USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
DEPARTAMENTO DE MICROBIOLOXÍA E PARASITOLOXÍA

Metagenomic study of autochthonous bacterial communities at different marine ecosystems affected by the *Prestige´*s oil spill in Galicia (NW-Spain). Biodegradation potential.

Jorge Alonso Gutiérrez

Vigo, 2009

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Thesis directed by Dr. Beatriz Novoa García and codirected by Prof. Antonio Figueras Huerta

Scientific memory presented by Jorge Alonso Gutiérrez in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy by the University of Santiago de Compostela

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INFORMAN:

Que la presente memoria adjunta, titulada "Metagenomic study of autochthonous bacterial communities at different marine ecosystems affected by the *Prestige´*s oil spill in Galicia (NW-Spain). Biodegradation potential", presentada por **D. Jorge Alonso Gutiérrez** para optar al grado de Doctor en Biología en la Universidad de Santiago de Compostela, ha sido realizada bajo su dirección y, reúne los requisitos necesarios para ser defendida ante el tribunal calificador.

Y para que así conste, firmamos la presente en Vigo a 6 de Diciembre de 2008.

Fdo. Dra. Beatriz Novoa García **Fdo. Dr. Antonio Figueras Huerta**

Dña. ALICIA ESTÉVEZ TORANZO, Doctora en Biología y Catedrática de Microbiología del Departamento de Microbiología y Parasitología de la Universidad de Santiago de Compostela.

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Summary

INTRODUCTION

Large scale marine oil spill accidents, have posed great threats and cause extensive damage to the marine coastal environments affecting fisheries, tourism and therefore economies of the affected sites.

NW coasts of Spain are next to a common route for maritime transport. This high traffic made Galician coasts be a hot point for oil spills. In fact, since the disaster of *Polycommander*, many other have occurred such as *Urquiola* (1976), *Andros Patria* (1978) or *Aegean Sea* (1992). Following this trend, the tanker *Prestige* (2002) released about 63,000 tonnes of heavy fuel being ranked as the 20th World largest oil spill hitherto. The spill affected more than 500 miles of the Spanish and even the French coastlines, resulting in an ecological disaster comparable or even worse than that of the *Exxon Valdez*.

Petroleum constituents are classified into four fractions: saturates, aromatics, resins and asphaltenes. Saturates are hydrocarbons containing no double bonds, while aromatics, with toxic carcinogenic effects, are composed of one or more aromatic rings. Resins and asphaltenes are too complex, big molecules of unknown structure. When petroleum is spilled in the sea, it spreads over the surface of the water and is subjected to many modifications that change its components and therefore physicochemical characteristics with time. This process, known as weathering, is mainly due to evaporation of the low molecular weight fractions, dissolution of the water-soluble components, mixing of the oil droplets with seawater, photochemical oxidation and microbial biodegradation. The susceptibility of petroleum hydrocarbons to microbial degradation is generally in the following order: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes > high-molecular-weight aromatics > Resins and Asphaltenes, which are considered recalcitrant to microbial degradation. The oil residue released by the *Prestige* was a heavy fuel oil lacking of the more labile fractions (22% aliphatics, 50% aromatics and 28% of resins and asphalthenes (Alzaga et al., 2004; http://csicprestige.iim.csic.es/informes/info01.pdf) and therefore resistant to weathering processes such as biodegradation.

Oil first came ashore in Galicia, where the predominantly rocky coastline was heavily contaminated and where mechanical methods, which typically recover no more than 10-15 percent of the oil after a major spill, were indeed not feasible. Fortunately, microorganisms capable of degrading petroleum hydrocarbons and related compounds are ubiquitous in marine habitats and therefore we could assume that the oil would be naturally dispersed and degraded by weathering processes with time. However, past surveys of heavily oiled sites have indicated that this is usually a slow process and oil deposits persist for many years. Defined as “the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes”, bioremediation offers one available oil

spill response to remove *Prestige*'s fuel deposited in big quantities at supralittoral areas of "Costa da Morte".

However, effectiveness of bioremediation is variable between applications and therefore still remains as a controversial issue. This variability depends on the interaction of three factors:

a) *The environmental characteristics of the polluted site.*

Nutrient levels, Tª, oxygen, etc. are factors, which have influence on biodegradation rates of the indigenous microbiota

b) *The type of oil spilled* (heavy fuel, light crude oil, gasoline, etc.).

Heavy fuels, such is the one from *Prestige*, are usually devoided of the most labile fractions and therefore pose more problems to biodegradation than light oils (with higher paraffin content).

c) *The biodegradation potential of the autochthonous hydrocarbon-degrading community of the affected habitat*.

There is no microorganism able to degrade all components from an oil mixture. In nature, biodegradation of a crude oil typically involves a succession of different hydrocarbon-degrading microbial communities.

Therefore, to know which nutrient or cofactor is going to be limiting for the biodegradation process of a specific type of oil, under the environmental conditions of the affected habitat, it is fundamental that we have detailed information about the autochthonous hydrocarbon-degrading consortium, performing such degradation processes, in order to design the most appropriate bioremediation treatment.

Owing to the highly persistent nature of *Prestige´*s cargo, the released oil drifted for extended periods with winds and currents, affecting diverse habitats along the NW Spanish coastline. In consequence, the present Thesis was aimed at studying the indigenous bacterial populations related with the *in situ* degradation of the *Prestige* fuel at two representative maritime environments of the Galician coast with distinct characteristics and, hence, distinct oil spill response strategies. Sediments and water from the Ría (Chapters I and II) and fuel paste from rocks and sand from "Costa da Morte" (Chapters III and IV) were sampled at different times after the *Prestige* oil spill.

MATERIALS AND METHODS

A combination of molecular (denaturing gradient gel electrophoresis (DGGE), 16S gene clone libraries and fluorescence in situ hybridization (FISH)) and culture-based techniques was used to assess the indigenous bacterial community structure and its potential and/or actual biodegradation capacity. In fact, combination of culture independent and culture-

based techniques has been shown in the present study as a powerful methodology in petroleum microbial ecology if we are aware of the limitations of each technique alone.

DNA identities (of the 16S rRNA gene) between sequences detected by molecular techniques from the oiled environments with those of previously described hydrocarbon-degrading strains, give us information about potential metabolism and degradation capacity of the studied community. However, such similarities of the 16S are just orientatitive of the actual catabolic capacity of a given community and must be interpreted with caution. In this sense, our studies did not remain as just taxonomic descriptions of microbial communities and many of the indigenous bacteria detected in the environment by means of molecular techniques as playing potential roles in the degradation of different fractions of the *Prestige´*s fuel in the environment, such *Alcanivorax*, *Rhodococcus*, *Citreicella*, *Dietzia*, *Sphingomonas*, etc. could be isolated and hence, further analysed with respect to its genetics, physiology and interaction with other members of the indigenous hydrocarbon-degrading bacterial consortia carrying out biodegradation pathways.

RESULTS AND DISCUSSION

Ría de Vigo

Environmental characteristics of the Ría de Vigo

The Ría de Vigo, (Galicia, NW Spain), is located in the northern boundary of the NW Africa upwelling system, and therefore in an area where upwelling–downwelling dynamics on the continental shelf (Arístegui et al., 2006) have a strong influence on its hydrography and, hence, on its plankton community structure and function. In fact, changes in bacterioplankton community structure and diversity were found throughout the year in Ría de Vigo, including the detection of new members in all major groups of bacteria.

The coastal upwelling induces the inflow of subsurface oceanic Eastern North Atlantic Central Water (ENAW) into the Ría. (Alvarez-Salgado et al., 1993). The incoming bottom current supplies the Galician estuaries with nutrients (mean values of Nitrogen (N) and Phosphorus (P) around 0.6 and 0.06 mg l^{-1} respectively; Nogueira et al. 1997) that causes the Ría de Vigo to be a ecosystem with a great ecological and economical value.

Cíes islands, part of the Natural Reserve “Illas Atlánticas”, are situated at the mouth of such ecosystem and mussel production in this region is the highest in Europe and one of the most intensive in the world, giving employment to 9000 people directly and 20000 indirectly (Figueiras et al., 2002).

Mussels and other shellfish growing in the Ría, filtrate water and accumulate pollutants. Therefore, Spanish authorities banned commercialization of shellfish yield from the Ría after the *Prestige* oil-spill with negative effects on the local economy. Primary oil-spill responses

(e.g. skimming of floating fuel, etc.) removed tones of fuel-oil from surface water but still lots of it sank and remained buried under sediments of the Natural Park from where it might be remobilized even today. Mechanical cleaning of the sediments could cause more harm than good since the seabeds of the National Park are delicate habitats with a high ecological value. Since bioremediation of water is not a feasible option (amendments would be diluted), and hand cleaning by scuba divers is a difficult and expensive task, the most feasible option left in such cases is to trust on natural attenuation of the environment. In spite of the critical role of bacterial assemblages in the oceanic food web, and therefore in the production of the Ría, there was no information available about the microbial communities growing at Ría de Vigo and even less of its biodegradation metabolism.

Bacterioplankton community structure of the Ría de Vigo

In contrast to previous results, which indicated that SAR11 clade members dominate surface bacterioplankton communities (Morris et al., 2002), in the Ría de Vigo, and other highly productive estuarine systems of the Iberian peninsula characterized by phytoplankton blooms (Henriques et al., 2004), *Roseobacter* members dominated the community (González et al., 2000) in detriment of SAR11, which is more adapted to the oligotrophic conditions of the open waters (Malmstrom et al., 2004, Rappé et al., 2002).

Different clades of *Roseobacter* lineage (NAC11-7, DC5-80-3, DG1128, CHAB-I-5, AS-21, etc.) and new clusters of SAR11, SAR86, *Betaproteobacteria*, etc. were detected in this study at specific seasons, which could likely reflect a seasonal trend. The different clades could have different abilities including biodegradation capacities (Sekine et al., 2006), or have different environmental preferences like the association with different phytoplankton species (Wagner-Dobler and Biebl, 2006; West et al., 2008).

Biodegradation potential of bacterioplankton communities of Ría de Vigo

When N and P are added in adequate quantities to cultures of seawater with crude oil as the only source of C and energy, *Alcanivorax* (Yakimov et al., 1998) and other 'professional hydrocarbonoclastic Gammaproteobacteria', such as the polyaromatic degrader *Cycloclasticus* (Chung and King, 2001), become dominant and, hence, the rate of biodegradation is strongly promoted (Harayama et al., 2004, Kasai et al. 2001, Kasai et al. 2002b, McKew et al., 2007, Roling et al. 2002). The fact that we were able to detect alkane-degrading *Alcanivorax* strain and *Cycloclasticus* sp., which responds to PAHs additions (Teira et al., 2007), from sediments and water of the Ría, showed that natural N, P and Oxygen levels present (Vilas et al. 2005) in the Vigo embayment are sufficient to promote the growth of such species playing critical roles in the natural attenuation of oil-polluted marine systems.

a) The chronic hydrocarbon pollution of the Ría (oil spillages, industrial and urban runoff, etc.); b) the favourable abiotic factors of the Ría for biodegradation metabolisms (high Oxygen and nutrient levels); c) the indigenous presence of key alkane- (*Alcanivorax* sp.) and PAH- (*Cycloclasticus* sp.) degrading species; d) the dominance and diversity of *Roseobacter* sequences (a genus previously related with oil biodegradation (McKew et al., 2007)); e) the high MPN population of alkane degraders in sediments (Alonso-Gutiérrez et al., 2008); f) the lack of effects of PAH addition on the indigenous bacterioplankton structure (Lekunberri et al., submitted; Lekunberri, 2008) and g) the fact that ongoing bioremediation was already noted at shoreline environments of the Ría (Medina-Bellver et al. 2005), support the theory of the existence of a preadapted hydrocarbon-degrading community in the Ría, which would use pollutants as a source of C and energy. Therefore, natural attenuation at Ría de Vigo is probably very effective and therefore it can be asumed that petroleum hydrocarbons dissolved in water after an oil-spill event would be readily consumed by the autochthonous bacterioplankton community.

Supralittoral Rocky shorelines of "Costa da Morte"

Oil spill response at "Costa da Morte"

After the decision was made to use bioremediation as a cleanup response to the *Prestige´*s oil-spill, different bioremediation commercial recipes were compared for its effectiveness at different rocky shorelines polluted by the accident. After different field trials, the oleophilic fertilizer S-200 proved to be the most successful and therefore it was used to clean unaccesible parts along the NW Spanish coasts where mechanical treatments were not feasible and/or advisable. Although its effectiveness was different between sites (Murado, M.A. et al., pers. comm.), there were no further assessments prior to its application at different sites. Such variability in treatment success was probably due to differences between the autochthonous bacterial communities.

Rock, sand and even water matrices, heavily polluted by fuel from *Prestige* one year before, were sampled at different places prior to S200 applications. Community analysis was performed with those matrices from supralittoral environments of "Costa da Morte" where the ensuing application of S-200 was succesfull. Therefore, results presented in this scientific memory give valuable information about the bacterial assemblages that positively respond to oleophilic fertilizer applications and will shed some light on the development of new bioremediation strategies.

Bacterial assemblages at "Costa da Morte" and its potential hydrocarbon-degrading ability

Fingerprinting analysis of the fuel accumulated at rocky shorelines under study showed a high degree of weathering which was consistent with the trends that follow biodegradation (Jiménez et al., 2006). Hence, as proposed for microbial communities from the Ría de Vigo, we can hypothesize that indigenous bacterial assemblages from "Costa da Morte" have indeed good biodegradation rates. This hypothesis is supported by the environmental conditions of the studied habitat and by its bacterial community structure.

"Costa da Morte" (Galicia, NW Spain) is also affected by the NW Africa upwelling system (Arístegui et al., 2006), and therefore is an area where nutrient levels (0.20 - 0.25 mg l^{-1} of Total N (Alvarez-Salgado et al., 2002)) are more suitable for biodegradation processes than those of the Sea of Japan (Total N~0,1 mg l^{-1}). In the first moments after an oil-spill event, marine bacterial communities become dominated by *Gammaproteobacteria* (Roling et al., 2002), nevertheless, when environmental factors are suitable for biodegradation processes, the oil is rapidly weathered and *Gammaproteobacteria*-dominated communities are quickly substituted by a more diverse community (Roling et al., 2002), like the one we found twelve months after the *Prestige* oil-spill. Around one year after the *Nakhodka* oil spill in the Sea of Japan, fuel paste samples of a similar composition to that of *Prestige*, were still dominated by *Gamma* and *Alphaproteobacteria* (Gram-negative) (Kasai et al., 2001; Maruyama et al., 2003), while, *Alphaproteobacteria* and Gram-positive *Actinobacteria* dominated our samples affected by *Prestige* after the same time.

The trophic structure of bacterial communities affected by the oil-spill presented a significantly higher percentage of alkane- and aromatic-degrading bacterial populations than non affected ones. Next to it, although rocks and sand are quite different substrates, community composition was quite similar. All of it suggested that fuel oil drove the composition and structure of the affected communities studied.

Many members of the described communities were previously associated with alkane- (e.g. *Xanthomonadaceae*, *Pseudoxanthomonas, Stenotrophomonas, Erythrobacter*, etc. (Chang et al., 2005; Young et al., 2007; Macnaughton et al., 1999; Roling et al., 2002)) and aromatic-degradation processes (e.g. *Citreicella* sp., Sphingomonadaceae spp., *Lutibacterium anuloederans*, described as a 2- and 3-ring PAH degrading bacteria which had a higher efficiency in the uptake of aromatics than *Cycloclasticus* species, etc. (Chung and King, 2001; McKew et al., 2007)). However, it was *Actinobacteria* (mainly *Corynebacterineae*) the dominant group carrying out *in situ* biodegradation processes of the *Prestige* fuel oil after one year of weathering. This distinct group of Gram-positive Actinobacteria, classified in the suborder *Corynebacterineae*, includes genera detected in the present study such as *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia* and *Rhodoccoccus*.

Molecular- and culture-based techniques showed *Rhodococcus*, as the most important alkane-degrader. It is well known that *Rhodococcus* is a genus with a remarkable metabolic diversity (Larkin et al., 2005) and able to produce biosurfactants which can enhance the activities of other degrading bacteria (Iwabuchi et al., 2002; Murygina et al., 2005; Van Hamme and Ward, 2001)). Other *Actinobacteria* such as *Gordonia, Dietzia*, and *Microbacterium*, with similar characteristics as *Rhodococcus*, were also detected in the community as degraders of linear and even branched alkanes (Rainey et al., 1995; Yumoto et al., 2002).

Further alkane degradation assays performed with the *Dietzia* strains isolated revealed interesting features of this Corynebacterium. This strain was able to produce hexadecene when growing on hexadecane. This represent a novel degradation pathway as described hitherto (van Beilen and Funhoff, 2007), which might be codify by novel gene sequences that we isolated from this strain (alkB and CYP153). Although this feature may have no use for bioremediation purposes it could be useful for bioconversion processes. In fact, isolation efforts are made not only for assessing the fate and effects of the spilled oil, but also for isolating bacteria that may contain novel degradation pathways useful for industry (Harayama et al., 2004; Van Hamme et al., 2003).

Corynebacterineae group might also play an important role in the degradation of PAHs since genus *Mycobacterium*, specialized in the degradation of adsorbed PAHs (Bastiaens et al., 2000), was detected in clone libraries in high proportion. The ability of *L. anuloederans* and *Mycobacterium* spp. to degrade fluorene and pyrene (Grifoll, M., pers. comm.), which are considered specially recalcitrant components of the fuel (Wammer and Peters, 2005), might explain the high abundance of this bacteria at heavy fuel samples under study, which devoid of the most easily biodegradable fractions.

Properties of Corynebacterineae

As opposed to *Gammaproteobacteria* (e.g. *Alcanivorax*, *Cycloclasticus*, *Thalassolituus*…) that dominates at first fast petroleum degradation processes (Kasai et al., 2002b), members of *Corynebacterineae* are never dominant at such stages (Margesin et al., 2003; Quatrini et al., 2008) being detected with higher frequency at resource limited environments where could play a key role in the *in situ* degradation of more recalcitrant components at long term intervals after an oil spill (Quatrini et al., 2008).

The results we obtained from the alkane degradation assays, performed with *Rhodococcus* and *Dietzia* strains isolated from the consortium, also supported this hypothesis. *Dietzia* spp. used long chain length (LCL) and branched alkanes only when the shorter ones were consumed. Probably this ability enables this genus to maintain its populations at long time periods after an oil-spill when the residual fuel has already enriched

in longer and more recalcitrant fractions. Next to it, hydrocarbon degrading genes, alkB and CYP153, and its respective hydroxilases, were constitutively expressed irrespective of the C source in both *Dietzia* and *Rhodococcus*. This explain why gram-positive degrading bacteria do not fluctuate in response to an oil spill (Margesin et al., 2003). On the contrary, Gammaproteobacteria, such as *Alcanivorax* grow efficiently in response to high HC inputs due to the inducible expression of its catabolic machinery (Yuste et al., 1998; Sabirova et al., 2006).

As a conclusion, stranded fuel oil at marine shorelines represents in itself a habitat which is subject of bacterial colonization and ensuing community succession, where Gram negative, Gammaprotobacteria, might be considered as *r* strategists and Gram positive, Actinobacteria, as *K* strategists growing at later stages of the biodegradation process due to its catabolic versatility (Larkin et al., 2005), which enable this group to grow on the most recalcitrant components of an oil in advanced state of weathering.

If the biodegradation metabolism of *K* strategists (Gram positive bacteria) is different from that of *r* strategists (Gram negative bacteria), as showed in the present study, it seems pretty obvious that the requirements to enhance their respective activities should be different in the same extent. In spite of this, bioremediation amendments are usually the same irrespective of the degree of oil weathering and succession stage, which was always unknown in the case of *Prestige* where bioremediation strategy were based on empirical data. Therefore, based on our observations and previous studies, different improvements are proposed for current bioremediation treatments to be applied at long-term oiled marine environments.

Potential improvements of bioremediation at "Costa da Morte" and similar habitats

Conclusions derived from the present work might be applied to other parts of the Spanish coast affected by the *Prestige* oil-spill since key members of the studied consortium (close to *Rhodococcus*, *Chromatiales*, *Rhodobacteriaceae, Roseobacter*-(*Citreicella*), *Erythrobacter* etc.) were also detected 400 km far at similar environments affected by the *Prestige* (Jiménez et al., 2007). Therefore, the community studied might be a kind of 'climax community' stablished at *Prestige* fuel after being weathered and enriched in recalcitrant fractions.

Microorganisms are able to produce biosurfactants that enhance oil degradation in the environment (Ron and Rosenberg, 2001, 2002). Mycolic acids are major and specific constituents of *Corynebacterineae*, which provide these gram-positive bacteria with an outer barrier that may explain both the limited permeability of their cell walls, its adhesion capacity and their general insusceptibility to toxic agents (Gebhardt et al., 2007). Although use of biosurfactants are usually not feasible as an oil spill response tool because are not cost-

effective to be produced, the use of synthetic mycolic acids, homologues to the natural ones and cheap to produce, was showed to be effective in enhancing the biodegradation capacity of oil-degrading *Rhodococcus* and other Actinobacteria (Linos et al., 2000; Lee et al., 2006). *Rhodococcus* itself produces surfactants which enhance biodegradation capacities of other members of the consortium (Iwabuchi et al., 2002) and therefore, the addition of synthetic mycolic acids to the current bioremediation amendments might improve its effectiveness at long-time oiled areas where the already weathered fuel are likely to be dominated by *Corynebacterineae* and other Actinobacteria.

In the present work we also showed important interactions between members of the hydrocarbon degrading consortia carrying out the *in situ* biodegradation of fuel from *Prestige*. Such interactions are too complex to try to study all of them. However, results from this study give some clues about possible positive interactions, which may be enhanced by bioremediation treatments.

Our results support the hypothesis previously made (McKew et al., 2007) that *Tistrella mobilis* is an opportunistic bacteria which grows on second metabolites (e.g. cathecol) derived from PAHs degradation processes carried out by the actual aromatic degraders. Now that we have isolated *Tistrella*, it would be interesting to study if the presence of this bacterium, which 'steal' intermediate catabolites from PAHs-degrading species, could accelerate the cleavage of new aromatic rings by the degrading bacteria in order to compensate such loss.

We also observed that *Rhodococcus* and mainly *Citreicella* species require some cofactor, probably a vitamin, to develop its alkane and aromatic degrading activities, respectively. Actinobacteria members of the consortia under study, such as *Dietzia*, can supply such cofactor. Application of these cofactors might be of interest in order to improve bioremediation treatments. Although detection of such cofactors might require a lot of work (yeast extracts contain a myriad of different vitamins, etc.), our observations could help in pointing further investigation at such respect.

Resumen

INTRODUCCIÓN

Los vertidos marinos de fuel a gran escala debidos a accidentes de buques petroleros, han planteado grandes amenazas y causado importantes daños a los ambientes costeros afectando a industrias pesqueras, al turismo y por lo tanto a las economías de los sitios afectados.

Las costas del Noroeste de España están junto a una importante ruta para el transporte marítimo. Este alto tráfico hace de las costas gallegas un punto caliente para los derramamientos de petróleo. De hecho, desde el desastre del *Polycommander*, muchos otros han ocurrido como por ejemplo el *Urquiola* (1976), el *Andros Patria* (1978) o el *Mar Egeo* (1992). Siguiendo esta tendencia, el petrolero *Prestige* (2002) se hundió frente a las costas gallegas virtiendo cerca de 63.000 toneladas de fuel pesado, lo que le sitúa como el vigésimo mayor accidente ocurrido en el mundo hasta la fecha. El vertido afectó a más de 500 millas de las costas españolas e incluso francesas, provocando un desastre ecológico comparable o aún peor que el del *Exxon Valdez*.

Los componentes del petróleo se clasifican en cuatro fracciones: saturados, aromáticos, resinas y asfaltenos. La proporción de estos componentes determina las características físicoquímicas del fuel como densidad, toxicidad, viscosidad, etc. La fracción saturada (también alcánica o alifática) son hidrocarburos que no contienen ningún doble enlace, mientras que la aromática son compuestos con uno o más anillos aromáticos (Hidrocarburos Aromáticos Policíclicos (HAPs)) que tienen efectos carcinógenos y tóxicos. Por último, las resinas y asfaltenos son macromoléculas muy complejas y de estructura desconocida.

Cuando el petróleo se derrama en el mar, se extiende por la superficie del agua y es sujeto a diversas modificaciones que cambian sus componentes con el tiempo y por lo tanto su comportamiento en el medio. Este conjunto de procesos, conocido como *weathering*, es principalmente debido a la evaporación de las fracciones de bajo peso molecular, a la disolución de los componentes solubles en agua, a la mezcla del fuel con agua de mar, a la oxidación fotoquímica y a la biodegradación microbiana. La susceptibilidad de los hidrocarburos del petróleo a la degradación microbiana está generalmente en el orden siguiente: alcanos lineales > alcanos ramificados > aromáticos de bajo peso molecular > alcanos cíclicos > HAPs de elevado peso molecular > Resinas y Asfaltenos, que se consideran recalcitrantes a la degradación microbiana.

El residuo de fuel vertido por el *Pretige* fue clasificado como un fuel pesado que carecía de las fracciones más lábiles (22% alifáticos, 50% aromáticos, 28% resinas y asfaltenos) (Alzaga et al., 2004; http://csicprestige.iim.csic.es/informes/info01.pdf) y por lo tanto más resistente a los procesos de *weathering* tales como biodegradación.

El petróleo primero llegó a las costas de Galicia que continuaron recibiendo grandes

cantidades de fuel durante largos periodos de tiempo. Los métodos mecánicos, que normalmente recuperan no más del 10-15 por ciento del fuel que llega a la costa, fueron en muchos casos no aplicables en la predominantemente rocosa y abrupta "Costa da Morte" gallega. Afortunadamente, los microorganismos capaces de degradar hidrocarburos y compuestos relacionados del petróleo son ubicuos en habitat marinos y por lo tanto podríamos asumir que el fuel se acabaría dispersando, degradando y desapareciendo de forma natural con el tiempo. Sin embargo, las observaciones previas en sitios fuertemente petroleados, como ocurrió en las costas gallegas, indican que esto es generalmente un proceso lento y los depósitos de petróleo persisten durante muchos años.

Definido como "el acto de agregar 'materiales' a los ambientes contaminados para causar una aceleración del proceso natural de biodegradación", la biorremediación se ofrece como una buena alternativa para la limpieza del fuel depositado en grandes cantidades tras la catástrofe del *Prestige* en el supralitoral gallego de la "Costa da Morte". Sin embargo, todavía sigue habiendo polémica en torno a la biorremediación puesto que su eficacia es variable entre diferentes aplicaciones. Esta variabilidad depende de la interacción de tres factores:

a) *Las características ambientales del sitio contaminado*.

Los niveles de nutrientes, oxígeno, Tª, etc. son factores que tienen influencia sobre la actividad biodegradadora de la microbiota indígena.

b) *El tipo de petróleo derramado (crudo ligero, fuel pesado, gasolina, etc.).*

Los combustibles pesados, tales como los que vertieron el *Prestige* o el *Nakhodka*, generalmente son pobres en las fracciones más inestables y por lo tanto plantean más problemas a la biodegradación que los crudos ligeros (con un mayor contenido de alcanos).

c) *El potencial de biodegradación de la población microbiana autóctona del habitat afectado.*

No hay microorganismo capaz de degradar todos los componentes de una mezcla del petróleo. En condiciones naturales, la biodegradación de un fuel implica normalmente la sucesión de diversas comunidades microbianas capaces de degradar diferentes hidrocarburos.

Por lo tanto, para diseñar el biorremedio más apropiado que potencie la tasa natural de biodegradación de la comunidad microbiana autóctona necesitamos tener la mayor información posible sobre dichos consorcios. Saber qué nutrientes, cofactores,… y en definitiva qué condiciones, dadas las características ambientales del habitat afectado y el tipo de fuel acumulado, son los óptimos para activar el metabolismo de dichas poblaciones degradadoras.

Debido a la naturaleza altamente persistente de la carga del *Prestige*, el fuel vertido

deambuló durante largo tiempo dirigido por los fuertes vientos y corrientes frente a la costa Noroeste de la Península Ibérica, afectando a diferentes hábitats de la misma. En consecuencia, la presente Tesis fue iniciada con el objetivo de estudiar las poblaciones bacterianas indígenas relacionadas con la degradación *in situ* del fuel del *Prestige* en dos ambientes marítimos representativos de la costa gallega con características distintas y, por lo tanto, con estrategias de respuesta diferentes frente al derramamiento. Sedimentos y agua de la Ría de Vigo (Capítulos I y II) y muestras de fuel acumulado sobre rocas y arena de la "Costa da Morte" (Capítulos III y IV) fueron muestreados en diferentes momentos tras la catástrofe del *Prestige*.

MATERIALES Y MÉTODOS

La combinación de técnicas moleculares (análisis del gen 16S mediante 'electroforesis en gel de gradiente desnaturalizante' (DGGE), 'librerías de clones' e 'hibridaciones *in situ'* (FISH)) y aquellas basadas en cultivo (número más probable (MPN), enriquecimientos con hexadecano, fenantreno, etc.), fue utilizada para determinar la estructura de la comunidad bacteriana autóctona y su capacidad potencial y/o real de biodegradación. De hecho, la combinación de técnicas cultivo-dependientes e –independientes (como las moleculares) usadas en el presente estudio ha demostrado ser una metodología de gran potencia en el campo de la ecología microbiana del petróleo, siempre y cuando seamos conscientes de las limitaciones de cada una de las técnicas de forma aislada.

Las identidades entre secuencias de ADN, del gen ribosomal ARN 16S, detectadas en los ambientes petroleados con las de especies bacterianas previamente relacionadas con la degradación de hidrocarburos, nos dan información sobre el metabolismo y la capacidad degradadora potencial de la comunidad estudiada. Sin embargo, tales semejanzas del 16S son simplemente orientativas de la capacidad catabólica real de una comunidad dada y se deben interpretar con precaución. En este sentido, nuestros estudios no se detuvieron en la simple descripción taxonómica de las comunidades microbianas. Muchas de las bacterias detectadas en los ambientes petroleados por medio de técnicas moleculares como potenciales agentes degradadores de diferentes fracciones del fuel del *Prestige*, tales como *Alcanivorax*, *Rhodococcus*, *Citreicella*, *Dietzia*, *Sphingomonas*, etc., se pudieron aislar y por lo tanto analizar en mayor profundidad respecto a su genética, fisiología e interacción con otros miembros de los consorcios bacterianos indígenas responsables de la biodegradación *in situ* del fuel del *Prestige*.

RESULTADOS Y DISCUSIÓN

Ría de Vigo

Características ambientales de la Ría de Vigo

La Ría de Vigo, (Galicia, NO España), está situada en el límite norte del sistema de afloramiento del NO Africano, y por lo tanto en un área donde las dinámicas de afloramiento-hundimiento sobre la plataforma continental (Arístegui et al., 2006) tienen una fuerte influencia sobre la hidrografía y, por lo tanto, la estructura y función de la comunidad planctónica de las Rías. De hecho, cambios en la estructura y la diversidad de la comunidad bacterioplanctónica de la Ría de Vigo fueron encontrados a lo largo del año (tabla II.3), incluyendo la detección de nuevos miembros en todos los grupos importantes de bacterias.

El afloramiento costero induce la afluencia de agua oceánica del este del Atlántico Norte (ENAW) en la Ría. (Alvarez-Salgado et al.,1993). Estas corrientes en profundidad proveen las aguas de las Rías gallegas con nutrientes (valores medios de nitrógeno (N) y fósforo (P) alrededor de 0.6 y 0.06 mg l^{-1} respectivamente; Nogueira et al.,1997) que hacen a la Ría de Vigo ser un ecosistema de gran valor ecológico y económico.

Las Islas Cíes, parte del Parque Natural “Illas Atlánticas”, se sitúan en la boca de la Ría de Vigo y la producción del mejillón en esta región es la más alta de Europa y una de las más importantes en el mundo, dando empleo a 9000 personas directamente y a 20000 indirectamente (Figueiras et al.,, 2002).

Los mejillones y otros moluscos que crecen en la Ría, son animales filtradores que acumulan sustancias contaminantes. Por lo tanto, las autoridades españolas prohibieron la comercialización de productos derivados de la Ría tras la catástrofe del *Prestige* provocando efectos negativos sobre la economía local. La respuesta primaria tras el vertido del *Prestige* mediante medios mecánicos (p.ej. colocación de barreras “booming”, barrido de fuel flotante “skimming”, recogida de fuel a mano por voluntarios, etc.) retiró varias toneladas de fuel. Sin embargo, gran parte de este fuel pesado se hundió y enterró bajo los sedimentos del Parque Natural desde donde puede ser remobilizado a día de hoy. La limpieza mecánica de estos sedimentos podría causar más daño que beneficio puesto que los fondos de este Parque son delicados habitats con un alto valor ecológico. Puesto que la biorremediación no es una opción factible (los tratamientos serían diluidos en el agua), y su limpieza a mano mediante buzos es una tarea cara y difícil, la opción más factible en tales casos es confiar en la atenuación natural del medio. A pesar del papel crítico de las comunidades bacterianas en la red de alimento oceánica, y por lo tanto en la producción de las Rías, hasta el presente estudio no había información disponible sobre dichas poblaciones y mucho menos de su potencial de biodegradación.

Estructura de la comunidad bacterioplanctónica de la Ría de Vigo

En contraste con la mayor parte de comunidades oceánicas superficiales dominadas por miembros del grupo SAR11 (Morris et al.,, 2002), miembros de *Roseobacter* dominaron el bacterioplancton de la Ría de Vigo en detrimento de SAR11, que se adapta más a las condiciones oligotróficas de las aguas abiertas (González et al., 2000, Malmstrom et al., 2004, Rappé et al., 2002). Es bastante probable que esta dominancia de *Roseobacter* sea una característica de ecosistemas del NO de la Península Ibérica influidos por afloraciones de fitoplacton (Henriques et al., 2004). Diversos clados pertenecientes al linaje de *Roseobacter* (NAC11-7, DC5-80-3, DG1128, CHAB-I-5, AS-21, etc.) y nuevos grupos dentro de SAR11, SAR86, *Betaproteobacteria*, etc. fueron detectados en este estudio en estaciones específicas, lo que podría reflejar una tendencia estacional. Los diferentes clados podrían tener diferentes habilidades incluyendo la capacidad de biodegradación (Sekine et al., 2006), o presentar diversas preferencias ambientales, como la asociación con distintas especies de fitoplancton (Wagner-Dobler y Biebl, 2006; West et al., 2008).

Potencial de biodegradación del bacterioplancton de la Ría de Vigo

Cuando se añaden N y P en cantidades adecuadas a cultivos experimentales de agua de mar con petróleo crudo como única fuente de C y energía, *Alcanivorax* (Yakimov et al., 1998) y otras Gammaproteobacteria consideradas 'profesionales de la degradación de hidrocarburos' (Harayama et al., 2004) como *Cycloclasticus* capaz de degradar compuestos poliaromáticos (Chung y King, 2001), aumentan rápidamente en número dominando la comunidad y, por lo tanto, promoviendo enérgicamente la tasa de biodegradación (Harayama et al., 2004, Kasai et al. 2001, Kasai et al. 2002b, McKew et al., 2007, Roling et al. 2002). El hecho de que hayamos detectado la presencia, en sedimentos y agua de la Ría de Vigo, de miembros de *Alcanivorax* degradadores de alcanos y *Cycloclasticus*, que responde a las adiciones HAP (Teira et al., 2007), indica que los niveles naturales de N, P y oxígeno presentes (Vilas et al. 2005) en este sistema de afloramiento son suficientes para promover el crecimiento de estas especies conocidas por jugar un papel fundamental en la atenuación natural de vertidos de petróleo en los sistemas marinos.

a) La contaminación crónica de hidrocarburos de la Ría (derrames de petróleo, industrial y de la escorrentía urbana, etc); b) los factores abióticos favorables de la Ría para el metabolismo de bacterias dgradadoras de hidrocarburos (altos niveles de oxígeno y nutrientes); c) la presencia de forma natural de géneros considerados fundamentales en la degradación de alcanos (*Alcanivorax* sp.) HAPs (*Cycloclasticus* sp.); d) el predominio y la diversidad de secuencias *Roseobacter* (un género previamente relacionado con la biodegradación de petróleo (McKew et al., 2007)); e) la alta población (MPN) de degradadores de alcanos en los sedimentos de la Ría tras el vertido (Alonso-Gutiérrez et

al., 2008); f) la falta de efectos de los HAPs en la estructura del bacterioplancton autóctono (Lekunberri et al. *submitted*; Lekunberri, 2008) y g) el hecho de que procesos de biorremediación en curso ya se detectasen en el entorno de la Ría (Medina-Bellver et al. 2005), apoyan la teoría de la existencia de una comunidad autóctona preadaptada a la degradación de hidrocarburos en la Ría de Vigo, que pueden utilizar los contaminantes como fuente de C y energía. Por lo tanto, la atenuación natural en la Ría de Vigo es probablemente bastante efectiva y por lo tanto puede asumirse que los hidrocarburos disueltos en el agua tras la catástrofe podrán ser consumidos con relativa rapidez por la comunidad bacteriana autóctona.

Supralitoral rocoso de la "Costa da Morte"

Respuesta al vertido de petróleo del Prestige en la "Costa da Morte"

Tras la aplicación de métodos físicos de limpieza, se tomó la decisión de utilizar la biorremediación como una medida secundaria para la limpieza del fuel del *Prestige* acumulado en zonas de difícil acceso. La eficacia de diferentes formulaciones de carácter comercial fue comparada y después de diferentes ensayos sobre el terreno, el fertilizante oleofílico S-200 demostró ser el más efectivo y por lo tanto, se utilizó para limpiar diferentes zonas a lo largo de la costa NO de España donde los tratamientos mecánicos no eran viables y / o conveniente. Aunque su eficacia fue diferente entre las zonas tratadas (Murado, MA et al.. pers. comm.) no hubo más evaluaciones antes de su aplicación en diferentes sitios. Esa variabilidad en el éxito del tratamiento se debe probablemente a las diferencias entre las comunidades bacterianas autóctonas.

Roca, arena e incluso agua, fuertemente afectadas por el vertido del *Prestige* un año antes, fueron muestreados en diferentes lugares antes de la aplicación del S-200. Los análisis reflejados en el presente estudio de comunidades bacterianas pertenecen a muestras de roca y arena petroleadas de zonas de la "Costa da Morte", donde la consiguiente aplicación de S-200 fue exitosa. Por lo tanto, los resultados presentados en esta memoria científica proporcionan una valiosa información acerca de las comunidades bacterianas que responden positivamente a las aplicaciones de fertilizantes de tipo oleofílico y arroja luz sobre el desarrollo de nuevas estrategias de biorremediación.

Comunidades bacterianas autóctonas de la "Costa da Morte" y su potencial de degradación de hidrocarburos

El análisis de identificación (*fingerprinting*) del fuel acumulado en las costas rocosas en estudio mostró un alto grado de meteorización (*weathering*), que está en consonancia con las tendencias que siguen a la biodegradación (Jiménez et al., 2006). Por lo tanto, tal como

se hipotetizó para las comunidades microbianas de la Ría de Vigo, podemos concluir que las comunidades bacterianas de la "Costa da Morte" presentan una buena tasa de biodegradación del hidrocarburo acumulado. Esta afirmación es apoyada por las condiciones ambientales del hábitat afectado y la estructura de la comunidad bacteriana detectada.

La "Costa da Morte" (Galicia, NO España) también se ve afectada por el afloramiento del NO de África (Arístegui et al., 2006) y, por tanto, es un área donde los niveles de nutrientes (0,20 - 0,25 mg l-1 de N total (Alvarez -Salgado et al., 2002)) son más adecuados para los procesos de biodegradación que los del Mar del Japón (Total N ~ 0,1 mg l-1). En los primeros momentos después de un derrame de petróleo, las comunidades de bacterias marinas son dominadas por diferentes Gammaproteobacteria (Roling et al., 2002). Sin embargo, cuando los factores ambientales son adecuadas para los procesos de biodegradación, el petróleo es rápidamente degradado y las comunidades dominadas por Gammaproteobacteria son rápidamente sustituidas por una comunidad más diversa (Roling et al., 2002), al igual que la que encontrada doce meses tras el vertido del *Prestige*. Alrededor de un año después del accidente del petrolero *Nakhodka* en el Mar del Japón, muestras de petróleo de una composición similar a la del *Prestige*, aún estaban dominadas por Gamma y Alphaproteobacteria (bacterias Gram-negativas) (Kasai et al., 2001; Maruyama et al. , 2003), mientras que, Alphaproteobacteria y Actinobacteria (Gram-positivas) dominaban nuestras muestras afectadas por el *Prestige* después de el mismo tiempo.

La estructura trófica de las comunidades bacterianas afectadas por el vertido poseían un porcentaje significativamente mayor de poblaciones degradadoras de alcanos y aromáticos que las no afectadas. Por otro lado, aunque las rocas y arena son muy diferentes sustratos, la composición de las comunidades bacterianas observadas eran muy similares entre sí. Todo ello indica que el petróleo dirigió la composición final de las comunidades bacterianas observadas.

Muchos miembros de las comunidades detectados en este estudio fueron anteriormente asociados con la degradación de alcanos (*Xanthomonadaceae*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Erythrobacter*, etc (Chang et al., 2005; Joven et al., 2007; Macnaughton et al., 1999; Roling et al. , 2002)) y aromáticos (*Citreicella* sp., *Sphingomonadaceae* spp. *Lutibacterium anuloederans*, descrita como una bacteria degradadora de HAP de 2 y 3 anillos con una mayor eficiencia que *Cycloclasticus* spp. (Chung y King, 2001; McKew et al., 2007), etc). Sin embargo, fue Actinobacteria (principalmente miembros del suborden *Corynebacterineae*) el grupo clave en los procesos de degradación *in situ* del fuel del *Prestige* tras un año a la intemperie. Este grupo de bacterias Gram-positivas incluye

géneros detectados en el presente estudio, tales como *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia* y *Rhodoccoccus*.

Tanto las técnicas moleculares como las basadas en cultivo pusieron de manifiesto el papel preponderante del género *Rhodococcus*, como degradador de la fracción alcánica remanente del *Prestige*. Es bien sabido que *Rhodococcus* es un género con una notable diversidad metabólica (Larkin et al., 2005) y capaz de producir biosurfactantes que hacen el fuel más disponible y mejoraran las actividades de otras bacterias degradadoras (Iwabuchi et al., 2002; Murygina et al., 2005 ; Van Hamme y Ward, 2001)). Otras Actinobacteria con características similares a *Rhodococcus* y capaces de degradar alcanos lineales e incluso ramificados como *Gordonia*, *Dietzia* y *Microbacterium* (Rainey et al., 1995; Yumoto et al., 2002), también formaban parte de la misma comunidad bacteriana.

Ensayos de degradación realizados con las cepas aisladas de *Dietzia* revelaron interesantes características de esta Corynebacteria. La cepa estudiada fue capaz de producir hexadeceno cuando usaba hexadecano como fuente de carbono. Esto puede representar una nueva vía de degradación con respecto a lo descrito hasta el momento donde las moléculas de alcanos son oxidadas por un extremo (van Beilen and Funhoff, 2007). La información para realizar esta ruta catabólica podría estar codificada por genes aislados de esta cepa (alkB y CYP153) en el presente estudio. Aunque esta característica puede no tener utilidad para fines de biorremediación podría ser útil para procesos de bioconversión. De hecho, los esfuerzos realizados para aislar bacterias se hacen no sólo con el objetivo de evaluar su capacidad degradadora y sus requerimientos, sino también para aislar cepas que puedan contener nuevas vías de degradación de utilidad para la industria (Harayama et al., 2004; Van Hamme et al., 2003) .

El suborden Corynebacterineae también podría desempeñar directamente un papel importante en la degradación de los HAP puesto que el género *Mycobacterium*, especializado en la degradación de aromáticos adsorbidos al sustrato (Bastiaens et al., 2000), se detectó en las librerías de clones en alta proporción. La capacidad de *L. anuloederans* y *Mycobacterium* spp. para degradar fluoreno y pireno (Grifoll, M., com. pers.), que se consideran componentes especialmente recalcitrantes del petróleo (Wammer and Peters, 2005), podría explicar la alta proporción de estas bacterias en las muestras de fuel pesado bajo estudio, que carece de la mayor parte de las fracciones más susceptibles a la biodegradación.

Propiedades de Corynebacterineae

A diferencia de las Gammaproteobacteria (*Alcanivorax*, *Cycloclasticus*, *Thalassolituus*...) que dominan con sus altas tasas metabólicas en las primeras fases del proceso de degradación del petróleo (Kasai et al., 2002b), los miembros de *Corynebacterineae* nunca

son dominantes en esas etapas, detectándose con mayor frecuencia en entornos de recursos limitados (Margesin et al., 2003; Quatrini et al ., 2008). Este grupo debe desempeñar, por lo tanto, un papel clave en la degradación *in situ* de los componentes más recalcitrantes que dominan la composición del fuel acumulado a largo plazo después de un accidente (Quatrini et al., 2008).

Los resultados obtenidos mediante ensayos de degradación de alcanos, realizados con las cepas de *Rhodococcus* y *Dietzia* aisladas del consorcio degradador autóctono, también apoyan esta hipótesis. *Dietzia* spp. utilizó alcanos de cadena larga (LCL) y ramificados sólo cuando los de cadena corta habían sido consumidos. Probablemente, es esta capacidad la que permite a este género mantener sus poblaciones largos períodos de tiempo después de un derrame de petróleo cuando el combustible residual ya se ha enriquecido en aquellas fracciones menos susceptibles a la degradación. Además, los genes degradadores de hidrocarburos, alkB y CYP153, detectados en este estudio, y sus respectivas hydroxilasas, se expresaron constitutivamente independientemente de la fuente en C en ambos géneros (*Dietzia* y *Rhodococcus*). Esto explica por qué estas bacterias Gram-positivas, a pesar de degradar hidrocarburos, no varían en respuesta a un derrame de petróleo (Margesin et al., 2003). Por el contrario, las Gammaproteobacteria (Gram-negativas) como *Alcanivorax,* son capaces de aumentar en número rápidamente tras un vertido en respuesta al alto aporte de hidrocarburos gracias a la expresión inducible de su maquinaria catabólica (Yuste et al., 1998; Sabirova et al., 2006).

Como conclusión, podría considerarse que el fuel oil depositado en las costas marinas representa en sí mismo un hábitat que es objeto de colonización bacteriana y de la consiguiente sucesión de comunidades, donde las bacterias Gram negativas como Gammaproteobacteria podrían considerarse estrategas de la *r* mientras las Gram positivas (Actinobacteria) actuarían como estrategas de la *K* en etapas posteriores del proceso de biodegradación gracias a su versatilidad catabólica (Larkin et al., 2005) que les hace capaces de crecer a base de las fracciones más recalcitrantes del fuel en avanzado estado de degradación.

Si el metabolismo de biodegradación de los estrategas de la *K* (bacterias Gram positivas) es diferente al de los estrategas de la *r* (bacterias Gram negativas), como se ha demostrado en el presente estudio, parece bastante obvio pensar que los requisitos para activar cada uno de esos metabolismos debería ser de la misma forma, diferente. A pesar de ello, las enmiendas aplicadas suelen ser siempre las mismas independientemente del estado de biodegradación del fuel y de la etapa de sucesión. Esta información siempre fue desconocida en el caso del *Prestige* cuya estrategia de biorremediación se basó exclusivamente en resultados empíricos. Por lo tanto, en base a nuestras observaciones y a estudios anteriores, se proponen diferentes ideas para mejorar las actuales estrategias de

bioremediación y/o desarrollo de nuevas formulaciones de biorremediación que puedan ser de aplicación en zonas petroleadas a largo plazo en el medio ambiente marino.

Estrategias de bioremediación en la "Costa da Morte" y hábitats similares

Las conclusiones derivadas del presente trabajo podrían aplicarse a otras partes del litoral español afectadas por el vertido del *Prestige* ya que muchos de los miembros clave del consorcio estudiado (*Rhodococcus*, *Chromatiales*, *Rhodobacteriaceae*, *Roseobacter*-(*Citreicella*), *Erythrobacter* etc.) también se detectaron a 400 km de distancia en entornos similares afectados por el *Prestige* (Jiménez et al., 2007). Por lo tanto, la comunidad estudiada parece representar una especie de "comunidad clímax" dirigida por la presencia de fuel del *Prestige* tras haber sido degradado y enriquecido en sus fracciones recalcitrantes.

Los microorganismos son capaces de producir biosurfactantes que mejoran la biodisponibilidad y degradación del petróleo en el medio ambiente (Ron y Rosenberg, 2001, 2002). Los ácidos micólicos, son moléculas tensioactivas características del suborden *Corynebacterineae*, que proporcionan a estas bacterias Gram-positivas una barrera exterior que explica tanto la limitada permeabilidad de sus paredes celulares como su capacidad de adhesión y su baja susceptibilidad a agentes tóxicos (Gebhardt et al. 2007). Aunque el uso de biosurfactantes no suele ser una respuesta viable ante vertidos de petróleo dado que son caros de producir, la utilización de ácidos micólicos, análogos a los naturales pero sintetizados químicamente a bajo coste, se mostró eficaz a la hora de aumentar la capacidad de biodegradación de *Rhodococcus* y otras Actinobacteria degradadoras de hidrocarburos (Linos et al., 2000; Lee et al., 2006). *Rhodococcus* por sí misma produce agentes tensioactivos que mejoran las actividades biodegradadoras de los demás miembros del consorcio (Iwabuchi et al., 2002) y, por tanto, la adición de ácidos micólicos sintéticos a las actuales fórmulas biorremediadoras podría aumentar su eficacia a la hora de ser aplicadas en zonas de la costa petroleadas hace tiempo dado que sus comunidades degradadoras estarán probablemente dominadas por *Corynebacterineae*.

