Universitat Autònoma de Barcelona

Agricultural wastewater treatment by Trametes versicolor immobilized on wood in a rotating drum bioreactor

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PhD Thesis

PhD Program in Environmental Science and Technology

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PAQUI BLÁNQUEZ CANO and MONTSERRAT SARRÀ ADROGUER, associate professors from the Departament d'Enginyeria Química, Biològica i Ambiental of the Universitat Autònoma de Barcelona.

CERTIFY:

That the engineer EDUARDO BELTRÁN FLORES has carried out under our supervision the research work entitled "Agricultural wastewater treatment by *Trametes versicolor* immobilized on wood in a rotating drum bioreactor", which is presented in this memory and it constitutes his thesis to obtain the Degree of Doctor in Environmental Science and Technology from the Universitat Autònoma de Barcelona.

To whom it may concern and may record it for the appropriate purposes, we present the aforementioned thesis to the Escola de Doctorat of the Universitat Autònoma de Barcelona, signing this certificate at

Bellaterra (Cerdanyola del Vallès), September 2022

"La respuesta a nuestros mayores problemas puede estar justo bajo nuestros pies"

- Hongos Fantásticos (2019)

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Abstract

The deterioration of water quality by persistent organic pollutants (POPs) is a global concern. Even at low concentrations, these compounds pose a significant toxicological risk to the ecosystem and human health. Among POPs, pesticides are still indispensable in addressing multiple challenges worldwide, but most of them fail to interact with the target pest and are disseminated in water and other environmental compartments, potentially triggering detrimental effects on numerous organisms. Responding to this concern, the scientific community is currently investigating different technologies to remove pesticides from water resources. Among other alternatives, fungal treatment by white rot fungi (WRF) has become an attractive strategy because these organisms present a powerful enzyme system capable of degrading a wide range of POPs, including pesticides, and is considered an environmentally friendly and low-cost approach.

The present thesis aims to develop a fungal bioreactor using *Trametes versicolor* immobilized on wood chips to remove pesticides from agricultural wastewater (AW) in a long-term treatment under non-sterile conditions.

First of all, a rotating drum bioreactor (RDB) was set up to treat AW spiked with diuron, as a model pollutant, using *T. versicolor* immobilized on pine wood chips. Although good results were obtained in terms of pesticide removals, *T. versicolor* was progressively detached from the wood surface. Subsequently, another bioremediation study was conducted using *T. versicolor* immobilized on holm oak wood chips to remove two model pesticides (diuron and bentazon) in a column reactor. Sorption played a predominant role in the removal of both pesticides, which motivated a comprehensive sorption study. The pesticide-contaminated wood was subsequently treated by *T. versicolor* in a solid biopile-like system.

Secondly, AW spiked with diuron and bentazon was treated by *T. versicolor* immobilized on holm oak wood chips in the RDB for 225 days, optimizing some operational parameters to achieve the consolidation of the fungal biomass in the reactor and promising removal performances.

Additionally, a cross-sectional study was conducted to shed light on some key questions about fungal bioremediation, including the effect of limiting dissolved oxygen level on contaminant degradation, growth kinetics on wood, and organic matter removal by *T. versicolor*.

Based on gained experiences and knowledge, the RDB was applied to treat agricultural rinse wastewater (RW), comparing its performance with that obtained by both a well-established fungal bioreactor (fluidized bed bioreactor) and a consolidated physical-chemical technology (ozonation). In this regard, the RDB proved to be the best option between the two fungal reactors, whereas the comparative study between the fungal and the ozonation treatments showed that each technology had its own advantages and achieved better results depending on the studied parameter. Furthermore, a treatment train consisting of a first stage of fungal bioremediation followed by ozonation was performed, in which the advantages of each process were synergistically combined to offer greater flexibility in the relationship between effluent quality and operating costs.

Therefore, this study expands the knowledge on fungal treatment of pesticides and proposes a viable and promising solution to a real agricultural contamination issue.

Resumen

El deterioro de la calidad del agua por los contaminantes orgánicos persistentes (POPs, de sus siglas en inglés) es una preocupación mundial. Incluso a bajas concentraciones, estos compuestos suponen un riesgo toxicológico significativo para el ecosistema y la salud humana. Entre los POPs, los pesticidas siguen siendo indispensables para hacer frente a múltiples desafíos en todo el mundo, pero la mayoría de ellos no interactúan con la plaga objetivo y se diseminan en el agua y otros compartimentos ambientales, lo que puede desencadenar efectos perjudiciales en numerosos organismos. En respuesta a esta preocupación, la comunidad científica está investigando actualmente diferentes tecnologías para eliminar los pesticidas de los recursos hídricos. Entre otras alternativas, el tratamiento fúngico mediante hongos de pudrición blanca (WRF, de sus siglas en inglés) se ha convertido en una estrategia atractiva porque estos organismos presentan un sistema enzimático potente capaz de degradar una amplia gama de POPs, incluidos los pesticidas, y se considera un enfoque respetuoso con el medio ambiente y de bajo costo.

La presente tesis tiene como objetivo desarrollar un biorreactor fúngico utilizando *Trametes versicolor* inmovilizado sobre astillas de madera para eliminar pesticidas de aguas residuales agrícolas (AW, de sus siglas en inglés) en un tratamiento a largo plazo en condiciones no estériles.

En primer lugar, se instaló un biorreactor de tambor rotatorio (RDB, de sus siglas en inglés) para tratar AW dopada con diurón utilizando *T. versicolor* inmovilizado en astillas de madera de pino. Aunque se obtuvieron buenos resultados en cuanto a la eliminación de pesticidas, *T. versicolor* se desprendió progresivamente de la superficie de la madera. Posteriormente, se realizó otro estudio de biorremediación utilizando *T. versicolor* inmovilizado sobre astillas de madera de encina para eliminar dos pesticidas modelo (diurón y bentazona) en un reactor tipo columna. La sorción desempeñó un papel predominante en la eliminación de ambos pesticidas, lo que motivó un estudio de sorción exhaustivo. La madera contaminada con pesticidas fue tratada posteriormente por *T. versicolor* en un sistema sólido similar a una biopila.

En segundo lugar, AW dopada con diurón y bentazona fue tratada por *T. versicolor* inmovilizada sobre astillas de madera de encina en el RDB durante 225 días, optimizando algunos parámetros

operativos para conseguir la consolidación de la biomasa fúngica en el reactor y unos rendimientos de eliminación prometedores.

Además, se realizó un estudio transversal para arrojar luz sobre algunas cuestiones clave sobre la biorremediación de hongos, incluyendo el efecto de limitar el nivel de oxígeno disuelto sobre la degradación de contaminantes, la cinética de crecimiento en la madera y la eliminación de materia orgánica por parte de *T. versicolor*.

Sobre la base de las experiencias y los conocimientos adquiridos, se aplicó el RDB para tratar el agua residual de lavado agrícola (RW, de sus siglas en inglés), comparando su rendimiento con el obtenido por un biorreactor fúngico bien establecido (biorreactor de lecho fluidizado) y una tecnología físico-química consolidada (ozonización). En este sentido, el RDB demostró ser la mejor opción entre los dos reactores fúngicos, mientras que el estudio comparativo entre el tratamiento fúngico y el de ozonización mostró que cada tecnología tenía sus propias ventajas y lograba mejores resultados según el parámetro estudiado. Además, se realizó un tren de tratamiento consistente en una primera etapa de biorremediación fúngica seguida de ozonización, en el que se combinaron sinérgicamente las ventajas de cada proceso para ofrecer una mayor flexibilidad en la relación entre la calidad del efluente y los costos de operación.

Por lo tanto, este estudio amplía el conocimiento sobre el tratamiento fúngico de pesticidas y propone una solución viable y prometedora a un problema real de contaminación agrícola.

Resum

El deteriorament de la qualitat de l'aigua pels contaminants orgànics persistents (POPs, per les seves sigles en anglès) és una preocupació mundial. Fins i tot a concentracions baixes, aquests compostos suposen un risc toxicològic significatiu per a l'ecosistema i la salut humana. Entre els POPs, els pesticides continuen sent indispensables per fer front a múltiples desafiaments a tot el món, però la majoria no interactuen amb la plaga objectiu i es disseminen a l'aigua i altres compartiments ambientals, podent desencadenar efectes perjudicials en nombrosos organismes. En resposta a aquesta preocupació, la comunitat científica investiga actualment diferents tecnologies per eliminar els pesticides dels recursos hídrics. Entre altres alternatives, el tractament fúngic per fongs de podriment blanc (WRF, per les seves sigles en anglès) s'ha convertit en una estratègia atractiva perquè aquests organismes presenten un sistema enzimàtic potent capaç de degradar una àmplia gamma de POPs, inclosos els pesticides, i es considera un enfocament respectuós amb el medi ambient i de baix cost.

La present tesi té com a objectiu desenvolupar un bioreactor fúngic utilitzant *Trametes versicolor* immobilitzat sobre estelles de fusta per eliminar pesticides d'aigües residuals agrícoles en un tractament a llarg termini en condicions no estèrils.

En primer lloc, es va instal·lar un bioreactor de tambor rotatori (RDB, per les seves sigles en anglès) per tractar AW dopada amb diurón utilitzant *T. versicolor* immobilitzat en estelles de fusta de pi. Encara que es van obtenir bons resultats quant a l'eliminació de pesticides, *T. versicolor* es va desprendre progressivament de la superfície de la fusta. Posteriorment, es va realitzar un altre estudi de bioremediació utilitzant *T. versicolor* immobilitzat sobre estelles de fusta d'alzina per eliminar dos pesticides model (diurón i bentazona) en un reactor tipus columna. La sorció va exercir un paper predominant en l'eliminació de tots dos pesticides, fet que va motivar un estudi de sorció exhaustiu. La fusta contaminada amb pesticides va ser tractada posteriorment per *T. versicolor* en un sistema sòlid similar a una biopila.

En segon lloc, AW dopada amb diurón i bentazona va ser tractada per *T. versicolor* immobilitzada sobre estelles de fusta d'alzina al RDB durant 225 dies, optimitzant alguns paràmetres operatius per aconseguir la consolidació de la biomassa fúngica al reactor i uns rendiments d'eliminació prometedors.

A més, es va realitzar un estudi transversal per donar llum sobre algunes preguntes clau sobre la bioremediació de fongs, incloent l'efecte de limitar el nivell d'oxigen dissolt sobre la degradació de contaminants, la cinètica de creixement a la fusta i l'eliminació de matèria orgànica per part de *T. versicolor*.

Sobre la base de les experiències i els coneixements adquirits, es va aplicar el RDB per tractar l'aigua residual de rentat agrícola (RW, per les seves sigles en anglès), comparant el seu rendiment amb l'obtingut per un bioreactor fúngic ben establert (bioreactor de llit fluïditzat) i una tecnologia fisicoquímica consolidada (ozonització). En aquest sentit, el RDB va demostrar ser la millor opció entre els dos reactors fúngics, mentre que l'estudi comparatiu entre el tractament fúngic i el d'ozonització va mostrar que cada tecnologia tenia els seus avantatges i aconseguia millors resultats segons el paràmetre estudiat. A més, es va realitzar un tren de tractament consistent en una primera etapa de bioremediació fúngica seguida d'ozonització, en què es van combinar sinèrgicament els avantatges de cada procés per oferir més flexibilitat en la relació entre la qualitat de l'efluent i els costos d'operació.

Per tant, aquest estudi amplia els coneixements sobre el tractament fúngic de pesticides proposant una solució viable i prometedora a un problema real de contaminació agrícola.

List of abbreviations

ATCC	American Type Culture Collection
AU	Activity unit
AW	Agricultural wastewater
CFU	Colony-forming unit
CHLOR	Chlortoluron
COD	Chemical oxygen demand
CRT	Cellular retention time
CYP450	Cytochrome P450 system
DGGE	Denaturing gradient gel electrophoresis
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DMP	2,6-dymetoxyphenol
DW	Dry weight
EBCT	Empty bed contact time
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FBB	Fluidized bed bioreactor
FBR	Fixed bed reactor
HPCs	Heterotrophic plate counts
HPLC	High-performance liquid chromatography
HRT	Hydraulic residence time
IPM	Integrated pest management
LC-qTOF-MS	Time-of-flight mass spectrometry
LOD	Lower limit of detection
LOQ	Lower limit of quantification
MBR	Membrane bioreactor
MS	Mass spectrometry
OUR	Oxygen uptake rate

PBCB	Packed bed channel bioreactor
PCPs	Personal care products
POPs	Persistent organic pollutants
PVDF	Polyvinylidene difluoride
PYRI	Pyrimethanil
PhAC	Pharmaceutical active compound
RBD	Rotating drum bioreactor
RR	Recirculation ratio
RW	Rinse wastewater
SD	Standard deviation
SEM	Scanning electron microscope
sOUR	Specific oxygen uptake rate
TBP	Tributyl phosphate
THIA	Thiacloprid
TP	Transformation product
TSS	Total suspended solid
VSS	Volatile suspended solid
WRF	White-rot fungi
WW	Wet weight
WWTP	Wastewater treatment plant

Content

Chapter 4

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Chapter 1

General introduction

1.1. Water scarcity and pollution

Driven by population growth, economic development and changing consumption patterns, global water demand has increased six-fold over the past 100 years and continues to grow at a sustained rate of 1% per year (WWAP, 2020). Combined with the increasing demand pressure, climate change will generate or aggravate water stress in many regions. Climate change is responsible for the increasing frequency and magnitude of extreme weather events such as heat waves, fires, floods and droughts. In fact, extreme weather events have been identified as the top 1 high-impact risk over the next few years in the Global Risks 2022 Report of the World Economic Forum (WEF, 2022). These climatic adversities will accentuate the problem of water stress not only in arid or dry regions, limiting the quantity of available resources, but also in areas with intense and erratic rainfalls, where water quality can be significantly deteriorated (Pereira et al., 2002).

In Europe, water scarcity affects approximately 20 % of the European territory and 30 % of its population on average each year. The cost of economic damage caused by droughts is estimated to be up to 9.000 billion euros per year, not including the costs associated with the ecological damage. Annual renewable freshwater resources per inhabitant exhibited a decreasing trend in all regions (except in Eastern Europe due to emigration) over the period 1990-2017. The largest declines were recorded in Cyprus (-32%), Malta (-54%) and Spain (-65%), mainly attributed to decreasing precipitation and increasing temperature and evapotranspiration (EEA, 2021).

In addition to intense consumption and climate change, pollution is another stress factor that affects water quality and scarcity. Water pollution can be defined as "the addition of substances or energy forms that directly or indirectly alter the nature of the water body in such a manner that negatively affects its legitimate uses" (von Sperling, 2007). The sources of contamination are multiple, including industries, hospitals, households, farms, sewage treatment plants, etc. Approximately 80 % of municipal and industrial wastewater is estimated to be discharged into the environment without any prior treatment, with harmful effects on human health and ecosystems. This percentage is much higher in less developed countries, where wastewater management and sanitation are very deficient (WWAP, 2021).

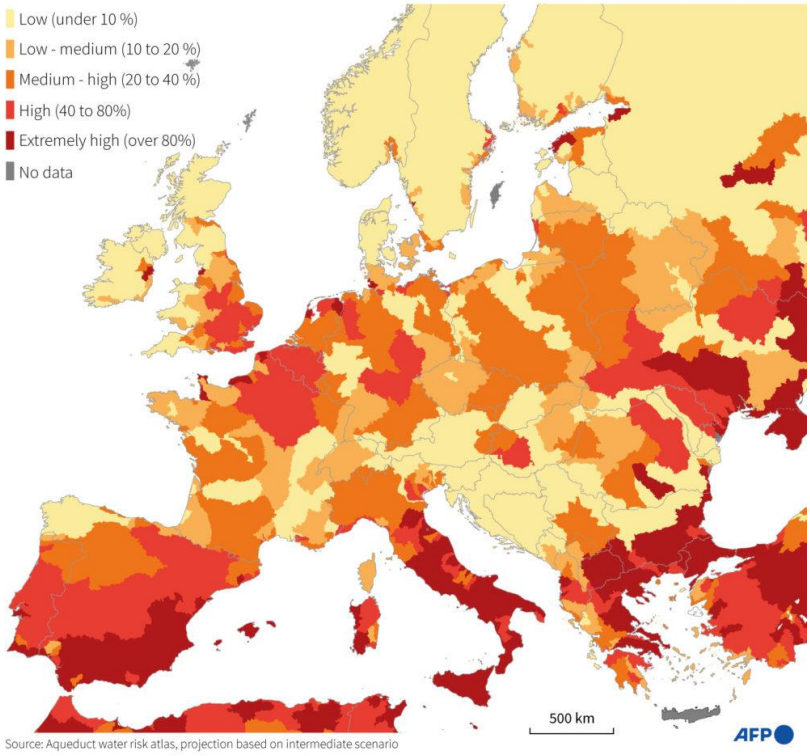


Fig. 1.1. Estimated water stress based on an intermediate scenario in Europe in 2030. Baseline water stress measures the ratio (in %) of total water withdrawals for human activities (including domestic, industrial, irrigation and livestock consumptions) to available water resources. Source: Aqueduct water risk atlas (WRI, 2019).

An example of water stress caused by pollution is a recent episode (May 2022) that occurred in Les Garrigues and Segrià (Catalonia), where nearly 20.000 people were deprived of water supply due to the detection of high levels of pesticides in the water treatment plant that supplies them. The pesticides detected are commonly used in agriculture in this area; thus they were believed to have been spread by the rains of the previous days (CCMA, 2022).

1.2. Persistent organic pollutants

The intense technological disruption achieved during the last decades has brought significant lifestyle benefits to modern societies. It is a hard task to imagine an advanced society without petrochemicals, agrochemicals, pharmaceuticals or industrial products that make such an important contribution to improving our lives. However, there is growing social awareness and concern about the risks associated with the release of chemicals into the environment (Harrison, 2014). According to The United Nations World Water Development Report 2017, urban,

industrial and agricultural activities are the main sources of wastewater, most of which is discharged into the environment without prior collection or treatment (World Water Assessmet Programme (WWAP), 2017).

Water contamination can be caused by various types of pollutants, which can be mainly classified into suspended solids, nutrients (nitrogen and phosphorus), pathogenic organisms, biodegradable organic matter and persistent organic pollutants (POPs). POPs can be defined as natural or anthropogenic chemicals that are considered recalcitrant, bioaccumulative and toxic to the environment and/or human health. The literature also refers to them as micropollutants, when found in the environment at low concentrations (μg L^{-1} or ng L^{-1}), and as emerging contaminants or contaminants of emerging concern. This expanding group comprises a wide range of compounds, including mainly organic dyes, pharmaceutical active compounds, personal care products, flame retardants and pesticides (Luo et al., 2014). Major sources of micropollutants include: domestic and hospital wastewaters; industrial effluents; runoff from farms (agriculture, livestock and aquaculture); and leachates from landfills (Barbosa et al., 2016). The released micropollutants can reach the aquatic environment and drinking water via several routes, as shown in Fig. 1.2.

a) Organic dyes

Many industries utilize synthetic dyes to color their products, such as those in the textile, leather, cosmetics, paper, printing and plastics sectors, among others. Effluents from these industries contain various kinds of natural or synthetic dyes. The most commonly used dyes are those that have bright colors, are easy to produce and apply to the fabric, and also have low cost; but their chemical structure also makes them easily soluble in water. (Sharma et al., 2011). Numerous synthetic dyes, particularly azo dyes, have proven to be toxic and have been banned in many countries. However, azo dyes are still one of the most widely produced and used dyes today because of their favorable properties and low cost. These compounds are very recalcitrant and are very difficult to remove from wastewater by conventional aerobic biological treatment (Bafana et al., 2011).

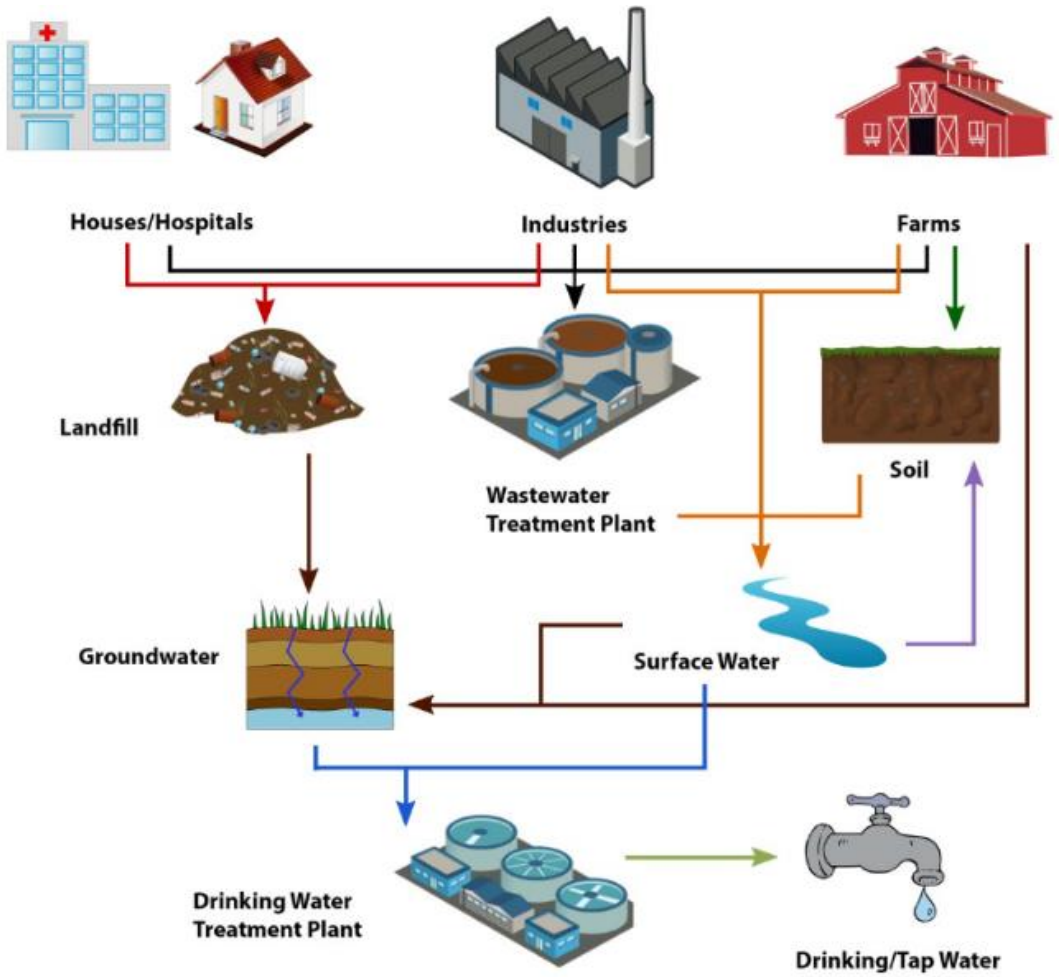


Fig. 1.2. Main sources and propagation pathways of micropollutants in the environment [adapted from Barbosa et al. (2016)].

b) Pharmaceutical active compounds

Pharmaceutical active compounds (PhACs) are bioactive molecules developed for medical purposes that are used in both human health and livestock. These compounds form a broad group that includes antibiotics, hormones, antidepressants, anticonvulsants, antihypertensives, nonsteroidal anti-inflammatory drugs, lipid regulators, etc (Cizmas et al., 2015). Hospitals are the main sources of PhCs, although pharmaceutical industries, animal waste, research activities and the disposal of expired drugs are also major contributors (Tiwari et al., 2017). These effluents are often discharged to municipal wastewater treatment plants (WWTPs). However, WWTP processes are not designed to treat these recalcitrant compounds. In fact, PhACs have been shown to adversely influence the performance of activated sludge treatment (Pauwels and

Verstraete, 2006). For this reason, WWTPs can release significant amounts of these pollutants into the environment, which have been shown to have detrimental effects on non-target organisms, such as alterations in reproductive function (Overturf et al., 2015). In addition, the widespread use of PhCs in medicine, veterinary medicine, agriculture and livestock has been a key factor in the development of antibiotic resistance, which poses a serious threat to public health (Frieri et al., 2017).

c) Personal care products

Personal care products (PCPs) are a group of organic compounds used as active ingredients in the formulation of a wide range of daily consumer products, such as hair dyes, cosmetics, lotions creams, deodorants, bath soaps, dental care products, shampoos, lipsticks, toothpaste, UV filters, detergents, sunscreens, fragrances and perfumes, among others. Unlike PhACs, PCPs are generally intended for external use and hence enter the environment directly and in larger amounts (Ray et al., 2020). Some PCPs have been reported to adversely affect the reproduction, growth and mortality of fish and invertebrates (Boxall et al., 2012) and have been shown to induce hormonal imbalances, endocrine and reproductive system disorders and diverse physiological effects in human beings, even at low concentrations (Montes-Grajales et al., 2017).

d) Flame retardants

Flame retardants are used to enhance the fire resistance of a wide range of consumer products, such as furniture, electronic castings, carpets, building materials, automotive components, insulation, textiles, etc. The proven persistence, bioaccumulation and toxicity of some halogenated flame retardants, mainly brominated flame retardants (such as polychlorinated biphenyls and polybrominated diphenyl ethers), has led to their restriction in some countries and their replacement by other more innocuous compounds such as phosphorous flame retardants (van der Veen and de Boer, 2012). Although their adverse effects on humans are more limited, phosphorous flame retardants are highly persistent compounds whose environmental impact has not yet been sufficiently documented (Wei et al., 2015).

1.3. Pesticides

1.3.1. Definition and classification

The International code of conduct on the distribution and use of pesticides created by the Food and Agriculture Organization of the United Nations (FAO) defines pesticides as "any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or regulating plant growth." (WHO and FAO, 2014). In brief, we can refer to pesticides as chemical compounds capable of protecting crops from pests. Pesticides can be categorized according to their intrinsic characteristics into different groups. The main classification is based on the target organism, but pesticide can also be classified according to their mode of entry, chemical nature, source of origin, formulation and toxicity, as shown in Table 1.1 (Yadav and Devi, 2017).

Table 1.1. Different classifications of pesticides and their main classes (Akashe et al., 2018; WHO, 2020; Yadav and Devi, 2017).

	Classes	**Characteristics**
Target organism	Insecticides, fungicides, bactericides, herbicides, acaricides, rodenticides, algaecides, larvicides, repellents, desiccants, ovicides, virucides, molluscicides, nematicides, avicides, moth balls, lampricides, piscicides, silvicides and termiticides	Substances intended to act selectively for each type of organism.
Mode of entry	Systemic	Chemicals that are absorbed by plants or animals and transfer to internal tissues.
	Contact (non-systemic)	Substances that need physical contact with the pest to be effective.
	Stomach poisons	Compounds that act through pest ingestion.
	Fumigants	Chemicals that kill pests by vapor respiration.
	Repellents	Repellents do not kill but just keep pests away.
Chemical nature	Organochlorine	Substances that cause disruption of the nervous system of the insects by an interference with different ion channels (such as sodium channels).
	Organophosphates	Acetylcholinesterase inhibitors. They are less persistent but more toxic to both vertebrates and invertebrates.

	Classes	Characteristics
	Carbamates	Carbamates have similar structure (derivatives of carbamic acid instead of phosphoric acid) and function (acetylcholinesterase inhibitor) than organophosphates.
	Synthetic pyrethroids	Neurotoxic compounds whose mechanism of action is the interaction with voltage-gated sodium channels in neurons.
	Neonicotinoid	Substances that block nicotinic acetylcholine receptors in the central nervous system of insects, causing overstimulation of their nerve cells, paralysis and death.
Source of origin	Chemical pesticides	Broad-spectrum synthetic pesticides.
	Biopesticides	Biologically derived pesticides (from animals, plants and microorganisms) that usually have higher specificity.
Formulation	Liquid	High purity oily solutions of pesticides mixed with an added emulsifier to allow for subsequent mixing with water.
	Powder	Finely ground dry powders consisting of active pesticide ingredients mixed with other dispersing agents.
	Granules	The active ingredient is mixed with inert clays to form particles of various sizes that require specialized dispersion equipment.
	Baits	The baits contain active ingredients mixed with a pest food or attractant.
	Dust	Powder is used as a carrier and is usually applied directly without mixing.
	Ultra-low volume liquid	Highly concentrated formulation, which usually contains close to 100 % of the active ingredient.
Toxicity, based on WHO classification	Class Ia – Extremely hazardous	Oral LD_{50}: < 5 mg·kg^{-1} body weight in rats. Dermal LD_{50}: < 50 mg·kg^{-1} body weight in rats.
	Class Ib – Highly hazardous	Oral LD_{50}: 5-50 mg·kg^{-1} body weight in rats. Dermal LD_{50}: 50-200 mg·kg^{-1} body weight in rats.
	Class II – Moderately hazardous	Oral LD_{50}: 50-2000 mg·kg^{-1} body weight in rats. Dermal LD_{50}: 200-2000 mg·kg^{-1} body weight in rats.
	Class III – Slightly hazardous	Oral LD_{50}: 2000-5000 mg·kg^{-1} body weight in rats. Dermal LD_{50}: > 2000 mg·kg^{-1} body weight in rats.
	Class IV – Unlikely to present acute hazard in normal use	Oral LD_{50}: ≥ 5000 mg·kg^{-1} body weight in rats.

1.3.2. Emission sources

Pesticides are one of the most important groups of chemical compounds in modern societies. The increase in world population and the economic growth experienced since the mid-20th century were largely due to the increasing expansion of intensive agricultural practices. Nowadays, the use of pesticides is still vital to meet the global food demand. Over the last decades, the use of pesticides has increased significantly worldwide, from 2 million tons in 2000 to more than 2.6 million tons in 2020 as shown in Table 1.2. Pesticides are intended for multiple uses in many industries, commerce, homes, parks and gardens, but the largest pesticide use is undoubtedly in the agricultural sector, which accounts for approximately two-thirds of the total (Atwood and Paisley-Jones, 2017).

Table 1.2. Use of pesticides between 2000 and 2020 (FAOSTAT, 2022).

Region	Annual pesticide use (tons)		Increase (%)
	2000	2020	
Africa	63894,15	105757,87	65,52
Asia	604147,71	658529,28	9,00
Caribbean	7545,02	10694,66	41,74
Central America	64000,90	90163,48	40,88
Europe	449646,32	468431,57	4,18
Spain	34597,00	43336,92	25,26
Northern America	469831,12	486732,44	3,60
Oceania	37776,00	70421,16	86,42
South America	350246,51	770393,77	119,96
World	2047087,73	2661124,23	30,00

However, a low proportion of the applied pesticides is believed to act effectively against target pests (Tudi et al., 2021). The remaining pesticides are released into the environment, many of which are considered persistent, bioaccumulative and toxic even at low concentrations (Chopra et al., 2011). Pesticides can enter the environment through diffuse (normal application in the fields) or point sources (through leaks or losses resulting from inappropriate pesticide management) (Neumann et al., 2002).

Leaves, flowers, seeds, pollen and nectar contaminated with pesticide residues can cause significant damage to non-target organisms. Pesticides can also accumulate in the soil, emitted

into the air or discharged into the aquatic environment, many of which can cause significant damage to the biosphere (Gavrilescu, 2005). Drift, volatilization and runoff are the three major pathways of pesticide dispersion to the environment. Drift is defined as the displacement of pesticide droplets or solid particles outside the application area. Some recommendations to reduce pesticide drift include calibrating and selecting proper nozzles and pressures on spray equipment, working in appropriate environmental conditions (wind, humidity and temperature), establishing an effective buffer zone and applying an appropriate droplet size. Solid and liquid pesticides applied to crops can volatilize very quickly and be blown by the wind even over long distances. The volatilization rate depends on pesticide formulas, meteorological conditions, application techniques, soil characteristics, etc. Runoff and leaching occur when rainfall follows pesticide application and can be major sources of surface and groundwater contamination (Pimentel and Peshin, 2014). The final fate of pesticides depends on the physical-chemical properties of the pesticides (vapor pressure, stability, solubility and pKa), the soil characteristics (pH, organic components, inorganic surfaces, soil moisture and soil microbiome), the plant species, the climatic conditions, the application method and dosage (Singh et al., 2018).

1.3.3. Occurrence of pesticides in water

Pesticides can enter the aquatic environment directly (e.g. accidental spills) and especially indirectly through runoff from surfaces and infiltration through the ground. Once diluted in water, pesticides can be broadly disseminated in the environment. The concentration of pesticides in water bodies can reach the magnitude of tens of milligrams per liter (Gavrilescu, 2005).

The occurrence of pesticides in the water resources of EU member countries has been a problem of growing concern during the last decade. One of the major sources of pesticide contamination are effluents from WWTPs. Although WWTPs act as a first barrier against the dissemination of pesticides in the environment, they are not specifically designed to treat these recalcitrant compounds and achieve low removal rates (Sutton et al., 2019). Likewise, water bodies near areas of intense agricultural activity are particularly prone to pesticide contamination. Increasing concentrations of pesticides have been detected in surface waters near agricultural fields (Ccanccapa et al., 2016; Köck-Schulmeyer et al., 2019; Pascual Aguilar et al., 2017; Postigo et al., 2021). Levels of pesticide contamination in water can be ranked in the following order:

cropland water > field ditch water > runoff > pond water > groundwater > river water > deep groundwater > sea water (Deknock et al., 2019; Masiá et al., 2013; Zhang et al., 2011). Another source of contamination that is becoming a concern is the generation of rinse wastewater (RW), which is the wastewater produced after the washing of containers and phytosanitary equipment, and which usually contains high concentrations of pesticides (Vela et al., 2019).

Given the environmental risk posed by the increasing occurrence of pesticides in water, the European Water Framework Directive and Groundwater Directive established a quality standard of 0.1 μg L^{-1} as the permissible limit for surface water and groundwater (EC, 2000a; EU, 2006). However, between 13 and 30 % of the samples analyzed in surface waters had one or more pesticides exceeding the threshold limit, with an increasing trend observed from 2013 to 2019, as shown in Fig. 1.3 (EC, 2021a). Periodic renewals of pesticide approvals for use in member states are carried out by the European Union (EU) and can be consulted in its updated pesticide database (EC, 2022). Despite being banned, recent studies have reported the detection of pesticides not approved by the EU in surface waters of some member states. This fact, rather than to their illegal use, has been attributed to the persistence of these compounds in the environment (Masiá et al., 2013; Pascual Aguilar et al., 2017).

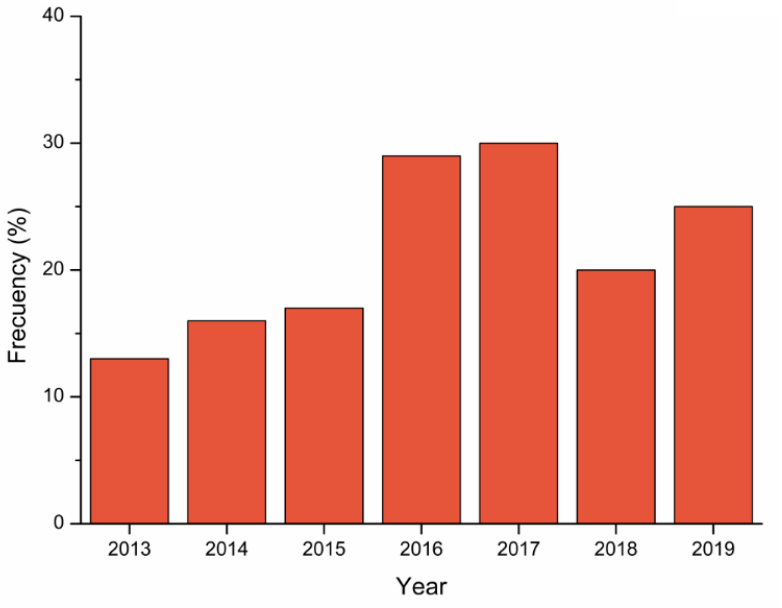


Fig. 1.3. Frequency at which pesticides detected at the monitoring points exceed thresholds from 2013 to 2019 (EC, 2021a).

This high dissemination of pesticides in the aquatic environment is clearly unsustainable and can cause serious environmental damage, thus alternative strategies that promote responsible management of crop protection are urgently required. In this regard, the European Commission (EC) adopted a package of proposals to align EU policies with the objective of reducing net greenhouse gas emissions by at least 55 % in 2030, known as the European Green Deal (EC, 2019). This program includes the target of reducing the use and risks of chemical pesticides by 50 % in 2030 through the Zero Pollution Action Plan (EC, 2021), the Farm to Fork Strategy (EC, 2020a) and the Biodiversity Strategy (EC, 2020b).

1.3.4. Toxicity of pesticides

Pesticides in water can affect aquatic organisms (such as invertebrates, plants, microorganisms, fish, or amphibians) either directly, i.e. by producing physiological changes in their organism due to their toxicity, or indirectly, e.g. by benefiting the proliferation of organisms that reduce the oxygen dissolved in the water (Shefali et al., 2021). Furthermore, pesticides can cross the biological membranes of phospholipids and bioaccumulate in the fatty tissues of many living beings. The concentration of these pesticides can increase progressively through various trophic levels of the food chain. This process is known as biomagnification and poses a significant risk to the environment (Chopra et al., 2011).

Pesticides also pose a significant public health risk as most of the human population is directly or indirectly exposed. Depending on the dose and contact time, pesticide exposure can be classified as intentional (accidental or suicidal), occupational (includes workers involved in the agricultural sector, such as farmers, applicators, transporters, dealers, and sellers of fruits and vegetables) and non-occupational (the majority of the population at the consumer level). Even at low concentrations, prolonged exposures to pesticides are believed to be associated with numerous health disorders, such as reproductive syndromes, respiratory dysfunctions, endocrine disruption, diabetes, neurological alterations and cancer (Rani et al., 2021; Sabarwal et al., 2018). Pesticides can also be degraded into transformation products (TPs) that can be even more toxic than the parent compounds (Ueyama et al., 2007). In addition, interactions between pesticides (and their parent compounds) can trigger synergistic mechanisms with unpredictable toxicological effects (Hernández et al., 2017).

1.4. Integrated Pest Management

Integrated pest management (IPM) is a coordinated approach that combines a set of sustainable agricultural practices to ensure effective pest control and crop safety. IPM is being widely promoted by the scientific community and is beginning to materialize in the pesticide regulatory policies of most developed countries. In fact, the EU has adopted the IPM strategy as a central pillar of action in the 2009 Directive on the sustainable use of pesticides (EC, 2009). The main principles of IPM can be summarized as follows:

1. Cultivation techniques, which include sowing dates and densities, under-sowing, crop rotation, conservation tillage, balanced fertilization, limitation of irrigation/drainage practices, etc.
2. Hygiene measures, such as routine cleaning of machinery and equipment, and responsible management of the resulting RW.
3. Pest monitoring using appropriate methods and tools to facilitate the design of the portfolio of protection measures to be implemented.
4. Biological, physical and other non-chemical techniques, which include the increasingly popular biopesticides, are encouraged over traditional chemical methods.
5. Synthetic pesticides with high selectivity and classified as low-risk compounds should be used at reasonable application doses and frequencies to minimize potential adverse effects on non-target organisms (including humans) and the development of pest resistance.

Therefore, the aim of the IPM strategy is not the eradication of pest populations, but to establish a compromise solution between environmental risk and agricultural production, while maintaining profits above the economic injury level. In any case, the design of an optimal pest management strategy involves close interaction between farmers, consultants, companies and retailers (Chandler et al., 2011).

1.4.1. Biopesticides

The IPM encourages the use of biopesticides, a contraction of biological pesticides, which are a type of crop protection product that encompasses microbial agents, bio-derived chemicals, plant-incorporated protectants and RNA interference pesticides. Approximately 75 % of the

biopesticides are based on *Bacillus thuringiensis*, used to control lepidopteran, dipteran and coleopteran insects (Samada and Tambunan, 2020). Evidence of the adverse effects of intensive use of synthetic pesticides has encouraged farmers to look for more environmentally friendly methods. Most traditional pesticides are broad-spectrum, i.e., they do not only act against the target organism but also against wide biodiversity (such as malathion, dimethoate, chlorpyrifos, etc.), which can lead to serious environmental and agricultural production costs. Once released into the environment, these compounds can remain unaltered for long periods and are easily transported over long distances and eventually bioaccumulated by surrounding flora and fauna (Burkhardt-Holm, 2011). When interacting with the receptor, pesticides can trigger toxic effects through multiple mechanisms, causing severe and sometimes fatal damage (Kumar et al., 2021).

In contrast, biopesticides are considered comparatively more selective and biodegradable, hence sustainable. This more controlled action considerably reduces the probability of pest resistance development. Another advantage of biopesticides is the lower cost associated with the discovery of new substances. In this regard, new biopesticides can be found by using only a few microorganisms, whereas synthetic pesticides require a more difficult and costly screening. For example, strains of the fungus *Talaromyces flavus* were extracted from strawberry crowns (*Fragaria spp*.) to evaluate the inhibitory effect on anthracnose caused by *Colletotrichum acutatum* and *Glomerella cingulata* (Ishikawa, 2013). It is estimated that companies have to screen about 160000 molecules before finding a single compound with potential application (Phillips McDougall, 2016).

However, despite the enormous potential of biopesticides, the scenario of complete replacement of synthetic pesticides seems to be still unattainable given their strong market position (Table 1.3). Biopesticides are also considerably more susceptible to aggressive environmental conditions, which may lead to their inactivation or degradation. Furthermore, the specific action of biopesticides, which ensures the safety of non-target biodiversity, also restricts their application to limited market niches. In fact, the most successful biopesticides on the market are those with a synthetic-like mechanism of action, causing similar environmental problems, such as *B. thuringiensis* (Glare et al., 2012). Biopesticides used for single pathogens require more expertise and dedication from farmers to design an adequate portfolio of techniques to guarantee

crop protection. This limited commercialization of biopesticides leads to higher production costs (Chandler et al., 2011).

Table 1.3. Main advantages and limitations of synthetic pesticides and biopesticides.

	Synthetic pesticides		Biopesticides	
New formulation	✗	Extremely low success rate and high cost	✓	Effective screening and low cost
Environmental risk	✗	Unspecific	✓	Selective
Persistence	✗	Recalcitrant	✓	Biodegradable
Development of pest resistance	✗	Relatively high	✓	Relatively low
General market acceptance	✓	User-friendly	✗	Advanced knowledge
Production	✓	Highly developed, low costs	✗	Early stage, high costs
Stability	✓	Very stable	✗	Susceptible to environmental conditions

Note: positive aspects are indicated with green ticks and negative aspects with red crosses.

Although not yet comparable to synthetic pesticides, the production and sales of biopesticides have followed a steady growth trend of about 10 % over the last decades (Moosavi and Zare, 2015). Farmers are increasingly aware of the medium and long-term benefits of applying the IPM strategies and, in particular, biopesticides. However, more emphasis must be placed on promoting the IPM benefits to a wider range of farmers, with manufacturers, suppliers, experts and regulatory authorities as key communicators. In the current context, these measures could support further market penetration of biopesticides and achieve a judicious mix between biopesticides and chemical pesticides to move towards a more harmonized and developed IPM. Until then, there is a significant risk of producing biopesticides with properties similar to those of synthetic pesticides, which may be equally toxic and persistent.

1.4.2. Management of materials exposed to pesticides: packaging, equipment and machinery

Accordingly, agricultural practices described by the IPM include the implementation of hygiene measures, i.e., the proper management of facilities and equipment that have come into direct contact with pesticides. In addition, the sustainable management of empty pesticide containers has been incorporated in the International Code of Conduct on the Management of Pesticides

(FAO-WHO, 2016). Therefore, particular caution should be taken when handling such equipment or containers, as inadequate practices may pose a high risk of point source contamination (Shukla et al., 2001).

Agrochemical containers usually present chemical residues after application and are classified as hazardous waste (EC, 2015). These products are distributed to consumers in many types of containers including films, nets and packaging. Most containers are made of plastic, with high-density polyethylene (HDPE), polypropylene (PP) or polyethylene terephthalate (PET) being the most commonly used materials, which are quite versatile and allow the production of multiple packing formats with considerable recyclable potential (Marnasidis et al., 2018). However, most agricultural plastic waste is still burned uncontrollably or buried in the soil of fields, assuming major risks in both cases. Uncontrolled burning is a very common practice that can release substances that are extremely harmful to the environment, compromising food and farmer safety. High temperatures reached can also harm soil quality and productivity by damaging the microbiome. In addition, burying the containers can lead to a significant loss of quality in soil characteristics and properties, contamination of food and clogging of water channels. The quality of buried materials may also be negatively affected, reducing their recyclable value (Blanco et al., 2018; Briassoulis et al., 2013). Empty containers of pesticides are even used for storing food and water in the most underdeveloped countries, posing a serious health risk (Jones, 2014).

In Europe, all these practices are illegal under the Landfill Directive 1999/31/EC (EC, 1999), which prohibits uncontrolled burying; the Incineration Directive 2000/76/EC (EC, 2000b), which forbids uncontrolled burning; and the Revised Waste Framework Directive 2008/98/EC (EC, 2008), which establishes the prohibition of uncontrolled waste disposal. The illegal practices used by farmers are mostly a consequence of the non-existent or inefficient management systems for agricultural plastic residues in most European countries, which in turn is caused by the lack of a European strategy for the management of such waste (Briassoulis et al., 2013).

Therefore, agricultural packaging residues are managed through different protocols depending on the legislation of each country. Some of the countries with more developed packaging return

systems are Germany (Pamira, 2017), France (Adivalor, 2018) and Spain (Sigfito, 2018), although each system has its particularities. The most relevant difference is regarding the category of the rinsed container, as in countries such as Germany and France it is declared as non-hazardous waste, whereas in Spain it is designated as hazardous waste (Picuno et al., 2020). This classification is important in determining the recycling value of such waste, as non-hazardous materials are considered raw resources rather than waste. In this regard, FAO and CropLife recommend recognizing rinsed containers as non-hazardous materials (CropLife, 2010).

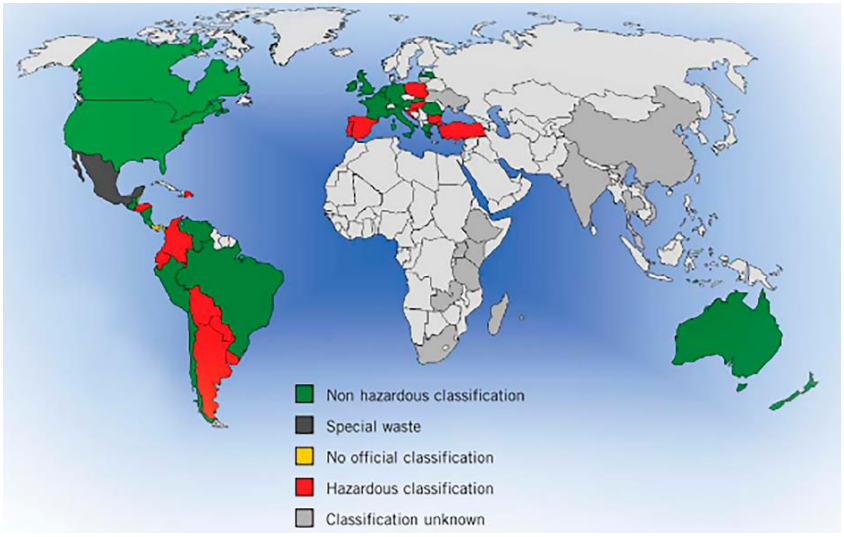


Fig. 1.4. Hazard classification of rinsed pesticide containers (CropLife, 2010).

The triple rinse technique for pesticide containers is recommended by the main international agencies, such as CropLife, FAO and WHO. This method applied immediately after emptying the containers can significantly reduce the risk of contamination (United Nations, 2021). However, when a plastic container is triple rinsed incorrectly or after a too long period, small imperfections or voids can be formed in the material increasing the roughness of the polymer surface, which facilitates the crystallization of toxic compounds and makes their removal difficult even by applying the triple washing procedure. Although the deterioration of the material does not pose any immediate risk to the environment, it may represent a major obstacle to the recycling process (Picuno et al., 2019).

Pesticide containers can be sent to specialized plants for treatment in some countries. For example, there is a non-profit association called Sigfito in Spain that manages empty containers (which may still contain pesticide residues) at the end of their useful life (Sigfito, 2018). For this purpose, Sigfito uses the triple rinse technique to wash the pesticides out of the container and allow subsequent recycling of the empty containers. In this regard, Sigfito is associated with two companies that deal with the waste produced: FCC Medioambiente (FCC Medio Ambiente, 2022), which is specialized in solid waste management, and SITA SPE IBÉRICA S.L.U. (Agbar, 2022), which are experts in dealing with semi-solid waste.

1.4.3. Management of rinse wastewater

Not only the cleaning of agrochemical containers but also of agricultural equipment and machinery can produce RW with a high pesticide content, which must be subsequently managed. Therefore, RW is actually a mixture of diluted pesticides, oils, solvents, commercial rinsing agents or any other compound. Some guidelines suggest the reuse of RW as an agrochemical product in the same agricultural field (EPA, 2012). Hence, rinsate would be reintroduced into the spraying equipment for redosing directly onto the same or adjacent fields (Shukla et al., 2001), or at the margins of the plantations (Life aquemfree, 2018). Reuse as a phytosanitary product is sometimes possible because the active ingredients are considered to be diluted below suboptimal concentrations (Kuo and Regan, 1999). In this respect, the rinsate is recommended to be reused in the same season and crop in which it was applied for the first time, avoiding mixtures and cross-contamination (Minnesota Department of Agriculture, 2016).

However, large farms can generate excessive volumes of RW for direct reuse. In this case, RW is accumulated in collection ponds for long periods to reduce its volume by evaporation, thus increasing pesticide concentration (Kuo and Regan, 1999). Despite volume reduction by evaporation, these waters may be difficult to transport due to their still large volume and elevated toxicity, thus onsite or decentralized treatment would be preferable.

1.4.4. Decentralized treatment of agricultural rinsate

An alternative to natural evaporation is to apply an optimized dehydration process followed by a centralized solid/semi-solid treatment. In Spain, a company called Syngenta has developed and installed several dehydration systems to manage RW from agricultural equipment that

generates solid by-products as waste (Syngenta, 2022). Although it is an upgraded approach, this system is still based on the reduction of water content, as in the case of the natural evaporation technique described above, and a centralized post-treatment of the paste or solid produced must be applied. Accordingly, the solid or paste that is generally stabilized by different chemical agents is finally deposited in landfills (BOE, 2020). Landfill management cannot be considered a genuinely environmentally friendly method and more sustainable alternatives must be explored. Decentralized treatment of RW is presumably the most appropriate strategy, although a viable treatment for this type of waste should be developed (Naveen et al., 2017; Saleh et al., 2020). This thesis focuses on the treatment of pesticide contamination of both agricultural field effluents and RW.

1.5. Treatment approaches for pesticide removal from water

In this scenario, the development of effective treatments for the removal of pesticides from agricultural wastewater (AW) is mandatory. Table 1.4 shows a summary of the main techniques used to remove organic pollutants, as well as the main advantages and limitations of each of them concerning investment and operational costs, removal efficiency, operability, reliability, pretreatment requirements, sludge production, environmental impact and generation of toxic by-products. They are commonly classified into physical, chemical and biological treatments according to their elimination strategy. The first two groups of treatments are frequently presented together as physical-chemical treatments due to the strong relationship between these phenomena (Marican and Durán-Lara, 2018). Physical-chemical treatments mainly include sorption, advanced oxidation processes (ozonation, photocatalysis, electrochemical oxidation, etc.) and membrane filtration, and have the advantage of being compact, fast and effective processes against pesticides. Among them, the most mature and widely implemented technologies for the full-scale treatment of pesticides in water have been ozonation, reverse osmosis, nanofiltration and activated carbon sorption processes (Dong et al., 2021; Martin-Gullon and Font, 2001; Reungoat et al., 2010; Yangali-Quintanilla et al., 2011). However, these approaches often involve high operational costs and the potential generation of residues or TPs (Ahmed et al., 2017).

In contrast, biological treatments, also known as bioremediation processes, are considered low-cost, environmentally friendly and efficient technologies, although they generally require longer treatment periods and add operational complexity due to biomass handling and maintenance (Marican and Durán-Lara, 2018). Among them, conventional activated sludge is a process traditionally used for the treatment of wastewater pollution, but it has demonstrated poor removal efficiency of pesticides and other recalcitrant POPs (Margot et al., 2015). Membrane bioreactor (MBR) is another common type of biological treatment technology in which activated sludge treatment is combined with a membrane separation process. Despite the improved results in terms of effluent quality and operational periods, these improvements are not good enough to economically justify the implementation of MRBs in municipal WWTPs (Grandclément et al., 2017). Recently, many studies have been reported on the bioremediation of pesticides in water using bioreactors inoculated with bacteria, algae or fungi (Saleh et al., 2020). Nevertheless, the scientific community is also increasingly convinced that water contaminated with complex mixtures of micropollutants, including pesticides, should be treated using hybrid technologies or treatment trains, in which the advantages of each technique are synergistically combined (Dhangar and Kumar, 2020).

Table 1.4. Main advantages and limitations of the technologies used to remove organic pollutants (Ahmed et al., 2017; Domingues et al., 2018; Harms et al., 2011; Judd, 2008; Luo et al., 2014; Marican and Durán-Lara, 2018; Sousa et al., 2022).

Technology	Advantages	Limitations
Physical-chemical methods		
Activated carbon	• Easy to scale-up • Dissolved organic carbon removal • Useful for removing POPs • Removal of residual disinfection/TPs	• Relatively high cost in energy and residues management • Activated carbon preparation is required • Post-treatment of activated carbon is required • Sorption capacity depends on the activated carbon and organic content characteristics.
Coagulation-flocculation	• Reduces turbidity through the aggregation of suspended solids • Increases sedimentation rate	• Not suitable for micropollutants • Large amount of sludge • Presence of coagulant residues in the aqueous phase
Chlorination	• Low cost	• Low capacity to remove POPs

Technology	Advantages	Limitations
	• Disinfection • Effective in oxidizing organic pollutants	• Formation of oxidation-products • The chlorine residues can be toxic to aquatic organisms
Ozonation	• Easy to scale-up • Fractional disinfection • Moderate energy requirements	• Formation of TPs and oxidation-products (such as bromate) • Requirement of a subsequent biological process to eliminate by-products
(Photo)catalytic oxidation	• Catalyst recycling • Can use solar radiation	• High cost of catalysts
UV treatment	• Simple process • Disinfection • Enhanced removal of POPs when combined with other technologies	• Formation of oxidation-products • Matrix pH adjustment required • High energy required
Electrochemical oxidation	• Degradation and mineralization of POPs • Compact design • Easy operation	• Not yet implemented on a full-scale application • Formation of by-products • High costs
Microfiltration and ultrafiltration	• Removal of pathogens • Useful for removing heavy metals • Low land requirement • Rejection of particles	• Not very effective in removing POPs (pore size usually 100 to 1000 times larger than micropollutants). • High operational cost • Periodic membrane cleaning and replacement
Nanofiltration	• Effective for treating WWTP effluents and saline water • Pre-treatment is usually necessary to remove solids • Partial removal of POPs • Low land requirement • Rejection of particles	• High energy demand • High membrane fouling and disposal issue • Periodic membrane replacement
Reverse osmosis	• Effective for treating WWTP effluents and saline water • Pre-treatment is usually necessary to remove solids • Can remove POPs • Low land requirement • Rejection of particles	• High energy demand • High membrane fouling and disposal issue • Corrosive effluent • Periodic membrane replacement
Biological methods		
Conventional activated sludge	• Relatively low investment and operational costs	• Low efficiencies in removing POPs

Technology	Advantages	Limitations
	• Relatively low land demand • Considered environmentally friendly	• The process can be sensitive to the toxicity of POPs • Not recommended when chemical organic demand (COD) is greater than 4000 mg O_2 L^{-1} • Large amount of sludge containing POPs
Membrane bioreactor (MBR)	• Effective for the removal of POPs • Relatively small plant size • Operation with relatively high organic loads	• Elevated energy consumption and investment cost • High fouling • Greater process complexity
Constructed wetlands	• Low energy consumption and operational and capital costs. • Efficient for removing POPs • Simplicity	• Clogging and sediment formation • Biofilm growth • Chemical precipitation • Seasonal dependent • High retention time and land requirement • A preliminary treatment is usually necessary
Microalgae-based treatment	• Considered a low-cost and environmentally friendly technology • Easy operation • No formation of metabolites • Biomass recovery for diverse purposes • Effective for treating POPs	• Low specificity • Aging • Difficult to separate biomass from effluent. • Seasonal-dependent process • Can require oxygen supply
Biofilter/biobeds	• Easy to scale-up • Compatible with bacterial contamination • Suitable for removing POPs • Low chemicals usage • Considered a low-cost and environmentally friendly technology	• Not indicated for high concentrations • Long operational periods (up to one year) • Difficult control operation • Long period of microbial acclimation is necessary • Generation of metabolites
Enzymatic treatment	• Biocatalyst recycling • Enzymes are biodegradable • Different substrates can be used	• Difficult to optimize • Requires information on POP degradation pathways • Enzymes groups limitations
Bacterial-based treatment	• Use pollutants as a growth substrate • Mobility in water • Fast metabolism and relatively short operational periods. • Particularly successful at eliminating chemicals with simple structures	• Preferably for waters with high content of pollutants that can be used as substrate. • Specific biochemical pathways, which limits their application to particular pollutants.

Technology	Advantages	Limitations
		• Can be aerobic organisms that require oxygen supply
Fungal-based treatment	• Effective for treating a wide range of POPs • Considered a low-cost and environmentally friendly technology • Powerful and nonspecific enzyme system • High resistance to toxicity • No prior acclimatization period is required • Capable of treating wastewater with high pollutant content	• Slow metabolism, high retention time is required • A relatively large area of land is needed • Biomass renewal is usually required • Matrix pH adjustment required • Not yet implemented on a full-scale application • Co-metabolic removal of micropollutants, thus requiring an additional carbon source • Aerobic organisms that require oxygen supply

1.6. Fungal treatment

1.6.1. Pesticide bioremediation by white rot fungi

The term white rot fungi (WRF) refers not to a taxonomic grouping, but to a set of fungal species (mainly basidiomycetes) that are capable of degrading lignin, which is a complex polyphenolic polymer that constitutes the structural support material of higher plants (e.g. trees). The name white rot alludes to the bleached appearance of the lignocellulosic substrate, e.g. wood, after being decomposed by these fungi.

