Universitat Autònoma de Barcelona

Microbial communities driving emerging contaminant removal. Impact of treated wastewater on the ecosystem

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January, 2018

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A thesis submitted in partial fulfillment of
the requirements for the degree of

PhD program in Microbiology

by

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With the approval of the supervisors,

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Bellaterra, January 2018

"I wish there was a way to know you're in the good old days before you've actually left them."

-Andy Bernard

This work has been funded by the Spanish Ministry of Economy and Competitiveness (project CTM2013-48545-C2-1-R), State Research Agency (project CTM2016-75587-C2-1-R), co-financed by the European Union through the European Regional Development Fund (ERDF) and supported by the Generalitat de Catalunya (Consolidated Research Groups 2014-SGR-559/2017-SGR-1762 and 2014-SGR-476/2017-SGR-14). Eloi Parladé Molist thanks Universitat Autònoma de Barcelona for the PIF pre-doctoral grant.

Abstract

Rising awareness of pollutants not previously detected or monitored (emerging contaminants) has brought new challenges to the scientific community focused in environmental remediation. This thesis aimed to assess the microbial communities responsible of emerging contaminant removal in a variety of human-engineered systems as well as to evaluate the impact of the resulting effluents when they are used as reclaimed water. Molecular microbiology methods were used along with bioinformatic tools to assess the development of enrichment cultures and bioreactors driven by bacteria, algae and fungi, capable of pollutant removal. The bacterial genera *Dehalogenimonas* and *Dehalobacterium* were identified as the responsible of organohalide degradation in two enrichment cultures and their concomitant microbiota was assessed to denote putative synergies. The specialized community developed in a pilot-scale photobioreactor was also elucidated during urban wastewater treatment, with efficient removal of pollutants and pharmaceuticals. Efficiency of enrichment cultures, obtained from natural environments, over algal pure cultures was demonstrated in the removal of estrogenic compounds. Moreover, filamentous cyanobacteria were linked to a fast and efficient recovery of photosynthetic biomass. The dynamics of indigenous bacteria, fungi, and the inoculated strain of *Trametes versicolor* were assessed in continuous bioreactors and fungal biopiles treating hospital wastewater and sewage sludge, respectively. Both the inoculated fungus and some bacterial communities were correlated to the removal of pharmaceutical families. Finally, the effect of effluents obtained from fungal treatment over soil ecosystems were compared to conventionally-treated wastewater in laboratory model systems (microcosms), showing up a similar behavior to the negative controls that did not contain pharmaceuticals.

Resum

Actualment, un dels nous reptes de la comunitat científica és fer front a l'increment de contaminants emergents als ambients naturals. La bioremediació s'ha perfilat com una bona alternativa per donar resposta a aquesta necessitat. El propòsit d'aquesta tesi ha estat estudiar les comunitats microbianes responsables de l'eliminació de contaminants emergents en diversos sistemes artificials, així com determinar l'impacte dels efluents resultants quan s'utilitzen com a aigua regenerada. Diferents tècniques moleculars i eines bioinformàtiques han permès caracteritzar els cultius d'enriquiment establerts, així com els bioreactors amb bacteris, algues i fongs, capaços d'eliminar contaminants. Els generes bacterians *Dehalogenimonas* i *Dehalobacterium* s'han identificat com a responsables de la degradació d'organohalonenats en dos cultius d'enriquiment i la seva microbiota acompanyant ha estat avaluada per posar de manifest possibles sinergies. Paral·lelament, s'ha caracteritzat la comunitat desenvolupada en un fotobioreactor a escala pilot durant l'eliminació de contaminants i productes farmacèutics a partir d'aigües residuals urbanes. Pel que fa a compostos estrogènics, s'ha demostrat una millor capacitat d'eliminació per part dels cultius d'enriquiment de microorganismes fototròfics en comparació amb cultius purs. A més a més, s'ha relacionat la presència de cianobacteris filamentosos amb una recuperació ràpida i eficaç de la biomassa fotosintètica. D'altra banda, s'ha analitzat la dinàmica de bacteris i fongs, així com de *Trametes versicolor* inoculat en bioreactors continus i biopiles fúngiques utilitzats per tractar aigües residuals hospitalàries i llots de depuradora. Tant el fong inoculat com algunes de les comunitats bacterianes indígenes s'han correlacionat amb l'eliminació d'alguns grups de fàrmacs. Finalment, s'ha avaluat l'impacte dels efluents obtinguts a partir de diferents tractaments d'aigües residuals hospitalàries irrigant microcosmos que mimetitzaven el sòl. Els resultats obtinguts han posat de manifest que els efluents fúngics es comporten de manera similar als controls negatius sense productes farmacèutics.

Acknowledgements

Gairebé quatre anys de tesi són difícils de resumir en uns paràgrafs d'agraïments. Sense intenció que s'allargui gaire, dedico primer de tot el meu treball a la meva família. Sobretot als meus pares, la meva germana i els meus avis, que em van donar el suport i recursos necessaris per fer possible que als 18 anys un jove Eloi s'independitzés a Cerdanyola i emprengués el viatge que l'ha dut fins aquí.

El bloc llarg d'agraïments va pels amics, la família que escull cadascú. Primer els de casa, que diuen, agraeixo en grup a tots els Moixernons. Gràcies en especial als que us heu preocupat sempre per si acabava ja o no la tesi, ja sabeu qui sou. A en Cristian, en Vila, en Marc, l'Uri i en Mardu per les reunions periòdiques on recordem els v/bells temps del RO. Als PV's també per tenir els braços oberts quan em presento després de mesos desaparegut.

Un cop fora de casa, agrair el suport incondicional dels companys de pis que m'han aguantat a Torrent 57 i Sant Antoni 52. En especial, en Marc, el Sebas, el Juanca, l'Arnau, el Toni i l'Alfredo (revisors express de la tesi). També als companys i amics de la carrera de microbiologia, per quatre anys fantàstics de créixer com a persona. En especial, una forta abraçada per l'Edu, la Jenni i la Marta. Entre tot això, em toca agrair a la Mercè que ens obrís les portes del seu laboratori a mi i l'Edu i que ens inspirés amb la seva passió per la ciència.

Parlant d'inspiració, la necessària per escriure la tesi (i fer jocs de taula) no sempre vé treballant; les millors pauses per dinar i desconectar no haurien estat possibles sense el Marc, l'Olivia, el Ferran, el Pablo, la Mara, el Jofre, el Samir, en Martí i la Débora. Repetint agraïment, gràcies a en Marc per fer que el laboratori sigui un lloc on sempre tinc ganes d'anar. A l'Oihane i la Jenni, per les mil ajudes en gestions i laboratori, a més de tota la gent del grup de microbiologia ambiental i molecular, no tindria prou espai per agrair-vos tots els favors!

Pel que fa al contingut de la tesi, les col·laboracions han sigut essencials perquè els capítols tinguin cara i ulls. Moltes gràcies a tota la gent de l'ICRA i el departament d'enginyeria química, biològica i ambiental per donar-ho tot i fer que puguem tenir uns articles fantàstics.

Després de la meva etapa de docent, no puc deixar d'agrair a tots els petits micros que han fet que em senti part de moltes promocions. En especial, Marina, Tamara i Clàudia per recordar-vos de mi quan era lluny. També per compartir cerveses, berenars i festes. Alex, menció especial per la teva infalibilitat quan hi ha un pla d'última hora.

A més, no m'estic oblidant de vosaltres, amb qui he compartit de la mà part d'aquest camí d'aventures, sense qui no seria el que sóc ara. Natàlia i Rocío, sobren les paraules.

Thanks to Jorge and Albert for opening Davis doors to me and making possible my stay. To Jordan for always keeping it chill and fun, Jon for the necessary common sense and advise, Rachel and Mike for the great trip to Tahoe, Seiji for his determination and making fencing possible for me, and Alec for fencing, chilling and partying with me when I had no one around. I also wanted to thank the Davis boardgamers, I felt always at home and enjoyed every single afternoon. Jason, thanks for inviting me to your place and review Gem Mayhem. Little things meant a lot to me. Special mention to my friend Rob, for being yourself and accompanying me in the adventures at the other side of the globe.

Last but not least, res de tot això no hauria estat possible sense l'empenta, motivació i oportunitat que la Núria i la Maira m'han donat a la universitat. Van confiar en mi al laboratori i m'han donat suport per seguir a l'investigació, fent possible la realitat d'aquesta tesi.

Abbreviations

ARG: Antibiotic Resistance Gene
ASV: Amplicon Sequence Variant
BES: 2-bromoethanesulfonate
CYP: Cytochrome P450
DCM: Dichloromethane
DCP: 1,2-dichloropropane
DDT: Dichlorodiphenyltrichloroethane
DGGE: Denaturing Gradient Gel Electrophoresis
E1: Estrone
E2: Estradiol
EC: Emerging Contaminant
EE2: Ethinylestradiol
EU: European Union
GMO: Genetically Modified Organism
HRT: Hydraulic Retention Time
HWW: Hospital Wastewater
IPBR: Industrial Photobioreactor
ITS: Internal Transcribed Spacer
OTU: Operational Taxonomic Unit
NAPL: Non-Aqueous Phase Liquid
PAH: Polycyclic Aromatic Hydrocarbon
PBR: Photobioreactor
PCR: Polymerase Chain Reaction
PhAC: Pharmaceutically Active Compound
qPCR: Quantitative Polymerase Chain Reaction
TSS: Total Suspended Solids
TWW: Treated Wastewater
UPGMA: Unweighted Pair Group Method with Arithmetic Mean
WRF: White-rot Fungi
WW: Wastewater
WWTP: Wastewater Treatment Plant

Contents

Chapter 1

General introduction and literature review

1.1 Emerging contaminants. Origin, sources and fate in the environment

First contamination records date from as far as two millennia ago, when Greek and Roman lead mining and smelting activities polluted the middle troposphere long before the Industrial Revolution [1]. Evidences were obtained from cumulative lead fallout to the Greenland Ice Sheet, that showed concentrations four times as great as natural values. Much later, the lack of environmental regulation during the industrial revolution resulted in unparalleled discharges of SO_2 and NO_2 gases that influenced the acid rain phenomenon [2, 3]. It was around this period when dichlorodiphenyltrichloroethane (DDT) was discovered by the Swiss chemist Paul Muller. This compound was the first important synthetic organic pesticide and the lack of knowledge of its side effects resulted in disastrous repercussions for wildlife [4,5] when it was popularized. DDT is one of the best examples of emerging contaminant (EC). Awareness over ECs was first raised by Rachel Carson with her book "Silent Spring", released in 1962 [6]. Although she was heavily criticized at the beginning for opposing to the use of DDT to eliminate mosquitoes and other pests, she was later proved right and DDT was banned [7].

Emerging contaminants, or "contaminants of emerging concern", are a group of chemicals that include substances of concern because of potential human or ecological health effects. They are not currently regulated in the environment due to lack of persistence, toxicity or bioaccumulation data [8]. Two main groups are considered within the term EC. First, newly synthesized chemicals or other substances not previously known that just recently started appearing in scientific literature. Second, contaminants known to exist and for which the environmental implications were not realized, so no monitoring was carried out.

Although there is not a list of all the chemicals considered EC, most of them are (or comprise) pharmaceuticals, personal care products, insect repellents, fragrances, cleaning products, detergents, flame retardants or chlorinated chemicals [9–12]. These products are produced worldwide on a 100,000 t scale [13]. Table 1.1 shows the main groups of EC and provides examples.

Table 1.1: Main types of emerging contaminants by functionality and examples of each group.

Group	Example
Pharmaceuticals	Carbamazepine, diclofenac
Personal care products	Benzophenone-3 (UV filter), galaxolide (fragance)
Flame retardants	Tetrabromobisphenol A
Nitrosamines	N-Nitrosodimethylamine
Surfactants	Nonylphenol
Plastifiers	Alkyl phosphate
Fuel additives	Methyl tert-butyl ether

In most cases, the entry point of these substances into the environment is through wastewater treatment plant (WWTP) discharges and reuse of sewage sludge [14–17]. Even following the mandatory European Union Directive for Urban Wastewater Treatment [18], WWTPs are not suited for the proper removal of EC [18–21]. Furthermore, some ECs present in treated WW and sewage sludge spread in agricultural soil can be transferred to the human food network by uptake into food crops or indirectly following uptake into forage crops [22–24]. Compared to other insoluble contaminants as polycyclic aromatic hydrocarbons (PAHs), EC are generally more polar

and soluble, conferring them greater capability to be taken up by roots and translocated within plants [22]. Other pathways through which EC can reach soil include manure and manure-derived products such as commercial mature compost and soil-based mediums for domestic gardens [25–28].

In addition to the challenge that removal or degradation of EC supposes, information regarding EC transformation products is scarce. Therefore, it is not known whether EC-derived metabolites could be, in some cases, even more toxic than the parent compounds. This problematic points to an evident need to expand the knowledge on EC abundances, transformation processes and removal in the environment and wastewater (WW) alike. While the project in which this thesis is framed aims to tackle all three points, the work described in this dissertation was carried out from a microbiological perspective. Specifically, this work encompasses the study of a variety of microbial-based removal processes featuring bacteria, algae and fungi in batch cultures and bioreactor systems designed for in situ and ex situ treatments, respectively. Precisely, chapters 4, 5 and 6 are centered around these three microbial groups capable of removing chlorinated alkanes, estrogens and pharmaceuticals, respectively.

1.1.1 Chlorinated alkanes

Heavily halogenated compounds such as those present in flame retardant products have been considered hazardous, recalcitrant, bioaccumulative and environmentally persistent ECs [29, 30]. and there are no described microorganisms able to degrade such complex compounds. However, biodegradation of less-halogenated compounds such as chlorinated alkanes (i.e. 1,2-dichloropropane and dichloromethane) is known to occur and are studied in this thesis.

Chlorinated compounds are naturally found in some marine environments [31] and become prevalent pollutants when industrial and human activities lead to alarming concentrations in groundwater and soil environments [32–37]. Traditionally, polychlorinated alkanes were employed as solvents, degreasing agents, paint removers or chemical intermediates in several industrial processes. Due to their abundant use and improper disposal, soils and groundwater alike still now suffer severe pollution.

Microorganisms are the main recyclers of halogenated organic compounds in the environment. A number of anaerobic bacteria have been described as capable of dechlorinating these compounds to non-toxic end-products [38]. Therefore, studying their metabolism is essential for the establishment of bioremediation strategies able to convert the pollutants into safe metabolites. To this day, microbial dehalogenation is known to occur through 4 different strategies [39–43]: i) Use of the chlorinated compound as a source of carbon and energy, ii) as electron acceptor in anaerobic respiration (i.e. dehalorespiration), iii) as detoxification mechanism or iv) through fortuitous reactions that bear no benefit to the microorganism (co-metabolism).

Unfortunately, biological degradation of contaminants to non-toxic end-products depends on multiple factors such as redox conditions, absence of inhibitory compounds or composition of indigenous microbiota, in some cases limiting natural bioremediation processes [38]. Non-appropriate conditions may lead to incomplete dechlorination and the consequent release of intermediate products. These intermediates can sometimes be even more toxic than the original compound, as it occurs

when vinyl chloride is formed from tetrachloroethylene [44].

A critical step in the degradation of organochlorines is the enzyme-mediated excision of the carbon-halogen bond. Biodegradation mechanisms are diverse, but the most efficient and widely studied is the dehalorespiration, first described by Mohn and Tiedje in the early 90's [45]. In any case, the structural properties of each compound ultimately determine the applicable mechanisms to achieve biodegradation.

Both dehalorespiration and some fermentation pathways use chlorinated compounds as the final electron acceptors and provide energy to the cell while yielding less or non-chlorinated organic compounds. This study therefore set out to assess the development of enrichment cultures capable of 1,2-dichloropropane dehalorespiration and also dichloromethane fermentation. These chlorinated pollutants are commonly found in polluted groundwater, so a better understanding of the indigenous communities responsible of its degradation can set the stage for the development of robust and sustainable bioremediation strategies.

1.1.2 Estrogenic compounds

Some estrogenic compounds are endocrine or hormone disruptors that act as mimickers, blockers or interferers of natural hormones at concentrations down to the order of ng/L [46–48]. While broad information is still scarce, adverse health effects on aquatic organisms were already reported more than two decades ago. Sumpter and Guillette documented the feminization of male and immature fish along with alterations in the gonads of alligators, both due to endocrine disruption [49,50].

In fact, numerous studies have demonstrated that estrogens such as estrone (E1), estradiol (E2) and ethinylestradiol (EE2) are responsible for numerous endocrine-disrupting effects in aquatic environments [51–55]. E1 and E2 are naturally produced in the body while EE2 is a synthetic compound used in birth control products such as pills, patches, rings or injectables. Human excretion is one of the main sources of these compounds in the environment and they have already been found in drinking water [56] in concentrations of 0.1 to 0.5 ng/L, sufficient to cause a strong biological impact [57].

The problematic not only resides in the presence of these compounds but in their activation state. Estrogens leave the human (or mammal) body in inactivated forms thanks to oxidation, deoxidation, hydroxylation or methylation processes. Unfortunately, upon reaching the WWTPs the bacterial sludge and remnant gut bacteria can turn the inactivated estrogens back to their original active form with sulfatase and β-glucuronidase enzymes [21, 58–60]. For this reason, removal of estrogenic compounds before reaching the WWTP is critical [13] and has been assessed in the project where this thesis is framed.

1.1.3 Pharmaceutically active compounds and antibiotic resistance genes

Within the large group of ECs, pharmaceutically active compounds (PhACs) encompass a variety of compounds of clinical use, among which antibiotics, anti-inflammatory drugs, beta-blockers,

X-ray media or lipid regulators are the most relevant. PhACs are generally found in aquatic environments due to human and animal excretion, landfill leaching or improper disposal [27, 61–64]. Concentrations of PhACs detected in the environment are sometimes sufficient to cause adverse effects [61]. Like in estrogens, the removal of these PhACs in WWTPs or purification plants is not guaranteed with the processes used nowadays and removal efficiencies are highly dependent on the physical and chemical properties of each individual compound. Concentration of the pharmaceuticals, geographical area and climate conditions play an important role in the fate of the contaminants. However the main sinks for PhACs are considered to be photodegradation and biodegradation [61].

Among PhACs, antibiotics are of particular interest due to their extended use and rising concern regarding acquired resistances in bacteria. Annual production of antibiotics was estimated by Kümmerer in 2009 to be around 100,000–200,000 t [65]. They were first used for therapeutic purposes in 1928 (when penicillin was discovered), employed since 1946 as growth promoters in animal farming, and finally even as legal food preservatives from 1955 until now [66–68]. Aside from animal-derived releases, hospital wastewater (HWW) is another major source of antibiotics in the environment [65] as it can harbor concentrations of antibiotics one order of magnitude higher than urban WW and up to 3 orders when compared to river water [63]. In general, 90% of a consumed antibiotic is excreted back into the environment in partial or non-metabolized forms [69, 70] and their environmental concentrations range from ng/L to g/L for aquatic and terrestrial environments, respectively [71].

It is a concern that the presence of antibiotics in the environment is tightly correlated with the increase of resistant bacteria and their associated antibiotic resistance genes (ARGs) [72–74]. Nowadays, ARGs are practically ubiquitous and have been found in WWTP effluents, lagoons, lakes, groundwater, rivers, seas, soil, sediments and even drinking water [75,76]. Antibiotics can facilitate the development of already resistant bacteria by eliminating competing susceptible populations or also by triggering stress-induced mutagenesis that increases the spontaneous mutation rate [77]. Once a mutation conferring an antibiotic resistance phenotype occurs, bacteria harboring the mutated gene will compete against wild-type ancestors and relative fitness will determine the outcome [78]. Mutant bacteria outcompeting the wild-type population will be able to spread the mutated allele in the bacterial community via vertical or horizontal gene transfer. Soil bacteria are considered an ancient reservoir of ARGs and through these mechanisms they are able to maintain (vertical transference) and disseminate (horizontal transference) resistances to the pathogens in the environment [79]. However, it should be stated that horizontal ARGs transfer ultimately depends on plasmid compatibility groups, phage specificity or nature of the transference [80–82].

From an analytic point of view, the detection of resistant bacteria and their ARGs is easier than measuring direct antibiotic concentration in environmental samples. Resistant bacteria can be cultured in media containing distinct antibiotic concentrations, whereas ARGs can be screened using primer-specific amplification or sequencing approaches (see chapter 7). Conversely, antibiotics in the environment are difficult to detect in low concentrations and typically require extraction steps that might bias or difficult a precise quantification. The main challenge that scientists of the field are facing is developing and testing appropriate analytic methods [83]. In this work, antibi-

otic concentrations are evaluated in chapters 5, 6 and 7 to study interactions with the microbial populations.

1.2 Environmental remediation

The remediation of environments consists in the removal of pollutant from natural matrices such as soil, sediments, groundwater or surface water. The methodology of choice to treat a contaminated matrix is subjected to the nature of the pollutant and type of matrix. A wide arrange of technologies for remediation are available nowadays that involve in situ or ex situ treatment with either physical, chemical or biological elements. A brief summary of the most popular technologies for remediation is presented in Table 1.2.

Table 1.2: Common technologies used for the remediation of polluted environments.

Technology	Type	Description
Chemical oxidation	in situ	Application of strong oxidants for total or partial degradation of pollutants.
Permeable reactive barriers	in situ	Subsurface emplacement of reactive materials to selectively capture or degrade pollutants.
Solidification/stabilization	in situ	Application of binder substance to avoid or reduce pollutant mobilization.
Surfactant-enhanced aquifer remediation	in situ	Solubilization of pollutant using surfactants and recovery in non-aqueous phase liquid.
Thermal desorption	in situ	Volatilization of pollutants, typical in soils.
Excavation/dredging	ex situ	Transport and confinement of contaminated soil in landfill.
Pump and treat	ex situ	Extraction of liquid matrix using pump and ex situ treatment.
Soil vapor extraction	ex situ	Vacuum removal of gases, including volatile organic compounds and treatment with activated carbon, heat or oxidation.
Bioremediation	in situ/ex situ	Utilization of biological processes to remove the pollutant.
Nanoremediation	in situ/ex situ	Use of nanoparticles to degrade or immobilize the pollutant via redox reactions or sorption, respectively.

Tackling the removal of contaminants in safe, sustainable and cost-efficient methodologies is one of the main challenges that scientists must face nowadays. Bioremediation arose as a promising cost-effective technology to solve this challenges using biological processes. In fact, back in 600 B.C. Romans had already promoted bioremediation for the first time when they directed wastewater into large tanks or pits, allowing microorganisms to consume the organic matter [84]. In the modern times, the concept of bioremediation was born in the late 60s, when the petroleum engineer George M. Robinson carried out a full-scale bioremediation by applying his custom bacterial mixtures into a commercial oil spill.

Nowadays, bioremediation could be defined as a process employing microorganisms or plants (phytoremediation) to remove, degrade, or contain toxic compounds. Within this possibilities, biodegradation is usually the preferred option because it implies that no recovery or post-treatment of the pollutant will need to be performed. While the term biodegradation is used to describe the bio-

transformation of one compound to another (usually less or non-harmful), the term mineralization is used for complete biodegradations yielding simple inorganic molecules such as carbon dioxide, ammonia or water. From this point onward, the term bioremediaiton will be used to address microorganism-based bioremediaiton, setting aside phytoremediation.

Although bioremediation efforts have been lately focused on hydrocarbons due to frequent pollution of soil and groundwater (more than 700 papers/year indexed in PubMed since 2005 that match "hydrocarbon bioremediation"), it should be reminded that the concept of bioremediation can be applied to the biological remediation of any matrix in the environment where pollutants, including EC, are present (i.e. soil, sediment, water bodies or air). To consider the application of a bioremediation process it is crucial to consider several key factors that can greatly affect its efficiency and can be summarized into:

- Contaminant structure: Complexity of the molecular structure.
- Contaminant concentration: While high concentrations of the compound can be toxic to the microbes, concentrations that do not reach certain threshold may be unable to trigger an enzymatic/metabolic response.
- Contaminant bioavailability: Contaminants strongly sorbed in solid matrices, sequestered by other molecules or in non-aqueous phase liquid (NAPL) forms have reduced bioavailability and are inherently harder to remove or degrade.
- Environmental conditions: Parameters such as redox potential, pH, nutrients, moisture and temperature are key factors that should be optimized to keep cells metabolically active.
- Microbial communities: Indigenous microbiota in the environment.

Furthermore, understanding of the biological mechanisms that drive biodegradation and the strategies that are available for a certain environment is also essential. Accordingly, a brief explanation is provided in sections 1.2.1 and 1.2.2, respectively.

1.2.1 Biological mechanisms

In the natural environment, microbial degradation is one of the key factors that determine the fate of many substances. The study of the microbes in bioremediation systems makes possible the selection of those with potential for the degradation. The microbiologist Martin Alexander was the first to outline the use of microbes for bioremediation of polluted sites. He introduced the principle of microbial infallibility [85] which states that no natural organic compound is totally resistant to biodegradation given the appropriate environmental conditions. Thus, in situations of nutrient scarcity microorganisms will typically exploit alternative (but less efficient) carbon and energy sources.

Unfortunately, a large proportion of the ECs are xenobiotic compounds that have been synthetized artificially and no metabolic pathways are expected to exist in the environment. However, biodegradation of some of these compounds can still occur in two situations: i) incorporation of the contaminant into an enzymatic pathway already present for another compound or ii) evolution of new pathways to become specific for the new compound. Both strategies should not be considered

independent but complementary [42,86]. In fact, the latter is a derivative of the first after suffering a mutation, genetic transference or amalgamation process.

One of the most interesting capabilities of microbial metabolism, and perhaps one of the least studied, is the metabolic synergy that microbes can establish. Either if the microorganisms involved in a mixed culture can grow by themselves or must remain in a consortia, the use of several strains in bioremediation can widen the range of pollutants available for degradation or increase the process yield [87–89]. However, not all mixed cultures can be considered a microbial consortium. The concept of consortium was first introduced by the German botanist Johannes Reinke in 1872 [90] and refers only to cultures where two or more organisms live in symbiosis. To set an example of the consortia importance, a case was reported where bacterial strains could degrade a recalcitrant pollutant (i.e. the herbicide mecoprop) while in consortium but could not survive when cultured individually with the same compound [91].

Unfortunately, the study of the mechanisms responsible of pollutant degradation is not an easy task. On one hand, metabolic pathways can be repressed if there is no incentive for cells to expend energy to carry out the targeted reaction (e.g. low toxicity or better carbon and energy sources available). On the other hand, several requisites must be met to quantify biodegradation: i) the microbial population has to be active, ii) the environmental conditions have to be adequate, iii) the contaminant should be available, iv) the removal mechanisms must remain limited or be known and v) the yield must be significant with respect to the hydraulic retention time (HRT), in the case of continuous bioreactors [92].

Considering the diverse microbial groups capable of biodegradation (e.g. Bacteria, Fungi or Algae) and given the large variety of ECs, the assessment of all the metabolic pathways involved in the bioremediation of these compounds is still a distant goal. Similarly to the case of chlorinated alkanes (Section 1.1.1), pathways can be effectively classified according to the purpose served for the microorganism. The most relevant biological mechanisms can be organized in three main categories:

1. Metabolization of the pollutant to obtain carbon or energy.

   Many studies have revealed the capacity of bacteria to metabolize pollutants to obtain carbon or energy. Organohalide respiration is probably the best example of bacteria obtaining energy from pollutants [93–96]. However, if the obtaining of carbon is also to be considered, fewer examples are available. Mägli *et al.* reported in 1996 [40] the capacity of *Dehalobacterium formicoaceticum* to grow utilizing dichloromethane (DCM) as source of carbon and energy. Another example is the ability of *Dehalobacter* sp. to also ferment DCM while in an enrichment culture [97]. Leaving dehalogenators aside, algae and cyanobacteria are also involved in the degradation of pollutants. Some mixotrophic species can dispense photosynthesis and activate the heterotrophic metabolism, capable of degrading pollutants in some species [98, 99].

2. Metabolization of the pollutant as a detoxifying mechanism.

   Coevolution of microorganisms, plants and animals during millions of years has allowed for the development of numerous toxins and specific detoxifying mechanisms for each one of them. One of the most important mechanisms is provided by the Cytochrome P450 monooxygenases

(CYPs), a superfamily of enzymes widely distributed among Eukarya, Archaea, Bacteria and even encoded in virus [100,101]. Although CYPs are implicated in the biosynthesis of essential compounds like ergosterol in bacteria and fungi [102], they have been extensively studied because of their leading role in detoxification and biodegradation of drugs, xenobiotics and other environmental pollutants [103–108]. Specifically, CYPs catalyze oxidation reactions employing a hemo group as a cofactor and are able to generate individual oxygen atoms from molecular oxygen [109]. The list of reactions carried out by CYPs includes hydroxylation, epoxidation, O-dealkylation, N-dealkylation, alcohol and aldehyde oxidation, oxidative dehalogenation and oxidative C-C bond cleavage [101,109].

3. Metabolization through fortuitous reactions.

   Cometabolism consists in the fortuitous degradation of a non-growth substrate (bears no benefit to the cell) in the obligate presence of a growth substrate or transformable compound. Not all cometabolism events are applicable to bioremediation of ECs or have been adequately studied. In this regard, fungi are the microorganisms that have been better characterized. Their ability to degrade structurally complex molecules (particularly white-rot fungi) has generally been attributed to their lignin-degrading enzyme system. They are extracellular non-specific enzymes jointly secreted into the environment by many fungi to break down macromolecular phenolic substrates into more assimilable compounds. The main enzymes belonging to this system are laccase, lignin peroxidase and manganese peroxidase [110–113].

   In cyanobacteria, the production of extracellular enzymes able to degrade pollutants non-specifically was also demonstrated by Wurster M *et al.* when phenol was degraded by a *Synechococcus* strain [114]. Moreover, both cyanobacteria and algae can also stimulate the degradation of pollutants indirectly by photosynthetically-mediated pH changes or high oxygen production [115]. In fact, fungi-algal co-cultures for the treatment of WW have been tested to improve biomass settling [116] but also to benefit EC degradation thanks to the fungal enzymes [117].

While the terms “Bioremediation” and “Biodegradation” are often used indistinctly in the literature, it is important to highlight that not all bioremediation strategies involve direct degradation of the contaminant. Other microbial-based or microbial-enhanced mechanisms include bioadsorption, bioabsorption, chelation, precipitation, mobilization, immobilization or changes in pH to affect the ionic state of the pollutant. Most of these mechanisms are well studied for metal and radionuclides bioremediation [118] but are not applicable to more complex substances (like many ECs).

When addressing EC removal, bioadsorption and bioabsorption (generally referred as biosorption) are probably the most popular mechanisms. They are highly dependent on the structure, functional groups or hydrophobicity of each pollutant, as well on the environmental conditions [92]. On the one hand, bioadsorption is a metabolically-independent physiochemical process that involves the adhesion of substances into microbial biomass. Not only alive cells but dead cells and cell fragments contribute to this mechanism, that is often referred as passive. On the other hand, bioabsorption consists in the uptake of substances by living cells through energy-dependent active transport [119,120]. In conclusion, adsorption and absorption are mechanisms to kidnap the pollutant inside the cell or in its surface, respectively, and facilitate its recovery from liquid matrices.

1.2.2 Bioremediation strategies

As presented in Table 1.2, two main strategies of bioremediation exist depending on the application site, namely in situ and ex situ. However, intrinsic bioremediation is often included as a third one, consisting in the inherent capacity of an environment to remove the pollutants without human intervention. Furthermore, in situ strategies can be subdivided, considering the nature of the intervention, in biostimulation or bioaugmentation. The combination of biostimulation and bioaugmentation can also be considered in challenging environments [121–123]. In some cases (as in sections 5.4 and 6.3), the term bioaugmentation refers to additional input of exogenous biomass during WW secondary treatment or biopile systems, respectively.

Biostimulation

Biostimulation can be defined as the bioremediation strategy that involves the modification of the environment to stimulate existing microorganisms capable of bioremediation. Thus, it implies modifying one or more of the previously mentioned key factors to enhance removal by microbial populations. This can be achieved in ways such as adding limiting nutrients [124], co-substrates [125], electron donors [126], electron acceptors [127] or carbon sources [127, 128] that could be naturally found in low abundances, constraining microbial activity. The main advantage of biostimulation is that the process is undertaken by autochthonous microorganisms, already present in the environment, that are well adapted to the local environment and evenly spread throughout the matrix.

Multiple challenges may arise when carrying out this bioremediation strategy. On one hand, adequate delivery of additives can be constrained by the nature of the treated environment. For example, in biostimulation of subsurface waters, a tight impermeable lithology can make it difficult for additives to spread evenly and fractures in the subsurface create preferential pathways where the additives will leak through. On the other hand, addition of nutrients might promote the growth of heterotrophic microorganisms that are not innate-degraders and will compete with the other local populations.

Bioaugmentation

In some cases, indigenous microbial populations may be unable to degrade the pollutant present in the environment due to the absence of the necessary metabolic pathways. Bioaugmentation tackles this problem by adding indigenous or allochthonous microorganisms to the polluted site to accelerate the removal of undesired compounds. Another situation under which bioaugmentation may be considered is when the concentration of microorganisms is low. The speed of decontamination is a critical factor in most situations so seeding with the appropriate strains can reduce the lag period to start the bioremediation process [129].

The main attributes desired in a microbial strain to be suitable for bioaugmentation include genetic stability, viability during storage, growth capacity, survival in hostile environments, and capacity to compete with indigenous microorganisms [130, 131]. In this regard, the use of genetically modified organisms (GMOs) is a promising strategy to attain adequate strains. However, release into the

environment is strictly forbidden in the European Union (EU) [132]. Ex situ bioremediation processes might consider using GMOs in confined systems but need to ensure afterwards that there is no release of the modified microorganisms into the environment.

1.3 Reclaimed water

Water abstraction is the permanent or temporary removal of water from rivers, canals, lakes, reservoirs or aquifers for human water management. It is a cause of water stress, fueled by increasing population (higher demand for irrigation and domestic purposes) and aggravated by drought events. Droughts have dramatically increased in the EU in the last 30-40 years and 11% of the European population (in 17% of the territory) had been affected by water scarcity by 2007 [133]. Moreover, the need for long term sustainable solutions arose in 2012, after the European Commission estimated a burden in the order of 100 billion euros caused by water shortages in the 1976–2006 period [134].

The environmental, social and economic benefits of reclaimed water have already been recognized and embedded within international, European and national strategies [135]. These include:

- Environmental improvements by alleviation of the pressure caused by water abstraction and reduced discharges of the WWTP into sensitive areas.
- Lower investment and energy costs compared to water supply alternatives like desalination or water transfer. Contributes to the reduction of greenhouse gas emissions.
- Reliable source water that is independent from seasonal droughts and weather variability. Can cover peaks of water demand.

However, if removal of ECs, resistant bacteria or antibiotic resistance genes cannot be guaranteed, caution must be taken when considering the reuse of water. As it has been reviewed in the previous sections, WWTP effluents are a main source of ECs in the environment and food network. The use of reclaimed water for domestic use or agricultural purposes should be halted until proper assessment of the available treatment processes is carried out.

As the development of a defined legal frame is still ongoing in the EU, reclaimed water experiments in this work (Chapter 6) focused in the use of reclaimed water for watering of forests, green areas or other uses non-accessible to the public (recreational). Such uses still require a tertiary treatment that is usually not available in WWTPs nor capable of removing all ECs. Advanced oxidation with ozone [136], other chemical oxidants [137] or sonolysis [138] have been investigated for the elimination of some pharmaceuticals.

At a national level, WWTPs in Spain usually comprise only primary and secondary treatments [139]. Spanish legislation of reused water demands filtration and UV treatment, followed by a maintenance disinfection using a low dose of sodium hypochlorite and the establishment of control points in the outtake and delivery points [140]. Required parameters for surveillance include *E. coli*, *Legionella*, *Salmonella*, *Taenia*, intestinal nematodes, total suspended solids (TSS), turbidity, total nitrogen and total phosphorus. Despite the control for pathogens is clearly strict, some au-

thors have studied the real effect of UV treatment in the removal PhACs (mainly in analgesics and antibiotics) and showed up that it was effective for a quantitative removal [56, 141–143].

To conclude, the problems addressed in this introduction are meant to highlight the need to further study and develop robust and cost-efficient technologies for bioremediation. This research must be conducted at all levels, from basic screening of new isolates with promising capabilities to the design, scale-up and optimization of new methods. Many of these points can be tackled from a microbiological point of view from within multidisciplinary groups. Hence, the following chapter raises the objectives for this thesis.

Chapter 2

Objectives

The main objective of the present thesis is to **assess the microbial communities responsible of emerging contaminant removal in a variety of human-engineered systems** and **evaluate the impact of resulting effluents on microbial communities when they are used as reclaimed water**. This objective is divided in the following specific goals:

- To identify the bacteria responsible of 1,2-dichlorometane dehalogenation in an enrichment culture obtained from river sediments and monitor the consortium developing process.
- To identify the bacteria responsible of dichloromethane dehalogenation in enriched membrane bioreactor slurry samples and assess the communities present in the consolidated consortium.
- To study the parameters shaping microbial communities in a pilot-scale photobioreactor inoculated with lake water for the treatment of urban wastewater and removal of pharmaceutically-active compounds.
- To establish effective strategies to perform algal bioaugmentation for the removal of estradiol.
- To assess the fate of *T. versicolor* during hospital wastewater treatment and elucidate the microbial communities dynamics in the system.
- To assess the fate of *T. versicolor* in a biopile treatment of sewage sludge and characterize the microbial assemblage.
- To evaluate the impact of reclaimed effluents from hospital wastewater treatment over soil communities in a microcosm system and assess whether irrigation can stimulate an increase in antibiotic resistance genes and mobile genetic elements.

Chapter 3

Materials and methods

3.1 Consumables, reagents and enzymes

3.1.1 Buffers, stock solutions and other reagents

Purchased reagents are presented along with its supplier in the annex (Table 10.1). Flocculating and coagulating reagents HyflocAC50 and HumolocDR3000 were kindly provided by Derypol, S.A. (Barcelona, Spain).

3.1.2 Sterilization of consumables and glassware

Irradiated micropipette tips with filter were used to prepare stock solutions. Trace metal mix A5 was sterilized by syringe filtration and all the rest of sterile reagents, glassware and consumables were autoclaved at 121°C for 20 min.

3.2 Culture media

Diverse culture media were employed througout the studies described in this thesis. For DCP-degrading cultures, the media prepared for *Dehalococcoides mccartyi* strain CBDB1 described elsewhere [144] was used. Briefly, the defined medium contained vitamins, trace elements, 5 mM sodium acetate as carbon source and titanium citrate (0.8 mM) or Na_2S 9 H_2O and L-cysteine (0.2 mM each) as reducing agent.

The medium used for the DCM-fermenting culture is based on the same media detailed above with some modifications. In this case, the medium contained carbon sources as acetate, pyruvate, fumarate and formate at 5 mM, and 200 mg $L^{-}1$ of yeast extract. It also contained vitamins, trace elements, 22.8 μM tungsten, 24.2 μM selenium, and Na_2S 9 H_2O and L-cysteine as reducing agents. [145] A bicarbonate solution (pH = 7) was used as buffer and resazurin was added as redox indicator.

Finally, the composition of the BG-11 variants used for this study is in accordance with the ATCC Media 616 and 617, as presented in table 3.1.

3.3 Experimental set up and sampling procedures

3.3.1 Enrichment cultures for chlorinated compound degradation

Enrichment culture for DCP degradation

Inoculum for the development of an enrichment culture able to dehalogenate 1,2-DCP was obtained from sediments of Besòs river estuary (4125'12.2"N 213'53.3"E, Barcelona, Spain) in November 2012. Samples were collected 15 cm below the surface, brought back to the lab, transferred to an anaerobic glovebox and used to set up the cultures on the same day. They were prepared in 100 mL glass serum bottles containing 6 g of sediment (wet weight) and 65 mL of anaerobically-prepared culture media (section 3.2). Serum bottles were sealed with Teflon-coated butyl rubber septa and

Table 3.1: BG-11 medium composition [146].

Component	Freshwater BG-11 (ATCC Medium 616)	Seawater BG-11 (ATCC Medium 617)
NaNO3	1.5 g	1.5 g
K2HPO4	0.04 g	0.04 g
MgSO4·7H2O	0.075 g	0.075 g
CaCl2·2H2O	0.036 g	0.036 g
Citric acid	0.006 g	0.006 g
Ferric ammonium citrate	0.006 g	0.006 g
EDTA (disodium salt)	0.001 g	0.001 g
Na2CO3	0.02 g	0.02 g
Trace metal mix A5[a]	1.0 mL	1.0 mL
Vitamin B12	-	1.0 g
NaCl	-	10.0 g
Distilled water	1 L	1 L

[a] Trace metal mix A5 contained: H_3BO_3 (2.86 g), $MnCl_2{\cdot}4H_2O$ (1.81 g), $ZnSO_4{\cdot}7H_2O$ (0.222 g), $NaMoO_4{\cdot}2H_2O$ (0.39 g), $CuSO_4{\cdot}5H_2O$ (79 mg), $Co(NO_3)2{\cdot}6H_2O$ (49.4 mg), Distilled water (1 L).

aluminum crimp caps and gassed with N_2/CO_2 (4:1, v/v, 0.2 bar overpressure) and H_2 (0.4 bar overpressure). 1,2-DCP was added using a syringe from a 3.2 mM stock solution in acetone to a nominal concentration of 50 μM. Culture bottles were prepared in triplicates and incubated at 25 ^{o}C under dark conditions without agitation. When the initial dose of contaminant was depleted, the same amount was supplied again in the bottle. Culture was transferred to fresh medium (10% v/v) after 80% of the second dose was consumed. For molecular microbiology analyses, all the volume of one bottle was filtered using Durapore membrane filters and immediately frozen. Filtration was conducted inside the fume hood to avoid exposition to traces of pollutant.

In order to exert some selective pressure in the cultures, two different strategies were performed: (1) a methanogenesis inhibitor (BES, 2-bromoethylsulfonate) was added in transfers from May to June 2014 (25 mM, final concentration) and maintained until February 2015 (5 mM, final concentration; table 3.2); and (2) a bacterial antibiotic (vancomycin, 50 μg mL^{-1}) was added in early enrichment transferences until Febraury 2015 (Table 3.2) to discard the presence of grampositive bacterial candidates described in the literature such as *Dehalobacterium*, able to reductively dechlorinate 1,2-DCP.

Three development stages were defined depending on reagents added to liquid enrichment cultures in keeping with both strategies used to decrease unwanted microbial load. Accordingly, establishment (E, vancomycin and high BES concentration), stabilization (S, vancomycin and low BES concentration) and consolidation (C, no additives) stages of the enrichment culture were designated (Table 3.2). The first stage (E) was defined to exert selective pressure to the community and allow the enrichment of the most suitable bacteria to degrade 1,2-DCP. S stage intended to maintain and acclimate the established community to high inputs of contaminant (up to 1500μM). Lastly, the purpose of C stage was to retain the developed community after removing the selective pressure.

Table 3.2: Enrichment culture samples over time and selective medium components present at each transference.

Sample	Date	Group[a]	Vancomicyn (μg mL^{-1})	2-BES (mM)
C1	May-14	E	50	25
C2	Jun-14	E	50	25
C3	Jan-15	S	50	5
C4	Feb-15	S	50	5
C5	Jul-15	C	0	0
C6	Nov-15	C	0	0
C7	Dec-15	C	0	0

[a] E, establishment; S, stabilization; C, consolidation.

Enrichment culture for DCM degradation

Two slurry samples were taken from different points of a membrane bioreactor (MBR) in a centralized industrial wastewater treatment plant (Barcelona, Spain). No stages were defined in this case. Addition of contaminants was carried out as described for the DCP culture, only with modifications in the medium composition [145] detailed in section 3.2. In this case, only 3–7% v/v inoculum was transferred to new bottles with fresh medium.

3.3.2 Cyanobacteria enrichment from microbial mats

Microbial mat samples were located in the Alfacs Peninsula at the southern spit of the Ebre Delta (4035'26.8"N 039'35.3"E, Spain) during the month of July 2015. Physico-chemical parameters of the seawater over the mats are presented in table 3.3.

Table 3.3: Physicochemical properties of the seawater above the microbial mats.

Parameter	Measure
Temperature	28.5 °C
Dissolved Oxygen	10.45 mg L^{-1}
Conductivity	51,590 μS cm^{-1}
Salinity	49.06 g L^{-1}
Total dissolved solids	46,800.00 mg L^{-1}
pH	8.19
Redox potential	218.2 mV

A total of 20 petri plates were used as corers and sunk to obtain samples at a depth of 1.5 cm. Sediment excess was removed using a clean knife and samples were refrigerated (4 °C) and brought back to the laboratory for further processing. Only the uppermost layer of the mats (2-3 mm) was used to prepare the inoculum for enrichment culture establishment. This upper green fraction was recovered from each plate with the help of sterile surgical blades and then gently homogenized in 0.9% NaCl solution and incubated in BG-11 (ATCC Medium 616) and marine BG-11 (ATCC Medium 617), two cyanobacteria-specific media. Media composition is provided in table 3.1. Three enrichments containing 280 mL of medium and 20 mL of homogenized inoculum were prepared in 500 mL Erlenmeyer flasks for each media. Cultures were named accordingly N0, N1, N2 (BG-11) and S0, S1, S2 (marine BG-11). Erlenmeyer 0 of each condition was maintained without the addition of hormones while erlenmeyer 1 was spiked with E2 and EE2 up to a concentration of 0.25 mg L $^{-1}$ for each hormone. Erlenmeyer 2 was spiked to the same concentration after growth

of was confirmed by increase in turbidity and pigments. Successive transferences were carried out using 10% v/v inoculum in 250 mL Erlenmeyer flasks under agitation and natural light. From the second transference and on, hormone concentrations were increased up to 0.5 mg L^{-1}. All samples were retrieved immediately after flask agitation using micropipettes inside the laminar flow hood. Micropipette tips were cut diagonally previous to sterilization to allow the retrieval of homogeneous volume.

3.3.3 Photobioreactor for secondary treatment and EC removal

A 1200 L multitube continuous photobioreactor (PBR) was set up in the roof of the School of Engineering building (Universitat Autònoma de Barcelona, Barcelona, Spain). Tubes were made of low density polyethylene and the other main components of propylene. Urban WW from toilets drainage of the Chemical, Biological and Environmental Engineering Department was directed to two settlers. From there, supernatant was collected and pumped by a peristaltic pump up to the PBR. Briefly, the PBR employed a paddle wheel to aerate and create a current between two distribution chambers that were connected by 8 tubes (4 for each direction). WW inlet and outlet were connected at opposing sides of the distribution chamber that contained the paddle wheel. A float switch was installed to avoid liquid overflow.

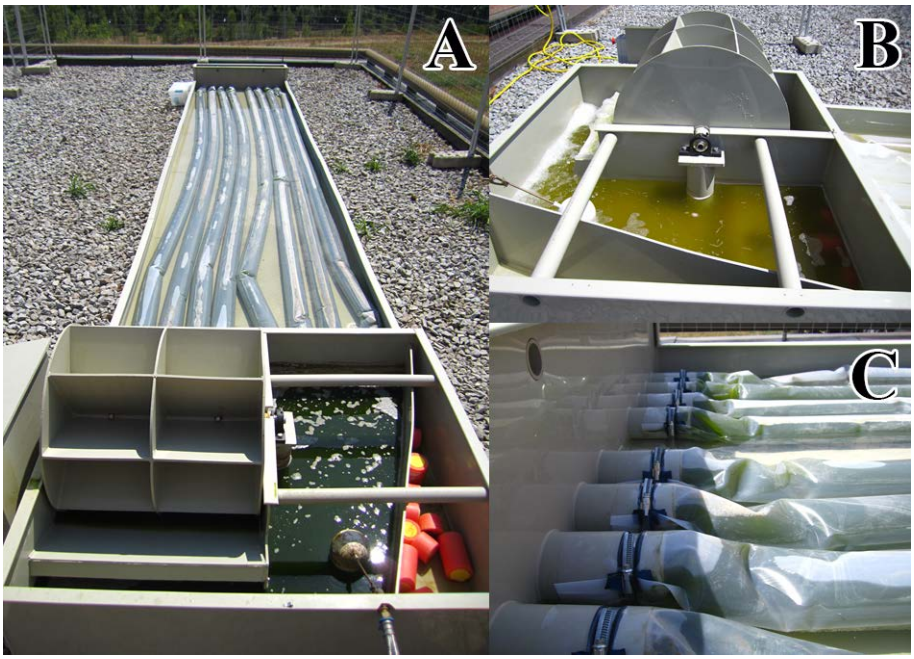

Figure 3.1: Photographs taken during PBR operation. A, General view of the PBR; B, Distribution chamber with the paddle wheel; C, Polyethylene tubes connecting the distribution chambers.