En el presente trabajo también se pusieron de manifiesto interacciones entre los miembros de la comunidad bacteriana autóctona que llevaba a cabo la biodegradación del fuel del *Prestige in situ*. Estas interacciones son de gran importancia en el proceso de degradación pero demasiado complejas para tratar de estudiar todas ellas. Los resultados de este estudio dan algunas pistas sobre posibles interacciones positivas que podrían ser aumentadas por los tratamientos de biorremediación.

Nuestros resultados apoyan la hipótesis anterior (McKew et al., 2007) de que *Tistrella mobilis* es una bacteria oportunista que crece a base de metabolitos secundarios (por ejemplo, catecol) derivados de los procesos de degradación de HAPs llevados a cabo por

las bacterias verdaderamente capces de romper el anillo aromático. Ahora que hemos aislado *Tistrella*, sería interesante estudiar si la presencia de esta bacteria, que 'roba' catabolitos intermedios del proceso de degradación, podría acelerar la rotura de nuevos anillos aromáticos por la bacteria degradadora con el fin de compensar esa pérdida .

También se observó que las especies de *Rhodococcus* y principalmente *Citreicella* requerían algún cofactor, probablemente una vitamina, para desarrollar sus actividades degradadoras de alcanos y aromáticos, respectivamente. Los miembros pertenecientes a Actinobacteria del consorcio en estudio, como por ejemplo *Dietzia*, pueden proporcionar tales cofactores. La aplicación de estos cofactores podría ser de su interés con el fin de mejorar los tratamientos de biorremediación. Aunque la detección de estos cofactores podría requerir una gran cantidad de trabajo (extractos de levadura contienen una multitud de diferentes vitaminas, etc), nuestras observaciones podrían orientar una investigación más a fondo en ese respecto.

要約

(Translation by Dr. Atsushi Yamazoe)

はじめに

海洋における大規模な油流出事故は、沿岸域にとって大きな脅威であり、漁業、観光等の経済に大きな影響を与え、大きな損害を引き起こす。スペインのNW海岸は、海上輸送の主要なルートの隣に位置する。このため、ガリシア海岸は石油流出事故の多発地点である。実際にPolycommander号の事故以来、Urquiola号 (1976), Andros Patria 号(1978) or Aegean Sea号(1992)による流出事故が起きている。この後で起きたタンカーPrestige号による事故(2002)では、世界第20番目の規模の石油流出と考えられ、およそ63,000トンの燃料重質油が放出された。その油流出は、スペイン、さらにフランスにまでおよぶ500マイル以上の海岸線に広がり、その生態学的影響はExxon Valdez号による油流出事故と同等かそれ以上のものであった。

石油成分は、飽和分、芳香族、レジンおよびアスファルテンの4つに大別される。飽和分は２重結合を持たない炭化水素であり、一方で芳香族は、一つもしくは２つ以上の芳香環から構成される炭化水素で、発ガン性物質である。レジンおよびアスファルテンは非常に複雑な未知の高分子構造をもつ物質である。石油は海に流出すると、水面に広がり、時間とともにその構成要素および物理化学的な特徴が変化する。この過程は風化とよばれ、流出油は主に低分子化合物の蒸発、水溶性物質の溶け込み、オイルドロップレット（油に海水が溶け込んだ細かい粒）の産生、光酸化と微生物生物分解という作用を受ける。

石油炭化水素の微生物分解の受けやすさは、通常、以下の順序： n-アルカン>分岐鎖アルカン>低分子量芳香族化合物>シクロアルカン>高分子芳香族化合物>レジンおよびアスファルテンであり、レジンおよびアスファルテンは最も微生物分解を受けにくい。Prestige号の事故による残存油は、微生物分解を受けやすい画分を含まない重い燃料油であり（22%の飽和分、50%の芳香族と28%のレジンおよびアスファルテン(Alzaga et al., 2004;http://csicprestige.iim.csic.es/informes/info01.pdf)、生物分解のような風化プロセスを受けにくい。

流出油は最初、ガリシアの海岸に漂着した。ガリシアの海岸はひどく汚染されたが、岩の多い海岸線だったため、大規模な流出事故の際に用いられる物理的な回収（一般的に流出油の10-15パーセントが回収できる方法）が可能ではなかった。
しかし、海には石油炭化水素と関連した化合物を分解できる微生物が普遍的に存在するため、我々は油が時間とともに風化によって分散および分解すると推定した。しかし、重質油に汚染された現場における過去の調査によると、その分解は遅く長期に渡って残存することが予想される。

「汚染現場に資材を投入し、天然に存在する生物的分解プロセスを加速させる行為」として定義されるバイオレメディエーションは、コスタ・デ・モルテにおけるPrestige号によってもたらされた大量の流出油に対するひとつの浄化方法である。

しかし、バイオレメディエーションによる効果については適用の面において、議論の地が残る。バイオレメディエーションの成功は、以下の３つの要因の相互作用に依存する。

（ア）汚染されたサイトの環境要因

栄養塩濃度，温度，酸素等の土着微生物叢の分解活性に影響を及ぼす要因.

（イ）汚染油の種類　（燃料重質油、軽質原油、ガソリン、その他）。

Prestige号から流出した油は重質燃料油であり微生物分解を受けやすい画分を含まないため、軽質油に比べバイオレメディエーションにおいて問題となる。

（ウ）汚染現場に生息する土着分解菌の分解能力

石油混合物のすべての成分を分解できる微生物は存在しない。自然では各種石油成分を分解する異なる微生物叢の移り変わりによって原油は分解される。

従って、最適なバイオレメディエーション処理を設計するためには、汚染現場環境においてある特定の油が分解される時にどのような栄養分もしくは生育因子が律速になるかを知る必要があり、そのためには、油の分解を行う土着の分解微生物叢の詳細な情報は不可欠である。

Prestige号からの流出油は、非常に残留性の高い性質のために風と海流に乗って長期間漂った。そして、スペイン北西の海岸線に沿って様々な生物の生息地に影響を及ぼした。

このような背景もあり、本論文は、それぞれが異なった特徴を持つために別々の油汚染対策の戦略が必要となるガリシアの代表的な二つの海岸において、Prestige号の燃料油の分解に関連する土着微生物叢の研究を目的とした。リア海岸においての海水および底泥（第Ｉ章および第II章）、そして、コスタ・ダ・モルテ海岸の岩に付着したペースト状の油および海砂（第III章と第IV章）は、Prestige号の流出事故後、別の時期に採取したものである。

材料と方法

培養に依存しない分子生物学的手法（DGGE法、16SrDNAクローンライブラリー法、FISH）と培養に依存した培養ベース手法の２種類の方法の組み合わせにより、土着微生物の菌叢解析、さらに土着微生物の持つ潜在的なまたは実際の油分解能力の解析を行った。実際のところ、この論文において分子生物学的手法と培養に依存した手法の組み合わせは、石

油微生物生態学において、それぞれの手法を別々に用いた場合におこるような制限がない、強力な方法であることが示された。

油汚染環境から分子生物学的手法により検出した16SrRNA遺伝子シーケンスと既知の石油分解菌のシーケンスを比較することによって、研究対象の菌叢が有する潜在的な石油分解能力を知ることができる。しかし、16SrDNA配列比較による解析は、あるサンプルの実際の分解能力を知るための最初のステップでしかなく、その解釈には注意が必要である。この意味において、我々の研究は、分子生物学的手法により環境中で検出される微生物叢や主要な土着菌の同定だけでなく、Prestige号の燃料の異なる石油成分の分解に関わる、例えば*Alcanivorax*、*Rhodococcus*、*Citreicella*、*Dietzia*、*Sphingomonas*属細菌等の潜在的な役割について述べた。これらの細菌は単離することができ、そして、さらにその遺伝子的、生理的解明や分解に関わる他の土着炭化水素分解菌群と相互関係について解明することができた。

結果および考察

リア・デ・ヴィゴ(Ría de Vigo)

リア・デ・ヴィゴ における環境要因

リア・デ・ヴィゴ（ガリシア、スペイン北西）は、アフリカ北西地方の噴出（下層の低温の水が表層に上昇する現象）の北の境界に位置し、このため、この地域では大陸棚）の上の湧昇循環が、プランクトンの構造や機能に強い影響を及ぼしている（Aristegui et al、2006。実際に、この地域においてバクテリアプランクトンの群衆構造の変化は一年を通じてみられ（表II.3）、主要なバクテリアの全てで新しいメンバーが検出されている。

沿岸域における噴出は、リアの表面下の海洋の東部地方の北大西洋中央水（ENAW）の流入を引き起こす(Alvarez-Salgado et al. 1993)。底層流の流入は、ガリシア河口域に栄養分を供給し（窒素（N）およびリン（P）の平均的な濃度は、それぞれ0.6および0.06mgl-1；Nogueira et al. 1997)、リアス・デ・ヴィゴに大きな生態学的・経済的な価値を及ぼす。

自然保護地区“Illas Atlánticas”の一部であるシエス島は、この豊かな生態系の入り口に位置し、この地域におけるムール貝の産出は、ヨーロッパで一位であり、世界でも最大なものの1つである。このため、直接的には9000人、間接的には20000人の雇用を支えている（Figueiras et al., 2002）。

リアに生育するムール貝と他の貝類は、水を濾過してそして汚染物質を蓄える。したがって、スペイン当局は、Prestige号による石油流出の後、リアにおける商業的な貝類の収

穫を禁止したため、地域経済に負の影響をもたらした。

初期の流出油対策（例えば、浮いている油のすくい取り等）により、海水表面から大量の燃料油が取り除かれたが、大量の油は沈み、現在まで自然公園の底質に埋もれたままである。水域のバイオレメディエーションが実行可能な選択ではないので（分解を促進するための添加物は薄められてしまう）、このような場合の残った唯一の選択は、自然減衰力を当てすることである。細菌群衆が海洋の食物網やリアの生物生産力に重要な役割を持っているにもかかわらず、リア・デ・ヴィゴにおける微生物集団やさらにその微生物による汚染物質分解に関する情報はない。

リア・デ・ビーゴにおける細菌叢

バクテリアプランクトンでは SAR11 群が大多数を占めるというこれまでの結果と比べ(Morris et al., 2002)、リア・デ・ヴィゴおよび植物プランクトンの異常発生で特徴づけられるイベリア半島の他の汽水域では、*Roseobacter*属が、貧栄養状態の外海に適応している SAR11 群を駆逐することで優占化してる(González et al., 2000, Malmstrom et al., 2004, Rappé et al., 2002)。

Roseobacter 系統のなかで異なる群（NAC11-7、DC5-80-3、DG1128、CHAB-I-5、AS-21等、図II.2）と新しい一群であるSAR11（A3,A4,図II.3）およびSAR86（IV図II.4）、そしてベータプロテオバクテリアが、季節変動を反映したためか特定の季節で検出された。異なる群の微生物には、異なる汚染物質分解能力を持つ可能性があること(Sekine et al.,2006)、または、異なる植物プランクトン種との連携のような異なる環境選択性がある(Wagner-Dobler and Biebl, 2006; West et al., 2008)。

リア・デ・ビーゴにおける潜在的な分解能力

原油を唯一の炭素源として加えた海水中に十分な窒素とリンが添加されると、*Alcanivorax* (Yakimov et al., 1998)や他の芳香族化合物分解菌である*Cycloclasticus*のようなガンマプロテオバクテリアの炭化水素利用菌が優占化し、微生物による分解が促進される(Harayama et al., 2004, Kasai et al. 2001, Kasai et al. 2002b, McKew et al., 2007, Roling et al. 2002)。我々が、ビーゴ湾の海水および底質で（栄養塩の添加なく）*Alcanivorax*や*Cycloclasticus*を検出した事実は、この地域において汚染物質の自然減衰に重要な役割を持つ微生物種の生育を促進するのに十分なレベルの窒素、リン、酸素濃度が存在することを示している。

a)リアにおける慢性的な汚染 （油の流出、工場や都市から流入等.）; b)バイオレメ

ディエーションに好都合な非生物的要因 (高酸素濃度および高栄養塩濃度); c)土着のアルカン分解菌 (*Alcanivorax* sp.) および PAH 分解菌(*Cycloclasticus* sp.)の存在; d)優占化した多用な *Roseobacter* 属細菌(本属の細菌は、油の分解に関連がある (McKew et al., 2007)); e)MPN法で検出した底質中 の高いアルカン分解菌数の存在 (Alonso-Gutiérrez et al., 2008); f)土着のバクテリアプランクトンの群集構造へのPAH化合物の添加による影響が見られない (Lekunberri et al., submitted; Lekunberri, 2008) そして、 g)リアの海岸において実施中のバイオレメメディエーション処理において、汚染物を炭素源・エネルギー源としてとして生育する微生物叢がすでに集積されている事 (Medina-Bellver et al. 2005)

流出油の物理的な回収は自然公園の繊細な環境に害を及ぼす恐れもあり、以上のようなことから、流出油の浄化には、水面に浮かんだ油をすくいとったあとの2時的な処理法としてナチュラルアテニュエーションを用いることが最適だと思われる。

潮上帯に位置する岩の多い海岸のコスタ・ダ・モルテ

コスタ・ダ・モルテにおける油汚染対策

Prestige号油流失事故の浄化にバイオレメディエーションを用いることが決定した後、岩が多い海岸線の別々の地域において、様々な商業用バイオレメディエーション方策の効果が比較された。いくつかの実地試験の後、親油性の肥料S-200を用いた処理が最も効果的なことがわかった。そして、それはスペイン北西の海岸において機械を用いた回収に不向きな汚染箇所において用いられた。その効果はサイト間で異なったが、(Murado, M.A. et al., pers. comm.)、その後の調査は行われなかった。そのような効果の差は、多分土着の細菌叢の違いによるものだと考えられる。

S-200の適用1年前に、異なる地点からPrestige号の流出油でひどく汚染された岩石、砂、海水サンプルが採取された。S-200の効果が確認されたコスタ・ダ・モルテの潮上帯において回収された岩石、砂、海水の微生物叢の解析が行われた。従って、これらの微生物叢の結果は、疎水性肥料の添加による微生物叢の変化を示すものであり、新たなバイオレメディエーション戦略の開発につながる。

コスタ・ダ・モルテにおける細菌群衆とその潜在的な炭化水素分解能

海岸線に残存していた油のフィンガープリント分析では、油の風化具合は微生物の分による傾向と一致している (Fig. III.4)(Jiménez et al., 2006)。このため、

我々は、コスタ・ダ・モルテのサンプルから検出された土着微生物叢は、石油に対する解能を持つと推測した。この推測は、現場の環境条件や微生物叢の結果からも支持される。

コスタ・ダ・モルテ（ガリシア、スペイン北西）も同様に、北西アフリカ噴出システム(Arístegui et al., 2006)、日本海より栄養塩濃度高く、バイオレメディエーションに適した地域である。

一般的に油流出直後の微生物叢は、ガンマプロテオバクテリアが優占化している。それでも、環境要因が微生物にとって分解に適した条件になると、油は急激に風化し、ガンマプロテオバクテリアが既に優占化している菌叢は、我々がPrestige号の事故12ヶ月後のサンプルで検出したものと同様に多様な菌層に遷り変わる(Roling et al., 2002)。日本海で起きたナホトカ号による流出事故1年後のペースト状油のサンプルでは、油の組成はPrestige号のものと似ていたが、その菌叢はグラム陰性菌のガンマおよびアルファプロテオバクテリアが優占化していた(Kasai et al., 2001; Maruyama et al., 2003)。一方で、汚染発生から同時期のPrestige号の流出事故サンプルでは、アルファプロテオバクテリアおよびグラム陽性のアクチノバクテリアが優占化していた。岩石と砂は非常に異なる担体であるにもかかわらず、微生物叢が類似していることは、流出油の種類が微生物叢に影響を与えていることを示唆する。

これまでの記述された微生物叢では、アルカン分解に関連するものとして *Xanthomonadaceae*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Erythrobacter* 属があり(Chang et al., 2005; Young et al., 2007; Macnaughton et al., 1999; Roling et al., 2002)、芳香族化合物の分解に関するものとして *Citreicella* 属., Sphingomonadaceae属.,*Lutibacterium anuloederans*（これらの菌株は、芳香環が2､3つPAHを化合物を分解し、*Cycloclasticus*属よりも芳香族化合物の取り込み効率が高い、Chung and King, 2001; McKew et al., 2007)の報告がある。しかし、Prestige号の流出事故1年後の原位置バイオレメディエーション処理過程で優占化している菌は、アクチノバクテリア(主に *Corynebacterineae* 科)であった。我々の研究で検出されたこの特徴的なグラム陽性菌のアクチノバクテリアは、*Mycobacterium*, *Dietzia*, *Williamsia*, *Gordonia*, および *Rhodoccoccus* 属等の種類であった。

我々の実験では分子生物学的手法と培養法により、アルカンの分解に最も重要な働きをするものとして*Rhodococcus*属を特定した。*Rhodococcus*属は、驚くべきほどの幅広い代謝能を持ち(Larkin et al., 2005)、ほかの分解菌の活性を高める生物的界面活性

剤を生産できることがよく知られている（Iwabuchi et al., 2002; Murygina et al., 2005; Van Hamme and Ward, 2001)。その他のアクチノバクテリアとして *Gordonia, Dietzia,* および *Microbacterium*属のような *Rhodococcus* 属と類似した特徴を持つ菌群を直鎖および分岐アルカンの分解菌として検出した。

*Dietzia*属の単離株を用いて行ったその後のアルカン分解分析で、このコリネバクテリウム属の面白い特徴が明らかになった。この菌株は、ヘキサデカンで培養した時、ヘキサデセンを産出することができた（図IV.8B）。これは、これまでに知られている経路(van Beilen and Funhoff, 2007)とは異なる新しい代謝経路であり、この代謝経路はこの単離菌から分離した新しい遺伝子（alkBとCYP153）によって行われているかも知れない。この新規代謝経路はバイオレメディエーションにおいては重要ではないかも知れないが、生物的物質変換プロセスにおいては役立つと思われる。実際に、分解菌の単離というのは、流出油の分解・浄化評価目的のみならず、産業に役立つ新規代謝経路を含む細菌の単離目的で行われる(Harayama et al., 2004; Van Hamme et al., 2003)。

吸着したPAH化合物の分解に強い *Mycobacterium*属（Bastiaens et al., 2000）がクローンライブラリー法において高い割合で検出されたことから、Corynebacterineae科グループは、PAH化合物の分解に重要な役割を持っているかも知れない。

また、燃料油の成分のうち分解されにくいフルオレンやピレン(Wammer and Peters, 2005)に対して *L. anuloederans* および *Mycobacterium*属細菌が分解能を持つことは(Grifoll, M., pers. comm.)、これら菌株が分解されにくい成分を含むPrestige号の流出油サンプルにおいて高い割合で検出された理由であるかも知れない。

Corynebacterineae科の特徴

初期の石油分解過程で優占化するガンマプロテオバクテリア（例えば*Alcanivorax*、*Cycloclasticus*、*Thalassolituus*）とは対照的に、Corynebacterineae科の細菌は決して石油の分解初期過程において優占化せず(Margesin et al., 2003; Quatrini et al.,2008)、油流出後、長時間経った後の分解されにくい成分が残存するような環境において、分解に重要な働きを持つため高い割合で検出されると考えられる（Quatrini et al., 2008)。石油分解のコンソーシアムから単離した*Rhodococcus* および *Dietzia* 属株を用

いたアルカンの分解試験の結果からも、上述の仮定が支持される。*Dietzia*属株は、直鎖の短いアルカンが消費された場合しか、直鎖の長いアルカンおよび分岐アルカンを利用しない。たぶん、本属のこの能力は、原油流出後、長時間経った後のような分子量が大きく分

解されにくい成分が残存する条件において高い菌数維持を可能にしていると考えられる。第二に、*Rhodococcus* および *Dietzia*属 では炭化水素分解遺伝子（alkB および CYP153）は、炭素源に関係なく恒常的に発現している。このことは、グラム陽性菌が油の流入に対して影響をうけないことを説明している(Margesin et al., 2003)。

この一方で、*Alcanivorax*のようなガンマプロテオバクテリアでは、その分解作用は誘導的であるため、炭化水素の流入に合わせて効率良く増殖する(Yuste et al., 1998; Sabirova et al., 2006)。

結論として、海岸線において一般的な燃料油は、細菌が定着し、そこで菌叢の移り変わりが起こりうる住処となる。そこで、グラム陰性菌は“ｒ”戦略をとる一方で、アクチノバクテリアのようなグラム陽性菌は、風化原油中の分解がされにくい成分を専門的に分解し、バイオレメディエーション過程の後半において増殖するような“ｋ”戦略をとる。“ｋ”戦略家（グラム陽性菌）の分解活動に必要な条件は、“r”戦略家（グラム陰性菌）の分解活動に必要な条件とかなり異なるということは、明白であったにもかかわらず、微生物叢の遷移について知られていなかったため、バイオレメディエーションのための添加物はいつも同じであった。

したがって、我々の実験結果と既報の研究に基づいて、長期間の油汚染環境に適用するために現行のバイオレメディエーション手法に対しての改善策が提案される。

コスタ・デ・モルテにおけるバイオレメディエーション手法の改良案

微生物は環境において油分解を促進する界面活性剤を生産することができる(Ron and Rosenberg, 2001, 2002)。ミコール酸は Corynebacterineae科の主要かつ特徴的な細胞の構成要素であり、グラム陽性菌の細胞壁の選択的透過性、粘着性、毒物に対する非感受性等の特徴を説明する外防壁を与える(Gebhardt et al., 2007)。生物的界面活性剤の使用は、コストが高いため、流出油の処理に用いることができない場合が時々あるが、生産コストが安い合成のミコール酸の使用は、油分解菌である *Rhodococcus* やその他のアクチノバクテリアの分解を促進する(Linos et al., 2000; Lee et al., 2006)。*Rhodococcus*属自身も、その他の菌株の分解を促進する界面活性剤を生産する(Iwabuchi et al., 2002)。したがって、合成ミコール酸の投入は、風化が進んだ、分解のされにくい流出油に対する資材として、原位置バイオレメディエーションを強化する良い戦略であると思われる。Prestige号の流出油で汚染された400ｋｍ離れたスペインの他の海岸においては、*Rhodococcus*, *Chromatiales*, *Rhodobacteriaceae*,

Roseobacter-(*Citreicella*), *Erythrobacter*属が検出されており(Jiménez et al., 2007)、本研究での結論がこの地域に応用できるかも知れない。

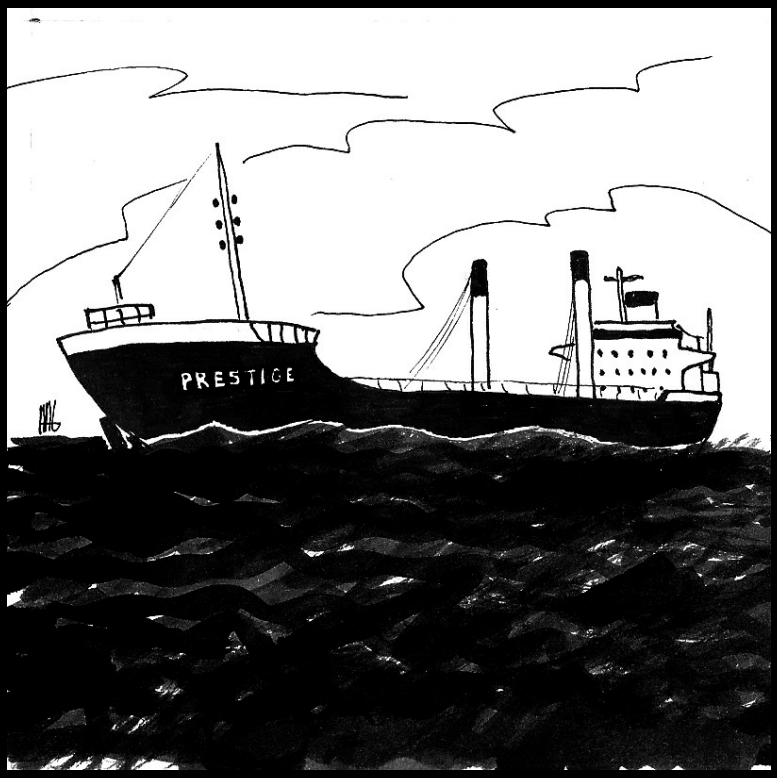
また、本研究の微生物叢はPrestige号による油流出後の風化が進み、難分解性の成分が残存したPrestige号流出油中の最終的な菌叢を表しているであろう。

本研究で我々は、現位置バイオレメディエーション処理において分解を担う微生物群の構成株の重要な相互作用について示した。このような株間での相互作用は、複雑すぎてすべてを明らかにすることはできない。しかし、本研究の結果は、バイオレメディエーション処置によって促進された正の相互作用について若干の手掛かりを与えると思われる。

我々の結果（図III.10A,B）は、「*Tistrella mobilis*は、PAHの分解で生じる代謝産物（例えばcathecol）を利用する日和見主義的な細菌である」(McKew et al., 2007)というこれまでの推定を支持している。

我々によって*Tistrella*,属が単離された今、この菌株を用いた研究で、この株がPAH分解菌から中間代謝産物を奪い、それによる損失を取り戻すために分解菌が新たな芳香環の開裂を行いPAH分解が促進されるということを研究すれば面白いと思われる。

我々はさらに、*Rhodococcus* と主に*Citreicella*属が、それぞれ、アルカンもしくはPAH化合物の分解能を発現するのにビタミン等の補因子を必要とすることも見いだした。本実験の微生物群の構成菌種である*Dietzia*属のようなアクチノバクテリアは自分自身で補因子を供給することができる。このような補因子の利用もバイオレメディエーション促進のために面白いかもしれない。補因子の特定は、重労働を必要とするかもしれないが、我々の発見は、補因子に関する更なる調査を助長することができると考えられる。



General Introduction

A. History of marine oil-spill accidents

Marine shorelines are important public and ecological resources that serve as a home to a variety of wildlife and provide public recreation. Modern society continues to rely on the use of petroleum hydrocarbons for its energy needs and it is estimated that between 1.7 and 8.8 million metric tons of oil are released into the world's water every year (NAS, 1985), of which more than 90% is directly related to human activities including deliberate waste disposal. Contrary to popular perception, only one eighth of the oil released into the aquatic environment is from tanker accidents, while most come from accidental spills during routine operations of extraction, transportation, storage, refining and distribution. However, large scale marine oil spill accidents, have posed great threats and cause extensive damage to the marine coastal environments and affecting fisheries, tourism and therefore to economies of the affected sites.

Table i below gives a brief summary of 20 major oil spills since 1967 (location is indicated in Fig. i). A number of these incidents, despite their large size, caused little or no environmental damage as the oil did not impact coastlines, which is why some of the names will be unfamiliar to the general public.

Table i. Major oil-spills since 1967. Four of them (bold-charactered) occurred at NW off Iberian Peninsula including *Prestige* oil-spill. (adapted from ITOPF; http://www.itopf.com/stats.html)

Position	Shipname	Year	Location	Spill Size (tonnes)
1	*Atlantic Empress*	1979	Off Tobago, West Indies	287000
2	*ABT Summer*	1991	700 nautical miles off Angola	260000
3	*Castillo de Bellver*	1983	Off Saldanha Bay, South Africa	252000
4	*Amoco Cadiz*	1978	Off Brittany, France	223000
5	*Haven*	1991	Genoa, Italy	144000
6	*Odyssey*	1988	700 nautical miles off Nova Scotia, Canada	132000
7	*Torrey Canyon*	1967	Scilly Isles, UK	119000
8	*Sea Star*	1972	Gulf of Oman, Wales	115000
9	*Irenes Serenade*	1980	Navarino Bay, Greece	100000
10	***Urquiola***	**1976**	**La Coruña, Spain**	**100000**
11	*Hawaiian Patriot*	1977	300 nautical miles off Honolulu	95000
12	*Independenta*	1979	Bosphorus, Turkey	95000
13	***Jakob Maersk***	**1975**	**Oporto, Portugal**	**88000**
14	*Braer*	1993	Shetland Islands, UK	85000
15	*Khark 5*	1989	120 nautical miles off Atlantic coast of Morocco	80000
16	***Aegean Sea***	**1992**	**La Coruña, Spain**	**74000**
17	*Sea Empress*	1996	Milford Haven, UK	72000
18	*Katina P.*	1992	Off Maputo, Mozambique	72000
19	*Nova*	1985	Off Kharg Island, Gulf of Iran	70000
20	***Prestige***	**2002**	**Off North-West coasts of Spain**	**63000**
35	*Exxon Valdez*	1989	Prince William Sound, Alaska, USA	37000

Although not even the twentieth but rather the 35th largest spill, the *Exxon Valdez* (1989) is included because it is maybe the best known of all for its acute and long term environmental impacts (Spies *et al*., 1996) on the wildlife of Prince William Sound, Alaska.

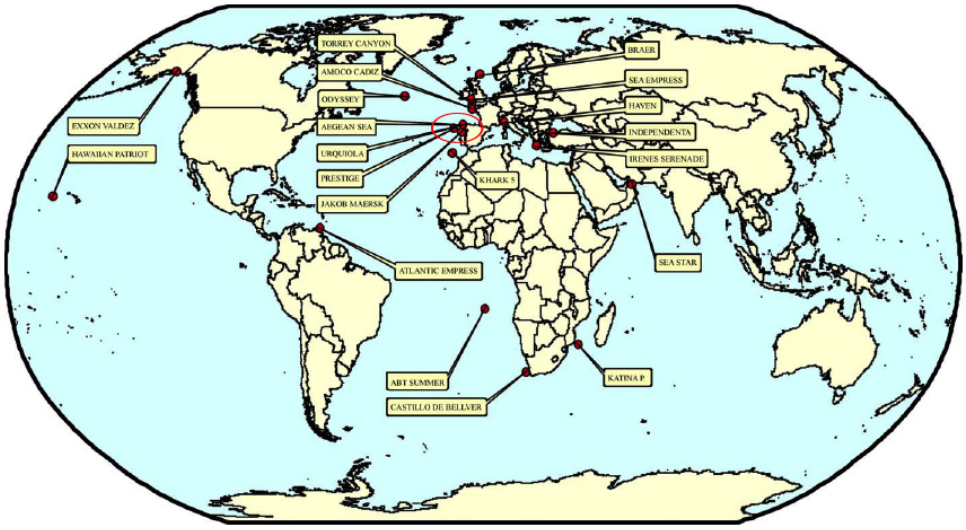


Fig.i. Location of the worst oil-spills. (from http://www.itopf.com/stats.html)

NW coasts of Spain are next to a common route for maritime transport. This high traffic made Galician coasts be a hot point for oil spills, as shown in the map made by The International Tanker Owners Pollution Federation Limited (ITOPF) (Fig. i). In fact, since the disaster of *Polycommander*, many other have occurred such as *Urquiola* (1976), *Andros Patria* (1978) or *Aegean Sea* (1992). Following this trend, during the afternoon of Wednesday 13 November 2002, the tanker *Prestige* (81,564 DWT), carrying a cargo of 77,000 tonnes of heavy fuel oil, suffered hull damage in heavy seas off northern Spain. The casualty was reportedly denied access to a sheltered, safe haven in either Spain or Portugal and so had to be towed out into the Atlantic. Stresses on the vessel make it break in two early on 19 November some 170 miles west of Vigo, and the two sections sank some hours later in water two miles deep (Fig. ii). In all, it is estimated that some 63,000 tonnes of heavy fuel were lost from the *Prestige* (Albaiges et al., 2006), being ranked as the 20th largest oil spill in history (Table ii). Next to it, the spill affected more than 500 miles of the Spanish and even the French coastlines, resulting in an ecological disaster comparable or even worse than that of the *Exxon Valdez*. Fisheries exclusion zones were put in place in Galicia shortly after the incident, banning virtually all fishing along about 90% of the coastline. All bans had been lifted by October 2003. The impact on fisheries in France was less extensive. In both countries, an impact on tourism was reported for 2003. Next to these and the oil-removal

costs, the Spanish authorities decided to remove the oil remaining in the wreck at an estimated cost of some €100 million.

Fig. ii. *Prestige´*s hull breakage just before sinking (Photo EFE).

Since 1974, The International Tanker Owners Pollution Federation Limited (ITOPF) has maintained a database of oil spills from tankers (http://www.itopf.com/stats.html), where it can be seen how the number of big oil-spills (>700 tonnes; like that of *Prestige*) has decreased in the last years (Fig. iii). However, because oil is so widely used, despite all the precautions, it is almost certain that oil spills and leakage will continue to occur. Thus, it is essential that we have effective countermeasures to deal with the problem.

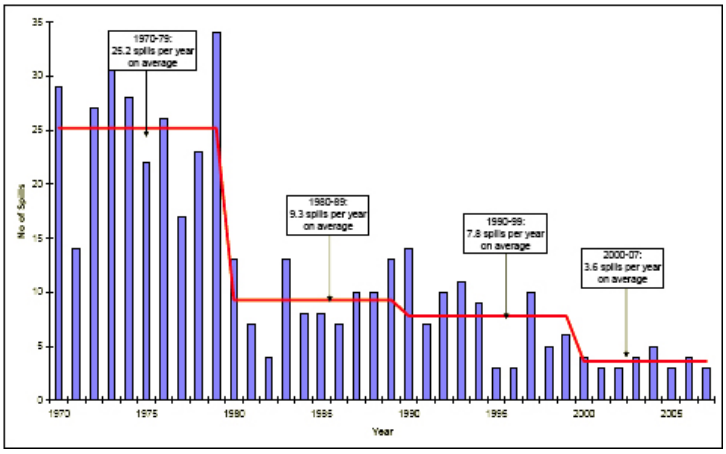


Fig. iii. Number of oil-spills over 700 Tonnes. (from http://www.itopf.com/stats.html)

B. Factors affecting clean-up strategies

Strategies for cleaning up an oil spill are greatly affected by a variety of factors, such as:

B1. the type of oil spilled,

B2. the characteristics of the spill site and

B3. the biodegradation capacity of the indigenous microbiota of the affected habitat.

B1. Physico-Chemical Properties of Crude Oil

Crude oils are very complex mixtures that exhibit a wide range of physical properties. Petroleum components may be classified into four major groups (Fig.iv) based on their differential solubility in organic solvents (Leahy and Colwell, 1990):

- Saturated hydrocarbons: Include normal and branched alkanes (isoprenoids) containing no double bonds which are further classified according to their chemical structure into *n*-alkanes or paraffins (C_nH_{2n+2}) and cyclic alkanes or naphthenes (C_nH_{2n}). They range in chain length from one to over 40 carbons and used to be the most abundant constituents in crude oils and the most readily biodegradable fraction.
- Aromatic hydrocarbons: Include monocyclic aromatics (e.g., benzene, toluene, and xylenes) and polycyclic aromatic hydrocarbons (PAHs) (e.g., naphthalene, anthracene and phenanthrene), which have two or more fused aromatic rings. PAHs are of environmental concern because they are potential carcinogens.
- Resins: Include polar compounds containing nitrogen, sulfur, and oxygen (e.g., pyridines and thiophenes). They are often referred to as NSO compounds.
- Asphaltenes: Consist of poorly characterized high molecular weight compounds that include both high molecular weight and poorly characterized hydrocarbons and NSOs. Metals such as Ni, V, and Fe are also associated with asphaltenes.

Relative composition in each of these components and the ambient temperature of the affected habitat influence important physical properties (e.g. density, viscosity, pour point, solubility in water…), which determine behavior of spilled oil. Understanding this behaviour is important with respect to oil fate, oil toxicity, bioremediation processes and therefore in determining the appropriate spill cleanup responses.

The oil residue released by the *Prestige* lacks of the more labile fractions (boiling point <300ºC). The product was a heavy fuel oil No. 6 with a density of 0.995 g · cm^{-3}, 2.28% S, 22% aliphatics, 50% aromatics and 28% of resins and asphalthenes (Alzaga et al., 2004) (http://csicprestige.iim.csic.es/informes/info01.pdf).

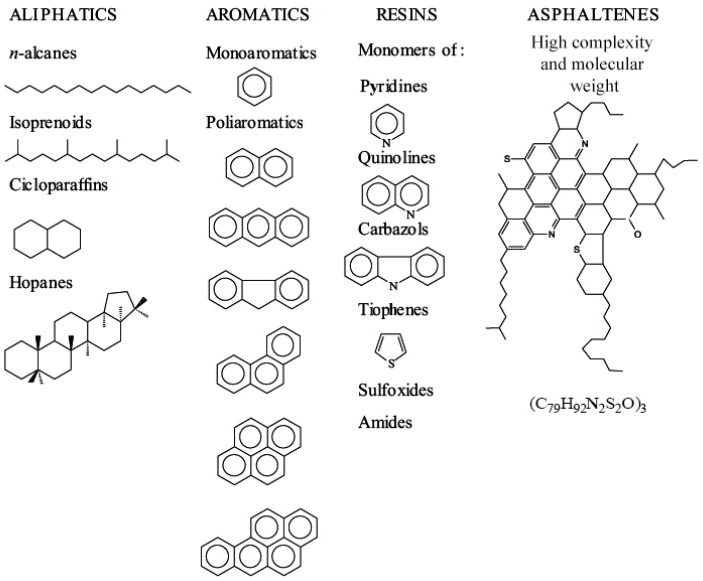


Fig. iv. Representative organic compounds found in crude oils. (modified from Vinas, 2005 and Zhu, *et al.* 2001).

B2. Characteristics of the Environment

When oil is introduced into the environment, it immediately goes through a variety of physical, chemical and biological changes known as a whole as weathering (Figure iv), which will alter oil composition and properties in ways that may affect its behaviour.

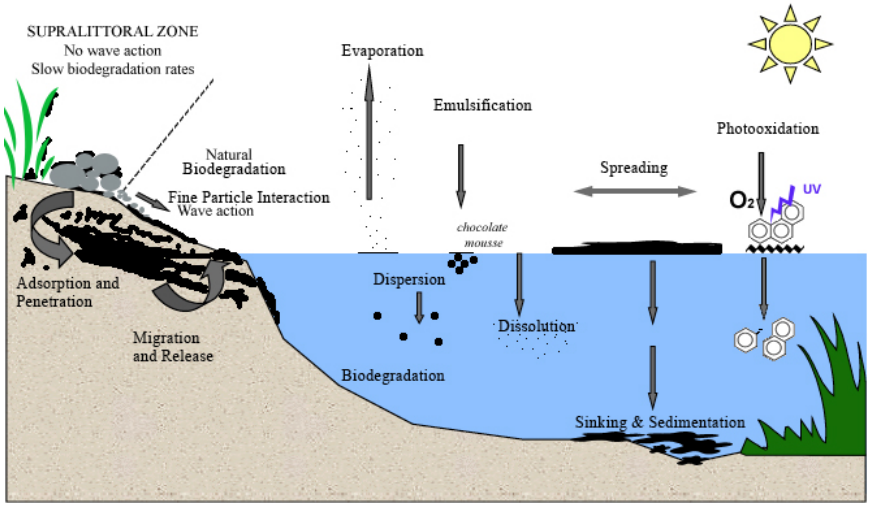


Fig. iv. Major weathering processes after an oil-spill (adapted from Zhu *et al.*, 2001)

The behavior of spilled oil in shoreline environments is primarily dependent on the properties of the shoreline, such as the porosity of the substrate and the energy of the waves acting on a shoreline. Higher wave exposure enhances both physical removal and weathering processes including biodegradation. Wave-swept rocky shores tend to recover from oil spills within a matter of months whereas supralittoral and estuarine environments may act as a petroleum sink for many years.

Large heavy fuel oil spills, such is the *Prestige*, are less usual than crude oils and exhibit a different fate as they are barely dispersed in the water column and mainly stranded on the shoreline or sedimented in the form of patches or tar aggregates; characteristics which hinder its natural biodegradation. Owing to the highly persistent nature of *Prestige´*s cargo, the released oil drifted for extended periods with winds and currents, travelling great distances. Oil first came ashore in Galicia, where the predominantly rocky coastline was heavily contaminated. Remobilisation of stranded oil and fresh strandings of increasingly fragmented weathered oil continued over the ensuing weeks, gradually moving the oil into the Bay of Biscay and affecting the north coast of Spain and the Atlantic coast of France, as far north as Brittany.

Biodegradation of oil is one of the most important processes involved in weathering and the eventual removal of fuel from the environment, particularly for the nonvolatile components of petroleum. Scientific studies are needed to cover various aspects of this process and the environmental factors that influence the rate of biodegradation at each habitat (such as temperature, pH, nutient and oxygen levels, etc.).

B3. Hydrocarbon-degrading microbiota at marine environments

Microorganisms capable of degrading petroleum hydrocarbons and related compounds are ubiquitous in marine, freshwater, and soil habitats. More than 200 species of bacteria, yeasts and fungi have been shown to degrade hydrocarbons although in the marine environment, bacteria are considered to be the predominant hydrocarbon-degraders (Floodgate, 1984).

The distribution of hydrocarbon-utilizing microorganisms is also related to the historical exposure of the environment to hydrocarbons. Those environments with a recent or chronic oil contamination will have a higher percentage of hydrocarbon degraders than unpolluted areas. In “pristine” ecosystems, hydrocarbon utilizers may make up less than 0.1% of the microbial community; and in oil-polluted environments, they can constitute up to 100% of the viable microorganisms (Atlas, 1981).

There is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil, not even a genetically modified “superbug”. In nature,

biodegradation of a crude oil typically involves a succession of different hydrocarbon-degrading microbial communities. Microorganisms from such consortia, classified as non-hydrocarbon utilizers, play indeed important roles in the eventual removal of petroleum from the environment. Petroleum degradation involves complex reactions where hydrocarbonoclastic species carry out the initial attack of the pollutant molecule generating intermediate compounds that may be subsequently utilized by a different group of organisms of the same community. This synergistic process results in further degradation of crude oil components (Karrick, 1977; Casellas et al., 1998).

As described earlier, petroleum components can be classified into four major groups which show differences in their susceptibility to biodegradation. Major metabolic pathways for many of these compounds have been well studied and documented (Van Hamme et al., 2003; Harayama et al., 2004):

- Saturates: In general, the *n*-alkanes are the most readily degraded components in a petroleum mixture. Branched alkanes are less readily degraded in comparison to *n*-alkanes and methyl branching increases the resistance to microbial attack.
- Aromatics: Aromatics are generally more resistant to biodegradation than most of saturates and their resistance increase with the level of complexity (number of fused rings). Monoaromatic hydrocarbons are easily biodegradable under aerobic conditions but in high concentration are toxic to some microorganisms due to their solvent action on cell membranes. The metabolism of PAHs is a more complex process than the metabolism of the aliphatic fraction where the initial bacterial dioxygenases from the PAHs metabolism, exhibited a lower specificity of substrate. PAHs with 2-4 rings are less toxic and still biodegradable. Frequently, the resulting oxidised PAHs require the intervention of another bacterial strain, which plays an important role in its degradation but cannot be detected as an aromatic hydrocarbon degrader. In this sense, it was previously described how the bacterial metabolism of fluorene needs the co-culture of two strains of which only one was able to degrade the aromatic while the other eliminates secondary metabolites produced by the former (Casellas et al., 1998). Probably this laboratory model reproduces a very frequent metabolic cooperation among different strains in the bacterial metabolism of PAHs *in situ*. PAHs with five or more rings can only be degraded through co-metabolism, in which microorganisms fortuitously transform non-growth substrates while metabolizing simpler primary substrates in the oil.
- Resins and asphaltenes: Compared to saturates and aromatics, very little is known about biodegradation of resins and asphaltenes due to their complex structures. They are considered as recalcitrant components.

In summary, the susceptibility of petroleum hydrocarbons to microbial degradation is generally in the following order: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes > high-molecular-weight aromatics > Resins and Asphaltenes.

Several marine bacteria capable of degrading petroleum hydrocarbons have been newly isolated. These are bacteria of the genera *Alcanivorax* (Yakimov *et al.*, 1998), *Cycloclasticus (*Dyksterhouse *et al.*, 1995), *Oleiphilus* (Golyshin *et al.*, 2002) and *Oleispira* (Yakimov *et al.*, 2003) within the γ-*Proteobacteria*. These bacteria use limited carbon sources with a preference for petroleum hydrocarbons and are thus 'professional hydrocarbonoclastic' bacteria. For example, *Alcanivorax* strains grow on n-alkanes and branched alkanes, but cannot use any sugars or amino acids as carbon sources. Similarly, *Cycloclasticus* strains grow on the aromatic hydrocarbons, naphthalene, phenanthrene and anthracene, whereas *Oleiphilus* and *Oleispira* strains grow on the aliphatic hydrocarbons, alkanoles and alkanoates.

C. Marine oil-spill response strategies

A number of approaches and technologies have been developed for controlling oil spills in marine shorelines.

These methods have been reviewed and described extensively in a number of technical documents, such as *Understanding Oil Spills and Oil Spill Response* (U.S. EPA, 1999). The most commonly used shoreline cleanup options are summarized in the following table (Table ii).

Table ii. Conventional Shoreline Clean-up Options (adapted from Zhu, *et al.*, 2001).

Category of Response Options	Example Technology
Physical method	Booming
	Skimming
	Manual removal (Wiping)
	Mechanical removal
	Washing (e.g. High pressure water)
	Sediment relocation/Surf-washing
	Tilling
	In-situ burning
Chemical mehod	Dispersants
	Demulsifiers
	Solidifiers
	Surface film chemicals
	*Biosurfactants
Natural method	Natural attenuation

(*This may be considered as bioremediation)

Physical removal, are the first response option and most of the released oil from a tanker is removed by these means before even reaching the coastline by booming and skimming. Manual and mechanical washing is also the first response to get rid of oil from coastlines, however it is estimated that only 10-15 percent is actually removed by these techniques.

Chemical methods pose concerns about its toxicity and long-term environmental effects; therefore they are commonly used just when the other methods are not effective. However, solidifiers can be used to facilitate recovery of oil from water by mechanical methods and dispersants, used at oiled coastlines, relocate the pollutant to open water where it can be diluted and more easily biodegraded. The use of extracellular polysaccharides and other biosurfactants from bacteria has been proved to be more effective than chemical surfactants in increasing bioavailability and, hence, enhancing biodegradation rates. They are selective, environmentally friendly, are not toxic and easily biodegradable in the marine environment (Ron and Rosenberg, 2001, 2002).

A major offshore cleanup operation was carried out using vessels from Spain and nine other European countries (including submarine *Nautile* from Ifremer (France); Fig.v). The

Fig. v. Shoreline and open-sea operations for the *Prestige´s* fuel oil removal (pictures from different sources (Diario el Mundo, Diario Faro de Vigo and Ifremer)).

response, which was probably the largest international effort of its kind ever mounted, was hampered by severe weather. The open-sea recovery operation off Spain reportedly removed almost 50,000 tonnes of oil-water mixture. However this, and the extensive

booming of estuaries and sensitive areas by the deployment of over 20km of physical barriers, failed to prevent extensive coastal contamination. Altogether approximately 1,900 km of shoreline were affected. The shorelines of Spain were largely cleaned manually by a workforce of over 5,000 military and local government personnel and volunteers (Fig. v). The process was slow, especially in rocky areas where access was difficult. A further problem was re-oiling of previously cleaned areas by re-mobilised oil. On the French Atlantic coast, the beach contamination took the form of numerous tar balls, which were relatively easy to remove. In total, some 141,000 tonnes of oily waste was collected in Spain and 18,300 tonnes in France. However a lot of oil remained at supralittoral zones and/or buried in sandy beaches where mechanical methods were not effective.

Natural attenuation allows oil to be removed and degraded by natural means (e.g. evaporation of lighter fractions such short alkanes, photooxidation and biodegradation). For some spills, it is probably more cost-effective and ecologically sound to leave an oil-contaminated site to recover naturally than to attempt to intervene, when natural removal rates are fast enough. Coastlines of Galicia (NW Spain), are located in the northern boundary of the NW Africa upwelling system, and therefore in an area where seawater is supplied with new, remineralized nutrients (Arístegui et al., 2006; Fraga, 1981; Figueiras et al., 2002), which may benefit the natural biodegradation rates of the spilled oil.

Marine shorelines have a wide range of sensitivities to oil and clean-up activities (Table iii) depending on the type of substrate, grain size, tidal elevation, exposure to wave and tidal energy, biological sensitivity, accessibility, etc. Bioremediation may be effective and cause

Table iii. Shoreline Environmental Sensitivity Index ESI ranking for habitats in marine shorelines (where 1 is least sensitive and 10 is most sensitive to oil and clean up actions) (from Zhu et al., 2001).

Environmental Sensitivity Index (ESI)	Shoreline Type
1	Exposed rocky shores
	Sea walls and piers
2	Exposed wave-cut platforms
3	Fine-grained sand beaches
4	Coarse grained sand beaches
5	Mixed sand and gravel beaches
6	Gravel beaches and riprap
7	Exposed tidal flats
8	Sheltered rocky shores
9	Sheltered tidal flats
10	Salt marshes and Mangroves

the least damage on both the moderately and the most sensitive shoreline types. However, its effectiveness is still uncertain due to the lack of sufficient research. As a rule of thumb we can say that for habitats with low ESI, mechanical methods and natural attenuation might be

sufficient as an oil spill response while for the most sensitive bioremediation is the best option since mechanical methods are not advisable.

D. Bioremediation as an oil-spill cleanup technology

Various types of microorganisms that are capable of oxidizing petroleum hydrocarbons are widespread in nature. Biodegradation is a particularly important weathering process that removes the non-volatile components of oil from the environment. However, this is a relatively slow process and may require months to years for microorganisms to degrade a significant fraction of an oil stranded within the sediments of marine environments. Defined as “the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes” (OTA, 1991), bioremediation is a green clean-up technology based on the premise that a large percentage of oil components are readily biodegradable in nature by the naturally occurring hydrocarbon-degrading bacteria (Atlas, 1984, 1981; Prince, 1993).

