UNIVERSITY OF THESSALY
Department of Biochemistry and Biotechnology

Study of the depuration efficiency and microbiology of biobed systems which receive pesticide contaminated effluents from various agro-food processing units

A thesis submitted by

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Study of the depuration efficiency and microbiology of biobed systems which receive pesticide contaminated effluents from various agro-food processing units

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Nothing in life is to be feared.
It is only to be understood.
Now is the time to understand more,
so that we may fear less

~Marie Sklodowska Curie

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Τέλος θα ήταν παράλειψή μου να μην ευχαριστήσω τους γονείς μου Βασίλη και Χρυσάνθη και τον αδερφό μου Δημήτρη, για την απεριοριστη υποστήριξη τους σε κάθε βήμα της ζωής μου

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BHI	Bulb Handling Industries
CBX	Carboxin
CHT	Chlorothalonil
FLD	Fludioxonil
FLX	Fluxapyroxad
FPI	Fruit Packaging Industries
IMZ	Imazalil
IPR	Iprodione
MET-M	Metalaxyl-M
MGE	Mobile Genetic Elements
SPI	Seed Producing Industries
TBZ	Thiabendazole

Chapter 1

General Introduction

1 PLANT PROTECTION PRODUCTS

1.1 DEFINITIONS, HISTORICAL BACKGROUND AND CONSUMPTION

Pesticides constitute a specific group of chemical or biological compounds that exert a preventive, controlling or inhibitory effect on the growth of harmful organisms or regulate plant growth (Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO), 2014). According to Directive 2009/128/EC the term pesticide includes (i) "plant protection products" (PPPs) that are used to improve yield and quality of agricultural production (Regulation (EC) No 1107/2009) and (ii) "biocides" that are used to control harmful organisms in non-agricultural practices (Directive 98/8/EC). Hereafter, with the term "pesticide" we are going to refer to PPPs, and both these terms are going to be used interchangeably.

Pesticide use is dated back to agriculture itself. Since ancient times, farmers have been employing numerous practices to enhance and preserve crop production like co-cultivation of venomous and nutritious plants at the same place for insect elimination by the toxic plants and usage of elemental sulfur, mercury or other substances for the prevention of fungal diseases (Tudi et al., 2021; Abubakar et al., 2020; Thrupp, 2000). The use of various natural compounds for pest control continued until 1870s, when inorganic synthetic materials became widely used like the Bordeaux mixture (copper sulfate and lime), arsenic (calcium arsenate and lead arsenate) and hydrogen cyanide. All of these compounds were highly toxic and relatively ineffective (Tano, 2011). The period after 1945 marked the era of synthetic pesticides with the discovery and use of Dichlorodiphenyltrichloroethane (DDT), captan, parathion and β-Hexachlorocyclohexane (BHC) in a wide range of pests (Unsworth, 2010). Their low cost enabled the widespread use of these compounds and, as a result, a raise in food production and decline of insect-bore diseases was observed, despite their disadvantages such as lack of selectivity, high rates of application and high toxicity. The decades of '70s and '80s were highlighted by the introduction of more selective pesticides such as the herbicide glyphosate and other commonly used organophosphate insecticides and fungicide families such as triazoles, imidazoles, pyrimidines and dicarboxamides (Andreazza and Scola, 2015; Unsworth, 2010). In the '90s, more selective compounds were developed which had better environmental and toxicological profiles and required lesser amounts per usage. Research into pesticides has continued until today to supply the global market with compounds characterized by improved selectivity, better resistance management and safer towards the user and environment.

In modern days, PPPs are routinely used against a wide range of pests, including plant pathogenic fungi, insects and weeds, so as to enhance crop production and preserve plant products during storage (Damalas and Eleftherohorinos, 2011). Intensification of agriculture, in order to meet the ever-growing consumer demands, has led to an increasing use of PPPs, both in the field and at postharvest level (Schreinemachers and Tipraqsa, 2012). PPP usage around the world has been on the incline in the last 29 years for Eastern Asia and South America reaching a plateau of 1,515,777.2 ± 338,625.7 and 478,835.6 ± 233,343.4 tn per year in 2010, whereas it has remained fairly stable for Europe, North America and Africa at 466,038.5 ± 23,184.4, 458,258.8 ± 26,749.5 and 76,403.6 ± 18,036.6 tn of PPP

consumption per year respectively (**Figure 1.1**, (FAO - Food and Agriculture Organization of the United Nations).

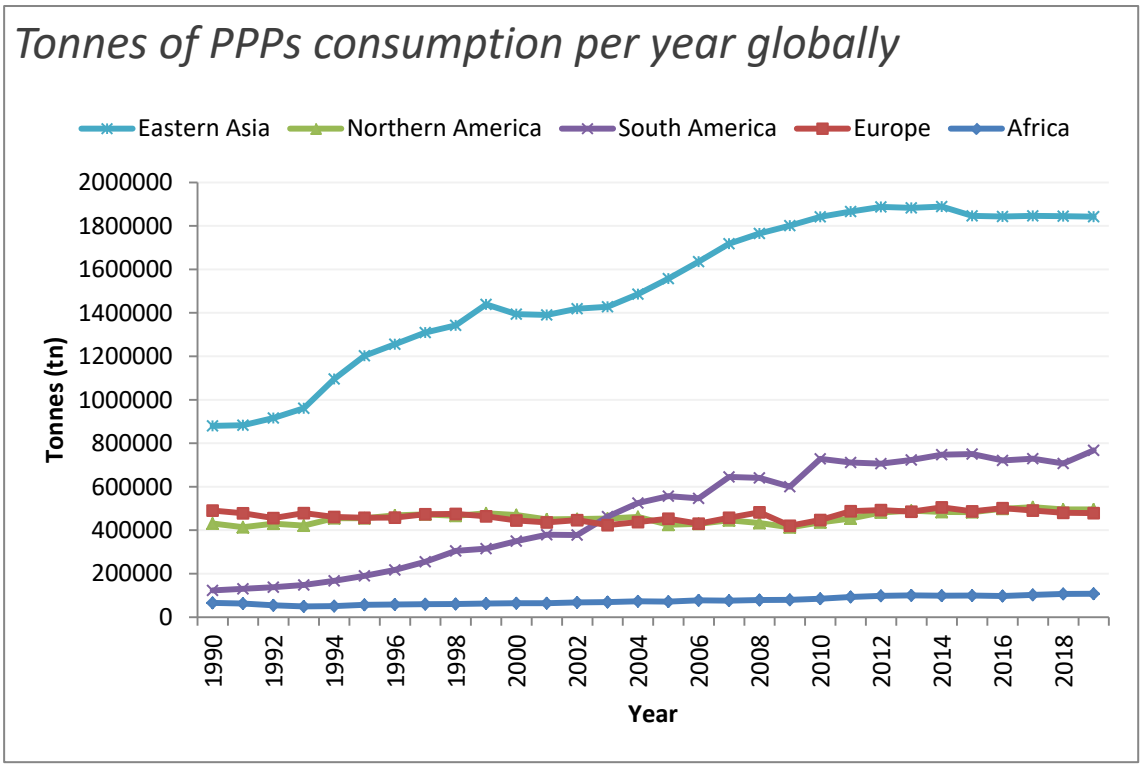

Figure 1.1. Total PPP quantities (tonnes) used in or sold to the agricultural sector for crops and seeds the past 29 years. Data source: (FAO - Food and Agriculture Organization of the United Nations)

Fungicides and bactericides usage in Europe has not changed a lot in the past 29 years, with an average of 190,508.8 ± 12,689 tn used in or sold to the agricultural sector for crops and seeds which correspond to 40.92 % of the total PPP consumption (**Figure 1.2 and 1.3**). Herbicides use follows covering 38.62% of the total PPP consumption (**Figure 1.2**). Herbicide use has shown an increase in Europe in the past decade with a maximum consumption of 218,010 tn recorded in 2012 (**Figure 1.3**). Insecticides constitute the third largest category of PPPs used in Europe with an average consumption of 54,857.1 ± 8,604 t, which corresponds to 11.78 % of the whole pesticide market (**Figure 1.2 and 1.3**). The remaining PPPs that were consumed in Europe the past 29 years correspond to 8.6 % of the total amount and include rodenticides, plant growth regulators, mineral oils, and other pesticides (**Figure 1.2**).

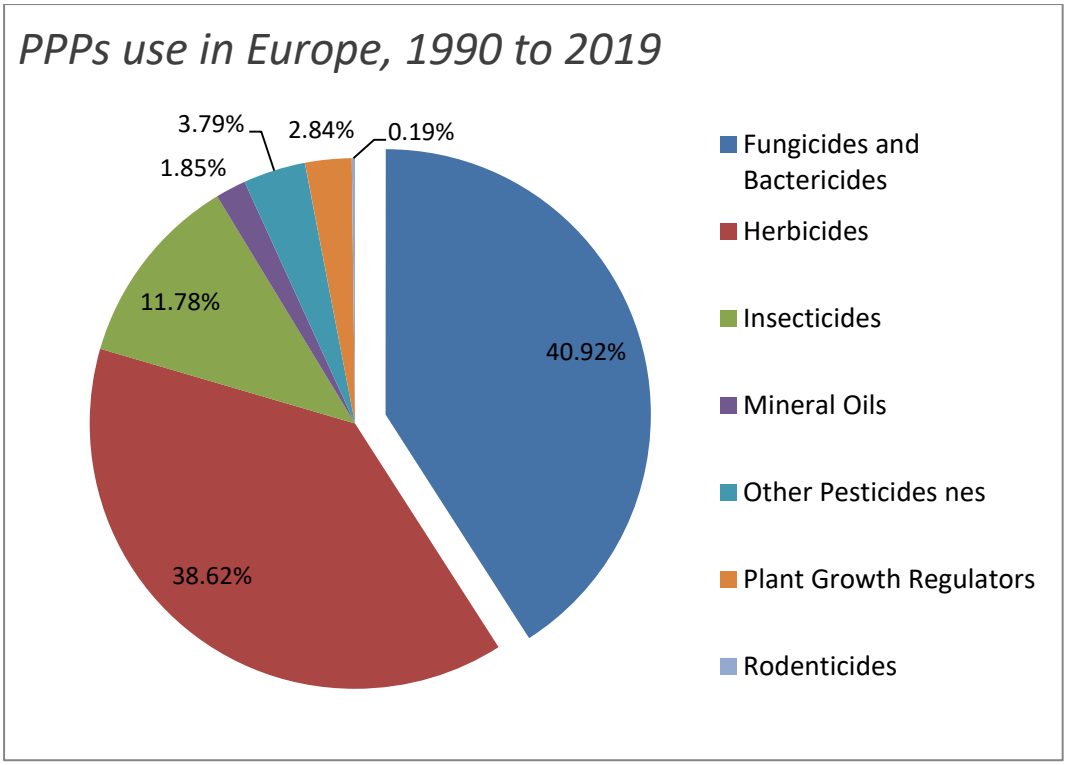

Figure 1.2. Percentages of PPPs used in or sold to the agricultural sector for crops and seeds, broken down by target organism and type category. Data source: (FAO - Food and Agriculture Organization of the United Nations)

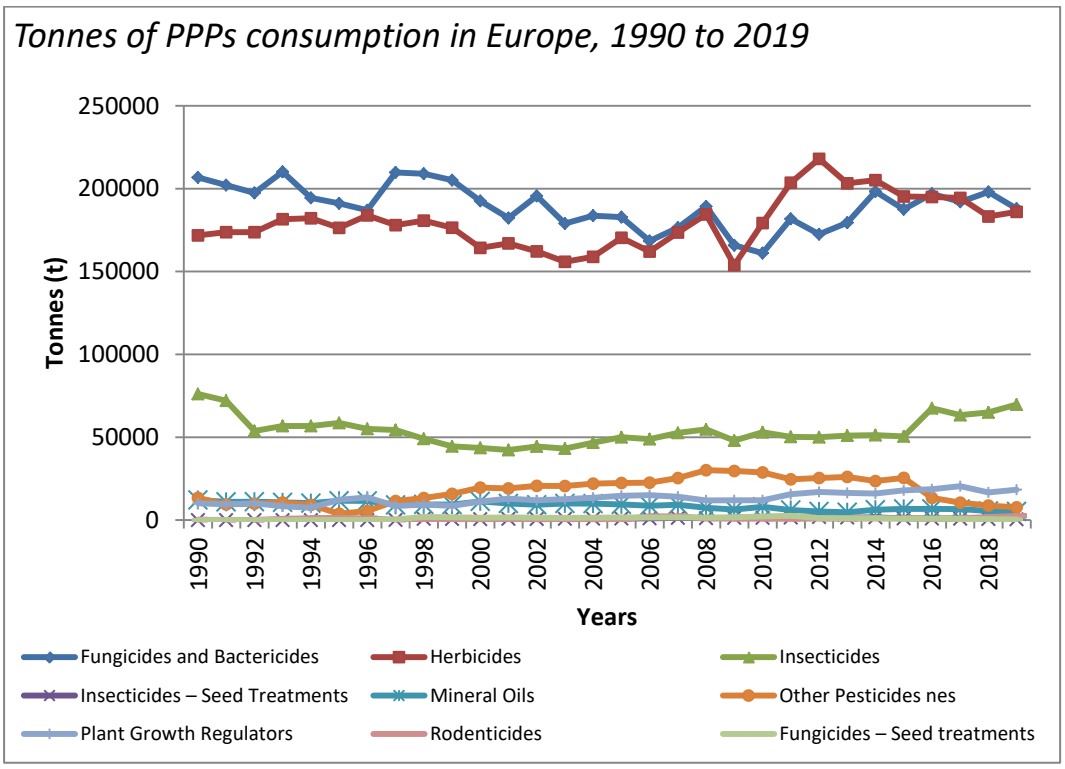

Figure 1.3. PPP quantities (tonnes) used in or sold to the agricultural sector for crops and seeds, broken down by target organism and type category. Data source: FAO - Food and Agriculture Organization of the United Nations

1.2 CLASSIFICATION

PPPs include a wide variety of chemical compounds that differ in target-organism, chemical identity, physicochemical properties and mode of action (Leong et al., 2020). Chemical identity and properties are valuable in determining the application approach, rate and the safety precautions implementation. Among the most common classification methods are the ones that rely on target-organism and chemical identity (**Table 1.1**).

Table 1.1 Indicative classification of PPPs according to target-organism and chemical identity

Target Organism	Chemical Identity	Example Compound
Fungicides	Anilides	Carboxin, Acetaminophen
	Benzimidazoles	Thiophanate-methyl, Thiabandazole, Carbendazim
	Carbamates	Mancozeb
	Chloronitriles	Chlorothalonil
	Dicarboxamide	Iprodione
	Imidazoles	Imazalil, Prochloraz
	Phenylamides	Metalaxyl, Mefenoxam
	Phenylpyrrole	Fludioxonil, Fenpiclonil
	Phthalimides	Captan
	Pyridine carboxamides	Fluxapyroxad, Penthiopyrad
	Strobilurins	Azoxystrobin
	Triazoles	Propiconazole
Herbicides	Carbamates	Carboxazole
	Chloroacetamides	Metolachlor, Acetochlor
	Organophosphates	Glyphosate
	Triazines	Atrazine
Insecticides	Carbamates	Carbofuran, Carbaryl, Oxamyl, Thiram
	Organochlorides	DDT, Lindane
	Organophosphates	Chlorpyrifos, Parathion, Diazinon, Malathion, Dimethoate
	Neonicotinoids	Imidacloprid, Thiamethoxam
	Pyrethroids	Permethrin, Deltamethrin, Cypermethrin

1.3 BENEFITS AND RISKS

The use of PPPs is considered an integral part of agricultural practices as it contributes to the increase in crop yields through (i) drastic reduction of weeds, which compete with crops for nutrients, (ii) suppression of diseases, which are mainly caused by plant pathogenic fungi and bacteria, and (iii) repression of insect pests that, together with pathogens, can result in huge economic losses by decreasing the amount of harvestable agricultural crops or stored produce (Abubakar et al., 2020). Increasing the agricultural yield is essential for meeting the demands of the ever-rising world population. Population numbers have risen dramatically in one century, from 1.5 billion in 1900 to about 6.1 billion in 2000, which equals to a 3 times greater increase than during the entire human history (Carvalho, 2017). The rapid population growth could not have been possible without a simultaneous growth in food production. PPP use results in enhancement of the production and availability of healthy foods like fruits and vegetables. Thus, PPPs are a significant contributor to alleviating hunger and providing an abundant supply of high quality food (Tudi et al., 2021). Improving nutrition has in the long term provided humans with better quality of life. PPPs application also helps in the preservation of wood and other useful materials from destruction by insects. Concerning human health, pesticides have been used for the control of mosquitoes that transmit *Plasmodium*, the causal agent of malaria, and flies that carry *Trypanosoma,* the causal agent of trypanosomiasis (also known as Sleeping Sickness). It is therefore apparent that the use of pesticides has significantly contributed to the well-being of mankind.

That being said, PPP use is usually accompanied with deleterious environmental and public health issues. Widespread application of PPPs in agricultural practices has led up to the generation of the serious issue of pesticide residues in environmental substrates and food, especially fresh produce (Farahy et al., 2021; Van Boxstael et al., 2013). PPPs are major environmental pollutants, seeing that their innate physicochemical characteristics allow their uncontrollable dispersion in the terrestrial and aquatic ecosystems (Carvalho, 2017). Continual utilization of PPPs contributes significantly to environmental pollution of the rhizosphere and bulk soil affecting soil structure, porosity, physicochemical properties and water retention capacity, eventually leading to high levels of soil impoverishment and erosion (Abubakar et al., 2020). Furthermore, introduction of pesticides in the environment inevitably leads to leakage into the water bodies and accumulation in groundwater or drinking water.

Many studies have demonstrated the potential of PPPs to non-target organisms (Liu et al., 2022; Zaller et al., 2021; Zhang et al., 2021; Adetunji et al., 2018; Katagi and Tanaka, 2016; Zhang et al., 2016). Non-target organisms are species that are unintentionally affected by pesticide application, being beyond the group of target organisms, and include plants, algae, birds, fish, beneficial insects, aquatic and soil microorganisms. The impact of PPPs on the activity of soil microorganisms results in negative effects in the decomposition of the organic matter, soil aeration and proper functioning of important geochemical cycles (nitrogen, carbon, phosphorus). In addition, pesticide consumption can lead to accumulation in the members of an ecosystem. Bioaccumulation refers to the presence of high concentrations of a compound in an organism, as a result of high intake to lower removal rates. Accumulation of pesticides in organisms of various trophic levels usually occurs via

direct uptake from water and though food, from the consumption of plant tissue, sediment and other members of the food chain.

Resistance development is another side effect of the continuous usage of PPPs. Evolution of resistant strains has already affected the efficiency of herbicides (Powles and Yu, 2010), fungicides (Lucas et al., 2015) and insecticides (Bass et al., 2015). It is estimated that about 10 billion US dollars are lost annually due to herbicide resistance (Palumbi, 2001). Moreover, models have shown that if insecticide resistance becomes widespread it could lead to 40% more malaria cases (Briët et al., 2013). Current evidence suggests that evolution of pesticide resistant organisms could surpass our ability to develop new and efficient pesticides (Gould et al., 2018).

PPPs also pose paramount threat to human health. Acute and chronic poisoning, neurobehavioral, carcinogenic, immunological, developmental and reproductive effects are amongst the most common consequences of exposure to PPPs. In order to get approval of use in the market, several criteria need to be taken into account including that no harm to human health or the environment is going to be caused through their use. Potential toxicity to humans is assessed through experiments using mainly mice and rats, and less often dogs and rabbits (Matthews, 2015). Studies of acute toxicity describe the effect of a single dose of the compound after oral, dermal and inhalation exposure in animals and determine the lethal dose 50 (LD_{50}) and lethal concentration 50 (LC_{50}), *i.e.* the dosage or inhaled concentration of a compound that is going to kill 50% of the dosed population (Gad, 2014). Acute toxicity in humans refers to the effect of a single exposure or repeated exposure over a short time (e.g. an accident during mixing or applying pesticides) and symptoms range from headache, fatigue and diarrhea to slowed heartbeat, seizures or unconsciousness (Ogg et al., 2018). Displayed on **Table 1.2** are acute toxicity hazard categories as described by the World Health Organization (WHO).

Table 1.2. Acute toxicity hazard categories according to WHO (World Health Organization and Safety, 2010)

WHO Class	**LD_{50} for the rat (mg/Kg body weight)**		**No of pesticides**
	Oral	**Dermal**	
Ia – Extremely hazardous	< 5	< 50	29
Ib – Highly hazardous	5 - 50	50 - 200	58
II – Moderately hazardous	50 - 2000	200 - 2000	250
III – Slightly hazardous	Over 2000		145
U – Unlikely to present acute hazard	5000 or higher		195

Chronic toxicity refers to the effect of long-term exposure to low doses of an active compound. Studies of chronic toxicity on rats usually last for 2 years (Matthews, 2015). Chronic exposure in humans takes years for the development of symptoms and possible adverse effects include cancer, nerve damage, endocrine system irregularities and reproductive disorders. PPPs exposure has commonly been associated with cancer development (Lerro et al., 2021; Matich et al., 2021; Alavanja, 2009; Greenburg et al., 2008; Samanic et al., 2008), with several compounds being classified in Groups 1 – “Carcinogenic

to humans", 2A – "Probably Carcinogenic to humans" and 2B – "Possibly Carcinogenic to humans" by the International Agency for Research on Cancer (IARC) ("List of Classifications – IARC Monographs on the Identification of Carcinogenic Hazards to Humans"). Prolonged exposure to pesticides can also cause changes in the function of the central, peripheral and autonomous nervous system resulting in neuropsychiatric disorders (depression, anxiety and mood disorders) and suicidal behavior to workers or farmers that are in contact with them on a daily basis (Freire and Koifman, 2013). The relationship between pesticide exposure and reproductive issues has been extensively studied because of the profound effect and life-long impact on human health (Wang et al., 2016; Frazier, 2008). PPPs belonging to organochlorines, organophosphates, carbamates, pyrethroids and various herbicides and fungicides have been associated with adverse reproductive effects like reduced fertility, spontaneous abortion, birth defects and fetal growth retardation (Mnif et al., 2011; Frazier, 2008). Several PPPs also possess strong affinity to steroid hormone receptors, specifically androgens and estrogens, acting as agonists and antagonists and disrupting the action and concentration of the natural hormones (Leong et al., 2020). Thus, these endocrine disruptor compounds can negatively affect reproductive and sexual development, especially on fetuses, infants and children (Sharpe, 2006).

1.4 ENVIRONMENTAL FATE AND POLLUTION

PPPs fate in environmental substrates is strongly associated with (I) their physicochemical properties according to which we can determine whether a compound is likely to be transferred in the soil (octanol-water partition coefficient, $LogP_{ow}$), water (water solubility, mg/L) or the air (vapor pressure, mPa), (II) the properties of the site including climate, soil type and structure, depth and structure of the groundwater and vegetation (Saha et al., 2019; Gjettermann et al., 2009; Gregoire et al., 2009) and (III) the agricultural practices which involve among others application techniques, quantities and frequency of application, irrigation and crop rotation regimes (Romanazzi et al., 2020; Andreazza and Scola, 2015; Balderacchi et al., 2013; Fait et al., 2010).

PPPs enter the environment through point and non-point (or diffuse) sources of pollution (Balderacchi et al., 2013; Neumann et al., 2002). Diffuse sources of pollution refer to movement of PPPs in soil, surface and ground water systems after being applied in the field (Harrison et al., 2019), while point sources concern on-farm activities like accidental spills during spray mixture preparation (tank filling, faulty equipment, waste disposal) (Sharma et al., 2020; Fait et al., 2007) and agro-food processing industry's effluents that make use of PPPs (Ccanccapa et al., 2016; Belenguer et al., 2014; Masiá et al., 2013)

Upon entering the environment, PPPs follow two paths, migration (movement), or degradation. Migration refers to transfer from its initial point of introduction and involves processes like volatilization, spray drift, surface runoff, leaching and adsorption (Fait et al., 2007).

1.4.1 MIGRATION (Volatilization, Leaching and Surface runoff)

Volatilization is the conversion from solid or liquid to gas form. Factors that affect significantly the volatilization of a PPP include its evaporation potential (vapor pressure), the weather conditions of the site e.g. temperature, humidity and air movement, and, if the pollutant is in a soil matrix, the soil properties such as texture, organic matter content and moisture level (Tudi et al., 2021). Airborne PPPs have a very high dispersion potential as they can be carried on air currents for very long distances (Gavrilescu, 2005). Spray drift is another form of airborne movement from the application site via spray droplets. Zhang et al., have shown that flight speed and altitude of unmanned aerial vehicle (drone) applications have a significant effect in pesticide droplet dispersion (Zhang et al., 2020). Surface runoff and leaching refer to the transportation of PPPs to surface water and groundwater systems over a slopping surface and vertically through the soil profile respectively. Runoff is often observed when the PPPs fail to be adsorbed on the soil colloids due to the high water volumes which could not be effectively infiltrated by the surface soil. Runoff is promoted by over-irrigation, high soil moisture content, slope of the point of introduction and amount and timing of rainfall (Tudi et al., 2021). Leaching is the vertical movement of pesticides and is highly influenced by soil properties and adsorption/desorption processes (Peña et al., 2020).

1.4.2 ADSORPTION/DESORPTION

Adsorption/desorption is the leading process that determines the distribution of PPPs in soil and controls their availability for other transportation or transformation processes. Essentially, adsorption is a phenomenon where pesticides bind to soil particles (Peña et al., 2020). Due to its importance, adsorption of plentiful PPPs has been extensively studied (Qisse et al., 2020; Smalling et al., 2018; Álvarez-Martín et al., 2016; Gulkowska et al., 2016; Papadopoulou et al., 2016). Soil properties that greatly affect the adsorption potential of a soil include pH, salinity, moisture and organic matter content. The latter is of paramount importance for pesticide adsorption, as it is the main adsorption surface of non-polar compounds and it has also been found to be interacting with polar compounds. (De Wilde et al., 2009; Wauchope et al., 2002). Practices that modify these factors to some extent may impact the adsorption/desorption process and, consequently, the PPPs polluting potential and effectiveness against pests (Peña et al., 2020). Pesticide properties define the adsorption outcome as well, with hydrophobicity and water solubility being the most important factors. It has been demonstrated that hydrophobic compounds strongly adsorb on the soil colloids and organic matter (Delgado-Moreno et al., 2017). Another type of adsorption is uptake by the plant tissue were PPPs compounds are transferred to the plant, usually through their root system (Romero et al., 2019).

1.4.3 DEGRADATION

Degradation is the most significant process of pesticide removal from environmental matrices and it can be quantified (its rates) through the concept of half-life, *i.e.* the time needed for the degradation of half of the applied amount of a pesticide compound from the

treated environment. Degradation refers to the breakdown or modification of the chemical structure of PPP compounds by abiotic or biotic pathways. The complete breakdown of compounds into inorganic components is termed mineralization. The degradation of pollutants is strongly influenced by adsorption which decides the fraction of the pollutant that is available in the soil solution and prone to degradation by biotic or abiotic means.

1.4.3.1 ABIOTIC DEGRDATION

Abiotic degradation includes photo-degradation, the breakdown of pesticides by sunlight radiation, and chemical degradation, which involves chemical reactions that happen spontaneously in environmental matrices. Photo-degradation can only occur on the surface of soil and water systems, the air or the foliage of the treated plants as it is heavily depended on light intensity (Gavrilescu, 2005). Nevertheless, its remediation potential has been studied for plenty PPPs (Huang et al., 2021; Zhou et al., 2021; Mahapatra et al., 2017; Remucal, 2014). Chemical degradation includes different reactions such as ionization, hydrolysis and oxidation-reduction and is usually dependent on the pH of the environmental system. Other factors that influence the efficiency of chemical degradation processes include temperature, oxygen and moisture levels.

Chemical reactions, coupled or not with photo-degradation processes, have been studied extensively for the depuration of wastewaters or remediation of pesticide contaminated sites. Studies mainly focus on advanced oxidation techniques like (photo-) Fenton processes (Fakhri et al., 2020; García-Estrada et al., 2020; Santiago et al., 2018b; Gar Alalm et al., 2015) and Ti-O_2 based photocatalysis (Molla et al., 2020; Sraw et al., 2018; Cruz et al., 2017; Xing et al., 2014; Jiménez et al., 2013). Ozone treatment has also been studied (Bisaria et al., 2021; Genena et al., 2011). The main drawback of recruiting abiotic reactions is the production of transformation products that sometimes are equally or more toxic than the parent compounds (Huang et al., 2021; Santiago et al., 2018a, 2013; Sirtori et al., 2014). Other setbacks in the implementation of abiotic processes for the removal of pesticides is the high energy requirements, high operational costs and the possible use of additional reagents, like electrolytes (Titchou et al., 2021; Sirés et al., 2014) or rapid replacement of installed equipment e.g. the electrode whose efficiency and half-life are reduced due to deposition of organic material on its surface.

1.4.3.2 BIOTIC DEGRDATION

Biotic or microbial degradation is the most important process that defines the persistence of PPPs in environmental matrices. Microbial degradation is attributed to the adaptation of microorganisms in soils with extensive exposure to pesticides, due to their genetic plasticity and quick generation times (Upadhyay et al., 2019) through which they develop pesticide.-catabolic genes and pathways (Bouteh et al., 2021; Russell et al., 2021; Zhu et al., 2020; Jaiswal et al., 2019; Jeffries et al., 2018; Widada et al., 2002) or through horizontal gene transfer, by acquiring mobile genetic elements like plasmids or transposons that carry relevant catabolic genes (French et al., 2020; Rios Miguel et al., 2020; Storck et al., 2020; Dealtry et al., 2014). Factors influencing microbial degradation include the structure of

the microbial community, salinity, carbon and nitrogen sources, presence of oxygen, temperature, soil moisture content and pH (Bhatt et al., 2019; Cycoń et al., 2017; Megharaj and Naidu, 2017; Wu et al., 2014). Biodegradation has been extensively studied for the bioremediation of pesticide-polluted sites (Mishra et al., 2021, 2020; Cycoń et al., 2017) and many organisms with pesticide degrading abilities have been isolated and characterized (Pandey and Choudhury, 2021; Zhou et al., 2021; Ambreen et al., 2020; Perruchon et al., 2017, 2016; Sharma et al., 2016; Perruchon et al., 2015; Karpouzas et al., 2000).

1.5 PESTICIDE REGULATORY FRAMEWORK

Pesticides are important environmental pollutants, as their innate physicochemical properties enable their dispersion in various environmental matrices (Carvalho, 2017). European Union (EU) recognized the polluting potential of pesticides and included many of them in the list of priority hazardous substances in the field of water policy (2455/2001/EC). The substances that are included in the list are aimed to be used progressively less until complete cessation or phasing out of discharges, emissions and losses in the water systems (2455/2001/EC). Recently, many pesticides were added in the "Persistent Organic Pollutants" list through REGULATION 2019/1021 which means that they are prohibited, should be withdrawn as soon as possible and their manufacturing, placing in the market and use is restricted (2019/1021).

EU addressed PPPs introduction in the market and use in Directive 2009/128/EC on establishing a framework for sustainable use of pesticides and in Regulation 1107/2009 concerning the placing of plant protection products in the market. The main aim of Directive 2009/128/EC is to achieve a sustainable use of PPPs by reducing the risks and impacts of their use in human health and the environment, and reduce dependency on the use of PPPs by promoting the use of integrated pest management and of alternative approaches or crop protection techniques. Regarding the latter, certain measures that should be put in place include monitoring the use of PPPs, training of professional users, distributors and advisors, informing the general public and raising awareness on the risks of PPP use in human health, non-target organisms and the environment.

Application of pesticides involves both the active and inert ingredients, which may not have pesticidal activity, but facilitate the efficiency of the active compound by enhancing the penetration into the target organism and its toxic action (Andreazza and Scola, 2015). Addressing that, EU adopted Regulation 1107/2009 that concerns authorization and placing in the market of PPPs in their commercial formulation, which contains the active substances, safeners, synergists, adjuvants and co-formulants.

Moreover, EU is constantly reviewing the approval of usage and is re-evaluating the active substances in order to make sure that they continue to fulfill the approval criteria (*Regulation (EU) 2020/1740*). As of now, 454 active substances, safeners and synergists are approved, 66 are pending, 927 are not approved and 17 have not been assessed at EU level yet ("EU Pesticides Database (v.2.2) Search Active substances, safeners and synergists," Accessed 2021-11-15).

Certainly, precautionary measures for proper pesticide use have not been undertaken only by the EU. Since 1975 the WHO has been publishing guidelines for classification of pesticides by hazard with regular revisions every few years, in order to support the effort for appropriate management of pesticides (World Health Organization, 2020). The hazard referred to by the guideline concerns the acute oral and dermal risk to health of the compound and its formulations. The list currently consists of 29 compounds in the Class Ia, 58 compounds in Class Ib, 250 compounds in Class II, 145 compounds in Class III, 195 compounds in Class U and 253 compounds believed to be obsolete or discontinued for use (**Table 2**, World Health Organization, 2020). Moreover, Environmental Protection Agency (EPA) of the United States is responsible for regulating PPPs and ensuring that they do not pose risks to human and animal health and to the environment by evaluating them and making sure they meet current scientific and regulatory standards through registration and reviews of registration processes.

2 AGRO-FOOD PROCESSING INDUSTRIES

The use of PPPs in the post-harvest treatment of plant products is of paramount importance as it ensures product quality during storage and minimizes losses due to pathogens. Postharvest handling is the connecting link between the farmers and the consumers and is associated with the implementation of handling technologies to minimize losses between harvest and consumption and to maintain quality characteristics, such as nutritional value, appearance, texture and flavor (Shree and Kumari, 2019). Fresh fruits and vegetables are prone to microbial spoilage due to their high moisture content and wide range of organic substrates. Therefore, their postharvest treatment with pesticides by agro-food processing industries is of paramount importance. Moreover, other plant propagating materials like seeds and bulbs also require pesticide treatment to control diseases and pests affecting seeds and seedlings. Further down, a few examples of agro-food processing units are described along with examples of fungicides that are commonly used by them.

2.1 SEED PRODUCING INDUSTRIES

Agriculture relies on pesticides, not only in terms of protecting the plant and plant products but the seed as well. Seed treatment constitutes a major economic market sector worldwide since the 1960s when large-scale commercial utilization of seed coating for field-scale precision agriculture began (Ma, 2019; Pedrini et al., 2017). Seed treatment involves technologies to (I) improve germination or seedling growth, (II) facilitate planting through enhancing seed appearance and handling characteristics, (III) deliver materials required at the time of planting like PPPs, micro-nutrients, plant growth regulators and growth stimulants and (IV) remove weak or dead seeds (Halmer, 2008). Coating is the placing of an artificial outer layer of fungicide or insecticide formulations in order to protect the seed and seedling by controlling or repelling harmful organisms (Halmer, 2008). The coating technology used to apply colorants and PPPs onto seeds is termed “film coating”, and it includes spraying large amounts of the coating liquid and subsequent or concurrent drying. Commonly, batches of seed are treated multiple times to build up an even film layer or a multi-layer in the case where a different component is used in each successive coating.

Multi-layer coats are usually applied to separate one component from another, to protect the seeds or those who handle the seeds or to control the release of an active ingredient after planting (Halmer, 2008). Hence, the wastewaters produced by seed producing industries generate effluents, loaded with high amounts of fungicidal components.

2.1.1 FUNGICIDES USED IN SEED PRODUCING INDUSTRIES

2.1.1.1 CARBOXIN

5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide or carboxin (CBX) is a carboxanilide fungicide that is commonly used for seed coating in cereals and crops (Ayesha et al., 2021; Lamichhane et al., 2020; Srinivas et al., 2017).

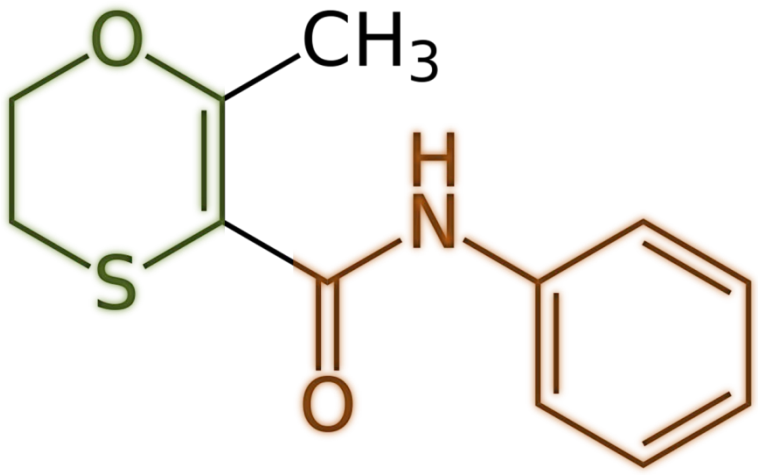

Figure 1.4 Chemical structure of CBX. The 1,4-oxathiin and anilide groups are colored green and red respectively

CBX was the first succinate dehydrogenase inhibitor that was introduced in the market in 1969 and is still in use (S. Li et al., 2021; Shively and Mathre, 1971). It is a systemic fungicide that is recommended for use in small grain cereals (wheat, soybean, barley, oat) against stem rot, whose causal agent is *Sclerotium rolfsii* (Rakholiya, 2015; Akgül et al., 2011; United States, Environmental Protection Agency, 2004), rust which is caused by basidiomycetes and rhizoctonia diseases (Yanase et al., 2007). CBX was first registered in the USA in 1968 and its authorization has been re-approved constantly until today. In EU, the approval period of CBX is in force until 31 May 2023 ("REGULATION 2019/324).

According to WHO classification of pesticides by hazard, CBX is placed in Group III of slightly hazardous compounds (World Health Organization, 2020). Conclusion on peer review of CBX risk assessment performed by EFSA, illustrated that CBX is accountable for sensitization by skin contact, low acute toxicity through oral, dermal and inhalation routes, and no skin or eye irritation (EFSA, 2010a). Regarding its ecotoxicity, CBX poses low risk to non-target arthropods, soil microorganisms and plants (EFSA, 2010a).

CBX is slightly hydrophobic with log octanol-water partition coefficient (Log P_{ow}) of 2.3 and solubility in water of 134 mg/L at 20^{o}C, pH = 7 (**Table 1.3**). So it is expected to be moderately mobile in a range of soils. Indeed, K_f and K_{Foc} values in a variety of soils range

from 1.56 – 2.71 to 123 – 213 ml/g respectively (**Table 1.3**). It is also not expected to move to the air as it is not a volatile compound (vapor pressure 0.02 mPa at 20^{o}C) (**Table 1.3**). CBX is not persistent in soil with half-lives of 0.06 – 1.68 days in lab studies and 0.3-11 days in field studies and it is prone to photo-degradation (0.1 days) (**Table 1.3**).

Table 1.3. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized or not for organic carbon) of the fungicide CBX (PPDB - Pesticide Properties Database. Accessed on 2021-11-15).

Property-Fate	**Value**	**Interpretation**
Water solubility (mg/L)	134	Moderate
Vapor pressure (mPa)	0.02	Non-Volatile
Log P_{ow}	2.3	Low
K_f (ml/gr)	1.56 – 2.71	Mobile
K_{foc} (ml/gr)	123 – 213	Mobile
$DT_{50\ lab}$ (days)	0.07 – 1.68	Non-persistent
$DT_{50\ field}$ (days)	0.23 – 3.49	Non-persistent
$DT_{50\ photolysis}$ (days)	0.1	Non-persistent

The main transformation products of CBX include carboxin sulfoxide, which is its main transformation product, and oxycarboxin. Similar to its parent compound, oxycarboxin possesses fungicidal activity (Dębska et al., 1979; Mathre, 1970) and similar physicochemical properties and environmental fate, albeit higher persistence with mean DT_{50} values of 21.2 and 42.3 days in lab and field studies respectively. Carboxin sulfoxide possesses low fungicidal activity, it is rather stable in water, mobile (K_{Foc} = 96 ml/g) and persistent ($DT_{50\ Lab}$ = 39.5 days) in soil (DellaGreca et al., 2004; PPDB - Pesticide Properties Database. Accessed on 2021-11-15). The risk to soil-dwelling organisms for both transformation products is considered as low, but they are harmful to aquatic organisms (EFSA, 2010a).

2.1.1.2 METALAXYL-M

Methyl N-(methoxyacetyl)-N-(2,6-xylyl)-D-alaninate or metalaxyl-M (MET-M) or Mefenoxam is the R-enantiomer of the chiral compound metalaxyl. MET-M is a systemic acylalanin fungicide that is commonly used as soybean seed treatment against oomycetes like *Globisporangium sp.* (Molin et al., 2022) or as a disease suppressor of *Phytophthora nicotianae*, which is the causal agent of foliar diseases on herbaceous annual plants (Hu et al., 2008), and of *Phytophthora infestans* that causes late blight in potato (Randall et al., 2014). More than 23000, 59000, and 54000 m^3 of metalaxyl-M were used in Australia, EU, and USA respectively, in 2017 (Lamichhane et al., 2020). MET-M mode of action involves disruption of fungal nucleic acid synthesis by inhibiting uridine incorporation by RNA polymerase I system (Randall et al., 2014; Fisher and Hayes, 1982). MET-M was first registered in USA at 1996 (United States, Environmental Protection Agency, 1996) although its racemic mixture had already been in the market for 17 years (United States, Environmental Protection Agency, 1994).

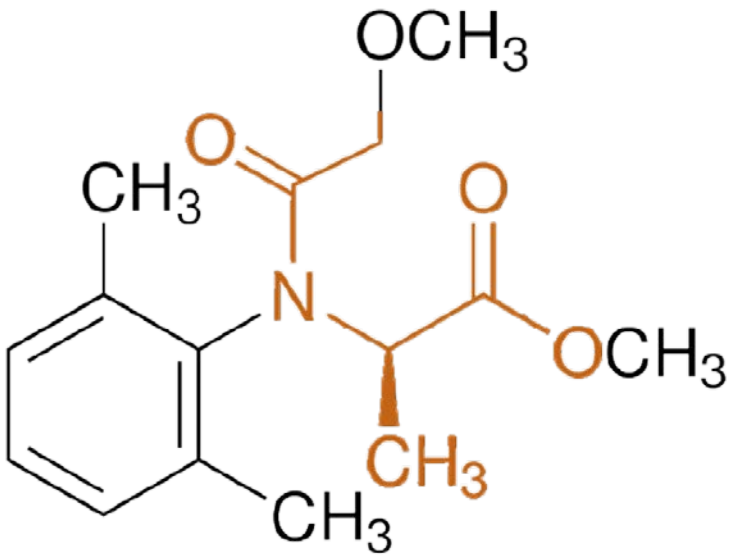

Figure 1.5 Chemical structure of MET-M. The acylaniline group is colored red

MET-M causes low to moderate acute toxicity when administered orally, dermally or by inhalation, and it does not provoke skin sensitization or irritation. No potential accumulation has been observed as well (EFSA, 2015). According to EPA, it is classified as a low and very low toxicity compound, but it is a moderate irritant in rabbits' eyes (United States, Environmental Protection Agency, 1994). MET-M poses low risk to non-target soil-dwelling and aquatic organisms (EFSA, 2015)

MET-M is a hydrophilic compound (Log Pow = 1.61) and remarkably soluble in water (26000 mg/L) (**Table 1.4**). It is not expected to be found in the air as its vapor pressure is low (3.3 mPa) (**Table 1.4**). MET-M is mobile in soil with K_f and K_{foc} values ranging between 0.07 – 8.01 and 20 – 284 ml/g respectively (**Table 1.4**). Moreover, MET-M is not prone to photo-degradation and it is moderately persistent in the environment with mean DT_{50} values of 6.5 and 14.1 days in lab and field studies respectively (**Table 1.4**).

Table 1.4. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized K_{foc} or not K_f for organic carbon) of the fungicide MET-M (PPDB - Pesticide Properties Database. Accessed on 2021-11-15).

Property-Fate	**Value**	**Interpretation**
Water solubility (mg/L)	26000	High
Vapor pressure (mPa)	3.3	Non-Volatile
Log P_{ow}	1.61	Low
K_f (ml/gr)	0.07 – 8.01	Moderately Mobile
K_{foc} (ml/gr)	20 - 284	Moderately Mobile
$DT_{50\ lab}$ (days)	1.38 – 73.1	Moderately persistent
$DT_{50\ field}$ (days)	9.3 – 30.9	Moderately persistent
$DT_{50\ photolysis}$ (days)	-	Stable

Under dark aerobic conditions, MET-M undergoes demethylation of the ester group and transforms to R-demethylmetalaxyl (Masbou et al., 2018) which exhibits low to high persistence (4.1 – 200 days) (EFSA, 2015) and to a lesser degree to the transformation

products CGA67868 and SYN546520 which exhibit low (1.6 – 4.9 days) and moderate to high (42-288 days) persistence respectively (EFSA, 2015).

2.1.1.3 FLUXAPYROXAD

3-(difluoromethyl)-1-methyl-N-(3',4',5'-trifluorobiphenyl-2-yl)pyrazole-4-carboxamide or fluxapyroxad (FLX) is a carboxamide fungicide commonly used for coating of soybean seeds (Marburger et al., 2017; Gaspar et al., 2014), barley (McLean et al., 2019), sugarcane (Wayment et al., 2021), canola (Fraser et al., 2020), cotton (Copeland et al., 2016) and other cereals. Its broad-spectrum efficiency made it a top seller fungicide in 2018, with 470$ million dollar sales (S. Li et al., 2021).

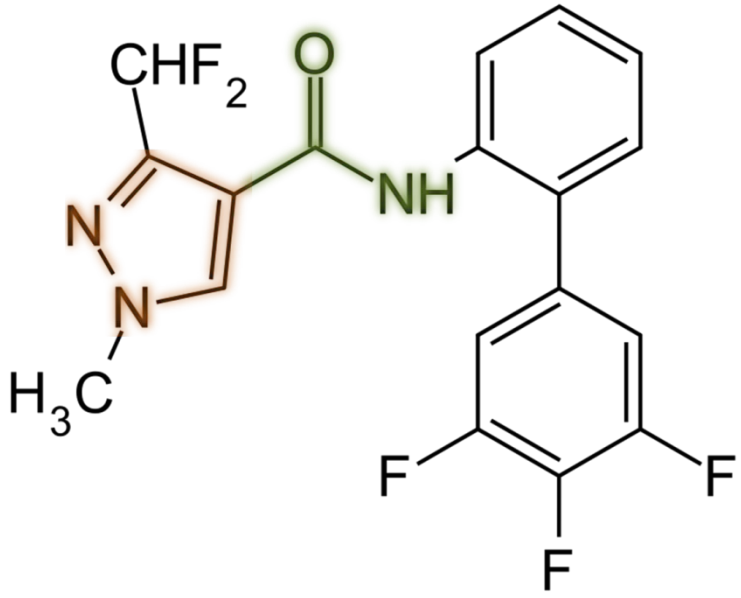

Figure 1.6. Chemical structure of FLX. The pyrazolium ring and the carboxamide group are colored red and green respectively

Its mode of action involves inhibition of the succinate dehydrogenase in complex II of the mitochondrial respiratory chain, afflicting cell respiration and energy production (S. Li et al., 2021). FLX is a newfound fungicidal compound with less than 10 years in the market as it was authorized for use in 2012 in both USA and EU (EFSA, 2012; United States, Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, 2012)

It is portrayed as slightly hazardous by WHO (World Health Organization, 2020). It causes low acute toxicity when administered orally, dermally or by inhalation but it does not induce any eye or skin irritation (EFSA, 2012). FLX is classified as “Not likely to be Carcinogenic to Humans” according to EPA’s Final Guidelines for Carcinogen Risk Assessment (United States, Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, 2012). Concerning its ecotoxicity, FLX risk for non-target organisms was described as low (EFSA, 2012).

FLX is a hydrophobic compound (Log Pow = 3.13) with particularly low water solubility at 3.44 mg/L (**Table 1.5**). Due to its hydrophobic nature, FLX tends to bind strongly to soil colloids and organic matter, which renders it immobile with mean K_f and K_{foc} values of 8.3 and 728 ml/gr respectively (**Table 1.5**). FLX is also persistent in soil with mean DT_{50} values of 183 and 181.5 days in lab and field studies respectively. It is not expected to be transferred into the air as it is not a volatile compound (2.7×10^{-6} mPa) (**Table 1.5**).

Table 1.5. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized K_{foc} or not K_f for organic carbon) of the fungicide FLX (PPDB - Pesticide Properties Database. Accessed on 2021-11-15).

Property-Fate	Value	Interpretation
Water solubility (mg/L)	3.44	Very low
Vapor pressure (mPa)	2.7×10^{-6}	Non-Volatile
Log P_{ow}	3.13	High
K_f (ml/gr)	2.5 – 17.9	Slightly Mobile
K_{foc} (ml/gr)	320 – 1101	Non-Mobile
$DT_{50\ lab}$ (days)	53.0 – 424	Persistent
$DT_{50\ field}$ (days)	38.9 – 370	Persistent
$DT_{50\ photolysis}$ (days)	-	Stable

Two soil transformation products of FLX, M700F001 and M700F002, were detected in soil studies performed with ^{14}C-labelled FLX in the pyrazole ring. M700F002 showed high persistence, equivalent to FLX, with DT_{50} values in soil ranging between 131 – 197 days under aerobic conditions in lab studies. In contrast, M700F001 is not persistent with DT_{50} values ranging between 2.3 - 10 days. All transformation products pose low toxicity risk to non-target organisms (EFSA, 2012).

2.2 FRUIT PACKAGING PLANTS

Fresh fruit and vegetables are important quality foods that have high nutritional and sensory (flavor, texture, appearance) value. Given the fact that these harvested commodities should remain fresh during storage, they are susceptible to spoilage and reduction of the physiological appearance (browning). According to FAO, it is estimated that approximately 14% of the food produced in the world in 2016 was lost in the supply chain after harvest and before the retail level (United Nations Environment Programme, 2021).

The intensity of the production systems and the high demand for quality food has driven members of the supply chain (growers, storage operators and processors) to employ a variety of practices to ensure a high level of effective pest control such as appropriate pre-harvest treatments, proper harvest and handling practices, effective sanitation of fruit and storage facilities, appropriate post-harvest antifungal treatments and adequate precautions during fruit storage and transportation (Schirra et al., 2011; Palou et al., 2009). Common high-incidence postharvest diseases in fruits include gray mold (caused by *Botrytis cinerea*), blue mold (caused by *Penicillium expansum*), sour rot (caused by *Geotrichum candidum*) and rhizopus rot (caused by *Rhizopus stolonifer*).

2.2.1 FUNGICIDES USED IN FRUIT PACKAGING PLANTS

2.2.1.1 FLUDIOXONIL

4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile or fludioxonil (FLD) is a broad-spectrum non-systemic phenylpyrrole fungicide that is commonly used for post-harvest treatment of citrus (D'Aquino et al., 2013, 2007) and drupe fruits (Förster et al., 2007) to control gray mold (caused by *Botrytis cinerea*). FLD is also used in bulb vegetable handling for the control of various fungal pathogens like *Botrytis narcissicola* which is the causal agent of neck rot (Chastagner and DeBauw, 2011) and *Fusarium sp.* that cause Fusarium basal rot in *Allium* species (Le et al., 2021).

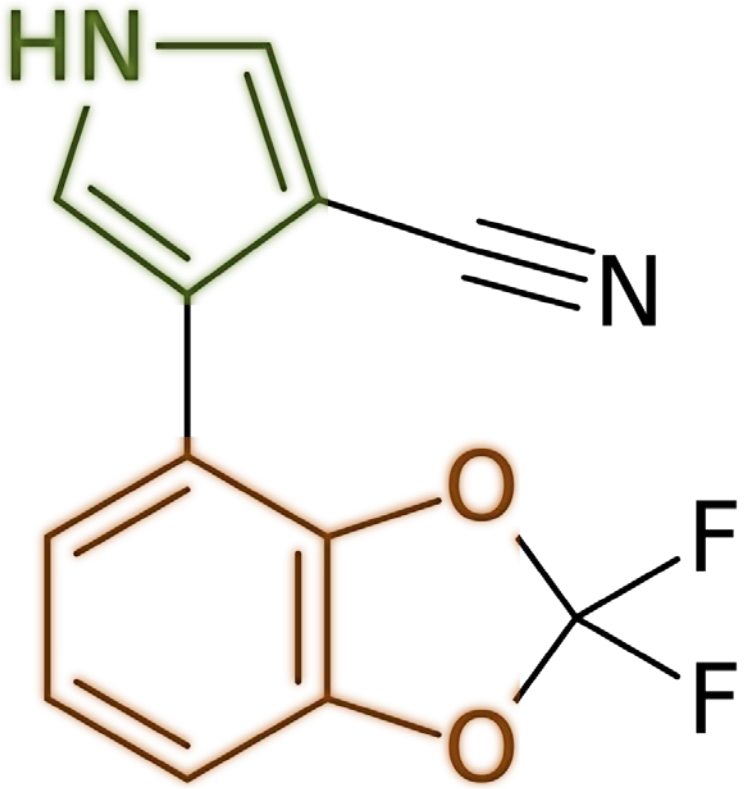

Figure 1.7. Chemical structure of FLD. The phenyl and pyrrole rings are colored red and green respectively

FLD is a synthetic analog of the natural antifungal compound pyrrolnitrin that was introduced to the market in 1990s (Kilani and Fillinger, 2016), for the control of *Botrytis cinerea*. Soon it was found to be a valuable tool for the control of most main postharvest diseases on a wide range of fruits (Schirra et al., 2011; Rosslenbroich and Stuebler, 2000). In EU it has been approved for use since 2008. Its mode of action includes inhibition of class III hybrid histidine kinase which controls the HOG (High Osmolarity Glycerol) response pathway, resulting in overproduction of glycerol and cell death by increased intercellular hydrostatic pressure (Brandhorst and Klein, 2019; Lawry et al., 2017; Lew, 2010).

FLD is classified as “Unlikely to Present Acute Hazard in Normal Use” by WHO (World Health Organization, 2020). It is characterized by negligible acute toxicity via oral, dermal or inhalation route and causes no skin and eye irritation (EFSA, 2007). Moreover the risk to insects, arthropods, earthworms and overall non-target soil organisms is considered low (EFSA, 2007).

FLD is hydrophobic (Log P_{ow} = 4.12) with water solubility as low as 1.8 mg/L (**Table 1.6**). It is not expected to be transferred into the air as it has very low vapor pressure (3.9 x 10^{-4} mPa) (**Table 1.6**). Similar it is considered rather immobile in soil with mean K_f and K_{foc} values of 3312 and 132100 ml/g respectively (**Table 1.6**). FLD is also a highly persistent fungicide in environmental matrices, with mean DT_{50} values in soil lab and field studies at 164 and 16 days respectively while it is resistant to aqueous hydrolysis (**Table 1.6**). The relatively lower DT_{50} of FLD in field studies is attributed to its high photo-degradation capacity, with a DT_{50} of 10 days (**Table 1.6**).

Table 1.6. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized K_{foc} or not K_f for organic carbon) of the fungicide FLD. (PPDB - Pesticide Properties Database. Accessed on 2021-11-15)**.**

Property-Fate	**Value**	**Interpretation**
Water solubility (mg/L)	1.8	Very Low
Vapor pressure (mPa)	3.9 x 10^{-4}	Low Volatility
Log P_{ow}	4.12	High
K_f (ml/gr)	290 – 7300	Non-Mobile
K_{foc} (ml/gr)	7500 – 210,000	Non-Mobile
$DT_{50\ lab}$ (days)	119-365	Persistent
$DT_{50\ field}$ (days)	8 – 43	Non-Persistent
$DT_{50\ photolysis}$ (days)	10	Moderately fast

During photo-degradation processes FLD is mainly transformed to three products, CGA 192155, CGA265378 and CGA 339833, which all result from oxidation of the pyrrole ring (EFSA, 2007). Transformation products of FLD show low to moderate persistence in soil with DT_{50} values ranging from 9 to 24 days for all three compounds. The transformation products of FLD are highly mobile in soil matrices, although further research is required about their environmental fate (EFSA, 2007). Finally, all three transformation products pose low toxicity to aquatic organisms (EFSA, 2007).

2.2.1.2 IMAZALIL

(RS)-1-(β-allyloxy-2,4-dichlorophenylethyl)imidazole or imazalil (IMZ) or enilconazole is a systemic imidazole fungicide that is commonly employed to control a wide range of fungal diseases in fruits (Schirra et al., 2011). It is a racemic mixture of enantiomers which recent studies have shown to have moderately different bioactivity (Li et al., 2019).

