Universitat Autònoma de Barcelona

DEGRADATION OF PHARMACEUTICAL COMPOUNDS BY MICROALGAE: PHOTOBIOREACTOR WASTEWATER TREATMENT, BIOMASS HARVESTING AND METHANIZATION

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- PhD Thesis -

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CERTIFIQUEM:

Que l'enginyera química ANDREA HOM DÍAZ ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental, el treball que amb el títol "Degradation of pharmaceutical compounds by microalgae: Photobioreactor wastewater treatment, biomass harvesting and methanization" es presenta en aquesta memòria, la qual constitueix la seva Tesi per a optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I per a què se'n prengui coneixement i consti als efectes oportuns, presentem a l'Escola de Postgrau de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat.

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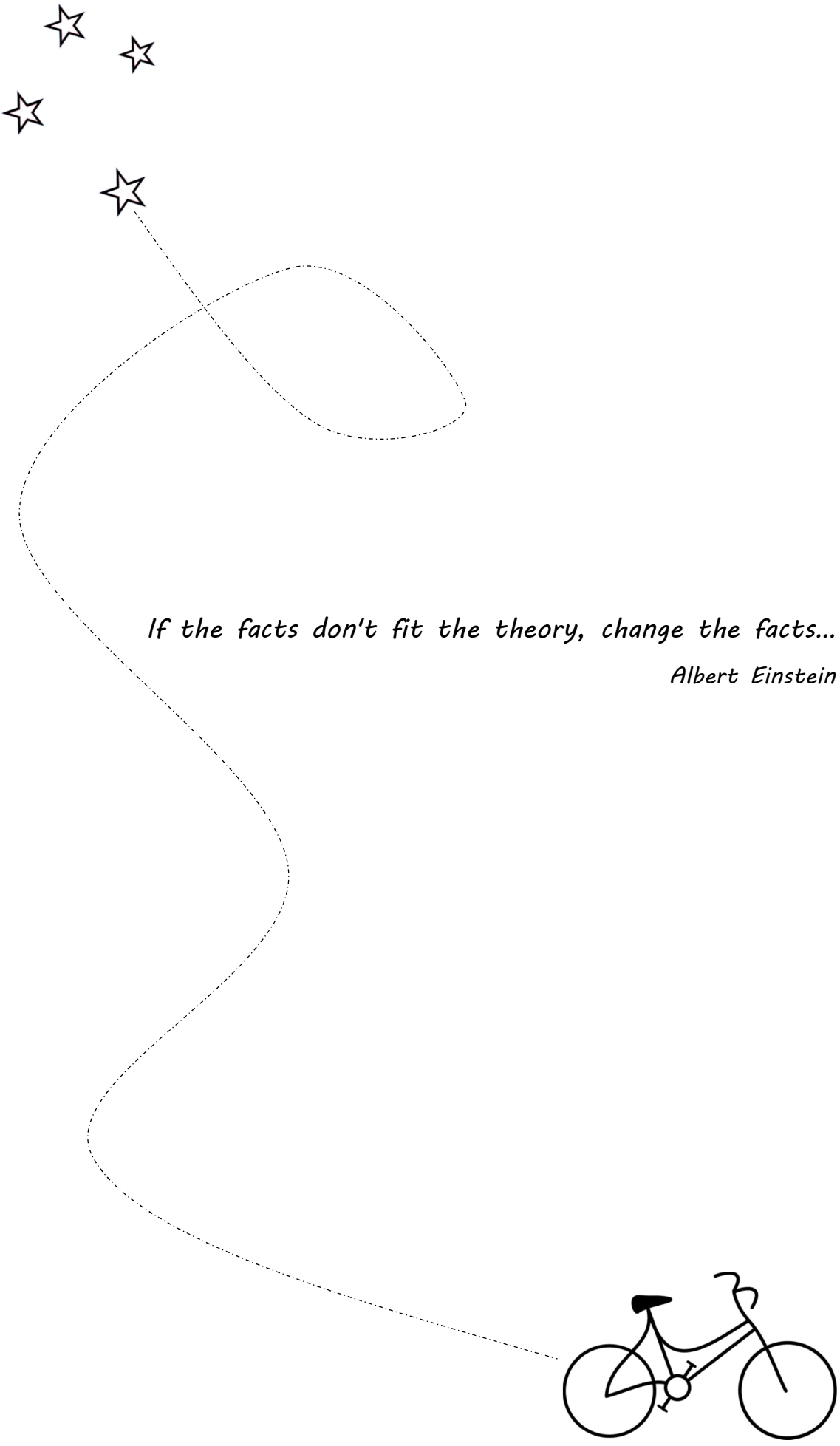

If the facts don't fit the theory, change the facts...

Albert Einstein

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ABSTRACT

Emerging contaminants (ECs) are a wide range of organic compounds detected in many environmental compartments that have raised an increasing interest in the scientific community due to their ubiquitous presence in the environment and their difficult degradation. Even though their environmental concentration is usually in the range of ng/L to µg/L, they still represent a threat to human health and environment since they are excreted with urine and faeces either as active substances or metabolites, because they are not completely assimilated. Among emerging contaminants, endocrine disrupting compounds (EDCs) and pharmaceutical active compounds (PhACs) are of major concern. It is widely accepted that the main source to the environment are the effluents of wastewater treatment plants (WWTP), where conventional activated sludge treatments are not able to degrade most of them being able to reach surface, groundwater and subsequently, drinking water. Therefore, alternative treatments should be found. One of those alternatives might be the use of microalgae by taking advantage of their capacity for wastewater treatment removing the nutrients contained in the wastewater and further microalgal biomass conversion into biofuels.

The present thesis assesses different factors related to microalgal degradation of emerging contaminants and wastewater treatment. As well as the study of one of the major bottlenecks on microalgal systems, the harvesting, which has the advantage to clarify the treated wastewater and concentrate the microalgal biomass for further biogas production. Pure microalgal cultures and real microalgal effluents have been considered.

First of all, individual degradation of two estrogenic compounds has been studied. The two estrogenic compounds evaluated have been recently incorporated in the priority substances in the Water Framework Directive: 17α-ethinylestradiol (EE2) and 17β-estradiol (E2). Their degradation by *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* pure cultures at laboratory scale conditions has been monitored. To obtain further insights in the mechanism of degradation the transformation products have been identified.

Moreover, the removal of a mixture of 10 PhACs (9 antibiotics and an antidepressant) has also been assessed in pure microalgal cultures at laboratory scale conditions. Transformation products from three chosen PhACs have been identified. One of the antibiotics, ciprofloxacin, was further evaluated. The removal mechanisms have been studied in real algal ponds in both, laboratory and pilot scale reactors.

On the other hand, an algal photobioreactor (PBR) treating urban wastewater has been designed and operated during 6 months. Its performance has been monitored and different operating conditions have been tested. Furthermore, PhACs removal has been evaluated during the steady state and microbial diversity has been identified. The estrogenic compound E2 removal in the PBR has been studied.

The importance of microalgal harvesting has been highlighted in the thesis. Three different harvesting techniques (i.e., natural sedimentation, coagulation-flocculation and the novel technique of co-pelletization using *Trametes versicolor* fungus) have been evaluated using two real microalgal effluents and a pure *Chlamydomonas reinhardtii* suspension.

Finally, methanization of exhausted biomass has been conducted. Prior the anaerobic digestion process microalgal biomass has been submitted to an enzymatic pretreatment for its cell wall solubilisation. Specific and non-specific enzymes have been tested as well as the synergistic effects between an enzymatic mixture. The study is completed valorising fungal biomass coming from the treatment of effluents.

RESUM

Els contaminants emergents (ECs) són un ampli grup de compostos orgànics detectats en diversos compartiments ambientals que degut a la seva presència en el medi ambient i la seva difícil degradació han aixecat un gran interès en la comunitat científica. Tot i que la seva concentració normalment està compresa entre els ng/L i els µg/L, poden representar una amenaça per a la salut humana i el medi ambient ja que són excretats per la orina i els fems, ja sigui en forma de compost actiu o com a metabòlit, ja que no són completament assimilats. D'entre tots els emergents, els compostos disruptors endocrins (EDCs) i els principis actius dels fàrmacs (PhACs) generen especial preocupació. Està àmpliament acceptat que la seva principal font d'entrada al medi ambient és a través dels efluents de les plantes depuradores (WWTP), on els tractaments convencionals de llots actius no són capaços de degradar-ne la majoria, sent capaços d'arribar a les aigües superficials, subterrànies i, posteriorment, a l'aigua potable. Per tant, s'han de buscar tractaments alternatius. Un d'aquests tractaments podria ser l'ús de les microalgues aprofitant la seva capacitat pel tractament d'aigües residuals, eliminant els nutrients de l'aigua residual i la seva posterior conversió en biocombustibles.

Aquesta tesi avalua diferents aspectes relacionats amb la degradació de contaminants emergents i tractament d'aigües amb microalgues. A més de l'estudi d'un dels principals colls d'ampolla dels sistemes de microalgues, la collita, que té l'avantatge de clarificar l'aigua residual tractada i concentrar la biomassa algal per a la seva conversió a biogàs. Tant, cultius purs com efluents de microalgues s'han considerat.

Primerament, s'ha estudiat la degradació individual de dos compostos estrogènics. Aquests dos compostos estrogènics s'han introduït recentment a la llista de substàncies prioritàries de la directiva de l'aigua: 17α-etinileestradiol (EE2) i 17β-estradiol (E2). La seva degradació s'ha monitoritzat a partir dels cultius purs de *Chlamydomonas reinhardtii* i *Pseudokirchneriella subcapitata* a nivell de laboratori. Per tal d'obtenir més informació sobre els mecanismes de degradació s'ha fet la identificació dels productes de transformació.

A més, la degradació d'una mescla de 10 fàrmacs (9 antibiòtics i un antidepressiu) s'ha avaluat en cultius purs de microalgues a escala laboratori. S'ha realitzat la identificació dels productes de transformació de tres PhACs seleccionats. Posteriorment, un dels antibiòtics, la ciprofloxacina, s'ha analitzat. Els seus mecanismes d'eliminació han estat estudiats en sistemes algals, tant en reactors a escala laboratori com en escala pilot.

Per altra banda, s'ha dissenyat un fotobioreactor (PBR) d'algues tractant aigua residual urbana i en operació durant 6 mesos. S'ha monitoritzat el seu seguiment i s'han provat diferents condicions d'operació. A més, un cop a l'estat estacionari l'eliminació de PhACs s'ha estimat i s'ha caracteritzat la població microbiana. També s'ha estudiat l'eliminació del compost estrogènic E2 en el PBR.

En aquesta tesi també es remarca la importància de la collita de microalgues. Tres tècniques de collita (i.e., sedimentació natural, coagulació-floculació i la tècnica innovadora de co-pel·letització utilitzant el fong *Trametes versicolor*) s'han aplicat en dos efluents reals de microalgues i a una suspensió pura de *Chlamydomonas reinhardtii*.

Finalment, s'ha dut a terme la metanització de biomassa exhausta. Abans del procés de digestió anaeròbia, per tal de solubilitzar la paret cel·lular les algues han estat sotmeses a un pretractament enzimàtic. S'han provat enzims específics i no específics, així com l'efecte combinat utilitzant una mescla d'enzims. Aquest estudi es completa amb la valorització de biomassa fúngica que prové del tractament d'efluents.

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Section 3. EMERGING CONTAMINANTS REMOVAL BY MICROALGAE

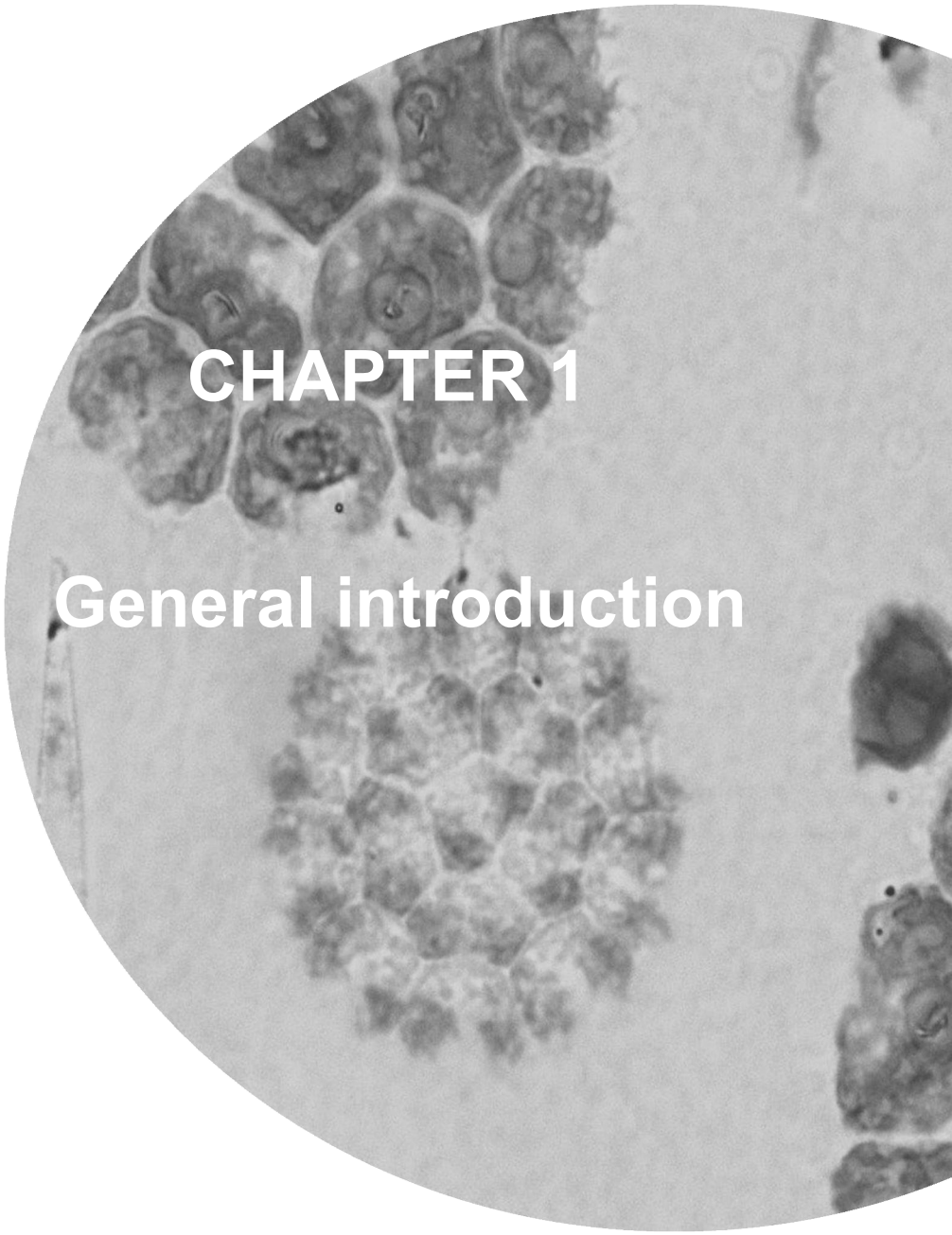
Section 4. BIOMASS VALORISATION

LIST OF ACRONYMS AND ABBREVIATIONS

ADC	Anaerobic digestion centrate
AD	Anaerobic digestion
ADP	Adenosine diphosphate
AOP	Advanced oxidation process
ASP	Aquatic species programme
ATP	Adenosine triphosphate
AZM	Azithromycin
BA	Bioaugmentation assay
BMP	Biochemical methane potential
BOD	Biological oxygen demand
BPA	Bisphenol A
CAS	Conventional activated sludge
CH_4	Methane
COD	Chemical oxygen demand
CPX	Ciprofloxacin
CRM	Clarithromycin
CRT	Cellular residence time
CW	Constructed wetland
DGGE	Denaturing gradient gel electrophoresis
DMP	2,6-dimetoxyphenol
DMSO	Dimethylsulfoxide
DO	Dissolved oxygen
E1	Estrone
E2	17β-estradiol
EC	Emerging contaminant
EDC	Endocrine disrupting compound
EE2	17α-ethinylestradiol
EOM	Extracellular organic matter
EPS	Extra polymeric substances
ERM	Erythromycin
ESI	Electrospray ionisation
FQ	Fluoroquinolone
GC	Gas chromatography
GM	Growth medium
GSI	Global solar irradiance
H_2	Hydrogen
HPLC	High-performance-liquid-chromatography
HPM	Pipemidic acid
HRAP	High rate algal pond
HRT	Hydraulic retention time
IPBR	Industrial photobioreactor
LOD	Limit of detection
LOQ	Limit of quantification
MBR	Membrane bioreactor
MBT	Mechanical biological treatment
MLOD	Method limit of detection

MW	Molecular weight
MWH	Microwave hydrolysis
NF	Nanofiltration
NFX	Norfloxacin
OD	Optical density
OFMSW	Organic fraction municipal solid waste
OFX	Ofloxacin
P	Phoshorous
PAH	Polycyclic aromatic compound
PAR	Photosynthetically active radiation
PBR	Photobioreactor
PCR	Polymerase chain reaction
PE	Polyethylene
PEF	Photosynthetic Efficiency
PEW	Primary effluent wastewater
PFO	Potassium ferric oxalate
PhAC	Pharmaceutical active compound
PP	Propylene
PPCP	Pharmaceutical and personal care products
PVC	Polyvinyl chloride
RITE	Research institute of innovative technology
RO	Reverse osmosis
SMP	Stoichiometric methane potential
SPD	Sulphapyridine
T	Temperature
TBEP	tris(2-tris(chloroisopropyl)phosphate
TC	Total carbon
TFC	Turbulent flow chromatpgraphy
TIC	Total inorganic carbon
TLS	Trilaminar sheath
TMP	Theoretical methane potential
TMT	Trimethoprim
TN	Total nitrogen
TOC	Total organic carbon
TP	Transformation product
TPh	Total phosphorous
TS	Total solids
TSS	Total suspended solids
UF	Ultrafiltration
UV	ultra violet
VFA	Volatile fatty acids
VFX	Venlafaxine
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WSP	Waste stabilisation pond
WW	wastewater
WWTP	Wastewater treatment plant

GENERAL ASPECTS



CHAPTER 1

General introduction

1 General introduction

1.1 Emerging contaminants

Environmental pollution, especially in waters, is increasing concern among the population being more frequent news about painkillers or birth control hormones found in waters. Is there any danger? How will these contaminants affect fish and other wildlife? Should we do something? What should we do? Many water contaminants are the subject of regulations that protect water quality (priority substances), but many more fall into the category of substances for which we do not know the answer to these basic questions. These include substances that have been called emerging contaminants (ECs). Because they are so numerous, diverse and ubiquitous, they are frequently lumped into categories that describe their purpose, use or other characteristic. Some common categories are pharmaceuticals, personal care products, plasticizers, flame retardants, pesticides, etc. The United States Geological Survey (USGS) provides a useful definition of ECs: "*any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects. In some cases, release of emerging chemical or microbial contaminants to the environment has likely occurred for a long time, but may not have been recognized until new detection methods were developed. In other cases, synthesis of new chemicals or changes in use and disposal of existing chemicals can create new sources of emerging contaminants*".

Effects of ECs on human and ecosystem health are largely unknown, and relatively little is known about the ways they travel through the environment or how they may be transformed or degraded in the course of their travels. Moreover, the fact that they do not appear individually, but as a complex mixture, which could lead to unwanted synergistic effects, increases the scientific concern about the need to better understand their occurrence, fate and ecological impact (Petrie et al., 2015). Some studies have shown that even very low exposure to certain ECs can have important impacts on biological systems; estrogenic compounds released to the environment can lead to

feminisation of male fish (Sumpter, 1995), antimicrobial resistance due to the antibiotics exposure is increasing and becoming an issue of concern (Cabello, 2006; Sapkota et al., 2008), reduction of macroinvertebrate diversity in rivers (Muñoz et al., 2009), are some examples of ECs ecotoxicological effects. Moreover, transformation products (TPs) of some compounds such as alkylphenols are even more toxic than the parent products (Soares et al., 2008).

Conventional activated sludge (CAS) treatment plants typically remove organics and pathogens, converting the mass of sewage to common gases and water. These facilities are not designed to remove all ECs so they can be discharged into the environment with treated wastewater effluent, recycled water, and wastewater plant sludge. Emerging contaminants have been detected in surface and ground water (Kümmerer, 2009a, 2009b; Petrovic et al., 2003a). This issue becomes crucial when the wastewater treatment plant (WWTP) effluent is reused for irrigation in agriculture or other purposes. It is necessary to assess the behaviour, fate, and health risks these compounds pose.

Among the emerging contaminants, pharmaceutical active compounds (PhACs) and endocrine disrupting compounds (EDCs) are of special concern. PhACs are designed to invoke specific biological effects in humans or animals at low doses. They include a wide variety of therapeutic families such as: antibiotics, psychiatric drugs, analgesics/anti-inflammatories, tranquilisers, β-blockers and diuretics, and each of them is composed of different chemicals (Petrovic et al., 2003a). They are developed to remain in the human body for an adequate period of time to reach their therapeutic effect, which means low biodegradability (Boxall et al., 2004). Contaminants classified as EDCs are substances that alter functions of the endocrine system and consequently adversely affect the health of exposed organisms (UNEP and WHO, 2013). EDCs include, among others, plasticizers, pesticides, fungicides, surfactants, flame retardants, and PhACs like hormones.

1.1.1 Sources, occurrence and fate in the environment

There are several possible sources and routes for the occurrence of ECs in the aquatic environment (Figure 1.1), however the vast majority of them come from untreated urban wastewaters and WWTP effluents, due to the continuous

entrance of ECs into the aquatic environment. (Jurado et al., 2012; Kümmerer, 2009b; Luo et al., 2014a; Petrovic et al., 2003a).

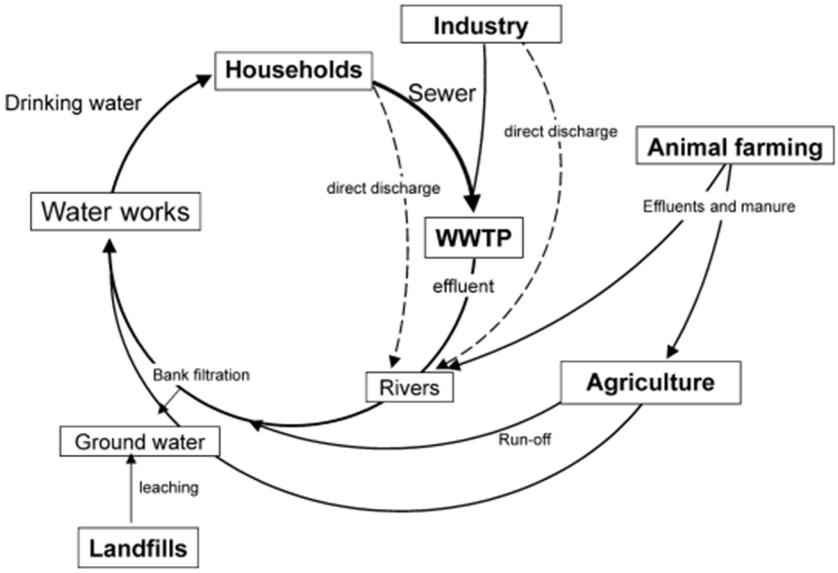


Figure 1.1 Pathways of exposure for ECs (Petrovic et al., 2003a).

PhACs are highly consumed by humans and animals (including their use for: livestock treatments, treatments of companion animals and aquaculture treatments) and partially metabolize them. The non-metabolized part is excreted, as unchanged active compound or in form of metabolites, in the urine and faeces ending up in a wastewater (WW) collection system. A high proportion of these drugs are usually consumed in households, healthcare centres and veterinary facilities (Heberer, 2002; Jones et al., 2005). In livestock farming and aquiculture, direct release may occur (Ellis, 2006). Some unused, surplus or expired drugs may be disposed according to especial waste management. Pharmaceutical industries can also contribute to the release of PhACs to the environment, although scarce data is available (Sim et al., 2011).

The major EDCs release source to the environment are industrial effluents. WWTP have also been identified as one of the main sources of EDCs release to freshwater ecosystems (Petrovic et al., 2002).

Groundwater, according to directive 2006/118/EC, is the largest body of fresh water in the European Union, but it is also the most sensitive, may suffer pollution from many sources, including water leakage from sewer and septic systems, seepage from rivers and application of fertilizers and agrochemicals, among others (Foster, 2001; Vázquez-Suñé et al., 2010). As a result, a wide

range of organic pollutants can be found in aquifers posing a risk to groundwater quality (Wolf et al., 2004). Several studies have proven that some pharmaceuticals are discharged into receiving water bodies (rivers, lakes and seas), which may be used as sources of drinking water (Gros et al., 2012; Ibáñez et al., 2013; Jurado et al., 2012). Hughes et al. (2013) carried out a vast study on pharmaceutical occurrence in freshwaters at national, regional, and global scales and high antibiotic concentrations were found in freshwaters, the highest concentration was reported for the antibiotic ciprofloxacin, with maximum concentration of 6.5 mg/L.

The capability of pharmaceutical ingredients to be absorbed and to interact with living organism makes them a potential hazard for the whole ecosystem (Sangion and Gramatica, 2016). PhACs and EDCs have also been detected in sludges, soils and sediments (Vicent et al., 2013). Sorption on sludge largely depends on their physico-chemical properties, e.g. hydrophobic compounds are more likely to partition in the organic portion of sludge. Although sorption separates compounds from wastewater, it does not result to their elimination from the sludge and therefore not a means of compound removal and new process have to treat them for their removal (Semblante et al., 2015; Verlicchi and Zambello, 2015). Ecosystems of urbanized estuaries are directly threatened by organic contaminants due to direct and in-direct inputs derived from enhanced human activity. Several studies have been carried out to evaluate the occurrence and distribution of diverse PhACs and ECs in river sediments or even coastal areas (e.g., Ebre River (Spain), Pearl River (China), coastal waters off southwestern Taiwan or Belgium) (Claessens et al., 2013; Ferreira da Silva et al., 2011; Jiang et al., 2014; Pintado-Herrera et al., 2016). So far, literature has focused on the environmental threats due to the existence of ECs in sludge-amended soils and several countries have set limit values and have suggested practices to prevent harmful effects on soil, vegetation, animals and humans (Alvarenga et al., 2015; Verlicchi and Zambello, 2015). Limited data soil toxicity is available, in most of the relevant articles the potential risk for the soil environment has been estimated using only aquatic toxicity data (González et al., 2010; Martín et al., 2015, 2012). However, recently a case study based in Greece has been published estimating the potential

environmental risks from the disposal of sewage sludge containing ECs in soil (Thomaidi et al., 2016).

Regarding the presence of pharmaceutical and personal care products (PPCPs) in the environment, the key point is to elucidate if so low concentrations actually possess human or environmental risk or not. Ecosystems as a whole require decades to centuries to recover from contamination. Even today little is known about this subject. There are many types of possible effects of xenobiotics to the aquatic biota. Despite acute toxicity and typical carcinogenic and mutagenic effects, activation of some receptor pathways can cause severe damage to organisms and their ecosystem by altering reproduction, hormonal and/or circulatory systems (Lapworth et al., 2012; Pal et al., 2010; Stuart et al., 2012). Moreover, an especial case is that of the antibiotics, which not only can cause toxic effects to the biota but also there is a growing concern about generation and spread of resistances.

Several attempts for risk assessment and prioritization of emerging contaminants have been performed (Hernando et al., 2006; Murray et al., 2010; Verlicchi et al., 2012). However, the scarcity of data on environmental concentrations, the few species used to identify toxic effects and the issue of the toxicity of mixtures, among others make that a reliable assessment of the potential risk of those compounds is not possible yet. Therefore, more environmental toxicity studies are needed in order to perform an appropriate risk assessment. The Water Framework Directive in its article 16, foresees, at European Union level, a list of priority substances which present a significant risk to or via the aquatic environment, and establishes criteria for the selection of such substances and environmental quality standards. The current list and its related environmental quality standards have been established by Decision No 2455/2001/EC and Directive 2008/105/EC. On January 31st 2012 the Commission legislative proposal for amending the list of priority substances, as the outcome of a review that considered the risks posed by some 2000 substances according to their levels in surface waters, and their hazardousness, production and use. Among the proposed 15 additional priority

substances the pharmaceutical substances (proposed for the first time) included are: 17α-ethinylestradiol (EE2), 17β-estradiol (E2) and diclofenac (EWA, 2012).

1.1.2 Treatments for PhACs and EDCs removal

Most current WWTPs are not designed to treat ECs and a high portion and their metabolites can escape elimination in WWTPs and enter the aquatic environment via sewage effluents, so other treatments are required (Petrovic et al., 2003a). Furthermore, we should be aware that EDCs and PPCPs possess a wide range of chemical properties and thus success in removal varies greatly depending upon their particular properties (Bolong et al., 2009). The partial or complete closure of water cycles is an essential part of sustainable water-resource management and some treatments have been proposed. The most studied have been physico-chemical treatments, although increasing concern is focused on biological treatments.

1.1.2.1 Physico-chemical treatments

In recent years the development and implementation of technologies such as advanced oxidation processes (AOPs), membrane filtration and activated carbon adsorption have been studied to improve removal efficiency of emerging contaminants.

AOPs, which use a combination of ozone with other oxidation agents (UV radiation, hydrogen peroxide, TiO_2) have been highly documented (Fagan et al., 2016; Oller et al., 2011). Differences among treatments have been also detected for the same compound removal. Gimeno et al. (2016) found better results for photocatalytic ozonation than for single ozonation. Filtration technologies, such as nanofiltration (NF), ultrafiltration (UF) and reverse osmosis (RO), have also been highly studied (Dolar et al., 2012; Secondes et al., 2014; Urtiaga et al., 2013). The removal of ECs via adsorption has been studied using activated carbons, zeolites, metal-oxide nanoparticles, clays, and a few other materials (Baccar et al., 2012; Cabrera-Lafaurie et al., 2014; Rivera-Jiménez and Hernández-Maldonado, 2008; Snyder et al., 2007; Sotelo et al., 2014; Yu et al., 2009). However, the performance or capacity has been limited due to size and uniformity of the adsorbent pores, difficulties in achieving

effective functionalization to increase selectivity, and in some instances, even operational costs.

Most of these techniques are applied for PhACs removal, instead from PhACs removal from effluents.

1.1.2.2 Biological treatments

Biological alternative strategies to CAS treatment for the removal of ECs are being studied. They are usually less expensive and present lower energy consumption than physico-chemical ones.

The most popular is the use of membrane bioreactors (MBRs) and it is considered the most promising development in microbiological wastewater treatment. It is already implemented in many WWTPs, although it was not initially designed for that purpose but for space-saving and reduction of sludge production. It was thought that the higher cellular residence time (CRT) and the acclimation of microorganisms could lead to higher removal percentages. Four steroidal hormones (estrone, 17α-ethynylestradiol, 17β-estradiol and 17β-estradiol-17-acetate) were observed to be greater than 90% removed together with other significant hydrophobic compounds, whereas hydrophilic compounds were particularly resistant to MBR treatment (Nguyen et al., 2014). The removal of some antibiotics has also been described in these systems (Dorival-García et al., 2013). It is important to notice that none of these works carried out the identification of TPs, so, part of the ECs removal could be due to sludge sorption.