WRF can break down lignin by means of their nonspecific and potent enzyme system, which is composed of extracellular enzymes, including laccase (EC 1.10.3.2), manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14), dye decolorizing peroxidase (EC 1.11.1.19), versatile peroxidase (EC 1.11.1.16), etc., and the intracellular enzyme system cytochrome P450 (Zhuo and Fan, 2021). The sophisticated oxidative system together with the strong resistance to the toxicity of WRF make them versatile in degrading a wide range of organic pollutants, such as dyes, industrial chemicals, pharmaceuticals, flame retardants, pesticides, etc (Maqbool et al., 2016; Mir-Tutusaus et al., 2018a). Accordingly, multiple articles have demonstrated the high degradation capacity of WRF towards different pesticides (Mori et al., 2021; Rodríguez-Rodríguez et al., 2017). Among them, *Trametes versicolor* has proven to be one of the most

effective species in eliminating pesticides, as well as other xenobiotics (Hu et al., 2020b; Marco-Urrea et al., 2009).

Despite the promising results of pesticide degradation obtained with WRF, certain limitations still make their application on an industrial scale unfeasible (Table 1.4). One concern is the need for nutrient addition. WRF usually degrade micropollutants co-metabolically, because although these compounds can be assimilated by fungi, they are generally present at too low concentrations to satisfy their metabolic demands and additional nutrient sources (mainly C and N) are required (Pointing, 2001). This poses a challenge for full-scale application, as the cost of adding glucose and nitrogen could be high and would also increase the chemical oxygen demand (COD) and nitrogen load. This drawback can be partially mitigated by adding the nutrients at the same consumption rate (Mir-Tutusaus et al., 2017).

Nevertheless, the addition of easily assimilated nutrient sources (such as glucose) can promote bacterial contamination, which has been identified as the main bottleneck of fungal technology. When operating under non-sterile conditions other microorganisms, mainly bacteria, can compete for substrate consumption and exert pressure on WRF survival, which in turn reduces the lifetime and treatment efficiency of the operations performed in fungal reactors (Mir-Tutusaus et al., 2016). Different strategies have been used to benefit the prevalence of fungi over bacteria, such as adjustment of C/N ratio and pH (Badia-Fabregat et al., 2016), biomass renovation (Blánquez et al., 2006) and fungal immobilization (Hai et al., 2013), among others.

In this regard, the scientific community seems to have reached a certain consensus on the advantages of fungal immobilization in bioremediation processes, as it overcomes some of the common limitations in fungal treatments, including fungal growth on reactor walls and accessories, foam production and biomass washout by decoupling the cellular retention time (CRT) from the hydraulic retention time (HRT) (Mir-Tutusaus et al., 2018a). There are basically two types of fungal immobilization: autoimmobilization as pellets and immobilization on carriers. Carriers can be either inert (e.g. polyurethane foam cubes) or non-inert (e.g. wood) (Zhuo and Fan, 2021). Among non-inert carriers, fungal immobilization on lignocellulosic substrate seems to attract special interest as they present multiple benefits:

a) Lignocellulosic substrate serves both as a support and as a source of nutrients, avoiding substrate competition with bacteria. This strategy is promising to address the issue of bacterial contamination and enhance the performance of fungal reactors when operating under non-sterile conditions (Hu et al., 2021).

b) It is a low-cost resource that could even be obtained from by-products generated in timber industries, making the treatment more sustainable and environmentally friendly.

c) Furthermore, wood is a porous material with high sorption potential, which combined with the bioremediation capacity of WRF increase the overall removal efficiency of the treatment (García-Vara et al., 2021).

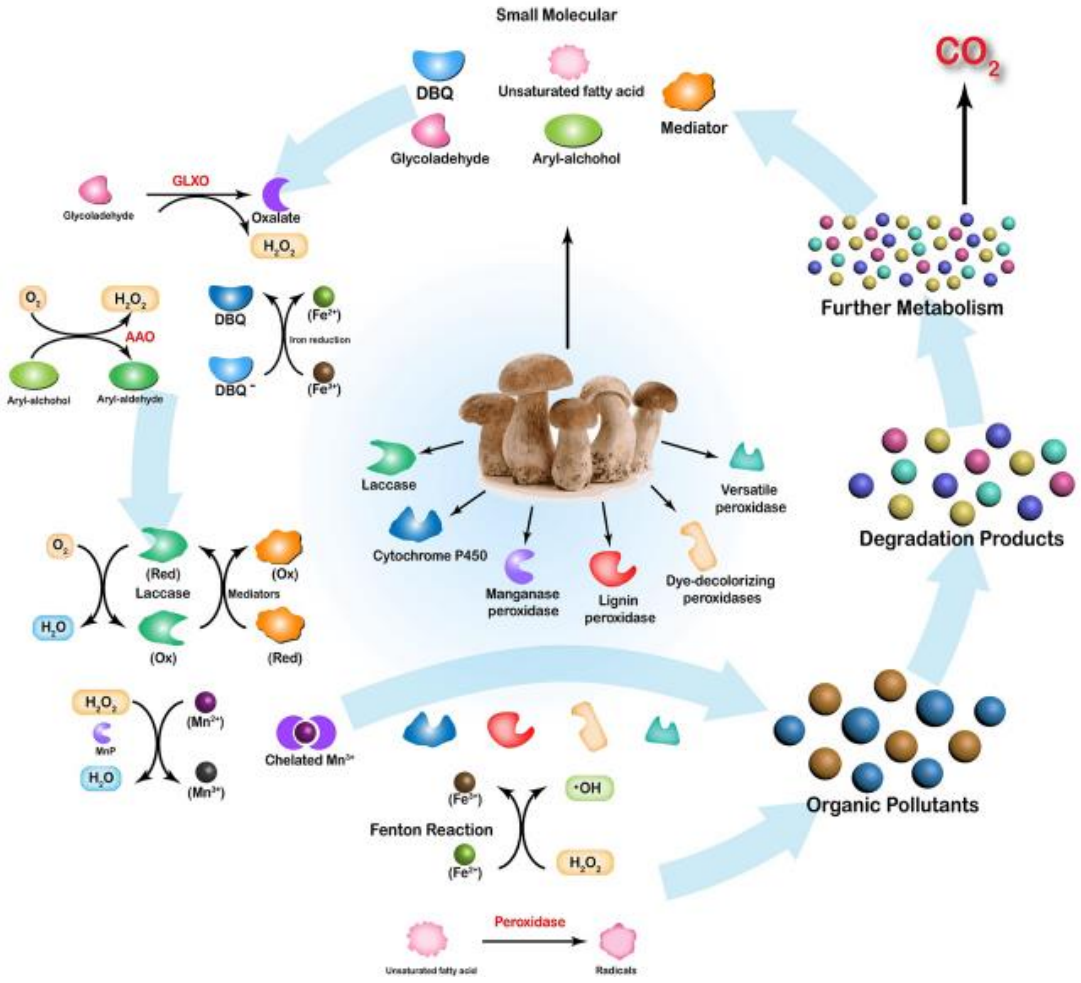


Fig. 1.5. Pathways of organic pollutant degradation by WRF (Zhuo and Fan, 2021).

1.6.2. Fungal reactor

In recent years, bioremediation using WRF has attracted increasing interest because of its unique characteristics, which include a powerful enzymatic system capable of degrading numerous pollutants, such as pesticides, and its strong resistance to toxicity (Gao et al., 2010). Accordingly, several fungal bioreactors have been investigated to remove pesticides (Table 1.5). For example, Nguyen et al. (2013) explored the degradation of several pesticides in a fungus-augmented MBR inoculated with *T. versicolor*, achieving removals of 29 % ametryn, 11 % atrazine, 65 % clofibric acid, 57 % fenoprop, 92 % pentachlorophenol and 20 % propoxur. In continuous mode, more than 85 % aerobic biodegradation of chlorpyrifos by *Aspergillus sp.* was reported in packed bed bioreactors (Yadav et al., 2015). *Aspergillus niger* was also able to continuously degrade 60 % of the herbicide atrazine from wastewater in a bioreactor (Marinho et al., 2017). One of the most widely used WRF is *T. versicolor* due to its proven ability to degrade multiple xenobiotics. For example, clofibric acid was biodegraded by this fungus in an air-pulsed fluidized bioreactor operated in continuous mode, eliminating 80 % of the compound (Cruz-Morató et al., 2013b). The immobilization strategy has already been explored for pesticide treatment in several fungal bioreactors. Hu et al. (2021) recently compared the pesticide removal efficiencies achieved by *T. versicolor* immobilized on pine wood in sequencing batch mode in two different bioreactors, a fluidized-bed and a trickled bed reactor. The latter reactor showed higher yields even for lower contact times, which was partially attributed to the sorption capacity of the wood.

Table 1.5. Reported evidence of pesticide abatement by using fungal bioreactors

Reactor	Fungus	Real/synthetic water	Matrix	Sterility	Operating period	HRT	pH	Initial concentration	Pesticide	Removal (%)	Source
Bottle reactor	*Aspergillus niger*	Synthetic water	Vishniac solution	Sterile	8 days	Batch	3,5	30 mg L^{-1}	Atrazine	72	Marinho et al. (2017)
Bottle reactor	*Trametes versicolor*	Real water	WWTP effluent	Sterile	12 hours	Batch	4.5	350 µg L^{-1}	Atrazine	0	Shreve et al. (2016)
Bottle reactor	*Trametes versicolor*	Real water	WWTP effluent	Sterile	12 hours	Batch	4.5	350 µg L^{-1}	DEET	0	Shreve et al. (2016)
Fixed-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	20 days	1 day	4.5	10 mg L^{-1}	Bentazon	6	Beltrán-Flores et al. (2021)
Fixed-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	40 days	3 days	4.5	10 mg L^{-1}	Bentazon	18	Beltrán-Flores et al. (2021)
Fixed-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	20 days	1 day	4.5	10 mg L^{-1}	Diuron	42	Beltrán-Flores et al. (2021)
Fixed-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	40 days	3 days	4.5	10 mg L^{-1}	Diuron	61	Beltrán-Flores et al. (2021)
Fluidized-bed bioreactor	*Trametes versicolor*	Synthetic water	Defined medium	Sterile	7 days	Batch	4.5	4 mg L^{-1}	Acetamiprid	20	Hu et al. (2021a)
Fluidized-bed bioreactor	*Trametes versicolor*	Synthetic water	Defined medium	Sterile	24 days	4 days	4.5	160 µg L^{-1}	Clofibric acid	80	Cruz-Morató et al. (2013)
Fluidized-bed bioreactor	*Trametes versicolor*	Synthetic water	Defined medium	Sterile	7 days	Batch	4.5	4 mg L^{-1}	Imidacloprid	65	Hu et al. (2021a)
Fluidized-bed bioreactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	30 days	3 days	4.5	10 mg L^{-1}	Bentazon	37	Hu et al. (2021)
Fluidized-bed bioreactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	30 days	3 days	4.5	10 mg L^{-1}	Diuron	30	Hu et al. (2021)

Reactor	Fungus	Real/synthetic water	Matrix	Sterility	Operating period	HRT	pH	Initial concentration	Pesticide	Removal (%)	Source
MBR	*Trametes versicolor*	Synthetic water	Malt extract-based	Non-sterile	110 days	2 days	4.5	5 µg L^{-1}	Fenoprop	57	Nguyen et al. (2013)
MBR	*Trametes versicolor*	Synthetic water	Malt extract-based	Non-sterile	110 days	2 days	4.5	5 µg L^{-1}	PCP*	92	Nguyen et al. (2013)
MBR	*Trametes versicolor*	Synthetic water	Malt extract-based	Non-sterile	110 days	2 days	4.5	5 µg L^{-1}	Propoxur	20	Nguyen et al. (2013)
Membrane bioreactor	*Trametes versicolor*	Synthetic water	Malt extract-based	Non-sterile	110 days	2 days	4.5	5 µg L^{-1}	Ametryn	29	Nguyen et al. (2013)
Membrane bioreactor	*Trametes versicolor*	Synthetic water	Malt extract-based	Non-sterile	110 days	2 days	4.5	5 µg L^{-1}	Atrazine	11	Nguyen et al. (2013)
Membrane bioreactor	*Trametes versicolor*	Synthetic water	Malt extract-based	Non-sterile	110 days	2 days	4.5	5 µg L^{-1}	Clofibric acid	65	Nguyen et al. (2013)
Packed bed	*Aspergillus sp.*	-	-	Sterile	45 days	-	7	180-250 mg L^{-1}	Chlorpyrifos	90	Yadav et al. (2015)
Packed-bed channel bioreactor	*Trametes versicolor*	Synthetic water	Tap water	Non-sterile	49 days	3 days	4.5	10 mg L^{-1}	Diuron	89	Beltrán-Flores et al. (2020)
Packed-bed channel bioreactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	35 days	3 days	4.5	10 mg L^{-1}	Diuron	94	Beltrán-Flores et al. (2020)
Packed-bed reactor	*Verticilium sp. and Metacordyceps sp.*	Synthetic water	Define medium	Sterile	10 days	9.8 hours	5.5	50 mg L^{-1}	Atrazine	59	Levio-Raiman et al. (2021)
Packed-bed reactor	*Verticilium sp and Metacordyceps sp.*	Synthetic water	Define medium	Sterile	10 days	9.8 hours	5.5	50 mg L^{-1}	Chlorpyrifos	85	Levio-Raiman et al. (2021)
Packed-bed reactor	*Verticilium sp and Metacordyceps sp.*	Synthetic water	Define medium	Sterile	10 days	9.8 hours	5.5	50 mg L^{-1}	Iprodione	96	Levio-Raiman et al. (2021)
Rotating drum bioreactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	225 days	3-5 days	4.5	10 mg L^{-1}	Bentazon	14	Beltrán-Flores et al. (2022)

Reactor	Fungus	Real/synthetic water	Matrix	Sterility	Operating period	HRT	pH	Initial concentration	Pesticide	Removal (%)	Source
Rotating drum bioreactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	16 days	3 days	4.5	10 mg L^{-1}	Diuron	61	Beltrán-Flores et al. (2020)
Rotating drum bioreactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	225 days	3-5 days	4.5	10 mg L^{-1}	Diuron	33	Beltrán-Flores et al. (2022)
Slurry reactor	*Bjerkandera adusta*	Real water	Slurry	Sterile	30 days	Batch	4.5	25 mg kg^{-1}	α-HCH*	95	Quintero et al. (2007)
Slurry reactor	*Bjerkandera adusta*	Real water	Slurry	Sterile	30 days	Batch	4.5	25 mg kg^{-1}	β-HCH*	66	Quintero et al. (2007)
Slurry reactor	*Bjerkandera adusta*	Real water	Slurry	Sterile	30 days	Batch	4.5	25 mg kg^{-1}	Lindane	95	Quintero et al. (2007)
Slurry reactor	*Bjerkandera adusta*	Real water	Slurry	Sterile	30 days	Batch	4.5	25 mg kg^{-1}	δ-HCH*	79	Quintero et al. (2007)
Trickle-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	30 days	3 days	4.5	10 mg L^{-1}	Bentazon	48	García-Vara et al. (2021)
Trickle-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	36 days	3 days	4.5	10 mg L^{-1}	Diuron	63	Hu et al. (2020)
Trickle-bed reactor	*Trametes versicolor*	Real water	Agricultural wastewater	Non-sterile	30 days	3 days	4.5	10 mg L^{-1}	Diuron	69	Hu et al. (2021)

*HCH is hexachlorocyclohexane and PCP is pentachlorophenol

Chapter 2

General objectives

The main goal of the present thesis is to develop a long-term continuous treatment of agricultural wastewater using the white-rot fungus *Trametes versicolor* immobilized on wood chips. In order to achieve this goal, the following specific objectives were formulated:

- To study the sorption phenomenon of pesticides of different chemical nature on wood chips.
- To set up a rotating drum bioreactor with *T. versicolor* immobilized on wood chips for the treatment of spiked agricultural wastewater under non-sterile conditions, in continuous mode and for a long-term period.
- To develop a biopile-type reactor for the treatment of old polluted wood chips.
- To assess the effect of the limiting oxygen level on pollutant degradation by *T. versicolor* and to analyze the evolution over time of dissolved oxygen level at various points within the RDB.
- To apply the rotating drum bioreactor for the treatment of real agricultural wastewater (rinse wastewater), and to compare its performance with that obtained both by a fluidized bed bioreactor (FBB), which is a fungal reactor that has consistently reported good results in the degradation of other pollutants, and by ozonation, which is one of the most widely used physical-chemical technologies in the treatment of micropollutants.

Chapter 3

General materials and methods

3.1. Lignocellulosic material

Two different lignocellulosic materials were used in the present work: pine wood pallets (*Pine sp.*) and holm oak wood (*Quercus ilex*). The pine wood pallets were kindly provided by Timgad S.A. (Barcelona, Spain), while the holm oak wood was collected from the forest located in Vidreres (Girona, Spain). They were stored at room temperature until use, when they were crushed and sieved, collecting the intermediate fraction of wood chips between the standardized sieves of 16 and 7.10 mm. Afterwards, the wood chips were completely immersed in tap water and autoclave at 120 °C for 30 minutes, after which they were separated with a strainer. The bulk density of the wood was calculated using Eq. (3.1) (ASTM, 2018):

$$\rho_b = \frac{m_s}{V_b} \qquad (3.1)$$

where V_b is the volume occupied by the bed of wood chips (mL), m_s (50 g), measured in a 500 mL graduated cylinder. The bed porosity (ϕ) was determined by Eq. (3.2):

$$\phi = \frac{V_w}{V_b} \qquad (3.2)$$

where V_w is the volume of water (mL) that was poured into the into the graduated cylinder until the wood chips were completely submerged. The true density was calculated by Eqs. (3.3) and (3.4):

$$\rho_t = \frac{m_s}{V} \qquad (3.3)$$

$$V = V_b - V_w \qquad (3.4)$$

Being V the volume occupied by the wood chips (mL).

3.2. Agricultural and rinse wastewaters

Agricultural wastewater (AW) was collected from drainage channels located in the Parc Agrari Baix Llobregat (Gavà, Spain). This area has an intense agricultural activity where multiple pesticides have recently been detected at very low concentrations. The pesticide presence pattern of this sampling point (SW10) was described by Postigo et al. (2021). The collection date, location and other information are summarized in Table 3.1. A coagulation-flocculation pre-treatment was required to erase algae from AW III. Coagulant ferric chloride (40 mg L^{-1})

and flocculant PROSEDIM CS 209 (2 mg L^{-1}) were added according to the following sequence: 2 min coagulation at 200 rpm, 15 min flocculation at 20 rpm and 30 seconds sedimentation.

Fig. 3.1. Agricultural drainage channel in Gavà, Barcelona (41°16'36.0"N 2°01'10.5"E, Spain).

RW (table 3.1) was collected from an artificial pond designed to accumulate wastewater produced during the washing of spraying equipment and agricultural machinery within the framework of the Sustainable Plant Protection program of the Institute of Agrifood Research and Technology (IRTA) in association with the Mas Badia Foundation (La Tallada d'Empordà, Spain). All of them were stored at 4 ºC until use.

Table 3.1. Information on the agricultural and rinse wastewaters.

ID	Date of collection	Location	Experiment	Spiking
AW I	25/09/2018	Gavà	Chapter 4	Yes
AW II	05/02/2019	Gavà	Chapter 4	Yes
AW III	09/07/2020	Gavà	Chapter 5	Yes
AW IV	06/10/2020	Gavà	Chapter 5	Yes
AW V	09/12/2020	Gavà	Chapter 5 & 6	Yes
RW I	11/09/2020	Mas Badia	Chapter 7	No
RW II	24/08/2021	Mas Badia	Chapter 8	No

3.3. Chemicals and reagents

3.3.1. Micropollutants

Analytical standards of diuron, bentazon, thiacloprid (THIA), chlortoluron (CHLOR), azoxystrobin (AZO), tebuconazole (TEBU), pyrimethanil (PYRI) and tributyl phosphate (TBP) were purchased from Sigma-Aldrich (Barcelona, Spain). Commercial herbicide KAOS-B (48 % bentazon) was supplied by SAPEC AGRO (Barcelona, Spain). The isotopically labeled standards used in the present work were of high purity grade (≥ 98 %). Stock solutions (10 mg mL^{-1}) of the pesticides intended for fungal treatment were prepared by dilution in acetonitrile, while those used for analytical purposes were prepared by diluting the standard compounds in methanol at 100 mg L^{-1}.

3.3.2. Other chemical compounds and reagents

High-performance liquid chromatography (HPLC) grade methanol, ethanol and acetonitrile, formic acid of high purity grade (≥ 98 %), and ferric chloride were acquired from Merck (Darmstadt, Germany). PROSEDIM CS 209 was obtained from Degrémont Iberia (Spain). Purified laccase of *T. versicolor* was purchased from Sigma-Aldrich (Barcelona, Spain). D(+)-Glucose was supplied by Acros Organics (New Jersey, USA). Ammonium chloride was acquired from Scharlau (Barcelona, Spain). Cyclohexane was obtained from Labkem (Barcelona, Spain). Oxalic, oxamic and maleic acids (≥ 99%) were purchased from Sigma-Aldrich. All other chemicals used were of analytical grade and purchased from Sigma-Aldrich (Barcelona, Spain).

3.4. Microorganisms and culture conditions

3.4.1. Media

The malt extract medium (20 g L^{-1}) was used to prepare agar plates and mycelial suspensions. The defined growth medium was prepared according to a modified method of Kirk et al. (1978), by using glucose and ammonium tartrate as carbon and nitrogen sources, respectively. Thiamine was added directly to the medium after autoclaving the reactor. The defined maintenance medium was modified from Cruz-Morató et al. (2013a), in which macronutrients and micronutrients were also provided, and it was used in the experiments conducted in the stirred

tank reactor (Chapter 6). All the media were autoclaved for 30 min at 121 ºC before use. Table 3.2 and Table 3.3 describe the detailed composition of the media.

Table 3.2. Composition of the maintenance and growth media.

	Concentration	
	Growth medium	Maintenance medium
Glucose (g L^{-1})	8	2
Ammonium tartrate (mg g pellets DW^{-1} day^{-1})	-	2
Ammonium chloride (g)	2.1	-
Macronutrients (mL L^{-1})	100	100
Micronutrients (mL L^{-1})	10	10
Thiamine (mg L^{-1})	10	-

Table 3.3. Composition of macronutrients and micronutrients in the defined media.

Micronutrients	Concentration (g L^{-1})	Macronutrients	Concentration (g L^{-1})
Nitrile triacetic acidic	1.5	KH_2PO_4	20
$MgSO_4 \cdot 7H_2O$	3.0	$MgSO_4 \cdot 7H_2O$	5
$MnSO_4 \cdot H_2O$	0.5	$CaCl_2$	1
NaCl	1.0		
$FeSO_4 \cdot 7H_2O$	0.1		
$CoSO_4$	0.1		
$ZnSO_4 \cdot 7H_2O$	0.1		
$CaCl_2 \cdot 2H_2O$	0.1		
$CuSO_4 \cdot 5H_2O$	0.01		
$AlK(SO_4)_2 \cdot 12H_2O$	0.01		
H_3BO_3	0.01		
Na_2MoO_4	0.01		

3.4.2. Fungal strain

T. versicolor ATCC 42530 was purchased from the American Type Culture Collection. Fungal strains were maintained by subculturing every 30 days on malt extract agar plates (2 % w/v) at pH 4.5 and 25 °C. A mycelial suspension of *T. versicolor* was prepared according to a previous method described by Font et al. (2003). Briefly, four 1 cm^2 agar plugs were cut from a Petri dish

and used to inoculate 500 mL Erlenmeyer flasks containing 150 mL malt extract medium. The culture was maintained with orbital agitation (135 rpm) at 25 ºC during 6 days of incubation. Afterwards, the fungal biomass was separated from the media by means of a strainer and subsequently homogenized with a T25 digital ULTRA-TURRAX® disperser (IKA GmbH, Germany) and stored in a 0.80 % (w/v) NaCl solution at a relation of 1:1 (v/v). The resultant mycelial suspension could be immediately used for inoculation or stored at 4 ºC under sterile conditions.

3.4.3. Pellet preparation

Fungal pellets were produced by inoculating 2.7 mL L^{-1} of the mycelial suspension into 10 L of defined growth medium in an air-fluidized glass bioreactor working under sterile conditions. The pH was maintained at 4.5 by adding 1 M HCl or 1 M NaOH. Dissolved oxygen (DO) was measured to ensure adequate aeration. Fluidized conditions were achieved by introducing a 1 s air pulse every 4 s through a solenoid valve placed at the bottom part of the reactor. The aeration rate was set at 0.8 L min^{-1} and the temperature was maintained at 25 ºC (Borràs et al., 2008).

3.4.4. Fungal immobilization

Likewise, the mycelial suspension was used as inoculum to produce the immobilized fungal culture. Thus, 0.25 mL g wood DW^{-1} of mycelial suspension was inoculated onto the wood chips inside a polyvinyl chloride box that was subsequently covered with aluminum foil. The fungal culture was grown under sterile conditions at room temperature: for 17 days (Chapter 5, 7 and 8) and 20 days (Chapter 4, Topic 4.1) in the RDB; 17 days in the FBR (Chapter 4, Topic 4.2); and at 25 ºC: for 24 days in the RDB (Chapter 6) and 50 days in the FBR (Chapter 6).

Fig. 3.2. *T. versicolor* immobilized on *Q. ilex* wood after 17 days of culture under sterile conditions in a polyvinyl chloride box.

3.5. Fungal reactors

A total of 6 different reactors were used: a rotating drum bioreactor (RDB), a fixed bed reactor (FBR), a biopile-like reactor (BR), a fluidized bed bioreactor (FBB), a stirred tank bioreactor (STB) and a stirred tank reactor with ozone supply (STR).

3.5.1. Rotating drum bioreactor

The RDB was constructed with a methacrylate tube (length: 45.0 cm x radius: 4.2 cm) supported on a polyvinylchloride gutter (length: 51.0 cm x radius: 7.0 cm). The wastewater was contained within the channel while the colonized wood chips were placed inside the tube. The tube was provided with multiple 8 mm diameter holes to ensure adequate contact between the biomass and the wastewater and it was covered with a plastic mesh to prevent biomass loss. Approximately 30 % of the biomass was submerged in the liquid phase, while the rest was in direct contact with air. The inner tube was connected to an electric motor (Worm Gear Motor, model: 4632-370, 12V) that rotates to alternate the submerged biomass fraction. The working volume was approximately 2.3 L. No additional substrate or nutrient was added to the reactor. Fig. 3.3 shows (a) a picture of the setup and (b) the schematic representation. The RDB was operated with different configurations depending on the experiment. These configurations are described in more detail in the specific methodology of each chapter.

a) **b)**

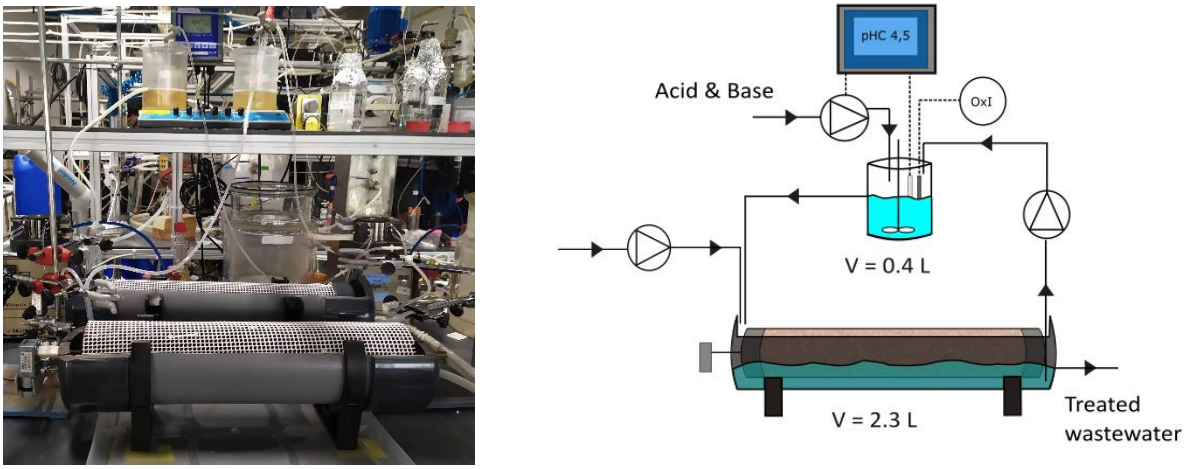


Fig. 3.3. Rotating drum bioreactor: a) actual reactor setup; b) schematic representation.

3.5.2 Fixed bed reactor

The FBR consisted of a cylindrical methacrylate tube (4.4 cm diameter) that contained the fungal biomass. Two columns with different lengths were operated: 28.5 cm in Chapter 4 (volume = 0.433 L) and 62 cm in Chapter 6 (volume = 0.900 L). The reactor was fed with an upward stream of AW, which was initially adjusted to pH 4.5. A series of ports were installed at different heights of the column (depending on the experiment) to withdraw samples (Chapter 4) or to measure DO (Chapter 6). Fig. 3.4 presents (a) an overview of the setup and (b) the schematic illustration.

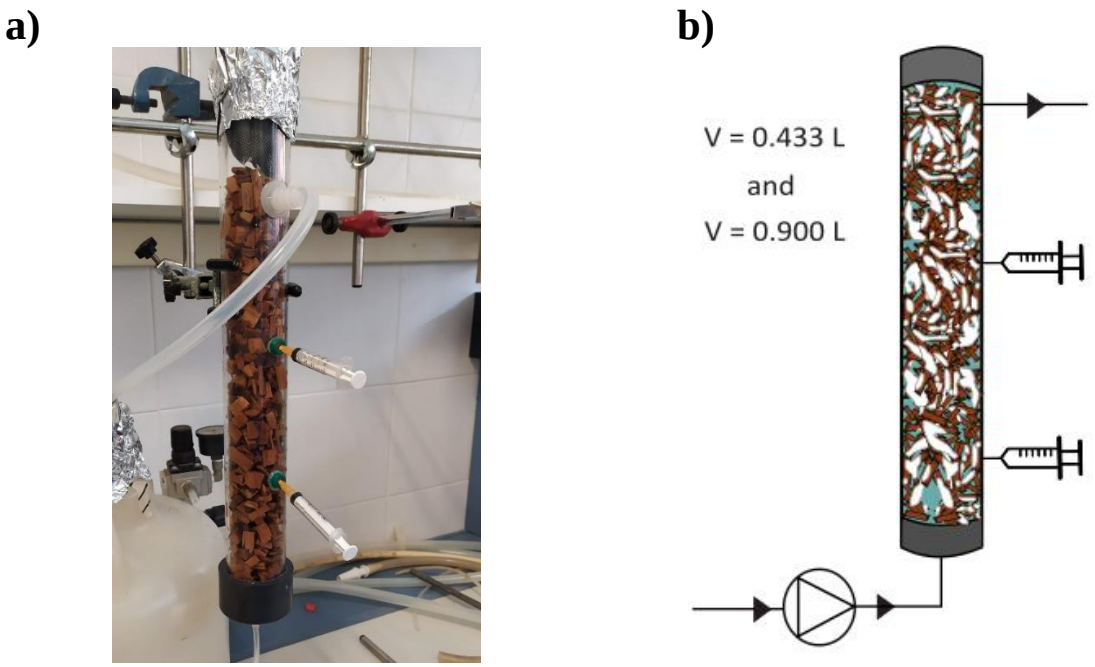


Fig. 3.4. Fixed bed column reactor: a) actual reactor setup; b) schematic representation.

3.5.3. Biopile-like reactor

The biopile was used for the treatment of pesticide-contaminated by-products (old colonized wood). In this regard, 30 g of polluted solid by-products (in triplicate) were treated in Scott glass bottles (Duran, Inc; 250 mL, 95 x 105 mm) equipped with one port screw cap opened with a passive air intake through a filter (0.45 µm). Three different strategies were implemented depending on the experiment: inoculation of fresh *T. versicolor* on wood (wood from the control reactors), re-inoculation and non-re-inoculation of fresh *T. versicolor* on old wood containing pre-grown fungus. In inoculation or re-inoculation strategies, 7 mL of mycelial suspension was added as fresh inoculum. The culture was maintained at a constant temperature of 25 ºC under static and non-sterile conditions. The duration of the treatment depended on each particular experiment and is specified in each chapter. Fig. 3.5 illustrates (a) an image of the biopile and (b) its schematic representation.

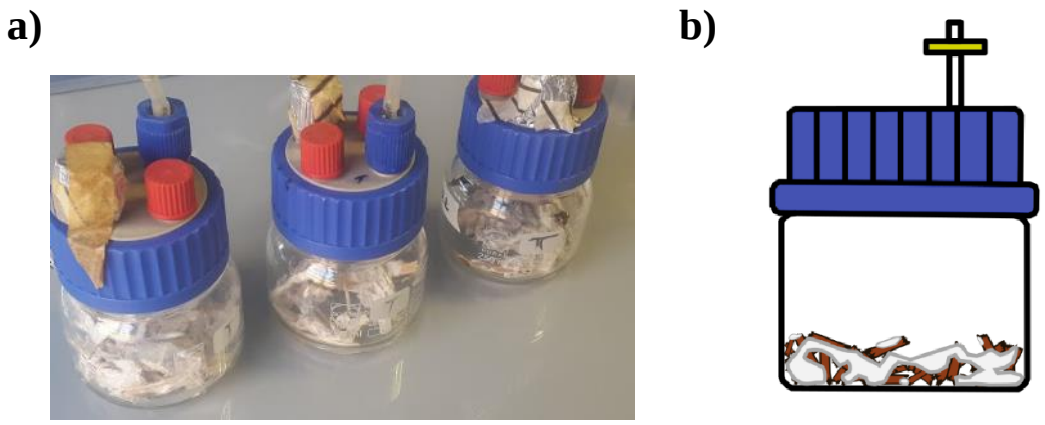


Fig. 3.5. Biopile-like reactor: a) actual reactor setup; b) schematic representation.

3.5.4. Fluidized bed bioreactor

The glass FBB consisted of a 7.5 cm diameter body and a considerably wider diameter head of 13.5 cm. The useful volume was 1.5 L. Fluidized conditions were achieved by introducing a 1 s air pulse every 4 s at a flow rate of 0.8 L min^{-1} through a solenoid valve placed at the bottom part of the reactor. The air pulses passed through a porous plate generating small bubbles that diffused through the liquid phase. The reactor head was equipped with several ports that were used for nutrient addition, foam collection, pH monitoring, acid and base inlet, air outlet system and sample collection. The pH was controlled at a constant value of 4.5 by adding 1 M NaOH or 1 M HCl. The FBB was employed in the comparative study (Chapter 7). Fig. 3.6 shows (a) a picture of the setup and (b) the schematic representation.

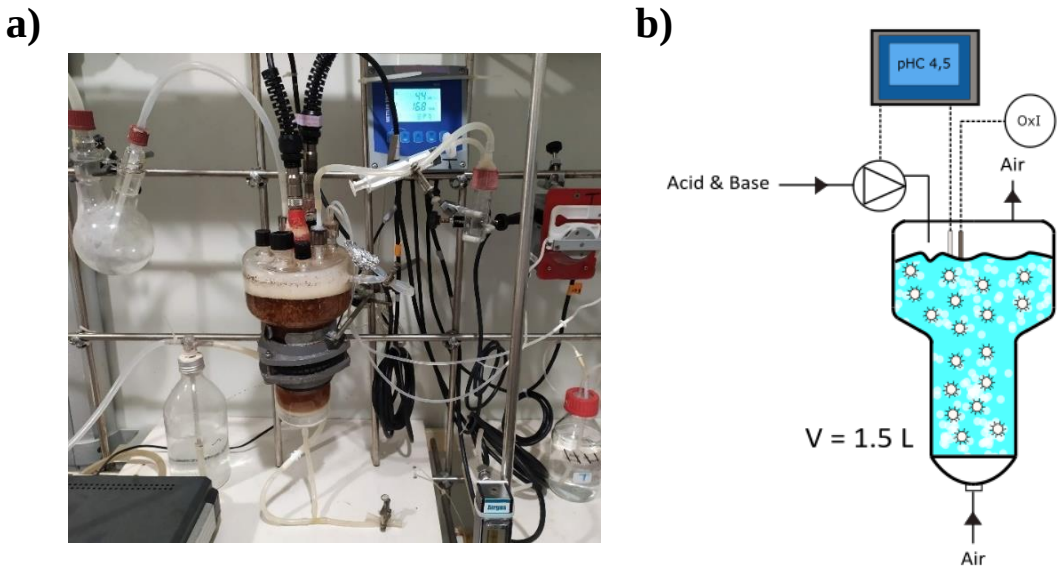


Fig. 3.6. Fluidized bed bioreactor: a) actual reactor setup; b) schematic representation.

3.5.5. Stirred tank bioreactor

The stirred tank bioreactor Applikon model EZ-Control (Applikon Biotechnology, Netherland) had a maximum useful volume of 2 L. The top of the reactor was closed with a metal lid that was equipped with several ports for the oxygen, pH and temperature probes. For oxygen control, the system was equipped with a pump that supplied filtered air through a sparger to the bottom of the reactor. DO levels were set at 5, 10, 15 and 30 %, depending on the experiment, so that the aeration rate was automatically adjusted to maintain these percentages, keeping a constant mechanical agitation of 200 rpm. The system also incorporated a pH control, which added 1 M HCl or 1 M NaOH via two peristaltic pumps to maintain a pH of 4.5. The temperature of the medium was fixed at 25 ºC by circulating water through the external jacket of the reactor, whose temperature was controlled with the aid of an external cooler. A foam trap was also installed at the gas outlet. In addition, the reactor also included a metallic tube connected to a 10 mL vial for sampling. This reactor was used to study the effect of DO in Chapter 6. The stirred tank setup and its schematic representation are shown in Fig. 3.7 (a) and (b) respectively.

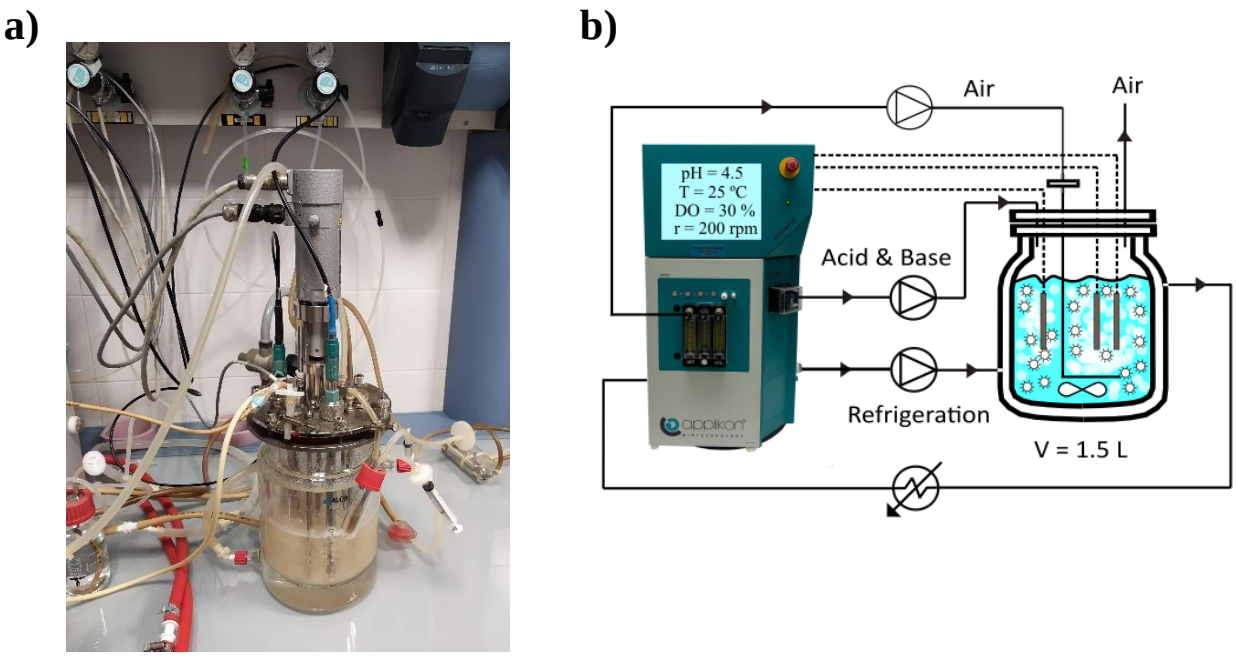


Fig. 3.7. Stirred tank reactor: a) actual reactor setup; b) schematic representation.

3.5.6. Stirred tank reactor with ozone supply

A crystal stirred tank reactor was used in ozonation studies (Chapter 8). This reactor had several ports at the top part: an O_3 inlet, a gas outlet and a sample port. The wastewater was magnetically stirred at 400 rpm. Ozone was generated from pure oxygen in a BMT 802X O_3 generator (BMT Messtechnik, Germany) and was introduced into the reactor through a ceramic diffuser at a constant concentration (50 g Nm^{-3}) and flow rate (150 Ncm^3 min^{-1}). A BMT 964 ozone analyzer (BMT Messtechnik, Germany) was used to monitor the O_3 concentration in the outlet gas stream. The gaseous outlet of the reactor was connected to a bottle with a potassium iodide solution to remove the O_3 leaving the reactor. Fig. 3.8 shows (a) a picture of the setup and (b) the schematic representation.

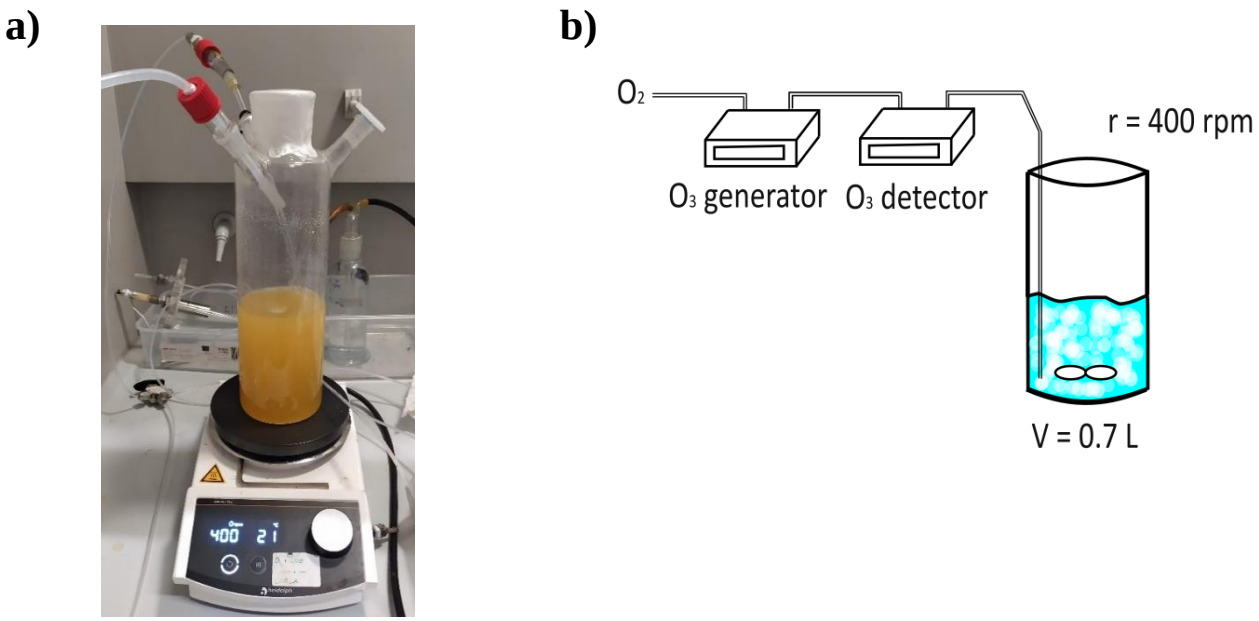


Fig. 3.8. Ozone stirred tank reactor: a) actual reactor setup; b) schematic representation.

3.6. Analytical methods

3.6.1. Pesticide analysis in agricultural wastewater

Residual concentrations of diuron and bentazon were quantified by high-performance liquid chromatography with UV detection (HPLC-UV). Liquid samples were initially filtered through Millipore Millex-GV PVDF filters (0.22 µm). Analyses were performed using a Dionex Ultimate 3000 HPLC system equipped with a UV detector operating at 254 nm. The separation was conducted in a C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 5 µm, 4.6 mm × 150 mm) at 30 ºC with a mobile phase consisting of 0.01 % formic acid solution (A) and acetonitrile (B). Gradient elution started with 35 % B from 0 min to 0.5 min, linearly rose to 45 % from 0.51 min to 12 min, decreased to 35 % for 1 min and finally maintained at 35 % for 2 min (García-Vara et al., 2021). The flowrate and sample injection volume were 0.9 mL min^{-1} and 40 µL, respectively. A limit of detection of 0.5 mg L^{-1} was obtained under these analytical conditions. Under these conditions, the retention times were approximately 9.03 and 9.75 min for diuron and bentazon, respectively.

3.6.2. Pesticide analysis in agricultural rinsed wastewater

Pesticides present in the RW were identified and quantified by the Servei d'Anàlisi Química at the Universitat Autònoma de Barcelona. The methodology is explained in detail in Annex A.

3.6.3. Pesticide extraction from wood and quantification

Pesticides were extracted from the old wood chips according to a modified method of Köck-Schulmeyer et al. (2013). Specifically, wet wood chips were first dried for approximately 24 h at room temperature (T ≈ 25 °C) and then crushed until homogenization by an A 11 basic Analytical mill. A total of 2 g of wood powder and 0.5 g of diatomaceous earth were mixed and introduced in a 22 mL cell. Solid-phase extraction was performed with an ASE® 200 Accelerated Solvent Extractor by injecting a mixture of formic acid (1 %, v/v) and acetone:dichloromethane (ACN:DCM, 1:1) as mobile phase. The extraction conditions were prefixed as follows: pressure 100 bar, temperature 100 °C, 2 cycles and 5 min static time. The resulting extract was evaporated with nitrogen to dryness, reconstituted with 10 or 5 mL methanol and filtrated (0.22 μm, PVDF) before HPLC analysis. This analysis was conducted in triplicate.

3.6.4. Laccase activity

Laccase activity was measured as an indicator of fungal activity according to Wariishi et al. (1992). Briefly, laccase activity was monitored through the variation in absorbance produced by the oxidation of 2,6-dimethoxyphenol (DMP). The absorbance was measured in a Varian Cary 3 UV/Vis spectrophotometer at 30 °C and 468 nm for 2 min. Activity units per liter (AU L^{-1}) are defined as the amount of DMP (in μM L^{-1}) oxidized per minute. The molar extinction coefficient of DMP was 24.8 mM^{-1} cm^{-1}.

3.6.5. *Vibrio fischeri* bioluminescence inhibition test (Microtox® test)

A toxicity test was performed using the acute toxicity bioassay kit from Modern Water (London, UK). In brief, the test is based on the attenuation of *Vibrio fischeri* bioluminescence after 5 and 15 min of exposure to selected dilutions of the samples, previously adjusted to pH 7. Toxicity was expressed as toxicity units (TU). Samples were analyzed in triplicate.

3.6.6. Biomass

3.6.6.1. Dry weight measurement

The dry weight (DW) of the fungal pellets was obtained after filtration with Whatman GF/C glass fiber filters (Whatman, Maidstone, England) and drying at 105 ºC to a constant weight.

3.6.6.2. Ergosterol extraction and measurement

The amount of *T. versicolor* immobilized on wood was determined by a modified method described by Novotný et al., (1999). The lignocellulosic culture was triturated with an analytical mill (A 11 basic, IKA GmbH, Germany). Subsequently, 0.5 g homogenized sample, 1 mL cyclohexane and 3 mL KOH-methanol solution (10 %, w/v) were added in a test tube to extract ergosterol by sonication for 15 min (50/60 Hz, 360 W) followed by heating in a thermal bath at 70 °C for 90 min. Afterwards, 1 mL of distilled water and 2 mL of cyclohexane were introduced into the test tube, which was vortexed for 30 seconds and centrifuged at 3500 rpm for 5 minutes. The supernatant organic phase was separated while the aqueous phase was washed twice with 2 mL cyclohexane. The organic phases were mixed and then evaporated to dryness under N_2. The dried ergosterol residue was reconstituted with 1 mL methanol at 40 ºC for 15 min, vortexed for 30 seconds, poured into an Eppendorf vial and centrifuged at 6000 rpm for 3 min. Afterwards, 0.9 mL of the supernatant was transferred to amber vials to be analyzed by a Dionex 3000 Ultimate HPLC equipped with a UV detector working at 282 nm and a C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 5 μm, 4.6 mm × 150 mm). The analytical method used was as follows: isocratic methanol elution at 1 mL min^{-1}, injection volume of 40 μL and constant oven temperature at 35 ºC. Under these conditions, the retention time was approximately 7.59 min. Ergosterol was measured in milligrams per gram of solid dry weight (mg g DW^{-1}). This analysis was conducted in triplicate.

3.6.7. Glucose concentration measurement

The glucose concentration was measured with a biochemistry analyzer YSI model 2700 (Yellow Spring Instruments & Co., USA) after filtration of the sample through a 0.22 μm Millipore Millex-GV PVDF filter.

3.6.8. Wastewater characterization

Conductivity was measured using a CRISON MicroCM 2100 conductometer. Color was analyzed by absorbance at a wavelength of 650 nm using a UNICAM 8625 UV/VIS spectrometer. COD and ammonia concentrations were monitored using the commercial kits LCK 114 and LCK 014 or LCK 314 and LCK 303, respectively (Hach Lange, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to the standard methods 2540 D and 2540 E, respectively, while heterotrophic plate counts (HPCs) were quantified following the standard method 9215 and expressed as the colony-forming units per mL (CFU mL^{-1}) (Rice et al., 2017). Dissolved organic carbon (DOC) was analyzed following standard procedure 5310 B of the Standard Methods for Examination of Water and Wastewater by using an Analytik Jena multi N/C 2100S/1analyzer and a Shimadzu TOC-L, respectively (APHA et al., 1988). Biological oxygen demand after 5 days (BOD_5) was performed as indicated in ISO 5815 (ISO, 1989). Chloride, nitrite, nitrate, phosphate and sulphate anions were quantified by a Dionex ICS-2000 ionic chromatograph equipped with a Dionex IonPac AS18-HC column (250 mm x 4 mm), which was operated at 1 mL min^{-1} with a 13 mM KOH solution. Samples were analyzed in triplicate.

3.6.9. Fungal community analysis

This analysis was developed by the Environmental Microbiology Group of Dr. Maira Martínez-Alonso and Dr. Núria Gaju, belonging to the Department of Genetics and Microbiology of the Autonomous University of Barcelona (Bellaterra, Spain). The methodology is explained in detail in Annex B.

3.7. Isotherm and kinetic equations

The kinetic equations of the pseudo-first, pseudo-second, Elovich and intra-particle diffusion models are shown in Table 3.4, while the Langmuir, Freundlich and Temkin isotherm models are given in Table 3.5.

Table 3.4. Summary of kinetic models and their linear forms.

Kinetic model	Non-linear form		Linear form	
Pseudo-first model	$q_t = q_e\left(1 - e^{-k_1 t}\right)$	(3.5)	$\ln(q_e - q_t) = \ln q_e - K_1 t$	(3.9)
Pseudo-second model	$q_t = \frac{k_2 q_e^2 t}{1 + k_2 q_e t}$	(3.6)	$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t$	(3.10)
Elovich	$q_t = b \ln(abt)$	(3.7)	$q_t = \frac{1}{b}\ln(ab) + \frac{1}{b}\ln t$	(3.11)
Intra-particle diffusion model	$q_t = K_{id} t^{1/2} + C$	(3.8)	$q_t = K_{id} t^{1/2} + C$	(3.12)

Parameters: q_e is the equilibrium adsorbed amount per gram of sorbent (mg g^{-1}), q_t the pesticide adsorption per gram of adsorbent (mg g^{-1}) at any time t (h), k_1 the pseudo-first rate constant (h^{-1}), k_2 the pseudo-second rate constant (g mg^{-1} h^{-1}), a the initial adsorption rate (mg g^{-1} h^{-1}), b is a coefficient associated to the activation energy and surface coverage (mg g^{-1}), K_{id} is the intra-particle diffusion rate constant (mg g^{-1} $h^{-1/2}$) and C the intercept.

Table 3.5. Summary of isotherm models and their linear forms.

Isotherm	Non-linear form		Linear form		
Langmuir	$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e}$	(3.13)	Langmuir-1	$\frac{1}{q_e} = \frac{1}{K_L q_{max}} \frac{1}{C_e} + \frac{1}{q_{max}}$	(3.18)
			Langmuir-2	$\frac{C_e}{q_e} = \frac{1}{q_{max}} C_e + \frac{1}{q_{max} K_L}$	(3.19)
			Langmuir-3	$q_e = -\frac{1}{K_L}\frac{q_e}{C_e} + q_{max}$	(3.20)
			Langmuir-4	$\frac{q_e}{C_e} = -K_L q_e + K_L q_{max}$	(3.21)
			Langmuir-5	$\frac{1}{C_e} = K_L q_{max} \frac{1}{q_e} - K_L$	(3.22)
Freundlich	$q_e = K_F C_e^{1/n}$	(3.14)		$\log q_e = \log K_F + \frac{1}{n}\log C_e$	(3.23)
	$K_F = \frac{q_{max}}{C_0^{1/n}}$	(3.15)			
Temkin	$q_e = B \ln(A C_e)$	(3.16)		$q_e = B \ln A + B \ln C_e$	(3.24)
	$B = \frac{RT}{A}$	(3.17)			

Parameters: q_e is the equilibrium adsorbed amount per gram of adsorbent (mg g^{-1}), q_{max} the Langmuir maximum adsorption capacity (mg g^{-1}), K_L the energy of adsorption (L mg^{-1}), C_e the pesticide concentration at equilibrium (mg L^{-1}), K_F the adsorption capacity (units depend on n value), C_0 the initial pesticide concentration (mg L^{-1}), n the adsorption intensity (unitless), R is the ideal gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature (K) and A is the Temkin equilibrium constant (L mg^{-1}).

Chapter 4

Fungal bioremediation of agricultural wastewater in short-term treatments

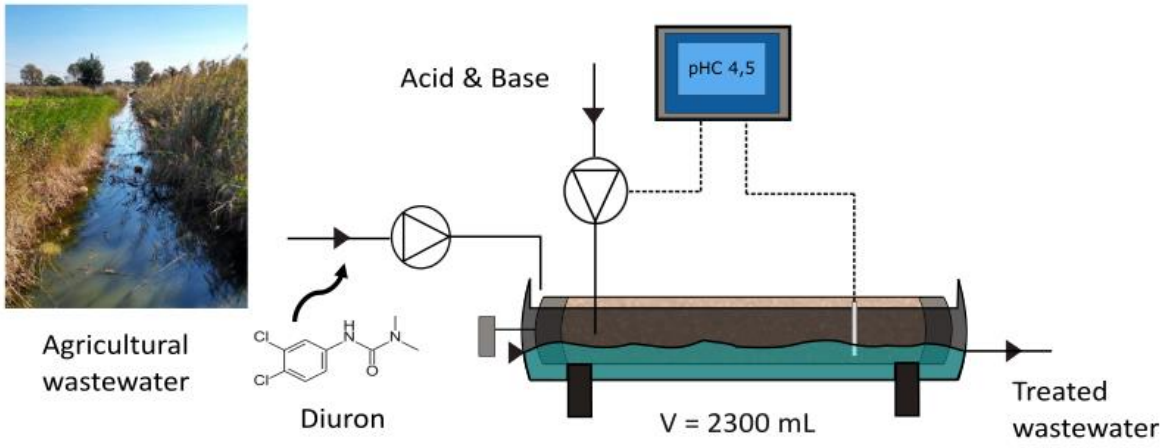
This chapter focuses on a first approach to AW treatment using *T. versicolor* immobilized on wood. Two main topics are discussed in this chapter:

Topic 4.1. Removal of diuron from agricultural wastewaters by *Trametes versicolor* immobilized on pine wood in simple channel reactors. *T. versicolor* is immobilized on pine wood, and a rotating drum bioreactor was used to treat AW spiked with diuron. A study of diuron sorption on holm oak wood is also included.

Topic 4.2. Pesticide bioremediation by *Trametes versicolor*: Application in a fixed bed reactor, sorption contribution and bioregeneration. In this section, pine wood is replaced by holm oak wood, and AW, spiked with diuron and bentazon, is treated in a column reactor. The old wood is regenerated in a biopile-like reactor. A detailed sorption study of diuron and bentazon on holm oak wood is also included.

Topic 4.1. Removal of diuron from agricultural wastewaters by *Trametes versicolor* immobilized on pine wood in simple channel reactors.

Based on the homonymous article *Beltrán-Flores, E., Torán, J., Caminal, G., Blánquez, P., Sarrà, M., 2020. Sci. Total Environ. 728, 1–10. https://doi.org/10.1016/j.scitotenv.2020.138414*



4.1.1. Introduction

Diuron is the common name for N-(3,4-dichlorophenyl)-N,N-dimethyl-urea, which is a phenyl urea herbicide extensively used to control germinating grasses, broadleaf weeds and mosses. This compound inhibits photosynthesis by blocking electron transfer in photosystem II in a broad spectrum of plants and photosynthetic microorganisms. Diuron is applied to many crops, especially cereals, but also in non-agricultural areas for the maintenance of railways, gardens, roads, parks, etc. (Giacomazzi and Cochet, 2004; Tixier et al., 2001).

Nevertheless, the use of diuron can cause serious environmental and public health problems due to its high persistence and toxic effects on living beings. Diuron has a recalcitrant structure with a half-life of approximately 328 days in soil (Jury et al., 1983). Once applied to the target field, rainfall can transport diuron through runoff and leaching from the soil to nearby water bodies (Langeron et al., 2014; Rupp et al., 2006). Aquatic organisms are especially susceptible to diuron exposure, but animal studies have also revealed carcinogenic effects of diuron on rats and cytotoxic and potentially genotoxic damage in human cells (Huovinen et al., 2015).

The United States Environmental Protection Agency has also identified diuron as a "known/likely" human carcinogen considering several types of carcinomas developed in rats (US EPA, 2003). Diuron is included in the list of priority substances in Directive 2013/39/EU in the Field of Water Policy of the EC Directive 2000/60/EC (EC, 2013). This directive urges the Member States to progressively reduce the emission and release of such substances. It also demands compliance with the EC Directive 98/83/CE, which requires Member States to comply with the concentration limit of 100 ng L^{-1} for individual species of pesticides in water intended for human consumption (EC, 1998). Accordingly, declining trends in diuron concentrations have been detected in some areas (Palma et al., 2014), probably due in part to the EC Decision 2007/417/EC to exclude diuron from Annex I of Directive 91/414/EEC for authorized phytosanitary products in the EU (EC, 2007; Palma et al., 2014). However, high concentrations have been recently detected in natural water resources, especially near crop fields and after rainfall, reaching values up to 150 ng L^{-1} (Ccanccapa et al., 2016; Lapworth and Gooddy, 2006).