Operation was initiated the 10th of July (2015) using an inoculum of 100 L of lake water from Pantà de Can Borrell (4127'05.8"N 206'41.1"E, Barcelona, Spain). Microalgal growth period consisted of the 10 days following inoculation and hydraulic tests were carried out until September 4. The monitored periods (I and II) were set at two different HRT. Period I comprised from September 14 to October 16, 2015. Period II comprised October 20 until December 20, 2015. Whenever samples were needed for microbial diversity characterization, they were taken directly from the distribution chambers into sterile crystal flasks.

3.3.4 Fungal bioreactor

An air-pulsed fluidized bed bioreactor was used for fungal treatment of HWW. To overcome the burdens of bacterial growth in the fungal bioreactor that lead to the fungus death after some weeks of operation, a coagulation-flocculation pretreatment was applied to the HWW before entering the reactor. This strategy, along with a partial biomass renovation (one third of the biomass renovated weekly), [147] allowed mid-term operation of the fungal bioreactor. The strain employed was *Trametes versicolor* ATCC® 42530^{TM}. It was maintained on 2% malt agar [148] slants at 25 °C until use and was routinely recultured. Whenever fungal pellets were necessary, they were obtained as previously reported [149] by inoculating mycelial suspension in a sterile glass air-pulsed fluidized bioreactor with a defined medium [150] and controlled pH at 4.5.

HWW was collected directly from the sewer manifold of Sant Joan de Déu Hospital (Barcelona, Spain) during September 2015 (HWW1), October 2015 (HWW2) and October 2016 (HWW3). Physico-chemical characterization of the effluents was provided elsewhere [151]. Coagulant and flocculant agents employed were HyflocAC50 and HumolocDR3000, respectively, kindly provided by Derypol S.A. (Barcelona, Spain). Bioreactor operation was carried out at a HRT of 3 days and growth-limiting levels of Glucose and NH_3Cl [151] to avoid increase of chemical oxygen demand (COD) in the effluent. Taking into account the partial biomass renovation, cellular retention time was of 21 days. A summary of the operation is provided in figure 3.2.

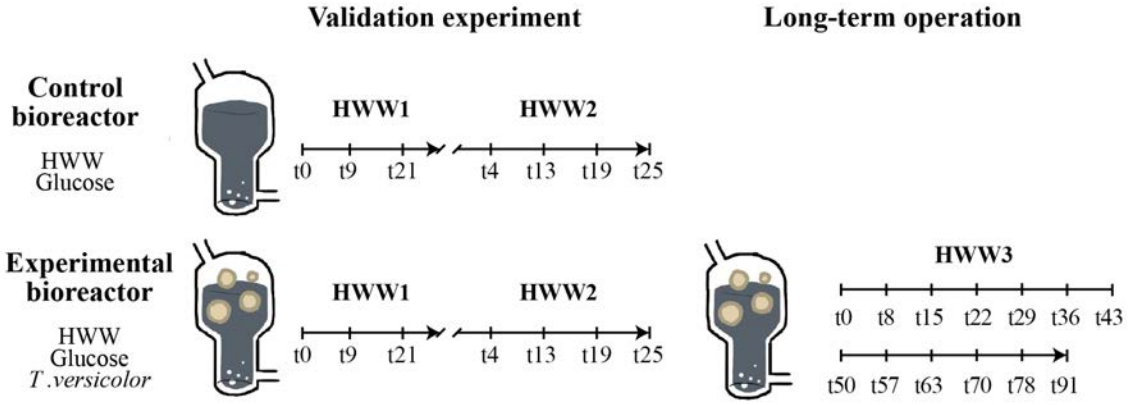

Figure 3.2: Schematic representation of each bioreactor operation and samples taken for microbiological analysis (time points). Arrows indicate the duration of each HWW treatment.

Two sample types were obtained from the *Trametes versicolor* fungal bioreactors:

- Supernatant samples were collected directly from the bioreactor, stored at 4 °C and transported to the lab. Samples were then filtered through 0.22 μm GV Durapore® membrane filters and the filters were stored at –80 °C.
- Pellet samples were centrifuged at 14.000 rpm to remove their liquid fraction and then stored at –80 °C. During the second operation of the bioreactor, pellet samples were instead lyophilized.

Furthermore, effluent resulting from HWW3 treatment was collected directly from the bioreactor outlet using a crystal bottle immersed in ice to preserve the effluent until enough volume was

available. This effluent was further treated using active sludge (section 3.3.6) and employed in the soil irrigation microcosms (section 3.3.7).

3.3.5 Fungal biopiles

Fungal biopiles were composed by dry WWTP sludge, pine bark, and the fungal inoculum (the same strain from section 3.3.4). Dry sewage sludge was collected after anaerobic digestion, dehydration and thermal drying stages in El Prat de Llobregat WWTP (Barcelona, Spain) during January 2014. Commercial decorative pine bark (*Pinus halepensis*) was bought from a local supplier, pruned in small pieces no longer than 1.5 cm and kept at room temperature to be used as bulking material. Mycelial suspension of *T. versicolor* to inoculate the biopiles was prepared according to Blanquez *et al.* [152] and resulting fungal biomass was homogenized and stored in a saline solution (0.85% NaCl) at 4 °C.

Biopiles were prepared in 250 mL Schott bottles equipped with 4-port screw caps. One of the caps was kept open with a 0.45 μm filter for passive air intake. 6 g of the sterile lignocellulosic substrate (20 min at 120 °C) were inoculated with 2 mL of mycelial suspension and humidity was set at 60%. After 7 days of incubation at 25 °C, biopiles were prepared mixing the substrate and fungus with 14 g of non-sterile dry WWTP sludge. Homogenization was performed via circular agitation and biopiles were incubated at 25 °C and 60% humidity. Half of the biopiles were re-inoculated with the mycelial suspension after 22 days of incubation. The same volume of sterile water was added to the other half. Control cultures were maintained in parallel under the same conditions but without *T. versicolor* inoculation. Triplicate cultures were sacrificed for analysis at each sampling day (i.e. days 0, 22 and 42 for controls and days 0, 10, 22 and 42 for experimental biopiles). A schematic representation of the set-up is provided in figure 3.3.

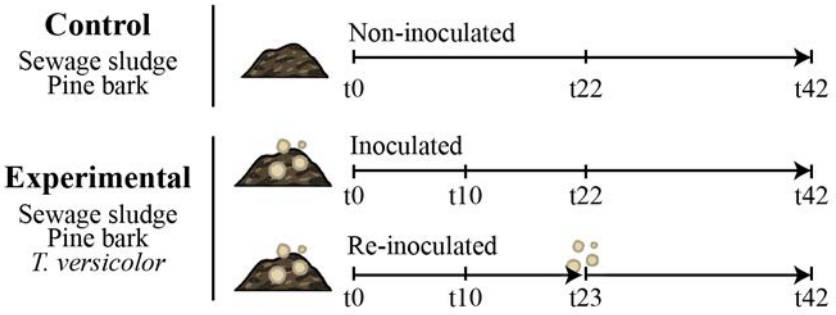

Figure 3.3: Schematic representation of the set-up and bioaugmentation strategies applied to biopiles. Sampled time-points are indicated in each arrow.

3.3.6 Secondary treatment with activated sludge

Activated sludge treatments to mimic conventional WWTP treatment were carried out at room temperature using a set of 2 L glass bottles placed on a magnetic stirrer plate. Permanent aeration was provided using stone air diffusers. Activated sludge was obtained from an urban WWTP in Sabadell (Barcelona, Spain), concentrated by centrifugation and then added to the HWW up to a total suspended solids concentration of 5 g L^{-1}.

3.3.7 Soil irrigation microcosms

Two different microcosm systems were designed to assess the impact of reclaimed water over soil communities. In both cases, aerobic, batch microcosms were prepared with soil collected (upper 10 cm) from a small market garden in Manlleu (Barcelona, Spain) using a sterile hand shovel. Soil was air-dried, sieved (2 mm), and an aliquot was preserved at –80°C. Three effluents were tested in these experiments: Effluent 1 (EFF1) was raw HWW equivalent to HWW3 from section 3.3.4; collected from the sewer manifold of Josep Trueta Hospital (Girona, Spain) and frozen to preserve its compounds until microcosm preparation. Effluent 2 (EFF2) was the reclaimed effluent obtained after treating EFF1 with activated sludge (section 3.3.6). Finally, effluent 3 (EFF3) was the resulting effluent of EFF1 going through the fungal bioreactor (section 3.3.4) and the activated sludge. COD values of EFF2 and EFF3 were <125 mg L^{-1} O_2, in accordance with the European Council Directive 91/271/EEC.

Short-term experiment

Short-term microcosms (45 days) followed two different irrigation strategies (unique and multiple) and were prepared in duplicates in 250 mL flasks with 50 g of soil and 100 mL of each effluent EFF1, EFF2 and EFF3. The resulting 12 Erlenmeyer flasks were placed in an orbital shaker at 50 rpm. Controls were prepared in duplicate with the same soil but irrigated with sterile distilled water. Experimental bottles following the unique irrigation strategy were left undisturbed, while the multiple irrigation strategy involved the weekly renovation of 10 mL of the liquid fraction (after 10-minute settling) with fresh effluent. Cotton caps were added to the Erlenmeyer flasks to allow for aeration. Slurry samples (74% water content w/w) were collected weekly while manually agitating the flasks to obtain homogeneous samples. At least 2 mL of slurry were collected from each replicate and sampling date and stored in Eppendorf tubes at –80 °C until processing. Only the pellets obtained by centrifuging of slurry samples at 8,000 g for 3 min were used in downstream analyses.

Mid-term experiment

On the other hand, mid-term microcosms (90 days) were set up in glass recipients and reproducing the soil environment. The discarded fraction of sieved soil (diameter less than 2 mm) was placed at the base of the recipients, covering a depth of 4 cm. Then, sieved soil was placed on top forming a layer of 4 cm more. Three replicates for each of the four conditions tested (i.e. control, EFF1, EFF2 and EFF3) were prepared and only the multiple irrigation strategy was followed this time. Each microcosm was watered weekly with 20 mL of effluent or distilled water. Samples were taken at the beginning (day 0) and end (day 90) of the experiment from a depth of 2 cm using sterile corers with the help of spatula. Samples were stored in sterile aluminum foil at –80 °C until processing.

3.4 Analytical methods

3.4.1 Analysis of 1,2-dichloropropane and dichloromethane

Gas chromatography analyses were performed by Siti Hatijah and Alba María Trueba as described elsewhere [95, 145] for DCP and DCM, respectively. A consumption·time ratio was calculated for the DCP-degrading culture at each defined stage to reflect the degradation capabilities of the culture in a freshly inoculated bottle. For this purpose, the time required for a culture to consume the first dose of pollutant (up to 100 μM) was registered from at least 5 bottles of each stage (consumption ratio). Additionally, the highest consumption attained in each stage was also calculated (consumption peak).

3.4.2 Analysis of 17-estradiol and 17-ethynylestradiol

Hormone concentrations were measured by Andrea Hom-Díaz in the Chemical, Biological and Environmental Engineering Department, School of Engineering (Universitat Autònoma de Barcelona, Spain) following the protocol proposed by Blánquez *et al.* [117].

3.4.3 Protein and chlorophyll *a* determination

A general estimate of the biomass in the cultures based on protein concentration was obtained via Bradford protein assay [153]. On the other hand, photosynthetic biomass was determined by chlorophyll *a* quantification [154] after protein extraction with methanol.

3.4.4 Analysis of pharmaceuticals

PhACs were analyzed by the Catalan Institute for Water Research (ICRA; Girona, Spain) using the method described by Gros *et al.* [155]

3.5 Molecular techniques

3.5.1 Genomic DNA and RNA extraction

All genomic DNA extractions from liquid and solid samples were conducted using the PowerWater® DNA Isolation Kit and PowerSoil® DNA Isolation Kit (Qiagen), respectively, following manufacturer instructions. The protocol provided by the manufacturer was also followed for soil RNA extractions using the Total RNA extraction kit (Qiagen) and retrotranscription into cDNA using the cDNA synthesis kit protoscript II (New England BioLabs). TURBO DNAse free kit (Ambion) was used to degrade DNA throughout the RNA extraction.

3.5.2 Quantification of DNA samples

DNA was quantified by different methods depending on the sample purpose. Polymerase chain reaction (PCR) products for Denaturing gradient gel electrophoresis (DGGE) were quantified

using reference ladders and the software Quantity One (Bio-Rad). All other DNA quantifications or quality checks were carried out using Nanodrop^{TM} 2000 Spectrophotometer (Thermo Fisher Scientific). Qubit 3.0 Fluorometer (Thermo Fisher Scientific) was acquired in the last period of the project and used to quantify DNA and RNA in the impact of effluents assays and long-term operation of the *T. versicolor* bioreactor.

3.5.3 PCR, primers and programs

PCR is nowadays an indispensable technique in molecular biology that allows the amplification of a fragment of DNA several orders of magnitude. In this work, 16S rRNA gene, 18S rRNA gene and Internal Transcribed Spacer (ITS) regions were used as markers for bacteria, eukaryotes and fungi, respectively. PCR and real-time qPCR reactions were performed in Bio-Rad thermocyclers S1000 and C1000, respectively.

Primers for conventional PCR and real-time qPCR targeting Bacteria, Eukarya or Fungi marker genes are presented in table 3.4. A GC-clamp (5'- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C -3') was included in one primer of each pair when used for DGGE samples. Primers used for real-time qPCR targeting antibiotic resistance genes and mobile elements are presented in table 3.5.

Table 3.4: Primers used for the amplification of Bacteria, Eukarya and Fungi marker genes.

Primer[a]	Sequence (5' to 3')	Specificity	Target site[b]	Reference
27F	AGAGTTTGATCMTGGCTCAG	Universal	9–27	[156]
341F	CCTACGGGAGGCAGCAG	Bacteria	341–357	[157]
518R	ATTACCGCGGCTGCTGG	Bacteria	542–518	[157]
907MR	CCGTCAATTCMTTTGAGTTT	Universal	907–926	[158]
1492R	GGTTACCTTGTTACGACTT	Universal	1510–1492	[156]
BL-DC-142F	GTGGGGGATAACACTTCGAAAGAAGTGC	*Dehalogenimonas*	143–170	[159]
BL-DC-1351R	AACGCGCTATGCTGACACGCGT	*Dehalogenimonas*	1,383–1,362	[159]
mod-BL-DC-1243F	GGYACAATGGGTTGCCACCGG	*Dehalogenimonas*	1,255–1,275	[160]
CYA359F	GGGGAATTTTCCGCAATGGG	Cyanobacteria	359–378	[161]
CYA781R(a)	GACTACTGGGGTATCTAATCCCATT	Cyanobacteria	805–781	[161]
CYA781R(b)	GACTACAGGGGTATCTAATCCCTTT	Cyanobacteria	805–781	[161]
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Fungi	-	[162]
ITS2R	GCTGCTTTCTTCATCGATGC	Fungi	-	[162]
EF4F	GGAAGGGRTGTATTTATTAG	Fungi	-	[163]
ITS4R	TCCTCCGCTTATTGATATGC	Fungi	-	[163]
Euk1AF	CTGGTTGATCCTGCCAG	Eukarya	4–20	[164]
Euk516R	ACCAGACTTGCCCTCC	Eukarya	563–548	[164]
TvqF	CACGCTCTGCTCATCCACTCT	*Trametes versicolor*	-	[165]
TvqR	GCAGAATGCTCCCGTTAAGG	*Trametes versicolor*	-	[165]
Arch349F	GYGCASCAGKCGMGAAW	Archaea	-	[166]
Uni1392R	ACGGGCGGTGTGTRC	Universal	1407–1390	[156]
Dhc1F	GATGAACGCTAGCGGCG	*Dehalococcoides*	1–17	[167]
Dhc264R	CCTCTCAGACCAGCTACCGATCGAA	*Dehalococcoides*	288–264	[168]
dcpA-360F	TTGCGTGATCAAATTGGAGCCTGG	*dcpA*	-	[169]
dcpA-1449R	TTTAAACAGCGGGCAGGTACTGGT	*dcpA*	-	[169]
panF	GGGCTYAACCTGGGAACTGCATTC	*Pandoraea*	954–975[c]	[170]
QpanR	CGGTCAACTTCACGCGTTAGCTWCG	*Pandoraea*	1093–1074[c]	This study

[a] R (reverse) and F (forward) designations refer to primer orientation in relation to the gene.
[b] *E. coli* numbering of 16S rRNA nucleotides.
[c] *Pandoraea* numbering of 16S rRNA nucleotides.

Table 3.5: Primers used for the amplification of antibiotic resistance genes and mobile elements.

Primer[a]	Sequence (5' to 3')	Specificity	Reference
Intl1-a-FW	CGAAGTCGAGGCATTTCTGTC	Integrase gene of class 1 integrons	[171]
Intl1-a-RV	GCCTTCCAGAAAACCGAGGA	Integrase gene of class 1 integrons	[171]
ermB-FW	AGCCATGCGTCTGACATCTA	Azithromycin	[172]
ermB-RV	CTGTGGTATGGCGGGTAAGT	Azithromycin	[172]
ermF-FW	TTTTCTGGGAGGTTCCATTG	Azithromycin	[172]
ermF-RV	TTTCCGAAATTGACCTGACC	Azithromycin	[172]
mph(A)-FW	AGTTCGTGGTGAACGACAAG	Azithromycin	[172]
mph(A)-RV	AGTCGATCATCCCGCTGAC	Azithromycin	[172]
sul1-FW	CGCACCGGAAACATCGCTGCAC	Sulfamethoxazole	[173]
sul1-RV	TGAAGTTCCGCCGCAAGGCTCG	Sulfamethoxazole	[173]
sul2-FW	TCCGATGGAGGCCGGTATCTGG	Sulfamethoxazole	[173]
sul2-RV	CGGGAATGCCATCTGCCTTGAG	Sulfamethoxazole	[173]
dfrA-q-FW	TTCAGGTGGTGGGGAGATATAC	Trimethoprim	[171]
dfrA-q-RV	TTAGAGGCGAAGTCTTGGGTAA	Trimethoprim	[171]

[a] R (reverse) and F (forward) designations refer to primer orientation in relation to the gene.

Regarding the amplification protocols, different step time and annealing temperatures were chosen depending on the primers used, specificity, target size and downstream applications (e.g. DGGE samples require GC-clamps). All reactions were set to remain forever at 15 °C after the main amplification steps. Thermocycler programs are provided as follows (Table 3.6) along with the associated primers:

Table 3.6: Thermocycler protocols used for each primer pair combination. All conventional PCR programs included a 5 min pre-denaturing step at 94 °C and a final elongation at 72 °C for 5 min.

Primer pair	Denaturing		Annealing		Elongation		Cycles
	T (°C)	time	T (°C)	time	T (°C)	time	
ITS1F-ITS2R	94	0:30	55	0:30	72	0:30	35
EF4-ITS4	94	0:30	55	0:30	72	0:30	35
Euk1AF-Euk516R	94	0:30	60	0:40	72	1:00	35
27F-907R	94	0:30	52	$0{:}30 \rightarrow 1:00^{a}$	72	1:30	30
CYA359F-CYA781R(a+b)	94	1:00	60	1:00	72	1:00	35
341F-907MR	94	1:00	$65 \rightarrow 55^{b}$	1:00	72	3:00	$20{+}15^{c}$
Arch349F-Uni1392R	94	1:00	57	1:00	72	1:30	30
dcpA360F-dcpA1419R	94	0:30	60	0:45	72	2:10	30
BLDC142F-BLDC1351R	94	1:00	63	0:45	72	1:00	35
Dch1F-Dhc264R	94	1:00	65+59	1:00	72	1:00	$13{+}20^{d}$
BLDCmod1243F-BLDC1351R	98	0:05			68.2	0:10	31
panF-QpanR	98	0:10			60	0:30	40
TvqF-TvqR	95	0:15			60	1:00	40
27F-907R	94	1:00			60	1:00	45
341F-518R	95	0:10			60	0:10	40
ARG (ermB and ermF)	94	1:00			58	1:00	45
ARG (Intl1, mph(A), sul1, sul2 and dfrA)	94	1:00			60	1:00	45

[a] +1 second per cycle.
[b] -0.5 °C per cycle.
[c] 15 additional cycles with 55 °C annealing.
[d] 13 13 cycles with 65 °C annealing and 20 additional cycles with 59 °C annealing.

3.5.4 Purification and concentration of DNA samples

In order to remove PCR inhibitors or avoid unspecific amplifications, commercial kits were employed to improve the quality of DNA samples. Removal of inhibitors from DNA extractions was performed with OneStep^{TM} PCR Inhibitor Removal Kit (Zymo Research). Purification and concentration of amplicons was done using DNA Clean Concentrator^{TM} (Zymo Research). Amicon® Ultra Centrifugal Filters (Millipore) were employed to merge and concentrate PCR products. Finally, Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research) and QIAquick Gel Extration Kit (Qiagen) were used to recover DNA bands from agarose gels.

3.5.5 Denaturing gradient gel electrophoresis

Genetic fingerprinting techniques generate a profile of microbial communities based on direct analysis of PCR products amplified from environmental DNA. Details of Denaturing gradient gel electrophoresis (DGGE) applied to microbial ecology were provided elsewhere [157]. In brief, the PCR products obtained from the amplification of microbial or environmental DNA are electrophoresed on a polyacrylamide gel containing a linear denaturing gradient obtained by the mixture of urea and formamide. Sequence variation determines the melting behavior of PCR amplicons and these stop migrating at distinct positions, producing a unique profile for each different microbial community.

In the present work, DGGE was performed using the Dcode Universal Mutation Detection System (Bio-Rad). Unless otherwise specified, all electrophoresis conditions remained constant for all the analyses except for the denaturing gradient, that depended on the primer set used. Gels were cast using 6% acrylamide and run at 60 ^{o}C and 75 V during 16 h (960 min) in 1× Tris acetate-EDTA. The standard denaturing gradients were 30-70% for bacteria (341F-907R) and 15-55% for fungi (ITS1F-ITS2R). A GC-clamp was included in the sequence of one primer of the pair to avoid complete denaturation of the resulting PCR products.

3.5.6 DNA visualization

Electrophoresis for DNA extraction and PCR product visualization were run on 1–2% agarose gels at 90 V for 30–50 min in 1× PBE buffer. If product size or concentration were calculated, Low Mass Ladder and High Mass Ladder markers were loaded into the gel for PCR products and total DNA extractions, respectively. 50 bp Ladder from Invitrogen and BioLabs were used as marker in DGGE gels. All samples loaded in agarose or acrylamide gels contained 10% v/v of loading buffer. All gels were stained using ethidium bromide and visualized under UV light in the Bio-Rad Universal Hood II.

3.5.7 Clone libraries analysis

The preparation of clone libraries allowed the sequencing of longer fragments of marker genes for a robust and practical taxonomical identification. Specifically, primers used for clone libraries were 27f–1492r, 27f–907r and Arch349f–Uni1392r.

First of all, amplification of the desired fragment from the DNA extract was carried out. Then, amplicon size and specificity were checked by electrophoresis in 1.5% agarose gels. If a purification step was needed, all PCR product was run in a 1.5% agarose gel and the specific band was recovered using a scalpel and further extracted with a comercial kit. Once quality DNA samples were obtained, libraries were constructed using the TOPO® TA Cloning® Kit (ThermoFisher) and following the manufacturer indications. 1 μL of culture was used as template for PCR reactions prior to sequencing. Clone libraries were kept at 4 °C during the analysis and stored at –80 °C indefinitely.

3.5.8 Real-time qPCR

Real-time quantitative PCR (qPCR) has been thoroughly used in many fields to measure abundance and expression of either taxonomical or functional genes. qPCR uses either intercalating fluorescent dye (e.g. SYBR Green) or fluorescent probes (TaqMan) to measure the accumulation of amplicons in real time after each PCR cycle. Increase in amplicon concentration is measured in the early exponential phase of amplification and it enables the quantification of genes or transcripts if they are proportional to the starting template concentration.

In this work, qPCR assays were performed using a CFX96 Real-Time System (Bio-Rad) in 20 μL reactions containing 1× ssoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.3–0.5 μM of each primer and 9–36 ng of sample DNA. Primer concentrations varied following the recommendations of the original authors (section 3.5.3) and amplification programs were specific for each primer pair and are listed in section 3.5.3. A melting curve analysis followed each PCR reaction to assess product specificity. Melting curves were generated measuring the fluorescence between 65 and 95 °C with increments of 0.5 °C each cycle and a dwell time at each temperature of 5 s. Reactions were always run in triplicates and non-template controls were included in each assay. Calibration curves were prepared using either plasmids containing the desired fragment or purified PCR products of known length. At least six serial dilutions were prepared independently in triplicates. Reaction efficiency was always between 92 and 105%.

Raw data processing was performed using the Bio-Rad CFX Manager software. Moreover, starting copies in each standard reaction for the calibration curve were calculated using the following formula (DNA concentration in ng·μL^{-1} and fragment size in base pairs):

$$\frac{DNA\ starting\ copies}{\mu L} = \frac{\left(DNA\ concentration \times 10^{-9}\right)\left(6.022 \cdot 10^{23}\right)}{\left(660 \times fragment\ size\right)\left(dilution\ factor\right)}$$

3.5.9 DNA sequencing

For recovered DGGE bands and clone libraries, Sanger sequencing was carried out by Macrogen (South Korea). On the other hand, all 16S metagenomic studies were carried out using the Illumina MiSeq platform at the facilities of Sistemas Genómicos (Paterna, Spain). Illumina sequencing was performed using a paired-end (2×250 bp) approach and primers described by Caporaso *et al.* [174] targeting the V4 region of the 16S small subunit (SSU) rRNA.

3.6 Bioinformatic analysis

3.6.1 Fingerprinting data

DGGE fingerprinting profiles were processed and analyzed using InfoQuest™ FP software (Bio-Rad). The introduction of samples in the system and preparation for analysis was carried out as follows:

1. TIFF-formatted DGGE images were imported into the software as densitometric curves, cropped and colour-inverted.
2. Lanes containing markers and samples were defined manually and a spectral analysis was performed. Background and Wiener cut-off scales obtained in the spectral analysis were then used for background subtraction and least square filtering, respectively.
3. Standard marker bands were defined in the respective lanes alongside internal markers (optional) and the gel was normalized.
4. Bands were defined in each sample through the "auto search bands" function. Minimum profiling, minimum area and shoulder sensitivity values were left as default. Manual revision of the bands was always performed and corrections were applied if necessary as false positives (nonexistent bands) can appear due to impurities in the gel.
5. Lanes were exported to the software database and samples of interest were selected to perform band-matching. Optimization and band comparison values were also left default and results were subjected to manual revision.
6. Binary or quantitative band-matching matrix was exported.

Unless otherwise specified, clustering analysis of the comparisons was also performed in InfoQuest™ FP using the Dice coefficient and area sensitive method. Jaccard coefficient was occasionally used for the sake of comparing DGGE data with other techniques. Dendrograms were generated using the UPGMA algorithm and exported in newick format for processing with the FigTree software. The rest of analyses including the calculation of richness (S_{obs}) and diversity (Shannon) estimates were conducted in R [175].

3.6.2 Sequencing data

Sequences produced from either Sanger or high throughput sequencing require quality control procedures before analysis. These quality control varied depending on the sequence origin or intended use. While high-throughput sequences did not manual pre-processing, sequences produced from Sanger sequencing (i.e. from DGGE and clone library approaches) were processed as follows:

1. Raw AB1-formatted files were explored using FinchTV 1.4.0 (Geospiza, Inc; http://www.geospiza.com).
2. Initial and final regions containing sequencing artifacts were trimmed out manually and each sequence was revised to detect low-quality fragments and errors in the automatic assignation of the nucleotides.
3. Sequences were exported in the FASTA format, aligned in BioEdit 7.2.5 (Hall 1999) and trimmed of the regions belonging to the primers used for sequencing.

4. Taxonomic identification of the sequences was either performed manually using BLAST [176] or automatically with MOTHUR [177] or R [178]. Automated taxonomic identification was performed using the SILVA database (release 123) as reference.

MOTHUR

MOTHUR is an open source software that was used in this work to analyze samples with an elevated number of sequences (i.e. clone libraries and high throughput sequencing data). Two analysis pipelines were used with MOTHUR depending on the sample source. 16S metagenomic data were processed according to the standard operating procedure (SOP) recommended by MOTHUR developers [179]. An extract of the main steps and settings in the protocol is provided below. The *summary.seqs* command was used after each step to obtain information of the last outputted FASTA file.

MOTHUR SOP for metagenomic data from Illumina MiSeq

1. Prepare contigs, trim and check quality
   (a) make.contigs
   (b) screen.seqs (Maximum ambiguities = 0; maximum length = 300 bp)
   (c) unique.seqs
   (d) count.seqs
   (e) align.seqs (Flip = true)
   (f) screen.seqs (Start = 11895; End = 25318; Maximum homopolymers = 8)
   (g) filter.seqs (Vertical = true; Trump = .)
2. Cluster sequences
   (a) unique.seqs
   (b) pre.cluster (Diffs = 2)
3. Chimera check
   (a) chimera.uchime (Dereplicate = true)
4. Assing taxonomy and process OTUs
   (a) classify.seqs (Taxonomy = SILVA database; Cutoff = 80)
   (b) remove.lineage (Taxon = Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)
   (c) dist.seqs (Cutoff = 0.20)
   (d) cluster
   (e) make.shared (Label = 0.03)
   (f) classify.otu
   (g) phylotype
   (h) make.shared

(i) classify.otu

(j) get.oturep

MOTHUR SOP for the grouped analysis of clone library sequences

1. Sequence clustering (quality check previously performed manually)
   (a) align.seqs (Flip = true)
   (b) dist.seqs (Output = lt)
   (c) cluster (Cutoff = 0.10; precision = 100)
   (d) bin.seqs
   (e) get.oturep (Label = 0.03)
2. Chimera check
   (a) chimera.uchime (Dereplicate = true)
   (b) get.oturep
3. Assign taxonomy and calculate diversity estimates
   (a) classify.seqs
4. Assing taxonomy and process OTUs
   (a) classify.seqs (Taxonomy = SILVA database; Cutoff = 80)
   (b) remove.lineage (Taxon = Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)
   (c) dist.seqs (Cutoff = 0.20)
   (d) cluster summary.single (Calc = nseqs-sobs-coverage-chao-shannon; subsample = "min n°")
5. Define groups and process OTUs
   (a) make.group (Groups = "group names")
   (b) count.seqs
   (c) make.shared (Label = 0.03)
   (d) get.sharedseqs
   (e) get.oturep
   (f) tree.shared
   (g) summary.shared
   (h) clearcut
   (i) venn (Permute = true, nseqs = true, groups = "group names"; label = 0.03)

R environment

While R is a software typically used for statistical analyses, in this occasion it is also used to process raw sequences as any other popular software like MOTHUR [177] or QIIME [180]. The protocol developed by Callahan *et al.* [181] using the Divisive Amplicon Denoising Algorithm 2 (DADA2) [182] was used in this work to filter, dereplicate, identify chimeras and merge paired-end reads of Illumina data consisting of multiple samples. One of the peculiarities of the method rests in the sequences clustering into Amplicon Sequence Variants (ASV); meaning that sequences are grouped by 100% similarity instead of the classical OTU clustering at 97% identity. This stringent requisite would not be possible without the denoising process, that predicts and corrects sequencing errors to ensure robust and reliable results [182]. The phyloseq package [183] was used in combination of the authors recommended protocol to simplify data handling. An extract of the main steps in the protocol is provided as follows:

R SOP for metagenomic data from Illumina Miseq

1. Install packages (CRAN and BioConductor)and load libraries
   (a) library(BiocStyle)
   (b) library(dada2)
   (c) library(DECIPHER)
   (d) library(ggplot2)
   (e) library(gridExtra)
   (f) library(knitr)
   (g) library(phangorn)
   (h) library(phyloseq)
   (i) library(vegan)
2. Check quality of forward and reverse reads
   (a) plotQualityProfile
3. Sequence trimming
   (a) fastqPairedFilter
4. Infer sequence variants data
   (a) derepFastq
   (b) dada (predict errors with subset of samples)
   (c) plotErrors
   (d) dada
   (e) mergePairs
   (f) makeSequenceTable
   (g) removeBimeraDenovo

5. Assign taxonomy
    (a) assignTaxonomy
    (b) assignSpecies
6. Construct phylogenetic tree
    (a) getSequences
    (b) AlignSeqs
    (c) phyDat
    (d) dist.ml
    (e) NJ
    (f) Pml
    (g) Update
    (h) optim.pml
7. Load metadata and build the phyloseq object
    (a) read.csv
    (b) phyloseq

3.6.3 Functional Annotation of Prokaryotic Taxa

Whenever functional prediction was carried out, FAPROTAX (Functional Annotation of Prokaryotic Taxa) was the tool of choice [184]. It is a database that maps prokaryotic clades, at the genera or species level, to established metabolic or other ecologically relevant functions. Over 80 functions are available although some categories might be redundant or encompass others (e.g. chemoheterotrophy includes aerobic chemoheterotrophy and fermentation).

3.6.4 Sequence submission

Trimmed and quality filtered sequences produced by Sanger were submitted to the National Center for Biotechnology Information (NCBI) GenBank. Illumina fastq raw files were submitted to the NCBI Sequence Read Archive (SRA).

3.6.5 Graphing software

Figures were generated either with SigmaPlot (version 11.0) or the R package [185] ggplot2. Adobe Illustrator CS6 was used to create composite figures.

3.7 Statistical analyses

Statistical analyses were performed using R [175] through RStudio [186] and microbial ecology data analyses using the vegan package [187]. Whenever assessing differences in means (t-test) or

performing analysis of variance (ANOVA), equality of variances was first assessed using Levene's test. Data not following a normal distribution ($p \leq 0.05$) were then analyzed using non-parametric methods such as Wilcoxon-Mann-Whitney or Kruskal-Wallis test. Regarding microbial community structure, differences between two or more groups of samples were tested with ANOSIM and influence of mapping variants were explained using ADONIS output. For high-throughput sequencing data, normalization of reads accounting for sequencing depths was performed using the metagenomeSeq package [188].

A Regression on Order Statistics (ROS) approach was used with the package NADA [189] when dealing with left-censored data in PhAC removal (sections 6.2.1 and 6.2.1), namely: below limit of detection (BLD) and below limit of quantification (BLQ). When ROS was not possible, values BLD and BLQ were considered to have a concentration BLD/2 and BLQ/2, respectively [190].

Chapter 4

Dehalogenating bacteria

The work described in this chapter was carried out thanks to the collaboration with Siti Hatijah Mortan (UAB) and Alba María Trueba Santiso (UAB), both in charge of the cultures and chemical analyses.

4.1 Introduction

Uncontrolled disposal of industrial residues and release of WWTP effluents into the environment have led to the pollution of groundwater by organochlorines in numerous countries, including those from the EU [191]. Although the occurrence of chlorinated ECs has been better characterized in wastewater and surface water environments compared to groundwater [192], exchanges between groundwater and surface water have been already demonstrated [193, 194]. The treatment of contaminated aquifers or groundwater reservoirs is typically carried out through permeable reactive barriers or treatment walls, a cost-effective technology. This technology was first reported by Mc Murthy and Elton [195] and is based on the construction of permanent, semi-permanent or replaceable units across the flow path of a contaminant plume to exert a physical, chemical or biological treatment.

For the purpose of biological bioremediation of contaminated aquifers, laboratory scale-systems to evaluate microbial capabilities to detoxify organochlorinated compounds represent an encouraging strategy. In this sense, mixed microbial cultures are promising approaches based on synergistic metabolic processes among the microbes present in a given scale-system to promote near or complete pollutant detoxification. Understanding the biological pathways and microbial interactions involved in the bioremediation process is of special interest to develop highly specialized enrichment cultures with broad bioremediation capabilities. This knowledge would also provide insights into the metabolic requirements of the microbes that could help to formulate improved culture media.

This chapter focuses in the development of two enrichment cultures with great potential to be used in bioremediation and capable of dechlorinating the alkanes 1,2-dichloropropane (DCP) and dichloromethane (DCM). In the first case, an enrichment culture containing *Dehalogenimonas* sp. was able to completely dechlorinate DCP into propene. In the other case, *Dehalobacterium* sp. was found in another environmental enrichment culture that readily metabolized DCM to acetate and formate.

4.2 *Dehalogenimonas* and 1,2-dichloropropane

DCP was widely employed over the last decades as solvent and chemical intermediate in the production of tetrachloromethane and tetrachloroethylene (TCE). Nowadays it is still present in some adhesives, sealants, coating products, inks, toners and washing products [196]. 1,000–10,000 t per year are manufactured and/or imported in the European Economic Area [196] and human exposure to even low concentrations of the DCP are enough to suppose a health risk [196, 197]. While its carcinogenic potential has not undergone a complete evaluation and determination, the substance is identified as a possible cause of cancer [196]. In fact, in 2013 the Japanese government recognized a causal relationship of DCP long exposure with bile duct cancer, further proved by Kumagai *et al.* [198].

DCP can be partially cometabolized under aerobic conditions by some bacteria, producing less chlorinated alkanes. Interestingly, the complete dechlorination of DCP has only been reported for organohalide respiring bacteria (ORB) that use the pollutant as a terminal electron acceptor and transform the DCP into propene through a dichloroelimination process [199–201]. Only a handful of isolated strains can obtain energy from the dechlorination of DCP without the cooperation of other species. These strains are: *Dehalogenimonas alkenigignens* IP3-3, *D. lykanthroporepellens* BL-DC-8 and BL-DC-9, *Dehalococcoides mccartyi* RC and KS, and *Desulfitobacterium dichloroeliminans* DCA1 [199, 202–204].

4.2.1 Enrichment culture establishment and pollutant consumption

In the present work, a mixed culture initiated from freshwater estuarine sediments was successively transferred to new culture media containing sodium acetate as sole carbon source and H_2 as the electron donor under anaerobic conditions in order to evaluate both the degrading capability of DCP by the active bacteria and the selective effect DCP and culture conditions over the bacteria present. Sediments were collected from the Besòs River estuary in Barcelona (Spain). The site was chosen based on previous works reporting the contamination of the area with polychlorinated naphthalenes, short-chain chlorinated paraffins and polychlorinated biphenils coming from a WWTP located at the mouth of the same river [205]. The presence of this pollutants was expected to provide a potential niche for many ORB.

First steps after the consumption of DCP was confirmed consisted in the determination of which microorganism oversaw the dehalogenation process. As exposed above, only three genera of bacteria are known to metabolize DCP into propene by themselves. With the addition of *Dehalobacter* genus to the list, able to dehalogenate DCP while in consortium [206], a total of four candidates had to be considered. *Desulfitobacterium* and *Dehalobacter* were initially discarded because the enrichment culture maintained the degradation of DCP after the addition of vancomycin (50 μg mL^{-1}), a known inhibitor of grampositive bacteria (both genera belong to the grampositive order Clostridiales). As there was no evidence of interference over growing dehalogenating microbes by the antibiotic, vancomycin was further kept in the culture conditions together with BES to maintain the positive selection. DiStefano *et al.* [207] already reported no inhibition in TCE dechlorination with mixed cultures when vancomycin was employed as long as H_2 was supplied directly as electron source.

Determination of which ORB was (or were) present in the culture was carried out via conventional PCR reactions using genus-specific primers for *Dehalococcoides* and *Dehalogenimonas*. Results confirmed the presence of *Dehalogenimonas* sp. by the presence of a unique amplicon of the expected size ($\sim$ 1240 bp) while discarding *Dehalococcoides sp.* (Figures 4.1A and 4.1B). Furthermore, the presence of the *dcpA* gene in the culture was also confirmed via PCR (Figure 4.1C). *dcpA* is the gene that codifies for the reductive dehalogenase in charge of the transformation of DCP to propene and was first described for members of the *Dehalococcoides* genus [169].

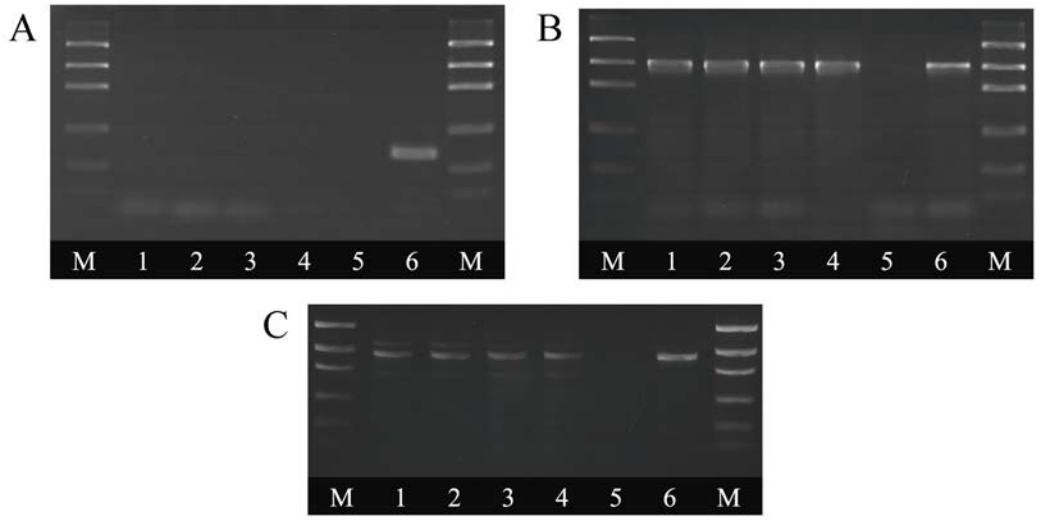

Figure 4.1: Detection of *Dehalococcoides* (A) and *Dehalogenimonas* (B) 16S rRNA genes and the *dcpA* gene (C). M: DNA low mass ladder; 1-4: template containing genomic DNA from the culture; 5: negative control; 6: positive control (A: *Dehalococcoides mccartyi* CBDB1; B-C: *Dehalogenimonas lykanthroporepellens* BL-DC-9).

The implication of *Dehalogenimonas* sp. in the organohalide removal was further proved using stable carbon isotope fractionation by Martín-González *et al.* [95], where the increase in *Dehalogenimonas* 16S rRNA copies was coupled to the consumption of DCP. The dihaloelimination reaction of 1,2-DCP to propene occurrs as follows:

$$C_3H_4Cl_2 + 2H^+ + 2e^- \longrightarrow CH_2CHCH_3 + 2Cl^- + 2e^-$$

The enrichment culture establishment process lasted 3 years and one month, from November 2012 to December 2015. Samples for the microbial characterization of the culture were taken for the first time in May 2014 and until December 2015 due to the low degradation yield obtained initially, not sufficient to consider it an efficient microbial assemblage. Culture procedures and DCP measurements are detailed in the materials and methods chapter, sections 3.3.1 and 3.4.1, respectively. An overview of the culture establishment timeline and the individual and cumulative consumption attained by each experimental bottle is presented in figure 4.2.

During the whole monitoring process, a total of 16,200 μM of DCP were consumed by the enrichment culture over 32 transferences. To compare the cumulative consumption, the consumption ratio (introduced in section 3.4.1), the consumption peak and the copies of *Dehalogenimonas* 16S rRNA gene (real-time qPCR detailed in section 3.5.8), results were compiled in table 4.1 for each stage. The table shows a slight decrease in the consumption ratio (5.86 to 5.12 μM day^{-1}) after the first E stage that was sharply recovered in the latter C stage (8.06 μM day^{-1}). Contrarily, consumption peak experienced a leap from 14.4 to 50.0 M·day^{-1} from stage E to S that correlated with a 7-fold increase in *Dehalogenimonas* concentration. The enhancement is justified either by *Dehalogenimonas* being able to grow in higher densities or by the release of selective pressure in the last stage (table 3.2). Precisely, BES slowed the dechlorination rate in previous work [208]

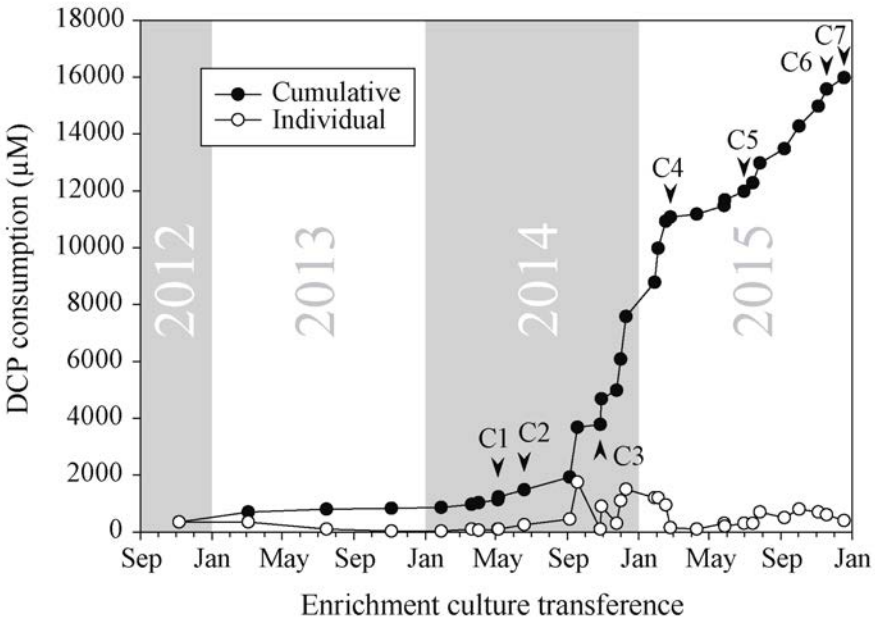

Figure 4.2: Cumulative (•) and individual (○) DCP consumption in each experimental bottle transference. Arrows indicate the seven transferences sampled for microbial community characterization.

with no apparent effects over community composition. The same authors disclosed changes among bacterial species and a slight decrease in dechlorination after the application of Vancomycin. Finally, highest consumption peak reached the maximum value (81.0 μM day^{-1}) in the C stage and by then *Dehalogenimonas* concentration remained stable.

Table 4.1: Enrichment culture consumption values along with the concentration estimate of *Dehalogenimonas*.

Stage[a]	Cumulative consumption (μM)	Consumption ratio (μM·day^{-1})	Consumption peak (μM day^{-1})	*Dehalogenimonas* 16S rRNA gene (10^6 copies mL^{-1})
E	1,680	5.861.9	17.4	0.510.08
S	11,880	5.121.4	50	3.710.25
C	16,180	8.064.2	81	3.550.26

[a] [a] E establishment, S stabilization, C consolidation.

4.2.2 Microbial characterization of the enrichment culture

Two PCR-based molecular approaches were tested in the present work in order to characterize the microbial assemblage: DGGE and clone library analysis. DGGE approach employed the primer pair 341f–907r while clone libraries covered a broader region with the primers 27f–907r. Primers chosen for clone libraries were decided based on their match with different microbial groups. 1492r is typically used in clone libraries to obtain a long sequence but was disregarded as it did not match the *Dehalogenimonas* genus, known to be abundant in the enrichment. To conclude, Illumina sequencing was performed with samples from the last two transferences to obtain more information of the microbial assemblage in the consolidated stage.

Sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers KX870887—KX870907 and KX882142—KX882636, respectively.

In the first place, DGGE fingerprinting profiles were obtained for each of the seven samples and a 100% band coverage was attained recovering and sequencing 21 prominent bands from 14 different positions in the gel (Figure 4.3A). Band sequences were deposited in the NCBI GenBank database under accession numbers KX870887—KX870907. Retrieved sequences were affiliated to 5 different phyla (Chloroflexi, Proteobacteria, Spirochaetes, Tenericutes and Synergistetes) including 5 known genera (*Dehalogenimonas*, *Azonexus*, *Geobacter*, *Desulfovibrio* and *Sphaerochaeta*) and 2 unclassified phylotypes from Firmicutes and Synergistetes. Information of band taxonomic affiliation and relative abundances is provided in Table 4.2. Moreover, cluster analyses were performed to determine the similarity and stability of the enrichment culture over time (Figure 4.3B). Clear differences were observed among the three stages. Samples from the first stage (E) were clearly different from the rest, as observed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) topology and dissimilarity value of 69.8% (Figure 4.3B). Furthermore, the late samples (C6 and C7) from the last stage C were equal, indicating that consolidation was reached.