Although mechanical methods are the first response option, they typically recover no more than 10-15 percent of the oil after a major spill (OTA, 1990). Since its successful application after the 1989 *Exxon Valdez* spill (Bragg *et al*., 1994; Prince *et. al*., 1994b), bioremediation has emerged as a polishing step after physical cleanup options have been applied.

Bioremediation has several advantages over conventional technologies. First, the application of bioremediation is relatively inexpensive. For example, during the cleanup of the *Exxon Valdez* spill, the cost of bioremediating 120 km of shoreline was less than one day’s costs for physical washing (Atlas, 1995). Bioremediation is also a more environmentally benign technology since it involves the eventual degradation of oil to mineral products (such as carbon dioxide and water), while physical and chemical methods typically transfer the contaminant from one environmental compartment to another. The success of oil spill bioremediation depends on our ability to establish and maintain conditions that favor enhanced oil biodegradation rates in the contaminated environment. There are two main approaches to oil spill bioremediation:

- *bioaugmentation,* in which known oil-degrading bacteria are added to supplement the existing microbial population, and
- *biostimulation*, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting cosubstrates, and/or by alterations in environmental conditions (e.g. surf-washing, oxygen addition by plant growth, etc.).

Biostimulation is a more effective approach because the addition of hydrocarbon

degrading microorganisms will not enhance oil degradation more than simple nutrient addition (Swannell et al., 1996; Diez et al., 2005; Vinas et al., 2005). The failure of bioaugmentation in the field may be attributed to environmental factors such as predation by protozoans, the oil surface area, or scouring of attached biomass by wave activity. However, the most likely reason is that the added bacteria seem to compete poorly with the indigenous population (Venosa *et al*., 1992).

Fortunately, oil-degrading microorganisms are ubiquitous in the environment, and they can increase by many orders of magnitude after being exposed to crude oil (Atlas, 1981; Lee and Levy, 1987, Pritchard and Costa, 1991). Therefore, in most environments, there is usually no need to add hydrocarbon degraders. In certain circumstances that have not been well defined, when the indigenous bacteria are incapable of degrading one or more important contaminants, addition of microbial inocula may be considered. Genetically engineered organisms are not likely to be used in the near or even distant future.

Bioremediation, like other technologies, also has its limitations. Oil bioremediation involves highly heterogeneous and complex processes and its success depends on having the appropriate microorganisms in place under suitable environmental conditions and nutrient/cofactor requirements. Its operational use are determined by the composition of the oil spilled, the characteristics of the affected environment (O_2 levels, pH, N/P levels…) and the biodegradation properties of its autochthonous microbial population. Bioremediation is also a relatively slow process, requiring weeks to months to take effect, which may not be feasible when immediate cleanup is demanded. Concerns also arise about potential adverse effects associated with the application of bioremediation agents, like toxicity of bioremediation agents themselves and metabolic by-products of oil degradation (Swannell *et al*., 1996).

E. Environmental factors affecting oil biodegradation

When oil spills occur in the environment, the rate of oil biodegradation is also greatly influenced by the characteristics of the contaminated environment. Major environmental factors affecting oil biodegradation are described:

E1. <u>Weathering</u>

Weathering processes (Fig. iv) have profound effects on oil biodegradation. Evaporation of volatile oil components removes the toxic low-molecular-weight components and dispersion increase the oil surface area therefore benefiting microorganisms because growth of oil degraders occurs almost exclusively at the oil-water interface. The formation of oil-in-water emulsions through the microbial production and release of biosurfactants has

also been found to be an important process in the uptake of hydrocarbons by bacteria and fungi (Singer and Finnerty, 1984). However, weathering processes can also reduce oil surface (e.g. emulsification) and increase the more recalcitrant fractions therefore hindering biodegradation rates.

E2. Temperature

The ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 15 to 20ºC in marine environments. However, it also depends on the indigenous microbial population. In cases where a psychrophilic consortium has been established, good biodegradtion rates can be observed at low temperatures (Zhu et al., 2001).

E3. Oxygen

Aerobic conditions are generally considered necessary for extensive degradation of oil hydrocarbons in the environment since major degradative pathways for both saturates and aromatics involve oxygenases (Cerniglia, 1992).

Anaerobic oil degradation is an exciting field of research because some studies demonstrated that in some marine sediments, PAHs and alkanes can be degraded under sulfate-reducing conditions at similar rates to those under aerobic conditions (Caldwell *et al.*, 1998; Coates *et al*., 1997). However, this process normally occur only at negligible rates, as reviewed by Atlas (1981), leading to the conclusion that the environmental importance of anaerobic hydrocarbon degradation can be discounted. Anaerobic biodegradation of oil in the environment still requires further studies since a lot of oil remains buried under anoxic conditions.

E4. Nutrients

In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are utilized in the conversion of 1 g of hydrocarbon to cell materials (Rosenberg and Ron, 1996). When a major oil spill occurs the supply of carbon is dramatically increased while nitrogen and phosphorus becomes limiting factors for oil degradation (Atlas, 1984; Leahy and Colwell, 1990). In marine environments, nutrient limitation is generally correlated to the low background levels of nitrogen and phosphorus in seawater (Floodgate, 1984).

Since weathering processes, temperature of the water or oxygen levels are nearly or completely unfeasible to control, bioremediation usually aim at increasing nutrient levels by,

for example, adding fertilizers. One of the main challenges associated with biostimulation in oil-contaminated coastal areas is maintaining optimal nutrient concentrations in contact with the oil. Design of nutrient delivery systems that overcome the washout problems characteristic of marine environments include oleophilic fertilizer formulations. Oleophilic fertilizers such Inipol EAP 22 (microemulsion containing urea as a nitrogen source, lauryl phosphate (the phosphorus source), 2-butoxy-1-ethanol as a surfactant, and oleic acid to give the material its hydrophobicity) became popular after showed its efectiveness at cobble beaches affected by the *Exxon Valdez* oil-spill (Pritchard and Costa, 1991; Pritchard et al., 1992). The rationale for this strategy is that oil biodegradation mainly occurred at the oil-water interface and oleophilic fertilizers are able to adhere to oil and provide nutrients at the oil-water interface.

The stormy weather and tough swell at the time of the *Prestige*'s oil-spill caused that the fuel were stranded even at the supralittoral and unaccesible parts of the affected coastlines where mechanical removal were not feasible. Besides it, wave action rarely reaches such zones, therefore hindering natural biodegradation rates. In such cases, bioremediation using oleophilic fertilizers (e.g. S-200) were chosen to biostimulate indigenous hydrocarbon-degrading microbiota at the affected seashore of some inaccessible parts of "Costa de la Muerte" (Galicia, Spain) achieving a visual acceleration of the natural biodegradation processes (Murado, CSIC http://csicprestige.iim.csic.es/desarro/biorrem/; data not published). An enhancement of biodegradation, specially of heavier alkanes and more alkylated polyciclic aromatic hydrocarbons (PAHs), were described in the treatment of the *Prestige* oil using the same formulation *in vitro* and in field conditions (Jiménez et al., 2006). The oleophilic fertilizer S-200 was demonstrated to be the best option to increase biodegradation rates of the *Prestige* oil at supralittoral zones. Therefore bioremediation offered one available option for the *Prestige*'s oil spill response.

However, the use of bioremediation remains a controversial issue, as its effectiveness varies with different oil spills (Harayama et. al, 2004). In this sense, efectiveness of bioremediation treatments after the *Prestige´*s oil spill in the field was also variable between sites (Murado, pers. comm.). Differences in autochthonous microbial communities were likely to be related with such variation among biodegradation rates. In the present study we studied the indigenous microbial consortia of oiled matrices where the subsequent application of S-200 showed an enhancement of the biodegradation rate.

Although the addition of nitrogen and phosphorus nutrients has been shown to accelerate the speed of biodegradation, oil bioremediation is a complex process and very little is known about the biological processes involved in the clean-up of contaminated marine environments. Therefore to understand the scope and strategies of oil bioremediation, it is essential to first understand the hydrocarbon-degrading bacterial

communities, its mechanisms of oil biodegradation and the factors that control its rate.

Important advances in microbial ecology have enabled the identification of some microbial populations effective in the degradation of hydrocarbons in natural environments. However, very little information about the vast majority of marine bacteria that remain uncultured are available, and more efforts should be made to isolate oil-degrading or oil-emulsifying bacteria which could be further investigated concerning their physiology requirements for enhancing their growth after an oil spill (Van Hamme et al., 2003; Harayama et al., 2004). Thus, before the application of *in situ* bioremediation (for a given place and type of oil) is important to search for hydrocarbon degrading indigenous microorganisms to study the conditions promoting their growth in the affected habitat in order to improve or develop new bioremediation amendments.

Currently, one of the major challenges in the application of oil bioremediation is the lack of guidelines regarding when and how to use this technology. Scientific data for the support of an operational guidelines document has recently been provided from laboratory studies and fields trials carried out by the U.S. Environmental Protection Agency (EPA), University of Cincinnati, and Fisheries and Oceans Canada. (Zhu *et al.*, 2001). Most of this introduction is actually based in such document.

F. Bioremediation response plan

As a summary, the major steps in a bioremediation selection and response plan in a specific habitat should include:

F1. Pre-treatment assessment

This step involves the evaluation of whether bioremediation is a viable option based on:

- the type of oil that has been spilled
- the presence of hydrocarbon-degrading microorganisms
- concentrations of background nutrients,
- the type of shoreline that has been impacted,
- other environmental factors (pH, T^a, O_2, accessibility of the site, logistics…)

F2. Design of treatment and monitoring plan

After the decision is made to use bioremediation, further assessments and planning are needed prior to the application. This involves selection of the rate-limiting treatment agents (e.g., nutrients), determination of application strategies for the rate-limiting agents, and design of sampling and monitoring plans.

To decide which nutrient or cofactor is going to be limiting for the biodegradation process it is fundamental that we know as much as possible about

the autochthonous microbial community. Interactions between members of the bacterial consortia carrying out biodegradation pathways will give us clues about how to enhance its degradation metabolisms.

F3. Assessment and termination of treatment

Chemical, toxicological, and ecological analysis.

Objectives

In spite of the long oil spill history in Galicia, very little is known about the indigenous microbiota associated to bioremediation processes and even less related with heavy fuel oil-spills. Among the very few works on this topic, the studies made after the *Nakhodka* oil spill in the Sea of Japan retrieved information about microbial populations adapted to the heavy fuel oil from this tanker. Although the fuel was of a similar composition to that of the *Prestige*, the coastal environment from the the Sea of Japan and the NW Atlantic coasts of Spain are quite different so different species and degradation rates would be expected.

Fuel-oil from *Prestige* affected a wide range of maritime habitats along the NW Spanish coastline and, hence, study the bacterial community of all of them would be an unfordable task. Therefore, the present Thesis was aimed at studying the indigenous bacterial populations related with the *in situ* degradation of the *Prestige* fuel at two representative maritime environments of the Galician coast with distinct characteristics:

a) The "Ría de Vigo", which represents a common estuarine-like ecosystem characteristic of the coasts of the NW of Iberian Peninsula known as 'Rías'. Water was sampled at different seasons since are upwelling ecosystems with highly variable hydrographic conditions.
b) Shoreline from "Costa da Morte", which is quite homogenous and covers a big extension of the coastline of Galicia (Fig. III.1). They are abrupt, well oxygenated rocky shorelines with though swell. During the *Prestige* oil-spill, stormy weather facilitated the accumulation of heavy fuel at the supralittoral zone where wave action rarely reachs.

Different matrices, sediments and water from the Ría (Chapters I and II) and fuel paste from rocks and sand from "Costa da Morte" (Chapters III and IV) were sampled at different times after the *Prestige* oil spill. A combination of molecular (denaturing gradient gel electrophoresis (DGGE), 16S gene clone libraries and fluorescence in situ hybridization (FISH)) and culture-based techniques was used to describe the hydrocarbon-degrading communities and to isolate some of its components to further study its physiology.

The information derived from these studies is summarized in this scientific memory which will help in the design of future, more effective bioremediation treatments for the coasts of Galicia.

Chapter I:

Culturable bacteria associated to recently affected sediments by the *Prestige´*s oil-spill.

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I.1 ABSTRACT

Sediments sampled next to the Cíes Islands, a natural reserve of the National Park of Atlantic Islands in Ría de Vigo (Spain), three months after the first tide of fuel from the *Prestige* tanker arrived, were chemically and microbiologically characterized. The chemical analysis of the sediments, using oil fingerprinting techniques, showed the occurrence of fresh *Prestige* fuel oil with a background of older hydrocarbon contamination.

The cultured bacterial community of the contaminated sediments harbored a high population of total heterotrophs and alkane degraders and a small proportion of aromatic degrading bacteria. Based on a partial 16S ribosomal RNA gene sequence data, thirty-seven different bacterial strains isolated in diluted marine agar were detected. Most of them were classified as members of *Gammaproteobacteria* groups (59%) and *Alphaproteobacteria* (21%), although members of *Bacteroidetes* (10%) and of *Firmicutes* (10%) were also found.

Among the different identified bacteria, some of them have been previously described as fuel oil degrading species such as *Alcanivorax, Shewanella, Vibrio, Pseudoalteromonas* and *Marinomonas*. Seven independent isolates were able to grow with hexadecane. However, these seven strains are grouped under the same phylotype based on the 16s rRNA gene sequence and were closely related to *Alcanivorax borkumensis* (*Gammaproteobacteria*). No aromatic degrading activities were detected among the culturable bacteria. The presence of fresh fuel without a detected level of biodegradation suggests that *Alcanivora*x was an early colonizer after the oil spill of *Prestige* tanker. To our knowledge, the isolation of *Alcanivorax* from environmental samples without the aid of an enrichment procedure has not been previously reported. Our results suggest the ability of *Alcanivorax* to compete and coexist with other heterotrophic marine bacteria in oil polluted marine environments rich in N and P.

I.2 INTRODUCTION

In November 2002, the oil tanker *Prestige* broke up and sank 120 nautical miles off the Galician coast (NW Spain) during a storm, leading to one of the largest known oil spills in the region. More than 80% of the 77,000-ton tanker's cargo is thought to have been spilled. The product was a heavy fuel oil No. 6 with a density of 0.995 g · cm^{-3}, 2.28% S, 22% aliphatics, 50% aromatics and 28% of resins and asphalthenes (Alzaga et al. 2004). Large heavy fuel oil spills are less usual than crude oils and exhibit a different fate as they are barely dispersed in the water column and mainly stranded on the shoreline or sedimented in the form of patches or tar aggregates.

The *Prestige* oil spread onto several thousand kilometers of the Spanish and French coasts arriving in the first week of December 2002 to Vigo. A lot of beaches and many natural reserves, such as the National Park of the Atlantic Islands, have been affected since then.

The ecological importance of the islands prompted the development of the present work to obtain information about the natural microbiota present in surface sediments after the *Prestige* oil spill and to study its potential for natural attenuation processes on heavy fuel oil biodegradation. Chemical and microbiological analyses provide valuable data for assessing the intrinsic bioremediation capabilities of the sediments.

Bioremediation, defined as the act of adding nutrients or bacteria to contaminated environments to cause an acceleration of the natural biodegradation processes, is recognized as an appropiate oil spill response tool (Swannell et al. 1996). Adding N and P (biostimulation) to the environment has been a common bioremediation strategy to favour the growth of degrading bacteria. The use of allochthonous petroleum degrading bacteria (bioaugmentation) obtained from enriched laboratory cultures has not yet been shown to be better than the stimulation of the autochthonous species of degraders (Swannell et al. 1996, Diez et al. 2005, Vinas et al. 2005a). Very recently, an enhancement of biodegradation, especially of heavier alkanes and more alkylated polycyclic aromatic hydrocarbons (PAHs), has been described in the treatment of the *Prestige* oil using an oleophilic fertilizer in vitro and in field conditions (Jiménez et al. 2006, Jiménez et al. 2007).

Before the application of *in situ* bioremediation it is advisable to search for indigenous hydrocarbon degrading microorganisms. There is limited information about the vast majority of oil-degrading marine bacteria that remain uncultured (Van Hamme et al. 2003) and even less is known about microbial species selected in marine sediments contaminated with heavy fuels.

To our knowledge, only the experiments conducted after the *Nakhodka* oil spill, retrieved information about microbial populations adapted to heavy fuel oil. The evolution of microbial

communities of polluted samples from natural beaches and water was studied, and later subjected to enrichment in batch culture. Under these conditions, adapted microorganisms were more easily grown in isolation (Kasai et al. 2002b). Different molecular approaches mainly involving 16S rRNA analysis such as PCR/DGGE (Kasai et al. 2001), clone libraries and specific oligonucleotide probes (Maruyama et al. 2003) were used in order to follow the changes in the degrading community. All these studies reached similar conclusions which could be summarized as follows: a) the total population of bacteria was almost stable and there were no long term effects in its composition due to the accident; b) the degraders of oil components showed a maximum population level immediately after the oil spill; c) predominant oil-degrading community was composed of bacteria which closely resembled the aliphatic hydrocarbon decomposer *Alcanivorax borkumensis,* followed by aromatic degrading bacteria *Cycloclasticus pugetii* and *Sphingomonas*.

Several marine bacteria capable of degrading petroleum hydrocarbons have been recently described such as *Alcanivorax* (Yakimov et al. 1998), *Oleiphilus* (Golyshin et al. 2002), *Oleispira* (Yakimov et al. 2003), *Cycloclasticus* (Dyksterhouse et al. 1995) and *Marinobacter* (Gauthier et al. 1992). This genus and few others which are obligate consumers of oil hydrocarbons has been named as obligate hydrocarbonoclastic bacteria (OHCB) which bloom after a pollution event. However, the types of hydrocarbon degrading bacteria that may bloom depend on the latitude/temperature, salinity, redox and other physico-chemical factors (Yakimov et al. 2007). Indeed, it has been recently showed that also biological factors, such as bioturbation, may favour the development of oil degrading communities in polluted environments (Cuny et al. 2007).

Alcanivorax borkumensis was isolated for the first time at the North Sea using *n*-tetradecane as the sole carbon and energy source (Abraham et al. 1998, Yakimov et al. 1998). In a relatively short period of time the *Alcanivorax* genus has been isolated or detected by molecular techniques from samples taken from different places such as the Atlantic Ocean, the Mediterranean Sea, the North Sea, the Sea of Japan, the South China Sea, and the Antarctic (Golyshin et al. 2003).

It is an aerobic organism which has been defined as “professional hydrocarbonoclastic bacteria” since it is an obligate consumer of alkanes and branched alkanes unable to grow on sugars or aminoacids (Harayama et al. 2004, Head et al. 2006, Yakimov et al. 2007). *Alcanivorax* usually exists in low numbers in unpolluted waters and rapidly increases in oil-polluted waters and coastlines (Harayama et al. 1999, Kasai et al. 2001, Syutsubo et al. 2001). Its capacity to grow on branched alkanes seems to be the reason why this bacteria become the major bacterial population in oil-contaminated sea water in the presence of an adequate proportion of N and P (Hara et al. 2003).

Like most of degrading species, *Alcanivorax* members had been isolated using enriched

media in fuel or any oil fraction (Bruns & Berthe-Corti 1999, Liu & Shao 2005) or in a very oligotrophic medium (Fernandez-Martinez et al. 2003) in order to avoid competition with heterotrophic marine bacteria, which usually outcompetes degrading species. *Alcanivorax* has also been isolated from heavy oil polluted samples from the *Nakhodka* oil spill in Japan. Again, *Alcanivorax* could only be isolated from oiled sand, gravel and seawater, after growing the polluted samples in nutrient enriched cultures (Kasai et al. 2001, Kasai et al. 2002b, Roling et al. 2002). These experiments were performed *in vitro* or under artificial enrichment conditions so its results cannot be directly extrapolated to the real environment. However, to answer the question of which microorganisms have particular significance for the removal of hydrocarbons from the environment it could be useful to obtain evidence from culture-based studies, which define the catabolic capabilities of candidate organisms and indicate the qualitative and quantitative importance of particular organisms in situ (Head et al. 2006).

In the present work, we report on a chemical and culture-based microbiological study of marine sediments affected by the *Prestige* heavy fuel oil spill, three months after the accident. Chemical fingerprinting was used to detect polluted sediments verifying that no cross contamination from different sources other than the *Prestige* fuel occurred. The most recently polluted sediments were directly used for a microbiological prospection using non-selective media for the isolation of degrading bacteria. As far as we know, no previous study has tried to isolate degrading strains directly from heavy oil polluted environmental samples without any prior enrichment procedure.

I.3 MATERIALS AND METHODS

I.3.1 Sampling

Surface sandy-gravel sediments next to Cíes Islands (42º 13, 50 N/ 08º 53, 57 W, National Park of Atlantic Islands, Vigo, Spain) (station 1, Fig. I.1) were collected by CIS (Centro de Investigaciones Submarinas, Vigo) scuba-divers at the subtidal zone (4 meters of depth), three months after the first *Prestige* black tide reached the islands. Samples were collected from the upper 10 cm of sediment by means of sterilized flasks.

Once collected, samples were stored at 4ºC and sent to the laboratory, where those for chemical analyses where kept frozen (-20ºC). Microbiological analysis of station 1 was conducted immediately after sampling. Other samples were collected at the same time for chemical analysis around the Islands at the stations shown in Figure I.1, using a box-core dredge (10 x 16 cm) and recovering the surface layer (1-2 cm, approx. 300g).

I.3.2 Chemical analysis

The sediment samples (about 5 g) were spiked with the surrogates (anthracene-d_{10}, pyrene-d_{10} and benzo[*a*]pyrene-d_{12}) and extracted with a Pressurized Solvent Extraction apparatus (Applied Separations, Allentown, PA, USA) with hexane-acetone (1:1) at 110 ºC for 10 min in 3 cycles. The recovered extracts were treated overnight with recently activated copper for elemental sulphur removal, then carefully evaporated to near dryness, and dissolved with 0.5 mL of *n*-hexane for further fractionation into aliphatic and aromatic hydrocarbons.

Fractionation was performed by column chromatography with 1 g anhydrous sodium sulphate (top), 6 g neutral alumina (middle) (activated at 350 ºC, 5% water deactivated) and 6 g silica gel (bottom) (activated at 120 ºC and 5% water deactivated). Two fractions were collected, i) aliphatic hydrocarbons, eluted with 20 ml of *n*-hexane, and ii) polycyclic aromatic hydrocarbons with 40 ml of *n*-hexane : dichloromethane (80:20). The collected extracts were concentrated and analyzed by gas chromatography coupled to mass spectrometry (GC-MS) using a Thermo-Electron Corporation system (Austin, TX, USA) in the electron impact (EI) mode at 70 ev. The gas chromatograph was equipped with a split/splitless injector (splitless time: 0.80 min, split flow 50 mL/min) and a HP-5 MS capillary column of 30 m x 0.25 mm id x 0.25 mm film (J&W Scientific, Folsom, CA, USA). The initial column temperature was held for 1 min at 70 °C, programmed at 15°C/min to 150°C/min and then at 6°C to a final temperature of 320°C which was held for 10 min. The carrier gas was helium at a constant flow of 1.2 mL/min. The injector temperature was 310ºC and transfer line and ion source were held at 250 and 200 ºC, respectively. Data were acquired in the full scan mode from 50 to 490 amu (2.4 scans/second) with 5 min of solvent delay and processed by the X-calibur software.

The peak areas of the target analytes were measured in the reconstructed ion chromatograms at m/z 85 for aliphatics, m/z 217-218 for steranes, m/z 191 for hopanes and the molecular ion for the aromatic hydrocarbons. Recoveries ranged from 70 to 110% except for naphthalene that were from 40 to 60% due to its higher volatility. The relative standard deviation (RSD) of the molecular biomarker indices was < 5%.

I.3.3 Bacterial isolation

Results from chemical analysis showed station 1 as the most polluted. Sediments from this place were used to isolate bacteria. It seems that the sea currents or any new inputs, which still occurred at the time of sampling, makes this part of the estuary hold a greater amount of pollution compared with neighbouring areas.

Ten-fold dilutions of the sediments from station 1 were spread directly onto agar plates containing 1/5 strength of Marine broth (Cultimed) and 1,5% (w/v) agar. Colony forming units (Cfus hereafter) were determined after incubation at 20ºC for 7 days in order to assess the most abundant culturable bacteria. For a morphological and biochemical characterization,

pure cultures were obtained from the most abundant culturable bacteria streaking them on Marine agar and storing them in Marine broth + 15% Glycerol at –80ºC for subsequent analysis.

I.3.4 Enumeration of heterotrophic and hydrocarbon–degrading microbial populations

Bacterial counts from the surface sediments were performed using the miniaturized most probable number (MPN) method performed in a 96-well microtiter plates with eight replicate wells per dilution (Wrenn & Venosa 1996). Total heterotrophs were counted in tryptone soy broth (TSB), aliphatic degraders were counted in mineral medium called BMTM (Hareland et al. 1975) containing 1 g *n*-hexadecane L^{-1} and aromatic hydrocarbon degraders were counted in BMTM containing a mixture of phenanthrene (0.5 $g \cdot L^{-1}$), fluorene, anthracene and dibenzothiophene (each at a final concentration of 0.05 $g \cdot L^{-1}$). All the media were supplemented with 3% NaCl. Hydrocarbon stocks were previously filtered through Teflon filters (Fluoropore 0.22 µm PTFE, Millipore).

BMTM mineral medium were composed by 18,6 mM $K_2HPO_4 \cdot 3H_20$; 7,2 mM $NaH_2PO_4 \cdot H_20$; 37 mM NH_4Cl; 0,8 mM $MgSO_4 \cdot 7H_20$; 43 µM $FeSO_4 \cdot 7H_20$; 17,8 µM $MnSO_4 \cdot H_20$; 10,5 µM $ZnSO4 \cdot 7H_20$; 4,2 µM $CoCl_2 \cdot 6H_20$ and 0,5 mM Nitrilotriacetic acid disodium salt.

I.3.5 Isolation and identification of hydrocarbon-degrading bacteria

All isolated bacteria were screened for hydrocarbon degradation capabilities by growth in different selective media. Two media were used: one for the detection of alkane-degradation and another to detect aromatic-degradation activity. Both were tested on each cultured bacteria using solid medium (Petri plates) and liquid medium (96-well microtitre sterile plates). Solid medium was used for the detection of alkane degraders on parafilm sealed Petri plates containing mineral medium agar (BMTM 3% NaCl solidified with purified agar) with sterile cotton soaked in hexadecane on their lids as the sole source of C and energy as previously described (Sei et al. 2003). For aromatic degraders isolation, naphthalene pebbles, instead of cotton, were used. The screening was carried out in duplicate for each. A negative control (bacteria growing only on purified agar) and a positive control in marine broth, were also included in the analysis.

The second test used to detect hydrocarbon-degrading strains was carried out in liquid mineral medium (BMTM + 3% NaCl) containing either *n*-hexadecane or PAHs mixture at the same concentrations described above for MPN enumeration. The strains were cultured overnight at room temperature in TSB. Cells were harvested by centrifugation (4000 × g for 15 min), washed twice, and finally suspended in mineral medium (BMTM+3% NaCl) to reach an optical density (OD) around 0.5 (determined using a multiscan spectrophotometer (Labsystems) at 620 nm. A total of 20 µl of suspended cells was used for the inoculation of two wells per plate. Plates with BMTM without hydrocarbons were inoculated with each strain and were used as negative controls.

I.3.6 DNA extraction and PCR amplification of the 16S rRNA gene

Colonies were picked up from the pure cultures, suspended in 100 µL of sterile milliQ water (Sigma-Aldrich Co.), boiled for 10 min, and centrifuged for 5 min at 12000g. Then, 2 µL of the supernatant was used as template DNA for PCR analysis. The complete 16S rRNA gene was amplified using primers F27 and R1492 as previously described (Edwards et al. 1989, Lane 1991). All PCR amplifications were performed with a Perkin-Elmer GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA). PCR reaction (25 µl) included 10mM Tris; 50mM KCl (pH 8.3); 2.5 mM $MgCl_2$; 400 µM of each deoxynucleotide; 1.25 U of *Taq* DNA polymerase (Amplitaq; PE Applied Biosystems, Foster City, CA), 0.4 µM of each primer, and 100 ng of template DNA. The reaction mixtures were subjected to a hot start (5 min at 95ºC) and after that to the following thermal cycling parameters:

(i) 5 min at 95°C; (ii) 40 cycles, with one cycle consisting of 30 seconds at 96°C for denaturation, 30 seconds at 55°C for annealing, and 1.5 min at 72°C for elongation; and (iii) a final extension step of 10 min at 72°C. PCR products were visualized in a 1% (w/v) agarose gel in 1X TAE buffer stained with ethidium bromide (0,6 mg/ml).

I.3.7 Sequencing and phylogenetic analysis

In order to eliminate the excess of primers and dNTPs for sequencing reactions, the PCR products were digested at 37°C for 1 hour using Shrimp Alkaline Phosphatase (SAP) (1U/µl) and Exonuclease I (ExoI) (10U/µl) (U.S.B. Corporation, Cleveland, OH, USA). The enzymes were afterwards inactivated by heating the samples at 80°C for 15 min.

Sequencing was accomplished using the ABI Prism Big Dye Terminator cycle-sequencing reaction kit (version 3.1) and an ABI Prism 3700 automated sequencer (PE Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. 16S rRNA genes were full sequenced in both directions using a set of six universal primers F27, R1492, F341, R907 (Edwards et al. 1989, Lane 1991), PSL and PSR (Campbell et al. 1995). Sequences were inspected, corrected and assembled into a single consensus sequence for each phylotype. After that, the sequences were examined with the BLAST search alignment tool comparison software (BLASTN)(Altschul et al. 1990) to detect the closest bacterial group to each strain among GenBank database.

Sequences from all phylotypes were aligned with reference sequences obtained from GenBank using ClustalX (Thompson et al. 1997). The alignment obtained was transferred to MacClade (Maddison & Maddison 2003) and was modified using the software GBlocks (Castresana 2000) to eliminate poorly informative regions of the DNA alignment, which are convenient to delete prior to phylogenetic analysis.

The alignment obtained was finally edited using the MacClade program and directly transferred to version 4.0b10 of PAUP*software (Swofford 2000). ModelTest software version 3.6 (Posada & Crandall 1998) was run as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment. We incorporated the best-fit model of nucleotide evolution, calculated by ModelTest, into software PAUP* and PHYML (Guidon & Gascuel 2003), which uses a single, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Finally, the trees created by PHYML and PAUP were edited using the software TreeViewX (Page 1996).

I.3.8 Nucleotide sequence accession numbers

The 37 bacterial 16Sr DNA sequences reported in this paper are available under GenBank accession numbers EU195920 to EU195956.

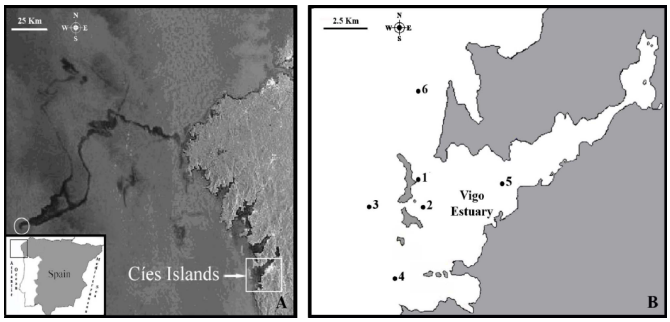


Fig. I.1.A: Satellite image (ENVISAT,http://envisat.esa.int/asar_oil_spill/) of the fuel reaching the Galician coast on November the 17th, 2002, from the *Prestige´s* wreck (white circle). **B**: Detailed map of Vigo Estuary, with the sampling stations (1-6) around the Cíes islands.

I.4 RESULTS

I.4.1 Chemical analysis

The GC profiles of the aliphatic fraction of the subtidal sediment of the Cíes Islands (Station 1, Fig. I.2) exhibited clear evidence of a fresh petrogenic contamination, based on occurrence of the homologous series of C_{17}-C_{40} *n*-alkanes overlying an unresolved complex mixture of hydrocarbons. In contrast, the profiles corresponding to the other samples (e.g. Station 3; Fig. I.2) exhibited the general features of rather pristine coastal environments, with the predominance of terrestrial biogenic (higher plant) C_{25}-C_{33} *n*-alkanes with odd-even

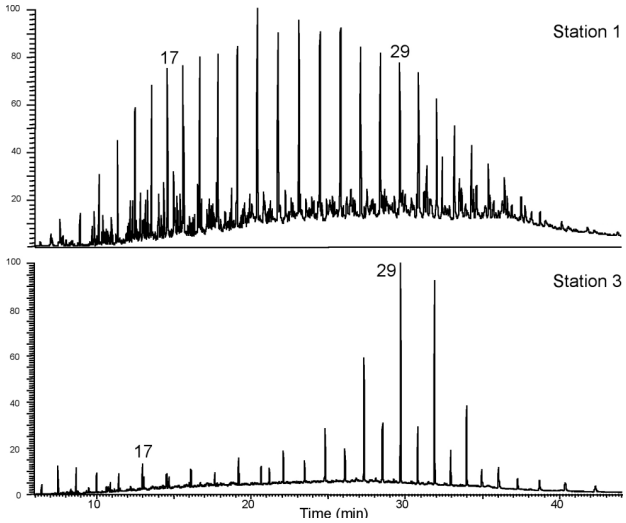


Fig. I.2. Representative profiles of the sediment aliphatic fractions (m/z 85) in stations 1 and 3. Peak numbers indicate the number of carbon atoms of *n*-alkanes. Stations correspond to those indicated in Figure I.1. Station 3 is used as a model for the rest of stations (2, 4, 5 and 6), which showed similar profile.

carbon number predominance and an almost absent unresolved complex mixture of hydrocarbons (Tolosa et al. 1996).Station 5 is an exception, located inside the Ría de Vigo, possibly influenced by local hydrocarbon inputs. Confirmation of the presence of *Prestige* oil in the Cíes Islands samples was obtained by a detailed study of the fossil biomarkers, namely steranes and triterpanes, currently used for oil spill fingerprinting (Daling et al. 2002). The diagnostic molecular parameters (Fig. I.3, Table I.1) indicate a clear correspondence with those of the fuel oil for Station 1, whereas Station 5 (inside the estuary) exhibits a pattern clearly different from the others.

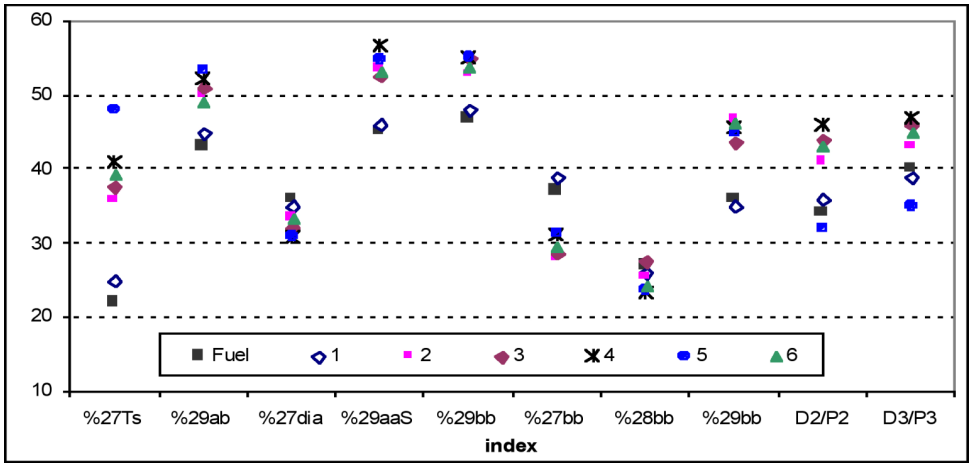


Fig. I.3. Source diagnostic ratios of aliphatic and aromatic hydrocarbons from the *Prestige* oil and the sediment stations shown in Figure I.1. Index definitions are indicated in Table I.1.

Table I.1. Diagnostic ratios used a source and weathering indicators for the *Prestige* oil samples in sediments.

Index	Definition	Structures
%27Ts	100*Ts/(Ts+Tm)	Ts: 18α(H)-22,29,30-trisnorneohopane Tm: 17α(H)-22,29,30-trisnorhopane
%29αβ	100*29αβ/(29αβ++30αβ)	29αβ: 17α(H),21β(H)-30-norhopane + 30αβ: 17α(H),21β(H)-hopane
%32αβS	100*32αβS/(32αβS+32αβR)	32αβ: 17α(H),21β(H)-bishomohopane (22S and 22R)
%27dia	100*27d(R+S)/[27d(R+S)+27ββ(R+S)]	27d: 13β(H),17α(H)-diacholestane (20S and 20R) 27ββ: 14β(H),17β(H)-cholestane (20R and 20S)
%29ααS	100*29ααS/(29ααS+29ααR)	29αα: 24-ethyl-14α(H),17α(H)-cholestane (20S and 20R)
%29ββ(R+S)	100*29ββ(R+S)/[29ββ(R+S)+ 29αα((R+S)]	29ββ: 24-ethyl-14β(H),17β(H)-cholestane (20R and 20S)
%27ββ	100*[27ββ((R+S)]/[27ββ(R+S)+ 28ββ(R+S)+ 29ββ(R+S)]	27ββ: 14β(H),17β(H)-cholestane (20R and 20S)
%28ββ	100*[28ββ(R+S)]/[27ββ(R+S)+ 28ββ(R+S)+ 29ββ(R+S)]	28ββ: 24-methyl-14β(H),17β(H)-cholestane (20R and 20S)
%29ββ	100*[29ββ(R+S)]/[27ββ(R+S)+ 28ββ(R+S)+ 29ββ(R+S)]	29ββ: 24-ethyl-14β(H),17β(H)-cholestane (20R and 20S)
D2/P2	100*D2/(D2+P2)	dimethyldibenzothiophenes (D2) and phenanthrenes (P2)
D3/P3	100*D3/(D3+P3)	trimethyldibenzothiophenes (D3) and phenanthrenes (P3)

Concurrently, the aromatic fraction of the Cíes sediments was dominated by the series of the petrogenic alkyl phenanthrenes and dibenzothiophenes (Table I.2) whereas the other stations exhibited distributions consistent with mixed petrogenic-pyrolytic sources, common in coastal sediments of urban/industrial areas and attributed to chronic runoff inputs. These are characterized by similar proportions of 2 to 4 aromatic ring alkylated components and

Table I.2. Average PAHs concentration (in mg/kg of sediment) of sediments from Cíes Islands and surrounding areas (stations as in Fig. I.1).

PAH*	Cíes Islands St.1	St. 2	St. 3	St. 4	St. 5	St. 6
N	1	1	1	-	20	2
N1	2	1	10	2	83	6
N2	5	3	24	5	125	17
N3	33	2	25	5	110	14
P	30	1	7	2	46	3
A	2	-	1	2	11	-
P1	237	6	10	3	152	14
P2	575	10	17	5	195	17
P3	460	12	12	6	232	15
D	31	-	6	5	24	1
D1	146	3	8	2	42	6
D2	320	7	14	4	86	12
D3	310	8	11	5	115	12
Fl	13	2	10	4	62	4
Py	81	2	7	3	49	3
BaA	11	1	3	1	21	2
C	19	2	4	2	37	3
C1	80	2	4	2	42	6
C2	75	3	4	2	45	12
C3	54	3	3	2	34	13
B(b+k)Fl	2	4	11	5	113	7
BePy	6	2	4	2	43	4
BaPy	4	3	3	1	41	5
Per	2	4	7	2	48	15
DBA	1	2	-	1	12	3
IPy	1	3	4	2	36	4
BPer	2	3	3	2	34	4
Total	2503	90	213	77	1858	204

* N: naphthalene, P: Phenanthrene, A: Anthracene, Fl: Fluoranthene, Py: Pyrene, D: Dibenzothiophene, BaA: Benz[*a*]anthracene, C: Chrysene, B(b+k)Fl: Benzo[*b+k*]fluoranthene, BePy Benzo[*e*]pyrene, BaPy: Benzo[*a*]pyrene, DBA: Dibenz[*ah*]anthracene, BPer: Benzo[*ghi*]perylene, IPy: Indeno[1,2,3-*cd*]pyrene. N1-N3, P1-P3, D1-D3 and C1-C3 are the methyl to trimethyl derivatives.

the series of parent 4- to 6-ring PAHs, encompassing the benzo[*b*]+[*k*]fluoranthenes and benzo[*a*]+[*b*]pyrenes (Broman et al. 1988, Tolosa et al. 1996).

The PAH concentrations reported in Table I.2 indicated moderate pollution in the subtidal samples of the Cíes Islands and low levels in other areas (77-213 mg/kg dry wt), on the order of those found in the continental shelf (Franco et al. 2006), with the exception of Station 5, which inside the Ría de Vigo had higher levels (1858 mg/kg dw). The ratios of C_2 and C_3 dibenzothiophenes and phenanthrene/anthracenes (D2/P2 and D3/P3), proposed for differentiating sources of spilled oils in sediments (Douglas et al. 1996), also support the presence of the *Prestige* oil in the Cíes sediment samples (Fig. I.3).

I.4.2 Enumeration of heterotrophic, alkane-degrading and polyaromatic-degrading microbial populations

At Station 1, the MPN results (Fig.I.4) suggest a bacterial community composed by a

moderately high most probable number of heterotrophs (10^5 MPN· $gram^{-1}$ of sediment) of which an almost 70 % (10^4-10^5 MPN · g^{-1}) belonged to the alkane degrading population. The aromatic degrading bacteria were also present although in a much lower proportion (around 10^3 MPN.g^{-1}).

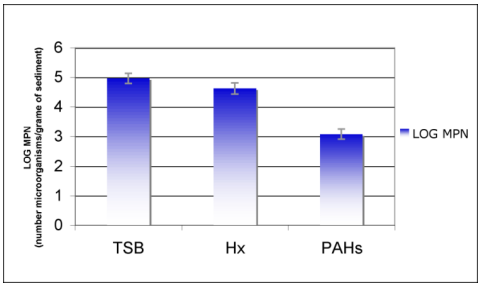


Fig. I.4. Measures of Most Probable Number (MPN) of bacteria per gram of sediment for each trophic community (TSB: total heterotrophic community; Hx: alkane degrading population; PAHs: aromatic degrading bacteria) as LOG of (nº microorg. / gr sediment). Standard deviations are represented by vertical lines at the top of the bars.

I.4.3 Culturable bacteria isolation

Ten-fold dilutions of the sediments were spread onto marine agar 1/5. More than 60 different bacteria were randomly isolated from the most diluted plates in order to include the most frequent culturable bacteria present in the polluted sediments. Only 51 isolated bacterial strains were able to maintain their viability throughout the whole experiment.

I.4.4 Bacterial identification and phylogenetic analysis

A total of 51 16S rRNA gene sequences were first compared with each other to detect identical sequences by using Clustal X. Of these, 37 were different (Table I.3) and were used to construct a whole alignment with sequences of known type species. After the first modifications in MacClade (cutting off left and right ends of the alignment), the number of characters in the alignment (approximately 1300) was reduced to only 737 after using GBlocks to make the alignment more suitable for phylogenetic analysis. Type strains from genera belonging to the phyla *Proteobacteria* and *Firmicutes* and members of the *Bacteroidetes* were used. The total alignment of 87 sequences (37 phylotypes plus type strain sequences) was used to estimate the taxonomic position of phylotypes by maximum likelihood, following the Tamura-Nei (TrN) (Tamura 1993) model recommended by ModelTest.

Phylogenetic analysis showed that most of our bacteria belonged to the *Gammaproteobacteria* (29 out of 37), followed by the *Alphaproteobacteria* (7 out of 37), *Bacteroidetes* (4 out of 37) and *Firmicutes* (4 out of 37). The highest richness was found in *Gammapro*teobacteria group with strains of the genera *Marinomonas, Shewanella, Vibrio, Psychrobacter, Alcanivorax* and *Pseudoalteromonas*. Among the *Alpharoteobacteria*, strains

Table I.3. Results from the Phylogenetic tree and BLASTN comparison (performed on November 2006) of our 51 sequences with those stored in the GeneBank. The table contains the names and accession numbers of the most similar species to each of the 51 studied isolates.

Phylogenetic Group	Closest Organism from GenBank database (accession no.) in Phylogenetic tree	Identities %	Isolate
Gammaproteobacteria (59%)			
Alteromonadaceae	Marine bacterium Tw-5 (AY028200)	99	P72
13.8 %	Marine bacterium Neptune (AY082669)	98-99	P77 = P86
	Alteromonadaceae bacterium R2 (AF539784)	98-99	P76 / P78´/ P82 / P120
Shewanellaceae	*Shewanella baltica* (AF173966)	99	P80 / P96 / P97 = P105
9.8 %	*Shewanella colwelliana* (AY653177)	100	P117
Pseudoalteromonadaceae	*Pseudoalteromonas* sp. YASM-7 (DQ173045)	99	P94
7.8 %	*Pseudoalteromonas* sp. SM9913 (AY305857)	99	P102
	Pseudoalteromonas sp. ARCTIC-P16(AY573035)	99	P108 =* P118
Vibrionaceae	*Vibrio* sp. V798 (DQ146994)	99	P90
5.8 %	*Vibrio* sp. Da4 (AF242272)	99	P121
	Bacterium CWISO12 (DQ334350)	99	P124
Oceanospirillales	*Marinomonas aquimarina* 11OM3 (AJ843079)	97	P111
5.8 %	*Marinomonas primoryensis* (AY771708)	97	P115
	Oceanospirillum multiglobuliferum (AB006764)	93	P95
Moraxellaceae	*Psychrobacter* sp. B-5161 (DQ399762)	99	P116
2%			
Alcanivoraceae	Gamma proteobacterium ST1 (AB005655)	99-100	P75 = P78Hx =P79n =
14%	(*Alcanivorax borkumensis*)		P83 = P84Hx = P99n = = P101n
Bacteroidetes (10%)			
	Dokdonia donghaensis DSW-21 (DQ003277)	99	P79
	Winogradskyella sp. BC1 (DQ356490)	98	P101
	Marine bacterium BBFL7 (AY028207)	99	P84 = P100
	Brumimicrobium mesophilum (DQ660382)	97	P99
Alphaproteobacteria (21%)			
Rhodobacteraceae	*Roseobacter* sp. 14III/A01/004 (AY576690)	100	P73
21%	*Rhodobacteraceae* bacterium 197 (AJ810843)	98	P87 = P92
	Octadecabacter orientus KOPR…(DQ167247)	97	P104
	Agrobacterium meteori (D88527)	99	P128
	Roseobacter gallaeciensis (AJ867255)	97	P74
	Roseobacter sp. 3008 (AM110967)	96	P78 = P81 = P85 = P88
	Roseobacter sp. JL-126 (AY745859)	96	P123
Firmicutes (10%)			
	Bacillus sp. NT N86 (AB167010)	99	P122
	Staphylococcus pasteuri CV5 (AJ717376)	100	P106
	Staphylococcus pasteuri ZA-b3 (AF532917)	99-100	P107 = P126
	Bacillus sp. 19493 (AJ315061)	95	P109

* '=' between two strains means that its sequences are identical. *P108 and P118 differ in 2 b.p.

similar to the genus *Roseobacter* and others of difficult adscription were detected. Four strains were similar to members of *Bacteroidetes* group, e.g. the genera *Brumimicrobium, Dokdonia*, *Winogradskyella* and *Cytophaga*, whilst the rest showed high similarity to *Firmicutes*, *e.g. Bacillus* and *Staphylococcus*.

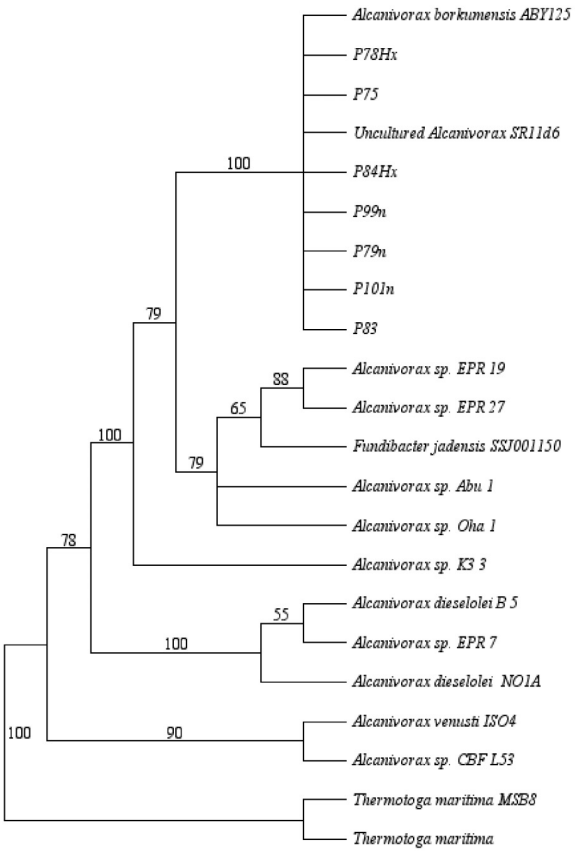


Fig. I.5. Maximum Parsimony phylogeny (based on complete 16S rDNA sequences) for the positive alkane degrading phylotypes. The tree were performed using PAUP* software following the Tamura-Nei+I+G model of nucleotide evolution and likelihood settings (Rates = gamma; Shape = 0.6768; Pinvar = 0.4386) recommended by Model Test. Bootstrap values on nodes (100 replicates) are only shown when are bigger than 50%. *Thermotoga* sp. were used to root the tree.

I.4.5 Screening of degrading capability and phylogenetic analysis.