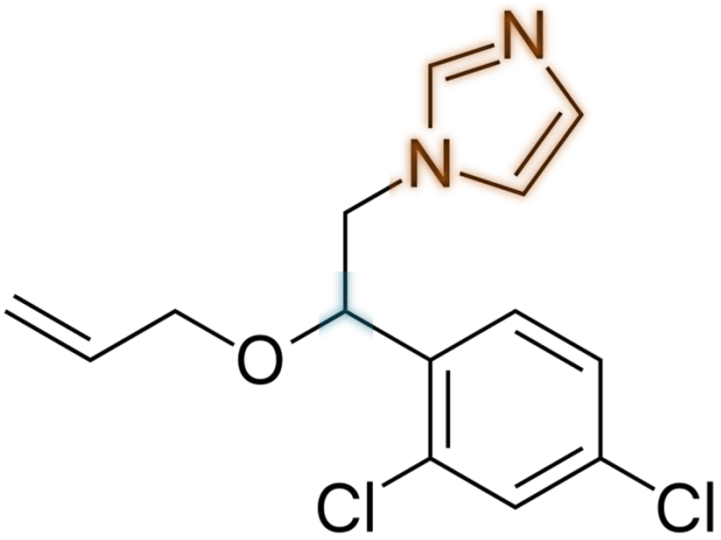

Figure 1.8. Chemical structure of IMZ. The imidazole ring is colored red and the chiral carbon is colored blue

IMZ is one of the most commonly used fungicides in post-harvest treatment of citrus for the preservation of the fruits during storage, shipping and marketing. It is especially effective against *Penicillium digitatum* that causes green mold to citrus fruits (Kellerman et al., 2016; Erasmus et al., 2013; Schirra et al., 2011), but it has also been found to control a variety of fungal pathogens in post-harvest treatment of horticultural crops (Schirra et al., 2011). IMZ disrupts fungal membrane function by inhibition of the demethylation step in the biosynthesis of ergosterol (Rozhon et al., 2019; Siegel and Ragsdale, 1978).

It was first introduced at 1983 and has been continuously approved for use in USA since then. In EU, it was first approved for use in 1997 and has also been continuously re-approved until 2024. EFSA proposed particular safety measures to be taken in order to ensure prevention of the exposure of the environment to IMZ. As a result EFSA proposed that its authorization is done under the clause that "*management measures tailored to local practice and legislation should be undertaken…to control the waste disposal of spent application solution and prevent accidental spillage or equipment wash water entering sewers or surface water drains"* (EFSA, 2010b)

IMZ is a moderately hazardous compound according to WHO (World Health Organization, 2020). It is toxic by oral and inhalation routes (rat LC50 227 mg/kg_{bw} and 1.84 mg/L respectively), while it has a low dermal toxicity. It is also a severe eye irritant (EFSA, 2010b). Studies suggest that IMZ is not genotoxic or teratogenic and does not have a toxic effect to mammals reproduction (EFSA, 2010b). However, liver and thyroid carcinomas have been demonstrated in mice and rats exposed to IMZ, respectively (United States, Environmental Protection Agency, 2005).

IMZ is a relatively hydrophobic compound with Log P_{ow} = 2.56 and water solubility of 184 mg/L at 20^{o}C (**Table 1.7**). It is has low volatility (0.158 mPa) and is not expected to be mobile in soil with mean K_f and K_{foc} values of 126.9 and 4753 ml/g respectively (**Table 1.7**). IMZ is a moderately persistent compound with mean DT_{50} in soil of 76.3 and 6.4 days in lab and field studies respectively (**Table 1.7**). Its low persistence in field studies could be

attributed to its fast photo-degradation, with a half-life of 6.1 days after continuous irradiation (**Table 1.7**).

Table 1.7. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized K_{foc} or not K_f for organic carbon) of the fungicide IMZ. (PPDB - Pesticide Properties Database. Accessed on 2021-11-15).

Property-Fate	Value	Interpretation
Water solubility (mg/L)	184	Moderate
Vapor pressure (mPa)	0.158	Low
Log P_{ow}	2.56	Low
K_f (ml/gr)	38.2 – 195.3	Non-Mobile
K_{foc} (ml/gr)	2,080 – 8,150	Non-Mobile
$DT_{50\ lab}$ (days)	43.9 – 128	Moderately persistent
$DT_{50\ field}$ (days)	5.7 – 7.1	Non-persistent
$DT_{50\ photolysis}$ (days)	6.1	Moderately fast

The main transformation product of IMZ is R14821 or imazalil-M, with almost 10% formation under aerobic conditions in the dark (R. Li et al., 2021; Matsumoto, 2001). IMZ-M is toxic to water flee (Daphnia magna), green algae (Pseudokirchneriella subcapitata) and zebrafish (Danio rerio) (Li et al., 2019)

2.3 BULB HANDLING INDUSTRIES

Postharvest application of fungicides for the control of fungal diseases is employed for several major bulb vegetables, the most significant of which are onions and garlic bulbs (Shree and Kumari, 2019; Patón et al., 2017; Sintayehu et al., 2011). Bulb vegetables are characterized by underground growth, vertical shoots and modified leaves which are used as food storage organs by the dormant plants (Shree and Kumari, 2019). Onions, garlic, chives, leeks and shallots are a few important bulb vegetables. Fungicides are often used in bulb dip treatments prior to planting to reduce infestation of emerging shoots and bulb (Chastagner and DeBauw, 2011), and postharvest to ensure quality preservation during storage (Sintayehu et al., 2011; Naik et al., 2007). Major postharvest diseases of onion include black mold, blue mold and gray mold caused by *Aspergillus niger, Penicillium sp.* and *Botrytis sp.* respectively (Ji et al., 2018; Prajapati, 2015; Shanmugam, 2006).

2.3.1 FUNGICIDES USED IN BULB HANDLING INDUSTRIES

2.3.1.1 CHLOROTHALONIL

Tetrachloroisophthalonitrile or chlorothalonil (CHT) is a non-systemic, broad spectrum, organochlorine fungicide used for the control of fungal disease in bulbs, including onion downy mildew caused by *Peronospora destructor* (Araújo et al., 2017)

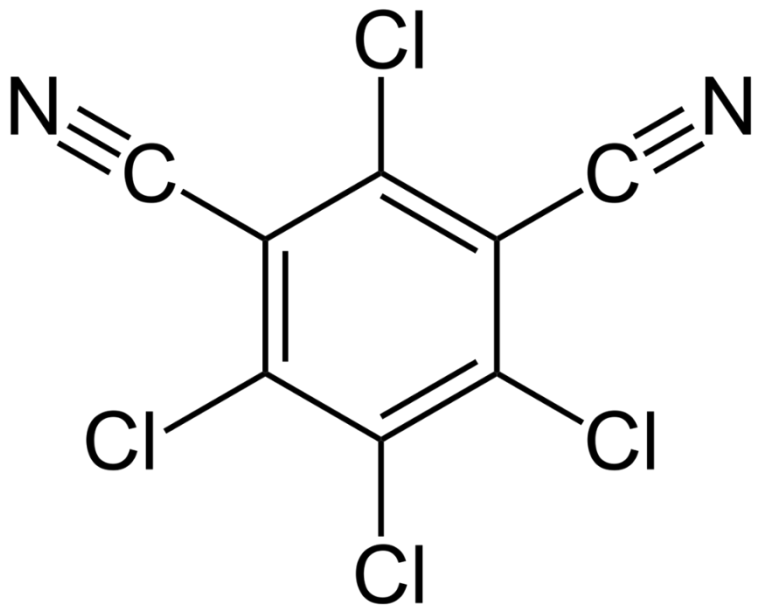

Figure 1.9. Chemical structure of CHT.

CHT mode of action involves prevention of spore germination and zoospore activity, but the exact mechanism remains unknown. Generally, CHT's antifungal properties are attributed to inactivation of cell sulfydryl enzymes, which are important in cellular respiration (Sherrard et al., 2003). Tillman et al. proposed that CHT mode of action includes formation of glutathione-CHT derivatives which result in inhibition of thiol-dependent enzymes (Tillman et al., 1973). Gallagher et al, demonstrated that CHT induction of glutathione involves increased cysteine uptake and increased gamma-glutamylcysteine synthetase activity (Gallagher et al., 1992). Therefore, CHT has been described as having multiple sites of action, which makes it an effective tool in disease management as it is complicated for target-organisms to develop resistance.

CHT was introduced in 1965 and first registered in the USA in 1966 for use on turfgrass and later in potato and other vegetable and orchard crops (United States, Environmental Protection Agency, 1999). CHT comes in the market as a standalone or as a mixture with other pesticide compounds in a great number of PPPs. In the EU, CHT was approved for use in 2005 in the context of Directive 91/414 /EEC (EUROPEAN COMMISSION, 2006) but its approval was not renewed in 2019, due to increasing concerns regarding tis genotoxicity and carcinogenicity effect and its risk to non-target organisms especially amphibians and fish (EUROPEAN COMMISSION, 2019). In addition, concerns were identified with regards to the transformation products of CHT, which pose a high risk for groundwater pollution (EUROPEAN COMMISSION, 2019).

According to WHO, CHT is classified as "Unlikely to Present Acute Hazard in Normal Use" (World Health Organization, 2020) based on the International Programme on Chemical Safety (International Programme on Chemical Safety, 1996). Peer review of pesticide risk assessment conducted by EFSA in 2018 concluded that CHT presents low acute toxicity via oral or dermal routes but it is very toxic when administered through inhalation and irritates the respiratory tract (EFSA, 2018). Moreover, it may cause serious eye damage and allergic skin reaction but it is not a skin irritant (EFSA, 2018). CHT is classified in carcinogenicity

category 1B - “May cause cancer” and it is unlikely to present neural and immune system toxicity, genotoxicity and endocrine disruption of androgen, estrogen and thyroid hormones. (EFSA, 2018).

CHT is a relatively hydrophobic compound with Log P_{ow} of 2.94 (**Table 1.8**). Its water solubility is as low as 0.81 mg/L, rendering it basically non soluble to aqueous solutions. It is not expected to be detected in the air, with a vapor pressure of 0.076 mPa (**Table 1.8**). Because of its very low water solubility, the affinity of CHT for soil adsorption is high with K_f and K_{foc} values being 3 – 74.1 and 330 – 7000 respectively (**Table 1.8**). Despite its strong adsorbance in soil, it is a non-persistent compound with mean DT_{50} values of 3.53 and 17.9 days in lab and field studies respectively. It is also prone to photo-degradation with a half-life of 0.72 days (**Table 1.8**).

Table 1.8. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized K_{foc} or not K_f for organic carbon) of the fungicide CHT. (PPDB - Pesticide Properties Database. Accessed on 2021-11-15).

Property-Fate	**Value**	**Interpretation**
Water solubility (mg/L)	0.81	Very Low
Vapor pressure (mPa)	0.076	Low
Log P_{ow}	2.94	Moderate
K_f (ml/gr)	3 -74.1	Slightly Mobile
K_{foc} (ml/gr)	330 – 7000	Slightly Mobile
$DT_{50\ lab}$ (days)	0.256 – 19	Non-Persistent
$DT_{50\ field}$ (days)	7.44 – 28.4	Non-Persistent
$DT_{50\ photolysis}$ (days)	0.72	Fast

CHT has been shown to transform into various products with different toxicological and environmental profiles. Transformation product R182281 (or 4-hydroxy-2,5,6-trichloroisophthalonitrile or 4-hydroxychlorothalonil) is worth noting as it seems to be the predominant metabolite in crops (Wu et al., 2014). Wu et al. have shown that repeated CHT application in greenhouse soil may lead to accumulation of both CHT and 4-hydroxy-chlorothalonil (Wu et al., 2014). It has been shown to cause acute toxicity after oral administration. Further studies showed that 4-hydroxychlorothalonil exhibits moderate to very high persistence in soil, with DT_{50} values ranging from 38 days to 609, and medium to high mobility in soil with mean K_{Foc} value of 484 ml/g (EFSA, 2018).

2.3.1.2 THIABENDAZOLE

2-(thiazol-4-yl)benzimidazole or thiabendazole (TBZ) is a popular systemic benzimidazole fungicide.

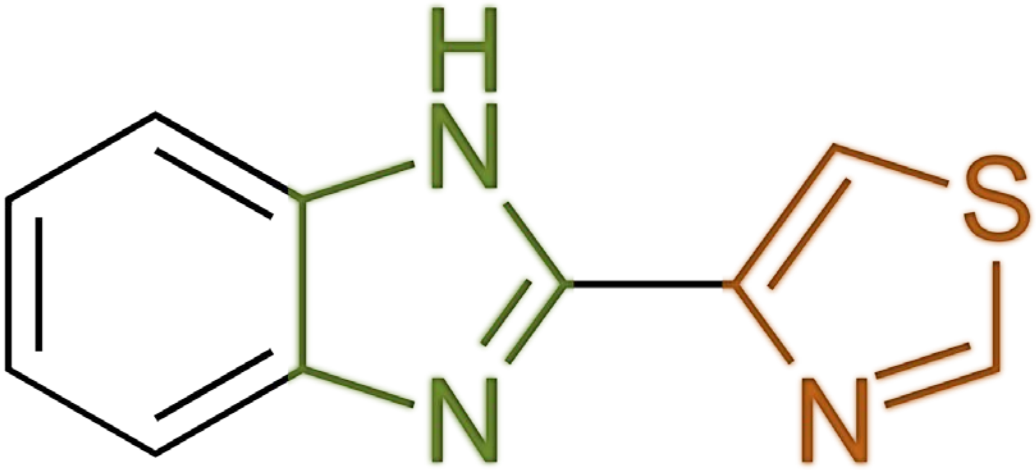

Figure 1.10. Chemical structure of TBZ. The thiazole and imidazole ring are colored red and green respectively.

TBZ is used for the control of neck rot, caused by *Botrytis narcissicola* (Chastagner and DeBauw, 2011), and narcissus basal rot, caused by *Fusarium oxysporum f.sp. narcissi* (Hanks, 1996) in commercial daffodil. TBZ contributes to complete inhibition of fungal cell mitosis by interfering with β-tubulin and, therefore, with microtubule assembly (Ishii, 1992; Davidse and Flach, 1978).

TBZ was first introduced by Merc Chemical Co. as an anthelminthic drug for human and livestock roundworm infestation. However, it was widely used a fungicide for the control of plant pathogenic fungi, especially during storage. It was first authorized as a fungicide in the USA in 1969 by Merck and Company, Inc. Currently 62 PPPs are registered in the USA whose active ingredient is TBZ (United States, Environmental Protection Agency, 2002). TBZ has been authorized for use in EU until 2032 (EUROPEAN COMMISSION, 2016) with the condition that postharvest industries will adequately collect and treat wastewater that contain TBZ residues.

TBZ is classified as slightly hazardous of acute risk to health by the WHO (World Health Organization, 2020). It shows low acute toxicity when administered orally, dermally and via inhalation, and no irritation to skin and eyes. TBZ does not have genotoxic potential neither it poses risk to reproduction or development (EFSA, 2014).

TBZ is a hydrophobic compound with Log P_{ow} = 2.39 and water solubility of 30 mg/L (**Table 1.9**). It is non-volatile with a vapor pressure of 5.3 x 10^{-4} mPa vapor pressure (**Table 1.9**). TBZ is slightly mobile in soils with K_f and K_{foc} values ranging between 13 – 100 and 580 – 3921 ml/gr respectively (**Table 1.9**). The adsorption of TBZ in soil has been positively correlated with organic matter content. TBZ is a major environmental pollutant on account of its high persistence in the environment with more than a year and 1000 days half-life in lab and field studies respectively (**Table 1.9**).

Table 1.9. The physicochemical properties (water solubility, vapor pressure, octanol-water partition coefficient log) and the environmental fate parameters (DT_{50}, Freundlich adsorption coefficient normalized K_{foc} or not K_f for organic carbon) of the fungicide TBZ. (PPDB - Pesticide Properties Database. Accessed on 2021-11-15).

Property-Fate	Value	Interpretation
Water solubility (mg/L)	30	Low
Vapor pressure (mPa)	5.3×10^{-4}	Low
Log P_{ow}	2.39	Low
K_f (ml/gr)	13 – 100	Slightly Mobile
K_{foc} (ml/gr)	580 – 3,921	Slightly Mobile
$DT_{50\ lab}$ (days)	>365	Highly Persistent
$DT_{50\ field}$ (days)	>1,000	Highly Persistent
$DT_{50\ photolysis}$ (days)	1.2	Moderately Fast

3 TREATMENT OF WASTEWATERS FROM AGRO-FOOD PROCESSING INDUSTRIES

Agro-food processing industries often use fungicides to preserve produce quality during storage and minimize losses due to pathogens. Post-harvest treatment of fruits, bulb vegetables and seeds results in the production of fungicide-contaminated effluents. The uncontrolled environmental release of these effluents will result in deterioration of natural resources, will impose toxicity to aquatic fauna and flora, and will eventually threat human health. As a matter of fact many studies have linked the detection of pesticides in surface water, sediments, groundwater and biota with the presence of intensive agro-industrial activities like citrus fruit packaging plants (Ccanccapa et al., 2016; Masiá et al., 2013). Improper handling of agro-industrial effluents, including direct discarding in agricultural land (land-spreading) and natural water bodies may cause severe environmental pollution with potentially persistent and toxic compounds (Papadopoulou et al., 2018). Moreover, discharge in municipal wastewater treatment systems can result in accumulation of fungicides as the generic microbial community of these systems is not able to efficiently remove persistent fungicides like IMZ and TBZ contained in the above agro-industrial effluents (Campo et al., 2013).

Thus, depuration of these effluents before environmental release should be a priority of industries that make use of pesticides. Nevertheless, an efficient and affordable method for pesticide depuration has not been introduced so far. Various methods have been studied for the depuration of these wastewaters that are mainly categorized to abiotic and biotic methodologies.

3.1 ABIOTIC WASTEWATER TREATMENT METHODOLOGIES

A plethora of abiotic processes have been studied for their efficiency in the depuration of pesticide contaminated wastewaters, including adsorption, evaporation, photocatalytic degradation and advanced oxidation processes.

Adsorption is a promising technique due to its low cost and operational simplicity which involves flexibility of usage, easy regeneration, sludge-free operation and no involvement of toxic intermediates (Yadav et al., 2021; Ahmad et al., 2010). However, most of the relevant research has been focused on removal of a single compound which is not applicable to real-world practices which mostly require the removal of diverse contaminants (N'Diaye et al., 2019; Ali, 2018; Njoku et al., 2014). Moreover, most of these studies concern aquatic solutions of pesticides and not actual wastewaters. High organic matter content, COD and inorganic salts that generally characterize effluents from agro-food industries, compete with pesticides for adsorption sites and tamper with the adsorption material (Wang and Wang, 2016).

Evaporation ponds have also been studied on account of their simplicity and cost-effectiveness especially for implementation in regions with high solar radiation levels (Amoatey et al., 2021). However, their application is hindered by environmental concerns arousing from overflow of wastewater, leakages via liners and evaporation of toxic compounds which may have an impact in flora and fauna including humans (Amoatey et al., 2021).

Advanced oxidation processes (AOP) have been thoroughly studied as useful pesticide-contaminated wastewater treatment technologies due to their ability to degrade a wide variety of organic compounds. Chemical oxidation refers to the use of oxidizing agents, such as the highly active hydroxyl radicals ($OH^{\cdot}$), that cause the decomposition of pesticide pollutants into biodegradable compounds, harmless products or CO_2, water and inorganics (mineralization) (Morillo and Villaverde, 2017; Quiroz et al., 2011). Other oxidizing agents that are commonly used are ozone, hydrogen peroxide (H_2O_2) and chlorine chemical compounds like hypochlorites, chlorine and chlorine dioxide (Pavel and Gavrilescu, 2008). AOP is the combination of these oxidants with iron salts, semiconductors and/or ultraviolet-visible irradiation in order to increase their depuration efficiency (Morillo and Villaverde, 2017). (Photo-)Fenton processes and titanium dioxide (TiO_2) photocatalysis are the most typical AOP techniques.

The Fenton process has been commonly recalled for the depuration of pesticide contaminated effluents (Lopez-Loveira et al., 2019; Gar Alalm et al., 2015). Santiago et al. studied the efficiency of Fenton-based processes for the mineralization of IMZ contained in deionized water, simulated wastewater and effluents from the postharvest treatment of banana fruit. They concluded that the Fenton reactions coupled with UV irradiation (photo-Fenton) were the most suitable approaches for the treatment of IMZ-contaminated wastewaters due to the high mineralization rates observed and the lower iron (Fe(II)) and H_2O_2 requirements compared to the Fenton procedure (Santiago et al., 2016). In a follow up study, Santiago et al. studied the factors that influence the efficiency of Fenton process and found that the most significant factor that determined the process was Fe(II) (Santiago et al., 2018a). Moreover, they verified higher mineralization with photo-Fenton processes and they estimated an operational cost of 2.06 €/m^3 for the treatment of 10 m^3 IMZ-contaminated wastewater (Santiago et al., 2018a). García-Estrada et al, demonstrated the potential of metallurgical copper slag in the depuration of TBZ through heterogeneous photo-Fenton-like reaction (García-Estrada et al., 2020). López-Loveira et al., showed the potential of photo-

Fenton processes when coupled with biological processes for the mineralization of IMZ in artificial wastewater (López-Loveira et al., 2017). Despite their high removal efficiency, (photo-) Fenton processes are characterized by several drawbacks including (i) high costs, (ii) risks related to H_2O_2 provision, storage and transport, (iii) accumulation of iron sludge, (iv) wasting reactions that reduce mineralization efficiency and (v) the potential formation of toxic intermediate compounds (Sirés et al., 2014).

TiO_2-based photocatalysis is a better studied AOP technique (Santiago et al., 2018b; Cruz et al., 2017; Xing et al., 2014; Lhomme et al., 2008). TiO_2 is a low cost, non-toxic and efficient catalyst for the depuration of pesticides from wastewaters (Bano et al., 2021). During TiO_2-based photocatalysis, H_2O_2 molecules are separated into two hydroxyl radicals, which in turn interact with pesticide compounds. Molla et al. demonstrated the potential of TiO_2-based photocatalysis against a water solution of the insecticide diazinon, with complete mineralization after 30 hrs of irradiation (Molla et al., 2020), whereas Sraw et al. tested the removal efficiency of TiO_2 coated clay beads for the insecticide monocrotophos and noted 74% mineralization (Sraw et al., 2018). Jiménez-Totozintle et al. showed the potential of biotreatment in combination with $TiO2/H_2O_2$ solar photocatalysis in the depuration of effluents from a citrus industry that contained the fungicides IMZ, TBZ and acetamiprid (Jiménez-Totozintle et al., 2015). The main drawbacks of this methodology include (i) the high costs of the system construction and the high electrical energy requirements, (ii) the gradual reduction of the removal efficiency associated with the deposition of organic material on electrode's surface that shortens its lifetime, (iii) the possible need for use of additional reagents, like electrolytes or H_2O_2 and (iv) the formation of slurry or toxic and reactive pesticide transformation products that need further treatment (Titchou et al., 2021; Sirés et al., 2014; Santiago et al., 2013).

Another concern regarding the efficiency of AOP treatment methods is that they have been tested mostly against wastewaters containing very low pesticide concentrations (μg/L) which is orders of magnitude below those found in agro-industrial effluents (Jiménez et al., 2013). Also, the majority of these studies were performed with distilled water instead of actual wastewaters which contain high organic matter and inorganic salts, that tend to interact with free radicals, reducing the mineralization of pesticide compounds (Bisaria et al., 2021; Brame et al., 2015). In fact, the few studies on real effluents are limited to fruit-packaging activities (García-Estrada et al., 2020; López-Loveira et al., 2017; Santiago et al., 2016; Jiménez-Totozintle et al., 2015; Jiménez et al., 2013; Santiago et al., 2013) and a single study to seed coating effluents (Wen et al., 2018). Finally, for most industries the implementation of advanced treatment technologies for the depuration of pesticide contaminated effluents is practically impossible due to their high operational and capital cost expenses.

3.2 BIOTIC WASTEWATER TREATMENT METHODOLOGIES

The use of macro- and microorganisms in wastewater depuration has been proposed as an ecofriendly, low-cost alternative to chemical methodologies.

Phytoremediation is a biotic approach used for the depuration of pesticide-contaminated sites and it has been studied in regard to its efficiency and cost-effectiveness (Tarla et al., 2020). Plants can remove pesticides from wastewater by (i) root uptake and accumulation, (ii) vapor uptake from the surrounding atmosphere, (iii) diffusion through

plant surfaces, especially in floating leaf or free-floating plants and (iv) releasing enzymes and exudates for the direct degradation of pesticides or the enhancement of the degradation potential of the rhizosphere microbial community (Negrete-Bolagay et al., 2021).

Among the plants that have been tested for their depuration potential is industrial hemp (*Cannabis sativa*) which can effectively remove contaminants due to its porous and hydrophilic surface structure and tolerance to pesticides (Wu et al., 2021). Lv et al. demonstrated the removal of IMZ and tebuconazole in saturated constructed wetland mesocosms planted with 5 wetland plant species. The authors proposed two possible transformation pathways, either inside the wetland plants after uptake or in the mesocosm bed substrate by plant-stimulated microbial community (Lv et al., 2016). Hwang et al demonstrated the complete removal of atrazine in a wetland planted with *Canna flaccida* (Hwang et al., 2020). Even though phytoremediation is a sustainable method for the removal of pesticides from environmental matrices, it does not come without problems. The rather low transformation rates of most pesticides in plants, the potential phytotoxicity of several pesticides and need for addition of extra C and N sources are amongst the major limitations of this method (Ibañez et al., 2016).

Biopurification systems (BPS) offer a simple, low cost and efficient solution for the depuration of pesticide-contaminated effluents. BPS can remove pesticide through adsorption and biodegradation processes (Vandermaesen et al., 2016). Several types of Biopurification systems have been developed like the biofilter, the Phyotbac ® and the biobed (De Wilde et al., 2007). The first reported biobed was used in Sweden for the handling of spillages during filling or washing of sprayers (Torstensson, 2000). Since then, many studies have explored the potential of biobeds for the treatment of pesticide-contaminated wastewaters (Lescano et al., 2022; Kumari et al., 2021; Vischetti et al., 2020; Karas et al., 2015; Vischetti et al., 2012; Karanasios et al., 2010a).

A shared feature of all BPS types and the key to their success is the organic packing material, the biomixture, a biologically active substrate that serves as pesticide adsorbent and as a source of an active microbial community able to effectively degrade a wide pesticide range (Domínguez-Rodríguez et al., 2021; Bergsveinson et al., 2018; Fogg et al., 2003). The biomixture typically consists of soil, a lignocellulosic material and a humified organic substrate which reflects locally available material (De Wilde et al., 2007).

Soil provides the microbial community and adsorption sites and should be rich in humus but low in clay content so as to allow high bioavailability of pesticides. Sniegowski and Springael studied the possibility of using pesticide-primed soil that is expected to carry pesticide-degrading microbes and showed improved degradation capacity of both parent compound and transformation products (Sniegowski and Springael, 2015).

Lignocellulosic materials act as a carbon (C) and nitrogen (N) source for the microorganisms and stimulate the degradation of pesticides by promoting the production of broad-spectrum lignin-degrading enzymes (Karas et al., 2011). The most popular lignocellulosic material is straw, but depending on locally cultivated crops other materials have been tested as well like branches and stalks from grape pruning, olive leaves, corn

cobs, citrus peels and sunflower residues (Romero et al., 2019; Karanasios et al., 2010b; Vischetti et al., 2008)

The humified material contributes an additional C and N source and numerous sites for pesticide sorption. Moreover, it helps maintaining aerobic conditions and regulates the pH and water holding capacity. Peat was the humified material of choice for the original biomixture (Torstensson, 2000) but other materials like composts and spent mushroom substrate (SMS) have gained popularity as well. Various composted materials have been employed as biomixture components that mirror local agricultural practices like olive leaf and tree pruning, vine pruning, grape marc, cotton residue and seed, sheep manure or spent coffee grounds (Omirou et al., 2012; Fenoll et al., 2011; Karanasios et al., 2010a; Monaci et al., 2009). Lately, SMS has been has been examined in many studies for pesticide depuration purposes (Alves et al., 2022; Álvarez-Martín et al., 2016; Rodríguez-Cruz et al., 2012). SMS is the readily available, complex organic residue that remains after mushroom harvest (Marín-Benito et al., 2016; Herrero-Hernández et al., 2011). It is generated in large quantities by mushroom farms and its utilization in the frame of circular economy will contribute to tackle pesticide pollution in a sustainable way. Many studies have demonstrated the potential of SMS as a component of the biobed packing material (Karas et al., 2016b; Gao et al., 2015; Marín-Benito et al., 2012; Karanasios et al., 2010a)

Often, full-scale biobed systems are covered with a layer of grass which helps in regulating the moisture of the biobed system by creating an upward transport of water. Furthermore, it acts as an adsorption site of pesticide molecules and it produces root exudates which may contribute to metabolic processes (Romero et al., 2019; Castillo et al., 2008).

Generally, biobeds consist of a meter deep pit, with is filled with the organic packing material. The bottom of the biobed can be sealed (lined biobed system) or not (inlined biobed system). An immediate asset of the lined system is the prevention of pesticide leaching to the bulk soil and potentially to groundwater. However sealing the bottom of the biobed system could result in imbalances in the biomixture moisture status, with drier surface layer (0-10 cm) and saturated deep layers which in turn will result in decreased microbial biomass (De Wilde et al., 2007).

The microbiome of the biobed packing material is crucial for its efficiency, as it is responsible for the removal of pesticides. Several methods have been previously employed to elucidate the role of microbial communities in the depurating capacity of biobed systems, such as measurement of microbial biomass carbon and microbial respiration (Marinozzi et al., 2013; Omirou et al., 2012; Karanasios et al., 2010b; Vischetti et al., 2008), estimation of the activity of a plethora of enzymes shown to be associated with pesticide transformation (Romero et al., 2019; Karas et al., 2016a; Marín-Benito et al., 2012; Karanasios et al., 2010b) and assessment of the abundance of phylogenetically distinct microbial taxa or genes via qPCR (Karas et al., 2016b). Despite the amount of literature, the structure and dynamics of the biobed microbiome remain hugely underexplored, and only recently has the microbial community composition of biobed systems been determined by amplicon sequencing approaches (Bergsveinson et al., 2018; Holmsgaard et al., 2017) and metagenomics and

metatranscriptomic analyses (Russell et al., 2021). Holmsgaard et al. in their study in an operational biopurification system, showed via pyrosequencing of 16S rRNA gene fragment that the microbial community was dominated by Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes and diverse response were observed on account of wastewater application. (Holmsgaard et al., 2017). Bergsveinson et al. studied four biobeds systems with different design and variable applied pesticides. They found, through Illumina sequencing of 16S rRNA gene fragment (bacteria) and ITS2 genomic region (fungi), that all biobed systems shared a core microbiome, which included the majority of most abundant bacterial phyla, such as *Proteobacteria*, *Actinobacteria*, *Bacteroidota* and *Chloroflexi*, and the fungal orders, like *Hypocreales*, *Sordariales* and *Pleosporales* (Bergsveinson et al., 2018). Lastly, Russell et al. in their study of microbial communities of two biobed systems by metagenomics analysis also demonstrated dominance of *Proteobacteria* (Russell et al., 2021).

Beyond the phylogenetic composition of the biobed microbiome, the occurrence, distribution and activity of genes responsible of pesticide biodegradation have also been addressed. Biopurification systems are considered a hot spot of mobile genetic element and horizontal gene transfer. Previous studies have shown that biobeds that treat pesticide-contaminated effluents are enriched in mobile genetic elements. Dealtry et al. studied the dynamics of MGEs in a biopurification system and reported high abundance of plasmids belonging to IncP-1, IncP-7, IncP-9, IncQ and IncW groups (Dealtry et al., 2014) while Stork et al, in their study of isoproturon degradation genes pdmAB also concluded that pesticide application induces high transposition of pesticide-catabolic traits between the members of the bacterial community (Storck et al., 2020). Dunon et al. demonstrated high abundance of IncP-1 and *IS*1071 due to pesticide treatment in an on-farm biopurification system (Dunon et al., 2013) and, in a later study, they demonstrated via metagenomics analysis, the role of *IS*1071 as a carrier of catabolic genes and a contributor of the shift of the microbial community towards pesticide biodegradation (Dunon et al., 2018).

The depuration potential of biobed systems can be greatly enhanced by inoculating with pesticide-degrading microorganisms. Karas et al. demonstrated increased performance in pilot biobed systems bioaugmented with bacteria able to rapidly degrade *ortho*-phenyl phenol, diphenylamine and TBZ (Karas et al., 2016b). Campos et al, showed accelerated biodegradation of iprodione (IPR) by an IPR-degrading *Arthrobacter* with or without the assistance of rhizosphere bacteria. Madrigal-Zúñiga et al showed increased performance of a biomixture bioaugmented with a white-rot fungus, *Trametes versicolor,* against the insecticide/nematicide carbofuran (Madrigal-Zúñiga et al., 2016).

4 AIMS OF THE THESIS

Agro-food processing industries use large amounts of fungicides in order to preserve produce quality during storage and transport (Omirou et al., 2012). The uncontrollable release of these effluents presents serious pesticide point-source pollution and poses a great environmental threat due to the high persistence and toxicity of the pesticides contained in those effluents on non-target organisms (Carvalho, 2017).

Through the years, many depuration methodologies have been studied but most of them have been limited to effluents from fruit packaging activities, while only one reported on the treatment of effluents from seed coating activities (Wen et al., 2018). Even though, abiotic methodologies have been studied for the removal of pesticides from agro-industrial effluents, their full implementation has not been accomplished due to their high operational costs and technological requirements, the formation of toxic by-products and poor results concerning mineralization (Sirés et al., 2014). In the absence of affordable and efficient alternative solutions, agro-food processing industries rely on certified companies for the ex situ treatment of their wastewaters as toxic effluents at a particularly high cost (0.7 – 3 € per L). Alternatively they discharge their effluents to municipal wastewater treatment facilities, which have limited pesticide removal capacity (Campo et al., 2013) or in adjacent agricultural land (land-spreading) and water systems compromising environmental quality (Papadopoulou et al., 2018). All of the above manifest the need for development and application of low-cost and appropriate methodologies for the decontamination of pesticide-contaminated agro-food wastewaters.

Biological treatment systems like biobeds constitute a sustainable, economically viable and efficient solution. Based on the above, numerous studies have been conducted in Mediterranean countries for the depuration of effluents from fruit packaging industries by biobed systems (Delgado-Moreno et al., 2017; Omirou et al., 2012; Vischetti et al., 2012). Likewise, the laboratory of Plant and Environmental Biotechnology has been studying the utilization of biobed systems for the depuration of pesticide-contaminated effluents generated by local fruit packaging plants in lab assays (Campos et al., 2017; Karas et al., 2016a, 2015) and full scale pilot biobed systems (Karas et al., 2016b). Despite the amount of literature, little is known about the removal potential of biobeds against recently introduced fungicides like FLD. Moreover, since the majority of research has been focused to fruit packaging industry effluents, nothing is known about the efficiency of biobeds against effluents produced by seed coating and bulb dipping activities which are characterized by low COD values and high concentrations of fungicides.

The high depuration efficiency of biobeds is attributed to the microbes of the biobed packing material. Nevertheless, the microbial communities that develop in the biobed while it treats pesticide-contaminated effluents are yet unexplored. Up to date, the majority of research has been focused in evaluating microbial activity and biomass, without pointing out any connections between specific microbial groups and pesticide degradation (Romero et al., 2019; Diez et al., 2017; Marinozzi et al., 2013; Coppola et al., 2011). Considering the importance of microbes to the removal of pesticides, bioaugmentation of biobed packing material with pesticide degrading microorganisms is a promising practice that ensures high efficiency of the depuration process (Karas et al., 2016b). The laboratory of Plant and Environmental Biotechnology already contains a collection of bacteria that degrade fungicides commonly used by fruit packaging plants like the microbial consortium that degrades TBZ (Perruchon et al., 2017), *Pseudomonas putida* that can rapidly degrade diphenylamine (Perruchon et al., 2015), *Sphingomonas haloaromaticamans* that can metabolize *ortho*-phenylphenol and *Arthrobacter* and *Paenarthrobacter* sp that can degrade iprodione (Katsoula et al., 2020; Campos et al., 2015).

Considering all the above, the main aims of this thesis were:

- **to assess the capacity of biobeds for the depuration of pesticide contaminated effluents from seed producing, bulb handling and fruit packaging industries ,**
- **to explore the composition of the microbiome of biobed systems and its response to continuous pesticide exposure,**
- **to isolate microorganisms with the ability to degrade recalcitrant fungicides contained in those effluents**

This main aim was achieved through a series of studies involving microcosm, leaching column studies and enrichment cultures. Hence we first determined in lab microcosm studies the dissipation and adsorption of fungicides contained in the different agro-industrial effluents in an SMS-based biomixture (Chapter 2). Secondly, we employed a column leaching study to determine the capacity of the SMS-based biomixture to retain the pesticides contained in the different agro-industrial effluents and we explored the composition and response of the biobed microbiome under realistic pesticide loading conditions using advanced amplicon sequencing approaches (Chapter 3). Finally, we isolated a fungal strain with the ability to degrade IMZ, the first microorganism able to degrade this recalcitrant fungicide, and characterized its degrading capacity under different laboratory and bioengineering conditions (Chapter 4). This thesis offers the necessary background research needed for an extension of the use of biobeds for the treatment of pesticide-contaminated effluents from various agro-food industries and provides an efficient IMZ-degrading microorganism which could be exploited as an inoculum in biobeds, for optimization of their performance towards IMZ-contaminated effluents, or in biological wastewater treatment units treating higher wastewater volumes than biobeds.

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Chapter 2

Expanding the use of biobeds: Degradation and adsorption of pesticides contained in effluents from seed-coating, bulb disinfestation and fruit-packaging activities

The work presented in Chapter 2 is included in the scientific paper:

Papazlatani, C.V., Karas, P.A., Tucat, G., Karpouzas, D.G., 2019. Expanding the use of biobeds: Degradation and adsorption of pesticides contained in effluents from seed-coating, bulb disinfestation and fruit-packaging activities. Journal of Environmental Management 248, 109221. https://doi.org/10.1016/j.jenvman.2019.06.122

1 INTRODUCTION

Pesticides constitute one of the most important group of environmental pollutants with several of them listed as priority pollutants of natural water resources (European Union, 2016). The pollution of surface water and groundwater systems by pesticides occurs by nonpoint and point sources. The later are major contributors of water pollution and occur during the implementation of poor agricultural practices while mixing and loading of pesticide solutions or when washing the sprayers (Cooper et al., 2016; Helweg et al., 2002; Müller et al., 2002). Recent studies suggested that agro-food industries that use pesticides act as point sources for the contamination of surface water systems (Ccanccapa et al., 2016; Masiá et al., 2013). Such industries include (a) fruit packaging plants that use fungicides like thiabendazole (TBZ) and fludioxonil (FLD) to protect fruits from fungal infestations during storage (Cerioni et al., 2017; D'Aquino et al., 2013), (b) seed producing industries which coat seeds with fungicides like carboxin (CBX), metalaxyl-M (MET-M), fluxapyroxad (FLX), and FLD (Rothrock et al., 2012) and (c) bulb handling industries which employ bulb dipping in dense solutions of chlorothalonil (CHT), TBZ, and FLD to prevent fungal infestations (Cooper et al., 2016; Clarkson and Hanks, 2012; Chastagner and DeBauw, 2011). The environmental peril by the uncontrolled release of the pesticide-contaminated wastewaters produced by those agro-industries has been acknowledged by the European Commission, which enforced the implementation of relevant wastewater treatment strategies (European Union, 2004). Despite that, little progress has been made on the treatment of such effluents. The few studies available have been limited to the treatment of wastewaters from fruit-packaging plants (Santiago et al., 2018a, 2018b; López-Loveira et al., 2017; Jiménez et al., 2013), while a single study recently reported on the treatment of wastewaters from seed-coating activities (Wen et al., 2018). Abiotic processes (i.e. Fenton, photo-Fenton processes, TiO2 catalysis) combined or not with biological processes could eliminate pesticides contained in effluents from fruit-packaging plants. However the efficiency of these treatment methods was assessed at μg L−1 pesticide concentration levels, which are orders of magnitude below those commonly found in the aforementioned wastewaters (Jiménez et al., 2013). In addition, there are concerns about the production of oxidized intermediates with higher toxicity than the parent compounds (Santiago et al., 2018a; Sirtori et al., 2014). On top of that, nothing is known about the treatment of the effluents produced by seed-coating and bulb-dipping activities or the efficiency of such treatments on the removal of pesticides recently introduced in fruit-packaging plants like FLD.

Biopurification systems like biobeds were initially proposed by Omirou et al. (2012) as a cost-effective and efficient solution for the depuration of wastewaters from the fruit-packaging industry. They are simple biological systems initially introduced for the depuration of wastewaters produced by on-farm activities (Castillo et al., 2008). In their simplest form, biobeds constitute a pit on the ground lined with an impermeable layer and a drainage at the bottom, which is filled with an organic substrate (named "biomixture") composed of soil, straw and a humified material (i.e. compost or peat) (De Wilde et al., 2007). The biomixture, the core of those systems, supports a highly diverse and catabolically versatile microbial community (Bergsveinson et al., 2018; Holmsgaard et al., 2017) and has high retention capacity for pesticides that are not biodegradable (Castillo and Torstensson, 2007).

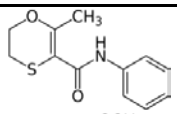
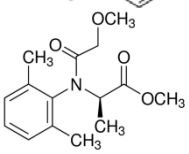
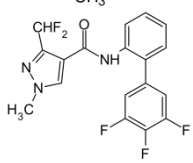
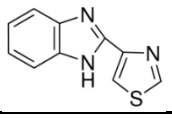
The main aim of this study was to evaluate the degradation and adsorption of pesticides contained in effluents from seed coating, bulb dipping, and fruit-packing activities on a biomixture and comparatively on the soil used for the preparation of the biomixture. The choice of the tested biomixture, composed of soil, straw, and spent mushroom substrate (SMS), was based on previous studies which had shown its high degradation capacity for TBZ, imazalil, diphenylamine, and ortho-phenylphenol used in fruit-packaging plants (Karas et al., 2015). This constitutes the first evaluation step towards the implementation of biobeds for the treatment of such effluents (Chapter 3).

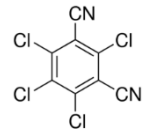
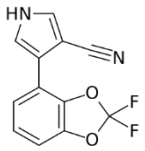
2 MATERIALS AND METHODS

2.1 PESTICIDES

Analytical standards of CBX (99.9% Pestanal ®), MET-M (98.4%, Pestanal ®), FLD (99.9% Pestanal ®), TBZ (99% Pestanal ®) and CHT (99.7% Pestanal ®) were purchased from Sigma-Aldrich, while FLX (99.9%, BAS 700 F) was provided by BASF Hellas. The chemical structures and the physicochemical properties of the studied pesticides are given in **Table 2.1**. Pesticide stock solutions (1000 mg L−1) in methanol (for CBX, MET-M, FLD and TBZ) or acetonitrile (for FLX and CHT) were prepared and used for the preparation of serial dilutions with concentrations ranging from 0.1 to 10 mg L−1, which were used for analytical purposes. The commercial formulations VITAVAX® 20/20 FS (CBX), APRON® 350 ES (MET-M), CELEST® 2,5 FS (FLD), SYSTIVA® 33.3 FS (FLX), TECTO® 50 SC (TBZ) and DACONIL® 500 SC (CHT) were used in the degradation study.

Table 2.1 The physicochemical properties (water solubility, vapor pressure, Log octanol-water partition coefficient (Log Pow)) and environmental fate parameters (Degradation Time 50% (DT50), freundlich adsorption coefficient normalized for organic carbon content (Kfoc)) of the pesticides used in the current study (adapted by the ("PPDB - Pesticide Properties Database. Accessed on 2021-11-15").

Pesticide	Chemical structure	Water Solubility (mg L^{-1})	Vapor Pressure (mPa)	Log Pow	DT50 (days)	Kfoc (mL g^{-1})
Carboxin		134	0.02	2.3	0.07‑1.68	123‑213
Metalaxyl-M		26000	3.3	1.71	1.38‑73.1	20‑284
Fluxapyroxad		3.44	2.70E-06	3.13	53‑424	320‑1101
Thiabendazole		30	5.30E-04	2.39	558-1000[a]	580‑3921

Chlorothalonil		0.81	7.60E-02	2.94	0.44-31.6	330-7000
Fludioxonil		1.8	3.90E-04	4.12	119-365	7500-210000

[a]Data from field studies

2.2 SUBSTRATES PREPARATION

The soil used in the current study was collected from the top 20 cm of a field site of the Hellenic Agricultural Organization-DEMETER in Larisa, Greece (39°63'27''N, 22°36'74''E). The soil was sieved (2mm mesh) to homogenize and stored at 4 °C until used. Wheat straw was acquired from a local farm in Larissa, Greece and SMS was obtained from a Pleurotus ostreatus edible mushroom production unit (Mpoulogeorgos, Trikala, Greece). Straw and SMS were chopped to small pieces (ca. 1-2 cm) and they were then mixed with soil at volumetric ratios of 25% straw, 50% SMS and 25% soil. Upon its preparation, the biomixture was left to mature at room temperature for a month. During this time, it was regularly mixed and hydrated. The physicochemical properties of the individual components and the final biomixture were determined as described in Karas et al., (2015) (**Table 2.2**).

Table 2.2 The physicochemical properties of the individual components and the biobed packing material used in the current study

Materials	pH	Organic Carbon (%)	Total N (%)	C/N
Soil	7.56	1.05	0.13	8.1
Straw	7.15	79.2	0.80	99
Spent Mushroom Substrate	6.83	71.0	1.20	59.2
Biobed packing material	7.10	29.3	0.30	97.7

2.3 PESTICIDES DEGRADATION IN BIOMIXTURE AND SOIL

2.3.1 EXPERIMENTAL SETUP

A bulk load (6.6 kg) of biomixture and soil were prepared and separated into 11 samples (600 g). The first five samples from each matrix (soil and biomixture) were individually treated with aqueous solutions of CBX, MET-M, FLX, TBZ, and CHT aiming to final concentrations of 52, 28, 34, 20 and 50 mg of the active substance kg^{-1} of solid matrix (dry weight) respectively. The next three samples from each matrix were treated with pesticides mixtures, in accordance with their industrial use: (a) CBX + MET-M (cotton seed-coating) (b) CBX+MET-M+FLX+FLD (cotton and wheat seed-coating) and (c) TBZ+CHT+FLD (bulb-dipping). The nominal concentrations of CBX, MET-M, FLX, TBZ and CHT in the matrices treated with pesticide mixtures were as given above (individually applied in the studied matrices), while for FLD the nominal concentrations of 10 and 20 mg kg^{-1} were used representing its concentration in effluents obtained from seed-coating and bulb-dipping activities,

respectively. The three final samples from each matrix were treated with aqueous solutions of FLD aiming to concentrations of 10, 20 and 150 mg FLD kg^{-1} matrix d.w. reflecting a direct disposal scenario of FLD-containing wastewaters produced by seed-coating, bulb-dipping, and fruit-packaging activities, respectively.

The nominal concentration levels used in the degradation study were calculated based on a realistic loading scenario of a 30 m^3 biobed, which receives in total 2, 3 and 10 m^3 of wastewaters from seed-coating, bulb-dipping and fruit-packing activities respectively. Regarding seed-coating the recommended dose rates of FLD, MET-M, FLX and CBX, are 200, 40, 50 and 150 mL of the formulation (see above) in 500 mL water per 100 kg of seeds, respectively. Based on previous measurements of our laboratory in wastewaters produced by a local seed-coating industry, approximately 1% of the applied pesticides ends up in the wastewaters. For the bulb dipping activities the recommended concentration levels of the studied pesticides in the treatment solutions were 275 mg L^{-1} for CHT, 110 mg L^{-1} for TBZ and 90 mg L^{-1} for FLD. Finally, the recommended concentration level of FLD in the treatment solution used in fruit packing plants is 3000 mg L^{-1} of which ca. 10% ends up in the wastewater (in accordance with our laboratory measurements in relevant wastewaters) considering loss of the fungicide due to solution recirculation and retention on the surface of the treated fruits.

Upon pesticides application the treated matrices were mixed by hand to homogenize and the moisture content was adjusted to 40% of the water holding capacity. All treated samples were then further separated into 27 sub-samples (20 g each placed in aerated plastic bags) which were incubated in the dark at 25 °C for 100 days. The moisture content of the treated matrices was maintained by regular additions of deionised water. Immediately after pesticide application and at fixed intervals thereafter, triplicate sub-samples from each treatment were removed and stored at −20 °C until analyzed for pesticide residues.

2.3.2 PESTICIDES DEGRADATION KINETICS

The four kinetic models proposed by the FOCUS workgroup on pesticide degradation kinetics (FOCUS, 2006) were used for calculating degradation kinetics. The single first order (SFO) kinetic model and the biphasic models hockey-stick (HS), first order multi-compartment (FOMC) and double first order in parallel (DFOP) model were used. Details on the mathematical formulas used by each model are given in **Supplementary Table 2.S1**. The χ^2 test as well as visual inspection and the distribution of the residuals were used as criteria to assess the agreement between calculated and observed data for a given fit. In all cases, the kinetic model selected to describe the degradation data was the one with the lower χ^2 value and the best fitted residuals to the calculated curve. The analysis was carried out in the R (R Core Team, 2018) Studio version 3.4.4, utilizing the package mkin (Ranke, 2019) version 0.9.47.1.

2.4 PESTICIDES ADSORPTION IN BIOMIXTURE AND SOIL

2.4.1 EXPERIMENTAL SETUP

The adsorption of the studied pesticides in biomixture and soil was determined with the standard batch equilibrium method according to the OECD guideline 106 (OECD, 2000). Soil and biomixture were air-dried and stored at room temperature until used. Stock solutions of each pesticide (10000 μg mL^{-1}) in acetone or methanol (only TBZ) were prepared using analytical standards. Preliminary kinetic studies at a single concentration level (6 mg L^{-1} for CBX, MET-M, FLX and TBZ; 0.6 and 0.4 mg L^{-1} for FLD and CHT, respectively) were employed to determine the most appropriate substrate:solution ratios and equilibration times for each pesticide (results given in **Supplementary Table 2.S2**. For the determination of the adsorption parameters appropriate amounts of the pesticide stock solutions were dissolved in 0.01M $CaCl_2$ for the preparation of a series of solutions with pesticide concentrations of 2, 4, 6, 8, and 10 mg L^{-1}. Sole exceptions were CHT and FLD for which the concentration levels ranged from 0.1 to 0.8 and 0.2 to 1.0 mg L^{-1}, respectively, to account for their low water solubility. In all cases the amount of organic solvent in the solution phase never exceeded 0.1%. For each pesticide 15 samples of soil or biomixture (1-2 g d.w.) were placed in glass flasks and mixed in triplicates with appropriate volumes of the different pesticide solutions to achieve the selected solid:solution ratios (listed in **Supplementary Table 2.S2**). Blank samples containing only pesticide solution and no soil or biomixture were also included to assess pesticides' stability and the absence of potential pesticide adsorption on the glass surfaces. All samples were shaken in an orbital shaker (200 rpm) at room temperature until equilibrium was reached (8-12 h, see **Supplementary Table 2.S2**), centrifuged at 7500 rpm for 5 min and the supernatant collected was used for the determination of pesticide levels.

2.4.2 ADSORPTION DATA ANALYSIS

The linear form of the Freundlich equation (**Eqn 1**) was used to describe the adsorption of pesticides on soil and biomixture:

$$log^{ads}K_s\,(eq) = log^{ads}K_F + 1/n * log^{ads}C_{aq} \qquad \textbf{(Eqn 1)}$$

where $^{ads}C_s$ (eq) is the amount of the test substance absorbed (μg g^{-1}) in equilibrium,

$^{ads}C_{aq}$ (eq) is the adsorbate equilibrium concentration (μg g^{-1}),

$^{ads}K_F$ is the Freundlich adsorption coefficient and

1/n is the freundlich equation exponent (OECD, 2000; Tran et al., 2017)

2.5 PESTICIDE RESIDUE ANALYSIS

2.5.1 PESTICIDES EXTRACTION FROM AQUEOUS SAMPLES

A liquid-liquid extraction method was used for the extraction of pesticides from the aqueous samples obtained from the adsorption study. For CBX, MET-M, and FLD extraction, 2 mL of the aqueous samples were mixed with 4 mL of methanol, while for TBZ 2 mL of the aqueous samples was mixed with 6 mL of methanol. FLX and CHT were extracted as described above

with the only exception that 4 mL of acetonitrile instead of methanol were used. The mixtures obtained were shaken vigorously for 30 s and the extract was passed through a 0.45 μm syringe filter (PTFE Syringe Filter). The filtrate was collected and stored at 20 °C until analyzed.

2.5.2 PESTICIDES EXTRACTION FROM SOIL AND BIOMIXTURE

CBX, FLD, and MET-M were extracted from 5 g of biomixture and soil with 20 mL of methanol. The mixture was agitated for 1 h in an orbital shaker at 300 rpm and then centrifuged at 7500 rpm for 5 min. The supernatant was collected and stored at −20 °C. For the extraction of FLX and CHT the same procedure was followed with the sole difference that acetonitrile instead of methanol was used. TBZ extraction was performed as described by Karas et al. (2015). In all cases, the final extracts were passed through a syringe filter 0.45 μm (PTFE Syringe Filter) and analyzed by HPLC.

2.5.3 VALIDATION OF THE PESTICIDES EXTRACTION METHODS

Analyses of 0.01M $CaCl_2$ samples fortified with each of the studied pesticides at three concentration levels (10, 1 and 0.1 mg L^{-1}) assessed the efficiency of the extraction method used. In accordance, analyses of soil and biomixture samples fortified at three concentration levels (10, 1 and 0.1 mg kg^{-1}) evaluated the efficiency of the respective extraction methods. Triplicates for each compound and concentration level were extracted as described above and the pesticide levels were determined by HPLC. The mean percentage recoveries for CBX, MET-M, FLX, FLD and CHT in soil samples were 91.7%, 91.4%, 105.2%, 110.0% and 89.7%, respectively (CV≤12.4%), while the corresponding values in biomixture samples were 89.2%, 87.5%, 95.7%, 81.9% and 101.3% (CV≤14.1%) respectively. Similarly, the mean percentage recoveries for CBX, MET-M, FLX, FLD, and CHT in the aqueous samples were 80%, 92.1%, 105.0%, 98.2% and 89.3% respectively (CV≤18.1%).

2.5.4 HPLC ANALYSIS

All extracts were analyzed in a Shimatzu HPLC-DAD system equipped with a Grace Smart RP C18 (150mm×4.6 mm). CBX and FLD were detected at 207 nm using a mobile phase of methanol/water with different strengths (60/40 and 70/30 v/v respectively) and retention times of 5.6 and 5.8 min respectively. MET-M was detected at 202 nm using a mobile phase of 65/35 methanol/water solution (by volume) with a retention time of 5.5 min. FLX and CHT were detected at 230 nm using a mobile phase of 70/30 acetonitrile/water (plus 0.1% phosphoric acid for CHT) and showed retention times of 3.8 and 3.5 min respectively. TBZ was detected at 254 nm using a mobile phase of 39/60.5/0.5 acetonitrile/water/25% NH3 solution (by volume) and had a retention time of 3.6 min. In all cases a flow rate of 1 mL min^{-1} was used, except for CHT for which the flow rate was adjusted to 1.4 mL min^{-1}.

2.6 STATISTICAL ANALYSIS

Significant differences (level of significance 5%) in pesticides degradation rates (kdeg and k1) in biomixture vs soil were determined with student's t-test. The confidence intervals of the degradation rates obtained by fitting the kinetic models to the degradation data were converted to standard deviations using the **Eqn 2**:

$$SD = \sqrt{N} \times (Upper\ limit - Lower\ limit)/3.92 \quad \textbf{(Eqn 2)}$$

where N=sample size and 3.92 is the standard error for a 95% confidence interval. Correlations between adsorption (kf) and degradation parameters (DT50) and between adsorption (kf) and pesticide properties (water solubility, Log Pow) were determined by Pearson's and Spearman's correlation tests using the R Studio version 3.5.0 (R Core Team, 2018).

3 RESULTS

3.1 PESTICIDE DEGRADATION

3.1.1 DEGRADATION OF PESTICIDES USED IN SEED-COATING ACTIVITIES

The degradation patterns of the different pesticides used in seed-coating activities are presented in **Figure 2.1**. The SFO model in most cases provided adequate fit to the degradation data with the exceptions of MET-M and CBX, where the HS model provided a better fit to their degradation pattern (**Table 2.3**). CBX was the least persistent compound with DT_{50} values ranging from 2.7 days in biomixture, to 12.6 days in soil. MET-M (DT_{50} = 31.3 – 74.8 days) and FLD (42.4 – 152.2 (extrapolated) days) showed moderate persistence. FLX was the most persistent compound with extrapolated DT_{50} values ranging from 142.9 days in biomixture to 784 days in soil.

Pesticides degradation was faster in the biomixture compared to soil. This was clear for CBX and FLD at both individual ($p < 0.05$) and combined application schemes ($p < 0.05$ for CBX when co-applied with MET-M). For example, FLD showed DT_{50} values of 42.4–52.9 days in the biomixture and 92.9–152.2 days in soil (Table 3). MET-M showed significantly higher DT_{50} ($p < 0.05$) in soil compared to the biomixture only when co-applied with the other seed-coating pesticides. This is nicely illustrated for MET-M with DT_{50} values of 35.3 and 47.5 days in the biomixture when applied with the other seed-coating pesticides (quadruple mixture) or just with CBX (double mixture) respectively, compared to its corresponding soil DT_{50} values of 74.8 and 57.7 days (**Table 2.3**).