The conventional activated sludge process used in WWTPs is not specifically designed to completely remove most pesticides. Several alternative treatments have been proposed to deal

with these kinds of recalcitrant compounds (Köck-Schulmeyer et al., 2013b). They are commonly classified into physical, chemical and biological treatments according to their elimination strategy. The first two groups of treatments are frequently presented together as physical-chemical treatments, which mainly include sorption, advanced oxidation processes (ozonation, photocatalysis, etc.) and membrane filtration (Marican and Durán-Lara, 2018). However, these technologies may be unfeasible for treating micropollutants from wastewater, as they can require expensive regeneration processes, high energy or catalyst consumption and additional post-treatments of the rejected streams (Prieto-Rodriguez et al., 2012; Taheran et al., 2016).

Among biological treatments, bioremediation using white-rot fungi (WRF) has been proposed as a promising alternative to degrade a wide range of xenobiotics, such as PhACs (Haroune et al., 2014), organic dyes (Baccar et al., 2011), PCPs (Rodarte-Morales et al., 2011) and pesticides (Mir-Tutusaus et al., 2014). In particular, *T. versicolor* is a fungus of special interest for bioremediation purposes since it has an unspecific enzymatic system composed of a family of ligninolytic extracellular enzymes (laccase and lignin, manganese and versatile peroxidases) and an intracellular enzymatic system known as cytochrome P450 (Asgher et al., 2008).

Despite the great potential of WRF, there is a critical drawback that can complicate its implementation on an industrial scale. Treating real wastewater matrices under nonsterile conditions negatively influences the enzymatic activity and survival of the fungus due to pressure exerted by other microorganisms, mainly bacteria. Operating under nonsterile conditions normally leads to shorter operation times and decreased degradation performance (Mir-Tutusaus et al., 2016).

Different strategies have been adopted to favor the predominance of fungi over bacteria, such as biomass renovation (Blánquez et al., 2006), adjustment of the C/N ratio and pH (Badia-Fabregat et al., 2016), and fungus immobilization (Li et al., 2015), among others. There are two types of immobilization strategies: autoimmobilization (fungal pellets) and immobilization onto supports (Mir-Tutusaus et al., 2018a). In particular, immobilization on natural substrates, such as wood from timber industry wastes, presents remarkable possibilities, since such substrates can be used as low-cost carriers without adding any other source of nutrients. Additionally,

wood substrates have a complex structure that is highly resistant to bacterial attacks. This material is especially convenient for the treatment of wastewater with low organic content, such as AW, in which the addition of external nutrients is typically mandatory (Torán et al., 2017).

This work aimed to develop and evaluate an automated channel-type reactor, known as a rotating drum bioreactor (RDB), to treat diuron-spiked AW by using *T. versicolor* immobilized on pine wood. Given the porous nature of wood, diuron sorption on pine wood was also studied both in batch mode, studying isothermal and kinetic models, and in successive batch experiments.

4.1.2. Materials and methods

4.1.2.1. Agricultural wastewater

Agricultural wastewater (AW I in Section 3.2, Chapter 3) was spiked with 6, 8 and 10 ppm diuron for single-batch, successive sorption and bioremediation experiments, respectively.

Table 4.1. Physicochemical characterization of the AW. Values are means ± standard deviation for triplicate samples.

Parameter	Value
pH	7.67 ± 0.04
Conductivity (mS cm^{-1})	2.25 ± 0.07
Color (650 nm)	0.05 ± 0.01
Chloride (mg Cl L^{-1})	570.5 ± 3.8
Sulphate (mg S L^{-1})	51.2 ± 0.1
Nitrite (mg N L^{-1})	2.8 ± 0.1
Ammonia (mg N L^{-1})	0.1 ± 0.1
TSS (mg L^{-1})	6 ± 1
COD (mg O2 L^{-1})	32 ± 1
DOC (mg C L^{-1})	16 ± 1
Heterotrophic plate count (CFU mL^{-1})	$4.8 \cdot 10^5 \pm 2.7 \cdot 10^4$

4.1.2.2. Sorption study

Various amounts of pine wood sorbent (5, 10, 20, 30, 40 and 60 g) were added to a series of 1 L Erlenmeyer flasks that were previously filled with 200 mL spiked wastewater. Spiked wastewater (6 ppm) was initially adjusted to pH 4.5. Continuous orbital shaking of 135 rpm and

a constant temperature of 25 ºC were maintained for almost 3 days (70 h) until equilibrium was reached. A total of 10 samples were withdrawn throughout the experiment to determine the remaining pesticide concentrations by HPLC. Eq. (4.1) and Eq. (4.2) were used to calculate diuron sorption at any time t and equilibrium, respectively:

$$q_t = \frac{(C_0 - C_t)\,V}{m} \quad (4.1)$$

$$q_e = \frac{(C_0 - C_e)\,V}{m} \quad (4.2)$$

where C_0, C_t and C_e (mg L^{-1}) are the diuron concentrations at initial time, any specific time t, and at equilibrium respectively, V (L) is the solution volume and m (g) is the mass of wood used in each experiment. The results obtained were fitted to the kinetic and isotherm models presented in Section 3.7 (Chapter 3).

4.1.2.3 Effect of successive sorption cycles

The sorption process in batch mode was replicated seven times for 20 g wood. At the end of each batch (at equilibrium), the wastewater was discarded and replaced by fresh spiked wastewater, thus restoring the initial pesticide concentration (8 ppm). However, the same wood chips (20 g) were retained and preserved to be reused in consecutive batches. A total of 8 liquid samples of 1 mL were withdrawn in each cycle, which were subsequently analyzed by HPLC.

4.1.2.4. RDB configuration

An RDB (Chapter 3, Section 3.5.1) was set up for the treatment of AW spiked with 10 ppm diuron. A total of 350 g DW of inoculated wood was introduced in the inner tube, which was operated at a constant rotation speed of 6 rpm. The wastewater spiked was continuously pumped from the influent tank to one RDB side, whereas the effluent stream was drained by overflow from the other RDB extreme. The pH was controlled to 4.5 by adding either 1 M HCl or 1 M NaOH directly into the channel. The system was continuously operated with an HRT of 3 d. Liquid samples were taken from the reactor effluent to measure diuron concentration, laccase and HPCs.

4.1.3. Results and discussion

4.1.3.1. Relation between contact time and diuron sorption

Pine wood was chosen as the immobilization support since Torán et al. (2017) had previously shown that *T. versicolor* exhibited higher biomass growth (in terms of ergosterol content) on this material than on other lignocellulosic substrates (pine bark, walnut shells and hazelnut shells). The apparent and true bulk densities were estimated to be 0.17 ± 0.01 g mL^{-1} and 0.55 ± 0.03 g mL^{-1}, respectively, by Eq. (3.1) and Eq. (3.3) of Section 3.1 (Chapter 3). A porosity of 69.3 ± 2.4 % was obtained for pine wood using Eq. (3.2) of Section 3.1 (Chapter 3). The correlation between wet and dry weights was 2.3 ± 0.2. These results are consistent with previous works (Kim et al., 2016; Vo et al., 1995).

Fig. 4.1 shows the sorption curves of diuron on pine wood chips over time. The system approached equilibrium in approximately 48 h for all sorbent amounts added (5, 10, 20, 30, 40, and 60 g). Higher wood doses increased the total diuron elimination but decreased the amount of diuron sorbed per unit of wood. Diuron sorption on pine chips was mainly attributed to the chemical affinity for organic components of wood, such as lignin (Rodríguez-Cruz et al., 2009).

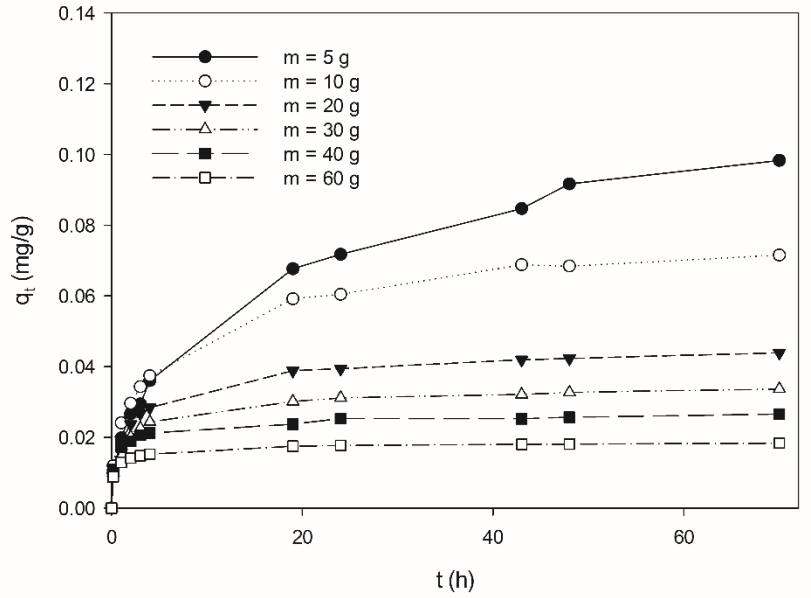


Fig. 4.1. Effect of contact time on diuron sorption for several sorbent doses at 25 ºC (C_0 = 6 mg L^{-1}).

Firstly, the sorption rose sharply since diuron molecules occupied the vacant sites of the external surface of the wood. As the external surface became saturated, the sorption rate progressively decreased since the solute encountered resistance in diffusing through the porous structure of the wood to be sorbed on the internal surface of the sorbent (Baccar et al., 2012). Significantly lower q_t values were obtained compared to other studies using similar carbon-based materials (De Gisi et al., 2016). However, this result was attributed to the higher wood-wastewater mass ratio used in the present work.

4.1.3.2. Sorption isotherms

The experimental equilibrium data were fitted to several isotherm models, which describe the possible sorption mechanisms and facilitate the subsequent design of the sorption process. The Langmuir, Freundlich and Temkin models were investigated. The isotherm equations were also linearized and plotted to determine the most representative model and its characteristic parameters from the slope and intercept. Five different Langmuir linear forms were used, as significant variations in their fits and parameter values were previously reported (Baccar et al., 2013). The linear forms of the isotherms are available in Chapter 3, Table 3.5.

The Langmuir model proposes sorption on finite sites and the formation of a single monolayer without chemical interaction between sorbate molecules (Langmuir, 1916). The empirical Freundlich model considers sorption on heterogeneous surfaces and is more suitable for low concentrations (Appel, 1973; Freundlich, 1909). Finally, the Temkin isotherm model assumes chemical interactions between molecules and a linear decrease in binding energy as vacant sites are filled (Aharoni and Ungarish, 1977; Temkin and Pyzhev, 1940).

The linear correlation coefficient R^2 and the normalized standard deviation Δq (%) were used to evaluate the correlation between the experimental data and the sorption isotherms. Δq is described by Eq. (4.3).

$$\Delta q\ (\%) = 100\sqrt{\frac{\sum\left(\frac{q_{exp}-q_{cal}}{q_{exp}}\right)^2}{N-1}} \qquad (4.3)$$

where q_{exp} and q_{cal} are the experimental and calculated amounts of diuron sorbed at equilibrium, respectively, and N is the number of measurements. A higher R^2 indicates a better fit between

the linear form and the experimental data. Furthermore, a lower Δq represents a stronger correlation between the experimental and predicted data (Baccar et al., 2013).

Table 4.2 summarizes the main parameters and fit coefficients of the isotherm models. Although Langmuir-1, Langmuir-5 and Freundlich obtained the highest R^2 (0.993), the Freundlich model exhibited a slightly lower Δq (5.245 %) and thus was considered to be the best model describing the equilibrium data. The Freundlich isotherm has been selected to represent pesticide sorption on carbon-based sorbents in previous studies (Laohaprapanon et al., 2010; Rodriguez-Cruz et al., 2007). Concerning the Freundlich parameters, a q_{max} of 0.154 mg g^{-1} was estimated with Eq. (3.15), which is in good agreement with those reported in the literature (Iqbal and Ashiq, 2007). Nevertheless, a further discussion of the resulting q_{max} is proposed in Section 4.1.3.4. The parameter 1/n is related to the sorption susceptibility of the system and can indicate favorable ($0 < 1/n < 1$), difficult ($1/n > 1$) or irreversible sorption ($1/n = 0$) (Q. Yang et al., 2017). In this case, 1/n revealed favorable sorption (1/n = 0.922) of diuron onto pine wood chips.

Table 4.2. Parameters, correlation coefficients and errors of Langmuir, Freundlich and Temkin isotherm models for diuron sorption onto pine wood chips at 25 ºC.

Isotherms	Parameters			
Langmuir	K_L (L mg^{-1})	q_{max} (mg g^{-1})	R^2	Δq (%)
Langmuir-1	0.057	0.548	0.993	5.782
Langmuir-2	0.046	0.677	0.423	5.758
Langmuir-3	0.112	0.305	0.387	7.200
Langmuir-4	0.043	0.711	0.387	5.789
Langmuir-5	0.051	0.610	0.993	5.757
Freundlich	n	q_{max} (mg g^{-1})	R^2	Δq (%)
	1.085	0.154	0.993	5.245
Temkin	A	B	R^2	Δq (%)
	2.113	0.044	0.938	13.637

4.1.3.3. Sorption kinetics

Sorption kinetics provide valuable information, such as the solute removal rate and the reaction pathway, which allows the subsequent design and scale-up of a sorption system. The pseudo-first- and pseudo-second-order, intraparticle diffusion (IPD) and Elovich models were

tested. The linear and non-linear kinetic equations of these models are shown in Chapter 3, Table 3.4. The pseudo-first and pseudo-second-order models consider chemisorption to be the dominant mechanism. In contrast, the IPD model identifies diffusion as the rate-limiting step. Between these two opposing models, the Elovich model describes an intermediate behavior with no clearly dominant mechanism (Wu et al., 2009).

The linear forms of these models were plotted to obtain the particular parameters of each model from the slope and the intercept. The best correlation to the experimental data was again selected according to R^2 and Δq. The fitting results for the kinetic models are presented in Table 4.3. Although the pseudo-second order model gave the highest R^2 fits for all sorbent doses, the Elovich model more accurately described the experimental data based on Δq. The only exception was in the experiment with m = 5 g, in which the IPD model showed a better fit. However, this result could be explained by the fact that the system did not reach full equilibrium for m = 5 g in the period under study. It is probable that over a longer period, the system would reach equilibrium, and the Elovich model would again provide the best fit. The Elovich model is appropriate for heterogeneous sorbents and liquid-solid systems, and recognizes the effect of surface coverage on the sorption rate over time. Moreover, the values of the kinetic parameters are in agreement with previous studies of micropollutant sorption on porous carbon-based materials (Emeniru et al., 2017; Tseng et al., 2003).

Table 4.3. Correlation coefficients, error and kinetic parameters of Pseudo-first order, pseudo-second order, Elovich and IPD model for diuron sorption on pine wood chips (25 ºC).

		Sorbate amount, m (g)					
		5	10	20	30	40	60
	$q_{e,exp}$ (mg g^{-1})	0.098	0.072	0.044	0.034	0.027	0.018
Pseudo-first order kinetic model	K_1 (h^{-1})	0.049	0.064	0.062	0.063	0.155	0.072
	$q_{e,cal}$ (mg g^{-1})	0.084	0.051	0.025	0.017	0.010	0.006
	R^2	0.977	0.974	0.936	0.910	0.801	0.864
	Δq (%)	57.029	65.229	74.634	78.810	86.340	87.228
Pseudo-second order kinetic model	K_2 (g mg^{-1} h^{-1})	1.525	4.368	14.901	20.667	38.528	80.421
	$q_{e,cal}$ (mg g^{-1})	0.103	0.073	0.044	0.034	0.026	0.018
	R^2	0.986	0.997	0.999	0.999	0.999	1.000
	Δq (%)	27.955	25.059	22.848	22.659	23.111	20.238
Elovich equation	a (mg g^{-1} min^{-1})	0.066	0.116	0.181	0.342	2.476	6.156
	b (g mg^{-1})	62.581	92.648	171.029	251.454	411.628	659.810
	R^2	0.935	0.981	0.995	0.991	0.967	0.964
	Δq (%)	56.400	16.975	2.642	3.426	5.889	4.992
IPD model	k_{id} (mg g^{-1} $h^{-1/2}$)	0.012	0.008	0.004	0.003	0.002	0.001
	C (mg g^{-1})	0.008	0.015	0.013	0.012	0.012	0.009
	R^2	0.978	0.903	0.811	0.745	0.611	0.557
	Δq (%)	12.577	20.745	22.858	19.337	16.633	15.436

4.1.3.4. Effect of successive sorption cycles on the sorption capacity

Successive sorption experiments were performed to verify the real sorption capacity of pine wood and to compare it to the q_{max} predicted by the Freundlich model. In addition, this system can be representative of continuous sorption processes in real scenarios, with relatively low solute concentrations and high sorbent doses. In this study, seven cycles in batch mode were completed by retaining the initial 20 g of pine wood chips and re-suspending these chips in 0.2 L of fresh spiked wastewater with 8 ppm diuron. The cumulative sorption of diuron is shown in Fig. 4.2.

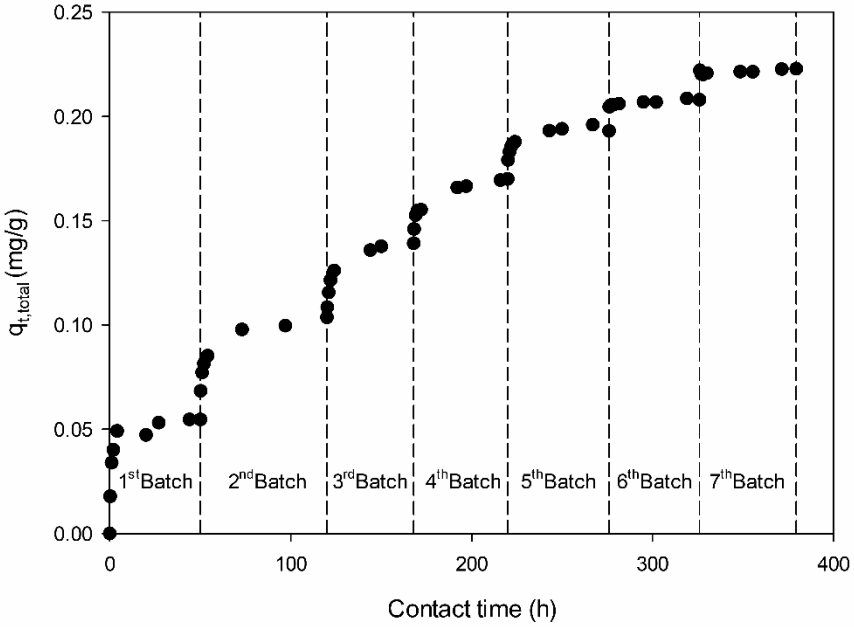


Fig. 4.2. Effect of successive cycle batches on diuron sorption over time.

In this case, a higher concentration of 8 ppm instead of 6 ppm was used to guarantee diuron detection in the HPLC (lower limit of detection of 0.5 ppm). As expected, increasing the initial diuron concentration led to higher sorption on the wood chips compared to that in the batch sorption process presented in Section 4.1.3.1 (0.05 instead of 0.04 mg g^{-1}). Once equilibrium was reached in the first batch of the successive sorption experiment, the treated wastewater was discarded and a new 0.2 L sample of wastewater containing 8 ppm diuron was poured inside the same Erlenmeyer flask to start the second batch. In the second batch, the wood was able to sorb more diuron from the wastewater. This result indicated that the wood had reached equilibrium in the first batch but its sorption capacity was not exhausted. The same process was repeated for 7 cycles. Although the sorption capacity of the wood decreased with each new cycle, the wood was not completely saturated after 7 batches.

Note that the Freundlich model estimated a q_{max} of 0.154 mg g^{-1}, but this level was exceeded after 4 cycles. The Freundlich model was initially selected because it provided the best fit based on the R (0.993) and Δq (5.757 %) values obtained in the batch experiment. However, the results of the successive batch process revealed that the Freundlich model could not accurately predict q_{max}. In fact, q_{max} is conventionally determined from the Langmuir model instead of by applying Eq. (3.15) to the Freundlich model (Baccar et al., 2013). In this regard, the Langmuir model

(Langmuir-5) obtained the same R and a Δq (5.245 %) insignificantly higher than that of the Freundlich model and can be also considered a good approach. Since the Langmuir model showed an adequate fit, its estimated q_{max} was also compared with the experimental data. Langmuir-5 provided a q_{max} of 0.610 mg g^{-1}, which was more consistent with the results of the successive sorption experiment. For that reason, the Langmuir model was selected as the most representative isotherm.

4.1.3.5. Rotating drum bioreactor treating diuron in spiked real wastewater

The objective of this section is to develop a fungal reactor effective in treating AW, whose design is adapted to a possible *in-situ* application in agricultural fields. In this respect, Torán (2018) had already proposed a packed bed channel bioreactor (PBCB) as an alternative system to a trickle-bed reactor, since the latter would require the installation of additional structures to support the column, complicating its implementation in rural areas. Based on the excellent performance shown by the PBCB, an RDB was proposed in the present study to automate the process. Manual mixing was replaced by continuous and automated rotation of the inner tube of the RDB by means of an electric motor. In addition, the outlet pump was discarded and the effluent left the reactor by overflow.

Fungal RDBs have been previously used for enzyme production (Colla et al., 2017; Domínguez et al., 2001) and biological desulfurization (Şener et al., 2018), and a similar rotating drum biological contactor inoculated with *white-rot fungus* has been applied for decolorization (Šíma et al., 2016). Recently, a rotating biological contactor containing immobilized *T. versicolor* was implemented to remove pharmaceutical compounds from urban wastewaters (Cruz del Álamo et al., 2020). However, this is the first time that a fungal RDB has been used to treat pesticides in real wastewaters.

Continuous rotation at 6 rpm was employed to ensure adequate oxygen diffusion and good mixing throughout the reactor. However, *T. versicolor* was progressively detached from the wood surface during treatment, probably due to the generation of significant shear forces that damaged the mycelial structure of the fungus (Zhong, 2010). This fact indicated that the rotation speed was too high to maintain the fungus immobilized on the pine wood over time. The rotation

speed also produced foam inside the inoculated reactor, which has been identified as a major issue in fungal treatments (Espinosa-Ortiz et al., 2016).

Despite the gradual detachment of the fungus, the treatment was maintained for 16 days. During this period, the average diuron elimination was slightly higher for the inoculated reactor (61 % ± 3 %) than for the control reactor (55 % ± 7 %). Diuron removal decreased over time in the control reactor, which was probably due to progressive wood saturation (Torán et al., 2017). In contrast, the removal rate of the inoculated reactor was more stable, remaining above 60 % after 16 days of treatment. A significant difference of up to 20 % in terms of diuron removal was achieved between both reactors at the end of the process. The process was stopped after 16 days because *T. versicolor* seemed to have been significantly washed out from the substrate surface.

A lower average removal (61 %) was observed compared to that obtained in the PBCB (94 %), even between the control reactors. This difference was mainly attributed to the different wood/wastewater doses used between the PBCBs and the RBDs. For the RBD, a dose almost 7 times lower was used (152 g DW L^{-1} instead of 1000 g DW L^{-1}), but diuron removal did not decrease proportionally, and only an average reduction of 14 % was obtained between the control reactors. This result may probably be due to the fact that there was a more effective contact between the wood chips and the AW driven by the continuous rotation. Stirring speed has been shown to influence not only the sorption kinetics but also the sorption equilibrium of organic compounds on carbon-based materials (Kuśmierek and Świątkowski, 2015). Higher removal efficiencies were also obtained by Torán et al. (2017) when working with *T. versicolor* immobilized on pine chips in a packed bed reactor for the removal of ibuprofen (90 %) and ketoprofen (80 %), but a similar removal efficiency was obtained for naproxen (60 %). Nevertheless, in that case, a higher dose of 240 g wood L^{-1} was also employed.

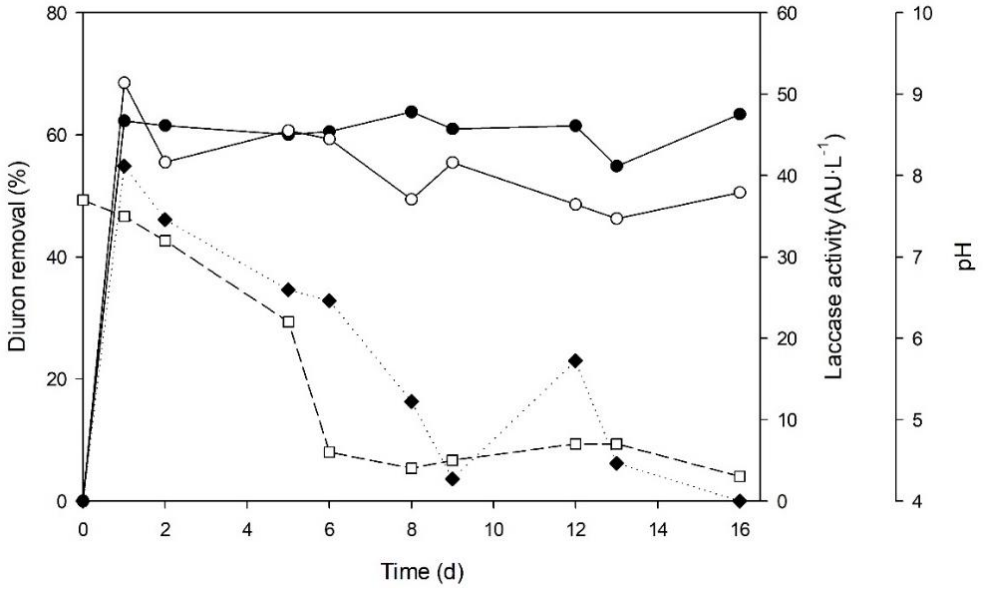


Fig. 4.3. Diuron removal profiles for the inoculated (black dots) and the control (white dots) RBDs, laccase activity (black rhombuses) and pH evolution (white squares) for inoculated RDB.

Laccase was measured as an indicator of fungal activity, being reduced from 41 AU L^{-1} to almost zero after 16 days. However, laccase depletion was not accompanied by a lower yield of diuron removals. This result may be due to the fact that not only laccase, but also other extracellular enzymes or the intracellular enzyme system cytochrome P450 may be involved in the degradation of this micropollutant (Marco-Urrea et al., 2009). Similar laccase levels and evolution over time were also detected by Torán et al. (2017) when working with *T. versicolor* immobilized on wood chips. In the PBCB, a laccase peak of 62 AU L^{-1} was detected on the 5^{th} day and afterwards declined to approximately 30 AU L^{-1} and 4 AU L^{-1} after 17 and 35 days, respectively (Torán, 2018). The lower levels of laccase activity detected in the RDB were mainly attributed to a lower initial biomass/wastewater ratio together with biomass washout from the wood.

The initial pH of the wastewater was not adjusted in the RDB at the beginning of the treatment. This decision was based on the natural ability of *T. versicolor* to acidify the medium observed in the PBCB experiment by Torán (2018). As shown in Fig. 4.3, the pH was progressively reduced during the first days of the process. However, the pH control was set at 4.5 on day 5 to ensure optimal operational conditions for *T. versicolor* to reverse and attenuate the fungal losses derived from the high rotation speed.

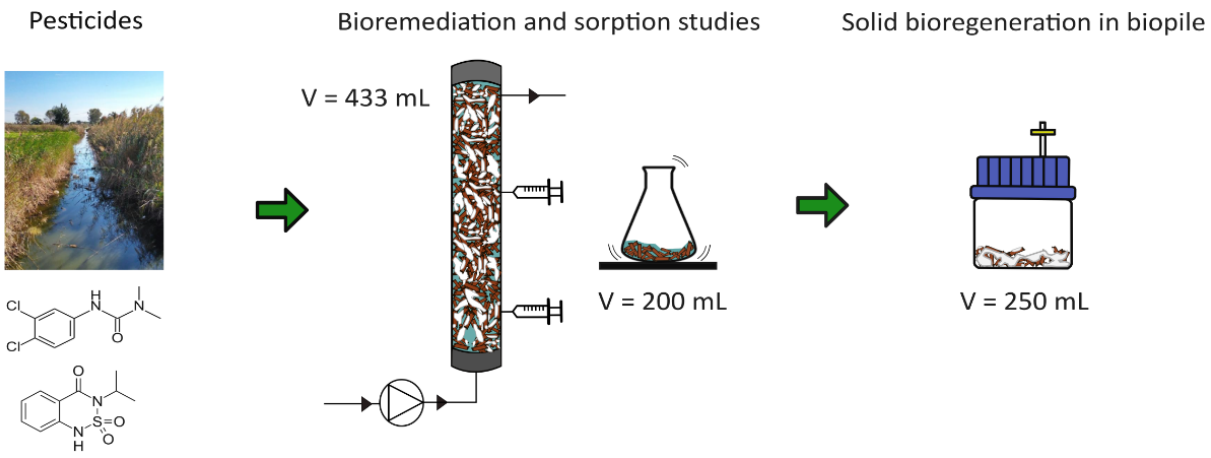
HPCs rose from an initial $4.8 \cdot 10^4 \pm 2.7 \cdot 10^4$ CFU mL^{-1} to $5.4 \cdot 10^5 \pm 5.0 \cdot 10^4$ CFU mL^{-1} after 16 days, whereas no significant variation was observed in the case of the PBCB. This greater bacterial proliferation was probably associated with both the lower biomass/wastewater dose employed in the RDB and the superficial removal of *T. versicolor* from the pine wood chips. Mir-Tutusaus et al. (2016) obtained a 100-fold increase in HPCs after only 3 days of treating non-sterile hospital wastewater with *T. versicolor* in a fluidized reactor. This notable increase was promoted by the addition of glucose and NH_4Cl as the main source of nutrients for the fungus, since these compounds are easily assimilable by bacteria. A more moderate increase in HPCs was achieved in continuous mode with a preliminary coagulation-flocculation step. In the present study, the HPCs increase was limited, and costs associated with nutrient supply and pre-treatment were avoided.

4.1.4. Conclusions

T. versicolor immobilized on wood chips was used to treat AW in simple channel bioreactors. According to the sorption isotherm, the Freundlich model gave the best fit for diuron sorption on the wood chips. However, successive sorption experiments revealed inconsistencies between the q_{max} predicted by the Freundlich model and the experimental results. In contrast, the experimental data fitted the q_{max} predicted by the Langmuir model, which was selected as the most representative one. Regarding kinetics, the Elovich model showed the best fit, suggesting that there was no clearly predominant sorption mechanism. The automated RDB effectively removed diuron from the spiked real wastewater, requiring 7 times lower wood amount than the PBCB. However, continuous rotation washed a significant amount of the biomass from the wood. Further research should further explore the effect of the main process variables on removal efficiency, analyze complex mixtures of pesticides at real concentrations, use mixtures of wood substrates, and finally scale up the process.

Topic 4.2. Pesticide bioremediation by *Trametes versicolor*: Application in a fixed bed reactor, sorption contribution and bioregeneration

Based on the homonymous article: Beltrán-Flores, E., Sarrà, M., Blánquez, P., 2021. Sci. Total Environ. 794, 1–11. https://doi.org/10.1016/j.scitotenv.2021.148386



4.2.1. Introduction

Over the last decades, the growing occurrence of pesticides in water resources has become a worldwide concern. Many of them are persistent, bioaccumulative and toxic to human beings and the environment, even at low concentrations (Ding et al., 2012; Juraske and Sanjuán, 2011). In fact, some pesticides are associated with serious health effects, such as cancer, endocrine disruption, and reproductive toxicity (Damalas and Eleftherohorinos, 2011; Kim et al., 2017). Although pesticides are generally released from diffuse sources, significant concentrations of pesticides have been reported in surrounding aquatic environments close to crop fields (Palma et al., 2014). Pesticides can enter water bodies by runoff or leaching through the soil (Triegel and Guo, 2019), especially during fumigation periods and after rainfall (Rabiet et al., 2010). Consequently, intensive agricultural practices have been associated with the occurrence of a wide range of pesticides in northeast Spain (Ccanccapa et al., 2016; Köck-Schulmeyer et al., 2019). In particular, multiple pesticides have been recently detected in several surface water locations of the lower Llobregat River basin (Barcelona, Spain). In this region, there are extensive agricultural areas cultivated with different crops, especially tomatoes in summer and artichokes in winter (Postigo et al., 2021).

The increasing occurrence of pesticides in agricultural waters calls for the development of abatement strategies. Physical-chemical strategies present several disadvantages for pesticide removal. For example, advanced oxidation and ozonation treatments involve high operational costs and the potential formation of TPs that can be even more toxic than the parent compounds (Rivera-Utrilla et al., 2013). In other approaches such as membrane filtration or sorption, contaminants simply move from one medium to another, and further treatments are still required (Taheran et al., 2016). In this context, bioremediation by white-rot fungi (WRF) emerges as a promising technique due to the nonspecific and complex enzymatic system used by these microorganisms, which can degrade a wide variety of micropollutants (Mir-Tutusaus et al., 2018a). The enzyme system consists of intracellular enzymatic complexes, such as the cytochrome P450, and extracellular lignin-modifying enzymes, such as laccase (Asgher et al., 2008). Through this sophisticated metabolism, WRF have demonstrated its ability to reduce the toxicity level of the effluent by degrading many micropollutants along with some of their TPs (Cruz-Morató et al., 2013a; Hu et al., 2020b; Mir-Tutusaus et al., 2017).

However, the application of this technology is still a challenge when operating in long-term operations under non-sterile conditions (Mir-Tutusaus et al., 2019). Other microorganisms, e.g. bacteria, can compete to consume the existing substrate and decrease fungal growth and enzymatic activity over time. In this regard, two indicators are frequently used to monitor fungal activity: laccase and ergosterol. Laccase is utilized as an indicator of extracellular enzymatic activity involved in the degradation of multiple micropollutants (Maqbool et al., 2016). By contrast, ergosterol is a sterol compound found in fungal cell membranes that can be used as an indicator of viable and active biomass (Gutarowska and Zakowska, 2009). To address the problem of microbial contamination, several strategies have been adopted in different fungal bioreactors that favor fungal dominance. This includes operation at optimal fungal pH, the use of nitrogen limiting conditions, partial biomass renewal and fungal immobilization on lignocellulosic support (Espinosa-Ortiz et al., 2016; Mir-Tutusaus et al., 2018b). The latter strategy is particularly interesting when treating AW, which generally contains insufficient carbon content to maintain fungal metabolism and thus requires the addition of an external carbon source. In this respect, lignocellulosic materials can not only serve as a sustainable and low-cost support of immobilization but also as a selective carbon source for WRF, thus preventing bacteria competition for substrate (Li et al., 2015). Recent studies have also documented enhanced pesticide removals and longer treatment periods in AW bioremediation using immobilized WRF (Torán et al., 2017; Torán, 2018).

Nonetheless, lignocellulosic supports are generally porous materials that can contribute significantly to the overall removal in fungal treatments (Hu et al., 2020b). In this case, the bioremediation process should be accompanied by kinetic and isotherm studies of the support, thus characterizing the sorption phenomenon and discerning between the sorption and biodegradation contributions (Baccar et al., 2012; Beltrán-Flores et al., 2020, Topic 4.1 of Chapter 4). Furthermore, effective treatment and mitigation strategies should be implemented to manage solid by-products, i.e., old contaminated wood, thereby preventing the release of harmful chemicals into the environment.

The main purpose of the present work was to investigate the removal of diuron and bentazon from AW by immobilized *T. versicolor* on *Q. ilex* wood. The AW was collected from the sampling point SW10 reported by Postigo et al. (2021), where multiple pesticides have been

recently detected. Among them, diuron and bentazon were selected as model compounds, as both are considered to be of particular concern according to the Directive 2008/105/EC on Environmental Quality Standards of the EC (EC, 2008b), and the EC has banned the use of diuron in the EU (EC, 2007). In this regard, *T. versicolor* has shown an excellent ability to degrade both pesticides. In fact, *T. versicolor* proved to be the most efficient species degrading diuron and its major metabolite (García-Vara et al., 2021; Hu et al., 2020b). Considering these findings, *T. versicolor* was the WRF selected to perform the bioremediation experiments in the present study. For this purpose, *T. versicolor* was previously grown on *Q. ilex* wood chips, which served simultaneously as a substrate and support. *Q. ilex* is known to be a natural substrate for *T. versicolor* and a quite abundant wood in the Mediterranean forest. Afterwards, the wood with the pre-grown fungus was used to set up an FBR to treat the AW continuously. Consistent with the significant role played by sorption, a further comprehensive sorption analysis was conducted by evaluating column breakthrough curves, together with batch mode kinetic and isotherm studies. The single batch sorption study was complemented with a sorption experiment in repeated batches to determine the maximum sorption capacity of the wood. Finally, solid residues (old wood and old colonized wood) were treated by *T. versicolor* in a biopile-like system through different inoculation strategies.

4.2.2. Materials and methods

4.2.2.1. Agricultural wastewater

The physicochemical characteristics of the AW used in this experiment (AW II) are shown in Table 4.4.

Table 4.4. Physicochemical characterization of the AW. Values are means ± standard deviation for triplicate samples.

Parameter	Value
pH	7.85 ± 0.04
Conductivity (mS cm^{-1})	1.73 ± 0.03
Color (650 nm)	0.02 ± 0.01
Chloride (mg Cl L^{-1})	268.1 ± 1.4
Sulphate (mg S L^{-1})	115.9 ± 1.6
Nitrite (mg N L^{-1})	2.4 ± 0.1
Ammonia (mg N L^{-1})	1.8 ± 0.2
TSS (mg L^{-1})	8 ± 1
COD (mg O2 L^{-1})	40 ± 2
Heterotrophic plate count (CFU mL^{-1})	$1.2 \cdot 10^5 \pm 1.6 \cdot 10^4$

4.2.2.2. Pesticide removal by *T. versicolor* immobilized on wood in a fixed bed reactor

A total of 160 g DW of inoculated wood (Chapter 3, Section 3.4.4) was used to set up an FBR. Another reactor was operated in parallel with non-inoculated wood chips as a control. The experiment was conducted at room temperature, approximately 25 ºC. The AW was adjusted to pH 4.5 and spiked with an initial concentration of 10 ppm diuron and bentazon. Neither diuron nor bentazon was initially detected in the original wastewater matrix by HPLC-UV above the lower limit of detection (LOD = 0.5 ppm). The empty bed contact time (EBCT) of the columns was fixed at 1 and 3 days. At selected times, 1 mL aliquots were withdrawn at three different heights of the columns (Z = 8.5, 18.5 and 28.5 cm) and analyzed by HPLC-UV to quantify the remaining pesticide concentration.

The overall pesticide removal q_{total} (mg) can be calculated by integrating the area under the breakthrough curve, as described in Eq. (4.4), where Q (mL min^{-1}) is the volumetric flow rate, t_{total} (min) is the total time, C_{ad} is the pesticide removal in terms of concentration (mg L^{-1}) and t is time (min)

$$q_{total} = \frac{Q}{1000} \int_{t=0}^{t=t_{total}} C_{ad} dt \quad (4.4)$$

The maximum capacity of the column q_{eq} (mg g^{-1}) and the total amount of pesticides entering the columns m_{total} (mg) are given by Eq. (4.5) and Eq. (4.6) respectively:

$$q_{eq} = \frac{q_{total}}{m} \quad (4.5)$$

$$m_{total} = \frac{C_0 Q t_{total}}{1000} \quad (4.6)$$

Variable C_0 is the initial concentration of the pesticides (mg L^{-1}) and m is the amount of wood (g). Finally, the removal percentage Y (%), and EBCT (min) at each height were calculated from the Eq. (4.7) and Eq. (4.8), respectively:

$$Y = \frac{q_{total}}{m_{total}} x100 \quad (4.7)$$

$$EBCT = \frac{V}{Q} \quad (4.8)$$

Variable V (mL) is the volume occupied by the fixed bed.

4.2.2.3. Sorption study and successive sorption cycles

A sorption study was conducted as described in Section 4.1.2.2. In this case, the AW was spiked with 10 ppm diuron and bentazon and the contact time was 50 h. The sorption capacity of the pesticides by the wood was also evaluated in several successive cycles as explained in Section 4.1.2.3. In this experiment, 30 g of wood and initial concentrations of 10 ppm diuron and bentazon were used.

4.2.2.4. Fungal treatment in a biopile system

Three different inoculation strategies were implemented: inoculation on wood, re-inoculation and non-re-inoculation on previously colonized wood (Chapter 3, Section 3.5.3). Inoculation was performed on wood wastes (30 g) produced in the single sorption experiment (Section 4.2.2.3), the successive sorption experiment (Section 4.2.2.3) and the column experiment (Section 4.2.2.2). By contrast, re-inoculation and non-re-inoculation strategies were applied to treat old colonized wood obtained from the experimental column. Afterwards, the solid samples were withdrawn and the remaining pesticide content (Chapter 3, Section 3.6.3) and ergosterol (Chapter 3, Section 3.6.6) were measured, both of them in triplicate.

4.2.2.5. Mathematical models for column breakthrough curves

Chu et al. (2020) has recently demonstrated that the Bohart-Adams, Thomas and Yoon and Nelson models are mathematically equivalent. Thus, these models can be fitted to the Chu logistic model, as shown in Table 4.5.

Table 4.5. Breakthrough curve models and their non-linear and linear forms of the Chu logistic model.

Mathematical models	Non-linear forms	General parameters	General linear form
Bohart-Adams model	$\frac{C_t}{C_0} = \frac{1}{1+\exp\left(\frac{k_{BA}N_0L}{u} - k_{BA}C_0t\right)}$ (4.9)	$a = \frac{k_{BA}N_0L}{u}$ (4.12) $b = k_{BA}C_0$ (4.13)	
Thomas model	$\frac{C_t}{C_0} = \frac{1}{1+\exp\left(\frac{k_{Th}q_0m}{Q} - k_{Th}C_0t\right)}$ (4.10)	$a = \frac{k_{Th}q_0m}{Q}$ (4.14) $b = k_{Th}C_0$ (4.15)	$\ln\left(\frac{C_0}{C_t} - 1\right) = a - bt$ (4.12)
Yoon–Nelson model	$\frac{C_t}{C_0} = \frac{1}{1+\exp(k_{YN}\tau - k_{YN}t)}$ (4.11)	$a = k_{YN}\tau$ (4.16) $b = k_{YN}$ (4.17)	

Variables k_{BA} ($cm^3\ mg^{-1}\ min^{-1}$), k_{Th} ($cm^3\ mg^{-1}\ min^{-1}$) and k_{YN} (min^{-1}) are the kinetic parameters of the corresponding models, N_0 ($mg\ cm^{-3}$) the saturation concentration, L (cm) the bed height, U_0 ($cm\ min^{-1}$) the superficial velocity, q_0 ($mg\ g^{-1}$) the adsorption capacity, Q ($cm^3\ min^{-1}$) is the volumetric flow rate, m (g) the wood amount, τ (min) the time required to reach 50 % adsorbate breakthrough, C_0 ($mg\ cm^{-3}$) the initial concentration and C_t ($mg\ cm^{-3}$) the concentration at any time t (min).

The Bohart-Adams model [Eq. (4.9)] is based on the surface reaction theory, which correlates C/C_0 with the contact time in a continuous system. The model also considers axial dispersion and mass transfer to be negligible, with the adsorbent capacity and the adsorbate concentration being the main driving forces. The Thomas model [Eq. (4.10)] assumes plug flow movement within the column. This model was proposed to follow the Langmuir isotherm and pseudo-second order kinetic without axial dispersion. The Yoon-Nelson equation [Eq. (4.11)] is a simple breakthrough model that describes the relationship between the probability of adsorption of each single adsorbate molecule, and the probability of breakthrough within the bed.

Two different parameters were analyzed to evaluate the correlation between the experimental data and the breakthrough curve models: linear correlation coefficient R^2 and normalized standard deviation Δq (%). Δq is defined by Eq. (4.3). The parameters y_{exp} and y_{cal} are the experimental and calculated values of the variables of interest, (being $y = C_t/C_0$ in this case), and N is the number of measurements analyzed. R^2 expresses the fit between the calculated

parameters and the linearized form of the models, and the experimental data. Δq indicates the correlation between the experimental and predicted values. The higher R^2 and lower Δq values represent a stronger model fit (Baccar et al., 2013).

4.2.3. Results and discussion

4.2.3.1. Pesticide removal by *T. versicolor* immobilized on wood in a fixed bed reactor

In this case, pine wood (used in Topic 4.1, Chapter 4) was replaced by holm oak wood (*Q. ilex*). Although Torán et al. (2017) demonstrated that pine wood was a better immobilization support for *T. versicolor* than other lignocellulosic substrates (pine bark, nutshell and hazelnut shell), this fungus is more commonly found on hardwood species in nature, such as *Q. ilex*, than on conifers (Cotter, 2014). *Q. ilex* wood chips presented the following characteristics: apparent and real densities of 0.27 ± 0.01 g mL^{-1} and 0.89 ± 0.02 g mL^{-1}, respectively, and a porosity of 62.4 ± 0.5 % (Chapter 3, Section 3.1). The WW/DW ratio was 2.5 ± 0.1. These results are consistent with previous works (Kim et al., 2016; Vo et al., 1995).

Fig. 4.4 (A-D) shows the breakthrough behavior at different bed depths and EBCTs. Breakthrough curves are generally used to describe the sorption profile in column reactors, as shown in Fig. 4.4 (B) and (D). However, in this study, the removal profile in the experimental columns, Fig. 4.4 (A) and (C), was also studied to ascertain the contribution of bioremediation and sorption. In this regard, the comparison of the breakthrough curves revealed that sorption played a key role in the overall removal.

Table 4.6. The physical-chemical properties of the investigated pesticides.

Pesticide	Type	Group	CAS number	Solubility at 20 ºC (mg L^{-1})	Log K_{ow}
Diuron	Herbicide	Phenylurea	330-54-1	37.4	2.68
Bentazon	Herbicide	Benzothiazinone	25057-89-0	570	-0.46

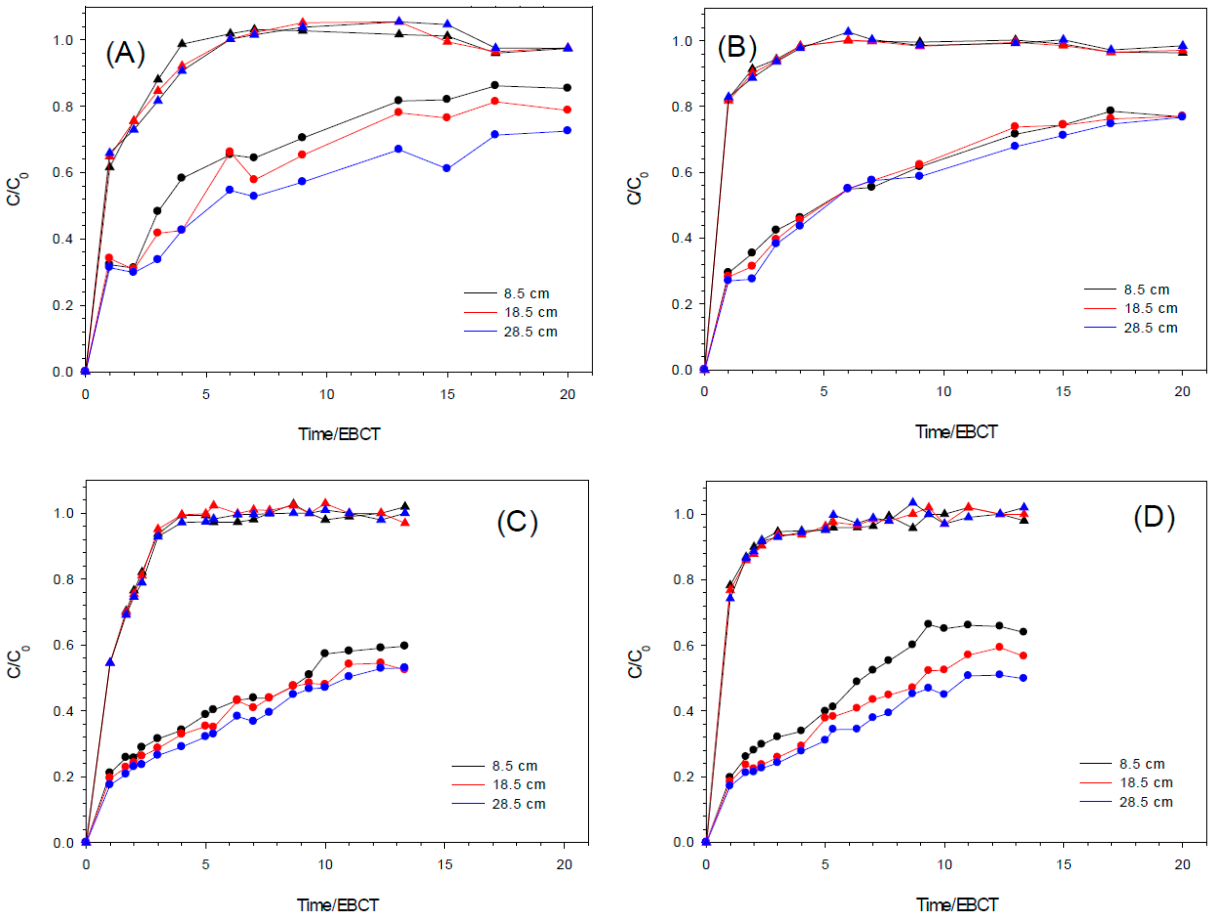


Fig. 4.4. Column breakthrough curves at three different heights (8.5, 18.5 and 28.5 cm) for an EBCT of 1 day in the experimental (A) and control (B) reactors, and an EBCT of 3 days in the experimental (C) and control (D) reactors. Dots and triangles represent diuron and bentazon respectively. C_0 is the initial concentration of the pesticides in the liquid phase (10 ppm), C_t is the pesticide concentration in the liquid phase at any time and EBCT is the empty bed contact time.

Sorption was significantly higher for diuron, as indicated by the removal percentage parameter (Y) in Tables 4.7 and 4.8. The sorption capacity of bentazon was exhausted, under the specified operating conditions, after approximately 5 EBCT in both cases. In contrast, the bed maintained around 25 and 50 % diuron sorption for EBCTs of 1 and 3 days, respectively. The higher affinity of diuron for wood was mainly attributed to the more hydrophobic nature of this compound (Table 4.6). In this regard, the effect of bed height on pesticide removal was only appreciable for diuron. As expected, a higher column height resulted in longer contact times between diuron and the fixed bed and thus higher removal efficiencies (Ahmad and Hameed, 2010). A similar

effect was obtained by increasing the EBCT from 1 to 3 days, entailing lower breakthrough curve slopes and higher overall removals in all cases (Chen et al., 2012).

Table 4.7. Main parameters of the fixed bed columns with an EBCT of 1 day, and diuron and bentazon removals (Y) after 20 days of treatment obtained from the breakthrough curves analysis.

Reactor	Pesticide	Z (cm)	EBCT (days)	m (g)	q_{total} (mg)	q_{eq} (mg g^{-1})	Y (%)
Exp	Diuron	8.5	0.30	47.7	20.753	0.435	28.78
Exp	Diuron	18.5	0.65	103.9	24.023	0.231	33.32
Exp	Diuron	28.5	1	160	30.146	0.188	41.81
Exp	Bentazon	8.5	0.30	47.7	3.540	0.074	6.31
Exp	Bentazon	18.5	0.65	103.9	3.932	0.037	6.05
Exp	Bentazon	28.5	1	160	4.175	0.026	5.93
Control	Diuron	8.5	0.30	47.7	26.439	0.555	36.74
Control	Diuron	18.5	0.65	103.9	26.524	0.255	36.79
Control	Diuron	28.5	1	160	28.298	0.177	39.25
Control	Bentazon	8.5	0.30	47.7	1.999	0.042	5.55
Control	Bentazon	18.5	0.65	103.9	2.087	0.020	5.85
Control	Bentazon	28.5	1	160	1.942	0.012	5.11

Variables: Z is bed height, EBCT the empty bed contact time, m the wood mass, q_{total} the overall pesticide removal, q_{eq} the maximum capacity of the column and Y the pesticide removal.

Table 4.8. Main parameters of the fixed bed columns with an EBCT of 3 days, and diuron and bentazon removals (Y) after 40 days of treatment obtained from the breakthrough curves analysis.

Reactor	Pesticide	Z (cm)	EBCT (days)	m (g)	q_{total} (mg)	q_{eq} (mg g^{-1})	Y (%)
Exp	Diuron	8.5	0.89	47.7	32.439	0.680	55.91
Exp	Diuron	18.5	1.95	103.9	34.156	0.329	58.86
Exp	Diuron	28.5	3	160	35.617	0.223	61.38
Exp	Bentazon	8.5	0.89	47.7	3.271	0.164	16.43
Exp	Bentazon	18.5	1.95	103.9	3.184	0.160	15.99
Exp	Bentazon	28.5	3	160	3.518	0.177	17.67
Control	Diuron	8.5	0.89	47.7	29.514	0.618	50.86
Control	Diuron	18.5	1.95	103.9	33.561	0.323	57.84
Control	Diuron	28.5	3	160	36.211	0.226	62.41
Control	Bentazon	8.5	0.89	47.7	1.996	0.100	10.03
Control	Bentazon	18.5	1.95	103.9	2.129	0.107	10.69
Control	Bentazon	28.5	3	160	2.183	0.110	10.96

Variables: Z is bed height, EBCT the empty bed contact time, m the wood mass, q_{total} the overall pesticide removal, q_{eq} the maximum capacity of the column, Y the pesticide removal.

In addition, slightly higher removals were obtained in the experimental reactor compared to the control reactor for bentazon, especially for an EBCT of 3 days. Being a more polar compound, bentazon probably exhibited limited affinity to the sorbent and, consequently, was more exposed to the extracellular enzyme system of *T. versicolor*. Indeed, laccase activity was found to be higher for an EBCT of 3 days throughout the treatment, as shown in Fig. 4.5. In his regard, laccase has recently proved to be involved in bentazon degradation (García-Vara et al., 2021), whereas discrepancies have been found in the case of diuron (Coelho-Moreira et al., 2018; Hu et al., 2020b). The laccase profile showed an initial peak and then started to decrease to zero after 16 and 22 days for EBCTs of 1 and 3 d, respectively. Although the presence of laccase indicates the enzymatic activity of the fungus, its absence does not necessarily entail the inactivation or death of the fungus (Mir-Tutusaus et al., 2017). Ergosterol was measured at the beginning and end of the column experiment with an EBCT of 1 day, obtaining 0.1222 ± 0.0119 and 0.1058 ± 0.0062 mg g DW^{-1} of ergosterol, respectively. Thus, 86.5 % of the initial fungal biomass remained active on the wood after 20 days of treatment.

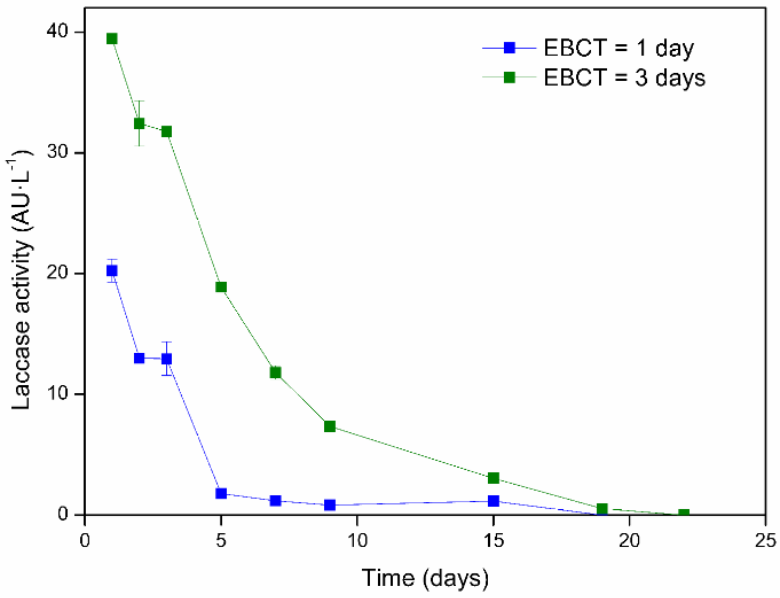


Fig. 4.5. Laccase activity over time in the FBRs with EBCT of 1 and 3 days.

4.2.3.2. Breakthrough curve modeling

The design and optimization of a column sorption process require mathematical approximations to describe and predict the column breakthrough curve. Several models have been developed

for the analysis of column systems, being the Bohart-Adams, Thomas and Yoon-Nelson the most common approaches.

Some authors have considered the Bohart-Adams, Thomas and Yoon-Nelson models to be mathematically independent. Consequently, these researchers discerned which model was more appropriate based on marginal differences in terms of R^2 (Ahmed and Hameed, 2018). Specifically, the Bohart-Adams model has been traditionally expressed as an exponential equation and thus, it is unable to fit the typical S-shaped or sigmoidal functions of the breakthrough curves. As a result, the Bohart-Adams model had been unfairly criticized for providing a poorer fit quality. Nevertheless, Chu has recently demonstrated that the mathematical formulas of all three models are equivalent and thereby can be expressed in terms of the same logistic growth function (Table 4.5) (Chu, 2020).

The linearized forms of the equations are shown in Table 4.5, and the fitting results along with the model constants are shown in Table 4.9. The specific constants of each model and the correlation coefficients were determined by linear regression analysis. Note that these models are generally used to describe the sorption profile of a column reactor. Nevertheless, in the present study, the models were also employed to analyze the removal profile of the experimental reactor for comparative purposes. In this regard, good fitting qualities were obtained in all cases, except the control reactor for bentazon. This finding can be ascribed to the more polar behavior of bentazon, which is poorly adsorbed by wood chips (Tables 4.6 and 4.7).

Generally, as the bed height increased, the kinetic parameter values tended to decrease. The only notable exception was observed when modeling the control reactor with an EBCT of 1 day for bentazon, which was attributed to its low R^2. Likewise, the saturation concentration ($N_{0)}$ and adsorption capacity (q_0) decreased with increasing the bed height, which is in agreement with Bakka et al. (2020). As expected, the time required to reach 50 % adsorbate breakthrough (τ) increased with the bed height as longer contact times were required. In addition, increasing the EBCT from 1 to 3 days (reducing Q) significantly decreases the kinetic parameters, and N_0, q_0 and τ increases in all cases, which has been reported elsewhere (Bayat et al., 2018).

Table 4.9. Fitting results and parameters of Bohart-Adams, Thomas and Yoon-Nelson models using Chu logistic equation.