Fungi have also been considered as potential microorganisms for ECs removal in real effluents. In particular promising results have been reported on the use of ligninolytic fungi. Estrogenic compounds were effectively removed using *T. versicolor* (Blánquez and Guieysse, 2008). Cruz-Morató et al. (2014) pointed out that 46 out of the 51 detected PhACs and EDCs in hospital WW were partially to completely removed from a treatment carried out in a batch fluidized bed bioreactor under non-sterile conditions inoculated with the white rot fungi *Trametes versicolor*. Badia-Fabregat et al. (2016) also used the same fungal strain for the removal of PhACs present in veterinary hospital WW, 66% removal was achieved in a non-sterile batch bioreactor and good removal

percentages were obtained in a short continuous mode operation. Although more research is needed this is a promising process. The use of the fungi on solid-state medium has also successfully reported for PhACs removal on sludge (Rodriguez-Rodriguez et al., 2011; Vicent et al., 2013).

Artificial wetlands have also been proposed for the removal of emerging contaminants due to the cost-effective technology: simplicity of operation and maintenance, low environmental impact, low or no energy cost, low waste production and ability to be integrated into the landscape (Ávila et al., 2014b). However, they have some drawbacks, such as the high land-use and degradation percentages similar to those obtained with CAS treatments (Li et al., 2014; Matamoros and Bayona, 2006; Verlicchi and Zambello, 2014). Although good removal have been observed for some ECs (Ávila et al., 2015, 2014a, 2014b; Matamoros et al., 2007a).

The use of microalgae is another one of these alternatives and their application as decontaminating agent together with WW treatment has been gaining concern. This treatment is explained in detail in the next section as it is the one studied in this thesis.

1.2 Microalgae

1.2.1 Algae history

The study of algae (phycology) has been widely considered since the 1930's when the first publications appeared (Chu, 1943, 1942). However, 160 years ago the first laboratory working on microalgae cultures appeared (Cohn, 1850; Famintzin, 1871), works on larger scale algal cultures began in United States of America (USA) in 1948-1950 and the first significant outdoor pilot plant studies on the production of *Chlorella* were carried out in 1951 (Massachusetts, USA) (Image 1.1), but was not until the 1950's that the commercial farming cultures appeared (Image 1.1). Algae are defined as organisms with a thallus not differentiated into roots, stem and leaves, contain chlorophyll *a* and able to use solar energy and fix carbon dioxide (CO_2) to produce compounds (Borowitzka and Moheimani, 2013; Lee, 2008). Microalgae have been described as

microscopic algae *sensu stricto* and in applied phycology the term is referred to microscopic eukaryotic algae and oxygenic photosynthetic bacteria (i.e., cyanobacteria). The origin of applied phycology date back to the establishment of a culture of *Chlorella* by Beijerinck (1890). *Chlorella* is the most well-known and reported microalgae, including the first book published about its culture from laboratory to pilot scale (Buriew, 1953).

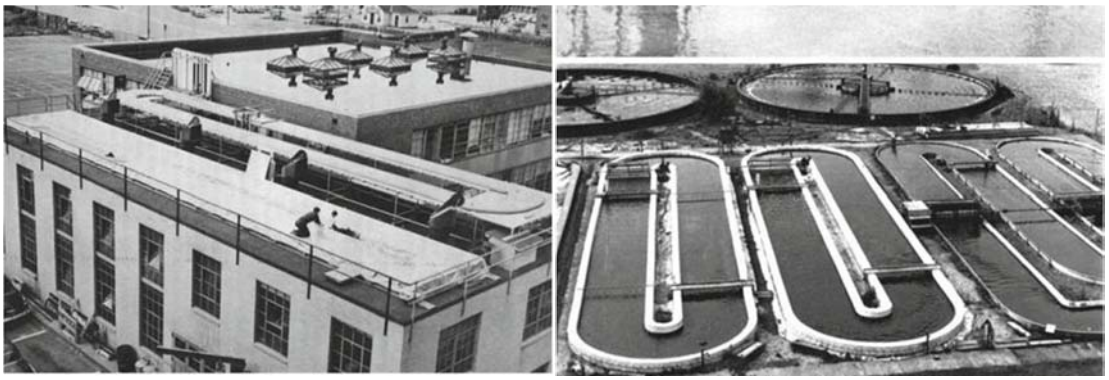


Image 1.1 Early large-scale algae culture systems. Left: tube-type reactors on the roof of the building at Cambridge, Massachusetts, USA in 1951. The first unit is in operation and the second is under construction (Anon, 1953). Right: Outdoor algae ponds at the Gesellschaft für Strahlen-und Umweltforschung, Dortmund, Germany. Raceway ponds are 20 m lond and circular ones 16 m diameter (Soeder, 1976).

Over the years, microalgae uses have been changing according to human necessities and the knowledge obtained. Microalgae have been considered as a promising new source of biomass, complementing the conventional agricultural crops. Moreover, the fact that they do not require fertile land for the cultivation is a great advantage, as well as most of the biomass can be valorised, due to high content of proteins and lipids and a low content of structural carbohydrates (Lam and Lee, 2012). During the first decades of 20th century, microalgae were thought as a possible source of food, solving world nutrition, thus the aim of the firsts works was to use the green alga *Chlorella* for large scale production of food (Buriew, 1953; Stanford Research Institute, 1950). However, during this early period of algal cultivation, technological advances and nutritional evaluations received major attention, having an effect on the aspirations related with the use of algae as supplementary food for human consumption, due to the widespread criticism on the exploitation of unconventional protein sources and microbial proteins. Therefore, a change in the use of algae was adopted and an extensive research was carried out

looking for other applications (e.g., animal feed, biofertilizers, or soil conditioners). During that period of time, techniques for algal cultivation were successfully developed, obtaining great results and widening the knowledge (i.e., photosynthesis, biology, nutrition, physiology, engineering, inhibiting parameters). However, problems came up, especially with large scale production and the requirements for the co-operation of microbiology with other scientific disciplines (e.g., engineering, economy, analytical chemistry, etc.). Microalgae can supply several nutrients (vitamins, antioxidants, highly digestible proteins and essential fatty acids) and research has been focused in that area creating tablets, powder, liquids or food containing microalgae strains (*Spirulina* and *Chlorella*) because they can contribute to improve nutritional health and well-being (Petracci et al., 2009; Plaza et al., 2008). The first commercial large scale culture of *Chlorella* commenced in the early 1960's in Japan, followed by a harvesting and culturing facility of *Spirulina* in Mexico (early 1970's). Since that moment, several plants were built mainly located in Asia and USA, producing great amounts of *Chlorella*, *Spirulina* and *Dunaliella salina* as a source of β-carotene (Dodd, 1979).

In the 1960's decade the need to find out new alternatives to energy sources arose, considering microalgae production as a source of renewable energy due to its conversion into valuable biomass. Oswald and colleagues at the University of California, focused on the large scale culture of algae for biomass production and for WW treatment (Oswald and Golueke, 1960; Oswald et al., 1957). An algal pond of 10^6 L of capacity was constructed. From that moment several ponds were constructed carrying out microalgal research projects. For example, a 300 m^2 WW treatment algal pond located in Jerusalem was modelled on Oswald's design by Shelef et al. (1973).

During the same decade, Oswald and Golueke (1960) proposed as source of energy microalgal biomass to produce biofuels. That statement, together with previous reports (Benemann, 1978) confirming Oswald and Golueke (1960) results lead to the development of the "Aquatic Species Programme" (ASP, 1980) (US Department of Energy) that considered algae as competitors with fossil fuels, as they contain lipids convertible to fuels. The findings lead to the large scale algae cultivation during long periods of time, where the engineering

and economic issues were scarce, not limiting the operation of the system. On that time, it was demonstrated that laboratory strain selection was not as productive as the strains grown spontaneously in the ponds (Sheehan et al., 1998). *Botryoccus braunii* was discovered as a long-chain hydrocarbon producer, because of the high presence of lipids, it was compared to fossil oils and it was considered a source of renewable fuels. However, its slow growth dismissed it as a candidate for commercial biofuels production (Casadevall et al., 1985; Wake and Hillen, 1980).

In the 1990s an innovative programme including projects at the Research Institute of Innovative Technology for the Earth (RITE) was developed by the Japanese Ministry of International Trade and Industry through the New Energy and Industrial Technology Developments Organisation. The objective of the programme was to find out clean methods of biological fixation of CO_2 based on the effective integration of photosynthesis functions of microorganisms (i.e., bacteria and microalgae). From that moment, the firsts publications on the subject appeared (Hanagata et al., 1992; Kurano et al., 1995). New findings were discovered, the growth of microalgae for biofuels using power station flue gas as a CO_2 source was the most remarkable one (Negoro et al., 1992, 1991) as well as different ways to upgrade biogas production (Toledo-Cervantes et al., 2016; Yan et al., 2016).

From that time microalgae were considered as a potential source of fermentable substrate, obtaining three different biofuels: biogas, biodiesel and biohydrogen.

Biogas is the product of the anaerobic digestion of organic matter and can be obtained from domestic sewage, industrial waste, wastewater, animal waste, organic fraction municipal solid waste (OFMSW) or aquatic biomass, such as macro and microalgae (Gunaseelan, 1997; Omer and Fadalla, 2003).

Biodiesel is the conversion of the fatty acids that microalgae produce. It is a renewable, biodegradable, nontoxic and environmentally friendly fuel. Biodiesel has the advantage that it emits 78% less CO_2 when burned, 98% less sulphur and 50% less of particulate matter emissions (Brown and Zeiler, 1993).

Biologically hydrogen gas (H_2) production was widely studied in the 1970's, however the firsts discoveries began in the 1940's when the production of H_2

was possible from unicellular green algae cultures under light irradiation (Gaffron and Rubin, 1942; Gaffron, 1939). Production can be increased according to the carbon content in the biomass; also the culture should be in closed systems, allowing the capture of hydrogen gas (Benemann, 1997). This biomass can be burned to produce energy because the calorific value of these microorganisms is greater than that of some charcoals.

According to the chemical composition of microalgae, the biomass may have several applications, apart from the biofuels described. Algae have been considered for providing both novel biologically active substances and essential compounds for human nutrition or pharmaceutical industry, increasing their interest. The production of drugs by microalgae strains is still in development, some can produce antibiotic, antifungal or antiviral substances. While others produce substances which have an important role in the prevention of oxidative stress-related diseases (i.e., cancer and neurological disorders), among others (Custódio et al., 2015, 2012; Richmond, 1990). Pigments from microalgae have been used as natural colorants in food products, and in some countries artificial dyes have been replaced by biodyes (Pandey et al., 2014). Bioplastics could also be obtained from microalgae, since they are able to produce polyhydroxyalkanoates (natural polyesters consisting of units of hydroxyalkanoic acids with similar properties to petrochemical plastics) (Jau et al., 2005). Some microalgal strains are able to fix nitrogen, which is why they have been studied as a potential source for biofertilizers applied on the agriculture.

Little research has been focused on removal of ECs in microalgal-based systems, despite thousands of communities worldwide depend on algal pond systems for WW treatment (Norvill et al., 2016). As previously described, the most popular microalgal-based systems for WW treatment are open systems with high hydraulic retention times (HRT). Microalgae have been previously described as good candidates for heavy metals removal (Perales-Vela et al., 2006; Wilde and Benemann, 1993), then the interest of these microoganisms for ECs removal has been considered. Laboratory scale studies include removal of a wider range of ECs, while at pilot scale or full scale the ECs studied are more limited.

Regarding laboratory scale studies, the aromatic compound salicylate has been demonstrated to be effectively removed in an algal-bacterial microcosm at laboratory scale by Borde et al. (2003). Cephalosporins removal has been studied using a novel system combining alga-activated sludge and high removal percentages were achieved (>89%) (Guo and Chen, 2015).

Concerning pilot scale and full scale studies the antibiotic tetracycline removal has been evaluated by de Godos et al. (2012) in a high rate algal pond (HRAP), results demonstrated that the main removal mechanisms was photodegradation. Recently, Matamoros et al. (2015), studied the removal of 26 ECs contained in WW that entered into a 470 L HRAP, the removal efficiency ranged from negligible removal to more than 90% depending on the compound.

A more exhausted research is included in each chapter introduction.

1.2.2 Microalgal systems

A conventional microalgae production system consists of (1) growth and cultivation, (2) biomass harvesting and dewatering, (3) extraction/conversion of the biomass to the product of interest (Figure 1.2). This process should be energetically, environmentally and economically sustainable.

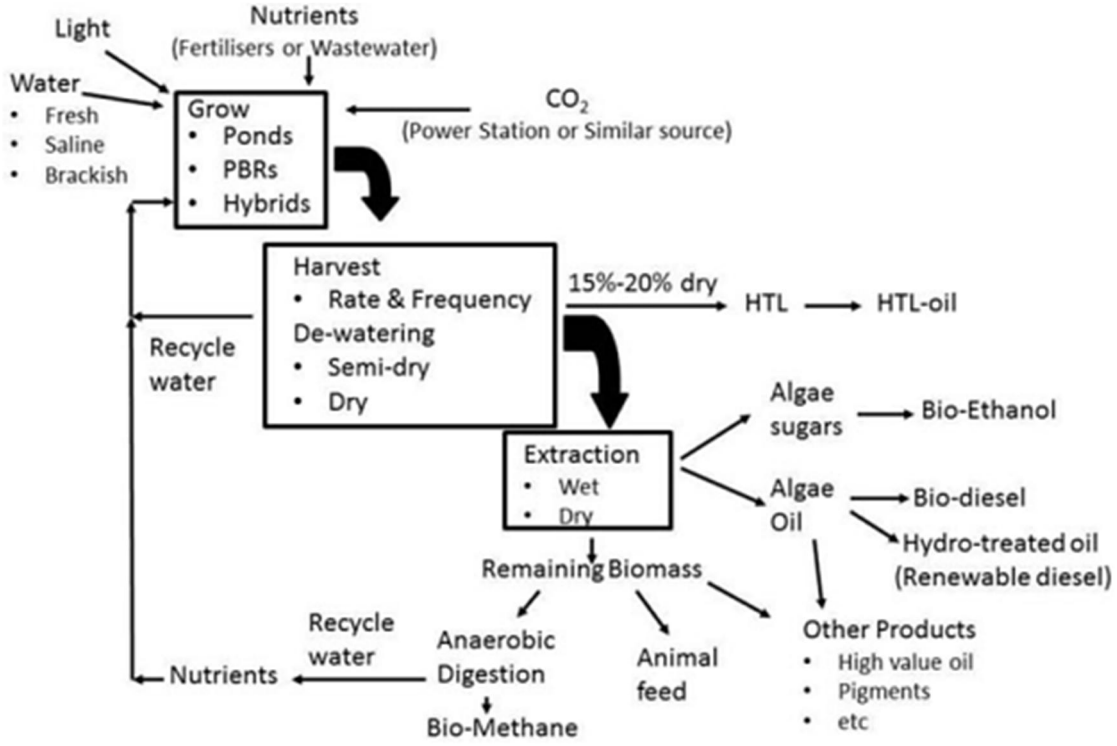


Figure 1.2 Microalgae production flow sheet (Moheimani et al., 2015).

1.2.2.1 Microalgal growth and cultivation

Under phototrophic growth conditions, microalgae absorb solar energy and assimilate CO_2 from the air and nutrients from aquatic habitats. The efficiency of photobioreactors (PBRs) is determined by the integration of light capturing, light transportation, light distribution and light utilization by microalgae through photosynthesis (Zijffers et al., 2008). But there are several factors affecting microalgae growth.

Carbon source is usually the most critical and microalgae could be grown under photoautotrophic (use of inorganic compounds, e.g., CO_2, bicarbonates), heterotrophic (use of organic carbon in the presence or absence of light supply) and mixotrophic (combination of the previous) conditions (Chojnacka and Noworyta, 2004; Xu et al., 2006). Nitrogen is the main nutrient, a limitation of it induces lipid formation since proteins are decomposed and converted to energy-rich products (i.e., lipids) (Hu et al., 2008). Nitrogen starvation is a common condition when biofuel from microalgae is the main purpose. The type of light source and the intensity could be critical factors for microalgal growth. The saturation light intensity needs to be distributed throughout the entire culture, but when shading effects occur inside the photobioreactor, mixing can reduce this effects (Chen et al., 2008). Outdoor systems have temperature variations that greatly depend on the light exposure (i.e., day/night cycle) and seasonal changes (Wang et al., 2012). The pH of the culture medium affects the biochemical reaction characteristics of microalgae. pH variations occur due to CO_2 consumption, when CO_2 from the gas phase is transferred into the culture medium, some of the CO_2 gas will dissolve and become soluble phase (HCO_3^-) which highly depends on the culture pH, the optimal has been described to be in the range of 7-9 (Miller et al., 1990; Wang et al., 2012). pH increases with photosynthetic drawdown of carbon and decreases overnight with respiration (Sutherland et al., 2015).

The choice of the reactor is one of the main factors that influence the productivity of microalgal biomass. Microalgae can be grown in two main types of systems: closed photobioreactors and open ponds.

Closed systems

Much attention has been paid to closed photobioreactors due to the high productivity achieved in cultures. Many designs have been included at laboratory or pilot scale (Image 1.2): vertical reactors, flat plate, annular, plastic bags, green wall panel, tubular. However, the scale up is limited by the light irradiation, the shading effect is higher and efficiency may be decreased (Ugwu et al., 2008). These systems are the most suitable for pure microalgae strains culture and its design must be carefully optimized for each individual strain according to its unique physiological and growth characteristics. Closed algal systems are not exposed to the atmosphere and are covered with a transparent material or contained within transparent tubing. One of the main advantages from that kind of photobioreactors is that there is no evaporation, as well as the contamination risk is reduced, moreover, CO_2 losses are more limited and are highly efficient for its fixation. All this facts lead to more reproducible conditions. Photosynthetic efficiency is increased, thus higher production of biomass. However, some of these systems may have a high initial cost for its construction and operating and maintenance may also be expensive, limiting the use of such systems for the production of high-value algal products (Chen et al., 2011). Usually, a specific microalgal strain is cultivated in order to obtain the desired product, this is the main reason why different types of closed photobioreactors have been developed in recent years. One of the firsts algal closed photobioreactors was built by Gudin (1976) as a pilot plant with a total working volume of 6.5 m^3. The plant was operated for several years culturing *Porphyridium cruentum* (productivity: 20-25 g/(m^2·d)) for the production of an exocellular sulphated polysaccharide used as a thickening agent.

Vertical column PBRs are made of vertical tubing with a gas sparger system installed at the bottom of the reactor as agitation system. In this category bubble column and airlift reactors are included. Flat panel PBRs are simple and advantageous in terms of mass production. Circulation is achieved by means of rising air bubbles. Nevertheless, high area is required and difficulties in cleaning arise. In order to reduce equipment costs, transparent bags (i.e., plastic) have been located on rigid frames (Tredici and Rodolf, 2004). Tubular systems are widely used, they are made of transparent pipes with small internal diameters,

which hurdles the cleaning. Circulation is provided by means of an air pump. The increase of working volume can easily be achieved by extending the tube length.

Image 1.2 Microalgal closed photoreactors. Top: vertical column; Bottom left: flat panel; Bottom right: horizontal tubular.

Open systems

Open ponds are the most usual setting for large scale outdoor microalgae cultivation due to their low cost of investment and operational capital. The major commercial production of algae is based on open channels stirred with a paddlewheel (raceway) which are less expensive and easier to build and operate. However, there are high contamination risks and low productivity, induced mainly by poor mixing regime and light penetration and the difficulty to control operating conditions. Evaporation losses have to be considered in open systems, temperature fluctuations, nutrient and light limitation, inefficient homogenization and lower biomass production yield (Brennan and Owende, 2010). These kinds of systems are the most suitable for WW treatment.

Three main types of algal ponds have been used for wastewater treatment, those are facultative ponds, maturation ponds, and high rate algal ponds (HRAP) (Image 1.3). Facultative and maturation ponds are often collectively referred to as waste stabilization ponds (WSP) or WW treatment lagoons and are widely used (Crites et al., 2006; Heaven et al., 2003; Shilton and Walmsley, 2005). The main difference among them is the depth as well as the origin from the influent. While facultative ponds are 1-2 m deep, they receive raw WW or anaerobic waste effluent, maturation ponds are shallower, 1-1.5 m deep and receive effluents from conventional WW treatment, there are considered on the tertiary treatment from WWTP, with the main objective of pathogen removal (Norvill et al., 2016). A major disadvantage of those systems is the relatively large land requirement compared with electromechanical treatment systems (e.g., activated sludge) (Craggs et al., 2012).

HRAPs retain the advantages of conventional ponds (simplicity and economy) but overcome many of their drawbacks (poor and highly variable effluent quality, limited nutrient and pathogen removal), and have the added benefit of recovering wastewater nutrients as harvestable algal/bacterial biomass for beneficial use as fertiliser or biofuel (Craggs et al., 2012). Moreover, the recycling of the medium has been implemented in order to reduce evaporation issues and enhance the reutilization of the nutrients from the culture (Handler et al., 2012). This system is considered the cheapest method of large scale microalgal production, HRAPs only require low power and are easy to maintain and clean (Chisti, 2008; Ugwu et al., 2008). They are between 0.2 and 0.5 m deep and pond water is mixed by means of a paddle wheel to ensure homogenization of culture in order to stabilize the microalgal growth and productivity; may be constructed of concrete, glass fiber or membrane (Brennan and Owende, 2010). The influent they receive comes from a previous waste treatment pond, effluents following screening or settling processes, or even raw WW (Norvill et al., 2016). HRAP combined with gravity settling pretreatment (e.g., primary clarifier) of raw wastewater (to remove organic solids) and post treatment of HRAP effluent (to remove algal/bacterial biomass), followed by additional effluent polishing if required would fit within the footprint of an existing two-pond oxidation pond system (Craggs et al., 2012). HRAPs are operated

under long HRT (8-14 d) to enhance phototrophs growth, which will provide oxygen for aerobic biodegradation and consume CO_2 during the day due to the photosynthesis. Moreover, daily variations in pH and dissolved oxygen (DO) occur due to the change in sunlight intensity and therefore, photosynthetic activity and pond temperature have a direct influence on the maintenance of cell density limiting the growth (García et al., 2006; Harun et al., 2010; Picot et al., 1993). Pond performance may also be highly affected between seasons due to environmental conditions and the ratios of algal and bacterial biomass dominance will be affected (García et al., 2000a; Mara and Pearson, 1998). Algae produce oxygen and organic acids for bacteria, and bacteria produce CO_2 and vitamins for algae, although competition for nutrient may be present (Liu et al., 2012; Norvill et al., 2016). Since the increasing awareness for ECs occurrence in WW, HRAPs have been studied for its removal (García et al., 2006; Matamoros et al., 2015).

Image 1.3 Microalgal open systems. Left: facultative pond; Right: High Rate Algal Pond.

1.2.2.2 Microalgal biomass harvesting

A major economic hurdle is the separation (harvesting) of the microalgae from their growth medium and then concentrate them, as the costs of harvesting typical account for 20–30% of the total production cost (Christenson and Sims, 2011; Gudin and Therpenier, 1986; Molina Grima et al., 2003). Harvesting is considered the bottleneck process for the downstream production of biofuels and other algal end products and research is still focused on that area in order to develop a reliable and cost effective method. Microalgal separation from the

aqueous growth medium is difficult as the low density of the cultures have a similar specific gravity to that of their medium and the small cell size. Moreover, hydrophobicity of the culture, salinity and medium composition could influence the separation method (Barrut et al., 2013; Milledge and Heaven, 2013; Uduman et al., 2010). Danquah et al. (2009a) reported that microalgal surface is negatively charged and the cells carry algogenic organic matter, which keeps stable their dispersed rate. Harvesting plays an important role in the energy requirement for the thickening process, as a consequence, different methods have been studied, namely: centrifugation, filtration, membrane separation, gravity sedimentation, flocculation, flotation, co-pelletization (Barros et al., 2015; Chen et al., 2011; Gerardo et al., 2015; Milledge and Heaven, 2013; Uduman et al., 2010). However, these methods have either technical or economical limitations such as: energy costs, flocculant toxicity, non-feasible scale up, density of the biomass slurry, fouling during membrane filtration, and methods are both species and product specific (Molina Grima et al., 2003; Oh et al., 2001; Rawat et al., 2013). An ideal harvesting process for full scale operation should be effective for the majority of microalgal strains and should allow the achievement of high biomass concentration, while reducing the costs of operation, energy and maintenance, as well as it must be non-toxic or non-contaminant for the biomass and allow the WW reuse of the culture medium (Danquah et al., 2009a, 2009b). Several authors reported advantages and drawbacks of the various methods for microalgal biomass harvesting (Barros et al., 2015; Milledge and Heaven, 2013).

A brief description of the most studied methods will be described as follows.

Sedimentation is commonly applied due to its simplicity and low costs, although it is a very slow process. Some advantages include the recycling of the water rich in nutrients, no potentially toxic chemicals are used and microalgae structure maintains its integrity because of the absence of turbulent flows or high pressures (Gerardo et al., 2015; Rawat et al., 2013). The process is described by Stoke's Law, gravitational forces cause solid particles to separate from a liquid of different density when frictional force becomes equal to the net gravitational force (Shelef and Sukenik, 1984). The main disadvantage of the process is low cell recovery because settling velocity varies between species

(density and radius of microalgae cells influence on the settling velocity), moreover, it can be altered within the same specie (Chen et al., 2011; Milledge and Heaven, 2013). Average settling velocities have been proposed by several authors ranging from 0.024 to 2.2 m/d (Barros et al., 2015; Gerardo et al., 2015; Milledge and Heaven, 2013). This method has not been widely used for separation due to the low recovery obtained and it has not yet been achieved on a large scale, only in combination with other harvesting techniques (Uduman et al., 2010).

Coagulation-flocculation is a process in which dispersed particles are aggregated together to form large particles for settling, thus overcoming the hurdle of repulsion with equicharged particles (Chen et al., 2011; Gerardo et al., 2015). Microalgae are kept in suspension due to the negative surface charge (Cheng et al., 2010). It has been suggested as a superior method to separate algae as it can handle large quantities and a wide range of microalgae species (Uduman et al., 2010). The ideal flocculant should be inexpensive, nontoxic and effective in low concentrations, preferably be derived from non-fossil fuel sources, sustainable and renewable (Molina Grima et al., 2003). Chemical flocculation commonly used in WW treatment implies the use of metal salts. To remove metal salts microalgae have been proven to be cost-effective, nevertheless, they may be toxic, contaminating the end product, they are highly sensitive to the pH level and are not feasible for all microalgal species (Chen et al., 2011; Molina Grima et al., 2003; Schlesinger et al., 2012). Also, they can have negative effects on microalgal viability and can colour and modify the growth media, which may prevent the recycling and reuse (Papazi et al., 2010; Schenk et al., 2008). Polymers can also be used, although they are more expensive, contamination and influences downstream are largely avoided (Laamanen et al., 2016). pH induced flocculation is also a recurrent technique, it has been described that microalgae can spontaneously flocculate when pH increases above 9 (Laamanen et al., 2016; Spilling et al., 2011). The medium pH rises naturally during cultivation due to the consumption of CO_2 (Uduman et al., 2010). Electro-coagulation-flocculation has also been studied by several authors obtaining good results with lower energy requirements than centrifugation (Poelman et al., 1997; Uduman et al., 2010; Vandamme et al.,

2011) Finally, flocculants derived from renewable animal material have also been studied. The most widely applied is chitosan due to its versatility on an extensive range of freshwater microalgae and it does not contaminate the microalgal biomass, but dosages are considerably higher than with synthetic flocculants (Divakaran and Pillai, 2002; Harith et al., 2009; Molina Grima et al., 2003). Recently, research has also been focused on the use of natural flocculants, such as *Ecotan* and *Tanfloc* studied by Gutiérrez et al. (2015), obtaining 90% biomass recovery.

Centrifugation is based on phase separation of microalgal biomass from aqueous solution when a centripetal force is applied based on the cell size and density difference between the biomass and the medium (Gerardo et al., 2015; Rawat et al., 2013). It is widely used for almost all strains obtaining high recovery rates, moreover is free from coagulants, flocculants or chemicals (Gerardo et al., 2015; Laamanen et al., 2016; Milledge and Heaven, 2013). However, due the energy requirement the operation is costly and makes the harvesting using centrifugation unfeasible at full scale and its application is limited to high-value products, although less energy consumption would be achieved if a combination of techniques are applied for pre-concentrating the microalgal culture (Barros et al., 2015; Milledge and Heaven, 2013; Rawat et al., 2013). Another limitation of such operation is the cell structure damage, and valuable materials will be lost into the medium due to the exposure of microalgae cells to high gravitational and shear forces (Barros et al., 2015; Chen et al., 2011; Gerardo et al., 2015).

Flotation is another harvesting method often defined as “inverted” sedimentation and used in WW treatment. Gas bubbles are fed to the broth which provide the lifting force needed for particle transport and separation, as long as particles in suspension are hydrophobic, in order to be attached to gas bubbles (Barros et al., 2015). In order to increase its efficiency, usually the addition of flocculants, coagulants or surfactants is adopted (Gerardo et al., 2015). It is more effective than sedimentation and faster. Moreover, lower space is required, operation times are relatively low, presents a small footprint and it can be applied on a diverse number of species once the operation is optimized (Barros et al., 2015; Henderson et al., 2010; Rubio et al., 2002). Depending on the bubble

production process energy requirement could be increased (Gerardo et al., 2015; Milledge and Heaven, 2013).

Filtration is based on a solid-liquid separation process where a semi-permeable filter acts as a barrier and a pressure is applied to force the liquid through the membrane. Several processes have been described depending on the microalgal cell size as well as the end product. The main drawback for all of them is membrane fouling and clogging, it occurs as a result of pore blocking and cake formation, reducing its application at full scale (Gerardo et al., 2015; Rawat et al., 2013). Micro-filtration and ultrafiltration have been described for small microalgae cells harvesting, but they require high energy and intermittent membrane replacement, increasing the operational costs (Molina Grima et al., 2003; Rawat et al., 2013, 2011).

New biological technologies have been recently considered. That is the case of flocculation of microalgae by adding bacteria, in that process an addition of extra organic substrate is required to enable their growth, but no chemicals are added. Microalgae and bacteria may form aggregations that are able to settle quicker than single microalgae due to their larger size, and there is no need of coagulant or flocculant addition (Barros et al., 2015; Van Den Hende et al., 2014a, 2011a). However, more research is needed in order to get through the disadvantages such as the influence of dominant microalgae species or WW type (Van Den Hende et al., 2014b, 2011a, 2011b). Similarly, fungal assisted microalgal flocculation has been described as an effective system for microalgal harvesting and successfully applied in WW treatment (Gultom and Hu, 2013). Some fungal species can pelletize entrapping the microalgal cells, this facilitates its harvesting by simple sieve filtration or sedimentation in most cases. Those techniques are sustainable and contribute to the effective WW treatment (Zhou et al., 2012).