In most cases, the application of seed-coating pesticides in double or quadruple mixtures resulted in higher DT_{50} values. In particular FLX ($p < 0.001$) and FLD ($p < 0.05$), showed significantly higher DT_{50} values in soil treated with the pesticides mixtures (DT_{50} > 365 and 54.9 – 152.2 days) compared to their individual applications (DT50 = 142.9 – 150.4 and 42.4 – 92.9 days). An exception to this was CBX whose degradation was either not significantly affected (biomixture, p=0.521) or significantly accelerated (soil, $p < 0.05$) when applied in quadruple pesticide mixtures (DT50 3.0 and 8.2 days, respectively) (**Table 2.3**).

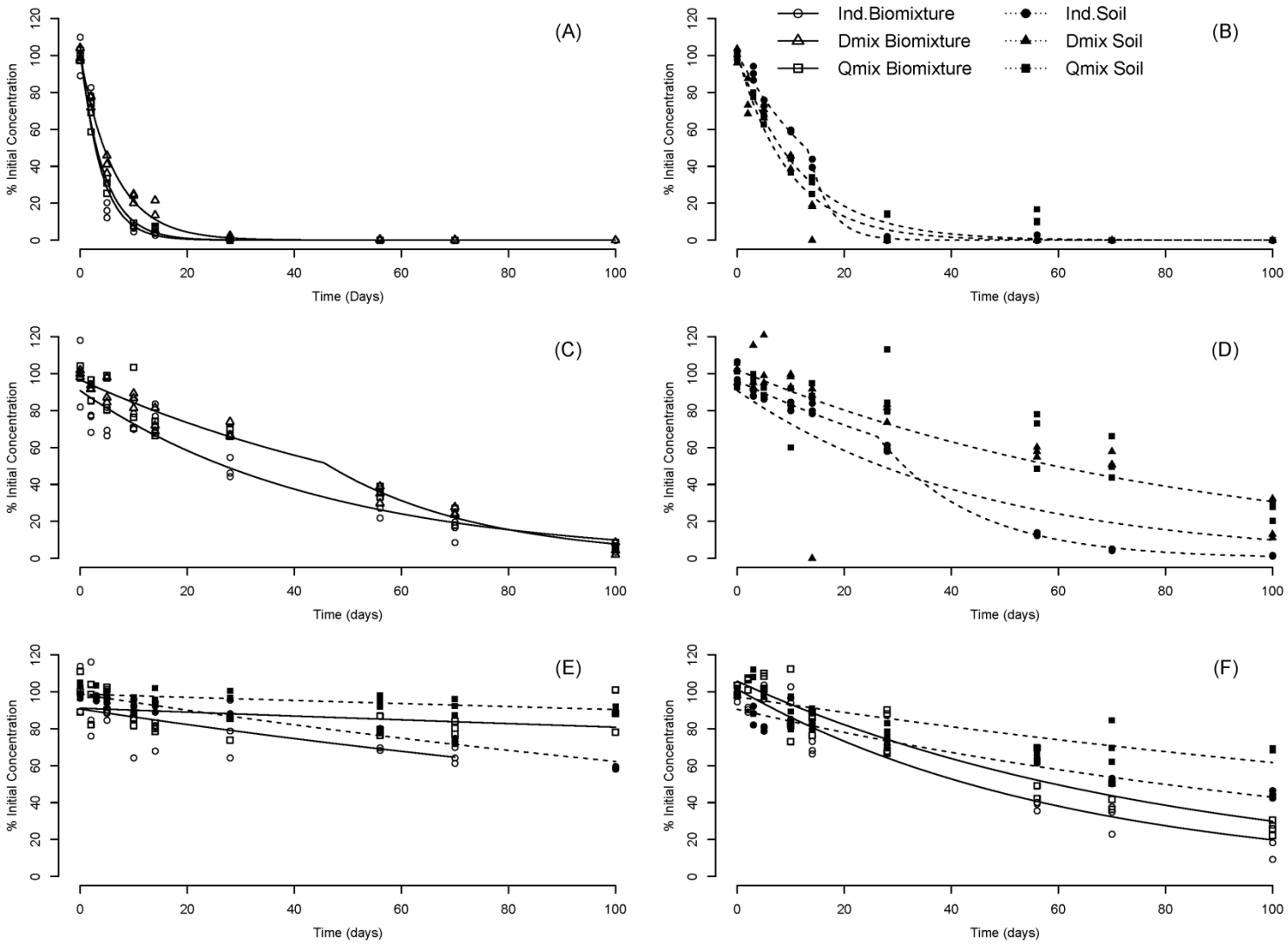


Figure 2.1 The degradation of carboxin in biomixture (A) and soil (B), metalaxyl-M in biomixture (C) and soil (D), fluxapyroxad (E) and fludioxonil (F), all used in seed-coating activities. Degradation of pesticides in biomixture (BMX) is designated with open symbols and solid lines and in soil with closed symbols and dashed lines. Pesticides where applied individually (Ind) (○,●) or co-applied in double (Dmix, carboxin + metalaxyl-M, △, ▲) or quadruple mixtures (Qmix, carboxin + metalaxyl-M + fluxapyroxad + fludioxonil, □, ■) Symbols represent data and lines the theoretical degradation kinetics.

3.1.2 DEGRADATION OF PESTICIDES ISED IN BULB-DIPPING ACTIVITIES

The degradation patterns of the different pesticides used in bulb-dipping activities are presented in **Figure 2.2**. The SFO provided the best fit to the degradation data of FLD (**Table 2.3**). The degradation of CHT was best described by the SFO (individual application) or the HS model (combined application scheme). Regarding TBZ, its degradation in the biomixture and soil was best described by the SFO and the HS model respectively. CHT was the least persistent compound both in biomixture (DT_{50} = 2.3 – 2.6 days) and soil (DT_{50} = 11.8 – 16.7 days). TBZ showed intermediate persistence (DT_{50} = 43.3 – 199.3 days), and FLD, this time applied at a nominal dose of 20 mg kg^{-1}, was the most persistent pesticide of the bulb-disinfestation industry (DT_{50} = 85.7 – 254.7 days) (**Table 2.3**).

All pesticides used in bulb-dipping activities showed shorter DT_{50} values in biomixture compared to soil ($p < 0.05$) regardless of the application scheme employed (individual or mixtures). For example the DT_{50} values of CHT in the biomixture, for individual and mixed application schemes, were 2.6 and 2.3 days respectively, compared to their corresponding DT_{50} values of 16.7 and 11.8 days ($p < 0.01$) in soil. Co-application of TBZ and FLD with CHT increased the persistence of the first two compounds in both tested matrices. Hence the DT_{50} of TBZ increased significantly from 45.2 and 43.3 days, when applied individually in the biomixture ($p < 0.05$) and in the soil ($p < 0.01$) respectively, to 119 and 199.3 days when applied as a mixture with the other two fungicides. On the contrary the persistence of CHT was not significantly affected ($p > 0.05$) by its co-application with TBZ and FLD (**Table 2.3**).

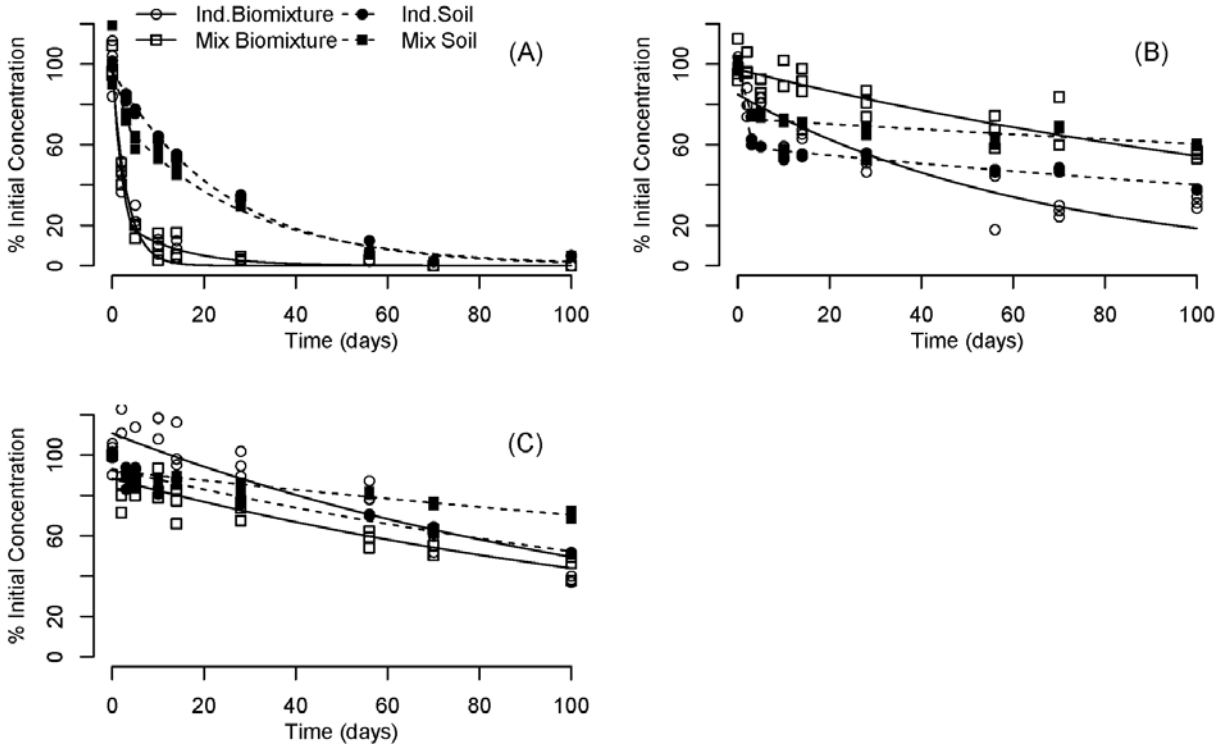


Figure 2.2 The degradation of chlorothalonil (A), thiabendazole (B) and fludioxonil (C) used in bulb-dipping activities in biomixture (BMX) (open symbols, solid line) and soil (S) (closed symbols, dashed line) when applied individually (Ind) (○,●) or in mixture (Mix) (chlorothalonil +thiabendazole + fludioxonil, □, ■). Symbols represent data and lines the theoretical degradation kinetics.

3.1.3 THE DEGRADATION OF FLD IN SOIL AND BIOMIXTURE

The degradation of FLD when applied at 150 mg kg^{-1}, simulating the disposal of effluents from the fruit-packaging industry was best described by the SFO model (**Figure 2.3, Table 2.3**). At this high application rate FLD showed a significantly higher ($p < 0.05$) DT_{50} in the biomixture (107.6 d) compared to its individual application in the same material at lower rates (10 and 20 mg kg^{-1}) as seed-coating (42.4 days) or bulb-dipping fungicide (85.7 days). The same dose-dependent degradation pattern was also evident in soil with significantly lower DT_{50} values ($p < 0.01$) observed at the lowest dose rates (10 and 20 mg kg^{-1}). The DT_{50} of FLD was over-doubled in soil (276.2 days) compared to the biomixture (107.6 days).

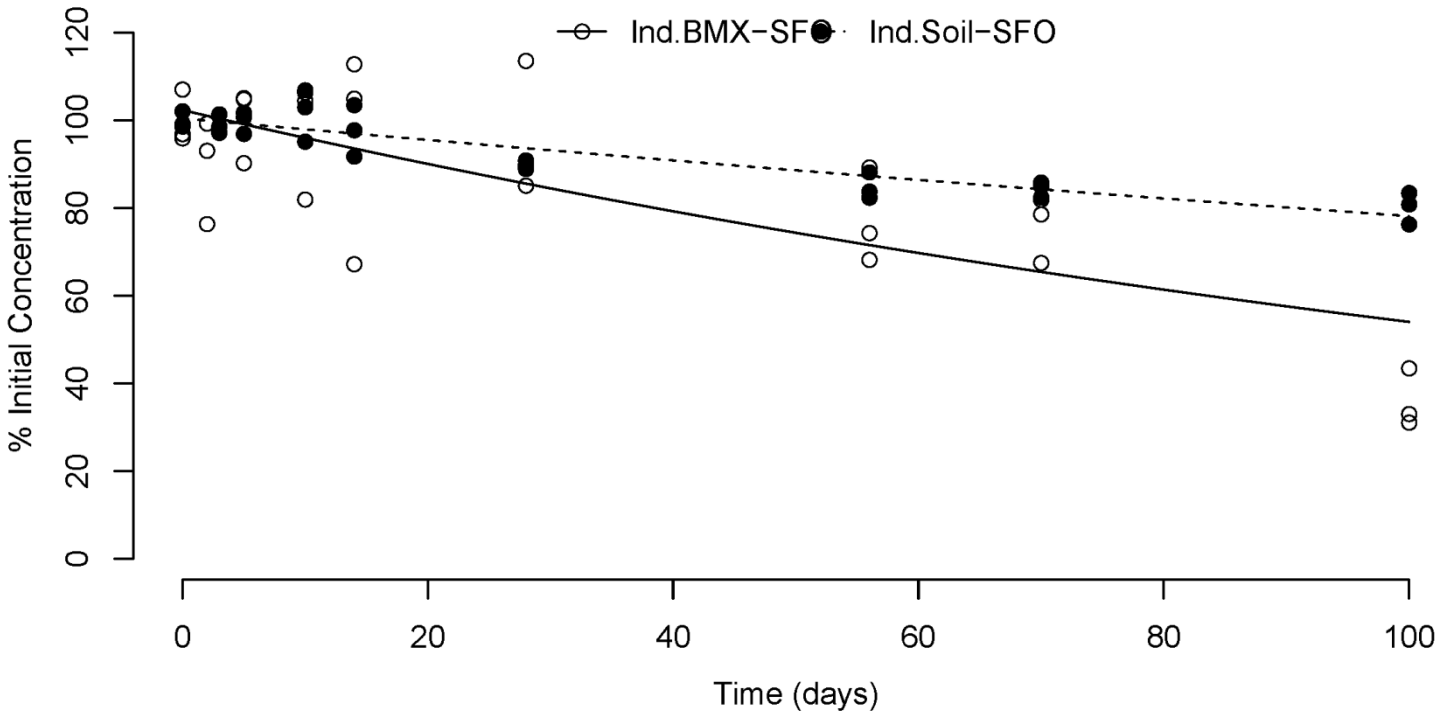


Figure 2.3 The degradation of fludioxonil when applied at 150 mg kg^{-1} in biomixture (○) and soil (●).

Table 2.3 The degradation kinetic parameters (DT50, kdeg or k1/k2 and χ2) of carboxin (CBX), metalaxyl-M (MET-M), fluxapyroxad (FLX), chlorothalonil (CHT), thiabendazole (TBZ) and fludioxonil (FLD) in biomixture and soil, applied singly (Individual) or in mixtures (Double or Quadruple) as calculated by fitting the most appropriate kinetic model. The type of agro-industrial activities associated with each pesticide treatment is also provided.

Activities	Application	Biomixture						Soil					
		Model	χ^2 (%)	K_{deg} (d^{-1})	k_1 (d^{-1})	k_2 (d^{-1})	DT_{50} (days)	Model	χ^2 (%)	K_{deg} (d^{-1})	k_1 (d^{-1})	k_2 (d^{-1})	DT_{50} (days)
Seed Coating	CBX Individual	SFO	23.1	0.262	-	-	2.65	HS	5.12	-	0.054	0.2639	12.6
	CBX Double	SFO	5.85	0.156	-	-	4.44	SFO	13.7	0.104	-	-	6.67
	CBX Quadruple	SFO	5.67	0.229	-	-	3.03	SFO	8.16	0.085	-	-	8.18
	MET-M Individual	SFO	10.76	0.022	-	-	31.3	HS	3.16	-	0.014	0.056	31.9
	MET-M Double	HS	4.15	-	0.014	0.035	47.5	SFO	11.93	0.012	-	-	57.7
	MET-M Quadruple	SFO	6.85	0.02	-	-	35.3	SFO	8.78	0.009	-	-	74.8
	FLX Individual	SFO	5.05	0.005	-	-	142.9	SFO	1.71	0.001	-	-	150.4
	FLX Quadruple	SFO	6.06	0.001	-	-	576.5	SFO	1.08	0.0009	-	-	784.1
	FLD Individual	SFO	7.88	0.016	-	-	42.4	SFO	4.61	0.007	-	-	92.9
	FLD Quadruple	SFO	4.96	0.013	-	-	54.9	SFO	4.92	0.005	-	-	152.2
Bulb Dipping	CHT Individual	SFO	10.03	0.264	-	-	2.63	SFO	4.27	0.042	-	-	16.7
	CHT Mixed	HS	10.05	-	0.296	0.046	2.34	HS	5.48	-	0.103	0.034	11.8
	TBZ Individual	SFO	11.53	0.015	-	-	45.2	HS	2.35	-	0.16	0.003	43.3
	TBZ Mixed	SFO	3.37	0.006	-	-	119.0	HS	2.84	-	0.096	0.002	199.3
	FLD Individual	SFO	7.89	0.008	-	-	85.73	SFO	3.41	0.006	-	-	120
	FLD Mixed	SFO	5.45	0.007	-	-	98.37	SFO	2.86	0.003	-	-	254.7
Fruit Packaging	FLD Individual	SFO	8.34	0.006	-	-	107.6	SFO	1.80	0.003	-	-	276.2

3.2 PESTICIDES ADSORPTION

Pesticide adsorption in all cases was adequately described by the Freundlich equation (r^2 > 0.90) (**Figure 2.4**). MET-M was the least adsorbed compound with K_f values of 1.15 and 3.2 mL g^{-1} in soil and biomixture, respectively (**Table 2.4**). CBX and FLX showed intermediate adsorption affinity with K_f values of 12.4 and 7.5 mL g^{-1} in soil respectively, compared to 22.97 and 41.8 mL g^{-1} in biomixture (**Table 2.4**). CHT showed moderate to high adsorption affinity with K_f values of 14.76 and 94.9 mL g^{-1} in soil and biomixture, respectively. TBZ and FLD showed the strongest adsorption with K_f values of 26.6 and 31.2 mL g^{-1} in soil and 104.6 and 123.3 mL g^{-1} in the biomixture (**Table 2.4**). All pesticides showed multiple folds higher adsorption affinity in the biomixture (K_f = 3.23 – 123.3 mL g^{-1}) vs soil (K_f = 1.15 – 31.2 mL g^{-1}).

Correlation tests identified significant positive correlations between the $logP_{ow}$ of the studied pesticides and their K_f values in both biomixture (0.740, $p < 0.05$) and soil (0.780, $p < 0.05$), and a significant negative correlation between the water solubility of the studied compounds and their K_f values in biomixture (-0.886, $p < 0.01$) and soil (-0.771, $p < 0.05$).

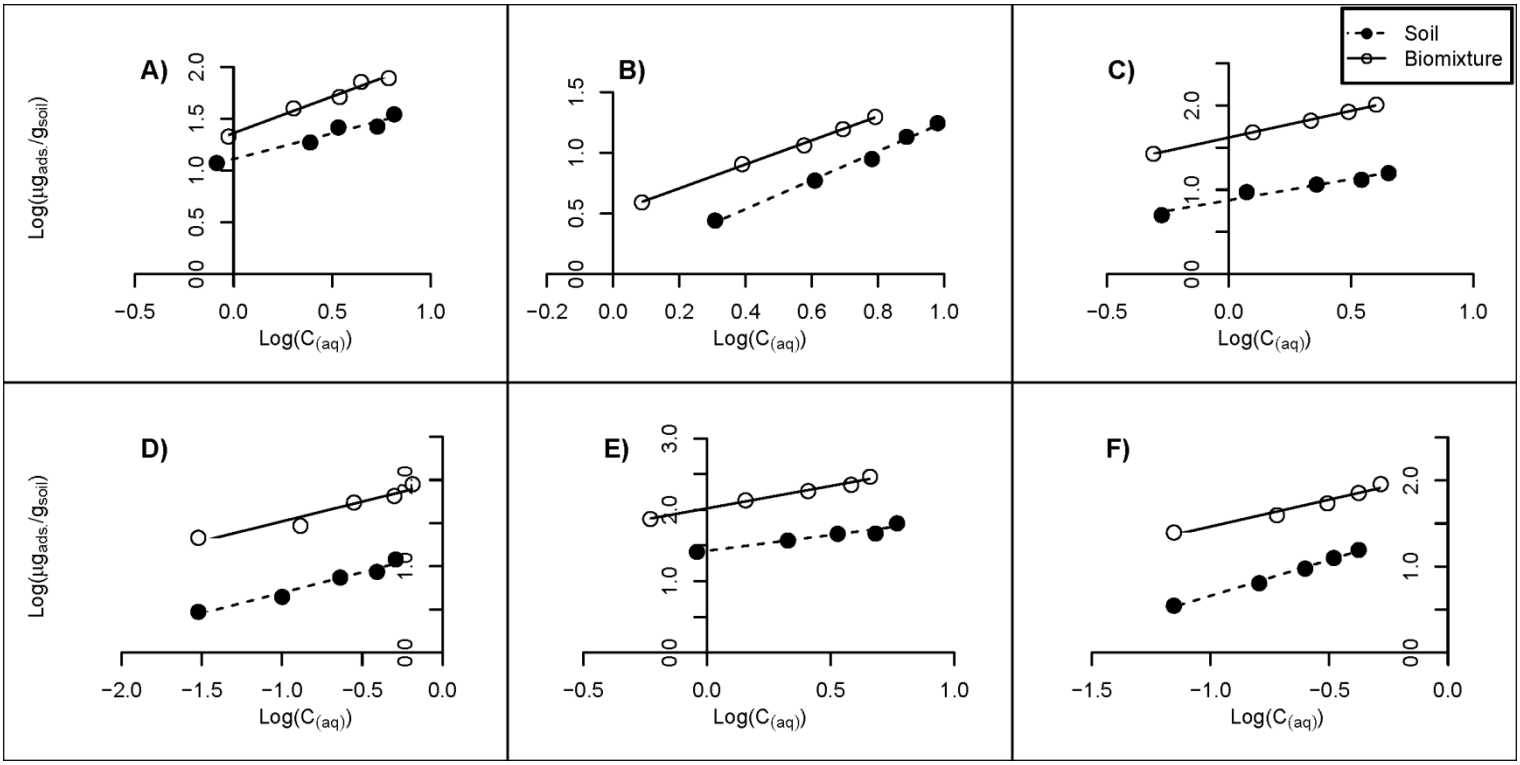


Figure 2.4 Adsorption isotherms of carboxin (A), metalaxyl-M (B), fluxapyroxad (C), chlorothalonil (D), thiabendazole (E) and fludioxonil (F) in soil (●) and biomixture (○). Each point is the mean of three replicates ± the standard deviation. Symbols represent data and lines the theoretical degradation kinetics.

Table 2.4 The adsorption parameters (K_f, n) calculated by using the Freundlich equation to describe the adsorption of carboxin (CBX), metalaxyl-M (MET-M), fluxapyroxad (FLX), chlorothalonil (CHT), thiabendazole (TBZ) and fludioxonil (FLD) in biomixture and soil.

Pesticides	Biomixture				Soil			
	r^2	K_F (mL g^{-1})	K_{Foc} (mL g^{-1})	n	r^2	K_F (mL g^{-1})	K_{Foc} (mL g^{-1})	n
CBX	0.982	22.97	78.4	1.416	0.961	12.9	1183.34	2.011
MET-M	0.998	3.23	11.03	1.595	0.993	1.15	109.30	2.260
FLX	0.999	41.76	142.5	1.582	0.960	7.53	716.76	1.994
CHT	0.933	94.94	324	1.595	0.970	14.76	1406.00	1.199
TBZ	0.987	104.60	357.1	1.011	0.935	26.60	2532.86	0.839
FLD	0.969	123.30	420.7	2.183	0.995	31.20	2972.00	2.100

4 DISCUSSION

4.1 PESTICIDE DEGRADATION IN BIOMIXTURE AND SOIL

The lack of established, efficient, and economically viable methods for the treatment of pesticide-contaminated agro-industrial wastewaters renders the development of systems for their detoxification a necessity. Bio-based treatment systems like biobeds appear as a possible and sustainable solution. In this context we tested the degradation and adsorption of pesticides contained in such agro-industrial effluents in a biomixture, known to be effective in the degradation of pesticides used in fruit-packaging industry (Karas et al., 2016), and comparatively in soil, a matrix considered as the natural buffer for pesticides released in the environment.

The four fungicides used in seed-coating activities showed the same order of persistence in biomixture and soil. CBX was the least persistent, followed by MET-M, while FLD and FLX were the most persistent compounds. Our findings are in agreement with previous degradation studies in soil, while literature data for their degradation in biomixtures are available only for MET-M and FLD. Regarding CBX, previous studies reported a rapid degradation in soil within 10 – 14 days, although, DT_{50} values were not calculated (Balasubramanya and Patil, 1980; Chin et al., 1970), while recent regulatory studies reported soil DT_{50} at 20 °C of 0.6 – 1.68 days (EFSA, 2010). Regarding MET-M, Karanasios et al., (2010b) tested its degradation in a biomixture composed of soil, straw and SMS from *Agaricus bisporus* when applied in a mixture with 7 other pesticides and reported a DT_{50} of 34.7 days, which is similar to the DT_{50} (35.4 days) observed in the current study when MET-M was applied in quadruple mixture (**Table 2.3**). The degradation of FLX in biomixtures has not been studied before. However, previous degradation studies of FLX in soil are in line with our findings with a DT_{50} = 157.6 days (Li et al., 2015). Similarly, Wu et al., (2015) studied the soil degradation of FLX at different application rates (0.75 and 7.5 mg kg-) and reported DT_{50} values of 158 and 385 days, respectively.

Similarly to the seed-coating pesticides, the fungicides used in bulb-dipping activities showed the same order of persistence in both studied matrices. CHT was the least persistent, while TBZ and FLD showed moderate to high persistence. Concerning CHT the

DT_{50} values observed are within the range reported in the literature. For example, DT_{50} values ranged from<1 day to 13.1 days in soil (Regitano et al., 2001; Souza et al., 2017) and from 2 to 12.2 days in biomixtures (Fogg et al., 2003a, 2003b) (Fogg et al., 2003a, 2003b). Similarly, the DT_{50} values of TBZ observed in our study (45.2 and 43.3 days in soil and biomixture, respectively) are in agreement with previous studies. In particular Karas et al., (2015) studied the degradation of TBZ in a biomixture, with the same composition as in our study, and in soil and reported DT_{50} values of 28.3 and 31.7 days respectively.

FLD was the sole pesticide that is used in all agro-industrial activities, although, at different application rates. In this frame we tested its degradation at various application rates which represent its use in seed coating, bulb-dipping and fruit-packaging activities, respectively. The degradation of FLD in both matrices followed a dose-dependent pattern with increasing DT_{50} values at increasing dose rates. A series of degradation studies by the same group reported DT_{50} values of 50 and 115.5 days for FLD in a biomixture treated at rates of 20 (Coppola et al., 2011) and 50 mg kg^{-1} (Marinozzi et al., 2013) respectively. Several previous studies in soil and biomixtures have reported similar dose-dependent degradation patterns for CHT, isoproturon, iprodione and terbuthylazine (Fogg et al., 2003b; Karanasios et al., 2012). This has been attributed to a potential inhibitory effect of pesticides (Vischetti et al., 2008) or their transformation products (Fogg et al., 2003b) to the microbial community resulting in reduced biodegradation rates.

A faster degradation of CBX, FLD, TBZ and CHT in biomixture compared to soil was evident when pesticides were applied individually. The superior degradation capacity of the biomixture over soil extended to all studied pesticides when the application of pesticide mixtures was considered. Our findings come to support several previous studies which have documented the higher degradation capacity of biomixtures over soil for a wide range of pesticides (Lescano et al., 2018; Karanasios et al., 2010a, 2010b; Fogg et al., 2003a, 2003b) (Fogg et al., 2003a, 2003b; Karanasios et al., 2010a, 2010b; Lescano et al., 2018).

Another interesting aspect was the degradation behavior of the different pesticides when applied individually or in mixtures, the latter representing a scenario more relevant to the practices followed by the different agro-industries. In line with previous studies, the application of pesticides in mixtures delayed the degradation of the individual compounds in most cases (Fogg et al., 2004, 2003a). Exceptions to this were CBX and CHT which showed similar degradation rates when applied individually or in mixtures with the other pesticides. The decrease in the degradation of TBZ and FLD when co-applied with CHT, but not of CHT itself when co-applied with these fungicides, suggest an inhibitory effect of the latter to the degradation of TBZ and FLD. Previous studies have reported an inhibitory effect of CHT to the degradation of other co-applied pesticides in biomixtures (Fogg et al., 2003b) and in soil (Singh et al., 2002; Chen et al., 2001). This has been attributed to the production of hydroxy-CHT, the main soil derivative of CHT, which is known to have adverse effects on soil microorganisms (Zhang et al., 2016; Wu et al., 2014). Another deviation from the general trend was the degradation of MET-M which was hindered by the co-presence of the other pesticides used in seed-coating activities (ie. CBX, FXD, FLX) only in soil and not in the biomixture. In line with this Fogg et al., (2003b) observed that the inhibitory effect of CHT to isoproturon degradation was evident only in soil but it was buffered in the biomixture.

4.2 PESTICIDE ASDORPTION IN BIOMIXTURE AND SOIL

The adsorption of the pesticides contained in effluents from different agro-food industries in a biomixture and in soil was also determined. In line with several previous studies, all tested pesticides, regardless of their physicochemical properties, showed weaker adsorption affinity in soil compared to the biomixture (Karanasios et al., 2010a, 2010b; Kravvariti et al., 2010; Henriksen et al., 2003). The higher adsorption capacity of the biomixture compared to soil has been attributed to its higher organic carbon content which provides more adsorption sites for the non-polar pesticides (De Wilde et al., 2009). Soil organic matter constitutes the main adsorption surface for non-polar pesticides, although polar pesticides also interact with the soil organic matter surfaces to a lower extent (Wauchope et al., 2002).

With the exception of MET-M and TBZ no data are available regarding the adsorption of the studied pesticides on biomixtures. In line with previous benchmarking research (Wauchope et al., 2002, Weber et al., 2004), the adsorption affinity in both studied matrices increased with the lipophilicity of the studied compounds as it was illustrated by the significant positive correlation between K_f and Log P_{ow} and conversely the negative correlation betwenn K_f with water solubility. METM, which was the most hydrophilic compound (**Table 2.1**), showed the lowest adsorption affinity with its K_f values for the biomixture being at the lower part of the range (4.8–16 mL g^{-1}) reported previously in other biomixture (Karanasios et al., 2010a, 2010b). CBX, which was the next more hydrophilic compound, was moderately to weakly adsorbed with its K_f values being higher than soil K_f values (1.61–2.71 mL g^{-1}) reported previously (EFSA, 2010). FLX showed moderate adsorption with its soil K_f values being within the range reported in regulatory studies performed in a range of soils (2.5–15.2 mL g^{-1}) (EFSA, 2012), and in line with previous studies, which reported organic carbon-depended adsorption of FLX (Gulkowska et al., 2016). CHT showed high adsorption affinity in both tested matrices with soil K_f values, which were at the lower part of the range reported in other studies (17.7–1357 mL g^{-1}) (Piwowarczyk and Holden, 2012; Patakioutas and Albanis, 2002). TBZ and FLD were two most strongly adsorbed compounds. Previously Karas et al. (2015) verified the stronger adsorption of TBZ in biomixtures over soil and reported Kf values which were in agreement with the current study. Regarding FLD, its soil K_f values were at the lower part of the range reported in the literature (62 - 213 mL g^{-1}).

Pearson's and Spearman's correlation tests between adsorption (K_f) and DT_{50} values for the different pesticides provided correlation coefficient of 0.063 (p=0.906) and 0.143 (p=0.803), respectively, suggesting no significant interplay between adsorption and degradation. Previous studies have also reported limited interactions between adsorption and degradation of pesticides in soil (Beulke et al., 2005) and biomixtures (Karanasios et al., 2010b) and suggested that the influence of adsorption on pesticides degradation largely depends on the physicochemical properties of the pesticide and the processes dominating the degradation of the pesticide in the environment (Kravvariti et al., 2010; Kah et al., 2007).

5 CONCLUSIONS

In the present study, we investigated the degradation and adsorption potential of a biomixture against pesticides which are used in various agro-industries and hence they have high potential of being present in the respective agro-industrial effluents. All pesticides exhibited higher adsorption affinity in the biomixture compared to soil. This did not negatively affect the degradation of pesticides which appear to be significantly faster, for most pesticides, in the biomixture compared to soil, regardless of the mode of application (individually or in mixtures). Co-application of pesticides in double, triple or quadruple mixtures, relevant to their practical use, resulted in a delay in the degradation of most pesticides. This was more prominent in soil compared to biomixture, while CHT had the most pronounced inhibitory effect on the degradation of other compounds (TBZ and FLD). Overall, our findings provide initial evidence for the potential of the tested biomixture to retain and degrade pesticides used in seed-coating, bulb-dipping and fruit-packaging activities. Follow up experiments at full-scale biobed systems packed with the studied biomixture are expected to unravel the full potential of these systems to depurate pesticide-contaminated effluents produced by those agro-food industries.

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Chapter 3

Using biobeds for the treatment of fungicide-contaminated effluents from various agro-food processing industries: microbiome responses and mobile genetic element dynamics

The work presented in Chapter 3 is included in the scientific paper:

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1 INTRODUCTION

Pesticides constitute major environmental pollutants, as their innate physicochemical characteristics and mode of application facilitate their dispersion, resulting in soil, surface water and groundwater pollution (Carvalho, 2017). The high polluting potential of pesticides was acknowledged early by the European Commission, as demonstrated by the inclusion of several pesticide active ingredients in the list of priority pollutants of water resources (2455/2001/EC).

Agro-food processing industries that use plant protection products (PPPs) constitute a serious point-source for the contamination of the natural water resources (Masiá et al., 2013; Ccanccapa et al., 2016; Bao et al., 2021). These include seed-producing industries (SPI), which treat seeds with systemic fungicides like carboxin (CBX), metalaxyl-M (MET-M) and fluxapyroxad (FLX) (Lamichhane et al., 2020; Ayesha et al., 2021), bulb handling industries (BHI) which immerse bulbs into dense solutions of fungicides like chlorothalonil (CHT), thiabendazole (TBZ) and fludioxonil (FLD) (Chastagner and DeBauw, 2011; Araújo et al., 2017; Bansal et al., 2018) and fruit-packaging industries (FPI) that make use of fungicides like imazalil (IMZ) and fludioxonil (FLD) for the control of fungal infections of fruits during storage (Cerioni et al., 2017; Matrose et al., 2021). Taking into consideration the environmental risk stemming from the improper management of the pesticide-contaminated effluents produced by these industries, the European Commission enforced the implementation of appropriate wastewater management practices (Regulation 2019/1021).

Different treatment processes have been tested to date for the depuration of these wastewaters with variable results. A few studies have tested the efficiency of abiotic transformation processes, such as advanced oxidation techniques like TiO2-based photocatalysis (Jiménez et al., 2013; Xing et al., 2014; Cruz et al., 2017; Sraw et al., 2018; Molla et al., 2020) and photo-Fenton processes (Gar Alalm et al., 2015; Santiago et al., 2018; Fakhri et al., 2020; García-Estrada et al., 2020) for the removal of pesticides from these agro-food effluents. Others have used combinations of abiotic and biological processes (Sánchez Peréz et al., 2014; Jiménez-Tototzintle et al., 2015; Lopez-Loveira et al., 2019; Bernardelli et al., 2021). Despite the promising results of some of these methods their full implementation has not been accomplished due to several reasons including (i) high costs of installation and operation, (ii) high chemical addition requirements, (iii) possible sludge formation and (iv) production of toxic pesticide transformation products which might require further treatment (Santiago et al., 2013; Sirés et al., 2014; Bisaria et al., 2021; Ganiyu et al., 2022). Moreover, with the exception of a few recent studies (e.g. Rezende et al., 2021), the vast majority of all these studies were performed with distilled water artificially contaminated with pesticides instead of real agroindustrial effluents. The organic matter and inorganic salts that are present in the industrial effluents act as “radical scavengers” reducing the depuration efficiency of systems based on advanced oxidation and photo-oxidation techniques (Brame et al., 2015; Bisaria et al., 2021).

Biological treatment could provide a solution to the depuration of pesticide - contaminated effluents (De Wilde et al., 2007). Previous studies have demonstrated the potential of biobeds to treat pesticide-contaminated effluents, produced (i) at agro-

industrial level in FPIs (Omirou et al., 2012; Karas et al., 2016b; Carniel et al., 2020; Dias et al., 2021) and agrochemical producing industries (Lescano et al., 2022) and (ii) at on-farm level by pesticide handling activities in vineyards (Romero et al., 2019) and olive orchards (Delgado-Moreno et al., 2017). The depuration efficiency of biobeds largely relies on their packing material, a biological active substrate that act as pesticide adsorbant and as a source of an active microbiota able to effectively degrade the pesticides contained in the effluents (Vandermaesen et al., 2016; Holmsgaard et al., 2017; Bergsveinson et al., 2018). Typically, the packing material consists of soil, lignocellulosic material e.g. straw, and a humified organic substrate like peat or compost. Spent mushroom substrate (SMS) has been studied lately for its effect in the environmental fate of pesticides and its use in biobeds (Herrero-Hernández et al., 2011; Rodríguez-Cruz et al., 2012; Álvarez-Martín et al., 2016; Marín-Benito et al., 2016; Alves et al., 2022). Karas et al. demonstrated the high depuration capacity of SMS-based biobed packing material so, in the present study, a biomixture composed of SMS, straw and soil was used (Karas et al., 2015).

The role and contribution of microbial communities colonizing biobed systems in the removal of pesticides has been monitored using various measurements like microbial biomass carbon (Vischetti et al., 2008; Marinozzi et al., 2013), microbial respiration (Karanasios et al., 2010b; Omirou et al., 2012; Marinozzi et al., 2013), total hydrolytic activity (Karanasios et al., 2010b; Romero et al., 2019), activity of manganese peroxidase (MnP) (Karanasios et al., 2010b; Karas et al., 2016a), laccase (Karanasios et al., 2010b; Karas et al., 2016a), lignin peroxidase (Karanasios et al., 2010b), dehydrogenase (Marín-Benito et al., 2012; Romero et al., 2019), β-glucosidase (Romero et al., 2019), acid phosphatase (Romero et al., 2019), urease (Romero et al., 2019), ortho-diphenol oxidase (Romero et al., 2019), and the abundance of phylogenetically distinct microbial taxa or genes involved in the degradation of aromatic compounds via qPCR (Karas et al., 2016b). However, it was only recently that the composition of the biobed microbiome was determined using amplicon sequencing approaches (Holmsgaard et al., 2017; Bergsveinson et al., 2018). Metagenomic and meta-transcriptomic analysis of biobed systems suggested an enrichment of biobeds in genes encoding for enzymes known to be involved in the degradation of pesticides like peroxidases, monooxygenases and hydroxylases (Russell et al., 2021). Similar previous studies also reported the enrichment of biobeds with genes involved in the degradation of substituted phenylurea herbicides like pdmA (Storck et al., 2020), libA and hylA (Horemans et al., 2016). Beyond the phylogenetic composition of the biobed microbiome, recent studies have showed that biobeds are evolutionary hotspots of novel pesticide transformation pathways (Storck et al., 2020) with mobile genetic elements (MGE), collectively called the mobilome, playing a significant role in this evolutionary process (Dealtry et al., 2014; Dunon et al., 2018).

The main aims of our study were (i) to evaluate the capacity of biobed systems to decontaminate effluents from various agro-food processing industries in a realistic biobed loading context, (ii) to investigate the effect of the continuous pesticide load on microbial succession in biobed systems and (iii) to explore the presence and dynamics of different parts of the mobilome during the operation of biobed systems. We hypothesized that the continuous discharge of pesticides will have a strong effect on microbial succession and will stimulate MGE abundance and dynamics in biobed systems. To explore the objectives and

hypotheses set out we employed an experiment on pilot biobed systems subjected to regular treatment with pesticide-contaminated effluents originated from fruit-packaging plants, seed-producing and bulb-handling industries. The efficiency of biobeds was monitored through regular analysis of pesticides in the collected effluents, while the processes involved in pesticide dissipation were determined through measurement of pesticide levels on the different biobed layers at the end of the study, enabling mass balance analysis. Microbial succession and the dynamics of MGE during biobed operation were monitored through amplicon sequencing and q-PCR respectively. The application of relevant statistical tools enabled the identification of microorganisms responsive to pesticide addition and provided hints about biobed operational conditions.

2 MATERIALS AND METHODS

2.1 CHEMICALS AND WASTEWATERS

Analytical standards of CBX (99.9% Pestanal®), MET-M (98.4% Pestanal®), TBZ (99% Pestanal ®), CHT (99.7% Pestanal), FLD (99.9% Pestanal®) and IMZ (99.3% Pestanal) were purchased from Sigma-Aldrich (Merck), while FLX (99.9% BAS 700 F) was provided by BASF Hellas. Fungicide stock solutions (1000 mg L^{-1}) in MeOH (CBX, MET-M, TBZ, FLD and IMZ) and acetonitrile (CHT and FLX) were prepared from the analytical standards and further used for analytical purposes. Commercial pesticide formulations of TBZ (TECTO® 50 SC), CHT (DACONIL® 500 SC), FLD (SCHOLAR® 230 SC) and IMZ (FUNGAZIL® 500 EC) were used for the preparation of aqueous pesticide solutions applied as wastewaters of BHI and FPI. The concentrations of TBZ, CHT and FLD in the BHI artificial wastewaters were 2.3, 8.1 and 3.2 mg L^{-1} respectively. Similarly, the concentrations of FLD and IMZ in the corresponding FPI wastewaters were 46 and 75 mg L^{-1} respectively. SPI effluents were kindly provided by the seed producing industry BIOS Agrosystems SA and they were diluted before application in the biobed systems to achieve pesticide concentrations of 5.14, 9.5 and 8.7 mg L^{-1} for CBX, MET-M and FLX respectively. These nominal concentrations of the fungicides in the artificial wastewaters and the diluted SPI wastewater, as applied in the biobed systems, were calculated to simulate a realistic loading scenario of a 30-m^3 biobed filled with the studied packing material having a bulk density of 0.6 t m^{-3}. The main physicochemical characteristics of the studied pesticides are given in **Tables 2.1 and 1.7**.

2.2 BIOBED PACKING MATERIAL PREPARATION

Soil, without any recent pesticide use, was collected from the top 20 cm of the field site of the Hellenic Agricultural Organization – DEMETER in Larisa, Greece (39°38’01.2”N 22°22’26.2”E), sieved (2 mm) to remove rock and plant material, and stored at room temperature until use. The soil was characterized as clay loam (37% sand, 31% clay and 32% slit). Wheat straw was acquired from a local farm in Larissa, chopped to small pieces (ca. 1–2 cm) with a blender homogenizer and stored at room temperature. Pleurotus ostreatus SMS was provided by the mushroom farm “Mpoulogeorgos” (Trikala, Greece) after two harvest cycles. SMS was chopped to small pieces (ca. 1–2 cm) and mixed with straw and soil at volumetric ratios of 50% SMS, 25% straw and 25% soil. Upon being thoroughly mixed, the biobed packing material was left to mature at room temperature for a month, during which

it was regularly hydrated and further mixed. Physicochemical properties of the individual components and the final biobed packing material are listed in **Table 2.2**. The pH of the packing material was stable throughout the experimental period at 7.59±0.25. The absence of residues of the studied pesticides in the starting material was verified by HPLC analysis as is described below.

2.3 EXPERIMENTAL SETUP OF COLUMN BIOBED SYSTEMS

The potential of biobeds to remove fungicides from effluents of three different agro-food processing industries was explored in column biobed systems. In particular, 12 PVC columns of 90 cm length and 12.5 cm internal diameter, were packed, from bottom to top, with a 7 cm layer of thoroughly washed gravel, 80 cm layer of packing material and a 3 cm layer washed gravel at the top to ensure uniform distribution of the wastewaters at the surface of the column and to avoid disturbance of the packing material surface. A metal sieve was placed at the bottom of each column to prevent passage of the packing material in the leachate. Plastic funnels and 5 L collection bottles were placed below the columns to collect the leachates. Sampling points of 2 cm diameter were opened along the column length at 20, 50, and 80 cm (top-down), to collect packing material for microbial community and MGE analysis during column operation. Sampling points were sealed with corks afterwards (**Figure 3.1**).

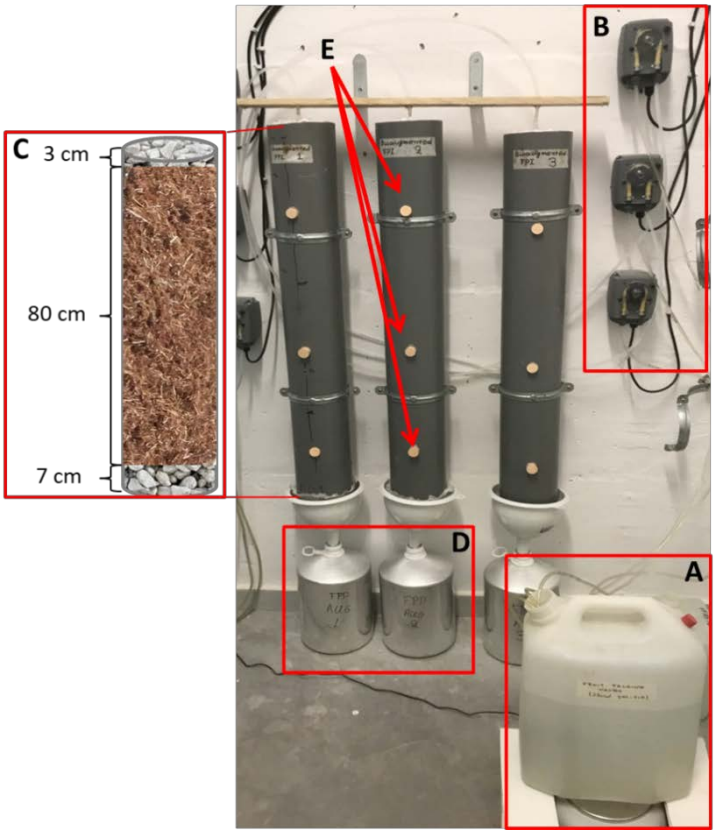


Figure 3.1 A photo and schematic representation of the setup of the column biobed used in our study. Wastewaters were stored in a plastic tank agitated continuously with a magnetic stirrer (A), the wastewater was transferred to the columns through peristaltic pumps (B). Each PCV column was packed (from top to bottom) with 3 cm of gravel, 80 cm of biobed packing material and 7 cm of gravel (C). The leachates from each biobed were collected in bottles placed underneath the column biobeds (D). Sampling points along the columns for the collection of packing material were sealed with corks (E).

Wastewaters from agro-industries were transferred into 15 L tanks and applied through peristaltic pumps at the top of each column for 5 min/6 times per day, to achieve simultaneous application of 500 mL day^{-1}. For each wastewater type we had devoted triplicate columns, while the final three columns received the same volume of pesticide-free water to serve as control for microbiome and MGE analysis. The tanks were refilled weekly with fresh wastewater and fungicide concentrations were monitored before and after filling to determine the true amount of fungicide applied in each column. The volume of leachate collected from each column was measured at 3-day intervals and a 200 mL sample was removed and stored at −20 °C for analysis of pesticide residues. At 21 and 60 days after the start of the wastewater application, samples of packing material from the inner part of the columns were collected with sterile tweezers from the designated sampling points along the column length and stored at −20 °C before analyzed.

The column operation was continued for a total of 103 days. At the end of the treatment period, the columns were left to drain for 6 days (until day 109). They were then dismantled, and the packing material was sectioned into 3 segments corresponding to the 0–20, 20–50, and 50–80 cm horizons from the top. Samples from each layer were stored at−20 °C for pesticide residue analysis and DNA extraction. The amounts of fungicides that was detected (i) in the leachates collected throughout the study (from day 4 to day 109), and (ii) in the packing material at the end of the study (day 109) were used for calculating the efficiency of biobeds and for mass balance analysis. The amount of pesticide dissipated (degraded and/or not being extractable with organic solvents) was determined by mass balance analysis after deducting from the total amount of pesticide applied in the column throughout the study (from day 0 to day 104), the total amount of pesticide found in the leachate (leached) throughout the study (from day 4 to day 109) and the total amount of pesticide recovered from the packing material at the end of the study (day 109) (retained).

2.4 FUNGICIDE RESIDUE ANALYSIS

2.4.1 FUNGICIDE EXTRACTION FROM AQUEOUS SOLUTIONS

A liquid-liquid extraction method was employed for the recovery of fungicide residues from the wastewater and leachate samples. CBX, MET-M and FLX, were extracted by mixing 2 mL of the aqueous samples with 2 mL of MeOH. TBZ, CHT and FLD were recovered using the same extraction method with the sole difference of adding H_3PO_4 2% v/v in the mixture. For the extraction of FLD and IMZ, 5 mL of aqueous sample were thoroughly mixed with 20 mL of chloroform ($CHCl_3$) in a separatory funnel for 20 min. The organic phase was collected and the aqueous phase was re-extracted with additional 20 mL $CHCl_3$. The organic phases from the two extraction steps were combined and evaporated to dryness in a rotary evaporator. The extract was resuspended in 2 mL MeOH, passed through a syringe filter (PTFE 0.45 μm, Whatman) and stored at−20 °C until analysis.

The methods used for the analysis of the pesticide residues in water samples were validated as follows. Leachates collected from columns receiving pesticide-free water were fortified at three concentration levels (2, 0.5 and 0.05 mg L^{-1}) with mixtures of the studied fungicides relevant to their industrial use, i.e. SPI mixture (CBX, MET-M and FLX), BHI mixture (TBZ, CHT and FLD) and FPI mixture (FLD, IMZ). The recoveries of all the studied

pesticides at the three concentration levels were between 90.1 and 112.2% and the precision of the extraction methods as determined by the relative standard deviation (RSD) was ≤20%. Specifically, mean percentage recoveries for CBX,MET-M, FLX, TBZ, CHT, IMZ and FLD when applied with the BHI and FPI mixture were 99.99 ± 5.59%, 95.32 ± 13.04%, 90.11 ± 10.82%, 112.18 ± 5.40%, 99.40 ± 11.38%, 102.62 ± 22.17%, 93.57 ± 11.12% and 105.82 ± 19.69% respectively and the corresponding RSDs were 5.59, 13.68, 12.01, 4.81, 11.45, 19.61, 11.88 and 18.41.

2.4.2 FUNGICIDE EXRACTION FROM BIOBED PACKING MATERIAL

Fungicide residues were extracted from the biobed packing material as described previously by Papazlatani et al. (2019). Briefly, CBX, MET-M and FLD were extracted from 5 g of packing material with 20 mL of MeOH and shaking in an orbital shaker at 200 rpm for 2 h for CBX and MET-M and 1 h for FLD. FLX and CHT were recovered by mixing 5 g of packing material with 20 mL of acetonitrile (ACN) and agitation for 1 h. In both protocols, the extract was subsequently centrifuged for 5 min at 8500 rpm and the clear supernatant was collected, filtered through a PTFE syringe filter (0.45 μm) and stored at −20 °C. TBZ and IMZ were extracted according to Karas et al. (2015).

2.4.3 HPLC ANALYSIS

Fungicide extracts were analyzed in a Shimadzu HPLC-DAD system equipped with an Athena C18 column (150 mm×4.6 mm) (ANPEL Laboratory Technologies) at a flow rate of 1 mL min^{-1}. Fungicides CBX, MET-M and FLX contained in the effluents from the SPI were detected in one run at 207, 202 and 230 nm respectively, using a mobile phase of MeOH/H_2O 55/ 45 v/v, with retention times of 5.5, 18.1 and 24.5 min respectively. Fungicides FLD and IMZ, contained in the effluents from FPI, were detected at 207 and 204 nm respectively with retention times of 8.1 and 19.5 min using a mobile phase of MeOH/H_2O 65/35 v/v. Fungicides TBZ, FLD and CHT, contained in the BHI effluents, were detected at 210, 210 and 230 nm respectively using a gradient elution with solvents A=acetonitrile and B=H_2O+0.1% H_3PO_4. The mobile phase was initially composed of 30% of A (0–5 min). It was then linearly increased to 70% of A from 5 to 15 min, kept constant for 1 min, and then returned to the initial condition in 2 min where it was maintained for 7 min. Under these conditions, the retention times of TBZ, FLD and CHT were 5.5, 18.2 and 24.5 min respectively.

External calibration curves were constructed by the injection of matrix matched standard solutions of each of the studied pesticides (concentration levels ranging from 0.05 to 10 mg L^{-1}) and they were used for pesticide quantification. Matrix-matched standard solutions were prepared by diluting the methanolic or acetonitrile working solutions of each of the studied compounds in leachates collected from biobeds drained with pesticide-free water and negligible matrix effects were evident (<10%). All pesticides showed high linearity (r^2 > 0.99) in the concentration range tested.

2.5 DNA EXTRACTION FROM THE BIOBED PACKING MATERIAL

DNA was extracted from the biobed packing material using the DNeasy PowerSoil™ Pro DNA kit (Qiagen, Germany) following the manufacturers' instructions. DNA quality was assessed via electrophoresis on 0.8% agarose gels. DNA concentration was evaluated via fluorometer measurement with Qubit v.2.

2.6 MICROBIAL COMMUNITY COMPOSITION AND DYNAMICS

The composition of the bacterial and fungal communitywas determined via multiplex amplicon sequencing with Illumina HiSeq technology (Admira health, New Jersey) that generated 250 bp paired-end reads. Primer sets 515f (GTGYCAGCMGCCGCGGTAA) – 806r (GGACTACNVGGGTWTCTAAT) (Walters et al., 2016) and fITS7 (GTGARTCATCGAATCTTTG) – ITS4 (TCCTCCGCTTATTGATATGC) (Ihrmark et al., 2012) were used for the amplification of the V4 region of the 16S rRNA gene of bacteria and the ITS2 genomic region of fungi, respectively. Thermal cycling conditions are given in **Table 3.1**, whereas indexing sequences of primers 515f and ITS4 can be found in **Supplementary Table 3.S1**. In total, two indexed amplicon libraries were prepared.

Table 3.1 The primers, sequences and thermocycling conditions used for amplicon sequencing analysis of the bacterial and fungal communities

Primer	Thermocycling Conditions	Sequence (5' – 3')	Target	Reference
515f 806r	98°C for 10 s, 50°C for 30 s, 72°C for 30 s (25 + 7 cycles) [b]; 72°C for 10 min	NNNNNNNNN**GT**GTGYCAGCMGCCGCGGTAA[a] GGACTACNVGGGTWTCTAAT	Bacterial V4 region of the 16S rRNA gene	Walters et al., (2016)
fITS7 ITS4	98°C for 10 s, 55°C for 30 s, 72°C for 30 s (25 + 7 cycles) b; 72oC for 10 min	GTGARTCATCGAATCTTTG NNNNNNNNN**GA**TCCTCCGCTTATTGATATGC[a]	Fungal ITS2 genomic region	Ihrmark et al., (2012)

[a] The sample index (consecutive Ns) and linker (bold letters) prior to the extension bases in the forward or reverse primer are indicated. Indexed sequences are listed in Supplementary Table 2

[b] The first number in parentheses indicates the number of cycles performed in the first PCR where the unindexed primers were used, while the second number indicates the additional cycles performed in the sample indexing PCR.

Sequence pre-analysis consisted of de-multiplexing with Flexbar version 3.0.3 (Dodt et al., 2012). Sequencing quality screening, chimera removal, alignment to reference databases and generation of the Amplicon Sequence Variant (ASV) matrices were performed with the dada2 package (Callahan et al., 2016a) of the R version 4.0.5 (R Core Team, 2020) as previously suggested (Callahan et al., 2016b). Silva SSU taxonomic dataset version 138 (McLaren, 2020) formatted for dada2, and UNITE general fasta release version 8.2 (Abarenkov et al., 2020) were used for the classification of the V4 16S rRNA and ITS2 amplicons respectively. The coverage of the microbial diversity was assessed through rarefaction curves prepared with the vegan package (Oksanen et al., 2019). The microbiome package (Lahti and Shetty, 2012) was used to calculate measures of α-diversity like Shannon (Spellerberg and Fedor, 2003) and inverse Simpson (Hill, 1973), the observed richness (S) and the Pielou's evenness (Pielou, 1966). The parametric ANOVA or non-parametric Kruskal-Wallis analyses of variance followed by Tukey's or Fisher's least-significant-difference post hoc test respectively, were used to assess the differences of α-diversity indices and the ASVs differential abundance between time points, along the column length and between industrial wastewater treatments. Analysis of variance was performed with the agricolae package v.1.3.3 (de Mendiburu, 2020). β-Diversity was evaluated via canonical correspondence analysis (CCA, ter Braak and Verdonschot, 1995) and redundancy analysis (RDA, Israels, 1984), depending on the first axis of detrended correspondence analysis (Lepš and Šmilauer, 2003). Permutational analysis of variance (Anderson, 2017) that accompanied

aforementioned β-diversity analyses, was performed with pairwise Adonis package, version 0.0.1 (Arbizu, 2020). Spearman's correlation analysis (Hollander and Wolfe, 1973) between ASV abundance at the end of column operation and pesticide residues that were retained in the different biobed horizons were performed with the R stats package (R Core Team, 2020). Demultiplexed, unassembled sequence data are publicly available in the National Centre for Biotechnology Information (NCBI) under Bioproject accession number PRJNA787598.

