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Improving the sewage sludge and wastewater quality through myco-remediation

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Thesis abstract

Pollutants are one of the many problems at the center of our society, especially in urban contexts where they can enter superficial and groundwater through wastewater treatment plants. Fungi, particularly Wood Decay Fungi (WDF), are a well-known group of organisms with great adsorbing potential and the capability to produce a wide spectrum of enzymes. These enzymes allow them to specifically interact with recalcitrant pollutants such as heavy metals and pharmaceuticals, removing them from the environment and/or degrading them.

In this study, several fungal strains were collected from two wastewater treatment plants in North Italy and from forests in Italy and Spain. These strains were isolated and identified using ITS-based molecular analysis. They represent a source of potentially exploitable and useful species for myco-remediation of heavy metals from sewage sludges and pharmaceuticals from wastewater.

Among the isolated species, 11 WDF strains were chosen and tested for their capabilities to grow on different types of sewage sludges. The three best strains were used for heavy metal adsorption tests on pre-thickening sludge. In parallel, seven WDF strains were tested in a defined liquid medium spiked with pharmaceuticals (Diclofenac, Ketoprofen, Paracetamol, and Irbesartan) to test their degradation capabilities. The good results led to focusing the research on pharmaceuticals and wastewater rather than on heavy metals and sewage sludges.

The strains with the best pharmaceutical degradation—*Ganoderma resinaceum*, *Perenniporia fraxinea*, and *Irpex lacteus*—alongside new strains, were then tested against 14 compounds in discharge wastewater to verify their degradation potential in a real water matrix. [*Omissis*] was chosen as the strain to test for the following experiments because it proved to be the most effective and versatile strain at degrading the target compounds.

Several experimental campaigns were performed to understand key parameters such as the type of wastewater, the optimal mycelium-water contact time, and the best inoculation method.

The results obtained have pointed out that: 1) incoming wastewater is the most suitable substrate for myco-remediation; 2) [*Omissis*] achieved the best degradation results at 48 and 72 hours from its inoculation; 3) the best-performing type of inoculum is a mixture of mycelium and culture broth. Among the compounds analyzed, the most degraded are [*Omissis*]. Other compounds are also degraded but to a lesser extent.

This information was needed to start the currently ongoing experimentation at a pilot plant scale and to understand the obstacles and critical issues that this type of research must address. Pilot plant experimentation confirmed the results obtained at the laboratory scale, achieving between 24 and 48 hours an average degradation percentage of [*Omissis*].

With this project, a precious strain was selected capable of withstanding competitors and environmental stress and degrading a wide number of pharmaceuticals. This represents a valuable starting point for further studies on the topic and valuable knowledge to keep exploring its potential in myco-remediation.

Introduction

The present PhD thesis in Earth and Environmental Sciences (XXXVI° Cycle) is the result of three years of work at the Micology Laboratory of University of Pavia. This research was funded by Regione Lombardia within Project CE4WE (Circular Economy for Water and Energy) and was done in collaboration with A2A Life Company and CAP Holding Spa. Project CE4WE aims to develop new technologies, knowledge and specific expertise for sustainable water cycle management. In this specific scenario, my PhD project was focused on sewage sludge and wastewater pollutants and their removal with the helping hand of mycology and fungi.

The introduction has the task of explaining the two pillars on which my research is based: the world of Wood Decay Fungi (WDF) with their properties and characteristics and the issue of emerging environmental pollutants. Finally, it will be made clear how these two seemingly unrelated elements can interact together with the concept of Myco-remediation, which serves as a starting point for a potentially concrete environmental intervention.

Experimental procedures, results, critical issues, and future perspectives will all be covered in the following chapters.

Chapter 1 reports the fundamental activity at the base of mycology: the collection of fungal samples, with the aim of obtaining a wide choice of strains to test hypotheses. The collection is followed by other important activities such as isolation in pure culture, identification and conservation. In this work, fungal strains were obtained from natural environments in Italy and Spain and directly from different spots in two wastewater treatment plants in North Italy.

In Chapter 2 the case of study is presented and the preliminary experiments applied to chosen fungal strains that led to the first results on heavy metals are described. These tests served to settle and build the base knowledge of the research and to bring out potential critical issues.

In Chapter 3 preliminary results on the degradation of pharmaceuticals products are discussed. These results led to a series of experimental campaigns on real wastewater. Six WDF strains were tested in discharge wastewater to see which one performed best. The selected strain was the subject of the following experiments to identify the most effective fungi-pharmaceuticals contact time, inoculation mode, and type of wastewater most suitable for subsequent pilot plant experimentation. The first pilot plant experimentation is described as it is a critical stage that must be addressed and refined before to the actual implementation in the wastewater treatment plant. This section also described issues, obstacles and future perspectives.

✓ Wastewater and pollutants

Pollution could be defined as the presence of substances, heat or energy in environmental media whose nature, location, or quantity produces undesirable environmental effects (UNdata). Pollution can be classified into three major categories: air pollution, water pollution and soil pollution. Recently other "minor" categories emerged, such as noise, visual, and light pollution.

Pollution is caused by pollutants defined as substances, solid, liquid or gaseous, of natural or human origins that, once introduced in the environment, have direct or indirect undesired effects on organisms and natural resources. Pollutants can be classified based on different parameters and aspects, such as their nature, source or effects on the environment (Mathew *et al*., 2017). They can also be classified by degradability into degradable or non-persistent pollutants (organic waste, domestic sewage, fertilizers and agricultural vegetal waste), persistent pollutants that can be degraded in long periods of time (pesticides, industrial chemicals and plastics) and non-degradable pollutants (heavy metals and radioactive waste) (Bharucha, 2005).

The problem related to pollutants is mostly tied to human activities and the urban environment, the main sources of all those substances that could become pollutants. Urban, industrial and agricultural settings are the main sources leading to pollution of air, water and, also through the latter, soil. Closely related to urban context is the case of water pollution. Water represents a vector that, passing through wastewater treatment plants (WWTPs), carries pollutants from houses and industries to waterways, rivers, seas, but also cultivated fields.

In such a context WWTPs have the key role to collect wastewater and, through several treatments, to remove organic and inorganic substances such as debris, sands, oils, nutrients and pathogens, returning clean water, with an advantage to the environment and the public health (Figure 1) (Padilla-Rivera *et al*., 2016; Margot *et al*., 2015).

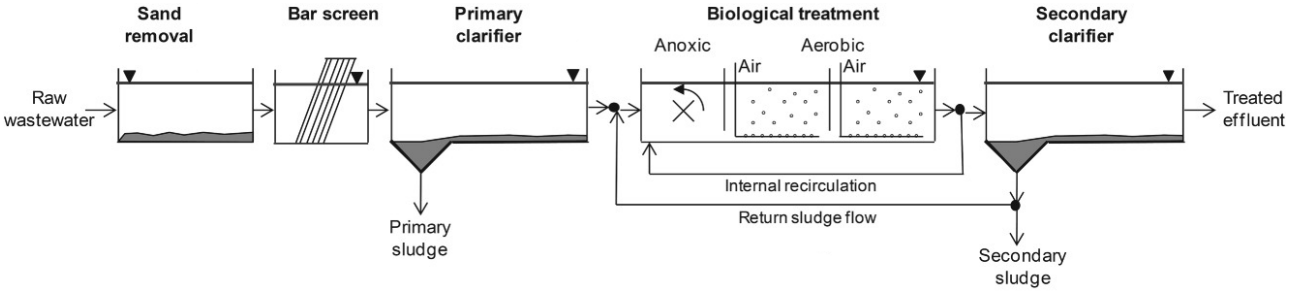

Figure 1- Base scheme of how a Wastewater Treatment Plant works (Margot *et al*., 2015).

Wastewater is defined as "a complex matrix containing significant concentrations of solids (total solids 350–1200 mg/l), dissolved and particulate matter (chemical oxygen demand 250–1000 mg/l), microorganisms (up to 109 number/mL), nutrients, heavy metals and micro-pollutants" (Warwick *et al*., 2013). Wastewater composition strongly depends on factors such as human and industrial activities, lifestyle, sewage technical and juridical frameworks, seasonality of microorganisms, urban's surrounding context and urban runoff (Henze & Comeau, 2008; Muller *et al*., 2020; Singh & Gupta, 2016; LaMartina *et al*., 2021). All these factors contribute to the possible presence and concentration of micropollutants in wastewater, including heavy metals and metalloids, hydrocarbons, pesticides, pharmaceuticals, personal care products, micro and nano-plastics and others. WWTPs are not designed to remove these micropollutants, unless through indirect means by sorption onto particulate matter, biotransformation by microorganisms already present in depuration phases, volatilization and photolysis or hydrolysis. For this reason, some micropollutants can pass through the depuration processes unscathed, concentrating in water and sewage sludges and pouring into surface and groundwater and in soil (Abbasi *et al*., 2022; Polinska *et al*., 2021, Margot *et al*., 2015, Balbaki *et al*., 2017).

Currently in Italy, the control of pollutants in waters leaving treatment plants is limited to specific substances and chemical parameters as reported in "DECRETO LEGISLATIVO 3 aprile 2006, n. 152" (https://www.gazzettaufficiale.it/dettaglio/codici/materiaAmbientale). Emission limits are divided in two categories, waters from urban WWTPs and waters from industrial WWTPs. Parameters

subjected to emissions limits control in discharge water are reported in Table 1.

Table 1 – Parameters for which there are emission limits in urban and industrial wastewater.

Parameters subjected to emissions limits control			
Urban wastewater discharged in surface water bodies		**Urban wastewater discharged in sensible areas**	
BOD_5		Total phosphorous (P)	
COD		Total nitrogen (N)	
Suspended solids			
Industrial wastewater discharged in surface water bodies, sewage and soil			
Ph	Temperature	Colour	Smell
Bod_5	Cod	Suspended Solids	Coarse Materials
Aluminium	Arsenic	Barium	Boron
Cadmium	Total Chromium	Chromium Vi	Iron
Manganese	Mercury	Lead	Nickel
Copper	Selenium	Tin	Zinc
Total Cyanides	Free Active Chlorine	Sulfides	Sulfites
Chlorides	Fluorides	Total Phosphorus	Ammonia Nitrogen
Nitrous Nitrogen	Nitric Nitrogen	Animal/Vegetable Fats and Oils	Total Hydrocarbons
Phenols	Aldehydes	Aromatic Organic Solvents	Nitrogenous Organic Solvents
Total Surfactants	Phosphorus Pesticides	Total Pesticides	-aldrin; dieldrin
-endrin; isodrin	Chlorinated Solvents	*Escherichia coli*	Acute Toxicity Assay

As can be seen in the table above, the substances that are currently being controlled are a limited list that excludes other important and potentially harmful micropollutants. This issue also applies to those waters and sludges that are intended for their use in agriculture for irrigation or fertilization of fields. In Italy the legal limits of substances that could be present in sewage water for reuse in agriculture are reported in "Decreto 2 maggio 2006" (https://www.gazzettaufficiale.it/eli/id/2006/05/11/06A04475/sg), while those for sewage sludge use are reported in "DECRETO LEGISLATIVO 27 gennaio 1992, n. 99" (https://www.gazzettaufficiale.it/eli/id/1992/02/15/092G0139/sg). In the present work sewage sludge limits taken into consideration are those in effect in Regione Lombardia and reported in "DECRETO N. 6665 Del 14/05/2019, atto n° 263 ". All the above decrees include emission lists composed by basic chemical parameters, such as BOD and COD, heavy metals and metalloids, some organic pollutants, micronutrients, and biological parameters such as the presence of *Escherichia coli* (Migula, 1895) Castellani & Chalmers and *Salmonella* spp.

Heavy metals and metalloids are pollutants of particular relevance being natural elements which include some fundamental for biochemical and physiological processes, such as Cu, Fe and Zn. However, they can still be harmful if their concentration exceeds certain levels. Human activities, including industry, mining, urban and sewage wastewater and certain pest control chemicals, can lead to accumulation or prolonged exposure to certain heavy metals and metalloids. Plants and fungi can accumulate these elements through processes like cell wall binding, chelation or compartmentalization, initially causing physiological and genetic problems. These substances can result in various health issues for humans, such as damage to several organs and, through prolonged exposure, physical, muscular, and neurological degenerative processes (Jaishankar *et al*., 2014; Rai *et al*., 2019).

The most important and /or highly concentrated heavy metals are reported in Table 2, along with their effects on human health.

Table 2- Most common heavy metals, their usage in production and industry and their effects on human health.

Heavy metal	Usage	Effects
Cd	By-product of zinc production, rechargeable batteries, special alloys and plastics production	Cause acute and chronic intoxications, osteoporosis, lung, bones and kidney damage. Can remain in soils and sediments for decades.
Cr	Metallurgy industries, electroplating, production of paints and pigments, tanning, wood preservation, chemical production, pulp and paper production	Formation of ulcers, DNA damage. Plant chlorosis and necrosis and inhibition of seed germination
Ni	Ingredient for metal production, cigarettes and detergents	Cancer, respiratory failure and dermatitis
Pb	Gasoline and house paint, plumbing pipes, storage batteries, cosmetics	Mental retardation, birth defects, psychosis, autism, hyperactivity, paralysis, muscular weakness, brain damage, kidney damage and may cause death

The health risks associated with accumulating substances in cultivated and commonly consumed plants and crops are well-known and closely linked. Due to these risks, the use of water and sludge for agricultural purposes, along with the heavy metal content in discharge waters, is subject to very strict limits.

What remains excluded from current legal limits are the so-called emerging pollutants—natural or artificial compounds such as pharmaceuticals and personal care products. These substances can enter various ecosystems and accumulate in food chains, posing a critical issue for the health of animals and humans. Emerging pollutants end up in wastewater, and as a result, they are found in superficial and groundwaters in quantities on the order of nanograms and micrograms. Termed 'emerging' pollutants, these substances have only recently come to light due to advancements in detection methods. The challenge with these emerging pollutants, being new substances, lies in the lack of knowledge regarding their identification and negative health effects. Consequently, there are currently no legal limits regulating their emissions (Ivshina *et al*., 2018; Stefanakis *et al*., 2020; Bell *et al*., 2011). The only existing tool for monitoring such substances and understanding their potential effects on ecosystems is the so-called Watch List (Directive 2013/39/EU). Through continuous monitoring, substances that may potentially harm water status are added to the list, updated every two years (Giardina *et al*., 2021). This list encompasses hormones, personal care products, some of the most commonly found pharmaceuticals in water, as well as various pesticides, insecticides, herbicides, and fungicides (Table 3).

Table 3 - List of substances added to the Watch List, type of substance and year they were added to the list.

Substance name	Type of substance	Watch List
Ethinyl estradiol	Semisynthetic estrogen	2015 (n°1)
Estrone; Estradiol	Estrogen; Steroid hormone	2015 (n°1)
Diclofenac	Nonsteroidal anti-inflammatorial	2015 (n°1)
Butylated Hydroxytoluene	Lipophilic (fat-soluble) organic compound, primarily used as an antioxidant additive	2015 (n°1)
Octinoxate	Cinnamate ester. Common ingredient in sunscreen and other skin care products to minimize DNA photodamage	2015 (n°1)
Erythromycin; Elarithromycin; Azithromycin	Macrolid antibiotics	2015 (n°1)
Methiocarb	Synthetic carbamate ester, used for pesticides	2015 (n°1)
Imidacloprid; Tiacloprid; Tiametoxam; Clotianidin; Acetamiprid	Neuro-active insecticides	2015 (n°1)
Oxadiazon	Aromatic ether	2015 (n°1)

Triallate	Herbicide	2015 (n°1)
Amoxicillin	Antibiotic	2018 (n°2)
Ciprofloxacin	Antibacterial	2018 (n°2)
Metaflumizone	Insecticide	2020 (n°3)
Sulfamethoxazole	Antibiotic	2020 (n°3)
Trimethoprim	Antibacterial and antiprotozoal	2020 (n°3)
Venlafaxine; Desvenlafaxine	Antidepressant	2020 (n°3)
Clotrimazole; Fluconazole; Imazalil; Ipconazole; Metconazole; Miconazole; Penconazole; Procloraz; Tebuconazole; Tetraconazole	Antimycotics	2020 (n°3)
Dimoxystrobin	Fungicide	2020 (n°3)
Famoxadone	Fungicide	2020 (n°3)

Since one of the problems associated with these substances is their ability to bioaccumulate in the environment, the greatest threat is that most of the negative effects of these pollutants have yet to be studied. A significant example of emerging pollutants is pharmaceuticals, defined as synthetic molecules designed to produce a therapeutic effect on the body (Bottoni *et al*., 2010). Once assumed, these compounds are excreted through feces and urine as a mixture of metabolites and substances that are often unchanged. The accumulation of pharmaceuticals in the environment can lead to the phenomenon of antimicrobial resistance. Antimicrobial resistance is caused by continuous exposure to pharmaceuticals such as antibiotics and antivirals, leading to the development of specific resistances, through genetic mutations, in microorganisms such as bacteria, viruses, and others. This results in greater difficulty in treating pathologies derived from this type of resistant organisms (Giardina *et al*., 2021). The phenomenon is also tied to the fact that part of the substances passing through the human body are not metabolized, and what is expelled includes both the original molecule and the metabolized one, both capable of resisting degradation and bioaccumulating (Tijani *et al*., 2013).

Heavy metals and pharmaceuticals are good examples, respectively, of well-known and strictly controlled pollutants, and emerging pollutants whose dynamics are not yet completely clear. The issue of heavy metals, as elements of an already established threat, and the issue of pharmaceuticals, whose problem has arisen and is developing in recent years, constitute the primary focus of this PhD thesis. In addition to these two examples, the literature on the subject is replete with other case studies involving pollutants whose problems should not be underestimated, such as hydrocarbons, pesticides, and microplastics. These pollutants, sharing similar issues with heavy metals and pharmaceuticals, can easily be found in wastewater, soils, and surface water, accumulating and posing a potential threat to human health and ecosystems.

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants consisting of multiple benzene rings. They can originate from natural sources, such as volcanic activities, and from artificial sources, including petroleum refining, chemical production, and industrial processing. PAHs are commonly found in wastewater and surface waters. Due to their high hydrophobicity and low aqueous solubility, their deposition and adsorption in the soil are increased. Their dispersion in the environment makes them dangerous pollutants, with studies reporting health effects such as various types of cancer, heart diseases, and immune system suppression (Zhang *et al*., 2019; Patel *et al*., 2020; Zango *et al*., 2020).

Pesticides constitute another category of pollutants that share a similar pathway with hydrocarbons. Pesticides are chemical substances with different molecules and chemical-physical properties, used to eliminate or interrupt the normal life cycle of certain organisms (e.g., insecticides, fungicides, herbicides). Their primary use is in agriculture to protect crops from harmful organisms or increase plant productivity. These substances then leach into water and soil, posing a health risk, as some pesticides are likely mutagenic, and others can cause poisoning, cancers, and other syndromes (Hassan *et al*., 2020; Saleh *et al*., 2020).

PAH, phenols, pesticides and their derivatives are considered priority pollutants and therefore their presence and concentration are constantly monitored. As already mentioned in Table 1, these

pollutants are some of the parameters subject to legal limits for water discharges. Wastewater treatment plants have the duty to regulate their presence, removing or lowering the amount of these pollutants through different methods and technologies. For PAH and phenols, different treatments can be employed, each with its own pros and cons, such as coagulation, flocculation, bioremediation, chemical oxidation, photocatalytic degradation, adsorption and others (Zango *et al*., 2020). Pesticides are treated similarly through chemical tretaments aimed to hydrolize the contaminants (coagulation, ozonation, fenton treatment), photochemical degradation, active carbons and/or the use of membrane bioreactors for pesticides difficult to degrade (Saleh *et al*., 2020).

PAHs and pesticides are well-known pollutants with precise regulations aimed at solving or containing the problem. Among the emerging pollutants, in addition to the already-discussed pharmaceuticals, there is the increasingly evident problem of microplastics. Microplastics are defined as plastic-derived fragments that are less than 5mm in size. These fragments are easily dispersed in the environment and can enter food chains, accumulating and posing a serious risk to living beings. It is reported that the presence of microplastics in soil and water can have adverse effects on plants by altering their growth and becoming a stress factor, and on animals by causing obstruction and organ damage. Since some microplastics may derive from products that are mixed with other chemicals, they can easily become carriers of toxic substances, pathogens, and other pollutants (Chia *et al*., 2021; Temporiti *et al*., 2022). As considered emerging pollutants, there is currently no regulation over the content of microplastics in waters. However, some traditional treatments used in wastewater treatment plants can reduce microplastics' concentration. The desanding and primary sedimentation processes are already capable of removing from 20 to 90 % of microplastics, but the removal efficiency greatly depends on the size and weight of the fragments. Even the biological process of activated sludge, the basis of biological water treatment, can reduce the content of microplastics, as bacteria and microorganisms can ingest them, creating aggregates that are more easily removed in the secondary and/or tertiary treatment phases (Khan *et al*., 2022).

✓ The challenge of Myco-remediation

Pollution is a constant and pressing concern; therefore, the continuous search for solutions to eliminate hazardous compounds and prevent their spread in the environment has become essential. Wastewater treatment plants primarily focus on macro pollutants, oils, microorganisms, and nutrients, while their efforts directed towards micropollutants (e.g., heavy metals) and emerging pollutants (e.g., pharmaceuticals) remain somewhat limited. Depuration processes can partially decrease the content of emerging pollutants through mechanisms such as dilution, sorption, photolysis, volatilization, flocculation, or sedimentation.

Traditional methods for micropollutant control and removal are based on physical and/or chemical methods, including membrane filtration technologies, Fenton oxidation processes, activated carbon-mediated adsorption, photocatalysis, electrochemical oxidation, photocatalytic membrane processes, ultrafiltration, and ozonation. However, these methods are often expensive or do not guarantee the removal of certain substances (Ahmed *et al*., 2021). For instance, in wastewater derived from urban sewage, pharmaceutical molecules are partly degraded by traditional depuration techniques. However, some of these molecules, more or less untouched, may reach the end of the water line and enter natural waterways. The efficiency of removing these substances is also highly variable due to conjugation/deconjugation processes that can occur, transforming pharmaceutical molecules into their metabolites through metabolization in the human body and vice versa in wastewater (Zhou *et al*., 2019).

Over the past 50-60 years, the concept of bioremediation has become increasingly popular as an alternative technique to be used, either alone or coupled with conventional techniques, to address the problem of environmental pollutants. “Bioremediation” could be defined as the use of microorganisms and/or their metabolic processes to remove, reduce, eliminate and/or transform pollutants from soil, water and air (Boopathy, 2000). It’s a technique first employed in 1960 by a scientist named George Robinson that tried to clean up an oil spill in California employing microorganisms; since then the interest and applied research in this field has been expanding more and more (Omokhagbor Adams *et al*., 2015).

Compared to conventional techniques, bioremediation can be applied both *in situ* and *ex situ*, depending on the type of contamination, application feasibility, and context. *In situ* remediation is performed directly at the site where pollution occurred. It is usually less expensive, but the choice of application depends strongly on site-specific factors such as geography, and natural and non-natural environmental parameters that can alter or disturb the remediation efficiency. *Ex situ* remediation involves translocating the pollutant or the contaminated matrix to a location where it can then be treated, making it a potentially expensive method (Azubuike *et al*., 2016). Bioremediation, in general, is less expensive than other techniques and preserves the natural state of the site where it is applied. Compared to other methods, bioremediation is better accepted by public opinion and can be used alongside traditional physical or chemical methods (Boopathy, 2000). Microorganisms commonly used in bioremediation studies include bacteria, archaea, algae, or fungi, either individually or in consortia (Abatenh *et al*., 2017). The strength of these organisms lies in their ability to tolerate different concentrations of pollutants and enzymatically degrade recalcitrant molecules.

Regarding the use of fungi for bioremediation, the correct term is 'mycoremediation,' which refers to the use of fungal organisms for the removal and/or degradation of pollutants. The use of fungi for environmental applications has garnered significant interest, with the number of publications on the subject nearly tripling in the past five years, owing to their potential and diverse applications (Rajhans *et al*., 2021; Anderson *et al*., 2016; Singh *et al*., 2020; Noman *et al*., 2019).

The effectiveness of fungi in this field is given by their peculiar growth which, by creating a vast network of hyphae, ensures that the surface area/volume ratio is optimal for passive removal mechanisms (bioadsorption) and at the same time ensures the effectiveness of their enzymes for active degradation mechanisms (biodegradation). Fungi are also able to tolerate high concentrations of heavy metals and adapt rapidly to changes in pH and temperature making them versatile and resilient organisms (Akhtar *et al*., 2020).

The most studied pollutants in the field of myco-remediation are heavy metals, and organic pollutants such as hydrocarbons and pharmaceuticals.

Fungi are organisms with exceptional metallo-resistance, i.e. the ability to survive heavy metal and metalloid induced toxicity through morphological, physiological and/or genetic properties and/or

through environmental processes to modify their toxicity. This ability comes from the number of interactions that fungi can enact with metals and metalloids (Figure 2).

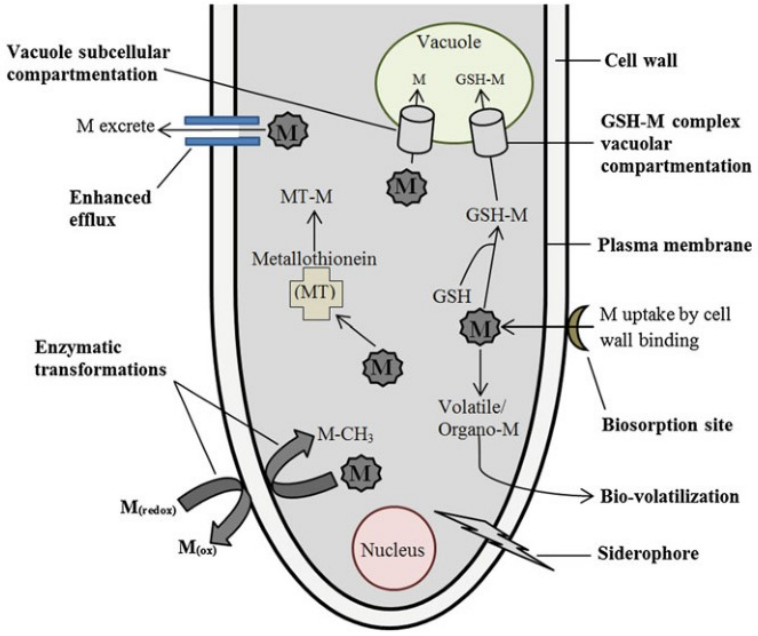

Figure 2- Fungal-metal interaction and detoxification mechanisms (Chan *et al*., 2016).

- Precipitation and complexation: production of extracellular molecules or substances that can chelate metal ions or precipitate them into oxalates, sulfates and phosphates. Precipitation of metals reduces their bioavailability.
- Intracellular uptake and detoxification: binded metals can be uptaked into cells where different mechanisms can be put in place to reduce the toxic effects of certain elements. Methylation is a reaction where the metal is transferred to a methyl group altering his solubility, volatility and toxicity. Metallothioneins and phytochelatins are compounds able to bind metal-ions and reducing their production of reactive oxygen species.
  Another method of detoxification is related to the compartmentalization of metals and metalloids within the vacuole, regulating their concentration in the cytosol.
- Cell wall binding: cell wall represents the first point of contact between the fungal cell and the environment. In particular, the cell wall is rich in potential binding sites provided by its main constituents. Polysaccharides, peptides, chitin and chitosans have different functional groups such as carboxyl, hydroxyls and amides which have strong binding properties towards metal-ions. This type of binding occurs through a passive, reversible, physical-chemical mechanism, not linked to the metabolism of the organism. This, unlike previous detoxification mechanisms, also allows dead fungal biomass to be utilized for myco-remediation of metals and other environmental pollutants (Chan *et al*., 2016; Gadd, 1994; Abbas *et al*., 2014).

Effectiveness in removing heavy metals is related to several factors, first of all the species that is being used, as different species have different binding sites with certain affinities toward metal ions. In the study carried out by Gabriel *et al*., 1994 a certain selectivity was found for *Stereum hirsutum* (Willd.) Pers. toward Pb and for *Ganoderma applanatum* (Pers.) Pat. toward Cd, Al, and Ca. Among Basidiomycetes *Pleurotus ostreatus* (Jacq.) P. Kumm. is one of the most studied species due to its rapid growth and ease of adaptation and is capable of remove a wide range of metals such as Cd, Mg, Hg, Cr, Pb, Ni, Zn (Akhtar *et al*., 2020; Kaphai *et al*., 2017; Dulai *et al*., 2015). *Agaricus bisporus* is another deeply studied species particularly active toward Cd (II), Cr (VI), Zn (II), Pb, Hg, Fe, Cu, Mn (Hamba & Tamiru, 2016; Ertugay & Bayhan, 2008; Nagy *et al*., 2017; Tüzen *et al*., 1998). Other species used extensively in this field are micro fungi such as *Aspergillus niger* Tiegh., *Saccharomyces cervisiae* (Desm.) Meyen and genus *Rhizopus* (Akhtar *et al*., 2020; Danesh *et al*., 2013;

Hanif & Bhatti, 2015; Danhkhar Hooda, 2011).

Not only does species-specific selectivity play a key role in the removal of heavy metals and metalloids, but also a number of chemical and environmental parameters such as:

- Type of biomass: free or immobilized biomass is an important difference based on the type of application and the role that the organism may plays on the matrix to be treated. How the biomass is grown also affects the contact surface available for the biosorption process. Live or dead biomass has implications for the type of interaction between organism and pollutant and thus whether the biological process is active or passive. Another factor could be also if the biomass is pre-treated, natural or engineered.
- Physicochemical parameters: temperature is a crucial factor for fungal growth. Each species has a different temperature optimum that enable the most efficient growth: lower or higher temperatures can stop mycelium growth or damage the organism. Acidity is an important factor both for vital parameters of the fungus and for the biosorption process. Just as with temperature, each species can tolerate a certain pH range, outside of which the growth and metabolic functions of the organism are greatly altered. Biosorption is pH dependent as acidity alters the effectiveness of binding sites, metal solubility and the activity of functional groups.
- Others: biomass concentration, initial ion concentration and the affinity between metal and biosorbent are other parameters that can substantially alter the effectiveness of metal uptake (Abbas *et al*., 2014; Danhkhar Hooda, 2011).

Fungal activity towards organic pollutants follows different processes and dynamics than those described for heavy metals and metalloids. When discussing mycoremediation applied to organic pollutants, such as pharmaceuticals in the case of this PhD thesis, several mechanisms come into play for their removal. The processes regulating fungus-pharmaceutical interactions can be either simple biosorption (a passive mechanism) or biodegradation (an active mechanism), with the latter exploiting the range of enzymes produced by fungi (Noman *et al*., 2019).

Biosorption of pharmaceuticals is based on their hydrophobicity: highly hydrophobic molecules are better degraded through the combination of biosorption and extracellular enzymes, while hydrophilic molecules are mainly removed by biodegradation. Compounds bonded to the cell wall are then degraded by secreted extracellular enzymes, resulting in the production of pharmaceutical degradation sub-products or metabolites, or they are transported into the cell and degraded by intracellular enzymes.

Similar to heavy metals, several parameters make fungi particularly efficient at degrading pharmaceuticals and other organic pollutants.

- The non-specificity of their enzymes allows them to act on a wide range of substances. More in detail, enzymatic degradation can be divided into two type, one operated by extracellular enzymes, such as Laccase, Manganese Peroxidase, Versatile Peroxidases and Lignin Peroxidase and one operated by intracellular enzymes such as the cytochrome P450 enzyme system.
- Access to pollutants by the fungal mycelium is essential in order to achieve a greater surface area for enzymes action and also a greater effectiveness of the passive processes that bind compounds to the fungal cell wall. It is therefore important to use fungal species or strains capable of rapid growth, both to have a greater hyphal network and to be able to counter possible competitors, such as bacteria, fungi, and other microorganisms, in order to apply myco-remediation on larger scales or on real non-sterile matrixes.
- Fungal enzymes are capable to degrade compounds in substrates poor of nutrients and in a wide pH range between 3 and 9 (Naghdi *et al*., 2019).

An important factor to take into account, on which the degradation of substances by fungal enzymes depends, is the chemical composition of the compound itself, particularly the functional groups. For this purpose, functional groups can be divided into two categories, electron donating and electron

withdrawing groups. Electron donating functional groups such as hydroxyl (–OH), amine ($–NH_2$), alkoxy (–OR), alkyl (–R) and acyl (–COR) characterize compounds reported to be degraded by fungi with high efficiency. On the contrary difficult to degrade compounds are associated with electron withdrawing groups such as amide ($–CONR_2$), carboxylic (–COOH), halogen (–X), and nitro (–NO2) (Yang *et al*., 2013). The breaking of these bonds leads to the formation of metabolites or by-products that could have similar persistency and/or toxicity compared to the parental pharmaceutical.

✓ Wood Decay Fungi

Fungi are amazing organisms found all over the world, thriving in various environments, from common settings like forests and cities to extremely hostile ones such as glaciers, deserts, mines, and deep-sea bottoms (Newsham, 2012; Selbmann *et al*., 2013). Their remarkable ability to adapt and survive has led fungi to inhabit almost every habitat. Currently, about 150,000 species of fungi have been identified and classified, while various studies estimate the total richness in fungal species to be between 1 and 13 million. In recent years, the discovery rate has been around 2000 new species per year, with *Agaricales*, *Pleosporales* and *Hypocreales* representing the orders with the largest number of species discovered (Bhunjun *et al*., 2022).

Fungi are eukaryotic heterotrophic organisms, meaning they need to acquire organic compounds from the environment for their metabolic functions. Fungi can exist in unicellular forms or be organized into a network of filamentous cells called hyphae. They can be essentially divided into two main groups: micro-fungi and macro-fungi, based on their reproductive structures, which can remain microscopic, such as conidiophores, or form large and macroscopic structures, such as sporocarps (Arnolds, 1992).

This work is mainly focused on macroscopic Wood Decay Fungi (WDF), organisms that utilize the main components of the plant cell wall for their metabolism. WDF are saprotrophic and/or parasitic organisms of plants. In forest ecology, they play a crucial role in degrading organic and lignocellulosic matter, promoting the recirculation of nutrients and elements, especially nitrogen and carbon (Lonsdale *et al*., 2008).

The type of degradation they carry out, at the expenses of the host plant, is what distinguishes them into three macro-categories: brown rot, white rot and soft rot fungi. The first one denotes a selective degradation of cellulose and hemicellulose, leaving a crumbly dark brown residue, the second one performs a complete degradation of all wood components, making the substrate bleached, spongy and frayed and the last one carries out mostly a superficial degradation (Schwarze, 2007; Goodell *et al*., 2008). The type of degradation and aggressiveness of the fungus, with associated damage toward the host, varies greatly from species to species and depends on different factors such as their enzymatic production, conditions for secretion, alternative non-enzymatic pathways and environmental conditions (Girometta *et al*., 2020). Two main enzymes categories involved in wood degradation are hydrolyses and oxido-reductases of which the former is involved in the degradation of cellulose and hemicelluloses, and the latter in the degradation of lignin (Bucher *et al*., 2004; van den Brink, 2011).

Cellulose, composed by D-glucose unities linked by β-1,4-glycosidic bonds, are degraded by cellulases, a class of enzymes that could link to different and specific sites of cellulose fibers through a carbohydrate binding module. Cellulases are divided in endoglucanases, exoglucanases and β-glucosidases. Endoglucanases act upon the amorphous regions, severing β-1,4-glycoidic bonds releasing chain ends, while exoglucanases removes tetrasaccharides or disaccharides from exposed chains. β-glucosidases hydrolyse glucose dimers to glucose.

Hemicellulose forms a complex matrix with other components of the cell wall, so the joint action of different types of enzymes is required for its degradation: xylanases (endo-1,4-β-xylanase and exo-1,4-β-xylosidase), mannanases (endo-β-1,4-mannanase and exo-β-1,4-mannosidase) and a series of accessorial enzymes.

Lignin degradation occurs thanks to lignin-peroxidases, manganese peroxidases and laccases. Lignin peroxidases can oxidize a wide range of compounds in the presence of H_2O_2 but can't bind directly to lignin due to its size being too large for the active site of the enzyme. In order to attack lignin, peroxidases generate small radicals capable to enter lignin matrix and oxidize it. Manganese-peroxidases, in the presence of H_2O_2, oxidize a Mn^{2}+ ion into Mn^{3}+, that is stabilized by organic acids. The Mn^{3}+ion can penetrate the lignified cell wall where it oxidizes phenolic and non-phenolic lignin components.

Laccases are monomeric, dimeric, or tetrameric glycoproteins, containing multiple copper atoms that oxidize phenolic compounds (in defined conditions also non-phenolic compounds), polyphenols, methoxy-substituted phenols or diamines, forming radicals that can repolymerize or cause depolymerization (Andlar *et al*., 2018; Hofrichter, 2002).

In addition to those already mentioned, fungi, both micro and macro, are capable to produce a

wide variety of other enzymes, which constitute about 50 % of the enzymes on the market. Enzymes can be classified into six main groups (El Gendi *et al*., 2022):

- Oxidoreductases: catalyse oxidation/reduction (redox) reactions and play a key role in host-specific virulence and in protection against host defences and environmental stresses (Yu *et al*., 2020). Lignin peroxidases and manganese-peroxidases are two of the most important enzymes, especially for white-rot fungi that use them for lignin degradation.
- Transferases: catalyse processes essential for amino acids and protein synthesis in cells. Glutathione transferases is particularly important for fungi thanks to its wide substrate specificity and the potential to detoxify a wide range of toxic compounds.
- Hydrolases: catalyse the hydrolysis of the substrate through the addition of water. Fungi produces many hydrolases such as proteases, amylases, lipases, and cellulases. The latter is fundamental for WDF for cellulose degradation together with exo- and endo-glucanasi and cellobiohydrolases. Another important enzyme of this category are chitinases due to the fact that, after cellulose, chitin is one of the most widespread polymers in nature.
- Lyases: catalyse the addition or elimination reaction with the formation of a new ring structure or new double bonds. Fungal pathogenicity depends also on lyases, in particular on pectin lyase, that is involved in plant cell wall degradation.
- Isomerases: catalyse the conversion of two isomers of the same compound.
  Most important fungal isomerases are glucose/xylose isomerase that converts D-glucose and D-Xylose into D-fructose and D-Xylulose.
- Ligases: catalyse the formation of a new bond between two molecules to form a new compound and their main role is to modify the cellular nucleic acid content.

Thanks to their enzymatic and metabolic activity, new application frontiers have opened to the world of fungi such as industrial, environmental and biomedical. In industry, mushrooms find application in: 1) food industries to reduce acrylamide formation in some production lines or to improve yield and/or extraction of products for beverages (exploiting mostly species of genus *Aspergillus*); 2) pulp and paper industries use mainly species of genus *Aspergillus*, *Fusarium* and *Trichoderma* to improve quality and properties of pulp and detoxify and remove undesired elements from paper; 3) textile industries, mainly genus *Aspergillus* and *Trichoderma*, for the bioscouring, use of pectinases to remove pectine and waxes from fibres, cotton fibres and cellulose fabrics and to remove or recover certain substances. Fungi find application also in biomedical field for the secretion of enzymes with antimicrobial, anticancer and antioxidant effects or for their use in the production of substances with such properties (El Gendi *et al*., 2022).

The use of fungi for environmental applications is a topic that has captured enormous interest, with the number of publications on the subject nearly tripling in the past 5 years. Wood Decay Fungi (WDF), in particular, are employed for the bioremediation and biodegradation of undesired compounds and pollutants, including pharmaceuticals, heavy metals, hydrocarbons, and dyes (Akhtar *et al*., 2020).

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PhD project aims

The objective of this PhD project is to apply the concept of Myco-remediation to sewage sludges and wastewater, aiming to improve their quality by removing heavy metals and/or degrading pharmaceuticals.

To achieve this goal, several steps will be taken:

1) Collect and acquire different fungal species and strains to obtain a diverse pool of fungal strains for selection based on performance.
2) Test the strains for their ability to grow on sludge and remove heavy metals.
3) Evaluate the strains for their capability to degrade pharmaceuticals under controlled laboratory conditions.
4) Select the best-performing strains capable of functioning in real matrices, such as wastewater, and conduct tests to determine the optimal conditions for the fungal-based treatment.
5) Test the best strains at a pilot plant scale.

A graphical abstract of the PhD project is provided in Figure 3.

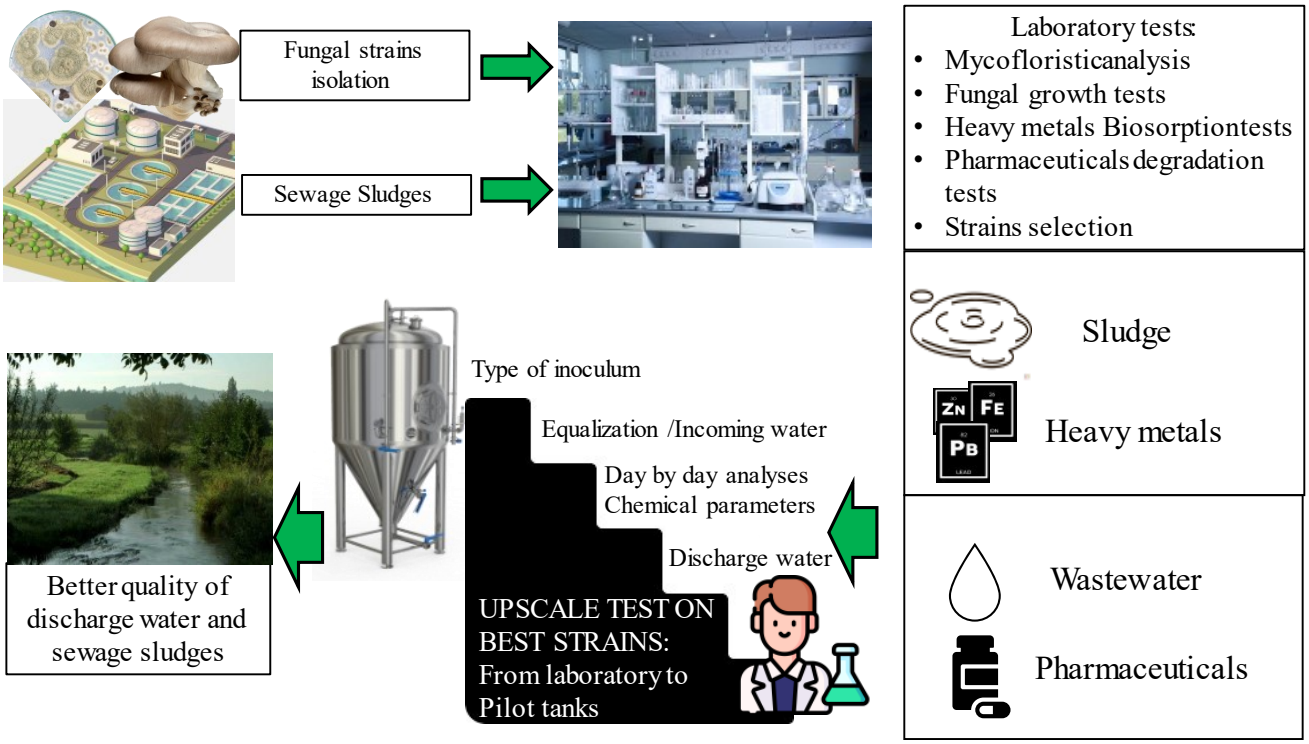

Figure 3- PhD project's graphical abstract.

The present PhD project was supported by Regione Lombardia, POR FESR 2014-2020—Call HUB Ricerca e Innovazione, Progetto 1139857 CE4WE: Approvvigionamento energetico e gestione della risorsa idrica nell'ottica dell'Economia Circolare (Circular Economy for Water and Energy). All steps of this PhD project, from strain collection to experiment design and analyses, were done in collaboration with two wastewater treatment companies: CAP Holding s.p.a. and A2A Life Company. The two companies, leaders in the water treatment sector in Lombardia (Italy), not only provided their knowledge and experience on the topic but also provided the substrate (wastewater, sewage sludge, etc.) for each experiment. They also provided funds to perform the analyses of pharmaceuticals in wastewaters.

To do so several goals were set for each year of PhD:

1st year objectives

- Collection of fungal strains from two wastewater treatment plants: Mycofloristic analysis → morphological and DNA identification of isolated strains → conservation in the MicUNIPV research fungal culture collection;

- Growth tests on sewage sludges → Initial fungal strains selection;
- Heavy metals biosorption tests;
- Pharmaceuticals degradation tests;
- Choice of substrate and analytes on which to focus research.

Collection, isolation and identification of fungal samples has continued throughout all three years of the PhD project. Collection and characterization of some WDF strains used in this PhD project were performed in collaboration with Marco Cartabia PhD project, MOGU S.r.l and project "MYCO-ADVANCED LEATHER MATERIALS (MATER)" funded by Fondazione Cariplo & Regione Lombardia, grant n° 2018-1765.

2nd year objectives

- To test WDF strains in discharge water from two different wastewater treatment companies to evaluate their degradation capabilities → select the most promising species;
- To test selected species to understand the best contact time between fungal mycelium and discharge water. Treated wastewater physical/chemical parameters were analysed;
- To test the best strains selected in incoming wastewater.

3rd year objectives

- To test the type of inoculum that achieves better degradation results and higher mycelium resistance against other microorganisms;
- To test the best strain degradation capabilities at pilot plant scale;
- Erasmus Traineeship to collect new fungal strains for future experiments and applications.

Chapter 1. From field to laboratory: sampling and fungal strains isolation

Applied mycology establishes its foundation through research culture collections. Among their various activities, culture collections preserve and utilize cultures and specimens for scientific research and industrial exploitation. By maintaining a diverse collection of fungal strains, it becomes possible to test different species and genera, each possessing distinct characteristics and capabilities.

The initial phase of this PhD project involved extensive bibliographical research and the sampling of new fungal strains, encompassing micro-fungi such as molds, yeasts, pseudo-yeasts, and Wood Decay Fungi (WDF). This sampling is complemented by essential activities including the isolation of mycelium in pure culture and the identification of strains, as illustrated in Figure 4.

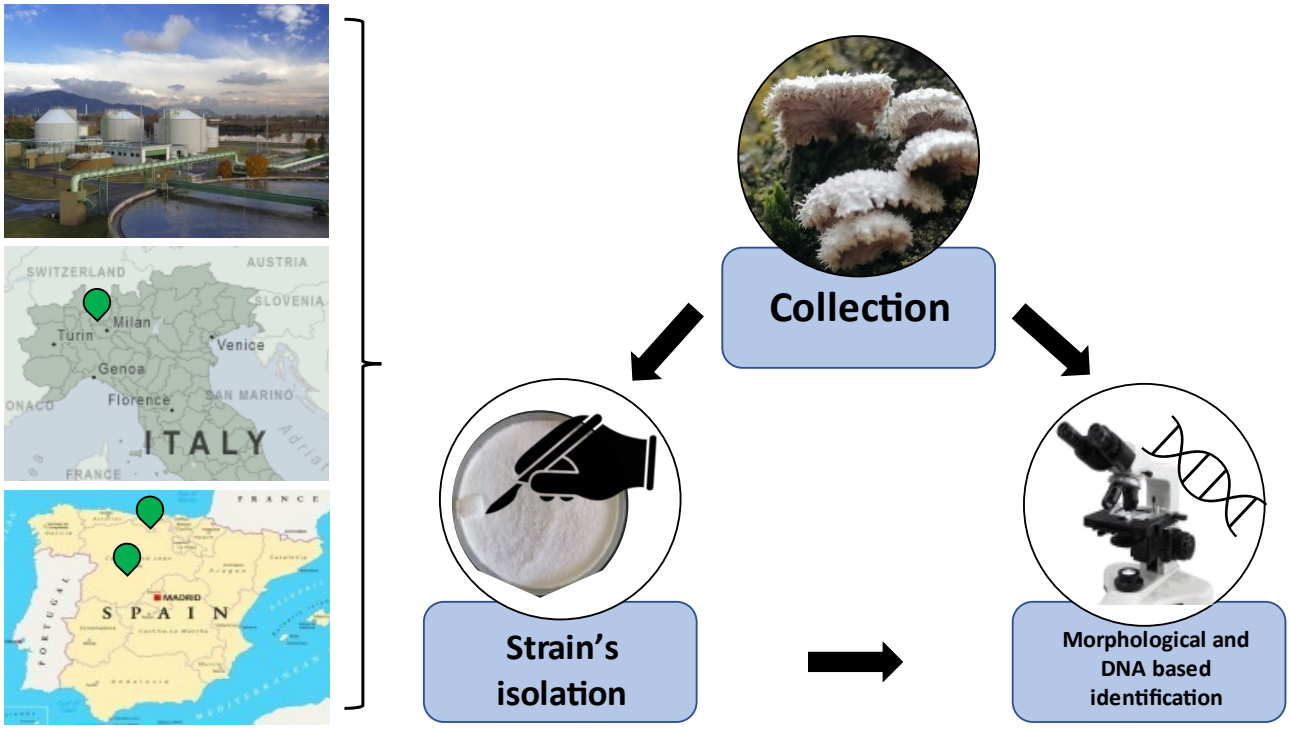

Figure 4- Schematization of the whole process of collection, isolation and identification of fungal strains.