Only 7 strains belonging to the same phylotype out of the 37 marine agar-culturable bacteria showed alkane-degrading activity, growing on hexadecane vapours and in liquid medium BMTM supplemented with hexadecane as the sole source of C and energy. Because of our interest in alkane-degraders, a specific phylogenetic analysis was carried out by means of parsimony criterion with bootstrap values. The phylogenetic tree (Fig. I.5) grouped the seven strains belonging to the same phylotype as *Alcanivorax borkumensis* with a boostrap value of 100. However, no PAHs-degrading bacteria could be detected among any of the 37 isolated strains.

I.5 DISCUSSION

The oiled sediment selected for isolation of bacteria in the current study presented a high degree of fresh hydrocarbon pollution characterized by fingerprint indexes of unweathered fuel from the '*Prestige'* spill and a high preservation of the *n*-alkane series (Fig. I.2), which may still have supported the bloom of obligate hydrocarbonoclastic bacteria at the time of sampling.

Petroleum pollution may stimulate the growth of hydrocarbon-degrading microorganisms that usually exist in low abundance in sediments, shifting the community structure of the affected sites. Previous studies have reported dominance of *Gammaproteobacteria* in bacterial communities from oil-affected marine habitats (Kasai et al. 2001, Roling et al. 2002, Roling et al. 2004, Hernandez-Raquet et al. 2006). However, the samples in these experiments had been recently oiled (Roling et al. 2002) or retrieved from places where natural attenuation proceeded slowly, such as the Japan Sea (Kasai et al. 2001).

However, communities from samples enriched with nutrients, or from places where high nutrient levels (0.8 mg l^{-1} of total nitrogen) naturally occur (Macnaughton et al. 1999), presented *Gammaproteobacteria*-dominated communities, which were rapidly substituted by *Alphaproteobacteria* due to higher rates of biodegradation. Disappearance of *Gammaproteobacteria* was linked to a lost of *Alcanivorax* dominance in the community, and this change in the community was faster when amended with nutrients (Roling et al. 2002, Roling et al. 2004). In most cases in which *Alcanivorax* spp. have been found to be dominant in oil-impacted environments, samples were analysed within days of the oil pollution or bioremediation event. In samples retrieved more than 8 weeks after the start of the experiments (Kasai et al. 2001, Maruyama et al. 2003), no such dominance was detected. In these cases, it is possible that the *Alcanivorax* spp. bloom had already ocurred (Macnaughton et al. 1999), or that the nutrient levels and alkane percentage of the spilled oil, critical for the growth of this genus, were too low (*Nakhodka*, Sea of Japan) (Kasai et al. 2001, Kasai et al. 2002b). All this is consistent with the observation that, after an initial rapid increase in population size, *Alcanivorax* spp. decline to much lower numbers within weeks (Syutsubo et al. 2001, Roling et al. 2002, Roling et al. 2004)

Polluted samples considered in the present study were retrieved 3 months after the last fuel input from the '*Prestige'*. Since low amount of alkanes were present in the '*Prestige'* oil and high biodegradation rates were expected in the Ría (Medina-Bellver et al. 2005), such a length of time would have already produced the *Alcanivorax* bloom long before sampling. Indeed, degradation of the fuel present in the rest of stations was quite advanced, compared to that at Station 1. Sea currents probably transported buried oil from other places to Station

1, supplying its sediments with unweathered oil (Fig. I.3) rich in alkanes (Fig. I.2), which enabled the growth and, thus, the isolation of *Alcanivorax* at such station.

Most of the isolated bacteria found in Cíes sediments were identified as members of the *Gammaproteobacteria* (59%) (Table I.3). Members of *Alteromonas*, *Shewanella, Pseudoalteromonas, Vibrio, Marinomonas, Psycrobacter and Alcanivorax* were identified. Although only the 7 strains (Table I.3) from the phylotype close to *Alcanivorax borkumensis* (Fig. I.5) were able to degrade alkanes, hydrocarbon-degrading strains have been previously described in all the isolated genera. For example, phenanthrene- and chrysene-degrading activities have been observed among members of the genera *Vibrio, Pseudoalteromonas* and *Marinomonas* (Melcher et al. 2002). *Shewanella* spp. able to grow on crude oil, tetradecane, naphthalene (Gentile et al. 2003) or catechol (Kato et al. 2001) have also been detected. Concerning the *Alphaproteobacteria* group, among the 4 isolated strains, P73 corresponded to *Roseobacter* sp. (100%) and strain P128 belonged to the genus *Agrobacterium* (99%) (Table I.3). Some species of these 2 genera have been described as polycyclic aromatic hydrocarbons degraders (Mahmood & Rao 1993, Widdel & Rabus 2001). Finally, 4 non-hydrocarbon-degrading strains, belonging to the *Bacteroidetes* and *Firmicutes* groups, were isolated. Only some species of the genus *Staphylococcus* has been previously described as naphthalene degraders (Zhuang et al. 2003).

The PAHs present in the '*Prestige'* fuel were mainly of high molecular weight (3 or more aromatic rings) leading to a low PAH bioavailability. The PAHs concentrations measured at the time of sampling were very low (around 2.5 $\mu g \cdot g^{-1}$ for Station 1; Table I.1). The small population (10^3 MPN g^{-1}) of PAH-degrading bacteria detected could be explained by this low concentration and bioavailability. The fact that no aromatic-degrading activity was detected among the isolated bacteria did not necessarily mean that they do not play a role in the biodegradation processes. Microbial communities are mainly naturally selected by its metabolic cooperation. Although several members of the microbial community could be secondary hydrocarbon degraders, their success could be related to co-metabolism, supply of growth factors for solubilization of substrates and/or elimination of toxic metabolites (Bouchez et al. 1995, Kanaly et al. 2002). In a previous study on a hydrocarbon-degrading co-culture, the importance of a non-degrading strain that eliminates an intermediate metabolite produced by the hydrocarbon-degrading strain was demonstrated (Casellas et al. 1998). Obviously, taking into account that the specific richness of the present study has been detected on diluted marine agar-culturable bacteria, other non-culturable species could fulfill critical roles in the global microbial community, as we recently described in a hydrocarbon degrading consortium (Vinas et al. 2005b). All but one of the isolated genera exhibited an absence of hydrocarbon degrading capabilities. The low amount of degrading

bacteria isolated was expected from the beginning, since no selective media was used to isolate degrading bacteria.

The Russian tanker '*Nakhodka'* released heavy oil, with a composition very similar to that of the '*Prestige'* spill, which covered more than 500 km of the Japanese coastline. The levels of N and P (0.1 mg l^{-1} and 0.01 mg l^{-1}, respectively) in the Sea of Japan are relatively small, which may have hindered *Alcanivorax* from dominating the microbial community. When N and P were added in adequate quantities to cultures of seawater with crude oil as the only source of C, *Alcanivorax* became dominant and the rate of biodegradation was strongly promoted (Kasai et al. 2001, Kasai et al. 2002b, Roling et al. 2002). However, this effect could only be shown in batch cultures and was not observed *in situ*, due to low levels of naturally occurring nutrients.

Since the oil from the '*Nakhodka'* had a similar composition to that from the *Prestige* (heavy fuel) and *Alcanivorax* phylotypes were isolated in high proportion without nutrient amendments in the Vigo estuary, it can be hypothesized that the environmental conditions along the Atlantic coast could be more suitable for biodegradation than those in the Sea of Japan (Total N~0,1 mg l^{-1}). In fact, ongoing natural bioremediation has already been observed to occur along Galician coasts (Medina-Bellver et al. 2005).

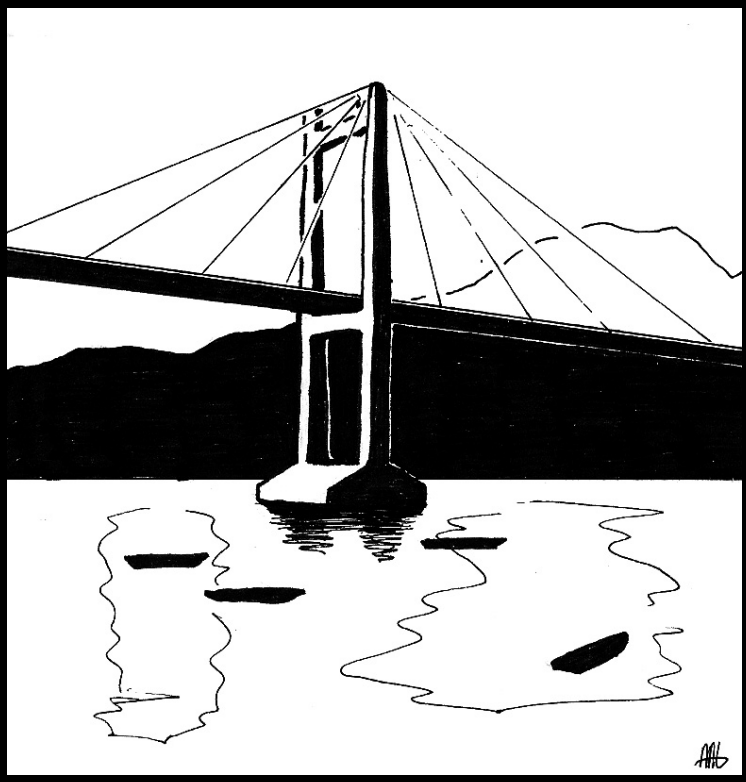
In the Ría de Vigo, in addition to the boundary conditions that usually determine the patterns of circulation in estuarine systems, an additional factor must be taken into account. The coastal upwelling, as a consequence of the wind regime over the adjacent shelf, induces the inflow of subsurface oceanic Eastern North Atlantic Central Water (ENAW) into the esturies of Galicia. This inflow has a major influence on their hydrography (Blanton et al. 1987, Prego & Fraga 1992, Alvarez-Salgado et al. 1993). During the upwelling and as a consequence of the circulation pattern, part of the biomass that is produced inside the estuary is transported offshore by the outgoing surface current. Part of this exported organic matter is remineralized either in the water column or on the bottom of the continental shelf (Fraga 1981), and therefore the incoming bottom current supplies the Galician estuaries not only with new nutrients but also with remineralized nutrients through a feedback mechanism (Figueiras et al. 1986, Alvarez-Salgado et al. 1993). This fertilizing process causes the Vigo estuary to be a highly productive ecosystem that processes a considerable amount of dissolved N and P (mean values around 0.6 and 0.06 mg l^{-1}, respectively, reaching even higher concentrations at certain times of the year) (Nogueira et al. 1997), which is 6 times higher than the total amount of N and P in the Sea of Japan. Furthermore, the sediments from Station 1 were sandy-gravel with a low organic matter content and thus well oxygenated (Vilas et al. 2005), enhancing the degradation capacity of the aerobic *Alcanivorax* (Yakimov et al. 1998).

Both the high proportion of alkane-degrading population (≈70% of the total MPN

bacteria) and the presence of *Alcanivorax* as the only *n*-alkane degrader could reflect an initial shift in the microbial community towards a new assemblage more adapted to hydrocarbon contamination, as previously observed in microcosm experiments (Roling et al. 2002). Since 14% of the isolates were classified as *Alcanivorax borkumensis*, even though this is a 'professional hydrocarbonoclastic bacteria', it could be concluded that a high proportion of this genus should be present in the sediments sampled to be able to compete and grow on the marine agar we used for isolation. The same method for isolation was used to count growing cells of *Alcanivorax* in oiled-seawater supplemented with fertilizers (Kasai et al. 2002b), and good correlation between colonies and direct cell counts by fluorescent *in situ* hybridization (FISH) (Syutsubo et al. 2001) was obtained. Different genotypes of *Alcanivorax* might be adapted to environments with different nutrient levels (Head et al. 2006), suggesting that the strain isolated in this study could be more adapted to competence under high nutrient levels. Although this hypothesis needs confirmation, such ability would be a good feature for bioremediation purposes.

Many previous studies have stated that *Alcanivorax* could play a critical role in the natural cleaning of oil-polluted marine systems, since the natural attenuation processes were accelerated when this species began to become dominant (Kasai et al. 2002b). In fact, our results are in agreement with the enrichment cultures made after the '*Nakhodka'* oil spill in the Sea of Japan (Kasai et al. 2002b, Maruyama et al. 2003), but, in our case, no enrichment was necessary. The presence of fresh fuel (preservation of the *n*-alkanes series, Fig. I.2 and I.3) and the high nutrient availability normally present in the Ria de Vigo supported the growth of *Alcanivorax,* allowing its presence in quantities sufficient to be isolated without enrichment, even when competing with other heterotrophic bacteria. Unfortunately, the present study did not yield data showing whether any acceleration is actually happening. However, the presence of *Alcanivorax* could be used as a bioindicator correlated with high rates of biodegradation. Indeed, the capacity of indigenous bacteria from the Galician coast to degrade the *Prestige* oil has been already confirmed (Medina-Bellver et al. 2005).

The isolation of *Alcanivorax* strains directly from oiled sediments is the first ecological evidence of the high natural abundance of this group of bacteria in the event of an oil-spill. Since the addition of fertilizers to the seawater would have no effect on the concentration of N and P, the use of bioremediation, understood as the artificial addition of nutrients, is not advisable. Hydrocarbon-degrading populations seem to grow quite well by themselves under the environmental conditions of the Ría de Vigo. However, at less exposed areas where the conditions are not so favorable the bioremediation amendments could be effective.



Chapter II:

Bacterioplankton associated to the upwelling ecosystem of “Ría de Vigo”. Stational variability and bioremediation potential.

Submitted to:

Environmental Microbiology

II.1 ABSTRACT

CARD-FISH and clone libraries indicated that *Roseobacter*, followed by *Bacteroidetes* and some gammaproteobacterial groups such as SAR86, dominated the composition of baterioplankton in Ría de Vigo in detriment of SAR11 (a major lineage almost absent in Ría de Vigo, which commonly dominates ocean waters). We hypothesize that high nutrient inputs, characteristic of this environment throughout the year, are the reason for such community structure.

Since we sampled 4 times during the year we could evaluate also the pronounced seasonal changes within each community component particularly for the *Roseobacter* lineage. We suggest that this seasonality in the coastal upwelling ecosystem of Ría de Vigo (NW-Spain) was associated to the characteristic phytoplankton communities of the four different hydrographical situations: winter mixing, spring bloom, summer stratification and autumn upwelling. These variable oceanographic conditions are the most likely reason for the pronounced seasonality observed of known and novel sequences found among all major marine bacterial lineages. The spring community was mainly dominated by two *Roseobacter* clades previously related with phytoplankton blooms. The rest of the seasons presented communities with higher diversity where we detected possibly-novel sequences related to *Roseobacter*, SAR11 and particularly SAR86.

The high diversity of *Roseobacter*, a genus previously related with hydrocarbon degradation and the detection of the 'professional hydrocarbonoclastic bacteria´ *Cycloclasticus* could reflect an intrinsic potential for oil biodegradiation in this ecosystem.

II.2 INTRODUCTION

Coastal upwelling systems are some of the more productive areas in the world oceans, contributing significantly to the export of primary production to the ocean interior. The Ría de Vigo, (Galicia, NW Spain), is located in the northern boundary of the NW Africa upwelling system, and therefore in an area where the along-shore winds interact with the coastal topography to generate upwelling–downwelling dynamics (cross-shore Ekman transport) on the continental shelf (Arístegui et al., 2006). The upwelling season in this ecosystem occurs on average from March to September while downwelling conditions predominate the rest of the year (Figueiras et al., 2002).

The coastal upwelling induces the inflow of subsurface oceanic Eastern North Atlantic Central Water (ENAW) into the estuaries of Galicia generating a circulation pattern where part of the biomass produced inside the estuary is transported offshore to be remineralized before coming back into the Rías (Alvarez-Salgado *et al.* 1993). This fertilizing process is responsible for the high productivity of the Ría de Vigo, reflected in the high shellfish production of this ecosystem (Figueiras et al., 2002).

Marine microbial communities play critical roles in carbon and nitrogen cycling through their influence on the formation and fate of dissolved organic matter (Azam et al., 1994) and many other processes. Despite its importance, little is known about the bacterioplankton neither in the coastal upwelling ecosystem of Ría de Vigo nor in ecosystems of similar characteristics. Previous studies showed that the number of heterotrophic culturable bacterioplankton (CFU) was strongly affected by upwelling events (Zdanowski and Figueiras, 1999), but the dynamics of the culturable bacteria might have little to do with those of the dominant bacteria.

Six bacterial clades account for 84% of the bacterioplankton 16S genes recovered from seawater around the world, as shown in a recent review paper (Fuhrman and Hagström, 2008). Among them, the SAR11 and *Roseobacter* clades from *Alphaproteobacteria,* and the *Gammaproteobacteria* SAR86, have been found to be abundant in coastal waters (González et al., 2000; Morris et al., 2002; Alonso-Sáez et al., 2007). Some of these groups are comprised almost completely of clone sequences of uncultured bacteria. In fact, SAR86 isolates have not yet been obtained, in spite of its abundance (Eilers et al., 2000). In contrast, *Roseobacter* is considered the most readily cultivated of these major marine lineages and recent studies have also revealed a high diversity of clusters in this and in the SAR11 lineages (Field et al., 1997; Buchan et al., 2005).

Few traits are representative of the entire clade, making *Roseobacter* a heterogeneous lineage formed hitherto by at least 17 genera, 36 described species, and literally hundreds of uncharacterized isolates and clone sequences. *Roseobacter* are often more abundant in

bacterial communities associated with marine algae, including natural phytoplankton blooms (González et al., 2000; Zubkov et al., 2002; Wagner-Dobler and Biebl, 2006). Following the thorough revision of Buchan et al. (Buchan et al., 2005), 13 major clusters were established and allowed to determine some linkages between characteristic habitats or ecological niches and the different clusters. For instance, the DC5-80-3 cluster is characteristic from oceanic waters, the OCT cluster is related to cold waters, the NAC11-7 cluster is commonly associated with algal blooms, etc. Although 16S rRNA gene sequence data alone are not a reliable predictor of ecological niche, a good classification of clone sequences can give us clues about the functional activities of clone clusters.

We provide a first insight into the bacterioplankton diversity in the Ría de Vigo using culture-independent molecular techniques. Samples were taken at 4 different oceanographic conditions characteristic of this ecosystem (Table II.1): spring bloom, summer stratification, autumn upwelling and winter mixing. Relatively extensive clone libraries, supported with CARD-FISH with common probes were used to describe the diversity and seasonal trends of abundance of the dominant marine bacterial groups.

Table II.1. Sampling site characteristics at each sampled season.

	Sampling characteristics			
	SPRING	SUMMER	AUTUMN	WINTER
Sampling date (mm/ dd/ yy)	03/ 02/ 05	07/ 02/ 05	09/ 20/ 05	01/ 27 /06
Water temperature (ºC)	10.5 ± 0.0	20.8 ± 0.0	15.4 ± 0.0	12.4 ± 0.0
Salinity	35.48 ± 0.02	35.02 ± 0.01	35.73 ± 0.00	35.60 ± 0.00
Dissolved inorganic nitrogen (DIN) in µM	4.40 ± 0.08	0.58 ± 0.11	5.66 ± 0.73	7.70 ± 0.44
Dissolved inorganic phosporus (DIP) in µM	0.52 ± 0.00	0.15 ± 0.01	0.51 ± 0.08	0.48 ± 0.02
Silicate (SiO_4) in µM	3.17 ± 0.04	0.59 ± 0.05	0.41 ± 0.02	3.72 ± 0.15
Particulate Organic Carbon (POC) in µM	16.1 ± 1.1	23.4 ± 0.3	33.9 ± 1.6	9.2 ± 0.6
Particulate organic nitrogen (PON) in µM	2.7 ± 0.2	3.4 ± 0.2	6.2 ± 0.4	1.3 ± 0.1
Prokaryotic abundance (PA) in ´10^5 cell ml^{-1}	7.2 ± 0.3	11.7 ± 0.5	17.4 ± 0.2	5.8 ± 0.3
Chlorophyll a concentration (mg m^{-3})	3.2 ± 0.2	1.9 ± 0.1	10.6 ± 0.7	0.5 ± 0.0

Since the Ría de Vigo is subject to continuous pollution from industrial and urban sewage we specifically looked for specialized hydrocarbonoclastic bacteria in the clone libraries and used a specific FISH probe (Maruyama et al., 2003) to detect genus *Cycloclasticus*, previously identified as a major player in the degradation of low molecular weight soluble aromatic hydrocarbons (LMW-PAHs) (Chung and King, 2001).

II.3 MATERIALS AND METHODS

II.3.1 Sampling

The Ría de Vigo is a coastal embayment that is driven completely by marine water mass circulation, and has minor freshwater influence. We sampled a position in the middle of the Ría (42º 13.9 'N; 8º 51.0' W) at the 4 most relevant periods of the seasonal plankton cycle in the coastal NE Iberian Atlantic waters: the spring bloom (in March 2005), summer stratification (July 2005), autumn upwelling (September 2005), and winter mixing (January 2006). The water was collected with large (1.5 m in diameter and 2 m deep) bags, which were filled from their bottom through a 200 μm mesh, in order to exclude mesozooplankton. Once filled, the bags were closed with a stopper and transported to shore to be then used as initial water of mesocosm experiments which are not reported here (Teira et al., 2007; Teira et al., 2008). The clone libraries were done with the initial water before addition of any supplement, but after a few hours of mesocosm fill-up. We lost the initial sample of March 2005, and used instead the control (no additions) sample of day 1. Since little changes were observed in bacterial abundance between times 0 and 1 (Lekunberri, 2008; Lekunberri et al., submitted), and since the DGGE analyses of the other three amplified samples revealed no visible changes between times 0 and day 1 (details not shown), we consider this sample to be also representative of the *in situ* community at this time of the year.

II.3.2 Collection of community DNA.

Microbial biomass was collected by sequentially filtering around 8 L of seawater through a 3 μm pore size Polycarbonate filter (Poretics) and a 0.2 μm Sterivex filter (Durapore, Millipore), using a peristaltic pump. The Sterivex units were filled with 1.8 mL of lysis buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M sucrose) and kept at -80ºC. Microbial biomass was treated with lysozyme, proteinase K and sodium dodecyl sulphate, and the nucleic acids were extracted with phenol and concentrated in a Centricon-100 (Millipore), as previously described (Massana et al., 1997).

II.3.3 Construction of 16S rRNA gene clone library

An almost complete 16S rRNA gene was PCR amplified using primers F27 and R1492 (Edwards et al., 1989; Lane, 1991). PCR reaction (25 μl) included 10mM TrisHCl [pH 8.3] ; 50mM KCl [pH 8.3]; 2.5 mM $MgCl_2$; 200 μM of each deoxynucleoside triphosphate; 1.25 U of AmpliTaqGold DNA polymerase (PE Applied Biosystems, Foster City, CA), 0.4 μM of each primer and 100 ng of the metagenomic DNA extracted from seawater samples. The reaction mixtures were subjected to a hot start (5 min at 95ºC) and after that to the following thermal cycling: (i) 5 min at 95°C; (ii) 40 cycles, with one cycle consisting of 30 seconds at 96°C for denaturation, 30 seconds at 54°C for annealing, and 1.5 min at 72°C for elongation; and (iii) a final extension step of 10 min at 72°C. This cycle were performed with a Perkin-Elmer GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA).

PCR products of approximately 1500 bp were cloned into pCR®2.1-TOPO® vector and transformed into competent *Escherichia coli* TOP 10F´ cells, following manufacturer's protocol of TOPO T/A Cloning Kit (Invitrogen).

PCR products of each clone using primers M13F and M13R were separated by electrophoresis in a 1% (w/v) agarose gel in 1×TAE buffer stained with ethidium bromide and photographed immediately under UV light using Gel Doc XR system and Quantity One software (Bio-Rad, Hercules, CA). A 100 bp ladder was used as a size marker (Promega).

One hundred different clones with the correct size insert, were sequenced in both directions using overlapping primers F341 and R907 (Edwards et al., 1989).

II.3.4 Clone library coverage estimations

Operational taxonomic units (OTUs) were

defined for the sequences under study to facilitate its classification. Those sequences with more than 97% (or 99%) identity were considered as belonging to the same OTU (Table II.2). Two estimates were used to calculate the coverage of our clone libraries: the Good and Chao et al.'s coverages. The proportion of ribotypes in a clone library of infinite size that would be represented in a smaller library can be estimated from a nonparametric calculation as the clone coverage defined by Good. Chao et al.'s coverage estimator is nonparametric and based on the abundance of the dominant OTUs in the community. The sample coverage C_{ACE} is estimated as the proportion of sequences in relatively rare OTUs that occur more than once in a library.

Moreover, the S_{Chao1} estimator is derived from the number of OTUs appearing either once or twice in a given library, and is therefore particularly suitable for data sets in which most OTUs are relatively rare in the library. The S_{Chao1} nonparametric estimator yields an unbiased estimate of the probable total number of OTUs present in the source assemblage (Lee and Chao, 1994). We performed these calculations to evaluate the coverage of our clone libraries (Table II.2). The diversity inside each major lineage of marine bacteria was estimated as the number of different OTUs per number of clones classified as members of such group (Table II.3):

$$H = \left(\frac{number\ of\ OTUs}{number\ of\ clones} \right) \times 100$$

II.3.5 Sequencing and Phylogenetic analyses

Partial 16S rRNA gene sequences using primers F341, R907 (Edwards et al., 1989) were obtained, edited and analysed as previously described (Alonso-Gutiérrez et al., 2008). Check Chimera program of the Ribosomal Database Project (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) were used to detect heteroduplex that were eliminated from analysis. After that, sequences were examined with the BLAST search alignment tool comparison software (BLASTN) to detect the closest bacterial sequences to each within the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Alignments with reference sequences obtained from GenBank were performed using ClustalW (http://align.genome.jp/), edited with MacClade 4.06 and directly transferred to version 4.0b10 of PAUP* software (Sinauer Associates Inc., Sunderland, MA). ModelTest software version 3.6 (Posada and Crandall, 1998) was run together with PAUP*, as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment. ModelTest examines maximum likelihood models ranging from simple to complex. The best-fit models of nucleotide evolution, calculated by ModelTest and PAUP*, were incorporated into software PHYML (Guidon and Gascuel, 2003), which uses a single, fast and accurate algorithm to estimate large phylogenies by Maximum Likelihood. Same alignments were also employed to generate phylogenies by Maximum Parsimony and Neighbour-Joining (NJ) using PAUP*. Finally, the trees created by PHYML were edited using the software FigTree v1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

To properly describe such complex communities, several phylogenetic analysis were performed in a hierarchical way. First a general phylogenetic tree (using reference sequences from all *Eubacteria*, details not shown) was performed to get a better view of the phylogenetic adscription of each clone. Alignments for preliminary classification of sequences containing numerous and too divergent taxa were modified by GBlocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). This computer program eliminates poorly aligned positions and divergent regions of the DNA alignment, which are convenient to delete prior to phylogenetic analysis. Secondly, those clones that clustered together in the general tree were separately analysed with the closest reference sequences described in previous studies such as those of the *Roseobacter* lineage (Buchan et al., 2005), SAR11 (Field et al., 1997) or SAR86 clades

(Suzuki et al., 2001; Sabehi et al., 2004). These phylogenies classify all clones in major groups already described, detect possible new clusters, and suggest seasonal trends of each group detected in the Ría de Vigo.

II.3.6 CARD-FISH

5 mL samples were fixed with 0.2-μm filtered paraformaldehyde (2% final conc.) and subsequently stored at 4ºC in the dark for 12-18 h. Thereafter, each sample was filtered through a 0.2 μm polycarbonate filter (Millipore, GTTP, 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP, 0.45 μm), washed twice with Milli-Q water, dried and stored in a microfuge vial at -20ºC until further processing in the laboratory.

The *in situ* abundance of different bacterial populations was determined using Catalysed Reported Deposition-Fluorescence *In Situ* Hybridisation (CARD-FISH) with oligonucleotide probes specific for the domain *Eubacteria* (EUB338) (Amann et al., 1990), *Alpha*- (ALF968) (Glockner et al., 1999), *Beta*- (Bet42a) (Manz et al., 1992) and *Gammaproteobacteria* (GAM42a) (Manz et al., 1992) subclasses, the *Bacteroidetes* group (CF319a) (Manz et al., 1996), the *Roseobacter* lineage (Ros537) (Eilers et al., 2001), the SAR11 cluster (SAR11-A1 and SAR11-441R) (Field et al., 1997; Morris et al., 2002), the SAR86 cluster (SAR86-1249) (Eilers et al., 2000), and the hydrocarbon-degrading *Cycloclasticus* bacteria (CYPU829) (Maruyama et al., 2003). The filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme (Pernthaler et al., 2002; Teira et al., 2004), were cut in sections and hybridized at 35 ºC with horseradish peroxidase (HRP)-labeled oligonucleotide probes during a minimum of 2-4 hours. Tyramide-Alexa488 was used for signal amplification (30-40 minutes) as previously described (Teira et al., 2004). We used 55% of formamide for all probes except for ALF968, SAR11A1 and SAR11-441R (45 % formamide). The hybridization for these 3 probes was done overnight (Alonso-Sáez et al., 2007). Cells were counter-stained with a DAPI-mix (5.5 parts of Citifluor [Citifluor, Ltd.], 1 part of Vectashield [Vector Laboratories, Inc.] and 0.5 parts of PBS with DAPI (final concentration 1 μg mL^{-1}).

The slides were examined with a Leica DMBL microscope equipped with a 100-W Hg-lamp and appropriate filter sets for DAPI and Alexa488. More than 800 DAPI-stained cells were counted per sample. For each microscope field, 2 different categories were enumerated: (i) total DAPI-stained cells, (ii) cells stained with the specific probe. Negative control counts (hybridization with HRP-Non338) averaged 0.5% and were always below 1.5% of DAPI-stained cells. The counting error, expressed as the percentage of standard error between replicates, was < 2% for DAPI counts and < 9% for FISH counts.

EUB338 probe detected 81±5 % of total DAPI-stained cells, and the average number of membrane nonpermeable cells determined with physiological probes in the samples was 82%, thus indicating a probable low representation of *Archaea* in the Ría.

II.3.7 Nucleotide sequence accession numbers

The nucleotide sequences identified in this study have been deposited in the GenBank database under accession numbers EU600492-EU600587 for spring library clones (locus 1_XX); EU600588-EU600681 for summer library clones (locus 2_XX); EU600397-EU600491 for autumn library clones (locus 3_XX); EU600682-EU600775 for winter library clones (locus 4_XX).

II.4 RESULTS

II.4.1 Clone library coverage estimations

At least 95 clones were analysed in each of the libraries corresponding to the four different hydrographical situations. Sequences of different length, ranging from 550 to more than 800 bp, were obtained after eliminating low quality peaks and joining the sequences obtained with each primer. Some of them included 16S rRNA genes from phytoplankton plastids which were excluded from further work (Table II.2). We used a minimum of 97% and 99% similarity threshold to group sequences in the same OTU (Table II.2). The Good and Lee-Chao (C_{ACE}) estimators indicated that our clone libraries covered on average 80% and 66%, respectively, of the total bacterioplankton richness present in Ría de Vigo for a 97% threshold (Table II.2). $S_{Chao1,}$ as expected, presented higher values for a similarity threshold of 99%.

Table II.2. Clone library characteristics

	Values for OTU at >97% / >99% similarity threshold			
	SPRING	SUMMER	AUTUMN	WINTER
Number of clones sequenced	96	96	96	96
Number of clones discarded as Chimeras	0	2	1	2
Number of clones discarded as Plastids	2	1	2	5
Number of clones analyzed	94	93	93	89
Observed OTU richness	27 / 40	36 / 54	39 / 54	38 / 49
S_{Chao1} estimates	49 / 81	54 / 125	47 / 92	55 / 82
Good´s coverage estimate (%)	81 / 72	77 / 57	83 / 65	79 / 66
C_{ACE} coverage estimate (%)	45 / 54	66 / 46	80 / 65	74 / 58

II.4.2 The *Roseobacter* lineage

FISH results showed that this group was the dominant at the four sampling times. The highest abundance occurred in spring and then reached a minimum in autumn and winter (Fig. II.1). The FISH abundances were pretty similar to clone frequencies in the libraries, where most clones were related to *Roseobacter* (30-70 %, Table II.3).

Table II.3. Relative abundance (CARD-FISH, F% ± standard error, in blue), and clone library characteristics (Frequency (C%), number of OTUs, and Diversity (H)) observed for each major group of marine bacteria found at each season. F% data for SAR11 are the average of the results obtained with the two different probes used (A1 and 441R; Fig. II.1)

	SPRING				SUMMER				AUTUMN				WINTER			
	FISH	CLONE LIBRARY			FISH	CLONE LIBRARY			FISH	CLONE LIBRARY			FISH	CLONE LIBRARY		
Major groups	F%	C%	OTUs	H	F%	C%	OTUs	H	F%	C%	OTUs	H	F%	C%	OTUs	H
Alphaproteobacteria	28±1	86	17	40	25±1	73	19	53	18±2	53	12	51	15±3	52	17	61
Roseobacter	52±7	70	11	23	43±5	52	14	42	28±5	30	8	46	28±8	29	10	54
SAR11	5±0.5	2	3	-	0.5±0	0	2	n.a.	2±0.5	12	2	55	1±0.3	11	4	60
Gammaproteobacteria	7±2	6	4	67	11±1	13	9	75	6±1	15	5	71	16±5	27	9	71
SAR86	5±0.5	0	0	n.a.	15±3	9	2	75	20±2	11	2	70	5±1	1	1	-
Cycloclasticus	0±0	0	0	n.a.	0±0	0	0	n.a.	0.1±0	0	0	n.a.	1±0.5	3	1	-
Bacteroidetes	14±2	5	4	80	27±3	11	5	80	23±3	23	16	86	29±6	6	4	100
Betaproteobacteria	3±1	2	1	-	0.2±0	0	0	n.a.	3±1	0	0	n.a.	1±0.2	2	1	-
Verrucomicrobia	n.a.	0	0	n.a.	n.a.	3	3	-	n.a.	2	1	n.a.	n.a.	0	0	n.a.
Actinobacteria	n.a.	0	0	n.a.	n.a.	0	0	n.a.	n.a.	1	1	n.a.	n.a.	0	0	n.a.
Spirochaetes	n.a.	0	0	n.a.	n.a.	0	0	n.a.	n.a.	0	0	n.a.	n.a.	1	1	-
Unclassified bacteria	n.a.	1	1	-	n.a.	0		n.a.	n.a.	6	4	65	n.a.	12	6	73

Diversity indices were measured only when the percentages of clones were ≥5%.
n.a: not measured or not applicable.

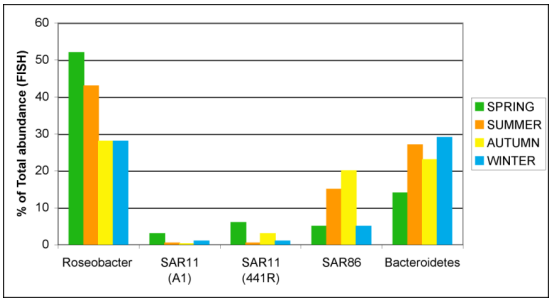


Fig. II.1. Relative abundances (using CARD-FISH) of the most interesting groups of bacteria detected in the Ría de Vigo at the different times of the year. Oligonucleotide probes represented cover part of the *Bacteroidetes* group (CF319a) (Manz et al., 1996), the *Roseobacter* lineage (Ros537) (Eilers et al., 2001), the SAR11 cluster (SAR11-A1 and SAR11-441R) (Field et al., 1997; Morris et al., 2002) and the SAR86 cluster (SAR86-1249) (Eilers et al., 2000). In the case of the SAR11 clade of *Alphaproteobacteria,* two different probes were used (A1 (Field et al., 1997) and 441R (Morris et al., 2002)). The CARD-FISH results for other groups of bacteria with lower representation in the community (e.g. *Betaproteobacteria* (Bet42a) (Manz et al., 1992) and *Cycloclasticus* (CYPU829) (Maruyama et al., 2003)) are presented in Table 3. Percentages from FISH of each group are referred to the total counts of *Eubacteria* positive cells (with probe EUB338 (Amann et al., 1990)).

Phylogenetic analysis of the *Roseobacter*-related sequences, suggested the presence of already described clusters (Fig. II.2, black characters) but also of new clusters, composed exclusively by sequences derived from this study (Fig. II.2 grey, red characters). Only the VIGO-ROS-A cluster contained three subclades with bootstrap values high enough to be proposed as a potential new cluster inside *Roseobacter* (red colour in Fig. II.2). The rest of them had bootstrap values lower than 60 or were classified between different lineages preventing its definition as new *Roseobacter* clades (grey colour in Fig. II.2).

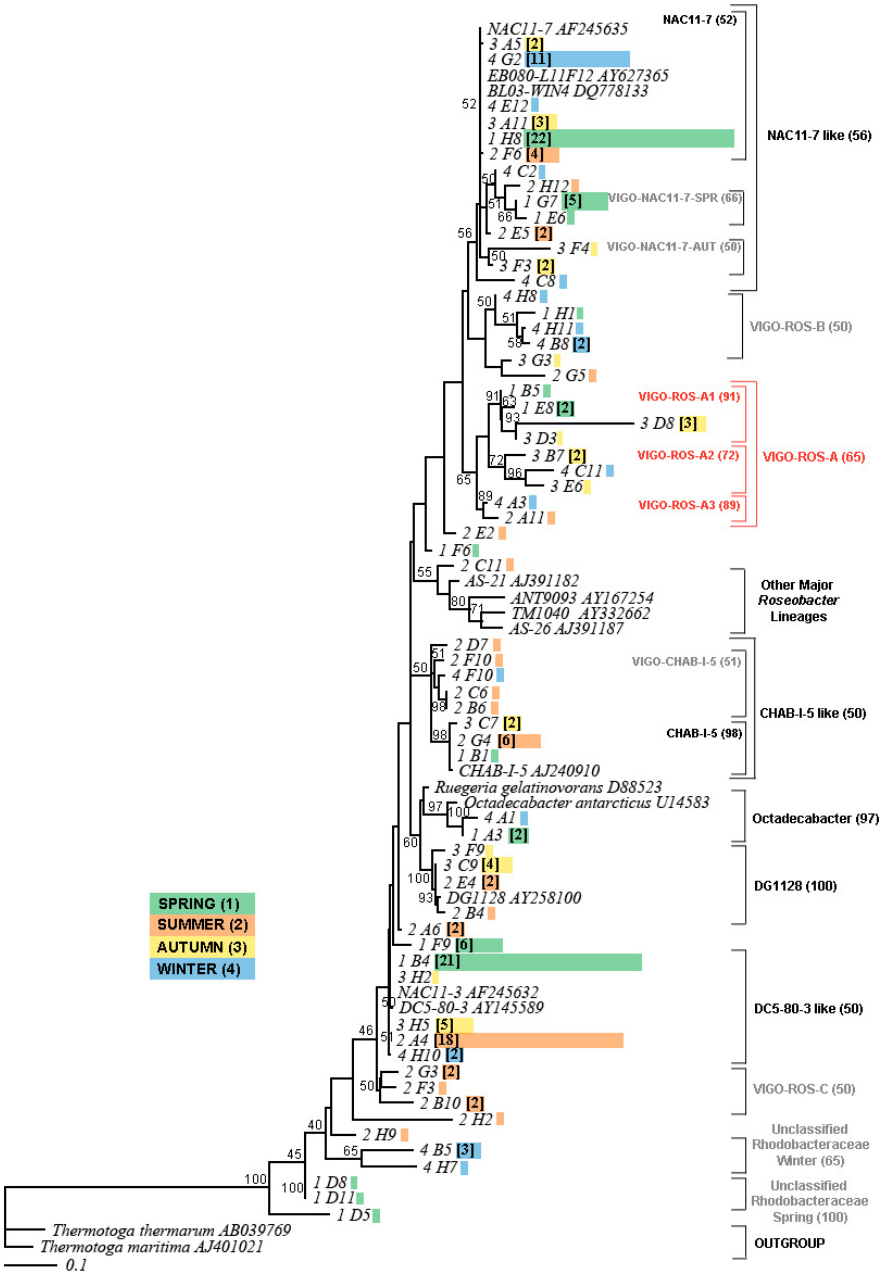


Fig. II.2. Maximum likelihood phylogenetic tree of the clones related with major *Roseobacter* clades as described by Buchan et. al., (Buchan et al., 2005). Clones belonging to already described clades are named in black characters, while clades composed exclusively by sequences derived from this study are in grey or red characters. Phylogenies are based on alignments of 650 bp. Parameters for searching trees in Phyml were selected by ModelTest and PAUP using the Akaike… (continue next page)

Fig. II.2 (cont.) …information criterion (AIC) (Model of Nucleotide evolution: GTR+I+G; Pinvar: 0.2630). Bootstrap values of ≥ 50% are shown at branch nodes (1000 iterations). Values in square brackets to the right of some clones represent the number of identical sequences in the same library (same season). Bars of different colour and length represent respectively the origin and relative frequency of each clone sequence as shown in the legend. *Thermotoga* spp.were used as outgroup.

Phylogenetic analysis also suggested some seasonal tendencies for the different clusters observed. For example, two different 16S rRNA gene sequences close to *Roseobacter*, with the highest frequencies among all, were mainly detected at two specific seasons. One of them, closely (99-100%) related to the uncultured *Roseobacter* strain DC5-80-3 (AY145589), was detected in 21 and 18 clones of the spring and summer libraries respectively (represented by clones 1B4 and 2A4 in Fig. II.2). The other sequence, 99-100% identical to NAC11-7 (AF245635), was represented by 22 clones in the spring library and 11 in the winter one (see clones 1H8 and 4G2 in Fig. II.2). The DG1128 clade appeared in summer and autumn not being detected the rest of the year. Although present in all libraries, CHAB-I-5 was mainly detected in summer. Three clusters: VIGO-ROS-C and two unclassified *Rhodobacteraceae*, only distantly related with the DC5-80-3 clade, were characteristic of summer, winter and spring season respectively (grey colour in Fig. II.2). Other subclades with lower bootstrap values also seemed to appear at specific seasons such as VIGO-NAC11-7-SPR, /-AUT and VIGO-CHAB-I-5, that were mainly detected in spring, autumn and winter respectively (grey colour in Fig. II.2). Other *Roseobacter* groups, which do not appear in Fig. II.2, formed well-defined clusters in a broader phylogenetic tree of *Rhodobacteraceae* (not shown). Among them, clones close to *Loktanella* were mainly detected in winter but appeared throughout the year while those close to *Jannaschia* were detected only in summer. Sequences classified as AS21-cluster appeared in all libraries except spring. Other clades, such as *Ruegeria-Silicibacter*, TM1040, *Roseivivax-Salipiger*, ANT9093, *Sulfitobacter-Staleya-Oceanibulbus*, *Citricella-Leisingera*, *Roseovarius* or *Rhodobacter* did not show up in our libraries.

II.4.3 SAR11

Both FISH and clone libraries revealed a low detection of this group in the Ría during the whole year. FISH results obtained with two different FISH probes, showed a constantly low population (around 3% on average, Fig. II.1). However, SAR11 clone sequences were mainly detected in autumn and winter (about 10-12 % of the library, Table II.3) with low and no representation in the spring and summer libraries, which was exactly opposite to what was found for the most abundant *Roseobacter* clones (NAC11-7 and DC5-80-3) (Fig. II.2). A phylogenetic tree was constructed to classify our SAR11 clone sequences using the

reference clusters already described by Field et al. (Field et al., 1997) (Fig. II.3).

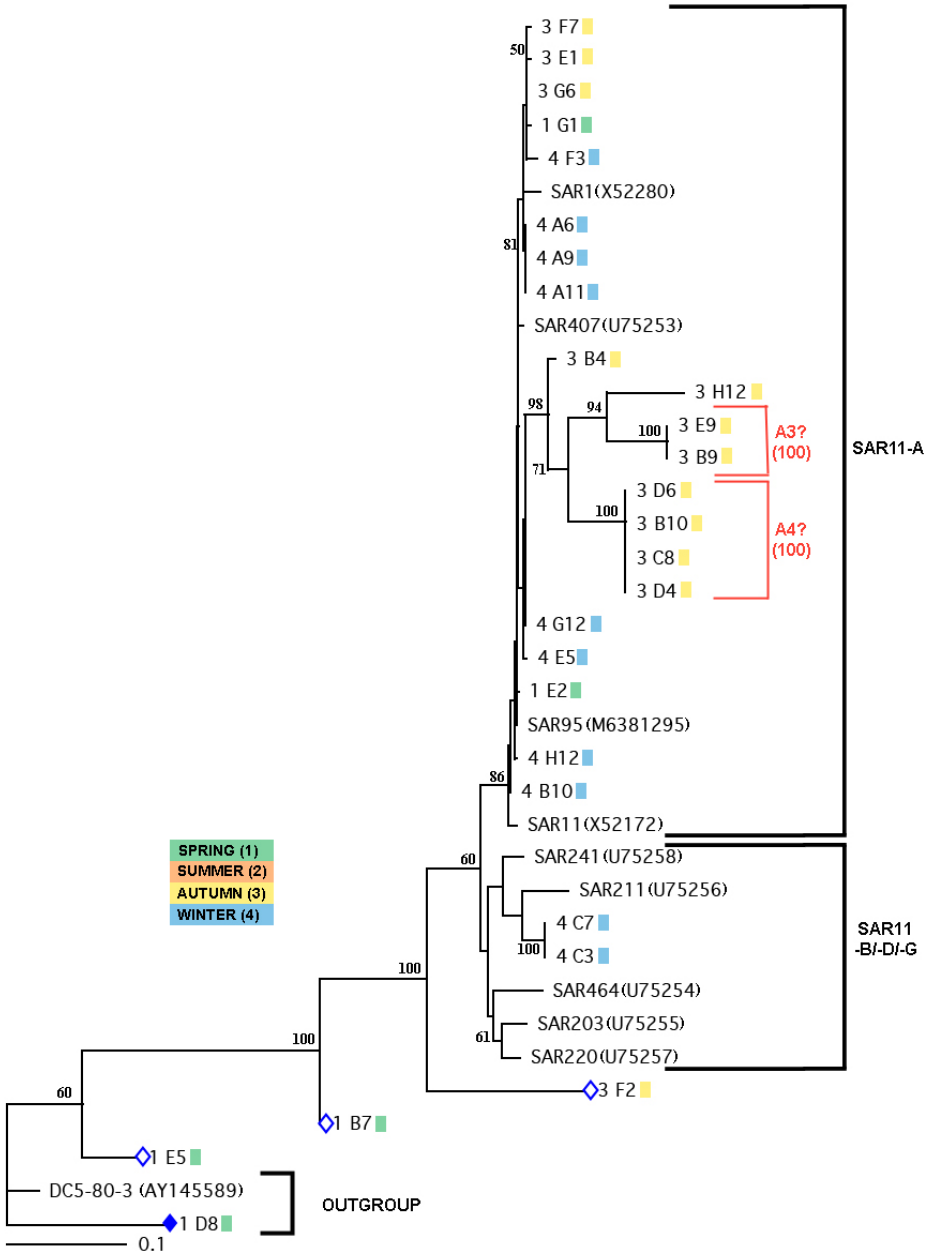


Fig. II.3. Maximum likelihood phylogenetic tree of the clones related with major SAR11 clades as described by Field et.al. (Field et al., 1997). Clones belonging to already described clades are named in black characters, while clades composed exclusively by sequences derived from this study are in grey or red characters. Phylogenies are based on alignments of 730 bp. Parameters… (continue next page)

Fig. II.3 (cont.) …for searching trees in Phyml were selected by ModelTest and PAUP using the Hierarchical Likelihood Ratio Test (hLRTs) (Model of Nucleotide evolution: GTR+I+G; Pinvar: 0.4519). Bootstrap values of ≥ 50% are shown at branch nodes (1000 iterations). Bars of different colour and length represent respectively the origin and relative frequency of each clone sequence as shown in the legend. Unfilled diamonds indicate sequences of difficult adscription between clades of *Roseobacter* and SAR11. Clade DC5-80-3 and clone D8 of *Roseobacter* (Fig.3) were used as outgroup.

Approximately half of these autumn and winter clones were represented by SAR1 (X52280)-like sequences (A1-subcluster containing SAR11, Fig. II.3). The rest of the autumn clones formed several possibly new subclusters within cluster A with a high bootstrap support (Fig. II.3, clade A3? and A4? in red). The other half of the winter clones, although belonged to the same subcluster A, were closer to SAR95 (M6381295) and SAR11 (X52172). The other large SAR11 clusters (-D/-B/-G) were only represented by two winter clones that did not cluster with any of the reference sequences used. Other sequences of difficult adscription (marked with diamonds) were left as unclassified *Alphaproteobacteria,* since they appeared between the SAR11 cluster and the *Rhodobacteraceae* family (Fig. II.3).

II.4.4 *Gamma- and Alphaproteobacteria*

Gammaproteobacteria was composed by diverse groups (*Alteromonas* spp., *Glaciecola* spp., *Saccharophagus* spp., *Oleispira* spp. and even *Cycloclasticus* spp.) and sequences from *Alphaproteobacteria* were mainly composed by *Roseobacter* and SAR11 related clones, as shown in Table II.3. However, FISH results for both general groups were likely biased by the relatively low coverage of the general phyla probes used since the amount of total *Gammaproteobacteria* and *Alphaproteobacteria* detected was less than the number of SAR86 and *Roseobacter* alone, respectively.

II.4.5 SAR86

Both FISH and clone libraries showed the same seasonal trend for this group of *Gammaproteobacteria*. Relative abundances of ca. 20% were detected by FISH in summer and autumn, while the group was almost absent from winter and spring communities. The highest abundance and diversity of this group was found in the autumn library, where at least one novel clade of SAR86 seemed to exist (Fig. II.4). A high bootstrap value of 98 and its position between clades SAR86 I, II and III, support the description of a new cluster inside SAR86 named as SAR86_IV? (Fig. II.4, red colour).