1.2.2.3 Extraction and further downstream process

Low cost scalable extraction methods for lipids and sugars for subsequent conversion to added value products at industrial scale are still in development. Most of the methods are at bench scale and there is a high energy requirement that unbalances the process. Cell disruption is essential for efficient extraction

of desired components that constitute the microalgal cell; mechanical (i.e., bead mills, sonication, autoclaving, among others) and non-mechanical methods (freezing, osmotic shock, enzymatic digestion, use of solvents or acid or base reactions) have been widely applied (Fon Sing et al., 2013). Lipid extraction is mandatory for biofuel production, once the lipids are extracted biodiesel is produced through a transesterification reaction in methanol with an acidic or an alkaline catalyst (Kim et al., 2013a). Pigments extraction is also of great importance, microalgae have three main pigments: (1) phycobiliproteins, that are high-value natural products with actual and/or potential biotechnological applications in nutraceuticals and pharmaceuticals, food and cosmetic industries as well as in biomedical research and clinical diagnostics (Manirafasha et al., 2016); (2) chlorophyllins, that have been used to control body odour of geriatric patients and dietary supplement, several studies found that chlorophyll and chlorophyllin have also antimutagenic and anticarcinogenic action (D'Alessandro and Antoniosi Filho, 2016); (3) β-carotene, that is an orange-yellowish pigment with growing demand, being used as colorant for food or nutritional supplement (D'Alessandro and Antoniosi Filho, 2016).

1.2.3 Microalgal-based industries in Spain

Some microalgal-based industries have started up in Spain and the microalgal market is in increasing development. The most important ones are going to be presented, as well as some WWTP which have incorporated microalgal units as part of their process.

AlgaEnergy is a technology-based company specialized in the field of microalgae biotechnology. It is managed by a team of entrepreneurs and scientists with solid track, proven success and extensive experience within their respective fields. The company is located in Madrid. The mission is to develop novel products derived from microalgae and to produce them in an economically profitable way. Its activities are focused on developing natural, sustainable and effective solutions for the following sectors: aquaculture, agriculture, nutrition, cosmetics, biofuels and technology ("AlgaEnergy," 2014).

Aqualgae develops complete solutions for microalgae production or research, from the simple supply of starter cultures and scale up solutions to

photobioreactors and industrial production plants. The company is located in A Coruña. Their products and services include: production of large scale microalgae plants, the design of photobioreactors and race ways, provide and implement all the process control and analytical equipment as well as all type of materials and laboratory equipment needed to cultivate, count, analyse microalgae, they offer easy solutions to scale up cultures, they provide strains and microalgae starter cultures as well as culture media, finally their service include scientific and technical consultancy (“Aqualgae,” 2016).

Algamoil was the first the continuous algae production system worldwide, set in Italy. Its aim is the production of vegetable oil from microalgae cultures to make biofuels and other subproducts. In 2015 it was stablished in Madrid (“Algamoil,” 2015).

Fitoplancton marino was stablished in 2002 in Cadiz and its mission is to improve the health of society by providing healthy products and functional ingredients derived from microalgae. The company generates high value-added products from microalgae with the commitment to be sustainable and economically viable. They are specialised in aquaculture, aquariology, cosmetics and human nutrition (“Fitoplancton marino,” 2002).

Algalimento is stablished in the Canary Islands and offers products based on microalgae, aiming aquaculture, aquariums needs animal and humans food. It is open to collaborate with other companies in the food industry, pharmaceutical and nutraceutical for the development of high value-added products (“Algalimento,” 2015).

Greenaltech is a company based in Barcelona composed by a team of scientists in green and molecular biology, microalgae, genomics, bioinformatics, chemistry and engineering who have created a powerful technology platform to discover and manufacture bioactives from microalgae. Some uniquee natural molecules with massive potential for the cosmeceutical, nutraceutical and pharmaceutical markets have been identified (“Greenaltech,” 2014)

ASN is one of the major *Spirulina* producers in Europe. Mountain spring water from Sierra Espuña (Murcia) is used for its cultivation, then it is naturally dried

and a wide range of products are commercialized (e.g., tablets, powder) (“ASN Leader,” 2016) (Image 1.4).

Image 1.4 Left: Raceway for Spirulina cultivation; Right: Commercial Spirulina (Courtesy of A.S.N. Leader S. L.).

Some electric companies have also been introduced into the algal based technologies. This is the case of the electric company Endesa, which in 2011 launched a sustainable initiative combining the CO_2 fixation by microalgae from industry emissions with biofuels production from its biomass.

The water company *Aqualia* was the promoter of the *All-gas* project, which aim is to convert algae into energy using wastewater. It was successfully grown its crop of algae biomass in Cadiz. The biomass obtained shows a particularly high-energy potential relative to its digestibility level, with a methane production capacity of around 200-300 L of gas per kilogram of biomass processed by anaerobic digestion. The microalgae also allow the purification of wastewater to a high standard. The biofuel produced by the *All-gas* project is expected to power 200 vehicles by 2016.

1.3 Biomass valorisation in effluent treatment systems

The production of renewable fuels is becoming increasingly important as the supply of petroleum reserves diminish and environmental consequences resulting from fossil fuel combustion become more severe. Fuels produced from biomass have the potential to reduce reliance on petroleum resources and reduce greenhouse gas emissions.

Microalgae have been identified as a promising renewable energy feedstock because of their high photosynthetic efficiency and improved land use. This effective conversion of solar energy results in biomass productivities that are significantly greater than terrestrial biofuel feedstocks (Mandal and Mallick, 2009; Minowa et al., 1995; Oswald, 1995). However, some limitations arise, including biomass massive production, low cost harvesting methods and low energy conversion downstream processes.

Biofuel from microalgae is obtained from the extraction of certain organic macromolecules that compose microalgal cells (Figure 1.3). For instance, lipids are extracted from biomass for biodiesel production, while carbohydrates are used for the production of bio-hydrogen and bioethanol. On the other hand, biogas may be either produced from the whole biomass residue and also could be used after lipid extraction for biodiesel production, although methane (CH_4) production is reduced.

Several authors pointed out that biogas generation, throughout anaerobic digestion was the most practical process for converting algae into energy, in this process wet biomass can be used and, therefore, no extensive drying methods are required (Campbell et al., 2009; Sialve et al., 2009; Wiley et al., 2011).

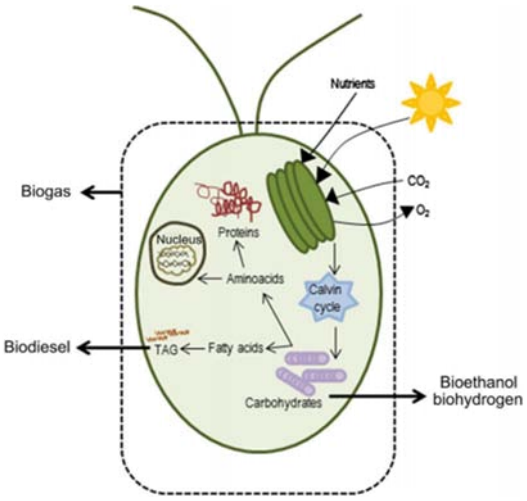


Figure 1.3 Biofuel production from microalgae (Passos et al., 2014a).

1.3.1 Anaerobic digestion

The anaerobic digestion (AD) process consists on the biological oxidation of organic matter by the action of specific microorganisms (in the absence of

atmospheric oxygen). Organic matter is converted into stable end products at the same time, and the process generates biogas (mainly composed of 65% methane and 35% carbon dioxide, other gases such as nitrogen, nitrogen oxides, hydrogen, hydrogen sulphide and ammonia are also formed in less than 1% (Angelidaki and Sanders, 2004)) that can be valorised energetically. Anaerobic digestion occurs in four main steps (Figure 1.4): hydrolysis, acidogenesis, acetogenesis and methanogenesis, obtaining biogas. Biogas is a renewable and versatile bioenergy source used for electricity and heat cogeneration.

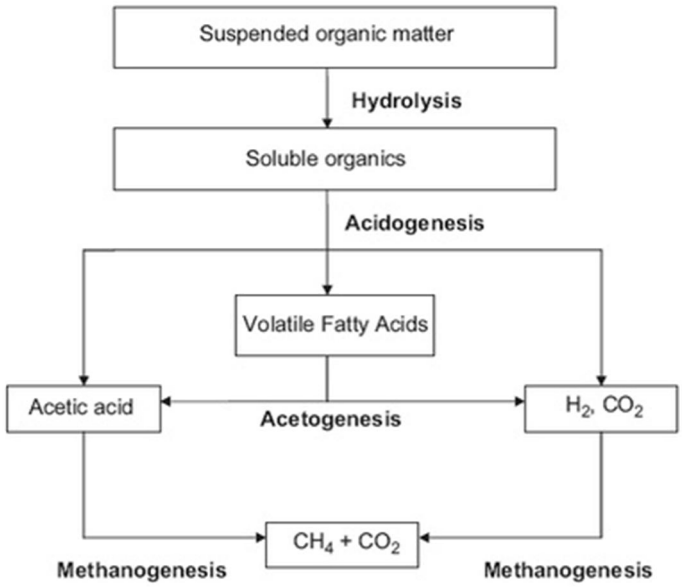


Figure 1.4 Subsequent steps in the anaerobic digestion process for biogas production (Appels et al., 2008)

Despite the successive steps, hydrolysis is generally considered as rate limiting during the biomass treatment. The hydrolysis step degrades both insoluble organic material and high molecular weight compounds such as lipids, polysaccharides, proteins and nucleic acids, into soluble organic substances (e.g., amino acids and fatty acids). The components formed during hydrolysis are further split during acidogenesis, the second step. Volatile fatty acids (VFA) are produced by acidogenic (or fermentative) bacteria along with ammonia (NH_3), CO_2, H_2S and other by-products. The third stage in AD is acetogenesis, where the higher organic acids and alcohols produced by acidogenesis are further digested by acetogens to produce mainly acetic acid as well as CO_2 and H_2. This conversion is controlled to a large extent by the partial pressure of H_2 in the mixture. The final stage of methanogenesis produces methane by two

groups of methanogenic bacteria: the first group splits acetate into methane and carbon dioxide and the second group uses hydrogen as electron donor and carbon dioxide as acceptor to produce methane (Appels et al., 2008).

1.3.1.1 Biochemical methane potential test

Biochemical methane potential (BMP) test is a measure of sample biodegradability and was developed to estimate in a relatively simple and reliable method the ultimate conversion and associated methane yield of organic substrates under anaerobic conditions. It involves batch incubation of a substrate under ideal conditions for anaerobic decomposition and the determination of the methane potential of substrates. (Chynoweth et al., 1993; Owen et al., 1979).

Methane potential or methane yield could be defined as the amount of methane that can be potentially produced when a certain substrate or waste is biodegraded under anaerobic conditions, it is expressed as L CH_4/g VS, L CH_4/g COD or L CH_4/g dry matter (volatile solids (VS), chemical oxygen demand (COD)). It is of great interest before implementing anaerobic digestion at full scale, since the test assessess the potential, adequacy and viability of the anaerobic treatment of such wastes of interest (Labatut et al., 2011). Substrate biodegradability depends on several physical, chemical and physiological factors reviewed by Angelidaki and Sanders (2004). Moreover, temperature, intermediate biodegradation compounds, the type of substrate or the ratio used have to be considered since they may affect the final BMP results (Cavaleiro et al., 2008; Labatut et al., 2011; Raposo et al., 2006).

Complete and detailed protocols to assess the biodegradability and methane potential of WW and organic wastes have been developed throughout the years: Field et al. (1988), Hansen et al. (2004), Owen et al. (1979). However, some modifications have been implemented by other authors in order to improve the methodology and adapt it to the solid substrates (Martín-González et al., 2010).

During the BMP assay, biogas production is monitored by measurements of volume of produced biogas at a constant pressure (volumetric methods) or measurements of exerted pressure at a constant volume (manometric

methods). On the other hand, biogas composition is nowadays determined off-line by gas chromatography (GC) techniques. The methane contribution resulting from sample decomposition is determined by subtracting background values obtained from seed-blanks, from the sample totals. Besides methane potential determination, the biodegradable fraction of wastes can be evaluated combining experimental methane potential values from the BMP assays with COD analysis (Labatut et al., 2011).

Some drawbacks appear from the BMP assays, the major bottleneck is the time required, more than 21 d, although a theoretical equation has been proposed by Symons and Buswell (1933) as a faster alternative when the composition of the waste is known. However, theoretical determinations assume the total conversion of organic matter into methane and carbon dioxide, therefore, theoretical methane potential values will be overestimated. Moreover, factors such as low biodegradability of some compounds, fraction of substrate used for cell synthesis and mass transfer difficulties between substrate and biomass are not considered (Angelidaki and Sanders, 2004). Other calculations are based in the components composition of waste (lipids, carbohydrates, proteins and other poorly biodegradable compounds) (Gunaseelan, 2007).

Besides time requirements, it has been widely applied to determine the ultimate methane production from a variety of feedstocks including: manure, lignocellulosic residues, microalgae, organic fraction matter, (Alzate et al., 2012; Ehimen et al., 2011; Gunaseelan, 2004; Krishna Kafle and Chen, 2016; Liu et al., 2015; Martín-González et al., 2010).

1.3.2 Biogas from biomass

More attention is focused on organic wastes and waste sludge from industrial sources, since they have been underutilized resources for biogas recovery. BMP is an easy technique for evaluating the feasibility of a substrate for its valorisation and could be applied to all organic industrial wastes. Some may reduce the plant costs if the methane produced is used, and others should be co-digested with other substrates to enhance methane production.

Labatut et al. (2011) determined the biomethane yields and biodegradability of 30 organic substrates from food (cheese whey, plain pasta, meat pasta, used vegetable oil, ice cream, fresh dog food, cola beverage, raw cabbage, raw potatoes), invasive aquatic plants (frogbit, water chestnut, Eurasian milfoil, water celery, chara) and others (switchgrass, corn silage, corn leachate, mouthwash, suspended and settled fat, oil and grease) using the BMP assay. The highest methane production was obtained from used vegetable oil, whereas switchgrass presented the lowest methane production. Substrates highly rich in lipids and easily-degradable carbohydrates exhibited higher methane potential, whereas more recalcitrant, lignocellulosic-materials presented lower methane yields. Residues from a fish cannery, a beer brewer and bread past its expiration date obtained from a market, were used for biogas potential determination by Krishna Kafle et al. (2013), obtaining good potential values. Slaughterhouses and meat processing industries generate large amounts of wastes and by-products, these wastes are typically rich in fats and proteins, therefore representing a good substrate for biogas production and several authors have conducted the BMP assay to determine the feasibility of the substrate (Cavaleiro et al., 2013; Pitk et al., 2012; Salminen and Rintala, 2002). Agricultural biomass is a relatively broad category of biomass that includes the food based portion (oil and simple carbohydrates) of crops (such as corn, sugarcane, beets) and the non-food based portion (complex carbohydrates) of crops (such as the leaves, stalks, and cobs of corn stover, orchard trimmings, rice husk, straw), perennial grasses, and animal waste. Large quantity of crops residues are produced annually worldwide and often dumped in open environment, such wastes could be valorised and converted into methane as many authors reported (Chandra et al., 2012; Kaparaju et al., 2009; Lei et al., 2010; Tong et al., 1990)

Fruit juice industry produces of high amounts of wastewater and the feasibility of anaerobic co-digestion two juice-based beverage industrial wastes was investigated by Hosseini Koupaie et al. (2014), with final results that could end up on a 37% cost reduction for the industry.

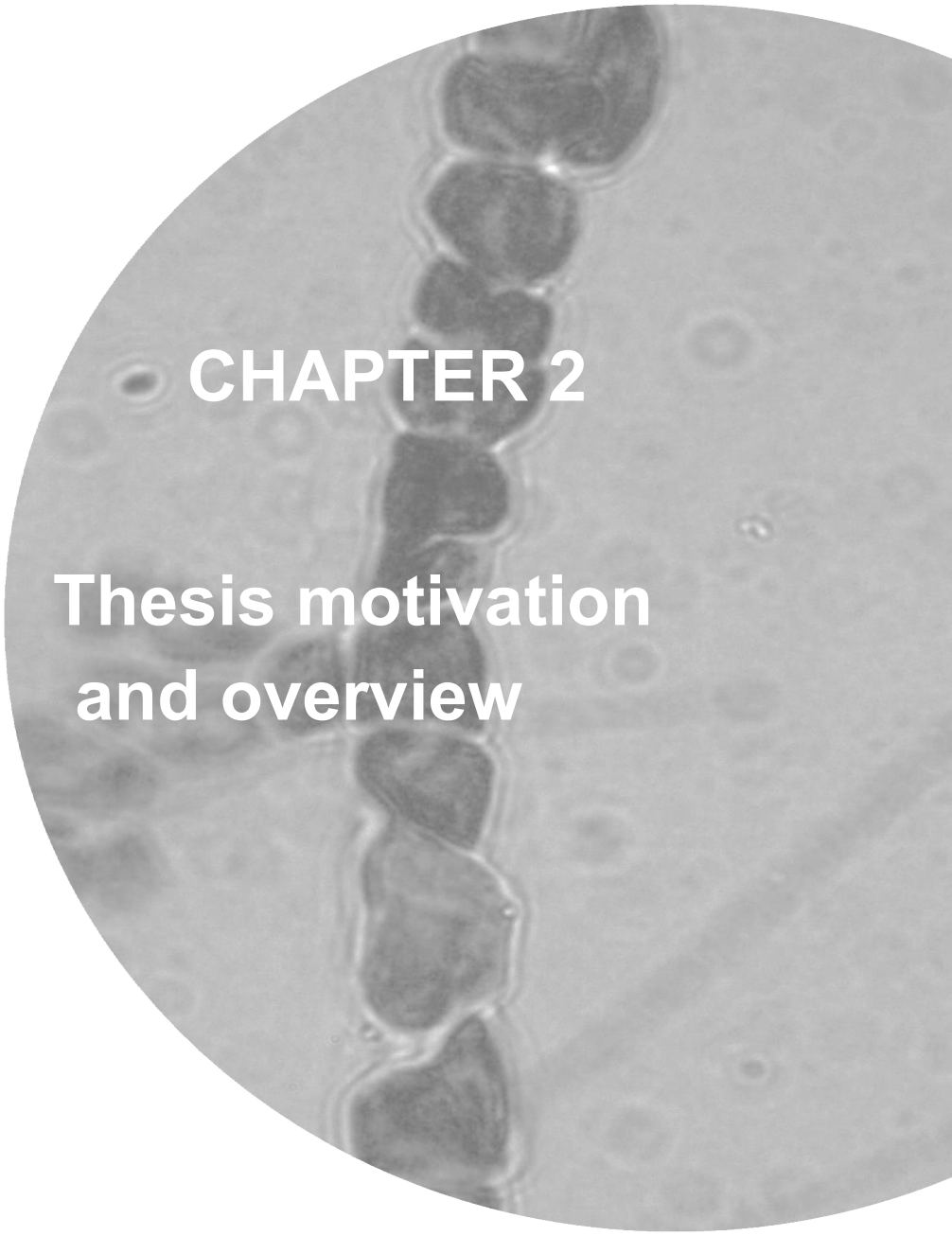
The first microalgal biomass anaerobic digestion published study appeared in the 1950’s and it was concluded that algal biomass were suitable for anaerobic

digestion (Golueke et al., 1957). Since then, microalgal biomass has become one of the feedstock for anaerobic digestion and its reported specific methane yield was within the range of 50–1197 mL CH_4/g VS and positive effect was observed in the co-digestion process which used microalgae as one of the feedstocks together with: glycerol, swine slurry, waste paper, corn silage (González-Fernández and Sialve, 2012; Olsson and Fallde, 2014; Ometto et al., 2014; Schwede et al., 2013)

Microbial digestion may be limited by the structure and chemical composition of the cell wall of some microalgae species resulting in significantly lower methane yields than expected (Atkinson et al., 1972; Burczyk et al., 1999). The resistance of microalgae cell walls is attributed to the formation of hardly biodegradable polymers, namely sporopollenin and algaenan (González-Fernández and Sialve, 2012). In general, the decrease of the cell degradation correlated well with the amount of biogas produced. The species with a high degree of decomposition and low amount of indigestible residues (*Chlamydomonas reinhardtii, Dunaliella salina, Arthrospira platensis* and *Euglenagracilis*) showed higher amounts of biogas production compared to the species with a lower degree of decomposition and higher amount of indigestible residues (*Chlorella kessleri* and *Scenedesmus obliquus*) (Mussgnug et al., 2010). If the algal biomass does not result from any cell disruption process, the cell walls could strongly modulate this aspect by protecting the cell against the enzymes produced by the anaerobic consortium and the accessibility to cell disintegration is most likely a major factor for the efficiency of fermentative biogas production (Mussgnug et al., 2010; Sialve et al., 2009). For this reason, pretreatment techniques have been used to solubilise particulate biomass and improve the anaerobic digestion rate and extent.

Pretreatment of a substrate prior to anaerobic digestion allows to significantly improve its biodegradability while acting on its physico-chemical properties: this step makes the organic matter more accessible to the anaerobic microflora and thus more easily degraded for biogas production (González-Fernández and Sialve, 2012; Sialve et al., 2009). The effectiveness of the pretreatment depends on the characteristics of microalgae, i.e., the toughness and structure of the cell wall, and the macromolecular composition of cells.

Pretreatment processes can be divided into four categories: thermal, mechanical, chemical and biological processes, which have quite different efficiency in improving the digestion process. Thermal pretreatments are regarded as the most effective on microalgae cell disruption, furthermore, do not require chemicals, which may contaminate biofuel products (Passos et al., 2014a). Biomass is solubilised by means of heat application, ranging from 50 to 270 ºC, although the optimal temperature depends on the substrate characteristics (Carrère et al., 2010). Mechanical pretreatments were distinguished as less dependent on microalgae species, but required a higher energy input if compared with thermal, chemical and biological methods (Passos et al., 2014a). They act by directly breaking cells through a physical force, which implies a high energy input. Some examples are ultrasounds, and microwaves, reporting better results for microwaves rather than for ultrasounds (Passos et al., 2015a). Chemical ones have been proved successful, particularly when combined with temperature. However, the use of chemicals, i.e., acidic or alkaline pretreatments, may contaminate downstream processes (Passos et al., 2014a; Bohutskyi et al., 2014; Mendez et al., 2013). Biological pretreatments have been recently studied because they are a promising alternative to energy-consuming pretreatments. It consists of utilization of microbes and enzymes to disrupt biomass and release intracellular material which enhances methane production rate (Gavala et al., 2004; Leal et al., 2006). Enzyme selection is based on the cell wall composition and it is a crucial characteristic for good biogas yields. Moreover, enzyme/microalgae ratio influences the enzyme activity efficiency; high loading of microalgae may result in high viscosity due to release of insoluble matter which in turn can hinder enzymatic activity (Rosgaard et al., 2007). Additionally, pH, temperature and exposure time have to be considered for appropriate performance avoiding enzyme denaturalisation (González-Fernández and Sialve, 2012). Recently, in order to decrease the costs involved on this pretreatment technique, enzymes have been immobilised on supporting materials (Fu et al., 2010). Up to date, literature in this subject is scarce, but it has been demonstrated that the use of microalgal cell wall specific enzymes, such as cellulase, increases the methane yield (Ehimen et al., 2013).



CHAPTER 2

Thesis motivation and overview

2 Thesis motivation and objectives

2.1 Research motivation

Emerging contaminants (ECs) are an issue of great concern and despite the efforts from the scientific community there is not a completely efficient solution for the removal of these compounds in wastewater (WW). The ideal system should be easy to operate, with low costs of investment as well as during its operation, simple, versatile, sustainable and environmentally friendly. Microalgae have high potential for WW treatment, and this kind of systems have been applied at full scale in some communities. However, ECs removal has not been fully investigated in these systems. Moreover, microalgal biomass can be valorised, transforming it into biofuels. Those processes are still under development, due to the high operating costs of microalgae harvesting. Taking into account the microalgal potential and the society requirements, the ideal picture of a microalgae process could be a closed system in which; wastewater feeds an algal pond, microalgae remove the nutrients for its growth as well as the emerging contaminants (several mechanisms may occur: biodegradation, sorption or photodegradation). This kind of process will also contribute to reduce CO_2 footprint. The effluent will be conducted to the harvesting unit, which should be optimized for this system obtaining an algal concentrate and a "clean" supernatant which can be reused or released to the environment since it will attain the limits of the legislative regulation. The concentrate will be conducted to the downstream process for its valorisation; the easiest and cheapest option will be biogas production. Taking into account the weak points of the proposed closed system the main research motivations are:

- To study the capacity of microalgae for emerging contaminants removal, comparing pure strains with real algal/bacterial consortiums in a photobioreactor (PBR) treating urban WW and to identify the processes occurring during the pharmaceutical active compounds (PhACs) degradation.

- To apply different harvesting processes for furtherly valorise the microalgal concentrate in anaerobic digestion processes.

2.2 Thesis overview

This thesis is divided in five sections and twelve chapters.

Section 1 includes the general aspects and it is formed by 2 chapters. Chapter 1 comprises a general introduction to the three main topics of the thesis including a state of the art: emerging contaminants, microalgae and biomass valorisation. In Chapter 2 the main thesis objectives are presented.

In Section 2, Chapter 3 is included, describing the general materials and methods along the thesis. The specific materials and methods used are presented in each chapter.

Sections 3 and 4 include the main results from the experimental work.

Section 3 includes Chapter 4 to 8 and in general could be described as the study of emerging contaminants removal from microalgal systems. Chapter 4 comprises a batch study using pure microalgae strains for estrogenic compounds removal, different conditions were conducted in order to determine the removal mechanisms and some transformation products were identified. Chapter 5 methodology is similar to Chapter 4, although instead of estrogenic compounds a mix of 9 antibiotics and an antidepressant were studied, sterile conditions were performed and transformation products identification was carried out for 3 chosen compounds. In Chapter 6 the removal of the antibiotic ciprofloxacin was evaluated using a real microalgal suspension, the work included laboratory and pilot scale experiments as well as batch experiments for the removal mechanisms determination. In Chapter 7 the performance of an outdoor continuous pilot scale microalgal photobioreactor is designed and evaluated. The performance of the pond for urban wastewater treatment has been monitored and PhACs removal has been quantified. Chapter 8 presents 24 h batch studies carried out in a pilot scale photobioreactor. An estrogenic contaminant was spiked and the removal was evaluated and compared with results from Chapter 4. The results obtained in batch at laboratory scale helped to determine the removal mechanisms occurring in the system.

Section 4 includes the downstream from microalgal systems and it is composed by Chapter 9 to 11. In Chapter 9 different microalgae harvesting techniques have been studied using different microalgal biomass. Microalgal biomass valorisation producing biogas from anaerobic digestion process is presented in Chapter 10, including different biomass pretreatments, prior to the anaerobic digestion process. Chapter 11 includes the anaerobic digestion of fungal biomass.

Section 5 includes Chapter 12 where the main conclusions of the thesis are exposed, being an overview of the main achievements of the thesis.

Finally, the references and the curriculum vitae of the author, including the list of publications and author's contributions, are presented at the end of the thesis.

2.3 Objectives

In order to accomplish the research motivations, the following specific objectives have been formulated:

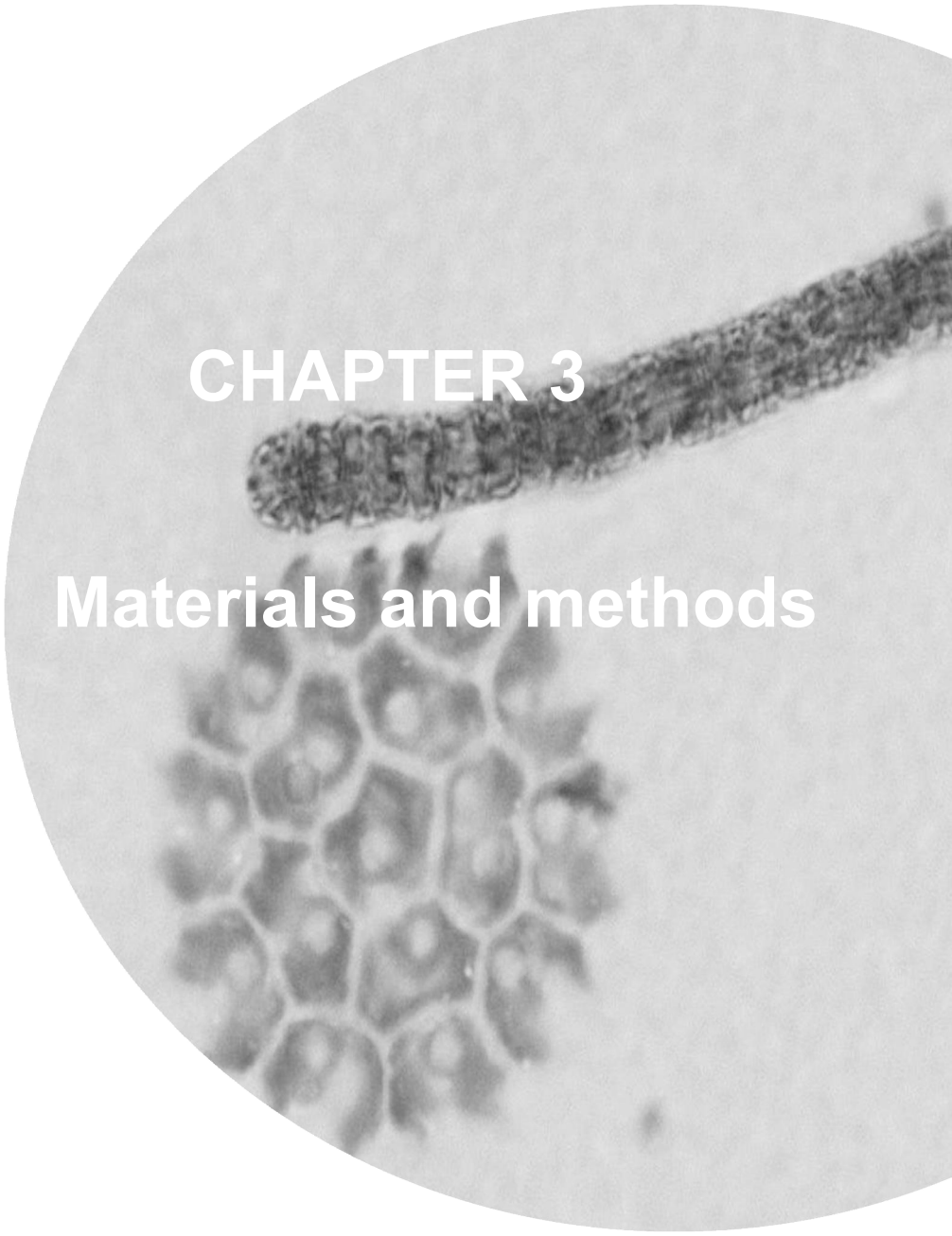
- To evaluate the microalgal capacity for emerging contaminants removal depending on the strain and contaminant (Chapter 4 and 5).
- To determine the removal mechanisms occurring during microalgal wastewater treatment containing emerging pollutants (Chapter 4, 5 and 8).
- To use microalgae as an alternative for treatment wastewaters and decontaminating WW containing conventional nutrients and emerging contaminants, (Chapter 6, 7 and 8).
- To assess different harvesting techniques in different microalgal effluents in order to approach to a reliable and cost effective method (Chapter 9).
- To evaluate biogas production from microalgal biomass and to improve microalgal biomass solubilisation prior anaerobic digestion using enzymatic pretreatments (Chapter 10).

