UNIVERSITÀ
POLITECNICA
DELLE MARCHE

Ph.D. in Life and Environmental Sciences

XXXIV cycle

Curriculum: Civil and Environmental Protection

Assessing the diversity of microbial assemblages and their bioremediation potential of chronically contaminated marine sediments

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Abstract

Chemical contamination of coastal marine sediments is a widespread phenomenon and represents a major concern for biodiversity and ecosystem health. Bioremediation is an environmental-friendly strategy gaining increasing attention for its potential to clean-up contaminated marine sediments. In this PhD thesis, first of all, I provided an overview of the current knowledge and perspectives on the bioremediation of marine sediments, based on literature review. Then I assessed the diversity of microbial assemblages in different chronically contaminated sediments of the Bagnoli-Coroglio, Mar Piccolo of Taranto and Falconara Marittima areas (all of them included in the list of Sites of National Remediation Interest) and their relationships with the level and typology of chemical pollutants. I tested the efficiency of bioremediation strategies based on inorganic nutrient addition and bioaugmentation approaches using selected bacterial or fungal consortia or both, previously isolated and identified, on PAH degradation in sediments displaying different contamination level and I investigated changes in metal partitioning and microbial diversity due to biotreatments. Results presented here suggest that chemical contaminants can have an important role in shaping prokaryotic diversity, potentially by selecting tolerant/resistant microbial taxa. Sediments of Falconara Marittima host microbial taxa with a high bioremediation capacity toward PAHs. These microbial taxa, including both bacteria and fungi, once isolated and growth on selected media, can be effective for the bioremediation of Bagnoli sediments highly contaminated with PAHs. Despite findings reported in this study do not allow disentangling the relative importance of the allochthonous vs. autochthonous microbial taxa on the biodegradation of PAHs, they provide new insights on bacterial-fungal interactions occurring during bioremediation of highly contaminated marine sediments. Overall, these results suggest that biotreatments based on selected bacterial and/or

fungal consortia or a combination of both could be an effective strategy to significantly reduce in a relatively short time PAH contamination of marine sediments, possibly leading to alternative management options compared to dredging and landfill disposal.

Abstract

La contaminazione dei sedimenti marini è una problematica ampiamente diffusa a livello globale, con importanti ripercussioni sia di natura ecologica sia di natura socio-economica. Le strategie biologiche basate sulla capacità dei microrganismi di degradare e/o trasformare i contaminanti in composti inerti e/o meno tossici, vengono sempre più applicate nel campo della bonifica ambientale, data la loro maggiore compatibilità ambientale ed i minori costi di trattamento. In questa tesi di dottorato, prima di tutto, ho effettuato un'estesa ricerca bibliografica sulle attuali conoscenze e le potenzialità applicative delle strategie di biorisanamento su sedimenti marini contaminati. Quindi ho valutato la diversità delle comunità microbiche nei sedimenti marini contaminati delle aree di Bagnoli-Coroglio, Mar Piccolo di Taranto e Falconara Marittima (tutte incluse nell'elenco dei Siti di Bonifica di Interesse Nazionale, SIN) e le loro relazioni con il grado e tipologia dei contaminanti chimici presenti. Ho testato l'efficienza di strategie di biostimolazione basate sull'aggiunta di nutrienti inorganici sui tassi di degradazione degli idrocarburi policiclici aromatici (IPA) in sedimenti a diverso livello di contaminazione, isolando e caratterizzando da tali campioni specifici ceppi batteri e fungini utilizzati per esperimenti di *bioaugmentation*. Ho inoltre studiato i cambiamenti della ripartizione dei metalli e della diversità microbica dovuti ai bio-trattamenti. I risultati di questa ricerca suggeriscono che i contaminanti chimici possono avere un ruolo importante nell'influenzare la struttura di comunità dei procarioti, potenzialmente selezionando taxa microbici tolleranti/resistenti. I sedimenti di Falconara Marittima ospitano taxa microbici con un'elevata capacità di biodegradazione degli IPA. L'aggiunta di taxa batteri e fungini isolati dai sedimenti marini di Falconara Marittima a campioni di sedimento altamente contaminati da IPA, prelevati nell'area di Bagnoli, è in grado di determinare una significativa riduzione delle

concentrazioni degli IPA, compresi i congeneri maggiormente tossici. Sebbene i risultati di questo studio non consentano di distinguere tra l'importanza relativa dei taxa microbici alloctoni e autoctoni sulla biodegradazione degli IPA, questi forniscono nuove conoscenze sulle interazioni batterico-fungine che si verificano durante il biorisanamento di sedimenti marini altamente contaminati. Complessivamente, questi risultati suggeriscono che i bio-trattamenti basati su consorzi batterici e/o fungini selezionati o su una combinazione di entrambi potrebbero costituire una strategia efficace per ridurre significativamente, in tempi relativamente brevi, la contaminazione da IPA dei sedimenti marini, portando possibilmente a opzioni di gestione alternative rispetto al dragaggio e allo smaltimento in discarica di tali matrici.

Aims of the thesis

The aims of this Ph.D. thesis are:

1. providing an overview of the current knowledge and perspectives on the bioremediation of marine sediments, based on literature review

2. assessing the diversity of microbial assemblages in different contaminated sediments and their relationships with the level and typology of chemical pollutants;

3. testing the efficiency of biostimulation strategies for the remediation of sediments displaying different concentrations of chemical contaminants;

4. isolation and identification of bacterial and fungal taxa and assessment of their bioremediation potential;

5. testing the efficiency of bioaugmentation approaches based on bacterial and fungal consortia on sediments characterized by different concentrations of contaminants;

6. assessing changes of microbial diversity in relation with bioremediation performance of contaminated marine sediments.

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Chapter 1

General Introduction

1.1 The microbial bioremediation potential of contaminated marine ecosystems

Coastal areas are sites facing intense ecological stress due to human activities. Among these, the inorganic and organic contaminants released in marine ecosystems can cause adverse biological effects, impairing biodiversity and ecosystem good and services (Chapman, 2007; Krek et al., 2018; Yu et al., 2019). Amongst different pollutants, heavy metals and petroleum hydrocarbons are the most harmful to ecosystem and human health (Buah-Kwofie et al., 2018; Chen et al., 2018; Fuentes-Gandara, 2018; Loflen et al., 2018). Among chemical contaminants which are recovered in the coastal environment, heavy metals and polycyclic aromatic hydrocarbons (PAHs) represent some of the most ubiquitous and spread (Cao et al., 2019; Fleming et al., 2006; Ke et al., 2017; Saeedi et al., 2012), and occur primarily as a result of anthropogenic inputs. Heavy metals are highly persistent and may exert toxic effects at all levels of biological organization, from cells to population and community structure, by altering enzymatic functioning and metabolic pathways (Agarwal, 2009; Chapman, 2007; Dell'Anno et al., 2009; Dell'Anno et al., 2003; Lloyd, 2003). Heavy metals have been reported to affect cellular organelles and components such as enzymes involved in metabolism, detoxification and damage repair; for instance, heavy metals can interact with DNA and nuclear proteins, causing DNA damage and conformational changes that may lead to cell cycle modulation, carcinogenesis or apoptosis (Beyersmann & Hartwig, 2008; Wang & Shi, 2001). Special attention has been devoted in understanding metal speciation and repartition in different

geochemical phases in which the heavy metals occur in order to determine their bioavailability and thus their toxicity (Maah, & Yusoff, 2012).

Polycyclic aromatic hydrocarbons (PAHs), containing two or more fused benzene rings are aromatic compounds produced mainly during combustion in natural and anthropogenic processes, and are typically found in high concentrations on industrial sites, particularly those associated with the petroleum, gas-production, and wood-preserving industries (Wilson & Jones, 1993). They cause concern as environmental pollutants because some are carcinogens and mutagens, and have a high potential for biomagnification through the aquatic food web due to their lipophilic nature. All these types of contaminants accumulate in coastal marine sediments representing their main depository, where they can exert a significant influence on the benthic biota (Rocchetti et al., 2012).

Different physicochemical techniques have been reported to reduce pollutant concentrations from waste waters and contaminated sediments by performing chemical precipitation, ion-exchange, reverse osmosis, electro-dialysis, and ultrafiltration and chemical, electrochemical and thermal strategies respectively (Crini & Lichtfouse, 2018; Lofrano et al., 2017; Peng et al., 2018). Each treatment has its own advantages and constraints not only in terms of cost, but also in terms of efficiency, feasibility, and environmental impact. There is therefore an urgent need to find sustainable and eco-compatible solutions for the remediation of contaminated sediments in situ. International policies are increasingly seeking management alternatives able to reduce sediment handling interventions, especially by promoting eco-compatible technologies for the decontamination of polluted matrices (WFD 2000/60 EU; European Marine Strategy Framework Directive 2000).

The fate of pollutants in coastal marine sediments largely depends on microbial activities (Duran et al., 2015). Polluted environments undergo physical, chemical and biological

processes known as “natural attenuation” that refers to the reduction of the mass, toxicity, mobility, volume and/or concentrations of pollutants (Agency United States Environmental Protection, 1999; Wang & Tam, 2019). Microbial communities represent a great candidate in remediation of challenging multi-contaminated environments thanks to their wide physiological/metabolic plasticity (Duran et al., 2008; Paisse et al., 2011). Indeed, microbes are able to produce diverse enzymes and compounds able to transform chemicals to a safer or lesser toxic compounds (Das & Dash, 2014; Dash & Das, 2012). Microbes are, thus, the major players in controlling and determining the fate of xenobiotics under both aerobic and anaerobic conditions (Bamforth & Singleton, 2005; Cason et al., 2019; Leahy & Colwell, 1990; Lösekann et al., 2007) with differences in their metabolism’s kinetics. Another important factor for pollutants degradation is represented by their chemical nature. Alkanes can be degraded more easily than branched alkanes or multiple-ringed aromatic hydrocarbons (Alexander 1965; Bagby et al., 2017). Moreover, a single large oil slick offers a smaller area for microbes to access compared to numerous small-sized oil slicks which are easier to digest thanks to the higher rate of diffusion through the oil-water interface (Zaki et al., 2015). The degradation rate is mostly affected by the availability of nutrients, such as nitrogen and phosphorus, the two most limiting factors for hydrocarbon metabolism (Calvo et al., 2009; McKew et al., 2007), but even sulphur and potassium availability can affect bioremediation rates (Evans et al., 2004). Other factors like water temperature, oxygen concentration, sediment particle size and mineralogical composition influence bioremediation rate (Leahy & Colwell, 1990; Slater et al., 2005). Crude oil degradation have been reported as faster in warm water since heat promotes the breakdown of the spilled petroleum that becomes more available to oil-degrading microbes (Maculay, 2014). Low temperatures, instead, inactivate microbial metabolism causing the shutdown of transport channels of cells and slow down cytoplasm flow processes (Yang et al.,

2009). Silt-clay sediments limit gas and solute exchanges and reduce microbe-hydrocarbon interactions, which is a prerequisite for biodegradation processes (Ahmad et al., 2019). The bioremediation performance depends also on pH, since microbial metabolism is typically effective at pH values around 6-8 (Ayangbenro & Babalola, 2017; N. Das & Chandran, 2011). Moreover, microorganisms exploit their ability for metals removal from solution through either enzymatic or non-enzymatic mechanisms (Iyer et al., 2005; Rajendran et al., 2003; Vala, 2010). Hydrocarbons' biodegradation is made possible by different classes of microbial enzymes. The main bioremediation-related enzymes are the oxidoreductases and the hydrolyses. The former comprises Mono- and Di-oxygenase, Laccase, Peroxidase, while the latter includes Cellulase and Protease (Karigar & Rao, 2011). Lipase, another hydrolase family enzyme, is not directly involved in the detoxification of hydrocarbons, but its enhanced expression level seemed to be a great indicator of biodegradation efficiency (Kadri et al., 2018; Margesin et al., 2000; Margesin et al., 1999; Riffaldi et al., 2006).

The aerobic degradation of PAHs in both bacteria and fungi have in common the initial step consisting in the introduction of atmospheric oxygen to the aromatic nucleus. The reaction of hydroxylation of an aromatic ring can be catalyzed by P-450 monooxygenase enzymes, used by bacteria and fungi, dioxygenase enzymes (enzyme consisting of reductase, ferredoxin and terminal oxygenase subunits) typical of bacteria, while lignin and manganese peroxidases and laccase are utilized by white rot fungi (Dell'anno et al., 2020; Shahsavari et al., 2019). However, consortia grant the best degradation rate thanks to the different enzymes and pathways they can share. Indeed, fungal hyphae easier penetrate contaminated soil in reaching pollutants and initiating the metabolism of High Molecular Weights PAHs. Therefore, bacteria seem to dominate communities in older PAH-polluted sites, also thank to capacity to produce

biosurfactants and biofilm that enhance PAH bioavailability and so the degradation (Cébron et al., 2008).

The use of biosurfactants represents a promising approach in treating polluted environments (Dell'Anno et al., 2018; Radmann et al., 2015). Indeed, the amphiphilic moieties of surfactants favor bioremediation processes by promoting the partitioning of the hydrophobic contaminants into internal hydrophobic cores of surfactant micelles, facilitating the detachment of pollutants from the sediments (Y. Li & Lee, 2019). Nikolopoulou and colleauges (2013) showed the effectiveness of a particular class of biosurfactants, rhamnolipids, in the remediation of marine crude oil contaminated matrixes. Indeed, the addition of rhamnolipids to a solution of crude oil and sand has led, after 15 days, to a degradation yield of 30% for fluorene, ca. 20% for phenanthrene and 10% for dibenzothiothene.

The halotolerant *Pseudomonas aeruginosa* (AHV-KH10) produce a biosurfactant which allowed biodegradation of diesel up to 70% (Pourfadakari et al., 2021). Higher removal rate for diesel degradation have been shown by the biosurfactants producer *Paracoccus* sp. MJ9, capable of removing up to 80% of diesel oil in about 5 days (Xu et al., 2020). Total petroleum hydrocarbon removal rate reached up to 80% in contaminated matrices by adding a mixture of rhamnolipids, biochar and nitrogen (Wei et al., 2020). This result suggests that a combined use of biosurfactants and compounds capable of stimulating the metabolism of the autochthonous microbial component may be an effective solution to increase bioremediation processes.

Hydrocarbons biodegradation rates are influenced by complex abiotic and biotic interactions, which are not completely clear yet. Fedorak and Westlake, (1981) reported the highest biodegradation rate represented by aromatic hydrocarbons, while Head and colleagues, (2006) showed a more rapid attack of saturated than aromatic hydrocarbons during the degradation of crude oil. This discrepancy underlines the need to feed our knowledge to be able to manage an

environmental clean-up designing the proper bio-treatment strategies. The rate of the reactions are mainly driven by oxygen availability, but there are many other impacting factors such as temperature, pH, nutrients (Zhou & Crawford, 1995). Kinetic models can be a useful tool for the prediction of the effect of these factors in addiction to forecast the residual contaminants concentration during bioremediation management (Bayen et al. 2009; Yang et al., 2008; Zhang et al., 1998). A great effort has been devoted to studying how different parameters (e.g. oxygen and redox oscillations) affect the degradation capacity of microorganisms in different environments, such as estuarine and tidal ones, but also in microcosms and bioreactors (Cravo-laureau & Duran, 2014; Duran & Cravo-laureau, 2016; Duran et al., 2015; Militon et al., 2015; Vitte et al., 2013). The results show a wide range of possibilities that, as already highlighted by Borch and colleagues a decade ago, only permit to formulate hypothesis and suppositions (Borch et al., 2010; Duran et al., 2015). Although the aerobic process is faster, more specific and complete (Haritash & Kaushik, 2009), microorganisms in anaerobic condition are able to take advantage of different electron acceptor than the oxygen, in order to stimulate their metabolism (Meckenstock et al., 2004; Rothermich et al., 2002). In anoxic marine sediment the biodegradation is carried out through different electron-accepting pathways using nitrate, iron, or sulfate as their terminal electron acceptor or by fermentation (Finke et al., 2007; Jørgensen, 1982; Shin et al., 2019). Sulfate-reducing bacteria are reported as the most spread and performing in anoxic contaminated sediments because sulfate is more abundant in seawater (Coates et al., 1996; Rothermich et al., 2002). A great number of genera of microorganisms have been studied for their capacity to oxidase low molecular weight PAH (composed by 2-3 aromatic rings), while at the increasing of the number of PAH's rings there is a decrease in the genera (Juhasz & Naidu, 2000). Microbes have evolved elaborate metal resistance systems to heavy metals since marine environment naturally contains these pollutants (deep-sea vents)

(Williams & Silver, 1984). The adaptation to metals is based on a great variety of resistance like chromosomal, transposon and, mostly, plasmid-mediated systems (Bennett, 2008). Heavy metals cannot be degraded, but undergo redox reactions influencing the solubility and speciation in which that element occurs and so their toxicity (Alomary & Belhadj, 2007; Tack & Verloo, 1995). Microbial tolerance mechanisms to heavy metals consist in transportation across cell membrane, accumulation on cell wall, intra- and extra-cellular entrapment, formation of complexes and, the most attractive aspect in the remediation field, redox reactions (Ahemad, 2012). The extracellular metal sequestration implies the avoidance of metal entry and consists in chelation and cell-wall binding, while the intracellular sequestration aims to bind metals to proteins or other ligands to prevent it from damaging the metal sensitive cellular targets like proteins or other ligands (Anahid et al., 2011; Sanyal et al., 2005). Microorganisms lack of a control system for lipophilic compounds that passively invade cells, while they have specialized and widespread uptake or avoidance mechanisms for ions and hydrophilic compounds. Sikkema and colleagues reported that hydrocarbons having hydrophobic nature are quite harmful for common soil and water microbes, since they are accumulated in the membrane structures resulting in membrane integrity loss (Sikkema et al., 1995). Microorganisms can modify the composition of their cell wall and/or cell membrane to protect essential components. In case of heavy metals presence they can alter the production of membrane channel proteins drastically reducing the metals permeability (Rouch et al., 1995). Bioaccumulation is a dynamic process of uptake of an element or a compound within an organism and subsequent concentration of it. Microbial accumulators are very important in remediation field as they can chelate and detoxify heavy metals besides to utilize specific enzymatic pathways useful in degrading toxic organic compounds (Banerjee et al., 2013; Injal & Raut, 2010; Sathendra et al., 2018). Several bacterial species have been reported to

bioaccumulate metal cations extracellularly, e.g. *Klebsiella aerogenes*, *Pseudomonas putida* and *Arthrobacter viscosus* (Ayangbenro & Babalola, 2017; Ilyas et al., 2017). Bai and Abraham, in 2002, discovered the implication of the amino group of the cell walls of *Rhizopus nigricans* in Cr(VI) binding from solution, while, three years later Taboski and colleauges, studied the marine fungi *Corollospora lacer*a and *Monodictys pelagica* discovering their capacity to accumulate lead and cadmium extracellularly in mycelia (Bai & Abraham, 2002; H. Singh, 2006; Taboski, Rand, & Pio, 2005). Gram-positive bacteria accumulate much higher concentrations of heavy metals on their cell walls respect to Gram-negative bacteria and the reason lies in the differences in the structure of their cell wall. Gram-positive have glycoproteins on their cell wall suggesting more potential binding sites than the lipopolysaccharides which compose the outer membrane of gram-negative (Das et al., 2008; Rani & Goel, 2009). Apart from the bacterial cell wall extracellular polysaccharides have also been reported to accumulate heavy metals, but it varies from one bacterial species to the other (Yee & Fein, 2001).

As bacteria, also fungi have been described for their ability to produce particular enzymes (e.g., catalases, peroxidases, laccases) able to degrade organic contaminants and/or to detoxify inorganic contaminants (Durairaj et al., 2015; Morel et al., 2013). The main fungi described as capable to degrade aromatic hydrocarbons belong to the genera *Aspergillus, Curvularia, Drechslera, Fusarium, Lasiodiplodia, Mucor, Penicillium, Rhizopus, Trichoderma* (Balaji et al., 2014; Chang et al., 2016; Lladó et al., 2013), while strains such as *Aspergillus niger, A. flavus and A. foetidus* , *Cryptococcu*s, *Penicillium* and *Curvularia* have been demonstrated to tolerate and effectively immobilize metals such as Pb, Hg, and U through biosorption processes (Chakraborty et al., 2013; Deshmukh et al., 2016; Kurniati et al., 2014; Mumtaz et al., 2013). Unfortunately, the accumulation rate in the coastal marine sediment of highly toxic and

persistent compounds underlines the insufficient effect of natural attenuation in decreasing the anthropogenic pollution (Chapelle et al., 2000).

In the 1960s George M. Robinson first utilized microbes for remediation purpose giving rise to modern bioremediation that consist in the use of living organisms, primarily microorganisms, to transform or degrade contaminants into non-hazardous or less hazardous chemicals (Adams et al., 2015; Leung, 2004). Remediation by enhanced natural attenuation, which involves improving nutrient, aeration, and moisture content have recorded many successes towards pollutant removal (Chikere et al., 2017; Odukoya & Lambert, 2015). Different laboratory and field experiments have utilized bioremediation approaches based on biostimulation and bioaugmentation strategies (Atlas, 1991; Bento et al., 2005; Nikolopoulou & Kalogeraki, 2016). Bioaugmentation is the introduction of specific microbial strains selected for their intrinsic remediation capacity, while biostimulation consists in the addiction of nutrients useful in enhancing the hydrocarbonoclastic and metal-detoxifying microbes metabolism (Atlas & Bragg, 2009; Cervantes-gonzález et al., 2019; Venosa & Zhu, 2003). These strategies represent two common and efficient bioremediation approaches in contaminated sediments through which it is possible to speed up the microbial efficiency (Floodgate, 1995; Prince, 1993; Chang, 2019). The isolation of microbial taxa directly from contaminated sites is expected to be more effective and useful for the remediation of polluted marine sediment since their adaptation to this kind of environment (Dell'anno et al., 2020; Dell'Anno et al., 2021). The use of allochthonous microbes may require the manipulation of the natural environment to gain optimal performance (e.g., changing oxygen and/or nutrient concentration, pH, S, T; Garbisu et al., 2017). Since most of the organic contaminants have low solubility in aqueous media, it is important to understand the degrading microbes' mechanisms of uptake to develop strategies to promote or accelerate their pollutants accession enhancing bioremediation processes.

Despite microbes lack the degradative pathway for complete degradation of certain newly introduced synthetic chemicals, there is evidence that microorganisms have the capacity to evolve such catabolic systems over time (Singh & Ward, 2004). When it does not naturally happen, a strategy could be to perform genetic manipulation designing superior biocatalysts combining catabolic segments from different organisms within one recipient to create complete catabolic pathways (Pieper & Reineke, 2000). However, the exploitation of genetically modified microorganism is regulated by a strict world-wide regulation and their utilization in field is often stifled (Drobník, 1999; Miller, 1997; Sayler & Ripp, 2000). Microbial communities subjected to bioremediation management undergo the selection of certain strains, like hydrocarbonoclastic ones, both in natural polluted environments, *in situ* (McKew et al., 2007; Zhao et al., 2019), and in enriched microcosm experiments, *ex situ* (Castle et al., 2006; Coulon et al., 2007; Liu et al., 2018; McKew et al., 2007). The application of bioremediation's strategies has the advantage of being non-invasive, relatively cost-efficient and, although it is not the fastest restoring process, the establishment of facilitation within microbial communities seemed to be very effective (April et al., 2000; Dean-ross et al., 2002; Flocco et al., 2019; Mckew et al., 2007; Megharaj & Naidu, 2017; Yu et al., 2005). The degradation mechanisms are significantly different between fungi and bacteria (Cerniglia, 1993). The marine hydrocarbon-degrading bacteria predominantly detected in chronically polluted environment belong to the Gammaproteobacterial genera of *Alcanivorax*, *Cycloclasticus*, *Marinobacter*, *Thalassolituus*, *Oleispira*, and *Oleiphilus*. Interestingly they represent just a minority in pristine areas (Head et al., 2006; Wang & Tam, 2019). In aquatic systems, fungi and yeasts tend to have higher rate of hydrocarbon degradation in sediment than bacteria. The most reported hydrocarbon-degraders yeast-like form of fungi are *Candida*, *Rhodotorula*, *Aureobasidium* and *Sporobolomyces* while for what concern filamentous fungi *Penicillum*, *Aspergillus* and

Cladosporium are the most frequently isolated as hydrocarbon-degraders (Atlas & Cerniglia, 1995). Studies on biodegradation performed by consortia systems established an enhanced adaptation capability and an increase in the degradation rate of about 65% and more of the total petroleum hydrocarbon level (Bhattacharya et al., 2019; Markiewicz et al., 2014; Mukherjee & Bordoloi, 2011). The use of consortia is confirmed an efficient and trustworthy way to mineralize/detoxify pollutants. These systems not only are able to detoxify pollutants through enzymes, but their power resides in the ability to mineralize the toxic intermediate metabolites, potentially liberated in the first remediation step, into least/reduced toxic compounds (Kumar et al., 2018). There are many ecotoxic risks associated to PAHs such as the high mutagenic and carcinogenic effect in microorganisms and its harmful aftermaths in animals when carried into the respiratory system through inhalation (Dong et al., 2018; Liu et al., 2019). The scientific community is now focusing the attention especially on the biologically degradable contaminant's classes, as well as on the secondary metabolites production derived by microbial degradation activity, since it is not possible to exclude the release of even more toxic compounds (Tomei & Daugulis, 2013). Microorganisms living in polluted coastal areas can be capable of several remediation activities, and it is thus useful to try to isolate, identify and characterize them by using traditional as well as next-generation microbiological methods and molecular analyses.

1.2 Cultural-based techniques and molecular approaches for investigating pollutant-degrading microbes

Cultural-based techniques allow studying the physiology and metabolism of the isolated microorganisms, although resulting in a microbial abundance underestimation. In fact, due to the limited laboratory conditions, only about 1% of the environmental microbial community is

commonly cultured (Brockman, 1995; Connon & Giovannoni, 2002; Handelsman et al., 2002; Lloyd-Jones et al., 1999) as confirmed by the molecular approach (Ward et al., 1990). The underestimation is not the only cultural-based gap. Discrepancies between *ex situ* and *in situ* pollutant-degrading bacteria have been reported (Watanabe, 2001; Watanabe & Baker, 2000; Watanabe & Hino, 1996). Molecular approach, based on DNA analyses, permit to assess microbial community structure and dynamics besides to provide details regarding functional diversity (Laureto et al., 2015). The direct study of environmental samples through molecular approaches (namely, metagenomics), allows characterizing until 99.9% of the entire microbial community, bypassing the need for isolating and culturing individual microbial cells. Jones and colleagues, thanks to molecular approach assessed new fungal lineage *Cryptomycota*, still unknown to science and known only from environmental sequences (Jones et al., 2011; Sette et al., 2013). Metagenomics also allows to identify functional genes of non-culturable microorganisms, preserving the *in situ* microbial community composition and its metabolic status that instead could undergo artificial biases by culturing (Gillan et al., 2015; Widada et al., 2002). In the context of pollution, researchers are interested in exploring the adaptive mechanisms to the environment and explaining the mechanism of synergy between microorganisms (Yan et al., 2021). Moreover, the molecular approach permits to investigate the change in microbial community composition during bioremediation experiments (Geiselbrecht et al., 1996; Miralles et al., 2007). This approach gave the possibility to identify microbial taxa mainly involved in detoxification and hydrocarbon degradation (Harayama et al., 2004; Head et al., 2006; Yakimov et al., 2005). The most common sequencing approach is targeted metagenomics, which processes in parallel great amounts of target genes. The ribosomal RNA (rRNA) represents the evolutionary clock of election due to its presence in all organisms, in addition to be really conserved but different enough to measure the evolutionary

distance (Perito & Cavalieri, 2018). Bacteria and Archea rRNA standard gene is the 16S, while for fungi the most prominent fungal phylogenetic markers are the 28S and the 18S rRNA gene sequences, as well as the ITS region (fungal internal transcribed spacer) (Edgar, 2018). In particular the most available sequenced data are the 18S rRNA gene sequences (Banos et al., 2018; Panzer et al., 2015). Among the sequencing technologies, the Sanger method is relatively expensive, slow but specific, while the Next Generation Sequencing typically produces lower-quality and shorter sequences, but rapidly and cheaply in huge amounts, thus representing the most powerful approach to obtain a more complete view of the overall diversity within environmental samples (Metzker, 2010; Sanger et al., 1977; Su et al., 2012). Moreover, sequencing technology has made considerable progress in terms of accuracy in recent years (Ji et al., 2019). Together, classic microbiology and molecular methods will provide a more comprehensive interpretation of the in situ microbial community, also and above all its response to natural attenuation processes and an applied bioremediation strategy (Brockman, 1995). Besides the possibility to boost microbe's remediation power supplying them with nutrients and other compounds essential for their metabolism, researchers are investigating genetically engineered microorganism able to degrade specific contaminants (Ghosal et al., 2016). Indeed, the research of bioremediation of petroleum pollution has entered the molecular age going more and more deeper in the mechanisms (Haro & De Lorenzo, 2001; Hennessee & Li, 2016; Hu et al., 2020; Xiong et al., 2019). Synthetic biology is having much attention as well as application in research studies. It refers to the design and modification of biological systems according to specific goals, for example bioremediation (Luo et al., 2015). Metabolic engineering can modify, transform and optimize cell metabolic pathways through multi-gene recombination technology. Beyond the possibility to change cell characteristics, enhancing for example the resistance of natural microorganisms to adapt to harsh natural environments, metabolic

engineering is capable to construct new metabolic pathways (Singh et al., 2008). Scientists were not satisfied with the efficiency of degradation by genetically engineered strains at the beginning, due to the lack of regulatory information on the metabolic network (de Lorenzo, 2009). In recent years, there has been progress in understanding the various pathways and principles of microorganisms' metabolism, which captivate the attention for bioremediation purpose (Bouhajja et al., 2017; Yamaga et al., 2010). The first step in construction of genetically engineered strains is to find the appropriate model microorganism about which the genome information and metabolic network are clear, enabling better gene manipulation (Nelson et al., 2002). Microbial composition is affected by the environmental conditions, including pollution, for which the crucial factor is the ability of the microorganism to survive to and possibly degrade pollutants. Microorganisms in contaminated sites undergo natural evolution, but at low speed that metabolic engineering can effectively improve (Liu et al., 2019; Pieper & Reineke, 2000). Enhancing the catalytic activity already possessed by microorganisms, genetic engineering can also provide brand new functions not peculiar for the host (Li et al., 2019), not only for what concern degradation ability but also the enhancement of microbes' tolerance to harsh environment (Fernández et al., 2012). It is almost impossible to create a "super-microorganism" able to complete the degradation of all hydrocarbons (Bodor et al., 2020). The effect of multiple bacteria on the same substrate is better than that of single bacteria (Noble et al., 2011). For this reason, combining microorganisms with different tolerance and different degradation systems can both reduce the environmental negative impact and achieve faster and better degradation power (Kuppusamy et al., 2016; Tzintzun-Camacho et al., 2012). The CRISPR technique, for example, relies on an enzyme called Cas9 that uses a guide RNA molecule to home in on its target DNA, then edits the DNA to disrupt genes or insert desired sequences. The method, behind its incredible power, hides a big ethical problem because of its

impossibility to trace "whether something has been mutated conventionally or genetically engineered", making difficult its regulation (Ledford, 2015).

1.3 Bioremediation approaches for the cleanup of contaminated marine sediments

In aquatic systems, sediments act as a sink of pollutants. A risk may occur in case of contaminants' resuspension that could lead to adverse effects at any level of the ecosystem if accumulated in organisms (Catania et al., 2015; Mcgenity et al., 2012). In the current scenario, remediation strategies based on biological methods are considered very promising, due to their eco-sustainability and cost-effectiveness, taking advantage of autochthonous microorganisms naturally selected from polluted sites able to degrade or detoxify contaminants. In order to enhance the remediation performance of microorganisms there are different strategies to take in account. Biological methods offer the opportunity to boost microbial remediation activity through bioaugmentation and biostimulation approaches. The other way is based on chemical-physical methods attempting to simplify the biological reactions acting on the contaminated matrix, like solvent extraction, surfactant addition, thermal and/or oxidative pretreatments (Boopathy R., 2000; Tomei & Daugulis, 2013). Bioremediation approaches can be considered successful only if sustainable. Laboratory investigation represents a fundamental step through which monitoring possible negative effects, minimizing undesirable environmental consequences. It is thus fundamental to consider the factors able to influence microbial activities in order to have a positive impact in the bioremediation process (Azubuike et al., 2016; Smith et al., 2015). Bioremediation technologies can be classified as *ex situ* and *in situ,* depending on where the treatment process takes place. For this reason, land farming is not itself an *in situ* or *ex situ* strategy, it depends from the possibility to perform or not in loco activities

like tillage and irrigation to stimulate autochthonous microorganisms (Azubuike et al., 2016). Since both strategies have pros and cons, site characterization and treatability studies are fundamental when developing a bioremediation strategy. *Ex situ* techniques represent the best approach from the predictability and efficiency point of view, permitting to treat different classes of pollutants and showing reproducibility in its success, thanks to the possibility to monitor and control the different phases of the processes (Maila & Cloete, 2005; Philp & Atlas, 2005). However, the risks of possible contamination spreading during the initial activities (e.g., dredging, excavation, handling, transport of hazardous material) must be considered, as well as the relevant costs. The main *ex situ* bioremediation techniques are based on biopile and bioreactors. These techniques aim to exploit microorganisms' remediation capabilities through their stimulation with nutrient amendment, aeration and turning of the sediment. Larsen and colleagues have been conducted an *ex situ* experiment utilizing bacteria encapsulated in alginate beads in order to protect them from protozoa's predation. The results were not very promising, showing cell inhibition due to the release of tannins production during alginate lysis (Larsen et al., 2009). In another study it was confronted the biostimulation and bioaugmentation efficiency in a landfarming *ex situ* treatment. The results showed that stimulated indigenous microbiome at a polluted site will likely outperform any allochthonous consortium (Fodelianakis et al., 2015). Khudhur and colleagues confirmed the efficiency of biostimulation underlining, however, some critical issues. They report a reduction in pollutant concentration in biostimulated samples compared with the ones undergoing the natural attenuation. However, in association with the decrease of pollutant, the treated samples showed greater toxicity due to the addiction of nutrients, underlining the need to better understand the best set up to avoid such harmful effects (Khudur et al., 2015). In contrast, the cost-effective *in situ* bio-remediation techniques reduce at minimum the sediment mobilization, increasing the environmental

sustainability and gaining greater public acceptance (Das & Chandran, 2011; Higgins et al., 1997; Vidali, 2001). The main *in situ* strategies are represented by bioventing, bioslurping, biosparging, phytoremediation. Except for phytoremediation that is based on the use of plant, these other methods are based on oxygen supply, differing in different zone treated with air injection and in the way the soil vapor is collected (e.g. extraction with vacuum-enhanced pumping). Another promising method for *in situ* bioremediation recently proposed is represented by electrochemistry (Cheng et al., 2019). In this case, the microbial stimulation is achieved by providing electron donors and acceptors by water electrolysis. Hydrogen is produced at the cathode (-) and serves as an electron donor, while at the anode (+), oxygen is produced and utilized as an electron acceptor (Mangold, 2014). The main problem in treating marine sediment *in situ*, is the very unstable environment respect to the land. In the last years, researchers worked hard trying to find a way to entrap microbes and utilize them in marine environment. Chen and colleagues demonstrated that immobilized-microorganism technique (IMT) is a useful approach for reduce soil contamination with PAHs (Chen et al., 2012). Few years ago, Zhao and colleagues performed in situ bioremediation using zeolite, a bacterial adsorbent, in combination of oil-degrading bacteria (*Bacillus spp.*) and wrapping agents (γ-PGA or chitosan). After 70 days the oil spilled pollutants removal rates were >50% in different marine sampling sites, indicating the effectiveness of this method (Zhao et al., 2018). Another interesting approach for the study of the microbial remediation was chosen by Genovese and colleagues. The aim of their study was to simulate an oil spill in a mesocosm treated by in situ aeration of sediments. The results confirmed the selection of specialized hydrocarbon-degrading microorganisms and a drastic depletion of pollutants, confirming that *in situ* aeration can be an efficient strategy to recover petroleum-contaminated marine sediments (Genovese et al., 2014).