EBCT (days)	Reactor	Pesticide	L (cm)	Bohart-Adams model		Thomas model		Yoon-Nelson model		R^2	Δq
				k_{BA} x 10^2 (cm^3 mg^{-1} min^{-1})	N_0 x 10^2 (mg cm^{-3})	k_T x x 10^2 (cm^3 mg^{-1} min^{-1})	q_0 x 10^2 (mg g^{-1})	k_{YN} x x 10^4 (min^{-1})	τ (min)		
0.30	Exp	Diuron	8.5	1.125	10.755	1.125	29.129	0.943	5552.19	0.89	16.44
0.65	Exp	Diuron	18.5	0.996	6.711	0.996	18.177	0.835	7541.16	0.87	13.41
1	Exp	Diuron	28.5	0.795	6.541	0.795	17.717	0.666	11323.03	0.89	11.56
0.30	Control	Diuron	8.5	0.922	16.722	0.922	45.292	0.773	8633.19	0.94	9.45
0.65	Control	Diuron	18.5	0.957	8.113	0.957	21.973	0.802	9115.90	0.91	11.88
1	Control	Diuron	28.5	0.942	5.940	0.942	16.087	0.790	10281.51	0.91	13.75
0.30	Exp	Bentazon	8.5	8.186	6.097	8.186	16.513	7.756	2785.28	0.83	20.23
0.65	Exp	Bentazon	18.5	7.071	3.040	7.071	8.234	6.699	3022.89	0.77	22.32
1	Exp	Bentazon	28.5	6.884	2.043	6.844	5.533	6.484	3129.25	0.76	22.65
0.30	Control	Bentazon	8.5	5.884	4.422	5.884	11.976	5.575	2019.99	0.56	18.78
0.65	Control	Bentazon	18.5	5.946	2.107	5.946	5.708	5.634	2095.37	0.57	19.08
1	Control	Bentazon	28.5	6.647	1.377	6.647	3.729	6.298	2109.12	0.63	18.93
0.89	Exp	Diuron	8.5	0.247	39.412	0.247	106.745	0.268	47118.19	0.95	11.09
1.95	Exp	Diuron	18.5	0.233	20.434	0.234	55.344	0.253	53169.39	0.91	11.84
3	Exp	Diuron	28.5	0.248	13.898	0.248	37.641	0.269	55708.64	0.95	11.07
0.89	Control	Diuron	8.5	0.313	31.531	0.313	85.399	0.340	37695.76	0.90	13.06
1.95	Control	Diuron	18.5	0.280	18.500	0.280	50.106	0.304	48137.26	0.93	11.87
3	Control	Diuron	28.5	0.247	14.270	0.247	38.648	0.269	57200.05	0.93	11.40
0.89	Exp	Bentazon	8.5	4.476	4.911	4.476	13.302	3.999	7136.41	0.84	15.67
1.95	Exp	Bentazon	18.5	4.541	2.253	4.541	6.102	4.057	7124.64	0.85	15.41
3	Exp	Bentazon	28.5	4.217	1.512	4.217	4.094	3.768	7364.09	0.81	16.11
0.89	Control	Bentazon	8.5	3.833	3.911	3.833	10.591	3.425	5682.12	0.62	15.48
1.95	Control	Bentazon	18.5	3.741	1.874	3.741	5.075	3.343	5926.27	0.61	15.74
3	Control	Bentazon	28.5	3.808	1.217	3.808	3.295	3.403	5927.89	0.63	15.51

Being k_{BA} (cm^3 mg^{-1} min^{-1}), k_{Th} (cm^3 mg^{-1} min^{-1}) and k_{YN} (min^{-1}) the kinetic parameters of the corresponding models, L (cm) the bed height, q_0 (mg g^{-1}) the adsorption capacity, τ (min) the time required to reach 50 % adsorbate breakthrough, and EBCT the empty bed contact time (days).

Owing to sorption contribution was predominant compared to fungal biodegradation (Section 4.2.3.1), limited differences in the model coefficients were consistently observed between the experimental and control columns in all cases. Therefore, lignocellulosic materials, such as wood chips, can not only serve as immobilization support and substrate in fungal treatments but also play a key role in micropollutant removal. This finding motivated a complete sorption study, which is presented in Sections 4.2.3.3-4.2.3.6. Moreover, given both pesticides were mostly not biodegraded but transferred from the liquid phase to the wood chips, an additional treatment of the solid phase is proposed in Section 4.2.3.7.

4.2.3.3. Correlation between contact time and pesticide sorption

Sorption kinetics and isotherm studies were required to accurately characterize the sorption phenomenon. Fig. 4.6 presents the profiles corresponding to both diuron and bentazon sorptions on *Q. ilex* wood chips over time. The system reached equilibrium in approximately 48 h for all sorbent doses applied (5, 10, 20, 30, 40, 60 g). Fig. 4.6 (A) shows q_t of approximately one order of magnitude higher than that in Fig. 4.6 (B), indicating that *Q. ilex* exhibited a higher affinity for diuron than for bentazon. Therefore, *Q. ilex* sorption capacity depended on the chemical nature of the pesticide, such as polarity (diuron K_{ow} = 2.68; bentazon K_{ow} = -0.46) (Luo et al., 2014).

As expected, the higher the sorbent doses used, the lower the pesticide sorptions per unit mass of wood at equilibrium. Nevertheless, in the case of the sorption curve of 5 g for bentazon, desorption was observed after 27 h. Again, this phenomenon can be mainly related to the limited affinity of bentazon for hydrophobic organic components of wood, such as lignin (Rodríguez-Cruz et al., 2009). Firstly, the sorption rose sharply since pesticide molecules occupied the vacant sites of the external surface of the wood. As the external surface became saturated, the sorption rate progressively decreased since the solute encountered resistance in diffusing through the porous structure of the wood to be adsorbed on the internal surface of the sorbent (Baccar et al., 2012). Lower q_t values were shown compared to other studies using carbon-based sorbents (De Gisi et al., 2016), which was attributed to the higher wood-wastewater proportion used in the present work.

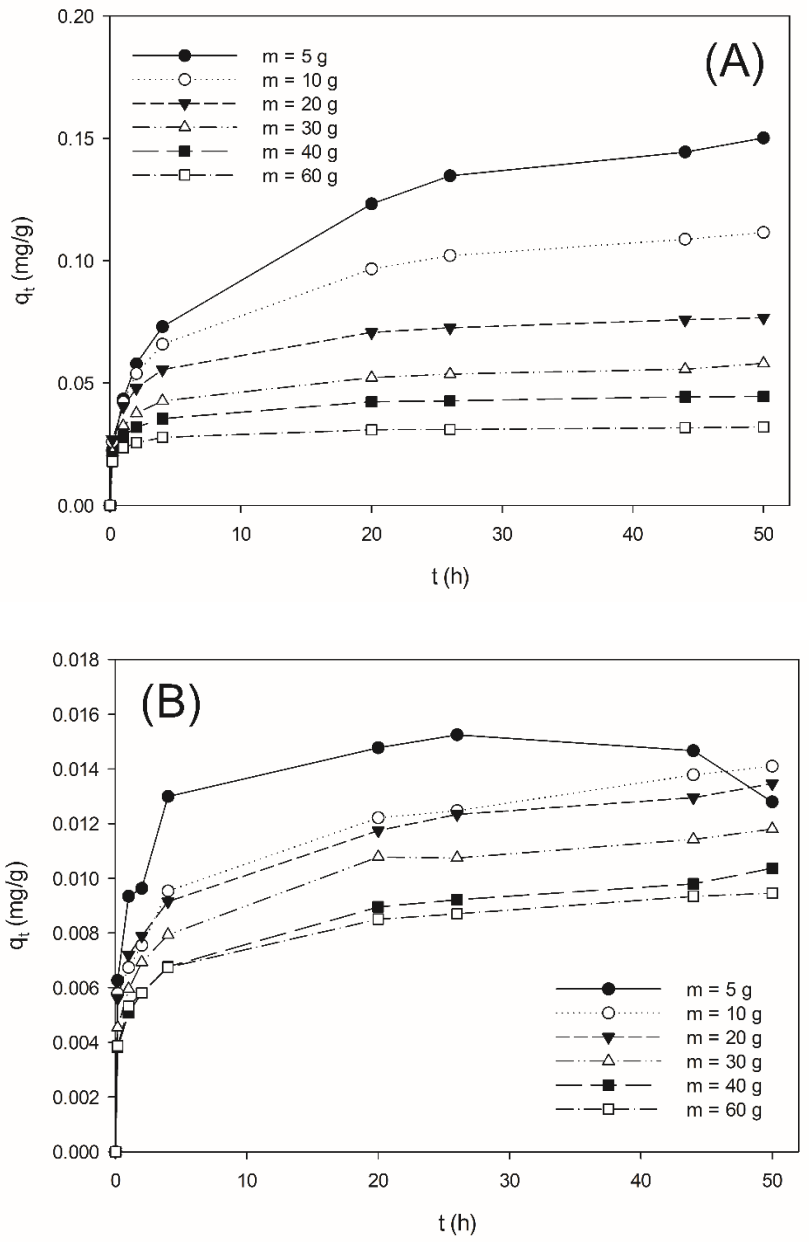


Fig. 4.6. Effect of contact time on diuron (A) and bentazon (B) sorption for different sorbent amounts (m) with a C_0 = 10 mg L^{-1} and at T = 25 ºC. The variable q_t is the pesticide sorption per gram of wood (mg g^{-1}) at any time t.

4.2.3.4. Sorption kinetics

Sorption kinetics are generally used to determine the mechanism or pathway of a sorption process along with the solute uptake rate. Sorption kinetics provide useful information for accurate design and full-scale application. The pseudo-first and pseudo-second order, Elovich, and Intra-particle diffusion models were investigated in this study. The linear and non-linear

kinetic equations are presented in Chapter 3, Table 3.4. In brief, the pseudo-first and pseudo-second order models assume that the chemisorption mechanism is the dominant process, whereas the intra-particle diffusion model considers diffusion to be the rate-limiting step. The Elovich model has been proposed as an intermediate model between the previous ones, in which no mechanism is clearly dominant (Wu et al., 2009).

The characteristic parameters of each kinetic model were calculated from the slope and intercept values through the regression of the linear forms presented in Chapter 3, Table 3.4. R^2 and Δq [Eq. (4.3)] were also analyzed and considered to select the best correlation with the experimental data. Table 4.10 shows the fitting results for the kinetic models. The Elovich equation obtained the best fit in all cases except for m = 5 g. Although the pseudo-second order model showed a slightly better R^2 fit, the Elovich model achieved a significantly lower Δq. The relatively low R^2 (0.849) obtained for m = 5 g is probably related to the poor affinity of bentazon for wood together with the low wood amount used in this case. The Elovich model considers that the adsorption rate decreases with increasing surface coverage, being mainly valid for heterogenous sorbents and liquid-solid systems. Moreover, the kinetic values obtained are in agreement with previous studies about the sorption of dyes and other organic compound sorption on carbon materials (Emeniru et al., 2017; Tseng et al., 2003). The Elovich model was also found to be the best approach considering R^2 and Δq, obtaining kinetic values of a similar order of magnitude to those presented for diuron sorption on pine wood in Topic 4.1 of Chapter 4.

Table 4.10. Pseudo-first order, pseudo-second order, Elovich and IPD kinetic model parameters, correlation coefficients and error values for diuron and bentazon sorptions on *Q. ilex* wood chips at 25ºC.

		Sorbate amount, m (g)					
		5	10	20	30	40	60
Sorbate		Diuron	Diuron	Diuron	Diuron	Diuron	Diuron
	$q_{e,exp}$ (mg g^{-1})	0.150	0.112	0.077	0.058	0.044	0.032
Pseudo-first order kinetic model	K_1 (h^{-1})	0.072	0.079	0.091	0.064	0.117	0.090
	$q_{e,cal}$ (mg g^{-1})	0.118	0.078	0.044	0.029	0.023	0.011
	R^2	0.985	0.980	0.965	0.886	0.957	0.883
	Δq (%)	61.848	67.355	74.565	80.890	76.523	87.376
Pseudo-second order kinetic model	K_2 (g mg^{-1} h^{-1})	1.981	4.052	10.699	14.994	28.162	61.408
	$q_{e,cal}$ (mg g^{-1})	0.156	0.114	0.078	0.058	0.045	0.032
	R^2	0.995	0.997	0.999	0.998	1.000	1.000
	Δq (%)	26.859	27.075	25.285	26.059	23.499	21.381
Elovich equation	a (mg g^{-1} min^{-1})	0.199	0.309	0.972	1.204	3.053	39.822
	b (g mg^{-1})	41.584	62.445	110.711	158.987	230.126	417.159
	R^2	0.968	0.984	0.996	0.997	0.993	0.978
	Δq (%)	27.071	12.221	2.555	1.446	2.207	3.137
IPD model	k_{id} (mg g^{-1} $h^{-1/2}$)	0.020	0.014	0.008	0.006	0.004	0.003
	C (mg g^{-1})	0.020	0.024	0.025	0.020	0.018	0.016
	R^2	0.951	0.903	0.788	0.755	0.682	0.556
	Δq (%)	14.999	14.295	15.490	15.827	15.937	16.175
Sorbate		Bentazon	Bentazon	Bentazon	Bentazon	Bentazon	Bentazon
	$q_{e,exp}$ (mg g^{-1})	0.015	0.014	0.013	0.012	0.010	0.009
Pseudo-first order kinetic model	K_1 (h^{-1})	0.089	0.074	0.066	0.070	0.059	0.085
	$q_{e,cal}$ (mg g^{-1})	0.006	0.009	0.007	0.007	0.006	0.005
	R^2	0.581	0.957	0.932	0.929	0.920	0.958
	Δq (%)	85.069	73.461	78.076	76.989	77.604	75.098
Pseudo-second order kinetic model	K_2 (g mg^{-1} h^{-1})	-566.052	40.873	51.332	59.791	59.040	79.974
	$q_{e,cal}$ (mg g^{-1})	0.014	0.014	0.013	0.012	0.010	0.010
	R^2	0.989	0.996	0.997	0.998	0.996	0.998
	Δq (%)	33.479	31.073	30.078	28.497	30.031	28.759
Elovich equation	a (mg g^{-1} min^{-1})	0.959	0.182	0.269	0.154	0.114	0.211
	b (g mg^{-1})	691.774	635.520	698.663	744.016	849.477	986.471
	R^2	0.849	0.957	0.981	0.983	0.982	0.993
	Δq (%)	10.052	10.036	5.911	6.231	6.622	3.485
IPD model	k_{id} (mg g^{-1} $h^{-1/2}$)	0.001	0.002	0.001	0.001	0.001	0.001
	C (mg g^{-1})	0.006	0.004	0.004	0.003	0.003	0.003
	R^2	0.598	0.831	0.798	0.812	0.930	0.779
	Δq (%)	19.571	13.314	13.780	13.829	13.515	14.324

Parameters: $q_{e,cal}$ is the calculated amount of pesticide adsorbed per gram of adsorbent at equilibrium (mg g^{-1}), k_1 the pseudo-first rate constant (h^{-1}), k_2 the pseudo-second rate constant (g mg^{-1} h^{-1}), a the initial adsorption rate (mg g^{-1} h^{-1}), b is a coefficient associated to the activation energy and surface coverage (mg g^{-1}), K_{id} is the intra-particle diffusion rate constant (mg g^{-1} $h^{-1/2}$) and C the intercept.

4.2.3.5. Sorption isotherms

Sorption isotherms show the partition ratio of a defined quantity of sorbate between the liquid and solid phases at equilibrium, for a given mixture and constant temperature. Fitting the experimental equilibrium data to a suitable isotherm model provides valuable information about the possible sorption mechanism and the favorability of the process, which in turn facilitates the design and scale-up of any sorption system. The sorption equilibrium data were correlated with three well-known isotherm models, the Langmuir, Freundlich, and Temkin models. The isotherm equations were converted into their linear forms and plotted to calculate the particular parameters of each model from the slopes and the intercepts. In the case of the Langmuir isotherm, five different linearization methods were applied, as significant variations in parameter values and fits have been previously reported (Baccar et al., 2013). The isotherm methods and their linear forms are shown in Chapter 3, Table 3.5.

The Langmuir model assumes homogeneous monolayer adsorption over a finite number of adsorption sites and no chemical interactions among sorbate molecules (Langmuir, 1916). The Freundlich model is an empirical expression that takes into account the surface heterogeneity and the exponential energy distribution of the active sites, being appropriate for low concentrations (Appel, 1973; Freundlich, 1909). Finally, the Temkin isotherm model considers the interaction between sorbate molecules and a linear decrease in binding energy as surface saturation increases (Aharoni and Ungarish, 1977; Temkin and Pyzhev, 1940).

The results of the model fits are shown in Table 4.11. The Freundlich model showed the best fit for both diuron (R^2 = 0.989 and Δq = 6.057 %) and bentazon (R^2 = 0.981 and Δq = 10.920 %) equilibrium isotherms. The Freundlich model has been previously selected to model pesticide sorption on porous carbon-based sorbents (Laohaprapanon et al., 2010; Rodriguez-Cruz et al., 2007). In addition, the maximum sorption capacity q_{max} can be estimated by applying Eq. (3.15) to the Freundlich Eq. (3.14). Consequently, q_{max} of 0.161 and 0.014 mg g^{-1} were obtained for diuron and bentazon sorptions respectively, which is in agreement with previous works using similar sorbent doses (Beltrán-Flores et al., 2020, Topic 4.1 of Chapter 4; Iqbal and Ashiq, 2007). Note that the theoretical maximum sorption capacity of diuron was approximately 10 times higher than that of bentazon. However, a more detailed discussion of the resulting q_{max} is proposed in Section 4.2.3.6.

Table 4.11. The Langmuir, Freundlich and Temkin parameters, correlation coefficients and error values for diuron and bentazon sorptions on *Q. ilex* wood chips at 25 ºC.

Isotherms	Diuron				Bentazon			
Langmuir	K_L (L mg^{-1})	q_{max} (mg g^{-1})	R^2	Δq (%)	K_L (L mg^{-1})	q_{max} (mg g^{-1})	R^2	Δq (%)
Langmuir-1	0.144	0.266	0.976	8.521	84.472	0.012	1.000	18.582
Langmuir-2	0.095	0.368	0.851	6.425	1.184	0.014	0.830	17.870
Langmuir-3	0.131	0.289	0.746	8.012	82.126	0.012	0.840	19.365
Langmuir-4	0.098	0.361	0.746	6.659	68.958	0.013	0.840	20.576
Langmuir-5	0.129	0.290	0.976	7.734	84.470	0.012	1.000	18.582
Freundlich	n	q_{max} (mg g^{-1})	R^2	Δq (%)	n	q_{max} (mg g^{-1})	R^2	Δq (%)
	1.299	0.161	0.989	6.057	1.877	0.014	0.999	10.920
Temkin	A	B	R^2	Δq (%)	A	B	R^2	Δq (%)
	1.529	0.058	0.945	11.443	89343	$9.225 \cdot 10^{-4}$	0.859	18.237

Variables: q_{max} is the Langmuir maximum adsorption capacity, K_L the energy of adsorption, R^2 the linear correlation coefficient, Δq the normalized standard deviation, n the adsorption intensity, B = RT/A, where R is the ideal gas constant (8.314 J mol^{-1} K^{-1}), T is the temperature (K) and A is the Temkin equilibrium constant (L mg^{-1}).

4.2.3.6. Effect of successive sorption cycles on sorption capacity

A successive sorption process was performed with a total of six sorption cycles in batch mode. Repeated sorption processes are frequently found in literature concerning the evaluation of regeneration or desorption efficiencies of a contaminated sorbent (Alizadeh Fard et al., 2017; Sathishkumar et al., 2015). However, in the present study, the successive sorption experiment was conducted to assess the total sorption capacity for two model pesticides. The results of the accumulative sorptions of diuron and bentazon are shown in Fig. 4.7.

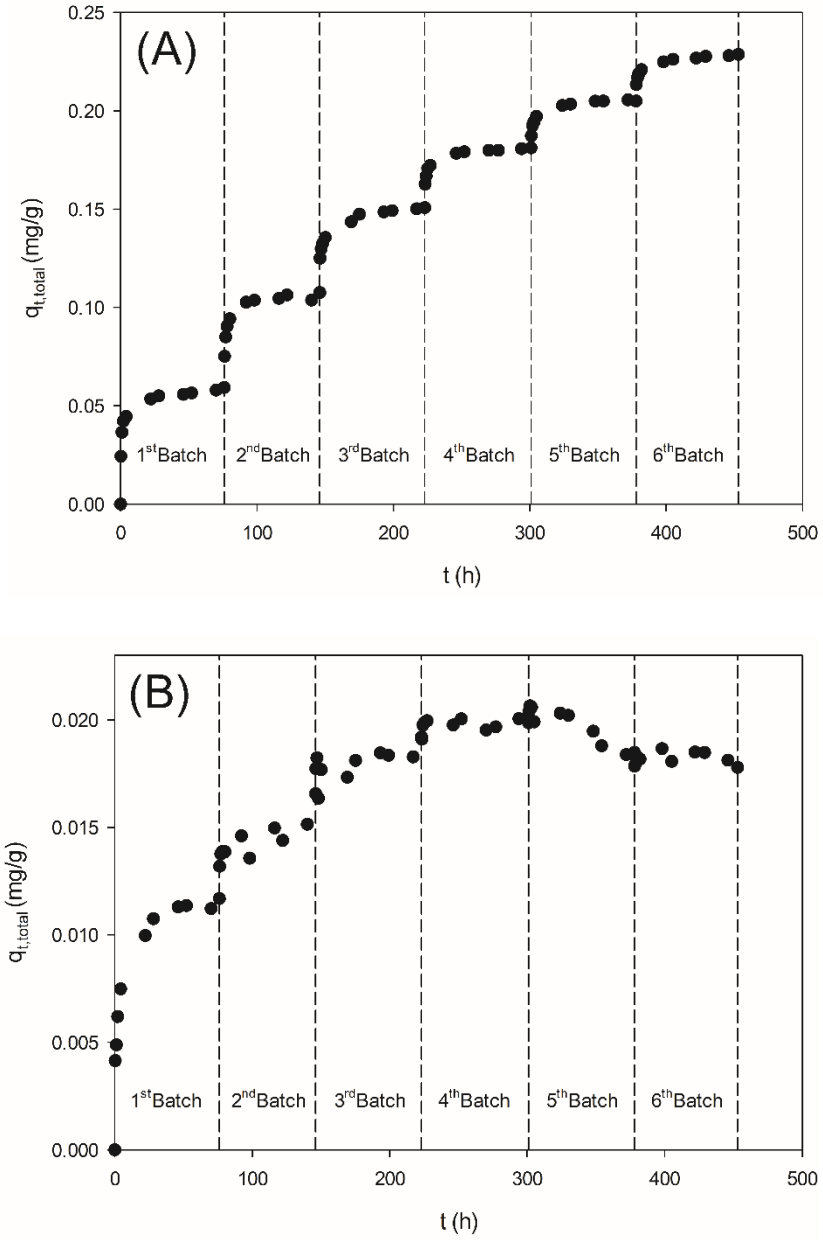


Fig. 4.7. Effect of successive cycle batches on diuron (A) and bentazon (B) sorption over time. The variable q_t is the pesticide sorption per gram of wood (mg g^{-1}) at any time t.

The sorption capacity reached at equilibrium in the first batch was exceeded in the second and successive batches for both pesticides, as shown in Fig. 4.7. Although equilibrium between the liquid and solid phase was reached at the end of each batch, the addition of fresh water at the initial concentration of pesticides (10 ppm) increased the sorption per gram of wood. In the case of bentazon, the wood seemed to reach its maximum sorption capacity (for the given conditions)

in the sequential batches, even being partially desorbed after 4 cycles (12 days). In any case, q_{max} estimated by the Freundlich model (Section 4.2.3.5) were exceeded in the sequential batch experiment and the FBR (Section 4.2.3.1). In section 4.1.3.4 (Topic 4.1, Chapter 4), the Langmuir model was used instead of the Freundlich model to calculate q_{max} of diuron on pine wood, since this estimation was more in line with the results observed in the sequential batch. In this case, although q_{max} for holm oak wood (both for diuron and bentazon) were not exceeded during the sequential batch, a higher sorption capacity was observed during the FBR experiment (Section 4.2.3.1). Therefore, the maximum sorption capacity of the holm oak wood exceeded that predicted by the models.

Fig. 4.8 shows the evolution of the sorption capacity over six consecutive batches. The sorption capacity of wood gradually decreased for both compounds as the sorbent material became saturated. However, wood chips still exhibited a remaining sorption capacity of approximately 35 % diuron at the end of the process, which highlights the remarkable sorption capacity of this lignocellulosic material for more hydrophobic pesticides (Zamora et al., 2000).

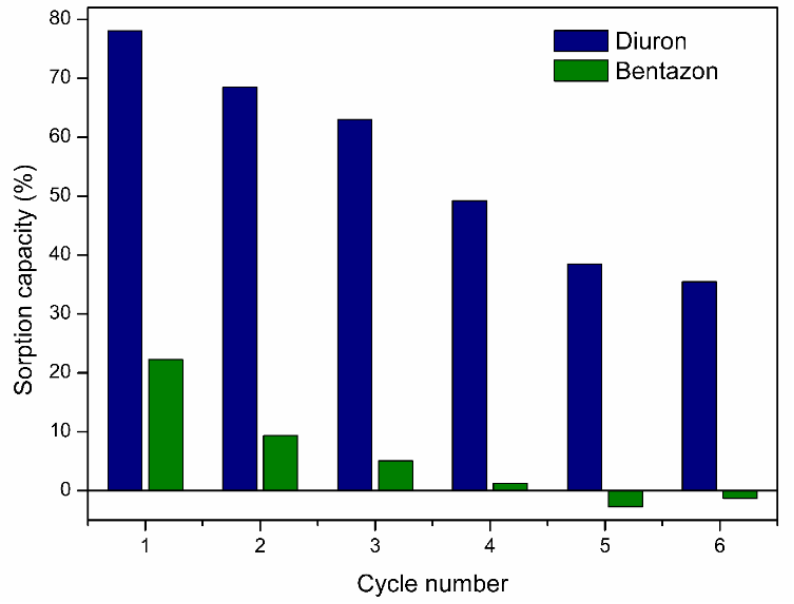


Fig. 4.8. Effect of successive cycle batches on the relative sorption capacity of wood for diuron (blue bars) and bentazon (green bars).

4.2.3.7. Solid treatment of wood in a biopile system

Wood has proven to be a porous material with excellent sorption capacity. Thus, an additional remediation strategy should be explored to ensure pesticide removal from the solid phase. Accordingly, a solid-phase treatment was conducted by inoculating *T. versicolor* on the polluted wastes in a biopile-like system. The solid by-products had previously been subjected to either the column (Section 4.2.3.1) or the sorption experiments (Sections 4.2.3.3-4.2.3.6). In this regard, three different strategies were evaluated: inoculation on old wood as well as re-inoculation and non-re-inoculation of previously colonized wood wastes. Extraction recoveries of 86.1 % and 86.8 % were obtained for diuron and bentazon, therefore factors of 1.16 and 1.15 were applied to correct data deviation, respectively. Table 4.12 shows the initial concentrations detected in the solid phase, and the degradation rates and yields achieved in each biopile experiment.

Various initial concentrations were detected depending on the previous application of the solid by-products. As expected, the lowest initial concentrations were obtained in the wood produced in the single sorption experiment. The highest initial concentrations were found in the colonized wood obtained from the experimental column. This concentration was higher than the concentration detected in the wood of the control column, evidencing a significant contribution of the fungal biofilm to the overall removal, as described elsewhere (Hu et al., 2020a).

Table 4.12. Degradation of diuron and bentazon contained in the polluted wood achieved by *T. versicolor* at different initial concentrations and operating conditions in a biopile system after 27 days of treatment. Values are means ± standard deviation for triplicate samples.

Experiment	Biopile conditions	Pesticide: diuron			Pesticide: bentazon		
		Initial concentration ± SD (mg g wood DW^{-1})	Degradation ± SD (%)	Degradation rate ± SD (mg g wood DW^{-1} day^{-1}) $x10^3$	Initial concentration ± SD (mg g wood DW^{-1})	Degradation ± SD (%)	Degradation rate ± SD (mg g wood DW^{-1} day^{-1}) $x10^4$
Sorption in batch (1 batch)	Inoculated	0.0514 ± 0.0014	92.50 ± 0.35	1.76 ± 0.01	0.0112 ± 0.0026	90.02 ± 2.72	3.73 ± 0.11
Successive sorption batch (6 batches)	Inoculated	0.2050 ± 0.0094	19.74 ± 5.09	1.50 ± 0.39	0.0146 ± 0.0004	43.23 ± 3.41	2.33 ± 0.18
Control column (EBCT = 1 day)	Inoculated	0.1715 ± 0.0153	24.32 ± 4.27	1.55 ± 0.01	0.0154 ± 0.0003	29.03 ± 4.84	1.66 ± 0.27
Experimental column (EBCT = 1 day)	Non-re-inoculated	0.2265 ± 0.0081	22.19 ± 4.09	1.86 ± 0.34	0.0171 ± 0.0013	39.11 ± 2.04	2.48 ± 0.13
	Re-inoculated	0.2265 ± 0.0081	30.37 ± 3.87	2.55 ± 0.27	0.0171 ± 0.0005	64.48 ± 2.40	4.09 ± 0.15

Results show that both pesticides were bioavailable to interact with the fungal enzyme system, obtaining significant concentration decreases in all cases. The highest relative degradation was obtained for the old wood resulting from the sorption experiment with 1 batch (Section 4.2.3.3). High diuron and bentazon relative degradations of about 93 and 90 %, respectively, were reached after 27 days in the biopile-like treatment. However, the relative degradation depends on the initial concentration of the pesticides in the wood. Thus, these high relative degradations are probably due to the low initial concentration in the wood wastes produced in the single batch sorption experiment (see initial concentrations in Table 4.12). In fact, the re-inoculation strategy proved to be the most effective approach in terms of degradation rate. The enhanced performances showed in the re-inoculated biopile (E-R) were attributed to higher fungal contents, as shown in Fig. 4.9.

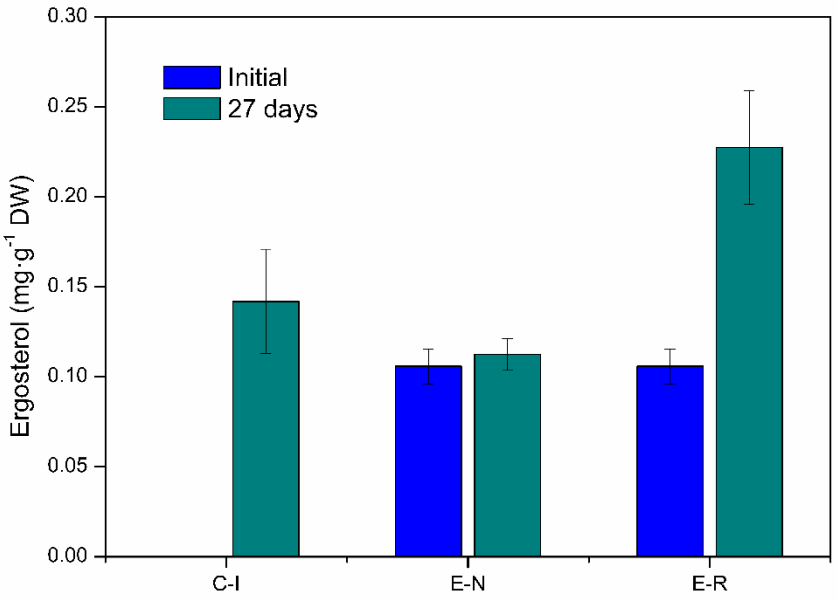


Fig. 4.9. Biomass, expresses as ergosterol content per unit of dry weight, detected in the wood chips in the biopile-like treatment. Bars are the averages and error bars are the standard deviations of three replicates. Blue bars are the initial ergosterol content of the solid waste and green bars are the ergosterol content of the solid waste in the biopile after 27 days of treatment. Origin of the by-products: C-I is wood from the control column inoculated; E-N and E-R represent by-products from the experimental column non-re-inoculated and re-inoculated, respectively.

The E-R approach resulted in a higher biomass content than those obtained in both the non-re-inoculation (E-N) and the inoculation (C-I) strategies. In this respect, the presence of pre-grown

fungus on the wood could favor a better adaptation and growth of the fresh fungus. Thus, these results indicate that the combination of both fungal treatments, the experimental column reactor followed by the biopile system with re-inoculation strategy, provided the best results in terms of fungal content and pesticide degradation. Results also suggest the potential of this technique to reuse polluted substrate in successive sorption/bioregeneration cycles, although longer operational periods would be desirable to increase treatment efficiency. This strategy would reduce wood requirements in full-scale applications while maintaining notable removal efficiencies and biomass growths. In addition, for full-scale applications, wood could also be supplied by some industries that can generate it as a by-product or waste. Thus, wood could be considered a more sustainable immobilization support than other materials, for example, the commonly used polyurethane foam (Mir-Tutusaus et al., 2018a).

Interestingly, the C-I strategy showed a higher biomass growth than in the E-N case. The limited rate of fungal growth achieved in the E-N biopile was attributed to the biomass aging process, which is generally overcome by periodic biomass renewals or re-inoculations, as in the case of the E-R (Blánquez et al., 2006). Nevertheless, the degradation rate was higher in the E-N than in the C-I, probably due to the low biomass content presented in the initial stage of the inoculated biopile.

To the best of the authors' knowledge, this is the first time that fungal-based technology has been implemented to bioregenerate sorbents contaminated with micropollutants. Fungal biopile systems have been traditionally used for pollutant removal from either soil or sewage sludge. In most cases, a lignocellulosic material (typically wheat straw) was previously colonized with a fungus under sterile conditions to be later mixed with the target residue. For example, Borràs et al. (2011) used wheat straw as a fungal carrier to treat soil contaminated with naproxen, obtaining approximately 50 % reduction after 20 days of culture. Llorens-Blanch et al. (2018) achieved an average drug removal of 66.45 % from sewage sludge in a biopile system with *T. versicolor* immobilized on pine wood working under non-sterile conditions. Rodríguez-Rodríguez et al. (2010) also used *T. versicolor* to completely remove naproxen from solid sewage sludge by using wheat straw as substrate, achieving over 48 % carbamazepine degradation. Rodríguez-Rodríguez et al. also obtained high average removals for several UV filters (80 %), brominated-flame-retardants (81 %) and pharmaceuticals (86 %) at similar initial

concentrations and removal rates. Nonetheless, 39 % (w/w dry basis) of fresh biomass was initially required as substrate, and the biopile treatment was maintained for 42 days with weekly homogenization (Rodríguez-Rodríguez et al., 2014). In the present biopile system, no additional source of nutrients or weekly mixing was required for 27 days of treatment.

Therefore, although the *T. versicolor* immobilized on wood was maintained (86.5 %) during the treatment of AW in an FBR, sorption on wood was found to be the predominant mechanism of pesticide removal. Thus, the solid by-products were further treated in a biopile-like reactor. In fact, the combination of the FBR followed by the biopile system provided the best results in terms of fungal content and pesticide degradation, which in turn allows the possibility of reusing wood in several treatment/bioregeneration cycles. However, future research should also focus on exploring different inoculum amounts, fungal consortia and reactor configurations, and move toward full-scale application.

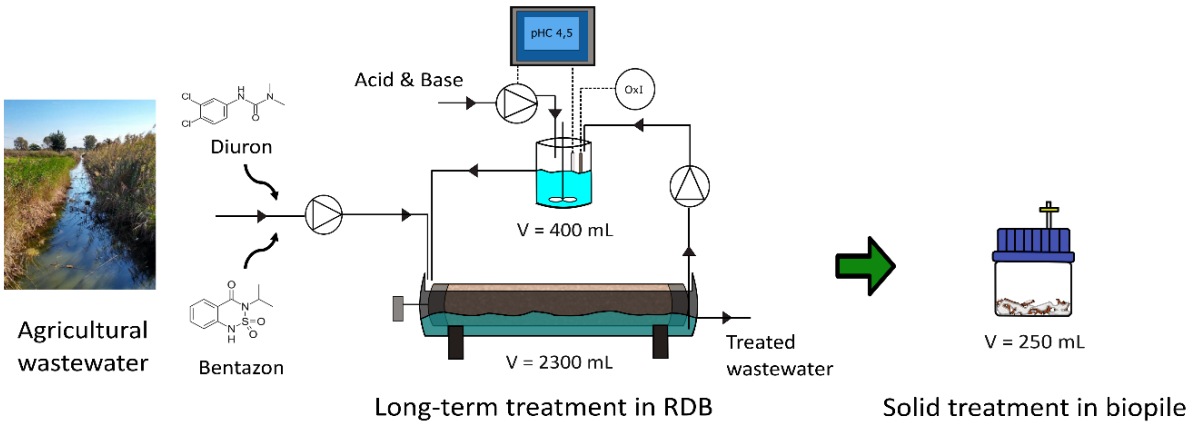
4.2.4. Conclusions

T. versicolor immobilized on wood chips was found to be effective in removing two target pesticides from AWs in an FBR. Although wood was initially selected to serve as a substrate and support for the fungus, its sorption capacity was found to be predominant compared to fungal biodegradation. This finding motivated a comprehensive sorption analysis, including the breakthrough curves of the FBR, and the isotherm and kinetic studies in Erlenmeyer flasks. The sorption capacity of the wood was really high, even exceeding the maximum capacity predicted by the models. Finally, polluted solid wastes were treated by *T. versicolor* in a biopile-like system reaching degradations of up to 93 % after 27 days of treatment. The combination of the inoculated column with the re-inoculated biopile showed the best results in terms of fungal biomass and pesticide degradation.

Chapter 5

Fungal bioremediation of agricultural wastewater in a long-term treatment: biomass stabilization by immobilization strategy

Based on the homonymous article: Beltrán-Flores, E., Pla-Ferriol, M., Martínez-Alonso, M., Gaju, N., Blánquez, P., Sarrà, M., 2022. J. Hazard. Mater. 439, 1–11. https://doi.org/10.2139/ssrn.3991524



5.1. Introduction

Pesticides are a broad group of chemical compounds used to improve agricultural production. However, pesticides have a high potential to drift into aquatic environments after application through storm-water discharges, surface runoff and leaching from agricultural areas (Vryzas, 2018). Thus, water bodies located near intensive agricultural fields are more vulnerable to pesticide contamination (Deknock et al., 2019). Furthermore, most pesticides are considered persistent compounds that, depending on their solubility, can remain dissolved in water or bioaccumulate in biota (Andrade et al., 2021). Long-term exposure to pesticides can lead to undesirable and harmful effects on the environment and human health. Even at low concentrations, chronic exposure to pesticides can pose a serious health threat not only to agricultural workers but also to the general population (Leong et al., 2020).

In this scenario, the development of effective treatments for the removal of pesticides in AW is mandatory. Physical-chemical methods have the advantage of being well-established, fast and effective processes against pesticides. However, these processes generate residues (such as in sorption) or toxic TPs (such as in single ozonation), and they are considered relatively expensive (Ahmed et al., 2017). In contrast, bioremediation has emerged as a cost-effective, sustainable, and efficient alternative, although it typically requires longer treatment times and involves more complications related to biomass maintenance (Azubuike et al., 2020).

Among other microorganisms, white-rot fungi (WRF) have attracted attention for their unique and versatile metabolism, which is capable of degrading a wide range of xenobiotics through the activity of their intracellular and extracellular enzyme systems (Zhuo and Fan, 2021). In addition, WRF have shown excellent resistance to highly toxic waters without any prior acclimatization process, unlike other biological treatments such as conventional activated sludge (Mir-Tutusaus et al., 2017). Nevertheless, fungal treatment still presents major challenges: nutrient addition, foaming, biomass aging and microbial contamination (Mir-Tutusaus et al., 2018a). In this regard, immobilization on lignocellulosic materials has been proposed as a promising strategy to address some of these operational limitations. Immobilization prevents mycelial dispersion, which frequently causes foaming and blocking, and allows separation between HRT and CRT. In addition, lignocellulosic materials, such as wood, provide a specific

source of nutrients for fungi that facilitates operation under non-sterile conditions (Torán et al., 2017).

A previous scientific paper used *T. versicolor* immobilized on wood to treat AW in a rotating drum bioreactor (RDB) (Beltrán-Flores et al., 2020, Topic 4.1 of Chapter 4). This reactor was operated for a short period to evaluate the feasibility of the treatment, but some critical drawbacks, such as the loss of immobilized biomass, were detected. However, these problems may be solved by implementing favorable operational strategies to consolidate biomass immobilization, thus enhancing pesticide degradation, in a long-term continuous treatment. Therefore, the aim of the present study was to set up an RDB using *T. versicolor* immobilized on *Q. ilex* wood chips to remove diuron and bentazon from AW over a long-term period. Throughout the treatment, the rotation frequency and HRT were modified to analyze their effect on the bioreactor performance, including biomass maintenance and removal efficiency. A comprehensive study of the fungal populations was also conducted to evaluate the relative abundance of *T. versicolor,* as well as other prominent members of the fungal community throughout the treatment until a stable community with degrading capability was achieved.

5.2. Materials and methods

5.2.1. Agricultural wastewater

AW III was used in Period I (P-I), whereas AW IV and V was treated in Period II (P-II) and Period III (P-III). As explained in Section 3.2 of Chapter 3, AW III was previously subjected to a coagulation and flocculation process to erase algae. The physicochemical characteristics of the AW are summarized in Table 5.1. In all cases, the AW was adjusted to pH 4.5 and 10 ppm diuron and bentazon (KAOS-B) were added from the stock solution (10 mg mL^{-1} in ACN) to allow detection by HPLC-UV.

Table 5.1. Physicochemical characterization of the AW. Values are means ± standard deviation for triplicate samples.

Parameter	AW III	AW III after pre-treatment	AW IV & V
pH	7.27 ± 0.08	6.65 ± 0.10	8.06 ± 0.02
Conductivity (mS cm^{-1})	2.66 ± 0.04	2.48 ± 0.04	3.17 ± 0.01
Color at 650 nm	0.09 ± 0.01	0.02 ± 0.01	0.10 ± 0.01
Chloride (mg Cl L^{-1})	13.7 ± 0.2	708.2 ± 45.4	653.7 ± 6.1
Sulphate (mg S L^{-1})	79.8 ± 1.1	59.9 ± 3.2	86.3 ± 1.6
Nitrite (mg N L^{-1})	0.3 ± 0.1	5.9 ± 0.4	0.4 ± 0.1
Nitrate (mg N L^{-1})	0	0	0
Ammonia (mg N L^{-1})	0	0	0
TSS (mg L^{-1})	25 ± 7	2 ± 1	16 ± 2
VSS (mg L^{-1})	24 ± 6	3 ± 1	11 ± 2
COD (mg O_2 L^{-1})	144 ± 6	27 ± 1	44 ± 4
DOC (mg C L^{-1})	32 ± 1	10 ± 1	16 ± 1
Heterotrophic plate count (CFU mL^{-1})	$1.19 \cdot 10^5 \pm 1.77 \cdot 10^4$	$4.99 \cdot 10^4 \pm 4.34 \cdot 10^3$	$3.50 \cdot 10^4 \pm 1.50 \cdot 10^4$

5.2.2. Agricultural wastewater treatment

Two rotating drum bioreactors (RDB) were operated in parallel, one of the reactors was assembled with wood initially colonized by *T. versicolor*, which is the experimental reactor (E-RDB), while the other reactor was set up only with wood as a control (C-RDB). The feed tank was stirred during the entire operation, from which water was pumped to one side of the reactor. The water left the reactor from the other side by overflow. The inner tube rotated one and a half turns every either 4 or 24 h (depending on the period) to ensure aerobic conditions. An external recirculation loop (4.7 L day^{-1}) was required for pH adjustment and DO measurement, which were performed in a recirculation tank (≈ 0.4 L). The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. The DO level was monitored using a CyberScan 600 Series Waterproof Handheld (Eutech Instruments). The DO level remained above 30 % in the recirculation tank throughout the treatment.

Liquid samples were withdrawn from the reactor effluent for analysis of pesticide concentration, laccase, color, COD, HPCs, and fungal community analysis. Solid samples were taken at the beginning and end of each period to measure ergosterol level, fiber content, SEM and fungal population. The reactors were successfully operated in continuous mode for 225 days. However,

the treatment can be divided into three stages according to the different operational conditions implemented in each period, as shown in Table 5.2.

Table 5.2. Values of the main operating variables in each treatment period.

Period	Time (days)	Rotation frequency	Wood amount (g DW)	HRT (d)
P-I	0-93	1.5 turns every 4 h	315	3
P-II	93-186	1.5 turns every 24 h	210 (old) + 335 (fresh) = 545	3
P-III	186-225	1.5 turns every 24 h	545 (old)	5

P-I, P-II and P-III are periods I, II and III, respectively.

5.2.3. Fungal treatment in a biopile system

A total of 105 g of polluted wood chips and colonized wood chips were withdrawn from the control and experimental RDBs, respectively, after 93 days of treatment. Afterwards, 30 g of contaminated wood was treated by *T. versicolor* in a biopile system per duplicate using three different strategies: inoculation on wood, re-inoculation and non-re-inoculation on wood containing pre-grown fungus (Chapter 3, Section 3.5.3). Solid samples were withdrawn after 27 and 48 days. Afterwards, the solid samples were withdrawn and the remaining pesticide content (Chapter 3, Section 3.6.3) and ergosterol (Chapter 3, Section 3.6.6) were measured.

5.2.4. Analytical methods

5.2.4.1. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images were acquired on a Zeiss EVO MA 10 scanning electron microscope. The pretreatment of the sample was performed by the Microscopy Service of the Autonomous University of Barcelona (Bellaterra, Spain), using the protocol shown in Annex C.

5.2.4.2. Fiber content analysis

Cellulose, hemicellulose and lignin contents were determined by the method of Van Soest et al. (1991). These analyses were performed by the Laboratory of Agriculture and Animal Production, Department of Animal and Food Science, Faculty of Veterinary Medicine,

Autonomous University of Barcelona (Bellaterra, Spain). The detailed methodology is described in Annex C.

5.3. Results and discussion

5.3.1. Colonization of wood chips

Q. ilex wood chips presented the following characteristics: apparent and real densities of 0.27 ± 0.01 g mL^{-1} and 0.89 ± 0.02 g mL^{-1}, respectively, and a porosity of 62.4 ± 0.5 % (Beltrán-Flores et al., 2021, Section 4.2.3.1 of Chapter 4). The WW/DW ratio was 2.5 ± 0.1. Afterwards, *T. versicolor* was inoculated on *Q. ilex* wood in a sterile box, reaching 0.26 mg g wood DW^{-1} of ergosterol content after 17 days of culture. This value was considerably higher than that obtained in a screening assay by Torán et al. (2017), in which up to 0.031 mg g wood DW^{-1} were reported using pine wood as immobilization support after 9 days of culture. These results can be explained by the fact that, in nature, *T. versicolor* is more commonly found on hardwood species, such as *Q. ilex*, than on conifers (Cotter, 2014). Indeed, this factor may have contributed to *T. versicolor* detachment from pine wood in the RDB studied in Section 4.1.3.5 (Chapter 4).

5.3.2. RDB performance

5.3.2.1. Biomass content

The initial treatment conditions are those indicated as P-I in Table 5.2, corresponding to the first 93 days of treatment. The main objective of P-I was to evaluate the adaptability of the fungus to the real environmental conditions found in an RDB. In this regard, an RDB had been previously used for the treatment of AW by *T. versicolor* pre-grown on lignocellulosic substrate (Beltrán-Flores et al., 2020, Topic 4.1 of Chapter 4). However, the fungus was apparently detached from the wood surface throughout the treatment. In that case, the loss of biomass was mainly attributed to the application of an excessive continuous rotation speed (6 rpm). High rotation speeds can cause undesirable damage to the mycelial structure of fungi (Zhong, 2010). Consequently, in P-I of the present study, the rotation frequency was considerably reduced from 6 rpm to 1.5 turns every 4 hours.

The time-course of ergosterol content was monitored to quantify the presence of fungal biomass over the studied period. Despite this adjustment, a significant decrease in fungal biomass was

observed after 78 days of treatment, when only 20 % of the initial fungal content remained immobilized on the wood (Table 5.2). P-I was maintained until 93 days to give the fungus a chance to recolonize the wood, as occurred in a previous study after significant decreases in biomass (Rodríguez-Rodríguez et al., 2010), but the original ergosterol level was not recovered.

The substantial loss of biomass that occurred in P-I was compensated by replacing part of the polluted wood with fresh inoculated wood chips to the E-RDB. The same procedure was performed in the C-RDB, but in this case, replacing the contaminated wood with fresh wood chips. Subsequently, the rotation frequency was reduced to 1.5 turns every 24 hours (6 times lower than in P-I) during the next 93 days of P-II (186 days in total). As shown in Table 5.3, the ergosterol level was successfully maintained during the first 78 days of P-II. This result showed evidence that static conditions favored the preservation and growth of the fungal biomass. By reducing the turning frequency to 24 h, the non-submerged biomass fraction probably had a longer recovery period from micropollutant exposure and submersion that benefited fungal immobilization and growth.

In the 186-day sample, a significant drop in ergosterol level was detected. However, this decrease was attributed to an incident in the control system on day 181, which resulted in a decrease of the water pH from 4.5 to 3.3 for 24 h. It is well known that a too acidic pH inhibits enzymatic production and fungal growth (Mir-Tutusaus et al., 2018a). After this period, this technical failure was solved and the pH was reset to 4.5. In P-III, the HRT was increased from 3 to 5 days, thus keeping the water longer in the reactor. In this case, a remarkable fungal recolonization of the wood was achieved after only 44 days of P-III (225 days in total). This rapid recolonization was probably the result of the consolidation of the established fungal community (Section 5.3.2.2) as a result of operating in more static conditions, both due to the lower rotation frequency and the longer residence time of the AW in the reactor.

Table 5.3. Fungal biomass presented on the wood chips, in absolute and relative terms, throughout the treatment.

Period	Time (days)	Ergosterol amount ± SD (mg g wood DW^{-1})	Remaining biomass ± SD (%)
I	0	0.26 ± 0.02	100
I	48	0.12 ± 0.02	44 ± 6
I	78	0.05 ± 0.01	20 ± 2
I	93	0.05 ± 0.01	21 ± 1
II	93	0.15 ± 0.01	56 ± 4
II	123	0.12 ± 0.01	45 ± 2
II	141	0.13 ± 0.02	50 ± 8
II	171	0.15 ± 0.01	59 ± 0
II	186	0.09 ± 0.01	36 ± 1
III	186	0.09 ± 0.01	36 ± 1
III	225	0.25 ± 0.03	97 ± 13

* Results are shown as mean values and corresponding standard deviations for triplicate measurements.

Variations in the fungal biomass content throughout the treatment detected via ergosterol analysis were also visualized by SEM. As shown in Fig. 5.1 (a) and (b), wood chips were densely colonized by *T. versicolor* mycelial biomass under sterile conditions. Afterwards, the colonized wood was used to set up an RDB under non-sterile environment in the operational conditions of P-I. As demonstrated by the ergosterol results, the fungal mycelial density was considerably reduced after 93 days of treatment (Fig. 5.1 (c)). However, a similar extent of colonization was recovered after partially renewing the biomass in the reactor and implementing the modifications of the operational conditions in P-II and P-III (Fig. 5.1 (d)).

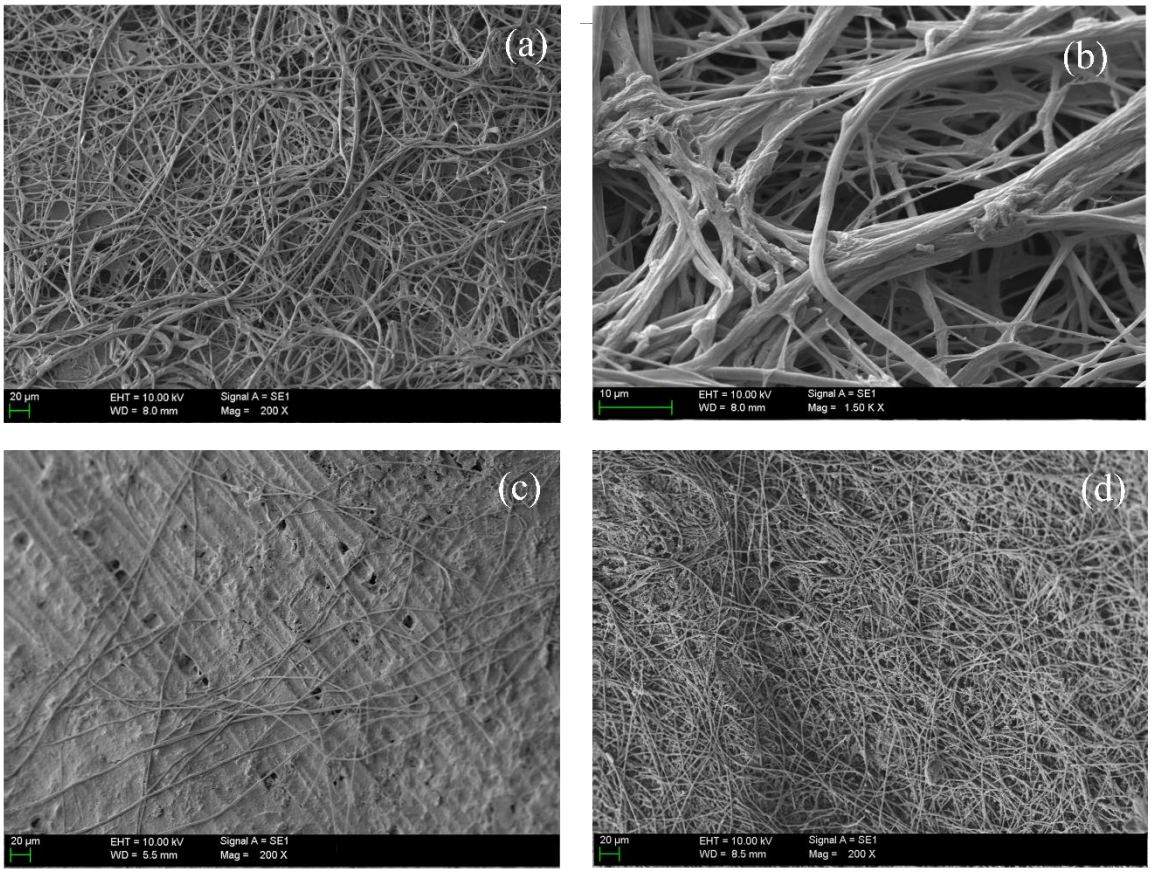


Fig. 5.1. SEM images of initial colonized wood for magnifications of 200 X (a) and 1500 X (b), after 93 days for 200 X (c) and 230 days for 200 X (d).

5.3.2.2. Fungal community assemblage

The fungal community dynamic of both reactors and the behavior and persistence of *T. versicolor* after inoculation was evaluated by DGGE analysis and prominent band sequencing, which were conducted by the Environmental Microbiology Group of the Autonomous University of Barcelona. For this reason, this thesis only includes a short presentation of the main conclusions obtained in this study. In brief, studies of the lignocellulosic support and effluent showed higher fungal diversity in the C-RDB than in the E-RDB. This suggests that inoculation of *T. versicolor* in the E-RDB contributed to a more stable, less diverse community of fungal species, as it dominated the competition for space and nutrients (Annex B).

In order to assess the presence of *T. versicolor* throughout the experiment, prominent bands of the gels were sequenced, allowing the calculation of the relative abundance of each phylotype present in solid and liquid samples from both reactors. In the wooden substrate from the E-RDB, the relative abundance of *Trametes* sp. decreased to 30 % at the end of P-II, but from this point onwards it remained constant (Table B.1, Annexes). This suggests that the reactor reached a stable state where *Trametes* sp. coexisted with two more evenly distributed fungal phylotypes: *Dipodascus* sp. and *Rhodotorula* sp. Both genera have been described in biodegradation studies (De Hoog and Smith, 2011; Dusengemungu et al., 2020), and *Rhodotorula* sp. was also detected in another reactor inoculated with *T. versicolor* (del Álamo et al., 2022). Regarding the C-RDB, a clear dominance of *Penicillium* sp. was detected in the early stages, but up to 8 different phylotypes were detected at the end of the treatment, with no evidence of having reached a stable fungal community.

In the effluent, *T. versicolor* was detected at all stages of the E-RDB, whereas it was absent in the C-RDB (Table B.2, Annexes). However, *Trametes* relative abundance decreased in the E-RDB effluent as the experiment progressed. This result, together with the results obtained in the wooden matrix analysis, suggests that the inoculated fungus was consolidated over time in the E-RDB, remaining attached to the lignocellulosic matrix and thus reducing its washout from the system. As the experiment progressed, *Dipodascus* sp. was established as the dominant phylotype in the effluent of the E-RDB, representing up to 90 % of the fungal diversity at the end of the experiment (De Hoog and Smith, 2011; Dusengemungu et al., 2020; Kawai and Hu, 2009). Therefore, the present study demonstrates the ability of *T. versicolor* immobilized on wood to survive under non-sterile conditions during long-term treatments. Although the relative abundance of this fungus was reduced, a stable consortium capable of maintaining its degradation capacity over time was assembled.

5.3.2.3. Evolution of bacterial counts

The HPC results are presented as the logarithm of CFU per mL in Fig. 5.2. Bacterial counts remained at a lower level in the E-RDB compared to the C-RDB throughout the entire treatment, especially during P-II and P-III. Therefore, bacterial contamination was successfully controlled in the E-RDB, indicating that the immobilization on *Q. ilex* wood can be an appropriate strategy to limit bacterial growth. In other systems where glucose is used instead of wood for fungal

maintenance, higher bacterial growths have been frequently reported (Hu et al., 2021; Mir-Tutusaus et al., 2017).

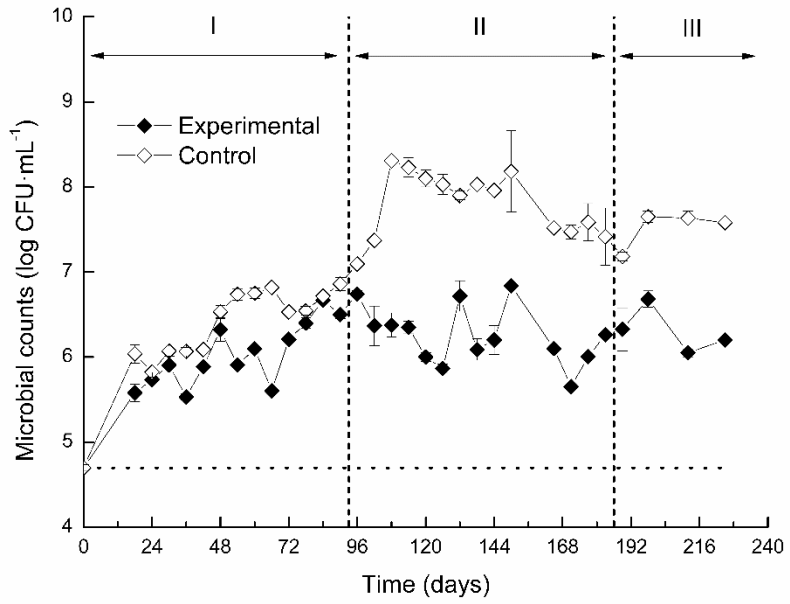


Fig. 5.2. HPCs in the AW throughout the continuous treatment in the RDB. White and dark dots are the HPCs of the effluent streams in the control and experimental reactors, respectively. Vertical dashed lines divide each operating period (I: Period I, II: Period II and III: Period III), and the horizontal dotted line is the HPC level of the wastewater effluent. Results are shown as mean values and corresponding standard deviations for duplicate measurements.

