zonation, we observed the MPN of aerobic hydrocarbon degraders to be highest in the upper zone, as expected, decreasing dramatically with depth to reach similar values as NRB in the anoxic zone (Figure 1.3, Table S1.4a). Nitrate reducing bacteria counts were slightly higher than aerobe counts in the upper oxidized layer, and also decreased considerably in the deeper layers (Figure 1.3a, Table S1.4a), consistent with nitrate reduction and denitrification being facultative processes (Philippot, 2005), and in accordance with other marine sediment profiles (Gao *et al*., 2010). IRB able to degrade aromatic compounds were barely detectable (below 50 cells/ml). Iron determinations showed values below 0.3% (w/w) in the sediments (Table 1.1), with maximal amounts generally found in the upper zone. However, most of the Fe(III) forms present in the sediments are unavailable to microbial reduction (Boyd and Ellwood, 2010). This probably limits this type of metabolism, explaining the absence of such culturable fraction.

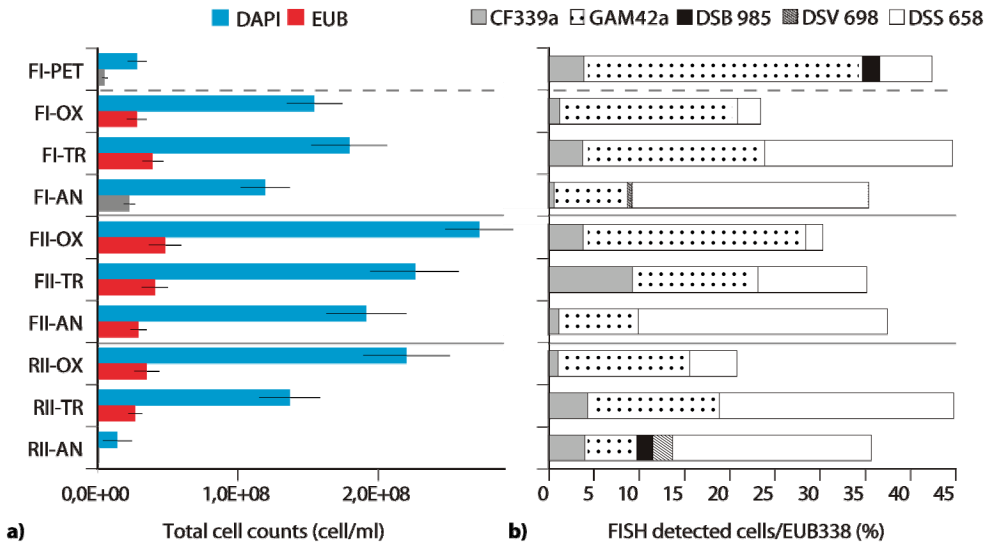


Figure 1.4. Absolute DAPI cell counts and percentage of *Proteobacteria* and *Cytophaga* over *Eubacteria* in the different depth zones of sediment collected from Figueiras beach in 2004 (FI) and from Figueiras and Rodas beach in 2007 (FII and RII, respectively). PET, tar aggregate collected at Figueiras in 2004; OX, TR and AN refer to the oxidized, transition and reduced zones, respectively, as described in the text (see Figure 1.1). The FISH probes used were the EUB338 mix targeting Bacteria, GAM42a targeting *Gammaproteobacteria*, DSS658 targeting *Desulfobacterarceae*, DSV698 targeting *Desulfovibrionaceae,* DSB985 targeting *Desulfobacter*, and CF339a targeting the phylum *Bacteroidetes* (Amann *et al*., 1990; Manz *et al*., 1996; Manz *et al*., 1998). No *Archaea* were detected in the samples with ARCH915 probe. Numerical values (average of triplicate samples ± SD) can be found in Supplementary Table S1.5a.

As expected, the highest counts were obtained for SRB, which represented the most relevant fraction of culturable organisms (Figure 1.4a; Table S1.4a). SRB able to oxidize aromatic compounds showed a different picture from the other metabolisms assayed and were more abundant in the transition zone, in accordance with previous findings of the highest proportion of SRB being at 12-18 cm depth (Jorgensen, 1982). Total values of aromatic oxidizers suggest than in the marine sediments studied, SRB are possibly the main players in aromatic hydrocarbon degradation. Although aerobic PAH degraders were abundant in the oxic zone, this only represents a thin upper layer in the sediment, in contrast to the redox influence zone where SRB are most active, which constitutes an at least 20 cm-wide sand horizon.Under sulfate reducing conditions, naphthalene and toluene where used by a higher proportion of the bacterial population, while naphthalene oxidizers prevailed among the NRB and IRB. Surprisingly, under aerobic conditions, the highest MPN counts were obtained with anthracene as substrate, which was ten times less abundant than naphthalene (Figure 1.4a; Table S1.4a). The MPN of SRB growing on acetate was high and increased slightly in the transition zone, while the MPN of acetate oxidizing NRB was lower and decreased in that horizon (Table S1.4a).

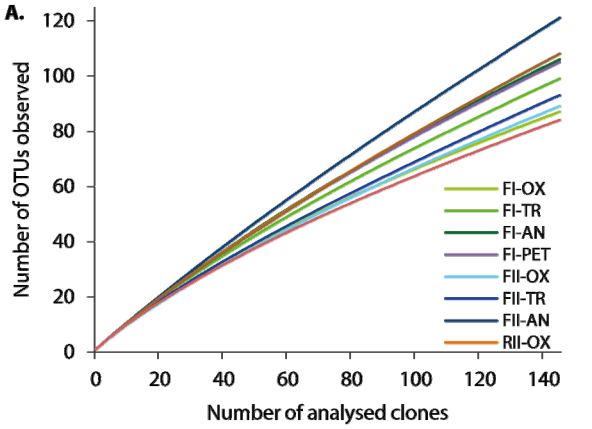
Table 1.2. Cultivable fraction of anaerobic naphthalene degrading population in the potentially active population of the sediment samples.

		%	SRB (%)			NRB (%)		
		EUB/DAPI	Naft/EUB	Tol/EUB	Ben/EUB	Naft/EUB	Tol/EUB	Ben/EUB
FI	OXI	18.11	0.012	0.003	0.006	0.003	0.001	0.002
	TR	21.78	0.018	0.018	0.014	0.001	0.001	0.001
	AN	18.56	0.020	0.020	0.014	0.001	0.001	0.001
	PET	17.33	**0.267**	**0.158**	**0.267**	**0.019**	**0.014**	**0.006**
FII	OX	17.67	0.002	0.001	0.001	0.001	0.001	0.001
	TR	18.00	0.011	0.002	0.002	0.001	0.001	0.001
	AN	15.17	0.004	0.001	0.001	0.002	0.001	0.001
RII	OX	15.83	0.001	0.001	0.001	0.003	0.001	0.001
	TR	19.33	0.017	0.004	0.001	0.001	0.001	0.001
	AN	8.50	**0.245**	**0.182**	**0.109**	**0.018**	**0.006**	**0.009**

Data from highly polluted samples are marked in bold.

Cell abundances detected by DAPI staining was low in all samples (below 2 x 10^8 cells per cm^{-3} of sediment, Figure 1.4a) as compared to other coastal sandy sediments of the Mediterranean and Wadden Seas and the Atlantic Ocean (Llobet-Brossa *et al*., 1998; Rusch *et al*., 2003; García-Martínez *et al*., 2009). The number of cells was generally highest in the transition zone and lowest in the reduced zone, as previously described in other sediments at that period of the year (Rusch *et al*., 2003). The percentage of DAPI-stained cells that could be hybridized with the bacterial EUB338 (I-III) probes varied between 21.8% in the transition zone and about 18% in both oxidized and anoxic zones (Table 1.2). These low yields of FISH detection as compared to DAPI counts could indicate a low metabolic state of a high proportion of the bacterial population (Rosselló-Mora *et al*., 1999). Interestingly, within the potentially active population as determined by FISH with EUB338 probe, the fraction of culturable toluene and naphthalene oxidizing SRB reached 0.2 ‰ in the deeper layer, in the same range as the acetate degrading population (Table 1.2). These values were one order of magnitude higher than the fraction of naphthalene oxidizing NRB.

Additional FISH probes specifically targeting *Gammaproteobacteria*, *Desulfobacteraceae*, *Desulfovibrionaceae*, *Desulfobacter*, *Bacteroidetes* and *Archaea* were tested. Figure 1.4b shows an uneven distribution of the bacterial groups through depth. *Desulfobacteraceae* and *Gammaproteobacteria* were the predominant clades, the fraction of *Deltaproteobacteria* increasing with depth while that of *Gammaproteobacteria* decreased (Figure 1.4b, Table S1.5a). *Desulfovibrio* were only detected in the reduced zone, and no *Archaea* could be detected in our samples (Figure 1.4b). *Archaea* could be expected in deeper layers of in the sediment, in the sulfate-methane transition zone where sulfate is depleted and methanogenesis predominates (Holmer and Kristensen, 1994).



B.

	FI-OX	FI-TR	FI-AN	FI-PET	FII-OX	FII-TR	FII-AN	RII-OX	RII-TR	RII-AN
Good's index	0.57	0.47	0.42	0.44	0.54	0.53	0.28	0.38	0.41	0.58

Figure 1.5. A) Rarefaction curve for 16S rRNA genes of microbial populations from the sediment sampled at Figueiras beach in 2004 (FI) and from Figueiras and Rodas beach in 2007 (FII and RII, respectively). PET, tar aggregate collected at Figueiras in 2004; OX, TR and AN refer to the oxidized, transition and reduced zones as described in the text (see Table S1.1). B) Good's Index generated using MOTHUR. Clones were grouped into phylotypes at a level of ≥97% sequence similarity.

To analyze the community composition along the depth profile we constructed a 16S rRNA gene library of each depth zone, and sequenced 146 whole-length 16S rRNA gene clones from each of them. Phylogenetic diversity in the samples from the different depth profiles was determined in the rarefaction curves, and sequencing effort completeness was assessed with the Good's Coverage estimator, which gave diversity coverage between 57% and 42% (Figure 1.5). In most cases, the reconstruction of the clones in the frame of the available databases affiliated the organisms present in the sediments as uncultured marine bacteria. For most of the clones (65%), the closest relatives (≥97% similarity) were related to sequences of bacteria detected in marine sediments and polluted sites, but also from extreme environments like cold seeps (Figure 1.6). Figure 1.7 summarizes the phylotype composition of each layer. Members of the phylum *Proteobacteria* predominated in all horizons, ranging from 66% in the oxidized zone to more than 71% in the reduced zone. The distribution of clones showed a clear depth-dependent pattern. The percentage of *Deltaproteobacteria* increased with depth, being the most abundant class in the reduced zone, while the relative presence of *Gammaproteobacteria* decreased concomitantly. These observations were in agreement with the FISH results (Figure 1.4). Phylotype distribution within the *Deltaproteobacteria*class showed a predominance of *Desulfobacteraceae*, especially in the deeper sample, followed by the order *Myxococcales*, which was more represented in the oxidized horizon (Figure 1.8). *Acidobacteria*, the third most abundant group, were more represented in the deeper samples. As expected, the presence of *Cyanobacteria* was highest in the upper layer, but still constituted a 2.5 % of the 16S sequences in the dark, reduced zone.

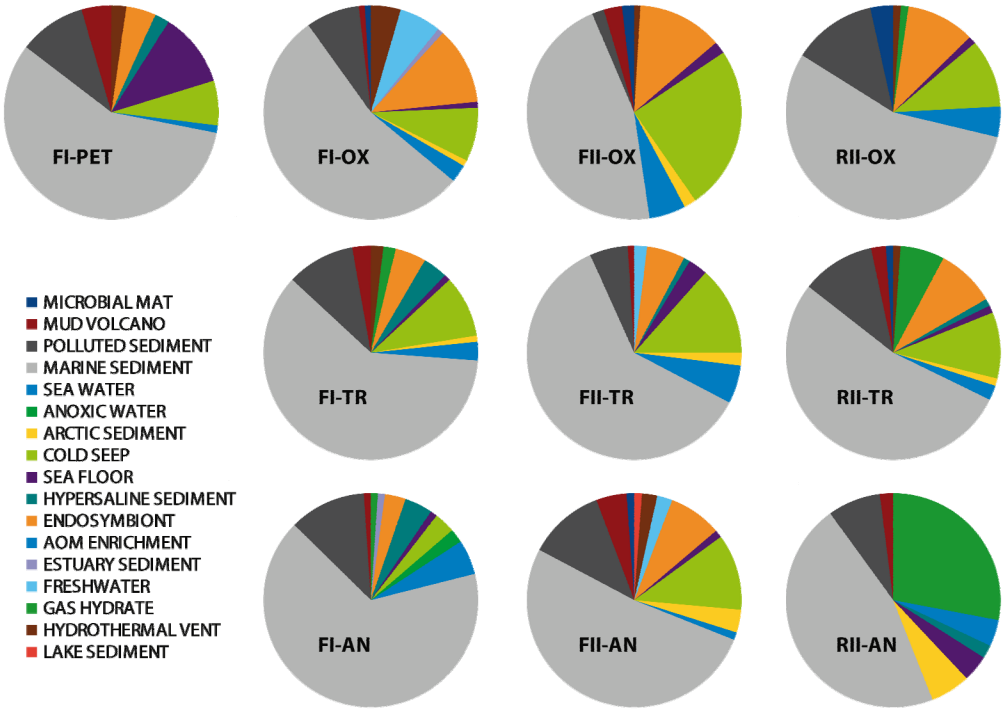


Figure 1.6. Distribution of environmental origin of the closest relatives found in the 16S rRNA gene libraries with at least 97% similarity.

Bacterial population associated with a residual oil sample (tar aggregate)

During 2004 sampling process, a residual oil patch buried within the sediment was detected and collected for analysis (FI-PET core, 20 cm long). The highest counts of aromatic oxidizing SRB and NRB were obtained in this sample. Total cell counts (DAPI) of the sample showed 6-10 times lower values than those obtained in the surrounding Figueiras sand where the PET core was collected (Figure 1.4a). The proportion of EUB338 FISH-targeted cells was lower than in the other samples (17%), although the fraction of culturable aromatic oxidizing SRB among the putative active bacteria population was highest (0.26% for naphthalene) (Table 1.2, Figure 1.3a), suggesting a high metabolic activity towards the contaminant in this sample, where naphthalene and derivatives constituted the major PAHs fraction (Table S1.3b). *Gammaproteobacteria* was the most retrieved group in the clone library of this sample, with about 40% of all the sequences (Figure 1.7), and was also the most targeted group by FISH (30%) (Figure 1.4b). On the other hand, about 22% of the clone sequences affiliated to the class *Deltaproteobacteria* (Fig 1.7). *Desulfobacteraceae* constituted about 16% in the library clones, but the FISH probes against this group only detected 7.5% of the total EUB-targeted population. It is remarkable that the probes used *a priori* in the microscopy counting just covered 65% of all the

Desulfobacteraceae sequences obtained in this work, as revealed by the probe match of the ARB package (Table S1.5b). This explained most of the discrepancy between FISH and 16S library results.

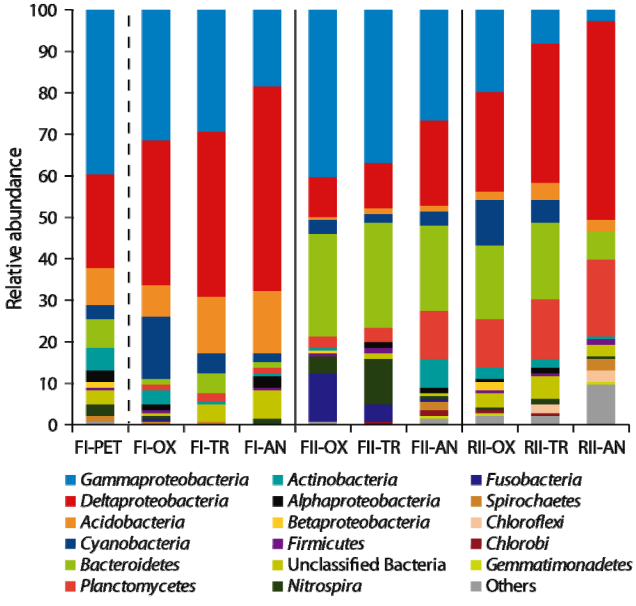


Figure 1.7. Cumulative plot of bacterial phyla detected in the different depth zones of sediment collected at Figueiras beach in 2004 (FI) and at Figueiras and Rodas beach in 2007 (FII and RII, respectively). PET, tar aggregate collected at Figueiras in 2004; OX, TR and AN refer to the oxidized, transition and reduced zones, respectively, as described in the text (see Figure 1.1).

The proportion of culturable PAH-oxidizing NRB within the active population as determined with EUB338 probe was also one order of magnitude higher than in the remaining Figueiras samples (0.019 %) (Table 1.1). Interestingly, the MPN of aerobes was very low, below the values obtained in the deeper Figueiras layer, and no alkane degraders were detected in this case.

Bacterial community analysis and biodegradation potential in 2007 samples

In June 2007, the sampling campaign was repeated in Figueiras beach, and was extended to the nearby Rodas beach, which was originally less influenced by the spill (Figure 1.1). However, petroleum aggregates intermingled with the sand were detected in the cores collected in this area, and they were processed as above.

The MPN of aromatics oxidizing SRB in the samples taken in Figueiras showed a similar pattern along sediment depth as previously observed, except that total values were lower for toluene and benzene oxidizers (Figure 1.3b, FII samples). Surprisingly, the proportion of anthracene oxidizers was higher than in the previous sampling, although the presence of this aromatic was not detected in these sediments (Table S1.3b). Similarly, NRB also included a high proportion of anthracene oxidizers. However, the NRB population followed a different distribution pattern as in the previous samples, showing similar abundance values through depth that could be related to the maintained high concentrations of

nitrate in the transition and reduced zones, not observed in other samples (Figure 1.3b, Table 1.1). As in the first sampling, the presence of hydrocarbon degrading aerobes was highest in the upper layer, although total values were three times higher than in 2004. Analysis of the three 16S rRNA gene libraries constructed from these samples showed a phylotype distribution that strongly differed from the pattern observed in 2004 (Figure 1.7). Although the phylum *Proteobacteria* still constituted the most abundant group, its relative abundance was below 50%. Among them, *Gammaproteobacteria* was the dominant group, and the proportion of *Deltaproteobacteria* was significantly lower in these samples, even in the deeper layer. The phylum *Bacteroidetes*, which was poorly represented in the 2004 samples, had a significant presence in this case, its abundance slightly decreasing with depth. This group was mainly composed of *Flavobacteria*- and *Sphyngobacteria*-like strains. The group *Acidobacteria* was almost undetectable and *Fusobacteria* and *Nitrospira* phylotypes were more abundant than previously. The noticeable presence of this last group, related to nitrite oxidizing activity in sediments (Satoh *et al*., 2007), could explain the relative high levels of nitrate in the transition and reduced zones of this site (Table 1.1). The FISH results confirmed the abundance of *Gammaproteobacteria* in FII samples, although the proportion of *Deltaproteobacteria* targeted by the probes used (DSS658, DSV698, DSB985) was higher than expected from the clone library results, especially in the deeper samples (Figure 1.4b).

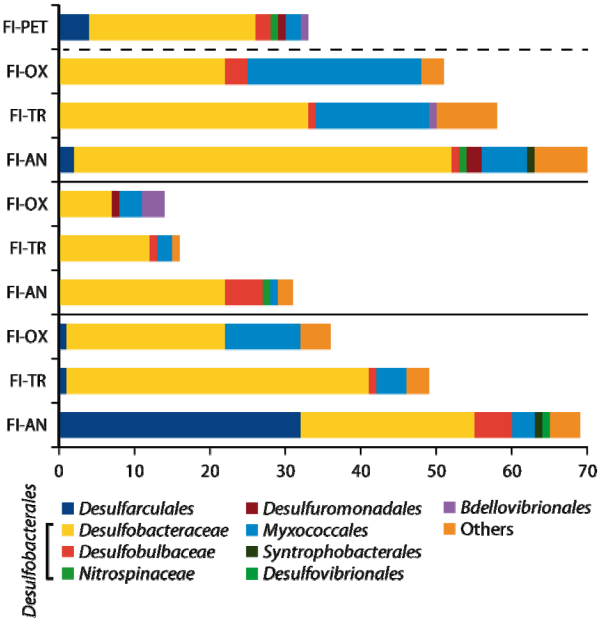


Figure 1.8. *Deltaproteobacteria* distribution detected in the 16S rRNA gene libraries from sediments samples collected at Figueiras beach in 2004 (FI) and at Figueiras and Rodas beach in 2007 (FII and RII, respectively). PET, tar aggregate collected at Figueiras in 2004; OX, TR and AN refer to the oxidized, transition and reduced zones, respectively, as described in the text (see Figure 1.1). "Others" includes Sh765B, Sva0485 and 10Bav-F6 group.

A similar analysis was carried out with the cores collected in the Rodas beach in 2007, which presented a tar aggregate at the bottom. The hydrocarbon composition of the oil patch was completely different from the oil aggregates collected in 2004 in the Figueiras

beach, suggesting a different origin of the contamination (Tables S1.2-S1.3, Figure 1.2). Interestingly, aromatic oxidizing SRB were equally abundant in the transition and reduced zones, contrarily to previous observations of a decrease with depth. This observation probably reflects the high concentration of aromatics in the deeper layers. The MPN counts of these samples were at the same level as in the transition zone of the Figueiras samples, except for the anthracene oxidizing population, which was especially high in this sediment (Figure 1.3b). Both the total cell counts (DAPI) and the proportion of EUB338 FISH-targeted cells were the lowest observed in this work, reflecting the strong toxic effect of the contaminant that seemed to promote a depletion in total cell abundances and putative active bacteria. As in FI-PET, the fraction of culturable aromatic oxidizing SRB among the putative active bacteria population was high for all aromatic tested, especially for naphthalene and benzene (0.26%) (Table 1.2). On the other hand, the distribution of aromatic degrading NRB in this beach followed the same pattern as FI samples, with highest counts in the oxidized zone and decreasing values towards the bottom. The abundance of hydrocarbon degrading aerobes was highest in the oxidized zone, but total values were lower than in FII samples (Figure 1.3b). A phylotype analysis of the 16S rRNA gene libraries revealed the lowest proportion of *Gammaproteobacteria* among all samplings, which decreased to less than 5% in the deeper layer. This decrease was confirmed in the FISH analysis using the GAM42a probe. The proportion of *Deltaproteobacteria* was high in this case, and increased towards the bottom. This was also the case for *Planctomycetes*, which were more abundant than in Figueiras samples. On the other hand, the phylum *Bacteroidetes* was abundantly represented, and decreased with depth. *Cyanobacteria*, which were abundant in the oxidized layer, decreased in the transition zone and were not detected in the deeper, anoxic zone. The Good's index for this group of samples gave diversity coverage between 0.58 and 0.28. As all libraries were constructed with an identical number of clones, these results indicate that FII-AN horizon was the most diverse of all studied (Figure 1.5).

1.4. Discussion

Relatively few studies have investigated the bacterial communities associated with hydrocarbon polluted coastal sandy sediments (Edlund *et al*., 2006; Paissé *et al*., 2008), especially in the NW Atlantic coast of the Iberian Peninsula. To our knowledge, this is one of the first characterizations of an anaerobic microbial community in subtidal coastal sediments affected by the *Prestige*'s accidental oil spill, since most microbiological studies targeted the aerobic population present in sea water and tidal beach sediment, focusing on cultivable isolates able to degrade specific compounds (Varela *et al*., 2006; Mulet *et al*., 2011). In the *Prestige* spill, oil burial by the action of beach dynamics was the main

mechanism of sediment pollution dispersal, resulting in intense subsurface oiling at several meter depths (Albaigés *et al*., 2006). Our analysis was centred on the heavily contaminated Figueiras beach, and on tar aggregates detected in Rodas sediment, and was carried out with samples taken 1.5 and 4.5 years after the accident. The different hydrocarbon composition of similar tar aggregates detected in 2004 in Figueiras and in 2007 in Rodas suggested that, in addition to the *Prestige* spill, these coastal environments had been exposed to additional sources of contamination, an expected situation in this area of intense sea shipping. Naphthalene and naphthalene derivatives were among the major components of the *Prestige* crude oil, and were also predominant in our samples. Previous work had shown that the higher solubility of naphthalene (N) increased naphthalene to methylnaphthalenes (N1) ratio from 1:3 in the crude oil to 1:1 in the soluble phase (González *et al*., 2006). It was surprising that in all samples except the tar aggregates, naphthalene was at least one order of magnitude less abundant than 2-methylnaphthalene (2MN, the main component of the N1 fraction in our samples). This could be partially due to a stronger biological activity towards naphthalene than towards its methylated derivative.

As expected, growth of aromatic oxidizers was observed in all samples with different electron acceptors. Sulfate reduction was the predominant type of respiration connected to hydrocarbon oxidative capacities, and SRB constituted the prevalent populations. Their relevance was maximal in the transition zone (FI-TR and FII-TR), as described previously in different types of non-polluted sediments (Llobet-Brossa *et al*., 2002; Bühring *et al*., 2005). However, the presence of high concentrations of hydrocarbons in deeper layers could modify this distribution, as in RII-samples, where cultivable SRB counts in the reduced zone reached levels similar to the transition zone due to the presence of the tar aggregate. Thus, the abundance of aromatic oxidizing SRB seemed to rely on both the presence of appropriate redox conditions and on the availability of suitable carbon sources. We observed the highest counts of aromatic oxidizers in the most contaminated samples (FI-PET and RII-AN), in contrast to the low total cell counts. It should be taken into account that only a fraction of viable cells are detected in the cultivation approach, thus the values of aromatic oxidizers obtained in these sediments are probably underestimated. MPN counts of SRB in Figueiras sediments were lower in 2007, which was confirmed by a lower proportion of *Deltaproteobacteria* in the 16S rRNA gene libraries.

Analysis of the 16S rRNA gene libraries obtained from all the samples revealed differences in the composition of the SRB population according to the contamination level: we used UniFrac to compare our samples using principal coordinate analysis (PCoA) based on the *Deltaproteobacteria* sequence phylogenetic affiliations obtained with ARB reconstructions (Ludwig *et al*., 2004; Lozupone *et al*., 2006). Principal component P1 and P2 could explain 33% of the variation and clearly grouped all samples except RII-AN, FI-PET and FII-OX (Figure 1.9a), suggesting that the degree of contamination was one of the major

causes selecting for specific populations within this class. Most *Deltaproteobacteria* sequences in the clustered samples belong to the order *Desulfobacterales*, mainly represented by *Desulfobacteraceae*. The ecological importance of this SRB group has been established (Leloup *et al*., 2009), but few studies report their presence in coastal polluted sediments (Paissé *et al*., 2008; Rosano-Hernández *et al*., 2012). A different situation was observed in RII-AN sample where, 46% of the sequences in RII-AN sample were affiliated to the order *Desulfarculales*, a group barely present or absent in the remaining samples except the also highly contaminated FI-PET, where these sequences reached 12% of the *Deltaproteobacteria* sequences (Figure 1.8). This order is represented by only one species with a single isolate, *Desulfarculus baarsii*, with the capability of complete oxidation of organic compounds to CO_2 (Sun *et al*., 2010). However, the 39 sequences in our samples affiliated with this group only showed an average 86% identity with *D. baarsii*, in accordance with most environmental sequences described so far belonging to this group. The isolated location of FII-OX in the PCoA analysis can be attributed to the low number of *Deltaproteobacteria* in this sample (14 sequences out of 146) and the presence of a relatively high proportion of *Bdellovibrionales* (3/14), which were absent in most of the remaining samples.

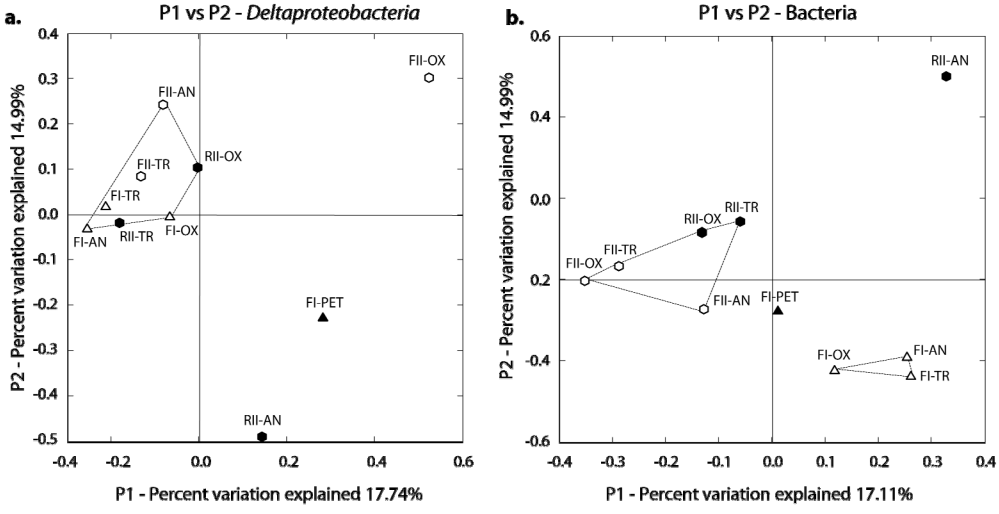


Figure 1.9. PCoA principal coordinate analysis of distances between OTUs present in the sediment samples collected from Figueiras beach in 2004 (FI) and from Figueiras and Rodas beach in 2007 (FII and RII, respectively). The percentage of the variation between the samples by principal coordinates is indicated on the axes. a) *Deltaproteobacteria*, b) All phylotypes.

A detailed analysis of *Deltaproteobacteria* sequences in all samples showed the significant presence in the sediments of strains belonging to the order *Myxococcales*, which represented the second group of *Deltaproteobacteria* in importance in these sediments (Figure 1.8). *Myxobacteria* have been generally associated with terrestrial environments,

although an increasing number of sequences of marine origin have been described in recent years (Jiang *et al*., 2010; Brinkhoff *et al*., 2011). Some cultured *Myxobacteria* are unique among prokaryotes for their singular and complex life style. By excreting hydrolytic enzymes they are able to degrade complex polymers, and they possess bacteriolytic properties (Shimkets *et al*., 2006). This group was present in all the sampled sites, especially in FI-OX sample, where they constituted as much as 45% of the *Deltaproteobacteria* sequences. In all cases, the abundance of this phylotype decreased through depth, in accordance with the finding of marine *Myxobacteria* predominantly in oxic or oxidized sediments (Brinkhoff *et al*., 2011). Our observation reinforces the possibility of this group of marine *Myxobacteria* also having an aerobic metabolism, as most terrestrial strains do (Shimkets *et al*., 2006). However, we cannot discard other metabolisms, as fermentative, respiratory denitrifier or metal reducer, also frequent in oxidized sediments. On the other hand, the abundance of *Myxobacteria* in marine sediments has been linked to grain size, and seems to be maximal in fine-grained sands (Brinkhoff *et al*., 2011). In our case, this was in accordance with a higher abundance of these sequences in the finest sand samples observed, corresponding to FI sampling (Table S1.1). A phylogenetic analysis of the *Myxococcales* sequences available in databases showed that marine *Myxobacteria* appear affiliated in several independent deeply branched clades, which indicates that this group was composed by different distant lineages able to colonize marine environments (Jiang *et al*., 2010). The closest relative of all except seven *Myxobacteria* sequences retrieved in our libraries were uncultured organisms from marine samples. Interestingly, most of them clustered within one of the main marine clusters described by Jiang *et al.* (2010) and Brinkhoff *et al.* (2012), clusters 13 and marine *Myxobacteria* cluster, respectively (Figure 1.10). No clear correlation between abundance of *Myxococcales* sequences in the different samples and the concentration of hydrocarbons was observed. Furthermore, sequences belonging to the same phylogenetic branch were retrieved from all horizon libraries.

Although nitrate reduction was the second most important respiration type linked to a degradative metabolism, NRB counts were one order of magnitude lower than those of sulfate reducers, and in the same range as aerobes. Diffusion from overlaying water and nitrification processes occurring in the oxic layer are the two major sources of nitrate in the sediment (Koike and Sorensen, 1988). Accordingly, in most cases nitrate concentration was relatively high in the oxidized and transition layers and decreased towards the bottom (Table 1.1). Therefore, the higher counts of NRB were observed in the upper layer, in agreement with denitrification being a facultative process (Gao *et al*., 2010). The only exception was FII sampling, where high counts of NRB were also observed in the transition and bottom layers, reflecting the relatively high concentration of nitrate in these horizons (FII in Figure 1.3b, Tables 1.1 and S4b). The presence of a significant population of NRB suggests they could play a relevant role in the biodegradation processes in sediments.

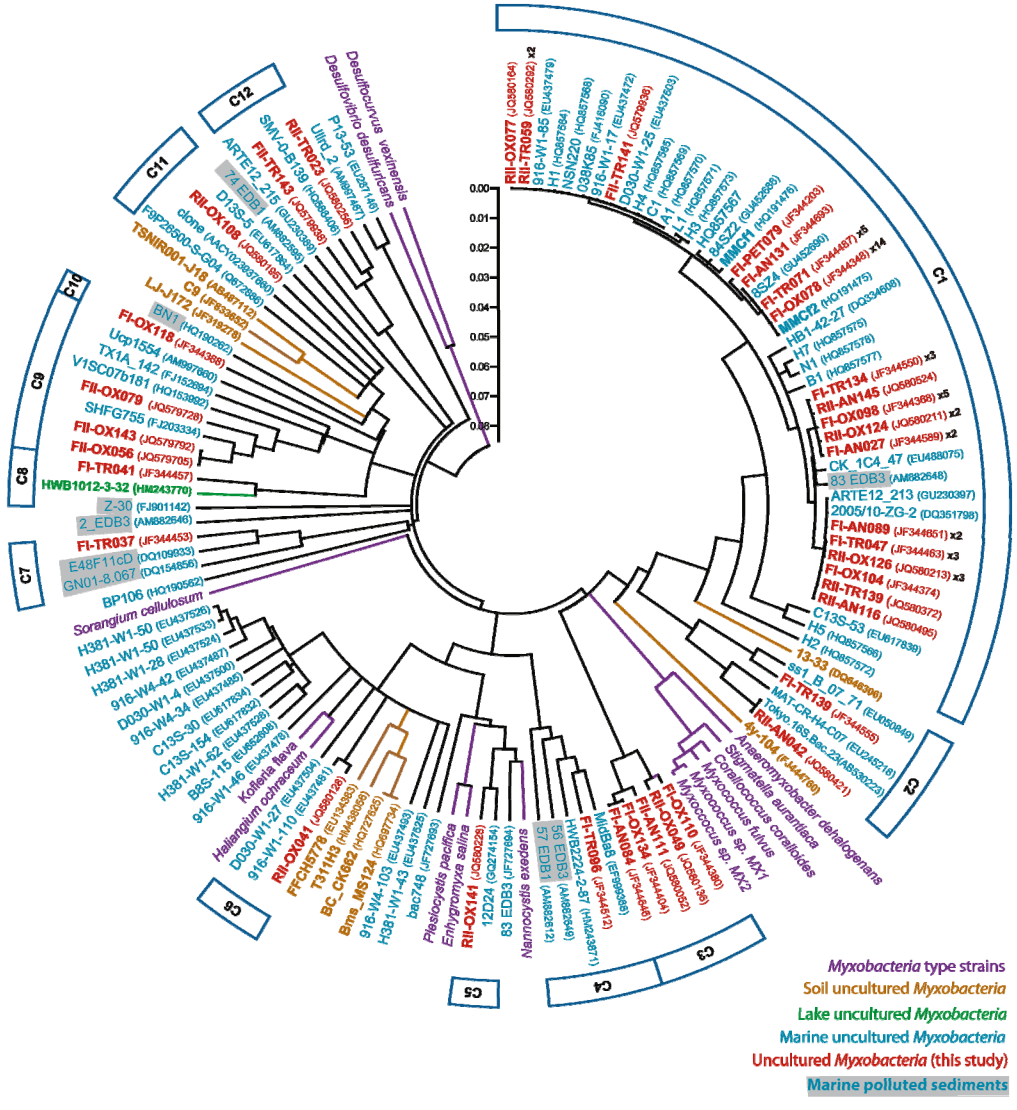


Figure 1.10. Neighbour-joining tree of *Myxobacteria* based on 16S rRNA gene sequences available in the SSU r108 database (SILVA database). A representative sequence belonging to the same OTU from each sample was selected, together with its closest relatives from marine and terrestrial environments; the number of retrieved sequences in each OTU is indicated (xn). Sequences from Brinkhoff *et al.* (2011) were aligned with the SINA-online tool and added to the database. The circle structure of the tree was made using MEGA 5.05. Clusters delimited in the tree have a phylogenetic distance of at least 10% respect to their terrestrial counterparts. Only full length sequences were considered. GenBank sequence accession numbers are given in parentheses. Different colors indicate the sample origin, as specified in the legend.