Chapter 2

Patterns and drivers of benthic prokaryotic diversity in coastal systems chronically contaminated by present and past industrial activities

2.1 Introduction

Anthropogenically induced chemical contamination has now reached a global dimension (Halpern et al., 2007; Jackson, 2001; Muralikrishna & Manickam, 2017). Compounds such as polycyclic aromatic hydrocarbons and heavy metals due to their toxicity are a major concern in terms of detrimental, biological and ecological effects. In marine ecosystems, sediments are a major repository for contaminants, which can alter benthic biodiversity, food web structure and ecosystem functioning. This applies especially to coastal areas subjected to high anthropogenic inputs deriving from multiple sources such as industrial effluents and continental run-off (Perrodin et al., 2013; Salomons & Brils, 2004). Depending on contamination levels and their bioavailability, pollutants can have negative effects at different levels of biological organization from cells to individuals, populations and communities (Arienzo et al., 2017; Bertocci et al., 2019; Cesar et al., 2009; Gambi et al., 2020; Losi et al., 2013; Mestre et al., 2018; Moreno et al., 2009; Trifuoggi et al., 2017). In marine sediments, prokaryotes play a key role in C cycling, nutrient regeneration processes and energy transfer to higher trophic levels (Barker Jorgensen, 2006; Roberto Danovaro et al., 2008). Therefore, although so far prokaryotes are not identified as a priority for ecological monitoring of the impact of chemical contaminants (R. Danovaro et al., 2020), the analysis of benthic prokaryotic diversity and activity can provide important

insights on the effects of contamination on biogeochemical cycles and on benthic trophodynamics (Franzo et al., 2016; Sun et al., 2013).

Some studies have investigated the potential effects of chemical contaminants on microbial assemblages in coastal marine sediments. High metals concentrations have been reported to inhibit benthic bacterial metabolism and turnover (A. Dell'Anno et al., 2003), as well as to influence the microbial diversity by selecting for more tolerant taxa (Gillan et al., 2005; Jeanbille et al., 2016). At the same time, prokaryotes can modify the fate of pollutants in the sediments by influencing speciation and mobility of toxic metals (Fonti et al., 2015) and degradation processes of organic contaminants (Head et al., 2006; Acosta-González & Marqués, 2016 and references therein).

Investigations carried out on polluted sediments have reported a low diversity and richness of prokaryotic assemblages (Quero et al., 2015), and the dominance of few taxa (Sun et al., 2012), but this is not a general rule since other studies reported no significant changes in bacterial richness after long term exposure to chemical contaminants (Besaury & Ghiglione, 2014; Gillan et al., 2005; Jeanbille et al., 2016). The combined effects of multiple contaminants on prokaryotic diversity and assemblage composition are still largely unknown (Misson et al., 2016; Tangherlini et al., 2020), since the majority of studies in coastal sediments have been performed with the aim of isolating and characterizing pollutant-degrading bacteria (hydrocarbonoclastic bacteria) and to evaluate their potential for sediment remediation (Acosta-González & Marqués, 2016; Lee et al., 2018; W. W. Li & Yu, 2015), rather than to understand their ecological responses.

In this study, we collected sediment samples from three different areas chronically contaminated by present and past industrial activities to investigate, through next generation sequencing, prokaryotic biodiversity. This study aims at improving knowledge on the potential

effects of different concentrations and typologies of chemical contaminants on benthic prokaryotic diversity and assemblage composition.

2.2 Materials and methods

2.2.1 Study areas and sampling

Sediment sampling has been carried out in the Sites of Remediation National Interest" (SNI) of Bagnoli-Coroglio bay, Falconara Marittima and Mar Piccolo of Taranto (Figure 2.2.1.1). The Bagnoli-Coroglio bay (Gulf of Naples, Tyrrhenian Sea) is a heavily contaminated coastal area by metals and hydrocarbons released from industrial activities started in the early 1900 and ended at the beginning of nineties. In particular, industrial activities in the Bagnoli-Coroglio area, mainly represented by a steel plant using fossil coal, iron and limestone, began in 1905 and ended at the beginning of nineties when the plant was closed (De Vivo & Lima, 2008). Here, sediment contamination by heavy metals is particularly high (Romano et al., 2004, 2009) as well as contamination by polycyclic aromatic hydrocarbons with concentrations three to four orders of magnitude higher than those reported from several marine benthic ecosystems worldwide (Arienzo et al., 2017). Sediments are dominated by coarse sand and sandy silt on the littoral shelf, fine sand at the margin of the gulf and silty and silty-clay particles in the central basin (Bertocci et al., 2019 and references therein).

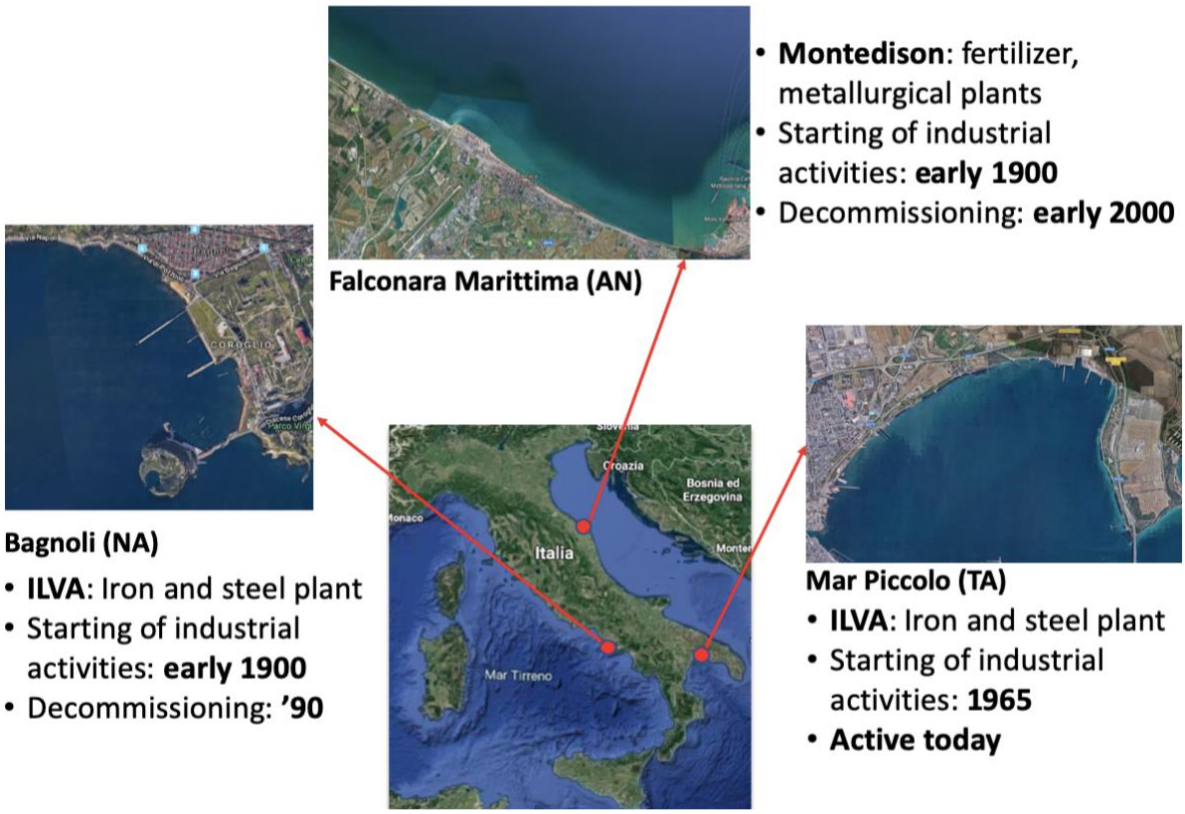

Figure 2.2.1.1. Location of the SNI with indication of the main industrial activities historically present.

The SNI of Falconara Marittima (Italy), located on the western coast of the central Adriatic Sea (Mediterranean Sea) has an extension of 1200 ha, and represents one of the areas with high risk of environmental crisis. The SNI also includes a marine area, located to the north, in front of the former Montedison plant, and to the south in front of the mouth of the Esino river and the API, an oil refinery (used since the 1940s as a refining and storage station for petroleum products).

The investigated area overlooks the former Montedison industrial plant, whose activity dates back to 1919 when the production of superphosphate started. In 1944, the factory was absorbed by the Montecatini Company, and used as a warehouse store by the British Royal Army Service Corps. From 1966 to 1990 (decommissioning year) the chemical pole has produced fertilizers using pyrite and phosphorous, which have been reported to contaminate soil and groundwater

of the surrounding area, together with heavy metals, fluorides, hydrocarbons and PAHs (Legambiente, 2021). The Mar Piccolo of Taranto is included in the list of SNI, being a chronically polluted area (Cardellicchio et al., 1997; Petronio et al., 2012; Quero et al., 2015) due to the presence, since decades, of the largest steel production plant in Europe. It also hosts a variety of other sources of pollutants, among which oil refineries, a large naval base (including a military ship-yard) and intense maritime traffic, and has received in the past considerable amounts of sewage from several pipes discharges (Cardellicchio et al., 1997). The Mar Piccolo of Taranto (Ionian Sea) is an inner, semi-enclosed basin which communicates with the adjacent Mar Grande through two channels, termed "Navigabile" and "Porta Napoli" (Petronio et al., 2012). The Mar Piccolo of Taranto displays a restricted circulation and extends for a total surface area of 20.7 km^2. It is structured into two inlets, the "First Inlet" (having the maximum depth of 13 meters) and the "Second Inlet" (maximum depth 8 meters; Cardellicchio et al., 2007). In terms of the hydrographic characteristics, this site can be compared to a brackish lake. Salinity is influenced by the input of freshwater originating by small tributary rivers and freshwater springs called "Citri" (Cavallo et al., 1999). Sediment samples were collected at 15 stations in the Bagnoli-Coroglio bay, 6 Stations in Falconara Marittima (of which 3 inside and 3 outside of the SNI), and 8 stations in Mar Piccolo of Taranto (Figure 2.2.1.2A-C).

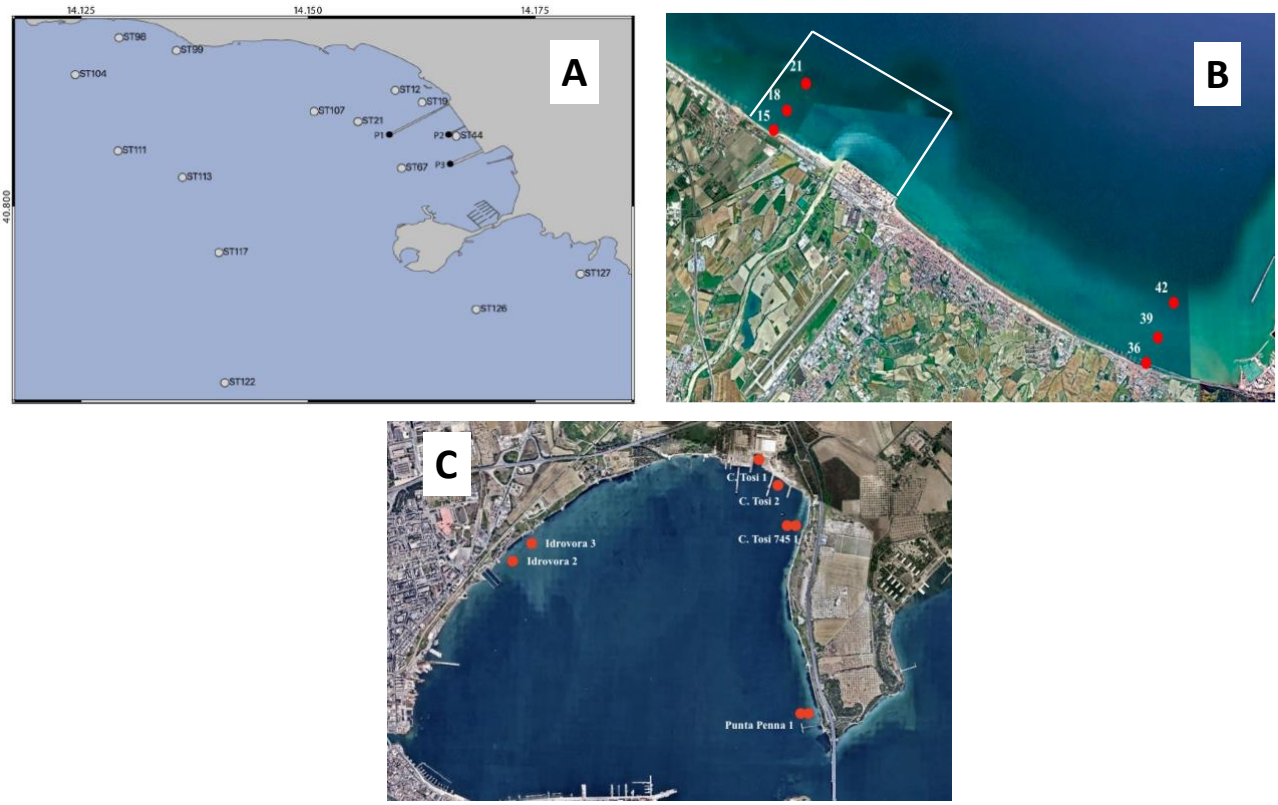

Figure 2.2.1.2. Location of sampling stations of sediments in each of the investigated areas: A) Bagnoli-Coroglio, Falconara Marittima (B) and Mar Piccolo of Taranto (C).

2.2.2 Chemical analyses

Aliphatic hydrocarbons C>12 (heavy hydrocarbons) were determined by gas chromatography equipped with a flame ionization detector (GCFID), according to the method proposed by the Italian Institute for Environmental Protection and Research (ISPRA, 2011), which also contain the definition of "Hydrocarbons with C>12". Polycyclic aromatic hydrocarbons (PAHs) were extracted from the sediment samples according to 3545A EPA method and analyzed by gas chromatography-mass spectrometry (GC-MS; EPA 8270D). Sixteen different congeners were analyzed including naphthalene, acenaphthene, fluorene, acenaphthylene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3,-cd]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i] perylene. Total PAH concentrations were obtained by the sum of the

concentrations of these 16 congeners. The concentrations of metals in the sediment were determined after microwave assisted acid digestion with a mixture of HNO_3, HF, H_2O_2 (EPA method 3052) and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). Iron was analyzed by inductively coupled plasma-optical emission spectrometry (ICP-OES), after the same acid digestion. Mercury was analyzed by Atomic Absorption Spectrometer AMA-254, according to EPA method 7473.

2.2.3 Prokaryotic diversity analysis

DNA was extracted in two replicates from 1 g of sediment from each analyzed sample by using the DNeasy PowerSoil Kit (MO BIO). Libraries were prepared from each replicate and sequencing was performed with all libraries on a single Illumina MiSeq flowcell by LGC Genomics GmbH (Berlin, Germany) using the primer pairs 515F–Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'; Corinaldesi et al., 2019; Parada et al., 2016). After primer removal through the cutadapt plugin within QIIME2 (Bolyen et al., 2019; Martin, 2011), trimmed reads were submitted to the DADA2 pipeline within QIIME2 (Bolyen et al., 2019; Callahan et al., 2016) to identify biologically-meaningful sequences, remove potential chimeras and produce final representative Amplicon Sequence Variants (ASVs), trimming forward reads at 250 and reverse reads at 220 bps, corresponding to an average quality value > 30 (PHRED score). For the taxonomic affiliation of ASVs, a reference database was first created within QIIME2 by trimming the SILVA database v132 (Quast et al., 2013) to the region amplified by sequencing primers; representative ASVs were then compared to the reference database using the proposed SciKit Learn-based approach (Bolyen et al., 2019). Representative sequences were subsequently aligned using the MAFFT aligner within the QIIME2 suite (Nakamura et al., 2018). The

alignment was masked and has been utilized for the construction of a midpoint-rooted phylogenetic tree using the FastTree software (Price et al., 2010). To minimize biases due to different sequencing depths among samples, the ASV table was randomly subsampled to the same number of sequences (9000). This normalized table was subsequently used to produce a table of the relative abundance of prokaryotic taxa at Family level and, along with the phylogenetic tree inferred from the representative sequence alignment, to produce alpha- and beta-diversity indices for each sample (Corinaldesi et al., 2019). We defined "exclusive ASVs" those ASVs present in a single site, "shared ASVs" those present in more than one site, and "core ASVs" those shared among all the sites investigated in this study (following the nomenclature proposed in C. Corinaldesi et al., 2018). A bipartite ASV-sample network was constructed using the normalized ASV table with the Gephi v0.9.2 tool (Bastian et al., 2009) to visualize the core, shared and exclusive ASVs in our dataset. Statistical tests on the prokaryotic assemblage structure were also performed using the R (v4.0.5; RC Team, 2013) package GGPubr (https://rpkgs.datanovia.com/ggpubr/), particularly with the *compare_means* procedure, to test by Wilcoxon's tests, after BH correction of p-values, the significance of differences in family abundances across the dataset. The R package vegan (Dixon, 2003) was then used to calculate the influence of pollutants on the structure of the prokaryotic assemblage at family level through a combination of dbRDA analyses and permutational analysis of variance by the *dbRDA* and *adonis2* functions.

2.3. Results and discussion

High concentrations of chemical contaminants in marine sediments can induce detrimental effects on abundance, biomass and diversity of benthic assemblages, from prokaryotes to megafauna (Gambi et al., 2020; M. Y. Sun et al., 2012). In the present study, we observed clear

differences in the concentrations of the different chemical pollutants both among and within the benthic systems analyzed. Sediments of the Bagnoli-Coroglio bay were characterized by extremely high concentrations of total aliphatic hydrocarbons C>12 (on average 308±88 mg kg^{-1}, range: 5-1083 mg kg^{-1}; Figure 2.3.1) and polycyclic aromatic hydrocarbons (on average 164±55 mg kg^{-1}, range: 0.4-746 mg kg^{-1}; Figure 2.3.2). Also, sediments collected in the Mar Piccolo of Taranto were generally highly contaminated by total aliphatic hydrocarbons C>12 (on average 78±35 mg kg^{-1}, range: 0.24-270 mg kg^{-1}; Figure 2.3.1) and polycyclic aromatic hydrocarbons (on average 6.2±2.2 mg kg^{-1}, range: 0.06-17 mg kg^{-1}, Figure 2.3.2), despite to a minor extent of those of the Bagnoli-Coroglio bay. Falconara Marittima sediments were generally characterized by low concentrations of total aliphatic hydrocarbons C>12 and polycyclic aromatic hydrocarbons concentrations (on average 3.5±2.1 mg kg^{-1} and 0.62±0.61 mg kg^{-1}, respectively) (Figure 2.3.1 and Figure 2.3.2), typically encountered in moderately polluted coastal areas (Baumard et al., 1998; Berto et al., 2009; Bihari et al., 2007). In the different benthic systems analyzed, contaminant concentrations displayed a wide spatial variability, with values of heavy metals (Table 2.3.1) and PAHs in several stations of Bagnoli-Coroglio close to the main source of contamination and in some benthic sites of the Mar Piccolo above those expected to induce detrimental biological effects (Long et al., 1995). With a few exceptions, the sediments of Falconara Marittima were characterized by pollutant concentrations lower than those expected to determine detrimental biological effects, suggesting a high self-depuration/natural attenuation capacity occurring over time after the decommissioning of the industrial plants.

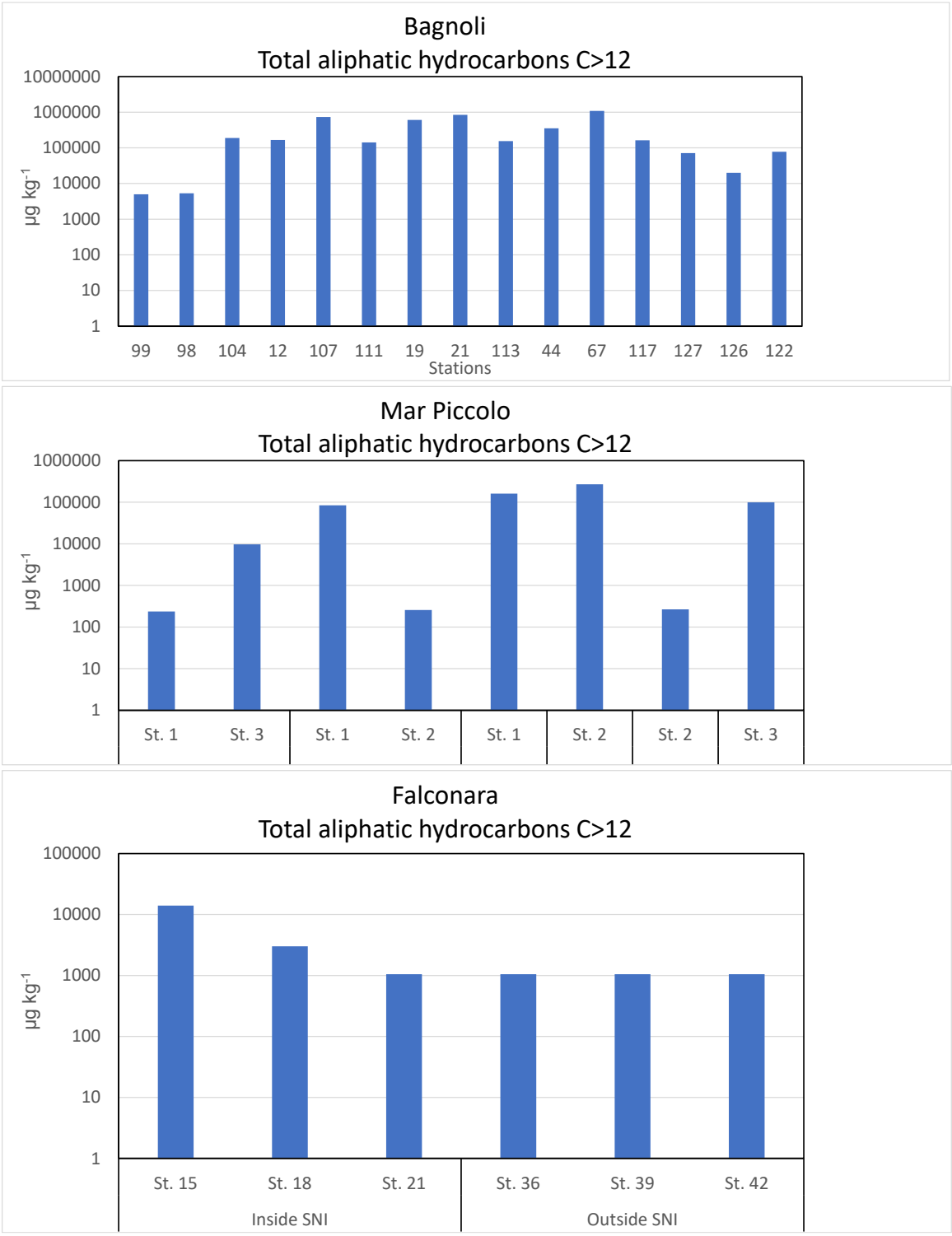

Figure 2.3.1. Total aliphatic hydrocarbon C>12 concentrations in the sediments collected in different stations of the three investigated areas. Please note that the y axis is set in log scale.

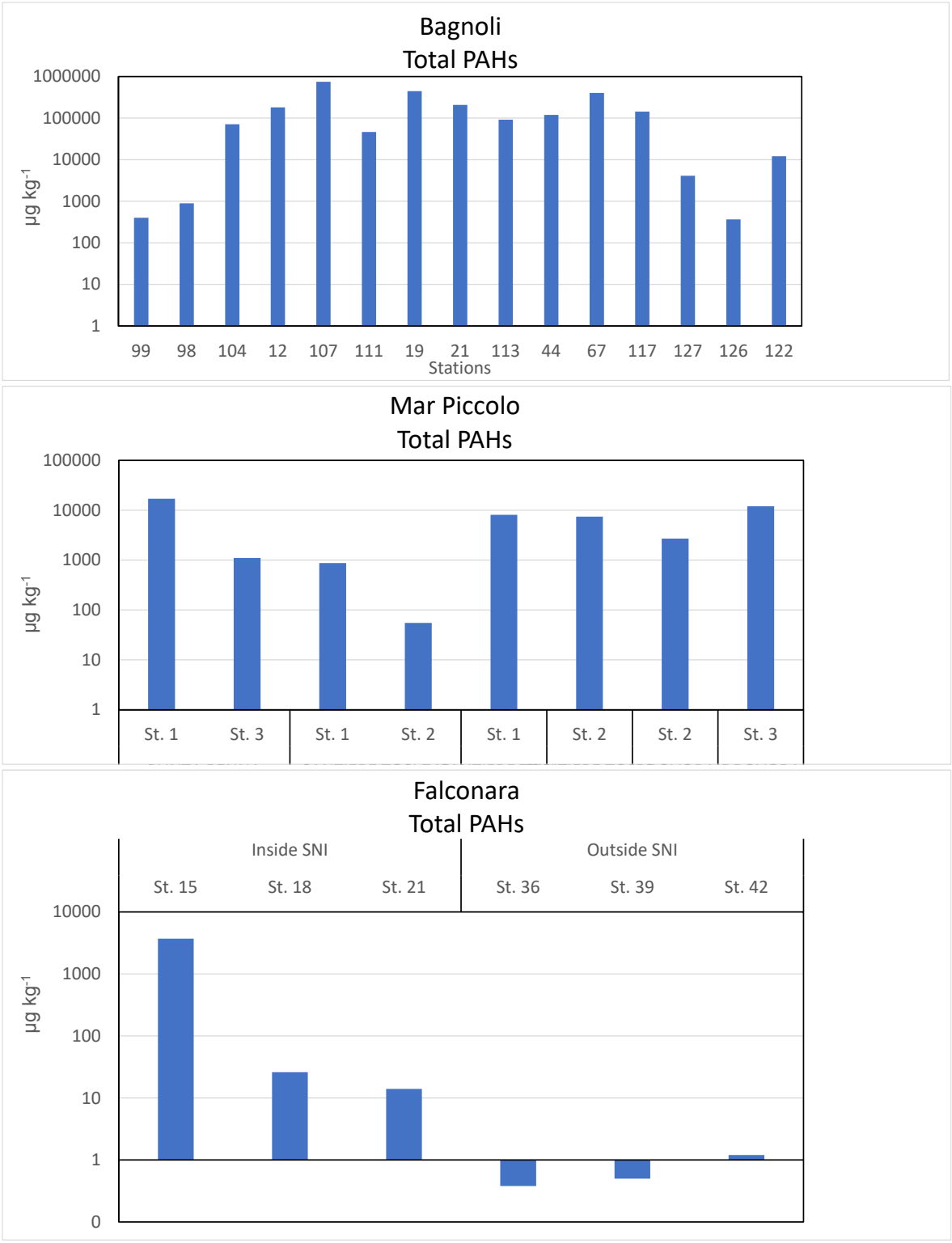

Figure 2.3.2. Total polycyclic aromatic hydrocarbon (PAH) concentrations in the sediments collected in different stations of the three investigated areas. Please note that the y axis is set in log scale.

Table 2.3.1. Heavy metal concentrations in the sediments of the different stations of the three sampling areas (Bagnoli-Coroglio, Mar Piccolo of Taranto and Falconara Marittima).

Area	Station	Al	As	Cd	Cr	Cu	Fe	Hg	Ni	Pb	V	Zn
		mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Bagnoli-Coroglio	99	77374,4	31,7	0,35	58,4	8,0	38402,7	0,01	11,1	47,3	108,0	114,9
Bagnoli-Coroglio	98	87204,7	37,7	0,33	28,1	7,2	32696,9	0,02	6,8	57,6	86,1	103,9
Bagnoli-Coroglio	104	66416,2	42,2	0,46	27,9	22,6	38974,5	0,34	8,7	126,3	86,6	310,5
Bagnoli-Coroglio	12	75999,0	75,2	0,38	20,8	15,6	55005,0	0,13	7,4	124,3	93,9	258,8
Bagnoli-Coroglio	107	68185,3	83,7	0,54	16,9	17,5	36351,5	0,15	7,3	127,4	84,1	293,3
Bagnoli-Coroglio	111	40760,3	37,5	0,50	47,7	36,5	40305,6	0,56	15,3	144,6	102,6	353,1
Bagnoli-Coroglio	19	55024,2	104,2	1,92	35,8	35,3	120304,7	0,59	13,6	314,1	91,3	745,0
Bagnoli-Coroglio	21	71222,0	64,8	1,09	15,7	28,3	41556,4	0,47	8,0	197,0	87,9	431,2
Bagnoli-Coroglio	113	62030,2	38,1	0,64	55,6	46,9	46466,5	0,82	15,3	199,2	101,6	489,5
Bagnoli-Coroglio	44	53300,2	48,2	1,54	31,8	30,6	182480,5	0,35	15,7	295,0	88,5	739,7
Bagnoli-Coroglio	67	54988,2	70,6	4,82	73,6	108,7	119179,8	1,73	24,5	785,7	119,9	1729,0
Bagnoli-Coroglio	117	59634,2	42,3	0,50	59,1	49,6	65040,7	0,77	17,6	176,2	101,5	477,2
Bagnoli-Coroglio	127	59919,2	21,7	0,39	12,7	15,3	27596,0	0,11	5,2	69,5	77,8	148,5
Bagnoli-Coroglio	126	83022,7	54,3	0,36	24,8	12,3	41750,0	0,03	11,5	61,1	144,0	154,0
Bagnoli-Coroglio	122	66018,9	22,3	0,43	67,8	44,7	37830,2	0,58	28,3	118,3	107,8	206,1
Mar Piccolo - Punta Penna	St. 1	1000,0	2,9	0,05	4,5	5,0	1800,0	0,21	3,5	9,6	6,8	15,0
Mar Piccolo - Punta Penna	St. 3	1200,0	3,3	0,05	15,0	7,1	2200,0	0,67	8,4	14,0	8,3	21,0
Mar Piccolo - Cantiere Tosi 745	St. 1	6200,0	6,6	0,31	17,0	76,0	9000,0	0,44	18,0	28,0	16,0	110,0
Mar Piccolo - Cantiere Tosi 745	St. 2	12000,0	4,1	0,11	34,0	26,0	16000,0	0,14	58,0	14,0	19,0	61,0
Mar Piccolo - Cantiere Tosi 1	St. 1	3600,0	18,0	0,29	19,0	80,0	15000,0	1,00	13,0	120,0	22,0	150,0
Mar Piccolo - Cantiere Tosi 2	St. 2	8600,0	14,0	0,24	42,0	150,0	21000,0	1,20	36,0	140,0	30,0	260,0
Mar Piccolo - Idrovora	St. 2	1900,0	4,9	0,08	8,6	8,6	3900,0	0,24	13,0	9,7	10,0	26,0
Mar Piccolo - Idrovora	St. 3	2800,0	5,4	0,16	10,0	14,0	5300,0	0,38	10,0	15,0	13,0	47,0
Falconara - inside SNI	St. 15	760	1,7	0,053	2,3	2	1900	0,011	2,9	1,4	2,5	6,4
Falconara - inside SNI	St. 18	3100	8,8	0,1	10	3,9	9700	0,018	13	4,2	10	18
Falconara - inside SNI	St. 21	4200	8,8	0,096	13	5,3	12000	0,018	16	4,8	12	22
Falconara - outside SNI	St. 36	1300	7,2	0,072	4,4	1,8	5100	0,012	5,4	2,8	5,1	8,3
Falconara - outside SNI	St. 39	3600	9,8	0,1	13	4,3	11000	0,013	15	5	12	21
Falconara - outside SNI	St. 42	3800	9,7	0,098	12	5,1	11000	0,018	14	4,9	12	20

In the present study, we compared the diversity of prokaryotic assemblages in benthic systems characterized by different levels of contamination. This analysis highlighted significant differences in terms of prokaryotic ASV richness between the Bagnoli and Taranto and between Bagnoli and Falconara areas (Figure 2.3.3). The Bagnoli area was characterized by the lowest richness values (752), whereas the Taranto area by the highest ones (1272).