5.3.2.4. Pesticide removal

The AW was collected from the sampling point SW10 presented by Postigo et al. (2021). However, the existing pesticides were found at very low concentrations, undetectable by HPLC-UV. For this reason, two of the pesticides that had been reported at this sampling point, diuron and bentazon, were selected as model compounds and were spiked to the real matrix of AW at a detectable concentration of 10 ppm. Moreover, both pesticides have been associated with potential health problems and have been considered of particular concern according to the Directive 2008/105/EC on Environmental Quality Standards of the EC (EC, 2008). In fact, the use of diuron is not approved in most EU Member Countries according to EC 1107/2009 (EC, 2009; Lewis et al., 2016).

Time-course profiles of diuron and bentazon concentrations are presented in Fig. 5.3. However, to facilitate interpretation of the results, pesticide concentrations were converted into relative removals and grouped into 15-day periods in Fig. 5.4. As can be seen, the elimination efficiencies achieved in the E-RDB were clearly higher than those in the C-RDB for both pesticides throughout the treatment, demonstrating the favorable contribution of *T. versicolor* to the removal capacity of the system, reaching removal peaks of 54 % diuron and 48 % bentazon.

The average removal of diuron was clearly higher than that of bentazon throughout treatment in both the E-RDB and C-RDB. This phenomenon was partly related to the different sorption capacity of *Q. ilex* for each pesticide. Diuron has proven to have a higher affinity for *Q. ilex* than bentazon, which was related to the different hydrophobic properties of the compounds (Beltrán-Flores et al., 2021, Topic 4.2 of Chapter 4). Thus, wood not only serves as immobilization support and a substrate for fungus but can also play a significant role in pesticide removal by sorption. However, the removal in the C-RDB cannot be attributed exclusively to sorption, as other fungi detected in the microbial community (such as *Dipodascus* sp. and *Rhodotorula* sp., Section 5.3.2.2) have been described in bioremediation studies (De Hoog and Smith, 2011; Dusengemungu et al., 2020).

A partial renovation of the colonized wood was required to recover the biomass washed out of the reactor and the treatment capacity of the system from 93 days onwards (Section 5.3.2.1). In this regard, the highest removal efficiencies were achieved at the beginning of P-I and P-II, when fresh (colonized) wood was added to the system. Changes in operating conditions enhanced biomass retention, and as a result, the E-RDB maintained significant treatment capacity for a longer period (225 days). In contrast, the C-RDB even showed bentazon desorption from 60-75 days onwards in P-I, and from 165-180 days onwards in P-II. Therefore, inoculation of *T. versicolor* improved treatment efficiency while extending the useful operational period of the system.

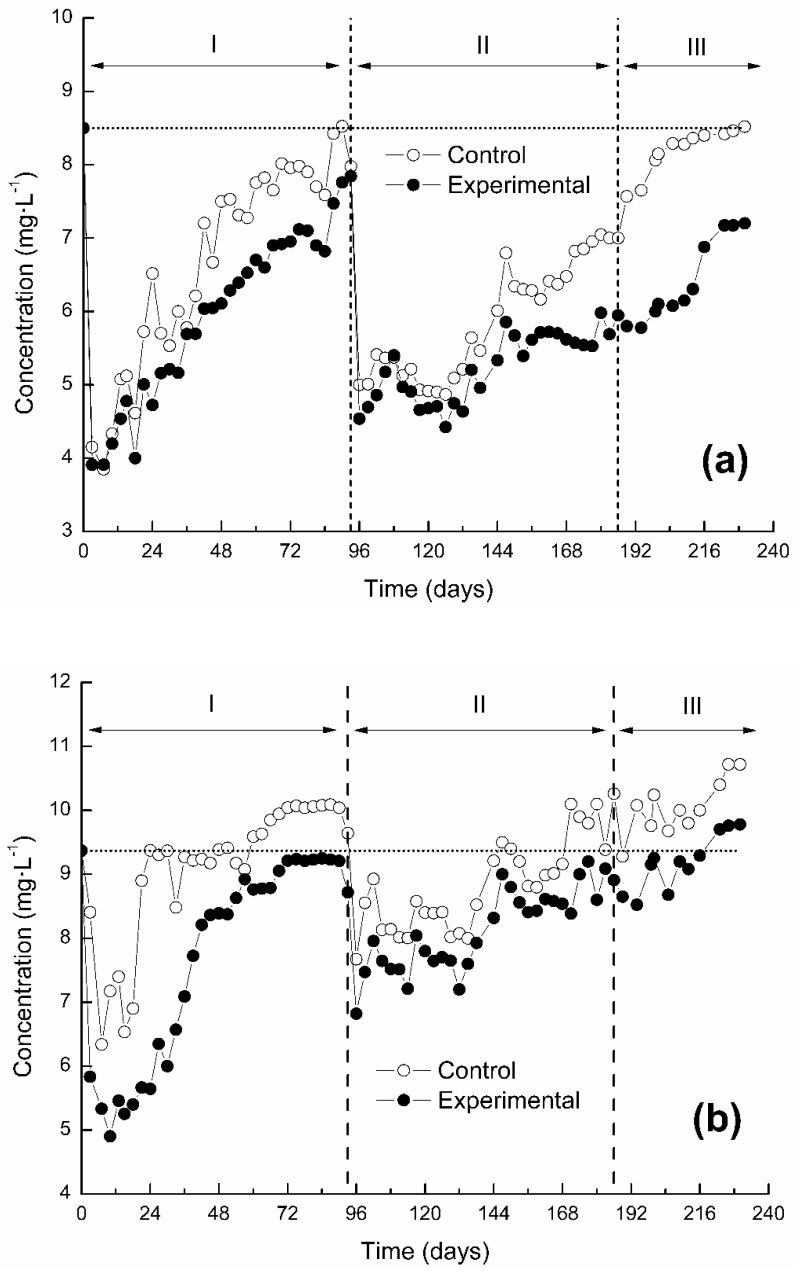


Fig. 5.3. Concentration profile of diuron (a) and bentazon (b) during the continuous treatment in the RDB. White and dark dots are the concentration values of the control and experimental reactors, respectively. Vertical dashed lines divide each operating period (I: Period I, II: Period II and III: Period III), and horizontal dotted lines are the influent concentrations.

Of particular interest is the maintenance of diuron removal capacity in the E-RDB in P-II and P-III, being considerably more stable over time than in P-I. In contrast to P-I, where E-RDB and C-RDB removals seemed to follow similar trends, a divergence was observed in the last two periods. This phenomenon was mainly attributed to the higher amount of fungal biomass immobilized on the wood in the E-RDB as a consequence of the reduction of the reactor rotation frequency in P-II and the increase of the HRT from 3 to 5 days in P-III. In P-I, the E-RDB achieved up to 12.6 % more diuron removal than the C-RDB (days 45-60). In P-II, biomass maintenance was consolidated and increased diuron removal by up to 13.9 % (days 165-180). Finally, increasing HRT prolonged treatment time, resulting in an improvement of up to 25.1 % (days 195-210) diuron removal in the E-RDB compared to the C-RDB.

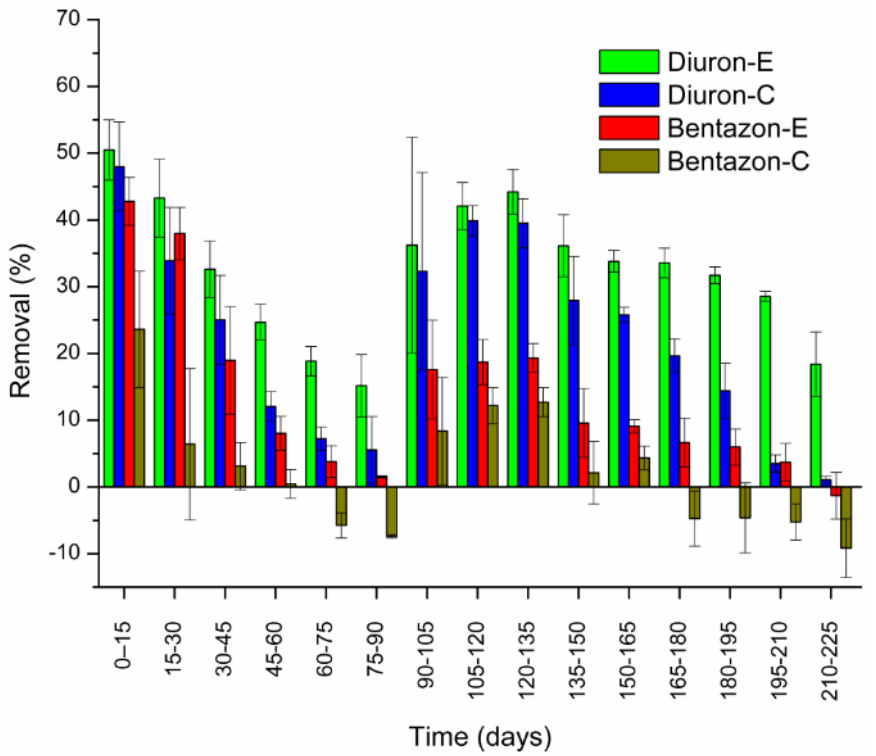


Fig. 5.4. Time-course profiles of diuron and bentazon removals grouped in 15-day periods obtained in the E-RDB and the C-RDB. The error bars represent the standard deviations for the specified periods.

In the case of bentazon, its removal could be influenced by the presence of laccase, which was only detected at the beginning of P-I and P-II, when fresh biomass was introduced into the reactor (Fig. 5.5). Recently, laccase has been reported to be involved in bentazon biodegradation (García-Vara et al., 2021), while there is no clear consensus for diuron (Coelho-Moreira et al., 2018; Hu et al., 2020b). Pinheiro et al. (2020) recently reported that laccase production by *T. versicolor* was favored under static conditions. However, in the present study, the activity of this enzyme remained low when working under more static conditions (in P-II and P-III). Nevertheless, the lack of laccase does not necessarily entail fungal inactivation, as *T. versicolor* possesses an intracellular enzyme system capable of degrading these types of compounds (Mir-Tutusaus et al., 2017).

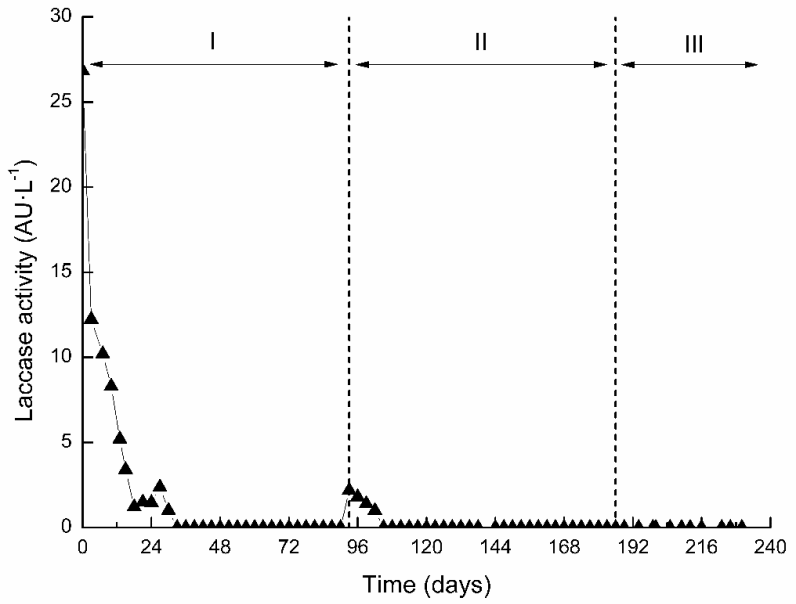


Fig. 5.5. Time-course profile of laccase activity during the continuous treatment. Vertical dashed lines divide each operating period (I: Period I, II: Period II and III: Period III).

5.3.2.5. COD and color

The initial COD of the AW increased considerably from 27 and 44 mg O_2 L^{-1} in P-I and P-II & III, respectively, to approximately 3000 mg O_2 L^{-1} when diuron and bentazon were spiked at 10 ppm. This increase was mainly attributed to the use of acetonitrile as a solvent for the stock solutions of each pesticide (10 mg mL^{-1} of stock solution) since its theoretical contribution to the COD of the AW (2451 mg O_2 L^{-1} of AW) is much higher than that of diuron

(13 mg O_2 L^{-1} of AW) and bentazon (15 mg O_2 L^{-1} of AW). However, as shown in Fig. 5.6, COD was rapidly reduced by about half in the first days of treatment in both reactors, indicating that acetonitrile was easily removed. However, after this drastic decrease, the COD values of both reactors were still considerably higher than the COD of the original wastewater. This difference was also associated with the extraction of some soluble compounds from the wood: initially, a large amount of these extracts was released (which was deduced from the intense color of the AW in the early stage), but as the treatment progressed, their contribution to COD became less important (Lacorte et al., 2003). In P-III, the COD level rose slightly due to the increase in HRT from 3 to 5 days.

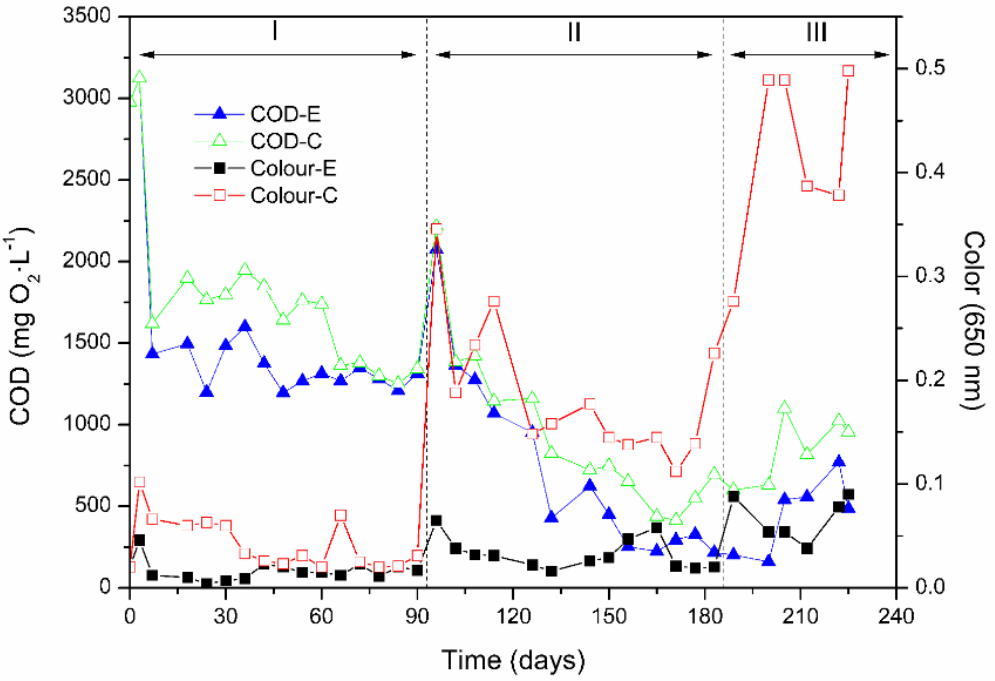


Fig. 5.6. COD and color profiles of the AW during the continuous treatment in the RDB. Blue and green triangles are the COD concentrations of the effluent streams in the E-RDB and C-RDB, respectively, while the black and red squares are the color levels of the effluent streams in the E-RDB and C-RDB, respectively. Vertical dashed lines divide each operating period (I: Period I, II: Period II and III: Period III).

Contradictory results have been previously reported regarding the COD reduction capacity of *T. versicolor* (Cruz-Morató et al., 2013a; Souza et al., 2014). In the present study, a better performance was observed in the E-RDB compared to the C-RDB, suggesting that *T. versicolor* along with the microbial consortium contributed to reducing COD. In this regard, although COD

remained above the original levels (27 and 44 mg O_2 L^{-1}) throughout the treatment, the E-RDB showed considerably lower COD than the C-RDB, with average removals of 14, 23 and 51 % in P-I, P-II and P-III, respectively.

Similar results were obtained concerning color analysis. A considerable increase in color was detected when the wastewater came in contact with the wood owing to the extraction of some soluble compounds that give the characteristic dark color to *Q. ilex* wood. As shown in Fig. 5.6, the addition of fresh wood in P-II and the increase of HRT in P-III led to more intense colors in the C-RDB, while remaining approximately constant in the E-RDB. Thus, the E-RDB showed higher decolorization over time, reaching average eliminations of 62 % in P-I, 82 % in P-II and 84 % in P-III compared to the C-RDB. Therefore, the E-RDB proved to be efficient in terms of COD and color removal.

5.3.2.6. Variation of fiber content in wood

A fiber analysis was conducted to assess the wood consumption by the fungal biomass. The chemical composition of each fiber type is given in Table 5.4. For a better interpretation of the results, the ash fraction was considered inert and used as a basis to calculate the relative content of each fiber with respect to the initial wood content (t = 0), thus obtaining the percentage reduction of each component.

Table 5.4. Comparison of fiber composition between the wood chips from the E-RDB after 225 days of treatment and the original wood.

Components	Composition (% DW^{-1})		Reduction (%)
	E-RDB (t = 0 days)	E-RDB (t = 225 days)	
NDF (%)	84.34 ± 0.25	83.60 ± 0.01	-
ADF (%)	60.51 ± 0.03	60.62 ± 0.11	-
ADL (%)	15.53 ± 0.31	13.13 ± 0.08	-
Hemicellulose	23.83 ± 0.28	22.98 ± 0.12	8.55 ± 0.10
Cellulose	44.98 ± 0.34	47.49 ± 0.19	-0.12 ± 0.01
Lignin	11.68 ± 0.32	9.07 ± 0.09	26.36 ± 0.72
Ash	3.85 ± 0.01	4.06 ± 0.01	0
Wood mass	-	-	5.17 ± 0.01

* Results are shown as mean values and corresponding standard deviations for triplicate measurements.

As shown in Table 5.4, the degradation capacity of the fungal consortium changes depending on the fiber type. Interestingly, the most highly degraded compound was lignin. Unlike cellulose and hemicellulose, which are relatively easily biodegradable polysaccharides used as substrates in the primary metabolism of multiple fungi and other microorganisms, lignin is an extremely recalcitrant compound and only seems to be biodegradable through secondary metabolism by the ligninolytic system of some fungi. Since lignin oxidation results in no energy gain, the main objective of ligninolytic fungi in secreting lignocellulosic enzymes (primally lignin peroxidase, manganese peroxidase and laccase) is to access the internal polysaccharides of the wood (Pointing, 2001). These results are in line with the analysis of fungal communities presented in Section 5.3.2.2, as *Trametes, Rhodotorula* and *Dipodascus* are ligninolytic fungi (Hainal et al., 2012; Parveen et al., 2022).

This analysis also showed a slight loss of wood mass during the treatment. This result supports the hypothesis that the increase in COD was caused by the release of soluble organic matter from wood. However, since wood decomposition was relatively low, substrate replacement was found to be unnecessary to ensure the survival of *T. versicolor*. Therefore, fresh wood would only be required to restore the sorption capacity of the system.

5.3.3. Solid-phase treatment in a biopile system

In treatments involving sorption, not only the removal performance in the liquid phase but also the fate of the polluted sorbent should be studied. Thus, additional remediation approaches should be explored to reduce the pesticide content in the old colonized wood. Accordingly, a solid-phase treatment was performed in a biopile-like system by inoculating *T. versicolor* onto the old (colonized) wood. Samples were withdrawn from the E-RDB (old colonized wood) and C-RDB (old wood) after 93 days of treatment (at the end of P-I), to be subsequently treated in the biopile for up to 48 days. In this regard, three different strategies were assessed: wood inoculation with *T. versicolor* (C-I), no re-inoculation (E-N) and re-inoculation of colonized wood (E-R). Extraction recoveries of 86.1 % and 86.8 % were obtained for diuron and bentazon, therefore factors of 1.16 and 1.15 were applied to correct data deviation, respectively.

Although a similar experiment was performed in a recent study (Beltrán-Flores et al., 2021, Topic 4.2 of Chapter 4), the old colonized wood had been subjected to a treatment period of only 20 days and the biopile treatment period had been limited to 27 days. The use of more realistic operational periods both in the RDB (93 days), which in turn change the characteristics of the wood and therefore the post-treatment performance, and in the biopile (48 days), can provide more reliable results and useful information for further full-scale application.

Fig. 5.7 shows the results of the biopile experiment after 27 and 48 days of treatment. As expected, higher residual concentrations were detected for diuron, which as a more hydrophobic compound tends to be sorbed to a greater extent by wood. Interestingly, higher initial concentrations were found in the colonized wood than in the wood without fungus. This fact evidenced a significant sorption contribution of the fungal biofilm to the overall removal, which has been previously reported (Hu et al., 2020a).

A mass balance of the total amount of both pesticides introduced into the reactor showed that the experimental sorbed amount was found to be considerably lower, 0.207 mg g wood DW^{-1} for diuron and 0.010 mg g wood DW^{-1} for bentazon, compared to those reported in the above-mentioned study, being of up to 0.680 mg g wood DW^{-1} for diuron and 0.177 mg g wood DW^{-1} for bentazon (Beltrán-Flores et al., 2021, Section 4.2.3.1 of Chapter 4). These results suggest that the wood lost some of its sorption capacity when subjected to long-term treatment, probably as a result of the consumption and degradation of this substrate by the fungal consortium.

Fig. 5.7 shows that the relative elimination of bentazon is higher than that of diuron throughout the treatment. For example, for E-I and 48 days, relative removals were 95 % for bentazon and 45 % for diuron. However, the biodegradation kinetics of both pesticides were also estimated to compare the treatment efficiency with the literature. Table 5.5 shows the biodegradation kinetic constants and rates for both pesticides, which were found to be clearly higher for bentazon. It is feasible to assume that bentazon, being a more polar molecule, has a lower diffusion capacity inside the wood, and thereby higher bioavailability to interact with the microbial consortium.

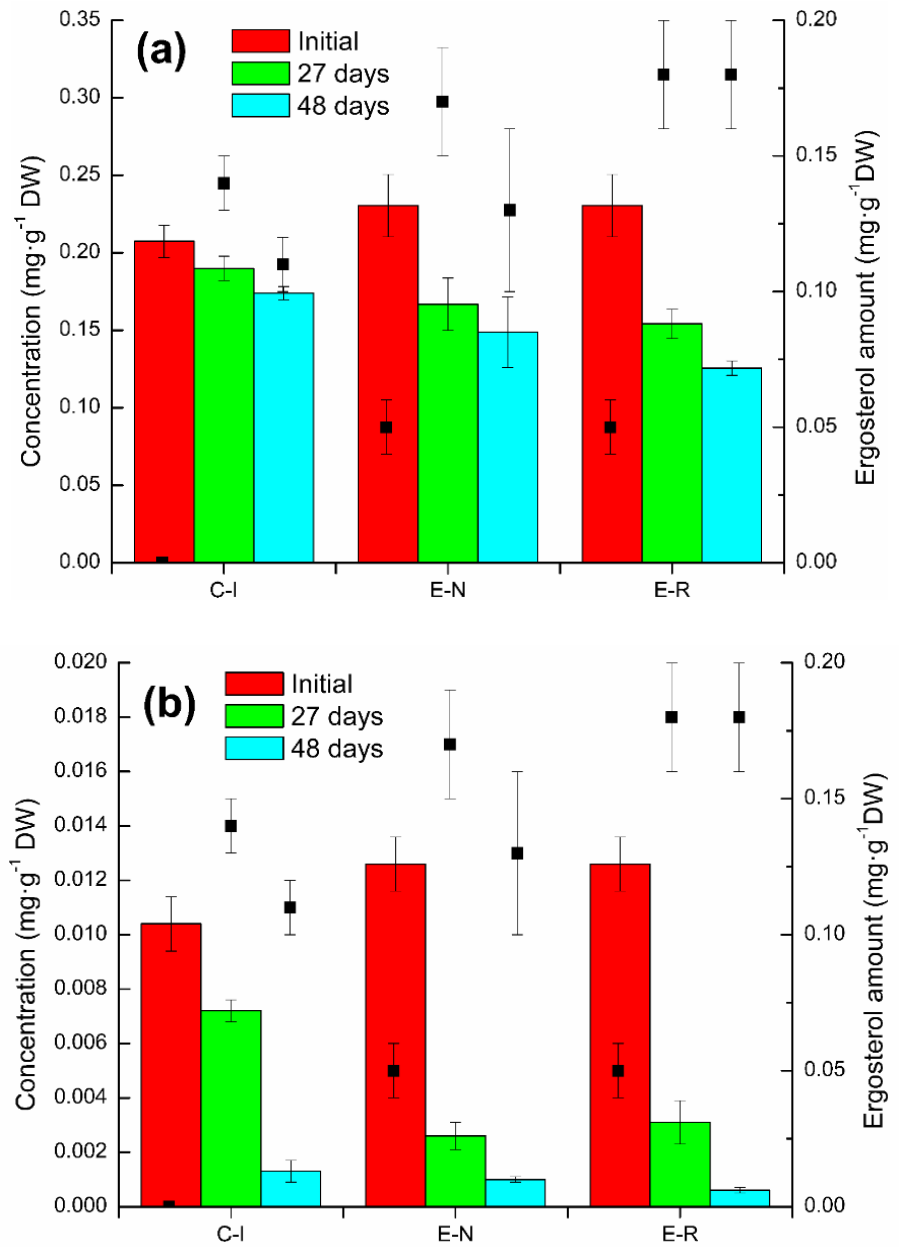


Fig. 5.7. Evolution of residual concentration of diuron (a) and bentazon (b), and ergosterol content in the biowastes during the biopile treatment. Average residual concentrations are represented with color bars after 0 (blue), 27 (green) and 48 (yellow) days of treatment and their error bars indicate the standard deviation (n = 2). Black squares are the average ergosterol level and their error bars show the standard deviation (n = 3). Biopile conditions: C-I corresponds with wood inoculated; E-N with biowastes without re-inoculation; E-R with biowastes with re-inoculation.

Table 5.5 also shows that E-R was the most efficient strategy in terms of degradation rate, followed by E-N and C-I. However, the degradation performance of the E-R was only slightly higher than that achieved by the E-N, which could question the viability of re-inoculation. Similar results of diuron degradation rates were reported in another solid-phase biopile treatment with *T. versicolor* (Beltrán-Flores et al., 2021, Section 4.2.3.7 of Chapter 4). Regarding bentazon, the removal efficiency was lower compared to that obtained in Section 4.2.3.7, but the wood had been previously subjected to a much shorter period of treatment, 20 days instead of 93 days used in the present study. This result may be due to the fact that older wood may constitute a worse substrate for the fungus, thus depressing its metabolic activity in the biopile treatment. In any case, the application of the E-R was justified considering ergosterol content, being more stable than in the E-N, which showed a significant decrease after 48 days (Fig. 5.7). The partial decomposition of the wood along with the presence of pre-grown fungus probably benefited the adaptation and growth of the fresh inoculum. Thus, these results indicate that the combination of both fungal treatments, the E-RDB process followed by the biopile system with re-inoculation strategy, provided the best results in terms of fungal content and pesticide degradation. This finding is consistent with that obtained in section 4.2.3.7, where the best strategy was found to be the combination of the column inoculated with *T. versicolor* and the re-inoculated biopile.

Table 5.5. Biodegradation rates and first-order kinetic constants of diuron and bentazon removals from old (colonized) wood by the microbial consortium using different inoculation strategies in a solid-phase biopile treatment.

	Biopile conditions		
	Inoculated (C-I)	Non-re-inoculated (E-N)	Re-inoculated (E-R)
Pesticide: Diuron			
Degradation rate (mg g wood DW^{-1} day^{-1}) x 10^3	0.70 ± 0.03	1.73 ± 0.42	2.22 ± 0.41
R^2	1.00	0.94	0.97
k x 10^2 ($days^{-1}$)	0.37 ± 0.03	0.93 ± 0.18	1.28 ± 0.15
R^2	0.99	0.96	0.99
Pesticide: Bentazon			
Degradation rate (mg g wood DW^{-1} day^{-1}) x 10^3	0.19 ± 0.05	0.25 ± 0.08	0.26 ± 0.07
R^2	0.94	0.90	0.94
k x 10^2 ($days^{-1}$)	4.19 ± 1.92	5.30 ± 0.37	6.29 ± 0.74
R^2	0.86	1.00	0.99

* Results are shown as mean values and corresponding standard deviations for duplicate measurements.

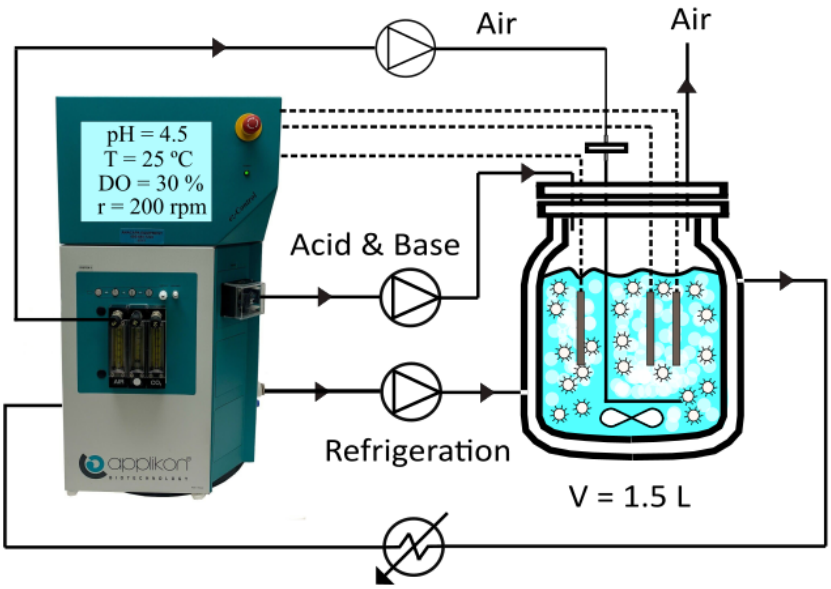
These results suggest that *T. versicolor*-based biopile can be a useful tool for the bioremediation of polluted substrate, thus allowing its reuse as immobilization support. This technology would reduce substrate requirements in full-scale applications, while maintaining remarkable removal efficiencies and biomass growths. Wood could also be acquired as a by-product or waste from timber industries in the case of full-scale application, making the process even more sustainable, especially compared to other materials commonly used for fungal immobilization such as polyurethane foam (Mir-Tutusaus et al., 2018a).

5.4. Conclusions

AW was successfully treated by *T. versicolor* immobilized on *Q. ilex* wood chips in a rotating drum bioreactor during a long-term process (> 7 months). Inoculation of *T. versicolor* on *Q. ilex* wood chips and adjustment of the operational parameters led to the consolidation of a fungal consortium in the reactor, composed of *T. versicolor*, *Dipodascus* sp. and *Rhodotorula* sp., with enhanced degradative capacity. Decreasing rotation and increasing HRT improved total biomass retention and thus, pesticide removal over a longer operational period. Significant COD and color removals were also obtained compared to the control reactor. Afterwards, solid by-products exposed to the long-term process were successfully treated by *T. versicolor* in a biopile-like system. The combination of the inoculated RDB with the re-inoculated biopile showed the best results in terms of fungal biomass and pesticide degradation. Therefore, unlike previous studies, biomass was maintained, high biodegradation (not only sorption) was achieved, contaminated by-products were successfully treated after a long period of exposure, and in addition, a detailed microbial study was included confirming fungal consolidation in the reactor. These results demonstrated that the use of the appropriate substrate, treatment system and operating conditions allow fungal technology to be implemented under non-sterile conditions during long-term processes, thus being ready for full-scale applications.

Chapter 6

Assessing the effect of the limiting oxygen level on pollutants degradation and the capacity of organic matter reduction by fungi



6.1. Introduction

Micropollutants in the aquatic environment have become a worldwide issue of rising environmental concern. These compounds constitute a diverse and expanding group of anthropogenic and natural pollutants that include pharmaceuticals, personal care products, steroid hormones, industrial chemicals, pesticides and flame retardants, among others (Luo et al., 2014). Even though these xenobiotics are generally detected at low concentrations, many of them raise significant toxicological concerns in both wildlife and human beings (Leong et al., 2020; Liu et al., 2020). Therefore, remediation techniques must be developed and applied to remove these toxic compounds from the environment.

The most established micropollutant treatments are physical-chemical, as they are considered relatively fast, simple and effective methods against micropollutants. However, these techniques have important limitations, such as the formation of residues in sorption, fouling in membrane technology, and high operational costs and the generation of TPs in advanced oxidation processes (Benner et al., 2013). In contrast, bioremediation is considered a sustainable and environmentally friendly technology that, despite possible technical difficulties related to biomass maintenance, presents increasing evidence of its potential application in micropollutant abatement. Particularly, WRF are basidiomycetes known for their ability to degrade a wide range of micropollutants through their powerful enzyme system, which is composed of extracellular enzymes, such as laccase, and the intracellular enzyme system known as cytochrome P450 (Zhuo and Fan, 2021). Among WRF, *T. versicolor* is considered one of the best candidates because of its excellent degradation efficiency for many xenobiotics (Mir-Tutusaus et al., 2018a).

Like other WRF, *T. versicolor* is an aerobic fungus whose growth and metabolic activity have traditionally been considered to be affected by the DO concentration in the medium (Maqbool et al., 2016; Rau et al., 2009). However, some investigations have shown that this fungus can operate under anaerobic conditions, e.g. in the conversion of xylose to ethanol (Okamoto et al., 2014), or even degrade recalcitrant compounds such as industrial dyes (Chen and Yien Ting, 2015). In any case, no study has quantitatively determined the lower level at which a WRF (e.g.

T. versicolor) is oxygen limited in liquid media. The DO content is an essential parameter since it might lead to significant energy consumption and, consequently, excessive operational cost.

Another key parameter in wastewater treatment is COD. WRF generally degrade recalcitrant micropollutants via co-metabolism, thus requiring an easily assimilated carbon supply for maintenance. In addition, micropollutant concentrations are too low to support metabolic requirements (Badia-Fabregat et al., 2015). Nevertheless, some WRF, such as *Pleurotus ostreatus* and *Phanerochaete chrysosporium*, have been reported to successfully assimilate intrinsic COD from high organic load wastewaters without the addition of supplementary nutrients (Fountoulakis et al., 2002; Lu et al., 2009). In contrast, *T. versicolor* has shown a doubtful ability to reduce COD (Badia-Fabregat et al., 2017; Torán et al., 2017), although there is still scarce evidence and many information gaps to investigate.

Therefore, the aim of this study was to determine the DO concentration at which *T. versicolor* was oxygen-limited in terms of pollutant degradation, where bentazon and tributyl phosphate (TBP) were used as target compounds. Two different types of chemicals, a pesticide (bentazon) and a flame retardant (TBP), were chosen to cross-sectionally study the effect of DO on the degradation of micropollutants. Bentazon was chosen as the target pesticide because it is rapidly degraded by *T. versicolor* and has a low sorption effect (García-Vara et al., 2021). Besides, bentazon has been associated with potential health problems and is considered of particular concern according to the Directive 2008/105/EC on Environmental Quality Standards of the EC (EC, 2008). Meanwhile, TBP has been shown to pose toxicological risks in several organisms (Lin, 2009; Yan et al., 2020). The study regarding the effect of the DO concentration on pollutant degradation was also used to evaluate whether two well-established reactors in fungal bioremediation, an FBR and an RDB, worked under non-limiting conditions. In addition, this study analyses *T. versicolor* kinetic growth and capacity to remove organic matter (in terms of COD) under sterile conditions.

6.2. Materials and methods

6.2.1. Agricultural wastewater

The characteristics of the AW used are shown in Table 6.1.

Table 6.1. Physicochemical characterization of the AW. Values are means ± standard deviation for triplicate samples.

Parameter	Value
pH	8.06 ± 0.02
Conductivity (mS cm^{-1})	3.17 ± 0.01
Color at 650 nm	0.10 ± 0.01
Chloride (mg Cl L^{-1})	653.7 ± 6.1
Sulphate (mg S L^{-1})	86.3 ± 1.6
Nitrite (mg N L^{-1})	0.4 ± 0.1
Nitrate (mg N L^{-1})	0
Ammonia (mg N L^{-1})	0
TSS (mg L^{-1})	16 ± 2
COD (mg O_2 L^{-1})	44 ± 4
DOC (mg C L^{-1})	16 ± 1
Heterotrophic plate count (CFU mL^{-1})	$3.50 \cdot 10^4 \pm 1.50 \cdot 10^4$

6.2.2. Stirred tank bioreactor: biodegradation

A total of 1.5 L of maintenance medium (Chapter 3, Section 3.4.1) was sterilized at 120 ºC for 30 minutes. During operation, glucose and ammonium tartrate were supplied at the consumption rate, approximately 1 g L^{-1} d^{-1} and 7 mg $L^{-1}d^{-1}$ (2 mg g pellets DW^{-1} d^{-1}), respectively. The glucose and ammonium tartrate stock solutions were 0.3 g mL^{-1} and 3.2 mg mL^{-1}, respectively. The pH was set to 4.5 and controlled using 1 M HCl and NaOH. The temperature was fixed at 25 ºC. DO levels were set at 5, 10, 15 and 30 %, depending on the experiment, so that the aeration rate was automatically adjusted to maintain these percentages, keeping a constant agitation of 200 rpm.

A total of 80 g L^{-1} wet weight of pellets (equivalent to 3.5 g DW L^{-1}) were inoculated into the reactor. In each cycle, a pulse of 10 ppm bentazon or 5 ppm TBP from their respective stock solutions (10 mg mL^{-1} in methanol) was injected into the bioreactor every 24 or 48 hours, respectively. A total of 3 cycles were performed for each DO level, which means a total injection of 30 ppm bentazon and 15 ppm TBP. Separate experiments were conducted for each DO level, i.e., with fresh medium and biomass. Samples of 2 mL withdrawn from the bioreactor were

centrifuged at 15.000 g for 1 min to separate the supernatant from the pellets. The resulting supernatant was used to measure glucose, laccase and pesticide concentration.

6.2.3. Stirred tank bioreactor: sOUR estimation

The specific oxygen uptake rate (sOUR) measures the rate at which oxygen is consumed by a microorganism/consortium and is defined as the mass of oxygen consumed per unit of fungal biomass and time (g O_2 g biomass DW^{-1} day^{-1}). For this purpose, 1 L of maintenance medium (without micropollutants) was saturated above 50 % DO, when aeration was stopped. The sOUR was obtained by dividing the value of the slope of the linear regression of the DO concentrations over time by the biomass amount. The operating conditions were the same as in Section 6.2.2: the medium was previously autoclaved, pH was controlled at 4.5, agitation was maintained constant at 200 rpm and 3.5 g L^{-1} DW pellets were added.

6.2.4. Stirred tank bioreactor: organic matter removal

Regarding the study of the organic matter removal, the consumption rate of glucose was compared with that of another more complex source of COD, sodium acetate. For this purpose, 1 L of maintenance medium was treated but replacing glucose with an equivalent amount of sodium acetate in terms of COD (2 g O_2 L^{-1}). Thus, a pulse of acetate was added in the medium from a stock solution of 0.2 g mL^{-1}. Once the acetate was almost consumed, a new acetate pulse was injected, obtaining two complete cycles. In addition, a control experiment with glucose was conducted. In these cases, the DO level was maintained at 30 % and the medium was not spiked with any micropollutant. Samples of 2 mL withdrawn from the bioreactor were centrifuged at 15.000 g for 1 min to separate the supernatant from the pellets. The resulting supernatant was used to measure glucose or acetate. The operating conditions were the same as in Sections 6.2.2 and 6.2.3: the medium was previously autoclaved, pH was controlled at 4.5, agitation was maintained constant at 200 rpm and 3.5 g L^{-1} DW pellets were added.

6.2.5. Growth kinetics of *T. versicolor* on holm oak wood

The fungus was inoculated onto the wood inside a polyvinyl chloride box as described in Chapter 3, section 3.4.4. During the growing period, solid samples were taken to measure the ergosterol content (Chapter 3, Section 3.6.6).

6.2.6. Fixed bed reactor

An FBR was assembled with 250 g DW of inoculated wood. The reactor was fed with an upward stream of AW that was initially adjusted to pH 4.5. The flow rates were fixed at 12, 23 and 40 mL min^{-1}. DO measurements were performed by inserting the oxygen probe through a series of holes, plugged with septums, which were made in the column. Thus, the DO was measured at 0, 7 (P-1), 19 (P-2), 34 (P-3) and 49 (P-4) cm height (Fig. 6.1) using an OXROB10 robust oxygen probe coupled to a FireSting-PRO (4 channels) fiber-optical multi-analyte meter (Pyroscience, Germany). In this case, the sOUR was calculated for each reactor section according to Eq. (6.1) and Eq. (6.2).

$$sOUR = \frac{[DO_E - DO_S] \cdot Q}{M_{biomass} \cdot 1000} \qquad (6.1)$$

$$M_{biomass} = \frac{X_{ergo}}{R_{E-B}} \cdot M_{wood} \qquad (6.2)$$

where DO_E and DO_S are the DO concentrations at the inlet and outlet of each section (mg O_2 L^{-1}), respectively, Q the flow rate (L day^{-1}), $M_{biomass}$ the fungal biomass of each section (g DW), X_{ergo} the ergosterol content in the wood (mg ergosterol g wood DW^{-1}), R_{E-B} is the correlation between ergosterol mass and fungal biomass of *T. versicolor* determined by Rodríguez-Rodríguez et al. (2010), and corresponds to 6.61 (mg ergosterol g biomass DW^{-1}), and M_{wood} the wood mass in each section (g).

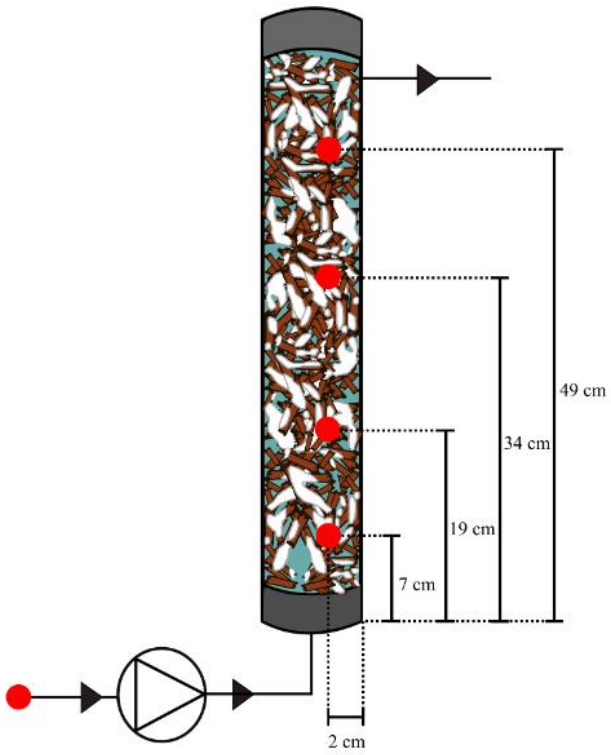


Fig. 6.1. Representation of the FBR and DO measurement points

6.2.7. Rotating drum bioreactor

DO concentrations were analyzed in an RDB previously reported in the literature (Beltrán-Flores et al., 2022, Chapter 5). Accordingly, the feed tank was stirred during the entire operation, from which water was pumped to one side of the reactor. The water left the reactor from the other side by overflow. An external recirculation loop (4.7 L day^{-1}) was required for pH adjustment, which was performed in a recirculation tank (≈ 0.4 L). The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. A total of 545 g DW of inoculated wood was introduced into the inner tube, which was rotated one and a half turns every 24 hours. Aeration was initially provided through a diffuser located in the reactor until reaching oxygen saturation. Afterwards, aeration was turned off, and the dynamic profile of DO uptake was monitored until equilibrium, which was reached after 30 h of operation. The oxygen level was measured using the same equipment as in Section 6.2.6 at 3 different points of the reactor: inlet, intermediate, and outlet; and at two depths: 4 and 6 cm (reactor dimensions are length: 51 cm x radius 7 cm), as shown in Fig. 6.2.

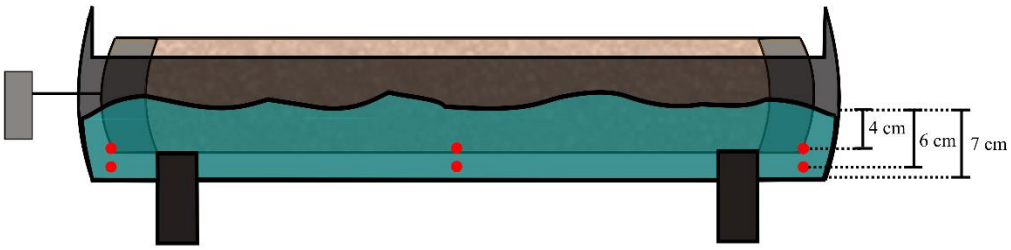


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Fig. 6.2. Representation of the RDB and DO measurement points

6.2.8. Acetate measurement

The samples were filtered prior measurement with 0.22 µm pore-size Millipore Millex-GV PVDF filters and, when required, diluted with ultrapure water to have concentration between the calibration ranges. Acetate was measured by HPLC (Ultimate 3000, Dionex Integrion HPLC, USA) calibrated to measure volatile fatty acids (VFA) in the range of 20 to 1000 mg L^{-1} (acetate, propionate, formate, isobutyrate, valeric, butyrate and iso-valeric). The separation was performed in an ICSep ICE-CPREGEL 87H3 column (height: 150 mm, diameter: 7.8 mm, Transgenomic, Omaha, NE, USA) at 30 ºC working with a sulphuric acid (6 mM H_2SO_4) as mobile phase pumped at a constant flow rate of 0.5 mL min^{-1}. The HPLC was equipped with a refractive index detector (Waters 2410) with an IR of 1024 and an autosampler (Ultimate 3000 Autosampler). Under these conditions, the retention time was approximately 18.31 min.

6.3. Results and discussion

6.3.1. Stirred tank bioreactor

6.3.1.1. Effect of dissolved oxygen on the biodegradation of two model micropollutants.

Experiments were conducted at different DO levels in order to evaluate the effect of oxygen on the fungus degradation ability. The DO set-point was fixed between 5 and 30 % saturation. This operational range was established based on the fact that most WWTPs work above 25 % saturation, which is considered a non-oxygen-limited region (Du et al., 2018). Hence, a saturation of 30 % DO was initially selected to work under non-oxygen limiting conditions, at

which *T. versicolor* was supposed to maintain its intrinsic degradation capacity intact, while lower DO levels of 15 %, 10 % and 5 % were chosen to assess the effect of oxygen limitation on degradation activity.

The relative remaining concentrations of the pollutants are shown in Fig. 6.3. *T. versicolor* was only able to completely eliminate both micropollutants in all cycles studied when working at 30 % DO. This result indicates that the maximum performance of the fungus was accomplished at the highest DO level. However, the degradation efficiencies obtained when working at 30 % and 15 % DO were clearly better than those achieved under more restricted oxygen conditions. In this regard, Leisola et al. (1983) and Li et al. (2016) demonstrated that the presence of oxygen was a critical factor in the removal of lignin and carbamazepine respectively by the WRF *P. chrysosporium*. Nevertheless, *T. versicolor* was able to partially degrade both compounds, bentazon and TBP, even at a very low DO level of 5 %, which is in line with other research, in which the fungus remained metabolically active in anaerobic environments (Chen and Yien Ting, 2015), e.g. being able to bioconvert xylose to ethanol without oxygen (Okamoto et al., 2014).

This finding was further analyzed by studying the biodegradation kinetic constants of bentazon and TBP, as shown in Table 6.2. According to these results, the fungus was found to be in an oxygen-limiting region when the DO decreased below 15 %, i.e., 10 % and 5 %. In this region, the degradation capacity was dramatically reduced by up to 84 %. In contrast, it cannot be clearly concluded whether the fungus is oxygen-limited when the DO was restricted to 15 % since the biodegradation rate was only moderately reduced for bentazon and remained high for TBP. At 30 % DO, *T. versicolor* was even able to completely remove bentazon at 10 ppm after 6 h and TBP at 5 ppm after 32 h.

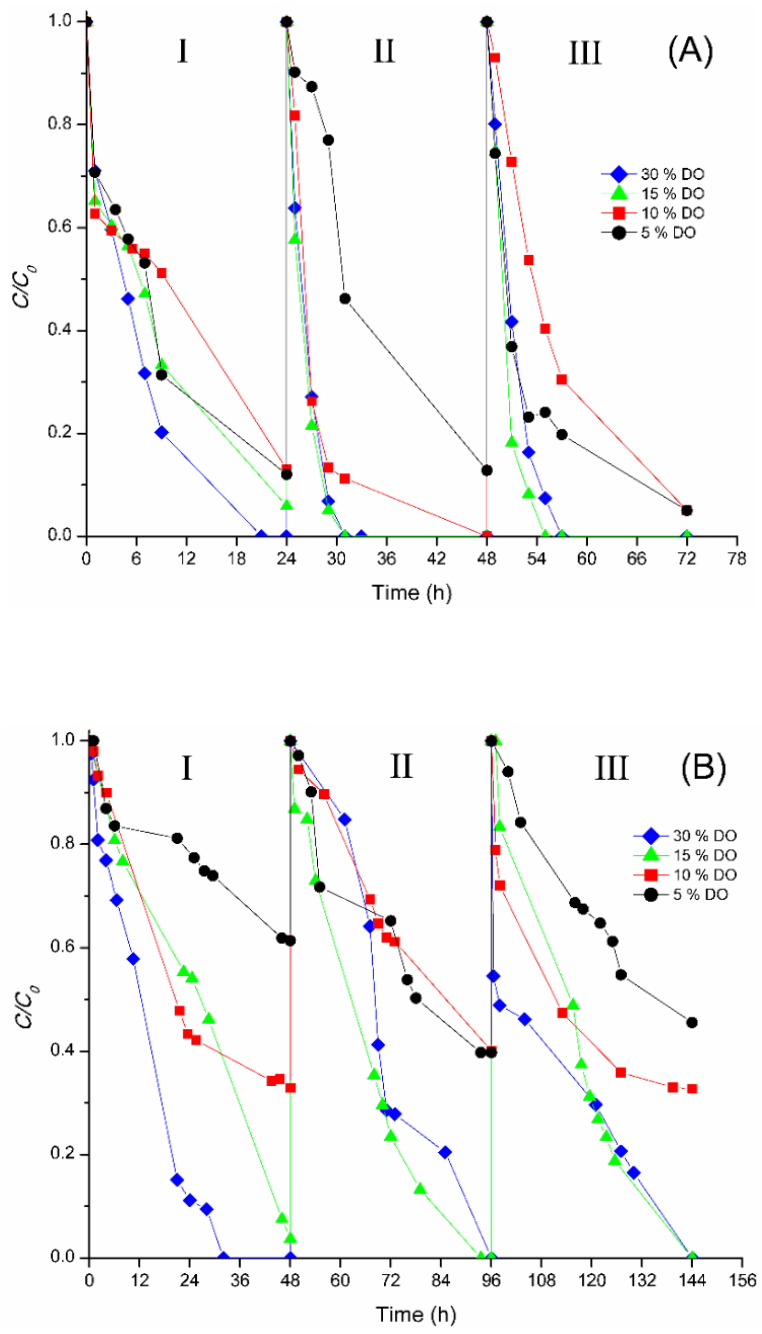


Fig. 6.3. (A) Bentazon concentration profiles for three batches of 24 h and (B) TBP concentration profiles for three batches of 48 h at different DO levels: blue rhombuses are 30 % DO, green triangles are 15 % DO, red squares are 10 % and black dots are 5 %. Initial concentrations are 10 ppm bentazon and 5 ppm TBP.

Table 6.2. First-order kinetic constants of bentazon and TBP biodegradation at four different DO levels (30, 15, 10 and 5 %) and three cycles. Average kinetic constants and degradation decline were calculated from the regression of the point cloud of all cycles.

	k (h^{-1})							
	Bentazon				TBP			
DO (%) Cycle	30	15	10	5	30	15	10	5
1	-0.287	-0.119	-0.086	-0.095	-0.125	-0.099	-0.026	-0.010
2	-0.397	-0.355	-0.296	-0.086	-0.077	-0.106	-0.019	-0.020
3	-0.348	-0.397	-0.126	-0.144	-0.082	-0.051	-0.027	-0.017
Average (point cloud)	-0.335 (R^2 = 0.914)	-0.288 (R^2 = 0.789)	-0.164 (R^2 = 0.767)	-0.109 (R^2 = 0.798)	-0.096 (R^2 = 0.753)	-0.096 (R^2 = 0.847)	-0.025 (R^2 = 0.898)	-0.015 (R^2 = 0.790)
Degradation decline (%)	0	14.0	51.04	67.5	0	0	74.0	84.4

Furthermore, laccase activity was analyzed as an indicator of fungal activity. This extracellular enzyme was only monitored for TBP since a recent study has shown that, although laccase is slightly involved in bentazon degradation, the main mechanism of degradation is via cytochrome P450 (García-Vara et al., 2021). Fig. 6.4 shows the evolution of laccase activity for each DO value. Enzyme activity reached maximum values at 30 % DO, while it was substantially limited as DO decreased, especially below 15 %. These results show that the enzymatic activity of *T. versicolor*, and thus its degradation potential, is inhibited when working near anoxic conditions. In this regard, Pinheiro et al. (2020) evaluated laccase production by *T. versicolor* in three different reactors, detecting higher activity values when aeration was incorporated into the system. Therefore, laccase production was found to be correlated to DO concentration during the process, which is in good agreement with the results of the present study. Additionally, a higher DO concentration may not only lead to an increase in laccase production by *T. versicolor* but could also enhance degradation efficiency, as oxygen is an essential electron acceptor in certain laccase-mediated oxidation reactions (Ortner et al., 2015).

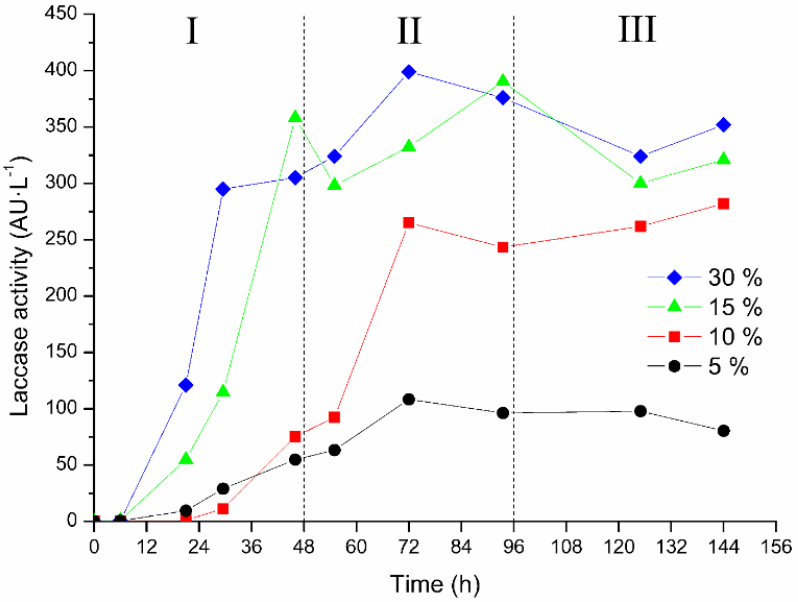


Fig. 6.4. Time course profile of laccase activity for TBP degradation at different DO concentrations: blue rhombuses at 30 % DO, green triangles at 15 % DO, red squares at 10 % and black dots at 5 %.

Bettin et al. (2020) also reported maximum laccase activity working at 30 % DO for another WRF, *Pleurotus sajor-caju* PS-2001. When the DO concentration was above 30 % DO, laccase

production was reduced while favoring biomass growth. In the present study, biomass growth was limited by the use of a maintenance medium (2 g L^{-1} of glucose) instead of a growth medium (8 g L^{-1} of glucose), remaining in all cases below 4 g DW L^{-1} of fungal biomass at the end of the experiments (initial biomass concentration was 3.5 ± 0.4 g DW L^{-1}).

Therefore, the DO level was found to be a critical variable to control and, according to these results, at least 15 % DO is desirable to remain the maximum degradative potential of *T. versicolor*. Aeration represents the largest contribution to the costs of most WWTPs (Rosso et al., 2008), thus reducing the oxygen content from 30-25 % (typically used in WWTP) to 15 % can result in large energy savings. This is the first study that quantitatively determine the DO level at which *T. versicolor*, as an example of WRF, is oxygen-limited in a bioremediation process.

6.3.1.2. Specific oxygen uptake rate estimation

A fundamental parameter for the design of any aerobic bioprocess is the sOUR. The sOUR can be estimated from the oxygen uptake of the fungus in a specific period. This study requires working under non-oxygen limiting conditions. In this case, the DO level was set at approximately 52 %. Afterwards, the aeration of the system was turned off, obtaining a constant decreasing trend of DO concentrations. From the slope of its linear regression, the oxygen consumption was found to be 2.9952 % min^{-1} (Fig. 6.5). Taking into account that the oxygen saturation (100 % DO) is 8.3 mg O_2 L^{-1} (at 25 ºC) and that the inoculated biomass was 3.5 g DW L^{-1} (at 25 ºC), a sOUR of 0.1023 g O_2 g biomass DW^{-1} day^{-1} was obtained. This consumption was predictably lower than that (1.39-2.05 g O_2 g $biomass^{-1}$ day^{-1}) obtained by Thiruchelvam and Ramsay (2007) since in the latter case a growth medium was used instead of a maintenance medium.