- To find out new alternatives to energy sources, considering microalgae production as a source of renewable energy due to its conversion into valuable biomass (Chapter 10).

- To evaluate biogas production from exhausted biomass used in non-conventional wastewater treatment (Chapter 11).

Section 2

PROCEDURES



CHAPTER 3

Materials and methods

3 Materials and methods

3.1 Microorganisms

3.1.1 Microalgae

Chlamydomonas reinhardtii (UTEX ID 2243), *Chlorella sorokiniana* (UTEX ID 1663), and *Pseudokirchneriella subcapitata* (UTEX ID 1648) were supplied in agar tubes, whereas *Dunaliella tertiolecta* (UTEX ID LB999) was supplied in liquid media. All of them were from the culture collection at the University of Texas (UTEX) and were maintained by subculturing on Petri dishes in their specific growth medium and agar (1.5%) medium and stored at 4ºC.

3.1.2 Fungus

Trametes versicolor (ATCC#42530) was obtained from the American Type Culture Collection and was maintained by subculturing on Petri dishes in malt extract (2%) and agar (1.5%) medium and stored at 25ºC.

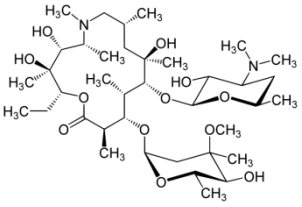
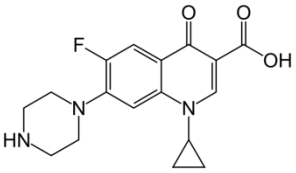
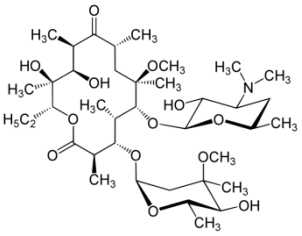
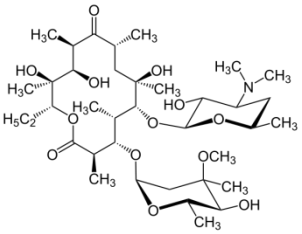
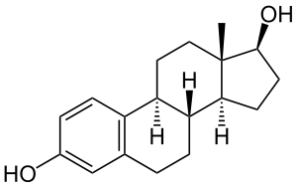
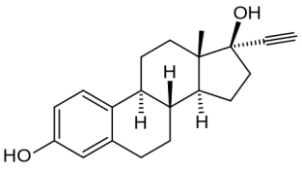
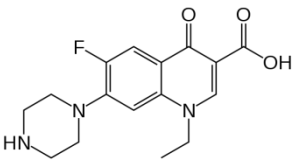
3.2 Chemical compounds and reagents

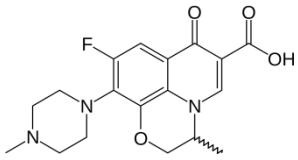
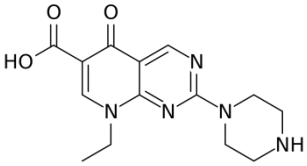
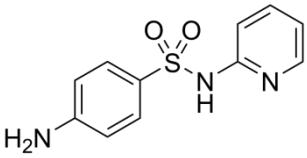
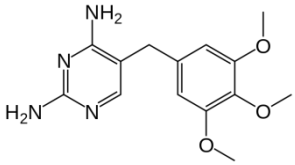
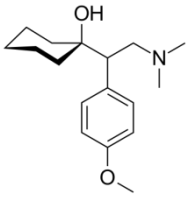
3.2.1 Selected contaminants

3.2.1.1 Contaminants for microalgal degradation studies

The selected contaminants for the different experiments of degradation by microalgae can be found in Table 3.1 with their physico-chemical characteristics, chemical structure and supplier.

Table 3.1 Physico-chemical characteristics, chemical structure and supplier for the selected contaminants studied in this thesis.

Compound	Abbreviation	Chemical structure	CAS number	Supplier	Purity
Azithromycin	AZM		83905-01-5	Sigma-Aldrich	≥95%
Ciprofloxacin	CPX		85721-33-1	Sigma-Aldrich	≥98%
Clarithromycin	CRM		81103-11-9	Sigma-Aldrich	≥98%
Erythromycin	ERM		114-07-8	Sigma-Aldrich	≥99%
17β-estradiol	E2		50-28-2	Sigma-Aldrich	≥98%
17α-ethinylestradiol	EE2		57-63-6	Sigma-Aldrich	≥98%
Norfloxacin	NFX		70458-96-7	Sigma-Aldrich	≥98%

Compound	Abbreviation	Chemical structure	CAS number	Supplier	Purity
Ofloxacin	OFX		82419-36-1	Sigma-Aldrich	99.8%
Pipemidic acid	HPM		51940-44-4	Sigma-Aldrich	≥98%
Sulfapyridine	SPD			Sigma-Aldrich	≥99%
Trimethroprim	TMT		738-70-5	Sigma-Aldrich	≥99%
Venlafaxine	VFX		93413-69-5	UPS	≥98%

Estrogenic compounds stock solutions were prepared at 1 g/L and dissolved in acetone.

Ciprofloxacin stock solution was prepared at 1 g/L and dissolved in 0.1% formic acid.

Antibiotics and venlafaxine stock solutions were prepared separately and dissolved in dimethyl sulfoxide (DMSO).

3.2.1.2 Contaminants for analytical measurements

All antibiotic standards for Chapter 5 study were obtained at high purity grade (>90%). Erythromycin (ERM), azithromycin (AZM), clarithromycin (CRM), ofloxacin (OFX), ciprofloxacin (CPX), trimethoprim (TMT), norfloxacin (NFX), pipemidic acid (HPM), sulfapyridine (SPD); and the internal standards ofloxacin-d3, erythromycin-N,N^{13}C and ciprofloxacin-d8 hydrochloride hydrate were purchased from Sigma-Aldrich, whereas sulfamethoxazole-d4 and azythromycin-d3 from Toronto Research Chemicals (Ontario, Canada).

The 80 pharmaceutical standards for emerging contaminants determination in Chapter 7 were purchased from Sigma-Aldrich, US Pharmacopeia, European Pharmacopeia and Toronto Research Chemicals. The isotopically labelled compounds were purchased from Sigma-Aldrich, CDN isotopes (Quebec, Canada), and Toronto Research Chemicals (Ontario, Canada).

All standards used for Chapter 5 and 7 experiments were of high purity grade (>90%). Standard solutions were prepared on a weight basis in methanol (at a concentration of 1000 mg/L) and stored at -20 ºC. Working standard solutions containing all pharmaceuticals were prepared in methanol/water (10:90, v/v) as well as isotopically labelled internal standards mixtures used for internal standard calibration. Ultra-pure water, acetonitrile and methanol LiChrosolv grade were supplied by Merck (Darmstad, Germany).

3.2.2 Chemical products and reagents

Salts and reagents

Salts and reagents for medium preparation suppliers are specified in the medium composition tables. All chemicals used were of analytical grade.

Glucose, ammonium tartrate dibasic, malt extract, laccase and other chemicals were purchased from Sigma-Aldrich (Barcelona, Spain).

Enzymes

Cellulase was provided with the commercial name of Celluclast, glucohydrolase with the commercial name of Glucanex and xylanase with the commercial name of Shearzyme by Novozymes Spain SA.

Coagulants and flocculants

The coagulants used were: 40 % $FeCl_3$ (20 g/L) and 8 % Al_2O_3 (3 g/L, AC50 (2.5 mL AC50/250mL H_2O) they were subministered by Aquazur Iberia S.L. (Madrid, Spain). Properties are presented in Table 3.2.

The flocculants used were: DR3000 (2.5 mL DR3000/1000 mL H_2O) and GO2030 (2.5 mL GO2030/250 mL H_2O) subministered by Aquazur Iberia S.L. (Madrid, Spain). Properties are presented in Table 3.3.

Table 3.2 Coagulants properties.

Coagulant	Appearance	Density (g/cm^3)	Viscosity (cP)	pH	Price (€/kg)
$FeCl_3$	Reddish liquid	1.42	9	2.5	0.05
Al_2O_3	Colourless transparent liquid	1.33	n.a. [a]	2.5	0.30

[a] *n.a.: data not available*

Table 3.3 Coagulants properties.

Flocculant	Appearance	Density (g/cm^3)	Viscosity (cP)	pH	Ion strength	Price (€/kg)
DR3000	White milky liquid	1.20	<600	3.0 – 4.1	Medium cationicity (35%)	2.3
GO2030	White milky liquid	1.20	<3000	3.0 – 5.0	High anionicity (30%)	2.0

Solvents

All the solvents were of high purity grade. High-performance-liquid-chromatography (HPLC) grade acetonitrile was supplied by Merck (Darmstadt, Germany) and acetone by Sigma-Aldrich (Barcelona, Spain).

Endocrine disruptor chemicals

The rest of the endocrine disruptor chemicals (EDCs) analysed were of high purity level, and detail information about them can be found elsewhere (Gorga et al., 2013).

3.3 Media and culture conditions

3.3.1 Media for microalgae and culture conditions

Microalgae were transferred from Petri plaques or agar tubes to Erlenmeyer flasks using a Kolle handle. The picked algal colony was resuspended in 10 mL of specific medium in 25 mL Erlenmeyer flasks. Microalgae were maintained under 16 h light conditions (172 ± 18 µmol/(m^2 s)) irradiance level, measured by a light meter (LI.189, LI-COR Quantum/Radiometer/Photometer, USA)) at a controlled temperature (25 ± 1ºC) and a gentle stirring on a shaker platform (30 rpm) (orbital shaker Kuhner, LS-X, Switzerland) until the culture was visually green, the agitation speed was increased to 120-130 rpm. Afterwards, part of the culture was transferred to a 50 mL Erlenmeyer flask containing 25 mL of medium and the algal volume transferred from the initial Erlenmeyer flask was replaced with growth medium. That procedure was continuously repeated until the desired algal concentration and volume was obtained.

Microalgae were grown in their specific or general microalgae growth medium (GM), specified in the Materials and methods from each chapter. Growth medium, trace elements solutions and vitamins solutions are defined in the following tables.

Growth mediums and Petri plaques were prepared following the instructions from Box 3.1. Algal growth mediums are defined in Table 3.4, Table 3.5, Table 3.6 and Table 3.7. Trace solutions are presented in Table 3.8, Table 3.9, Table 3.10, Table 3.11 and Box 3.2.

Vitamin solutions were prepared following the procedures from Box 3.3. Vitamin B_{12}, biotin and thiamine solutions composition are presented in Table 3.12.

Box 3.1 Microalgal medium and Petri plaques preparation procedures.

For 1 L total:

- To approximately 850 mL of ultrapure H_2O, add each of the components in the order specified (except vitamins) while stirring continuously.
- Adjust the pH
- Bring the total volume to 1 L with ultrapure H_2O
- Cover and autoclave medium
- Allow to cool down
- Add vitamins (if required)
- Store at refrigerator temperature

For 1.5% agar medium: add 15 g of agar to the flask; do not mix

Table 3.4 BG-11 medium composition for P. subcapitata culture (Li et al., 2011).

Component	**Concentration (mg/L)**	**Supplier**
Glucose	1500	Quemivita
Na_2CO_3	20	Panreac
$NaNO_3$	1500	Sigma-Aldrich
$Na_2MgEDTA$	1	Sigma-Aldrich
Ferric ammonium citrate	6	Panreac
Citric acid	6	Merck
$CaCl_2 \cdot 2\ H_2O$	36	Panreac
$MgSO_4 \cdot 7\ H_2O$	75	Sigma-Aldrich
K_2HPO_4	30.5	Panreac
H_3BO_3	2.86	Panreac
$MnCl_2 \cdot 4\ H_2O$	1.81	Panreac
$ZnSO_4 \cdot 7\ H_2O$	0.222	Panreac
$CuSO_4 \cdot 5\ H_2O$	0.079	Fisher Chemical
$CoCl_2 \cdot 6\ H_2O$	0.050	Alfa Aesar
$NaMoO_4 \cdot 2\ H_2O$	0.391	Panreac

Table 3.5 P49 medium composition for C. reinhardtii culture (UTEX, 2012).

Component	Concentration (mg/L)	Supplier
$MgSO_4 \cdot 7\ H_2O$	40	Sigma-Aldrich
Na_2glycerophosphate $\cdot 5\ H_2O$	50	Sigma-Aldrich
KCl	50	Panreac
Glycylglycine	50	Merck
P IV metal solution	6 [a]	-
NH_4NO_3	100	Sigma-Aldrich
$CaCl_2 \cdot 2\ H_2O$	74	Panreac
Yeast extract	200	Scharlau
Triptone	400	VWR
Vitamin B_{12}	1 [a]	-
Vitamin biotin	1 [a]	-
Vitamin thiamine	1 [a]	-

[a] *volume concentrations (mL/L)*

Table 3.6 TAP medium composition for microalgae culture.

Component	Concentration (g/L)	Supplier
Tris	2.42	Sigma-Aldrich
TAP salts solution	25	-
Phosphate solution	0.375 [a]	-
Hutner's trace elements	1.0 [a]	-
Glacial acetic acid	1.0 [a]	Sigma-Aldrich

[a] *volume concentrations (mL/L)*

Table 3.7 Modified Bristol medium composition for microalgae culture.

Component	Concentration (mg/L)	Supplier
$NaNO_3$	250	Sigma-Aldrich
$CaCl_2 \cdot 2\ H_2O$	25	Panreac
$MgSO_4 \cdot 7\ H_2O$	75	Sigma-Aldrich
K_2HPO_4	75	Panreac
KH_2PO_4	175	Panreac
NaCl	25	VWR
P-IV metal solution	6 [a]	-
Vitamin B_{12}	1 [a]	-
Vitamin biotin	1 [a]	-
Vitamin thiamine	1 [a]	-

[a] *volume concentrations (mL/L)*

Table 3.8 PII metal solution composition for microalgae growth medium preparation.

Component	Concentration (mg/L)	Supplier
EDTA disodium salt $\cdot 2\ H_2O$	1000	Sigma-Aldrich
H_3BO_3	1140	Panreac
$FeCl_3 \cdot 6\ H_2O$	49	Sigma-Aldrich
$MnSO_4 \cdot H_2O$	164	Panreac
$ZnSO_4 \cdot 7\ H_2O$	22	Panreac
$CoCl_2 \cdot 6\ H_2O$	4.8	Alfa aesar

Table 3.9 P-IV metal solution for microalgae growth medium preparation.

Component	Concentration (mg/L)	Supplier
$Na_2EDTA \cdot 2\ H_2O$	750	Sigma-Aldrich
$FeCl_3 \cdot 6\ H_2O$	97	Sigma-Aldrich
$MnCl_2 \cdot 4\ H_2O$	41	Merck
$ZnCl_2$	5	Merck
$CoCl_2 \cdot 6\ H_2O$	2	Alfa aesar
$Na_2MoO_4 \cdot 2H_2O$	4	Panreac

Table 3.10 TAP salts and phosphate solution composition for TAP medium.

TAP salts solution		Phosphate solution	
Component	**Concentration (g/L)**	**Component**	**Concentration (g/L)**
NH_4Cl	15	K_2HPO_4	288
$MgSO_4 \cdot 7\ H_2O$	4	KH_2PO_4	144
$CaCl_2 \cdot 2\ H_2O$	2		

Table 3.11 Hutner's trace solution composition for TAP medium.

Component	Amount (g)	Water (mL)	Supplier
EDTA disodium salt	50	250	Panreac
$ZnSO_4 \cdot 7\ H_2O$	22	100	Panreac
H_3BO_3	11.4	200	Panreac
$MnCl_2 \cdot 4\ H_2O$	5.06	50	Merck
$CoCl_2 \cdot 6\ H_2O$	1.61	50	Alfa aesar
$CuSo_4 \cdot 5\ H_2O$	1.57	50	Fischer chemical
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	1.1	50	Sigma-Aldrich
$FeSO_4 \cdot 7\ H_2O$	4.99	50	Panreac

Box 3.2 Hutner's trace solution preparation for TAP medium.

Mix all solutions except EDTA. Bring to boil, and then add EDTA solution. The mixture should turn green. When everything is dissolved, cool to 70ºC. Keeping temperature at 70, add 85 mL hot 20% KOH solution (20 grams / 100 ml final volume). Do NOT use NaOH to adjust the pH.

Bring the final solution to 1 liter total volume. It should be clear green initially. Stopper the flask with a cotton plug and let it stand for 1-2 weeks, shaking it once a day. The solution should eventually turn purple and leave a rust-brown precipitate, which can be removed by filtering through two layers of Whatman#1 filter paper, repeating the filtration if necessary until the solution is clear. Store refrigerated or frozen convenient aliquots. Some people shorten the time for formation of the precipitate by bubbling the solution with filtered air.

If no precipitate forms, the solution is still usable. However, you might want to check the pH in this case and adjust it to around 7.0 using either KOH or HCl as needed.

To prepare sulphur-free trace elements for hydrogen generation, the sulphate salts can be replaced with equimolar chloride salts ($ZnCl_2$ 10.0 g; $CuCl_2 \cdot 2\ H_2O$ 1.00 g; $FeCl_2 \cdot 4\ H_2O$, 3.60 g).

Box 3.3 Vitamins solutions for microalgal mediums preparation procedures.

For 200 mL total:

- Prepare 200 mL of HEPES buffer
- Adjust the pH to 7.8
- Add vitamin, wait until fully dissolved
- Sterilize by 0.45 µm Millipore filter
- Store in dark at freezer temperature

Table 3.12 Vitamins solutions composition.

Vitamin B_{12}		**Vitamin biotin**		**Vitamin thiamine**	
Component	**Amount (mg)**	**Component**	**Amount (mg)**	**Component**	**Amount (mg)**
HEPES buffer [a]	2400	HEPES buffer [a]	2400	HEPES buffer [a]	2400
Vitamin B_{12} [a]	27	Biotin [a]	5	Thiamine [a]	67

[a] *Supplier: Sigma-Aldrich*

3.3.2 Media for Trametes versicolor, culture conditions and pellets production

Pellets production was done as previously described by Font et al. (2003). In the protocol, two steps can be differentiated: firstly the formation of mycelium and afterwards the growth of fungal pellets. At every moment, sterility conditions were kept. For that purpose, all media and material were sterilised by autoclaving them 30 min at 121ºC.

For mycelium formation, four 1 cm^2 cubes of fungal growing area of Petri plates were cut and inoculated in a 500 mL Erlenmeyer flask with 150 mL of growing media (malt extract 20 g/L adjusted to pH 4.5). After 5-6 days at 25 ±1 ºC and 135 rpm (r=25 mm) of orbital shaking, the mycelium was formed. It was separated from the media by means of a strainer and triturated with a homogenizer Ystral GmgH X/10/20 in an 8 g/L NaCl solution at a relation 1:1 v/v. The resultant suspension can be immediately used for the formation of pellets or kept at 4 ºC.

For the formation of pellets, 1 mL of the triturated mycelia suspension was added to 1 L Erlenmeyer with 250 mL of growing media. The culture was

maintained during 5-6 days at orbital agitation (135 rpm) and 25ºC, to finally obtain the 2-3 mm pellets. Final pellets were separated from the media with a strainer, washed with MilliQ water and resuspended in an 8 g/L NaCl solution at a relation 1:1 v/v. They can be immediately used or kept at 4 ºC for a maximum of one month.

For laccase production for the enzymatic pretreatment experiments (Chapter 10), a defined media (Table 3.13 and Table 3.14), modified from (Kirk et al., 1978), with glucose, ammonium, dimethyl succinic acid and macronutrients and micronutrients, adjusted at pH of 4.5 was used (Blánquez et al., 2004).

Table 3.13 Composition of defined medium for T. versicolor culture.

Component	Concentration (g/L)	Supplier
Glucose	8	Quemivita
Ammonium tartrate dibasic	3.3	Sigma-Aldrich
2,2-dimethyl succinic acid	3.3	Sigma-Aldrich
Macronutrients	10 [a]	-
Micronutrients	1 [a]	-

[a] *volumetric concentrations (mL/L)*

Table 3.14 Composition of macronutrients of the defined medium for T. versicolor.

Component	Concentration (g/L)	Component	Concentration (g/L)
Nitrile triacetic acid	1,5	$ZnSO_4 \cdot 7\ H_2O$	0.1
$MgSO_4 \cdot 7\ H_2O$	3,0	$CuSO_4 \cdot 5\ H_2O$	0.01
$MnSO_4 \cdot H_2O$	0,5	$AlK(SO_4)_2 \cdot 12\ H_2O$ [a]	0.01
NaCl	1,0	H_3BO_3	0.01
$FeSO_4 \cdot 7\ H_2O$	0,1	Na_2MoO_4	0.01
$CoSO_4 \cdot 7\ H_2O$ [a]	0,2		

[a] *Supplier: Panreac*

Table 3.15 Composition of micronutrients of the defined medium for T. versicolor.

Component	Concentration (g/L)
KH_2PO_4	20
$MgSO_4 \cdot 7\ H_2O$	5
$CaCl_2$	1

3.3.3 Media for BMP tests

Methanogenic activity assays from Chapter 11 were carried out using an anaerobic basic medium containing nutrient solution, trace metals solution and sodium sulfide solution. Table 3.16 presents the composition of the solutions.

Table 3.16 Composition of additional medium solutions for methanogenic activity assays.

Macronutrients		**Trace elements**	
Component	**Concentration (g/L)**	**Component**	**Concentration (g/L)**
NH_4Cl	170	$FeCl_3 \cdot 4\ H_2O$	2
KH_2PO_4	37	$CoCl_2 \cdot 6\ H_2O$	2
$CaCl_2 \cdot 2\ H_2O$	8	$MnCl_2 \cdot 4\ H_2O$	0.5
$MgSO_4 \cdot 4\ H_2O$	9	$CuCl_2 \cdot 2\ H_2O$ [b]	0.03
		$ZnCl_2$	0.05
		H_3BO_3	0.05
		$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.09
		$Na_2SeO_3 \cdot 5\ H_2O$ [c]	0.1
		$NiCl_2 \cdot 6\ H_2O$ [c]	0.05
Sulphur solution		EDTA [d]	0.1
Component	**Concentration (g/L)**	HCl 36% [e]	1 [a]
$Na_2S \cdot 9\ H_2O$	0.1	Resarzurin [c]	0.5

[a] volumetric concentration (mL/L); [b] Supplier: Merck; [c] Supplier: Sigma-Aldrich; [d] Supplier: Panreac; [e] Supplier: Scharlau

The theoretical calculated values for the composition of the required substrates solution (acetic, propionic and butyric acids) for methanogenic activity assays are described in Table 3.17.

Table 3.17 Composition of substrates solution (100 g VFA_{COD}/kg).

Component	Concentration (g acid/g COD) [a]	Volume (mL/L)	Commercial density (kg/L)	Supplier
Acetic acid (C_2)	1.067	66	1.05	Sigma-Aldrich
Propionic acid (C_3)	1.514	14	0.99	Panreac
Butyric acid (C_4)	1.818	2.3	0.96	Merck

[a] *pH 7 (adjust using NaOH)*

3.4 Analytical methods

3.4.1 17β-estradiol and 17α-ethinylestradiol determination

To determine the E2 and EE2 concentrations used in Chapter 4 and 8, 1 mL samples were taken from each Erlenmeyer flask and extracted with acetone. The samples were diluted 1/1 (v:v) with the solvent, mixed in a vortex (Zx^3,Velp Scientifica, Italy) and left for 15 minutes in the ultrasonication device (Branson 3510, Netherlands) before being mixed again and filtered (Millex-GV, PVDF, 0.22 µm, Millipore). The filtered samples were analysed using a Dionex 3000 Ultimate HPLC (Barcelona, Spain) equipped with an ultraviolet (UV) detector at 220 nm (Table 3.18). The column temperature was 30ºC, and a sample volume of 20 µL was injected from a Dionex autosampler. Chromatographic separation was achieved using a GraceSmart RP-18 column. The mobile phase consisted of ultrapure water (A) and acetonitrile (B); the analysis was performed isocratically (60% A) at 1 mL/min. The retention times for E2 and EE2 were 15 min and 21 min, respectively. The quantification limit for both compounds was 0.125 mg/L.

Table 3.18 Specifications for chromatographic analysis of E2 and EE2.

Parameter	Specification
Chromatograph	Ultimate 3000
Detector	Variable wavelength detector
Column	Phenomenex Luna 18 5u 250 mm x 4.6 mm
Carrier liquid	Acetonitrile (40%) & Ultrapure water (pH 7) (60%)
Flow maximum	1 mL/min
Inject volume	20 µL
Oven temperature	30 ºC
UV_VIS_1_Wavelenght	220 nm
Data collection rate	2.4 Hz
Run time	25 min

3.4.2 Antibiotics and venlafaxine determination

For the determination of the 9 antibiotics and the antidepressants studied in Chapter 5, The system used for analysis consisted in a chromatograph coupled to a TSQ Vantage triple quadrupole mass spectrometer containing two high-pressure mixing quaternary pumps Accela 1250 (loading and eluting pumps). The on-line extraction was performed in a Cyclone (50 × 0.5 mm, 60 µm particle size, 60 A pore size; Thermo Fisher Scientific, Franklin, MA) and the compound were separated using a Kinetex Biphenyl (50 × 2.1 mm, 2.6 µm particle size; Phenomenex, Torrance, CA). Acetonitrile (A), acetonitrile:IPA:acetone (45:10:45) (B) and water (C) mobile phases were chosen for loading and cleaning TFC column (loading pump), and formic acid 0.1% in methanol (A) and formic acid 0.1% in water (B) mobile phases were selected for analytical separation (eluting pump). Water samples were directly injected into the chromatographic system by loading 20 µL. A summary of the gradient programmed is presented in ST 1 for a total chromatographic run time of 6 min.

The conditions for MS were designed as follows: spray voltage, 3500V; capillary temperature, 300ºC; vaporizer temperature, 350ºC; shealth gas preassure, 40 arb; ion sweep gas pressure, 0 arb; collision gas pressure, 1.5 mTorr; Q1 peak width, 0.70 (FWHM); Q3 peak width, 0.70 (FWHM). Data processing and

system operation were controlled by the Xcalibur 2.2 software computer program (Thermo Scientific, San Jose, CA). Data acquisition was performed in selected reaction monitoring mode for their corresponding precursor and product ions.

3.4.3 Ciprofloxacin determination

Ciprofloxacin concentration determination for Chapter 6 experiments was quantified using 4 mL filtered samples (0.22 µm Millipore sterile filters) transferred into HPLC vials and analysed using a Dionex 3000 Ultimate HPLC (standard volume injected of 50 µL), a Kinetex Core-shell C-18 150mm*4.6mm column with 2 µm packing, 100Å pores, eluted with 1 mL/min flow with gradient between Solvent A (acetonitrile with 0.1% formic acid) and Solvent B (water with 0.1% formic acid). Solvent A started at 3%, increasing to 55% at 4.5 min, 95% from 4.7-6.9 min, decreasing to 3% at 7 min (total run time 9 min). The column was maintained at 25°C. CPX was detected by fluorescence (Em 445 nm and Ex 274 nm) at 4.5 min retention time (Table 3.19). The limit of quantification was 0.01 mg/L.

Table 3.19 Specifications for chromatographic analysis of CPX.

Parameter	**Specification**
Chromatograph	Ultimate 3000
Detector	Variable wavelength detector
Column	Kinetex Core-shell C-18 150 mm x 4.6 mm
Carrier liquid	acetonitrile 0.1% formic acid/ H_2O 0.1% formic acid
Flow maximum	1 mL/min
Inject volume	50 µL
Oven temperature	25 ºC
Fluorescence emission	445
Fluorescence excitation	274
Data collection rate	2.4 Hz
Run time	9 min

3.4.4 Pharmaceutical active compounds determination

For PhACs identification from Chapter 7, Gros et al. (2012) methodology was applied for preparation and quantification analyses. Briefly, samples were filtered through 1 µm glass fibre followed by 0.45 µm membrane filters (Whatman, United Kingdom). Afterwards they were extracted by using Oasis HLB cartridges (60 mg, 3mL) and conditioning them with 5 mL of methanol followed by 5 mL of HPLC-grade water. 25 mL and 50 mL of samples from input and output containing Na_2EDTA at a final concentration of 0.1% (g solute/g solution) and surrogates standards at 200 ng/L and 100 ng/L were added respectively. After loading all the cartridges, target compounds were eluted with 6 mL of pure methanol and evaporated to dryness under gentile nitrogen stream and reconstituted with 1 mL of methanol/water (10:90, v/v). Finally, 10 µL of a 1 ng/µL standard mixture containing the corresponding isotopically labelled standards were added in the extracts previous to the analysis.

Chromatographic separation was performed in Scheduled MRM^{TM} with a Waters Acquity UPLC liquid chromatography system coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer with turbo ElectroSpray Ionisation source. Both positive and negative ionization modes were explored using an Acquity HSS T_3 column (50 mm × 2.1 mm i.d, 1.8 µm particle size) and an Acquity BEH C_{18} column (50 mm × 2.1 mm i.d, 1.7 µm particle size) purchased from Waters Corporation. For positive and negative ionization mode methanol and 10 mM formic acid/ammonium formiate (pH 3.2) at a flow rate of 0.5 mL/min and acetonitrile 5 mM ammonium acetate/ammonia (pH 8) at a flow rate of 0.6 mL/min were used.

3.4.5 Identification of endocrine disruptors compounds

The identification of 26 endocrine disruptors compounds (EDCs) and related compounds from Chapter 4, were analysed using an EQuan on-line sample enrichment system (Thermo Fisher Scientific) with turbulent flow chromatographic columns before normal chromatographic separation. The chromatograph was coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA). More detailed information can be found elsewhere (Cruz-Morató et al., 2014).

3.4.6 Identification of transformation products

3.4.6.1 Estrogenic compounds

For the identification of known transformation products (TPs) from estrogenic compounds (Chapter 4), the strategy followed was based on the so called non-target analysis of known compounds (Llorca and Rodríguez-Mozaz, 2013). For this reason, an in-house library was built based on the accurate masses of the TPs of E2 and EE2 described in the literature as generated from different water treatments. This library was used during the screening of the samples to tentatively identify any suspected TP.