2.7 ANALYSIS OF THE DYNAMICS OF MGE IN BIOBEDS

Beyond amplicon sequencing analysis, we determined, via q-PCR, the dynamics of MGE known to be involved in the transmission of pesticide degrading genes like (i) the integrase gene of Class-1 integrons (*intI*1 gene) commonly associated with antibiotic resistance genes (Stalder et al., 2014) and IncP-1 plasmids carrying pollutant degrading genes (Martinez et al., 2001; Dealtry et al., 2014); *intI*1 has been proposed as a general marker of environment human disturbance (Gillings et al., 2015), (ii) the *IS*1071 insertion sequence (*tnpA* gene) (Dunon et al., 2018) (iii) plasmids incP-1ε (*trfA* gene) and incP-1 (*korB* gene) (Dealtry et al., 2014).

The abundance of *intI*1 and of total bacteria was determined via q-PCR using primers HS463aF/HS464R and Eub338/Eub518 respectively. This allowed the normalization of *intI*1 copies to the copies of the 16S rRNA gene. Both genes were quantified using a CFX96 connect detection system (Bio-Rad, Germany) in a reaction volume of 10 µL containing 1× KAPA SYBR FAST qPCR master Mix (2×) Universal (KAPA Biosystems, USA), 400 nM of each primer for *intI*1 gene or 200 nM of each primer for the 16S rRNA gene, 0.4 ng/µL BSA, 5 ng of template DNA (for both target genes) and MilliQ ddH_2O up to the final volume. Briefly, the qPCR thermocycling program involved an initial denaturation at 95 °C for 3min followed by 35 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s (the extension step was excluded in the case of the 16S rRNA gene) with detection of the fluorescence signal after each cycle.

The abundance of *tnpA* (*IS*1071), *korB* (IncP-1), *trfA* (IncP-1ε) were determined using a TaqMan q-PCR assay. Assays were run in 10 µL reaction using the KAPA Taq DNA polymerase (KAPA Biosystems, USA) on an Applied Biosystem Quant Studio 5 real-time thermal cycler (ThermoFisher Scientific, USA). The reaction contained 1 U of KapaTaq polymerase, 400 nM of each dNTP, 1×Buffer for KapaTaq with $MnCl_2$, appropriate volume of each primer/probe, 0.4 ng/µL BSA and MilliQ ddH_2O up to the final volume. The thermal program of the Multiplex TaqMan qPCR starts with an initial denaturation step at 95 °C for 5min followed by 40 cycles of 95 °C for 15 s, 54 °C for 15 s and 60 °C for 60 s with detection of the fluorescence signal of each fluorophore probe at the end of each cycle. The 16S rRNA gene was also quantified in the Multiplex TaqMAn qPCR assays (primer set BACT1369F/PROK1492R) to normalize abundance data to the copy numbers of the 16S rRNA gene. All qPCR data were transformed to copies per g of dry weight packing material and then normalized by dividing with the copies of the 16S rRNA gene per g of dry weight biobed packing material. Primers and thermal cycling conditions are given in **Table 3.2**.

Table 3.2 The primers and TaqMan probes used for the determination of the abundance of mobile genetic elements (MGE) via qPCR using either Sybr and Multiplex Taqman protocols as indicated below

Primer/probe name	Sequence (5' – 3')	Target gene/MGE	Amplicon size (bp)	TaqMan Probe 5' Fluorophore / 3' Quencher	Final qPCR Concentrations (nM)	References
		Sybr protocol				
HS463aF	CTGGATTTCGATCACGGCACG	*intI1*	473		400	Hardwick et al., (2008)
HS464R	ACATGCGTGTAAATCATCGTCG				400	
Eub338f	ACT CCT ACG GGA GGC AGC AG	16S rRNA	180		400	Fierer et al., (2005)
Eub518r	ATT ACC GCG GCT GCT GG				400	
		Taqman protocol				
korB-F	TCATCGACAACGACTACAACG	*korB* / IncP-1	118		400	Jechalke et al., (2013)
korB-Fz	TCGTGGATAACGACTACAACG				200	
korB-R	TTCTTCTTGCCCTTCGCCAG				400	
korB-Rge	TTYTTCYTGCCCTTGGCCAG				200	
korB-Rd	TTCTTGACTCCCTTCGCCAG				200	
korB-Pr	TCAGYTCRTTGCGYTGCAGGTTCTCVAT			5' FAM, 3' TAMRA	300	
korB-Pgz	TSAGGTCGTTGCGTTGCAGGTTYTCAAT			5' FAM, 3' TAMRA	300	
IS-F	GCTTGGTCACTTCTGGGTCTTC	*tripA* / *IS*1071	180		400	Dunon et al., (2013); Providenti et al., (2006)
IS-R	CTATGCCCGTCTATCGTTACCC				400	
tp_tripA	TCTTGAAGCCTTTGCTGG CCAGAGTA			5' HEX, 3' Eclipse	200	
trfAε941f	ACGAAGAAATGGTTGTCCTGTTC	*trfA* / IncP-1ε	74		400	Heuer et al., (2012)
trfAε1014r	CGTCAGCTTGCGGTACTTCTC				400	
trfAε965tp	CCGGCGACCATTACAGCAAGTTCATTT			5' ROX, 3' BHQ1	400	
BACT1369F	CGGTGAATACGTTCYCGG	16S rRNA	127		400	Suzuki et al., (2000)
PROK1492R	GGWTACCTTGTTACGACTT				300	
TM1389F	CTTGTACACACCGCCCGTC			5' Cy5, 3' BHQ3	200	

3 RESULTS

3.1 LEACHING OF FUNGICIDES

MET-M showed the highest leaching potential, among the fungicides contained in the effluents from the SPI, with its total amount recovered in the effluent corresponding to 6.83% ± 4.77 of the total applied amount. MET-M was detected in the leachate at day 11 with its levels remaining stable until day 68 when an increase in its amounts in the leachates (>2 mg) until the end of the study was observed (**Figure 3.2A**). FLX was first detected in the leachates at day 76 and it was continuously detected until the end of the study. Its total amount detected in the leachate throughout the study was 3.86% ± 4.13 of the total amount applied. Carboxin (CBX) on the other hand was detected sporadically and in low amounts, overly accounting for 1.65% ± 0.71 of the total amount applied (**Figure 3.2A**).

FLD, TBZ and CHL, contained in effluents produced by the BHI, showed low leaching potential (**Figure 3.2B**). FLD showed the lowest leaching potential with only 0.03%±0.03 of the total amount applied being detected in the leachates. TBZ and CHT were detected in the leachates at low amounts with their total amount leached being 2.35% ± 0.45 and 1.17% ± 0.25 respectively. Interestingly, TBZ and CHT showed a different leaching pattern compared to the fungicides contained in the effluents from the other two agro-industries, with their residues being detected in the leachates mostly during the first 80 days.

IMZ and FLD, contained in the effluents from FPI, showed a low leaching potential. The former was detected in the leachates in miniscule amounts, accounting for 0.02% ± 0.01 of the total amount applied, while the latter was mainly detected in the leachates collected at the final 10 days, summing to 0.32% ±0.31 of the total amount applied (**Figure 3.2C**).

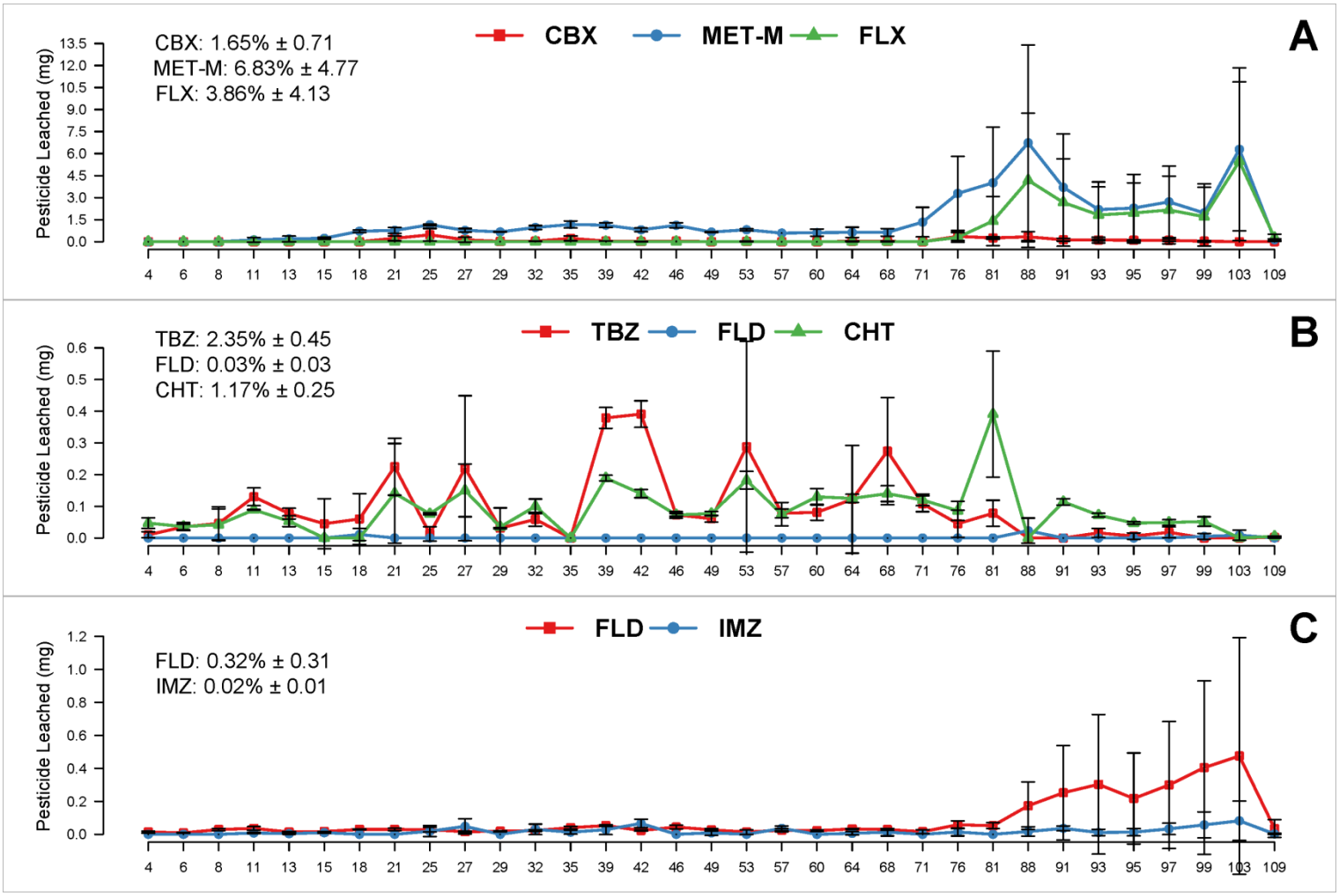


Figure 3.2 Pesticide amounts detected in the leachates of the column biobeds receiving wastewaters from (A) seed-producing industries, (B) bulb-handing industries (C) fruit-packaging industries. Each value is the mean of three measurements obtained from three replicate columns. Error bars represent the standard deviation of the mean. The % of the total applied amount of each pesticide that was detected in the leachates is shown as an inserted table in each graph

3.2 MASS BALANCE ANALYSIS OF FUNGICIDES

Mass balance analysis was employed to identify the primary environmental fate processes that are operative in the biobed systems: dissipation vs retention by the biobed packing material.

3.2.1 SEED PRODUCING EFFLUENTS

Regular monitoring of the fungicide levels in the SPI wastewaters applied on the biobeds allowed us to calculate with accuracy the total amount of CBX, MET-M and FLX applied in the columns during the whole experimental period which summed to 161.1, 708.1 and 567.8 mg respectively. The total amounts of CBX, MET-M and FLX detected in the leachates for the whole experimental duration were 2.7 ± 1.1, 48.4 ± 33.7 and 21.9 ± 23.5 mg respectively, while the total amount of CBX, MET-M and FLX retained by the biobed and it was recovered at day 109 was 19.0 ± 9.6, 3.7 ± 1.7 and 503.6 ± 19.7 mg respectively. Based on mass balance

analysis we showed that the total amount of CBX, MET-M and FLX dissipated during the study was 139.4 ± 10.6, 656 ± 32.2 and 42.2 ± 3.8 mg respectively.

CBX and MET-M showed significantly higher dissipation levels (86.53% ± 6.59 and 92.64% ± 4.55 respectively) compared to FLX which was mostly retained by the biobed packing material (88.7% ± 3.47 vs 11.82% ± 5.98 and 0.53% ± 0.235 for CBX and MET-M respectively) (**Figure 3.3**). When looking at the distribution of pesticide residues inside the column, FLX was mostly retained at the top 20 cm (65.38% ± 0.47), while the remaining 29.8% ± 7.3 and 4.8% ± 7.37 were detected in the 20–50 and 50–80 cm horizons respectively (**Figure 3.4**). CBX and MET-M showed a higher mobility with 33.9% ± 5.24 and 26.14% ± 11.47 respectively being detected in the surface horizon (0–20 cm) and 34.27% ± 11.72 and 46.91% ± 1.61 respectively being detected in the deeper horizon (50–80 cm) (**Figure 3.3**).

3.2.2 BULB HANDLING INDUSTRY EFFLUENTS

The total amount of FLD, TBZ and CHT loaded in the biobed columns during the whole study duration, as determined by regular analysis of the levels of pesticides in the wastewaters applied in the columns, was 149.3, 126.6 and 242.5 mg respectively. The total amount of FLD, TBZ and CHT leached during the whole experimental duration was 0.049 ± 0.05, 3.0 ± 0.6 and 2.8 ± 0.6 mg, whereas the total amount of FLD, TBZ and CHT retained in the packing material and recovered at day 109 was 65.8±28.5, 30.1±19.1 and 24.7±16.6 mg respectively. Mass balance analysis showed that the total amount of FLD, TBZ and CHT dissipated during the study equals to 83.5 ± 28.5, 93.5 ± 19.7 and 215.0 ± 17.2 mg respectively.

The three fungicides showed not statistically significant differences in their levels of dissipation which varied from 55.9% ± 19.1 for FLD to 73.9% ± 15.56 and 88.7% ± 7.11 for TBZ and CHT respectively (**Figure 3.3**). Conversely, FLD showed significantly higher retention in the biobed packing material (44.1% ± 19.1) compared to TBZ (23.8 ± 15.1%) and CHT (10.2%±6.9). All fungicides contained in these wastewaters showed limited mobility in the columns (**Figure 3.3**). Hence, from the total amount retained in the biobeds 84.5% ± 5.1, 96.4% ± 0.87 and 92% ± 3.7 of FLD, CHT and TBZ respectively were retained in the top 20 cm (**Figure 3.4**). The latter is in line with the low amounts of these pesticides recovered in the leachates; 0.03% ± 0.03 for FLD, 1.17% ± 0.25 for CHT and 2.35% ± 0.45 for TBZ.

3.2.3 FRUIT PACKAGING INDUSTRY EFFLUENTS

The total amount of FLD and IMZ loaded in the biobeds during the whole study duration was 884.5 and 2554.5 mg respectively. The total amount of FLD and IMZ found in the leachate throughout the study was 3.5 ± 3.6 and 0.6 ± 0.3 mg respectively, and the total amount that was retained in the packing material and recovered at day 109 was 732.1 ± 86.4 and 1573.6 ± 404.3 mg respectively. Mass balance analysis showed that the total amount of FLD and IMZ dissipated during the experimental duration was 148.8 ± 89.9, and 908.3 ± 404.7 mg respectively.

FLD and IMZ showed low dissipation in the biobeds with only 16.8%± 10.17 and 38.4% ± 15.8 being dissipated respectively (**Figure 3.3**). The two fungicides were mostly retained by the biobed packing material (82.8% ± 9.8 for FLD and 61.60% ± 15.8 for IMZ) but showed different mobility patterns. FLD showed a more uniform distribution with 64.9%±

6.88, 25.44% ± 11.46 and 9.7% ± 7.3 detected in the top, middle and lower horizons respectively (**Figure 3.4**). Whereas 95% ± 3.45 of the amount of IMZ retained in the biobeds was detected at the topmost horizon. In line with all the above, FLD and IMZ exhibited low leaching potential, with only 0.4%±0.4 and 0.02%±0.01 of the total applied amounts respectively being detected in the leachates (**Figure 3.3**).

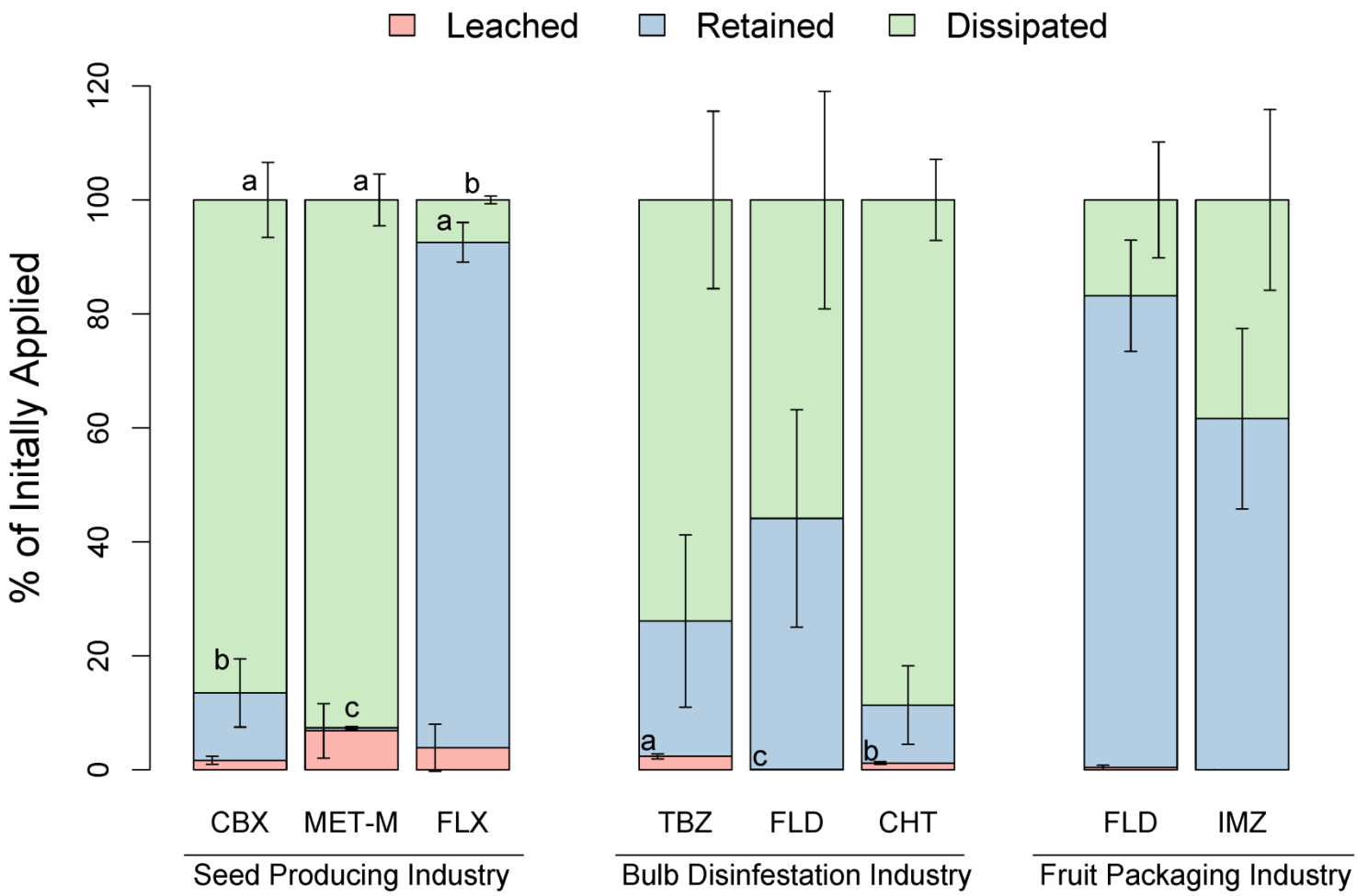


Figure 3.3 Mass balance analysis in the column biobed systems of the pesticides contained in effluents from the (a) seed producing industry (carboxin (CBX), metalaxyl-M (MET-M), fluxapyroxad (FLX); (b) bulb handling industry (thiabendazole (TBZ), fludioxonil (FLD), chlorothalonil (CHT); (c) fruit-packaging industry (fludioxonil (FLD) and imazalil (IMZ)). Error bars represent the standard deviation of the mean of measurements obtained from three replicate columns per treatment. Lower case letters indicate statistically significant differences between pesticides contained in the same wastewater for the same process (leached, retained or dissipated).

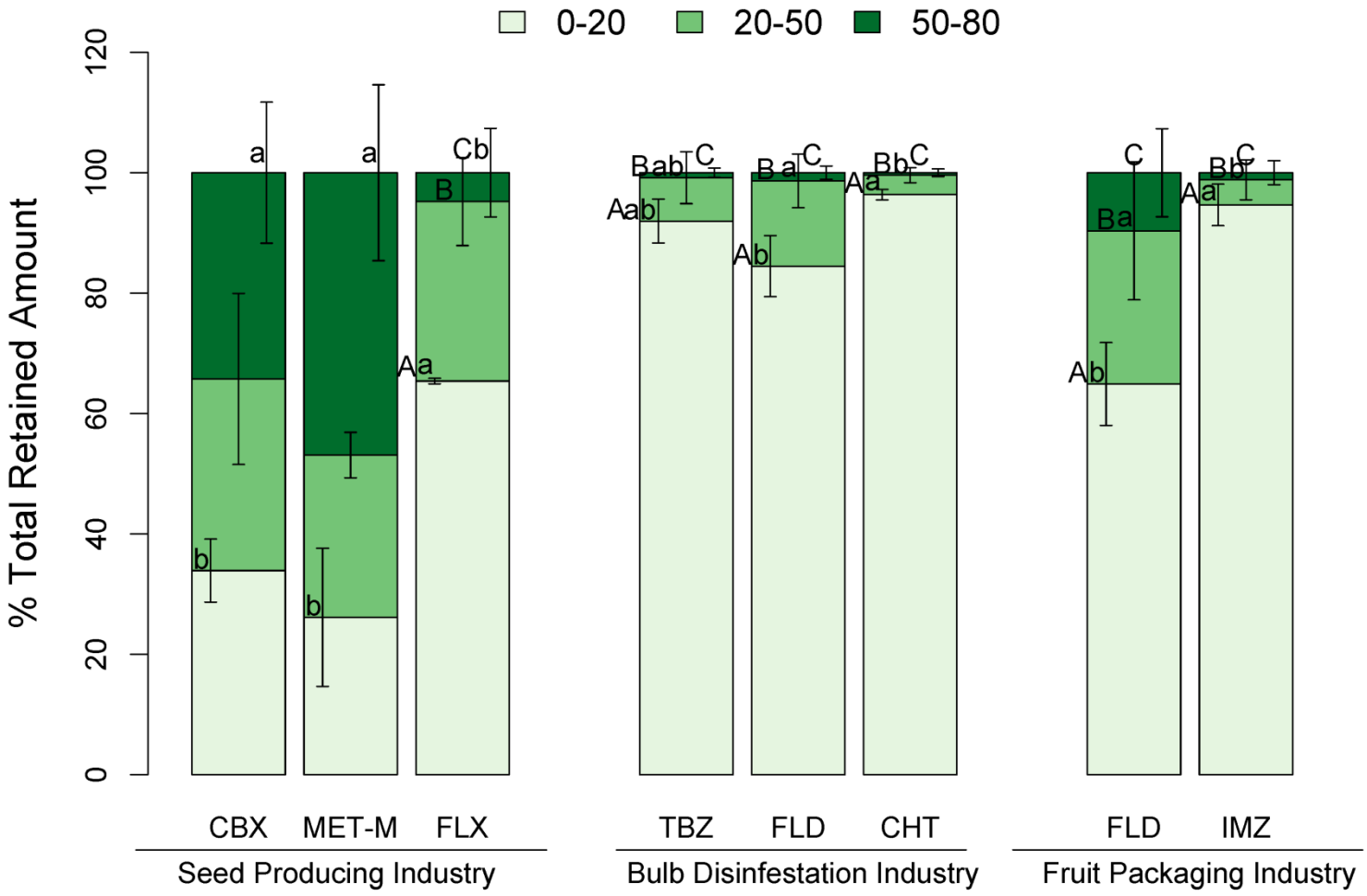


Figure 3.4 The distribution of pesticide residues that were detected in the biobed packing material at the end of the study in the three column horizons (0-20, 20-50 and 50-80 cm from the top). Lower case letters indicate statistically significant differences between pesticides contained in the same wastewater for each column horizon, while upper case letters indicate statistically significant differences between column horizon for each analyzed pesticide.

3.3 MICROBIAL COMMUNITY COMPOSITION AND DYNAMICS

Total bacteria and fungi sequencing effort provided on average of 56,709 ± 45,674 and 30,639 ± 12,032 sequences respectively. After quality control analysis, 1832–161,203 and 3627–35,890 high quality sequences per sample were obtained, for bacteria and fungi respectively. Rarefaction curves reached a plateau in all samples indicating that the sequencing effort provided adequate coverage of the microbial diversity (**Figure 3.5**).

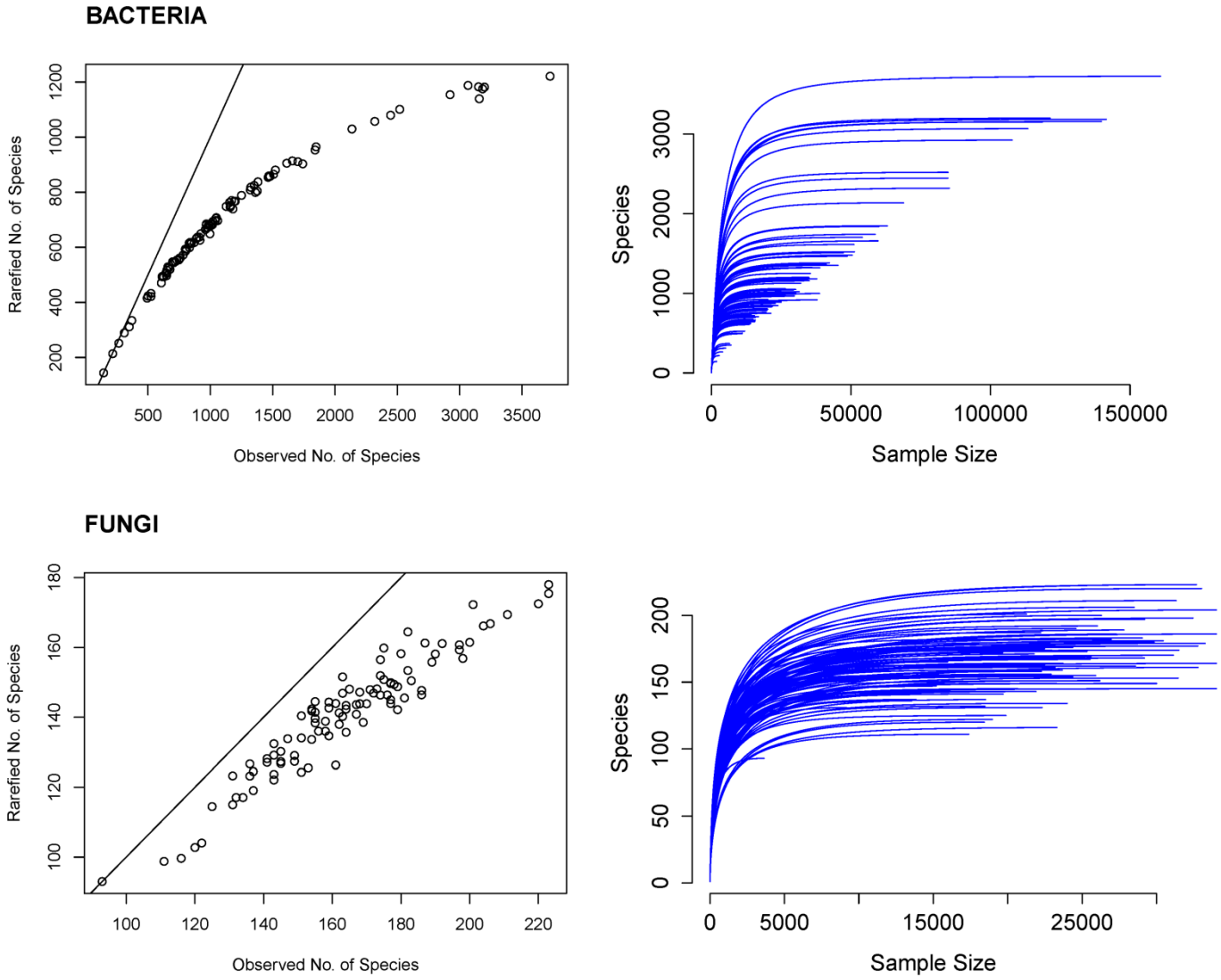


Figure 3.5 Scatterplots of the observed and rarefied number of ASVs (left panels) and rarefaction curves of the diversity coverage of our sequencing effort (right panels) for bacteria (top) and fungi (bottom).

Bacterial communities were dominated by *Proteobacteria*, *Bacteroidota* and *Actinobacteriota* with an average relative abundance of 38.22%±6.16, 17.40%±4.97 and 14.29%±8.70 respectively (**Figure 3.6**). *Acidobacteriota* relative abundance showed a temporal increase ranging from 0.38% ± 0.29 to 11.13% ± 2.75 in all treatments (**Figure 3.6**). Fungal communities were dominated by *Sordariomycetes* (71.94%±8.65) (**Figure 3.7**). Other fungal classes that were present in appreciable numbers were *Agaricomycetes* (7.58% ± 5.56), *Aphelidiomycetes* (4.16% ± 3.52), and *Pezzizomycetes* (3.97% ± 6.35) (**Figure 3.7**).

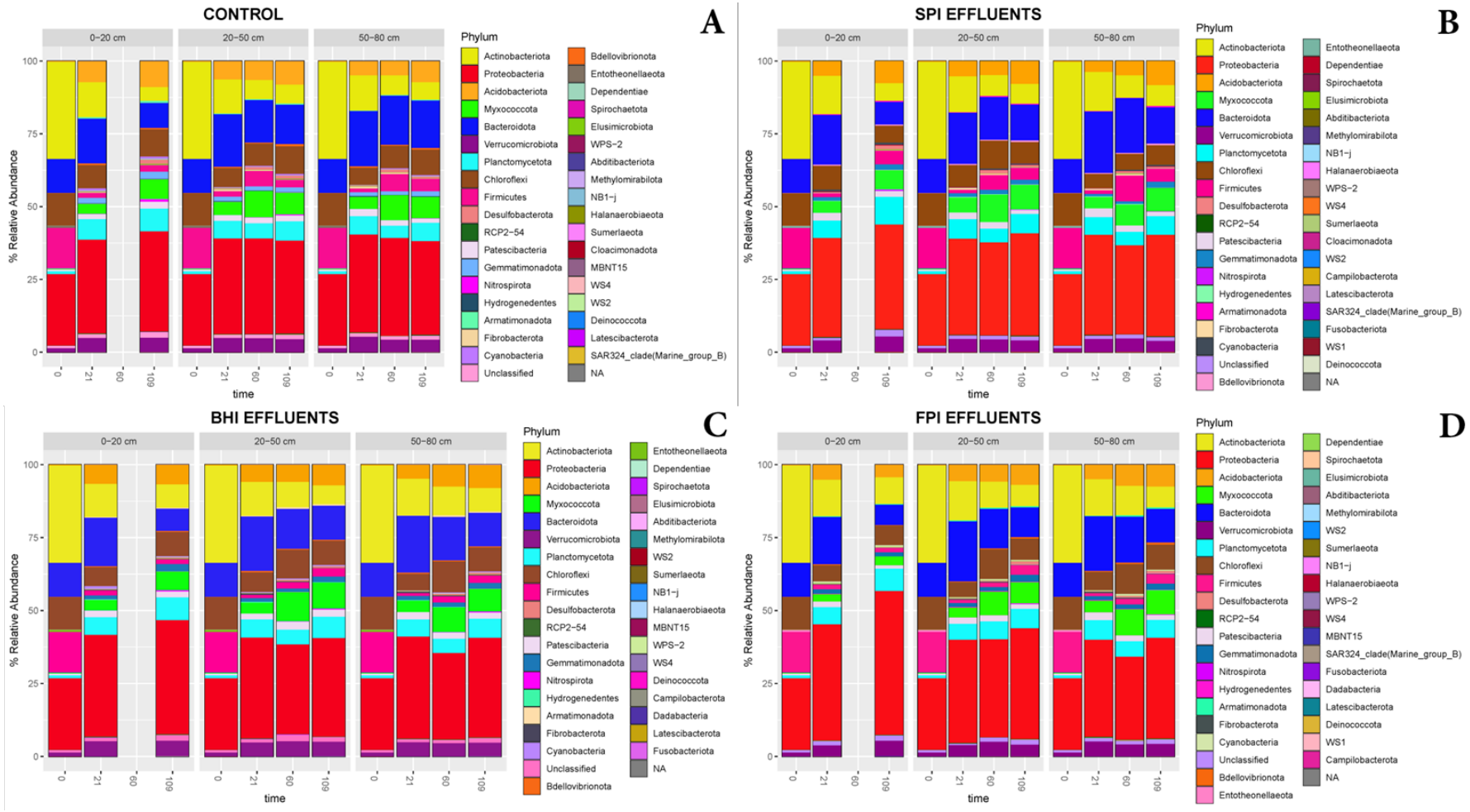


Figure 3.6 The composition of the bacterial community in the different horizons (0-20, 20-50 and 50-80 cm) of pilot biobed systems receiving either pesticide-free water (A) or pesticide-contaminated effluents from (B) Seed producing Industries (SPIs), (C) Bulb-handling industries (BHIs) or (D) Fruit packaging Industries (FPIs) at different time intervals along the 109 experimental days.

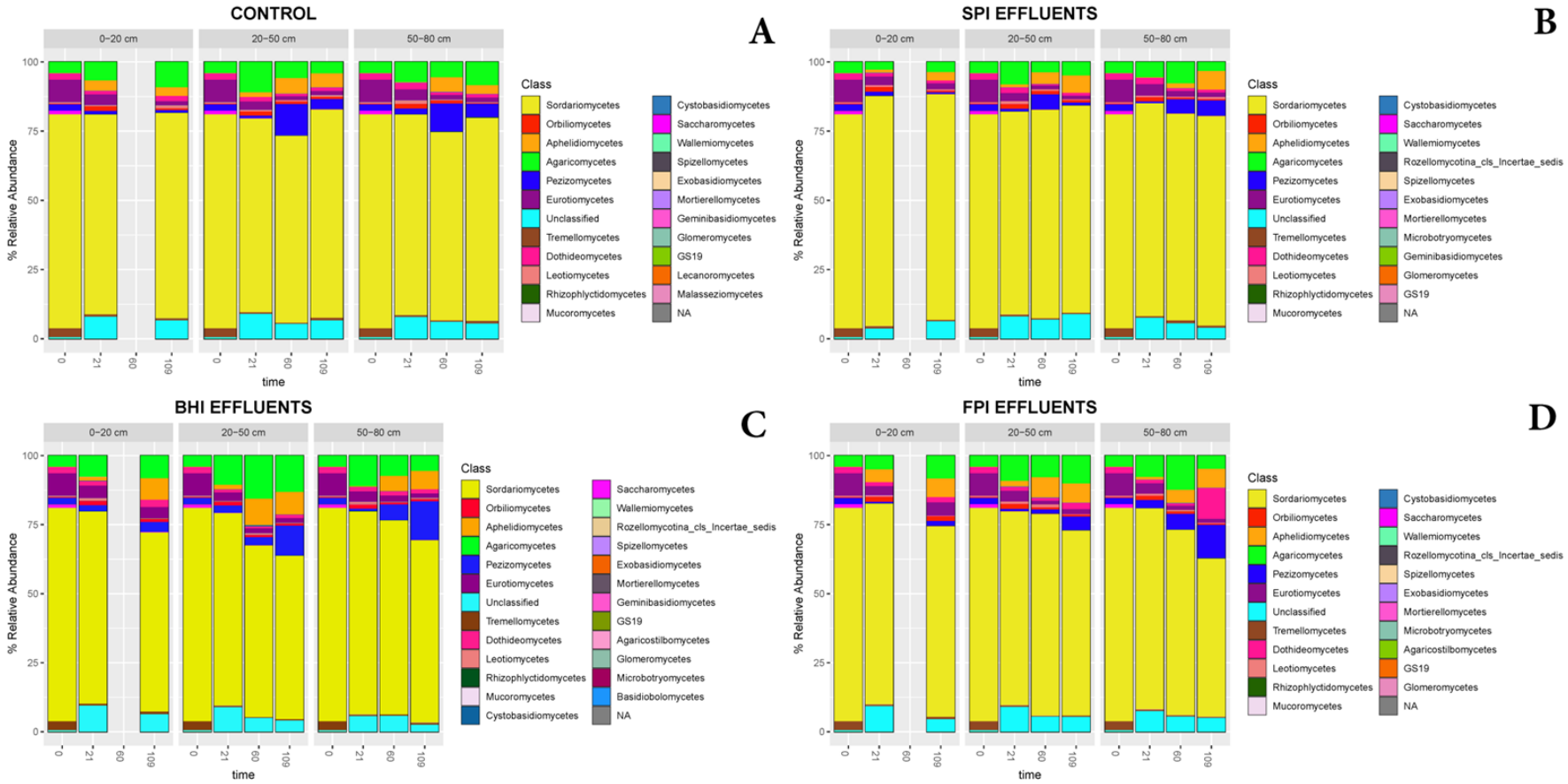


Figure 3.7 The composition of the fungal community in the different horizons (0-20, 20-50 and 50-80 cm) of pilot biobed systems receiving either pesticide-free water (A) or pesticide-contaminated effluents from (B) Seed producing Industries (SPIs) (C) Bulb-handling industries (BHI) or (D) Fruit packaging Industries (FPIs) at different time intervals along the 109 experimental days.

3.3.1 FACTORS AFFECTING THE α-DIVERSITY OF THE BACTERIAL AND FUNGAL COMMUNITIES

Biobeds supported a rich and diverse bacterial community with a mean observed ASV number per sample of 1134±659, and mean Shannon's and inverse Simpson diversity index values of 6.51 ± 0.52 and 545.4 ± 264 respectively (**Table 3.3**). Finally, Pielou's index averaged at 0.95±0.01 suggesting highly even bacterial communities. Statistical analysis revealed no significant effects of wastewater treatment and depth on the different α-diversity indices. Whereas time, as a main factor had a significant effect on the α-diversity indices.

Fungal communities showed low species richness, ranging from 93 to 223 observed ASVs per sample (**Table 3.3**). Regardless of wastewater treatment and column depth the values of both diversity indices showed significant temporal decrease from 3.71 ± 0.16 to 3.21 ± 0.27 (Shannon's) and from 17.24 ± 3.36 to 9.76 ± 2.87 (inverse Simpson's) on days 21 and 109 respectively. Pielou's evenness values ranged from 0.63 ± 0.04 to 0.73 ± 0.02 suggesting moderately uneven communities characterized by dominant species. Community evenness showed significant temporal changes as well, with higher values observed at 21 days. As with bacterial communities, wastewater composition did not significantly affect the α-diversity of the fungal community.

Table 3.3 The α-diversity indices of the bacterial and fungal community in biobed samples collected at different time points and biobed horizons. Each value is the mean of 12 samples ± standard deviation. Values followed by the same letters are not statistically different at 5% level.

COMMUNITY	DAY	HORIZON	OBSERVED RICHNESS	SHANNON DIVERSITY	INVERSE SIMPSON DIVERSITY	PIELOU'S EVENESS
BACTERIA	**21**	**0-20**	899.5 ± 429.23 bcd	6.31 ± 0.63 bc	495.29 ± 231.07 ab	0.95 ± 0.01 a
		20-50	925.83 ± 362.93 bcd	6.37 ± 0.6 bc	510.49 ± 192.27 ab	0.95 ± 0.01 a
		50-80	871.83 ± 297.4 cd	6.36 ± 0.42 bc	485.19 ± 165.82 ab	0.95 ± 0.01 a
	60	**20-50**	961.42 ± 348.52 abcd	6.44 ± 0.44 abc	506.29 ± 163.27 ab	0.95 ± 0.01 a
		50-80	820.67 ± 234.82 d	6.32 ± 0.3 c	454.04 ± 136.19 b	0.95 ± 0.01 a
	109	**0-20**	1681 ± 1015.16 abc	6.86 ± 0.63 ab	755.68 ± 379.21 a	0.95 ± 0.01 a
		20-50	1604 ± 1001.76 ab	6.83 ± 0.57 ab	711.19 ± 349.4 a	0.95 ± 0.01 a
		50-80	1665.83 ± 899.41 a	6.88 ± 0.62 a	757.14 ± 356.08 a	0.95 ± 0.01 a
FUNGI	**21**	**0-20**	168.58 ± 26.68 abc	3.58 ± 0.19 b	14.88 ± 3.9 b	0.7 ± 0.03 b
		20-50	178.92 ± 19.88 a	3.79 ± 0.1 a	18.63 ± 2.79 a	0.73 ± 0.02 a
		50-80	177.67 ± 29.02 a	3.76 ± 0.08 a	18.21 ± 1.94 ab	0.73 ± 0.02 a
	60	**20-50**	165.92 ± 14.54 abc	3.5 ± 0.18 b	15.23 ± 3.14 ab	0.68 ± 0.03 bc
		50-80	176.25 ± 16.13 ab	3.55 ± 0.14 b	14.63 ± 3.02 bc	0.69 ± 0.02 bc
	109	**20**	152.42 ± 16.98 abc	3.21 ± 0.29 c	8.53 ± 2.32 d	0.64 ± 0.05 de
		20-50	144 ± 25.86 c	3.29 ± 0.26 c	11.11 ± 3.09 cd	0.66 ± 0.05 cd
		50-80	150.17 ± 19.52 bc	3.14 ± 0.25 c	9.63 ± 2.75 d	0.63 ± 0.04 e

3.3.2 FACTORS AFFECTING THE β-DIVERSITY OF THE BACTERIAL AND FUNGAL COMMUNITIES

The factors affecting the β-diversity of the microbial communities in biobed systems were further examined. Time, column horizon and wastewater origin exerted weak but significant effects on the β-diversity of the bacterial and fungal community ($p = 0.01$) (Fig. 3). CCA revealed that time and column horizon accounted for 8.8%of the total variance in bacterial communities (**Figure 3.8A**), unlike wastewater origin which explained only 3.2% of the variance (**Supplementary Figure 3.S1**). Pairwise comparison of the bacterial community composition between the different column horizons showed no statistically significant differences at 21 days (**Figure 3.8A**). However significant differences in the bacterial community between different horizons emerged at later sampling days. At 60 days, significant differences in the bacterial community between the 20–50 and the 50–80 cm horizons were observed, while at day 109 strong differences were evident between the top (0–20 cm) and the bottom (50–80 cm) horizon ($p < 0.05$).

Similar to bacteria, the fungal community was strongly affected by the factors time and column horizon (**Figure 3.8B**), whereas the wastewater origin showed no significant effects ($p = 0.188$) (**Supplementary Figure 3.S1**). RDA showed that time and column horizon together accounted for 30.4% of the total variance ($p=0.01$) (**Figure 3.8B**). Significant differences in the composition of the fungal communities between the top (0–20 cm) and the deeper horizons (20–50 cm and 50–80 cm) were recorded at 21 days, which were still visible at 109 days ($p < 0.05$). In the absence of samples from the top horizon at 60 days, a significant difference in the composition of the fungal community in the two other horizons (20–50 cm vs 50–80 cm) was observed.

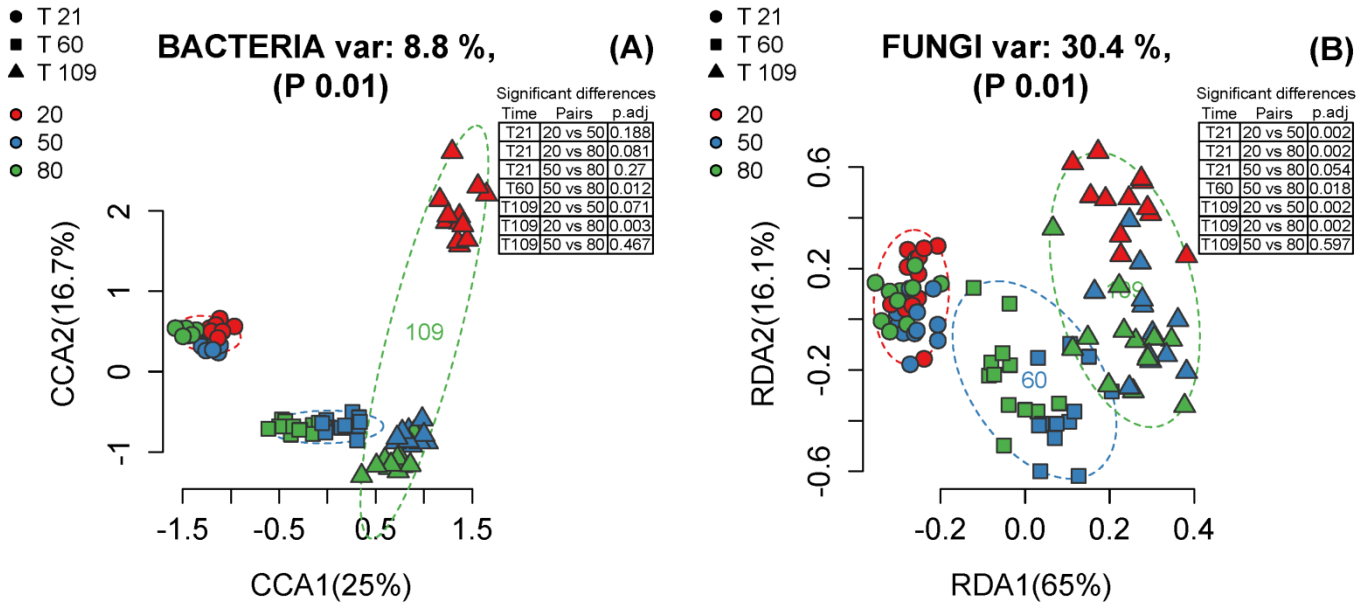


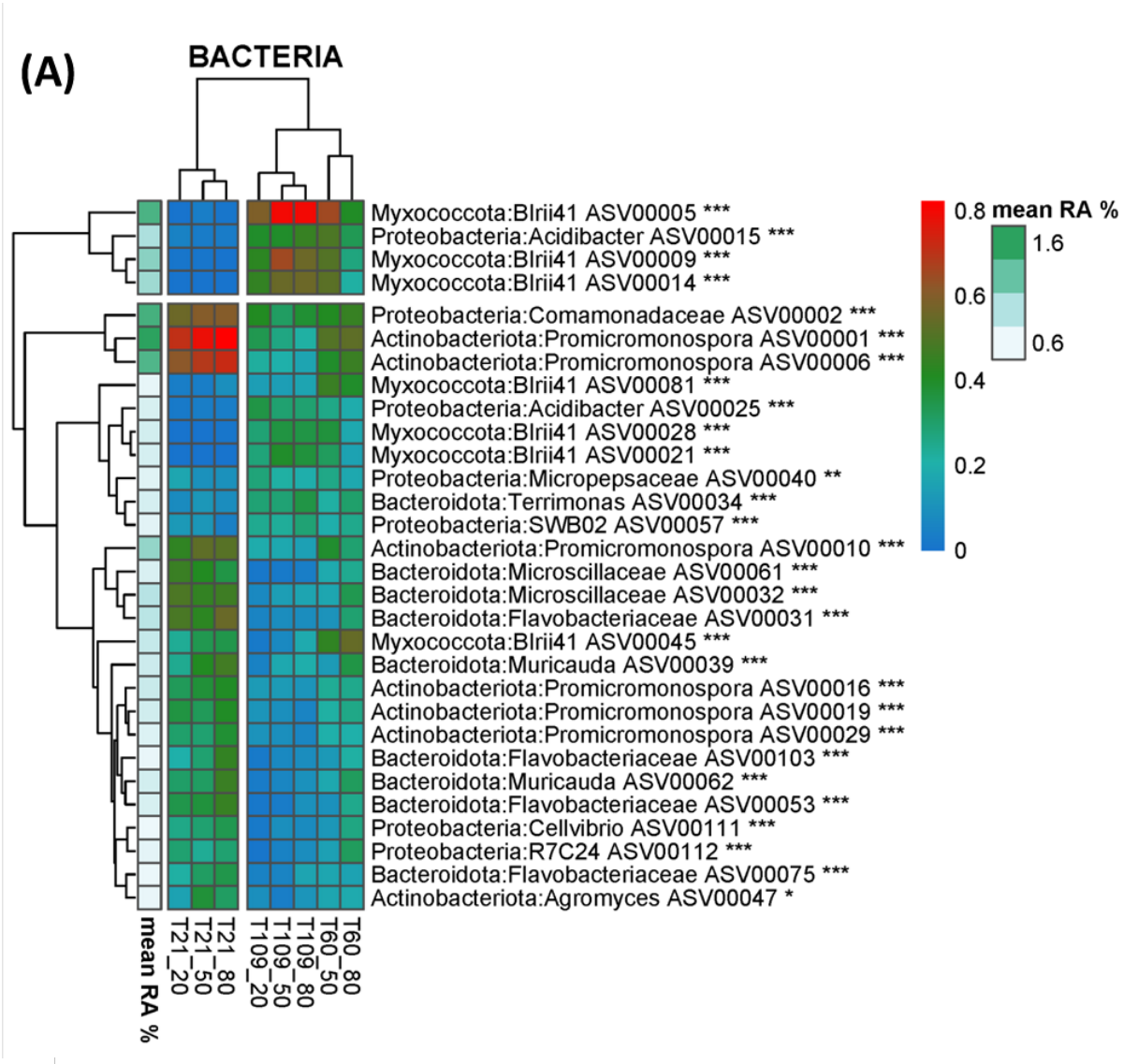
Figure 3.8 Canonical Correspondence Analysis (CCA) and redundancy analysis (RDA) of the bacterial (A) and fungal (B) communities colonizing the different horizons of the biobed systems. Samples were ordinated according to sampling time and horizon. Inserted tables display pairwise comparisons of the microbial communities in the different horizons at each time point.

3.3.3 TIME- AND HORIZON SPECIFIC DIFFERENTIAL ABUNDANCE OF ASVS

Based on the strong effects of time and column horizon on the composition of the microbial communities in biobed packing material, the bacterial and fungal communities were screened for ASVs that showed a consistent and significant response to these two factors. In total, 128 bacterial and 120 fungal ASVs were identified, whose abundance was significantly changed along time and column horizons.

Some clear and consistent taxon level temporal patterns in bacterial abundance were evident. Seven ASVs belonging to *Promicomonospora*, which were among the most abundant ones, three ASVs belonging to *Microscillaceae*, and two ASVs affiliated to *Confluentibacter* showed a significant decrease in their relative abundance with time regardless of the treatment and the column depth (**Figure 3.9A; Supplementary Figure 3.S2**). Conversely, nine ASVs belonging to *Myxococcota*, which were among the most dominant ASVs, three ASVs belonging to Acidibacter, three ASVs belonging to *Terrimonas*, four ASVs belonging to *Arenimonas*, six ASVs belonging to Proteobacteria SWB02, and eight ASVs affiliated to *Anaerolinae*/*Chloroflexi* showed a significant increase in their relative abundance with time (**Figure 3.9A; Supplementary Figure 3.S2**).

Regarding fungi, ASV0001 affiliated to *Stachybotrys chartarum* dominated the fungal community with a mean relative abundance of 18% in all samples, and its relative abundance significantly increased with time (**Figure 3.9B; Supplementary Figure 3.S3**). A similar temporal pattern was evident for other dominant ASVs like *Immersiella* and *Aphelidiomycota*. On the other hand, ASVs belonging to *Schizothecium* (9), *Lasiosphaeriaceae* (6), *Sarocladium* (3) *Microascaceae* (5) and different Basidiomycota (5) genera, *Pleurotus*, *Coprinus* and *Efibulobasidium* showed a clear decrease in their relative abundance with time (**Figure 3.9B; Supplementary Figure 3.S3**).



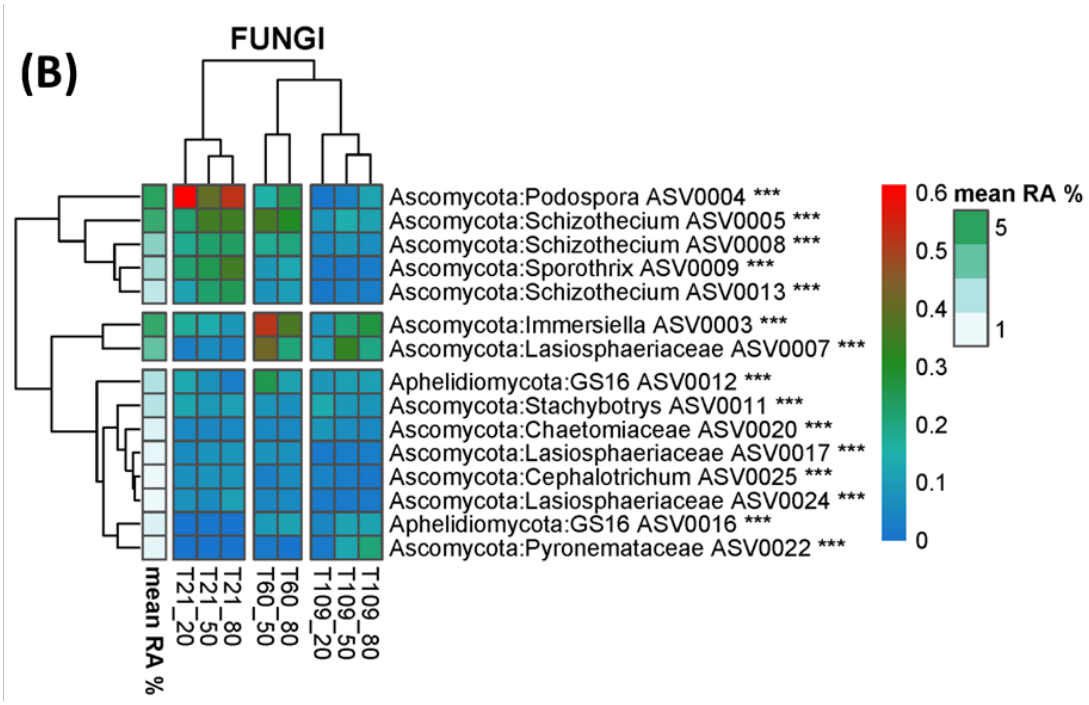


Figure 3.9 Heatmaps showing bacterial (A) and fungal (B) ASVs that showed significant differential abundance in the different biobed horizons along the different time points. It should be noted that the most dominant fungal ASV *Stachybotrys chartarum* was not displayed to avoid masking other differentially abundant ASVs.

3.3.4 ASV ABUNDANCE AND PESTICIDE RESIDUE CORRELATION

The amplicon sequencing dataset was further explored for significant correlations between ASV abundance and pesticide residues that were detected in the different horizons of the biobed packing material at the end of the study period. Several ASVs showing increasing relative abundance with increasing pesticide residues (positive correlations) or decreasing relative abundance with increasing pesticide residues (negative correlations) were detected. CBX residues did not show any significant correlations with the relative abundance of bacterial ASVs. MET-M was positively correlated with *Cellulosimicrobium*, *Proteobacteria-R7C24* and *Bacteroidota*, while negative correlations were observed with *Saprospiraceae*, *Myxococcota-Blrii41*, *Rhodocyclaceae*, *Arenimonas*, *Azospira* and Bryobacter (**Figure 3.10A; Supplementary Figure 3.S4**). FLX was positively correlated with *Chloroflexi*-A4b, *Blastocatellaceae*, *Steroidobacter* and *Candidatus_Solibacter*, but negatively correlated with *Gammaproteobacteria-R7C24*, *Myxococcota-Blrii41*, *Planococcaceae*, *Paludibacteraceae*, *Cellulomonadaceae*, *Terrimonas*, *Muricauda* and *Luteitalea*, (**Figure 3.10A; Supplementary Figure 3.S4**). Regarding fungicides used in BHI, CHT and FLD residues showed a significant negative correlation with an ASV belonging to *Microscillaceae* (**Figure 3.10A; Supplementary Figure 3.S5**). Both fungicides used in FPI were negatively correlated with ASVs belonging to *Pseudoflavitalea* and *Chryseolinea* and positively correlated with ASVs belonging to *Pseudomonadaceae*, *Tahibacter*, *Ferrovibrio* (2) *Methylibium* (3), *Ralstonia* and *Sphingopyxis* (**Figure 3.10A; Supplementary Figure 3.S6**).