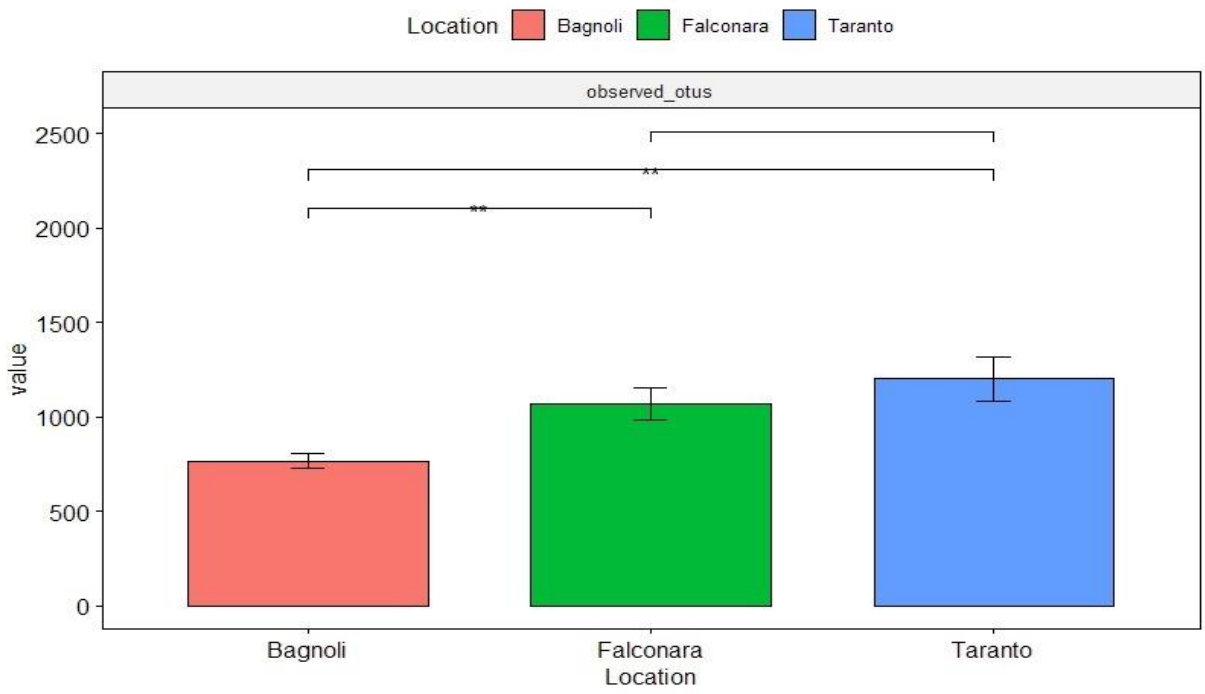

Figure 2.3.3. Comparison of ASV richness in the sediments of the three different benthic areas. Average values and standard deviations are reported.

Considering all samples from each area, the Bagnoli area displayed the highest value of "exclusive" (i.e. only present in that area) ASVs (10982), followed by the Taranto and Falconara areas (8758 and 5327; Figure 2.3.4). A total of 411 ASVs were shared among all areas, representing ca. 1.5% of the total number of ASVs found across all the sampling sites (26954). Overall, ASV richness values for the whole areas (which account for the total number of ASVs found among all samples within that area) were higher for the Bagnoli area (12181) than for the others (10164 and 6907 for the Taranto and Falconara areas). These results suggest that the high level of contamination within the Bagnoli area, despite reducing the overall richness within single site, might drive the development of site-specific communities by selecting contaminant-resistant prokaryotic taxa (Tangherlini et al., 2020). Interestingly, the presence of a low, but not negligible fraction of ASVs common to all areas investigated,

suggests the presence of a “core” set of widespread pollution-tolerant or pollution-resistant prokaryotic taxa.

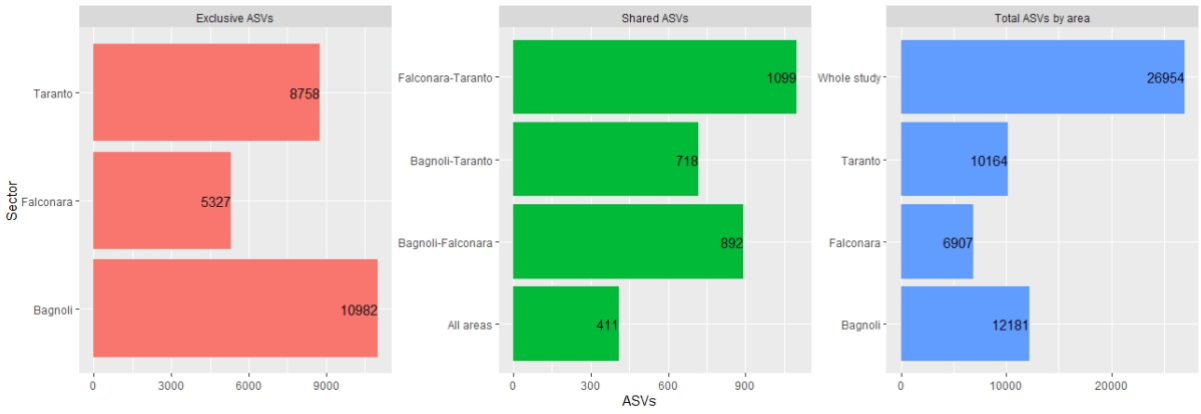

Figure 2.3.4. Exclusive, shared and total ASV richness in the sediments of the three different benthic areas.

Analysis of taxonomic diversity highlighted that the Bagnoli and Falconara areas were mainly characterized by a high abundance of Bacteria belonging to the *Woesiaceae*, *Pirellulaceae*, *Thermoanaerobaculaceae* and *Flavobacteriaceae* families, along with uncultured *Actinomarinales* in Falconara. The Taranto sediments were mainly characterized by *Desulfosarcinaceae*, *Flavobacteriaceae*, *Thermoanaerobaculaceae* and *Anaerolineaceae* families (Figure 2.3.5). Statistical analyses highlighted that 8 taxa were found to have significantly different (p-values < 0.05) abundances among areas: *Woeseiaceae* and *Pirellulaceae* were more represented across the Bagnoli and Falconara areas, while uncultured *Actinomarinales* and Subgroup_22 were more represented in the Falconara area (Table 2.3.2). Indeed, members of the *Thermoanaerobaculaceae* family were already found to be highly represented within highly-contaminated sites (Kristensen et al., 2021; Lehosmaa et al., 2021). Members of some of the other widespread families (e.g. *Woeseiaceae*) have been shown to

encompass a wide range of different metabolisms, thus potentially providing adaptations to a broad range of conditions within the marine environment (Lehosmaa et al., 2021; Mußmann, et al., 2017), thus explaining their presence at both high and low contamination levels.

Figure 2.3.5. Main bacterial families encountered in the sediments of the three investigated benthic areas.

Table 2.3.2. Output of the pairwise statistical tests carried out to evaluate the significance of the difference in abundances of taxa between locations.

Taxon	Location 1	Location 2	BH-adjusted p-value	Significance	Method
f__Woeseiaceae	Bagnoli	Taranto	1,70E-05	****	Wilcoxon
f__Woeseiaceae	Falconara	Taranto	0,0026	****	Wilcoxon
f__Pirellulaceae	Bagnoli	Taranto	0,04	***	Wilcoxon
f__Pirellulaceae	Falconara	Taranto	0,0071	****	Wilcoxon
f__Desulfosarcinaceae	Bagnoli	Taranto	5,30E-05	****	Wilcoxon
f__Desulfosarcinaceae	Falconara	Taranto	0,0041	****	Wilcoxon
o__Actinomarinales (uncultured)	Bagnoli	Falconara	1,10E-06	****	Wilcoxon
o__Actinomarinales (uncultured)	Falconara	Taranto	0,00065	****	Wilcoxon
f__Sandaracinaceae	Bagnoli	Taranto	0,024	***	Wilcoxon
c__Gammaproteobacteria (uncultured)	Falconara	Taranto	0,00026	****	Wilcoxon
f__Subgroup_22	Bagnoli	Falconara	0,005	****	Wilcoxon
f__Desulfocapsaceae	Bagnoli	Falconara	0,028	***	Wilcoxon
f__Desulfocapsaceae	Bagnoli	Taranto	0,001	****	Wilcoxon

Prediction of the functional potential of prokaryotic taxa across the three areas highlighted fermentation and nitrification as the most represented, although the Taranto area appeared to be enriched in taxa involved in respiration of sulphur compounds (Figure 2.3.6). Interestingly,

sequences belonging to taxa involved in aromatic compound degradation were not abundant across the three areas. Indeed, the presence of several nitrogen-dependent taxa within the Bagnoli area (e.g. *Nitrosopumilaceae*), concomitant with high level of contamination of these sites, might imply that prokaryotes involved in the biogeochemical N cycle of nitrogen (e.g. through ammonia oxidation) are also favored by the local environmental conditions, including the low overall environmental quality (R. Zhang et al., 2021), although these metabolic relationships should be better investigated in the future.

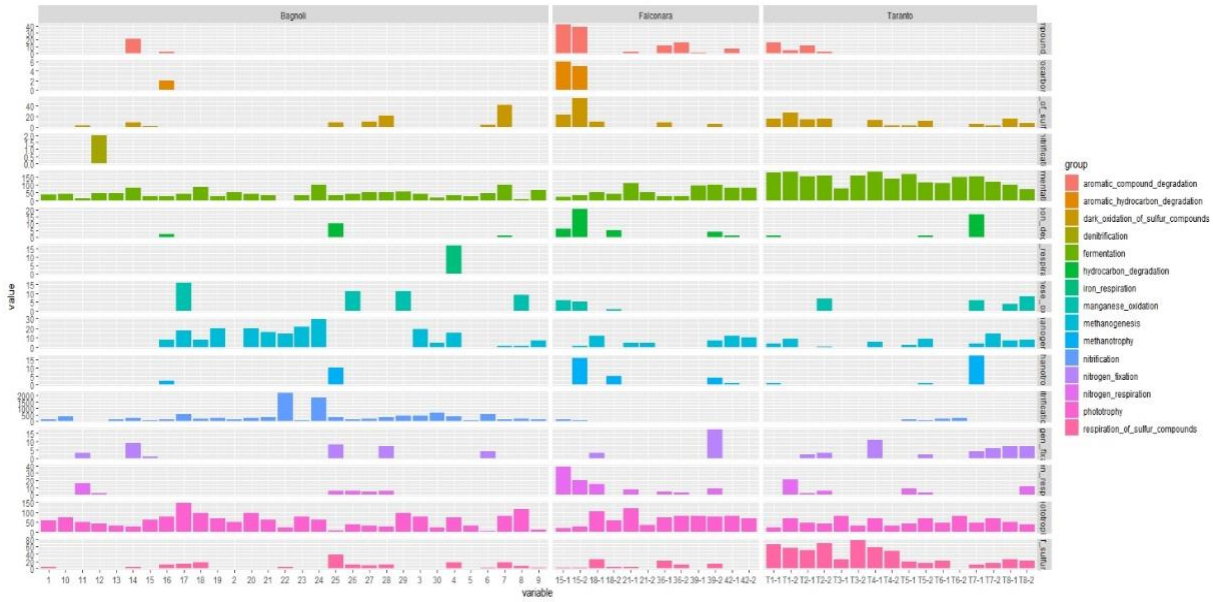

Figure 2.3.6. Prediction of the functional potential of the prokaryotic taxa present in the sediments of the investigated benthic areas.

In the present study, we carried out a distance-based redundancy analysis (dbRDA) of the prokaryotic assemblage composition (in terms of differential sequence abundance using the Bray-Curtis distance) in relation with the contaminants present in the sediment. The dbRDA plot showed that the prokaryotic assemblages in each area were separated from each other, with a few stations of the Falconara Marittima area overlapping with those of Mar Piccolo of Taranto

(Figure 2.3.7). Prokaryotic assemblage composition was significantly related with heavy metal contents, whereas no significant relationships were found either with aliphatic hydrocarbon C>12 or PAH concentrations.

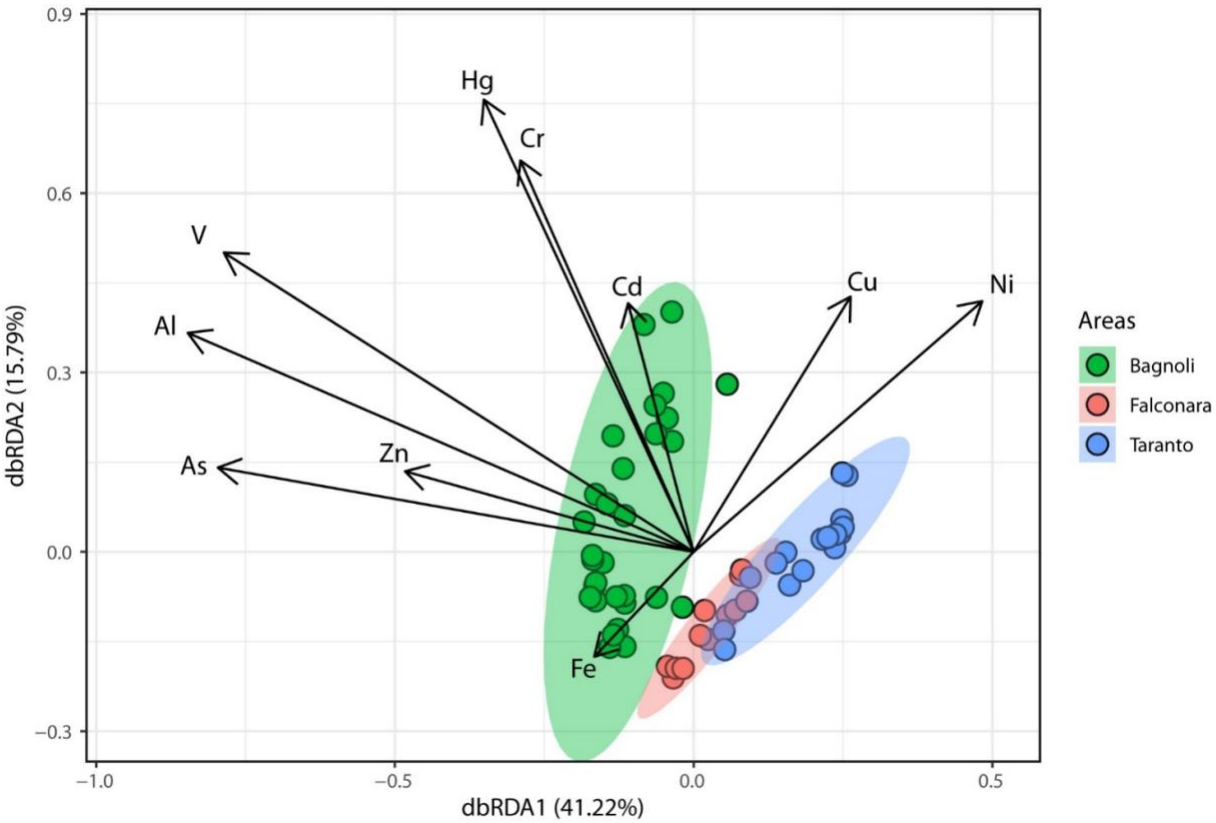

Figure 2.3.7. Output of the distance-based redundancy analysis (dbRDA) carried out on the prokaryotic assemblage composition (in terms of different sequence abundance using the Bray-Curtis distance) of the stations of the three benthic areas in relation with the contaminants present in the sediments. Pollutants not contributing to significantly explaining the variation are not shown in the plot.

In addition, multivariate multiple regression analysis revealed that the main potential drivers influencing prokaryotic assemblage composition were represented by metals, instead of PAHs and hydrocarbons (Table 2.3.3), although explaining 42% of the total variation. Thus, other factors acting at the local scale, such as trophic availability, habitat heterogeneity, competition

and predation processes, may have an additional role in shaping the patterns of prokaryotic diversity in the different systems investigated.

Table 2.3.3 Output of the *adonis2* function carried out to assess the significance of the pollutants on the samples investigated.

Variable	**Sum of Sqs**	**R2**	**F**	**Pr(>F)**	**Significance**
Al	1	0,15309	13	0,001	***
As	0,3099	0,03351	3	0,001	***
Cd	0,2559	0,02767	2	0,005	**
Cr	0,3058	0,03307	3	0,004	**
Cu	0,3448	0,03729	3	0,002	**
Fe	0,1824	0,01972	2	0,038	*
Hg	0,3288	0,03555	3	0,003	**
Ni	0,3163	0,0342	3	0,001	***
Pb	0,1875	0,02027	2	0,072	ns
V	0,2195	0,02373	2	0,026	*
Zn	0,2111	0,02282	2	0,038	*
Hydrocarbons C>12	0,1205	0,01304	1	0,3	ns
Total PAHs	0,1054	0,0114	0,9382	0,491	ns
Residual	5	0,53465			
Total	9	1			

Overall, findings reported in this study suggest that chemical contaminants, even after decades from the end of their release, influence to a certain extent the diversity of the benthic prokaryotic

assemblages. The different pollution levels along with their variability across the different benthic areas can be an important factor promoting diversification of the benthic bacterial and archaeal assemblages, likely selecting those lineages more adapted to specific mixtures of different contaminants. These results open new perspectives for understanding of the long-term effects of chemical contamination on the benthic prokaryotic assemblages and on the key ecological processes they mediate.

Chapter 3

Sediments of the National Remediation sites can contain microbial taxa highly efficient for hydrocarbon degradation: the case study of Falconara Marittima

3.1. Introduction

Coastal zones represent key areas of interactions between ocean and land, and provide a wide range of ecosystem goods and services such as carbon and nutrient recycling, climate regulation, food provision and grounds for tourism recreational activities (Barbier, 2012, 2017; Costanza et al., 1997; Turner & Schaafsma, 2015; Yanes et al., 2019). However, the rapid urbanization and industrialization of coastal zones and the consequent marine pollution, habitat degradation, alien species invasion and alteration of trophic the food webs are determining the loss of these goods and services (Todd et al., 2019). The Mediterranean Sea hosts more than 200 petrochemical plants, energy installations and chemical plants along its coasts (Civili, 2010) and only less than 1% of the total area remains relatively unaffected by human activities (Micheli et al., 2013). In most cases, despite the closure of industrial plants for since many years and the ceased release of pollutants into the sea and/or the implementation of environmental regulations, the contamination still persists (Croudace & Rothwell, 2015). Marine sediments represent the final sink for all contaminants, which can have deleterious effects on marine life at all levels of biological organization (Gambi et al., 2020; Mele et al., 2020; Tangherlini et al., 2020). Among chemical contaminants, polycyclic aromatic hydrocarbons (PAHs) are of particular concern because some are carcinogens and mutagens,

and have a high potential for biomagnification through the aquatic food web due to their lipophilic nature. Thus, there is an urgent need to find sustainable and eco-compatible solutions for the remediation of marine sediments contaminated by PAHs. Bioremediation is an environmental-friendly strategy gaining increasing attention for its potential to clean-up hydrocarbon contaminated marine sediments (Akcil et al., 2015; Beolchini et al., 2010; P. Chen et al., 2017; A. Dell'Anno et al., 2012; Head et al., 2006; Kronenberg et al., 2017). Several field and laboratory experiments demonstrated that the biodegradation of hydrocarbons in the sediments can be accelerated through the addition of inorganic nutrients and/or different electron acceptors/donors able to stimulate the autochthonous microbial assemblages (Atlas & Bragg, 2009; Atlas & Cerniglia, 1995; A. Dell'Anno et al., 2012; Dell'anno et al., 2020; Head et al., 2006; Head & Swannell, 1999; Kalantary et al., 2014; Kasai et al., 2002). However, PAHs in aged contaminated sediments can be recalcitrant to biodegradation due to adsorption to sediment particles, which reduces availability for microbial degradation (Dandie et al., 2010). Therefore, optimization of bioremediation strategies can be needed to reclaim historically contaminated sediments. At the same time, aged contaminated marine sediments can host specific prokaryotic taxa which can have a high natural attenuation capacity (i.e. contributing to the reduction of contamination level over time), thus potentially representing optimal candidates to be exploited for enhancing bioremediation performance through bioaugmentation strategies (Dell'Anno et al., 2021; Jacques et al., 2008; Yu et al., 2005 and references therein). In this study, we tested the efficiency of biostimulation strategies for PAH degradation on marine sediments collected in the National Remediation Site of Falconara Marittima and their effects on prokaryotic abundance and diversity. We also investigated natural attenuation capacity of the allochthonous microbial assemblages and we finally isolated and identified

different microbial taxa suitable for bioaugmentation interventions to be carried out in other benthic systems contaminated with PAHs.

3.2 Material and Methods

3.2.1 Study area and sediment sample collection

Sediment samples were collected in the framework of the project BIOBLUETECH, financially supported by Fondazione CARIVERONA, at two coastal stations, one of which (hereafter defined station 15) located within the National Remediation Site (SNI) of Falconara Marittima and the other (hereafter defined station 36) located few km far in between the SNI and the Ancona harbor. These two stations have been selected to test bioremediation performance on sediments potentially characterized by different concentrations and typologies of congeners of PAHs and different allochthonous microbial assemblages.

The SNI of Falconara Marittima (Italy), located on the western coast of the central Adriatic Sea (Mediterranean Sea) has an extension of 1200 ha, and represents one of the areas with high risk of environmental crisis. The SNI also includes a marine area, located to the north, in front of the former Montedison plant, and to the south in front of the mouth of the Esino river and the API, an oil refinery (used since the 1940s as a refining and storage station for petroleum products).

The investigated area overlooks the former Montedison industrial plant, whose activity dates back to 1919 when the production of superphosphate started. In 1944, the factory was absorbed by the Montecatini Company, and used as a warehouse store by the British Royal Army Service Corps. From 1966 to 1990 (decommissioning year) the chemical pole has produced fertilizers using pyrite and phosphorous, which have been reported to contaminate soil and groundwater

of the surrounding area, together with heavy metals, fluorides, hydrocarbons and PAHs (Legambiente, 2021).

Overall, the coastal area where sediments have been collected is characterized by multiple sources of contamination deriving from the ex-industrial site Montedison (which operated from 1900 for a century before the decommissioning in the early 2000), an oil refinery operating since the early 1940s, an international airport, a river outflow, a dense urban area and an important harbor (Carletti et al., 2014; Tirabassi et al., 2005).

Sediment samples have been collected at the two stations by a Van Veen grab and the top 10 cm used for bioremediation experiments.

3.2.2 Set up of biostimulation experiments

The biostimulation experiments were performed in 250 mL Erlenmeyer flasks containing 100 g wet sediment samples of each station and 50 mL pre-filtered (0.2 μm) and autoclaved seawater. Replicate microcosms (n = 3 for each station) were added with $(NH_4)2SO_4$ and K_2HPO_4 (final concentrations of 50 and 5 mM of nitrogen and phosphorus, respectively). The final concentrations of N and P were defined on the basis of the organic carbon content in the sediment according to a molar C:N:P ratio of 100:10:1 (Beolchini et al., 2010; Dell'Anno et al., 2020). Additional microcosms (n = 3 for each station) prepared in the same way, but without inorganic nutrient addition, were used as controls. To investigate the efficiency of the autochthonous microbial assemblages in PAH biodegradation, additional microcosms containing the sediment from the two stations were added with inorganic nutrients and with a mixture of PAHs (i.e. phenanthrene, pyrene and benzo(a)pyrene). In particular, 150 μg of phenanthrene, pyrene and benzo(a)pyrene were added to microcosms containing sediment from

station 15 and station 36 and 50 μg of phenanthrene, pyrene and benzo(a)pyrene were added to sediment collected at station 36.

The addition of 150 μg of a mix of phenanthrene, pyrene and benzo(a)pyrene to sediment of station 15 simulate an increase of such congeners, ca. three folds of those analytically determined, whereas the addition of 150 μg of a mix of phenanthrene, pyrene and benzo(a)pyrene to sediment of station 36 simulate an increase of such congeners, of ca. six folds of those analytically determined. Finally, the addition of 50 μg of a mix of phenanthrene, pyrene and benzo(a)pyrene to sediment of station 36 simulate the contamination level by such congeners present in the sediment of station 15.

Overall, 21 microcosms were prepared and subsequently incubated for two months at room temperature. Sediment sub-samples were collected at the beginning of the experiment (T0) and after 1 (T1) and 2 months of incubation (T2) for the determination of polycyclic hydrocarbon concentrations (PAHs), microbial abundance and biomass, and microbial diversity. Additional sediment-subsamples were collected for the isolation of bacterial and fungal taxa.

3.2.3 Polycyclic aromatic hydrocarbon determinations

Polycyclic aromatic hydrocarbons (PAHs) were extracted from the sediment samples according to 3545A EPA method and analyzed by gas chromatography-mass spectrometry (GC-MS; EPA 8270D). Twenty different congeners were analyzed including naphthalene, acenaphthene, fluorene, acenaphthylene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3,-cd]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i] perylene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i] pyrene and dibenzo[a,l]pyrene. Total PAH concentrations were obtained by the sum of the concentrations of these 20 congeners.

3.2.4 Prokaryotic abundance analysis

For the determination of the prokaryotic abundance, sediment slurries (2.5 mL) were treated with pyrophosphate (5 mM final concentration) and ultrasound (three times for 1 min; Danovaro et al., 2002). For prokaryotic counts, sub-samples (1 mL) were diluted 50–100 times, stained with SYBR Green (0.01% final concentration) and filtered on Anodisc 0.2 μm pore size filters. Filters were analyzed under epifluorescence microscopy using a Zeiss Axioplan microscope equipped with a 50-W lamp. Ten to 50 fields were viewed at 1000 × magnification and a minimum of 400 cells were counted. Prokaryotic counts were normalized to sediment dry weight after desiccation (60 ◦C, 24 h).

3.2.5 Prokaryotic diversity analysis

DNA has been extracted from two replicates of sediment sub-samples collected during the time-course experiments. The preliminary step, before DNA extraction, consisted in the removal of extracellular DNA based on three washing solutions (WS) at different concentration: WS1, 50 mM Tris-HCL, pH 8.3; 200 mM NaCl; 5 mM Na2EDTA; 0,05% Triton X-100; WS2, 50 mM Tris-HCL, pH 8.3; 200 mM NaCl; 5 mM Na2EDTA; WS3, 10 mM Tris-HCL, pH 8.3; 0,1 mM Na2EDTA. The subsequent DNA extraction was performed utilizing the DNeasy PowerSoil Kit, QUIAGEN, following the manufacture's procedure. Sequencing libraries were prepared from each replicate and sequencing was performed with all libraries on an Ion Torrent chip (using the in-house facilities provided by Stazione Zoologica di Napoli "Anton Dohrn") using the primer pairs 515F–Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'; Parada et al., 2016; Corinaldesi et al., 2019).

After primer removal through the cutadapt plugin within QIIME2 (Bolyen et al., 2019; Martin, 2011), trimmed reads were submitted to the DADA2 pipeline within QIIME2 (Bolyen et al.,

2019; Callahan et al., 2016) using the *denoise-pyro* plugin over a length of 250 bp (Callahan et al,. 2016). Taxonomic inference was performed through the *classify-consensus-vsearch plugin* (Rognes et al., 2016) using the SILVA database (v138; Quast et al., 2013). For the taxonomic affiliation of ASVs, a reference database was first created within QIIME2 by trimming the SILVA database v138 (Quast et al., 2013) to the region amplified by sequencing primers. A first overview of the taxonomic structure of the assemblage was obtained after normalizing the ASV table to 95000 sequences. ASVs from the normalized table were then clustered in modules of co-occurring sequences through the SCNIC plugin (Shaffer et al., 2020); we then removed all ASVs not belonging to at least 2 samples and with less than 200 sequences and then clustered co-occurring ASVs into modules through the SCNIC tool. Modules accounting for at least 10000 sequences and were kept for further analyses.

3.2.6 Microbial isolation and identification

Sediment sub-samples collected from controls and treated samples were used for the isolation of bacterial and fungal taxa, which was carried out using marine agar for bacteria and YPD (containing rifampicin to avoid bacterial contamination) for fungi.

Isolates of the different microbial taxa were used for DNA extraction, which was carried out by the DNeasy Blood & Tissue kit (QIAGEN), according to the manufacture's procedure. The DNA was quantified and then amplified using primer sets targeting 16S rRNA gene for bacteria and ITS1 for fungi. The amplicons were then sequenced by Sanger method.

3.3. Results and discussion

3.3.1 Efficiency of PAH degradation

The sediments collected at the two stations were characterized by different concentrations of total PAHs, with values much higher in the sediment of station 15 (ca. 0.73 mg kg-1) than those of stations 36. On the basis of total PAH concentrations, sediment of station 15 can be classified as moderately polluted when compared to other costal benthic systems (Dell'anno et al., 2020). However, the high concentrations of benzo(a)pyrene, the main congener present in the sediment, pose ecological concern, being a mutagenic and cancerogenic compound.

Time-course experiments carried out on sediment samples collected at station 15 revealed after two months of incubation a remarkable drop of total PAH concentrations both in the microcosms without any amendment and in the microcosms containing inorganic nutrient and the mix of PAHs. (Figure 3.3.1.1). These results indicate that the autochthonous microbial assemblages present in the sediment of station 15 can have a high natural attenuation capacity toward PAHs, even in the absence of additional nutrient supply. Such microbial assemblages are also able to degrade to a large extent not only the PAHs already present into the sediment, but also, if adequately stimulated by inorganic nutrient, the additional inputs of PAH congeners.

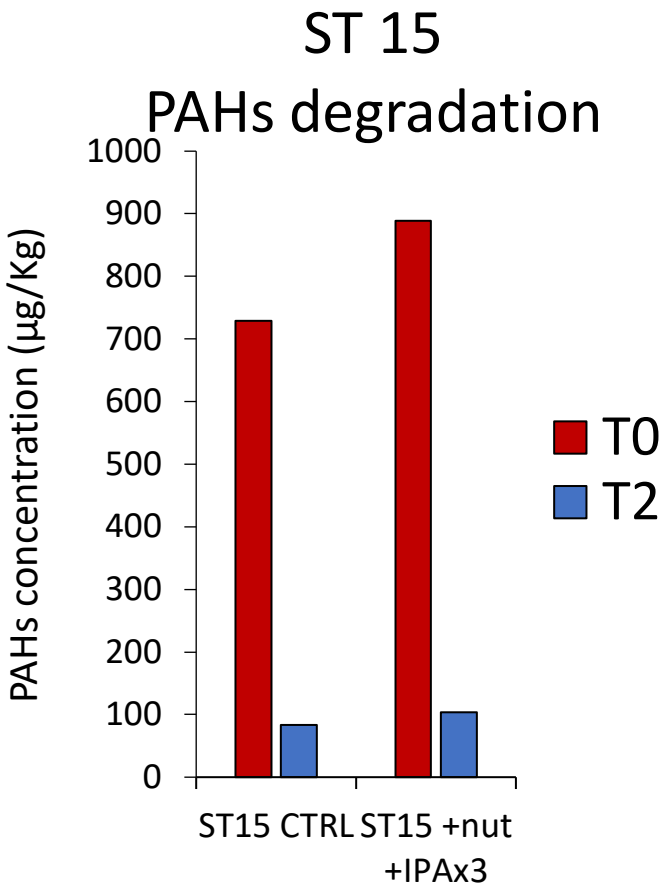

Figure 3.3.1.1 Total PAH concentrations at the beginning (T0) and at the end (T2) of the time-course experiment carried out using sediment collected at station 15 without any amendment (ST15 CTRL) and supplied with nutrients and 150 µg of a mix of different PAH congeners (ST15 + nut + PAHx3).

It is generally assumed that low molecular weight PAHs are degraded faster and to a minor extent than high molecular weight PAHs (Beolchini et al., 2010). This was not the case of the present study, since also benzo(a)pyrene underwent high degradation along with other most abundant congeners phenanthrene, pyrene, and anthracene after two months of sediment incubation without any external supply (i.e. control microcosms; Figure 3.3.1.2). Indeed, the relative contribution of such compounds strongly decreased over time (Figure 3.3.1.3), suggesting a microbial selective degradation towards specific PAH congeners. This result

reinforces previous findings that reported fast degradation rates of specific PAHs, including high molecular weight compounds (Dell'Anno et al., 2020).

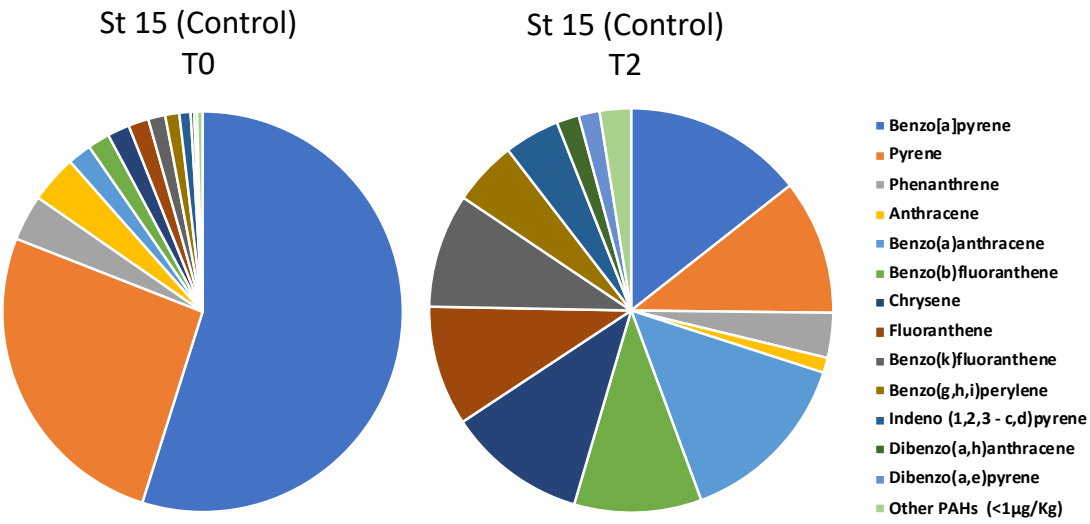

Figure 3.3.1.2 Contribution of the different PAH congeners to the total PAH concentrations at the beginning and at the end of the experiment carried out on sediment samples of station 15 without any addition.