Finally, the population of IRB was almost undetectable, consistent with the possible limited availability of this acceptor in the oceans. Although *in situ* respiration rates were not determined, our results point to the coexistence of diverse respiratory metabolisms in the

different horizons, as an indication of a considerable overlap in electron acceptors in the analyzed depths, as previously suggested (Canfield and Thamdrup, 2009).

FISH results revealed that a high proportion of the bacterial population was potentially inactive, as reflected by the low detection rates. This was especially conspicuous in the most heavily polluted sample RII-AN, and to a lesser extent in FI-PET, probably because of the toxic effect of the contaminant that diminished the total cell abundances, but promoted specific sulfate reducing metabolisms (Suárez-Suárez *et al*., 2011). In fact, a strong positive response of a significant fraction of the community towards the incoming carbon source could be inferred from the high proportion of aromatic oxidizers within the population, which increased by a factor of ten in the highly contaminated sediments (FI-PET, RII-AN, Table 1.2). The diversity in the two highly polluted samples as determined in the rarefaction curves showed different values: RII-AN was the least diverse sample, while FI-PET rarefaction curve paralleled those among the most diverse samples (Figure 1.5). It seems that although petroleum contamination certainly exerts a strong selective pressure, FI-PET and RII-AN communities are subjected to additional factors and geochemical conditions that affect diversity.

The metabolic repertoire of populations is the mirror image of species diversity present in the sediments. The coordinate activity of this diversity determines the capability of response of such communities against environmental changes or stresses. Altogether, the community structure showed a great plasticity, with substantial differences between the two sampling times, suggesting sediments were constantly adapting to changing environmental factors. We performed a PCoA of all sequences obtained from the two samplings. Principal coordinates P1 and P2 explained almost equal amounts of the variation in the data (17.11% and 14.99% of the variation, respectively) (Figure 1.9b). PC1 separated samples according to the sampling times except for the highly polluted RII-AN, which positioned at the positive end of both PC, and FI-PET, which had values near zero for both PC. Figueiras beach community structure was initially dominated by *Gamma-* and *Deltaproteobacteria*, while in 2007 the phylum *Bacteroidetes*, almost absent in 2004, was a main component of the community. Within this class, *Flavobacteria*, and to a lesser extent *Sphyngobacteria*, were predominant in 2007 samples. *Bacteroidetes* are generally associated with fermentative metabolism related to labile high-molecular-weight organic matter degradation under conditions of high nutrient concentrations, and could respond to summer upwelling of algal blooms, or to fermentation processes (Rosselló-Mora *et al*., 1999; Gomez-Pereira *et al*., 2010). Their exact function in the ecosystem is unknown, but it has been connected to the degradation of phytoplankton-derived polymers like peptidoglycan, proteins, and different types of polysaccharides (Gomez-Pereira *et al*., 2012). In 2007 the overall species distribution in Rodas and Figueiras was similar, despite the 600 m distance between the two sampling sites and the slight differences in sand

characteristics (Table S1.1). However, there was little overlap of operational taxonomic units (OTUs) between samples, although the OTUs shared by all the samples were also the most abundantly represented in the libraries.

Generally, the extent of subsurface pollution after oil spill incidents tends to be underestimated (Bernabeu *et al*., 2009). The remediation of these long-term polluted, hardly accessible, areas will depend on the activity of the natural populations present at each contaminated site. We could envisage that after a sudden income of high concentrations of a new carbon source such as a crude oil spill, with toxic effects on bacterial integrity, bacterial communities will react according to their resistance capacity and to their metabolic abilities to use specific oil components as substrate. As we have shown, in this process the availability of different electron acceptors will definitely influence the redox activities prevailing in each case, resulting in an enrichment of the fitter and best degrader strains.

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Suplementary material

(Chapter I)

Table S1.1. Samples description with some geophysical parameters.

	Sample*		Depth (cm)	D[a]	P[b]	Sand grain size (%)[c]				Sorting[d] (σ_1)	Median size D_{90}(mm)
						coarse	medium	fine	very fine		
2004	FI	OX[1]	2-5	1.65	0.38	1.2 ± 0.6	17.9 ± 3.4	24.5 ± 3.7	**55.0 ± 2.2**	0.643	0.335
		TR[2]	11-14	1.65	0.38	0.8 ± 0.0	17.7 ± 1.3	30.4 ± 0.4	**50.6 ± 1.2**	0.595	0.330
		AN[3]	32-35	1.63	0.39	2.7 ± 1.7	29.7 ± 3.6	27.6 ± 5.1	**38.7 ± 0**	0.718[ms]	0.382
		PET[4]	1-15	1.74	0.35	0.8 ± 0.2	**82.4 ± 71**	7.4 ± 3.7	8.8 ± 3.8	0.393[ws]	0.373
2007	FII	OX[1]	2-5	1.64	0.38	6.3 ± 0.6	30.7 ± 1.9	**44.7 ± 0.0**	15.6 ± 1.9	0.554	0.373
		TR[2]	12-15	1.64	0.38	12.4 ± 1.5	26.7 ± 4.7	**40.6 ± 1.7**	18.0 ± 1.4	0.677	0.491
		AN[3]	32-35	1.62	0.39	11.6 ± 0.6	32.1 ± 1.3	**39.6 ± 0.1**	13.4 ± 1.3	0.655	0.589
	RII	OX[1]	2-5	1.56	0.42	32.9 ± 2.5	**53.6 ± 2.7**	7.0 ± 0.0	2.5 ± 0.0	0.562	0.661
		TR[2]	9-13	1.54	0.42	28.3 ± 1.9	**51.4 ± 1.5**	9.9 ± 0.8	5.6 ± 0.59	0.653	0.660
		AN[4]	23-27	1.77	0.34	11.0 ± 0.1	**75.3 ± 0.1**	5.5 ± 0.7	2.8 ± 0.3	0.640	0.682

* Samples were taken by triplicate except for FI-PET.

[a] Density (variances below ± 0.03)

[b] Porosity (variances below ± 0.01)

[c] Wentworth scale. The most abundant grain size in each sample is in bold.

[d] Sorting coefficient calculated with GRADISTAT v 8.0

[ms] moderately sorted; [ws] well sorted

[1] Nitrogenous zone (probably oxygen influenced). White sand.

[2] Transition zone (nitrogenous-sulfidic zone).Mixed white-grey sand.

[3] Sulphidic zone. Grey sand. Sulfide odor.

[4] Gray sand mixed with residuary petroleum.

Table S1.2a. Aliphatic hydrocarbon composition in the sediments from 2004 sampling

Compound	FI (ppm)				Prestige Oil (µg/g)[2]
	OX	TR	AN	PET	
nC10	0.16 ± 0.23	0.16 ± 0.12	nd	nd	no data
nC11	4.11 ± 1.82	1.96 ± 0.56	0.26 ± 0.16	5.62 ± 5.07	no data
nC12	0.38 ± 0.08	0.34 ± 0.09	0.16 ± 0.07	5.49 ± 3.43	365
nC13	0.75 ± 0.4	0.47 ± 0.08	0.32 ± 0.16	9.10 ± 3.43	550
nC14	0.43 ± 0.12	0.36 ± 0.10	0.28 ± 0.13	12.06 ± 3.42	657
nC15	2.22 ± 1.59	1.11 ± 0.27	0.90 ± 0.52	9.28 ± 2.02	665
nC16	0.55 ± 0.23	0.35 ± 0.08	0.32 ± 0.16	9.48 ± 2.93	623
nC17[1]	2.38 ± 1.64	1.20 ± 0.37	1.06 ± 0.59	24.78 ± 12.22	751
nC18	nd	0.44 ± 0.13	0.43 ± 0.18	10.69 ± 2.47	487
nC19	1.30 ± 0.7	0.83 ± 0.29	0.76 ± 0.40	10.54 ± 5.58	656
nC20	nd	nd	nd	8.91 ± 3.28	681
Phytane	nd	nd	nd	10.11 ± 3.89	382
nC21	nd	nd	nd	8.72 ± 2.23	676
nC22	nd	nd	nd	9.63 ± 2.29	594
nC23	nd	nd	nd	10.09 ± 1.24	720
nC24	nd	nd	nd	12.01 ± 3.37	795
nC25	nd	nd	nd	9.37 ± 0.97	804
nC26	nd	0.15 ± 0.21	nd	10.66 ± 1.18	827
nC27	nd	nd	nd	9.64 ± 1.56	653
nC28	nd	nd	nd	9.15 ± 1.21	538
nC29	nd	nd	nd	9.31 ± 1.32	484
nC30	nd	nd	nd	9.53 ± 1.50	661
nC31	nd	nd	nd	8.89 ± 0.64	564
nC32	nd	nd	nd	8.23 ± 0.84	400
nC33	nd	nd	nd	7.75 ± 0.59	377
nC34	nd	0.79 ± 1.12	nd	7.28 ± 0.90	351
nC35	nd	nd	nd	7.19 ± 1.76	459
nC36	nd	nd	0.55 ± 0.78	3.42 ± 1.74	365
nC37	nd	nd	nd	3.36 ± 0.33	550
nC38	nd	nd	nd	3.04 ± 0.31	657

[1] Sum of nC17 and pristane
[2] Data from Alzaga *et al.*, 2004
nd: not detected

Table S1.2b. Aliphatic hydrocarbon composition in the sediments from 2007 sampling

Compound	FII (ppm)			RII (ppm)		
	OX	TR	AN	OX	TR	AN
nC10	0.37	nd	nd	nd	nd	nd
nC11	2.40 ± 0.20	3.00 ± 0.08	1.83 ± 0.37	2.26 ± 0.41	1.18 ± 0.31	4.46 ± 0.69
nC12	0.37 ± 0.04	1.10 ± 0.10	0.36 ± 0.05	0.31 ± 0.03	45.59 ± 19.7	nd
nC13	0.65 ± 0.07	0.80 ± 0.06	nd	0.48 ± 0.04	nd	1.08 ± 0.07
nC14	0.45 ± 0.03	0.47 ± 0.07	0.33 ± 0.03	0.46 ± 0.01	1.13 ± 0.17	1.47 ± 0.10
nC15	1.33 ± 0.12	2.46 ± 0.22	1.37 ± 0.03	1.18 ± 0.15	1.89 ± 0.36	3.13 ± 0.40
nC16	0.32 ± 0.07	0.52 ± 0.05	0.33 ± 0.01	0.41 ± 0.03	0.77 ± 0.04	nd
nC17[1]	1.42 ± 0.09	2.70 ± 0.39	1.59 ± 0.21	1.13 ± 0.07	0.73 ± 0.04	nd
nC18	0.57 ± 0.07	0.64 ± 0.03	0.38 ± 0.04	0.42 ± 0.02	nd	13.20 ± 1.02
nC19	1.04 ± 0.09	1.83 ± 0.23	1.04 ± 0.14	0.81 ± 0.17	1.53 ± 0.23	4.74 ± 1.18
nC20	nd	nd	nd	nd	0.68 ± 0.03	nd
Phytane	nd	nd	nd	nd	nd	nd
nC21	nd	nd	nd	nd	0.42 ± 0.05	8.01 ± 0.83
nC22	nd	nd	nd	nd	nd	11.11 ± 1.97
nC23	nd	nd	nd	0.19 ± 0.01	nd	19.21 ± 1.54
nC24	nd	nd	nd	0.59 ± 0.11	nd	53.58 ± 8.92
nC25	nd	nd	nd	0.27 ±0.01	nd	84.73 ± 7.38
nC26	0.52 ± 0.06	nd	nd	nd	nd	155.54 ± 8.33
nC27	nd	nd	nd	nd	nd	225.1 ± 22.0
nC28	nd	nd	nd	nd	nd	271.1 ± 18.09
nC29	nd	nd	nd	nd	nd	339.08 ± 30.9
nC30	nd	nd	nd	nd	nd	290.61 ± 26.4
nC31	nd	nd	nd	nd	nd	404.51 ± 8.42
nC32	nd	nd	nd	nd	nd	478.40 ± 64.0
nC33	nd	nd	nd	nd	nd	475.76 ± 43.4
nC34	2.68 ± 0.39	nd	1.48 ± 0.17	1.59 ± 0.09	nd	415.6 ± 24.81
nC35	nd	nd	nd	nd	nd	422.45 ± 9.10
nC36	nd	nd	nd	nd	nd	408.83 ± 28.3
nC37	nd	nd	nd	nd	nd	300.66 ± 39.7
nC38	nd	nd	nd	nd	nd	165.31 ± 12.4

[1] Sum of nC17 and pristane
nd: not detected

Table S1.3a. PAH composition in the sediment samples from 2004 sampling.

Compound	Fl (ppm)				Prestige Oil (µg/g)[1]	Sand[2] (ppb)
	OX	TR	AN	PET		
N	0.08 ± 0.03	0.08 ± 0.02	0.07 ± 0.02	4.31 ± 2.91	375	0.7
N1[3]	1.43 ± 0.42	0.62 ± 0.19	0.06 ± 0.03	19.09 ± 9.67	1076	1.0
N2	dnq	nd	nd	dnq	1232	1.2
N3	nd	nd	nd	37.3 ± 22.0	1017	2.6
N4	nd	nd	nd	19.4 ± 7.19	no data	no data
D	nd	nd	nd	0.72 ± 0.53	107	<dl
Ph	nd	0.03 ± 0.02	0.02 ± 0.00	1.74 ± 0.24	339	1.43
Ph1	nd	nd	nd	3.96 ± 2.24	892	1.8
Ph2	nd	nd	nd	dnq	886	3.0
Ph3	nd	nd	nd	dnq	592	5.7
Ph4	nd	nd	nd	dnq	no data	no data
A	nd	0.18 ± 0.10	0.02 ± 0.00	0.52 ± 0.53	<ld	<dl
Py	nd	nd	nd	nd	117	3.6
Py1	nd	nd	nd	dnq	no data	12.3
Bfl	nd	nd	nd	dnq	no data	no data
Fl	nd	nd	nd	dnq	nq	<dl

Naphthalene (**N**), 1-methylnaphthalene (**1-MN**), 2-methylnaphthalene (**2-MN**), dimethylnaphthalene (**N2**), trimethylnaphthalene (**N3**), tetramethylnaphthalene (**N4**), dibenzothiophene (**D**), Phenanthrene (**Ph**), methylphenanthrene (**Ph1**), Dimethylphenanthrene (**Ph2**), Trimethylphenanthrene (**Ph3**), Tetramethylphenanthrene (**Ph4**), Anthracene (**A**), Pyrene (**Py**), Methylpyrene (**Py1**), Benzofluorene (**Bfl**), Fluoranthene (**Fl**)

Data in ppm (µg/g)
[1] Data from *Prestige* oil, Alzaga *et al*., 2004.
[2] Data at 61 cm depth (µg/Kg) from Bernabeu *et al*., 2009
[3] Sum of 1-MN and 2-MN.
nd: not detected.
nq: not quantified
<dl: below limit of detection
dnq: detected but not quantified because of the absence of internal standards

Table S1.3b. PAH composition in the sediment samples from 2007 sampling

Compound	FII (ppm)			RII (ppm)		
	OX	TR	AN	OX	TR	AN
N	0.05 ± 0.02	0.05 ± 0.01	0.16 ± 0.60	0.07 ± 0.03	0.82 ± 0.15	64.8 ± 8.31
N1[3]	1.92 ± 0.48	2.64 ± 0.65	1.47 ± 0.13	2.25 ± 0.06	2.47 ± 0.49	101.9 ± 16.2
N2	nd	nd	nd	nd	nd	dnq
N3	nd	nd	nd	nd	nd	167.3 ± 27.0
N4	nd	nd	nd	nd	nd	45.6 ± 11.8
D	nd	nd	nd	nd	nd	nd
Ph	nd	nd	nd	nd	0.24 ± 0.17	35.7 ± 7.39
Ph1	nd	nd	nd	nd	nd	<dl
Ph2	nd	nd	nd	nd	nd	dnq
Ph3	nd	nd	nd	nd	nd	dnq
Ph4	nd	nd	nd	nd	nd	dnq
A	nd	nd	nd	nd	0.22 ± 0.04	5.7 ± 1.7
Py	nd	nd	nd	nd	nd	nd
Py1	nd	nd	nd	nd	nd	nd
Bfl	nd	nd	nd	nd	nd	nd
Fl	nd	nd	nd	nd	nd	nd

Naphthalene (**N**), 1-methylnaphthalene (**1-MN**), 2-methylnaphthalene (**2-MN**), dimethylnaphthalene (**N2**), trimethylnaphthalene (**N3**), tetramethylnaphthalene (**N4**), dibenzothiophene (**D**), Phenanthrene (**Ph**), methylphenanthrene (**Ph1**), Dimethylphenanthrene (**Ph2**), Trimethylphenanthrene (**Ph3**), Tetramethylphenanthrene (**Ph4**), Anthracene (**A**), Pyrene (**Py**), Methylpyrene (**Py1**), Benzofluorene (**Bfl**), Fluoranthene (**Fl**)

Data in ppm (µg/g)
[1] Data from *Prestige* oil, Alzaga *et al*., 2004.
[2] Data at 61 cm depth (µg/Kg) from Bernabeu *et al*., 2009
[3] Sum of 1-MN and 2-MN.
nd: not detected.
nq: not quantified
<dl: below limit of detection
dnq: detected but not quantified because of the absence of internal standards

Table S1.4a. Most Probable Number Counts of hydrocarbon oxidizing bacteria from the first sampling.

electron acceptor	C source	FI							
		OX		TR		AN		PET	
		MPN counts	95% C.I.	MPN counts	95% C.I	MPN counts	95% C.I	MPN counts	95% C.I.
sulfate ($x10^3$)	**A**	0.5	0.2-1.1	0.4	0.2-0.9	0.1	0.07-0.4	12.8	3.2-50.2
	N	3.3	1.5-7.3	7.1	3.4-14.7	4.4	2.1-9.2	12.8	3.2-50.2
	T	0.7	0.3-1.4	7.2	3.5-14.9	4.4	2.1-9.2	7.6	0.2-26.8
	B	1.7	0.3-5.0	5.4	2.6-11.2	3.1	1.3-6.8	12.8	3.2-50.2
	Ac	6.1	3-12.6	8.4	4.0-17.5	5.5	2.6-11.4	48.9	3.4-57.5
nitrate ($x10^2$)	**A**	1.1	0.5-2.6	0.8	0.3-1.6	0.3	0.1-0.8	14.1	3.4-57.5
	N	7.7	3.7-16	2.6	1-1-6.0	1.2	0.5-2.6	9.1	2.5-33
	T	0.9	0.4-2.1	0.4	0.2-1.0	0.7	0.3-1.6	6.6	1.9-23
	B	4.8	2.3-10.1	0.8	0.4-1.7	0.7	0.3-1.4	3.0	0.7-12.2
	Ac	35.2	16-76.5	18	7.7-41.8	46.7	22-97.6	141	34-575
FeOH (x10)	**A**	-	-	-	-	-	-	3.0	0.7-12.3
	N	2.1	0.8-5.2	1.6	0.5-4.3	1.1	0.3-3.5	3.0	0.7-12.3
	T	1.1	0.3-3.6	1.1	0.3-3.6	1.1	0.3-3.5	2.4	0.5-10.9
	B	0.3	0.04-2.4	0.3	0.04-2.4	0.3	0.04-2.4	4.8	1.3-17.4
	Ac	11.4	5.2-24.5	9.3	4.4-19.5	3.3	1.5-7.3	30.6	7.6-122
oxygen ($x10^2$)	**A**	57.3	34.3-95.8	29.6	18.1-48.5	7.5	4.6-12.5	1.4	0.04-4.4
	N	26.1	16-42.7	10.5	6.3-17.5	2.1	1.2-3.8	2.27	0.08-0.5
	Ph	15.4	9.2-25.6	6.2	3.8-10.2	4.3	2.6-7.6	2.27	0.08-0.5
	Py	28.6	17.5-46.8	4.7	2.9-7.7	4.6	2.8-7.6	1.4	0.0
	Hx	10.5	6.2-17.4	2.2	1.3-3.9	2.1	1.2-3.8	-	-
	Un	15.4	9.2-25.6	3.5	2.1-5.9	3.5	2.1-5.9	-	-

Anthracene (**A**); Naphthalene (**N**); Toluene (**T**); Benzene (**B**); Phenathrene (**Ph**), Pyrene (**Py**), Hexane (**H**), Undecane (**Un**), Acetate (**Ac**). No growth (-)

Table S1.4b. Most Probable Number Counts of hydrocarbon degrading bacteria from the second sampling.

electron acceptor	C source	FII						RII					
		OX		TR		AN		OX		TR		AN	
		MPN counts	95% C.I.	MPN counts	95% C.I.	MPN counts	95% C.I.	MPN counts	95% C.I.	MPN counts	95% C.I.	MPN counts	95% C.I.
sulfate ($x10^3$)	**A**	0.3	0.1-0.9	2.7	0.9-7.4	0.5	0.2-1.4	0.5	0.2-1.4	4.6	1.8-11.4	4.8	1.9-11.8
	N	1.0	0.4-2.5	4.6	1.8-11.4	1.2	0.4-3.1	0.2	0.07-2.6	4.6	1.8-11.4	2.7	0.9-7.4
	T	0.2	0.08-0.6	0.9	0.3-2.4	0.3	0.1-0.8	0.2	0.07-	1.0	0.4-2.5	2.0	0.7-5.7
	B	0.2	0.07-0.5	0.7	0.3-1.8	0.4	0.1-1.2	0.3	0.1-0.9	0.3	0.1-0.8	1.2	0.4-3.2
	Ac	2.7	0.9-7.4	3.4	1.3-8.9	8	3.3-19.6	2.0	0.7-5.7	7.6	3.1-18.6	8.6	3.5-21.2
nitrate ($x10^2$)	**A**	2.7	0.9-7.4	0.7	0.2-1.6	4.6	1.8-11.4	3.9	1.5-9.9	3.9	1.5-9.9	1.0	0.4-2.5
	N	3.0	1.-8.2	3.0	1.1-8.1	5.8	2.4-14.1	10.3	4.8-25.8	1.2	0.4-3.1	2.0	0.7-5.7
	T	3.4	1.3-8.9	1.4	0.5-3.8	1.1	0.4-2.9	1.0	0.4-2.5	0.6	0.2-1.6	0.7	0.3-1.7
	B	0.9	0.3-2.4	0.5	0.2-1.3	0.7	0.3-1.9	3.9	1.5-9.9	1.0	0.4-2.6	1.0	0.4-2.5
	Ac	7.01	2.9-16.9	3.9	1.5-9.9	10.3	4.8-25.8	8.8	3.6-21.7	5.8	2.4-14.1	5.9	2.4-14.3
FeOH (x10)	**A**	-	-	-	-	-	-	-	-	-	-	-	-
	N	4.6	1.8-11	-	-	3.0	1.1-8.1	-	-	5.9	2.4-14.3	3.0	1.1-8.1
	T	-	-	1.9	0.6-6.1	-.	-	-	-	-	-	-	-
	B	-	-	0.5	0.07-3.8	-	-	-	-	-	-	-	-
	Ac	7.6	3.1-18.6	18.8	6.6-53.2	76.8	31.7-186	4.8	1.9-12	20.5	7.2-57.9	14.1	5.2-38.1
oxygen ($x10^2$)	**A**	9.8	5.3-18.4	3.5	1.8-6.5	0.85	0.3-2.2	7.4	4.0-13.8	1.3	0.6-3.0	0.3	0.09-1.5
	N	11.2	5.9-20.9	7.6	4.3-14.7	1.8	0.8-3.7	6.4	3.5-11.7	5.6	3.1-10.4	0.9	0.4-2.4
	Ph	9.8	5.3-18.4	2.6	1.3-5.5	0.5	0.1-1.8	4.7	2.5-8.6	2.9	1.5-5.5	0.3	0.09-1.5
	Py	7.2	3.9-13.3	1.1	0.4-2.7	0.1	0.02-1.8	4.7	2.6-8.7	0.3	0.09-0.1	-	-
	Hx	9.8	5.3-18.4	2.9	1.5-5.5	0.5	0.1-1.8	4.7	2.6-8.7	0.6	0.01-0.1	-	-
	Un	14.4	7.7-26.8	3.4	1.8-6.4	1.0	0.4-2.5	7.5	4.0-13.8	1.0	0.4-2.5	0.18	0.02-1.3

Anthracene (**A**); Naphthalene (**N**); Toluene (**T**); Benzene (**B**); Phenathrene (**Ph**), Pyrene (**Py**), Hexane (**H**), Undecane (**Un**), Acetate (**Ac**). No growth (-)

Table S1.5a. FISH counts of bacterial cells in the sediment samples

Sample			Cells (mean ± SD)		Percentage of hybridized cells					
			DAPI ($x10^7$ ml^{-1})	EUB 338 ($x10^6$ ml^{-1})	EUB/ DAPI	CF33 9a/ EUB	GAM42a/ EUB	DSV/ EUB	DSS/ EUB	DSB/ EUB
2004	FI	OX	15.4 ± 2.0	27.7 ± 7.5	18.11	1.17	19.68	nd	2.53	nd
		TR	17.9 ± 2.6	39.0 ± 7.0	21.77	3.70	20.12	nd	20.75	nd
		AN	11.9 ± 1.9	22.3 ± 5.7	18.55	0.55	8.09	0.55	26.11	nd
		PET	2.8 ± 0.6	4.8 ± 1.6	17.33	3.84	30.76	nd	5.76	1.92
2007	FII	OX	27.1 ± 5.1	47.9 ± 1.5	17.66	3.75	24.57	nd	1.88	nd
		TR	22.6 ± 4.4	40.6 ± 9.9	18.0	9.20	13.87	nd	12.09	nd
		AN	19.1 ± 3.8	29.0 ± 8.4	15.16	1.0	8.79	nd	27.5	nd
	RII	OX	22.0 ± 5.4	34.9 ± 7.9	15.83	1.0	14.55	nd	5.22	nd
		TR	13.6 ± 2.7	26.4 ± 6.8	19.33	4.26	14.5	nd	25.9	nd
		AN	1.39 ± 0.4	1.1 ± 0.5	8.50	3.96	5.75	2.17	21.92	1.78

nd= not detected

Archaea were not detected with probe ARCH915

Table S1.5b. Efficiency of probes against 16S rRNA gene libraries

	Probe	Clones in the library[1] (a)	Positive matches (b)	Matches against positive target (c)	Unspecific matches (b-c)	Non detected (a-c)
FI-PET	DSV 698	1	0	0	-	1(100%)
	DSS 658	17	15	11	4 (17%)	6(35%)
	DSB985	6	6	6	-	-
FII-OX	DSS 658	7	9	7	2 (2%)	-
FII-TR	DSS 658	12	14	12	2 (16%)	-
FII-AN	DSS 658	22	24	21	3 (13%)	1 (4,5%)

[1] According to SILVA classification.

Chapter II

Microbial diversity of marine oil-polluted coastal sediments: *state of the art*.

2.1 Introduction

Microbial biodegradation is the major environmental process affecting the fate of hydrocarbons in both terrestrial and aquatic ecosystems. Autochthonous microorganisms have become the basis for many *in situ* bioremediation strategies in contaminated sites. Describing and understanding the variables affecting hydrocarbon biodegradation process, i.e. the involved pathways, the degradation kinetics and the optimal degradation conditions, are important to evaluate if natural attenuation would be feasible in the impacted ecosystems (Watanabe *et al.*, 2002; Lu *et al.*, 2011b). The description of the diversity carrying out these processes is vital not only for the basic scientific knowledge, but also to help implement effective bioremediation projects through the analysis of the dynamics and structure of microbial communities.

Different lines of evidence confirm that microbial communities actively respond to the presence of hydrocarbons in marine sediments: most probable number counts of hydrocarbon oxidizers are highest in polluted sites (Bachoon *et al.*, 2001; Beazley *et al.*, 2012; Mortazavi *et al.*, 2013); more active groups within autochthonous bacterial populations are detected (Kolukirik *et al.*, 2011; Suárez-Suárez *et al.*, 2011); the diversity and abundance of functional genes related to biodegradation are higher in comparison with pristine environments (van der Meer, 2006; Beazley *et al.*, 2012; Acosta-González *et al.*, 2013a). However, important negative effects on the microbial communities are also observed: the total cell counts can be drastically reduced despite percentages of bacterial active populations remaining stable (Suárez-Suárez *et al.*, 2011; Acosta-González *et al.*, 2013b).

The response of microbial communities in oil-polluted sediments depends on environmental factors like temperature, petroleum composition, time of exposure and quantity of contaminants present in the sediment. In addition the availability of electron acceptors could result in a switch between anaerobic/aerobic redox conditions, which seems to favor hydrocarbon degradation (Cravo-Laureau *et al.*, 2011; Das and Chandran, 2011; Rocchetti *et al.*, 2011; Liu and Liu, 2013). The particular sediment's geochemistry and its geophysical characteristics greatly influence the microbial distribution (Kolukirik *et al.*, 2011). A recent survey analyzing available data from the global International Census of Marine Microbes (ICoMM) suggested that marine environments harbor a persistent microbial seed (Gibbons *et al.*, 2013), although fluctuations in the environmental conditions can modify the structure of the microbial communities. If exposure to pollution is prolonged in time, microbial populations become more adapted. Evidence suggests that the previous contamination history is a determining factor in the bacterial population response to the presence of contaminants. The absence of a prior contact with pollutants limits the response capacity to oil contamination in pristine sediments (Head and Swannell,

1999; Païssé *et al.*, 2010), although with exceptions (Suárez-Suárez *et al.*, 2011). On the other hand, once the substrates have been consumed (or become less available) the numbers of cultivable contaminant oxidizers decrease (Head *et al.*, 2006). Active biodegradation of residual petroleum apparently decreases and even ceases after a prolonged period of time, due to organic matter preference and to the prevalence of non-biodegradable recalcitrant oil fractions (Díez *et al.*, 2005; Slater *et al.*, 2005; Pearson *et al.*, 2008). Finally, grazing activities of protists and the meiofauna alter the size of bacterial oxidizers populations, causing positive or negative effects on the microbial biodegradation activities when sediments are exposed to pollutants (Näslund *et al.*, 2010; Stoeck and Edgcomb, 2010). Bioturbation can have a positive influence and stimulate biodegradation by distributing contaminants through the sediments (Timmermann *et al.*, 2011).

Aerobic oxidizing bacteria are widespread in nature but the low availability of oxygen in the subsurface of sediments limits their metabolic activity (Holliger *et al.*, 1997). Initially, natural attenuation of hydrocarbons in anoxic environments was reticent to be recognized due to the absence of clear evidence of *in situ* anaerobic degradation (Harayama *et al.*, 1992; Harayama *et al.*, 1999). However, in groundwater systems, iron and nitrate respiration are frequently the dominant metabolism associated to biodegradation (Christensen *et al.*, 2000; Winderl *et al.*, 2007). In coastal sediments, the dominant respiration process is through sulfate reduction (Skyring, 1987) and shelf sediments could be responsible for up to 90% of global marine sulfate reduction (Jorgensen, 1982). This process is the recurrent metabolism associated with hydrocarbon degradation in marine sediments, especially below the superficial zone of oxygen influence (Coates *et al.*, 1997; Lu *et al.*, 2011b). Nitrate reduction coupled to natural attenuation of hydrocarbons as an alternative metabolism has not been extensively characterized in polluted sediments and is expected to be limited by the availability of nitrate (MacRae and Hall, 1998; Lu *et al.*, 2011a). In polluted subtidal sediments, nitrate reduction has been reported to take place *in situ* at a minor scale (Acosta-González *et al.*, 2013b). However, studies demonstrating the importance of nitrate reduction over sulfate reduction in simulated experiments have shown the potential of denitrification in bioremediation practices in marine habitats (Lu *et al.*, 2012). Therefore, in sediments receiving an important influx of nitrate from anthropogenic sources, the activity of nitrate reducing bacteria (NRB) could be significantly increased and thus their degradative capacities accelerated. The addition of nitrate to accelerate the bioremediation process in marine sediments has been tested with positive results (MacRae and Hall, 1998; Rockne and Strand, 1998). Fermentative metabolism could be important for the degradation mediated by syntrophic interactions (Head *et al.*, 2006). Addition of iron (Lovley and Phillips, 1987) or manganese oxides (Canfield *et al.*, 1993) to marine sediments switched the sulfate reduction process, but biodegradation was apparently not stimulated during the presence of terminal electron acceptors different to sulfate (Coates *et al.*, 1996).

Coastal sediments include beaches (littoral zones where tidal, intertidal and subtidal zones are differentiated), wetlands, mangroves, estuaries and salt marshes along the coastline of continents. Characterization of the microbial diversity in oil affected beaches has predominantly focused intertidal rather than subtidal zones, and most studies mainly analyzed the bacterial communities from the upper oxic-influenced sediment layer. In order to describe the diversity and structure of microbial communities in habitats from coastal sediments, we decided to review and compare different available information. We compiled and reviewed i) the cultivated diversity and ii) the diversity studies from marine polluted sites available in the literature; and we analyzed diversity data from iii) classical molecular approaches, and iv) next generation sequencing (NGS) data to determine if diversity patterns could be detectable.

2.2. Representatives of cultivable diversity

Degraders are generally less metabolically versatile in marine habitats as compared to terrestrial habitats (Yakimov *et al.*, 2007). Many bacterial strains with degradative capacities have been isolated from the environment. However, in most cases they have been shown to only make a minor contribution to the actual *in situ* degradation of the contaminants, and to be phylogenetically distant from the bacteria identified using molecular methods (Harayama *et al.*, 1999; Watanabe, 2001). It is generally accepted that identifying and isolating the specific degraders in the environment is not an easy task, and several methods to improve the isolation of degrading strains have been proposed (Huang *et al.*, 2008; Bollmann *et al.*, 2010). In the case of marine sediment, hydrocarbon oxidizing isolates are not abundant as compared with soils or aquifers (Jin *et al.*, 2012). A list of the bacterial species isolated from coastal marine sediments described in the literature as able to oxidize hydrocarbons aerobically and anaerobically is provided in Table 1. Aerobic degraders were usually isolated from intertidal sediments, where generally only the first centimeters of the sediment column had been examined. Isolated strains belong mainly to the *Gammaproteobacteria*. Strains of *Alteromonas* were reported as key players of aerobic hydrocarbon oxidation in tidal sediments (Jin et al., 2012). *Acinetobacter* isolates degraded more than 90% of oil compounds compared to control cultures (Kostka *et al.*, 2011). Genera like *Cyclocasticus* and *Alcanivorax* are widespread in coastal polluted sediments, associated to the degradation of aromatic and aliphatic compounds respectively (Geiselbrecht *et al.*, 1998; Kasai *et al.*, 2002; Harayama *et al.*, 2004). *Cyclocasticus* is a genus restricted to marine habitats and the type species *C. pugetti* was able to degrade aerobically many aromatic compounds (Dyksterhouse *et al.*, 1995; Staley, 2010). Analysis of *Alcanivorax* genome revealed this genus is highly adapted to the degradation of petroleum and cannot use sugars or amino acids as carbon sources (Yakimov *et al.*, 1998). *Alcanivorax dieseoli* could

degrade more than 90% of oil compare and prevailed in oil-contaminated seawater and sediments oxidizing a broad range of alkanes (Hara *et al*., 2003; Kostka *et al*., 2011). In Gulf of Mexico supratidal sediments affected by the *Deep Water Horizon* (DWH) spill, *Alcanivorax* populations increased up to ten times and were especially abundant in the heaviest polluted samples (Kostka *et al*., 2011). In contrast to *Alcanivorax*, *Marinobacter*, *Vibrio* and *Acinetobacter* strains can utilize a broad range of oil-carbon sources (Kostka *et al*., 2011). Denitrifying *Pseudomonas* have been isolated from intertidal sediments (Mulet *et al*., 2011). *Bacteroidetes* are recurrent inhabitants of polluted coastal sediments, although only the species *Yeosuana aromativorans*, a strict aerobic degrader, has been isolated (Kwon *et al*., 2006; Kim and Kwon, 2010). Pure cultures of *Firmicutes* alkane and naphthalene degraders, or consortia growing in oil-enrichments were eventually obtained from coastal polluted and unpolluted sediments (Engelhardt *et al*., 2001; Zhuang *et al*., 2002; Zhuang *et al*., 2003).