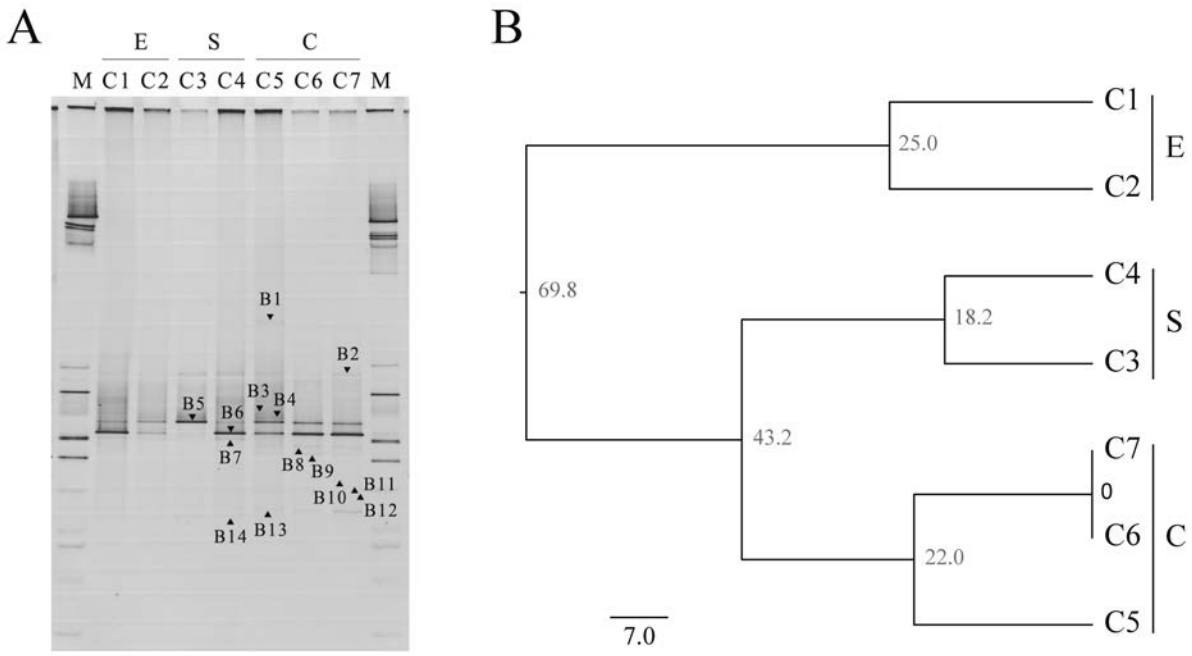

Figure 4.3: A. DGGE fingerprint containing samples C1 to C7 flanked with markers (Mixed 50 bp ladders, Invitrogen and BioLabs). Unique phylotypes (▲) were labelled B1 to B14. B. Cluster analysis of the DGGE profile. Similarity was calculated with Jaccard coefficient, clustering was done using the UPGMA and dissimilarity values are displayed for each clade level.

In the second place, three 16S rRNA gene clone libraries were constructed from samples grouped in the three defined development stages. Sequences were deposited in NCBI's GenBank database under accession numbers KX882142—KX882636. Coverage values ranged from 96 to 98% and species richness (Sobs) and diversity indices (Shannon) slightly varied for the three clone libraries (Table 4.3). Phylotypes from the early E stage showed higher richness and diversity when compared to S and C enrichment conditions. As expected after treatment with inhibitors, the overall tendency was a decrease in both microbial richness and diversity over time. The Operational

Table 4.2: Taxonomic affiliation of DGGE bands according to SILVA database. Relative band intensity is presented for each stage after normalization according to 16S rRNA gene copy number information [209].

Taxonomical group	Genus	Band	Normalized relative band intensity (%)		
			E	S	C
Chloroflexi	*Dehalogenimonas*	B3, B4, B5	70.3	64.8	57.6
Betaproteobacteria (beta)	*Azonexus*	B6	27	28.5	35.5
Deltaproteobacteria (delta)	*Geobacter*	B7, B8, B9	1.7	2.4	2.5
Deltaproteobacteria (delta)	*Geobacter*	B7, B8, B9	1.7	2.4	2.5
Deltaproteobacteria (delta)	*Desulfovibrio*	B14	0.5	1.1	0.4
Spirochaetes	*Sphaerochaeta*	B1, B11, B12, B13	0.5	0.3	2.1
Synergistetes	Unclassified	B10	0	0	0.4
Tenericutes	Unclassified	B2	0	3	1.5

Taxonomic Unit (OTU)-based approach was carried out at a 97% cutoff, resulting in 25 OTUs, 11 of which (44%) were singletons and further discarded from the analysis. The three defined stages were considered to perform a rank abundance analysis of defined OTUs (Figure 4.4). About 82% of the analyzed clones belonged to OTUs 1 (50%) and 2 (32%). Phylogenetic analysis of the defined OTUs revealed that these two main groups of sequences belonged to the genera *Azonexus* and *Dehalogenimonas*, respectively. It is worth mentioning that other less abundant OTUs (i.e. OTU 9, 11 and 12) were also phylogenetically associated to *Azonexus*. Following up in terms of abundance, OTUs 3 (97% similar to *Geobacter lovleyi*) and 6 (99% similar to *G. sulfurreducens*) accounted for 7% of the analyzed clones. In less abundance, OTUs 4 and 7 (4% of clones) were found to be closely affiliated with *Desulfovibrio* and OTU 5 (2% of clones) to *Sphaerochaeta*, a recently described genus within the phylum Spirochaetes neither displaying helical morphology nor motility [210]. The 7 remaining OTUs accounted for a 4% of the total number of clones and belonged to distinct taxonomical groups.

Finally, Illumina MiSeq characterization of the consolidated culture provided a deeper and robust insight (99.9% coverage) on the bacterial community composition developed at the end of the enrichment process. Raw sequence reads are available at NCBI Sequence Read Archive (SRA) under accession number SRR4279905. Bioinformatic processing was carried out with MOTHUR as indicated in section 3.6.2.

Table 4.3 presents the α-diversity richness and diversity indexes, matching perfectly with the results obtained from clone libraries. Moreover, a rank abundance plot was constructed (Figure 4.4) discarding all singleton OTUs (174 out of 238; 73.1%) revealing that a roughly 95% of the sequences were represented by the first 4 OTUs, with individual relative abundance values of 68, 10, 9 and 7%, respectively. Taxonomical affiliations of the abundant OTUs were consistent with the previous results in DGGE and clone libraries. *Azonexus*, *Dehalogenimonas*, *Geobacter* and *Desulfovibrio* corresponded to OTUs 1 to 4, respectively. It's worth to mention that the resulting fragment from the Illumina analyses (250 bp long) was not long enough for a proper taxonomic affiliation of the OTU 3, which belonged to an unclassified strain from order Desulfomonadales where the genus *Geobacter* is located. It is the only case where manual indexation was carried out after confirming the match in BLAST [176].

Table 4.3: Information of clone libraries and Illumina data. Coverage, richness and diversity indicators are represented for each analyzed sample.

Sample[a]	Clones	Coverage (%)	Sobs[b], [c]	Shannon[b], [c]
E-CL	125	96	14.0±0.2	1.7±0.0
S-CL	124	96.8	12.0±0.0	1.4±0.0
C-CL	246	98.4	9.2±1.2	1.2±0.1
C-IS	236,527	99.9	238±0.0	1.2±0.0

[a] CL Clone library, IS Illumina sequencing, E Establishment, S Stabilization, C Consolidation.
[b] values were determined at a 97% cut-off.
[c] alpha-diversity estimators for the clone libraries (samples CL) were calculated over a subset of 124 random clones to avoid biases between libraries due to different sampling efforts.

As expected, two distinct molecular approaches (and a final characterization with a third one) provided different and deep insights on the microbial composition, as reflected by the different relative abundance of a given genera in the DCP-degrading enrichment culture. Diversity and shifts in the community structure of the growing enrichment due to culture transfers were evidenced by plotting relative abundance results from the three molecular techniques (i.e., DGGE, clone libraries and Illumina sequencing; Figure 4.6).

Influence of vancomycin and BES over the community was evident, as grampositive bacteria did not develop in the enrichment culture and neither methanogenic or non-methanogenic Archaea were detected after the 25 mM BES (data not shown). A genus level approach was used when possible in order to describe the bacterial community. *Dehalogenimonas* and *Azonexus* were the dominant genera (80 — 97% of the total community) in the culture independently of the stage while the rest of genera combined never exceed 20% of the community (DGGE: 3 – 8%, clone libraries: 9 – 17%, Illumina sequencing: 20%). In general, the relative abundance of the main genera in the culture showed an inverse pattern, whereas *Azonexus* increased over time, *Dehalogenimonas* decreased over time with minimum values at C stage. Low-abundant groups (individually accounting for <10%) as *Desulfovibrio* and *Geobacter* were stable in S and C stages, whereas at the initial stage of the enrichment differences arose depending on the sequencing technique. In comparison to DGGE and clone libraries, Illumina sequencing evidenced a higher contribution of low retrieved groups like *Geobacter* and *Desulfovibrio*. Underrepresented groups (relative abundance values below 1%) were clustered in a single category, which contained 3 bacterial groups for DGGE and clone libraries and up to 23 for Illumina dataset. In the Illumina data, *Azonexus* genus reached a relative abundance of 50% of the community while *Dehalogenionas* accounted for a roughly 30%. It must be taken into account that Illumina sequencing was only performed using the last two transfers (i.e. C6 and C7), so some slight differences at the final stage with respect to DGGE and clone libraries were expected. For example, an increase in *Desulfovibrio* and *Azonexus* relative abundances occurred at the same time that *Dehalogenimonas* contribution diminished. Detailed information in relation to all detected groups is presented in Table 4.4.

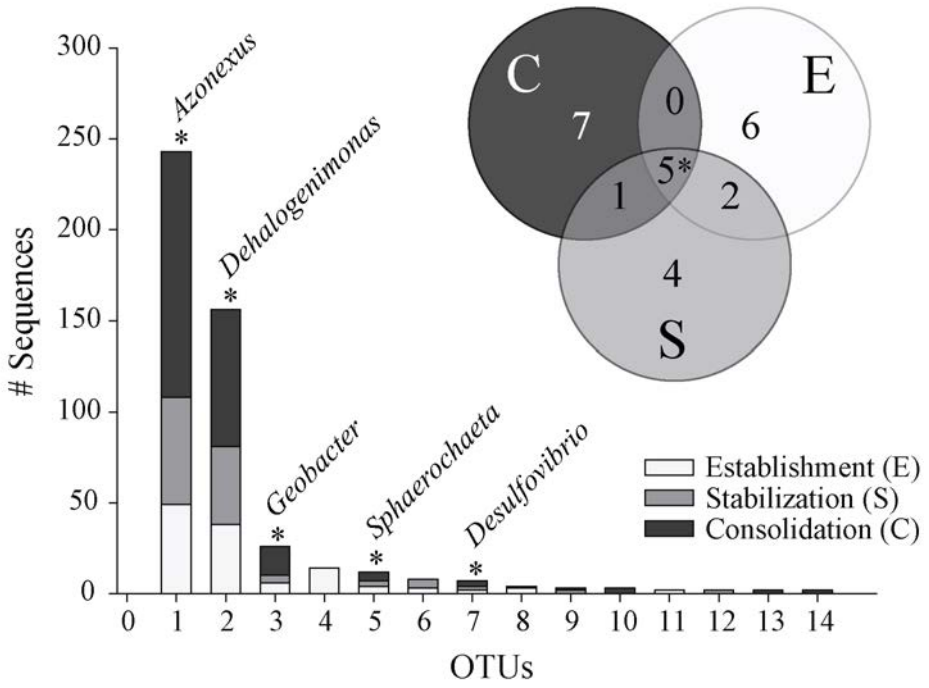

Figure 4.4: Rank abundance plot from clone libraries data showing the assigned OTUs (97% cut-off) and their abundance in samples grouped according to development stage of the enrichment culture. Singletons are not represented. The inset represents Venn diagrams showing the shared OTUs among the three development stages.

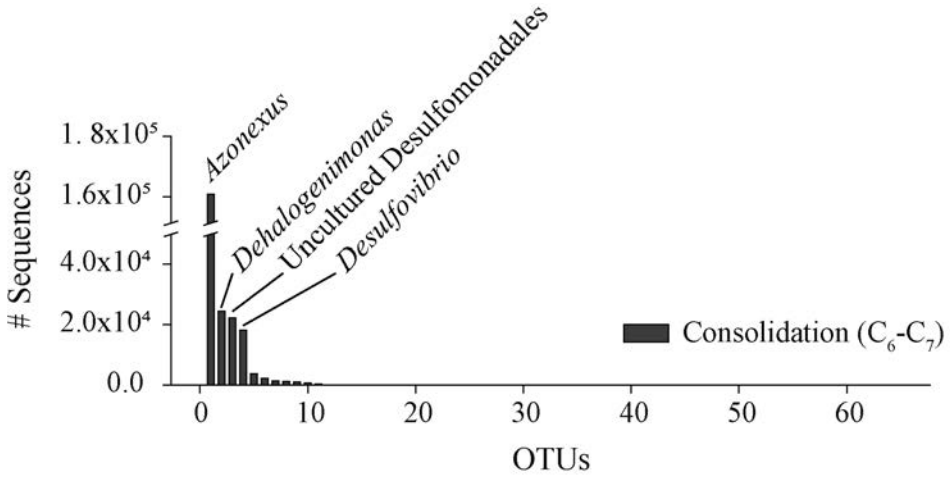

Figure 4.5: Rank abundance plot of the OTUs resulting from the high throughput sequencing of the latest samples C6 and C7.

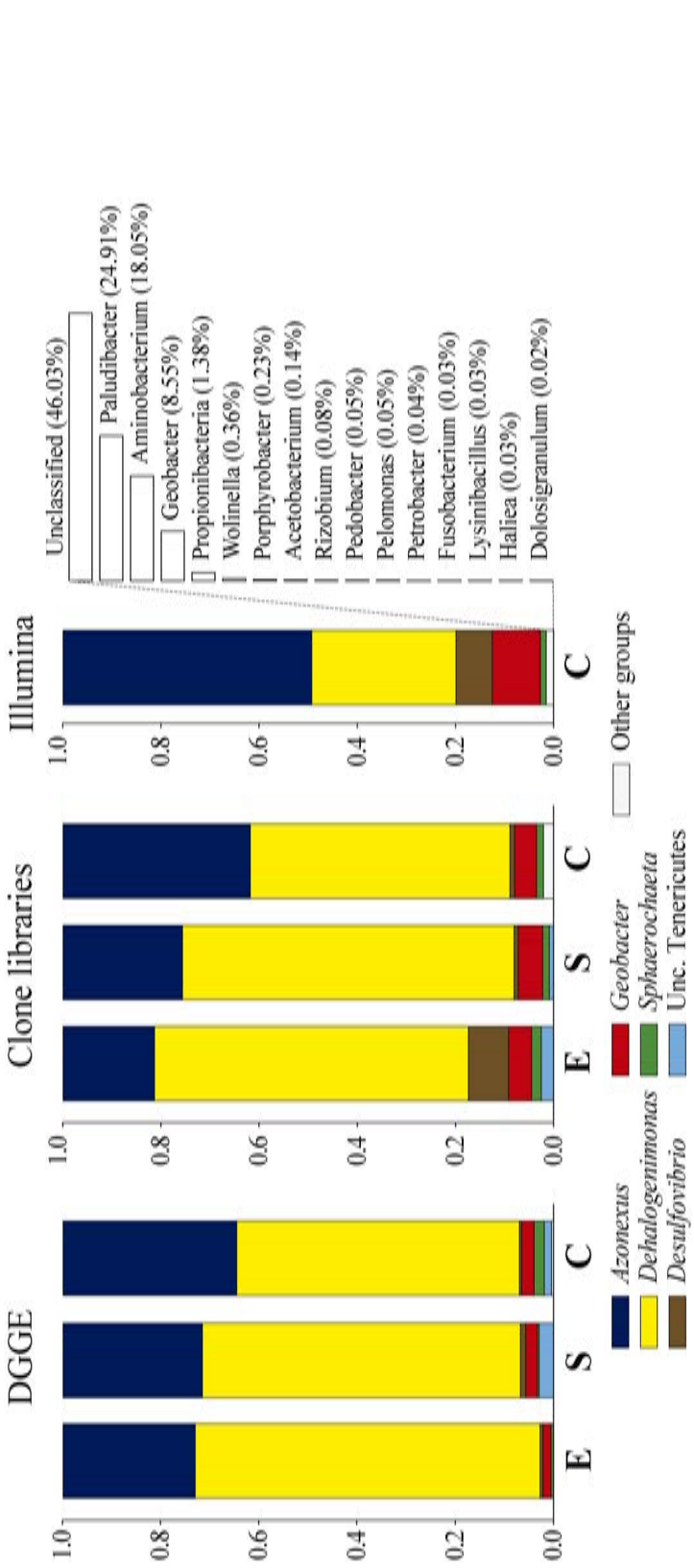

Figure 4.6: Normalized [209] relative abundance of the bacterial communities analyzed with different techniques. E, Establishment; S, Stabilization; C, Consolidation.

Table 4.4: Taxonomic affiliation of the retrieved Illumina sequences from the consolidation (C) stage. Number of sequences is presented along with the normalized relative abundance.

Taxonomical group		Nº Sequences	Relative abundance (%)
Actinobacteria	Unclassified Propionibacterium	52	0.0215
Bacteroidetes	*Paludibacter*	962	0.3869
	Pedobacter	2	0.0008
	Unclassified	4	0.0015
Chloroflexi	*Dehalogenimonas*	24359	29.3907
Cyanobacteria	Unclassified	2	0.0009
Firmicutes	*Acetobacterium*	9	0.0022
	Dolosigranulum	2	0.0003
	Lysinibacillus	4	0.0005
	Unclassified Clostridiales	8	0.0015
	Unclassified Veillonellaceae	1138	0.2496
Fusobacteria	*Fusobacterium*	2	0.0005
Alphaproteobacteria	*Porphyrobacter*	3	0.0036
	Rizobium	3	0.0012
Betaproteobacteria	*Azonexus*	160972	50.7109
	Pelomonas	2	0.0008
	Petrobacter	2	0.0006
	Unclassified Comamonadaceae	16	0.0064
	Unclassified Oxalobacteraceae	5	0.003
Gammaproteobacteria	*Haliea*	2	0.0004
	Unclassified Pseudomonadaceae	2	0.0005
Deltaproteobacteria	*Desulfovibrio*	21835	7.3796
	Geobacter	22469	9.9334
	Unclassified Desulfovibionales	16	0.006
Epsilonproteobacteria	*Wolinella*	14	0.0056
Spirochaetes	*Spirochaeta*	2250	1.1651
Synergistetes	*Aminobacterium*	697	0.2803
Bacteria	Unclassified	1521	0.4454

It is a complicated task to evaluate our results based on previously published works, as information in relation to other *Dehalogenimonas*-abundant consortia is either scarce or poorly detailed [204, 211, 212] and there seems to be no resemblance to other DCP-degrading microbial assemblages [202] in the literature. The clustering of C stage samples from DGGE fingerprinting (Figure 4.3B) and the faster growth of the culture (data not shown), together with the improvement observed in the early degradation ratio, strongly support the successful consolidation of the degrading mixed culture. In this sense, to author's knowledge this is the first in-deep characterization of a microbial assemblage developed around the ORB genus *Dehalogenimonas* able to degrade DCP.

To evaluate the reliability of the obtained results from three different techniques, all representative sequences from each methodology were aligned, trimmed to the maximum comparable length (ca. 250 bp) and clustered altogether in OTUs at a 97% cut-off value. OTUs containing the most dominant groups were shared between all three methodologies as seen in the Venn diagram (Figure 4.7).

Variability in low-abundant groups was attributed to detection limit inherent to the DGGE, where species with abundances lower than 1% are hardly detected [157]. Moreover, differences in DGGE primer affinities for *Geobacter*, *Desulfovibrio*, *Sphaerochaeta* and uncultured Mollicutes groups were discarded because they showed higher affinities for all groups except for Mollicutes when checked through TestPrime [213]. The presence of *Geobacter* species in our enrichment culture, and particularly the dechlorinator *G. lovleyi* (OTU 3 from clone libraries exhibited a 97% similitude to *G. lovleyi*), is a common characteristic with other published works [214,215] that employed inoculums from either creek sediments or contaminated soil and groundwater.

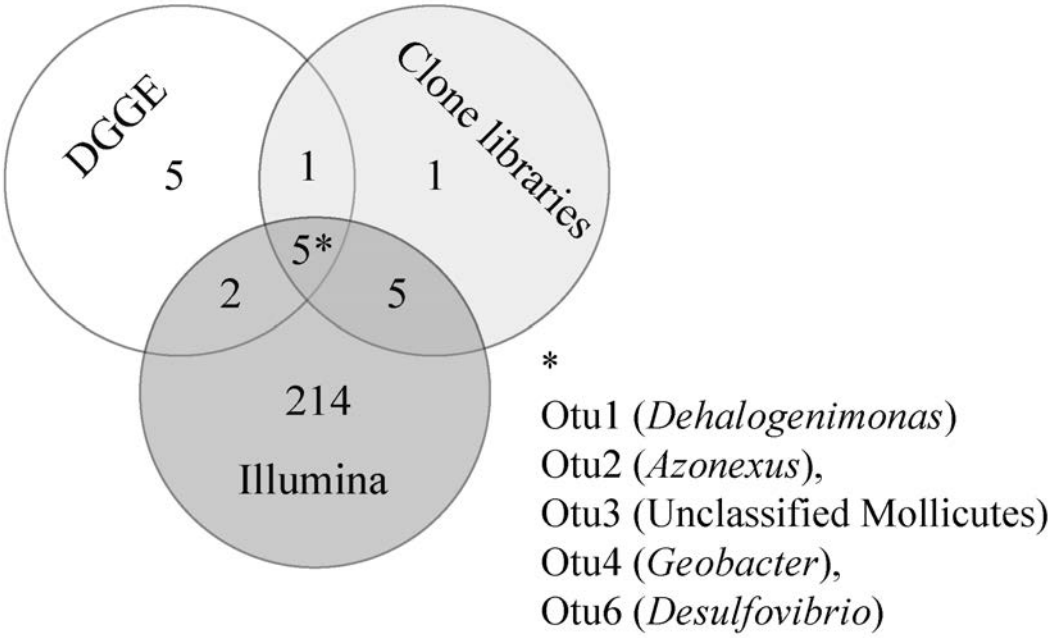

Figure 4.7: Venn diagram of the OTUs retrieved (97% cut-off) from representative sequences of each methodology.

Along with *Dehalogenimonas*, *Azonexus* was the most abundant genus throughout the culture consolidation. Interestingly, OTUs associated with *Azonexus* displayed 99% identity (BLAST) to the nitrogen-fixing *Azonexus caeni* (NR_041017) [216]). Although nitrogen was provided in the medium through NH_4Cl, its exhaustion likely restrained the growth of non-diazotrophic bacteria after some time of activity. Previous works [217] in oil-polluted soils described enhancements in bioremediation when nitrogen-fixing bacteria were present in the environment. Reportedly, metabolic synergies were also responsible for the improvement so they should not be disregarded in the case of DCP, as *Azonexus* is part of the metabolically versatile Rhodocyclaceae family. In the DCP-degrading culture, at the end of the analyzed period, we detected the presence of microbes belonging to the *Sphaerochaeta* genus (ca. 1.5%), typically found in association with *Dehalococcoides* [210] but not yet described in association with *Dehalogenimonas* species. It has been hypothesized that *Sphaerochaeta* could provide with substrates (e.g. acetate and H2) or protection against short-term oxygen exposure to *Dehalococcoides* [218]. However, oxygen protection must be ruled out for *Dehalogenimonas* due to their intrinsic short-term O_2 exposure tolerance [201]. Furthermore, Iasur-Kruh *et al.* [219] suggested a synergy between *Sphaerochaeta* and *Pelobacter* (close relative to *Geobacter*); when *Sphaerochaeta* was absent from the culture, dehalogenation activity was no longer detected.

Pure culture isolation attempts of *Dehalogenimonas* strains by dilution to extinction approaches with still dechlorinating activity provided insights onto which community members were closely associated to the ORB. In this sense, only *Desulfovibrio* and an uncultured Veillonellaceae representative remained accompanying *Dehalogenimonas* in low abundance (12 and 1%, respectively) when a clone library analysis was performed (ca. 90 clones, 95% coverage at 97% cutoff; data not shown) in the same conditions as described above. This tight association with *Desulfovibrio* likely allowed the reported density increase of *Dehalogenimonas* as it was described for the ORB *Dehalococcoides ethenogenes* 195 [220].

In conclusion, results evidence a specialized community developed in the culture along with the organohalide-respiring *Dehalogenimonas*. Main abundant groups as *Geobacter*, *Sphaerochaeta* and *Desulfovibrio* were presumptively either suppliers of essential factors or growth enhancers. These results further direct towards an implementation of analytical approaches to analyze the contribution of each individual genus at a metabolic level. For example, in the particular case of *Azonexus*, evaluation of its ability to supply nitrogen compounds to the rest of the community would be of interest to assess the viability of the enrichment culture in simplified media.

4.3 *Dehalobacterium* and dichloromethane

As DCP, dichloromethane (DCM) is also used in adhesives, sealants, coatings and washing products. Moreover, it is also present in biocides, plant protection products, cosmetics and personal care products [196]. Around 100,000–1,000,000 t per year are manufactured and/or imported in the European Economic Area and the substance is suspected of causing cancer [196] and inhibit the growth and reproducibility of aquatic organisms [221, 222] among other affections. Although the use of DCM is declining with time, the discharges are still significant and it is frequently found polluting WW or subsurface waters of industrial areas [223]. Some microbes like *Acinetobacter* sp. and facultative methylotrophic bacteria have been described to degrade DCM in specific conditions [224,225] and when methanogenesis is also inhibited [226]. *Dehalobacter* and *Dehalobacterium* are the only two known genera capable of fermenting DCM and use it as sole source of organic carbon and energy while producing formate and acetate [40, 227]. In particular, *Dehalobacterium formicoaceticum* can also degrade dibromomethane when DCM is present in the medium [228].

4.3.1 Enrichment culture development and microbial characterization

The inoculum for the establishment of an enrichment culture consisted of two slurry samples taken at different points of a membrane bioreactor (MBR) from an industrial wastewater treatment plant. The plant was located in Santa Perpetua de Mogoda (Barcelona, Spain) and received influents containing many halogenated compounds such as DCM, chloropropane, monochlorobenzene or tetrachloroethylene. Thus, it was expected to find numerous microbes adapted to the presence of these pollutants and even capable of degrading some of them. Sampling was carried out in November 2014 and the manipulation procedures were as described for the DCP-degrading enrichment. When DCM dechlorination activity was detected, the identification of the strain with degradation ability was carried out by DGGE and is reported in Trueba-Santiso *et al.* [145]

Briefly, enrichment cultures were subjected to a dilution-to-extinction approach in order to facilitate the isolation of the active dechlorinators. Moreover, cultures in semi-solid media (1% low melting point agar) yielded different colony morphologies that were recovered and inoculated back in liquid medium. These cultures (the diluted enrichment and three colony-derived cultures) were then maintained for three transferences and two of them showed dehalogenation activities while the other two did not. DGGE was performed using the primer set 341f-907r (Figure 4.8) and profiles revealed the presence of *Acetobacterium* sp. in all samples while the band belonging to genus *Dehalobacterium* only appeared in the DCM-degrading bottles.

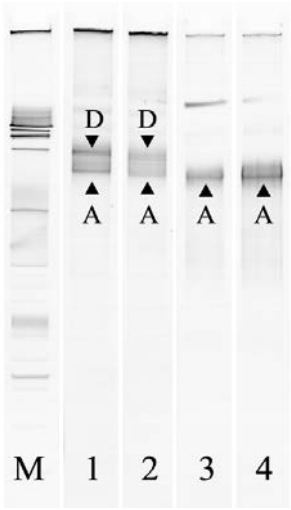

Figure 4.8: Bacterial DGGE profiles of serially diluted enrichment cultures. M: mixed 50bp ladder DNA markers. Lane 1: enrichment culture showing DCM activity corresponding to the 10^{10} diluted terminal positive tube. Lane 2: culture from an agar shake tube colony showing activity against DCM. Lanes 3 and 4: cultures from agar shake tube colonies without DCM degrading activity. Band A: *Acetobacterium* sp. Band D: *Dehalobacterium* sp.

The culture establishment process was only assessed in this case in the initial and end points of the process, comprising a total of 15 transferences since November 2014 until November 2015. Compared to the DCP-degrading consortium, this *Dehalobacterium*-driven enrichment culture attained higher degradation yields in a brief period. While the first inoculum needed more than 30 days to consume 10 mM of DCM (oral communication), latter transferences were able to amend up to 2000 μM in 10 days. At this point, the culture was considered stable due to the robust and reproducible DCM degradation [145]. Characterization of the bacterial community developed in the consolidated culture was performed via high throughput sequencing of the 16S rRNA gene V4 region. Three replicates of the culture were considered along two samples from the inoculation sources (slurry 1 and 2) for sequencing using the Illumina platform. Raw reads are available in SRA under the accession number SRR4422954. Bioinformatic procedure was carried out with R and details are presented in section 3.6.2.

Taxonomical affiliation of the obtained sequences revealed that Bacteroidetes, Deinococcus-Thermus, Proteobacteria, Tenericutes were the main Phyla represented in the source samples from which the enrichment culture was then developed. To unveil the drastic changes undergone in the culture establishment, abundant ($\geq$0.01 relative abundance) amplicon sequence variants (ASVs) in the slurry and the culture were presented at the genus level (if possible) in a composite plot (Figure 4.9) and ordered following abundance in the slurry.

Although three different *Dehalobacterium* ASVs were detected, all of them matched the same sequence from *Dehalobacterium formicoaceticum* strain DMC complete genome (Accession n^o CP022121.1) with 100, 99 and 100 % identity, respectively. Interestingly, none of the top 19 ASVs from the source slurry were maintained in the developed culture. Except for *Wolinella*, the other main genera driving the culture (*Acetobacteirum*, *Dehalobacterium* and *Desulfovibrio*) had relative abundances below 0.01 in the slurry. While exposure to DCM seems to promote the anaerobic metabolism and has been linked to the presence of *Desulfovibrio* and *Acetobacterium* [229], the specific medium composition was likely involved in the selection too. To elucidate the roles of the four members of the assemblage, it is important to remember that formate and acetate are produced in the following reaction:

$$3CH_2Cl_2 + CO_2 \longrightarrow 2HCOO^- + CH_3COO^- + 6Cl^- + 9H^+$$

Acetobacterium and *Wolinella* can both use formate as a substrate it in reactions that are key for its growth [230–235]. Furthermore, acetic acid produced by *Acetobacterium* can eventually turn into more acetate, which is profitable for *Wolinella* due to its capacity to use acetate as carbon source when polysulfide is available as final electron acceptor [236]. Regarding *Desulfovibrio*, it can use formate and H_2 [42, 237] and has been classified as a putative DCM dechlorinator [229]. Furthermore, sulphites and sulphates (formed from sodium sulphide, added as reducing agent) serve *Desulfovibrio* as electron acceptors [42].

As a general overview, acetate and formate produced by the culture during the dechlorination of DCM can be used as biostimulators of other ORB (i.e. *Dehalogenimonas* or *Desulfospirillum*) because they serve as electron donors. In fact, the combination of different ORB to bioremediate aquifers contaminated with multiple organochlorinated compounds supposes a cost-effective option recently started gathering many interests.

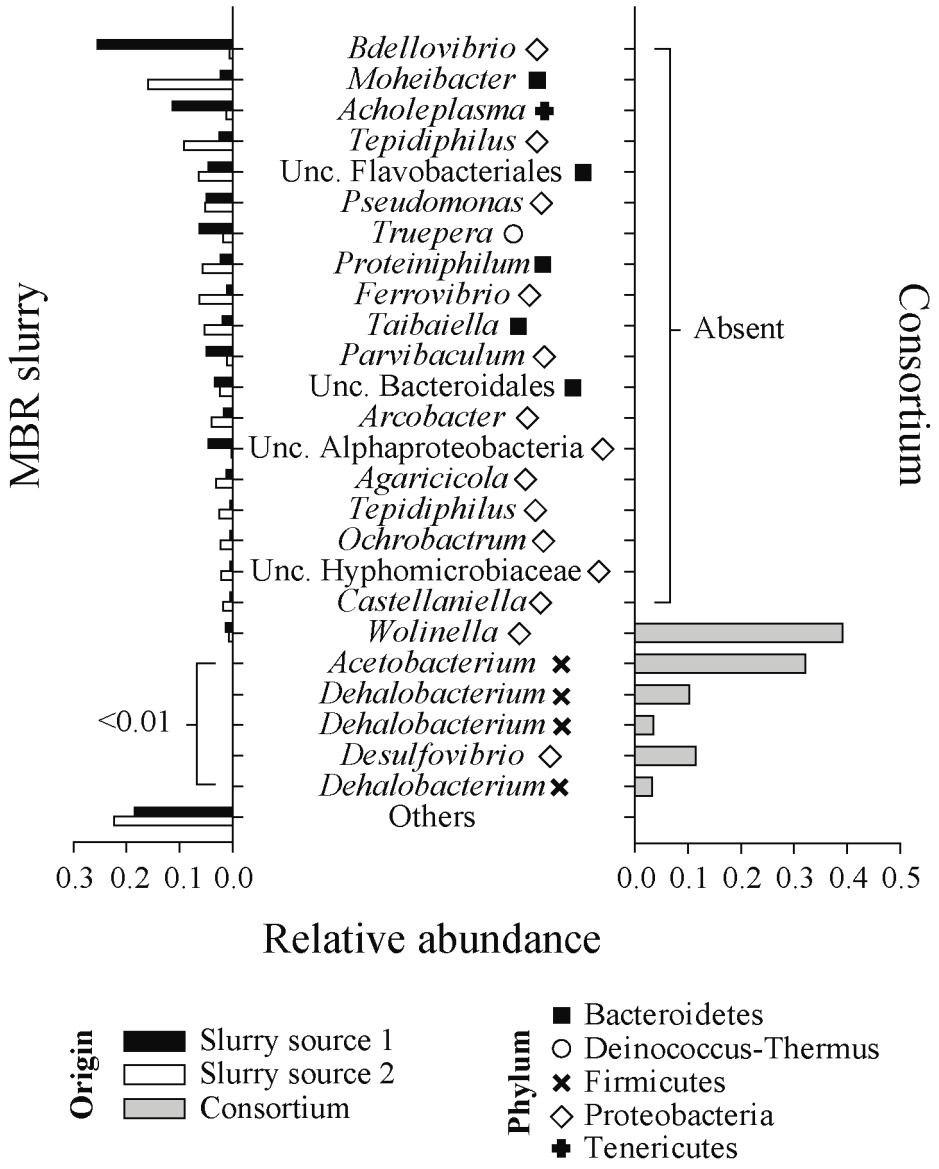

Figure 4.9: Relative abundance of the microbial taxa present in the inoculum sources and enrichment culture at the genus level. Closest affiliation was provided for sequences of unknown genus and only taxa with relative abundance 0.01 are presented. Abundance bars are colored according to the sample origin and phylum affiliations are indicated with symbols.

Chapter 5

Photobioreactor treatment

The work described in this chapter was carried out thanks to the collaboration with Andrea Hom Díaz (UAB) and Adrián Jaén Gil (ICRA), in charge of the PBR operation and pharmaceutical analysis, respectively.

5.1 Introduction

The use of microalgae in WW treatment is in rising trend due to its capacity to remove nutrients and organic matter in association with native WW bacteria [238]. As oxygenic phototrophs, microalgae obtain energy from sunlight, assimilate CO_2 (from the atmosphere or generated by bacteria) and release O_2 profitable for bacteria. Moreover, they clear inorganic nutrients such as N and P and are even capable of metal and EC removal [92, 239, 240]. Elimination of EC by microalgae in pure culture is indeed effective [241–243] but assessment of ECs in microalgal-technologies at a higher level of implementation is scarce [240, 244, 245]. Furthemore, microalgal biomass has wide post-application potential such as to obtain biofuels, biopolymers, metabolites or other bioproducts [246–249].

In this chapter, the performance and microbial community shifts of a tubular microalgal reactor are evaluated during wastewater treatment in different seasonal periods and regimes for pharmaceutically active compounds (PhACs) degradation. Furthermore, a biomass increase strategy (bioaugmentation) was tested ex situ with the aim to improve the degradation of the E2 hormone. Finally, the findings of the study directed the research into the screening of cyanobacteria in natural environments. Despite being considered useful in organic pollutant degradation and bioremediation of metals [99, 250–252], scarce information is available regarding its potential for EC removal [253].

5.2 Photobioreactor operation and removal

A pilot-scale photobioreactor (PBR) with capacity for 1200 L was set up with the objective to treat urban WW coming from a faculty building in the Universitat Autònoma de Barcelona (Barcelona, Spain). Two operation periods were defined and hydraulic retention times were set at 8 and 12 days for period I and II, respectively. The work from this section covers operation until December 2015. However, the PBR was maintained during a third period for further studies. Detailed description of the PBR and its operation can be found in section 3.3.3 and in Hom-Diaz *et al.* [254].

Throughout the operation, total suspended solids (TSS) determination was used as biomass indicator (Fig. 5.1A). TSS in the WW were almost negligible and values remained constant until the end of the operation. During operation period I, the average TSS value was 376±120 mg L^{-1} and the minimum and maximum were 153 and 594, respectively. In comparison, data from period II were always lower with an average of 298±114, a minimum of 123 and a maximum of 513.

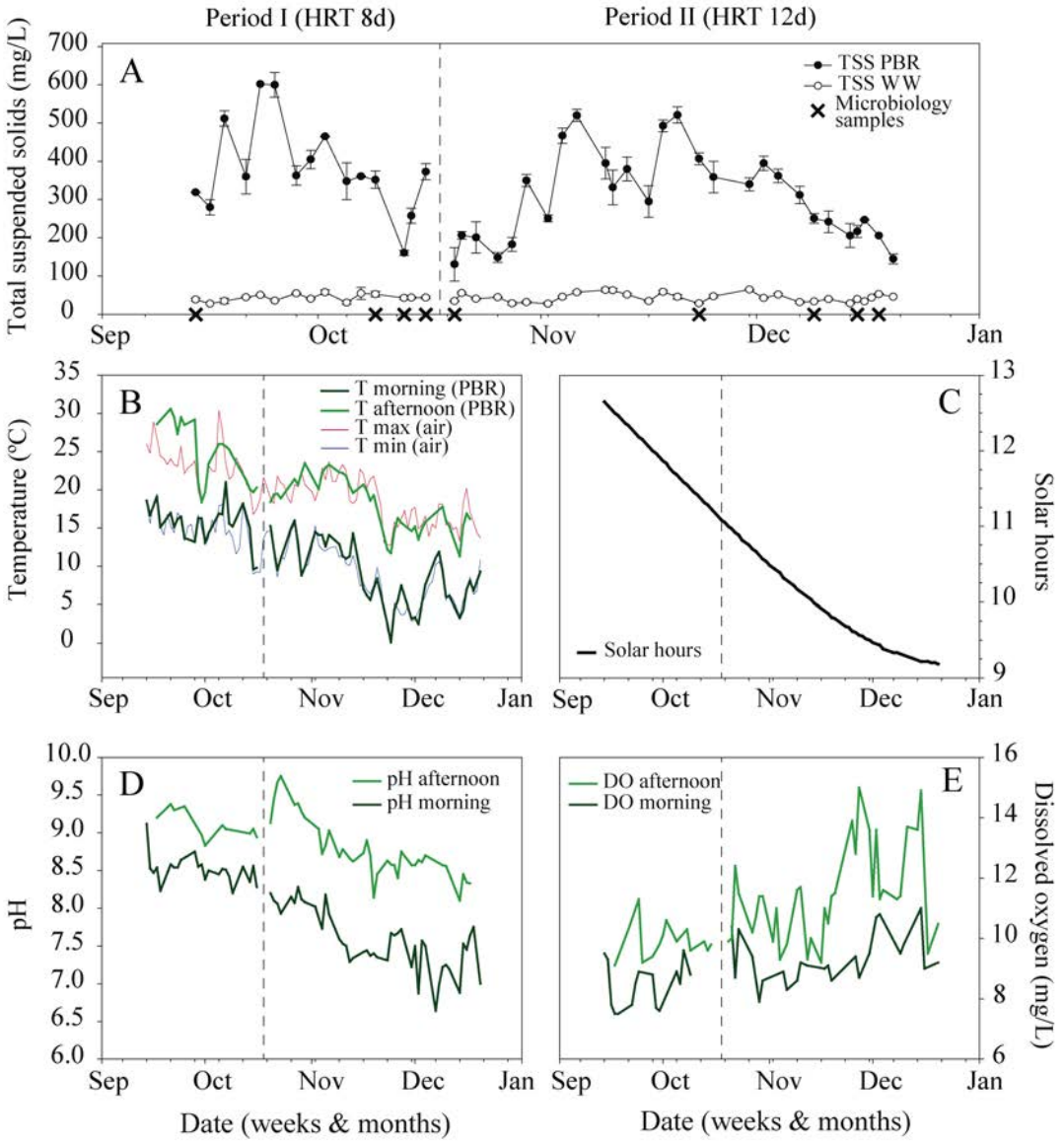

Figure 5.1: Performance and environmental parameters measured during the PBR operation periods I and II. A, samples taken throughout operation for microbial diversity determination and TSS in the WW and PBR; B, morning and afternoon temperature in the PBR along with environmental maximum and minimum temperatures; C, Solar hours; D, pH in the morning and afternoon; E, Dissolved oxygen in the morning and afternoon.

The main parameters to be considered for the monitoring of the PBR were temperature, solar irradiance, pH and dissolved oxygen (DO). The first two are environmental variables that greatly influence biological activity while the two latter provide information of how the PBR is working because they are influenced by photosynthetic activities [255, 256].

First, temperature is the most determinant parameter for the growth of algal biomass [255–257]. As expected, the temperature of the PBR in the afternoon and morning measurements matched almost perfectly with the maximum and minimum environmental temperatures, respectively (Fig. 5.1B). In fact, when maximum temperatures (internal or environmental) were compared with TSS,

significant positive correlations were observed, confirming the mentioned importance of temperature (Table 5.1). To put it as an example, the worst effect over biomass was a temperature drop during the second period (November 22, 2015) when temperature dropped to 0 °C. Cold temperatures, in fact, are the main bottleneck for the efficient implementation of PBRs. Studies have already introduced the fell down of biomass due to low temperatures [258, 259] and others specifically addressed the issue for the production of algal biofuel [260]. Similarly, solar irradiation is also essential for photosynthetic communities. Daily solar hours are presented in figure 5.1C. Although in this case there was no significant correlation between TSS and solar hours (r=0.29, p-value=0.056), the effect of decreasing light hours was assessed previously by other authors [261].

In relation to pH and DO (Fig. 5.1D-E), parameters influenced by photosynthetic activities were studied early in the morning and in the afternoon to assess the activity at night and day periods, respectively. During the day (afternoon measure) O_2 levels raised along with the pH due to the release of gaseous oxygen from photosynthesis. This oxygen combined with CO_2 and generated bicarbonate ions that raised pH consequently. On the contrary, overnight (morning measure) O_2 was consumed by trophic activity but not replenished, lowering DO levels and also pH because $HCO3^-$ could not be formed without O_2. Overall, correlation analyses (Table 5.1) showed that pH, temperatures and solar hours were all strongly and positively correlated. TSS only correlated positively to high temperatures (i.e. T max and T afternoon) and negatively to morning DO. Unexpectedly, DOs exhibited a negative correlation with all the other parameters but between themselves (i.e. morning DO and afternoon DO). Despite microalgae were the responsible for oxygen production, gases solubility is lower in warm water. Moreover, elevated temperatures might have also accelerated bacterial metabolism, resulting in an overall higher consumption of oxygen.

Table 5.1: Correlations of the measured parameters during the PBR operation. Significance is expressed as follows: '***' p≤0.001, '**' p≤0.01, '*' p≤0.05, '' p>0.05.

	pH morning	T morning	DO morning	pH afternoon	T afternoon
T morning	**0.73*** **				
DO morning	**-0.47** **	**-0.49** **			
pH afternoon	**0.72*** **	**0.60*** **	-0.21		
T afternoon	**0.75*** **	**0.81*** **	**-0.57*** **	**0.62*** **	
DO afternoon	**-0.49*** **	**-0.70*** **	**0.46***	**-0.37***	**-0.61*** **
T max	**0.65*** **	**0.72*** **	**-0.49** **	**0.51*** **	**0.86*** **
T min	**0.73*** **	**0.96*** **	**-0.52*** **	**0.61*** **	**0.83*** **
TSS	**0.13**	0.11	**-0.51***	-0.05	**0.41***
solar hours	**0.90*** **	**0.80*** **	**-0.54*** **	**0.74*** **	**0.82*** **

	DO afternoon	T max	T min	TSS	solar hours
T max	**-0.48*** **				
T min	**-0.71*** **	**0.77*** **			
TSS	-0.18	**0.33***	0.09		
solar hours	**-0.58*** **	**0.76*** **	**0.83*** **	0.29	

Data regarding the removal of nutrients (mainly in the form of ammonium and phosphate) and pollution indicators is presented in table 5.2. Ammonium nitrogen ($NH4^+$-N) was removed efficiently during both periods, mainly to be used for protein synthesis by algae [262]. Total phosphorus (P) removal was low (44%) in period I and improved up to 82% by period II. All removal values except P in period I complied with the minimum removal established by the European Commission directive 98/15/EC [263]. In any case, the remaining concentration of P in period I was in accor-

dance with the limit value (2 mg L^{-1}) defined by the same directive, so the system was valid for WW treatment. Pollution indicators such as chemical oxygen demand (COD), total carbon (TC) and total organic carbon (TOC) were measured and showed distinct trends during both periods. Briefly, COD removal was above 80% in period I and slightly below 70% in period II, while TC removal behaved identically but inversely in both periods. TOC removal was almost identical (75%) in both periods. Interestingly, no correlation (except for a weak negative correlation for P removal) was observed comparing TSS and the removal of nutrients and pollution indicators (Table 5.2). This fact suggests that free-living bacteria not forming part of algal aggregates (particles under 2 μm are not represented by TSS) were more determinant for the removal of nutrients and pollutants than algae [264] .

Table 5.2: Average removal percentages of nutrients during operation period I and II along with correlation of TSS with the removal percentages. Significance is expressed as follows: '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$, '' $p > 0.05$.

% Removal	N	P	COD	TC	TOC
Period I	90.86±5.63	43.50±10.66	81.93±3.93	66.09±3.16	75.22±4.13
Period II	95.85±4.39	82.28±8.69	67.87±15.34	83.67±5.26	74.92±12.76

Correlation	N	P	COD	TC	TOC
TSS	-0.1254	-0.42434*	0.2436	-0.14573	0.153337

Finally, moving on to the removal of EC, a total of 81 PhACs were analyzed in both the WW influent and PBR effluent and 17 of them were detected in one or both operating periods (Figure 5.2). In this work, the detected compounds were grouped in four functional groups, namely Analgesics and Anti-inflammatories, Antibiotics, Psychiatric drugs, and Other PhACs. In-depth study of PhACs present during the PBR operation was published recently [254] by other members in our research team and a detailed table is provided in the annex (Table 10.2). Major differences were observed either between the influent concentrations of the two periods or the individual concentration of the compounds in each family (Fig. 5.2). For example, the most abundant family was analgesics and anti-inflammatories and its influent concentrations varied in more than 16,500 ng L^{-1} from period I to II (Fig. 5.2A). Alternatively, antibiotics were far less abundant but their concentration was doubled from period I to II (from 3.079 to 6.617 ng L^{-1}) and the main contributor to the total abundance in the first period (ciprofloxacin) was replaced by ofloxacin in the second one (Fig. 5.2B). Concerning psychiatric drugs and other PhACs, higher concentrations were also found in period II influent and Lorazepam and Atenolol were the most abundant compounds from each group, respectively. Different population needs and varying consumption of water per capita could explain the observed differences between both periods.

Regarding the removal of PhACs by families, total removal and average removal were calculated separately for each period of operation (Table 5.3). On one hand, total removal was calculated considering the sum of all the substances in each family. Hence, PhACs found in high concentrations contribute more to the total removal value. On the other hand, the average of each compound removal was also presented along with the standard deviation in order to get glimpse of how the compounds in each group behaved. Promising results were obtained for Analgesics and anti-inflammatories (Fig. 5.2A) and other PhACs groups (Fig. 5.2D), with total removals ranging 94-96% and 84-93%, respectively. Poor removal of Naproxen (10.2%) and Ketoprofen (36.2%) in period I, along with Salicylic acid (33.4%) in period II contributed to the low average observed for

Analgesics and anti-inflammatories. In contrast, limited removal percentages were obtained for the antibiotics (53 and 70%) and psychiatric drugs (57 and 59%) taking into account total removal. Average removals were equal or higher than the total removal in both families, pointing out that some of the most abundant compounds were poorly removed from the WW (e.g. Ciprofloxacin, Ofloxacin and Lorazepam).