Concerning fungi, CBX and MET-M residues showed significant positive correlations with ASVs belonging to *Rozellomycota* and *Ascomycota* respectively (**Figure 3.10B; Supplementary Figure 3.S7**), while FLX residues were negatively correlated with ASVs belonging to Basidiomycota (*Coprinellus*, *Sordariomycetes*, *Pezizales* and *Podospora*) and positively correlated with ASVs of *Rozellomycota*, *Phialophora*, *Chaetomium*, *Aspergillus* and *Arthrographis* (**Figure 3.10B; Supplementary Figure 3.S7**). TBZ, FLD and CHT, all contained in the effluents from bulb handling industries, showed positive correlations with *Rozellomycota*, *Aspergillus*, *Cyphellophora*, *Trichoderma* and *Schwanniomyces* (**Figure 3.10B; Supplementary Figure 3.S8**). FLD and IMZ, contained in effluents from FPI, showed significant positive correlations with *Rozellomycota*, *Cyphellophora*, *Dactylella*, *Cephalotrichum* and *Exophiala*. Whereas an *Immersiella* ASV showed significant negative correlation with FLD and IMZ (**Figure 3.10B; Supplementary Figure 3.S9**).

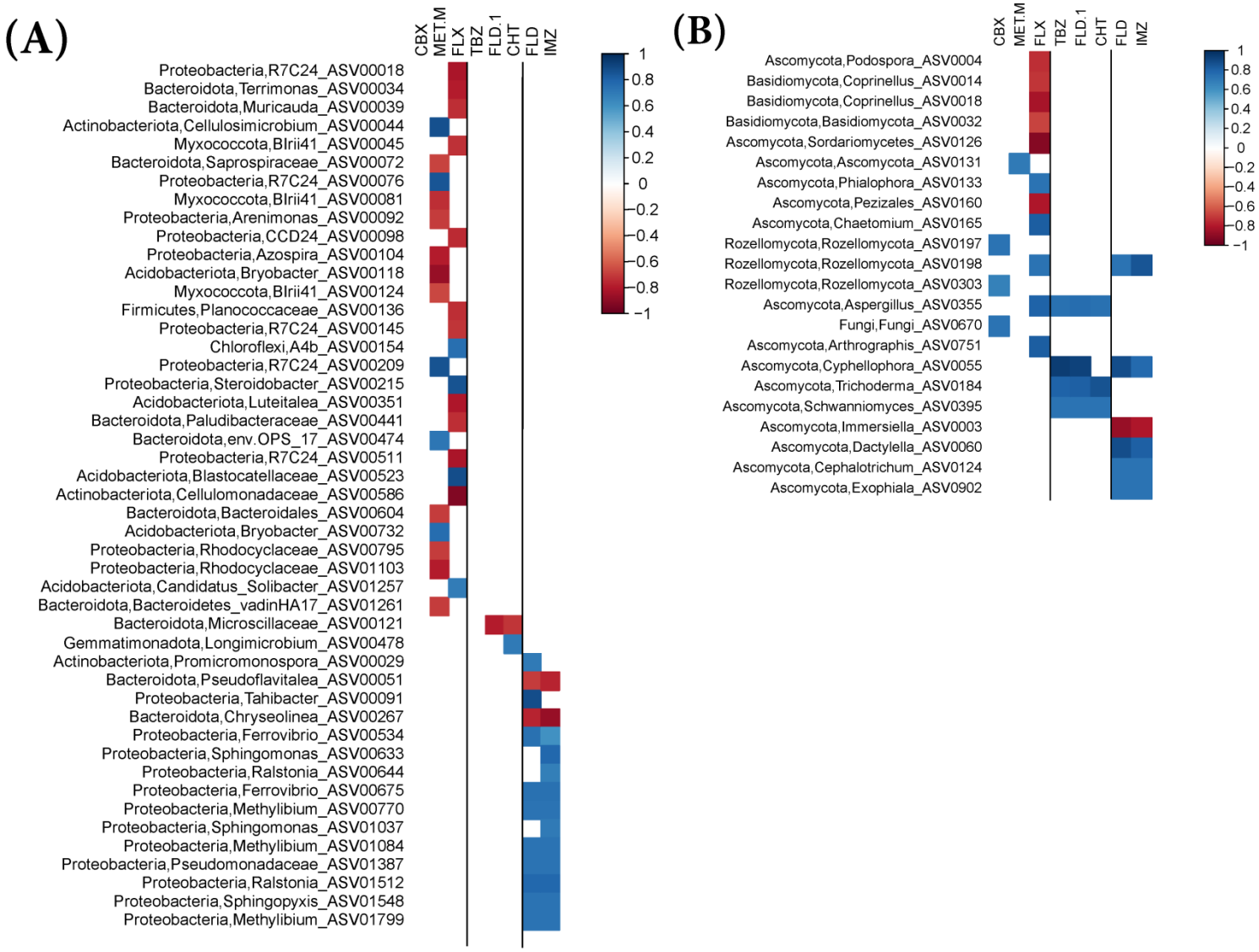


Figure 3.10 Heatmaps showing significant positive or negative correlations (Spearman's rho correlation coefficient is shown on the side) between the abundance of bacterial (A) and fungal (B) ASVs and fungicide residues as determined at the end of the study in the biobed packing material.

3.4 ANALYSIS OF THE DYNAMICS OF MGE IN BIOBEDS

Before wastewater application *intI1* and *IS*1071 were the only MGEs that were detected in the biobed packing material at 0.05 ± 0.01 and 0.131 × 10^{-2} ± 0.05 × 10^{-2} relative copies respectively. Wastewater treatment and column depth did not show a significant effect, as main factors, on the relative abundance of any of the MGEs studied, whereas significant temporal changes were evident for *intI1*, *IS*1071 and *trfA* (**Figure 3.11; Supplementary Figure 3.S10**). More specifically the relative abundance of *intI1* significantly increased with time from 2.32 × 10^{-3} ± 1.22× 10^{-3} copies per g at 21 days to 4.39 × 10^{-3} ± 3.09×10^{-3} copies per g at 109 days (**Supplementary Figure 3.S10**). Regarding *IS*1071 and *trfA*, a temporal increase was observed with average copies ranging from0.17±0.28 on day 21 to 3680.52 ± 20,350.93 on day 109 for *IS*1071 and 0.835 × 10^{-4} ± 3.54 × 10^{-4} on day 21 to 891 ± 4650 relative copies on day 109 for *trfA* (**Supplementary Figure 3.S10**). The most dramatic

increase for both MGEs was evident in the columns receiving effluents from BHI, corresponding to a raise in the relative abundance of *IS*1071 from0.06 ± 0.06 at day 21 to 13,685.4±40,611.2 at day 109 and of *trfA* from2.57 × 10−3± 6.99×10^{-3} at day 21 to 3140±9230 at day 109 (**Figure 3.11**). No significant changes in the abundance of *korB* were observed during the study.

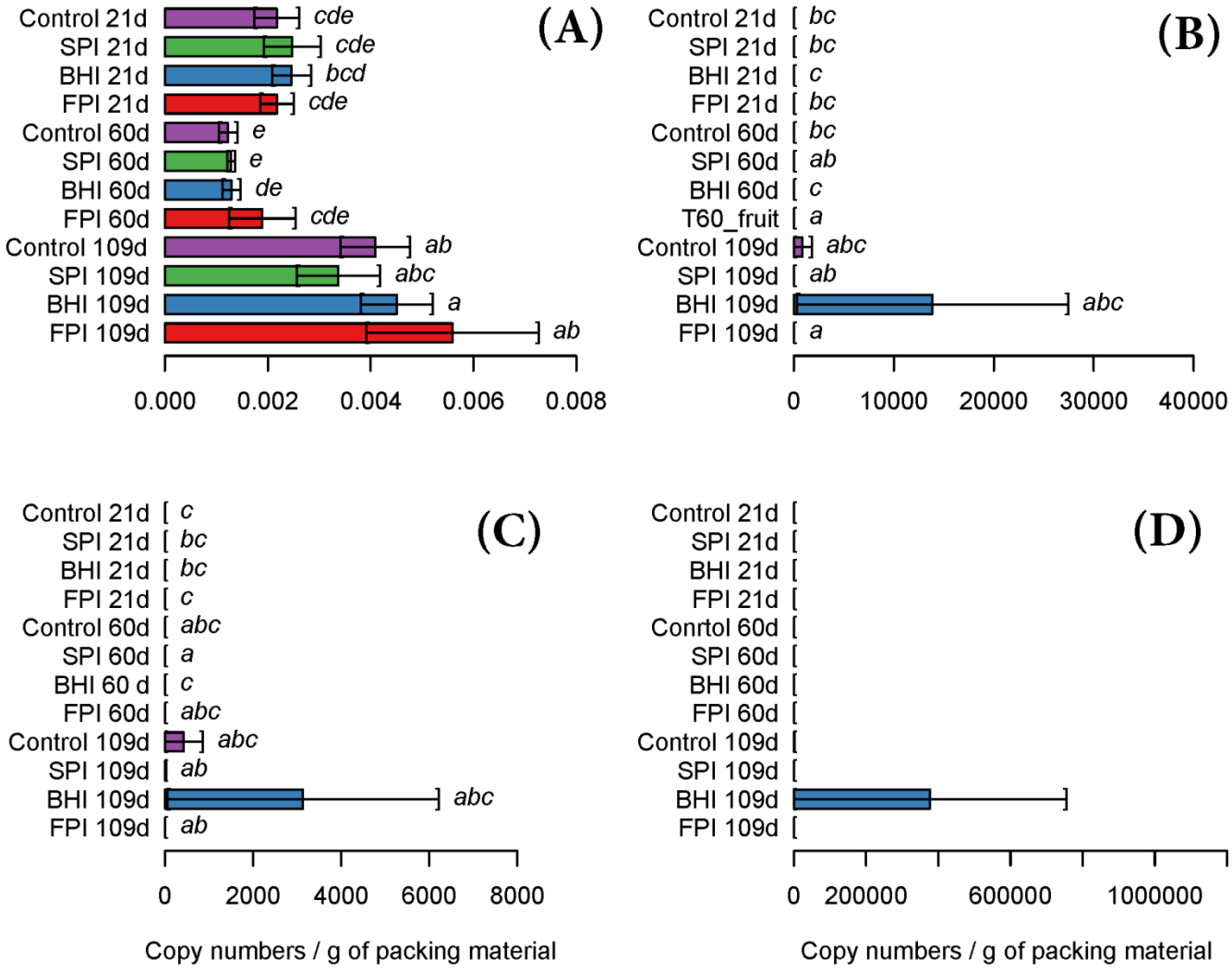


Figure 3.11 The relative abundance of (A) *intI1*, (B) *IS*1071, (C) *korB* and (D) *trfA* (expressed as copy numbers per gram of biobed packing material (dry weight) normalized to the copy numbers of the 16S rRNA gene) in biobeds receiving effluents from seed-producing industries (SPIs), bulb handling industries (BHIs), fruit packaging industries (FPIs) and in biobeds treated with no pesticide containing effluents (control). Each bar represents the mean of 9 samples (6 in the case of 60 days) ± standard error. Bars designated by the same lower case letters are not significantly different at the 5% level.

4 DISCUSSION

4.1 THE PERFORMANCE OF BIOBED SYSTEMS

The capacity of biobeds to treat effluents from different agro-food processing industries that make use of fungicides was assessed in a biobed column study. The capacity of biobeds to remove CBX, MET-M and FLX from wastewaters produced by SPIs was initially examined. FLX was the sole fungicide that was mostly retained in the biobed packing material in line with its high sorption affinity onto organic matter (Gulkowska et al., 2016; Ou and Latin, 2018). On the other hand, MET-M and CBX were mostly dissipated by the biobeds in line with the rapid degradation of these two chemicals in soil (Balasubramanya and Patil, 1980; EFSA, 2010, 2015) and variable biobed packing materials (Karanasios et al., 2010a; Papazlatani et al., 2019). The high leaching potential of MET-M, the highest among the fungicides tested, is in accord with its high hydrophilic character, the highest among the tested fungicides (**Table 2.1**), which suggest low adsorption affinity. Indeed previous studies in biobed packing material of various compositions reported MET-M K_{foc} values ranging from 7.81 to 213 L kg^{-1} (Karanasios et al., 2010a,b; Vischetti et al., 2020). The high mobility of MET-M has been also demonstrated in a BiomassBed system receiving washates from a sprayer-tank for two years, where it was the least retained fungicide (Vischetti et al., 2012). The late leaching pattern of all SPI fungicides might be associated with the high adsorption affinity of FLX which could have saturated the sorption sites of the biobed packing material during the first 70 days of the experiment and hence favored the leaching of the surplus of pesticides applied on the columns.

Regarding pesticides contained in effluents from BHI, CHT, TBZ and FLD were mainly dissipated or retained by the biobed packing material, with the former being the most important process. The three fungicides showed limited mobility as it is suggested by their low leached amount and the concentration of their residues at the top 20 cm of the biobeds (over 90% of the total amount retained). Our findings are in accord with previous studies by Karas et al. (2016b) who reported nearly 87% dissipation of TBZ in pilot biobeds packed with a bioorganic material identical to the one used in present study. Similarly Gao et al. (2015) and Fogg et al. (2004a) studied the dissipation of CHT in biobeds packed with different packing materials, and reported high degradation percentages and limited mobility.

Regarding FLD and IMZ contained in effluents from FPI, biobeds effectively removed both fungicides from the effluents with more than 99.8% of the total applied amounts retained or dissipated with the former being the dominant process. Omirou et al. (2012) and Karas et al. (2016a) studied the capacity of biobeds to retain and dissipate fungicides contained in effluents from FPIs. In line with our study, they observed a negligible leaching of IMZ (0.09–0.22% of total applied amount), with the amount of IMZ retained by the biobed packing material being detected at the top 20 cm of the biobed system. Similarly to IMZ, previous studies have also demonstrated the high adsorption affinity and low mobility of FLD in soil and biobed packing material, which is in line with our findings (Fenoll et al., 2011; Vischetti et al., 2012).

4.2 THE COMPOSITION OF BIOBEDS MICROBIOME

We further determined the composition of the bacterial and fungal communities in biobed systems, followed microbial succession during their operation and identified how microbial communities colonizing the biobed packing material respond to continuous exposure to agro-industrial effluents of variable pesticide composition.

The bacterial community of biobeds was dominated by *Proteobacteria*, *Actinobacteria* and *Bacteroidota* in line with previous studies (Holmsgaard et al., 2017; Bergsveinson et al., 2018). Russell et al. (2021) in their metagenomic analysis of microbial communities in two operating biobed systems also demonstrated dominance of Proteobacteria. The fungal community was dominated by Sordariomycetes of the orders *Hypocreales* and *Sordariales* which is in accord with previous studies by Bergsveinson et al. (2018).

Interestingly wastewater treatment did not have a significant influence on the diversity of the bacterial and fungal communities which showed a remarkable resilience. Previous studies by Bergsveinson et al. (2018) also suggested that the microbiome of biobeds was resilient and was not affected by biobed design or operation parameters. The composition of the bacterial community in the pilot biobeds showed clear temporal patters and further varied at the different biobed horizons. Hence, bacterial communities showed limited variation with depth at start (21 d), but they largely differentiated from the top to the bottom horizon at the end of the study (109 days). Similar temporal patterns were evident also for the fungal community although this time the differences in the composition of the fungal community along depth were evident from the first sampling day and persisted for the whole experimental duration. The earlier differentiation of the fungal community along the biobed depth could not be attributed to the nature of the pesticides applied (fungicides) since the same pattern was also evident in the non-treated columns. This differentiation is probably driven by the early establishment of microaerophilic conditions in the deeper layers of the biobed system which are expected to affect mostly the fungal rather than the bacterial community which is more easily adapted to low oxygen conditions (Reith et al., 2002). Earlier column studies by Fogg et al. (2004b) reported that high water loadings could result in saturation and the establishment of microaerophilic conditions at the deeper layers and a slowdown of pesticide degradation.

Considering that the pesticide composition of the wastewater did not significantly affect the bacterial and fungal communities, we attempted to identify the bacterial and fungal taxa whose abundance showed consistent temporal patterns. Bacteria belonging to *Myxococcota*, *Acidibacter*, *Terrimonas*, *Arenimonas* and *Chloroflexi* showed a consistent increase with time. The *Myxococcota* ASVs identified in our study were affiliated to the *Myxoccota Blrii41* family which have been identified as dominant member of the bacterial community in aerobic green waste composts (Cai et al., 2018). Members of this phylum are notorious predators in soil swarming their prey (Waite et al., 2020). However recent metagenomic analysis of uncultured *Myxococcota*, like members of the Blrii41 family (Petters et al., 2021), from non-soil environments appeared to be strict anaerobes with lack of predation capacities (Murphy et al., 2021). This along with the significant increase in the abundance of *Anaerolinae*/*Chloroflexi* and *Acidibacter* ASVs with time provides further

support to our earlier suggestion about the establishment of microaerophilic conditions in the biobed which favored facultative or strict anaerobes like *Chloroflexi*/*Anaerolinae* (Sun et al., 2015; Gschwend et al., 2020), *Acidibacter* (Falagán and Johnson, 2014) and *Myxococcota*.

Regarding fungi, consistent temporal decreases in the relative abundance of *Schizothecium* and to several basidiomycetes belonging to *Pleurotus*, *Coprinus* and *Efibulobasidium* were noted. Members of the genus *Schizothecium* are coprophilus fungi that predominate in organic-rich substrates like the biobed packing material (Zheng et al., 2021). The time – dependent reduction in the relative abundance of *Basiodiomycetes* might be associated with the prevalence of conditions in the biobed systems which disfavor their proliferation. In a similar column study with the same packing material Karas et al. (2016a) showed that the high water loading of biobeds with fungicide-containing effluents have detrimental effects on the survival and activity of *Pleurotus ostreatus* colonizing the spent mushroom substrate at the onset of the experiment. On the other hand, dominant fungi in the biobed packing material appear to increase in abundance with time. These included *Stachybotrys chartarum*, previously isolated from soil and artificial cellulose-containing materials (Elanskii et al., 2004; Jie et al., 2013), *Immersiella* and *Scutellinia*, both being saprotrophic fungi. The latter has been previously shown to increase in abundance under soil compaction, where air permeability and gas diffusion are reduced (Longepierre et al., 2021), conditions which are expected to prevail at the latter stages of our biobed leaching column study.

Finally, positive and negative correlations between the relative abundance of members of the bacterial and fungal community and pesticide residues in the biobed packing material were identified. Positive correlations between pesticide residues and microbial abundance were hypothesized to signify microorganisms that are either involved in the degradation of the pesticide or not affected by the studied pesticide and they increase to fill voids left by microorganisms sensitive to pesticides. In contrast, negative correlations reflect the response of microorganisms that are sensitive to pesticides. FLD and IMZ residues were positively correlated with bacterial taxa that are known to possess pesticide degrading members, like *Pseudomonadaceae*, *Comamonadaceae*, *Tahibacter*, *Sphingomonas*, *Sphingopyxis* and *Ferrovibrio* (Boon et al., 2001; Lu and Lu, 2018; Bai et al., 2020; Kumar et al., 2021). CHT was positively correlated only with a member of the genus *Longimicrobium*, shown to have a positive correlation with iprodione residues in previous studies (Katsoula et al., 2020).MET-M resulted in enrichment of the packing material with *Cellulosimicrobium* and *Terrimonas*, previously reported as degraders of 2,4,5-trichloro-phenoxyacetic acid (Korobov et al., 2018) and FLD (Mavriou et al., 2021), respectively. MET-M was negatively correlated with *Saprospiraceae*, in line with previous studies which showed that members of the family were also sensitive to exposure to fungicides like carbendazim and tricyclazole (Shi et al., 2019).

Regarding fungi, significant positive correlations between *Rozellomycota*, *Aspergillus*, *Cyphellophora* and many applied fungicides were evident. *Rozellomycota* are commonly detected in anoxic habitats (Grossart et al., 2016) and possess the enzymatic toolbox for lignin degradation (Song et al., 2019). *Cyphellophora europaea* is known to be involved in the degradation of nicotinic acid (vitamin B3) and hydrocarbons (Bokor et al.,

2021; Radwan and Ruiz, 2021), while *Aspergillus* has been reported as degrader of carbendazim, mancozeb and metolachlor (Sanyal and Kulshrestha, 2004; Ahlawat et al., 2010). Other taxa that are positively correlated with fungicides contained in the effluents of FPI include strains able for lignocellulose degradation i.e. *Cephalotrichum* (Zanellati et al., 2020) and opportunistic black yeasts that thrive in hydrocarbon-rich environments, i.e. *Exophiala xenobiotica* (De Hoog et al., 2006; Isola et al., 2013) All three pesticides used in bulb dipping activities were positively correlated with *Trichoderma* genus, whose degradation potential has already been demonstrated against various antibiotics (Manasfi et al., 2020) and pesticides (Katayama and Matsumura, 1993; Tripathi et al., 2013; Sharma et al., 2016; Manasfi et al., 2020). It should be clarified that correlations between the abundance of phylogenetically distinct microbial units and pesticide residues do not constitute absolute proof of the involvement of the specific microorganisms in associated functions (e.g. pesticide degradation), and this should be verified via culture-dependent or shotgun metagenomic and meta-transcriptomic approaches.

4.3 THE DYNAMICS OF MGES IN BIOBEDS

Finally, the dynamics of MGEs known to be involved in the transmission of pesticide degrading genes were determined. It was hypothesized that treatment of biobeds with high pesticide loads will stimulate genetic exchanges between members of the bacterial community towards the evolution and dissemination of novel pesticide degradation pathways (Dealtry et al., 2014). In contrast to our hypothesis, no significant differences in the relative abundance of *Intl1*, *IS*1071 and IncP-1 and IncP-1ε, all known to be associated with the horizontal gene transfer of pesticide-degrading genes (Popowska and Krawczyk-Balska, 2013; Dunon et al., 2018) were observed. Instead, a temporal increase in the abundance of most MGEs tested was evident, regardless of the treatment employed in the different columns. This might be associated with the establishment with time of abiotic and biotic conditions in biobed systems, beyond pesticide-imposed selection pressure, that stimulated genetic exchange between members of the microbial community (Aminov, 2011). Such conditions could be associated with the gradual decomposition of the high organic matter content of the biobed packing material with time that might release nutrients and easily assimilable carbon sources supporting the active metabolic state of bacteria required for horizontal gene transfer (van Elsas and Bailey, 2002; Heuer and Smalla, 2012). Still the exact etiology for the temporal increase in MGE abundance in our biobed systems warrants further research.

5 CONCLUSIONS

Our study showed that biobed systems could effectively remove pesticides from effluents produced by SPIs, BHIs and FPPs, by employing different processes depending on the polarity and biodegradability of the chemicals contained in the effluents. More lipophilic substances were mostly retained by the biobed packing materials while the more polar and biodegradable pesticides were dissipated. Biobeds supported a microbial community whose composition was not affected by pesticide exposure and the pesticide composition of the different agro-industrial effluents used. Instead the microbial community showed clear temporal patterns along the different biobed horizons, an effect most probably driven by

the establishment of microaerophilic conditions upon water saturation of biobeds, facilitating the establishment of anaerobic and facultative anaerobic bacteria. Measurements of selected MGE dynamics in the biobed systems did not concur with our hypothesis for their increasing abundance in biobeds receiving pesticide contaminated effluents comparing to control. Further studies using shotgun metagenomics targeting both genomic and plasmid DNA will provide insights into the role of horizontal gene transfer and MGEs in the evolution of pesticide catabolic pathways.

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Chapter 4

Isolation, characterization and industrial application of a *Cladosporium herbarum* fungal strain able to degrade the fungicide imazalil

The work presented in Chapter 4 is included in the scientific paper:

Papazlatani, Christina V., Maria Kolovou, Elisabeth E. Gkounou, Konstantinos Azis, Zografina Mavriou, Stefanos Testembasis, George S. Karaoglanidis, Spyridon Ntougias, and Dimitrios G. Karpouzas. 2022. "Isolation, Characterization and Industrial Application of a Cladosporium Herbarum Fungal Strain Able to Degrade the Fungicide Imazalil." Environmental Pollution, October, 119030. https://doi.org/10.1016/j.envpol.2022.119030

1 INTRODUCTION

Imazalil ((RS)-1-(β-allyloxy-2,4-dichlorophenethyl)imidazole, IMZ) is a systemic imidazole fungicide used mainly for the post-harvest treatment of pome and citrus fruits. It is also registered for use in protecting tomatoes grown in artificial substrates and as seed treatment on cereals (EFSA, 2010). Its mode of action involves the disruption of the cell membrane function of pathogenic fungi, by inhibiting the demethylation step in the biosynthesis of ergosterol (Khan et al., 2001; Siegel and Ragsdale, 1978). IMZ is persistent in soil and in riverine water/sediment systems with DT_{50} values of 41-135 days and 161-165 days, respectively (EFSA, 2011). It is strongly adsorbed in soil organic matter (K_{Foc} = 4753 – 7942.9 ml g^{-1}) (Karas et al., 2015; EFSA, 2010), which deems it immobile in soil with low risk for groundwater contamination (Karas et al., 2016a; Omirou et al., 2012; EFSA, 2011). Regarding toxicity, IMZ has been listed by the US Environmental Protection Agency as "likely to be carcinogenic to humans" (EPA, 1999), while recent studies in mice suggested that IMZ can act as an endocrine disruptor (Jin et al., 2019) and a hepatotoxic factor (Jin et al., 2018). IMZ is moderately toxic to off-target aquatic organisms, like invertebrates and fish (EFSA, 2011), although other studies have reported considerable toxicity to aquatic micro-invertebrates (Castillo et al., 2006) and zebrafish (Jin et al., 2016). IMZ is not toxic to soil microorganisms (Papadopoulou et al., 2016), but it was toxic to the earthworm *Eisenia andrei* (Pereira et al., 2020).

Postharvest application of IMZ results in the formation of large wastewater volumes of high fungicide concentrations (10 – 50 mg L^{-1}) (Santiago et al., 2018a, 2016). Acknowledging the environmental risk from the release of these wastewaters, the European Commission granted authorization to IMZ under the clause that appropriate waste management treatment processes would be put into place by the users to ensure minimum exposure of natural water resources to IMZ (EFSA, 2010). Pilot systems using advanced oxidation processes, like photo-Fenton (Santiago et al., 2018a, 2016), TiO_2-based photocatalysis (Santiago et al., 2018b, 2013; Jiménez et al., 2013) or ozone treatment (Genena et al., 2011) were evaluated for the removal of IMZ and other fungicides from agro-industrial effluents and showed high efficiency. However, several reasons have hampered the full-scale implementation of these systems including (i) the reduction of the efficiency and shortening of the electrode half-life, due to deposition of organic material on its surface, (ii) the high electric energy requirements and cost of systems construction (iii) the possible use of additional reagents, like electrolytes (Titchou et al., 2021; Sirés et al., 2014) and (iv) the possible production of oxidized pesticide transformation products that are equally or more toxic than the parent compounds (Santiago et al., 2013).

In the absence of treatment systems, fruit-packaging plants (FPPs) discharge their effluents in municipal wastewater treatment systems. However, the generic microbial community of these systems fails to remove IMZ and other persistent fungicides contained in the above agro-industrial wastewaters (Campo et al., 2013). Based on all these, IMZ is now considered a major contaminant of surface water systems in fruit-producing regions of Europe (Fonseca et al., 2019; Ccanccapa et al., 2016; Masiá et al., 2013) and beyond (Castillo et al., 2006). Biobased processes have also been tested for the treatment of these effluents.

Constructed wetlands were studied as a possible method for the removal of IMZ from wastewaters and showed promising results (Lv et al., 2016), although their efficiency was tested at μg L^{-1}, which are far lower than the IMZ levels encountered in wastewaters from FPPs. Omirou et al., (2012) and Karas et al., (2016b) used on-farm biopurification systems for the treatment of these effluents and showed high removal efficiencies, despite the fact that IMZ was not degraded, but it was adsorbed in the packing material of these systems. Considering all the above, it is anticipated that biological treatment systems, based on tailored-made microbial inocula with advanced degradation capacities against the target fungicides, might be an optimum solution for the depuration of these effluents (Perruchon et al., 2017).

Although microbes for the rapid degradation of fungicides contained in effluents from FPPs, like thiabendazole (TBZ), ortho-phenylphenol (OPP) and iprodione (IPR), have been previously isolated (Perruchon et al., 2017, 2016; Campos et al., 2015), no microorganisms with the capacity to effectively degrade IMZ have been reported yet. López-Loveira et al., (2017) first isolated an IMZ-tolerant bacterial consortium from the sludge of a system receiving effluents from a FPP. The consortium was able to degrade up to 50% of 500 mg L^{-1} IMZ in the presence of extra carbon sources. Karas et al., (2011) showed that the white-rot fungus Trametes versicolor was able to degrade 10 mg L^{-1} of IMZ, but failed to degrade a 5-fold higher concentration of the fungicide.

In this frame, we aimed (1) to isolate and identify microorganisms with the capacity to effectively dissipate IMZ, (2) to characterize their degradation capacity against IMZ under variable conditions relevant to their potential application in the treatment of agro-industrial effluents, (3) to assess its efficiency to establish and effectively remove IMZ from wastewaters when used as inoculum in an immobilized cell bioreactor. Considering that the isolated IMZ-degrading microorganism belonged to the genus *Cladosporium* (teleomorph: *Mycosphaerella*), that encompass both pathogenic and saprotrophic members (Crous, 2009), and its potential practical use as starting inoculum in bioreactors established in FPPs, we tested its pathogenicity in citrus and pome fruits.

2 MATERIALS AND METHODS

2.1 CHEMICALS

Analytical standards of IMZ (99.3% Pestanal ®), fludioxonil (FLD) (99.9% Pestanal ®), TBZ (99.3% Pestanal ®), OPP (99.6% Pestanal ®), IPR (99% Pestanal®) and its main derivative 3,5 dichloro-aniline (3,5-DCA) (≥99.7%) were purchased from Fluka/Sigma-Aldrich. Analytical standards were used for the preparation of pesticide stock solutions (1000 mg L^{-1}) in methanol that were utilized for pesticide quantification as described below. Commercial formulations of the pesticides including FUNGAZIL® 500 EC (IMZ), SCHOLAR 230 SC (FLD), TECTO 50% ® SC (TBZ), FOAMER® 20 EC (OPP) and ROVRAL 50 SC (IPR) were used for the preparation of aqueous solutions of the pesticides, which were further used in liquid culture studies. The antimicrobial agents, chloramphenicol and nystatin, were purchased by Sigma-Aldrich. Working solutions of chloramphenicol (25 g L^{-1}) and nystatin (30 g L^{-1}) were prepared in ethanol and DMSO, respectively.

2.2 GROWTH MEDIA

The isolation and routine cultivation of IMZ-degrading microorganisms was performed in (i) a minimal salt medium supplemented with nitrogen, casein hydrolysate (0.15 gr L^{-1}), glucose (0.5% w/v) (MSMN) and (ii) potato dextrose broth (PDB). Both media were amended with IMZ, added as a filter sterilized aqueous solution prepared with the use of the commercial formulation. MSMN was prepared as described in Karpouzas and Walker, (2000a). Solid versions of these media were prepared by adding 15 gr L^{-1} agar. Unless otherwise stated, all liquid cultures were incubated in the dark at 25°C in an orbital shaker at 200 rpm.

2.3 ISOLATION OF IMZ-DEGRADING MICROORGANISMS

A range of substrates, all characterized by heavy exposure to IMZ, were tested as sources for the isolation of IMZ-degrading microorganisms through enrichment cultures. These substrates included (a) a soil from a disposal site receiving effluents from a FPP in Cyprus (IMZ level at sampling was 15.7 μg g^{-1}), (b) sediment from the pipes discharging the wastewater of a FPP in Arta, Greece to a nearby field site (IMZ level at sampling was 10.3 μg g^{-1}), (c) sediment from the drencher of a FPP in Larissa, Greece (IMZ level at sampling was 0.105 μg g^{-1}), (d) soil collected from a disposal site of a FPP in Agia, Larissa, Greece (IMZ levels ranging from 0.6 to 6.4 μg g^{-1}), (e) sludge from municipal aerobic sewage sludge systems in the regions of Tyrnavos and Agia known to receive effluents from FPP. However, it should be noted that at the time of sampling no residues of IMZ were detected in the sludge. Enrichment cultures inoculated with the above matrices did not result in an appreciable degradation of IMZ with the sole exception of soil (a), which was further used for the isolation of IMZ-degrading microorganisms.

To stimulate its IMZ-degrading microbiota, soil (a) was treated with a fresh addition of IMZ (5 μg g^{-1} dry soil) and incubated under aerobic conditions in the dark at 25°C. When more than 50% degradation of IMZ was observed in the soil, 1 g of soil was used for the inoculation of enrichment cultures of MSMN supplemented with 10 mg L^{-1} IMZ as described by Karpouzas et al., (2000b). In total, four enrichment cycles were undertaken. At the point where more than 50% degradation of IMZ in the fourth enrichment cycle had occurred, 150 μl were spread on IMZ-amended (20 mg L^{-1}) MSMN and PDA plates. The latter was included based on preliminary microscopy observations of the prolific growth of fungi in the enrichment cultures. All plates were incubated at 25°C in the dark, for 4 days. Ten and twelve morphologically distinct colonies were picked from MSMN and PDA plates respectively and were inoculated in fresh MSMN and PDB liquid media supplemented with 20 mg L^{-1} IMZ. Duplicate samples per medium were not inoculated to serve as abiotic controls. All cultures were incubated as described above, and the degradation of IMZ was determined at day 7 by HPLC.

2.4 MOLECULAR IDENTIFICATION OF THE IMZ-DEGRADING MICROORGANISM

DNA was extracted from fresh cultures of the isolated fungus in PDB using the CTAB method (Doyle and Doyle, 1987). DNA quality was assessed via electrophoresis and its concentration was determined via Qubit 2.0 fluorometer (Thermo Scientific). DNA from pure cultures was used for the amplification of the ITS genomic region using primers ITS1F and ITS4 (**Supplementary Table 4.S1**) and KAPA Taq polymerase (KapaBiosystems, Wilmington, Massachusetts). The amplification product was subsequently purified (Nucleospin II clean-up kit, Macherey-Nagel, Germany), cloned into the pGEM-T® Easy plasmid vector system (Promega, Madison, USA) and transformed into *Escherichia coli* DH5a competent cells, following standard procedures (Sambrook and Green, 2012). White colonies of the transformed bacteria were selected for plasmid extraction with the NucleoSpin Plasmid kit (Macherey-Nagel, Germany) and were sequenced via the Sanger method in Cemia S.A., Larissa, Greece. Sequences were aligned in UNITE database, version 8.0 (Nilsson et al., 2019), with mothur v.1.42.3 (Schloss et al., 2009). Moreover, obtained sequences were aligned against selected fungal phyla with MUSCLE (Edgar, 2004) and the alignment was further edited by Gblocks (Castresana, 2000) before phylogenetic analysis. A phylogenetic tree was prepared with phyML, version 3.1 (Guindon et al., 2010), using the General Time Reversible (GTR) model with 100 bootstrap analysis.

2.5 VERIFICATION OF THE ROLE OF THE FUNGUS IN THE DEGRADATION OF IMZ

To confirm the key role of the fungal isolate on the degradation of IMZ, we assessed the degradation capacity of the isolated fungus in the presence of the antifungal agent nystatin and the antibacterial agent chloramphenicol. For this purpose, 15 liquid cultures of the IMZ-degrading microorganism were prepared in PDB and they were amended with aqueous solutions of IMZ, aiming to a nominal fungicide concentration of 50 mg L^{-1}. Triplicate cultures were amended with DMSO and ethanol solutions of nystatin and chloramphenicol respectively, aiming to nominal concentrations of 50 and 100 mg L^{-1}, or remained untreated. In addition, duplicate cultures received the same amount of DMSO or ethanol without antimicrobial agents to consider possible negative effects of the solvents in the degradation capacity of the isolate. The media were inoculated with 2 x 10^3 conidia mL^{-1} collected from a fresh fungal culture. Abiotic controls without fungal addition were prepared for all tested treatments. IMZ concentration in liquid cultures was determined regularly via HPLC-PDA.

2.6 TESTING THE PATHOGENICITY OF THE IMZ-DEGRADIND FUNGUS ON FRUITS

A prerequisite for any further practical application of the IMZ-degrading fungus in the depuration of agro-industrial effluents is the verification that it carries no potential pathogenicity against fruits treated by fruit-packaging plants. Hence, we examined the potential of the isolated fungal strain to infect and cause disease in apple (cv. 'Fuji'), pear

(cv. 'Crystalli') and orange (cv 'Arta') fruits. In total, 24 apple, 24 orange and 19 pear fruits were surface-sterilized by immersing for 2 min in a 70% EtOH solution. All fruits were further rinsed with sterile distilled water, placed in plastic containers, lined with wet paper towels. Fungal conidia were collected from an active culture of the fungus on PDA using a sterile cotton swab and suspended in 2 ml sterile distilled water solution containing 0.1% (v/v) Tween 80 (PanReac Applichem, Germany). Spore suspension's density was adjusted to 2 x 10^6 conidia mL^{-1}, using a hemocytometer (Neubauer, Hamburg, Germany). Each fruit was subjected to two inoculation methods, with and without wounding. Wounds (3 mm in depth) were created on the surface of half of the fruits from each category, using a sterile needle. Fifty (50) µL of conidial suspension were transferred on the wound or unscathed surface. The containers with the fruits were covered with lids, to ensure high relative humidity (RH>90%) and incubated at 22^oC for 7 days. At the end of the incubation period, disease incidence (%) was measured by counting the number of infected fruits. Pathogenicity was confirmed by re-culturing on PDA plates.

2.7 CHARACTERIZATION OF THE DEGRADATION POTENTIAL OF THE IMZ-DEGRADING FUNGUS

2.7.1 INOCULUM PREPARATION

All liquid culture experiments described below were inoculated with conidia, harvested from growing fungal cultures on solidified or liquid media. Briefly, agar plates were inoculated with 700 µL of a fresh growing liquid culture of the fungus. The plates were then incubated at 25^oC, in the dark for 2-3 weeks. Conidia were collected from plates with sterile 0.85% NaCl, pelleted by centrifugation at 8,000 rpm, 16^oC for 30 min, counted using a hemocytometer and their concentration was adjusted to the desired levels with addition of sterile 0.85% NaCl (Skiada et al., 2019).

2.7.2 ASSESSMENT OF THE GROWTH AND DEGRADATION CAPACITY OF THE FUNGAL ISOLATE AT INCREASING IMZ CONCENTRATIONS

The ability of the fungal isolate to grow and degrade IMZ was evaluated under increasing concentrations of the fungicide in both PDB (nutrient rich) and MSMN (minimal) growth media. Liquid cultures were amended with appropriate volumes of aqueous solutions of IMZ prepared from its commercial formulation, in order to achieve final concentrations of 0, 20, 50 and 100 mg L^{-1}. For each tested concentration, 12 liquid cultures were prepared, by inoculation with approximately 2 x 10^3 conidia mL^{-1}. Duplicate non-inoculated controls per medium were also included to determine the abiotic degradation of IMZ. All cultures were incubated at 25^oC and 160 rpm in the dark. At four different time points after the onset of the experiment (**Table 4.1**), three flasks were removed from incubation and used for (i) the determination of IMZ concentration by HPLC-PDA and (ii) the measurement of fungal growth by determination of the dry weight of the fungal biomass.

Table 4.1 Time after inoculation when determination of IMZ concentration and fungal growth took place.

Growth medium	IMZ concentration (mg L^{-1})	Days after inoculation
MSMN	0	3, 7, 10, 14
	20	3, 5, 7, 10
	50	3, 7, 10, 14
	100	7, 14, 21, 28
PDB	0	3, 7, 10, 14
	20	3, 4, 5, 7
	50	3, 7, 10, 14
	100	7, 14, 21, 28

2.7.3 ASSESSMENT OF THE CAPACITY OF THE FUNGUS TO DEGRADE OTHER FUNGICIDES

We further tested the capacity of the isolated fungus to degrade the fungicides FLD, TBZ, OPP, IPR and its persistent transformation product 3,5-DCA. These compounds are used along with IMZ in the post-harvest treatment of fruits and they are expected to co-occur with IMZ in the wastewaters of the fruit-packaging plants. The biodegradation of the abovementioned fungicides was monitored in MSMN amended with appropriate volumes of aqueous solutions of the fungicides, aiming at final concentrations of 50 mg L^{-1}. For each pesticide, three cultures were inoculated with *ca.* 10^6 conidia mL^{-1}, while two further cultures were not inoculated to serve as abiotic controls. All cultures were incubated in the dark at 25°C, 160 rpm. The degradation of the tested fungicides was determined by regular measurement of their concentration in the liquid cultures.

2.7.4 DEGRADATION DATA ANALYSIS

The degradation rates of IMZ were calculated with the single first order (SFO) kinetic model and the biphasic Hockey-Stick (HS) model based on the recommendations of the FOCUS workgroup on pesticide degradation kinetics (FOCUS, 2006). The model showing the best fit to the degradation data was selected based on the χ^2 test (χ^2 value < 15) and visual inspection of the distribution of the residuals compared to the observed data. The analysis was carried out in the R Studio version 4.0.0 (R Core Team, 2020), utilizing the package mkin version 0.9.50.2 (Ranke, 2019).

2.7.5 FUNGAL GROWTH MEASUREMENTS AND KINETICS

The fungal biomass collected at various time points by filtration through cheesecloth, was air dried at 70°C for approximately 16 h and the dry weight was determined. The exponential growth rate was deduced from the growth curve and was described by the equation:

$$\mu x = \frac{dx}{dt}$$

2.7.6 STATISTICAL ANALYSIS

Pairwise comparison analysis between the different degradation coefficients and growth rates, were carried out with Welch's t-test, which assumes the variances to be unequal. The linear correlation among the concentration of IMZ and fungal biomass for every

experimental treatment was assessed through estimation of Pearson's correlation coefficient.

2.8 PERFORMANCE OF THE IMZ-DEGRADING FUNGUS IN A BIOREACTOR SYSTEM

2.8.1 BIOREACTOR SETUP

We eventually evaluated the capacity of the isolate to effectively decontaminate agro-industrial effluents that contained IMZ under bioreactor conditions. An immobilized cell bioreactor of 550 mL volume was set up, containing a 150 ml column of porous glass beads. Adequate oxygen supply, i.e. 4 mg L^{-1}, was provided to the immobilized biomass by inserting an air diffuser on the upper part of the bioreactor and recirculating the effluent in an upflow manner (Mavriou et al., 2021). The bioreactor was inoculated with 50 mL of 4.8 x 10^4 conidia mL^{-1} of the fungal strain and operated under a constant hydraulic retention time (HRT) of 10 days. The biosystem was fed with synthetic wastewater containing 200 mg L^{-1} IMZ and a salt and trace elements solution (Mavriou et al., 2021) for an operating period of 105 days.

2.8.2 MEASURMENT OF THE PHYSICOCHEMICAL PROPERTIES OF THE BIOREACTOR SYSTEM

During bioreactor operation, influent and effluent physicochemical characteristics were determined at various time-points. Electrical conductivity (EC) and pH were determined with a Crison CM35 probe and a HANNA HI 98191 meter, respectively. Chemical oxygen demand (COD) concentrations were measured based on the "Standard Methods for the Examination of Water and Wastewater" (Clesceri et al., 1998). Total Kjeldahl nitrogen (TKN) was estimated after acid digestion, ammonia distillation and titration of ammonium nitrogen (NH_4^+-N) content (Clesceri et al., 1998). A column containing Cd-copperized granules was used to achieve nitrates reduction to nitrites, which were further measured spectrophotometrically at 453 nm using sulfanilamide/(1-naphthyl)ethylenediamine-dihydrochloride as indicator (Clesceri et al., 1998). The concentration of IMZ in the influent and effluent were determined in a HPLC-PDA system (ECOM, Czech Republic) equipped with a 5 UniverSil C18 250 × 4.6 mm column (Fortis, UK). IMZ was eluted isocratically with a mobile phase of 75/25, v/v acetonitrile/H_2O at flow rate of 0.8 mL min^{-1}.

2.8.3 DETERMINATION OF MICROBIAL COMPOSITION AND DYNAMICS IN THE BIOREACTOR

Beyond physicochemical traits, we determined the succession of the microbial community colonizing the bioreactor system and further investigated the capacity of the fungal inoculum to colonize and establish itself in the immobilized cell bioreactor. The abundance of total bacteria and fungi along the bioreactor operation was determined via q-PCR. For the total fungal abundance, the 18S rRNA gene was amplified with the primer set FF390-FR1 using a SYBR Green protocol (**Supplementary Table 4.S1**). For the total bacterial abundance, the 16S rRNA gene was amplified with the primers Eub338 and Eub518, following a SYBR Green protocol (**Supplementary Table 4.S1**).

The composition of the microbial community was determined at samples collected at 3, 24, 46 and 68 days after inoculation. These time points were selected in order to examine the start-up period needed for the adaption of the microbial biomass in the bioreactor which was achieved with three to five HRTs. After this time the bioreactor was operating under steady state conditions. DNA extraction from Siran beads was performed after freezing and pestling to dust, using the NucleoSpin Tissue kit (Macherey-Nagel, Germany), following the manufacturer's protocol. A lyticase step was included, where 100 U lyticase were added in each treated sample. The fungal community composition was determined via sequencing of the ITS genomic region with the primer set P-ITS1- P-ITS4 with a HotStarTaq Plus Master Mix kit (Qiagen) (**Supplementary Table 4.S1**) and amplicon sequencing was performed in Miseq Illumina 2x300bp paired-end platform at "Mr DNA" (USA). The bacterial community composition was determined via amplification of the V4 region of the 16S rRNA gene with the primers 515f and 806r with Q5 High Fidelity DNA polymerase (New England Biolabs) (**Supplementary Table 4.S1**), where the PCR reaction was performed for 28 cycles, followed by a second PCR for 7 cycles with barcoded primers for multiplexing the amplicons, and sequences were obtained via HiSeq Illumina Rapid Mode 2x250bp paired-end reads (Admira health, New Jersey). More details about our amplicon sequencing analysis are provided in Vasileiadis et al. (2018).

2.8.4 DATA ANALYSIS

Sequence pre-analysis consisted of de-multiplexing with Flexbar, version 3.0.3 (Dodt et al., 2012). Consequently, the dada2 version 1.18.0 (Callahan et al., 2016a) package of the R software (R Core Team, 2020) was used for quality control, sequencing errors, PCR introduced chimeras, and the generation of the final ASV matrices as previously suggested (Callahan et al., 2016b). UNITE general fasta release version 8.2 (Abarenkov et al., 2020) and Silva SSU taxonomic dataset version 138 (McLaren, 2020) formatted for dada2 were used for classification of the ITS and 16S rRNA amplicons, respectively.

The microbiome package v1.12.0 (Lahti and Shetty, 2012) of the R software was used to calculate measures of α-diversity, like the Shannon diversity index (Spellerberg and Fedor, 2003), the inversed Simpson index (Hill, 1973), the observed richness (*S*) and the Pielou's evenness index (Pielou, 1966).

2.9 FUNGICIDE RESIDUE ANALYSIS

2.9.1 FUNGICIDE EXTRACTION FROM LIQUID CULTURES

Fungicides were extracted from growth media with a liquid-liquid extraction method. Briefly, for IMZ and FLD, 200 µL of the liquid culture were mixed with 800 µL methanol. The mixture was vortexed for 10 sec and centrifuged for 5 min, at 13,300 rpm at room temperature and the supernatant was used for HPLC analysis. For TBZ and OPP, we followed the protocols described by Perruchon et al., (2017) and Perruchon et al., (2016), respectively, whereas for IPR and 3.5-DCA, the protocol described by Campos et al., (2017) was followed.

2.9.2 HPLC ANALYSIS

All extracts were analyzed in a Shimatzu HPLC-DAD system equipped with an Athena C18 column (150mm × 4.6 mm) (ANPEL Laboratory Technologies) with a flow rate of 1 mL min^{-1}. IMZ was detected at 204 nm, with a retention time of 5.1 min, using a mobile phase of methanol : water at 80 : 20 v/v with 0.25% ammonia solution. FLD, IPR and 3.5 DCA were detected at 207, 220 and 220 nm respectively, with a retention time at 5.6, 4.3 and 3.7 min respectively, using mobile phases of 70:30 v:v methanol:water for FLD and 70:30 v:v acetonitrole:water for IPR and 3,5 DCA. TBZ and OPP were detected at 210 and 245 nm, using a mobile phase of acetonitrile:water:25% NH_3 solution at volumetric ratios of 25:74:1 for TBZ and 60:39.5:0.5 for OPP. Under these chromatographic conditions, the retention times were 7.4 and 4.6 min, respectively.

3 RESULTS

3.1 ISOLATION AND IDENTIFICATION OF THE IMZ-DEGRADING FUNGUS

Enrichment cultures showed a rapid degradation of IMZ in both media. Selected colonies tested in the corresponding IMZ amended media revealed that only one of the selected strains, a fungal strain forming velvety, olivaceous green colonies, was able to degrade IMZ within 7 days. Pure cultures in PDB verified the rapid degradation and growth of the fungal isolate. Sequencing of the ITS region showed that the isolated strain clustered within the genus *Cladosporium* showing highest homology to *Cladosporium herbarum*, and its teleomorph *Mycosphaerella tassiana* (bootstrap value: 84) (**Figure 4.1**).

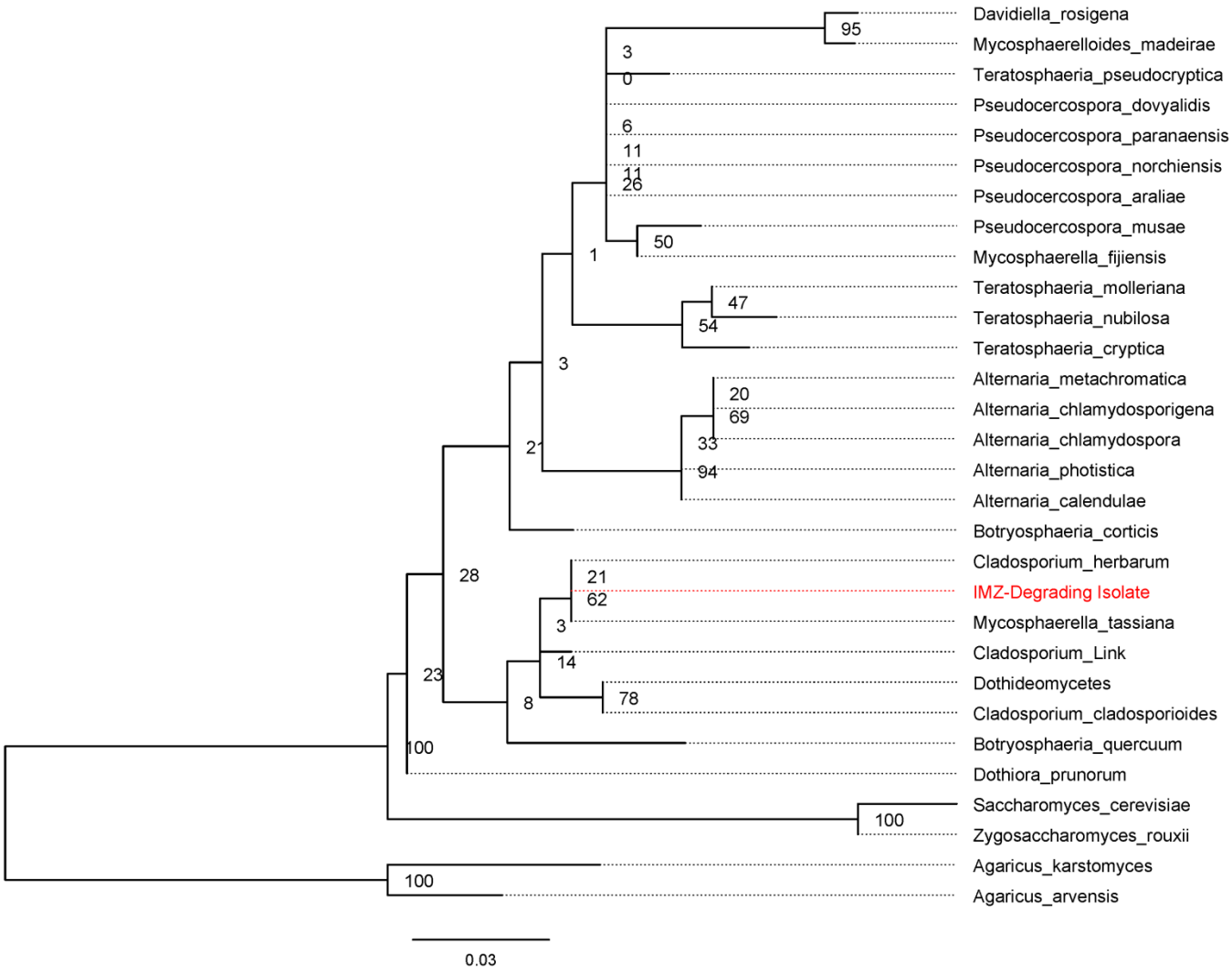

Figure 4.1 Phylogenetic relationships of the fungus isolated in the current study with other closely related species, after performing muscle alignment (Edgar, 2004), and estimating maximum likelihood phylogenies with phyML (Guindon et al., 2005).

3.2 VERIFICATION OF THE DIRECT INVOLVEMENT OF THE FUNGAL ISOALTE IN THE DEGRADATION OF IMZ

We further checked the hypothesis that the fungal isolate was the sole degrader of IMZ or if there was involvement of bacterial strains that were not removed during the various purification steps. To test this, we determined the degradation of the fungicide by *C. herbarum* in liquid cultures in the presence of nystatin (fungicide) or chloramphenicol (bactericide). In the presence of nystatin, a total inhibition of IMZ degradation was observed (K_{deg} = 2e-12 mg IMZ d^{-1}) (**Figure 4.2**), compared to chloramphenicol, whose presence did not significantly alter the biodegradation rate of IMZ (K_{deg} = 0.14 mg IMZ d^{-1}) compared to the control, which was not amended with any biocide (K_{deg} = 0.16 mg IMZ d^{-1}) (**Figure 4.2**). These results verified that the main degrader of IMZ was *C. herbarum* and negated the role of potential bacterial contaminants.

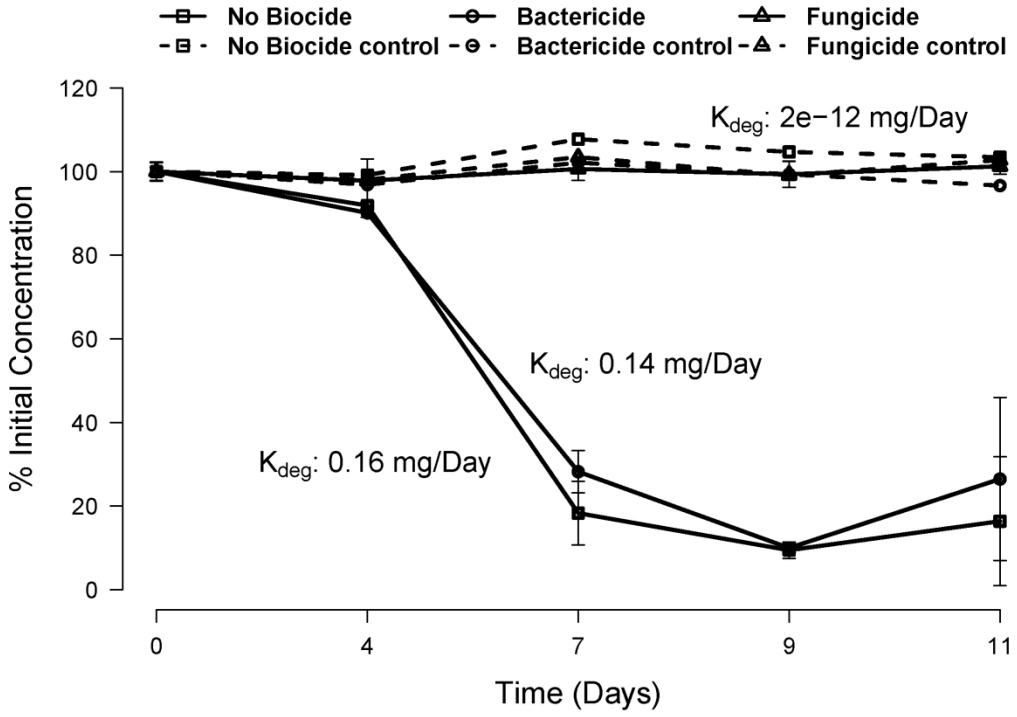

Figure 4.2 The degradation of imazalil (IMZ) by *Cladosporium herbarum* (solid lines) and in the abiotic control (dashed lines) in MSMN supplemented with nystatin (fungicide, red), chloramphenicol (bactericide, green) or in the absence of any biocide (no biocide, blue). Each value is the mean of three replicates with error bars representing the standard deviation of the mean. The degradation rates of IMZ in the different treatments, as calculated by fitting the data to the single first order kinetic model, are also shown.