To achieve this, several sampling campaigns were conducted with the aim of supplementing the strains available in the MicUNIPV research fungal culture collection of DSTA, University of Pavia, with new species and strains from various taxonomical groups. The MicUNIPV collection currently comprises 600 different strains, primarily from the Basidiomycota division, with a predominant focus on Wood Decay Fungi (WDF) within the polyporoid group. This group of fungi includes several species suitable for myco-remediation studies due to their enzymatic potential. However, it is crucial to consider other phyla that could also be potentially useful, such as corticioid fungi and species from the Ascomycota division. For these reasons, multiple sampling campaigns and studies have been conducted throughout the project:

1) A mycofloristic analysis to assess the fungal community composition of two wastewater treatment plant, coupled with fungal strains isolation;
2) A collection and characterization of WDF strains from northern Italy, in collaboration with Marco Cartabia PhD project;
3) A collection and characterization of polyporoid and corticioid fungal strains from Spain in collaboration with Salamanca University.

The initial step involved understanding the extent of fungal presence within wastewater treatment plants (WWTPs). This process, known as mycofloristic analysis, is essential for comprehending the fungal groups commonly found in WWTPs and identifying species adapted to this artificial environment. By obtaining a comprehensive understanding of the fungal communities in each

tank and treatment phase, the feasibility of applying myco-remediation without altering the community composition is assessed. As an example, *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg is the most common species found in both WWTPs analysed. Utilizing this species for bioremediation purposes would not significantly disturb its already prevalent presence within the tanks. Conversely, the use of underrepresented or potentially pathogenic species to humans or plants could disrupt the fungal community or cause environmental damage. The mycofloristic analysis also facilitates the collection and isolation of the sampled organisms. In the case of micro-fungi, the sampling process begins with collecting the matrix they are expected to inhabit, such as wastewater and sludge. Multiple sampling campaigns were conducted between late 2019 and early 2021 at various locations along the water and sludge line and during different seasons. This approach ensures observing variations in the fungal community not only spatially but also over time. Water and sludge collected were diluted and spread onto petri dishes containing culture medium (malt extract 2 % is a common culture medium for fungi as it contains a lot of nutrients for their growth). Dilutions are a fundamental practice with micro-fungi that enables the observation of the growth of both slow and fast-growing species. Once fungal colonies appear on the plates, an initial morphological identification is conducted. Macroscopic characteristics of the colony make it easy to distinguish moulds from yeasts and pseudo-yeasts. However, an observation under an optical microscope is necessary for an initial identification. Subsequently, a sterile fragment of the colony is collected and placed in a glass tube containing new growth culture medium, sealed with a cotton cap to maintain sterility while allowing gas exchanges. These labeled glass tubes are incubated at 25°C. These procedures ensure the acquisition of a pure culture of the fungus of interest starting from its colony, free from contaminations by other organisms.

The sampling and isolation of fungi that possess a macroscopical fruiting body, such as the previously mentioned WDF, follows different procedures from those of micro-fungi. If micro-fungi have soil or water as their natural growth environment, WDF grow on plants and/or on plant material. For this reason, fieldwork related to fruiting bodies sampling starts with the identification of suitable environments to host these organisms: forests, natural parks, and in some cases urban environments. The search for specimens is done simply by walking within the designated area of interest and locating basidiomata on living and dead trees and logs/vegetal remains. Once a specimen is found, if the condition is such that isolation can be performed (not visibly compromised or rotting), it is collected and information such as the habitat, the geographic coordinates, and the host species is recorded. Information regarding the host is particularly important because some species of fungi grow only on particular host species. Host information can be critical for an accurate morphological identification of the fungal specimen. Morphological identification, performed with manuals and dichotomous keys, must be confirmed by a molecular DNA identification based on the ITS region, the barcoding region for fungi (Harnelly *et al*., 2022).

After collection and initial morphological identification, the crucial step in applied mycology occurs: mycelium isolation in pure culture. Isolation in pure culture entails obtaining a culture, either in petri dishes or glass tubes, that contains only the mycelium of the species of interest, free from contamination. Obtaining the strain in pure culture is fundamental for mycological collections and serves as the starting point for any application based on fungi.

Isolating Wood Decay Fungi (WDF) in pure culture can be achieved in various ways. If the basidiomata is thick enough, as seen in species like *Fomes fomentarius* (L.) Fr. or *Pleurotus ostreatus*, direct isolation is possible. This process is carried out under sterile conditions, either under a biological hood or near a flame. A piece of basidiomata is taken from its context, the interior of the fruiting body, and transferred into a petri dish containing Malt Extract Agar culture media + Chloramphenicol (antibiotic). The section of basidiomata must be obtained from a part of the fruiting body not in direct contact with the environment, such as the hymenophore, to prevent contamination by other organisms. On the other hand, if the basidiomata is thin and does not allow for direct isolation, as is the case with corticioid fungi, isolation from spores is performed. In this study, spores from corticoid fungi were obtained using two different methods explained in Chapter 1.3.

Following collection and isolation, another fundamental step is conservation. Conservation allows the strains to remain vital and ready for use even after long periods of time. First of all, the

mycelium of each strain is transplanted from the plate in which the first isolation occurred into new and numerous petri dishes, to ensure to have multiples and identical copies of the same strain. This procedure is performed periodically to refresh the cultures but also for applications purposes. A newly transplanted mycelium will be in an active growth condition and will therefore perform much better compared to an older mycelium. For these reasons in this study, before every experiment, new transplants were made for each strain involved.
Once obtained different copies of a strain, they can be conserved in different ways:

- Short to medium conservation at 4°C: this type of conservation is fast and simple and it is used to keep the strains vital and ready for use from a few months to one year;
- Long conservation at -80°C: this type of conservation requires an initial sample preparation to avoid damage due to freezing but allows the biological material to remain viable for several years.

All strains used in the present PhD project are currently conserved in two mycological collections:

- MicUNIPV Research Fungal Strain Culture Collection of DSTA, University of Pavia;
- Mogu Research Fungal Strain Collection (MRFSC).

The scientific articles presented later in this chapter describe the three sampling campaigns that were carried out.

In Buratti *et al*., 2022 (Chapter 1.1), the first sampling campaign in the two wastewater treatment plants is described. In this work a total of 151 fungal strains belonging to 107 different morphotypes were isolated from the water treatment line. Almost all of the isolated strains are micro-fungi, with *Penicillium*, *Talaromyces*, *Aspergillus*, *Trichoderma*, *Trichosporon sensu lato* being the most frequent genera. Among them, two strains of species belonging to the WDF group were also found: *Bjerkandera adusta* (Willd.) P. Karst. (not mentioned in the paper) and *Irpex latemarginatus* (Durieu & Mont.) C.C. Chen & Sheng H. Wu (cited in the manuscript also by its previous name *Oxyporus latemarginatus* (Durieu & Mont.) Donk). *B. adusta* is an already known species for its potential in the field of myco-remediation (Aydin *et al*., 2016; Covino *et al*., 2016; Dhiman *et al*., 2020).

Most of the WDFs used in the PhD project were either already available in the MicUNIPV collection or were collected during the sampling campaign described in Cartabia *et al*., 2022 (Chapter 1.2). In this study, a total of 96 different strains belonging to 76 different species were sampled and identified through morphological and molecular means.

Between October 2022 and January 2023, another sampling campaign was conducted in collaboration with Salamanca University (Spain). The objective of this work was to expand the selection of strains potentially useful for applications in the field of myco-remediation. While many of the species tested in this field belong to the polyporoid group, those in the corticioid group are still relatively understudied, despite being equally interesting for their enzymatic potential. Therefore, with this work, we aimed to broaden the selection of available strains for future applications, with the goal of testing and discovering new species for the degradation and/or removal of pollutants. In this study, 120 basidiomata were collected from two biogeographic areas of Spain: the Mediterranean zone in the center of Spain and the Eurosiberian oceanic climate zone in the north of the Iberian Peninsula (Figure 4). These samples were morphologically identified, and, wherever possible, the isolation of mycelium in pure culture was performed from spores or directly from basidiomata. The mycelium of 55 strains was successfully isolated and further confirmed by ITS-based molecular identification of DNA. The results of this research are reported in the third paper presented in Chapter 1.3, Buratti *et al*., 2023b, currently in draft form and submitted to the peer-reviewed scientific journal 'Forests' (MDPI).

Each of the two sampling campaigns is accompanied by a description of the mycelium obtained in pure culture. Each strain was characterized by observing the macroscopic features of the colony (e.g., color and appearance of the aerial mycelium), the microscopic features of its hyphal component (e.g., size and peculiar structures), and the growth rate.

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1.1 Fungal Diversity in Two Wastewater Treatment Plants in North Italy

The following paragraph has been extrapolated from the scientific article by "Buratti, S., Girometta, C. E., Baiguera, R. M., Barucco, B., Bernardi, M., De Girolamo, G., Malgaretti, M., Oliva, D., Picco, A.M., Savino, E. (2022). Fungal diversity in two wastewater treatment plants in North Italy. Microorganisms, 10(6), 1096.

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Abstract: In urban wastewater treatment plants, bacteria lead the biological component of the depuration process, but the microbial community is also rich in fungi (mainly molds, yeasts and pseudo-yeasts), whose taxonomical diversity and relative frequency depend on several factors, e.g., quality of wastewater input, climate, seasonality, and depuration stage. By joining morphological and molecular identification, we investigated the fungal diversity in two different plants for the urban wastewater treatment in the suburbs of the two major cities in Lombardia, the core of industrial and commercial activities in Italy. This study presents a comparison of the fungal diversity across the depuration stages by applying the concepts of α-, β- and ζ-diversity. Eurotiales (mainly with *Aspergillus* and *Penicillium*), Trichosporonales (*Trichosporon sensu lato*), Saccharomycetales (mainly with *Geotrichum*) and Hypocreales (mainly with *Fusarium* and *Trichoderma*) are the most represented fungal orders and genera in all the stages and both the plants. The two plants show different trends in α-, β- and ζ-diversity, despite the fact that they all share a crash during the secondary sedimentation and turnover across the depuration stages. This study provides an insight on which taxa potentially contribute to each depuration stage and/or keep viable propagules in sludges after the collection from the external environment.

Keywords: urban wastewater**;** fungi; diversity; depuration

1. Introduction

Wastewater treatment technology has a long story; the first tests on depuration by activated sludges were attempted by Ardern and Lockett in 1914 [1].

According to Italian law (Decreto Legislativo 3 Aprile 2006, n. 152) [2], currently, wastewater depuration discriminates among urban wastewater (domestic wastewater possibly mixed with industrial wastewater and rainwash water), domestic wastewater (from domestic activities and human metabolism only) and industrial wastewater (from any productive and/or commercial activity, different from domestic activity and rainwash water).

As schematized by ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale) [3], a typical treatment plant for urban and domestic wastewater treatment is composed of different sectors basically referred to as preliminary treatment (debris and oil removal), primary treatment (reduction of total suspended solids), secondary treatment (reduction of biodegradable organic matter and colloids by activated sludge), tertiary treatment (reduction of nutrients, mainly nitrogen and phosphorous, which have not been removed yet by microbial metabolism), and disinfection (to reduce microbes before final discharge in stream/river). The plant sectors are therefore different from each other as concerns the quantity and composition of suspended solid particles, pH, microbial competition, dissolved O_2, C/N ratio, fluid perturbation/agitation, residence time of the water and sludges [4–6].

Based on the above, wastewater depuration plants can host different microbial (and fungal) communities in different environmental conditions depending on the

peculiar structure of the plant itself, climate, land cover and human activities in the catchment area and number of inhabitants [7–10]. The latter variable is related to the definition of "population equivalent". A population equivalent of one person means "the organic biodegradable load having a five-day biochemical oxygen demand (BOD5) of 60 g of oxygen per day" [2,11]. The population equivalent is a basic unit to size a treatment plant and to provide it with the most suitable technology; this also affects the composition and structure of the whole microbial community.

To date, the depuration stages that significantly involve a biological activity, i.e., the activated sludges, rely on selected bacteria strains naturally mixed with autochthonous ones. Bacteria are assumed to be the most efficient and easily self-sustainable microbes that are able to crash the dissolved nitrogen in the enormous volumes of wastewater to be treated daily in urban and metropolitan areas [12,13]. On the other hand, neither fungi- nor algae-based technologies have been applied yet beyond laboratory scales despite their great potential [14].

Fungi are a well-represented component of the wastewater microbial community that can prove to be very useful and exploitable organisms thanks to their multiple capabilities [15]. Fungi can easily adapt to hostile environments and rapidly changing conditions, for instance different types of municipal and industrial wastewaters, sites strongly polluted by hydrocarbons, acid substrates, or low level of oxygen [4,16,17]. Fungi have been studied and exploited for their production of extra-cellular enzymes (e.g., laccase and peroxidase) capable of degrade complex and potentially hazardous molecules as pesticides, hydrocarbons, dyes, and pharmaceuticals [18–23]. Some species can also accumulate and bio-concentrate heavy metals and other elements [24–27].

Even if many studies are still needed, mycoflora in wastewater treatment plants could help the denitrification process, the removal of nutrients and the reduction of suspended solids. Hyphae of filamentous fungi tend to strengthen sludge flocks, making them larger and with irregular shapes and thus improving the active sludge process [28]. The fungal community in wastewater environments is highly variable, but a core of common shared genera is reported [5,29–31]. *Penicillium*, *Candida* and *Geotrichum* species are the most represented followed by a more variable group with *Trichoderma*, *Trichosporon* and *Rhodotorula*. Fungal taxa variation in treatment plants also depends on season and temperature: fungal diversity seems to differ between summer and winter season. Some taxa such as *Penicillium*, *Trichoderma*, *Acremonium* and *Aspergillus* are more represented in the warm months [32,33].

Taking all of this into consideration, the aim of the present work was to qualitatively characterize the fungal diversity at different stages of the depuration process in an area never investigated before. Two plants for the treatment of urban wastewater located in Lombardia, the most densely populated region and the core of productive and commercial activities in Italy, were chosen. The treatment plants in metropolitan and peri-metropolitan areas thus receive remarkable inputs throughout the whole year [34] and provide significant study cases for highly inhabited areas. Besides, the study area has a subcontinental climate with a sharp difference between summer and winter seasons that can influence fungal diversity as well.

Such a characterization aims therefore to provide a scenario of the variation in diversity patterns across the different environmental conditions in the depuration process, pointing out which taxa are the most represented or unexpected instead. This work on ecological diversity in Italian plants is preliminary to subsequent studies on the possible functional role of the fungal species present.

1. Materials and Methods

1.1. Structure of Wastewater Treatment Plants

The following treatment plants for urban wastewater were examined (the full names are not available due to security reasons):

- Plant 1, managed by CAP Holding; this plant is located in the South-West sector of the Metropolitan City of Milan; it caters for a population equivalent of 320,000 people and treats an average wastewater volume of 100,000 m^3 day^{-1};
- Plant 2, managed by A2A Ciclo Idrico; this is located in Eastern Lombardia; it caters for a population equivalent of 296,000 people and treats an average wastewater volume of 70,000 m^3 day^{-1}.

A basic scheme of the water treatment process is reported in Figure 1.

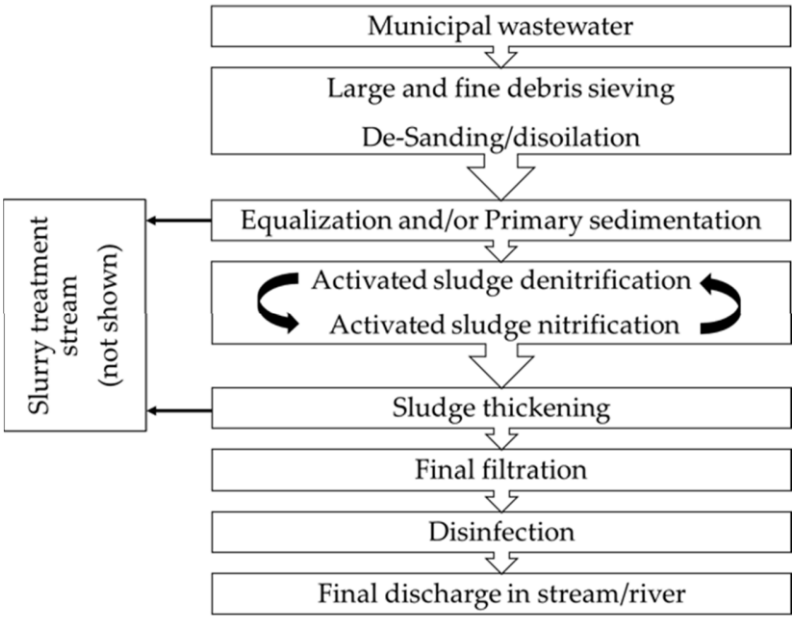


Figure 1. Basic scheme of water treatment in the plants. Only the water treatment stream is shown in detail.

The structures of Plant 1 and Plant 2 are slightly different from each other; therefore, the two sampling transects do not perfectly overlap and only some stages (namely, the activated sludge) are properly comparable. The two plants were consequently analyzed separately; the codes corresponding to each depuration stage in the Plants are schematized in Table 1.

Table 1. Treatment stages examined in this study.

Scheme	Plant	Code in this Study
Primary sedimentation of input wastewater	1	1-PSed
Activated sludge–denitrification	1	1-Denitro
Activated sludge–oxidation	1	1-Oxy
Filtration input–discharge post-secondary sedimentation	1	1-Filt. input
Post ozonation	1	1-End
Equalization of input wastewater	2	2-Equal
Activated sludge–denitrification	2	2-Denitro
Activated sludge–oxidation	2	2-Oxy
Filtration input	2	2-End

1.2. Sampling and Isolation in Pure Culture

Samples of water and samples of sludge (i.e., water with 5–8 g L^{-1} solids suspension) were collected between November 2018 and May 2020. Samples were manually shaken for at least 1 min per bottle in order to resuspend all the particulate and homogenize the propagules distribution. Serial dilution in physiological solution (NaCl 0.9%) was axenically performed by using 1 mL as the basic unit according to the scheme in Table 2.

Table 2. Dilution scheme for wastewater and sludge samples.

Stage	Bulk	1:10	1:100	1:1000	1:10,000
1-PSed	x	x	x	x	
1-Denitro			x	x	
1-Oxy		x	x	x	
1-Filt. input			x	x	x
1-End			x	x	x

2-Equal	x	x	x	x	
2-Denitro			x	x	
2-Oxy		x	x	x	
2-End			x	x	x

Bulk and diluted samples were spread in triplicate onto PDA (potato dextrose agar, Biokar Diagnostics), 15 cm diameter Petri dishes and incubated in the dark at room temperature for 28 days. PDA was prepared according to the manufacturer's instructions (Biokar Diagnostics, 3.9%) and 150 ppm chloramphenicol (Fagron) were added before auto- clave sterilization. Mycofloristic surveys were performed weekly to allow the propagules to overcome any latency period.

In every weekly survey, real-time approximate identification (morphotype approach) based on morphology was carried out by means of stereomicroscope (Zeiss Stemi 2000C) and optical microscope (Zeiss Axioplan). The morphotype approach represents a first, basic step to organize the identification workload when dealing with apparently numerous taxa and little survey time, either for fungi or other organisms [35,36].

At least two cultures per morphotype were isolated in a glass tube containing PDA (as above), corked with raw cotton and incubated at room light and temperature. Pure cultures were morphologically checked to validate the morphotype.

1.3. Molecular Identification of Selected Strains

Based on the strain set obtained as above, at least one isolated morphotype per each plant was selected for further molecular identification.

DNA extraction was obtained by means of a Nucleospin Plant II kit (Macherey-Nagel) according to the manufacturer's instructions. Due to the great variety in mycoflora, PCR amplification concerned the ITS region only; on the other hand, the ITS region is regarded as an efficient barcode for most fungal taxa [37–39]. ITS1-ITS4 primers were used for filamentous fungi (including mycelia sterilia too), whereas ITS5-ITS4 primers were used for yeasts and pseudo-yeasts [40]. Further details of the complete identification protocol are reported in Girometta et al. (2020) [41].

1.4. Estimation of Ecological Parameters

The wastewater flow in the treatment plants under examination is mainly unidirectional and the two plants share only one significant re-pumping line from Oxy to Denitro. The most water proceeds from the discharge of the activated sludge to the final dep- uration stages and discharge in the stream. This allows for the approximation of the data structure to a spatial environmental gradient whose sample selection scheme is directional
from a point source [42].

Based on the General concepts of α-, β-, γ- and ζ-diversity, the composition and structure of the communities in each depuration stage were investigated and compared along the depuration process, i.e., stage by stage.

In order to estimate the α-diversity in each depuration stage, Simpson's evenness and Pielou's regularity were compared as suggested by Mouillot et Leprêtre (1999) [43] and calculated as summarized by Bullini et al. (1998) [44].

$$E = \sum^{s}_{i=1} p^2_i \,/ S \quad (1)$$

where: p_i = fraction of individuals of the species i in the overall individual population; S = overall number of species in the populatin = γ diversity

As summarized by Baselga (2010) [45], "β-diversity is the variation of species com- position of assemblages", i.e., the variation between depuration stages in this context. The β-diversity partitioning was investigated based on pairwise (nearest neighbor) presence- absence models by Jaccard's and Simpson's indices [46].

According to Hui and McGeoch (2014) [47], "ζ-diversity is the number of species shared by a given number of sites and provides a measure of turnover for each combination of i sites". Analogous to β-diversity, ζ-diversity was normalized based on Jaccard's assumptions [42,45].

As a whole, β-diversity and ζ-diversity were calculated as follows:

Jaccard's dissimilarity

$$\beta cc = (b + c)/(a + b + c) \quad (2)$$

Jaccard's distance

$$\beta rich = |b - c|/(a + b + c) \quad (3)$$

Simpson's turnover

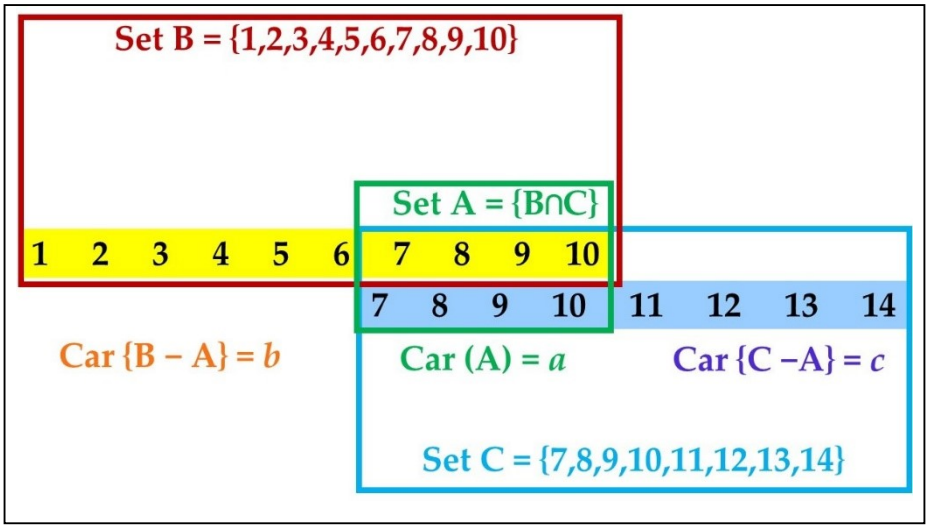
$$\beta\text{-}3 = \beta cc - \beta rich \quad (4)$$

Normalized ζi-diversity

$$(i = 2) = \zeta 2 = a/(a + b + c) \quad (5)$$

where: a is the number of taxa (cardinality) in set A = {B ∩ C}, i.e., a = Car (A); b = Car {B −A}; and c = Car {C − A}, given two neighbor sites A and B (Figure 2).

Figure 2. Basic generic example scheme of the data structure as applied in β-diversity and ζ-diver- sity formulae. The hypothetical example considers two neighbor sites including 10 and eight species, respectively.



2. Results and Discussion

2.1. Sampling, Isolation in Pure Culture and Identification

From the whole pool of fungal taxa sampled, 60 morphotypes from Plant 1 and 47 from Plant 2 were successfully isolated in pure culture.

The morphotype approach generally fails in discriminating most yeast species from each other, and the same happens for arthrosporigenous pseudo-yeasts. Yeasts and pseudo-yeasts must be therefore sampled more intensely than moulds.

ITS-based molecular identification of the selected strains resulted in acceptable dis- crimination for all of the morphological categories under examination (yeasts, spori- genous filamentous, mycelia sterilia).

All fungal taxa sampled in this study are reported in Table 3: genera identification was carried out by morphological approach, and species identification was achieved by ITS-based molecular analysis. Taxonomy check on MycoBank [48].

Table 3. Sampled fungal taxa with reference to the depuration stage of provenance.

Fungal Taxa	Author	Depuration Stages of Provenance
Acremonium spp.	Link	1-Psed, 1-Denitro, 1-End; 2-Equal, 2-Denitro, 2-Oxy
Alternaria spp.	Nees	1-Denitro, 2- Equal
Apiotrichum domesticum	(Sugita, A. Nishikawa & Shinoda) Yurkov & Boekhout	2-Oxy
Apiotrichum montevideense	(L.A. Queiroz) Yurkov & Boekhout	2-Equal
Apiotrichum laibachii	(Windisch) Yurkov & Boekhout	1-Psed, 1-Denitro, 1-Oxy

Aspergillus flavus	Link	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input; 2-Equal, 2-Denitro, 2-Oxy
Aspergillus fumigatus	Fresen.	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input, 1-End; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Aspergillus niger	Tiegh.	1-Psed, 1-Oxy, 1-Filt. Input, 1-End; 2-Equal, 2-Denitro, 2-Oxy
Aspergillus tubingensis	Mosseray	1-Oxy
Aspergillus spp.	P. Micheli ex Haller	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input; 2-Oxy, 2-End
Candida pseudolambica	M.T. Sm. & Poot	1-Denitro
Cladosporium spp.	Link	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input, 1-End; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Chaetomium sp.	Kunze	2-Oxy
Chrysosporium tropicum	J.W. Carmich.	1-Denitro, 1-Oxy, 1-Filt. Input
Cosmospora butyri	(J.F.H. Beyma) Gräfenhan	2-Equal
Cutaneotrichosporon cutaneum	(Beurm., Gougerot & Vaucher bis) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input; 2-Equal, 2-Oxy
Cutaneotrichosporon jirovecii	(Frágner) Xin Zhan Liu, F.Y. Bai	1-Oxy; 2-Equal, 2-Oxy
Cutaneotrichosporon mucoides	(E. Guého & M.T. Sm.) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout	1-Psed, 1-Denitro, 1-Oxy
Debaryomyces hansenii	(Zopf) Lodder & Kreger	1-Psed
Dipodascus fermentans	(Diddens & Lodder) P.M. Kirk	1-Denitro
Diutina neorugosa	(Paredes, Deanna A. Sutton, Cano & Guarro) Khunnamw., Jindam., Limtong & Lachance	1-Psed
Engyodontium sp.	de Hoog	2-Equal
Exophiala lecanii-corni	(Benedek & G. Specht) Haase & de Hoog	2-Equal, 2-End
Fusarium fujikuroi	Nirenberg	1-Oxy
Fusarium oxysporum	Schltdl.	2-Denitro
Fusarium spp.	Link	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Fusicladium sp.	Bonord.	2-Equal
Geotrichum candidum	Link	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input, 1-End; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Geotrichum fragrans	Morenz	2-Oxy
Geotrichum spp.	Link	1-Psed, 1-Denitro, 1-Oxy 2-Equal, 2-Denitro, 2-Oxy, 2-End
Graphium sp.	Corda	1-Psed
Mucor spp.	Fresen.	1-Psed, 1-Denitro, 1-Oxy; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Oxyporus latemarginatus	(Durieu & Mont.) Donk	1-Oxy
Phialophora spp.	Medlar	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input
Phycomyces sp.	Kunze	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input
Phoma spp.	Sacc.	1-Denitro, 1-Filt. Input; 2-Equal
Penicillium albocoremium	(Frisvad) Frisvad	1-Oxy
Penicillium crustosum	Thom	2-Equal
Penicillium expansum	Link	2-Oxy
Penicillium griseofulvum	Dierckx	1-Oxy, 1-Equal
Penicillium olsonii	Bainier & Sartory	1-Psed
Penicillium verrucosum	Dierckx	1-Oxy
Penicillium spp.	Link	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input, 1-End; 2-Equal, 2-Denitro, 2-Oxy
Purpureocillium lilacinum	(Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson	1-Oxy; 2- Equal, 2-Denitro, 2-Oxy, 2-End

Rhizopus oryzae	Went & Prins. Geerl.	1-Psed, 1-Denitro, 1-Oxy; 2-Equal, 2-Denitro, 2-Oxy
Rhodotorula glutinis	(Fresen.) F.C. Harrison	1-Psed, 1-Denitro, 1-Oxy, 1-End; 2-Equal, 2-End
Rhodotorula mucilaginosa	(A. Jörg.) F.C. Harrison	1-Oxy
Sampaiozyma ingeniosa	(Di Menna) Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout	1-Psed
Scedosporium dehoogi	Gilgado, Cano, Gené & Guarro	2-Oxy
Scheffersomyces spartinae	(Ahearn, Yarrow & Meyers) Kurtzman & M. Suzuki	1-Denitro
Scopulariopsis brevicaulis	(Sacc.) Bainier	1-Psed, 1-Denitro; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Sporobolomyces spp.	Kluyver & C.B. Niel	2-Equal 2-Denitro
Talaromyces flavus	(Klöcker) Stolk & Samson	2-Equal
Talaromyces spp.	C.R. Benj.	2-Equal
Trichoderma asperellum	Samuels, Lieckf. & Nirenberg	1-Psed, 1-Denitro, 1-Oxy; 2-Equal, 2-Oxy
Trichoderma citrinoviride	Bissett	2-Oxy
Trichoderma harzianum	Rifai	1-Psed
Trichoderma saturnisporum	Hammill	2-Denitro
Trichoderma virens	(J.H. Mill., Giddens & A.A. Foster) Arx	1-Denitro
Trichoderma spp.	Pers.	1-Psed, 1-Denitro, 1-Oxy, 1-End; 2-Equal, 2-Denitro, 2-Oxy, 2-End
Trichosporon asahii	Akagi ex Sugita, A. Nishikawa & Shinoda	1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input
Verticillium sp.	Nees	2-Oxy
Yarrowia lipolytica	(Wick., Kurtzman & Herman) Van der Walt & Arx	2-Denitro
Zygoascus polysorbophila	(Kurtzman) Nagats., Kiyuna & Sugiy.	2-Denitro
Other yeasts		1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input, 1-End; 2-Equal, 2-Denitro, 2-End
Sporigenous fungi		1-Psed, 1-Denitro, 1-Oxy, 1-Filt. Input
Mycelia sterilia		1-Psed, 1-Oxy, 1-Filt. Input; 2-Denitro

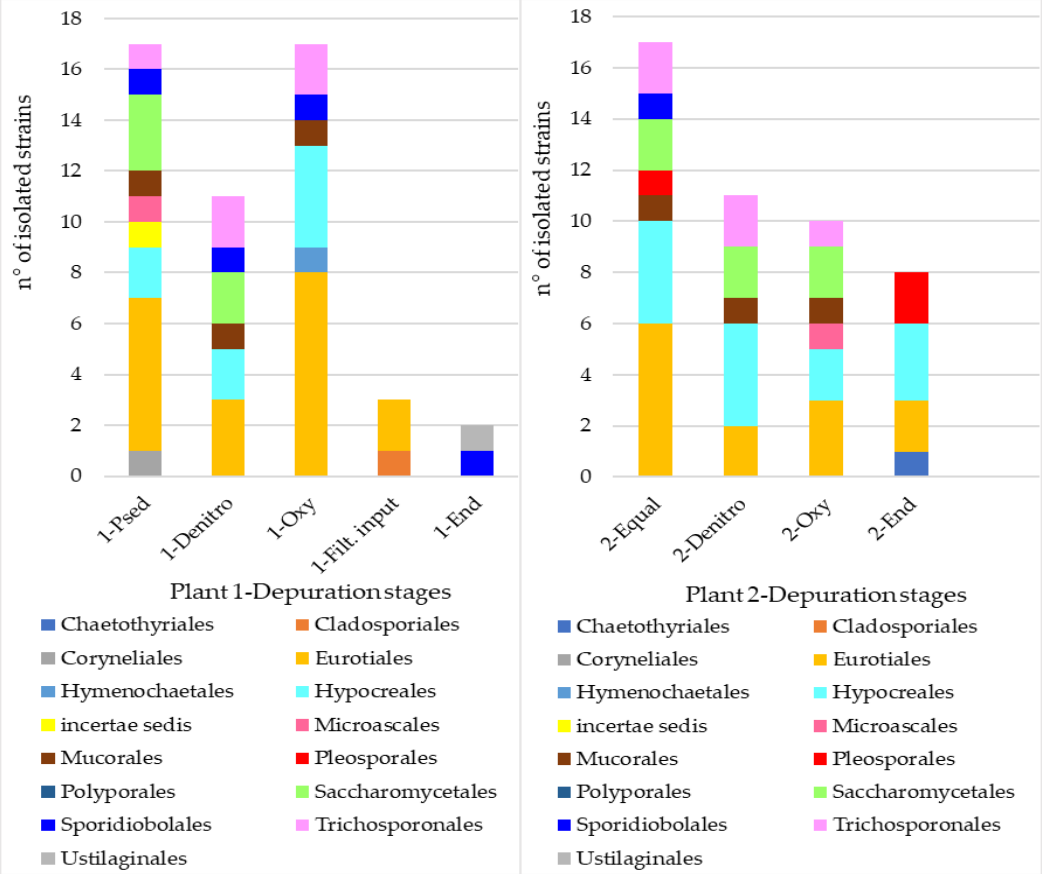
Certain fungal taxa were found in almost every stage of depuration: genera such as *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Mucor*, *Penicillium* and *Trichoderma* are generally found in water and soil and their spores are constantly present in the air [49,50]. These fungi follow the stream across the depuration stages and their availability as environmental contaminants can explain why some taxa are present even after the oxidation and disinfection process, as they are easily sampled by chance.

Family *Trichosporanaceae* is also well represented by species of the genera *Apiotrichum*, *Cutaneotrichosporon* and *Trichosporon*, once all are grouped in the latter [51]. These fungi are yeast and yeast-like organisms generally isolated from soil and environment and some species also from human and animal skin [52]. Species such as *Apiotrichum domesticum*, *Apiotrichum montevideense, Cutaneotrichosporon mucoides* and *Trichosporon asahii* are potentially pathogenic and of clinical importance [52,53] but from this study it emerges that even if these fungi are found in different depuration stages, they are successfully eliminated by the depuration process, as they are no longer found in post-ozonation (1-End) and in Filtration input (2-End).

2.2. Diversity Patterns at Fungal Order Scale

By merging morphotypes with results from molecular identifications, the isolated strains show the diversity pattern reported in Figure 3, giving us the composition of the fungal community throughout both treatment plants. The single data were grouped at the taxonomic level of order to better compare the mycoflora present both in the different depuration stages in the two plants and during the four seasons.

As expected, diversity in wastewater in the first sampling stages (1-PSed and 2-Equalization) is more affected by external propagule sources, both from the urban areas and agricultural systems. Eurotiales, Hypocreales, Saccharomycetales and Trichosporonales are the Orders mainly sampled.



(**a**) (**b**)

Figure 3. Diversity pattern of the isolated strains at an Order scale in Plant 1 (**a**) and Plant 2 (**b**) with reference to the depuration stage of provenance.

Among all isolated strains, Eurotiales is the most represented in both the treatment plants and in almost all depuration stages. In this study, Eurotiales include *Aspergillus* and *Penicillium or Talaromyces*, as well as *Paecilomyces*. It should be noted that the nomenclatural distinction between *Penicillium* and *Talaromyces* has been adopted, despite the fact that they are the anamorph and teleomorph of the same taxon, respectively. This decision was taken to preserve the information about the occurrence of species which are known to reproduce sexually as well.

Furthermore, Hypocreales constitutes the base of the fungal community in this study, as species belonging to this order have been sampled in three out of five depuration stages in Plant 1 and in all stages in Plant 2. *Fusarium* and *Trichoderma*, which are very common in agricultural systems and soils, are the most represented species. A special mention is deserved by *Trichoderma*, whose species play an important role in soil ecology due to their competition and hyperparasitism versus phytopathogens (mainly fungi and nematodes).

Moreover, *Trichoderma* species stimulate plant defense induction [54]. Here, five species were detected: *T. harzianum* and *T. virens*, *T. citrinoviride* and *T. saturnisporum*, and *T. asperellum* (the most common species in the present sampling) [55]. The widespread *T. asperellum* is particularly interesting because only in 1999 it was recognized to be a different species from *T. viride*; however, since then *T. asperellum* has been increasingly detected in agricultural soils. This is likely to also be due to its above mentioned application as a biocontrol agent [56].

Despite the fact that a quantitative approach is out of the scope of the present work, it can be noted that *Fusarium* species are less represented than its major

antagonists in the soil, i.e., *Trichoderma* species; this is important since both *F. oxysporum* and *F. fujikuroi* are severe phytopathogens [57].

Cosmospora, typically developing an *Acremonium*-like morphology, is phylogenetically close to *Fusarium*. Here, the genus is represented by *C. butyri*, which is related to lipid-rich substrates [58].

According to the recent taxonomic revision of the genus *Paecilomyces*, the species *P. lilacinus* now is named *Purpureocillium lilacinum* and it has switched from Eurotiales to Hypocreales [59].

Saccharomycetales includes fungi that are well-known to be common in wastewater, where they degrade simple polysaccharides and fatty acids. In the present work, eight genera belonging to Saccharomycetales were detected: *Candida*, *Dipodascus*, *Diutina*, *Galactomyces*, *Geotrichum*, *Scheffersomyces*, *Yarrowia* and *Zygoascus*. *Geotrichum*, which is found worldwide in air, soil, water, sewage, as well as in plants, besides being found in human feces, was the most representative, as it concerns morphotype frequency and spatial colonization in a Petri dish, displaying most pseudo-yeast morphology.

The order Trichosporonales resulted in three Genera phylogenetically very close to each other and belonging to *Trichosporon sensu lato*, i.e., *Apiotrichum*, *Cutaneotrichosporon* and *Trichosporon sensu stricto*. *Trichosporon s.l.* morphotype proved to be very common and displayed both budding and arthrospore formation. As for Saccharomycetales, *Trichosporon s.l.* is also commonly represented in soil and in water; however, its trophic spectrum also includes keratinolysis and thus degradation of hair(s) and skin in wastewater [9,60,61].

As a whole, yeasts and pseudo-yeasts generally are over-represented in wastewater treatment plants as they are favored by the abundance of organic matter and, compared to filamentous fungi, are facilitated in growth by the asexual mode of reproduction (buds and arthrospores). Filamentous fungi in continuous wastewater flow are often hampered in sporulation and mycelia can produce forms of resistance such as chlamydospores [62].

2.3. Seasonal Variation

Fungal community composition at the Order scale shows a seasonal variation, with similar results between the two plants (Figure 4).

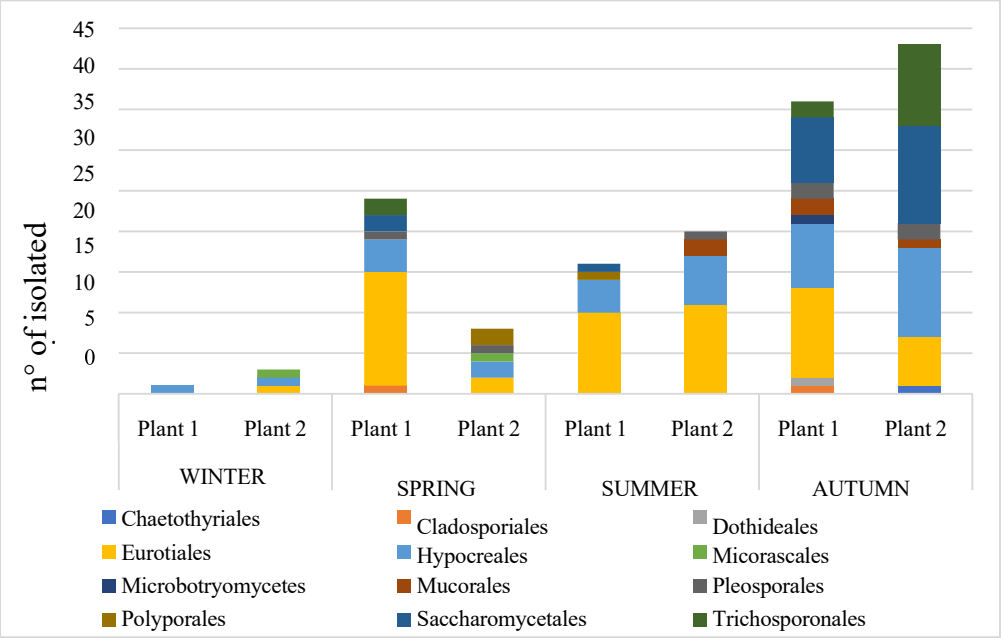


Figure 4. Seasonal variation of the isolated strains at an Order scale.

A higher number of isolates was found in summer and autumn compared to winter and spring. Orders follow this trend as well.

Eurotiales (mainly *Aspergillus* and *Penicillium* species) and Hypocreales (mainly *Fusarium* and *Trichoderma* species) are confirmed to be the most represented in the two plants across the whole year. Saccharomycetales with *Geotrichum* species and

Trichosporanales are also frequent, especially in autumn. Other orders are less represented and were sporadically isolated compared to the others.

As a whole, the wastewater environment seems to host a wider and more diversified community in summer and autumn compared to the other seasons: these results confirm what is as also reported in other works [32,33].

Seasonal variation of isolated strains is probably related to conditions of humidity and temperature, with rainy and warmer months characterized by a more diverse fungal community. This relation is also supported by the metereological data of Lombardia: April, May, June, September, October and November are the months with most average millimeters of precipitation and with average temperatures between 15 °C and 20 °C [63].

2.4. Diversity Indices

Since a wastewater treatment plant is composed of different systems and environ- mental conditions, different community structures are expected in each depuration stage.

Simpson's evenness and Pielou's regularity describe how each taxon is representative within the community based on the ratio between the taxon individuals and the over- all number of individuals.

Evenness indices by Simpson (1949) [64] and Pielou (1966) [65] are compared in Fig- ure 5. The substantial disagreement between the two indices suggests that Pielou's regularity, (a derivation from Shannon-Weaver's index), is not truly informative in this case since the small sample size highlights the bias [43]. As the community structure evolves towards increasing diversity loss, zero inflation is a bias factor to take into account [66].

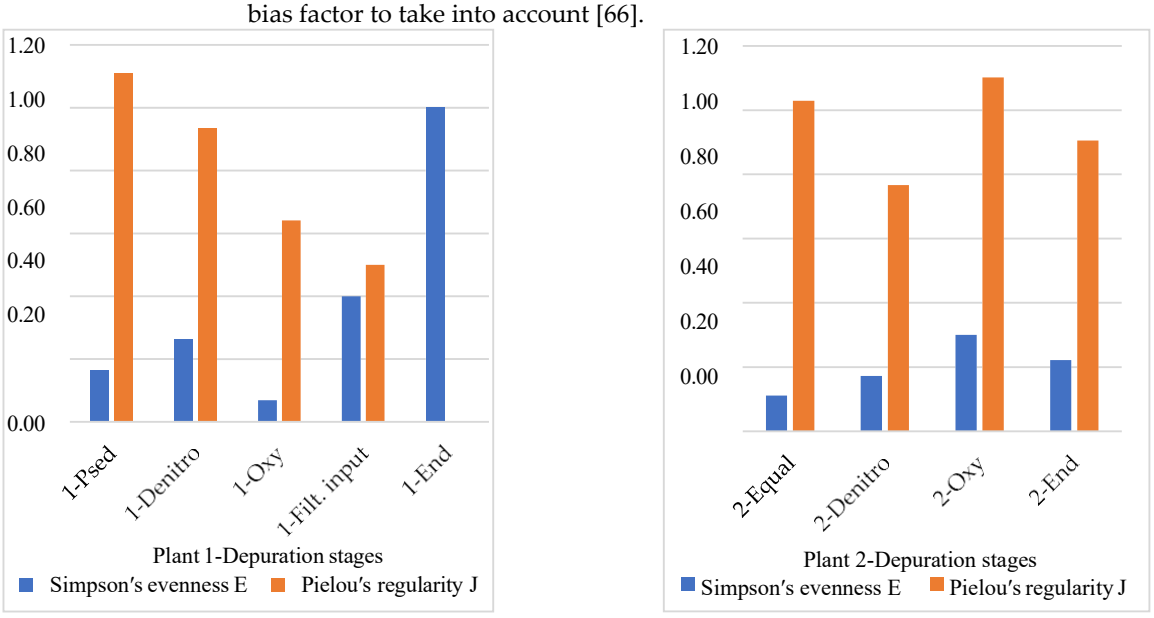


Figure 5. Simpson's evenness and Pielou's regularity at an Order scale in different depuration stages of Plant 1 (**a**) and Plant 2 (**b**).

Concretely, the different structure of the two plants and sedimentation pools in particular may explain the differences in the taxa occurrence and repartition along the depuration stages. The final stage in Plant 1 loses diversity (Simpson's evenness 100) with only few species represented, while Plant 2 appears to be favoured in preserving more propagules until the final stages.

As mentioned, β-diversity described the compositional change of the community. The above discussed data suggest that β-diversity partitioning is governed by pairwise presence/absence models. Jaccard's indices and Simpson's turnover, i.e., normalizations of raw ζ-diversity [42], are reported in Figures 6 and 7 for Order and genus scale, respectively.