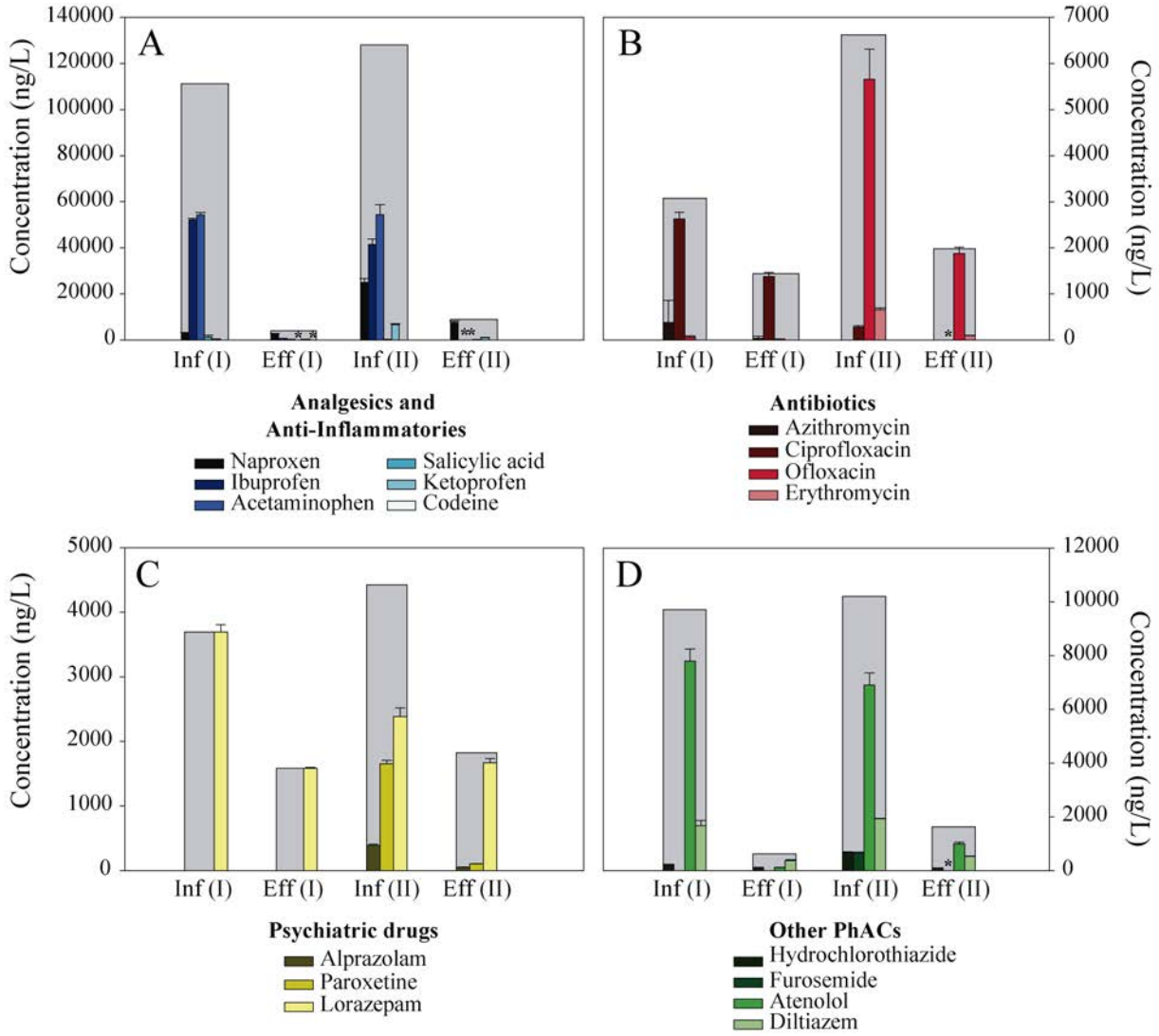

Figure 5.2: Influent (Inf) and effluent (Eff) total concentration of PhAC families (gray bars) and its compounds during the operation periods I and II. A, Analgesics and anti-Inflammatories; B, Antibiotics; C, Psychiatric drugs; D, Other PhACs.

5.3 Photobioreactor diversity assessment

Few studies have focused on the microbial communities in open ponds [265, 266]. In this work, the eukaryotic and cyanobacterial communities developing in the PBR were studied via DGGE while bacteria were characterized using high-throughput sequencing of the V4 region of the 16S rRNA gene using the Illumina platform.

Table 5.3: Total and average removal percentages of each PhAC family during periods I and II.

	Period I		Period II	
	Total removal (%)	Average removal (%)	Total removal (%)	Average removal (%)
Analgesics and anti-inflammatories	96.26	70.84±38.45	92.98	77.44±27.73
Antibiotics	53.2	68.03±20.6	69.97	83.34±15.82
Psychiatric drugs	57.22	57.2±0	58.84	70.4±35.13
Other PhACs	93.58	73.37±27.31	83.91	85.41±10.82

5.3.1 Eukarya and Cyanobacteria

Eukaryotic diversity throughout the operation of the PBR was assessed in a 20-45% denaturing gradient DGGE with a specific voltage of 100 V along with the primer set Euk1AF-Euk516R. Samples were analyzed and sequenced altogether with the ones from the removal improvement experiments (section 5.4). One DGGE was performed for the characterization of Cyanobacteria using the primer set CYA359F-CYA781R(a+b) and 30-70% denaturing conditions. Representative band sequences were submitted to the GeneBank database under the accession numbers KY076627—KY076664 and KY073310—KY073315 for Eukarya and Cyanobacteria, respectively. Raw reads from Illumina sequencing were deposited in SRA under the accession number SRR6436219.

Time-points sampled are detailed in table 5.4 and represented in the time-scale along with TSS (Figure 5.1A). Briefly, duplicates were taken for each sample except for the inoculum and the PBR was sampled after establishing a new HRT, during each steady state and after a freezing event (sample t6, Table 5.4).

Table 5.4: Sampling points during the PBR operation and details of the operation parameters.

	Sample	HRT (days)	Operation day (Month)	Description
	Lake		(Sept.)	Inoculum
Period I	t0	8	0 (Oct.)	Start HRT 8 d
	t1	8	25 (Oct.)	Steady state
	t2	8	29 (Oct.)	Steady state
	t3	8	32 (Oct.)	Steady state
Period II	t5	12	36 (Nov.)	Start HRT 12d
	t6	12	39 (Nov.)	After freezing
	t7	12	55 (Dec.)	Steady state
	t8	12	61 (Dec.)	Steady state
	t9	12	64 (Dec.)	Steady state

DGGE fingerprinting profiles of the PBR operation samples are presented in figure 5.3. Sequencing of the representative bands from 15 phylotypes (considering both PBR operation and the experiments for biomass improvement DGGEs) provided a 96% band coverage. Retrieved sequences belonged to 6 different phyla (Blastocladiomycota, Cercozoa, Chlorophyta, Chytridiomycota, Ciliophora and Lophotrocozoa) including 11 genera (*Acutodesmus*, *Brachionus*, *Chlorella*, *Desmodesmus*, *Gaertneriomyces, Paraphysoderma*, *Pseudospongiococcum*, *Rhogostoma*, *Scenedesmus*, *Uronema* and *Vorcicellides*); two phylotypes could not be assigned a taxonomy further than Eukarya. Additional information regarding band sequences is presented in table 5.5.

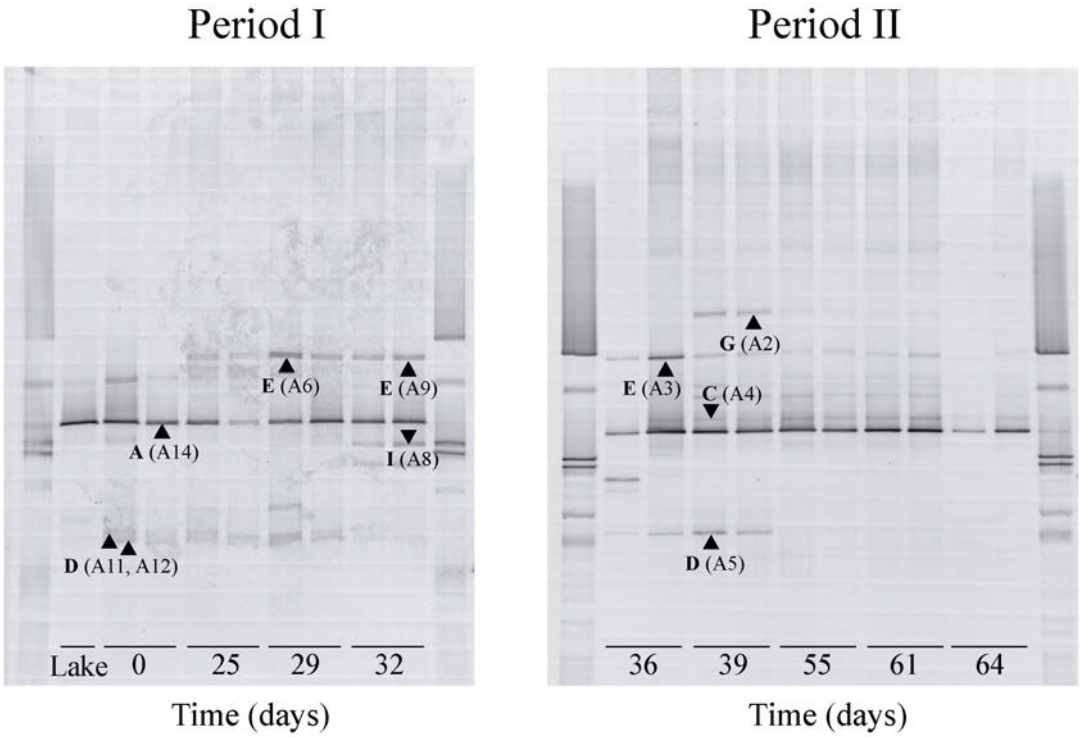

Figure 5.3: DGGE profiles of eukaryotic communities detected in the PBR during periods I (left) and II (right). Representative bands recovered are indicated (▲) and labelled following the band codes showed in table 5.5. Details of each band are provided in table 5.5.

Table 5.5: Phylogenetic affiliations of the sequences obtained from each DGGE band phylotype.

Phylotype (band)	Closest relative (BLAST)	Accession nº	Phylum	Similarity (%)
A (A14; B1-5)	*Chlorella*	KM985375	Chlorophyta	100
B (B6)	*Scenedesmus*	KT279469	Chlorophyta	100
C (A4; B7)	*Pseudospongiococcum*	KU057947	Chlorophyta	100
D (A5,11,12; B8-12)	*Brachionus*	KT729747	Lophotrochozoa	100
E (A3,6,9; B13-18)	*Paraphysoderma*	KJ563218	Blastocladiomycota	100
F (B19)	*Vorticellides*	JQ723993	Ciliophora	100
G (A2, B20)	*Rhogostoma*	LC032468	Cercozoa	99
H (B21)	Uncultured eukaryote	KT252432	Eukarya	94
I (A8; B22-26)	Uncultured eukaryote	JF775023	Eukarya	99
J (B27,28)	*Scenedesmus*	AB255365	Chlorophyta	99
K (B29)	*Acutodesmus*	KP726267	Chlorophyta	99
L (B30,31)	*Uronema*	KM020180	Chlorophyta	100
M (B32-34)	*Desmodesmus*	AB917135	Chlorophyta	100
N (B35,36)	*Scenedesmus*	KT279469	Chlorophyta	100
O (B37,38)	*Gaertneriomyces*	EF024210	Chytridiomycota	96

Looking straight at the gel profiles, it was evident that a single intense band (A: *Chlorella*) was present in all samples, surrounded by more (e.g. D and E) or less (e.g. C and I) persistent bands along the PBR operation. In order to accurately evaluate differences in the community structure, a Bray-Curtis based non-metric multidimensional scaling (NMDS) was performed grouping samples by operations (Fig. 5.4). This figure denotes a clear separation between both operating periods (I and II), meaning that eukaryotic community shifted from one period to another. As expected, the Lake inoculum was closer to the samples corresponding to period I due to short period of time

between sampling and start up of operation; in fact, grouped close together to sample t0 from period I. Also, communities tended to be proximal within themselves during period II, indicating more stability.

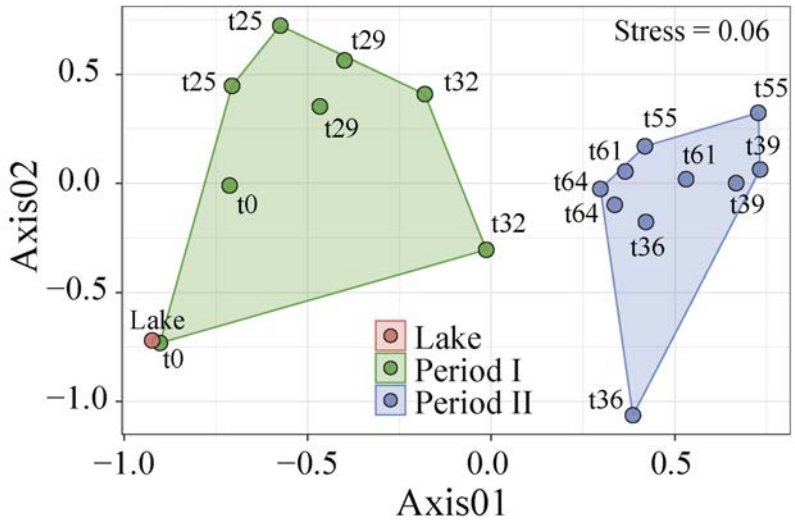

Figure 5.4: NMDS ordination (Bray-Curtis dissimilarity) of samples during the PBR operation. Stress is included within the graph. Sample replicates were included for each sample.

Moreover, taxonomy was then assigned to the band-matching DGGE matrix and a relative abundance plot was constructed (Fig. 5.5). Non-identified bands (4%) were not considered for this plot and duplicates from each time point were plotted next to each other. On the whole, duplicates appeared to be consistent all along the operation. *Chlorella* was the only phototrophic eukaryote that was detected along all the PBR operation. On average, its relative abundance was 0.8±0.14 and did not fall beneath 0.42 in any sample. *Chlorella* is a mixotrophic algae, (capable of both autotrophic and heterotrophic growth) cultured at industrial scale since the early 1960s. It has been broadly studied due to its applications in WW treatment, bioremediation, biofuel production, pigments, cosmetics or nutrition among others [267–269]. The other microalgae detected belonged to genus *Pseudospongiococcum* and was mainly present during the second period of operation but at a low abundance (0.05-0.13). Although not much information is available regarding this genus, Tell *et al.* reported in 2011 [270] that almost 20% of the taxa they registered from the same family (Chlorococcaceae) were restricted to cold temperatures. It is likely that the detected strain belonged to a psychrotolerant or psychrophilic species, as it increased right after the PBR freezing (day 39) and its abundance showed a strong negative correlation ($p<0.05$) with PBR temperatures in the morning (-0.7) and afternoon (-0.8).

Setting aside photosynthetic communities, *Paraphysoderma* was noticeable in the results during most part of the first period, reaching relative abundances as high as 0.28, and then remained in the PBR until the end of operation. Its persistence since the appearance by day 25 was not surprising, as the phylotype detected belonged to the algae-parasiting species *Paraphysoderma sedebokerense*; this fungal species are epibiotic parasites of algae that were described in 2008 [271] and have been related to the loss of biomass in industrial facilities across the world, stirring the development of patented applications for its control [87, 272]. *Vorticellides* (ciliate) and *Brachionus* (planktonic rotifer) phylotypes were found in moderate abundance, at least in half of the samples analyzed, with average abundances of 0.04±0.06 and 0.07±0.04, respectively. Although *Brachionus* can eat

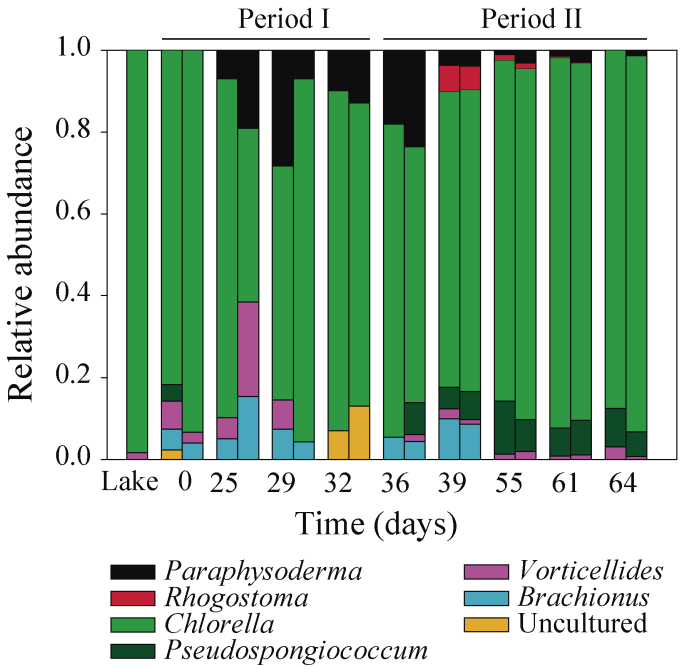

Figure 5.5: Relative abundance of the eukaryotic genera detected in the PBR. Sample replicates were included for each sample.

ciliates such as *Vorticellides*, both genera feed on microorganisms and are involved in the control of microbial populations. Despite not much information is available on *Vorticellides*, it is closely related to *Vorticella*, of which *Chlorella* is the main endosymbiont. It is likely that a fraction of the detected *Chlorella* were actually found inside the ciliate individuals instead of free-living.

To extract the main elements making up the differences during each operation period and bring out strong patterns in the dataset, a principal component analysis (PCA) was performed (Fig. 5.6). Data were previously standardized to have variables with equal means and standard deviations but different ranges. Samples from each period clearly separated in two non-overlapped groups with PC1 axis explaining 45.3% of variance between sample groups. Environmental variables such as temperature, solar hours, DO and pH were the main contributors to PC1, along with the relative abundance of *Pseudospongiococcum*. As detailed in section 5.2, environmental variables are all interrelated so it was not strange that they were responsible of most variance.

As it was mentioned in section 5.1, the PBR was operated for a third period not studied in this section. However, visual differences were observed in the algal biomass between periods I–II and III. Microalgal biomass in periods I and II was mainly composed of settling flocs while in the last operation the PBR biomass consisted of free-living microalgae and few settling flocs. Because no phylotypes corresponding to filamentous microorganisms were detected in the eukarya DGGE that could justify the presence of the settling flocs, another DGGE approach was performed with few samples from different time points to reveal the cyanobacterial diversity. Despite the presence of multiple bands in each sample analyzed (Fig. 5.7), band sequencing revealed the presence of only two phylotypes (P and Q) from genera *Phormidium* and *Leptolyngbya* (Table 5.6). Both

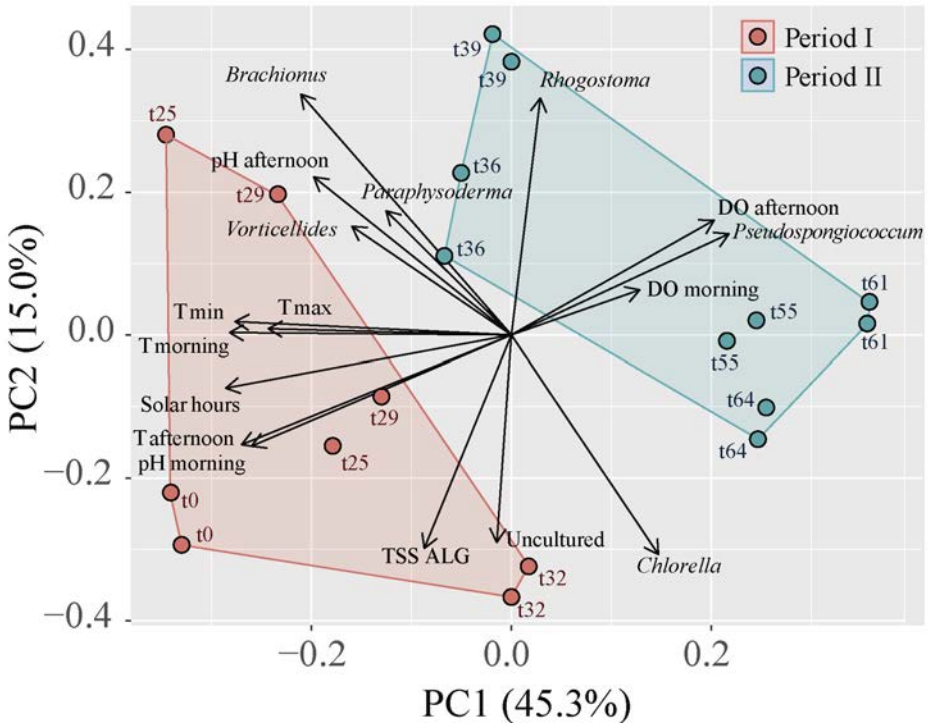

Figure 5.6: Principal component analysis from the DGGE fingerprinting and environmental variables. Sample distribution (PCA score) and loadings (arrows) indicate the direction of its maximum change. Samples were colored and grouped according to the operating period.

genera belong to filamentous cyanobacteria responsible for the formation of the settling flocs. The presence of settling flocs is actually preferred over free-living microorganisms because they facilitate the biomass recovery process.

Table 5.6: Phylogenic affiliation, accession numbers of the closest relatives and sequence similarity of the Cyanobacteria DGGE bands.

DGGE bands	Closest relative (NCBI database)	Accession nº	Phylum	Similarity (%)
P (B39-42)	*Phormidium*	AB183566	Cyanobacteria	99
Q (B43-44)	*Leptolyngbya*	LN997861	Cyanobacteria	100

5.3.2 Bacteria

WW typically harbors an important bacterial richness [273] so a DGGE approach was not considered due to the difficulty to discriminate bands in complex fingerprints. Instead, samples were pooled according to its origin for 16S metagenomic sequencing. The V4 region of the 16S rRNA gene was sequenced using Illumina MiSeq. As previously stated, all bioinformatic and statistical analyses were performed in R and detailed information is available in section 3.6.2.

Establishment of the bacterial communities from the lake inoculum to the PBR was first assessed by plotting the relative abundances of each phyla (Fig. 5.8). As it is common, Proteobacteria phylum was the most abundant both in the lake and in the PBR. Its further division into classes provided insight on the acclimation process. A great proportion of the Gammaproteobacteria in

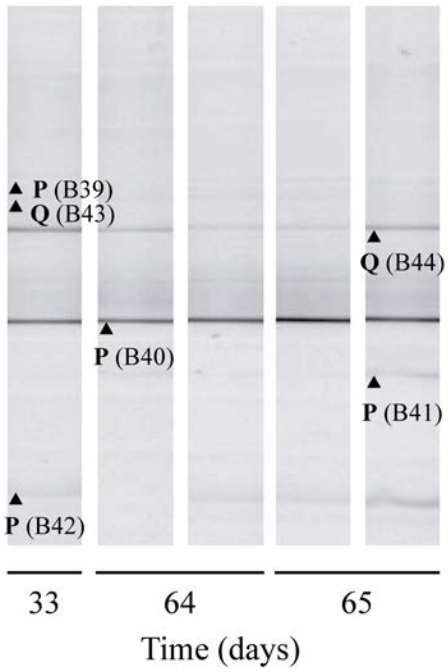

Figure 5.7: Cyanobacterial DGGE profiles of samples from the PBR operation in period I and II. Sequenced DGGE bands are indicated (▲) along with their code and phylotypes (P-Q) indicated in table 5.6.

the inoculum (0.22) was lost after establishment of the PBR bacterial assemblage (0.02-0.04); as opposed to Betaproteobacteria, which incremented its relative abundance from 0.03 to 0.24 and 0.32 in PBR-I and II, respectively. Aside from the operational conditions, presence of PhACs in the influent WW of the PBR might have contributed to the selection of bacteria from the Betaproteobacteria class, as they are known for its versatile capacity to degrade pollutants [274]. In fact, abundance of these bacteria in the influent WW (not sampled) could also justify the observed increase. Further exploration of Betaproteobacteria representatives revealed that only one ASV was lost in the lake-to-PBR transition, belonging to an unidentified genus from the Comamonadaceae family. At the same time, 17 ASVs were acquired in the PBR operation and 7 of those were unique of period II.

Stepping out of the Proteobacteria group, the most abundant phyla were Bacteroidetes, Cyanobacteria, Verrucomicrobia, Planctomycetes and Gemmatiomonadetes. Relative abundances of these phyla also experienced changes from the inoculum to the PBR. One of the most marked variations was the decrease of Bacteroidetes, going from 0.3 to 0.17 and 0.15 in PBR-I and II, respectively. Regarding Cyanobacteria, relative abundance in the PBR dramatically increased from 0.10 (Lake) to 0.30 (PBR-I). This increase was reversed in period II, when low temperatures and limited sunlight likely affected the phototrophic bacteria, reducing its relative abundance back to 0.13.

Interestingly, Spirochaetae and Fusobacteria phyla were not detected in the PBR while they were present in the lake inoculum. Their total disappearance could not be confirmed but a significant decrease in its abundance was indisputable. Oppositely, Chloroflexi, Synergistetes, Chlorobi,

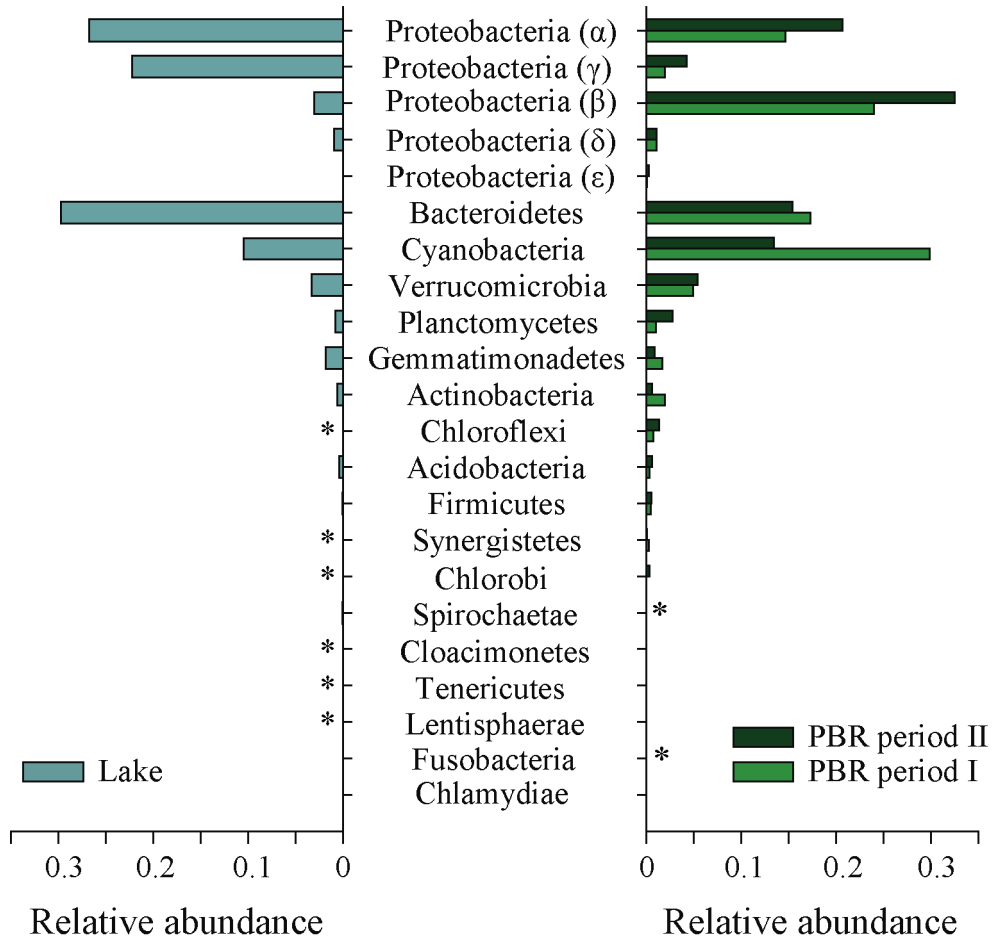

Figure 5.8: Relative abundance of the bacterial phyla present in the Lake and PBR during periods I and II. Proteobacteria were broken down to the Class level. Abundance bars are colored according to the sample origin and an asterisk (*) is used to indicate the absence of sequences from each phylum.

Cloacimonetes, Tenericutes and Lentisphaerae phyla were detected only in the PBR. Bacteria from this groups were either coming from the WW influent or from air sedimentation into the distribution chambers.

Bacterial communities developing in PBRs depend to a high extent on the algae's extracellular products but they are not necessarily algal symbionts [275]. As seen before, in this particular study *Chlorella* was the main microalgae in the system. Accordingly, thorough revision of the available work on *Chlorella* symbionts [276–284] allowed the preparation of a list of microorganisms with capacity to stimulate positively the algae's growth. Sequences from the metagenomic study were screened for the symbionts (Fig. 5.9) described in the literature and 24 matches were found, belonging to 6 different genera. The most abundant symbiont genera in the lake were *Flavobacterium* and *Sphingomonas*, but their relative abundances decreased in both operation periods of the PBR. On the contrary the other four genera (*Brevundimonas*, *Hyphomonas*, *Pseudomonas* and *Rhizobium*) either increased their relative abundance or were only detected in the PBR.

Furthermore, FAPROTAX [184] was used to assess functional traits of the bacteria in the PBR. To mend the differences caused by different sequencing depths in each sample, normalization of count number was performed using metagenomeSeq [188]. Results are presented in table 5.7. First, four

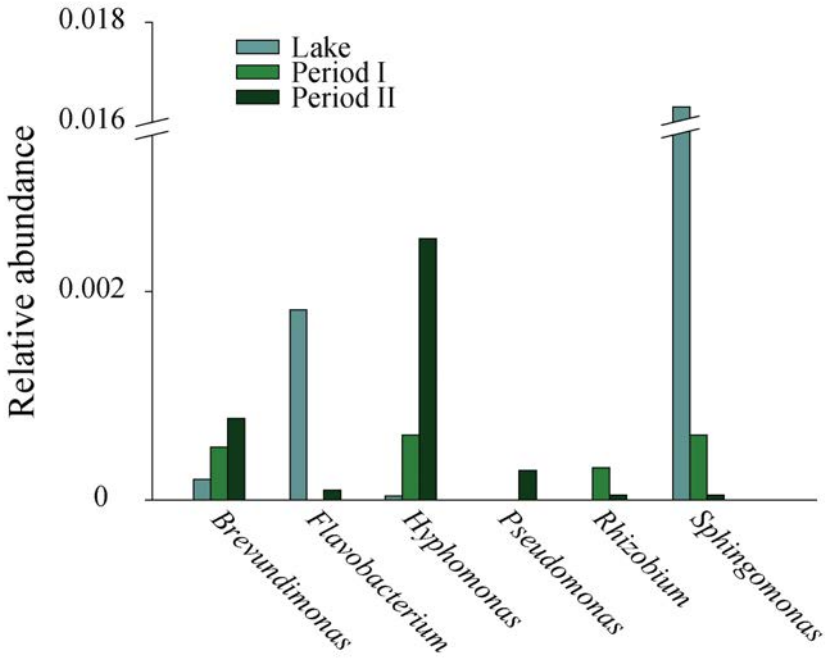

Figure 5.9: Relative abundance of *Chlorella* symbionts detected in the inoculum source and both periods of the PBR operation.

metabolic processes of the N cycle (nitrification, denitrification, N fixation and ammonification) were assessed and clear differences were observed between the lake inoculum and PBR. Briefly, no groups involved in nitrification and denitrification processes were detected in the lake but then appeared in the PBR samples. The continuous input of N through WW (mostly in form of urea, readily converted to ammonia) is likely responsible for the increase in these functions. In opposition, N fixation and ammonification were almost exclusive of the source sample and are precisely the responsible mechanisms that can ultimately provide ammonia to the system when its abundance is limited in the natural environment.

In addition, phototrophy, aerobic chemoheterotrophy and fermentation categories provide an overview of the general metabolic trends. As expected, phototrophy increased substantially in the transition from lake to PBR. Fermentation and aerobic chemoheterotrophy did not show the same patterns; while the first gradually increased over time, the second only increased in period II. To conclude, an important increase of exoparasitic and predatory bacteria in the PBR was revealed by FAPROTAX, in the line of previous findings regarding eukaryotic parasites and predators (section 5.3.1). Essentially, the increase of predators and parasites in closed systems is an inevitable outcome that hinders the growth of microalgae, especially in pure cultures [86,285]. In fact, they are the natural drivers of algal populations and diverse strategies for their control have been developed, including harvesting [285], physical disruption [286], biological control [287,288], selective breeding [289] or use of chemical agents [290,291].

5.4 Strategies for the improvement of hormone removal

As it was introduced in the first chapter, the release of hormones or their analogues into the environment is a concerning subject due to their endocrine-disrupting capacity. The popularity of

Table 5.7: FAPROTAX counts of normalized reads revealing abundance of bacteria involved in eight functional groups.

Sample	Lake	PBR-I	PBR-II
Nitrification	0	1.17	10.16
Denitrification	0	13.29	16.94
N fixation	2.7	0	1
Ammonification	4.52	0	0
Phototrophy	157.87	206	186.56
Aerobic chemoheterotrophy	373.93	319.5	504.43
Fermentation	70.08	81.49	116.28
Exoparasitic / predatory	4.27	25.69	16.36

contraceptive pills is probably one of the best examples of this problematic [292], as they can be formulated either with the natural hormone 17-estradiol (E2) or the synthetic estrogen ethinylestradiol (EE2), being the latter more recalcitrant to biodegradation. Neither of these substances is desired in the environment so the PBR microbial assemblage was used to test hormone biodegradability.

A laboratory-scale experiment was carried out to evaluate the impact that increasing biomass density with a specialized culture had over E2 removal [293]. Previous authors already reported differences in hormone removal depending on the microalgae strain used. As an example, Peng *et al.* [294] achieved better results in progesterone removal using *Scenedesmus obliquus* before *Chlorella pyrenoidosa*. In this work, a culture obtained from an industrial PBR (IPBR) was used to increase the density of the PBR biomass (presented in section 5.1) in laboratory-scale conditions. E2 was chosen for this study at a final concentration of 2 mg L^{-1}. Two configurations were tested (3:1 and 1:1 v/v of PBR and IPBR biomass, respectively) and biomass from period III of the PBR (not studied previously) was used as control group. Killed controls were also included. Eukaryotic diversity was assessed simultaneously to the PBR operation (section 5.3.1). Primers and DGGE conditions were also the same.

Samples analyzed in the bioaugmentation assay exhibited a total of 11 phylotypes and fingerprinting profiles are presented in figure 5.10. The lake inoculum was only included as a reference. Detailed information of representative bands is available in section 5.3.1 (Table 5.5).

Microbial communities of interest were principally the ones found in the control and IPBR biomass. 3:1 and 1:1 ratios were just mixtures of these two sources and its likeness to the original samples is evident by looking the band patterns. In terms of bacterial richness and diversity, the original PBR biomass showed the higher values compared to the rest of the samples (Table 5.8). Higher richness in bioaugmented samples (i.e. 3:1 and 1:1) could be expected due to the mixture of biomass with different composition. On one hand, the IPBR biomass was only composed by *Desmodesmus* (Chlorophyta) and *Gaertneriomyces* (Chytridiomycota) genera. On the other hand, control biomass (PBR, period III) was composed by microorganisms from the Blastocladiomycota (*Paraphysoderma*), Cercozoa (*Rhogostoma*) and Chlorophyta (*Acutodesmus*, *Chlorella*, *Pseudospongiococcum*, *Scenedesmus* and *Uronema*) phyla.

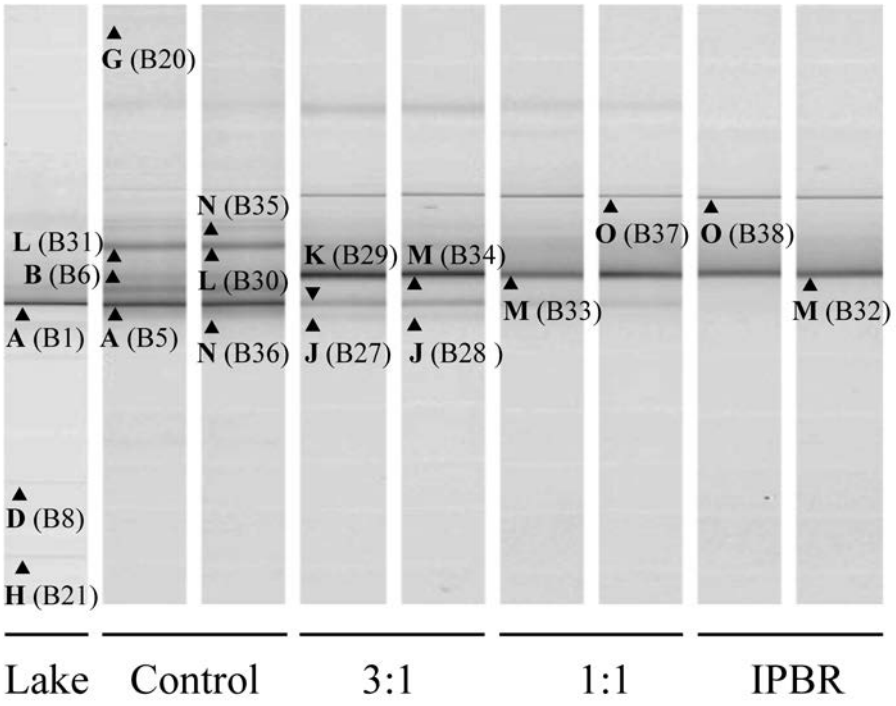

Figure 5.10: Eukaryotic DGGE profiles of the samples from the bioaugmentation assay. Recovered bands (▲) were labelled according to its phylotype. Detailed information is presented in table 5.5. Lake, Inoculum samples; Control, PBR biomass; IPBR, Industrial PBR biomass; 3:1 and 1:1 ratios are Control:IPBR (v/v).

Removal assay results were summarized in table 5.9. The observed tendency was that as the TSS increased, E2 concentration decreased. Near-complete removal was achieved in the presence of IPBR biomass, whereas the control only attained 88% removal at the end of the assay. Removal rate in each condition was expressed with the k value (Table 5.9), reiterating that as the IPBR biomass ratio grew higher, E2 removal rate also increased. Finally, removal yield was calculated considering the initial biomass concentration because it was different for all three conditions. Results indicated that the highest E2 removal yield (4.52·10-4 mg E2/mg TSS·h) was obtained by the control biomass, despite not reaching complete removal. 3:1 ratio followed with a yield of 3.21·10-4 mg E2/mg TSS·h and at last, 1:1 ratio with 2.83·10-4 mg E2/mg TSS·h. These demonstrated that PBR biomass was more efficient in removing E2 compared to the IPBR. Aside from *Chlorella* and *Pseudospongiococcum*, already present in operation periods I and II, *Acutodesmus*,

Table 5.8: Richness, diversity (Shannon) and evenness (Pielou) estimates from samples in the bioaugmentation assay.

Sample	richness	diversity	evenness
PBR-A	9	1.85	0.84
PBR-B	9	1.76	0.80
3:1-A	6	1.32	0.74
3:1-B	6	1.29	0.72
1:1-A	4	1.01	0.73
1:1-B	4	0.85	0.61
IPBR-A	2	0.66	0.95
IPBR-B	2	0.64	0.92

Scenedesmus and Uronema were reported for the first time in the control biomass from period III. The first two genera are closely related with the *Desmodesmus* strain in the IPBR, belonging to the Scenedesmaceae family. Considering this, bioaugmentation achieved higher TSS levels (i.e. higher cell density) but was unable to introduce metabolic diversity into the consortium.

Table 5.9: Kinetic rate constants and E2 removal yield for lab-scale batch assays. Control experiment (no bioaugmentation); 3:1 and 1:1 volumetric ratio bioaugmentation experiments (PBR:IPBR).

Parameter	Control	3:1	1:1
TSS (mg/L)	162	258	353
Final removal (%)	88	99	100
k (h-1)	0.084	0.191	0.318
r2	0.968	0.945	0.913
E2 removal yield[a]	$4.52{\cdot}10^{-4}$	$3.21{\cdot}10^{-4}$	$2.83{\cdot}10^{-4}$

[a] [a] E2 removal yield units: mg E2 removed (mg TSS·h)$^{-1}$.

Bioaugmentation has been previously used to increase biodiesel production with microalgae with high lipid content [295] but this is the first work in which bioaugmentation was conducted to remove ECs. Despite the addition of *Desmodesmus* sp. could not increase the E2 removal yield in this study, previous authors reported the capacity of this genus for estrogenic compound removal while achieving elevated removal percentages (88-100%) [242]. Results from this study confirm that the higher the biomass, the faster the E2 removal. Therefore, an increase of the biomass through bioaugmentation can improve removal via biodegradation and biomass sorption mechanisms, although the latter are hard to quantify.

Another factor to be taken into account when planning a bioaugmentation is the quality of the inoculum source. Precisely, the fungi *Gaertneriomyces* (band O, figure 5.3, table 5.5) was abundant in the IPBR biomass and many of its species are able to parasite a wide range of hosts including diatoms, microalgae or large algae [296,297]. As it was discussed previously, parasites and predators can alter the population dynamics of PBR systems or even suppose their demise [285] if their composition is fragile. The most susceptible communities will be the ones with less diversity and evenness, and thus, less capable of recuperating from perturbances. Going by this example, pure algal cultures might be easy to model but also extremely susceptible to disruption in the presence of a specific predator/parasite. On the opposite, rich and diverse communities as the ones in the PBR might be harder to study but are stable and less susceptible to antagonists.

5.5 Screening to find cyanobacterial degraders

Cyanobacteria can bioremediate metal and hydrocarbon-contaminated sites but have not been screened for estrogenic-degrading capabilities. So far, only eukaryotic microalgae have been found capable of such feat. For this reason, the search for cyanobacterial strains with similar capacities was also conducted.

Microbial mats are horizontally-stratified and self-sustained microbial communities found in benthic ecosystems [298, 299]. They are shaped by physiochemical gradients and cyanobacteria are found abundantly in its uppermost layer along with other photosynthetic microorganisms [299].

The aim of this study is to screen a naturally occurring microbial mat from Ebre Delta for suitable candidates to be used in wastewater treatment bioreactors with capability of hormone degradation. The sampling location was chosen because of continuous exposure to pollutants (including EC) coming from the same river [300,301] and the research group experience in microbial mats [302–305]. Cyanobacteria-specific liquid enrichments were carried out along an acclimation to E2 and EE2 hormones. Sampling, processing and enrichment procedures are described in section 3.3.2 while media composition is provided in section 3.2.

Freshwater and seawater variants of the BG-11 medium were used and growth occurred in both media regardless of the presence of hormones. Preliminary assays (data not shown) suggested that E2 was removed in both enrichment cultures but EE2 was recalcitrant and hardly removed. Further assays focused in the non-saline BG-11 enrichment, which would be expected to work better in urban wastewater.

Hormone removal assay was performed using the fourth consecutive transfer of culture N2 (see section 3.3.2 for more details) under light and dark conditions and including heat-killed controls. Hormones E2 and EE2 were spiked in the cultures at a final concentration of 0.5 mg L^{-1} each. Samples were taken at days 0, 1, 2, 3, 5, 7 and 14 for microbial characterization and quantification of hormones, chlorophyll *a* and total protein. A sonication step was included in the protocol to avoid hormone sorption into the biomass. Although E2 was detected at days 3 and 5 for light and dark conditions, results revealed that only 2 days were needed for near-complete removal of the hormone (Fig. 5.11A). As suspected, EE2 was hardly removed and its concentration only decreased when exposed to light in both experimental and killed cultures. Most part of the removal occurring in the killed controls was caused by photodegradation, confirming that biodegradation was taking place in the experimental flasks because removal was higher than photodegradation values. In parallel, monitoring of biomass indicators (i.e. chlorophyll *a* and total protein) revealed that the growth of the microbial assemblage was limited under dark conditions (Fig. 5.11B). In the presence of light, production of chlorophyll *a* increased to 27 ng mL^{-1} and total protein to 12 μg mL^{-1}; both parameters triplicated its initial values. On the other hand, only slight increases of protein concentration were detected when light was absent and chlorophyll *a* remained constant. A chlorophyll *a*/total protein ratio was calculated (Fig. 5.11C) to evidence that phototrophic communities were comparable in the beginning but they were ultimately favored in flasks exposed to natural light.

DGGE with the bacterial primer set 341f-907r revealed the presence of prominent bands from phyla Actinobacteria, Bacteroidetes and Proteobacteria (data not shown). Contrarily to what was expected, Cyanobacteria were not detected with the primer set used, but three eukaryotes from the Chlorophyta phylum were detected instead thanks to plastid DNA. Although it is known that Cyanobacteria are capable to adapt to strong selective pressure, their absence in the final enrichment could be explained by the selection of populations well adapted to freshwater.

Nevertheless, E2 removal kinetics were identical under light and dark conditions, suggesting the implication of non-phototrophic bacteria in the process. Still, photosynthetic communities growing under light could not be discarded as degraders, as supported by parallel works [293]. Next steps

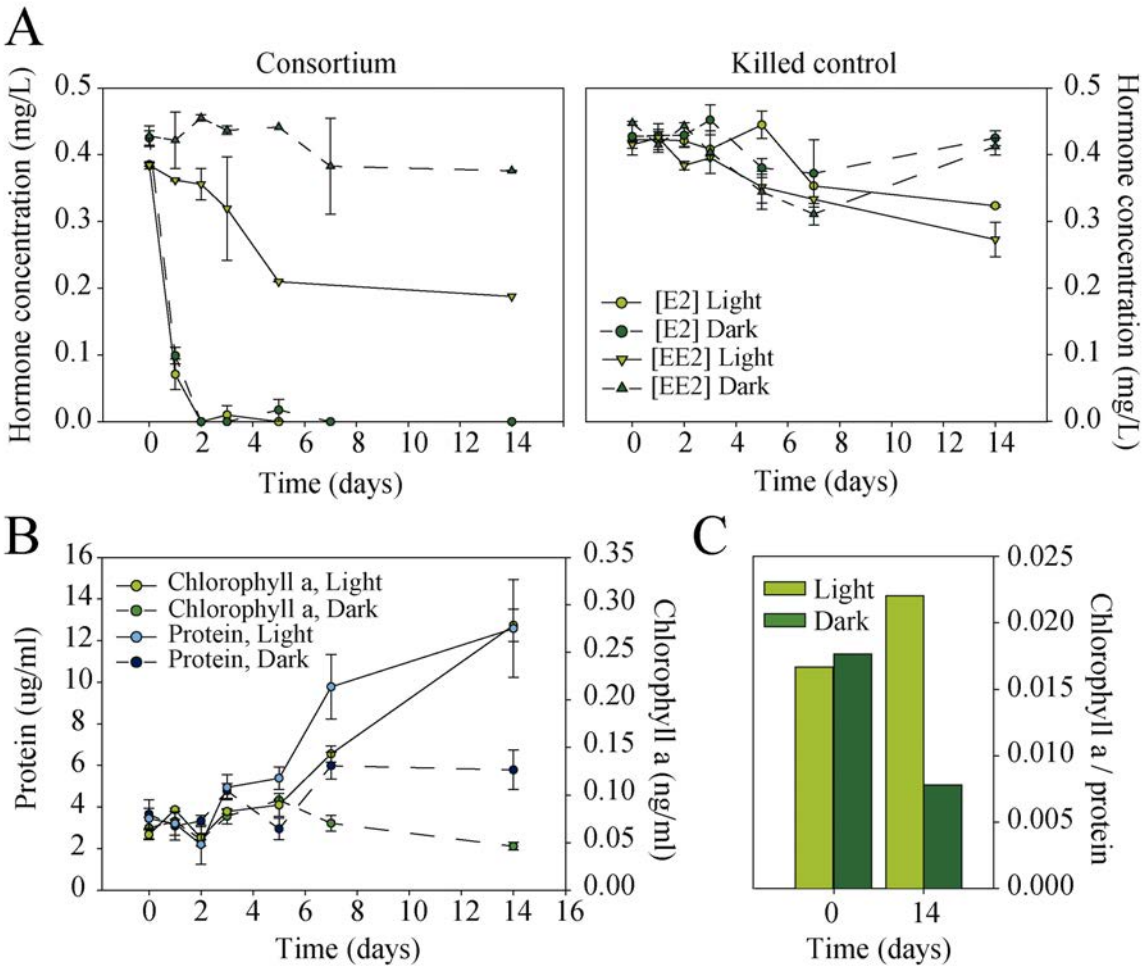

Figure 5.11: A. Hormone concentration (E2 and EE2) in the consortium (left) and killed controls (right) during the removal assay. B. Representation of the total protein and chlorophyll *a* concentration over time. C. Chlorophyll *a*/protein ratio at the beginning and end of the assay.

in the line of research should focus in both finding sources of freshwater cyanobacteria adapted to pollution and scaling-up the enrichment cultures from flasks to laboratory bioreactors that would enable a bioaugmentation strategy.

Chapter 6

Fungal bioremediation

The work described in this chapter was carried out thanks to the collaboration with Josep Anton Mir Tutusaus (UAB), Guillem Llorens Blanch (UAB) and ICRA, in charge of the fungal bioreactor operation, fungal biopiles and pharmaceutical analysis, respectively.

