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Mycoremediation per la degradazione di idrocarburi inquinanti

Mycoremediation for degrading hydrocarbon pollutants

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Abstract

Nowadays waste products derived from human life and industrial activities represent a serious problem in the world, due to their difficulty of disposal and recovery. Although in the past little importance has been given to environmental quality, the last two decades have been characterized by an increasing awareness on the topic; nevertheless, pollution from many organic substances such as hydrocarbons, remains a mostly unsolved issue, because these compounds are often recalcitrant and show high levels of toxicity.

In recent years, the scientific interest for hydrocarbons is mainly related to their recognized carcinogenic action resulting from the metabolic transformations of these compounds into diol-epoxides, molecules able to bind to DNA and to induce genetic mutations (Man *et al.*2013).

These recalcitrant substances are known to enter the environment frequently and in large scale via several routes (Singh, 2006). One of the major routes is the spill on the ground, often due to neglected maintenance of structures dedicated to production, storage and distribution; as concerns terrestrial environments, these phenomena may determine some harmful effects such as surface water pollution through runoff, groundwater pollution by leachate, evaporative atmospheric pollution, sublimation or wind drift, thus resulting in decreases in crop yields, poisoning through the food chain and impact on the landscape. Another route includes production, storage and transportation which represent a hazard due to accidental releases of petroleum compounds, mostly in marine environments, especially involving bottom sediments.

Currently, restoring areas polluted by hydrocarbon products usually requires some chemical, physical, and biological treatments. Among these, biological treatments are more suitable for sustainable and economic applications because traditional remediation techniques for the treatment of hydrocarbon compounds are often limited by application costs and low efficiency.

Hence, the present study proposes a sustainable and economic biotechnology aimed at allowing the recovery and disposal of soils and marine sediments contaminated by hydrocarbons. The project represents a response to the need for providing a biotechnological protocol devoted to safeguard the environment andterritory and to allow the reclamation of sites contaminated by substances which have been deemed harmful to human health. As it is known, some fungal species are extremophiles, able to adapt to adverse environmental conditions (both for abiotic factors and for nutrient deficiency) which would be limiting for most living organisms (Gadd, 2007); this feature makes some fungi an important tool to biodegrade pollutant compounds

(Kumar *et al.* 2011). For these reasons, as concerns the hydrocarbon pollution of soils and marine sediments, the present study proposes an alternative solution to the traditional technologies of rehabilitation and environmental remediation, through the study, isolation, identification, and exploitation of those fungi able to degrade these toxic compounds. Several experiments reported in the literature demonstrate that fungi have an ability to metabolize and degrade many hydrocarbon compounds, such as oils, petroleum derivatives, and polycyclic aromatic hydrocarbons (Cerniglia *et al.* 2010; Harms *et al*. 2011; Al-Jawhari *et al.* 2014; Reyes-César *et al.* 2014; Marco-Urrea *et al.* 2015).

The studies conducted so far provide a general overview about fungal remediation activity, contributing to support the hypothesis of the possible large-scale application of some fungi in the degradation processes of organic pollutants. The research is aimed at assessing the sustainability of these innovative biotechnologies and at deepening the knowledge of the factors that may affect the activity of some fungal species or strains within different matrices contaminated by different types of hydrocarbons. The study is the result of a multidisciplinary approach involving mycologists, geologists, chemists, and oceanographers.

The thesis is organized as follows. Chapter 1 presents the hydrocarbons focusing on polycyclic aromatic hydrocarbons (PAHs); it describes the traditional remediation techniques which are currently used and defines the role of fungi in the degradation of recalcitrant organic compounds and in the remediation of contaminated soils and waters (mycoremediation). Chapter 2 details the aims of the thesis, while in Chapter 3 the materials and methods are listed and described.

Chapter 4 deals with the mycological characterization of a marine area polluted by hydrocarbon compounds in relation to different substrates (biotic and abiotic), depths and marine currents, to identify and select a pool of fungal species adaptable to extreme polluted environments, which could be used for bioremediation purposes.

Chapter 5 shows the results of the investigations on 15 fungal species isolated from a real oily slime and screened to assess their ability to degrade a PAH mixture. The most suitable fungal strains were employed in the *in vitro* degradation tests.

Chapter 6 investigates, through pilot-scale experiments, the role that fungi can play as tools for the remediation of real polluted matrices contaminated by petroleum derivatives.

Finally, Chapter 7 outlines the conclusions, showing the significant importance of the fungal exploitation for recovering marine and terrestrial areas contaminated by hydrocarbons. Furthermore, some hints for future works and applications are provided.

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Chapter one

INTRODUCTION

1.1 Hydrocarbons and PAHs

Hydrocarbons are organic compounds with low solubility in water, consisting in carbon and hydrogen atoms, (Pretsch *et al.* 2000); they can be divided in hydrocarbons with high or low molecular weight; those with a low molecular weight (C <12) are gaseous at room temperature, while those with high molecular weight (C> 12) are liquid or solid and they are generally very recalcitrant.

Among hydrocarbon compounds, PAHs (Polycyclic Aromatic Hydrocarbons) represent undoubtedly the most recalcitrant; these are a large group of widespread environmental pollutants (Clemente *et al.* 2001; Jiang *et al.* 2009) distributed in aquatic environments (Shi *et al*. 2005), sediments, and soils (Lima *et al.* 2005). They are waste products derived from an incomplete combustion of wood and fossil fuels, produced by cooking operations and gasification processes, especially in coal processing; they are also produced by other anthropic sources of incomplete combustion such as automobile exhaust, thermal power plants, waste incineration and industrial emissions (Jones *et al.* 1989; Benner *et al.* 1990; Clemente *et al.* 2001). Furthermore, these compounds are derived from some natural sources such as oil seeps, ancient sediment erosion and are naturally formed during grassland and forest fires or volcanic eruptions (IARC, 1983).

PAHs and other hydrocarbon compounds, of natural and anthropic origin, end up in the atmosphere and then fall back on specific environmental compartments, such as soils, compromising their protective, productive and ecological functions (Samanta *et al.* 2002). From the soil, hydrocarbon products infiltrate in the water sources and may reach the phreatic layer, thus propagating in the surrounding areas, potentially far away from the main source of pollution (Figure 1).

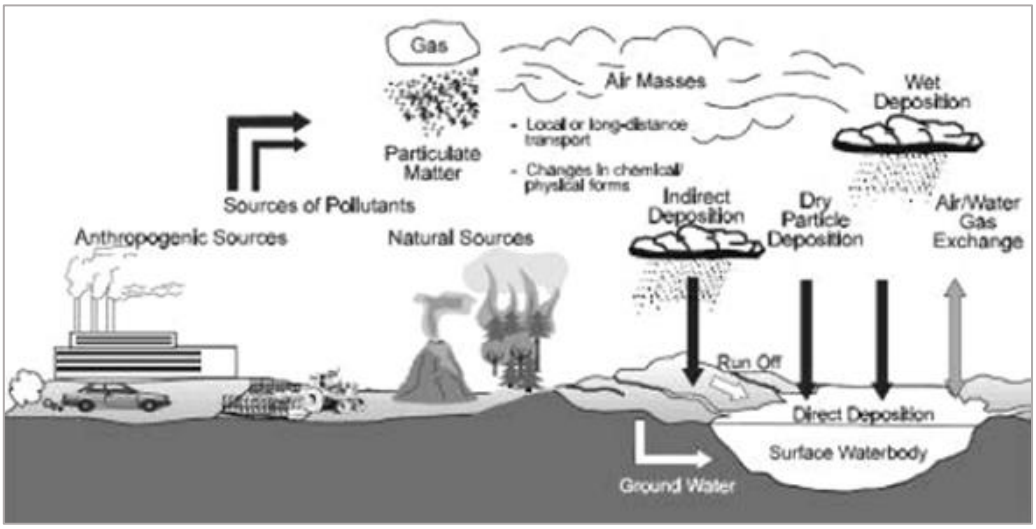


Fig. 1. Hydrocarbons and PAHs dispersion in the atmosphere and their deposition on soil and water (Source: U.S. EPA 2000).

Due to the environmental persistence and the carcinogenic potential, these compounds have been widely studied by various researchers (Matsubara *et al.* 2006; Wang *et al.* 2013; Farrington *et al.* 2014). In this scenario it is important to focus research on innovative remediation technologies aimed to reduce the propagation and persistence of these toxic compounds in the biosphere, in order to limit their negative effects on the environment and on human health.

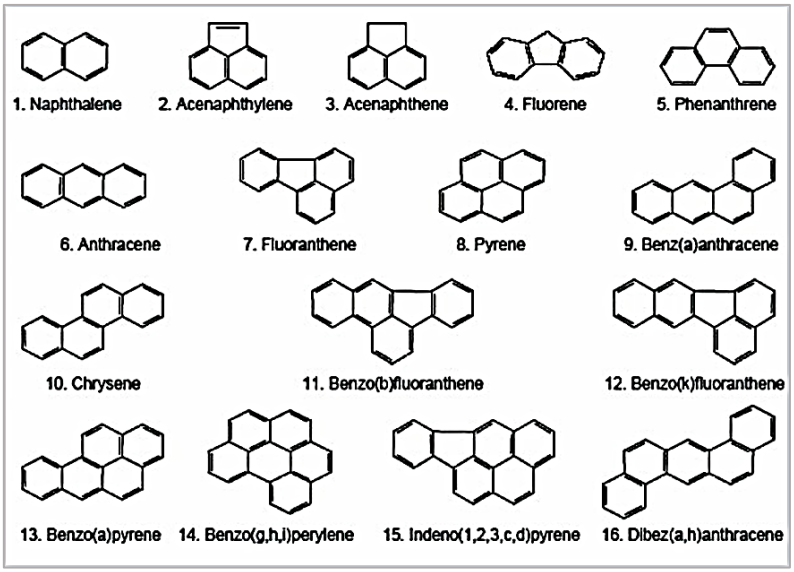


Fig. 2. Main PAHs according to the U.S. EPA

PAHs	CAS-Number	Cancer for the human (IARC, 2016)	Main pollutants according to the EPA (USA)	Classification according to Annex VI, Regulation (CE) No 1272/2008
Benzo(a)pyrene	50-32-8	1	X	X (carc.1B)
Dibenzo(a,h)anthracene	53-70-3	2A	X	X (carc.1B)
Benzo(a)anthracene	56-55-3	2B	X	X (carc.1B)
Benzo(b)fluoranthene	205-99-2	2B	X	X (carc.1B)
Benzo(k)fluoranthene	207-08-9	2B	X	X (carc.1B)
Chrysene	218-01-9	2B	X	X (carc.1B)
Indeno(1,2,3,c,d)pyrene	193-39-5	2B	X	
Benzo(g,h,i)perylene	191-24-2	3	X	
Naphthalene	91-20-3	2B	X	X (carc. 2)
Anthracene	120-12-7	3	X	
Acenaphthene	83-32-9	3	X	
Fluoranthene	206-44-0	3	X	
Fluorene	86-73-7	3	X	
Phenanthrene	85-01-8	3	X	
Pyrene	129-00-0	3	X	
Acenaphthylene	208-96-8	3	X	

IARC classification:

Group 1: proven carcinogenicity
Group 2A: probable carcinogenicity
Group 2B: possible carcinogenicity
Group 3: insufficient data to classify it as a human carcinogen

Table1. Main PAHs and carcinogenic groups reported in the IARC classification (2016).

1.2 Traditional remediation techniques

In recent years, the number of remediation methods available has considerably increased; traditional remediation techniques are used to remove many types of recalcitrant organic pollutants and consist in particular chemical, physical and biological treatments (Riser-Roberts 1998; Sarkar *et al.* 2005).

Among these, isolation or encapsulation (Sheoran *et al.* 2008), chemical-physical restoration (Gustavson *et al.* 2000; Riding *et al.* 2013) and thermal restoration (Chou *et al.* 2009) are the most common techniques that are nowadays used to remediate polluted matrices.

Isolation or encapsulation techniques are based on confining the polluted matrix to reduce the pollutant propagation by building physical barriers around it; subsequently some products are applied on the barriers in order to avoid the diffusion of the pollutants in surface water and groundwater.

Chemical-physical remediation techniques are instead based on the use and exploitation of the physical / chemical properties of the pollutants: chemical treatments include redox reactions that transform pollutants into less toxic or less mobile compounds.

Physical treatments, on the other hand, are based on specific systems aimed at separating the contaminants from the solid or liquid matrices to obtain a concentrated solution that will then be destined for a final specific treatment. In this scenario, the most used techniques are vapor extraction, air injection, soil washing, electrokinetic treatment, chemical and permeable reactive barriers. Another very popular category of traditional remediation techniques is the thermal treatments; among the thermal remediation techniques, the following are used:

- Incineration process: this technique consists in complete oxidation of the combustible part of the waste. The heat produced by this combustion process may be employed for both electric and thermal energy production. This process occurs in incinerators, that are characterized by energy recover.
- Thermic desorption: this is a process based on soil treatments aimed to evaporate the polluting substances. This treatment technique is best fit to treat volatile and semi-volatile pollutants and is focused on vaporizing and separating the organic pollutants from the soil (Henner *et al.* 1997).
- Pyrolysis: this technique involves the chemical decomposition of organic materials induced by heat in the absence of oxygen (González-Pérez *et al.* 2014; Lam *et al.* 2016); it occurs at temperatures above 430°C and it is useful to break organic macromolecules into smaller and simpler structures more easily attacked by some microorganisms.

The mentioned techniques are currently the most widely used in remediation treatments as they are the ones that allow quicker reclamation; however, in addition to being very expensive, they often cause high environmental impact and may interact negatively with natural degradation processes.

1.3 Bioremediation techniques

Bioremediation of organic compounds is a technology leading to the transformation or degradation of organic contaminants in non-toxic or less toxic compounds by some extremophile microorganisms (Vidali, 2001). In recent years, this ecological technique has been used in several sites worldwide, with varying degrees of success (Farhadian *et al.* 2008; Wolicka *et al.* 2009; Perelo *et al.* 2010) but unfortunately, little is yet known about techniques, advantages and disadvantages of the processes involved.

Researchers have developed different bioremediation techniques aiming at restoring polluted environments with low application costs compared to traditional remediation techniques. In this scenario, the

autochthonous microorganisms play an important role in the degradation of polluting substances (Verma *et al.* 2016), because they have supposedly developed many adaptations useful for their survival and growth in the polluted matrix.

Two types of biodegradation are currently known for bioremediation: among these, the *biotransformation* process is an important remediation technique implying the use of biological systems to produce chemical alterations in contaminating macromolecules, in order to degrade them to non-toxic compounds (Pimentel *et al.* 2011; Kebamo *et al.* 2015). Another bioremediation model is *biomineralization* can be rather defined as the complete biodegradation of organic materials into inorganic constituents such as CO_2 or H_2O_2 (Mann 2001; Simkiss 2012).

However, it is known that the effectiveness of bioremediation depends on different chemical/physical parameters such as the nature of the pollutant, the size and depth of the contaminated matrix and the degree of contamination; these are some variables that may have negative or positive feedback on the response of the involved organisms.

Bioremediation techniques aimed to remove recalcitrant organic compounds such as hydrocarbons, PAHs, oils and similar, can be employed *in-situ* or *ex situ,* in aerobic or anaerobic conditions.

In-situ techniques are the most widely applied due to lower operating costs and environmental disturbance; this technology leads to waste treatments on site by the exploitation of specific organisms, not pathogenic and often autochtonous, (Verma *et al.* 2016) adding specific nutrients and oxygen to the polluted matrix (Juwarkar *et al.* 2010; Azubuike *et al.* 2016) to increase their growth rate and degradation efficiency.

The *ex situ* bioremediation techniques consist of excavating or removing the contaminated soil and subject it to biological treatment. It involves pretreating the soil with water, specific nutrients, additives and pH modifications in order to increase the growth of autochthonous microorganisms.

The most important bioremediation *in situ* treatments are:

- Bioventing

This treatment is the *in-situ* process with a wider application; it involves supplying air and nutrients to the contaminated soil through wells or vents, in order to stimulate the growth of indigenous microorganisms

(Philp *et al.* 2005). This technique is often used to restore small and shallow areas contaminated by light spilled petroleum products (Höhener *et al.* 2014) and takes place with optimal soil conditions such as pH 6-8 and temperatures between 0 and 40˚C.

- Biosparging

The biosparging technique involves the injection of pressurized air below the water table in order to increase the groundwater oxygen concentrations and enhance the rate of biodegradation; in this scenario some pioneriing organisms such as bacteria and fungi represent an important tool of remediation both for their ecological plasticity and for the adaptability to extreme environmental conditions. Biosparging is widely used in treating aquifers contaminated by petroleum products, especially benzene, toluene, ethylbenzene and xylene (Kao *et al.* 2008).

- Bioaugmentation

Bioaugmentation is the application of indigenous, allochthonous or even genetically modified microorganisms to hazardously polluted waste sites, to accelerate the removal of recalcitrant compounds (Mrozik *et al.* 2010). This technique is employed in small-scale remediation of polluted sites, and it is mainly applied to restore municipal wastewater in order to restart activated sludge bioreactors (Herrero and Stuckey, 2015).

The most important bioremediation *ex situ* treatments are:

- Biopiles

In this technique, the excavated soil is mixed with additives and placed on different layers in aboveground containers (Gomez *et al.* 2014). This process is generally used for the treatment of soils contaminated by fuel hydrocarbons (Dias *et al.* 2015; Azubuike *et al.* 2016), non-halogenated VOCs, halogenated VOCs, SVOCs and pesticides. It is a process in which compost is formed into piles and aerated with blowers or vacuum pumps.

- Bioreactors

These apparatuses are often employed to treat large quantities of organic pollutants *ex situ* through chemical processes that involve organisms able to degrade specific contaminants; the use of bioreactors containing residues of soil polluted by crude oil is very useful, as its functioning allows to keep track of changes in the dynamics of the microbial population, thus allowing easy characterization of the main bacterial or fungal communities involved in the processes of bioremediation (Chikere *et al.* 2012; Zangi-Kotler *et al.* 2015). In some cases, a pretreatment is required such as soil washing or physical extraction before the matrix is placed in a bioreactor.

1.4 Microorganisms involved in degradation processes of hydrocarbons compounds and PAHs

Some microorganisms such as fungi, bacteria, algae and plants play an important role in the detoxification of polluted matrices (Chaudhry *et al.* 2005; Das *et al.* 2011; Chekroun *et al.* 2014); as regards hydrocarbons products and PAHs, it is known that bacteria and fungi are the main degraders of oil spilled in environment.

It has been shown that bacteria have evolved tolerance and internal pH control strategies, which have enabled them to survive and colonize contaminated soils and water. In 1988, some researchers published the first study on the isolation of a bacterium from an environment polluted by PAHs (Heitkamp *et al.* 1988) and proved its effectiveness in the degrading of fluoranthene, pyrene, 1-nitropyrene, 3-methylcholanthrene and 6-nitrochrysene. Also, in 1988, Mahaffey *et al.* presented the first direct demonstration of ring fission during HMW PAH biodegradation (Kanaly *et al.* 2000).

Several bacteria are potential agents of hydrocarbon degradation such as *Pseudomonas, Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia, Mycobacterium and Sphingomonas*, as reported by Chekroun *et al.* (2014).

Another emerging technology intended to resolve a wide variety of environmental pollution problems, including the cleanup of soils and groundwater contaminated with hydrocarbons, is the phytoremediation; this biotechnique is focused on the specific use of extremophile plants to remove contaminants from the matrix, or facilitate the immobilization or degradation of the pollutants (McIntyreet *et al.* 2003; Huang *et al.* 2004; Pilon-Smits, 2005). Algae have been employed in the remediation of hydrocarbon oil pollution; it has

been proved that these aquatic organisms may degrade a large percentage of the hydrocarbons in motor and crude oil (Walker *et al.* 1975).

Vascular plants can also used in hydrocarbon phytoremediation; in this context the association among rhizosphere, bacteria and fungi is very effective in restoring the ecological and environmental balances of the polluted areas (Harvey *et al.* 2002). One of the main benefits of this innovative technique is that plants can be subsequently harvested, processed and disposed of without great expense, thus phytoremediation could be easily applied to the treatment of numerous contaminated sites.

It has been recently proved that also some fungal species may be employed for bioremediation purposes with excellent effectivity and low application costs (D'Annibale *et al.* 2006; Okparanma *et al.* 2011). Fungi are plastic organisms, able to colonize very different environments, penetrating the substrates and adapting to stressful environmental conditions (such as acidic pH and lack of nutrients). It is known that some fungal organisms can use a wide range of different carbon sources and are capable to transform hydrocarbon compounds into non-toxic compounds (Verdin *et al.* 2004; Cerniglia *et al.* 2010) through specific enzymes, such as laccase, lignin peroxidase, and manganese peroxidase (Baborová *et al.* 2006; Reyes-César *et al.* 2014; Marco-Urrea *et al.* 2015).

Mycoremediation is a technique consisting in the use of fungi to clean up polluted sites, and it represents one of the most innovative and cost-effective biotechnologies, that could be improved in order to be used as a support or even to replace traditional recovery methods; nevertheless, this technology can have some disadvantages e.g. long application times, degradation efficiency dependant on seasonality and incomplete knowledge of the degradation mechanisms regarding xenobiotic compounds.

1.5 Role of fungi in bioremediation of hydrocarbons compounds and PAHs

Fungi are chemoheterotrophic organisms able to employ different ecological strategies (symbiont, pathogens or saprotrophs), colonize substrates of various nature, including those polluted by organic compounds (Gadd 2004), and often capable of surviving in environmental conditions which are extreme for most organisms (Burford *et al.* 2003; Gadd 2004).

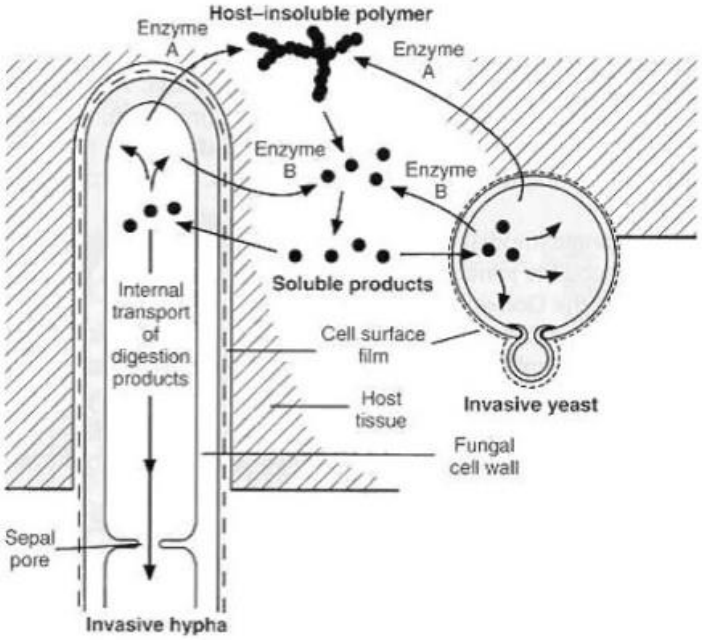


Fig. 3. Fungal metabolism scheme (Deacon, 2006).

Some fungi may degrade organic compounds, such as hydrocarbons, by specific enzymes which allow a rapid degradation of the pollutant down to its mineralization (Cerniglia 1993). Among the fungi able to degrade hydrocarbons the ligninolytic ones are best fit to remediate polluted areas; some fungi are indeed known for their ability to degrade lignocellulose (Pointing *et al.* 2000; Sánchez *et al.* 2009), playing an important role in the biochemical transformation of detritus through the production of extracellular enzymes (Guillén *et al.* 2005) (Figure 3). These enzymes may degrade not only lignin but also several recalcitrant environmental compounds that have structural analogies with it, such as hydrocarbons compounds (Baborová *et al.* 2006; Haritash *et al.* 2009).

These features, together with their ecological plasticity, enable fungi to be a potential tool for the remediation of contaminated areas. Numerous studies were carried out in order to assess which enzymes are involved in the hydrocarbon degradation, proving that are the main extracellular enzymes secreted by lignin

peroxidase, manganese peroxidases and laccase fungi that degrade these toxic compounds (Verdin *et al.* 2004; Baborová *et al.* 2006; Cerniglia *et al.* 2010).

The application of fungal technology to the remediation of polluted areas holds promise since 1985, when the white-rot fungus *Phanerochaete chrysosporium* was found to be able to degrade several important environmental pollutants (Bumpus *et al.* 1985; George *et al.* 1989; Cripps *et al.* 1990; Hammel *et al.* 1992; Kirby *et al.* 1995). Since then several strains of white-rot fungi, such as *Pleurotus ostreatus, Irpex lacteus, Trametes versicolor,* have been demonstrated to attack some recalcitrant organopollutants including hydrocarbon compounds; moreover, it has been shown that, in addition to ligninolytic fungi, also some extremophile fungal organisms can use these substances as alternative carbon sources for their growth (Verdin *et al.* 2004; Cerniglia *et al.* 2010), revealing adaptability to adverse environmental conditions (Reyes-César *et al.* 2014; Marco-Urrea *et al.* 2015); among these, *Cunninhamella elegans* has proven able to degrade some PAHs compounds such as chrysene, pyrene, fluorantene, benzo(a)pirene in less toxic products (Pothuluri *et al.* 1995). *Amorphoteca resinae* is another fungal species know for the ability to grow in presence of hydrocarbon pollution and to degrade PAHs; in the last two decades, this fungus has been repeatedly found growing in vehicle tanks, causing malfunctions and damage to engines. Several other species of microfungi have been found to be able to metabolize these compounds, such as *Aureobasidium* (Sihag *et al.* 2014), *Rhodotorula* (Cerniglia *et al.* 2010, Sihag *et al.* 2014), *Sporobolomyces* (Sihag *et al.* 2014), *Geotrichum* (Giraud *et al.* 2001; Cerniglia *et al.* 2010) *Rhizopus* (Cerniglia *et al.* 2010; Fernández-Luqueño *et al.* 2011). *Absidia* (Garon *et al.* 2004) and *Aspergillus* spp. (Ye *et al.* 2011).