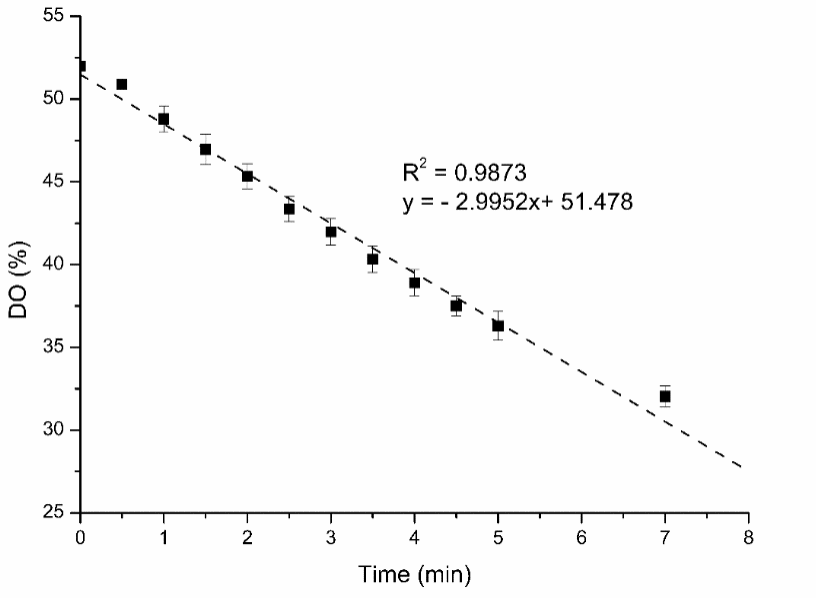


Fig. 6.5. Oxygen uptake rate for *T. versicolor*. Dots are mean values and error bars are the standard deviation for triplicate tests.

6.3.1.3. Organic matter removal

WRF degrade micropollutants mainly by co-metabolism, as these fungi require a more abundant carbon source for their maintenance (Font et al., 2003). Carbon sources that have been traditionally used as WRF substrates are glucose, which is an easily assimilable nutrient (Espinosa-Ortiz et al., 2016), straw and wood, which are natural substrates for these fungi (Topic 4.2). These substrates are generally required because the intrinsic organic matter in the water usually consists of complex compounds that are difficult to be assimilated by WRF. In fact, COD reduction from wastewater by WRF is inconsistent and scarcely reported (Palli et al., 2016; Pokhrel and Viraraghavan, 2004). In this section, the ability of *T. versicolor* to reduce COD under sterile and non-oxygen-limited conditions was evaluated. For this purpose, acetate (sodium acetate) was added to the medium as a complex carbon source, which is commonly used in synthetic waters to simulate COD (Yang et al., 2019). In this case, no pesticides or other organic compounds were added to the medium and DO was set at 30 % to operate in the non-oxygen limiting region, preventing these factors from influencing acetate consumption.

Fig. 6.6 shows the results of two degradation experiments using either acetate and glucose (control) as COD sources. As expected, *T. versicolor* was able to completely consume glucose

in a short period, obtaining a decreasing trend that was adjusted to a straight line (R = 0.9953) whose slope was 83.28 mg O_2 L^{-1} h^{-1}. For 3.5 g pellets DW L^{-1}, the glucose consumption rate was 15.86 mg O_2 g pellets DW^{-1} h^{-1}. Interestingly, the fungus was also able to consume an equivalent amount of acetate in terms of COD, although over a considerably longer period (approximately 130 versus 26 h). Once consumed, a second acetate pulse was added to verify whether the fungus was able to maintain its metabolic activity over time in an additional cycle. In the second pulse, *T. versicolor* was also able to almost completely degrade acetate for the same period (130 h). The decreasing acetate trends of both cycles were fitted to straight lines, giving a slope of 16.23 mg O_2 L^{-1} h^{-1} (R = 0.9567) for the first pulse, and 15,91 mg O_2 L^{-1} h^{-1} (R = 0,8953) for the second pulse. For 3.5 g pellets DW L^{-1}, the acetate consumption rates were 3.09 and 3.03 mg O_2 g pellets DW^{-1} h^{-1} for the first and second cycle, respectively. Therefore, the consumption rate of acetate was approximately 5 times lower than that of glucose, which was attributed to the greater complexity of the former compound, but *T. versicolor* was able to assimilate a complex carbon source for 264 h (11 days).

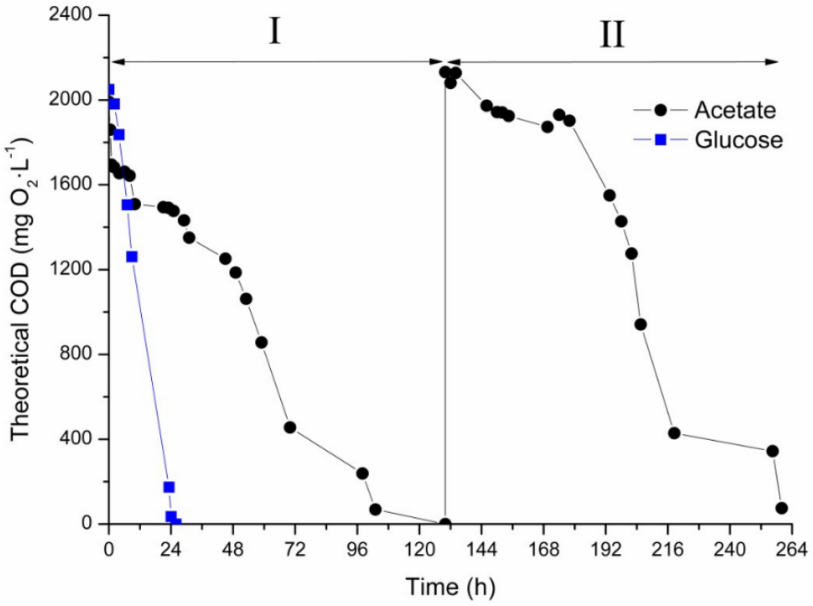


Fig. 6.6. Acetate (black circle) and glucose (blue circle) profiles in terms of COD level over time.

In addition, soluble COD analyses were performed at the beginning and end of each cycle to ascertain whether the acetate was completely mineralized or whether TPs were formed. In the

first cycle, soluble COD was reduced from approximately 2000 to 892 mg O_2 L^{-1}. This result indicates that although *T. versicolor* was able to completely remove acetate, some TPs that contribute to COD were probably generated. Anyway, *T. versicolor* was found to be able to reduce COD by 55 % at the end of the first cycle. After spiking the medium with approximately 2 g L^{-1} of acetate at the beginning of the second cycle, the soluble COD increased to 3072 mg O_2 L^{-1}, which progressively decreased throughout this last cycle to 1480 mg O_2 L^{-1} (52 % removal). These results contrast with other studies that have reported the inability of *T. versicolor* to reduce COD. In this regard, Badia-Fabregat et al. (2017) studied several fungal treatments under non-sterile conditions in continuous operational mode with veterinary hospital wastewater and reverse osmosis concentrate with COD concentrations of 245-264 mg O_2 L^{-1} and 65 mg O_2 L^{-1} respectively. In both cases, significant increases in COD concentration were reported, which were attributed to the possible production of metabolites. Anastasi et al. (2012) examined the treatment of textile wastewater by different fungal strains and concluded that fungal treatment was more effective in decolorization than in COD reduction. In particular, *T. versicolor* was the worst performing strain in terms of COD reduction, considerably raising the initial level. Hu et al. (2021) also reported a considerable increase in COD concentration during the treatment of AW by *T. versicolor* in an FBB. In this case, the COD increase was attributed to the addition of antifoam (Tween 80). By comparing the results of the reported studies with those obtained in the present work, it can be deduced that although the fungus is capable of consuming complex organic matter, hence reducing COD, the efficiency of the treatment can depend on other factors, such as the characteristics of the organic content, the matrix, the operating conditions and the reactor type.

6.3.2. Growth kinetics of *T. versicolor* on *Q. ilex* wood.

Q. ilex wood has proven to be a suitable immobilization support for *T. versicolor* (Topic 4.2 and Chapter 5). In previous chapters, *T. versicolor* had been inoculated on *Q. ilex* wood in a sterile box until apparently complete colonization was achieved. However, even though the wood seems to be completely covered by fungal biomass after approximately 17 days, the fungus can continue to grow in the wood, increasing the density of both external and internal mycelial networks. In this regard, fungal biomass amount is traditionally quantified by ergosterol content, which is a sterol found in the membrane cells of fungi and microalgae (Gutarowska and

Zakowska, 2009). A growth kinetics study can establish a correlation between the cultivation period and the biomass amount.

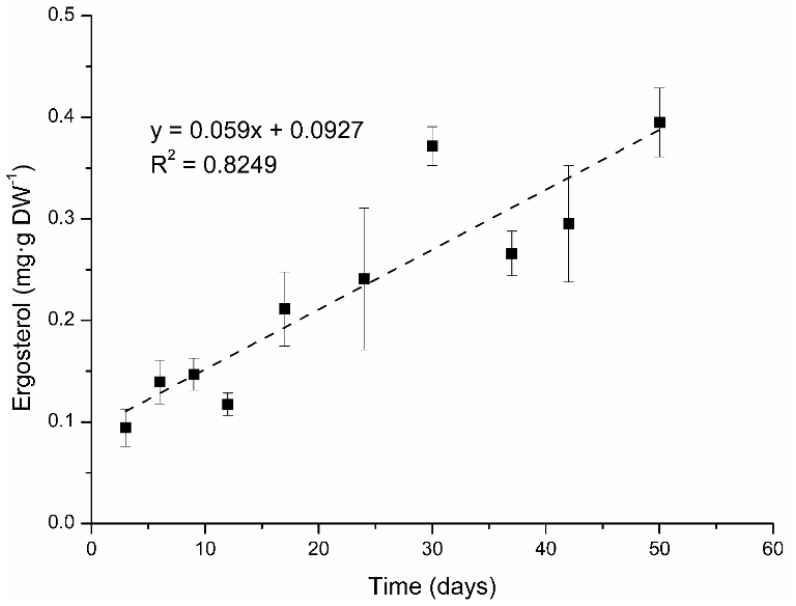


Fig. 6.7. Growth of *T. versicolor* on *Q. ilex* wood expressed in terms of ergosterol content. Dots are mean values and error bars are the standard deviation for triplicate cultures. The dashed line represents the linear regression.

Fig. 6.7 shows the increasing trend of ergosterol content over 50 days. These results indicate that the fungus kept growing after completely covering the visible surface of the wood (first 17 days). Like other microorganisms in substrate-limited environments, fungi often exhibit a growth trend that can be approximated by Monod's equation, which describes a first phase of exponential growth, followed by deceleration (Tišma et al., 2010). Even though single hyphae usually extend at a constant rate, lateral branch hyphae can induce exponential growth of filamentous fungi. Nonetheless, exponential growth in batch cultures can be limited by nutrient depletion or hyphae overgrowth (Gow and Gadd, 1995). In this case, *T. versicolor* maintained constant growth throughout the cultivation period (0.059 mg ergosterol g wood DW^{-1} day^{-1}), without reaching the deceleration phase. Growth at a constant rather than exponential rate may be due to the creation of multiple microcolonies during the inoculation process: exponential growth of hyphae from one microcolony may be limited by encountering hyphae from another microcolony (Ikasari and Mitchell, 2000). However, *T. versicolor* still had enough wood to sustain its growth after 50 days of cultivation. The colonized wood produced in the growth phase

was used in Sections 6.3.3 and 6.3.4. In each case, the wood presented a different biomass content, which was produced according to the cultivation periods described in Fig. 6.7.

6.3.3. Fixed bed reactor

The wood was inoculated with *T. versicolor* in a box under sterile conditions, reaching 0.395 ± 0.034 mg g wood DW^{-1} of ergosterol content after 50 days of culture. Fig. 6.8 shows the DO evolution over time in the FBR working in continuous mode for different flow rates. The results showed that the DO consumption rate of the system varied over time until reaching the equilibrium (maximum). This transition period was attributed to the interaction between water and residual air during the column filling stage. This evolution was hardly observed when working at a flow rate of 40 mL min^{-1} (Fig. 6.8 (C)) as the column was rapidly filled up.

Fig. 6.9 shows two graphs that allow a better interpretation of the results in the steady-state. In this case, the steady-state results in the FBR have been unified to form point cloud plots that allow a better correlation between variables. Fig. 6.9 (A) shows that there is a strong correlation ($R^2 = 0.968$) between the EBCT and the DO level in the reactor. The higher the EBCT, the longer period the water remained inside the reactor, and thus, the more DO was assimilated by *T. versicolor*. This correlation indicates that an FBR with the characteristics described in the present study operated under limiting DO concentrations (i.e., less than 15 % DO, Section 6.3.1.1) from 10.4 min of EBCT onwards. These results show that, even if the influent is fed at 100 % saturation, an FBR may require internal aeration in order to guarantee aerobic conditions for the fungus.

The sOUR can be calculated from DO results, the level of ergosterol in the wood and the amount of wood in each reactor section [Eq. (6.1) and (6.2)]. DO consumption was basically attributed to the activity of *T. versicolor* since the presence of other microorganisms was considered negligible. Different sOURs were obtained for each flow rate and reactor section studied. As shown in Fig. 6.9 (B), an exponential correlation ($R^2 = 0.947$) was found between the DO concentration and the sOURs of the system. Thus, the existence of different sOURs was mainly attributed to the DO level measured in each reactor section. These results contrast with those obtained in the stirred-tank reactor (Section 6.3.1.2). In the latter case, sOUR was considered constant in the non-oxygen limiting region. However, it should be noted that in the stirred-tank

reactor there was a much better oxygen transfer than in the FBR. In the FBR, low DO liquid interfaces or even dead zones were probably created around the fungus with diffusional and oxygen transfer limitations that, as a consequence, restricted oxygen availability to the fungus (Garcia-Ochoa et al., 2010).

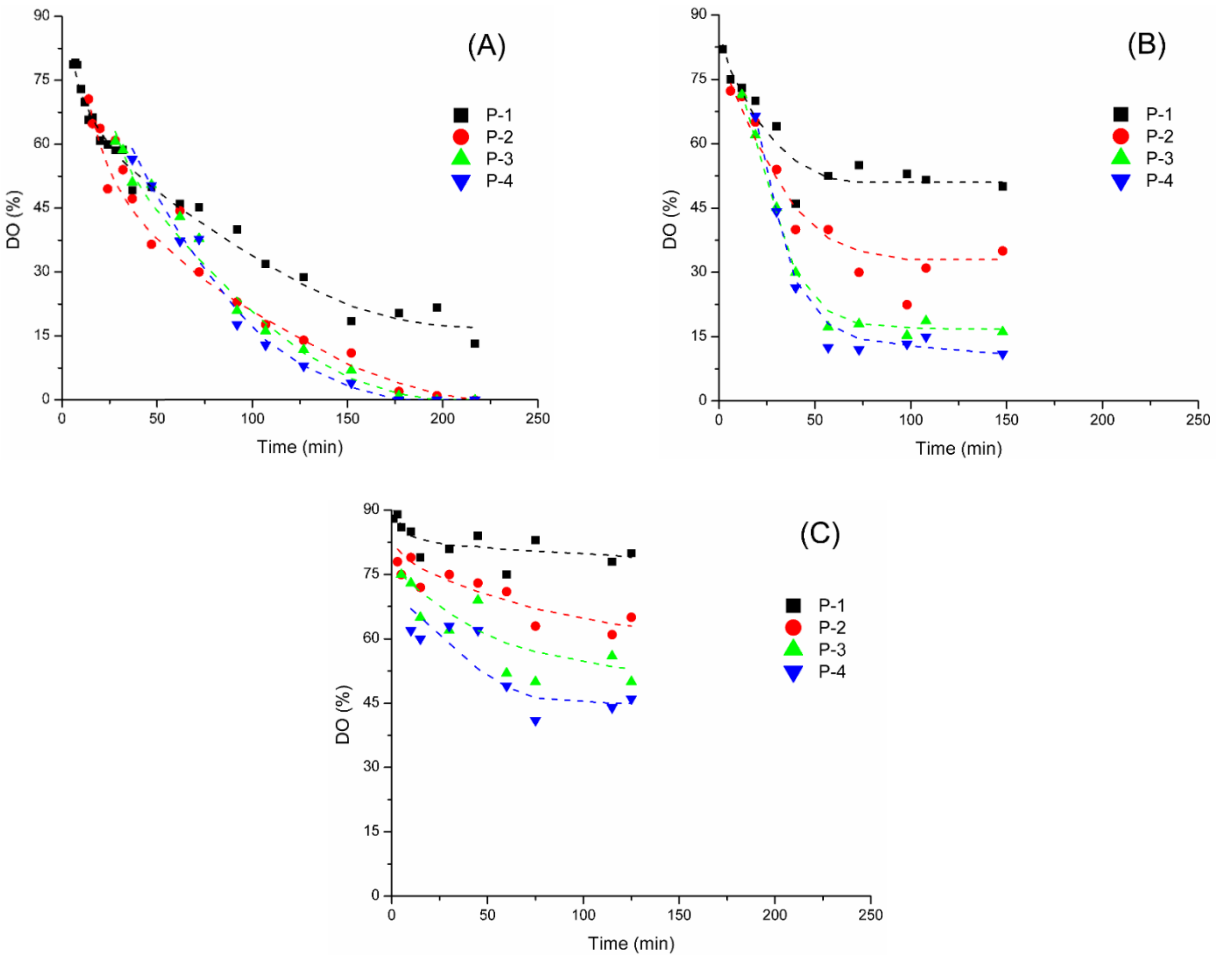


Fig. 6.8. DO depletion profiles in the FBR at flow rates of 12 (a), 23 (b) and 40 (c) mL min^{-1}. The reactor measurement points were located at 7 (P-1), 19 (P-2), 34 (P-3) and 49 (P-4) cm heights.

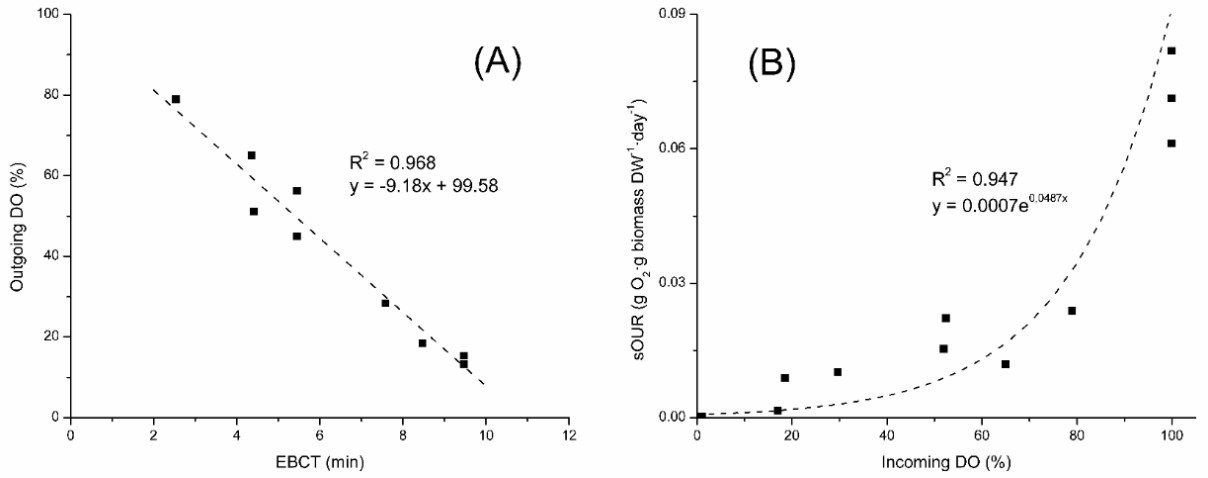


Fig. 6.9. Correlations between outgoing DO and EBCT (a), and between sOUR and incoming DO. Dashed lines are linear (a) and exponential (b) regressions.

6.3.4. Rotating drum bioreactor

The RDB was inoculated with wood colonized by *T. versicolor*, which was previously cultured for 24 days (0.2409 mg ergosterol g wood DW^{-1}). The RDB is a reactor with a rotating mechanism that maintains aerobic conditions using two strategies: alternation of the submerged biomass and aeration of the liquid medium by agitation.

Consequently, high speeds and long periods of rotation promote aeration and thus the DO saturation in the system. In this regard, a constant and relatively high rotational speed (12 rpm) maintained high DO saturation (8-6.5 mg O_2 L^{-1}) in a rotating biological contactor inoculated with *T. versicolor* immobilized on polypropylene discs (del Álamo et al., 2022). In contrast, continuous rotation (6 rpm) has also been reported to produce fungus detachment from wood (Topic 4.1), which is probably attributed to the generation of shear forces that damage the mycelial structure or/and to a better performance of the fungus in this substrate when working under static conditions. Regarding the latter, the study developed in Chapter 5 showed better biomass maintenance when the rotation frequency was changed from 1.5 turns every 4 h to every 24 h. Accordingly, Pinheiro et al. (2020) reported that lower agitation speeds favored the production of laccase in submerged fermentation of *T. versicolor* using agro-industrial wastes as carbon sources, obtaining the best results when working under static conditions. Although operating under more static conditions could favor biomass immobilization on wood and thus increased treatment yield, the lack of agitation may cause the submerged biomass to be oxygen

limited. In this Section, DO was measured at different points of the reactor (at the inlet, middle and outlet) to evaluate whether the fungus was under limiting conditions in the RDB.

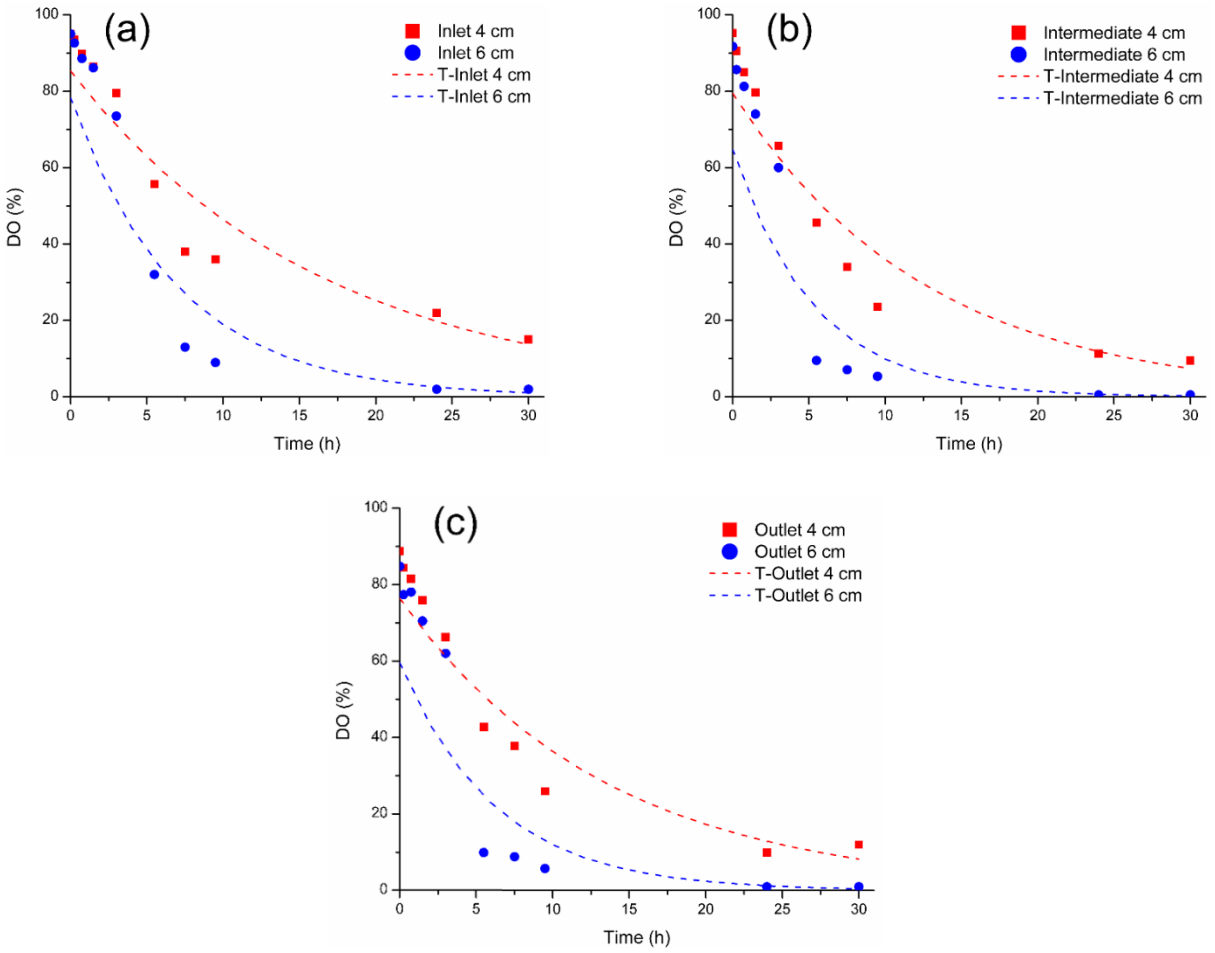


Fig. 6.10. DO depletion profiles measured at two depths (4 and 6 cm) at the inlet (A), intermediate zone (B) and outlet (C) of the RDB. Dashed lines are the curves described by the fitted first-order kinetics (T: theoretical).

DO depletion profiles were fitted to first-order kinetics (Table 6.3). The oxygen limiting conditions (15 %) were reached at all studied points of the RDB before 30 h of operation. According to the kinetics, oxygen limiting conditions were reached for the first time in the RDB (intermediate point, 6 cm depth) after approximately 7.8 h; and at all studied points after 28.5 h. Furthermore, rotation performed after 24 h (1.5 turns) did not result in an appreciable increase in DO concentrations. These results indicate that if the RDB is operated under the same conditions for a period longer than 7.8 h (conservative scenario) without additional aeration, the submerged fungal fraction can be oxygen limited. However, fungal biomass has proven to

remain active and immobilized on wood in the RDB for a long period of operation (Chapter 5). These results indicate that the alternation of the submerged fraction may mitigate the effects of anoxic conditions and contribute to maintaining the metabolic activity of the fungus.

Although DO concentrations were considerably reduced after 30 h in the channel, it remained around 82 % in the recirculation tank. In Topic 4.1 (Chapter 5) the DO concentration was periodically monitored in the recirculation tank of the RDB and it remained above 30 % throughout the treatment. This higher DO level in the recirculation tank compared to the channel may be due to aeration during liquid dripping in the recirculation tank, a larger liquid-air contact surface and stirring of the recirculation tank. Accordingly, measurements were also conducted in the feed tank, obtaining an average value of 34.5 ± 4.1 % DO, but it is feasible to assume that the actual DO concentration at which the effluent entered the reactor was significantly increased by dripping.

Table 6.3. Kinetics of DO consumption in the RDB

	Inlet		Intermediate		Outlet	
Depth (cm)	4	6	4	6	4	6
Kinetic rate (h^{-1})	$6.09 \cdot 10^{-2}$	$1.42 \cdot 10^{-1}$	$7.93 \cdot 10^{-2}$	$1.88 \cdot 10^{-1}$	$7.41 \cdot 10^{-2}$	$1.60 \cdot 10^{-1}$
R^2	0.927	0.910	0.933	0.912	0.922	0.882

DO concentrations varied as a function of depth and axial location, as demonstrated by fitting the data to a first-order kinetics of oxygen depletion (Table 6.3). The consumption curves were approximately fitted to first-order kinetics, from which the kinetic constants were calculated. DO decrease was faster at 6 cm than at 4 cm depth, probably due to worse oxygen diffusion, and at the middle point than at the extremes of the RDB, which was attributed to a lower air-water contact surface.

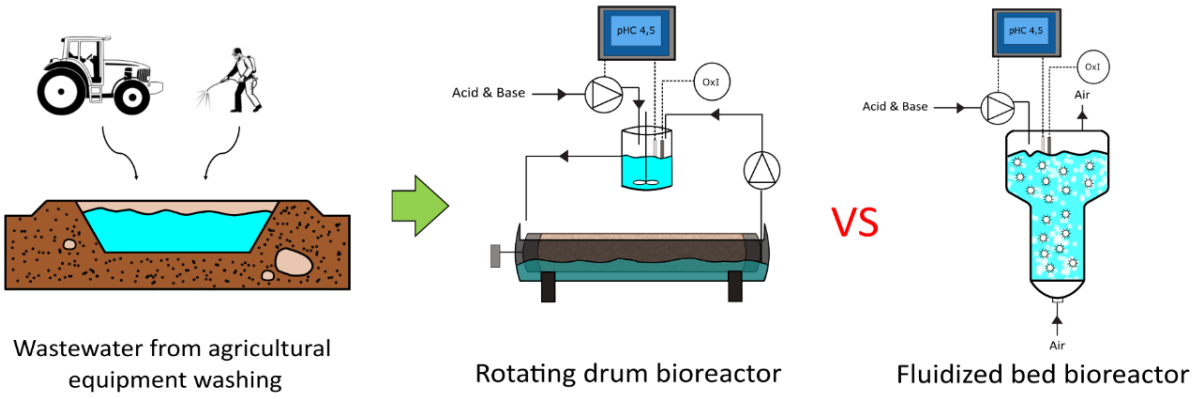
These results indicate that the submerged biomass operates under oxygen limiting conditions from the first 24 h onwards (in some points even earlier), and therefore, the incorporation of an additional aeration system in the RDB would be highly recommended to enhance the treatment performance. This improvement should be explored in future work on the RDB, along with the evaluation of operational costs, prior to its eventual implementation at full-scale.

6.4. Conclusions

T. versicolor was able to completely remove bentazon at 10 ppm after 6 h and TBP at 5 ppm after 32 h under non-limiting oxygen conditions. The limiting DO concentration was found to be 15 % of saturation (1.3 mg O_2 L^{-1}). Based on this result, two well-studied reactors in fungal bioremediation, the FBR and RDB, were found to be oxygen limited for an EBCT longer than 10.4 min and an operational period longer than 7.8 h, respectively. In case these limits are exceeded, aeration should be added to ensure full aerobic conditions. Moreover, *T. versicolor* was able to assimilate acetate as a complex carbon source, without requiring the addition of any supplementary nutrients. This result indicates that *T. versicolor* is not only able to remove micropollutants co-metabolically but also to consume complex organic matter as a substrate, thus decreasing the COD of the broth. Therefore, our findings suggest that bioremediation using *T. versicolor* is a promising strategy to degrade different micropollutants and reduce COD when working in the non-limiting oxygen conditions.

Chapter 7

Fungal treatment of agricultural rinse wastewater comparison between two operational strategies



7.1. Introduction

The main effects of climate change, i.e., growth in atmospheric CO_2 levels, temperature rise and changes in precipitation patterns, have been shown to lead to increased pest activities on crops. Changes in the dynamics of pest influx and crop disease threaten to cause significant agricultural production and economic losses, and compromise global food supply and security (Singh, 2015). In addition, a growing world population and rising living standards require a greater effort from the primary sector to meet global food demand. In this context, the use of pesticides is necessarily unavoidable to ensure pest control and food production worldwide (Popp et al., 2013).

Nevertheless, pesticides are becoming a major global concern owing to their overuse in agricultural activities, environmental persistence, high mobility, bioaccumulation and toxic effects (Sharma et al., 2019). For these reasons, good practices in the agricultural sector should be subjected to clear regulations regarding the correct management and application of pesticides, comprising the prohibition of highly toxic pesticides, the selection of specific pesticides for each type of pest and crop, and the application of reasonable doses. In this respect, particular emphasis should be placed on rinse wastewater (RW), also known as rinsate or agricultural washing wastewater, which is generated on farms when washing agricultural machinery and equipment. Afterwards, RW is usually deposited in collection ponds, avoiding dilution by rainfall and leaks during handling that could contaminate the surrounding soil (EC, 2009c). Finally, accumulated RW should be treated by an *in-situ* process specifically designed for this application before discharge.

Over the last few decades, various physical, chemical and biological technologies have already been implemented for pesticide elimination from wastewater. However, there are several limitations that affect the treatment of pesticides by physical-chemical methods, such as the transfer of these compounds from liquid to solid phase without real degradation (e.g. sorption), high operational costs (e.g. membrane filtration) and the potential formation of TPs (e.g. ozonation) (Ahmed et al., 2017). In contrast, bioremediation is gaining increasing attention in recent decades as an environmentally friendly, efficient and low-cost approach (Marican and Durán-Lara, 2018). In particular, bioremediation using WRF seems to be a very promising

technology for RW treatment. RW is usually produced at relatively low volumes, which is desirable in biological processes using WRF as these microorganisms generally require relatively long HRTs. Furthermore, RW is generated at high concentrations of multiple pesticides, which can be treated by WRF due to their excellent resistance to elevated toxicities. Consequently, WRF do not require prior acclimatization as in other biological processes such as conventional activated sludge. The powerful enzyme system of WRF allows the removal of a wide range of organic compounds, including pesticides, considered recalcitrant in other treatment systems (Mir-Tutusaus et al., 2018a).

Fungal reactors have been extensively studied for wastewater treatment (Espinosa-Ortiz et al., 2016). During the last years, the scientific community seems to have reached a certain consensus on the benefits provided by biomass immobilization in fungal reactors, as it overcomes some of the most common limitations in fungal treatments, including fungal growth on reactor walls and accessories, foam production and biomass washout by decoupling the cellular retention time (CRT) from the hydraulic retention time (HRT) (Mir-Tutusaus et al., 2018a). There are basically two types of fungal immobilization: autoimmobilization as pellets and immobilization on carriers. Carriers can be either inert (e.g. polyurethane foam cubes) or non-inert (e.g. wood) (Zhuo and Fan, 2021).

In this regard, one potentially applicable system for RW treatment is an air-pulsed FBB using *T. versicolor* pellets, which is a well-established fungal reactor that has been successful in treating micropollutants at lab-scale even for a long-term period (Mir-Tutusaus et al., 2019). However, operating an FBB has several operational limitations, such as bacterial contamination (since glucose is added as a carbon source) and foaming (as forced aeration is required) (Mir-Tutusaus et al., 2018a). Alternatively, an RDB with *T. versicolor* immobilized on wood has shown good performance in treating pesticides from AW and its long-term viability has also been previously demonstrated (Beltrán-Flores et al., 2022, Chapter 5). Immobilization on lignocellulosic materials such as wood has some advantages: sustainability, low cost, use of a specific substrate for fungi (limiting bacterial competition) and micropollutant sorption (Hu et al., 2020b; Torán et al., 2017). Furthermore, the design of the RDB has been specifically conceived for *in-situ* application in agricultural fields. However, special emphasis should be placed on the sorption capacity of wood and the development of an effective solid post-treatment

of the old contaminated wood, together with the DOC increase resulting from the release of soluble organic compounds into the water (Beltrán-Flores et al., 2022, Chapter 5).

Therefore, a comparative study between both reactors is required to elucidate whether fungal immobilization strategy, i.e., pellets (auto-immobilization) or immobilization on lignocellulosic material, is more viable for RW treatment. The present work aimed to set up an FBB and an RDB using *T. versicolor* as inoculum for treating RW from pesticide application equipment, and compare their performances and applicability considering different perspectives.

7.2. Materials and methods

7.2.1. Agricultural rinse wastewater

Physicochemical characteristics of the RW are summarized in Table 7.1.

Table 7.1. Physicochemical characterization of the RW. Values are means ± standard deviation for triplicate samples.

Parameter	Values
pH	7.82 ± 0.11
Conductivity (mS cm^{-1})	5.63 ± 0.21
Color at 650 nm	0.17 ± 0.05
Chloride (mg Cl L^{-1})	797.3 ± 2.4
Sulphate (mg S L^{-1})	334.8 ± 4.6
Nitrite (mg N L^{-1})	0.1 ± 0.1
Nitrate (mg N L^{-1})	0.3 ± 0.1
Ammonia (mg N L^{-1})	60 ± 1
TSS (mg L^{-1})	75 ± 19
VSS (mg L^{-1})	48 ± 10
COD (mg O_2 L^{-1})	2088 ± 46
DOC (mg C L^{-1})	707 ± 14
Heterotrophic plate count (CFU mL^{-1})	$3.01 \cdot 10^6 \pm 5.66 \cdot 10^5$
Toxicity (TU)	13.60 ± 1.01

7.2.2. Rotating drum bioreactor

In this case, the reactor was fed in sequential batches for two periods of 17 days and the inner tube was rotated one and a half turns every 24 h. An external recirculation loop (4.7 L day^{-1})

was required for pH adjustment and DO measurement, which were performed in a recirculation tank (≈ 0.4 L). The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. The DO level was monitored using a CyberScan 600 Series Waterproof Handheld (Eutech Instruments). The DO level remained above 30 % in the recirculation tank throughout the treatment. In this case, an inner tube with a larger diameter (radius of 6 cm instead of 4.2 cm) was used, containing a total of 1100 g DW of colonized wood. Liquid samples were taken from the reactor effluent for analyses of pesticide concentration (Annex A), laccase, color, COD, HPCs, toxicity, phytotoxicity and fungal community analysis. Solid samples were withdrawn at the end of the treatment to measure the remaining pesticide content (Chapter 3, Section 3.6.3) and fungal communities (Annex B).

7.2.3. Fluidized bed bioreactor

A total of 2.2 g L^{-1} DW pellets (WW/DW = 23 ± 3 for triplicate measurement) were transferred to an FBB with a useful volume of 1.5 L, but only 1.3 L of RW was added in anticipation of possible foaming. The RW was previously autoclaved at 121 ºC for the experiment under sterile conditions. The reactor was operated in batch mode for 17 days at 25 ºC and pH 4.5. Glucose and NH_4Cl were fed for *T. versicolor* maintenance at a molar C/N ratio of 7.5 (Mir-Tutusaus et al., 2017). The same analyses performed for the liquid samples in the RDB (Section 7.2.2) were replicated for those of the FBB.

7.2.4. Solid-phase treatment in a biopile-like system

A total of 30 g DW of by-products (in triplicate) obtained at the end of the second batch of the RDB were treated under non-sterile conditions and 25 ºC for 27 days. Afterwards, the solid samples were withdrawn to measure the remaining pesticide content (Chapter 3, Section 3.6.3).

7.2.5. Analytical techniques

The phytotoxicity of the RW was analyzed through a seed germination test of tomato (*Solanum lycopersicum*) by exposing 10 seeds to 10 mL of each sample (triplicate) in Petri dishes for 24 hours. Subsequently, each group of 10 seeds was placed on a Whatman Nº1 filter of 70 mm diameter inside Petri dishes for 10 days of incubation at room temperature and exposure to natural light. The filters were pre-humidified with 3 mL distillate water. Relative seed germination (SG), relative root elongation (RE) and germination index (GI) were determined as

described elsewhere (Rodriguez-Rodriguez et al., 2011). The same parameters were measured in distilled water as germination controls.

$$SG = \frac{seeds\ germinated}{seeds\ germinated\ in\ control}\ x\ 100 \qquad (7.1)$$

$$RE = \frac{mean\ root\ length}{mean\ root\ length\ in\ control}\ x\ 100 \qquad (7.2)$$

$$GI = \frac{SG\ x\ RE}{100} \qquad (7.3)$$

Fungal communities were studied following the methodology presented in Annex B. The identification and quantification of the pesticides were performed as shown in Annex A.

7.3. Results and discussion

7.3.1. Rinse wastewater characteristics

The main characteristics of this RW compared to other agricultural and urban wastewater was the high content of organic matter in terms of COD or DOC detected (Table 7.1), and as a consequence of the eventual decomposition of this organic matter, slightly higher levels of ammonium and bacteria counts (Beltrán-Flores et al., 2021, Section 4.2.2.2 of Chapter 4; Metcalf and Eddy, 2003). This study analyses not only the treatment efficiency concerning the removal of pesticides, but also the evolution of other characteristic parameters to determine the effluent quality.

7.3.2. Pesticide identification and quantification

Liquid chromatography coupled to time-of-flight mass spectrometry (LC-qTOF-MS) is a useful technique for qualitative analysis and identification of organic micropollutants (Arsand et al., 2018). High mass resolution along with the acquisition of mass profile data allowed the screening of non-targeted compounds.

Unknown compounds were identified through exact mass analysis. LC-qTOF-MS analysis shows high specificity, allowing the pesticide identification by the characterization of the

monoisotopic mass with a maximum error of ± 2.5 mDa. The detected exact masses were close to those of the following common pesticides: thiacloprid (THIA), chlortoluron (CHLOR), azoxystrobin (AZO) and tebuconazole (TEBU). Moreover, the isotope pattern validated the identification of such pesticides. In this regard, the experimental isotopic patterns match the predicted isotope distributions as shown in Table 7.2.

Table 7.2. Compounds identified in the RW based on HRMS library searching.

Compound	Chemical Formula [M]	Experimental Monoisotopic Mass (Da) $[M+H]^+$	Experimental Isotope ions (R.A.) $[M+H]^+$	Theoretical Isotope ions (R.A.) $[M+H]^+$	Mass error (mDa)*
Thiacloprid	$C_{10}H_9ClN_4S$	253.0305	253.0305 (100.0)	253.0309 (100.0)	-0.4
			254.033 (12.2)	254.0333 (13.1)	-0.4
			255.0276 (38.0)	255.0279 (37.3)	-0.4
			256.0295 (4.5)	256.0303 (4.8)	-0.8
Chlortoluron	$C_{10}H_{13}ClN_2O$	213.0785	213.0785 (100.0)	213.0789 (100.0)	-0.4
			214.0811 (11.0)	214.0819 (11.7)	-0.8
			215.0758 (33.0)	215.0761 (32.8)	-0.4
			216.0784 (3.5)	216.0790 (3.8)	-0.5
Azoxystrobin	$C_{22}H_{17}N_3O_5$	404.1231	404.1234 (100.0)	404.1240 (100.0)	-0.6
			405.1256 (23.5)	405.1272 (25.2)	-1.6
			406.1280 (4.1)	406.1298 (4.0)	-1.8
Tebuconazole	$C_{16}H_{22}ClN_3O$	308.1522	308.1522 (100.0)	308.1524 (100.0)	-0.2
			309.1546 (18.3)	309.1554 (18.7)	-0.8
			310.1493 (33.6)	310.1499 (33.8)	-0.6
			311.1513 (5.5)	311.15267 (6.1)	-1.1

R.A., relative abundance (%).

*Mass error = $(m/z_{experimetal} - m/z_{theoretical})\cdot 1000$

Afterwards, commercial reagents of each pesticide were spiked to the RW to verify that the chromatogram peaks of the standard compounds matched those detected in the original matrix. After confirmatory analysis, initial pesticide concentrations were quantified obtaining a total of 40.55 mg L^{-1} of pesticides, consisting of 19.17 mg L^{-1} THIA, 7.42 mg L^{-1} CHLOR, 4.47 mg L^{-1} AZO and 9.49 mg L^{-1} TEBU. Although these concentrations are relatively high for agricultural water (Köck-Schulmeyer et al., 2019), they are reasonable considering that the RW comes from the washing of agricultural equipment that had been in direct contact with pesticides.

THIA is a neonicotinoid insecticide that acts by disrupting the nervous system of these organisms. This pesticide was included in the watch list of substances in Decision 2015/495/EU (EC, 2015), as it was suspected to pose a high risk to human health as endocrine disrupting, neurotoxic and carcinogenic compounds. Recently, the EC banned THIA by not renewing its license for use in the EU (EC, 2019b). CHLOR is an herbicide belonging to the phenylurea class that acts by the inhibition of photosynthetic electron transport. It is moderately toxic to most aquatic species, birds and worms, although toxicity to mammals is considered low. AZO is a broad-spectrum systemic fungicide that acts on the target organism by inhibiting spore germination and has been recognized to have physiological impact on some aquatic organisms. TEBU is a triazole fungicide that affects the biosynthesis of the phytohormone gibberellin, thus inhibiting seed germination and plant growth. It is classified as a possible carcinogen (rating C) on the U.S. Environmental Protection Agency's Office of Pesticide Programs list of carcinogens. These last three pesticides are currently approved for use in the EU under EC Directive 1107/2009 (EC, 2009), but AZO and TEBU are currently under study. AZO has been included in the watch list of substances in Decision 2022/1307/EC and TEBU was first included in the watch list of substances in Decision 2020/1161/EC, but due to insufficient high-quality monitoring data TEBU has remained on the watch list in Decision 2022/1307/EC (EC, 2022, 2020).

Table 7.3. The physical-chemical properties of the investigated pesticides.

Pesticide	Type	Group	CAS number	Solubility at 20 ºC (mg L^{-1})	Log K_{ow}
Thiacloprid	Insecticide	Neonicotinoid	111988-49-9	185	1.26
Chlortoluron	Herbicide	Phenylurea	15545-48-9	70	2.41
Azoxystrobin	Fungicide	Strobilurin	131860-33-8	6	2.50
Tebuconazole	Fungicide	Triazole	107534-96-3	36	3.70

7.3.3. Comparison between bioreactors

7.3.3.1. Pesticide removal performance

Two different fungal immobilization strategies were used: auto-immobilization (pellets) in the FBB and immobilization on wood chips in the RDB. In the RDB, *Q. ilex* wood chips presented a porosity of 62.4 ± 0.5 %, a WW/DW ratio of 2.5 ± 0.1, and real and apparent densities of 0.27 ± 0.01 g mL^{-1} and 0.89 ± 0.02 g mL^{-1} respectively (Section 4.2.3.1, Chapter 4). The initial ergosterol content was approximately 0.121 ± 0.018 mg g wood DW^{-1}. Considering that 1100 g of colonized wood chips were transferred to the reactor and the ergosterol-biomass correlation for the selected strain is 6.61 mg g biomass DW^{-1} (Rodríguez-Rodríguez et al., 2010), a total of 20 g DW of biomass were introduced to the reactor. However, only 30 % of the biomass was continuously submerged in the reactor, which is 2.2 g biomass DW L^{-1}. Hence, an equivalent pellet inoculum was used in each FBB experiment.

Pesticide removal profiles in the FBB and RDB treatments are shown in Fig. 7.1. The FBB operation was conducted under both sterile (FBB-S) and non-sterile (FBB) conditions to evaluate the effect of microbial contamination on the treatment performance. Although non-sterility can benefit the efficiency of fungal treatment owing to the positive synergies created by the microbial consortium, e.g. by metabolite degradation, non-sterile conditions can also exert pressure on the WRF survival, which in turn can reduce the fungal activity and duration of the process (Mir-Tutusaus et al., 2018a). In this case, the FBB-S was more efficient than the FBB concerning total pesticide removal, reaching 88 and 51 %, respectively. This result confirms that other microorganisms, such as bacteria, can compete for substrate and reduce fungal degradation activity over time (Mir-Tutusaus et al., 2019). However, from a practical point of view, the FBB can be considered a better option than the FBB-S given that sterilization is a costly process that should be avoided at full-scale applications, and negligible bacterial proliferation was observed in the FBB (Section 7.3.3.3.) while maintaining high removal efficiency.

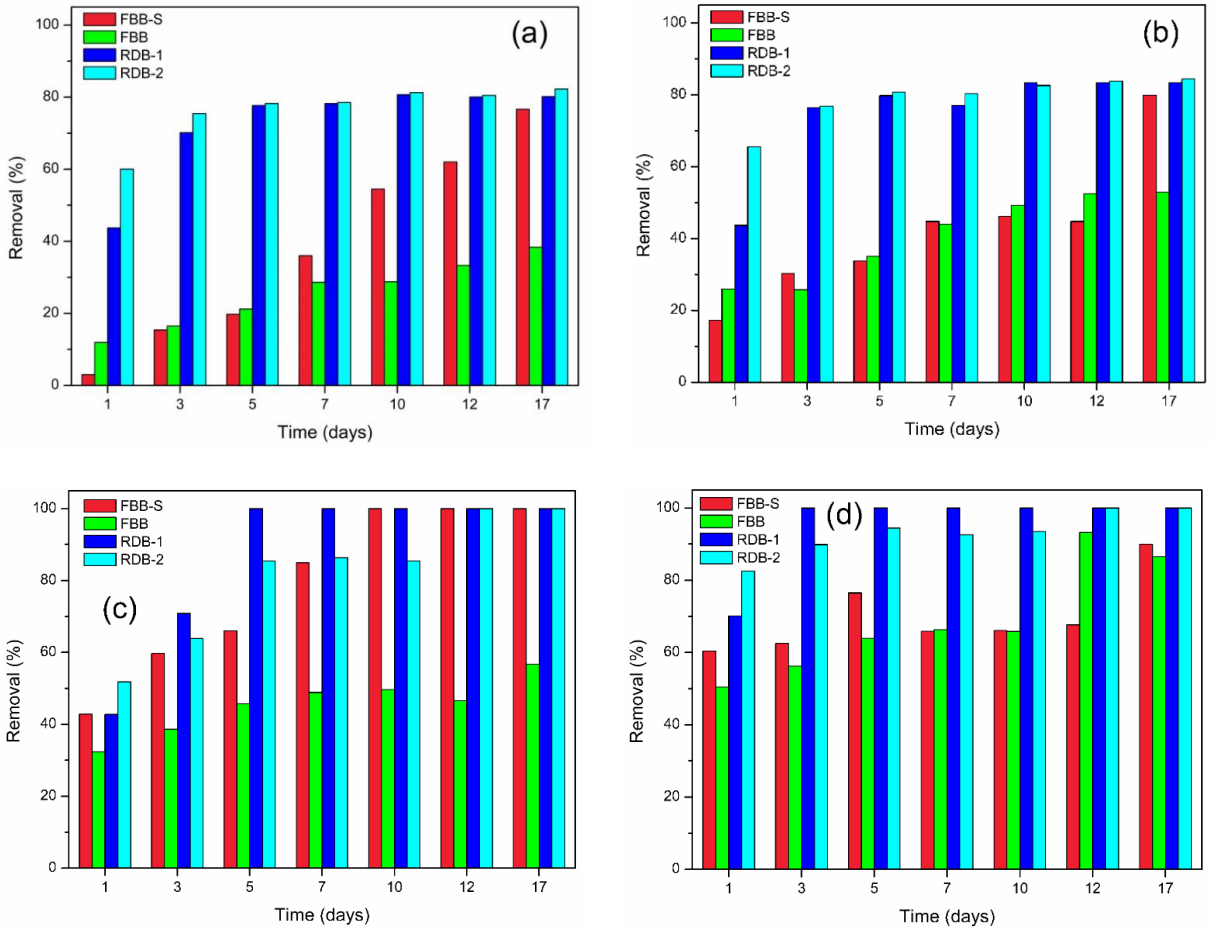


Fig. 7.1. Pesticide removals achieved for THIA (a), CHLOR (b), AZO (c) and TEBU (d) in each experiment.

Alternatively, the RW was also treated by *T. versicolor* immobilized on wood in an RDB, which has shown good results in treating AW under non-sterile conditions (Beltrán-Flores et al., 2022, Chapter 5). As shown in Fig. 7.1, higher elimination yields were obtained in the RDB, reaching up to 85 % of total removal, compared to the FBB (51 %). In fact, equilibrium was not reached in the FBB during the 17 days of treatment, while the RDB showed maximum removals after the first 5 days. This result is particularly interesting for future full-scale reactor applications, as operating periods, and thus reactor volumes, could be considerably reduced. The removals obtained in this experiment were higher than those reported in other RDBs in previous chapters (Chapters 4 and 5) mainly for two reasons: in this case a larger amount of biomass was added in the RDB (1100 vs. 315-545 g biomass DW) and the wastewater remained for a longer period inside the reactor (17 days vs. an HRT of 3-5 days). In any case, a significant amount of

pesticides could be sorbed on the wood in the RDB (Beltrán-Flores et al., 2021, Topic 4.2 of Chapter 4), which motivated the solid phase study presented in Section 7.3.4.

THIA was the pesticide with the lowest removal performances in both systems, being 38 % and 80 % for the FBB and RDB, respectively. In contrast, both the FBB (86 %) and the RDB (100 %) achieved high TEBU removals. Thus, although WRF have been reported to remove both pesticides (Chan-Cheng et al., 2020; Mori et al., 2021), in the present study *T. versicolor* was clearly more efficient removing TEBU than THIA.

Since the RDB showed the best results in terms of pesticide removal, as well as other parameters studied in Section 7.3.3.3, such as COD, color and bacterial count, a second batch was conducted in this system to evaluate its performance in a sequential treatment. In this regard, pesticide removal yields (87 % total removal) comparable to those achieved in the first batch (85 % total removal) were obtained, demonstrating the viability of the RDB for sequential/continuous treatments, as also shown in Chapter 5.

7.3.3.2. Toxicity

The capacities of both reactors to remove the pesticides detected in the RW were evaluated in Section 7.3.3.1. However, other undetected compounds or potential metabolites may also contribute to the overall toxicity of the effluent, thus acute toxicity and phytotoxicity analyses were also required (Marco-Urrea et al., 2009). Initial toxicity of 13.6 TU was considerably reduced in both reactors (Table 7.4). As occurred in the case of the detected pesticides (Section 7.3.3.1), sterile conditions favored the reduction of toxicity in the FBB, denoting a better degradation activity of the fungal consortium in the absence of competing microorganisms. However, even under non-sterile conditions, the removal capacity of the RDB prevailed over that of the FBB-S. In any case, toxicity values were lower than the wastewater discharge limit (25 TU) established in Catalonia (DOGC, 2003).

Table 7.4. Toxicity and phytotoxicity of the treated and original RW. Values are means ± standard deviation (n = 3).

Wastewater	Operating conditions	Toxicity (TU)	Phytotoxicity		
			Relative seed germination SG (%)	Relative root elongation RE (%)	Germination index GI (%)
Pond	-	13.6 ± 1.0	52.9 ± 5.2	69.6 ± 4.7	36.9 ± 3.2
FBB-S	Sterile	6.0 ± 0.3	88.2 ± 20.6	82.28 ± 15.5	72.6 ± 17.8
FBB	Non-sterile	8.6 ± 2.8	76.5 ± 31.8	82.9 ± 11.9	63.4 ± 11.8
RDB-1	Non-sterile	2.2 ± 0.3	111.8 ± 40.9	93.7 ± 20.2	104.7 ± 29.2
RDB-2	Non-sterile	2.9 ± 0.1	61.8 ± 27.0	78.5 ± 1.7	48.5 ± 4.3

Similar results were reflected in the phytotoxicity test. The RDB effluent caused less impact on tomato seed germination and growth than that of the FBB. Actually, the RDB effluent achieved even better SG and GI results than distillate water, which was probably ascribed to the release of some nutrients that considerably stimulate seedling growth, such as NH_4^+ and K^+ (Loffredo et al., 2016). However, partial inhibition was obtained in the RDB-2 compared to the RDB-1, indicating a certain deterioration of the effluent quality throughout the treatment. Since pesticide concentrations were even lower in the RDB-2 than in the RDB-1, the increased toxicity was related to the formation of some metabolites. In this respect, a possible metabolite of THIA with 200-fold higher toxicity in vertebrates has been previously reported (Casida, 2011). This phenomenon should be further investigated in future works.

7.3.3.3. Monitoring of bioreactors

As shown in Sections 7.3.3.1 and 7.3.3.2, the RDB was clearly more efficient than the FBB concerning toxicity abatement. However, there are other parameters to consider in selecting the best reactor for RW treatment, such as laccase activity, COD, color and microbial counts.

Laccase is a typical indicator used to evaluate enzyme activity in fungal treatments (Mir-Tutusaus et al., 2017). The RDB reached substantially higher laccase activity levels than the FBB during the treatment, with activity peaks of 16 and 2 AU L^{-1}, respectively (Fig. 7.2). Furthermore, growing patterns were observed for the RDB and FBB-S, while the FBB showed a decreasing trend from day 5 onwards, evidencing the negative effect of contamination on the enzyme metabolism of the pelleted fungus. Interestingly, this laccase depletion was not

observed in the RDB under non-sterile condition, indicating that the immobilization on wood chips played a vital role in maintaining the enzymatic activity of the fungal consortium. Laccase production was mainly attributed to *Trametes sp.* and *Phanaerochaete sp.*, which are the only two genera of WRF detected in the fungal consortium (Section 7.3.5) capable of synthesizing this enzyme (J. Yang et al., 2017). In this regard, Torán et al. (2017) reported low laccase activity for reactors based on both pelleted and colonized-wood, but in the latter case, lower extent of fungal colonization (according to ergosterol test) had been achieved during the fungal growth stage. Hu et al. (2021) obtained slightly higher laccase levels in an FBB with *T. versicolor* pellets compared to a trickle-bed reactor (TBR) with colonized wood, but this fact was mainly attributed to poor aeration inside the TBR. Furthermore, laccase activity was significantly higher in the second batch of the RDB, which evidenced that the immobilized fungal community was able to increase its enzyme activity as it adapted to non-sterile conditions. The laccase activity in this experiment was higher than those reported in other RDBs in previous chapters (Chapters 4 and 5), probably due to the addition of a larger amount of colonized wood and the implementation of a longer residence time of the wastewater inside the reactor.

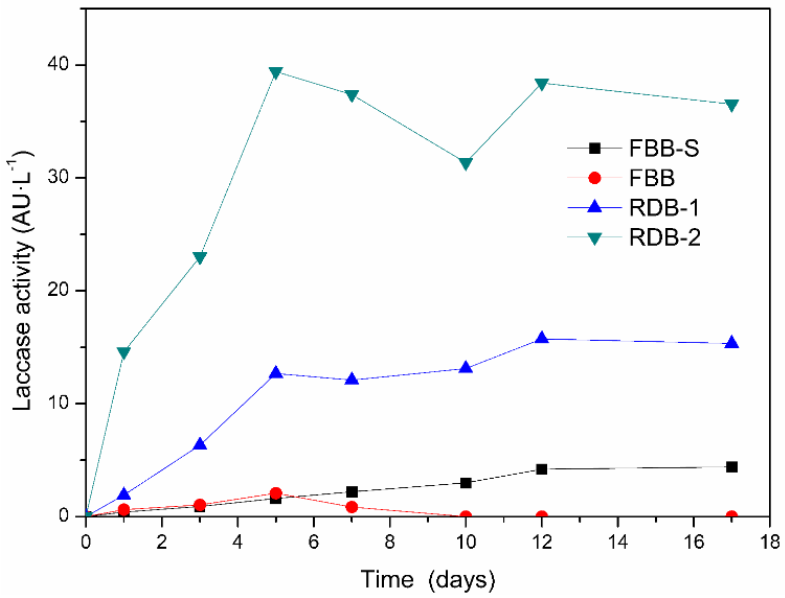


Fig. 7.2. Time-course profile of laccase activity in each experiment.

COD, color and HPCs of the treated effluents are presented in Table 7.5. Contradictory results regarding the COD reduction capacity of *T. versicolor* have been previously reported (Cruz-

Morató et al., 2013; Souza et al., 2014). In this study, COD increased significantly in both reactors, but to a lesser extent in the case of the RDB. In the RDB, the main contribution to COD was probably the release of organic matter from the wood chips (Lacorte et al., 2003). In the case of the FBB, COD increase was attributed to the addition of antifoam Tween 80 (also known as polysorbate 80, theoretical COD = 2207 mg O_2 mL^{-1}). In this respect, fungal treatments involving forced aeration are known to cause foaming (Font et al., 2003). Consequently, antifoam was only required in the FBB. More antifoam was required in the case of the FBB-S (7 mL) compared to the FBB (5 mL) probably owing to the more intensive metabolic activity demonstrated by *T. versicolor* under sterile conditions, which in turn increased COD level in the FBB-S. Regarding the RDB, COD was substantially lower in the second batch (RDB-2), which indicates that a significative part of the soluble organic compounds of the wood were extracted during the first cycle. Moreover, some of the soluble organic matter of the wood was probably consumed by *T. versicolor* and the associated fungal consortium instead of being released into the liquid phase (Beltrán-Flores et al., 2022, Chapter 5). In that study, the COD of the inoculated reactor was found to be substantially lower than that of the control reactor, with this difference becoming more noticeable over time. Therefore, these results are a positive sign for the RBD application in large-scale during long-term operations.