6.1 Introduction

The main sources of PhACs in the ecosystem are WWTP effluents and sewage sludge, as already introduced in the first chapter (section 1.1.3) . In the first case, PhACs are not removed during primary and secondary treatments and reach the environment once the treated water is discharged [19]. In the second case, sludge generated as a residue during the solid-liquid separation in these primary, secondary, and even tertiary treatments accumulates high concentrations of PhACs [306,307] and are in sometimes used as low-cost fertilizers without an adequate treatment. The regulation framework in Spain for agricultural use of WWTP sludges is currently regulated by Order AAA/1072/2013, affecting the Royal Decree 1310/1990 [308]. This regulations demand a control for diverse physiochemical parameters, metals concentration or bacterial pathogens; however, neither EC nor ARG evaluations are yet considered.

The discharge of untreated hospital wastewater (HWW) into the sewage network is the main cause why PhACs are found in elevated concentrations in WWTPs. For this reason, on-site treatment of HWW before it is discharged into urban sewage is a promising strategy to remove these recalcitrant compounds [309]. In the last years, advanced treatments like activated carbon, advanced oxidation (e.g. UV radiation or ozone), ion exchange or membrane filtration have been developed in order to decrease the presence of ECs in wastewater [310, 311]. Nevertheless, costs associated to these technologies and lack of experience in the field still hinder its proper implementation.

In this sense, fungal bioremediation arose as an economical and sustainable alternative. In particular, white-rot fungi (WRF) have been deeply studied in the removal of EC and have the capability to degrade PhACs as it has been demonstrated in spiked media and raw effluents [312–315]. In this project, the use of the WRF *T. versicolor* is studied as a viable option for the bioremediation of both HWW and sewage sludge. The presence of diverse mechanisms involved in the degradation of PhACs (e.g. cytochrome P450, peroxidase or laccase) make *T. versicolor* an optimal candidate for the establishment of bioremediation systems.

The aim of the chapter is to evaluate the fate of *T. versicolor* during the operation, elucidate the microbial assemblages developing in treatment systems and assess the feasible interactions established among indigenous microbiota.

6.2 Continuous treatment of hospital wastewater

The ability of *T. versicolor* to grow in form of pellet makes it suitable to establish a continuous bioreactor to treat HWW using a fluidized bed. Unfortunately, commensal bacteria suppose an important drawback that interfere the operation with active fungus during prolonged periods. Bacteria are abundant in HWW and exert competitive pressure in the reactor, ultimately out-

matching *T. versicolor*. To avoid this outcome, two strategies were applied in the bioreactor: i) The implementation of a pretreatment coagulation-flocculation step to decrease the bacterial load in the water and ii) the partial renovation of *T. versicolor* biomass to stabilize the pellet age [147].

6.2.1 Bioreactor set-up and performance

In order to verify the effectivity of the implemented strategies (i.e. coagulation-flocculation and partial biomass renovation), a validation experiment was performed previous to the long-term operation.

The validation consisted in the operation of control and experimental bioreactors in parallel, treating the same HWWs. Nutrients were added to both bioreactors but only the experimental was inoculated with *T. versicolor* pellets. HWWs (i.e. HWW1 and HWW2) were obtained in consecutive months from the Sant Joan de Déu hospital (Barcelona, Spain) and treated sequentially in an operation that lasted 56 days (29 days with HWW1 and 27 days with HWW2). The long-term continuous treatment of HWW3 lasted for 91 days. HWW3 was obtained from the same source as in the validation experiment one year after. Further details regarding bioreactor operation are provided in section 3.3.4.

Validation experiment

Laccase activity and glucose consumption were monitored during the treatment (data not shown). Although laccase activity was not detected in the first period of operation (HWW1), maybe due to the interference of some compound present in the HWW, when fungal biomass was recovered from the reactor the laccase activity could still be measured in an ex situ assay. In fact, some authors recommend the purification of the enzyme prior to activity measurements [316]. During the second period (HWW2), irregular peaks over 45 U L^{-1} were noted at different days; however, no activity was detected in the control bioreactor (data not shown). While laccase activity is an indicator of fungal activity, high removal capacity was reported at low laccase activity.

Glucose concentration remained close to zero during all the operation because it was added at par with the consumption rate. The addition of glucose in the control bioreactor resulted in accumulation during the first two weeks, as there was no fungus capable of consuming it. Accumulated glucose concentration lead to the growth of HWW-native microorganisms that kept glucose levels insignificant after two weeks. While no significant increase in COD was reported in the experimental reactor, both COD and TSS levels in the bioreactors did not descend to the European Union standards of 125 mg L^{-1} and 35 mg L^{-1}, respectively [18]. Nevertheless, the treatment was only designed to decrease PhACs load and the resulting effluent was meant to go through a regular WWTP after discharge to the sewer network.

Regarding PhACs removal, 34 out of the 81 PhACs analyzed were detected during the treatments (Annex, tables 10.3 and 10.4). Detailed description of the PhACs detected was reported by Mir-Tutusaus *et al.* [151]. This work focused on the removal of PhAC families linked to the communities present at each stage of the operation. Overall, concentration of pharmaceuticals was higher in HWW1 compared to HWW2. Out of the four families defined (analgesics and anti-inflammatories,

antibiotics, psychiatric drugs and others) the most abundant compounds were from the analgesics and anti-inflammatories group (Table 6.1), followed by others, antibiotics and psychiatric drugs. A Venn diagram with the shared PhACs in each HWW is presented in figure 6.1. It should be stated that the sampled hospital possessed an important psychiatric pavilion, that explains the unusually high concentration of psychiatric drugs.

Table 6.1: Pharmaceuticals concentration in the hospital wastewater effluents grouped by therapeutic group.

Therapeutic group	HWW1 ($ng \cdot L^{-1}$)	HWW2 ($ng \cdot L^{-1}$)	HWW3 ($ng \cdot L^{-1}$)
Analgesics and anti-inflammatories	46,061.87	26,404.07	34,049.92
Antibiotics	4,783.03	909.6	134.04
Psychiatric drugs	1,080.18	732.11	7,174.03
Other PhACs	9,203.06	3,019.93	4,168.55
Total	61,128.14	31,065.71	45,526.54

Following, figure 6.2 presents the removal percentages of each PhAC family along the validation experiment. Analgesics and anti-inflammatories were the most abundant in the influent but also the best efficiently removed by both experimental and control bioreactors (Fig. 6.2). Setting aside the first sample from day 9, when the bioreactors were just reaching the steady state, a 76% or higher removal was always attained. Interestingly, the control bioreactor showed slightly better results, especially during the HHW2 treatment, when removal rates ranged 94–99%. To understand this trend, it should be considered that both fungi and bacteria (also present in the control bioreactor) are capable of degrading compounds from this family [317].

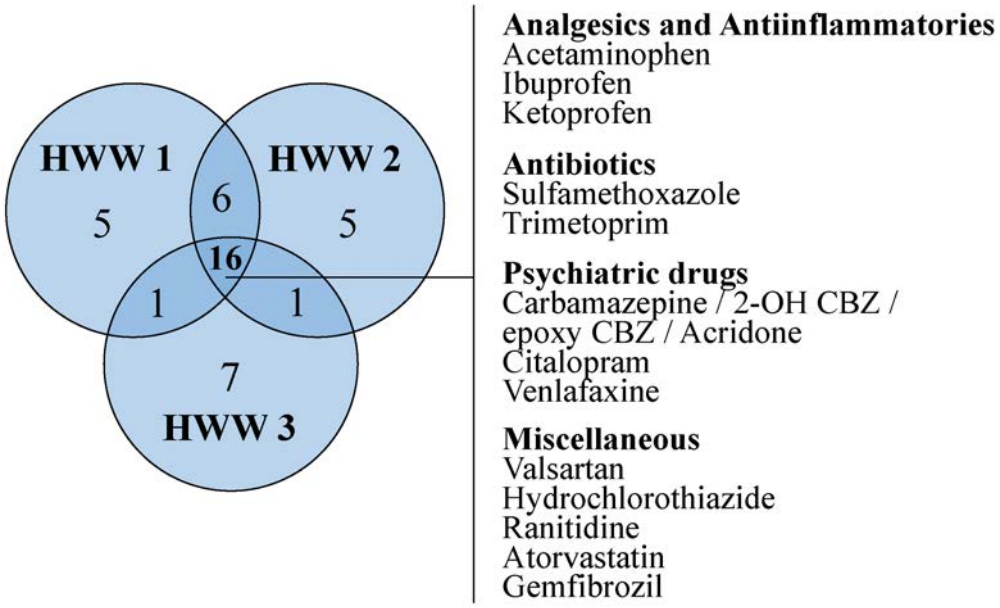

Figure 6.1: Venn diagram displaying the number of unique and shared PhACs among the HWW sampled. A list of those compounds shared in all three sources is presented at the right side of the figure.

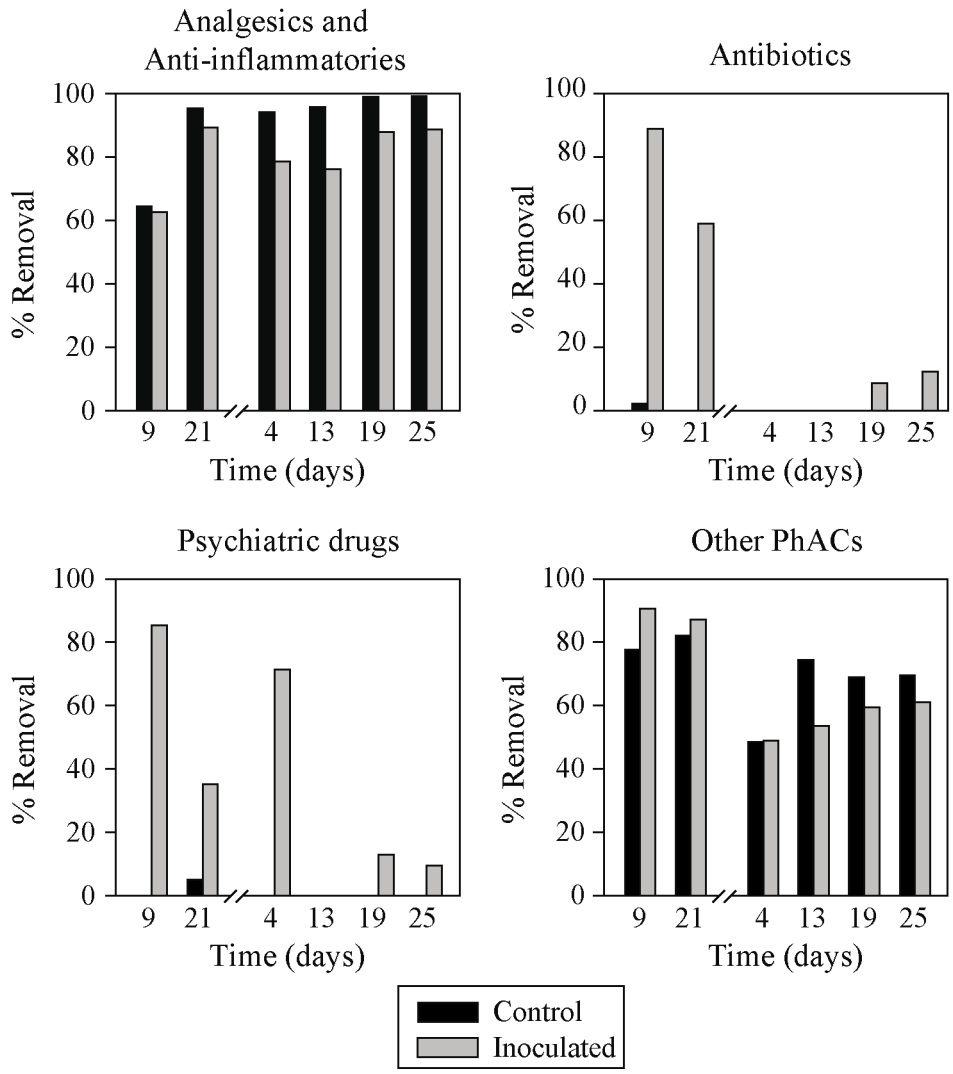

Figure 6.2: Time-course removal percentages of analgesics and anti-inflammatories (upper left), antibiotics (upper right), psychiatric drugs (lower left) and other PhACs (lower right) in the control (black) and inoculated bioreactor (grey) during the validation experiment. Treatment of HWW1 belongs to the first two points (days 9 and 21) while HWW2 is represented after the break in the x axis (days 4,13, 19 and 25).

In the case of antibiotics (Fig. 6.2), removal was not significant in the control bioreactor whereas *T. versicolor* was able to remove 90% of the initial load in the experimental bioreactor. Unfortunately, capacity decreased to 60% at day 21 and was kept at minimum levels (0–13%) during the whole treatment of HWW2. Similarly, no significant removal of psychiatric drugs was observed in the control bioreactor (Fig. 6.2) but encouraging results were obtained in the *T. versicolor*-inoculated reactor (from 86% removal to 35%) treating HWW1. The same trend is repeated for the treatment of HWW2, in which the initial 72% removal decreases to values ranging 0–13%. This tendency could be linked to microbial abundance patterns of other microorganisms present in the matrix. Precisely, correlations between bacterial groups and removal percentages are assessed in section 6.2.2.

PhACs not belonging to any of the above groups were assigned to "Other PhACs". The inoculated bioreactor proved to be efficient in removing most of these compounds in HWW1 (87–91% removal) and performed slightly worse in HWW2 (49–61% removal). In this case, the control bioreactor exhibited equivalent results, just slightly worse in HWW1 than in HWW2. The great diversity in the nature of the substances hinders the assessment of key points linked to its degradation; however, as control and experimental results are comparable, it is likely that either HWW-native bacteria or fungi are also capable of degradation.

Overall, the PhAC families that were best removed were analgesics and anti-inflammatories and other PhACs. Aside from degradation, processes as bioadsorption and bioabsorption might be implicated in the removal of these families, as they are precisely the most abundant in terms of concentration. Finally, a complementary toxicity assay (Microtox acute toxicity bioassay) reported in Mir-Tutusaus *et al.* [151] indicated absence of toxicity in the experimental bioreactor.

Long-term experiment

Laccase activity and glucose concentration were measured during the long-term operation of the bioreactor (data not shown). During the first 60 days of operation, constant peaks of laccase activity around 50 U L^{-1} were detected on a weekly basis, likely corresponding to the partial biomass renovation. After a faint activity reported in day 63, laccase activity remained insignificant until the end of the experiment. Glucose concentration remained insignificant during all the treatment as it was added at consumption rate (data not shown).

Moreover, real-time qPCR with specific primers for *T. versicolor* Internal Transcribed Spacer (ITS) region (TvqF-TvqR) was used to assess the fate of the fungus in the pellets (Fig. 6.3A) and liquid matrix (Fig. 6.3B). Detailed information about the primers and qPCR procedures is presented in sections 3.5.3 and 3.5.8, respectively.

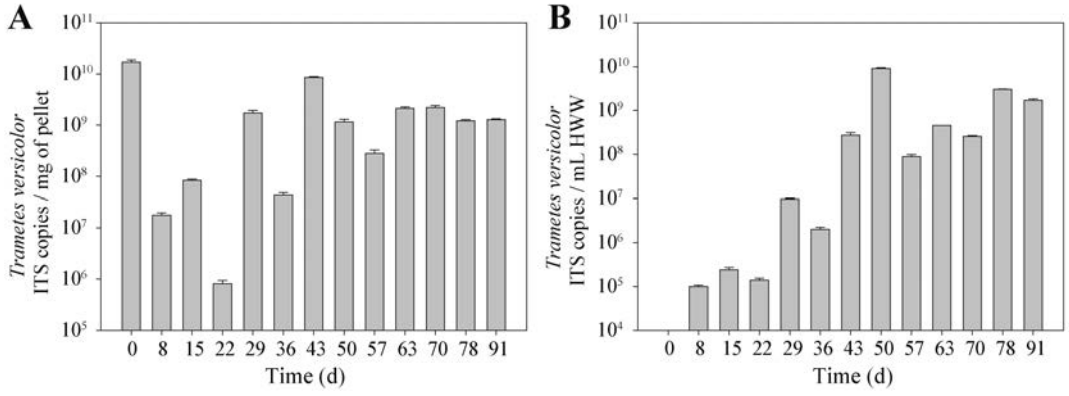

Figure 6.3: Real-time qPCR quantification of *T. versicolor* ITS copies during the bioreactor operation in the pellets (A) and liquid matrix (B). Standard deviation of qPCR reaction triplicates is included. Liquid matrix sample from day 0 did not amplify.

Regarding removal of PhACs, 25 out of 81 PhACs were detected, with similar concentrations to the previous HWW1 and HWW2 (Table 6.1). Antibiotic concentrations were exceptionally low while on the contrary psychiatric drugs were almost 7 times higher than in HWW1. Time-course

removal profiles are presented in figure 6.4 and comprise samples from days 15 to 91, when the bioreactor was in the steady state. Sample from day 8 was not included as the bioreactor had still not reached the steady state (after at least three times the HRT of 3 days). Detailed information is available in the annex (Table 10.5).

As expected from the already satisfactory results in the validation experiment, analgesics and anti-inflammatories were removed efficiently during all the long-term operation with an average removal of 77% and values ranging 42–93%.

Antibiotics were removed at lower rates compared to analgesics and after a gap of two weeks with no removal on days 22 and 29, a modest but steady degradation was attained (37–57%) until the end of the experiment. In this case, coinciding removal values in some samples are due to normalization of left-censored data (section 3.7).

Accordingly, psychiatric drugs and other PhACs removal behaved similarly in the long-term experiment as they did in the validation. Removal was stable within ranges of 55-68% and 60-70% removal for psychiatric drugs and other pharmaceuticals, respectively, until day 50. From that point onward, a gradual decrease in removal occurred simultaneously until all-time low removals of 19.5% and 37.6% were reached for psychiatric drugs and others, respectively. It is adequate to state that these families include a wide variety of compounds that are rarely present in the environment, at least compared to the other groups, and degradation pathways might not exist yet. In addition, recalcitrant compounds are known to be within the psychiatric drugs or other drugs families. Thus, it is likely that the observed removal is high in the beginning due to sorption onto fresh biomass that was renewed weekly, following partial renewal strategy [147].

When laccase activity (data not shown) and PhAC removal were compared, no correlation was found that would suggest a key role of this enzyme. In fact, other degradation pathways from WRF such as the cytochrome P450 mechanism are also involved in the transformation of pharmaceutical compounds [152, 315, 318]. For this reason, although the role of laccase in PhAC degradation is well studied [319, 320], we suggest that the absence of laccase enzymatic activity should not be used as an indicator of removal decay in bioremediation systems with WRF.

Regarding the presence of *T. versicolor* during the operation, fungal pellets ITS copies were at its highest concentration in day 0 ($1.7{\cdot}10^{10}$), when the pellets were fresh. This concentration then fluctuated during the first half of operation and by day 43 it almost stabilized around 10^9 ITS copies per mg of pellet. On the other hand, ITS copies in the liquid matrix were initially undetectable (no amplification) or with values around 10^5 until day 22. Afterwards, ITS concentration gradually increased over time, reaching a peak of $9{\cdot}10^9$ copies per mL by day 50 and fluctuating between $8.8{\cdot}10^7$ and $3{\cdot}10^9$ until the end of the operation. This increasing tendency is explained by fungal cells detaching from the pellets as these aged in the bioreactor. While the age of the pellets was maintained by the re-inoculation strategy, mycelium in the supernatant accumulated over time. Eventually, progressive detachment of fungal mycelium from the pellets combined with the partial biomass restoration strategy seemed to produce a balance after day 63 between *Trametes* in the supernatant (10^9 ITS copies $\cdot$ mL^{-1}) and in the pellet (10^9 ITS copies $\cdot$ mg pellet $^{-1}$). While some variability of *T. versicolor* ITS copies in pellets was expected due to colonization by

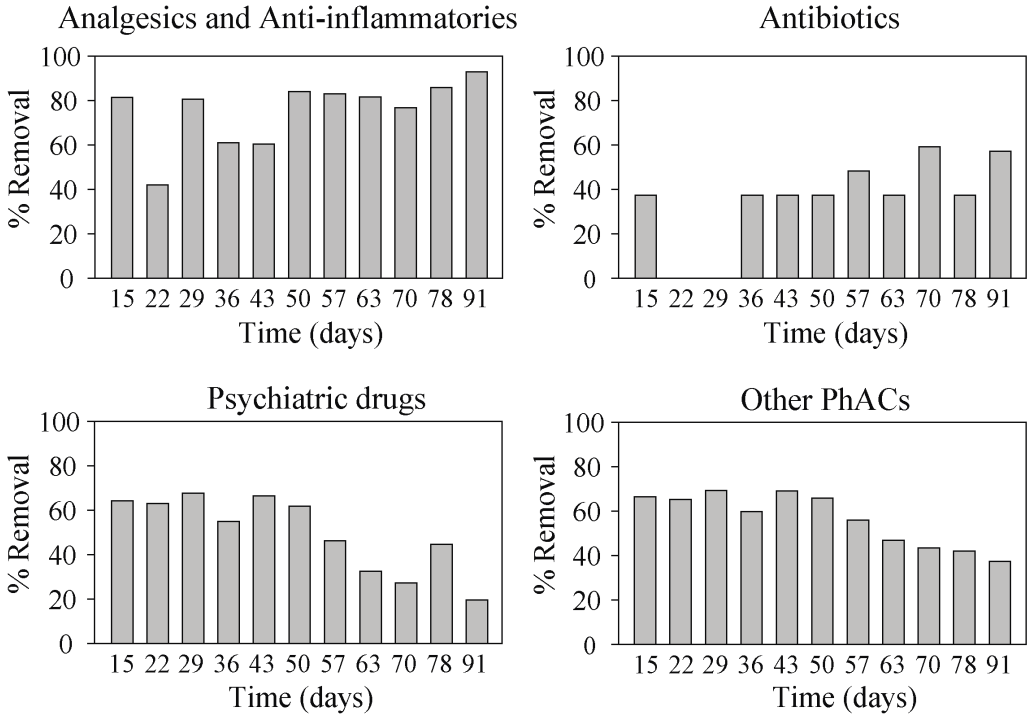

Figure 6.4: Time-course removal percentages of analgesics and anti-inflammatories (upper left), antibiotics (upper right), psychiatric drugs (lower left) and other PhACs (lower right) during the long-term experiment.

other microorganisms or adhesion of particulate solids, concentration in day 22 was unusually low. This sharp decrease coincided with a hiatus in antibiotics removal (described below in this section) and a pronounced increase in the relative abundance of the Alphaproteobacterium *Ochrobactrum* (details in section 6.2.2).

To better understand the qPCR results, it should be taken into account that genes encoding ribosomal RNA and ITS in eukaryotic cells occur in tandem repeats thousands of copies long [321]. While the exact number of copies for each taxon is not known, it is safe to assume that a single *T. versicolor* cell can be represented at least by 10^3 ITS copies. Going by this assumption, no more than 250 cells per mL were present in the liquid matrix the first 22 days of operation. Likewise, the highest concentration attained was probably around 10^7 cells per mL of supernatant or g of pellet.

6.2.2 Microbial communities in the bioreactor

The analysis of both bacterial and fungal populations developed in the validation and long-term operation experiments was carried out via DGGE. Fingerprinting profiles of the pellets in the inoculated bioreactor were also obtained for comparison with the liquid matrix. The primer set 341f-907r was chosen for the amplification of bacteria and a nested approach using the primer sets EF4f-ITS4r and ITS1f-ITS2r was chosen for fungi. Details regarding the amplification protocols and electrophoresis conditions are provided in sections 3.5.3 and 3.5.5, respectively.

A total 8 DGGE gels were obtained, each containing fungal or bacterial samples from each operation (validation and long-term). All representative sequences obtained from the DGGE were submitted to the GeneBank database under the accession numbers KX530041—KX530058, KX523866—KX523887, MF683211—MF683229 and MF682288—MF682321 for fungi in the validation experiment, bacteria in the validation experiment, fungi in the long-term experiment and bacteria in the long-term experiment, respectively.

Fungi

Fungal DGGE profiles from each experiment and condition along with the details of recovered bands are provided in table 6.2 and figure 6.5, respectively. In total, 32 prominent bands were excised and sequenced, obtaining band coverages of 97% (validation) and 98% (long-term). Unidentified bands were not considered in further analyses. Only the phyla Ascomycota and Basidiomycota were represented in both operations and 9 genera were encompassed within those phyla: *Candida*, *Fusarium*, *Isaria*, *Phialemoniopsis* and *Trichoderma* from to the Ascomycota phylum and *Asterotremella*, *Clitopilus*, *Trametes* and *Tremella* from the Basidiomycota phylum. The band-matching matrix with quantitative data was linked to the sequencing results to better assess and comment the evolution of fungal communities and generate relative abundance graphs at the genus level (Fig. 6.6). Real-time qPCR results, PhAC concentrations, and removal rates were integrated in the same graph (Fig. 6.6) to facilitate the interpretation of results.

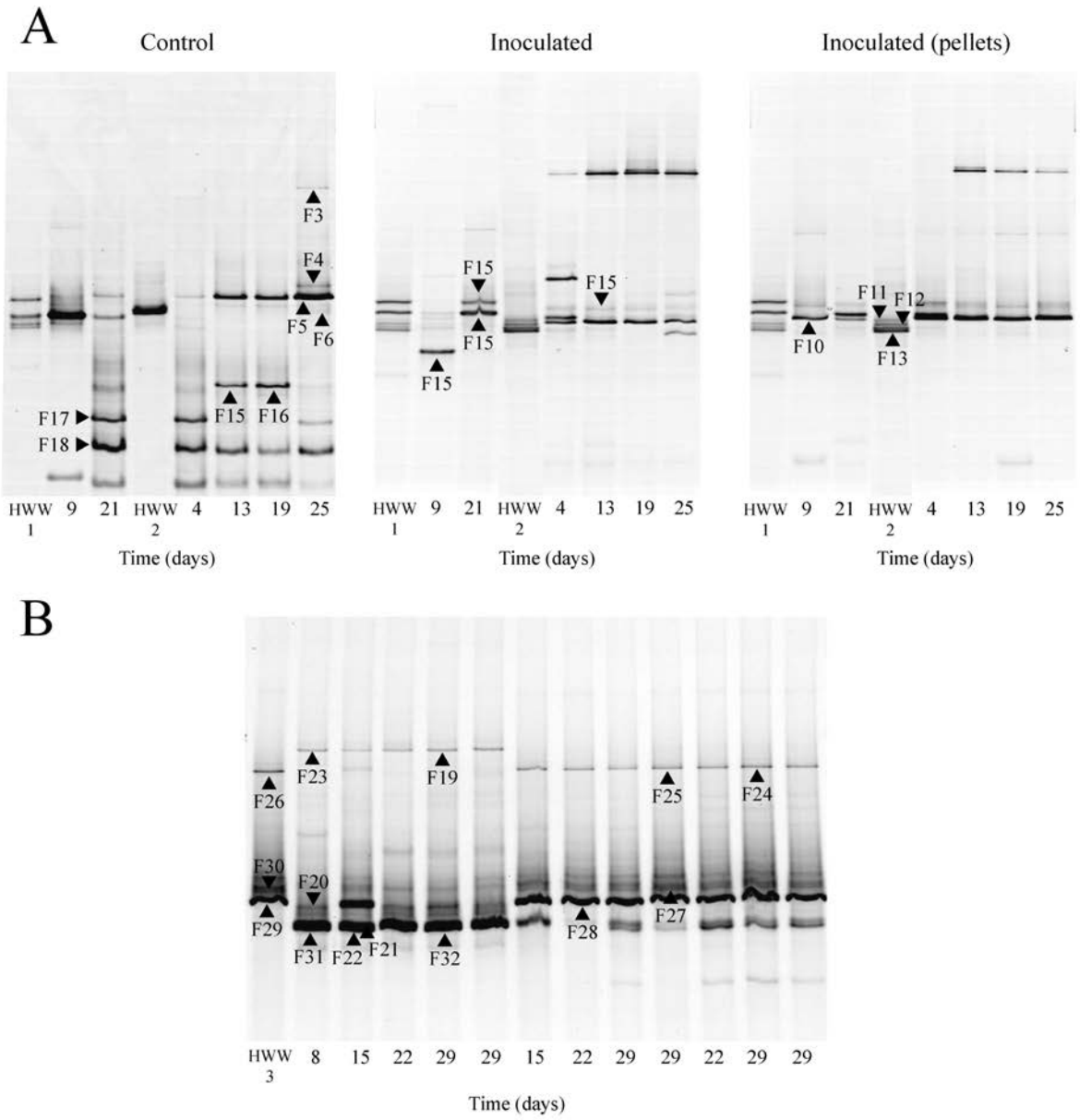

Figure 6.5: DGGE profiles of fungal communities detected in the validation (A) and long-term (B) operation of the bioreactor. Representative bands recovered are indicated (▲) and labelled following the codes provided in table 6.2

Table 6.2: Phylogenetic affiliations of fungal ITS sequences obtained from the DGGEs of the validation operation.

DGGE band	Phylogenetic affiliation (Phylum)	Closest cultured BLAST match	Accession number	Similarity (%)
F01-02	Ascomycota	*Candida*	KJ722419	100
F03-06	Ascomycota	*Phialemoniopsis*	AB278180	98
F07, F9	Basidiomycota	*Asterotremella*	KC118118	100
F08, F10	Basidiomycota	*Trametes*	KR261581	100
F11-13	Ascomycota	*Isaria*	FN548150	99
F14	Basidiomycota	*Tremella*	KP986514	100
F15	Ascomycota	*Trichoderma*	KR856224	100
F16-18	Ascomycota	*Trichoderma*	KR856224	100
F19-23	Ascomycota	*Fusarium*	KY582114	100
F24-30	Basidiomycota	*Trametes*	KY949632	100
F31	Ascomycota	*Fusarium*	KU361576	100
F32	Ascomycota	*Fusarium*	KT269793	97

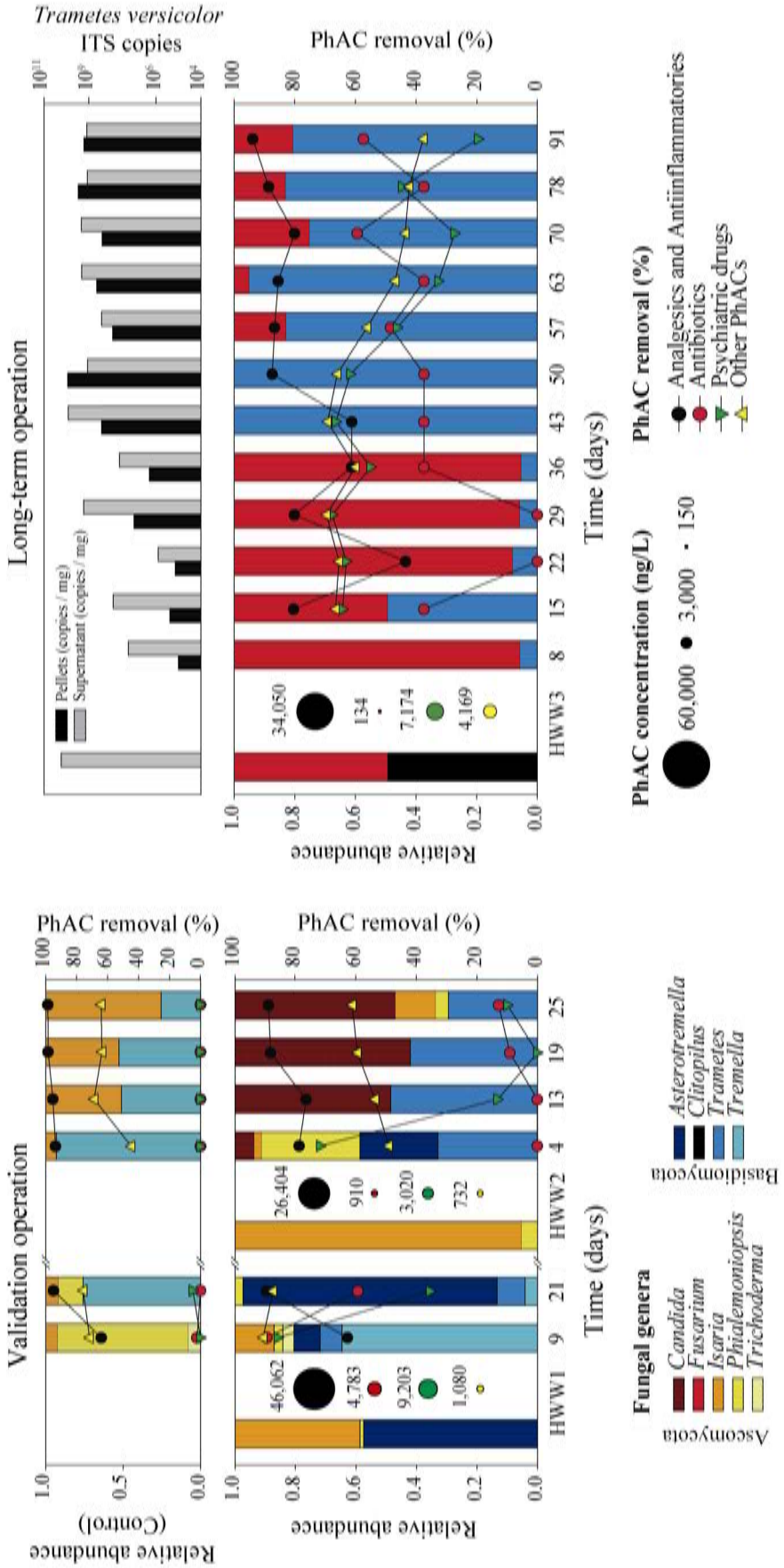

Figure 6.6: Relative abundance of fungal sequences at the genus level from the validation (left) and long-term (long) experiments. PhAC initial concentration are represented by spheres and removal percentages are presented in form of solid lines and colored points indicating different PhAC families. qPCR results are provided to compliment long-term operation.

Globally looking at the validation experiment, a notable heterogeneity existed between fungal communities in each bioreactor and influent (HWW1 and HWW2). A Venn diagram displaying the number of unique and shared genera in each HWW treatment is presented in figure 6.7. As an exception to the mentioned heterogeneity, *Trametes* was present in all supernatant samples because individual cells detached from the inoculated *T. versicolor* pellets.

The control bioreactor was dominated sequentially in time by *Phialemoniopsis*, *Tremella* and *Isaria* (the latter likely due to its high abundance in HWW1 and HWW2). Parallelly, the presence of *T. versicolor* pellets in the experimental bioreactors likely influenced the communities and neither of the three genera mentioned above achieved high abundances for a prolonged period of time. Peaks in the relative abundance of *Tremella* (0.65) and *Asterotremella* (0.84) were observed during the treatment of HWW1 in days 9 and 21, respectively. While the relative abundance increase in genus *Asterotremella* was expected due to its abundance in HWW1, *Tremella* representatives were not detected in the HWW. Nevertheless, it should be noted that only two samples (from days 9 and 21) were taken during the first part (HWW1) of the validation experiment and thus a frame of the operation is overlooked in these results. The second part of the validation experiment was mostly dominated by *Candida* and *Trametes*, achieving relative abundances from 0.07 to 0.58 and 0.30 to 0.49, respectively.

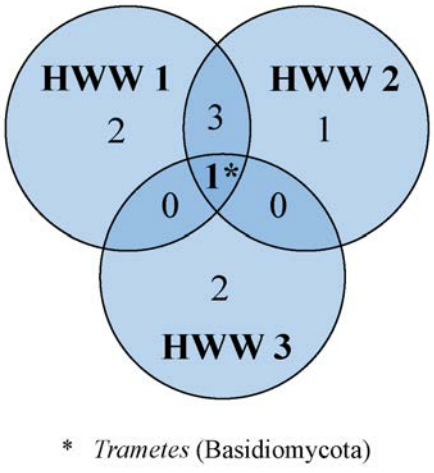

Figure 6.7: Venn diagram displaying fungal unique and shared genera along the treatment of each HWW.

During the long-term operation, *Clitopilus* was present in the influent but did not develop in the liquid matrix, at least enough to be detectable by the PCR-DGGE approach. On the contrary, *Fusarium* representatives rapidly colonized the supernatant of the bioreactor with abundances between 0.51 and 0.95 until day 36, when the highest value was attained. Precisely, it was after day 36 that *Fusarium* was completely displaced by *Trametes* in the supernatant and was only able to come back after that point to a maximum relative abundance of 0.25. In this sense, the late increase of *Trametes* could be predicted by the qPCR results. As it was mentioned already in section, the detachment of *T. versicolor* mycelia from the introduced pellets and its accumulation as free-living cells in the supernatant could explain the outcome shared in the three HWW treatments.

In terms of fungal richness and diversity, no trends were shared by the different operations or removal efficiencies. Aside from *Trametes*, that was inoculated for its known biodegradative potential, *Trichoderma* was the only fungal genus also capable of degrading PhACs as carbamazepine or clarithromycin [322]. Moreover, the capacity of *Trichoderma* to bioremediate heavy metals and polyethylene was already reported [323–325]. Among the rest, only *Candida*, *Fusarium* and *Isaria* are known to bioremediate oil-contaminated soils, sludges, and insecticides, respectively [326–328].

Bacteria

Having considered fungi, bacteria thriving in operations were also analyzed. The corresponding DGGE profiles and band sequence details are presented in table 6.3 and figure 6.8, respectively. A total of 49 bands were recovered, providing coverages of 97% (validation) and 90% (long-term). Again, unidentified bands were not included in further analyses. Up to 23 different genera from phyla Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria were identified during experiments (Table 6.3). In contrast to fungi and due to the higher number of identified genera, description of trends and data representation were performed at the phylum level. However, phylum Proteobacteria encompasses an enormous functional diversity so it was subdivided to the class level. Relative abundance graphs were also prepared and integrated with the qPCR and PhAC concentration and removal results (Fig. 6.9).

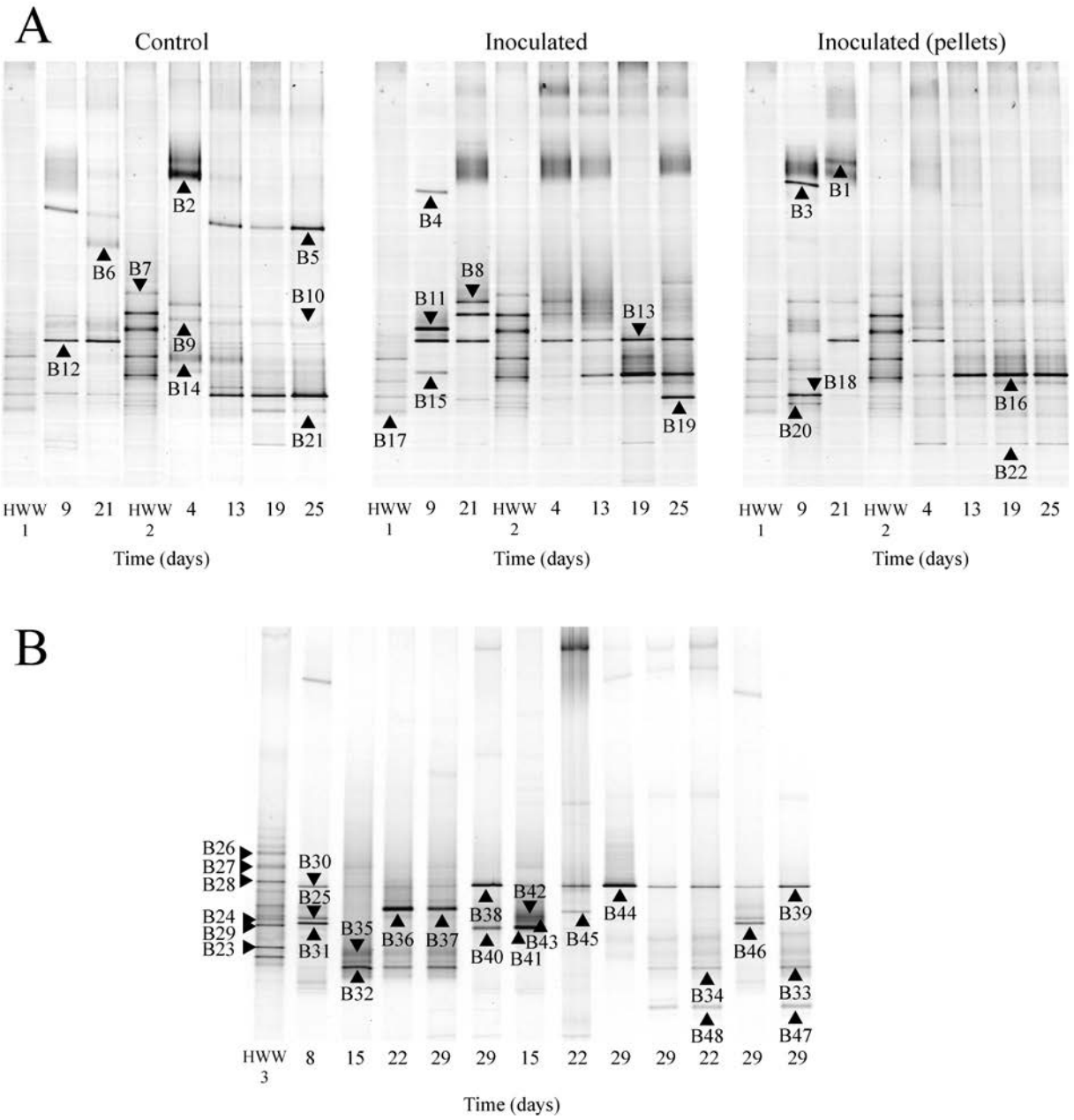

Figure 6.8: DGGE profiles of bacterial communities detected in the validation (A) and long-term (B) operation of the bioreactor. Representative bands recovered are indicated (▲) and labelled following the codes in table 6.3

Table 6.3: Phylogenetic affiliations of bacterial 16S rRNA gene sequences obtained from the DGGEs of the validation and long-term operation.

DGGE band	Phylogenetic affiliation (Phylum)[a]	Closest cultured BLAST match	Accession number	Similarity (%)
B01	Bacteroidetes	*Flavobacterium*	KT354259	100
B02	Bacteroidetes	*Flavobacterium*	JF915323	99
B03-04	Bacteroidetes	*Elizabethkingia*	LN995715	100
B05	Bacteroidetes	*Chryseobacterium meningosepticum*	AF207076	100
B06	Bacteroidetes	*Bacteroides*	NR113070	95
B07	Firmicutes	*Faecalibacterium*	HQ457025	100
B08	Bacteroidetes	*Dyadobacter*	LN890052	100
B09	Bacteroidetes	*Dyadobacter*	DQ207362	100
B10	Betaproteobacteria	*Microvirgula*	LN997979	100
B11	Firmicutes	*Lactococcus*	KU942499	100
B12	Betaproteobacteria	*Burkholderia*	KT862889	100
B13	Betaproteobacteria	*Pandoraea*	LN995687	100
B14	Cyanobacteria	Cyanobacterium TDX16	KJ599678	95
B15	Alphaproteobacteria	*Acetobacter*	KR261398	99
B16	Alphaproteobacteria	*Rhizobiu.*	KT387839	100
B17	Betaproteobacteria	*Comamonas*	LN558648	99
B18	Betaproteobacteria	*Comamonas*	KT716080	99
B19	Firmicutes	*Paenibacillus.*	JX469414	99
B20	Gammaproteobacteria	*Stenotrophomonas*	KR922087	100
B21	Alphaproteobacteria	*Magnetospirillum*	KM289194	99
B22	Actinobacteria	Microbacteriaceae bacterium	KR082269	100
B23	Betaproteobacteria	*Comamonas*	KX279654	100
B24	Betaproteobacteria	*Delftia*	MF156902	100
B25	Betaproteobacteria	*Delftia*	KX980470	97
B26	Firmicutes	*Faecalibacterium*	AY169429	96
B27	Gammaproteobacteria	*Acinetobacter*	JN849077	97
B28	Firmicutes	*Acutalibacter*	CP021422	97
B29	Betaproteobacteria	*Paraburkholderia*	KY992888	96
B30	Betaproteobacteria	*Pandoraea*	CP010431	100
B31	Gammaproteobacteria	*Stenotrophomonas*	MF442269	97
B32-34	Gammaproteobacteria	*Raoultella*	MF455198	98–100
B35	Gammaproteobacteria	*Raoultella*	MF429591	100
B36-37	Alphaproteobacteria	*Ochrobactrum*	LC150701	100
B38-39	Betaproteobacteria	*Pandoraea*	CP010897	99
B40-43	Gammaproteobacteria	*Luteibacter*	KY938100	100
B44	Betaproteobacteria	*Burkholderia*	MF383417	100
B45	Bacteroidetes	*Pedobacter*	EF204468	96
B46	Gammaproteobacteria	*Stenotrophomonas*	KY910087	100
B47-48	Alphaproteobacteria	*Azospirillum*	CP012406	99
B49	Bacteroidetes	*Flavobacterium*	FJ447541	98

[a] [a] Class level was used to better classify the phylotypes belonging to Proteobacteria.

As a general trend, all bioreactors harbored communities highly similar to the ones in the respective HWW treated. Contrarily to fungi, bacteria were able to establish in the bioreactor without much influence from the inoculated fungal pellets due to its high concentration in the HWW, even after the flocculation pre-treatment [151].

During the validation experiment three groups stood out for their elevated relative abundances. First, Betaproteobacteria were persistent along the operation in both conditions (i.e. control and experimental) and HWWs. While day 4 in the HWW2 control treatment remains an exception (in which Betaproteobacteria were absent), the relative abundance of the class ranged 0.24–0.45 and 0.46–0.81 in the experimental and control operations, respectively. Class Betaproteobacteria is precisely well known for the broad variety of metabolic pathways that their members harbor [274].

Second, Alphaproteobacteria exhibited a peculiar tendency during the treatment of HWW2 where they thrived during the mid-late operation but were not detected in the influent. Highest abundance occurred coincidently around day 19 in both bioreactors. Strains from this class were likely introduced in the validation experiment bioreactor during the HWW1 treatment and were not able to develop until the matrix conditions changed due to a new influent (HWW2).

Third, Bacteroidetes phylum was found in all samples except in day 19 of HWW2 treatment (experimental). Abundances of its representatives were among 0.09-0.60 and 0.15-0.83 in the experimental and control bioreactors, respectively. In this case Bacteroidetes were found in both HWW. Concerning the remaining groups, members of the Firmicutes were abundant in HWW2 but appeared briefly and at random in both bioreactors and HWW treatments. Also, Actinobacteria were present in restricted abundances in most of the samples although they were not detected in any HWW. Lastly, Cyanobacteria were unexpectedly present in both wastewaters but were only found during the treatment of HWW2 in both experimental and control conditions.

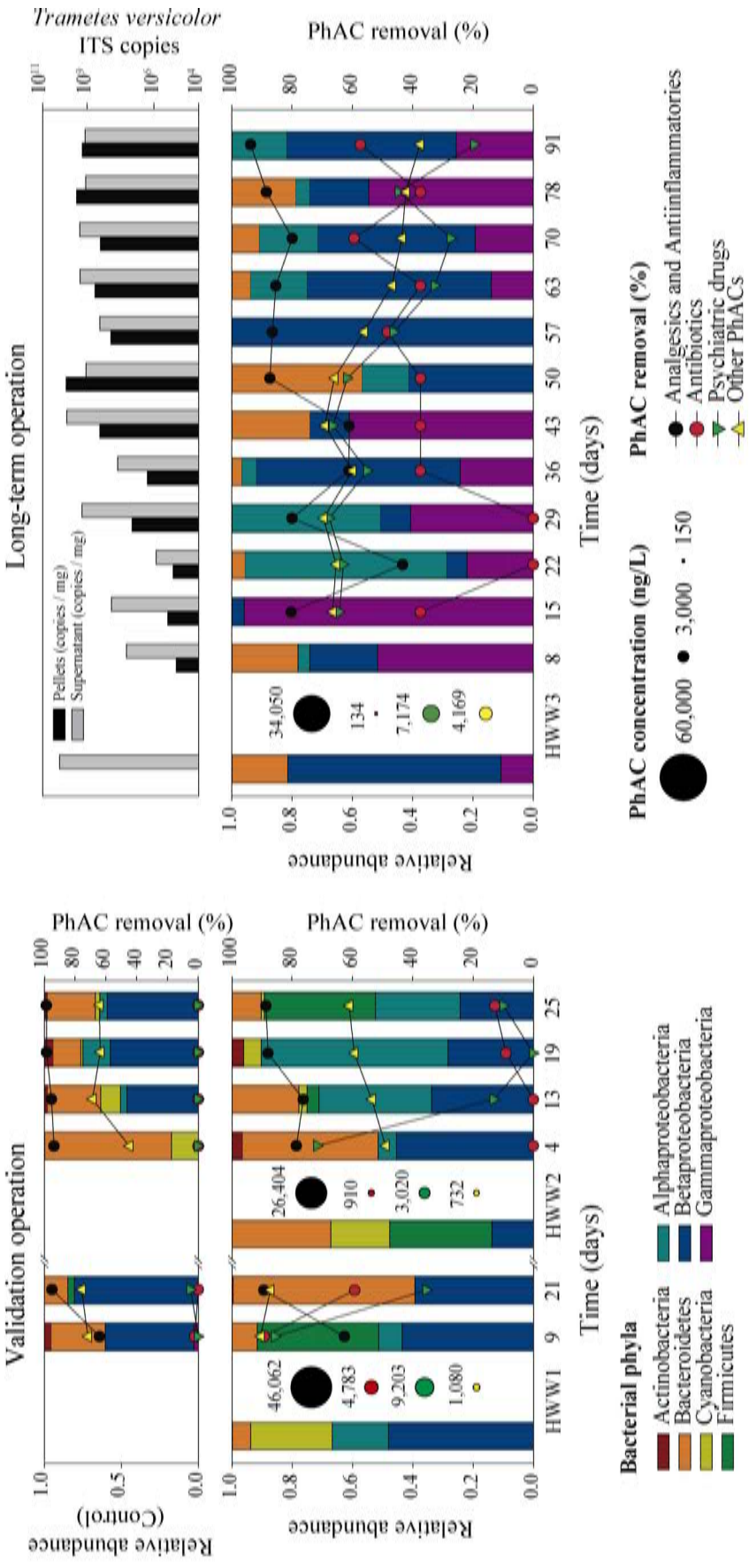

Figure 6.9: Relative abundance of bacterial sequences from the validation (left) and long-term (long) experiments. PhAC initial concentration are represented by spheres and removal percentages are presented in form of solid lines and colored points indicating different PhAC families. qPCR results are provided to compliment long-term operation.