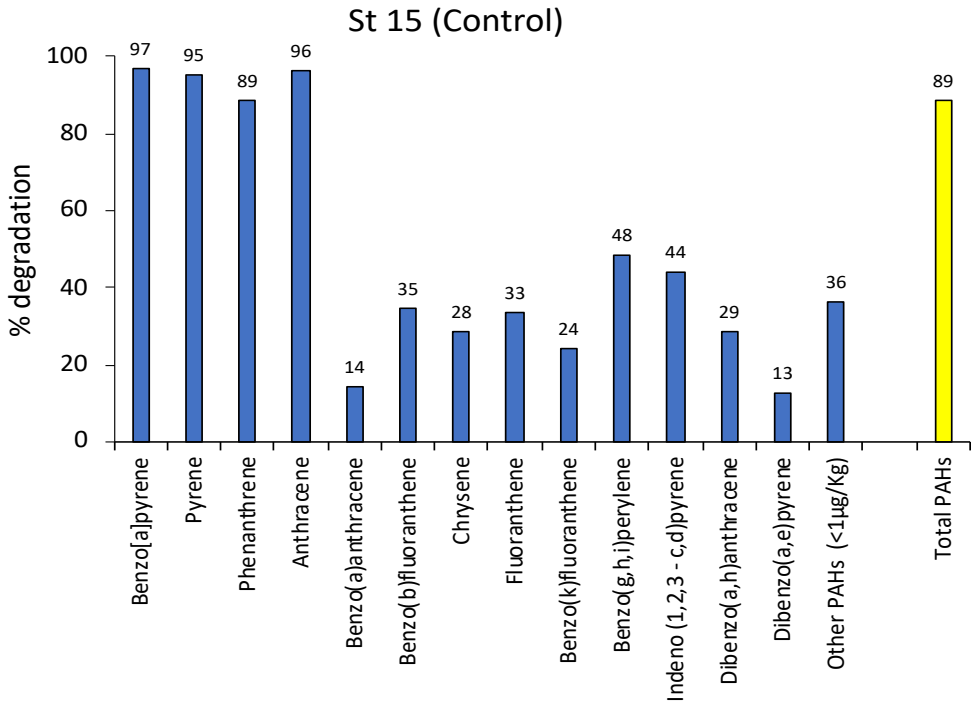

Figure 3.3.1.3 Degradation yield, expressed as percentage, of the different PAH congeners after two months of incubation of sediment samples of station 15 without any addition.

Limited changes occurred over time in PAH composition of sediments of station 15 spiked with inorganic nutrients and a mix of PAH congeners (Figure 3.3.1.4). The majority of the PAH congeners, was, indeed, degraded at a similar extent (with values ranging from 84 to 96%; Figure 3.3.1.5), indicating that an external input of nutrient and PAHs can promote similar rates of degradation of a wide spectrum of PAHs present in the sediment.

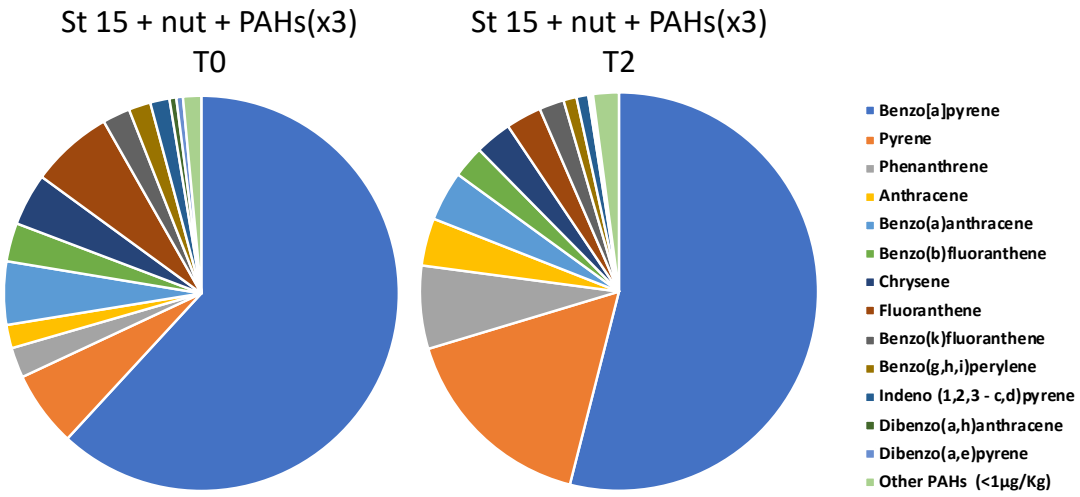

Figure 3.3.1.4 Contribution of the different PAH congeners to the total PAH concentrations at the beginning and at the end of the experiment carried out on sediment samples of station 15 treated with inorganic nutrients and a mix of PAHs.

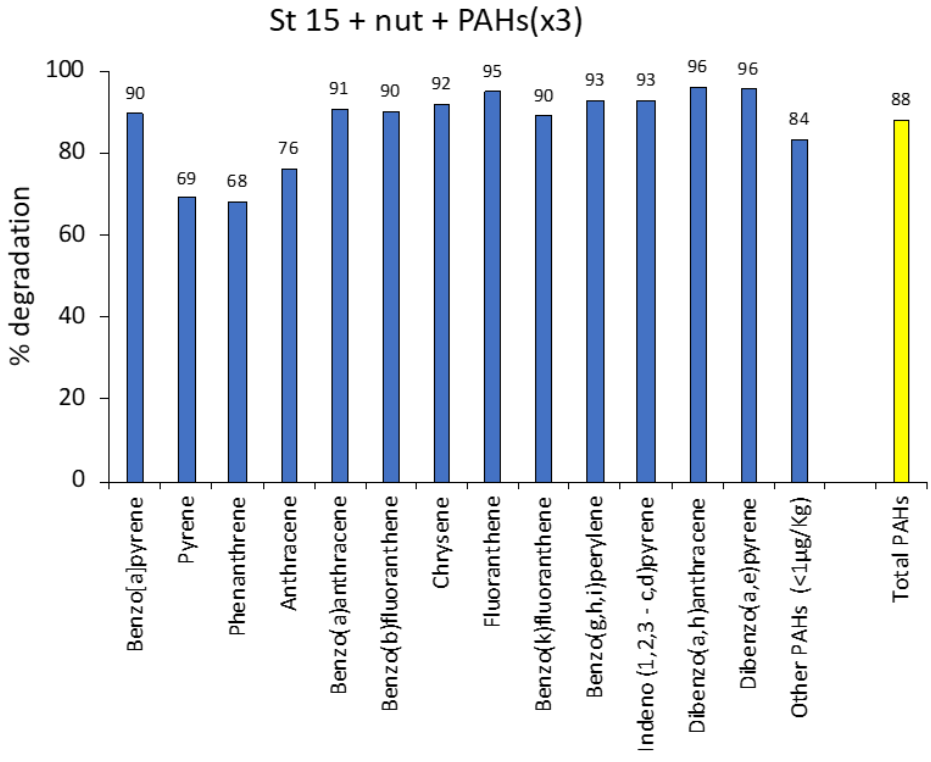

Figure 3.3.1.5 Degradation yield, expressed as percentage, of the different PAH congeners after two months of incubation of sediment samples of station 15 supplied with inorganic nutrients and a mix of PAHs.

Despite the two experimental conditions promoted degradation rates of PAH congeners to a different extent, they were highly effective for the removal of total PAHs, with an overall biodegradation yield close to 90%. In the experiments carried out with sediment of stations 36 without any addition (i.e. control), it was not possible to calculate the percentage of PAHs removal since all PAH congeners were present in very low concentrations and in most cases below the analytical detection limits. Time-course experiments carried out with sediments of station 36 supplied with inorganic nutrients and a mix of PAHs at different concentrations revealed a decrease of total PAH concentrations after two months of incubation (Figure 3.3.1.6). Such a decrease was much higher in the sediments supplied with the lowest amounts of PAHs (54% vs 35%). In particular, at the lowest amounts of added PAHs, biodegradation yield of benzo(a)pyrene was equivalent to 54%, whereas no biodegradation of such congener was observed at the highest amounts of added PAHs, despite other congeners were efficiently degraded (Figure 3.3.1.7). These results suggest that above certain values of contamination by PAHs, microbial-degradation processes can proceed preferentially toward specific congeners, leading potentially to an accumulation of other un-degraded congeners in the benthic systems.

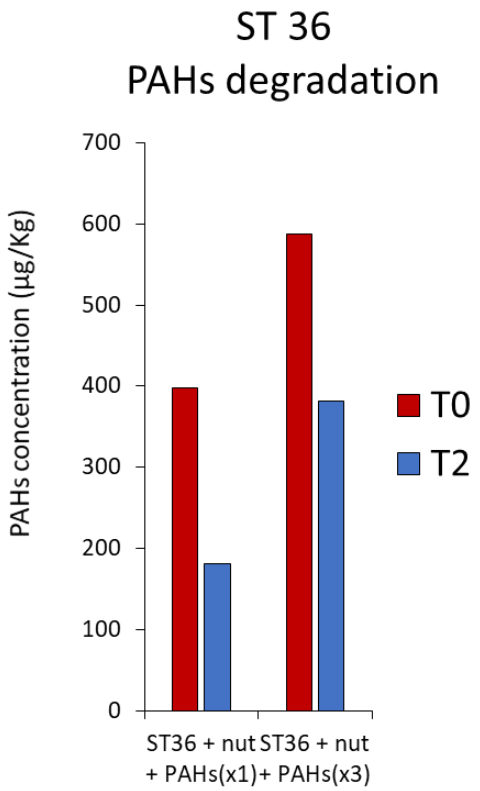

Figure 3.3.1.6 Total PAH concentrations at the beginning (T0) and at the end (T2) of the time-course experiment carried out using sediment collected at station 36 supplied with nutrients and 50 (ST36 + nut + PAHx1) and 150 µg of a mix of different PAH congeners (ST36 + nut + PAHx3).

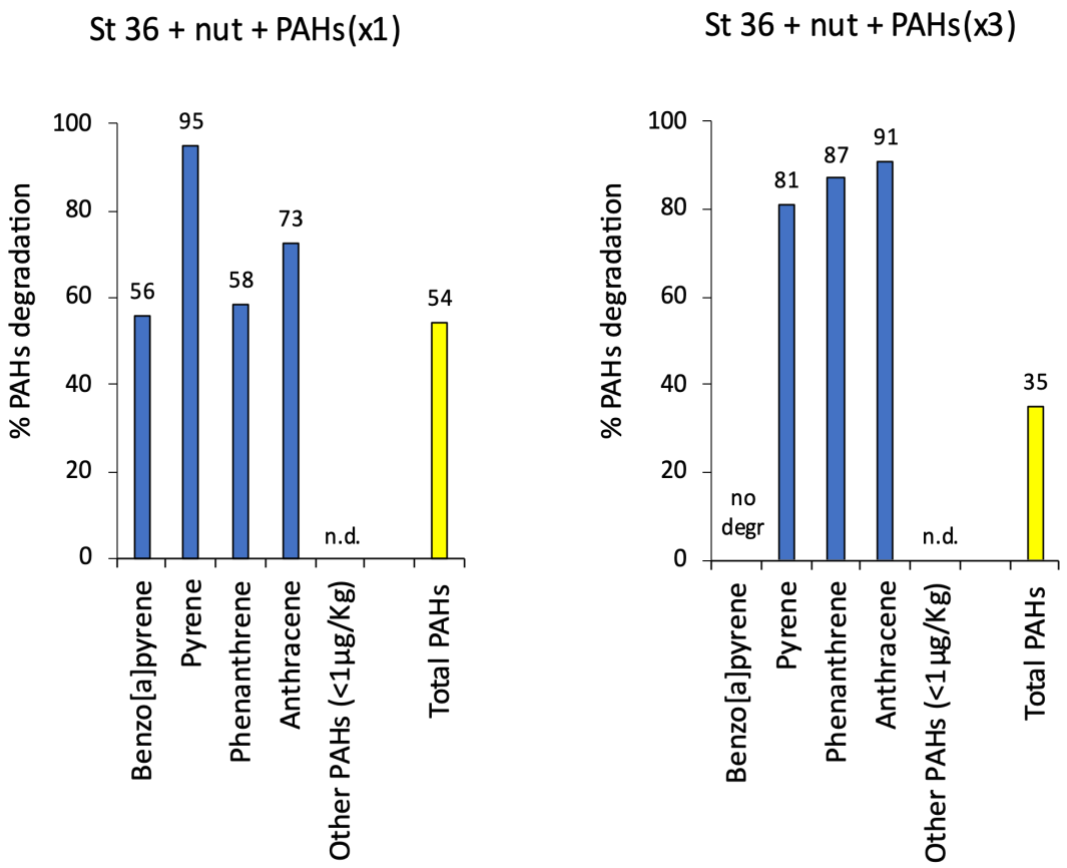

Figure 3.3.1.7 Degradation yield, expressed as percentage, of the different PAH congeners after two months of incubation of sediment samples of station 36 supplied with inorganic nutrients and a mix of 50 (ST36 + nut + PAHx1) and 150 µg (ST36 + nut + PAHx3) of PAHs.

3.3.2 Responses of prokaryotic abundance and diversity during time-course experiments

The sediments of station 15 and 36, despite the different concentrations of total PAHs were characterised by similar values of prokaryotic abundances (Figure 3.3.2.1), typically encountered in other benthic coastal areas (A. Dell'Anno et al., 2003). There is evidence that prokaryotes are a dynamic component of benthic assemblages, able to respond rapidly to changes in environmental conditions and inputs of nutrients and contaminants. For istance, inorganic nutrient addition, commonly used to increase the bioremediation yield of contaminated marine sediments, has been repeatedly reported to increase prokaryotic

abundance by stimulating microbial growth (Beolchini et al., 2010; Dell'Anno et al., 2012; Dell'Anno et al., 2020). At the same time, the addition of PAHs to the sediment can reduce the prokaryotic standing stocks, by selecting the most tolerant taxa able to resist or degrade hydrocarbons. In the present study, neither the sediments used as controls or sediments supplied with inorganic nutrients and a mix of PAHs displayed significant changes of prokaryotic abundances over time. The lack of significant changes over time in prokaryotic standing stocks of the different treated microcosms suggest that inorganic nutrient supply can counteract the toxic effects potentially induced by an input of PAHs, even at concentrations much higher than those already present in the sediment.

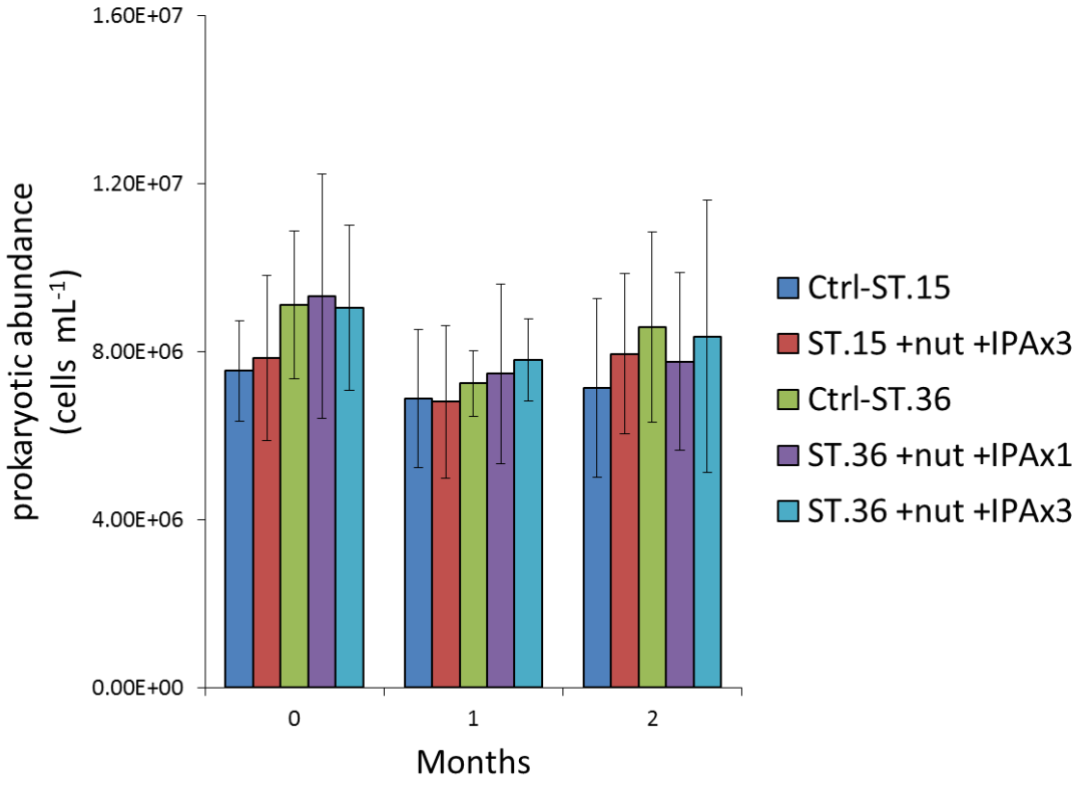

Figure 3.3.2.1. Changes of prokaryotic abundance during time-course experiments carried out using sediments collected at the station 15 and 36.

Conversely to prokaryotic standing stocks, prokaryotic diversity analysis revealed notable changes in the taxonomic composition occurring over time (Figure 3.3.2.2). In particular, the assemblage composition of samples from station 36 and 15 at the beginning of the experiment was similar, but at the end of the experiment, all samples displayed a low degree of similarity to each other, regardless of station and treatment. These results reinforce previous findings reporting major shifts in prokaryotic assemblage composition during bioremediation experiments (Fonti et al., 2015; Rocchetti et al., 2012). In the present study we found that *Pirellulacee* and *Woeseiaceae* were, overall, the most represented prokaryotic families across the whole dataset, whereas taxa such as *Nitrosopumilaceae*, *Flavobacteriaceae* and *Rhodobacteriaceae* showed remarkable shift in the abundance according to treatment and time. Indeed, these results can be explained also in terms of the wide range of possible metabolic pathways encoded by the different members of both *Pirellulaceae* and *Rhodobacteriaceae*, which make them generalist families (Chen et al., 2021), while taxa such as *Nitrosopumilaceae*, which show specific adaptations to the utilization of oxygen and nitrogen (Zhang et al., 2021), might respond differently to treatments.

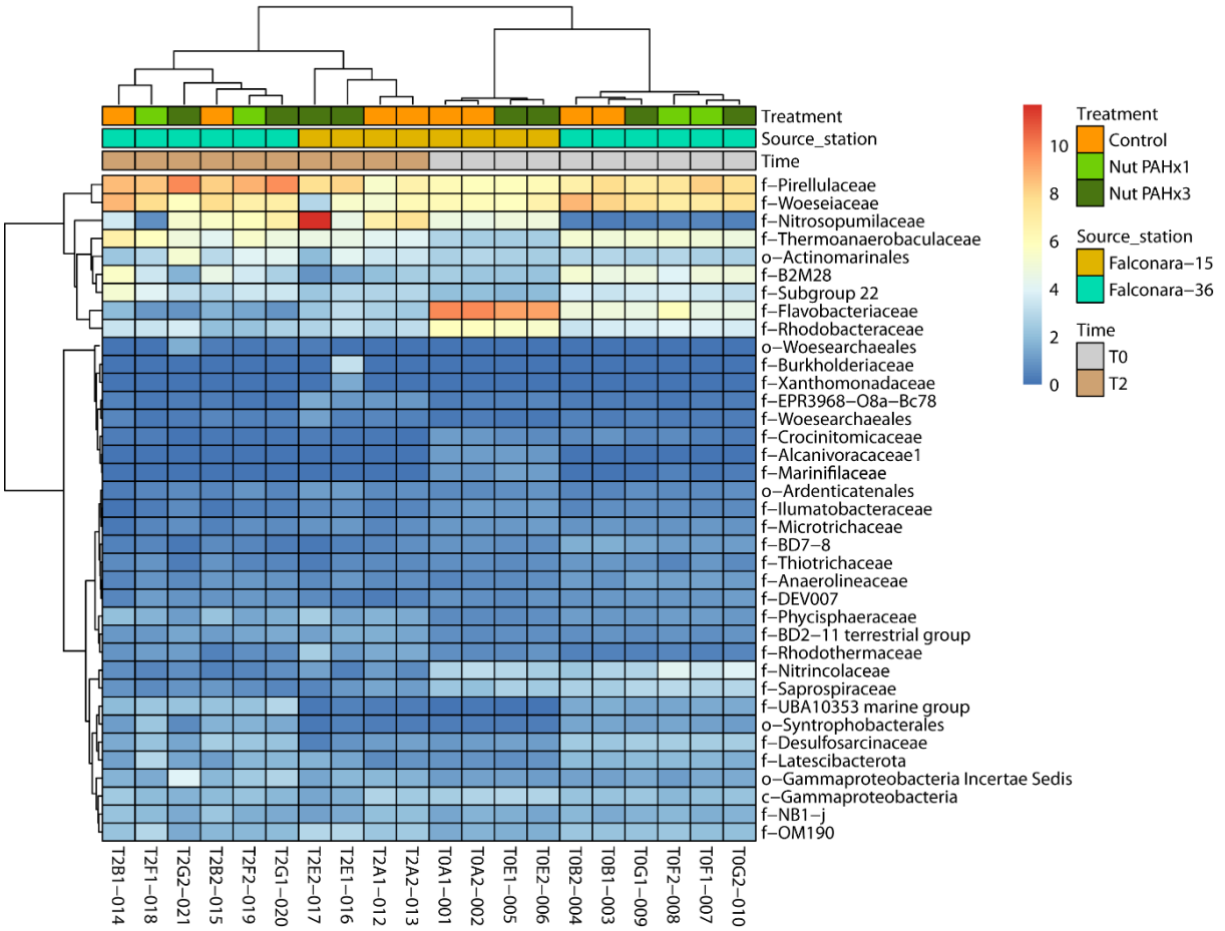

Figure 3.3.2.2. Prokaryotic community composition (at Family level) at the beginning of the incubation and after two months in the sediment of station 15 and 36 used for the different experiment. Tile colors refer to the abundance of sequences affiliating to each taxa (as a percentage over the total number of sequences).

The SCNIC clustering procedure led to identification of 286 co-occurring ASV modules for a total of 2033 ASVs. Of the 286 co-occurring ASV modules, 33 of which represented at least 10000 sequences. Thirteen of these 33 modules interestingly were represented by more than 20000 sequences each, but only 4 showed high value of ASV richness (>120 ASV, Figure 3.3.2.3).

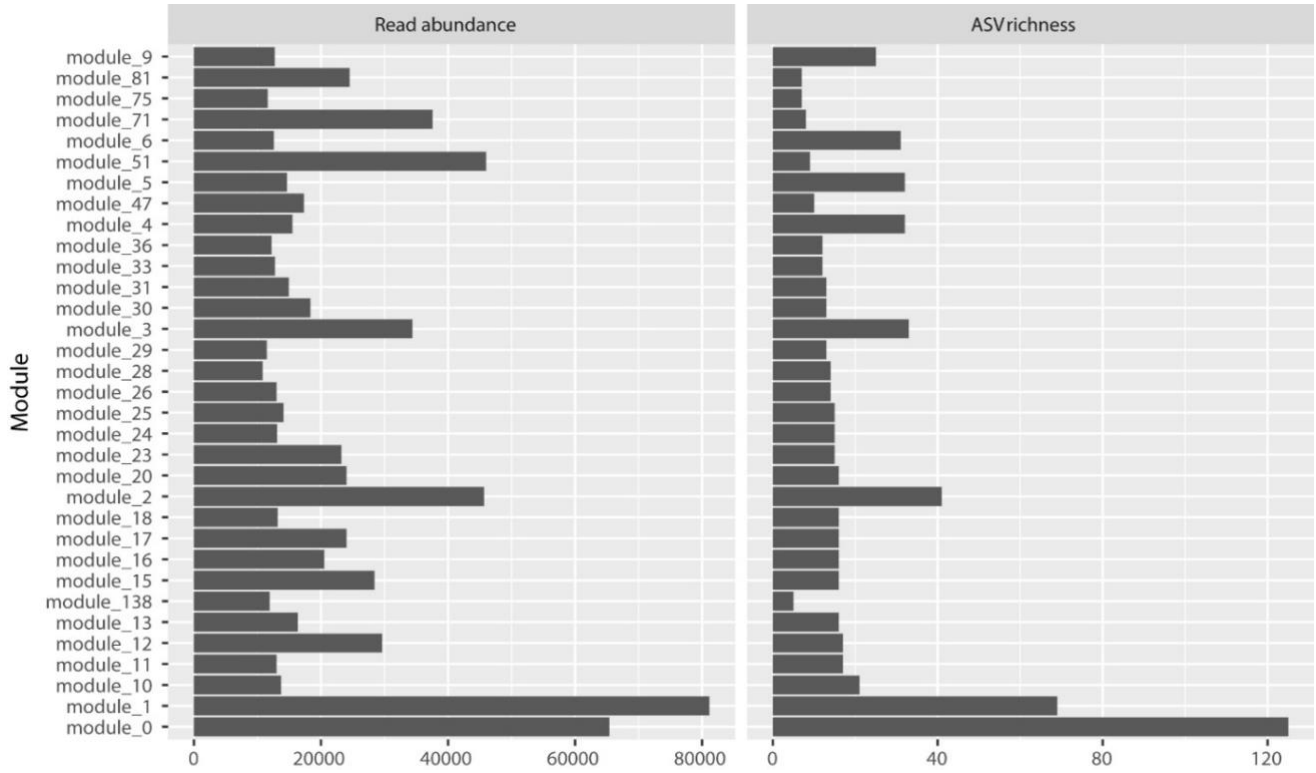

Figure 3.3.2.3 Sequence abundance and ASV richness of principal co-occurring ASV modules in different samples.

The analyses of the co-occuring ASV modules revelead that the two sediments investigated were characterized by very different prokaryotic assemblages, dominated by module 1 (St. 36) and 0 (St. 15). At the begenning of the experiment (T0), both controls and treatments showed a great homogeneity in their prokaryotic assemblages. Instead, at the end of the experiment (T2) clear differences between the two stations and between controls and treatments were found. In particular, sediments of station 15 were enriched in modules 51 and 3, with control samples retaining the starting abundances of modules 81 and 4, while sediments of station 36 were characterised by the presence of modules 1, 51, 31 and 9 more abundant in the treatments than in the controls (Figure 3.3.2.4). The structural simplification provided by the module approach allowed us to better appreciate the differences between samples, which appear to be limited to a few modules encompassing a wide range of taxa.

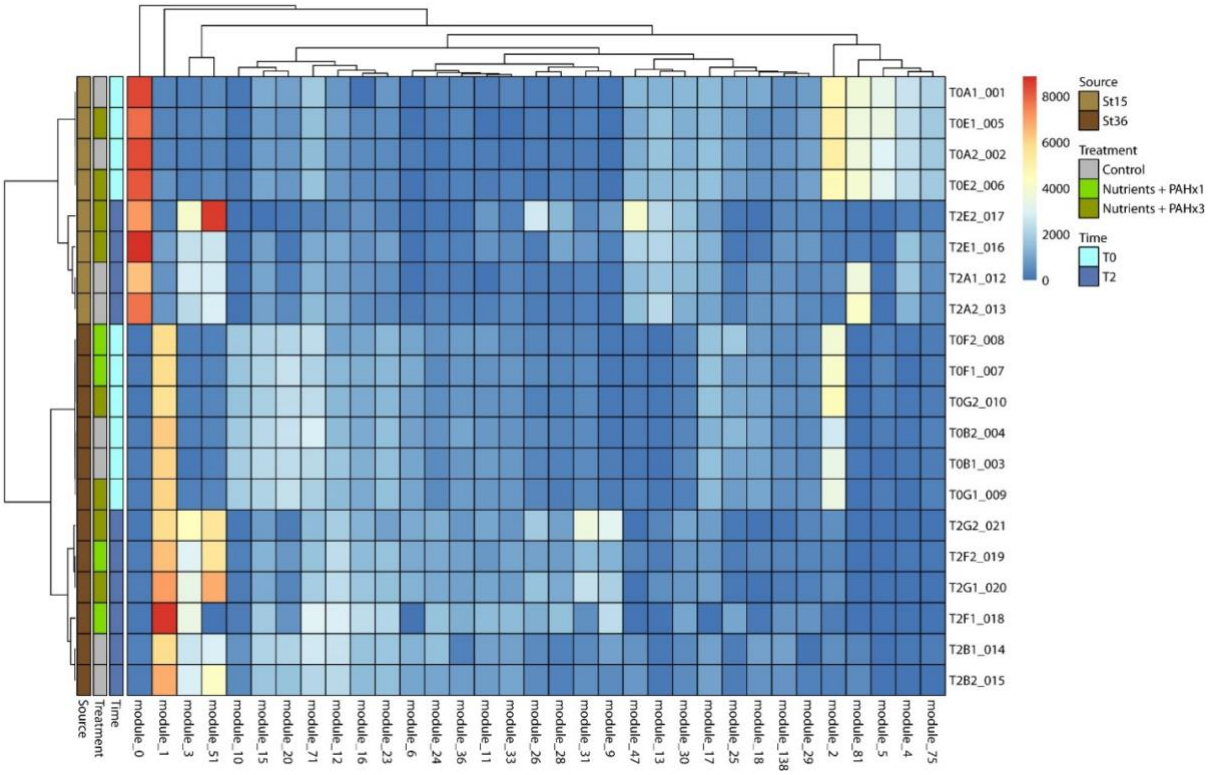

Figure 3.3.2.4. Relative abundance of modules of co-occurring ASV at the beginning of the incubation and after two months in the sediment of station 15 and 36 used for the different experiment.

Focusing on modules showing significant changes, results highlighted that both modules 0 and 1 (more abundant, respectively, in sediments of station 15 and 36) were collecting ASV belonging to prokaryotic classes including Acidimicrobiia, Alphaproteobacteria, Bacteroidia, Gammaproteobacteria and Planctomycetes, differing for the esclusive presence/absence of several other classes (e.g., Actinobacteria, Desulfobacteria, Ignavibacteria, OM190, Phycisphaerae, Syntrophobacteria, Thermoleophilia and Vicinamibacteria in module 1). Modules 9 and 31 (more abundant in treatments at T2 of sediments of station 36) comprised mainly ASVs of the prokaryotic classes Babeliae, BD2-11, BD7-11, Dadabacteriia, Nanoarchaeia and SAR324 (associated with hydrothermal plumes). Modules 3 and 51 (mostly associated with T2 in both stations 15 and 36) were mainly characterized by ASVs of the the

prokaryotic classes Nitrososphaeria, Polyangia, Subgroups 22 and 26. Module 2 (which decreased in abundance at T2) comprised mainly ASVs of the the prokaryotic classes Alpha-/Gamma-proteobacteria, Bacteroidia and Verrucomicrobiae (Figure 3.3.2.5). This approach has been previously utilized in studies carried out in heavy metal contaminated sites (e.g. mine soils; Chun et al., 2021), in which it allowed to identify clusters of prokaryotes with different degrees of resistance/tolerance to metal pollution; the application of this approach to our investigation allowed us to identify clusters of prokaryotic taxa which clearly differ in abundance between the two sediments investigated regardless of time and treatment (modules 0 and 1) and by time (modules 3, 51, 2). This suggests that relationships between taxa within our experimental setup was influenced by both the original prokaryotic community and the experimental treatments.

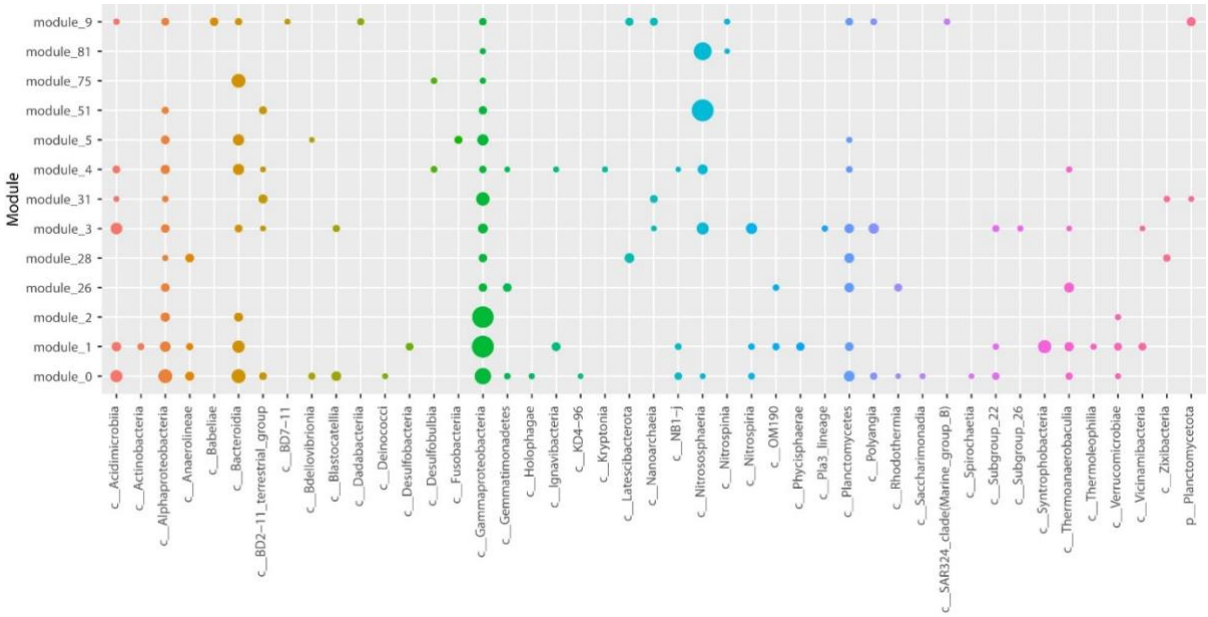

Figure 3.3.2.5**.** Taxonomic composition at class level of each of the main co-occuring ASV modules identified in the Falconara sediments during the time-course experiment.