The main physical factors known to influence the efficacy of fungal biodegradation are:

- Temperature (high temperature favors chemical and biochemical reactions).
- Humidity, that is essential to increase the fungal activity (60% humidity is often the ideal parameter).
- Oxygen. The presence of oxygen is essential: not only it allows oxidative processes to degrade part of the hydrocarbons, but it also promotes the fungal growth and activity.
- pH (to restore acidic or basic soils specific fungal strains, adapted to particular pH values, may be employed).

- C, N, P (the presence and availability in the soil of macronutrients such as carbon, nitrogen and phosphorus are a determining factor that regulates the hydrocarbon degradation by autochthonous fungal communities).
- Soil particle size (fungi generally have a better degradation capacity on gravel and sand than on clay, as their hyphae cannot expand into soils presenting particle sizes < 0.002 mm).
- Bioavailability of pollutants.

Although it is known that the physical factors mentioned above may influence the fungal degradation efficiency, little is yet known about fungal degradation mechanisms regarding hydrocarbons, and the knowledge about the application times of mycoremediation techniques on a large scale is still incomplete; hence, further research is needed to increase the information we possess about this innovative biotechnology, and to improve its *in situ* applicability.

1.6 References

AI-Jawhari IFH. 2014. Ability of some soil fungi in biodegradation of petroleum hydrocarbon. J Appl Environ Microbiol. 2(2):46-52.

Azubuike CC, Chikere CB, Okpokwasili GC. 2016. Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World J Microbiol Biotechnol. 32(11):180.

Baborová P, Möder M, Baldrian P, Cajthamlová K, Cajthaml T. 2006. Purification of a new manganese peroxidase of the white-rot fungus Irpex lacteus, and degradation of polycyclic aromatic hydrocarbons by the enzyme. Res Microbiol. 157(3):248-253.

Benner Jr BA, Bryner NP, Wise SA, Mulholland GW, Lao RC, Fingas MF. 1990. Polycyclic aromatic hydrocarbon emissions from the combustion of crude oil on water. Environ Sci Technol. 24(9):1418-1427.

Bumpus JA, Tien M, Wright D, Aust SD. 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science. 228(4706):1434-1436.

Burford EP, Fomina M, Gadd GM. 2003. Fungal involvement in bioweathering and biotransformation of rocks and minerals. Mineral Mag. 67(6):1127-1155.

Cerniglia CE, Sutherland JB. 2010. Degradation of polycyclic aromatic hydrocarbons by fungi. In Handbook of hydrocarbon and lipid microbiology. Springer Berlin Heidelberg. 2010:2079-2110

Cerniglia CE. 1993. Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotech. 4(3):331-338.

Chaudhry Q, Blom-Zandstra M, Gupta SK, Joner E. 2005. Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment (15 pp). Environ Sci Pollut R. 12(1):34-48.

Chekroun KB, Sánchez E, Baghour M. 2014. The role of algae in bioremediation of organic pollutants. Int Res J Public Environ Health. 1(2):19-32.

Chikere CB, Chikere BO, Okpokwasili GC. 2012. Bioreactor-based bioremediation of hydrocarbon-polluted Niger Delta marine sediment, Nigeria. 3 Biotech. 2(1):53-66.

Chou JD, Wey MY, Chang SH. 2009. Study on Pb and PAHs emission levels of heavy metals-and PAHs-contaminated soil during thermal treatment process. J Environ Eng. 136(1):112-118.

Clemente AR, Anazawa TA, Durrant LR. 2001. Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. Braz J Microbiol. 32(4):255-261.

Cripps C, Bumpus JA, Aust SD. 1990. Biodegradation of azo and heterocyclic dyes by Phanerochaete chrysosporium. Appl Environ Microbiol. 56(4):1114-1118.

D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M. 2006. Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. Appl Environ Microbiol. 72(1):28-36.

Das N, Chandran P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol Res Inter. 2011.

Dias RL, Ruberto L, Calabró A, Balbo AL, Del Panno MT, Mac Cormack WP. 2015. Hydrocarbon removal and bacterial community structure in on-site biostimulated biopile systems designed for bioremediation of diesel-contaminated Antarctic soil. Polar Biol. 38(5):677-687.

Farhadian M, Vachelard C, Duchez D, Larroche C. 2008. In situ bioremediation of monoaromatic pollutants in groundwater: a review. Bioresource Technol. 99(13):5296-5308.

Farrington JW, Takada H. 2014. Persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and plastics: Examples of the status, trend, and cycling of organic chemicals of environmental concern in the ocean. Oceanography. 27(1):196-213.

Fernández-Luqueño F, Valenzuela-Encinas C, Marsch R, Martínez-Suárez C, Vázquez-Núñez E, Dendooven L. 2011. Microbial communities to mitigate contamination of PAHs in soil—possibilities and challenges: a review. Environ Sci Pollut R. 18(1):12-30.

Gadd GM. 2004. Mycotransformation of organic and inorganic substrates. Mycologist. 18(2):60-70.

Gadd GM. 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol Res. 111(1):3-49.

Garon D, Sage L, Wouessidjewe D, Seigle-Murandi F. 2004. Enhanced degradation of fluorene in soil slurry by Absidia cylindrospora and maltosyl-cyclodextrin. Chemosphere. 56(2):159-166.

George EJ, Neufeld RD. 1989. Degradation of fluorene in soil by fungus Phanerochaete chrysosporium. Biotechnol Bioeng. 33(10):1306-1310.

Giraud F, Guiraud P, Kadri M, Blake G, Steiman R. 2001. Biodegradation of anthracene and fluoranthene by fungi isolated from an experimental constructed wetland for wastewater treatment. Water Res. 35(17):4126-4136.

Gomez F, Sartaj M. 2014. Optimization of field scale biopiles for bioremediation of petroleum hydrocarbon contaminated soil at low temperature conditions by response surface methodology (RSM). Int Biodeter Biodegr. 89(2014):103-109.

González-Pérez JA, Almendros G, De la Rosa JM, González-Vila FJ. 2014. Appraisal of polycyclic aromatic hydrocarbons (PAHs) in environmental matrices by analytical pyrolysis (Py–GC/MS). J Anal Appl Pyrol. 109(2014):1-8.

Guillén F, Martínez MJ, Gutiérrez A, Del Rio JC. 2005. Biodegradation of lignocellu-losics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol. 8(2005):195-204.

Gustavson KE, Harkin JM. 2000. Comparison of sampling techniques and evaluation of semipermeable membrane devices (SPMDs) for monitoring polynuclear aromatic hydrocarbons (PAHs) in groundwater. Environ Sci Technol. 34(20):4445-4451.

Hammel KE, Gai WZ, Green B, Moen MA. 1992. Oxidative degradation of phenanthrene by the ligninolytic fungus Phanerochaete chrysosporium. Appl Environ Microbiol. 58(6):1832-1838.

Haritash AK, Kaushik CP. 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J Hazard Mater. 169(1-3):1-15.

Harms H, Schlosser D, Wick LY. 2011. Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol. 9(3):177.

Harvey PJ, Campanella BF, Castro PM, Harms H, Lichtfouse E, Schäffner AR., Werck-Reichhart D. 2002. Phytoremediation of polyaromatic hydrocarbons, anilines and phenols. Environ Sci Pollut R. 9(1):29-47.

Heitkamp MA, Cerniglia CE. 1988. Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl Environ Microbiol. 54(6):1612-1614.

Henner P, Schiavon M, Morel JL, Lichtfouse E. 1997. Polycyclic aromatic hydrocarbon (PAH) occurrence and remediation methods. Analusis. 25(9-10):M56-M59.

Herrero M, Stuckey DC. 2015. Bioaugmentation and its application in wastewater treatment: a review. Chemosphere. 140(2015):119-128.

Höhener P, Ponsin V. 2014. In situ vadose zone bioremediation. Curr Opin Biotech. 27(2014):1-7.

Huang XD, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM. 2004. A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environ Pollut. 130(3):465-476.

IARC. 1983. Polynuclear Aromatic Compounds, Part 1: Chemical, Environmental and Experimental Data, vol. 32. International Agency for Research of Cancer, Lyon, France.

Jiang YF, Wang XT, Wang F, Jia Y, Wu MH, Sheng GY, Fu JM. 2009. Levels, composition profiles and sources of polycyclic aromatic hydrocarbons in urban soil of Shanghai, China. Chemosphere. 75(8):1112-1118.

Jones KC, Stratford JA, Waterhouse KS, Furlong ET, Giger W, Hites RA, Schaffner C, Johnston AE. 1989. Increases in the polynuclear aromatic hydrocarbons content of as agricultural soil over the last century. Environ. Sci. Technol. 23(1):95-101.

Juwarkar AA, Singh SK, Mudhoo A. 2010. A comprehensive overview of elements in bioremediation. Rev Environ Sci Bio. 9(3):215-288.

Kanaly RA, Harayama S. 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol. 182(8):2059-2067.

Kao CM, Chen CY, Chen SC, Chien HY, Chen YL. 2008. Application of in situ biosparging to remediate a petroleum-hydrocarbon spill site: Field and microbial evaluation. Chemosphere. 70(8):1492-1499.

Kebamo S, Tesema S, Geleta B. 2015. The role of biotransformation in drug discovery and development. J Drug Metab Toxicol. 6(196):2.

Kirby N, Mc Mullan G, Marchant R. 1995. Decolourisation of an artificial textile effluent by Phanerochaete chrysosporium. Biotechnol Lett. 17(7):761-764.

Kumar A, Bisht BS, Joshi VD, Dhewa T. 2011. Review on bioremediation of polluted environment: A management tool. Int J Environ Sci. 1(6):1079.

Lam SS, Liew RK, Jusoh A, Chong CT, Ani FN, Chase HA. 2016. Progress in waste oil to sustainable energy, with emphasis on pyrolysis techniques. Renew Sust Energ Rev. 53(2016):741-753.

Lima ALC, Farrington JW, Reddy CM. 2005. Combustion-derived polycyclic aromatic hydrocarbons in the environment—a review. Environ Forensics. 6(2):109-131.

Man YB, Kang Y, Wang HS, Lau W, Li H, Sun XL, Giesy JP, Chow KL, Wong MH. 2013. Cancer risk assessments of Hong Kong soils contaminated by polycyclic aromatic hydrocarbons. J. Hazard. Mater. 261(2013):770–776.

Mann S. 2001. Biomineralization: principles and concepts in bioinorganic materials chemistry (Vol. 5). Oxford University Press on Demand.

Marco-Urrea E, García-Romera I, Aranda E. 2015. Potential of non-ligninolytic fungi in bioremediation of chlorinated and polycyclic aromatic hydrocarbons. New biotechnol. 32(6):620-628.

Matsubara M, Lynch JM, De Leij FAAM. 2006. A simple screening procedure for selecting fungi with potential for use in the bioremediation of contaminated land. Enzyme Microb Tech. 39(7):1365-1372.

McIntyre T. 2003. Phytoremediation of heavy metals from soils. In Phytoremediation (pp. 97-123). Springer Berlin Heidelberg.

Mrozik A, Piotrowska-Seget Z. 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol Res. 165(5):363-375.

Okparanma RN, Ayotamuno JM, Davis DD, Allagoa M. 2011. Mycoremediation of polycyclic aromatic hydrocarbons (PAH)-contaminated oil-based drill-cuttings. Afr J Biotechnol. 10(26):5149-5156.

Perelo LW. 2010. In situ and bioremediation of organic pollutants in aquatic sediments. J Hazard Mater. 177(1-3):81-89.

Philp JC, Atlas RM. 2005. Bioremediation of contaminated soils and aquifers. In Bioremediation (pp. 139-236). American Society of Microbiology.

Pilon-Smits E. 2005. Phytoremediation. Annu. Rev. Plant Biol. 56(2005):15-39.

Pimentel MR, Molina G, Dionísio AP, Maróstica Junior MR, Pastore GM. 2011. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnology research international, 2011.

Pointing SB, Hyde KD. 2000. Lignocellulose-degrading marine fungi. Biofouling. 15(1-3):221-229.

Pothuluri JV, Selby A, Evans FE, Freeman JP, Cerniglia CE. 1995. Transformation of chrysene and other polycyclic aromatic hydrocarbon mixtures by the fungus Cunninghamella elegans. Can J Botany. 73(S1):1025-1033.

Pretsch E, Buehlmann P, Affolter C, Pretsch E, Bhuhlmann P, Affolter C. 2000. Structure determination of organic compounds (p. 108). Berlin: Springer-Verlag.

Reyes-César A, Absalón ÁE, Fernández FJ, González JM., Cortés-Espinosa DV. 2014. Biodegradation of a mixture of PAHs by non-ligninolytic fungal strains isolated from crude oil-contaminated soil. World J Microbiol Biotechnol. 30(3):999-1009.

Riding MJ, Doick KJ, Martin FL, Jones KC, Semple KT. 2013. Chemical measures of bioavailability/bioaccessibility of PAHs in soil: fundamentals to application. J Hazard Mater. 261(2013):687-700.

Riser-Roberts E. 1998. Remediation of petroleum contaminated soils: biological, physical, and chemical processes. CRC press.

Samanta SK, Singh OV, Jain RK. 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol. 20(6):243-248.

Sánchez C. 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv. 27(2):185-194.

Sarkar D, Ferguson M, Datta R, Birnbaum S. 2005. Bioremediation of petroleum hydrocarbons in contaminated soils: comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ Pollut. 136(1):187-195.

Sheoran V, Sheoran AS, Poonam P. 2008. Remediation techniques for contaminated soils. Environ Eng Manag J. 7(4):379-387.

Shi Z, Tao S, Pan B, Fan W, He XC, Zuo Q, Xu FL. 2005. Contamination of rivers in Tianjin, China by polycyclic aromatic hydrocarbons. Environ Pollut. 134(1):97-111.

Sihag S, Pathak H, Jaroli DP. 2014. International Journal of pure & applied bioscience. Int. J. Pure App. Biosci. 2(3):185-202.

Simkiss K, Wilbur KM. 2012. Biomineralization. Elsevier. p.337.

Singh H. 2006. Mycoremediation: fungal bioremediation. John Wiley & Sons, Hoboken.p.592.

Verdin A, Sahraoui ALH, Durand R. 2004. Degradation of benzo [a] pyrene by mitosporic fungi and extracellular oxidative enzymes. Int Biodeter Biodegr. 53(2):65-70.

Verma JP, Jaiswal DK. 2016. Book review: advances in biodegradation and bioremediation of industrial waste. Front Microbiol. 6(2016):1555.

Vidali M. 2001. Bioremediation. an overview. Pure Appl Chem. 73(7):1163-1172.

Walker JD, Colwell RR, Petrakis L. 1975. Degradation of petroleum by an alga, Prototheca zopfii. Appl Microbiol. 30(1):79-81.

Wang XT, Miao Y, Zhang Y, Li YC, Wu MH, Yu G. 2013. Polycyclic aromatic hydrocarbons (PAHs) in urban soils of the megacity Shanghai: occurrence, source apportionment and potential human health risk. Sci Total Environ. 447(2013):80-89.

Wolicka D, Suszek A, Borkowski A, Bielecka A. 2009. Application of aerobic microorganisms in bioremediation in situ of soil contaminated by petroleum products. Bioresource Technol. 100(13):3221-3227.

Ye JS, Yin H, Qiang J, Peng H, Qin HM, Zhang N, He BY. 2011. Biodegradation of anthracene by Aspergillus fumigatus. J Hazard Mater. 185(1):174-181.

Zangi-Kotler M, Ben-Dov E, Tiehm A, Kushmaro A. 2015. Microbial community structure and dynamics in a membrane bioreactor supplemented with the flame retardant dibromoneopentyl glycol. Environ Sci Pollut R. 22(22):17615-17624.

Chapter two

AIMS AND SCOPES

2.1 General aims

The general objective of the work is to define and evaluate the applicability of a protocol that involves a set of fungal species to recover marine and terrestrial areas contaminated by hydrocarbon compounds and PAHs. Hence, the research was conducted on different types of matrices; the degradative efficiency of some fungi on an oily slime from the Port of Genoa and on gravel and clay soil matrices contaminated by petroleum derivates was evaluated.

2.2 Specific aims

In the last two decades, many studies on hydrocarbons was performed about fungal degradation activity (April *et al.* 1998; Sutherland, 2004; Marco-Urrea *et al.* 2015; Zafra *et al.* 2015); these researches support the hypothesis that some fungal strains could be employed in the degradation processes of organic pollutants. The researches on mycoremediation conducted so far, as also underlined by D'Annibale *et al.* (2006), have focused on the production of artificial matrices and substrates, subsequently contaminated with the specific recalcitrant organic compounds; hence the degradative capacities of most fungal strains have been tested only *in vitro*. Therefore, investigations are necessary to assess the actual role of fungi in the decontamination of polluted matrices, in non-sterile conditions using the native matrices from the contaminated sites.

The objectives of this work are the following:

- mycological characterizations and chemical-physical analysis (pH, T, density, viscosity, chemical composition) of soils and matrices contaminated by both hydrocarbon compounds and PAHs;

- isolation and selection of native fungal strains, among those present in the selected matrix, by means of the modified Gams plate dilution method (1987) for isolating viable strains;

- cryopreservation at -20 °C and -80 °C of the fungal strains isolated from environments exposed to hydrocarbon pollution, for bioremediation purposes.

- identification of the isolated fungal strains with a polybasic approach (macro-micromorphological, physiological and molecular analysis);

- identification of possible Italian industrial partners, active in the recovery and disposal of hydrocarbon products;

-*in vitro* screenings to select the most resistant autochthonous strains, among those isolated from hydrocarbon-polluted matrices;

- preparation of mesocosms to identify a pool of fungi able to remove recalcitrant hydrocarbons from real non-sterile matrices;

- chemical analysis of the matrices after the fungal treatment to test the degradation efficiency.

The study is based on the hypothesis that isolating autochthonous organisms allows the identification of fungal strains that are particularly adapted to live in presence of contaminants. Moreover, the work is aimed at improving the knowledge on the correlation between fungi and mycological parameters in hydrocarbon contaminated areas, in order to investigate the possible application of fungi in bioremediation.

2.3 References

April TM, Abbott SP, Foght JM, Currah RS. 1998. Degradation of hydrocarbons in crude oil by the ascomycete Pseudallescheria boydii (Microascaceae). Can J Microbiol. 44(3):270-278.

Boopathy R. 2000. Factors limiting bioremediation technologies. Bioresource Technol. 74(1):63-67.

Burford EP, Fomina M, Gadd, GM. 2003. Fungal involvement in bioweathering and biotransformation of rocks and minerals. Mineral Mag. 67(6):1127-1155.

D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M. 2006. Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. Appl Environ Microb. 72(1):28-36.

Gams W, van der Aa HA, van der Plaats-Niterink AJ, Samson RA, Stalpers JA. 1987. (C.B.S.) Centraalbureau voor Schimmelcultures. Course of Mycology 3rd ed. Baarn: Institute of the Royal Netherlands Academy of Arts and Sciences. 136p.

Hyde KD, Jones EG, Leaño E, Pointing SB, Poonyth AD, Vrijmoed LL. 1998. Role of fungi in marine ecosystems. Biodivers Conserv. 7(9):1147-1161.

Marco-Urrea E, García-Romera I, Aranda E. 2015. Potential of non-ligninolytic fungi in bioremediation of chlorinated and polycyclic aromatic hydrocarbons. New biotechnol. 32(6):620-628.

Smith GW, Weil E. 2004. Aspergillosis of gorgonians. In Coral Health and Disease (pp. 279-287). Springer, Berlin, Heidelberg.

Sutherland JB. 2004. Degradation of hydrocarbons by yeast and filamentous fungi. Fungal biotechnology in agricultural, food and environmental applications. 417-429.

Zafra G, Moreno-Montaño A, Absalón ÁE, Cortés-Espinosa DV. 2015. Degradation of polycyclic aromatic hydrocarbons in soil by a tolerant strain of Trichoderma asperellum. Environ Sci Pollut R. 22(2):1034-1042.

Chapter three

MATERIALS AND METHODS

3.1 Generic protocol of fungal isolation from environments polluted by hydrocarbons

Fungi were isolated from extreme environments such as the waters of the Genoa Port, marine sediments, an oily slime or soils contaminated by hydrocarbons. The isolation of vital fungal strains was carried out by using different modified plate dilution techniques (Greco et al. 2018) employing various media coltures such as MEA (Malt Extract Agar), MEAs (Malt Extract Agar Seawater), PDA (Potato Dextrose Agar), RB (Rose Bengal), and a "cheap preparation" added with specific antibiotics (Figure 1) to allow the exclusive growth of the selected fungi and to minimize the costs for possible industrial treatments.

To isolate vital fungal strains, the contaminated matrices were diluted with sterile water 1:10 and shaken mechanically for 3 min to 300 rpm. The suspension was further diluted by factors of 10. Later, 1 ml of polluted suspension was plated into Petri dishes containing 20 ml of undercooled media often presenting specific antibiotics, such as chloramphenicol, to avoid the bacterial growth.

Later, Petri dishes were incubated at 24±1 °C in the dark for 28 days. The plates were checked weekly and the number of fungal colonies grown subsequently was counted after 4 weeks.

MEA	
Malt extract	20 g
Peptone	1 g
Glucose	20 g
Agar	20 g
Chloramphenicol	0.04 g
MEAs	
Malt extract	20 g
Peptone	1 g
Glucose	20 g
Agar	20 g
Chloramphenicol	0.04 g
PDA	
Potato flakes	22 g
Glucose	20 g
Agar	20 g
RB	
Peptic digest of animal tissue	5 g
Dextrose	10 g
Monopotassium phospate	1 g
Magnesium sulfate	0.5 g
Rose Bengal	0.025 g
Dichloran	0.002 g
Agar	15 g
CHEAP preparation	
Saccharose	20 g
Peptone	1 g
Chloramphenicol	0.04 g

Fig. 1. Culture media used for the fungal isolation, referred to 1 L of H_2O.

3.2 Polybasic approach to fungal identification

The isolated fungal strains were identified with a polybasic approach which determined the fungal micro- and macromorphological features, the different trophic and physiological requirements and molecular analyses.

The isolated strains were preserved in axenic cultures by using test tubes containing MEA medium in the culture collection of the Mycological Laboratory of DISTAV (University of Genoa, Italy).

To allow future mycoremediation researches, all the fungal strains were stored at 4 °C and cryopreserved, in triplicate, at -20 and -80°C in refrigerate fungal chambers.

3.2.1 Morphological identification

The fungal strains were identified by observing their micro- and macromorphological characteristics and their different trophic and physiological requirements. Morfological identification was carried out by using specific taxonomical keys (Raper and Fennel, 1977; Korneup and Wansher, 1978; Pitt, 1979; Malloch, 1981; Klich, 2002; Samson and Frisvad, 2004) and then by optical microscopy (10×/0.30 to 40×/0.75), according to conventional mycology methods.

3.2.2 Molecular identification

Following the morphological identification of the fungal species, the identities of the isolated fungi were confirmed using nuclear DNA extraction, PCR amplification and DNA sequencing. The genomic DNA was extracted from 100 mg of fresh fungal culture using a modified CTAB method (Doyle and Doyle, 1987) consisting in the amplification of the β- tubulin gene using Bt2a and Bt2b primers (Glass and Donaldson, 1995) and the ITS region amplification using universal primers ITS1F/ITS4 (Gardes and Bruns, 1993). The PCR products were purified and sequenced using MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Sequencher® version 5.2 (sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA). Taxonomic assignment of the sequenced samples was carried out using the BLASTN algorithm to compare the sequences obtained in the present study with the GenBank database (https://www.ncbi.nlm.nih. gov/genbank/).

3.3 References

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull Bot Soc Am. 19:11–15.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 2:113– 118.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microb. 61(4):1323–1330.

Greco G, Cecchi G, Di Piazza S, Cutroneo L, Capello M, Zotti M. 2018. Fungal characterisation of a contaminated marine environment: the case of the Port of Genoa (North-Western Italy). Webbia. 73(1):97–106.

Klich MA. 2002. Biogeography of Aspergillus species in soil and litter. Mycologia. 94(1):21–27.

Kornerup A, Wanscher JH. 1978. Methuen handbook of colour. 3rd edn London: Eyre Methuen.

Malloch D. 1981. Moulds, their isolation, cultivation, and identification. University of Toronto Press.

Pitt J. 1979. The Genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. New York (NY): Accademic Press.

Raper KB, Fennell DI. 1977. The Genus Aspergillus RE Krieger Publishing Company. Huntington, New York.

Samson RA, Frisvad JC. 2004. Penicillium subgenus Penicillium: new taxonomic schemes and mycotoxins and other extrolites. Stud Micol. 49(49):1–260.

3.4 Preamble to the experiments described in the following chapters

In the following chapters, tests *in vitro* and in mesocosms were carried out to draft a protocol devoted to recover both terrestrial and marine environments contaminated by hydrocarbon compounds; to this purpose, some fungal strains from hydrocarbons polluted environments was isolated and checked.

