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DECAPAGE PROJECT: HYDROCARBON DEGRADATION IN COASTAL SEDIMENTS*

The effect of oil spills on the bacterial diversity and catabolic function in coastal sediments: a case study on the *Prestige* oil spill

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Abstract The accident of the *Prestige* oil tanker in 2002 contaminated approximately 900 km of the coastline along the northern Spanish shore, as well as parts of Portugal and France coast, with a mixture of heavy crude oil consisting of polycyclic aromatic hydrocarbons, alkanes, asphaltenes and resins. The capacity of the autochthonous bacterial communities to respond to the oil spill was assessed indirectly by determining the hydrocarbon profiles of weathered oil samples collected along the shore, as well as through isotope ratios of seawater-dissolved CO_2, and directly by analyses of denaturing gradient gel electrophoresis fingerprints and 16S rRNA gene libraries. Overall, the results evidenced biodegradation of crude oil components mediated by natural bacterial communities, with a bias towards lighter and less substituted compounds. The changes observed in the *Proteobacteria*, the most abundant phylum in marine sediments, were related to the metabolic profiles of the sediment. The presence of crude oil in the supratidal and intertidal zones increased the abundance of *Alpha-* and *Gammaproteobacteria*, dominated by the groups *Sphingomonadaceae*, *Rhodobacteraceae* and *Chromatiales*, whilst *Gamma-* and *Deltaproteobacteria* were more relevant in subtidal zones. The phylum *Actinobacteria*, and particularly the genus *Rhodococcus*, was a key player in the microbial response to the spill, especially in the degradation of the alkane fraction. The addition of inorganic fertilizers enhanced total biodegradation rates, suggesting that, in these environments, nutrients were insufficient to support significant growth after the huge increase in carbon sources, as evidenced in other spills. The presence of bacterial communities able to respond to a massive oil input in this area was consistent with the important history of pollution of the region by crude oil.

Keywords In situ hydrocarbon biodegradation · Marine sediments · Alcanivorax · Bacterial community response · Prestige · Anaerobic hydrocarbon degradation · Alkanes · Aromatics · PAHs · North Atlantic

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Introduction

Marine oil spills are recurrent incidents of varying magnitudes, in general caused by accidents in the production and transport of petroleum and its by-products around the world. They became important during the past decades, especially in the coastal zones of the European Atlantic area, a major oil tanker transport route considered the highest oil spill hotspot area worldwide (Vieites et al. 2004). In particular, the northwestern region of the Spanish coast has been continuously affected by these oil spills, among others: the *Polycommander* (1970; 15,000 t), the *Monte-Urquiola* (1976; 101,000 t), the *Andros Patria* (1978; 60,000 t), the *Aegean Sea* (1992; 67,000 t) and the *Erika* (1999; 31,000 t) (CEDRE 2013). In November 2002, the oil tanker *Prestige* sank at 155 miles off the northern Spanish coast, releasing an estimated 64,000 t of heavy oil that contaminated the coastline along approximately 900 km of Galicia and the Bay of Biscay, affecting Spain, France and to a lesser extent, Northern

Portugal. As a consequence, in total more than 600 sandy beaches were polluted by crude oil along the Spanish Atlantic coast (Fernández-Fernández et al. 2011).

Incidents of these magnitudes have important consequences on the autochthonous microbial communities, which suffer drastic changes in structure and function. Although the specific changes observed are characteristic of each contamination event, general patterns are observed. Most information available on the specialized hydrocarbon degraders blooming after an oil spill initially originated from culture-based approaches aimed at isolating marine bacteria able to degrade specific hydrocarbons. Numerous genera of the *Gammaproteobacteria*, such as members of the *Oceanospirillales* as *Alcanivorax* (Yakimov et al. 1998), *Thalassolituus* (Yakimov et al. 2004) and *Oleispira* (Yakimov et al. 2003), and members of the *Thiotrichales* such as the genus *Cycloclasticus* (Dyksterhouse et al. 1995), and many others (see Yakimov et al. (2007) for an extensive review on marine hydrocarbon degraders), were first isolated and identified as highly specialized hydrocarbon degraders from enrichment cultures on specific hydrocarbon mixtures. The relevance of these strains, as part of the response of the autochthonous bacterial communities to the presence of hydrocarbons in marine environments, was repeatedly observed using culture-independent approaches both in field experiments and in laboratory microcosms when simulating such oil spills. Numerous bacterial strains belonging to the abovementioned taxonomic groups could be isolated from various marine environments (Syutsubo et al. 2001; Kasai et al. 2002; Maruyama et al. 2003; Röling et al. 2004), demonstrating their ubiquity.

Since the *Exxon Valdez* spill in Alaska in 1989, the largest marine spill in the USA at that time, such important oil spills throughout the world have promoted intensive research to understand how the marine microbial communities respond to oil spills, with the final goal of developing bioremediation strategies for the recovery of the most extensively affected areas (Atlas and Hazen 2011). In the case of the *Exxon Valdez* spill, emphasis was placed on implementing appropriate remediation protocols for a continuous treatment of the shoreline, taking advantage of the stimulation of the capacity for natural attenuation of the autochthonous bacterial communities. Research efforts focused on biodegradation by tracing the changes in the chemical composition of crude oil, although no special attention was paid to the specific microbes involved in the process (Atlas and Hazen 2011). Field tests showed that nutrients such as nitrogen and phosphorus were the limiting factors for biodegradation, and that the addition of fertilizers containing both elements significantly increased the biodegradation rates (Atlas and Bragg 2009). A similar approach was used after the *Sea Empress* incident on the coastline of Wales in 1996 and showed that slow-release fertilizers could be used to increase in situ bioremediation in the affected areas (Swannell et al. 1996).

Molecular approaches to identify and quantify the marine microbial populations in areas affected by an oil spill are relatively recent. Molecular tools were first used after the *Nakhodka* spill into the Japan Sea in 1997 (Kasai et al. 2001). The analysis by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene sequences detected differences within the predominant bacterial phyla in seawater and in samples of oil paste. In the seawater samples members of the *Bacteroidetes*, *Alphaproteobacteria* and *Cyanobacteria* were predominant, whilst the bacterial groups found in the oil paste were related to the known hydrocarbon degraders such as *Sphingomonas subarctica* and *Alcanivorax borkumensis* (Kasai et al. 2002). In a coastal area heavily contaminated after the spill, the oil-degrading community was dominated by a strain closely related to the aromatic hydrocarbon degrader *Cycloclasticus pugetii*, which accounted for some 25 % of the bacterial population, followed by the alkane degrader *A. borkumensis* that reached up to 7 % of the community (Maruyama et al. 2003).