The samples were analysed using an on-line turbulent flow chromatography system coupled to a hybrid linear ion trap – high resolution mass spectrometer LTQ Orbitrap (TFC-LTQ Orbitrap). An Aria TLX-1 chromatographic system (Thermo Fisher Scientific) was used for purification and separation purposes. This system was composed of a PAL auto sampler and two mixing quaternary pumps (eluting pump and loading pump). The entire system was controlled via Aria software, version 1.6, under Xcalibur 2.2 software. The compounds were extracted using on-line turbulent flow chromatography (TFC) based on an earlier published work by Gorga et al. (2013) for the analysis of endocrine disruptors. The on-line extraction was performed in a Cyclone chromatographic column (50 × 0.5 mm, 60 µm particle size, 60 Å pore size; Thermo Fisher Scientific, Franklin, MA), and the compounds were separated using a Hypersil GOLD analytical column (50 × 2.1; 3 µm; Thermo Fisher Scientific, Franklin, MA). The extraction process consisted of two main steps using the Focus Mode operative mode. First, 10 µl of sample was introduced into the TFC column at 1.5 mL/min with acidified water with formic acid (0.1%), where the analytes of interest were retained in the active pore sites while the rest of the matrix was discharged to the waste. In the second step, the compounds were desorbed from the TFC column onto the analytical column through a normal LC gradient with water (0.1% formic acid) and acetonitrile. The total run time for each injection was 10 min.

The chromatograph was coupled to a hybrid linear ion trap-Fourier Transform Mass Spectrometry analyser (LTQ-Orbitrap VelosTM, Thermo Fisher Scientific),

equipped with a diverter valve (used to divert to waste unwanted portions of chromatographic runs) and an Electrospary Ionisation (ESI) source. The diverter valve was used with three valve positions: from 0 to 1.5 min, the flow was discharged to the waste; from 1.50 to 7.75 min, the valve was switched to injection mode; and from 7.75 to 10.00 min, the valve was switched again to the waste. The ionisation of the compounds was performed in the negative mode after a pre-screening in the positive mode in which no different chromatographic peaks were detected. Mass calibration and mass accuracy checks were performed prior to every sample run with LTQ ESI Negative Ion Calibration Solution (Thermo Fisher Scientific), and the mass accuracy was always within an error of ±5 ppm. The ionisation voltage was set at 3 kV with the sheath gas flow at 35, auxiliary gas flow at 10, S-Lens RF level at 60% and the capillary temperature and the source heater temperature at 450 °C. The samples were acquired using two different acquisition methods in parallel: 1) the first method was triggered through full scan within a mass-to-charge (m/z) range of 100 to 700 m/z at a resolving power of 60,000 FWHM. 2) The second experiment was performed using a data-dependent analysis based on the MS fragmentation of the m/z detected in the 1st experiment that coincided with the series of masses listed in the in-house library built by the authors. The ions were isolated in the ion trap with a width of 2.0 Da, and the collision induced a dissociation activation type (Q = 0.250 and an activation time of 30 ms), with normalised collision energy (35) detected at an HRMS of 7,000. Xcalibur 2.2 (Thermo Scientific) software was used for data interpretation.

Data processing was carried out with Exact Finder 2.5 software, which was used to automatically detect the compounds described in the literature and listed by the authors in an in-house library. The identification of compounds was based on double bound equivalents, with errors within ±5 ppm; when possible, the MS2 fragmentation was interpreted for each compound.

3.4.6.2 Antibiotic compounds

For the tentative detection of antibiotics transformation products (TPs) (Chapter 5), the samples were analysed by using on-line turbulent flow chromatography coupled to a hybrid high resolution mass spectrometer LTQ-Orbitrap (Thermo

Fisher Scientific Company; Villebon-France). 1 mL of samples were freeze-dried and reconstituted in 100 µL of pure water. Samples were injected directly to an on-line TurboFlow LC system coupled to a hybrid linear ion trap high resolution Orbitrap LC-LTQ-OrbitrapVelos (Thermo Fisher Scientific Company; Villebon-France) equipped with a Turbo Ion Spray source. Same chromatographic columns, volume injection and mobile phases were used as explained in 3.4.2, a total chromatographic run time of 19 min. Positive and negative ionization modes were investigated. As no results were found for negative mode analysis, data processing was performed for positive mode only. The samples were acquired in full-scan within a mass-to-charge (m/z) range of 100-1000 m/z at a resolving power of 60000 FWHM and fragmenting the 10 most intense ions detected at a resolving power of 7500 FWHM. The entire system was controlled via Aria software, version 1.6, under Xcalibur 2.1 software. Other MS conditions were designed as follows: spray voltage, 3500V; capillary temperature, 300ºC; sheath gas pressure, 40 arb; and aux gas flow rate, 20 arb. The data obtained was processed by applying the suspect screening methodology described in previous section (3.4.6.1 for estrogenic compounds) and Jaén-Gil et. al. (in preparation). For this purpose, an in-house library was built from literature for data processing to detect any suspect TPs in C*hlamydomonas reinhardtii* and C*hlorella sorokiniana* microalgae water samples.

3.4.7 Volatile Fatty Acids determination

Volatile fatty acids (VFAs) (acetic, propionic, butyric, valeric and n-valeric acids) were determined by gas chromatography (Chapter 11) following a similar methodology as Li et al. (2010). The reactor samples were centrifuged (30 min, 13,500 min^{-1}; Beckman Coulter Inc., Brea, CA, USA), filtered (Whatman GF/A 0.45 µm; GE Healthcare Life Sciences, Buckinghamshire, UK), and mixed (1:1, v/v) with a solution of 0.2% dimethyl propanoic acid in formic acid. The VFA concentrations were quantified using the same gas chromatograph mentioned above with a flame ionization detector equipped with a Teknocroma column (25% NPGA, 2% H_3PO_4, and 2.7 m × 1/8”; Sant Cugat del Vallès, Spain). Nitrogen was used as the carrier gas at 230 kPa, and the oven, injector, and

detector temperatures were set at 130, 250, and 260 °C, respectively. Sample injection was performed manually, and the injection volume was 1 µL. The run time of the sample analyses was 33 min, and the detection range was 0.5–8 g/L. Volatile fatty acids are not detected under 0.5 g/L. Specifications of the chromatographic method are described in Table 3.20.

Table 3.20 Specifications for chromatographic analysis of VFA.

Parameter	Specification
Chromatograph	Hewlett Packard (HP 5890)
Detector	Flame ionization detector (FID)
Column	Teknocroma (25% NPGA, 2% H_3PO_4) 2.7m x 1/8”
Carrier gas	Nitrogen (230 kPa)
Oven temperature	130 ºC
Injector temperature	250 ºC
Detector temperature	260 ºC
Sample volume	1 µL
Injection details	Manual
Detection range	0.5-8 g/L
Run time	33 min

3.4.8 Biogas composition

The biogas composition (methane and carbon dioxide) (Chapter 11) was analysed using a gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA, USA) with a thermal conductivity detector equipped with a Supelco Porapak Q column (3 m × 3.2 mm, Palo Alto, CA, USA) using helium at 338 kPa as the carrier gas. The oven temperature was fixed at 70 °C, the injector temperature was fixed at 150 °C, and the detector temperature was fixed at 180 °C. The sample was injected manually using an injection volume of 100 µL. The run time lasted 3 min and resulted in a detection range of 0–100% (v/v). The results are expressed at standard temperature (0ºC) and pressure (1 atm) (mL CH_4). Details are shown in Table 3.21.

Table 3.21 Specifications for chromatographic analysis of biogas.

Parameter	Specification
Chromatograph	Hewlett Packard (HP 5890)
Detector	Thermal conductivity detector (TCD)
Column	Supelco Porapack Q (250 ºC) 3.0m x 1/8”
Carrier gas	Helium (338 kPa)
Oven temperature	70 ºC
Injector temperature	150 ºC
Detector temperature	180 ºC
Sample volume	100 µL
Injection details	Manual
Detection range	0-100% (v/v)
Run time	3 min

3.5 Laboratory analysis

3.5.1 Organic matter

Organic matter: total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS) were analysed according to Standard Methods (APHA et al., 1999) in order to calculate biomass concentration. An aliquot of microalgal suspension was filtered through a pre-weighed standard glass microfiber filter 1.6 µm (GF/A, Whatman, USA) and dried at a constant temperature of 105 ºC until constant weight. For volatile solids measurements the sample was ignited at 550 ºC.

3.5.2 Optical density

Microalgal growth was followed during all of the experiments by measuring the optical density (OD) of the algal culture at 400 nm for *S. capricornutum* (for Chapter 4 experiments). Then, all cultures were measured at 683 nm for *C. reinhardtii*, *C. sorokiniana, P. subcapitata* and *Dunaliella tertiolecta* using a

UNICAM 8625 UV/VIS spectrophotometer for Chapter 4 experiments and the rest Hach Lange DR3900. Optical density parameters were converted into algae dry weight (TSS) for each sample.

For Chapter 6 experiments OD was measured on a spectrophotometer (Helios-Alpha (Thermo Scientific, USA) at 683 nm.

3.5.3 Fungal dry weight

Biomass pellets dry weight was determined after vacuum-filtering the cultures through pre-weighted glass-fiber filters 1.6 µm (GF/A, Whatman, USA). The fungal amount was determined as the constant weight at 100 ºC following Standard Methods (APHA et al., 1999).

3.5.4 pH and conductivity

Conductivity was determined by a CRISON MicroCM 2100 conductimeter and pH by a pHmeter CRISON MicropH 2001.

Dissolved oxygen (DO), pH, and temperature during Chapter 7 and 8 experiments were recorded using an in situ multimeter: pH PCE-PHD 1 (PCE Instruments, Albacete, Spain).

DO, pH, and temperature during Chapter 6 experiments were recorded using an in situ multimeter: Orion Star A326 multi-meter (Thermo Fisher Scientific Inc, USA)).

3.5.5 Ions

The $N\text{-}NH_4^+$ concentration and chemical oxygen demand (COD) were analysed by using commercial kits LCH303 and LCK114 or LCK314 respectively (Hach Lange, Germany).

The $N\text{-}NH_4^+$ and total phosphorous concentration during Chapter 7 and 8 experiments was measured using Analyzer Y15 (Biosystems, Barcelona, Spain).

Chloride, nitrate, nitrite and sulphate anions were quantified by a Dionex ICS-2000 ionic chromatograph and using a Dionex ICS-2000 Ion Chromatograph

(Dionex Corporation, Sunnyvale, USA) equipped with a Dionex IonPac AS18-HC column (250 mm x 4 mm) and for Chapter 6 measurements an AS11-HC column (250 mm x 4 mm) eluted at 1 mL/min with a 13 mM KOH aqueous solution.

3.5.6 Total carbon and total nitrogen

Total carbon (TC), total organic carbon (TOC), total inorganic carbon (TIC), were measured with a TIC/TOC Analyzer (Model 1020A, OI Analytical, USA) equipped with a non-dispersive infrared detector and a furnace maintained at 680 ºC.

TC, TOC, TIC, TN were recorded using a TOC analyser (Shimadzu, TOC-L and TNM-L units) for Chapter 6 experiments.

3.5.7 Glucose analysis

Glucose concentration was measured with a biochemical analyser YSI 2700 SELECT (Yellow Spring Instruments) in the concentration range 0-20 ± 0.04 g/L. Analysis is based on the enzymatic reaction of glucose oxidation to oxygen peroxide through the enzyme glucose oxidase immobilized in a membrane and the subsequent reduction of oxygen peroxide in a platinum anode, that converts the signal in electric intensity.

3.5.8 Laccase activity

Laccase activity was measured through the oxidation of 2,6-dimetoxyphenol (DMP) by the enzyme laccase in a modified version of the method for the determination of manganese peroxidase of (Kaal et al., 1993). The analysis process is based on the measure of the absorbance variance at 468 nm at 30 ºC during 2 min in a Varian Cary 3 UV/Vis spectrophotometer. The reaction was done with 600 µL of sample, 200 µL of sodium malonate 250 mM at pH 4.5 and 50 µL of DMP 20 mM. Activity units per litre (U/L) are defined as the amount of DMP in micromoles per litre which are oxidised per minute (µmol DMP/(L min)). The molar extinction coefficient of DMP was considered as 24.8 1/(mM cm) (Wariishi et al., 1992).

3.5.9 Actinometry

The actinometry method used to quantify average light intensity for the laboratory scale HRAPs and indoor beaker batch tests using potassium ferric oxalate was adapted from (Hatchard and Parker, 1956) the light spectrum was measured by an Optronics OL756 spectroradiometer (Orlando, FL, USA).

A solution of 0.006 M potassium ferric oxalate (PFO) in 0.1 N H_2SO_4 was prepared and added to the beakers at the normal working volume. 0.5 mL samples were taken at regular times over 60 min, and immediately added to 9.5 mL 0.1 N H_2SO_4, 5 mL buffer (0.6 N sodium acetate with 0.36 N H_2SO_4), and 2 mL phenanthroline monohydrate (2.72 g/L); the solution was made up to 25 mL with RO water, mixed, transferred to a cuvette, and after a 30 min wait absorbance readings were taken at 510 nm.

A calibration curve for iron (II) concentration was prepared, using $FeSO_4$ solution from 0 to 0.0003 M in 0.1 N H_2SO_4.

The rate of production of iron (II) in the ponds was converted to total irradiance received using the following equation:

$$I_0 = \frac{\beta}{q} N_A \frac{1}{A} \frac{hc}{\int_\lambda \lambda * f(\lambda) * (1 - T_\lambda)} \qquad \text{Eq. 3.1}$$

Where q is the quantum efficiency (1.21 (Hatchard and Parker, 1956), N_A is Avogrado's number (6.02 * 10^{23} mol^{-1}), h is Plank's constant (6.6 * 10^{-34} J.s), c is the speed of light in a vacuum (3 * 10^8 m/s), λ is the wavelength of the light, f(λ) is the fraction of the light energy emitted by the light bulb (Viva-lite®) in each interval [λ to λ+dλ], T_λ is the transmittance of the PFO at wavelength λ, and A is the surface area (m^2). β is calculated from the slope [Fe^{2+}]/time (s), multiplied by the total volume.

3.6 Microbial communities analysis

3.6.1 DNA extraction

Water samples were filtered in 0.22 µm pore GV Durapore membrane filters (Millipore) right after collection and stored at -80ºC until further processed altogether. DNA extraction was conducted using PowerWater®DNA Isolation Kit (MO BIO Laboratories, Inc.).

For eukarya polymerase chain reaction (PCR) about 10 ng of extracted DNA were used as the template in which eukaryotic 18S ribosomal DNA (rDNA)-specific primers were used. The tested denaturing gradient gel electrophoresis (DGGE) set of primers were Euk1A and Euk516r-GC (Table 3.22), which amplify a fragment approximately 560 bp long. The PCR mixtures (50 µL) contained each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.5 µM, 2.5 U of *Taq* DNA polymerase (Invitrogen), and the PCR buffer supplied with the enzyme. The PCR program for the primer set A included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 60 s. During the last cycle, the length of the extension step was increased to 5 min. An aliquot of the PCR product was electrophoresed in a 1.5% agarose gel, stained with ethidium bromide, and quantified by using a standard (Low DNA Mass Ladder; Invitrogen).

Table 3.22 Oligonucleotide sequences used.

Primer	Sequence (5' to 3')	Saccharomyces cerevisiae positions	Specificity	Reference
Euk1A	CTGGTTGATCCTGCCAG	4 to 20	Eukarya	(Díez et al., 2001)
Euk516r-GC[a]	ACCAGACTTGCCCTCC	563 to 548	Eukarya	(Díez et al., 2001)

[a] The GC clamp sequence is:
CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.

3.6.2 Denaturing gradient gel electrophoresis

DGGE was performed with the Dcode Universal Mutation Detection System (Bio-Rad). PCR products were loaded directly onto 6% (wt/vol) polyacrylamide gels containing a 20-45% chemical denaturing gradient and run in 1x TAE (40 mM Tris acetate [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). Gradients were formed with 6% (wt/vol) acrylamide stock solutions (acrylamide-N,N'-methylenebisacrylamide, 37:1) where the 100% denaturing solution contained 7 M urea (Bio-Rad) and 40% [vol/vol] formamide (Merck) deionized with AG501-X8 mixed-bed resin (Bio-Rad). Electrophoresis was performed at a constant voltage of 75 V and a temperature of 60°C for 16 h. After electrophoresis, the gels were incubated for 30 min in Milli-Q water containing ethidium bromide (0.5 mg/ liter), rinsed for 30 min with Milli-Q water, and photographed with UV transillumination (302 nm) with Universal Hood II (Bio-Rad) (Muyzer et al., 1993).

3.6.3 Sequencing

Prominent bands were excised using micropipette tips and re-amplified. These amplified DNA fragments from gel bands were placed in 96-well plates and sequenced by Macrogen Inc (South Korea).

Dendrogrames were calculated using the software InfoQuest FP 4.50 and plotted in FigTree v1.4.2.

3.7 Calculations

3.7.1 BMP tests and methanogenic activity assays

The daily biogas production was monitored measuring the pressure increase in the headspace of each reactor by means of a pressure switch manometer. The pressure data was then converted to a volumetric data thanks to the following Eq 3.2 that expresses the produced volume of biogas at standard conditions for temperature and pressure (0°C, 1 atm).

$$V_{STP} = \frac{T_{ST} \cdot P_R \cdot V_R}{P_{SP} \cdot T_R} \qquad \text{Eq. 3.2}$$

Where:

V_{STP} is the volume of biogas at standard conditions, [mL].

P_R is the pressure inside the batch reactor, [atm]

V_R is the volume of the reactor's headspace, [mL].

T_{ST} is the temperature at standard conditions, [K].

P_{SP} is the pressure at standard conditions, [atm].

T_R is the temperature inside the batch reactor, [K].

The daily methane production (V_{CH4} in mL_{CH4}) is obtained by sampling gas from the headspace of the vessel with a syringe and subsequent analysis of methane content by GC (TCD detection). In this way the volume of methane is calculated as the volume of biogas times the methane percentage registered in the gas-chromatograph (Eq. 3.3).

$$V_{CH_4} = V_{STP} \cdot CH_4(\%) \qquad \text{Eq. 3.3}$$

The BMP value (mL_{CH4}/g $VS_{substrate}$) is then determined as the net cumulate methane production (calculated as the sum of the daily volumes and obtained by subtracting the methane production of the blank assays from the methane production of each batch reactor containing substrate sample) divided by the g VS of substrate added at the initial time (Eq 3.4).

$$BMP = \frac{V_{CH_4,sub\&in} - V_{CH_4,in}}{g\ VS_{sub}} \qquad \text{Eq. 3.4}$$

Methanogenic activity rate is calculated using the following equation (Eq. 3.5).

$$CH_{4,activity} = \frac{R}{V_{eff} \cdot VS_{initial} \cdot CF} \qquad \text{Eq. 3.5}$$

Where:

R is the methane production rate [L_{CH4}/d], corresponding to the first medium or maximum slope in the graphic representation of the cumulative methane production versus time.

V_{eff} is the effective volume, [L].

$VS_{initial}$ is the initial inoculum concentration, [g VS/L].

CF is the theoretical conversion factor, 395 mL CH_4/ g COD at 35ºC.

3.7.2 Methane production potential

The theoretical stoichiometric methane potential (SMP) was calculated through the equation given by Symons and Buswell (1933).

$$C_aH_bO_cN_d + \left(\frac{4a-b-2c+3d}{4}\right)H_2O \rightarrow \left(\frac{4a+b-2c-3d}{8}\right)CH_4 + \left(\frac{4a-b+2c+3d}{8}\right)CO_2 + dNH_3$$

Eq. 3.6

The SMP was calculated using Eq. (2),

$$SMP = \frac{1}{8}\left(\frac{4a+b-2c-3d}{12a+b+16c+14d}\right)V_m \qquad \text{Eq. 3.7}$$

Where V_m is the molar volume of methane at standard temperature and pressure.

$$C_nH_aO_b + \left(\frac{4n-a-2b}{4}\right)H_2O \rightarrow \left(\frac{4n+a-2b}{8}\right)CH_4 + \left(\frac{4n-a+2b}{8}\right)CO_2 \quad \text{Eq. 3.8}$$

$$B_{o,th}\left[\frac{L\,CH_4}{g\,C_nH_aO_b}\right] = \frac{1}{8}\left(\frac{4n+a-2b}{12n+a+16b}\right)V_m \qquad \text{Eq. 3.9}$$

Where V_m is the molar volume of methane at standard temperature and pressure.

Fungal considerations for methane production potential

Fungal proteins, lipids and carbohydrates empirical formula was based on Møller et al. (2004) study ($C_5H_7O_2N$, $C_{57}H_{104}O_6$ and $C_6H_{10}O_5$, respectively).

Finally the theoretical methane production (TMP) was calculated using the formula previously reported by Prajapati et al. (2014c).

$$TMP = \frac{1}{100}\left(SMP_{protein}\cdot \%_{protein} + SMP_{lipid}\cdot \%_{lipid} + SMP_{carbohydrate}\cdot \%_{carbohydrate}\right) \qquad \text{Eq. 3.10}$$

Where the SMP of protein, lipid and carbohydrates are 0.496, 1.014 and 0.415 L CH_4/g VS, respectively. The percentages of each compound are based on a study carried out by Ma et al. (2015) of *Trametes hirsuta* and extrapolated to the

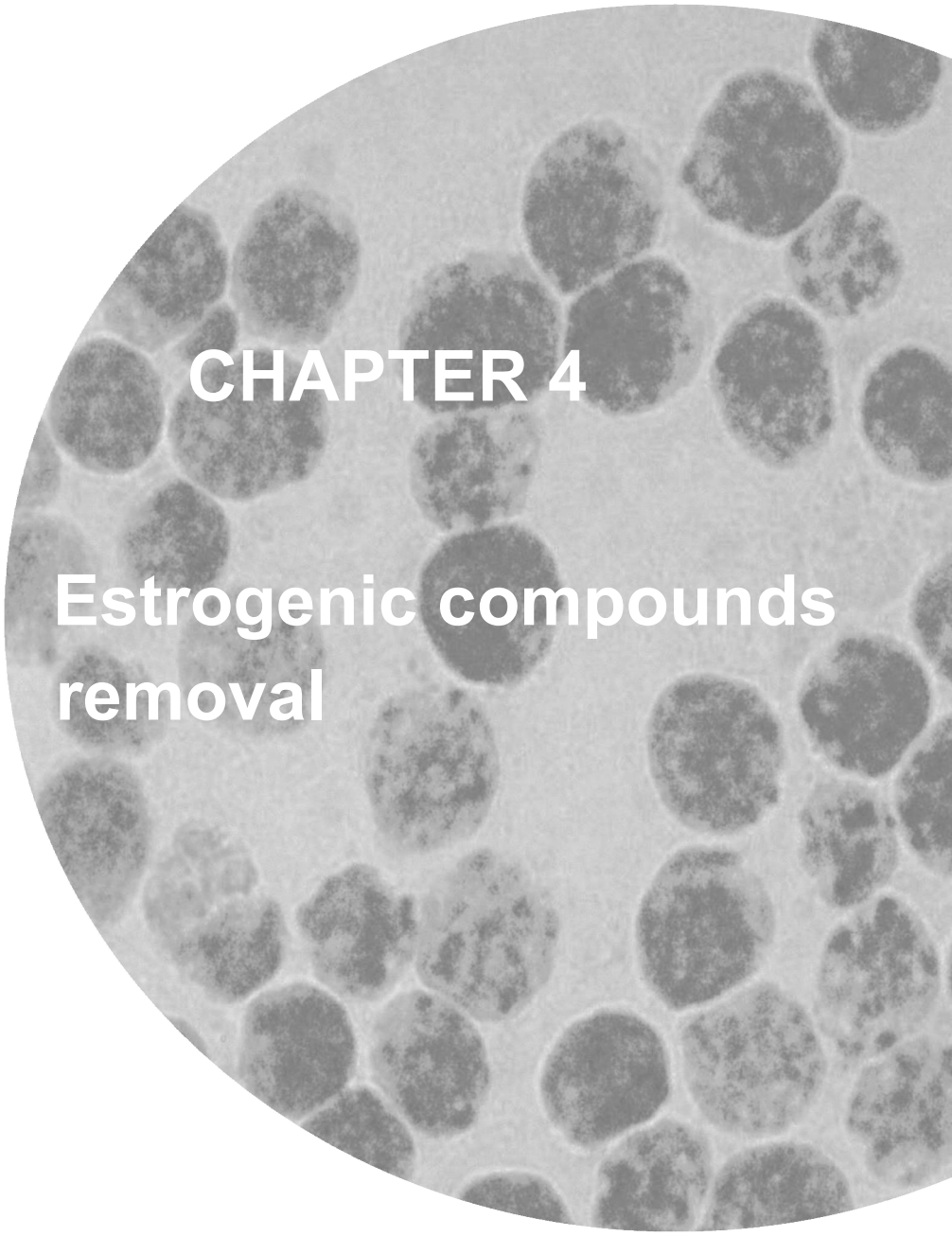
present fungi, *T. versicolor* (28.69% proteins including aminoacids, 1.15% lipids, 64.27% carbohydrates including fructose, glucose trehalose, arabinose and mannitol, 0.275% nucleotides (not considered for methane production) and 5,47% ash (not included in methane production).

3.8 Statistical analysis

All the experiments were performed in triplicate unless otherwise stated. The standard deviation and means were analysed for significance using the software Sigmaplot through one way ANOVA. Holm-Sidak method was used to compare the significance of differences among tested algae at *p* values of <0.05 results are reported as either mean ± standard deviation or error bars.

Section 3

EMERGING CONTAMINANTS REMOVAL BY MICROALGAE



CHAPTER 4

Estrogenic compounds removal

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4 Estrogenic compounds removal

Abstract

Pseudokirchneriella subcapitata and Chlamydomonas reinhardtii were tested for possible biodegradation of the hormones 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) culturing them in defined media and cultured in anaerobic digester centrate (ADC). Neither ADC nor the hormones had a negative or toxic effect on the microalgae growth but ADC enhanced it. E2 and EE2 biodegradation was evaluated under different culture conditions. After 7 days of treatment, between 88% and 100% of E2 was removed by P. subcapitata. Overall, 42 and 54% of the removal was attributed to biodegradation processes, while the rest of the removal was due to adsorption onto the microalgal biomass. For EE2, removals between 60 and 95%, depending on the culture conditions, were achieved, with biodegradation accounting for 20-54% of the removal. E2 and EE2 were completely removed in the experiments performed with C. reinhardtii, except for EE2 in the presence of ADC, which removal accounted for 76%. However, C. reinhardtii presented higher adsorption percentages: 86% and 71% after 7 days for E2 and EE2, respectively. Transformation products (TPs) of E2 and EE2 generated in each treatment were also determined. Two TPs were tentatively proposed as degradation products of E2 and EE2 by the microalgae. In addition, the removal of 26 endocrine disruptors and related pollutants present in the centrate were also monitored: bisphenol A was completely removed, whereas tris(2-butoxyethyl) phosphate was only removed in the absence of hormones.

4.1 Introduction

Estrogenic activities in wastewater treatment plant (WWTP) effluents have previously been described by several authors and were attributed to the presence of natural estrogens, such as 17β-estradiol (E2), and synthetic estrogens, such as 17α-ethinylestradiol (EE2) (Chang et al., 2011; Jarošová et al., 2014a). These compounds are excreted in the urine by women and therefore end up in wastewater (WW) effluents. They can cause severe damage because of their endocrine disrupting effects, provoking several negative effects on the environment and human and wildlife health, especially in aquatic environments, even at concentrations below 10 ng/L (Rudder et al., 2004). E2 and EE2 have recently been included as priority substances in the European Commission (European Commission, 2007) due to their high estrogenic potency. E2 and EE2 have been described as the most important micropollutants in municipal WWTPs (Rudder et al., 2004). Although some

urban WWTPs have been reported to remove up to 98% of E2 (Servos et al., 2005; Snyder et al., 1999) determined that EE2 is not removed. Due to the low biodegradability of these compounds, they are found in the environment (Esteban et al., 2014). The fate of hormones during sludge treatment has been poorly documented. Only a few studies have reported the fate and behaviour of estrogenic hormones in anaerobic sludge treatment at laboratory scale and full scale experiments (Ifelebuegu, 2011; Muller et al., 2010). In these studies, it was found that in full scale experiments, the removal percentages were lower (18-32% for E2 and 10-15% for EE2) and that the removal mechanisms were mainly sorption onto sludge biomass. Another study reported removal efficiencies of approximately 50% in sludge from anaerobic digesters (Paterakis et al., 2012). Hamid and Eskicioglu (2013) assessed the fate of steroidal hormones during sludge anaerobic digestion pre-treated by microwaves and reported removal efficiencies between 50 and 70%.

Because of the difficulty of removing hormones, there is a need to find a treatment to eliminate these compounds before they are discharged into surface waters to prevent any toxic effects in the environment. Several studies have been conducted to find an appropriate treatment for the removal of E2 and EE2; some examples of treatments are the use of manganese oxide (Rudder et al., 2004), sorption of EE2 on activated charcoal (Kumar and Mohan, 2011), and advanced techniques such as photo-catalysis (Ohko et al., 2002). Biological studies have employed fungi, such as *Trametes versicolor* and *Ganoderma lucidum* (Blánquez and Guieysse, 2008), or bacteria, such as *Sphingobacterium* sp. JCR5 (Haiyan et al., 2007). Microalgae can be used as low-cost systems for pollutant elimination treatments, through photooxidation and/or biodegradation. Della Greca et al. (2008) studied the removal and biotransformation of EE2 in a synthetic medium using the microalgae *P. subcapitata* and found that EE2 was transformed into 3 products ($C_{20}H_{24}O_3$, $C_{20}H_{23}O_4N$ and $C_{20}H_{24}O_3$). The conversion yield was enhanced as cell density increased. In addition, Abreu et al. (2012) and Singh et al. (2011) have reported that microalgae are able to grow in wastewater and exhibit high nutrient removal efficiencies.

In the present work, a microalgae cultivation study was developed using two microalgae strains, *Pseudokirchneriella subcapitata* (also known as

Selenastrum capricornutum and *Rhapidocelis subcapitata*) and *Chlamydomonas reinhardtii*, to evaluate their potential to biodegrade two estrogens growing on centrate from anaerobic digestion sludge. Due to the nitrogen and phosphorous content, several wastewater (urine, dairy, urban and digestate) can serve as a major nutrient source for microalgae cultivation (Abreu et al., 2012; Li et al., 2011a; Singh et al., 2011). *P. subcapitata* was selected as model specie because of its ubiquitous occurrence and easy cultivation. *C. reinhardtii* has been previously tested to be grown on wastewater centrate (Li et al., 2011a).

4.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

4.2.1 Anaerobic digester centrate

Anaerobic sludge was collected from an urban WWTP located in 'El Prat de Llobregat' (Barcelona, Spain), and the anaerobic digester centrate (ADC) was obtained by centrifuging at 3000 rpm for 15 minutes. The ADC used in the study was characterised (Table 4.1) and stored at -20 ºC until the set up of the experiments.

Table 4.1 ADC characterization prior use.

Parameter	Value
pH	7.86
TSS (mg/L)	202
COD (mg/L)	1150
$N\text{-}NH_4^+$ (mg/L)	153

4.2.2 Experimental set up

For E2 and EE2 removal *C. reinhardtii* was grown on P49 Medium (Table 3.5) and *P. subcapitata* on BG-11 Medium (Table 3.4) (Li et al., 2011a). The effluent used in the experiment was ADC (Table 4.1).