Considering that sulfate reduction is the dominant respiratory metabolism found in anoxic marine sediments, it is not a surprise that anaerobic degraders isolated from coastal sediments are almost limited to the *Deltaproteobacteria* class (Table 1) (Nunoura *et al*., 2007). Considerable advances in the current knowledge on the biochemistry and genetics of anaerobic degradation of alkanes, toluene and naphthalenes in sulfate reducing bacteria (SRB) have been obtained through the characterization of the strains *Desulfobacula toluolica* tol2, *Desulfatibacillum alkenivorans* AK-01 and the SRB NaphS2/NaphS3 (Rabus and Widdel, 1995; So and Young, 1999; Musat *et al*., 2008). The genus *Desulfatibacillum* is an example of the specialization of *Deltaproteobacteria* in marine sediments: all isolated species from this genus have degradative capacities. *Desulfotignum*, *Desulfobacter*, *Desulfatibacillus*, *Desulfosalina*, and *Desulfococcus* were detected in enriched oil-enrichments from microbial mats (Abed *et al*., 2011). Isolation has been restricted to the dominant metabolisms (sulfate reduction in this case) and successive transfers of enrichments are necessary to further reduce the effects of alternative carbon and energy sources different to the tested hydrocarbons (Chang *et al*., 2005).

To date, *Ferroglobus placidus*, isolated from Mediterranean hydrothermal vents, is the only archaeal species able to anaerobically degrade hydrocarbon compounds -other than methane- (Tor and Lovley, 2001). Nevertheless, many aerobic hydrocarbon oxidizing *Archaea* have been successfully isolated from marine coastal sediments: *Haloferax* sp. HA1, *Halobacterium* sp. HA-3 and *Halococcus* sp. HA-4 aliphatic and aromatic oxidizers were isolated from hypersaline coastal areas polluted after the first Gulf war (Al-Mailem *et al*., 2010), arqueobacteria EH4 that degraded eicosane, pristane, some *n*-alkanes and PHAs was isolated from salt marsh (Bertrand *et al*., 1990).

Table 2.1. Bacterial species described ion the literature (type strain or first isolate) isolated from marine habitats able to oxidize hydrocarbon compounds aerobically and anaerobically.

Strain	Taxonomic affiliation*	Hydrocarbon source	Sampling site	Reference
Aerobic bacteria				
Ochrobactrum sp. BAP5	*Brucellaceae (A)*	phenanthrene, benzo[a]pyrene, pyrene and fluoranthene	Marine sediment, Xiamen (China)	Wu *et al.*, 2009
Kordiimonas gwangyangensis GW14-5^T	*Kordiimonadaceae (A)*	benzo[a]pyrene and pyrene	Marine sediment, Gwangyang Bay (Korea)	Kwon *et al.*, 2005
Sphingomonas sp. 2MPII	*Sphingomonadaceae (A)*	2-methylphenanthrene	Polluted marine sediment, Gulf of Fos (France)	Gilewicz *et al.*, 1997
Novosphingobium pentaromativorans US6-1^T		PAHs of two-five rings	Estuarine sediment, Ulsan Bay (Korea)	Sohn *et al.*, 2004
Alcanivorax borkumensis SK2^T	*Alcanivoracaceae (G)*	n-hexadecane	Marine sediments, Western-Elms harbour (North Sea)	Yakimov *et al.*, 1998
Alcanivorax borkumensis P84Hx			Subtidal sediment, Cies Islands (Spain)	Alonso-Gutiérrez *et al.*, 2008
Alcanivorax jadensis T9^T		pristane, tetradecane and hexadecane	Intertidal zone. North Sea coast (Germany)	Bruns and Berthe-Corti, 1999; Fernández-Martínez *et al.*, 2003
Alteromonas sp. SN2	*Alteromonadaceae (G)*	naphthalene, phenanthrene, anthracene and pyrene	Oil-contaminated tidal flat sediment (Korea)	Jin *et al.*, 2012
Marinobacter hydrocarbonoclasticus SP17^T		Tetradecane, hexadecane, eicosane, heneicosane, pristane, phenyldecane and phenanthrene.	Polluted sediments, Gulf of Fos (France)	Gauthier *et al.*, 1992
Marinobacter nanhaiticus D15-8W^T		naphthalene, phenanthrene and anthracene	Marine sediment (South China sea)	Gao *et al.*, 2013
Thalassolituus oleivorans MIL-1^T	*Oceanospirillaceae (G)*	n-alkanes (C7 to C20) and their oxidized derivatives	Marine sediment. Harbour of Milazzo (Italy)	Yakimov *et al.*, 2004b
Neptunomonas naphthovorans NAG-2N-126		2,6-dimethylnaphthalene and phenanthrene.	Polluted sediments, Eagle Harbor (USA)	Hedlund *et al.*, 1999

Oleiphilus messinensis ME102^T	*Oleiphilaceae (G)*	n-alkanes (C11 to C20) and their oxidized derivatives	Marine sediment, Messina harbor (Italy)	Golyshin *et al.*, 2002
Cycloclasticus pugetii PS-1^T	*Piscirickettsiaceae (G)*	biphenyl, naphthalene, anthracene, phenanthrene, salicylate, toluene.	Marine sediment, Puget Sound (Washington)	Dyksterhouse *et al.*, 1995
Pseudomonas sp. Al1	*Pseudomonadaceae (G)*	1-heptadecene	Polluted sediment Lavera, Gulf of Fos (France)	Gilewicz *et al.*, 1991
Vibrio cyclotrophicus P-2P44	*Vibrionaceae (G)*	Naphthalene, 2-methylnaphthalene and phenanthrene.	Marine polluted sediments, Eagle Harbor (USA)	Hedlund and Staley, 2001
Yeosuana aromativorans GW1-1^T	*Flavobacteriaceae (Bt)*	benzo[a]pyrene and pyrene.	Estuarine sediment, Gwangyang Bay, (South Sea)	Kwon *et al.*, 2006
Bacillus naphthovorans MN-003	*Bacillaceae (F)*	naphthalene	Oil-contaminated marine sediments (Singapur)	Zhuang *et al.*, 2002
Planomicrobium alkanoclasticus MAE2	*Planococcacceae (F)*	n-alkanes C_{11} to C_{33}.	Intertidal marine sediment, Stert Flats, Bridgewater Bay (UK)	Engelhardt *et al.*, 2001; Dai *et al.*, 2005
Denitrifying bacteria				
Alphaproteobacterium Th1	*Rhodobacteraceae (A)*	Toluene	Romanian coast (Black Sea), marine sediment	Alain *et al.*, 2012
Thauera sp. TS-11	*Rhodocyclaceae (B)*	Toluene	Tidal flat near an industrial site (Korea)	An *et al.*, 2004
Marinobacter sp. 2sq31	*Alteromonadaceae (G)*	Squalene	Carteau cove, Gulf of Fos (France), marine sediment	Rontani *et al.*, 2002
Halomonas sp. Co12	*Halomonadaceae (G)*	Toluene	Horumersiel harbor (North Sea). marine sediment,	(Alain *et al.*, 2012)
Halomonas sp. DT-T		Toluene, *m*-xylene.	Muddy sediment,Le Dourduff en Mer (France)	
Denitrifer strain TT-Z	*Incertae sedis (G)*		Terenez beach (France), subtidal sandy sediment,	
Pseudomonas sp. NAP-3-1	*Pseudomonadaceae (G)*	Naphthalene	Eagle Harbor (USA), polluted marine sediment	Rockne *et al.*, 2000
Vibrio sp. NAP-4	*Vibrionaceae (G)*			

Sulfate reducing bacteria				
Desulfatibacillum sp. Pnd3	*Desulfobacteracea (D)*	n-alkanes C_{14} to C_{17}	Marine mud (Venice, Italy), marine sediment	Aeckersberg *et al.*, 1998
Desulfatibacillum alkenivorans AK-01		n-alkanes C_{13} to C_{18}	Arthur Kill channel (NY), Petroleum-contaminated estuarine sediment	So and Young L, 1999
Desulfatibacillum alkenivorans PF2803^T		n-alkenes C_8 to C_{23}	Oil-polluted sediments, Fos Harbour (France)	Cravo-Laureau *et al.*, 2004
Desulfatibacillum aliphaticivorans CV2803^T		n-alkanes C_{13} to C_{18}	Hydrocarbon-polluted marine sediments, Gulf of Fos (France)	Cravo-Laureau *et al.*, 2005
Desulfobacula toluolica Tol2^T		Toluene	Marine sediments, Eel Pond (Massachusetts)	Rabus *et al.*, 1993
Desulfobacula phenolica Ph01		Phenol, benzoate, 4-Hydroxybenzoate	Marine mud (North Sea coast)	Bak and Widdel, 1986; Kuever *et al.*, 2001
Desulfotignum balticum SAX^T			Marine coastal sediment (Baltic Sea)	Drzyzga *et al.*, 1993; Kuever *et al.*, 2001
Desulfobacterium aniline Ani1^T		Aniline and dihydroxybenzenes	Marine mud (North Sea)	Schnell *et al.*, 1989
Deltaproteobacterium sp. PL12		*n*-hexane, *n*-decane	Oil polluted sediment, Shuaiba (Kuwait)	Higashioka *et al.*, 2009
Desulfosarcina PP31		*p*-xylene	Marine sediments from Shuaiba, Kuwait	Higashioka *et al.*, 2012
sulfate-reducing bacterium NaphS2	*Desulfarculaceae (D)*	Naphthalene, 2-methylnaphthalene, benzoate.	Horumersiel harbor (North Sea), marine sediments	Galushko *et al.*, 1999
sulfate-reducing bacterium NaphS3			Black sediment, Mediterranean lagoon, Étang de Berre (France)	Musat *et al.*, 2008

Alcanivorax borkumensis SK2, *Cycloclasticus pugetii* PS-1, *Vibrio cyclotrophicus* P-2P44 and *Marinobacter hydrocarbonoclasticus* SP17 are facultative anaerobes able to reduce nitrate, but anaerobic hydrocarbon degradation has not been tested. Pubmed search was done with the phrases: "marine sediment" and "hydrocarbon". ** Family classification. Parenthesis indicated the corresponding phylum level: *Alphaproteobacteria* (A), *Betaproteobacteria* (B), *Gammaproteobacteria* (G), *Deltaproteobacteria* (D), *Firmicutes* (F) and *Bacteroidetes* (Bt).

Unfortunately, the degradative capacities of many marine strain isolated from non-polluted samples have only been examined in a few cases (Guo *et al.*, 2007). In some cases, different strains were isolated from marine polluted enrichments, but their hydrocarbon degradation capacities were not determined (Fu *et al.*, 2011). However, genome analysis of bacteria not considered to hold degradative capacities confirmed the presence of genes homologous to those coding for hydrocarbon degradation pathway enzymes (Head *et al.*, 2006; Acosta-González *et al.*, 2013a). Improving the techniques used for the isolation of degrading strains and the increasing metagenomic analysis of polluted *vs*. unpolluted samples will contribute to increase the current knowledge about the functional diversity available to respond to the presence of contaminants in these environments.

2.3. Microbial diversity analyzed by classical 16S rRNA gene analysis

Microbial communities benefit from the presence of pollutants if they are able to degrade any of the petroleum-components and to survive hydrocarbon toxicity. Biodegradation is increased by the presence of high bacterial diversity rather than by the selection of specific taxa (Dell'Anno *et al.*, 2012). The species richness in polluted samples is similar or sometimes higher than in pristine samples (Dos Santos *et al.*, 2011; Acosta-González *et al.*, 2013b), but in cases where the level of contamination is high, it could decrease drastically (Torsvik *et al.*, 1996; Wang and Tam, 2011; Acosta-González *et al.*, 2013b).

Efforts to describe the biogeography of marine microbial communities started only recently (Hilyard *et al.*, 2008; Zrafi-Nouira *et al.*, 2009; Zinger *et al.*, 2012; Gibbons *et al.*, 2013). Such studies aimed at comparing diversity among ecosystem types (and locations) within one realm (e.g. marine coastal or deep sediments). The comparative analysis of the microbial diversity between polluted sites from marine coastal sediments is crucial to measure the impact of pollution on microbial communities. In a previous work, we analyzed the response of subtidal sediments bacterial communities to the presence of hydrocarbon contamination. In order to compare our results with the situation in similar sites exposed to contamination, we performed a search in the databases for works characterizing the biodiversity of hydrocarbon polluted coastal sediment. The compilation was done by searching published works in PubMed and Scopus (search keywords: coastal sediment/marine sediment AND pollution/oil/petroleum/spill). We compared the sequences from available works targeting the three main zones of several coastal sediments: supratidal microbial mats in the Gulf of Mexico analyzed after the *Deep Water Horizon* (DWH) spill (Kostka *et al.*, 2011); microcosm and mangrove sediments from oil-polluted intertidal (Dos Santos *et al.*, 2011; Mortazavi *et al.*, 2013) and subtidal zones (Beazley *et al.*, 2012; Acosta-González *et al.*, 2013b; Liu and Liu, 2013). Figure 2.1 shows the

compilation of the available data. As expected, in all samples the phylum *Proteobacteria*, which represented between 85 and 45% of the populations, persisted as the major and common phylogenetic group in marine sediments. *Gamma-* or *Deltaproteobacteria* were the more abundant groups, as it was also observed in pristine coastal sites. This phylum is the largest phylogenetic, metabolic and morphologic lineage of the domain *Bacteria*. The dominance of the classes *Delta-*, *Gamma- and Alphaproteobacteria* in polluted coastal sediments is not a surprise since their members are widely recognized as important hydrocarbon degraders under aerobic and anaerobic conditions (Kersters *et al.*, 2007).

Although many species included in the *Betaproteobacteria* class are able to oxidize hydrocarbon compounds under aerobic and denitrifying conditions, members of this group were less abundant in coastal sediments, contrasting with their significant prevalence in soil environments (Parales, 2010). *Epsilonproteobacteria* is a group commonly described in extreme habitats like hydrothermal deposits and deep sediments (Zrafi-Nouira *et al.*, 2009). It has not been associated with degradative responses of bacterial communities in polluted coastal sediments but as observed in Figure 2.1, its abundance was important in some sites. *Zetaproteobacteria* is a recently described monophyletic group deeply rooted in the phylum *Proteobacteria* and frequently found in deep-sea environments (Militon *et al.*, 2010). As *Zetaproteobacteria* include many iron-oxidizing species, their detection in coastal sediments is plausible, although rarely reported (McBeth *et al.*, 2011).

Other important phyla were detected in the contaminated sites, although they did not show a similar distribution pattern to that observed for *Proteobacteria. Actinobacteria, Acidobacteria, Planctomycetes* and *Bacteroidetes* were abundant taxa in polluted sediments and have been reported as minority communities in a global diversity survey of marine coastal sediments (Hilyard *et al.*, 2008). The *Firmicutes* seemed to appear at initial stages of pollution (sample C, D, E and G). A common observation from almost all studies was the detection of great fractions of unclassified organism belonging to *Bacteria*. Diversity patterns of microbial communities inhabiting polluted coastal sediments appeared to be determined by the niche type (tidal, intertidal and subtidal) rather than by the contamination *per se* (Figure 2.1), although pollution seemed to influence the abundance and structure of the communities.

Some general features characterizing each zone could be extracted from these data and also from the literature. Although the literature addressing polluted marine sites is abundant, only a few published data could be used in our analysis, for different reasons: in some cases, part or all the sequences had not been deposited and were not available (Pearson *et al.*, 2008); generally, when the Sanger approach was used, only a few clones per sample were sequenced (it was rare to find more than a hundred sequenced clones per

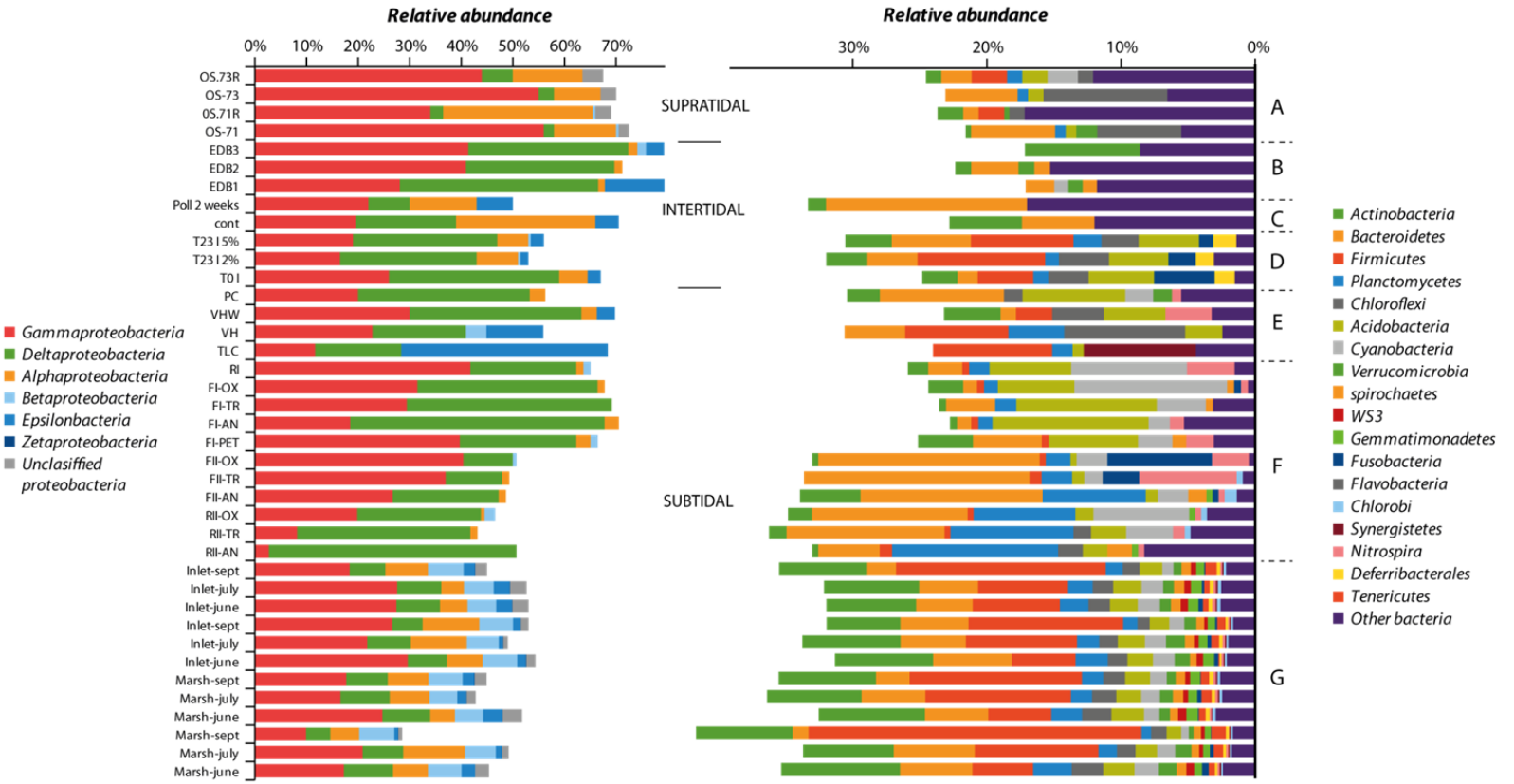

Figure 2.1. Comparison of phyla distribution of bacterial communities in diverse polluted marine sediments environments. Left side: The abundant phylum *Proteobacteria* is dissected at the class level. Right side: Non-*Proteobacterial* phyla. Data correspond to studies of samples from: supratidal microbial mats (A) (Kostka *et al.*, 2011), microcosm and mangroves sediments from intertidal zones (D) (Dos Santos *et al.*, 2011) and subtidal oil-polluted sediments (E, F, G) (Beazley *et al.*, 2012; Acosta-González *et al.*, 2013b; Liu and Liu, 2013). "Other bacteria" include unclassified bacteria.

sample); to compare clone libraries, ADRA or T-RFLP approaches were used rather than 16S rRNA gene sequencing; sometimes, results were presented as merged data from polluted and non-polluted experiments (Mortazavi *et al.*, 2013). Therefore, data obtained using alternative approaches were considered.

Supratidal and intertidal sediments. *Gammaproteobacteria* and to a lesser extent *Alphaproteobacteria* were dominant in these samples. It is known that these classes were key players in the aerobic degradation of oil layers deposited in Gulf of Mexico supratidal sediments affected by the DWH spill. There, *Alcanivorax* populations increased up to ten times and were especially abundant in the heaviest polluted samples (Kostka *et al.*, 2011). In some intertidal sediments the detected *Gammaproteobacteria* seemed to have a lesser contribution to *in situ* biodegradation than *Alphaproteobacteria* (Dell'Anno *et al.*, 2012; Mortazavi *et al.*, 2013). *Alphaproteobacteria*, *Planctomycetes* and *Deltaproteobacteria* were found in anoxic layers of heavily polluted intertidal microbial mats (Abed *et al.*, 2011), while *Firmicutes* was the most abundant class in sediments with an artificially and moderately oil-polluted sediments (Köchling *et al.*, 2011).

Subtidal sediments. *Gammaproteobacteria* were found in a considerable proportion in both intertidal and subtidal sediments (Figure 2.1). *Gammaproteobacteria* include many NRB and their members can reach layers below the oxic influence zone. In many samples, this group represented the dominant population of the polluted sediments (e.g., Inlet samples). However, it has been described that sulfate-reducing bacteria belonging to the *Deltaproteobacteria* was the prevailing group in reduced zones from polluted subtidal sediments, and biodegradation below superficial sediment layers was dependent of this metabolism (Rothermich *et al.*, 2002; Miralles *et al.*, 2007). Within this class, *Desulfobacteraceae* (*Desulfobacterales*) was frequently reported in polluted intertidal and subtidal sediments (Suárez-Suárez *et al.*, 2011; Rosano-Hernández *et al.*, 2012). Other orders within *Deltaproteobacteria* were also present. In some cases unclassified clusters were dominant over *Desulfobacteraceae* and *Desulfobulbaceae* (Zhang *et al.*, 2008). *Epsilon* and the recently described *Zetaproteobacteria* class have been detected using Geoship analysis (Liu and Liu, 2013) but a role in biodegradation was not established.

Besides *Proteobacteria*, the group *Cytophaga* spp., *Flavobacteria*, *Bacteroidetes* (CFB) has been reported as the most active microbial population detected by FISH in subtidal oil-polluted sediments (Acosta-González *et al.*, 2013b) (Figure 2.1). Groups like *Actinobacteria*, *Bacteroidetes*, and *Chloroflexi* disappeared in polluted microcosms from intertidal sediments (Mortazavi *et al.*, 2013) while they were maintained or they increased in subtidal polluted sediments (Figure 2.1) (Beazley *et al.*, 2012; Acosta-González *et al.*, 2013b; Liu and

Liu, 2013). Studies in Black Sea sediments reported an increase of *Firmicutes* concomitant with an increase in the pollution level (Kolukirik *et al.*, 2011).

Estuarine systems. *Proteobacteria* (*Gamma-*, *Delta-*, *Alphaproteobacteria*) dominated bacterial communities in polluted estuarine environments but *Acidobacteria* were also abundant and important in the response to contamination (Dos Santos *et al.*, 2011; Gomes *et al.*, 2013; Sun *et al.*, 2013). Sulfate reducing *Desulfococcus* species from estuarine and subtidal sediments were associated to hydrocarbon degradation in the anoxic zones (Miralles *et al.*, 2007; Gomes *et al.*, 2013). *Gammaproteobacteria* genus *Marinobacterium*, *Marinobacter* and *Cycloclasticus* increased in oil amended microcosms (Dos Santos *et al.*, 2011). Strains from the genus *Pseudomonas* and Gram-positive related bacteria isolated from the rhizosphere of mangrove plants were able to degrade many aromatic compounds aerobically (Daane *et al.*, 2001). Other metabolisms different to sulfate reduction can co-occur in estuaries. A high diversity of iron reducing bacteria in the polluted Scheldt estuary (Northwest Europe) associated to great quantities of available iron (Fe3+) has been reported, but nothing was done to assess their potential role in biodegradation (Lin *et al.*, 2007).

Archaea. *Archaea* have been detected in intertidal and subtidal polluted sediments, but no direct connection to biodegradation processes was associated to their presence (Köchling *et al.*, 2011; Suárez-Suárez *et al.*, 2011). The importance of *Archaea* in anaerobic hydrocarbon degradation was demonstrated when the specific inhibition of methanogens in a methanogenic enrichment decreased the degradation rate of naphthalene and phenanthrene (Chang *et al.*, 2006). The establishment of syntrophic consortia of methanogens and hydrocarbon-degrading acetogenic bacteria (sulfate reducers) could explain the presence of *Archaea* in polluted zones (Zengler *et al.*, 1999; Miralles *et al.*, 2010). The co-occurrence of methanogenesis and sulfate/nitrate reduction in some samples from the upper sediment zone of Marmara Sea suggests that the syntrophic relations of *Archaea* are not confined to the sulfate-methane transition zone of polluted sediments (Kolukirik *et al.*, 2011). *Archaea* communities in subtidal unpolluted sediments were dominated by *Crenarchaeota*, whereas *Euryarchaeota* (relate to methanogens) became dominant when oil-treatment was applied (Miralles *et al.*, 2010).

It's important to note that the majority of studies in the literature were restricted to the first centimeters of the sediment profile. Studies below 20 cm were less frequent and similar depth profiles were not always available for comparison. Therefore, the highest abundance in our analysis of *Gammaproteobacteria*, generally associated with oxic-anoxic transition conditions, was not a surprise. In our studies of polluted sediment diversity, many

16S rRNA sequences were closer to sequences found in other polluted sites, and this is a common trend observed by other researchers.

As mentioned above, the main problem encountered when analyzing the literature was the heterogeneity of the data. It is highly recommended that the characterization of a polluted site also include in the analysis similar non-polluted samples from close locations. For simulation experiments, the ideal situation would be to simulate spill conditions *in situ*, although *ex situ* micro- or mesocosms are a recurrent and acceptable alternative. Finally, cautions should be taken to avoid mixing terms like microcosm and enrichments.

2.4. Analysis of next generation sequencing data.

Data generated by any NGS technology (preferentially 454 pyrosequencing) show notable differences when compared with data generated with classical Sanger approaches. Before the appearance of NGS, a few groups, dominated by *Proteobacteria*, had been described as common inhabitants of marine polluted environments. As discussed in previous sections, the analysis of the microbial diversity in polluted sediments from coastal ecosystems by classical methodologies weighted up the presence of the *Proteobacteria* and other phyla, but did not allow the detection of diversity patterns at lower taxonomic levels. The importance of *Proteobacteria* and other groups in marine sediment samples was confirmed by NGS data, but more precise information could be obtained with this approach. For instance, the relative high abundance of *Marinobacter* was demonstrated at initial stages of oil inputs, and progressively decreased when degradation occurred (Dos Santos *et al.*, 2011), demonstrating the specific importance of *Marinobacter* species within the *Gammaproteobacteria* group.

To improve the description of the bacterial diversity in the samples analyzed in chapter I, we decided to perform a massive sequencing of the V1-V3 region of 16S rRNA gene of the samples from the *Prestige* affected beach described in chapter I (except FI-OX) using 454 GLX Roche pyrosequencing. The Rodas sample, collected from an unaffected beach in the same island, was also included; this sample has been used in the analysis of the functional genes (chapter III). We compared these data with the results obtained previously in the full-length 16S rRNA gene cloned libraries (Figure 2.2). As expected, more phylogenetic groups could be detected with the pyrosequencing approach, although thegeneral picture was comparable at the phyla level. *Gammaproteobacteria, Deltaproteobacteria* and *Acidobacteria* appeared largely overestimated by the Sanger approach, while *Alphaproteobacteria, Actinobacteria* and *Chloroflexi* were underestimated. *Epsilonproteobacteria*, previously undetected, represent up to 4% of amplicons in FI-PET sample.

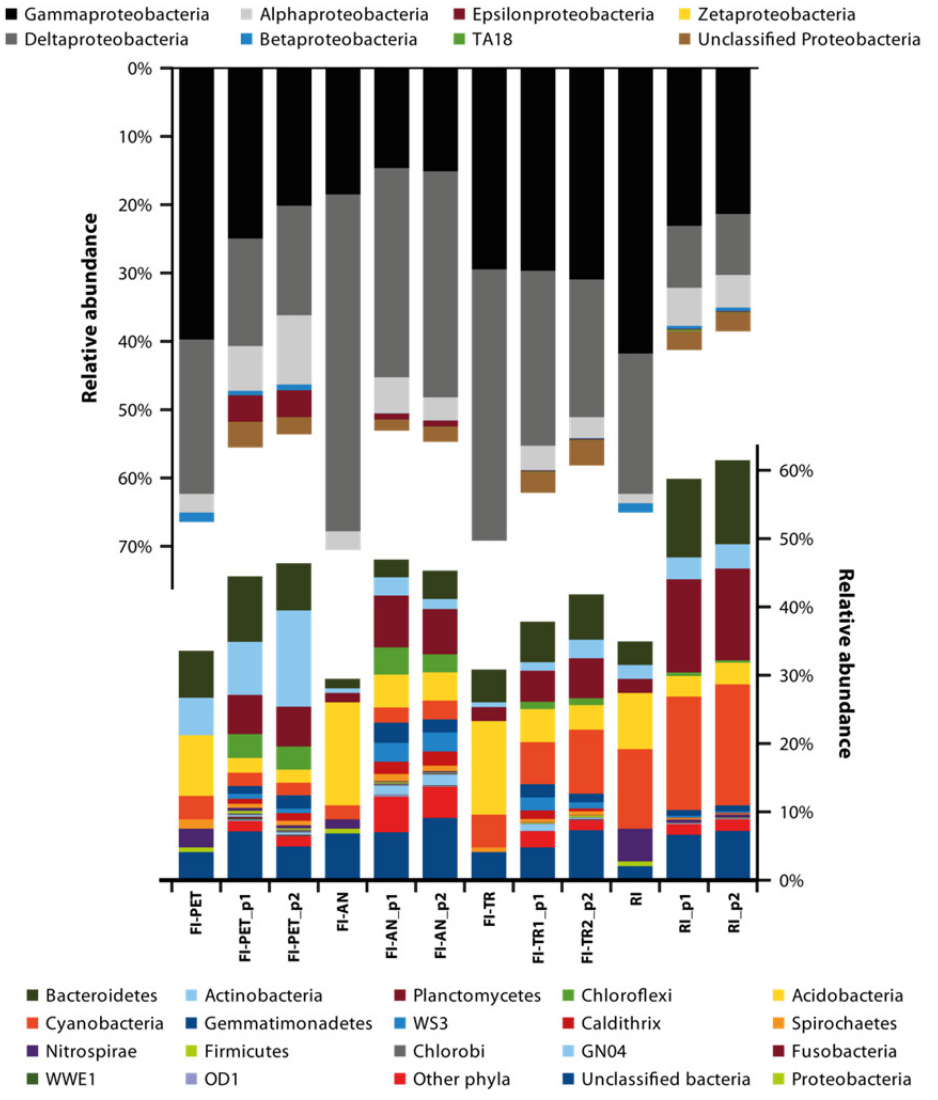

Figure 2.2 Comparison of phyla distribution between whole length 16S rRNA gene Sanger libraries and V1-V3 amplicon pyrosequencing (two replicates from different DNA extractions, labeled -p1 and -p2) from samples analyzed in chapter I. Top, comparison of *Proteobacteria* classes distribution. Bottom, non proteobacterial phyla. Other phyla, only detected in the pyrosequencing approach, included *Armatimonadetes*, *Deferribacteres*, *Fibrobacteres*, *Tenericutes*, *Verrucomicrobia*, *Lentisphaerae* together with many phyla without cultivable representatives.

The NGS results were further compared with similar data from polluted and pristine sites available in MG-RAST database (Meyer *et al.*, 2008). Unfortunately, search of comparable datasets from marine polluted and pristine samples (sequences of the V1-V3 region) in the MG-RAST database only detected one similar study, from a polluted estuarine in Australia (Sun *et al.*, 2013). The comparison of this study with our samples is shown in Figure 2.3. Samples from polluted sediments from estuarine and Cíes Islands affected by

the *Prestige*'s oil spill appeared separated by both principal coordinates (38%). Thus, there was no evidence of polluted samples clustering independently of the sample origin; principal coordinates clearly separated samples by ecosystem type. It is worth noting that the pristine sample from the Cíes Islands was separated from the polluted samples from the same location, indicating an effect of pollution on microbial communities.

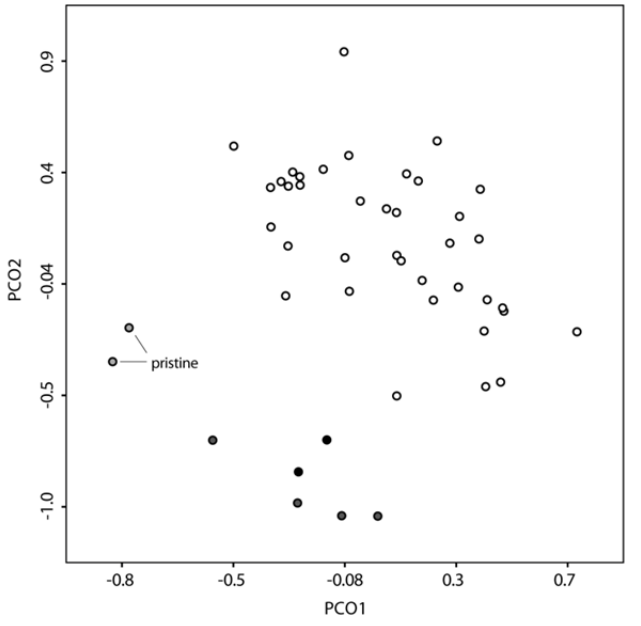

Figure 2.3. PCoA analysis of amplicons obtained by 454 pyrosequencing of V1-V3 16S rRNA region deposited in MG-RAST. The following microbial communities from polluted sediments available in MG-RAST were compared: i) subtidal estuarine samples from Australia (white) (Sun *et al.*, 2013); ii) subtidal slightly (dark gray) and heavily (black) polluted samples from a *Prestige* oil spill affected beach (Figueiras); and iii) pristine samples collected at the same sampling time from a beach close to Figueiras (Pristine, light gray). The data were compared using the MG-RAST tools and SSU database (SILVA) using a maximum e-value of 1e-3, a minimum identity of 60 %, and a minimum alignment length of 15 bp for RNA databases.

To extent the analysis, we searched for NGS amplicons studies in the NCBI SRA (Sequence Read Archive) database. Most of the retrieved studies belong to currently unpublished data (see section 5.2 of experimental procedures). PCoA plots were constructed using unweighted (Figure 2.4a) and weighted (Figure 2.4b) variables. Suggested clustering of samples from polluted and pristine sites is indicated with red and blue lines, respectively. Neither coordinate (PC1 or PC2) *per se* could separate these clusters in the unweighted or weighted PCoA. However, clustering of polluted samples was better explained when the abundance of taxa was considered, i.e. in the weighted analysis (29.1% explained compared with only 14.36% in the unweighted analysis). This suggested that, if the abundance enhances clustering among polluted samples, then the most abundant groups should be common to the majority of the samples (red circles, Figure 2.4b).

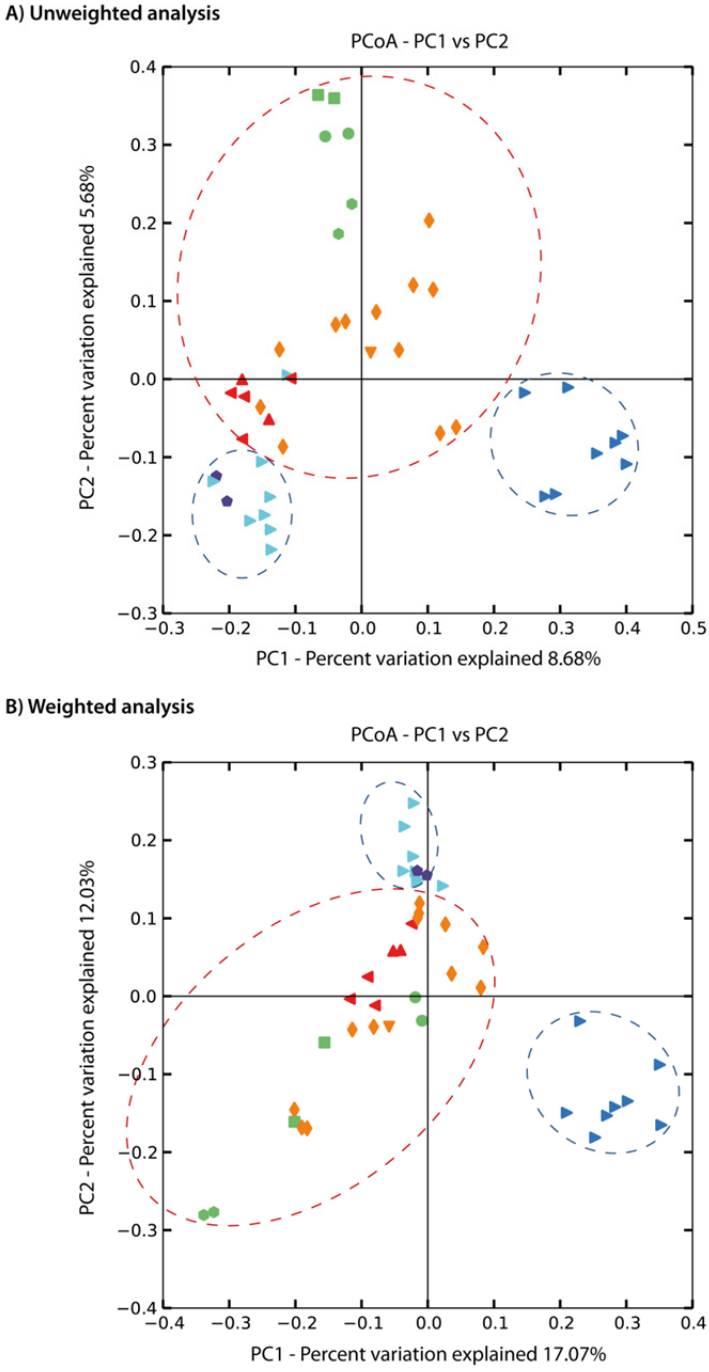

Figure 2.4. PCoA analysis comparing 16S rRNA V1-V3 region amplicons obtained from polluted and pristine subtidal sediments. Each color represent a sample site: light blue (Fundy Bay, Atlantic Canada), dark blue (King Island, Antartic Ocean) purple (Rodas beach, Atlantic Ocean, Spain) correspond to pristine samples. Orange (Gulf of Mexico, USA), red (Figueiras beach, Spain) and green (Figueiras beach, Microcosms) correspond to spill affected sediments. Unweighted (A) and weighted (B) analysis are shown.