Recently, the Deepwater Horizon (DWH) blowout in 2010 in the Gulf of Mexico resulted in a massive oil discharge (680, 000 t). This provided a unique opportunity for the well-prepared scientific community to apply high-throughput molecular microbiology tools and to carry out a thorough analysis of the marine microbial community response towards the impact of hydrocarbons on it, and its role in the bioremediation of crude oil (revised in Kimes et al. 2014). The results evidenced a fast and dynamic response of the autochthonous microorganisms to the presence of oil-derived hydrocarbons. The important shifts in the community structure observed over time were connected to changes in the presence and bioavailability of the individual components of oil hydrocarbons. Unlike that of other spills, the oil from the Macondo 252 well contained a high proportion of light hydrocarbon compounds such as medium-chain *n*-alkanes, monoaromatic compounds (BTEX) and polycyclic aromatic hydrocarbons (PAHs), which made it initially more easily biodegradable than the more recalcitrant heavy fractions of crude oil that constituted the bulk of the spills discussed above (Atlas and Hazen 2011). The changes in the bacterial communities in response to this spill were characteristic of the differently affected ecosystems. Results from samples taken 4 to 7 weeks after the spill in the plum at 1100 m depth of the water column revealed a significant increase in total bacterial biomass, concomitant with the blooming of a novel species of the *Gammaproteobacteria* belonging to the *Oceanospirillales* and taxonomically related to *Oleispira* (Hazen et al. 2010). The new species accounted for more than 90 % of the retrieved 16S rRNA gene sequences in certain samples (Redmond and Valentine 2012). This

species was shown to be primarily involved in the aerobic degradation of alkanes (Mason et al. 2012). The dominant community then shifted towards *Cycloclasticus* and *Colwellia*. This last genus was probably favoured by its capacity to degrade short-chain gaseous hydrocarbons such as methane, ethane and propane, the most important constituents of that plum (Mason et al. 2014). These groups disappeared when the Macondo 252 well was finally capped and were replaced by a diverse community in which *Flavobacteria*, *Rhodobacteraceae*, *Alteromonadaceae* and methylotrophic bacteria were the most abundant groups (Redmond and Valentine 2012; Dubinsky et al. 2013). Subsequent samplings undertaken between 6 months and more than 1 year after the accident showed the recovery of a community structure reminiscent of the initial, non-polluted, situation. The community response in the surface water was similar, but a more diverse and variable community dominated by *Alpha-* and *Gammaproteobacteria* was found in the oil slicks observed on the surface of the water. Finally, in the oil spill-affected coastal sands and sediments, miles away from the spill well, an increased cell density similar to that observed in the plum was detected. In this case, *Alpha-* and *Gammaproteobacteria* were prevalent, including an important transient blooming of *Alcanivorax* (Kostka et al. 2011). Members of the *Rhodobacteraceae* and *Alteromonadaceae* were more abundant in these contaminated samples, from which hydrocarbon degrading *Roseobacter* and *Marinobacter* strains could be isolated. All these groups, together with representatives of the *Pseudomonadales* also abundant in the polluted sands, were shown to be active in hydrocarbon degradation (Lamendella et al. 2014).

In the case of the *Prestige* oil spill in 2002, research efforts were designed to analyse the fate of oil, its impact on the different coastal communities and the response of these microbial communities towards the presence of huge amounts of oil components, among others. Both culture-dependent and molecular techniques were used to identify the most abundant bacterial taxa blooming after the spill. The most popular and affordable molecular approaches available at the moment of the spill were DGGE fingerprinting analysis and classical 16S rRNA gene libraries. Here, we revise the hitherto published studies which analysed the impact of the spill on the coastal environments, and especially those aimed at understanding the microbial response to the *Prestige* spill in these habitats.

Nature and fate of the spilled oil from the *Prestige*

The *Prestige* oil slick was made up of heavy and complex mixtures of saturated (22 %) and aromatic (50 %) hydrocarbons, and of asphaltenes and resins (28 %). The aromatic fraction was mainly composed of naphthalene and phenanthrene and their alkyl derivatives, whilst the saturated fraction consisted of cyclic and linear hydrocarbons of variable length (CSIC 2003a; Alzaga et al. 2004; Albaigés et al. 2006). Furthermore, it contained some trace metals such as nickel, vanadium, copper and zinc (CSIC 2003b; Santos-Echeandía et al. 2005). A part of the spilled oil was drifting on the water surface for several months until it finally landed on the coastline (Franco et al. 2006). An important fraction of the oil slick was eliminated through intensive mechanical extraction. However, for the most affected beaches, a long-term persistence of oil residues in intertidal and subtidal areas was monitored. There, the strong hydrodynamic activity provoked burying and resurfacing of their heavy constituents (Bernabeu et al. 2013).

Crude oil exposed to marine environments undergoes a series of weathering processes of different natures, among which evaporation, dissolution, photo-oxidation and biodegradation produce the strongest changes in its chemical composition (Harayama et al. 1999). Chemical analyses of the oil-polluted samples were undertaken to obtain the oil molecular fingerprint which is required to determine both the source of oil and the type and degree of weathering. Detailed analyses of the hydrocarbon profiles in sediment and water column samples collected during the first year after the *Prestige* wreck, however, could barely evidence the presence of hydrocarbon compounds originating from this spill above the background of chronic pollution. These apparently low hydrocarbon levels of the water-soluble fraction (WSF) were attributed to the low water solubility and the heavy nature of the spilled oil (Blanco et al. 2006; Franco et al. 2006; Salas et al. 2006). Only in the most severely impacted zones, in the Costa da Morte area (Galicia) (Franco et al. 2006), the results obtained from the analyses of those samples suggested some clear contribution from the *Prestige* accident above the local background of pollution. The spilled crude oil was finally deposited in the form of high-molecular-weight hydrocarbon-rich tarballs onto surface sediments, a part of which became buried in the sand along the shoreline (Franco et al. 2006). The analyses of these aggregates clearly indicated that they originated from the *Prestige* spill (Alonso-Gutiérrez et al. 2009; Acosta-González et al. 2013a).

Impact of the *Prestige* spill on coastal environments

A variety of habitats were affected by the *Prestige* spill, ranging from supralittoral, littoral (intertidal and supratidal) and sublittoral (subtidal) levels, to oceanic and bathyal environments. These included important fisheries as well as other highly diverse biological communities (Castanedo et al. 2006; Juanes et al. 2007). Oil toxicity is primarily attributed to the aromatic fraction, particularly to PAHs, which represent the main constituents of the *Prestige* oil, and which are potentially mutagenic, carcinogenic and teratogenic (Albers 2003;

Barros et al. 2014). Marine ecosystem compartments predominantly impacted by oil spills are in general the seaside flora and fauna, especially birds, fish, whales, crustaceans and bivalve molluscs, among others. Humans also constitute an exposed population whose health may be potentially deteriorated by the noxious properties of crude oil. The toxic effect of the *Prestige* spill has been reviewed extensively, with special emphasis on the macrofauna (Junoy et al. 2005; Moreno et al. 2013) and on human health (Aguilera et al. 2010; Zock et al. 2011).

An overall impression which could be drawn out from toxicity analyses was that the toxic effect of the spill on planktonic organisms was weak (Penela-Arenaz et al. 2009), an observation which could be attributed to the low water solubility of most of the oil components, as was also determined during the chemical analysis of the WSF. Varela et al. (2006) observed an acute accumulation of some of the constituents of oil in plankton, although its origin was much more traced to the local chronic pollution than to the *Prestige* oil spill. Only in those samples collected from the severely affected Costa da Morte, the origin of the accumulated oil could be traced, and as such assigned to the *Prestige* spill. During the spring bloom of plankton in 2003, no noticeable effects of the spill on the biomass of phytoplankton and its primary production rates were detected above the inherent variability of the planktonic cycles (Salas et al. 2006; Varela et al. 2006). Bode et al. (2006) also found that the abundance of bacterioplankton was within the range observed before the spill although bacterial activity was significantly enhanced, especially during the post-spill winter and summer. The high production rates and an increase in the utilisation of oxygen during the winter after the spill were interpreted as a sign of biodegradation of oil-derived hydrocarbons (Bode et al. 2006).