With reference to the long-term operation, dominance of the Proteobacteria was evident throughout the 91 days of treatment. Again, Betaproteobacteria were present in all samples and their relative abundance was especially high from day 50 onward (0.2). In fact, 71% of the band intensities in the DGGE belonged to this metabolically diverse class, reaching even 100% in day 57.

Alphaproteobacteria and Gammaproteobacteria were the remaining classes of the Proteobacteria phylum that were detected in the long-term experiment. Similar to Betaproteobacteria, their presence was observed in almost all samples (9 and 10 out of 12, respectively) although they greatly differed in terms of relative abundance. Alphaproteobacteria relative abundance was 0.15 (Min: 0.05; Max: 0.67) while Gammaproteobacteria doubled the value with an average of 0.32 (Min: 0.14; Max: 0.96). Interestingly, the peak abundance of Alphaproteobacteria was on the same days as it happened in the validation experiment (day 22) and took place at the same time that *Trametes* concentrations were at its minimum. The group was also not detected in the source influent.

From the remaining phyla detected in the validation assay, only Bacteroidetes was found in this long-term operation. Bacteria from this phylum appeared intermittently, reaching in three occasions peaks of relative abundance over 0.2.

At the phylum and class levels, correlations were assessed and the only significance found was the one of Gammaproteobacteria with the removal of psychiatric drugs ($r=0.64$, $p<0.01$). Focusing on the most abundant and persistent genera within the group, *Luteibacter*, *Raoultella* and *Stenotrophomonas* had overall abundances of 0.22, 0.52 and 0.22, respectively, covering 96% of the total Gammaproteobacteria. While it is true that many authors have reported the biodegradation of the anticonvulsant Carbamazepine [243,315,322,329], works involving other psychiatric drugs are scarce [330,331] and none related to *Luteibacter*, *Raoultella* or *Stenotrophomonas*. Despite the lack of evidences regarding psychiatric drug degradation, the potential of these genera should not be disregarded, for they are able to degrade a wide variety of substances such as insecticides [332–335], antitumorals [336], anti-inflammatories [337] or synthetic polymers [338].

To get a grasp of the bacterial genera that are unique or shared between treatments, a Venn diagram was constructed (Fig. 6.10). Pairwise comparison showed that HWW1 and HWW2 shared up to 4 genera between them but none with HWW3. The results are not surprising considering that HWW1 and HWW2 were obtained from the collector with only one month of difference compared to the one year separation with HWW3. However, letting aside individual comparisons, three genera were in common in the three treatments, namely *Flavobacterium* (Bacteroidetes), *Comamonas* and *Pandoraea* (Betaproteobacteria).

Flavobacterium is widely distributed in water, wastewater and hospital environments [339] but no implication was evidenced with the removal of any pharmaceutical. In fact, numerous members of this genus are opportunistic pathogens [340] and its representatives were likely shared in all treatments due to their ubiquity.

In contrast, both *Comamonas* and *Pandoraea* could have an influence on the removal processes, either positively or negatively, as their abundance during the operations exhibited significant corre-

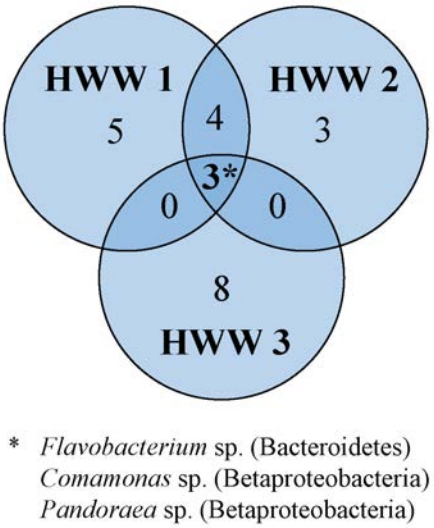

Figure 6.10: Venn diagram displaying bacterial unique and shared genera along the treatment of each HWW.

lations with psychiatric drugs and antibiotics removal. In the first case, *Comamonas* was negatively correlated to psychiatric drug removal (r= -0.53 p<0.01). Concretely, the genus was abundant during the control treatment in the validation experiment and in the end of the long-term experiment, when none or low psychiatric drug removal was observed, respectively. Implication of *Comamonas* in the removal decrease could be explained by antagonistic relationships with microorganisms responsible of removal or by the simple ability to outcompete them. As it can be seen in figure 6.1, only three psychiatric drugs (i.e. Carbamazepine, citalopram and venlafaxine) and the transformation products (TPs) of Carbamazepine (CBZ) were present in all the operations. Transformation of CBZ by *Comamonas* and appearance of its TPs would justify the observed decrease in Psychiatric drug removal. However, both CBZ and its TPs increased in the last stages of the long-term operation (data not shown) so the hypothesis was discarded. There is no record of *Comamonas* degrading psychiatric drugs yet; however, some of its species -including pathogens [341]- can degrade estrogens such as 4-chlorophenol or tetrabromobisphenol [342–344]. In the second case, *Pandoraea* had a strong positive correlation with antibiotics removal (r= 0.60 p<0.01). Bacteria from genus *Pandoraea* are known for their metabolic versatility and some strains are capable of organohalogenate, PAH and lignin degradation [345–347]. Soil is the usual habitat for many of its species but the genus was described in 2000 [348] from patients suffering cystic fibrosis and it is precisely in the clinical field that many species have been characterized for multiple antibiotic resistance. In fact, antibiotic susceptibility tests revealed resistance of *Pandoraea* isolates to beta-lactams, ciprofloxacin and even colistin, a last resort antibiotic [349–351]. While more studies would be necessary to assess to which degree *Pandoraea* is involved in antibiotic degradation, its ability to thrive in presence of antibiotics surely provides fitness advantages in front of other species.

To confirm the presence of this genus beyond relative abundance, a qPCR approach was used to quantify the copies of *Pandoraea* 16S rRNA gene in the liquid matrix and pellet. Due to the lack of pre-designed qPCR primers for this genus, a reverse primer (QpanR) was designed using

primer3 and BLAST [352] to work in combination with panF [170]. Specificity of panF–QpanR was checked with Silva TestPrime 1.0. [213] and 89% of *Pandoraea* entries were covered while the only additional match was <0.5% of the Burkholderiaceae family, of which *Pandoraea* is member.

Presence of *Pandoraea* was successfully confirmed and differences in abundance of the 16S rRNA gene between liquid matrix and pellet were of 1 to 3 orders of magnitude, generally following the same trends (Fig. 6.11). In the beginning, *Pandoraea* slowly decreased in the liquid until day 42, where a drastic decrease of 4 orders of magnitude was observed. Precisely, at day 42 *Luteibacter* was thought responsible of a bloom in the bioreactor (61% relative abundance of total bacteria) in which the matrix and pellets turned pink [353]. After the fast recovery of the system thanks to the pellet renewal strategy, *Pandoraea* also recovered and kept an increasing tendency until the end of operation. Justifying this situation, it should be pointed out that pellets in the bioreactor might serve as a bacterial reservoir in the liquid matrix. While *Pandoraea* rRNA copies decreased dramatically in the liquid matrix, pellet copies did not even decrease two orders of magnitude and could serve as an inoculum source for bacteria to colonize again the the liquid matrix.

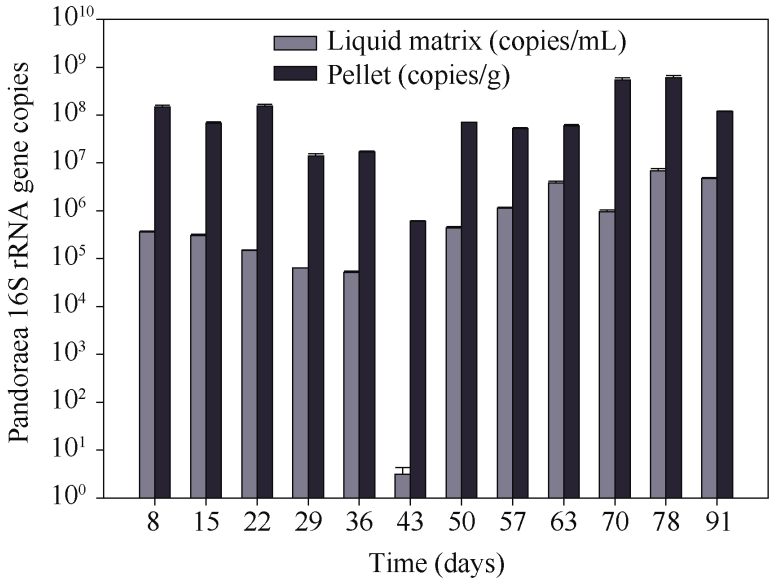

Figure 6.11: Real-time qPCR quantification of *Pandoraea* sp. 16S rRNA copies in the long-term operation bioreactor. Quantification was performed in liquid and pellet samples and expressed as copies per mL of matrix and g of dry pellet, respectively.

As it was mentioned in the introduction of section 6.2.2, microbial communities present in the pellets were also analyzed to assess whether colonization of bacteria or fungi other than *Trametes* occurred and could be relevant. As it could be expected from the *Pandoraea* qPCR results, DGGEs showed that communities in the pellets were mostly a reflex of the supernatant (Figs. 6.5 and 6.8). An exception to this general trend was the non-detection of *Phialemoniopsis* despite being in both HWW and in most supernatant samples. In contrast, Actinobacteria and Alphaproteobacteria groups were slightly favored in the pellets and Bacteroidetes and Firmicutes showed the most differences between pellet and supernatant samples. These results point that it would be of interest to perform in-depth studies of the pellets microbial communities after some time of treatment to see up to which point they can act of reservoirs of non-abundant taxa.

6.3 Solid-phase biopiles

The sludge is the main residue generated in WWTPs during solid-liquid separations in primary, secondary and tertiary treatments [354]. The use of this sludge in agriculture and forestry processes as a low-cost fertilizer supposes an important valorization method. Nonetheless, as it was stated in introduction of this chapter, WWTP sludge typically contains elevated concentrations of PhACs that released into the environment suppose a risk for both human and animal health. For this reason, a proper treatment process should be applied to ensure the safety of sludge re-use.

In a regular WWTP, sludge is typically stabilized via heat drying or pH increase and then thickened via centrifugation, filtration or water evaporation. Microbial load is already reduced in the stabilization step but processes such as irradiation or pasteurization can be used to further decrease the microbial concentration [355, 356]. As of today, most of these processes are not capable of removing ECs [357, 358]; in this sense, fungal biopiles are a promising alternative in which the sludge and fungi are mixed with a bulking material to provide aeration, give structure and serve as a substrate for the fungal inoculum [359–361]. The advantage provided by this methodology also relies in the minimum maintenance and inputs required, which make biopiles a cost-effective processes for long-time treatment [362–364].

Previous studies using *T. versicolor* and straw as substrate to treat WWTP sludge reported the absence of the fungus after 22 days of operation [365]. To overcome this problem and improve the treatment, biopiles were prepared in this case using pine bark as bulk material, despite being harder to degrade and hydrolyze, because it serves as a better bulking material that allows the scale-up of biopiles. Furthermore, the fate of *T. versicolor* was assessed using real-time qPCR and microbial communities were analyzed using PCR-DGGE and sequencing approach.

6.3.1 Set-up and performance

In a similar manner to section 5.4, the deliberate inoculation of specialized biomass (the WRF *T. versicolor*) was considered as biomass addition of an artificial system. More specifically, two bioaugmentation strategies were studied during the treatment of real WWTP sludge. In the first case, experimental biopiles were inoculated with *T. versicolor* and incubated 42 days. In the second case, a re-inoculation at day 22 was performed in half of the experimental samples. Control biopiles without the fungal inoculum were also included and pine bark was always used as substrate and bulk material due to its low economical value and abundance in the region (NE of the Iberian Peninsula). Triplicate samples were sacrificed for analysis at days 0, 10, 22 and 42 (0, 22 and 42 for the control biopiles). For more information see section 3.3.5.

As in the fungal bioreactor, activity was monitored through laccase activity, but this proved to be nil after day 10 (unpublished data). In any case, the highest activity registered (0.007±0.002 U g^{-1}) was still 1 to 4 orders of magnitude lower than the activity achieved in other biopiles [365,366], although they used straw as substrate, which is more easily degraded. Furthermore, phenolic compounds in pine bark and competition with the sludge autochthonous microorganisms could also inhibit laccase production. In any case, it was already stated that other mechanisms such as CYP

might be the ones implicated in the EC degradation.

Detailed description of the PhACs detected is provided in the annex (Table 10.6) and was reported in Llorens-Blanch et al. work [367]. Briefly, 19 out of 45 analyzed PhACs were detected in the biopiles at day 0. Total concentration was of 430.79±103.26 ng per gram of biopile. Some of the standard deviations presented [367] were high, up to 70% of the measured concentration value such as previously described by Radjenović *et al.* [368]. This fact is inherent to the heterogeneity of solid samples and the extraction procedures are limiting steps that imply elevated dispersion and variability between replicates.

As in sections 6.2.1 and 6.2.1, PhACs were grouped by families (i.e. Analgesics and anti-inflammatories, antibiotics, psychiatric drugs and other drugs). The drug concentration for each family and time point is presented in figure 6.12. Unlike in the bioreactor treatment, where only influent and effluent were analyzed, PhAC concentration in this study was assessed in the solid matrix of the same biopiles. Therefore, adsorption of the pollutants onto biomass or other surfaces was not accounted as removal because a solid phase extraction procedure was performed as described elsewhere [155]. PhACs removal percentages in this case were most likely due to transformation, degradation or volatilization processes.

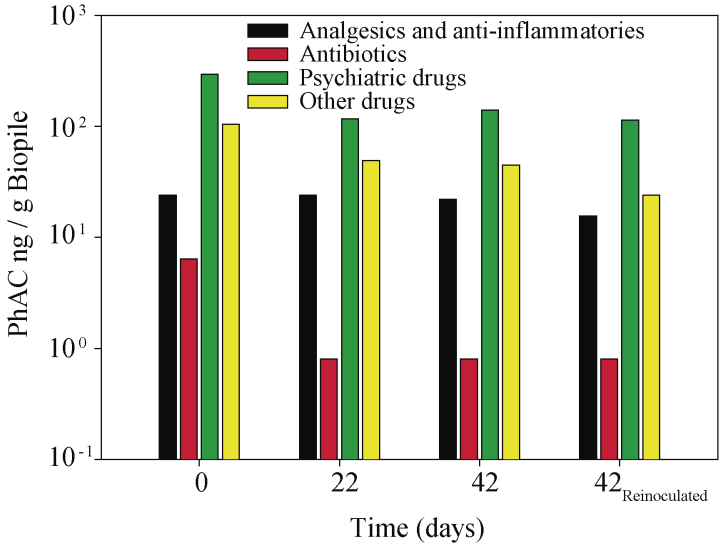

Figure 6.12: Total concentration of each PhAC family in the biopiles at 0, 22 (previous to reinoculation) and 42 days. Individual concentrations and deviations are available in the annex table 10.6.

Starting with analgesics and anti-inflammatories, they represent a small portion of the total PhAC concentration and are not efficiently removed, contrary to what was observed in HWW treatment [151]. While concentration at day 22 remains constant (24.2 ng g^{-1}), by day 42 it is decreased to 22.1 and 15.6 ng L^{-1} in the inoculated and reinoculated biopiles, respectively. Precisely, because compounds of this family are easily removed, they were probably degraded during the WW treatment and only a low portion of recalcitrant compounds remained in the sludge.

In the antibiotics family, Sulfamethoxazole was the only compound detected and the concentration of the group was only 6.4 ng g^{-1} at day 0. A decrease of concentration down to 0.81 ng g^{-1} was

reported after 22 days of treatment, with no further improvement by day 42. Other works featuring *T. versicolor* in biopile systems reported degradation efficiencies of Clarithromycin of the same order as in (82 to 85%) after 42 days of treatment, despite initial concentration was five times higher.

Interestingly, psychiatric drugs were the most abundant family of pharmaceuticals in the sludge, making up for almost 70% of the total PhACs concentration. The initial concentration was of 295.8 ng g^{-1} and decreased to 117.2 ng g^{-1} by day 22; there was a great variability in the removal rates of each compound, ranging from 0 to 100% and with a mean value of 60%. By day 42, the total concentration of Psychiatric drugs was slightly lower (113.8 ng g^{-1}) in the re-inoculated biopiles and an increase over day 22 was noted in the inoculated biopiles (141 ng g^{-1}). This increase is probably due to the heterogeneity in solid samples, as already stated above in this section. Some pollutants included in the psychiatric drugs group such as carbamazepine are known to be highly recalcitrant but still degradable by some fungi species including *T. versicolor* [365, 369, 370].

Lastly, the remaining compounds not grouped in the previous families were grouped as "other PhACs". Drugs belonging to this family amounted 104.4 ng g^{-1} and were steadily removed when *T. versicolor* was reinoculated, achieving 53 and 77% removal by days 22 and 42, respectively. On the contrary, biopiles in which the fungus was not reinoculated only managed to reduce the concentration of other PhACs by 4% (4 ng g^{-1}) in the last 20 days.

Rodriguez-Rodriguez *et al.* [365] reported the loss of fungal activity by day 22 but could not confirm the disappearance of the inoculated *T. versicolor*. In this study, a qPCR assay was performed to elucidate the fate of the same fungus in the biopiles. Three samples from initial (t0) and both inoculated and re-inoculated end points (t42 and t42Reinoculated) were analyzed and results are shown in figure 6.13. Considering the average of the three replicates, around $2{\cdot}10^7$ ITS copies were detected at day 0 and only $5.9{\cdot}10^4$ and $1.2{\cdot}10^4$ for day 42 in inoculated and reinoculated biopiles, respectively. As it was suspected, *T. versicolor* did not completely disappear from the biopiles but its concentration was reduced between 3 and 4 orders of magnitude, even when re-inoculated. Bearing in mind the assumption made in the previous section regarding ITS copies in eukaryotic cells, results imply that only 10 to 60 *T. versicolor* cells were present per gram of biopile regardless of re-inoculation.

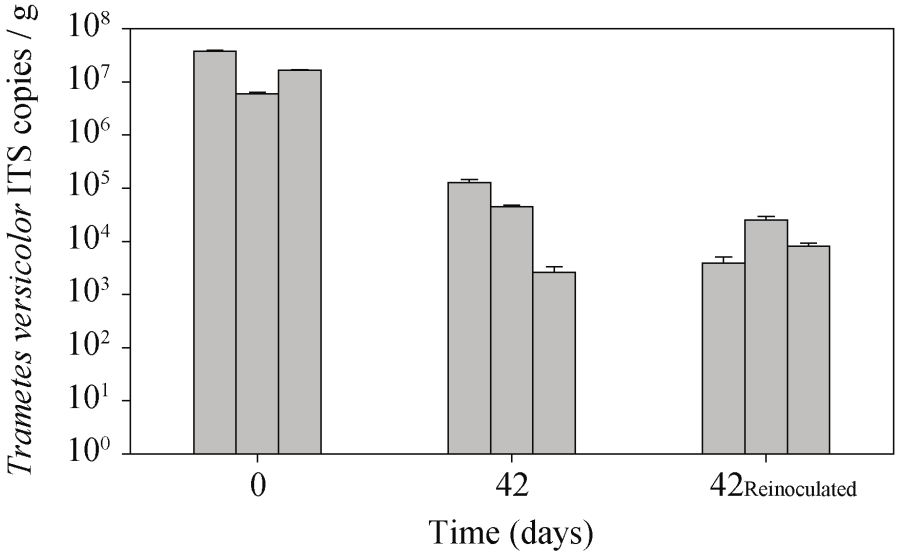

Figure 6.13: Real-time qPCR quantification of *T. versicolor* ITS copies in the biopiles at the beginning (day 0) and end (day 42) of the experiment for inoculated and re-inoculated biopiles.

6.3.2 Microbial communities in the biopiles

Aside from the performance and presence of *T. versicolor* in the biopiles, the assessment of microbial communities (bacteria and fungi) in the biopiles was assessed by means of DGGE. Information on how microbial communities change through time and react to the *T. versicolor* inoculation of pre-grown mycelium could provide useful information of the occurring positive or negative interactions.

Fungal and bacterial fingerprints were obtained using the primer sets and protocols described in the fungal bioreactor treatment (section 6.2.2). Characterization was carried out in 5 different time points for experimental inoculated and reinoculated cultures: 0, 10, 22, 23 (only reinoculated) and 42. Non-inoculated biopiles (control group) were only sampled at days 0, 22 and 42. DGGE fingerprints are presented in figures 6.14A and 6.14B for fungi and bacteria, respectively. Detailed information of the recovered bands is presented in tables 6.4 and 6.5, and sequences were deposited in the GenBank database under the accession numbers MF398478—MF398488 and MF383380—MF383399 for Fungi and Bacteria, respectively.

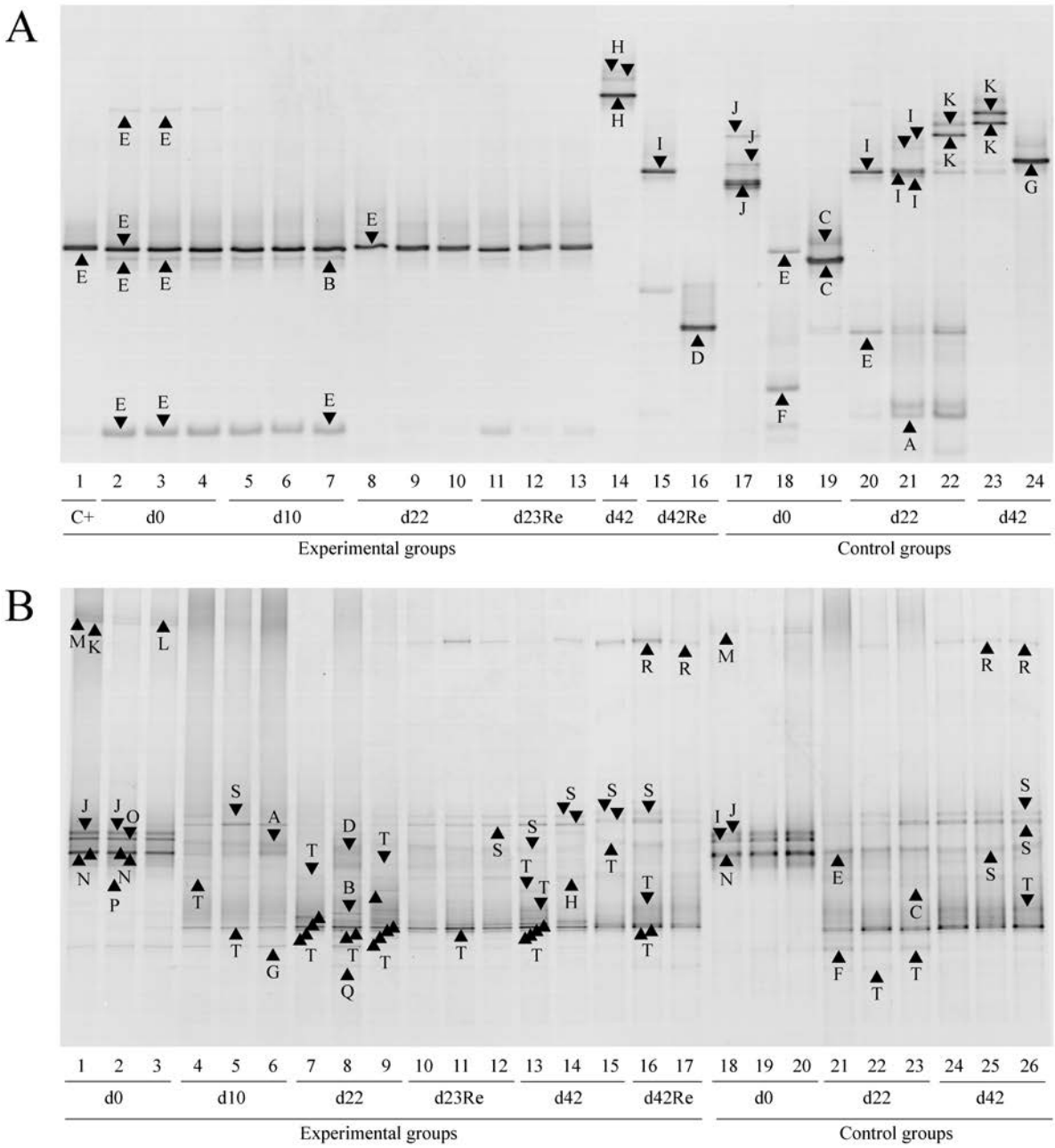

Figure 6.14: DGGE fingerprints of fungal (A) and bacterial (B) populations. Samples from inoculated biopiles (experimental groups) are divided in days 0, 10, 22, 23 and 42. Re-inoculated biopiles belong to the groups d23Re and d42Re. The same codes were used for non-inoculated biopiles (control groups) but no samples were collected at day 10. Recovered bands (▲) were labelled following the codes in table 6.4.

The study of fungal DGGEs revealed the almost-complete predominance of *Trametes* in the experimental biopiles from days 0 to 23 (6.15A). During this period, sequences from other fungi were masked by *Trametes* and fell behind the DGGE detection limit. After the loss of *Trametes* by day 42 (confirmed by qPCR), diverse fungal genera such as *Meyerozyma*, *Coriolopsis* and *Wickerhamomyces* were detected. Precisely, these samples from day 42 were completely dominated by each one of the genus mentioned above. This heterogeneity could be explained by a fungal growth in form of clusters or aggregates where only one fungal genus was present or at least highly abundant at a local level, masking the other groups.

Table 6.4: Sequence information for the DGGE bands obtained analysing the fungal community.

DGGE band (lane/s)	Phylogenetic affiliation (Phylum)	Closest cultured BLAST match	Accession nº	Similarity (%)
A (20-22)	Ascomycota	*Aspergillus*	KP975532	100
B (7)	Ascomycota	*Acremonium*	KF669512	99
C (19)	Ascomycota	*Pseudallescheria*	KP132722	100
D (16)	Basidiomycota	*Coriolopsis*	AY684172	99
E (1-13; 19-22)	Basidiomycota	*Trametes*	KP761168	100
F (18)	Basidiomycota	*Peniophora*	LN808982	100
G (22-24)	Ascomycota	*Meyerozyma*	KR054629	100
H (14)	Ascomycota	*Wickerhamomyces*	KJ451713	99
I (15; 20-22)	Ascomycota	*Meyerozyma*	KR085964	100
J (17)	Basidiomycota	*Rhodotorula*	LN833560	100
K (22-23)	Basidiomycota	*Trichosporon*	KP658861	99-100

Table 6.5: Sequence information for the DGGE bands obtained analysing the bacterial community.

DGGE band (lane/s)	Phylogenetic affiliation (Phylum)[a]	Closest cultured BLAST match	Accession nº	Similarity (%)
A (3-6)	Firmicutes	*Bacillus*	LN774422	94
B (8)	Firmicutes	*Bacillus*	KP670289	97
C (23)	Firmicutes	*Sporosarcina*	HQ603002	98
D (8)	Firmicutes	*Staphylococcus*	KT261256	99
E (21-23)	Firmicutes	*Staphylococcus*	KR732655	95
F (21-23)	Actinobacteria	*Brevibacterium*	LC082101	100
G (1-6; 8; 10; 12; 14-17)	Actinobacteria	*Brevibacterium*	LC068966	100
H (10-15)	Alphaproteobacteria	*Brevundimonas*	KP895785	97
I (18-20)	Firmicutes	*Clostridium*	AB971795	98
J (1-3;18-20)	Firmicutes	*Clostridium*	KF528156	94
K (1-3)	Firmicutes	*Clostridium*	KJ722507	98
L (1-3)	Firmicutes	*Clostridium*	EU089965	97
M (1-3;18-20)	Firmicutes	*Clostridium*	KJ722512	99
N (1-3;18-20)	Firmicutes	*Clostridium*	AB610575	99
O (1-3)	Firmicutes	*Clostridium*	FJ424481	100
P (1-3;18-20)	Firmicutes	*Intestinibacter*	NR_027573	97
Q (8-17)	Actinobacteria	*Dietzia*	KR181931	98
R (9-17; 22-26)	Bacteroidetes	*Salinimicrobium*	HG008896	95
S (4-17; 21-26)	Bacteroidetes	*Pedobacter*	NR_117231	97
T (2-17; 21-26)	Gammaproteobacteria	*Lysobacter*	DQ490982	100

[a] [a] Class level was used to better classify the phylotypes belonging to Proteobacteria.

Focusing in bacteria, relative abundance graphs are presented in figure 6.15C and 6.15D. First and foremost, bacterial communities in the experimental and control groups displayed very similar patterns, reflecting apparent indifference to the fungus inoculation. At the phylum level Firmicutes were abundant at day 0 and lost representation as operation went on despite remaining present in all samples.

Except for *Staphylococcus*, mainly present at the end of the operation, all the detected Firmicutes (i.e. *Bacillus*, *Clostridium*, *Sporosarcina* and *Intestinibacter*) were spore-formers that could survive the heat treatment of the sludge and remain viable to develop when the biopiles were prepared. In fact, rapid bacterial shifts that occurred during the first days of operations are likely due to the oxygenation of the sludge after mixture with the lignocellulosic co-substrate.

Interestingly, Gammaproteobacteria was represented by *Lysobacter* that behaved in the opposite way: it was not detected at the beginning of the biopiles but sharply increased its representation by days 22 and 42 in both experimental (Inoculated 0.76±0.06; Re-inoculated 0.70±0.13) and control groups (0.55±0.10). Other groups such as Actinobacteria, Bacteroidetes and Alphaproteobacteria slightly gained abundance in day 22 and 42 but always remained below 0.3 relative abundance.

Strangely, *Trametes* was abundant by day 22 but not detected by day 42, regardless if re-inoculation was carried out. This fact suggests that the microbial communities developing in the biopiles could be involved in this outcome by negatively affecting *Trametes* once they attain certain concentrations (after day 22). Representatives of the fungal genus *Meyerozyma* were present in a re-inoculated sample at day 42 (Fig. 6.15A) and in all control samples from day 22 onward (Fig. 6.15B), when *Trametes* allegedly lacked the colonizing ability in the experimental biopiles. Despite genus level was used in this study to ensure taxonomic accuracy, BLAST analysis of the sequences with best quality revealed a 100% identity with the yeast *Meyerozyma guilliermondii*. Isolates from this species (formerly *Pichia gilliermondii*; anamorph *Candida guilliermondii*) can metabolize wood-derivates such as xylose and hemicellulose hydrolysates [371,372] and have been widely studied for its ability to inhibit the growth of decay-related fungi [373,374], making it a suitable candidate to inhibit *T. versicolor*. Methods of action of this yeast include attachment to the antagonist hyphae, competition for nutrient and production of extracellular cell wall-degrading enzymes [373–377]. Interestingly, *Wickerhamomyces anomalus* is another former *Pichia* (*Pichia anomala*) that was found as the only representative in day 42 from the inoculated biopiles (Fig. 6.15A). This yeast is known for its strong antimicrobial activity induced by Killer Toxins [378].

On the other hand, the Gammaproteobacteria *Lysobacter* was absent in the beginning of the operation and was abundant from day 22 onwards, point in which the re-inoculation of *T. versicolor* was unproductive. *Lysobacter* species are generally known as biocontrol agents and especially fungal suppressors. In fact, numerous fungal-antagonizing mechanisms have been described in *Lysobacter* including specific antibiotics, chitinases and secondary metabolites as the Heat-Stable Antifungal Factor (HSAF) [379–382].

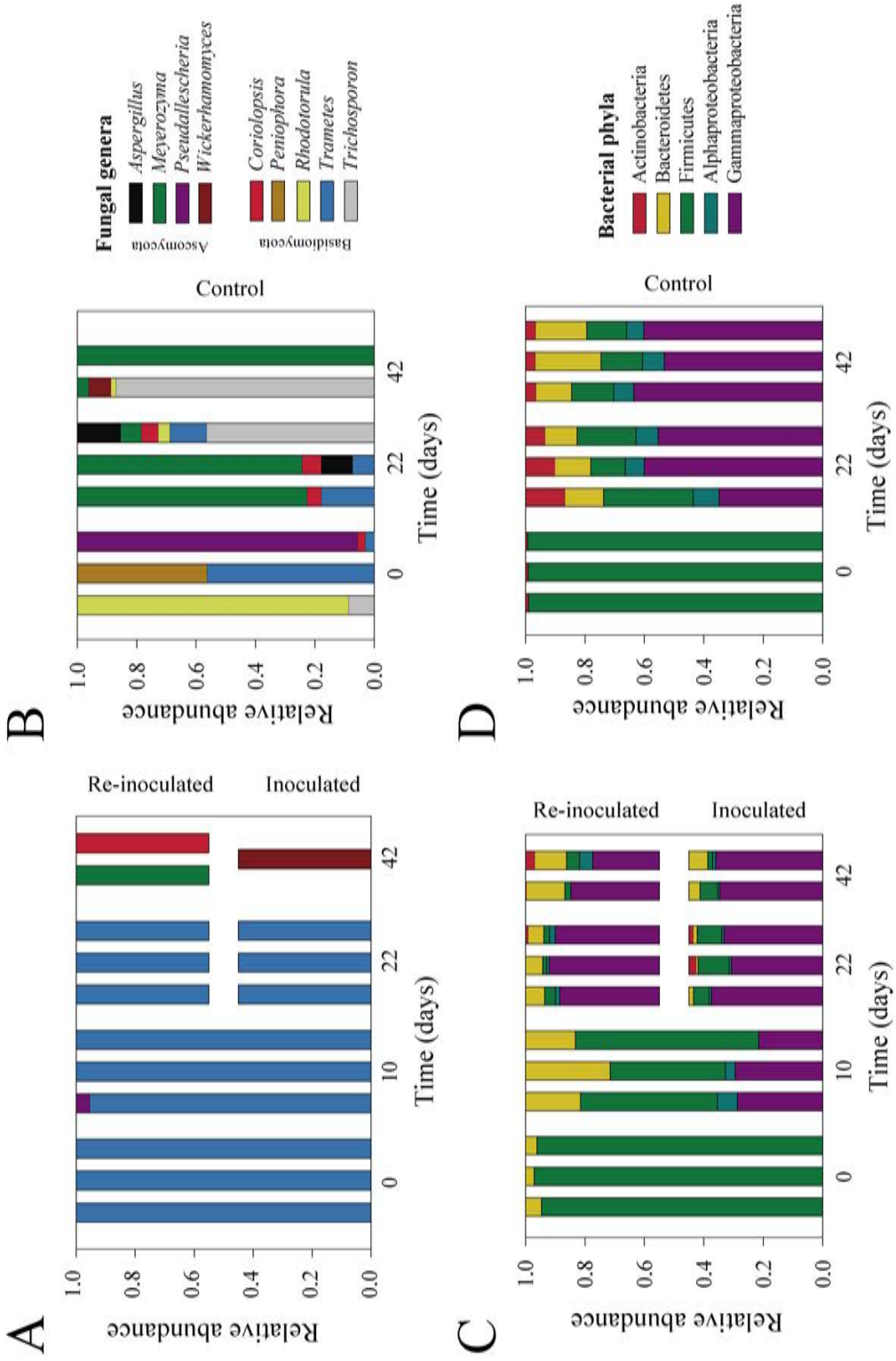

Figure 6.15: Relative abundance of fungal (A, B) and bacterial (C, D) sequences calculated from the band intensities in the DGGEs of experimental (A, C) and control (B, D) biopiles.

While it is true that the inoculation with high concentrations of *T. versicolor* would undoubtedly give a fitness advantage to microorganisms such as *Meyerozyma* and *Lysobacter* (justifying its enrichment in the biopiles), both genera were also abundant in the controls with no mycelium inoculation so they are also efficient in the colonization of new environments and ensuring their prevalence over time. The development of new strategies to favor *T. versicolor* or antagonize its competitors is much required if the viability of fungus is desired at long term. The most sustainable and economic-friendly alternative would be the search for specific substrates that only *T. versicolor* can use, avoiding at least the direct competition for nutrients in the biopiles.

Chapter 7

Impact of effluents on the ecosystem

7.1 Introduction

In the upcoming years, water reuse will be an essential measure to reduce pressure over the environment, establish reliable water sources and cut energy-derived costs from other supply alternatives like water transfer or desalination. The personal consumption of reclaimed water is still not regulated inside the EU, although some of its member states have issued their own guidelines, regulations or legislative frameworks [383–388]. Meanwhile, the reuse of water for agriculture and green area watering is currently in the spotlight. In both cases, soil matrix is critical to the ecosystem as it acts as a water filter and growth medium. It harbors billions of organisms and sustains the whole terrestrial trophic network. Traditionally, the main goal in the risk assessment of effluent reuse was to ensure that no pathogens or detectable pollutants were present in the watered matrix; however, now we know that ECs (including ARGs and mobile genetic elements) must also be controlled to mitigate effects in animal development or increase of resistant strains, against which antibiotics are rapidly losing the war [389, 390].

As stated in the introduction, we are still far from ensuring a 100% safe reuse of water and the first step towards this goal involves focusing in systems not directly tied to human and animal consumption such as maintenance of green areas, with less demanding regulation.

This chapter aims to elucidate the impact of different HWW effluents (i.e. the main source of ECs and ARGs in WWTPs) over the microbial communities in a soil ecosystem. To do so, evaluation through the construction of microcosms was proposed. To the author's knowledge, no works have yet focused on the use of HWW treated with a fungal bioreactor to reduce the EC load before regular treatment. This study aims to set the first stones in this field and to obtain knowledge that can be extrapolated to more complex systems.

7.2 Effluents

Effluents used in this work were collected in October 2016 from Sant Joan de Déu Hospital (Barcelona, Spain). Three types of effluent were defined depending on the treatment applied:

- Effluent 1 (EFF1): Raw wastewater directly collected from the sewer manifold. Equivalent to HWW3 used in the long-term fungal treatment from chapter 5.
- Effluent 2 (EFF2): Treated wastewater using activated sludge from a WWTP.
- Effluent 3 (EFF3): Treated wastewater using the fungal continuous bioreactor (section 6.2.1) and then activated sludge from a WWTP.

Effluent obtention and secondary treatment with activated sludge are detailed in sections 3.3.4 and 3.3.6, respectively. Pharmaceuticals present in each effluent were analyzed per triplicate following the same methodology [155] described in the previous chapter and classified in four groups (Analgesics and anti-inflammatories, antibiotics, psychiatric drugs and other PhACs) according to their functionality.

The PhAC load of each effluent and family is presented in figure 7.1. 43 out of the 81 PhACs analyzed were detected in at least one sample replicate. Overall, total pharmaceutical loads were of 41,648, 24,092, and 13,145 ng L^{-1} for Effluents 1, 2 and 3, respectively. For more detailed information, see table 10.7 in the annex. In terms of total abundance, analgesics and anti-inflammatories take the lead, followed by psychiatric drugs, other PhACs and antibiotics, respectively. Analgesics and antiinflammatories were significantly reduced after conventional treatment (EFF2) and flocculation-coagulation-*T.versicolor* strategy (EFF3). Illustrating the problematic with PhACs already mentioned throughout this work, neither antibiotics nor psychiatric drugs or other PhACs were reduced after conventional treatment. However, the complete treatment with activated sludge and the fungal bioreactor yielded good results, reducing the overall concentration of all families except for the other PhACs.

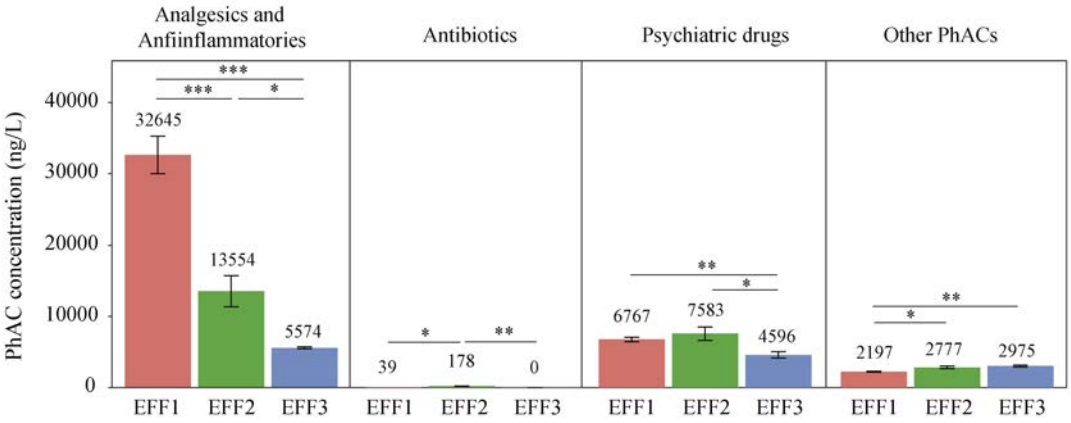

Figure 7.1: Pharmaceutical load of each family of compounds in the effluents used for the impact studies. Significant differences in PhACs concentration were indicated as follows: '***' p≤0.001, '**' p≤0.01, '*' p≤0.05, 'o' p≤0.1, '' p>0.1.

7.3 In vitro short-term impact assay

The first impact assay was designed to be a short-term study of 45 days to evaluate the possible impact of the effluents following two irrigation strategies: unique and multiple. Microcosms were prepared in form of slurry and shifts in the microbial communities were assessed via DGGE (bacteria and fungi) and 16S metagenomics (bacteria).

Microcosms were prepared in 250 mL Erlenmeyer flasks, following the set up proposed by Fahrenfeld *et al.* [391]. A graphical representation of the experimental set-up is provided in figure 7.2 and detailed procedure is available in section 3.3.7. Briefly, 12 experimental flasks (3 effluents × 2 irrigation strategies × 2 replicates) prepared with 50 g of sieved soil (2 mm) and 100 mL of each effluent were kept in agitation and monitored for 45 days, sampling 2 mL of slurry on a weekly basis. A control was also included in duplicate and irrigated with sterile distilled water.

7.3.1 Community analysis

Evaluation of community shifts was carried out with the samples from the initial (t0) and final (t45) points because few variations were expected to happen at a weekly scale. Bands from bacterial

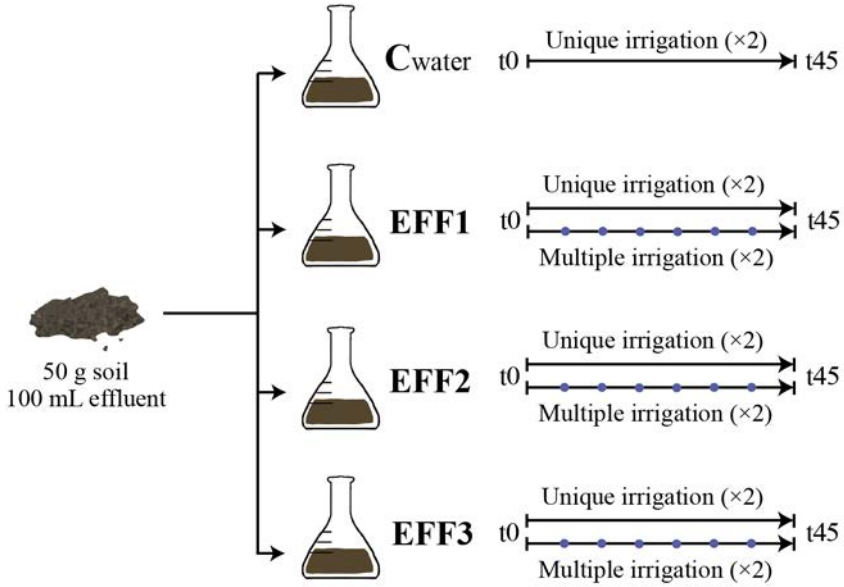

Figure 7.2: Schematic representation of the short-term experiment set up. Arrows represent the duration of the experiment, time points (t0 and t45) indicate when sampling was done and blue dots indicate the weekly irrigation.

and fungal DGGEs were not recovered in this short-term study because it only aimed to assess the community shifts caused by the presence of pharmaceuticals in the effluents. Nevertheless, a descriptive metagenomic analysis of the 16S rRNA gene (V4 region) was performed to assess taxonomic-related changes not elucidated by DGGE.

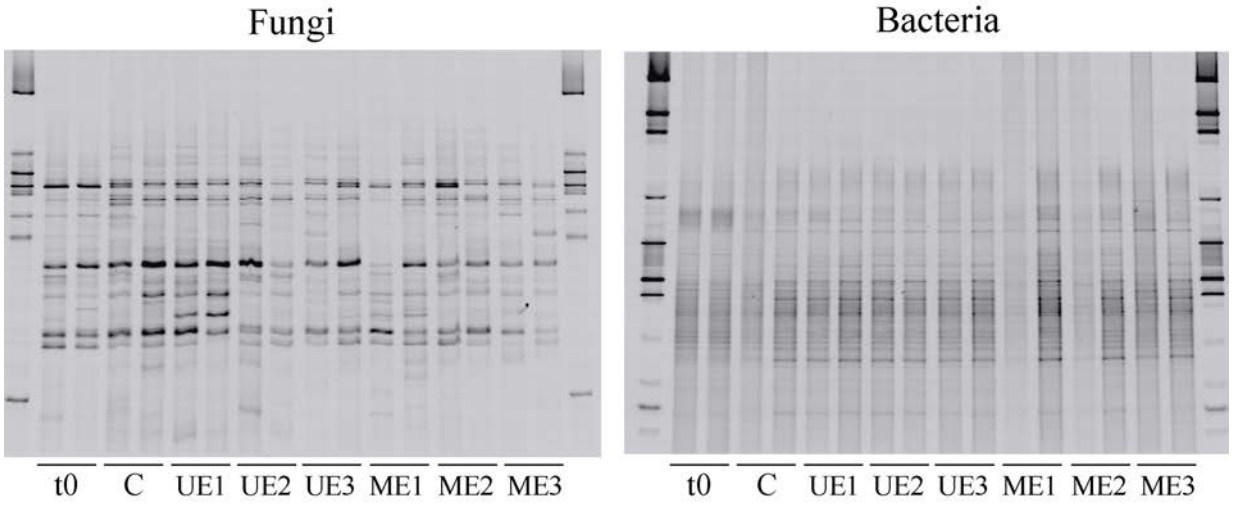

Figure 7.3: DGGE profiles of fungal (left) and bacterial (right) communities in the short-term impact assay. t0, initial samples at day 0; C, Control; U, Unique irrigation; M, Multiple irrigation; E1, EFF1; E2, EFF2; E3, EFF3. Except for t0, the rest of the samples belong to day 45.

Fungi

The nested approach using the primer sets EF4-ITS4 and ITS1f-ITS2 was chosen for fungal DGGEs. Samples considered for analysis were two replicates from t0 and all duplicates from t45. Supposedly, all t0 samples were expected to yield the same results so only the control was chosen for this time point. The resulting profile is presented in figure 7.3 (left).

Bands were not recovered in this case but a total of 30 phylotypes were identified in the band-matching. Richness and diversity (Shannon) estimates were calculated for each sample and the results ranged 10 to 16 and 1.31 to 2.29, respectively. Moreover, the effect of time (t0 and t45), effluent type (C, EFF1, EFF2 and EFF3) and irrigation strategy (C, unique and multiple) over richness and diversity was assessed using a t-test or a one-way analysis of variance (ANOVA). Surprisingly, the only significant result found (p=0.019) was an increase in the fungal richness from t0 to the end of the experiment.