To determine whether some taxa were relevant in shaping the structure of microbial communities in the samples, a PCoA analysis was recalculated considering taxonomic categories at the order level and including abundance in the analysis (Figure 2.5). The size of the grey spheres in the picture is proportional to the mean relative abundance of taxa across all samples (please note that this is not related to the absolute taxa abundance in the whole analysis). The majority of taxa with the highest relative abundance were close to

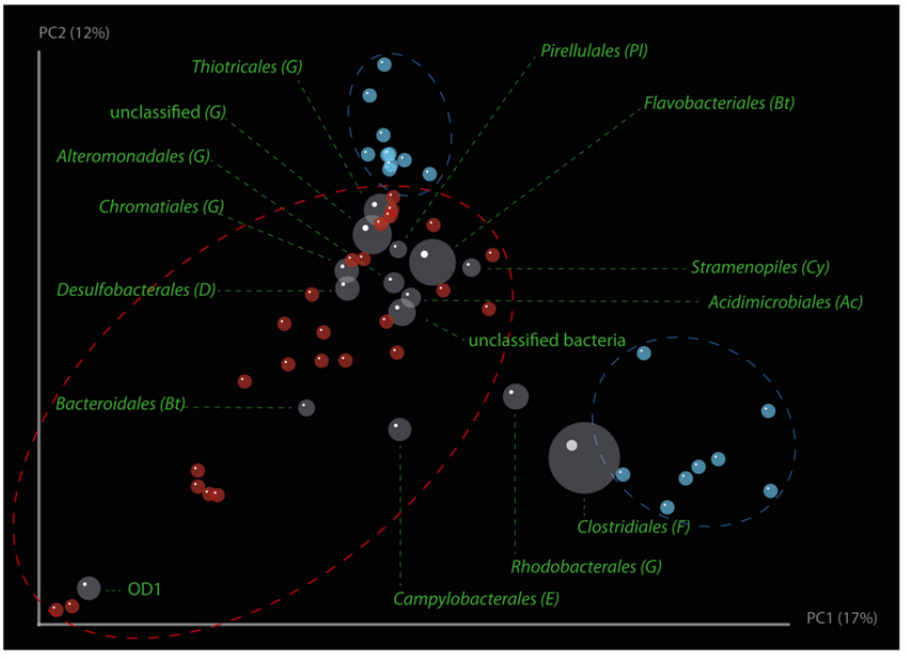

Figure 2.5. Sub-plot from the weighted distance matrix, considering the fifteen most prevalent taxa (at the order level) in different areas of the PCoA plot. The size of the gray sphere is proportional to the mean relative abundance of the represented taxon across all samples. Blue and red points correspond to samples from polluted and pristine sites, respectively, as in Figure 2.4.

polluted samples. *Firmicutes*, which was closest to one pool of pristine samples, had the highest relative abundance. Therefore, as suggested above, abundance of some taxa determined the clustering of polluted samples. Polluted samples were mainly represented by *Proteobacteria* and *Bacteroidetes* sequences. Analyzing taxonomic sub-levels, we could see that some specific groups contributed to the clustering. *Thiotricales* and unclassified *Gammaproteobacteria*, together with *Flavobacteriales* (*Bacteroidetes*) were the orders that contributed most to the community structure of polluted samples. Other important orders were *Alteromonadales* and *Chromatiales* (*Gammaproteobacteria*) and *Desulfobacterales* (*Deltaproteobacteria*), groups which included many oxidizer species extensively characterized in biodegradation studies. The samples from the microcosm experiment with

naphthalene (Chapter IV) were mainly composed of sequences related to OD1 candidate division.

2.5. Concluding remarks.

A succession of changes takes place when sediment microbial communities are exposed to a contamination event. Assessing which main factors determine these changes is not an easy task. Populations of microbes fluctuate following pollution inputs depending on their capacity to resist hydrocarbon toxicity or to utilize them as carbon source, but other unknown variables are also relevant. Thus, contaminants alter the structure and function of bacterial communities as the continuous exposure to pollutants selects for bacterial populations that govern the altered habitats (Harayama *et al*., 2004). Variations in microbial abundance have been observed after the release of petroleum inputs in marine sediments (Powell *et al*., 2003; Yakimov *et al*., 2004a). However, the implementation of bioremediation strategies did not always register changes in the community structure (Kim *et al*., 2008), suggesting a previous adaptation of the communities to pollution. The PCoA analysis presented here (Figure 2.4) suggested that the microbes involved in the degradation of hydrocarbons in distant sediment samples were related among them and revealed that if lower taxonomic levels were evaluated, the observed differences between samples were more consistent.

Results of some published and unpublished works seem to be considerably influenced by the use of enrichment cultures, which could be expected since not all natural conditions can be simulated in the laboratory. Probably microcosm experiments conducted *in situ* or *ex situ* would be a better option to determine the changes in the microbial diversity and community structure. Early works showing fingerprinting analysis carried out using classical microbial ecology tools demonstrated changes in microbial communities as a consequence of pollution events. However, just a few studies used multivariate analysis to demonstrate the relationships between community composition and environmental variables (Cetecioğlu *et al*., 2009). Despite identifying some fluctuating populations, they showed just a small part of a complex environment. Unfortunately, these types of data, which are profuse, are not manageable for comparison with current technologies addressing 16S rRNA sequences. Although NGS allow access to unexplored diversity, it still presents great inconvenient. Development of more powerful and user-friendly bioinformatics tools and standardization of sequence format in the different databases are urgently required.

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Chapter III

Diversity of benzylsuccinate synthase-like (*bssA*) genes in hydrocarbon-polluted marine sediments suggests substrate-dependent clustering

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3.1. Introduction

Marine habitats are continually exposed to hydrocarbon pollutants that are released through natural seepage and also generated by anthropogenic activities. Many bacteria have adapted the ability to degrade low molecular weight hydrocarbons to carbon dioxide and water. Thus, biodegradation of contaminants mediated by autochthonous microorganisms has become the basis for bioremediation strategies in contaminated sites. Application of such technologies requires preceding efforts to identify the relevant players in the remediation process and assessment of the optimal attenuation conditions. Until recently, these strategies were primarily based on the exploitation of aerobic processes performed by aerobic bacteria, but many exposed ecological niches, such as marine sediments, are under permanent anoxic conditions (Rockne and Strand, 1998; Brune *et al.*, 2000). Thus, the anaerobic catabolism of hydrocarbons is of crucial importance for natural attenuation (Chakraborty and Coates, 2004; Grossi *et al.*, 2008). Investigations of the biochemistry, genetics and physiology of anaerobic hydrocarbon degradation are essentially centered on the degradation of mono-aromatic compounds and *n*-alkanes (Spormann and Widdel, 2000; Heider, 2007), naphthalene (Zhang and Young, 1997), and 2-methylnaphthalene (DiDonato *et al.*, 2010; Selesi *et al.*, 2010).

The initial steps in the anaerobic degradation of hydrocarbons are considerably diverse between microorganisms (Heider, 2007). Still, activation of a broad range of aromatic hydrocarbons converges into a few major central metabolites, which are further dearomatized and channeled to the central cell metabolism. Fumarate addition, the initial and key step in toluene degradation, is a general activation strategy in many anaerobic aromatic activation pathways. The reaction is catalyzed by the glycyl radical enzyme benzylsuccinate synthase (Bss), and the reaction product is further converted in several steps to the final key metabolite benzoyl-CoA (Heider, 2007). Benzylsuccinate synthase is composed of three different subunits. The large subunit (*bssA* gene) included in the pyruvate formate-lyase (PFL) family of glycyl-radical enzymes, contains the active-site determinants that characterizes this family, which consists of a conserved glycine motif located near the C-terminal end of the subunit, and a conserved cysteine residue in the middle of the protein sequence (Selmer *et al.*, 2005). Benzylsuccinate synthase catalyzes the addition of the methyl group of toluene to a fumarate co-substrate to produce (R)-benzylsuccinate (Biegert *et al.*, 1996; Beller and Spormann, 1997; Leuthner *et al.*, 1998).

Several hydrocarbons have been shown to undergo a similar reaction as main step in their degradation. For example, anaerobic degradation of *o*- and *m*-xylenes, *m*- and *p*-cresols, and ethylbenzene are initiated by BssA-like activity (Beller and Spormann, 1997; Krieger *et al.*, 1999; Müller *et al.*, 1999, 2001; Kniemeyer *et al.*, 2003). A similar fumarate addition catalyzed by a 2-naphthylmethylsuccinate synthase, coded by the *nmsA* gene, was

shown to be the first step in the degradation of 2-methylnaphthalene (2MN) by sulfate-reducing bacteria (SRB) (Musat *et al*., 2009; Selesi *et al*., 2010). SRB and nitrate-reducing bacteria (NRB) also activate *n*-alkanes degradation in a reaction resembling BssA-dependent toluene activation, rendering 1-methylalkyl-succinates (Kropp *et al*., 2000; Rabus *et al*., 2001). The *masD* and *assA* genes, encoding the large subunit of this enzyme, were simultaneously described in the denitrifier *Azoarcus* sp. HxN1 (Grundmann *et al*., 2008) and the SRB *Desulfatibacillum alkenivorans* AK-01 (Callaghan *et al*., 2008), respectively.

PCR amplification of catabolic genes has been proposed to assess anaerobic BTEX metabolisms *in situ* (Beller *et al*., 2008). Although a limited number of *bssA* sequences from pure cultures are available to date (Coschigano *et al*., 1998; Leuthner *et al*., 1998; Achong *et al*., 2001; Kane *et al*., 2002; Kube *et al*., 2004; Shinoda *et al*., 2004; Shinoda *et al*., 2005), *bss*A-like sequences have been detected in many enrichment cultures and consortia able to degrade hydrocarbons (Botton *et al*., 2007; Washer and Edwards, 2007; Callaghan *et al*., 2010). The strong conservation of *bssA* sequences and the recurring detection of this gene in hydrocarbon contaminated environments has promoted its use as an indication of hydrocarbon degradation at these sites (Beller *et al*., 2002; Winderl *et al*., 2007; Beller *et al*., 2008; Oka *et al*., 2011; Pilloni *et al*., 2011; Staats *et al*., 2011). Sequence analysis revealed greater than expected gene diversity, likely related to local distribution, substrate specificity and dominant respiration strategies (Winderl *et al*., 2007). Orthologues of a*ssA* (*masD*) gene were recently found in pure cultures of sulfate-reducing and denitrifying *Proteobacteria* (Callaghan *et al*., 2008; Zedelius *et al*., 2011), and in a thermophilic methanogenic community (Mbadinga *et al*., 2012). Analysis of retrieved sequences revealed that *assA* sequences grouped into a distinct cluster that was separated from canonical *bssA* sequences. Aditionally, four *nmsA* sequences retrieved from SRB isolates or sulfate-reducing enrichment cultures (Musat *et al*., 2009; DiDonato *et al*., 2010; Selesi *et al*., 2010) clustered together with *nmsA* sequences recently detected in environmental samples of different origin (von Netzer *et al*., 2013). The majority of these sequences were retrieved from hydrocarbon contaminated aquifers and river sediments; very little is known regarding *bssA*-like genes present in marine environments, and the studies targeting the effect of pollution on the microbial communities in subtidal sediments are scarce.

Indigenous microbial communities play a significant role in hydrocarbon degradation of oil-polluted sediments, as evidenced by a great repertoire of metabolic genes that have evolved to oxidize these hydrocarbons. The presence and diversity of the functional genes in the environment could identify the biodegradative potential of microbial communities. In this study, we analysed for the first time the *bssA*-like gene diversity of subtidal marine sediment samples collected from a heavily polluted beach affected by the *Prestige* oil spill (Atlantic Islands' National Park, Spain), where the oxidative capacities of microbial communities has already been demonstrated (Alonso-Gutiérrez *et al*., 2008; Acosta-

González *et al.*, 2013). To determine time-exposure effect on the functional diversity dynamics, crude oil or naphthalene contamination was simulated in microcosms from sediments collected in Alcúdia Bay (Mallorca, Mediterranean Sea). The resulting *bssA* gene diversity was compared with all available sequences to provide a comprehensive analysis of the global *bssA* gene distribution. Our data suggested that contamination had a positive influence on the functional gene diversity. The observed grouping of BssA-like homologues into consistent clusters was apparently related to substrate specificity (toluenes, methylnaphthalenes and alkanes).

3.2. Experimental procedures

***Site description and microcosm preparation*.** Sediment samples were collected from an Atlantic Island beach (Figueiras) in Northern Spain, which was affected by the *Prestige* oil spill in November 2002. Sediment cores (50 cm) were collected in May 2004 by scuba-divers at nine meters depth with 50 cm long cores, and an additional 30 cm core was collected at a place where a petroleum spot, intermingled with the sediment, was found by the diving team (FI-PET) (Acosta-González *et al.*, 2013). Cores were kept intact at 4ºC until processed. Three sections were selected for analysis: the upper oxic layer (2 to 5 cm, FI-OX), a transition layer (11 to 14 cm, FI-TR) and a sulphidic zone (32 to 35 cm, FI-AN). An additional core from a neighboring less affected beach (Rodas beach, RI) was also analyzed. Microcosm experiments were set up in June 2007 with sediments collected at a second site in Alcúdia Bay (Mallorca, Spain) within a pristine seabed. Three cores (50 cm) were collected as above at twelve meters depth. Two of them were artificially contaminated with naphthalene (M-NAPH) or crude oil (M-OIL). A third core remained untreated and served as a control (M-CON). The cores were sealed with duct tape and incubated at 22°C in the dark for four months. The sediment section at 12 to 16 cm was used for the analysis.

***Bacteria enumeration*.** Most probable number (MPN) counts of hydrocarbon oxidizing bacteria were estimated in an artificial seawater minimal medium as described previously (Acosta-González *et al.*, 2013). Anaerobic medium was amended with either acetate or an aromatic compound (toluene, benzene, naphthalene or anthracene) to a final concentration of 1 mM, or with a crude oil drops (only for Mallorca samples) as the sole carbon source. Either 10 mM sulfate, 20 mM ferrihydrite or 20 mM manganese (only for Mallorca samples) were added as electron acceptors to the media. All tubes were incubated in the dark at 22°C for 6 months.

***Hydrocarbon analysis*.** Hydrocarbons were extracted from duplicate frozen sediment aliquots (2 ml) and Deuterated Mix 37 (manufactured by Dr. Ehrenstorfer) and 5a-

cholestane (Aldrich) were added as internal standards before the extraction. Hydrocarbon analysis was determined by GC-MS as previously described (Acosta-González *et al.*, 2013). A static headspace gas chromatographer mass spectrometer (Varian 450GC 240MS with CTC CombiPal autosampler) was used for the determination of BTEX in contaminated sediments (Shin, 2012).

***Nucleic acid extraction*.** The SDS-based DNA extraction method was used with duplicate samples of frozen sediment as described by Zhou and colleagues (1996) with some modifications as described in section 4.2.2 (experimental procedures).

Amplification of benzylsuccinate synthase alpha-subunit gene (bssA). Primers described previously (Winderl *et al.*, 2007) were used to amplify the alpha-subunit of benzylsuccinate synthase gene (*bssA*). Primers 7772f and 8542r, that successfully amplified partial *bssA* genes of expected size, were chosen for the gene library preparations**.** As unspecific amplicons were obtained, the amplified fragment with the expected size was separated onto a 15 cm-long agarose gel (1.5% w/v) and extracted with the QIAGEN Gel extraction Kit. Purified fragments were cloned in pGEM-T vector (Promega) and 70 positive clones were selected for sequencing.

***Sequence analysis*.** Methods for sequence analysis are detailed in section 5.3 (experimental procedures).

3.3. Results and discussion

Sample characterization.

In order to assess the diversity of the *bssA* gene as an indication of anaerobic hydrocarbon degradation, samples were collected from Figueiras beach (FI samples), a long-term oil-contaminated beach in northern Spain, and from sediment microcosms collected in Mallorca (Mediterranean sea) and spiked with naphthalene or crude oil (M samples). BTEX (benzene, toluene, ethylbenzene and xylenes) compounds were only detected in two samples: the oil contaminated microcosms in Mallorca (M-OIL), and the petroleum patch in Figueiras sediment (FI-PET) (Fig. 3.1a and Table S2a). The crude oil contamination was composed of aliphatic and aromatic hydrocarbons, with a high proportion of naphthalene and alkylated derivatives (Fig. 3.1b, Table S2b). The diversity and aromatic biodegradation potential in samples collected from the Atlantic Island has already been described (Acosta-González *et al.*, 2013). *Delta-* and *Gammaproteobacteria* constituted more than 60% of the retrieved 16S rRNA gene sequences, and the proportion of

Deltaproteobacteria increased towards the bottom of the sediment core while *Gammaproteobacteria* were predominant in FI-PET. The highest counts of aromatic oxidizing bacteria were obtained using sulfate-reducing conditions with benzene, toluene or naphthalene as a carbon source (Table S3.3). The microbial diversity and potential for sulfate reduction-dependent hydrocarbon degradation in the sediments where M-samples were collected was verified previously (Suárez-Suárez *et al.*, 2011). As observed in the Atlantic Islands samples, *Gamma-* and *Deltaproteobacteria* were the most abundant communities in these sediments. After four months of incubation, the MPN of sulfate-reducing bacteria (SRB) determined in the three cores indicated an oil-dependent stimulation of the bacterial populations in all carbon sources assayed. The highest counts were observed with acetate, and the community able to use crude oil exhibited counts within this range (Table S3.3). In contrast, the naphthalene treatment was toxic to most microbial populations, except for those that were able to oxidize naphthalene or crude oil, which increased by four-fold and two-fold, respectively. Thus, as observed in the Atlantic Island sediments (Acosta-González *et al.*, 2013), the bacterial communities able to use crude oil carbon sources increased in response to high pollution levels, despite a large decrease in the total cell counts.

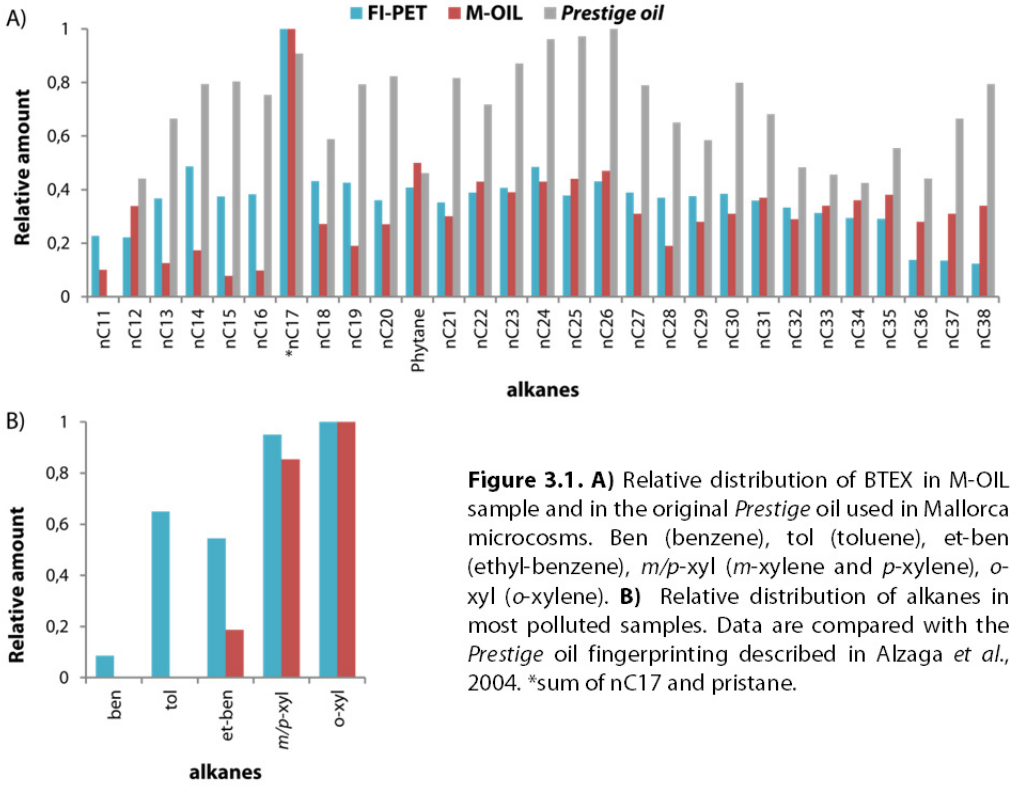


Figure 3.1. A) Relative distribution of BTEX in M-OIL sample and in the original *Prestige* oil used in Mallorca microcosms. Ben (benzene), tol (toluene), et-ben (ethyl-benzene), *m/p*-xyl (*m*-xylene and *p*-xylene), *o*-xyl (*o*-xylene). **B)** Relative distribution of alkanes in most polluted samples. Data are compared with the *Prestige* oil fingerprinting described in Alzaga *et al.*, 2004. *sum of nC17 and pristane.

Detection of *bssA*-like genes in the sediments.

We tested several previously designed primer pairs to monitor *bssA* genes in sediment samples (Winderl *et al.*, 2007). Only primers 7772f and 8542r consistently produced PCR amplification products with DNA extracted from sediment, rendering a product with an expected fragment size of approx. 794 bp. The amplified sequence covered the gene region that codes for part of the active site of the enzyme, including the conserved cysteine residue where the highly reactive thiyl radical is formed. Eight *bssA* gene libraries were constructed from DNA isolated from each sediment sample, and seventy clones from each library were sequenced. As previously described, non *bssA*-related DNA fragments of the same size were also obtained using these primers, which represented up to one third of the analyzed sequences (Table 3.1) (Winderl *et al.*, 2007; Herrmann *et al.*, 2009).

Table 3.1. Specificity of the *bssA* probe in each sample.

		Number of clones[1]	
Site	**Sample**	**(+)**	**(-)**
Mallorca	**M-CON**	51	19
	M-NAPH	44	26
	M-OIL	50	20
Figueiras	**FI-PET**	68	2
	FI-OX	49	21
	FI-TR	51	19
	FI-AN	53	17
	RI	43	27

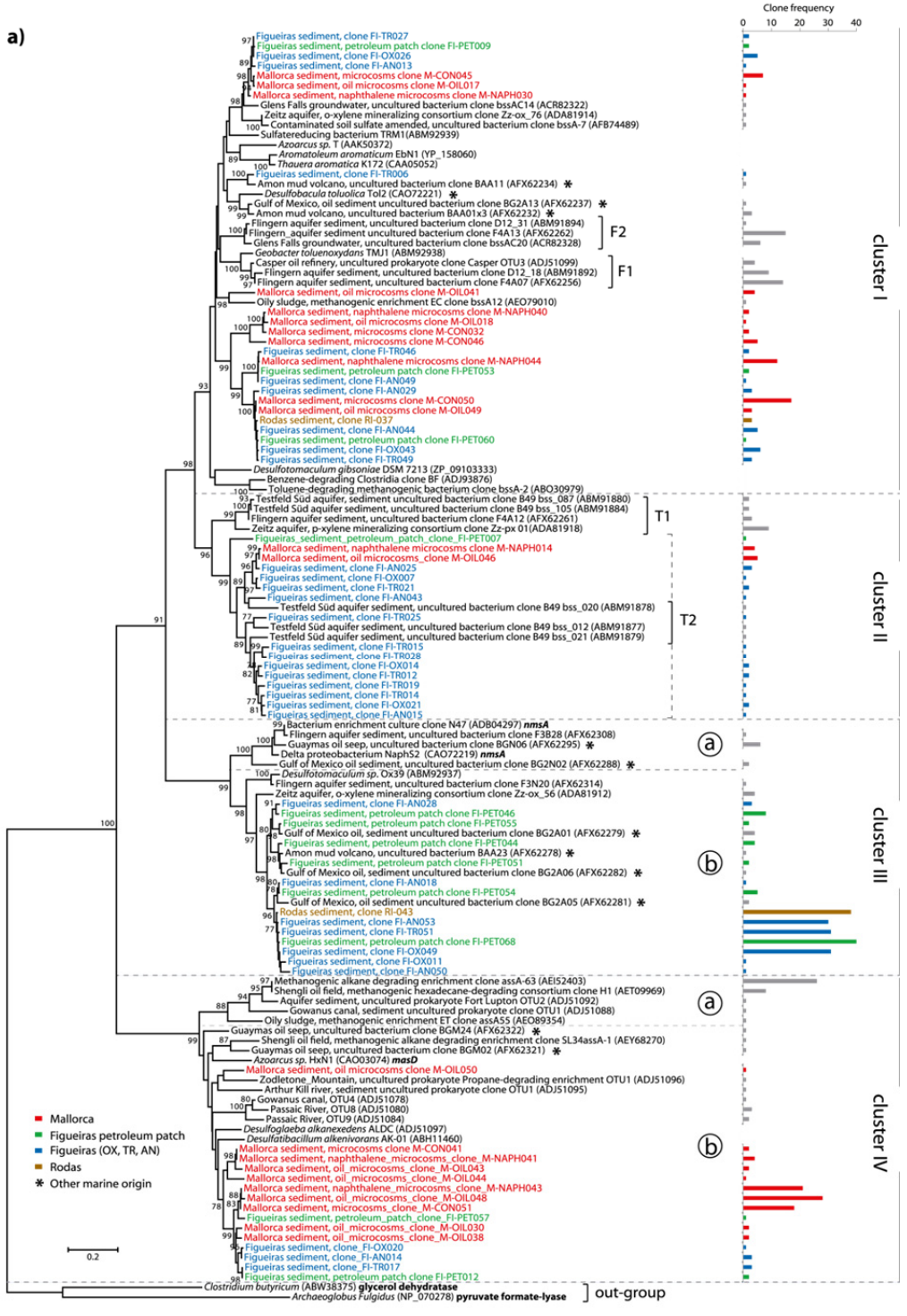
[1](+) *bssA*-like sequences; (-) unrelated, non-*bssA* sequences

Sequence analysis of the PCR products revealed fragments of two different lengths: the expected 794 bp fragment, and a group of 773 bp-long fragments that also showed strong similarity to the *bssA* gene. The amplified *bssA* sequences from the eight libraries were translated into amino acids sequences (BssA) and compared with available BssA sequences; a phylogenetic tree of the amplified and database sequences is provided in Figure 3.2a. Four main clusters that grouped sequences sharing 75% amino acid similarity were identified. Principal coordinate analysis (PCoA) based on the phylogenetic reconstruction of all BssA-like sequences grouped sequences in the same four clusters (Figure 3.3a); coordinates P1 and P2 accounted for 30% and 21,7% of the variance, respectively.

Cluster I included the canonical, toluene-specific BssA sequences (*bssA sensu stricto*), present in well-characterized toluene degrading strains that belong mostly to the

Proteobacteria group, as well as environmental sequences retrieved from natural samples, including hydrocarbon polluted aquifers and aquifer sediments, oilfields, sludge and polluted soils. All 794 bp sequences retrieved from marine sediments (60 from Mallorca samples and 57 from the Atlantic Islands samples), were located within this cluster. The 773 bp sequences were distributed in the remaining clusters. Cluster II comprised several environmental sequences retrieved from *p*-xylene enrichment cultures and included the previously described clusters T1 and T2 of unidentified BssA-homologues, which constituted two distinct groups within this cluster (Winderl *et al.*, 2007). Sequences included in Cluster II were associated with the *in situ* oxidation of BTEX compounds, including *p*-xylene, in sediment enrichments, both under sulfate-reducing conditions (Winderl *et al.*, 2007; Herrmann *et al.*, 2009). Only 26 of our sediment sequences clustered within this group and were primarily retrieved from the Atlantic Island contaminated sediments. Separation between clusters I and II could not be attributed to a distant phylogenetic origin of the sequences. Cluster I included a *bssA*-like sequence (EHG03004, annotated as formate-C-acetyltranferase) from the non-*Proteobacteria Desulfotomaculum gibsonie* DSM 7213 genome (GI:355360296; AGJQ01000002.1) (Figure 3.2a, see Figure 3.2b). Sequences closely related to this hypothetical *D. gibsonie bssA* gene were also found in environmental samples where methanogenesis was prevalent, and where a *Clostridium* counterpart was suggested to be involved in toluene degradation (Washer and Edwards, 2007; Fowler *et al.*, 2012). Moreover, several sequences in cluster II were retrieved from environments dominated by sulfate-reducing conditions, where hydrocarbon oxidizers were associated with *Proteobacteria*-dominant communities (Winderl *et al.*, 2007; Herrmann *et al.*, 2009).

Two sub-groups were distinguishable in Cluster III: the first one (cluster IIIa), defined as the *nmsA sensu stricto* cluster (von Netzer *et al.*, 2013), included sequences from oil-polluted marine sites, contaminated aquifer sediments (von Netzer *et al.*, 2013), and 2MN-specific *nmsA* gene products recently described in some SRB isolates and enrichments able to degrade naphthalene and 2MN (DiDonato *et al.*, 2010; Selesi *et al.*, 2010). The second sub-group (cluster IIIb) comprised sequences obtained from the Atlantic Islands sediments and a few additional sequences of marine origin, the putative *bssA* gene from the non-proteobacterial strain *Desulfotomaculum* sp. Ox39 and sequences retrieved from enrichments able to grow on *o*-xylene as the only carbon source. Although we could amplify *nmsA* related sequences from the *Prestige*-contaminated FI-samples, it is worth noting that, in the current study, the 7757f and 8857r primers were unsuccessful in amplifying the *nmsA* sequence from the *Deltaproteobacteria* strain NaphS2, as previously described for the well characterized 2MN-degrading consortium N47 (von Netzer *et al.*, 2013). Finally, cluster IV corresponded to sequences annotated as AssA. Two clusters could be distinguished within this group: cluster IVa comprised several environmental sequences


a)
Clone frequency
0
10
20
30
40
Figueiras sediment, clone FI-TR027
Figueiras sediment, petroleum patch clone FI-PET009
Figueiras sediment, clone FI-OX026
Figueiras sediment, clone FI-AN013
Mallorca sediment, microcosms clone M-CON045
Mallorca sediment, oil microcosms clone M-OIL017
Mallorca sediment, naphthalene microcosms clone M-NAPH030
Glens Falls groundwater, uncultured bacterium clone bssAC14 (ACR82322)
Zeitz aquifer, o-xylene mineralizing consortium clone Zz-ox_76 (ADA81914)
Contaminated soil sulfate amended, uncultured bacterium clone bssA-7 (AFB74489)
Sulfatereducing bacterium TRM1(ABM92939)
Azoarcus sp. T (AAK50372)
Aromatoleum aromaticum EbN1 (YP_158060)
Thauera aromatica K172 (CAA05052)
Figueiras sediment, clone FI-TR006
Amon mud volcano, uncultured bacterium clone BAA11 (AFX62234) *
Desulfobacula toluolica Tol2 (CAO72221) *
Gulf of Mexico, oil sediment uncultured bacterium clone BG2A13 (AFX62237) *
Amon mud volcano, uncultured bacterium BAA01x3 (AFX62232) *
Flingern aquifer sediment, uncultured bacterium clone D12_31 (ABM91894)
Flingern_aquifer sediment uncultured bacterium clone F4A13 (AFX62262)
Glens Falls groundwater, uncultured bacterium clone bssAC20 (ACR82328)
F2
Geobacter toluenoxydans TMJ1 (ABM92938)
Casper oil refinery, uncultured prokaryote clone Casper OTU3 (ADJS1099)
Flingern aquifer sediment, uncultured bacterium clone D12_18 (ABM91892)
Flingern aquifer sediment, uncultured bacterium clone F4A07 (AFX62256)
F1
Mallorca sediment, oil microcosms clone M-OIL041
Oily sludge, methanogenic enrichment EC clone bssA12 (AEO79010)
Mallorca sediment, naphthalene microcosms clone M-NAPH040
Mallorca sediment, oil microcosms clone M-OIL018
Mallorca sediment, microcosms clone M-CON032
Mallorca sediment, microcosms clone M-CON046
Figueiras sediment, clone FI-TR046
Mallorca sediment, naphthalene microcosms clone M-NAPH044
Figueiras sediment, petroleum patch clone FI-PET053
Figueiras sediment, clone FI-AN049
Figueiras sediment, clone FI-AN029
Mallorca sediment, microcosms clone M-CON050
Mallorca sediment, oil microcosms clone M-OIL049
Rodas sediment, clone RI-037
Figueiras sediment, clone FI-AN044
Figueiras sediment, petroleum patch clone FI-PET060
Figueiras sediment, clone FI-OX043
Figueiras sediment, clone FI-TR049
Desulfotomaculum gibsoniae DSM 7213 (ZP_09103333)
Benzene-degrading Clostridia clone BF (ADJ93876)
Toluene-degrading methanogenic bacterium clone bssA-2 (ABQ30979)
Testfeld Süd aquifer, sediment uncultured bacterium clone B49 bss_087 (ABM91880)
Testfeld Süd aquifer sediment, uncultured bacterium clone B49 bss_105 (ABM91884)
Flingern aquifer sediment, uncultured bacterium clone F4A12 (AFX62261)
Zeitz aquifer, p-xylene mineralizing consortium clone Zz-px 01(ADA81918)
T1
Figueiras_sediment_petroleum_patch_clone_FI-PET007
Mallorca sediment, naphthalene microcosms clone M-NAPH014
Mallorca sediment, oil microcosms clone M-OIL046
Figueiras sediment, clone FI-AN025
Figueiras sediment, clone FI-OX007
Figueiras sediment, clone FI-TR021
Figueiras sediment, clone FI-AN043
Testfeld Süd aquifer sediment, uncultured bacterium clone B49 bss_020 (ABM91878)
Figueiras sediment, clone FI-TR025
Testfeld Süd aquifer sediment, uncultured bacterium clone B49 bss_012 (ABM91877)
Testfeld Süd aquifer sediment, uncultured bacterium clone B49 bss_021 (ABM91879)
T2
Figueiras sediment, clone FI-TR015
Figueiras sediment, clone FI-TR028
Figueiras sediment, clone FI-OX014
Figueiras sediment, clone FI-TR012
Figueiras sediment, clone FI-TR019
Figueiras sediment, clone FI-TR014
Figueiras sediment, clone FI-OX021
Figueiras sediment, clone FI-AN015
Bacterium enrichment culture clone N47 (ADB04297) nmsA
Flingern aquifer sediment, uncultured bacterium clone F3B28 (AFX62308)
Guaymas oil seep, uncultured bacterium clone BGN06 (AFX62295) *
Delta proteobacterium NaphS2 (CAO72219) nmsA
Gulf of Mexico oil sediment, uncultured bacterium clone BG2N02 (AFX62288) *
a
Desulfotomaculum sp. Ox39 (ABM92937)
Flingern aquifer sediment, uncultured bacterium clone F3N20 (AFX62314)
Zeitz aquifer, o-xylene mineralizing consortium clone Zz-ox_56 (ADA81912)
Figueiras sediment, clone FI-AN028
Figueiras sediment, petroleum patch clone FI-PET046
Figueiras sediment, petroleum patch clone FI-PET055
Gulf of Mexico oil, sediment uncultured bacterium clone BG2A01 (AFX62279) *
Figueiras sediment, petroleum patch clone FI-PET044
Amon mud volcano, uncultured bacterium BAA23 (AFX62278) *
Figueiras sediment, petroleum patch clone FI-PET051
Gulf of Mexico oil, sediment uncultured bacterium clone BG2A06 (AFX62282) *
b
Figueiras sediment, clone FI-AN018
Figueiras sediment, petroleum patch clone FI-PET054
Gulf of Mexico, oil sediment uncultured bacterium clone BG2A05 (AFX62281) *
Rodas sediment, clone RI-043
Figueiras sediment, clone FI-AN053
Figueiras sediment, clone FI-TR051
Figueiras sediment, petroleum patch clone FI-PET068
Figueiras sediment, clone FI-OX049
Figueiras sediment, clone FI-OX011
Figueiras sediment, clone FI-AN050
Methanogenic alkane degrading enrichment clone assA-63 (AEI52403)
Shengli oil field, methanogenic hexadecane-degrading consortium clone H1 (AET09969)
Aquifer sediment, uncultured prokaryote Fort Lupton OTU2 (ADJ51092)
Gowanus canal, sediment uncultured prokaryote clone OTU1 (ADJ51088)
a
Oily sludge, methanogenic enrichment ET clone assA55 (AEO89354)
Guaymas oil seep, uncultured bacterium clone BGM24 (AFX62322) *
Shengli oil field, methanogenic alkane degrading enrichment clone SL34assA-1 (AEY68270)
Guaymas oil seep, uncultured bacterium clone BGM02 (AFX62321) *
Azoarcus sp. HxN1 (CAO03074) masD
Mallorca sediment, oil microcosms clone M-OIL050
Zodletone_Mountain, uncultured prokaryote Propane-degrading enrichment OTU1 (ADJ51096)
Arthur Kill river, sediment uncultured prokaryote clone OTU1 (ADJ51095)
Gowanus canal, OTU4 (ADJ51078)
Passaic River, OTU8 (ADJ51080)
Passaic River, OTU9 (ADJ51084)
Desulfoglaeba alkanexedens ALDC (ADJ51097)
Desulfatibacillum alkenivorans AK-01 (ABH11460)
Mallorca sediment, microcosms clone M-CON041
Mallorca sediment, naphthalene_microcosms_clone_M-NAPH041
Mallorca sediment, oil_microcosms_clone_M-OIL043
Mallorca sediment, oil_microcosms_clone_M-OIL044
Mallorca sediment, naphthalene_microcosms_clone_M-NAPH043
Mallorca sediment, oil_microcosms_clone_M-OIL048
Mallorca sediment, microcosms_clone_M-CON051
Figueiras sediment, petroleum_patch_clone_FI-PET057
Mallorca sediment, oil_microcosms_clone_M-OIL030
Mallorca sediment, oil_microcosms_clone_M-OIL038
Figueiras sediment, clone_FI-OX020
Figueiras sediment, clone_FI-AN014
Figueiras sediment, clone_FI-TR017
Figueiras sediment, petroleum patch clone FI-PET012
b
Clostridium butyricum (ABW38375) glycerol dehydratase
Archaeoglobus fulgidus (NP_070278) pyruvate formate-lyase
out-group
Mallorca
Figueiras petroleum patch
Figueiras (OX, TR, AN)
Rodas
* Other marine origin
0.2
cluster I
cluster II
cluster III
cluster IV