Laboratory assays were performed to estimate the toxicity of the WSF of the *Prestige* oil which gave controversial results that were attributed to the different methods used to prepare this WSF in the different laboratories. Some authors reported the absence of significant effects in different toxicity tests using microalgae and Cladocera (Navas et al. 2006). Others found a significant reduction in larval growth of sea urchins, success of mussel embryogenesis and the survival of copepods and fish (Saco-Álvarez et al. 2008). Furthermore, the toxicity of the *Prestige* WSF prepared in the laboratory was comparable to the toxicity of natural seawater samples collected during the first days after the spill, which completely inhibited the embryogenesis of bivalves and sea urchins (Beiras and Saco-Álvarez 2006).

The in situ toxicity of oil residues was determined in the swash and dry sand zones, two areas severely impacted by the oil spill (de la Huz et al. 2005), which were also severely disturbed by clean-up activities that involved the removal of sand (Penela-Arenaz et al. 2009). A general decrease in the abundance and richness of the macrofauna related to the degree of pollution was observed in the intertidal and supratidal zones of all of the studied beaches. The most impacted taxa were the isopod genus *Eurydice* and the polychaete *Scolelepis squamata*, although this last organism seemed to be more influenced by clean-up activities than by the presence of oil (Junoy et al. 2005). Reduced diversity indexes were generally observed for polychaetes, insects and semi-terrestrial crustaceans in all samples, whilst the changes in the diversity of marine crustaceans were either positive or negative, according to the specific site (de la Huz et al. 2005). On the other hand, the distribution of benthonic communities was not modified by the presence of tar aggregates on the bottom of the Galician continental shelf, after the accident (Serrano et al. 2006).

As an alternative approach to the determination of the potential impact of the spill, the toxic effect of the polluted sediments was also determined using biomarker organisms in laboratory assays. Impacts on subtidal and shoreline sediment samples from moderately affected beaches collected a few days, a month and 10 months after the spill were relatively low as determined by the burrowing behaviour of clams, success in embryogenesis, photosynthesis and growth tests of microalgae (Franco et al. 2006), which correlated with the moderate toxicity of water collected in the affected area (Marino-Balsa et al. 2003). The long-term toxicity which resulted from the spill and which was analysed 4 years after the accident showed the absence of acute toxicity, concomitant with the decrease of PAH concentration in the sediments. However, the accumulation of PAHs was detected in the tissues of benthic organisms, suggesting that PAHs were still present in the food chain (Morales-Caselles et al. 2008).

Finally, only those sediment samples directly affected by an oil patch suffered a severe decrease in microbial abundance (Acosta-González et al. 2013a). The impact of the oil on the microbial abundance in the shoreline habitats was negligible, although significant changes in the trophic structure of bacterial communities were observed (see below) (Alonso-Gutiérrez et al. 2009).

Evidence for in situ biodegradation

The first evidence for in situ biodegradation of the spilled oil from the *Prestige* accident-affected shoreline came from direct measurements of the stable carbon isotope ratio of total dissolved inorganic carbon (DIC) in water samples taken 3 months after the spill from heavily contaminated beaches of Galicia (Medina-Bellver et al. 2005). The DIC $^{12}CO_2/^{13}CO_2$ ratio in some samples indicated degradation of a very $\delta^{13}C$-negative source such as the *Prestige* crude oil ($\delta^{13}C=-30.6‰$), suggesting that the bacteria present in the contaminated water were able to transform crude oil components into inorganic carbon. An increase in the most probable

number (MPN) enumeration values above 10^5 cells/ml for undecane, naphthalene and phenanthrene degraders in water samples collected 1 month after the spill further supported this idea (Medina-Bellver et al. 2005). Similarly, the analysis of the hydrocarbon profile of surface sediments and of phytoplankton collected during the early summer of 2003 suggested some extended catabolic degradation of petrogenic hydrocarbons, although the origin of the contaminant could not always clearly be attributed to the *Prestige* spill (Salas et al. 2006).

Chemical analyses of the spilled oil were often carried out in the frame of bioremediation experiments which were implemented to test the efficiency of biostimulation strategies. In this context, a detailed characterization of the weathering properties of the *Prestige* oil was carried out both in situ in a selected polluted beach and under controlled laboratory conditions, in order to define the changes that different weathering processes produced on the hydrocarbon profile of the spilled oil (Díez et al. 2005; Jiménez et al. 2006). To assess the presence of characteristic constituents of the *Prestige* oil components in the samples, sterane and triterpane (m/z 191) fossil markers were analysed in detail. Among the molecular markers proposed as oil fingerprints, Jimenez et al. (2006) found that the depletion of diasteranes and C27 sterane components, together with a decrease in the 14α(H),17α(H) steranes versus the 14β(H),17β(H) isomers in the aliphatic fraction, were the best indicators for biodegradation in these samples. Overall, these data proved to be useful for the interpretation of the analytical results obtained from environmental samples in subsequent studies (Franco et al. 2006; Salas et al. 2006; Alonso-Gutiérrez et al. 2008, 2009; Bernabeu et al. 2013). The hydrocarbon profile of intertidal sediment and cobblestone samples from polluted beaches showed depletion of short chain *n*-alkanes and aromatics, a possible indication for the presence of an autochthonous bacterial population of diverse metabolic capacities towards hydrocarbons (Gallego et al. 2006; Jiménez et al. 2006). Monitoring of these beaches after several months showed further depletion of *n*-alkanes and of 3 to 4 ring PAHs, in some cases to negligible levels, a fact which was confirming this idea. The biodegradation of the oil was clearly enhanced in the presence of the oleophilic fertilizer S200 (Jiménez et al. 2006), which resulted in a significant increase in the microbial population able to grow with crude oil as the only carbon source (Gallego et al. 2006).

An extensive survey was carried out along the northern Spanish shore during the first year after the spill to determine the fate of the spilled oil by analysing the hydrocarbon profile of more than 200 tarballs collected from the water surface and onshore. In general, the hydrocarbon profile of the samples suggested that biodegradation was not dominant in the weathering process (Díez et al. 2007). The degradation of *n*-alkanes was only noticeable after a 2 months lag period and did not progress extensively over time. This was attributed to the compact nature of the spilled oil, which limited its bioavailability. In the case of the aromatic fraction, degradation of oil components was not even. A clear trend towards a faster degradation of lighter or less substituted compounds and a preference for certain substituent positions in the aromatic rings were observed: Isomers of derivatives of naphthalene and phenanthrene with β-substituents were preferentially degraded. Altogether, the results suggested a limited biodegradation and a significant persistence of the spilled oil in the sea. On the other hand, oil samples collected 1 year after the spill from an oil-coated cobblestone beach showed the almost total depletion of alkylnaphthalenes which was attributed to evaporation and washing. However, a significant depletion in the higher fractions of *n*-alkanes and a reduction of β-substituted PAH isomers were taken as a clear indication of microbial degradation (Alonso-Gutiérrez et al. 2009).

During the evaluation of various bioremediation agents on artificially crude oil-painted granite tiles placed on the supratidal and intertidal areas of a Galician beach, Fernández-Álvarez et al. (2007) observed a general depletion of total hydrocarbons in both the control and treated tiles, as determined gravimetrically. This was explained as a natural attenuation process which was taking place, although photochemical oxidation might also have contributed to the observed loss of PAHs of high molecular weight. Only the treatment with sunflower biodiesel seemed to accelerate the depletion of residual aliphatic and aromatic fractions to some extent (Fernández-Álvarez et al. 2007).