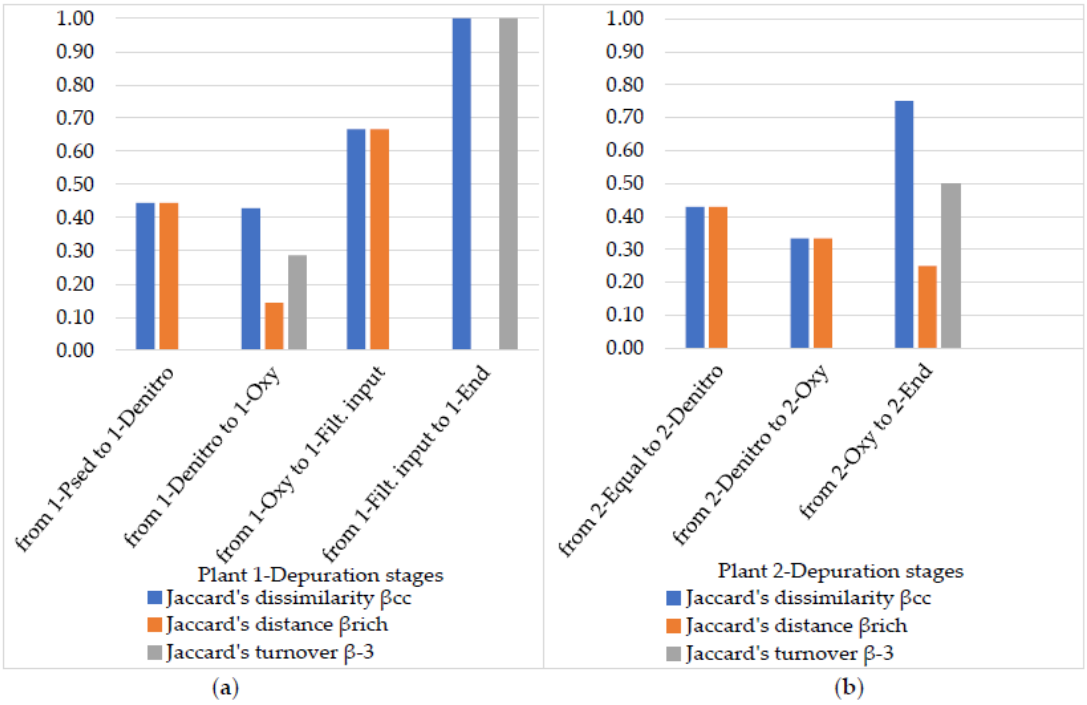
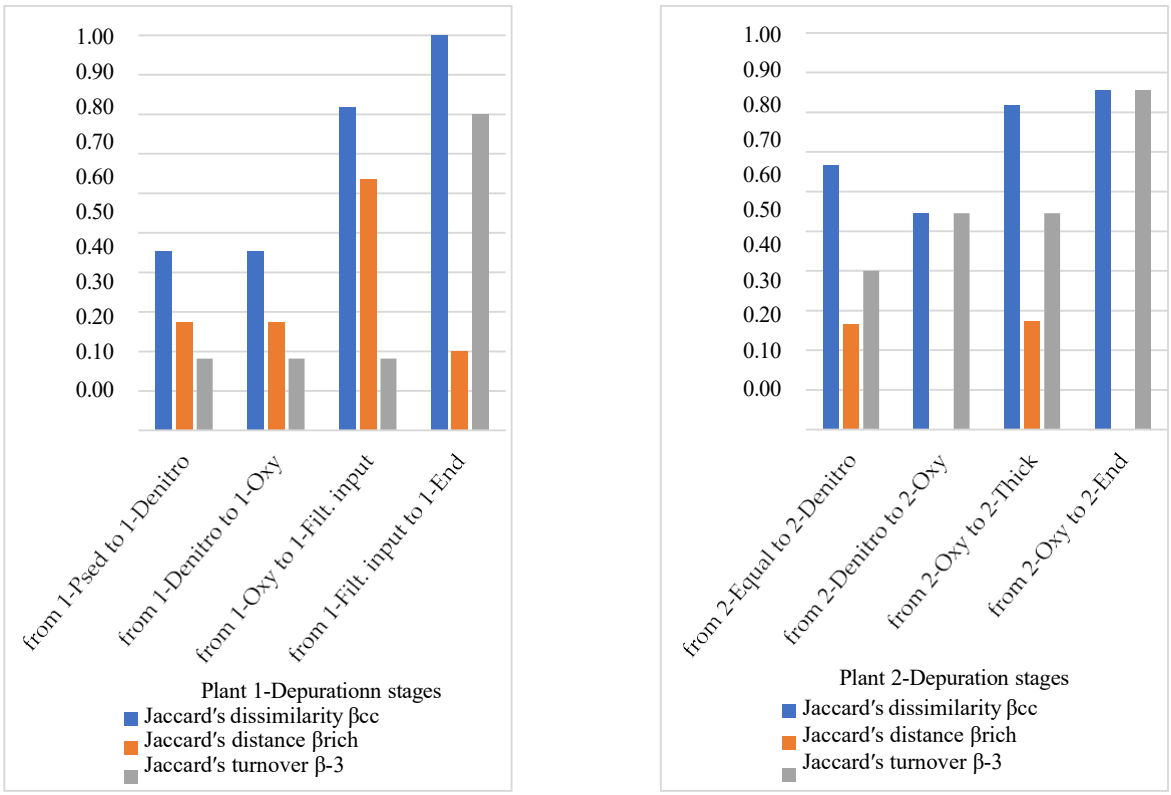


Figure 6. Pairwise distance and directional turnover based on Jaccard's models at an Order scale in different depuration stages in Plant 1 (**a**) and Plant 2 (**b**).

Based on the order scale, Jaccard's dissimilarity βcc increases in Plant 1 by a pairwise comparison of depuration stages, whereas the Jaccard's turnover β-3 based on absolute species number doesn't show a clear trend, except for the final stage. The dissimilarity trend in Plant 2 is less regular. In Plant 1, the severe constraints before the final discharge provoke a dramatic increasing in turnover. The Jaccard's distance βrich in Plant 1 is highest when crossing from 1-Oxy to 1-Filt. Input, despite the turnover, is null, as the number of isolated strains is higher compared to the previous stage; however, it is not the same in Plant 2, where the distance is less variable.



(**a**) (**b**)

Figure 7. Pairwise distance and directional turnover based on Jaccard's models at a genus scale in different depuration stages in Plant 1 (**a**) and Plant 2 (**b**).

As expected, the genus scale is more informative than the order scale, although only four orders are represented by three or more genera. Based on genus scale, Jaccard's dis- similarity βcc in Plant 1 particularly increases when crossing from 1-Oxy to 1-Filt. input and then furtherly increases to final discharge. More interestingly, Plant 1 has a constantly low Jaccard's turnover β-3 except when crossing to the final discharge, whereas the turnover in Plant 2 is always around 0.5. The Jaccard's distance βrich is similar among the stages in both the plants, except for the stage from 1-Oxy to to 1-Filt. input in Plant 1 (that explains the most dissimilarity observed by βcc) and the stage from 2-Denitro to 2-Oxy in Plant 2 (although the communities are qualitatively different due to turnover). In Plant 2, turnover has a remarkable role in preserving the diversity.

The activated sludge seems therefore to be a critical stage that imposes environmental constraints with particular concern to oxygenation. In many depuration plants there are two backward re-pumping lines: the first is from the oxidation pool to the denitrification one (this is both the case of Plant 1 and Plant 2), and the second is from the final filtration to the denitrification one (this is the case of Plant 1 only). Such a partial bi-directional flow favors the community homogenization at least between the activated sludge and the sedimentation stage.

Notwithstanding this, the stage from the activated sludge to the secondary sedimentation provokes, as mentioned, a dramatic decline in microbial richness, since the super- natant is impoverished in nutrients with respect to the sunk slurry particles. As expected, and most important in the depuration process, the final discharge furtherly destroys the microbial community in the water. As a whole, such a loss can be seen both as the result of microbial quantitative reduction and consequent qualitative sampling bias.

This is consistent with normalized ζ-diversity at a genus scale that clearly shows similar dynamics in Plant 1 and Plant 2. The stage from the initial input to the activated sludge represents a first bottleneck more in Plant 2 than in the Plant 1. The ζ-diversity is in fact lower when passing from the 2-Equal to 2-Denitro (ζ-diversity 0.33) than from 1-PSed to 1-Denitro (ζ-diversity 0.55). This was unexpected because

the wastewater in 2-Equal is very similar to 2-Denitro, whereas 1-Psed is a further intermediate stage between the initial input and the denitrification.

In both the treatment plants the output from the activated sludge (1-Oxy and 2-Oxy) encounters a bottleneck where the number of shared taxa (i.e., ζ-diversity) crashes (Plant 1 ζ-diversity 0.18; Plant 2 ζ-diversity 0.14). In Plant 1 the output from the activated sludge undergoes secondary sedimentation; the resulting supernatant (1-Filt. input) is therefore significantly depleted of particles as well as fungal propagules. As a whole, ζ-diversity between 1-Oxy and 1-Filt. input relies on sharing taxa in Eurotiales (Figure 3).

In Plant 2, the output from the activated sludge undergoes two different processes which result in similar values of ζ-diversity. When passing from 2-Oxy to 2-End ζ-diver- sity relies on sharing taxa in Hypocreales and Eurotiales (i.e., true moulds), which are very common in the environment and may therefore be represented even at the exit of the depuration process.

Actually, the taxa sampled after the ozonation process are to be meant as sampled by chance due to the environmental availability of propagules outside the ozonation com- pound itself instead of the failure of the depuration process. It should be kept in mind that ζ-diversity is a similarity measure based on diversity instead of population size, therefore it does not at all imply considerations about the depuration success.

The depuration principle as meant by Italian law [2,6] aims at "disinfection" instead of "sterilization", meaning that microbial contamination is accepted on condition it is below the safety threshold indicated by the law itself. Nevertheless, it is noteworthy that such thresholds concern bacteria only (namely the coliforms *Escherichia coli*, *Enterococcus* spp., *Clostridium perfringens*, and *Pseudomonas aeruginosa*) whereas no fungal propagules are monitored by default [67]. This is due to the fact that the depuration process basically relies on the bacterial activity more than the fungal one. Moreover, there is another issue hampering the ability to apply to the fungi the same qualitative and quantitative surveys routinely applied to bacteria: filamentous fungi grow much slower even in optimal conditions [68]. However, as previously discussed, filamentous fungi show a severely limited reproduction in sludges and slurries of depuration plants, whereas yeasts and pseudo- yeasts are more favoured but their populations are crashed by the conventional disinfection methods [6] as well as the bacterial ones, particularly when adopting sieving biomembranes [34,69]. This means that the discharged water from a depuration plant provides a negligible fungal inoculum into the receiving stream. Nevertheless, periodic surveys on the fungal propagules in the discharged water may suggest what is the most efficient disinfection method when projecting future plants or restructuring/adjusting the existent ones.

3. Conclusions

Wastewater treatment plants are composite systems where different fungal communities are hosted depending on the specific conditions of each depuration stage. The diversity pattern in input strongly affects the community in first stages (primary sedimentation and activated sludge), but is radically changed in secondary sedimentation, i.e., after the activated sludge stage and the separation of spent microbial particles and residual nutrients from the supernatant. As expected, the cyclic flow between denitrification and nitrification systems contributes to homogenize the communities in activated sludge despite the difference in oxidation conditions.

From a qualitative mycofloristic perspective, Eurotiales, Hypocreales and Tricho- sporonales, as well as Saccharomycetales, are the most represented orders in all the dep- uration stages, mainly including genera such as *Penicillium* or *Talaromyces*, *Aspergillus*, *Trichoderma*, *Trichosporon sensu lato* and several yeasts and pseudo-yeasts such as *Ge- otrichum*. Despite the fact that Plant 1 and Plant 2 show different diversity patterns, the above mentioned taxa are basically represented in both.

The ITS region approach resulted in acceptable discrimination based on cross-

check on the output by Mycobank Molecular ID. ITS is regarded as a suitable barcode region when dealing with surveys on a wide spectrum of fungi. Further selected markers may be introduced to confirm specific identification within complex Genera such as *Penicillium* or *Talaromyces* and *Trichoderma* as well as to investigate sub-specific diversity.

The wastewater fungal community is an often ignored, but equally represented, part of the microbial community. Deepening the knowledge about fungal species' presence and fluctuation across depuration stages and seasons can help in better understanding their role in the depuration process and how to exploit them in synergy with the bacterial component. This work also highlights the importance of periodic sampling campaigns to monitor the fungal community not only in the different depuration stages but also into the final water stream.

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and S.B.; investigation, C.E.G., S.B. and R.M.B.; resources, A.M.P., D.O., M.M., B.B. and G.D.G.; data curation, C.E.G., S.B., B.B. M.B. and G.D.G.; writing—original draft preparation, C.E.G. and S.B.; writing—review and editing, E.S., A.M.P., M.B., B.B. and G.D.G.; visualization, C.E.G. and S.B.; su- pervision, E.S., D.O., B.B. and G.D.G.; project administration, D.O., A.M.P. and E.S.; funding acquisition, D.O., A.M.P. and E.S. All authors have read and agreed to the published version of the manuscript.

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1.2 Lignicolous Fungi Collected in Northern Italy: Identificationand Morphological Description of Isolates

The following paragraph has been extrapolated from the scientific article by Cartabia, M., Girometta, C. E., Baiguera, R. M., Buratti, S., Babbini, S., Bernicchia, A., & Savino, E. (2022). Lignicolous fungi collected in northern Italy: Identification and morphological description of isolates. Diversity, 14(5), 413.

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Abstract: In recent years, fungi, particularly lignicolous fungi, have been re-considered as a source for biotechnological and industrial applications. Lignicolous basidiomycetes are the most effective at degrading wood, particularly cellulose, hemicelluloses and lignin, which are among the most resistant biopolymers. This study aims to constitute a research collection of lignicolous fungal strains that are useful for further studies and applications in different production fields. The basidiomata used to isolate the strains in a pure culture were, firstly, identified through macroscopic and microscopic characteristics integrated with ecological data. To obtain pure cultures of dikaryotic mycelia, 96 different strains of *Agaricomycetes* belonging to 76 different species and related to 51 genera (18 families and 5 orders) were isolated using a malt extract agar (MEA) medium enriched with hydrogen peroxide. The identity of the isolated strains was then confirmed by molecular analysis through the sequencing of the internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster. All the strains are currently conserved using different methods, and their vitality is periodically tested.

Keywords: fungal strain isolation; lignicolous fungi; research fungal strain collection

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1. Introduction

Fungi are an essential, fascinating and useful group of organisms with biotechnological potential for pure and applied research as well as for industrial exploitation [1]. Filamentous fungi have been used for more than a century as versatile and highly productive organisms. Nowadays, fungi, especially basidiomycetes, can be used in many different applications: in the medicinal field as immunostimulants and food supplements; in pharmacology as a source of bioactive compounds against human and animal diseases (e.g., antibacterial antibiotics, antifungals, antiviral agents, anti-cancer agents, anti-diabetes, controllers of cardio-vascular diseases, etc.); in agriculture as biocontrol agents against fungi, insects, nematodes, weeds, etc., as low-impact food and protein sources and to enhance crops and forestry; and for commodities such as cosmetics, preservatives, enzymes and textile dyes [1].

Both scientific research and industrial applications require not only constant material but also simple and fixed conditions. This allows for a better control of the variables that can influence the output of the research, providing an understanding of which parameters can modify the result and how. Being able to reproduce the same experiment is a key principle of scientific research, and it is fundamental for industrial applications to have standard production processes that consistently produce identical products.

Fungal strain diversity represents a genetic resource that should be preserved. For this reason, certified collections have been created. Fungal culture collections are of primary

importance in order to deepen our knowledge of the taxonomy, species distribution and officinal properties and to investigate the potential applications of fungi [2]. Moreover, collections can play a role in preserving biodiversity and conserving endangered species ex situ.

Culture collections are a fundamental source for researchers at an international level, and the exchange and availability of quality-guaranteed, authenticated pure cultures are increasingly in demand. The World Federation for Fungal Collections has provided detailed guidelines [3] which aim to ensure collections are of high quality, from the origin to the conservation, and the availability of each strain. In comparison to the past, this is made relatively easy by the increasing accessibility of standardized equipment (including sterile hoods, refrigerators, freezers, etc.) and labware for the isolation, safe culturing and preservation of strains. Nowadays, some major variables make the observation of the culture characteristics reproducible over time and comparable among different work and laboratories. They are: the use of commercial culture media instead of the home-made older ones; the use of conventional Petri dishes, vessels and sealing tools (plastic film or paper adhesive tape alongside the "evergreen" raw cotton for tubes), which differently affect the gas exchange and dehydration; the use of incubators to keep the growth temperature constant or finely tuned.

Some major culture collections around the world include the CBS-KNAW, the All-Russian Collection of Microorganisms (VKM) and the Agro-food & Environmental Fungal Collection (BCCM/MUCL) [4–6]. In addition, the Project MIRRI (Microbial Resource Research Infrastructure) is a tool used within the European Union to build a pan-European platform to coordinate the access to individual resources (not only fungal) and promote the above-mentioned quality standards [7].

Besides the well-established fungal culture collections which can afford the requirements for the conformity to WFFC standards, many universities and small research centres all over the world have their own culture collections [8–10]. These strains can be consideredan important source of biological and genetic material because they are geographically widespread, and their contribution could be significantly representative of the biodiversity of local ecosystems [11,12]. These small collections could thus represent the initial stage in the development of an official collection accessible to the scientific community in the future. This would allow for comparisons among different species, or different strains belonging to the same species, that had been isolated from different substrates, environments or geographical areas. This type of information is often required because the biochemical differences between them could be relevant [13–16].

The Culture Collection of the University of Pavia (MicUNIPV) has its roots in the former Laboratory of Cryptogamic Botany (the first of its kind in Italy), founded in the 19th century by Santo Garovaglio. Nowadays, the Department of Earth and Environmental Sciences of the University of Pavia is an associated member of the MIRRI Italian Node. By keeping a multi-focus approach, the current Laboratory of Mycology has developed a wide collection of both micromycetes and macromycetes, among which there is a continuously increasing collection of wood decay species [2].

The isolation and study of wood decay species has a particularly strong cultural background in Asia, where the use of these fungi has a long tradition [17,18]. In the Italian landscape, only a few other culture collections have devoted part of their effort to wood decay fungi, namely: MUT—Mycotheca Universitatis Taurinensis; SAF—University of Palermo Mycotheca; PeruMyc—Department of Chemistry, Biology and Biotechnology, University of Perugia; AQUI—University of L'Aquila; ColD-Collection of DISTAV—University of Genova; and BUCC—Bologna University Culture Collection. This list may not be exhaustive, since wood decay fungi have been increasingly gaining the interest of researchers for different base and applied purposes (e.g., FBL—Fungal Biodiversity Lab, Sapienza University of Roma).

The aim of this work was to sample as many species as possible within lignicolous Basidiomycota from different environments in northern Italy in order to isolate fungal

strains useful for further studies and applications in different fields. A consequential goal of this work was to provide a detailed morphological description of each strain, which can support both applied and pure research.

2. Materials and Methods

2.1. Basidiomata Sampling

The fieldwork was carried out in different geographical areas of northern and central Italy (the Piemonte, Lombardia, Liguria and Lazio regions). In order to collect as many lignicolous species as possible, different environments were investigated (Table 1).

Table 1. Environments investigated for field sampling in northern Italy. Ecoregional sections and subsections, as in Blasi et al. [19].

Ecoregional Section	Ecoregional Subsection	General Description of the Environment	Main Plant Species
Central and Eastern Alps	Pre-Alps	coniferous mixed forests	*Pinus sylvestris, Quercus robur, Castanea sativa*
Central and Eastern Alps	Pre-Alps	thermophilous broadleaf forest	*Quercus pubescens, Cornus mas, Ostrya carpinifolia*
Central and Eastern Alps	Pre-Alps	fresh broadleaved forests	*Fagus sylvatica, Fraxinus excelsior, Carpinus betulus, Quercus robur, Robinia pseudoacacia*
Western Alps	North-Western Alps	mountain forest	*Picea abies, Abies alba, Larix decidua, Salix spp., Sorbus montanus* and *Alnus alnobetula*
Italian part of Ligurian-Provencal Province	Italian part of Ligurian-Provencal Province	Mediterranean scrub	*Quercus ilex, Myrtus communis, Pistacia lentiscus*
Central and Eastern Alps	Pre-Alps	urban and suburban environments (tree lined roads, parks, private and public gardens)	Not applicable

In order to obtain the greatest possible species diversity, some areas were more intensively sampled than others; therefore, the effort to collect and isolate strains was not the same for all sites [20].

2.2. Fungal Strains Isolation

In this study, the isolation effort was focused on dykariotic mycelia only; no isolation from basidiospores was attempted.

Only actively growing basidiomata were collected. Where possible, the cleanest samples were completely or partially harvested (depending on their size and local rarity) using a knife and touching them as little as possible.

The collected portion was placed into aluminum foil to keep it clean until the laboratory work.

In order to avoid destroying the basidiomata of *Fomitopsis officinalis*, its mycelium was directly isolated in the field using a sterilized scalpel and the flame of a lighter. This precaution was taken because this species, whose growth is very slow and mostly restricted to protected areas, was assessed as endangered by the Global Fungal Red List Initiative [21,22].

The protocol generally used to isolate the mycelia from wild basidiomata [23–25] was slightly modified according to Rush Wayn [26]. To isolate the fungal strains, Petri dishes of 90 mm diameter were prepared using 2% malt extract agar (MEA) with 6 mL L^{-1} of a solution of 3% hydrogen peroxide in order to reduce the spore germination of the contaminants.

Based on the references above, the classic withdrawal of a piece of context under sterile conditions was applied for thick-context species (with thicknesses greater than 2 mm). For treating thin-context species, the humid chamber method was applied. To establish a humid environment where the mycelium could regrow for a couple of days, the harvested basidiomata were placed at 10 °C in the dark inside small plastic boxes (humid chambers) on soaked paper. The fresh mycelium that developed in the humid chambers was transferred in sterile conditions under a laminar flow hood into the Petri dishes.

2.3. Basidiomata and Fungal Strains Identification

The identification of the collected basidiomata was carried out by macro- and micromorphological identification through dichotomous keys [27–33]. Microscopy was executed using a Paralux monocular microscope.

Furthermore, the main microscopic characteristics of the isolated mycelia were observed based on Stalpers et al. [23], with reference to colony colour, colony edge, mat morphology, clamps and the presence/absence of chlamidospores.

Besides the morphological investigations, the molecular identification of isolates was needed to confirm the species identity. Firstly, to produce a sufficient amount of dry biomass, each strain was put into a 200 mL Erlenmeyer flask containing 50 mL of 2% malt extract (ME) solution and grown for 10 days at 25 °C in the dark and in static. The biomass was then collected with forceps in sterility, placed in glass tubes at 18 °C and freeze- dried (Buchi lyovapor L-200). The DNA was extracted following the NucleoSpin Plant II protocol and then amplified by a Polymerase Chain Reaction (PCR) using the DreamTaq PCR Green Master Mix and the primer pair ITS1 and ITS4. The PCR was performed as follows: denaturation (95 °C) 5 min + 30 s; annealing (50 °C) 45 s; elongation (72 °C) 1 min. All the steps were repeated for 35 cycles, after which the final elongation (72 °C, 10 min)was carried out [2].

The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sent to Macrogen (The Netherlands) for sequencing. The obtained sequences were assembled, corrected and subsequently analysed by BLAST and Molecular ID searches by respectively using the GenBank (NCBI) [34] and MycoBank (CBS) [35] databases. The taxonomic assignments were based on similarity to reference sequences of these databases.

MycoBank [35] was used as the reference for the taxonomy and systematics.

2.4. Fungal Strain Conservation

The isolated strains were stored under different environments: storage in Petri dishes and tubes with 2% MEA at 4 °C; storage on colonised filter paper discs submerged in sterilised and demineralized water in water vials at 4 °C [36]; and cryopreservation at −80 °C.

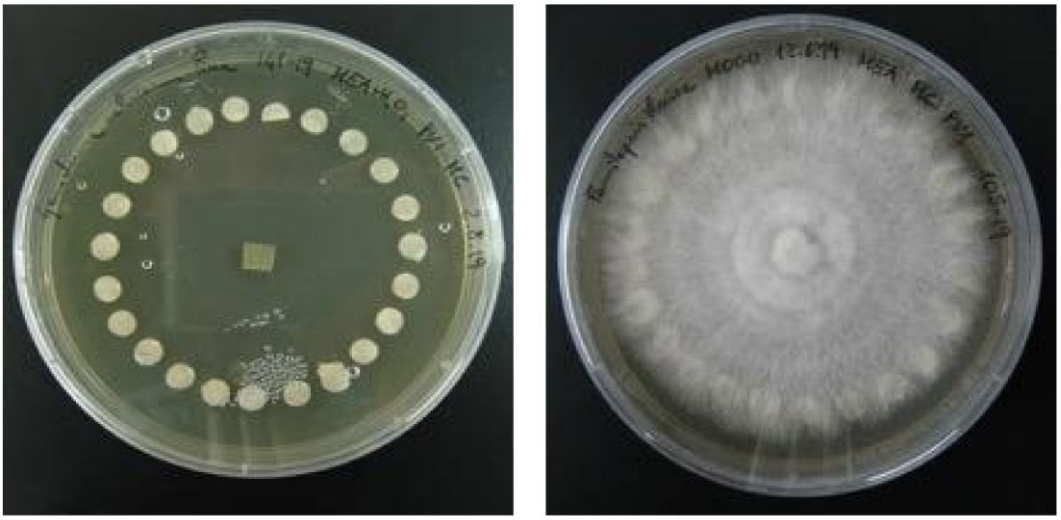
2.4.1. Conservation on Paper Discs

For the disc preservation, the sterilised paper filter discs of 5 mm diameter were placed into a 2% MEA Petri dish and consequently colonised by the growing mycelium (Figure 1a,b). The colonised discs were then moved in sterile polypropylene vials containing demineralized water and stored at 4 °C in the dark.

To verify the vitality of the strains, after 18 months, colonised discs were removed from the water under sterile conditions and back-cultured in the MEA Petri dishes at 25 °C in the dark. Analogous back-cultures were set up to test the vitality of the strains in the Petri dishes and tubes at 4 °C. For cultures kept at −80 °C, vitality was tested for random strains only.

2.4.2. Cryoconservation

The cryoconservation protocol has an initial step in which the strains are inoculated in a flask with a liquid medium (ME 2%). After 7 days, or after good mycelium production, the biomass can be stored. Operating under sterile conditions, the mycelium was withdrawn from the flask and placed in a 10 mL tube containing a 15% solution of glycerol. The mycelial suspension in the glycerol solution was then homogenised by vortexing at 3000 rpm for 30 s. Mycelium homogeneous cutting was obtained by adding broken microscopy cover slides which had previously been autoclaved. Then, 1 mL of the suspension was placed in1.5 mL sterile cryotubes. For each fungal strain, four replicates were stored at −80 °C.



(**a**) (**b**)

Figure 1. (**a**) Petri dish with paper discs before the mycelium growth; (**b**) the paper discs were colonised by the mycelium and can be moved to vials for storage.

All the strains are currently maintained in the research fungal collections of Mogu S.r.l (MRFC) and of the University of Pavia (MicUNIPV).

2.5. Morphological Description of Pure Cultures

The mycelia of each strain were described based on MEA cultures in 90 mm Petri dishes, incubated at 25 °C in the dark and checked every 48 h. The inoculum came from a 10-day-old mother colony and was placed at the edge of the plate to allow the colonyto expand over the whole dish diameter [37]. The radius was measured using a calliper(0.1 mm resolution). The growth rate (mm day^{-1}) was calculated for each strain on day 7 of growth and reported as the average of the three replicates. The uncertainty from random error (the absolute uncertainties of the individual measures) was calculated according to Harris [38].

Besides the basic visual inspection of macromorphology, the main micromorphological characters were examined on day 7 of growth (or day 15 for very slow growing strains) by a Zeiss Stemi 2000-C stereoscope and by a Nikon LABOPHOT-2 microscope. The mycelia were mounted in lactophenol cotton blue or lactophenol-acid fuchsin for optical microscopy.

3. Results

3.1. Basidiomata Sampling

The main purpose of this study was not to carry out a blind scan of different ecosystems but, instead, to precisely identify certain species known to grow in a particular habitat type or in a specific place. This was possible thanks to long-term data which have been collected and registered by a local mycological group concerned with mushroom species growing in the Varese province (Italy) since 1990 [39].

The principal types of habitats that constitute the landscapes in northern Italy, and those present in the province of Varese, were investigated during specific sampling campaigns. Fresh broadleaf forests are the most represented habitat, and 38 strains (40%) were isolated from the species collected there. Urban and suburban environments also contained many lignicolous species, leading to the isolation of 27 strains (28.4%) (Figure 2). Even if there are fewer trees in urban areas than in natural environments, many species of lignicolous basidiomycetes grow in urban settings. Trees in public parks, private gardens and along roads can host fungi as they are older and generally in poor health due to the low-quality growing conditions, over-pruning and wounds caused by cars or root cutting for excavations, etc. Furthermore, in these urban areas, the species of tree that usually grow in different environments can coexist.

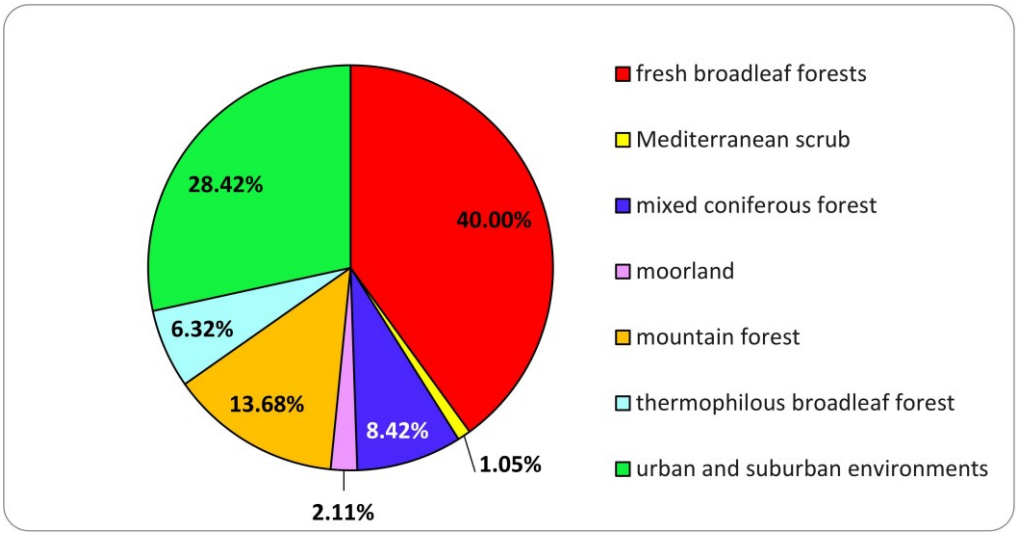


Figure 2. Distribution of the isolates among the explored habitats where the basidiomata originated.

In total, 26 genera of trees on which the fungal species were growing could be identified (Figure 3). In particular, the majority of the collected basidiomata were growing on *Quercus* spp. (12%).

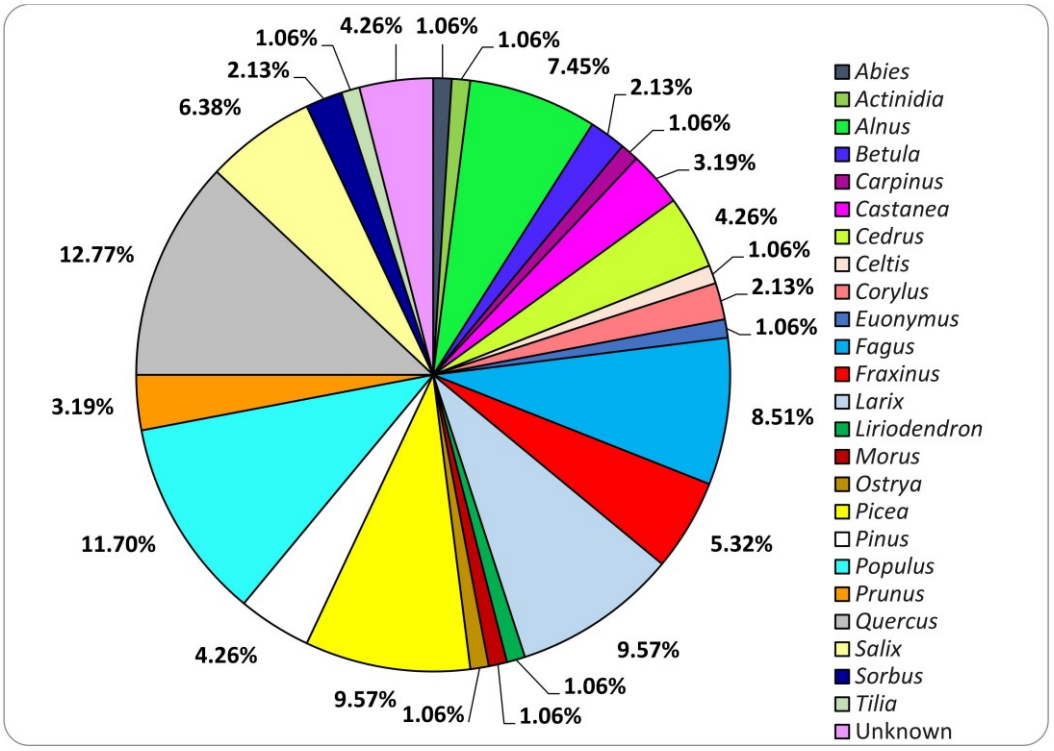


Figure 3. Tree genera hosting the original basidiomata from which the strains were isolated.

3.2. Fungal Strains Collection

The mycelium in pure culture has been successfully isolated from 96 out of the 103 basidiomata collected (93.2%). The molecular confirmation of the morphological identification showed that all the isolated strains belong to *Agaricomycetes*, namely, 76 different species from 51 genera, 18 families and 5 orders (*Agaricales*, *Gloeophyllales*, *Hymenochaetales*, *Polyporales* and *Russulales*) (Table 2).

Table 2. Taxonomy of the isolated strains and reference to the code within the Mogu S.r.l research fungal collection (MRFC). Taxonomy relies on MycoBank [35].

Order	Family	Species	MRFC Code
Agaricales	*Mycenaceae*	*Panellus stipticus* (Bull.) P. Karst.	183-21
	Strophariaceae	*Cyclocybe cylindracea* (DC.) Vizzini & Angelini	187-21
Gloeophyllales	*Gloeophyllaceae*	*Gloeophyllum odoratum* (Wulfen) Imazeki	077-18
		Neolentinus lepideus (Fr.) Redhead & Ginns	132-19
		Neolentinus schaefferi (Weinm.)	190-21
Hymenochaetales	*Hymenochaetaceae*	*Fomitiporia mediterranea* M. Fisch.	079-18
		Fomitiporia mediterranea M. Fisch.	082-19
		Fuscoporia contigua (Pers.) G. Cunn.	085-19
		Fuscoporia contigua (Pers.) G. Cunn.	130-19
		Fuscoporia torulosa (Pers.) T. Wagner & M. Fisch.	063-18
		Inonotus radiatus (Sowerby) P. Karst.	053-18
		Phylloporia ribis (Schumach.) Ryvarden	049-18
Polyporales	*Dacryobolaceae*	*Postia tephroleuca* (Fr.) Julich	211-21
	Fomitopsidaceae	*Antrodia* sp.	074-18
		Antrodia cfr. alpina (Litsch.) Gilb. & Ryvarden	134-19
		Cyanosporus alni Niemelä & Vampola	071-18
		Daedalea quercina (L.) Pers.	089-19
		Flavidoporia pulvinascens (Pilát) Audet	193-21
		Fomitopsis betulina (Bull.) B.K. Cui, M.L. Han & Y.C. Dai	042-18
		Fomitopsis iberica Melo & Ryvarden	004-18
		Fomitopsis iberica Melo & Ryvarden	104-19
		Fomitopsis officinalis (Vill.) Bondartsev & Singer	143-19
		Fomitopsis pinicola (Sw.) P. Karst.	087-19
		Fomitopsis pinicola (Sw.) P. Karst.	117-19
		Fomitopsis pinicola (Sw.) P. Karst.	124-19
		Neoantrodia serialis (Fr.) Audet	111-19
		Niveoporofomes spraguei (Berk. & M.A. Curtis) B.K. Cui, M.L. Han & Y.C. Dai	156-19
		Osteina obducta (Berk.) Donk	147-19
		Osteina undosa (Peck) B.K. Cui, L.L. Shen & Y.C. Dai	162-19
	Grifolaceae	*Grifola frondosa* (Dicks.) Grey.	210-21
	Incrustoporiaceae	*Skeletocutis amorpha* (Fr.) Kotl. & Pouzar	171-19
		Tyromyces chioneus (Fr.) P. Karst.	158-19
	Irpicaceae	*Irpex lacteus* (Fr.) Fr.	076-18
		Irpex lacteus (Fr.) Fr.	160-19
		Irpex latemarginatus (Durieu & Mont.) C.C. Chen & Sheng H. Wu	109-19
	Ischnodermataceae	*Iscnoderma benzoinum* (Wahlenb.) P. Karst.	195-21

Table 2. *Cont.*

Order	Family	Species	MRFC Code
Polyporales	*Laetiporaceae*	*Laetiporus sulphureus* (Bull.) Murrill	188-21
		Phaeolus schweinitzii (Fr.) Pat.	136-19
	Meruliaceae	*Abortiporus biennis* (Bull.) Singer	064-18
		Bjerkandera adusta (Willd.) P. Karst.	101-19
		Vitreoporus dichrous (Fr.) Zmitr.	083-19
		Phlebia rufa (Pers.) M.P. Christ.	186-21
	Phanerochaetaceae	*Antrodiella faginea* Vampola & Pouzar	169-19
		Porostereum spadiceum (Pers.) Hjortstam & Ryvarden	102-19
		Terana caerulea (Schrad. ex Lam.) Kuntze	177-19
	Polyporaceae	*Cerrena unicolor* (Bull.) Murrill	145-19
		Coriolopsis gallica (Fr.) Ryvarden	086-19
		Coriolopsis trogii (Berk.) Domanski	027-18
		Daedaleopsis confragosa (Bolton) J. Schröt.	155-19
		Daedaleopsis tricolor (Bull.) Bondartsev & Singer	028-18
		Daedaleopsis tricolor (Bull.) Bondartsev & Singer	148-19
		Dichomitus campestris (Quél.) Domanski & Orlicz	168-19
		Dichomitus squalens (P. Karst.) D.A. Reid	012-18
		Fomes fomentarius (L.) Fr.	066-18
		Fomes fomentarius (L.) Fr.	091-19
		Fomes fomentarius (L.) Fr.	179-19
		Ganoderma adspersum (Schulzer) Donk	106-19
		Ganoderma adspersum (Schulzer) Donk	007-18
		Ganoderma adspersum (Schulzer) Donk	036-18
		Ganoderma adspersum (Schulzer) Donk	097-19
		Ganoderma adspersum (Schulzer) Donk	112-19
		Ganoderma applanatum (Pers.) Pat.	045-18
		Ganoderma carnosum Pat.	161-19
		Ganoderma carnosum Pat.	191-21
		Ganoderma lucidum (Curtis) P. Karst.	037-19
		Ganoderma lucidum (Curtis) P. Karst.	137-19
		Ganoderma resinaceum Boud.	046-18
		Ganoderma resinaceum Boud.	120-19
		Ganoderma resinaceum Boud.	209-21
		Ganoderma valesiacum Boud.	196-21
		Irpiciporus pachyodon (Pers.) Kotl. & Pouzar	175-19
		Lenzites betulinus (L.) Fr.	088-19
		Perenniporia fraxinea (Bull.) Ryvarden	122-19
		Picipes melanopus (Pers.) Zmitr. & Kovalenko	159-19
		Polyporus alveolaris (DC.) Bondartsev & Singer	096-19
		Polyporus badius (Pers.) Schwein.	093-19
		Polyporus corylinus Mauri	192-21

Table 2. *Cont.*

Order	Family	Species	MRFC Code
Polyporales	*Polyporaceae*	*Polyporus squamosus* (Huds.) Fr.	094-19
		Pycnoporus cinnabarinus (Jacq.) P. Karst.	174-19
		Sarcoporia polyspora P. Karst.	172-19
		Trametes gibbosa (Pers.) Fr.	054-18
		Trametes hirsuta (Wulfen) Pilát	067-18
		Trametes hirsuta (Wulfen) Pilát	144-19
		Trametes suaveolens (L.) Fr.	061-18
		Trametes suaveolens (L.) Fr.	070-18
		Trametes versicolor (L.) Lloyd	139-19
		Trichaptum abietinum (Pers. ex J.F. Gmel.) Ryvarden	133-19
		Truncospora atlantica Spirin & Vlasák	078-18
		Yuchengia narymica (Pilát) B.K. Cui, C.L. Zhao & Steffen	176-19
Russulales	*Bondarzewiaceae*	*Heterobasidion abietinum* Niemelä & Korhonen	069-18
		Heterobasidion annosum (Fr.) Bref.	065-18
	Hericiaceae	*Laxitextum bicolor* (Pers.) Lentz	166-19
	Peniophoraceae	*Peniophora quercina* (Pers.) Cooke	090-19
	Stereaceae	*Stereum hirsutum* (Willd.) Pers.	073-18
		Stereum sanguinolentum (Alb. & Schwein.) Fr.	127-19

As reported above, MycoBank [35] was used as the only reference in this study. However, in comparison with Index Fungorum [40] and part of the literature, nomenclaturalissues are still being debated, mainly for the following species: *Polyporus badius* (Pers.) Schwein., also known as *Picipes badius* (Pers.) Zmitr. & Kovalenko, and *Polyporus squamosus* (Huds.) Fr., also known as *Cerioporus squamosus* (Huds.) Quél according to Mycobank and Bernicchia & Gorjon (2020) [28].

The family of *Polyporaceae* is the most represented in the collection: almost 50% of the isolated species belong to it (Figure 4). For orders, *Polyporales* is by far the most represented: 55.6% of the isolated families belong to it (Figure 5).

Among all of the species listed in Table 2, 80% are considered white rot agents, while 20% are brown rot.

3.3. Basidiomata and Fungal Strains Identification

The molecular identification of the strains confirmed the morphological identification of the collected basidiomata and disentangled the uncertain identity of poorly differentiated samples (primordia of *Fomitiporia contigua* 085-19, *Ganoderma adspersum* 007-18, *Daedaleopsis tricolor* 028-18) or species that are very similar to each other (*Fomitiporia mediterranea*, *Cyanosporus alni*, *Postia tephroleuca*, *Dichomitus squalens*, *Porostereum spadiceum*).

The identifications of two strains (*Antrodia* sp. and *Antrodia cfr. alpina*) were still uncertain, and these could not be unequivocally identified. In particular, the basidioma macrocharacteristics and the host tree suggest that the strain was *A. alpina* (e.g., its change to red in KOH and its growth on *Larix decidua*), but the molecular analysis on the ITS region does not exclude *A. xantha*. Therefore, the strain 134-19 is referred to as *Antrodia cfr. alpina*. Further molecular markers (e.g., factor 1-α and LSU) are needed to confirm the identities of these strains [41].

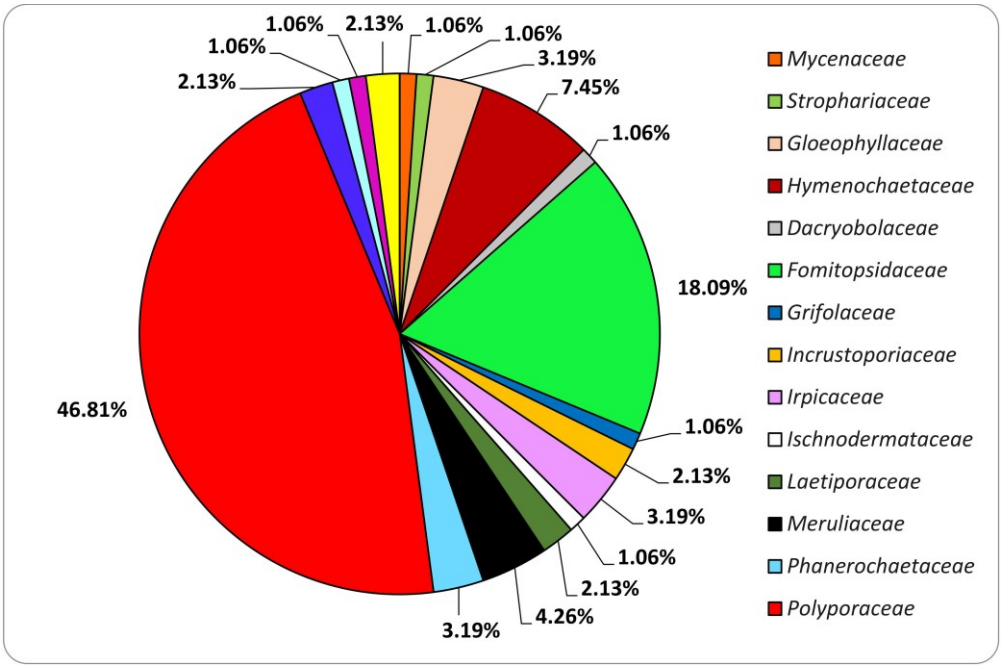


Figure 4. Families represented in the set of isolated strains included in this study.

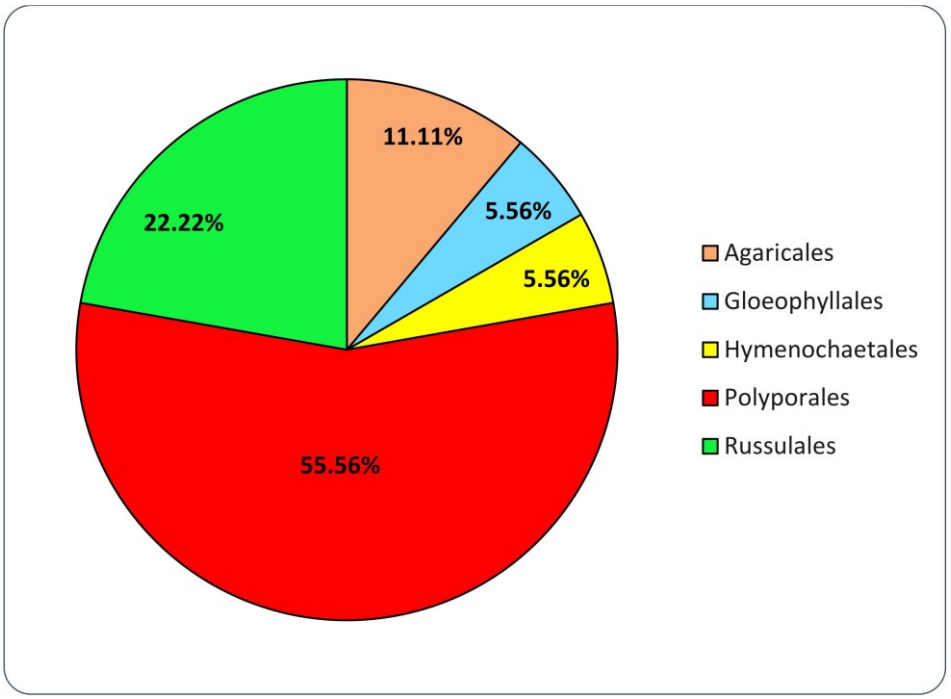


Figure 5. Orders represented in the set of isolated strains included in this study.

The molecular analysis was important for *Fomitiporia mediterranea*, which can only be distinguished from *Fomitiporia punctata* using ITS sequences, since all the microscopical and macroscopical elements are the same. MycoBank reported the *Laetiporus* genus in *Fomitopsidaceae* because *Laetiporaceae* is considered invalid. Nevertheless, *Phaeolus* is placed in *Laetiporaceae*. We decided to follow Justo et al. (2017) [42] and consider *Laetiporaceae* Julic 1989 [33] valid, so *Laetiporus* could be included in this family along with *Phaeolus*.