3.3 PATHOGENICITY TESTS

Species of the genus *Cladosporium* and its teleomorph *Mycosphaerella* have adopted various lifestyles from saprotrophic to plant pathogenic (Crous, 2009). With this in mind and based on our intention to utilize this fungus as microbial inoculum in fruit-packaging plants (FPPs) wastewater treatment systems, we assessed the pathogenicity of our fungal isolate against fruits commonly processed by fruit-packaging plants, i.e. apples, pears and oranges. Artificially inoculated apples and orange fruit showed no disease symptoms (**Figure 4.3**). Regarding pears, artificial inoculations resulted in rot symptoms appearance at an incidence of 10% and 55% when the surface was unscathed or wounded, respectively. Lesion diameter on symptomatic fruit ranged from 1 to 1.7 cm. However, in all the above cases, the fungus that was recovered from the lesions after cultivation in PDA plates was identified as *Penicillium* sp.

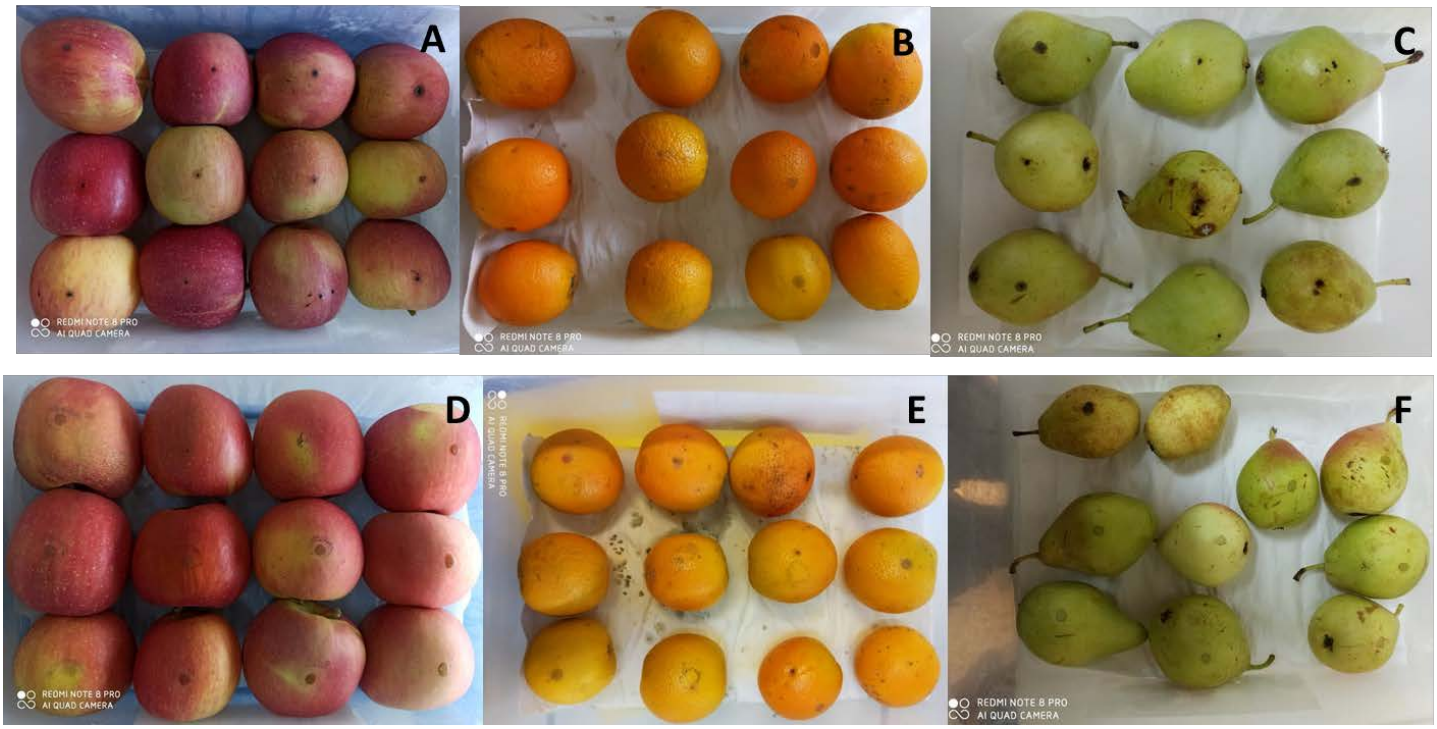

Figure 4.3 Artificially inoculated apple (cv. Fuji), orange (cv Arta) and pear (cv Crystallia) fruit with the fungus *Cladosporium herbarum*, 10 days after inoculation. Panels A, B and C show fruits that were wounded prior to inoculation, whereas panels D, E and F show fruits that were not wounded prior to inoculation.

3.4 CHARACTERIZATION OF THE BIODEGRADATION POTENTIAL OF THE IMZ-DEGRADING FUNGUS

3.4.1 ASSESSMENT OF THE GROWTH AND DEGRADATION CAPACITY OF THE ISOLATE AT INCREASING IMZ CONCENTRATIONS

The degradation of IMZ was best described by the SFO kinetic model in all tested conditions. The degradation rate of IMZ showed a dose – dependent pattern that was decreasing with increasing IMZ concentration and ranged from 0.032 to 0.153 mg IMZ d^{-1} in MSMN and from 0.074 to 0.145 mg IMZ d^{-1} in PDB (**Table 4.2**, **Figure 4.4**). This dose-dependent pattern in the degradation rates of IMZ was statistically significant only in MSMN.

In accordance with the decreasing degradation rates at higher concentration levels, we observed a clear dose-dependent decrease in fungal growth, as determined by the fungal biomass produced, with increasing concentrations of IMZ (**Figure 4.4**). In MSMN, we observed a significant ($p<0.05$) decrease in the growth rates of the fungus from 0.012 g d^{-1} when cultured in absence of IMZ to 0.007 and 0.003 g d^{-1} when cultured in the presence of 50 and 100 mg L^{-1} IMZ, respectively Whereas a significant rise in fungal growth rates were observed (0.016 g d^{-1}) when cultured in the presence of 20 mg L^{-1} of IMZ (**Table 4.2**). In PDB, this effect was still evident with the growth rates of the fungus being reduced from 2.37 g d^{-1} in the absence of IMZ to 0.203 ($p<0.05$), 0.134 ($p<0.05$) and 0.035 ($p<0.05$) g d^{-1} when the fungus was grown in the presence of 20, 50 and 100 mg L^{-1} of IMZ, respectively (**Table 4.2**). Correlation analysis showed a strong negative correlation between IMZ concentration and fungal biomass in MSMN ($r = -0.93$, $p<0.05$) and a weaker, but still significant effect in PDB ($r = -0.42$, $p<0.05$).

Table 4.2 The degradation rates of imazalil IMZ and the growth rates of Cladosporium herbarum in MSMN and PDB at increasing concentrations of the fungicide.

IMZ treatment	Degradation rate (mg IMZ d^{-1})	Growth rate (g d^{-1})
MSMN		
0 mg L^{-1}		0.012 ± 0.001
20 mg L^{-1}	0.153	0.016 ± 0.001
50 mg L^{-1}	0.092	0.007 ± 0.001
100 mg L^{-1}	0.032	0.003 ± 0.000
PDB		
0 mg L^{-1}		2.370 ± 0.350
20 mg L^{-1}	0.100	0.203 ± 0.024
50 mg L^{-1}	0.145	0.134 ± 0.083
100 mg L^{-1}	0.074	0.035 ± 0.000

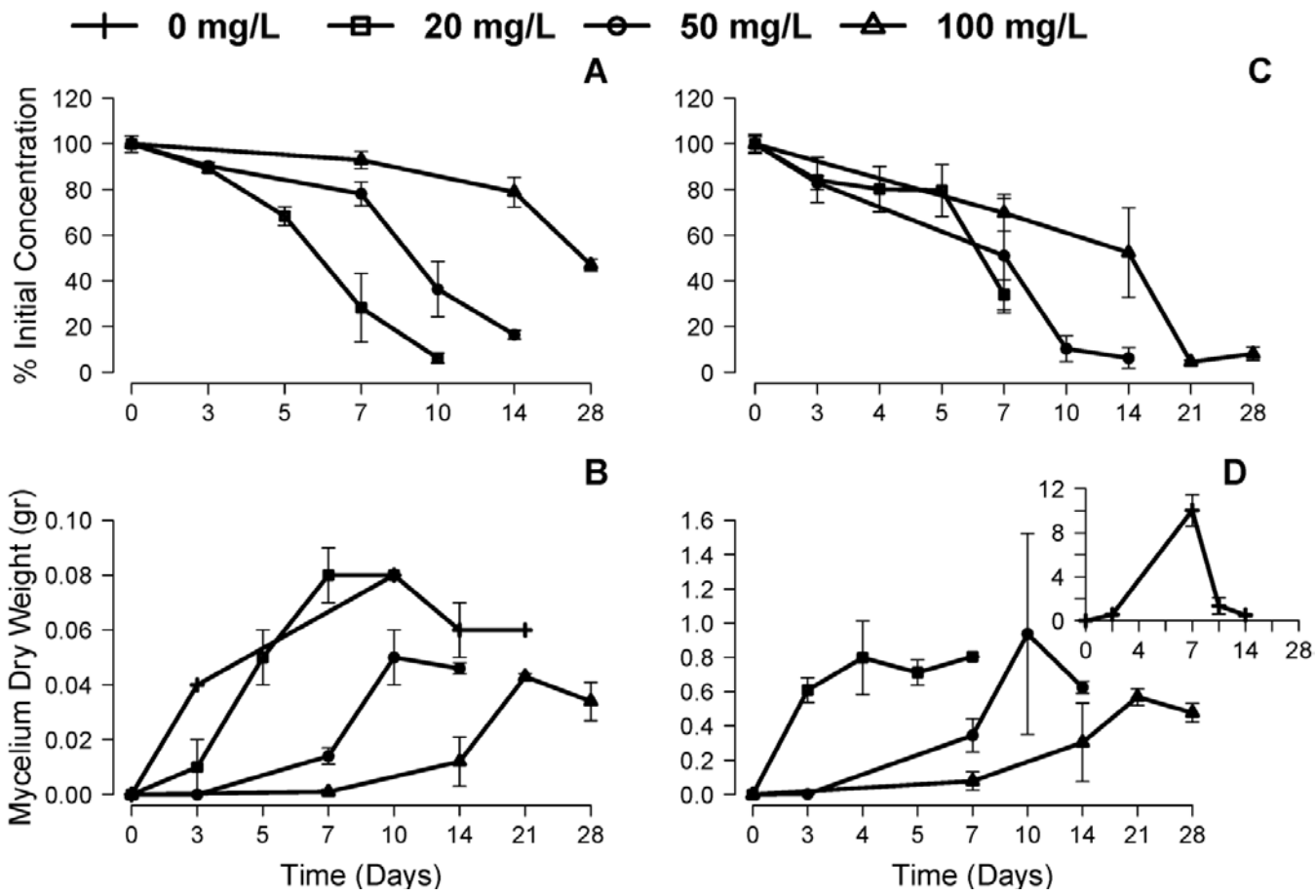

Figure 4.4 The degradation of increasing concentrations of imazalil (IMZ) (A and C) and estimation of fungal growth by measuring the dry weight of mycelial biomass produced (B and D) in MSMN (A and B) and PDB (C and D). The graph in panel (D) shows the growth of the fungus in PDB in the absence of IMZ.

3.4.2 ASSESSMENT OF THE CAPACITY OF THE ISOLATE TO DEGRADE OTHER FUNGICIDES

We determined the capacity of the fungal strain to degrade other fungicides used in FPPs that are, hence, expected to co-occur with IMZ in the relevant wastewaters. The fungus failed to degrade OPP (**Figure 4.5**), whereas it degraded partially or fully the other fungicides tested. *C. herbarum* was able to partially degrade FLD and TBZ, with degradation rates of 0.0062 mg d^{-1} (SFO model) and 0.33 mg d^{-1}/0.0067 mg d^{-1} (HS model) respectively (**Table 4.3**). The degradation of FLD and TBZ in the abiotic controls was negligible. Regarding iprodione (IPR), we observed similar degradation rates in the inoculated samples (K_{deg} = 0.109 mg d^{-1}) and the abiotic control samples (K_{deg} = 0.134 mg d^{-1}), suggesting that the degradation of IPR under the conditions employed was mostly abiotic (**Table 4.3**). In parallel, we followed the formation and degradation of 3,5 DCA, the major transformation product of IPR, in liquid cultures. Interestingly, we observed a significantly higher degradation rate of 3,5-DCA (k_{deg} = 0.687 mg d^{-1}) in the inoculated cultures compared to non-inoculated controls (k_{deg} = 0.079 mg d^{-1}).

Table 4.3 The degradation rates of the various fungicides treated with Cladosporium herbarum in MSMN, as calculated by fitting the single first order (SFO) or the Hockey Stick (HS) model.

Fungicides	Model fitted	Degradation rates (mg d^{-1})
Imazalil (IMZ)	SFO	0.106
Fludioxonil (FLD)	SFO	0.0062
Thiabendazole (TBZ)	HS	0.330/0.0067[a]
Ortho-phenylphenol (OPP)	SFO	0.00057
Iprodione (IPR)	SFO	0.109
3,5-Dichloroaniline (3,5-DCA)	SFO	0.687

[a] k_1 = 0.330 mg d^{-1} and k_2 = 0.0067 mg d^{-1}

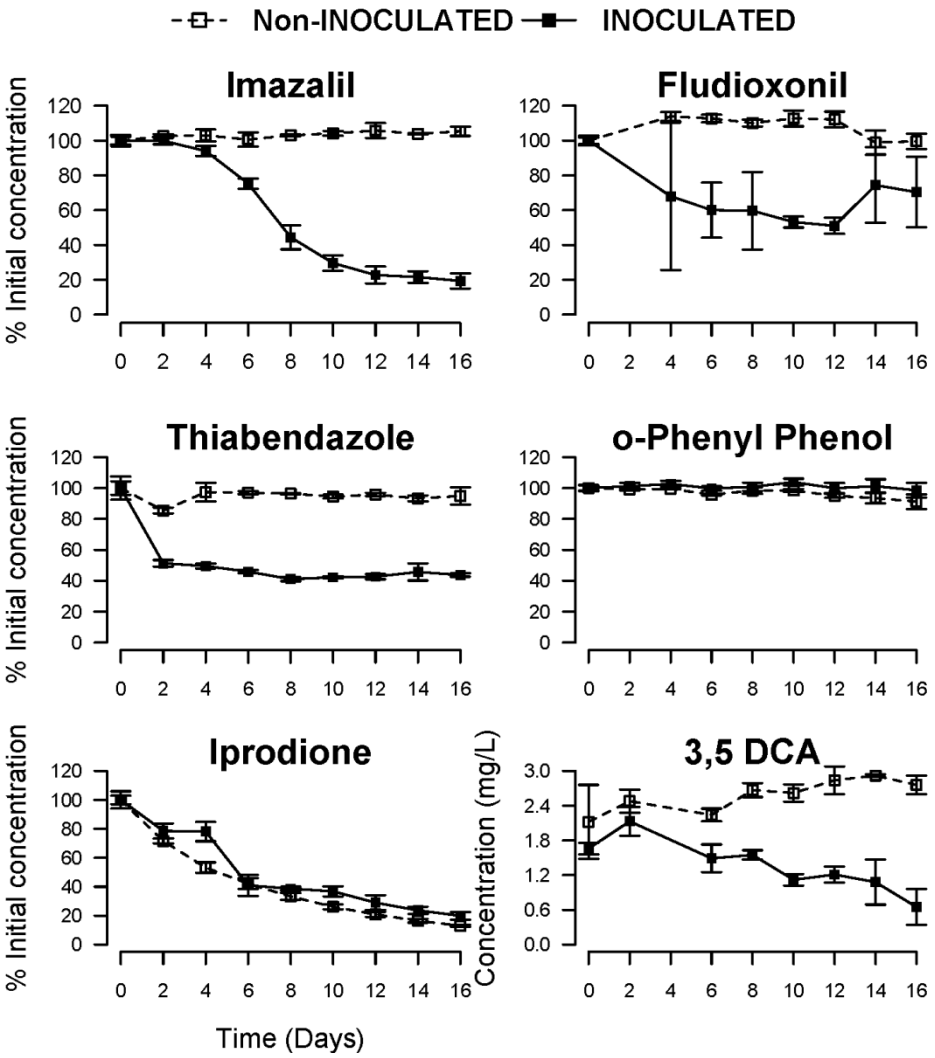

Figure 4.5 The degradation of imazalil (IMZ), fludioxonil (FLD), thiabendazole (TBZ), ortho-phenylphenol (OPP), iprodione (IPR) and 3,5-dichloraniline (3,5-DCA) in MSMN either inoculated (solid line, black square) or not inoculated (dashed line and empty squares) with the IMZ-degrading fungus Cladosporium herbarum. Each value is the mean of triplicates with error bars representing the standard deviation.

3.5 PERFORMANCE OF THE IMZ-DEGRADING FUNGUS IN A BIOREACTOR SYSTEM

3.5.1 BIOREACTOR PERFORMANCE – PHYSICOCHEMICAL MEASUREMENTS

The concentration of IMZ decreased during the operation of the immobilized cell bioreactor, from 200 mg L^{-1} in the influent to 8.19 ± 0.79 mg L^{-1} in the effluent, with an average removal efficiency above 96% (**Figure 4.6A**). pH was increased during the process from 6.94 ± 0.03 in the inflow to 7.37 ± 0.03 in the outflow (**Figure 4.6B**). Influent COD was 657 ± 53 mg L^{-1} (**Figure 4.6C**), while effluent total and soluble COD (tCOD and sCOD) averaged at 116 ± 3 and 103 ± 3 mg L^{-1}, respectively, representing a tCOD removal efficiency of 81.7 ± 0.6%. EC in the influent was 3.09 ± 0.01 mS cm^{-1} compared to the effluent, where it gradually rose to 3.69 ± 0.03 mS cm^{-1} during the startup stage and stabilized to this value thereafter (**Figure 4.6D**). TKN in the influent was 18.13 ± 0.26 mg L^{-1}, while the average TKN in the effluent reached to 3.00 ± 0.30 mg L^{-1} (**Figure 4.6E**), corresponding to TKN removal efficiency of 83.60 ± 1.50%. Negligible concentrations of NH_4^+-N and NO_3^--N, i.e. 0.25 ± 0.06 and 0.12 ± 0.02 mg L^{-1} respectively, were determined in the effluent, whereas no NO_2^--N was detected (**Figure 4.6E**)

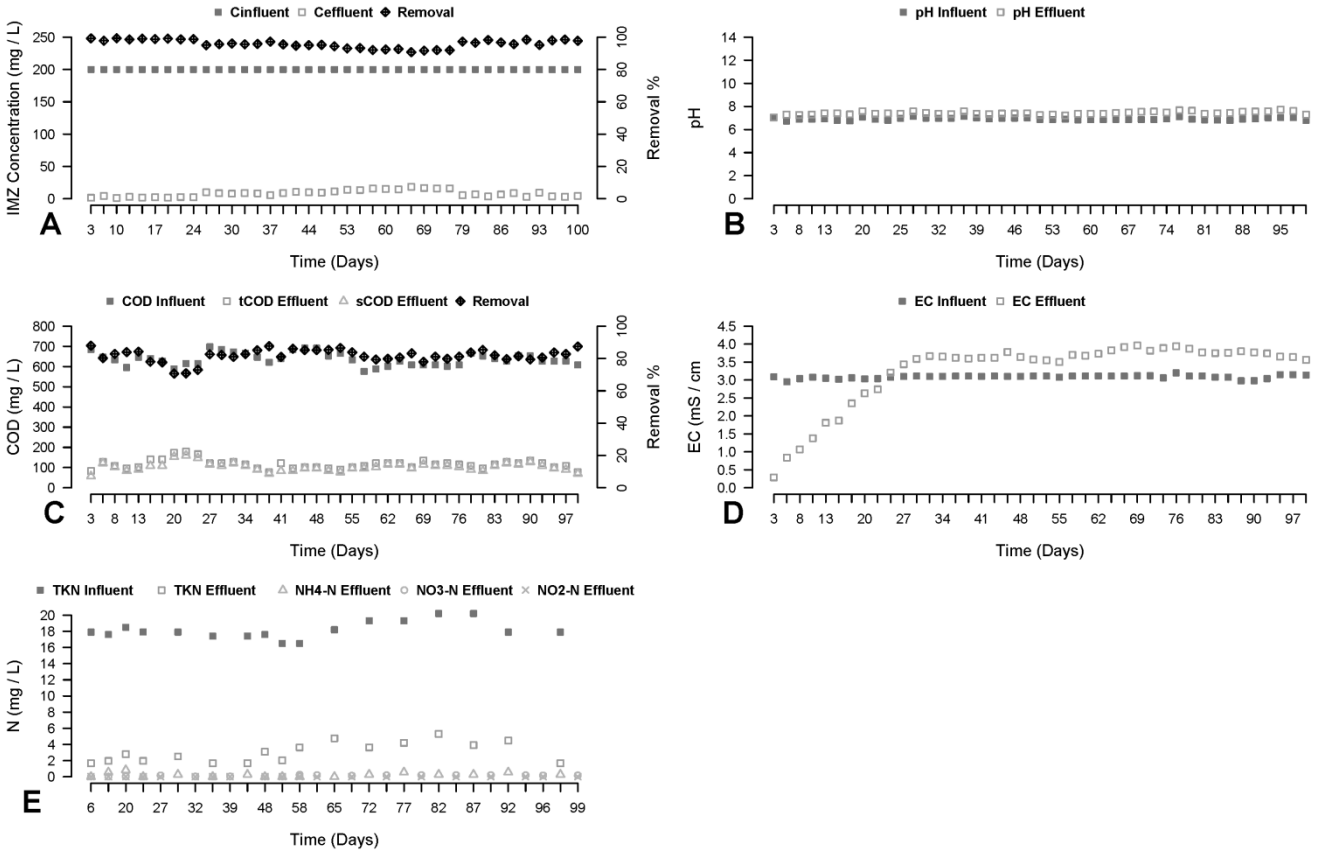

Figure 4.6 The performance of the immobilized cell bioreactor inoculated with the imazalil (IMZ)-degrading fungal strain Cladosporium herbarum. (A) the concentration of IMZ in the influent and the effluent and the removal efficiency of the biosystem, (B) the pH in the influent and the effluent, (C) the total and soluble COD (tCOD, sCOD) concentrations in the influent and the effluent, (D) the Electrical Conductivity (EC) in the influent and the effluent and (E) the levels of TKN in the influent and the effluent, and the concentrations of NH4+-N, NO2--N and NO3--N in the effluent.

3.5.2 MICROBIAL COMMUNITY ANALYSIS IN THE BIOREACTOR

We first followed the dynamics of fungi and bacteria in the bioreactor via q-PCR. Fungal and bacterial numbers showed an initial peak at the onset of the process (3 days) reaching to 1.2 x 10^5 and 1.2 x 10^6 gene copies per ng DNA and declined thereafter to 2.1 x 10^4 and 3 x10^5 respectively (**Figure 4.7a**). We further looked at the relevant contribution of bacteria and fungi in the microbial community and we noted a dominance of bacteria throughout the process with the ratio of 18S rRNA to 16S rRNA copies being less than 0.2 at all-time points **(Figure A3b**). The ratio displayed a significant increase ($p < 0.05$) until day 46 and return to the initial levels by day 68.

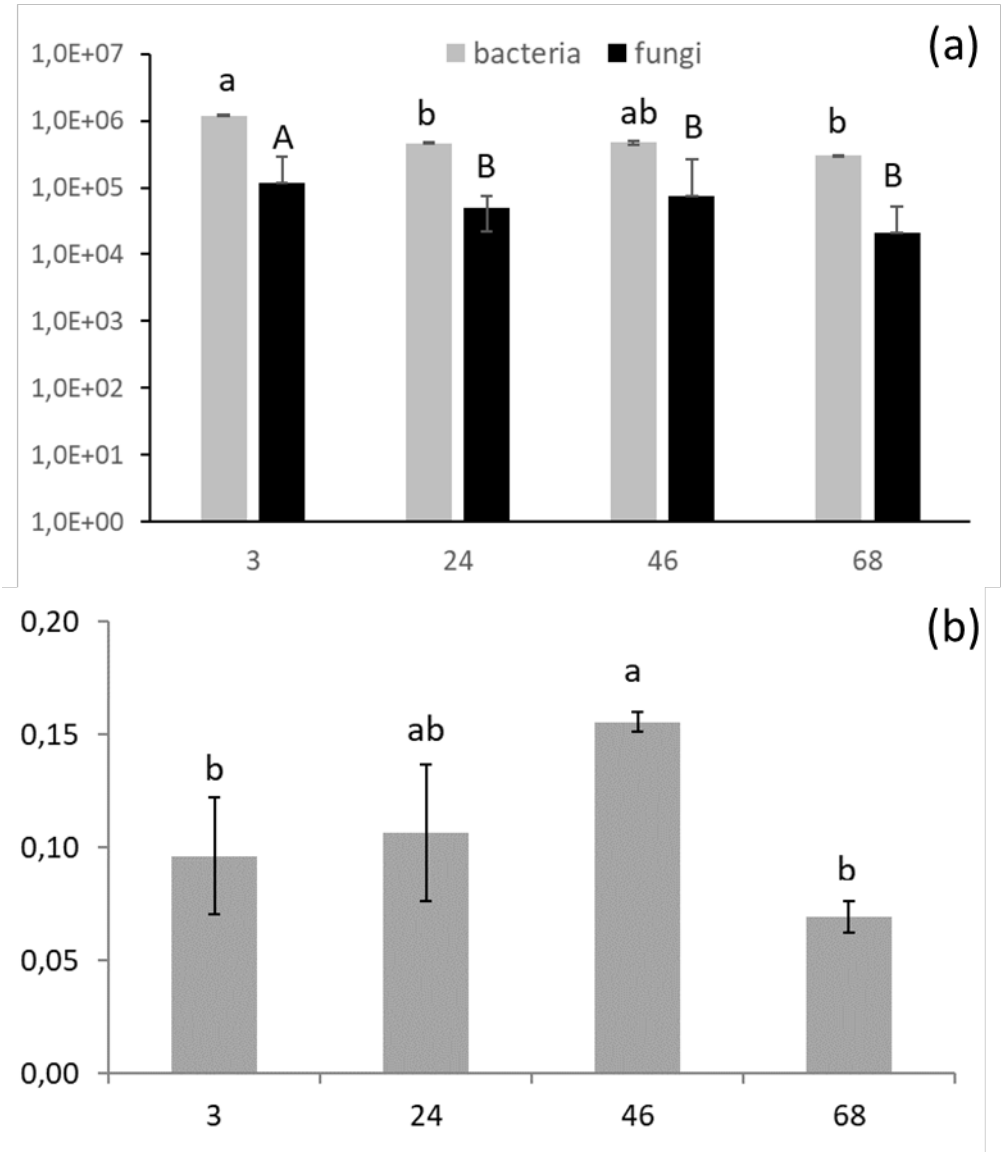

Figure 4.7 (a) The dynamics of fungi and bacteria in the immobilized cell bioreactor inoculated with the IMZ-degrading strain Cladosporium herbarum as determined by q-PCR analysis of the 18S rRNA and 16S rRNA gene, respectively. Within each microbial group, bars designated by the same letter are not significantly different at the 5% level, (b) The ratio of the copy numbers of the fungal 18S rRNA and bacterial 16S rRNA genes is also presented. Bars designated by the same letter are not significantly different at the 5% level.

We subsequently monitored microbial succession in the bioreactor using amplicon sequencing. The mean values of α-diversity indices like Shannon and inverse Simpson for the fungal community were 0.656 and 1.314 respectively (**Table 4.4**), suggesting the establishment of a fungal community with limited diversity. Comparison of the α-diversity values for the fungal community at the different time points suggested no statistically significant differences between the various time points examined. Pielou's evenness values varied from 0.175 ± 0.0167 to 0.216 ± 0.0185 suggesting the establishment of a greatly uneven fungal microbial community composed of dominant or rare species. Further investigation of the relative abundance of the most dominant ASV in each sample showed values of 85.5 to 88.8 % that did not significantly differ between time points (**Table 4.4**). Analysis of the composition of the fungal microbial community revealed that the most dominant species, showing relative abundance of 98.1 to 99.9%, belonged to *Cladosporium herbarum* (**Figure 4.8A**).

In contrast to fungi, the values of the α-diversity indices like Shannon's (5.02 ± 0.07 to 4.81 ± 0.04) and inverse Simpson (146.2 ± 10.2 to 66.15 ± 2.8) for bacteria (**Table 4.4**) suggested the establishment of a diverse bacterial community. Both indices showed a significant decline along the operation of the bioreactor. Pielou's evenness index of all samples averaged at 0.853, indicating a rather even bacterial community. At phylum level, the bacterial community was dominated by Proteobacteria (88.14 ± 6.44), throughout the operation of the bioreactor, equally composed of Alphaproteobacteria (orders *Rhizobiales, Sphingomonadales*), Betaproteobacteria (orders *Caulobacterales, Burkholderiales*) and Gammaproteobacteria (orders *Pseudomonadales, Xanthomonadales*) (**Figure 4.8B** and C).

Table 4.4 The α-diversity indices of the fungal and bacterial community in the immobilized cell bioreactor treating IMZ-contaminated effluents

FUNGI

TIME	OBSERVED	SHANNON	INVERSE SIMPSON	PIELOU'S EVENNESS	DOMINANT RELATIVE ABUNDANCE
3	35 ± 9.07 a	0.608 ± 0.0697 a	1.268 ± 0.0485 a	0.175 ± 0.0167 a	0.888 ± 0.0176 a
24	27 ± 2.08 a	0.601 ± 0.105 a	1.298 ± 0.0637 a	0.183 ± 0.0336 a	0.877 ± 0.0211 a
46	22 ± 1.15 a	0.668 ± 0.0615 a	1.328 ± 0.0429 a	0.216 ± 0.0185 a	0.866 ± 0.0143 a
68	34.667 ± 2.85 a	0.746 ± 0.00825 a	1.361 ± 0.0114 a	0.211 ± 0.00367 a	0.855 ± 0.00392 a

BACTERIA

TIME	OBSERVED	SHANNON	INVERSE_SIMPSON	PIELOU'S EVENNESS
3	472.333 ± 29 a	5.42 ± 0.0648 a	146.235 ± 10.2 a	0.881 ± 0.002 a
24	355.333 ± 21.1 b	5.022 ± 0.0666 b	89.062 ± 5.45 b	0.856 ± 0.00287 ab
46	312.333 ± 15.1 b	4.851 ± 0.0179 c	66.146 ± 2.79 c	0.845 ± 0.00894 b
68	332.333 ± 11.8 b	4.81 ± 0.0388 c	73.519 ± 4.98 bc	0.829 ± 0.0112 b

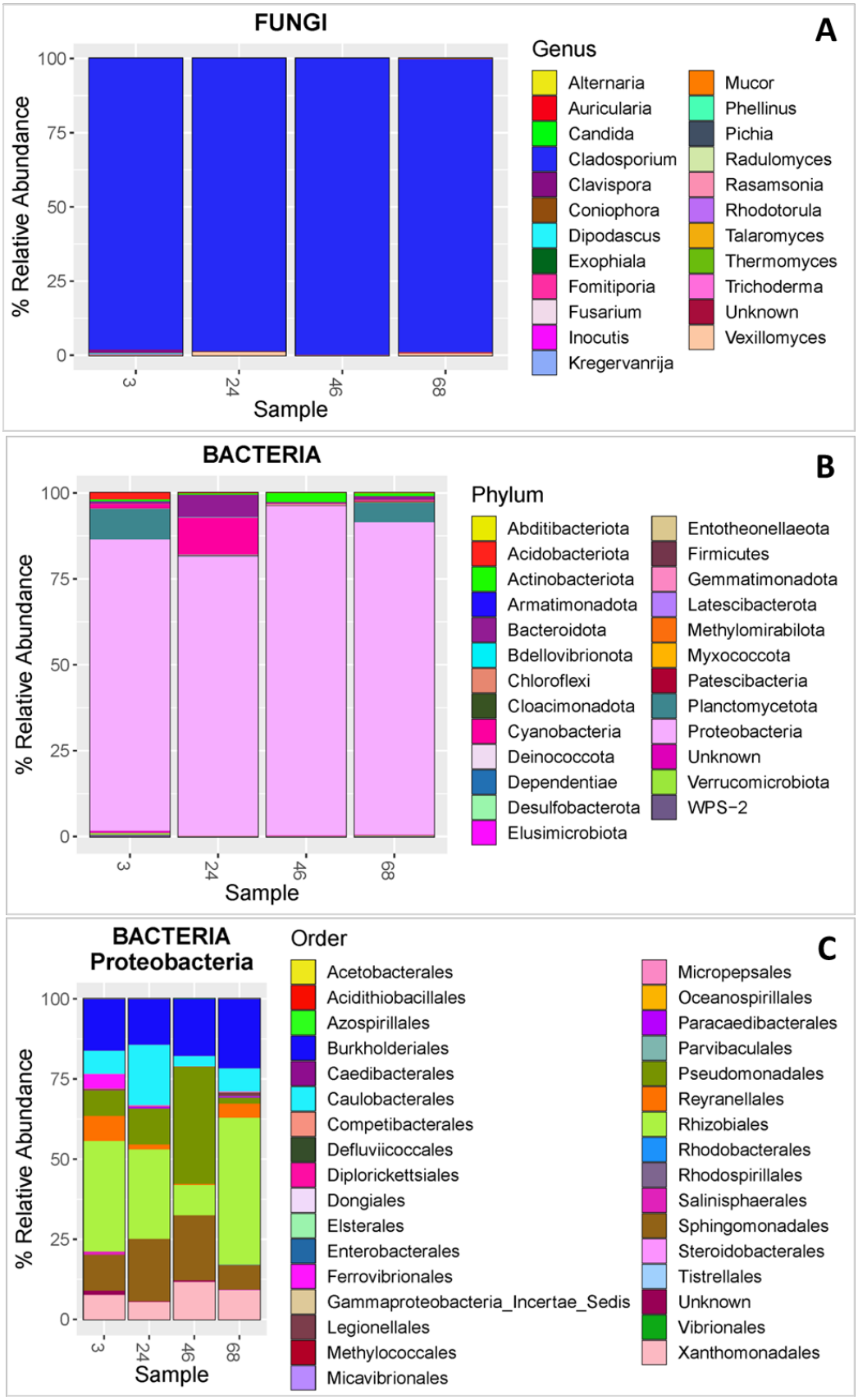

Figure 4.8 The composition of the fungal (at genus level) (A) and bacterial (at phylum level) (B) community in a bioreactor inoculated with the IMZ-degrading fungus *Cladosporium herbarum* along the 105-day operation period. The composition of the phylum Proteobacteria, which dominated the bacterial community is further displayed at the order level (C).

4 DISCUSSION

IMZ is considered a major environmental threat as a result of (i) its widespread use in fruit-packaging plants (FPPs), (ii) the lack of processes for the on-site treatment of the IMZ-contaminated agro-industrial effluents, (iii) the limited capacity of the municipal wastewater treatment systems to remove it from the effluents and (iv) its demonstrated environmental persistence and toxicity. In this frame, we aimed to isolate microorganisms able to degrade IMZ and tested their potential for use as tailored-made inocula in biological wastewater treatment systems.

Enrichment cultures from a soil with demonstrated exposure to IMZ resulted in the isolation of a fungal strain, phylogenetically assigned to *C. herbarum* that was able to effectively dissipate IMZ in the presence of extra C and N sources. To our knowledge, this is the first report of a microorganism able to effectively degrade IMZ. López-Loveira et al., (2017) isolated from sewage sludge previously exposed to IMZ, a bacterial consortium, consisting of *Chryseobacterium*, *Staphylococcus*, *Burkholderia* and *Burkholderia cepacia* strains, that was able to partially degrade high concentrations of IMZ (500 mg L^{-1}). However, the role of the individual members of the consortium on the degradation of the fungicide was not clarified.

Previous studies have identified bacteria that were able to degrade fungicides used in fruit-packaging plants like TBZ (Perruchon et al., 2017), OPP (Perruchon et al., 2016) and IPR (Campos et al., 2015), whereas it is the first time that a fungal strain able to dissipate a persistent fungicide, like IMZ, is reported. Several fungal strains that possess the ability to degrade herbicides (Dao et al., 2019; Koroleva et al., 2015; Ellegaard-Jensen et al., 2014, 2013; Pinto et al., 2012) and insecticides (Bhatt et al., 2020; Oliveira et al., 2015; Kamei et al., 2011; Sagar and Singh, 2011) have been previously isolated, whereas the corresponding list when regards fungicides is certainly shorter. Bending et al., (2002) showed that the white-rot fungus *Stereum hirsutum* degraded by 64.6% the fungicide metalaxyl in liquid cultures. Pinto et al., (2012) demonstrated the biodegradation potential of *Fusarium oxysporum*, *Aspergillus oryzae*, *Lentinula edodes*, *Lecanicillium saksenae* and *Penicillium brevicompactum* against difenoconazole, with the latter showing the best performance. (Karas et al., 2011) showed that the white-rot fungus *Trametes versicolor* was effective in degrading phenolic compounds, like diphenylamine and OPP, used in FPPs, but achieved only partial degradation of TBZ and IMZ.

Members of the genus *Cladosporium* have been previously reported to degrade recalcitrant organic pollutants including pesticides. Strains of *C. cladosprioides* and *C. oxysporum* were identified as main degraders of chlorpyrifos (Chen et al., 2012) and endosulfan (Mukherjee and Mittal, 2005) respectively, while Chen et al., (2011) reported the isolation of a *Cladosporium* strain, which was able to degrade various pyrethroids and their transformation products. *C. herbarum* is considered a saprotroph, which under stress conditions could exhibit pathogenesis in various fruit crops (Barbosa et al., 2001), whereas its teleomorph *M. tassiana* is a putative pathogen of *Cruciferaceae* (Petrie and Vanterpool, 1978) and date palm (Commitee on Standardization of Common Names for Plant Diseases, 1988). Based on the above and considering the practical implications of the use of *C. hebarum* in FPP, we assayed the pathogenicity of our isolate against fruits. We noted that *C.*

hebarum did not exhibit pathogenicity to apples, oranges and pears, suggesting that it could be safe to use it as inoculum in biological wastewater treatment units implemented in FPPs.

We further explored the impact of parameters expected to affect the dissipation performance of the fungus under practical conditions, like IMZ concentrations, and its capacity to degrade other fungicides commonly used along with IMZ in FPPs. The fungus was able to degrade IMZ in both culture media at concentration levels up to 100 mg L^{-1}, which are over-double (20-50 mg L^{-1}) those often encountered in effluents from fruit-packaging plants (Santiago et al., 2018a, 2013). The fungus showed a dose-dependent decrease in its growth rates, which was reflected in a reciprocal reduction in the degradation rates of IMZ. These results suggest that *C. herbarum* is effectively degrading IMZ, but not in a growth-linked manner. Instead, it seems to be adversely affected by the fungicide at higher concentration levels, still degrading IMZ at a slower rate. IMZ is a broad-spectrum fungicide used for the control of fungi belonging to *Helotiales* (*Botrytis cinerea, Gloeosporium* sp.) (Vorstermans and Creemers, 2007), *Eurotiales* (*Penicillium* sp.) (Erasmus et al., 2015), *Pleosporales* (*Alternaria* sp.) and *Botryosphaeriales* (*Diplodia* sp.) (Ben-Yehoshua et al., 1987), all being Ascomycetes like *C. herbarum*. Our findings suggest that the dissipation of IMZ by *C. herbarum* is a detoxification rather than a growth-linked process. On-going transcriptomic analysis will shed light into the genetic mechanism driving the response of the fungus to IMZ exposure.

We further evaluated the capacity of the fungus to degrade other fungicides used along with IMZ in FPPs, hence are expected to co-occur with IMZ in the agro-industrial effluents. *C. hebarum* showed a remarkable capacity to partially degrade FLD and TBZ, considered resistant to biodegradation (EFSA, 2014, 2007), unlike OPP, which although it is generally more amenable to biodegradation (Karas et al., 2015; Körner et al., 2000), it was not degraded by the fungus. OPP is used as a broad-spectrum disinfectant in agriculture and in cosmetic products (SCCS (Scientific Committee on Consumer Safety), 2015), hence its toxicity on *C. herbarum* is not surprising. Regarding IPR, its dissipation in the abiotic control did not allow us to verify the degrading capacity of *C. herbarum* against this fungicide. IPR, like all dicarboxamide fungicides, is prone to abiotic hydrolysis in neutral to alkaline pH conditions (Campos et al., 2015; Szeto et al., 1989), like the pH of our growth media (pH 6.7). Still, *C. herbarum* showed a remarkable capacity to degrade 3,5-DCA, the main transformation production of IPR (Campos et al., 2017). This capacity of *C. herbarum* is particularly interesting considering that 3,5-DCA is the most recalcitrant DCA isomer (Yao et al., 2011) and all IPR-degrading bacteria isolated to date do not have the capacity to degrade 3,5-DCA (Yang et al., 2018; Campos et al., 2017; Athiel et al., 1995). Further studies using mixed microbial community approaches, by combining *C. herbarum* and *Paenarthrobacter*, an IPR-degrading bacterium of our group (Katsoula et al., 2020), will be explored for the effective removal of iprodione and its derivatives. Overall, our findings suggest that *C. herbarum* could be used as a starting inoculum for the biological treatment of effluents from FPPs containing most fungicides, but not OPP, which is not compatible with this fungus.

Eventually, we directly assessed the capacity of the fungus to remove IMZ from wastewaters under bioengineering conditions by using an immobilized cell bioreactor. The inoculated bioreactor showed high removal efficiency (>96%) compared to other biological

treatment systems, like biobeds, whose dissipation efficiency varied from 72 to 95.7% (Karas et al., 2016a). The removal efficiency observed is expected to minimize the risk for environmental dispersal of IMZ and hence limit its adverse effects on aquatic organisms. In addition to its depuration performance, we followed the establishment and survival of *C. herbarum* and the overall microbial succession in the bioreactor. The fungus was able to establish successfully, and dominate the fungal community in the bioreactor, comprising >98% of the overall fungal propagules detected in the bioreactor throughout the study. Still, the established microbial community was dominated by bacteria, which comprised of approximately > 80% of the microbial population. Proteobacteria prevailed in the bacterial community of the bioreactor and particularly representatives of *Sphingomonadales, Burkholderiales* and *Pseudomonadales* orders. Bacteria belonging to these orders are known for their capacity to degrade pesticides in general, and fungicides used in FPPs in particular. For example, a *Sphingomonas haloaromaticamans* strain (Perruchon et al., 2016) and a *Sphingomonas*-based bacterial consortium (Sotirios et al., 2020) were able to degrade OPP and TBZ respectively, while *Pseudomonas* and *Burkholderia* spp. were previously reported as diphenylamine degraders (Campos et al., 2015; Shin and Spain, 2009). Moreover, two constituents of the bacterial consortium that was identified by López-Loveira et al. (2017) to tolerate and slowly degrade IMZ belonged to *Burkholderia*.

5 CONCLUSIONS

We report the first isolation of a fungal strain, identified as *C. herbarum*, able to degrade IMZ. The degrading capacity of the fungus was impaired at high concentration levels, suggesting that the degradation of IMZ is a detoxification instead of a growth-linked process. Still, the fungus was able to degrade IMZ at concentration levels higher than those expected to occur in agro-industrial effluents. The fungal isolate achieved appreciable degradation of other fungicides expected to co-occur with IMZ in agro-industrial effluents, except OPP, and exhibited successful establishment and high removal efficiency of IMZ when used as a starting inoculum in an immobilized cell bioreactor receiving IMZ-contaminated effluents. All our findings suggest that *C. herbarum* has high potential for use as a tailored-made inoculum in biological treatment systems for the removal of IMZ from agro-industrial effluents, whose treatment constitute a menace for FPPs. Further genomic, transcriptomic and metabolomic analysis will shed light into the degradation mechanism of IMZ by *C. herbarum* and facilitate the further optimization of the relevant bioprocesses.

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Chapter 5

General Discussion, Conclusions and Future Perspectives

1 GENERAL DISCUSSION

Agro-food processing industries often resort to the use of immense amounts of fungicides in order to ensure high quality and minimize losses of fresh plant products during storage and transport (Damalas and Eleftherohorinos, 2011). This practice results in the production of large volumes of pesticide-contaminated wastewaters which constitute serious environmental concern on account of the high environmental persistence and toxicity of the pesticides contained in those effluents (Carvalho, 2017). Authorities have posed precautionary measures to prevent the uncontrollable release of agro-industrial effluents by enacting restrictions in their use under the clause of proper wastewater management (EFSA, 2010; EUROPEAN COMMISSION, 2016). Even though agro-food processing industries should implement measures for the depuration of their effluents, the lack of efficient and cost-effective treatment methods, has forced them to follow improper and environmentally harmful disposal practices (Campo et al., 2013; Papadopoulou et al., 2018). Biological treatment systems like biobeds constitute an affordable, effective and sustainable solution for the treatment of agro-industrial effluents. Numerous studies have shown the potential of biobeds to treat wastewaters from fruit packaging industries (FPI), yet the use of biobeds for the depuration of effluents from other agro-food processing industries, like seed producing (SPI) and bulb handling (BHI) industries is still unexplored. Biobeds owe their depuration capacity to the microbial communities of the packing material although the microbial composition of these systems and the dynamics of the mobile genetic element (MGE) that are expected to be involved in the selection of novel catabolic traits against pesticides are not yet adequately explored. Optimization of the performance of biobed systems against persistent and particularly mobile chemicals could be implemented with bioaugmentation of biobeds with tailored-made microbial inocula specialized in the degradation of the target pesticides (Karanasios et al., 2012; Karas et al., 2016). **Within this frame, in the present thesis we aimed to (i) explore the potential of biobed systems to treat pesticide-contaminated effluents produced by agro-food industries beyond FPI including BHIs and SPIs, (ii) determine the microbial composition of the biobeds microbiota and identify the factors driving microbial succession in biobed systems during their operation (iii) monitor the dynamics of MGE, known to play a role in the dispersal of pesticide catabolic traits, in biobed systems during their operation and (iv) isolate microorganisms able to degrade one of the most persistent fungicide contained in these agro-industrial effluents, IMZ, with the potential to be used as inoculum in following biological treatment systems like biobeds and dedicated bioreactors**.

To determine the potential of biobeds to treat these agro-industrial effluents we followed a step-by-step experimental approach which first involved lab microcosm experiments to determine the potential of biobed packing materials to degrade or adsorb the pesticide contained in those effluents. So, in **Chapter 2** we determined the degradation and adsorption of fungicides used in SPI (carboxin (CBX), metalaxyl-M (MET-M), fluxapyroxad (FLX), fludioxonil (FLD)), BHI (chlorothalonil (CHT), thiabendazole (TBZ), FLD) and FPI (FLD) in a biobed packing material composed of 25% soil, 25% straw and 50% SMS and comparatively in soil with no previous pesticide application. Pesticides were applied in the different substrates in mixtures and individually to simulate realistic exposure

conditions. A first and important observation was the significantly higher degradation rates of pesticides in the biobed packing material compared to soil. Application in double, triple and quadruple mixtures delayed the dissipation of fungicides in both matrices, with this effect being more noticeable in soil rather than in the packing material. Adsorption study showed that all tested fungicides exhibited higher adsorption affinity in the biobed packing material in comparison to soil. Overall these findings suggested a superior degradation and adsorption potential of biobed packing material compared to soil for the fungicides contained in the various agro-food industrial effluents.

In Chapter 3 we employed a column biobed experiment, using the same biobed packing material as in Chapter 2, in order to assess depuration efficiency of biobed systems for the treatment of wastewaters from different agro-food processing industries in a realistic loading scenario. We noted that biobed systems could effectively depurate effluents produced by SPI, BHI and FPI, with different processes being responsible for fungicide removal depending on polarity and biodegradability of the compounds; lipophilic substances like FLX were mostly retained in the biobed packing material, whereas polar ones like CBX were mostly degraded. In addition, we noted that most polar chemicals like MET-M were leached to a greater extend compared to the other compounds contained in the effluents.

In the same experimental set up, we envisaged to follow microbial succession and MGE dynamics in the biobed systems using amplicon sequencing and q-PCR approaches. We tested the hypothesis that the continuous exposure of the biobeds microbiome to high pesticide loads will affect the microbial community and will promote the dissemination and dynamics of MGE. We found that the biobed packing material sustained a rather resilient bacterial and fungal community composed mostly of *Proteobacteria* and *Sordariomycetes* respectively that were not affected by applied wastewater treatments. Instead the microbial community of biobeds showed clear temporal patterns along the different biobed horizons. This was probably induced by microaerophilic conditions upon water saturation of the packing material, as we noticed significant increase in the abundance of facultative or strict anaerobic bacteria like *Chloroflexi*/*Anaerolinae*, *Acidibacter* and *Myxococcota.* Regarding MGEs like *Intl1*, *IS*1071 and IncP-1 and IncP-1$^{\varepsilon}$, our hypothesis was not verified since we did not observe significant increases in the relative abundance of the above mentioned MGEs in response to continuous exposure to pesticides. Instead, we observed a temporal increase in the abundance of most MGEs tested, which presumably is related with the establishment of biotic or abiotic conditions, beyond pesticide-related pressure that facilitate this temporal pattern. Overall, biobed systems supported a resilient microbial community which did not appear to respond to pesticide exposure but instead is changing in response to abiotic and biotic conditions established gradually in the biobed systems with time.

Finally in Chapter 4 we aimed to isolate microorganisms that could degrade IMZ, one of the most persistent fungicide contained in those effluents, with the prospect of using this microorganism as inoculum in wastewater biological treatment systems. Bioaugmentation of the packing material with specialized degrading inocula could result in further enhancement of the efficiency of biobed systems. The laboratory of Plant and Environmental Biotechnology already contains a collection of bacteria that degrade fungicides commonly used by fruit packaging plants but no single microorganism has ever been isolated able to

degrade the widely used and persistent fungicide imazalil (IMZ). In this scope, enrichment cultures from soil receiving IMZ-contaminated effluents from FPIs led to the isolation of a *Cladosporium herbarum* strain which showed degradation of IMZ in both nutrient rich and selective media. *C. herbarum* posed no infection potential on fruit commonly processed by FPIs, an important trait for its exploitation in on-site treatment of FPI effluents. *C. herbarum* was able to degrade IMZ in nutrient rich and selective media at concentration levels up to 100 mg/L, which are higher than those expected to occur in agro-industrial effluents, but its growth and degrading capacity were reduced at increasing IMZ concentrations, implying that the degradation of IMZ is a detoxification mechanism instead of a growth-linked process. Study of the adaptation of the isolate to the presence of other fungicides that are commonly used by FPIs and are, thus, expected to co-occur with IMZ in the effluents showed partial dissipation of FLD, TBZ and 3,5 dichloroaniline (3,5DCA), a toxic transformation product of iprodione degradation (IPR), unlike *ortho*-phenylphenol (OPP) in the presence of which *C. herbarum* showed inadequate growth and, therefore, degradation capacity. Lastly, the ability of *C. herbarum* to depurate IMZ-contaminated effluents was assessed in a benchtop bioreactor study where high removal efficiency (>96%) was observed. Amplicon sequencing analysis showed that *C. herbarum* was able to successfully establish and dominate the fungal community throughout the study. All of the above support the high potential of *C. herbarum* for the removal of IMZ, which could be employed for the bioaugmentation of biobed systems and further enhancement of their performance against IMZ-contaminated effluents, or in biological wastewater treatment units that treat very high wastewater volumes.

2 CONCLUSIONS

Overall, our findings lead to several important conclusions for the potential use of biobeds in the depuration of agro-industrial effluents:

- Biobeds are efficient in the removal of fungicides contained in effluents from agro-food industries and could be used in the treatment of these effluents
- Biobeds support a resilient microbial community whose composition showed temporal patterns most probably driven by the establishment of conditions that favour the proliferation of microaerophilic microorganisms after 100 days of operation
- The first IMZ-degrading microorganism, a fungal strain of *C. herbarum*, was isolated and showed high capacity to remove IMZ from effluents under both laboratory and bioreactor conditions where it became established during the wastewater treatment process

3 FUTURE PERSPECTIVES

The current thesis contributed strong evidence for the efficiency of biobed systems for the treatment of pesticide-contaminated effluents from various agro-food industries and provided a fungal strain capable of IMZ-degradation Meanwhile, new research challenges have risen, which are to be addressed in the future, regarding:

I. Identification of the genetic arsenal of *C. herbarum* involved in the dissipation of IMZ. This will be achieved through the following steps which are under way:
    a. Genomic analysis of *C. herbarum*, already been performed in the platforms of Illumina (short reads, high accuracy) and MinION (long reads, high error rate), and genome assembly and functional annotation is on-going.
    b. A transcriptomic analysis of the fungal isolate in the presence/absence of IMZ has been undertaken and the analysis of the transcriptome of *C. herbarum* is expected to shed light into the genetic pathways activated during dissipation of IMZ.
    c. A shotgun LC-MS/MS of the fungal culture during degradation of IMZ (inoculated and non-inoculated with *C. herbarum*) has been performed in collaboration with the Analytical Chemistry Laboratory in National Kapodistrian University of Athens by the group of Prof. N. Thomaidis and preliminary evidence identified three transformation products whose confirmation and quantification is under way and is going to be confirmed.

II. Application of *C. herbarum* in biobed systems or in biological wastewater treatment units for the depuration of IMZ-contaminated effluents. The application of *C. herbarum* in biobed systems has been already performed and the analysis of the data are under way. Regarding the application of *C. herbarum* in biological wastewater treatment systems, the fungal strain has been have already applied as a tailored-made inoculum in a full scale bioreactor system established in the premises of Poulis S.A. in Larissa, Greece receiving IMZ-contaminated effluents from this FPI, and its removal efficiency is under way.

III. The utilization of *C. herbarum* in combination with iprodione-degrading bacteria of the genus *Paenarthrobacter*, available in the Laboratory of Plant and Environmental Biotechnology, for the construction of synthetic microbial consortium for optimization of the degradation of the fungicide iprodione. This combination will facilitate the mineralization of iprodione which is currently transformed by bacteria to 3,5-dichloroaniline, a rather toxic compound and the most persistent dichloroaniline isomer which is effectively transformed by *C. herbarum.* First tests with this synthetic microbial consortium have provided promising results while further optimization is required to maximize degradation performance.

IV. The application of shotgun metagenomic and plasmidomic approaches in biobed systems towards a better understanding of the functional and evolutionary potential of the biobed microbial community.

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EXTENDED SUMMARY

Agro-food processing industries use large amounts of fungicides to ensure availability of fresh plant products during storage and transport. These include seed-producing industries (SPI), which treat seeds with systemic fungicides like carboxin (CBX), metalaxyl-M (MET-M) and fluxapyroxad (FLX), bulb handling industries (BHI) which immerse bulbs into dense solutions of fungicides such as chlorothalonil (CHT), thiabendazole (TBZ) and fludioxonil (FLD), and fruit-packaging industries (FPI) that make use of fungicides like imazalil (IMZ) and fludioxonil (FLD) for the control of fungal infections of fruits during storage. As a result, they generate large amounts of pesticide-contaminated effluents which constitute serious environmental threats due to the high environmental persistence and toxicity of the pesticides contained in them. Despite relevant regulation, the lack of efficient and cost-effective treatment methods has pushed agro-food industries into improper and environmentally harmful disposal practices.

Through the years, many depuration methodologies have been studied, but their full implementation has not been achieved due to poor results concerning mineralization, high operational costs and formation of toxic by-products. Biological treatment systems like biobeds could provide an efficient and sustainable solution to the depuration of pesticide-contaminated effluents. Some recent studies have demonstrated the efficient decontamination of FPI effluents in biobeds, while the use of biobed systems for the treatment of SPI and BHI effluents is still not explored. The high depuration capacity of biobeds is attributed to the microbiome of the packing material, but the composition and succession of the microbial communities and the dynamics of mobile genetic elements (MGE) involved in the dispersion of pesticide-catabolic genes in the bacterial community of the packing material during biobed operation, are not yet adequately explored. The performance of biobed systems against persistent and particularly mobile pesticides can be greatly enhanced by bioaugmentation of the packing material with tailored-made microbial inocula specialized in the degradation of target compounds. With these in mind, we aimed (i) to provide evidence for the capacity of biobed systems to depurate pesticide-contaminated effluents from SPI, BHI and FPI, (ii) to shed some light on the composition of biobed microbiome and identify factors that drive microbial succession during biobed operation, (iii) to provide insight on the occurrence and distribution of MGEs in biobed systems during operation and (iv) to isolate microorganisms able to degrade IMZ, a highly persistent fungicide that is widely used by agro-food processing industries, especially FPI, with the potential to be used in future biological treatment systems such as biobeds and bioreactor units.