In the most severely affected areas of the Galician shore, crude oil from the spill also reached salt-marshes located in some estuaries. In these particular coastal ecosystems composed of loamy sand and loam which were covered predominantly by rushes and different types of grass (Andrade et al. 2004), hydrocarbon profile analyses revealed that in a period of 4 years, more than 85 % of the saturated and aromatic fraction had naturally been degraded, and that the efficiency of the process was dependent of the thickness of the fuel-oil crusts that covered the soil (Vega et al. 2009).

Cultivation-independent analysis of the bacterial communities in oil-polluted beaches

Different culture-independent approaches were used to directly analyse the bacterial diversity present in marine sediments and beaches affected by the *Prestige* oil spill. Figure 1 summarizes the distribution of phylogenetic groups found in different studies on several affected beaches, which are discussed below. The dominance in all cases of the phylum *Proteobacteria* was evident and agrees with the global analysis of the bacterial diversity in coastal sediments (Zinger et al. 2011). Among *Proteobacteria*, the classes *Gamma*- and *Alphaproteobacteria* were dominant in supratidal samples, together with *Bacteroidetes* and *Actinobacteria*, as observed

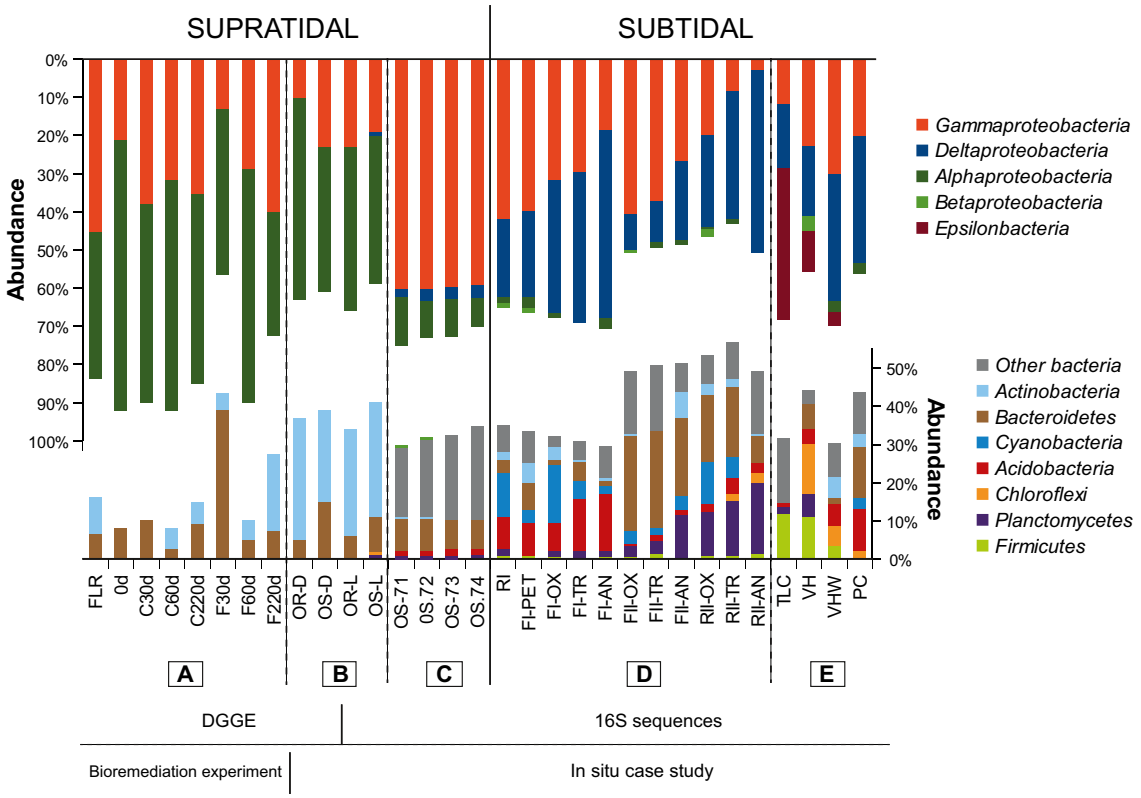

Fig. 1 Composition of bacterial communities present in different sediments affected by the *Prestige* oil spill. Data from comparable polluted sites (*C* and *E*) are included for comparison. From left to right: *A* Supratidal sediments on the Cantabrian coast, Spain (Jiménez et al. 2007): FLR, *Prestige* oil residue; C0d, C30d and C220d are controls of the bioremediation assay evaluated at 0, 30 and 220 days, respectively, whilst F0d, F30d and F220d correspond to the fertilized plots evaluated in the same experiment at 0, 30 and 220 days. *B* Supratidal samples form rocks and sand at Faro Lariño (Spain) affected by the *Prestige* spill (Alonso-Gutiérrez et al. 2009): OR, Oiled Rocks; OS, Oiled Sand; D, DGGE analysis; L, 16S rRNA gene libraries. *C* 16S rRNA gene NGS (pyrosequencing) libraries from DWH spill-affected Pensacola Beach sand samples collected on July 2, 2010 (Kostka et al. 2011). OS71, heavily polluted sites; OS72, moderately polluted site; OS73 and OS74, non-polluted sites. *D* Subtidal sediments affected by the *Prestige* spill in Cies Islands (Galicia, Spain) (Acosta-González et al. 2013a): RI, non-polluted Rodas beach; FI-PET, tar aggregate from Figueiras beach collected in 2004; FI-OX/FI-TR/FI-AN, samples from oxic, transition, and anoxic zones of Figueiras beach sediments collected in 2004; FII-OX/FII-TR/FII-AN, samples from oxic, transition, and anoxic zones of Figueiras beach sediments collected in 2007; RII-OX/RII-TR/RII-AN, samples from oxic, transition and anoxic zones of Rodas beach sediments with oil at 30 cm depth. *E* Subtidal sediments from Victoria Harbor, Hong Kong (Dyksterhouse et al. 1995): VH and VHW, polluted sites; TLC, adjacent open oceanic site; PC, Estuary discharge affected

in other spills (Kasai et al. 2001; Kostka et al. 2011). One year after the spill, the bacterial communities in rock and sand samples severely affected by the spill both in Cantabria (Bay of Biscay) and Costa da Morte were dominated by *Alphaproteobacteria*, ranging between 40 and 70 % of the total bacterial population, followed by *Gammaproteobacteria* and *Actinobacteria*, with varying proportions according to the specific site (Fig. 1(A, B)) (Jiménez et al. 2007; Alonso-Gutiérrez et al. 2009). Despite the distance between the two sites, several relevant phylotypes in the two communities were identical or closely related, suggesting that the observed response was likely to be a common pattern in the extended polluted area. The main representatives of the *Gammaproteobacteria* were the members of the order *Chromatiales*, and among the *Alphaproteobacteria*, the families *Sphingomonadaceae* and *Rhodobacteraceae* were the most abundant taxa. In general, sand communities with lower hydrocarbon levels were more diverse (Jiménez et al. 2007), and bacterial communities in the oil paste-covered rock surfaces were predominantly associated with alkane degradation, whilst the communities in oil-contaminated sand (small oil drops scattered among sand grains) were related to the degradation of both aliphatic and aromatic fractions (Alonso-Gutiérrez et al. 2009). The analysis by DGGE detected the presence of members of the *Alphaproteobacteria* previously related to hydrocarbon degradation, such as *Lutibacterium anuloederans*, reported to degrade 2 and 3 ring PAHs (Chung and King 2001), and *Actinobacteria* related to *Mycobacterium* spp., reported to mineralize the much more recalcitrant 3 to 4 ring PAHs (Willumsen et al. 2001). This may explain their presence in samples where the easily degradable hydrocarbons had already been depleted (Alonso-Gutiérrez et al. 2009). Among the *Actinobacteria*, the suborder *Corynebacterinae*, and especially the genera *Rhodococcus*

and *Dietzia*, was repeatedly found in the 16S rRNA gene libraries and dominated the cultivable aerobic alkane degrading community (Table 1, see below). *Actinobacteria* are especially resistant to dry and resource-limited conditions and were suggested to be involved in the degradation of both the long-chain alkane and aromatic fractions in these