The genus *Ganoderma* P. Karst. is a taxonomical group whose strains are among the most difficult to distinguish [27]. Nevertheless, the reported strains in Table 2 have been unequivocally identified. In particular, the morphological discrimination between *G. applanatum* and *G. adspersum* basidiomata could be difficult if the collected specimensare too young. However, *G. adspersum* could be identified from mycelium layers among tube

strates, but if the basidioma is less than two years old, this feature cannot be observed. In this case, molecular analyses are always required to be sure of their exact identification. *Ganoderma resinaceum,* when grown on *Salix* or *Alnus* close to water, sometimes has long stipes and presents a thinner context when compared to the specimens usually growing on *Quercus* spp. This can mean that it resembles *G. lucidum* morphologically, but these two species are well separated by molecular analysis. On the other hand, for the identification of *G. carnosum*, it was more critical to use molecular methods over morphological identification because different but equally supported (>97%) identification alternatives were produced by the comparative analysis of the ITS sequences in both Mycobank [35] and NCBI [34].The molecular analysis failed to discriminate the isolated strain from *Ganoderma valesiacum* (which has a white context and grows on *Larix decidua* only) as well as from *Ganoderma oregonense* and *Ganoderma tsugae*, (two North American species) [43,44]. In addition, *G. valesiacum* and *G. carnosum* present quite different mycelia on MEA: the first pigments quite quickly and forms a thin layer of hyphae, whereas the second forms a white, thicker and faster growing mycelium. Further studies are ongoing to clarify whether they can be treated as a single species or whether they should be considered different entities

Finally, for *Cyclocybe cylindracea*, the identification of the taxonomic situation is still uncertain. As shown in Vizzini et al. [45], two well supported clades exist. Furthermore, two names are accepted: *Cyclocybe aegerita* (V. Brig.) Vizzini and *Cyclocybe cylindracea* (DC.) Angelini & Vizzini, but no typus is assigned to them (according to personal communication with Vizzini). It is probable that the two accepted names will be assigned to the two existing clades of this collective species.

As it can be observed in Table 2, only a small number of corticioid strains have been isolated, even though they are abundant and occur frequently in nature. It is particularly difficult to isolate them properly since they not only have a very thin context but are also rarely found clean and actively growing. Among the corticioid species, the mycelia of *Terana caerulea* and *Porostereum spadiceum* were isolated with success. The basidiomata of these two species are quite common in nature, but they are thin and close to the ground, so the strains in pure culture are not so common. It is difficult to maintain these two species on artificial media. Storage using paper-filter disks at 4 °C has proven to be effective, as the two species were able to regrow after 18 months of storage.

Among the isolated strains, *Dichomitus squalens*, *Fomitopsis iberica*, *Niveoporofomes spraguei*, *Ganoderma carnosum*, *Ganoderma valesiacum*, *Fomitopsis officinalis*, *Polyporus corylinus* and *Sarcoporia polyspora* are considered uncommon or rare species with a scattered distribu-tion, at least in Italy, as reported in the *Checklist of Italian fungi—Basidiomycota* [46] and in Bernicchia & Gorjon [29].

Some species are host-specific, such as *G. valesiacum* and *F. officinalis*, which are strictly associated with *Larix decidua*. On the contrary, other strains, even if not common, showed a very large spectrum of hosts: in particular, *F. iberica* could grow on both angiosperms and gymnosperms. Notably, this species was found exclusively in urban parks. Other species that grow preferably in urban areas are *F. mediterranea*, *P. fraxinea*, *G. adspersum* and *G. resinaceum*. *Ganoderma carnosum*, which usually grows in *Abies alba* forests, was found in two different public parks on decayed coniferous stumps.

Due to the decision to isolate the mycelia from fresh and actively growing basidiomata, only a small number of collected samples could not be isolated: *Fistulina hepatica, Pleurotus dryinus, Serpula himantioides, Meruliopsis taxicola, Dendropolyporus umbellatus*, *Rigidoporus sanguinolentus, Neofavolus suavissimus*, *Favolaschia calocera* and *Hericium cirrhatum*. This was because molds or bacterial contaminations were always present, overgrowing the target mycelium even with the addition of hydrogen peroxide.

3.4. Morphological Description of Pure Cultures

The main characteristics of the mycelia in pure culture are reported in Table 3.

Table 3. Morphological description of the strains in pure culture and the average growth rate of the three replicates calculated at 7 days after inoculation (absolute uncertainty from random error ± 0.17 mm).

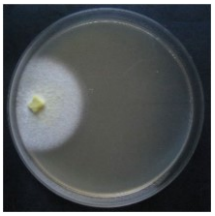
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Abortiporus biennis (Bull.) Singer	064-18	Colony colour white. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony. Aerial mycelium < 1.5–5 µm wide, submerged mycelium < 1.5–3 µm wide. Clamps present. Chlamydospores present.	8.1		[23]
Antrodia alpina (Litsch.) Gilb. & Ryvarden	134-19	Colony colour white to yellowish-ochraceous. Reverse darkened. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat downy-felty. Aerial mycelium 1.5–3 µm wide, submerged mycelium < 1.5–3 µm wide. Clamps absent. Anastomosis present.	0.7		
Antrodiella faginea Vampola & Pouzar	169-19	Colony uncoloured to white. Reverse bleached. Colony edge submerged to appressed, edge line fringed, marginal hyphae fimbriate. Mat farinaceous-velvety. Aerial mycelium 1.5–3 µm wide, submerged mycelium < 1.5–3 µm wide. Clamps absent.	1.4		
Bjerkandera adusta (Willd.) P. Karst.	101-19	Colony white. Reverse bleached. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat cottony-woolly-floccose. Aerial mycelium 3–5 µm wide. Clamps present.	10.3		[23]
Cerrena unicolor	145-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose. Aerial mycelium 1.5–3 µm wide, submerged mycelium 3–5 µm wide. Clamps absent.	8.3		[23]
Coriolopsis gallica (Fr.) Ryvarden	086-19	Colony uncoloured to white. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat felty. Aerial mycelium < 1.5–3 µm wide. Clamps present.	4.9		[23]

Table 3. *Cont.*

Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Coriolopsis trogii (Berk.) Domanski	027-18	Colony colour white to cream. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat silky to felty. Aerial mycelium < 1.5–5 µm wide, submerged mycelium < 1.5–5 µm wide. Clamps present.	4.8		[23]
Cyanosporus alni Niemelä & Vampola	071-18	Colony uncoloured. Colony edge appressed to raised, edge line compact, marginal hyphae dense. Mat downy. Submerged mycelium 1.5–3 µm wide. Clamps absent.	0.3		
Cyclocybe cylindracea (DC.) Vizzini & Angelini	187-21	Colony white to brownish. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat silky-woolly. Aerial mycelium < 1.5–5 µm wide. Clamps present.	3.3		[47]
Daedalea quercina	089-19	Colony white to cream. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat felty. Aerial mycelium < 1.5–3 µm wide. Clamps present.	3.0		[23,48]
Daedaleopsis confragosa (Bolton) J. Schröt.	155-19	Colony white to brown. Reverse darkened. Colony edge appressed to raised, edge line compact, marginal hyphae dense. Mat woolly-floccose, zonated. Aerial mycelium 1.5–3 µm wide, submerged mycelium 3–5 µm wide. Clamps present.	3.1		[23,48,49]
Daedaleopsis tricolor (Bull.) Bondartsev & Singer	148-19	Colony white to brown. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony. Aerial mycelium 1.5–3 µm wide. Clamps absent.	5.2		[49]
Dichomitus campestris (Quél.) Domanski & Orlicz	168-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat downy, zonated. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–7.5 µm wide. Clamps present.	1.9		

Table 3. *Cont.*

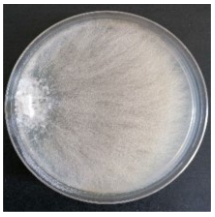
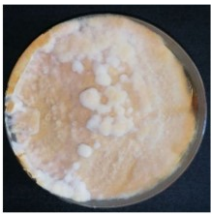
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Dichomitus squalens (P. Karst.) D.A. Reid	012-18	Colony white-cream. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat floccose-felty. Aerial mycelium 1.5–3 µm wide. Clamps present. Chlamydospores present.	5.4		[23]
Flavidoporia pulvinascens (Pilát) Audet	193-21	Colony uncoloured to white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat woolly. Aerial mycelium 3–7.5 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	0.7		
Fomes fomentarius (L.) Fr.	179-19	Colony white. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat woolly-felty. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3. Clamps present.	6.6		[23,48–50]
Fomitiporia mediterranea M. Fisch.	079-18	Colony yellowish-ochraceous. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat cottony, zonated. Aerial mycelium < 1.5–3 µm wide, submerged mycelium 3–5. Clamps absent. Hyphae with some oil drops.	2.30		[51]
Fomitopsis betulina (Bull.) B.K. Cui, M.L. Han & Y.C. Dai	042-18	Colony white. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose. Aerial mycelium 1.5–5 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	4.4		[23,48]
Fomitopsis iberica Melo & Ryvarden	104-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat silky-floccose. Aerial mycelium < 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present. Hyphae with some oil drops. Chlamidospores present, but rare.	6.0		

Table 3. *Cont.*

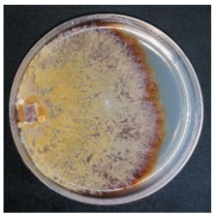
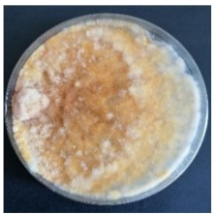
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Fomitopsis officinalis (Vill.) Bondartsev & Singer	143-19	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose-plumose. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present. Chlamydospores present.	1.3		[23,48]
Fomitopsis pinicola (Sw.) P. Karst.	117-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose. Aerial mycelium < 1.5–5 µm wide. Clamps present.	3.5		[23,48–50]
Fuscoporia contigua (Pers.) G. Cunn.	085-19	Colony brownish. Colony edge appressed, edge line compact, marginal hyphae dense. Mat silky-crustose. Aerial mycelium 1.5–5 µm wide. Clamps absent.	0.9		[23]
Fuscoporia torulosa (Pers.) T. Wagner & M. Fisch.	063-18	Colony yellowish. Reverse darkened. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony. Aerial mycelium 1.5–5 µm wide, submerged mycelium 1.5–5 µm wide. Clamps absent. Mycelium with skeletal hyphae.	0.3		[23]
Ganoderma adspersum (Schulzer) Donk	036-18	Colony white to yellow-ochraceous. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat downy. Aerial mycelium < 1.5–5 µm wide. Clamps present. Mycelium with skeletal hyphae and oil drops.	3.7		[23,50]
Ganoderma applanatum (Pers.) Pat.	045-18	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat silky-velvety-crustose. Aerial mycelium 3–5 µm wide. Clamps present. Saline crystals present.	4.8		[23,48,50]

Table 3. *Cont.*

Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Ganoderma carnosum Pat.	161-19	Colony white to yellowish-ochraceous. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony-floccose-crustose. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present. Saline crystals present.	4.8		
Ganoderma lucidum (Curtis) P. Karst.	137-	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony. Submerged mycelium 1.5–3 µm wide. Clamps present.	2.6		[23,48,50,52,53]
Ganoderma resinaceum Boud.	046-18	Colony white. Reverse bleached. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat farinaceous. Aerial mycelium < 1.5–3 µm wide, submerged mycelium < 1.5–3 µm wide. Clamps present. Chlamidospores abundant. Anastomosis present.	7.5		[23,50]
Ganoderma valesiacum Boud.	196-21	Colony white-ochraceous. Colony edge appressed, edge line fringed to bayed, marginal hyphae fimbriate. Mat downy-farinaceous. Aerial mycelium <1.5–5 µm wide, submerged mycelium <1.5–3 µm wide. Clamps present.	0.8		
Gloeophyllum odoratum (Wulfen) Imazeki	077-18	Colony white-cream. Reverse darkened. Colony edge appressed-raised, edge line fringed, marginal hyphae fimbriate. Mat downy-woolly. Aerial mycelium 1.5–5 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	0.2		[23]
Grifola frondosa (Dicks.) Grey.	210-21	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat cottony-woolly. Aerial mycelium < 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	3.2		[23,48,53]

Table 3. *Cont.*

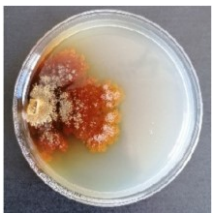
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Heterobasidion abietinum Niemelä & Korhonen	069-18	Colony white-yellowish. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat farinaceous-granular to floccose. Aerial mycelium 1.5–7.5 µm wide. Clamps absent. Chlamidospores present. Numerous basidia.	2.4		
Heterobasidion annosum (Fr.) Bref.	065-18	Colony white to yellow-ochraceous. Colony edge submerged, edge line fringed, marginal hyphae fimbriate. Mat silky-floccose. Aerial mycelium < 1.5–5 µm wide, submerged mycelium < 1.5–7.5 µm wide. Clamps absent. Chlamidospores present.	1.3		[23,48]
Inonotus radiatus (Sowerby) P. Karst.	053-18	Colony brownish. Colony edge submerged, edge line fringed, marginal hyphae bayed. Mat downy. Aerial mycelium 1.5–5 µm wide, submerged mycelium 1.5–3 µm wide. Clamps absent. Hyphae with oil drops.	1.1		[23,48]
Irpex lacteus (Fr.) Fr.	076-18	Colony white. Reverse bleached. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat silky-woolly. Aerial mycelium 1.5–7.5 µm wide, submerged mycelium 1.5–7.5 µm wide. Clamps absent. Mycelium with skeletal hyphae.	8.0		[23]
Irpex latemarginatus Durieu & Mont.	109-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat cottony to floccose. Aerial mycelium 1.5–3 µm wide, submerged mycelium 3–5 µm wide. Clamps absent. Hyphae with oil drops. Anastomosis present.	9.8		[23]
Irpiciporus pachyodon (Pers.) Kotl. & Pouzar	175-19	Colony white. Reverse bleached. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat cottony to floccose. Aerial mycelium < 1.5–5 µm wide. Clamps present. Skeletal hyphae present. Chlamidospores present.	4.5		[23]

Table 3. *Cont.*

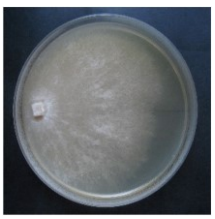
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Ischnoderma benzoinum (Wahlenb.) P. Karst.	195-21	Colony uncoloured to white. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate-bayed. Mat downy-velvety. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	3.3		[23]
Laetiporus sulphureus (Bull.) Murrill	188-21	Colony cream. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate. Mat farinaceous-granular-floccose. Aerial mycelium 1.5–5 µm wide, submerged mycelium 5–7 µm wide. Chlamydospores abundant.	2.6		[23,48,53]
Laxitextum bicolor (Pers.) Lentz	166-19	Colony white-cream. Reverse darkened. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose to farinaceous. Aerial mycelium 1.5–3 µm wide, submerged mycelium < 1.5–7 µm wide. Clamps present. Mycelium with skeletal hyphae.	3.4		[23]
Lenzites betulinus (L.) Fr.	088-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat cottony-floccose to felty. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	3.1		[23,48,54]
Neoantrodia serialis (Fr.) Audet	111-19	Colony uncoloured to white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat downy-cottony-floccose. Aerial mycelium < 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps absent. Saline crystals present. Hyphae with some oil drops.	2.3		[23,48]
Neolentinus lepideus (Fr.) Redhead & Ginns	132-19	Colony white. Reverse darkened. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate. Mat cottony-woolly-felty. Aerial mycelium 1.5–3 µm wide, submerged mycelium < 1.5 µm wide. Clamps present.	3.3		[48]

Table 3. *Cont.*

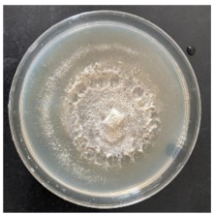
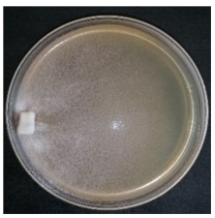
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Neolentinus schaefferi (Weinm.) Redhead & Ginns	190-21	Colony white. Reverse darkened. Colony edge appressed to raised, edge line even, marginal hyphae dense. Mat farinaceous. Aerial mycelium < 1.5–3 µm wide, sumberged mycelium 1.5–3 µm wide. Clamps present. Chlamydospores present. Hyphae with oil drops.	2.5		
Niveoporofomes spraguei (Berk. & M.A. Curtis) B.K. Cui, M.L. Han & Y.C. Dai	156-19	Colony white. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat cottony-floccose. Aerial mycelium 1.5–3 µm wide, submerged mycelium 3–5 µm wide. Clamps present but scarce. Chlamidospores abundant.	3.1		
Osteina obducta (Berk.) Donk	147-19	Colony uncoloured to slight brownish. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat absent to downy. Aerial mycelium < 1.5–7 µm wide, submerged mycelium < 1.5–3 µm wide. Clamps present.	0.3		[23]
Osteina undosa (Peck) B.K. Cui, L.L. Shen & Y.C. Dai	162-19	Colony uncoloured. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat downy. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present.	0.2		
Panellus stipticus (Bull.) P. Karst.	183-21	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat downy-cottony-floccose. Aerial mycelium < 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps absent. Saline crystals present. Hyphae with oil drops.	1.5		
Peniophora quercina (Pers.) Cooke	090-19	Colony white. Reverse bleached. Colony edge submerged-appressed, edge line fringed, marginal hyphae fimbriate. Mat downy to cottony-woolly-floccose. Aerial mycelium 3–5 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present. Saline crystals present.	4.0		[23]

Table 3. *Cont.*

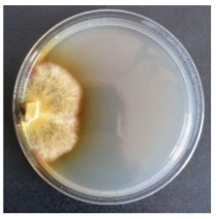
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Perenniporia fraxinea (Bull.) Ryvarden	122-19	Colony white, with a pink gradient. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony-felty. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present. Chlamidospores present.	3.4		[23,48]
Phaeolus schweinitzii (Fr.) Pat.	136-19	Colony yellowish-ochraceous. Reverse darkened. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose. Aerial mycelium 1.5–5 µm wide. Clamps present.	2.4		[23,48]
Phlebia rufa (Pers.) M.P. Christ.	186-21	Colony white-cream. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose-plumose. Aerial mycelium 1.5–3 µm wide. Clamps present. Saline crystals present.	8.5		[23]
Phylloporia ribis (Schumach.) Ryvarden	049-18	Colony yellow-ochraceous. Reverse darkened. Colony edge raised, edge line compact, marginal hyphae fimbriate. Mat cottony. Aerial mycelium 1.5–3 µm wide. Clamps absent. Anastomosis present.	0.1		[23]
Picipes melanopus (Pers.) Zmitr. & Kovalenko	159-19	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony-woolly-floccose. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–5 µm wide. Clamps present. Saline crystals present.	0.7		[23]
Polyporus alveolaris (DC.) Bondartsev & Singer	096-19	Colony white. Reverse bleached. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate. Mat cottony-woolly. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps present. Saline crystals present.	4.3		[23,48]

Table 3. *Cont.*

Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Polyporus badius (Pers.) Schwein.	093-19	Colony white. Colony edge appressed to submerged, edge line fringed, marginal hyphae dense. Mat downy to cottony-woolly. Aerial mycelium 1.5–3 µm wide, submerged mycelium 1.5–3 µm wide. Clamps absent.	3.0		[23]
Polyporus corylinus Mauri	192-21	Colony white. Reverse bleached. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat felty. Aerial mycelium < 1.5–3 µm wide, submerged mycelium < 1.5–3 µm wide. Clamps present. Chlamidospores abundant.	4.9		
Polyporus squamosus (Huds.) Fr.	094-19	Colony white to brownish. Reverse bleached. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat farinaceous-floccose. Aerial mycelium 3–5 µm wide, submerged mycelium 1.5–5 µm wide. Clamps present.	1.1		[23,48,55]
Porostereum spadiceum (Pers.) Hjortstam & Ryvarden	102-19	Colony white. Reverse bleached. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat plumose. Aerial mycelium <1.5–3 µm wide, submerged mycelium <1.5–3 µm wide. Clamps present.	6.0		
Postia tephroleuca (Fr.) Julich	211-21	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate to slighty bayed. Mat farinaceous-felty-velvety. Aerial mycelium 1.5–5 µm wide, sumberged mycelium 1.5–5 µm wide. Clamps present. Saline crystals present.	1.2		[23]
Pycnoporus cinnabarinus (Jacq.) P. Karst.	174-19	Colony white to orange-reddish. Reverse bleached. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate. Mat woolly-velvety. Aerial mycelium 1.5–3 µm wide, sumberged mycelium 1.5–3 µm wide. Clamps present.	4.3		[23,48]

Table 3. *Cont.*

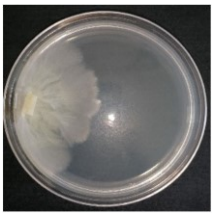
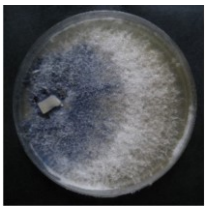
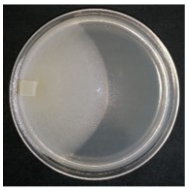
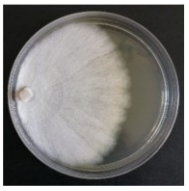
Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Sarcoporia polyspora P. Karst.	172-19	Colony uncoloured to whitish. Colony edge appressed, edge line compact-bayed, marginal hyphae dense. Mat silky. Aerial mycelium 3–5 µm wide, sumberged mycelium 1.5–3 µm wide. Clamps present.	0.3		
Skeletocutis amorpha (Fr.) Kotl. & Pouzar	171-19	Colony uncoloured. Colony edge appressed, edge line fringed-bayed, marginal hyphae fimbriate. Mat downy. Sumberged mycelium 5–7.5 µm wide. Clamps absent.	0.2		[23,48]
Stereum hirsutum (Willd.) Pers.	073-18	Colony white to orange. Colony edge raised, edge line fringed, marginal hyphae fimbriate. Mat cottony-floccose. Aerial mycelium < 1.5–7.5 µm wide, sumberged mycelium < 1.5 µm wide. Clamps present. Skeletal hyphae present.	9.6		[23,49,55]
Stereum sanguinolentum (Alb. & Schwein.) Fr.	127-19	Colony white to ochraceous. Colony edge raised, edge line fringed, marginal hyphae fimbriate-bayed. Mat downy. Sumberged mycelium 1.5–3 µm wide. Clamps rare.	0.9		[23,48]
Terana caerulea (Schrad. ex Lam.) Kuntze	177-19	Colony white to blue. Reverse bleached. Colony edge appressed to submerged, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose. Aerial mycelium 3–7.5 µm wide. Clamps present.	4.3		
Trametes gibbosa (Pers.) Fr.	054-18	Colony white. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat floccose. Aerial mycelium < 1.5–3 µm wide, sumberged mycelium < 1.5–3 µm wide. Clamps present.	6.6		[23]
Trametes hirsuta (Wulfen) Pilát	067-	Colony white to cream. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat woolly-floccose. Aerial mycelium < 1.5–5 µm wide. Clamps present.	4.7		[23,48,55]

Table 3. *Cont.*

Species	MRFC Code	Mycelium Description	Average Growth Rate (mm day^{-1})	Colony Morphology on MEA (2%)	References
Trametes suaveolens (L.) Fr.	070-18	Colony white. Reverse bleached. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony-woolly-floccose. Aerial mycelium 1.5–3 µm wide. Clamps present.	7.5		[23,48]
Trametes versicolor (L.) Lloyd	139-19	Colony white. Reverse bleached. Colony edge appressed to raised, edge line fringed, marginal hyphae fimbriate. Mat downy-floccose. Aerial mycelium < 1.5–3 µm wide, submerged mycelium < 1.5. Clamps present.	6.9		[23,48,55]
Trichaptum abietinum (Pers. ex J.F. Gmel.) Ryvarden	133-19	Colony uncoloured. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony. Aerial mycelium < 1.5–5 µm wide, sumberged mycelium < 1.5–3 µm wide. Clamps present. Chlamydospores present.	0.9		
Truncospora atlantica Spirin & Vlasák	078-18	Colony white. Colony edge raised, edge line even, marginal hyphae dense. Mat farinaceous. Aerial mycelium < 1.5–3 µm wide, sumberged mycelium 1.5–3 µm wide. Clamps present. Chlamydospores present. Hyphae with oil drops.	0.8		
Tyromyces chioneus (Fr.) P. Karst.	158-19	Colony white. Colony edge appressed, edge line fringed, marginal hyphae fimbriate. Mat cottony-floccose. Aerial mycelium 1.5–5 µm wide, sumberged mycelium 1.5–3 µm wide. Clamps present.	2.7		
Yuchengia narymica (Pilát) B.K. Cui, C.L. Zhao & Steffen	176-19	Colony white. Reverse bleached. Colony edge appressed to raised, edge line fringed. Mat downy- farinaceous-granular to floccose-plumose. Aerial mycelium 1.5–5 µm wide, sumberged mycelium 1.5–3 µm wide. Clamps absent. Chlamydospores abundant.	2.0		
Vitreoporus dichrous (Fr.) Zmitr.	083-19	Colony uncoloured. Colony edge submerged, edge line bayed, marginal hyphae fimbriate. Mat sumberged. Submerged mycelium 1.5–3 µm wide. Clamps present.	3.8		[23,48]

Besides the morphological characteristics reported in Table 3, some additional features could be observed later (i.e., when the colonies were over 15 days old) and are reported as follows:

- *Phylloporia ribis* showed thin, up to 1 cm long, crystals. The nature of these peculiar structures is unknown and could be worthy of further investigation (Figure 6);
- The brown rot agents *Gloeophyllum odoratum*, *Neolentinus lepideus*, *Fomitopsis officinalis*, *Antrodia cfr. Alpina* and *Fomitopsis iberica* and the white rot agents *Fuscoporia contigua* and *Polyporus squamosus* produced a non-localized MEA colour change to darker hues;
- *Neolentinus lepideus* pure cultures developed a strong and pleasant anisate smell, similar to the basidiomata;
- *Abortiporus biennis* and *Peniophora quercina* produced dark-reddish exudates.

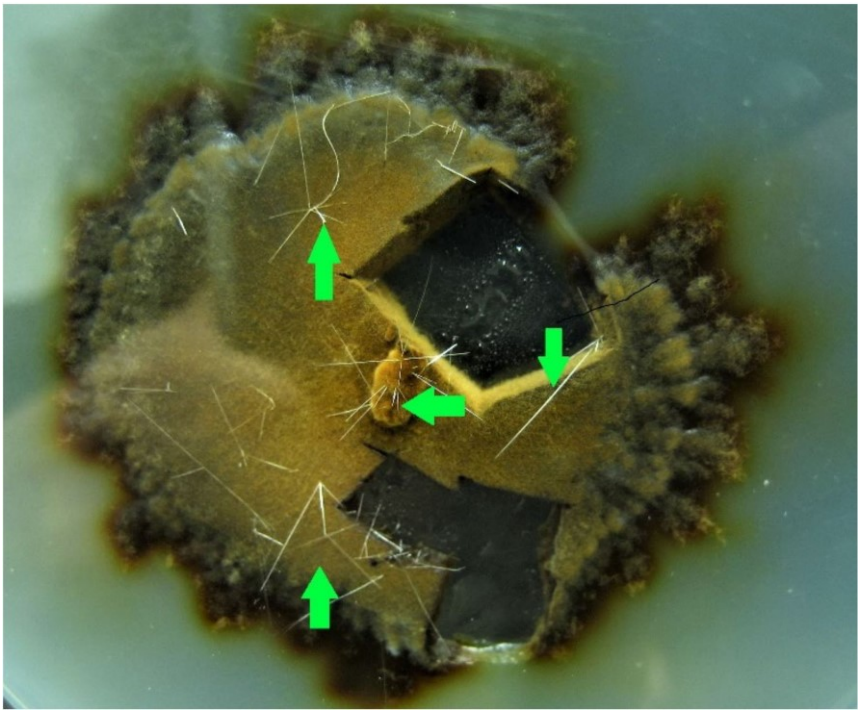


Figure 6. Pure culture of *Phylloporia ribis* showing white 1 cm-long crystals, a number of which are indicated by the green arrows.

Exudates are recurrent in A. biennis and P. quercina according to both the literature [23] and the authors' previous experience.

Regarding the mycelium characteristics, all of the strains related to the *Ganoderma* genus had a very compact and thin-layered mycelium. On the other hand, *Agaricales* had a fluffy and inconsistent mycelium when compared to *Polyporales*.

The strains belonging to *Hymenochaetaceae* (*Fuscoporia, Phylloporia, Fomitiporia, Inonotus*) produced a coloured mycelium in the Petri dish: *Fuscoporia* and *Fomitiporia* showed a brownish, thick mycelium, whereas *Inonotus* and *Phylloporia* presented a thin, yellowish mycelium with extrusions in agar dark-brown compounds. A number of other strains present a coloured mycelium: *Antrodia cfr. alpina* has a sulphur-yellow mycelium; *Stereum hirsutum* has a mycelium that is light orange; *Terana caerulea* has a mycelium that starts out white before becoming an intense blue colour; and *Pycnoporus cinnabarinus* has an orange-reddish mycelium reflecting the colour of its basidiomata.

The important characteristics for the biotechnological application of fungal strains are the consistency of the mycelium production and the growth rate in culture. A few strains presented thin (or transparent), inconsistent mycelia (*I. latemarginatus, Polyporus squamosus, Stereum sanguinolentum, Terana caerulea*). Others showed a very slow growth rate: *Antrodia cfr. alpina, Fomitopsis officinalis, Osteina obducta* and *Cyanosporus alni* among

brown rot agents; and *Fuscoporia torulosa, Inonotus radiatus, Phylloporia ribis* and *Skeletocutis amorpha* among white rot agents.

The cultures of *Laetiporus sulphureus* and *Fomitopsis officinalis* have a dusty surface due to the production of asexual spores.

Some other species, such as *Abortiporus biennis, Coriolopsis gallica and C. trogii, Daedaleopsis confragosa, Fomes fomentarius, Fomitopsis iberica and F. pinicola, Ganoderma carnosum* and *G. lucidum, Irpex lacteus, Irpiciporus pachyodon, Lenzites betulinus, Polyporus alveolaris, Stereum hirsutum, Trametes gibbosa, T. hirsute* and *T. suaveolens* presented a fast-growing and homogeneous tough colony.

3.5. Fungal Strains Conservation

To date, all of the isolated strains have resulted in successful conservation thanks to the application of combined storage methods.

Based on the back-cultures, all of the strains are maintained alive after the classic storage in MEA (Petri dish or tube) at 4 °C.

It has been demonstrated that all the isolated strains are maintained alive for at least 18 months in water vials on paper-filter discs at 4 °C, but not all regrew immediately when transferred to a new MEA Petri dish. Of particular note is the case of *Osteina undosa* on 162-19 colonized filter paper discs. After 18 months of storage in water vials at 4 °C, the discs were placed on MEA Petri dishes for strain refreshment. The mycelium started to grow again only after 7 months of total inactivity at 25 °C.

All the randomly back-cultured strains removed from –80 °C were able to regrow on MEA.

4. Conclusions

Strains isolated in pure culture from lignicolous fungi are a powerful tool for both pure and applied research.

The successful isolation ratio was very high in the developed method. In total, only 9 out of 103 strains could not be isolated (less than 10% of the total).

From the perspective of taxonomy and systematics, this work has achieved a remarkable stock of new strains from both common and rare species; such strains will be available for future studies and collaborations. The main outcome to be highlighted is therefore the possibility to fill a geographic gap by introducing strains from northern Italy in such future studies; this is particularly true for the rare/uncommon species that are often excluded or poorly represented in the experimental sets due to the lack of strains in pure culture.

Among the many possible applications of fungi, the characteristics of the mycelia are particularly important in the case of the formation of myco-materials. *Mycenaceae, Strophariaceae, Dacryobolaceae, Laetiporaceae* and *Bondartzewiaceae* suggest their inadequacy for producing materials based on fungi, as their colonies on artificial media are thin, slow-growing and formed of an inconsistent mycelium. The strains belonging to the *Fomitopsidaceae*, *Hymenochaetaceae*, *Irpicaceae*, *Meruliaceae*, *Phanaerochaetaceae*, *Polyporaceae* and *Stereaceae* fungal families seem to be the most suitable for myco-materials due to the high growth rates, homogeneity and stiffness of their mycelial colony.

This study provides the first step for further work on the selection of suitable fungal strains in order to obtain pure fungal materials or biocomposites based on fungi. Consistent with the results of this study, 21 different strains belonging to 20 species were selected from the strain set described above and examined as described in Cartabia et al. [38].

Author Contributions: Conceptualization, E.S. and M.C.; methodology, M.C., R.M.B., S.B. (Simone Buratti) and C.E.G.; validation, A.B.; investigation, M.C., S.B. (Simone Buratti) and R.M.B.; resources, S.B. (Stefano Babbini) and E.S.; data curation, M.C., C.E.G. and S.B. (Simone Buratti); writing—original draft preparation, M.C., R.M.B. and S.B. (Simone Buratti); writing—review and editing, C.E.G., E.S., S.B. (Simone Buratti), R.M.B. and M.C.; supervision, A.B., S.B. (Stefano Babbini) and E.S.; project administration, E.S. and R.M.B.; funding acquisition, S.B. (Stefano Babbini) and E.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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1.3 An Example of the Conservation of Wood Decay Fungi: The New Research Culture Collection of Corticioid and Polyporoid Strains of the University of Salamanca (Spain)

The following paragraph has been extrapolated from the scientific article by Buratti, S., Girometta, C. E., Savino, E., & Gorjón, S. P. (2023). An Example of the Conservation of Wood Decay Fungi: The New Research Culture Collection of Corticioid and Polyporoid Strains of the University of Salamanca (Spain). Forests, 14(10), 2029.

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Abstract: Over the last decade, fungal conservation has become an increasingly important topic, especially for species tied to forest ecosystems. Among these, wood decay fungi are a group of interesting species from ecological and applicative points of view. Culture collections represent an important tool for the conservation of species and research material. The aim of this study was to establish the first research culture collection of wood decay fungal strains, mainly corticioid and polyporoid species, at Salamanca University (Spain). From two areas of the Iberian Peninsula, a total of 120 basidiomata were collected and morphologically identified. From these, 55 strains were successfully isolated in pure culture and their identity was confirmed by DNA molecular analysis. The average growth rate of each strain was recorded and mycelium characteristics, such as colony morphology and microscopic features, were described. Notable strains in the collection included: (1) *Botryobasidium asperulum* and *Phlebia rufa* for taxonomical studies; (2) *Hericium erinaceus*, *Grifola frondosa* and *Pleurotus* species for medicinal properties; (3) *Irpex lacteus*, *Phanerochaete sordida* and *Trametes versicolor* for their degradation capabilities; (4) *Stereum gausapatum* and *Stereum hirsutum* for their applicative and enzymatic potential. The new fungal strain culture collection represents a valuable tool for the ex situ conservation of Mediterranean wood decay fungi.

Keywords: lignicolous fungi; corticicolous fungi; isolation in pure culture; growth rate; mycelium characteristics; ex-situ conservation; Mediterranean fungal diversity

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1. Introduction

Biodiversity conservation has emerged as a driving field in biology and environmental sciences; as a major reference for both scientists and institutions, the IUCN (International Union for the Conservation of Nature) has expanded its Red Lists to include an increasingly diverse range of organisms in order to address the global biodiversity crisis. Today, the IUCN estimates that "more than 42,100 species are threatened with extinction, that is still 28% of all assessed species" [1]. Fungi have one of the greatest ranges of biodiversity on Earth, with 2.2 to 3.8 million species, many of which have yet to be identified and described [2]. Nonetheless, fungal conservation has only recently been consolidated as a topic to address environmental policies, such as through the Global Fungal Red List Initiative (GFRLI) [3]. Everyone can register with the GRFLI in order to propose species for assessment: all the available data on the nominated species are included in a frame based on IUCN protocols and data can be edited and commented on while under assessment; assessment is also subjected to external review and then checked for approval by the chairs of IUCN fungal specialist groups; the final step is the inclusion decision by the IUCN Red List Unit.

Just like other life forms, several fungal species are experiencing threats caused by pollution, climate change, fragmentation and loss of habitats, loss of symbionts, over exploitation and habitat conversion [4]. Despite the fact that the great majority of fungal species has yet to be assessed, 73% out of the 597 fungal species currently on the global red list are found in forest ecosystems [5].

Fungal diversity in forests has different functions and trophic niches, including mycorrhizals, litter saprotrophs, lichenized fungi, etc. Wood decay fungi (WDF) have a major role since they break down the wood components and boost the nutrients cycle [6–8].

WDF are a broadly defined group of taxa able to effectively colonise and degrade different lignocellulosic substrates [9,10]. They are present at various stages of wood decay and include both pure (non-pathogenic) saprotrophs and opportunistic pathogens. Depending on the species (both fungal and host) and environmental conditions, the latter cause varying severity of damage to hardwood and sapwood. Such plasticity and variability in degradation, as well as their ecological role, make WDF an optimal research topic in forestry for monitoring the possible shifts in the environmental drivers that cause fungal proliferation and plant decline [11,12]. Furthermore, WDF are extensively studied in industrial biotechnology [13] for applications encompassing medicine and nutraceutics to biomaterials, bioremediation and the degradation of persistent organic pollutants [14–16]. As a result of the loss or anthropic change of forest habitats, fungal diversity is an ecological and biotechnological resource that is under increasing threat. The ex situ con- servation of fungal strains is thus critical for ensuring the availability of basic and applied research material. WFCC (World Federation for Culture Collections); WFCC-certified culture collections play an important role in this regard [17], as well as research centres and universities research collections, private individuals and companies who respectively collect strains for amateur or industrial purposes [18,19]. Since 1980, the WFCC has provided standards for culture collections in order to establish a global network of collections for ex situ conservation [20]. Therefore, culture collections are essential for collecting, characterising and studying fungal species, archiving their information and conserving strains for basic and applied research, as well as for industrial exploitation. Finally, collections are intrinsically representative of fungal diversity and species richness at various spatial scales (regional, national, etc.) [21]. The overall amount of private and institutional culture collections containing fungal strains around the world is unknown; WFCC has 104 partners broadly referring to "microbes" in general, not only fungi. Analogously, the MIRRI (Microbial Resource Research Infrastructure) "brings together 50+ microbial domain Biological Resource Centres (mBRCs), culture collections and research institutes from ten European countries" [22] but official partners in turn collect data from further collections owned by sub-partners. Several private companies also hold private collections, which are normally protected by industrial secrecy.

The periodical culture check is a major task in any culture collection in order to ascertain the vitality of the cultures themselves, update the nomenclature and check for changes in morphology or conflicts with previous identification. Culture checks are carried out by two methods: morphology and DNA sequencing. DNA sequencing is costly, but it ensures the culture is properly identified. Morphological identifications can be time-consuming but they are required to correctly identify specific cultures, especially if there is no conspecific reference in the DNA sequence database [23].

The Mycology Section in the Department of Botany and Plant Physiology of Salamanca University (Spain) owns a large collection of WDF *exsiccata* (SALA-Fungi). On the other hand, the Laboratory of Mycology in the Department of Earth and Environmental Sciences of Pavia University (Italy) has a wide research culture collection of WDF strains [24,25]. The aim of this work was to establish a new fungal research culture collection of WDF strains for the ex situ conservation of forest biodiversity in two areas of central and northern Spain. Culture morphology (including a macro- and micromorphological description) and molecular barcoding were used to identify fungal strains. Furthermore, for each strain, a brief overview of its application potential based on literature data has been included.

The work also includes basic guidelines for isolating corticioid fungi, which are difficult to isolate in pure culture due to their thin, resupinate context. The current study is the first step towards connecting the new collection to the collection network, which will increase the resources available for fungus conservation.

2. Materials and Methods

WDF strains are obtained by isolating mycelium in pure culture from sporophores collected in the wild (or cultivated). Only fungi from the phylum Basidiomycota (whose sporophores are known as basidiomata) were considered in this study. Thus, the initial step was to collect and identify basidiomata in forests.

2.1. Basidiomata Sampling

Basidiomata sampling was carried out between October and December 2022 in two areas of Spain (Figure 1): (1) 2.67 km^2 in Salamanca province, Castilla y León region (Mediterranean zone in the center of Spain); (2) 10.705 km^2 in Cantabria region (temperate oceanic climate zone in the north of the Iberian Peninsula) [26].

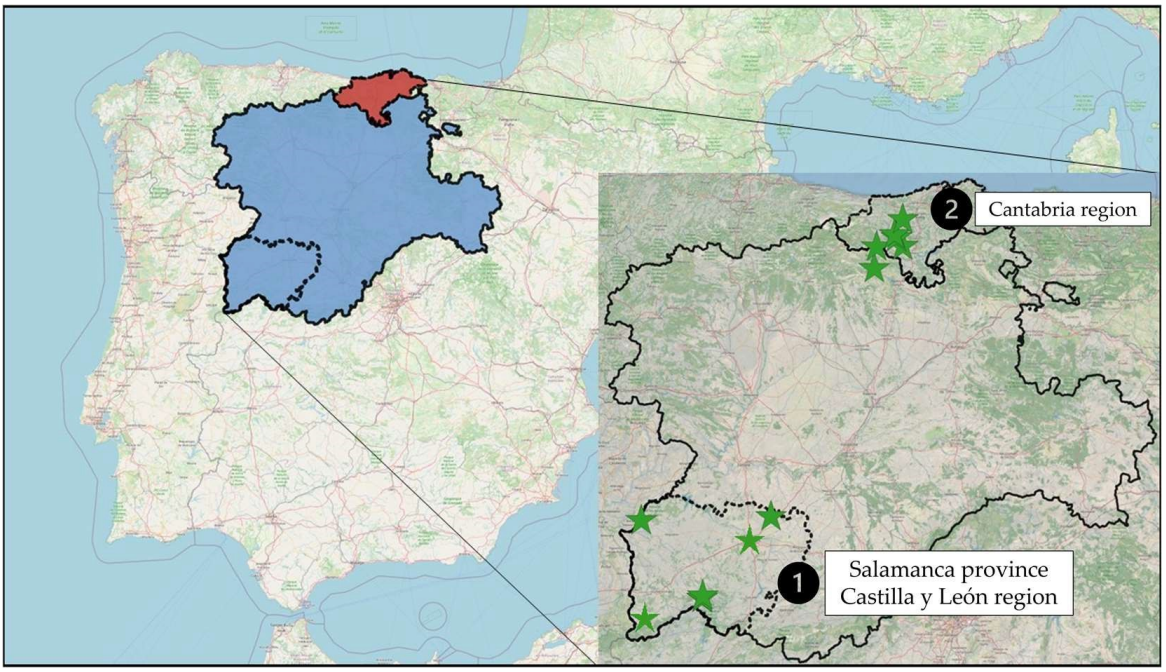


Figure 1. Sampling areas: (1) Salamanca province in Castilla y León region (blue); (2) Cantabria region (red). The dotted line within the Castilla y León region represents the borders of Salamanca province. Sampling sites are marked by stars. Source: Google Satellite and Open Street Map re-elaborated by the authors in QGIS 3.10.14.

All the samples were geolocalized by a smartphone (P30 lite, Huawei, Shenzen, China) GPS (Google Maps, Google, Mountain View, CA USA, based on cartographic data 2023 by the Instituto Geográfico Nacional, Madrid, Spain); basic information about the host plant and environmental features was recorded.

Basidiomata were completely or partially harvested using a knife and, in the case of some corticioid species, part of the woody substrate was drawn as well. The samples were then placed in paper packets and isolated in pure culture the same day or stored at 4 °C until processing. Basidiomata that were not used for isolation were dried at 40 °C before being packed and stored in dry conditions. All the *exsiccata* are currently deposited in SALA-Fungi herbarium under the acronym of the collector Sergio Pérez Gorjón (SPG).

2.2. Strain Isolation in Pure Culture

The isolation of mycelium in pure culture was performed by two different standard methods (slightly modified by the authors) depending on the specimen morphology [27,28].

For specimens with bulky or thick basidiomata, isolation was carried out from the context, i.e., the internal hyphal mass lying between the upper surface and the hymenial layer [29]. Little portions (typically 10 mm^3) of context were drawn and inoculated in Petri dishes with 2% malt extract agar (MEA) (Biokar Diagnostics, Allonne, France and VWR Chemicals, Milano, Italy) and 50 ppm chloramphenicol (VWR Chemicals, Milano, Italy) under sterile conditions, e.g., in a biological hood or in close proximity to a flame.

For specimens with thin or very small basidiomata, isolation was carried out from spores. Two methods were used to obtain spores. The first method involved hanging a piece of basidiomata or a piece of wood colonised by the fungus onto a microscope slide with the hymenial surface pointing downward. Wet clothing was draped over the experimental apparatus to generate a humid environment conducive to spore release. Released spores fallen onto the microscope slide were subsequently drawn by an inoculating loop. The inoculating loop was pre-soaked in sterile water to increase spore adhesion. The spores were finally spread out in Petri dishes with 2% MEA + chloramphenicol.

Analogously, the second method consisted of: (1) cutting a little square (usually <1 cm^2) piece of the hymenial layer or a gill from the specimen; (2) attaching it on the inside of the top of a Petri dish using vaseline (VWR Chemicals, Milano, Italy) as described and depicted in Choi et al. [30]; (3) incubating the Petri dish in an almost vertical position to allow spores to drop and spread all over the medium surface (this step usually takes 1–2 days).

Petri dishes were incubated at 25 °C in the dark (Biolog, Fratelli Galli, Fizzonasco, Italy) to allow mycelium growth or spore germination. The transplantation of colonies in new 2% MEA Petri dishes was performed as soon as germination was observed (usually by 1 week, depending on the species), whereas colonies grown from context pieces were allowed to colonize the whole dish.

Successfully isolated strains are currently conserved at 4 °C in the SALA-DNA Plant Biobank of the University of Salamanca (Spain) and at 4 °C and −80 °C in the MicU-NIPV Research Culture Collection of the University of Pavia (Italy). The cryopreservation procedure at −80 °C is described in Cartabia et al. [24].

Each strain has been assigned the same code number as its related specimen, along with the field data that goes with it.

2.3. Fungal Strain Morphological and Molecular Identification

Collected basidiomata were identified by micro- and macro-morphological features observed with an optical microscope (Leica DM750, Leica Microsystems, Wetzlar, Germany) and a stereomicroscope (LeicaEZ4, Leica Microsystems, Wetzlar, Germany) and through manuals and identification keys [29,31,32].

The morphological identification of isolated strains was further confirmed by molecular analysis (ITS region). Each strain was grown in a 100-mL flask containing 50 mL of 2% malt extract (ME, Biokar diagnostics, Allonne, France) solution and incubated at 25 °C in the dark (thermostat as above) until mycelial biomass was obtained. Biomass was sterilely drawn by tweezers and placed in 1.5-mL Eppendorf tubes for molecular identification; about 20 mg of dry biomass/sample was used for DNA extraction. DNA extraction was performed using a Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. DNA concentration was quantified using a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently adjusted to 25 ng/µL prior to PCR. Amplification of the ITS region was carried out using a DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and ITS1-ITS4 primers (Thermo Fisher Scientific). The complete PCR and purification protocol is described in Girometta et al. [25]. Purified samples were sequenced by Macrogen (Milano, Italy). DNA sequences were edited using Sequencher 5.0 and compared with those available in the GenBank [33] and MycoBank [34] databases.

2.4. Growth Tests and Description of the Fungal Strains

For each fungal strain, three 90-mm Petri dishes (2% MEA) were prepared; agar plugs with actively growing mycelium (surface of about 0.25 cm^2) were inoculated at the edge of the Petri dish to allow the fungal colony to expand across the entire diameter of the plate. This also enabled us to track its growth for longer periods of time, up to the entire plate coverage or 6 weeks (arbitrary deadline). Petri dishes were incubated at 25 °C in the dark. Mycelium growth was recorded daily with a calliper (0.1 mm resolution) as the distance between the inoculation point and the edge of the colony, measured over the diameter of the Petri dish. The growth rate was calculated as the average daily growth (mm/day) of the three replicates.