Seven different experimental conditions were established in triplicate, for each strain, to evaluate the growth as well as the estrogen degradation (Table 4.2) over 10 days. All experiments were carried out in 100 mL Erlenmeyer flasks containing an initial growth medium (GM) volume of 50 mL. Axenic culture control (A) and ADC culture control (F) were compared with conditions spiked with E2 and EE2, the axenic culture (B) and the ADC culture (G), respectively, in order to evaluate the estrogenic compounds removal by microalgal biomass using two different media: growth medium and ADC. A killed control (C) was performed to assess the contribution of sorption to the overall removal of hormones, in which microalgae were thermally inactivated (20 minutes at 121ºC). Two abiotic controls were carried out (D and E) containing sterilised medium without microalgae; they helped to determine whether light, evaporation and other physico-chemical processes had any effect on estrogen removal. Condition E was covered to be light preserved so that the contribution of photodegradation to the hormones removal could be determined. Unitary samples (2 mL) were taken daily and analysed.

Table 4.2 Different experimental conditions for P. subcapitata and C. reinhardtii and their respective abbreviations.

	Condition	Biomass	Hormones	Growth medium
A	Axenic Culture Control	Microalgae	-	GM
B	Axenic Culture	Microalgae	E2 + EE2	GM
C	Killed Control	Killed microalgae	E2 + EE2	GM
D	Abiotic 1	-	E2 + EE2	GM
E	Abiotic 2 (light protected)	-	E2 + EE2	GM
F	ADC Culture Control	Microalgae	-	GM+ADC
G	ADC Culture	Microalgae	E2 + EE2	GM+ADC

The initial microalgae concentration was 100 mg/L dry weight for both strains. Until the 3rd day, when biomass was at its exponential growth period (data not shown and in agreement with Mitra et al., (2012)), no modifications were made. On the 3rd day, Erlenmeyer flasks with experimental conditions (B, C, D, E and G) were spiked with E2 and EE2 at an initial concentration of 5 mg/L, respectively. Moreover, in Erlenmeyer flasks with conditions F and G, 10 mL of ADC was added on the same day.

4.3 Results and discussion

4.3.1 Growth results

Time-course profiles of *P. subcapitata* and *C. reinhardtii* cell growth are shown in Figure 4.1. It is observed that during the first growing period (from 0 to the 3rd day), all experimental conditions grew up at the same rate, achieving a biomass dry weight of 110 ± 3 mg/L for *P. subcapitata* and 266 ± 16 mg/L for *C. reinhardtii*. On the 3rd day, ADC and/or hormones were added, and the growth of the different microalgae under the different culture medium conditions was evaluated.

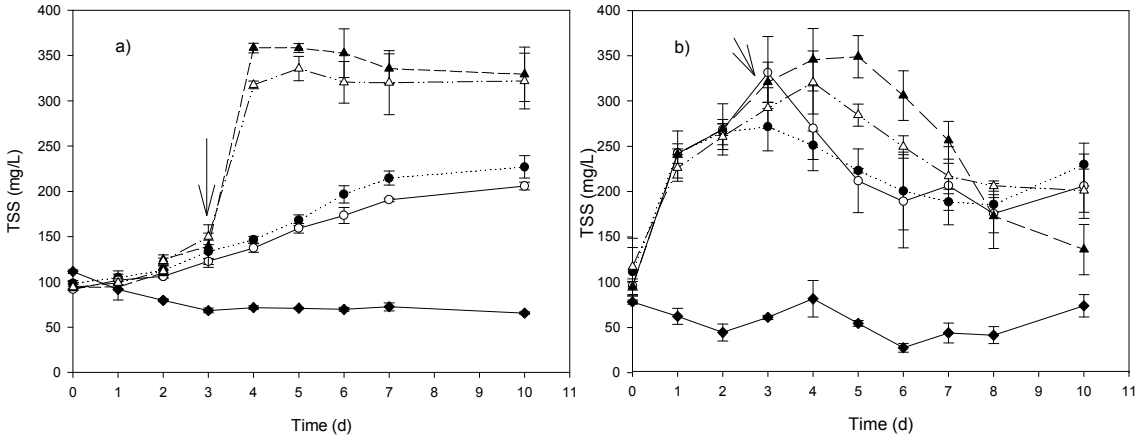


Figure 4.1 Time-course profile (10 days) of a) P. subcapitata growth and b) C. reinhardtii growth in terms of dry biomass: (●) *A, axenic culture;* (○) *B, microalgae with hormones;* (♦) *C, killed control;* (▲) *F, ADC culture without hormones;* (△)*G, ADC culture with hormones;* (↓) *hormones and/or ADC addition. Y-error bars represent the standard deviation.*

The results for *P. subcapitata* showed that growth was enhanced using ADC (conditions F and G), reaching 370 mg/L on the 4th day, whereas without ADC addition (conditions A and B), the biomass increased slightly reaching 150 mg/L. This behaviour is consistent with the results reported by several authors who studied the effect of different effluents on microalgae culture growth (Fenton and Ó hUallacháin, 2012; Mitra et al., 2012).

C. reinhardtii did not grow as much as *P. subcapitata* when ADC was added; the biomass on the 4th day was approximately 307 mg/L with ADC (F and G) and 302 mg/L without ADC (A and B). The difference between the strains could be attributed to the fact that the *P. subcapitata* GM contained glucose to establish mixotrophic cell cultivation using light and an organic carbon source. On the contrary, the *C. reinhardtii* medium did not contain carbon; hence, this strain was less capable of assimilating the carbon coming from the ADC effluent. Abreu et al. (2012) demonstrated that the mixotrophic growth is more efficient for microalgal production, while Moon et al. (2013) stated that *C. reinhardtii* growth under heterotrophic conditions is not beneficial.

From day 4th, *P. subcapitata* biomass was maintained until the end of the experiment; however, *C. reinhardtii* increased slightly for 2 days after ADC addition and then (from day 5) decreased. As Wang et al. (2012) proposed, successive carbon-nitrogen (C-N) feeding is the most suitable way to achieve higher cell concentrations and could be evaluated as another means of adding ADC when carbon and nitrogen are depleted.

E2 and EE2 were spiked (conditions B and G) on the 3rd day of the experiment to evaluate whether the estrogens had a negative effect on the microalgae growth. From Figure 4.1, comparing A with B and F with G, it can be observed that the growth was not affected when both hormones were added; the tendency was the same. Comparing the results for *P. subcapitata* when estrogens were added (condition B) with the culture (condition A), it can be observed that at the end of the experiment, the growth was only 9% lower in B than in A; however, on the 3rd day, before E2 and EE2 were added, the difference in the biomass concentration was 8% lower. This finding suggests that hormones do not affect strain growth. The same effect was observed under conditions F and G, cultures containing ADC. On the 3rd day, before hormone

spiking, the growth in F was 7% lower than that in G, but on the 10th day, the growth was approximately equal. Thus, neither ADC nor hormones inhibited the growth of *C. reinhardtii* or had a toxic effect; the growth decrease was attributed to nutrient depletion.

To disprove the idea of microbial growth being the main consequence of the growth due to the addition of non-sterile ADC, a sample was analysed under a microscope, and primarily microalgae strains were observed, so microbial growth in ADC was considered minor.

4.3.2 17β-estradiol and 17α-ethinylestradiol removal

During a 7 day period, after E2 and EE2 were spiked, both concentrations were monitored to determine the ability of each strain to eliminate these compounds. Figure 4.2 shows the results of E2 and EE2 removal. E2 and EE2 were below the detection limit for the *P. subcapitata* and *C. reinhardtii* controls (A and F), confirming that ADC does not contain detectable concentrations.

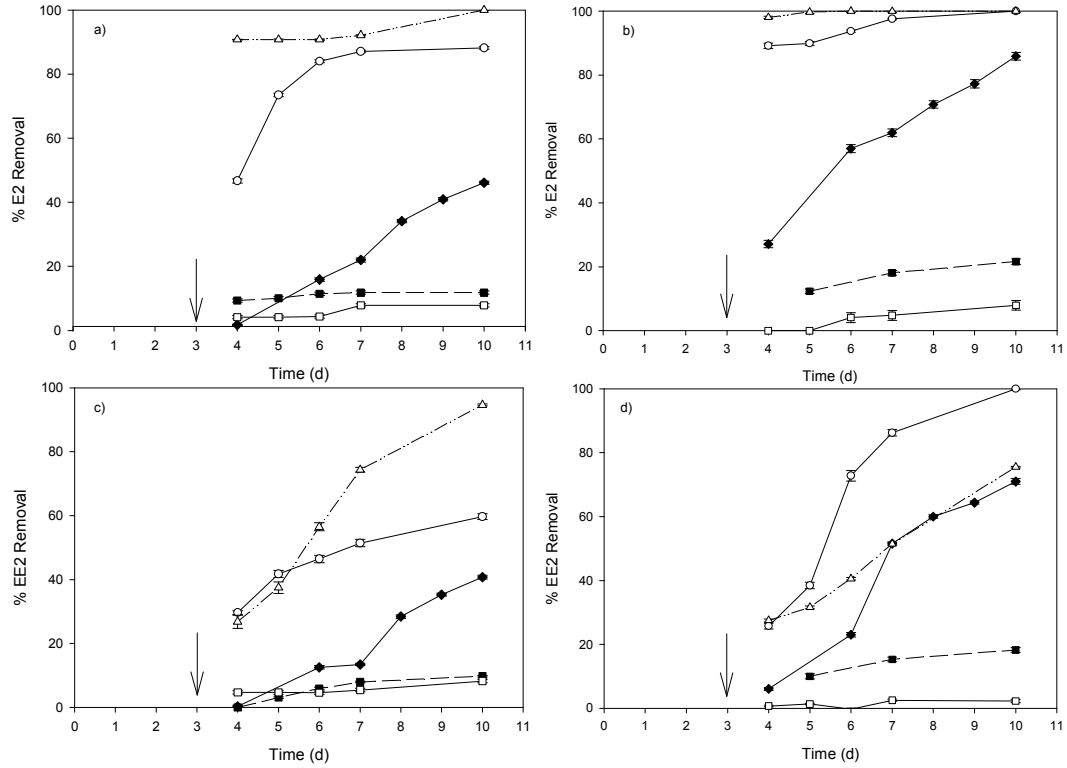


Figure 4.2 Time-course profile (10 d) of a) %E2 removal by P. subcapitata b) %E2 removal by C. reinhardtii c) %EE2 removal by P. subcapitata d) %EE2 removal by C. reinhardtii. (○) *B, microalgae with hormones;* (♦) *C, killed control;* (■) *D, abiotic culture with hormones;* (□) *E, abiotic culture with hormones and light protected;* (∆) *G, ADC culture with hormones;* (↓) *hormone and/or ADC addition.Y-error bars represent the standard deviation.*

The photodegradation (D) of both hormones was low, although it was higher in *C. reinhardtii*. Because the GMs were composed of different compounds, photodegradation could differ and therefore affect hormone removal. Evaporation losses (E) were low for both microalgae cultures.

In all graphs from Figure 4.2, it can be observed that the removal percentage increased over time. The highest percentages were observed for the E2 removal achieved by both strains, and both were higher than the killed control, which quantified the compound adsorption onto the biomass. Luo et al. (2014b) reported a higher adsorption capacity of dead algal cells than live cells due to changes in the cell membrane. At the end of the present study, the adsorption percentages were increased for both strains; as contact time increased, larger quantities were adsorbed. Shi et al. (2010) also reported that a portion of the estrogens was adsorbed on the live microalgae consortium surface, with great differences between the algae species despite similar initial biomass concentrations. Similar effects were reported for other pollutants such as polycyclic aromatic hydrocarbons (PAHs) tested on *P. subcapitata* and *Chlorella* sp. (Luo et al., 2014b). After 24 h, E2 and EE2 adsorption (C) on *P. subcapitata* was negligible (2% for E2 and 0% for EE2), whereas *C. reinhardtii* presented higher values (27% and 6% for E2 and EE2, respectively). The biodegradation percentages for E2 were 62% and 71% for conditions B and G, respectively, and 20% and 22% for EE2. EE2 adsorption onto the microalgae surface was lower than E2 adsorption; this effect is in accordance with the findings of Shi et al. (2010), who reported that the affinity of EE2 for the microalgae surface was lower than that of natural estrogens. The results are also in accordance with those obtained when other microorganisms were used, such as fungal biomass (Blánquez and Guieysse, 2008).

Table 4.3 shows the E2 and EE2 removal percentages at 24 h and 7 d after spiking. The hormone removal was related to an increase in biomass, and higher values of removal were achieved when growth increased. This increase occurred when ADC was added (experiment G), which enhanced *P. subcapitata* growth considerably (Figure 4.1). These percentages (>95%) were higher than the ones previously published by Shi et al. (2010), who reported a maximum percentage of 54% for E2 removal. On the other hand, *C. reinhardtii* E2 removal

was similar with and without ADC (experiment G and B) because the growth was exponential at the beginning due to assimilation of nutrients present in the culture. On the contrary, no increase in biomass was observed when ADC was added to the *P. subcapitata* culture. Twenty-four hours after ADC addition, E2 removal was similar for both strains: 0.012 mg $E2_{removed}$/mg biomass for *P. subcapitata* and 0.014 mg $E2_{removed}$/mg biomass for *C. reinhardtii*. Based on these results, it can be confirmed that E2 removal is related to biomass.

Table 4.3 E2 and EE2 removal percentages for P. subcapitata and C. reinhardtii 24 h after spiking and at the end of the experiment, 7 days after spiking. (B, microalgae with hormones; C, killed control with hormones; G, ADC culture with hormones).

	Pseudokirchneriella subcapitata				***Chlamydomonas reinhardtii***			
	E2 (%)		**EE2 (%)**		**E2 (%)**		**EE2 (%)**	
	24h	7d	24h	7d	24h	7d	24h	7d
B	47	88	30	60	89	100	26	100
C	2	46	0	41	27	86	6	71
G	91	100	27	95	98	100	28	76

EE2 is more stable than E2 and more persistent in the environment (Rudder et al., 2004), so it is more difficult to be removed by both strains. For experiment G, EE2 removal per mg of biomass was lower compared with E2 removal for both strains. This result is in accordance with Nadal et al. (2006), who found that the photodegradation rate was highly dependent on the molecular weight (MW), in the present work EE2 has a higher MW than E2. The EE2 removal values were 0.004 and 0.003 mg $EE2_{removed}$/mg biomass for the *P. subcapitata* and *C. reinhardtii* cultures, respectively. There were no significant differences in hormone removal between the experiments with and without ADC for both strains.

At the end of the experiment, E2 was completely removed by both strains, except by *P. subcapitata* in condition B (88%), where it seems that the biomass was not enough to remove E2 completely. The final EE2 removal percentages were consistent with the amount of biomass present. *P. subcapitata* removed a higher percentage of EE2 as biomass increased, in both the B and G

experiments, as did *C. reinhardtii*, which was capable of removing EE2 completely without an additional nutrient supply.

4.3.3 Endocrine disruptor degradation

Twenty-six endocrine disruptors and related compounds (caffeine, 1H-benzotriazole, 5-methyl-1H-benzotriazole, tris(2chloroethyl)phosphate, tris(chloroisopropyl)phosphate, levonorgestel, progesterone, tris(2-butoxyethyl)phosphate (TBEP), estriol-3-sulfate, estriol-16-glucuronide, estrone-3-sulphate, methylparaben, estriol, estrone-3-glucuronide, estradiol-17-glucuronide, ethylparaben, propylparaben, bisphenol A (BPA), benzylparaben, estradiol, estrone, diethylstilbestrol, trichlorocaraben, triclosan, octylphenol and nonylphenol) were analysed in conditions F and G at the time of spiking and at the end of the experiment. TBEP and BPA were the only ones detected (Figure 4.3).

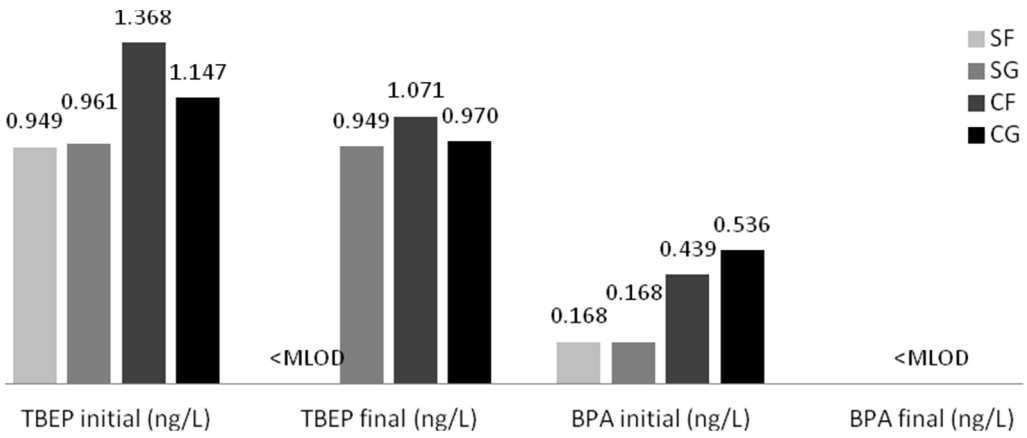


Figure 4.3 TBEP and BPA removal by microalgae (SF: P. subcapitata not spiked with hormones; SG: P. subcapitata spiked with hormones; CF: C. reinhardtii not spiked with hormones; CG: C. reinhardtii spiked with hormones; MLOD: Method Limit of Detection).

C. reinhardtii achieved TBEP removals of 15% and 22% in the presence and absence of E2 and EE2. On the other hand, TBEP was not significantly removed by *P. subcapitata* when E2 and EE2 were spiked, but it was removed below the method limit of detection (MLOD) in non-spiked samples. This is the first study reporting the removal of TBEP by microalgae. Therefore, further studies should be conducted to further investigate this phenomenon, although possible interactions between TBEP and hormones could have occurred. The

final concentrations of BPA were below the MLOD, so both strains were able to remove it. The results for BPA degradation by microalgae are in accordance with previous algal studies, such as the one by Gattullo et al. (2012), who reported 48% removal of BPA (4 mg/L initial concentration) with *Monoraphidium braunii* after 4 days of treatment. Li et al. (2009) performed similar experiments and achieved 88% removal with the lowest concentration tested (0.01 mg/L BPA) in a 16 day treatment with *Stephanodiscus hantzschii*.

4.3.4 Transformation products identified during microalgae treatments

Information about the degradation by-products of E2 and EE2 that is available in the literature was compiled to create an in-house library (as indicated in Chapter 3). This library (Table 4.4) was used to perform a screening analysis of the samples treated with microalgae using a LC-LTQ-Orbitrap. Those transformation products (TPs) tentatively detected in the samples together with their molecular mass and chemical structure are listed in Table 4.4 and Table 4.5.

Five TPs originating from E2 were tentatively identified in the experiments. The most abundant compound detected was estrone (TP1) in the experiments performed with both microalgae strains (B) and those with microalgae and ADC (G). It results from the oxidation of the alcohol at position 17. In this case, the compound was completely identified by comparing it (retention time and spectrum) with the standard compound. Estrone was identified as a degradation product of estradiol in *Chlorella vulgaris* cultures (Lai et al., 2002). It should be noted that this product was also detected in samples preserved under dark conditions (E), possibly due to some anaerobic and/or oxidation processes (Czajka and Londry, 2006; Zhao et al., 2008), degradation by proteobacterias (Pauwels et al., 2008), degradation by organisms present in water and activated sludge (Sarmah and Northcott, 2008; Ternes et al., 1999) and non-biological processes such as photolysis or oxidation (Mazellier et al., 2008; Zhao et al., 2008).

On the other hand, the generation of TP2, TP4 and TP5 cannot be attributed only to the microalgae, as they were detected in the killed controls as well (C). This finding indicates that this transformation can occur in non-biotic processes

and can be associated with photooxidation because dead cells have greater available binding sites (Luo et al., 2014b).

TP2 is originated from the hydroxylation of the aromatic ring during the treatments. However, it was not possible to identify the oxidation position, and two configurations were postulated for this compound (Table 4.4). TP2 was detected in previous studies performed with the fungi *Aspergillus alliaceus* (Williamson et al., 1985), as well as in ozonation experiments (Bila et al., 2007; Maniero et al., 2008).

TP3 has been described as one of the main metabolites of E2 (Dubey et al., 2001). However, this compound was only detected during the experiments carried out with *P. subcapitata* in conditions B and G.

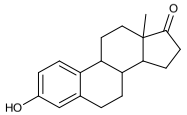
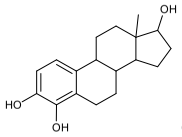
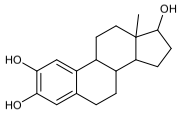
TP4 and TP5 were identified as possible transformation products in our study because two different chromatographic peaks (4.50 min and 5.00 min) were detected, in both microalgae experiments, for samples B and G, as well as in the killed control (C). However, it was not possible to differentiate between TP4 and TP5. The fragmentation patterns (during MS2 experiments) of these two compounds were exactly the same, as was their retention time according to the log P calculated by VCCLAB (Tetko et al., 2005; VCCLAB, 2005) (Table 4.4). TP4 and TP5 were identified as ozonation products in a previous work developed by Irmak et al. (2005).

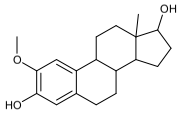
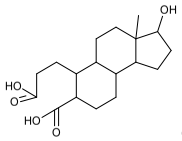
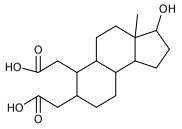
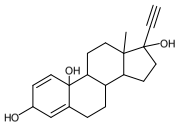
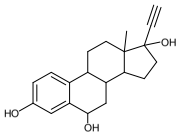
Concerning the degradation of EE2, 6 TPs were tentatively proposed. TP6 was postulated as a transformation product in condition B in the experiments with *C. reinhardtii*. This compound was also detected, among other transformation products, by Della Greca et al. (2008) during degradation studies of EE2 with different microalgae, including *P. subcapitata*. However, in our study with *P. subcapitata*, it was not possible to detect this compound. The difference in these results may be due to the different concentration of EE2 used during the experiments (5 mg/L in the present work *vs.* 100 mg/L by Della Greca et al. (2008)). TP7 was also postulated by Della Greca et al.(2008) as an EE2 biodegradation product of microalgae; in our study, however, it was detected in the killed control (C) with both microalgae strains, indicating the involvement of processes other than microalgal degradation.

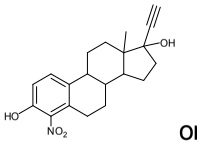
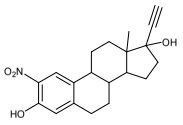
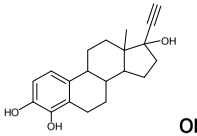
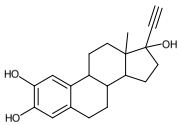
TP8 and TP9 have previously been reported to be transformation products of EE2 obtained via non-biotic nitration by ammonia-oxidising bacteria in studies carried out by Gaulke et al. (2008) and in various biotic and non-biotic degradation studies performed by Skotnicka-Pitak et al. (2008). Both were detected under different experimental conditions, with and without microalgae, with different results for each strain. The GM composition can lead to the formation of different radicals that identify different TPs in each culture. Luo et al. (2014b) grew two strains in the same GM and found the same TPs, concluding that the identification was independent of the strain. The presence of some of those TPs in the experiment C (killed control) suggests that the responsible for its generation was not only the microalgae.

TP10 and TP11 were detected, and their structure has been described in previously published works. However, it was not possible to distinguish the compounds by MS2 pattern or by their chromatographic retention time due to the similarity between the log P calculated by VCCLAB (Tetko et al., 2005; VCCLAB, 2005). Nonetheless, both compounds were detected in the experiments performed with *C. reinhardtii* (experiments B and G) as well as in experiment C with both microalgae. This last result could indicate that the generation of these compounds is not only due to the microalgae as Yi and Harper Jr. (2007) postulated in their nitrification studies.

Table 4.4 Transformation products of E2 and EE2 detected during microalgae experiments.

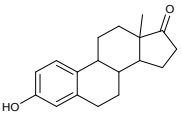
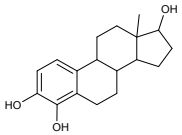
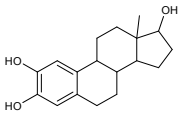
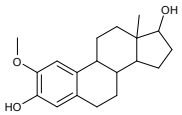
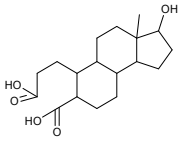
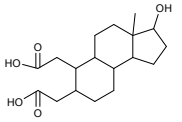
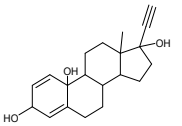
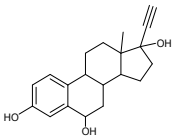
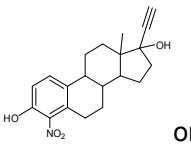
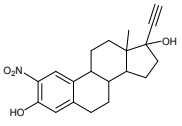
TP	Molecular structure	log P (VCCLAB, 2005; Wiley et al., 2000)	± SD	tr[a] (min)	Molecular formulae	Exact mass	Error (ppm)	Organism or process
1 (E2 TP)	Estrone 	3.65	0.32	7.06	$C_{18}H_{22}O_2$	270.1660	1.924 - 4.045	i) *Chlorela vulgaris* (Lai et al., 2002) ii) Anaerobic degradation in lake sediment or sludge (Czajka and Londry, 2006) iii) The strains belong to the α, β and γ-Proteobacteria (Pauwels et al., 2008) iv) Groundwater and river water biodegradation (Sarmah and Northcott, 2008) v) Activated sludge (Ternes et al., 1999) vi) Photolysis (Mazellier et al., 2008) vii) Oxidation (Zhao et al., 2008)
2 (E2 TP)	 or 	3.52	0.30	6.00	$C_{18}H_{24}O_3$	288.1770	1.899 - 3.772	i) Ozonation (Bila et al., 2007; Maniero et al., 2008) ii)*Aspargillus alliaceus* (Williamson et al., 1985)

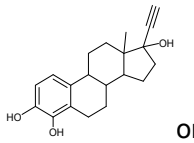
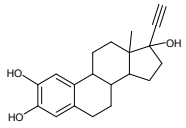
TP	Molecular structure	log P	± SD	tr[a] (min)	Molecular formulae	Exact mass	Error (ppm)	Organism or process
3 (E2 TP)		3.77	0.26	6.30	$C_{19}H_{26}O_3$	302.1930	2.906 - 3.615	Metabolites of estradiol (Dubey et al., 2001; Xiao et al., 2001)
4 (E2 TP)	 or	2.27	0.51	4.50	$C_{18}H_{24}O_5$	320.1670	2.140 - 3.038	Ozonation and O_3/UV (Irmak et al., 2005)
5 (E2 TP)		2.29	0.55	5.00	$C_{18}H_{24}O_5$	320.1670	2.072 - 3.038	
6 (EE2 TP)		2.14	0.71	5.22	$C_{20}H_{24}O_3$	311.16526	1.030	*Selenastrum quadricauda* (Della Greca et al., 2008)
7 (EE2 TP)		2.89	0.51	5.80	$C_{20}H_{24}O_3$	311.16526	2.359 - 3.874	*Ankistrodesmus braunii* (Della Greca et al., 2008)

TP	Molecular structure	log P	± SD	tr[a] (min)	Molecular formulae	Exact mass	Error (ppm)	Organism or process
8 (EE2 TP)		4.16	0.46	7.20	$C_{20}H_{23}O_4N$	340.15543	1.957 - 3.034	i) *N. europaea* and *Nitrosospira multiformis* bacteria (Gaulke et al., 2008)
or								
9 (EE2 TP)		4.16	0.53	7.70	$C_{20}H_{23}O_4N$	340.15543	2.316 - 3.213	ii) Biotic and abiotic degradation studies (Skotnicka-Pitak et al., 2008)
10 (EE2 TP)		3.67	0.56	6.23	$C_{20}H_{24}O_3$	311.16526	2.209 - 3.677	Nitrification processes (Yi and Harper Jr., 2007)
or								
11 (EE2 TP)		3.63	0.53	6.61	$C_{20}H_{24}O_3$	311.16526	2.239 - 3.972	

[a] *tr: retention time*

Table 4.5 Transformation products of E2 and EE2 detected during the 7 day batch experiments with the two microalgae tested (x: Experiments in which the transformation product was detected). More detailed information about the TPs can be found in Table 4.4.

TP	Molecular structure	*P. subcapitata*							*C. reinhardtii*						
		A	B	C	D	E	F	G	A	B	C	D	E	F	G
1 (E2 TP)	Estrone 		x			x		x		x			x		x
2 (E2 TP)	 or 		x	x	x			x		x	x				x
3 (E2 TP)			x					x							
4 (E2 TP)	 or		x	x				x		x	x				x
5 (E2 TP)			x	x				x		x	x				x
6 (EE2 TP)										x					
7 (EE2 TP)				x							x				
8 (EE2 TP)	 or		x	x	x			x							
9 (EE2 TP)			x	x	x										

TP	Molecular structure	*P. subcapitata*							*C. reinhardtii*						
		A	B	C	D	E	F	G	A	B	C	D	E	F	G
10 (EE2 TP)	 or			x						x	x				x
11 (EE2 TP)				x											x

4.1 Conclusions

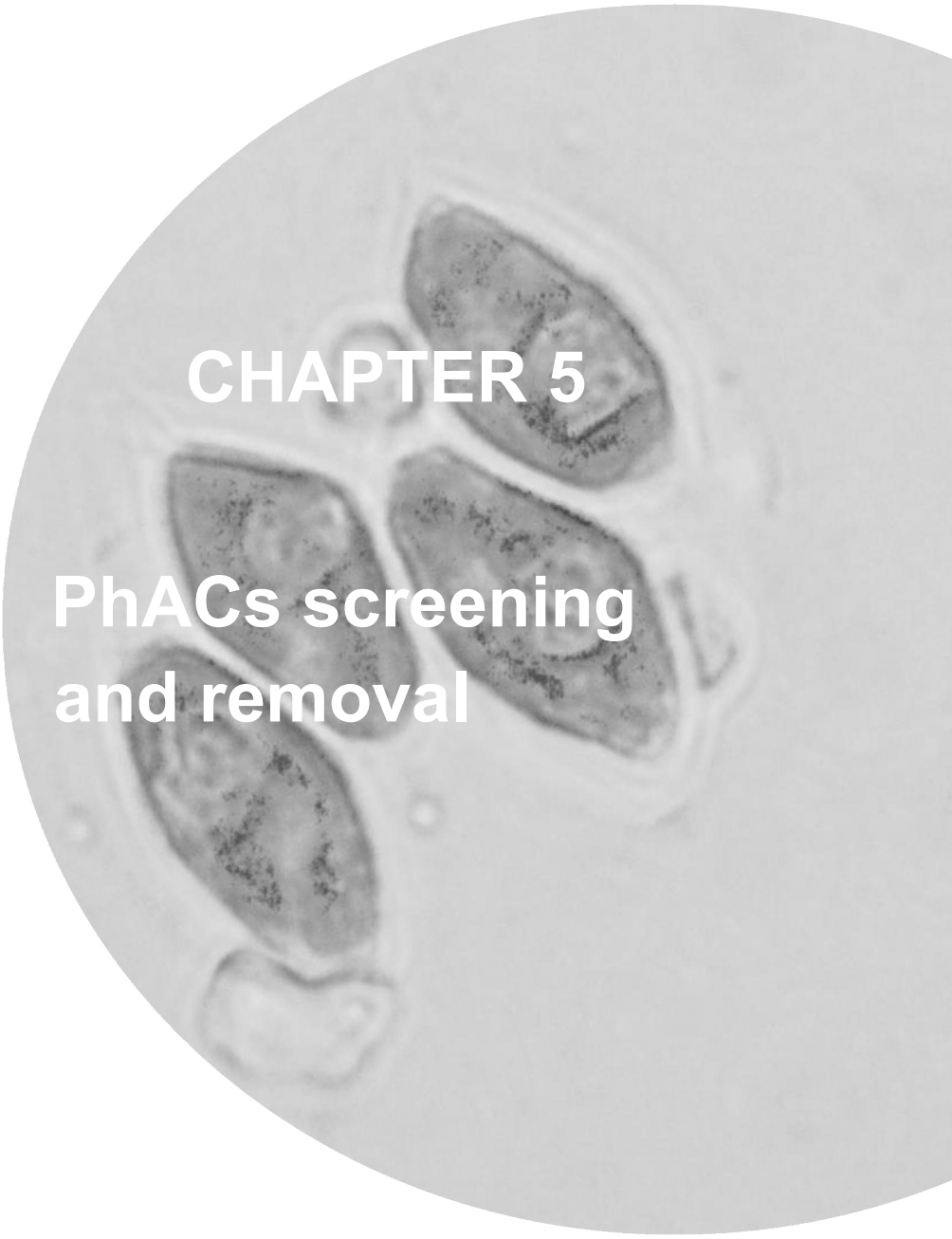
Pseudokirchneriella subcapitata and *Chlamydomonas reinhardtii* were grown in a general and specific medium, respectively, achieving maximum growth of 150 and 302 mg/L, respectively.