Wastewater coloration increased throughout the treatment in the RDB, being substantially lower in the second batch (Table 7.5). As with the COD, the color gain in the RDB was mainly attributed to the extraction of some organic compounds that give the characteristic dark color to *Q. ilex* wood. In the case of the FBB, a higher turbidity was also observed probably as a consequence of a significant loss of pellet morphology (Espinosa-Ortiz et al., 2016).

The HPC results are presented as the logarithm of CFU in Table 7.5. The HPCs remained constant in both reactors, indicating that bacterial contamination was successfully controlled. The addition of glucose in the FBB did not result in significant bacterial proliferation, contrary to what was reported in the same reactor by Hu et al. (2021), probably caused by the inhibitory effect of the relatively high toxicity of the RW on the bacterial community.

Table 7.5. COD, color and microbial counts of the RW throughout the treatment in the three different fungal reactors.

Operation time (day)	COD (mg O_2 L^{-1})				Color				Microbial counts [log (CFU mL^{-1})]			
	FBB-S	FBB	RDB_1	RDB_2	FBB-S	FBB	RDB_1	RDB_2	FBB-S	FBB	RDB_1	RDB_2
0	2088	2088	2088	2088	0.171	0.171	0.171	0.171	-	6.47	6.47	6.47
0*	5190	4802							-			
1	5600	5160	3709	3228	0.079	0.183	0.197	0.156	-			
3	6380	7392	4407	3517	0.080	0.170	0.296	0.172	-			
5	8360	10024	5340	3727	0.052	0.155	0.390	0.191	-	6.42	6.85	6.46
7	11448	10404	6006	3754	0.046	0.129	0.413	0.197	-			
10	13128	9296	6336	4068	0.039	0.212	0.427	0.228	-	6.48	7.02	6.10
12	13104	8120	6643	4158	0.040	0.310	0.302	0.247	-			
17	10476	8140	7076	4785	0.041	0.372	0.243	0.212	-	6.61	6.54	6.51

* Antifoam was initially added to prevent excessive foaming resulting from aeration. Microbial counts were measured in duplicate, with a standard deviation below 2 % in all cases.

Considering the remarkable good results of pesticide removal (Section 7.3.3.1), toxicity reduction (Section 7.3.3.2) and the stable values of the characteristic RW parameters obtained in this section, the RDB effluent was found to present an overall quality clearly superior to that from the FBB and the original RW. Furthermore, unlike the continuous glucose supplementation required in the FBB, the RDB only demanded the initial low-cost wood chips as substrate. Therefore, it is concluded that the RDB is the most promising treatment for further full-scale applications. This reactor could be used for the on-site treatment of the RW produced in the IRTA's agricultural fields (Mas Badía). In these fields, several pesticide applications are performed for each type of crop throughout the summer campaign. To avoid cross-contamination of pesticides between crops, the machinery is washed after each application, and the produced wastewater is accumulated in the collection pond. Accordingly, at the end of the campaign period, the collected wastewater could be treated in batch mode by a full-scale RDB for pesticide and toxicity abatement.

7.3.4. Solid-phase study and treatment

Studies involving sorption processes should not only evaluate micropollutant transfer from the liquid to the solid phase, but also the fate of the polluted sorbent. Hence, solid by-product obtained from the RDB were analyzed to determine the contributions of the sorption and biodegradation to the overall removal by means of pesticide mass balance. Sorption was found to be a crucial mechanism of pesticide elimination, being around 81 % THIA, 83 % CHLOR, 83 % AZO and 25 % TEBU. This remarkable sorption contribution of pesticides by wood had previously been reported in the literature (Hu et al., 2021). Sorption capacity of wood has been related to pesticide hydrophobicity, and therefore, affinity of these compounds for wood fibers (Beltrán-Flores et al., 2021, Topic 4.2 in Chapter 4). In the present study, CHLOR and AZO showed slightly higher sorption than THIA, which may be due to the fact that they are more hydrophobic compounds (Table 7.3). However, TEBU was the most apolar ($\log K_{ow} = 3.70$) and simultaneously the least sorbed pesticide, which was mainly attributed to its high biodegradability in the early stages of treatment, as shown in Fig. 7.1 (d). In other words, biodegradation was the fastest removal mechanism for TEBU, preventing its potential sorption on wood.

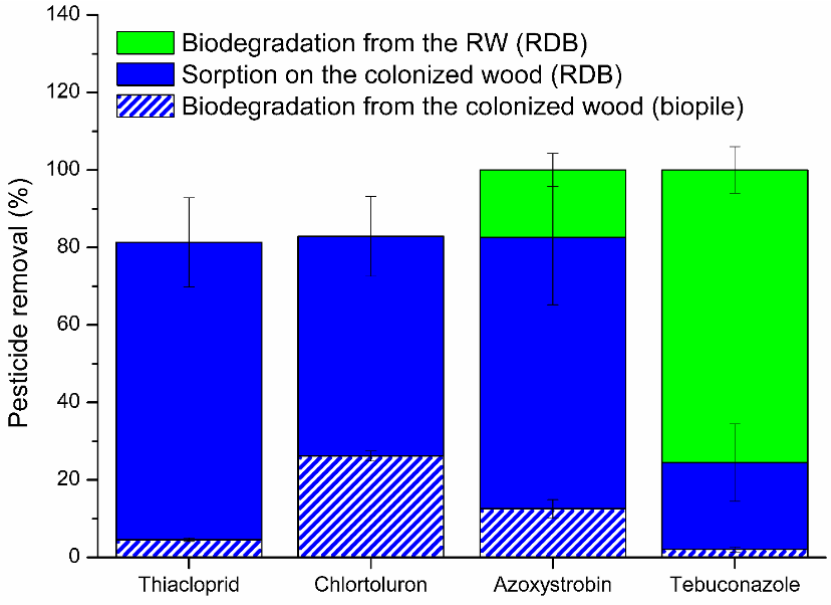


Fig. 7.3. Pesticide removal balance in the two-stage treatment consisting of the RDB and the biopile: biodegradation from the RW in the RDB (green bars), sorption on the colonized wood in the RDB (blue bars) and biodegradation from the colonized wood in the biopile reactor (bars with blue line pattern). The sum of the three bars corresponds to the total removal of each pesticide. Results are shown as mean values and corresponding standard deviations for triplicate measurements.

Solid by-products resulting from the RDB were subsequently treated in a biopile-like reactor during 27 days to evaluate the ability of the remaining fungus to biodegrade sorbed pesticides. Fig. 7.3 shows the results of the pesticide removal balance in the two-stage treatment consisting of the RDB and the biopile. The analysis of the biomass after the biopile treatment allowed the quantification of the pesticide biodegradation in the solid biopile, being 5 % THIA, 26 % CHLOR, 13 % AZO, 2 % TEBU. Although these percentages are apparently low, the total amount of pesticides removed is remarkable given their high content in the wood ($\approx$ 28 mg g wood DW^{-1}). Thus, biodegradation rates of the pesticides were also calculated to facilitate comparison with other literature results (Table 7.6). In this regard, a similar biodegradation rate of total pesticides (3.25 10^{-4} mg g wood DW^{-1} day^{-1}) was achieved in a previous fungal treatment of wood in a biopile system (Beltrán-Flores et al., 2021, Section 4.2.3.7 of Chapter 4). Longer operational periods, such as those commonly applied in compost-like systems, could considerably reduce the remaining pesticide content of the solid by-products (Rodriguez-Rodriguez et al., 2011). In addition, future research should explore other operational

strategies to improve removal efficiency, such as the addition of fresh colonized wood, re-inoculation of solid by-products, adjustment of the moisture content, modification of the inoculation method and wood crushing.

Table 7.6. Biodegradation of pesticides contained in the old colonized wood achieved by *T. versicolor* after 27 days of treatment. Values are means ± standard deviation for triplicate samples.

Pesticide	Initial concentration ± SD (mg g wood DW^{-1}) x 10^2	Biodegradation ± SD (%)	Biodegradation rate ± SD (mg g wood DW^{-1} day^{-1}) x 10^5
Thiacloprid	3.83 ± 0.40	4.56 ± 0.47	7.96 ± 0.82
Chlortoluron	1.51 ± 0.12	26.20 ± 1.76	17.67 ± 1.19
Azoxystrobin	0.91 ± 0.14	12.54 ± 2.54	5.10 ± 1.03
Tebuconazole	0.57 ± 0.08	2.06 ± 0.91	1.78 ± 0.79

7.3.5. Fungal community assemblage

PCR-DGGE analysis and prominent band sequencing were performed to evaluate the fungal diversity and dynamics of both reactors and the persistence of *T. versicolor* after inoculation. These analyses allowed the determination of the phylogenetic affiliations of every recovered band and the calculation of the relative abundance of each phylotype present in all samples analyzed. These studies were conducted by the Environmental Microbiology Group of the Autonomous University of Barcelona. For this reason, this thesis only includes a short discussion of the main findings obtained in this study.

Samples corresponding to RW displayed high fungal diversity, with 3 prominent genera representing more than half of the fungal community: *Penicillium* sp., *Phanaerochaete* sp. and *Meyerozima* sp. (Table B.6., Annexes). Both *Penicillium sp.* and *Phanaerochaete sp.* have been reported to degrade pesticides (Chen et al., 2021; Zehra et al., 2017). *Meyerozyma sp.* has also been documented to degrade other micropollutants such as textile dyes (Ali et al., 2021). *Trametes sp.* was not identified in any of the three replicates, indicating its absence from the initial RW. A lower population diversity was observed in the effluents of the RDB and the FBB (Table B.8, Annexes). In both cases, *Trametes sp.* was detected, but its presence in the RDB effluent was actually minor, whereas in the final sample of the FBB effluent *Trametes sp.* represents almost 50 % of the fungal diversity. This result suggests that the strategy of

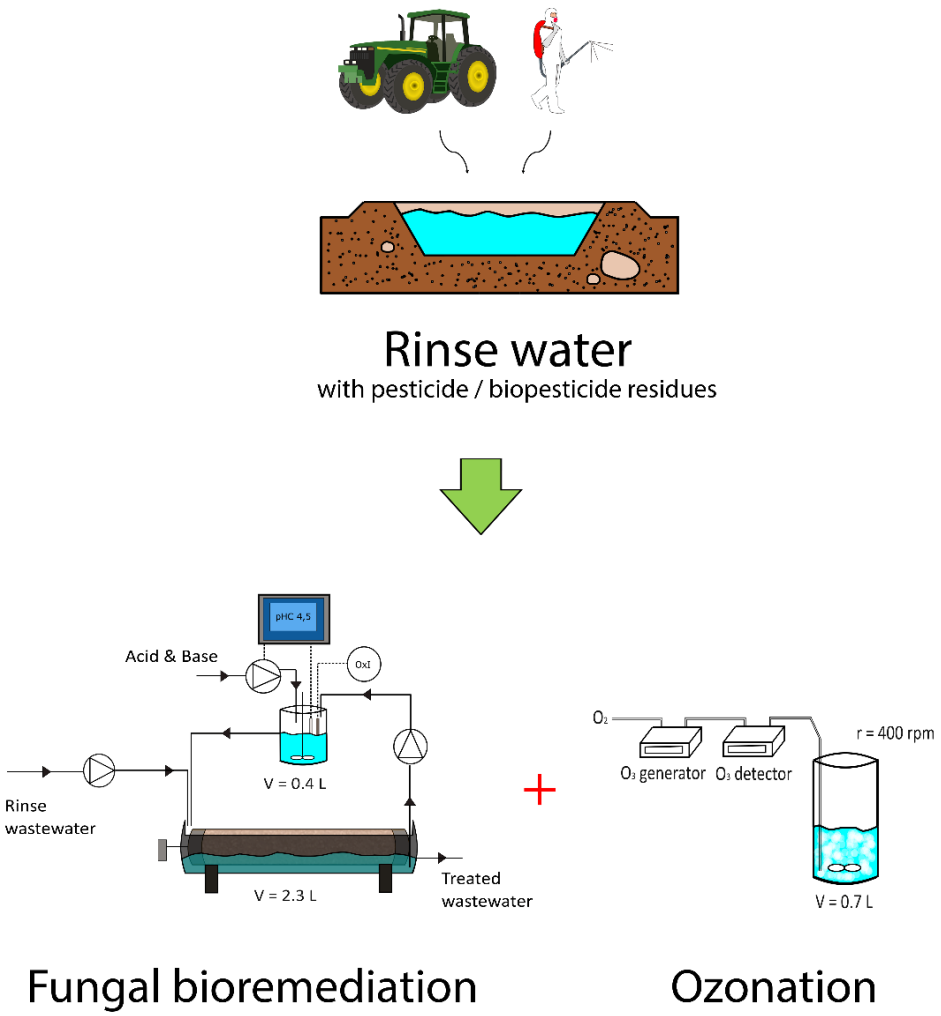
immobilization on wood used in the RDB was more effective in retaining biomass than the auto-immobilization (pellets) approach implemented in the FBB. Furthermore, the relative abundance of *Trametes sp.* decreased considerably when comparing the old wood obtained from the RDB and that treated in the biopile, indicating a certain proliferation of other fungi during the biopile treatment (Table B.5, Annexes). As indicated in section 7.3.4, the biopile reactor could incorporate different strategies to enhance the preservation of *T. versicolor* in future work, such as the addition of fresh wood, optimization of the moisture content of the wood, re-inoculation of biomass and/or wood crushing.

7.4. Conclusions

RW with an inherently high pesticide content was treated in two different fungal reactors. The RDB proved to be a better candidate than the FBB according to all studied parameters, including 87 % versus 51 % in pesticide removal, respectively. Fungal community study showed that *T. versicolor* was especially dispersed in the FBB, while this fungus was successfully immobilized in the RDB. In addition, old contaminated wood was treated with *T. versicolor* in a biopile-like reactor, achieving remarkable biodegradation rates of up to $17.67 \pm 1.19 \cdot 10^{-5}$ mg g^{-1} day^{-1}. These results suggest that the RDB is a promising approach for full-scale treatment of RW.

Chapter 8

Rinse wastewater treatment by combining fungal bioremediation and ozonation



8.1. Introduction

Pesticides comprise a broad group of organic compounds essential to maintain the current lifestyle of our societies. Pesticides, together with fertilizers and the use of appropriate machinery, improve crop varieties and enhance agricultural productivity by reducing losses caused by the proliferation of weeds, pests and diseases. These substances are also used to control the spread of disease vectors, such as malaria, in the transport sector, in the protection of wooden structures and buildings affected by termites or other insects, and in ornamental landscaping, parks and gardens (Aktar et al., 2009). Despite their essential status, the indiscriminate use of pesticides can cause serious and irreversible damage to the integrity of the environment and human health. Some studies have associated exposure to these compounds with the development of various human diseases, such as cancer, asthma and diabetes (Huang et al., 2019; Stoleski et al., 2019; Velmurugan et al., 2017). In addition, interactions between pesticides can trigger synergistic mechanisms with unpredictable toxicological effects (Hernández et al., 2017).

The EC has promoted the implementation of Integrated Pest Management (IPM) in the 2009/128/EC Directive on the sustainable use of pesticides (EC, 2009a), which calls for the use of sustainable agricultural practices. These principles include cultivation techniques (crop rotation, balanced fertilization, etc.), pest monitoring, use of biopesticides, selection of low-risk pesticides and dosages, and hygiene measures. Hygiene measures should cover the management of RW, which is generated after washing agricultural machinery and equipment that has been in contact with pesticides. In this regard, some studies and guidelines recommend reusing this wastewater as phytosanitary products in the same agricultural fields (EPA, 2012; Life aquemfree, 2018; Shukla et al., 2001). However, this strategy is unfeasible on large farms that produce significant volumes of RW. In this case, RW is usually accumulated in large collection ponds where it is concentrated by natural evaporation (Kuo and Regan, 1999). The management of this type of residue is still scarcely documented, so it is necessary to develop a treatment option specifically designed for this application, capable of reducing toxicity and, eventually, allowing reclaimed water reuse.

Among the chemical oxidation technologies, some of them conceptually known as advanced oxidation processes (AOPs), ozonation stands out as one of the most mature and consolidated options for the degradation of organic pollutants, including micropollutants, in urban and industrial wastewaters. Ozone is a molecule with high oxidative capacity and disinfectant potential (Von Gunten, 2003). When applied to water, ozone can directly attack the organic pollutants (mainly at low pH) or generate hydroxyl radicals ($^{\bullet}OH$, mainly at high pH), which are highly non-selective oxidative species, among other radicals that can be formed (Sharma et al., 2018). Ozone is an electrophilic molecule that specifically interacts with the high electron density sites in organic molecules, such as aromatic and unsaturated bonds, whereas the hydroxyl radical is highly reactive and its formation is recommended to mineralize the pollutants and minimize the potential TPs. The production of hydroxyl radicals can be enhanced in many ozonation treatments, either by adding some reagent (such as methanol or hydrogen peroxide) or by radiation (photolytic ozonation). Another alternative to reduce the amount of TPs in the treated wastewater is the use of active catalysts or photocatalysts (i.e., catalytic or photocatalytic ozonation, respectively). Nevertheless, all these approaches involve significant increases in costs that may be excessive for environmental applications (Derco et al., 2015). Moreover, these radicals can act inefficiently when treating waters with complex matrices, some being consumed by other species such as HCO_3^-, CO_3^{2-}, NO_3^-, NO_2^-, Cl^-, Br^-, and natural organic matter (Gomes et al., 2017).

Bioremediation is considered a safe, low-cost and sustainable technology that uses microorganisms, plants and enzymes to mitigate pollution. Among other microorganisms, WRF are gaining increasing attention due to their complex enzyme system, composed of both extracellular enzymes (mainly lignin peroxidase, manganese peroxidase, versatile peroxidase and laccase) and the intracellular system known as cytochrome P-450, which is capable of degrading a wide range of pollutants (Asgher et al., 2008). Fungi are microorganisms with a quite slow metabolism, thus fungal treatments require longer operating periods than other types of wastewater treatment techniques. For this reason, fungal technology is particularly suitable for the treatment of relatively small volumes of water, as is common in RW. In addition, WRF bioremediation also seems to be appropriate for treating RW (which are characterized by high pesticide concentrations and toxicity level), since WRF have demonstrated excellent resistance

to toxicity and thus do not require a prior acclimatization period as in other biological processes, such as conventional activated sludge (Mir-Tutusaus et al., 2017). Accordingly, the RDB has shown to be an interesting candidate for agricultural water treatment, as this reactor has demonstrated excellent performance in removing pesticides and its design has been adapted to *in-situ* application in agricultural fields (Chapters 5 and 7). Likewise, ozonation can be a viable alternative since it typically requires shorter treatment periods, and associated costs could be reduced by restricting the addition of other radical precursors, radiation and/or (photo)catalysts, i.e., by using single ozonation. In any situation, selecting the most appropriate technology would require a comparative study of multiple parameters, including pesticide elimination, generation of TPs, overall water quality and costs.

An alternative option is to implement a treatment train. Treatment trains can synergistically combine the advantages of several technologies, often achieving better results than those obtained by each treatment individually. In this regard, AOPs have been used as pre-treatment to oxidize biologically recalcitrant compounds and make wastewater pollution more biodegradable (Mansour et al., 2014). These pre-treatments usually pursue a low mineralization of the pollutants to avoid high operating costs. Afterwards, a biological stage can be applied to eliminate the potential TPs. However, several studies have also reported the strategy in the opposite direction, i.e., applying first a bioremediation treatment to reduce the pollutant load followed by an AOP stage to complete its elimination or even mineralization (Oller et al., 2011).

Therefore, the present work aimed to study the treatment of RW by ozonation, fungal bioremediation, and the combination of both technologies in a treatment train. Their performances were compared in terms of pesticide removal, generation of TPs, overall quality and economic costs. The treatment train consisted of a first stage of fungal bioremediation followed by ozonation. The fungal treatment was intended to reduce the concentration of pesticides and hence the ozonation time required in the subsequent stage, as ozonation has been suggested to be a more expensive technology.

8.2. Materials and methods

8.2.1. Agricultural rinse wastewater

Physicochemical characteristics of the RW are summarized in Table 8.1.

Table 8.1. Physicochemical characterization of the RW. Values are means ± standard deviation for triplicate samples.

Parameter	Value
pH	7.64 ± 0.15
Conductivity (mS cm^{-1})	10.98 ± 0.12
Color at 650 nm	0.71 ± 0.05
Chloride (mg Cl L^{-1})	1635.7 ± 2.7
Sulphate (mg S L^{-1})	784.7 ± 3.1
Nitrite (mg N L^{-1})	0
Nitrate (mg N L^{-1})	556.5 ± 0.1
Ammonia (mg N L^{-1})	133 ± 13
Total phosphorus (mg L^{-1})	14.3 ± 3.2
Phosphate (mg L^{-1})	0
TSS (mg L^{-1})	120.0 ± 15.3
BOD_5 (mg O_2 L^{-1})	135 ± 11
COD (mg O_2 L^{-1})	4263 ± 85
DOC (mg C L^{-1})	1527 ± 35
Heterotrophic plate count (CFU mL^{-1})	$21.0 \cdot 10^4 \pm 1.4 \cdot 10^4$
Toxicity (TU)	13.53 ± 2.27

8.2.2. Fungal treatment in a rotating drum bioreactor

Two RDBs were operated in parallel, the experimental reactor (E-RDB), which was assembled with wood initially colonized by *T. versicolor*, and the control reactor (C-RDB), which only contained wood. The E-RDB and the C-RDB were operated for 67 and 53 days, respectively, both with an HRT of 5 days. The inner tube rotated one and a half turns every 24 hours to alternate the submerged biomass fraction. A total of 545 g DW of colonized wood was introduced inside the inner tube. An external recirculation loop (4.7 L day^{-1}) was required for pH adjustment and DO measurement, which were performed in a recirculation tank (≈ 0.4 L). The RW was previously adjusted to pH 4.5 and then automatically controlled throughout the

treatment by adding either 1 M HCl or NaOH inside the recirculation tank. Aeration was introduced at the reactor inlet from day 28 onwards through a diffuser at 0.1 NL/min. DO was measured by using an OXROB10 robust oxygen probe coupled to a FireSting-PRO (4 channels) fiber-optical multi-analyte meter (Pyroscience, Germany). Samples of 3 mL were withdrawn throughout the treatment to measure the concentration of the pesticides (Annex A) and laccase activity (Chapter 3, Section 3.6.4). The effluent was stored in collection tanks for subsequent analysis and treatment by ozonation.

8.2.3. Stirred tank reactor with ozone supply

8.2.3.1. Ozonation experiments with spiked ultrapure water

Ultrapure water was spiked with 16 ppm thiacloprid (THIA), 34 ppm chlortoluron (CHLOR) and 46 ppm pyrimethanil (PYRI) and treated by ozonation [Fig. 8.1-1)] for 480 min in the stirred tank reactor presented in Section 3.5.6 (Chapter 3). Aliquots of 1 mL were withdrawn from the reactor at specific times (depending on the experiment) for further analysis of pesticide and concentrations of TPs. At the end of the experiment, liquid samples were taken for the analysis of color, COD BOD_5, DOC, toxicity and HPCs. The treatment was performed at room temperature (approximately 18 ºC). The test was performed in triplicate.

8.2.3.2. Ozonation treatment of rinse wastewater and RDB effluent

The original RW (RW II in Chapter 3) and the RDB effluent were treated by ozonation [Fig. 8.1-2) and 8.1-3), respectively] until complete degradation of the three studied pesticides (720 min) in the stirred tank reactor presented in Section 3.5.6 (Chapter 3). Aliquots of 1 mL, or 8 mL when analyzing DOC, were withdrawn from the reactor at specific times (depending on the experiment) for further analysis of pesticide and concentrations of TPs. At the end of the experiment, liquid samples were taken for the analysis of color, COD BOD_5, DOC, toxicity and HPCs. Two additional experiments were conducted for the treatment of the RDB effluent in which sodium hydroxide (NaOH) and methanol (MeOH) were added to evaluate their effect on the ozone treatment performance. Therefore, the RDB effluent was also treated by ozonation at an initial pH of 7.6 and with 2 % (v/v) MeOH. Again, 1 mL samples were taken at specific times (depending on the experiment) to measure pesticide and concentrations of TPs.

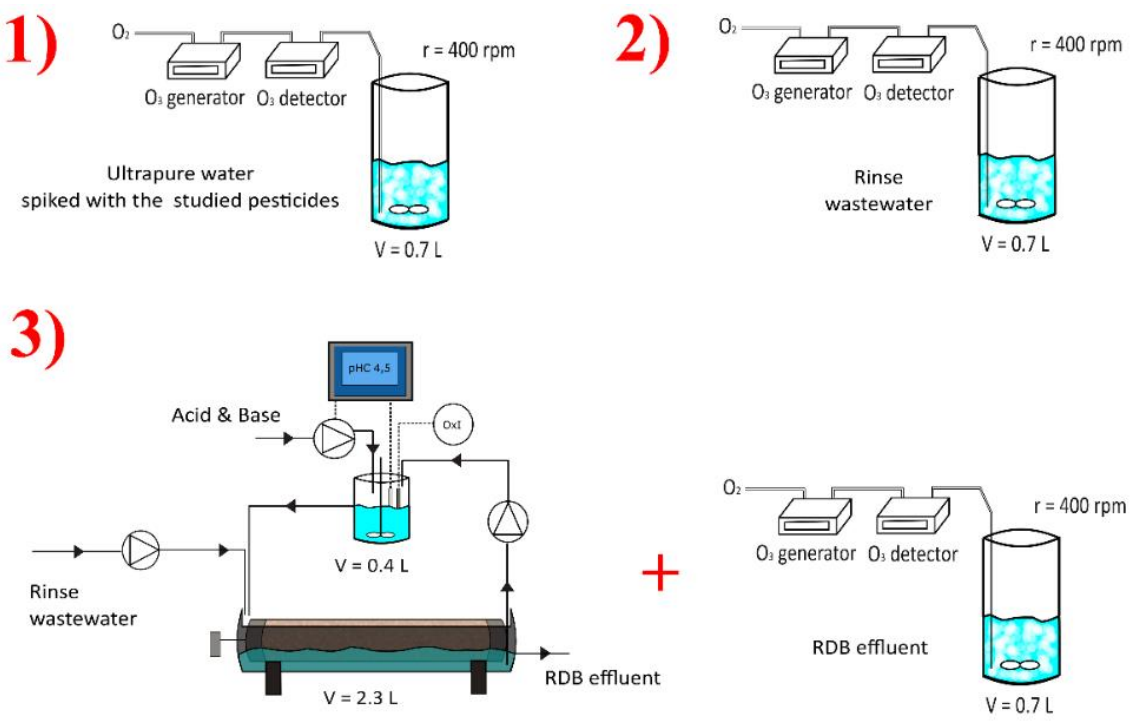


Fig. 8.1. Scheme of the different experiments: 1) ozonation of ultrapure water spiked with the studied pesticide in a stirred tank reactor, 2) ozonation of RW in a stirred tank reactor, and 3) treatment train consisting of a first stage of fungal bioremediation followed by a second stage of ozonation. The ozonation stage in treatment train 3) was studied in three different experiments: with the original RDB effluent, with the RDB effluent at pH 7.6, and with the RDB effluent with 2% (v/v) of MeOH.

8.2.4. Analytical techniques

The identification and quantification of the pesticides were performed as shown in Annex A. The concentrations of oxalic, oxamic and maleic acids were quantified as described elsewhere (Torres-Pinto et al., 2019). In brief, these aliphatic acids were measured by using a Hitachi Elite Lachrom HPLC equipped with a UV-vis detector and a 5 mM H_2SO_4 mobile phase through an Alltech OA-1000 Organic Acids column (300 mm). The eluent was pumped at a constant flow rate of 0.5 mL min^{-1}. The sample loop volume and detection wavelength were set at 30 µL and 200 nm, respectively. The LOD in ultrapure water was 0.20 and 0.06 ppm for oxalic and oxamic acids, respectively, and in the RW the LOD was 0.5 ppm for both oxamic and maleic acids. Under these conditions, the retention times were approximately 5.90, 8.50 and 9.10 min for

oxalic, maleic and oxamic acids, respectively. Other analyses performed in this study are described in Chapter 3.

8.3. Results and discussion

8.3.1. Rinse wastewater characteristics

The main characteristics of this specific RW (Table 8.1) compared to other agricultural and urban wastewaters were the high content of intrinsic organic matter detected in terms of COD (4263 ± 85 mg O_2 L^{-1}) and DOC (1527 ± 35 mg L^{-1}) and, as a consequence of the eventual decomposition of this organic matter, slightly higher levels of ammonium and bacteria counts were detected (Beltrán-Flores et al., 2021, Chapter 4; Metcalf and Eddy, 2003). Thus, this study not only focuses on pesticide removal but also analyzes these conventional parameters to evaluate the final effluent quality.

8.3.2. Pesticide identification and quantification

Liquid chromatography coupled to time-of-flight mass spectrometry (LC-qTOF-MS) is a useful technique for qualitative analysis and identification of organic pollutants (Arsand et al., 2018). High mass resolution along with the acquisition of mass profile data allowed the screening of non-targeted compounds.

Unknown compounds were identified through exact mass analysis. LC-qTOF-MS analysis shows high specificity, allowing the pesticide identification by the characterization of the monoisotopic mass with a maximum error of ± 2.5 mDa. The detected exact masses were close to those of THIA, CHLOR and PYRI. Moreover, the isotope pattern validated the identification of such pesticides. In this regard, the experimental isotopic patterns match the predicted isotope distributions as shown in Table 8.2. Afterwards, commercial reagents of each pesticide were spiked into the RW to verify that the chromatogram peaks of the standard compounds matched those detected in the original matrix. After the confirmatory analysis, initial pesticide concentrations were quantified as 9.77 mg L^{-1} THIA, 24.32 mg L^{-1} CHLOR and 27.54 mg L^{-1} PYRI. Thus, the total pesticide concentration in terms of total carbon was 38.47 mg C L^{-1}. Although these concentrations are relatively high for agricultural wastewater (Köck-

Schulmeyer et al., 2019), they are reasonable considering that the RW comes from the washing of agricultural equipment that had been in direct contact with pesticides.

Table 8.2. Compounds identified in the RW based on HRMS library searching.

Compound	Chemical Formula [M]	Experimental Monoisotopic Mass (Da) $[M+H]^+$	Experimental Isotope ions (R.A.) $[M+H]^+$	Theoretical isotopic ions (R.A.) $[M+H]^+$	Mass error (mDa)*
Thiacloprid	$C_{10}H_9ClN_4S$	253.0305	253.0305 (100.0)	253.0309 (100.0)	-0.4
			254.033 (12.2)	254.0333 (13.1)	-0.4
			255.0276 (38.0)	255.0279 (37.3)	-0.4
			256.0295 (4.5)	256.0303 (4.8)	-0.8
Chlortoluron	$C_{10}H_{13}ClN_2O$	213.0785	213.0785 (100.0)	213.0789 (100.0)	-0.4
			214.0811 (11.0)	214.0819 (11.7)	-0.8
			215.0758 (33.0)	215.0761 (32.8)	-0.4
			216.0784 (3.5)	216.0790 (3.8)	-0.5
Pyrimethanil	$C_{12}H_{13}N_3$	200.1176	200.1176 (100.0)	200.1182 (100.0)	-0.6
			201.1201 (15.1)	201.1211 (14.2)	-1.0

R.A., relative abundance (%).

*Mass error = $(m/z_{experimetal} - m/z_{theoretical})\cdot 1000$

THIA is a neonicotinoid insecticide whose mechanism of action is based on the alteration of the nervous system of the target organisms. It was included in the first watch list of substances in Decision 2015/495/EU (EC, 2015), as it is suspected to pose a high risk to human health as endocrine disrupting, neurotoxic and carcinogenic compounds. Recently, the EC banned THIA by not renewing its license for use in the EU (EC, 2019b). CHLOR is an herbicide belonging to the phenylurea class that acts by the inhibition of photosynthetic electron transport. It is moderately toxic to most aquatic species, birds and worms, although toxicity to mammals is considered low. PYRI is a broad-spectrum fungicide that inhibits methionine biosynthesis, which affects protein formation and subsequent cell division. This fungicide exhibits a moderate ecotoxicity and is considered a possible liver, kidney, adrenals, bladder and thyroid toxicant, and possible human carcinogen (Lewis et al., 2016). These last two pesticides (CHLOR and PYRI) are approved for use in the EU under EC Directive 1107/2009 (EC, 2009).

Table 8.3. The physical-chemical properties of the investigated pesticides.

Pesticide	Type	Group	CAS number	Solubility at 20 ºC (mg L^{-1})	Log K_{ow}
Thiacloprid	Insecticide	Neonicotinoid	111988-49-9	185	1.26
Chlortoluron	Herbicide	Phenylurea	15545-48-9	70	2.41
Pyrimethanil	Fungicide	Anilinopyrimidine	53112-28-0	110	2.84

8.3.3. Fungal treatment in a rotating drum bioreactor

Q. ilex wood chips presented a porosity of 62.4 ± 0.5 %, a WW/DW ratio of 2.5 ± 0.1, and real and apparent densities of 0.27 ± 0.01 g mL^{-1} and 0.89 ± 0.02 g mL^{-1}, respectively (Section 4.2.3.1, Chapter 4). The RW was successfully treated for 67 days in the E-RDB and 53 days in the C-RDB. In the non-inoculated C-RDB, the removal was mainly ascribed to the sorption of these pesticides onto the wood. The average eliminations during the first 33 days (until C-RDB equilibrium) were 45 % THIA, 50 % CHLOR and 57 % PYRI in the E-RDB, and 43 % THIA, 47 % CHLOR and 54 % PYRI in the C-RDB (Fig. 8.2). Therefore, it was concluded that sorption contribution was decisive during this period. The difference in elimination between E-RDB and C-RDB may be due to three main factors: 1) biodegradation by *T. versicolor*; 2) biodegradation by other microorganisms; 3) sorption by the biofilm (Chapter 7). Although sorption was high, part of the sorbed pesticides can be removed from the solid-phase during the treatment or by applying a biopile post-treatment, as done in Chapters 4, 5 and 7.

DO was measured with the aid of a standard oxygen probe in the recirculation tank, which remained above 30 % throughout the treatment, thus biomass was assumed to work under non-limiting conditions. However, from day 28th onwards, another DO probe was used, which was thin enough to be inserted directly into the channel. This probe revealed a very low oxygen level within the channel (< 5 %), which indicated the submerged biomass had been working under limiting condition (Chapter 6). For this reason, aeration was incorporated into the channel, maintaining the DO level near saturation (i.e., 100 %) for the remaining treatment period. In this respect, pesticide removal was subsequently reduced for both the E-RDB and the C-RDB (Fig. 8.2), possibly as a consequence of the partial evaporation of water caused by aeration, which reduced the amount of water in contact with the fungal biomass. However, pesticide

removal in the E-RDB increased from day 39th onwards, even surpassing the initial elimination yields, when wood sorption capacity was maximum. The average removals during the entire treatment period in the E-RDB were 45 % THIA, 49 % CHLOR and 53 % PYRI, corresponding to a total elimination in terms of DOC of 50.43 % (19.40 mg C L^{-1}). Similar removals were reported for two other pesticides, diuron (up to 54 %) and bentazon (up to 48 %), in the same RDB, with sorption being the predominant elimination mechanism (Beltrán-Flores et al., 2022, Section 5.3.2.4 of Chapter 5).

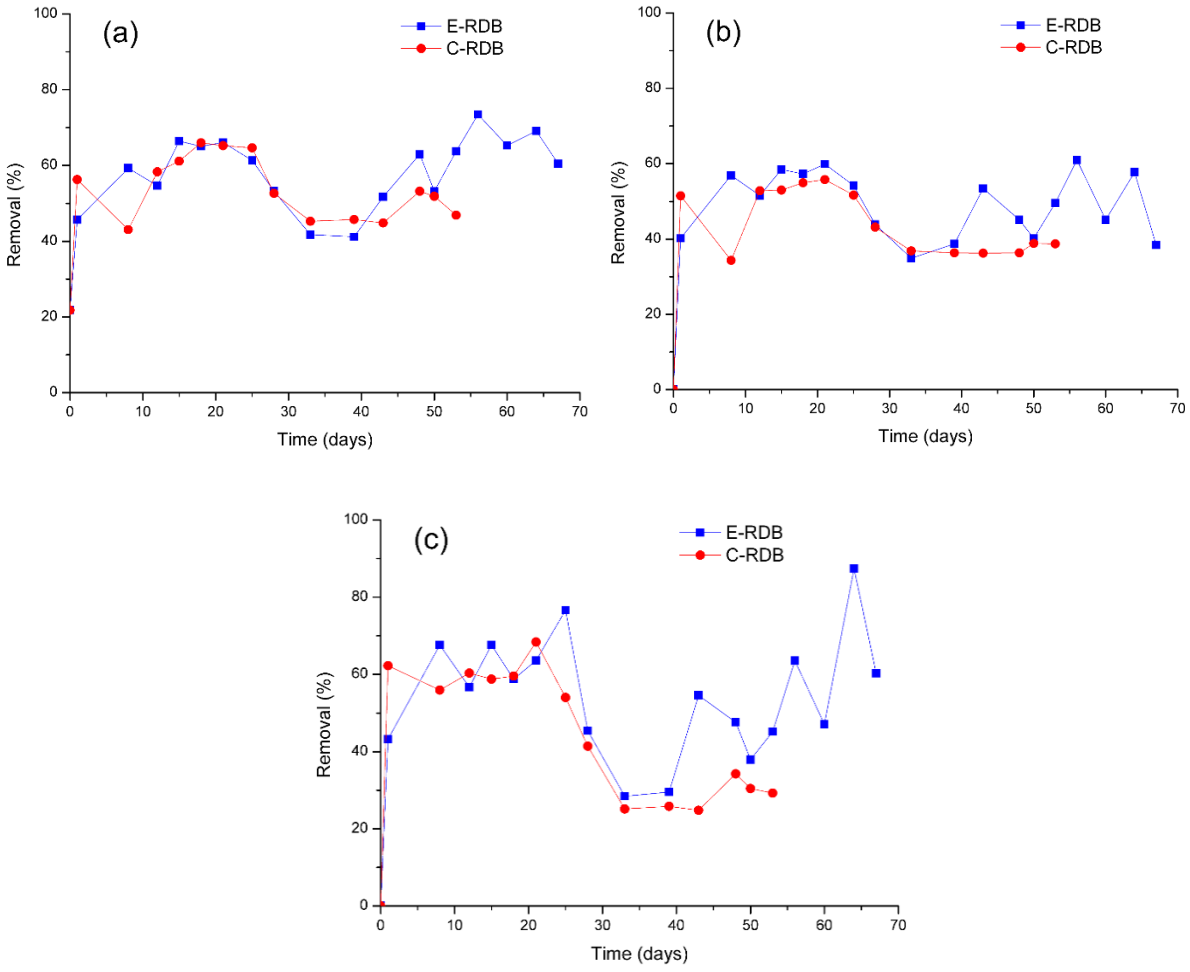


Fig. 8.2. THIA (a), CHLOR (b) and PYRI (c) removal profiles by *T. versicolor* immobilized on *Q. ilex* with an HRT of 5 days in the RDB. Blue squares and red circles are the removals by the experimental (E-RDB) and control (C-RDB) reactors, respectively.

Fig. 8.3 shows the results of laccase activity in the E-RDB. A laccase profile typical of fungal reactors was obtained, in which the activity peak was reached during the first few days and then progressively declined over time. However, laccase activity was not completely depleted but remained at around 0.53 AU L^{-1} after 67 days of operation. The fact that laccase was still present at the end of the experiment is a very positive result since in another similar experiment it reached zero in an even shorter period of 33 days (Beltrán-Flores et al., 2022, Section 5.3.2.4 of Chapter 5). The maintenance of laccase activity during such a long period (67 days) was partially attributed to the incorporation of aeration since it is well known that oxygen is required for the production of this enzyme. In this regard, Pinheiro et al. (2020) evaluated laccase production by *T. versicolor* in three different reactors, detecting higher activity values when aeration was incorporated into the system. Therefore, laccase production was found to be correlated to DO concentration during the process, which is in line with the results of the present study. Additionally, a higher DO concentration may not only lead to increased laccase production by *T. versicolor* but could also enhance pesticide degradation, as oxygen is an essential electron acceptor in certain laccase-mediated oxidation reactions (Ortner et al., 2015).

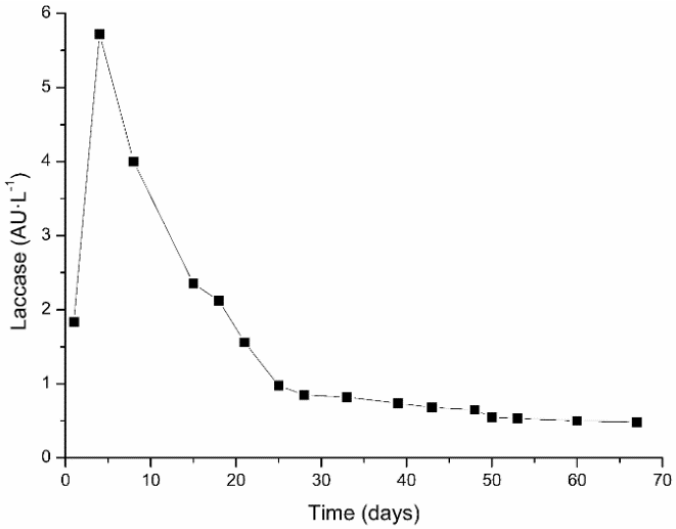


Fig. 8.3. Laccase activity of *T. versicolor* immobilized on *Q. ilex* wood chips in the E-RDB (HRT 5 days).

8.3.4. Ozonation in a stirred tank reactor

Before treating the RW, an experiment was conducted with ultrapure water spiked with the detected pesticides to evaluate their degradability by ozonation [Fig. 8.1-1)]. The initial concentrations at which the ultrapure water was spiked (approximately 16 ppm THIA, 34 ppm

CHLOR and 46 ppm PYRI) were slightly higher (although of the same order of magnitude) than those found in the RW (approximately 10 ppm THIA, 25 ppm CHLOR and 28 ppm PYRI). The degradation profiles of the three model pesticides are shown in Fig. 8.4. All three pesticides were completely removed (below the LOD) by ozonation: after 240 min for THIA, 180 min for CHLOR and 120 min for PYRI. These results are in line with previous studies, in which different AOPs had already been used to treat these pesticides. For instance, THIA (0.1 ppm) was treated by ozonation for 20 min, achieving eliminations around 40 %, 50 % and 65 % with doses of 0.5, 1.0 and 1.5 mg O_3 mg DOC^{-1} (Guo et al., 2020). This study also concluded that THIA was primarily abated by $^{\bullet}OH$ radicals rather than directly by O_3. Benitez et al. (2007) applied O_3 at constant 40 L h^{-1} for the treatment of CHLOR (1 µM, corresponding to 0.21 ppm), reporting eliminations over 60 % and 100 % for O_3 concentrations of $1 \cdot 10^{-5}$ M and $3 \cdot 10^{-5}$ M, respectively. In this case, CHLOR oxidation proved to be predominantly due to the action of radicals in the first stage, and direct ozonation in the second stage. In the case of PYRI, this pesticide was found to be highly reactive to O_3 in a previous study (Ochir et al., 2021). An equivalent molar amount of O_3 relative to PYRI (2 µM, corresponding to 0.4 ppm) was supplied, which was completely removed after 20 min. These results showed that the pesticides detected were successfully eliminated by ozonation, hence the next step was the treatment of the RW, in which these pesticides are intrinsically present.

Fig. 8.5 (a) shows the results of pesticide concentrations during the ozonation treatment of the original RW [Fig. 8.1-2)]. As occurred with the spiked ultrapure water, all three pesticides were completely removed using single ozonation. However, a significant longer treatment period of up to 720 min was needed in the case of RW [Fig. 8.5 (a)], instead of less than 240 min when treating ultrapure water with a higher initial concentration of these pesticides (Fig. 8.4). This slower degradation was attributed to the complex matrix of the RW, which contained a considerable amount of natural organic matter (Table 8.1) that probably interacted with ozone and/or its radicals, decreasing the treatment efficiency.

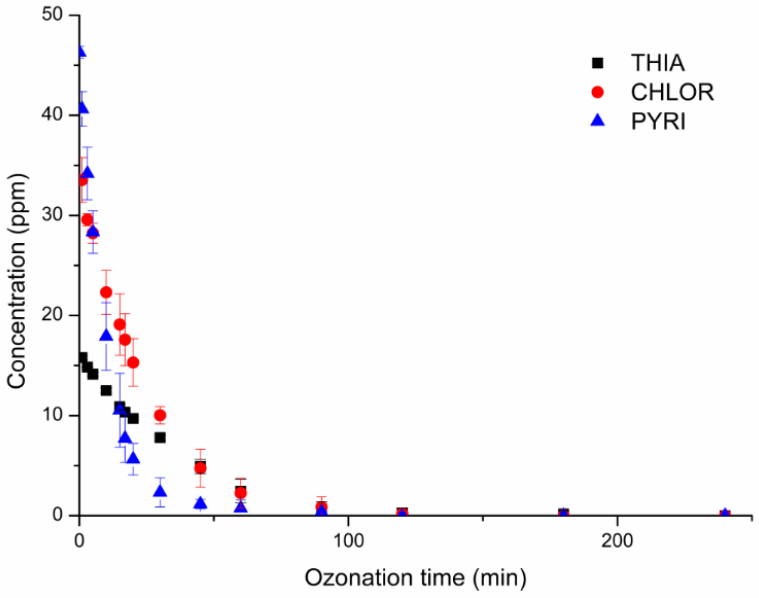


Fig. 8.4. Evolution of THIA, CHLOR, and PYRI for ozonation (O_3) with an inlet concentration of 50 g O_3 Nm^{-3} and flow rate of 15 Ncm^3 min^{-1} in the gas phase in experiments performed with spiked ultrapure water. Values are means ± standard deviation for triplicate samples.

On the other hand, Fig. 8.5 (b) shows the results of the pesticide concentrations during the ozonation treatment of the fungal-treated effluent [Fig. 8.1-3)]. The effluent produced throughout the fungal treatment in the RDB was stored in an accumulation tank to be subsequently treated by ozonation. For this reason, the initial concentrations in Fig. 8.5 (b) are lower than those in Fig. 8.5 (a). Despite the lower initial pesticide concentrations, the same treatment time (720 min) used for the original RW was required to completely remove the pesticides. This result may be due to the fact that during the RDB treatment, despite the decrease in pesticide concentration, some soluble compounds were extracted from the wood, increasing the content of natural organic matter (Table 8.5 in Section 8.3.8). This finding has also been described in Chapters 5 and 7.

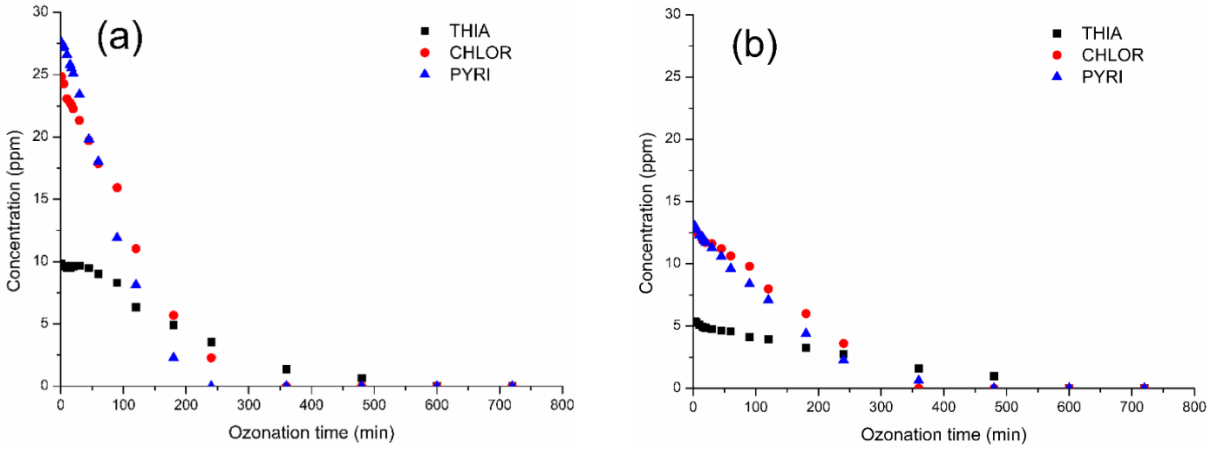


Fig. 8.5. Evolution of THIA, CHLOR, and PYRI for ozonation (O_3) with an inlet concentration of 50 g O_3 Nm^{-3} and flow rate of 15 Ncm^3 min^{-1} in the gas phase in experiments performed with (a) the original RW and (b) the fungal-treated RW.

In addition, the data were fitted to zero-order, first-order and second-order kinetic models to determine the best model and the respective degradation kinetics. In this regard, Fig. 8.6 shows the data of relative concentrations and the degradation kinetics with the best fit for each case. First-order kinetics were best adjusted to the results of the ultrapure water experiment, i.e., the degradation rate was directly proportional to the pesticide concentration. In contrast, the degradation of the pesticides was closer to zero-order kinetics for RW treatment, in which the degradation rate was independent of the pesticide concentration. These results were ascribed to the effect of natural organic matter content, rather than pH (spiked ultrapure water: pH 6.02; rinse wastewater: pH 7.64; RDB effluent: pH 4.50). In the case of RW and RDB effluent, the concentration of the pesticides had a negligible effect on the degradation rate because ozone (and its radicals) can also act non-selectively with the intrinsic organic matter of the matrix.

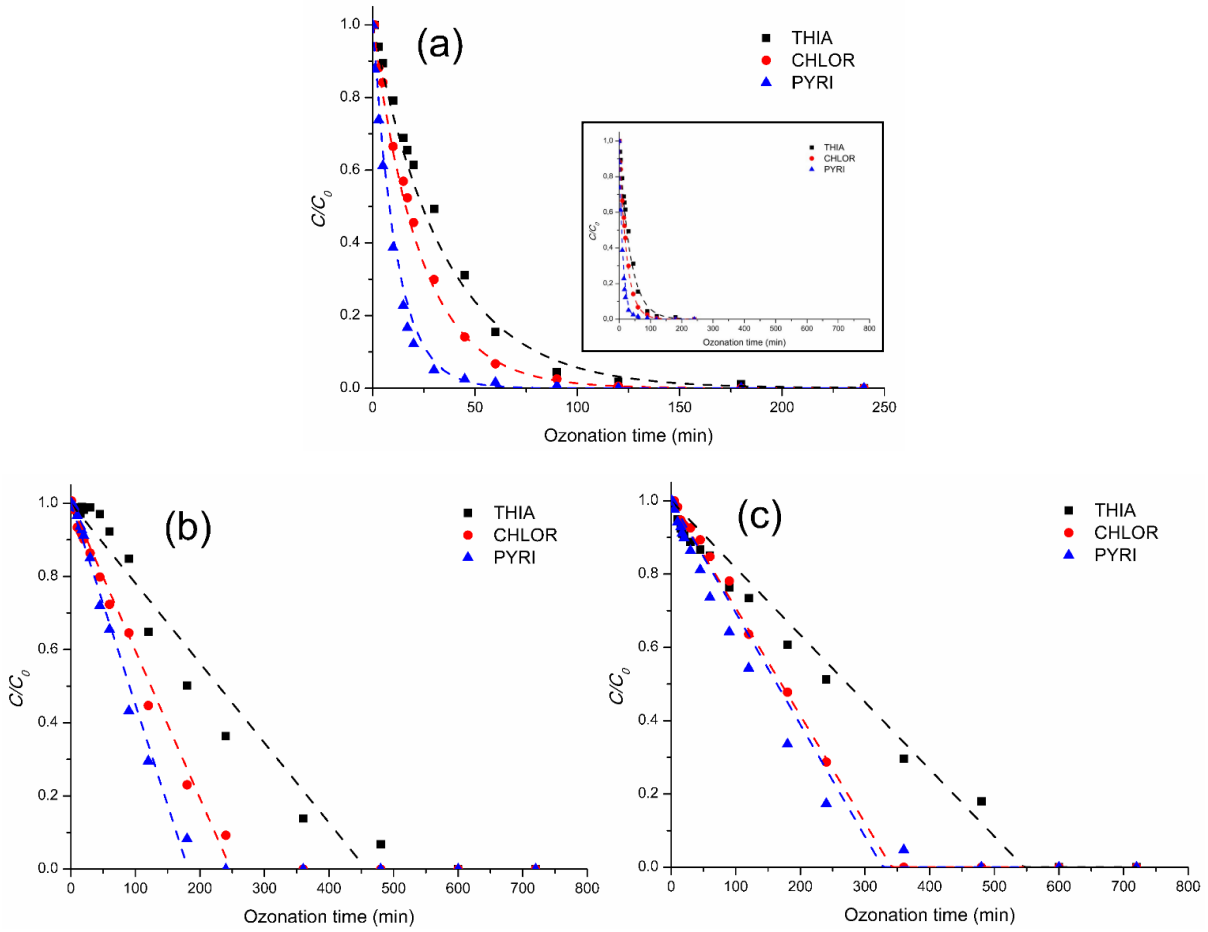


Fig. 8.6. Kinetics of degradation by ozonation (O_3) with an inlet concentration of 50 g O_3 Nm^{-3} and flow rate of 15 Ncm^3 min^{-1} of the gas phase in experiments performed with (a) ultrapure water (which includes a small figure showing the complete treatment period), (b) the original RW and (c) the fungal-treated effluent.

Table 8.4 shows the results of the best fits as well as the kinetic constants determined in each case. As can be seen, the reactivity in ozonation was PYRI > CHLOR > THIA. The higher reactivity of PYRI can be related to the nitrogen bridges between the phenyl and primidyl rings (Karaca et al., 2012). In addition, the kinetic constants for the degradation rates of the pesticides in the RDB effluent were lower than those in the RW, which was attributed to the higher organic content present in the former.

Table 8.4. Kinetic constants obtained for the fitting of pesticide degradation by ozonation.

Water	Kinetics	THIA	CHLOR	PYRI
Ultrapure water	First-order	R^2 = 0.964 k = 0.029 min^{-1}	R^2 = 0.998 k = 0.043 min^{-1}	R^2 = 0.977 k = 0.087 min^{-1}
Original RW	Zero-order	R^2 = 0.961 k = 0.022 mg L^{-1} min^{-1}	R^2 = 0.990 k = 0.098 mg L^{-1} min^{-1}	R^2 = 0.986 k = 0.152 mg L^{-1} min^{-1}
RDB effluent	Zero-order	R^2 = 0.983 k = 0.009 mg L^{-1} min^{-1}	R^2 = 0.994 k = 0.037 mg L^{-1} min^{-1}	R^2 = 0.963 k = 0.038 mg L^{-1} min^{-1}

8.3.5. Technology comparison in terms of pesticide removal

Fig. 8.7 shows the percentages of removal of the studied pesticides (in terms of DOC) achieved by direct ozonation of the original RW (blue squares) and by the treatment train, i.e., fungal bioremediation (in the RBD) followed by ozonation (green circles), during the ozonation period. The results of the treatment train (bioremediation + ozonation), started with an elimination value of 50.43 %, which corresponds to the average elimination achieved during the fungal treatment in the RBD (HRT = 5 days, treatment period = 67 days). Note that to achieve the same level of removal, direct ozonation required approximately 103 minutes. Comparing both curves, it can be deduced that bioremediation had a positive contribution during the first 168 minutes of the ozonation treatment when approximately 77 % removal was reached. From this period onwards, the removal efficiencies were practically identical in both cases, until reaching 100 % elimination. Therefore, this study suggests that the treatment train would only be appropriate when pesticide removals of less than 77 % are required. However, other factors should be considered to facilitate the selection between direct ozonation and the treatment train, such as TPs, toxicity, organic matter content and operating costs.

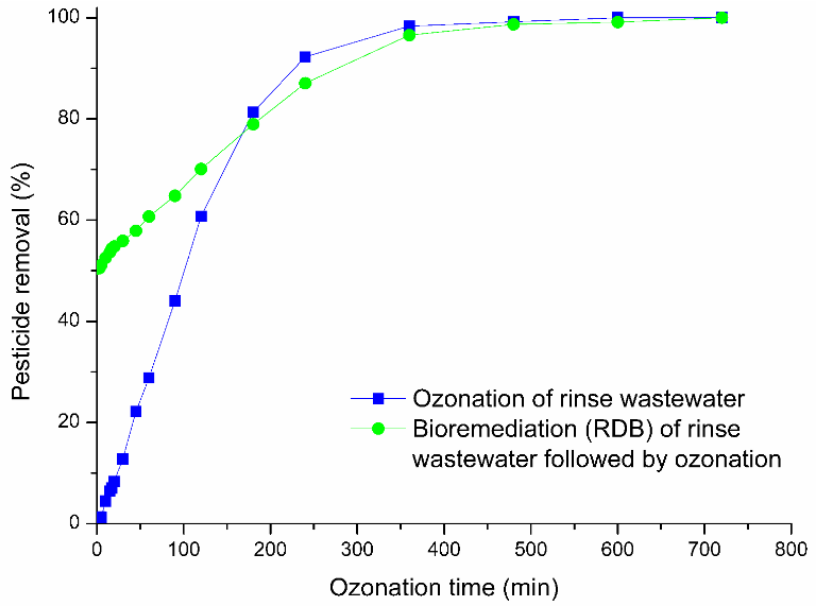


Fig. 8.7. Pesticide removals achieved by ozonation applied to the original RW (blue squares) and the fungal-treated effluent (green circles). The results only correspond to the ozonation period

8.3.6. Technology comparison in terms of transformation products

Ozonation is a process that is well known to generate TPs, including different types of low-molecular-weight carboxylic acids (Moreira et al., 2016). In this regard, three ozonation TPs (oxalic, oxamic, and maleic acids) were detected and monitored in the present study. Fig. 8.8 shows the evolution of the concentrations of the TPs during the ozonation experiment with ultrapure water. Ozonation of the spiked pesticides produced oxalic and oxamic acids (at quite similar concentrations), while no maleic acid was detected. Oxalic acid has been identified as one of the most typical TPs in the ozonation of several organic compounds. Oxamic acid is also common in the ozonation of organic compounds containing nitrogen functional groups and is considered to be an even more refractory TP than oxalic acid (Faria et al., 2008).