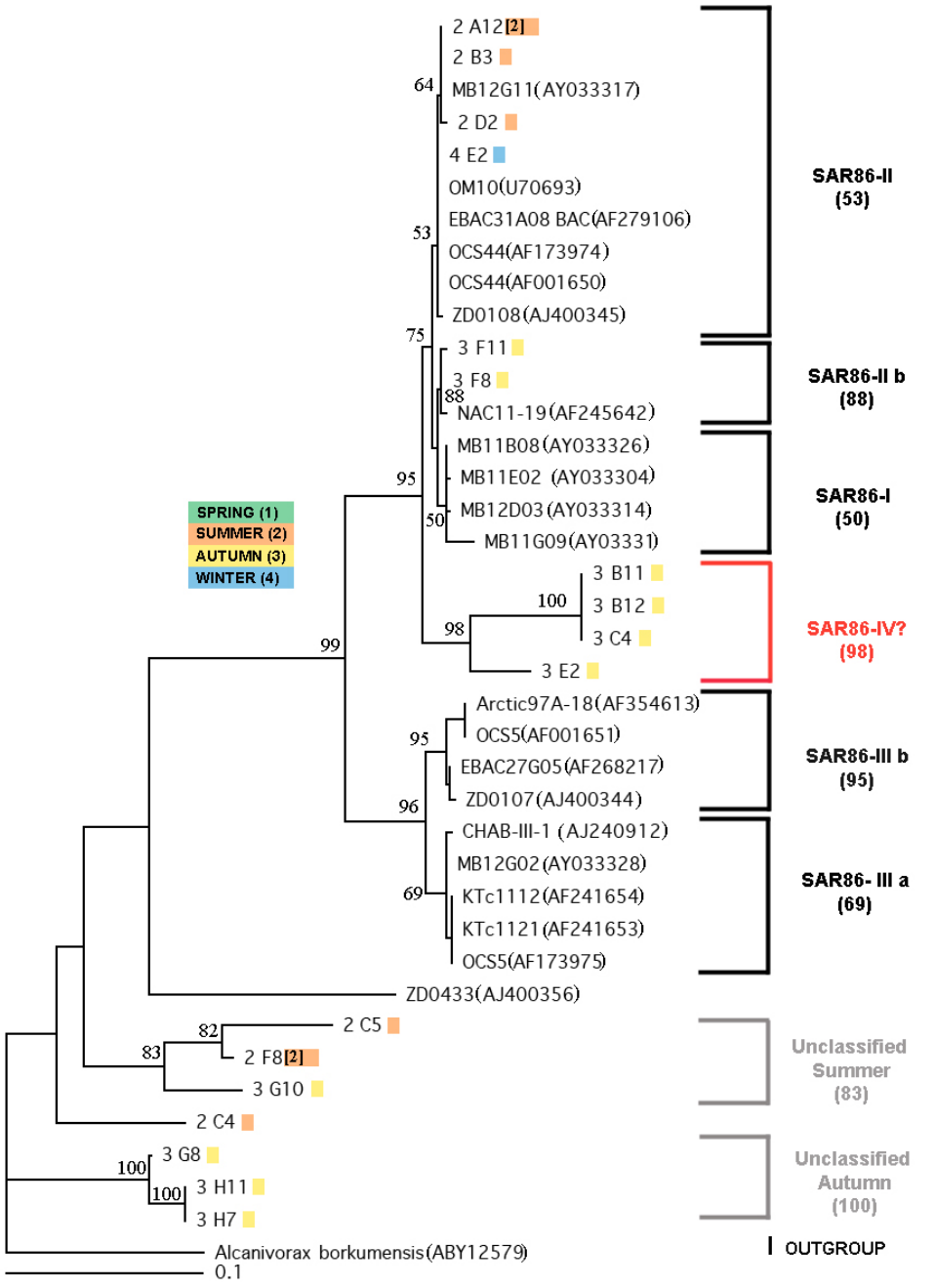


Fig. II.4. Maximum likelihood phylogenetic tree of the clones related with major SAR86 clades as previously proposed (Suzuki et al., 2001; Sabehi et al., 2004). Clones belonging to already described clades are named in black characters, while clades composed exclusively by sequences derived from this study are in grey or red characters. Phylogenies are based on alignments of… (continue next page)

Fig. II.4 (cont.) …600 bp. Parameters for searching trees in Phyml were selected by ModelTest and PAUP using the Akaike information criterion (AIC) (Model of Nucleotide evolution: GTR+I+G; Pinvar: 0.4683). Bootstrap values of ≥ 50% are shown at branch nodes (1000 iterations). Values in square brackets to the right of some clones represent the number of identical sequences in the same library (same season). Bars of different colour and length represent respectively the origin and relative frequency of each clone sequence as shown in the legend. The *Gammaproteobacteria, Alcanivorax borkumensis* (Y12579) was used as outgroup.

II.4.6 *Bacteroidetes*

FISH results showed a relatively constant, and high, contribution to the bacterial assemblage throughout the year (20±5% of DAPI counts) with the exception of spring, when it seemed to be outcompeted by the presence of *Roseobacter*. The libraries' results showed that the group was the most diverse with almost all clones being represented only once in each library (Table II.3). Sequences close to genus *Flavobacteria*, *Bacteroidetes*, *Polaribacter*, *Gelidibacter*, *Flexibacter*, *Cytophagales* and *Winogradskyella* were detected in Ría de Vigo. However, no interesting seasonal trends were observed except that *Winogradskyella* and *Polaribacter* could only be detected in autumn when the highest abundance of *Bacteroidetes* was observed.

II.4.7 *Betaproteobacteria*

Both FISH and clone library data indicated low relative populations, of around 2±1% throughout the year. The sequences detected in winter and spring were related with members of the *Methylophilaceae* family. However, the sequences from each season were quite different between them and those of spring, although close to members of this family, were likely to constitute a novel group inside the *Betaproteobacteria* (tree not shown).

II.4.8 *Verrucomicrobia and Actinobacteria*

Verrucomicrobia-related sequences were detected in summer and autumn (3±1 % of each clone library) but not the in rest of the year, while *Actinobacteria* were detected only during autumn (1% of the clone library). Sequences from both groups were close to uncultured species of undefined affiliation.

II.4.9 *Cycloclasticus*

Specific probes and clone library results showed exactly the same trend for this PAHs-degrading group in the microbial community (Table II.3). It appeared in winter while the rest of the year remained in almost undetectable numbers. All sequences from winter were identical and close (99-100%) to the species *Cycloclasticus spirillensus* (DQ659429).

II.5 DISCUSSION

II.5.1 Methodological considerations

Although clone library frequencies did not match with FISH percentages, results from FISH and libraries showed, in general, similar trends (Table II.3). Unspecificity and poor-coverage of FISH probes and cloning or PCR biases, such as the amplification positive bias for low G+C sequences, could be some of the reasons for such differences (von Wintzingerode et al., 1997). In general, results from FISH were considered as a quantitative tool in this study, while clone sequences were used to describe diversity.

The *Roseobacter*, SAR11 and *Cycloclasticus* probes used perfectly matched with all clone sequences classified as members of these groups (Table II.3). However, other probes like SAR86 presented serious discrepancies with sequences of this group detected in the Ría as discussed later. The worst case was found for the *Gamma* and *Alphaproteobacteria* groups, which were drastically underestimated by FISH counting (Table II.3). In these two cases, there was a considerable inconsistency between counts with the general probes (ALF968 and GAM42a) and the more specific probes (ROS537 and SAR86/1249). Amann and Fuchs recently analyzed the validity of these old group-specific probes, designed more than a decade ago (Amann and Fuchs, 2008). They found that the group coverage (percentage of group members that are identified relative to the total number of members in the group) for ALF968 and GAM42a probes is 81 and 76% respectively. Therefore if some non-detected members are particularly abundant in a sample, the underestimation using the general probe can be considerable as seemed to occur in the present study. Using the current rRNA database (SILVA) we found that 9% of the sequences (mainly from uncultured *Rhodobacteraceae*) detected with the ROS537 probe are not detectable with the ALF968 probe. *In silico* alignments of the ALF968 probe sequence showed perfect matched with most of the clones detected; however 20 out of the 80 different sequences classified as *Rhodobacteraceae* in this study did not match with the ALF968 probe. It is very likely that some of these non-detected members were particularly abundant in the Ría de Vigo, explaining the big discrepancy in FISH counts. In environments were SAR11 dominates over *Roseobacter*, such as Blanes Bay (Mediterranean Sea), FISH counts match fairly well the library data (Alonso-Sáez et al., 2007). The same occurred when checking *Gammaproteobacteria* probes: the vast majority (98 %) of sequences detected with SAR86/1249 were not detected with the GAM42a probe. This analysis demonstrates that the old general probes must be used with caution and point out the urgent need to refine group-specific probes using high-quality rRNA databases (Amann and Fuchs, 2008).

II.5.2 Bacterial diversity in the Ría de Vigo

Changes in bacterioplankton community structure and diversity were found throughout the year in Ría de Vigo (Table II.3), including the detection of new members in all major groups of bacteria.

Both, culture-independent (i.e., 16S rRNA-based) and culture-dependent studies have indicated that members of the *Alphaproteobacteria* belonging to the *Roseobacter* lineage are abundant in coastal (González and Morán, 1997; González et al., 1999) and open ocean environments where they are often found in association with phytoplankton (Prokic et al., 1998; González et al., 2000) and specially with dimethylsulphoniopropionate-producing algal bloom, probably playing a relevant role in the cycling of organic sulfur compounds (González et al., 2000).

In Ría de Vigo, the hydrographic conditions are highly variable throughout the year. During the upwelling season (from March to October) dissolved organic matter (DOM) accumulation occurs several days after each upwelling pulse, when phytoplankton growth is limited by inorganic nutrient depletion (Doval et al., 1997; Alvarez-Salgado et al., 1999). The *Roseobacter* lineage clearly dominated the bacterioplankton in all seasons having its higher contribution to community structure in spring, when the phytoplankton community was dominated by actively growing chain-forming diatoms (Teira et al., 2007). By contrast, despite the high chlorophyll-a concentration measured in autumn (Table II.1), the abundance of *Roseobacter* was lower than in spring. The high phytoplankton density found in September was actually related to the decay of a diatom bloom (the chlorophyll-a concentration dropped from 10.6 to <3 mg m^{-3} in less than 48 h) (Teira et al., 2007). The phytoplankton community dominated by flagellates in winter (M. Varela, pers. com.), due to high inorganic nutrient levels and low DOM concentrations (Doval et al., 1997; Alvarez-Salgado et al., 1999), might be the reason for the relative lower dominance of *Roseobacter* also at this time of the year.

The marked difference between seasons in the detection of the two major clades of *Roseobacter* (NAC11-7 and DC5-80-3) could likely reflect a seasonal trend. Both seemed to overlap in the spring phytoplankton bloom when intermediate oceanographic characteristics exist (moderate temperatures and high inorganic nutrient inputs) (Table II.1, Fig. II.2). The different clades could use different strategies, or have different environmental preferences, including association with different phytoplankton species (Wagner-Dobler and Biebl, 2006; West et al., 2008). In the summer, autumn and winter periods, when NAC11-7 and DC5-80-3 were relatively less dominant, the rest of *Roseobacter* clades (DG1128, CHAB-I-5, AS-21, etc.) and other lineages (SAR11, SAR86, etc.) were detected with higher frequency compared to the spring library (Fig. II.2, II.3 and II.4). To know whether this increase is the reflection of a relaxed competence with the two major groups of *Roseobacter* or simply due

to the higher probability of detecting rare clones when the dominants are not that abundant, more specific FISH probes should be designed to study the ecophysiology and follow seasonal trends of this heterogeneous group (Selje et al., 2004).

In contrast to previous results, which indicated that SAR11 clade members dominate surface bacterioplankton communities (Morris et al., 2002), in the Ría de Vigo they seem to be outcompeted by other groups, and present very low abundances throughout the year. In summer, SAR11 was not even detected by CARD-FISH (Table II.3) using SAR11-A1 probe (Field *et al.* 1997), which mainly targets surface members of this clade. This observation is consistent with a deep-water influence on the bacterial assemblage due to summer upwelling (Alvarez-Salgado *et al.* 1993). The low abundance of SAR11 in some coastal embayments was first reported in the estuarine environment of Ría de Aveiro (Portugal) (Henriques et al., 2004), which is located in the same coastline as Ría de Vigo (around 200 km from Vigo), being also impacted by the NW Africa upwelling system. These authors speculated with the possibility of technique-related bias, but with the clone libraries and CARD-FISH results obtained in our study it can be safely concluded that low SAR11 abundances are a characteristic feature of eutrophic bay environments from the Atlantic coast of the Iberian Peninsula. This feature had been previously observed in the Baltic Sea (Riemann et al., 2008) and in the low salinity part of Eastern North America estuaries (Kirchman et al., 2005). However, the low SAR11 contributions in the case of Ría de Vigo, which is a special coastal upwelling system different from an estuary, cannot be related to low salinity levels (Table II.1).

A seasonal abundance pattern of SAR11 opposite to *Roseobacter* in oligotrophic coastal waters has already been observed (Alonso-Sáez et al., 2007). In that study, *Roseobacter* were less abundant than SAR11 throughout the seasonal cycle and thrived only during chlorophyll a-rich periods (winter-spring). In the present study we observed a similar pattern but with the difference that SAR11 was always less abundant than *Roseobacter*. Recent studies have revealed a higher metabolic activity of *Roseobacter* in competing with SAR11 for nutrients under eutrophic conditions (Alonso and Pernthaler, 2006). The Vigo embayment, characterized by a high production and phytoplankton blooms following upwelling events throughout the year, provides a rich environment where *Roseobacter* members probably develop in detriment of SAR11 which are more adapted to the oligotrophic conditions characteristic of the open waters (Rappé et al., 2002). For example, *Roseobacter* has been proposed as the main mediator of the flux of dimethyl sulfonopropionate (DMSP) in coastal waters or during blooms (González et al., 2000), whereas in open ocean environments DMSP is mainly consumed by SAR11 (Malmstrom et al., 2004).

Gammaproteobacteria were present in low numbers as shown by FISH and clone libraries. However, interesting sequences of this class, belonging to the SAR86 cluster and to the hydrocarbon-degrading genus *Cycloclasticus,* were present in our samples associated to specific seasons.

The SAR86 lineage has also been found to be abundant within previously studied bacterioplankton communities (González et al., 2000; Suzuki et al., 2004). However, and as occurred with the other lineages, SAR86 members contributed little to community structure as compared to *Roseobacter* in Ría de Vigo. SAR86 reached maximum abundances in autumn when the relative abundance of *Roseobacter* was lowest. It is during its peak in population when the new cluster SAR86-IV was detected (Fig. II.4). This new group could be characteristic of the waters under study and the reason why SAR86 reached its maximum in autumn. During winter this group did not exist in high numbers in spite of the low abundance of *Roseobacter*.

Culture independent data generated in clone libraries and FISH revealed the presence of bacteria phylogenetically close to the species *Cycloclasticus spirillensus* in the Ría during the winter season. This species, firstly isolated from sediments, was described as a polyaromatic hydrocarbon-degrading bacteria (Chung and King, 2001).

The *Bacteroidetes* lineage was represented by a small number of really diverse clones with low similarity between them or to any sequence in the GenBank database (Table II.3). No phylogenies are detailed here since the low amount of clones in this study prevented from drawing any conclusion about this complex lineage. The *Bacteroidetes* group has been reported to dominate during bloom periods (Pinhassi and Hagstrom, 2000; Eilers et al., 2001; Mary et al., 2006) when high nutrient inputs could favour these organisms, as recently demonstrated (Alonso and Pernthaler, 2006). In our study a relatively constant high percentage proportion (23% as a mean from the FISH results) are present throughout the year. As it was proposed for SAR11, probably the low number of clones analyzed hindered the detection of more *Bacteroidetes* in the other libraries dominated by *Roseobacter*. In a system constantly provided with nutrients such as Ría de Vigo, *Roseobacter* and *Bacteroidetes,* better adapted to these situations (Alonso and Pernthaler, 2006), maintained a constantly high population along the year in contrast to typical open ocean organisms like SAR11.

Seasonality was also observed for *Betaproteobacteria*. This group of bacteria more frequent in fresh waters (Methé *et al.* 1998), were detected during the whole year in this estuarine ecosystem influenced by terrestrial runoffs. Only in summer this group was nearly absent (Table II.3), probably because upwelled oceanic water influenced the bacterioplankton community commonly present in the Ría. In winter, sequences almost identical to cluster OM43 were detected (99-100% of identity with U70704). This clade of

Betaproteobacteria, commonly associated with phytoplankton blooms, has recently been isolated and sequenced (Giovannoni et al., 2008) and the isolate (AAUX01000001) shared 98% of its sequence with our winter clones. However, in spring new sequences with less than 98% similarity to any previously known 16S sequence appeared and the phylogenetic analysis (tree not shown) suggested its classification as a novel group inside *Betaproteobacteria*.

II.5.3 Detection of new clusters

Information from review studies of major marine bacteria, such as *Roseobacter* (Buchan et al., 2005), SAR11 (Field et al., 1997) or SAR86 (Suzuki et al., 2001; Sabehi et al., 2004), and from our four clone libraries (retrieved from different oceanographical conditions) enabled us to detect new clades characteristic of specific seasons. This information should be further incorporated in the design of new, more specific, FISH probes to describe with higher precision the bacterial communities of upwelling ecosystems.

From the new clusters described in the trees performed, the most interesting ones belonged to SAR86 clones. Clusters SAR86-II, SAR86-I, SAR86-IIIa, b and its respective clones matched perfectly with the probe used. However, three of the clusters proposed had important mismatches with the sequence. For example, the proposed VIGO_SAR86-IV and the two unclassified groups (summer and winter) had 6-8, 5-7 and 4 bp mismatching respectively to the of the probe sequence. The last two clusters are not clearly related with the rest of SAR86, but cluster SAR-86-IV, in red colour, is clearly between described clusters of SAR86 from which is separated by a high bootstrap value. Tree topology and bootstrap support were also obtained by Parsimony and NJ methods (not shown). High bootstrap values and the large number of sequences of proposed new clusters in SAR11 and SAR86 clusters (A3?, A4? and IV? in Fig. II.3 and II.4) prompt the design of more specific probes to detect these new clusters proposed.

II.5.4 Bioremediation potential

In a thorough study performed in the Thames estuary (UK), *Cycloclasticus* seemed to dominate seawater microcosms spiked with single PAHs (McKew et al., 2007), and in the absence of any better competitor, bacteria of the genus *Cycloclasticus* seemed to be the key organism for polyaromatic hydrocarbon degradation in seawater (Kasai et al., 2002a; Maruyama et al., 2003; McKew et al., 2007; Teira et al., 2007). We detected this organism in the Ría de Vigo, both with FISH and clone libraries during winter. Probably the high maritime traffic and industrial and urban run-off to the Ría provide enough amounts of polyaromatics for *Cycloclasticus* to appear in detectable numbers. Thus, the water of the Ría seems to hold a preadapted hydrocarbon-degrading population without the experimental addition of

any pollutant, as has been recently proposed for the already polluted Thames estuary (McKew et al., 2007). In the same studies (Coulon et al., 2007; McKew et al., 2007), an important role of *Roseobacter* species for hydrocarbon degradation was also reported. The dominance of this lineage in Ría de Vigo could also include some yet-uncultured, species related with biodegrading activity. Although a more exhaustive monitoring is necessary to draw conclusions, genus *Cycloclasticus* apparently increased during the winter season in Ría de Vigo. It was previously suggested that temperature, more than nutrient addition, is what drives the degrading community composition and that degrading organisms such as *Oleispira*, *Cycloclasticus* or *Thalassolituus* are really efficient even at low temperatures (4ºC) (Coulon et al., 2007). In fact, the same winter water sample used in the present study was spiked in microcosms with increasing concentrations of LMW-PAHs and we could observe that the detection of *Cycloclasticus* (using the same probes as in the current study) increased proportionally to the initial concentration of the added PAHs (Teira et al., 2007). The winter and spring samples (colder samples, Table II.1) experienced a stronger increase of *Cycloclasticus* abundance than autumn and summer, thus supporting the conclusions of Coulon et.al. (Coulon et al., 2007). Furthermore, there is a combination of beneficial factors in winter (low temperatures and high N and P, Table II.1) that might allow a better development of these organisms and that would provide the ecosystem with the ability to degrade hydrocarbon pollution. A more likely explanation for this increase is a punctual spillage of contamination, which used to occur in the Ría de Vigo from different anthropogenic sources ("Environmental Observatory of Ría de Vigo" (Provigo)).

The same water, and therefore the same bacterioplankton assemblages described in this chapter, were used in a parallel study where the bacterial communities characteristic of each season were exposed to realistic oil additions (PAHs concentrations comparable to those at the time of the accident). Although the bacterial communities were severely affected by the environmental conditions, the punctual addition of PAHs did not have effects on the structure and function of any community except the one of the summer as shown by DGGE profiles (Lekunberri et. al, 2008; Lekunberri et. al, submitted). Therefore, it was hypothesized that if bacterial assemblages, commonly found in the Ría, were not affected by point additions of PAHs has to be because they were accustomed to the presence of similar hydrocarbon concentrations in the waters, that they could use and metabolize. Summer season is dominated by upweling events due to the dominance of North winds over the adjacent shelf. Therefore, during summer, water from upper layers of the Ría is leaving the system, being replaced by subsurface oceanic ENAW waters (Alvarez-Salgado *et al.* 1993). The absence of *Betaproteobacteria* and SAR11 in CARD-FISH analysis of the summer water (Table II.3) is consistent with a deep-water origin of the bacterial assemblage. Such community, previously surviving in deep oceanic waters was probably not adapted to the

allochthonous organic matter present in the Ría, explaining the observed changes in its diversity and function in the presence of PAHs.

Although our dataset alone is too small to draw general conclusions; a) the presence of this *Cycloclasticus* spp., which responds to PAHs additions (Teira et al., 2007), b) the appearance of alkane-degrading *Alcanivorax* species in high numbers after the *Prestige* oil-spill in sediments of the Ría (Alonso-Gutiérrez et al., 2008; Chapter I), c) the lack of effects of PAH addition on indigenous bacterioplankton structure (Lekunberri et al., submitted; Lekunberri, 2008) and e) the dominance of *Roseobacter*, previously related with hydrocarbon degradation (Coulon et al., 2007; McKew et al., 2007), support the theory of the existence of a preadapted hydrocarbon-degrading community in the Ría.

Chapter III:

Indigenous bacterial communities associated to supralittoral environments of “Costa da Morte” (NW-Spain) affected by the *Prestige* oil spill prior to bioremediation applications.

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Applied and Environmental Microbiology

III.1 ABSTRACT

The bacterial communities in two different shoreline matrices, such as rocks and sand from “Costa da Morte” (NW Spain), were investigated 12 months after being affected by the *Prestige* oil spill. Culture-based and culture-independent approaches were followed to compare the bacterial diversity present in these environments, compared with a non-oiled site.

A long-term effect of fuel over the microbial communities in the oiled sand and rock was suggested by the higher proportion of alkane and PAHs degraders and the differences in DGGE patterns when compared with the reference site. Class *Alphaproteobacteria* and *Actinobacteria* were the prevailing groups of bacteria detected in both matrices although the sand bacterial community exhibited higher species richness than the one of the rock.

Culture-dependent and -independent approaches suggested that *Rhodococcus* genus could play a key role in the *in situ* degradation of the alkane fraction of *Prestige* fuel together with other *Corynebacterineae*. Moreover, other members of this suborder such as *Mycobacterium* spp. together with *Sphingomonadaceae* bacteria (mainly *Lutibacterium anuloederans*) were related as well with degradation of the aromatic fraction of *Prestige* fuel. The multi-approach methodology applied in the present study allowed us to assess the complexity of autochthonous microbial communities related with the degradation of heavy fuel from *Prestige* and to isolate some of their components for a further physiology study. Since several *Corynebacterineae* members, related with degradation of alkanes and PAHs, were frequently detected in this and other supralittoral environments affected by the *Prestige* oil-spill along the NW Spanish coast, the addition of mycolic acids to bioremediation amendments is proposed to favour the presence of these degraders in long-term fuel polluted areas of similar characteristics.

III.2 INTRODUCTION

Since the *Polycommander* accident, many other oil spills such as *Urquiola* (1976), *Andros Patria* (1978) or *Aegean Sea* (1992) have occurred in the Galician coast (NW Spain) where an intense maritime traffic takes place. In November 13, 2002 the oil tanker *Prestige* sprang a leak off Finisterre Cape (Galicia, NW Spain) and six days later its oil tank broke up and sank 240 km west off Galicia. The spill of 60 000 tons of heavy fuel oil polluted 500 miles of the Spanish coast, reaching the French coasts. The "Costa da Morte" (NW Spain) was the most affected area (Albaiges et al., 2006). The oil residue released by the *Prestige* was devoided of the more labile fractions (boiling point <300ºC) having high contents of aromatic hydrocarbons (≈50%) as well as resins and asphaltenes (≈30%) (Alzaga et al., 2004).

Information about the autochthonous microbial populations at maritime oil polluted sites is scarce (Harayama et al., 2004). The studies carried out after the *Nakhodka* oil spill, with a similar chemical composition to that of *Prestige* fuel, gathered information on the marine microbial populations adapted to heavy fuel oil. Different molecular approaches, mainly involving 16S rRNA analysis such as PCR/DGGE (Kasai et al., 2001), clone libraries and specific oligonucleotide probes (Maruyama et al., 2003) were used in order to describe the bacterial community established at different environments and time intervals after the oil-spill.

The majority of previous studies were focused either on the isolation of few culturable degrading strains, or just on detecting 16S sequences of all bacteria present in polluted samples without gathering information on their physiology. As a consequence, more efforts should be made to understand community structures *in situ* and to isolate the key oil-degrading species present, with the aim to further investigate their requirements (Van Hamme et al., 2003; Harayama et al., 2004) that could be used in the development of new bioremediation.

In the present study, we report a microbiological analysis of a cobblestone beach from "Costa da Morte" (NW Spain), affected by the *Prestige* heavy fuel oil spill, twelve months after the last fuel stranding. Microbial community was examined thoroughly using a triple-approach method based on different cultivation strategies and culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) and the screening of 16S rRNA gene clone libraries.

III.3 MATERIALS AND METHODS

III.3.1 Sampling

On March 2004, 12 months after the last fresh fuel stranding from *Prestige*, oil-polluted samples were taken from the supralittoral zone of a cobblestone beach located next to "Faro Lariño" (42º 46, 25 N/ 09º 07, 30 W, Carnota, Spain) (Fig. III.1). Samples included small oil drops scattered among sand grains (OS) and fuel paste attached to rock surfaces (OR). The heavy fuel from *Prestige* attached to rock´s surfaces and interstices formed thick oil layers where different materials get attached. Non-oiled sand samples (NOS) from an adjacent zone were taken as controls. Samples were placed in sterilized glass jars and kept cool (4ºC) or frozen (-20ºC) until corresponding analyses (Fig. III.2).

III.3.2 Chemical analysis

To assess the degree of biodegradation of the sample and to verify that no cross contamination from different sources other than the *Prestige* fuel occurred, oil residues (1 g) were dissolved in 5.0 mL of dichloromethane (SupraSolv grade, Merck, Darmstadt, Germany), phase separated, and percolated through 2 g of anhydrous sodium sulfate. The organic extracts were carefully evaporated until dried up and one aliquot (5-10 mg) was dissolved in hexane and then fractionated in a previously conditioned (with 6mL hexane, Merck) cyanopropyl-silica solid-phase cartridge (SiO_2/CN, 1.0/0.5 g, 6 mL, Interchim, Montluçon, France), as reported elsewhere (Alzaga et al., 2004). The aliphatic and aromatic fractions were obtained by eluting with 4.0 mL of hexane (FI) and 5.0 mL of hexane-dichloromethane (1:1) (FII), respectively. Both fractions were then analyzed by GC-MS on a TRACE-MS Thermo Finningan TRACE-GC 2000 gas chromatograph (Dreieich, Germany) fitted with a HP 5MS (30 m x 0.25 mm id x 0.25 µm film) capillary column (J&W Scientific, Folsom, CA, USA).

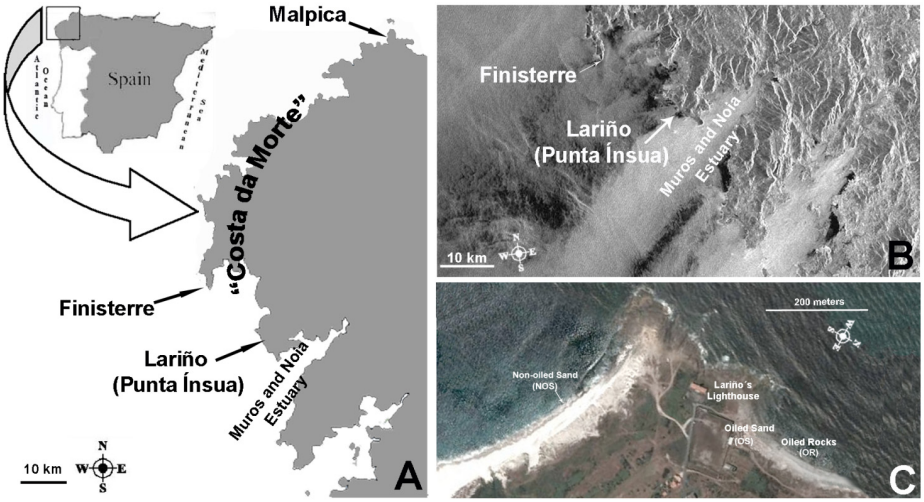

Fig. III.1. **A**: Detailed map of the NW coast of Spain (Galicia) known as "Costa da Morte" or "Costa de la Muerte" (from Muros and Noia Estuary to Malpica), which means "Coast of Death" for its though swell and weather. "Punta Ínsua" is the main landmark next to the sampling site. **B**: Satellite image (ERS SAR, http://earth.esa.int/ew/oil_slicks/galicia_sp_02/os_galicia_nov_02.html) acquired on the 13 January 2003 (orbit 40429 frame 2745) by the ESA (European Space agency). The rightmost dark areas along the coast are with high probability oil slicks affecting our sampling site at that time. **C**: Closer satellite image of the sampling sites location from GoogleMaps (http://maps.google.com/).

The extent of biodegradation for each compound was measured from the normalised peak area of the target analyte referred to that obtained from the same compound in the control sample (Albaiges et al., 2006; Jiménez et al., 2006). The peak areas of the target analytes were measured in the reconstructed ion chromatograms at m/z 85 for aliphatics, and at the corresponding molecular ion for the aromatics, as described elsewhere (Diez et al., 2005).

III.3.3 Microbial characterization

Samples were directly analyzed (OR, OS) or previously enriched using different fuel components such as alkanes or aromatics. Procedures and nomenclature are summarized in Fig. III.2 and Table III.1 respectively.

III.3.4 Enumeration of heterotrophic and hydrocarbon–degrading microbial populations

Bacterial counts of heterotrophs, *n*-hexadecane and PAH degraders were performed using a miniaturized most probable number (MPN) in 96-well microtiter plates with eight replicate wells per dilution as described elsewhere (Wrenn and Venosa, 1996; Alonso-Gutiérrez et al., 2008). All the media used (TSB and mineral medium BMTM (Alonso-Gutiérrez et al., 2008)) were corrected to reach 3% NaCl. MPN results are shown as means from triplicates and Student´s t-Test was used to compare them (Fig. III.6).

III.3.5 Isolation of culturable strains

Culturable microorganisms from OR (rock) and OS (sand) samples, and from enrichment cultures grown on phenanthrene or *n*-hexadecane were isolated onto different media (Table III.1). Culturable heterotrophs were isolated at 20ºC onto five fold diluted marine agar (MA 1/5) supplemented to maintain 3% NaCl. *n*-hexadecane and phenanthrene degraders were isolated onto mineral agar (BMTM Agar 3% NaCl) supplemented with *n*-hexadecane in vapour phase (Sei et al., 2003) or phenanthrene (0,1%) as a sole carbon and energy source, respectively (Table III.1). All isolated strains were stored at -80ºC in 20% (v/v) of glycerol for its subsequent analysis.

III.3.6 Screening of hydrocarbon-degrading capability of strains

All isolated strains were screened for their hydrocarbon-degrading capability of alkanes and aromatics either onto solid or liquid mineral medium, as previously described (Alonso-Gutiérrez et al., 2008).

To assess hydrocarbon-degrading capability in solid medium, mineral agar supplemented with *n*-hexadecane and phenanthrene was used as described above. Microtiter plates containing 200 µL per well of mineral media (BMTM 3% NaCl) and *n*-hexadecane, F1 or PAH mixture were used, as for MPN analysis, in liquid screenings. F1 is the aliphatic fraction (2.5 $g \cdot L^{-1}$) obtained from Casablanca crude oil (Vinas et al., 2002).

To inoculate the biodegradation assays, the strains were grown overnight at room temperature on TSB (3% NaCl). Cells were harvested by centrifugation at 4000g for 15 min, washed twice, and finally suspended in mineral medium (BMTM+3% NaCl) to reach an optical density (OD) of 0.5 (determined at 620 nm using a multiscan spectrophotometer (Labsystems)). Twenty µl of suspended cells were used for the inoculation of two wells per plate. Another plate with only mineral medium was inoculated as negative control. Only those wells with evident turbidity when compared to control plate were considered positive.

III.3.7 DNA extraction

Total community DNA was extracted from OR and OS samples following a bead beating protocol using a PowerSoil DNA soil extraction kit (MoBio Laboratories, Inc., Solano Beach, CA) following the manufacturer's instructions.

Genomic DNA from the heterotrophic population and from the ones related with alkane and aromatic degradation (-Hx and -PAHs respectively hereafter) was obtained from the 8 wells corresponding to the highest positive

dilution of MPN plates of OR and OS samples. Cells were harvested from wells, lysed with Sodium Dodecyl Sulphate (SDS 10%), Lysozyme and Proteinase K, treated with CTAB 10% (Cetyl Trimethylammonium Bromide) and freezed-thawed three times using liquid Nitrogen and a 65ºC bath. The extracted DNA was purified by Phenol-Chloroform-Isoamyl alcohol extraction, as previously described (Wilson, 1987; Bennasar et al., 1998).

III.3.8 DGGE

Genomic DNA from OR, OS, NOS, MPN microtiter plates and hydrocarbon degrading strains were subjected to DGGE analysis. The 16S rRNA gene hypervariable regions V3-V5 were amplified using primers 16F341-GC and 16R907 (Yu and Morrison, 2004). Primer F341-GC included a GC clamp at the 5´end (5´-CGCCCGCCGCGCCCCGCGCCCGTCCCGCC GCCCCCGCCCG-3´). In this case, PCRs were performed in a volume of 50 µl containing 1.25 U of Taq (TaKaRa ExTaq™ Hot Start Version; TaKaRa Bio Inc., Otsu, Siga, Japan), 1X ExTaq Buffer (2mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 0.5 µM of primers and 100 ng of template DNA. After 9 min of initial denaturation at 95°C, a touchdown thermal profile protocol was carried out, and the annealing temperature was decreased by 1°C per cycle from 65°C to 55°C; followed by 20 additional cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 55ºC, and 1.5 min of primer extension at 72°C, followed by 10 min of final primer extension at 72ºC.

Approximately 800 ng of purified PCR product was loaded onto a 6% (wt/vol) polyacrylamide gel that was 0.75 mm thick with denaturing gradients and ranged from 40 to 75% denaturant concentrations (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1×TAE buffer (40mMTris, 20mMsodium acetate, 1 mM EDTA; pH 8.4) using a DGGE-2001 system (CBS Scientific Company, Del Mar, CA) at 100 V and 60°C for 16 h. DGGE gels were stained with 1X TAE buffer containing SybrGold (Molecular Probes, Inc., Eugene, OR, USA). Predominant DGGE bands were excised with a sterile razor blade, suspended in 50 µl sterilized MilliQ water, stored at 4°C overnight, reamplified by PCR using primers F341-R907 and cloned using TOPO TA cloning kit (Invitrogen) as described below.

III.3.9 Analysis of DGGE images

Bacterial diversity and correlation principal-component analysis (PCA) of band types and relative peak area was calculated, as previously described (Vinas et al., 2005a), for the different DGGE profiles (i.e. OR, OS, NOS, OR-Hx, OR-PAH, OS-Hx and OS-PAH (Fig. III.7)) to consider possible shifts in the composition of the microbial populations. A dendrogram was constructed using the nearest neighbour cluster method with the Pearson product-moment correlation coefficients calculated from the complete densitometric curves for fingerprints of the different bacterial communities.

III.3.10 16S rRNA gene clone library

Almost complete 16S rRNA was amplified from OS and OR genomic DNA using primers F27 and R1492 as previously described (Edwards et al., 1989; Lane, 1991). PCR reaction (25 µl) included 10mM TrisHCl [pH 8.3]; 50mM KCl [pH 8.3]; 2.5 mM $MgCl_2$; 200 µM of each deoxynucleoside triphosphate; 1.25 U of AmpliTaqGold DNA polymerase (PE Applied Biosystems, Foster City, CA), 0.4 µM of each primer and 100 ng of DNA extracted from either OR or OS samples. The reaction mixtures were subjected to initial denaturation and enzyme activation step (5 min at 95ºC), 40 cycles, consisting of 30 seconds at 96°C, 30 seconds at 54°C and 1.5 min at 72°C, followed by an extension step of 10 min at 72°C.

PCR products were ligated into pCR®2.1-TOPO® vector and transformed into competent *Escherichia coli* TOP 10F´ cells, following manufacturer's protocol of TOPO T/A Cloning Kit (Invitrogen). A restriction fragment length polymorphism analysis (RFLP) of clones was performed to identify clone representatives of different enzyme restriction pattern, digesting the

PCR products separately with 5U of Alu I and Taq I (Amersham Biosciences, Uppsala, Sweden) for 3 h. at 37ºC and 65ºC respectively.

PCR products from recombinant clones and the resulting restriction enzyme fragment patterns were separated by electrophoresis in a 1% and 3% (w/v) agarose gel in 1× TAE buffer respectively, stained with ethidium bromide and photographed under UV light using Gel Doc XR system and Quantity One software (Bio-Rad, Hercules, CA). Those clones representatives of different enzyme restriction pattern were sequenced in both directions using internal primers F341 and R907 (Edwards et al., 1989).

III.3.11 Sequencing and phylogenetic analysis

Sequencing was accomplished using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) and an ABI PRISM™ 3700 automated sequencer (PE Applied Biosystems, Foster City, California, USA) following the manufacturer's instructions. 16S rRNA genes were partially sequenced in both directions using primers F341, R907 (Edwards et al., 1989). Sequences were inspected, assembled, subjected to the Check Chimera program of the Ribosomal Database Project (Maidak et al., 2000) and examined with the BLAST search alignment tool comparison software (BLASTN) (Altschul et al., 1990) to detect the closest bacterial group to each strain among GenBank database.

Sequences were aligned with reference ones obtained from GenBank and phylogenetic analysis were performed, as previously described (Alonso-Gutiérrez et al., 2008), to better classify the detected bacteria.

III.3.12 Nucleotide sequence accession numbers.

The 363 nucleotide sequences identified in this study have been deposited in the GenBank database under accession numbers EU374875-EU375237 (Table III.1).

Table III.1. Nomenclature of sequences retrieved in this study sorted by its methodological source. Isolation methodology for strains, accession numbers of the sequences as well as figures and tables referred to them are indicated for better understanding.

	OR Samples				OS Samples			
Methodology source	**Code**	**Accession numbers**	**Tables**	**Figures**	**Code**	**Accession numbers**	**Tables**	**Figures**
DGGE BANDS from PCR amplified-								
-Total DNA from sample	R n^{er}	EU375011-29	3,S1	7A,9A	S n^{er}	EU375139-51	3,S1	7A,9B
-DNA from MPN-Hx	RH n^{er}	EU375095-98	3,S2	7B,9A	SH n^{er}	EU375222-29	3,S2	7C,9B
-DNA from MPN-PAHS	RPb n^{er}	EU375137-38	3,S2	7B,9C	SP n^{er}	EU375230-37	3,S2	7C,9C
CLONE LIBRARY from PCR amplified-								
-Total DNA from sample	Rc n^{er}	EU375030-94	3,S3		Sc n^{er}	EU375152-221	3,S3	
STRAINS isolated with								
MA 1/5 from original samples	RP n^{er}	EU375099-136	2,S4		AP n^{er}	EU374875-933	2,S5	
MA 1/5 from highest dilution on MPN-Hx plates	RPH n^{er}		2,S4		APH n^{er}	EU374934-49	2,S5	
MA 1/5 from highest dilutions on MPN-PAHs plates	RPP n^{er}		2,S4		APP n^{er}	EU374950-68	2,S5	
BMTM + Hx from original samples	PDR n^{er}	EU374991-97	2,S4	9A	PDA n^{er}	EU374969-90	2,S5	9B
BMTM + Phe from enrichment cultures in Phe	PhR n^{er}	EU374998-5005	2,S4	9C	PhS n^{er}	EU375006-10	2,S5	9C

MA: Marine Agar, BMTM: mineral agar, Phe: Phenanthrene, Hx: *n*-Hexadecane,
MPN-Hx/-PAHs: Most Probable Number counting

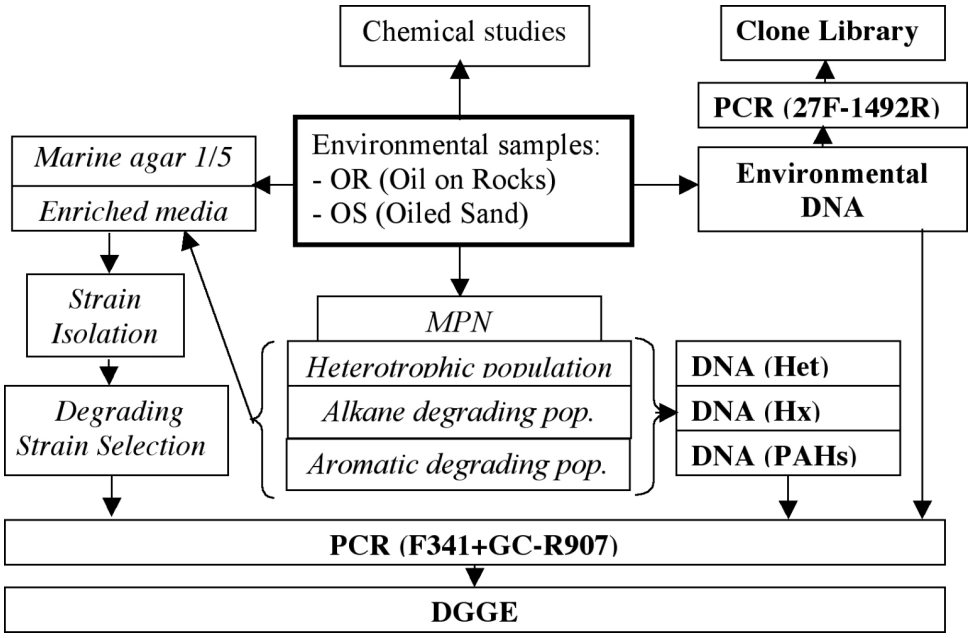

Fig. III.2. Flowchart diagram illustrating protocols used in this study. For both samples (OR and OS) chemical, *microbiological* and molecular analyses were made. A near full-length 16S gene PCR fragment from total DNA of samples was cloned. The clone library was screened using RFLP and different OTUs were sequenced. Templates for direct DGGE were either extracted DNA (directly from the environment or from the trophic populations grown in the MPN plates) or existing PCR products from degrading strains. The DGGE profiles from the total DNA of each sample and of its three trophic populations (heteretrophic, alkane- and aromatic-degrading) were compared to identify common bands between them. Degrading strains, isolated using different mediums, were individually screened using DGGE to detect those that comigrated with specific bands in the profiles. Sequences from the most interesting bands and strains were compared with those from the clone library to get a quantitative proportion of the different species found.

III.4 RESULTS

III.4.1 Chemical analysis

The GC profiles of the aliphatic fractions evidenced a petrogenic contamination, based on the occurrence of the homologous series of C_{15}-C_{40} *n*-alkanes overlying an unresolved complex mixture of hydrocarbons (Fig. III.3).

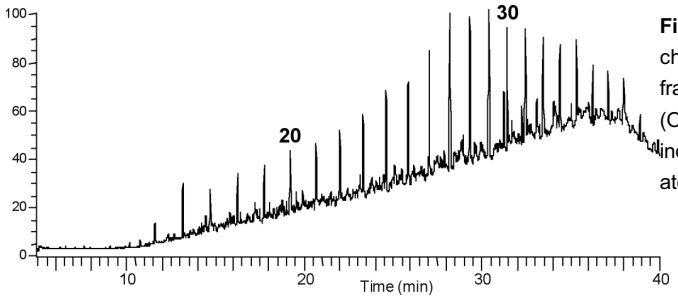

Fig. III.3. Total ion chromatogram of the aliphatic fraction of the oiled rock sample (OR). Numbers over the peaks indicate the number of carbon atoms of the *n*-alkane series.

The confirmation of the presence of the *Prestige* oil was obtained by a detailed study of the fossil biomarkers, namely steranes and triterpanes, currently used for oil spill fingerprinting (Daling et al., 2002). The diagnostic molecular parameters for the oiled samples indicated a clear correspondence with those of the fuel oil (Fig. III.4), whereas those of the control sand

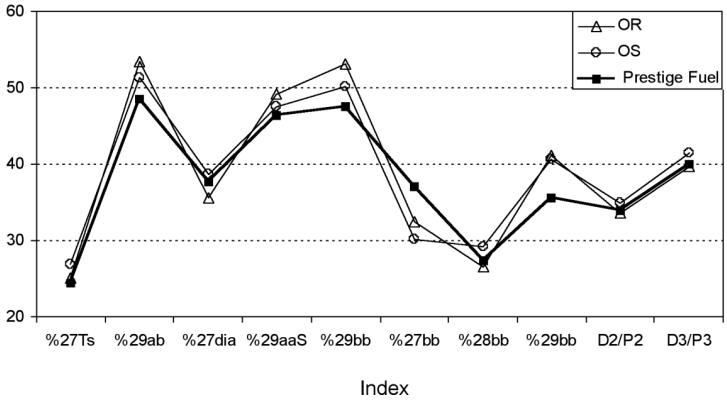

Fig. III.4. Source diagnostic ratios (Jiménez et al., 2006) of the *Prestige´*s fuel oil and the rock (OR) and sand (OS) polluted samples.
Index definitions can be found in Table I.1.

sample (NOS) exhibited a different pattern. The ratios of C_2 and C_3 dibenzothiophenes (D2 and D3) and phenanthrene/anthracenes (P2 and P3), proposed for differentiating sources of spilled oils in sediments (Douglas et al., 1996), also supported the presence of the *Prestige* oil in the collected samples (Fig. III.4).

The occurrence of biodegradation was assessed by the depletion of certain components with respect to those more refractory, such as triterpanes (e.g. hopane), and by changes of relative distributions within isomeric series (e.g. alkyl C1- and C2-phenanthrenes, dibenzothiophenes). In summary, the n-alkanes were severely depleted in the lower fraction (<*n*-C20) as a result of weathering, but also the higher ones which should be attributed to biodegradation (Fig. III.5A).

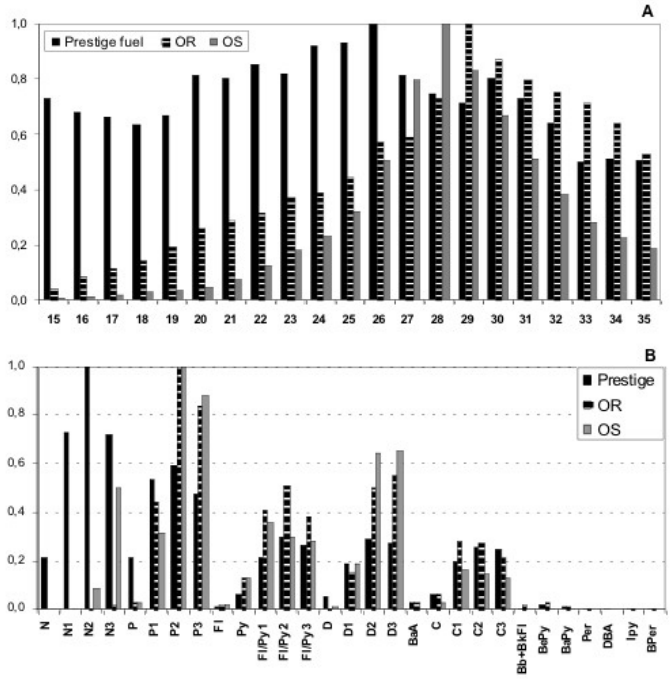

Fig. III.5. Relative distributions of *n*-alkanes (A) and polycyclic aromatic hydrocarbons (B) with respect to the major peak in the *Prestige* oil and rock (OR) and sand (OS) polluted samples.
N: naphthalenes,
P: phenanthrenes,
Fl/Py: fluoranthenes/pyrenes,
D: dibenzothiophenes,
BaA: Benz[a]anthracene,
C: chrysenes,
Bb/BkFl: benzo[*b*] and benzo[*k*]fluoranthene,
BePy: benzo[*e*]pyrene,
BaPy: benzo[*a*]pyrene,
Per: perylene,
DBA: dibenzanthracene.
IPy: indeno[1,2,3-cd]pyrene,
BPer: benzo[g,h,i]perylene.

III.4.2 Enumeration of heterotrophic, alkane-degrading and aromatic-degrading microbial populations.