Table 1 Bacterial groups isolated from the *Prestige* spill contaminated beaches

Organism[h]	Atlantic Islands[a]			Costa da Morte[b]			A Coruña[c]			A Coruña[d]			Costa da Morte[e]		
	N[i]	% deg.[f]		N[i]	% deg.[f]		N[i]	% deg.[f,g]		N[i]	% deg.[f]		N[i]	% deg.[f]	
		alk	PAH		alk	PAH		alk	PAH		alk	PAH		alk	PAH
Gammaproteobacteria	*30*			*75*			*7*			*6*					
Oceanospirillales	10	70		23	22					4	100				
(Alcanivorax)	(7)	100		(3)	100					(4)	100				
Alteromonadales	16			7			6	33	33	2	100				
Pseudomonadales	1			13	7	62	1						86	10	24
Xhantomonadales				32	47	47									
Vibrionales	3														
Thiotrichales							1								
Alphaproteobacteria	*11*			*38*			*17*			*3*					
Rhizobiales	1			7			5	40	20	2					
Rhodobacterales	10			6		17	8	12	12	1	100				
(Citreicella)				(1)											
Rhodospirillales				7			1								
(Thalassospira)				(2)	50		(1)		100						
(Tistrella)				(4)		100									
Sphingomonadales				18	22	67	1								
(Sphingomonas)				(12)		100									
Caulobacterales							2								
Actinobacteria				*64*											
Actinomycetales				64	84										
(Rhodococcus)				(46)	100										
(Dietzia)				(3)	100										
(Gordonia)				(3)	100										
Bacteroidetes	*5*			*11*			*6*			*1*					
Flavobacteriales	5			4	25					1	100				
Cytophagales				7			5								
Firmicutes	*5*			*3*			*1*			*1*					
Bacillales	5			3	33		1			1	100				

[a] February 2003, Alonso-Gutiérrez et al. 2008

[b] March 2004, Alonso-Gutiérrez et al. 2009

[c] Five-year enrichment, Vila et al. 2010

[d] Seventeen-month enrichment, Jiménez et al. 2011

[e] March 2004 to July 2005, Mulet et al. 2011

[f] Percentage of isolates for which hydrocarbon degradation (alkanes or PAHs) could be confirmed

[g] Growth on hydrocarbon compounds was only tested on solid media

[h] Within each order, the data for the most relevant genera are indicated in brackets

[i] Number of isolates

environments (Álvarez and Silva 2013). The dominance of the *Alphaproteobacteria* and *Actinobacteria* was again explained in terms of the biodegradation process reaching advanced stages where the gammaproteobacterial communities were less active (Alonso-Gutiérrez et al. 2009).

The addition of the commercial oleophilic fertilizer S200 for 220 days did not produce significant changes in the communities as compared with untreated controls, at least not at the high taxonomic levels: a gradual decrease in the dominance of the *Alphaproteobacteria* (from 70 to 72 % of the population at the beginning of the study to 30 to 50 % in the end) especially in the bio-stimulated plot, and a concomitant two- and three-fold increase in *Gammaproteobacteria* at the end of the experiment were observed in the control and fertilized treatments, respectively (Fig. 1(A)) (Jiménez et al. 2007). The abundance of *Bacteroidetes* and *Actinobacteria*, especially of the genus *Rhodococcus*, also increased over time. The use of the S200 fertilizer considerably enhanced the degradation of high-molecular-weight alkanes, PAHs and their alkylated derivatives when compared with the control plots, suggesting an important role for the *Gammaproteobacteria* in oil degradation under those conditions (Fig. 1(A)). A study of supratidal sediments affected by the DWH spill also reported an increase in the populations of the *Gammaproteobacteria* during the first 4 months after the spill, especially in the most heavily polluted samples, where the largest increase in the relative bacterial abundance corresponded to the genus *Alcanivorax* (Kostka et al. 2011) (Fig. 1(C)). The presence of *Alcanivorax*, however, was not detected in the Cantabria bioremediation experiment (Jiménez et al. 2007), but *Alcanivorax* strains able to degrade *n*-alkanes could be isolated from samples taken from the Atlantic Islands (Galicia) (Alonso-Gutiérrez et al. 2008). The failure to detect *Alcanivorax* might have been due to one of the PCR primers used (341F) which was not matching that genus, according to the TestProbe tool available in the Silva website (Quast et al. 2013). The initial abundance of *Chromatiales* and the final presence of *Rhodococcus* were associated with the different stages of the biodegradative process in which these groups took part: Whilst the *Chromatiales* were acting on crude oil components at the initial stages, *Rhodococcus* was principally active during the final stages (Abed et al. 2002; Kostka et al. 2011; Mortazavi et al. 2013).

The short-term response of bacterial communities towards the *Prestige* oil was also analysed in simulated experiments. Fluorescence in situ hybridization (FISH) analysis of seawater mesocosm experiments carried out in the waters of a Galician estuary showed that the addition of the WSF of the *Prestige* oil to the initial communities dominated by the groups *Bacteroidetes* and *Proteobacteria* resulted in an increase in the abundance of total prokaryotes, whilst *Alphaproteobacteria* were negatively affected by the treatment (Teira et al. 2007). The presence of high amounts of PAHs strongly stimulated the blooming of *Cycloclasticus*, which could reach up to 11 % of the bacterial community after 4 days, decreasing thereafter to stabilize at values higher than the initial levels (Teira et al. 2007). These results suggest that, as for *Alcanivorax*, the role of *Cycloclasticus* in hydrocarbon degradation was relevant in the initial stages of the spill when the levels of low-molecular-weight PAHs were high. It is worth noting that this strain was neither detected using PCR-based molecular tools (Alonso-Gutiérrez et al. 2009), nor in *Prestige* oil enrichment cultures (Vila et al. 2010).

Seven years after the *Prestige* accident, Reis et al. (2014) evaluated the biodegradation potential under laboratory conditions of the adapted microbial communities from an intertidal beach of the Costa da Morte where superficial tarballs had repeatedly been found. These samples were exposed to oil under various conditions of biostimulation. The results demonstrated that the addition of a nitrogen fertilizer enhanced the degradation of the total petroleum hydrocarbons (from 61 to 85 % degradation) in 15 days, whilst the degradation of the PAHs increased from 38 to 70 %, especially for anthracene and phenanthrene. The bacterial community structure changed under all of the tested conditions, as determined by phylum-targeted DGGE analyses. The main changes in the *Alphaproteobacteria* subclass seemed to correspond to the presence of the fertilizer, whilst the community structure of *Betaproteobacteria*, *Actinomycetales* and *Pseudomonas spp*. changed in accordance to the presence or absence of oil; strongly suggesting that these bacterial communities might have been an active part of the hydrocarbon degraders in these sediments (Reis et al. 2014).