Preliminary growth tests have been implemented to select the most suitable fungal strains to employ *in vitro* experiments. Moreover, to allow future large scale myco-treatments and evaluate fungal degradation efficiency, mesocosms have been prepared; mesocosms experiments proved highlight that environmental conditions may affect the degradation process performance.

At first, Port of Genoa was initially chosen as site of study. The area has been identified as an extreme environment, strongly compromised by pollution from hydrocarbon compounds such as heavy hydrocarbons, PAHs and oil products. In the area mycological studies on different biotic and abiotic substrates were carried out to allow a mycological characterization; this step proved to be very useful to select some hydrocarbon-tolerant fungi presenting high degradative capacity.

It is well known that some fungal species can live in extreme environmental conditions (lack of nutrients, acid or basic pH, presence of toxic elements), which make them able to survive and proliferate at high concentrations of pollutants, guaranteeing an effective degradation action. Hence, fungal characterization in the Port of Genoa was carry out to identify hydrocarbon-adapted strains for a fruitful exploitation in future mycoremediation treatments.

Later, subsequent experiments to restore soil and marine matrices contaminated by hydrocarbons substances were carried out; among soils, sand, gravel, clay and ground soils contaminated by heavy hydrocarbons were considered for both *in vitro* experiments and mesocosms. As concerns pollution of marine environments, an oily slime produced by ships was employed only *in vitro* experiments as matrix contaminated by PAHs.

In this scenario three case-studies have been described to characterize the mycobiota, to identify polluting factors that could influence the presence of some fungal strains compared to others, and to select other specific fungal species for future mycological applications.

Chapter four

CASE STUDY 1. FUNGAL CHARACTERIZATION OF A CONTAMINATED MARINE ENVIRONMENT: THE CASE OF THE PORT OF GENOA (NORTH-WESTERN ITALY)

4.1 Abstract

Nowadays, little information is available about if and how contaminants may impact on the presence of marine fungi. Indeed, many environmental factors may facilitate the presence of specific fungal taxa. This paper deals with the mycodiversity of 22 stations characterised by contaminated seawaters in the Port of Genoa (North-Western Italy). Several substrates were taken into account and 319 vital strains were isolated belonging to 20 genera and 47 species. The most common genera were *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma*. The fungal richness and mycodiversity were also evaluated and both appeared significantly high in most of these contaminated stations.

4.2 Introduction

Biodiversity represents 'the variability among living organisms from all sources, including terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species, and of ecosystems' (Millennium Ecosystem Assessment Board 2005). It is known that various levels of disturbance in an ecosystem may have different negative effects on the biological diversity (Sala *et al.* 2000; Bruno *et al.* 2003). In recent years, many mycological researches have investigated the microfungal communities of extreme environments contaminated by organic substances; in particular, scientists are beginning to examine regional patterns of fungal biodiversity (Rydin *et al.* 1997; Kuffer *et al.* 2005; Peay *et al.* 2007; Schmit *et al.* 2007; Pautasso *et al.* 2009), and there are some local studies about the impact of urbanisation on fungal communities (Pouyat *et al.* 1994; Baxter *et al.* 1999; Cousins *et al.* 2003; Tarvainen *et al.* 2003; Ochimaru *et al.* 2007; Pautasso *et al.* 2009; Di Piazza *et al.* 2017a). Although a number of scientists have commonly used the measure of biodiversity as a metric to evaluate the quality of an investigated habitat (Lake 1990; Costanza 1992; Vitousek 1994; Sala *et al*. 2000; Bruno *et al.* 2003; Di Piazza *et al.* 2017a; Di Piazza *et al.* 2017b; Mammola *et al.* 2017) (the higher the biodiversity, the higher the quality of the assemble of organisms populating the habitats), this appears incorrect while analysing the microfungal communities in contaminated areas. The Port of Genoa (North-Western Italy) represents a typical example of a polluted environment, because the local seawaters are subjected daily to the contamination by industrial wastes, polycyclic aromatic hydrocarbons (PAHs), oils and

similar. Moreover, this area presents extreme ecological parameters, especially the lack of oxygen and high values of phosphorus and nitrogen (Ruggieri *et al.* 2011). Our work aims to characterise marine fungal flora and evaluate the micodiversity in the contaminated areas of the Port of Genoa in order to verify the hypothesis that the presence of fungi may be positively correlated with human presence and organic pollution.

4.3 Material and methods

4.3.1 Study area

The sampling of biotic and abiotic substrates was conducted in the Port of Genoa (Figures 1 and 2). This area represents one of the most industrialised zones in North-Western Italy and extends for 10 km from west to east between the airport of Genoa 'Cristoforo Colombo'and the mouth of the Bisagno Torrent (Figure 1, 2). Every day, the Port is subjected to considerable traffic of both small and large vessels, and includes different terminals devoted to receiving goods of various kinds, among which are coal and crude oil carried by tankers. The waters inside the Port are rich in nutrients, faecal coliform, chlorophyll α and PAHs, and the bottom sediments are rich in metals, organic materials and PAHs due to the input of sewage and industrial discharges (Ruggieri *et al.* 2011; Cutroneo *et al.* 2014, 2015).

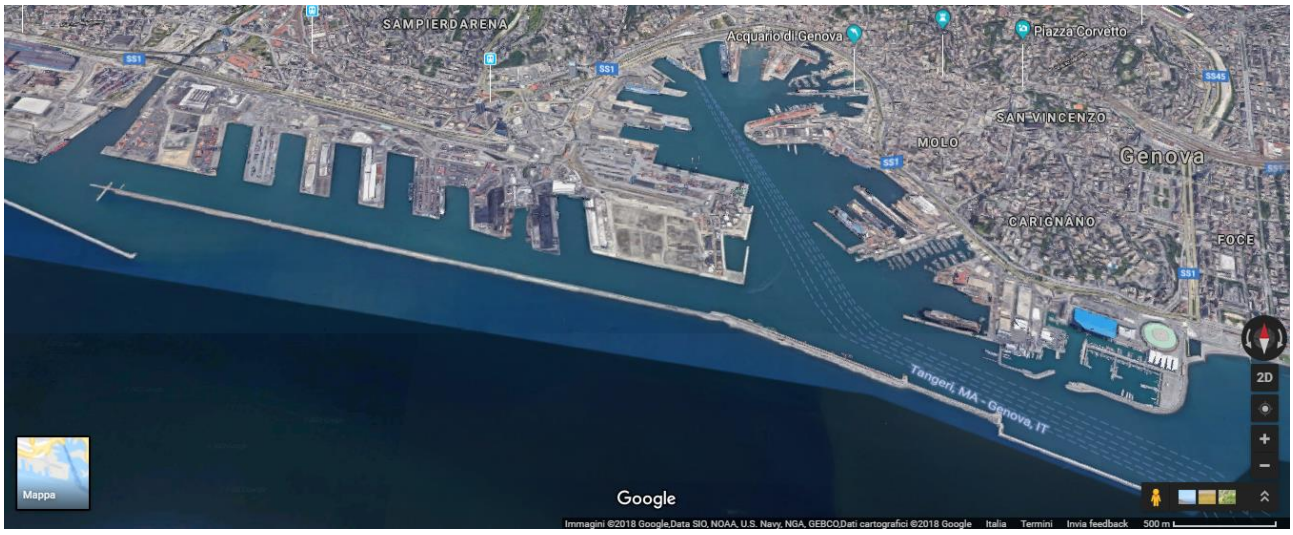


Fig. 1. Study area from satellite (2D).

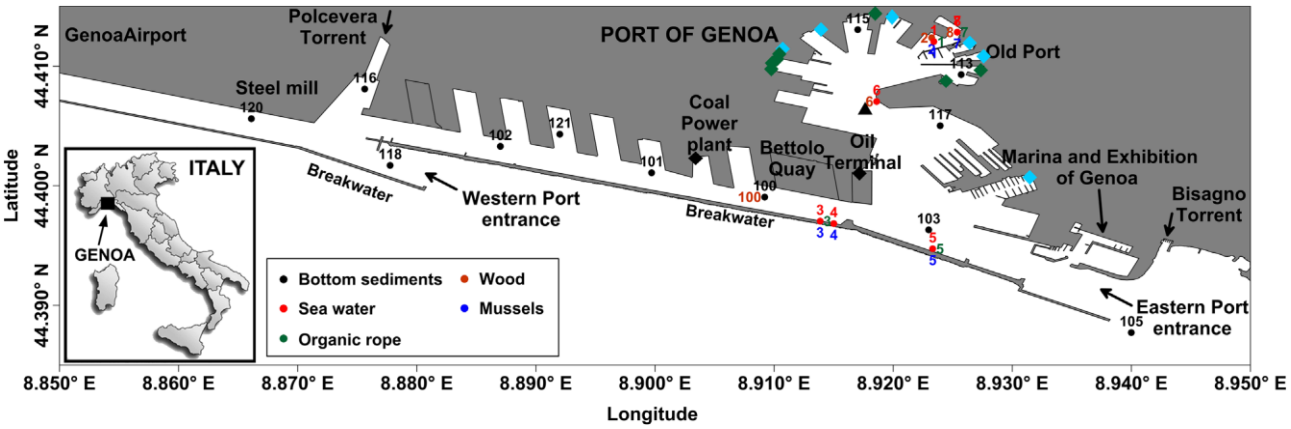


Fig. 2. Distribution of the sampling stations inside the Port of Genoa. The stations are divided in the different study substrates: bottom sediments in black, water in red, organic rope in green, wood in brown and mussels (shells and internal organs) in blue. The different discharges inside the port are shown with rhombuses and triangles: streams with cyan rhombus, surface water sewer with green rhombus, black waters with black rhombus and wastewater treatment plant discharge with black triangle.

4.3.2 Sampling of fungal strains

Marine fungal characterisation was performed analysing 36 samples from 22 different stations inside the Port of Genoa (Figure 2) during three different campaigns in the winter season, specifically from 1 December 2015 to 29 January 2016. For this first characterisation of the fungal community of the Port, we have chosen to sample during winter because it is the season with the lowest biological activity rate in seawaters and, therefore, the ideal period for the initial characterisation of the study area. The stations were selected near to industrial or urban discharges, docking points of cruise ships and the mouths of the Polcevera and Bisagno Torrents. In order to have a wide vision of microenvironments suitable for fungal growth, different kinds of substrate were collected: sediments were collected by a Van Veen grab from the superficial layer of the bottom; water was sampled with a Niskin bottle from the surface of the water column; organic substrates consisting of wood, mooring ropes and calcareous shells of *Mytilus galloprovincialis* Lamarck were collected in the sea surface. The samples were stored in sterilised polyethylene jars and ice boxes and brought to the laboratory for a complete analysis. Together with the samples, the chemical-physical characteristics of the seawater were measured close to the bottom (last 1 m of the water column) with a conductivity–temperature–depth (CTD) multiparametric probe (Idromarambiente) equipped with supplementary sensors: a turbidimeter (values in Formazin Turbidity Units, FTU; range 0–25 FTU), a

photosynthetically active radiation (PAR; expressed in μmol m^{-2} s^{-1}) sensor and a dissolved oxygen (in %) sensor. The salinity was determined using the Practical Salinity Scale.

4.3.3 Fungal strains isolation from different substrates

Specific methods were employed to isolate living fungi biologically adapted to marine contaminated waters, in order to select a pool of fungal strains to be used in future mycoremediation protocols. Fungal characterisation was performed by inoculating different organic and inorganic samples in Petri dishes. Different fungal strain isolation methods were adopted for each substrate typology.

Organic ropes

In sterile conditions under biological extractor with vertical laminar flow, the samples were washed with autoclaved seawater. For fungal strain isolation, organic ropes were cut into small pieces about 1 cm^2 and the fragments were inoculated in Petri dishes (diameter 9 cm) containing Rose Bengal seawater (RBs) culture media. To enable a better distribution of fungal propagules, the media surface was covered with 3 ml of autoclaved seawater and homogenously distributed by concentric agitation for 30 s. Afterwards, Petri dishes were incubated at 24±1 °C, in the dark, for 14 days and monitored weekly.

Wooden fragments

In sterile conditions, the samples isolated from wooden fragments were scratched and washed with sterile autoclaved seawater in order to eliminate the macroscopic impurities and the fouling organisms. Later, the samples were cut into small pieces about 2 cm^2. For fungal strain isolation, the fragments were inoculated in Petri dishes (diameter 9 cm) containing modified culture medium Malt Extract Agar seawater (MEAs) added with chloramphenicol antibiotic. The fragments of wood were covered with 3 ml of autoclaved seawater. In order to obtain a good homogenisation and distribution of fungal propagules, Petri dishes were shaken with concentric movements for 30 s. Petri dishes were incubated at 24±1 °C, in the dark, for 14 days and checked weekly.

Mytilus galloprovincialis

Samples of *M. galloprovincialis* were collected and immediately preserved at 4±1 °C to avoid the decomposition process. Later, in order to gather only representative data of the samples, the mollusks with

open and/or damaged shells were discarded; in this manner, each tested sample consisted of eight organisms with mass between 75 and 100 g.

Calcareous shells

M. galloprovincialis calcareous shells were scratched and washed with sterile seawater; 3 ml of washing water were homogeneously scraped on Petri dishes containing modified RBs culture medium. The Petri dishes were incubated at 24±1 °C for 2–3 weeks, in the dark, to allow an optimal growth of fungi.

Internal organs

An adequate number of mussels (c. eight organisms with mass between 75 and 100 g) were blended into little pieces about 0.5 cm^2 (Figure 3) to obtain a proper amount of flesh and intervalvular fluid. The fragments were inoculated on modified RBs culture medium and covered with 3 ml of autoclaved seawater to achieve a good distribution of fungal propagules.

Fig. 3. Mussel internal organ fragments used for microfungal isolation.

Bottom sediments

Microfungi were counted and isolated from the bottom sediments with a modified dilution plate technique (Greco *et al.* 2017) using two different culture media (MEAs and RBs). The dilution was obtained by mixing in vials 1 g of marine sediment with 100 ml of sterile water. Each marine sediment sample was plated in duplicate, once for each dilution (1:50.000 and 1:100.000). Finally, the plates were incubated at 24±1 °C in the dark for 14 days to allow an optimal growth of fungi.

Seawater

Water samples were shaken for ca. 2 min; in sterile conditions, under a biological extractor with vertical laminar flow, 1 ml of seawater suspension was homogenously spread on the surface of a MEA culture media. The plates were incubated for 14 days and the fungal colonies were counted weekly.

4.3.4 Identification of fungal strains

Independently from the type of substrate, isolated marine fungal species were cultured on different media, such as MEAs, RBs, Yeast Agar medium (CYA), Potato Dextrose Agar medium (PDA) and Oat Agar medium (OA) (Greco *et al.* 2017). Then a polyphasic approach based on physiological, morphological and molecular characteristics was used in order to identify the isolated strains. Genomic DNA was extracted from 100 mg of fresh fungal culture using a modified CTAB method (Doyle and Doyle, 1987). The PCR amplification of β-tubulin gene was performed using Bt2a and Bt2b primers (Glass and Donaldson, 1995) and ITS region amplification using universal primers ITS1F and ITS4 (White *et al.* 1990; Gardes and Bruns, 1993) was used to identify the most critical strains (Di Piazza *et al.* 2017b). The PCR protocol was as follows: one cycle of 5 min at 95 °C; 40 s at 94 °C; 45 s at 55 °C; 35 1-min cycles at 72 °C; one 10-min cycle at 72 °C. Later, PCR products were purified and sequenced using Macrogen Inc. (Seoul, Republic of Korea). The sequence assembly and editing were performed using SequencherR (Gene Codes Corporation, version 5.2). The taxonomic assignment of the sequenced samples was carried out using the BLASTN algorithm to compare the sequences obtained in the present study against the GenBank database. We took a conservative approach to species-level assignment (identity ≥ 97%) and we verified the accuracy of the results by also studying the macro- and micromorphological features of the colonies. The isolated fungal strains were conserved at 4 ± 1°C and cryopreserved at −20°C in the culture collection of the Mycological Laboratory of the Department of Earth, Environment and Life Sciences of the University of Genoa, and the sequences obtained were deposited in GenBank with accession numbers from MG650600 to MG650617 for the ITS, and from MG604350 to MG604359 for the btub sequences.

4.3.5 Data analysis

The biodiversity levels (*H'*) of each station were computed by the means of Shannon's biodiversity index calculated in $\log_2$ (Shannon 1948), where the probabilities are estimated as the ratio between the number of a specific colony forming unit (CFU) and the total number of colonies observed in all the plates.

4.4 Results

Our investigation revealed the presence of 319 CFU belonging to 20 genera and 47 species. The most recurrent fungal strains belonged to the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Trichoderma*. The most common fungal species were *Penicillium crustosum* (57 CFU), *Rhizopus arrhizus* (16 CFU), *Trichoderma harzianum* (15 CFU) and *Aspergillus carbonarius* (14 CFU). All the sampling stations, with a few exceptions (e.g. stations 117 and 121), were characterised by a high mycodiversity, as summarised in Table 1. In particular, station 100 showed the highest biodiversity ($H' = 4.10$), as highlighted by the results of the Shannon index (Table 1) with a number of strains belonging to 22 species (Table 2). The diversity values found in the studied stations ranged from 0 to 4.10 with a mean value of 2.33. Moreover, as concerns the different types of substrate sampled, the highest mycodiversity was observed in bottom sediments and internal organs of *M. galloprovincialis* (Figure 3). Table 3 reports the physical-chemical parameters of the seawater close to the bottom sediments sampling points.

Station	Number of species	Microfungal strains	Shannon's index (*H′*)
1	8	20	2.55
2	13	37	3.38
3	11	29	2.80
4	10	29	3.05
5	6	11	2.37
6	6	8	2.41
7	7	23	2.51
8	6	10	2.45
100	22	46	4.10
101	7	14	2.65
102	6	17	2.31
103	8	17	2.78
105	3	4	1.50
113	4	5	1.92
115	7	11	2.59
116	10	20	3.07
117	1	1	0.00
118	5	7	2.13
120	3	6	1.25
121	2	4	0.81

Table 1. Number of fungal species, microfungal strains and Shannon's biodiversity index (H′) for all the sampling stations.

4.5 Discussion

Among the isolates, the most recurrent fungal species were *Penicillium crustosum*, *Rhizopus arrhizus*, *Trichoderma harzianum* and *Aspergillus carbonarius. P. crustosum* is an important, pan-global contaminant of lipid- and protein-rich foods such as nuts, cheese or meat (Pitt 1979; Samson and Frisvad, 2004; Domsch *et al.* 2007). *P. crustosum* was often isolated from organic waste and compost for its capacity to grow in acidic substrates and, in recent years, it was isolated from glacial ice in extremely cold Polar Regions (Sonjak *et al.* 2006). *R. arrhizus* (Figure 4b) is a heterothallic fungus with a bipolar mating type, it is an opportunistic human and animal pathogen and represents the most common cause of mucormycosis (Goldstein *et al.* 2009);

R. arrhizus is mainly distributed in the tropical and subtropical zones in a wide variety of soils, and is an agent in the decay of vegetables, fruits, seeds and cow dung (Domsch *et al.* 2007). Moreover, this species was also isolated from forest soils and polluted still and running waters (Samson and Frisvad, 2004).

T. harzianum (Figure 4c) is a filamentous cosmopolitan fungus, commonly found in soils (Domsch *et al.* 2007) and in areas contaminated by heavy metals (Zotti *et al.* 2014; Cecchi *et al.* 2017a; Cecchi *et al.* 2017b). *T. harzianum* is used as a fungicide for seeds and soil treatment because, in general, it is considered a valuable biocontrol agent against several plant-pathogenic fungi (Komatsu 1976; Elad 2000).

A. carbonarius (Figure 4d) has a worldwide distribution (Abarca *et al.* 2004): it occurs in a great variety of substrata and is considered a common food spoilage fungus (Pitt 1979). Furthermore, *A. carbonarius* can grow on different plasticisers and related compounds and to degrade several hydrocarbons from fuel oil (Domsch *et al.* 2007). Among the fungal species less frequently found, it is worth noting the isolation of *Aspergillus sydowii* Thom & Church and *Gibberella zeae* Petch (Figure 4e). *A. sydowii* is a saprotrophic and pathogenic fungus that has been commonly isolated from soil and plant material (Domsch *et al.* 2007); in marine environments, it was isolated from the West Indian Sea (Geiser *et al.* 1998) and Australian coastal waters (Hayashi *et al.* 2016) and is indicated as a cause of aspergillosis of sea fan corals in the Caribbean Sea (Alker *et al.* 2001). The presence of *A. sydowii* within the Port of Genoa is thus the first record in the marine environment of the Mediterranean Sea, as highlighted by Greco *et al.* (2017). The filamentous fungus *G. zeae* (Figure 4e) is the agent of the head blight of wheat and, as such, has caused several billion dollars worth of damage over the past decade (Goswami *et al.* 2004). The presence of the *G. zeae* in the waters of the Port of Genoa may be due to the vessels transport, as in the case of *A. sydowii*. As concern the species diversity and variability ranges found by the Shannon index, they are similar to what has been found in other marine environments affected by human activities reported, for example, by Das *et al.* (2009) and Li *et al.* (2016), who have studied the microfungal diversity in the bottom sediments on the continental slope of the Bay of Bengal (India) and in the intertidal zone of the Chinese Seas, respectively.

Station 100, which presents the highest diversity, is localised in an area characterised by a high concentration of metals (such as As, V, Cu, Cd, Pb, Zn and Hg; Cutroneo *et al.* 2017) and PAHs (Cutroneo *et al.* 2015) because the station is in front of the coal power plant of Genoa, the disposal site of the dredged sediments (Bettolo Quay, Figure 2) and near the oil terminal, which receives coal and crude oil carried by tankers (Cutroneo *et al.* 2014). The higher micodiversity of such a polluted area confirms that environmental perturbing factors of groups of organisms may positively affect the growth and development of several fungal species, thus increasing the presence of marine fungi. The high microfungal abundance in bottom

sediments is probably due to the high concentration of organic matter and organic pollutants, helped by the seawater characteristics, such as low water temperature, salinity, dissolved oxygen, high turbidity and chlorophyll α (Table 3): indeed, all these parameters strongly impact the composition of microfungal communities (Li *et al.* 2016). The high concentration of microfungal communities in *M. galloprovincialis* internal organs is maybe due to the capacity of these mollusks to filter and retain large quantities of water and, consequently, particle matter, to be often used as biomarkers (Domouhtsidou *et al.* 2000; Stabili *et al.* 2005; Valavanidis *et al.* 2006; Vlahogianni *et al.* 2007).

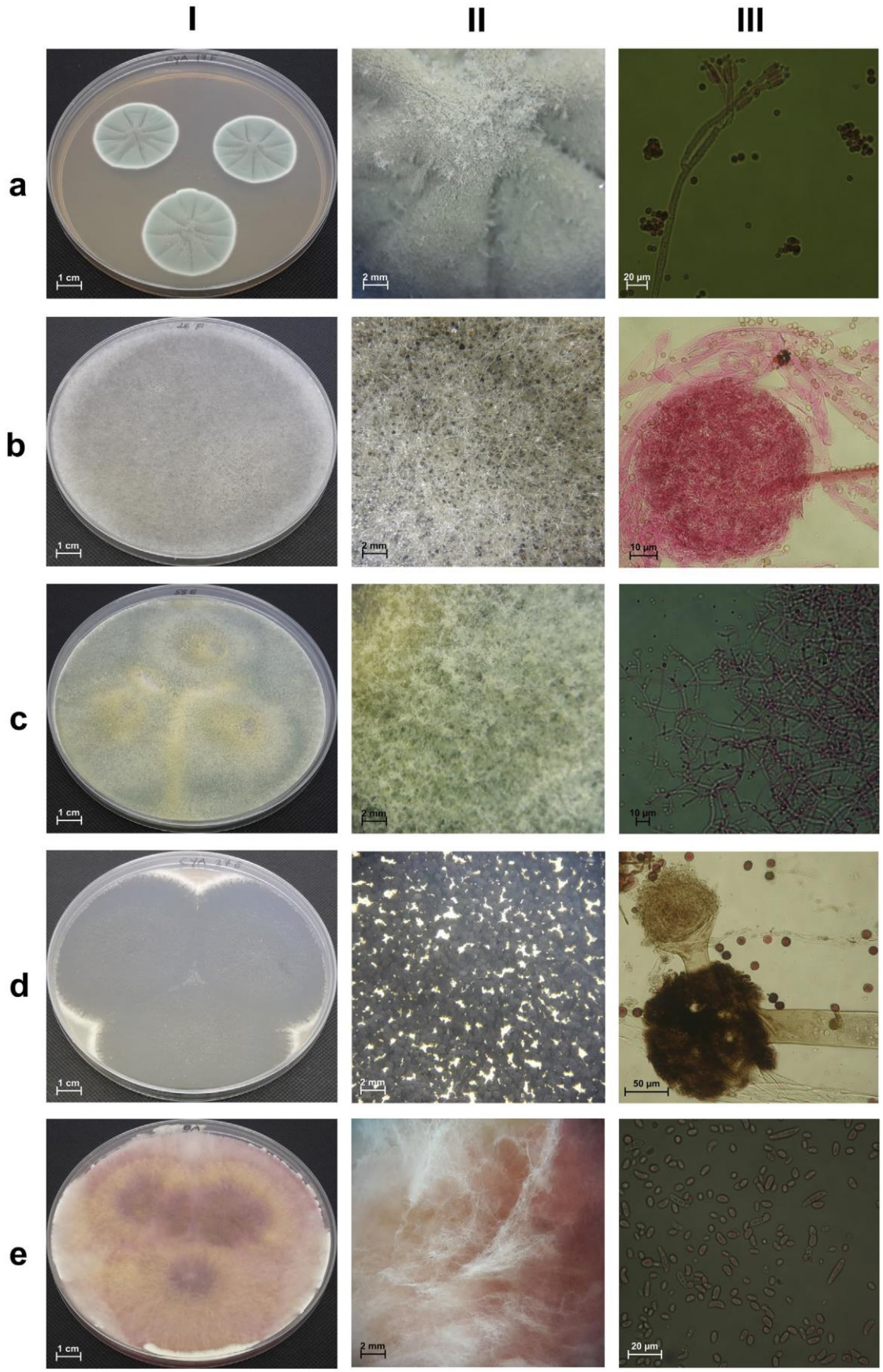


Fig. 4. (a) *Penicillium crostosum*; (b) *Rhizopus arrhizus*; (c) *Trichoderma harzianum*; (d) *Aspergillus carbonarius*; (e) *Gibberella zeae*. i: plate after seven days at 25°C; ii: details under stereomicroscope; iii: details under optical microscope (40×/0.75).