While the total heterotrophic bacteria in the oiled and non-oiled sands (OS and NOS) presented similar abundances (10^5-10^6 microorganisms per gram of sample), hydrocarbon degrading populations were 10-100 fold higher in the oiled sample compared to NOS (Fig. III.6). The alkane-related population found in OR and OS was also similar (around 10^4-10^5 microorganisms · gr^{-1}), accounting for more than 50% of the heterotrophic bacteria and always higher than the aromatic one. However, aromatic degrading bacteria were ten fold higher in the polluted sand (10^3 -10^4 microorganisms · gr^{-1}) than in the oiled rocks.

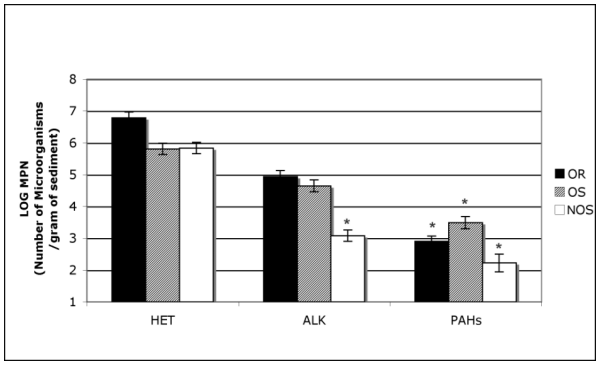

Fig. III.6. Most Probable Number (MPN) of heterotrophic (HET), alkane-degrading (ALK) and PAH-degrading populations (PAHs) in rock (OR), sand (OS) polluted samples compared with the non-oiled sand (NOS). Standard deviations (n=8) are represented by vertical lines at the top of the bars. *: Significantly different from the other two samples using Student´s t-Test. Polluted samples have similar populations, except for aromatic degraders, while unpolluted samples always have significantly lower degrading populations than polluted ones.

III.4.3 Isolation of culturable strains

Around 40 morphologically different strains were isolated on 1/5 strength marine agar (MA 1/5) directly from each sample (RPx and APx strains), whereas over 20 strains were isolated in the same medium from the MPN plates of populations related with *n*-hexadecane and aromatic hydrocarbons degradation (RPHx and RPPx strains from OR and APHx, APPx strains from OS; Table III.1).

Using selective media for isolation of alkane degraders (BMTM-agar + *n*-hexadecane), approximately 20 additional strains were isolated directly from the rock (PDRx strains) and the sand (PDAx strains) samples respectively. Finally, 15 additional strains for each sample were isolated in phenanthrene-agar from an enrichment culture grown on phenanthrene (0.05 % w/v) at 150 rpm and 25ºC for more than 2 weeks (strains PhRx and PhSx, Table III.1).

All strains isolated from polluted sites (RP and AP) were sequenced but in the other cases (RPH, RPP, APH, APP, PDA, PDR, PhR, PhS) only those strains suspected of having some degrading capacity or with the same migration length as any of the OR/OS related DGGE bands, were further analysed (Table III.1).

III.4.4 Screening of hydrocarbon degrading capability

Alkane-degrading activity was found in isolated strains from all environments (sand and rock) and, although the percentage of degraders varied depending on the media used, it was always dominated by *Actinobacteria* (Table III.2). In general, polluted sand samples (Table III.S5 in the supplemental material) presented a much higher percentage of

Table III.2. Percentage of major bacterial groups found in oiled rocks (OR) and sand (OS), depending on the methodology of study. Culture-dependent (isolation in MA 1/5 or in mineral medium supplemented with Hexadecane (Hx) or Phenanthrene (Phe); Table III.1) and molecular techniques (DGGE and Clone Library) are compared. DGGE percentages are based just on the proportion of bands excised without taking intensities into account. Bold percentages indicate the adscription of degrading strains.

	OR								
Phylogenetic Group	DGGE			CLONE LIBRARY		MPN in MA		Enrichment	
	TOTAL (OR)	ALK.DEG. (MPN-Hx)	ARO.DEG. (MPN-PAHs)		MA 1/5 (RP)	MPN-Hx (RPH)	MPN-PAHs (RPP)	Hx (PDR)	Phe (PhR)
Gammaproteobacteria	10%	25%		23%	48%				**18%**
Alphaproteobacteria	53%	25%	100%	43%	18%				**82%**
Bacteroidetes	5%			6%	18%				
Actinobacteria	32%	50%		30%	8%	**100%**	**100%**	**100%**	
Firmicutes					8%				
Proportion of alkane-degrading / total isolates					***0/38***	***2/21***	***1/24***	***23/32***	***0/15***
Proportion of PAHs-degrading / total isolates					***0/38***	***0/21***	***0/24***	***0/32***	***11/15***
Percentage of alkane° (or PAHs*) degrading strains					***0%***	***9,5%°***	***4%°***	***72%°***	***73%****

	OS								
Phylogenetic Group	DGGE			CLONE LIBRARY		MPN in AM		Enrichment	
	TOTAL (OS)	ALK.DEG. (MPN-Hx)	ARO.DEG. (MPN-PAHs)		MA 1/5 (AP)	MPN-Hx (APH)	MPN-PAHs (APP)	Hx (PDA)	Phe (PhS)
Gammaproteobacteria	23%	63%		19%	23% **12%**			**10%**	**67%**
Alphaproteobacteria	38%	37%	88%	39%	23%				**33%**
Deltaproteobacteria				1%					
Bacteroidetes	15%			9%	12%				
Actinobacteria	24%			30%	37% **88%**			**90%**	
Firmicutes			12%		5%				
Planctomycetes				1%					
Chloroflexi				1%					
Proportion of alkane-degrading / total isolates					***8/43***	***0/21***	***0/25***	***21/26***	***0/15***
Proportion of PAHs-degrading / total isolates					***0/43***	***0/21***	***0/25***	***0/26***	***9/15***
Percentage of alkane° (or PAHs*) degrading strains					***19%°***	***0%***	***0%***	***81%°***	***60%****

MPN-Hx/-PAHs: Most Probable Number counting plate of alkane/aromatic degrading related population using Hexadecane/PAHs as the only source of C and energy.
ALK.REL./ARO.REL.: Alkane/Aromatic degrading related population cultured in the MPN-Hx/MPN-PAHs plates.

hydrocarbon-degrading strains than polluted rock samples (Table III.S4 in the supplemental material) even using non-selective media (33% of AP compared to 2.6% of RP strains). As expected, the selective media (hydrocarbon-agar) was the best strategy to isolate alkane-degrading strains (72% and 81% out of PDR and PDA strains were alkane degraders; Table III.2) and almost the only way to isolate bacteria related with PAHs degradation (PhR/PhS strains). Eleven PhR and nine PhS isolates (representing 73% and 60% of the total) from the OS and OR phenanthrene enrichments, grew as a pure culture on PAHs. These strains belonged to only two species of *Pseudomonas* and *Sphingomonas* (Table III.3).

Table III.3. Summary table of the 16S rRNA sequences from DGGE bands, clones and degrading strains of the polluted rocks (OR) and sand (OS) samples. Designations and accession numbers for sequences and levels of similarity to related organisms are shown.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree[a]	DGGE Band	Clone Library %		Degrading Isolates %[b]		Importance for Bioremediation[c]	
			OR	OS	OR	OS	*This*	*Others* (Ref.)
Alcanivoraceae (γ)	*Alcanivorax spp.* (AM286690;DQ347532;AY258109;AY683537)		3/65	3/72		3/29°	Hx	(Kasai et al., 2002)
Chromatiales (γ)	Uncultured bacterium clone B101-25 (DQ001686)	S9		2/72				
	Uncultured bacterium clone E101-47 (DQ001646)		4/65					
	Uncultured *gamma proteobacterium* (DQ870518)	R15=S11	4/65	1/72				(Jiménez et al., 2007)
	Uncultured soil bacterium clone M54-Pitesti (DQ378269)	S8	1/65	4/72				
Halomonadaceae (γ)	*Halomonas* sp. M6-20C (AY730247)						$Hx^{?}$	(Pepi et al., 2005)
Pseudomonadaceae (γ)	*Pseudomonas spp.* (AY691188; AJ312176)				2/11*	6/9*	Phe	(Mueller et al., 1990)
Salinisphaeraceae (γ)	*Salinisphaera sp.* ARD M17 (AB167073)		6/65					
Xanthomonadaceae (γ)	*Dokdonella koreensis* DS-140 (AY987369)	SH2/SH3					$Hx^{?}$	
	Pseudoxanthomonas spadix IMMIBAFH-5 (AM418384)	RH4/SH5/SH6					$Hx^{?}$	(Young et al., 2007)
	Stenotrophomonas sp. KL1A1 (DQ208664)	SH8					$Hx^{?}$	(Kasai et al., 2001)
Erythrobacteraceae (α)	*Erythrobacter sp.* JL893 (DQ985055)	R16=SH7	1/65				$Hx^{?}$	(Rolling et al., 2002; Jiménez et al., 2007)
Hyphomicrobiaceae (α)	*Hyphomicrobium sp.* Ellin112 (AF408954)	S5						
Phyllobacteriaceae (α)	*Parvibaculum lavamentivorans* (AY387398)	R6/SH1	5/65	1/72				(Schleheck et al., 2004)
	Rhizobium loti (U50165)			4/72				
	Soil *Phyllobacteriaceae* bacterium (DQ099469)	S4						
Rhizobiaceae (α)	*Agrobacterium sp.* Mei-QS6 (EF090606)	SH4					$Hx^{?}$	
	Rhizobium sp. 28/2 (DQ310471)		2/65					Fani & Pini (unp.)
	Sinorhizobium sp. L1 (AJ879127)						$Phe^{?}$	Karvelis et.al.(unp)
Rhizobiales (α)	*Martelella mediterranea* MACL11 (AY649762)						$Phe^{?}$	
Rhodobacteraceae (α)	Uncultured *Rhodobacteraceae* bacterium (DQ870525)	R7=S7	3/65					(Jiménez et al., 2007)
	Citreicella sp. 2-2A (AB266065)	R9/R10/R11	1/65				$Phe^{?}$	Watanabe et al.(unp)
Rhodospirillaceae (α)	*Thalassospira* sp. DBT-2 (DQ659435)	RH3		1/72				Wang et.al. (unp)
	Tistrella mobilis (AB071665)	RPb1					$Phe^{?}$	(McKew et al., 2007)
Rhodospirillales (α)	*Rhodovibrio sp.* 2Mb1 (AY987846)	SP1/SP2					$Phe^{?}$	
Sphingomonadaceae (α)	*Lutibacterium anuloederans* (AY026916)	R13/R14=S10/S6	6/65	5/72				(Chung and King,2001)
	Novosphingobium spp (AY690709; AJ416411)		3/65	1/72				(Kaplan, 2004)
	Sphingomonas spp. (AY646154;AJ717392;AY690679;AB099636;AF282616)	SP5/SP6/SP8 RPb2=SP7	4/65	1/72	9/11*	3/9*	Phe	Ahn,Y.(unp) (Stolz et al., 2000)
	Sphingopyxis sp. FR1093 (DQ781321)	R12/R17						
Xanthobacteraceae (α)	*Xanthobacter autotrophicus T102* (U62888)	SP3					$Phe^{?}$	(Hirano et al., 2004)
Bacteriovoracaceae (δ)	*Bacteriovorax sp.* GSL4A1 (DQ536441)			1/72				
Flavobacteriaceae (B)	*Cellulophaga sp.* D3054 (DQ480142)	R18	1/65					
	Uncultured bacterium clone HB2-46-16 (DQ334636)	S12						
	Yeosuana aromativorans (AY682382)	S13		1/72				(Kwon et al., 2006)

Table III.3(cont.). Summary table of the 16S rRNA sequences from DGGE bands, clones and degrading strains of the polluted rocks (OR) and sand (OS) samples. Designations and accession numbers for sequences and levels of similarity to related organisms are shown.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree[a]	DGGE Band	Clone Library %		Degrading Isolates %[b]		Importance for Bioremediation[c]	
			OR	OS	OR	OS	*This*	*Others* (Ref.)
Dietziaceae (A)	*Dietzia spp.* (AB159036; X79290)		3/65		3/26°		Hx	(Yumoto et al., 2002)
Gordoniaceae (A)	*Gordonia polyisoprenivorans* (DQ154925)					3/29°	Hx	(Linos et al., 1999)
Microbacteriaceae (A)	*Leifsonia* spp. (AY771748) (DQ473536)			2/72				
	Microbacterium spp. VKM Ac-2048 (AB042083)		2/65					(Schippers et al., 2005)
	Microcella putealis CV2T (AJ717388)	R8						
Mycobacteriaceae (A)	*Mycobacterium* spp. (AY255478;AJ276274;AY235429; DQ372728)			5/72				(Bastiaens et al., 2000)
Nocardiaceae (A)	*Rhodococcus* sp. 5/1 (AF181689)	R1=S1=RH1/R3/R5	11/65	5/72	15/26°	14/29°	Hx	(Bej et al., 2000)
	Rhodococcus sp. MBIC01430 (AB088667)	R2=S2=RH2/S3	2/65	1/72	8/26°	8/29°	Hx	(Sekine et al., 2006)
	Rhodococcus opacus ML0004 (DQ474758)					1/29°	Hx	(Iwabuchi et al., 2002)
Streptomycetaceae (A)	*Streptomyces* sp. 6G9 (EF198882)						Hx[?]	
Williamsiaceae (A)	*Williamsia sp.* MT8 (AY894336)	R4		2/72				
Bacillaceae (F)	*Geobacillus stearothermophilus* S6090 (EF095714)	SP4					Phe[?]	(Meintanis et al., 2006)
Staphylococcaceae (F)	*Staphylococcus epidermidis* S09 (AY741152)						Hx[?]	
Planctomycetaceae (P)	Uncultured *planctomycete* clone SC3-24 (DQ289930)			1/72				
Thermomicrobiaceae (C)	*Thermomicrobium sp.* GR108 (DQ130040)			1/72				

γ, α and δ: *Gamma*, *Alpha*, and *Deltaproteobacteria*, respectively; B: *Bacteroidetes*; A: *Actinobacteria*; F: *Firmicutes*; P: *Planctomycetes*; C: *Chloroflexi*.
=: identical sequence. /: similar sequence.
°: Alkane degrading isolates. *: Aromatic degrading isolates.
?: related to alkane or aromatic degradation but need further study of its specific role in it
unp: Unpublished reference. Normally it refers to the authors of the closest sequence in GenBank (Accession Number).
[a] Sequences were matched with the closest relative from the GenBank database after BLAST and Phylogenetic analysis.
[b] Proportion each degrading species has been isolated relative to the total number of isolates able to degrade the same fraction in each polluted matrix.
[c] Compilation of the available data from "*This*" and "*Other*" studies about the degrading ability related to each 16S sequence. Information from "*This*" study comes from the strains isolated which gives positive results in the degradation tests for alkanes or aromatics, marked as "Hx" and "Phe" respectively. Although it is not totally appropriate to draw conclusions of physiological features from molecular data, references of "*Other*" studies reporting any relation of the sequence detected with biodegradation (degrading capacity, detection in hydrocarbon polluted samples…) are given.

III.4.5 DGGE profiles of total bacterial community

Cluster and PCA analysis of DGGE profiles indicated that bacterial communities from oiled samples (OR and OS) were quite similar between them while the non-oiled control (NOS) was the most distantly related (Fig. III.7 and III.8). Five OR DGGE bands R1, R2, R7, R14, R15 were respectively identical in sequence to OS DGGE bands S1, S2, S7, S10 and S11. Those bands were related to the following genera: *Rhodococcus,* uncultured *Rhodobacteriaceae, Lutibacterium* and *Chromatiales,* respectively. Additional bands from these and other genera related with oil degradation, such as *Citreicella* spp. (bands R9, R10, R11), *Sphingopyxis* spp. (bands R12, R17), *Erythrobacter* spp. (band R16) and *Yeosuana aromativorans* (band S13) were also found in total DGGE profiles (Table III.3 and III.S1 in the supplemental material).

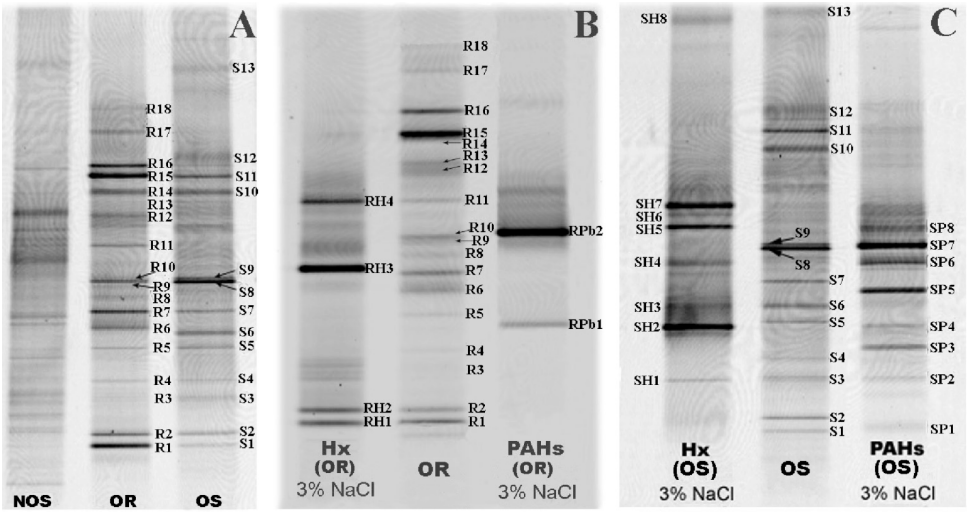

Fig. III.7. DGGE profiles of PCR-amplified 16S rRNA genes of bacterial communities from oiled samples compared with the one from control sand (NOS) (A). The hydrocarbon degrading bacterial populations from polluted rock (B) and sand (C) are compared with their total profiles showing a marked heterogeneity between the different matrices. Codes to the right (R1 to R18 and S1 to S13) indicate cut and sequenced bands from the total profiles OR and OS respectively (Table III.S1 in the supplemental material). Bands from the alkane degrading (RHn^{er} and SHn^{er}) and aromatic degrading population (RPn^{er} and SPn^{er}) of rock and sand respectively were sequenced as well (Table III.S2 in the supplemental material) finding higher "species richness" (number of bands) in the sand. Bacterial diversity (Table III.4), correlation principal-component analysis (PCA) and a dendrogram (Fig. III.8) were calculated from the complete densitometric curves of the different DGGE profiles (i.e. OR, OS, NOS, OR-Hx, OR-PAH, OS-Hx and OS-PAH) to consider possible shifts in the composition of the microbial populations.

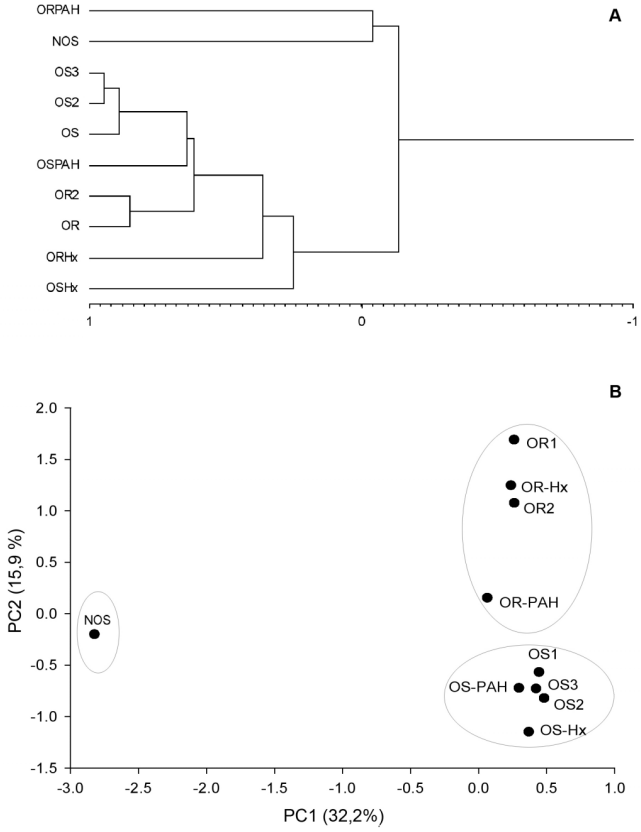

Fig. III.8. A. Cluster analysis from similarity matrix generated from denaturing gradient gel electrophoresis profiles (Fig. III.7) according Pearson product moment and the unweighted pair group method. **B.** Principal-component analysis (PCA) scatter plot (2D) of DGGE profiles (Fig. III.7). DGGE profile of OS samples were close to hydrocarbon degradares, whereas OR DGGE profile was more similar to alkane degraders than to PAH degraders. NOS: Non-oiled sand; OSnº: Oiled sand replicates; ORnº: oiled rock replicates. OR-Hx, OR-PAH, OS-Hx, OS-PAH : total DNA from highest MPN growing dillutions on Hexadecane and PAHs respectively.

III.4.6 DGGE profiles of presumably oil-degrading bacterial populations.

PCA analysis (Fig. III.8) of excised and non-excised bands from the different DGGE profiles (Fig. III.7 B, C) suggested that OR community members were mainly related with alkane degradation while the oiled sand community include members related with the degradation of both fractions. However, 16S rDNA sequences were obtained only from most conspicuous bands of the profiles and no common sequences could be confirmed between populations related with the degradation of alkanes (-Hx) and aromatics (-PAHs) (Table III.S2 in the supplemental material) and the total community (OR, OS) (Table III.S1 in the supplemental material). An exception was found with bands RH1 and RH2 from the presumably *n*-hexadecane-degrading population profile of the rock sample (OR-Hx). These bands were respectively identical to the *Rhodococcus* bands shared by the total community profiles of the oiled rock and sand (RH1=R1=S1 and RH2=R2=S2; Table III.3).

Higher diversity related to alkane and aromatic degradation was found in the sand than in the oiled rocks (Table III.4). However, some common bands within the OR-Hx profiles

Table III.4. Number of DGGE bands detected and Shannon Weaver diversity index calculated for each DGGE profile (Fig. III.7 and III.8).

	OR	OR2	OS	OS2	OS3	NOS	OR-Hx	OS-Hx	OR-PAH	OS-PAH
Shannon Weaver Diversity Inde:	1.24	1.06	1.26	1.33	1.25	1.38	0.91	1.03	0.46	1.07
DGGE bands ("richness")	20	18	22	22	24	27	11	13	6	16

NOS: Non-oiled sand; OSnº: Oiled sand replicates; ORnº: oiled rock replicates.
OR-Hx, OR-PAH, OS-Hx, OS-PAH : total DNA from highest MPN growing dillutions on Hexadecane and PAHs respectively.

(RH4) and OS-Hx (SH5, SH6) were found related to *Pseudoxanthomonas spadix* (99-100% of similarity) (Table III.3). Other *Xanthomonadaceae* genus related with alkane-degradation in OS were close to *Dokdonella koreensis* (bands HA2/HA3) and to *Stenotrophomonas maltophilia* (band SH8). *Alphaproteobacteria* genus *Erythrobacter* was detected as one of the most conspicuous of the OS-Hx profile (SH7).

The OR-PAHs DGGE profile was composed by only two dominant bands (Fig. III.7B), RPb1 and RPb2, corresponding to *Tistrella* and *Sphingomonas* genus, whereas eight bands could be excised and sequenced from the OS-PAHs DGGE profile (Fig. III.7C and Table III.S2 in the supplemental material). It is important to point out that band RPb2 (OR-PAHs) was identical to SP7 (OS-PAHs) being close to *Sphingomonas* spp. Another three similar sequences from the sand, bands PA5, PA6 and PA8, were also related to *Sphingomonas* genus (Table III.3).

III.4.7 Clone libraries

As explained in the following section, to obtain an image of the most abundant genera present in each matrix community (Table III.3 and III.S3 in the supplemental material) approximately 70 clones were sequenced for each sample (OR and OS).

III.4.7.1 Oiled rock sample total community (OR)

The main bacterial groups found in OR were Classes *Alphaproteobacteria* (43%) (*Parvibacalum* and *Lutibacterium* genera), *Actinobacteria* (28%) (*Rhodococcus, Dietzia* and *Microbacterium* spp.) and *Gammaproteobacteria* (23%) (*Salinisphaera, Chromatiales and Alcanivorax* spp.) (Table III.2). The most important genus was *Rhodococcus* represented by seven different sequences accounting for 20% of the total library. Two of such sequences, with the highest frequencies, were identical to DGGE bands R1 and R2 respectively (Table III.3). Phylogenetic analysis placed them close to species *Rhodococcus fascians* DSM20669 (99 and 98% of similarity respectively). A minor presence of clones related to *Alcanivorax* spp. (3 out of 65) was found, and only one of them (1/65) was identical to *Alcanivorax borkumensis.* Members close to *Chromatiales* group, *Parvibaculum and Lutibacterium*

genera detected in the DGGE profiles (Tables III.S1 and III.S2 in the supplemental material) were also found in the clone libraries (7-12% of clones; Table III.3 and S3 in the supplemental material). *Salinispahaera* genus (97-98%) although not detected by DGGE constituted a high percentage of the library (9%). The *Bacteroidetes* group (6 %) was also found, represented by four different genera (Table III.S3 in the supplemental material).

III.4.7.2 Oiled sand sample total community (OS).

A higher species richness was found in the OS community compared to the one of the rocks (OR) (Table III.4). The main bacterial groups were Class *Alphaproteobacteria* (38% of clones, including twenty different genera), Class *Actinobacteria* (30% of clones, enclosing *Rhodococcus* (8%) and *Mycobacterium* (7%) genera) and Class *Gammaproteobacteria* (19% of clones). In contrast to OR, OS sample contained other minor representatives such as *Deltaproteobacteria*, *Planctomycetes* and *Chloroflexi groups*. *Bacteroidetes* group also presented a notable number of species (Table III.S3 in the supplemental material). *Lutibacterium anuloederans* (96-99%) clones, similar to bands S10 and S6 (Fig. III.7A), accounted for a 6%. The majority of clones related to *Rhodococcus* was again identical to DGGE band S1 (99% similarity to *R. fascians* DSM20669). Most of *Gammaproteobacteria* was close to sequences of uncultured *Chromatiales* identical to bands S8 (6%) and S9 (3%), while *Alcanivorax* was detected again in low abundance (4%) and only one clone out of 72 was identical to *A. borkumensis*.

III.4.8 Bacterial isolates

Alkane degrading strains isolated from the OR sample belonged exclusively to *Rhodococcus* and *Dietzia* genera from Class *Actinobacteria* (Table III.S4 in the supplemental material). In fact, 26 out of the 32 positive alkane degrading strains matched exactly with either R1=S1=RH1 or R2=S2=RH2 bands´ sequences from DGGE belonging to *Rhodococcus* genus (Fig. III.9A), whereas 3 out of 32 were related to genus *Dietzia*. *Rhodococcus* strains “type 1” and “type 2” were, respectively, identical to *Rhodococcus* sp. 5/1 (AF181689) and 99% similar to *Rhodococcus sp.* MBIC01430 (AB088667) (Table III.3). *Dietzia* related strains (e.g. PDR4 or PDR22), close to *D. maris* (99-100%) and *D. psychralkaliphila* (99-100%), migrated close to *Rhodococcus'* bands (Fig. III.9A). Both are *Actinobacteria* genus characterized by a high G+C content and thus stability in its 16S sequences which migrated longer in the DGGE gel. The different strains of *Rhodococcus* seemed to grow very close in hexadecane culture, being undistinguishable and difficult to isolate. DGGE helped to detect those non-pure cultures such as PDR 23 which were a

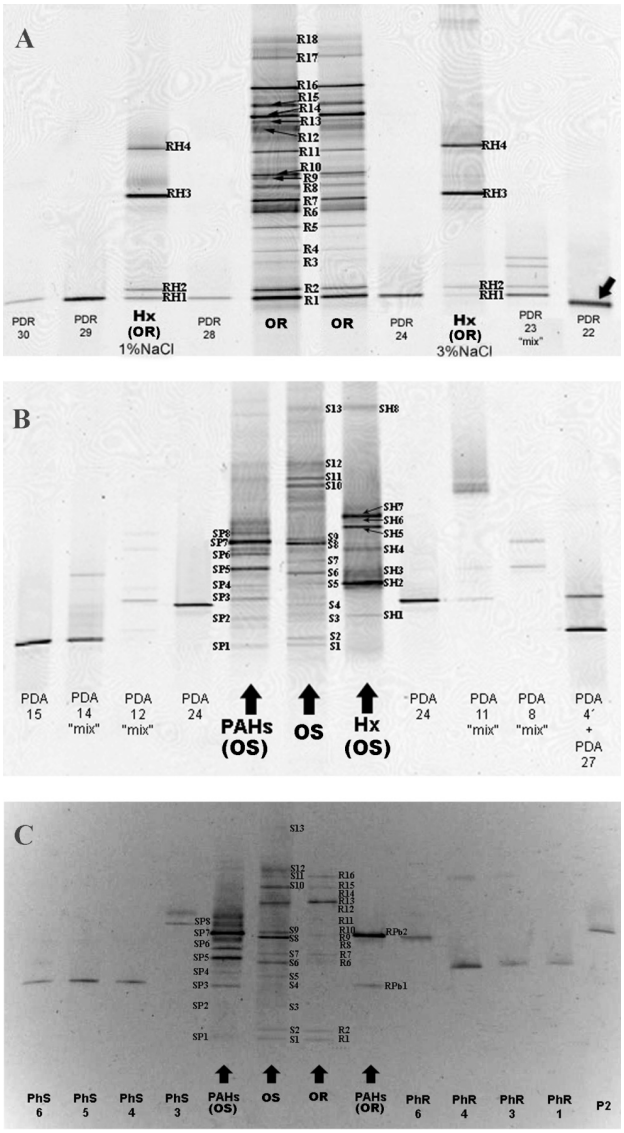

Fig. III.9.
DGGE of isolated alkane degrading strains playing major roles on *Prestige´*s crude oil degradation on rocks (A) and sand (B). The two upper bands of PDR23 "mix" are heteroduplex from the two *Rhodococcus'* sequences.
The few isolates related to PAHs degradation were compared with the DGGE profiles of sand and rock simultaneouslly (C).

mixture of the two *Rhodococcus* strains: "type 1" and "2" (Fig. III.9A). Strains were separated afterwards using marine agar where the different strains developed different colour and morphology. Although isolates, mainly from the enrichment cultures in hexadecane (PDA), also confirmed the dominance of *Rhodococcus* (16 out of 26; 80%) in the sand, a higher number of additional species related with alkane-degradation (e.g. *Gordonia, Erythrobacter*, *Stenotrophomonas* and *Alcanivorax* spp.; Fig. III.9B and Table III.S5 in the supplemental

material) could be isolated in the OS compared to the rocks (Fig. 9A and Table III.S4 in the supplemental material). Even though both isolates of *Erythrobacter* (99% similar to bands SH7 and R16, from the OS-Hx and OR DGGE profiles respectively) and *Stenotrophomonas* (identical to band SH8) were detected in the population related with alkane-degradation, no degrading ability could be confirmed, oppositely to *Dietzia*, *Rhodococcus*, *Gordonia* and *Alcanivorax* isolates which were able to grow on hexadecane as only source of C and energy (Table III.3).

All isolates related with PAHs degradation were obtained from phenanthrene enrichments (PhR and PhS strains) like *Sphingomonas*, *Pseudomonas stutzeri* and *Tistrella mobilis* (Fig. III.9C and Tables III.3 and III.2). One exception occurred with *Citreicella* strain RP3, which only could be isolated using 1/5 strength marine agar directly from fuel oil attached to rocks (OR). Aromatic degrading ability could be confirmed only in species of two genera, *Sphingomonas* and *Pseudomonas*. PAH-degrading strains of *Sphingomonas* were close to DGGE bands of OS-PAHs (SP5-SP8) and one of them was identical to bands RPb2 and SP7 (Table III.3). *Tistrella* isolates had identical sequence to DGGE band RPb1 (OR-PAHs) and therefore are related with the degradation of aromatics although no ability could be confirmed. Something similar occurred with *Citreicella* strain RP3, which had a 16S sequence close to DGGE bands R9-R10 and identical to band R11 and clone Rc10 (EU375056) from oiled rocks (Table III.3). The strain was close (99-100%) to *Citreicella* sp. 2-2A (AB266065), although no degrading activity could be observed in our strain with the methodology used.

III.5 DISCUSSION

III.5.1 Impact of the fuel over the microbial populations

The *Prestige* oil-spill did not affect bacterial abundances in the studied areas but induced deep changes in the trophic structure of bacterial communities. A similar situation was described after the *Nakhodka* oil spill, with a similar composition to the *Prestige* fuel, in the marine communities of the Japan Sea (Kasai et al., 2001; Maruyama et al., 2003). Although the communities were qualitatively different from non-oiled sand (NOS), those affected by the oil spill still conserved high species richness and diversity. These results agree with previous observations, in which the community diversity was dramatically reduced just after the pollution event, to progressively recover until pre-oiling levels, but with a different structure dominated by hydrocarbonoclastic bacteria (Roling et al., 2002; Hernandez-Raquet et al., 2006). In the present study, we could observe that although rocks and sand are quite different substrates, community composition were quite similar suggesting that fuel oil drives the structure of the affected communities. However, the higher species richness and diversity of OS communities detected by culture-dependent and independent methods, suggests that the environmental conditions in oiled rock surface, subject to daily contrasting temperatures and dryness, may require a more specialized microbial population to survive at such restricting conditions compared to the sand where a higher number of different bacteria can grow.

III.5.2 Predominance of taxonomical groups and microbial diversity

Most of previous studies have focused on the short-term effects of crude oil or its components on marine bacterial communities which usually became dominated by *Gammaproteobacteria* (Abed et al., 2002; Roling et al., 2002; Hernandez-Raquet et al., 2006) just after the oil-spill. In artificially oiled environments amended with nutrients, biodegradation rates were promoted and the first fast petroleum degradation processes, carried out by communities dominated by *Gammaproteobacteria* (e.g. *Alcanivorax*, *Cycloclasticus*, *Thalassolituus*…), were rapidly substituted by *Alphaproteobacteria* in less than a month (Roling et al., 2002). In the present work, as previously done after the *Nakhodka* oil spill in the Japan Sea (Kasai et al., 2001), we focused on the analysis of long term affected communities by heavy fuel oil. In the affected coasts of the Japan Sea, where natural attenuation proceeded slowly probably due to small amount of nutrients (Total N~0,1 mg l^{-1}), bacterial communities from oil paste were still dominated by *Gamma* and *Alphaproteobacteria* (Gram-negative) more than 12 months after the oil-spill (Kasai et al., 2001), indicating that oil from *Nakhodka* were still rich in those more biodegradable fractions due to slow degradation processes. However, *Alphaproteobacteria* and Gram-positive

Actinobacteria dominated our oiled-samples after the same time. Gram-positive bacteria do not respond to high hydrocarbon inputs (Margesin et al., 2003) and are never dominant just after an oil-spill, being detected in non-polluted areas (Macnaughton et al., 1999; Kloos et al., 2006) or in long weathered oil polluted environments (Quatrini et al., 2008). The differences observed between the molecular marker ratios of the original fuel oil and those of the oiled samples (Fig. III.4) are also consistent with the trends that follow biodegradation (Jiménez et al., 2006). The aromatic fraction exhibited a predominance of alkylnaphthalenes in the original oil that were almost lost in the collected samples mainly by water washing and evaporation (Fig. III.5B). However, microbial degradation was observed by the severe depletion of the n-alkanes fraction, even higher ones (Fig. III.5A), and the relative reduction of isomers with β-substituents such as the 2-/3-methyl phenanthrenes and dibenzothiophenes, within their respective series (Jiménez et al., 2006). This enrichment in more recalcitrant fractions of the fuel might explain the dominance of Gram-positive bacteria previously hypothesized as having roles in the degradation of such less biodegradable hydrocarbon classes (Quatrini et al., 2008).

Since the oil from *Nakhodka* had a similar composition to that from *Prestige* (heavy fuel) and time of sampling was the same, it seems likely that the higher amount of nutrients (0.20 - 0.25 mg l^{-1} of Total N of which 0.15 mg ml^{-1} are nitrate (Alvarez-Salgado et al., 2002)) supplied to the littoral of “Costa da Morte” (NW of Spain) by the NW Africa upwelling system (Figueiras et al., 2002) is the responsible for the observed differences in the biodegradation rates (Wrenn et al., 2006) and community composition.

Alcanivorax (Yakimov et al., 1998) dominates oil degrading communities when nutrients are supplied, but with normal levels of nutrients more diverse communities can exist (Kasai et al., 2002b; Roling et al., 2002). In a recent study we reported the presence of *A. borkumensis* in high numbers just after the *Prestige* oil-spill in sediments of “Ría de Vigo” (Alonso-Gutiérrez et al., 2008), where the alkane fraction was still abundant and had higher nutrient levels (0.6 mg l^{-1} of Total N) compared to “Costa da Morte” (0.20-0.25 mg l^{-1} of Total N) existed (Alvarez-Salgado et al., 2002). In this sense, members of the well described hydrocarbonoclastic genus *Alcanivorax* were still present in oiled rocks and sand but in very low numbers, as occurred at marine environments long time affected by heavy fuels (Kasai et al., 2001; Quatrini et al., 2008).

DGGE profile differences among the different trophic populations detected and the reduction in number of bands respect to the total profiles (Table III.4), suggested an important specialization of species roles in the biodegradation process of the fuel in both matrices.

III.5.3 Population related to alkane degradation

Culture independent and dependent analysis showed *Actinobacteria,* mainly *Rhodococcus* species, as the key alkane-degrading group of bacteria. *Rhodococcus* has been associated with the degradation of *n*-alkanes up to C_{36} (Whyte et al., 2002) and branched alkanes (Whyte et al., 1998) which are particularly abundant in the *Prestige* fuel (Diez et al., 2005). It is well known that *Rhodococcus* is a genus with a remarkable metabolic diversity (Larkin et al., 2005) and able to produce biosurfactants which can enhance not only the bioavailability of fuel components, but also the growth of other degrading bacteria (Iwabuchi et al., 2002; Murygina et al., 2005). *Dietzia* and *Microbacterium* species, detected exclusively at OR clone library, have been respectively described as alkane degraders including branched alkanes (Rainey et al., 1995; Yumoto et al., 2002) or related to oil degradation in hydrocarbon-polluted sites (Schippers et al., 2005; Hernandez-Raquet et al., 2006). Since *Dietzia, Microbacterium* and *Rhodococcus* belong to Class *Actinobacteria,* some common characteristic might explain the dominance of this group at the oiled rock (OR). In this sense, an interesting study which compared the different uptake of hydrocarbons by two *Pseudomonas* and *Rhodococcus* strains (Van Hamme and Ward, 2001), clearly showed how the hydrophobic surface developed by the latter allowed the growth of *Pseudomonas* attached to the oil surface increasing its degrading capacity. This capacity might explain the relative major presence of *Rhodococcus* in the oiled rocks compared to the sand since this ability could represent an important advantage to survive at such harsh environment.

Several *Xanthomonadaceae* members were detected in this study associated to the degradation of alkanes since they were detected in Hx- and PAHs- DGGE profiles (Fig. III.7B, C and Table III.3). In fact, with the exception of *Dokdonella koreensis* not previously related neither to alkane degradation nor to oil-polluted sites, other species (*Pseudoxanthomonas spadix* and *Stenotrophomonas maltophilia)* were previously associated with oil degradation and surfactant production (Chang et al., 2005; Young et al., 2007). Alphaproteobacterium genus *Erythrobacter*, commonly encountered after first fast degradation processes (Macnaughton et al., 1999; Roling et al., 2002), was detected as well as part of the population related with alkane degradation (OS-Hx). Since no ability to degrade hydrocarbons could be confirmed for any of these strains, they might play secondary roles in the degradation of this fraction in collaboration with *Actinobacteria*, mainly in the oiled sand where a higher diversity existed (Table III.4).

III.5.4 Populations related to aromatic degradation

As explained in the Introduction the metabolism of PAHs is a more complex process than the metabolism of the aliphatic fraction and frequently, the resulting oxidised PAHs require

the intervention of another bacterial strain, which plays an important role in its degradation but cannot be detected as an aromatic hydrocarbon degrader.

Strains related to *Sphingomonas* were isolated as phenanthrene-degrading strains in both matrices (Table III.3). The aromatic-degrading *Sphingomonas* isolated in this study were quite different from the single clone (Sc29) detected and thus no significance importance of these strains can be attributed to the *in situ* degradation of the fuel mix. However, two DGGE bands (R12 and R17) and 4,5% of OR library were related to *Sphingopyxis* and *Novosphingobium*, respectively (Table III.3); two genera formerly considered as *Sphingomonas* (Takeuchi et al., 2001). Moreover, oil paste from a beach affected by the *Nakhodka* oil-spill presented sequences related to *Sphingomonas subartica* (100%) which were proposed as playing roles in PAHs degradation (Kasai et al., 2001). Interestingly, other members of this family could play a central role in the degradation of the aromatic fraction of *Prestige* fuel in both matrices such as *Lutibacterium anuloederans* (95-100%) (two DGGE bands and around 7 % of clones of each sample; Table III.3). Although we could not isolate any strain of this species, *L. anuloederans* was described as a 2- and 3-ring PAH degrading bacteria which had a higher efficiency in the uptake of aromatics than *Cycloclasticus* species (Chung and King, 2001). Similar observations have been recently observed in a complete study performed in the Thames estuary (UK), where *Cycloclasticus* seemed to dominate seawater microcosms spiked with single PAHs except those containing fluorene where a sequence close to *L. anuloederans* was found (McKew et al., 2007).

Several clones close to different species of *Mycobacterium* spp. were detected in the oiled sand. All clones were different among them but some were close to the species *M. frederiksbergense* (98-100%), which has previously been described to mineralize the PAHs phenanthrene, fluoranthene and pyrene (Willumsen et al., 2001). *Mycobacterium* species are specialized in the degradation of adsorbed PAHs in soils (Bastiaens et al., 2000). However, the most frequently used PAH-degrading bacteria isolation methodology, including the one we used, is conducted in liquid media with agitation (Asconcabrera and Lebeault, 1993), so those strongly adhering bacteria may tend to escape from conventional isolation techniques (Vanloosdrecht et al., 1987; Bastiaens et al., 2000) as occurred in the present study. Fortunately, other research groups could obtain aromatic degrading isolates of *Mycobacterium* spp. from pyrene enrichments of *Prestige* polluted samples which were really close to OS (M. Grifoll, pers. comm.; http://otvm.uvigo.es/vertimar2007/comunicaciones/VEM2004-08556_ortega.doc).

Cycloclasticus has been proposed as the main PAHs degrader in many previous studies including some after the *Nakhodka* oil spill (Maruyama et al., 2003; McKew et al., 2007). However, those studies analyzed communities from seawater samples just after the oil-spill at first fast degradation processes when this and other *Gammaproteobacteria,* like

Alcanivorax, dominated the community, while oiled matrices under study have already suffered from weathering and biodegradation processes at the time of sampling. The ability of *L. anuloederans* and *Mycobacterium* spp. to degrade fluorene and pyrene, which are considered specially recalcitrant components of the fuel (Wammer and Peters, 2005), might explain the high abundance of this bacteria at heavy fuel devoid of the most easily biodegradable fractions.

III.5.5 Community dependent degrading activities

Low or no growth in hydrocarbon-agar was detected for pure isolates of *Rhodococcus* (1 and 2 type), *Tistrella* (PhS5A) and *Citreicella* (RP3). However, since these bacteria were related with the degradation of fuel fractions in this and other studies (Table III.3), a deeper analysis of their degrading capacity was performed. We studied two different hypotheses, that either i) these strains might need some kind of "growth factor" or "cofactor" (e.g. vitamins) to develop their degrading capacity or ii) have no degrading capacity but consume second metabolites produced by other hydrocarbonoclastic bacteria. In both cases we checked if isolated members of the community were able to provide this requirements.

Tistrella mobilis was previously detected in seawater microcosms spiked with PAHs mixtures. In that case, it was hypothesised that this species could have a secondary role in the degradation of catabolic intermediates of aromatic compounds, owing to their appearance only after 6 or 9 weeks of incubation (McKew et al., 2007). In the present work, isolates of this species (strain PhS5A) could not grow as pure isolates on PAHs with the methodology used. However, in combination with the degrading *Sphingomonas* isolated (strain PhS5B), a big growth of *Tistrella* could be observed (Fig. III.10A,B). Our observations suggest the existence of a metabolic collaboration between them, where *T. mobilis* probably grow on second metabolites derived from the phenanthrene degradation carried out by *Sphingomonas*. Tests performed in mineral medium with different PAHs or their most common catabolites produced during biodegradation, showed that *Tistrella* is an opportunistic bacteria which grows on second metabolites produced by *Sphingomonas*. *Tistrella* alone was not able to degrade even Naphthalene but degrade quite well Catechol (Fig. III.10E), which is a common intermediary metabolite of many aromatic degrading pathways surely produced by the *Sphingomonas* strain. This feature could explain why this non-degrading bacteria grows in such high numbers in the presence of aromatic degraders and why has been associated with aromatic degrading communities at the end of the degradation process (McKew et al., 2007).

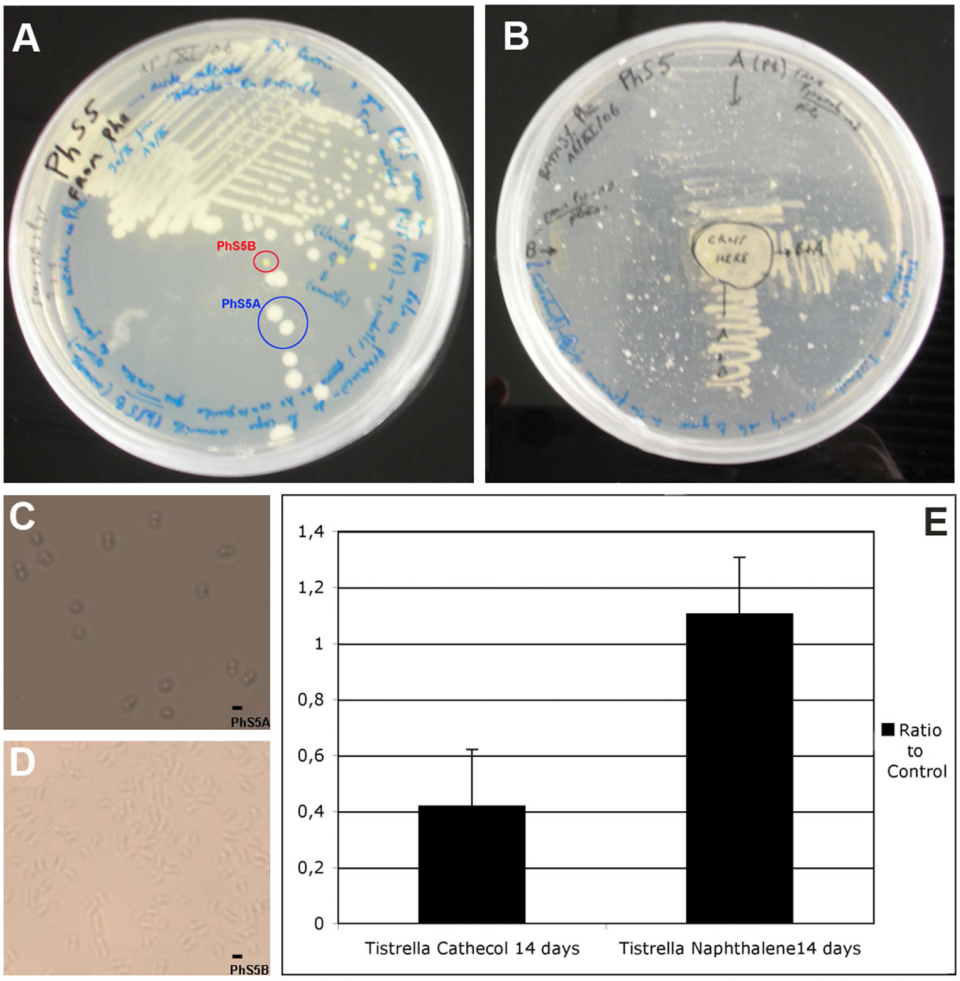

Fig. III.10. A: Cultivation on marine agar of a mixed culture of *Tistrella* (strain PhS5A; EU375009) and *Sphingomonas* (PhS5B; EU375008) previously grown in Arabian crude oil. Yellow colonies from the PAHs degrader *Sphingomonas,* were less abundant than white colonies from *Tistrella*. B: Even though *Tistrella* has no degrading capacity, it could grow in big numbers in phenanthrene-agar when grew next to *Sphingomonas* (see higher density from cross point in agar plate). C: Microphotography of *Tistrella* strain PhS5A (coccoidal cells). C: Microphotography of *Sphingomonas* strain PhS5B (bacillar cells). E. Degradation extent of Naphthalene (the simplest of the aromatic compounds) and Catechol (a common intermediary compound in aromatic biodegradation processes) by pure isolates of *Tistrella*. No degradation was observed for Naphthalene but a significant degradation respect to the control was observed for the Catechol.