In Chapter 2 we studied the degradation and adsorption, two major processes controlling the environmental fate of pesticides in biobed packing material composed of 25% soil, 25% straw and 50% spent mushroom substrate and comparatively in soil with no previous pesticide exposure. The degradation of CBX, MET-M, FLX, FLD, TBZ and CHT, was studied under individual and in-mixture application relevant to their industrial use, to simulate realistic exposure conditions, while FLD was also tested at different concentrations (10, 20, and 150 mg/kg) representing the dose rates used by the different industries. The

majority of fungicides, regardless of the mode of application, resulted in higher dissipation in the biobed packing material (DT_{50} = 2.34 – 142.9 days) than in soil (DT_{50} = 6.67 – 784.1 days). In most cases application in mixtures retarded fungicides' degradation, with CHT having the most pronounced inhibitory effect in the degradation of TBZ and FLD. FLD degradation showed a dose-dependent pattern with its DT_{50} increasing from 42.4 days at 10 mg/kg to 107.6 days (at 150 mg/kg). In addition, all pesticides showed higher adsorption affinity in the biomixture (K_f = 3.23 - 123.3 g mL^{-1}) compared to soil (K_f = 1.15-31.2 g mL^{-1}). The findings of Chapter 2 provided initial evidence of the depuration potential of biobeds against fungicides contained in effluents generated by SPI, BHI and FPI which allowed us to proceed with our research of biobed systems treating agro-industrial effluents.

Consequently in Chapter 3, we employed a biobed column experiment using the same packing material as in Chapter 2, in order to assess the efficiency of biobed systems to depurate agro-industrial effluents containing mixtures of fungicides in a realistic loading scenario. We demonstrated that the biobed columns could effectively retain and dissipate the fungicides contained in agro-industrial effluents with 93.1 - 99.98 % removal efficiency in all cases. Lipophilic substances like FLX were mostly retained in the biobed while more polar substances were mostly dissipated like CBX or showed higher leaching potential like MET-M. The effect of continuous effluent application in the packing material's microbiome was also explored in the same experimental setup through amplicon sequencing analysis. Contrary to our expectation, biobed column supported resilient bacterial and fungal communities, which were not affected by fungicide application but showed temporal patterns along the different horizons. Facultative or strict anaerobic bacteria like *Chloroflexi*/*Anaerolinae*, *Acidibacter* and *Myxococcota* showed significant increase in the abundance supporting the hypothesis that the temporal patterns were driven by microaerophilic conditions upon water saturation of the packing material. Lastly, we investigated the dynamics of MGE, namely *IntI1*, *IS*1071 and IncP-1 and IncP-1ε, expecting the continuous exposure to high pesticide loads will promote their dissemination. However, our hypothesis was not confirmed, as continuous wastewater application did not affect the dynamics of MGE in biobeds. Instead, we observed temporal increase in the abundance of most MGE tested, suggesting the influence of biotic or abiotic factors, beyond pesticide-related pressure. All in all, the findings of Chapter 3 reinforce the high potential of biobed systems for the depuration of agro-industrial effluents and showed that the packing material contains a resilient microbiome that is not affected by pesticide exposure, but responds to abiotic and biotic conditions that gradually develop in the biobed system.

Chapter 4 was dedicated in the isolation and characterization of a microorganism able to degrade the widely used and persistent fungicide IMZ, and in the investigation of its potential use as inoculum in biotic wastewater treatment systems. A *Cladosporium herbarum* strain capable of degrading IMZ was isolated via enrichment cultures from a soil that was receiving regular discharges of FPI's IMZ-contaminated effluents. The *C. herbarum* strain did not show any pathogenicity on fruits commonly processed by FPIs, a trait essential for its biotechnological exploitation in the treatment of FPI effluents. The isolate was able to degrade up to 100 mg/L of IMZ but its degrading capacity and growth was reduced at increasing IMZ concentrations in a dose-dependent manner, indicating that the degradation of IMZ is a detoxification mechanism instead of a growth-linked process. The isolated strain

was able to tolerate the presence other fungicides, which are commonly used by FPIs and are, thus, expected to co-occur with IMZ in their effluents, and showed partial dissipation of FLD, TBZ and 3,5 dichloroaniline (3,5DCA), a toxic transformation product of iprodione (IPR) degradation. On the contrary *ortho*-phenylphenol (OPP) inhibited the growth and, therefore, degradation capacity of the isolate. The ability of *C. herbarum* to depurate IMZ-contaminated effluents was assessed in a benchtop bioreactor fed with artificial IMZ-contaminated wastewater (200 mg L-1). Amplicon sequencing analysis showed that *C. herbarum* was able to successfully establish and dominate the fungal community of the bioreactor throughout the study and successfully removed >96% of IMZ. Overall, the findings of Chapter 4 demonstrate the high potential of *C. herbarum* to remove IMZ under lab and bioengineering conditions.

As a whole our study demonstrated the high potential of the use biobed systems for the depuration of fungicide-contaminated effluents from seed-producing, bulb-dipping and fruit packing industries. We also showed that the biobeds support a resilient microbiome, whose composition was most probably affected by microaerophilic conditions that gradually developed in the packing material. Lastly we reported the isolation of a *C. herbarum* strain with the capacity to degrade IMZ and examined its potential for the depuration of FPI effluents that contain IMZ.

ΠΕΡΙΛΗΨΗ

Οι μονάδες μεταποίησης αγροτικών προϊόντων χρησιμοποιούν μεγάλες ποσότητες μυκητοκτόνων ούτως ώστε να διασφαλίσουν την διαθεσιμότητα των φρέσκων αγροτικών προϊόντων κατά την αποθήκευση και μεταφορά. Τέτοιες μονάδες αποτελούν σποροπαραγωγικές μονάδες που επικαλύπτουν σπόρους με μυκητοκτόνα όπως τα carboxin (CBX), metalaxyl-M (MET-M) και fluxapyroxad (FLX), βιομηχανίες διαχείρισης βολβών οι οποίες εμβαπτίζουν βολβούς σε πυκνά διαλύματα μυκητοκτόνων όπως chlorothalonil (CHT), thiabendazole (TBZ) και fludioxonil (FLD), και τα συσκευαστήρια φρούτων που χρησιμοποιούν μυκητοκτόνα όπως τα imazalil (IMZ) και fludioxonil (FLD) για τον έλεγχο μυκητολογικών προσβολών των φρούτων κατά την αποθήκευση. Αποτέλεσμα αυτών των εφαρμογών, είναι η παραγωγή μεγάλου όγκου υγρών αποβλήτων επιβαρυμένων με γεωργικά φάρμακα τα οποία αποτελούν σημαντικό περιβαλλοντικό κίνδυνο λόγω της υπολειμματικότητας και της τοξικότητας των μυκητοκτόνων που περιέχονται σε αυτά. Παρά τους σχετικούς κανονισμούς, η έλλειψη αποτελεσματικών και οικονομικά εφαρμόσιμων μεθόδων για την διαχείριση των συγκεκριμένων αποβλήτων, έχει οδηγήσει τις αγρο-βιομηχανίες στην εφαρμογή ακατάλληλων και περιβαλλοντικά επιβλαβών πρακτικών απόρριψης των συγκεκριμένων υγρών αποβλήτων.

Διάφορες μέθοδοι απορρύπανσης έχουν μελετηθεί με τα χρόνια, αλλά η πλήρης εφαρμογή τους δεν έχει επιτευχθεί εξαιτίας χαμηλής αποτελεσματικότητας, δαπανηρής λειτουργίας και τον σχηματισμό τοξικών παραπροϊόντων κατά την διαχείριση των αποβλήτων. Τα βιολογικά συστήματα διαχείρισης των αποβλήτων, όπως οι βιοκλίνες, παρέχουν μια αποτελεσματική και αειφόρο λύση στην απορρύπανση αποβλήτων που περιέχουν γεωργικά φάρμακα. Η συντριπτική πλειοψηφία των διαθέσιμων μελετών αποδεικνύουν την αποτελεσματική απορρύπανση αποβλήτων από συσκευαστήρια

φρούτων, ενώ η χρήση βιοκλινών για την επεξεργασία αποβλήτων σποροπαραγωγικών μονάδων και μονάδων διαχείρισης βολβών δεν έχει διερευνηθεί. Η υψηλή ικανότητα των βιοκλινών να απομακρύνουν γεωργικά φάρμακα από υγρά απόβλητα αποδίδεται στο μικροβίωμα που αποικίζει το πληρωτικό υλικό των βιοκλινών. Παρόλα αυτά η σύνθεση και διαδοχή των μικροβιακών κοινοτήτων καθώς και η ποικιλότητα και η δυναμική μεταθετών στοιχείων που συμβάλλουν στην διασπορά γονιδίων καταβολισμού των γεωργικών φαρμάκων στην βακτηριακή κοινότητα κατά την λειτουργία της βιοκλίνης, δεν έχουν μελετηθεί επαρκώς. Η απόδοση των συστημάτων βιοκλινών έναντι υπολειμματικών ή εξαιρετικά κινητικών γεωργικών φαρμάκων μπορεί να αυξηθεί ιδιαίτερα μέσω βιοενίσχυσης του πληρωτικού υλικού με μικροβιακά εμβόλια, εξειδικευμένα στην διάσπαση των ενώσεων-στόχων. Σύμφωνα με τα παραπάνω, η παρούσα διδακτορική διατριβή είχε ως στόχο (i) την αξιολόγηση της ικανότητας των συστημάτων βιοκλινών να απορρυπαίνουν απόβλητα επιβαρυμμένα με γεωργικά φάρμακα που παράγονται από σποροπαραγωγικές μονάδες, βιομηχανιών διαχείρισης βολβών και συσκευαστηρίων φρούτων, (ii) την διερεύνηση της σύστασης του μικροβιώματος των βιοκλινών και της ταυτοποίησης παραγόντων που επηρεάζουν την μικροβιακή διαδοχή κατά την λειτουργία της βιοκλίνης, (iii) την μελέτη της εμφάνισης και κατανομής των μεταθετών στοιχείων στα συστήματα βιοκλινών κατά την λειτουργία τους και (iv) την απομόνωση μικροοργανισμών με την ικανότητα να διασπά το IMZ, ένα ιδιαίτερα υπολειμματικό μυκητοκτόνο το οποίο χρησιμοποιείται ευρέως από τα συσκευαστήρια φρούτων, στοχεύοντας στην πιθανή χρήση του σε συστήματα διαχείρισης υγρών αποβλήτων όπως βιοκλίνες και βιοαντιδραστήρες.

Στο Κεφάλαιο 2 μελετήσαμε την αποδόμηση και προσρόφηση, δύο κύριες διεργασίες που ελέγχουν την περιβαλλοντική τύχη των γεωργικών φαρμάκων, σε πληρωτικό υλικό βιοκλινών που αποτελείται από 25% έδαφος, 25% άχυρο και 50% εξαντλημένο υπόστρωμα καλλιέργειας μανιταριών, και σε έδαφος χωρίς πρότερη έκθεση σε γεωργικά φάρμακα. Η αποδόμηση των CBX, MET-M, FLX, FLD, TBZ και CHT μελετήθηκε μετά από εφαρμογή μεμονωμένα και σε μίγματα σύμφωνα με την χρήση τους από τις εκάστοτε αγρο-βιομηχανίες, ούτως ώστε να προσομοιωθούν ρεαλιστικές συνθήκες εφαρμογής, ενώ το FLD μελετήθηκε σε διάφορες συγκεντρώσεις (10, 20, και 150 mg/kg) αναπαριστώντας τις δόσεις που χρησιμοποιούνται από την κάθε αγρο-βιομηχανία. Η πλειοψηφία των μυκητοκτόνων, ανεξαρτήτου τρόπου εφαρμογής, αποδομήθηκαν ταχύτερα στο πληρωτικό υλικό των βιοκλινών (DT_{50} = 2.34 – 142.9 ημέρες) σε σύγκριση με το έδαφος (DT_{50} = 6.67 – 784.1 ημέρες). Στις περισσότερες περιπτώσεις η εφαρμογή των γεωργικών φαρμάκων σε μίγματα καθυστέρησε την αποδόμηση των μυκητοκτόνων, με το CHT να έχει την πιο αισθητή ανασταλτική επίδραση στην αποδόμηση των TBZ και FLD. Η αποδόμηση του FLD έδειξε ένα δοσο-εξαρτώμενο πρότυπο με τον χρόνο ημιζωής του να αυξάνεται από τις 42.4 ημέρες, όταν εφαρμόστηκε σε 10 mg/kg, σε 107.6 ημέρες όταν εφαρμόστηκε σε 150 mg/Kg. Επιπροσθέτως, όλα τα γεωργικά φάρμακα έδειξαν υψηλότερη προσρόφηση στο βιομίγμα (K_f = 3.23 - 123.3 g mL^{-1}) σε σύγκριση με το έδαφος (K_f = 1.15-31.2 g mL^{-1}). Τα ευρήματα του Κεφαλαίου 2 παρέχουν πρώτα στοιχεία για την δυνατότητα των βιοκλινών να απομακρύνουν μυκητοκτόνα που βρίσκονται στα απόβλητα σποροπαραγωγικών μονάδων, βιομηχανιών διαχείρισης βολβών και συσκευαστηρίων φρούτων, κάτι που μας επιτρέπει να προχωρήσουμε με τη περαιτέρω αξιολόγηση των συστημάτων βιοκλινών σε πιο ρεαλιστικά συστήματα φόρτισης.

Έτσι στο Κεφάλαιο 3, πραγματοποιήσαμε πείραμα στηλών έκπλυσης, πιλοτικές βιοκλίνες, που πληρώθηκαν με το ίδιο πληρωτικό υλικό όπως στο Κεφάλαιο 2, ούτως ώστε να αξιολογήσουμε την απόδοση των συστημάτων βιοκλινών στην απορρύπανση αποβλήτων αγρο-βιομηχανιών που περιέχουν μίγματα μυκητοκτόνων, σε ρεαλιστικές συνθήκες φόρτισης. Παρατηρήσαμε ότι οι βιοκλίνης μπόρεσαν να κατακρατήσουν και να διασπάσουν τα μυκητοκτόνα που περιέχονταν στα απόβλητα σε ποσοστό 93.1 - 99.98 %. Λιπόφιλες ενώσεις όπως το FLX κυρίως κατακρατήθηκαν στο πληρωτικό υλικό, ενώ πιο πολικές ενώσεις κυρίως αποδομήθηκαν όπως το CBX ή εκπλύθηκαν όπως το MET-M. Η επίδραση της συνεχούς εφαρμογής αποβλήτου στο μικροβίωμα του πληρωτικού υλικού διερευνήθηκε στην ίδια πειραματική εγκατάσταση μέσω ανάλυσης της αλληλουχίας προϊόντων ενίσχυσης DNA. Αντίθετα με την αρχική μας υπόθεση, οι βιοκλίνες υποστήριζαν ιδιαίτερα ανθεκτικές βακτηριακές και μυκητιακές κοινότητες, η σύσταση των οποίων δεν επηρεάστηκε από την εφαρμογή των μυκητοκτόνων, αλλά εμφάνισε ξεκάθαρα χρονικά μοτίβα μεταβολών στα διάφορα βάθη των στηλών έκπλυσης. Προαιρετικά ή υποχρεωτικά αναερόβια βακτήρια όπως τα *Chloroflexi*/*Anaerolinae*, *Acidibacter* and *Myxococcota* έδειξαν σημαντική αύξηση της αφθονίας τους ενισχύοντας την υπόθεση ότι οι χρονικές αυτές μεταβολές στην σύσταση της μικροβιακής κοινότητας των βιοκλινών οφείλονταν στην επικράτηση μικροαερόφιλων συνθηκών εντός των βιοκλινών λόγω κορεσμού του πληρωτικού υλικού με το υγρό απόβλητο. Τέλος, μελετήσαμε την αφθονία μεταθετών στοιχείων, όπως *IntI1*, *IS*1071, IncP-1 and IncP-1ε, στηριζόμενοι στην υπόθεση ότι η διαρκής έκθεση των βιοκλινών σε υψηλές συγκεντρώσεις γεωργικών φαρμάκων θα ενισχύσει την αφθονία τους και δη την διασπορά γονιδίων αποδόμησης των γεωργικών φαρμάκων στην βακτηριακή κοινότητα. Παρόλα αυτά η υπόθεσή μας δεν επαληθεύτηκε καθώς η συνεχής εφαρμογή του αποβλήτου δεν επηρέασε την αφθονία των μεταθετών στοιχείων. Αντίθετα παρατηρήσαμε αύξηση της αφθονίας των περισσότερων υπό μελέτη μεταθετών στοιχείων με τον χρόνο, υποδηλώνοντας την επικράτηση βιοτικών ή αβιοτικών παραγόντων που ενισχύσουν την διασπορά μεταθετών στοιχείων και γενικότερα τις γενετικές ανταλλαγές μεταξύ μικροοργανισμών. Εν κατακλείδι, όλα τα ευρήματα του Κεφαλαίου 3, ενισχύουν την άποψη ότι οι βιοκλίνες μπορούν να χρησιμοποιηθούν αποτελεσματικά για την απορρύπανση αποβλήτων αγρο-βιομηχανιών και έδειξαν ότι τα πληρωτικό υλικό συντηρεί ένα ιδιαίτερα ανθετικό μικροβίωμα το οποίο δεν επηρεάζεται από την έκθεση σε γεωργικά φάρμακα αλλά φαίνεται να αποκρίνεται σε βιοτικές και αβιοτικές συνθήκες που σταδιακά επικρατούν στη βιοκλίνη.

Το Κεφάλαιο 4 εστίασε στην απομόνωση και χαρακτηρισμό μικροοργανισμών με την ικανότητα να αποδομούν το ευρέως χρησιμοποιούμενο και υπολειμματικό μυκητοκτόνο IMZ, και στην διερεύνηση της δυνατότητας χρήσης του ως εμβόλιο σε βιολογικά συστήματα διαχείρισης αποβλήτων. Ένα στέλεχος του είδους *Cladosporium herbarum* με την ικανότητα να αποδομεί το IMZ απομονώθηκε μέσω καλλιεργειών εμπλουτισμού από έδαφος που δέχονταν απόβλητα συσκευαστηρίου φρούτων που περιείχαν IMZ. Το *C. herbarum* δεν έδειξε φυτοπαθογόνο δράση έναντι φρούτων που μεταχειρίζονται συχνά από συσκευαστήρια φρούτων, ένα αναγκαίο χαρακτηριστικό για την μετέπειτα χρήση του για την επεξεργασία αποβλήτων συσκευαστηρίων φρούτων. Το στέλεχος κατάφερε να αποδομήσει έως και 100 mg/L IMZ αλλά η αποδομητική του ικανότητα και η ανάπτυξή του μειώθηκαν με την αύξηση της συγκέντρωσης του IMZ με ένα

δοσο-εξαρτώμενο πρότυπο υποδεικνύοντας ότι η αποδόμηση του IMZ είναι ένας μηχανισμός αποτοξικοποίησης παρά συνδέεται με την παραγωγή ενέργειας. Το *C. herbarum* έδειξε αντοχή στην παρουσία και άλλων μυκητοκτόνων που χρησιμοποιούνται ευρέως από τα συσκευαστήρια φρούτων και αναμένεται να συνυπάρχουν στα απόβλητα μαζί με το IMZ, και αποδόμησε μερικώς ή πλήρως τα FLD, TBZ και 3,5 dichloroaniline (3,5-DCA), ένα τοξικό προϊόν της αποδόμησης του μυκητοκτόνου iprodione (IPR). Αντίθετα το *ortho*-phenylphenol (OPP) ανέστελλε πλήρως την ανάπτυξη του μικροοργανισμού και, κατά συνέπεια, την αποδομητική του ικανότητα. Η ικανότητα του *C. herbarum* να απορρυπαίνει απόβλητα που περιέχουν IMZ αξιολογήθηκε σε έναν βιοαντιδραστήρα εργαστηρίου στον οποίο παρέχονταν υγρά απόβλητα που περιείχαν 200 mg/L IMZ. Μεταταξινομική ανάλυση της κοινότητας των μυκήτων έδειξε ότι το στέλεχος *C. herbarum* κατάφερε να εδραιωθεί και να επικρατήσει της μυκητιακής κοινότητας του βιοαντιδραστήρα και να απομακρύνει επιτυχώς >96% του IMZ. Συνολικά, τα ευρήματα του Κεφαλαίου 4 επιδεικνύουν την δυνατότητα του *C. herbarum* να απομακρύνει το IMZ σε εργαστηριακές και βιομηχανικές συνθήκες.

Συγκεντρωτικά η παρούσα διατριβή απόδειξε την υψηλή προοπτική των βιοκλινών για την απορρύπανση αποβλήτων επιβαρυμένων με μυκητοκτόνα που παράγονται από διάφορες αγροτικές μεταποιητικές βιομηχανίες. Παράλληλα αποδείχτηκε ότι οι βιοκλίνες υποστηρίζουν ένα ιδιαίτερα ανθετικό μικροβίωμα, του οποίου η σύσταση και δομή φαίνεται ότι επηρεάζεται από την επικράτηση μικροαερόφιλων συνθηκών οι οποίες επικράτησαν σταδιακά στο πληρωτικό υλικό. Τέλος, αναφέρουμε για πρώτη φορά την απομόνωση ενός μικροοργανισμού με την ικανότητα να αποδομεί το IMZ, που ταυτοποιήθηκε ως *C. herbarum* και διερευνήσαμε την δυνατότητα χρήσης του για την απορρύπανση αποβλήτων που περιέχουν IMZ.

ANNEX I - SUPPLEMENTARY DATA OF CHAPTER 2

Supplementary Table 2.S1 The parameters and equations used by each of the four kinetic models used for the calculation of the dissipation kinetic parameters of the studied pesticides

Model	Equation	Parameters
SFO	$M = M_0e^{-kt}$	M_0 : Initial concentration K : Rate constant
Hockey - Stick	$M = M_0e^{-kt}$ for $t \leq t_b$ $M = M_0e^{-k1tb}e^{-k2(t-tb)}$ for $t > t_b$	M_0 : Initial concentration K_1,K_2 : Rate constants t : Breaking point (time at which the rate constant changes)
FOMC	$M = M_0 / (t/\beta + 1)^a$	M_0 : Initial consentration α, β : Shape and location parameters
DFOP	$M = M_1e^{-k1t} + M_2e^{-k2t}$	M_1, M_2 : Amount of chemical applied to each compartment K_1, K_2 : Rate constant for each compartment

Supplementary Table 2.S2 The substrate:solution ratios and equilibration times used to assess the adsorption of each pesticide

Pesticide	**Biomixture**		**Soil**	
	Substrate:Solution	**Equilibration Time**	**Substrate:Solution**	**Equilibration Time**
CBX	1:20	24	1:10	12
MET-M	1:5	8	1:5	8
FLX	1:20	8	1:5	12
TBZ	1:50	8	1:50	8
CHT	1:200	12	1:50	12
FLD	1:200	8	1:25	8

ANNEX II - SUPPLEMENTARY DATA OF CHAPTER 2

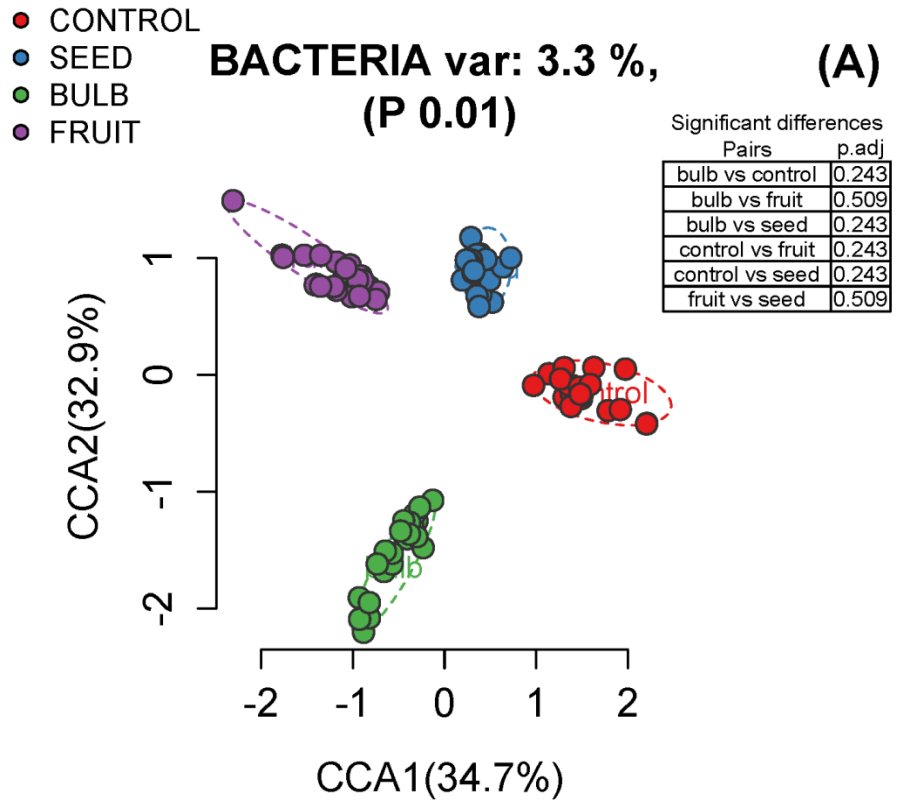
Supplementary Table 3.S1 . Amplicon library number, the sample code name and the forward (f) or reverse (r) primer index sequences (5' - 3') associated with them

Library	Sample ID	515f primer index	ITS4r primer index	Sample ID	515f primer index	ITS4r primer index
1st Library	T0 BMX A	TTCTTCTTCGT	TTATTACCGGA	T60 C1 50	TTCAATCGTGT	TTAGTACGTGA
	T0 BMX B	TTCTCAATGGT	TTATTAGGCGA	T60 C1 80	TTCAGGTATGT	TTAGATCCTGA
	T21 C1 20	TTGTCAGGTGT	TTATTGCGAGA	T60 C2 50	TTGTATCGAGT	TTAGATGAGGA
	T21 C1 50	TTGAAGTTCGT	TTATACTGGGA	T60 C2 80	TTGTGGTGTGT	TTAGACTACGA
	T21 C1 80	TTGCAACAAGT	TTATACCTCGA	T60 C3 50	TTGAGTCATGT	TTAGACATGGA
	T21 C2 20	TTGGACGACGT	TTATACGCAGA	T60 C3 80	TTGGTTGTCGT	TTAGAGTCAGA
	T21 C2 50	TTCTTCAAGGT	TTATAGACCGA	T60 B1 50	TATAAGCCAGT	TTAGCAGATGA
	T21 C2 80	TTCTCAGAAGT	TTATGTTCGGA	T60 B1 80	TTCTATGCAGT	TTAGCCTGTGA
	T21 C3 20	TTCAGTAAGGT	TTATGTGACGA	T60 B2 50	TTCAATGACGT	TTAGGTACAGA
	T21 C3 50	TTCGACAATGT	TTATGAAGGGA	T60 B2 80	TTCGTTCTAGT	TTAGGCGCCGA
	T21 C3 80	TTGTCGATAGT	TTATGAGCTGA	T60 B3 50	TTGTAATGGGT	TTCTTATGGGA
	T21 B1 20	TTGAAGGAAGT	TTATGCCATGA	T60 B3 80	TTGATAGCAGT	TTCTTACTCGA
	T21 B1 50	TTGCAGTATGT	TTATGGTGTGA	T60 S1 50	TTGAGAGTGGT	TTCTTAGCAGA
	T21 B1 80	TATATCAGGGT	TTAATTCGCGA	T60 S1 80	TTGGTATGAGT	TTCTTCAGTGA
	T21 B2 20	TTCTTGTCAGT	TTAATCCAGGA	T60 S2 50	TATAGTCTCGT	TTCTTCGACGA
	T21 B2 50	TTCATATGGGT	TTAATCGGTGA	T60 S2 80	TTCTAACAGGT	TTCTTGAAGGA
	T21 B2 80	TTCAGACTTGT	TTAATGTGGGA	T60 S3 50	TTCAACTAGGT	TTCTTGGTTGA
	T21 B3 20	TTCGAGCACGT	TTAATGCCTGA	T60 S3 80	TTCGTTGGTGT	TTCTATTCCGA
	T21 B3 50	TTGTGTATCGT	TTAATGGACGA	T60 F1 50	TTGTAAGTCGT	TTCTATAGGGA
	T21 B3 80	TTGACTATGGT	TTAACTTCCGA	T60 F1 80	TTGATCTTGGT	TTCTAACAGGA
	T21 S1 20	TTGCCTAGTGT	TTAACTAGGGA	T60 F2 50	TTGAGCCTCGT	TTCTACCGAGA

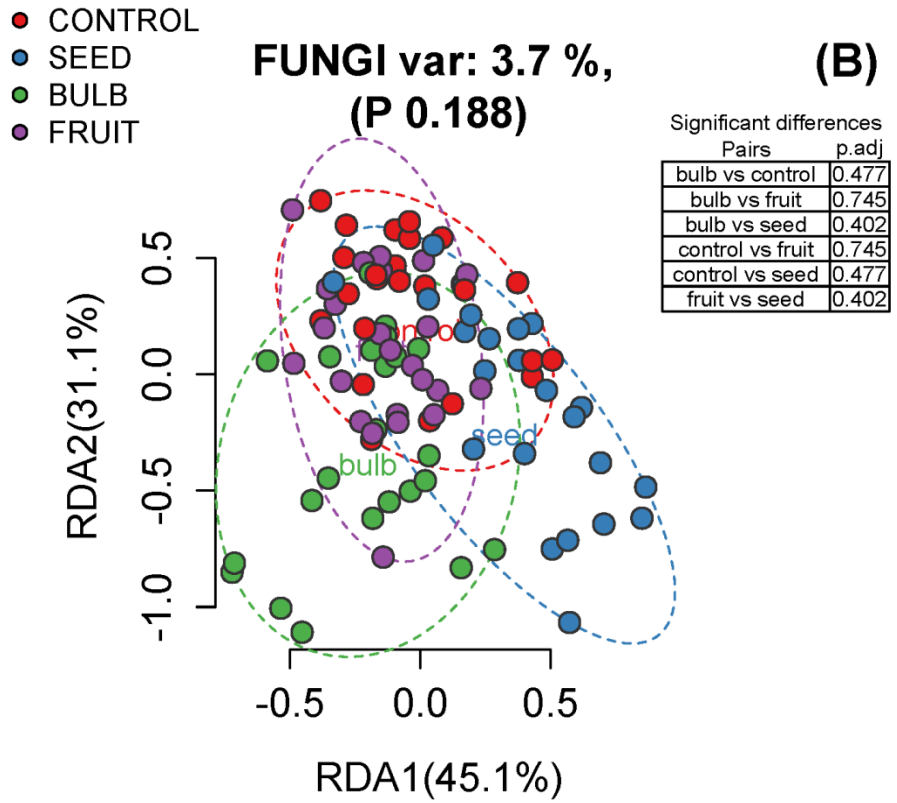
	T21 S1 50	TATATCGTCGT	TTAACAGTCGA	T60 F2 80	TTGGTCTATGT	TTCTAGTTGGA
	T21 S1 80	TTCTTGAGTGT	TTAACCTTGGA	T60 F3 50	TATAGACAGGT	TTCTAGCCTGA
	T21 S2 20	TTCATAGTCGT	TTAACCGAAGA	T60 F3 80	TTCTAGTTGGT	TTCTAGGAAGA
	T21 S2 50	TTCAGAGGAGT	TTAACGACAGA	T109 C1 20	TTCGTGATCGT	TTCTGACGTGA
	T21 S2 80	TTGTTCAGAGT	TTACTTACGGA	T109 C1 50	TTGTCTTCAGT	TTCTGCTCAGA
	T21 S3 20	TTGTGTGAAGT	TTACTTGTCGA	T109 C1 80	TTGATGAGGGT	TTCATTGTGGA
	T21 S3 50	TTGACGTGAGT	TTACTAGAGGA	T109 C2 20	TTGCATAAGGT	TTCATCTTCGA
	T21 S3 80	TTGCCTCACGT	TTACTCTGAGA	T109 C2 50	TTGGAACTTGT	TTCATGTCAGA
	T21 F1 20	TATATGCACGT	TTACTCCTTGA	T109 C2 80	TATAGCAACGT	TTCAGTTACGA
	T21 F1 50	TTCTTGGACGT	TTACTGGCAGA	T109 C3 20	TTCTCTAACGT	TTCAGTCCTGA
	T21 F1 80	TTCATCACAGT	TTACATTGCGA	T109 C3 50	TTCAAGGTTGT	TTCAGATTGGA
	T21 F2 20	TTCAGCAGTGT	TTACAGTAGGA	T109 C3 80	TTCGATGTGGT	TTCAGAAGAGA
	T21 F2 50	TTGTTCGTTGT	TTACAGGTTGA	T109 B1 20	TTGTCTCTTGT	TTCAGCTGTGA
	T21 F2 80	TTGTGACTAGT	TTACCTAACGA	T109 B1 50	TTGAACTCAGT	TTCAGGCTAGA
	T21 F3 20	TTGACGAATGT	TTACCTCTAGA	T109 B1 80	TTGCATGTTGT	TTCCTTCATGA
	T21 F3 50	TTGCCAATCGT	TTACCTGGTGA	NTC 1st A	TTGGACATAGT	TTCCTAATGGA
	T21 F3 80	TATAACGAGGT	TTACCATCGGA	NTC 1st B	TATAGGATGGT	TTCCTACGAGA
2nd Library	T109 B2 20	TTCTTCTTCGT	TTATTACCGGA	T109 S3 80	TTGCAGTATGT	TTATGGTGTGA
	T109 B2 50	TTCTCAATGGT	TTATTAGGCGA	T109 F1 20	TATATCAGGGT	TTAATTCGCGA
	T109 B2 80	TTCAGTTCAGT	TTATTCTCCGA	T109 F1 50	TTCTTGTCAGT	TTAATCCAGGA
	T109 B3 20	TTCGAATCAGT	TTATTCGTGGA	T109 F1 80	TTCATATGGGT	TTAATCGGTGA
	T109 B3 50	TTGTCAGGTGT	TTATTGCGAGA	T109 F2 20	TTCAGACTTGT	TTAATGTGGGA
	T109 B3 80	TTGAAGTTCGT	TTATACTGGGA	T109 F2 50	TTCGAGCACGT	TTAATGCCTGA
	T109 S1 20	TTGCAACAAGT	TTATACCTCGA	T109 F2 80	TTGTGTATCGT	TTAATGGACGA
	T109 S1 50	TTGGACGACGT	TTATACGCAGA	T109 F3 20	TTGACTATGGT	TTAACTTCCGA
	T109 S1 80	TTCTTCAAGGT	TTATAGACCGA	T109 F3 50	TTGCCTAGTGT	TTAACTAGGGA

	T109 S2 20	TTCTCAGAAGT	TTATGTTCGGA	T109 F3 80	TATATCGTCGT	TTAACAGTCGA
	T109 S2 50	TTCAGTAAGGT	TTATGTGACGA	NTC 2nd A	TTCATCACAGT	TTACATTGCGA
	T109 S2 80	TTCGACAATGT	TTATGAAGGGA	NTC 2nd B	TTCAGCAGTGT	TTACAGTAGGA
	T109 S3 20	TTGTCGATAGT	TTATGAGCTGA	T109 F1 20	TATATCAGGGT	TTAATTCGCGA
	T109 S3 50	TTGAAGGAAGT	TTATGCCATGA	T109 F1 50	TTCTTGTCAGT	TTAATCCAGGA

Supplementary Figure 3.S1 Canonical Correspondence Analysis (CCA) and redundancy analysis (RDA) of the bacterial (A) and fungal (B) communities colonizing the different horizons of the biobed systems treated with water (control), wastewater from seed producing industries (seed), wastewater from bulb handling industries (bulb) or wastewaters from fruit packaging industries. Samples were ordinated according to origin of the wastewater treatment. Inserted tables display pairwise comparisons of the microbial communities in the different treatments

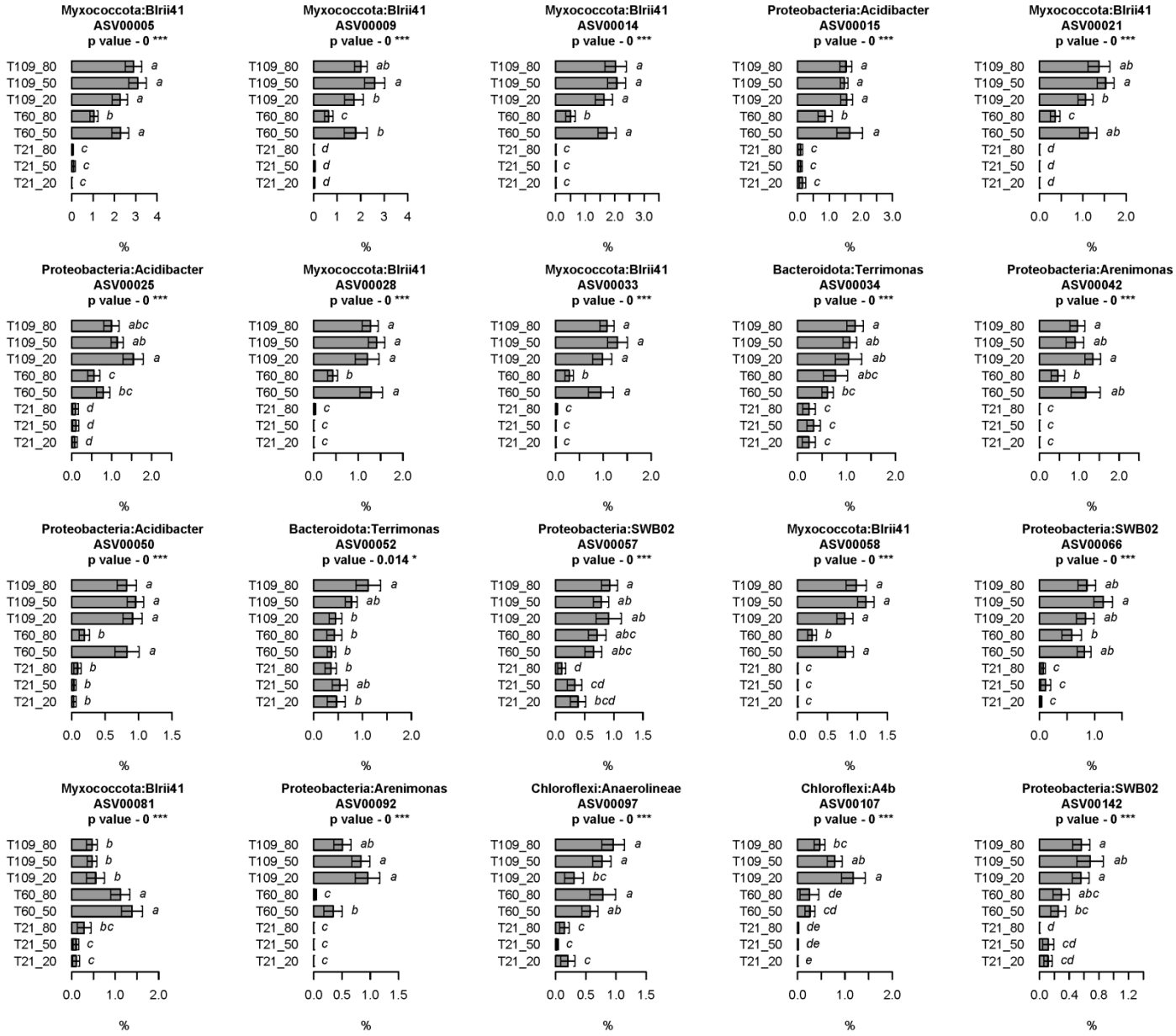


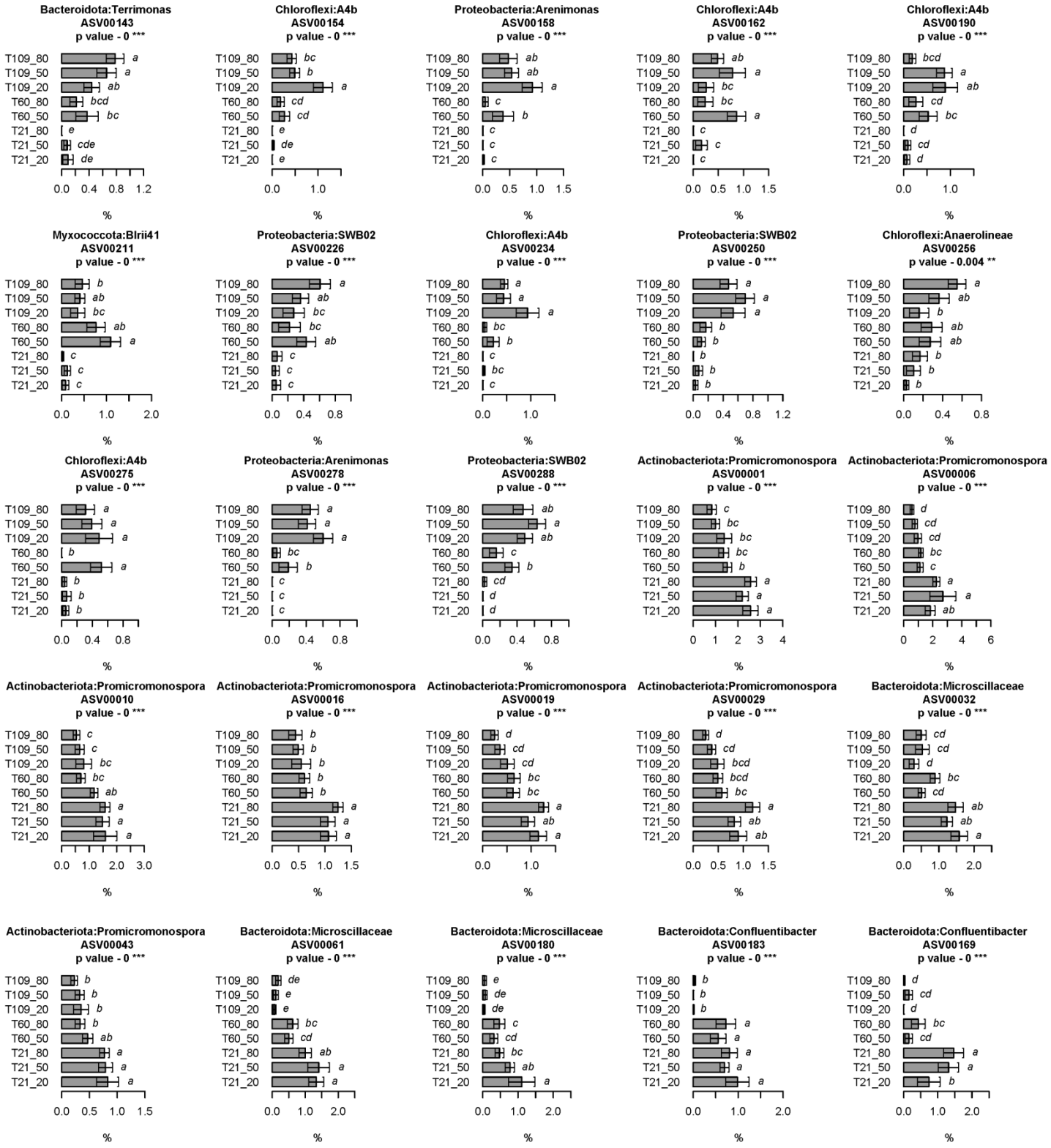
Significant differences	
Pairs	p.adj
bulb vs control	0.243
bulb vs fruit	0.509
bulb vs seed	0.243
control vs fruit	0.243
control vs seed	0.243
fruit vs seed	0.509



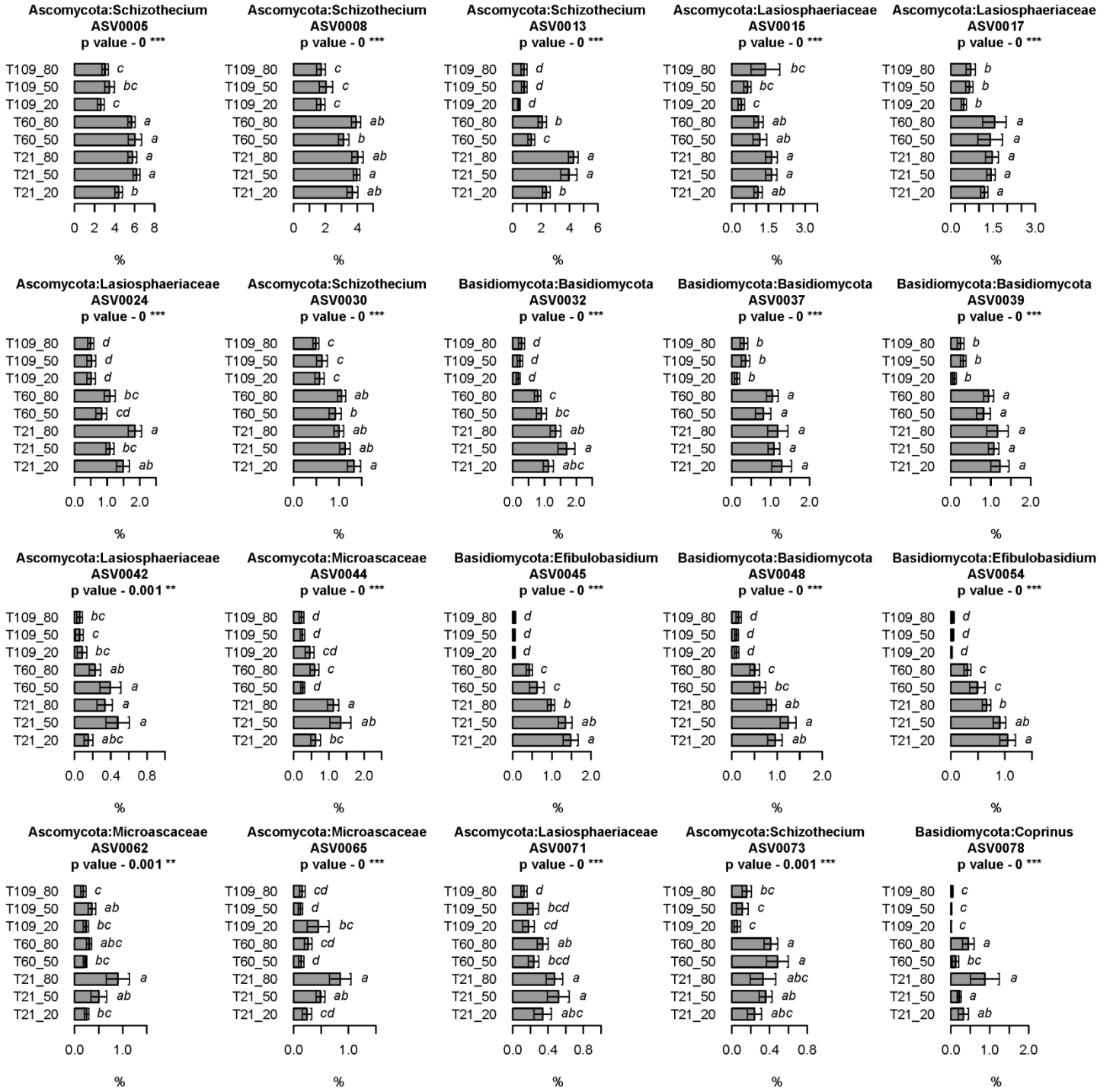
Significant differences	
Pairs	p.adj
bulb vs control	0.477
bulb vs fruit	0.745
bulb vs seed	0.402
control vs fruit	0.745
control vs seed	0.477
fruit vs seed	0.402

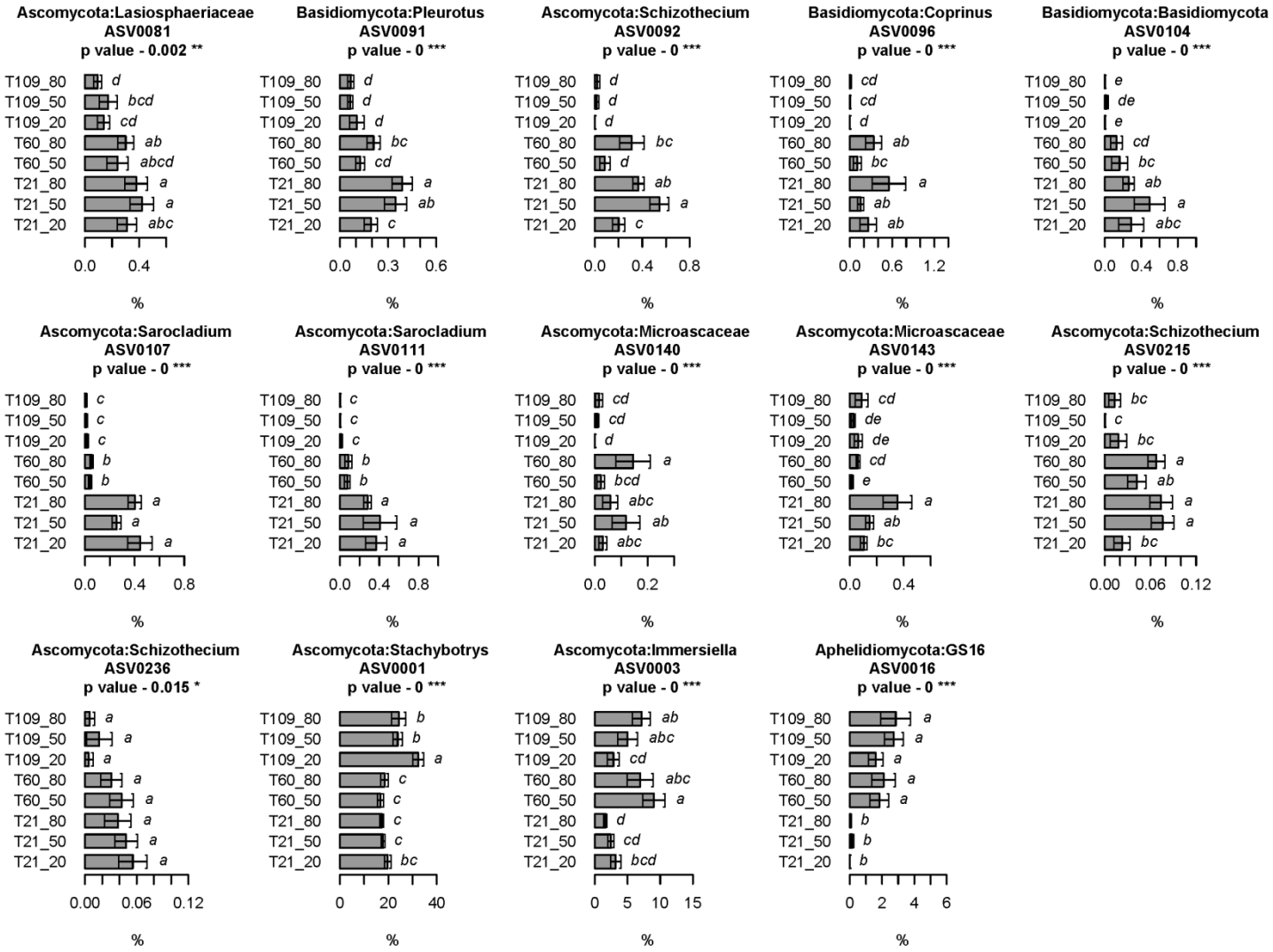
Supplementary Figure 3.S2 Barplots of bacterial ASVs that exhibit significant temporal patterns in their relative abundance. Each bar is the mean of 12 replicates ± standard error. Bars designated by the same letter indicate significant differences at the 5% level.