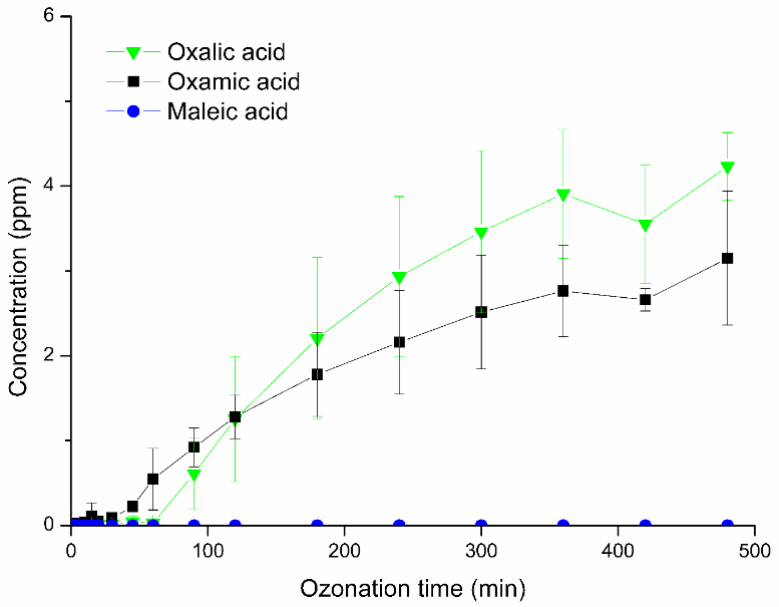


Fig. 8.8. Oxalic (green triangles), oxamic (black squares) and maleic (blue circles) concentrations over time in ozonation treatment with ultrapure water. Values are means ± standard deviation for triplicate measurements.

The same analysis of the TPs was performed for the RW treated by fungal bioremediation only (results not shown). Neither oxamic nor maleic acids were produced by fungal bioremediation. Maleic acid has been proposed as a potential metabolite in the degradation pathway of some pesticides by *T. versicolor*, although it has not yet been detected (Hu et al., 2022). No literature was found on the production of oxamic acid. Regarding oxalic acid, it is well known that WRF secrete oxalic acid during their secondary metabolism to degrade cellulose (Dutton et al., 1994), but this compound was not detected in the experiments with RW most probably due to the interferences with the matrix.

Fig. 8.9 shows the results obtained with single ozonation (red circles) and the treatment train (blue circles) applied to the RW (the results with UP water are also included for comparison purposes). Oxamic and maleic acids were detected in these experiments. Although oxalic acid was most probably also generated by ozonation of the RW, it could not be detected in the present work because it was interfered with the matrix in the chromatographic analysis. It is interesting to observed that maleic acid was produced by ozonation of the RW, which was not detected in the treatment of spiked ultrapure water, and thus its occurrence was attributed not to the oxidation of the identified pesticides, but to the degradation of other organic compounds contained in the matrix. Degradation of maleic acid by ozonation is known to produce glyoxylic

and formic acid, but none of these compounds were detected most probably because they were produced at very low concentrations and interfered with the signal of the matrix (Ramseier and von Gunten, 2009).

Fig. 8.9 also shows that the introduction of a fungal bioremediation process before the ozonation stage significantly reduced the production of oxamic and maleic acids. This favorable result was attributed to the powerful enzyme system of *T. versicolor*, which can degrade a wide range of organic compounds, thus limiting the production of potential metabolites (Mir-Tutusaus et al., 2018a). Therefore, these results support the use of the treatment train instead of direct ozonation to reduce the production of the studied TPs. A final stage of conventional activated sludge could also be applied, either *in-situ* in the agricultural field or in a WWTP, to achieve the complete elimination of these TPs (Nakamura et al., 2004).

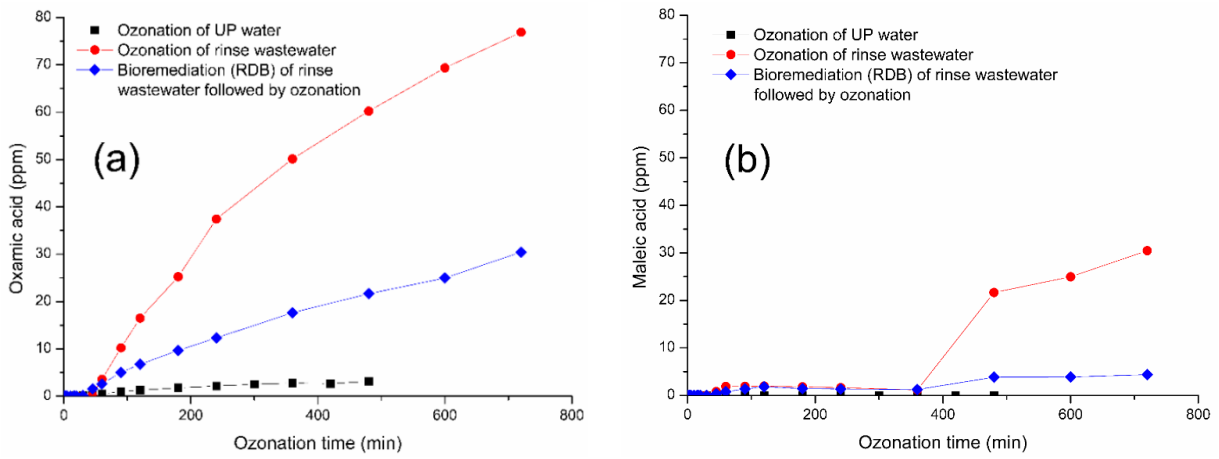


Fig. 8.9. Oxamic (a) and maleic (b) acids generated over time as TPs by the following treatments: ozonation of ultrapure water (black squares), ozonation of RW (red circles) and bioremediation of RW followed by ozonation (blue rhombuses).

8.3.7. Effect of increasing the pH or adding methanol

The effects of pH increase or adding MeOH on pesticide removals were evaluated since both strategies are considered to promote ozone decomposition and •OH radical formation (Buffle et al., 2006). For this purpose, (i) the pH of the RDB effluent (pH 4.5) was increased to that of the original RW by adding NaOH (pH 7.6), and (ii) maintaining the pH 4.5, a small volume of 2 % MeOH (v/v) was added to the RDB effluent (Ofori et al., 2018).

Fig. 8.10 (a) shows the degradation of the pesticides (in terms of DOC) and Fig. 8.10 (b) and (c) show the formation of TPs, i.e., oxamic and maleic acids, respectively. Adding MeOH down the degradation rate of the pesticides and, as a consequence, the oxamic acid formation was slightly slower. Regarding the pH, Ochir et al. (2021) recently demonstrated that the removal efficiency of pyrimethanil was strongly pH-dependent, rising from 64 % removal at pH 5 to 99 % at pH 7. Nevertheless, in the present study, the pH increase strategy marginally improved the rate of pesticide degradation and had little or no effect on the generation rate of low-molecular-weight carboxylic acids. Therefore, these strategies did not really reduce the treatment period required for the removal of these pesticides, nor the generation of TPs, and thus should be discarded in order to avoid higher costs.

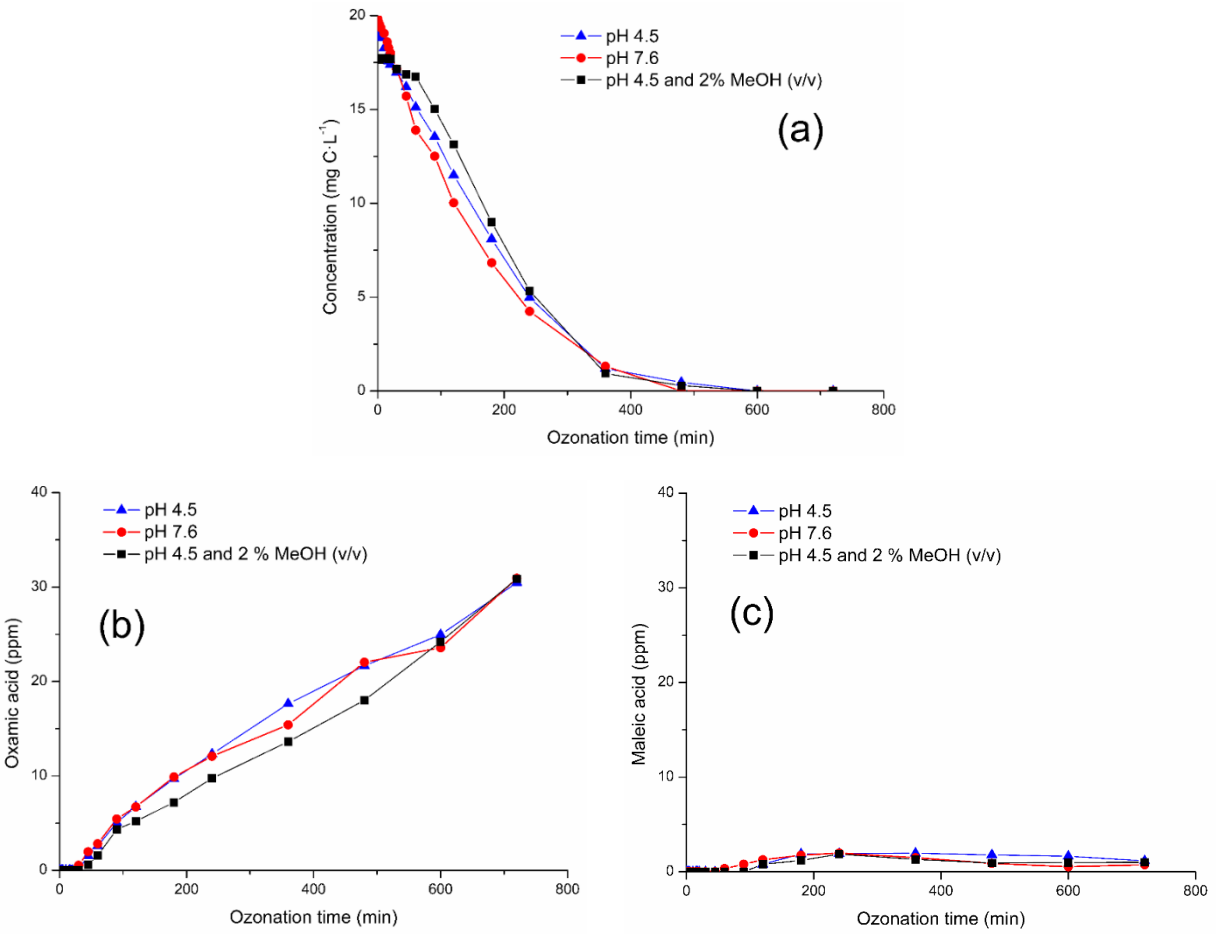


Fig. 8.10. Effects of pH increase (to pH = 7.6) and adding 2 % MeOH (v/v) on: (a) ozonation of pesticides (in terms of DOC), and (b) oxamic (b) and maleic acids concentrations.

8.3.8. Study of other parameters

Table 8.5 shows the results of other key parameters of the original RW and the effluent from each treatment. The initial pH of the original RW was 7.64, but it was maintained at 4.50 during the treatment in the fungal RDB. The pH was not controlled in any ozonation treatment, being spontaneously reduced from 7.64 to 4.38 and 3.12 in the direct ozonation of RW and the ozonation stage of the treatment train, respectively. The color was measured by absorbance, which increased during the bioremediation treatment. This increase was attributed to the extraction of soluble organic compounds from the wood, some of which give the characteristic dark color to *Q. ilex* wood (Chapter 5 and 7). This effluent was appreciably clarified by ozonation, which is a well-known process for removing color from the medium (Mezzanotte et al., 2013).

Table 8.5. Characterization parameters of the original RW and the differently treated effluents.

Sample	pH	Color (abs)	COD (mg O_2 L^{-1})	BOD_5 (mg O_2 L^{-1})	BOD_5/COD ratio	DOC (mg C L^{-1})	Toxicity (TU)	HPCs (CFU mL^{-1})
Original RW	7.64	0.713	4263	135	0.03	1527	13.53	$2.10 \cdot 10^5$
Biorem. effluent	4.50	1.068	11180	1588	0.14	2981	13.49	$6.82 \cdot 10^5$
O_3 effluent	4.38	0.811	2944	215	0.07	1072	16.37	< LOQ
Effluent from Biorem. + ozonation	3.12	0.654	10030	3050	0.30	2656	12.06	< LOQ

Note: LOQ is limit of quantification. In all cases, standard deviations below 5 % toxicity (triplicate) and 2 % HPCs (duplicate) were obtained.

One of the most important characteristics of this RW was its high organic matter content, especially if compared to typical agricultural wastewaters. In the literature, contradictory results have been reported on the ability of *T. versicolor* to remove COD (Cruz-Morató et al., 2013a; Section 6.3.1.3, Chapter 6). In the present work, the organic matter content was drastically increased during the bioremediation stage, again mainly attributed to the release of organic compounds from the wood. In contrast, ozonation was able to reduce the COD, especially in the case of direct ozonation of RW (from 4263 to 2944 mg O_2 L^{-1} in 720 min).

Similar conclusions were obtained when analyzing the DOC, which increased during the bioremediation process and decreased considerably with ozonation, particularly with direct

ozonation (from 1527 to 1072 mg C L^{-1} in 720 min). Ozonation has already been shown to completely or partially degrade parent compounds, but it is often unable to fully mineralize them due to the formation of various types of TPs, such as low-molecular-weight carboxylic acids (Moreira et al., 2015). Table 8.6 shows the theoretical contributions of the pesticides and the TPs to the total DOC, which was experimentally measured. Regarding the results of degradation with ultrapure water, it can be seen that ozonation completely degraded the pesticides after 4 h of treatment, but not the studied TPs even after 8 hours. In the case of the RW, the contribution of the pesticides and TPs detected to total DOC was very low, indicating the existence of significant unidentified organic matter.

Table 8.6. Pesticide and TPs contribution to the DOC during the ozonation treatment.

		Ozonation time (h)			
		0	4	8	12
Ozonation of UP water	Pesticides (mg C L^{-1})	59.9	0.0	0.0	-
	Oxalic acid (mg C L^{-1})	0.0	0.8	1.1	-
	Oxamic (mg C L^{-1})	0.0	0.6	0.9	-
	Maleic acid (mg C L^{-1})	0.0	0.0	0.0	-
Ozonation of RW	DOC (mg C L^{-1})	1527.2	1398.8	1114.0	1072.8
	Pesticides (mg C L^{-1})	38.5	3.0	0.3	0.0
	Oxalic acid (mg C L^{-1})	*	*	*	*
	Oxamic (mg C L^{-1})	0.0	10.1	16.2	20.7
	Maleic acid (mg C L^{-1})	0.0	0.6	1.6	1.8
Bioremediation of RW followed by ozonation	DOC (mg C L^{-1})	2980.8	2709.2	2700.4	2656.4
	Pesticides (mg C L^{-1})	19.1	5.0	0.5	0.0
	Oxalic acid (mg C L^{-1})	*	*	*	*
	Oxamic (mg C L^{-1})	0.0	3.3	5.8	8.2
	Maleic acid (mg C L^{-1})	0.0	0.0	0.7	0.5

Note: total DOC was obtained experimentally, while the DOC of oxamic, oxalic and maleic acid was calculated theoretically from their respective concentrations.
*Not determined due to interference with the matrix.

The BOD_5/COD (Table 8.5) showed that the original RW, the RDB effluent and the ozonated RW, were non-biodegradable (<0.2) (Metcalf and Eddy, 2003). The only effluent that was found to be moderately biodegradable was that resulting from the treatment train. This is an important finding in favor of the treatment train since the effluents obtained would require a further biological treatment according to current regulations in Catalonia, such as a conventional activated sludge treatment, which could be performed *in-situ* or in a WWTP (DOGC, 2003).

Regarding the toxicity (Table 8.5), no major changes were observed, being slightly reduced by bioremediation and somewhat increased by ozonation of the RW. The increased toxicity in ozonation was ascribed to the generation of some TPs, which can be even more toxic than the parent compounds (Cruz-Morató et al., 2013b). Interestingly, a more remarkable decrease in toxicity was obtained in the case of the treatment train, suggesting some synergistic effect when combining both technologies.

Finally, the number of colony-forming units of total heterotrophs (at 37 ºC) was also analyzed (Table 8.5). Bacterial counts rose slightly after 67 days of bioremediation treatment while remaining below the LOQ in the ozonation effluent. Ozone has already proven to be a good strategy for bacterial removal (Gorito et al., 2021). In this case, no bacterial regrowth was observed even after keeping the samples in the refrigerator for several weeks.

8.3.9. Technology comparison in economic terms

8.3.9.1. Fungal bioremediation costs

The most significant bioremediation costs were related to electricity consumption. In this case, the HRT used in the RDB was considered as the calculation base, i.e., 5 days, which is the time required to treat 2.7 L of RW. Bioremediation costs include inlet pumping (BP), recirculation pumping (BR), air pumping (BA), magnetic stirring of recirculated RW (BS), rotation of the inner tube (BRot) and preparation of the mycelial suspension (M), as shown in Eq. (8.1).

$$B = BP + BR + BA + BS + BRot + M \qquad (8.1)$$

Each of these costs is in turn composed of the power of the equipment (W) multiplied by the operating time (t). Note that laboratory-scale devices were used, which are generally less efficient than industrial equipment. In the case of inlet pumping consumption, the pump has a maximum flow rate of 0.60 L h^{-1} and the filling time was 4.50 hours (2.7 L), thus:

$$BP = BP_W \cdot BP_t = 5\,(W) \cdot 4.50\,(h) = 22.50\,Wh \qquad (8.2)$$

The recirculation flow rate was 4.7 L day^{-1}, so it can be considered that the pump, with a maximum flow rate of 0.6 L h^{-1} and a maximum power of 5 W, worked for 39.17 hours during 5 days.

$$BR = BR_W \cdot BR_t = 5\ (W) \cdot 39.17\ (h) = 195.85\ Wh \qquad (8.3)$$

Aeration was supplied from day 28 onwards, i.e., for 39 days, by using a general pump that worked for several installations. However, to simplify calculations, a small 0.5 W positive displacement electric pump was assumed to be used.

$$BA = BA_W \cdot BA_t = 0.5\ (W) \cdot 69.85\ (h) = 34.93\ Wh \qquad (8.4)$$

The agitation in the recirculation tank (BS) was active during the entire operating period, being its cost significant and thus taken into account in the calculations. However, agitation of the feed tank was performed punctually four times per day, hence it was considered negligible and was excluded from the cost estimation.

$$BS = BS_W \cdot BS_t = 2.5\ (W) \cdot 120\ (h) = 300\ Wh \qquad (8.5)$$

The power consumption resulting from the rotation of the internal drum was obtained as shown in Eq. (8.6).

$$BRot = BRot_W \cdot BRot_t = 19.20\ (W) \cdot 0.014\ (h) = 0.27\ Wh \qquad (8.6)$$

Mycelial suspension preparation requires autoclaving (MA) and orbital shaking (MOS) steps, as described in Section 3.4.2 (Chapter 3). Malt extract is an expensive reagent, but it can be perfectly substituted by defined medium, as demonstrated by Borràs et al. (2008). Other costs, such as the purchase of *T. versicolor* culture, were considered negligible.

$$M = MA + MOS \qquad (8.7)$$

It can be reasonably assumed that preparation of the mycelium suspension can be performed every 6 months, as the RDB has been shown to require low biomass renewal (Beltrán-Flores et al., 2022, Chapter 5). Therefore, these costs should be distributed over 6 months, and then the cost related to a period of 5 days should be computed. The autoclave operated for 2 hours, whereas the orbital shaker worked for 5 days (120 h).

$$MA = MA_W \cdot MA_t = 2000\ (W) \cdot \frac{2\ (h) \cdot 5\ days}{182.5\ days} = 109.59\ Wh \qquad (8.8)$$

$$MOS = MOS_W \cdot MOS_t = 30\ (W) \cdot \frac{120\ (h) \cdot 5\ days}{182.5\ days} = 98.63\ Wh \qquad (8.9)$$

$$M = MA + MOS = 208.22\ Wh \qquad (8.10)$$

Therefore, the bioremediation energy consumption accounted for one HRT unit (5 days) was as follows:

$$B = BP + BR + BA + BS + BRot + M = 22.50 + 195.85 + 34.93 + 300 + 0.27 + 208.22 = 761.77\ Wh \qquad (8.11)$$

Given that the average price of electricity in Spain is 0.28374 € $(kWh)^{-1}$ (July 2022) the cost of bioremediation for each 2.7 L of treated water (5 days) was calculated as shown in Eq. (8.12).

$$B = 761.77\ (Wh) \cdot 0.28374\ €\ (\text{kWh})^{-1} = 0.22\ € \qquad (8.12)$$

Therefore, the cost per liter treated by fungal bioremediation is 0.08 € L^{-1}, as shown in Eq. (8.13).

$$B = \frac{0.22\ (€)}{2.7\ (L)} = 0.08\ €\ L^{-1} \qquad (8.13)$$

The economic analysis focused on the ozonation period for both direct ozonation and the treatment train, thus the bioremediation cost of the treatment train was computed as a fixed cost (independent of the ozonation time) prior to the ozonation stage (Section 8.3.9.3 and 8.3.9.4).

8.3.9.2. Ozonation costs

The economic study focused on the ozonation period, thus time-dependent energy consumption and costs were treated as function of ozonation time. Ozonation costs (O) can be separated into costs related to energy consumption (OE) and oxygen supply (OO).

$$O = OE + OO \qquad (8.14)$$

The electrical costs of ozonation consisted of the consumptions of the pump (OP), the ozone generator (OG) and the magnetic stirrer (OS):

$$OE = OP + OG + OS \qquad (8.15)$$

In turn, each of these costs was calculated on the basis of power consumption (W) and working time (t). OP was considered a cost that was incurred during reactor filling prior to the beginning of treatment, and thus was independent of the operating time. OP_t was calculated as the division of the reactor volume (700 mL) by the maximum pump flow rate (600 mL h^{-1}).

$$OP = OP_w \cdot OP_t = 5\ (W) \cdot 1.17\ (h) = 5.85\ Wh \qquad (8.16)$$

$$OG = OG_w \cdot OG_t = 80\ (W) \cdot OG_t(h) = 80\ OG_t\ Wh \qquad (8.17)$$

$$OS = OS_w \cdot OS_t = 2.5\ (W) \cdot OS_t(h) = 2.5\ OS_t\ Wh \qquad (8.18)$$

Given that OG_t and OS_t are equal to the ozonation time (O_t), and knowing the average price of electricity in Spain, the electrical cost of ozonation can be expressed as follows:

$$OE = OP + OG + OS = 1.65 \cdot 10^{-3} + 2.27 \cdot 10^{-2} OG_t + 7.09 \cdot 10^{-4}\ OS_t = (1.65 \cdot 10^{-3} + 2.34 \cdot 10^{-2} O_t)\ € \qquad (8.19)$$

The oxygen cost (OO) was calculated by multiplying the oxygen flow rate (OO_Q) by the treatment time (O_t) and by the specific cost of oxygen (OO_c), as shown in Eq. (8.20). Oxygen consumption was 150 mL min^{-1} (9 L h^{-1}) and the price of oxygen was 50.20 € for each 10.6 m^3 bottle (0.00474 € L^{-1}).

$$OO = OO_Q \cdot O_t \cdot OO_c = 9\ (L\ h^{-1}) \cdot O_t(h) \cdot 0.00474\ (€\ L^{-1}) = 4.27 \cdot 10^{-2}\ O_t\ € \qquad (8.20)$$

Therefore, the total cost of ozonation was:

$$O\ (1 - batch) = OE + OO = 1.65 \cdot 10^{-3} + 2.34 \cdot 10^{-2} O_t + 4.27 \cdot 10^{-2}\ O_t = [1.65 \cdot 10^{-3} + 6.61 \cdot 10^{-2}\ O_t(h)]\ € \qquad (8.21)$$

This cost was relative to each ozonation batch, in which 0.7 L of RW was treated. However, the same calculation basis of 2.7 L was used in order to compare the ozonation and bioremediation processes. For this purpose, the ozonation cost was multiplied by a factor of 3.86.

$$O = [6.38 \cdot 10^{-3} + 2.55 \cdot 10^{-1}\ O_t(h)]\ € \qquad (8.22)$$

Changing O_t units from hours to minutes:

$$O = [6.38 \cdot 10^{-3} + 4.23 \cdot 10^{-3}\ O_t(min)]\ € \qquad (8.23)$$

Therefore, the cost per liter treated by ozonation can be calculated using the Eq. (8.24).

$$O = \frac{[6.38 \cdot 10^{-3} + 4.23 \cdot 10^{-3}\ O_t(min)]\ (€)}{2.7\ (L)} = [2.36 \cdot 10^{-3} + 1.57 \cdot 10^{-3}\ O_t(min)]\ €\ L^{-1} \qquad (8.24)$$

8.3.9.3. Treatment train costs

Treatment train costs (T) were calculated by adding the costs of fungal bioremediation [Eq. (8.12)] and ozonation [Eq. (8.23)], as shown in Eq. (8.25).

$$T = B + O = 0.22 + [6.38 \cdot 10^{-3} + 4.23 \cdot 10^{-3}\ O_t(min)] = \\ = [0.23 + 4.23 \cdot 10^{-3}\ O_t(min)]\ € \qquad (8.25)$$

Therefore, the cost per liter treated by the treatment train can be calculated using the Eq. (8.26).

$$T = \frac{[0.23+4.23\cdot10^{-3}\ O_t(min)]\ (€)}{2.7\ (L)} = [8.52 \cdot 10^{-2} + \ 1.57 \cdot 10^{-3}\ O_t(min)]\ € L^{-1} \qquad (8.26)$$

8.3.9.4. Comparative study

The costs per liter treated by the ozonation and treatment train previously calculated in Eq. (8.24) and Eq. (8.26), respectively, were used to obtain Fig. 8.11 (a). As can be seen, the analysis focuses on the ozonation period, so other costs prior to this stage were considered as initial fixed costs. In this regard, the ozonation costs start from practically zero, since only the cost of filling the stirred tank was taken into account and was computed before the ozonation period. In the case of the treatment train, the bioremediation cost (in addition to the cost of filling the stirred tank in the ozonation stage) was considered fixed and computed at the beginning of the ozonation treatment [Eq. (8.26)], as shown in Fig. 8.11 (a). The slopes of both equations [Eqs. (24) and (26)], and thus those of their respective representations in Fig. 8.11 (a), are equal since they correspond to the ozonation cost per liter of treated wastewater.

To simplify calculations, pesticide removal curves of Fig. 8.7 were considered to follow a linear trend during the first 180 minutes of ozonation, obtaining $R^2 = 0.9972$ for the ozonation process [Eq. (8.27)] and $R^2 = 0.9964$ for the treatment train [Eq. (8.28)]. The units of these linear equations of DOC elimination were transformed from % to mg C, obtaining the equations Eq. (8.29) for the ozonation treatment and Eq. (8.30) for the treatment train. Afterwards, the specific costs [Fig. 8.11 (b)] of the direct ozonation of RW were obtained by dividing Eq. (8.23) and Eq. (8.29), and the specific costs of the treatment train were calculated by dividing Eq. (8.25) and Eq. (8.30).

$$Removal\ of\ pesticide\ DOC\ by\ ozonation = 0.4711 \cdot O_t(min)\ \ (\%) \qquad (8.27)$$

$$Removal\ of\ pesticide\ DOC\ by\ the\ treatment\ train = \\ = 0.1621 \cdot O_t(min) + 50.43\ \ (\%) \qquad (8.28)$$

$$Removal\ of\ pesticide\ DOC\ by\ ozonation = 0.4893 \cdot O_t(min)\ \ (\text{mg C}) \qquad (8.29)$$

$$Removal\ of\ pesticide\ DOC\ by\ the\ treatment\ train = \\ = 0.1643 \cdot O_t(min) + 52.81\ \ (\text{mg C}) \qquad (8.30)$$

The specific costs of ozonation are initially high because the cost of filling the reactor (OP) was considered a fixed cost and was computed at time 0, when pesticide elimination was inexistent. However, as the reaction progressed, the direct ozonation process seemed to be a more cost-effective treatment than the treatment train for removing pesticides. This result is probably explained by the fact that during the bioremediation process part of the organic matter of the wood was extracted, increasing the DOC of the water, which also interacts with ozone molecules and the generated radicals. According to Fig. 8.11 (b), the specific cost of ozonation was higher during the first 84 minutes, but then it was exceeded by the specific costs of the treatment train.

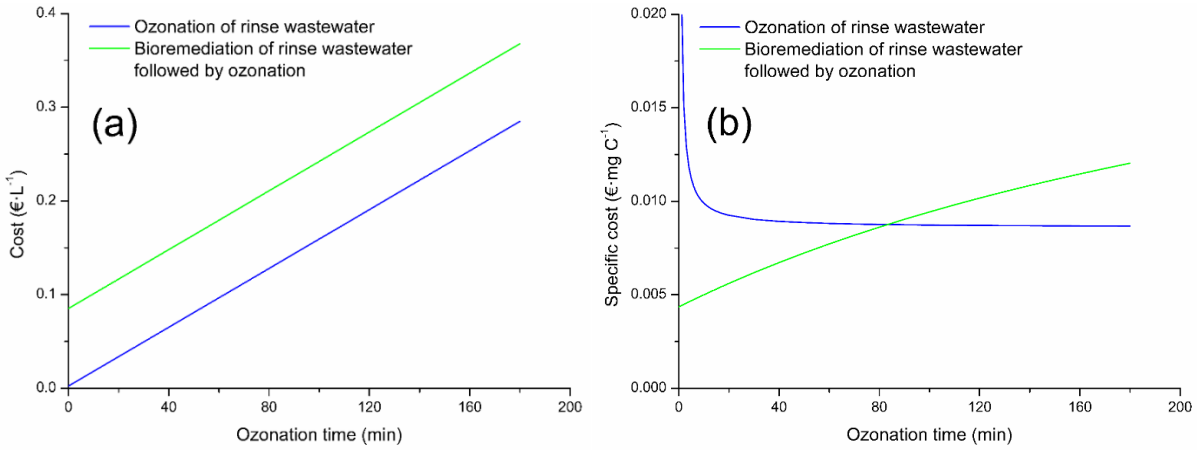


Fig. 8.11. Evolution of costs (a) and specific costs per mg of C removed (b) during the ozonation stage.

Another way of expressing these results that can further facilitate the selection between the different approaches is the correlation between the costs [Eqs. (8.23) and (8.25)] and the percentage of pesticides eliminated [Eqs. (8.27) and (8.28)], as shown in Fig. 8.12. For the same pesticide removal yield (≈ 50 %), the fungal bioremediation (0.22 €) proved to be 2.09 times cheaper than the ozonation treatment (0.46 €, Fig. 8.12), which corresponds to values of 4.20 10^{-3} € mg C^{-1} and $8.78 \cdot 10^{-3}$ € mg C^{-1}, respectively. However, bioremediation was only able

to remove 50 % (19.40 mg C L^{-1}) of the pesticides (38.47 mg C L^{-1} in the RW, Table 8.6). Ozonation was required to achieve higher removals, either directly applied or as the second stage in the treatment train. Fig. 8.12 indicates that the cost associated with the application of the treatment train was lower than that of direct ozonation for eliminations below 64 %. Therefore, these results show that the selection of any of these technologies in economic terms depends on the requirements for effluent quality. If the detected pesticides are supposed to be completely eliminated, direct ozonation is mandatory. In contrast, if the requirements for pesticide concentrations in the effluent are more flexible, which is often dictated by the current regulation, a treatment train (for eliminations between 50-64 %) or a bioremediation process (for elimination lower than 50 %) should be applied. However, it should be taken into account that the results of other studied parameters can also influence the selection of the most appropriate technology, such as effluent biodegradability and formation of TPs (Section 8.3.8).

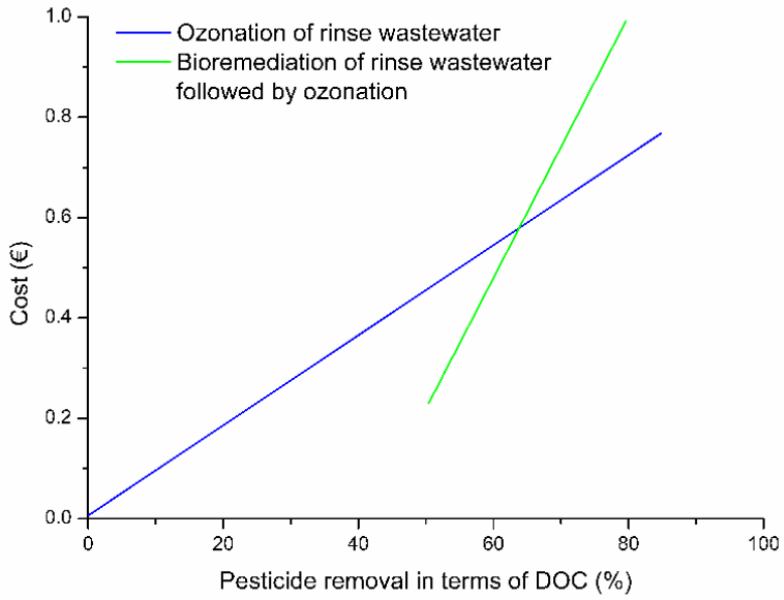


Fig. 8.12. Correlation between costs and pesticide removals in terms of DOC.

8.4. Conclusions

Fungal bioremediation achieved an average removal of the studied pesticides of 50 % (in terms of DOC) in a continuous RDB after 67 days (HRT = 5 days). The ozonation treatment in batch mode proved to be more effective in removing pesticides, achieving complete removal after 4 hours in ultrapure water, and after 12 hours in RW and RDB effluent (treatment train).

Bioremediation, unlike ozonation, did not produce any of the studied TPs, and even reduced their generation by ozonation when applied in an upstream stage in the treatment train. Regarding other conventional parameters, ozonation was more effective in reducing COD (31 %), DOC (30 %) and HPCs (100 %). Moreover, the only effluent that was found to be moderately biodegradable was that from the treatment train ($BOD_5/COD = 0.30$). The cost study showed that the fungal bioremediation treatment was more cost-effective ($4.20 \cdot 10^{-3}$ € mg C^{-1}) compared to ozonation ($8.78 \cdot 10^{-3}$ € mg C^{-1}). According to the economic cost study, fungal bioremediation would be recommended for pesticide removals below 50 %, the treatment train between 50-64 %, and direct ozonation for removals above 64 %. Therefore, the treatment train proved to be an interesting approach that integrates the advantages of both processes, thus offering more flexibility to the relationship between effluent quality and operating costs.

Chapter 9

General conclusions

Based on the results obtained throughout this thesis, some main conclusions can be drawn as shown below:

- The RDB with *T. versicolor* immobilized on holm oak wood is a good strategy for the long-term treatment of agricultural wastewater.
- Sorption by the lignocellulosic support played a predominant role in pesticide removal, especially for hydrophobic compounds.
- Holm oak wood proved to be a better lignocellulosic support than pine wood for *T. versicolor* immobilization in terms of growth and maintenance of fungal biomass.
- Lower rotation frequencies and higher hydraulic retention times in the RDB favored the maintenance of fungal biomass and the degradation efficiency of the studied pesticides in a long-term treatment.
- The biopile treatment proved to be a promising alternative for the treatment of old contaminated wood. The combination of the inoculated RDB with the re-inoculated biopile showed the best results in terms of fungal biomass content and pesticide degradation.
- *T. versicolor* was oxygen limited when the dissolved oxygen level was below 15 %, thus this variable should be carefully monitored in fungal bioreactors. In the case of the RDB, oxygen limiting conditions were reached after 7.8 h, thus the introduction of forced aeration is recommended. However, despite oxygen limiting conditions, fungal biomass has proven to remain active and immobilized on wood in the RDB even for a long-term treatment.
- A cross-sectional study showed that *T. versicolor* grew at a constant rate on holm oak wood during the growing stage (up to 50 days) and that the fungus can consume complex carbon sources such as acetate, thereby reducing COD.
- The RDB was found to be more efficient than the FBB in treating agricultural rinse wastewater. The comparative study between the fungal and ozonation technologies showed that each technique provided better results depending on the studied variable and that the combination of both technologies in a treatment train was a good approach to adapt the process to the desired effluent quality, minimizing operational costs.

Chapter 10

Future work

In future work, a proof-of-concept project is planned to validate the innovative value of the research conducted in this thesis and whether the techniques developed can be incorporated into the market and generate value for society. Specifically, we intend to apply the RDB at full scale and *in-situ* in agricultural fields for the treatment of rinse wastewater, taking into account the following considerations:

- The biopile-like treatment should be optimized, e.g. by adjusting the moisture content of the wood, the inoculation method of the fungal biomass and the wood size.
- The RDB effluent should be carefully characterized to assess whether it can be reused for irrigation, sent to WWTP or discharged directly into the environment in accordance with current legislation.
- Aeration is not mandatory but could improve the process efficiency.
- Alternative treatment trains can be investigated, e.g. by coupling a fungal bioremediation process to the concentrated effluent rejected from a membrane process (e.g. nanofiltration or reverse osmosis).

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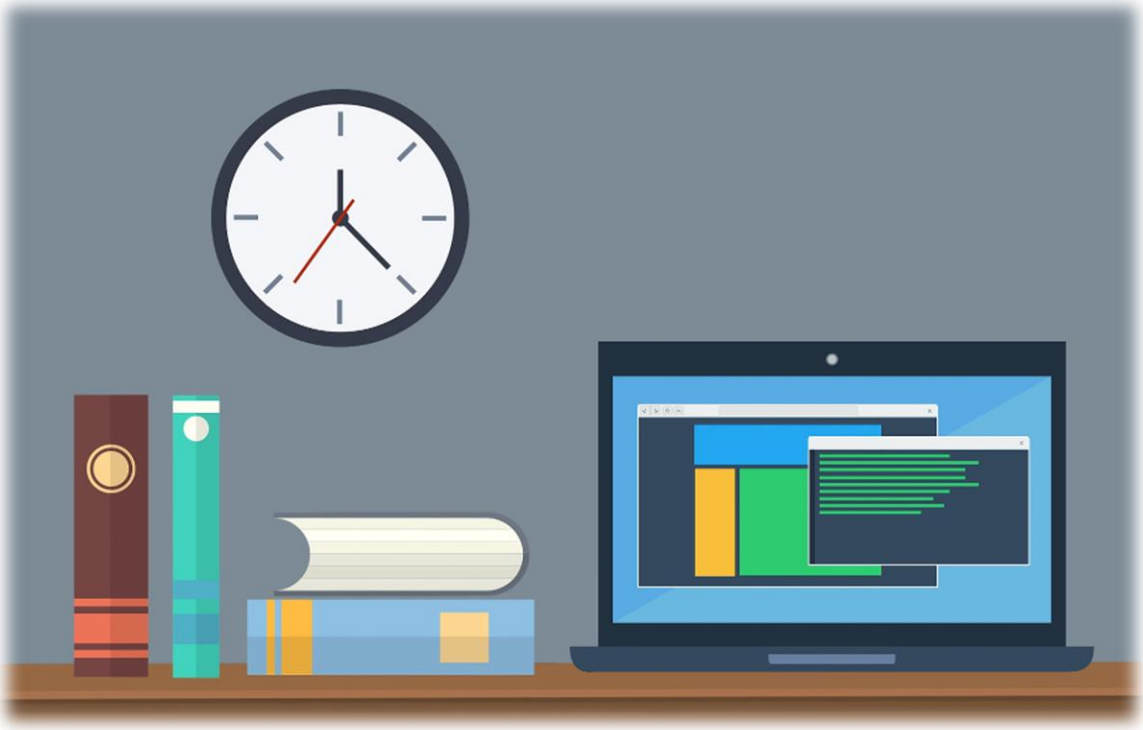

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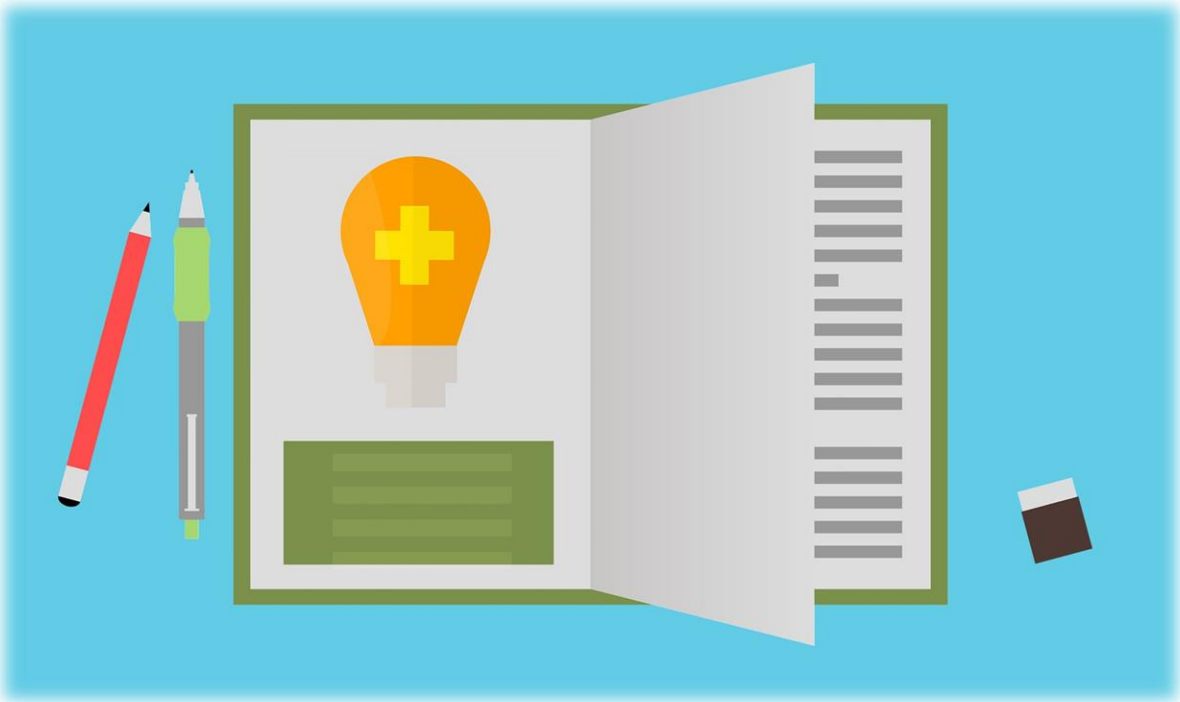

Paper published on indexed journals

- Beltrán-Flores, E., Torán, J., Caminal, G., Blánquez, P., Sarrà, M., 2020. The removal of diuron from agricultural wastewaters by *Trametes versicolor* immobilized on pinewood in simple channel reactors. Sci. Total Environ. 728, 1–10. https://doi.org/10.1016/j.scitotenv.2020.138414
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- Beltrán-Flores, E., Pla-Ferriol, M., Martínez-Alonso, M., Gaju, N., Sarrà, M.*, Blánquez, P. Fungal treatment of agricultural washing wastewater: comparison between two operational strategies. Journal of Environmental Management, under revision.
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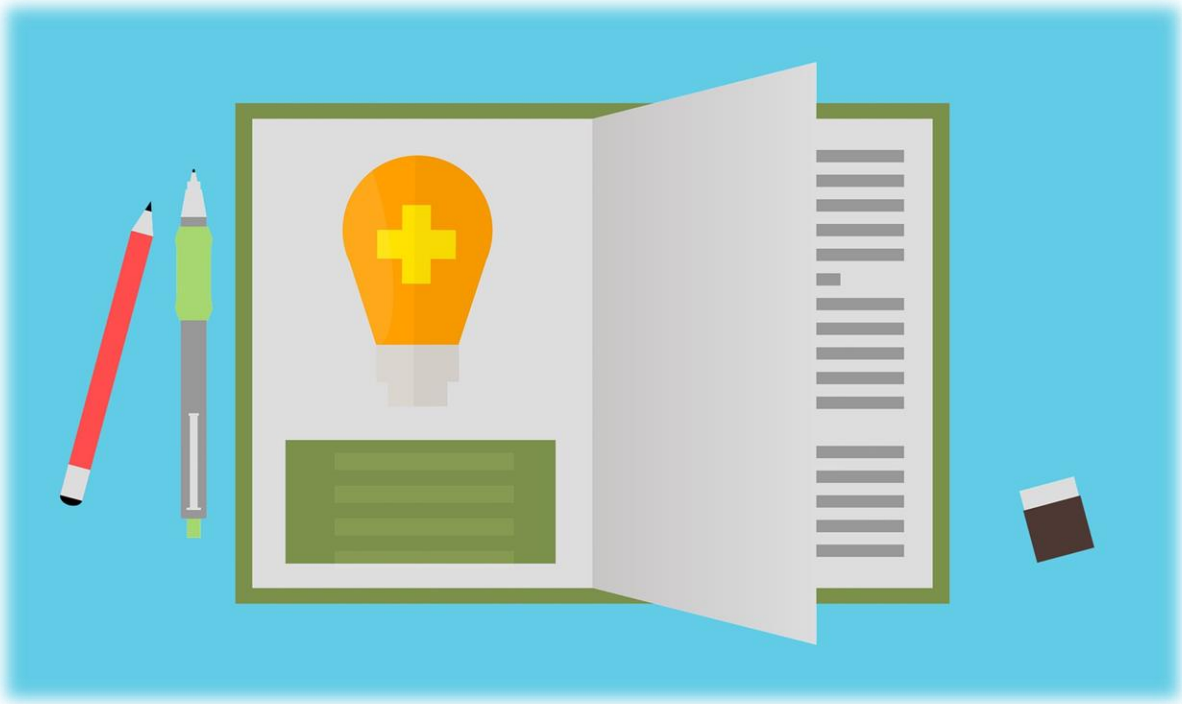
- Beltrán-Flores, E., Blánquez, P., Sarrà, M*, Silva, A.M.T. Rinse wastewater treatment by combining fungal bioremediation and ozonation. Water Research, under revision
- Beltrán-Flores, E., Sarrà, M.*, Blánquez, P. Rinse water management in the agricultural sector: A review. Science of the Total Environment, under revision.

Annexes



Annex A

Pesticide analysis for the real water

A proposal for the identification of the pesticides detected in the agricultural wastewater was performed by HPLC (HPLC, 1200RR, Agilent Technologies). The HPLC was equipped with a diode-array detector (DAD) and a microTOF-Q Mass Spectrometer (Bruker Daltonics, Bremen, Germany). Briefly, 1 mL of sample was withdrawn and filtered through a 0.22 µm Millipore Millex-GV PVDF filter. Chromatographic separation was performed using an C18 reversed-phase column (Phenomenex®, Kinetex® EVO C18 100 Å, 5 µm, 4.6 mm × 150 mm) with the following operating conditions: 30 ºC, eluent flow rate of 0.9 mL min-1 and injection volume of 40 µL. The mobile phase consisted of 0.01 % formic acid solution and acetonitrile, which was pumped isocratically at a ratio of 50:50.

Analytes were first detected by DAD using a wavelength of 252 nm. Afterwards, the eluent was split using a T-type splitter before entering the QTOF (split = 1:2). Thus, the flow rate reaching the ESI-Q-TOF-MS detector was 0.3 mL min^{-1}. Samples were analyzed in the positive electrospray mode (ESI). MS data were acquired at a m/z range of 50-1000 Da. Spray voltage was set at 2000 V and the drying gas temperature was 210 ºC. The capillary voltage of the ion source was 5000 V. The dry gas flow rate was set at 8.0 L min^{-1} and the nebulizer gas pressure was 4.0 bar. Nitrogen was used as both drying and nebulized gas. The pre-pulse storage time was 8 µs and the source transfer time was 65.0 µs. Instrument calibration was performed by using a 10 mM sodium formate solution.

The MS data were processed using Bruker Compass DataAnalysis 4.2 (Bruker Daltonics, Bremen, Germany). Candidate compounds were identified on the Chemspider website by their exact masses (Royal Society of Chemistry, 2015). Finally, confirmatory analyses and quantification were conducted using the analytical standards of the detected pesticides. In this case, the analysis was performed using the same analytical method in a Dionex Ultimate 3000 HPLC-UV. Under these conditions, the retention times were approximately 3.27, 4.29, 6.01, 7.87 and 8.23 min for THIA, CHLOR, PYRI, AZO and TEBU, respectively.

Annex B

DNA extraction and PCR-DGGE of the fungal community

Effluent and lignocellulosic support samples from the bioreactors were externally collected at regular intervals, immediately stored at 4ºC and delivered within a few hours. Liquid samples were then filtered through 0.22 µm filters (Durapore®: PDVF membrane, 47 mm diameter), using a vacuum filtration pump (Millipore). After that, dry filters were stored at -80ºC until DNA extraction. Solid samples, instead, were processed to separate the fungal mycelium from the wood chips by mechanical force (sterile forceps), followed by agitation (30 min., 200 RPM), and sonication (7 min.) in phosphate buffer (0.1 M, pH 7.0).

Total DNA extraction was then performed on filters using DNeasy® PowerWater DNA extraction kit (Qiagen), and on solid samples using DNeasy® PowerSoil DNA extraction kit (Qiagen) always following manufacturer instructions. After extraction, all DNA samples were stored at -20ºC until further processing. These samples were subsequently amplified by nested PCR to reduce non-specific products and increase sensitivity, using two sets of specific primers targeting fungal ITS (Internal Transcribed Spacer) region. The reaction mix was prepared on a final volume of 50 µL, containing 10-50 ng of DNA template, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 µM each of dATP, dCTP, dGTP and dTTP premixed, 12.5 pmoles of each primer and 0.5 U of Taq DNA polymerase enzyme (Invitrogen, Waltham, Massachusetts, USA).

The primer pair used for the first round of the nested PCR was EF4 and ITS4 (Gardes and Bruns, 1993), while primers ITS1F and ITS2R (White et al., 1990) were utilized for the second round. A GC-clamp (5'- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C -3') was attached to the 5’ end of the primer ITS1F to prevent complete denaturation for DGGE profiling. All PCR reactions were carried out in a Bio-Rad Thermocycler (model S1000) programmed for 35 cycles, each of 30s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, an initial denaturation step of 94 °C for 5 min, and a final extension step at 72 °C for 7 min. The specific amplification products were analyzed using agarose gel (1.5%) in 1x TBE buffer (Trisborate-EDTA) stained with 0.5 µg/mL ethidium bromide.

Afterwards, all amplification products were further studied by denaturing gradient gel electrophoresis (DGGE) to assess fungal genetic variations throughout the experiment and provide an estimate of richness and abundance of prominent members of the fungal assemblage. All DGGEs were performed in a "Dcode Universal Mutation Detection System" (Bio-Rad). Desired PCR products were loaded into 6% (wt/vol) polyacrylamide gels with a 15-55 % chemical denaturing gradient (100% denaturant contained 7 M urea and 40% (v/v) deionized formamide) and submerged in 1x TAE (40 mM Tris acetate at pH 7.4, 20 mM sodium acetate, 1 mM EDTA).

Electrophoresis was performed in all cases at a constant 75 V and a temperature of 60°C for 16 h using a Bio-Rad Power Pac 1000 power supply. The gels were inspected under UV illumination using a BioRad Universal Hood II Gel Doc™ XR System (BioRad) and photographed after staining them with ethidium bromide.

After visualization of DGGE profiles, prominent bands were cut, recovered, and stored at -20ºC in a buffer solution. Later, all excised bands were reamplified and sequenced at external facilities by Macrogen, Inc. (South Korea). The resulting sequences were compared with those in the Gen-Bank nucleotide database by using the Basic Local Alignment Search Tool (BLAST) to identify the closest known relatives (Altschul et al., 1990). Sequences determined in this study are available at the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers OL684714 through OL684779. InfoQuest™ FP (v4.5) software (Bio-rad, USA) was used to analyze the DGGE profiles and perform hierarchical cluster analysis by an unweighted pair group with mathematical averages (UPGMA) based on Euclidean distances. Subsequent analyses and statistics were performed using R version 4.1.2.

Table B.1. Relative abundance of different fungal genera detected on the lignocellulosic support in the RDBs at the studied stages (0, initial; I, t93; II, t186; III, t216). Symbols: "-", absent; "+", 0.01-20%; "++", 21-40 %; "+++", 41-60 %; "++++", 61-80 %; "+++++", ≥ 81 %.

	Period	***Cadophora***	***Meyerozyma***	***Trametes***	***Rhodotorula***	***Dipodascus***	***Penicillium***	**Others**
C-RDB	0	-	-	-	-	-	+++++	-
	I	++	++	-	-	-	++	++
	II	+	+++	-	++	-	+	+
	III	+	++	+	+	+	++	+
E-RDB	0	-	-	+++++	+	-	-	-
	I	-	+	+++	++	-	-	-
	II	-	-	++	++	++	-	-
	III	-	-	++	++	++	-	-

Table B.2. Relative abundance of different fungal genera detected in the effluents of the RDBs at the studied stages (t_0 PRE, initial RW; t_0 POST, RW after the coagulation-flocculation pre-treatment; I, 60 days; IIa, 120 days; IIb,186 days; III, 216 days). Symbols: "-", absent; "+", 0.01-20%; "++", 21-40 %; "+++", 41-60 %; "++++", 61-80 %; "+++++", ≥ 81 %.

	Period	***Cortinarius***	***Screlomularia***	***Dipodascus***	***Morteriella***	***Trametes***	***Penicilium***	***Cladosporium***	***Vanrija***	**Others**
RW	t_0 PRE	+	+	+++	+	-	+	-	-	-
	t_0 POST	-	+	+	-	-	+++	++	+	-
C-RDB	I	+	+	++++	-	-	+	-	-	-
	IIa	+	-	+	-	-	+++	-	+	-
	IIb	++	+	+	+	-	+	+	-	+
	III	+++	-	++	-	-	+	-	-	+
E-RDB	I	-	-	+++++	-	+	-	-	-	-
	IIa	-	-	+++	-	+	+	+	+	-
	IIb	-	-	+++++	-	+	-	-	-	-
	III	-	-	+++++	-	+	-	-	-	-

Table B.3. Shannon diversity and evenness indices corresponding to the different fungal genera detected on the lignocellulosic support of the RDBs at the studied stages (0, initial; I, 93 days; II, 186 days; III, 216 days).

	Period	Shannon	Evenness
C-RDB	0	-	-
	I	1,5173	0,9428
	II	1,4302	0,7982
	III	1,8208	0,8756
E-RDB	0	0,3139	0,4529
	I	0,8912	0,8112
	II	1,0964	0,9980
	III	1,0982	0,9996

Table B.4. Shannon diversity and evenness indices corresponding to the different fungal genera in the effluent of the RDBs at the studied stages (t_0 PRE, initial RW; t_0 POST, RW after the coagulation-flocculation pre-treatment; I, 60 days; IIa, 120 days; IIb,186 days; III, 216 days).

	Period	Shannon	Evenness
RW	t0 PRE	1,2811	0,7960
	t0 POST	1,1478	0,7132
C-RDB	I	0,7972	0,5751
	II	1,1087	0,7998
	IIb	1,9236	0,9251
	III	1,2241	0,8830
E-RDB	I	0,4790	0,6911
	II	1,1433	0,7104
	IIb	0,2544	0,3670
	III	0,1096	0,1581

Table B.5. Relative abundance of different fungal genera detected on the lignocellulosic support of the reactors. Symbols: "-", absent; "+", 0.01-20%; "++", 21-40 %; "+++", 41-60 %; "++++", 61-80 %; "+++++", ≥ 81 %.

Sample	*Trametes*	*Meyerozyma*	*Rhodotorula*	*Cystobasidium*	*Vishniacozyma*	*Phanaerochaete*	Others
RDB	++	++	+	+	+	+	+
Biopile	+	-	++	+	++	++	+

Table B.6. Relative abundance of different fungal genera detected in the effluent of the reactors. Symbols: "-", absent; "+", 0.01-20%; "++", 21-40 %; "+++", 41-60 %; "++++", 61-80 %; "+++++", ≥ 81 %.

Sample	*Meyerozima*	*Rhodotorula*	*Cystobasidium*	*Trametes*	*Phanaerochaete*	*Aspergillus*	*Cladosporium*	*Didymella*	*Penicillium*	Others
RW	+	+	+	-	+	-	+	+	++	++
FBB	-	-	-	+++	-	+	-	-	++	+
RDB-1	++	++	+	+	-	-	-	-	-	+
RDB-2	++	+++	-	+	-	-	-	-	-	+

Table B.7. Shannon diversity and evenness indices corresponding to the different fungal genera detected on the lignocellulosic support of the reactors.

Sample	Shannon	Evenness
RDB	1,7185	0,8264
Biopile	1,6908	0,8131

Table B.8. Shannon diversity and evenness indices corresponding to the different fungal genera detected in the effluent of the reactors.

Sample	Shannon	Evenness
RW	2,0111	0,7253
FBB	1,0502	0,6525
RDB-1	1,1423	0,5870
RDB-2	1,0892	0,7857

Annex C

C.1. Scanning electron microscopy

Surface growth of *T. versicolor* on wood was assessed by SEM. Solid samples were fixed on a 2.5 % glutaraldehyde solution with 0.1 M phosphate buffer (PB). Afterwards, post-fixation was performed using 1 % osmium vapor containing 0.8 % ferrocyanide in 0.1 M PB overnight. Afterwards, the samples were washed with ultrapure water and dehydrated in increasing concentrations of ethanol (50, 70, 90, 96 and 100 %). Finally, the samples were subjected to the critical point drying method (Baltec CPD030) and metallized with AuPd in order to improve the quality of images.

C.2. Fiber content analysis

Cellulose, hemicellulose and lignin contents were analyzed (triplicate) by the method of Van Soest et al. (1991). The fiber content study was performed using an Ankom200 Fiber Analyzer incubator (Ankom Technology, USA) by continuously introducing sulfuric acid (96 %), an acid detergent solution (consisting of sulfuric acid and cetyltrimethylammonium bromide) and a neutral detergent solution (containing SDS, disodium EDTA dihydrate, 10-hydrated sodium tetraborate, anhydrous dibasic sodium phosphate and triethylene glycol, pH 6.9–7.1). Ash was obtained after at least 3.5 h incineration in a muffle at 550 ºC. Fiber components were calculated as follows:

$$\text{Hemicellulose (\%)} = \text{NDF} - \text{ADF} \qquad \text{(C2.1)}$$

$$\text{Cellulose (\%)} = \text{ADF} - \text{ADL} \qquad \text{(C2.2)}$$

$$\text{Lignin (\%)} = \text{ADL} - \text{Ash} \qquad \text{(C2.3)}$$

where NDF represents the natural detergent fiber, ADF corresponds to acid detergent fiber and ADL means acid detergent lignin.