The colony macro-morphology (colour, mycelium aspect) and microscopical characters (such as hyphal morphology, hyphal diameter and peculiar structures) of isolated strains were described. Samples were observed in lacto-fuchsin using a Zeiss Stemi 2000-C (Zeiss, Oberkochen, Germany) stereomicroscope and a Nikon LABOPHOT-2 (Nikon, Minato, Tokyo, Japan) microscope. Descriptions are reported as a set of code numbers corresponding to defined characters (Table 1), adopting a comparable and simplified coding scheme as Nobles [35] and Stalpers [36].

Table 1. Code numbers and corresponding description of macroscopical and microscopical characters.

Macroscopical Characters			
Code	**Description**	**Code**	**Description**
1	Colony colour white, pale or transparent	11	Aerial mycelium felty: with mycelium cottony or wooly woven to form a compact surface
2	Colony colour yellow, ochraceous, brown or others (even if partially)	12	Aerial mycelium floccose: with little tufts of hyphae
3	Reverse of Petri dish colour unchanged	13	Colony lacunose: with depressions on the surface
4	Reverse of Petri dish colour bleached	14	Aerial mycelium plumose: with tufts composed of a central hypha from which smaller hyphae branch off
5	Reverse of Petri dish colour darkened	15	Aerial mycelium silky: with long and prostrate hyphae
6	Colony smooth and/or appressed and/or pellicular	16	Aerial mycelium subfelty: colony with a thin and prostrate mat, usually hardly visible
7	Aerial mycelium cottony: with hyphae spreading in all directions	17	Aerial mycelium velvety: with short and erected hyphae appressed together
8	Colony crusty, usually dark in colour	18	Aerial mycelium wooly: colony matted with long hyphae or groups of hyphae
9	Aerial mycelium downy: with short erected hyphae sparsely scattered	19	Colony mycelium submerged
10	Colony farinaceous in appearance		
Microscopical Characters			
Code	**Description**	**Code**	**Description**
20	Hyphae with clamps at all septa	28	Cystidia in vegetative mycelium
21	Hyphae simple-septate	29	Short projection or protuberances on cell-wall
22	Hyphae simple-septate with scattered or rare clamps	30	Oil or resinous drops on cell-wall
23	Hyphae with thin cell-wall	31	Hyphal knots or tangles
24	Hyphae with thick cell-wall	32	Hyphal swellings
25	Hyphae with cells closely packed forming a pseudoparenchyma	33	Absence of conidia and/or blastoconidia and/or arthrospores and/or chlamydospores
26	Hyphae with numerous short branches, curved branches or thick-walled nodes	34	Presence of conidia and/or blastoconidia and/or arthrospores and/or chlamydospores
27	Encrusted hyphae or hyphae with crystals	35	Presence of anastomosis/hyphal bridges

3. Results and Discussion

3.1. Basidiomata Sampling, Strains Isolation and Identification

The aim of the wood decay fungal sampling field activity was to collect basidiomata from as many species as possible in order to isolate them in pure culture and obtain a large number of strains. The sample was carried out in natural and semi-natural settings that were abundant in dead wood and WDF species.

The first sampling area location was in Salamanca province (Castilla y León), which has a Mediterranean climate and a high concentration of Pyrenean oak woods, pine forests and holm oak forests. The most frequent host species in this area are *Quercus pyrenaica* Willd. and *Pinus sylvestris* L.

The first sampling area location was in the Saja-Besaya Natural Park (Cantabria) and in the Parque Natural Montaña Palentina (Castilla y León, Palencia province), which has a Eurosiberian oceanic climate and is characterized by beech forests and pine forests (generally distinct from each other). The most frequent host species in this area are *Fagus sylvatica* L., *Quercus pyrenaica* and *Corylus avellana* L.

Consistent with the above, climate data indicated different conditions in the two examined areas over the sampling period. Namely, Castilla y Léon recorded 13.5–7.4–4.7 °C vs. 14.4–9.8–7.7 °C in Cantabria as average temperatures in October–November–December, respectively. Analogously, rainfall recorded 65–56–50 mm vs. 119–148–111 mm distributed in 7–7–6 vs. 11–12–11 rainy days. As a whole, the relative humidity was 66–79–82 % vs. 76–79–78% [26].

In the two areas, 120 basidiomata from 90 different species were collected.

Mycelium isolation in pure culture was tried on all acquired samples, either directly from basidiomata or spores. The isolation of 55 strains from 50 different species was achieved (Table 2). On all isolated strains, ITS-based genetic barcoding was performed and results were compared to the morphological identification of the basidiomata. Table 2 lists the accession numbers for all ITS sequences that have been deposited in GenBank. Some species were unable to be isolated due to basidiomata conditions, such as a lack of spore production or deterioration.

Table 2. Isolated strains currently present in the new culture collection. Code number of the collection, topographic data, number of isolated strains and GenBank accession numbers are reported.

Species	Specimen Code Number (SPG)	Host	Region (Spain)	Geographical Coordinates	N° of Strains	GenBank Accession Number
		Corticioid Fungi				
Athelia epiphylla Pers.	5316	*Quercus pyrenaica* (on *Hymenochaete tabacina)*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336245
Botryobasidium asperulum (D.P. Rogers) Boidin	5367	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336246
Byssomerulius corium (Pers.) Parmasto	5425	*Quercus ilex*	Castilla y León	41°08′10″ N 5°28′47″ W	1	OR336247
Crustomyces subabruptus (Bourdot and Galzin) Jülich	5387, 5400	*Fagus sylvatica*	Cantabria	43°07′37″ N 4°17′29″ W 43°06′21″ N 4°16′30″ W	2	OR336248 OR336249
Efibula tuberculata (P. Karst.) Zmitr. and Spirin	5315, 5368	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W 40°33′57″ N 6°08′08″ W	2	OR336250 OR336251
Gloeocystidiellum clavuligerum (Höhn. and Litsch.) Nakasone	5392	*Corylus avellana* (on *Hymenochaete corrugata*)	Cantabria	43°07′37″ N 4°17′29″ W	1	OR336252
Hericium erinaceus (Bull.) Pers.	5459, 5462, 5472	*Quercus pyrenaica*	Castilla y León	40°21′09″ N 6°46′51″ W	1	OR336253
Hymenochaete rubiginosa (Dicks.) Lév.	5312	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336254
Hyphoderma mutatum (Peck) Donk	5372	*Fagus sylvatica*	Castilla y León	43°02′31″ N 4°27′51″ W	1	OR336255
Hyphoderma transiens (Bres.) Parmasto	5402	*Fagus sylvatica*	Cantabria	43°07′37″ N 4°17′29″ W	1	OR336256
Megalocystidium leucoxanthum (Bres.) Jülich	5413, 5427, 5429	*Quercus ilex*	Castilla y León	41°08′10″ N 5°28′47″ W	1	OR336257

Merulius tremellosus Schrad.	5357	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336258
Peniophora quercina (Pers.) Cooke	5311, 5318	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336259
Peniophorella praetermissa (P. Karst.) K.H. Larss.	5366	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336260
Phanerochaete sordida (P. Karst.) J. Erikss. and Ryvarden	5342	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336261
Phlebia rufa (Pers.) M.P. Christ.	5323, 5370	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	2	OR336262 OR336263
Phlebiopsis crassa (Lév.) Floudas and Hibbett	5344, 5363	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336264
Porostereum spadiceum (Pers.) Hjortstam and Ryvarden	5356, 5358	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336265
Radulomyces molaris (Chaillet ex Fr.) M.P. Christ.	5343, 5364	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	2	OR336266 OR336267
Sertulicium granuliferum (Hallenb.) Spirin and Volobuev	5378	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336268
Stereum gausapatum (Fr.) Fr.	5419	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336269
Stereum hirsutum (Willd.) Pers.	5314, 5418	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336270
Stereum subtomentosum Pouzar	5379	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336271
Tulasnella violea (Quél.) Bourdot and Galzin	5340	*Quercus pyrenaica*	Castilla y León	40°32′58″ N 6°08′21″ W	1	OR336272
Vuilleminia comedens (Nees) Maire	5322	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336273
Vuilleminia coryli Boidin, Lanq. and Gilles	5381	*Corylus avellana*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336274
Xenasmatella vaga (Fr.) Stalpers	5371	*Fagus sylvatica*	Castilla y León	43°02′31″ N 4°27′51″ W	1	OR336275
Polyporoid fungi						
Ceriporia reticulata (Hoffm.) Domanski	5320	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336276
Daedaleopsis confragosa (Bolton) J. Schröt.	5386	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336277
Dichomitus campestris (Quél.) Domanski and Orlicz	5306	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336278
Fomitopsis pinicola (Sw.) P. Karst.	5348, 5398	*Fagus sylvatica*	Cantabria	43°07′37″ N 4°17′29″ W	1	OR336279
Gloeoporus dichrous (Fr.) Bres.	5307, 5308	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336280
Grifola frondosa (Dicks.) Gray	5436	*Quercus pyrenaica*	Castilla y León	40°21′09″ N 6°46′51″ W	1	OR336281
Hapalopilus rutilans (Pers.) Murrill	5309, 5313, 5314, 5315, 5316	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336282
Irpex lacteus (Fr.) Fr.	5345	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336283
Mycoacia gilvescens (Bres.) Zmitr.	5401	*Fagus sylvatica*	Cantabria	43°07′37″ N 4°17′29″ W	1	OR336284
Steccherinum bourdotii Saliba and A. David	5380, 5385	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336285
Steccherinum fimbriatum (Pers.) J. Erikss.	5369	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336286
Steccherinum ochraceum (Pers. ex J.F. Gmel.) Gray	5360, 5417	*Quercus pyrenaica*	Castilla y León	42°53′29″ N 4°29′13″ W	1	OR336287
Trametes betulina (L.) Pilát	5384	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336288
Trametes versicolor (L.) Lloyd	5382	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336289
Trichaptum abietinum (Pers. ex J.F. Gmel.) Ryvarden	5406	*Pinus sylvestris*	Cantabria	43°02′48″ N 4°12′48″ W	1	OR336290
Trichaptum biforme (Fr.) Ryvarden	5439	*Quercus pyrenaica*	Castilla y León	40°21′09″ N 6°46′51″ W	1	OR336291
Truncospora atlantica (Berk.) Spirin and Vlasák	5390	*Arbutus unedo*	Asturias	43°23′50″ N 4°31′57″ W	1	OR336292
Xylodon nespori (Bres.) Hjortstam and Ryvarden	5396	*Corylus avellana*	Cantabria	43°07′37″ N 4°17′29″ W	1	OR336293
Xylodon paradoxus (Schrad.) Chevall.	5317, 5319	*Quercus pyrenaica*	Castilla y León	40°33′57″ N 6°08′08″ W	1	OR336294
Xylodon spathulatus (Schrad.) Kuntze	5328	*Pinus sylvestris*	Castilla y León	40°32′52″ N 6°08′33″ W	1	OR336295
Agaricoid fungi						
Pleurotus eryngii var. ferulae (Lanzi) Sacc.	5411	*Ferula communis*	Castilla y León	41°06′28″ N 6°43′35″ W	1	OR336296

Pleurotus pulmonarius (Fr.) Quél.	5388	*Fagus sylvatica*	Cantabria	43°06′21″ N 4°16′30″ W	1	OR336297
Schizophyllum commune Fr.	5430	*Populus tremula*	Castilla y León	40°57′48″ N 5°41′00″ W	1	OR336298

According to the accepted differentiation between "corticioid" versus "polyporoid" species in the literature [29,31], twenty-six out of the fifty isolated species are corticioid, twenty-one are polyporoid and three are agaricoid. The corticioid group has a higher representation than the polyporoid group because the former are more numerous at the sampling sites. The only WDF agaricoid species sampled in this study were *Pleurotus eryngii* var. *ferulae*, *Pleurotus pulmonarius* and *Schizophyllum commune*.

Isolated strains belonging to nine distinct Orders; *Polyporales* (50%), *Russulales* (15%), *Agaricales* (13%) and *Hymenochaetales* (9%) have the highest proportion (Figure 2). At the family level, collected specimens are much more differentiated and record 25 different families, with *Polyporaceae* (13%), *Irpicaceae* (11%), *Steraceae* (7%), *Meruliaceae* (7%) and *Phanerochaetaceae* (7%) having the highest representation; the remaining 49% is made up of families that account for ≤5% of the total specimen amount (Figure 3).

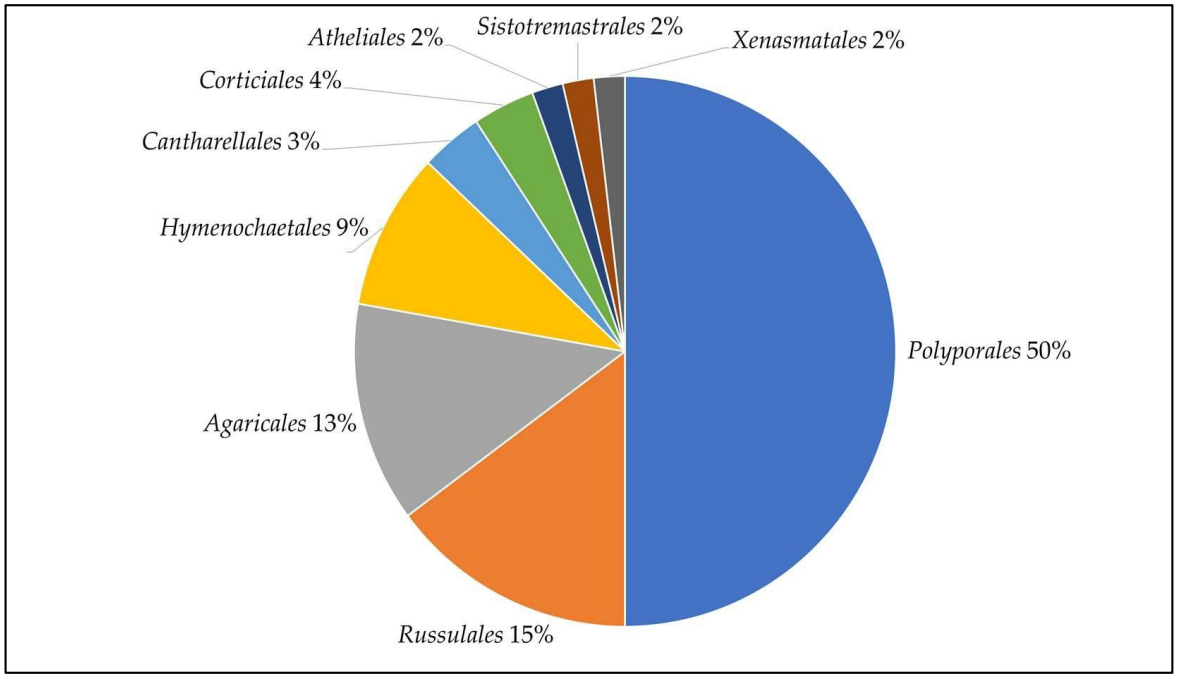


Figure 2. Division of isolated strains by Order rank.

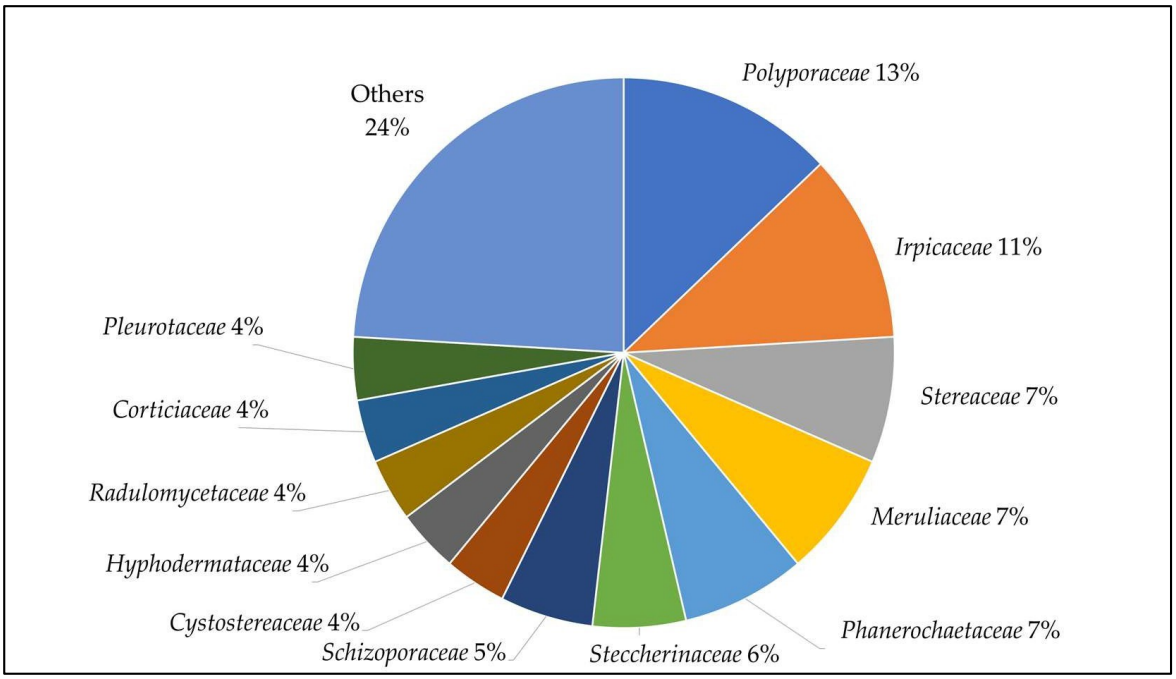


Figure 3. Division of isolated strains by Family rank.

3.2. *Description and Growth Rate of Isolated Strains*

The average growth rate (mm/day) of isolated strains calculated on three replicates is reported in Figure 4.

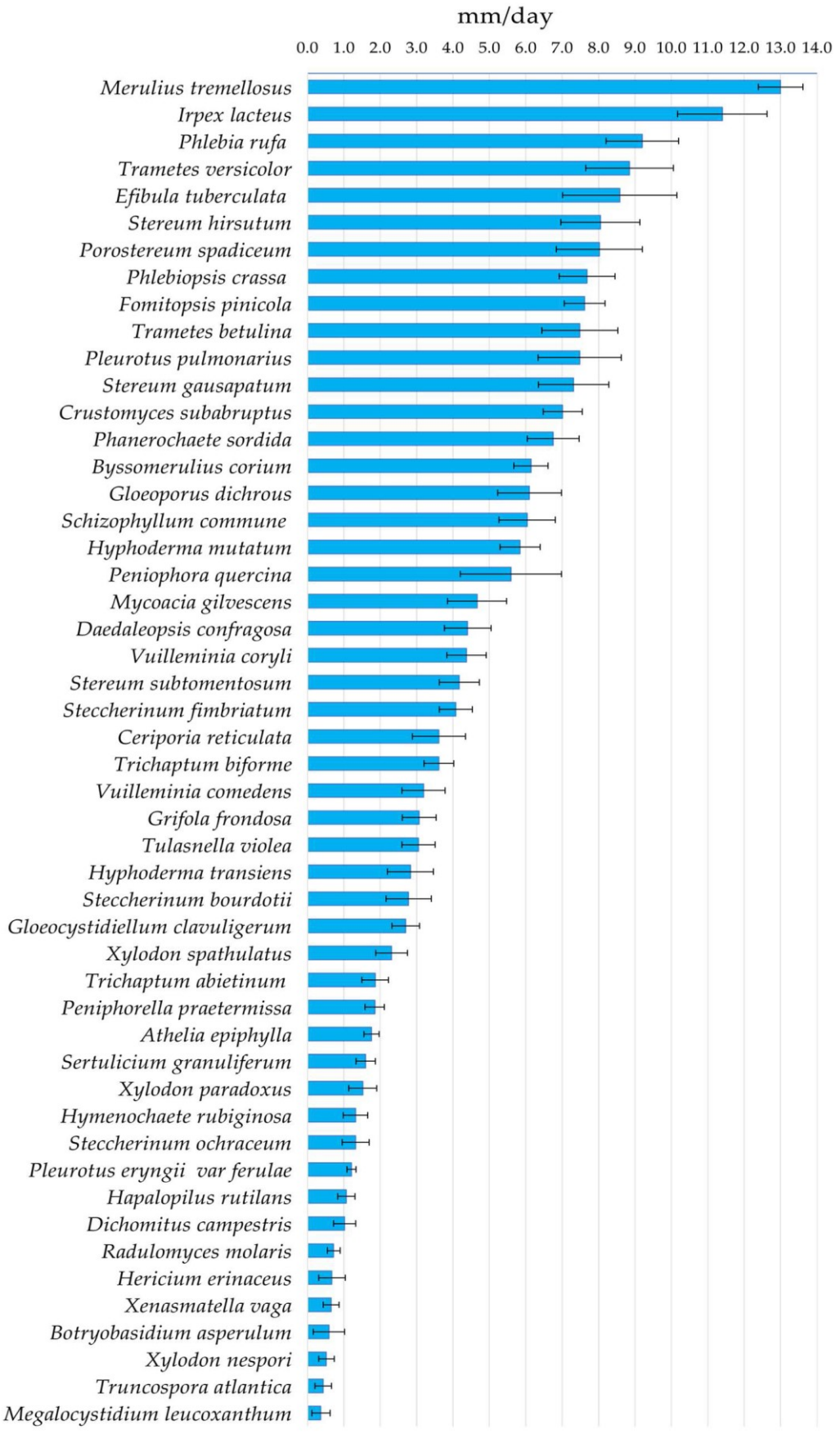


Figure 4. Average growth rate of isolated strains in mm/day. The bar indicates the standard error.

The isolated strains growth rates ranged from 0.4 mm/day to a 13 mm/day. The highest growth rates were reported by: *Merulius tremellosus* (13 mm/day), *Irpex lacteus* (11.4 mm/day), *Phlebia rufa* (9.2 mm/day), *Trametes versicolor* (8.9 mm/day), *Efibula tubercu- lata* (8.6 mm/day), *Stereum hirsutum* (8.1 mm/day) and *Porostereum spadiceum* (8 mm/day). On the contrary, the lowest growth rates were reported by: *Megalocystidium leucoxanthum* (0.4 mm/day), *Truncospora atlantica* (0.4 mm/day), *Xylodon nespori* (0.5 mm/day), *Botryobasidium asperulum* (0.6 mm/day), *Xenasmatella vaga* (0.7 mm/day), *Hericium erinaceus* (0.7 mm/day) and *Radulomyces molaris* (0.7 mm/day).

The growth rate on standard culture media serves as basic information that characterises strains in a culture collection and is functional to the strain selection process for application research. Any industrial biotechnology (including fungal biotechnology) must rely on strains that quickly and consistently grow. Myco-materials and bio-composites, for example, require fast-growing strains capable of producing consistent mycelium and a predictable colour [37]. Analogously, myco-remediation applications heavily rely on enzymatic activity; thus, basic knowledge of the species ecology, trophism and degradation type in the wild serves for further research, such as the characterization of the enzymes they can produce and optimisation of secretion conditions. However, the growth rate of a specific species is insufficient information in and of itself, because various strains may exhibit different degradation and growth rates. Similarly, intraspecific morphological changes can be noticed in several strains, such as variable aerial mycelium development or colour pattern. As a result, each detained strain can display a different potential and should be identified and characterised.

All strains were subjected to morphological descriptions of colony morphology and microscopical characteristics. Table 3 contains a description of each strain. Photos of colonies were taken with the entire Petri dish covered; in the case of incomplete colonisation or slow growing strains, a deadline of 6 weeks following inoculation was established.

Table 3. Microscopical characters, colony morphology and growth of isolated strains. Particularities or notable structures observed are reported in the "Notes" column. All of the photos are by Simone Buratti.

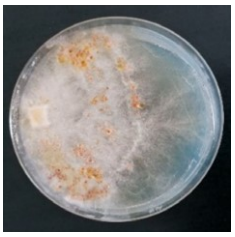
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Athelia epiphylla (SPG 5316)	1, 2, 4, 11, 16, 22, 23, 24, 33, 35	2.0–5.0		 Presence of brown sclerotia	[36]
Botryobasidium asperulum (SPG 5367)	2, 5, 6, 8, 9, 21, 22, 24, 32, 33	5.0–8.0			

Table 3. *Cont.*

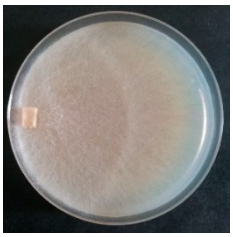
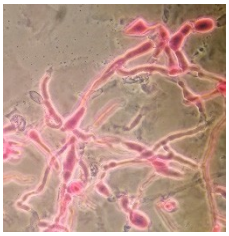
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Byssomerulius corium (SPG 5425)	1, 4, 6, 7, 9, 16, 18, 21, 23, 24, 31, 32, 33	2.0–5.0			
Ceriporia reticulata (SPG 5320)	1, 2, 3, 6, 9, 18, 21, 23, 32, 33	2.5–17.0		 Very noticeable hyphal swellings	[36]
Crustomyces subabruptus (SPG 5387)	1, 5, 12, 14, 18, 20, 23, 24, 26, 27, 33	1.5–7.0			
Daedaleopsis confragosa (SPG 5386)	1, 2, 5, 7, 11, 22, 23, 26, 33	1.0–2.5			[35,36,38]
Dichomitus campestris (SPG 5306)	2, 5, 6, 7, 9, 19, 20, 23, 24, 26, 32, 33	1.5–5.0			[24]
Efibula tuberculata (SPG 5315)	1, 3, 6, 9, 22, 23, 24, 25, 27, 32, 33	2.0–10.0			[36]

Table 3. *Cont.*

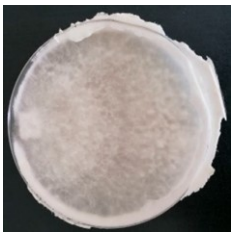
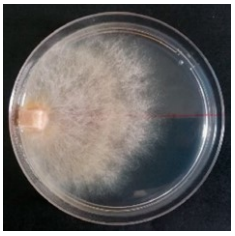
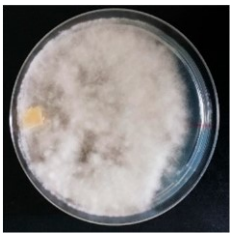
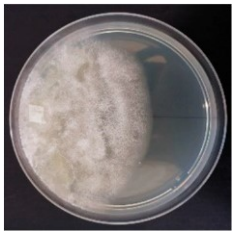
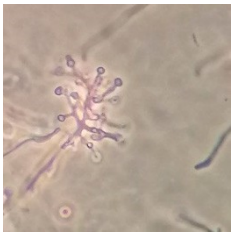
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Fomitopsis pinicola (SPG 5398)	1, 4, 7, 11, 18, 20, 23, 33	1.0–3.0			[24,35,36,38,39]
Gloeocystidiellum clavuligerum (SPG 5392)	1, 2, 3, 5, 6, 9, 10, 20, 23, 24, 26, 32, 33	2.0–3.5			[40]
Gloeoporus dichrous (SPG 5307)	1, 3, 6, 9, 12, 20, 23, 32, 33	1.5–4.0			[35,36]
Grifola frondosa (SPG 5436)	1, 3, 7, 11, 18, 22, 23, 24, 33	2.0–3.0			[24,36]
Hapalopilus rutilans (SPG 5309)	1, 2, 4, 9, 10, 16, 22, 23, 34	2.0–4.0		 Presence of blastoconidia	[35,36,38]
Hericium erinaceus (SPG 5459)	1, 3, 5, 6, 7, 12, 20, 23, 24, 30, 33	2.0–8.0			

Table 3. *Cont.*

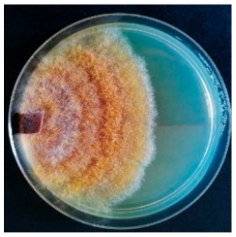
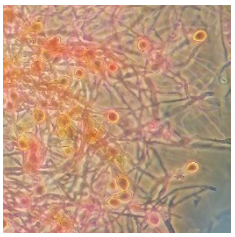
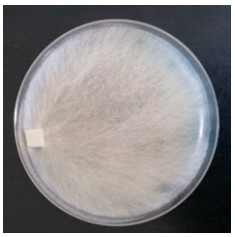
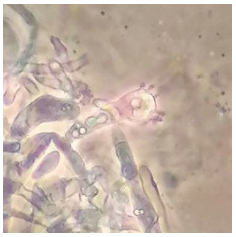
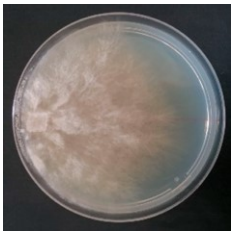
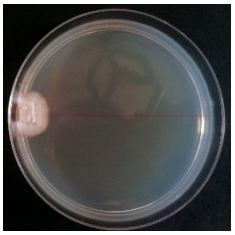
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Hymenochaete rubiginosa (SPG 5312)	2, 5, 7, 18, 21, 23, 24, 34	2.0–5.0		 Pigmented hyphae and terminal chlamydospores	[36]
Hyphoderma mutatum (SPG 5372)	1, 4, 6, 9, 18, 20, 23, 24, 32, 34	2.5–5.0		 Formation of basidia in vegetative mycelium	[35,36]
Hyphoderma transiens (SPG 5402)	1, 2, 5, 6, 7, 9, 10, 15, 20, 23, 24, 33, 35	2.0–5.0			
Irpex lacteus (SPG 5345)	1, 3, 4, 7, 18, 21, 23, 24, 32, 33, 35	1.2–7.0			[24,36,39]
Megalocystidium leucoxanthum (SPG 5413)	1, 5, 9, 10, 11, 21, 23, 24, 32, 33	2.0–5.0		Absence of clamps, probably due to isolation from spores leading to monokaryon mycelium	[36]
Merulius tremellosus (SPG 5357)	1, 4, 6, 7, 9, 21, 23, 24, 30, 32, 33	2.0–7.0			[36,39]

Table 3. *Cont.*

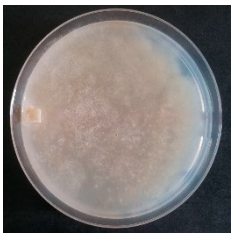
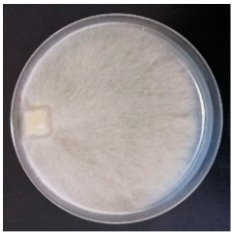
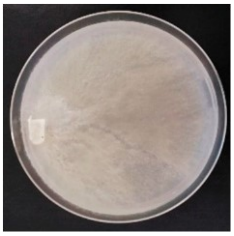
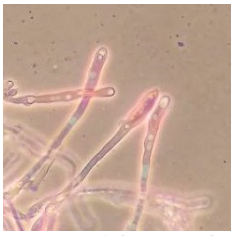
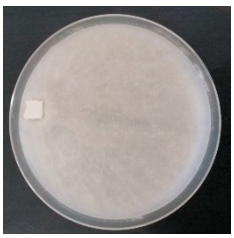
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Mycoacia gilvescens (SPG 5401)	1, 4, 6, 9, 14, 20, 23, 24, 27, 34	2.0–7.5			
Peniophora quercina (SPG 5318)	1, 5, 7, 22, 23, 24, 26, 33	1.5–5.0			[24,39]
Peniphorella praetermissa (SPG 5366)	1, 3, 7, 11, 18, 20, 23, 24, 26, 27, 32, 34	2.5–6.0			[36]
Phanerochaete sordida (SPG 5342)	1, 3, 6, 16, 21, 23, 24, 27, 34	2.5–8.0			[36]
Phlebia rufa (SPG 5370)	1, 2, 4, 6, 7, 9, 22, 23, 24, 26, 27, 28, 32, 34	2.0–7.5		 Presence of gloeocystidia in vegetative mycelium	[24,36]
Phlebiopsis crassa (SPG 5363)	1, 4, 7, 16, 21, 23, 24, 27, 33	1.0–7.5			

Table 3. *Cont.*

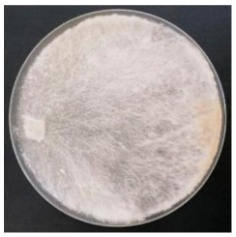
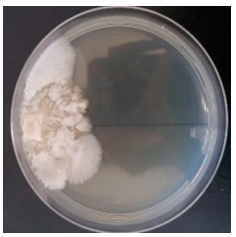
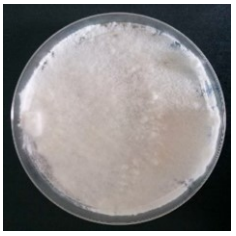
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Pleurotus eryngii var. ferulae (SPG 5411)	1, 3, 7, 11, 20, 23, 33	2.0–3.0			
Pleurotus pulmonarius (SPG 5388)	1, 3, 7, 11, 22, 23, 31, 34	1.0–3.0			
Porostereum spadiceum (SPG 5356)	1, 2, 4, 7, 18, 20, 23, 24, 26, 30, 33	1.5–5.0			[24]
Radulomyces molaris (SPG 5343)	1, 3, 5, 6, 7, 9, 20, 23, 24, 26, 32, 33, 35	2.0–3			[36]
Schizophyllum commune (SPG 5430)	1, 3, 4, 7, 11, 20, 23, 24, 29, 34	2.0–5.0			[24,35,36,38,41]
Sertulicium granuliferum (SPG 5378)	1, 2, 3, 6, 14, 20, 23, 30, 32, 34	2.0–7.0			

Table 3. *Cont.*

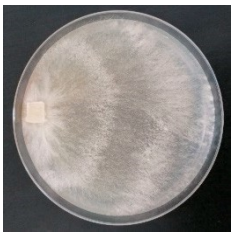
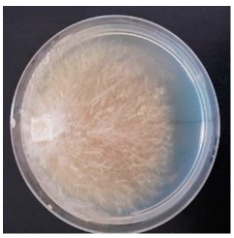
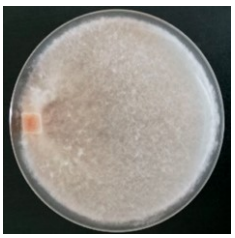
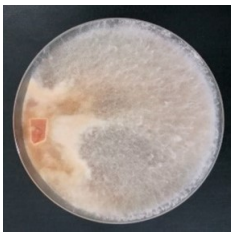
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Steccherinum bourdotii (SPG 5380)	1, 2, 4, 6, 9, 10, 20, 23, 24, 33	2.0–7.0			
Steccherinum fimbriatum (SPG 5369)	1, 3, 7, 12, 22, 23, 24, 32, 33	2.5–5.0			[36]
Steccherinum ochraceum (SPG 5360)	1, 2, 3, 6, 7, 16, 20, 23, 24, 26, 30, 32, 33	2.0–7.0			[36]
Stereum gausapatum (SPG 5419)	1, 2, 3, 7, 11, 12, 22, 23, 24, 30, 33	2.0–7.5			[36]
Stereum hirsutum (SPG 5314)	1, 2, 5, 7, 12, 16, 18, 22, 23, 24, 30, 33, 35	2.0–6.0			[24,36,38,41]
Stereum subtomentosum (SPG 5379)	1, 5, 7, 12, 22, 23, 24, 30, 33	2.0–5.0			[36]

Table 3. *Cont.*

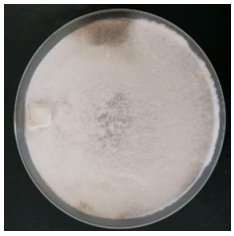
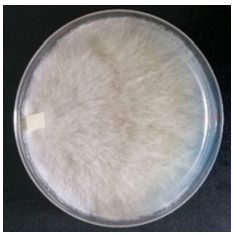
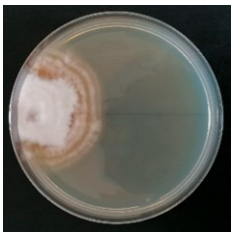
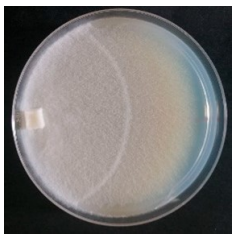
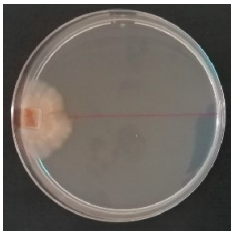
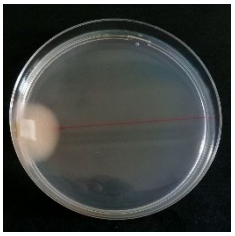
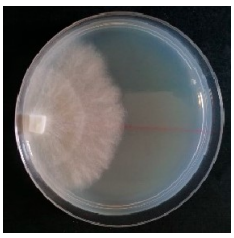
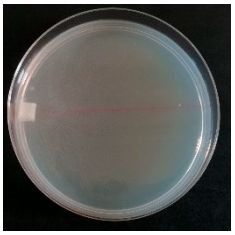
Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Trametes betulina (SPG 5384)	1, 3, 7, 11, 12, 17, 20, 23, 24, 33	1.5–3.0			[24,35,36,39,41]
Trametes versicolor (SPG 5382)	1, 4, 7, 16, 18, 20, 23, 24, 27, 33	2.0–3.0			[24,35,36,39,41]
Trichaptum abietinum (SPG 5406)	1, 3, 6, 7, 12, 16, 20, 23, 26, 27, 31, 32, 33	2.0–5.0			[24,35,36]
Trichaptum biforme (SPG 5439)	1, 4, 5, 18, 20, 23, 24, 33	2.0–5.0			[42]
Truncospora atlantica (SPG 5390)	1, 2, 5, 7, 9, 10, 11, 22, 23, 24, 26, 27, 31, 33	2.0–5.0			[24]
Tulasnella violea (SPG 5340)	1, 2, 4, 7, 11, 18, 20, 23, 24, 30, 32, 33, 35	2.0–5.0			[36]

Table 3. *Cont.*

Species	Cultural Characters	Hyphal Width (µm)	Photos of the Colonies in MEA	Notes	References
Vuilleminia comedens (SPG 5322)	1, 4, 12, 19, 22, 23, 27, 33	1.5–3.0			[36]
Vuilleminia coryli (SPG 5381)	1, 3, 7, 11, 20, 23, 32, 34	2.5–5.0			
Xenasmatella vaga (SPG 5371)	1, 2, 56, 9, 10, 20, 23, 26, 32, 33	1.5–4.0			[36]
Xylodon nespori (SPG 5396)	1, 3, 6, 9, 20, 23, 26, 31, 33	2.0–5.0			
Xylodon paradoxus (SPG 5317)	1, 4, 7, 9, 18, 20, 23, 32, 34	1.5–3.0		Presence of blastoconidia	[36]
Xylodon spathulatus (SPG 5328)	1, 3, 6, 9, 20, 23, 24, 27, 34	1.5–3.5		Colony almost transparent; presence of blastoconidia	

Most of the isolated strains had consistent colony morphology across the replicates and after transplantation. However, in other cases, the following cultural variables were observed:

- The mycelium of *Phlebiopsis crassa* turned purple after a few weeks of growing in isolation, which is also a basidiomata feature. This characteristic was lost in subsequent cultures;
- *Hericium erinaceus* exhibited two distinct morphologies: one that was more compact and consistently growing (as indicated in Table 3) and one with hyphae bundled to form aerial mycelium;
- *Truncospora atlantica* colonies are characterized by powdery mycelium that can either cover the entire colony, making it entirely white, or cover it partially, resulting in a darker-coloured zonation.

The mycological literature abounds with descriptions of basidiomata morphology, whereas descriptions of mycelium in pure culture are sparse, if not missing.

To manage a new culture collection, each strain should be provided with a document containing information on the original field specimen, descriptions and photographic records. Cultural descriptions and photos are tools for comparing and checking the state of cultures and their morphology, as well as any changes and degeneration symptoms, during regular cultural procedures such as culture refreshing or transplantation [43,44]. Further- more, such descriptions enable cross-validation of the response by molecular barcoding on pure cultures, as well as optimization of the barcoding effort itself.

It is noteworthy that approximately 25% of the species reported and described in this work still lack a description of their culture morphology. This reflects the general lack of knowledge about corticioid fungi, which is attributable to the highly specialized skills required for their identification. Because corticioids have often exiguous basidiomata and poor macromorphological traits, reference descriptions of isolates can boost confidence and provide an additional tool for proper identification and typification.

3.3. Notable Species in the New Strain Collection of Salamanca University: Issues and Potentiality

The new WDF strain collection of Salamanca University is a remarkable asset, since many corticioid species have, until now, been overlooked due to morphological cryptism and isolation difficulties. Even when several major culture collections in Europe (CBS, MUT, MUCL, LE-BIN/VKM) [45–48] are used as a reference, most corticioid species are only represented by a few strains, while others are completely absent. Compared to these references, this study isolated pure culture strains of *Botryobasidium asperulum*, *Crustomyces subabruptus*, *Sertulicium granuliferum* and *Mycoacia gilvescens* that were not present in other significant culture collections.

Although most of the strains currently held in the Salamanca University research culture collection are common species according to European florae, their conservation is crucial. According to the ex situ conservation principle, species that are common today may become rare and endangered in the future (declining population criterion). For instance, *Grifola frondosa* and *Hericium erinaceus* are fairly common species but are experiencing localised negative trends that are pushing them towards rarity. *Grifola frondosa* has been assigned the preliminary category of Least Concern (LC) at the global level and Near Threatened (NT) in Europe. This status was assigned because of population decline primarily due to habitat degradation. Similarly, *Hericum erinaceus* has been assigned Least Concern because it is still a common species in many countries, but is very rare in others. The population of this species is likewise declining due to habitat degradation [4]. Once more, it should be highlighted that most corticioid diversity is overlooked and therefore "data deficient" in terms of overall conservation status, habitat needs and population trends.

A summary of the most notable isolated species and the research opportunities available to them is listed below.

3.3.1. Species of Special Concern for Taxonomical and/or Biogeography Studies

Athelia epiphylla is considered a species complex and a challenging issue in taxonomy due to its very exiguous colonies and basidiomata, which are difficult to examine. This is why any novel strain in pure culture could be significant, especially when coupled with

morphological description, because sclerotia are supposed to be discriminant traits. The specimen in this study was found growing on a basidiomata of *H. rubiginosa* and lacked any distinguishing feature from *A. arachnoidea* (i.e., bisporic basidia), although the latter is frequently lichenicolous [32].

Botryobasidium asperulum is a species distinguished by small ellipsoid and asperulate basidiospores. According to Langer [49], it is an eminently tropical species that was first reported in Cuba and subsequently collected throughout tropical areas of Africa (Guadeloupe, Gabon, Kenya, Malawi and Ethiopia). *Botryobasidium stigmatisporum*, as Boidin and Gilles reported from La Reunion, has ornamented basidiospores as well, although larger. The occurrence of *B. asperulum* in Europe is controversial; Bernicchia and Gorjón [31] reported several specimens from the Mediterranean region, but European specimens may belong to *Botryobasidium laeve* (J. Erikss.) Parmasto according to Larsson and Ryvarden [32]. The latter species is reported from North Europe and is distinguished by larger, smooth basidiospores; however, the same authors argued that ornamentations are tricky discriminants since they are often difficult to discern. The size and shape of the basidiospores of our specimen meet the concept of *B. asperulum*, and the asperulate ornamentation is visible.

So far, no *B. asperulum* sequences have been deposited in GenBank (NCBI) or other databases. The majority of the sequences deposited in the genus *Botryobasidium* are from the species *B. laeve* and *B. subcoronatum*. As a result, there are no *B. asperulum* comparative sequences: the ITS sequence obtained in this study may be the first for this species.

More sequences of this species, preferably from tropical zones, could help to enlarge the pool in databanks and enable appropriate molecular identification in order to resolve the distribution and taxonomy difficulties discussed above.

Phlebia rufa is a common and sub-cosmopolitan species, although it is most likely a species complex with several sibling entities. Consequently, there is a significant deal of interest in collecting cultures to assesss sexual compatibility as well as sequencing for phylogenetic studies. Basidiomata are generally resupinate or slightly reflexed and the gelatinous hymenophore is irregularly poroid to merulioid, cream to brown in color [31]. *P. rufa* generally produces large and massive basidiomata that spread to cover the whole substrate, which is typically a large trunk or fallen branch. It is a very efficient decomposer that occurs early in wood degradation and causes intense white rot, giving the wood a spongy and soft character. Due to its great degradation potential and high growth rate, it is a good candidate for biodegradation and bioremediation processes.

Phlebiopsis crassa is a holotropical species that has just been discovered in temperate Europe [50,51]. *P. crassa* was only previously known in Europe from a Polish antique collection [52], and it is now likely extinct at the collecting site [53]. This species' reappearance in Southern Europe could be due to a recent import that has made it invasive or naturalised. *P. crassa* has violaceous, resupinate to reflexed basidiomata, as well as encrusted, brown cystidia and sometimes originates from the subiculum. It causes intense white rot that colonises small- and medium-sized branches in broadleaves. As a result, it is an excellent candidate for biodegradation procedures.

3.3.2. Species of Special Concern for Nutraceutical/Medicinal Properties

Grifola frondosa and *Hericium erinaceus* are becoming increasingly scarce in the wild [4] and are widely known for their therapeutic potential. *Grifola frondosa* is a well-known edible fungus not only for its high nutritional content and flavor, but also for its bioactive properties and application in therapeutic goods [54]. *Hericium erinaceus* is a renowned medicinal mushroom that has been studied for the treatment of neurodegenerative diseases as well as for its metabolites, bioactive compounds and numerous nutraceutical properties such as anticancer, antioxidant, antimicrobial, neuroprotective, immunomodulatory and anti-aging [55–57].

Pleurotus eryngii frequently interacts with *Apiaceae* roots, including *Eryngium* spp. In this study, a strain of *Pleurotus eryngii* var. *ferulae* was isolated from the roots of *Ferula communis* L. The variant *ferulae* is a popular edible fungus due to the huge size of its

basidiomata and the nutraceutical benefits of its metabolites [58]. A strain of *Pleurotus pulmonarius* was also isolated; while being less popular than other *Pleurotus* species such as *P. ostreatus*, this species is important for its edible and therapeutic characteristics [57,59,60]. Finally, *Pleurotus* species have also been proposed for the biodegradation of pollutants and recalcitrant substrates such as polycyclic aromatic hydrocarbons [61], crude oil [62], bisphenol A [63] and phenolic compounds in olive oil wastewater [64].

3.3.3. Species of Special Concern for Enzymatic and/or Degradation Potential

Irpex lacteus is a white rot agent with significant biotechnological potential for water and soil bioremediation [65,66]. *I. lacteus* eso-enzymes have been shown to digest polycyclic aromatic tri- to hexa-cyclic hydrocarbons [67], TNT [68], synthetic dyes [69,70] and a variety of other contaminants [65,66]. The strain isolated in this study grew at the second fastest rate, demonstrating its usefulness for biomass production.

Phanerochaete sordida is a common and widespread species in Europe, where it is found on the decaying wood of deciduous trees, rarely conifers. Because of its wide distribution, it is a highly polymorphic species [71]. *Phanerochaete sordida* and *Phanerochaete chrysosporium* are two of the most investigated species for pollutant degradation due to their high enzymatic efficiency and fast growth. Although *P. sordida* has received less attention than *P. chrysosporium*, it has been reported to degrade pollutants such as polycyclic aromatic hydrocarbons [72], neonicotinoid insecticides [73] and pharmaceuticals [74]. This is significant since *P. sordida* strains can be gathered from temperate, boreal regions, unlike *P. chrysosporium*.

Three common *Stereum* species were isolated: *Stereum hirsutum*, *Stereum gausapatum* and *Stereum subtomentosum*. Because they produce aggressive white rot, they are all recognised as pioneers in the early stages of wood degradation. *Stereum hirsutum* is the most common species, with a seemingly cosmopolitan distribution and high growth rate. Because of these factors, it may be a suitable option for myco-material technologies [37]. Furthermore, Goppa et al. [75] identified *S. hirsutum* as a possible novel food based on a range of compounds discovered in the mycelium, including a high choline content. Accord- ing to Jovic et al. [76], *S. gausapatum* strains can produce an array of enzymes including laccases, manganese-dependent peroxidases, multifunctional peroxidases and lignin peroxidases. Because of the potential for biodegradation, this makes *S. gausapatum* a species of particular interest. Both the strains of *S. gausapatum* and *S. hirsutum* obtained in this work grew faster than the average.