The picture of the microbial diversity in subtidal sediments affected by the *Prestige* oil spill was quite different from that obtained for supratidal and intertidal sediments (Acosta-González et al. 2013a). The anaerobic respiration, mainly represented by sulfate reduction, was the type of metabolism connected to biodegradation in sediments of the Atlantic Islands in 2004 and 2007. The dominance of sulfate reduction was clearly linked to the presence of *Deltaproteobacteria* communities. The distribution of classes of the *Proteobacteria* was controlled by the redox properties of the sediment profile: in the oxidized zone, *Gammaproteobacteria* (which include aerobes and facultative anaerobes) dominated, whilst in the transition zone and reduced zone, the *Deltaproteobacteria* (anaerobes) was the dominant group (Fig. 1(D)). 16S rRNA sequences of *Deltaproteobacteria* very similar to those found in the polluted subtidal sediments of the Atlantic Islands were also reported from polluted marine sediments (Stauffert et al. 2013), from hydrocarbon seeps associated with dodecane degradation from the mud sediments of the Amod Volcano (Genovese et al. 2014) and ^{13}C-toluene degraders from methanogenic enrichments from a contaminated aquifer (Fowler et al. 2014). It is important to note that the *Gammaproteobacteria* was the main group in samples of tar aggregate collected from the same site, as well as in oil residues of subtidal sediments (Fig. 1(D)) (Acosta-González et al. 2013a), which was also documented in samples from other spills (Kasai et al. 2001). Additional to the members of the *Proteobacteria*, other phyla seemed to play a relevant role in the biodegradation

processes occurring in situ in these anoxic hydrocarbon-polluted sediments. The increased presence of *Bacteroidetes*, *Actinobacteria* and *Firmicutes* communities, described as common inhabitants of coastal sediments, has been associated with advanced stages of oil degradation in *Prestige* oil-polluted sediments (Alonso-Gutiérrez et al. 2009; Acosta-González et al. 2013a).

In general, the richness of species in polluted subtidal sediments was higher than in pristine samples (Acosta-González et al. 2013a) as previously observed in other polluted sites (Dos Santos et al. 2011). However, in cases where the level of contamination was extremely high, the richness of species could decrease drastically (Torsvik et al. 1996; Wang and Tam 2011; Acosta-González et al. 2013a).

A general observation is that in anaerobic environments the degradation of complex molecules only occurs after the establishment of a microbial consortium, as it is the case for methanogenesis using long-chain alkanes (Zengler et al. 1999). Anaerobic sediments seem to harbour an autochthonous microbiota, especially of sulfate reducing bacteria, which are able to tackle an important fraction of the crude oil compounds (Suárez-Suárez et al. 2011; Acosta-González et al. 2013a). In this regard, attempts to culture crude-oil degrading sulfate reducers resulted in the enrichment of consortia composed of a variety of organisms: in one of the enrichments, which was maintained by repeated subculturing, members of the *Desulfobacteraceae* known to be hydrocarbon degraders co-occurred with relatives of the uncultured clade OP3, as yet unreported as anaerobic hydrocarbon degraders (Suárez-Suárez (2012)). *Epsilonproteobacteria* and *Firmicutes* were also found, but with lower abundance.

The impact of the *Prestige* oil on the microbial communities of pristine subtidal sediments was explored in situ by using a non-impacted, pristine sandy flat area in the north bay of Mallorca as a model (Suárez-Suárez et al. 2011). Several months of incubation with *Prestige* crude evidenced that, besides strong toxicity and deleterious effects on the autochthonous microbiota, the presence of *Prestige* oil also enhanced a subpopulation of the sulfate reducing bacterial community, as was observed by increased numbers of microscope counts, cultivability and sulfate reduction rates. As already observed in the impacted Galician coast, the Mediterranean sediments also contained autochthonous microorganisms able to bioremediate the crude oil in situ in anoxic sediments (Suárez-Suárez et al. 2011).

Enrichment and isolation of pure cultures of aerobic hydrocarbon-degrading microorganisms

In parallel to the molecular characterization of bacterial communities, a number of laboratory assays was carried out to identify cultivable aerobic bacteria present in the polluted sites and to explore their biodegradative activity. Culture-dependent analyses of tarballs collected from the Bay of Biscay 2 to 3 weeks after the spill identified strains belonging to the *Gammaproteobacteria*, *Firmicutes* and *Bacteroidetes*, common in oil-polluted marine environments, although their biodegradation capacities were not determined (Martín-Gil et al. 2004). The picture was similar in the superficial subtidal sediments of a heavily contaminated area in the Atlantic Islands 3 months after the spill. The cultivable aerobes were dominated by *Gammaproteobacteria*, followed by *Alphaproteobacteria*, *Bacteroidetes* and *Firmicutes* (Alonso-Gutiérrez et al. 2008). In these samples, the aerobes able to grow on *n*-alkanes reached 70 % of the total cultivable population, whilst the cultivable PAH-degraders were two orders of magnitude less abundant. Seven strains consistently growing with hexadecane as the sole carbon source were isolated, all of which were closely related to the hydrocarbonoclastic *A. borkumensis*, the predominant aliphatic hydrocarbon degrader detected blooming at early stages in similar oil spills (Table 1) (Kasai et al. 2002; Harayama et al. 2004; Kostka et al. 2011). No aromatic-degrading bacteria could be isolated in this study (Alonso-Gutiérrez et al. 2008). As already mentioned, the cultivation-independent analyses suggested that *Actinobacteria* also played a relevant role in alkane degradation after the *Prestige* spill (Alonso-Gutiérrez et al. 2009). In fact, an *n*-alkane-degrading species of the *Actinobacteria* such as *Rhodococcus fascians* (Jiménez et al. 2007), and several *Dietzia* strains (Alonso-Gutiérrez et al. 2009) could be isolated several months after the spill (Table 1). One of the *Dietzia* strains was shown to degrade *n*-alkanes ranging from C_{12} to C_{38} as well as branched-chain alkanes, probably through a novel alkane degrading pathway (Alonso-Gutiérrez et al. 2011). *Actinobacteria*, and especially the genera *Rhodococcus* and *Gordonia*, have been described as important players in long-chain alkane degradation under dry, aerobic and resource-limited conditions (Whyte et al. 1998; Yuste et al. 2000; Quatrini et al. 2008), which may explain the predominance of *Rhodococcus* in the weathered samples (Alonso-Gutiérrez et al. 2009) and which suggests a major contribution of this strain to the degradation of the *Prestige* high-molecular-weight alkanes (Jiménez et al. 2007). Although *A. borkumensis* was the predominant cultivable species isolated a short time after the spill (Alonso-Gutiérrez et al. 2008), its reduced presence in these late samples, which also had a poorer nutrient level, suggests a lower contribution to biodegradation at those stages, as already earlier described (Kasai et al. 2001).