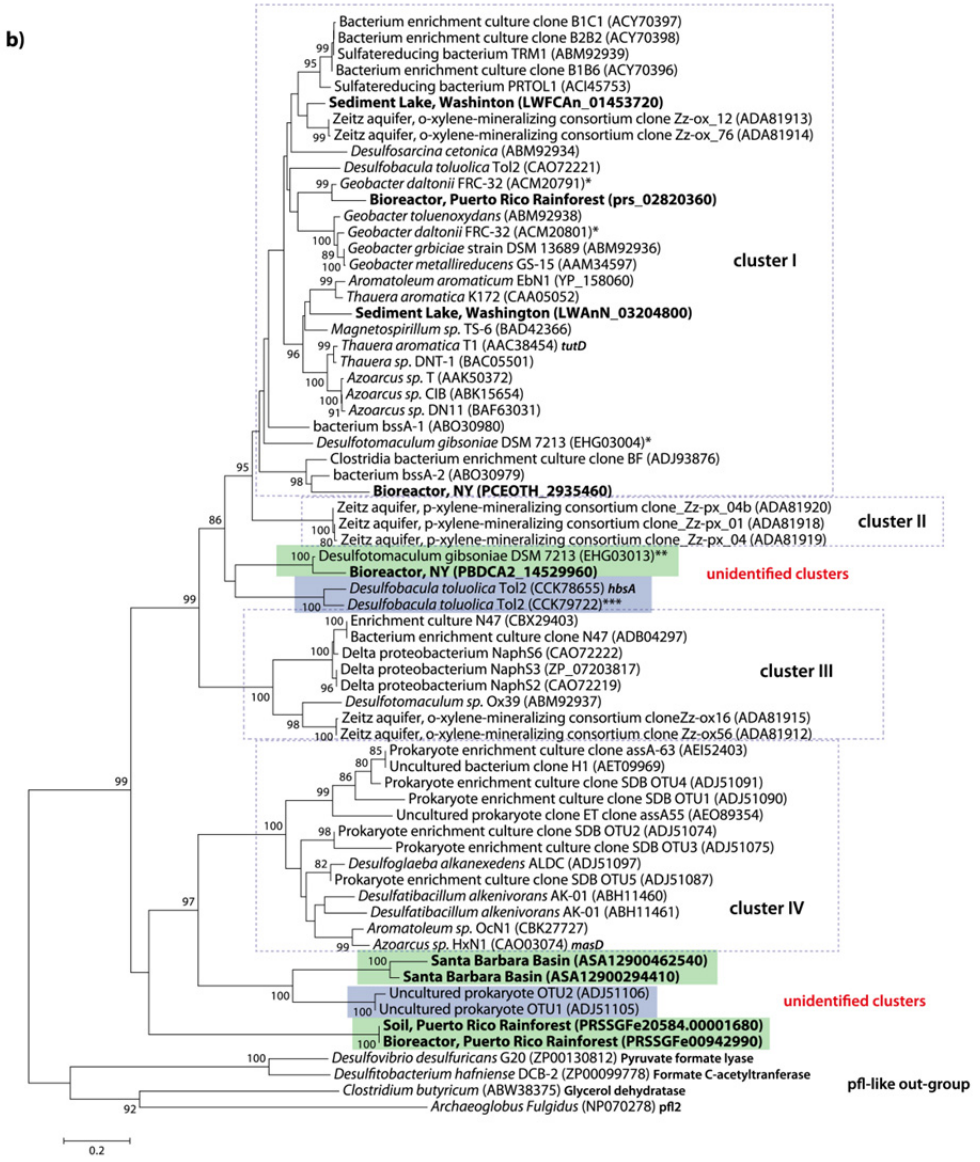


Figure 3.2. a) Phylogeny of partial BssA-like amino acid sequences retrieved from marine sediments from Figueiras beach (FI, blue), a petroleum patch detected in the sediment (FI-PET, green) and Rodas beach (RI, brown) in the Atlantic Islands, and sediment microcosms from Mallorca (M, red). Closest relatives, detected in anaerobic hydrocarbon oxidizing strains or enrichments, and putative environmental sequences available in the databases at the beginning of this study are included. Different depths corresponding to oxic (FI-OX), transition (FI-TR) and anoxic (FI-AN) zones and were analyzed in Figueiras. Mallorca sediments were treated with *Prestige*'s oil (M-OIL), naphthalene (M-NAP) and nothing (M-CON). Sequences in the databases retrieved from marine environments are marked with an asterisk. Numbers in parentheses represent protein GenBank accession numbers. A sequence cutoff of 5% of amino acid dissimilarity ($OTU_{0.05}$) was used to select the sequences and calculate the sequence frequency (showed as horizontal bars at the right). The tree was rooted with pyruvate formate lyase (PFL2) paralogues as out-group. **b)** Unidentified clusters found in our analysis. The phylogenetic tree shows all BssA-like sequences from isolated strains and enrichment cultures deposited in databases and sequences annotated as putative pyruvate-formate lyase (pfl) enzymes from genomes and metadata (metagenomes). The analysis suggests the existence of non-classified clusters

(highlighted in blue and green). Asterisks denote the sequences annotated as pfl from aromatic oxidizing strains that belong to cluster I (*) or unidentified cluster (**). A third sequence (BssA3) from *Desulfobacula toluolica* Tol2 annotated as BssA (Wöhlbrand *et al.*, 2013) groups in the HbsA cluster (***). The sequences retrieved from metagenomes are written in bold. Two sequences annotated as putative glycyl radical enzymes (Callaghan *et al.*, 2010) are included in the analysis (ADJ51105 and ADJ51106). Numbers in parentheses represents protein GenBank accession numbers or gene object ID (metagenomic data).

retrieved from a methanogenic octacosane-degrading enrichment (designated SDB) and from hydrocarbon-impacted aquifer sediments where the presence of methanogens in 16S rRNA libraries was reported as evidence of a methanogenic metabolism (Callaghan *et al.*, 2010). Cluster IVa also included sequences from a methanogenic consortium growing on different length alkanes, which had previously been annotated as AssA sequences in published (Wang *et al.*, 2012) and unpublished studies (Table S3.1). None of the sequences retrieved from our sediment samples clustered within this group. It is worth noting that *Archaea* were not detected in the Atlantic Island samples using fluorescence in situ hybridization (FISH) analysis (Acosta-González *et al.*, 2013). Cluster IVb comprised sediment sequences derived by the current study, previously described alkane-specific AssA sequences retrieved from well-characterized alkane-degrading strains, and several environmental sequences from hydrocarbon impacted sediments (Figure 3.2a). The only plausible factor that explained the separation in clusters IVa an IVb was the phylogenetic divergence between bacterial populations associated with methanogenic or non-methanogenic conditions. Some other sequences retrieved from the methanogenic enrichment culture SDB (Callaghan *et al.*, 2010) were found in cluster IVb. Thus, the methanogenic consortium in that culture probably had many bacterial counterparts responsible for hydrocarbon degradation.

Based on the literature review of the representative strains and enrichments identified in Figure 3.2a, the primary factor controlling the clustering of BssA-like sequence was likely the range of substrates that could be recognized by each sequence-encoded protein. P1 separated proteins that could degrade alkane from those degrading aromatics, while P2 separated methylnaphtalene from substituted mono-aromatic or alkane degrading enzymes. An apparent phylogeny-dependent clustering could also be observed for sequences with a known phylogenetic origin within each cluster.

The database search for *bssA* homologues also retrieved some entries that were not affiliated with any cluster despite their high homology with *bssA*-like sequences (Figure 3.2b). These sequences include a second *bssA* homologue in *D. gibsonie* DSM7213 genome (EHG03013), two putative PFL sequences amplified from a polluted aquifer, five metagenomic sequences derived from methane oxidizing archaeal communities in the Santa Barbara Basin, the recently described hydroxybenzylsuccinate synthase *hbsA* gene from *Desulfobacula toluolica* and a third bssA-like gene in this strain (Table S3.1 and Figure

3.2b). These sequences seemingly represent new phylogenetic branches of *bssA* homologues (Figure 3.2b) (Wöhlbrand *et al.*, 2013).

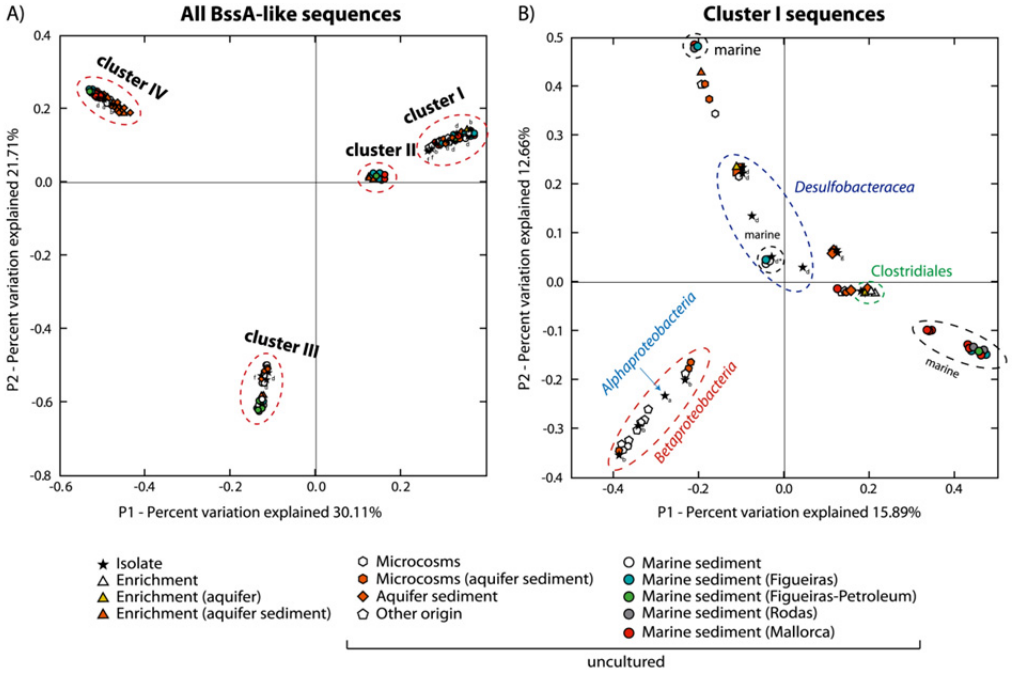


Figure 3.3. Unweighted PCoA plot based on distances between BssA-like sequences present in the sediment samples collected from marine sediments (Figueiras beach, Rodas beach and Mallorca) and available in the databases (see table S3.1). **A)** PCoA of all BssA-like sequences; **B)** PCoA of BssA Cluster I sequences. The percentage of the variation between the samples by principal components is indicated on the axes. Spots of sequences from bacteria isolates (stars) are labeled with a letter that indicates the taxonomic affiliation: *Alphaproteobacteria* (a), *Betaproteobacteria* (b), *Clostridiales* (c), *Geobacterales* (g) and *Delsulfobacterales* (d). Bacterial isolates from marine environments are marked with an asterisk. Colored circles indicate sample analyzed in this work. A sequence cutoff of 2 of amino acid similarity ($OTU_{0.02}$) was used to select the sequences for the analysis.

Diversity of *bssA*-like genes in marine sediments of two different origins.

In the different libraries, the proportion of sequences present in each cluster varied according to the sample origin. The highest proportion of AssA sequences was observed in the M-samples (Figure 3.4). The majority (94%) of sequences in the three gene libraries obtained from Mallorca grouped in either BssA cluster I (60 sequences) or AssA cluster IVb (81 sequences), most of which were derived from the M-OIL sample (Figure 3.4). The 9 remaining sequences were assigned to cluster II. In contrast to the Mallorca sediments, less than 4% of sequences assigned in the FI-samples were assigned to the AssA group. After four months of incubation of Mallorca sediments with crude oil, the increase in hydrocarbon levels was concomitant with a slight increase in the relative abundance of

assA sequences (Table S3.2b). Because the *Prestige* oil was composed of both alkanes and aromatic hydrocarbons, the stimulation of a specific degrader population suggests that the alkane degrading community responded more rapidly in these sediments; this conclusion was supported by a low nC18/phytane ratio (Fig. 3.1b) (Díez *et al.*, 2005). Still, the presence of *bssA*-like sequences in the untreated sample suggests a low basal level of hydrocarbons in this area (e.g., through sporadic bilge water discharges), which would sustain such microbial populations.

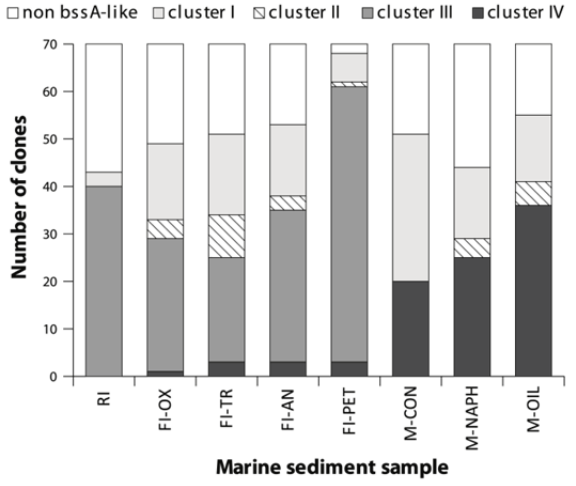


Figure 3.4. Frequency distribution of BssA-like sequence types in the different libraries constructed from sediment samples. Non bssA-like, white; cluster I, light grey; cluster II, streaked; cluster III, dark grey; cluster IV, black.

PCoA of all AssA sequences (cluster IV) identified cluster IVa at one end of the P2 coordinate (Figure 3.5). In addition a group formed primarily of sequences retrieved from Passaic aquifer sediments (Callaghan *et al.*, 2010) could be distinguished within cluster IVb. The phylogenetic tree showed that these sequences were more closely related to sequences from *Desulfatibacillum alkenivorans* AK-01 than to previously described environmental sequences retrieved from aquifer sediments (Figure 3.2a). This is consistent with the great abundance of *Deltaproteobacteria* in the Passaic samples and the detection of an active SRB population surviving on crude oil components. The minor differences observed between FI- and M- samples in cluster IVb suggest that these could belong to closely related microorganisms.

Independently of their environmental origin (geography, depth or pollution level), the sequences retrieved from the marine sediment samples clustered together and separately from the other BssA-like sequences. In cluster I marine sediment sequences were associated with previously described proteobacterial and environmental BssA sequences, forming in most cases separated clusters (Figure 3.2a). The few marine-derived sequences available in the literature (von Netzer *et al.*, 2013) formed a distinct cluster with the sole marine isolate *D. toluolica* Tol2 BssA and with clone FI-TR006 retrieved in this work. PCoA of all sequences belonging to cluster I, the two main coordinates separated marine samples in two main clusters. One cluster was close to *Deltaproteobacteria* and may represent

diverging marine organisms within this group; the second cluster could not be assigned to any phylogenetic group (Figure 3.3b). *Betaproteobacteria* formed a consistent cluster that contained no sediment sequence, and a group a sequences from Mallorca clustered with a central group related to *Clostridiales* isolates and sequences retrieved from methanogenic enrichments.

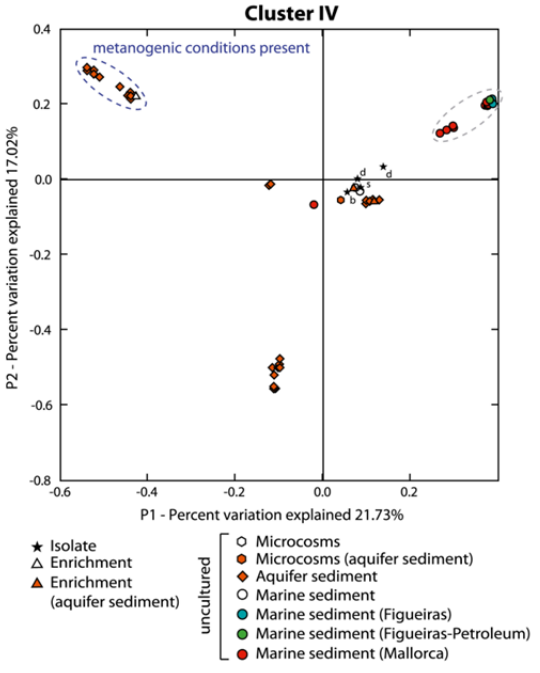


Figure 3.5. PCoA (Principal coordinate analysis) of distances between *assA* sequences present in the sediment samples collected from marine sediments (Figueiras beach, Rodas beach and Mallorca) and sequences available in the databases (see table S1). The positions of the sequences corresponding to isolates are labeled with a letter that indicates the taxonomic affiliation: *Betaproteobacteria* (b), *Desulfobacterales (Desulfatibacillum alkenivorans AK-01)* (d) and *Syntrophobacterales (Desulfoglaeba alkanexedens*) (s). The percentage of the variation between the samples by principal coordinate is indicated on the axes. A sequence cutoff of 2% of amino acid similarity ($OTU_{0.02}$) was used to select the sequences for the analysis.

On the other hand, clusters II and III were clearly dominated by sequences from the Atlantic Islands sediments. The majority of sediment sequences clustered together with sequences of marine origin and formed a distinct group within cluster III (Figure 3.2a). More than 90% of the retrieved sequences in the Rodas beach sample (RI), where no AssA-type sequences were detected, and almost 90% of the sequences from the petroleum aggregate sample (FI-PET) clustered within cluster III and close to the putative *Desulfotomaculum sp.* OX39 *bssA* sequence (Figure 3.4). Incubation of this *o*-xylene degrading strain with a mixture of *m*-xylene and *o*-xylene demonstrated that the degradation pathways of these two compounds was initiated by different fumarate-adding enzymes (Morasch *et al.*, 2004). It seems that the amplified sequence of this strain did not correspond to a toluene/xylene specific *bssA* gene but rather to a related *bssA*-like enzyme. Our attempts to grow *Desulfotomaculum sp.* OX39 with 2MN as the only carbon source were unsuccessful but there were strong indications that this strain could co-metabolize dimethylnaphthalenes when *m*-xylene was the major organic substrate (Morasch *et al.*, 2004). The main contaminants in the *Prestige*-affected sediments were naphthalene and alkylated derivatives (Table S3.2a and S3.2b (Alzaga *et al.*, 2004; Acosta-González *et al.*, 2013)),

including primarily methylated and dimethylated compounds. We thus associated the Atlantic Islands sequences in this cluster with the degradation of methylated naphthalenes. Interestingly, none of the sequences retrieved from the Mediterranean sediment microcosms were found in this group.

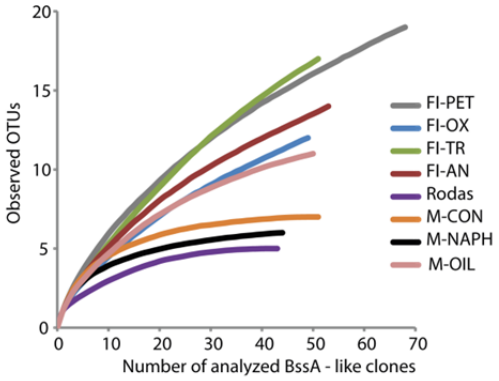


Figure 3.6. Rarefaction curves of the BssA-like sequences from sediments samples (FI, Figueiras; M, Mallorca) with a sequence cutoff of 2% of amino acid similarity ($OTU_{0.02}$).

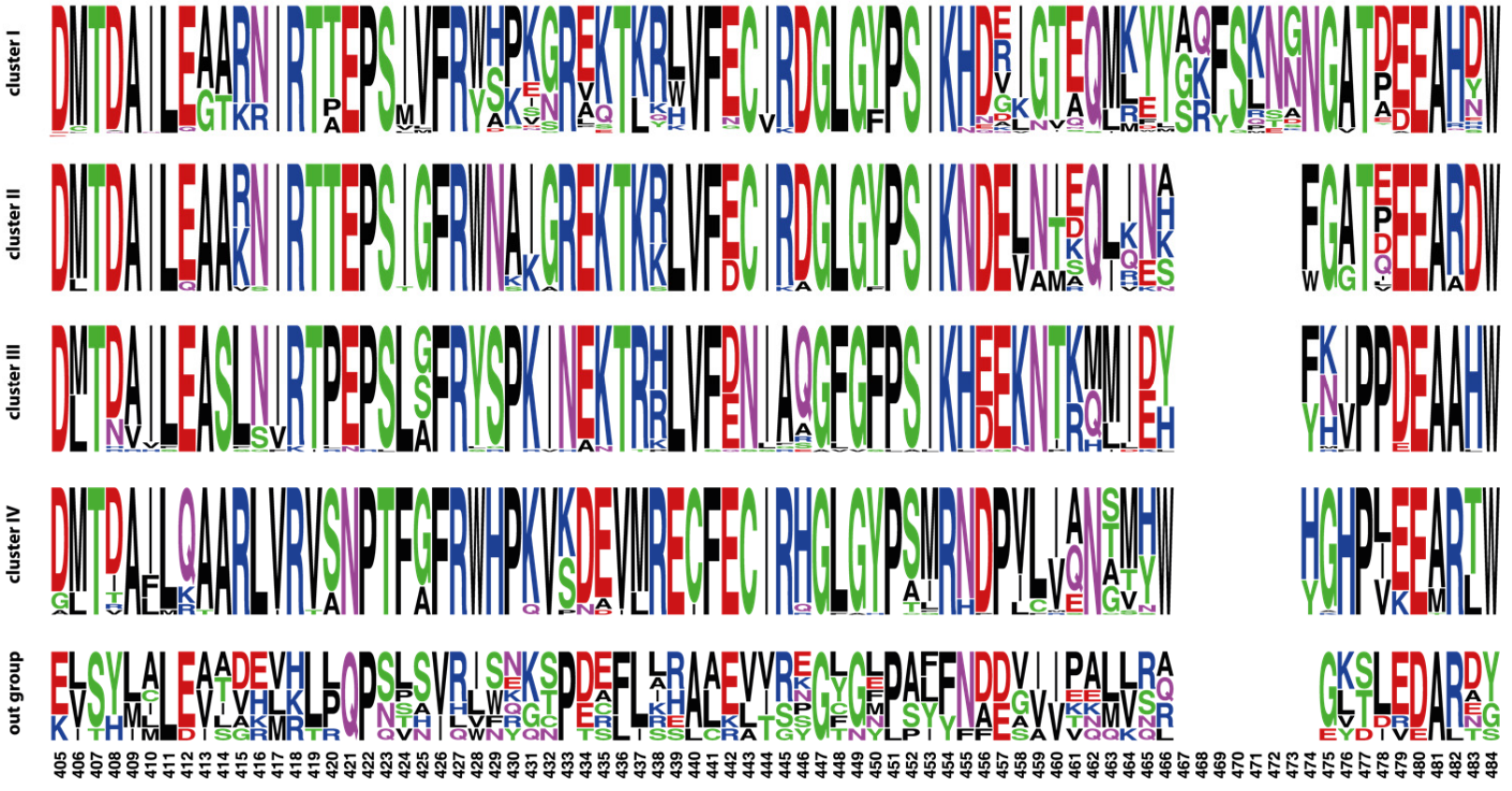
Rarefaction analysis of all marine sediment libraries showed that the diversity of the Mediterranean BssA-like sequences was low compared to those derived from the Atlantic Island samples, except for the oil polluted M-OIL sample, which were mainly represented in clusters I and IVb (Figure 3.6). Comparison of BTEX compounds in the original *Prestige* oil and in the oil treated microcosm indicated more rapid consumption of toluene and ethylbenzene relative to xylenes in these samples (Fig. 3.1a), which could explain the near absence of Mallorca sequences in cluster II. This suggests that the biodegradation of hydrocarbon contaminants may require a longer exposure period. FI-PET displayed the greatest sequence diversity (Figure 3.6), and included sequences that were distributed among all clusters except cluster IVa, as it was the case for the remaining FI-samples. In contrast, only two sequence types were observed in the RI sample collected from a less polluted beach in the same area: three sequences belonged to the same OTU in cluster I and the remaining sequences formed a single OTU in cluster III. This sample displayed the lowest diversity and also showed the higher proportion of unspecific amplified sequences. Altogether our data suggest that the stimulation of bacterial hydrocarbon oxidizers by the presence of pollutants resulted in a substantial increase in functional gene diversity as compared to non-polluted sites.

Protein sequence determinants of functional diversity.

The PCoA performed with all available BssA-like sequences clearly identified different clusters that could be related to the particular range of substrates utilized by each enzyme (Figure 3.3A). If true, alignment of the protein sequences in each cluster should reveal both similarities in the active site residues that are relevant for substrate specificity among

homologues sharing similar substrates, and major differences at these sites for enzymes that target different groups of substrate. The sequences in the amplified PCR fragments analyzed in this work included the active site of the enzyme, especially the conserved Cys residue directly involved in the abstraction of a hydrogen atom from the substrate (Selmer *et al.*, 2005). The binding pocket of the active site has been accurately defined by the structure of four crystallized proteins belonging to the radical enzyme family: *Clostridium butyricum* glycerol dehydratase (O'Brien *et al.*, 2004), *Archaeglobus fulgidus* PFL2 (Lehtiö *et al.*, 2006), *Clostridium scatologenes* hydroxyphenylacetate decarboxylase (Martins *et al.*, 2011) and *Escherichia coli* pyruvate formate-lyase (Becker *et al.*, 1999). The residue positions shaping this pocket have been identified in each structure and mapped in the multiple sequence alignment. Despite the relatively low homology between the four proteins (41-53%), the position of these residues in the structure was conserved and could be superimposed between structures (Martins *et al.*, 2011). To locate these positions in the BssA-like sequences, we built sequence logos for each cluster (Figure 3.7). Inspection of the different logos revealed a high degree of conservation between the sequences of the different clusters, and identified variable regions and residues. A gap of seven residues between positions 467 and 474 (all positions henceforth refer to *T. aromatica* K172 BssA numbering (Leuthner *et al.*, 1998)) distinguished all sequences with respect to cluster I. In addition cluster IV showed two single gaps at positions 508 and 528 and an insertion after position 603 with respect to the remaining sequences.

Positions 491 and 507 are two of the five main residues that shape the active site pocket (in addition to the reactive Cysteine) that were included in the amplified fragment. In addition, residues His536 and Glu637 of *C. scatologenes* hydroxyphenylacetate decarboxylase (corresponding to positions 509 and 611 in the alignment) were shown to interact with the substrate *p*-hydroxyphenylacetate (Martins *et al.*, 2011). To map these residues in the protein active site, we built a possible structure model of a representative protein sequence fragment from cluster III (FI-PET068) using *C. butyricum* glycerol dehydratase chain A (PDB 1r9d_A) as template (O'Brien *et al.*, 2004), with which it shared 47% homology (Figure 3.8). Several residues in the FI-PET068 sequence could not be modelled and are presented as a dotted black line. Glycyl radical enzymes share a common global protein topology, consisting of a ten-stranded α/β-barrel composed of two antiparallel five stranded sheets surrounded by α-helixes. The active site pocket is embedded in the core of this structure. The putative active site pocket positions were occupied by a different residue in the different cluster proteins and are summarized in Figure 3.8: position 491 was Ser in all clusters except cluster III, where it was Ala; positions 507 and 509, which flanked a characteristic gap in cluster IV, had a conserved Gly residue in all clusters except clusters IV, where the residues were Ser and Ala; position 611 was only conserved in cluster I proteins (Figure 3.8).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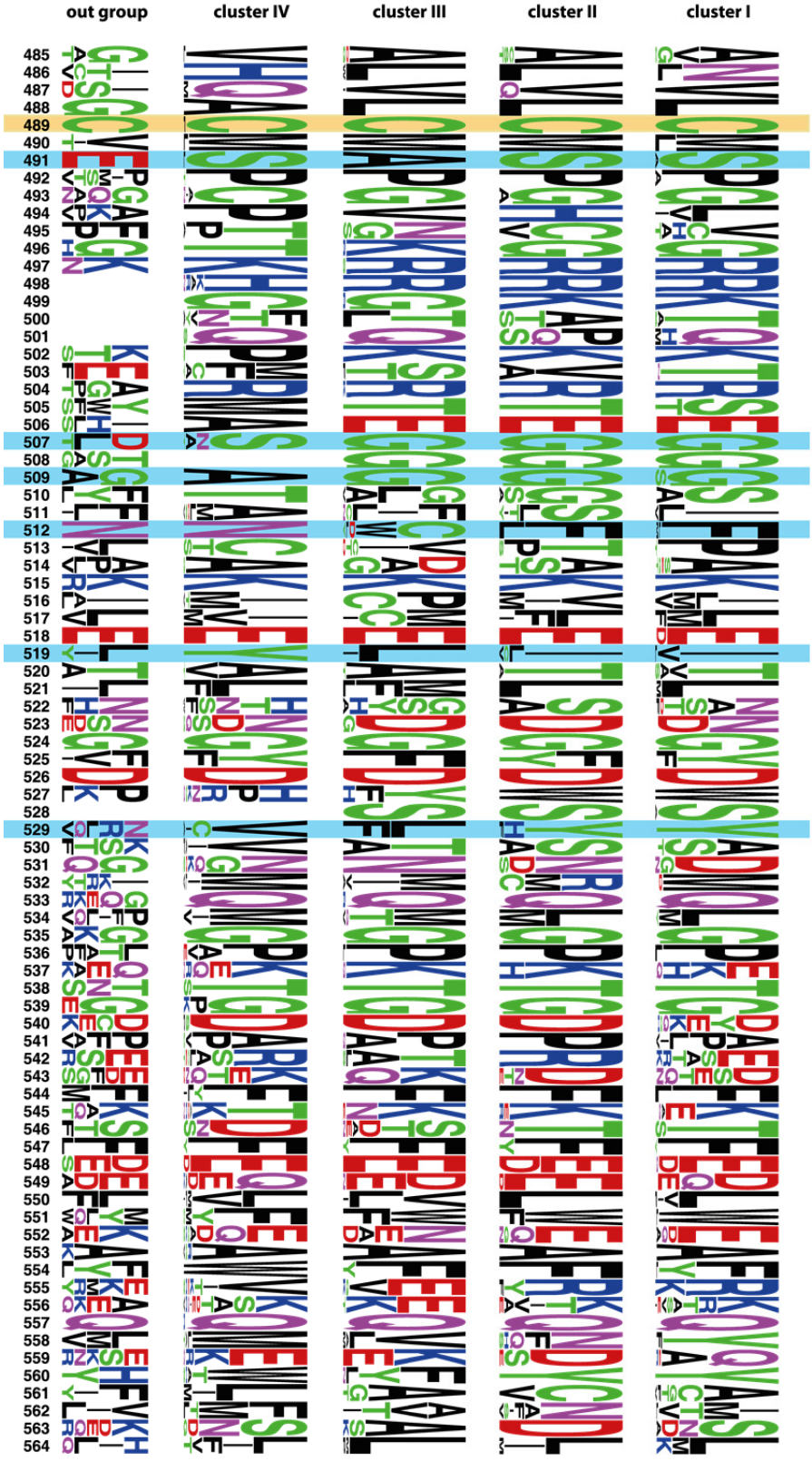
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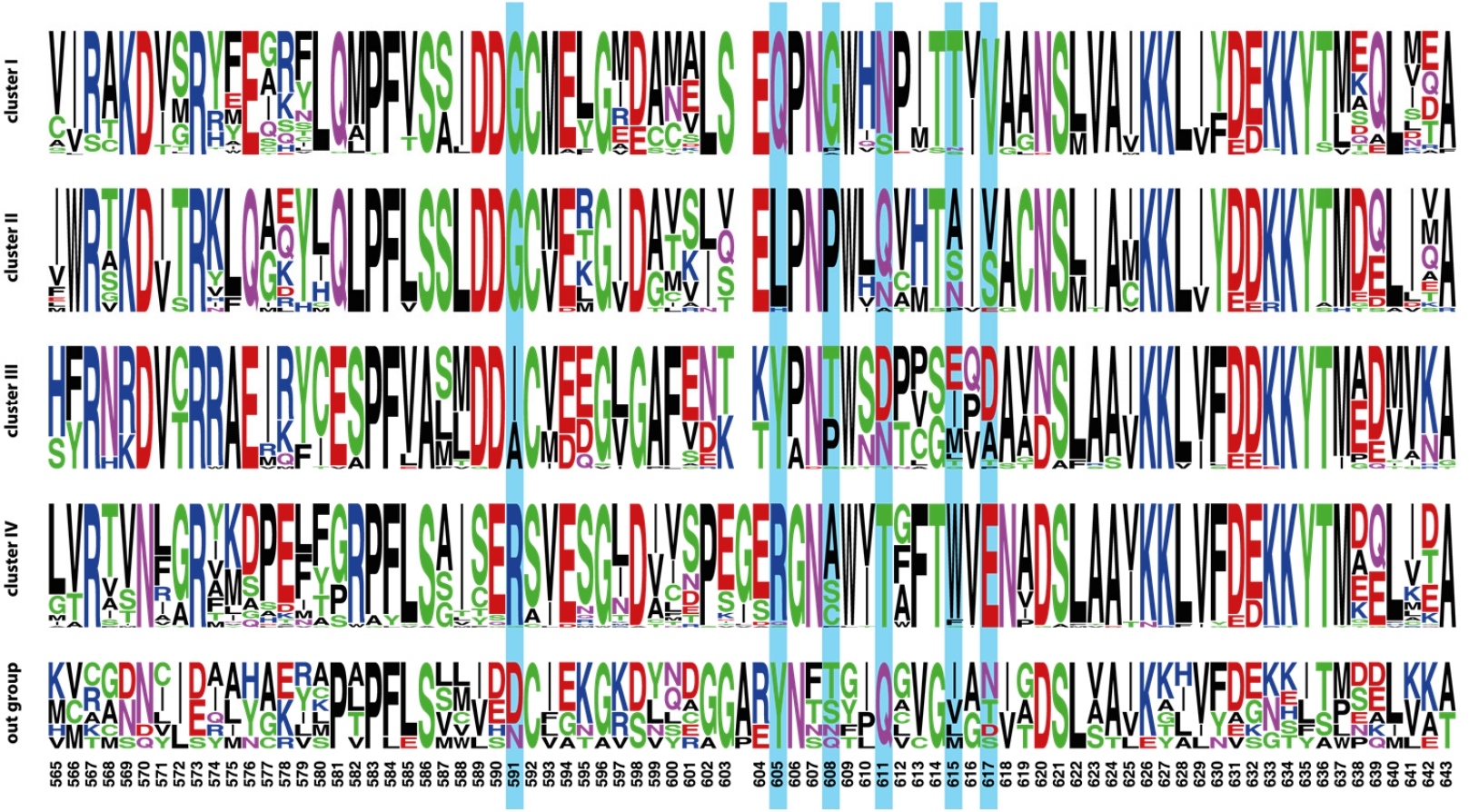
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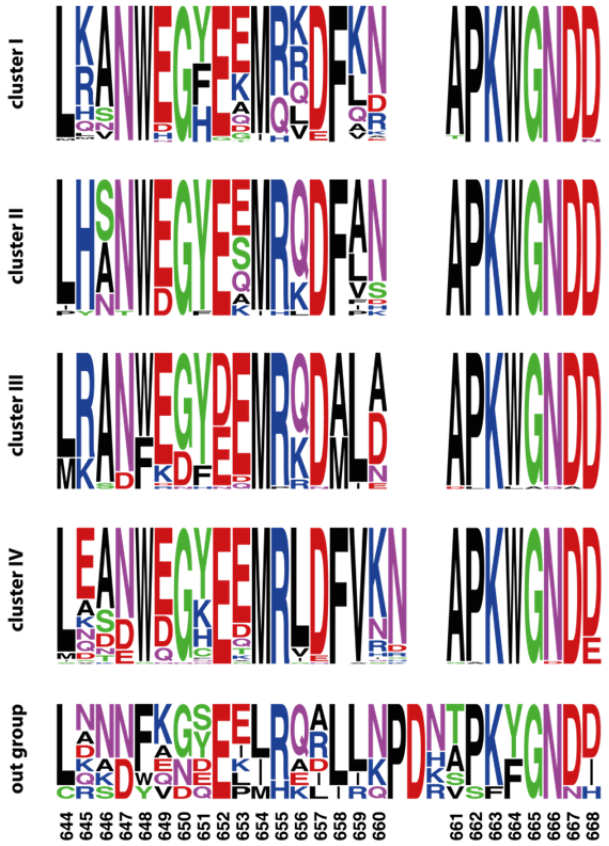
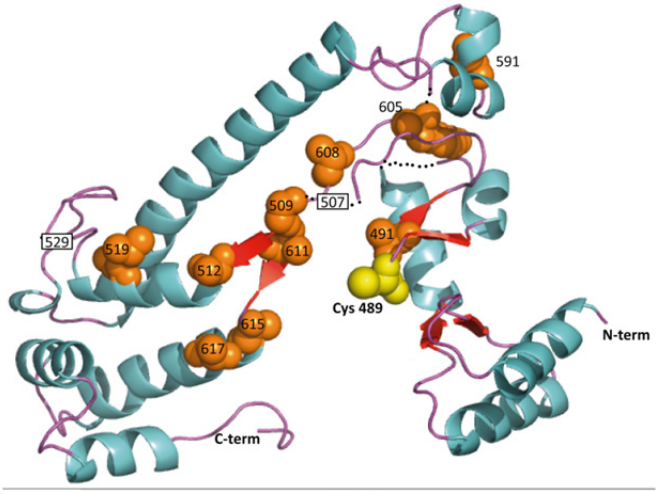


Figure 3.7. Alignment of the consensus sequences of the different clusters determined in the study showing the frequency profiles of amino acids at each position (logo). Position numbers refer to *Thauera aromatica* K172 sequence. The active site cysteine residue is highlighted in yellow, while the specificity relevant residues shown in Figure 4 are highlighted in blue.