	MICROFUNGAL STRAINS ISOLATED IN THE STUDY STATIONS IN THE DIFFERENT SUBSTRATES																																		
FUNGAL SPECIES	1OR	1W	1CS	1IO	1S	2OR	2W	2CS	2IO	2S	3OR	3W	3CS	3IO	3S	4CS	4IO	5OR	5CS	5IO	6W	7OR	7IO	8w	100	101	102	103	105	113	115	116	117	118	120	121	
Acremonium persicinum											1																									1
Aspergillus carbonarius			1	2			1		3													2	1			3		1								14
Aspergillus creber																									1			1								2
Aspergillus flavus							1						1												4											6
Aspergillus melleus																									4											4
Aspergillus ochraceus	2			1					1						1						3						1									9
Aspergillus oryzae																										2				1			1		3	7
Aspergillus penicilloides																1	1											3				1				6
Aspergillus sydowii															1				1	1												1	1			5
Aspergillus terreus																					1	1			7			1		1	1			1		13
Aspergillus tubigensis							3		1													4						4								12
Chaetomium globosum																							3		1											4
Chaetonium nigricolor																									1				1						1	3
Cordyceps memorabilis														1																						1
Engydontium album																									2	1										3
Eurotium niveoglaucum	1																																			1
Fusarium equiseti														1										2	2											5
Fusarium oxysporum																								1	1				2							4
Fusarium petroliphilum																								1	2											3
Fusarium solani																									1				1		1					3
Galactomyces candidum																		2		2	1				5							3				13
Galactomyces geotrichum																1											3				3	5				12
Galactomyces sp.																									1						1	2				4
Geotrichum candidum																									1	3				2		2				8
Gibberella zeae																			1								2									3
Gleotinia temulenta																									1											1
Humicola grisea								3																												3
Mucor racemosus											1																					3			4	8
Paecilomyces formosa																		1																		1
Penicillium antarticum						2															1							3			1					7
Penicillium atramentosum																								1	2									1			4
Penicillium bialowiezense																1																				1
Penicillium biourgeianum																1			1																	2
Penicillium commune	1										2										1	2	1	3								1				11
Penicillium concentricum																												1								1
Penicillium crustosum	2	3	1		2	1	1	1	2	3	2	2	3	1	2	3	3	1	1			5	3			1	4	3			3	1		3			57
Penicillium expansum	1					1										1	1				1	1			1												7
Penicillium Janthinellum																																1					1
Penicillium olsonii	1					1																									1						3
Pseudallescheria boydii																									4	1										5
Rhizopus oryzae	1		1			1	1	1			1	1	2	3		2	1										1										16
Rhizomucor pusillus											1		2												1										1	1	6
Rhizopus stolonifer																2	2								1												5
Sordaria fimicola																									2					1				1			4
Talaromyces amestolkiae																									1												1
Trichoderma harzianum						1		1	2							3	3							2		3											15
Trichoderma longibrachiatum						1	1	1	1							2	1										6										13
Trichoderma virens						1																															1
																																					319
	9	3	3	3	2	9	8	7	10	3	8	3	8	8	2	17	12	4	4	3	8	15	8	10	46	14	17	17	4	5	11	20	1	7	6	4	

OR = organic rope; W = wood; CS = calcareous shell of M. galloprovincialis; IO = internal organs of M. galloprovincialis;
S = seawater; stations from 100 to 121 are composed only of bottom sediments. To simplify the table, values equal to zero are omitted.

Table 2. Microfungal species isolated in the study stations from different substrates.

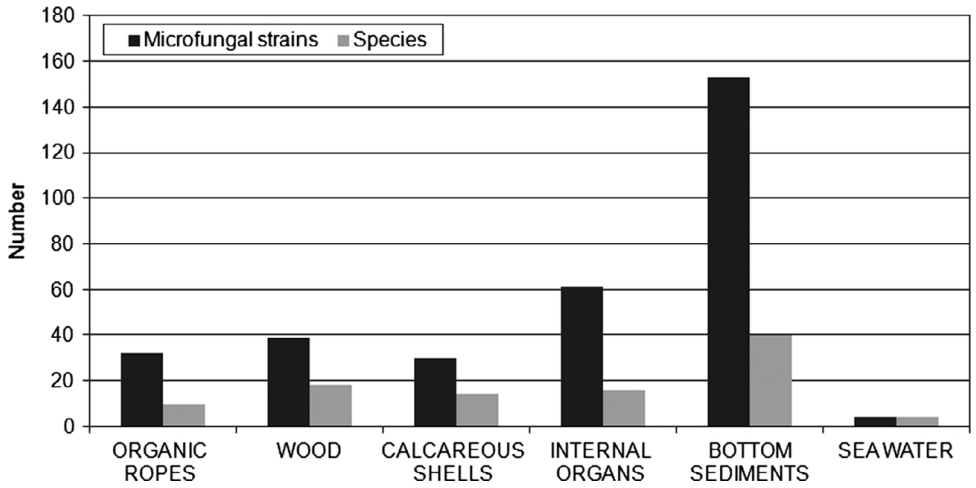


Fig. 5. Total number of microfungal strains (black column) and fungal species (grey column) found in the different substrates.

Stations	Fungal strains	Fungal species	T	O%	PAR	Tu	Chl	S
100	46	22	13.2	66.6	45.4	9.3	0.6	37.5
101	14	7	13.3	65.8	45.8	10.4	0.4	37.6
102	17	6	13.3	66.3	47.8	9.6	0.5	37.5
103	17	8	13.2	68.0	44.9	10.6	0.5	37.6
113	5	4	13.1	65.7	68.5	5.5	0.3	36.9
115	11	7	13.4	68.9	53.0	6.2	0.4	37.3
116	20	10	13.7	66.4	43.9	17.4	0.7	37.7
117	1	1	13.8	70.4	42.6	10.5	0.5	37.8
118	7	5	13.9	69.9	48.1	3.9	0.4	38.0
120	6	3	13.9	68.1	52.0	7.7	0.4	37.8
121	4	2	13.9	68.0	59.7	7.0	0.4	37.7

t = temperature; o% = dissolved oxygen; Par = photosynthetically active radiation; tu = turbidity; Chl = chlorophyll α; S = salinity.

Table 3. Physical-chemical parameters of the seawater close to the bottom.

4.6 Conclusions

This research revealed that anthropic interference and organic contamination may be one of the reasons for the increased mycodiversity in polluted marine environments; the study confirms that many isolated marine fungal strains are perfectly adapted to environmental contamination by organic and inorganic substances. Furthermore, this supports the idea that the autochthonous microfungi may be fruitfully exploited to accumulate or degrade many contaminating substances and therefore they could be employed in innovative and sustainable remediation techniques (Cecchi, Marescotti, *et al.* 2017; Cecchi, Roccotiello, *et al.* 2017; Di Piazza, Cecchi, *et al.* 2017). Hence, this preliminary study may represent the first step to drafting a

protocol aimed to employ autochthonous fungi for bioremediation purposes in seawaters and bottom sediments contaminated by industrial wastes, such as PAHs, metals and others.

4.7 References

Abarca ML, Accensi F, Cano J, Cabanes FJ. 2004. Taxonomy and significance of black aspergilli. A Van Leeuw J Microb. 86(1):33–49.

Alker AP, Smith GW, Kim K. 2001. Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean Sea fan corals. Hydrobiologia. 460(1–3):105–111.

Baxter JW, Pickett ST, Carreiro MM, Dighton J. 1999. Ectomycorrhizal diversity and community structure in oak forest stands exposed to contrasting anthropogenic impacts. Can J Botany. 77(6):771–782.

Bruno JF, Stachowicz JJ, Bertness MD. 2003. Inclusion of facilitation into ecological theory. Trends Ecol Evol. 18(3):119–125.

Cecchi G, Marescotti P, Di Piazza S, Zotti M. 2017a. Native fungi as metal remediators: silver myco-accumulation from metal contaminated waste-rock dumps (Libiola Mine, Italy). J Environ Sci Heal B. 52 (3):191–195.

Cecchi G, Roccotiello E, Di Piazza S, Riggi A, Mariotti MG, Zotti M. 2017a. Assessment of Ni accumulation capability by fungi for a possible approach to remove metals from soils and waters. J Environ Sci Heal B. 52 (3):1–5.

Costanza R. 1992. Toward an operational definition of ecosystem health. In: Costanza R, Norton BG, Haskel BD, editors. Ecosyst Health. Washington (DC): Island Press; p. 239–256.

Cousins JR, Hope D, Gries C, Stutz JC. 2003. Preliminary assessment of arbuscular mycorrhizal fungal diversity and community structure in an urban ecosystem. Mycorrhiza. 13(6):319–326.

Cutroneo L, Carbone C, Consani S, Vagge G, Canepa G, Capello M. 2017. Environmental complexity of a port: Evidence from circulation of the water masses, and composition and contamination of bottom sediments. Mar Poll Bull. 119:184–194.

Cutroneo L, Castellano M, Carbone C, Consani S, Gaino F, Tucci S, Magri S, Povero P, Bertolotto RM, Canepa G, Capello M. 2015. Evaluation of the boundary condition influence on PAH concentrations in the water column during the sediment dredging of a port. Mar Pollut Bull. 101(2):583–593.

Cutroneo L, Massa F, Castellano M, Canepa G, Costa S, Povero P, Tucci S, Capello M. 2014. Technical and public approaches to involve dredging stakeholders and citizens in the development of a port area. Environ Earth Sci. 72(8):3159–3171.

Das S, Lyla PS, Khan SA. 2009. Filamentous fungal population and species diversity from the continental slope of Bay of Bengal. India Acta Oecol. 35(2):269–279.

Di Piazza S, Baiardo S, Cecchi G, Ambrosio E, Paoli C, Vassallo P, Zotti M. 2017a. Microfungal diversity in the swash zone interstitial water (SZIW) of three Ligurian urban beaches (NW, Italy). Italian. J Mycol. 46(2017):8– 20.

Di Piazza S, Cecchi G, Cardinale AM, Carbone C, Mariotti MG, Giovine M, Zotti M. 2017b. *Penicillium expansum* Link strain for a biometallurgical method to recover REEs from WEEE. Waste Manage. 60(2017):596–600.

Di Piazza S, Isaia M, Vizzini A, Badino G, Voyron S, Zotti M. 2017c. First mycological assessment in hydrothermal caves of Monte Kronio (Sicily, southern Italy). Webbia. 72(2), 2017.

Domouhtsidou GP, Dimitriadis VK. 2000. Ultrastructural localization of heavy metals (Hg, Ag, Pb, and Cu) in gills and digestive gland of mussels, *Mytilus galloprovincialis* (L.). Arch Environ Con Tox. 38 (4):472–478.

Domsch KH, Gams W, Anderson TH. 2007. Compendium of soil fungi. 2nd ed. Taxonomically revised by Gams W. Etching: IHW-Verlag; p. 672.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull Bot Soc Am. 19:11–15.

Elad Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Prot. 19(8–10):709–714.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 2:113– 118.

Geiser DM, Taylor JW, Ritchie KB, Smith GW. 1998. Cause of sea fan death in the West Indies. Nature. 394:137–138.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microb. 61(4):1323–1330.

Goldstein EJ, Spellberg B, Walsh TJ, Kontoyiannis DP, Edwards J, Ibrahim AS. 2009. Recent advances in the management of mucormycosis: from bench to bedside. Clin Infect Dis. 48(12):1743–1751.

Goswami RS, Kistler HC. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol Plant Pathol. 5(6):515–525.

Greco G, Capello M, Cecchi G, Cutroneo L, Di Piazza S, Zotti M. 2017. Another possible risk for the Mediterranean Sea? *Aspergillus sydowii* discovered in the Port of Genoa (Ligurian Sea, Italy). Mar Pollut Bull. 122(2017):470–474.

Hayashi A, Crombie A, Lacey E, Richardson AJ, Vuong D, Piggott AM, Hallegraeff G. 2016. *Aspergillus sydowii* marine fungal bloom in Australian coastal waters, its metabolites and potential impact on Symbiodinium dinoflagellates. Mar Drugs. 14(3):59.

Komatsu M. 1976. Studies on Hypocrea, Trichoderma and allied fungi antagonistic to shiitake, Lentinus edodes (Berk.) Sins. Rep Tottori Mycol Inst. 13:1–113.

Kuffer N, Senn-Irlet B. 2005. Influence of forest management on the species richness and composition of woodinhabiting basidiomycetes in Swiss forests. Biodivers Conserv. 14(10):2419–2435.

Lake PS. 1990. Disturbing hard and soft bottom communities: a comparison of marine and freshwater environments. Aust J Ecol. 15:477–488.

Li W, Wang M, Bian X, Guo J, Cai L. 2016. A high-level fungal diversity in the intertidal sediment of Chinese seas presents the spatial variation of community composition. Front Micology. 7:2098–3110.

Mammola S, Di Piazza S, Zotti M, Isaia M. 2017. Humaninduced alterations of the cave mycobiota in an Alpine Show Cave (Italy, SW-Alps). Acta Carsologica. 46(1):111– 123.

Millennium Ecosystem Assessment Board. 2005. Ecosystems and human well-being: policy responses. Vol. 3. Washington (DC): Island Press.

Ochimaru T, Fukuda K. 2007. Changes in fungal communities in evergreen broad-leaved forests across a gradient of urban to rural areas in Japan. Can J Forest Res. 37(2):247– 258.

Pautasso M, Zotti M. 2009. Macrofungal taxa and human population in Italy's regions. Biodivers Conserv. 18(2):473–485.

Peay KG, Bruns TD, Kennedy PG, Bergemann SE, Garbelotto M. 2007. A strong species–area relationship for eukaryotic soil microbes: island size matters for ectomycorrhizal fungi. Ecol let. 10(6):470–480.

Pitt J. 1979. The Genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. New York (NY): Accademic Press.

Pouyat RV, Parmelee RW, Carreiro MM. 1994. Environmental effects of forest soil-invertebrate and fungal densities in oak stands along an urban-rural land use gradient. Pedobiologia. 38:385–399.

Ruggieri N, Castellano M, Capello M, Maggi S, Povero P. 2011. Seasonal and spatial variability of water quality parameters in the Port of Genoa, Italy, from 2000 to 2007. Mar Pollut Bull. 62(2):340–349.

Rydin H, Diekmann M, Hallingback T. 1997. Biological characteristics, habitat associations, and distribution of macrofungi in Sweden. Conserv Biol. 11(3):628–640.

Sala OE, Chapin FS, Armesto JJ, Berlow E, Bloomfield J, Dirzo R, Huber-Sanwald E, Huenneke LF, Jackson RB, Kinzig A, et al. 2000. Global biodiversity scenarios for the year 2100. Science. 287(5459):1770–1774.

Samson RA, Frisvad JC. 2004. Penicillium subgenus Penicillium: new taxonomic schemes and mycotoxins and other extrolites. Stud Micol. 49(49):1–260.

Schmit JP, Mueller GM. 2007. An estimate of the lower limit of global fungal diversity. Biodivers Conserv. 16(1):99– 111.

Shannon CE. 1948. A mathematical theory of communication. Bell Syst Tech J. 27:623–656.

Sonjak S, Frisvad JC, Gunde-Cimerman N. 2006. Penicillium mycobiota in Arctic subglacial ice. Microbial Ecol. 52(2):207–216.

Stabili L, Acquaviva MI, Cavallo RA. 2005. *Mytilus galloprovincialis* filter feeding on the bacterial community in a Mediterranean coastal area (Northern Ionian Sea, Italy). Water Res. 39(2):469–477.

Tarvainen O, Markkola AM, Strommer R. 2003. Diversity of macrofungi and plants in Scots pine forests along an urban pollution gradient. Basic Appl Ecol. 4(6):547–556.

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotox Environ Safe. 64(2):178–189.

Vitousek PM. 1994. Beyond global warming: ecology and global change. Ecology. 75(7):1861–1876.

Vlahogianni T, Dassenakis M, Scoullos MJ, Valavanidis A. 2007. Integrated use of biomarkers (superoxide dismutase, catalase and lipid peroxidation) in mussels *Mytilus galloprovincialis* for assessing heavy metals' pollution in coastal areas from the Saronikos Gulf of Greece. Mar Pollut Bull. 54(9):1361–1371.

White TJ, Bruns TD, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand D, Sninsky J, White T, editors. PCR protocols, a guide to methods and applications. Orlando (FL): Academic; p. 315–322.

Zotti M, Di Piazza S, Roccotiello E, Lucchetti G, Mariotti MG, Marescotti P. 2014. Microfungi in highly coppercontaminated soils from an abandoned Fe–Cu sulphide mine: Growth responses, tolerance and bioaccumulation. Chemosphere. 117:471–476.

Chapter five

CASE STUDY 2. MYCOREMEDIATION OF AN OILY SLIME CONTAINING A POLYCYCLIC AROMATIC HYDROCARBON MIXTURE

5.1 Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are waste products which today represent a serious problem in the world due to their high toxicity and difficult removal from the environment. For these reasons, they represent an important and challenging topic of study and research.

PAHs may be degraded through biotic pathways that include both aerobic and anaerobic degradation by bacteria, fungi, cyanobacteria and eukaryotic algae. In recent decades, fungi are proving very useful in the biodegradation of some of the more toxic PAHs, such as anthracene, pyrene, benzo[a]pyrene and fluorene. However, there is a lack of information from an application point of view. The paper sheds light on real-world polluted matrices that can be actually degraded by fungi.

In this paper, 15 fungal species were isolated from an oily slime deriving from waste products of naval activities and screened to assess their ability to degrade a PAH mixture. The most suitable fungal strains were employed in the degradation treatment.

A set of selected microfungi (including *Fusarium solani* and a fungal consortium of *Pseudallescheria boydii*, *Talaromyces amestolkiae* and *Sordaria fimicola*) was proved to better degrade PAHs than the other fungi considered. The greatest degradation activity was observed during the first week of treatment.

This study shows the significant relevance of exploiting native fungi to recover marine and terrestrial areas contaminated by PAHs. Moreover, the use of selected fungi isolated from the same contaminated substrate demonstrates to be highly effective in the mycoremediation of recalcitrant pollutants such as oily slime containing PAHs mixture.

5.2 Introduction

Nowadays, waste products derived from human life and industrial activity represent a serious problem in the world due to their difficulty of removal from the environment and disposal. Although in the past little importance has been payed to the environmental quality, the risk tied to Polycyclic Aromatic Hydrocarbon (PAH) contamination and its associated carcinogenic effects on human health is today widely recognized. Nevertheless, PAH pollution remains a very relevant problem because these compounds are often recalcitrant and highly toxic.

PAHs are a large group of environmental pollutants (Clemente *et al.* 2001; Jiang *et al.* 2009) distributed in aquatic environments (Shi *et al.* 2005), sediments, soils (Lima *et al.* 2005), and air (Abdel-Shafy *et al.* 2016). They are an incomplete combustion product of wood and fossil fuels, automobile exhausts, power generation plants, refuse burning, and industrial emissions (Jones *et al.* 1989; Clemente *et al.* 2001) and they can also derive from some natural sources, such as oil seeps, forest fire or volcanic eruptions (IARC 1983). PAHs end up in atmosphere and then fall back on specific environmental compartments, such as soil or water, compromising their productive and ecological functions (Samanta *et al.* 2002).

Nowadays the removal of PAHs from environmental compartments is very difficult; the effectiveness of removal of these organic pollutants is related to specific physical properties that usually influences both their behavior and persistence in the environment such as specific gravity (relative density), tendency to evaporation, viscosity and the pour point. Furthermore, these properties depend on chemical composition of the PAHs mixture, proportion of volatile compounds, and the presence / absence of more complex hydrocarbon chains.

In the removal plans of the PAHs, the solubility also plays an important role; for example, low molecular weight aromatic compounds and alkenes are more soluble in water than alkanes. Moreover, the water solubility of hydrocarbons decreases as the number of saturated substituents linked to the aromatic rings or to the double bonds increases. Finally, site-specific factors (temperature, pH, salinity, granulometry) can play a significant role in removing these compounds.

PAH have been extensively studied by various researches due to their environmental persistence, proven adverse effects on plants and soil micro-organisms, and the well-known carcinogenic and teratogenic effects on humans. The scientific interest for PAHs is mainly related to their recognized harmful action resulting from the metabolic transformations of these compounds into diol-epoxides, molecules able to bind to DNA and to induce genetic mutations (Man *et al.*2013).

In this scenario, a serious issue of PAH pollution is represented by oily slimes due to their often-high PAH concentration and difficult disposal; oily slime is steadily produced by container ships, oil tankers or merchant ships and disposed in dedicated systems where they are treated with chemical-physical techniques. While traditional remediation techniques for PAH treatment are often limiting due to their costs and poor efficiency and still produce a highly dangerous final waste, bioremediation has received wide acclaim

because it requires little energy and may efficiently detoxify recalcitrant organic compounds (Singh 2006). Conversion of PAHs can be induced by the action of specialized organisms, such as fungi and bacteria; in this context, several studies have been published dealing with the degradation efficiency of macrofungi (Márquez-Rocha *et al.* 2000; Han *et al.* 2004; Cajthaml *et al.* 2008; Patel *et al.* 2009). Only in the recent years, the attention was also addressed to the possible use of microfungi for bioremediation purposes (Di Piazza *et al.* 2017).

Some microfungal species have proven able to cope adverse environmental conditions and transform PAHs into non-toxic/fewer toxic compounds obtaining carbon sources necessary to satisfy their nutritional needs (Verdin *et al.* 2004; Cerniglia *et al.* 2010, Reyes-César *et al.* 2014; Marco-Urrea *et al.* 2015). Thereby, selected microfungal organisms may result efficient to metabolize PAHs. Specifically, some microfungi have enzymes (e.g. laccase, lignin peroxidase, and manganese peroxidase) playng a primary role in the PAH degradation (Baborová *et al.* 2006).

Among the microfungal genera that can play a significant role in the PAH degradation processes, there are: *Aureobasidium* (Sihag *et al.* 2014), *Rhodotorula* (Cerniglia *et al.* 2010; Sihag *et al.* 2014), *Sporobolomyces* (Sihag *et al.* 2014), *Geotrichum* (Giraud *et al.* 2001; Cerniglia *et al.* 2010) and *Rhizopus* (Cerniglia *et al.* 2010; Fernández-Luqueño *et al.* 2011) isolated from soil and water samples. Garon *et al.* (2004) found that *Absidia cylindrospora* Hagem removed about 90% fluorene in 12 days from a hydrocarbon-contaminated soil, whereas in the absence of the fungus the process took 24 days. Ye *et al.* (2011) showed that *Aspergillus fumigatus* Fresen was able to degrade anthracene: the anthracene molecular structure was modified into a series of compounds with lower toxicity levels as phthalic anhydride, anthrone, and anthraquinone.

The mayor novelty of the paper consists of checking whether real-world polluted matrices can be degraded by fungi. More specifically, we investigated which fungal strains, selected in the matrices themselves, can degrade hydrocarbons.

The matrices consisted of oily slimes from commercial vessel rich in PAHs and hydrocarbons. The use of autochthonous organisms (no foreign strains were employed) represents another intriguing aspect that may foster a possible exploitation of this kind of treatment on large-scale.

5.3 Materials and methods

5.3.1 Oily slime sampling and laboratory strategy

Samples of oily slime derived from commercial vessels and affected by high concentrations of PAHs were collected from December 2015 to February 2017 directly upon arrival at the treatment plant, stored in sterile glass jars and conserved in a fridge at 5 ± 1 °C to preserve their chemical and physical characteristics.

A specific protocol hereinafter discussed was employed to isolate from the oily slime vital fungal strains, biologically adapted to live in this highly contaminated substrate (Greco *et al.* 2017). The complete sequence of laboratory activities is diagrammatically illustrated in Figure 1.