Analysis of the relationships among prokaryotic taxa through co-occurrence networks, indeed, revealed that the structure of modules both in terms of taxonomy and network properties was related with enviromental stress levels (Hernandez et al., 2021). Overall, these findings suggest that different prokaryotic taxa can promote natural attenuation processes of PAH contamination in marine sediments and that several prokaryotic taxa belonging to different classes, besides Gammaproteobacteria that include known hydrocarbonoclastic bacteria (e.g. *Alcanivorax spp.*, *Cycloclasticus spp.;* Yakimov et al., 2005), may be actively involved in PAH degradation, potentially through syntrophic and mutualistic interactions between degrading and non-degrading oil bacteria (Head et al., 2006) and/or biotransformation processes through co-metabolism (Boopathy, 2000; Leahy & Colwell, 1990).

3.3.3 Isolation and identification of bacterial and fungal taxa with a high biodegradation performance

The experiments carried out in the present study allowed us also to isolate different bacterial and fungal taxa using specific substrates, which, once obtained as pure cultures, where taxonomically identified by molecular approaches based on the amplification of 16S rDNA and ITS1 regions and sequencing through Sanger method. Overall, 53 bacterial strains (Table 3.3.3.1) and 32 fungal strains (Table 3.3.3.2) were isolated and taxonomically identified at the lowest resolution level from the sediments used for the different experiments. Four bacterial strains were isolated from control samples of station 15, whereas all the other bacterial strains were isolated from sediment samples of station 15 and 36 spiked with a mix of PAHs. Twelve of fungal strains were isolated from sediments of stations 15 used as controls, and 17 from sediments of stations 36 and 15 spiked with PAHs.

Table 3.3.3.1. List of the bacterial strains isolated and taxonomically identified from the different experimental systems.

Experimental system	Bacterial identification number ID	Taxonomic identification (Sanger)
ST15 CTRL R1	5	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Vibrionaceae;Vibrio;
ST15 CTRL R1	7a	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Nitrincolaceae;Marinobacterium;
ST15 CTRL R1	7b	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 CTRL R1	8	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R1	63	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Vibrionaceae;Photobacterium;
ST15 +nut +IPAx3 R1	64	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Vibrionaceae;Photobacterium;
ST15 +nut +IPAx3 R1	65	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R1	66a	Bacteria;Firmicutes;Bacilli;Bacillales;Planococcaceae;Lysinibacillus;
ST15 +nut +IPAx3 R1	66b	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R2	67	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales; Rhodobacteraceae;Epibacterium;
ST15 +nut +IPAx3 R2	68	Low quality
ST15 +nut +IPAx3 R2	69	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPxA3 R2	70	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R2	71	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Aeromonadaceae;Oceanisphaera;
ST15 +nut +IPAx3 R2	72	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R3	73a	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Aeromonadaceae;Oceanisphaera;
ST15 +nut +IPAx3 R3	73b	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Marinobacteraceae;Marinobacter;
ST15 +nut +IPAx3 R3	74	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R3	75	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R3	76	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST15 +nut +IPAx3 R3	77	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx1 R1	78	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae;Halomonas;
ST36 +nut +IPAx1 R1	79	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae;Halomonas;
ST36 +nut +IPAx1 R1	80	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae;Halomonas;
ST36 +nut +IPAx1 R1	81	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae;Halomonas;
ST36 +nut +IPAx1 R2	82	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Oceanobacillus;
ST36 +nut +IPAx1 R2	83	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales; Rhizobiaceae;Nitratireductor;
ST36 +nut +IPAx1 R2	84	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;

ST36 +nut +IPAx1 R2	85a	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx1 R2	85b	Low quality
ST36 +nut +IPAx1 R2	86	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx1 R3	87	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Aeromonadaceae;
ST36 +nut +IPAx1 R3	88	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Aeromonadaceae;
ST36 +nut +IPAx1 R3	89	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx1 R3	90	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Aeromonadaceae;
ST36 +nut +IPAx1 R3	91a	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales; Rhizobiaceae;Nitratireductor;
ST36 +nut +IPAx1 R3	91b	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx3 R1	92	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Idiomarinaceae;Idiomarina;
ST36 +nut +IPAx3 R1	93a	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae;Halomonas;
ST36 +nut +IPAx3 R1	93b	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx3 R1	94a	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales; Rhizobiaceae;Nitratireductor;
ST36 +nut +IPAx3 R1	94b	Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacterales; Idiomarinaceae;Idiomarina;
ST36 +nut +IPAx3 R1	95	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx3 R2	96a	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales; Rhizobiaceae;Nitratireductor;
ST36 +nut +IPAx3 R2	96b	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx3 R2	97	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales; Rhizobiaceae;Nitratireductor;
ST36 +nut +IPAx3 R2	98a	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Bacillus;
ST36 +nut +IPAx3 R2	98b	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Marinobacteraceae;Marinobacter;
ST36 +nut +IPAx3 R2	99	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales; Rhizobiaceae;Nitratireductor;
ST36 +nut +IPAx3 R3	100a	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae; Bacillus;
ST36 +nut +IPAx3 R3	100b	Low quality
ST36 +nut +IPAx3 R3	101	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae; Halomonas;
ST36 +nut +IPAx3 R3	102	Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales; Halomonadaceae; Halomonas;

Table 3.3.3.2. List of the fungal strains isolated and taxonomically identified from the different experimental systems.

Experimental system	Fungal identification number (ID)	Taxonomic identification (Sanger)
ST15 CTRL R1	1	Trichocomaceae
ST15 CTRL R1	2	Trichocomaceae
ST15 CTRL R1	3	Microascaceae
ST15 CTRL R2	4	Piskurozymaceae
ST15 CTRL R2	5	Arachnomycetaceae
ST15 CTRL R2	6	Plectosphaerellaceae
ST15 CTRL R2	7	Trichocomaceae
ST15 CTRL R2	8	Microascaceae
ST15 CTRL R3	9	Phaeosphaeriaceae
ST15 CTRL R3	10a	Trichocomaceae
ST15 CTRL R3	10b	Chaetomiaceae
ST15 CTRL R3	11	Trichocomaceae
ST15 +nut +IPAx3 R1	32	Chaetomiaceae
ST15 +nut +IPAx3 R1	33	Microascaceae
ST15 +nut +IPAx3 R1	34	Hypocreales*
ST15 +nut +IPAx3 R1	35	Hypocreaceae
ST15 +nut +IPAx3 R2	36	Hypocreales*
ST15 +nut +IPAx3 R2	37	Low quality
ST15 +nut +IPAx3 R2	38	Hypocreaceae
ST15 +nut +IPAx3 R2	39	Hypocreaceae
ST15 +nut +IPAx3 R2	40	Sporormiaceae
ST15 +nut +IPAx3 R3	41	Piskurozymaceae
ST15 +nut +IPAx3 R3	42	Myxotrichaceae
ST15 +nut +IPAx3 R3	43	Hypocreales*
ST15 +nut +IPAx3 R3	44	Hypocreaceae
ST15 +nut +IPAx3 R3	45	Cordycipitaceae
ST15 +nut +IPAx3 R3	46	Trichocomaceae
ST15 +nut +IPAx3 R3	47	Plectosphaerellaceae
ST15 +nut +IPAx3 R3	48	Hypocreaceae
ST36 +nut +IPAx1 R3	49	Myxotrichaceae
ST36 +nut +IPAx1 R3	50	Hypocreaceae
ST36 +nut +IPAx3 R1	51	Hypocreaceae

The bacterial and fungal strains isolated in the present study are affiliated to family/genera comprising known taxa able to degrade hydrocarbons (Dell' Anno et al., 2021; Liu et al., 2017). As such, these strains are expected to be suitable candidates to be used for enhancing bioremediation performance of highly contaminated marine sediments through bioaugmentation strategies.

Chapter 4

Microbial consortia from low polluted coastal areas can be highly efficient in the biodegradation of polycyclic aromatic hydrocarbons in highly contaminated marine sediments

4.1 Introduction

Pollutants, such as heavy metals and metalloids and polycyclic aromatic hydrocarbons (PAHs) enter into the marine environment through multiple sources, including improper industrial discharges or waste disposal practices, atmospheric deposition and continental runoff. Such compounds can accumulate in high concentrations in marine sediments especially of coastal areas characterized by high anthropogenic pressure and low hydrodynamic regimes, posing a great concern for ecosystem and human health and demanding effective remediation interventions (Dell'Anno et al., 2020).

Various solutions based on chemical and electrochemical strategies have been developed for the remediation of contaminated marine sediments. Unfortunately, these methods have several disadvantages such as high costs and environmental impact, especially if applied *in situ*. For such reasons, international policies (e.g., the European Marine Strategy Framework Directive) are increasingly seeking alternative solutions limiting sediment handling interventions and promoting the decontamination of these matrices by using eco-compatible in situ technologies. Bioremediation strategies employing microorganisms for the remediation of contaminated environmental matrices are a promising alternative. Indeed, their efficiency in reducing contamination levels is noteworthy, together with their versatility to be used with different types

of contaminants and in different environmental contexts. Bioremediation mechanisms occur under aerobic or anaerobic conditions. The degradation of organic pollutants involves aerobic/anaerobic respiration and fermentative metabolism while transformation/sequestration of heavy metals (which do not undergo degradation) are based on bioaccumulation, biotransformation, and bioleaching activities (Fonti et al., 2015 and references therein). Generally, bioremediation processes can be enhanced by biostimulation of autochthonous assemblages (e.g., by adding different chemical compounds and or electron donors/acceptors) or by bioaugmentation, which consists of adding selected microorganisms capable of degrading or mobilizing contaminants.

Microbial taxa potentially beneficial for the bioremediation of contaminated sediments can be originally from the same area or can be isolated from other contaminated areas (Dell'Anno et al., 2020; Dell'Anno et al., 2021). The use of autochthonous microorganisms is expected to be more effective and ecologically friendly, since these are likely better adapted to the specific local environmental conditions than allochthonous microbes, which may require the manipulation of the natural environment to maximize their performance (e.g., changing oxygen and/or nutrient concentration; Garbisu et al., 2017). Among bacteria involved in bioremediation processes, most belong to the genera *Alcaligenes*, *Achromobacter*, *Acinetobacter*, *Alteromonas*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Flavobacterium*, *Pseudomonas* (Ojuederie and Babalola, 2017; Xu et al., 2018). Moreover, genera such as *Alcanivorax*, *Marinobacter*, *Thallassolituus*, *Cycloclasticus*, and *Oleispira* include hydrocarbonoclastic bacteria (OHCB) and are known for their specific ability to degrade hydrocarbons (Yakimov et al., 2007).

Besides bacteria, fungi have been described for their ability to produce particular enzymes (e.g., catalases, peroxidases, laccases) able to degrade organic contaminants and/or to immobilize inorganic contaminants (Durairaj et al., 2015; Morel et al., 2013). Fungi belonging to the genera

Aspergillus, *Curvularia*, *Drechslera*, *Fusarium*, *Lasiodiplodia*, *Mucor*, *Penicillium*, *Rhizopus*, *Trichoderma* were reported as able to degrade aromatic hydrocarbons (Balaji et al., 2014; Lladó et al., 2013). However, most of these studies were carried out to test the ability of the different microbial taxa to degrade hydrocarbons, rather than to investigate the performance of microbial consortia for the decontamination of polluted marine sediments.

The aim of the present study was to test bioremediation approaches based on bioaugmentation using different microbial strains isolated from relatively unpolluted coastal areas, but displaying high degradation capacity towards PAHs, in order to improve the efficiency of removal of such compounds from highly contaminated sediments collected in the Bagnoli-Coroglio bay (Southern Tyrrhenian Sea, Mediterranean Sea). The tested treatments were based on: (i) inocula of a bacterial consortium, (ii) inocula of a fungal consortium, (iii) inocula of both the abovementioned bacterial and fungal consortia. All these treatments were designed hypothesizing that microbial consortia having a wide range of degradation enzymes than single microbial taxa can enhance biodegradation performance also of the most refractory PAH congeners represented by high molecular weight compounds. Moreover, we tested potential changes induced by bioaugmentation strategies on metal mobility, as well as the overall effects of the bioaugmentation treatments on the autochthonous microbial assemblages.

4.2. Material and Methods

4.2.1 Study area and sediment sampling

Sediments sampling was carried out in the Bagnoli-Coroglio, located within the Gulf of Naples (Southern Tyrrhenian Sea). The Bagnoli-Coroglio is listed among the Italian contaminated Sites of National Interest for the high levels of chemical contamination due to industrial activities. Such industrial activities in the Bagnoli-Coroglio area, mainly represented by a steel plant using

fossil coal, iron and limestone, began in 1905 and ended at the beginning of nineties when the plant was dismissed (De Vivo & Lima, 2008; Qu et al., 2018). Previous investigations carried out in marine sediments in front of the plant report very high concentrations of different metals and PAHs due to the industrial activities (Albanese et al., 2010; Arienzo et al., 2017; De Vivo & Lima, 2008; Romano et al., 2009; Romanoet al., 2018; Trifuoggi et al., 2017). Sediment samples (from the sediment surface down to 50 cm depth) were collected in November 2017 in front of the industrial plant using a vibro-corer. After collection, sediment samples were homogenised, and sub-samples were collected for the analysis of polycyclic aromatic hydrocarbon and heavy metal concentrations, as well as of metals associated with different geo-chemical phases.

4.2.2 Microorganisms used for bioaugmentation experiments

Bacteria and fungi have been isolated and taxonomically identified through Sanger sequencing from relatively unpolluted sediments of Falconara Marittima (Central Adriatic Sea), included in the list of the National Remediation Sites. Overall, 53 bacterial and 32 fungal strains were isolated, from autochthonous microbial assemblages characterized by high natural attenuation capacity and biodegradation activities toward PAHs (see chapter 3). These microbial strains were further screened for their ability to growth in the presence of high concentrations of PAH and metals characterizing the Bagnoli-Coroglio sediments. In particular, 50 bacterial strains and 8 fungal strains were selected for carrying out bioaugmentation experiments. In particular, for bioaugmentation experiments, we used a consortium of bacterial strains belonging to 7 different orders, a consortium of fungal strains affiliated to 7 different orders and a mixed consortium of such bacterial and fungal strains (Table 4.2.2.1).

Table 4.2.2.1 Taxonomic details and number of bacterial and fungal strains isolated from Falconara sediment and utilized for bioaugmentation experiment.

Bacterial pool (n. strains used for each taxon)	Fungal pool (n. strains used for each taxon)
Aeromonadaceae (5)	Eurotiales (1)
Alteromonadales (2)	Filobasidiales (2)
Bacillales (22)	Glomerellales (1)
Enterobacterales (3)	Hypocreales (1)
Pseudomonadales (8)	Microascales (1)
Rhizobiales (9)	Onygenales (1)
Rhodobacterales (1)	Pleosporales (1)

4.2.3. Experimental procedures

The bacterial and fungal strains were grown up on specific media (marine agar broth for bacteria and YPD containing rifampicin to avoid bacterial contamination for fungi) up to reach a sufficient biomass. For bioaugmentation experiments, a ca. equal biomass of the different consortia to that present in the sediments of Bagnoli-Coroglio used was inoculated. Bioaugmentation experiments were performed in 500 mL Erlenmeyer flasks containing 150 g wet sediment samples and 75 mL

pre-filtered 0.2 μm and autoclaved seawater. Replicate microcosms were supplied separately with 2 mL of marine agar broth containing the bacterial consortium, 2 mL of 10% glucose, 1% yeast extract, 0.25% $(NH_4)_2SO_4$ and 0.15% K_2HPO_4 containing the consortium with the fungal spores and 2 mL of a mixed culture of bacterial and fungal consortia. Additional microcosms prepared in the same way, but without addition of bacterial and/or fungi, were used as controls. Since sediments of Falconara Marittima were characterized by high attenuation capacity of the autochthonous microbial assemblages toward PAHs, additional microcosms experiments were carried out by mixing 15 g of wet sediments from Falconara Marittima with 135 g wet sediments collected in the Bagnoli-Coroglio bay (ratio 1 to 10 w:wt) in the presence of 75 mL

of pre-filtered 0.2 μm and autoclaved seawater. All flasks were incubated at a constant temperature of 20 °C for 1 month. Subsamples were collected at the beginning of the experiment (T0), after 15 days (T1) and after 1 month (T2) for the determination of PAH concentrations, prokaryotic abundance and microbial diversity and heavy metal concentrations and metals associated with different geo-chemical phases at the beginning (T0) and at the end of the experiment (1 month).

4.2.4 Polycyclic aromatic hydrocarbon and heavy metal determinations

Polycyclic aromatic hydrocarbons (PAHs) were extracted from the sediment samples according to 3545A EPA method and analyzed by gas chromatography-mass spectrometry (GC-MS; EPA 8270D). Twenty different congeners were analyzed including naphthalene, acenaphthene, fluorene, acenaphthylene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3,-cd]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i] perylene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i] pyrene and dibenzo[a,l]pyrene. Total PAH concentrations were obtained by the sum of the concentrations of these 20 congeners.

Heavy metal content in the sediment was determined after acid digestion, as follows: dried sediment sub-samples were transferred in Teflon boxes, added with 5 mL fluoridric acid and 1 mL of ‘‘aqua regia” (i.e. HCl:HNO_3 = 3:1) incubated for 90 min at 150 °C. At the end of the incubation period, 5 mL of 10% boric acid were added and the obtained extracts were analysed by atomic absorption spectrophotometry and by inductively coupled plasma-atomic emission spectrometry. Metals distribution in different mineralogical fractions were determined by means of a selective extraction procedure, which utilized, sequentially, specific chemical reagents to extract heavy metals associated with different geochemical phases (Quevauviller,

1998). Four different fractions are considered: (i) the exchangeable and carbonate bound fractions (hereafter defined as exchangeable fraction), extracted utilizing 0.11 M acetic acid, pH 2.8; (ii) iron and manganese oxides fraction (i.e., reducible

fraction), extracted with 0.1 M NH_2OH, pH 2; (iii) organic and sulfide fraction (i.e., oxidizable fraction), extracted by hydrogen peroxide 30% and treated with ammonium acetate at pH 2, and (iv) the residual fraction, that remains in the residual solid, is determined by difference with the total metal content.

4.2.5 Prokaryotic abundance analysis

For the determination of the prokaryotic abundance, sediment slurries (2.5 mL) were treated with pyrophosphate (5 mM final concentration) and ultrasound (three times for 1 min; Roberto Danovaro, Manini, & Dell'Anno, 2002). For prokaryotic counts, sub-samples (1 mL) were diluted 50–100 times, stained with SYBR Green (0.01% final concentration) and filtered on Anodisc 0.2 μm pore size filters. Filters were analyzed under epifluorescence microscopy using a Zeiss Axioplan microscope equipped with a 50-W lamp. Ten to 50 fields were viewed at 1000x magnification and a minimum of 400 cells were counted. Prokaryotic counts were normalized to sediment dry weight after desiccation (60 °C, 24 h).

4.2.6 Microbial diversity analysis

DNA has been extracted from two replicates of sediment sub-samples collected during the time-course experiments. The preliminary step, before DNA extraction, consisted in the removal of extracellular DNA based on three washing solutions (WS) at different concentration: WS1, 50 mM Tris-HCL, pH 8.3; 200 mM NaCl; 5 mM Na2EDTA; 0,05% Triton X-100; WS2, 50 mM Tris-HCL, pH 8.3; 200 mM NaCl; 5 mM Na2EDTA; WS3, 10 mM Tris-HCL, pH 8.3; 0,1 mM

Na2EDTA. The subsequent DNA extraction was performed utilizing the DNeasy PowerSoil Kit, QUIAGEN, following the manufacture's procedure. Sequencing libraries were prepared from each replicate and sequencing was performed with all libraries on an Ion Torrent chip (using the in-house facilities provided by Stazione Zoologica di Napoli "Anton Dohrn") using the primer pair 515F–Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'; Parada et al., 2016; Corinaldesi et al., 2019) for prokaryotes and the primer pair ITS3F (5′- GCATCGATGAAGAACGCAGC-3′) and ITS4R (5′- TCCTCCGCTTATTGATATGC-3′) for fungi (Nilsson et al., 2019).

After primer removal through the cutadapt plugin within QIIME2 (Martin, 2011; Bolyen et al., 2019), trimmed reads from 16s sequencing libraries were submitted to the DADA2 pipeline within QIIME2 (Callahan et al., 2016; Bolyen et al., 2019) using the *denoise-pyro* plugin over a length of 250 bp (Callahan et al,. 2016); ITS sequencing libraries were subjected to trimming by means of the ITSxpress tool (Rivers et al., 2018) to only keep the fraction of sequence within the ITS2 region before being submitted to the DADA2 pipeline within QIIME2 (Callahan et al., 2016; Bolyen et al., 2019) using the *denoise-pyro* plugin without length trimming. Taxonomic inference was performed through the *classify-consensus-vsearch plugin* (Rognes et al., 2016) using the SILVA database (v138; Quast et al., 2013) for prokaryotes and the UNITE database for ITS sequences (Nilsson et al., 2019). For the taxonomic affiliation of ASVs, a reference database was first created within QIIME2 by trimming both databases to the region amplified by sequencing primers. A first overview of the taxonomic structure of both assemblages was obtained after clustering ASVs in modules of co-occurring sequences through the SCNIC plugin (Shaffer et al., 2020) after normalizing the ASV table to 4000 sequences for 16s libraries and 27000 sequences for ITS libraries. Differences in assemblage structure were analysed through the *adonis2* procedure in the *vegan* R package. ASVs from the non-

normalized tables were then removed when not belonging to at least 2 samples and with less than 100 sequences before running a second clustering to identify ASVs co-occurring into modules through the SCNIC tool. To test for the variation in module abundances over time, module abundances were centered log-ratio (CLR) transformed to account for compositionality (Gloor et al., 2017) and detrended over time using the *pracma* R package (Faust et al., 2015) before being submitted to the Rhythmicity Analysis Incorporating Non-parametric methods (RAIN; Thaben & Westermark, 2014) only keeping modules with significant (p-value < 0.05) and marked (log-transformed values <-5 and >5) periodicity (Coenen et al., 2020). ITS and 16S sequences obtained from the strains utilized in the bioaugmentation experiment were trimmed to the ITS2 region and to the region sequenced by NGS primers and then aligned to the ASVs identified in the present study through the MAFFT tool to identify the closest ASVs within our dataset, as a proxy to check for the abundance of inoculated strains during the experiment. The abundance of these ASVs was tracked over time and treated as above to identify the temporal trends of the inoculated strains.

4.3 Results and discussion

4.3.1 Biodegradation of PAHs and effects on metal mobility due to bioaugmentation treatments

Sediments collected in the present study for the bioremediation experiments were characterized by high concentrations of both PAHs and heavy metals, confirming previous findings obtained in the Bagnoli-Coroglio bay (Mercogliano et al., 2016; Qu et al., 2018; Romano et al., 2009). PAHs were largely dominated by high-molecular weight (HMW) compounds (accounting for >90% of the total PAH concentrations) with concentrations largely exceeding the threshold

levels established by the Italian law for the management of contaminated marine sediments. Overall, on the basis of the chemical hazard quotient (Regoli et al., 2019), the sediment used for bioremediation experiments was classified as severely contaminated.

The different microbial consortia utilized in the present study were highly efficient in the biodegradation of both low and high molecular weight PAHs, allowing to significantly reduce total PAH contamination after one month of incubation (Figure 4.3.1.1). The degradation yields of PAHs due to the addition of the different microbial consortia were rather similar and much higher than that previously reported for Bagnoli sediment treated with a single fungal taxon belonging to Aspergillus sp. (Dell'Anno et al., 2020). These findings indicate that bioaugmentation using microbial consortia (i.e., different fungi and bacteria taxa) can improve significantly the biodegradation efficiency of PAHs in contaminated marine sediments (Mikesková et al., 2012). Previous findings reported that marine sediments can host microbial assemblages with high natural attenuation capacity toward hydrocarbons (i.e. ability to degrade hydrocarbons over time; Dell'Anno et al., 2012; Dell'Anno et al., 2020). This was the case of the Falconara sediments, where the total PAH concentrations significantly decreased over time. Conversely, the autochthonous microbial assemblages of the Bagnoli sediments were inefficient in the degradation of PAHs whose concentrations remained stable over time. In such a case, the application of biostimulation or bioaugmentation strategies are required to promote degradation processes of PAHs. Mixing the sediments of Falconara and Bagnoli determined only a slight decrease of PAH concentrations suggesting that the combination of the two matrices in the tested proportions and related microbial assemblages contained therein is not a useful strategy for achieving remediation targets over a relatively short time scale. Overall, these results suggest that biotreatments based on selected bacterial and/or fungal strains or a combination of both could be an effective strategy to significantly reduce in a relatively short

time PAH contamination of marine sediments, possibly leading to alternative management options compared to dredging and landfill disposal.

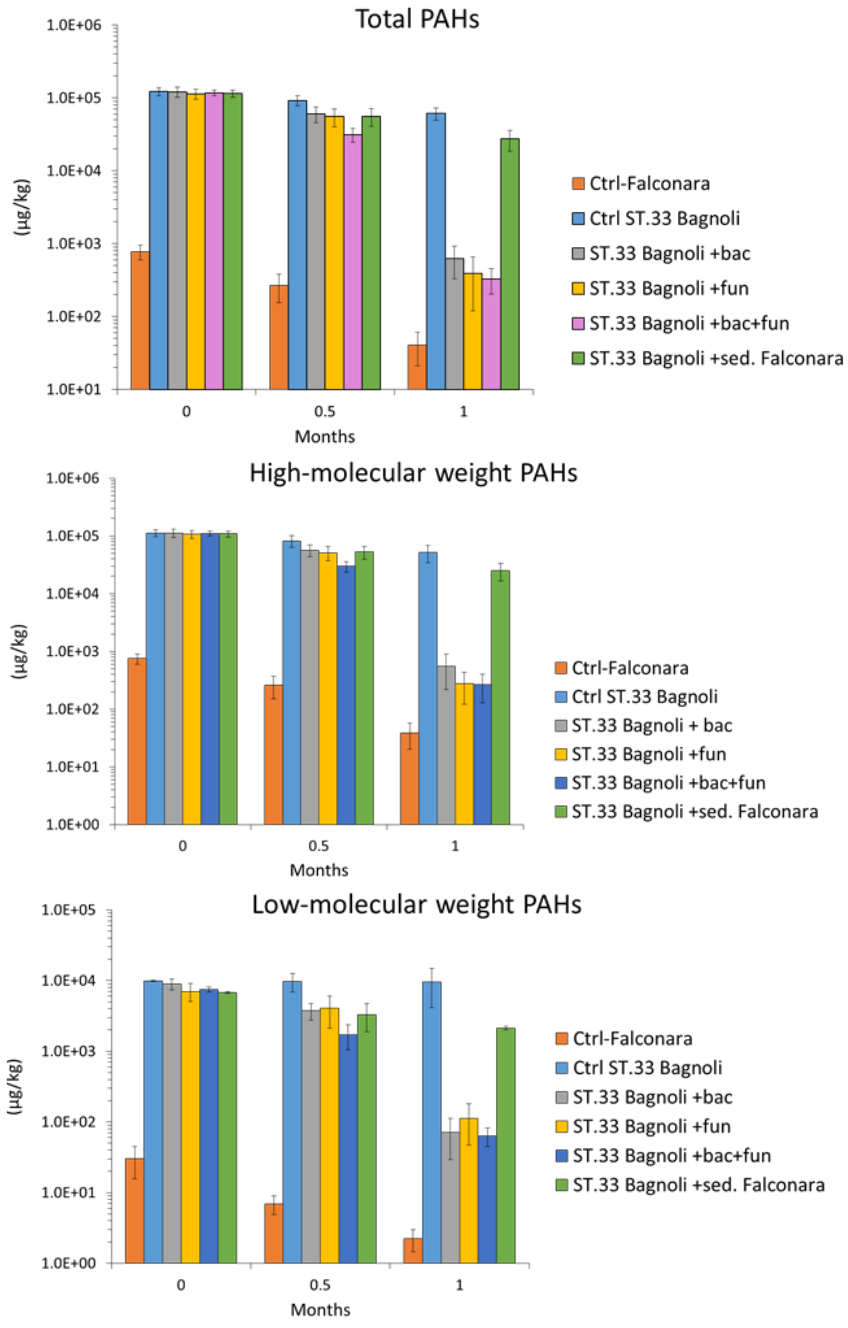

Figure 4.3.1.1. Temporal changes of total, low molecular wight and high molecular weight PAHs concentrations during bioaugmentation experiments carried out using the different microbial consortia. Values of PAHs in y-axis are in $LOG_{(10)}$ scale.

Increasing evidence suggests that biotreatments based on nutrient addition (both organic and inorganic) of contaminated sediments can determine significant changes in metal partitioning and mobility (Dell'Anno et al., 2009; Rocchetti et al., 2012; Fonti et al., 2015), but such an effect due to bioaugmentation still needs to be investigated (Dell'Anno et al., 2020).

Such aspect should be adequately considered for the ecotoxicological risk that may arise, especially if bioremediation approaches are applied *in situ*.

A previous study carried out on Bagnoli sediments reported major changes in the partitioning of metals, including an increase in the mobility of arsenic due to the addition of *Aspergillus sp*. (Dell'Anno et al., 2020). In the present study, no significant changes in the repartition of the heavy metals among the different geochemical fractions of the sediments were observed, independent of the biotreatment applied (Figure 4.3.1.2 and Figure 4.3.1.3). The lack of changes in the in the partitioning of metals observed in the present study can be due on one hand to the differences in the geochemical characteristics of the sediment analyzed and on the other hand, to potential interactions among the different microbial taxa used able to maintain the metals in their original repartition phases.

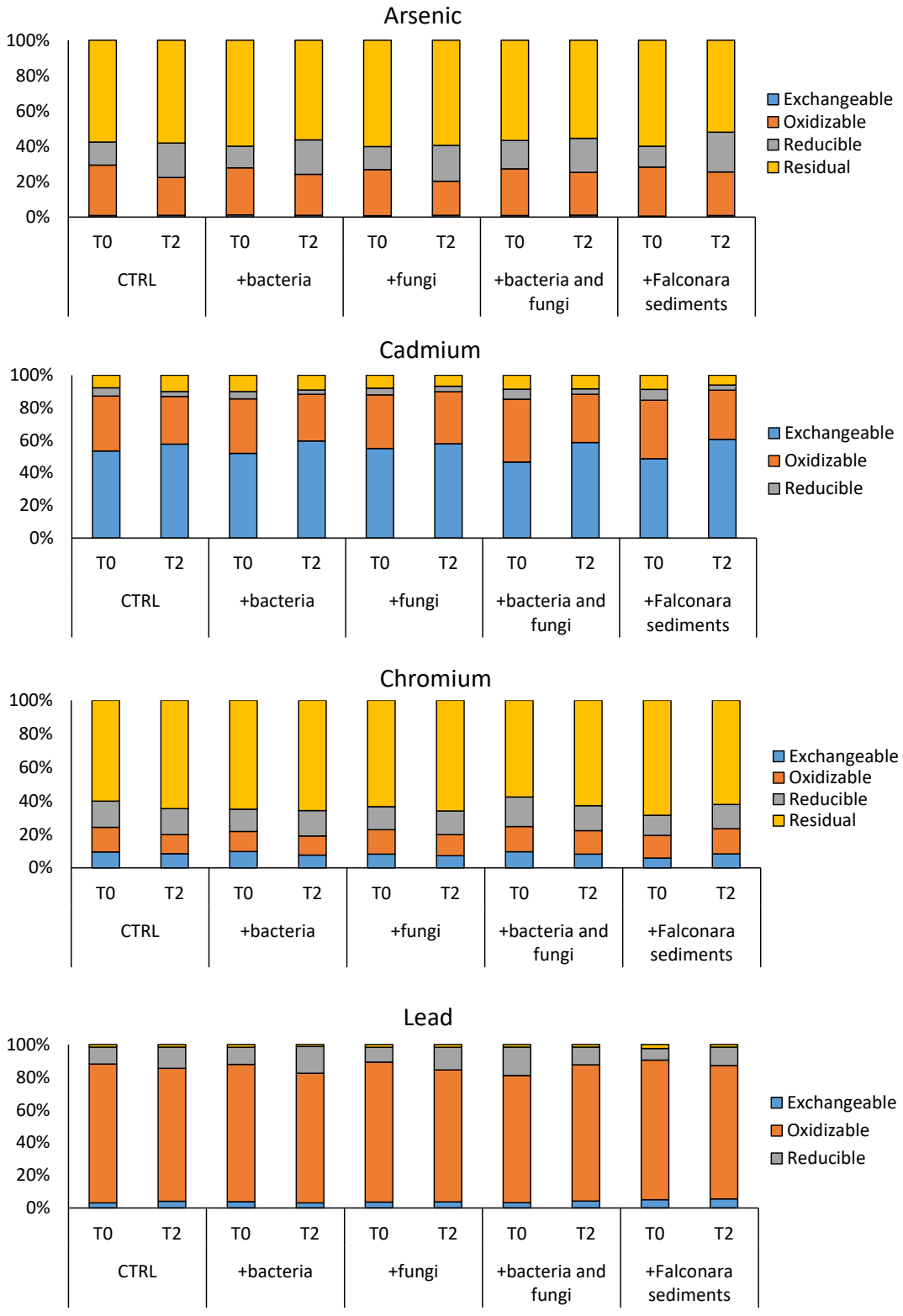

Figure 4.3.1.2. Repartition of As, Cd, Cr and Pb in the different geochemical fractions at the beginning and after 1 month of incubation of the Bagnoli sediments treated with the different microbial consortia.