Unlike for alkane degraders, the isolation of PAH degrading strains generally required enrichment cultivation on crude oil or on individual oil compounds. In fact, the degradation of PAHs of high molecular weight in the environment may require the cooperation of different species, which are sharing intermediate catabolites produced by some of them which then are being used as a substrate by others (McGenity et al. 2012). Only a *Citreicella* strain related to PAH degradation could be directly isolated from weathered

oil samples almost depleted of alkylnaphthalenes (Table 1) (Alonso-Gutiérrez et al. 2009). However, strains identified as *Pseudomonas stutzeri*, *Tistrella mobilis* and *Sphingomonas* spp. were isolated after enrichment on phenanthrene, although they were not detected by molecular methods in the original bacterial communities (Alonso-Gutiérrez et al. 2009). In contrast, sequences of the *Sphingomonadaceae* related to *Sphingopyxis* and *Novosphingobium* could be detected by DGGE in the original samples. Consistent aerobic PAH degradation in liquid medium was only confirmed for *Sphingomonas* species and *P. stutzeri* (Table 1). The genus *Sphingomonas* was also suggested to play an important role in PAH degradation after the *Nakhodka* spill (Kasai et al. 2001), although the actual relevance of the isolated *Sphingomonas* species from the *Prestige* oil spill, in in situ PAH biodegradation, could not be confirmed (Alonso-Gutiérrez et al. 2009). Finally, the *T. mobilis* strain isolated from the enrichment mentioned above was only able to grow on PAHs in the presence of the isolated *Sphingomonas* strain, suggesting a metabolic cooperation between the two strains (Alonso-Gutiérrez et al. 2009).

In an analysis, with the attempt of specifically targeting the *Pseudomonas* group in samples collected between 16 and 32 months after the spill, *Pseudomonas* strains able to grow on either hexadecane or naphthalene were easily isolated from intertidal sediments of polluted beaches of the Costa da Morte (Table 1) (Mulet et al. 2011). Most of the isolated naphthalene degraders were classified as *P. stutzeri*, whilst most hexadecane degraders were ascribed to *Pseudomonas alcaliphila*. *Pseudomonas aeruginosa* strains able to degrade long-chain alkanes were also isolated 5 years after the *Nakhodka* spill (Chaerun et al. 2004), and recently, an abundant *Pseudomonas* community was detected in sand samples collected from heavily affected beaches after the DWH spill. Direct metatranscriptomic analysis of the RNA isolated from these samples detected sequences mapping to PAH degrading genes from *Pseudomonas*, *Alteromonadales* and *Rhodobacterales*, highlighting their role in hydrocarbon degradation at these coastal sites (Lamendella et al. 2014). Attempts to isolate fungi from an oil-in-water emulsion of crude oil from the *Prestige* were unsuccessful (Martín-Gil et al. 2004).

Different simulation experiments were carried out to analyse the response of adapted microbial communities when transferred to specific hydrocarbon mixtures as sole carbon sources. Starting from a bacterial consortium enriched during 5 years on artificial seawater supplemented with *Prestige* oil, Vila et al. (2010) observed efficient degradation by the initial community of linear and branched alkanes, of PAHs up to five rings, and of many of their alkyl derivatives. DGGE analyses of subcultures established in either the aliphatic or aromatic fraction of the oil identified specific strains involved in the degradation of different hydrocarbons. The analysis of the community dynamics revealed a succession of bacterial groups associated with the biodegradation process. *A. borkumensis* dominated the community in the first week after the addition of oil, until depletion of the linear and branched-chain alkanes, and decreased thereafter, concomitant with an increase of the genus *Marinobacter* of the *Gammaproteobacteria* and of several genera of the *Alphaproteobacteria* such as *Maricaulis*, *Roseavarius*, *T. mobilis* and *Thalassospira*, all being involved in the degradation of low molecular weight PAHs. Thereafter, the degradation of the PAHs of higher molecular weight was carried out by the genera *Marinobacter* and *Methylophaga*, belonging to the *Gammaproteobacteria*. A similar initial enrichment of *Alcanivorax* and *Marinobacter* as primary degraders of the easiest degradable fractions after a spill was repeatedly observed (Kasai et al. 2002; Röling et al. 2004). Unfortunately, only very few of these strains could be isolated on these hydrocarbons as pure cultures (Table 1) (Vila et al. 2010).

A crude oil emulsion collected 5 months after the spill in a severely affected estuary in A Coruña (Galicia) was used as starting material for enrichment experiments to identify the relevant autochthonous hydrocarbon degraders in the sample (Jiménez et al. 2011). Again, 17 months of incubation in artificial seawater supplemented with inorganic nutrients strongly selected for *Alcanivorax* spp., which reached 66 % of the bacterial community as determined by 16S clone libraries. When incubated with fresh crude oil, the community was able to degrade almost half of the oil components after 2 months. A fast and intense change in the community structure was observed: *Alcanivorax* disappeared after day 20 whilst sequences belonging to the genera *Lutibacterium* and *Muricauda* were detected during the final stages (Jiménez et al. 2011). Some other groups remained constant throughout the whole process, such as *Thalassospira*, *Marinobacter hydrocarbonoclasticus*, as well as species of *Roseobacter*, *Parvibaculum* and *Alcaligenes*; they were suggested to play a relevant role in biodegradation. Strains of *Alcanivorax*, *Muricauda* and *Marinobacter* could be isolated and their capacity for hydrocarbon degradation confirmed (Table 1).

Finally, *Breoghania corrubedonensis*, belonging to a new genus of the *Alphaproteobacteria*, was identified from a subculture enriched on pyrene composed of 84 % of the *Alphaproteobacteria* (*Breoghania*, *Thalassospira*, *Paracoccus* and *Martelella*) and of 16 % of the genus *Gordonia*, belonging to the *Actinobacteria* (Gallego et al. 2010, 2014). However, the only aromatic growth substrate that *B. corrubedonensis* could use was protocatechuate, a common intermediate of several degradation pathways of aromatics (Gallego et al. 2010). The presence of this strain in the pyrene degrading consortium, and the impossibility to obtain any pyrene-degrading isolates from this consortium, clearly points towards a metabolic collaboration of uncultivable strains of this bacterial community. Molecular analysis of the

aromatic ring hydroxylating dioxygenase genes present in the consortium suggested that the above *Gordonia* species was the key player in pyrene degradation (Gallego et al. 2014).

Functional diversity related to hydrocarbon biodegradation

PCR-amplification of key genes of microbes active in bioremediation and their use as biomarkers represents a powerful method to assess the potential and activity of bacterial communities for biodegradation in environmental samples (Stapleton and Sayler 1998). Until recently, most of the functional genes analysed in environmental studies were related to aerobic pathways. Good markers to assess aerobic hydrocarbon degradation activity in environmental samples are *alkB*, coding for the alkane hydroxylase involved in aerobic alkane degradation (Heiss-Blanquet et al. 2005; Kloos et al. 2006), and *nahA*, encoding a component of the naphthalene dioxygenase (Debruyn et al. 2007). Anaerobic pathways have only recently received sufficient attention (Winderl et al. 2007; Acosta-González et al. 2013b; von Netzer et al. 2013). Although hydrocarbon degrading genes and pathways have been characterized from isolated marine microorganisms, the information about the functional diversity related to hydrocarbon degradation in marine polluted sediments is scarce compared to the information available from contaminated soils and aquifers. The presence of the *alkB* gene in marine environments was first detected in polluted subantarctic sediments (Guibert et al. 2012), and information about its presence in metagenomes, including those of marine origin, has only been recorded recently (Nie et al. 2014). In the case of the affected sediments from the *Prestige* oil spill, *alkB* sequences were detected in both the oxic and anoxic zones of the subtidal sediments, although the role of an aerobic gene function in a predominantly anoxic environment remained unexplained (Acosta-González 2013). These *alkB*-like sequences retrieved from marine environments were evolutionary distant from their soil counterparts, as earlier observed in polluted Antarctic sediments (Guibert et al. 2012).