Strain RP3, detected by culture dependent and independent methods in the oiled rocks, was close (99-100%) to *Citreicella* sp. 2-2A (AB266065). This strain, firstly isolated from seawater as a PAHs-degrading bacterium by Kodama, Y. and Watanabe, K. in 2006

(unpublished results), was detected with identical sequence as "Uncultured *Roseobacter* sp. (DQ870519)" in supralittoral rocks affected by the *Prestige* oil-spill more than 400 km far from our sampling site (Jiménez et al., 2007). However, no growth was observed in the aromatic-degradation test performed to RP3 in mineral medium with Phenanthrene. To check if RP3 need some "cofactor" to develop its degrading capacity, we compared its degradation capacity of the aromatic fraction of Arabian crude oil in the presence and absence of yeast extract (YE) or alkane degrading strains. YE added to this mineral medium enabled strain RP3 to degrade several PAHs as shown in Fig. III.11C. The tiny amount of

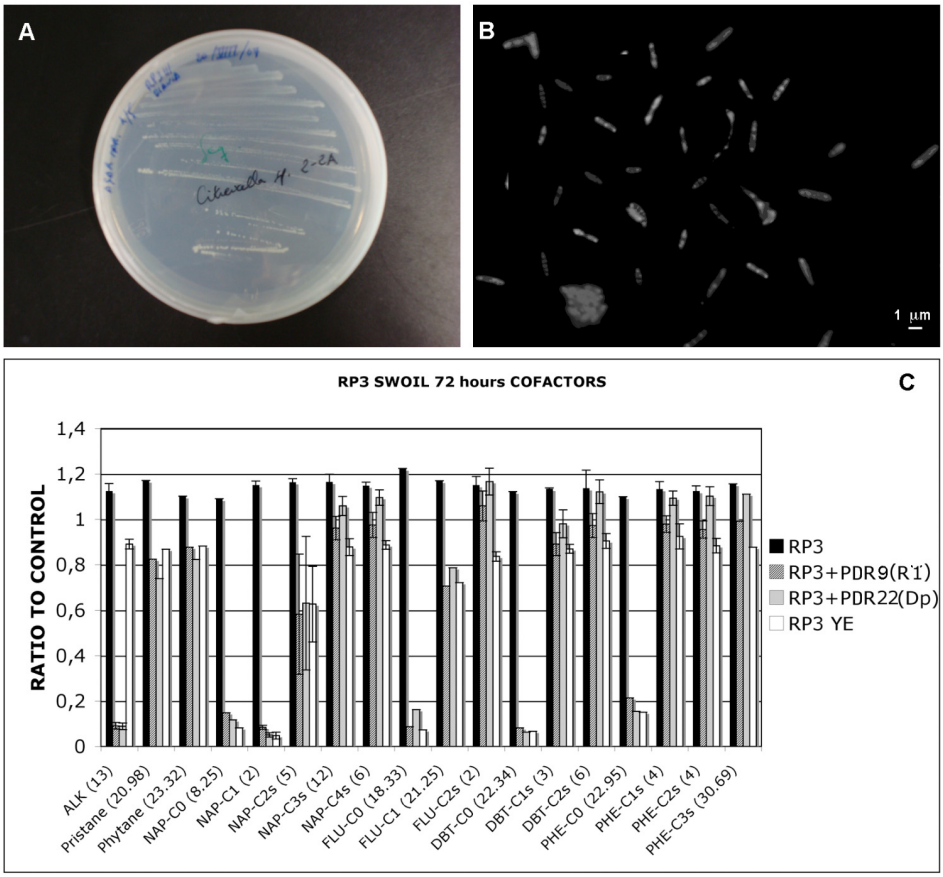

Fig. III.11. A: White colonies of *Citreicella* (strain RP3; EU375119) growing in MA 1/5. B: Microphotography of strain RP3 (spindle-shaped cells). C. Degradation extent, relative to control, of the aromatic fraction of the Arabian crude oil used as substrate for RP3 cells in the presence and absence of "cofactors" (YE (yeast extract), *Dietzia* (PDR22) or *Rhodococcus* `type 1´ (PDR9) strains).

YE added (0.01%) was unable to support bacterial growth by itself but provided the medium with a myriad of vitamins and other growth factors not present in the sea water. The same

degrading activity was observed when RP3 was cocultivated with strain PDR22 (*Dietzia* sp.) or PDR9 (*Rhodococcus* sp.). Therefore, since no degradation was observed for RP3 in the absence of YE or other strains, we concluded that genus *Citreicella* needs a "cofactor" to develop its PAHs degrading capacity, which is provided by the bacterial community associated to fuel.

Rhodococcus strains (PDR9 and PDA27) corresponding to bands type 1 and 2 also need some requirements, although not as stringently as *Citreicella*. When *Rhodococcus* strains were grown next to *Dietzia* in mineral medium and alkanes, its growth was much more active suggesting that degrading capacity of this abundant species in the community is enhanced by the presence of other bacteria that provide "cofactors". (Fig. III.12).

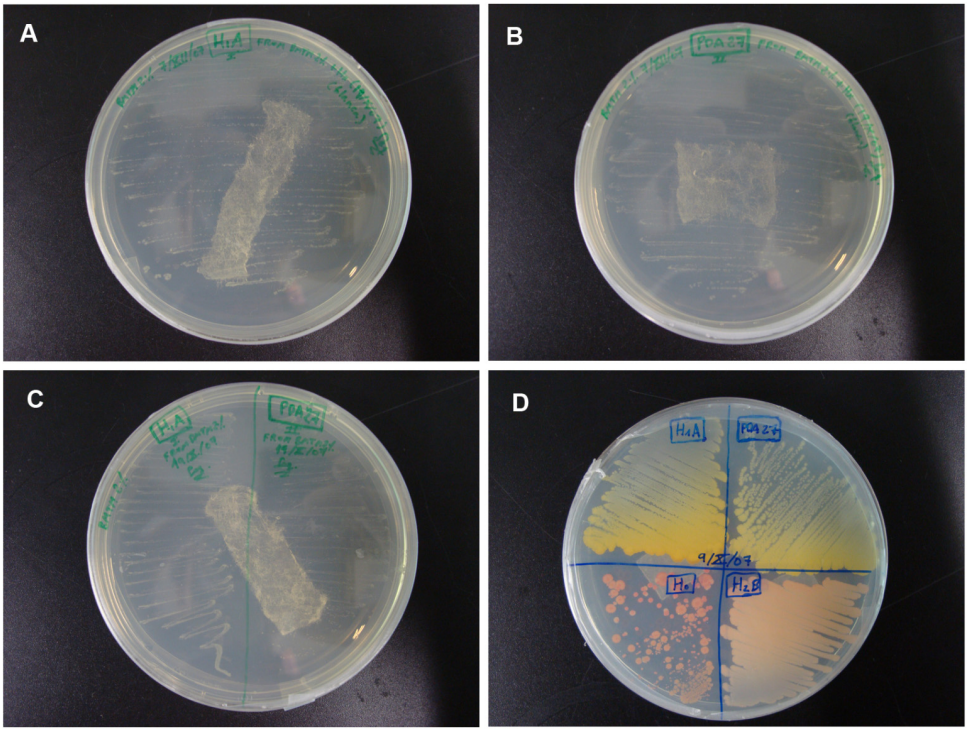

Fig. III.12. Strains grown in mineral agar with hexadecane as the only source of C and Energy; A: Monoculture of *Rhodococcus* "type 1" (strain PDR9; EU374994). B: Monoculture of *Rhodococcus* strain "type 2" (strain PDA27; EU374971). C: Coculture of the two *Rhodococcus* PDR9 and PDA27. D: Coculture of *Rhodococcus* (PDR9 and PDA27) and *Dietzia* strains (PDR4; EU374997 and PDR22; EU374995)). Only in this last case a strong growth of *Rhodococcus* strains could be observed. Besides it, colour and density of its colonies increased towards *Dietzia* indicating that the latter provides some kind of "cofactor", probably a vitamin, which enhances the growth of *Rhodococcus*.

III.5.6 Ubiquity of bacterial species

Sequences close to *Rhodococcus*, *Chromatiales*, *Rhodobacteriaceae, Roseobacter-* (*Citreicella*) and *Erythrobacter* detected at both samples under study (OR, OS), were respectively identical to Accession numbers DQ870544, DQ870518, DQ870525, DQ870519 and DQ870538 retrieved from another cobblestone beach affected by the *Prestige* (Jiménez et al., 2007). Next to it, several sequences found in our clone libraries shared at least 99% similarity with other DGGE bands detected in that study. What is more interesting is that DGGE profiles of that study became more similar to our OR and OS, at advanced stages of the degradation process (Jiménez et al., 2007) which agreed with our hypothesis. Although samples were taken from rock surfaces similar to our OR, the beach was situated more than 400 km far from our sampling point. Therefore, it seems that conclusions derived from the present work can be applied to other parts of the Spanish coast affected by the *Prestige* oil-spill.

III.5.7 Bioremediation amendments

Mycolic acids, very-long-chain (C30–C90) α-alkyl, β-hydroxy fatty acids, are major and specific constituents of a distinct group of Gram-positive bacteria, classified in the suborder *Corynebacterineae*, which includes genera detected in the present study such as *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia* and *Rhodoccoccus*. As opposed to Gram-negative bacteria, such *Pseudomonas* or *Alcanivorax*, that dominates at first fast petroleum degradation processes (Kasai et al., 2002b), members of this group are never dominant at such stages (Margesin et al., 2003; Quatrini et al., 2008) being detected with higher frequency at resource limited environments where could play a key role in the *in situ* degradation of more recalcitrant components at long term intervals after an oil spill (Quatrini et al., 2008). Unusually, these gram-positive bacteria contain an outer permeability barrier that may explain both the limited permeability of their cell walls and their general insusceptibility to toxic agents (Gebhardt et al., 2007) which has been related with an enhanced biodegradation capacity (Linos et al., 2000; Lee et al., 2006). Therefore, the addition of mycolic acids to bioremediation amendments applied to coasts with ecological features close to those of the affected Spanish areas and long term affected by a similar contaminant such as the *Prestige* fuel, might be a good strategy to enhance *in situ* degradation.

Chapter IV:

Alkane degrading ability of autochthonous *Corynebacterineae* detected as playing key roles in the degradation of the *Prestige´s* oil-spill.

Manuscript in preparation

IV.1 ABSTRACT

Two *Rhodococcus* (H1 and H2) and two *Dietzia* (H0 and H0B) strains isolated from the *Prestige* oil-spill, were able to degrade and grow on different chain-length *n*-alkanes up to C_{37} from Arabian crude oil used as the only source of C and energy. *Dietzia* strains also degraded branched alkanes (e.g. pristane and phytane) and strain H0B produced hexadecene when growing on hexadecane. Therefore, *Dietzia* has enzymes able to catalyse novel reactions against the alkane molecule. alkB and CYP153 (cytochrome P450) gene homologues, characterized as alkane hydroxylases, were amplified with degenerate primers, cloned and sequenced on both genus. Only one copy of alkB gene was detected in each genus, while CYP153 genes presented a higher diversity in *Dietzia* (three different genes in each strain compared to only one copy in *Rhodococcus*). This genetic diversity might be related with the singular degrading activities detected in *Dietzia* strain H0B. alkB and CYP153 genes from both genus appear to be constitutively expressed based on alkane degradation and RT-qPCR assays. Constitutively expression and diversity of alkane degrading genes is in agreement with previous observations that suggested Gram-positive bacteria act as *K* strategists during degradation processes.

IV.2 INTRODUCTION

Fuel and oil-derived compounds are one of the most common pollutants in the oceans and bioremediation, defined as the act of adding materials to contaminated environments to accelerate the natural biodegradation processes, is accepted as an appropriate response tool oil spill (Swannell et al., 1996; Venosa and Zhu, 2003). Fuel oils are complex mixtures of four groups of compounds (aliphatics, aromatics, resins and asphaltenes). The last two fractions are considered to be recalcitrant but microbial catabolic pathways for their biodegradation have already been investigated (Van Hamme et al., 2003) for the other two (with toxic effects). However, very little information about the vast majority of such indigenous marine bacteria is available, and more efforts should be made to isolate key-degrading bacteria which could be further investigated concerning their physiology requirements to favor their growth after an oil spill (Harayama et al., 1999; Van Hamme et al., 2003; Harayama et al., 2004). Although many groups of Gram-positive bacteria are known for its enzymatic versatility and great biotransformation and biodegradation potential of organic compounds, the information about its role in degradation processes at oil polluted environments is scarce (Larkin et al., 2005).

Although the ability to grow on medium (MCL) to long-chain-length (LCL) *n*-alkanes is a very common property of Gram-negative as well as Gram-positive bacteria, only members of the former, like *Pseudomonas putida* GPo1 (van Beilen et al., 2001) and more recently *Alcanivorax borkumensis* SK2 (Hara et al., 2004; Sabirova et al., 2006), has been studied in depth with respect to enzymology, genetics, as well as potential applications (van Beilen et al., 2002; van Beilen and Funhoff, 2007). In this sense, the most extensively characterized pathway for aerobic alkane-degradation are encoded by OCT plasmids carried by Gram-negative bacteria like *Pseudomonas* (van Beilen et al., 1992; van Beilen et al., 2001) where integral membrane non-heme diiron monooxygenases of the AlkB-type allows to grow on *n*-alkanes with carbon chain lengths from C_5 to C_{16} (van Beilen and Funhoff, 2007). These particulate alkane hydroxylases (pAHs), encoded by alkB genes, are found as well in a wide range of *Proteobacteria* and *Actinomycetales*. These pAHs requires two soluble electron tranfer proteins, named rubredoxin and rubredoxin reductase, which transfer e^- to the active site of the enzyme oxidizing alkanes to 1-alkanols. These products are then further metabolized by alcohol and aldehyde dehydrogenases to fatty acids that enter the central metabolism via the b-oxidation pathway. The other major group of enzymes related with terminal oxidation of medium (MCL) to long-chain-length (LCL) *n*-alkanes up to C_{16}, are cytochromes P450, mainly from family CYP153, which has been recently proposed as common in alkane-degrading eubacteria lacking integral membrane hydroxylases (van Beilen et al., 2006).

The present study reports the alkane degrading activity and its genetic basis from indigenous *Corynebacterineae* (genus *Rhodococcus* and *Dietzia*) proposed as playing key roles in the degradation of hydrocarbons at rocky shorelines affected by the *Prestige* oil-spill one year before (Chapter III). DAPI countings and GC-MS were used, respectively, to describe the growth-curve and to characterize the degree of fuel oil degradation of each strain through time under different conditions. Study of alkane degrading encoding genes homologous to alkB and CYP153 revealed new sequences from which primers were designed to study its expression under different conditions using RT-qPCR.

IV.3 MATERIALS AND METHODS

IV.3.1 Bacterial strains

Gram-positive actinobacteria from suborder *Corynebacterineae* were detected by culture dependent and independent methods (DGGE, clone libraries) as a major, naturally occurring, group in the degradation of different fractions of the *Prestige* oil accumulated at supralittoral rocky shorelines of the Spanish coast 12 months after the accident (Chapter III). Four strains, identified in that study as two *Rhodococcus* spp. (PDR9 (EU374994) and PDA27 (EU374971)) and two *Dietzia* spp. (PDR4 (EU374997) and PDR22 (EU374995)), were renamed as *Rhodococcus* strains H1 and H2 and *Dietzia* strains H0 and H0B, respectively, and subjected to a deeper phylogenetic analysis based on an almost complete fragment of 16S rRNA genes using primers F27 to R1492 as previously described (Alonso-Gutiérrez et al., 2008).

IV.3.2 Growth conditions

To obtain sufficient cell population for alkane degradation assays bacteria were cultured for 72 hours on seawater collected from the Pacific Ocean, 300 km off the coast of Tokyo (Japan) and supplemented with 1g of NH_4NO_3, 0.2 g of K_2HPO_4, 12 mg of $FeCl_3$ and 0.1 g of yeast extract per liter of seawater (SWsupp, hereafter) to which 0.5 % of a C source (namely pyruvate, hexadecane or hexadecanol) was added.

For nucleic acid extraction, cells were grown on BMTM-agar 2%NaCl (Alonso-Gutiérrez et al., 2008) using pyruvate (0.5%) or hexadecane, as the only source of C and energy, until early stationary phase.

IV.3.3 Alkane degradation activity assay

After pregrowth on each of the C sources, cells were centrifuged and resuspended in 3 mls of SWsupp to reach a high-density population ($10^{9\text{-}10}$ cells/ml). 1000 ppm of Arabian crude oil or the tested alkane (C_{14} or C_{16}) was added to the cells and extracted for GC-MS analysis after different incubation periods.

All strains were tested for its ability to degrade the different oil fractions, growing them on Arabian crude oil during 68 hours in 3 ml of SWsupp (Fig. IV.3) and checking the degree of oil degradation using gas chromatography-mass spectrometry (GC-MS). Initial inocula consisted on 150ml of stationary-phased grown bacteria and after the incubation period of 68 hours, hydrocarbons were extracted twice from the cultures with an equal volume of dichloromethane. Sodium sulfate was added to the dichloromethane extracts to dehydrate, and the supernatants were concentrated to approximately 100 ml by N_2 purging. The concentrated extracts were subjected to GC-MS using an Agilent 6890A Gas chromatograph with an Agilent 5973 mass selective detector (Agilent Technologies, USA) equipped with an Ultra 2 fused silica capillary column (25 m in length, 0.2 mm in diameter and 0.33 mm in film thickness; HP 19091B-102, Agilent Technologies, USA). The temperatures of the injection port, transfer line, MS source and quadrupole were maintained at 250, 250, 230 and 150˚C, respectively. The column temperature was increased from 80˚C to 320˚C at a rate of 5˚C/min for the first 48 min, and then kept at 320˚C for the next 20 min. Helium was used as the carrier gas at a constant pressure of 20 psi. All peak areas for hydrocarbons obtained with the GC-MS selected ion monitoring were normalized by dividing the peak area for that of 17a(*H*),21b(*H*)-hopane (Prince et al., 1994).

Alkane degrading activity of strains H1 (*Rhodococcus* sp.) and H0B (*Dietzia* sp.), pregrown on different C sources (pyruvate, hexadecanol or hexadecane), were measured using tetradecane (C14) or hexadecane (C16). 1000 ppm of the tested alkane (C_{14} or C_{16}) was added to the high-density cell culture and extracted for GC-MS analysis after different incubation periods up to 6 minutes. To confirm if the alkane degrading capacity of the strain was or not inducible, chloramphenicol (an antibiotic which inhibits the synthesis of proteins at translation level) was added to cells simultaneously with the tested HC. Strains H1

and H0B (previously checked as sensitive to this antibiotic at 0.1 g L^{-1}) could only degrade the added HC if the degrading enzymes had been previously expressed in the pregrowth period. The amount of the tested hydrocarbon which remained after a long incubation period (days) at 25ºC, and the metabolites produced were measured by GC-MS after extraction with dichloromethane (DCM) as mentioned above.

IV.3.4 DAPI counts

Different dilutions, in sterile water, of the original culture were fixed in a final volume of 1ml of 4% Paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, Japan). Fixed cells were stained using 1µg·ml^{-1} of 4'-6-Diamidino-2-phenylindole (DAPI) and filtered using Polycarbonate black membrane filter (0.2 mm pore size; Advantec Toyo, Ltd., Japan). The filters were examined under 100X amplification with a fluorescence microscope (Olympus IX81).

IV.3.5 Nucleic acid extraction

Cell paste was boiled on steril Milli-Q H_2O for 10 minutes to extract DNA from bacteria. For RNA isolation, cell paste was collected from the Petri plates (see growth media) on DEPC cleaned tubes and homogenized with Trizol reagent (Invitrogen, CA, USA) following the manufacturer's protocol with addition of three freeze-thawing cycles to better break cell walls. RNA was treated with Turbo-DNA free kit (Ambion, CA, USA) to remove contaminating DNA. Final concentration and purity were measured using NanoDrop (Thermo Fisher Scientific, MA, USA).

IV.3.6 Construction of gene clone library

PCRs were performed in a volume of 50 µl containing 1.25 U of Taq (TaKaRa ExTaq™ Hot Start Version; TaKaRa Bio Inc., Otsu, Siga, Japan), 1X ExTaq Buffer (2mM $MgCl_2$), 200 µM of each deoxynucleoside triphosphate, 100 ng of template DNA and 0.5 µM of primers alkB1-f/-r (Kloos et al., 2006) or P450-fw1/-rv3 (van Beilen et al., 2005) for the amplification of alkB and CYP153 gen homologs respectively. The reaction mixtures were subjected to the following thermal cycling parameters: (i) a hot start (9 min at 95ºC); (ii) 30 cycles, with one cycle consisting of 30 seconds at 96°C for denaturation, 30 seconds at 58°C for annealing, and 1 min at 72°C for elongation; and (iii) a final extension step of 10 min at 72°C. The thermal profile used for CYP153 genes was exactly the same but, in order to avoid appearance of unspecific bands, a touchdown thermal profile protocol was carried out during the first 10 cycles of step (ii), where the annealing temperature was decreased by 1°C per cycle from 65°C to 60°C followed by 20 additional cycles at 60ºC of annealing temperature. These cycles were performed with a Perkin-Elmer GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA).

PCR products were separated by electrophoresis in a 1% (w/v) agarose gel in 1×TAE buffer stained with ethidium bromide and photographed immediately under UV light using Gel Doc XR system and Quantity One software (Bio-Rad, Hercules, CA). 100 and 50 bp ladders were used as size markers (Promega).

Those PCR products with the expected size (around 550 bp and 350 bp for alkB and CYP153 genes respectively), were cloned into pCR®2.1-TOPO® vector and transformed into competent *Escherichia coli* TOP 10F´ cells, following manufacturer's protocol of TOPO T/A Cloning Kit (Invitrogen). Screening of clones with insert of the correct size was performed using primers M13F and M13R. Plasmid DNA (of 10 transgenic clones per gene and strain) was isolated following routine procedures stablished at our laboratory.

IV.3.7 Sequencing and Phylogenetic analyses

Excess of primers and dNTPs was removed by enzyme digestion using 10 and 1U of ExoI and SAP, respectively (Amersham Biosciences) at 37°C for 1 hour followed by inactivation at 80°C for 15 min.

Sequencing was accomplished using the ABI PRISM™ Big Dye Terminator Cycle Sequencing

Ready Reaction Kit (version 3.1) and an ABI PRISM™ 3700 automated sequencer (PE Applied Biosystems, CA, USA) following the manufacturer's instructions. Cloned alkB and CYP153 genes´ fragments were sequenced in both directions using vector primers M13F and M13R. The sequences were inspected for the presence of ambiguous base assignments, hand edited using Edit View (Applied Biosystems) and FinchTV (Geospiza Inc.) and finally assembled using GeneJockeyII (Taylor P.L. 1995, BIOSOFT) into a single consensus sequence. After that, the sequences were examined with the BLAST search alignment tool comparison software (BLASTN, BLASTx and tBLASTx) (Altschul et al., 1990) to detect the closest bacterial sequences within the GenBank database.

Amino acid sequences were inferred from nucleotide sequence data using the EMBOSS: Transeq program of EMBL-EBI (European Bioinformatics Institute, http://www.ebi.ac.uk/) and aligned using MAFFT: http://align.bmr.kyushuu.ac.jp/mafft/online/server/ (Katoh and Toh, 2008) with reference ones obtained from GenBank. The alignment obtained was edited in MacClade program (Maddison and Maddison, 2003) and directly transferred to version 4.0b10 of PAUP*software (Swofford, 2000). ProtTest software version 10.2 (Abascal et al., 2005) was run as a guide to determine the best-fit maximum likelihood (ML) model for the edited alignment. ProtTest examines models of protein evolution like Blosum62, Dayhoff, JTT etc. The best-fit models of protein evolution, calculated by ProtTest were incorporated into software PHYML (Guindon and Gascuel, 2003), which uses a single, fast and accurate algorithm to estimate large phylogenies by ML. Finally, the trees created by PHYML were edited using TreeViewX (Page, 1996).

IV.3.8 alkB & CYP153 expression studies

Expression of alkB and CYP153 genes isolated from *Rhodococcus* sp. H1 and *Dietzia* sp. H0B was assessed by Real-Time PCR (qPCR). RNA from hexadecane- and pyruvate-grown bacteria was isolated as described above and five µg from each were used to obtain cDNA by the Superscript II Reverse Transcriptase and random primer (Invitrogen) following the manufacturer's instructions. qPCR assays were performed using the 7300 Real Time PCR System (Applied Biosystems) with specific primers (Table IV.1). Each primer (0.5 µl; 10 µM) and the cDNA template (1 µl) were mixed with 12.5 µl of SYBR green PCR master mix (Applied Biosystems) in a final volume of 25 µl. The standard cycling conditions were 95 ºC for 10 min followed by 40 cycles of 95 ºC 15 s and 60 ºC 1 min. The comparative CT method (2-ΔΔ CT method) was used to determine the expression level of analyzed genes (Livak and Schmittgen, 2001). The expression of the candidate genes (alkB and CYP153) was normalized using DNApolIV primers (Sharp et al., 2007), able to amplify this housekeeping gene in our strains (H1 and H0B). Fold units were calculated dividing the normalized expression values of hexadecane-grown cells by those of the pyruvate-grown bacteria. Strain cultivation, RNA extraction and RT-PCR were performed twice in two independent experiments. For each gene and strain, three different q-PCR lectures were performed.

IV.3.9 Nucleotide sequence accession numbers

The nucleotide sequences identified in this study have been deposited in the GenBank database under accession numbers: FJ435349-50 (16S); FJ435353-55 (alkB) and FJ435356-62 (CYP153).

Table IV.1. Primers used in this study.

Primer	Sequence (5´-- 3´)	Bases number	Product size	Reference
alkB-1f	AAYACNGCNCAYGARCTNGGNCAYAA	26	≈550bp	(Kloos et al., 2006)
alkB-1r	GCRTGRTGRTCNGARTGNCGYTG	23		
CYP153 (P450fw1)	GTSGGCGGCAACGACACSAC	20	≈339 bp	(van Beilen et al., 2005)
CYP153 (P450rv3)	GCASCGGTGGATGCCGAAGCCRAA	24		
DNA pol IV fw	GACAACAAGTTACGAGCCAAGATC	24	≈27 bp	(Sharp et al., 2007)
DNA pol IV rv	CCTCCGTCAGCCGGTAGAT	19		
AlkB_H1_fw	GTGTTCGGTCCGATCGTATT	20		
AlkB_H1_rv	TCACGATGTGATCGGAGTTC	20	172 bp	This study
AlkB_H0B_fw	CTGATCGTCCAGGCAATCTT	20		
AlkB_H0B_rv	GCTGCAGGTGGTACAGGAAG	20	172 bp	This study
CYP153_H1_fw	CGATCAGTTCGACAAGCTCA	20		
CYP153_H1_rv	TGCGTACCACATGACCACTT	20	169 bp	This study
CYP153_H0_C4_fw	CGAGAAGCTCAAGTCCAACC	20		
CYP153_H0_C4_rv	CACATCACGACCTTGTCACC	20	153 bp	This study
CYP153_H0_UN_fw	CCAGTTCGAGAAGCTCAAGG	20		
CYP153_H0_UN_rv	CTGACGCATACCACATGACC	20	170 bp	This study

IV.4 RESULTS AND DISCUSSION

IV.4.1 Bacterial strains

The almost complete 16S rRNA gene from the four alkane-degrading strains under study showed that *Rhodococcus* strains H1 and H2 were both closer to *R. fascians* (99-100%) than to any other species. Although described as a phytopathogen (Crespi et al., 1992), it might have attained its alkane degrading capacity by the mobilization (horizontal transfer) of large linear plasmids which is a common feature of this genus (Larkin et al., 2006). In fact, plasmids sequenced from *R. erythropolis* PR4, which harboured many ORFs related with alkane degradation, were mosaic structures that might have resulted from repeated recombinations, insertions, deletions and transpositions (Sekine et al., 2006). Another common feature among *Rhodococcus* is the production of effective surfactants which can enhance the bioavailability of fuel components and growth of HC degrading bacteria (Iwabuchi et al., 2002; Murygina et al., 2005). In the present study we observed how *Rhdococcus* strains H1 and H2 developed a high hydrophobicity and penetrated the oil drops whereas *Dietzia* strains, like *Alcanivorax*, tend to grow attached to its surface (Fig. IV.1). H0 and H0B strains were close to *Dietzia maris* (99-100%) and *D. psychralkaliphila* (99-100%), respectively, (Rainey et al., 1995; Yumoto et al., 2002) described as alkane degrading bacteria including branched ones. Although different species of *Dietzia* are frequently related to environments polluted with hydrocarbons, (Brito et al., 2006; Kleinsteuber et al., 2006; von der Weid et al., 2006), few studies concerning its physiology and genetics has been performed hitherto compared to *Rhodococcus*.

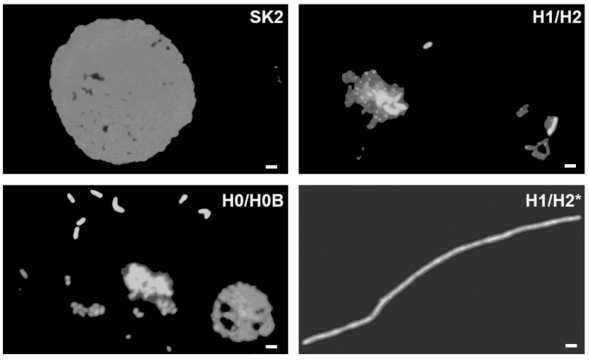

Fig. IV.1. Microphotography of DAPI-stained bacteria (100x) from different groups analyzed. SK2: *Alcanivorax borkumensis* strain SK2. H1/H2: *Rhodococcus* strains from this study. H1/H2*: different shape of *Rhodococcus* cells, which appear with higher frequency when yeast extract was added. H0B: *Dietzia* strains from this study. Bottom right scale bar: 1μm.

Both genera belong to suborder *Corynebacterineae* to which most of Gram-positive HC degraders belong (e.g. *Rhodococcus*, *Dietzia*, *Gordonia*, *Nocardia*, *Mycobacterium*…). Unusually, these Gram-positive bacteria contain an outer permeability barrier made of

mycolic acids (very-long-chain (C30–C90) a-alkyl, b-hydroxy fatty acids), which are major and specific constituents of *Corynebacterineae*. This feature may explain the limited permeability of their cell walls, the general insusceptibility of this group to toxic agents (Gebhardt et al., 2007) and its enhanced biodegradation capacity (Linos et al., 2000; Lee et al., 2006). As opposed to Gram-negative bacteria, such *Pseudomonas* or *Alcanivorax*, that dominates at first fast petroleum degradation processes (Kasai et al., 2002b), members of this group are never dominant (Margesin et al., 2003; Quatrini et al., 2008) at such stages being detected with higher frequency at resource limited environments where they could play a key role in the degradation of more recalcitrant components at long term intervals after an oil spill (Chapter III; Quatrini et al., 2008).

IV.4.2 Alkane degrading activity

Most Gram-negative HC degraders are able to grow on short and medium *n*-alkanes (Vomberg and Klinner, 2000), while some recent studies have highlighted the ability of actinobacteria, such as *Rhodococcus*, to degrade molecules from C_6 to C_{36} (van Beilen et al., 2002).

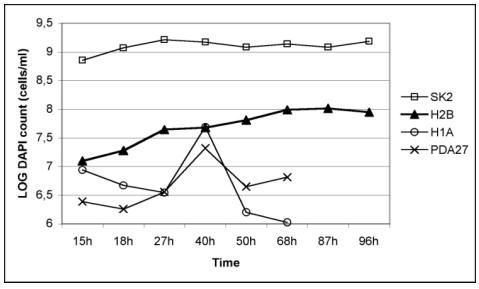

Fig. IV.2. Growth curves determined by DAPI counts of strains under study (*Rhodococcus* (H1A and PDA27) and Dietzia (H2B)) compared with *Alcanivorax borkumensis* SK2. No measures were made longer than 68 hours for *Rhodococcus* strains since the high hydrophobicity developed by the strains of this genus did not allowed an accurate counting. A partial view of the growth curves of the *Rhododoccus* strains is shown to point out the time (around 40 hours) when hydrophobibity is developed by the strains. Media used was composed of sea water supplemented with nutrients and Arabian crude oil (1000 ppm).

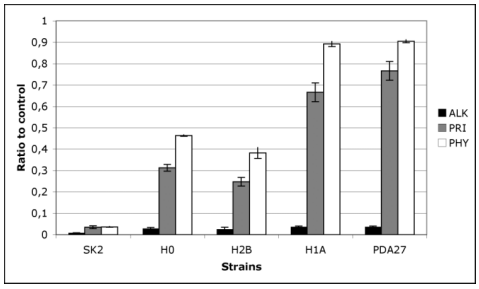

Fig. IV. 3. Degradation of alkanes from Arabian crude oil used as the only source of C and Energy for the different strains of *Dietzia* (H0 and H2B) and *Rhodococcus* (H1A and PDA27)) compared to *Alcanivorax borkumensis* strain SK2 after 68 hours incubation. No degradation of aromatics was detected for any of the isolates.
ALK: Summatory of *n*-alkane peak areas ranging from C_{12}-C_{24} relative to control.
PRI: branched alkane pristane (2,6,10,14-tetramethylpentadecane).
PHY: branched alkane phytane (2,6,10,14-tetramethylhexadecane).

All of our isolates were able to grow, at least, on medium (MCL) and long-chain-length (LCL) *n*-alkanes from C_{12} to C_{24} from Arabian crude oil (Fig. IV.3). *A. borkumensis* SK2 increased much more rapidly compared to our Gram-positive strains (Fig. IV.2 and IV.3), which do not increase so much in response to high HC levels, as previously described (Kasai et al., 2002b; Margesin et al., 2003). Probably longer alkanes could be used as

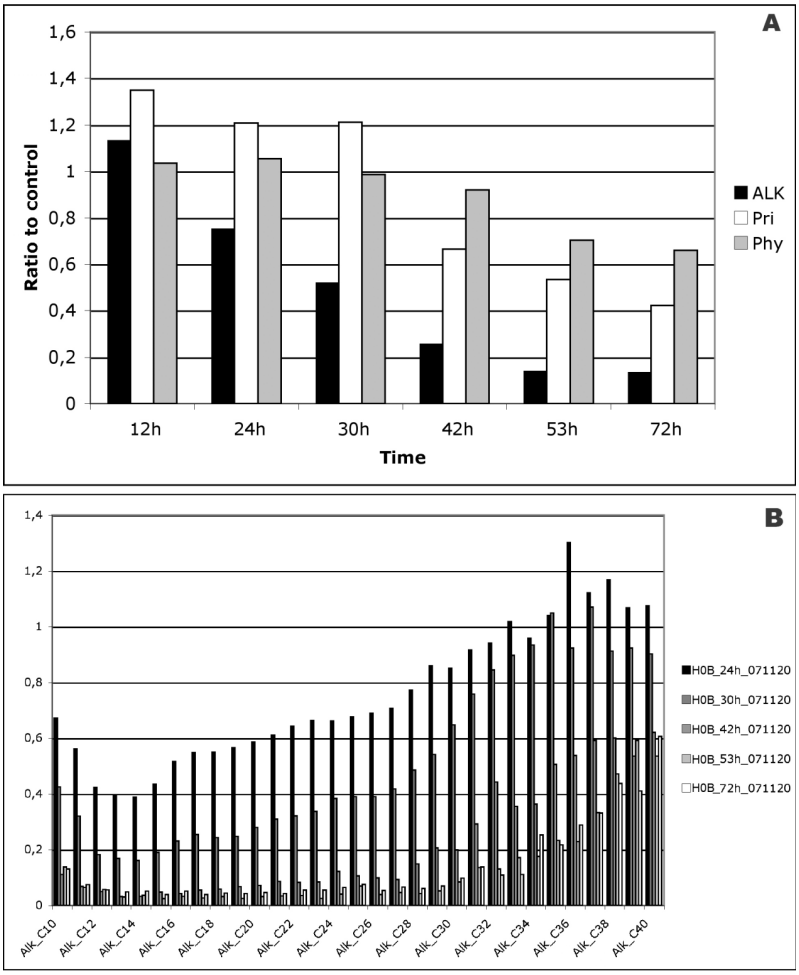

Fig. IV.4. Degradation of paraffins by *Dietzia* strain H2B during a 72 hours growth on an Arabian crude oil mixture used as the only source of C and Energy. **A.** Degradation of LCL *n*-alkanes (summatory of C_{10}-C_{40} peak´s areas respect to control) and branched alkanes (pristane and phytane). **B.** Detailed view of each n-alkane degradation during time. Degradation of longer n-alkanes (>C_{30}) and branched alkanes (mainly pristane) started between 30-42 hours culture, which coincided with early exponential phase growth of the strain as shown by DAPI counts (Fig. IV.2).

substrate but resolution of the peaks was not good enough. In this sense, analysis with a more sophisticated GC-MS (Hydrogen as carrier gas) was performed over Arabian crude oil degraded by *Dietzia* strain H0B at different time intervals. The analysis revealed a marked degradation of *n*-alkanes up to C24 during the first 30 hours (Fig. IV.4) at beginning of the exponential growth phase (Fig. IV.2). LCL *n*-alkanes up to C37 were degraded to a big extent (>60%) but its utilization did not started until middle exponential growth phase (between 30 and 42 hours, Figs. IV.2 and IV.4B). Also at this growth-phase started the utilization of branched alkanes (pristane and phytane) by *Dietzia* sp. H0B. Therefore, it seems that this species uses LCL and branched alkanes when the shorter ones are consumed. Probably this ability enables this genus to maintain its populations at long time periods after an oil-spill when the residual fuel has been enriched in longer and more recalcitrant fractions. Next to it, this strain was able to produce hexadecene when growing on hexadecane (Fig. IV.8B), which might be due to novel enzymes present in this species which start the oxidation process at the middle of the alkane chain instead of doing it at terminal or subterminal positions as described hitherto (van Beilen and Funhoff, 2007).

IV.4.3 alkB and cytochrome P450 of family CYP153 genes related with hydrocarbon degradation

All the nucleotide sequences were homologous to genes of alkane-hydroxylases and of cytochromes P450 (family CYP153) from Gram-positive isolates (McLeod et al., 2006; Stinear et al., 2008) or uncultured bacteria (Kubota et al., 2005) (Fig. IV.5 and IV.6).

Phylogenetic analysis revealed that AlkB coding genes from *Rhodococcus* strain H1 clustered (bootstrap of 83) with sequences detected in other strains of the same genus isolated on antartic environments (e.g. ABD36390 from Fani & Pini unp.; Fig. IV.5). In fact, the closest 16S bacterial sequence to H1 was *Rhodococcus* sp. 5/1 (AF181689) previously detected in Antartic waters as alkane degrader (Bej et al., 2000). Therefore our strain could be adapted to harsh environments with low temperatures and nutrient availability. AlkB sequences detected in *Dietzia* spp. H0 and H0B clearly grouped together (bootstrap value of 100), better than to any other known group of sequences. They were only distantly related with other sequences from *Rhodococcus*, indicating that this group might contain novel enzymes catalysing different reactions than previously described.

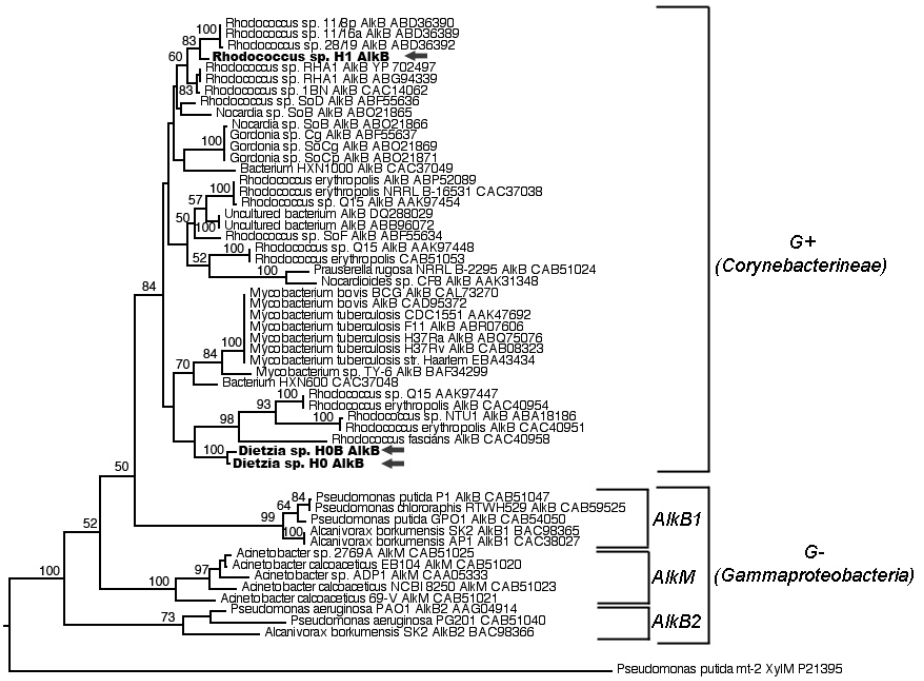

Fig. IV.5. Maximum likelihood phylogenetic tree of the genes homologous to alkB cloned from *Rhodococcus* (H1A) and *Dietzia* (H2B and H0) strains. Phylogenies are based on alignments (499 bp ≈ 166 aa) of AlkB protein sequences stored at GeneBank with the aminoacidic sequences deduced from translation of our clone sequences. Parameters for searching trees in Phyml were selected by ProtTest and PAUP using the Akaike information criterion (AIC) (Model of Nucleotide evolution: WAG+G; Pinvar: 0.08). Bootstrap values of ≥ 50% are shown at branch nodes (1000 iterations). Protein accession numbers are indicated at end of each sequence name while those derived from this study are marked with arrows. Xylene monoxygenase from *Pseudomonas putida* was used as outgroup.

CYP153 cytochromes from both *Rhodococcus* and *Dietzia* strains tended to cluster together than with any other sequence and were close to sequences derived from metagenomic studies of petroleum contaminated sites (Kubota et al., 2005). However, bootstrap values were always low due to the small size of the sequence analyzed and the high variability of these enzymes among bacteria. Therefore, from the phylogeny (Fig. IV.6) we can only assure that all of our cloned sequences belong to family CYP153A (bootstrap value of 84). However, it is worth to mention that one sequence cloned from *Dietzia* strain H0B (CYP153_H0B_uniq) clustered neither with our sequences nor with any previously described.

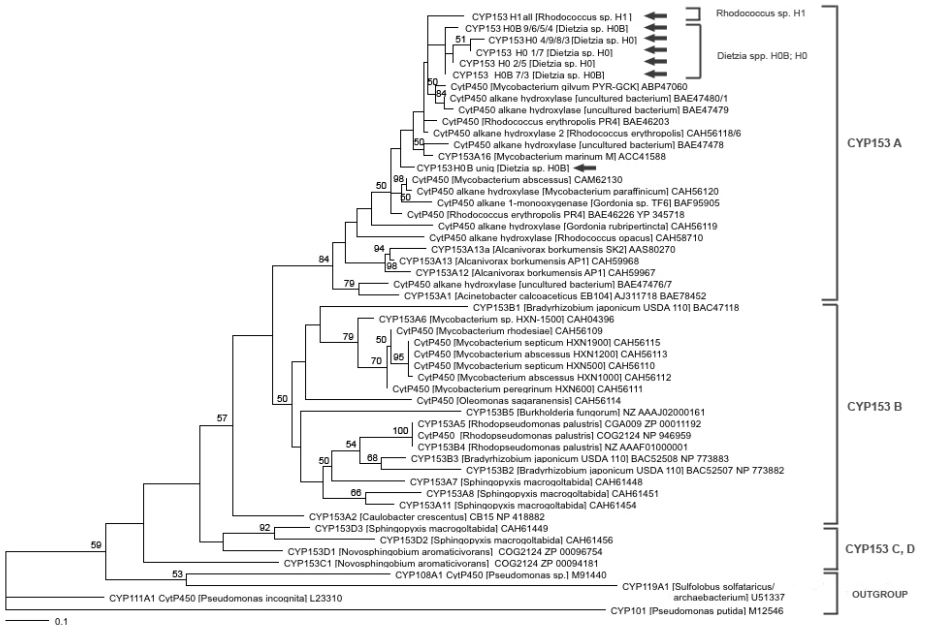

Fig. IV.6. Maximum likelihood phylogenetic tree of the genes homologous to CYP153 (cytochrome P450) cloned from *Rhodococcus* (H1A) and *Dietzia* (H2B and H0) strains. Phylogenies are based on alignments (296 bp ≈ 98 aa) of CYP153 protein sequences stored at GeneBank with the aminoacidic sequences deduced from translation of our clone sequences. Parameters for searching trees in Phyml were selected by ProtTest and PAUP using the Akaike information criterion (AIC) (Model of Nucleotide evolution: WAG+G; Pinvar: 0.06). Bootstrap values of ≥ 50% are shown at branch nodes (1000 iterations). Protein accession numbers are indicated at end of each sequence name while those derived from this study are marked with arrows. Different families of cytochromes P450 related to CYP153 were used as outgroup.

AlkB and CYP153 are completely unrelated enzymes which are found with highest diversity among Gram-positive bacteria where different coding genes are commonly found (Heiss-Blanquet et al., 2005; van Beilen and Funhoff, 2005; van Beilen et al., 2006). The functional meaning of multiple alkane hydroxylase systems is unknown and it was hypothesized that each enzyme is related to oxidation of a different range of alkanes or active during different growth phases (Marin et al., 2003). In this sense, the diversity of genes related to alkane-degradation found in *Dietzia* strain H2B could codify for new enzymes, which might be related with any of the special degrading abilities observed in such strain.

IV.4.4 Regulation of alkane degrading activity

Alkane degradation activity for both *Rhodococcoccus* and *Dietzia* strains seemed to be independent from the C source. No significant difference was observed for the alkB and CYP153 genes´ expression levels between the pyruvate and hexadecane-grown cells neither of *Dietzia* sp. H0B nor of *Rhodococcus* sp. H1A (Fig. IV.7). Although moderately high standard deviations prevents from drawing strong conclusions about the exact fold induction level, expression of all genes analyzed were certainly detected under both conditions irrespective of the C source used. Next to it, since RNA presence does not imply a higher enzyme level because regulation could exist at translation level, the constitutively expression of these enzymes was further confirmed by the GC-MS assays.

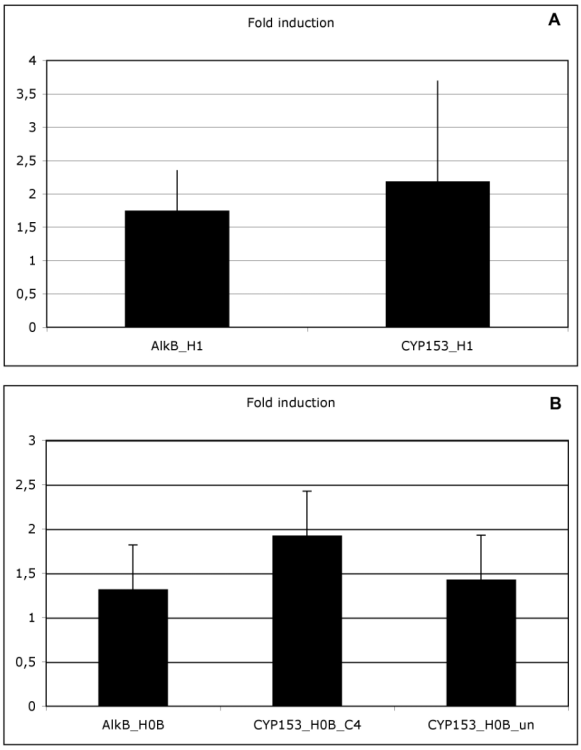

Fig. IV.7. Expression analysis of alkB and CYP153 genes from *Rhodococcus* sp. H1 (A) and *Dietzia* sp. H0B (B) using RT-qPCR. Bars represent fold induction of each gene expressed in the presence of hexadecane relative to the expression level when growing on pyruvate. No significant differences were observed for any of the genes analyzed, which were constitutively expressed irrespective of the substrate used. Expresion levels were previously normalized using a housekeeping gene (DNApolIV).

Addition of chloramphenicol did not hinder the degradation of alkanes in pyruvate-grown cells of *Rhodococcus* and *Dietzia* strains (Fig. IV.8), meaning that hydroxylases must have been expressed and synthesized during its growth in such C source. Besides this, degradation rates of pyruvate- compared to hexadecane-grown cells of both genus, without

addition of the antibiotic, were also similar (Fig. IV.9). Therefore, both genus seem to constitutively express alkane hydroxylases in the presence (e.g. hexadecane as C source) or absence (e.g. pyruvate as C source) of its substrate.

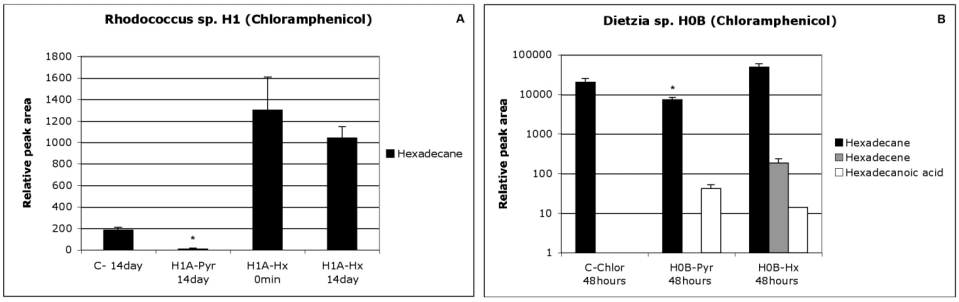

Fig. IV.8. Alkane degradation assay using antibiotic chloramphenicol with *Rhodococcus* sp. H1 (A) and *Dietzia* sp. H0B (B), pregrown on hexadecane (Hx) or pyruvate (Pyr). The amount of hexadecane which remained after 14 days and 2 days of incubation with strains H1A-Pyr and H0B-Pyr respectively, was significantly lower (*) than the original amount (C-) as measured by Student´s t-Test. Carry-over from previous culture avoid obtention of clear results with hexadecane-grown cells. Hexadecene was detected among the catabolites produced by *Dietzia* sp. H0B when growing on hexadecane. This has not been previously observed in any other alkane degrading bacteria.