Cluster	Position											
	491	507	509	611	512	617	591	615	608	529	605	519
I	Ser	Gly	Gly	Asn	Phe	Val	Gly	Thr	Gly	Tyr	Gln	Ile
II	Ser	Gly	Gly	X	Phe	X	Gly	X	Pro	Tyr	Leu	Ile
III	Ala	Gly	Gly	X	X	X	X	X	X	X	Tyr	Leu
IV	Ser	Ser	Ala	Thr	Asn	Glu	Arg	Trp	X	Val	Arg	Tyr

Figure 3.8. Mapping of the putative residues involved in substrate specificity in FI-PET068 BssA fragment model structure obtained using the crystal structure of *Clostridium butyricum* glycerol dehydratase chain A (1r9d_A) as template. Alpha-helices are represented as blue spirals and β-strands as red arrows. The active site Cys residue is shown as a yellow sphere. The sequence fragments (residues 474, 504 to 507 and 527 to 529) that were not resolved in the model are shown as black dotted lines. The relevant active site pocket residues and specificity-determining site (SDS) residues with highest score according to SPEER analysis are depicted as orange spheres except positions 507 and 529 located in a non-modeled sequence fragment, which are shown in a white rectangle. Positions are numbered according to *T. aromatica* K172 sequence (CAA05052). The table shows the conserved amino acid residue occupying the predicted cluster, where X means that the position was not conserved.

To detect additional positions that could be relevant to protein specificity (the so-called specificity determining sites, SDS), we used the SPEER methods, which can define protein subfamilies and predicts family protein functionally relevant residues (Chakrabarti *et al.*, 2007; Chakraborty *et al.*, 2012). Analysis of a multiple alignment of all available BssA-like sequences with SPEER software gave four different groups that matched the clusters previously defined in the phylogenetic tree and left the four unclassified sequences as singletons. The SPEER software could identify several residues predicted to be relevant for protein substrate specificity (Figure 3.8). All predicted SDS were located downstream of the conserved Cys in the active site. We observed stronger differences between the AssA group (clusters IV) and the remaining groups, such that all SDS residues in this group differed from the rest. Finally position 605, which was occupied by a different amino acid residue in each cluster, could distinguish the four subfamilies. The structural consequences of all these

changes are difficult to predict, but could result in slight adjustments in the active site cavity that would alter its properties (polarity, shape, volume) and could determine differences in the range of substrates recognized by the enzyme.

3.4. Concluding remarks

In this work we have been able to detect different analogues of the *bssA* gene, which reflect the strong potential for hydrocarbon degradation in these coastal sediments. The *bssA*-like gene diversity was correlated with the presence of pollutants like mono-aromatics and naphthalene derivatives. The long persistence of pollutants seemed to determine the presence of BssA-like variants; sequences retrieved from the Atlantic Islands sediments could be found in all clusters and were more diverse than Mallorca sequences. Mallorca samples were deliberately contaminated during a short incubation period and exhibited only small differences compared to non-polluted sediments. The global analysis of the *bssA* gene suggested a broad substrate-dependent clustering and the existence of new clusters of analogous enzymes mainly detected in metagenomic analyses. Sequences of marine origin were separated from the terrestrial homologues, indicating a geographical divergence within clusters. The description of new gene variants would require both enhanced probe design and the exploration of new environments, as this work has shown. Although the number of metagenomes in databases is increasing, the number of *bssA* sequences retrieved with this amplification-independent approach is limited. Thus, the detection of functional biomarkers will continue to be a good indicator of biodegradation potential.

Acknowledgements

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Suplementary material

(Chapter III)

Table S3.1. BssA-like sequences retrieved form the databases and used in the analysis.

bssA* like	**Protein ID**	**Seq.**	**Sample source**	**Contaminants****	**notes**	**Ref.**
Thauera aromatica strain T1	AAC38454	1	Untreated gasoline spill soil, California (USA)	Gasoline		Coschigano and Young, 1997
Thauera aromatica K172	CAA05052	1	Municipal sewage plant in Konstanz (Germany)		Anaerobic sludge	Leuthner *et al.*, 1998
Azoarcus sp. T	AAK50372	1	Anaerobic, toluene-degrading Laboratory aquifer column.	Toluene	Column filled with material from the interface of a river-groundwater infiltration site.	Achong *et al.*, 2001
Geobacter metallireducens GS15	AAM34597	1	Freshwater surficial bottom sediments, Potomac River.			Kane *et al.*, 2002
Aromatoleum aromaticum EbN1	YP_158060	1	Anoxic freshwater mud sampled in Bremen, Germany			Kube *et al.*, 2004
Thauera sp. DNT-1	BAC05501	1	Anaerobic sludge from a wastewater treatment plant			Shinoda *et al.*, 2004
Magnetospirillum sp. TS6	AD42366	1	Soils from damp ground and rice paddies		Denitrifying *Alphaproteobacteria*	Shinoda *et al.*, 2005
Azoarcus sp. CIB	ABK15654	1				Un[1]
Azoarcus sp. DN11	BAF63031	1	Contaminated subsurface aquifer, Kumamoto (Japan)	Gasoline-contaminated groundwater		Kasai *et al.*, 2007
Desulfosarcina cetonica DSM 7267	ABM92934	1	Oil recovery water, Azerbaijan			Winderl *et al.*, 2007
Geobacter grbiciae strain DSM 13689	ABM92936	1	Freshwater sediment, estuary of the Potomac River, USA.			
Desulfotomaculum sp. OX39	ABM92937	1	Sediment of a drilling core taken at a former gasworks plant near Stuttgart (Germany)	Monocyclic aromatics, PAHs, and heterocyclic compounds.		

Geobacter toluenoxydans TMJ1	ABM92938	1	Sediment from a near Stuttgart (Germany)	Tar-oil-contaminated site		
Sulfate reducer TRM1	ABM92939	1	Soil percolation column filled with material from a contaminated aquifer (Stuttgart)	BTEX		
Desulfobacula toluolica strain Tol2	CAO72221	1	Marine sediment, Eel Pond			
Sulfate-reducing bacterium PRTOL1	EU780921	1	Naval Air Station, Patuxent River	Aviation fuel-contaminated soil		Beller *et al.*, 2008
Georgfuchsia toluolica strain G5G6T	CBH30982	1	Polluted aquifer, Banisveld (The Netherlands)	BTEX-containing landfill leachate		Weelink *et al.*, 2009
Geobacter daltonii FRC-32	ACM20791 ACM20801	2	Contaminated shallow subsurface	mixed radionuclides, heavy metal and hydrocarbon contamination	Annotated as Formate C-acetyltransferase	Un[2]
Desulfotomaculum gibsoniae DSM 7213	EHG03004	1	freshwater mud		Annotated as pyruvate formate-lyase (PFL)	Un[3]
Bacterium bssA-2	ABO30979	1	Sand deposits at Pensacola aquifer (California)	Nitrogen heterocycles, PAHs and phenols	toluene-degrading methanogenic consortium	Washer and Edwards, 2007
Bacterium bssA-1	ABO30980	1				
Bacterium enrichment culture	ADA81912-ADA81917	6	heavily contaminated former industrial field site Zeitz (Germany)	BTEX	*o*-xylene-mineralizing consortium	Herrmann *et al.*, 2009
	ADA81918-ADA81925	8			*p*-xylene-mineralizing consortium	
Bacterium enrichment culture	ACY70396-ACY70398	3	BTEX-contaminated aquifer near Zeitz (Germany)	BTEX	Groundwater Enriched consortium Zz5-7	Jehmlich *et al.*, 2010
Clostridia enrichment (clone BF)	ADJ93876	1	Soil of a former coal gasification site in Gliwice, Poland	Bencene	benzene-degrading and iron-reducing clone	Abu Laban *et al.*, 2010
uncultured bacterium	ABM91877-ABM91886	10	Former gasworks site, Testfeld Süd near Stuttgart (Germany)	Tar-oil-contaminated	Contaminated aquifer sediments	Winderl *et al.*, 2007

	ABM91887-ABM91896	10	Contaminated Flingern aquifer sediments, Düsseldorf (Germany)			
	ABM91897-ABM91912	16	Gasworks site in Pasing, Munich (Germany)			
uncultured bacterium	ADG27767-ADG27785	19	Gasworks site, aquifer sediment, Testfeld Süd, (Germany)	Coal processing products, BTEX, PAHs	Toluene-degrading 'heavy' DNA (SIP)	Winderl *et al.*, 2010
uncultured prokaryote	ADJ51098-ADJ51100, ADJ51102, ADJ51103	5*	Oil refinery in Casper (Wyoming)	Fuel and liquid propane gas, gasoline, heavy fuel oil, kerosene, asphalt	Contaminated aquifer sediment	Callaghan *et al.*, 2010
	ADJ51101, ADJ51104	2	Gas condensate-contaminated Aquifer, South Platte alluvial aquifer, Fort Lupton (Colorado)	96% w/w C5-C15 compounds (18% w/w BTEX)		
uncultured bacterium	ACR82309-ACR82335	27	Groundwater, rural wooded area in South Glens Falls (NY)	Gas condensate, BTEX	Coal tar waste-contaminated groundwater	Yagi *et al.*, 2010
uncultured *Thauera sp.*	AFB74483, AFB74485-AFB74487	4	Granular sludge nitrate amended (Washington)	BTEX	Toluene degrading microcosms	Sun and Cupples, 2012
	AFB74484	1	Agricultural soil nitrate amended (Michigan)			
uncultured *desulfobacterium*	AFB74488	1	Contaminated soil sulfate amended (Michigan)			
uncultured bacterium	AFB74489	1				
uncultured bacterium	AEO79008-AEO79010	3	Oily sludge	Oil	Incubated for 500-days under methanogenic conditions without any carbon sources	Wang *et al.*, 2012
uncultured prokaryote	AEY68269	1	Production water from Shengli oilfield, Shangdong (China)	moderately heavy oil	Methanogenic enrichments cultures	Mbadinga *et al.*, 2012

uncultured bacterium	AFX62232-AFX62236	5	Amon mud volcano station 929			von Netzer *et al.*, 2012
	AFX62237	1	Gulf of Mexico, hydrocarbon seep dive 161			
	AFX62238-AFX62277, AFX73928-AFX73934	47	Contaminated Flingern aquifer sediments, Düsseldorf (Germany).	Tar-oil contaminated	Contaminated aquifer sediments. Sampling from 2009.	
nmsA* like	**Protein ID**	**Seq.**	**Sample source**	**Contaminants****	**notes**	**Ref.**
delta proteobacterium NaphS2	CAO72219	1	North Sea sediment (Germany)		Anaerobically incubated medium with sulfate and naphthalene.	Musat *et al.*, 2009
delta proteobacterium NaphS3	CAO72220	1	Etang de Berre marine sediment (France)			
delta proteobacterium NaphS6	CAO72222	1				
bacterium enrichment culture clone N47	ADB04297	1	contaminated aquifer near Stuttgart (Germany)		Sulfate reducing conditions with naphthalene.	Meckenstock *et al.*, 2000
uncultured bacterium	AFX62278	1	Amon mud volcano station 929			von Netzer *et al.*, 2012
	AFX62279-AFX62293	15	Gulf of Mexico, hydrocarbon seep dive 161			
	AFX62294-AFX62301	8	Guaymas hydrocarbon seep, dive 4573			
	AFX62302-AFX62320	19	Contaminated Flingern aquifer sediments, Düsseldorf (Germany).	Tar-oil contaminated	Contaminated aquifer sediments. Sampling from 2009.	
hbsA	**Protein ID**	**Seq.**	**Sample source**	**Contaminants****	**notes**	**Ref.**
Desulfobacula toluolica strain Tol2	CCK78655	1	Marine sediment, Eel Pond		Hydroxybenzylsuccinate synthase subunit A	Wöhlbrand *et al.*, 2012
	CCK79722	1			Annotated as *bssA* in the NCBI database	
assA	**Protein ID**	**Seq.**	**Sample source**	**Contaminants****	**notes**	**Ref.**

Desulfatibacillum alkenivorans AK01 (sulfate-reducing bacterium AK-01)	ABH11460 ABH11461	2	Estuarine sediment, Arthur Kill waterway (NY/NJ)	Oil contaminated sediments		Callaghan *et al.*, 2008
Desulfoglaeba alkanexedens ALDC	ADJ51097	1	Naval storage facility, VA (USA)	Oily wastewater		Callaghan *et al.*, 2010
Aromatoleum sp. OcN1	CBK27727	1	Sediment samples from ditches in Bremen (Germany)			Zedelius *et al.*, 2011
Azoarcus sp. HxN1	CAO03074	1				Grundmann *et al.*, 2008
prokaryote enrichment	ADJ51074, ADJ51075, ADJ51087, ADJ51090, ADJ51091	5	Marine sediments, Paletta Creek site in San Diego Bay (CA)	Oil	Methanogenic, octacosane ($C_{28}H_{58}$)-degrading enrichment culture	Callaghan *et al.*, 2010
uncultured prokaryote	ADJ51096	1	North of Zodletone Mountain in the Anadarko Basin, OK (USA)		Sulfate-reducing conditions with propane	
uncultured prokaryote	AEO89352-AEO89354	3	Oily sludge incubated for 500-days		Methanogenic conditions	Wang *et al.*, 2012
prokaryote enrichment	AEI52403-AEI52425	23			Methanogenic alkane degrading enrichment culture	Un[4]
uncultured bacterium	AET09969-AET09982	14	Contaminated soil, Shengli oil field, eastern China	Oil	Methanogenic hexadecane-degrading consortium enriched with crude oil	
uncultured prokaryote	ADJ51068, ADJ51077, ADJ51079-ADJ51081, ADJ51084-ADJ51086	8	Contaminated aquifer sediment, Passaic River (NJ)	Sites have been negatively impacted by petroleum spills		Callaghan *et al.*, 2010
	ADJ51076, ADJ51078,	5	Contaminated river sediment, Gowanus Canal (NY)			

	ADJ51083, ADJ51088, ADJ51089					
	ADJ51082, ADJ51094	2	Contaminated river sediment, Newtown Creek (NY)			
	ADJ51069-ADJ51072, ADJ51095	5	Contaminated river sediment, Arthur Kill waterway (NY/NJ)			
	ADJ51073, ADJ51092, ADJ51093	3	Gas condensate-contaminated aquifer, South Platte alluvial aquifer, Fort Lupton (Colorado)	96% w/w C5-C15 compounds and 18% w/w BTEX	Sulfate- reducing and methanogenic conditions	
	ADJ51096	1	Zodletone spring water, Anadarko Basin (OK)	Methane, ethane, and propane	Anoxic water	
uncultured bacterium	AFX62321-AFX62322	2	Guaymas hydrocarbon seep, dive 4573			von Netzer *et al.*, 2012
Unidentified clusters (annotated as pfl like)	**Protein ID**	**Seq.**	**Sample source**	**Contaminants****	**notes**	**Ref.**
uncultured prokaryote	ADJ51105-ADJ51106	2	Gas condensate-contaminated aquifer, South Platte alluvial aquifer, Fort Lupton (Colorado)	96% w/w C5-C15 compounds and 18% w/w BTEX	Sediments under sulfate-reducing and methanogenic conditions	Callaghan *et al.*, 2010
Desulfotomaculum gibsoniae DSM 7213	EHG03013	1	freshwater mud		Annotated as pyruvate formate-lyase (PFL)	Un[3]
Unidentified clusters (Metagenomic projects)	**Gene object ID**		**Sample source**	**Contaminants****	**notes**	**Ref.**
Poplar biomass microbial communities from Brookhaven National Lab, NY	PBDCA2_14529960		Bioreactor	Solid waste	Sample from total biomass decay community	IMG‡
Methane oxidizing archaeal communities, Santa Barbara Basin	ASA129_00294410		Marine sediment, Santa Barbara Basin (ANME Sed A12 9-12 cm)			
	ASA129_00462540					
Microbial communities from Puerto Rico rain forest that decompose	PRSSGFe_00942990		Soil			
	PRSSGFe2_0584.000016					

switchgrass	80				
Discharged *bssA* like[†] (Metagenomic projects)	**Gene object ID**	**Sample source**	**Contaminants[**]**	**notes**	**Ref.**
Microbial communities from Lake Washington	LWFCAn_01453720 LWAnN_03204800	Sediment, Lake Washington, Seattle (USA)		Methane and Nitrogen Cycles (Flow sorted aerobic + nitrate)	IMG[‡]
Green-waste compost microbial community from solid state bioreactor, Puerto Rico	prs_02820360	Compost	Solid waste		
PCE-dechlorinating microbial communities from Ithaca, NY	PCEOTH_2935460	Bioreactor	Tetrachloroethylene and derivatives		

[*] *bssA* like and *nmsA* like refers to BssA and NmsA *sensu lato* sequences, respectively.

[**] Reported contaminants in the isolation source

Unpublished references: Blazquez et al., 2006 (Un[1]), Lucas et al., 2009 (Un[2]), Lucas et al., 2011(Un[3]), Cheng et al., 2011[1] (Un[4])

[†] these sequences do not share ≥80% of the same region. They were included only for the analysis of Figure S6. [‡] IMG (Integrated Microbial Genomes Database)

Table S3.2a. BTEX composition in the sediment samples.

	BTEX compound (ppb)				
	Ben	**EtBen**	**Tol**	**m/p-xyl**	**o-xyl**
FI-PET	nd	nd	nd	blq	blq
M-OIL	nd	16.1 ± 2.1	nd	73.6 ± 13.5	86.2 ± 14.3
Prestige Oil (x10^3)	0.16 ± 0.02	1.26 ± 0.16	1.04 ± 0.11	1.82 ± 0.16	1.91 ± 0.12

Ben (bencene); **EtBen** (Ethylbencene), **Tol** (toluene), **m/p-xyl** (m-xylene and p-xylene), **o-xyl** (o-xylene)

nd = not detected

blq = below limit of quantification (<1 ppb)

Table S3.2b. PAHs composition in the sediment samples.

	PAH compound (ppm)					
	N	**1-MN**	**2-MN**	**N2**	**N3**	**N4**
RI	1.28 ± 0.96	nd	0.12 ± 0.17	nd	nd	nd
FI-OX	0.10 ± 0.04	0.12 ± 0.3	1.5 ± 1.09	nd	nd	nd
FI-TR	0.07 ± 0.02	nd	0.42 ± 0.21	nd	nd	nd
FI-AN	0.05 ± 0.01	nd	0.03 ± 0.01	nd	nd	nd
FI-PET	4.3 ± 1.91	2.52± 1.07	27.55 ± 6.1	22.5 ± 3.94	46.2 ± 13.2	25.1 ± 4.8
M-CON	0.0064± 0.00	nd	nd	nd	nd	nd
M-NAP	0.053± 0.00	nd	blq	nd	nd	nd
M-OIL	0.71± 0.22	0.07± 0.04	0.91± 0.37	0.21 ± 0.06	0.09 ± 0.02	nd
Prestige Oil[a]	345		1076[1]	1232	1017	no data

Naphthalene (**N**), 1-methylnaphthalene (**1-MN**), 2-methylnaphthalene (**2-MN**), dimethylnaphthalene (**N2**), trimethylnaphthalene (**N3**), tetramethylnaphthalene (**N4**).

[1] Sum of 1-MN and 2-MN.

[a] Data for the Prestige oil fingerprinting analysis described in Alzaga et al., 2004.

*Methylated compounds with C1, C2 and C3 groups

nd = not detected

blq = below limit of quantification (<1 ppb)

Table S3.3. Most Probable Number of aromatic degrading bacteria in the Mallorca sediment microcosms and Atlantic Islands sediments.

			Carbon source					
		Treatment	**Oil** (CI (95%))	**Ben** (CI (95%))	**Tol** (CI (95%))	**An** (CI (95%))	**Naph** (CI (95%))	**Ac** (CI (95%))
Mallorca	SRB (x10^3)	None	23 (10-170)	9.2 (1-17)	9.2 (1-17)	2.3 (0.6-8)	0.21 (0.0-0.7)	92 (10-170)
		Naph	42 (10-170)	4.2 (1-17)	4.2 (1-17)	2.3 (0.6-8)	0.92 (0.2-3)	42 (10-170)
		Oil	92 (10-170)	15 (10-170)	15 (10-170)	14 (10-170)	0.92 (0.2-3)	150 (10-170)
	IRB (x10)	None	-	-	-	-	-	230 (66-800)
		Naph	2.3 (0.6-8)	-	-	4.2 (1-17)	-	70 (16-300)
		Oil	-	2.3 (0.6-8)	0.92 (0.2-3.7)	-	-	210 (61-730)
	MnRB (x10)	None	2.3 (0.6-8)	-	0.92 (0.2-3.7)	-	-	210 (61-730)
		Naph	2.3 (0.6-8)	0.36 (0.05-2.5)	0.92 (0.2-3.7)	0.92 (0.2-3.7)	-	23 (6.6-80)
		Oil	-	0.36 (0.05-2.5)	2.3 (0.6-8)	-	-	150 (4.1-52)
Atlantic Islands[a]	SRB (x10^3)	FI[b]	nd	1.0 (1.9-49)	4.1 (1.0-27)	0.33 (0.1-0.9)	4.9 (3-7.1)	67 (44-100)
		FI-PET	nd	12.8 (3.2-50)	7.6 (2-26.8)	12.8 (3.2-50)	12.8 (3.2-50)	48.9 (3.5-57.5)
		RI	nd	1.3 (0.5-3.6)	0.03 (0.01-0.09)	0.02 (0.008-0.07)	0.8 (0.3-1.8)	3.5 (1.3-9)
	IRB (x10)	FI[b]	nd	0.3 (0.04-2.4)	1.1 (0.3-3.5)	-	1.6	8.0
		FI-PET	nd	4.8 (1.3-17.4)	2.4 (0.5-10.9)	3.0 (0.7-12.3)	3.0 (0.7-12.3)	30.6 (7.6-122)
		RI	nd	-	-	-	-	-

[a] Data taken from Acosta-González *et al.*, 2013 (Acosta-González *et al.*, 2013).
[b] Average values from FI-OX, FI-TR and FI-AN.
nd, not determined; -, no growth detected.

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Chapter IV

Preliminary metagenomic analysis of marine sediment bacterial communities from artificially polluted microcosms.

4.1. Introduction

The microbial metagenome is the largest gene reservoir in the biosphere and it is essential for the maintenance of the biogeochemical cycles in nature. Unfortunately, current estimations suggest that less than 1% of the microorganisms present in soil, sediments, water and other environments contributing to this reservoir have been isolated and cultivated under laboratory conditions. The genetic potential and main features of these uncultivable organisms remain thus unexplored. Recent microbiology and microbial ecology have experienced a fundamental change with the advent of the massive sequencing technologies and sequence analysis tools (Venter *et al.*, 2004). Initially, these new powerful techniques focused the identification of the whole genome sequence of particular organisms. However, their potential to analyze microbial communities as such, without the previous requirement for the isolation of the community components, was soon recognized. In this regard, a metagenome can be defined as the whole set of genomes of all individuals present in an environment or in a sample, independently of their characteristics or their abundance. From a conceptual viewpoint, metagenomics considers the microbial community present in a sample as a "functional unit", which reacts as a whole against the changes in its environment, and which is defined by its genetic pool. Thus, affiliating the functions present in the metagenome (i.e., the genes) to a particular microorganism may be considered secondary in current metagenomic analyses. Although increasing efforts in improving the bioinformatics analysis tools and the sequencing capacities are being made to reach the ideal goal of linking organisms to functions, current studies rather focus the comparison of different environments or conditions. The main objective is this case would be to identify what could be called the "metagenomic footprint" that characterizes each environment, and to analyze the global responses of the microbial population to each external stimulus.

Two alternative approaches can be used in metagenomic studies. Early works always used a previous cloning step where environmental DNA fragments were enriched by constructing metagenomic libraries in specialized vectors (Beja *et al.*, 2000; Venter *et al.*, 2004). However, the difficulties to obtain quality libraries from certain environmental samples and the increasing efficiency and price lowering of sequencing technologies such as 454 pyrosequencing have allowed the alternative approach of directly sequencing the environmental DNA, avoiding the previous cloning steps. Both approaches are sometimes used in parallel (Ghai *et al.*, 2010).

The "omics" revolution has provided microbial ecologists with huge quantities of novel information, the analysis of which represents a big challenge. Several bioinformatic tools have been developed to examine the microbial and functional diversity of metagenomes (De Filippo *et al.*, 2012). Microbial diversity, studied with phylogenetic

markers like ribosomal genes, can be analyzed with specialized software like MOTHUR (before DOTUR) or Qiime (Quantitative Insights Into Microbial Ecology) (Schloss *et al.*, 2009; Caporaso *et al.*, 2010). Reference phylogenies found in SILVA o GreenGenes databases can be used for the better assignation of microbial taxonomy to OTUs (McDonald *et al.*, 2012; Quast *et al.*, 2013). On the other hand, software for annotation of genomes and metagenomes are abundant. In fact, the most popular are presented as online platforms like MG-RAST, IMG/EM or CAMERA (Meyer *et al.*, 2008; Sun *et al.*, 2011; Markowitz *et al.*, 2012). MG-RAST utilizes different tools to classify genes in specific categories: **COG**s (Clusters of Orthologous Groups of proteins) is an attempt to do phylogenetic classification of proteins (Tatusov *et al.*, 2000); **KO** (Kegg Orthology) is a database of orthologous groups of genes that correspond to KEGG pathway nodes, BRITE hierarchy nodes, and KEGG module nodes (Kanehisa *et al.*, 2004); **NOG**s, the non-supervised database of orthologous groups from more than thousand organism; and Subsystems, a classification of genes based on functional roles (Overbeek *et al.*, 2005). Tools designed for *de novo* assembly of metagenomes are free-available in different repositories (Nagarajan and Pop, 2013) and examples are Meta-IDBA (Namiki *et al.*, 2012), metaVELVET (Namiki *et al.*, 2012) or Genovo (Laserson *et al.*, 2011). MIRA, one of the most used software for genome assembly, can also be used with metagenomes (Chevreux *et al.*, 2004). A critical step in the metagenome analysis is the previous sequence filtering, which includes demultiplexing when several samples are loaded in the same plate, filtering according to quality parameters, or any other property such as size or GC content. PRINSEQ can be used for filtering both fasta and fastq files (Schmieder and Edwards, 2011).

The objective of this work was to identify the main changes suffered by the microbial communities present in marine sediment when exposed to high concentrations of a contaminant mixture such as crude oil, or to a specific aromatic compound as naphthalene. We used the available massive sequencing tools to identify the constituents of the community and the relevant genetic functions present in each condition. To our knowledge, there are no published metagenomic studies conducted in this type of environment.

4.2. Experimental procedures

Microcosm setup. Three sediment cores collected from Figueiras beach (42°13'55N, 8°53'50W) during the 2007 campaign (Acosta-González *et al.*, 2013) were used to set up three microcosms artificially contaminated with naphthalene (FII-mNAP) or *Prestige*'s crude oil (FII-mOIL) as described in chapter III. The third core remained untreated and served as a

control (FII-mCON). The cores were sealed with duct tape and incubated at 18°C in the dark for eight months. The sediment section from 2 to 16 cm depth was used for the analysis.

Geochemical parameters and bacteria enumeration. Geochemical characterization, hydrocarbon and metabolites determinations, Most Probable Number (MPN) enumeration of bacteria, DAPI counts and fluorescent *in situ* hybridization (FISH) of the sediment samples were done as described in the methodology section. Intermediates like 2-NA (2-naphthoic acid), TH-2-NA (5,6,7,8-tetrahydro-2-naphthoic acid), HH-2-NA (Hexahydro-2-naphthoic acid) and MNA (2-naphthoic carboxylic acid) were determined by GC-MS after extracting the sample as described in the methodology section. For MPN, sulfate, nitrate, iron and manganese (10 mM) were used as electron acceptors; naphthalene, 2-methylnaphthalene, lactate and acetate (final concentration of 2 mM) or crude oil (20 µl per tube) were used as carbon sources. The cultivable fraction of aerobic bacteria growing on hydrocarbon sources (naphthalene, phenanthrene, pyrene, undecane and anthracene) was estimated in 96-well plates. Tubes (anaerobic growth, quintuplicate) and plates (aerobic growth, quintuplicate) were incubated at 18°C in the dark and growth was followed during one year. Tubes and wells were used for MPN calculations (quintuplicates). For FISH detection, Cy3 labeled probe mix EUB338 was used (Daims *et al.*, 1999). Sample FII-0 was taken at the microcosm setup time (time zero), and was included for comparison with the treatments through time.

Nucleic acid extraction. DNA extraction was performed on duplicate frozen aliquots (20g wet wt.) from each microcosm treatment. A lysis-based protocol of DNA extraction was used as previously described (Zhou *et al.*, 1996), with the indicated modifications (section 4.2.2., Experimental procedures). The integrity and yield of extracted nucleic acids were checked by standard agarose gel electrophoresis and SyberGreen staining. Nucleic acids were quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) and SyberGreen.

16S rRNA pyrosequencing. Partial amplification of the 16S rRNA gene was performed with the bacterial universal primers 6F and 532R that cover hypervariable regions V1-V3 using a coded-primer (tag) approach for multiplex pyrosequencing as indicated in table 4.2 (Experimental procedures). Each DNA obtained from the duplicate DNA extractions was independently amplified and several reactions (50 µl) were necessary to obtain the required concentration for pyrosequencing (20 µl, 20 ng/µl). To determine optimal PCR cycles and annealing temperatures for the primers, gradient PCR were performed with annealing temperatures ranging between 50°C and 60°C (TGradient, BIOMETRA). Equimolar amplicon concentrations were mixed, purified and subjected to

pyrosequencing using a Genome Sequencer FLX system (454 Life Sciences, Branford, CT) at CITIUS (University of Seville).

Sequencing of metagenomic DNA. Equal amounts of each DNA duplicates for each treatment (2 µg) were mixed and used for direct pyrosequencing in a 454FLX machine. Direct sequencing of DNA was done at GATC Biotech (Konstanz, Germany). DNA samples were used for construction of three tagged standard libraries for single read sequencing done on one half PicoTiterPlate in a 454 Roche GS FLX machine.

Amplicon pyrosequencing analysis. Two independent approaches were used in amplicons analyses: i) MG-RAST platform (Metagenomic Analysis Server), where semi-automatically data processing was used (Glass *et al.*, 2010) and, ii) Qiime (Quantitative Insights in Microbial Ecology), that allows the data processing with command line arguments (Caporaso *et al.*, 2010). For MG-RAST analysis, the original .sff files provided were directly submitted and the analysis was run with default parameters. For Qiime analysis, the original .sff file were extracted with the sff clip tool provided by MOTHUR (Schloss *et al.*, 2009) and the resulting .fasta and .qual files were used as imput file using the parameters described in Experimental procedures (section 6.4.) In both approaches, phylotype distribution at different taxonomic levels and PCoA exploratory analysis were done. OTUs were classified at ≥97% sequence similarity level.

Metagenome annotation and analysis. Statistics of metagenome pyrosequencing are shown in table S4.2.The raw data from each metagenome (.sff files) (FII-mCON, FII-mNAP and FII-mOIL) were submitted to the MG-RAST server to perform the annotation using the default parameters of the software (Meyer *et al.*, 2008).

4.3. Results and discussion

Biogeochemical characterization

The geochemical analysis of sediment samples at the beginning of the experiment (sample FII-0) gave similar results to those previously described for the same sampling campaign in Chapter I (Table 4.1). Increases in organic matter content observed in the three microcosms with respect to the starting point was unexpected, and could be the consequence of high microbial activity in the control sample, although the high contents in the contaminated samples probably reflected their high hydrocarbon content. Hydrocarbon concentrations before the addition of contaminants did not represent a

significant fraction of the total hydrocarbon concentration in both treated microcosms (naphthalene and oil).

Table 4.1. Geochemical parameters and hydrocarbon content of the microcosms

Sample	Total organic matter (mg/ml)	Hydrocarbons (ppm)[b]				SO_4^{2-}	NO_3^-	Fe[a] (%)
		Alk.	BTEX	Naph	2MN	mM[c]	µM[c]	
FII-0	4.42 ± 0.5	10.1	-	0.03	1.85	26.6 ± 0.5	59.7 ± 8.6	0.06
FII-mCON	12.8 ± 1.3	11.1	-	-	1.37	25.7 ± 0.8	54.4 ± 9.1	0.05
FII-mNAP	13.5 ± 2.3	13.5	-	9.41	0.54	25.0 ± 1.0	48.9 ± 12.3	0.01
FII-mOIL	29.8 ± 1.8	39	<dl	2.6	2.02	23.1 ± 0.9	42.4 ± 5.8	n.d

a. % of dry weight of sediment
b. Sum of total hydrocarbons detected in the analysis (see tables S2-S3)
c. Molarity in interstitial water
<dl: below limit of detection
n.d: not determined due to the high hydrocarbon content

As expected, the highest counts of MPN were obtained for the sulfate reducing bacteria in all samples (Figure 4.1). Sulfate and nitrate concentrations decreased with time in the FII-mOIL microcosm (Table 4.1), where the highest SRB and NRB viable counts were determined. In this sample, SR and NR oil-oxidizers counts were in the range of counts determined for acetate and lactate oxidizers. In the naphthalene treated microcosms, SRB counts were similar for N, 2MN and oil, but NRB counts were only considerably high for oil oxidizers. The control microcosm (without treatment) presented MPN counts similar to those in the reference sample at the beginning of the experiment (FII-0). Interestingly, in the treated microcosms, an increase of up to one order of magnitude in the MPN enumerations was observed when compared with control (FII-mCON) and previous reports from the same sediments samples (Acosta-González *et al.*, 2013).