An NMDS plot based on Bray-Curtis dissimilarity was then constructed using the relative abundance band matrix to assess changes at the community composition level (Fig. 7.4A,B and C. Overlapping between samples grouped by time, effluent and irrigation was observed in all cases and no significant differences were observed (ANOSIM R=0.09, p=0.375; R=0.11, p=0.147; R=0.01, p=0.427, respectively). However, while time (Fig. 7.4A) and irrigation (Fig. 7.4C) categories showed almost complete overlap, the grouping by effluent revealed certain differences between effluents (Fig. 7.4B). Community dissimilarity differences were then explored with "time", "effluent" and "irrigation" as sources of variation with a permutational multivariate analysis of variance using distance matrices (ADONIS, section 3.7). Results showed that low and moderate percentages of variation were explained by time (ADONIS R^2=0.11, p=0.016) and effluent type (ADONIS R^2=0.26, p=0.033), respectively.

A reflection should be made regarding how can the PhACs in each HWW affect fungal communities. In figure 7.4B, the three effluent clusters show a tendency to be arranged from left to right by Axis01 in order of decreasing total PhAC concentration. It was expected that control samples would cluster close to EFF3 because they did not contain pharmaceutical compounds. Instead, controls are close to EFF1 and distinguished from the other clusters by its position in the Axis02. This causality might be explained by pharmaceuticals that do not follow the general tendency and are found more abundant in EFF2 and EFF3 than in EFF1 (similar to the controls). A detailed search of individual PhAC concentrations revealed that three compounds (Levamisol, Hydrochlorothiazide and Propanolol) from the "Other PhACs" family matched with the desired profile (Table 7.1).

Table 7.1: Concentrations of three selected PhACs with concentration in EFF1 lower than in EFF2 and EFF3. bld: Concentration below limit of detection.

Compound (family)	Concentration (ng L^{-1})		
	EFF1	EFF2	EFF3
Levamisole (anthelmintic)	bld	291±18	933±52
Hydrochlorothiazide (diuretic)	511±67	607±174	911±72
Propranolol ($\beta - blocker$)	bld	18±3	238±25

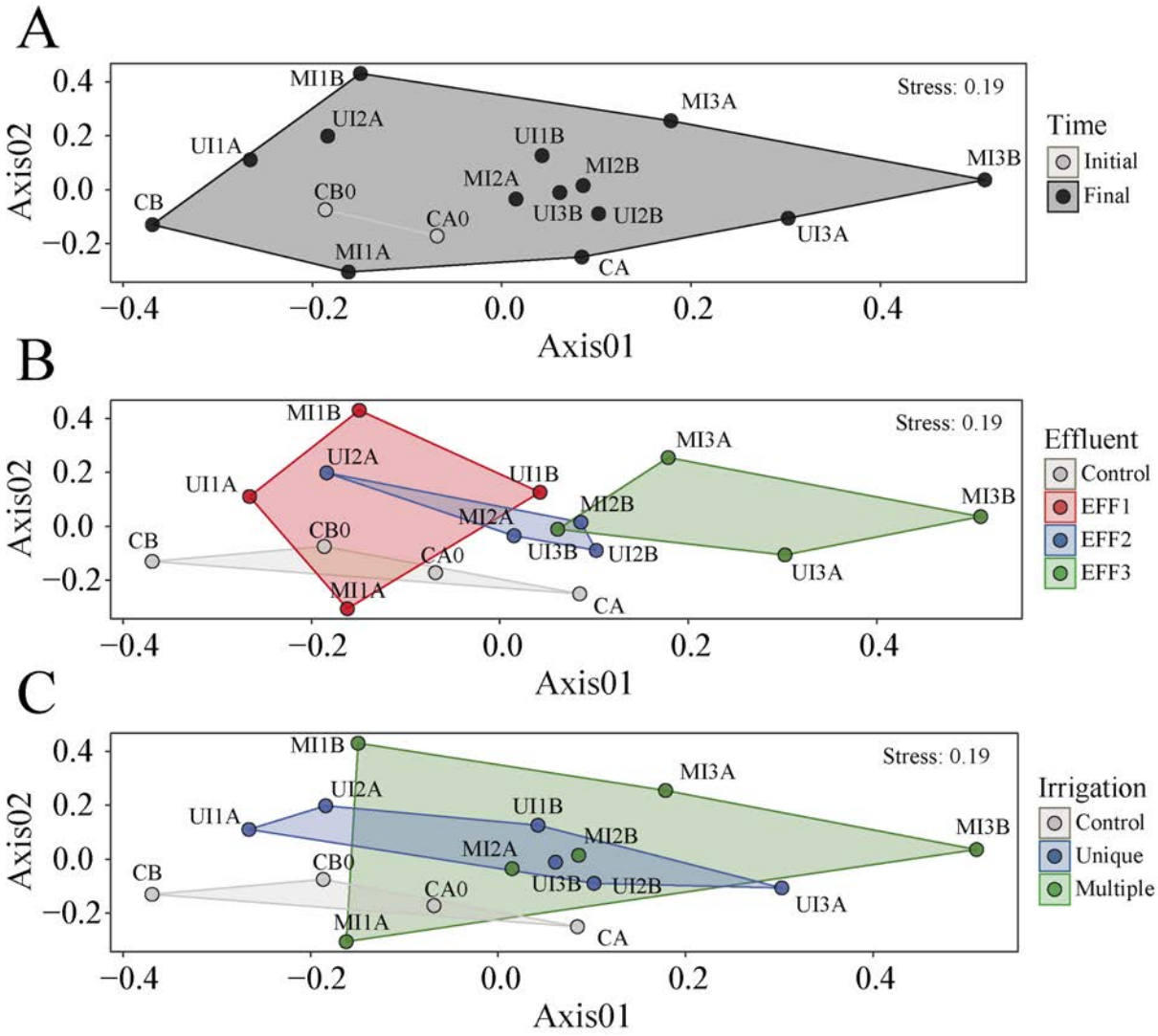

Figure 7.4: NMDS plots of fungal communities grouped by time (A), effluent (B) and irrigation strategy (C). Samples are identified by unique codes (C, control; UI, unique irrigation; MI, multiple irrigation; 1, effluent 1; 2, effluent 2; 3, effluent 3; 0, initial sample; A-B, replicates)

Perhaps the compound of more interest was levamisol, an antagonist of L-subtype nicotinic acetylcholine receptors from nematodes and helminths [392–394]. Although there are exceptions [395, 396], nematodes are generally predators of fungi and bacteria. As a speculation, the presence of levamisol in EFF2 and EFF3 could have altered the trophic network in the microcosm, reflected in the DGGE patterns. Alternately, levamisole is also known to have direct [397] and indirect [398] antifungal effects that could justify changes in the community structure .

Regarding the other compounds, only hydrochlorothiazide was found to have phototoxic effects against a model fungus [399]. Propranolol did not seem to have effects over fungi but instead was sensible itself to oxidation by white-rot fungi such as *T. versicolor* [400]. Perhaps, the capacity of some fungi to biotransform compounds like propranolol to active metabolites [401] could explain the increment in concentration of compounds not detected initially.

Bacteria

For the assessment of Bacteria, the same analytic procedure was followed as previously with the primer set 341f-907r. DGGE profile is depicted in figure 7.3 (right) and a total of 31 bands were identified in the fingerprints. However, richness and Shannon estimators were higher in bacteria, with values ranging 19–24 and 2.38–3.05, respectively. To put data into perspective, time, effluent and irrigation categories were considered again and significant differences were found in diversity over time (p=0.001) and richness depending on the effluent (p=0.0005). Shannon diversity increased as time passed from 2.59±0.03 to an overall average in t45 of 2.83±0.19. In the case of richness, the irrigation with raw HWW (EFF1) seemed to increase the phylotypes detected (23.5±1 versus the rest 20.9±0.7).

In this case, time, effluent and irrigation were used again to group the samples of the NMDS plot (Fig. 7.5A, B and C). Contrarily to the fungal communities, overlapping was only evident in bacteria when samples were grouped by effluent (Fig. 7.5B). Significant differences of bacterial communities were found considering time (ANOSIM R=0.77, p=0.004), effluent (ANOSIM R=0.21, p=0.028) and irrigation(ANOSIM R=0.38, p=0.001). When partitioning dissimilarities for the sources of variation, irrigation strategy was the stronger predictor of function (ADONIS R^2=0.35, p=0.001), followed by effluent type (ADONIS R^2=0.33, p=0.02) and time (ADONIS R^2=0.28, p=0.009). As expected, control samples from t0 were the ones responsible for the big disparities and greatly influenced the tests. Thus, both tests were re-run for effluent and irrigation categories without day 0 control samples, leaving irrigation as the only significant factor driving community differences (ANOSIM R=0.23, p=0.044 and ADONIS R^2=0.27 p=0.034). This findings made clear that bacteria were driven by disturbances in the environment (i.e. periodical renovation of effluents) while fungi were sensible to the composition of each effluent.

Setting DGGE aside, a metagenomic approach was conducted to go deeper in terms of diversity (from fewer samples) and to carry out a wide taxonomic characterization. Briefly, each sample included in the DGGE was also considered for Illumina sequencing of the 16S rRNA gene (V4 region). Duplicates of each sample were pooled in equimolar concentrations and sequenced at Servicios Genómicos (Paterna, Valencia). Raw reads were deposited into NCBI SRA platform under the accession numbers SRR6436576 (samples Ct0, UI1, UI2 and UI3) and SRR6436577 (samples Ct45, MI1, MI2 and MI3). Analysis of the sequences was carried out using R and dada2 as a denoising algorithm [182].

Richness and Shannon diversity were calculated for each sample (table 7.2) after normalization of the reads [188]. It was expected that the overall values were substantially higher than in DGGE due to the differences in resolution of the techniques. Aside from that, results denoted major differences in richness and Shannon estimates between t0 and all the rest of samples from t45. Furthermore, DGGE findings pointed out an increase of richness in samples irrigated with effluent 1 that is not mirrored in the Illumina results.

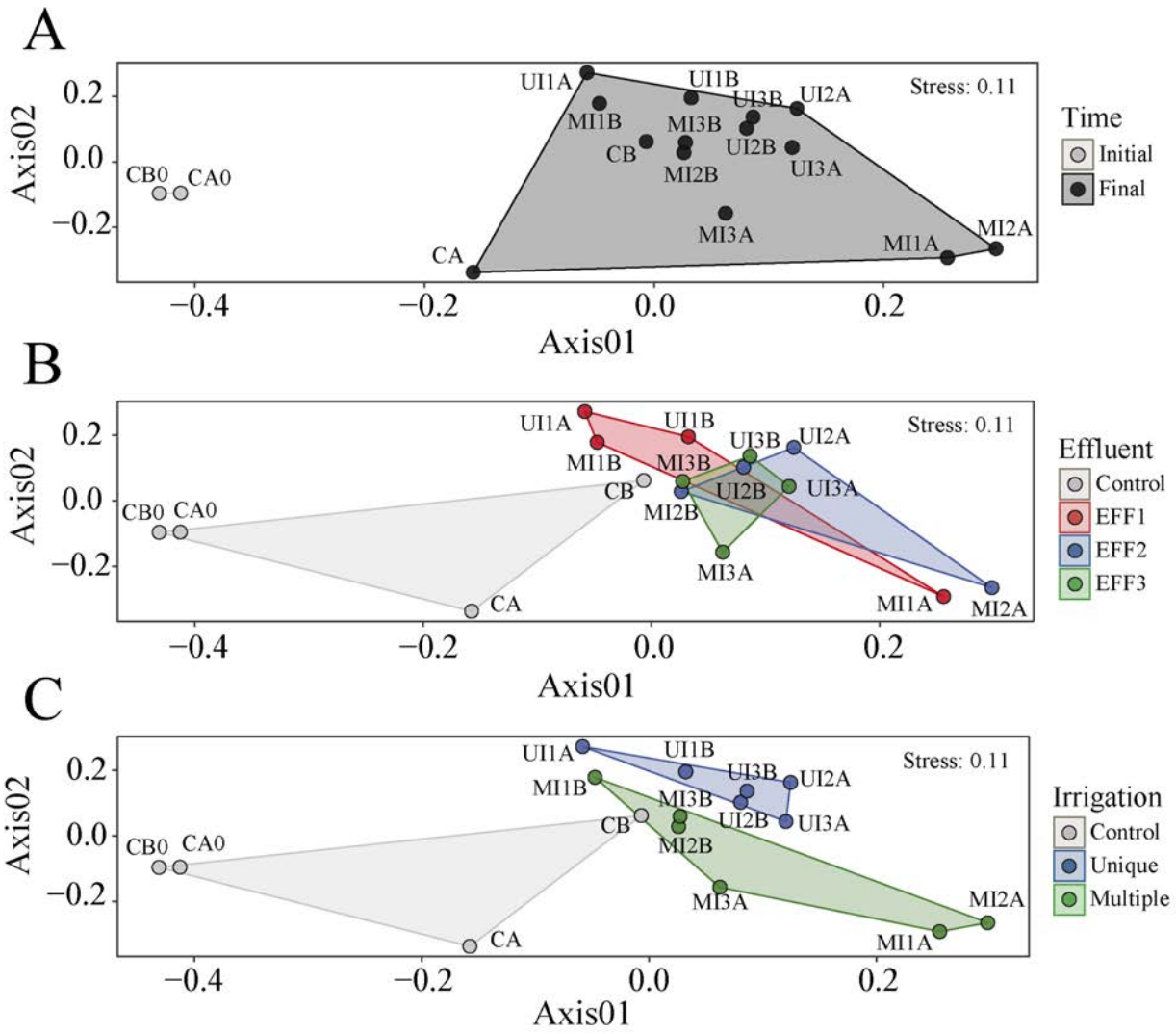

Figure 7.5: NMDS plots of bacterial communities grouped by time (A), effluent (B) and irrigation strategy (C). Samples are identified by unique codes (C, control; UI, unique irrigation; MI, multiple irrigation; 1, effluent 1; 2, effluent 2; 3, effluent 3; 0, initial sample; A-B, replicates)

To dig deeper into the differences caused by temporality, relative abundances of the most represented phyla were plotted in figure 7.6. Relative abundance representation of each phyla did not appear to be influenced by the type of effluent neither the irrigation system. Only in the case of Actinobacteria, relative abundance was higher in samples irrigated only once.

Interestingly, changes in richness and diversity were linkable to the behavior of the control (C) sample. Major differences in relative abundances were exclusively found comparing Ct0 and the rest of the samples. In fact, the control after 45 days (Ct45) was more similar to the irrigated samples than to itself at t0. Phyla Proteobacteria, Planctomycetes, Bacteroidetes and Verrucomicrobia had substantially lower relative abundances at the beginning of the experiment and were favored in all end-point samples. On the contrary, Actinobacteria and Firmicutes drastically lost representation (by 50% in some samples) by day 45. These two phyla are composed of many bacteria that thrive in soils and Actinobacteria were, by far, the most abundant phylum in the original soil. They are really important contributors to the soil systems and behave similarly to fungi by decomposing organic

Table 7.2: Richness and Shannon estimates of the Illumina samples from the short-term impact assay.

Sample	Richness	Shannon
C t0	1,274	7.049
C t45	2,932	7.767
MI1 t45	2,911	7.771
MI2 t45	3,109	7.832
MI3 t45	3,007	7.803
UI1 t45	2,974	7.781
UI2 t45	3,014	7.801
UI3 t45	3,016	7.805

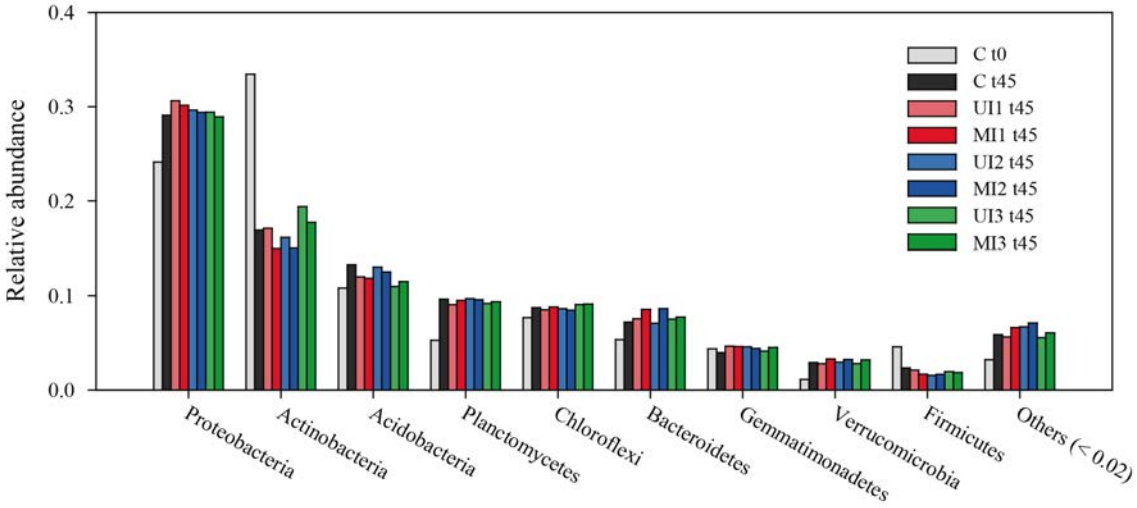

Figure 7.6: Relative abundance of the main microbial phyla in the short-term impact experiment. Phyla beneath 0.02 relative abundance were grouped in the "Others" category.

matter so plants can take up the resulting molecules. More in detail, orders Acidimicrobiales (Actinobacteria) and Bacillales (Firmicutes) were the most abundant of their respective phyla and also lost 54 and 60% of its representation, respectively.

Overall, the evidence from this study suggests that fungi were susceptible to the composition of the effluents while bacteria are more susceptible to the irrigation strategy employed. Putting facts into context, one of the explanations behind the community shifts was likely related to chemo-physical changes induced by the experimental design. Aside from PhACs contained in each effluent, the simple addition of water (either in control or effluents) changed the matrix from solid to liquid, consequently altering the water availability in the environment. This effect was taken into account in the design of the next assay, in which soil was kept as a solid matrix to better mimic the real environment. Furthermore, a dose increase during a longer period of time was also planned.

7.4 In vitro mid-term impact assay

Considering the premises set in the previous section, a second impact assay was set to last for 90 days and it was named mid-term impact assay. In this case, soil was used directly to prepare the microcosms and a higher dose of PhACs was evaluated by applying more effluent weekly. The main focus of this study was to evaluate how irrigation with reclaimed water affected bacterial

communities in terms of structure and acquisition of ARGs. Preparation of the microcosms is thoroughly described in section 3.3.7 and a brief summary is provided next, along with a graphical representation (Fig. 7.7). Sieved soil was distributed into glasses creating a 4 cm-deep layer over the non-sieved fraction, that was used as drain. Microcosms were set in triplicates and only the multiple irrigation strategy was followed. Effluent types were still the same as in the previous experiment, namely: control, EFF1, EFF2 and EFF3. A total of 12 microcosms resulted from the combination of variables (1 irrigation strategy × 4 effluent types × 3 replicates). Each experimental and control microcosm was watered weekly with 20 mL of sterile water or effluent during a period of 90 days. Samples were retrieved using sterile corers and a spatula.

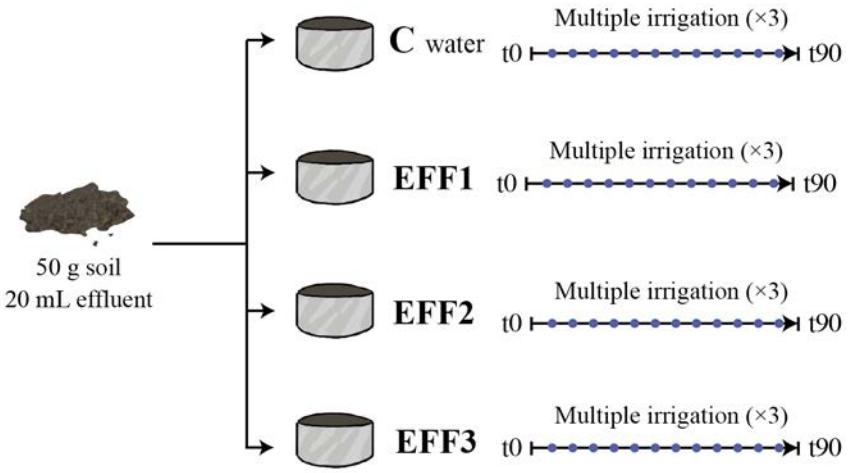

Figure 7.7: Schematic representation of the mid-term experiment set up. Arrows represent the duration of the experiment, time points (t0 and t90) indicate when sampling was done and blue dots indicate the weekly irrigation.

7.4.1 Community analysis

DNA and RNA extractions (section 3.5.1) were performed in all samples. Final cDNA yield after retrotranscription was low and only a few samples could be used in 16S rRNA metagenomics. In this case, DNA and cDNA samples were included in a 96-well plate and sent for Illumina sequencing to Michigan State University's Research Technology Support Facility (United States). Sequences belonging to 16S rRNA and 16S rRNA gene were processed in parallel with DADA2. Contaminant sequences were removed based on the ASVs contaminating the Mock community and reads were normalized to account for differing sequencing depths.

In the first place, general effects of the different treatments over microbial communities were evaluated by comparing the Shannon diversity indexes of all sample groups (Fig. 7.8A). As expected in the control microcosms, there were no changes in diversity ($p=0.26$) after the 90 days of periodic irrigation. However, all effluents caused a significant decrease in soil microbial diversity compared to the initial values ($p<0.0001$). The decrease was general for all effluents and slightly lower for EFF3, so the driving factor was shared between groups. Two explanations could fit this outcome: First, the variety of pharmaceutical compounds still present in the effluents (mainly in EFF1 and EFF2) could alter the soil-native populations. Second, irrigation with treated effluents increases

the organic matter in soil [402–404], enriching heterotrophic species and disrupting the balance of species. This option is supported by chemoheterotrophy predictions obtained using faprotax (Fig. 7.8B).

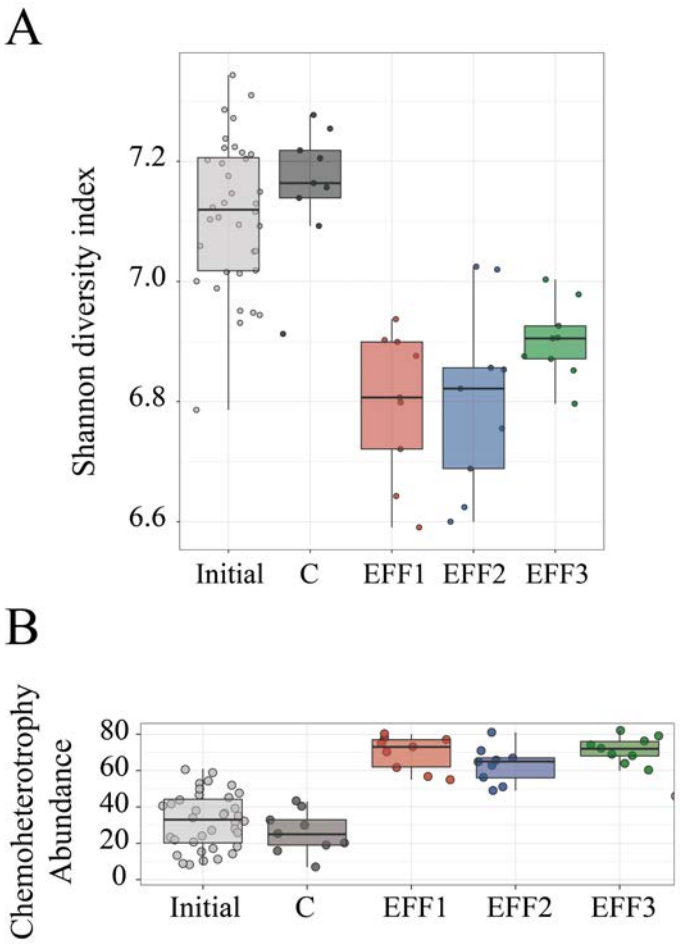

Figure 7.8: A, Shannon diversity of all samples grouped by effluent treatment. B, Normalized abundance of ASV related to chemoheterotrophy predicted via FAPROTAX.

To picture a clearer image of the structural changes occurring after the irrigation, an NMDS plot based on Bray-Curtis dissimilarities was prepared in figure 7.9. Samples belonging to the three effluents were the most distant ones from the initial point and grouped close together with some overlap. Time was found to strongly influence sample variations (ADONIS, R^2=0.28, p=0.001) and the control samples at the endpoint were clearly differentiated from the initial by both axis1 and axis2. This transition was assumed to be the natural succession of communities along time so further comparisons were conducted between control at the endpoint (just referred as Control) and effluents at the endpoint (EFF1, EFF2 and EFF3). Moreover, another curious fact was the arrangement of the effluents where EFF1 and EFF2 were closer to the control samples compared to EFF3. This fact indicated that low concentration of PhACs and COD not necessarily meant a closer resemblance to the water-irrigated soil.

Bacterial communities were then grouped at the order level and checked for significant differences between control and effluent-irrigated microcosms. Only variations >0.9% with a p<0.05 were considered (Fig. 7.10). According to the results, Acidobacteria subgroup 6, Myxococcales,

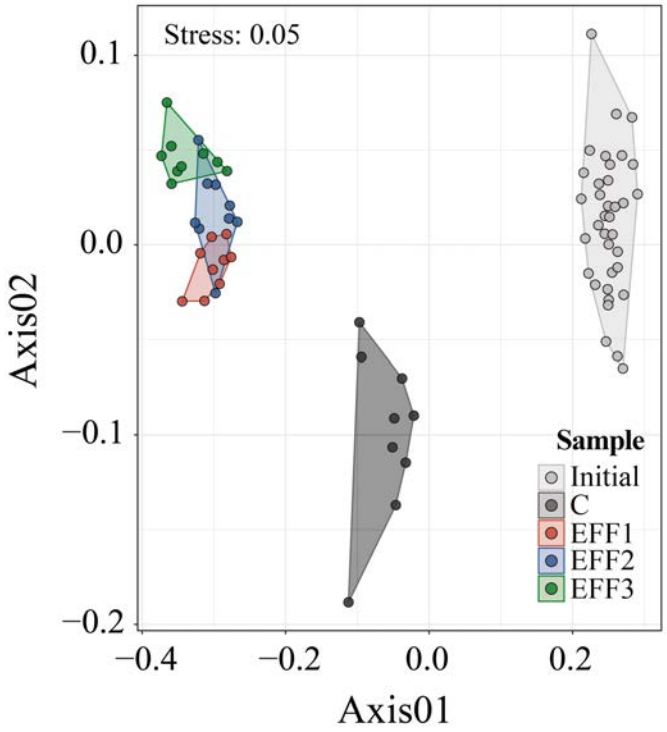

Figure 7.9: NMDS based on Bray-Curtis dissimilarities of community composition grouped by effluent treatment.

Gemmatimonadales and Sphingobacteriales were the bacterial orders that lost more representation when soil was irrigated with treated wastetwater (TWW). They are mostly comprised of bacteria that thrive in soils [405–410] so it was not strange to observe this important reduction after alteration with effluents. Acidobacteria subgroup 6 was the most affected by effluents in terms of proportion. A similar study of long-term soil irrigation [411] already described shifts in the Acidobacteria groups. In our case the Actinobacteria subgroups 3, 5, 7, 10 and 25 were all found to be underrepresented in the effluent-irrigated samples.

Reciprocally, orders Micrococcales, Cytophagales, Rhodospirillales and Sphingomonadales were enriched in the effluent-treated microcosms. Some of them like Cytophagales are not still well characterized [412] but are likely involved in the degradation of cellulose by extracellular enzymes. The most favored order Sphingomonadales (mainly represented by *Sphingomonas*) is known for the capacity of many of its strains to degrade aromatic compounds and are of interest in bioremediation [413] and even phytoremediation [414]. This could be an indicator of selective enrichment of bacterial strains due to the presence of PhACs with aromatic structures in all effluents (e.g. Ibuprofen, ketoprofen, carbamazepine and valsartan). Accordingly, an increase of Alphaproteobacteria (such as *Sphingomonas*) in irrigated soil was also reported by Wafula D. *et al.*. [411]. However, there was discrepancy with the claims of the study regarding a decrease of Actinobacteria, as they were enriched instead (in particular order Micrococcales).

At this point, only changes between control and effluents have been appraised due to their similarity in the NMDS plot. However, it is of particular interest to know the specific consequences of irrigation with each effluent as raw hospital wastewater (EFF1) should imply at least some differ-

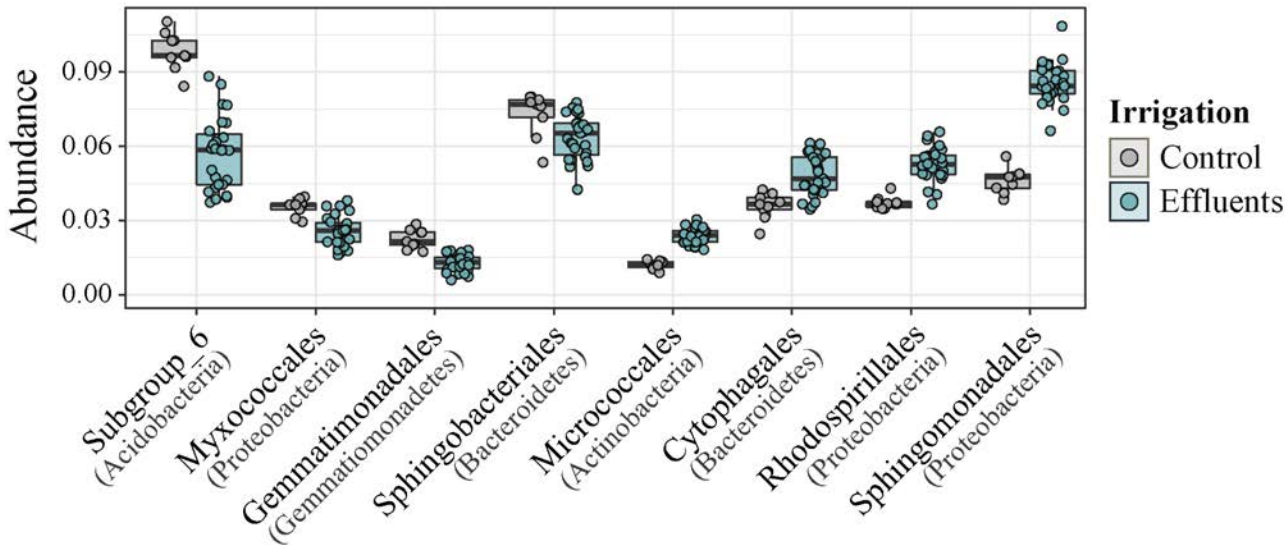

Figure 7.10: Significant differences ($p<0.05$, >0.9% variation) in bacterial relative abundances at the order level between control and effluent-irrigated soils. Phylum affiliation of each order is provided in brackets.

ences. To address this, relevant changes (>0.9%, $p<0.05$) between microcosms treated with each effluent were assessed at the order level and presented in figure 7.11. At first glance, Acidobacteria subgroup 6, Cytophagales and Sphingobacteriales decrease in those microcosms irrigated with TWW (EFF2 and EFF3). Most of these taxa were more abundant in the control samples (Fig. 7.10) and give meaning to the previously observed trend in which EFF1 was proximate to the control samples (Fig. 7.9). Furthermore, it is likely that HWW1 was the main contributor to the high abundance of Cytophagales in effluents reported in figure 7.10. Finally, EFF3 had higher abundance of Chthoniobacterales (Verrucomicrobia) compared to the rest of effluents and an increase in the Actinobacteria order Solirubrobacterales was also seen in the soil irrigated with TWW.

To know if the results reflect the real communities in the soil and are not artifacts caused by the incoming relic DNA found in the effluents (stored in frozen conditions), cDNA Illumina results were used to check if differences in abundance of genera were shared between DNA and rRNA. Briefly, all genera that changed significantly its abundances between control and effluent samples were searched in the RNA results and checked for the same qualitative change (increase or decrease). More than 90% of the genera behaved with the same pattern in RNA compared to DNA (data not shown). It should be admitted that differences at the genus level were not representative of the real community because most ASV were identified at the order level (84.6%), but only 32.4% were assigned a genus. However, this evaluation was not reliable at higher taxonomic ranks such as Order or Phylum. Thus, it served only as a validation method to ensure that environmental DNA from the effluents was not driving the microbial shifts described.

While studying the significant differences in taxon abundance entails valuable information of the processes running microbial succession in the soil microcosms, changes in functional capabilities might not be appreciated if not assessed specifically. For this reason, all samples were run through FAPROTAX to obtain a broad view of the predicted functional differences.

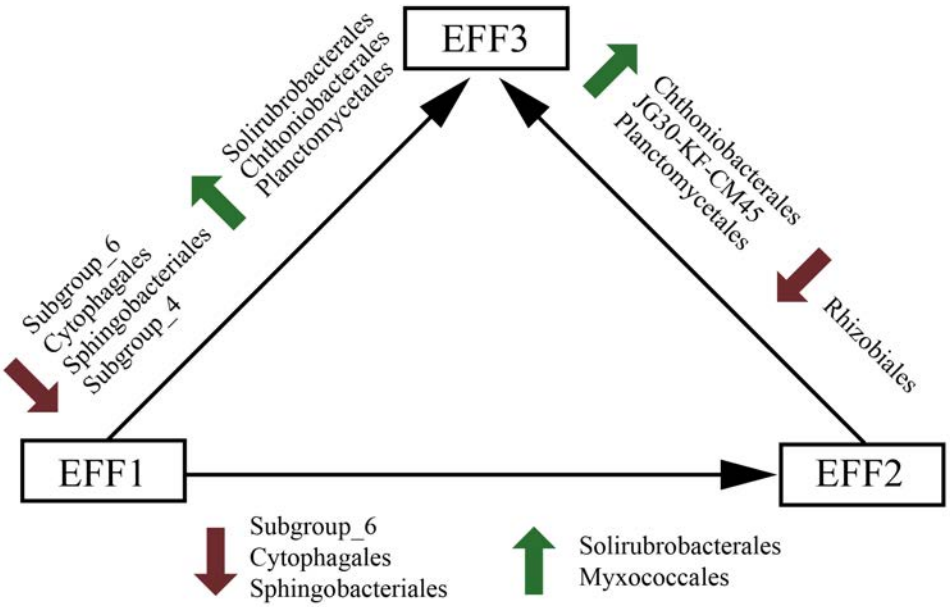

Figure 7.11: Significant differences ($p<0.05$, >0.9% variation) in bacterial abundances at the order level between effluents. Comparison between effluents are indicated following the sense of black arrows. Red and green arrows indicate the bacterial taxa that decrease and increase, respectively.

On one hand, the changes in the nitrogen cycle were investigated through broad categories as nitrogen fixation, nitrification and denitrification. Taxa capable of fixing nitrogen (Fig. 7.12A) were consistent in all microcosms at the endpoint. The only difference observed was an increase compared to the initial composition at day 0 ($U=81$, $p=0.02$), indicating that effluents had no impact over this function and only natural succession was responsible of differences. This was not the case of nitrification process, in which communities in soils irrigated with EFF1 and EFF2 had a poor presence of nitrificating bacteria (Fig. 7.12B) in comparison to the control (C) ($U=81$, $p<0.0001$ and $U=69$, $p=0.01$, respectively). Moreover, EFF1 microcosms experienced a decrease in denitrification (Fig. 7.12C) compared to C ($U=68$, $p=0.014$). The low abundance of nitrification meant that nitrite was scarce in the soil and less denitrification took place. Overall, it can thus be suggested that EFF1 contributes with organic matter that disrupts the N cycle [415], tightly coupled with the C cycle [416], and favors heterotrophs (as seen in figure 7.8) that surpass the microbes in charge of nitrification.

On the other hand, the presence of pathogens and bacteria from human gut was of interest due to the origin of the effluents used. Abundance of human gut microbiota of EFF1 samples (Fig. 7.13A) was higher compared to any of the controls (C, $U=4$, $p=0.001$; t0, $U=12$, $p<0.0001$). Precisely, no significant changes occurred between any other control and experimental group. This outcome was clearly expected because of EFF1 being raw HWW that did not receive treatment after direct contact with feces. Following a completely different trend, there was a natural attenuation of human pathogens (Fig. 7.13B) that took place during the 90 days of the experiment. Control, EFF1 and EFF2 experienced a decrease in abundance of human pathogens with reference to t0 ($U=318$ $p<0.0001$; $U=266$ $p=0.002$ and $U=257$ $p=0.006$, respectively) and controversially, the soil irrigated with the most treated effluent (EFF3) was the only one to cause a considerable increase in pathogens compared to C ($U=0$, $p<0.0001$) and even initial the samples ($U=44$, $p=0.0004$). The enrichment of pathogenic species such as the opportunistic *Pandoraea* was already reported

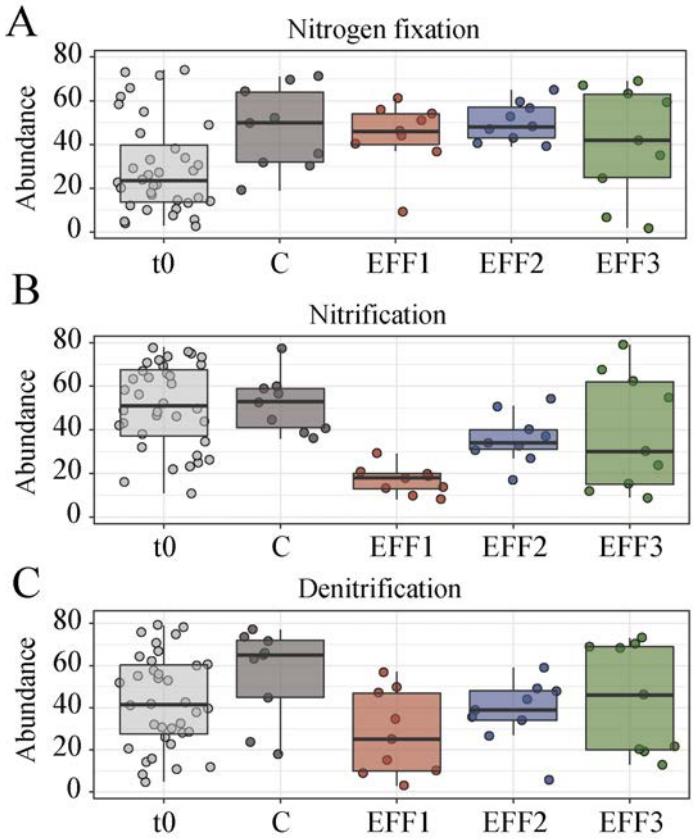

Figure 7.12: Normalized abundance of ASV related to nitrogen fixation (A), nitrification (B) and denitrification (C) predicted via FAPROTAX.

previously in section 6.2.2. EFF3 taxa that matched with "Human pathogens" category were retrieved from the FAPROTAX report and included 36 different ASV, 18 species and 12 genera from Firmicutes and Proteobacteria phyla. For detailed information see table 10.8 in the annex.

Concluding with this section, it has been demonstrated that irrigation with different effluents alters the natural succession of bacteria in soil in terms of community structure. Inter-effluent differences in PhACs had a much lower impact on microbial communities compared to the presence-absence of organic matter, reflected by COD. Compellingly, functional prediction provided insights into trends difficult to predict by manual revision of the data and pointed out sensitive issues related to pathogen enrichment. Furthermore, as introduced for *Pandoraea* in section 6.2.2, the acquisition of antibiotic resistances by clinical pathogens is a great problematic. Fortunately, it served to bring awareness of the importance of preventing ARG acquisition at global scale. Following the need to acquire more knowledge on ARGs increase and dispersion, this chapter also studied the influence of each microcosm irrigation in the total and relative abundance of ARGs.

7.4.2 Antibiotic resistances

Constant mentions to the increase of ARGs due to the release of effluents into the environment have been made throughout the chapters of this dissertation. However, ARGs and antibiotics are essential for the survival of many bacteria [417] and soil is by itself an extremely abundant environmental reservoir of ARGs [82, 417]. In this section, a selection of antibiotic resistance genes

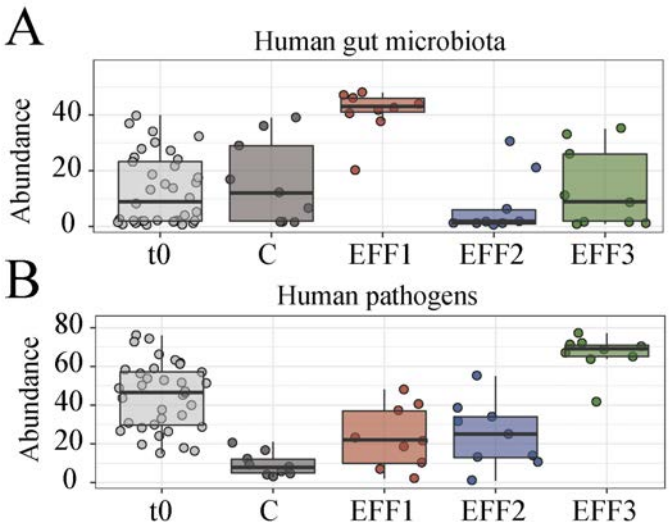

Figure 7.13: Normalized abundance of ASV related to human gut microbiota (A) and human pathogens (B) predicted via FAPROTAX.

and a mobile element were studied using real-time qPCR to assess whether the reuse of different effluents had an impact on their environmental presence.

Criteria for the selection of genes considered the antibiotics previously detected in the same hospital effluent [151] and suggestions from literature review [418] regarding environmental genes of interest. Six ARGs were studied in this assay belonging to sulfonamide resistance genes sul1 and sul2, erythromycin resistance genes ermB and ermF, macrolides resistance gene mphA and trimethoprim resistance gene dfrA. Furthermore, the abundance of a class I integrase (intl1) was also investigated.

Primers and qPCR programs used for the amplification of ARGs is detailed in section 3.5.3 of the Materials and methods. Aside from the absolute quantification of ARGs, bacterial abundance was also estimated (primers 341f-518r) to calculate abundances of ARGs relative to the 16S rRNA gene copy numbers.

To begin with, the initial concentration of all ARGs and integrase gene was calculated using all samples from day 0, before any treatment was applied (Table 7.3). Sulfonamides resistance gene sul1 was the most abundant resistance with $3.58{\cdot}10^{6}$ copies per gram of soil. Most of the other genes have abundances between 10^{3} and 10^{5} except for the trimethoprim resistance-coding dfrA gene ($5.9{\cdot}10^{1}$). Interestingly, both genes coding for erythromycin resistance (ermB and ermF) exhibited the same average abundance. Despite this study focuses in the impact of effluents over the ecosystem, baseline concentrations of antibiotic resistance genes are always expected in soil due to the high density of antibiotic-producing bacteria that favor their selection [419].

Relative and absolute copies of ARGs were merged in a composite plot 7.14 to allow for better intra and inter-gene comparison. Previous to the assessment of the impact, it should be mentioned that no significance was found in any of the means after abundance was normalized by 16S copies. The scale in which changes in concentration occur for ARGs was too small in comparison to the great variation inherent to 16S rRNA. Resulting abundances can still be compared descriptively, but due

Table 7.3: ARGs in the mid-term experimental microcosms previous to irrigation (t0). Abundance is expressed in copies per gram of soil ± standard deviation.

Gene	Abundance (copies g soil^{-1})
sul1	$3.58{\cdot}10^{6}$ ± 0.53
sul2	$9.40{\cdot}10^{4}$ ± 2.35
ermB	$1.40{\cdot}10^{3}$ ± 0.31
ermF	$1.40{\cdot}10^{3}$ ± 0.36
mphA	$1.56{\cdot}10^{5}$ ± 0.57
dfrA	$5.90{\cdot}10^{1}$ ± 4.43
intl1	$1.30{\cdot}10^{4}$ ± 0.21

to the great effect of 16S copies over ARG copies, standard deviation was high in all analysis. On the other hand, changes in the absolute number of ARG copies were evaluated with a paired t-test to account for peculiarities of each individual microcosm. Due to the restrictive results provided by the paired test, significance $p \leq 0.1$ was considered in the figures and discussion, as indicative of a fairly-significant result.

First of all, sulfonamide resistance gene sul1 (Fig. 7.14A) decreased in terms of absolute abundance in all samples. Still, some differences in significance could be appreciated due to and heterogeneous decrease between replicates (e.g E2). The same tendency is seen in the relative abundances of all experimental samples. It is likely that sul1 was not abundant or present in the original effluent (EFF1). Similarly, sul2 (Fig. 7.14B) also decreased in all samples and results were significant in all conditions except for EFF2, in which one of the replicates increased its concentration. In this case, all relative abundances appeared similar and only EFF3 seemed to stimulate a decrease in relative abundance, still non-significant.

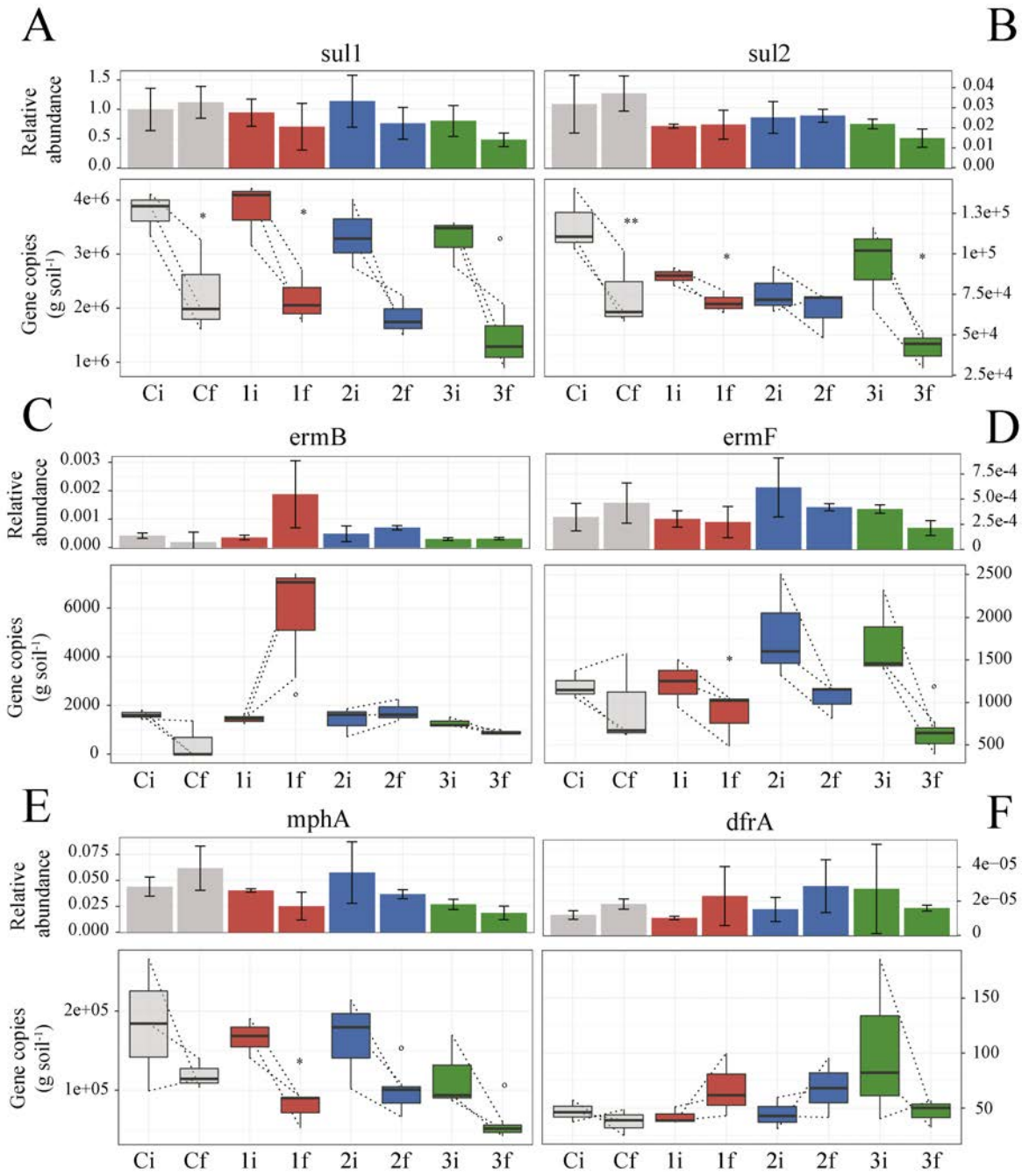

Figure 7.14: Absolute (below) and relative (above) abundances of ARGs conferring resistance to sulfonamide (A, sul1; B, sul2), erythromycin (C, ermB; D, ermF), macrolides (E, mphA) and trimethoprim (F, dfrA). C, control; 1, EFF1; 2, EFF2; 3, EFF3. i, initial sample from day 0; f, final sample from day 90. Relative abundances were calculated with 16S rRNA gene copy numbers. Initial and final values of each sample are linked by a dashed line. Significant differences are indicated as follows: '***' $p \leq 0.001$, '**' $p \leq 0.01$, '*' $p \leq 0.05$, 'o' $p \leq 0.1$, '' $p > 0.1$

Moving on to the erythromycin resistance genes, clear differences were noted between the two analyzed genes. No remarkable changes were noted in the abundance of ermB in the C, EFF2 and EFF3 microcosms (Fig. 7.14C). However, the raw effluent stimulated an important increase in gene copies per gram of soil, triplicating its abundance in two of the three replicates. This increase was also reflected in the relative abundances, although significance testing was hindered by the huge variance introduced by 16S copies. In regard to ermF, copy number decreased in average

for all samples, reproducing the tendency in the relative abundances. Strangely, aside from the increase in concentration in one of the samples from the control, relative abundance in the control also seemed to increase. Karkman *et al.* reported an enrichment of this same gene in a WWTP, especially accumulating in the sludge [307].

mphA is responsible for coding the macrolide 2'-phosphotransferase I, involved in the resistance against macrolides such as azithromicyn or erythromycin. Both relative abundance and total gene copies of mphA gene were clearly on a decreasing trend for all experimental conditions. This gene was clearly not abundant in the effluents but strange behaviour was observed again in the control, which did not change significantly and reflected a slight increase in relative abundance.