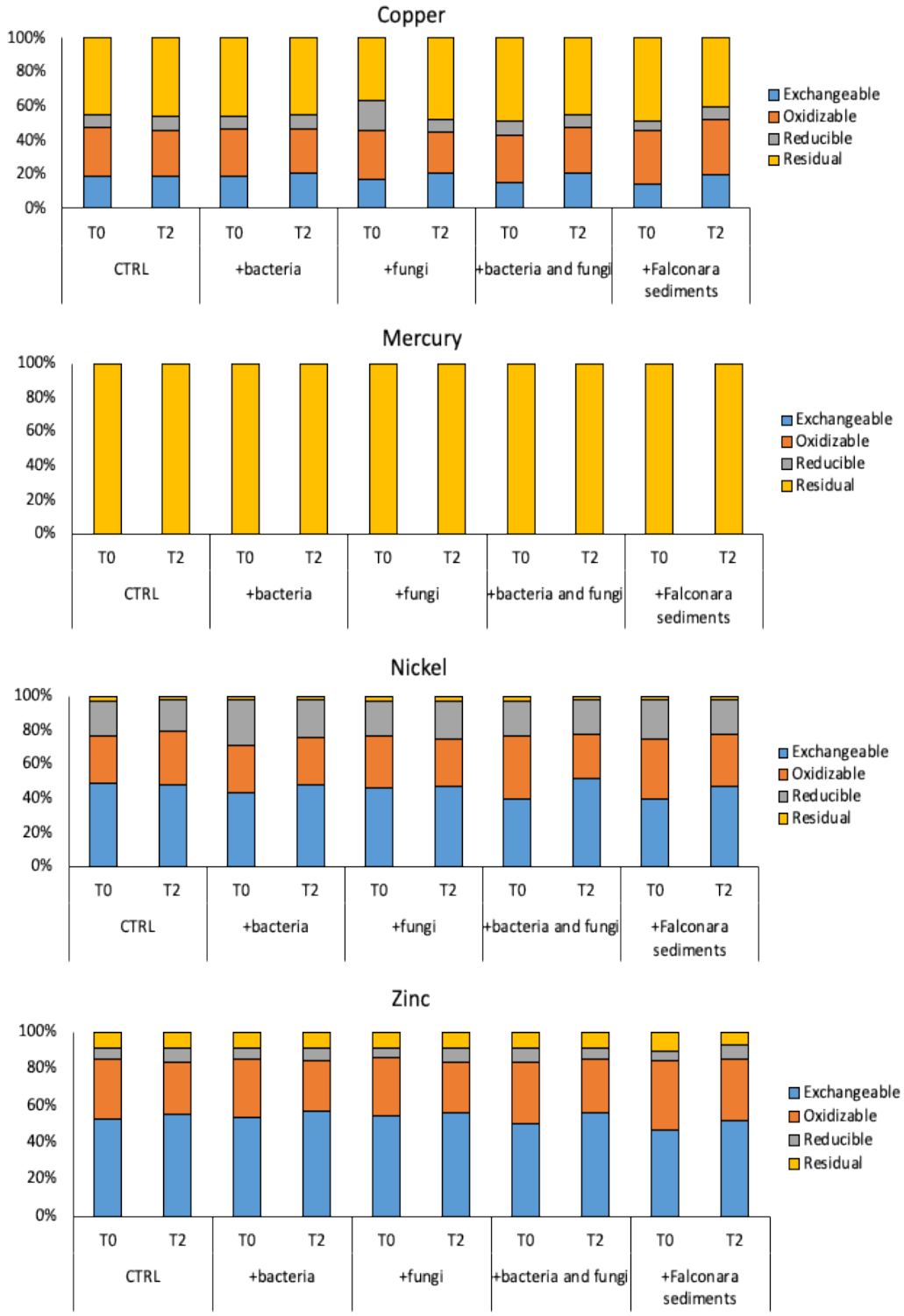

Figure 4.3.1.3. Repartition of Cu, Hg, Ni, Zn in the different geochemical fractions at the beginning and after 1 month of incubation of the Bagnoli sediments treated with the different microbial consortia.

4.3.2 Changes of prokaryotic abundance and microbial diversity due to biotreatments

The addition of the different bacterial taxa both alone and in combination with fungi in the sediments of Bagnoli did not determine significant changes of the overall benthic prokaryotic standing stocks (Figure 4.3.2.1). Thus, the increase of degradation of PAHs observed over time was independent by changes of prokaryotic standing stocks, conversely to what previously reported during biostimulation experiments based on nutrient addition of contaminated marine sediments (Beolchini et al., 2010; Dell'Anno et al., 2020).

At the same time, no significant differences were observed in the prokaryotic abundance among all sediments and treatments investigated.

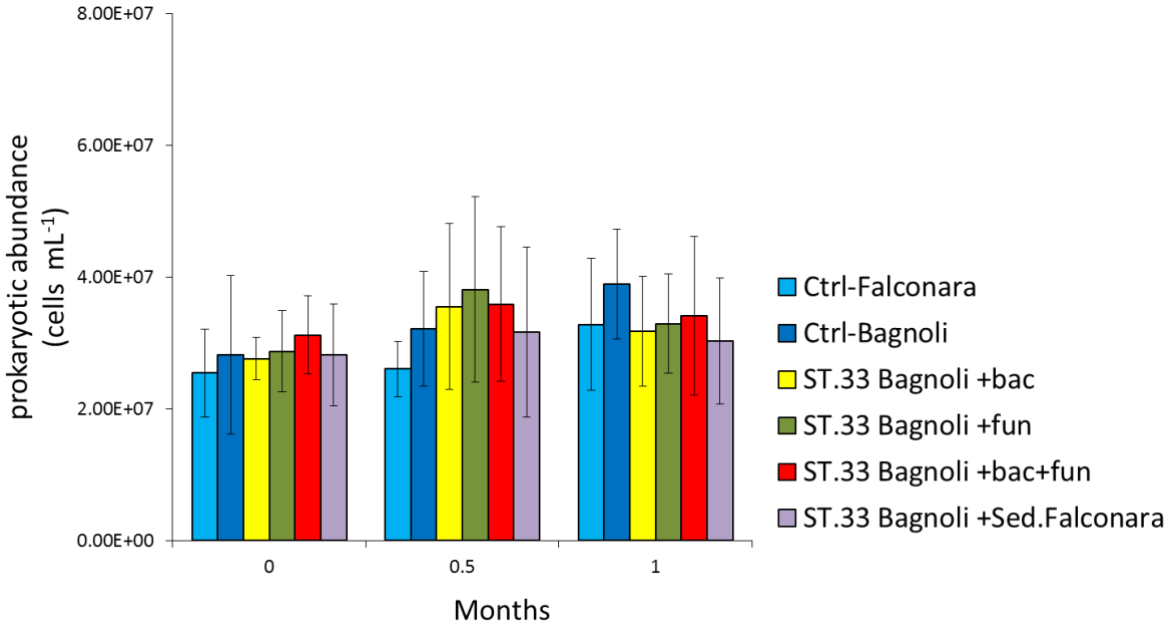

Figure 4.3.2.1. Temporal patterns of prokaryotic abundance in the sediments used for the different experiments.

Conversely to what observed for prokaryotic abundance, the analysis of prokaryotic diversity revealed notable differences between Falconara and Bagnoli sediments and among the different treatments and sampling intervals (Figure 4.3.2.2). At family level, no single taxon was shared

across the whole dataset and different prokaryotic taxa dominated in the different sample types and different sampling intervals. Statistical analyses highlighted that the variance of the prokaryotic assemblage composition was significantly explained by the treatment (explaining 20% of the variability), sampling station (explaining 10% of the variability) and time interval (explaining 8.2% of the variability). Sequences belonging to the family *Flavobacteriaceae* remarkably increased within bioaugmented samples of Bagnoli at T1 and T2, but not in the relative control.

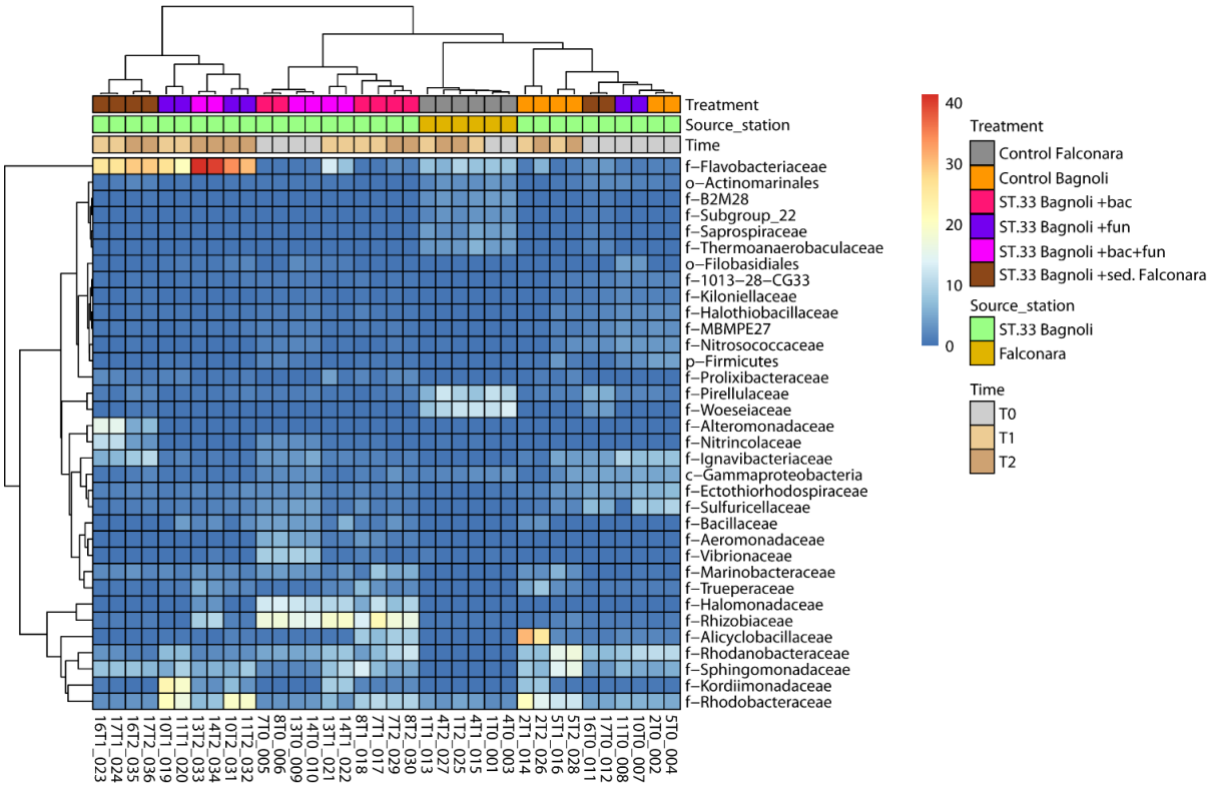

Figure 4.3.2.2. Changes of prokaryotic diversity at Family level during time-course experiment carried out in the present study.

The family *Flavobacteriaceae* includes both bacteria involved in the degradation and cycling of organic C, including hydrocarbons (Zouch et al., 2018), and thus likely involved in the degradation of PAH observed in the present study. Interestingly, high abundance of

Flavobacteriaceae were encountered in sediments amended with fungi, suggesting an association between fungi and members of this widespread prokaryotic family.

Taxonomic analysis of the fungal assemblages of the Bagnoli sediments highlighted the presence of sequences belonging to a limited number of taxa, mainly represented by *Piskurozymaceae* in samples inoculated by the fungal consortium and the mix of bacteria and fungi and by *Sympoventuriaceae* in samples not subjected to fungal addition. Interestingly, a high percentage of sequences found in the sediment of Falconara Marittima were not affiliated to known fungal taxa (Figure 4.3.2.3). Information about the relationships between fungi and bacteria in highly polluted marine sediments is lacking, and thus it is difficult to frame these results in a wider context. Despite this, our results suggest that members of the *Sympoventuriaceae* family are likely to represent a consistent fraction of the natural fungal assemblage within the Bagnoli sediments, while *Piskurozymaceae* (which were indeed isolated within this study) better respond to treatments and might also be associated with bacteria from the *Flavobacteriaceae* family (which were more abundant after fungal amendment).

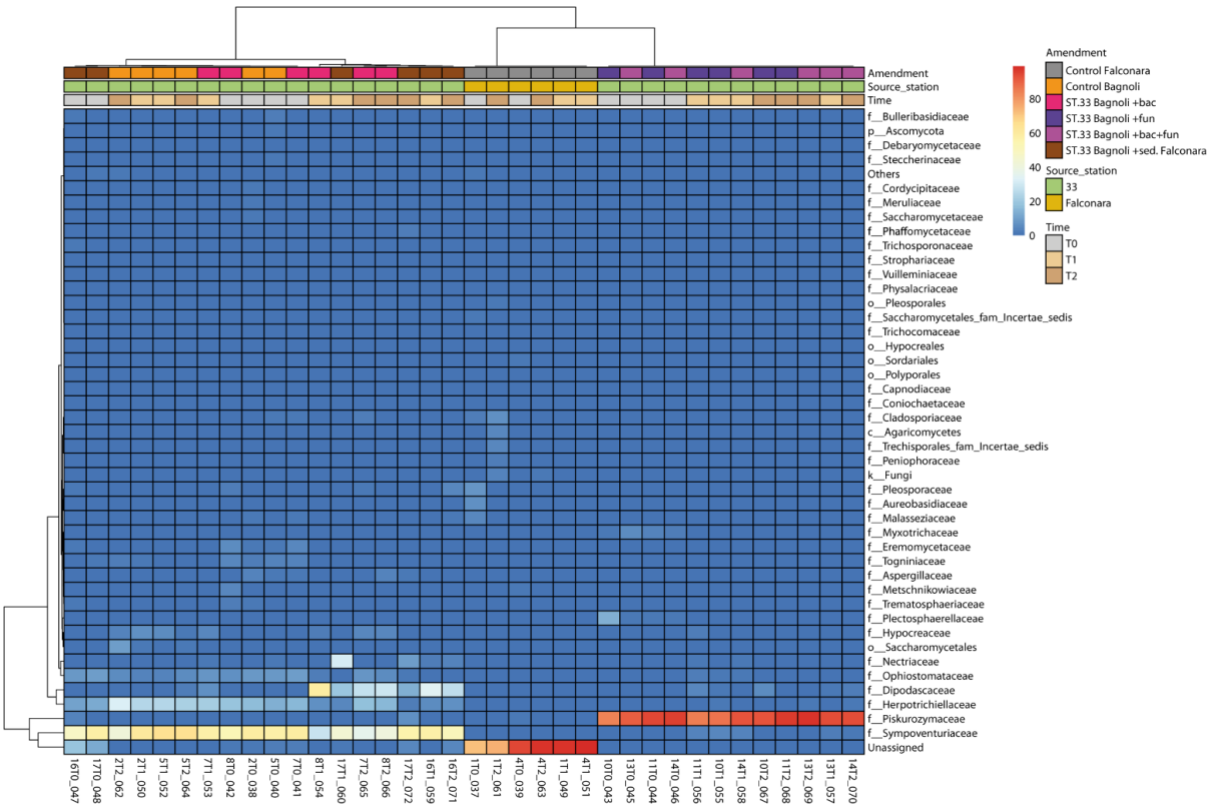

Figure 4.3.2.3. Changes of fungal diversity at Family level during time-course experiment carried out in the present study.

The analysis of co-occurring modules of prokaryotic ASVs allowed us to highlight the presence of potential prokaryotic consortia responsible of PAHs degradation in the sediment's samples subjected to bioaugmentation, and to further investigate the effect of different treatments on the prokaryotic assemblage composition.

In the Bagnoli sediments, the prokaryotic ASVs can be clustered in 64 different modules, 12 of which showing great level of variation. In particular, the abundance of modules 8 and 12 increased over time in the bioaugmented sediments, that of modules 4 and 10 significantly increased over time in the Bagnoli sediments when sediments from Falconara were added. Finally, module 13 showed an increase over time in the Bagnoli sediments added with the bacterial consortium (Figure 4.3.2.4).

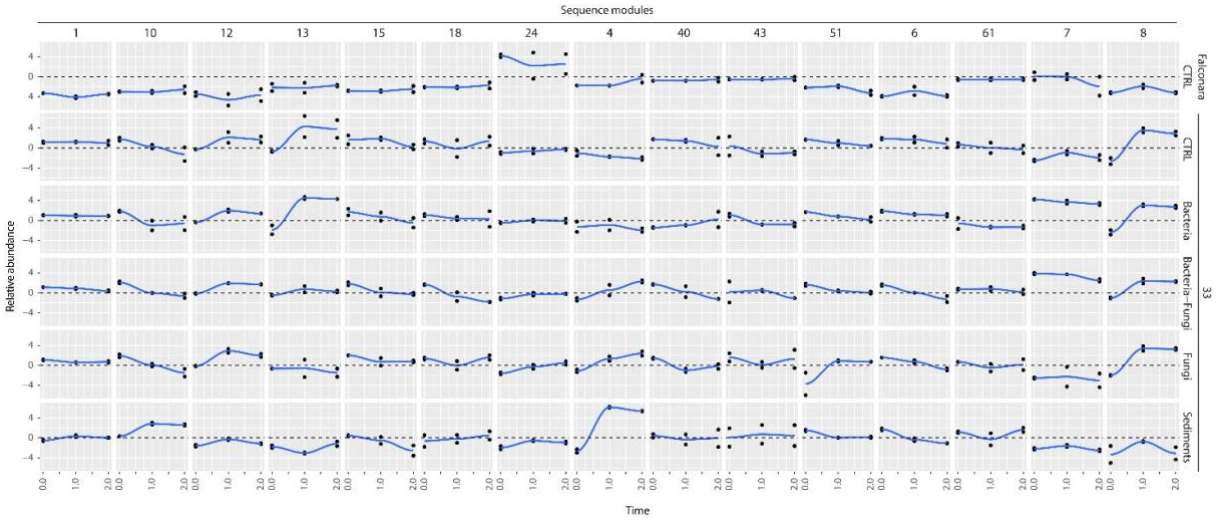

Figure 4.3.2.4**.** Relative abundances of each of the main prokaryotic co-occurring ASV modules in controls and treatments of the experiments carried out in the present study.

A detailed analysis of the main identified prokaryotic modules in bioaugmented sediments revealed that both modules 8 and 12 contained bacterial taxa mainly affiliated with *Acidihalobacteaceae*, *Holosporaceae*, *Hyphomicrobiaceae*, *Isosphaeraceae*, *Kordiimonadaceae*, *Mariprofundaceae*, *Prolixibacteraceae*, *Rhizobiaceae*, *Rhodobacteraceae*, *Sphingobacteraceae* and *Trueperaceae*. These taxa, whose relative contribution increase over time in all bioaugmentation treatments sediments, are metabolically diverse, including chemolitotrophic iron oxidizers (*Mariprofundaceae*, Moreira et al., 2014), PAH degraders (e.g. *Rhodobacteraceae*; Gutierrez et al., 2011) and acidophilic bacteria (Figure 4.3.2.5; Yang et al., 2016). Module 13 contained bacterial taxa mainly associated with *Alicyclobacillaceae*, *Bacillaceae*, *Desulfitobacteriaceae*, the latter comprising sulphate reducers encountered in anoxic conditions (Figure 4.3.2.5). Overall, these findings suggest that sediment bioaugmentation using microbial consortia can promote the development of a diversified bacterial assemblage, characterized by a wide range of metabolic functions, and that the module-based approach can be a useful strategy for the identification of microbial consortia

potentially able to cooperate for the degradation of complex mixtures of organic pollutants such as PAHs.

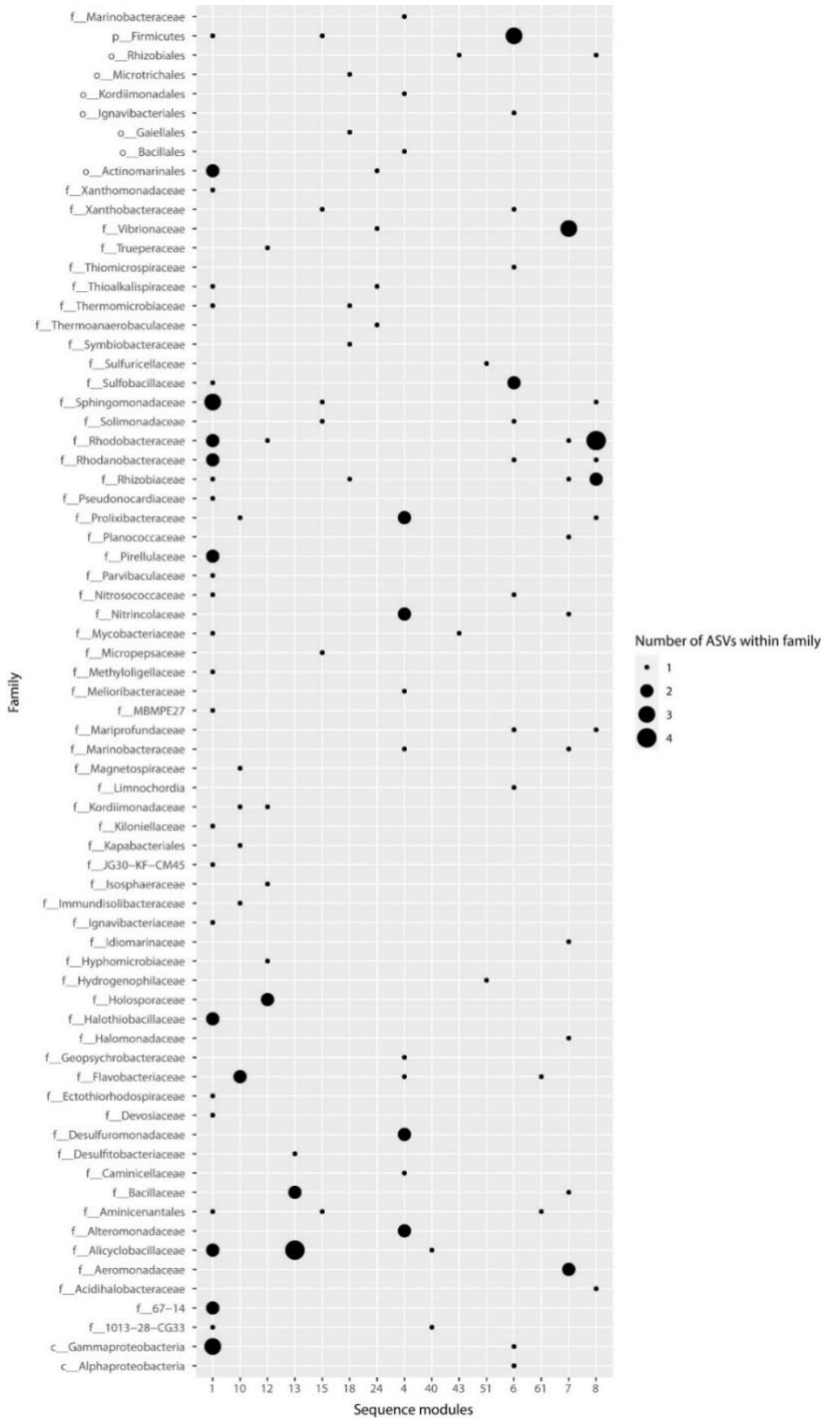

Figure 4.3.2.5. Taxonomic composition of each of the main modules of prokaryotic co-occurring ASVs identified in the present study.

In addition to the co-occurring modules of prokaryotic ASVs, we investigated the fungal co-occurring ASV modules, which, in the Bagnoli sediments, can be clustered in 68 different modules, 25 of which showing a great level of variation. In particular, sediments added with fungi and the mix of fungi and bacteria were characterized by a high relative abundance of fungal ASVs associated with the module 5, while fungal ASVs associated to module 26 appear to be stimulated by all bioaugmentation treatments; ASVs associated with modules 1 and 19 increased in abundance both in the control and after the addition of the bacterial consortium, but not in all the other biotreatments (Figure 4.3.2.6).

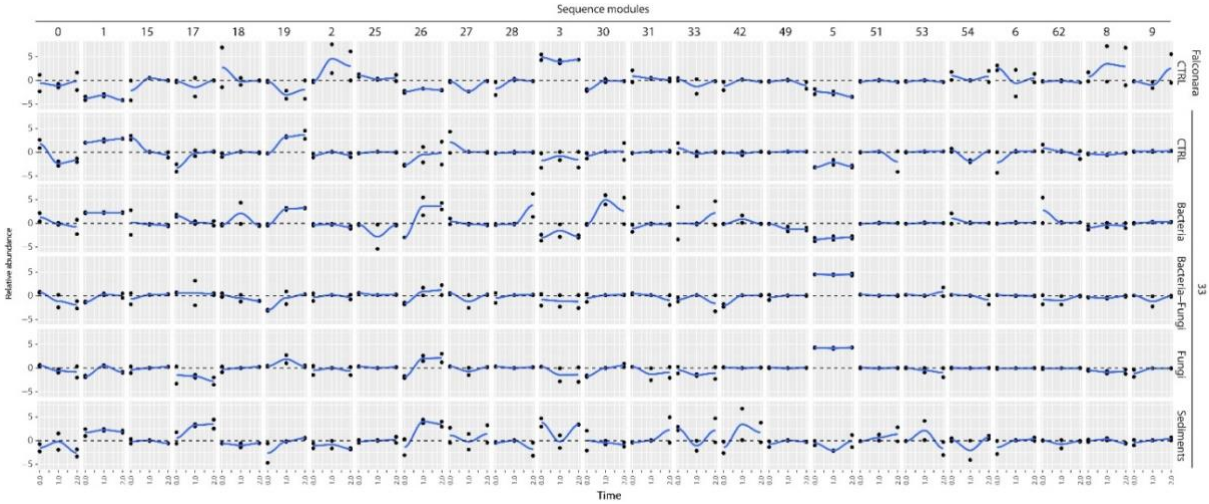

Figure 4.3.2.6**.** Relative abundances of each of the main fungal co-occurring ASV modules in controls and treatments of the experiments carried out in the present study.

Taxonomic analysis of the fungal modules revealed that module 5 was mainly represented by fungal taxa affiliated with *Piskurozymaceae*, *Sporormiaceae*, *Myxotrichaceae*, *Microascaceae* and *Aspergillaceae*, while module 26 showed only fungal ASVs related to *Sympoventuriaceae* and *Dipodascaceae* (Figure 4.3.2.7).

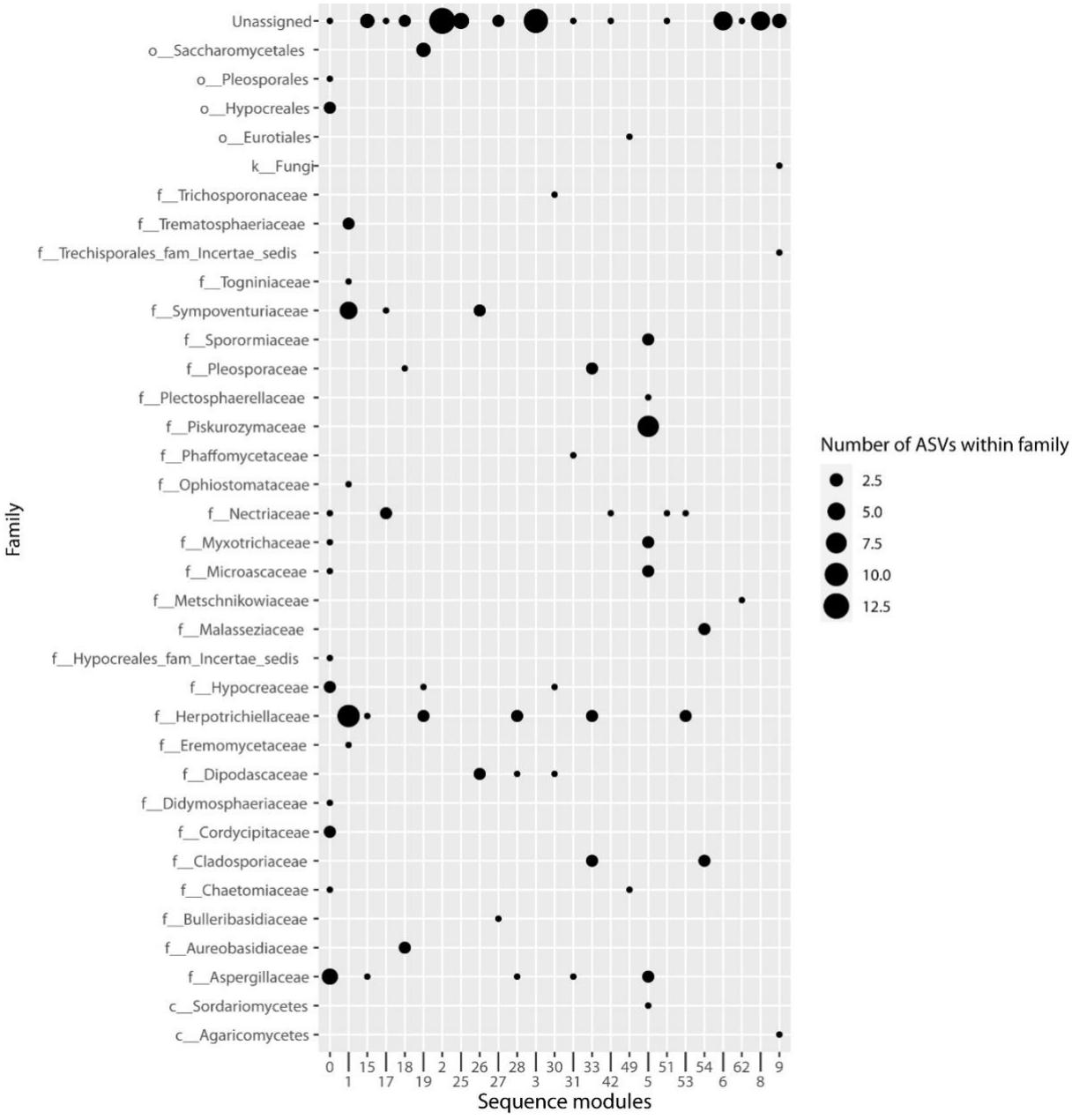

Figure 4.3.2.7**.** Taxonomic composition of each of the main modules of fungal co-occurring ASVs in controls and treatments of the experiments carried out in the present study.

Overall, despite the lack of information on the physiology of marine fungi in polluted marine ecosystems, data reported in the present study allowed to identify clusters of fungal taxa potentially able to degrade PAHs in contaminated marine sediments. The understanding of the interactions between fungi and prokaryotes and their implications on the degradation of organic

pollutants in contaminated marine ecosystems are still in their infancy (Dell'Anno et al., 2021) and require future research, but there are some preliminary evidence in contaminated soils that fungi and bacteria interactions may enhance the removal of organic pollutants exploiting the complex network of relationships between these two components (Jambon et al., 2018, and references therein; Ventorino et al., 2018).

4.3.3 Identification and monitoring of the prokaryotic and fungal taxa added to the sediments

To explore the potential direct role of the different microbial consortia added to the sediments in the biodegradation of PAHs, we compared bacterial and fungal ASVs obtained by NGS with sequences of 16S rDNA and ITS of the cultured bacterial and fungal strains. Such a comparison allowed to identify 22 prokaryotic and 16 fungal ASVs related to the strains inoculated in the sediments. It is worth to note that the number of fungal ASVs taxonomically identified by NGS is higher than that of fungal strains added to the sediments. This is likely due to the intra-species variability of the fungal ITS, which can lead to an overestimation of the actual number of fungal taxa added to the sediment.

Fourteen of the 22 prokaryotic ASVs related to the strains inoculated in the sediments were identified with confidence and were present at high abundance at the beginning of the time-course incubation experiments, but then strongly decreased over time (Figure 4.3.3.1a). The analysis carried out on the fungal ASVs related to the strains inoculated in the sediments revealed that 4 of 16 ASVs were identified with confidence and were present at high abundance at the beginning of the time-course incubation experiments and then remained rather constant over time (Figure 4.3.3.1b). Thus, these results suggest that the two allochthonous microbial components can respond differently once they are added to the sediments. The added bacterial strains might not tolerate the specific conditions present in the Bagnoli sediments and/or not

able to efficiently compete with the autochthonous microbial assemblages. Conversely, at least some of the added fungal strains might be able to survive and to adapt to the sediment conditions, with a higher ecological fitness when compared to other taxa.

Overall, despite findings reported in this study do not allow us to disentangle the relative importance of the allochthonous *vs.* autochthonous microbial taxa on the biodegradation of PAHs, they provide new insights on the potential of microbial consortia as a sustainable and eco-compatible bioremediation strategy for the reclamation of highly contaminated marine sediments.