Anaerobic digestion centrate addition enhanced microalgae growth due to the nutrient supply, *P. subcapitata* increased its growth 2.5 times, but ADC only increased *C. reinhardtii* growth 2%.

E2 and EE2 were shown to not be toxic for microalgae growth, at the concentration tested, and both strains were able to remove both estrogenic compounds within 7 days, although EE2 was more difficult to remove. The final removal values were 88-100% for *P. subcapitata* and 76-100% for *C. reinhardtii*. ADC addition enhanced hormone removal in the *P. subcapitata* culture because it increased the growth of the strain, and removal is directly related to the biomass in the culture; 89% biodegradation was observed 24 h after ADC was added.

Several degradation products of E2 and EE2 were detected. Only one TP was shown to be directly related to *P. subcapitata* degradation of E2, and another was found to be related with EE2 degradation in the *C. reinhardtii* culture. Other products were detected as a consequence of photodegradation.

Finally, in the experiments with ADC, BPA was completely removed by both strains, whereas TBEP was only degraded when hormones were not spiked.



CHAPTER 5

PhACs screening and removal

5 PhACs screening and removal

Abstract

Batch experiments with four microalgae strains in axenic conditions were conducted for 10 micropollutant (antibiotics and an antidepressant pharmaceutical active compounds) removal assessment. Different experimental conditions gave information about the removal mechanisms involved. Venlafaxine concentration remained constant along the study, none of the tested mechanisms occurred. Regarding the antibiotics, high removal percentages were obtained for Chlamydomonas reinhardtii and Chlorella sorokiniana cultures (>73% for sulphapyridine). Furthermore, high growth rates were obtained. Pseudokirchneriella subcapitata and Dunaliella tertiolecta gave lower removal percentages for most of the micropollutants tested (<48% and <56%, respectively). Photodegradation removal mechanism has been detected for fluoroquinolones, whereas high sorption values have been detected in pipemidic acid. Sulphapyridine removal could be partially biodegraded. Several removal mechanisms took place on macrolides and trimethoprim removal from the cultures. Transformation products were identified for 3 selected micropollutants (azithromycin, erythromycin and sulphapyridine).

5.1 Introduction

Microalgal-based technologies have been recently considered as potential systems for emerging contaminants (ECs) removal.

Most of the studies based on microalgae for ECs removal are focused in 1 or 2 ECs and usually at laboratory scale, including axenic cultures. Degradation of estrogenic compounds has been widely documented (Della Greca et al., 2008, 1996; Lai et al., 2002), also several authors used microalgae for aromatic compounds degradation (phenolic compounds, naphthalene, azo dyes, diaryl ethers) (Pinto et al., 2002; Semple et al., 1999; Todd et al., 2002). Biotransformation of 6 pharmaceutical active compounds (PhACs) (ibuprofen, diclofenac, metoprolol, trimethoprim, paracetamol and carbamazepine) on *C. sorokiniana* culture has been reported by de Wilt et al. (2016), however the biotransformation was attributed to the activity of natural present microorganisms in urine and anaerobically treated black water where the microalgae were grown. Therefore, it is still an issue of concern if microalgae can biodegrade those pollutants.

It is well known that antibiotics pose a significant risk to environmental and human health, even at low concentrations (Kümmerer, 2009a). In addition, the overuse and misuse of antibiotics in both, humans and animals, has led to the emergence of antibiotic-resistant bacteria and concern has been received on the residues for the environment and the toxicology effect (Dantas et al., 2008; Kasprzyk-Hordern et al., 2009; Pruneau et al., 2011), so new technologies are required for its removal. Antibiotics can be classified by either their chemical structure or mechanism of action (chloramphenicols, macrolides, tetracyclines, sulphonamides, fluoroquinolones, imidazoles, β-lactams and others (Ahmed et al., 2015)). De Godos et al. (2012) used a pilot scale high rate algal pond (HRAP) fed with synthetic wastewater (WW) for the removal of the antibiotic tetracycline. Dissolved tetracycline removal was stabilised around 69%, and the main removal mechanisms were attributed to sorption and photodegradation. Moreover, 4 freshwater green microalgae were employed by Zhou et al. (2014) to study the removal of various contaminants (including antibiotics) in real WW in Erlenmeyer flasks. Clarithromycin and roxithromycin were highly removed (>80%), however, lincomycin was poorly removed (<50%).

The aim of this study is to determine the microalgal capacity for the removal of 9 antibiotics from different categories and a persistent antidepressant commonly detected in WW effluents, venlafaxine. All 10 PhACs were spiked together, since it has been pointed out that a mixture of drugs may have a different mode of action. On the other hand, PhACs in real water samples may act together. Not only the absolute component concentrations are important but also their contribution of the mixture effect (Mater et al., 2014). Representative antibiotics have been assessed.

Three macrolide antibiotics (azithromycin (AZM), clarithromycin (CRM) and erythromycin (ERM)) were studied. Macrolide antibiotics are an important class of antibiotics and they are considered the most important antibacterial agents used in human medicine (Evgenidou et al., 2015). AZM, has been detected at high concentration on sludge (Jelic et al., 2012). Contrary to AZM, ERM is not stable in the aquatic environment and it is rapidly converted in the dehydrated product, consequently, the detection of ERM in wastewaters can proceed only via the detection of its dehydrated metabolite, ERM-H_2O, and according to the

literature, this transformation product (TP) does no longer exhibit antibiotic properties (Evgenidou et al., 2015). CRM has been detected to have high persistence in various environmental matrices and partial or no degradation during wastewater treatment has been obtained (Clara et al., 2005; Zhang et al., 2008). Trimethoprim (TMT) has been detected in wastewater treatment plants (WWTPs) influents at concentrations ranging from 0.14 to 1.3 mg/L and in effluents from 0.02 to 1.3 µg/L (Le-Minh et al., 2010).

Fluoroquinolones (FQ) are a significant group of antibiotics due to the high potential against pathogens and to the broad activity spectrum as has been reported by Andersson and MacGowan (2003) who evaluated the antibacterial spectrum and potency of ciprofloxacin (CPX), norfloxacin (NFX) and ofloxacin (OFX).

Pipemidic acid (HPM) is a commonly quinolone found in wastewaters. Removal efficiencies of quinolone derivatives from the aqueous phase during WW treatment have been reported to be usually high, for that reason the removal by microalgae strains has been assessed. Finally, sulphayridine (SPD), a sulphonamide antibiotic, has been included in the study. Sulphonamides are one of the most widely used antibiotics in humans and especially in animal husbandry and fish farming (Cháfer-Pericás et al., 2010; García-Galán et al., 2012).

The highly recalcitrant antidepressant venlafaxine (VFX) was also evaluated together with the antibiotics, because of its low degradation during WW treatment. It has been found in WW and in environmental waters (Baker and Kasprzyk-Hordern, 2013; Boix et al., 2015; Gracia-Lor et al., 2011). Approximately 29–48% of a venlafaxine dose is excreted in the urine within 48 h as the unconjugated metabolite O-desmethylvenlafaxine, while 5% is excreted as unchanged parent compound (Baselt, 2004; Howell et al., 1993), however, other authors reported that only a fraction of the drug was transformed to O-desmethylvenlafaxine under aerobic conditions, whereas under anaerobic conditions VFX was almost exclusively converted to O-desmethylvenlafaxine (Gasser et al., 2012). Some authors reported the depletion of VFX, with the 99.9% eliminated after 5 minutes of reaction, upon advanced oxidation with UV/H_2O_2 at laboratory conditions (García-Galán et al., 2016). An electrocatalytic

ozonation process, completely degraded venlafaxine under optimal reaction conditions (Li et al., 2015). Removals obtained in different WWTP were also low, ranging between 18 to 40% (Kasprzyk-Hordern et al., 2009; Metcalfe et al., 2010; Schlüsener et al., 2015).

Two of the microalgal strains selected for the study have been *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* since they are considered model organisms, several studies have been conducted using them and for the good results obtained in Chapter 4. The third strain selected has been *Chlorella sorokiniana*. *Chlorella* genus have been widely used, they have rapid grow rates and efficient WW nutrient removal (Kümmerer, 2008). Finally, *Dunaliella tertiolecta* has been selected because its potential for biodiesel production despite its slow growth (Arroussi et al., 2015).

5.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

5.2.1 Screening set up

Five different experimental conditions were established in triplicate for each strain to evaluate the growth as well as the PhACs removal over 14 days (Table 5.1). Small microalgae aliquots from grown cultures were introduced into Erlenmeyer flasks containing 100 mL of growth medium (GM). Experimental cultures (A and B) contained algae under light and dark conditions (24h). Abiotic controls (C and D) contained sterilised medium without algae under continuous light irradiation and darkness; they helped to determine whether light, evaporation, photodegradation and other physico-chemical processes had any effect on PhACs removal. A killed control (E) under 24 h light was performed to assess the contribution of sorption to the overall removal of pollutants, in which algae were thermally inactivated (20 minutes at 121 ºC).

0.1 mg/L of each PhAC, azithromycin (AZM), clarithromycin (CRM), erythromycin (ERM), trimethroprim (TMT), norfloxacin (NFX), ciprofloxacin

(CPX), ofloxacin (OFX), pipemidic acid (HPM), sulphapyridine (SPD) and venlafaxine (VFX) were spiked into the cultures. Unitary samples for PhACs removal determination were taken at time zero, 7 days after and at the end of the experiment (14 days). The samples were analysed by HPLC coupled to a triple quadrupole mass spectrometer.

Table 5.1 Experimental conditions for PhACs removal with microalgae strains and their respective abbreviations. (+ indicates the presence of PhACs and/or light; - indicates the absence of PhACs and/or light).

	Experimental condition	Culture	PhACs	Light
A	Experimental Light	Algae	+	+
B	Experimental Dark	Algae	+	-
C	Abiotic Light	-	+	+
D	Abiotic Dark	-	+	-
E	Killed Control	Killed algae	+	+

5.3 Results and discussion

5.3.1 Growth results

Time-course profiles of *C. reinhardtii, C. sorokiniana, D. tertiolecta* and *P. subcapitata* cell growth under light and dark conditions are shown in Figure 5.1. All experimental conditions grew up until the PhACs spiking (vertical arrow), after that moment growth variations between dark and light samples were observed.

Exponential growth of *Chlamydomonas reinhardtii* achieved a total suspended solids (TSS) value of 703 mg/L after 7 days of growth under light conditions, then the cell concentration decreased slightly until 450 mg/L at the end of the experiment. Whereas under dark conditions exponential phase ended on the 5th day achieving a TSS concentration of 314 mg/L, afterwards, cell concentration decreased until 150 mg/L at the end of the experiment.

Chlorella sorokiniana exponential growth phase under light conditions was similar to the *C. reinhardtii* culture. The exponential phase ended with a TSS

concentration of 673 mg/L, followed by light decrease and maintenance around 540 mg/L until the end of the experiment. There was no growth detected under dark conditions, TSS was constant with a slightly decrease during the following 14 experimental days.

Dunaliella tertiolecta growth did not have a pronounced exponential phase, and there was not a visible lag phase, that lack may be because the majority of the cells at the time when they were transferred to the culture media were in the growth phase. The growth increased until the end the experiment, a similar profile as Sánchez-Saavedra et al. (2010) published. However, dark samples immediately stopped growing since no light was irradiated, there was a rapid decrease at the beginning and then the tendency seemed to stabilise.

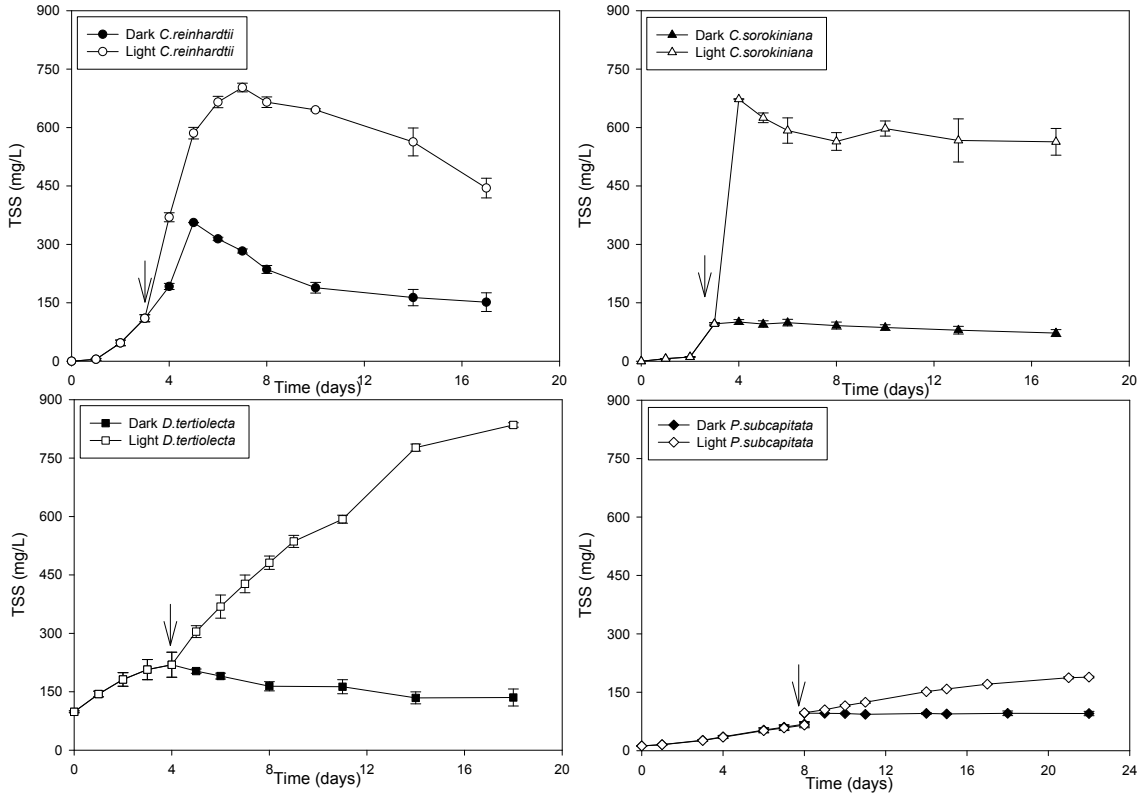


Figure 5.1 Time course growing profiles.(↓) *indicates the moment when PhACs were spiked to the culture.*

Pseudokirchneriella subcapitata growth was lower than for the rest of the strains. The maximum TSS obtained was 189 mg/L. *P subcapitata* under light kept growing despite the PhACs addition. However, some inhibition may be occurring decreasing the growth rate. Dark samples stopped growing and the biomass remained constant until the end of the experiment.

5.3.2 Antibiotics and venlafaxine removal

In this study the use of four microalgae strains has led to different removal percentages depending on the antibiotic tested. The antidepressant venlafaxine has also been considered.

Decrease on PhACs concentration is presented in the following figures (from Figure 5.2 to Figure 5.11). Clear differences on PhACs removal depending on the compound and the microalgae strain are observed.

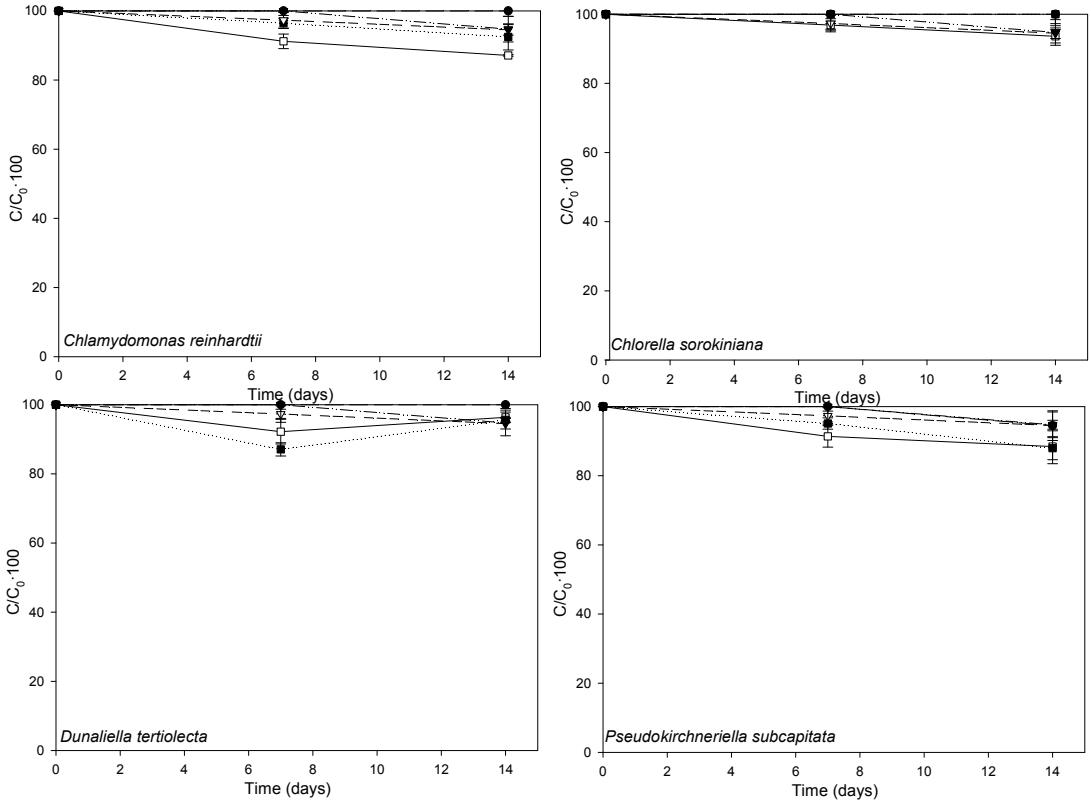


Figure 5.2 Venlafaxine (VFX) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

The persistence capacity of the antidepressant venlafaxine (Figure 5.2) has been confirmed, it has not been removed neither in abiotic controls, nor in microalgae cultures.

Figure 5.3 to Figure 5.5 show the macrolides AZM, CRM and ERM removal. From dark and light abiotic controls it is seen that photodegradation (light abiotic) and hydrolysis (dark abiotic) occurs, 33% and 20%, for AZM and ERM, respectively, although CRM hydrolysis is lower, 5%. Sorption onto the algal biomass depends on the strain, obtaining a wide range of percentages for the

same compound by the four microalgae strains. It is worth saying that results from the killed control may not be only due to sorption, since the microalgal biomass changed during the thermal process, the colour changed (from green to yellow-brown) and as more days passed, clearer the suspension was, allowing more light irradiance, whereas the microalgae culture had the shading effect due to cells in suspension and less light was irradiated inside the culture. In general, *C. reinhardtii* killed controls had high macrolides removals (42-64%), whereas they were poorly removed in *P. subcapitata* cultures (<23%). These results are in accordance with the ones obtained by Kobayashi et al. (2006) studied the removal of some antibiotics during WW treatment, concluding that AZM and CRM were not much removed, however, the adsorption onto the activated sludge was the main removal mechanism for AZM.

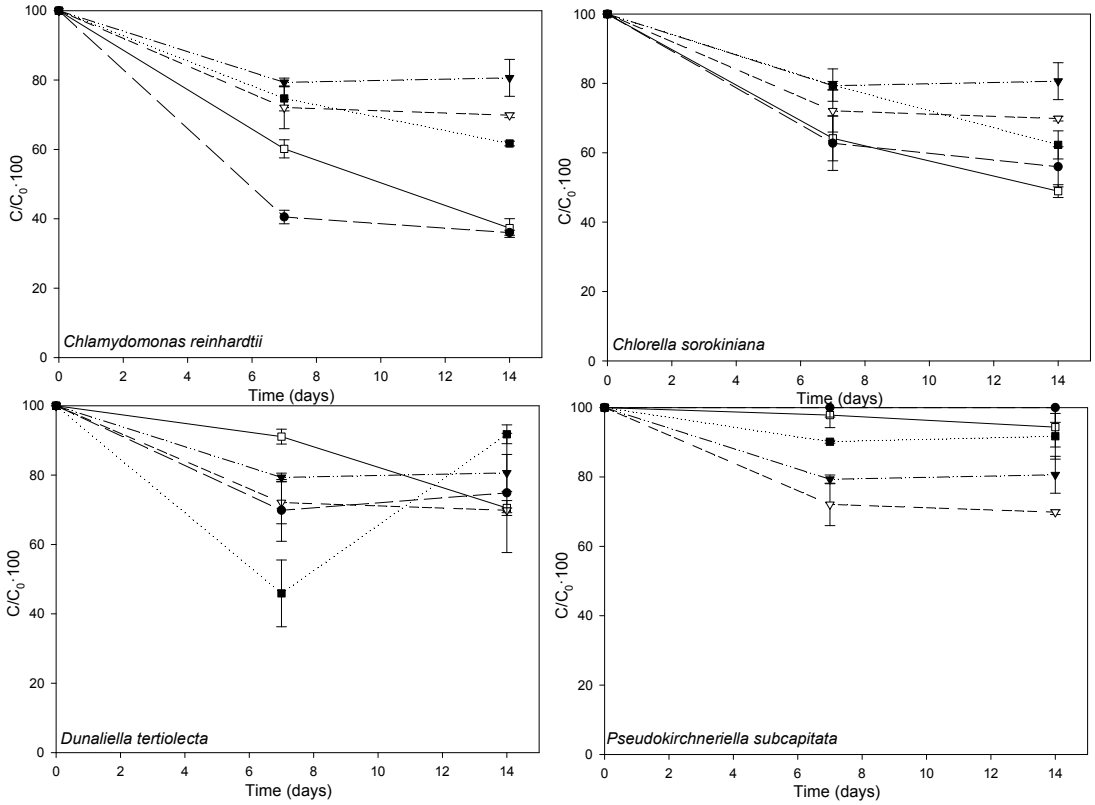


Figure 5.3 Azithromycin (AZM) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

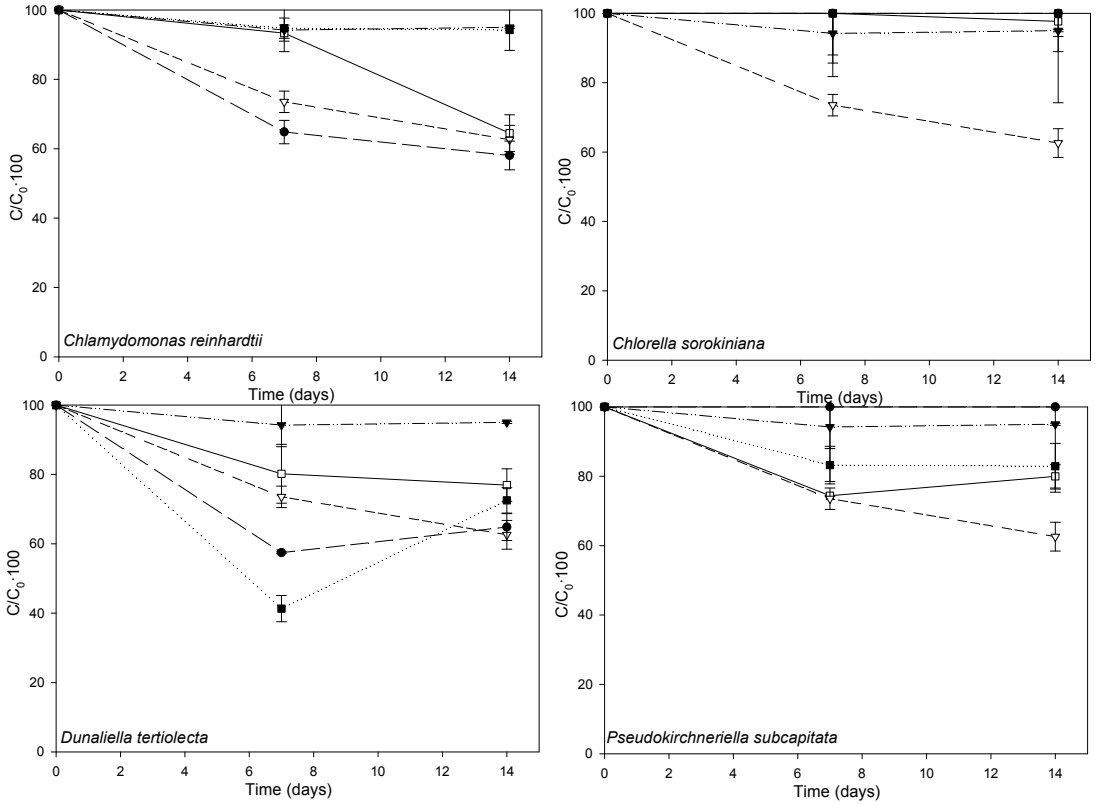


Figure 5.4 Clarithromycin (CRM) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

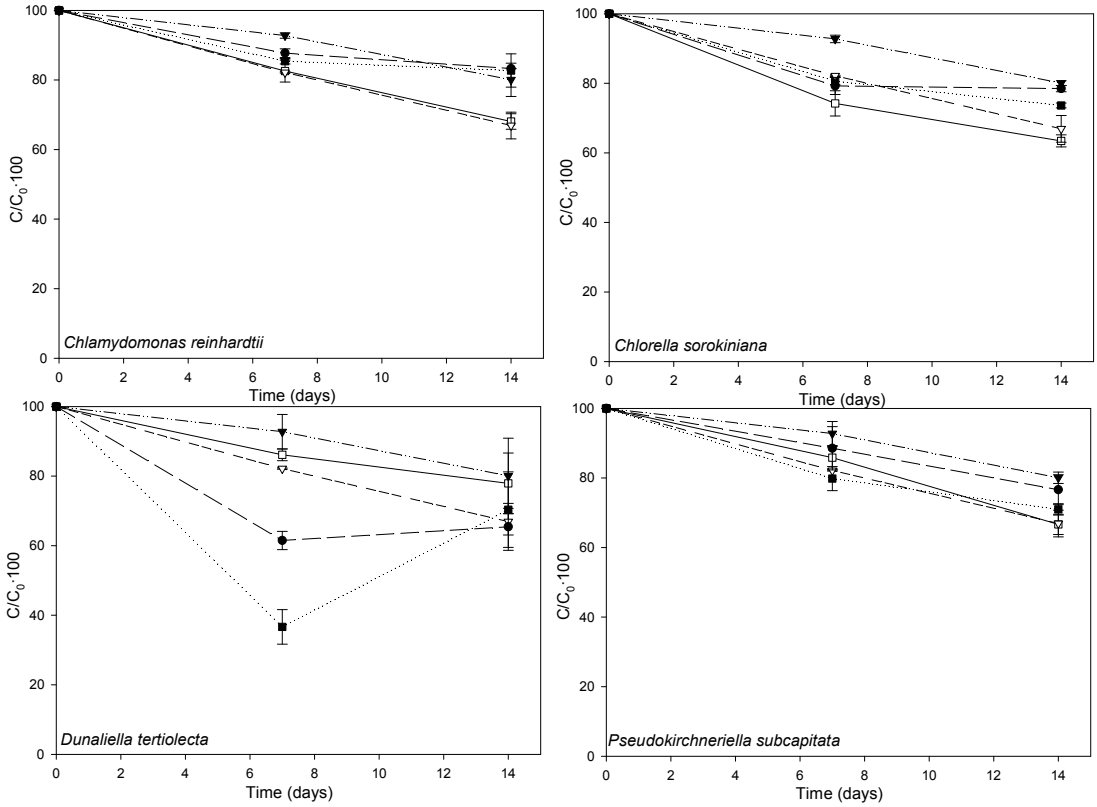


Figure 5.5 Erythromycin (ERM) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

Regarding microalgal removals, AZM (Figure 5.3) presented higher removal percentages than CRM and ERM (Figure 5.4 and Figure 5.5, respectively), except for *P. subcapitata* culture. Great AZM removal percentages were obtained for *C. reinhardtii* and *C. sorokiniana* cultures, 63 and 52%, respectively. Under the dark, removals were lower; in that case, part of the compound could be adsorbed onto the microalgal biomass. ERM removal percentages were similar for all microalgae strains, around 35%. Under the darkness; removals were reduced to 25-30%. ERM toxicity has been studied on *P. subcapitata* strain by Nie et al. (2013), obtaining that ERM may cause toxic effects on its antioxidant system, as well as other cycles that may inhibit the microalgae functions, so ERM removal may be affected. On the other hand, Campa-Córdova et al. (2006) evaluated the effect of ERM on *Isochrysis galbana* microalgae, it was not significantly affected at the test doses of 0.5, 1.0, 3.0, 6.0, 9.0, and 12.0 mg/L. With regards to CRM, lower removal percentages were observed (<36%), moreover, in *C. sorokiniana* culture no removal was detected.