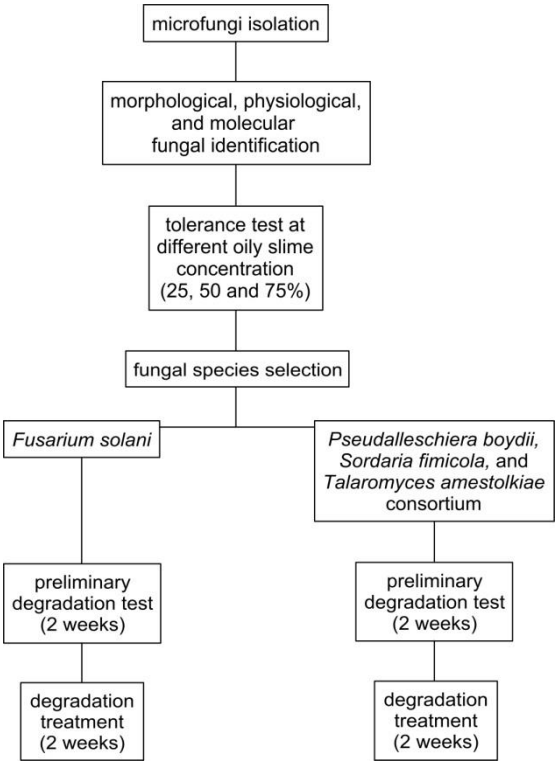


Fig. 1. Scheme of the complete sequence of laboratory activities.

5.3.2 Fungal isolation and identification

Microfungi were counted and isolated from the oily slime with a modified dilution plate technique (Greco et al. 2018) using a Malt Extract Agar Sea water (MEAs) culture medium. The dilution (performed as v/v) was obtained by mixing in sterile vials 0.5 g or 1 g of oily slime with 100 mL of sterile water. Each sample was plated in triplicate implementing 1:100 and 1:200 diluitions; later, serial diluitions till 1:50.000 and 1:100.000 were performed.

Fungal strains were identified by an integrated morphological and molecular approach, which determined both the micro- and macro-morphological characteristics using specific taxonomical keys (Pitt 1979; Klich 2002) and then by optical microscopy (10×/0.30 to 40×/0.75), that those molecular. The identities of the isolated fungal strains were confirmed using nuclear DNA extraction, PCR amplification and DNA sequencing. The genomic DNA was extracted from 100 mg of fresh fungal culture using a modified CTAB method (Doyle and Doyle 1987). Subsequently amplification of the β- tubulin gene using Bt2a and Bt2b primers (Glass and Donaldson 1995) and of the ITS region amplification using universal primers ITS1F/ITS4 (White et al. 1990) were performed. The PCR products were purified and sequenced using the DNA sequencing services of MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Sequencher® sequence analysis software (version 5.2, Gene Codes Corporation, Ann Arbor, MI USA). Taxonomic assignment of the sequenced samples was carried out using the Nucleotide Basic Local Alignment Search Tool (BLASTN) algorithm to compare sequences obtained in the present study with the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

Later, the fungal strains were maintained in axenic cultures of MEA at 4 ± 1°C and cryopreserved at −20°C in the culture collection of the Mycological Laboratory of the Department of Earth, Environment and Life Sciences of the University of Genoa (DISTAV) and the sequences obtained were deposited in GenBank with accession numbers from MK499445 to MK499452 and MK503926 for the ITS, and from MK519551 to MK519555 for the btub sequences.

5.3.3 Screening and degradation test with growth check

A screening test was carried out to select, among the isolates, the most suitable fungal strains for the oily slime treatment. To this purpose, the oily slime was homogenized and added to MEA medium at three different concentrations (25%, 50%, and 75%) in sterile 9-mm Petri dishes. Later, Petri dishes were inoculated with 1 ml of 10^8 conidial suspension of each isolated autochthonous fungal strain and incubated in the dark at 24 ± 1°C; fungal growth was monitored daily for a total of 14 days.

After the screening test, fungal strains having the higher oily slime tolerance were used in the degradation test employing the modified culture medium with the best fungal growth results in the screening test, that was the 25% of oily slime concentration. For the degradation test, two distinct fungal sets were considered: the former consists of only one selected species, while the latter is represented by a consortium of fungal species (fungal consortium, FC). Simultaneously, during the degradation tests, the fungal growth was checked (**Fig. 2**). Therefore, once inoculated (with 1 ml of 10^8 conidial suspension for both fungal sets) the Petri dishes containing 25% of homogenized oily slime concentration were incubated at 24 ± 1°C in the dark. The oily slime + culture medium contained in the Petri dishes was chemically analyzed at the starting time (t_0); the oily slime + biomass + culture medium was analyzed after 7 (t_1) and 14 (t_2) days. Five replicates for each fungal set and for each time (t_0, t_1, and t_2) were employed for the degradation test. For not changing the hydrocarbon concentrations and safety reasons, the modified culture medium containing the oily slime was not sterilized before the treatment. Therefore, to obtain valid chemical analyzes outcome, no control plates were considered due to the spontaneous growth of microorganisms in the oily slime, as also reported by He et *al.* (2014).

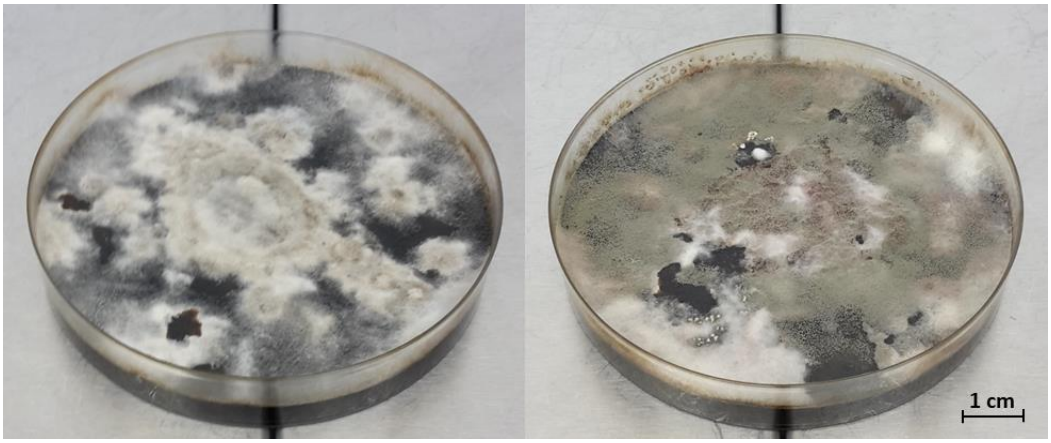


Fig. 2. The two fungal sets used: *Fusarium solani* (left) and fungal consortium (FC) (right) on modified culture media containing oily slime.

5.3.4 Chemical analysis and data treatment

To determine the degradation efficiency of mycological screening test and treatment, specific chemical analyses has been carried out. The chemical analysis of PAH hydrocarbon concentrations in Petri dishes was assessed by a dedicated laboratory of analysis though chromatography; in this regard the standard methods EPA 3550C + EPA 8270E and EPA 3550C + EPA 8015D were carried out. Samples were weighed and subjected to solvent extraction by sonication. The organic extract was subjected to instrumental analysis; a gas chromatograph with flame ionization detector (GC-FID) was employed to allow the heavy hydrocarbon (C>12) analysis. In this context, the chromatogram was elaborated by integrating the time group of the dodecane retention (C12) to the one of the tetracontane (C40) and quantified through an aging line with mineral oil standard. Moreover, a gas chromatograph with a mass spectrometer (GC-MS) was employed to allow the PAH analysis. Later, the analytes were separated, and the recognition of each PAH was verified considering the retention time and the mass spectrum.

The fungal degradation efficiency was evaluated using the following formula:

$$\mathbf{\Delta C} = \frac{\mathbf{C_0} - \mathbf{C_1}}{\mathbf{C_0}}$$

where **ΔC** is the fungal degradation efficiency, $\mathbf{C_0}$ is the initial oily slime concentration before the fungal treatment, and $\mathbf{C_1}$ is the final oily slime concentration after fungal treatment.

5.4 Results

From the mycological charachterization of oily slime 15 species belonging to 12 genera were isolated (Table 1); among the isolates, Penicillium and Trichoderma were the most represented genera with 3 and 2 species, respectively. All the species have been identified with morphological and molecular analysis.

The morphological analysis highlighted that none of the isolated fungal strains showed different characteristics than those mentioned by taxonomic keys used due to the microhabitat characterized by high

concentration of PAHs; thereby, both the micro- and the macroscopic fungal characters are resulted as typical of the described genera.

As concerns the growth and degradative tests carried out, among the isolated and identified strains, those showing tolerance to higher oily slime concentrations (50% and 75%) such F. solani, P. boydii, S. fimicola, and T. amestolkiae were selected and used to assess their ability to exploit oily slime as source of carbon for the growth. F. solani has been individually tested while P. boydii, T. amestolkiae, and S. fimicola have been used together as a FC in the degradative test.

In this context, we employed the generic culture media MEA that simply played the role of a starter, enabling fungal strains to develop up to a certain level at which fungi begin the degradation process.

Results of the growth rate of fungal species during the degradation test showed that the FC grew faster than F. solani in the first week of the treatment (Fig. 4) and that both the fungal sets reached at least 70% of the plate coverage during the two first weeks of test.

The employed species in the FC showed a good synergic ratio resistance to oily slime concentration of 25% and cooperating in the hydrocarbon degradation process.

The employed species in the FC, showed a good synergistic relationship, showing themselves to be vital throughout the duration of the test and cooperating in the hydrocarbon degradation process.

Starting from these results, the degradation treatment was carried out using a real oily slime sample for 2 weeks. Results show that after 14 days, both F. solani and FC revealed a great capability of degrading hydrocarbons In detail, total hydrocarbons decreased by 88% following mycological treatment with FC while by 83% with F. solani, acenaphthylene has been totally biodegraded both applying FC and F. solani (Fig. 5), naphthalene decreased by 90% both with FC and F. solani, the benzo[a]pyrene concentration diminished of 87% with FC while of 79% applying F. solani, benzo[e]pyrene has been biodegraded by 92% with FC while by 84% with F. solani, chrysene decreased by 81% with FC while by 75% following F. solani treatment, the pyrene concentration diminished of 76% with FC while of 69% applying F. solani, anthracene was degraded of 74% with FC while of 66% following F. solani treatment, phenanthrene decreased by 76% with FC while by 69% applying F. solani, while fluoranthene, fluorene and benzo[a]anthracene has been respectively degraded of 76%,77%, and 68% applying FC whereas of 68%, 71%, and 34% following F. solani treatment.

Table 1. Results of the fungal strains tested at concentrations of 25%, 50%, and 75% of oily slime. + = vital fungal strains; - = not vital fungal strains.

Fungal species	**75%**	**50%**	**25%**
Eurotium niveoglaucum (Thom & Raper) Malloch & Cain 1972	-	-	+
Penicillium antarcticum A.D. Hocking & C.F. McRae 1999	-	-	+
Pseudallescheria boydii (Shear) McGinnis, A.A. Padhye & Ajello 1982	+	+	+
Fusarium solani (Mart.) Sacc. 1881	-	+	+
Galactomyces geotrichum (E.E. Butler & L.J. Petersen) Redhead & Malloch 1977	-	-	+
Penicillium crustosum Thom 1930	-	-	+
Penicillium atramentosum Thom 1910	-	-	+
Gibberella zeae (Schwein.) Petch 1936	-	-	+
Sordaria fimicola (Roberge ex Desm.) Ces. & De Not. 1863	+	+	+
Aspergillus ochraceus G. Wilh. 1877	-	-	+
Talaromyces amestolkiae N. Yilmaz, Houbraken, Frisvad & Samson 2012	+	+	+
Mucor racemosus Bull. 1791	-	-	+
Rhizopus stolonifer (Ehrenb.) Vuill. 1902	-	-	+
Trichoderma harzianum Rifai 1969	-	-	+
Trichoderma longibrachiatum Rifai 1969	-	-	+

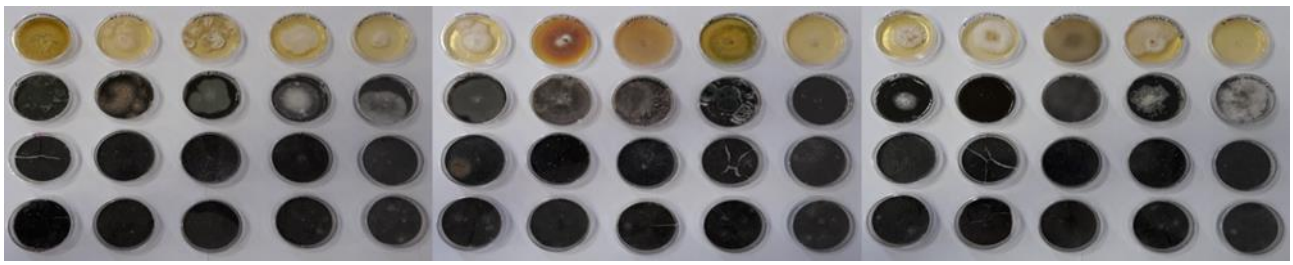


Fig. 3. Resistance test of 15 isolated fungal strains to oily slime with different concentrations. From the top to the bottom, control plates and plates with 25%, 50%, and 75% concentrations of oily slime.

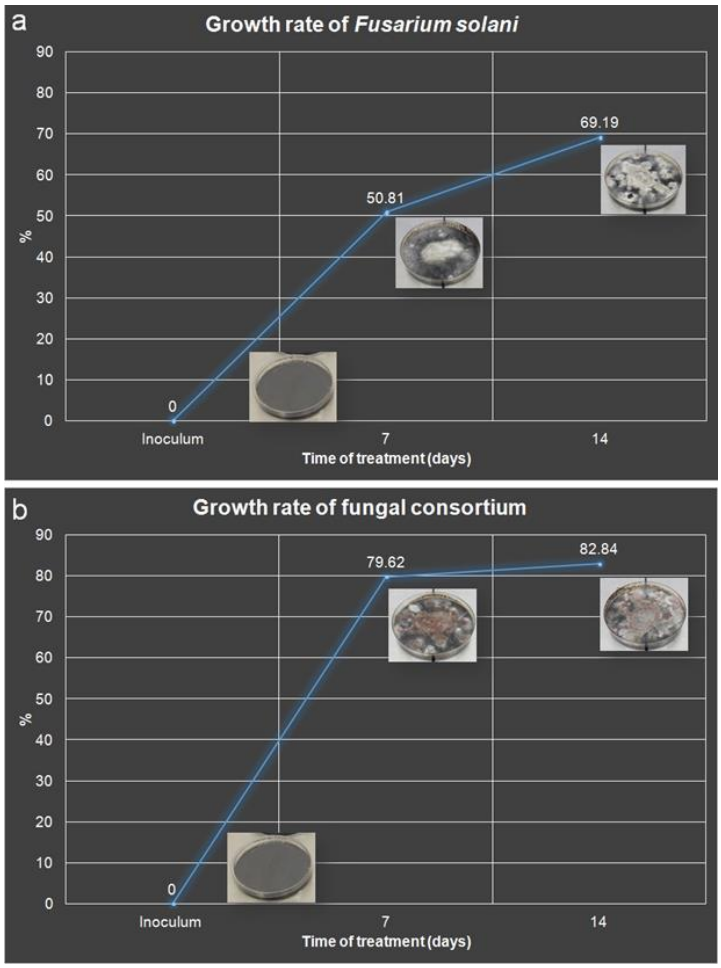


Fig. 4. Growth rate of fungal sets during the 14-days experiment. Above: *Fusarium solani*; below: fungal consortium of *P. boydii*, *T. amestolkiae* and *S. fimicola*.

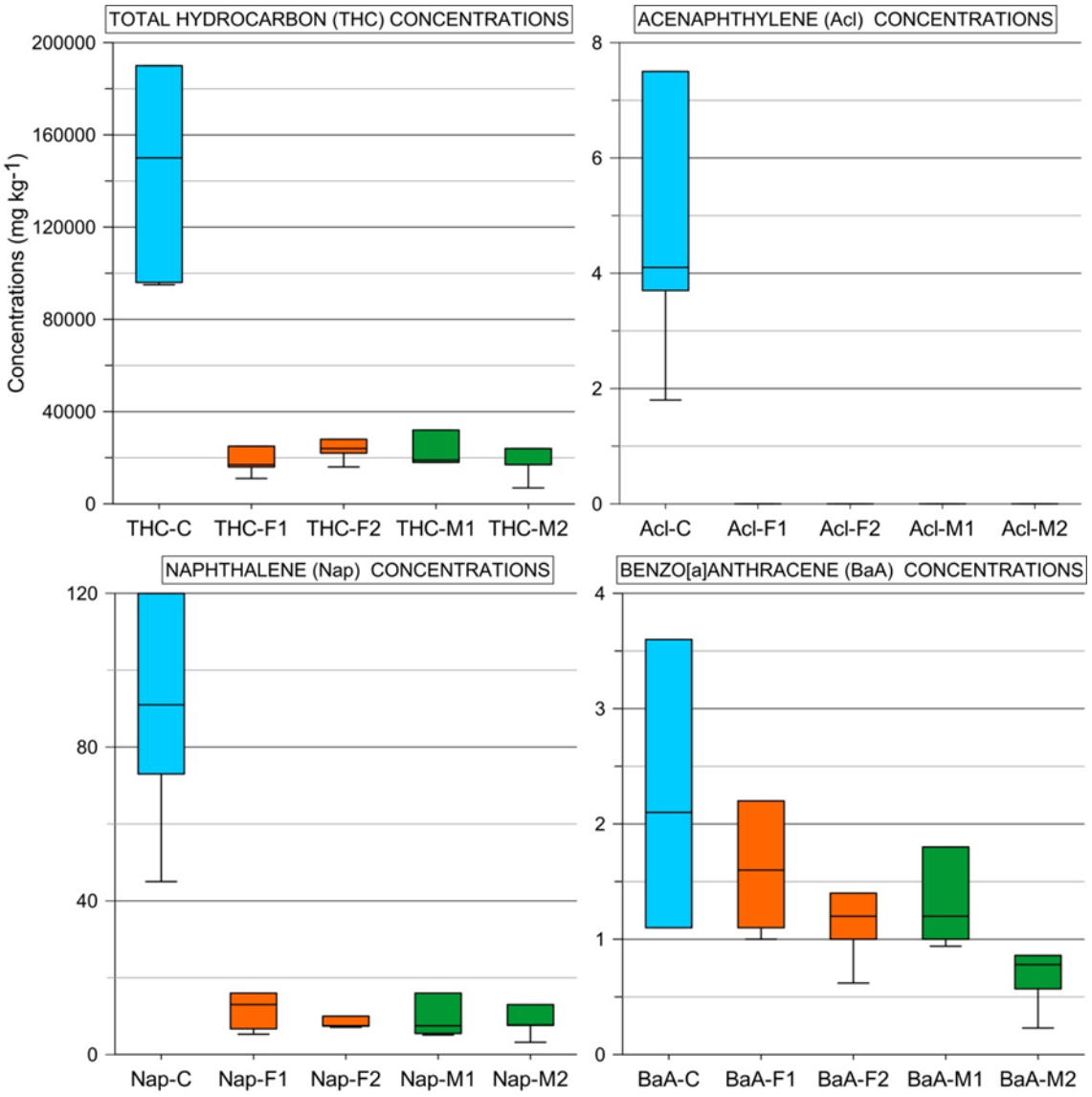


Fig. 5. Boxplots of total hydrocarbons (THC), acenaphthylene (Acl), naphthalene (Nap) and benzo[a]anthracene (BaA) in the control samples (C, 5 replies; blue) and in the samples treated with *Fusarium solani* (F; orange) and fungal consortium (FC; green) at the four treatment times (1: after 7 days, and 2: after 14 days of treatment).

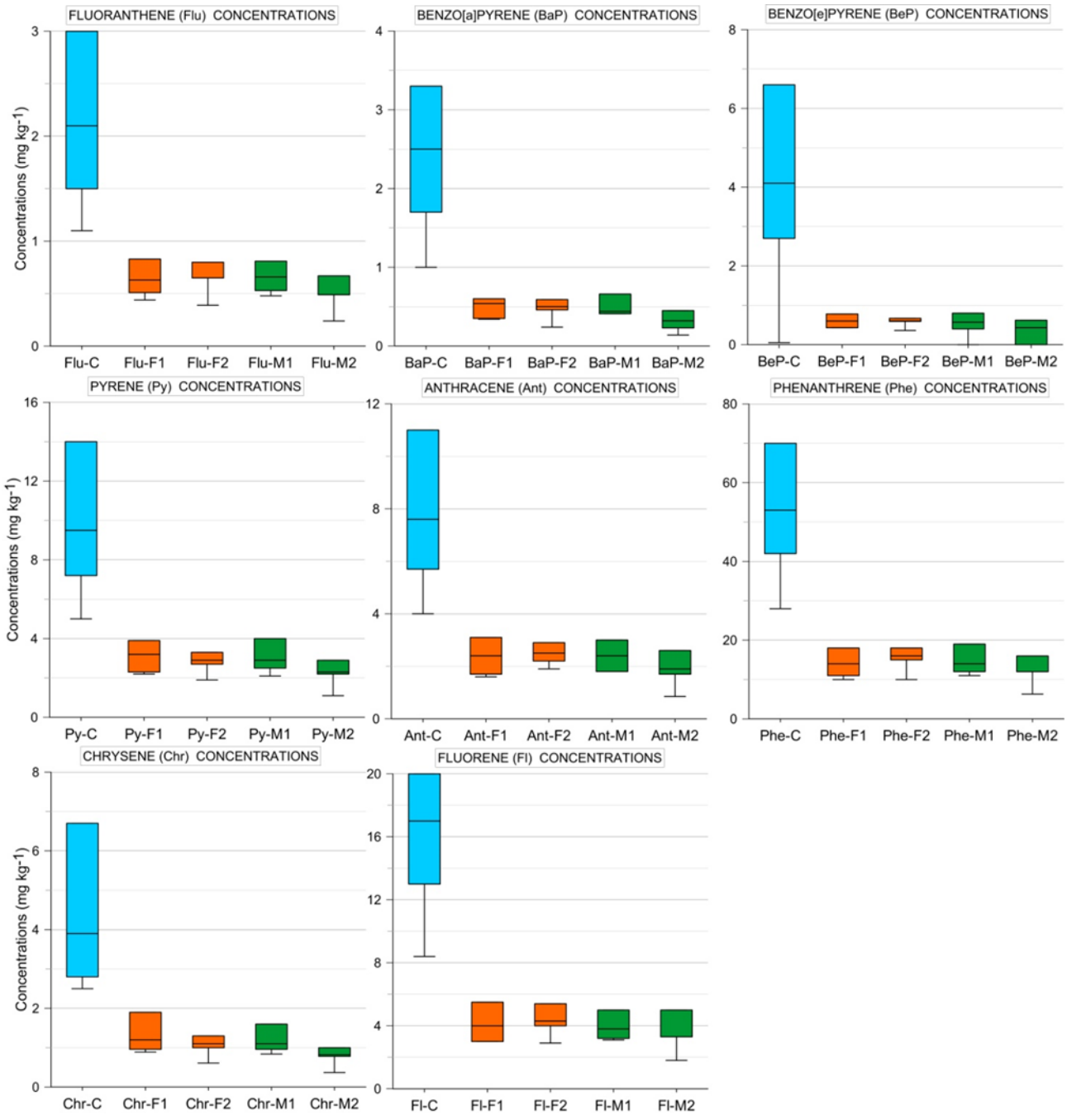


Fig. 6. Boxplots of benzo[a]pyrene (BaP), benzo[e]pyrene (BeP), pyrene (Py), anthracene (Ant), phenanthrene (Phe), chrysene (Chr), fluoranthene (Flu), fluorene (Fl) in the control samples (C, 5 replies; blue) and in the treated samples with *Fusarium solani* (F; orange) and fungal consortium (M; green) at the four treatment times (1: after 7 days, and 2: after 14 days of treatment).

5.5 Discussions

5.5.1 Fungal characterization

Among the isolated fungal strains, *Eurotium niveoglaucum* is identified as a fundamental agent of fermentation process of the indigenous Chinese dark tea by Mao *et al.* (2017); *Galactomyces geotrichum* is the most important mould for the dairy industry and is present in milk, cheeses and alcoholic beverage in which it is inserted to obtain characteristic aroma and taste for consumers (Grygier *et al.* 2017); *Gibberella zeae* is an important pathogen on major cereal crops such as wheat, barley, and maize (Son *et al.* 2011); *Rhizopus stolonifer* is commonly known as black bread mold and is an agent of plant disease (Bautista-Baños *et al.* 2014); *Trichoderma harzianum* is used as a fungicide in foliar application, seed treatment and soil treatment for the suppression of diseases generated by other fungal pathogens (Braun *et al.* 2018); *Aspergillus ochraceus* was isolated from Spanish and Brazilian grape samples (Pardo *et al.* 2005) and from coffee pulp (Romero-Borbón *et al.* 2018). *Penicillium antarcticum*, *Penicillium crustosum*, *Mucor racemosus*, and *Trichoderma longibrachiatum* have a worldwide distribution and colonize many habitats such as vegetation products, soil, and food.

The particularity and relative rarity of some fungi found in our oily slime sample, not widely distributed in the world, such as *E. niveoglaucum* found in the past only in the Chinese dark tea, can give us the opportunity to hypothesize the origin (China) of the ship from which the oily slime has been downloaded, the route that the ship makes during its stopovers or the goods that it carried.

5.5.2 PAH degradation

The results of the screening test highlighted the different growth response that each fungal species found in the oily slime has reached inside the studied matrix. At higher concentrations of oily slime (75%) 11 out 15 strains didn't grow due to limiting pollution conditions; these tested fungal species could have ended up in the original matrix by chance, and here may have lived in latent conditions, waiting for more suitable growth conditions. Otherwise, after their inoculation in the culture media having more assimilable sources of nutrients (at 25 and 50% of oily slime), they may have found suitable conditions for growth.