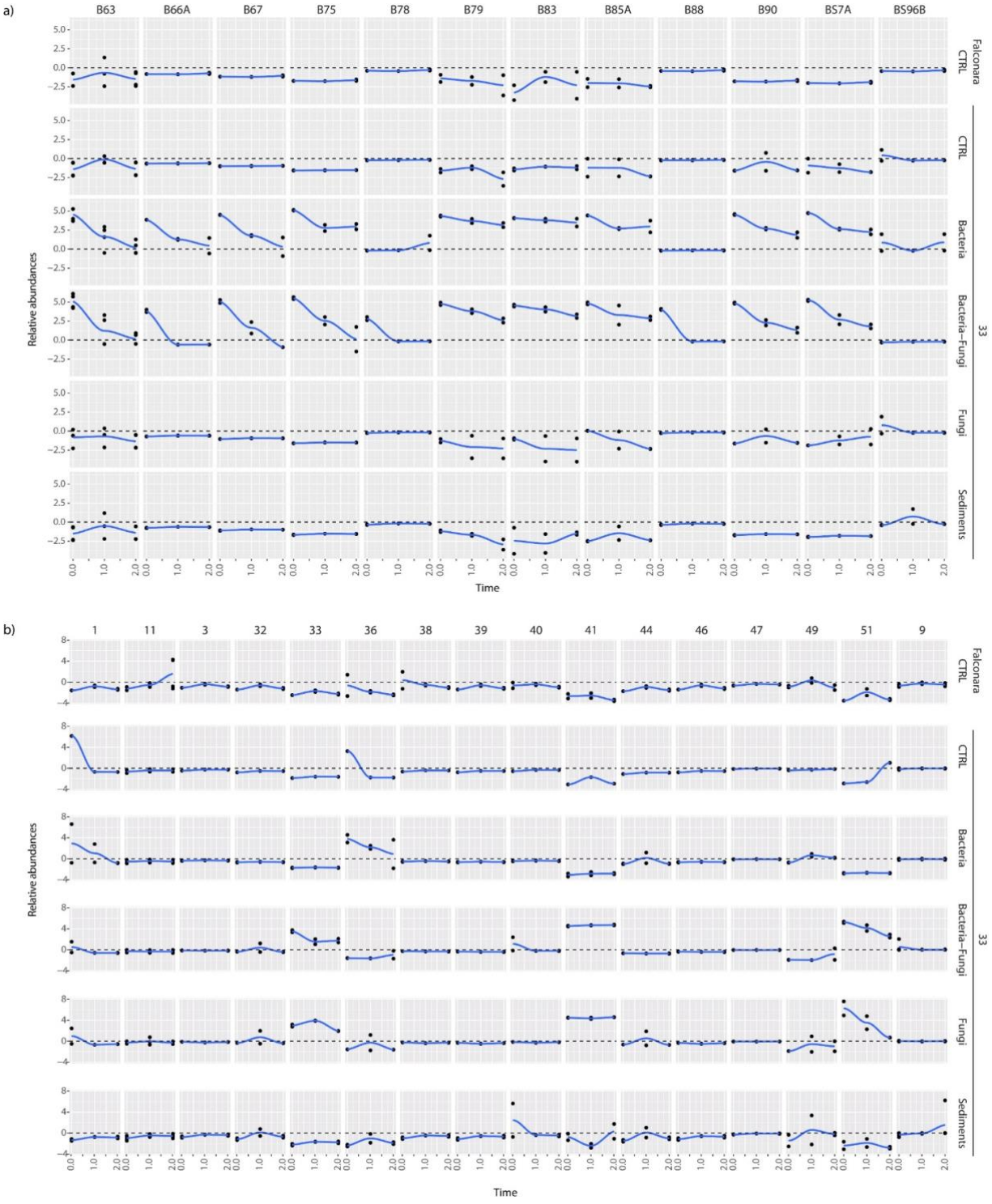

Figure 4.3.3.1. Changes of bacterial (a) and fungal (b) ASVs corresponding to sequences belonging to bacterial and fungal strains inoculated during the bioaugmentation experiment.

Chapter 5

Assessing the efficiency of selected microbial consortia for the bioremediation of marine sediments highly contaminated with polycyclic aromatic hydrocarbons

5.1 Introduction

Environmental contamination by polycyclic aromatic hydrocarbons (PAHs) and metals is reaching a global dimension and represents a serious risk for the sustainable provision of ecosystems' goods and services and for human wellbeing (Johnston et al., 2015). Chemical contamination can impair biological components at different trophic levels, from microbes to top predators (Gupta, 2019 and citations therein). Recent findings highlighted the presence of ca. 2.5 million potentially contaminated sites, only in Europe, two third of which polluted by hydrocarbons and metals (Liedekerke et al., 2014). Marine sediments of coastal areas characterized by high anthropogenic inputs and low hydrodynamic regimes can accumulate large amounts of PAHs and metals and require efficient remediation solutions (Bandowe et al., 2014; Das & Das, 2015; Sun et al., 2018). To date, different remediation techniques, such as physical, chemical, and biological, have been used for the removal of contaminants. Despite the fact that physical and chemical approaches have been practiced for decades, they still suffer from different drawbacks, including high costs and the undesirable generation of secondary pollutants (Dangi et al., 2019). By contrast, biological remediation (bioremediation,) in the form of microbe-based treatments, is a cost-effective, eco-friendly, and socially acceptable way to remove pollutants such as hydrocarbons (Ławniczak et al., 2020 and references therein;

Straube et al., 2003) from environmental matrices. Nevertheless, while culturable bacteria were isolated from contaminated sites since decades (Raymond et al., 1976), the approach of bioremediation has so far failed to provide convincible solutions in pollutant management. Classically, the majority of the studies performed in the field of bioremediation have aimed to isolate, culture, and characterize the organisms that are responsible for the remediation process (Borchert et al., 2021). While using such culture-based techniques has resulted in the identification of a number of microbes carrying out the biodegradation of specific contaminants (Malla et al., 2018 for examples), it suffers from important drawbacks. One is that more than 99% of the microorganisms present in the environment cannot be cultivated under laboratory conditions. This, known as the 'great plate count anomaly', has made challenging the recovery of specific isolates that are responsible for, or participate in, a given biodegradation process.

Synthetic microbial community is a promising method for constructing an artificial microbial community with function-specific species for bioremediation purposes (Jaiswal & Shukla, 2020 and citations therein). Synthetic microbial consortia may play important roles in bioremediation, as the division of labor in consortia is important for the degradation of complex mixtures of organic pollutants such as petroleum hydrocarbons, which usually requires multiple steps (Hays et al., 2015). The constructed synthetic consortia can serve as the seed culture for bioaugmentation of in situ bioremediation practice and for biodegradation in confined reactors as ex situ remediation approaches. To this regards, previous studies provided evidence that defined microbial consortia containing different taxa can be more effective than single isolates in the degradation of different PAHs from contaminated environmental matrices (e.g., Jacques et al., 2008; Tang et al., 2010).

Although bioremediation based on synthetic microbial consortia can be effective for removing hydrocarbons, this strategy could induce important changes in the partitioning, mobility and

bioavailability of heavy metals in the sediment, possibly increasing environmental risk (Dell'Anno et al., 2020). Therefore, an accurate risk analysis should be conducted to assess the contextual effects of the biotreatments on sediments characterized by mixed chemical contamination (due to the presence of both organic and inorganic contaminants).

The Bagnoli-Coroglio bay, in the Gulf of Naples (Tyrrhenian Sea, Mediterranean Sea), is a typical example of coastal area chronically contaminated by PAHs and metals, which have been released for decades by industrial activities and stopped at the beginning of nineties (Arienzo et al., 2017; Romano et al., 2004, 2008; Trifuoggi et al., 2017). Here, PAH concentrations can be three to four orders of magnitude higher than those reported from several marine benthic systems worldwide (Arienzo et al., 2017; de Vivo and Lima, 2018).

In this study, we tested the efficiency of different selected microbial consortia for PAH degradation on marine contaminated sediments collected in the Bagnoli-Coroglio bay by comparing: i) bioaugmentation performance of the addition of a bacterial consortium, ii) bioaugmentation performance of the addition of a fungal consortium and iii) bioaugmentation performance of the addition of a mixed consortium composed of both the above bacteria and fungi. At the same time, we investigated the effects of biotreatments on heavy metal partitioning, as well as on changes of the microbial diversity over time in the sediments supplied with the different microbial consortia.

5.2 Material and Methods

5.2.1 Study area and sediment sampling

The sediment samples used in the present study were collected in the Bagnoli-Coroglio within the Gulf of Naples (Southern Tyrrhenian Sea) at the south-eastern portion of the Pozzuoli Bay, about 10 km west of the city of Naples. The industrial activities in the Bagnoli-Coroglio area

began in 1905 and were mainly represented by a steel plant using coal, iron ores and limestone which were carried by ships to the coast as raw materials, transported by a conveyor belt to the plant and then processed. The iron production was increased until its interruption due to the Second World War in 1943. By this date, the plant was also enlarged, including the construction in 1930 of a northern long pier for large ships delivering raw materials, and a southern pier where the final products were loaded onto outgoing ships. The steel and iron production restarted in 1946 and lasted until 1990, when all activities ceased. Between 1962 and 1964, the marine area between the two piers was partially filled up with contaminated soil from the plant to obtain new space for enlargement. A comprehensive reclamation project has been recently developed, including a multidisciplinary assessment aimed at evaluating the ecological impacts of past industrial activities with reference to the natural and anthropogenic characteristics of the local environment (ABBaCo project, see http://www.gazzettaufficiale.it/eli/id/2017/03/08/17A01736/sg%20).

Previous analyses carried out on marine sediments collected in front of the abandoned industrial plants have reported very high concentrations of different metals and PAHs due to the industrial activities (De Vivo and Lima, 2008; Albanese et al., 2010; Arienzo et al., 2017; Romano et al., 2017, 2009; Trifuoggi et al., 2017).

For bioaugmentation experiments, sediment samples (from the sediment surface down to 50 cm depth) were collected in November 2017 in front of the industrial plant using a vibro-corer. After collection, sediment samples were homogenized, and sub-samples were collected for the analysis of polycyclic aromatic hydrocarbon and heavy metal concentrations.

5.2.2 Microorganisms used for bioaugmentation experiments

Bacteria and fungi have been isolated and taxonomically identified through Sanger sequencing from relatively unpolluted sediments of Falconara Marittima (Central Adriatic Sea), included in the list of the National Remediation Sites. Overall, 53 bacterial and 32 fungal strains were isolated, from autochthonous microbial assemblages characterized by high natural attenuation capacity and biodegradation activities toward PAHs (see chapter 3). These microbial strains were further screened for their ability to growth in the presence of high concentrations of PAHs and metals characterizing the Bagnoli-Coroglio sediments. In particular, 50 bacterial strains and 8 fungal strains were selected for carrying out bioaugmentation experiments. In particular, for bioaugmentation experiments, we used a consortium of bacterial strains belonging to 7 different orders, a consortium of fungal strains affiliated to 7 different orders and a mixed consortium of such bacterial and fungal strains (Table 5.2.2.1).

Table 5.2.2.1 Taxonomic details and number of bacterial and fungal strains isolated from Falconara sediment and utilized for bioaugmentation experiment.

Bacterial pool (n. strains used for each taxon)	Fungal pool (n. strains used for each taxon)
Aeromonadaceae (5)	Eurotiales (1)
Alteromonadales (2)	Filobasidiales (2)
Bacillales (22)	Glomerellales (1)
Enterobacterales (3)	Hypocreales (1)
Pseudomonadales (8)	Microascales (1)
Rhizobiales (9)	Onygenales (1)
Rhodobacterales (1)	Pleosporales (1)

5.2.3. Experimental procedures

In this study, the same experimental procedure previously applied on sediment samples collected in the Bagnoli-Coroglio was used to test the performance of the different microbial

consortia on sediment samples characterized by different concentrations and typologies of chemical contaminants.

In particular, the bacterial and fungal strains were grown up on specific media (marine agar broth for bacteria and YPD containing rifampicin to avoid bacterial contamination for fungi) up to reach a sufficient biomass. For bioaugmentation experiments, a ca. equal biomass of the different consortia to that present in the sediments of Bagnoli-Coroglio used was inoculated. Bioaugmentation experiments were performed in 500 mL Erlenmeyer flasks containing 150 g wet sediment samples and 75 mL pre-filtered 0.2 μm and autoclaved seawater. Replicate microcosms were supplied separately with 2 mL of marine agar broth containing the bacterial consortium, 2 mL of 10% glucose, 1% yeast extract, 0.25% (NH4)2SO4 and 0.15% K2HPO4 containing the consortium with the fungal spores and 2 mL of a mixed culture of bacterial and fungal consortia. Additional microcosms prepared in the same way, but without addition of bacterial and/or fungi, were used as controls.

All flasks were incubated at a constant temperature of 20◦C for 1 month. Subsamples were collected at the beginning of the experiment (T0), after 14 days (T1), 28 days (T2) and 90 days (T3) for the determination of PAH concentrations, prokaryotic abundance and microbial diversity and metals associated with different geo-chemical phases at the end of the experiment (i.e., after 3 months).

5.2.4 Polycyclic aromatic hydrocarbon and heavy metal determinations

Polycyclic aromatic hydrocarbons (PAHs) were extracted from the sediment samples according to 3545A EPA method and analyzed by gas chromatography-mass spectrometry (GC-MS; EPA 8270D). Twenty different congeners were analyzed including naphthalene, acenaphthene, fluorene, acenaphthylene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene,

chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3,-cd]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i] perylene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i] pyrene and dibenzo[a,l]pyrene. Total PAH concentrations were obtained by the sum of the concentrations of these 20 congeners.

Heavy metal content in the sediment was determined after acid digestion, as follows: dried sediment sub-samples were transferred in Teflon boxes, added with 5 mL fluoridric acid and 1 mL of ‘‘aqua regia” (i.e. $HCl:HNO_3$ = 3:1) incubated for 90 min at 150 °C. At the end of the incubation period, 5 mL of 10% boric acid were added and the obtained extracts were analysed by atomic absorption spectrophotometry and by inductively coupled plasma-atomic emission spectrometry. Metals distribution in different mineralogical fractions were determined by means of a selective extraction procedure, which utilized, sequentially, specific chemical reagents to extract heavy metals associated with different geochemical phases (Quevauviller, 1998). Four different fractions are considered: (i) the exchangeable and carbonate bound fractions (hereafter defined as exchangeable fraction), extracted utilizing 0.11 M acetic acid, pH 2.8; (ii) iron and manganese oxides fraction (i.e., reducible fraction), extracted with 0.1 M NH_2OH, pH 2; (iii) organic and sulfide fraction (i.e., oxidizable fraction), extracted by hydrogen peroxide 30% and treated with ammonium acetate at pH 2, and (iv) the residual fraction, that remains in the residual solid, is determined by difference with the total metal content.

5.2.5 Prokaryotic abundance analysis

For the determination of the prokaryotic abundance, sediment slurries (2.5 mL) were treated with pyrophosphate (5 mM final concentration) and ultrasound (three times for 1 min; Danovaro et al., 2002). For prokaryotic counts, sub-samples (1 mL) were diluted 50–100 times,

stained with SYBR Green (0.01% final concentration) and filtered on Anodisc 0.2 μm pore size filters. Filters were analyzed under epifluorescence microscopy using a Zeiss Axioplan microscope equipped with a 50-W lamp. Ten to 50 fields were viewed at 1000x magnification and a minimum of 400 cells were counted. Prokaryotic counts were normalized to sediment dry weight after desiccation (60 °C, 24 h).

5.2.6 Microbial diversity analysis

DNA has been extracted from sediment sub-samples collected during the time-course experiments. The preliminary step, before DNA extraction, consisted in the removal of extracellular DNA based on three washing solutions (WS) at different concentration: WS1, 50 mM Tris-HCL, pH 8.3; 200 mM NaCl; 5 mM Na_2EDTA; 0,05% Triton X-100; WS2, 50 mM Tris-HCL, pH 8.3; 200 mM NaCl; 5 mM Na_2EDTA; WS3, 10 mM Tris-HCL, pH 8.3; 0,1 mM Na_2EDTA. The subsequent DNA extraction was performed utilizing the DNeasy PowerSoil Kit, QUIAGEN, following the manufacture's procedure. Sequencing libraries were prepared from each replicate and sequencing was performed with all libraries on an Ion Torrent chip (using the in-house facilities provided by Stazione Zoologica di Napoli "Anton Dohrn") using the primer pairs 515F–Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'; Parada et al., 2016; Corinaldesi et al., 2019) for prokaryotes and the primer pair ITS3F (5′- GCATCGATGAAGAACGCAGC-3′) and ITS4R (5′- TCCTCCGCTTATTGATATGC-3′) for fungi (Nilsson et al., 2019). After primer removal through the cutadapt plugin within QIIME2 (Martin, 2011; Bolyen et al., 2019), trimmed reads from 16s sequencing libraries were submitted to the DADA2 pipeline within QIIME2 (Callahan et al., 2016; Bolyen et al., 2019) using the *denoise-pyro* plugin over a length of 250 bp (Callahan et al,. 2016); ITS sequencing libraries were subjected to trimming by means of the ITSxpress

tool (Rivers et al., 2018) to only keep the fraction of sequence within the ITS2 region before being submitted to the DADA2 pipeline within QIIME2 (Callahan et al., 2016; Bolyen et al., 2019) using the *denoise-pyro* plugin without length trimming. Taxonomic inference was performed through the *classify-consensus-vsearch plugin* (Rognes et al., 2016) using the SILVA database (v138; Quast et al., 2012) for prokaryotes and the UNITE database for ITS sequences (Nilsson et al., 2019). For the taxonomic affiliation of ASVs, a reference database was first created within QIIME2 by trimming both databases to the region amplified by sequencing primers. A first overview of the taxonomic structure of both assemblages was obtained after clustering ASVs in modules of co-occurring sequences through the SCNIC plugin (Shaffer et al., 2020) after normalizing the ASV table to 20000 sequences for 16s libraries and 10000 sequences for ITS libraries. Differences in assemblage structure were analysed through the *adonis2* procedure in the *vegan* R package. ASVs from the non-normalized tables were then removed when not belonging to at least 2 samples and with less than 100 sequences before running a second clustering to identify ASVs co-occurring into modules through the SCNIC tool. To test for the variation in module abundances over time, module abundances were centered log-ratio (CLR) transformed to account for compositionality (Gloor et al., 2017) and detrended over time using the *pracma* R package (Faust et al., 2015) before being submitted to the Rhythmicity Analysis Incorporating Non-parametric methods (RAIN; Thaben and Westermark, 2015) only keeping modules with significant (p-value < 0.05) and marked (log-transformed values <-5 and >5) periodicity (Coenen et al., 2020). ITS and 16S sequences obtained from the strains utilized in the bioaugmentation experiment were trimmed to the ITS2 region and to the region sequenced by NGS primers and then aligned to the ASVs identified in the present study through the MAFFT tool to identify the closest ASVs within our dataset, as a proxy to check for the abundance of inoculated strains during the experiment. The

abundance of these ASVs was tracked over time and treated as above to identify the temporal trends of the inoculated strains.

5.3 Results and discussion

5.3.1 Biodegradation of PAHs and effects on metal mobility due to bioaugmentation treatments

Sediments used for the bioaugmentation experiments were characterized by extraordinary high concentrations of total PAHs (ca. 315 mg kg^{-1}), ca. three times higher than those characterizing the sediments used for biotreatments (see chapter 4) and three to four orders of magnitude higher than those reported from several marine benthic systems worldwide (Arienzo et al., 2017; de Vivo and Lima, 2018). The PAH pool was almost exclusively accounted by high molecular weight congeners (accounting all together for 97% of the total PAH) represented mainly by fluoranthene (24%), pyrene (19%), and benzo[a]pyrene (9%).

Time-course experiments revealed that after 28 days only the addition of the consortium made by bacteria and fungi determined a significant decrease of PAHs when compared with untreated sediments (Figure 5.3.1.1). However, longer incubation determined a significant decreased of PAHs concentrations in all the bioaugmented sediments when compared with the controls. These findings suggest that the mix of the selected bacteria and fungi can promote a faster degradation than the consortia made by the single microbial components. We also found that the highest biodegradation yield of total PAHs at the end of the experiment was due the addition of the mix of bacteria and fungi (on average a decrease of 71% of the initial total PAH concentrations), followed by the use of the consortium made by only fungi (58%) or bacteria (46%). A previous study using a microbial consortium isolated from petrochemical landfarm site, containing 5 different bacterial taxa and one fungal taxon, reported a degradation efficiency

of 78% of anthracene, phenanthrene, and pyrene in soil in 70 days (Jaques et al. 2008). Similarly, aliphatic and aromatic hydrocarbons of crude oil were efficiently degraded by a defined microalgal–bacterial consortium containing four bacterial species and one oil-tolerant microalga (Tang et al., 2010). As such, consortia made by selected bacteria and microbial eukaryotes can be more effective in the biodegradation of PAHs not only when compared to single isolates, but also, as in our study, when compared with the efficiency of consortia made exclusively of fungi or bacteria.

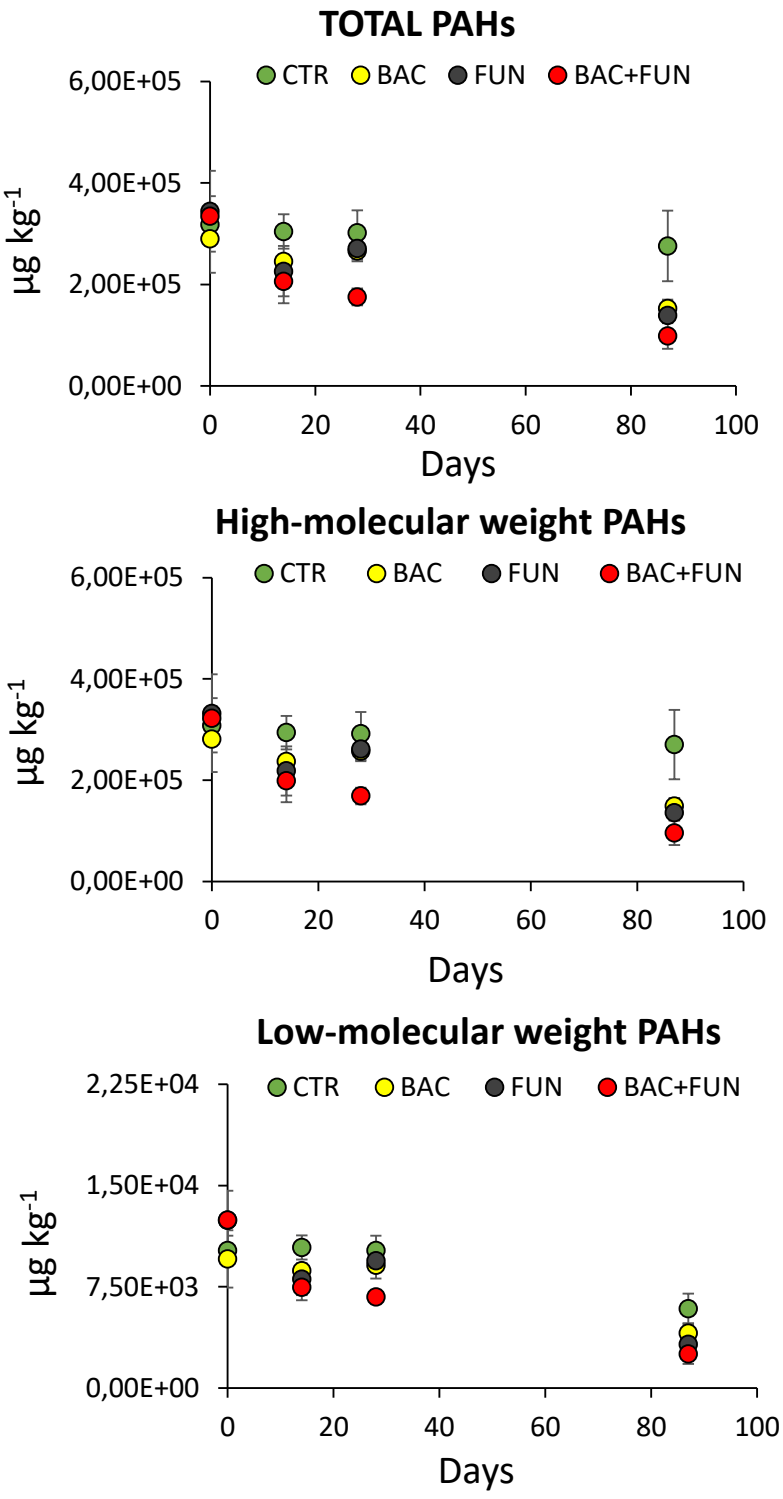

Figure 5.3.1.1. Temporal changes of total, low molecular wight and high molecular weight PAHs concentrations during bioaugmentation experiments carried out using the different microbial consortia. Values of PAHs in y-axis are in $LOG_{(10)}$ scale.

The analysis of constant decay rates of total PAHs provided further evidence of the different efficiency of the different consortia added to the contaminated sediments of Bagnoli-Coroglio. Based on PAH concentrations determined at each time interval during bioaugmentation experiment and assuming a first order degradation kinetic, we found that the constant decay rate of total PAHs was approximately >2-fold faster in the experiments with bacteria and fungi when compared to the sediments containing bacteria alone (Figure 5.3.1.2).

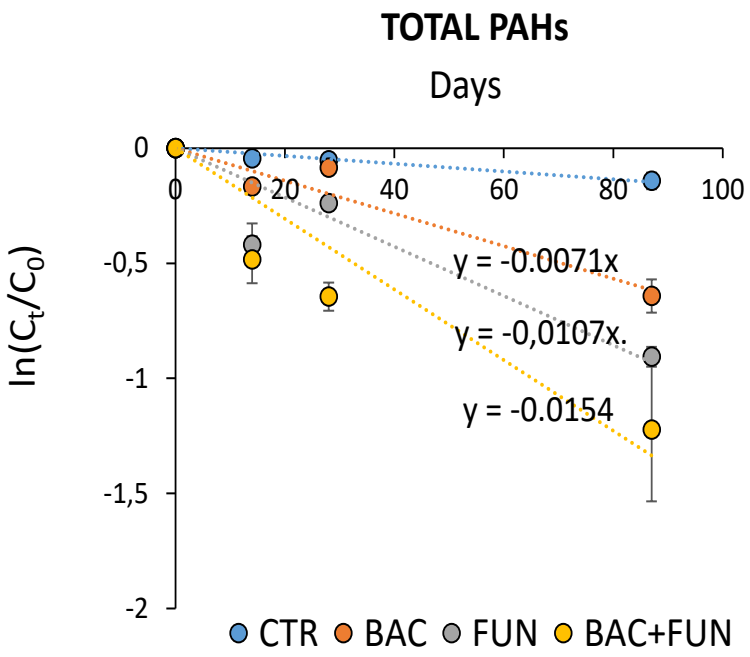

Figure 5.3.1.2. Decay rates of total PAHs calculated on the basis of their concentrations determined on Bagnoli sediments during bioaugmentation experiments carried out using the different microbial consortia.

Previous studies have reported a high potential of microbial assemblages to degrade high-molecular weight PAHs (Beolchini et al., 2010; Déziel et al., 1996; Ghosal et al., 2016; Perfumo et al., 2006), but information on degradation rates of the different congeners following bioremediation of chronically contaminated marine sediments is still largely lacking

(Dell'Anno et al., 2020). In the present study, we found that the dominant PAH congeners present in the Bagnoli sediments were degraded with fastest rates following the addition of fungal and bacterial consortium and slowest rates following the addition of bacteria alone (Figure 5.3.1.3). Besides this, no preferential degradation apparently occurred in any of the bioaugmentation treatment since the values of the decay constant of the different PAH congeners were very similar. Overall, these findings suggest that, despite the residual concentrations of total PAHs in the Bagnoli sediments at the end of the experiments were different depending on the bioaugmentation treatments investigated, their composition, at least for the dominant PAH congeners, remained rather similar.

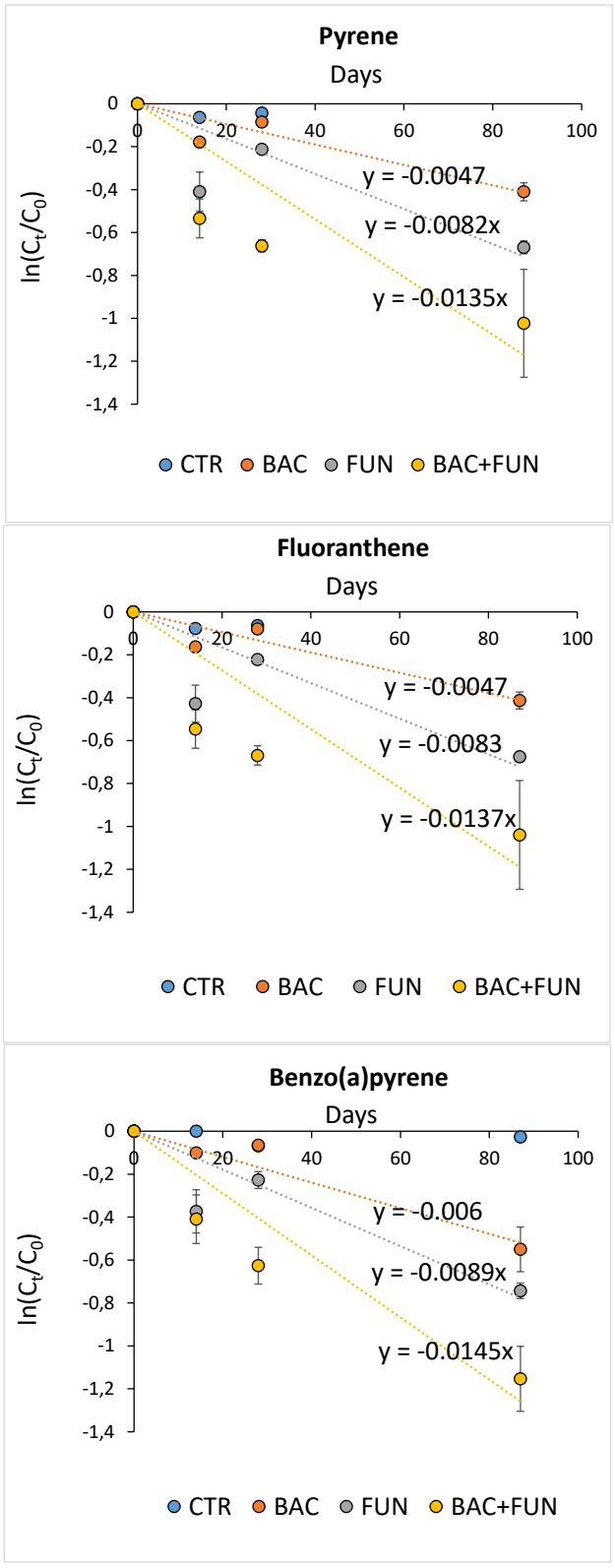

Figure 5.3.1.3. Decay rates of the main PAH congeners calculated on the basis of their concentrations determined on the Bagnoli sediments during bioaugmentation experiments carried out using the different microbial consortia.

The bioaugmentation treatment based on the addition of the consortium of bacteria and fungi was from one hand effective for PAH degradation and from the other have a negligible effect on the partitioning of heavy metals among the different geochemical phases when compared with the untreated sediments (Figure 5.3.1.4). Since the increasing of metal mobility due to biotreatments can represent a major ecological risk (Dell'Anno et al., 2020), these findings suggest that bioaugmentation treatment using such selected microbial consortia can represent a good compromise for the reduction of PAH contamination, avoiding the potential detrimental ecological effects due to the increase of metal mobility.

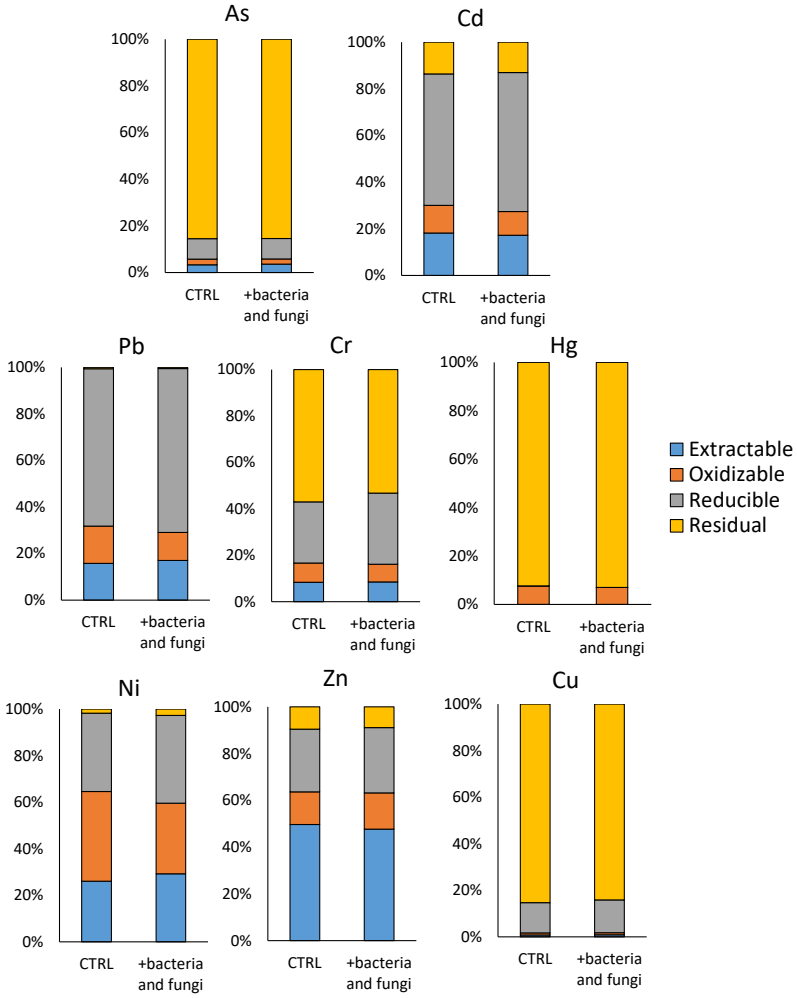

Figure 5.3.1.4. Comparison of the repartition of As, Cd, Pb, Cr, Hg, Ni, Zn and Cu in the different geochemical fractions of untreated (CTRL) and treated sediment samples with the bacterial and fungal consortium at the end of incubation (3 months).

5.3.2 Changes of prokaryotic abundance and microbial diversity due to biotreatments

Studies investigating bioremediation performance of contaminated marine sediments have repeatedly reported an increase of the prokaryotic standing stocks following the addition with inorganic and or organic nutrients (i.e. biostimulation; Beolchini et al., 2010; Dell'Anno et al., 2012; Dell'Anno et al., 2020), but information on responses of prokaryotic abundance to the addition of microbial consortia is lacking. In the present study, no significant differences of prokaryotic abundances were found either between control and treatments, or comparing the different treatments over time (Figure 5.3.2.1). Thus, bioaugmentation strategies using the microbial consortia selected in the present study apparently have no stimulation effects in terms of overall abundance of the prokaryotic assemblages of the Bagnoli sediments.