Trametes versicolor is one of the most widely utilized fungal species due to its degradative properties and enzymes, as well as its ability to produce several metabolites, including medicinal ones [57,77]. It is currently regarded as a cosmopolitan species, although it is a highly variable and possibly complex species difficult to discriminate [78]. It has a high degradative capacity, causing branches and trunks to break down during the early stages of wood degradation. The isolation of various strains could aid in the further taxonomical and physiological (e.g., enzymatic) characterization of this species.

Trichaptum abietinum is a pioneer species that produces small basidiomata on newly fallen *Picea* and *Abies* logs and stumps. It is a fast-growing saprotroph that degrades the sapwood of dead hosts, causing white pocket rot. On the other hand, *Trichaptum biforme* also colonizes the earlier wood stages but is associated with hardwoods. *Trichaptum biforme* displays laccase and peroxidase activity and can decolorize several aromatic dyes, implying that it has good bioremediation potential [79–82].

4. Conclusions

Culture collections, in addition to their fundamental role of conserving fungal material ex situ, allow for the sharing, exchange and application of strains for knowledge and scientific research.

The newly established strain collection of Salamanca University contributes to the ex situ conservation of Mediterranean WDF. The new fungal strains culture collection

of Salamanca University contains 55 strains of WDF (corticioid, polyporoid and a few agarics) from 50 different species, 25% of which have never been described in pure culture before. The collection also includes four strains belonging to species not found in other major European collections: *Botryobasidium asperulum*, *Crustomyces subabruptus*, *Sertulicium granuliferum* and *Mycoacia gilvescens*.

These strains represent a subset of Mediterranean fungal diversity and provide a solid basis for future pure and applied research.

Several strains in the collection could be used for taxonomic and species distribution research, as well as applied mycology.

Botryobasidium asperulum and *P. rufa* need further research: the former to solve the controversies about its presence in Europe, the latter to disentangle its species complex and its phylogenetics. *Botryobasidium asperulum* is likewise interesting because of the complete lack of DNA sequences in databases. The medicinal properties of *Hericium erinaceus*, *G. frondosa*, *P. eryngii* and *P. pulmonarius*, as well as the increasing number of new bioactive compounds discovered, make these species stand out. Many of the isolated species, such as *I. lacteus*, *P. sordida* and *T. versicolor*, have a broad applicative spectrum. Finally, the fast growth and/or enzymatic potential of *S. gausapatum* and *S. hirsutum* make them promising candidates for degradation and bioremediation applications.

Future research goals also include exploring further methods to preserve the viability and full physiological functionality of the strains, e.g., by growing them on suitable lignocellulosic substrates in axenic conditions.

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Chapter 2. Preliminary myco-remediation tests

2.1 Case of study: two wastewater treatment plants in Lombardia (Italy). Issues and possible fungi-based solutions

This PhD project is a part of the "Circular Economy for Water and Energy" (CE4WE) project, funded by Regione Lombardia. All the activities conducted within this project were in collaboration with two companies involved in urban wastewater treatment: 1) Cap Holding s.p.a. deals with the water treatment in the province of Milano (Lombardia); 2) A2A life company, in particular the "Ciclo idrico" section, overseeing with water treatment in the province of Brescia (Lombardia). For confidentiality reasons, the names of the water treatment plants operated by these companies are not disclosed in this PhD project. The case study outlined here focuses on managing pollutants within the water treatment plants of these companies. The wastewater treatment plants (WWTPs) involved in this project operate in areas designated for agricultural purposes but receive wastewater from nearby large cities. Consequently, addressing the issue of pollutants and their potential impact on crops and human health is of paramount importance.

In civil wastewater treatment plants, the primary objective is to achieve discharge water that meets specific environmental standards, as discussed earlier. To achieve this, undesirable substances such as suspended solids, pathogens, nutrients, and potential pollutants must be removed. One of the fundamental purification processes involves the activated sludge tank, exploiting the activity of the microbial community. This process generates a byproduct known as sewage sludge—an initially liquid matrix with a variable solids load that may contain undesirable substances such as pathogens, heavy metals, and organic compounds, as well as nutrients like nitrogen and phosphorous (Fytili et al., 2008). The sewage sludge undergoes further treatment processes aimed at reducing water content, resulting in a dry waste that is less costly to dispose of.

Disposal methods for sewage sludge include various options such as incineration, landfill, and agricultural reuse (European Environmental Agency, 2019). Data spanning from 2013 to 2015 indicate that in most European Union countries, the predominant method for sewage sludge disposal is agricultural use (49%), followed by incineration (25%), recultivation/land reclamation (12%), landfill (9%), and other methods (5%). Sewage sludge disposal statistics for each country are illustrated in Figure 5, extrapolated by EurEau, 'The European Federation of National Associations of Water Services.'

Figure 5- Sewage sludge usage in European countries (Years 2013-2015). Data extrapolation is not avaiable for Austria, Bulgaria, Croatia, Denmark, Ireland, Serbia and Switzerland.

As previously mentioned, sewage sludge retains valuable nutrients, making it an excellent

fertilizer and transforming waste into a valuable resource. However, it is crucial to consider that sewage sludge may also contain pollutants, necessitating strict regulations for its use in agriculture. As reported by Smith in 2009, compounds that can be found in sewage sludges may include:

- Detergent residues;
- Pharmaceuticals, antibiotics, endogenous hormones and synthetic steroids;
- Heavy metals;
- Persistent compounds from incomplete combustion of fossil fuels;
- Persistent compounds from domestic sources
- Persistent compounds prohibited from use/manufacture, but present in the domestic environment;
- Compounds discharged to sewer used in industrial processes or domestically (including solvents, flame retardants or compounds that leach from plastics and surfaces during end-use);
- Compounds with endocrine-disrupting potential.

The European Directive 86/278/EEC addressed the need to regulate the use of sewage sludge in agriculture, establishing concentration limits and analytical techniques for monitoring heavy metals (Cd, Cu, Ni, Pb, Zn, Hg, Cr), nitrogen, phosphorus, pH, dry matter, and organic matter. Each member country is responsible for transposing these European directives into its national regulations. In Italy, specifically in the Lombardia region, the directive has been transposed through DECREE No. 6665 issued on 14/05/2019. This decree classifies sewage sludges for agricultural use into two quality categories characterized by two different sets of limit values: 'high-quality sludge' and 'suitable sludge.' The designation 'high quality' indicates stricter, and therefore lower, limit values compared to 'suitable' sludge. The limit values encompass physical and chemical parameters (pH, dry matter, etc.), heavy metal content, nutrient levels (total nitrogen, total phosphorus, total potassium, etc.), organic pollutants (e.g., various hydrocarbons), and biological and microbiological parameters. Sludge controls are in place to prevent or at least minimize health issues and the accumulation of substances such as heavy metals in the environment and agricultural products, as previously described.

Most of the substances found in sewage sludges are transported by wastewater entering wastewater treatment plants. Some of these substances are captured in the solids formed during the treatment processes, and thus in sewage sludges, while others may remain in the wastewater. Water entering treatment plants may contain:

-Nutrients such as Nitrates and Phosphates which, if not properly removed, can lead to eutrophication of the water bodies in which they are deposited;

-Halogens such as Fluoride, Chloride and Bromide. The latter can cause serious damage to aquatic organisms and plants;

-Heavy metals, already widely discussed and strictly controlled for adverse health effects;

-Emerging organic pollutants, which include numerous categories of substances (pesticides, hydrocarbons, and dyes) including persistent pollutants such as pharmaceuticals, which can bioaccumulate in food chains and transfer globally (Madhav *et al*., 2020).

As already described in the introduction of this PhD thesis, emerging pollutants such as pharmaceuticals have an impact on water quality, although there are still no regulations and established legal limits, only observation lists exist (Watch List). Until regulations are enacted, it is crucial to explore effective solutions to reduce the concentration of pharmaceuticals in water discharged into the environment.

In addition to the common practice of reusing sludge as fertilizer in agriculture, there is also the growing practice of reusing treated water for irrigation. Consequently, it becomes evident that the presence of compounds like pharmaceuticals, if not adequately removed, could pose health risks to consumers of products irrigated through this method.

The use of fungi and their diverse activities emerges as a viable and sustainable solution to address both the heavy metal content in sewage sludge and the pharmaceuticals in wastewater. As mentioned earlier, the ability of fungal mycelium to adsorb heavy metals on the cell wall surface of hyphae can be harnessed to accumulate and remove heavy metals from solid or semi-solid substrates, such as sewage sludge. This process, called adsorption, is passive and occurs through contact between the mycelium and the substrate, allowing for the use of dead biomass. The trapped metals can subsequently be recovered from the fungal biomass using acids.

On the other hand, the degradation of pharmaceuticals requires an active process dependent on the production of exo-enzymes. Fungal enzymes are tasked with breaking down molecules, such as lignin and cellulose in the wild, to provide nutrients for the fungus' metabolism. When these enzymes come into contact with pharmaceuticals, they break the bonds of their molecules, with the ease of degradation depending on the type of bonds. In this case, the fungal biomass must be used alive to maintain active enzyme production.

2.2 WDF's growth capabilities in sewage sludges

From the sampling campaigns that were carried out, several fungal species were identified as potentially useful for their application in myco-remediation.

Species used for experimental activities were chosen based on 4 criteria:

1. rapid growth of mycelium and general good colonization in culture conditions;
2. degradation potential due to their enzymatic production or degradation effects observed on natural substrates (i.e. aggressiveness toward plant host or wood condition after fungus attack);
3. presence of literature data proving their use for bioremediation;
4. taxonomic vicinity with species already used in bioremediation. This was considered to evaluate whether congeneric species possess similar degradative capacities compared to better-known species.

Among the available fungal strains in MicUNIPV research culture collection the following strains were chosen for the experimental tests (Table 4).

Table 4 - Strains of potential interest for experimental applications and criteria for which they were chosen.

Species	Strain code	Rapid growth	Literature data	Natural degradation	Taxonomical vicinity	Publications
Bjerkandera adusta (Willd.) P. Karst.	Bj.a.1	X	X			Gupta et al.,2011; Balaes et al., 2013; Kathiravan et al., 2021
Ganoderma lucidum (Curtis) P. Karst.	G.l.3		X		X	Kathiravan et al., 2021
Ganoderma resinaceum Boud.	G.r.9	X	X	X		Balaes et al., 2013
Irpex lacteus (Fr.) Fr.	I.lc.3	X	X			Novotny 2009; Balaes 2013; Kathiravan 2021;
Oxyporus latemarginatus (Durieu & Mont.) C.C. Chen & Sheng H. Wu	R3100XA2	X			X	
Perenniporia fraxinea (Bull.) Ryvarden	P.f.31	X	X	X		Sturini et al., 2017
Perenniporia meridionalis Decock & Stalpers	P.m.1		X			Doria et al., 2014
Pleurotus eryngii (DC.) Quél.	Pl.e.1	X	X		X	Kapahi et al., 2017; Kathiravan et al., 2021;
Pleurotus ostreatus (Jacq.) P. Kumm.	Pl.o.5	X	X			Kapahi et al., 2017; Kathiravan et al., 2021;
Schizophyllum commune Fr.	S.c.1	X	X			Gupta et al., 2011
Trametes gibbosa (Pers.) Fr.	Tr.g.1	X			X	

Trametes versicolor (L.) Lloyd	Tr.v.1	X	X			Gupta et al., 2011; Kathiravan et al., 2021;

The key element that guided the choice of almost all fungal strains was the speed of growth. This parameter is fundamental to accelerate the initial stages of any application, such as those related to biomass production and/or inoculum preparation. The speed at which the mycelium develops also serves to ensure good colonisation of the substrate and, in the case of non-sterile substrates, a greater resistance to competing organisms. Another important element was the existence of previous data about other bio-remediation capabilities, such as for example if these species had already been used for studies on other environmental contaminants, thus showing a certain predisposition in enzyme production and pollutant degradation. Ecological notes also led to the selection of two species, *Ganoderma resinaceum* and *Perenniporia fraxinea*, for the visible degradation they carry out to the detriment of their host plant, thus showing considerable enzymatic potential. A few species, *Ganoderma lucidum, Oxyporus latemarginatus, Pleurotus eryngii* and *Trametes gibbosa*, were chosen because of their taxonomical vicinity to other largely exploited or well-studied species, namely *Ganoderma resinaceum*, *Irpex lacteus*, *Pleurotus ostreatus* and *Trametes versicolor*.

After the identification of a pool of potential interesting species, preliminary experimental tests were carried out with the aim of setting up and defining future experiments.

Preliminary tests are tests and trials that are carried out before the main experimental phase in order to understand whether the application is feasible and which direction to take to make it so. In the case of this PhD thesis, two possible lines of research were initially outlined: one focused on the removal of heavy metals from sewage sludges and one focused on the concentration reduction of pharmaceuticals from wastewaters.

The heavy metal experimental line had as targets substrate the sewage sludge, a solid or semi-solid matrix that is not easy to operate with, while the pharmaceuticals experimental line had as a target substrate the wastewater, a liquid matrix in which a variable load of suspended solids is present.

Three groups of preliminary tests were carried out:

1) As mentioned earlier, WDF possess eso-enzymes capable of degrading wood components, the products of which are then used by the organism for its own metabolism. These enzymes have a low specificity and can therefore degrade a wide range of even very complex substrates and use them for growth (Hamman, 2004; Subramanian *et al*., 2014). Since under laboratory conditions WDFs are commonly grown in liquid solutions, the focus for preliminary growth tests was mainly on solid sludge, as a substrate that could prove problematic. Therefore, the first test was to evaluate whether the chosen fungal strains could exploit sewage sludge as a growth substrate, evaluating their colonisation capabilities.
2) Myco-remediation applied to heavy metals and metalloids exploits the phenomenon of adsorption whereby certain elements bind to sites on the fungal cell wall. Heavy metals can then be transported inside the cell and bound to other molecules (Chen *et al*., 2022). This makes it possible to lower the concentration of these elements by removing the fungus from the polluted system. The aim of the second preliminary test was to assess whether the selected fungal strains were able to adsorb and thus remove heavy metals potentially present in the sludge.
3) In contrast to heavy metals, the process that leads to the reduction of pharmaceuticals concentrations in water is related to the activity of endo- and eso-enzymes secreted by fungi (Naghdi *et al*., 2018). Enzymes attack the pharmaceutical molecule, breaking its bonds and thus registering a lower concentration of the initial compound. The aim of the preliminary tests was to evaluate the ability of selected strains to lower the concentration of certain pharmaceuticals in defined culture media and under controlled conditions.

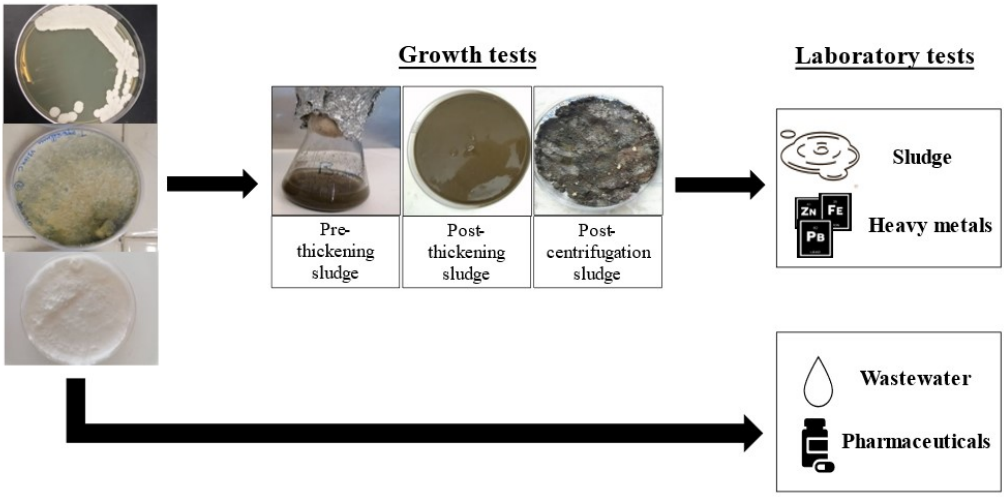

Figure 6- Graphical abstract to summarise the preliminary tests.

To better understand the potential of WDFs to remove heavy metals from sludges, several tests were performed. First of all, the growth capabilities of different fungal strains were tested on different types of sludges, in order to understand which were able to grow on this substrate and which were the fastest and most efficient at colonising it. Data obtained would also have been useful to identify which type of sludge would be the best for the application.

Therefore, 11 WDF strains (Table 5) were chosen for growth test on sludges.

Table 5- WDF strains tested.

Species	Strain code	Notes
Bjerkandera adusta	Bj.a.1_MicUNIPV	Saprotroph on dead and living deciduous trees; white rot fungus.
Bjerkandera adusta	V5100XC_MicUNIPV	Isolated through mycofloristic analysis from A2A WWTP.
Ganoderma lucidum	G.l.3_MicUNIPV	Saprotroph on deciduous trees, mainly oaks; white rot fungus. Isolated on *Quercus cerris.*
Ganoderma resinaceum	G.r.9_MicUNIPV	Saprotroph on living deciduous trees, mainly on *Quercus*; white rot fungus.
Lenzites warnieri Durieu & Mont.	L.w.5_MicUNIPV	Saprotroph on dead and living deciduous trees, white rot fungus. Isolated on *Quercus robur*.
Perenniporia fraxinea	P.f.31_MicUNIPV	Saprotroph on living deciduous trees or on large stumps; white rot fungus. Found on a wide range of host plants.
Pleurotus eryngii	Pl.e.1_MicUNIPV	Saprotroph on *Apiaceae*; white rot fungus.
Pleurotus ostreatus	Pl.o.5_MicUNIPV	Saprotroph on dead and living deciduous trees and rarely on coniferous trees; white rot fungus. Isolated on *Quercus robur*.
Schizophyllum commune	S.c.1_MicUNIPV	Saprotroph on dead deciduous trees; white rot fungus. Isolated on *Populus alba*.
Trametes gibbosa	Tr.g.1_MicUNIPV	Saprotroph on dead deciduous trees, rarely on living trees; white rot fungus. Isolated on *Salix alba*.

Trametes versicolor	Tr.v.1_MicUNIPV	Saprotroph on dead deciduous trees and rarely on coniferous trees; white rot fungus. Isolated on *Quercus robur*.

Strains selected for these tests are well-known species, characterized by fast growth and good colonization capabilities, both on natural substrates and on culture medium. Some of these species such as *B. adusta, P. ostreatus* and *T. versicolor* are also species already known for their myco-remediation potential.

Three types of sewage sludge were chosen for the tests: sludges taken before and after the thickening process and centrifuged sludge. Thickening process is a standard procedure in WWTPs aimed to increase the percentage of solid phase and at the same time reduce the liquid phase of the sludge. Sludge taken before the thickening process (Pre-thickening sludge) is a dense, but still liquid, matrix with a percentage of solids ranging from 0.6 % to 8 %. Sludge obtained after thickening process (post-thickening sludge) is a matrix with a lower water content and with a percentage of solids ranging from 2.5 % to 12 % (De Feo *et al*., 2014.) After thickening, the sludge undergoes centrifugation to further reduce the water content, thus obtaining a solid matrix: centrifuged sludge contains between 15 % and 50 % solids content (Novak ,2006). Thickening and centrifugation have the ultimate goal of reducing the final weight of the sludge. A schematized representation of WWTPs sludge line is reported in Figure 7.

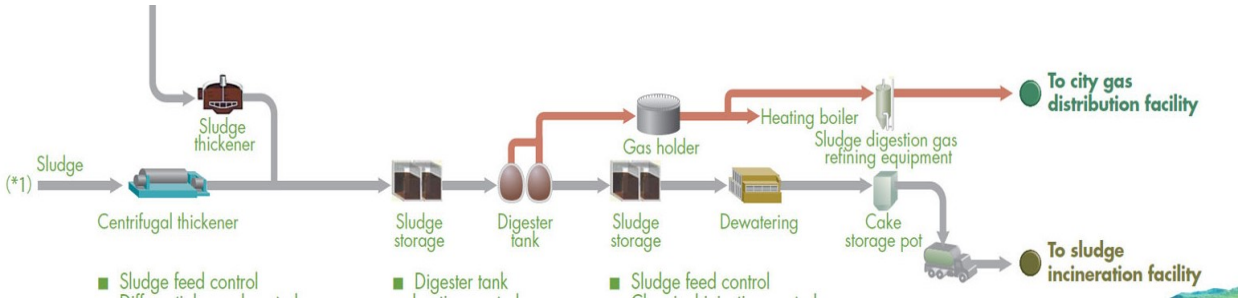

Figure 7- Overview of processes and treatments normally used in the WWTPs sludge treatment line (Yokogawa.com).

2.2.1 Materials and methods

Growth tests on centrifuged sludge were performed in two different conditions: 1) on sterilized sewage sludge as such; 2) on sterilized sewage sludge with the supplement of 1 % sawdust. The addition of sawdust was done to assess whether these fungi, which normally grow on woody substrates, needed a small content of lignocellulosic material to help them in the early stages of colonization. Growth on pre- and post-thickening sludge was tested on sludges taken from the two WWTPs partners in the project: post-thickening from the A2A WWTP and pre-thickening from CAP WWTP. All sludges were tested in axenic conditions (sludge was sterilized).

Each selected strain was grown in 100 mL flasks containing 50 mL Malt Extract Broth (ME) 2 % inoculated with 5 agar plugs with active growing mycelium. Flasks were incubated for 2 weeks at 24 °C in the dark.

Centrifuged sludge was put in sealed autoclavable bags and then manually mixed to homogenize it. In half of the bags 1 % w/w sawdust was added to evaluate if a nutritional starter was needed. The bags were sterilized for two subsequent days at 121 °C for 60 minutes. For each strain and for the control, petri dishes (15 cm diameter) were prepared. The day after the second sterilization, in a biological hood, 100 g of sludge were placed in each plate.

Pre- and post-thickening sludge, also called “slurry”, were put in flasks and sterilized for two

subsequent days at 121 °C for 60 minutes. For each strain and for the control, two 100 mL flasks were prepared. The day after the second sterilization, in biological hood, the slurry was homogenized, 50 mL were poured into each flask, inoculated and incubated at 25 °C for three weeks.

Mycelium obtained from the growth in the flasks was homogenized, obtaining a mycelium-medium suspension: inoculation of the sludge was made by withdrawing 4 mL of suspension and spraying it evenly on the centrifuged sludge and in the flask containing pre- and post-thickening sludge.

2.2.2 Results

A preliminary test was performed on sterile centrifuged sludge with a few selected species (*B. adusta*, *P. fraxinea*, *P. ostreatus* and *T. versicolor*) to verify if the growth tests procedure was functional at showing fungal growing capabilities on sludge. This preliminary test proved that WDF grow very well on this substrate forming a mycelium mat on the surface; *T. versicolor* was growing slow but at the end was able to colonize and consume the sludge.

Tests on centrifuged sludge were repeated on all the chosen strains: in this test some problems with sterilization occurred so there were some bacterial contaminations in the plates. Species like *B. adusta*, *P. ostreatus*, *T. gibbosa*, *T. versicolor* and *G. resinaceum* still managed to grow in the contaminated substrate. From these two tests it also emerged that sawdust supplementation as a nutrient starter doesn't help fungal growth, sometimes instead it can be detrimental, probably due to the fact that it represents another possible vector of contamination. For these reasons sawdust supplementation was discarded in the other tests.

Growth test results are reported in Table 6 as a qualitative data of sludge surface colonization.

Table 6- Growth tests results: 0) No growth 1) Sporadic growth 2) Partial colonization 3) ~50 % surface colonization 4) ~80 % surface colonization 5) Total colonization.

	Preliminary test – centrifuged sludge		**Centrifuged sludge**		**Slurry sludge**	
WDF strains	**Sludge as such**	**Sludge + 1%sawdust**	**Sludge as such**	**Sludge + 1%sawdust**	**Pre-thickening**	**Post-thickening**
B. adusta Bj.a.1		4	3-4	1	1	5
B. adusta V5100XC			2-3	1	1	5
G. lucidum G.l.3			0	0	1	0
G. resinaceum G.r.9			3	0	1	5
L. warnieri L.w.5			1	1	1	0
P. fraxinea P.f.31		5	0	0	1	5
P. eryngii Pl.e.1			0	2	1	0
P. ostreatus Pl.o.5	4		1-2	0	2	2-3
S. commune S.c.1			0	0	1	5
T. gibbosa Tr.g.1			1-2	1	1	5
T. versicolor Tr.v.1		5	0	1-2	2	1

Pre-thickening sludge was the substrate that gave the best result probably because was less dense, still liquid and therefore more similar to a cultural broth. Many strains managed to colonize the sludge forming a mycelium disk on its surface. On the contrary fungal growth has been limited on post-thickening sludge, forming only a thin mycelium layer on the surface; only *P. ostreatus* managed to form few mycelium flocks (Figure 8).

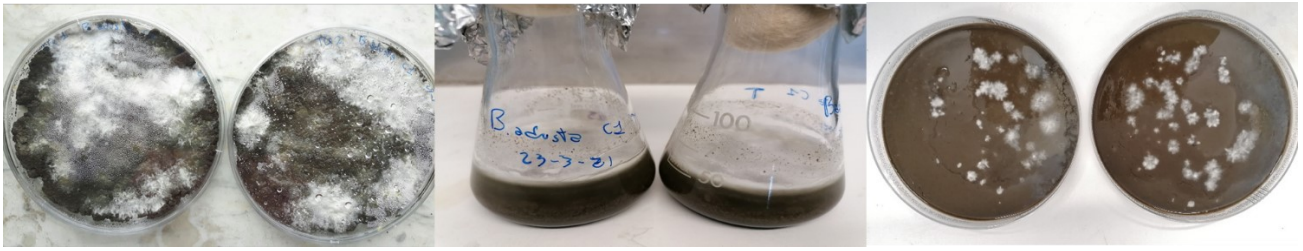

Figure 8- From left to right, WDF growth on: centrifuged sludge, pre-thickening sludge, post-thickening sludge.

Strains that shown the best surface coverage and fastest growth are *B. adusta* (both tested strains), *S. commune*, *T. gibbosa*, *G. resinaceum* and *P. fraxinea*. Based on these results, it was decided to use the pre-thickening sludge as the ideal substrate to test the removal of heavy metals by fungi, as it was the one that obtained the best colonization results.

2.3 Myco-remediation: fungal adsorbtion of heavy metals from sewage sludge

In collaboration with the Chemistry Department of University of Pavia (prof. A. Profumo research team) a preliminary experiment to evaluate fungi capabilities to adsorb/biosorb heavy metals was performed with the purpose to set a procedure and to obtain an initial feasibility indication. The aim of this experiment was to test whether a mass of mycelium placed in pre-thickening sludge was able to remove heavy metals (via adsorption and/or biosorption processes), reducing their concentration in the sludge (Figure 9).

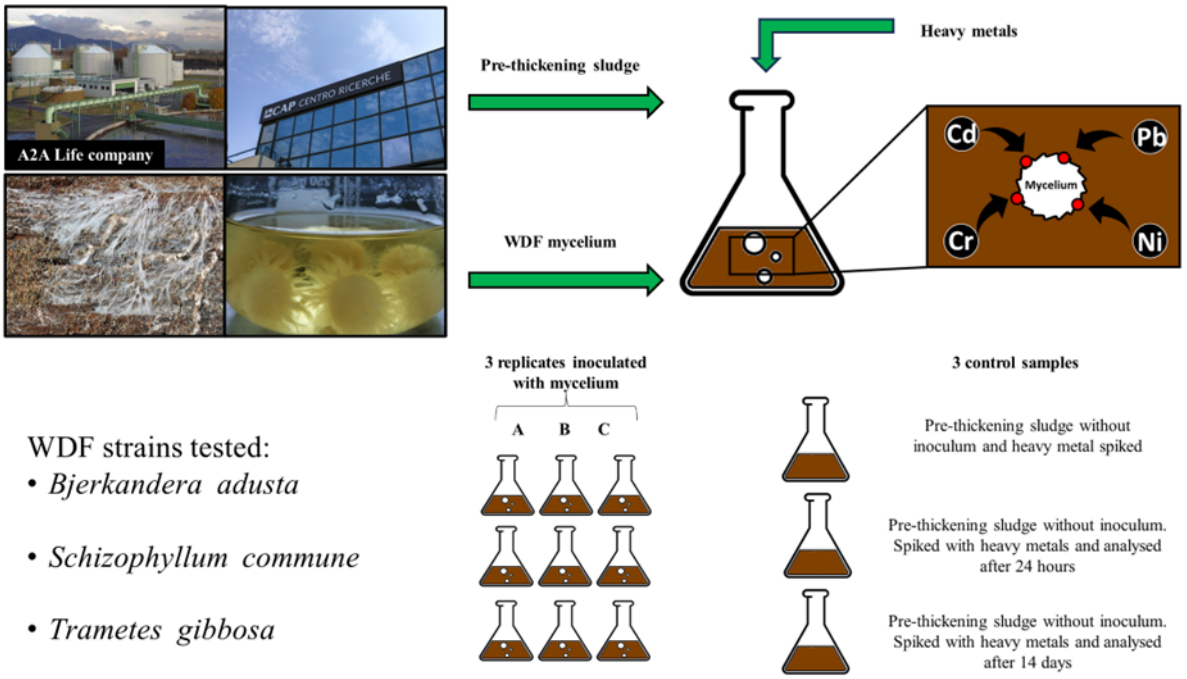

Figure 9- Graphical abstract of the preliminary test for mycelium based heavy metal adsorption.

2.3.1 Materials and methods

For this preliminary test the selected substrate was the pre-thickening sludge sampled from CAP and A2A WWTPs. This type of sludge, alongside the use of fungal biomass in the form of pellet (spherical mycelium structures), allows the mycelium surface, once submerged, to make full contact with the sludge and to be removed at the end of the experiment.

The slurry was sterilized through two cycles of autoclave at 121 °C for 60 minutes and for each trial 100 mL flasks containing 50 mL of sterile sludge were prepared. No previous data were available on the presence and concentration of heavy metals in the supplied pre-thickening sludge. A defined concentration of the elements of interest was spiked in the sludge to ensure a proper reading of the adsorption/biosorption phenomenon.

The concentrations of spiked heavy metals were chosen to simulate the suitability threshold for the sewage sludge use in agriculture (Table 7).

Table 7- Spiked heavy metals' concentrations.

	Cd (mg/L)	**Cr (mg/L)**	**Ni (mg/L)**	**Pb (mg/L)**
Suitability (Id)	0.6	6	9	22.5

Fungal strains chosen for this experiment were: *Bjerkandera adusta* (Bj.a.1_MicUNIPV), *Schizophyllum commune* (S.c.1_MicUNIPV) and *Trametes gibbosa* (Tr.g.1_MicUNIPV). All fungal strains were grown in 100 mL flasks containing 50 mL of Malt Extract 2 % inoculated with 5 agar

plugs taken from Petri dishes with active growing mycelium. Flasks were incubated on a shaker at 120 rpm at 25 °C. Flask agitation allows mycelium to grow in spherical structures (pellets), facilitating the harvest and the handling of the fungal biomass. Three 100 mL flasks containing 50 mL of slurry were prepared for each strain. Solutions of Cd, Cr, Ni and Pb were inoculated into each flask at the concentrations of heavy metals reported in the table above. This was repeated for the sludge of both WWTPs. Sludge spiked with heavy metals was let to rest for 24 hours before fungal inoculation.

Flasks were inoculated with mycelium as follows:

a) The content of flasks used for fungal biomass production was poured in a sterilized nylon to collect the mycelium pellets without the growth medium;
b) Pellets were washed with 500 mL of distilled water to remove Malt extract residues and then gently squeezed to remove excess water;
c) The biomass was weighed and divided into same weight aliquots;
d) In each flask 4g of mycelium (wet weight) were inoculated.

Three control flasks for each WWTP were prepared:

1) Flask containing slurry as such (not spiked) to evaluate the original content in heavy metals;
2) Flask containing slurry spiked with heavy metals and analysed after 24 hours;
3) Flask containing slurry spiked with heavy metals and analysed after 14 days.

All flasks were incubated at 25 °C in the dark in static conditions.

After incubation the mycelium was separated from the sludge. The sludge was mineralized in a microwave with nitric acid and analyzed at the ICP-MS. Microwave-assisted digestion was performed by a MarsXpress microwave system supplied by CEM (CEM s.r.l., Cologno al Serio, Italy), equipped with a 3 EasyPrep vessel carousel and internal temperature control. Each vessel contained 15 mL of sludge and 10 mL of nitric acid. Mineralization occurred for 20 minutes at 800 W power, 200 °C and 800 psi. Mycelium mineralization was achieved in Beckers containing nitric acid placed on heating plates. The mineralized samples (sludge and mycelium) were filtered through 0.45 µm paper filters and brought to a volume of 50 mL with ultra-pure water obtained by a Millipore MilliQ system. Samples were analysed by ICP-MS (quadrupole Elan DRC-e, PerkinElmer, Shelton, CT, USA) equipped with a standard ICP torch, cross flow nebulizer, nickel sampler, skimmer cones and dynamic reaction cell™ (DRC).

2.3.2 Results

The experiment revealed several critical issues related to the complexity of the substrate (pre-thickening sludge), which may have had negative consequences on the correct reading of the data.

1. The sludge is a non-homogeneous liquid suspension, so even when shaking the samples before aliquoting them, the weight of the 15 mL of sludge was variable. The average weight was about 14.64±0.87 g. Therefore, the concentrations are reported as µg/g in relation to the starting weight of the sludge.
2. Particulate matter from the sludge tends to adhere to the mycelium pellets. This becomes a problem as it is not possible to remove this particulate by washing the mycelium with water, as this procedure would also remove the metals adhered to the mycelium cell wall (Albert *et al*., 2018). This issue also implies a difficulty in precisely quantifying the concentration of metals adhered to and/or absorbed by the fungus and calculating the mass balance.

3. The sludge from the two WWTPs has different characteristics. Even within the same WWTP, the sludge may vary depending on the season and other factors. The sludge from CAP WWTP has the ability to buffer well against pH changes, not deviating from pH 7. The sludge from A2A WWTP doesn't possess the same ability therefore its pH dropped after the heavy metal spiking. To bring the pH back in line with other samples, additions of NaOH 1M were made.

Results are reported in Table 8 as the heavy metals concentrations in the untreated sludge (t_0), in sludge spiked with heavy metals after 24 hours (T1) and after 14 days (T14), and in sludge spiked and treated with the mycelium of the 3 fungal strains.

Table 8 – Heavy metals concentrations in untreated, spiked and treated sludge in the two WWTPs.

Pre-thickening Sludge	**Cd µg/g**		**Cr µg/g**		**Ni µg/g**		**Pb µg/g**	
CAP WWTP	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
T0	<		12.3	0.3	5.7	0.1	1.4	0.1
T1	0.40	0.06	15.6	1.2	14.3	0.9	14.8	1.6
T14	0.38	0.02	15.9	0.7	14.3	0.3	14.3	0.6
B. adusta	0.40	0.05	16.3	2.9	14.0	1.4	14.6	1.5
S. commune	0.40	0.00	16.8	0.1	13.8	0.2	14.5	0.1
T. gibbosa	0.34	0.13	13.5	4.2	12.0	2.2	12.5	3.3

Pre-thickening Sludge	**Cd µg/g**		**Cr µg/g**		**Ni µg/g**		**Pb µg/g**	
A2A WWTP	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
T0	<		0.2	0.04	0.1	0.05	<	
T1	0.3	0.01	4.5	0.1	54.2	1.40	11.0	0.4
T14	0.3	0.07	4.7	0.6	53.3	6.78	11.3	1.6
B. adusta	0.4	0.08	5.1	0.5	57.3	4.28	12.7	1.8
S. commune	0.6	0.07	7.0	0.7	66.4	3.78	18.1	1.6
T. gibbosa	0.4	0.01	4.8	0.1	53.8	1.08	12.2	0.2

The concentrations of metals found in the sludge after its contact with the fungal mycelium and those found in the control samples are similar. No data seem to indicate that this method leads to significant metal sequestration by the fungus. This behavior could, however, be caused by the long contact time, which after an initial adhesion of the metals on the cell wall could lead to their release into the sludge. Another possible cause can be attributed to the sludge tending to obstruct the mycelium leading to limited contact between the fungal hyphae and the entire volume of the sludge.

2.3.2 Conclusions

Although this preliminary experiment did not lead to any significant results it provided several indications. The complexity of the sludge matrix does not ease the study of the adsorbtion effect of tested strains. Sludge inhomogeneity and flocks obstructing the mycelium surface, preventing its contact with the rest of the sludge volume, limits the effectiveness of the fungal treatment. The sludge is a complex liquid matrix with high suspended solids that causes various complications to experimental procedures such as the samples preparation, the analytical readings and the mass balance calculations.Based on the results achieved with heavy metals and the promising preliminary data achieved with pharmaceuticals (Chapter 3.1) a decision was taken together with the two wastewater treatment companies: to abandon temporarily the heavy metal experimental line to focus the research efforts, time and resources on the pharmaceuticals degradation in wastewaters.

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Chapter 3. WDF capabilities to degrade pharmaceuticals in wastewater

Chapter 3 will illustrate all the experimental activities carried out on pharmaceuticals degradation in order to understand the different parameters for a correct application of myco-remediation.

The general objective is to start with laboratory experimentation and then transpose the results obtained to refine the application at pilot plant level. The pilot plant is a machinery that simulates, on a smaller scale, a wastewater treatment plant tank. This intermediate phase of the experimentation is crucial as it mediates the transition from the narrow laboratory scale to the broad scale of the entire water treatment facility.

As mentioned earlier, at this point of the PhD project, resources and research effort are focused on the study of pharmaceuticals and their degradability by WDF with the final aim to apply them in urban wastewater treatment plants.

To do so, it is important to understand:

1) the best fungal strain/s for this kind of application;
2) the type of wastewater to be used and therefore the WWTP tank in which the application will potentially take place;
3) the most effective type of fungal inoculum;
4) what are the dynamics and interactions between fungi and pharmaceuticals.

Therefore, a first experimental line (Figure 10) was set to select the best conditions before the application in the pilot plant.

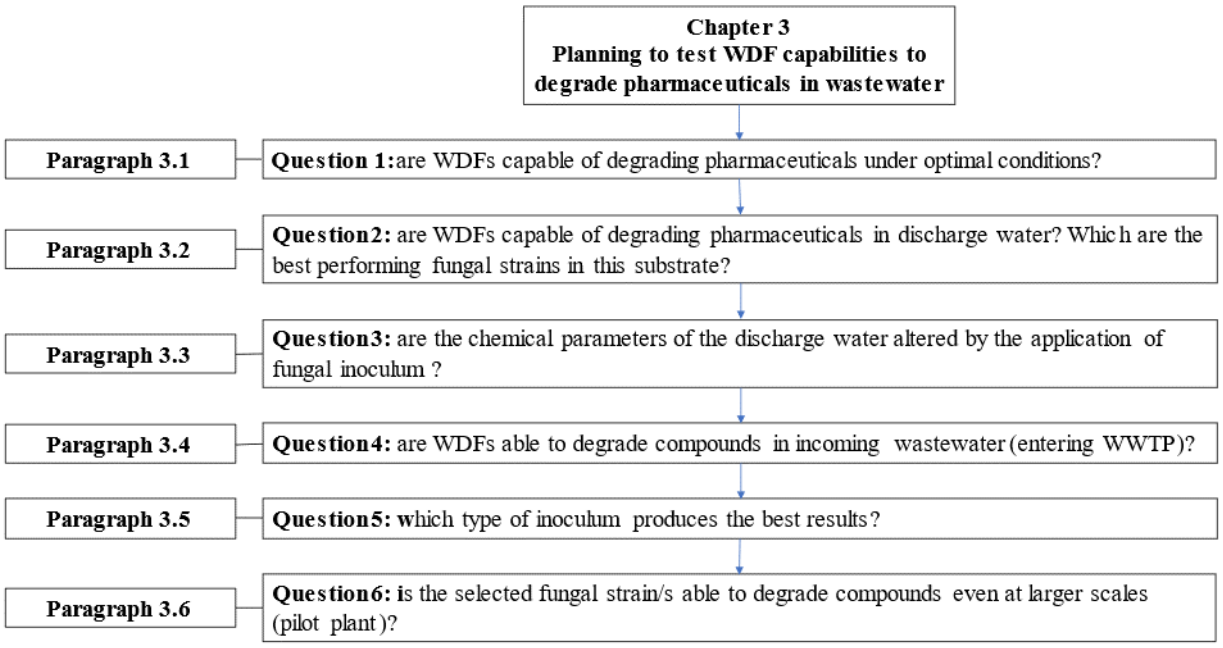

Figure 10- Scheme of the experimental campaigns aimed to identify the best fungal strain, type of inoculum and choice of wastewater tank for pharmaceuticals degradation.

3.1 Are WDFs capable of degrading pharmaceuticals under optimal conditions?

The degradation capabilities of wood decay fungi towards wide categories of pollutants have already been demonstrated by various studies (Vaksmaa *et al*., 2023). Even if the effects and degradative capacities of a given species are known, it is still important to investigate the individual fungal strains to be used, as in different contexts they may act differently. It is also important to test different species or taxonomically related species as their enzymatic production, and thus their ability to break the bonds of the target molecules, may also be different. As it will be discussed later, the structure of the pharmaceuticals molecules affects the degradative capability of the enzymes as certain bonds are more difficult to break than others (Yang *et al*., 2013).

The aim of this first experiment was to find out whether the chosen WDF possessed the ability to lower the concentration of chosen pharmaceuticals in a sterile and nutrient-rich environment (lab scale test).

To achieve this first goal, the following 7 WDF strains (belonging to MicUNIPV) were chosen:

1. *Bjerkandera adusta* (Bj.a.1)
2. *Ganoderma resinaceum* (G.r.9)
3. [*Omissis*]
4. [*Omissis*]
5. *Perenniporia fraxinea* (P.f.31)
6. *Perenniporia meridionalis* (P.m.1)
7. *Trametes gibbosa* (Tr.g.1)

Besides the criteria reported at the beginning of chapter 2, *B. adusta*, *G. resinaceum* and [*Omissis*] were chosen as they have been reported to degrade pollutants (Table 4); *T. gibbosa* and [*Omissis*] because are congeners with species studied for myco-remediation and *P. meridionalis* was taken into consideration due to its good production of Mn peroxidase, an useful enzyme for degradation processes (Doria *et al*., 2014, Girometta *et al*., 2017). *Ganoderma resinaceum* and *P. fraxinea* are very aggressive plant pathogens (Bernicchia and Gorjon, 2020) and they were included in the list of tested fungi as their aggressiveness in wild could be due to a strong enzyme production and thus they can achieve a good myco-remediation potential. If these two species proved to be excellent pharmaceuticals degraders, and given their nature as plant pathogens, it would become necessary to employ special measures to limit their dispersion in the environment.

For this preliminary investigation, 4 pharmaceuticals were chosen as target molecules: Diclofenac, Paracetamol, Ketoprofen and Irbesartan. The first three are among the most commonly used pharmaceuticals and therefore can be found in high concentrations in wastewater. Irbesartan, on the other hand, is a molecule that is difficult to degrade with conventional methods and which remains stable when subjected to photodegradation and to hydrolysis and oxidation under certain conditions (Husain *et al*., 2011; Ladhari *et al*., 2021).

3.1.1 Methods

Each fungal strain was cultured in 500 mL flasks containing 200 mL of 2 % Malt Extract (ME) liquid medium and inoculated with agar plugs (surface of about 1 cm^2) from plates with active growing mycelium (mycelium transplanted in new petri dishes 5 days before). Flasks were incubated for 10 days at 120 rpm at 25 °C. The agitation and the circular motion of flasks allows mycelium to grow forming spherical structures referred to as pellets. When growth was complete the content of each flask was drained and the pellets were washed with sterile distilled water, then gently squeezed to remove excess water.

For each strain, four 100 mL flasks containing 70 mL of ME 2 % were prepared (one for each pharmaceutical to be tested). In each of the four flasks, only one pharmaceutical was spiked to obtain the desired concentration of 10 µg/L. Flasks were inoculated with 1 g (wet weight) of mycelium pellets. A control flask for each pharmaceutical was prepared containing ME 2 % and the 10 µg/L

pharmaceutical solution in order to evaluate the pharmaceuticals' natural degradation. Each experiment was performed in triplicate and flasks were incubated in the dark at 25 °C in static (unstirred) conditions. An aliquot of liquid medium was taken from each flask after 24 h and after 7 days to analyse the pharmaceutical concentration. Samples were prepared by ultracentrifugation of 1 mL of each medium solution at 14,000 rpm for 5 min to remove fungal residues. The supernatants were analysed by high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS). Detailed parameters can be found in Buratti *et al*., 2023 (Chapter 3.8).

3.1.2 Results and discussion

The degradation of each pharmaceutical by the different fungal strains is shown in Table 9. The data are expressed as the percentage amount of pharmaceutical degraded compared to the control sample (malt extract spiked with the pharmaceutical but without fungal inoculum).

Table 9- Pharmaceuticals degradation percentages (mean of 3 replicates ± standard deviation) after 24 hours and after 7 days from fungal inoculum. Significantly different concentrations compared to the control are marked by * based on a t-test (* p-value < 0.05).

	Diclofenac		**Irbesartan**		**Ketoprofen**		**Paracetamol**	
Fungal strain	**24h**	**7d**	**24h**	**7d**	**24h**	**7d**	**24h**	**7d**
B. adusta	28 ± 12*	37 ± 25	-15 ± 11	3 ± 4	17 ± 4*	28 ± 3*	13 ± 10	100 ± 0*
G. resinaceum	38 ± 14*	73 ± 31	-6 ± 2	18 ± 1*	19 ± 4*	64 ± 12*	25 ± 4*	100 ± 0*
[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]
[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]	[*Omissis*]
P. fraxinea	52 ± 7*	49 ± 10*	-22 ± 3	22 ± 11	31 ± 13*	67 ± 11*	73 ± 14*	100 ± 0*
P. meridionalis	24 ± 13	21 ± 19	-13 ± 8	11 ± 3	7 ± 8	77 ± 0*	20 ± 10*	24 ± 13
T. gibbosa	7 ± 12	5 ± 13	-4 ± 8	11 ± 1	33 ± 1*	40 ± 2*	16 ± 7	100 ± 0*

Diclofenac concentration was lowered, with a statistically significant difference, by 5 out of 7 fungal strains. Among these, the highest degradation effects were recorded with *P. fraxinea*, 52 % degradation after 24 hours, and with [*Omissis*] and [*Omissis*], with a recorded degradation after 7 days of 93 % and 40 % respectively. Although *G. resinaceum* achieved an average degradation of 73 % after 7 days, and therefore the second highest for Diclofenac, it also showed a high standard deviation that makes this datum unreliable.

The degradation of pharmaceuticals depends on their molecular structure and the ease of fungal enzymes in breaking their bonds. Molecules with electron donating groups usually are more easily degraded by fungal activity than other molecules, while electron withdrawing functional groups make the molecule less oxidizable and thus more difficult to degrade (Yang *et al*., 2013). Diclofenac, even though it contains functional groups belonging to both categories, is reported to be a molecule easily degraded by fungal enzymes (Marco-Urrea *et al*., 2010; Dalecka *et al*., 2020).