The last ARG to be assessed was dfrA, coding for the protein dihydrofolate reductase, involved in the resistance to trimethroprim. In this case, no clear changes were observed in the control and even a slight reduction is hinted by relative abundance and gene copies of EFF3 microcosm. However, a non-significant increase is clearly appreciated for samples treated with EFF1 and EFF2. As it was stated, trimethoprim resistance was chosen because the antibiotic was commonly found in the studied HWW and precisely, for effluents 1, 2 and 3, the concentrations of the antibiotic were of 16 ng L^{-1}, 8 ng L^{-1}, and below limit of detection, respectively. These data point to a clear relationship between presence of the antibiotic and an increase of the absolute and relative abundance of its associated resistance gene. The fungal bioreactor treatment proved in this case to be an efficient way to deal with antibiotics with potential to increase its associated resistance genes.

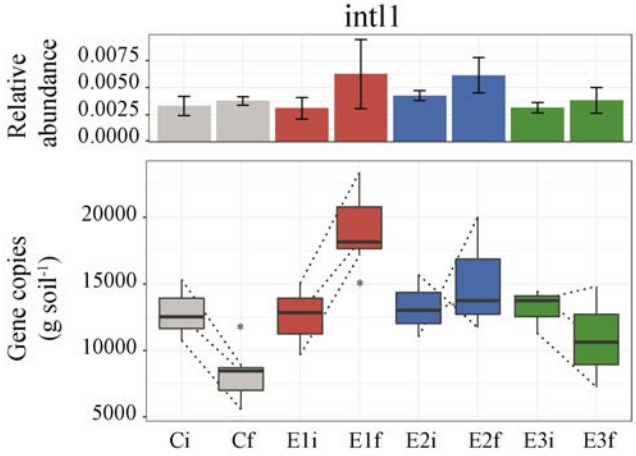

Figure 7.15: Absolute (above) and relative (below) abundance of the integrase gene intl1.

Finally, integrase gene abundances were presented individually in figure 7.15. Control treatment with distilled water decreased the number of copies significantly after 90 days of treatment. This gene is considered a marker of anthropogenic pollution [420] and a proportional effect is observed depending on the treatments applied to the effluent. First, the most polluted effluent (EFF1) significantly increased integrase gene copies during the treatment both in absolute copies and in relative abundance. Second, EFF2 showed mixed results for each replicate that ultimately did not cause neither an enrichment nor a decrease of intl1 copies. As in EFF1, intl1 relative abundance increased after the irrigation with this effluent. Third and last, EFF3 resulting from fungal-treatment and activated sludge showed an overall decrease of its1 copies, with relatively

stable relative abundances. Positive results for EFF3 resulting in attenuation of intl1 are highly promising because most potent ARGs in pathogens are often encoded on mobile elements [421–423].

Overall, the results were greatly favorable for the treatments applied to EFF3, which did not stimulate the increase of any gene and behaved similarly to the control, mitigating the resistance genes as time passed. In the case of EFF2 however, a better result was expected for an effluent treated simulating a conventional WWTP. For example Yang *et al.* reported that most of the antibiotic resistance genes were in the WWTP influent were removed (overall 98% reduction) in a metagenomic approach. Nonetheless, discrepancies still held between studies regarding the relative abundance to the 16S rRNA gene copy numbers [307, 424–427].

Chapter 8

General discussion

Emerging contaminants are encompassed in a catch-all group of extremely diverse substances both at the functional and structural level. They include substances such as pharmaceuticals, personal care products, endocrine-disrupting compounds or halogenated substances among many others. A great number of these pollutants are considered recalcitrant and poorly removed by conventional means of wastewater treatment [428–431]. This issue results in the release of EC into the environment, where they accumulate at concentrations ranging from nanogram per liter (e.g. carbamazepine) [151] up to milligram per liter (e.g. acesulfame) [432]. Moreover, the current high detection limits difficult the task of assessing both ECs presence and removal strategies in environmental matrices.

Having the previous statements in consideration, the combination of microbial species is a worthy option for the bioremediation these ECs that are blended in the environmental matrices [433]. Microorganisms are found in nature in mixed communities with complex metabolic networks, where they can degrade almost any compound thanks to metabolic synergies [85]. To unravel the functions carried by each population there is the need to combine knowledge on analytical chemistry, classical microbiology and molecular microbial ecology. Precisely, this is the multidisciplinary profile of the project in which this thesis is framed. Analytic procedures performed to obtain most of the data presented in this work would not have been possible without the work of each collaborator cited in the introduction of the experimental chapters.

The main focus of this thesis is to assess a variety of human-engineered communities involved in the degradation of ECs in confined spaces such as anaerobic flasks, bioreactors, photobioreactors or controlled biopiles. It is essential to remember that not only communities directly involved in pollutant transformation are of interest, but rather all the community developed around.

The first block of results was presented in chapter 4, where a DCP-degrading enrichment culture was characterized. The microbial ensemblage was driven by *Dehalogenimonas* and a synergy with *Sphaerochaeta* to obtain acetate and H_2 was suggested along with the capability of *Azonexus* and *Geobacter* to act as dehalogenation enhancers. Of these last two, only *Geobacter* strains have been use studied on their own for organochloride bioremediation capacity [214, 434]. In a similar manner, characterization of a DCM-degrading culture suggested that metabolites released by *Dehalobacterium* during dichloromethane fermentation could be used by its surrounding populations. However, their contribution to *Dehalobacterium* growth has not been elucidated yet. The robustness of this interactions could be determinant for the future success of the cultures in a field bioremediation. As it is economically unfeasible to formulate specific and anaerobic culture media to be supplied along with the consortium in a contaminated site, efforts should be directed to combine compatible strains to establish syntrophic synergies. As an example, recent works achieving high-rate reductive dehalogenation are based on bacterial mixtures [435] but, unfortunately, no efforts are directed into characterizing the present strains and understanding their interactions.

If inter-species relationships are already hard to characterize in consortia, the community developed in PBR takes the challenge two steps further by not being easily manipulable (like culture flasks are) and by constantly receiving an external input of WW, carrying its own native communities. Overall, results of the PBR operation were really positive because effluent resulting from the treatment compiled with the minimum removals required by the European Commission

directive 98/15/EC still in force [263] and a fairly good amount of PhACs were removed from the influent [254]. The most noticeable drawback was the vulnerability to seasonal variation; as when temperatures and sunlight decreased, so did the yield of the PBR. One solution to ensure a good treatment compensating for the slower metabolism of the microalgae is to extend the HRT, consequently prolonging the time needed to treat certain volume of WW. However, sustainable alternatives to maintain the PBR temperature and prevent yield loss have already been tested using heat waste (generated by machinery) [260, 436]. The downside of this approach is that it requires planning previous to the PBR construction to optimize the heat distribution system. Works in this direction are yet to assess viability of algal ponds or PBRs bigger than pilot-scale. Another possible solution could rely in a bioaugmentation strategy (as seen in section 5.4) with cyanobacteria or microalgae that were better adapted to low temperatures and could establish a synergy with the indigenous communities. On another line, handling of accumulated biomass should also be addressed. The effectivity of sorption processes depends on type of functional groups present on the algal cell wall [437–440]. Since some years ago, numerous studies have implemented biosorption solutions using dead algae biomass [441–443] for the removal of a variety of pollutants. Either if microalgal biomass is periodically removed and disposed or recovered for post-application purposes, a thoughtful treatment or proper disposal should be planned that accounts for the adsorbed and absorbed pollutants. Disposal considerations are usually assessed when dealing with metal bioremediation or toxic sludges [444]. However, algal biomass is not considered as hazardous by the Waste classification guidelines [445] (AU) or the European List of waste [446]. Only recently, dioxins, furans and/or PCB over certain concentrations were proposed to be included in the vigent european Commission Decision 2000/532/EC [446].

Concerning the bioremediation carried out using white rot fungi, wide expertise in sterile treatment of selected pollutants was already accumulated by the collaborators in the bioremediation group from the Chemical, Biological and Environmental Engineering Department (UAB) [312,313,315,318,365,400]. Thus, the chosen step for this project was to tackle the bioremediation of real HWW. The continuous treatment of HWW proved to be useful in removing most of the detected PhACs with relatively stable removal rates thanks to the implemented partial biomass renovation strategy [147]. The numerous studies in *T. versicolor* degradation that have been cited above demonstrated that degradation mechanisms in WRF are generally non-specific (laccase and peroxidase-dependant) and the mechanisms behind many reactions are yet to be found. However, not only fungi are to be held responsible for this success [151]. Some bacterial taxa growing in the same bioreactor were found strong and significantly correlated to the removal of psychiatric drugs and antibiotics. *Luteibacter*, *Raoultella* and *Stenotrophomonas* (class Gammaproteobacteria) were abundant in different moments of the operation and correlated altogether with psychiatric drug removal. On the other hand, *Pandoraea* was linked to antibiotics removal and is precisely resistant to a handful of them [349–351]. Independently of how it is able to resist antibiotics, controversy ensues at this point concerning bacteria harbouring drug-inactivating enzymes. Due to the abundance of antibiotics, organic matter (inherent of HWW) and glucose (to promote *Trametes* growth), bacteria harbouring ARGs are likely favoured in the bioreactor. Although they might be involved in antibiotic degradation, main point of removing antibiotics is to prevent the increase and dissemination of its ARGs in the environment. Is it ethical to let these resistant populations grow and be released into the sewage system? A reasonable solution rests in the application of membrane filters to retain bacterial and fungal biomass. Many studies [447–449] have already used MBR to decrease the ECs in HWW.

At this point, a handful of critical obstacles would remain in order to scale-up the system for a real implementation in the hospitals:

1. The volume capacity of the system to needs increase exponentially to keep up with the effluents generated daily.
2. The scale-up of the bioreactor would demand increasing volumes of nutrient for the supplementation of *T. versicolor* pellets.
3. The partial biomass renovation will be bothersome with the amount of pellets to be produced and replenished.
4. At a bioreactor scale, treated HWW was homogeneous as it was collected beforehand. However, in a full-scale continuous bioreactor the effluent entering the system would be constantly changing, challenging the stability of the fungus.

Many of these limitations might prevent the application of this effective technology in the real hospitals and for this reason the integration of the fungal treatment to a MBR would be really desirable. These MBR systems rely on a partial sludge re-inoculation strategy to maintain active communities that could be supplemented with WRF such as *T. versicolor*, mimicking the long-term treatment described in this chapter. Furthermore, the capacity of *T. versicolor* to operate in a MBR was already confirmed elsewhere [450].

A desire for clean effluents is the premise that motivated the last chapter in the thesis. The evaluation of effluents in a microcosm system was not expected to not provide absolute results on the water quality for reuse, but instead, guidance to where attention should be directed in further research and development of treatment technologies.

Although the results from the short-term impact assay generated data of interest regarding the influence of three effluents over microbial communities, the change of matrix from soil to slurry was the source of great part of the variability as demonstrated by the 16S metagenomic results. Still, despite the differences between t0 and t45 samples, significant trends were identified on differential effect of effluent type or irrigation strategy in fungal and bacterial communities.

Lucubration on the effect of PhACs over fungal communities was indeed included in the discussion of chapter 7. Nonetheless, numerical or statistical evidences were constrained by the lack of experimental data supporting specific results. Even with the intention to assess some of the observed results, the particularities of a complex matrix of pollutants and changing microbial communities make it impossible to fully replicate the environment in which a trend was identified. To put it as an example, the absence/presence of the antihelmintic levamisole was suggested to be influencing the fungal community structure. However, an in-vitro assay to confirm the results would not feature the same accompanying PhACs nor the unique community distribution of the original assay.

After re-formulating the experimental design of the impact assay to maintain a solid matrix and use more effluent volume, the mid-term impact assay was maintained for 90 days. Effluents influenced the community structure and diversity but no clear trends could be deducted as they seemed to drive bacterial communities to a similar endpoint. Precisely, behaviour was not entirely consistent with similar references [411]. Nevertheless, truly positive and promising results were

obtained in the assessment of mobile elements and ARG copies. According to the results of total copy abundance, the fungi-treated effluent EFF3 showed mirrored patterns to the control groups irrigated with distilled water. Because the fungal treatment was performed before secondary (conventional) treatment with active sludge, it probably cannot be labeled as a tertiary treatment. Yet, the practical effects of this pretreatment were those of a tertiary process capable of reducing the abundance of PhACs and even ARGs.

The use of cDNA (obtained from RNA) was reported in this chapter to be of use to ensure that environmental DNA from the effluents was not driving the observed changes in bacterial communities. However, a recent studies [451, 452] highlighted the impact of this relic DNA in soil microbial diversity estimates. Despite previous work [453] supporting that inactive cells can contain high rRNA concentrations and viceversa, rRNA is still useful to discriminate between dead cells and relic DNA from viable and active ones. In this sense, it was still of interest to see if differences between Phyla were important and consistent. Four DNA samples from time 0d were compared with their RNA counterparts (Fig. 8.1). Differences between DNA and RNA were present in all dominant phyla (>1% relative abundance). Basically, all samples revealed a major abundance of either rRNA copies (RNA) or rRNA gene copies (DNA) consistently for each phylum (except from Bacteroidetes and Planctomycetes, in which EFF3 and C samples behaved against the trend, respectively). On the one hand, Proteobacteria, Verrucomicrobia, Firmicutes and Cyanobacteria exhibited greater RNA abundances, linkable to viable and active representatives. On the other hand, Acidobacteria, Bacteroidetes, Planctomyces, Actinobacteria, Chloroflexi and Gemmatiomonadetes could be overstimated in the original study from section 7.4.1. In this case the comparison was made between relative abundance data but it would be of interest to design quantitative assays in the future to contrast with current knowledge on rRNA:rDNA gene ratios [452].

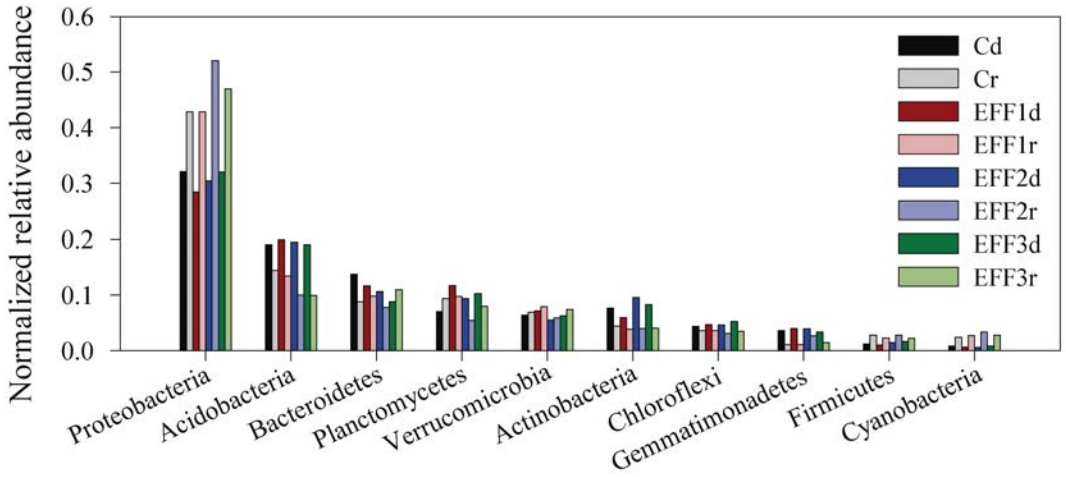

Figure 8.1: Normalized relative abundance of the abundant phyla (>1%) in paired DNA and RNA samples before the irrigation experiment. d, DNA; r, RNA.

As it was introduced in this discussion, no similarities should be expected between communities responsible of EC removal either because compounds are structurally different between themselves [454] or because the approaches described in this work were carried out with different configurations, scales and objectives. However, a global screening of all the groups encountered in this work was conducted to have a broad view of the communities in the main experiments. In

the first place, removal percentages and dominant taxa (top 3 abundant genera throughout each experiment) were compiled in table 8.1. Best PhAC removal results were obtained in PBR, specifically in the second period. The reason behind this good performance is probably the high HRT of the operation that allows for throughout removal. On the opposite side, biopiles did not seem to remove as efficiently as the other systems. However, it should be taken into account that the detected removal belongs to degradation (either by physico-chemical means or biologically) and not sorption. Precisely, in the PBR and fungal bioreactor an unknown fraction of the removal was likely due to sorption processes [245, 325, 438, 444].

Table 8.1: Summary of the average PhAC removal and dominant bacterial and eukaryotic communities (top 3 most abundant genera) of the main bioremediation strategies covered in the thesis. FBR, Fungal Bioreactor. AA, Analgesics and antiinflammatories; AB, Antibiotics; PD, Psychiatric drugs; Other, Other PhACs. Re: Re-inoculated biopiles.

	Removal (%)				Relative abundance (%)	
Treatment	AA	AB	PD	Other	Bacteria	Fungi/Eukarya
FBR (Validation)	80.6	28.4	35.9	66.8	*Pandoraea* (30.9) *Rhizobium* (22.3) *Dyadobacter* (13.9)	*Trametes* (28.2) *Candida* (28.8) *Asterotremella* (19.68)
FBR (Long-term)	89.5	23.1	32.5	59.7	*Pandoraea* (24.2) *Raoultella* (12.2) *Comamonas* (10.8)	*Trametes* (38.4) *Fusarium* (28.8) *Tremella* (16.6)
Biopiles (Inoculated)	8.7	87.5	52.3	57.0	*Lysobacter* (42.0) *Clostridium* (31.1) *Bacillus* (10.9)	*Trametes* (89.5) *Wickerhamomyces* (10.0) *Pseudallescheria* (0.5)
Biopiles (Re-inoculated)	35.4	87.5	61.5	77.0	*Lysobacter* (39.1) *Clostridium* (30.4) *Pedobacter* (12.1)	*Trametes* (81.4) *Coriolopsis* (9.1) *Meyerozyma* (9.1)
PBR (Period I)	70.8	68.0	57.2	73.4	*Thauera* (28.9) *Leptolyngbia* (26.0) *Oscillatoria* (11.8)	*Chlorella* (75.3) *Paraphysoderma* (10.6) *Vorticellides* (5.6)
PBR (Period II)	77.4	83.3	70.4	85.4	*Thauera* (37.0) *Leptolyngbia* (11.1) *Oscillatoria* (6.6)	*Chlorella* (81.0) *Pseudospongiococcum* (7.1) *Brachionus* (2.85)

In most cases, only the two most abundant genera made up more than 50% of the total abundance, indicating highly biased communities with few dominant taxa. At the phylum level, dominant taxa belonged mainly to Proteobacteria (Betaproteobacteria and Gammaproteobacteria especially) and Cyanobacteria. To go further, all 16S metagenomic data were pooled and analyzed altogether using R and dada2. Then, simplified relative abundance data from fungal continuous treatment was merged with the Illumina read counts and normalized altogether with metagenomeSeq. From the 31 Phyla detected, 27 were observed at least in one of the PBR-related samples, 19 in the dechlorinating cultures and just 5 in the fungal treatment (resolution limited by the DGGE approach). Aside from harboring the most abundant genera in the different operations, phylum Proteobacteria was detected in all environments so it could be compared in all studies, including the fungal bioreactor. The individual assessment of its classes provided interesting results (Fig. 8.2).

First of all, Betaproteobacteria were abundant in samples from the three treatment operational systems (i.e. dehalogenation, PBR and fungal treatment). Alphaproteobacteria and Gammaproteobacteria groups were detected at some point in all chapters too, and mostly remained abundant

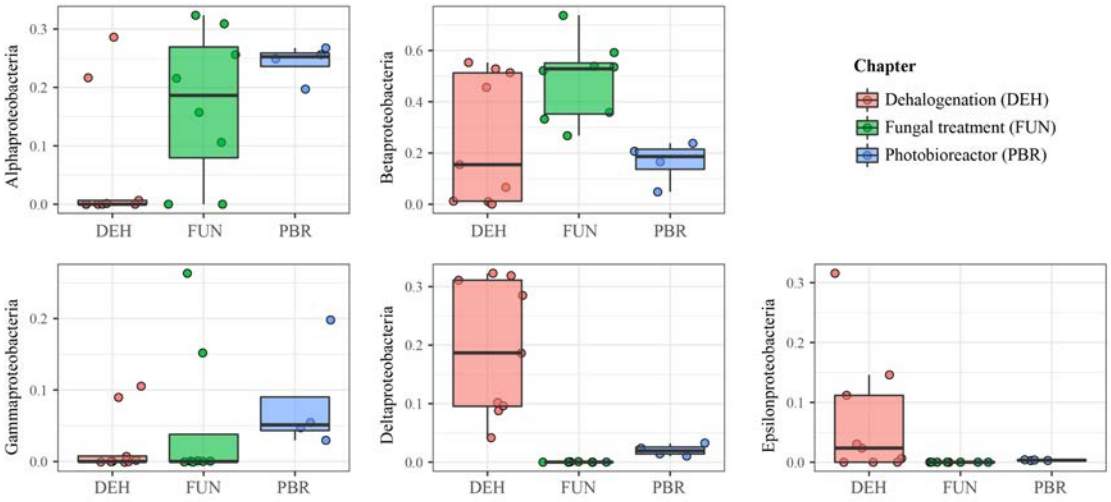

Figure 8.2: Normalized abundance of Proteobacteria classes in the samples from the three treatment chapters.

in the PBR. Interestingly, differences were evident in Deltaproteobacteria and Epsilonproteobacteria classes as they were almost exclusively found in the dehalogenalogenation samples.

High diversity and abundance of species have been highlighted throughout this thesis as favorable traits for the robust removal of pollutants and enhanced endurance against external stress (e.g. Photobioreactor bioaugmentaton in section 5.4). However, highly specialized communities tend to develop when the cultures are exposed to certain pollutants, inevitably drifting towards an uneven community (e.g. Illumina results from the DCP-degrading culture in section 4.2.2 and DCM-degrading culture in section 4.3.1). In this sense, it was the purpose of this work to address the nature of communities in different treatment systems, offering a broad view of the processes and refusing to label findings as either positive or negative. A lineup of the most relevant findings from the work presented in this dissertation were synthesized in the following chapter in form of conclusions.

Chapter 9

Conclusions and future prospects

9.1 Conclusions

The main concluding remarks of the present PhD project are listed below and grouped as a function of main experimental chapters.

I. Dehalogenating enrichment cultures

- *Dehalogenimonas* sp. was identified in sediment samples from an historically polluted area that showed capacity to degrade organochlorinated compounds.
- An enrichment culture established from those sediments was able to degrade increasing concentrations of 1,2-dichloropropane. *Dehalogenimonas* was found responsible of the dehalorespiration of 1,2-dichloropromane into propene.
- Bacteria with reported capacity to enhance dehalogenation or provide essential growth factors were found accompanying *Dehalogenimonas* in the enrichment cultures. Thorough study of the enrichment provided insight into the interactions between the populations and guidance for the formulation of efficient consortia for bioremediation.
- *Dehalobacterium* sp. was identified in slurry samples from an industrial membrane bioreactor capable of dichloromethane fermentation.
- Development of an enrichment culture from the slurry samples resulted in acute shifts of bacterial communities, favoring four minority strains to consolidate a highly efficient consortium for the degradation of dichloropropane.

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E. Parlade, S. Hatijah Mortan, M. Llirós, G. Caminal, N. Gaju, and M. Martinez-Alonso. Characterization of a Dehalogenimonas-driven Anaerobic Consortium Capable of 1,2-Dichloropropane Degradation. (In preparation)

II. Photobioreactor operation for urban wastewater treatment

- Temperature, irradiation and hydraulic retention time were the main drivers of microbial diversity in a pilot-scale photobioreactor dominated by *Chlorella* and able to remove organic matter, inorganic nutrients and pharmaceutical compounds.
- The presence of cyanobacteria from genera *Phormidium* and *Leptolyngbia* contributed to the formation of settling flocs that favor biomass harvesting.
- The photobioreactor biomass was more efficient in the removal of estradiol and had a higher richness and diversity, making it less susceptible to losses due to predation and parasitism.
- Laboratory-scale bioaugmentation with *Desmodesmus* sp. biomass from an industrial photobioreactor enhanced total estrogen removal through an increase of biomass concentration

E. Parladé, A. Hom-Diaz, P. Blánquez, M. Martínez-Alonso, T. Vicent, and N. Gaju, Effect of cultivation conditions on beta-estradiol removal in laboratory and pilot-plant photobioreactors by an algal-bacterial consortium treating urban wastewater. Water Research, vol. 137, 2018.

III. Fungal bioreactor for hospital wastewater treatment

- Hospital wastewater indigenous communities are able to remove analgesics, antiinflamatories and other pharmaceuticals in the bioreactor. However, only a bioreactor with *Trametes versicolor* pellets resulted in removal of antibiotics and psychiatric drugs.
- *T. versicolor* pellets in the bioreactor served as a reservoir for bacteria and contributed to the increase of the same fungus in the liquid matrix due to mycelia detachment.
- Supplementation of fungal bioreactors with glucose allowed the proliferation of *Pandoraea* sp., a multi-drug resistant opportunistic pathogen which correlated strongly with the removal of antibiotics. *Pandoraea* thrived in the liquid matrix and fungal pellets alike.
- Biopile treatment using *T. versicolor* for pharmaceuticals removal achieved higher removal rates for single compounds and for all the drugs simultaneously when it was re-inoculated with the fungus.
- The development of *T. versicolor* in the biopiles is hindered by the presence of the bacterium *Lysobacter* and the fungus *Meyerozyma guillermondii*, both specialized in antagonizing white-rot fungi through nutrient competition, direct adhesion or production of antagonizing metabolites.

J. Mir-Tutusaus, E. Parladé, M. Llorca, M. Villagrasa, D. Barceló, S. Rodríguez-Mozaz, M. Martínez-Alonso, N. Gaju, G. Caminal, and M. Sarrà. Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation- occulation pretreatment. Water Research, vol. 116, pp. 65-75, 2017.

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IV. Impact of treated wastewater on the environment

- Short-term irrigation of slurry microcosms with different treated hospital wastewater had a deep effect on fungal and bacterial assemblages. The effluent type drove fungal community changes while the irrigation strategy employed was more determinant to bacteria.
- Mid-term soil microcosm irrigation revealed a limited effect of pharmaceuticals over bacterial community structure and highlighted a more notable influence of temporal dynamics and dissolved organic carbon. Bacterial richness and diversity decreased after 90 days of irrigation.
- In terms of predicted functional groups, chemoheterotrophic bacteria were enriched in microcosms with raw HWW while an increase in pathogens was stimulated by effluents treated in the fungal bioreactor.
- Control microcosms and the *T. versicolor*-treated effluent behaved equivalently and did not increase the concentration of antibiotic resistance genes.
- Integrase and antibiotic resistance genes tested showed a decrease in abundance during the mid-term impact assay. Increases in copy concentrations only occurred when irrigation was performed with raw and conventionally-treated effluents.

E. Parladé, J. Mir-Tutusaus, G. Caminal, M. Sarrà, N. Gaju, M. Martínez-Alonso. Impact of reclaimed hospital wastewater on soil microbial communities. (In preparation)

9.2 Future prospects

Having attained important results in each chapter, it is clear that much remains to be done in all lines of this research.

First addressing the microbial assemblages for organohalide bioremediation, an attempt to isolate the most abundant strains could allow the study of metabolic synergies by co-culturing in nutrient-limited media. Assuming the success of the isolation, genome sequencing of obtained strains would provide unique insights on each strain.

Concerning the microalgal PBR, a year-long continuous operation would be of interest to fully understand the implication of biological and environmental parameters. Future research should also explore pharmaceuticals concentration in harvested biomass to determine the implication sorption and degradation processes.

While the operation of the fungal bioreactor is at an advanced stage, the precise mechanism of fungal degradation of PhACs remains yet to be elucidated. Continuous efforts are still needed on this direction along with the need to reduce the need of external nutrients, scale-up the system and restrain the release of microbial biomass.

Finally, in terms of impact of reused water on natural ecosystems, the challenge now is to extend the monitoring period in microcosms and consider larger-scale or in-situ evaluation of the fungal bioreactor effluents, which exhibited the best results.

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Chapter 10

Annex

Table 10.1: Consumables, reagents and enzymes purchased, along with their supplier.

Supplier	Product
Ambion	Turbo DNA-free kit.
Bio-Rad	Acrylamide, ammonium persulfate, Bradford reagent, AGr 501-X8 Resin and 50x TAE buffer
Fluka	BG-11 medium and trace metal mix A5.
Invitrogen	Taq polymerase, DNA ladders, deoxynucleotides and Qubit 3.0 reagents.
Merck	Durapore membrane filters.
New England BioLabs	50 bp ladder, cDNA synthesis kit protoscript II.
Panreac	Glycerol, sodium chloride and sodium hydroxide.
Conda	Tryptone soy agar, yeast extract and agarose D1 low EEO.
Sigma-Aldrich	Water for molecular use, 17-estradiol (E2), 17-ethynylestradiol (EE2) (>98% purity), HPLC grade acetone (≥99.9% purity), formamide, ethidium bromide, bromophenol-blue xylene-xyanole dye and kanamycin.

Table 10.2: Detected PhACs in the influent and effluent and removals during the PBR operation in periods I and II.

	Period I			Period II		
	Influent±SD (ng L^{-1})	Effluent±SD (ng L^{-1})	Removal (%)	Influent±SD (ng L^{-1})	Effluent±SD (ng L^{-1})	Removal (%)
Analgesic and anti-inflammatories						
Naproxen	2,945 ± 368	2,646 ± 239	10.2	25,043 ± 1,726	7,701 ± 623	69.2
Ibuprofen	52,091 ± 675	786 ± 14	98.5	41,450 ± 2,431	blq	blq
Acetaminophen	54,294 ± 988	412 ± 26	99.2	54,438 ± 4,262	bld	bld
Salicylic acid	1,349 ± 738	bld	bld	368 ± 12	245 ± 30	33.4
Ketoprofen	472 ± 52	301 ± 51	36.2	6729 ± 413	1,039 ± 75	84.6
Codeine	33 ± 1	bld	bld	2.085	bld	bld
Antibiotics						
Azithromycin	385 ± 481	43 ± 43	88.8	-	-	-
Ciprofloxacin	2,629 ± 142	1,377 ± 91	47.6	294 ± 28	bld	bld
Ofloxacin	65 ± 29	21 ± 5	67.7	,5662 ± 651	1,882 ± 133	66.8
Erythromycin	-	-	-	661 ± 42	100 ± 8	84.9
Psychiatric drugs						
Alprazolam	-	-	-	389 ± 20	49 ± 3	87.4
Paroxetine	-	-	-	1,652 ± 50	103 ± 4	93.8
Lorazepam	3,696 ± 114	1581 ± 16	57.2	2,383 ± 137	1,669 ± 64	30
Other PhACs						
Hydrochlorothiazide	228 ± 6	127 ± 2	44.3	686 ± 13	107 ± 1	84.4
Furosemide	-	-	-	669 ± 21	bld	bld
Atenolol	7,795 ± 447	115 ± 9	98.5	6906 ± 448	997 ± 72	85.6
Diltiazem	1,678 ± 189	381 ± 23	77.3	1935 ± 15	530 ± 16	72.6

blq: Below limit of quantification.
bld: Below limit of detection.

Table 10.3: Detected Analgesics and anti-inflammatories, antibiotics and psychiatric drugs in the fungal bioreactor validation experiment. Influent concentrations for HWW1 and HWW2 are presented along with the removal percentage in the experimantal (E) and control (C) bioreactors.

	HWW1			HWW2		
	Influent (ng L^{-1})	E removal ± SD (%)	C removal ± SD (%)	Influent (ng L^{-1})	E removal ± SD (%)	C removal ± SD (%)
Analgesic and anti-inflammatories						
Acetaminophen	>20,000	>99.3 ± 0	>85.7 ± 0	>20,000	99.5 ± 1	100.0 ± 0
Diclofenac	951.3	99.8 ± 0	95.9 ± 8	bld	-	-
Ibuprofen	>20,000	>85.5 ± 19	>88.7 ± 6	3,960.14	84.6 ± 2	93.0 ± 2
Ketoprofen	5,109.3	-3.6 ± 84	71.5 ± 20	2,432.33	-54.4 ± 72	79.8 ± 25
Phenazone	bld	-	-	9.23	-314.0 ± 139	-147.3 ± 192
Antibiotics						
Azithromycin	bld	-	-	45.36	-95.5 ± 107	88.5 ± 21
Ciprofloxacin	366.4	47.1 ± 25	-32.8 ± 67	266.90	-7.2 ± 16	-2.5 ± 29
Sulfamethoxazole	1,130.4	78.2 ± 9	29.0 ± 31	55.85	34.8 ± 76	-42.6 ± 87
Trimethoprim	748.3	52.3 ± 35	-27.8 ± 54	81.80	-26.9 ± 96	-42.1 ± 67
Ofloxacin	2537.4	71.1 ± 14	3.8 ± 21	459.39	-57.8 ± 168	-65.4 ± 54
Psychiatric drugs						
10.11-epoxyCBZ	673.2	43.6 ± 9	81.3 ± 30	1,816.91	25.4 ± 6	82.6 ± 35
2-hydroxyCBZ	1,661.1	99.9 ± 0	76.7 ± 46	>2,000	>74.9 ± 50	>18.3 ± 24
Acridone	126.0	-183.5 ± 43	74.5 ± 0	225.85	-295.8 ± 221	6.5 ± 62
Alprazolam	bld	-	-	10.04	-17.7 ± 41	4.4 ± 41
Carbamazepine	251.4	61.0 ± 19	16.0 ± 8	270.09	22.7 ± 7	11.6 ± 7
Citalopram	297.4	39.0 ± 18	-18.2 ± 5	351.00	-4.4 ± 38	-22.0 ± 14
Norfluoxetine	12.5	91.1 ± 0	-26.6 ± 233	bld	-	0
Olanzapine	131.5	98.7 ± 2	-44.6 ± 153	66.98	99.2 ± 0	-73.9 ± 104
Setraline	98.8	98.3 ± 0	98.3 ± 0	127.85	98.7 ± 0	55.1 ± 50
Razodone	36.3	98.0 ± 0	-112.5 ± 94	20.86	69.7 ± 0	29.7 ± 54
Venlafaxine	495.3	41.0 ± 41	-18.2 ± 7	146.18	-19.2 ± 79	-78.8 ± 46

blq: Below limit of quantification.
bld: Below limit of detection.

Table 10.4: Detected PhACs from the "others" group in the fungal bioreactor validation experiment. Influent concentrations for HWW1 and HWW2 are presented along with the removal percentage in the experimantal (E) and control (C) bioreactors.

	HWW1			HWW2		
	Influent (ng L^{-1})	E removal ± SD (%)	C removal ± SD (%)	Influent (ng L^{-1})	E removal ± SD (%)	C removal ± SD (%)
Other PhACs						
Thiabendazole	blq	70.0 ± 0	70.0 ± 0	9.10	97.7 ± 0	92.3 ± 0
Warfarin	10.0	94.8 ± 0	94.8 ± 0	bld	-	-
Valsartan	112.7	34.2 ± 22	43.4 ± 46	55.46	-44.6 ± 63	15.6 ± 18
Atenolol	59.4	14.3 ± 37	78.7 ± 23	154.45	67.5 ± 24	60.9 ± 19
Sotalol	251.6	-151.9 ± 29	-205.2 ± 65	bld	-10,442.4 ± 20885	-17,997.6 ± 35,995
Furosemide	bld	-664.4 ± 1431	-1,018.9 ± 2,124	161.88	97.6 ± 0	96.2 ± 3
Hydrochlorothiazide	58.4 ± 34	23.1 ± 14	650.78	10.9 ± 12	21.8 ± 2	
Tamsulosin	bld	-	-58.3 ± 117	7.30	41.6 ± 5	98.7 ± 0
Ranitidine	96.7 ± 3	89.9 ± 6	20.43	-2.4 ± 107	-77.3 ± 61	
Loratadine	1.2	46.9 ± 33	75.5 ± 0	bld	-89.6 ± 113	-116.7 ± 135
Atorvastatin	14.9	94.9 ± 7	90.1 ± 15	13.77	95.6 ± 2	71.1 ± 28
Gemfibrozil	6,364.1	100.0 ± 0	83.6 ± 3	1,921.34	85.1 ± 19	90.3 ± 19
Dexamethasone	121.8	77.2 ± 44	33.5 ± 76	bld	-34501.3 ± 23,481	-32,983.9 ± 23,315

blq: Below limit of quantification.
bld: Below limit of detection.

Table 10.5: Detected PhACs in the fungal bioreactor during long-term operation. Influent concentrations (HWW3) are presented along with weekly samples labeled by day.

Compound	HWW3	d8	d15	d22	d29	L36	d43	d50	d57	d63	d70	d78	d85	d91
Analgesics and anti-inflammatories														
Acetaminophen	6,942 ± 213	225	168	9,517	195	bld	bld	bld	296	145	128	bld	253	104
Diclofenac	1,650 ± 83	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	163
Ibuprofen	12,837 ± 1,860	5,004	1,708	2,914	1,025	1,066	3,486	1,416	1,530	2,574	1,855	bld	329	157
Ketoprofen	1,291 ± 154	38,676	4,712	3,785	2,446	1,254	2,654	2,064	2,407	2,130	3,732	1,273	1,436	1,479
Oxycodone	35 ± 0	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld
Salycilic acid	11,266 ± 4,467	2,665	1,019	630	2,395	10,388	5,170	1,162	740	1,213	809	3,267	606	453
Antibiotics														
Sulfamethoxazole	85 ± 24	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	22
Trimetoprim	25 ± 2	blq	blq	bld	blq	blq	blq	blq	blq	blq	bld	blq	bld	bld
Psychiatric drugs														
2-OH CBZ	658 ± 22	bld	bld	310	bld	bld	bld	bld	bld	bld	479	bld	860	bld
Acridone	246 ± 23	108	147	96	bld	193	280	248	97	138	86	200	138	164
Carbamazepine	2,004 ± 308	826	984	860	862	1,431	404	875	1,457	1,691	1,752	1,516	2,151	1,900
Citalopram	278 ± 51	bld	bld	bld	76	139	111	75	62	94	124	216	257	214
Diazepam	36 ± 5	bld	bld	121	51	bld	blq	bld	bld	bld	bld	bld	blq	blq
epoxy CBZ	1,342 ± 219	618	541	634	526	489	867	785	750	1,258	1,163	738	1,810	1,292
N-Desmethyl-venlafaxine	625 ± 15	bld	bld	bld	bld	bld	bld	bld	bld	bld	287	bld	434	350
O-Desmetyl-venlafaxine	926 ± 150	bld	267	221	190	166	bld	50	436	397	475	245	637	444
Trazodone	173 ± 41	bld	17	24	15	17	bld	11	24	bld	49	29	61	135
Venlafaxine	791 ± 122	bld	317	294	355	653	430	428	603	805	730	653	875	676
Other PhACs														
Valsartan	2,355 ± 37	799	875	884	808	808	828	829	780	860	877	2135	961	933
Metoprolol	97 ± 0	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld
Hydrochlorothiazide	743 ± 104	150	255	215	276	418	190	237	349	369	392	492	521	446
Tamsulosin	17 ± 0	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld
Ranitidine	57 ± 5	bld	bld	21	bld	bld	bld	bld	34	bld	25	bld	bld	bld
Atorvastatin	6 ± 0	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld	bld
Gemfibrozil	494 ± 9	318	28	57	bld	14	11	10	bld	9	bld	bld	9	12

blq: Below limit of quantification.
bld: Below limit of detection.

Table 10.6: Detected PhACs in Biopiles systems and its removal yields before and after re-inoculation.

	Initial concentration ± SD (ng g^{-1})	Removals ± RSD (%)		
	Biopiles set-up (0d)	Before re-inoculation (22d)	Non-re-inoculated (42d)	Re-inoculated (42d)
Analgesics and Anti-inflammatories				
Ibuprofen	19.38 ± 13.57	[a]	14.33 ± 0.14	35.96 ± 0.36
Oxycodone	4.45 ± 0.82	[a]	11.81 ± 0.12	70.29 ± 1.00
Codeine	0.34 ± 0.22	[a]	0.00 ± 0.00	[a]
Antibiotics				
Sulfamethoxazole	6.43 ± 0.95	87.48 ± 1.00	87.48 ± 1.00	87.48 ± 1.00
Psychiatric Drugs				
Citalopram	75.55 ± 10.46	42.76 ± 0.43	19.69 ± 0.20	35.45 ± 0.35
Sertraline	52.58 ± 5.69	92.97 ± 1.00	92.97 ± 1.00	92.97 ± 1.00
Fluoxetine	51.14 ± 8.39	81.53 ± 0.82	72.79 ± 0.73	75.16 ± 0.75
Paroxetine	42.13 ± 5.51	87.38 ± 0.87	79.32 ± 0.79	87.57 ± 0.88
Trazodone	34.91 ± 5.97	75.00 ± 0.75	53.79 ± 0.54	83.88 ± 0.84
Venlafaxine	29.64 ± 1.72	3.34 ± 0.03	[a]	16.69 ± 0.17
Carbamazepine	5.05 ± 0.69	[a]	[a]	0.42 ± 0.00
Olanzapine	4.79 ± 1.31	100.00 ± 1.00	100.00 ± 1.00	100.00 ± 1.00
Other PhACs				
Levamisol	5.95 ± 0.35	22.77 ± 0.23	21.90 ± 0.22	57.08 ± 0.57
Amlodipine	18.18 ± 5.43	43.45 ± 0.43	49.68 ± 0.50	82.78 ± 0.83
Diltiazem	6.91 ± 1.17	100.00 ± 1.00	100.00 ± 1.00	100.00 ± 1.00
Hydrochlorothiazide	8.98 ± 0.16	47.25 ± 0.47	82.30 ± 9.12	82.30 ± 9.12
Tamsulosin	7.09 ± 0.51	31.95 ± 0.32	15.71 ± 0.16	53.93 ± 0.54
Gemfibrozil	36.52 ± 11.32	60.74 ± 0.61	54.03 ± 0.54	69.13 ± 0.69
Atorvastatin	20.79 ± 3.23	83.01 ± 0.83	75.44 ± 0.75	93.80 ± 0.74

[a] Removal not assessed, final concentration was higher than the initial.

Table 10.7: PhAC concentrations in the effluents used to irrigate soil microcosms.

Compound	EFF1 ± SD (ng L^{-1})	EFF2 ± SD (ng L^{-1})	EFF3 ± SD (ng L^{-1})
Analgesics and anti-inflammatories			
Acetaminophen	9,836 ± 486	242 ± 96	bld
Codeine	22 ± 2	145 ± 22	bld
Diclofenac	1,502 ± 87	1,593 ± 193	bld
Ibuprofen	12,949 ± 878	9,188 ± 1,518	221 ± 16
Indomethacine	44 ± 0	bld	bld
Ketoprofen	1,891 ± 195	1,873 ± 351	4,833 ± 233
Meloxicam	6 ± 1	bld	7 ± 1
Naproxen	2,248 ± 195	2,096	bld
Phenazone	bld	46 ± 6	136 ± 6
Salycilic acid	4178 ± 1,521	466 ± 16	453 ± 49
Antibiotics			
Sulfamethoxazole	66 ± 0	170 ± 19	bld
Trimetoprim	17 ± 1	8 ± 0	bld
Psychiatric drugs			
2-OH CBZ	711 ± 26	619 ± 74	bld
Acridone	184 ± 13	67 ± 10	35 ± 0
Alprazolam	bld	10 ± 1	5 ± 0
Carbamazepine	2,031 ± 38	2,044 ± 246	732 ± 32
Citalopram	261 ± 29	133 ± 36	802 ± 42
Diazepam	31 ± 1	26 ± 6	bld
epoxy CBZ	1,319 ± 15	1,127 ± 140	619 ± 49
Fluoxetine	96 ± 0	bld	87 ± 10
Lorazepam	58 ± 0	75 ± 10	357 ± 219
N-Desmethyl-venlafaxine	320 ± 11	bld	274 ± 0
O-Desmetyl-venlafaxine	693 ± 91	2,406 ± 280	869 ± 86
Paroxetine	bld	bld	bld
Sertraline	167 ± 44	bld	170 ± 0
Trazodone	92 ± 16	193 ± 64	70 ± 7
Venlafaxine	907 ± 56	916 ± 139	898 ± 44
Other PhACs			
Atenolol	49 ± 1	29 ± 3	bld
Propanolol	bld	18 ± 3	238 ± 25
Sotalol	4 ± 0	13 ± 1	bld
Levamisol	bld	291 ± 18	933 ± 52
Irbesartan	bld	153 ± 20	bld
Valsartan	1,097 ± 25	1,056 ± 66	824 ± 35
Clopidrogel	bld	blq	5 ± 0
Hydrochlorothiazide	511 ± 67	607 ± 174	911 ± 72
Tamsulosin	bld	bld	25 ± 2
Desloratadine	bld	bld	30 ± 0
Ranitidine	39 ± 5	19 ± 0	27 ± 1
Bezafibrate	11 ± 0	bld	bld
Gemfibrozil	494 ± 9	601 ± 3	5 ± 0

blq: Below limit of quantification.
bld: Below limit of detection.

Table 10.8: Human pathogens detected with FAPROTAX in the samples from EFF3 of the mid-term impact of effluents experiment.

Phylum	Order	Genus and/or Species
Firmicutes	Bacillales	*Bacillus cereus*
Firmicutes	Clostrales	*Clostridium perfringens*
Firmicutes	Clostrales	*Clostridium perfringens*
Firmicutes	Clostrales	*Clostridium perfringens*
Firmicutes	Clostrales	*Clostridium sordellii*
Proteobacteria	Roseomales	*Roseomonas*
Proteobacteria	Roseomales	*Roseomonas*
Proteobacteria	Roseomales	*Roseomonas*
Proteobacteria	Roseomales	*Roseomonas*
Proteobacteria	Alcaliales	*Alcaligenes faecalis*
Proteobacteria	Arcobaales	*Arcobacter cryaerophilus*
Proteobacteria	Arcobaales	*Arcobacter cryaerophilus*
Proteobacteria	Escherales	*Escherichia coli*
Proteobacteria	Pantoeales	*Pantoea agglomerans*
Proteobacteria	Pantoeales	*Pantoea agglomerans*
Proteobacteria	Coxielales	*Coxiella*
Proteobacteria	Acinetales	*Acinetobacter*
Proteobacteria	Acinetales	*Acinetobacter*
Proteobacteria	Acinetales	*Acinetobacter*
Proteobacteria	Acinetales	*Acinetobacter guillouiae*
Proteobacteria	Acinetales	*Acinetobacter johnsonii*
Proteobacteria	Acinetales	*Acinetobacter lwoffii*
Proteobacteria	Acinetales	*Acinetobacter lwoffii*
Proteobacteria	Acinetales	*Acinetobacter lwoffii*
Proteobacteria	Moraxeales	*Moraxella*
Proteobacteria	Pseudoales	*Pseudomonas stutzeri*
Proteobacteria	Pseudoales	*Pseudomonas stutzeri*
Proteobacteria	Pseudoales	*Pseudomonas stutzeri*
Proteobacteria	Pseudoales	*Pseudomonas stutzeri*
Proteobacteria	Stenotales	*Stenotrophomonas*
Proteobacteria	Stenotales	*Stenotrophomonas*
Proteobacteria	Stenotales	*Stenotrophomonas*
Proteobacteria	Stenotales	*Stenotrophomonas acidaminiphila*
Proteobacteria	Stenotales	*Stenotrophomonas maltophilia*
Proteobacteria	Stenotales	*Stenotrophomonas maltophilia*
Proteobacteria	Stenotales	*Stenotrophomonas maltophilia*