This first phase of the study allowed us to identify and choice the most suitable fungal species for PAH degradation purposes. This result was already achieved in the degradation of PAHs and other chemical contaminants for example by Godoy *et al.* (2016) that explored the potential of fungi isolated from PAH polluted soil, and by Mouhamadou *et al.* (2013) that studied the potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls (PCBs).

The literature already highlighted this fungal characteristic, though the most of studies have faced this problem addressing the ability of fungi to degrade a single hydrocarbon compound and often starting from samples synthetized in laboratory. In this context, in fact, Verdin *et al.* (2004) demonstrated fungal ability to break up benzo[a]pyrene, similarly, Ravelet *et al.* (2000) and Romero *et al.* (2002) focused on fungal degradation of pyrene, and Wu *et al.* (2010) proved their fungal remediation activity on anthracene.

Starting from the similar carried out research by He *et al.* (2014) on a real oily slime containing aliphatics, aromatics, N-S-O compounds, asphaltenes and Total Petroleum Hydrocarbon, our work dealt with the fungal capability to degrade a real complex mixture of PAHs (oily slime) produced by human activity.

Moreover, compared to the studies conducted so far, the novelty of our research consists in suggesting a pool of native filamentous microfungal species able to degrade not only a single PAH but simultaneously a mixture of PAHs, in a short treatment time (2 weeks); the obtained results could open the way for future industrial applications.

Among the selected species *F. solani* has been employed both for its good resistance obtained by screening test and for its rapid growth rate on the modified culture medium; in addition, we have employ this fungal species for its well Known degrading-PAHs capacities reported in previous studies (Romero *et al.* 2002; Wu *et al.* 2010).

F. solani has cosmopolitan distribution and is active in decomposing cellulolytic plant substrates (Domsch and Gams 2007).

it has often been isolated from environments contaminated by PAHs (Romero *et al.* 2002; Wu *et al.* 2010; Mineki *et al.* 2015).

Wu *et al.* (2010) showed that *F. solani* isolated from PAH-contaminated mangrove environments in Ma Wan, Hong Kong removed anthracene and benzo [a] anthracene by 40 and 60% respectively, after 40 days of fungal treatment.

Romero *et al.* (2002) isolated *F. solani* from contaminated sediments of the industrial area near the YPF Oil Refinery. The sediments contained 1773 ppm total hydrocarbon and 159 ppm pyrene; in this study *F. solani* was highly active and metabolized pyrene as the sole source of carbon.

Hong *et al.* (2010) have isolated from petrol station soil five strains of *F. solani* showing over 60% degradation of pyrene supplied within 2 weeks.

In our experiment, in addition to *F. solani,* to evaluate the synergy relationship of more fungal species, it was tested a fungal consortium formed by strains having tolerance to the highest concentration of oily slime (75%) *(Pseudallescheria boydii, Talaromyces amestalkiae, Sordaria fimicola).*

As concern *Pseudallescheria boydii,* having part of the FC has a worldwide distribution, particularly in soil; it was isolated from polluted water, composted municipal waste and diesel fuel (Domsch and Gams 2007); It is a well-known saprotrophytic filamentous fungus recognized as a potent agent of infection (human pathogenicity code 2), in both immunocompromised and immunocompetent patients (Guarro *et al.* 2006; Lamaris *et al.* 2006). This fungus causes human infections called hyalohyphomycosis which are characterized by the growth of non-pigmented septate hyphae in the infected tissue (Ulfig *et al.* 2008); *P. boydii* can also cause invasive disease which can involve the central nervous system (Nesky *et al.* 2000).

However, its use in our tests is justified both by its resistance to the highest oily slime concentrations (50-75%) shown in the screening test carried out and by its good biodegradable potential (Cerniglia *et al.* 2010; Reyes-César *et al*. 2014); once tested the good hydroacruric degradation capacities of *P. boydii,* its pathogenicity may not be limiting for a possible industrial application because our goal is to open new avenue in mycological field aimed at investigating metabolic pathways and extracting enzymes involved in the degradation process.

The application of *Talaromyces amestolkiae* in the degradative test is justified by its adaptability to PAHs environmental contamination reported by Greco *et al.* (2018); which have found this species on PAH-contaminated marine sediments during a survey in the Port of Genoa (North-Western Italy).

Sordaria fimicola has a worldwide distribution though predominantly in temperate regions (Domsch and Gams 2007). It was mainly isolated from fresh droppings of horse, hare, rabbit, cow and many other mostly herbivorous animals (Domsch and Gams 2007); moreover it is Known to degrade various cellulose-containing substrates (Domsch and Gams 2007).

However, the use of *Sordaria fimicola* in the degration test is justified by its capacity lignin-degrading; Raghukumar *et al.* (2008) during a study about the treatment of colored effluents with lignin-degrading enzymes founded that *Sordaria fimicola* secreted MnP and laccase in seawater media.

Thus, this fungus belonging to the category of lignin-degrading fungi can be an important tool for bioremediation of matrices contaminated by PAHs; as is well documented, many ligninolytic micro-organisms can degrade PAHs because of the highly unspecific nature of their ligninolytic systems and the resemblance of the lignin structure with PAHs (Hong *et al.* 2010; Acevedo *et al.* 2011).

Our data show that both *F. solani* and FC revealed a high efficiency in degrading most PAHs, especially during the first week of treatment; moreover, FC compared to *F. solani* show a better efficiency in the degradation process of PAHs mostly in the second week of fungal mycological treatment.

PAHs defined as toxic to humans and aquatic organisms, persistent in the environment and bio accumulative such as benzo (a) pyrene, anthracene, fluorene have been visibly reduced following mycological treatment using both *F. solani* and F.C; the concentration of total hydrocarbons decreased by at least 83%, the acenaphthylene has been completely biodegraded while Pyrene concentration was reduced in a week of mycological treatment with both *F. solani* and F.C. below the current value of 5 mg / kg (value for sites for private public and residential green areas) provided for in Annex 4/14 - Annex 5 to Title V of Part Four of Legislative Decree 152/2006.

The obtained results could be considered to implement future applications in large scale considering that the degradability efficiency and the differences found in PAH degradation rate and time can depend on several factors that influence also the growth rate, sporulation and metabolite fungal production. Environmental factors such as oxygen concentration, nutrient accessibility, pH, temperature, humidity can affect the fungal growth and degradation activity (Vieira *et al.* 2018). Chemical characteristics of matrix such as chemical composition, bioavailability rate and liquid or solid phase can also affect the fungal degradation efficiency. In fact, the low bioavailability rate of PAHs tends to increase with aging, and this is a key-constraint to the fungal clean-up of PAH contaminated matrices (Covino *et al.* 2010). Moreover, fungal physiological factors, such as specific enzymatic activity, mass and size of fungi (Kadri *et al.* 2017) play a fundamental role in the PAH degradation. In fact, for example, *F. solani* has cellulosolitic capacity and produces specific lignin-degrading enzymes needful for the degradation process, as confirmed by the literature (Rafin *et al.* 2000,

2009; Veignie *et al.* 2004; Chulalaksananukul *et al.* 2006). These enzymes can degrade not only lignin but also several recalcitrant environmental pollutants, such as hydrocarbons and PAHs (Rafin *et al.* 2000, 2009; Veignie *et al.* 2004; Chulalaksananukul *et al.* 2006).

Our data relating the first week of treatment also show that the FC achieving a higher growth rate and hydrocarbon degradation efficiency than what was obtained with *F. solani*, highlighting the positive effect of the synergistic relationship among the species of the FC. This joint fungal action allowed obtaining similar results to those achieved by Anastasi *et al.* (2009), Balaji *et al.* (2014) and Chen *et al.* (2010).

5.6 Conclusions

This study proves the degradative capacity of filamentous fungi isolated from polluted oily slime to biodegrade PAH compounds. This work represents a contribution in the research of sustainable remediation techniques of polluted matrices and in the selection of specific PAHs-degrading fungal strains. Our results highlight the importance of selection of fungal strains for mycoremediation purposes, and the importance of isolating species already present in the matrix to be treated, to avoid the introduction of alien species and to employ organisms that are already adapted to those environments.

Starting from our results, it would be necessary to conduct further studies to identify the enzymes involved in PAH degradation and optimize the biodegradation process. The study of the PAH bioavailable fraction during the fungal degradation activity will be of fundamental importance to devise any appropriate protocols of fungal efficiency augmentation. Furthermore, a deeper investigation on the synergistic relationship between fungi and isolated bacteria may be relevant to better understand how the environmental variables can influence the various degradation process parameters. Finally, a draft protocol could be devised for developing a procedure able to exploit the synergistic relationship of fungi and bacteria for PAH biodegradation.

5.7 References

Abdel-Shafy HI, Mansour MSM. 2016. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. Egypt. J. Petroleum. 25(1):107–123.

Anastasi A, Coppola T, Prigione V, Varese GC. 2009. Pyrene degradation and detoxification in soil by a consortium of basidiomycetes isolated from compost: role of laccases and peroxidases. J. Hazard. Mater. 165(1–3):1229–1233.

Baborová P, Möder M, Baldrian P, Cajthamlová K, Cajthaml T. 2006. Purification of a new manganese peroxidase of the white-rot fungus Irpex lacteus, and degradation of polycyclic aromatic hydrocarbons by the enzyme. Res. Microbiol. 157(3):248–253.

Balaji V, Arulazhagan P, Ebenezer P. 2014. Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. J. Environ. Biol. 35(3):521–529.

Bautista-Baños S, Bosquez-Molina E, Barrera-Necha LL. 2014. Chapter 1 - *Rhizopus stolonifer* (Soft Rot). In: Barka, E.A., Clément, C. (eds) Plant Microbe Interactions, pp. 269–289. Research Signpost, Kerala.

Braun H, Woitsch L, Hetzer B, Geisen R, Zange B, Schmidt-Heydt M. 2018. *Trichoderma harzianum*: Inhibition of mycotoxin producing fungi and toxin biosynthesis. Int. J. Food Microbiol. 280(2018):10–16.

Cajthaml T, Erbanová P, Kollmann A, Novotný Č, Šašek V, Mougin C. 2008. Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus.* Folia Microbiol. 53(4):289–294.

Cerniglia CE, Sutherland JB. 2010. Degradation of polycyclic aromatic hydrocarbons by fungi. In: Handbook of hydrocarbon and lipid microbiology, pp. 2079–2110. Springer, Berlin Heidelberg.

Chen B, Wang Y, Hu D. 2010. Biosorption and biodegradation of polycyclic aromatic hydrocarbons in aqueous solutions by a consortium of white-rot fungi. J. Hazard. Mater. 179(1-3):845–851.

Chulalaksananukul S, Gadd GM, Sangvanich P, Sihanonth P, Piapukiew J, Vangnai AS. 2006. Biodegradation of benzo (a) pyrene by a newly isolated Fusarium sp. FEMS Microbiol. Lett. 262(1):99–106.

Clemente AR, Anazawa TA, Durrant LR. 2001. Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. Braz. J. Microbiol. 32(4):255–261.

Covino S, Cvancarova M, Muzikar M, Svobodova K, D'annibale A, Petruccioli M, Federici F, Kresinova Z, Cajthaml T. 2010. An efficient PAH-degrading Lentinus (Panus) tigrinus strain: Effect of inoculum formulation and pollutant bioavailability in solid matrices. J. Hazard. Mater. 183(1-3):669–676.

Di Piazza S, Cecchi G, Cardinale AM, Carbone C, Mariotti MG, Giovine M, Zotti M. 2017a. *Penicillium expansum* Link strain for a biometallurgical method to recover REEs from WEEE. Waste Manage. 60(2017):596–600.

Domsch KH, Gams W, Anderson TH. 2007. Compendium of soil fungi, 2nd taxonomically revised edition by W. Gams. IHW, Eching.

Doyle J, Doyle JL. 1987. Genomic plant DNA preparation from fresh tissue-CTAB method. Phytochem. Bull. 19(11):11–15.

Farrington JW, Takada H. 2014. Persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and plastics: Examples of the status, trend, and cycling of organic chemicals of environmental concern in the ocean. Oceanography. 27(1):196–213.

Fernández-Luqueño F, Valenzuela-Encinas C, Marsch R, Martínez-Suárez C, Vázquez-Núñez E, Dendooven L. 2011. Microbial communities to mitigate contamination of PAHs in soil—possibilities and challenges: a review. Environ. Sci. Pollut. R. 18(1):12–30.

Garon D, Sage L, Wouessidjewe D, Seigle-Murandi F. 2004. Enhanced degradation of fluorene in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. Chemosphere. 56(2):159–166.

Giraud F, Guiraud P, Kadri M, Blake G, Steiman R. 2001. Biodegradation of anthracene and fluoranthene by fungi isolated from an experimental constructed wetland for wastewater treatment. Water Res. 35(17): 4126–4136.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microb. 61(4):1323–1330.

Godoy P, Reina R, Calderón A, Wittich RM, García-Romera I, Aranda E. 2016. Exploring the potential of fungi isolated from PAH-polluted soil as a source of xenobiotics-degrading fungi. Environ. Sci. Pollut. Res. 23(20):20985–20996.

Greco G, Capello M, Cecchi G, Cutroneo L, Di Piazza S, Zotti M. 2017. Another possible risk for the Mediterranean Sea? *Aspergillus sydowii* discovered in the Port of Genoa (Ligurian Sea, Italy). Mar. Pollut. Bull. 122(1-2):470–474.

Greco G, Cecchi G, Di Piazza S, Cutroneo L, Capello M, Zotti M. 2018. Fungal characterisation of a contaminated marine environment: the case of the Port of Genoa (North-Western Italy). Webbia. 73(1):97–106.

Grygier A, Myszka K, Rudzińska M. 2017. Galactomyces geotrichum - moulds from dairy products with high biotechnological potential. Acta Sci. Pol. Technol. Aliment. 16(1):5–16.

Han MJ, Choi HT, Song HG. 2004. Degradation of phenanthrene by *Trametes versicolor* and its laccase. J. Microbiol. 42(2):94–98.

He YM, Duan, XG Liu, YS. 2014. Enhanced bioremediation of oily sludge using co-culture of specific bacterial and yeast strains. J. Chem. Technol. Biot. 89(11):1785–1792.

IARC (International Agency for Research on Cancer). 1983. Polynuclear aromatic compounds, part 1, chemical, environmental, and experimental data. IARC Monog. Eval. Carc. 33–451.

Jiang YF, Wang XT, Wang F, Jia Y, Wu MH, Sheng GY, Fu JM. 2009. Levels, composition profiles and sources of polycyclic aromatic hydrocarbons in urban soil of Shanghai, China. Chemosphere. 75(8):1112–1118.

Jones KC, Stratford JA, Tidridge P, Waterhouse KS, Johnston AE. 1989. Polynuclear aromatic hydrocarbons in an agricultural soil: long-term changes in profile distribution. Environ. Pollut. 56(4):337–351.

Kadri T, Rouissi T, Brar SK, Cledon M, Sarma S, Verma M. 2017. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review. J. Environ. Sci. 51(2017):52–74.

Klich MA. 2002. Biogeography of Aspergillus species in soil and litter. Mycologia. 94(1):21–27.

Lima ALC, Farrington JW, Reddy CM. 2005. Combustion-derived polycyclic aromatic hydrocarbons in the environment—a review. Environ. Forensics. 6(2):109–131.

Man YB, Kang Y, Wang HS, Lau W, Li H, Sun XL, Giesy JP, Chow KL, Wong MH. 2013. Cancer risk assessments of Hong Kong soils contaminated by polycyclic aromatic hydrocarbons. J. Hazard. Mater. 261(2013):770–776.

Mao Y, Wei BY, Teng JW, Huang L, Xia N. 2017. Analyses of fungal community by Illumina MiSeq platforms and characterization of Eurotium species on Liupao tea, a distinctive postfermented tea from China. Food Res. Int. 99(2017):641–649.

Marco-Urrea E, Garcia-Romera I, Aranda E. 2015. Potential of non-ligninolytic fungi in bioremediation of chlorinated and polycyclic aromatic hydrocarbons. New Biotechnol. 32(6):620–628.

Márquez-Rocha FJ, Hernández-Rodríguez VZ, Vázquez-Duhalt R. 2000. Biodegradation of soil-adsorbed polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. Biotechnol. Lett. 22(6):469–472.

Matsubara M, Lynch JM, De Leij FAAM. 2006. A simple screening procedure for selecting fungi with potential for use in the bioremediation of contaminated land. Enzyme Microb. Tech. 39(7):1365–1372.

Mouhamadou B, Faure M, Sage L, Marcais J, Souard F, Geremia RA. 2013. Potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls. Fungal Biol. 117(4):268–274.

Pardo E, Marin S, Sanchis V, Ramos AJ. 2005. Impact of relative humidity and temperature on visible fungal growth and OTA production of ochratoxigenic *Aspergillus ochraceus* isolates on grapes. Food Microbiol. 22(5):383–389.

Patel H, Gupte A, Gupte S. 2009. Biodegradation of fluoranthene by basidiomycetes fungal isolate *Pleurotus ostreatus* HP-1. Appl. Biochem. Biotech. 157(3):367–376.

Pitt JI. 1979. The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces. Academic Press Inc. Ltd., London.

Rafin C, Pottn O, Veignie E. 2000. Degradation of benzo[a]pyrene as sole carbone source by a non white rot fungus, *Fusarium solani*. Polycycl. Aromat. Comp. 21(2000):311–329.

Rafin C, Veignie E, Fayeulle A, Surpateanu G. 2009. Benzo[a]pyrene degradation using simultaneously combined chemical oxidation, biotreatment with *Fusarium solani* and cyclodextrins. Bioresource Technol. 100(12):3157–3160.

Ravelet C, Krivobok S, Sage L, Steiman R. 2000. Biodegradation of pyrene by sediment fungi. Chemosphere. 40(5):557–563.

Reyes-César A, Absalón ÁE, Fernández FJ, González JM, Cortés-Espinosa DV. 2014. Biodegradation of a mixture of PAHs by non-ligninolytic fungal strains isolated from crude oil-contaminated soil. World J. Microb. Biot. 30(3):999–1009.

Romero MC, Salvioli ML, Cazau MC, Arambarri AM. 2002. Pyrene degradation by yeasts and filamentous fungi. Environ. Pollut. 117(1):159–163.

Romero-Borbón E, Grajales-Hernández D, Armendáriz-Ruiz M, Ramírez-Velasco L, Rodríguez-González JA, Cira-Chávez LA, Estrada-Alvarado MI, Mateos-Díaz JC. 2018. Type C feruloyl esterase from *Aspergillus ochraceus*: A butanol specific biocatalyst for the synthesis of hydroxycinnamates in a ternary solvent system. Electron. J. Biotechn. 35(2018):1–9.

Samanta SK, Singh OV, Jain RK. 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol. 20(6):243–248.

Shi Z, Tao S, Pan B, Fan W, He XC, Zuo Q, Xu FL. 2005. Contamination of rivers in Tianjin, China by polycyclic aromatic hydrocarbons. Environ. Pollut. 134(1):97–111.

Sihag S, Pathak H, Jaroli D.P. 2014. Factors affecting the rate of biodegradation of polyaromatic hydrocarbons. Int. J. Pure App. Biosci. 2(3):185–202.

Singh H. 2006. Mycoremediation: fungal bioremediation. John Wiley & Sons, Hoboken.

Son H, Min K, Lee J, Raju NB, Lee YW. 2011. Meiotic silencing in the homothallic fungus *Gibberella zeae*. Fungal Biol. 115(12):1290–1302.

Veignie E, Rafin C, Woisel P, Cazier F. 2004. Preliminary evidence of the role of hydrogen peroxide in the degradation of benzo[a]pyrene by a non-white rot fungus *Fusarium solani*. Environ. Pollut. 129(1):1–4.

Verdin A, Sahraoui ALH, Durand R. 2004. Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. Int. Biodeter. Biodeg. 53(2):65–70.

Vieira GAL, Magrini MJ, Bonugli-Santos RC, Rodrigues MVN, Sette LD. 2018. Polycyclic aromatic hydrocarbons degradation bymarine-derived basidiomycetes: optimization of the degradation process. Braz. J. Microbiol.

Wang XT, Miao Y, Zhang Y, Li YC, Wu MH, Yu G. 2013. Polycyclic aromatic hydrocarbons (PAHs) in urban soils of the megacity Shanghai: occurrence, source apportionment and potential human health risk. Sci. Total Environ. 447(2013):80–89.

White TJ, Bruns T, Lee SJWT, Taylor JL. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18(1):315–322.

Wu YR, Luo ZH, Vrijmoed LLP. 2010. Biodegradation of anthracene and benz[a]anthracene by two *Fusarium solani* strains isolated from mangrove sediments. Bioresource Technol. 101(24):9666–9672.

Ye JS, Yin H, Qiang J, Peng H, Qin HM, Zhang N, He BY. 2011. Biodegradation of anthracene by *Aspergillus fumigatus*. J. Hazard. Mater. 185(1):174–181.

Yu J. 2013. The effect of pH value on the polycyclic aromatic hydrocarbon's degradation in sludge during biological aerobic fermentation process. Adv. Mater. Res. 664:72–76.

Chapter six

CASE STUDY 3. PILOT-SCALE MYCOREMEDIATION OF REAL POLLUTED MATRICES CONTAMINATED BY HYDROCARBON COMPOUNDS

6.1 Abstract

Pollution by hydrocarbon compounds, especially involving soils, is today an environmental problem of growing importance, which is often underestimated. The persistence of this kind of organic pollution depends on the level of biodegradability of the hydrocarbon compounds involved, on the microbiota of the polluted areas and on specific environmental factors, often influenced by seasonality, such as temperature, humidity, pH.

In this work, biodegradation of hydrocarbons has been performed using, in pilot-scale experiments, an oily-slime degrading fungal consortium and a lignivore macrofungus (*Pleurotus ostreatus* (Jacq.) P. Kumm. 1871) to restore three artificially contaminated soils, presenting different grain sizes.

The data obtained from mycological and chemical analyses showed a remarkable degradation efficiency, with a considerable influence of seasonality during the 9 months of fungal treatment; therefore, both *P. ostreatus* and the fungal consortium could represent an important mycoremediation tool on polluted soils, with optimal results especially in spring treatments.

6.2 Introduction

Hydrocarbons, the main components of gasoline, diesel and fuel oils, are among the most widespread pollutants of land, groundwater, surface water and seawater (Soclo *et al.* 2000; Zhou *et al.* 2003; Chen *et al.* 2004); as concerns the soil, hydrocarbon contamination is an increasing and underestimated problem, mostly in industrialized countries (Gennadiev *et al*. 2015). Hydrocarbon pollution is mostly due to accidental or intentional spills, or to the fallout and consequent deposition of airborne polluting substances (Singh 2006); these compounds may derive from car exhausts, domestic heating and various industrial activities (Jones *et al.*1989; Clemente *et al.* 2001). Hydrocarbons with lower molecular weight can volatilize in the atmosphere or be leached until they reach the stratum (Dror *et al.* 2002); those with high molecular weight, on the other hand, are strongly adsorbed to the clay-humus fraction of the soil (Rivas, 2006).

Many pollutants, once spilled on the surface of the soil, may undergo oxidative and photochemical degradations (Singh 2006); furthermore, leaching processes by meteoric or superficial water can cause

petroleum products to penetrate the ground and drain by gravity, being transported far from the polluting source.

These substances may be harmful both to the environment and to human and animal health, even entering the food chain (Matsubara *et al.* 2006; Farrington *et al.* 2014).

The granulometry of the polluted soil can greatly influence the degradability of hydrocarbon pollutants, which are often difficult to remove, especially from fine grained soils, by using regular soil washing treatments (Vaccari *et al.* 2009).

In recent years, researchers have tried to develop different solutions to the problem of hydrocarbon soil pollution, by proposing dedicated techniques aimed to favor the degradation processes of these recalcitrant substances (Namkoong *et al.* 2002; Rahman *et al.* 2002; de Nardi *et al.* 2005; Bento *et al.* 2005; Tsai *et al.* 2009; Beškoski *et al.* 2011); anyway, many studies report techniques that result in an incomplete degradation.

It is well-known that numerous soil microorganisms, particularly bacteria (Das *et al.* 2011), yeasts and fungi (Sutherland 2004), can utilize a wide range of different carbon sources, and are thus capable to transform petroleum hydrocarbon compounds into nontoxic or less toxic substances (Cerniglia *et al.* 2006).

Microbial breakdown of hydrocarbon pollutants is generally a very effective but rather slow process; in this context, a solution aimed at speeding up the degradation process of such recalcitrant organic compounds could be the use of a consortium of microorganisms, rather than a single organism (Greco *et al.* 2018).

Fungi can be considered an important microbiological resource for the bioremediation of soils; it has been proved that some fungi, such as white-rot fungi and lignicolous microfungi, are able to degrade recalcitrant hydrocarbons by secreting cellulolytic enzymes such as lignin peroxidase, manganese peroxidase and laccases (Pointing *et al.* 2001; D'Annibale *et al.* 2005; Acevedo *et al.* 2011).

Researchers are focusing their studies on the hydrocarbon degradation capacity of several microfungi; is has been shown that some fungi such as *Fusarium solani* (Greco *et al.* 2018), *Fusarium oxysporum* (Verdin *et al.* 2004), *Trichoderma asperellum, Trichoderma viride* (Verdin *et al.* 2004; Husaini *et al.* 2008), *Rhizopus* sp., *Penicillium funiculosum* (Mancera-López *et al.* 2008) and *Aspergillus sydowii* (Al-Jawhari *et al.* 2014) are among the best species for degrading hydrocarbon compounds.