Irbesartan was the most resistant to degradation by fungal activity. The presence of an amide group, electron withdrawing functional group, in the molecule could be the reason why the degradation rates obtained turned out to be very low. In addition, in some cases, higher concentrations were obtained and therefore negative degradation rates were recorded. This apparent increase effect can be caused by several factors that can alter the analytical readings, such as Irbesartan low water solubility or changes of pH caused by fungal metabolism that can alter the solubilisation and ionisation. Among fungal strains tested, only *G. resincaeum* and *O. latemarginatus* managed to achieve a small reduction in Irbesartan concentration (18 %), although only 7 days after fungal inoculation. *Perenniporia fraxinea* also achieved a 22 % concentration reduction, although not statistically significant.

Among strains tested on Ketoprofen 6 out of 7 achieved a statistically significant difference in concentration compared to the control sample. Although the degradation percentages obtained at

24 hours were quite low, the most significant were obtained at 7 days, achieving on average 69 % degradation. The highest degradation percentages were achieved at 7 days, in decreasing order, by *P. meridionalis* (77 %), [*Omissis*] (69 %), *P. fraxinea* (67 %) and *G. resinaceum* (64 %). Ketoprofen is a molecule that contains a carboxyl group, electron withdrawing functional group, and so, as is also the case with Irbesartan, its structure may be the reason why a significant decrease of the pharmaceutical concentration was only achieved at 7 days.

While Diclofenac was found to be the most easily degraded pharmaceutical after 24 hours, Paracetamol had the highest degradation percentages after 7 days. All strains managed to obtain a statistically significant concentration decrease compared to the control, with 4 out of 7 strains achieving complete pharmaceutical degradation (100 %) after 7 days, namely *B. adusta*, *G. resinaceum*, *P. fraxinea* and *T. gibbbosa*. Another noteworthy strain was [*Omissis*] which managed to obtain an 80 % degradation of Paracetamol. These high degradation rates can be explained with the molecular structure of Paracetamol that contains two electron donating functional groups and therefore making the molecule bonds easily attacked by fungal enzymes.

3.1.3 Conclusions

The results obtained show that the 7 WDF tested possess the ability to lower the concentration of the chosen pharmaceuticals, albeit with variable quantities and rates depending on the fungal strain, type of pharmaceutical and contact time.

The degradation percentages obtained after 24 hours from the fungal inoculum (mycelium pellets) have generally proved to be low, with the exception of those obtained from *P. fraxinea* which proves to be an efficient strain even in a short time. The highest concentration reductions were obtained at 7 days especially for Paracetamol and Ketoprofen. *G. resinaceum*, [*Omissis*], *P. fraxinea* and *B. adusta* showed the best performances. Irbesartan was partially degraded only by *G. resinaceum* and [*Omissis*].

The results obtained in controlled conditions provided an indication of which fungal strains are worth investigating to test degradation capabilities on real substrates such as wastewater, where the presence of multiple polluting molecules and microbial competition must be considered. For further experimentations using wastewater as substrate, *B. adusta* (Bj.a.1), *G. resinaceum* (G.r.9), [*Omissis*], [*Omissis*] and *P. fraxinea* (P.f.31) were selected.

3.2 Are WDFs capable of degrading pharmaceuticals in discharge water? Which are the best performing fungal strains in this substrate?

The following experiment was performed on a real aqueous substrate while, in general, literature data refer either to controlled laboratory conditions (see Chapter 3.1) or to synthetic substrates simulating wastewaters.

The first test was planned with the aim of removing any pharmaceuticals still present in the treated water before its release into the environment and in waterways. Treated water, henceforth referred to as discharge water, is a clear matrix that must respect well-defined parameters (see Table 1).

It was planned to carry out the test using both sterilized and not sterilized discharge water, in order to compare the results with the data obtained previously (3.1) and to evaluate the action of the fungal inoculum in a real substrate containing other microorganisms too. In this first experiment, 6 fungal strains were chosen to be tested for their degradative capacity towards 14 compounds chosen as target molecules. The same compounds were used in all the subsequent experiments. The aim of the experiments is summarized in the following scheme (Figure 11).

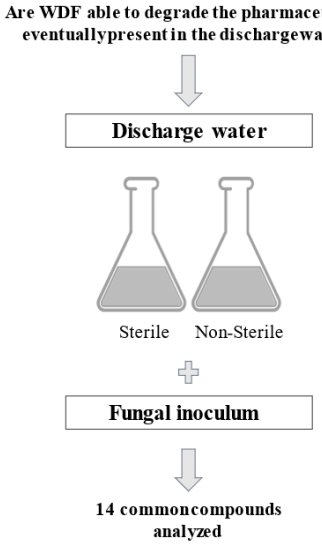

Figure 11-Schematization of the experiment aim.

3.2.1 Methods

Based on the results of previous experiment 6 fungal strains were chosen:

[*Omissis*].

The 14 compounds chosen as target molecules are reported in Table 10. These compounds are commonly used pharmaceuticals except for 5-Methyl-Benzotriazole that has different uses.

Table 10- List of target compound chosen to test fungal degradation.

Compound	CAS number	Compound usage
5-Methyl-Benzotriazole	136-85-6	Not an actual pharmaceutical but a corrosion inhibitor and ultraviolet light inhibitor, used for aircraft de-icing agents, plastic stabilizers, anti-fogging agents, pharmaceuticals, fungicides, paints and coatings
Amisulpride	71675-85-9	Antipsychotic and antidepressive agent
Azithromycin	83905-01-5	Antibiotic
Carbamazepine	298-46-4	Anticonvulsant and analgesic
Clarithromycin	81103-11-9	Antibiotic
Diclofenac	15307-86-5	Non-steroidal anti-inflammatory agent with antipyretic

		and analgesic actions
Gabapentin-Lactam	64744-50-9	Transformation product of gabapentin (anti-epileptic)
Irbesartan	138402-11-6	Non peptide angiotensin II antagonist with antihypertensive activity
Ketoprofen	22071-15-4	Anti-inflammatory analgesic and antipyretic
Lamotrigine	84057-84-1	Antiepileptic and analgesic
Metoprolol	51384-51-1	Beta-adrenergic antagonist and antihypertensive
Ofloxacin	82419-36-1	Antibiotic
Propyphenazone	479-92-5	Non-steroidal anti-inflammatory and non-narcotic analgesic
Sulfamethoxazole	723-46-6	Antibiotic

The experiment was performed as follows:

[*Omissis*].

1) After 48 hours a water sample was taken from each flask and analysed by Eurolab Analysis and Results Srl. (Vicenza) by a liquid chromatograph coupled to a mass spectrometer (LC-MS/MS) to determine the concentration of the 14 compounds. The analytical limit of quantification was 10 ng/L.

Experimental set and flasks preparation is schematized in Figure 12.

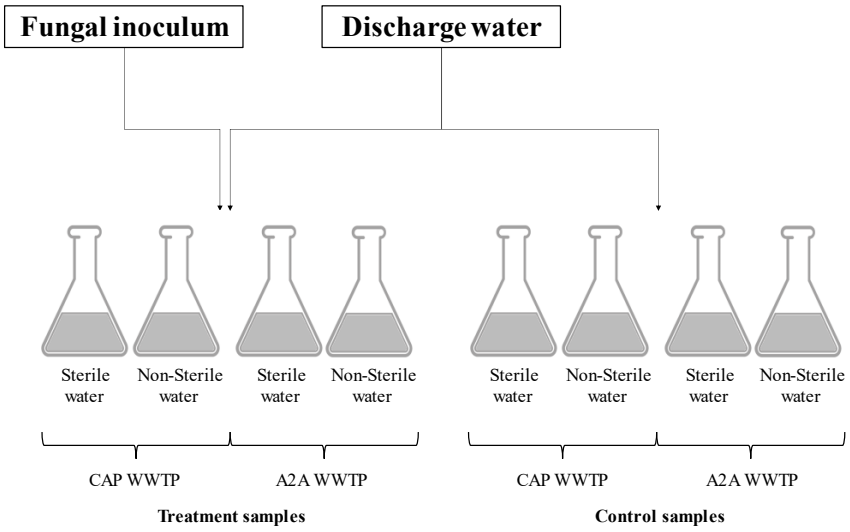

Figure 12- Schematization of the experimental set.

3.2.2 Results and discussion

The actual compounds concentrations found in the discharge water are considered sensitive data and belong to the two wastewater treatment companies (CAP and A2A), therefore concentrations cannot be disclosed as such. Data reported below are expressed as the percentage reduction of the 14 compounds' concentration compared to those in the respective control sample.

Degradation percentages for each WWTP and for sterile and non-sterile discharge waters are reported in Table 11-14.

Table 11- Degradation percentages (compared to control) obtained by each fungal strain after 48 hours in CAP WWTP sterile discharge water.

[*Omissis*]

Table 12- Degradation percentages (compared to control) obtained by each fungal strain after 48 hours in CAP

WWTP non-sterile discharge water.
[*Omissis*]

Table 13- Degradation percentages (compared to control) obtained by each fungal strain after 48 hours in A2A WWTP non-sterile discharge water.
[*Omissis*]

Table 14- Degradation percentages (compared to control) obtained by each fungal strain after 48 hours in A2A WWTP non-sterile discharge water.
[*Omissis*]

The discharge waters supplied by the two WWTPs had differences in the most represented compounds and in their starting concentrations. One of the critical issues with this type of work is the non-reproducibility of the substrate in different experiments as the wastewater has different contents depending on the season, the water catchment area and can differ due to uncontrollable variables. Furthermore, it is not possible to store the water for future uses as storage, whether at room temperature or at controlled low temperatures, alters the water parameters. In both WWTPs, [*Omissis*] was the most concentrated compound. In the water sample from CAP WWTP [*Omissis*] showed the highest concentrations. In the water sample from A2A WWTP, the most concentrated compound were [*Omissis*].

It can be observed that degradation percentages were, in most cases, higher in sterilised discharge water than in non-sterilised water. Looking at the pharmaceuticals individually, an higher number of fungal strains managed to reduce at least 20 % of the concentration in sterile discharge water compared to when tested in non-sterile water. This phenomenon can easily be explained by the fact that there are no other microorganisms in the sterile water, which represent for the fungus both competition for available resources and a source of stress as some organisms may feed on the fungus hyphae.

In CAP discharge water, the most easily degraded compounds, those with the highest degradation percentages, were: [*Omissis*] in sterile discharge water and [*Omissis*] in non-sterile discharge water. The situation in A2A discharge water was similar, where the most degraded compounds were: [*Omissis*] in sterile discharge water and [*Omissis*] in non-sterile discharge water. [*Omissis*] were the two most degraded compounds in both WWTPs in non-sterile water, and therefore under the most unfavourable conditions. These two compounds possess electron donating functional groups in their molecular structure, in particular amine groups ($-NH_2$), which makes them easily degradable molecules. On the other hand, for some compounds such as Diclofenac, Irbesartan and Ketoprofen, the opposite behaviour was observed, i.e. an apparent increase in concentration with negative degradation percentages. This phenomenon is also reported in other studies and could be caused by desorption and/or deconjugation processes. The first phenomenon involves the liberation into the water of compounds trapped in suspended solids by the activity of microorganisms (bacteria, fungi or others). The liberation of the entrapped compounds (also thanks to the mechanical activity of hyphae) increases the concentration and leads to an underestimation of degradation percentages. The second phenomenon that could occur is deconjugation. Pharmaceuticals that enter the human body (parent compounds) could transform in one of their metabolites to increase the absorbtion of the compound by the organism. Both the parent and metabolized (also called conjugated) pharmaceuticals are excreted from the body and therefore could be found in wastewaters. The deconjugation process could transform the conjugated metabolites back to their parental form, leading to an increase of the target compound concentration (Lishman *et al*., 2006; Celiz *et al*., 2009; Brown and Wong, 2015; Brown and Wong, 2018). These two phenomena occur naturally in wastewater and therefore were not observed in the experiment described in the previous section.

Observing the individual performance of the fungal strains, two in particular appear to be the best performing: [*Omissis*]. These two strains were able to obtain on average an higher degradation percentages compared to the other strains and a good degradation on a greater number of compounds,

even in non-sterile discharge water.

[*Omissis*] achieved good degradation in CAP discharge water for the following compounds: [*Omissis*]. In A2A discharge water, good degradation percentages were achieved in for: [*Omissis*].

[*Omissis*] achieved good degradation in CAP discharge water for the following compounds: [*Omissis*]. In A2A discharge water, good degradation percentages were achieved for: [*Omissis*].

Even if the degradative activity of the two best strains was often very similar, [*Omissis*] turned out to be more versatile as it degraded compounds on which the activity of [*Omissis*] was more limited (for example [*Omissis*]) (Figure 13).

[*Omissis*]

Figure 13- [*Omissis*] and [*Omissis*] degradation percentages (compared to control) in sterile and non-sterile discharge water of after 48 hours from inoculation.

3.2.3 Conclusions

The results obtained confirm that WDF are able to degrade emerging pollutants such as pharmaceuticals in real wastewater. WDF strains tested were able to reduce the concentration of the chosen target compounds. Some pharmaceuticals proved to be more easily degraded by fungal enzymes ([*Omissis*]), while others proved to be more problematic ([*Omissis*]).

The comparison of sterile and non-sterile water served to show how fungi are able to carry out degradation even in non-sterile substrates and with conditions potentially hostile for fungal survival and activity. Since, however, sterilisation of the substrate is an unfeasible procedure for the scale-up process and the data on non-sterile water was encouraging, it was decided to use exclusively non-sterile water for subsequent experiments.

Among the 7 fungal strains chosen, [*Omissis*] and [*Omissis*] proved to be the best in terms of the amount of compound degraded and the number of compounds their degradation affected. However, of these two, [*Omissis*] proved to be the best performing. For this reason, [*Omissis*] was the selected strain for the subsequent experiments aimed at optimizing and refining the procedures for the pilot plant scale up.

Once the effect of WDF on the tested compounds has been clarified and the best performing strain has been selected, a number of parameters must be verified.

1) The substrate used, discharged water, is a delicate part of the water treatment process as it is the water that is discharged into natural waterways and therefore subjected to legal restrictions and limitations. For this reason, it must be verified that the fungal treatment does not alter the physical/chemical parameters of the water, rendering it no longer compliant with the law limitations.

2) To verify fungal activity, the concentration of the 14 compounds was analysed only 48 hours after inoculation. It is therefore needed to verify the concentration trend, also in view of the desorption and deconjugation phenomena, by carrying out analyses over a longer period of time.

3.3 Are the physical/chemical parameters of the discharge water altered by the application of fungal inoculum?

The experiments carried out in chapter 3 have a final purpose: to collect data in order to optimise the application of myco-remediation on a pilot plant scale. Once the best strain was selected, the focus was set on the application optimisation and on its feasibility. The analysis carried out 48 hours after inoculation gave the right indications as to whether or not there was activity on the target compounds but gave no information on how the concentrations of these compounds change over time. For this reason, it was decided to carry out an experiment using only non-sterile discharge water, analysing the same 14 compounds and taking water samples after 24 hours, 48 hours, 72 hours and 7 days from the mycelium inoculation. This was done to understand not only the trend of the concentrations but also the best mycelium-wastewater contact time. In this experiment, in addition to the control sample (non-inoculated sample analysed at the same times of the treated samples), another control was added: a water sample analysed just after its collection from the treatment plants, thus representing the sample at time zero (t_0).

It is also of fundamental importance to understand whether the fungal biomass and its activity can considerably alter the main parameters of the discharge water. An alteration of the water that brings the physical/chemical parameters close to or beyond the legal limits would render the application immediately unfeasible. Therefore, analyses were carried out on some fundamental parameters of the discharge water. The newly tested parameters and the respective emission limits (as reported in DECRETO LEGISLATIVO 3 aprile 2006, n. 152) are reported in Table 15.

Table 15- Tested parameters and emission limits depending on the potential of the treatment plant, expressed in Equivalent Population (EP).

	Emission limits (mg/L)	
Tested parameters	**10000-100000 EP**	**>100000 EP**
Ammoniacal nitrogen	/	/
Conductivity	/	/
Total Nitrogen	15	10
Total phosphorus	2	1
	2000-10000 EP	**>10000 EP**
Chemical Oxygen Demand (COD)	125	125
Total suspended solids (SST)	35	35

3.3.1 Methods

The preparation procedures of the biomass, of the flasks containing the discharge water, and inoculation procedures are the same described in section 3.2.1. The only differences are that this time only non-sterile water was used and 4 flasks were prepared for each sampling time: two for each WWTP, one inoculated with mycelium pellets and one not inoculated (control sample).

At each sampling time (24 hours, 48 hours, 72 hours and 7 days) two water samples were taken: the first was analysed by Eurolab s.r.l. to evaluate the concentration of the 14 target compounds, the second was analysed by A2A Ciclo idrico to evaluate the physical/chemical parameters of the discharge water.

3.3.2 Results and discussion

Data reported are expressed as the percentage reduction of the concentration of 14 compounds compared to the concentration in the Time zero (t_0) control sample and in the control sample taken at the corresponding sampling time. Degradation percentages for each WWTP and compounds concentration trends are reported in Table 16 and Figure 14 for CAP WWTP and in Table 17 and

Figure 15 for A2A WWTP.

Table 16- Compounds degradation percentages (compared to the reference control sample: T0 control sample analysed after its collection from the WWTP; 24h,48h,72h and 7d control sampled analysed after the indicated time) in CAP discharge water inoculated with [*Omissis*].

[*Omissis*]

[*Omissis*]

Figure 14- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in CAP discharge water: BLUE LINE control sample non-inoculated water, ORANGE LINE water inoculated with [*Omissis*] mycelium.

Table 17- Compounds degradation percentages (compared to the reference control sample: T0 control sample analysed after its collection from the WWTP; 24h,48h,72h and 7d control sampled analysed after the indicated time) in A2A discharge water inoculated with [*Omissis*].

[*Omissis*]

[*Omissis*]

Figure 15- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in A2A discharge water: BLUE LINE control sample non-inoculated water, ORANGE LINE water inoculated with [*Omissis*] mycelium.

In the previous experiment [*Omissis*] were the most degraded compounds.

The data reporting the high degradation of [*Omissis*]were confirmed also in this experiment. In CAP discharge water the degradation of [*Omissis*]continued after the 48-hour threshold with an average degradation respectively of [*Omissis*]. In A2A discharge water these two compounds were effectively degraded ([*Omissis*]) only after 7 days, due to a different behaviour of the concentration trends compared to those reported in CAP discharge water. In this experiment [*Omissis*] were degraded at lower rates compared to the previous data. Their concentration variation also shows an increase in both the control and the treated sample after the 48-72 hours threshold.

It should also be noted that, in this experiment and only in CAP discharge water, [*Omissis*], which was the most degraded molecule in the previous experiment, has very low degradation percentages. This is explained by the fact that, this time, in the water coming from CAP WWTP the concentration of the pharmaceutical was initially already very low and close to the analytical limit of quantification. This leads to an incorrect interpretation of the degradation percentages, making them unreliable. [*Omissis*] analysed in A2A discharge water is confirmed to be the most easily degraded compound by fungal activity.

In some cases, such as [*Omissis*] (only in CAP water), the concentration of the control samples was lower than those in the treated samples, while in [*Omissis*] the control sample concentration tends to rise at 7 days.

In many samples, the compounds appear to follow a trend with a rise in concentration up to 48 hours and a decrease at 72 hours, after which the concentration either remains stable or tends to rise again at 7 days. This variation in concentrations is also found in control samples where the trend overlaps or is 24 hours out of phase compared to trend of treated samples. Thus, the two phenomena of desorption and deconjugation may also occur in the absence of the fungus and may be accentuated in its presence.

Analyses carried out on the physical/chemical parameters of the discharge water (Table 18) revealed some critical issues. It was found that some parameters, once treatment with fungal mycelium had begun, increased to levels near the legal limits. In particular, total nitrogen (48h), total phosphorus (24-48h), COD (24-48h) and total suspended solids (48h only in CAP water) reach high values compared to the control samples. This significant alteration of parameters makes fungal treatment in the discharge water risky, as it could render the treated water no more compliant with

the legal limits.

Table 18- Chemical/physical parameters of treated and un-treated discharge water samples.
[*Omissis*]

A further observation, which also emerged in the previous experiment, is that the presence of mycelium in the water stimulates the formation of a biofilm composed of microorganisms (mainly bacteria and protozoa) that tends to slowly damage the mycelium. Discharge water is a substrate relatively poor in nutrients and this may affect the mycelium's ability to survive and withstand against microbial competition. It is therefore necessary to ensure that the fungus has more nutrients to grow and that it doesn't represent a source of nutrition for other organisms.

3.3.3 Conclusions

The analysis of the concentration of the 14 compounds over a time span of 7 days yielded interesting results but also revealed some critical issues. It appears that in most cases the contact time leading to the highest degradation percentages is between 48 and 72 hours. The concentration of the compounds, however, tends to vary considerably, probably due to desorption and/or deconjugation effects that also occur in the control samples. Concentration trends also vary depending on the compound and on the source of the discharge water.

The analysis of the physical/chemical parameters of the discharge water also showed how the activity of the mycelium tends to alter considerably the water parameters compared to those of the control samples, making this type of application on discharge water risky and not feasible.

Therefore, it was decided to change the substrate from the discharge water (coming out of the treatment plant) to the water entering the treatment plant. In this substrate, for an eventual application in the treatment plant, changes in physical/chemical parameters would be less impactful as still at the beginning of the water treatment line. This substrate is also richer in nutrients available for the microorganism and the fungus which could therefore be able to survive better.

3.4 Are WDFs able to degrade compounds in incoming wastewater (entering WWTP)?

The new substrate chosen to test [*Omissis*] activity is here called "incoming water", the wastewater sampled after the initial screening phase. In this phase the wastewater from the sewers passes through a metal grate where wastes and coarse solids are removed. The incoming water of the two WWTPs, however, differs slightly due to the path it follows and the structure of the treatment facility. CAP incoming water can be taken directly after the initial solids screening, whereas A2A incoming water can only be taken once it reaches the equalization tank, where the water contents are diluted and homogenized. Unlike discharge water, incoming water is no longer a clean matrix but a turbid one, as it contains a higher amount of suspended solids but also more nutrients and microorganisms.

The main objectives of this experiment are two: 1) to verify the activity of [*Omissis*] in the non-sterile incoming water, analyzing the concentrations of the 14 target compounds at the same sampling times used in the previous experiment; 2) evaluate the effect of two different types of inoculum, one composed by only mycelium and one by mycelium and its culture broth. Analyses on the physical/chemical parameters of the incoming water were repeated as well.

3.4.1 Methods

[*Omissis*]

1) A water sample from each WWTP was analysed just after its collection from the treatment plant, thus representing the sample at time zero (t_0).
2) Flasks were incubated at 25 °C in the dark in static conditions.
3) At each sampling time a water sample was taken from the corresponding flask and the concentration of the 14 target compounds was analysed by Eurolab s.rl. A water sample was taken for the analysis of the water physical/chemical parameters as well.

[*Omissis*]

Figure 16- Schematization of the experimental set.

3.4.2 Results and discussion

Data reported are expressed as the percentage reduction of the 14 compounds' concentration compared to the concentration found in the Time zero (t_0) control sample and in the control sample taken at the corresponding sampling time. Results obtained from samples inoculated with culture broth were corrected taking into account the dilution factor. Degradation percentages for each WWTP and compounds concentration trends are reported in Table 19 and Figure 17 for CAP WWTP and in Table 20 and Figure 18 for A2A WWTP.

Table 19- Compounds degradation percentages (compared to the reference control sample: T0 control sample analysed after its collection from the WWTP; 24h,48h,72h and 7d control sampled analysed after the indicated time) in CAP incoming water inoculated with [*Omissis*]. Type of inoculum is reported as follows: only mycelium (myc) and mycelium + culture broth (myc+CB).

[*Omissis*]

[*Omissis*]

Figure 17- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in CAP incoming water: BLUE LINE control sample non-inoculated water, ORANGE LINE water inoculated with [*Omissis*] mycelium and its culture broth, GREY LINE water inoculated with only [*Omissis*] mycelium.

Table 20- Compounds degradation percentages (compared to the reference control sample: T0 control sample analysed after its collection from the WWTP; 24h,48h,72h and 7d control sampled analysed after the indicated time) in A2A incoming water inoculated with [*Omissis*]. Type of inoculum is reported as follows: only mycelium (myc) and mycelium + culture broth (myc+CB).

[*Omissis*]

[*Omissis*]

Figure 18- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in A2A incoming water: BLUE LINE control sample non-inoculated water, ORANGE LINE water inoculated with [*Omissis*] mycelium and its culture broth, GREY LINE water inoculated with only [*Omissis*] mycelium.

The results obtained from the analysis of incoming water revealed that this substrate does not constitute a problem for the fungus activity, with degradation rates in some cases higher than those obtained in the discharge water. The degradation in incoming water achieved positive results but slightly different between the two WWTP due to the composition diversity of the water.

In CAP incoming water, it was observed that:

-[*Omissis*] is degraded very well by the mixed mycelium and culture broth inoculum with a decrease in concentration that remains stable at [*Omissis*] until 72 hours, after which it starts to increase.

-[*Omissis*] was gradually degraded up to [*Omissis*] at 7 days in water samples treated with the mixed inoculum.

-[*Omissis*] concentration was also reduced by [*Omissis*] in 7 days by the mixed inoculum.

-[*Omissis*] were confirmed as some of the most degraded compounds with degradation percentages respectively of [*Omissis*] (after 48 hours) and [*Omissis*] (after 7 days) achieved by the mixed inoculum.

-[*Omissis*] were present in the incoming water at very low concentrations, close to the analytical limit of quantification, so the degradation rates are not reliable.

-[*Omissis*] showed higher concentrations in the treated samples compared control samples.

In CAP incoming water the mixed mycelium/culture broth inoculum showed an increased degradative capability on [*Omissis*] compared to those recorded in discharged water.

In A2A incoming water, it was observed that:

-[*Omissis*] was degraded by [*Omissis*] after 24 and 48 hours by the mixed inoculum.

-[*Omissis*] concentration was reduced by [*Omissis*] in the first 24 hours by the mixed inoculum.

-[*Omissis*] concentration was about [*Omissis*] lower compared to control for the entire duration of the experiment in water samples treated by the mixed inoculum.

-[*Omissis*] was reduced by [*Omissis*] in the first 24 hours by mixed inoculum while [*Omissis*] was reduced by [*Omissis*] in 24 hours by both mycelium-only and mixed inoculum.

-As regards [*Omissis*] similar data to those achieved in CAP incoming water were obtained, with degradation percentages respectively of [*Omissis*] (after 48 hours) and [*Omissis*] after 7 days, both achieved by the mixed inoculum.

- [*Omissis*] achieved [*Omissis*] degradation after 72 hours by mixed inoculum and [*Omissis*] after 72 hours by mycelium-only inoculum.

-In A2A incoming water [*Omissis*] showed higher concentrations in the treated samples compared to control samples.

In A2A incoming water the new mixed inoculum achieved higher degradation percentages for almost all compound, with the exception of [*Omissis*], compared to those obtained in discharge water.

The samples treated with the mixed inoculum gave better results than those with only the mycelium, with lower compounds concentration recorded in the first 72 hours. With some compounds it seems that the better degradation capabilities given by the culture broth also increase the effects of desorption and/or deconjugation phenomena, while these are less present in the control samples and in those inoculated with only mycelium. This is particularly visible after 72 hours where the compounds concentration tends to rise again.

Observing the concentration trends in the samples inoculated with only mycelium and in the samples with the mixed inoculum, two specular trends are recorded in the first 72 hours. For some compounds, in the presence of only mycelium, the concentration increases up to 48 hours and then

decreases afterwards. This can be explained by the fact that the mycelium still has to produce the enzymes and therefore initially the only active process available is the mechanical one by hyphae, which break up the particulate releasing the compounds entrapped. On the contrary, in some compounds the presence of the mixed inoculum causes the concentration to decrease in the first 48-72 hours. This is probably because the possible presence of enzymes in the culture broth immediately starts the degradative process lowering the concentrations. After 72 hours the desorption and deconjugation processes increase the concentrations again.

The advantage of an inoculum with mycelium and its culture broth is that enzymes are already present as they are released by the mycelium during the growth/incubation process. Introducing enzymes into the substrate accelerates the degradation of the compounds in the water, giving the mycelium an advantage both in terms of its degradative capabilities and in terms of survival. It has been observed that the mycelium in the presence of its own culture broth seems to better resist the competition of other microorganisms, with mycelium pellets still well formed after 7 days. In samples where only the mycelium was present, after 7 days, the pellets were very small in size and less in quantity (Figure 19). The inoculation of the culture broth potentially still rich in nutrients constitutes an advantage both for the fungus and for the other microorganisms. However, the already available nutrients contained in the cultural broth allows the mycelium to survive better and to keep producing biomass.

Figure 19- Flasks after 7 days containing: 1) Incoming water without fungal inoculum; 2) Incoming water with mycelium only inoculum 3) Incoming water inoculated with mycelium and its own culture broth.

Physical/chemical parameters of water samples also show a difference between the two types of inoculum, as reported in Table 21. The changes that occur in the water of the two WWTPs after fungal inoculation are similar. The effects caused by inoculation with the culture broth are much more pronounced since nutrients increase the activity of the fungus and of the other microorganisms. There is a reduction in ammoniacal nitrogen and a simultaneous increase in total nitrogen and total phosphorus. Due to the nutrients introduced by the mixed inoculum, the increase in fungal biomass led to a significant increase in COD and total suspended solids. The reduction of the pH to more acidic values in samples inoculated with mixed inoculum may be caused by the fungus trying to create an hostile environment for bacterial proliferation.

Table 21- Average values of chemical/physical parameters of control samples and treated samples with the two types of inoculum.

[*Omissis*]

3.4.3 Conclusions

With this experiment, the degradative activity of [*Omissis*] was confirmed also on the incoming water, achieving lower concentrations of the target compounds compared to those obtained in the discharge water.

The use of two different types of inoculum showed how inoculating the culture broth to support the mycelium proved useful in improving the degradative efficacy, short-term action and survival of the mycelium. The variation in the concentrations of the target compounds showed that the best time for an application using the new type of inoculum (mixed) is at 48 -72 hours. After this time interval an increase in compounds concentrations is observed.

The use of the mixed inoculum has both pros and cons. The advantages of mixed inoculum are: 1) it acts within 48 hours from the inoculum; 2) the degradation percentages are higher than those obtained with mycelium-only inoculum; 3) the fungus can survive in the incoming water and withstand better the microbial competition. The cons of mixed inoculum are: 1) the desorption and/or deconjugation effects seems to be more pronounced; 2) the values of certain physical/chemical water parameters are greatly affected.

Three questions, however, still need to be clarified: is it the combined effect of mycelium and culture broth that achieve efficient substrate degradation, or does the mycelium component play a minor role? Could the variable trend in compound concentrations be due to the fact that a flask is used for each sampling time? What data would be obtained if samples were taken at different times from the same flask?

3.5 Which type of inoculum produces the best results?

With the previous experiment a new type of inoculum was tested, which combines the mycelium with its cultural broth, obtaining good degradation results. However, it is necessary to clarify how the individual components of this inoculum interact with the incoming water. Therefore, in this experiment the analysis on the incoming water was repeated using 3 different types of [*Omissis*] inoculum: one with only mycelium, one with only the culture broth and one with the mixture of culture broth and mycelium. The aim was to evaluate whether the cultural broth alone can degrade the compounds or if the mycelium is also necessary to ensure a good degradation activity.

It also emerged that during the 7 days, the concentration of the compounds tends to be variable. In the previous experiments the water was taken from the two WWTPs and then inserted into various flasks, one flask for each sampling time. To exclude that the variation of the concentrations could be due to preparation of the different flasks (for example caused by the heterogeneity of the water poured in the different flasks) it was decided to change the experimental set. In this experiment, the water samples for each sampling time were taken from the same flask.

3.5.1 Methods

[*Omissis*]

A water sample from each WWTP was analysed just after its collection from the treatment plant, thus representing the sample at time zero (t_0);
Flasks were incubated at 25 °C in the dark in static conditions. At each sampling time a water sample was taken and the concentration of the 14 target compounds was analysed by Eurolab s.r.l.
In this experiment, flasks containing 1 L of incoming water were used to ensure that sufficient water volume was still present after each sampling.

3.5.2 Results and discussion

Data reported are expressed as the percentage reduction of the 14 compounds' concentration compared to the concentration found in the Time zero (t_0) control sample and in the control sample taken at the corresponding sampling time. Results obtained from samples inoculated with culture broth were corrected taking into account the dilution factor. Degradation percentages for each WWTP and compounds concentration trends are reported in Table 22 and Figure 20 for CAP WWTP and in Table 23 and Figure 21 for A2A WWTP.

Table 22- Compounds degradation percentages (compared to the reference control sample: T0 control sample analysed after its collection from the WWTP; 24h,48h,72h and 7d control sampled analysed after the indicated time) in CAP incoming water inoculated with [*Omissis*]. Type of inoculum is reported as follows: only mycelium (myc), only culture broth (CB) and mycelium + culture broth (myc+CB).

[*Omissis*]

[*Omissis*]

Figure 20- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in CAP incoming water: BLUE LINE control sample non-inoculated water, ORANGE LINE water inoculated with *Irpex lacteus* mycelium and its culture broth, GREY LINE water inoculated with only [*Omissis*] mycelium, YELLOW LINE water inoculated with only [*Omissis*] culture broth.

Table 23- Compounds degradation percentages (compared to the reference control sample: T0 control sample analysed after its collection from the WWTP; 24h,48h,72h and 7d control sampled analysed after the indicated time) in A2A incoming water inoculated with [*Omissis*]. Type of inoculum is reported as follows: only mycelium (myc), only culture broth (CB) and mycelium + culture broth (myc+CB).

[*Omissis*]

[*Omissis*]

Figure 21- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in A2A incoming water: BLUE LINE control sample non-inoculated water, ORANGE LINE water inoculated with *Irpex lacteus* mycelium and its culture broth, GREY LINE water inoculated with only [*Omissis*] mycelium, YELLOW LINE water inoculated with only [*Omissis*] culture broth.

The results obtained differ depending on the WWTP. The results obtained in CAP incoming water differ from those obtained previously, unlike those of A2A. This may be due to the fact that A2A incoming water comes from the equalization tank, which therefore makes the samples more homogeneous with each other. CAP incoming water, which does not undergo this type of process, is more prone to changes due to differences in incoming water content.

In CAP incoming water, good results were obtained compared to control samples for [*Omissis*].

As concern [*Omissis*], the culture broth-only inoculum achieved higher degradation percentages ([*Omissis*]) up to 48 hours compared the other types of inoculum. The culture broth-only inoculum is the best performing also against [*Omissis*] in the first 72 hours, with the maximum degradation percentage achieved of [*Omissis*] at 24 hours. In contrast, the mixed inoculum performed better against [*Omissis*] with a degradation of [*Omissis*] in the first 48 hours, after which the effect of culture broth-only seems to be higher. Mixed inoculum recorded against [*Omissis*] a degradation of [*Omissis*] in the first 24 hours, followed by an increase in concentration probably due to the release of the compound from the particulate, and another [*Omissis*] degradation at 7 days. [*Omissis*] achieved very high degradation percentages, with no difference between the mixed inoculum and the culture broth-only inoculum, both achieving better results compared to mycelium-only inoculum. [*Omissis*], as for the other experiments, had a low starting concentration, so degradation percentages are not reliable. The results obtained in CAP incoming water differ from those obtained in previous experiments where [*Omissis*] were degraded. In CAP incoming water better results were achieved with culture broth-only inoculum compared to the mixed inoculum. With this type of inoculum, the phenomena that cause a rise of compounds concentration in the 24-48 hours time span were reduced. The apparent greater efficacy of this inoculum could therefore be due to desorption and/or deconjugation phenomena occurring less, also thanks to the lack of the hyphae mechanical action.

In A2A incoming water good degradation percentages were recorded for [*Omissis*]. These results are in line with those found in the previous experiment. [*Omissis*] was degraded by [*Omissis*] by the mixed inoculum between 48 and 72 hours. Both the mixed inoculum and the culture broth-only inoculum showed similar degradation percentages in the first 24-48 hours for [*Omissis*], after which the degradation achieved by the mixed inoculum was higher. [*Omissis*] recorded two different concentration trends for the mixed and culture broth-only inoculum: the former decreases the concentration by [*Omissis*] in 72 hours but then an increase is observed; instead the latter keeps lowering the concentration up to [*Omissis*] up to 7 days. [*Omissis*] is degraded well by both the mixed and culture broth-only inoculum, but the first one achieved higher degradation percentages up to 72 hours. Degradation of [*Omissis*] reaches good percentages ([*Omissis*]) only with mixed inoculum at 72 hours. As regards [*Omissis*], the culture broth-only inoculum reaches higher degradation percentages only at 24 hours compared to mixed inoculum, than the latter achieves higher degradation percentages up to [*Omissis*]. As it happens with CAP incoming water, [*Omissis*] in A2A incoming water is degraded equally by mixed inoculum and culture broth-only inoculum up to 87 % at 72 hours. In A2A incoming water the most efficient inoculum is the mixed one. The reduction effect achieved by culture broth-only inoculum related to those phenomena that increase the concentration observed in CAP incoming water, is not present in this instance. The broth-only inoculum achieves higher degradation percentages in short times (first 24 hours) where the degradation effect is caused by enzymes already present in the inoculum, while the mixed inoculum become more efficient at longer times, probably due to enzymes produced by the mycelium.

3.5.3 Conclusions

Among the three types of inoculum tested, the mycelium-only inoculum achieved the worst

degradation results, coupled with poor mycelium conditions after the first 72 hours. Combining the results obtained from the incoming water of both WWTPs, both the culture broth-only inoculum and the mixed inoculum achieved high degradation percentages on a good number of compounds. Between these two, the mixed inoculum still seems the most promising as it combines the effectiveness of the enzymes already present in the culture broth with their continuous production by the mycelium. The effects of desorption and deconjugation are still present even in the samples with the only culture broth and continue to be the main critical issue for this type of application.

Once the best fungal strain, the ideal substrate and the best type of inoculum have been identified, is it possible to take this application from laboratory scale to a scale where the volumes of water to treat are larger (pilot plant)? Does this type of fungal-based treatment retain its effectiveness at pilot plant scale?

3.6 Is the selected fungal strain/s able to degrade compounds even at larger scales (pilot plant)?

The main problem with applied research in myco-remediation is being able to transpose the results obtained on a laboratory scale to reality, in this case to the water treatment plant. This step is the most critical one, as the research had to move from a controlled environment, such as the laboratory, to an environment where variables control is limited and where new issues can emerge. To do this, it was decided to test the research findings on an intermediate scale between the laboratory and the treatment plant scale, where larger volumes of water can be used while still being able to control some parameters. Thanks to the CE4WE project and in collaboration with the two project partners CAP s.r.l. and A2A life company, two pilot plants were built, one for each company (Figure 22).

Figure 22- CAP pilot plant on the left and A2A pilot plant on the right.

These machineries are equipped with a tank that can hold up to 500 L of water, equipped with a central paddle agitator (60-100 rpm) and 3 air jets (maximum flow rate 24 L/min) whose task is to keep the tank's content agitated and aerated. The tank is also equipped with an internal grid, sensors to monitor the status of parameters such as pH and oxygenation and is placed in a container whose internal temperature can be regulated.

The objective of the first pilot plant trial is therefore to reproduce the degradative effect of [*Omissis*] mycelium and cultural broth on the 14 target compounds, in a larger volume of water and under non-sterile conditions. The experiment carried out on the pilot plant was a preliminary test to verify the feasibility of the application under these new conditions and to evaluate the effect of already known and possible new issues.

3.6.1 Methods

The first pilot plant trial was performed only in the A2A pilot plant, therefore only A2A incoming water was used. From previous results, the best type of inoculum involves the use of a culture medium, which is why it was decided to inoculate a mycelium suspension in culture broth.

[*Omissis*]

4) Before the start of the experiment, the pilot plant tank was disinfected by filling it with water and NaOH for 24 hours.
   [Omissis]
5) Samples of water were taken before inoculum (t_0), immediately after inoculum (to obtain a before/after inoculation comparison), after 24 hours, 48 hours, 72 hours and 7 days. Concentration of the 14 target compounds were analysed by Eurolab s.r.l.

3.6.2 Results and discussion

[*Omissis*] growth in the pilot plant has been continuous for the first 72 hours, forming a

consistent layer of mycelium on the inner grid of the tank. A water sample was taken to quantify the biomass produced, which was 1.97 g/L of dry mass. After the third day a strong presence of other microorganisms started to develop weakening and consuming part of the mycelium present. The use of a more complex and rich culture broth supplying the fungus not only with sugars, via Malt Extract, but also vitamins, via Yeast Extract, helped it to survive and to grow for the first few days.

Degradation percentages of the target compounds are reported in Table 24, corrected taking into account the dilution factor and compared them to control (t_0). Compounds concentration variations are reported in Figure 23.

Table 24- Compounds degradation percentages (compared to T0 before inoculation) in A2A incoming water inoculated with [*Omissis*].

[*Omissis*]

[*Omissis*]

Figure 23- Compounds concentrations (y-axis) as time passes (n° of hours, x-axis) in A2A incoming water in pilot plant. The graph shows two points for t = 0, the first representing the concentration before inoculation (red dot), the second the concentration after inoculation.

The concentration trends of 10 out of 14 compounds reported a drop just after inoculation, after which gradually rise again up to 24 hours. This phenomenon can be explained either by an immediate enzymatic action, as reported in some studies (Alharbi *et al*., 2019; Marco-Urrea *et al*., 2010a; Marco-Urrea *et al*., 2010b), by an adhesion of the compounds to the mycelium cell wall at the time of inoculation, or by their combined effect. The hypothesis of such an evident adhesion effect to the cell wall could be linked to inoculum preparation that, this time, involved the blending of the mycelium to develop numerous pellets. An higher number of smaller pellets greatly increases the contact surface area compared to few bigger pellets, as those used s inoculum previously. After inoculum insertion, the compounds into the water are probably adsorbed on the fungal cell wall, thus registering a drop of compound concentration in the water, and then start to be degraded either by extracellular or intracellular enzymes (Tormo-Budowski *et al*., 2021).

In most samples, in the first 24 hours, the concentration rises probably due to the effects of desorption and deconjugation that start to happen. These effects are still not very evident probably because the rise in concentration is partially countered by the enzymatic degradation. At 48 hours from inoculation, another drop in concentrations occur, reporting the highest degradation rates after those recorded just after inoculation. Between 48 and 72 hours there is a rapid increase in concentrations of many compounds, which then gradually slows down to 7 days. This probably happens because the fungal mycelium, and therefore its activity, begins to deteriorate due to microbial competition and therefore enzymatic degradation can no longer counteract the increase in concentrations due to the desorption and deconjugation phenomena.

Compared to the t_0 sample collected prior to inoculation, high degradation rates were found, with very similar concentration trends, for the following compounds:

[*Omissis*].

From these data, it can be observed that [*Omissis*] had a good degradative capability in the time between inoculation and 48 hours. The degradation percentages obtained are higher than those obtained previously with a mean global degradation (excluding negative values) of 56 %, a mean degradation at 24 hours of 68 % and a mean degradation at 48 hours of 71 %. These high degradation values can be attributed to two main factors: 1) to a greater richness of the cultural broth and a greater oxygenation of the water given by agitation and aeration; 2) to a greater mycelium-water contact surface given by the smaller and more numerous mycelium pellets and by the agitation.

Almost all compounds showed consistent and almost perfectly overlapping concentration trends. On the other hand, [*Omissis*] had different trends compared to the others and in which, with the exception of the last one, the increases and decreases in concentrations are much more pronounced.

3.6.3 Conclusions

The first test carried out on a pilot plant scale achieved positive results. [*Omissis*], when applied at larger volumes and using the same inoculum-wastewater ratios, manages to maintain its degradative capability. After treatment the concentrations of the compounds obtained were much lower than those found at t_0 in the untreated sample, with degradation rates often exceeding 50 %. However, these percentages could be overestimated due to the lack of control samples for times subsequent to t_0. It is also important to understand what happens in the moments following the inoculum insertion into the incoming water, in order to better understand the sudden drop in concentration that was observed.

This first experiment on a pilot plant revealed two critical issues reported also in similar studies and experiments.

1) The core of this type of application is to keep the necessary biological processes active, in this case enzymatic degradation, while keeping the exploited organism alive and in good conditions. Due to the type of target substrate, i.e. incoming water, which contains a strong microbial and contaminant component, and due to the impossibility of sterilize it given the enormous cost this would entail (Gao *et al., 2008)*, the survival of the fungal mycelium becomes paramount. Excluding a sterilisation of the substrate, which remains one of the main obstacles of this application (Mir-Tutusaus *et al*., 2018), the only solution remaining is to obtain an already strong and active inoculum. For this reason, during the previous trials, the inoculum with culture broth was always the most efficient, because it contained a mycelium that had the nutrients to grow and become competitive with the other microorganisms. In this first trial we have seen that this solution works. The process can still be optimised as the fungus only manages to remain healthy and active in degradation for a few days, after which the other micro-organisms, which also take advantage of the nutrients given by the inoculum, become preponderant.

2) The previous point is closely linked to the second problem that emerged, namely the cost of the nutrients needed to obtain a competitive fungal biomass. All the nutrients that are used for inoculum preparation have a cost that, for a proper real application, must not exceed the actual economic gain from the myco-remediation treatment. It is therefore necessary, to be able to continue the research and experimentation at the pilot plant level and beyond, to be able to optimise the culture media to reduce the relative costs. In a circular economy perspective, this could be done by using agricultural and/or industrial waste to replace expensive nutrients such as sugars with cheaper ingredients that the fungus can still use for its own growth.

3.7 What happens to the pharmaceutical concentrations just after fungal inoculation?

The results of the first pilot plant experimentation confirmed the degrading capabilities of [*Omissis*] observed at laboratory scale. However, adding a sampling time just after the inoculation phase showed a sudden drop in concentrations that could not be observed in previous experiments, as the first sampling took place 24 hours after inoculation. To better understand this phenomenon, it was decided to carry out a further experiment at laboratory scale to understand what happens to the compound concentration trends in the minutes following inoculation. An experiment in flasks was therefore repeated, with a similar method to those done previously, but with very close sampling times, i.e. after 10, 20, 30, 60 minutes and 24 hours after inoculation.

The aim of this experiment was to understand whether the initial decrease in concentration is due to the mycelium acting as an adsorbent for the compounds or whether it is due to the action of the enzymes potentially already present and active in the culture broth.

3.7.1 Methods

The fungal biomass was obtained from flasks prepared and incubated as described in section 3.4.1 (point 2 and 3). In this experiment, only incoming water from A2A WWTP was used as substrate. Two water samples were analysed just after their collection from the treatment plant, thus representing two replicates of the time zero (t_0) sample. [*Omissis*]. A sample of water was taken from each flask after 10 minutes, 20 minutes, 30 minutes, 60 minutes and 24 hours from the inoculation. Samples were analysed to evaluate the concentration of the 14 target compounds by Eurolab s.r.l.

3.7.2 Results and discussions

Concentration trends of the 14 target compounds are reported in Figure 24.

[*Omissis*]

Figure 24- Compounds concentrations (y-axis) as time passes (n° of minutes, x-axis) in A2A incoming water: BLUE LINE control sample non-inoculated water, YELLOW LINE water inoculated with [*Omissis*] mycelium and its culture broth, GREY LINE water inoculated with only [*Omissis*] culture broth.

From the concentration profiles, the compounds can be divided into two groups: a first group consisting of [*Omissis*] in which the concentrations of the inoculated water were higher than those in the control sample and therefore not very informative for the purposes of this experiment, and a second group where already after the first 10 minutes, a decrease of the concentration is detected. In this second group, in all samples there is a sudden drop in concentration in the first 10 minutes (up to percentages of 44-79 %) which then fluctuates and slowly rises again at 24 hours. In the interval covering the first 10 minutes, the concentration of [*Omissis*] is reduced by approximately [*Omissis*] more with the mixed inoculum than with the broth-only inoculum. In [*Omissis*] the initial reduction is similar for both inoculum types. Considering only those samples in which there is a degradation effect, thus excluding those in the first group, it can be said that the rapid decrease in concentration immediately following inoculation is mainly attributable to the degradative effect of the enzymes contained in the culture broth. For some compounds, however, this rapid decrease is more pronounced with the use of the mixed inoculum, so this minor difference may also be due to their adsorption on the cell wall of the fungal hyphae.