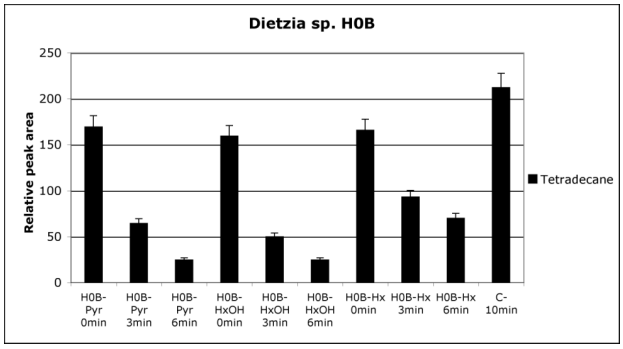

Fig. IV.9. Speed of degradation of tetradecane (C14) of pyruvate-, hexadecanol- and hexadecane-grown *Dietzia* cells, represented as Pyr, HxOH and Hx respectively. Three measures at 0, 3 and 6 minutes indicate that the proportion of tetradecane degraded at each time interval was irrespective of the C source. * Cells previously grown in hexadecane (Hx) carry over some of this substrate as detected by GC-MS (result not shown). This is probably the explanation to the slightly lower degradation of tetradecane by Hx cells.

The physical factors, by removing the most volatile HC compounds, and the heavy nature of fuel from *Prestige*, may have enriched the microbial community with those

populations capable of utilizing the heaviest components of the contaminants (Sei et al., 2003). This study reports the constitutively expression of alkane hydroxylase related genes in two actinobacteria genera of the *Corynebacterineae* suborder to which most of gram positive HC degraders belong (e.g. *Rhodococcus*, *Dietzia*, *Gordonia*, *Nocardia*, *Mycobacterium*…).

Constitutively expressed alkane degrading genes observed in the present study, agree with previous observations where gram-positive GC-rich bacteria, adapted to low nutrient levels, do not fluctuate in response to the addition of a C source such as HC from an oil spill (Margesin et al., 2003). Gram positive alkane degraders have been detected in uncontaminated soils (Heiss-Blanquet et al., 2005; Kloos et al., 2006) and in a recent field bioremediation assay performed in a cobblestone beach affected by *Prestige* fuel oil, *Rhodococcus* was found in both control and fertilized plots (Jiménez et al., 2007). Therefore, we suggest that Gram positive bacteria act as *K* strategists as opposed to Gram negative bacteria, such as *Pseudomonas* and *Alcanivorax*, which grow efficiently in response to high HC levels in the event of an oil spill, thus being considered as *r* strategists. In fact, induction of alkane degrading enzymes in the presence of its substrate was observed in these species (Yuste et al., 1998; Sabirova et al., 2006).

General Discussion

A. Methodological considerations

Results from our studies proved that the combination of molecular techniques such as PCR based (DGGE and clone libraries) with PCR independent (FISH) give more trustable results, which support stronger hypothesis. In the study of bacterioplankton structure of the Ría de Vigo, results from FISH were considered as a quantitative tool, while clone sequences were used to improve high-quality rRNA databases and further design new group-specific probes which could be used in further ecophysiological studies of novel clusters detected in the water. However, FISH is not a suitable technique to study oil communities (Harayama et al., pers. comm.), especially those of *Prestige´*s heavy fuel (self experience, Chapter IV). Many hydrocarbon-degrading bacteria develop a high hydrophobicity growing attached to the oil and the oil itself is autoflurescent, making accurate FISH countings almost impossible.

Combination of culture-independent and culture-based techniques has been shown in the present study as a powerful methodology in petroleum microbial ecology if we are aware of the limitations of each technique alone.

DNA identities (of the 16S rRNA gene) between sequences detected by molecular techniques from the oiled environments with those of previously described hydrocarbon-degrading strains, give us information about potential metabolism and degradation capacity of the studied community. However, even strains of the same species are known to posses quite different characteristics (e.g. infective capacity, antibiotic resistance and also degradation of a specific pollutant are frequently coded by genes carried in plasmids which are specific of some strains (Larkin et al., 2006)). Therefore, such similarities of the 16S are just an approximation of the actual catabolic capacity of a given community and must be interpreted with caution. In this sense, our studies did not remain as just taxonomic descriptions of microbial communities related with degradation processes of the fuel. Indeed, many of the indigenous bacteria detected in the environment by means of molecular techniques as playing potential roles in the degradation of different fractions of the *Prestige´*s fuel in the environment, such *Alcanivorax*, *Rhodococcus*, *Citreicella*, *Dietzia*, *Sphingomonas*, etc. could be isolated. Hence, the isolated hydrocarbon-degrading strains could be further analysed with respect to its genetics, physiology and interaction with other members of the indigenous hydrocarbon-degrading bacterial consortia carrying out biodegradation pathways.

B. Environments affected by the *Prestige* oil-spill and its biodegradation capacity

B.1 RÍA DE VIGO

B.1.a Environmental characteristics

The Ría de Vigo, (Galicia, NW Spain), is located in the northern boundary of the NW Africa upwelling system, and therefore in an area where upwelling–downwelling dynamics on the continental shelf (Arístegui et al., 2006) have a strong influence on its hydrography and, hence, on its plankton community structure and function. In fact, changes in bacterioplankton community structure and diversity were found throughout the year in Ría de Vigo (Table II.3), including the detection of new members in all major groups of bacteria.

The coastal upwelling induces the inflow of subsurface oceanic Eastern North Atlantic Central Water (ENAW) into the Ría. (Alvarez-Salgado et al. 1993). The incoming bottom current supplies the Galician estuaries with nutrients (mean values of Nitrogen (N) and Phosphorus (P) around 0.6 and 0.06 mg l^{-1} respectively; Nogueira et al. 1997) that causes the Ría de Vigo to be a ecosystem with a great ecological and economical value.

Cíes islands, part of the National Park “Illas Atlánticas”, are situated at the mouth of such ecosystem and mussel production in this region is the highest in Europe and one of the most intensive in the World, giving employment to 9000 people directly and 20000 indirectly (Figueiras et al., 2002, Figueras, 2007).

Mussels and other shellfish growing in the Ría, filtrate water and accumulate pollutants. Therefore, Spanish authorities banned commercialization of shellfish yield from the Ría after the *Prestige* oil-spill with negative effects on the local economy. Primary oil-spill responses (e.g. skimming of floating fuel, etc.) removed tones of fuel-oil from surface water but still lots of it sank and remained buried under sediments of the Natural Park from where it might be remobilized even today. Mechanical cleaning of the sediments could cause more harm than good since the seabeds of the National Park are delicate habitats with a high ecological value. Since bioremediation of water is not a feasible option (amendments would be diluted), and hand cleaning by scuba divers is a difficult and expensive task, the most feasible option left in such cases is to trust on natural attenuation of the environment. In spite of the critical role of bacterial assemblages in the oceanic food web, and therefore in the production of the Ría, there was no information available about the microbial communities growing at Ría de Vigo and even less of its biodegradation metabolism.

B.1.b Bacterioplankton community structure

In contrast to previous results, which indicated that SAR11 clade members dominate surface bacterioplankton communities (Morris et al., 2002), in the Ría de Vigo, and other

highly productive estuarine systems of the Iberian peninsula characterized by phytoplankton blooms (Henriques et al., 2004), *Roseobacter* members dominated the community (González et al., 2000) in detriment of SAR11, which is more adapted to the oligotrophic conditions of the open waters (Malmstrom et al., 2004, Rappé et al., 2002).

Different clades of *Roseobacter* lineage (NAC11-7, DC5-80-3, DG1128, CHAB-I-5, AS-21, etc. in Fig. II.2) and new clusters of SAR11 (A3?, A4?, in Fig. II.3), SAR86 (IV? in Fig.II.4), *Betaproteobacteria*, etc. were detected in this study at specific seasons, which could likely reflect a seasonal trend. The different clades could have different abilities including biodegradation capacities (Sekine et al., 2006), or have different environmental preferences like the association with different phytoplankton species (Wagner-Dobler and Biebl, 2006; West et al., 2008).

B.1.c Biodegradation potential

When N and P are added in adequate quantities to cultures of seawater with crude oil as the only source of C and energy, *Alcanivorax* (Yakimov et al., 1998) and other 'professional hydrocarbonoclastic Gammaproteobacteria', such as the polyaromatic degrader *Cycloclasticus* (Chung and King, 2001), become dominant and, hence, the rate of biodegradation is strongly promoted (Harayama et al., 2004, Kasai et al. 2001, Kasai et al. 2002b, McKew et al., 2007, Roling et al. 2002). The fact that we were able to detect alkane-degrading *Alcanivorax* strain and *Cycloclasticus* sp., which responds to PAHs additions (Teira et al., 2007), from sediments and water of the Ría, showed that natural N, P and Oxygen levels present (Vilas et al. 2005) in the Vigo embayment are sufficient to promote the growth of such species playing critical roles in the natural attenuation of oil-polluted marine systems.

a) The chronic hydrocarbon pollution of the Ría (oil spillages, industrial and urban runoff, etc.); b) the favourable abiotic factors of the Ría for biodegradation metabolisms (high Oxygen and nutrient levels); c) the indigenous presence of key alkane- (*Alcanivorax* sp.) and PAH- (*Cycloclasticus* sp.) degrading species; d) the dominance and diversity of *Roseobacter* sequences (a genus previously related with oil biodegradation (McKew et al., 2007)); e) the high MPN population of alkane degraders in sediments (Alonso-Gutiérrez et al., 2008); f) the lack of effects of PAH addition on the indigenous bacterioplankton structure (Lekunberri et al., submitted; Lekunberri, 2008) and g) the fact that ongoing bioremediation was already noted at shoreline environments of the Ría (Medina-Bellver et al. 2005), support the theory of the existence of a preadapted hydrocarbon-degrading community in the Ría, which would use pollutants as a source of C and energy. Therefore, natural attenuation at Ría de Vigo is probably very effective and therefore it can be asumed that petroleum hydrocarbons dissolved in water after an oil-spill event would be readily consumed by the autochthonous

bacterioplankton community.

B.2 SUPPRALITTORAL ROCKY SHORELINES OF "COSTA DA MORTE"

B.2.a Oil spill response at "Costa da Morte"

After the decision was made to use bioremediation as a cleanup response to the *Prestige´*s oil-spill, different bioremediation commercial recipes were compared for their effectiveness at different rocky shorelines polluted by the accident. After different field trials, the oleophilic fertilizer S-200 proved to be the most successful and therefore it was used to clean unaccesible parts along the NW Spanish coasts where mechanical treatments were not feasible and/or advisable. Although its effectiveness was different between sites (Murado, M.A. et al., pers. comm.), there were no further assessments prior to its application at different sites. Such variability in treatment success was probably due to differences between the autochthonous bacterial communities.

Rock, sand and even water matrices, heavily polluted by fuel from *Prestige* one year before, were sampled at different places prior to S200 applications. Community analysis was performed with those matrices from supralittoral environments of "Costa da Morte" where the ensuing application of S-200 was succesfull. Therefore, results presented in this scientific memory give valuable information about the bacterial assemblages that positively respond to oleophilic fertilizer applications and will shed some light on the development of new bioremediation strategies.

B.2.b Natural attenuation at "Costa da Morte"

Fingerprinting analysis of the fuel accumulated at rocky shorelines under study (Fig. III.4) showed a high degree of weathering which was consistent with the trends that follow biodegradation (Jiménez et al., 2006). Hence, as proposed for microbial communities from the Ría de Vigo, we can hypothesize that indigenous bacterial assemblages from "Costa da Morte" have indeed good biodegradation rates. This hypothesis is supported by the environmental conditions of the studied habitat and by its bacterial community structure.

"Costa da Morte" (Galicia, NW Spain) is also affected by the NW Africa upwelling system (Arístegui et al., 2006), and therefore is an area where nutrient levels (0.20 - 0.25 mg l^{-1} of Total N (Alvarez-Salgado et al., 2002) are more suitable for biodegradation processes than those of the Sea of Japan (Total N~0,1 mg l^{-1}). In the first moments after an oil-spill event, marine bacterial communities become dominated by *Gammaproteobacteria* (Roling et al., 2002), nevertheless, when environmental factors are suitable for biodegradation processes, the oil is rapidly weathered and *Gammaproteobacteria*-dominated communities are quickly

substituted by a more diverse community (Roling et al., 2002), like the one we found twelve months after the *Prestige* oil-spill. Around one year after the *Nakhodka* oil spill in the Sea of Japan, fuel paste samples of a similar composition to that of *Prestige*, were still dominated by *Gamma* and *Alphaproteobacteria* (Gram-negative) (Kasai et al., 2001; Maruyama et al., 2003), while, *Alphaproteobacteria* and Gram-positive *Actinobacteria* dominated our samples affected by *Prestige* after the same time.

B.2.c Bacterial assemblages and potential hydrocarbon-degrading ability

The trophic structure of bacterial communities affected by the oil-spill presented a significantly higher percentage of alkane- and aromatic-degrading bacterial populations than non affected ones. Next to it, although rocks and sand are quite different substrates, community composition was quite similar. All of it suggested that fuel oil drove the composition and structure of the affected communities studied.

Many members of the described communities were previously associated with alkane- (e.g. *Xanthomonadaceae*, *Pseudoxanthomonas, Stenotrophomonas, Erythrobacter*, etc. (Chang et al., 2005; Young et al., 2007; Macnaughton et al., 1999; Roling et al., 2002)) and aromatic-degradation processes (e.g. *Citreicella* sp., Sphingomonadaceae spp., *Lutibacterium anuloederans*, described as a 2- and 3-ring PAH degrading bacteria which had a higher efficiency in the uptake of aromatics than *Cycloclasticus* species, etc. (Chung and King, 2001; McKew et al., 2007)). However, it was *Actinobacteria* (mainly *Corynebacterineae*) the dominant group carrying out *in situ* biodegradation processes of the *Prestige* fuel oil after one year of weathering. This distinct group of Gram-positive Actinobacteria, classified in the suborder *Corynebacterineae*, includes genera detected in the present study such as *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia* and *Rhodoccoccus*.

Molecular- and culture-based techniques showed *Rhodococcus*, as the most important alkane-degrader. It is well known that *Rhodococcus* is a genus with a remarkable metabolic diversity (Larkin et al., 2005) and able to produce biosurfactants which can enhance the activities of other degrading bacteria (Iwabuchi et al., 2002; Murygina et al., 2005; Van Hamme and Ward, 2001)). Other *Actinobacteria* such as *Gordonia, Dietzia*, and *Microbacterium*, with similar characteristics as *Rhodococcus*, were also detected in the community as degraders of linear and even branched alkanes (Rainey et al., 1995; Yumoto et al., 2002).

Further alkane degradation assays performed with the *Dietzia* strains isolated revealed interesting features of this Corynebacterium. This strain was able to produce hexadecene when growing on hexadecane (Fig. IV.8B). This represent a novel degradation pathway as described hitherto (van Beilen and Funhoff, 2007), which might be codified by novel gene sequences that we isolated from this strain (alkB and CYP153). Although this feature may

have no use for bioremediation purposes it could be useful for bioconversion processes. In fact, isolation efforts are made not only for assessing the fate and effects of the spilled oil, but also for isolating bacteria that may contain novel degradation pathways useful for industry (Harayama et al., 2004; Van Hamme et al., 2003).

Corynebacterineae group might also play an important role in the degradation of PAHs since genus *Mycobacterium*, specialized in the degradation of adsorbed PAHs (Bastiaens et al., 2000), was detected in clone libraries in high proportion. The ability of *L. anuloederans* and *Mycobacterium* spp. to degrade fluorene and pyrene (Grifoll, M., pers. comm.), which are considered specially recalcitrant components of the fuel (Wammer and Peters, 2005), might explain the high abundance of this bacteria at heavy fuel samples under study, which devoid of the most easily biodegradable fractions.

B.2.d Properties of *Corynebacterineae*

As opposed to *Gammaproteobacteria* (e.g. *Alcanivorax*, *Cycloclasticus*, *Thalassolituus*…) that dominates at first fast petroleum degradation processes (Kasai et al., 2002b), members of *Corynebacterineae* are never dominant at such stages (Margesin et al., 2003; Quatrini et al., 2008) being detected with higher frequency at resource limited environments where could play a key role in the *in situ* degradation of more recalcitrant components at long term intervals after an oil spill (Quatrini et al., 2008).

The results we obtained from the alkane degradation assays, performed with *Rhodococcus* and *Dietzia* strains isolated from the consortium, also supported this hypothesis. *Dietzia* spp. used long chain length (LCL) and branched alkanes only when the shorter ones were consumed. Probably this ability enables this genus to maintain its populations at long time periods after an oil-spill when the residual fuel had already enriched in longer and more recalcitrant fractions. Next to it, hydrocarbon degrading genes, alkB and CYP153, and its respective hydroxilases, were constitutively expressed irrespective of the C source in both *Dietzia* and *Rhodococcus*. This explains why gram-positive degrading bacteria do not fluctuate in response to an oil spill (Margesin et al., 2003). On the contrary, Gammaproteobacteria, such as *Alcanivorax* grow efficiently in response to high HC inputs due to an inducible expression of its catabolic machinery (Yuste et al., 1998; Sabirova et al., 2006).

As a conclusion, stranded fuel oil at marine shorelines represents in itself a habitat which is subject of bacterial colonization and ensuing community succession, where Gram negative, Gammaprotobacteria, might be considered as *r* strategists and Gram positive, Actinobacteria, as *K* strategists growing at later stages of the biodegradation process due to its catabolic versatility (Larkin et al., 2005), which enable this group to grow on the most recalcitrant components of an oil in advanced state of weathering.

If the biodegradation metabolism of *K* strategists (Gram positive bacteria) is different from that of *r* strategists (Gram negative bacteria), as showed in the present study, it seems pretty obvious that the requirements to enhance their respective activities should be different in the same extent. In spite of this, bioremediation amendments are usually the same irrespective of the degree of oil weathering and succession stage, which was always unknown in the case of *Prestige* where bioremediation strategy were based on empirical data. Therefore, based on our observations and previous studies, different improvements are proposed for current bioremediation treatments to be applied at long-term oiled marine environments.

B.2.e Potential improvements of bioremediation at "Costa da Morte" and similar habitats

Conclusions derived from the present work might be applied to other parts of the Spanish coast affected by the *Prestige* oil-spill since key members of the studied consortium (close to *Rhodococcus*, *Chromatiales*, *Rhodobacteriaceae, Roseobacter*-(*Citreicella*), *Erythrobacter* etc.) were also detected 400 km far at similar environments affected by the *Prestige* (Jiménez et al., 2007). Therefore, the community studied might be a kind of 'climax community' stablished at *Prestige* fuel after being weathered and enriched in recalcitrant fractions.

Microorganisms are able to produce biosurfactants that enhance oil degradation in the environment (Ron and Rosenberg, 2001, 2002). Mycolic acids are major and specific constituents of *Corynebacterineae*, which provide these gram-positive bacteria with an outer barrier that may explain both the limited permeability of their cell walls, their adhesion capacity and their general insusceptibility to toxic agents (Gebhardt et al., 2007). Although use of biosurfactants are usually not feasible as an oil spill response tool because are not cost-effective to be produced, the use of synthetic mycolic acids, homologues to the natural ones and cheap to produce, was showed to be effective in enhancing the biodegradation capacity of oil-degrading *Rhodococcus* and other Actinobacteria (Linos et al., 2000; Lee et al., 2006). *Rhodococcus* itself produces surfactants which enhance biodegradation capacities of other members of the consortium (Iwabuchi et al., 2002) and therefore, the addition of synthetic mycolic acids to the current bioremediation amendments might improve its effectiveness at long-time oiled areas where the already weathered fuel are likely to be dominated by *Corynebacterineae* and other Actinobacteria.

In the present work we also showed important interactions between members of the hydrocarbon degrading consortia carrying out the *in situ* biodegradation of fuel from *Prestige*. Such interactions are too complex to try to study all of them. However, results from

this study give some clues about possible positive interactions, which may be enhanced by bioremediation treatments.

Our results support the hypothesis previously made (McKew et al., 2007) that *Tistrella mobilis* is an opportunistic bacteria which grows on second metabolites (e.g. cathecol) derived from PAHs degradation processes carried out by the actual aromatic degraders. Now that we have isolated *Tistrella*, it would be interesting to study if the presence of this bacterium, which 'steal' intermediate catabolites from PAHs-degrading species, could accelerate the cleavage of new aromatic rings by the degrading bacteria in order to compensate such loss.

We also observed that *Rhodococcus* and mainly *Citreicella* species require some cofactor, probably a vitamin, to develop their alkane and aromatic degrading activities, respectively. Actinobacteria members of the consortia under study, such as *Dietzia*, can supply such cofactor. Application of these cofactors might be of interest in order to improve bioremediation treatments. Although detection of such cofactors might require a lot of work (yeast extracts contain a myriad of different vitamins, etc.), our observations could help in pointing further investigation at such respect.

Conclusions

1) Combination of culture-independent and culture-based techniques has been shown in the present study as a powerful methodology in petroleum microbial ecology.

2) The isolation of *Alcanivorax* strains directly from oiled sediments is the first ecological evidence of the high natural abundance of this group of bacteria in the event of a real oil-spill.

3) The Ría de Vigo is an upwelling-affected coastal ecosystem of changing environmental conditions throughout the year, which drive the bacterioplankton community structure and function and probably the appearance of novel clusters of bacteria (e.g. SAR11, SAR86, *Betaproteobacteria*, etc.).

4) High nutrient levels and/or competence with *Roseobacter* are probably the cause for the almost absence of the SAR11 lineage throughout the year in Ría de Vigo and other 'Rías' of NW Iberian Peninsula.

5) Indigenous, probably preadapted hydrocarbon-degrading, bacterioplankton communities growing under favorable environmental conditions of Ría de Vigo (high nutrient and oxygen levels) support 'Natural attenuation' as the best secondary oil spill response at this ecosystem.

6) *Prestige´*s fuel accumulated after one year at rocky shorelines of "Costa da Morte" showed a high degree of weathering due to biodegradation probably due to a succession of effective hydrocarbon-degrading autochthonous bacterial communities of "Costa da Morte".

7) Heavy fuel from *Prestige* was dominated, after some months of weathering and biodegradation processes, by *Actinobacteria* (mainly suborder *Corynebacterineae*; e.g. *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia* and *Rhodoccoccus*), which may play key roles in the degradation of specific, more recalcitrant fractions of heavy fuels at later stages of the biodegradation process.

8) *Dietzia* strain H0B was able to produce hexadecene when growing on hexadecane, which represent a novel alkane degradation pathway that might be codified by novel gene sequences that we isolated from this strain (alkB and CYP153).

9) Hydrocarbon degrading genes, alkB and CYP153, and its respective hydroxilases, were constitutively expressed irrespective of the C source in both *Dietzia* and *Rhodococcus*. This explain why gram-positive degrading bacteria do not fluctuate in response to an oil spill (Margesin et al., 2003).

10) The addition of synthetic mycolic acids and cofactors, such as vitamins, to current bioremediation treatments applied to long-weathered, recalcitrant, oil-spills, usually dominated by Actinobacteria, might be a good strategy to enhance its *in situ* biodegradation rates.

Conclusiones

1) La combinación de técnicas moleculares y de cultivo, usada en el presente estudio, ha demostrado ser de gran utilidad y eficacia en estudios de ecología microbiana relacionada con el petróleo.

2) El aislamiento de cepas de *Alcanivorax,* a partir de sedimentos contaminados por el vertido del *Prestige*, es la primera evidencia de su presencia en alto número tras un vertido real de petróleo.

3) La Ría de Vigo es un ecosistema de alto valor ecológico afectado por episodios de afloramiento que hacen variar sus características a lo largo del año. Esta variabilidad en su hidrografía parece ser la causa de los cambios estacionales observados en la estructura y composición del bacterioplancton (incluida la aparición de nuevos grupos dentro de SAR11, SAR86, *Betaproteobacteria*, etc.).

4) Los altos niveles de nutrients y/o competencia con miembros de *Roseobacter* son la causa más probable de la casi ausencia de miembros de SAR11 en las aguas de la Ría de Vigo y de otras ´Rías´ de la Península Ibérica.

5) Las comunidades de bacterioplancton autóctonas, probablemente preadaptadas al uso y degradación de hidrocarburos, que crecen bajo las condiciones favorables de la Ría de Vigo (altos niveles de nutrients y oxígeno), apoyan la 'atenuación natural' como una buena medida secundaria de respuesta ante un vertido de petróleo.

6) El fuel del *Prestige* acumulado tras un año sobre rocas del supramareal de la "Costa da Morte" mostró un alto nivel de meteorización debido a procesos de biodegradación. Esto indica que comunidades bacterianas autóctonas de la "Costa da Morte", con diferentes capacidades de degradación de hidrocarburos, han tenido que sucederse para alcanzar tal grado de degradación.

7) El fuel pesado del *Prestige* estaba dominado, tras algunos meses de procesos de degradación, por *Actinobacteria* (ppte. suborden *Corynebacterineae*; p.ej. *Mycobacterium*, *Williamsia*, *Gordonia*, *Dietzia* and *Rhodoccoccus*). Este grupo parece jugar un papel clave en la degradación de las fracciones del petróleo más recalcitrantes que dominan las últimas fases del proceso de degradación.

8) La cepa de *Dietzia* H0B fue capaz de producir hexadeceno a partir de hexadecano, lo que indica la existencia de una nueva ruta de degradación de alcanos. Las enzimas responsables podrían estar codificadas por nuevas secuencias génicas (alkB y CYP153) que también detectamos en este estudio.

9) Los genes relacionados con la degradación de hidrocarburos (alkB y CYP153) y sus respectivas enzimas (hidroxilasas), se expresaron de forma constituva independientemente de la fuente de C usada, tanto en *Rhodococcus* como en

Dietzia. Esto explica por qué las poblaciones de bacterias degradadoras Gram positivas no fluctuan en respuesta a un vertido (Margesin et al., 2003).

10) La adición de ácidos micólicos sintéticos y de cofactores, como vitaminas, a las actuales fórmulas biorremediadoras que se aplican sobre petróleos ya degradados, enriquecidos en fracciones menos biodegradables y normalmente dominados por *Actinobacteria*, puede ser una buena estrategia para aumentar su eficacia.

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Supplemental Material

Supplemental Table III.S1. Results from the BLASTN comparison (performed on March 2007) of the DGGE bands' sequences from the total profiles (Fig. III.4A) of polluted rocks and sand samples with those stored in the GeneBank. The table contains the names and accession numbers of the closest organisms to each band.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	OR band
Nocardiaceae (A)	*Rhodococcus sp.* NPO-JL-61 (AY745831)	99	R1
Nocardiaceae (A)	*Rhodococcus sp.* 11/16a (DQ310479)	99	R2
Nocardiaceae (A)	*Rhodococcus sp.* 28/19 (DQ310477)	99	R3
Williamsiaceae (A)	*Williamsia sp.* MT8 (AY894336)	98	R4
Nocardiaceae (A)	*Rhodococcus sp.* 28/19 (DQ310477)	98	R5
Phyllobacteriaceae (α)	*Mesorhizobium sp.* W33 (AB192408)	95	R6
Rhodobacteraceae (α)	Uncultured *Rhodobacteraceae* bacterium (DQ870525)	99	R7
Microbacteriaceae (A)	*Microcella putealis* CV2T (AJ717388)	99	R8
Rhodobacteraceae (α)	*Citreicella sp.* 2-2A (AB266065)	96	R9
Rhodobacteraceae (α)	*Citreicella sp.* 2-2A (AB266065)	99	R10
Rhodobacteraceae (α)	*Citreicella sp.* 2-2A (AB266065)	99	R11
Sphingomonadaceae (α)	*Sphingopyxis sp.* FR1093 (DQ781321)	99	R12
Sphingomonadaceae (α)	*Lutibacterium anuloederans* (AY026916)	99	R13
Sphingomonadaceae (α)	*Lutibacterium anuloederans* (AY026916)	99	R14
Chromatiales (γ)	Uncultured *gamma proteobacterium* (DQ870518)	99	R15
Erythrobacteraceae (α)	*Erythrobacter sp.* JL893 (DQ985055)	98	R16
Sphingomonadaceae (α)	*Sphingopyxis sp.* FR1093 (DQ781321)	99	R17
Flavobacteriaceae (B)	*Cellulophaga sp.* D3054 (DQ480142)	97	R18
			OS band
Nocardiaceae (A)	*Rhodococcus sp.* NPO-JL-61 (AY745831)	99	S1
Nocardiaceae (A)	*Rhodococcus sp.* 11/16a (DQ310479)	99	S2
Nocardiaceae (A)	*Rhodococcus sp.* 11/16a (DQ310479)	95	S3
Phyllobacteriaceae (α)	Soil *Phyllobacteriaceae* bacterium (DQ099469)	91	S4
Hyphomicrobiaceae (α)	*Hyphomicrobium sp.* Ellin112 (8ª) (AF408954)	96	S5
Sphingomonadaceae (α)	*Lutibacterium anuloederans* (3ª) (AY026916)	96	S6
Rhodobacteraceae (α)	Uncultured *Rhodobacteraceae* bacterium (DQ870525)	99	S7
Chromatiales (γ)	Uncultured soil bacterium clone M54 (DQ378269)	99	S8
Chromatiales (γ)	Uncultured bacterium clone B101-25 (DQ001686)	99	S9
Sphingomonadaceae (α)	*Lutibacterium anuloederans* (3ª) (AY026916)	99	S10
Chromatiales (γ)	Uncultured *gamma proteobacterium* (DQ870518)	99	S11
Flavobacteriaceae (B)	Uncultured bacterium clone HB2-46-16 (DQ334636)	95	S12
Flavobacteriaceae (B)	*Yeosuana aromativorans* (AY682382)	97	S13

*A: Actinobacteria, B:Bacteroidetes, α: Alphaproteobacteria, γ: Gammaproteobacteria. Id.: Identities

Supplemental Table III.S2. Results from the BLASTN comparison (performed on March 2007) of the DGGE bands´ sequences from the hydrocarbon degrading profiles of polluted rocks (Fig. III.4B) and sand samples (Fig. III.4C) with those stored in the GeneBank. The table contains the names and accession numbers of the closest organisms to each band.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	OR-Hx
Nocardiaceae (A)	*Rhodococcus sp.* NPO-JL-61 (AY745831)	99	RH1
Nocardiaceae (A)	*Rhodococcus sp.* 11/16a (DQ310479)	100	RH2
Rhodospirillaceae (α)	*Thalassospira sp.* DBT-2 (DQ659435)	98	RH3
Xanthomonadaceae (γ)	*Pseudoxanthomonas spadix* (AM418384)	99	RH4
			OR-PAHs
Rhodospirillaceae (α)	*Tistrella sp.* D6-30 (AM403200)	98	RPb1
Sphingomonadaceae (α)	*Sphingomonas xenophaga* (AB099636)	100	RPb2
			OS-Hx
Phyllobacteriaceae (α)	*Parvibaculum lavamentivorans* (AY387398)	95	SH1
Xanthomonadaceae (γ)	*Dokdonella koreensis* DS-140 (AY987369)	94	SH2
Xanthomonadaceae (γ)	*Dokdonella koreensis* DS-140 (AY987369)	99	SH3
Rhizobiaceae (α)	*Agrobacterium sp.* Mei-QS6 (EF090606)	96	SH4
Xanthomonadaceae (γ)	*Pseudoxanthomonas spadix* (AM418384)	99	SH5
Xanthomonadaceae (γ)	*Pseudoxanthomonas spadix* (AM418384)	99	SH6
Erythrobacteraceae (α)	*Erythrobacter sp.* JL893 (DQ985055)	92	SH7
Xanthomonadaceae (γ)	*Stenotrophomonas sp.* 3C_5 (AY689032)	99	SH8
			OS-PAHs
Rhodospirillales (α)	*Rhodovibrio sp.* 2Mb1 (AY987846)	92	SP1
Rhodospirillaceae (α)	*Rhodovibrio sp.* 2Mb1 (AY987846)	93	SP2
Xanthobacteraceae (α)	*Xanthobacter viscosus* (AF399970)	98	SP3
Bacillaceae (Firmicutes)	*Geobacillus stearothermophilus* (EF095714)	98	SP4
Sphingomonadaceae (α)	*Sphingomonas* sp. 3Y (AY646154)	99	SP5
Sphingomonadaceae (α)	*Sphingomonas* sp. 3Y (AY646154)	99	SP6
Sphingomonadaceae (α)	*Sphingomonas xenophaga* (AB099636)	99	SP7
Sphingomonadaceae (α)	*Sphingomonas* sp. 3Y (AY646154)	98	SP8

* A: Actinobacteria, α: Alphaproteobacteria, γ: Gammaproteobacteria. Id.: Identities

Supplemental Table III.S3. 16SrDNA clone library results from rock (OR) and sand (OS) polluted samples. Frequency is indicated for each clone respect to the total analysed at each environment.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	**OR**	**OS**
Gammaproteobacteria				
Alcanivoraceae	*Alcanivorax borkumensis* SK2 (AM286690)	99	1/65	1/72
	Alcanivorax sp. clone SR11d6 (AF548762)	99		1/72
	Alcanivorax sp. Shm-2 (2ª) (AB058675)	99		1/72
	Alcanivorax sp. B-1146 (DQ347532)	99	2/65	
Chromatiales	Uncultured bacterium clone (AF468314)	91		1/72
	Uncultured bacterium clone (DQ001686)	99		2/72
	Uncultured soil bacterium clone (DQ378269)	99	1/65	4/72
	Uncultured bacterium clone (DQ001646)	93	4/65	
Halomonadaceae	*Halomonas sp.* mp1 partial (AJ551115)	100	1/65	
Pseudoalteromonadaceae	*Pseudoalteromonas sp.* (DQ789375)	99		1/72
Pseudomonadaceae	*Pseudomonas putida* (DQ458961)	93		1/72
	Uncultured bacterium clone (DQ334639)	92		1/72
	Uncultured soil bacterium clone (DQ123668)	99		1/72
Salinisphaeraceae	*Salinisphaera sp.* ARD M17 (AB167073)	97	6/65	
Alphaproteobacteria				
Aurantimonadaceae	*Fulvimarina litoralis* (AY178863)	99		1/72
	Aurantimonas coralicida (AJ786361)	99	1/65	
Caulobacteraceae	*Brevundimonas sp.* (AJ227793)	96		1/72
Erythrobacteraceae	*Erythrobacter sp.* JL-475 (DQ104409)	94	1/65	
Hyphomicrobiaceae	*Pedomicrobium fusiforme* (Y14313)	97		1/72
Parvularculaceae	*Parvularcula bermudensis* (AF544016)	92		1/72
Phyllobacteriaceae	*Mesorhizobium sp.* BNC1 (CP000390)	92		1/72
	Parvibaculum sp. W7-12 (DQ659442)	92		1/72
	Parvibaculum lavamentivorans (AY387398)	98	5/65	
	Rhizobium loti (U50165)	94		4/72
Rhizobiales	Uncultured organism clone (DQ395481)	97		1/72
Rhizobiaceae	*Rhizobium sp.* 28/2 (DQ310471)	99	2/65	
Rhodobacteraceae	Arctic sea ice bacterium (AF468367)	96	3/65	
	Citreicella sp. 2-2A (AB266065)	99	1/65	
	Hyphomonas polymorpha (AF082796)	97		1/72
	Paracoccus sp. MBIC3024 (AB008115)	95		1/72
	Phaeobacter daeponensis (DQ981486)	90		1/72
	Rhodobacter litoralis (AY563033)	93	1/65	
	Rhodobacter sp. DQ12-45T (EF186075)	95	1/65	1/72
	Rhodobacteraceae bacterium (AM403233)	96		1/72
	Roseobacter sp. JL985 (DQ985046)	94	1/65	
	Roseobacter sp. COL2P (DQ659415)	93		1/72
	Salipiger bermudensis (DQ178660)	92	1/65	
	Silicibacter pomeroyi DSS-3 (CP000031)	95		1/72
	Sulfitobacter dubius (AY180102)	99	1/65	
	Sulfitobacter sp. S-8 (DQ978989)	99		1/72
	Uncultured *Sulfitobacter sp.*clone (AY697912)	99		1/72
Rhodospirillaceae	*Thalassospira sp. DBT-2 (DQ659435)*	99		1/72
Sphingomonadaceae	*Lutibacterium anuloederans (AY026916)*	96	5/65	4/72
	Novosphingobium hassiacum (AJ416411)	97		1/72
	Novosphingobium sp. HZ11 (AY690709)	94	3/65	
	Porphyrobacter sp. J3-AN66 (DQ454121)	99		1/72
	Sphingomonas sp. GC14 (AY690679)	99	1/65	1/72
	Uncultured bacterium clone F9 (AY375133)	96	1/65	

Supplemental Table III.S3(cont.). 16SrDNA clone library results from rock (OR) and sand (OS) polluted samples. Frequency is indicated for each clone respect to the total analysed at each environment.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	OR	OS
Deltaproteobacteria				
Bacteriovoracaceae	*Bacteriovorax sp.* GSL4A1 (DQ536441)	99		1/72
Bacteroidetes				
Flavobacteriaceae	*Aequorivita antarctica* S4-8 (AY771732)	94		2/72
	Aureimonas pelagi (EF108217)	91	1/65	
	Cellulophaga sp. D3054 (DQ480142)	97	1/65	
	Formosa algae (AY771766)	95	1/65	
	Gelidibacter algens (AF001367)	95		1/72
	Subsaxibacter broadyi P7 (AY693999)	97		1/72
	Vitellibacter sp. D7-13 (AM403205)	97		1/72
Flexibacteraceae	*Flexibacter sp.* D12-42.1 (AM403239)	99		1/72
	Flexibacter tractuosus (AB078076)	98	1/65	
Actinobacteria				
	Uncultured forest soil bacterium (AY913337)	97		1/72
Actinosynnemataceae	*Saccharothrix flava* (AF114808)	90		1/72
Cellulomonadaceae	*Cellulomonas sp.* CR1-1 (AY205295)	99		1/72
Dietziaceae	*Dietzia psychralcaliphila* (AB159036)	99	1/65	
	Dietzia sp. BBDP42 (DQ337506)	98	1/65	
	Dietzia sp. CNJ898 PL04 (DQ448696)	96	1/65	
Microbacteriaceae	*Leifsonia rubra* (AY771748)	98		1/72
	Leifsonia sp. Wged11 (DQ473536)	98		1/72
	Microbacterium esteraromaticum (AB099658)	94	1/65	
	Microbacterium sp. (AB042083)	95	1/65	
Mycobacteriaceae	*Mycobacterium canariasense* (AY255478)	99		1/72
	Mycobacterium frederiksbergense (AJ276274)	99		1/72
	Mycobacterium sacrum BN 3151 (AY235429)	100		2/72
	Mycobacterium sp. O228YA (DQ372728)	97		1/72
Nocardiaceae	*Rhodococcus fascians* SE59 (AY771765)	99	1/65	
	Rhodococcus sp. MBIC01430 (AB088667)	98		1/72
	Rhodococcus sp. NPO-JL-61 (AY745831)	99	6/65	5/72
	Rhodococcus sp. 11/16a (DQ310479)	95	2/65	
	Rhodococcus sp. 28/19 (DQ310477)	96	2/65	
	Rhodococcus sp. P_wp0233 (AY188941)	99	1/65	
	Rhodococcus sp. SGB1168-118 (AB010908)	100	1/65	
Nocardioidaceae	*Aeromicrobium marinum* (AY166703)	99		1/72
	Aeromicrobium sp. Gsoil 098 (AB245394)	95		1/72
Sporichthyaceae	*Sporichthya polymorpha* (AB025317)	95		1/72
Williamsiaceae	*Williamsia sp.* KTR4 (DQ068382)	99		2/72
Planctomycetes				
Planctomycetaceae	Uncultured *planctomycete* clone (DQ289930)	98		1/72
Chloroflexi				
Thermomicrobiaceae	*Thermomicrobium sp.* GR108 (DQ130040)	96		1/72

Supplemental Table III.S4. Strains isolated from the rock sample (OR) using different media. Their relative abundances and the percentage of alkane and aromatic degrading strains (bottom) for each strategy of isolation are indicated.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	OR				
			MA 1/5 (RP)	MPN in MA		Enrichment	
				MPN-Hx (RPH)	MPN-PAHs (RPP)	Hx (PDR)	Phe (PhR)
Gammaproteobacteria			**47,37%**				
Halomonadaceae (γ)	*Cobetia* sp. MACL02 (EF198244)	100	1/38				
	Halomonadaceae bacterium MRN515 (DQ993335)	99	5/38				
	Halomonas sp. 7020 (AM111022)	99	1/38				
	Halomonas sp. B-2046 (DQ347535)	100	3/38				
	Halomonas sp. M6-20C (AY730247)	100	1/38[a]				
	Uncultured *Halomonas* sp. YSSX2 (EF190073)	100	4/38				
Idiomarinaceae (γ)	*Idiomarina* sp. BSw10081 (EF191024)	100	1/38				
Moraxellaceae (γ)	*Psychrobacter aquimaris* KOPRI24929 (EF101547)	100	1/38				
Pseudomonadaceae (γ)	*Pseudomonas* sp. MG-1 (DQ981492)	100	2/38				
	Pseudomonas argentinensis CH01 (AY691188)	99					2/15*
Alphaproteobacteria			**18,42%**				
Aurantimonadaceae (α)	*Aurantimonas coralicida* WP1 (AJ786361)	100	1/38				
Rhizobiaceae (α)	*Sinorhizobium* sp. L1 (AJ879127)	95					2/15*[a]
Rhizobiales (α)	*Martelella mediterranea* MACL11 (AY649762)	98	1/38				
Rhodobacteraceae (α)	*Citreicella* sp. 2-2A (AB266065)(DGGE bands R9-11)	98	1/38[a]				
Rhodobacteraceae (α)	*Jannaschia pohangensis* H1-M8 (DQ643999)	98	1/38				
Rhodospirillaceae (α)	*Thalassospira lucentensis* DSM 14000T (AM294944)	98	1/38				
Sphingomonadaceae (α)	*Sphingomonas* (*Sphingopyxis*) *baekryungensis* (AY608604)	100	2/38				
	Sphingomonas sp. AC83 (AJ717392)(DGGE band RPb2)	100					2/15*
	Sphingomonas sp. KH3-2 (AF282616)	100					7/15*
Bacteroidetes			**18,42%**				
Flavobacteriaceae	Uncultured *Bacteroidetes* (AJ318193)	93	1/38				
	Sufflavibacter litoralis IMCC 1001 (DQ868538)	98	1/38				
	Cytophaga sp. 4301-10/1 (AJ542652)	100	2/38				
	Cytophaga sp. MBIC01539 (AB086623)	100	2/38				
Actinobacteria			**7,89%**				
Promicromonosporaceae	*Promicromonospora vindobonesis* V-45T (AJ487302)	99	3/38				
Dietziaceae	*Dietzia psychralcaliphila* (AB159036)	99				1/32°	
	Dietzia sp. CNJ898 PL04 (DQ448696)	99				2/32°	
Nocardiaceae	*Rhodococcus* sp. 5/1 (AF181689) (DGGE band R1)			2/21°		13/32°	
	Rhodococcus sp. MBIC01430 (AB088667)(DGGE band R2)				1/24°	7/32°	
Firmicutes			**7,89%**				
Bacillaceae	*Bacillus hwajinpoensis* (AB274755)	99	2/38				
Staphylococcaceae	*Staphylococcus epidermidis* S09 (AY741152)	100	1/38				
	Proportion of alkane-degrading strains (°)		***0/38***	***2/21°***	***1/24°***	***23/32°***	***0/15°***
	Proportion of PAHs-degrading strains (*)		***0/38***	***0/21****	***0/24****	***0/32****	***11/15****

Id.: Identities.
MA 1/5: Marine agar diluted 5 times.
MPN in MA: Positive dilutions from the most probable number of alkane (MPN-Hx) and aromatic degrading (MPN-PAHs) microtiter plates were sawn on MA 1/5.
Hx and Phe: Isolates from alkane and PAHs enrichment cultures using hexadecane or phenanthrene as the only source of C and Energy.

Supplemental Table III.S5. Strains isolated from the sand (OS) using different media. Its relative abundances and the percentage of alkane and aromatic degrading strains (bottom) for each strategy of isolation are indicated.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	OS				
				MPN in AM		Enrichment	
			AM 1/5 (AP)	MPN-Hx (APH)	MPN-PAHs (APP)	Hx (PDA)	Phe (PhS)
Gammaproteobacteria			**23,3%**				
Alcanivoraceae (γ)	*Alcanivorax* sp. DG881 (AY258109)	100	1/43[a]				
	Alcanivorax dieselolei B-5 (AY683537)	99				2/26[a]	
Alteromonadaceae (γ)	*Marinobacter bryozoanae* 50-11T (AJ609271)	99	1/43				
	Marinobacter koreensis (AB274772)	100	1/43				
	Microbulbifer hydrolyticus (AJ608704)	99	1/43				
Halomonadaceae (γ)	*Halomonas* sp. B-4011 (DQ347541)	94				1/26[a,?]	
Idiomarinaceae (γ)	Uncultured *Idiomarina* sp. DS071 (DQ234155)	99	1/43				
Pseudoalteromonadaceae (γ)	*Pseudoalteromonas* sp. BSw10020 (DQ789376)	100	1/43				
	Pseudoalteromonas sp. YACS7 (DQ658914)	99	1/43				
Pseudomonadaceae (γ)	*Pseudomonas* sp. XQY-51 (DQ200846)	99	1/43				
	Pseudomonas stutzeri 24a51 (AJ312176)	100					6/15*
	Uncultured *Pseudomonas* sp. SIMO-4042 (DQ295987)				1/25[a,?]		
Xanthomonadaceae (γ)	*Lysobacter concretionis* Ko07 (AB161359)	99	2/43				
	Stenotrophomonas sp. KL1A1 (DQ208664)			15/21[a,?]	15/25[a,?]		
Alphaproteobacteria			**23,3%**				
Aurantimonadaceae (α)	*Aurantimonas coralicida* (AJ786361)	100	1/43				
Erythrobacteraceae (α)	*Erythrobacter* sp. JL893 (DQ985055)(DGGE band SH7)	100	4/43[a,?]				
Rhizobiales (α)	*Martelella mediterranea* MACL11 (AY649762)	99					2/15*[,?]
Rhodobacteraceae (α)	*Loktanella* sp. ice-oil-484 (DQ521395)	98	1/43				
	Paracoccus sp. TDMA-10 (AB264130)	100	1/43				
	Roseobacter sp. LZXC16 (DQ659063)	97	1/43				
	Roseovarius sp. DG944 (AY258090)	99	1/43				
Rhodospirillaceae (α)	*Rhodocista* sp. JZHS37 (DQ658977)	99	1/43				
	Thalassospira sp. DBT-2 (DQ659435)	99				1/26[a,?]	
	Tistrella mobilis (AB071665)(DGGE band RPb1)	99					4/15*[,?]
Sphingomonadaceae (α)	*Sphingomonas* sp. KH3-2 (AF282616)	99					3/15*
Bacteroidetes			**11,6%**				
Flavobacteriaceae	*Flavobacteriaceae* bacterium BSD RB 42 (AY259505)	96	1/43				
	Gaetbulibacter saemankumensis SMK-45 (AY883939)	97	1/43[a,?]				
Flexibacteraceae	*Algoriphagus* (*Rhodovirga*) *chordae* (AJ575265)	100	1/43				
	Algoriphagus antarcticus S4-9 (AY771733)	100	1/43				
	Algoriphagus locisalis MSS-171 (AY835923)	100	1/43				

Supplemental Table III.S5(cont.). Strains isolated from the sand (OS) using different media. Its relative abundances and the percentage of alkane and aromatic degrading strains (bottom) for each strategy of isolation are indicated.

Phylogenetic Group	Closest Classified Organism from GenBank database (accession no.) in Phylogenetic tree	Id. %	OS				
			AM 1/5 (AP)	MPN in AM MPN-Hx (APH)	MPN in AM MPN-PAHs (APP)	Enrichment Hx (PDA)	Enrichment Phe (PhS)
Actinobacteria			**37,2%**				
Cellulomonadaceae	*Cellulomonas* sp. UFZ-B529 (AF237956)	100	1/43				
Gordoniaceae	*Gordonia polyisoprenivorans* (DQ154925)	99				3/26°	
Microbacteriaceae	*Agrococcus baldri* (AB279548)	100	1/43				
	Clavibacter sp. LZXC44 (DQ659089)	100	2/43				
	Microbacterium keratanolyticum OU01 (DQ118082)	100	1/43				
	Microbacterium sp. G16 (EF091839)	100	1/43				
Nocardiaceae	*Rhodococcus* sp. 5/1 (AF181689) (DGGE band S1)	100	5/43°			9/26°	
	Rhodococcus sp. MBIC01430 (AB088667)(DGGE band S2)	100	2/43°			6/26°	
	Rhodococcus opacus ML0004 (DQ474758)	99				1/26°	
Nocardioidaceae	*Nocardioides* sp. A14N (AB251600)	100	1/43				
Promicromonosporaceae	*Promicromonospora* sp. 6727 (DQ487013)	100	1/43				
Streptomycetaceae	*Streptomyces* sp. 6G9 (EF198882)	100	1/43°?			1/26°?	
Firmicutes			**4,6%**				
Bacillaceae	*Bacillus hwajinpoensis* (AB274755)	100	1/43				
	Bacillus sp. 2216.25.2 (AB094471)	100	1/43				
Staphylococcaceae	*Staphylococcus epidermidis* S09 (AY741152)	100				1/26°?	
	Proportion of alkane-degrading strains		***8/43°***	***0/21°***	***0/25°***	***21/26°***	***0/15°***
	Proportion of PAHs-degrading strains		***0/43****	***0/21****	***0/25****	***0/26****	***9/15****

Id.: Identities.
MA 1/5: Marine agar diluted 5 times.
MPN in MA: Positive dilutions from the most probable number of alkane (MPN-Hx) and aromatic degrading (MPN-PAHs) microtiter plates were sawn on MA 1/5.
Hx and Phe: Isolates from alkane and PAHs enrichment cultures using hexadecane or phenanthrene as the only source of C and Energy.

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