The naphthalene treatment drastically decreased (85%) the total cell numbers determined by DAPI staining (Table 4.2). The high naphthalene concentrations in this microcosm were apparently extremely toxic to the active populations of bacteria (EUB/DAPI counts). The toxicity was not so extreme in the oil treatment, which lost 33% of the total population but kept the fraction of potentially active bacteria similar to the control microcosm. However, despite this toxicity, the relative abundance of hydrocarbon oxidizers was higher in both treatments than in the untreated microcosm. The presence of naphthalene increased the fraction of naphthalene oxidizers, and to a lesser extent of 2MN and oil oxidizers. Oil treatment produced the highest increase in specific hydrocarbon oxidizer populations, probably due to the diversity of the hydrocarbon present in the mixture. Altogether, these results suggest that the input of a new carbon source strongly

promotes the activity of bacterial subpopulations within the community, despite being toxic to an important fraction of bacteria (Table 4.2).

Table 4.2. Total cell numbers (DAPI) and percentage of potentially active bacteria (%EUB/DAPI) determined by FISH with EUB 338 probe.

	DAPI (cells x10^{-7})	%EUB /DAPI	SRB (%)*					NRB (%)*				
			Naft	2MN	Oil	Ace	Lac	Naft	2MN	Oil	Ace	Lac
FII-0**	24.5 ± 2.3	16.42	0,001	0,001	0,004	0,002	0,001	0,000	0,000	0,001	0,000	0,001
FII-mCON	29.1 ± 1.9	14.18	0,001	0,002	0,003	0,002	0,003	0,000	0,000	0,001	0,000	0,001
FII-mNAP	4.31 ± 0.8	9.43	0,073	0,031	0,042	0,102	0,065	0,003	0,002	0,020	0,020	0,005
FII-mOIL	19.7 ± 2.3	16.60	0,043	0,068	0,371	0,464	0,281	0,002	0,003	0,022	0,026	0,007

Data from highly polluted samples are marked in bold.
*percentage of cultivable cells (MPN) over bacterial counts
**sample at time 0.

The naphthalene:2MN ratio in the original *Prestige* oil was 1:3 (Barrett *et al.*, 2011), while in the oil treated microcosm it was 1:1, suggesting a faster degradation of the methylated PAH. This was probably the consequence of the higher abundance in FII-mOIL microcosm of 2MN oxidizers (both SRB and NRB) than of naphthalene oxidizers (Figure 4.1). Naphthoic acid, the first intermediate in the naphthalene anaerobic degradation pathway in SRB (Zhang and Young, 1997), was detected by GC-MS analysis only in the naphthalene treated samples (58 ± 4 ppb ml^{-1} sed). Other possible intermediates mentioned in the Methods section were not detected with this method in any of the samples.

Bacterial community diversity

Ribosomal 16S RNA gene amplicon analysis

DNA was extracted from the three microcosms after eight months incubation and the 16S rRNA gene V1-V3 region was amplified with specific primers and pyrosequenced. The resulting 16S rRNA sequences were analyzed with the Qiime software pipeline (Caporaso *et al.*, 2010). Phylogenetic analysis of the resulting sequences revealed significant differences in bacterial diversity between treatments. Figure 4.2 shows the main phyla distribution, and depicts separately the proteobacterial classes. The results were also compared with the reference pyrosequencing data from Figueiras and Rodas samples (FI and RI, see Chapter II). In the untreated and oil-amended microcosms, *Proteobacteria* dominated as expected (see discussion in previous chapters), and this phylum constituted more than 60% of the amplicons. Within this group, *Alphaproteobacteria* abundance in the FII-mOIL microcosm increased at least twice with respect to the control microcosm, becoming the most abundant class in this sample. In our previous characterization of Figueiras sediment,

Alphaproteobacteria were not the most abundant group but, interestingly, were more abundant in the most polluted sample analyzed (FI-PET, Figure 4.2). It seems that *Alphaproteobacteria* could be relevant in the first steps of a contamination episode, as also deduced from similar observations in short-term polluted experiments (Bordenave *et al.*, 2007; Abed *et al.*, 2011; Kostka *et al.*, 2011). The relative abundance of *Deltaproteobacteria*

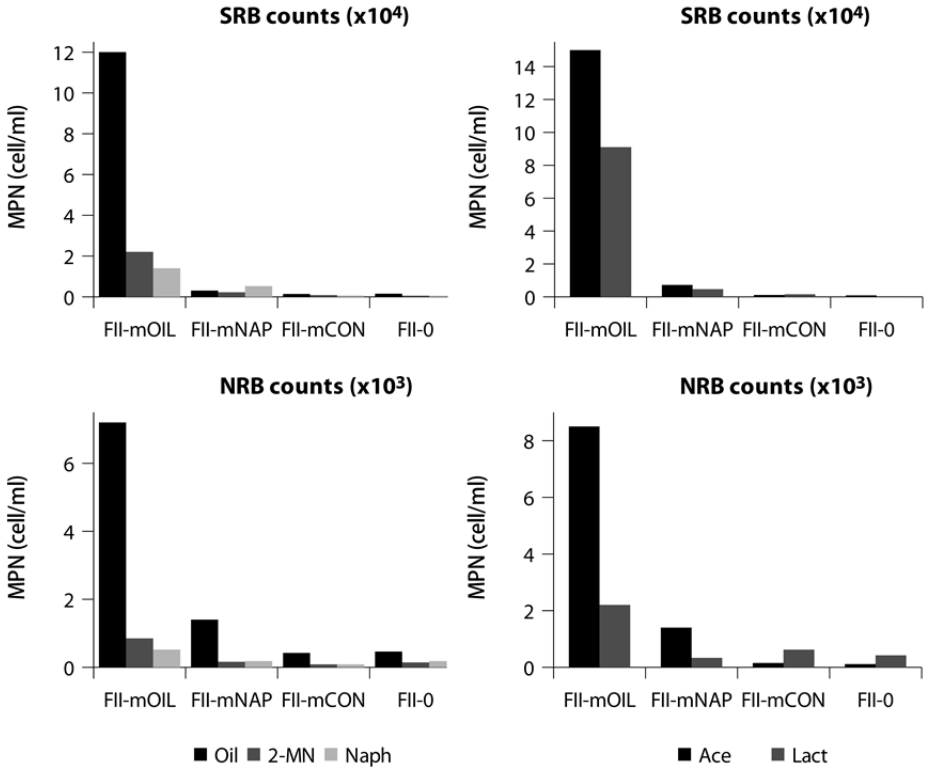

Figure 4.1. Most probable number of SRB and NRB able to grow on different carbon sources. Left) Aromatic carbon sources: Naph, naphthalene; 2MN, 2-methylphthalene; Oil, *Prestige* fuel. Right) Other carbons sources: Ace, Acetate; Lact, Lactate. Counts were calculated from quintuplicate cultures.

ranged between 17-23% in both microcosms. The distribution of members of this class in the original samples from Figueiras and Rodas sediments were apparently more dependent on the redox conditions, since *Gamma-* were more abundant than *Deltaproteobacteria* in the less reduced superficial layers. However, although the analyzed zone comprised oxidized/reduced conditions, the proportion of *Deltaproteobacteria* was highest in the oil-polluted microcosm. The small size of the gammaproteobacterial communities in all microcosms, which barely reached 10% of the amplicons, was significantly lower in these samples than in the original Figueiras sediments from both sampling periods (see Chapter I and II), suggesting that the microcosm setup produced a specific decrease of members of this class. *Epsilonproteobacteria* had a notable presence in the unpolluted samples, and

drastically decreased in the naphthalene and oil polluted microcosms. Although this class has been commonly detected in other marine environments, its presence in coastal

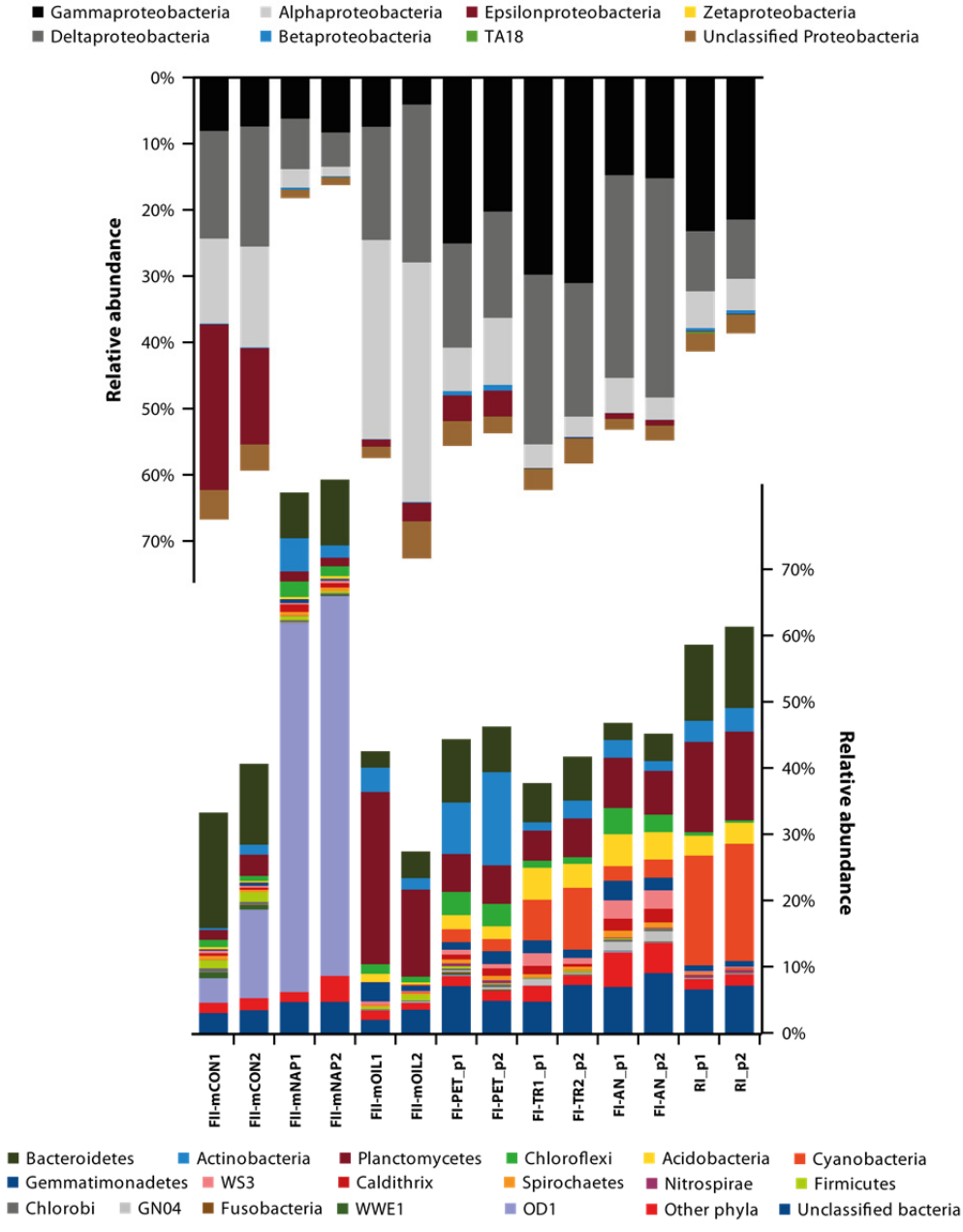

Figure 4.2 Phyla distribution among 16S rRNA amplicons (region V1-V3) from the microcosms. Previous samples characterized in Chapter I and II are shown for comparison. Top, *Proteobacteria* classes. Bottom, non-proteobacterial phyla. "Other phyla" includes *Armatimonadetes*, *Deferribacteres*, *Synergistetes*, *Fibrobacteres*, *Tenericutes*, *Verrucomicrobia*, *Lentisphaerae* together with many phyla with no cultivable representatives.

sediments is usually less important. In fact, in the Figueiras sediments sampled in the first campaign, *Epsilonproteobacteria* sequences were practically absent except in the polluted FI-PET. Contrarily of what we see in the treated microcosms, in a few cases a relevant presence of *Epsilonproteobacteria* sequences has been detected in polluted marine sediments or aquifers (Paissé *et al.*, 2008; Bozinovski *et al.*, 2012).

The phylum *Bacteroidetes* was less abundant in the oil-polluted microcosm, and slightly decreased in the naphthalene treatment with respect to the untreated microcosm. Abundance of this group thus seemed to be affected at initial stages of contamination, when they became less abundant (FII-mOIL). The extensive fermentative metabolism displayed by these microorganisms to break down compounds not easily degradable by other metabolisms could explain why they were not selected at initial contamination stages. The phylum *Planctomycetes* showed a significant increase in the oil-polluted microcosm, but important fractions of this phylum were also found in the Figueiras and Rodas sediments, especially in the oxidized zone of the sediment.

The most streaking result, however, was the dramatic difference observed in the naphthalene treated microcosm as compared with the control. In contrast to the previously discussed microcosms, naphthalene treatment selected totally different microbial communities, where *Proteobacteria* represent only 16% (in average) of the amplicons. *Gammaproteobacteria* and *Deltaproteobacteria* represented the most abundant classes within this phylum. The candidate division OD1 dominated this microcosm with 64% (in average) of all 16S rRNA sequences. OD1, OP11 and SR1 groups were originally included within OP11 candidate division by 16S rRNA analysis (Smith *et al.*, 2012). However recently, concatenated marker gene analysis of environment recovered genomes from *Microgenomates* (OP11), *Parcubacteria* (OD1) and *Gracilibacteria* (GN02) suggested the monophyly of the group, and the superphylum *Patescibacteria* (from *patesco* (Latin), meaning bare) was proposed to cluster the divisions, reflecting the reduced metabolic capacities of these lineages (Rinke *et al.*, 2013). OP11/OD1 division appears globally distributed in marine and freshwater environments, and has been reported in polluted sites from aquifers and Yellow river sediments (Dojka *et al.*, 1998; Yu *et al.*, 2011). It has recently been associated to high dissolved organic carbon content suboxic layers of boreal lakes, and to suboxic ponds (Briee *et al.*, 2007; Kobayashi *et al.*, 2012). It is worth noting that this group was almost absent in the original Figueiras sediments. Its predominant presence in naphthalene-treated microcosm could be related to the increase in total organic matter resulting from cell death and/or to a higher resistance to naphthalene toxicity (Table 4.1). Other changes in the phyla composition were evident: *Cloroflexi* slightly increases their abundance in the naphthalene (NAP) and oil (OIL) treatments; *Acidobacteria* only emerged in the oil-treatment; and *Cyanobacteria*, which were almost absent, were surely not favored by the dark incubation of the microcosms. *Firmicutes*, frequently reported in marine

polluted and unpolluted sediments, were not present in the naphthalene treatment, although their abundance in the other microcosms constitute up to 2.5% of the amplicons.

Binning analysis by MG-RAST

The 16S rRNA gene amplicons analyzed in the preceding section with Qiime were also analyzed with the MG-RAST platform. In parallel to the amplicon approach, the DNA extracted from the microcosms was also subjected to whole genome pyrosequencing. The resulting sequences were processed and annotated through the MG-RAST server. This platform can perform a binning analysis by assigning every sequence read or contig to a specific organism (or OTU) based on the best alignment obtained in a blast search, and it is generally used to infer the microbial diversity present in the analyzed sample when 16S rRNA gene sequences are not available.

Binning of the microcosm metagenome was calculated with MG-RAST by comparing the sequences trough the M5 non-redundant (M5NR) protein database. The binning obtained for each metagenomes at the domain level is depicted in Figure 4.3. More than 77% of sequences were assigned to *Bacteria* except in the NAP metagenome, where sequences annotated as *Archaea* reached 12%. Between 10-20% of the sequences in the microcosms corresponded to unassigned annotations. The binning analysis confirmed the dominance of *Proteobacteria* in the control (CON) and OIL samples (Figure 4.4).

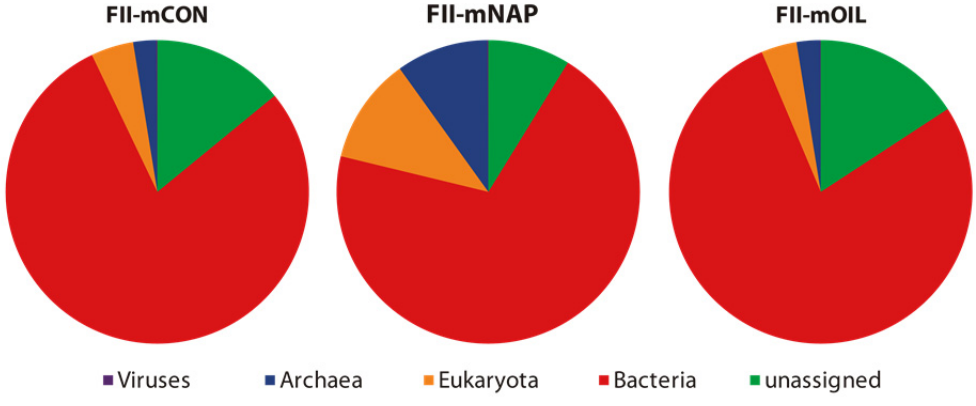

Figure 4.3. Binning distribution at domain level among the studied metagenomes

The taxonomic distribution obtained with both methods (MG-RAST amplicon analysis and MG-RAST binning) were compared. Figure 4.4 shows that binning analysis specially favored *Proteobacteria*, especially *Beta-* and *Gamma-* classes, less abundant according to Qiime and MG-RAST amplicon analysis. The binning analysis also suggested that most sequence annotations belonged to this phylum in the naphthalene microcosm (Figure 4.4).

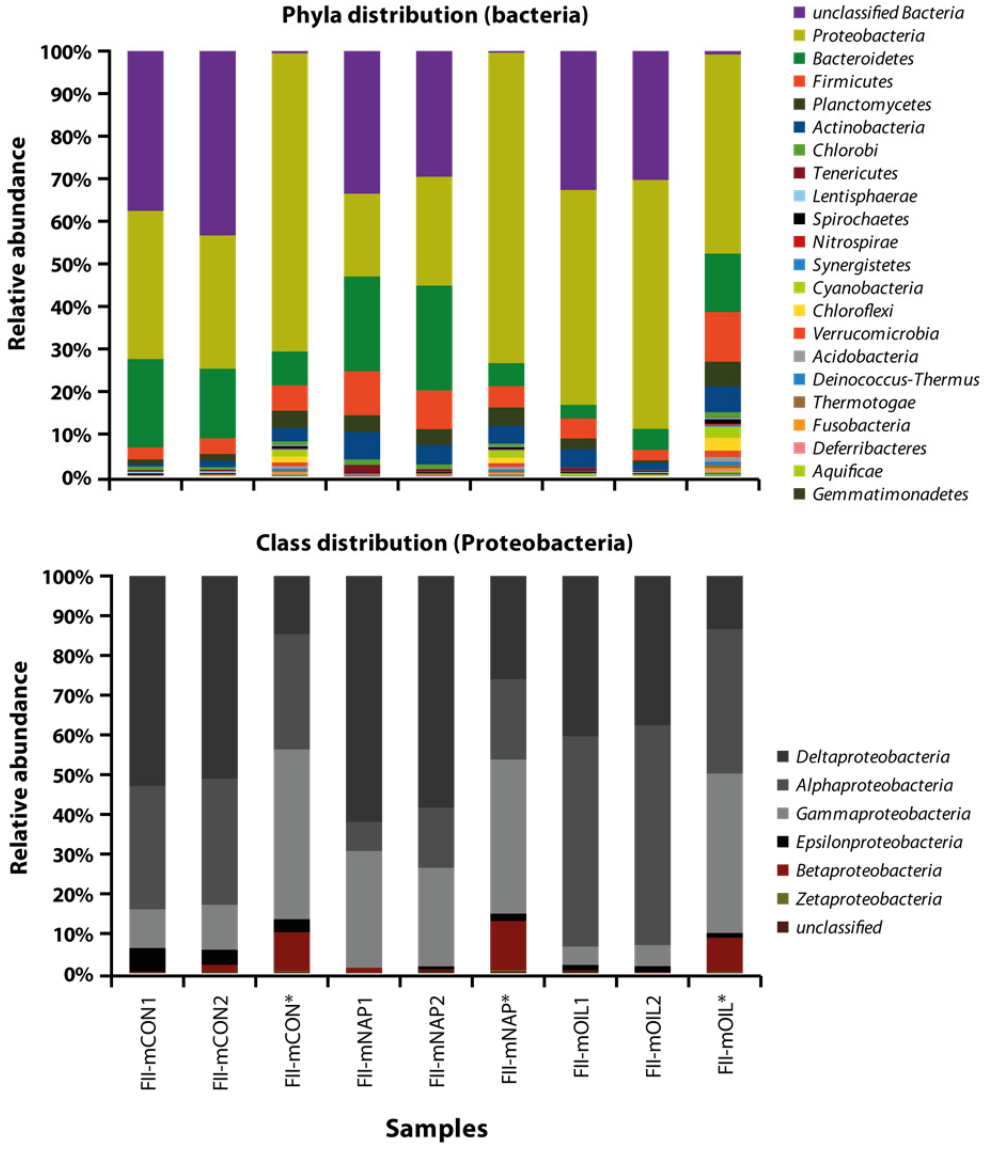

Figure 4.4. Phylotype distribution of microbial communities in microcosms according to MG-RAST. The 16S rRNA amplicons (made by duplicates for each treatment) were compared to SSU database (SILVA) using a maximum e-value of 1e-5, a minimum identity of 60 %, and a minimum alignment length of 15 measured in bp for RNA databases. Binning results of the annotated metagenomes is used for comparison of denoted with an asterisk.

The absence in the databases of gene annotations from the OD1 clade, and in general from many uncultured "candidate" divisions, biased binning classification towards the best possible match in the databases, which not always reflected the real situation. In contrast, annotations corresponding to *Bacteroidetes* reflected the 16S rRNA results. Binning overestimated the abundance of *Planctomycetes* in the FII-mCON and FII-mNAP

metagenomes. *Firmicutes* were also overrepresented in all samples, and also in the MG-RAST amplicon analysis. The details of the amplicon pyrosequencing processing in MG-RAST can be found in table S4.1.

In our hands, Qiime taxonomic assignation was more accurate when done with the GreenGenes database because uncultivated and "rare" groups seemed not to be extensively considered in SSU or RDP (Ribosomal database project) default options used by MG-RAST. Great differences in the distribution of clades at the phylum level were observed, and Qiime assignations included more taxonomic groups.

Metagenome analysis.

A relatively large fraction of the sequences failed to pass the QC filter, although compared to other metagenomic projects it was not excessive (Table S4.2). A high proportion of the passed sequences (more than 57%) could not be annotated by the RAST server and curiously, the lowest percentage of annotated sequences corresponded to the naphthalene treated microcosm (FII-mNAP) (Figure S4.1): using a maximum e-value threshold of 10^{-3}, 41.1% and 52.7% of the CON and OIL metagenome sequences could be annotated, respectively, but only 24.5% were annotated in the NAP metagenome. This indicated that the putative function of the majority of the genes from the naphthalene metagenome was unknown, which was probably connected to the abundance of OD1 candidate division bacteria (*Parcubacteria*) in this sample. The genomic background of this group has only recently started to be deciphered (Wrighton *et al.*, 2012), and therefore the annotation of related sequences through comparison with current databases remains insufficient. Interestingly, the best annotations were obtained with the oil-treated metagenome.

Functional analysis according to MG-RAST Subsystem categories

We compared the gene complement between treated and untreated samples using the GO subsystem categories. Substantial differences between metagenomes were evident: the FII-mNAP metagenome was the most different sample based on the abundance of subsystem categories (Figure 4.5, Table 4.3). The artificially oil-polluted and the unpolluted control microcosms were more related between them at the functional level, suggesting that these sediments were somehow pre-adapted to oil pollution. However, these two samples were considerably different at the taxonomic diversity level (Figure 4.2). This could imply that the changes during the oil treatment that determined the main differences with the control (a marked decrease in the *Epsilonproteobacteria* and *Bacteroidetes* groups and an increase in *Alphaproteobacteria* and *Planctomycetes*) mainly

affected specific functional genes, leaving unchanged the global gene content. On the other hand, as both samples shared taxonomic groups frequently found in sediments and annotation databases mainly comprise information belonging to these groups (*Delta-Gamma-* or *Alphaproteobacteria*, which are more represented in the databases than *Epsilonproteobacteria* or *Bacteroidetes*), differences between these samples would not be easily detected. As microbial communities from the naphthalene microcosm were undoubtedly different, we expected that functional gene analysis would show many differences when compared with the other experimental conditions.

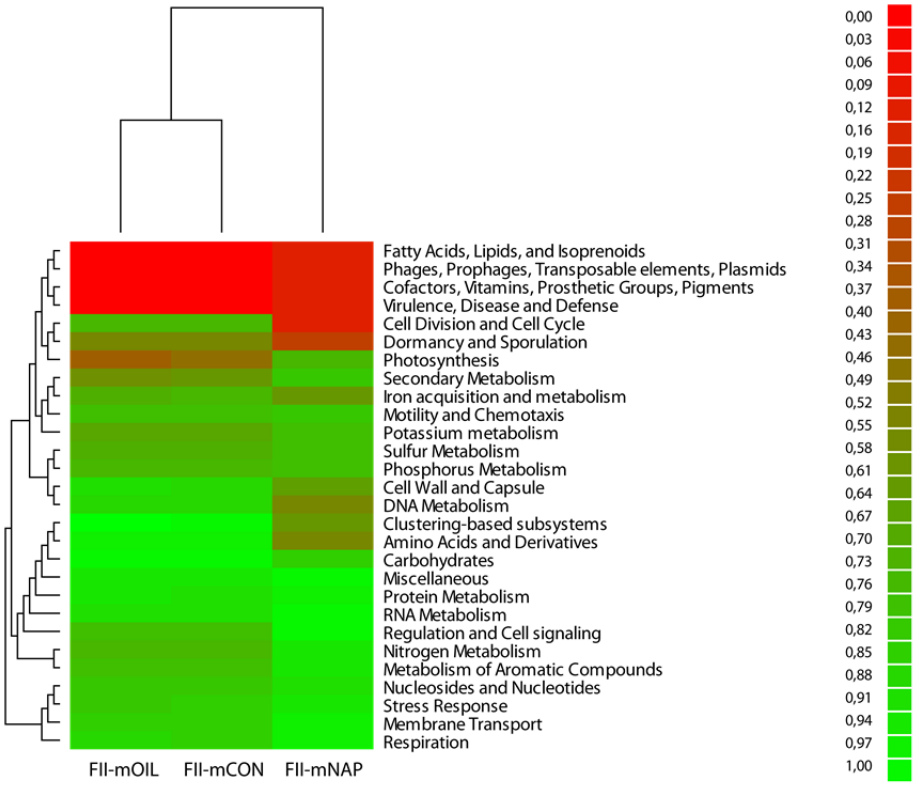

Figure 4.5. Heatmap showing abundance of level 1 subsystems annotations of metagenomes.

The heatmap analysis of subsystem annotations revealed some specific differences that are analyzed below. In the NAP metagenome, the category "cell division/cell cycle" only represented a small fraction of the retrieved sequences. As this category represents universal information contained in basically all organism genomes, the marked underscore of this category was unexpected. Since cell division is a *sine-qua-non* characteristic of living prokaryotes, we could speculate that organisms present in this sample that belong to this phylum use non-conventional machineries to carry out this function. Dormancy/sporulation genes were scarce in all metagenomes, and especially in the NAP-treated sample. Sporulation could be linked to the low abundance of gram positive clades

(*Firmicutes* and *Actinobacteria*) in these samples. A deeper analysis of these categories was not possible because the sub-level descriptions (levels 2 and 3) have not yet been defined.

Annotations related to "cell wall and capsule" were also infrequent in the NAP metagenome. The recent sequencing of nine complete genomes from single cells of *Parcubacteria*/OD1 uncultured organisms showed a striking total absence of cell envelope-related and LPS (lipopolysaccharide) synthesis genes. In this sense, a link between *Patescibacteria* (wich includes *Parcubacteria*)and *Terrabacteria* (*Firmicutes* and *Tenericutes*, *Cyanobacteria*, *Chloroflexi*, *Actinobacteria*, *Thermi* and *Armatimonadetes*) was noticeable since both superphyla shared monoderm-like (single membrane) or atypical gene complements (Rinke *et al.*, 2013). We might suggest that the low frequency of LPS and cell envelope genes in the NAP metagenome was owing to the considerable presence of *Parculobacteria* organisms (>50%). In addition, the genes associated to these functions in *Parcubacteria* communities inhabiting Figueiras sediments would be far distant relatives to known genes, and classification by current analysis against the databases would be inadequate to identify them. Early characterization of and OD1-related contig provided evidence of low sequence similarity of OD1 putative gene products to their orthologs (Elshahed *et al.*, 2005). In any case, the gene distribution according to GO categories indicated that the percentage of this category in the NAP sample was the lowest among the metagenomes (Figure 4.6).

With respects to the secondary metabolism, differences in the heatmap were detected, again the NAP-treated microcosm showing the most different pattern. Interestingly, the nine sequenced *Parcubacteria* genomes available also showed a markedly weak presence of genes related to amino acid, nucleotide and sugar metabolism, as well as electron transport chain. Only those genes related to environment response and movement, and also to hydrogen metabolism, showed a normal-to-high abundance (Rinke *et al.*, 2013). This is consistent with our results in the OD1-phylum rich NAP-treated microcosm, where the GO functional categories related to amino acid, DNA and cofactor metabolisms were significantly lower than in the other two microcosms, while genes related to regulation and cell signalling were more abundant (Figure 4.6). Hydrogenase-related sequence abundance in the NAP metagenome was up to 10 fold higher than in the other metagenomes. The FII-mNAP metagenomic annotations revealed a large presence of sequences included in the respiration category (10.3%), twice greater than in FII-mCON (5.1%) and FII-mOIL (4.8%) metagenomes. In the reference *Parcubacteria* genomes, sequences related to electron transport chain were absent, suggesting a limitation of the cellular respiration processes. Unexpected capacities specific to sediment communities could be suggested within the OD1/*Parcubacteria* group.

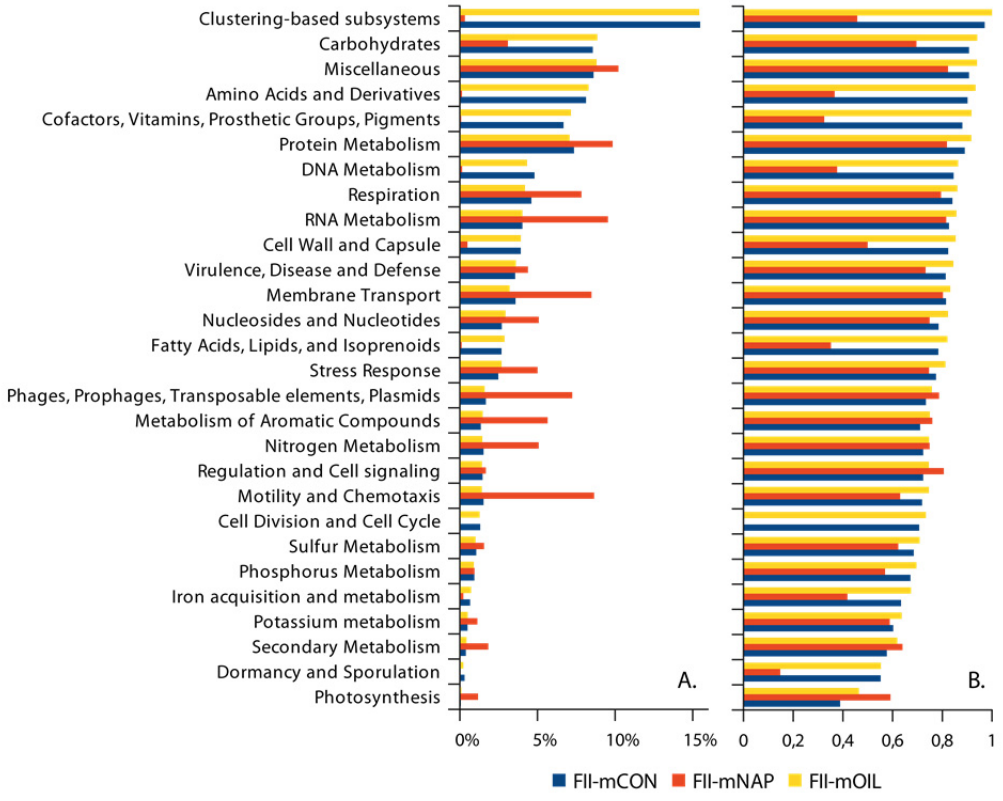

Figure 4.6. Distribution of functional categories according to GO terms (Gene Ontology). The data was compared to Subsystems (MG-RAST) using a maximum e-value of 1e-3, a minimum identity of 50 %, and a minimum alignment length of 15 aa. A) Raw data values, and B) Data normalized to values between 0 and 1 (MGRAST V3 Normalization).

Genes related to Metazoan cell defense and cellular differentiation were present in similar percentages, indicating no effect of the treatments on these organisms. Biosynthesis of phenylpropanoids, a function normally linked to plant metabolism, was over-represented in the treated microcosms, especially in the mNAP sample. Cinnamic acid degradation gene abundance was high in the NAP treatment (Table 4.3).

Photosynthesis genes rose up in the NAP sample, which was not consistent with the almost complete absence of *Cyanobacteria* 16S rRNA amplicons in this FII-mNAP microcosm (and also in the remaining samples). The detection of sequences related to denitrification steps confirmed the presence of NRB in these samples (Table 4.3). However, nitrate reduction metabolism did not seem to be important for naphthalene degradation in the naphthalene treatment, where low counts of naphthalene oxidizers were obtained (Figure 4.1).

Table 4.3. Detailed differences based in subsystems categories annotations among studied metagenomes. Number of sequences (and percentages over the total annotations) of hits with a minimal 1^{-3} e-value are shown. The numbers of unique sequences within the annotation are shown in brackets.