Recent studies have shown that fungal consortia seem to work better than the application of individual fungal species, because their synergistic action can speed up the process of degradation (Anastasi *et al.* 2009; Chen *et al.* 2010; Balaji *et al.* 2014).

Furthermore, it should be highlighted that a lot of *in vitro* studies have been conducted, but few applicative tests in soil mesocosms have been carried out; real matrices are indeed characterized by an important variability of environmental factors (Kadri *et al.* 2017) that interfere with the experimentation, making data interpretation often more difficult.

Our study is aimed to test, with a pilot-scale experimentation, the effectiveness of a pool of selected fungi in remediating polluted soil matrices.

Starting from the above considerations, our work was designed employing *Pleurotus ostreatus* and a microfungal consortium, to test their degradation effectiveness during 9 months of pilot-scale fungal treatments; this contributes to selecting a pool of fungal species suitable for the degradation of petroleum products and to the drafting of a protocol for large-scale applications.

6.3 Material and methods

Samples of polluted soils were collected in October 2017, inside an industrial area in north-western Italy, near an urban waste storage site; using a garden shovel and a rubberized mechanical shovel, aliquots of soil belonging to 3 different granulometric categories (mixed soil, ground and clay) were sampled, for a total of 450 kg. Later, the fraction size <2cm was selected by mechanical sieving and 5000 mg/kg of a solution containing gasoline (20%), diesel oil (40%) and fuel oils (40%) was added to each different soil.

Each artificially contaminated soil was mixed in a cement mixer and placed, according to the different grain size, in 12 aliquots containing 30 kg, kept at room temperature.

The fungal species employed to arrange the inoculum were the following: *Pleurotus ostreatus* (Jacq.) P. Kumm. 1871, that has been individually tested, and *Fusarium solani* (Mart.) Sacc. 1881, *Pseudallescheria boydii* (Shear) McGinnis, A.A. Padhye & Ajello 1982, *Talaromyces amestolkiae* N. Yilmaz, Houbraken, Frisvad & Samson 2012, *Paecilomyces formosus* Sakag., May. Inoue & Tada ex Houbraken & Samson 2009 that have been used together, as a fungal consortium; the selected species, taken from the mycological

collection of the Genoa mycoteque (DISTAV), were identified with a polybasic approach which determined the micro- and macromorphological characteristics using specific taxonomical keys (Raper and Fennel, 1977; Korneup and Wansher, 1978; Pitt, 1979; Malloch, 1981; Samson and Frisvad, 2004) and then by optical microscopy (10×/0.30 to 40×/0.75). The identities of the selected fungal strains were confirmed using nuclear DNA extraction, PCR amplification and DNA sequencing. The genomic DNA was extracted from 100 mg of fresh fungal culture using a modified CTAB method (Doyle and Doyle, 1987). The morphological identifications were confirmed by amplification of the β- tubulin gene using Bt2a and Bt2b primers (Glass and Donaldson, 1995) and the ITS region amplification using universal primers ITS1F/ITS4 (Gardes and Bruns, 1993). The PCR products were purified and sequenced using MACROGEN Inc. (Seoul, Republic of Korea). Sequence assembly and editing were performed using Sequencher® version 5.2 (sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA). The taxonomic assignment of the sequenced samples was carried out using the BLASTN algorithm to compare the sequences obtained in the present study with the GenBank database (https://www.ncbi.nlm.nih. gov/genbank/).

The soils samples were inoculated with a saccharose solution containing 1 x 10^8 conidia/ml suspension for the fungal consortium, and a PDA (Potato Dextrose Agar) liquid culture of *Pleurotus ostreatus* mycelium*;* to allow a better mycelial growth the staging areas were irrigated daily by spraying 100 ml of water per box, while to guarantee an adequate oxygenation the matrices were weekly turned over with a sterile shovel.

The mesocosms were analyzed monthly over 9 months to monitor the degradation process resulting from the fungal treatment.

Fig. 1. A) Sample of artificially contaminated soil mixed in a cement mixer; **B)** Pilot-scale experiments with real polluted matrices; **C)** Mycological solution containing fungal inocula.

6.3.1 Chemical analyses

For each box, samples were taken by coring in three different points, and the three carrots obtained were consecutively mixed to form one sample to analyze. Chemical analyses were carried out in accordance with Italian D. Lgs n. 152/2006, Part Four, Title V, Annex 5, Table 1. As concerns the C < 12 hydrocarbon analyses, samples were analyzed based on raw material using the EPA 5021A 2003 method for sample preparations; organic compound analysis was instead carried out using the headspace method. The instrumental reading was performed employing the EPA 8260C 2006 method, based on the use of gas flame chromatography (FID).

The C > 12 hydrocarbon samples were dried at 40°C in a muffle-furnace to eliminate humidity and were sieved at 2mm to remove the skeleton (particle size between 2 mm and 2 cm). Subsequently, the soil fraction under 2mm was chemically analyzed; the results were then compared to those of the skeleton fraction. In this scenario the EPA 3550C 2007 and EPA 8270D 2007 methods were employed: these methods imply the hydrocarbon extraction in n-hexane with ultrasound, the purification of the extract with Florisil and the instrumental reading by gas chromatography FID.

6.4 Results

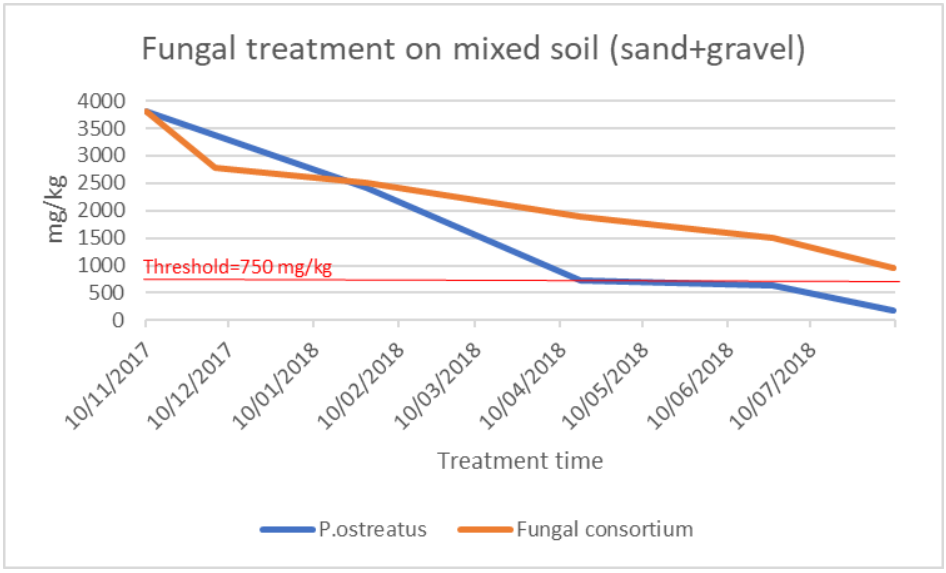
The data from the chemical analyses have shown that both *Pleurotus ostreatus* and the microfungal consortium are very efficient in the degradation of the tested hydrocarbon compounds, sensibly reducing the level of pollutants in the three soil matrices we examined. The best results were obtained by *Pleurotus ostreatus*, which has shown a greater biodegradation capacity than our fungal consortium on all the three different evaluated matrices.

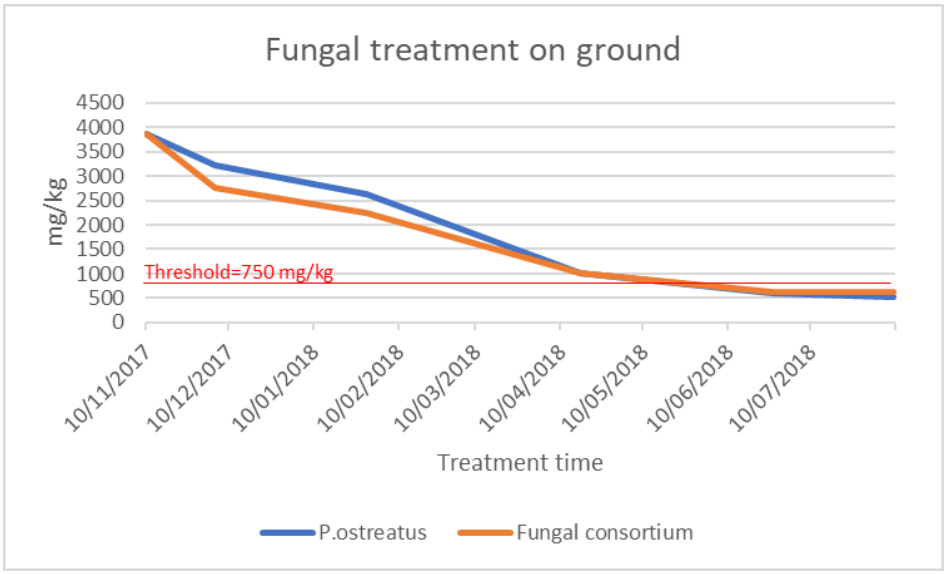
On the mixed soil matrix, after 5 months of fungal treatment, *P. ostreatus* has reduced pollution values to below 750 mg/kg, the current legal limit for hydrocarbons in industrial areas, while the fungal consortium has degraded the hydrocarbon compounds down to the value of 960 mg/kg, well below the starting value of 5000 mg/kg.

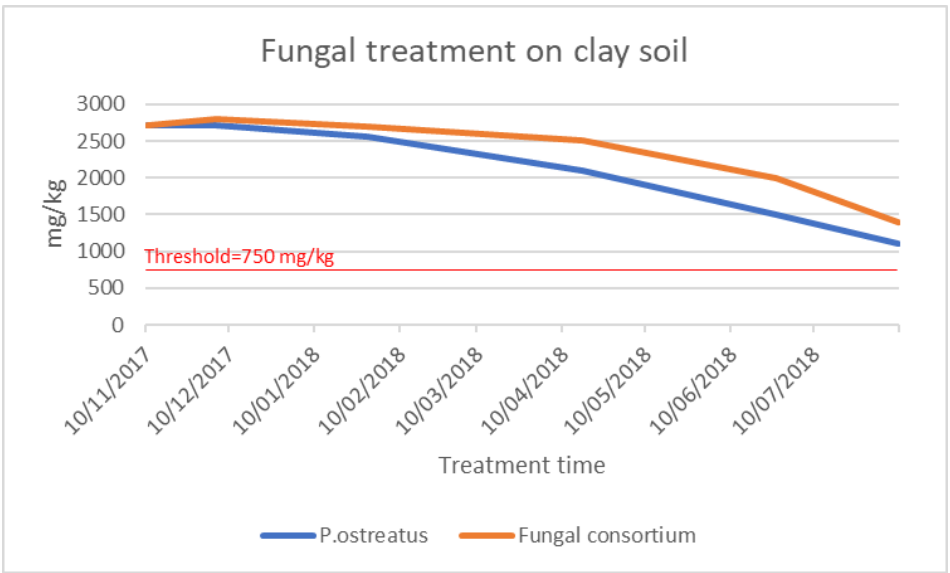
Seasonal differences in the degradation efficiency have been observed: *P. ostreatus* has proved more effective in spring and winter, while the fungal consortium has degraded more in spring and autumn.

On the ground matrix, after 7 months of fungal treatment both *P. ostreatus* and the fungal consortium have reduced hydrocarbon compounds to below 750 mg/kg, with a higher degradation efficiency in spring and autumn, showing a percentage peak above all in the spring season.

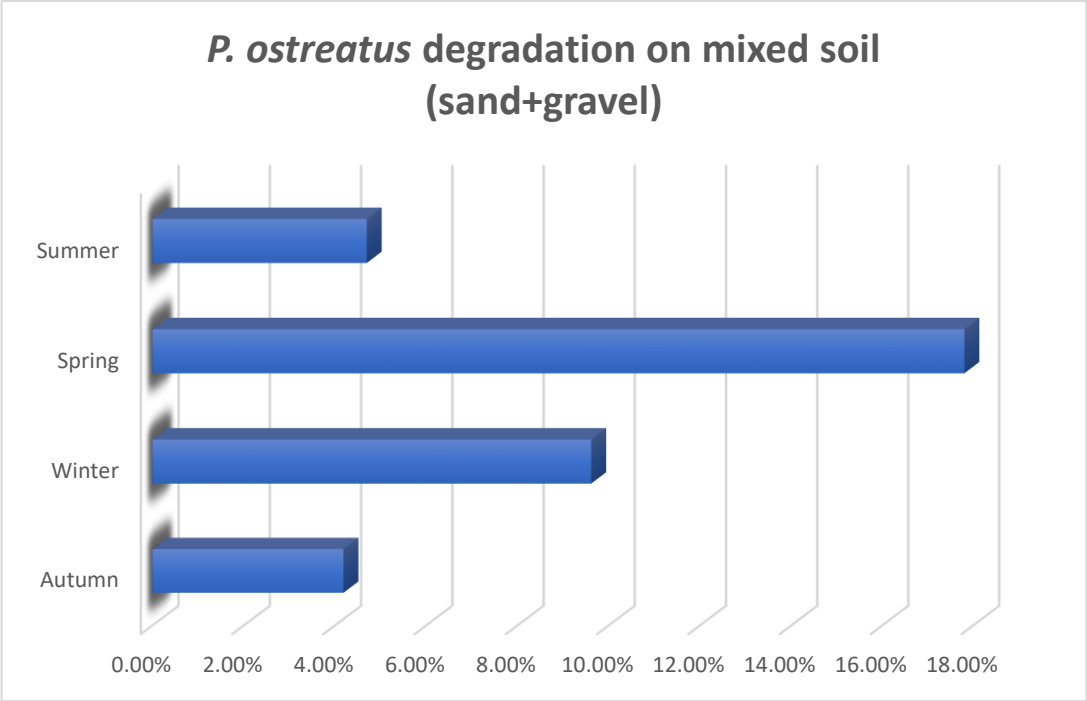
On the clay soil matrix, after 9 months of fungal treatment, both *P. ostreatus* and the fungal consortium have sensibly reduced the hydrocarbon concentrations, but not below the legal threshold currently set for industrial areas: as concerns *P. ostreatus*, the concentration of the pollutants has lowered to 1100mg/kg while the F.C. has reduced the hydrocarbon level down to 1400 mg/kg. On this soil matrix, both *P. ostreatus* and the F.C. showed a higher degradation efficiency in spring and summer.

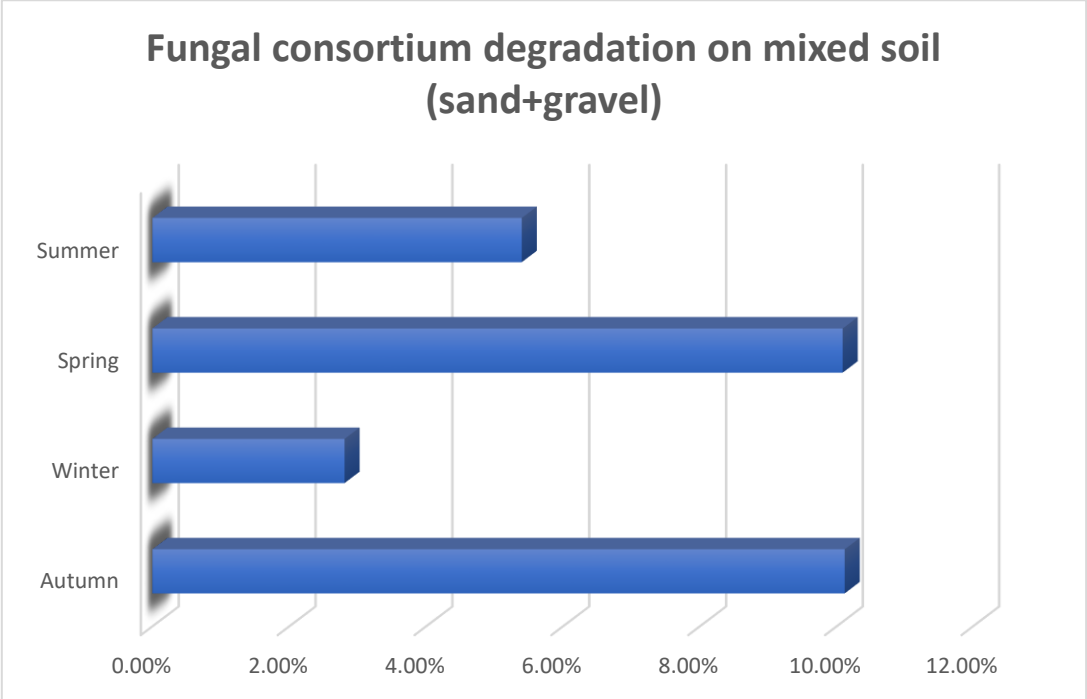




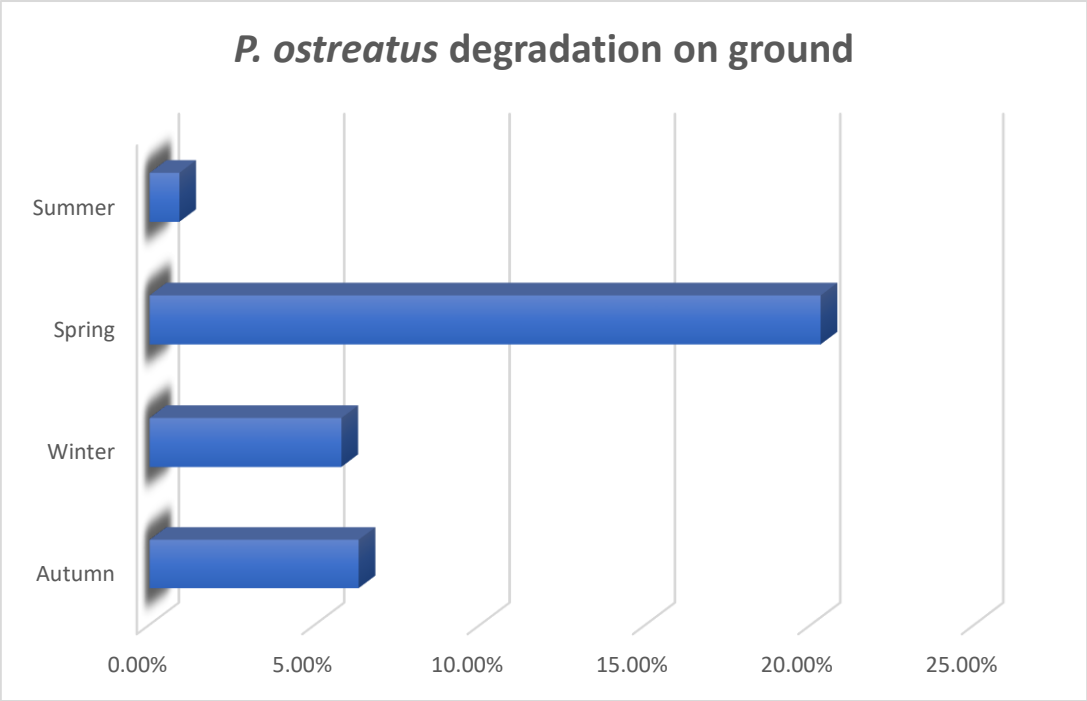


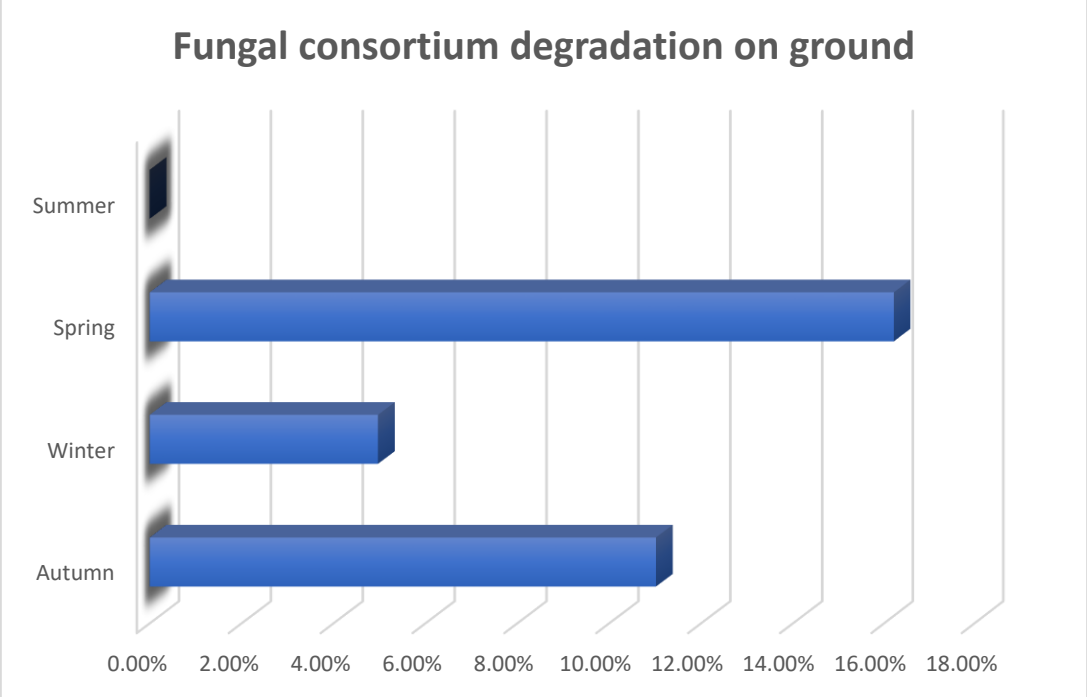
Trends about F.C. (fungal consortium) and *Pleurotus ostreatus* treatment for nine months.



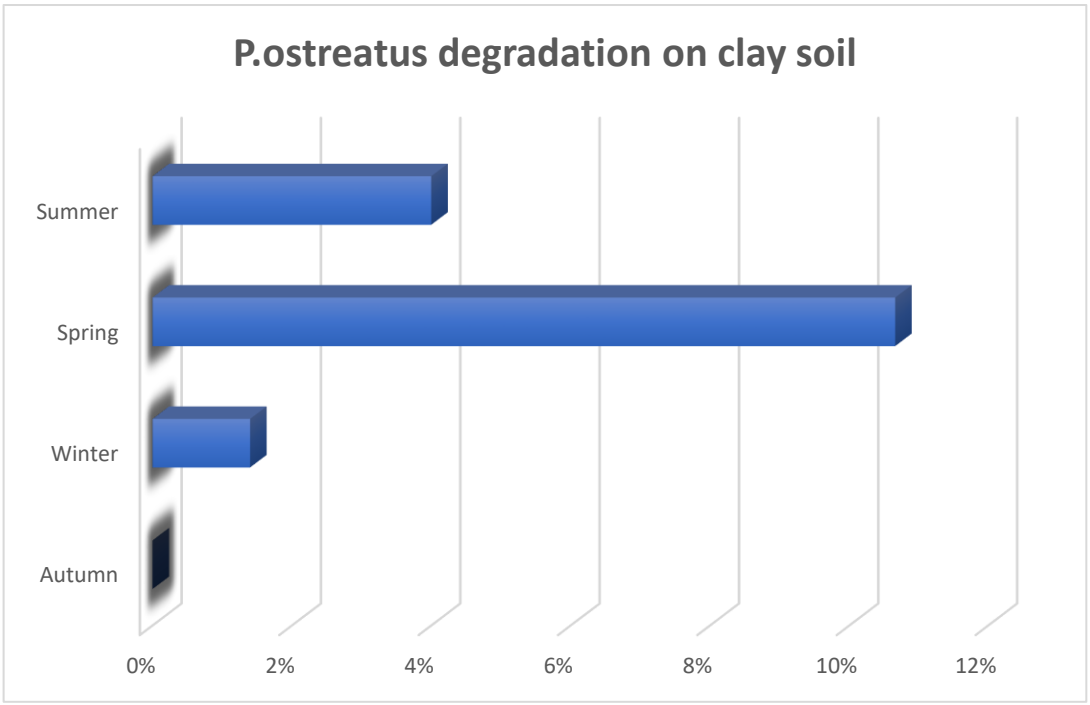


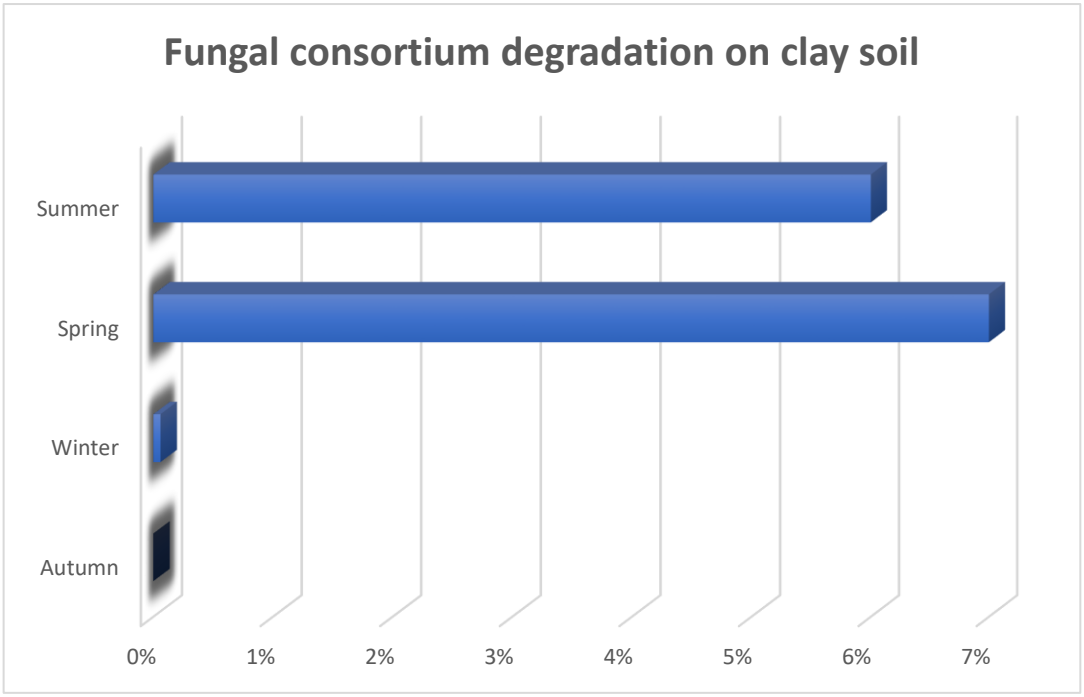
A. Seasonal performance of *P. ostreatus* treatment on mixed soil.
B. Seasonal performance of F.C. treatment on mixed soil.