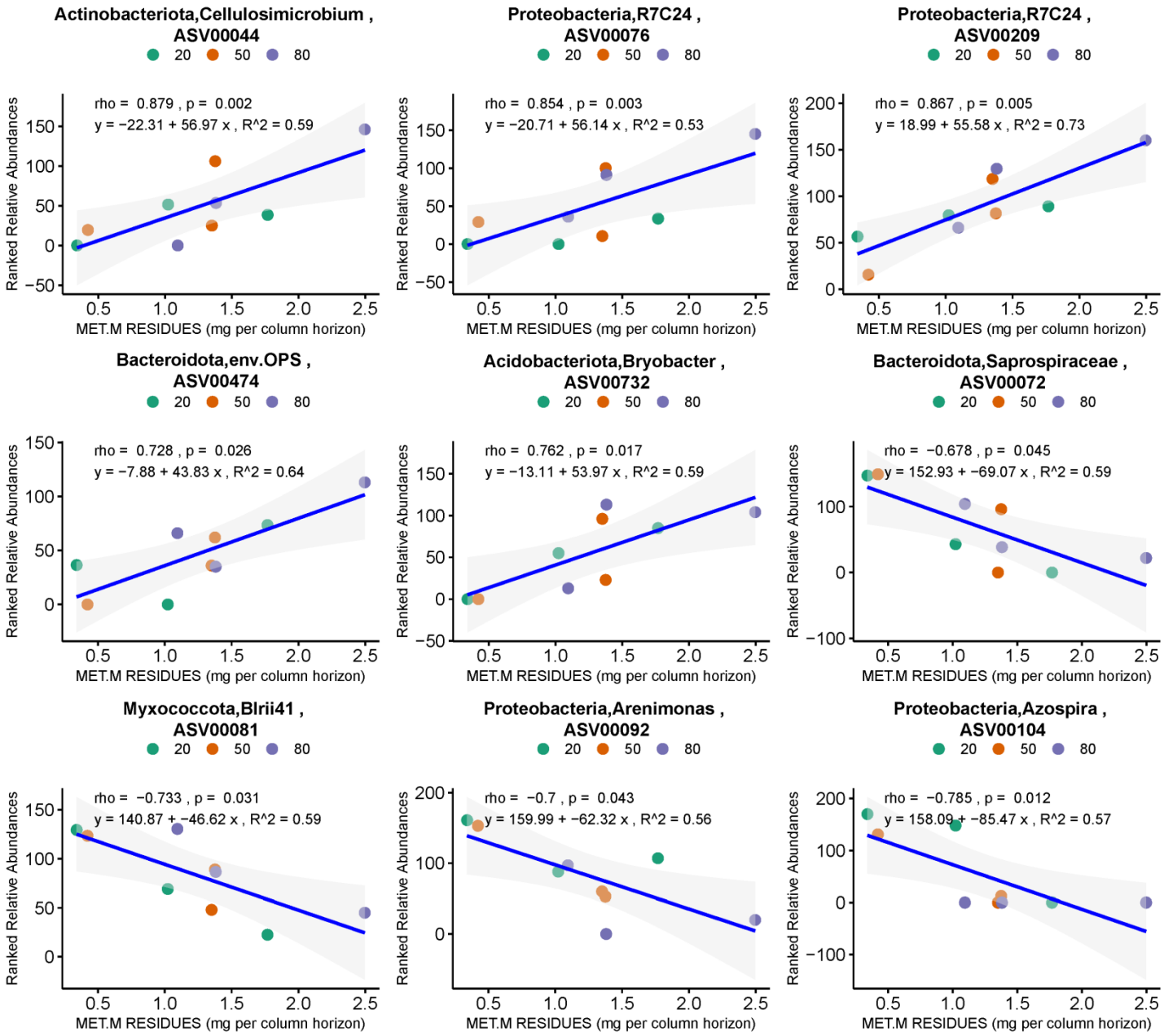


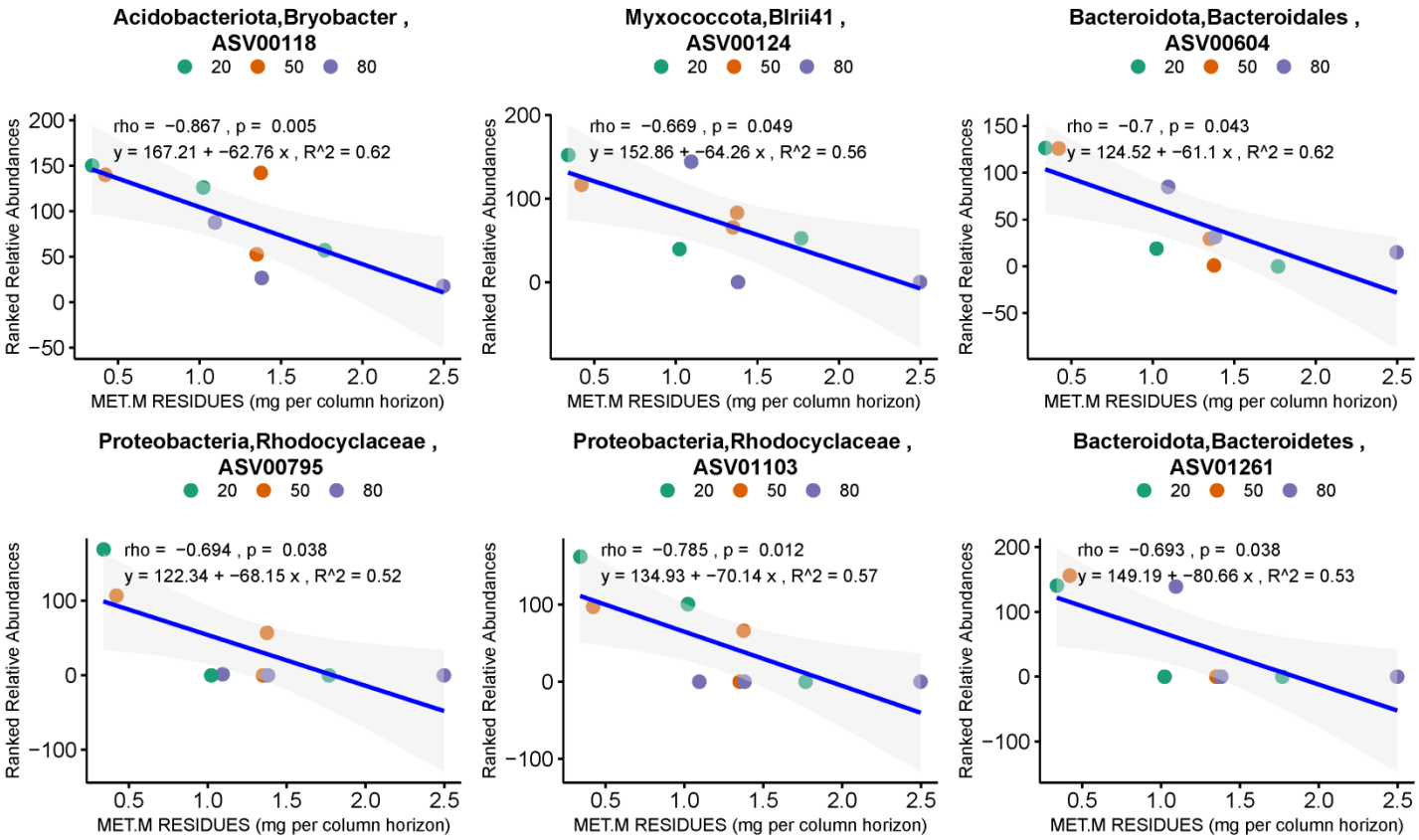
Supplementary Figure 3.S3 Barplots of fungal ASVs that exhibit significant temporal patterns in their relative abundance. Each bar is the mean of three replicates ± standard error. Bars designated by the same letter indicate significant differences at the 5% level

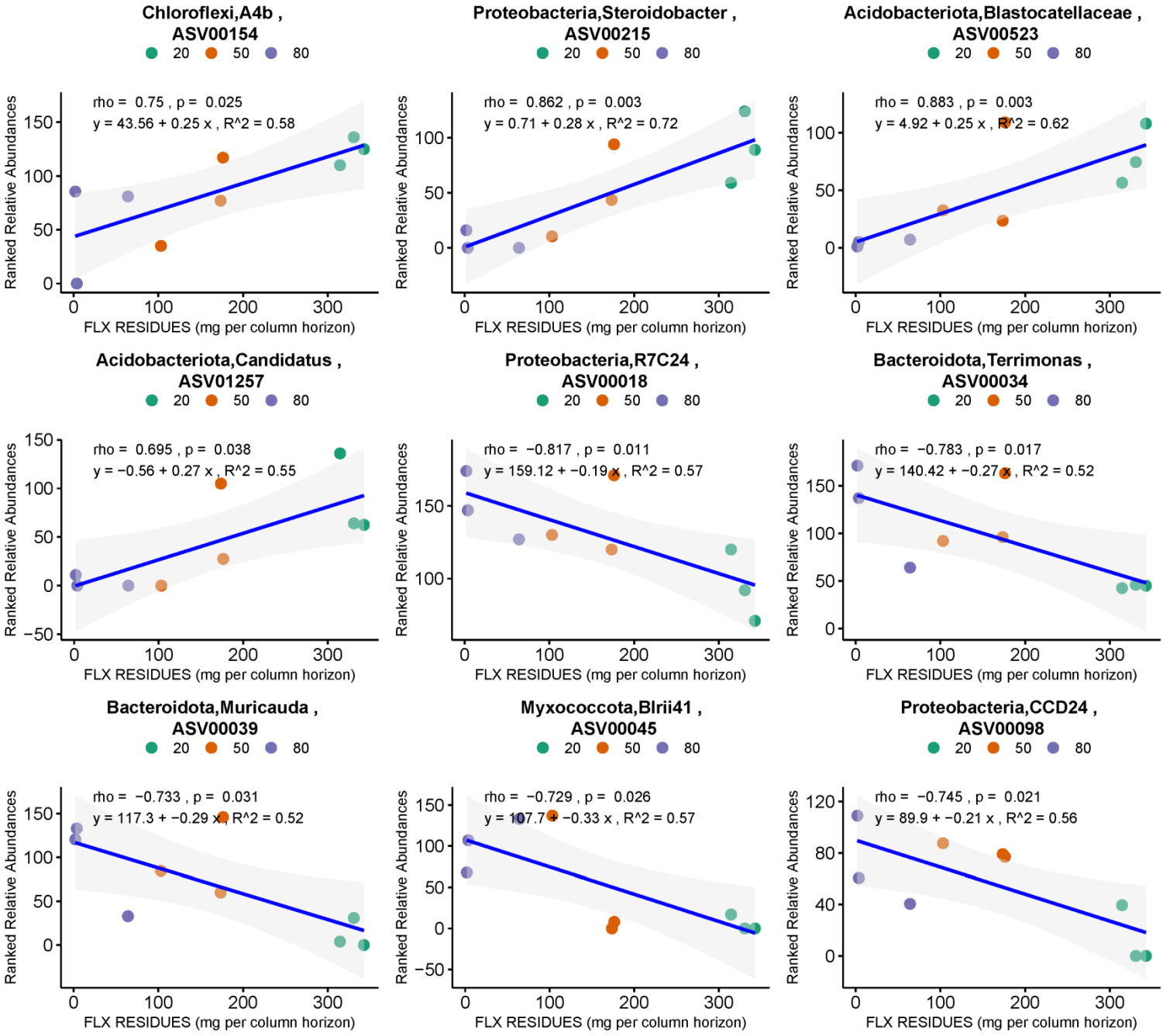


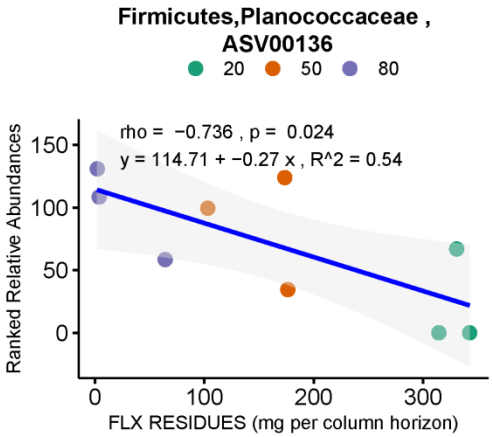


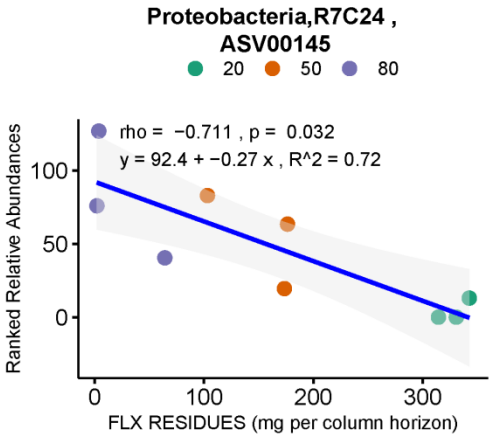
Supplementary Figure 3.S4 Scatterplots of bacterial ASVs whose relative abundance showed significant positive or negative correlation with the residues of metalaxyl M (MET-M) and fluxapyroxad (FLX) in columns receiving wastewaters from the seed producing industry (SPI).

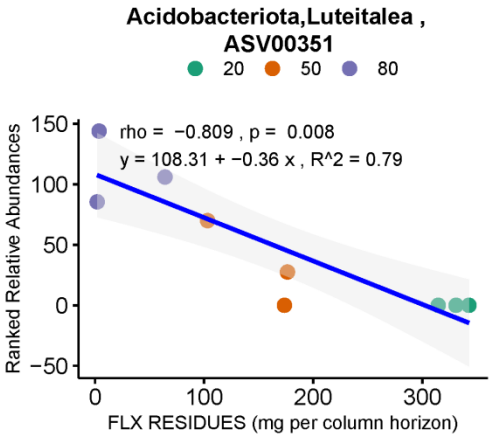


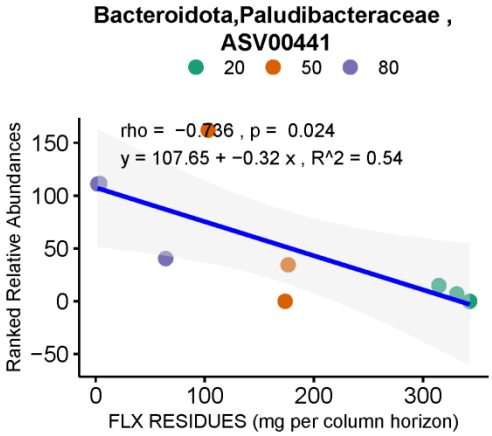


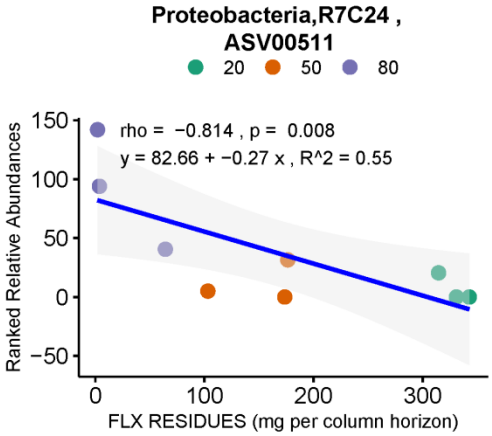


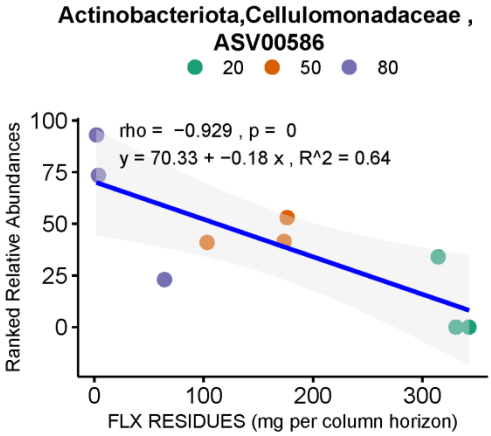




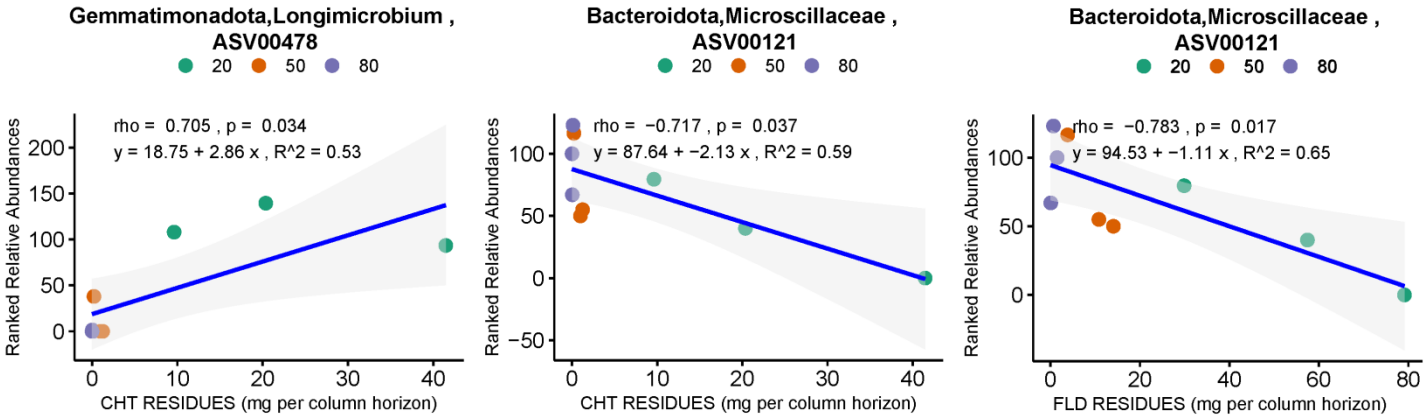




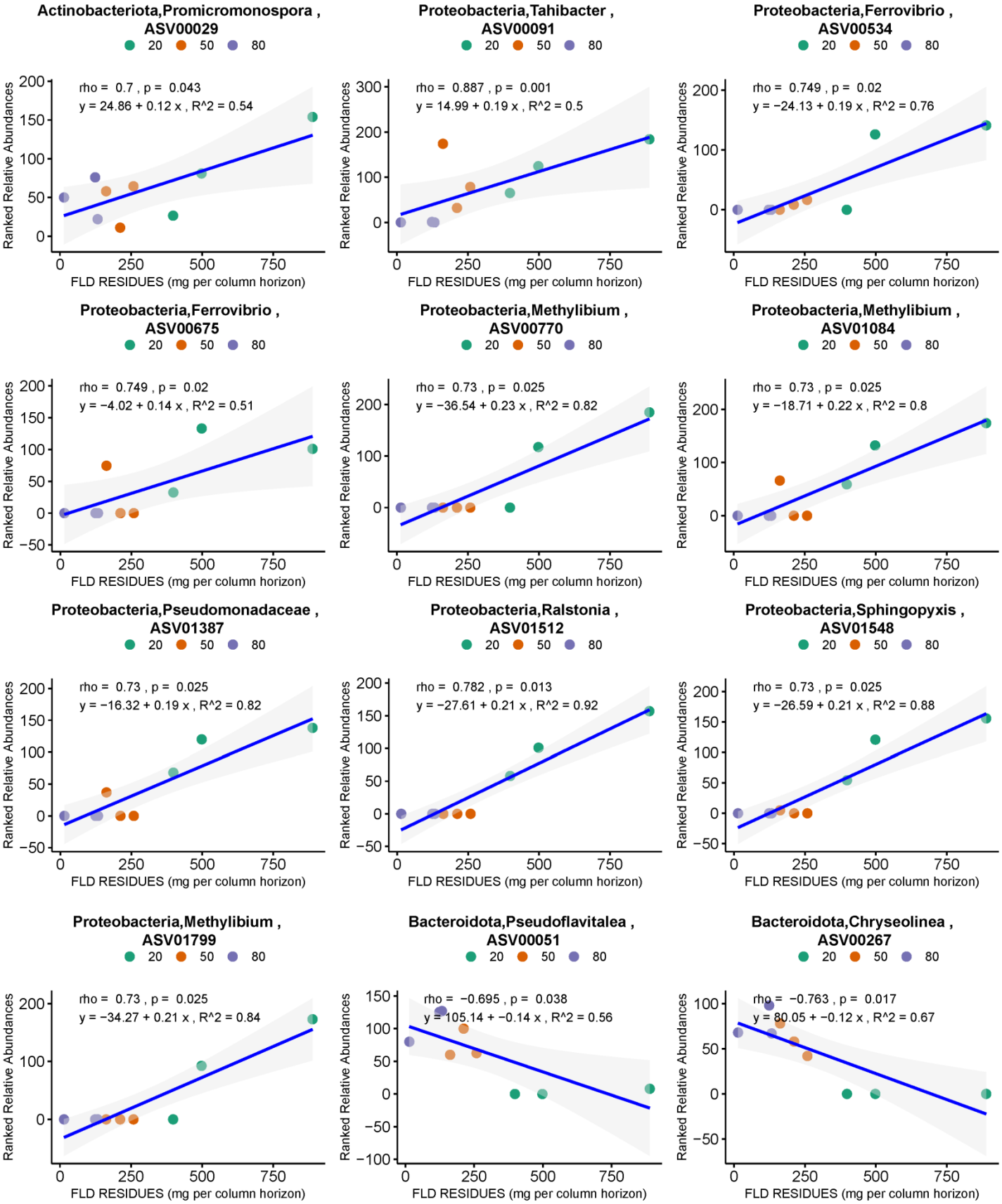


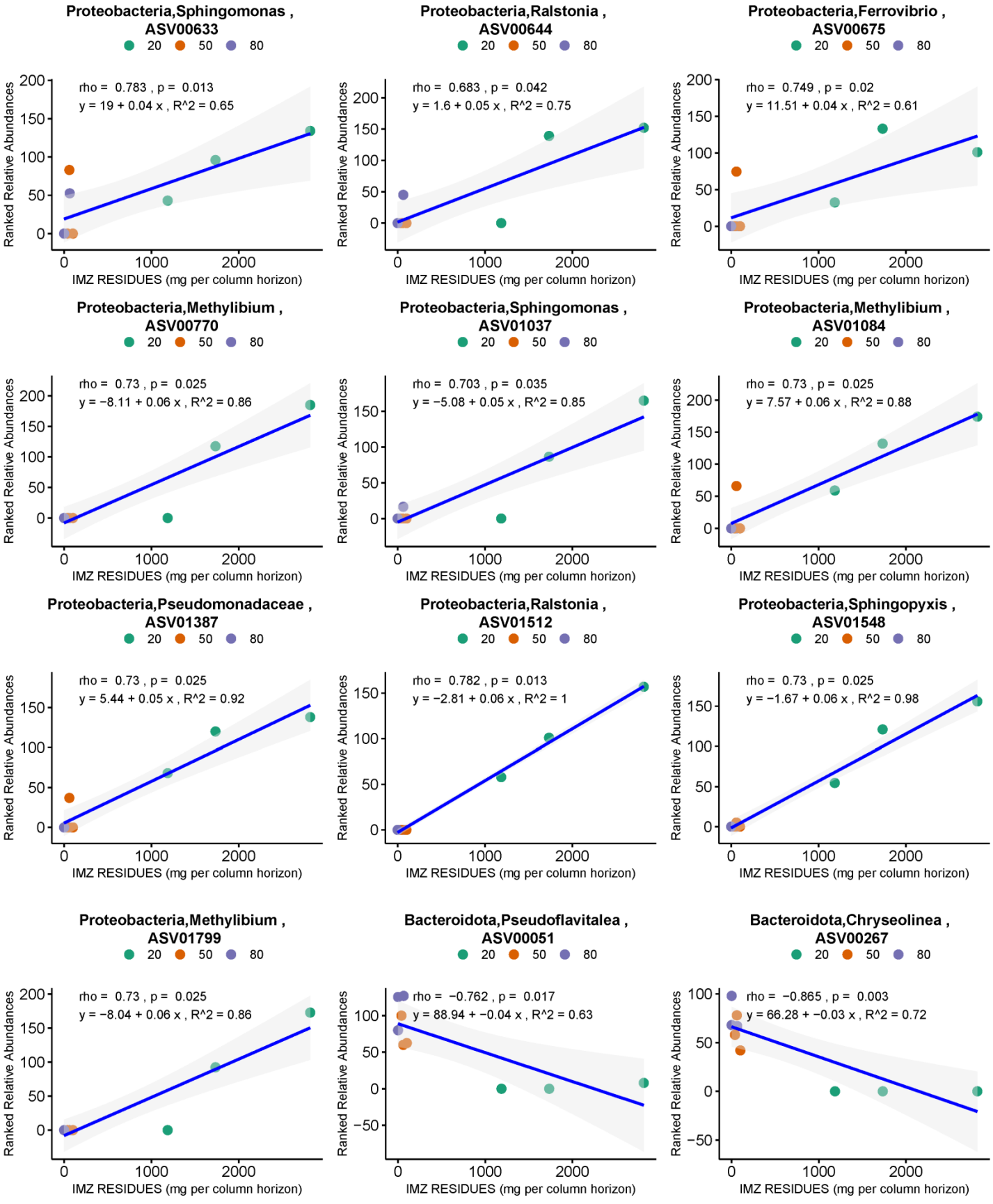


Supplementary Figure 3.S5 Scatterplots of bacterial ASVs whose relative abundance showed significant positive or negative correlation with the residues of Chlorothalonil (CHT) and fludioxonil FLD in columns receiving wastewaters from the bulb handling industry (BHI).

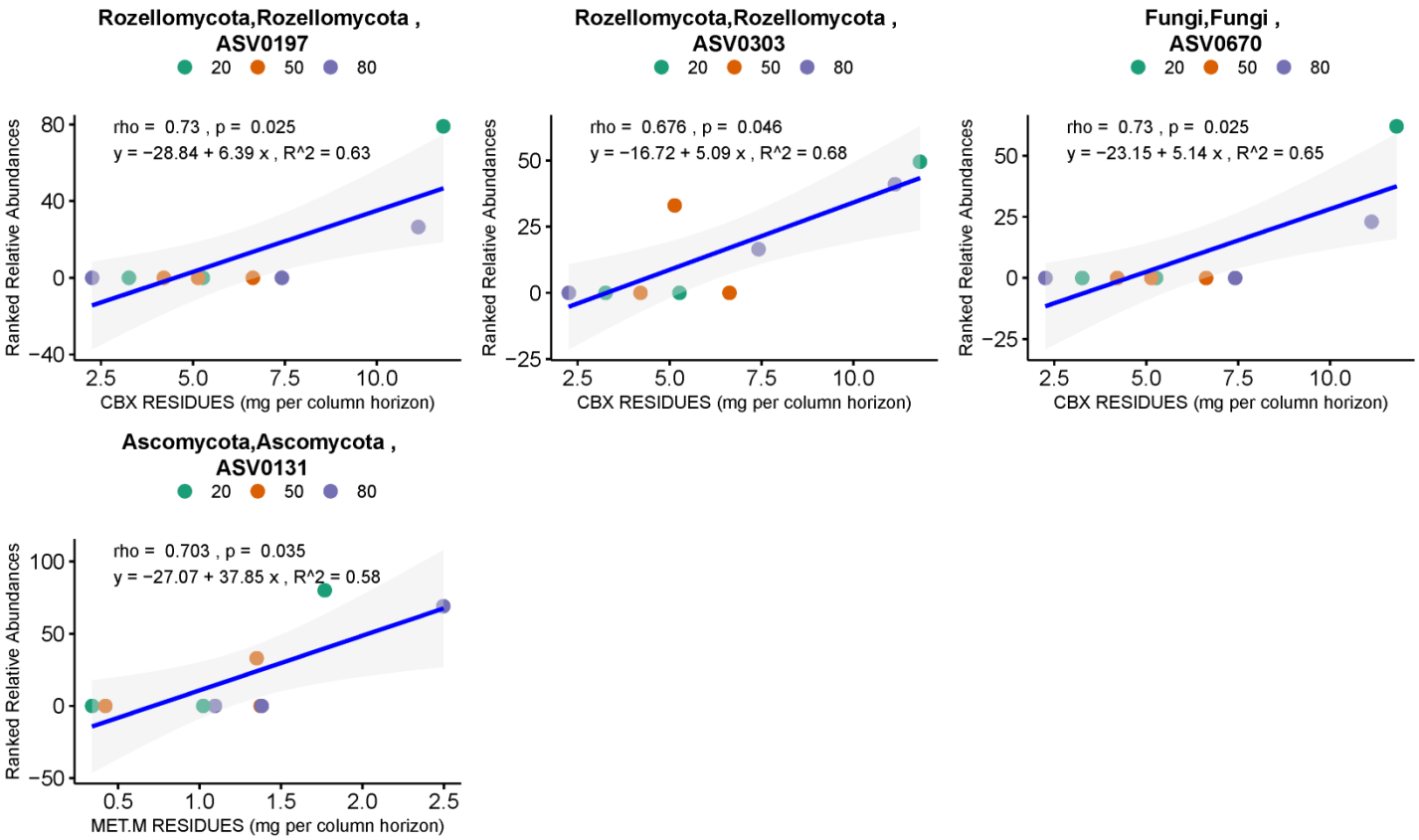


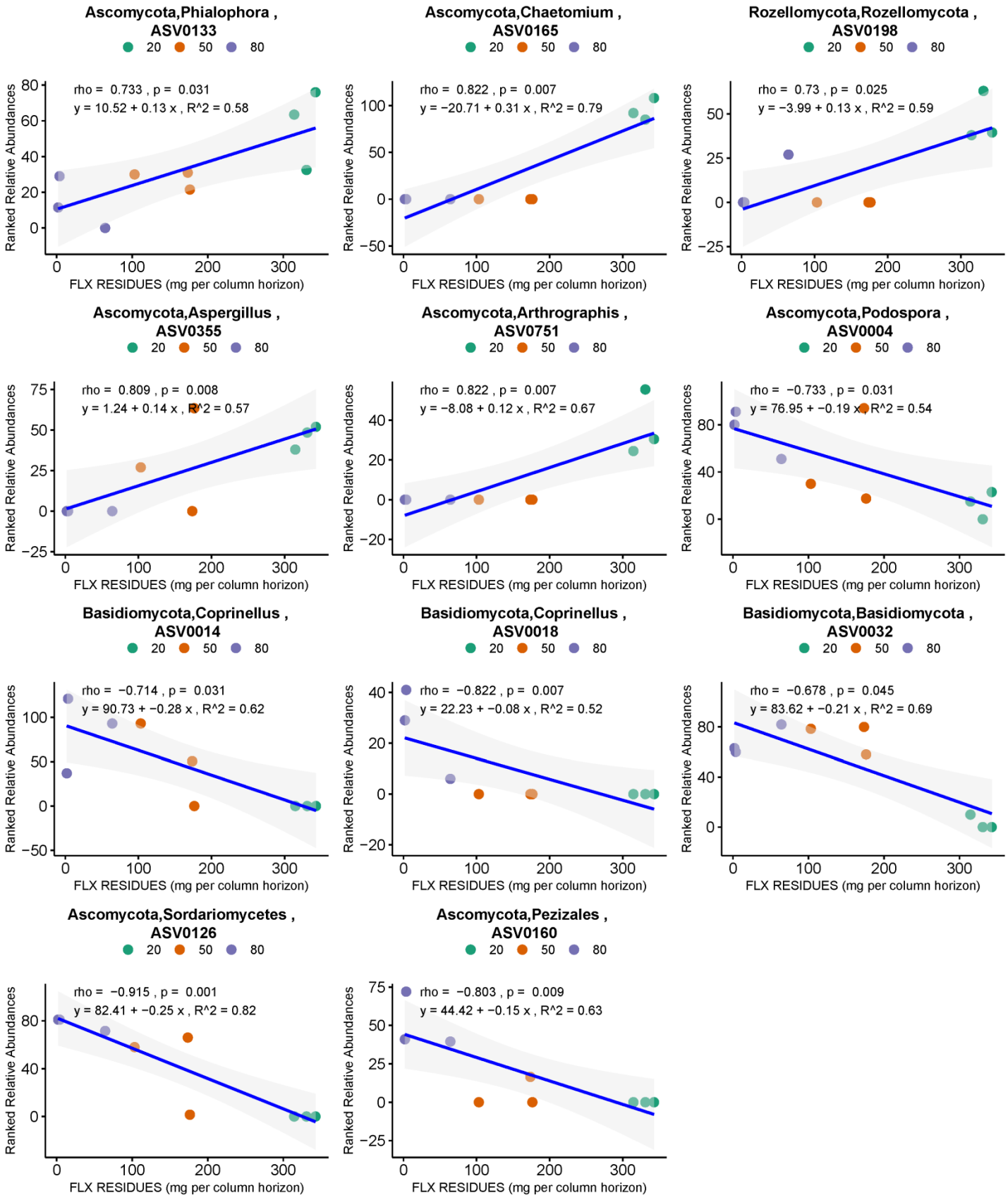
Supplementary Figure 3.S6 Scatterplots of bacterial ASVs whose relative abundance showed significant positive or negative correlation with the residues of fludioxonil (FLD) and imazalil (IMZ) in columns receiving wastewaters from the fruit packaging industry (FPI)



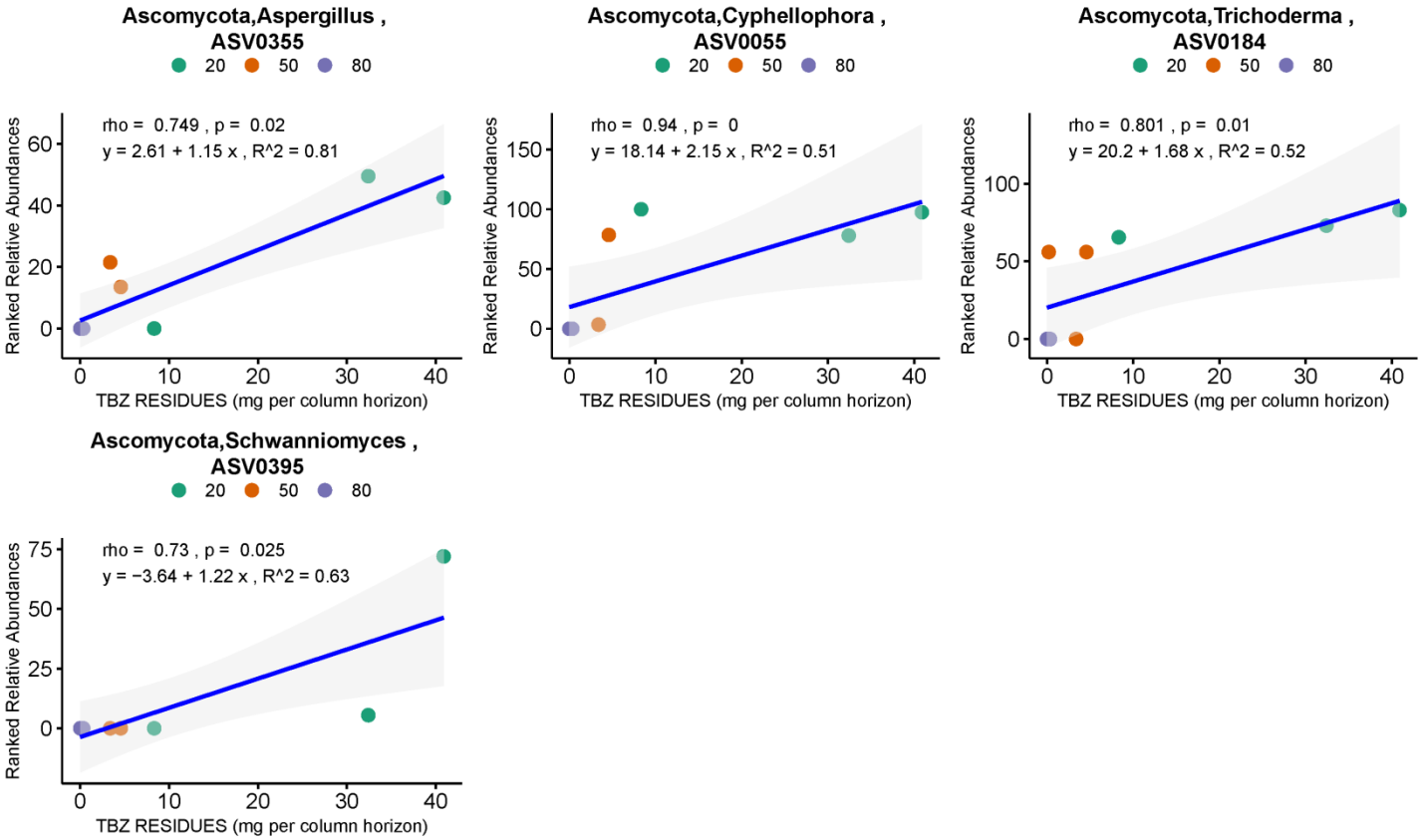


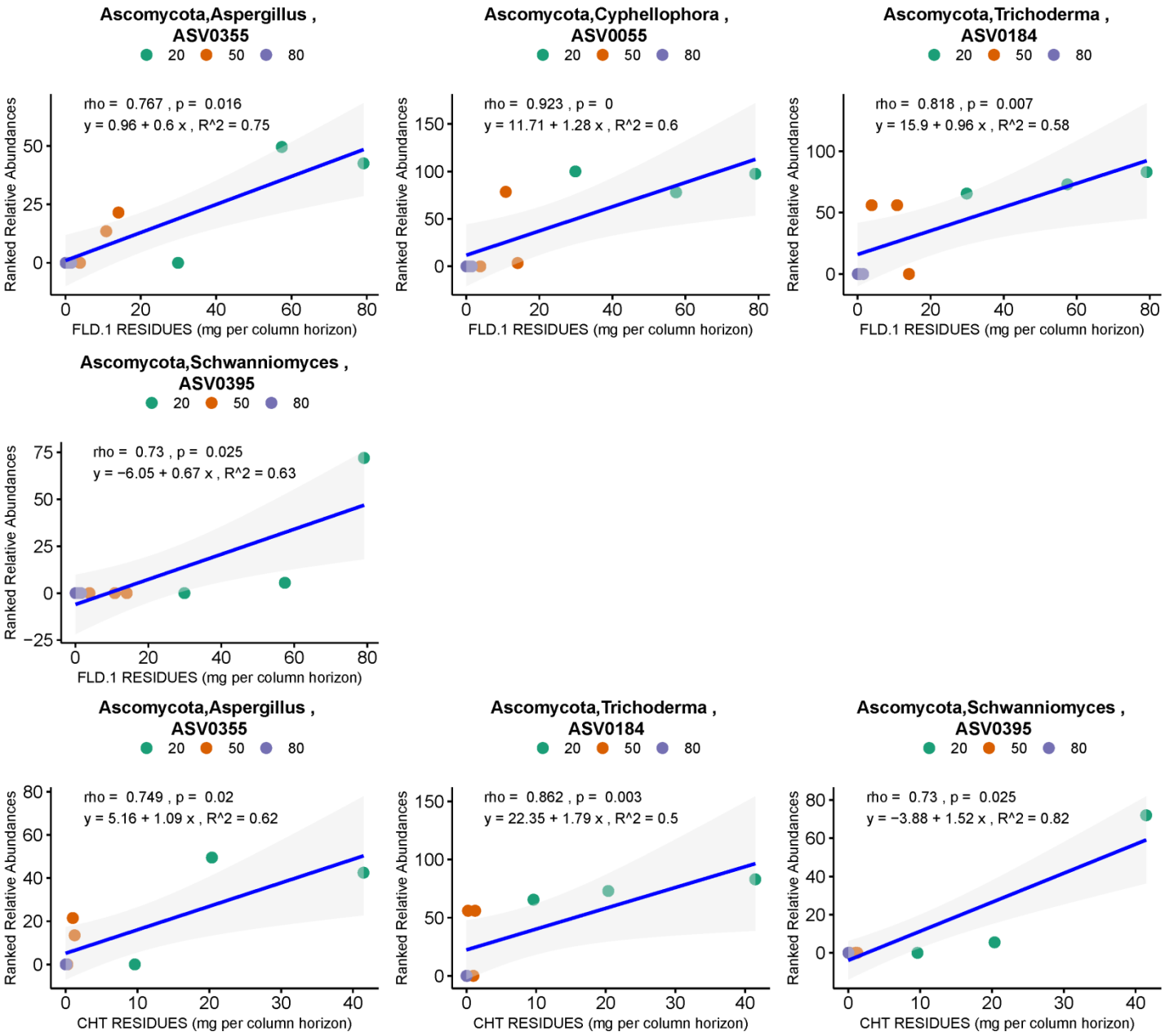
Supplementary Figure 3.S7 Scatterplots of fungal ASVs whose relative abundance showed significant positive or negative correlation with the residues of carboxin (CBX), metalaxyl M (MET-M) and fluxapyroxad (FLX) in columns receiving wastewaters from the seed producing industry (SPI)



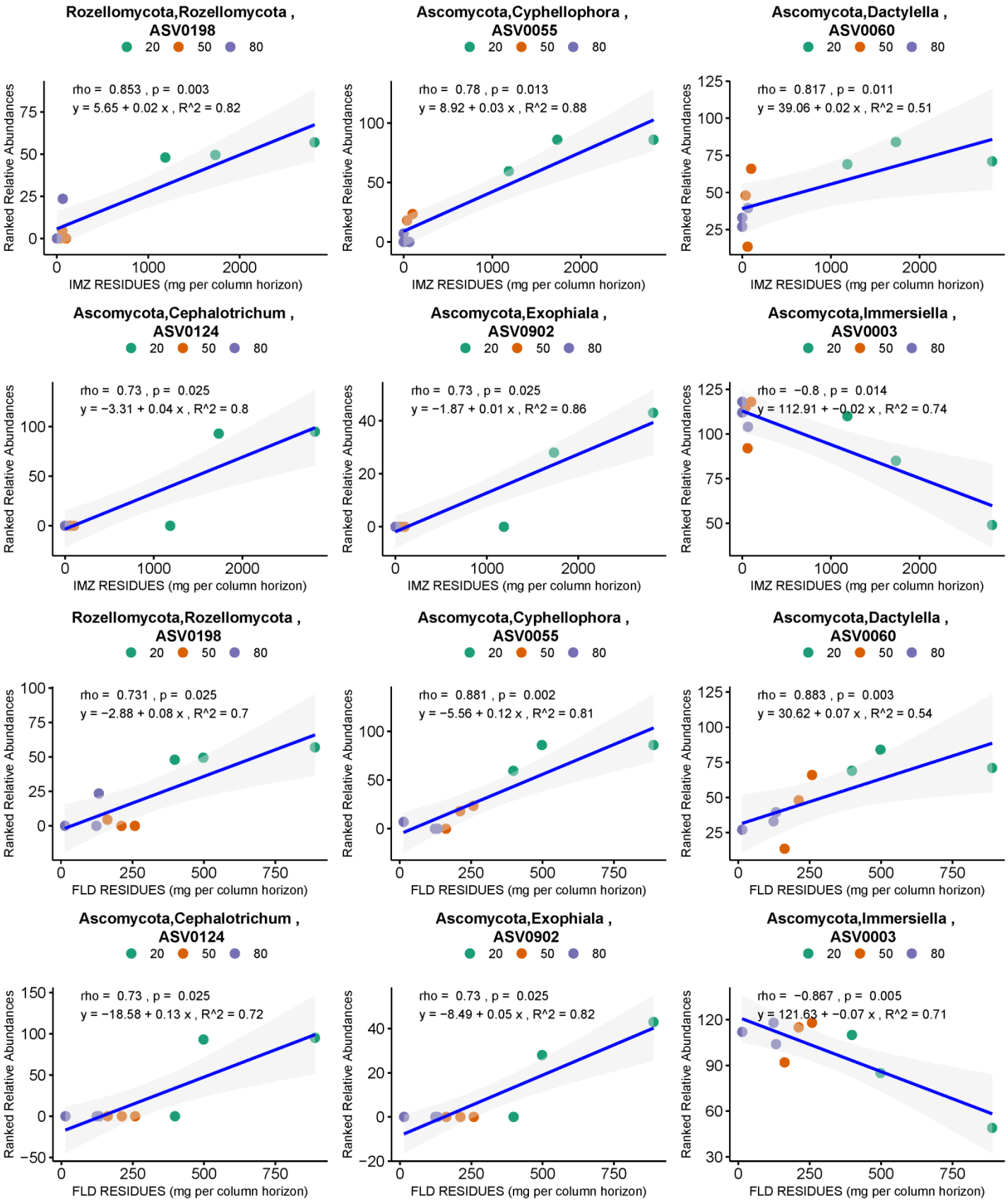


Supplementary Figure 3.S8 Scatterplots of fungal ASVs whose relative abundance showed significant positive or negative correlation with the residues of thiabendazole (TBZ), fludioxonil (FLD) and chlorothalonil (CHT) in columns receiving wastewaters from the bulb handling industry (BHI).

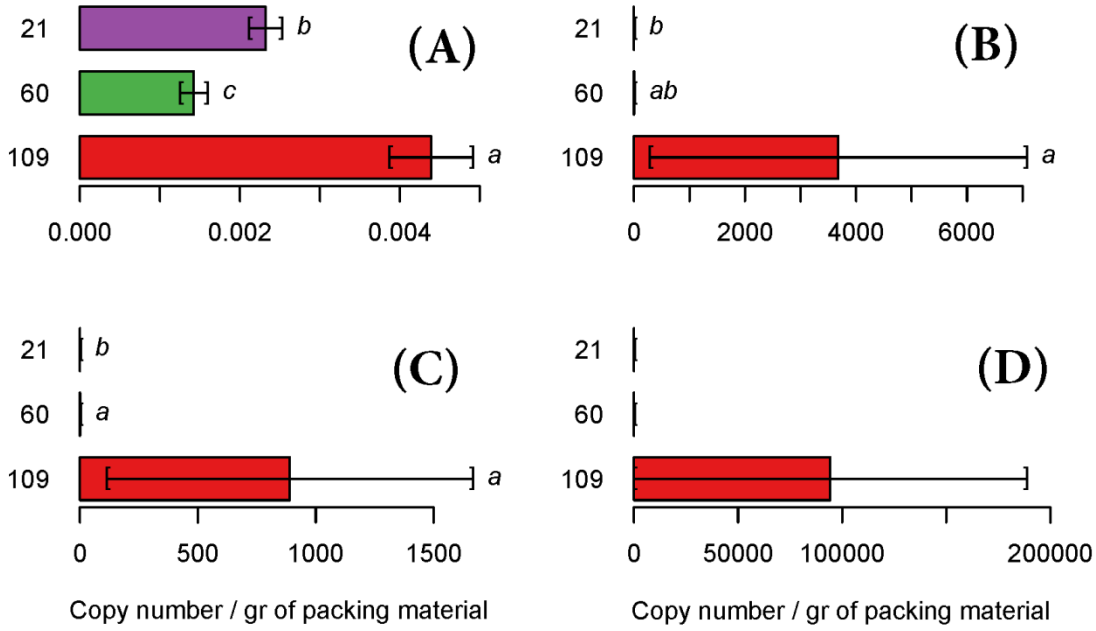




Supplementary Figure 3.S9 Scatterplots of fungal ASVs whose relative abundance showed significant positive or negative correlation with the residues of imazalil (IMZ) and fludioxonil (FLD) in columns receiving wastewaters from the fruit packaging industry



Supplementary Figure 3.S10 The temporal changes in the relative abundance of (A) *intI1*, (B) *IS*1071, (C) *korB* and (D) *trfA* (expressed as copy numbers per gram of biobed packing material (dry weight) normalized to the copy numbers of the 16S rRNA gene) in biobeds, regardless of wastewater treatment and biobed horizon. Each bar represents the mean of 36 samples ± standard error. Bars designated by the same lower case letters are not significantly different at the 5% level.



Annex III - SUPPLEMENTARY DATA OF CHAPTER 4

Supplementary Table 4.S1 The primers, sequences and thermocycling conditions used in the current study

Primer	Thermocycling Conditions	Sequence (5' – 3')	Use	Reference
ITS1F ITS4	95°C for 5 min; 95°C for 30 sec, 55°C for 30sec, 72°C for 1 min (35 cycles); 72°C for 10 min	TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC	Molecular identification of fungal isolate	(White et al., 1990)
FF390 FR1	95°C for 3 min; 95°C for 15 sec, 50°C for 35sec, 72°C for 10 sec (40 cycles); **Melting curve**: 65-95°C, increments of 0.1°C sec-1	CGA TAA CGA ACG AGA CCT AIC CAT TCA ATC GGT AIT	q-PCR fungal community in bioreactor	(Prévost-Bouré et al., 2011)
Eub338 Eub518	95°C for 3 min; 95°C for 15 sec, 60°C for 20sec (35 cycles); **Melting curve**: 65-95°C, increments of 0.1°C sec-1	ACT CCT ACG GGA GGC AGC AG ATT ACC GCG GCT GCT GG	q-PCR bacterial community in the bioreactor	(Fierer et al., 2005)
P-ITS1 P-ITS4	94°C for 3min; 94°C for 30 sec, 53°C for 40sec, 72°C for 1 min (30 cycles); 72°C for 10 min	TCC GTA GGT GAA CCT GCG G TCC TCC GCT TAT TGA TAT GC	Amplicon sequencing analysis fungal community	(White et al., 1990)
515f 806r	98°C for 10 s, 50°C for 30 s, 72°C for 30 s (25 + 7 cycles) [b]; 72°C for 5 min	NNNNNNNNNGTGTGYCAGCMGCCGCGGTAA[a] GGACTACNVGGGTWTCTAAT	Amplicon sequencing analysis bacterial community	(Walters et al., 2016)

[a] The sample index (consecutive Ns) and linker (bold letters) prior to the extension bases in the forward or reverse primer are indicated.

[b] The first number in parentheses indicates the number of cycles performed in the first PCR where the unindexed primers were used, while the second number indicates the additional cycles performed in the sample indexing PCR.

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Home Address	Markt 2, 6701CZ, Wageningen, Netherlands
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EDUCATION

2017-2022 PhD degree at the laboratory of Plant and Environmental Biotechnology, Department of Biochemistry and Biotechnology, University of Thessaly
Dissertation Title: *"Study on the depuration efficiency and microbiology of biobed systems which receive pesticide-contaminated wastewaters from agrofood processing units"*

2016-2017 Master's Degree at Biotechnology – Nutrition and Environment, Department of Biochemistry and Biotechnology, University of Thessaly.
Title of Master's thesis: "*Mapping of the main soilborne plant pathogenic fungi in soil from protected crops from Crete, Peloponnese and western Greece"*
Graduation Grade: 8.5

2010-2015 Bachelor studies at University of Thessaly, Department of Biochemistry and Biotechnology
Title of thesis: "*The effect of fumigant formulation PALADIN® in the population of selected soilborne phytopathogenic fungi"*
Graduation grade: 7,96

SCHOLARSHIP

24/04/2018-24/04/2021 State Scholarships Foundation (IKY), Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project "Strengthening Human Resources Research Potential via Doctorate Research" (MIS-5000432)

WORK EXPERIENCE

11-13/10/2018 Volunteering 1) as an Evaluation Coordinator, 2) in the preparation of an exhibit about the history of chocolate and 3) participating at the exhibit of the laboratory of Plant and Environmental Biotechnology at the first Thessaly Science Festival, in The Mill of Pappas, Larissa.

2017	Volunteering in the project "Biology goes to kindergarten". A project about visiting kindergarten and elementary schools to educate students about the researcher's job.
01/09/2014-28/02/2015	Participation on the Erasmus Plus European Programme in the laboratory of Phytopathology in Wageningen University, Netherlands. Performed study on "*Bacterial endophytes of tomato leaves: 1) Antifungal properties and 2) Influence of leaf health on microbiome"*

PERSONAL SKILLS AND COMPETENCES

Technical Skills	Molecular Techniques: Polymerase Chain Reaction (PCR, Real time PCR), Construction of Clone Libraries, Ligation Reaction, Transformation of Bacterial Cells, DNA Clean Up from PCR and agarose gel, Agarose Gel Preparation and DNA Electrophoresis, Preparation of multiplex amplicon sequencing libraries using barcoded primers Classical Microbiology: Culture Media Preparation and Inoculation, Isolation of Pure cultures, Isolation of microorganisms from various environmental habitats Microscopy: Optical microscopy Analytical Techniques: Liquid-liquid and solid-liquid extraction of organic molecules, Use of Rotary evaporator, Use of HPLC/DAD system
Computer Skills:	Basic knowledge of Bioinformatics: Analysis of Illumina DNA sequencing data, Accompliced user of R, Competent user of Bash scripting / Linux terminal Excellent use of MS Office's Word, Excel, Powerpoint
Languages	Greek: Native English: Fluent

CONFERENCES/SEMINARS ATTENDANCE

16-18 December 2021	9th Conference of Hellenic Scientific Society of Microbiokosmos
Place	Agricultural University of Athens, Greece
08 December 2021	FEMS Microbiology Ecology Webinar on Approaches, Methods and Challenges in Microbiome Research
20-23 April 2021	Microbiome Data Analyses Workshop, Hasselt University (Webinar)
22 January 2021	4th Webinar of Hellenic Scientific Society of Mikrobiokosmos
08 December 2020	DRomics virtual training course of the ROVALTAIN Foundation
06-09 October 2020	2nd International Conference on Microbial Ecotoxicology

(Virtual Edition)

18-20 April 2019	8th Conference of the Hellenic Scientific Society of Mikrobiokosmos
	Workshop on Analysis of Microbial NGS data
Place	FORTH/ICE-HT Conference Centre, Patra
15-18 November 2018	Hellenic Bioinformatics Conference 2018,
	Workshop on RNA-seq and ChIP Seq data analysis
Place	Thessalonika concert hall, Thessaloniki
24-28 June 2018	Summer School of Mikrobiokosmos: "The role of microbiome in ecosystem functioning, food security, human health and environmental protection
Place	Conference and Cultural Center of the Univ. of Thessaly, Pelion
20-17 February 2018	Workshop on genome assembly.
Content	Sequencing technologies, Genome assembly methods, Practical on Escherichia coli O157:H7 genome assembly
Place	Department of Biochemistry and Biotechnology, University of Thessaly, Larissa
14-15 June 2017	Workshop on metagenomics.
Content	Application of meta-omics on water and land environments, Data processing.
Place	Conference and Cultural Center of the Univ. of Thessaly, Pelion
7-9 April 2017	7th Symposium of the Scientific Society of MIKROBIOKOSMOS,
Place	National Hellenic Research Foundation, Athens
31 October 2014	Royal Dutch Society for Microbiology (KNVM) fall meeting,
Place	Royal Artis Zoo, Amsterdam
6-8 December 2013	Congress of the Hellenic Society of Biochemistry and Molecular Biology,
Place	Eugenides Foundation, Athens

PRESENTATIONS ON CONFERENCES AND SEMINARS

Pedrinho A, Karas P, Papadopoulou E, Papazlatani C, Wittenberg G, Karpouzas D, Assessing the toxicity of biopesticides on soil microbiota using a modification of the OECD 216 test, 9th Conference of the Hellenic Scientific Society of Mikroviokosmos (Poster)

Papazlatani C, Kolovou M, Gkounou E, Azis K, Mavriou Z, Testembasis S, Karaoglanidis G, Karas P, Tsetsekos G, Katsoula A, Ntougias S, Karpouzas D Isolation, characterization and application of a *Mycosphaerella tassiana* fungal isolate for the removal of imazalil from agro-industrial effluents, 9th Conference of the Hellenic Scientific Society of Mikroviokosmos (Poster)

Testempasis S, Papazlatani C, Theocharis S, Karas P, Koundouras S, Karpouzas D, Karaoglanidis G, Insights into the effects of agronomical management practices in

Aspergillus incidence and carposphere's microbial communities of grapevine, 9th Conference of the Hellenic Scientific Society of Mikroviokosmos (Poster)

Papadopoulou E, Bachtsevani E, Papazlatani C, Rousidou C, Lampronikou E, Menkissoglu-Spiroudi U, Nicol G, Ehaliotis C, Karpouzas D, Comparative evaluation of the efficacy of quinone imine, dicyandiamide (DCD), nitrapyrin, and 3,4-dimethylpyrazole phosphate (DMPP) to inhibit nitrification under different temperature and pH conditions, 9th Conference of the Hellenic Scientific Society of Mikroviokosmos (Poster)

Bachtsevani E., Papazlatani C, Rousidou C, Lampronikou E, Tsiknia M, Vasileiadis S, Menkissoglu-Spiroudi U, Ehaliotis C, Philippot L, Nicol G, Karpouzas D, Papadopoulou E, Beyond on-target effects of potential and established nitrification inhibitors on the soil microbiota, 9th Conference of the Hellenic Scientific Society of Mikroviokosmos (Oral by Bachtsevani E.)

Christina V. Papazlatani, Maria Kolovou, Panagiotis A. Karas and Dimitrios G. Karpouzas Isolation and characterization of a *Mycosphaerella tassiana* isolate able to rapidly degrade the recalcitrant fungicide Imazalil, 4th Webinar of Hellenic Scientific Society of Mikrobiokosmos (Oral)

Christina V. Papazlatani, Maria Kolovou, Panagiotis Karas, Dimitrios G Karpouzas (2020) Isolation and characterization of a Mycosphaerella tassiana strain able to rapidly degrade the recalcitrant fungicide Imazalil, 2nd International Conference on Microbial Ecotoxicology (Virtual Edition) (Oral poster communication)

Panagiotis Karas, Christina Papazlatani, Dimitrios Karpouzas (2020) Exploring the potential of biobeds to treat the pesticide-contaminated effluents from various agro-food industries: insights into the role of microbiome and plasmidome, 2nd International Conference on Microbial Ecotoxicology (Virtual Edition) (Poster communication)

Papazlatani C., Perruchon C., Katsoula A., Lagos S., Papadopoulou E.S., Vasileiadis S., Karas P.A., Karpouzas D.G (2019) Isolating bacteria able to rapidly degrade fungicides used in fruit packaging industry: Tailored made inocula for the treatment of relevant agro-industrial effluents, 8th Conference of the Hellenic Scientific Society of Mikroviokosmos (poster)

Papazlatani V.C., Karas A.P., Tucat G., Karpouzas G.D. (2019). Expanding the use of biobeds: Dissipation and adsorption of pesticides contained in effluents from seed-coating, bulb disinfestation and fruitpackaging activities. 8th Conference of the Hellenic Scientific Society of Mikroviokosmos (poster)

Karas P.A., Papazlatani C., Tucat G.*, Karpouzas D.G. (2016) Testing biobeds as a possible solution for the biodepuration of pesticide contaminated wastewaters produced by various agro-industries. III Latin American Biobed Workshop, September 2017, Brazil

Katsoula A., Papazlatani C., Papadimitriou A., Rousidou C., Papadopoulou K. K., Karpouzas D. G. (2015) Estimation of the population levels of soil-born fungal plant pathogens in soils from greenhouses in Western Greece via q-PCR, 6th Symposium of the Scientific Society of MIKROBIOKOSMOS (poster)

A. Ruaud, B. Nieuwesteeg, C. Papazlatani, H. Gibriel, R. Nijland (2015) Endophytic bacteria of tomato: A source of antifungal and antibacterial compounds. (Poster) 6th Congress of European Microbiologists (FEMS 2015), 7-11 June, Maastricht

PUBLICATIONS IN PEER REVIEWED JOURNALS

Evangelia Papadopoulou, Ph.D., Eleftheria Bachtsevani, Christina Papazlatani, Constantina Rousidou, Antonios Brouziotis, Eleni Lampronikou, Myrto Tsiknia, Sotirios Vasileiadis, Urania Menkissoglu-Spiroudi, Constantinos Ehaliotis, Laurent Philippot, Graeme W. Nicol, Dimitrios G. Karpouzas The effects of quinone imine, a new potent nitrification inhibitor, dicyandiamide and nitrapyrin on target and off-target soil microbiota. Microbiology Spectrum. **Under review**

Papazlatani, Christina V., Panagiotis A. Karas, Eleni Lampronikou, and Dimitrios G. Karpouzas. 2022. “Using Biobeds for the Treatment of Fungicide-Contaminated Effluents from Various Agro-Food Processing Industries: Microbiome Responses and Mobile Genetic Element Dynamics.” Science of The Total Environment 823 (March): 153744. https://doi.org/10.1016/j.scitotenv.2022.153744

Papazlatani, Christina V., Maria Kolovou, Elisabeth E. Gkounou, Konstantinos Azis, Zografina Mavriou, Stefanos Testembasis, George S. Karaoglanidis, Spyridon Ntougias, and Dimitrios G. Karpouzas. 2022. “Isolation, Characterization and Industrial Application of a *Cladosporium herbarum* Fungal Strain Able to Degrade the Fungicide Imazalil.” Environmental Pollution, October, 119030. https://doi.org/10.1016/j.envpol.2022.119030

Bachtsevani, Eleftheria, Christina V. Papazlatani, Constantina Rousidou, Eleni Lampronikou, Urania Menkissoglu-Spiroudi, Graeme W. Nicol, Dimitrios G. Karpouzas, and Evangelia S. Papadopoulou. 2021. “Effects of the Nitrification Inhibitor 3,4-Dimethylpyrazole Phosphate (DMPP) on the Activity and Diversity of the Soil Microbial Community under Contrasting Soil PH.” Biology and Fertility of Soils, October. https://doi.org/10.1007/s00374-021-01602-z

Papazlatani, Christina V., Panagiotis A. Karas, Guillermo Tucat, and Dimitrios G. Karpouzas. 2019. “Expanding the Use of Biobeds: Degradation and Adsorption of Pesticides Contained in Effluents from Seed-Coating, Bulb Disinfestation and Fruit-Packaging Activities.” Journal of Environmental Management 248 (October): 109221.

https://doi.org/10.1016/j.jenvman.2019.06.122

Papazlatani, C., C. Rousidou, A. Katsoula, M. Kolyvas, S. Genitsaris, K. K. Papadopoulou, and Dimitrios G. Karpouzas. 2016. “Assessment of the Impact of the Fumigant Dimethyl Disulfide on the Dynamics of Major Fungal Plant Pathogens in Greenhouse Soils.” European Journal of Plant Pathology 146 (2): 391–400. https://doi.org/10.1007/s10658-016-0926-6