Level 1	Level 2	Level 3	FII-mCON		FII-OIL		FII-NAP	
Photosynthesis	Electron transport and photophosphorylation	Photosystem I	0	-	0	-	51 (36)	0,23%
		Photosystem II	0	-	1	0,00%	88 (69)	0,40%
	Light-harvesting complexes	Bacterial light-harvesting proteins	0	-	0	-	36 (30)	0,16%
	-0	Bacteriorhodopsin	0	-	1	0,00%	42 (34)	0,19%
Cell Division and Cell Cycle	-0	Bacterial Cytoskeleton	405 (335)	0,70%	428 (345)	0,56%	0	-
		Control of cell elongation - division cycle in Bacilli	23 (18)	0,04%	30 (25)	0,04%	0	-
		Cyanobacterial Circadian Clock	16	0,02%	22	0,02%	0	-
		Macromolecular synthesis operon	205 (166)	0,35%	301 (226)	0,39%	0	-
		Two cell division clusters relating to chromosome partitioning	182 (157)	0,31%	248 (209)	0,32%	0	-
Dormancy and Sporulation	-0	Persister Cells (Cell division inhibitor)	10 (10)	0,01%	26 (24)	0,03%	0	-
		Sporulation Cluster (Transcription-repair coupling factor)	99 (55)	0,17%	126 (91)	0,16%	0	-
Secondary Metabolism	Aromatic amino acids and derivatives	Cinnamic Acid Degradation	21(19)	0,03%	27(25)	0,03%	43 (39)	0,19%
	Bacterial cytostatics, differentiation factors and antibiotics	Phenazine biosynthesis	3 (3)	0,00%	10 (10)	0,01%	19 (19)	0,08%
		2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone and other bacterial morphogens	0	-	0	-	120 (109)	0,55%
	Active compounds in metazoan cell defense	-0	74 (74)	0,12%	119 (84)	0,15%	21 (17)	0,09%
	Biosynthesis of phenylpropanoids	-0	2 (2)	0,00%	26 (26)	0,03%	109 (92)	0,50%
	Plant Hormones	Auxin biosynthesis	82 (69)	0,14%	98 (80)	0,13%	121 (94)	0,55%
		Auxin degradation	46 (31)	0,08%	55 (40)	0,07%	2 (2)	0,00%
Cell Wall and Capsule	Capsular and extracellular polysaccharides	Vibrio Polysaccharide (VPS) Biosynthesis	63 (57)	0,11%	81 (74)	0,10%	0	-
		others	1085 (974)	1,89%	1252 (1056)	1,65%	4 (4)	0,01%
	Cell wall of Mycobacteria	Mycolic acid synthesis	155 (150)	0,27%	230 (217)	0,30%	0	-

	Gram-Positive cell wall components	Vibrio Core Oligosaccharide Biosynthesis	57 (57)	0,10%	82 (74)	0,10%	62 (58)	0,28%
		Lipopolysaccharide-related cluster in *Alphaproteobacteria*	89 (73)	0,15%	121 (92)	0,16%	0	-
		-0	910 (762)	1,59%	1240 (956)	1,64%	42 (31)	0,19%
	Peptidoglycan Biosynthesis	-0	841 (711)	1,47%	1071 (868)	1,41%	0	-
	Recycling of Peptidoglycan Amino Acids	-0	110 (86)	0,19%	135 (122)	0,17%	0	-
DNA Metabolism	CRISPs	CRISP	20 (17)	0,03%	31 (24)	0,04%	0	-
	CRISPs	CRISP Cmr Cluster	1 (1)	0,00%	0	-	28 (25)	0,12%
	DNA recombination	-0	54 (49)	0,09%	81 (72)	0,10%	0	-
	DNA repair	-0	1709 (1323)	2,98%	1958 (1490)	2,59%	4 (4)	0,01%
	DNA replication	DNA replication, archaeal	55 (47)	0,09%	83 (55)	0,11%	0	-
	DNA replication	Plasmid replication	47 (44)	0,08%	63 (63)	0,08%	0	-
	DNA uptake, competence	-0	79 (59)	0,13%	85 (74)	0,11%	0	-
Amino Acids and Derivatives	-0	-0	8639 (6976)	15,1%	11411 (8809)	15,1%	39 (34)	0,17%
Regulation and Cell signaling	Programmed Cell Death/ Toxin-antitoxin Systems	-0	62 (58)	0,10%	64 (59)	0,08%	391 (321)	1,79%
	Proteolytic pathway	-0	8 (3)	0,01%	14 (3)	0,01%	414 (327)	1,90%
	Quorum sensing/biofilm formation	-0	47 (38)	0,08%	90 (70)	0,11%	1000 (749)	4,58%
	Regulation of virulence	-0	212 (157)	0,37%	214 (189)	0,28%	7 (6)	0,03%
	Signal transduction in Eukaryotes	P38 MAP kinase pathways	0	-	0	-	122 (85)	0,56%
Nitrogen Metabolism	-0	Ammonia assimilation	339 (251)	0,59%	446 (318)	0,59%	239 (173)	1,09%
	-0	Cyanate hydrolysis	17 (17)	0,03%	31 (23)	0,04%	13 (13)	0,06%
	-0	Denitrification	116 (88)	0,20%	130 (95)	0,17%	41 (36)	0,18%
	-0	Dissimilatory nitrite reductase	52 (36)	0,09%	75 (57)	0,09%	73 (63)	0,33%
	-0	Nitrate and nitrite ammonification	239 (163)	0,41%	263 (173)	0,34%	526 (300)	2,41%
	-0	Nitric oxide synthase	13 (12)	0,02%	31 (30)	0,04%	24 (23)	0,11%
	-0	Nitrogen fixation	75 (54)	0,13%	72 (60)	0,09%	248 (229)	1,13%
	-0	Nitrosative stress	43 (35)	0,07%	69 (55)	0,09%	6 (5)	0,02%

Biodegradation potential in Subsystem categories

The subsystem classification of aromatic metabolism focuses the aerobic degradation pathways, better characterized and extensively studied for many years, so the picture we obtained was enriched in aerobic enzyme genes. A detailed list of the gene categories involved in aromatic degradation pathways is shown in Table 4.4. The abundance of metagenomic sequences of enzymes involved in the aerobic and anaerobic pathways stood out in the naphthalene amended metagenome (6.5%) compared with control (1.9%) and oil (2.1%) metagenomes. However, all samples harbored representatives in almost all categories. No substantial differences between sequences from oil-polluted (FII-mOIL) and unpolluted (FII-mCON) treatments were observed. It is remarkable that the specific anaerobic metabolism of toluene and ethylbenzene pathway was detected at a high frequency in the naphthalene treatment. As discussed previously, microbial communities changed considerably when the naphthalene treatment was applied. Although the biodegradative potential of the candidate division *Parculobacteria*/OD1 has not been established, the specific changes observed in the metabolic enzymes related to biodegradation of aromatic compounds suggest they could be involved in the process. Recently, ^{13}C-labeled monochlorobencene induction of ground water microcosms revealed the presence of one *Parculobacteria* and one *Comamonadaceae* (*Betaproteobacteria*) related sequence in the heavy fraction of DNA, suggesting a possible role of these organisms in the anaerobic degradation of this compound (Martinez-Lavanchy *et al.*, 2011). Genes related to aromatic degradation have not been detected so far in the few available genomes of *Parculobacteria*/OD1 (Rinke *et al.*, 2013), but the role of *Parculobacteria* in aromatic biodegradation in our experiment cannot be discarded.

Table 4.4. Aromatic Compounds Metabolism (level 1 of Subsytems categories) compared among metagenomes. Main differences are indicated in bold.

Level 2	Level 3	FII-mCON		FII-mOIL		FII-mNAP	
-0	Benzoate transport and degradation cluster	104 (79)	0,18%	86 (72)	0,11%	53 (47)	0,24%
	Cresol degradation	0	-	2 (2)	0,00%	0	-
	Gentisare degradation	21 (20)	0,03%	29 (28)	0,03%	77 (60)	**0,35%**
	Phenylacetyl-CoA catabolic pathway (core)	29 (26)	**0,05%**	52 (45)	**0,06%**	1 (1)	0,00%
	Toluene 4-monooxygenase (T4MO)	3 (1)	0,00%	0	-	0	-
	carbazol degradation cluster	11 (9)	0,01%	13 (11)	0,01%	42 (28)	**0,19%**
	p-cymene degradation	0	-	1 (1)	0,00%	20 (19)	0,09%
Anaerobic degradation of aromatic compounds	Acetophenone carboxylase 1	3 (3)	0,00%	0	0,00%	20 (20)	0,09%
	Anaerobic benzoate metabolism	94 (89)	0,16%	160 (138)	0,21%	72 (66)	0,33%
	Anaerobic toluene and ethylbenzene degradation	3 (3)	0,00%	5 (3)	0,00%	129 (101)	**0,59%**
	Hydroxyaromatic decarboxylase family	0	-	0	-	3 (3)	0,01%
Metabolism of central aromatic intermediates	4-Hydroxyphenylacetic acid catabolic pathway	19 (19)	0,03%	40 (27)	0,05%	7 (7)	0,03%
	Catechol branch of beta-ketoadipate pathway	51 (50)	0,08%	87 (70)	0,11%	14 (14)	0,06%
	Central meta-cleavage pathway of aromatic compound degradation	35 (31)	0,06%	35 (33)	0,04%	48 (37)	**0,22%**
	Homogentisate pathway of aromatic compound degradation	52 (44)	0,09%	71 (60)	0,09%	25 (25)	0,11%
	N-heterocyclic aromatic compound degradation	29 (21)	0,05%	43 (30)	0,05%	80 (63)	**0,36%**
	Protocatechuate branch of beta-ketoadipate pathway	57 (54)	0,10%	89 (74)	0,11%	136 (107)	**0,62%**
	Salicylate and gentisate catabolism	41 (38)	0,07%	44 (43)	0,05%	52 (49)	**0,23%**
Peripheral pathways for catabolism of aromatic compounds	Benzoate catabolism	24 (21)	0,04%	44 (35)	0,05%	63 (54)	**0,28%**
	Biphenyl Degradation	30 (24)	0,05%	38 (31)	0,05%	208 (166)	**0,95%**
	Chloroaromatic degradation pathway	30 (30)	0,05%	46 (39)	0,06%	22 (19)	0,10%
	Chlorobenzoate degradation	7 (6)	0,01%	7 (7)	0,00%	7 (6)	0,03%
	Naphtalene and antracene degradation	10 (10)	0,01%	25 (22)	0,03%	101 (76)	**0,46%**
	Phenol hydroxylase	3 (3)	0,00%	3 (3)	0,00%	13 (11)	0,06%
	Phenylpropanoid compound degradation	14 (14)	0,02%	27 (24)	0,03%	44 (42)	**0,20%**
	Quinate degradation	15 (12)	0,02%	23 (18)	0,03%	16 (12)	**0,07%**
	Salicylate ester degradation	9 (8)	0,01%	6 (6)	0,00%	24 (20)	**0,11%**
	Toluene degradation	3 (3)	0,00%	4 (3)	0,00%	24 (19)	**0,11%**
	n-Phenylalkanoic acid degradation	381 (330)	0,66%	595 (510)	0,78%	113 (70)	0,51%
	p-Hydroxybenzoate degradation	5 (5)	0,00%	8 (6)	0,01%	0	-

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Suplementary material

(Chapter IV)

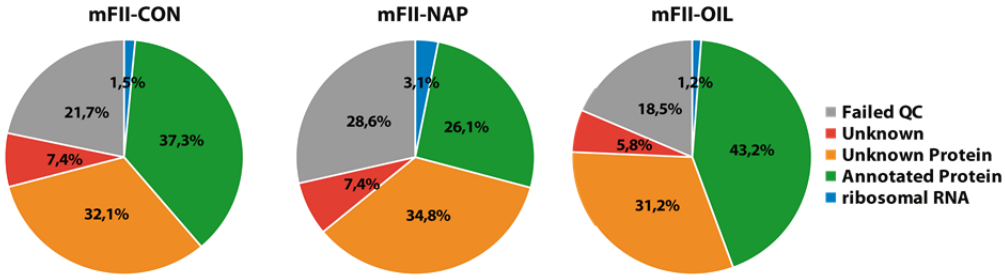

Figure S4.1. Sequence breakdown of the uploaded sequences from the metagenomes. Failed QC represents the percentage of sequences that failed to pass the QC pipeline of MG-RAST, it means, the pre-processing and de-replication steps. Percentages according to the default parameters used by MG-RAST for process the metagenome projects.

Table S4.1. Statistics of amplicons datasets analyzed by MG-RAST.

	Parameter	FII-mCON1	FII-mCON2	FII-mNAP1	FII-mNAP2	FII-mOIL1	FII-mOIL2
Upload	Sequences	11,305	12,963	9,623	13,499	11,952	7,366,936
	bp Count	6,203,328	7,100,439	5,227,112	7,335,057	6,494,928	13,548
	Average Length (bp)	548 ± 63	547 ± 69	543 ± 51	543 ± 52	543 ± 63	543 ± 64
	Mean GC percent	52 ± 3 %	52 ± 2 %	52 ± 1 %	52 ± 2 %	56 ± 2 %	54 ± 2 %
Post QC	Sequences Count	11,183	12,840	9,571	13,404	11,838	4,797,761
	bp Count	4,200,221	4,802,435	3,884,159	5,485,762	4,151,742	13,389
	Average Length (bp)	375 ± 193	374 ± 200	405 ± 187	409 ± 195	350 ± 192	358 ± 192
	Mean GC percent	52 ± 2	52 ± 3	52 ± 2 %	51 ± 3 %	55 ± 2 %	54 ± 3 %
Predicted rRNA Features		1,982	2,439	1,704	2,507	1,868	2,874
Identified rRNA Features		1,621	1,843	1,249	1,839	1,371	2,372

Table S4.2. Statistics of metagenomes analyzed by MG-RAST.

	Parameter	FII-mCON	FII-mNAP	FII-mOIL
Upload	Sequences	176,923	159,231	189,025
	bp Count	93,135,376	85,117,571	98,770,407
	Average Length (bp)	526.418	534.554	522.526
	Mean GC percent	50.6 ± 1.0 %	47.7 ± 1.1%	52.3 ± 0.9%
Post QC	Sequences Count	159,299	142,757	173,254
	bp Count	46,000,478	37,680,114	55,538,437
	Average Length (bp)	288.768	263.946	320.561
	Mean GC percent	50.8 ± 1.0%	47.1 ± 1.2%	51.8 ± 1.0%

General discussion

Marine coastal ecosystems could be continuously subjected to impacts of oil spills, considered one of the most serious environmental problem affecting marine environments. Human populations have rapidly expanded in these areas during the last decades to the point that more than one third of the human population currently lives in close proximity to coastal areas. Indispensable socioeconomic and recreational services, in addition to network connections for people and trade activities, are seriously affected when coastal habitats are contaminated. Moreover, oils spills have potential long-term adverse effects on wildlife, and environmental recovery may take several years or decades. The *Prestige* oil spill affected Atlantic coastlines from Galicia to the Basque Country in Spain, and reached beaches in France (CEDRE, 2002; Albaigés *et al.*, 2006). While most of the crude oil deposited in the accessible surface of the shore was cleaned-up by extensive removal techniques, large oil deposits accumulated in the sediments, interspersed with the sand (Bernabeu *et al.*, 2006). Bioremediation-based recovery of these highly polluted areas requires an understanding of the response of the indigenous microbial communities to the contamination event, and an estimate of their functional potential to carry out the biodegradation of the contaminants. Unlike other environments such as aquifers and soils affected by pollution, the ecological role of the microbial communities inhabiting submerged polluted sediments has been less studied. We therefore decided to investigate the influence of massive crude oil contamination on the microbial population of coastal subtidal sediments in the Cíes Islands (NW coast of Spain). An area heavily affected by the spill was sampled 18 and 53 months after the *Prestige* tanker sank in order to conduct different analysis. Three sediment depths, corresponding to oxidized, transition states (oxidized/reduced conditions) and reduced horizons, were examined. In addition, microcosm's experiments were designed to reproduce the contamination event under laboratory conditions. Microbial communities were studied using culture-dependent and classical molecular methods, and also by metagenomic surveys coupled to high throughput sequencing technologies.

Within the sediment, the distribution of bacterial communities depended on the dominant redox condition in each zone. Counts of different metabolic populations correlated with the dominant electron acceptor present in each case, as follows: aerobic oxidizer fraction was highest in the upper zone and decreased dramatically with depth. Aromatic oxidizing nitrate reducing bacteria (NRB) counts were slightly higher than aerobes in the oxidized layer, and also decreased considerably with depth. Iron reducing bacteria were barely detectable. As expected, the highest counts were obtained for sulfate reducing bacteria (SRB), which represented the most relevant fraction of aromatic oxidizers, being maximal at 12-15 cm depth (transition zone) (Figure 1.3 and Table S1.4). This distribution is consistent with what has been observed in studies of unpolluted sediments

(Llobet-Brossa *et al.*, 2002; Bühring *et al.*, 2005), but differs from other studies wherein polluted superficial layers were reported as more active for biodegradation (Miralles *et al.*, 2007). In any case, as superficial sediment depths were preferentially analyzed in the majority of published works, we did not have many references to compare with our data.

In our research, the main response of the bacterial community to the presence of hydrocarbon pollutants was a significant decay in viability, which was extreme when naphthalene was used as the single contaminating compound, as determined by total cell DAPI counts. However, both FISH counts of specific taxonomic groups and MPN enumeration of hydrocarbon oxidizers revealed the stimulation of -populations specialized in the degradation of the incoming carbon source, especially of SRB (Figure 1.3 and 1.4). A similar response to hydrocarbons was observed in Mediterranean mesocosms, where crude oil and/or naphthalene negatively influenced the total microbial community concomitant with an increase in SRB abundance and sulfate reduction rates (Miralles *et al.*, 2007; Suárez-Suárez *et al.*, 2011). In addition, previous exposure to hydrocarbons seems to be a key factor in determining the capacity of marine sediments to anaerobically degrade PAHs: sediments from polluted sites have been used as inoculum of sediments with minor pollution levels, resulting in the stimulation of SRB populations with biodegradative capacities. On the other hand, pristine sediments exposed to naphthalene enhanced their degradative response compared with non-exposed sediments (Coates *et al.*, 1997; Hayes *et al.*, 1999).

Overall, our results also suggested that the bacterial communities present in the sediment were pre-adapted to face the presence of hydrocarbons: the counts of hydrocarbon oxidizers were highest in the most polluted samples, although counts were also high in less polluted sediments (Figure 1.3) and control untreated microcosms from both the Atlantic Ocean (Table 4.2) and Mediterranean Sea (Table S3.3); anaerobic degradation marker genes were detected in all these samples and correlated with exposure to specific pollutants (Figure 3.2, Table 3.1); the functional diversity and biodegradation potential as determined in the metagenomic analysis revealed little differences between the control and oil treated sediment microcosms (Figure 4.5). This was expected, since existing studies have established that coastal habitats are areas frequently polluted with hydrocarbons, mainly derived from anthropogenic activities, and ranges from low to moderate pollution levels are correlated to the proximity to urbanized areas (Readman *et al.*, 2002; Tolosa *et al.*, 2004; Gonul and Kucuksezgin, 2012; Inomata *et al.*, 2012). The main source of marine oil pollution derives from ship operation at a global scale (Gertler *et al.*, 2010; Rogowska and Namieśnik, 2010). However, oil spills provoke high levels of hydrocarbon contamination in specific places. Therefore, in many parts of the marine realm, microbial communities are familiarized with the presence of hydrocarbons and some populations take advantage of their metabolic capacities to survive in these environments.

Bacterial diversity inhabiting *Prestige*-affected sediments as determined by classical whole-length 16S rRNA gene analysis showed a predominance of *Gamma*- and *Deltaproteobacteria*, and FISH counts confirmed this result (Figure 1.3 and 1.7). Some samples from the first campaign were "re-sequenced" using a massive approach, and the V1-V3 16S rRNA gene region was further pyrosequenced. The results at the high taxonomic levels using either of the approaches agreed (Figure 2.2). *Delta*- and *Gamma*- dominance was confirmed and the distribution was according to the redox conditions and was consistent with MPN and FISH results (Figure 1.3, 1.4 and 1.7). However, differences between samples were highlighted when deeper taxonomic levels were considered. In the classical approach, *Desulfobacteraceae* was the most abundant group within the *Deltaproteobacteria*, followed by sequences affiliated with the order *Myxococcales* (Figure 1.8). This result was similar in the pyrosequencing approach. All *Myxococcales* sequences retrieved from 16S rRNA libraries were related to marine sequence and the biggest clade was affiliated with a myxobacterial clade reported as abundant in marine environments (Brinkhoff *et al*., 2012). Interestingly, sequences affiliated to the order *Desulfarculales* constituted half of the *Deltaproteobacteria* sequences retrieved from the heaviest contaminated sample (RII-AN). PCoA (Principal Coordinates Analysis) of 16S rRNA gene libraries suggested fluctuation in the community distribution with time (Figure 1.9). Changes in the abundance of certain groups such as *Bacteroidetes* contributed to these observed differences. Although a predominance of certain metabolic types in each horizon could be delimited, a considerable overlap in the use of electron acceptors was observed, confirming that each selected zone could be influenced by more than one respiratory metabolism. Altogether, our results evidenced the presence in these sediments of a microbial community with the potential to respond against hydrocarbon contamination, consistent with the long pollution history of the site.

To extend the analysis of microbial diversity, we used similar tools to analyze available data from polluted and unpolluted sites. An unweighted PCoA plot was constructed (Figure 2.4a) and suggested clustering according to polluted or pristine sample origin, but neither PC1 nor PC2 principal coordinates explained such grouping alone. Unlike the unweighted analysis, the weighted PCoA showed a close clustering among samples from polluted sites (Figure 2.4b). In the polluted samples, organisms within the most abundant groups were closer related among them than in the unpolluted ones (Figure 2.4b). This suggests that the abundance of certain taxa mainly determine the similarities found among polluted sites. Some diversity patterns were evident at higher taxonomic levels (e.g. *Proteobacteria*), but were not determinant to describe the basal/common microbiota found in polluted sediments. When lower taxonomic ranks were taken into account, more relevant differences were evident and some groups within *Proteobacteria* (*Desulfobacterales* in *Deltaproteobacteria*, *Alteromonadales* and *Chromatiales* in *Gammaproteobacteria*) or within

Bacteroidetes (*Flavobacteriales*) could be associated with the presence of pollution (Figure 2.5).

The response of the bacterial communities to hydrocarbon was also analyzed at specific functional levels. To that end, we selected as functional marker a widely used gene coding for benzylsuccinate synthase (*bssA*), a central enzyme in anaerobic aromatic degradation (Winderl *et al.*, 2007). A common activation mechanism through fumarate addition operates in the anaerobic degradation pathways of most aromatics and n-alkanes compounds (Tierney and Young, 2010). The use of degenerated primers allowed us to efficiently amplify the *bssA* genes but also the different *bssA* gene variants in our samples (figure 3.2a). Both natural sediments affected by the *Prestige* spill and artificially contaminated microcosms were screened for the presence of *bssA*-like genes. The aforementioned adaptation to pollution and the response capacities were also correlated at this level. The presence of *bssA* homologues was detected in all sediment samples that were analyzed, but clear differences were observed among them. In addition to the classical *bssA* sequences that were targeted, we were able to detect sequences homologous to naphthylmethylsuccinate synthase gene (*nmsA*) and alkylsuccinate synthase gene (*assA*), the *bssA* homologues for anaerobic 2-methylnaphthalene and alkane degradation, respectively (Callaghan *et al.*, 2008; Musat *et al.*, 2008). The detection of *bssA*-like variants was determined by the persistence, level and type of pollution present in the marine samples. The observed gene diversity was lower in the Mallorca sediments, which were dominated by *assA*-like sequences (Figure 3.4 and 3.6). In contrast, the Atlantic Islands samples, which were highly contaminated with a methylnaphthalene-rich crude oil showed a high proportion of *nmsA*-like sequences (figure 3.1 and 3.4). Some of the detected genes were phylogenetically related to *Deltaproteobacteria* communities, described as the predominant hydrocarbon degraders at these sites, and also probably to *Desulfobacterales*. Differences between all detected *bssA*-like genes described to date indicate a separation between marine and terrestrial sequences, and further subgrouping according to taxonomic affiliation (Figure 3.3). Global analysis suggested that *bssA*-homologues appeared to cluster according to substrate-specificity (Figure 3.3 and 3.5). To our knowledge, only one study of *bssA*-like genes in marine sediments has been carried out but not as extensively as the analysis presented here (von Netzer *et al.*, 2012) and none of the published works reported the simultaneous detection of all *bssA*-gene variants in the same sample.

Once the degradative potential had been inferred, the microbial diversity characterized and the functional gene diversity of enzymes from hydrocarbon degradation pathways described, we wanted to complete the global picture of microbial communities in the polluted sediments through the metagenomic comparison of experiments that

simulate sediment pollution with crude oil and naphthalene in a controlled system (microcosm setup, chapter IV). Although our comparative analysis of metagenomes was preliminary, the results offered some interesting observations. We initially expected stronger differences between metagenomes, especially between the control and both naphthalene and oil amended microcosms. However, the differences between the control and oil samples were barely detectable. Oil pollution did not have as great an effect on microbial communities as compared to naphthalene (Figure 4.2). Two reasons could explain this observation: i) the time scale for the experiment (8 months) was too short to affect oil-treated microcosm, and ii) the crude oil constituents, more toxic than naphthalene, were less bioavailable, hence producing a lower toxic effect (relative to naphthalene) at least in the experiment's time scale. The observed changes in the community structure of OIL-amended microcosms with respect to CON suggest that the exposure time of the experiment was sufficient to produce an effect on the microbiota. It seems also that naphthalene *per se* did not cause the differences between treatments, because the aromatic fraction of the *Prestige* crude oil used in the experiment was mainly composed by naphthalene and naphthalene derivatives (approx. 22-25%). In fact, the numbers of naphthalene oxidizers were very similar between both treatments (Figure 4.1 and Table 4.2). Rather, NAP microcosm had 3-5 times higher naphthalene concentrations (Table 4.1). Thus concentration seemed to be critical for the toxic effect, although the absence of additional carbon sources could also be detrimental. The presence of naphthalene *per se* could benefit some of the common microbial populations in both treatments, but if naphthalene was present with other oil components, as in the oil-treated microcosm, specialized populations would be equally selected and then the differences in diversity and functional genes between the two polluted microcosms would be evident.

Both the metagenomic survey and the specific detection by PCR of degradation enzymes were useful and complementary for the description of the functional diversity. Metagenomics let us describe non-amplified regions of the genes by PCR, or to identify potentially new functions, as detected in a global search for *bssA*-related enzymes in the databases (Figure 3.2b). The specific detection of functional genes with probes enabled a better comparison of different samples and studies, as well as the assessment of the diversity richness. Few studies about metagenomes of polluted sediments have been published to date. Both taxonomic and specific functions apparently rose owing to the presence of pollutants. In the case of microcosms, we interestingly observed the highest presence of genes related to biodegradation in the naphthalene treatment. As discussed, if naphthalene selected for specific microbial populations, this compound seemed to stimulate also the degradative functions of aromatic oxidizer populations. Low significant differences between metagenomes from the control and oil-treated samples suggested

that microbial communities were similar and, as mentioned above, this implies that microbial community functional genes did suffer an important change because they were already adapted to oil pollution. In fact, in the last 35 years several tanker-spill accidents have hit the Galician coasts: *Prestige* (77.000 tons of oil, in 2002), *Aegean Sea* (74.000 tons of oil, in 1992), *Cason* (1.100 tons of chemicals, in 1987), *Good Lion* (80 tons of bunker fuel, in 1983), *Andros Patria* (210.000 tons of oil, in 1978), *Urquiola* (100.000 tons, in 1976) are among the most important. However, despite being "adapted" to pollution, microbial population changes should be evidenced after severe oil input to the sediments, and in fact some differences could be noticed. For instance, *Alphaproteobacteria* and *Planctomycetes* rose in the oil-treated microcosm after 8 months incubation. The abundance of *Alphaproteobacteria* in the unpolluted and oil-treated microcosm contrasted with their minor detection in previous *in situ* diversity surveys (Chapter I and II). This abundance was unexpected because this class had been rather reported in intertidal sediments, where anoxic conditions fluctuate and are not as severe as in subtidal sediments. As *Alphaproteobacteria* were reported at initial stages of pollution by many researchers, we suggest that *Alphaproteobacteria* played a similar role in the oil-amended microcosm (Yakimov *et al.*, 2004; Païssé *et al.*, 2010; Mortazavi *et al.*, 2013). However, the relevant presence of this group in the unpolluted microcosm could also be attributed in part to a microcosm setup effect. The appearance of previously undetected OD1 phylum in the control and NAP-treated microcosms indicates that oil-treatment affects the abundance of this group of organism. *Epsilonproteobacteria* seemed to be severely affected by pollution because this group was not present in the NAP-microcosm, and was much reduced in the oil-microcosm (less than 5%).

Probably the most striking result from the metagenomic survey was the unexpected prevalence of *Parcubacteria* in the naphthalene-treated microcosm. This was directly detected through 16S rRNA survey, but could also be inferred from the metagenome functional analysis. This sample showed typical features recently described for genomes belonging to this phylum (Elshahed *et al.*, 2005; Wrighton *et al.*, 2012; Rinke *et al.*, 2013): the low presence of LPS and cell envelope genes, the weak presence of genes related to amino acid, nucleotide and sugar metabolism, the normal-to-high abundance of genes related to regulation and cell signaling (Figure 4.6), and the abundance of hydrogen metabolism-related sequences. An exception would be the abundance in the NAP-microcosm of several annotated sequences associated with cellular respiration functions and a considerable proportion of aromatic degradation-related sequences, which were barely found and not detected in previously characterized *Parcubacteria* genomes, respectively (Rinke *et al.*, 2013). Although a connection between this phylum and aromatic degradation has only been cautiously suggested (Martinez-Lavanchy *et al.*, 2011; Yu *et al.*, 2011; Pearce *et al.*,

2012), it is tempting to suggest that their notorious presence in naphthalene-polluted sediments is related to the degradation of this compound. Alternatively, a particular resistance capacity of *Parcubacteria* to this PAH could explain the marked enrichment of this group in the sample, and would suggest the existence of particularly resistant (atypical) cell envelopes. The finding of OD1-related sequences in our samples was especially interesting because this group is one of the taxonomically described phylum for which no cultivable representative is available. Organisms belonging to this phylum are generally detected in organic matter-rich water environments, where the oxygen concentration becomes limiting or absent (Briee *et al.*, 2007; Borrel *et al.*, 2010; Kobayashi *et al.*, 2012). Few studies reported the presence of OD1/*Parcubacteria* in soils (Ren *et al.*, 2011; Yu *et al.*, 2011). Therefore, their presence in marine sediments, and especially in naphthalene polluted samples, was unexpected. Speculative differences found among *Parcubacteria*/OD1 communities from NAP metagenome and the described *Parcubacteria*/OD1 genomes could reflect adaptation strategies to sediment/water media, but exploration of more habitats is necessary to verify this hypothesis. A deeper analysis of the metagenomic sequences could reveal interesting features about this group. We believe that in this metagenomic analysis, many differences between the samples are being neglected because annotations are based on the current knowledge from well-described groups, like *Proteobacteria*, and less extensively studied groups like OD1 and *Epsilonproteobacteria* are missing in the annotation. In fact, binning analysis showed a different picture of phylogenetic phyla distribution than the one determined by 16S rRNA (Figure 4.4) and this is principally due to the absence of annotations of less studied groups. The high percentage of successful annotations in the oil-treated sample probably relies on the abundance of *Proteobacteria*, where well characterized classes *Alpha*, *Gamma* and *Delta* dominate.

Overall, this study tried to address many questions regarding the responses of microbial communities inhabiting polluted sediments by applying different methodologies. Attempts to identify the specific populations active towards these compounds using *in situ* stable isotope probing with ^{13}C-naphthalene or RNA extraction were unsuccessful. Enrichment cultures to isolate species or consortia able to degrade aromatic compounds, especially naphthalene, are going on in our laboratory. It would be interesting to construct a fosmid-cloned metagenome of the NAP microcosm, to achieve a better understanding of *Parcubacteria* genomics and clarify the role of this group in biodegradation. The comparison of 16S rRNA sequences from regions other than V1-V3 will support the global analysis of microbial diversity patterns in polluted and unpolluted coastal sediments.

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Conclusiones

I. La contaminación ocasionada por el vertido accidental del *Prestige* tuvo como consecuencia la selección de poblaciones microbianas específicas, el aumento del número de oxidadores aerobios y anaerobios de hidrocarburos y una disminución del número total de células como resultado de la exposición a concentraciones altas de petróleo. Las comunidades bacterianas dominantes correspondieron, básicamente a *Deltaproteobacteria* y *Gammaproteobacteria* (*Proteobacteria).*

II. El metabolismo predominante asociado a la degradación de hidrocarburos fue la sulfato-reducción, llevada a cabo por especies de *Desulfobacterales* (*Deltaproteobacterias*). Para nuestro entender, por primera vez se describió la presencia de especies de *Myxoccocales* (*Deltaproteobacteria*) en sedimentos costeros contaminados. Este grupo generalmente abunda en zonas de influencia óxica, y su rol en la biodegradación anaerobia en sedimentos se desconoce.

III. Las diferencias en la diversidad de las distintas comunidades bacterianas detectadas en los sedimentos analizados fueron debidas principalmente al tiempo de exposición al contaminante y a los niveles de contaminación presentes en cada muestreo.

IV. La presencia de altas concentraciones de naftaleno como único contaminante en microcosmos resultó altamente tóxica para la mayoría de comunidades, y seleccionó mayoritariamente organismos de una división candidata (*Parculobacteria*/OD1) reportada con mayor frecuencia en ambientes acuáticos subóxicos. Aunque nuestros datos tentativamente indican un papel de dicho grupo en la respuesta degradadora frente a compuestos aromáticos, éste no está confirmado. La abundancia de *Alphaproteobacteria* dentro de *Proteobacteria* se asoció a las primeras fases de contaminación de los microcosmos.

V. El análisis global de la diversidad microbiana presente en sedimentos costeros sugiere que una gran fracción de las comunidades bacterianas son más similares entre sí en ambientes contaminados que en ambientes aparentemente no contaminados. Si se analizan jerarquias taxónomicas inferiores (orden o familia), las asociaciones entre comunidades y contaminación son más evidentes, permitiendo proponer patrones de distribución más específicos.

VI. La presencia de genes específicos de degradación anaerobia de hidrocarburos aromáticos en los sedimentos es reflejo del historial de contaminación de los sitios

examinados: los sedimentos con un largo historial de contaminación, como los de las Islas Atlánticas, presentan una mayor diversidad funcional de enzimas relacionadas no sólo con degradación de tolueno (*bss*A), sino también con la degradación de alcanos (*ass*A) y derivados del naftaleno (*nms*A).

VII. La abundancia de los genes de degradación anaerobia está relacionada con la especificidad por el sustrato asociado a cada tipo de gen detectado y a la concentración de contaminantes específicos en las muestra, especialmente evidente en la riqueza de genes tipo *nms*A en las muestras de las islas Atlánticas contaminadas por el crudo del *Prestige*, muy rico en derivados del naftaleno.

IX. El análisis de los metagenomas de los microcosmos sugiere que las comunidades presentes en los sedimentos están pre-adaptadas a la presencia de hidrocarburos y que los cambios son más evidentes a nivel taxonómico que a nivel funcional.

Diseño de la cubierta:

Annsofi Bourgogne

http://arretederever-deux.blogspot.fr/

arretederever@gmail.com

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Avenida de Madrid 20, Granada, España.

El vertido accidental del *Prestige* ocurrido en 2002 contaminó zonas costeras desde Galicia hasta el País Vasco, alcanzando incluso algunas zonas de Francia. Las labores manuales de limpieza se llevaran a cabo principalmente en las zonas litorales, de fácil acceso, mientras que en los sedimentos sub-litorales (sumergidos) las labores de recuperación fueron más complicadas. Comparada con suelos o acuíferos contaminados, la ecología microbiana de estos ambientes ha sido poco estudiada. Para entender la respuesta de la microbiota autóctona frente al vertido del *Prestige*, decidimos analizar muestras de un área contaminada a los 18 y 53 meses de ocurrida la catástrofe. Se analizaron tres profundidades del sedimento: la zona superficial con más influencia del oxígeno; la zona de transición (condiciones oxidantes/reducidas) y la zona anaerobia. Además, realizamos experimentos en microcosmos para simular y comparar eventos de contaminación accidental. Las comunidades microbianas se caracterizaron mediante el recuento de degradadores aerobios y anaerobios, el análisis de la diversidad microbiana mediante microscopia de fluorescencia (FISH) y la secuenciación del gen ARNr 16S, la detección de genes de degradación anaerobia de hidrocarburos, y finalmente el análisis funcional mediante metagenómica.

El metabolismo dominante asociado a la degradación de hidrocarburos fue la sulfato-reducción, como era de esperar en este tipo de ambientes anóxicos. Los altos recuentos de oxidadores de hidrocarburos aerobios y anaerobios pusieron en evidencia el potencial degradador de las comunidades que habitan estos sedimentos. Paralelamente, la presencia de genes específicos de degradación anaerobia de compuestos aromáticos (tolueno y metil-naftaleno) y alifáticos (alcanos) corroboró el potencial degradador y además, permitió correlacionar la presencia de dichos genes con los tipos de contaminantes más comunes en las muestras. La toxicidad de los hidrocarburos produjo una disminución en el número de células, y resultó especialmente aguda en el caso del microcosmos contaminado con naftaleno. A pesar de ello, el porcentaje de la fracción cultivable de organismos oxidadores de hidrocarburos fue alta y aumentó en los casos de mayor contaminación. Las proteobacterias, especialmente las clases *Deltaproteobacteria* y *Gammaproteobacteria*, dominaron las librerías de ARNr 16S de los sedimentos. Por otra parte, la diversidad de las comunidades microbianas de los microcosmos cambió drásticamente en comparación al resultado obtenido de la caracterización in situ, especialmente en el tratamiento con naftaleno, donde más del 60% de las secuencias correspondían a un grupo no cultivable, OD1/*Parculobacteria*, poco descrito en sedimentos marinos aunque sí en ambientes anóxicos como aguas profundas de lagunas o biodigestores.

De manera general, los resultados indican que las comunidades microbianas están pre-adaptadas a la contaminación. El historial de contaminación de estos sedimentos es el determinante de la capacidad de degradación de las comunidades microbianas. Los resultados descritos anteriormente permiten predecir una respuesta potencialmente activa de la microbiota frente a la contaminación en los sedimentos contaminados por el vertido del *Prestige*.

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