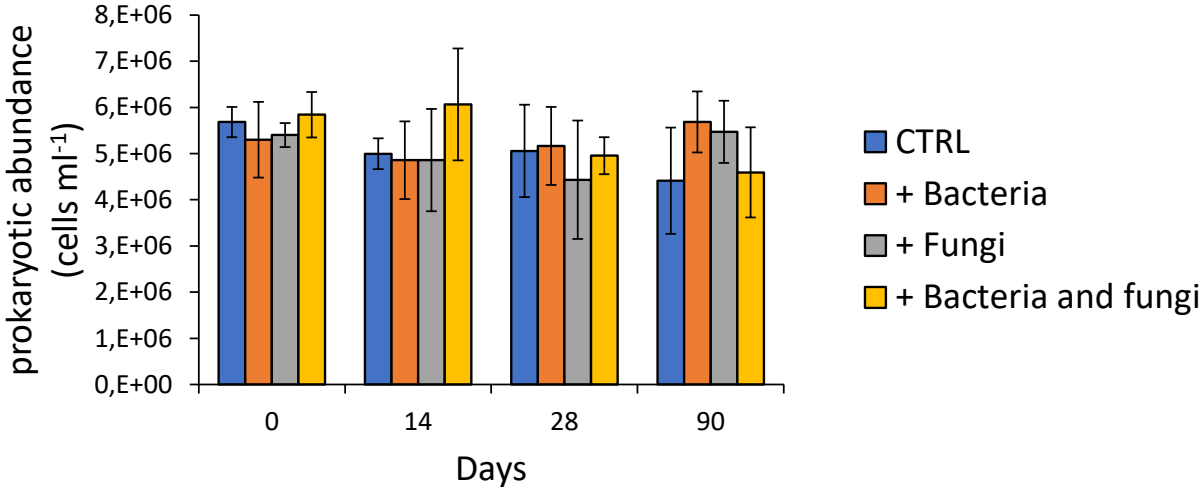

Figure 5.3.2.1. Temporal patterns of prokaryotic abundance of untreated (CTRL) and treated sediment samples with the different microbial consortia.

Although no significant changes were found in prokaryotic abundances, major differences in the taxa composition between untreated and treated sediments and among treatments were observed (Figure 5.3.2.2). In particular, no single taxon was found to be widespread across the

entire dataset. During the time-course experiments, the abundance of sequences affiliating with the *Flavobacteriaceae* family both in the untreated and treated sediments increased. Such family includes heterotrophic bacteria able to use organic resources, including hydrocarbons (Zouch et al., 2018), thus potentially involved in natural attenuation processes toward PAH contamination occurring in untreated sediments or in the enhanced biodegradation of PAHs occurring in bioaugmented sediments.

During the first 14 days of incubation, the bacterial assemblage composition of sediment samples supplied with the bacterial consortium was distinctly separated from the others, while it clustered with the other samples with increasing incubation time. These findings indicate that in the short term the addition of the bacterial consortium can promote a shift of the bacterial assemblage composition, which however is much less appreciable at longer time scale when compared with the other samples. At longer incubation times, in each sample, the abundance of taxa affiliated with *Alicyclobacillaceae*, *Nitrosopumilaceae* (which are sensitive to oxygen and nitrogen concentrations; Zhang et al., 2021)*, Aeromonadaceae*, *Halomonadaceae* and *Vibrionaceae* decreased. Overall, statistical analyses highlighted that both incubation time and amendment type explained significantly (p-value < 0.05) 35% of the variability of the prokaryotic assemblage composition. Compared with the results obtained from the previous bioaugmentation experiment (see chapter 4), in this study changes in the relative abundances of specific taxa of the prokaryotic assemblages were more evident, with relative abundances of each family varying widely across the different treatments. Despite this, all samples tend, over time, to converge on a *Flavobacteriaceae*-dominated bacterial assemblage.

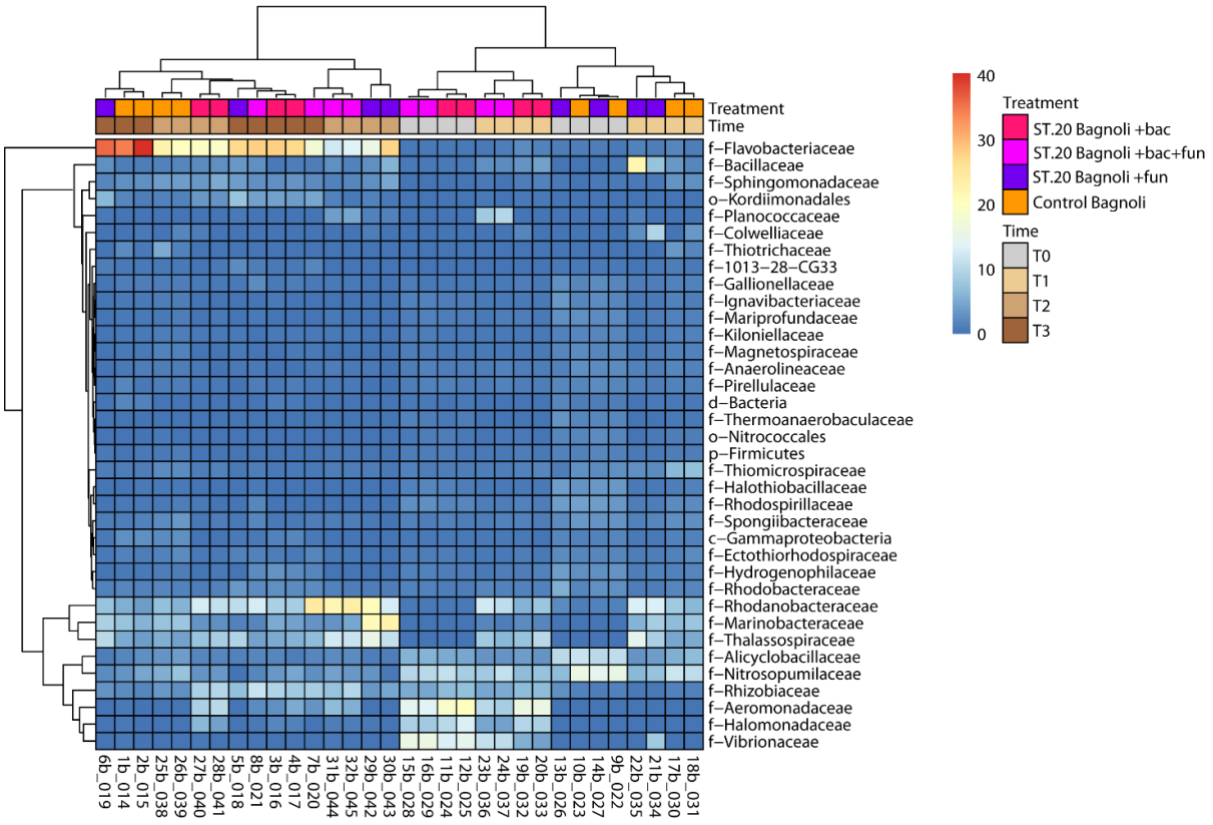

Figure 5.3.2.2. Changes of prokaryotic assemblage composition at family level during time-course experiment carried out in the present study.

Taxonomic analysis of the fungal assemblage composition revealed that most of the sequences belonged to a limited number of taxa, mainly *Piskurozymaceae* and *Herpotrichiellaceae* in samples added with fungi, and *Sordariomycetes* in sediment samples amended by only bacteria (Figure 5.3.2.3). High abundances of sequences affiliated with the fungal family *Piskurozymaceae* were also identified in the previous bioaugmentation experiment based on fungal addition carried out on different sediment samples collected in Bagnoli-Coroglio in relation with high abundances of sequences of bacteria of the *Flavobacteriaceae* family (see chapter 4). However, results from the time-course experiments carried out in the present study revealed that the relative abundance of this fungal taxon appear to be negatively related to the relative abundance of bacteria belonging to *Flavobacteriaceae*, suggesting a differential

response of these two microbial components depending on the specific sediment characteristics and autochthonous microbial components present therein.

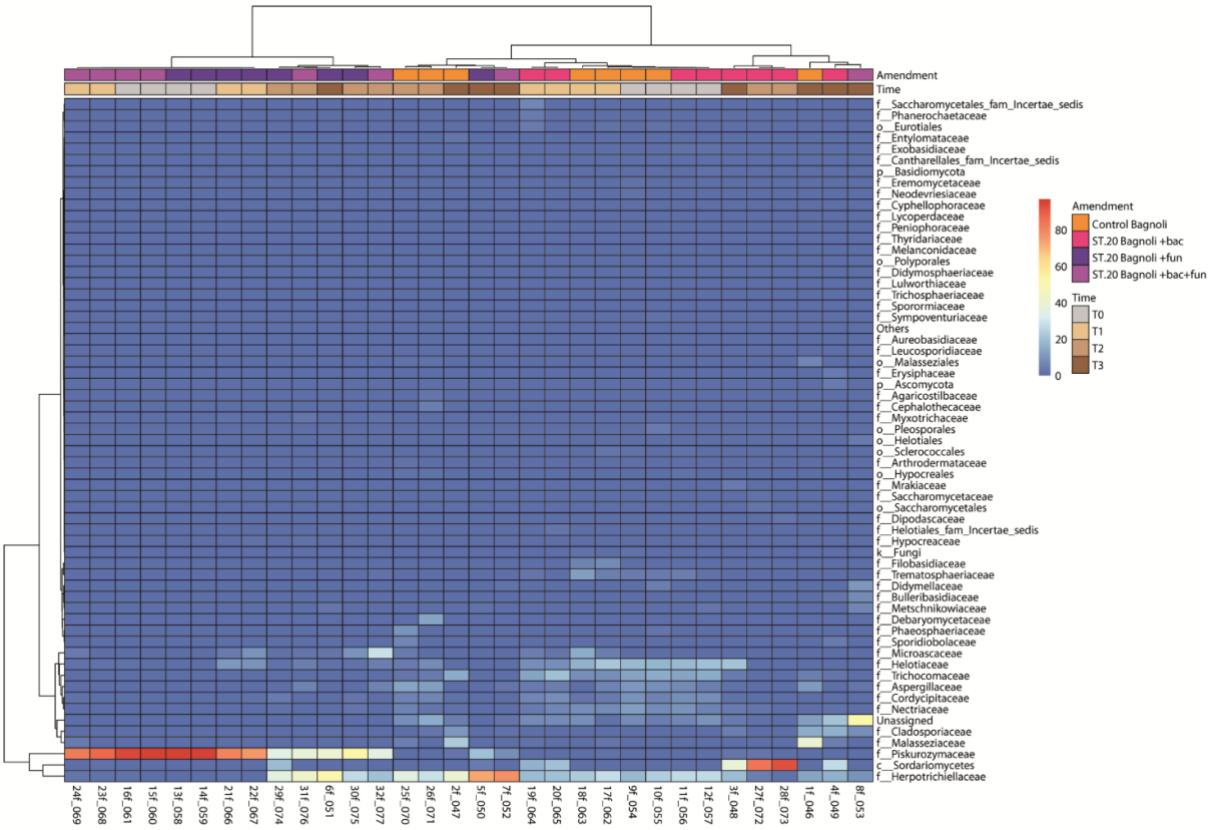

Figure 5.3.2.3. Changes of fungal assemblage composition at family level during time-course experiment carried out in the present study.

The analysis of co-occurring modules of prokaryotic ASVs allowed us to identify 248 different modules, 30 of which showing a major degree of variation in the dataset. The module-based approach was used to better characterize the real ASV abundance, reducing the bias introduced by rarefaction and normalization and to provide more compelling insights on changes in microbial assemblage composition occurring among treatments and over time (Gloor et al., 2017). Modules 17 and 19 showed high relative abundances in untreated sediment samples, whereas the relative abundance of module 3 was high and stable over time in the sediments

supplied with the bacterial consortium. Other modules showed no clear patterns, suggesting a high variability of prokaryotic assemblages occurring over time depending both on natural succession of prokaryotic diversity and sediment treatment (Figure 5.3.2.4).

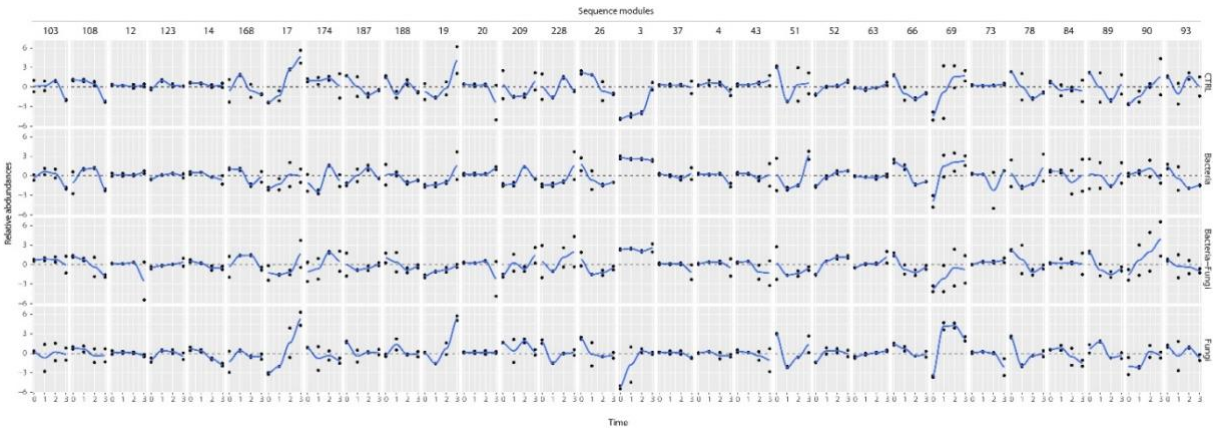

Figure 5.3.2.4 Relative abundances of each of the main prokaryotic co-occurring ASV modules in untreated and treated sediments during the time-course experiments carried out in the present study.

Taxonomic analysis of the ASVs contained in the main identified prokaryotic modules is reported in Figure 5.3.2.5. Such analysis revealed that the module 3 was characterized by a high number of ASVs related to *Aeromonadaceae*, *Halomonadaceae* and *Vibrionaceae* at the beginning of the experiment, whose relative abundance, however, strongly decreased over time.

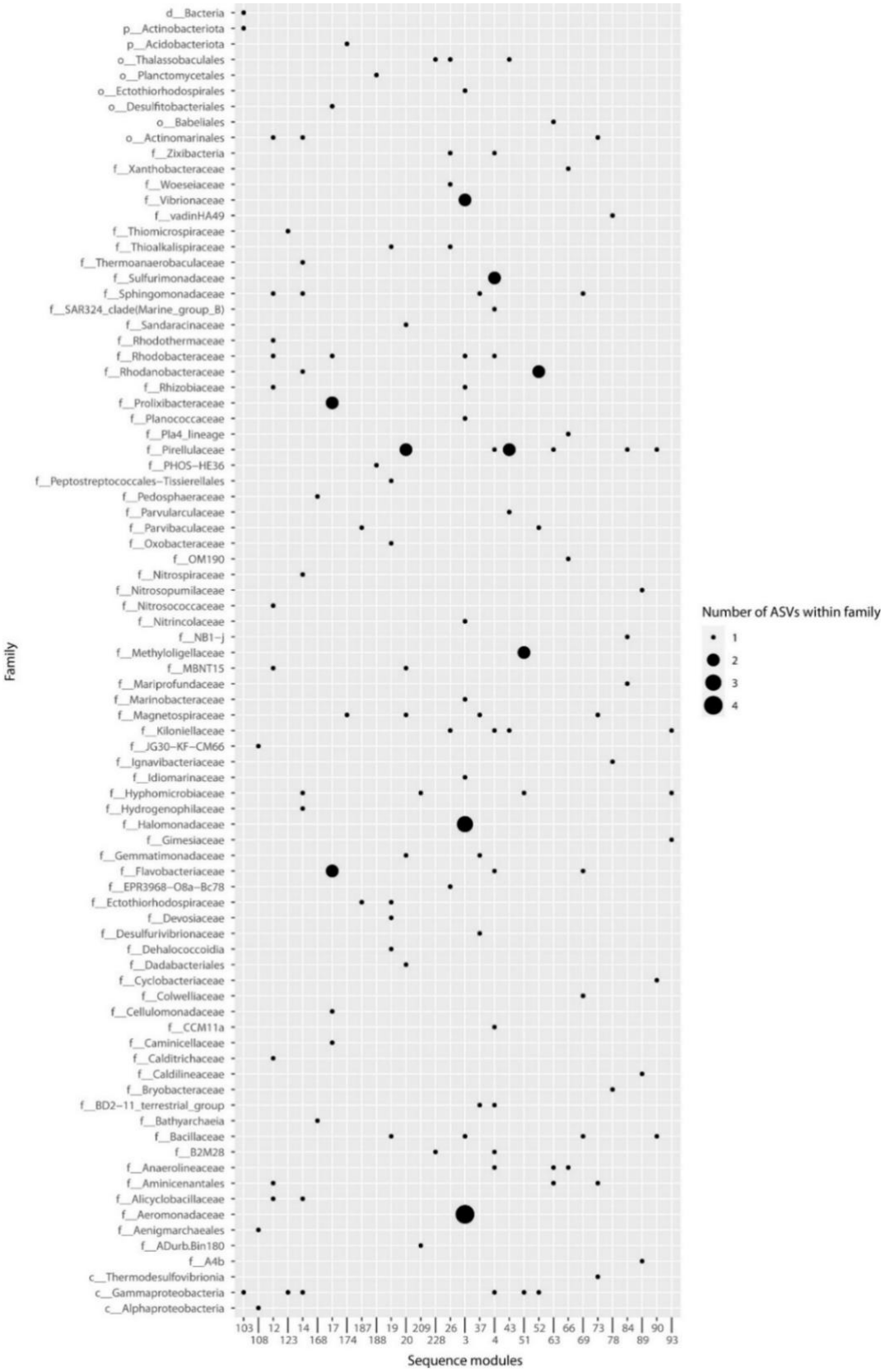

Figure 5.3.2.5. Taxonomic composition of each of the main modules of prokaryotic co-occurring ASVs identified in the present study.

Besides prokaryotic ASV modules, we also investigated the fungal co-occurring ASV modules, which, were grouped in 32 different modules, 10 of which showing a great level of variation in the dataset (Figure 5.3.2.6). In particular, the abundance of ASVs associated to module 0 were higher at the beginning of the experiment in sediment samples amended with the fungal consortium or with the consortium of bacteria and fungi when compared with untreated sediments. Such module 0 contained high number of ASVs related to *Piskurozymaceae*, *Sporormiaceae*, *Myxotrichaceae*, *Microascaceae* and *Aspergillaceae* (Figure 5.3.2.7). However, their abundances strongly decreased with increasing incubation time. At the same time, the abundance of ASVs associated to module 8, which was very low at the beginning of the experiment increased with time in sediment samples amended with the fungal consortium or with the consortium of bacteria and fungi. These results suggest that fungal assemblages are sensitive to the experimental conditions applied and can respond differently over time to bioaugmentation approaches.

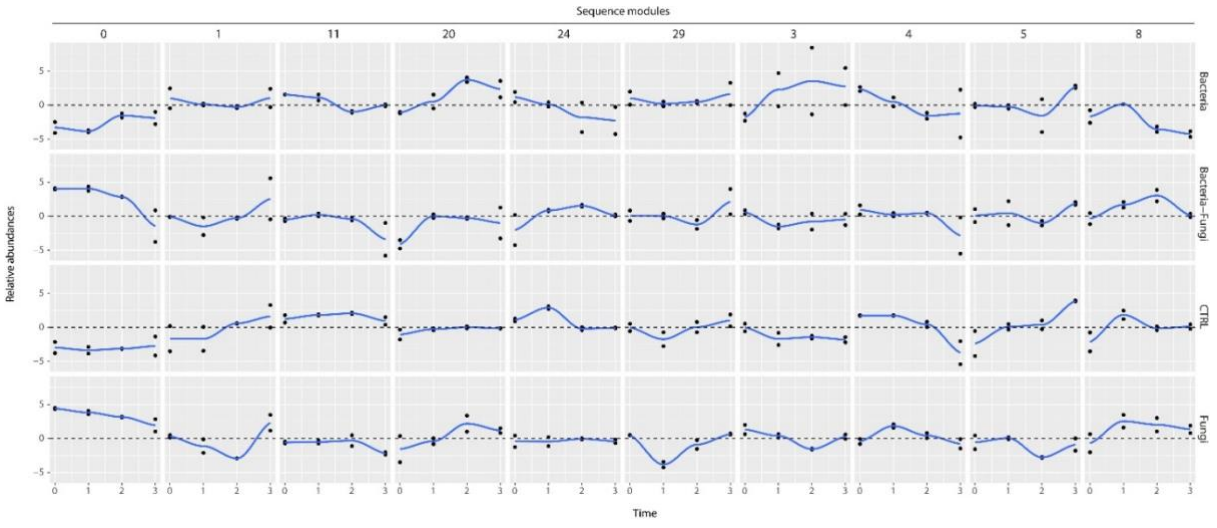

Figure 5.3.2.6. Relative abundances of each of the main fungal co-occurring ASV modules in untreated and treated sediments during the time-course experiments carried out in the present study.

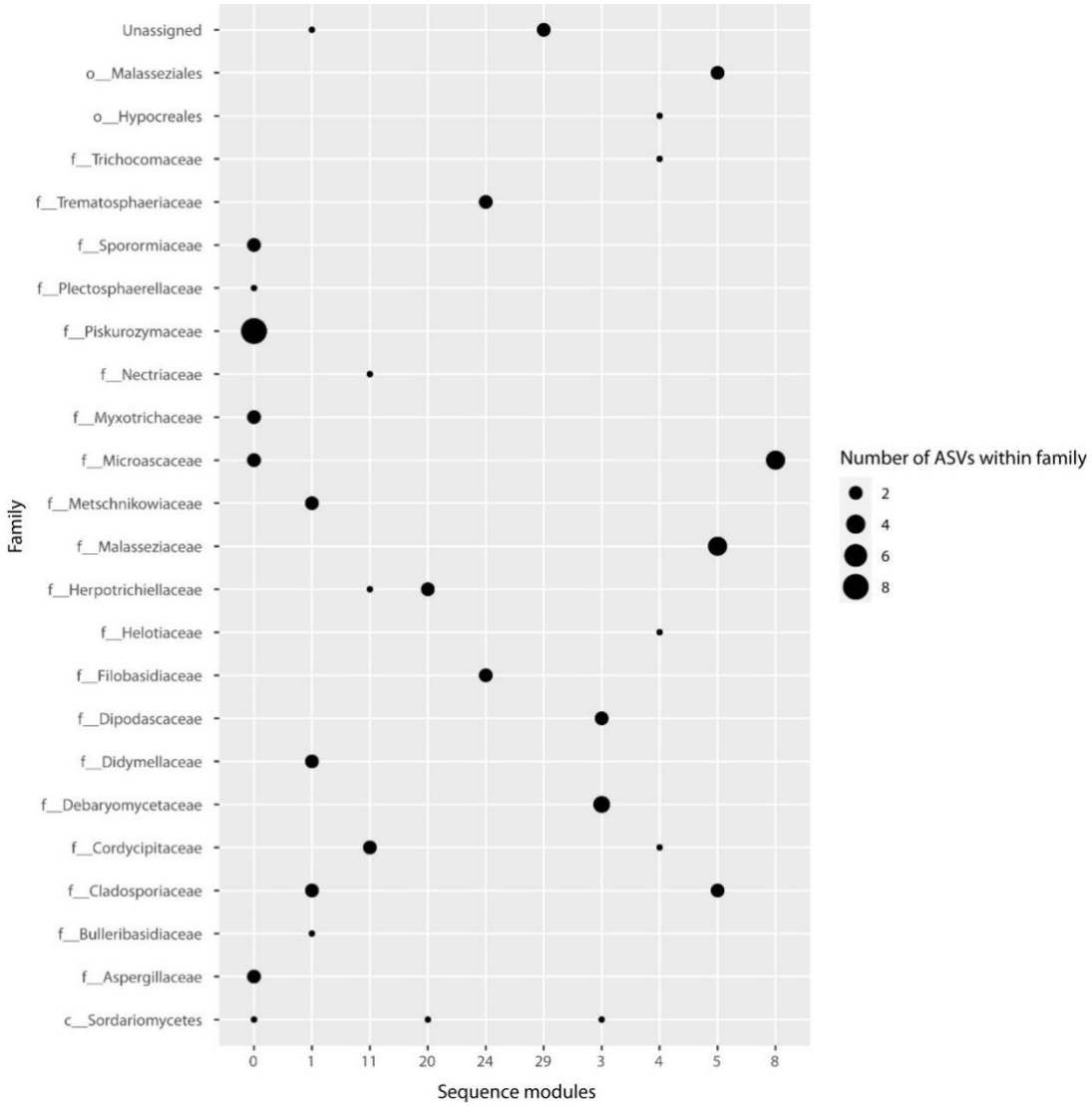

Figure 5.3.2.7**.** Taxonomic composition of each of the main modules of fungal co-occurring ASVs in untreated and treated sediments during the time-course experiments carried out in the present study.

5.3.3 Identification and monitoring of the prokaryotic and fungal taxa added to the sediments

To explore the potential effects of the different microbial consortia added to the sediments in the biodegradation of PAHs, we compared bacterial and fungal ASVs obtained by NGS with sequences of 16S rDNA and ITS of the cultured bacterial and fungal strains. Such a comparison allowed to identify 24 prokaryotic and 11 fungal ASVs as potentially affiliating with the added

taxa (Figure 5.3.3.1a,b). The relative abundances of ASVs affiliating with bacterial strains B63, B67 and B79 added to the sediments alone or with fungi were high at the beginning of the experiments and then strongly decreased with increasing incubation time. Conversely, other abundant ASVs affiliating with bacterial strains B83, B85A, B88 and B90 showed a remarkable stability over time. We also found that ASVs affiliating with fungal strains 33, 40 and 41 added to the sediments alone or with bacteria were high at the beginning of the experiments and then strongly decreased with increasing incubation time, whereas other fungal strains such as 51, although showing low relative abundances at the beginning of the experiments, increased over time.

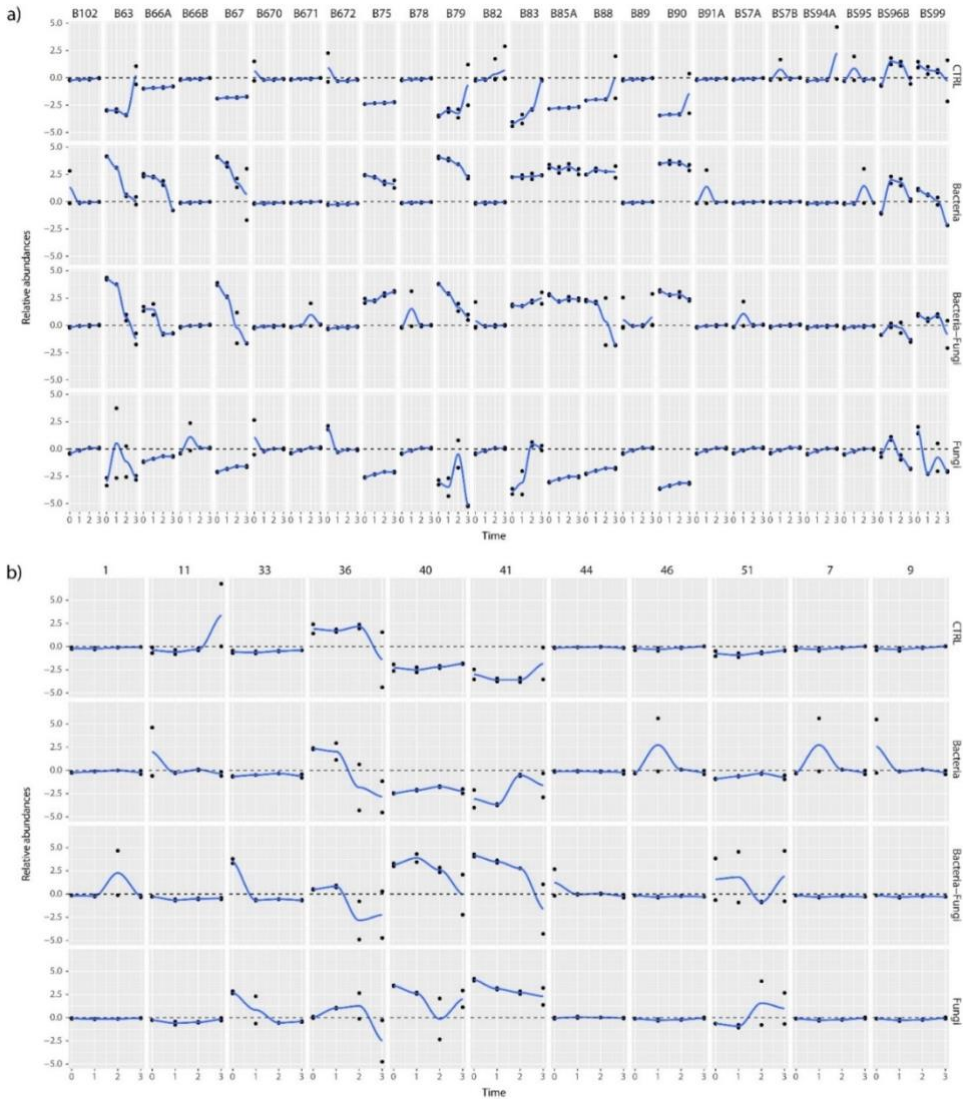

Figure 5.3.3.1**.** Changes of bacterial (a) and fungal (b) ASVs corresponding to sequences belonging to bacterial and fungal strains inoculated during the bioaugmentation experiment.

Overall, these results suggest that the different bacterial and fungal strains added to the Bagnoli sediments can have different fitness and ability to cope with the harsh environmental conditions and/or with competition processes with the other microbial components, thus potentially contributing to a different extent in the biodegradation processes of PAHs. Although further studies are needed to understand the complex interactions among different microbial components, findings reported here suggest that bioaugmentation strategies based on the

addition of selected fungal-bacterial consortia can be effective for the bioremediation of marine sediments highly contaminated with PAHs.

General conclusions

The analysis of the tree investigated areas included in the list of Sites of National Remediation Interest (Bagnoli, Mar Piccolo of Taranto and Falconara Marittima) highlights the presence of rich and diverse benthic prokaryotic assemblages (as number of ASVs) even in sediments displaying very high concentrations of organic and inorganic pollutants. This result suggests that the harsh environmental conditions related to chemical contamination do not influence to a major extent the number of prokaryotic taxa encountered. Each investigated site within the same areas are characterized by several exclusive ASVs suggesting that local conditions can promote prokaryotic turnover diversity resulting in a high diversity of the whole benthic area (gamma diversity). All investigated areas were characterized by a not negligible fraction of shared prokaryotic ASVs, suggesting the presence of a "core" set of widespread pollution-tolerant or pollution-resistant prokaryotic taxa. Among contaminants analyzed, only metals influence, to a certain extent, the benthic prokaryotic assemblage composition, likely selecting those lineages more adapted to tolerate/resist to metal contamination.

The sediments of the Site of National Interest of Falconara Marittima host microbial taxa highly efficient for hydrocarbon degradation, which in turn could be responsible of the relatively low levels of contamination observed in that area. Biostimulation experiments carried out on Falconara sediments based on inorganic nutrients addition revealed high PAH degradation yield comparable to that observed without nutrient amendments. However, the addition of inorganic nutrients promoted the degradation of the different PAH congeners at a similar extent, whereas in the un-treated sediments a preferential degradation of the more abundant PAHs occurred. We found that *Pirellulacee* and *Woeseiaceae* were, overall, the most represented prokaryotic families across the whole dataset, whereas taxa such as *Nitrosopumilaceae*, *Flavobacteriaceae*

and *Rhodobacteriaceae* showed remarkable shift in the abundance according to treatment and time. Indeed, these results can be explained also in terms of the wide range of possible metabolic pathways encoded by the different members of both *Pirellulaceae* and *Rhodobacteriaceae*, which make them generalist families, while members of *Nitrosopumilaceae*, involved in specific functions related to N cycling, might respond differently to treatments. In any of the Falconara sample, we did not observe the presence of known hydrocarbonoclastic bacteria, suggesting that a variety of still un-identified bacterial taxa can promote PAH degradation, possibly through syntrophic and mutualistic interactions between degrading and non-degrading oil bacteria and/or biotransformation processes through co-metabolism.

Bioaugmentation experiments carried out on Bagnoli sediments characterised by different PAH concentrations (ca. 110 and 330 mg kg^{-1} total PAHs) using a mix of bacteria, a mix of fungi and a mix of both previously isolated and identified from Falconara sediments were effective in the degradation of these organic pollutants. In particular after 1 month of biotreatment of the sediment contaminated by ca. 110 mg Kg^{-1} PAHs, its oncentrations decreased more than two orders of magnitude with a degradtion efficiency close to 99%, independent by the typology of the added microbial consortia (i.e. only bacteria or fungi or a mix of both). In the sediments characterised by a initial total PAH contamination of ca. 330 mg Kg^{-1}, biotreatments carried out using the same bioaugmentation approach allowed us to significantly reduce hydrocarbon concentration after 3 months of incubation, with the highest degradation efficiency in mesocosms containing the mix of bacteria and fungi (ca. 71%), followed by those containing the mix of fungi or bacteria alone (58% and 46%, respectively). Conversely to what previously reported, the different bioagumenation experiments carried out on Bagnoli sediments did not determine major changes in the repartitrion of heavy metals among the different geochemical fractions and thus on their bioavalability and potential toxicity. This aspect is particular relevant

for the ecotoxicological risk that may arise, especially if bioremediation approaches are applied *in situ*. In the different bioaugmented sediments, an increase of *Flavobacteriaceae* was generally observed, whereas a variety of fungi belonging to different families were encountered. During the time-course experiments we checked through metabarcoding of the overall bacterial and fungal assemblages present in the sediments, sequences belonging to the added bacterial and fungal taxa in order to explore their potential role in the biodegradation of PAHs. Such analysis revealed that different bacterial and fungal strains added to contaminated sediments can have different ability to cope with the harsh environmental conditions and/or with competition processes with the autochthonous microbial components, thus potentially contributing to a different extent in the biodegradation of PAHs.

Overall findings reported in this study indicate that bioaugmentation strategies based on the addition of selected fungal-bacterial consortia can be effective for the bioremediation of marine sediments highly contaminated with PAHs, possibly opening alternative management options respect to common practices based on dredging and landfill disposal.

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