The assessment of hydrocarbon degraders in different anoxic environments has successfully been achieved through the detection of specific functional markers, such as genes coding for the large subunit of the synthase of benzylsuccinate (*bssA*, of anaerobic degradation of toluene (Winderl et al. 2007), naphthylmethylsuccinate (*nmsA*, of 2-methylnaphthalene degradation (von Netzer et al. 2013) and alkylsuccinate (*assA*, of *n*-alkane degradation) (Callaghan et al. 2010) which, due to their high sequence identity can, in some cases, be all amplified with the same primer pair (Acosta-González et al. 2013b). The diversity of these enzymes in marine sediments was described for the first time in 2013 in sediments of the Gulf of Mexico, in hydrocarbon seeps in Norway (von Netzer et al. 2013) and in sediments affected by the *Prestige* spill in the Atlantic Islands, as well as in Mediterranean microcosm experiments amended with *Prestige* oil (Acosta-González et al. 2013b). An unexpected diversity of the genes was observed in the *Prestige* oil spill-affected samples from Galicia (Table 2). The distribution of *bssA*-like analogues (*nmsA* and *assA*) in these samples was associated with the chemical composition of the oil present in the analysed samples; the *nmsA* sequences were predominant in the Galician libraries, which correlated with the high concentrations of methylnaphthalenes in the polluted sediments. In contrast, the proportion of *assA* sequences was very low, in accordance with the undetectable amounts of *n*-alkanes in these sediments. The results were compared with those obtained from Mediterranean sediments treated during 4 months with the *Prestige* oil. In this case, the diversity of the *bssA*-like gene sequences was lower, consistent with a longer period of adaptation of Galician microbial communities to this pollution. As for *alkB* sequences, the *bssA*-like marine sequences were only distantly related to their counterparts from soils and aquifers (Acosta-González et al. 2013b).

General conclusions

It is now well established that following a massive delivery of oil into a marine environment, the presence and bioavailability of the different constituents of the oil trigger a series of changes in the bacterial community structure that are observed as a succession and fluctuation in the predominant populations involved in the biodegradation of the different oil compounds (Harayama et al. 2004), where the relative abundance of the different specialized groups varies over time whilst the

Table 2 Presence and diversity of functional genes in subtidal sediments affected by the *Prestige* spill (Atlantic Islands, 2004)

	Number of retrieved sequences/($OTU_{0.05}$)[a]				
Gene	RI[b]	FI-OX[c]	FI-TR[c]	FI-AN[c]	FI-PET[d]
alkB[e]	80/(15)	80/(13)	80/(10)	80/(11)	80/(7)
bssA (cluster I)[f]	3/(1)	16/(2)	17/(4)	15/(4)	6/(3)
bssA (cluster II)[f]	0	4/(3)	9/(7)	3/(3)	1
nmsA like[f]	40/(1)	28/(3)	22/(3)	32/(3)	58/(5)
assA like[f]	0	1	3/(1)	3/(1)	3/(3)

[a] Number of OTUs with a sequence cutoff of 5 % amino acid dissimilarity ($OTU_{0.05}$).

[b] RI: non-polluted subtidal sediments collected at Rodas beach.

[c] FI-OX/FI-TR/FI-AN: subtidal sediment from oxic, transition, and anoxic zones of Figueiras beach.

[d] FI-PET: petroleum patch detected in the sediment at Figueiras beach.

[e] Acosta-González, unpublished.

[f] Modified from Acosta-González et al. 2013b.

specific oil components are being degraded. Although the overall picture obtained after the *Prestige* spill met this general pattern, some particularities characterized this spill. Despite the huge amounts of oil released into the sea, the extremely heavy nature of the oil mixture resulted in almost undetectable amounts of *Prestige* oil in the water soluble fractions, whilst most of it remained as part of subsequently formed tar aggregates, or became firmly attached to the sediment (Franco et al. 2006). Because the potential of microorganisms to sense and transform pollutant molecules depends on their accessibility and bioavailability (Megharaj et al. 2011), the response of the microbial communities after the *Prestige* spill was not as intense as that one observed in spills releasing much better bioavailable constituents of oil, such as those from the recent DWH spill (Kimes et al. 2014). However, the analysis of hydrocarbon composition, DIC isotope ratios, microbial counts and microbial community structures clearly evidenced an important response of the autochthonous populations, which showed a high potential for biodegradation. A clear preference in the biodegradation for lighter and less substituted compounds was repeatedly observed (Alonso-Gutiérrez et al. 2009). The presence in the affected areas of bacterial communities able to respond to a massive oil input is consistent with the important history of pollution of the region.

As described in reports on other marine spills, several bacterial taxa appeared as the most relevant players in the response to the outburst of the crude oil. Marine communities of coastal sediments are dominated by the phylum *Proteobacteria* (Zinger et al. 2011), and as expected the *Proteobacteria* was the most abundant phylum in all of the sediment samples analysed in the affected areas of the *Prestige* oil spill. The presence of oil modified the distribution of the different classes within this group, although changes were restrained by the metabolic profile of the sediment (oxic and anoxic), and the ecological properties of each zone (texture, nutrient levels etc.). Polluted supratidal and intertidal zones were generally dominated by *Alpha*- and *Gammaproteobacteria* (Jiménez et al. 2007; Alonso-Gutiérrez et al. 2009), whilst subtidal sediments were dominated by *Delta*- and *Gammaproteobacteria* (Acosta-González et al. 2013a). *Sphingomonadaceae* and *Rhodobacteriaceae* dominated among the *Alphaproteobacteria*, whilst the *Chromatiales* was the prevailing group among the *Gammaproteobacteria*. Although *Alcanivorax*, an ubiquitous genus blooming early after a spill, was also detected three months after the *Prestige* spill (Alonso-Gutiérrez et al. 2008), its relevance in long-term polluted samples was low (Alonso-Gutiérrez et al. 2009), and its presence could only be confirmed after enrichment (Alonso-Gutiérrez et al. 2008; Vila et al. 2010). However, other strains belonging to the *Oceanospirillales* could be isolated from polluted samples (Alonso-Gutiérrez et al. 2009). Furthermore, the presence of an *Alcanivorax* community prepared to rapidly respond to oil inputs together with other known hydrocarbon degrading taxa was demonstrated in simulation experiments (Vila et al. 2010; Jiménez et al. 2011). Unfortunately, the initial phases (days to weeks) in the community response to the spill were not directly analysed in the case of the *Prestige* spill and could only be inferred from controlled spill simulations. A comparable fast response of *Alcanivorax* to oil inputs was observed with similar approaches after the *Nakodka* spill (Kasai et al. 2002) and in model microcosms (Röling et al. 2002). Besides the *Preoteobacteria*, the *Actinobacteria* and particularly the genus *Rhodococcus* were the key players in the microbial response to the spill, especially in the degradation of the alkane fraction. As for previous spills, the addition of fertilizers enhanced the biodegradation rates, reinforcing the general idea that, in natural marine environments, inorganic nutrients such as phosphate and nitrogen are insufficient to support good bacterial growth and biodegradation after a sudden increase in carbon sources (Harayama et al. 2004; Atlas and Hazen 2011). Finally, the response of anaerobic bacterial communities of subtidal sediments to an oil spill was analysed for the first time. This environment was severely affected by the spill and showed a strong decay in the total bacterial community, paralleled by an important increase in the bacterial population which is metabolically active towards hydrocarbons, among which the *Deltaproteobacteria* were the main players, as expected. The high diversity of genes encoding anaerobic hydrocarbon degrading enzymes in the area was related to the composition of hydrocarbons of the spilled oil.

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