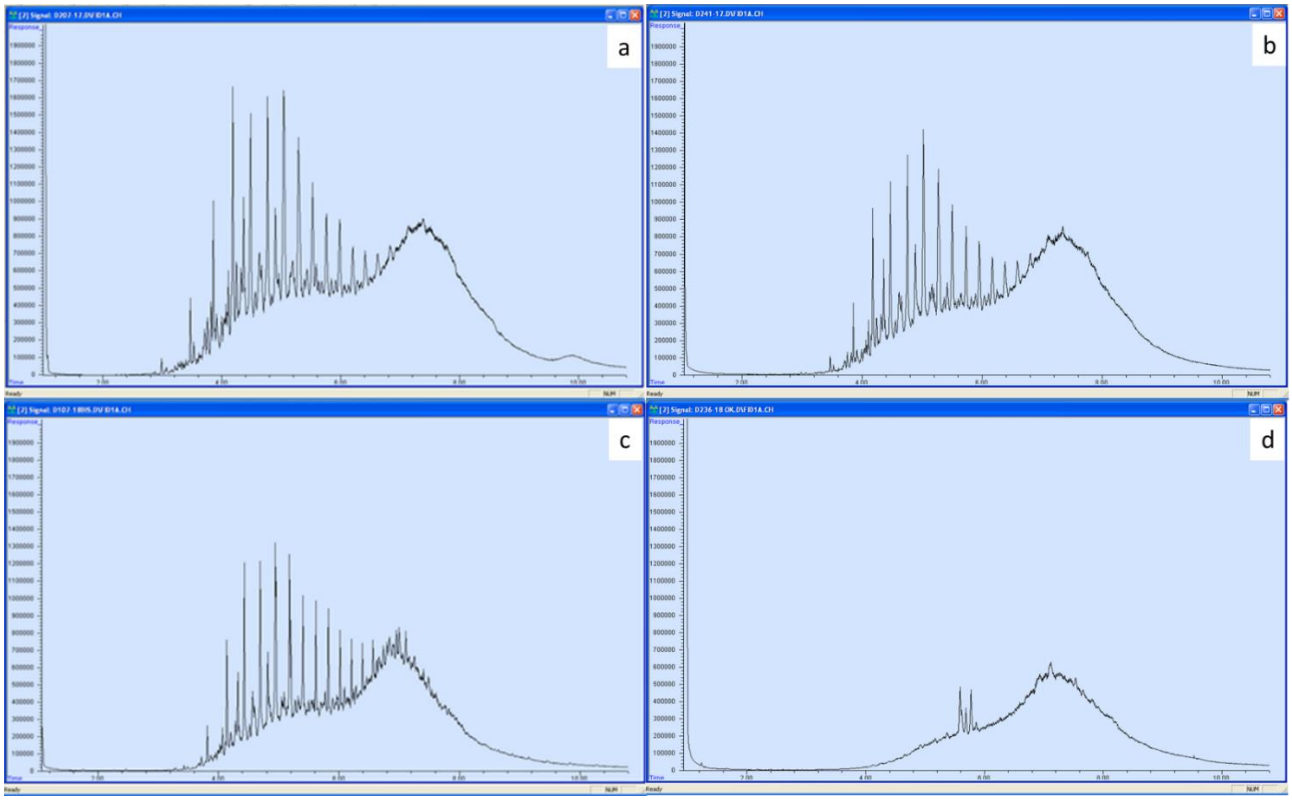


C. Seasonal performance of *P. ostreatus* treatment on ground.
D. Seasonal performance of F.C. treatment on ground.





E. Seasonal performance of *P. ostreatus* treatment on clay soil.
F. Seasonal performance of F.C. treatment on clay soil.



a. Gas chromatogram of diesel and fuel oil trends following treatment of mixed soil with *P. ostreatus*, in autumn. b. Gas chromatogram of diesel and fuel oil trends following treatment of mixed soil with *P. ostreatus*, in winter. c. Gas chromatogram of diesel and fuel oil trends following treatment of mixed soil with *P. ostreatus*, in spring. d. Gas chromatogram of diesel and fuel oil trends following treatment of mixed soil with *P. ostreatus*, in summer.

6.5 Discussions

Pleurotus ostreatus and a microfungal consortium (F.C.) were employed on real non-sterile contaminated soils to evaluate the biodegradation of hydrocarbon compounds: a 9 months pilot-scale fungal treatment has been performed on three different soil matrices.

We decided to employ a selected fungal pool that already proved effective for the remediation of hydrocarbon compounds; these fungal species were previously isolated from an extreme environment presenting hydrocarbon contaminations (Greco *et al.* 2018) and were maintained in the culture collection of the Mycological Laboratory of the Department of Earth, Environment and Life Sciences of the University of Genoa (DISTAV), for mycoremediation purposes.

Mostly cellulolytic fungi such as *F. solani* (in the consortium) and *P. ostreatus* were selected, because they have proven more suitable for the removal of recalcitrant organic compounds such as hydrocarbons, which have molecules with a complex chemical structure, often like the lignin molecules. Some fungi belonging to genera *Aspergillus ssp.* (Cortes-Espinosa *et al.* 2006), *Fusarium spp.* (Potin *et al.* 2004), *Trichoderma ssp*. (Mollea *et al.* 2005) and *Penicillium spp.* (Saraswathy and Hallberg, 2005) are known to be good producers of complex ligninolytic extracellular enzymes such as laccases and peroxidases, that are effective in degrading several aliphatic and aromatic compounds, due to the non-specificity of these enzymatic systems (Tekere *et al.* 2005; Tortella *et al.* 2005).

In the present experimentation we decided to use *P. ostreatus* for its well known ability to degrade hydrocarbon compounds, while the fungal consortium was employed because, in recent years, several researches employing fungal consortia have shown a more vehement hydrocarbon degradation, mostly in *in vitro* applications*;* according to many studies, indeed, the synergistic action of multiple fungi can intensify and facilitate the degradation processes of these recalcitrant compounds (Anastasi *et al.* 2009; Chen *et al.* 2010; Balaji *et al.* 2014).

To reduce the high costs of large-scale applications, a cheap modified culture medium, based on water added with saccharose, was employed; it has proved to be very effective for promoting the growth of the selected fungi. This modified culture medium simply played the role of a starter, enabling the mycelia to develop up to a certain level, at which the fungi begin the degradation process.

The data from the chemical analyses showed how the efficiency of fungal degradation varies according to seasonality; it is known that some physical parameters such as temperature and humidity have influence on the fungal biological cycle and on fungal activity; the optimal temperature for mycelial growth is in a range between 24 and 37 °C, while the *optimum* for humidity is about 60% (Deacon, 2006).

The data we collected during this experimentation show that both the macro-fungus *P. ostreatus* and the fungal consortium (F.C.) could be considered as important tools for the bioremediation of polluted sandy, gravelly and clayey soils, with the maximum efficiency in spring treatments. Moreover, *P. ostreatus* has proved suitable to remediate sandy and gravelly soils in winter, cultivated soils in autumn and clay soils in summer, while the fungal consortium could be employed to restore sandy, gravelly and cultivated soils in autumn, clay soils in summer.

The fungal treatment on clay matrices resulted in a lower degradation efficiency, probably due to the typically compact substrate presenting low porosity and low oxygen circulation; these conditions can indeed be limiting for the fungal growth, which has shown a low development in the first two seasons of fungal treatment (autumn and winter).

6.6 Conclusions

Our pilot-scale experiments provide the evidence of the applicability of seasonal large-scale mycoremediation and select some species rather than others for the restoration of polluted soils, according to the seasons and the soil granulometry.

Moreover, this research is proposed as a step forward, from the *in vitro* studies previously performed by most researchers in this field, to a pilot-scale study on actual soil matrices.

Many studies show that fungal efficiency in degrading hydrocarbons compounds may be improved by the presence of some specific bacterial species; it has also been reported that, although bacteria alone can start to break down a hydrocarbon mixture, twice that amount can be degraded when bacteria and fungi are both present (Boonchan *et al.* 2000). Hence, in future pilot-scale experiments, the synergism between fungi and bacteria should be thoroughly investigated, as it could be an effective tool to increase the efficiency of hydrocarbon degradation, compared to what could be achieved by individual organisms.

Therefore, starting from the data we have obtained, in the future it will be necessary to provide for the drafting of a practical protocol for industrial applications that sees both fungi and bacteria as actors in the degradation processes of recalcitrant organic compounds that are harmful to the human health and to the environment.

6.7 References

Acevedo F, Pizzul L, del Pilar Castillo M, Cuevas R, Diez MC. 2011. Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus Anthracophyllum discolor. J Hazard Mater. 185(1):212-219.

AI-Jawhari IFH. 2014. Ability of some soil fungi in biodegradation of petroleum hydrocarbon. J Appl Environ Microbiol. 2(2):46-52.

Anastasi A, Coppola T, Prigione V, Varese GC. 2009. Pyrene degradation and detoxification in soil by a consortium of basidiomycetes isolated from compost: role of laccases and peroxidases. J Hazard Mater. 165(1-3):1229-1233.

Balaji V, Arulazhagan P, Ebenezer P. 2014. Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. J Environ Biol. 35(3):521-529.

Bento FM, Camargo FA, Okeke BC, Frankenberger WT. 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource technol. 96(9):1049-1055.

Beškoski VP, Gojgić-Cvijović G, Milić J, Ilić M, Miletić S, Šolević T, Vrvić MM. 2011. Ex situ bioremediation of a soil contaminated by mazut (heavy residual fuel oil)–A field experiment. Chemosphere. 83(1):34-40.

Boonchan S, Britz ML, Stanley GA. 2000. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl Environ Microb. 66(3):1007-1019.

Cerniglia CE, Sutherland JB. 2006. Relative roles of bacteria and fungi in polycyclic aromatic hydrocarbon biodegradation and bioremediation. Fungi Biogeochem. Cycles, 24, 182.

Chen B, Wang Y, Hu D. 2010. Biosorption and biodegradation of polycyclic aromatic hydrocarbons in aqueous solutions by a consortium of white-rot fungi. J Hazard Mater. 179(1-3), 845-851.

Chen B, Xuan X, Zhu L, Wang J, Gao Y, Yang K, Lou B. 2004. Distributions of polycyclic aromatic hydrocarbons in surface waters, sediments and soils of Hangzhou City, China. Water Res. 38(16):3558-3568.

Clemente AR, Anazawa TA, Durrant LR. 2001. Biodegradation of polycyclic aromatic hydrocarbons by soil fungi. Braz. J. Microbiol. 32(4):255–261.

Cortés-Espinosa DV, Fernández-Perrino FJ, Arana-Cuenca A, Esparza-Garcia F, Loera O, Rodríguez-Vázquez R. 2006. Selection and identification of fungi isolated from sugarcane bagasse and their application for phenanthrene removal from soil. J Environ Sci Heal A. 41(3):475-486.

D'Annibale A, Ricci M, Leonardi V, Quaratino D, Mincione E, Petruccioli M. 2005. Degradation of aromatic hydrocarbons by white-rot fungi in a historically contaminated soil. Biotechnol Bioeng. 90(6):723-731.

Das N, Chandran P. 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol Res Inter. 2011.

De Nardi IR, Ribeiro R, Zaiat M, Foresti E. 2005. Anaerobic packed-bed reactor for bioremediation of gasoline-contaminated aquifers. Process Biochem. 40(2):587-592.

Deacon JW. 2006. Fungal Biology. Malden, US.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull Bot Soc Am. 19:11–15.

Dror I, Gerstl Z, Prost R, Yaron B. 2002. Abiotic behavior of entrapped petroleum products in the subsurface during leaching. Chemosphere. 49(10):1375-1388.

Farrington JW, Takada H. 2014. Persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and plastics: Examples of the status, trend, and cycling of organic chemicals of environmental concern in the ocean. Oceanography. 27(1):196–213.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol Ecol. 2:113– 118.

Gennadiev AN, Pikovskii YI, Tsibart AS, Smirnova MA. 2015. Hydrocarbons in soils: Origin, composition, and behavior. Eurasian Soil Sci+. 48(10):1076-1089.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microb. 61(4):1323–1330.

Greco G, Cecchi G, Di Piazza S, Cutroneo L, Capello M, Zotti M. 2018. Fungal characterisation of a contaminated marine environment: the case of the Port of Genoa (North-Western Italy). Webbia. 73(1):97-106.

Husaini A, Roslan HA, Hii KSY, Ang CH. 2008. Biodegradation of aliphatic hydrocarbon by indigenous fungi isolated from used motor oil contaminated sites. World J Microbiol and Biotechnol. 24(12):2789-2797.

Jones KC, Stratford JA, Tidridge P, Waterhouse KS, Johnston AE. 1989. Polynuclear aromatic hydrocarbons in an agricultural soil: long-term changes in profile distribution. Environ. Pollut. 56(4):337–351.

Kadri T, Rouissi T, Brar SK, Cledon M, Sarma S, Verma M. 2017. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review. J Environ Sci. 51, 52–74

Kornerup A, Wanscher JH. 1978. Methuen handbook of colour. 3rd edn London: Eyre Methuen.

Malloch D. 1981. Moulds, their isolation, cultivation, and identification. University of Toronto Press.

Mancera-López ME, Esparza-García F, Chávez-Gómez B, Rodríguez-Vázquez R, Saucedo-Castaneda G, Barrera-Cortés J. 2008. Bioremediation of an aged hydrocarbon-contaminated soil by a combined system of biostimulation–bioaugmentation with filamentous fungi. International Biodeterioration & Biodegradation. 61(2):151-160.

Matsubara M, Lynch JM, De Leij FAA M. 2006. A simple screening procedure for selecting fungi with potential for use in the bioremediation of contaminated land. Enzyme Microb. Tech. 39(7):1365–1372.

Mollea C, Bosco F, Ruggeri B. 2005. Fungal biodegradation of naphthalene: microcosms studies. Chemosphere. 60(5):636-643.

Namkoong W, Hwang EY, Park JS, Choi JY. 2002. Bioremediation of diesel-contaminated soil with composting. Environ Pollut. 119(1):23-31.

Pitt J. 1979. The Genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. New York (NY): Accademic Press.

Pointing S. 2001. Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biot. 57(1-2):20-33.

Potin O, Rafin C, Veignie E. 2004. Bioremediation of an aged polycyclic aromatic hydrocarbons (PAHs)-contaminated soil by filamentous fungi isolated from the soil. Int Biodeter Biodegr. 54(1):45-52.

Rahman KSM, Banat IM, Thahira J, Thayumanavan T, Lakshmanaperumalsamy P. 2002. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresource Technol. 81(1):25-32.

Raper KB, Fennell DI. 1977. The Genus Aspergillus RE Krieger Publishing Company. Huntington, New York.

Rivas FJ. 2006. Polycyclic aromatic hydrocarbons sorbed on soils: a short review of chemical oxidation based treatments. J Hazard Mater. 138(2):234-251.

Samson RA, Frisvad JC. 2004. Penicillium subgenus Penicillium: new taxonomic schemes and mycotoxins and other extrolites. Stud Micol. 49(49):1–260.

Saraswathy A, Hallberg R. 2005. Mycelial pellet formation by Penicillium ochrochloron species due to exposure to pyrene. Microbiol Res. 160(4):375-383.

Singh H. 2006. Mycoremediation: fungal bioremediation. John Wiley & Sons.

Soclo HH, Garrigues PH, Ewald M. 2000. Origin of polycyclic aromatic hydrocarbons (PAHs) in coastal marine sediments: case studies in Cotonou (Benin) and Aquitaine (France) areas. Mar Pollut Bull. 40(5):387-396.

Sutherland JB. 2004. Degradation of hydrocarbons by yeast and filamentous fungi. Fungal biotechnology in agricultural, food and environmental applications, 417-429.

Tekere M, Read JS, Mattiasson B. 2005. Polycyclic aromatic hydrocarbon biodegradation in extracellular fluids and static batch cultures of selected sub-tropical white rot fungi. J Biotechnol. 115(4):367-377.

Tortella GR, Diez MC, Durán N. 2005. Fungal diversity and use in decomposition of environmental pollutants. Crit Rev Microbiol. 31(4):197-212.

Tsai TT, Kao CM, Surampalli RY, Chien HY. 2009. Enhanced bioremediation of fuel-oil contaminated soils: laboratory feasibility study. J Environ Eng. 135(9):845-853.

Vaccari M, Riganti V, Collivignarelli MC, Zanaboni S. 2009. Recupero e valorizzazione del residuo limo-argilloso da lavaggio di suoli contaminati da idrocarburi – Parte I: vincoli autorizzativi e normativi. Recycling. 13(4):32-37.

Verdin A, Sahraoui ALH, Durand R. 2004. Degradation of benzo [a] pyrene by mitosporic fungi and extracellular oxidative enzymes. Int Biodeter Biodegr. 53(2):65-70.

Zhou JL, Maskaoui K. 2003. Distribution of polycyclic aromatic hydrocarbons in water and surface sediments from Daya Bay, China. Environ Pollut. 121(2):269-281.

Chapter seven

CONCLUSIONS AND GENERAL REMARKS

The results obtained allowed the microfungal characterization of polluted seawaters in the Port of Genoa and the evaluation of the possible correlations between the presence of marine microfungi and the hydrocarbon pollution characterizing the area. Other hydrocarbon-tolerant fungal species have been isolated from extreme polluted environments and employed in growth and degradation experiments to restore incoherent materials, such as oily slimes or soils of different lithologies contaminated with petroleum products. The experiments have shown the effective viability of strains isolated in extreme environments and their active role in degradative processes of recalcitrant compounds. The selected fungi were able to degrade PAHs and hydrocarbons compounds in both *in vitro* and mesocosm experiments.

Hence, the thesis confirms extremophile fungi as candidates for biotechnological applications, such as the bioremediation of seawater or soils polluted by hydrocarbon compounds. The fungi of polluted areas, adapted to survive in limiting and toxic conditions, indeed represent an important sustainable tool for the restoration of compromised habitats.

Nevertheless, at present there is a lack of information about mechanisms to remove hydrocarbons during fungal treatments, specifically in large scale restoration. Thus, a multidisciplinary approach is required to fully develop this potential; a better knowledge of the factors controlling hydrocarbon bioavaialability and mycodegradation and their optimization is also necessary to enhance mycoremediation in marine and terrestrial environments. Moreover, further investigations on enzymes involved in fungal degradation processes can represent an important target of future research in order to optimize the application times of mycoremdiation on large scale and to open new perspectives on fungal hydrocarbon metabolisms.

Finally, a mixed culture of fungi and bacteria may be further effective for restoration on large-scale sites due to their different, but complementary, ability to degrade hydrocarbons. An accurate investigation on the synergistic relationship between fungi and bacteria may be relevant to better understand the variables that affect degradation processes, to improve and speed up bio-treatments.

PUBLICATIONS

International papers

Di Piazza S, Zotti M, Barranco R, Cecchi G, **Greco G,** Ventura F. 2018. Post-mortem fungal colonization pattern during 6 weeks: two case studies. *Forensic science international*.

Greco G, Cecchi G, Di Piazza S, Cutroneo L, Capello M, Zotti M. 2018. Fungal characterisation of a contaminated marine environment: the case of the Port of Genoa (North-Western Italy). Webbia. 73(1):97-106.

Greco G, Capello M, Cecchi G, Cutroneo L, Di Piazza S, Zotti M. 2017. Another possible risk for the Mediterranean Sea? Aspergillus sydowii discovered in the Port of Genoa (Ligurian Sea, Italy). Mar Pollut Bull. 122(1-2):470-474.

Capello M, Carbone C, Cecchi G, Consani S, Cutroneo L, Di Piazza S, **Greco G,** Tolotti R, Vagge G, Zotti M. 2017. A mycological baseline study based on a multidisciplinary approach in a coastal area affected by contaminated torrent input. Mar Pollut Bull. 119(1):446-453.

Greco G, Di Piazza S, Gallus L, Amaroli A, Pozzolini M, Ferrando S, Bertolino M, Scarfì S, Zotti M. First description of a fatal fungal infection by *Aspergillus tubingensis* on the marine sponge *Chondrosia reniformis*. Submitted.

Greco G, Di Piazza S, Cecchi G, Cutroneo L, Capello M, Zotti M. Mycoremediation of oily slime containing a polycyclic aromatic hydrocarbon mixture. Submitted.

Cecchi G, Cutroneo L, Di Piazza S, **Greco G,** Vagge G, Zotti M, Capello M. Fungi as tool of polluted port sediment remediation. Submitted.

Rosatto S, Roccotiello E, **Greco G,** Cecchi G, Zotti M, Vezzulli L, Mariotti M. Rhizosphere response to nickel in a facultative hyperaccumulator". Submitted.

International congresses

Zotti M, Di Piazza S, Cecchi G, **Greco G,** Rosa E, Pucillo G. Perché la caratterizzazione micologica di matrici inquinate?. Expo Remtech 2018 Remediation Technologies. Ferrara, 19-21 Settembre 2018.

Greco G, Di Piazza S, Cecchi G, Cutroneo L, Faga M, Capello M, Zotti M. Mycoremediation of oily slime containing a polycyclic aromatic hydrocarbon mixture. 6th International Conference on Sustainable Solid Waste Management. Naxos Island, 13-16 Giugno 2018.

Cecchi G, Cutroneo L, Di Piazza S, **Greco G,** Vagge G, Zotti M, Capello M. Fungi as tool of polluted port sediment remediation. 6th International Conference on Sustainable Solid Waste Management. Naxos Island, 13-16 Giugno 2018.

Greco G, Capello M, Cecchi G, Di Piazza S, Cutroneo L, Carbone C, Tolotti R, Consani S, Vagge G, Zotti M. Marine-derived fungi as potential indicators of sediment quality and bioremediation tool: preliminary study", 10th International SedNet Conference. Genova, 14-17 Giugno 2017 **(2°Poster Prize).**

Greco G, Capello M, Cecchi G, Cutroneo L, Di Piazza S, Zotti M. Aspergillus sydowii in the Port of Genoa (Liguria NW Italy). 112° Congresso della Società Botanica Italiana (IV International Plant Science Conference). Parma, 20-23 Settembre 2017.

Greco G, Cecchi G, Di Piazza S, Mariotti MG, Zotti M. Fungi: New Biotechnology For Waste Management. IWIW 2016 - International Workshop on Industrial Waste. Genova, 17 Febbraio 2016.

Zotti M, Carbone C, Cecchi G, Consani S, Cutroneo L, Di Piazza S, Gabutto G, **Greco G,** Vagge G, Capello M. Mycodiversity In Marine Sediments Contaminated By Heavy Metals: Preliminary Results. European Geosciences Union General Assembly, 2016. Vienna, 17-22 Aprile 2016.

Ventura F, Di Piazza S, Cecchi G, **Greco G,** Bellini E, Zotti M. A Mycrofungal Colonisation of a Corpse Oral Cavity. IALM International Symposium. Venezia, 21-24 Giugno 2016.

Rosatto S, Roccotiello E, Cecchi G, Di Piazza S, **Greco G,** Zotti M, Mariotti MG. A rhizosphere approach to mitigate soil erosion and pollution. III International Plant Science Conference (IPSC). Roma, Tor Vergata, 21 - 23 September 2016.

National congresses

Di Piazza S, Cecchi G, **Greco G,** Ventura F, Zotti M. I funghi nella micologia forense: il caso del cadavere mummificato. UMI 2018, Congresso Nazionale dell'Unione Micologica Italiana. Siena, 6-8 Settembre 2018.

Cecchi G, Di Piazza S, **Greco G,** Cutroneo L, Capello M, Zotti M. Funghi per risanare sedimenti portuali contaminati. UMI 2018, Congresso Nazionale dell'Unione Micologica Italiana. Siena, 6-8 Settembre 2018.

Zotti M, Cecchi G, **Greco G,** Di Piazza S, Bellini E, Ventura F. I funghi dei cadaveri. UMI 2016, Congresso Nazionale dell'Unione Micologica Italiana. Aquila, 12-13 Settembre 2016.

Greco G, Capello M, Cecchi G, Cutroneo L, Di Dio M, Di Piazza S, Vagge G, Zotti M. Microfunghi Dei Sedimenti Del Porto Di Genova. UMI 2016, Congresso Nazionale dell'Unione Micologica Italiana. Aquila, 12-13 Settembre 2016.

Rosatto S, Roccotiello E, Cecchi G, Di Piazza S, **Greco G,** Zotti M, Mariotti MG. Phytoremediation: Un Approccio Rizosferico. III Workshop nazionale Bonifica, Recupero Ambientale ESviluppo Del Territorio: esperienze a confronto sul Fitorimedio. Roma, 17-18 marzo 2016.