3.7.3 Conclusions

The brief experiment performed served to clarify an effect observed during the pilot plant experiment. The results obtained reported a sudden decrease in the concentration comparable between culture broth-only and mixed inoculum samples. In a few compounds a lower concentration was observed in samples inoculated with the mixed inoculum. From the results obtained, it is therefore

plausible that the immediate degradation effect recorded after fungal inoculation, both in this experiment and in the pilot plant, is due mainly to the enzymes present in the culture broth. A partial contribution to this effect may also be due to the adsorption of compounds on the mycelium cell wall.

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3.8 Are two plant pathogens WDFs capable of degrading pharmaceuticals under optimal conditions and in discharge water?

In section 3.1, it was discussed how the best performing strains, among those tested, were *I. lacteus* (I.lc.3), *G. resinaceum* (G.r.9) and *P. fraxinea* (P.f.31). *G. resinaceum* and *P. fraxinea* pose a possible ecological risk if they are used and exploited in sensitive systems such as WWTPs. The spread of spores or propagules in the environment potentially could lead to an increase in plants infected with these two plant pathogens. Therefore, for ecological and pathogenicity reasons, these strains were discarded for the wastewater treatment plant experimentation. These species, although they are aggressive plant pathogens, showed a great degradative capability against pharmaceuticals. Their degradative capabilities can still be exploited for wastewater myco-remediation if precautions are observed or if certain methods are applied to reduce/nullify their dispersion. Such methods could be mycelium immobilisation on scaffolds or isolation and immobilisation of their enzymes. For future studies, the application of enzymes is certainly an area to be explored, as immobilised enzymes are more effective and remain stable against possible alterations related to changes in pH and temperature or to the presence of other substances. For these reasons, it was decided to carry out a preliminary test to evaluate if these two strains could degrade pharmaceuticals also in discharge water in an experiment similar to the one described in section 3.2. This test is necessary to understand whether the application of their immobilised mycelium or their enzymes is a viable and feasible in wastewater. The experiment and the results are reported below in Buratti *et al*., 2023 alongside results already described in section 3.1.

Ganoderma resinaceum and *Perenniporia fraxinea:* Two Promising Wood Decay Fungi for Pharmaceutical Degradation

The following paragraph has been extrapolated from the scientific article by Buratti, S., Rinaldi, F., Calleri, E., Bernardi, M., Oliva, D., Malgaretti, M., De Girolamo, G., Barucco, B., Girometta, C.E, Savino, E. (2023). Ganoderma resinaceum and Perenniporia fraxinea: Two Promising Wood Decay Fungi for Pharmaceutical Degradation. Journal of Fungi, 9(5), 555.

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Abstract: Wood decay fungi (WDF) are a well-known source of enzymes and metabolites which have applications in numerous fields, including myco-remediation. Pharmaceuticals are becoming more problematic as environmental water pollutants due to their widespread use. In this study, *Bjerkandera adusta*, *Ganoderma resinaceum*, *Perenniporia fraxinea*, *Perenniporia meridionalis* and *Trametes gibbosa* were chosen from WDF strains maintained in MicUNIPV (the fungal research collection of the University of Pavia) to test their potential to degrade pharmaceuticals. The degradation potential was tested in spiked culture medium on diclofenac, paracetamol and ketoprofen, three of the most common pharmaceuticals, and irbesartan, a particularly difficult molecule to degrade. *G. resinaceum* and *P. fraxinea* were found to be the most effective at degradation, achieving 38% and 52% (24 h) and 72% and 49% (7 d) degradations of diclofenac, 25% and 73% (24 h) and 100% (7 d) degradations of paracetamol and 19% and 31% (24 h) and 64% and 67% (7 d) degradations of ketoprofen, respectively. Irbesartan was not affected by fungal activity. The two most active fungi, *G. resinaceum* and *P. fraxinea*, were tested in a second experiment in discharge wastewater collected from two different wastewater treatment plants in northern Italy. A high degradation was found in azithromycin, clarithromycin and sulfametoxazole (from 70% up to 100% in 7 days).

Keywords: myco-remediation; pharmaceuticals; wastewater; diclofenac; paracetamol; ketoprofen

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1. Introduction

Wood decay fungi (WDF) in forest ecosystems play a key role in the degradation of lignocellulosic organic matter, promoting nutrient availability and carbon cycling [1,2]. Based on the type of degradation they perform, WDF can be divided into three main categories: brown rot fungi capable of degrading cellulose and hemicellulose but not lignin, white rot fungi which can degrade all wood components and soft rot fungi that are limited to the degradation of superficial wood [3,4]. The degradation capacity of WDF is enabled by the production of a large spectrum of extracellular enzymes, both ligninolytic and non- ligninolytic, along with other secondary metabolites. Cellulose hydrolysis occurs mainly through exo- and endo-glucanases and cellobiohydrolases; hemicellulose is hydrolyzed mostly by xylanases, glucosidases and mannanases; while lignin is mainly degraded by lignin-peroxidases, manganese peroxidases and laccases [3,5]. Some secondary metabolites also play a key role as cofactors or mediators in the degradation of cell wall components, as enzymes such as peroxidases and laccases are too large to penetrate through cell wall pores. Some metabolites such as veratryl alcohol, oxalate, chlorinated anisyl and chlorinatedhydroquinone act as mediators or as substrates in reactions catalyzed by lignocellulosicenzymes or play important roles in the production of other substances that can help thedegradation process, such as extracellular H_2O_2 production [6,7].

The discovery of the potential for enzymatic and metabolite degradation by WDF has led to the exploitation of fungi and their enzymes in different fields of application [8,9]. Some WDF are well known and have already been used for centuries for their medicinal and nutraceutical properties. Recently, interest has been rising in their potential application in other fields, such as design, cosmetics and textiles, and in the circular economy for sustainable reuse of water resources or waste of various kinds. In the last few years, fungi have been investigated for use in the production of new materials, so called myco-materials [10], recycling of agro-industrial wastes [11,12] and particularly for enzyme-centered degradation of pollutants such as plastics, hydrocarbons, pesticides, dyes and pharmaceuticals in soil and water [9,13,14].

Some pollutants have become more problematic with the recent COVID-19 pandemic due to the large increase in single-use plastics, such as masks and gloves, and the increased consumption of pharmaceuticals and antibiotics [15]. Pharmaceuticals and their metabolites enter wastewater treatment plants where they are not completely eliminated, and the residual concentration is discharged in superficial and ground water [16–18]. Nowadays, the European Union specifies quality standards and priority contaminants but there are no concentration limits for pharmaceuticals in discharge waters [19]. A watch-list exists, which is updated every two years, to monitor emerging contaminants and their possible effects on the environment and human health [20].

WDF have the potential to simultaneously reduce pharmaceuticals and other pollutants in water in a sustainable way thanks to their production of a variety of enzymes. Some species of fungi such as *Tramtes versicolor* (L.) Lloyd, the most used and researched species, *Pleurotus ostreatus* (Jacq.) P. Kumm. and *Phanerochaete chrysosporium* Burds. have been widely studied and have proven to be effective on some pharmaceutical compounds such as diclofenac, ketoprofen and ibuprofen [21–26]. Among WDF, many species are yet to be studied and may have the capability to degrade pharmaceuticals and other contaminants. It is important to investigate their potential to further extend the range of applications for myco-remediation.

Many studies have identified *Bjerkandera adusta* (Willd.) as a promising species for remediation/degradation applications [27–29]. In addition, *Perenniporia meridionalis* (Decock and Stalpers) has already been shown to have good Mn peroxidase activity [30], and *Trametes gibbosa* (Pers.) Fr. could prove to be of interest if it is found to possess similar capabilities as *T. versicolor*. In nature, *Ganoderma resinaceum* Boud. and *Perenniporia fraxinea* (Bull.) Ryvarden are plant saprotrophs and pathogens capable of easily degrading wood matter and both are aggressive towards the host [31,32]. This ability could be translated into a strong enzymatic action that could be exploited for myco-remediation, bringing to light interesting new results. *P. fraxinea* is also a relatively poorly studied species and its qualities and possible applications have only recently been discovered. *P. fraxinea* has been used for carotenoid extraction and it has been established that this species can produce enzymes with fibrinolytic properties that can be used in medicine against thrombosis. Moreover,

certain strains of *P. fraxinea* have shown the ability to degrade some dyes and to tolerate and accumulate heavy metals such as Cd, Hg and Cu [33–36].

The Fungal Research Culture Collection MicUNIPV of the University of Pavia (Italy) maintains 600 fungal strains belonging to 130 different species, and many of these strains still have unexplored potential.

The aim of this study is to provide an indication of which WDF species may be promising for future myco-remediation applied studies or for exploitation of enzymes.

The first experiment investigated the pharmaceutical degradation potential of *B. adusta*, *G. resinaceum, P. fraxinea*, *P. meridionalis* and *T. gibbosa* in a sterile liquid culture medium spiked with diclofenac, paracetamol and ketoprofen, three of the most common pharmaceuticals found in wastewaters, and irbesartan, a molecule that is difficult degrade in conventional wastewater treatment methods. Irbesartan is an orally active lipophilic pharmaceutical used to treat hypertension and diabetic nephropathy, characterized by a high permeability and low solubility and is commonly detected both in groundwater and in drinking water. Irbesartan is also a molecule that remains stable when subjected to photodegradation and to hydrolysis and oxidation under certain conditions. A number of experiments on the degradability of sartans have also shown that irbesartan has one of the lowest degradation percentages [37,38]. The second experiment investigated the degradation potential of the two best strains previously identified in wastewater discharged from two different wastewater treatment plants (WWTPs).

1. Materials and Methods

1.1. Choice of Wood Decay Fungi (WDF)

The *Bjerkandera adusta*, *Perenniporia fraxinea*, *Perenniporia meridionalis* and *Trametes gibbosa* strains belonging to the Fungal Research Culture Collection MicUNIPV of Department of Earth and Environmental Sciences, University of Pavia (Italy), and a strain of *Ganoderma resinaceum* belonging to MOGU Srl (MOGU's Fungal Strain Collection—MFSC) were chosen based on a literature review.

All the strains were previously obtained from basidiomata collected in northern Italy, isolated in pure culture, identified by ITS rDNA analyses as reported by Cartabia et al. 2022 [39] and maintained in 2% malt extract agar medium (MEA, Biokar diagnostics, Allonne, France and VWR Chemicals, Milano, Italy) at 3 °C.

1.2. Pharmaceuticals and Discharge Wastewater

Diclofenac sodium DCF (CAS: 15307-79-6), irbesartan IRS (CAS: 138402-11-6), ketoprofen KET (CAS: 22071-15-4) and paracetamol PCT (Acetaminophen CAS: 103-90-2) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Discharge wastewater was taken at the end of water line in two urban wastewater treatment plants in Lombardy, northern Italy, hereinafter referred to as WWTP1 and WWTP2.

The pharmaceuticals tested in discharged wastewater are listed in Table 1. All discharged wastewater samples were analysed by Eurolab Analysis and Results Srl.

Table 1. Pharmaceuticals and molecules tested in discharged wastewater by Eurolab Analysis and Results Srl. Abbreviations used in this study and classification and CAS numbers for each compound are reported (Pubchem.com, accessed on 14 January 2023) [40].

Name	Abbreviation	Classification	CAS Number
5 Methyl-Benzotriazole	5 MB	Corrosion inhibitor and ultraviolet light inhibitor, used for aircraft de-icing agents, plastic stabilizers, anti-fogging agents, pharmaceuticals, fungicides, paints and coatings	136-85-6
Amisulpride	AMS	Antipsychotic and antidepressive agent	71675-85-9
Azithromycin	AZM	Antibiotic	83905-01-5
Carbamazepine	CBZ	Anticonvulsant and analgesic	298-46-4
Clarithromycin	CLR	Antibiotic	81103-11-9
Diclofenac	DCF	Non-steroidal anti-inflammatory agent with antipyretic and analgesic actions	15307-86-5
Gabapentin-Lactam	GBL	Transformation product of gabapentin (anti-epileptic)	64744-50-9
Irbesartan	IRS	Nonpeptide angiotensin II antagonist with antihypertensive activity	138402-11-6
Ketoprofen	KET	Anti-inflammatory analgesic and antipyretic	22071-15-4
Lamotrigine	LMT	Antiepileptic and analgesic	84057-84-1
Metoprolol	MPL	Beta-adrenergic antagonist and antihypertensive	51384-51-1
Ofloxacin	OFX	Antibiotic	82419-36-1
Propyphenazone	PRP	Non-steroidal anti-inflammatory and non-narcotic analgesic	479-92-5
Sulfamethoxazole	SMX	Antibiotic	723-46-6

1.3. Experimental Procedure in Liquid Culture Medium

For each fungal strain, 500 mL flasks containing 200 mL of 2% malt extract (ME) liquid medium were prepared by inoculating agar plugs (surface of about 1 cm^2) from plates with active growing mycelium. Flasks were incubated for 10 days at 120 rpm at 25 °C. Mycelium grew in agitation conditions with spherical structures, referred to as pellets. The content of each flask was drained and the pellets were washed with sterile distilled water then gently squeezed to remove excess water.

For each strain, four 100 mL flasks containing 70 mL of ME 2% were prepared (one for each pharmaceutical to be tested). In each of the four flasks, only one pharmaceutical was spiked to obtain the desired concentration of 10 µg/L and then flasks were inoculated with 1 g (wet weight) of pellets.

A control flask for each pharmaceutical was prepared containing ME 2% and the 10 µg/L solution of the compound in order to evaluate the pharmaceuticals' natural degradation.

Each experiment was performed in triplicate and flasks were incubated in the dark at 25 °C in static (unstirred) conditions.

An aliquot of liquid medium was taken from each flask after 24 h and after 7 days to analyse the pharmaceutical concentration.

To ensure that the variation in drug concentration was not due to pharmaceutical adsorption on the fungal cell wall, parallel tests with dead mycelium were carried out. Agar plugs with active growing mycelium were inoculated in 100 mL flasks with 70 mL of ME 2% and incubated for 7 days. At the end of incubation, flasks were autoclaved at 120 °C for 30 min to kill the fungal biomass. The flasks were allowed to cool to room temperature and then 10 µg/L of pharmaceutical was added.

1.4. Analytical Procedures

Samples were prepared by ultracentrifugation of 1 mL of each medium solution at 14,000 rpm for 5 min to remove fungal residues. The derived supernatants were analysed by high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS).

Analyses were performed by an ExionLC system equipped with a degasser, a quaternary pump, an autosampler and a thermostated column compartment (SCIEX, Framingham, MA, USA) and a X500QTOF mass spectrometer (SCIEX, Framingham, MA, USA) with an ESI source. The LC-MS system was controlled by SCIEX OS software (1.7 version).

Diclofenac samples were analysed using an Eclipse XDB-C18 column (5.0 µm, 4.6 × 150 mm, Agilent Technologies, Santa Clara, CA, USA), while for irbesartan, ketoprofen and paracetamol, a XTerra® MS C18 column (5.0 µm, 2.1 × 250 mm, Waters, Milford, MA, USA) was used.

A column temperature of 25 °C, an injection volume of 20 µL and isocratic elution were applied to all samples.

The mobile phases were composed of 0.1% formic acid in water/acetonitrile (50:50, *v*/*v*) for diclofenac or 0.1% formic acid in water/0.1% formic acid in acetonitrile for the other compounds (65:35, *v*/*v* for irbesartan and ketoprofen and 97:3, *v*/*v* for paracetamol).

The flow rate was set at 1 mL/min for diclofenac samples and 0.3 mL/min for the other pharmaceuticals.

The following MS parameters were applied: curtain gas, 30 psi; ion source gas 1, 40 psi; ion source gas 2, 50 psi (diclofenac) or 45 psi (irbesartan, ketoprofen and paracetamol); temperature, 450 °C (diclofenac) or 350 °C (irbesartan, ketoprofen and paracetamol); polarity, positive; ion spray voltage, 5500 V; CAD gas, 7; TOF start mass, 100 Da; TOF stop mass, 1000 Da; accumulation time, 1 s; declustering potential, 60 V; and collision energy, 10 V.

The pharmaceutical degradation percentages were calculated as follows:

$$100 - \frac{\text{pharmaceutical area in the sample}}{\text{average pharmaceutical area in the 3 control samples}} \times 100$$

For each analyte, the limit of detection (LOD) was calculated from the signal/noise (S/N) ratios of samples at different concentrations, normalized for their concentrations (C) as follows:

$$\text{LOD} = \text{C}\frac{3}{\text{S/N}}$$

where S/N = 2H/h, as defined by USP [41]. The reported LODs were calculated as average values of six analyses for each analyte. The LOD was 1.36 µg/L for diclofenac, 0.22 µg/L for irbesartan, 0.79 µg/L for ketoprofen and 1.72 µg/L for paracetamol.

1.5. Experimental Procedure in Wastewater

The two best performing strains from the experiment in sterile liquid culture medium were tested in discharge wastewater, a clear water matrix with a small and variable quantity of solid particulates. For each fungal strain, flasks for pellet formation were prepared as described in Section 2.3.

For each strain, four 1 L flasks with 400 mL of discharge wastewater were prepared, one for each WWTP and one for each sampling time (24 h and 7 days). Each flask was inoculated with 5 g (wet weight) of pellets.

A control flask for each WWTP and sampling time was prepared containing only discharge wastewater. A sample was also taken at the moment of water collection directly from the treatment plant (T0 sample).

Flasks were incubated in the dark at 25 °C in static conditions.

A 50 mL aliquot of liquid medium was taken from each flask after 24 h and after 7 days and analysed by Eurolab Analysis and Results Srl with LC-MS/MS to determine the pharmaceuticals' concentration.

2. Results

2.1. Sterile Liquid Culture Medium

The degradation results obtained from each strain are reported in Figure 1.

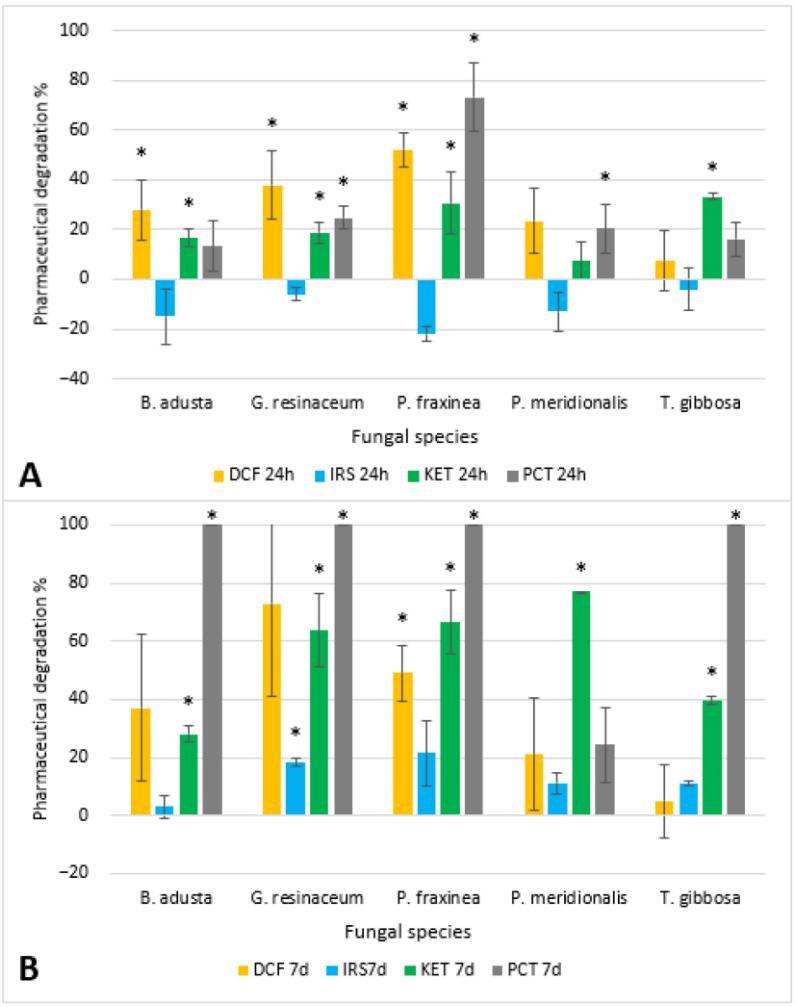


Figure 1. Pharmaceutical degradation percentages after 24 h (**A**) and after 7 days (**B**) for each strain tested. Significantly different concentrations compared to the control are marked by * based on a *t*-test

(* p-value < 0.05).

Diclofenac was reduced by up to 50% in 24 h by *P. fraxinea* and more than 50% by *G. resinaceum* and *P. fraxinea* in 7 days. No strain was able to decrease Irbesartan more than 30% even after 7 days. Ketoprofen was degraded in the range of 10–30% in 24 h and more than 60% by 3/5 strains in 7 days. Only one strain, *P. fraxinea,* was capable of halving the paracetamol concentration in 24 h, while after 7 days 4/5 strains achieved its complete degradation.

The results obtained from samples inoculated with dead mycelium showed similar concentrations as the malt extract control, thus excluding the adsorption component from pharmaceutical degradation.

2.1.1. Diclofenac

P. fraxinea and *G. resinaceum* reported higher diclofenac degradation percentages (52% and 38%, respectively) in the first 24 h compared to other strains; *T. gibbosa* had the lowest degradation capabilities (5–7%). *G. resinaceum* seemed to achieve an even greater diclofenac degradation after 7 days, but the standard deviation was too high to consider this result reliable. *P. fraxinea* was the only strain where the diclofenac concentration, after fungal activity, was statistically different from the malt extract control (p value < 0.05), both after 24 h and after 7 days. *B. adusta* and *G. resinaceum* had a statistically significant effect onlyin the 24 h samples due to the high standard deviation of the 7 day sample. In all strains tested, there were no statistically significant differences in concentrations between 24 h and 7 days.

2.1.2. Irbesartan

Irbesartan exhibited a very low degradation rate compared to the other pharmaceuticals tested, with an apparent increase in pharmaceutical concentrations at 24 h. The highest degradation percentage was obtained by the use of *P.fraxinea* (22%), followed by *G. resinaceum* (18%), even if the former had a high variability compared to the latter. *G. resinaceum* is also the only strain that had a statistically different irbesartan concentration compared to control, though after 7 days.

There was a statistically significant difference between concentration registered after 24 h and 7 days in *G. resinaceum*, *P. fraxinea* and *P. meridionalis*.

2.1.3. Ketoprofen

The strains that exhibited a high ketoprofen degradation after 24 h were *T. gibbosa* (33%) and *P. fraxinea* (31%). After 7 days of incubation, the highest degradation was achieved by *P. meridionalis* (77%), followed by *P. fraxinea* (66%) and *G. resinaceum* (64%). The strain with the overall lowest degradation was *B. adusta*.

The concentration of ketoprofen was statistically different in samples treated with *B. adusta*, *G. resinaceum*, *P. fraxinea* and *T. gibbosa*, at both incubation times. In *P. meridionalis* samples, the difference was significant only after 7 days.

In all samples except *T. gibbosa*, a 7 day incubation time achieved higher degradation percentages than those achieved after 24 h, with statistically different values from the control.

2.1.4. Paracetamol

Paracetamol degradation tests yielded better long-term results compared to other pharmaceuticals. Degradation at 24 h was achieved with optimal results only by *P. fraxinea* (73%), while at 7 days, all strains, except *P. meridionalis*, achieved almost complete degradation, lowering the paracetamol concentration below the analytical LOD. Degradation percentages were statistically different from the control at 24 h in *G. resinaceum*, *P. fraxinea* and *P. meridionalis* and at 7 days in *B. adusta*, *G. resinaceum*, *P. fraxinea* and *T. gibbosa*.

Concentrations between 24 h and 7 day samples were statistically different only in *B. adusta*, *G. resinaceum* and *T. gibbosa*.

2.2. Discharge Wastewater

The two best performing strains from the sterile liquid culture medium experiment were *G. resinaceum* and *P. fraxinea*. They were tested in discharge wastewater to see how the degradation performance obtained in sterile conditions could be altered in a real water matrix containing a mixture of pharmaceuticals and other organisms.

The concentration of pharmaceuticals in wastewater is never constant, but rather subjected to variations related to multiple factors such as the season. Table 2 shows the ranges of concentrations of compounds recorded in the two WWTPs examined.

Table 2. Pharmaceuticals and molecules tested, with minimum and maximum concentrations found in the discharge water of WWTPs under study.

Molecule	WWTP 1 Concentration Range (ng/L) (Min–Max)	WWTP 2 Concentration Range (ng/L) (Min–Max)
5 Methyl–Benzotriazole	3228–13,089	331–1159
Amisulpride	28–36	33–130
Azithromycin	254–458	120–277
Carbamazepine	197–362	130–325
Clarithromycin	32–227	29–90
Diclofenac	198–2914	224–898
Gabapentin–Lactam	54–118	267–465
Irbesartan	223–638	55–240
Ketoprofen	14–199	22–112
Lamotrigine	80–186	143–320
Metoprolol	63–83	32–82
Ofloxacin	124–204	23–190
Propyphenazone	<10–22	<10–11
Sulfamethoxazole	<10–226	38–176

The percentages of degraded molecules compared to the T0 control for each strain and each WWTP are reported in Figure 2. The degradation percentages by fungal activity are reported net of degradation occurring in the control sample. The T0 control is a non-fungal inoculated sample, chosen to verify the normal degradation of compounds due to other biotic or abiotic factors.

The pharmaceuticals most affected by degradative activity were AZM, CLR and SMX, with noticeable effects (degradation percentages between 73% and 100%) only after 7 days, except for SFX whose degradation was between 61% and 85% after 24 h. Negative degradation—an increase in the concentration of the compound—was registered for some pharmaceuticals, with AMS and IRS being affected the most (−57– −71%).

G. resinaceum achieved better degradation results compared to *P. fraxinea* in 24 h for 7/14 molecules (PRP values are the same in both strains) in WWTP1 wastewater and 8/14 molecules (PRP values are the same in both strains) in WWTP2 wastewater. On the contrary, *P. fraxinea* achieved higher degradation percentages after 7 days for 13/14 compounds (SMX values are the same in both strains) in WWTP1 wastewater and 7/14 compounds (CLR and PRP values are the same in both strains) in WWTP2 wastewater. *G. resinaceum* obtained, on average, higher degradation values in discharge wastewater from WWTP2, while *P. fraxinea* had higher degradation in discharge wastew- ater from WWTP1.

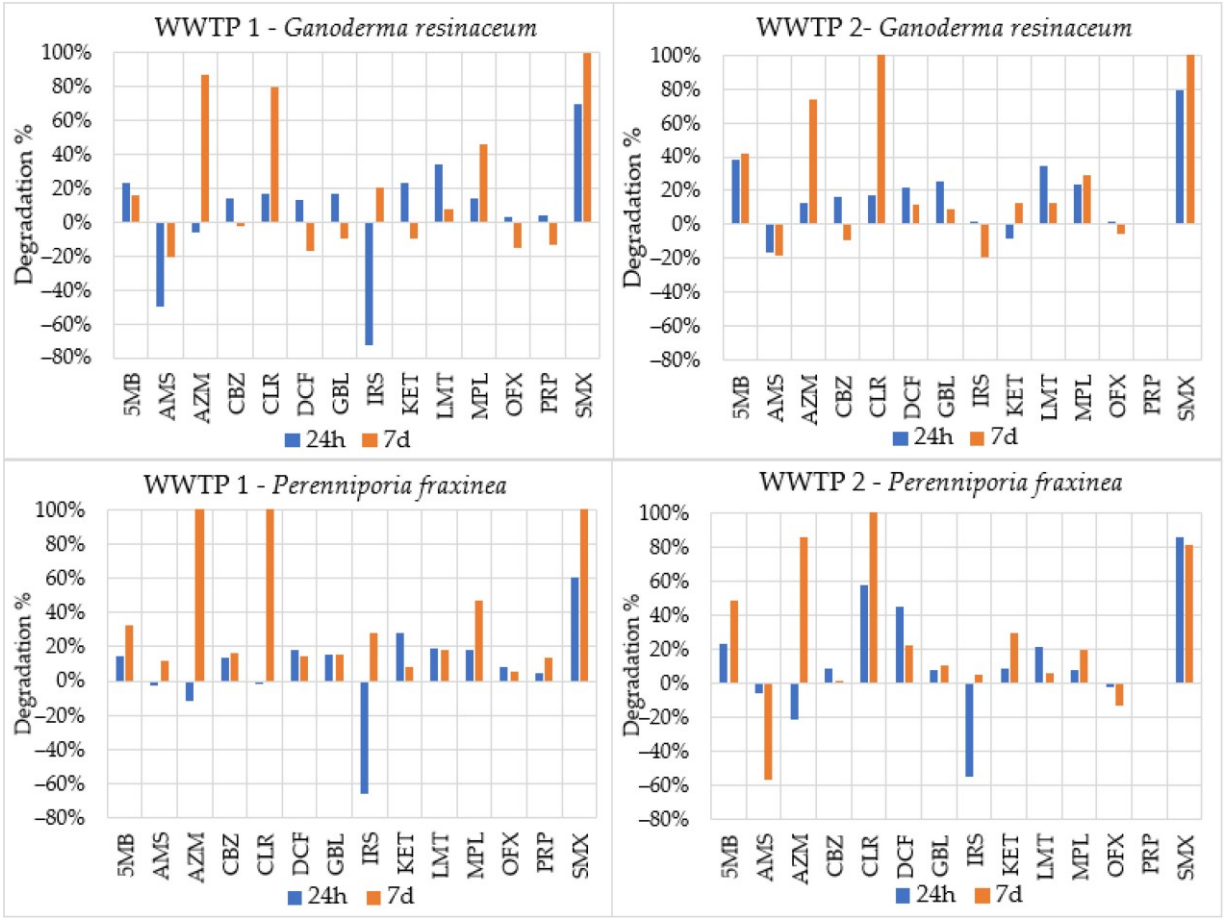


Figure 2. Pharmaceutical and molecule degradation percentages after 24 h and 7 days from fungal inoculum compared to the T0 control.

DCF, IRS and KET achieved comparable values to those obtained in sterile liquid culture medium experiment only in certain cases:

- DCF: after 24 h in WWTP2 discharge wastewater inoculated with *P. fraxinea*;
- IRS: after 24 h in WWTP1 discharge wastewater for both strains;
- KET: after 7 days in WWTP1 discharge wastewater for both strains.

In all the other cases, the degradation percentages in wastewater were lower compared to the liquid culture medium.

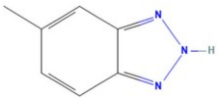
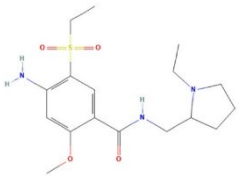
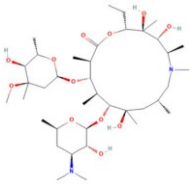
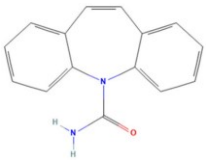
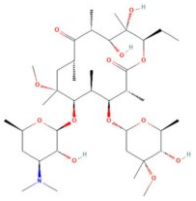
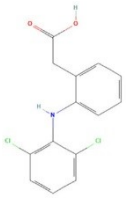
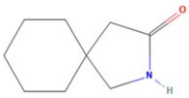
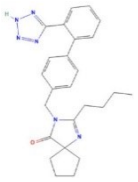
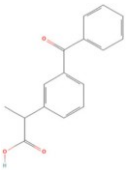
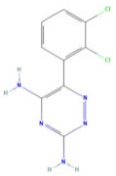
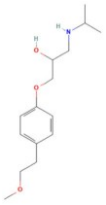
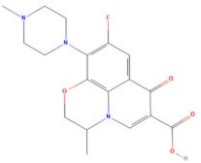
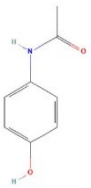
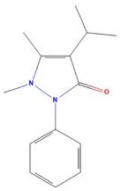
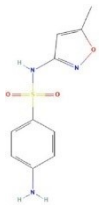
3. Discussion

The results obtained indicated that some of the tested species possess potential for degradation, as they were capable of breaking down pharmaceuticals even in a short time period.

Diclofenac appears to be the easiest degraded pharmaceutical in a short-term incubation, while paracetamol was the most easily degraded over long periods.

The ease of degradation by fungi likely depends on their enzymes and the type of bonds the target molecule possesses. Molecules with electron donating groups, such as amine and hydroxyl groups, are reported to be removed more efficiently by white rot fungi, while electron withdrawing functional groups, such as carboxylic, chlorine and amide groups, makes the molecule less oxidizable and thus more difficult to degrade [42]. The chemical structures of the pharmaceuticals and molecules analyzed are reported in Table 3.

Table 3. Name and chemical structures of the tested compounds (Pubchem.com).

Name	Chemical Structure	Name	Chemical Structure
5 Methyl-Benzotriazole		Amisulpride	
Azithromycin		Carbamazepine	
Clarithromycin		Diclofenac	
Gabapentin-Lactam		Irbesartan	
Ketoprofen		Lamotrigine	
Metoprolol		Ofloxacin	
Paracetamol		Propyphenazone	
Sulfamethoxazole			

In the liquid culture medium experiment, the fact that paracetamol has both a hydroxyl and a secondary amine group explains the high degradation percentages obtained [25,42]. Complete degradation of paracetamol was also achieved in other studies with *T. versicolor* in the same time span [23,26].

On the contrary, ketoprofen contains a carboxyl group, while irbesartan has an amide group. Their chemical structure could be the reason why the tested strains were capable of degrading ketoprofen only after 7 days, while they were not able to substantially lower the irbesartan concentration even after longer periods. Irbesartan also showed an apparent increase (negative degradation percentage), probably due to fungal metabolites that could alter the pH of the solution. This might affect the solubility and ionization of irbesartan and have an impact on detection in mass spectrometry. This compound is also characterized by a much lower solubility in water compared to the other tested pharmaceuticals and it therefore might be more affected by the matrix effect. Ketoprofen needs a longer contact time to achieve a good degradation and this is also confirmed by other studies, in which *Trametes versicolor* showed a high degradation (80%) of ketoprofen after 21 days, while *P. ostreatus* degraded it by 36% in 2 days [22,25,27,43].

For diclofenac, a high degradation achieved in 24 h was also confirmed by numerous studies that report its highly efficient removal by fungi [22,25,44]. The diclofenac degradation in our samples was lower compared to other works, where synthetic or defined liquid media treated with WDF *Trametes versicolor*, *Pleurotus ostreatus* or *Bjerkandera adusta* showed a very high degradation (80–99%) of diclofenac in a time range between 1 and 7 days.

Among strains tested in liquid culture media, *P. fraxinea* was the species with the greatest myco-remediation potential, followed by *G. resinaceum.* The mycelium of these two fungi achieved, in almost every experiment, the highest degradation percentage compared to other species. Even though both species were found to be excellent degraders, *P. fraxinea* is preferable for application due to the reduced variability of the results obtained compared to those of *G. resinaceum*. Compared to other highly performing fungal strains largely tested in the literature, such as the aforementioned *T. versicolor*, these two strains reach higher degradation percentages in the first 24 h for ketoprofen and paracetamol, acting faster compared to other known strains. On the contrary, their enzymatic action on diclofenac does not achieve the high degradation rate found in the literature.

Irbesartan still remains an understudied molecule regarding myco-remediation and there is insufficient data to make a comparison.

Of particular interest is that *P. meridionalis*, although not performing well in general, had specific degradative activity for ketoprofen, reaching 77% of degradation after 7 days. Further studies are needed to understand the specificity of its enzymes towards ketoprofen.

The good degradation of diclofenac and ketoprofen obtained in liquid culture media was not achieved in discharge wastewater, where degradation was overall low. This could be explained by enzyme activity focusing more on easily degradable molecules such as, in this case, azithromycin, clarithromycin and sulfamethoxazole. Azithromycin and clarithromycin are two molecules with a high number of electron-donating groups; the former has five hydroxyl groups and two tertiary amine groups and the latter has four hydroxyl groups and a tertiary amine group. Sulfamethoxazole is reported to reach generally high levels of degradation. Diclofenac removal is usually efficient but highly variable, ranging from a few hours to a few days depending on the fungal species used and enzyme type [42].

For some pharmaceuticals, in particular those degraded by *G. resinaceum*, such as CBZ, DCF, GBL, KET, OFX and PRP, after an initial degradation of the compound, an increase in concentration (negative degradation percentages) was found in 7 day samples. This phenomenon could be explained with desorption and/or deconjugation processes. Desorption involves the liberation of pharmaceuticals and other compounds from suspended solids and particles present in the water, often due to microorganism activity. Deconjugation concerns the metabolization of pharmaceuticals in the human body that transforms them into better absorbing compounds. In the end, both the parent and metabolized (conjugated)

pharmaceuticals are excreted from the body and can be found in wastewaters. These metabolites could undergo deconjugation processes that transform them back to the parent compound, thus leading to an increase in concentration [26,45–48]. In this case, enzymes appear to act first on pharmaceuticals already present in wastewater and then to desorb those in the suspended solids. Desorption of pharmaceuticals from suspended solids and deconjugation phenomena are also present in the control sample without fungal inoculum, but their effect is limited when compared with inoculated samples in which the degradative action on the suspended solids by enzyme activity and also by mechanical action of hyphae is more pronounced.

The opposite effect was registered with amisulpride and irbesartan, where an initial increase in concentration, probably due to the above-mentioned phenomena, was followed by a decrease over the 7 day period. In this case, because these two molecules are difficult to degrade, enzymes seem to first act on the particulates, releasing desorbed pharmaceuticals and causing an increase in their concentration, and only then degrading them.

The overall low degradation performances compared to culture liquid medium are probably also due to the discharge water, which consists of a relatively clean matrix containing a few nutrients that the fungus can use for metabolism and enzyme production. Furthermore, several studies report that substances commonly found in wastewater, such as NaCl, sulfides, halides, organic compounds and heavy metals, can alter or partially inhibit the activity of some enzymes. Competition for nutrients with bacteria and other microorganisms can slow fungal growth and reduce its efficiency to produce enzymes, thus decreasing the degradation of pollutants [23,49,50].

Compared to other microorganisms often used for bioremediation of waters such as algae, *P. fraxinea* shows the potential to achieve a higher degradation of diclofenac if we compare the 45% degradation achieved in WWTP2 in 24 h to the 22–79% in 5–9 days achieved by some algae species [51]. *G. resinaceum* and *P. fraxinea* achieve higher clarithromycin degradation (80–100%) in 24 h, whereas algae achieve the same in 7 days. Metroprolol degradation by these two fungi is lower (47%) compared to some algae species that could reach 99% removal. Sulfamethoxazole is a pharmaceutical almost completely degraded (99–100%) by *G. resinaceum* and *P. fraxinea*, while degradation by algae varies between 46% and 100% [51,52]. Carbamazepine is an example of a pharmaceutical of which both these fungal strains and algae struggle to degrade, with a maximum of 16% for *P. fraxinea* and 14–30% for algae [53].

4. Conclusions

WDF are proven to be of value in the degradation of emerging contaminants. Among the strains chosen and tested, *Perenniporia fraxinea* and *Ganoderma resinaceum* were found to be able to lower the concentrations of diclofenac, ketoprofen and paracetamol with high degradation percentages. *P. fraxinea* is the most promising strain, with a 52% diclofenac degradation (24 h), a 67% ketoprofen degradation (7 days) and a 73% paracetamol degradation in 24 h, increasing to 100% after 7 days.

The poor degradability of irbesartan, probably due to the compound's chemical structure and functional groups, was reconfirmed in this study. Indeed, only *G. resinaceum* was able to achieve a low but significant reduction.

In discharge wastewater, even though some pharmaceuticals remain tough to remove, the enzymatic activities of *G. resinaceum* and *P. fraxinea* could still be exploited to degrade AZM, CLR and SMX (from 70% up to 100%).

This study identified two species, relatively new to this field of mycology, that can be exploited for various applications. Myco-remediation of pharmaceuticals in water and wastewaters can be problematic due to the strong pathogenic nature of the two strains identified. Therefore, their exploitation for remediation purposes should be performed in a way that limits spores and propagule propagation in the environment, for example, through mycelium or enzyme immobilization techniques.

Further studies are needed to understand (1) the best contact time to optimize the pharmaceutical degradation between 24 h and 7 days, (2) the strategies that could be implemented to reduce the problem related to their pathogenicity and (3) which growth conditions can improve their degradative performance.

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Conflicts of Interest: The authors declare no conflict of interest.

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Conclusions

Pollutants remain a central and topical issue with repercussions on human health, environmental conditions and living organisms. Anthropogenic activities and the urban environment are the source of numerous pollutants that can pass through water treatment plants, ending up in watercourses and accumulating in the environment and therefore in organisms. Science in recent decades has been doing more and more to find sustainable solutions to this problem, in some cases with the help of organisms themselves. This is the case with so-called bioremediation or, in the case of this PhD thesis, myco-remediation which exploits the ability of fungi to adsorb pollutants onto cell wall components or to degrade them through enzymatic action.

In this PhD project, the action of fungi was used to address the case study of two urban wastewater treatment companies. This study involves two lines of research: one aimed at the removal of heavy metals from sewage sludge and one focused on degrading and lowering the concentration of pharmaceuticals in the water of the treatment plants. The ultimate goals of the project were: 1) to obtain a fungal strain capable of achieve the objectives described above; 2) to find a way to upscale the results obtained at laboratory scale to pilot plant; 3) setting the basis of the researched application for future use in wastewater treatment plants.

Firstly, the fungal community in the wastewater treatment plants was assessed, obtaining both an overview of the presence of fungi in the different tanks and a collection of fungal strains. This study provided a better understanding of the fungal community dynamics of the two treatment plants and of the species presence and variation among the different phases of water treatment. The first part of the project was aimed at obtaining a large pool of both microscopic and wood decay fungal strains. Three collections of strains were obtained through different sampling campaigns: one of micro fungi (mostly moulds, yeasts and pseudo-yeasts) isolated from the two treatment plants, one of WDF strains (with a majority of polyporoids) isolated from specimens collected from northern Italy, and one of WDF strains (with a majority of corticioids) isolated from specimens collected in Spain. For the experimental part of the project, it was decided to use WDF strains chosen for their high growth rate, their enzymatic production and/or their already known degradative capabilities.

The second step was to select the strains to be used for the removal of heavy metals from sludge, by evaluating their growth capabilities on different substrates. The growths capabilities were tested on three types of sludge differing in the percentage of suspended solids and water content. Thanks to the results obtained, the strains with the best growth were selected and the pre-thickening sludge was identified as the ideal substrate for preliminary tests. Heavy metals removal tests were therefore carried out by placing the mycelium of selected strains in contact with the pre-thickening sludge, enriched with Cd, Cr, Ni and Pb. The evaluation of the heavy metal concentrations in the sludge over a 14-day period was not positive, as the final concentrations did not differ between treated and untreated samples. This result led the study to focus on the second line of research, pharmaceuticals degradation.

The capabilities of the chosen strains to degrade four pharmaceuticals of interest were tested under sterile and controlled conditions. The chosen strains of *Bjerkandera adusta*, *Ganoderma resinaceum*, *Irpex lacteus*, *Oxyporus latemarginatus* and *Perenniporia fraxinea* achieved good degradation results towards Diclofenac, Ketoprofen and Paracetamol. In contrast, Irbesartan proved to be difficult to degrade. The results obtained from this preliminary test show that the chosen strains possess a degradative action on pharmaceuticals.

The next step of the project, crucial for the final experimentation, was to test the degradation of 14 target compounds in wastewater and thus under non-sterile conditions and in the presence of other organisms and contaminants. In the first experiment the degradation performance of different strains was tested and 3 particularly efficient strains were identified. Among these, [*Omissis*] was the best performing. All subsequent experiments were aimed at understanding which parameters would optimise the application and degradative effect of [*Omissis*] in wastewater. The following parameters were therefore identified.

- ✓ Ideal substrate: wastewater entering water treatment plants is a substrate rich in particles,

nutrients and other substances, alterations to the physical/chemical parameters of the water can be mitigated in the subsequent treatment phases.

- ✓ Contact time: all experiments evaluated the concentrations of the 14 target compounds over a 7-day period. The results showed that fungal activity achieves higher degradation rates in the interval between 48 and 72 hours.
- ✓ Inoculum type: degradation effects were tested using different inoculum types including fungal mycelium, enzyme-rich culture broth and a mixture of the two. It was therefore observed that the mixed inoculum of mycelium and culture broth not only increased the degradation effect, but thanks to the nutrients still present in the broth, ensured a greater survival of the mycelium against microbial competition.
- ✓ Most degraded compounds: among the 14 compounds tested those most easily degraded by fungal activity were [*Omissis*]. In contrast to the preliminary test results [*Omissis*] are hardly degraded in wastewater.

With the above-mentioned parameters obtained, the last step of the project was carried out: up-scaling the myco-remediation process to a larger scale, in this case to a pilot plant. A degradation test was then carried out in the pilot plant in 60 L of wastewater, obtaining similar results to those obtained in the laboratory, confirming the efficacy of [*Omissis*] in lowering the concentration of pharmaceuticals in wastewater. A sudden lowering of concentrations just after inoculation was also noted, probably due to the immediate action of the enzymes but also to a probable adsorption of the compounds onto the mycelium.

In conclusion, this study verified the possibility to use and apply WDF strains for the degradation of compounds (mainly pharmaceuticals) in substrates such as urban wastewater. During the project a considerable number of fungal strains were isolated. These strains represent a valuable source of research materials thanks to their already known or yet to be discovered application potential.

The results obtained with this PhD project have also brought to light numerous opportunities for future research and perspectives to deepen and learn more about certain aspects of this type of application:

- During the sampling campaign in Spain several corticioid fungi were isolated. This sometimes-overlooked group of fungi consists of species that in wild show a good degradation of wood components that can be exploited in enzyme based myco-remediation. Other interesting species to study are those isolated from the wastewater treatment plants since some works in literature report their ability to degrade or accumulate substances including pollutants and nutrients. These characteristics combined with their adaptation to the wastewater treatment plant environment makes them a possible research topic worth investigating.

-*Ganoderma resinaceum* and *Perenniporia fraxinea* are two species, among those tested on pharmaceuticals, that achieved high degradation rates. However, their nature as aggressive plant pathogens makes their use impossible in wastewater treatment plants, as their accidental propagation could lead to environmental damage. Nevertheless, a possible solution would be to try their application by immobilising their mycelium or isolating and immobilising their enzymes in scaffolds.

- During the experimentation using wastewaters, a number of phenomena leading to an unexpected variation in pharmaceuticals concentration have been observed. It would therefore be interesting to further investigate these relatively unstudied phenomena to find out causes and possible solutions. Similarly, another aspect that could not be assessed within the PhD project, but which must necessarily be addressed, is the identification and toxicity evaluation of pharmaceuticals degradation by-products. In order to apply myco-remediation to pharmaceuticals in wastewater it must be verified that the by-products that are generated are less toxic compared to the starting compounds.

-Pilot plant experimentation is still in its initial stages, but to make the application feasible it must become economically viable. In this regard, another research perspective is to try to optimise the preparation procedures and the components for growing and produce the fungal biomass. The mycelium growth and inoculum preparation parameters can still be improved to ensure better biomass production, while the components of the culture broth can be replaced with cheaper ones (e.g.

agricultural or other type of waste).

Myco-remediation applied to emerging pollutants is still an experimental practice, that can offer interesting research opportunities. This PhD project has brought this type myco-remediation closer to its possible future application, of which, however, many aspects can and must still be clarified and investigated.

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