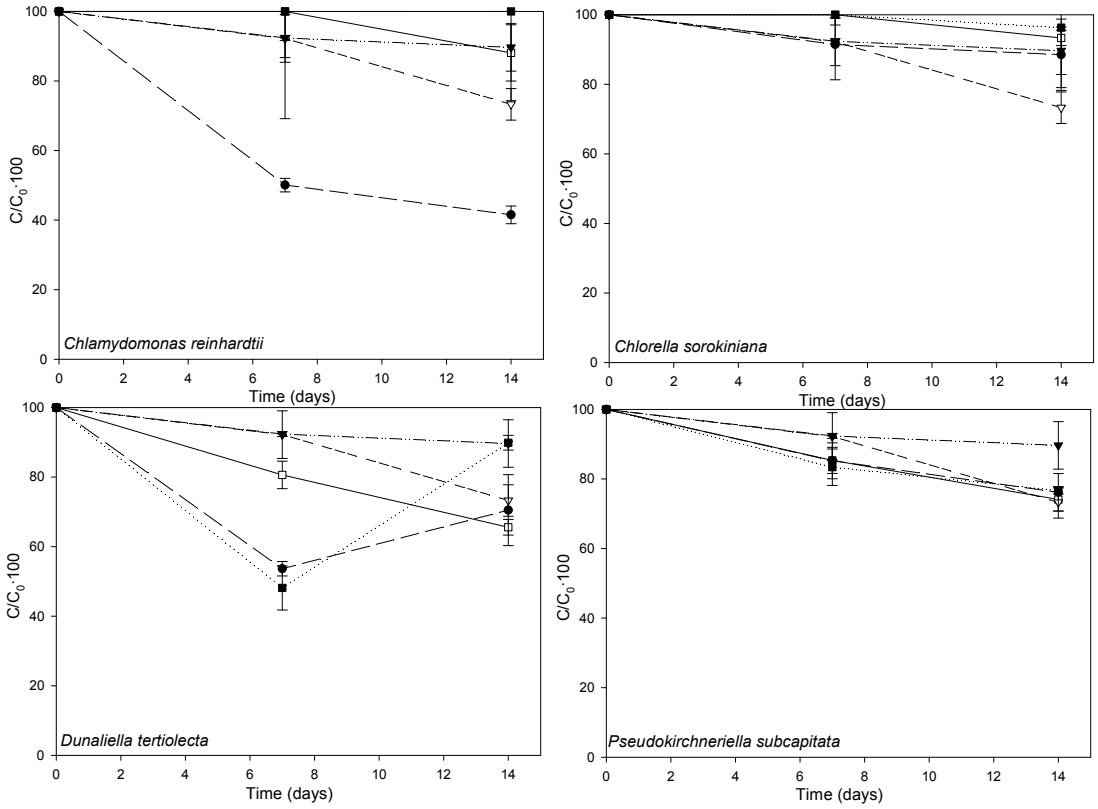


Figure 5.6 Trimethoprim (TMT) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

TMT removal is presented in Figure 5.6. Under abiotic conditions 10% of the compound is lost, whereas photodegradation can contribute up to 17%. On killed controls removal can be increased to 30%, although *C. reinhardtii* doubles that value. Regarding live conditions, most of the strains do not experience removal, except *P. subcapitata*, which the same percentage is removed from light and dark conditions. In that case, TMT may be adsorbed onto *P. subcapitata* cell surface, whereas for the other strains only little sorption occurs and the removal percentages may be due to biodegradation or molecule conversion due to light of exudates secreted by the microalgae. In several studies it has been found that TMT is quite resistant to biological wastewater treatment (Göbel et al., 2007; Lindberg et al., 2006), while other studies reported a partial removal of TMT ranging from 40 to 50% (Batt et al., 2006; de Wilt et al., 2016; Radjenovic et al., 2009) or even an almost complete removal (Kovalova et al., 2012). Biodegradation has been observed and it is often attributed to removal under aerobic (nitrifying) conditions (Batt et al., 2006; Eichhorn et al., 2005), which leads to the possibility that the removal percentages obtained in the present study can be carried out by microalgae degradation. However, low biodegradability has been proved by Yu et al. (2011), 27% was removed via biodegradation, and 40% was removed from the aqueous phase by bio-sorption or sorption, during the 14 days experiment using immobilized cell process, whereas volatilization and hydrolysis were negligible. Jewell et al. (2016) studied the variability of TMT removal in an activated sludge system treating municipal wastewater, it was found that the transformation products formation was influenced by the spike concentration. Some of the TPs were formed due to hydroxylations and several subsequent redox reactions, some by oxidation and some TPs were identified as photodegradation products of TMT. However, some authors pointed out that apart from biotransformation, photolysis is a possible mechanism (de Wilt et al., 2016; Ryan et al., 2010), whereas other stated the need for its oxidation prior to the biological treatment (Mansour et al., 2015). Lower removal percentages, ranging from 20 to 30%, have been detected in biofilm systems (Escolà Casas et al., 2015a, 2015b).

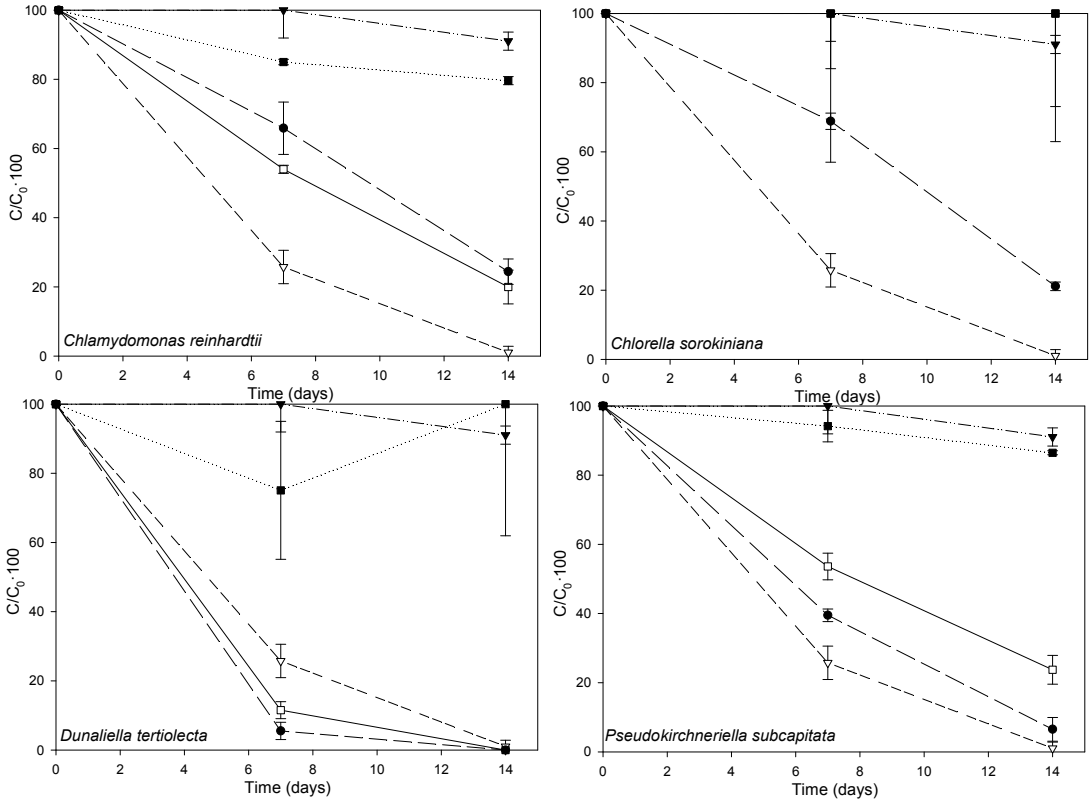


Figure 5.7 Ciprofloxacin (CPX) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

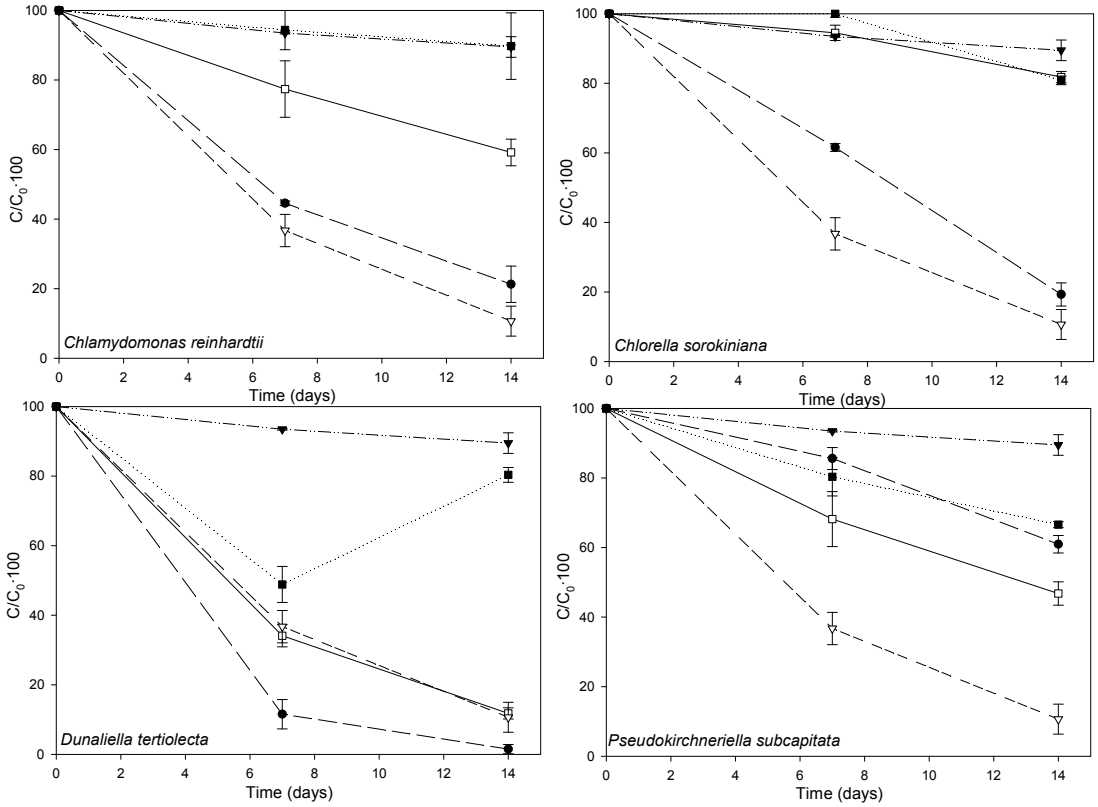


Figure 5.8 Ofloxacin (OFX) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

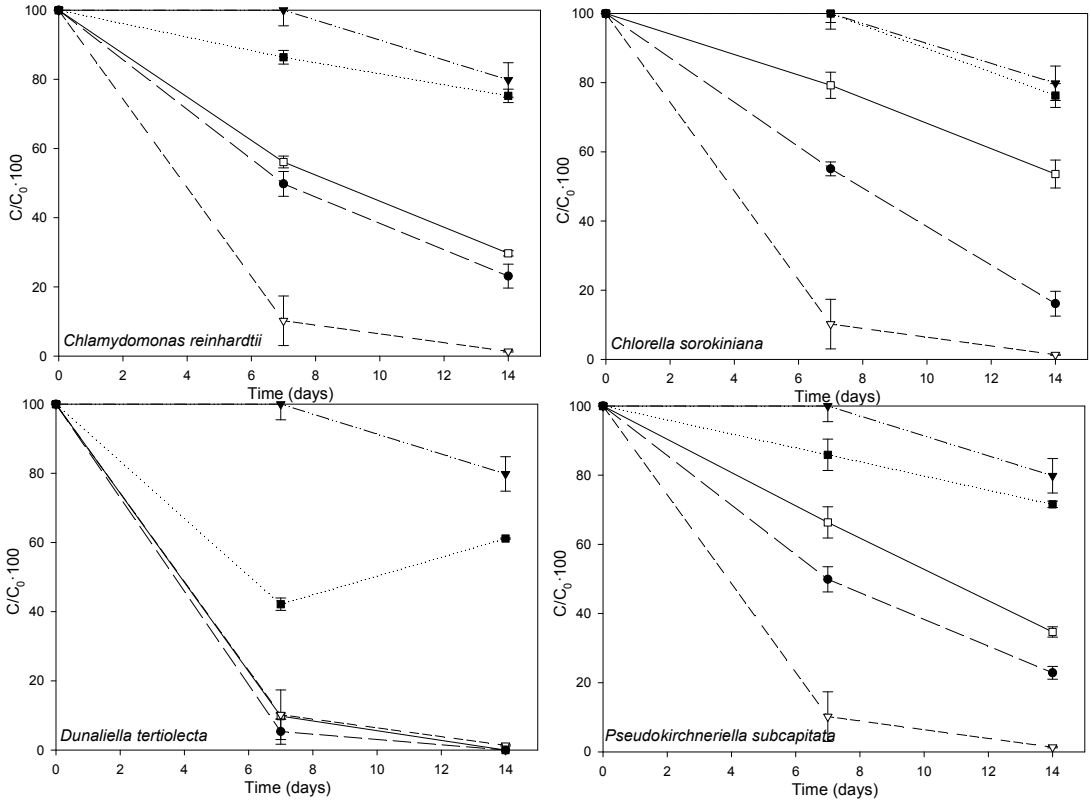


Figure 5.9 Norfloxacin (NFX) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

Ciprofloxacin, ofloxacin and norfloxacin were the FQs tested by the four microalgae strains, results are shown in Figure 5.7, Figure 5.8 and Figure 5.9. Photodegradation had a great impact on the three of them, >89% at the end of the study. Loses under dark conditions were accounted for less than 20%. Those results show how photosensitive these compounds are, as has been previously mentioned (Córdoba-Díaz et al., 1998). Babić et al. (2013) studied the photodegradation of CPX and NFX on different matrices and the results showed that they were degraded very quickly, solar irradiation contributed significantly to the degradation of fluoroquinolones, thus photodegradation is an important elimination process for FQs in the environment. In killed control cultures, high percentages were obtained for most of the strains (>76%), light and sorption onto algae surface have a great impact on FQs. Biodegradation was evaluated on the pure microalgae cultures. Under dark conditions removal percentages were lower than for light ones. *D. tertiolecta* obtained the higher removal percentages for all three FQ studied under light conditions, whereas under the darkness the removals were lower. *C. sorokiniana* had the worst FQ

removal percentages, lower than <46%. Zhang et al. (2012a) studied the photodegradation of NFX in an aqueous solution containing the microalgae strain *Chlorella vulgaris*. The presence of *C. vulgaris* enhanced NFX removal. They proposed that algae can generate some lower molecular weight organics that may produce the hydroxyl radical under UV irradiation, as well as some enzymatic reactions held on the algae surface. Increasing the algae concentration, more NFX was removed in a shorter period of time, that same behaviour was reported by Dorival-García et al. (2013) on quinolones removal from sludge. The amount of sludge encouraged the sorption of the antibiotics, favoring their removal from waters. NFX and OFX exhibited the highest sorption potentials, CPX had lower capability to sorb onto sludge. Magdaleno et al. (2015) evaluated the toxicity effect of 6 antibiotics, CPX among them, obtaining that CPX was highly toxic for *P. subcapitata*. The growth results for that strain in this work were not very high (Figure 5.1), and may be due to toxic effects of the spiked PhACs. CPX removal percentage under the dark was 77%.

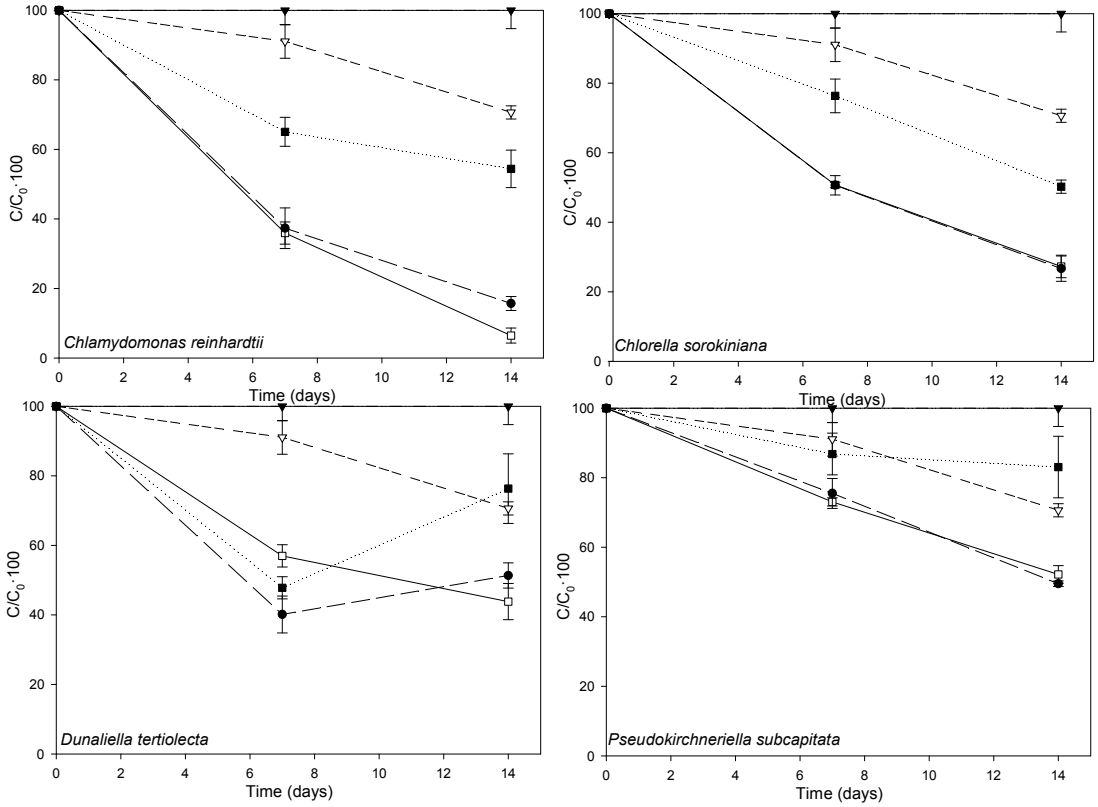


Figure 5.10 Sulphapyridine (SPD) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

Figure 5.10 shows the results for SPD removal from control and experimental conditions. Abiotic controls reported that 30% was photodegraded, since under the darkness conditions no loses were detected. SPD complete removal was almost achieved in *C. reinhardtii* culture, 93%, part of it could be attributed to microalgae biodegradation (47%), since 46% was removed from the live dark culture. Nevertheless, lower biodegradation percentages may be considered by *D. tertiolecta* and *P. subcapitata* cultures, 32 and 31%, respectively. Although there is no complete removal, 56 and 48%, sorption percentages are lower 24 and 17%, respectively. Literature on SPD removal is scarce, the study conducted by García-Galán et al. (2012) quantified the total amount of 16 sulphonamides in influent and effluent waters and sewage sludge of several WWTPs located in Spain, focusing in their removal and overall mass balance during the treatment. SPD was detected with the highest frequencies of detection and at the highest concentrations. The aqueous removal of SPD in that study was about 72%.

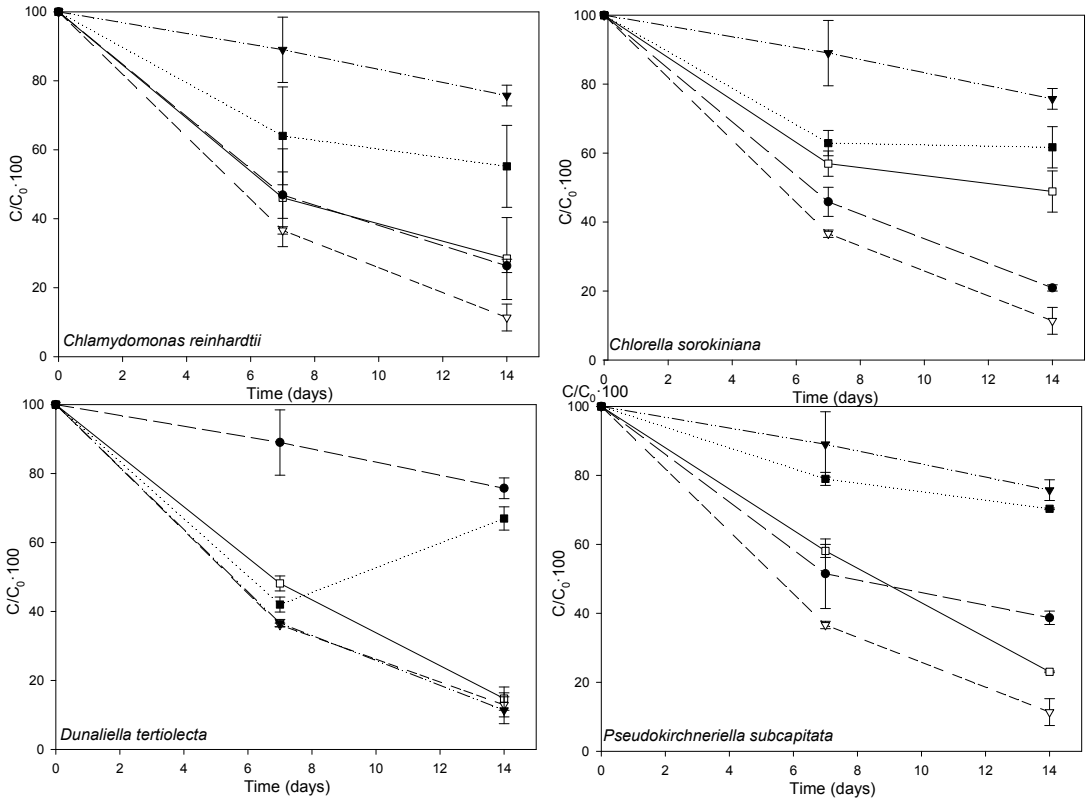


Figure 5.11 Pipemidic acid (HPM) removal along the time for each microalgae strain. (□) *microalgal biomass light;* (■) *microalgal biomass dark;* (♦) *killed control light;* (▽) *abiotic control light;* (▼) *abiotic control dark. C is the PhAC concentration and C_0 the initial PhAC concentration.*

Pipemidic acid (HPM) is a commonly found quinolone in wastewaters, the results obtained are presented in Figure 5.11. High removal percentages have been obtained for photodegradation and sorption at the end of the experiment, 89% and >61%, respectively. Dorival-García et al. (2013) demonstrated that quinolones were eliminated quickly by sorption in the first 60 min from the beginning of the experiments, and thus sorption equilibrium was rapidly reached. 85% removal was obtained for *D. tertiolecta* culture under the light. *C. reinhardtii* and *P. subcapitata* showed similar percentages, 71 and 77%, respectively, whereas *C. sorokiniana* only presented 51% removal. Live dark conditions removal percentages were below 45%.

The use of pure microalgae strains for antibiotics removal can be effective, since removal of such compounds is achieved, even though, most of the antibiotics tested are photosensitive and photodegradation is the major mechanisms occurring in the system. This is in accordance with other authors who stated that photolysis appears to be a major removal mechanism in algae treatment systems (de Wilt et al., 2016). Table 5.2 shows the removal percentages for the 4 microalgae strains and the 10 PhACs spiked under live and light conditions. Although microalgae may not contribute on all PhACs biodegradation, the fact of being treated in algal ponds, where illumination is required for biomass growth could enhance the removal of light-sensitive micropollutants. Moreover, microalgal biomass produced could be further used (de Wilt et al., 2016).

In general terms, *C. reinhardtii* and *C. sorokiniana* present better PhACs removal than *P. subcapitata* and *D. tertiolecta*. *P. subcapitata* presents the lowest removal values (e.g., 6% for AZM and <26% for TMT or CRM). Moreover, the TSS concentration obtained at the end of the experiment was lower than the other microalgae strains (Figure 5.1). Although *D. tertiolecta* culture could efficiently remove some of the PhACs (e.g., 85% for HPM, 56% for SPD), the low growth becomes a drawback. Then, *C. reinhardtii* and *C. sorokiniana* become a feasible option of PhACs removal, good growth rates and high PhACs removal percentages are achieved.

Table 5.2 PhACs removal percentage for live conditions under the light, 7 and 14 days after the spiking for the 4 microalgae strains.

	C. reinhardtii		***C. sorokiniana***		***D. tertiolecta***		***P. subcapitata***	
Compound	**7 d**	**14 d**	**7 d**	**14 d**	**7 d**	**14 d**	**7 d**	**14 d**
AZM	40	63	36	52	9	30	2	6
CRM	7	36	0	3	20	23	26	20
ERM	17	32	25	37	14	22	14	33
TMT	0	12	0	7	19	34	15	26
CPX	46	80	92	-	8	100	46	77
OFX	23	41	5	18	46	88	32	53
NFX	44	70	21	46	90	100	34	65
SPD	64	93	49	73	43	56	27	48
HPM	54	71	43	51	52	85	42	77
VFX	9	13	3	6	8	4	9	12

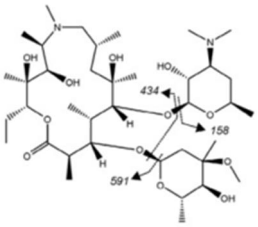
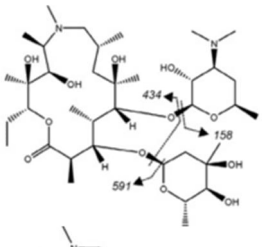
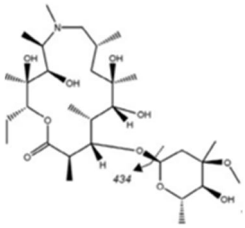
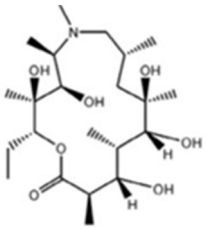
5.3.3 Transformation products identification

Revelation of TPs is a key aspect for understanding the possible removal mechanisms of micropollutants and to assess whether toxic or refractory products remain after transformation.

This part of the study includes the identification of TPs for 3 of the antibiotics tested to determine which removal mechanism may take place in the system. The identification of the TPs has been focused on *C. reinhardtii* and *C. sorokiniana* cultures.

AZM was selected since more than 50% was removed from both microalgae cultures, 63% for *C. reinhardtii* and 52% for *C. sorokiniana*. However, abiotic loses were detected, 30% under the light and 20% in the darkness. Three TPs were identified from AZM (Table 5.3), two of them were detected in *C. reinhardtii* culture, whereas the three of them in *C. sorokiniana* culture (Table 5.4).

Table 5.3 AZM TPs identification.

Compound	Molecular ion $[M+H]^+$	Molecular ion $[M-H]^-$	Proposed molecular structure	Reference
AZM $C_{38}H_{73}N_2O_{12}$	749.5158	747.5012		(Tong et al., 2011)
TP74 $C_{37}H_{71}N_2O_{12}$	735.5001	733.4856		(Tong et al., 2011)
TP78 $C_{30}H_{58}NO_{10}$	592.4055	590.3909		(Tong et al., 2011)
TP80 $C_{22}H_{44}NO_7$	434.3112	432.2967		(Tong et al., 2011)

TP74 and TP78 were found in abiotic controls (light and dark) meaning that they did not result from biodegradation. AZM photodegradation has been previously studied on different matrices. Tong et al. (2011) concluded that AZM was susceptible to undergoing indirect photolysis under simulated solar radiation, 7 TPs were identified, and 3 of them had the same characteristics as the ones identified in this study. The proposed mechanism for TP74 formation was a CH_2 reduction in the aminosugar or cladinose, whereas, TP78 resulted from the

aminosugar removal, involving the elimination of one of the two nitrogen atoms in the AZM molecule (Tong et al., 2011). TP80 was not found in abiotic controls, which is in disagreement with Tong et al. (2011), who proposed that TP80 was originated from the sequential elimination of both the cladinose and the aminosugar. TP80 detection in this study was different for both strains. In *C. sorokiniana* culture it was only detected in the presence of algae and light, fact that may let think that biodegradation of AZM is taking place. However, in *C. reinhardtii* culture it was detected in the presence of biomass, live and killed, cultures.

Table 5.4 TPs from AZM detection in microalgal cultures.

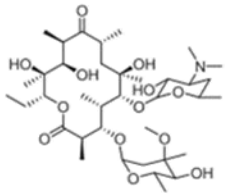
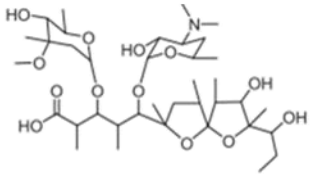
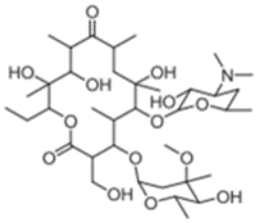
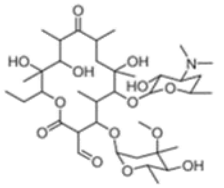
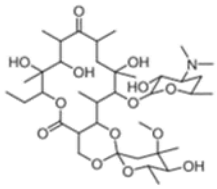
		TP74		**TP78**		**TP80**	
Time (d)	Condi-tion	*C. reinhardtii*	*C. sorokiniana*	*C. reinhardtii*	*C. sorokiniana*	*C. reinhardtii*	*C. sorokiniana*
0	A	×	×	-	×	×	×
	B	×	×	-	×	×	×
	C	×	×	-	×	×	×
	D	×	×	-	×	×	×
	E	×	×	-	×	×	×
7	A	✓	✓	-	✓	✓	×
	B	✓	✓	-	×	✓	×
	C	✓	×	-	✓	×	×
	D	✓	✓	-	✓	×	×
	E	✓	✓	-	×	✓	×
14	A	✓	✓	-	×	✓	✓
	B	✓	×	-	×	✓	×
	C	✓	✓	-	✓	×	×
	D	×	✓	-	✓	×	×
	E	✓	✓	-	×	✓	×

✓ indicates TP detection; × indicates no TP detection; - indicates no detection in the microalgal culture

Despite ERM removal percentage results from live and light conditions were not encouraging, the removal mechanisms are interesting since it is a widely prescribed antibiotic and it has been reported to be found at high concentrations (63 µg/kg, dry weight (Ding et al., 2011)), and also, 60% could be discharged on the effluent after WW treatment (Yan et al., 2014). 30% of the ERM in the solution can be photodegraded, while around 20% sorbed onto the microalgal cell surface. Removal percentage increased in live and light conditions up to 32

and 37% for *C. reinhardtii* and *C. sorokiniana*, respectively. Three main mechanisms may be occurring: photodegradation, sorption and biodegradation. Three TPs were detected and identified from ERM (Table 5.5); two of them were detected in each microalgae culture (Table 5.6).

Table 5.5 ERM TPs identification.

Compound	Molecular ion $[M+H]^+$	Molecular ion $[M-H]^-$	Proposed molecular structure	Reference
ERM $C_{37}H_{68}NO_{13}$	735.47630	733.46175		(Llorca et al., 2015)
TP85 $C_{37}H_{68}NO_{13}$	735.47630	733.46175		(Llorca et al., 2015)
TP87 $C_{37}H_{68}NO_{14}$	750.46340	748.44885		(Llorca et al., 2015)
TP88 $C_{37}H_{66}NO_{14}$	748.44790	746.73335	 	(Llorca et al., 2015)

Flickinger and Perlman (1975) stated that the bacteria *Pseudomonas* 56 can synthetize an enzyme system capable of degrading ERM. Several decades later, Kim et al. (2002) purified and characterised an erythromycin esterase from *Pseudomonas* sp. GD100 isolated from an aquaculture sediment, since it has been found that some proteobacteria were highly resistant to ERM due to their

constitutive erythromycin esterases. The results obtained confirmed that ERM was enzymatically removed by ERM esterase. Llorca et al. (2015) conducted an enzymatic treatment using EreB esterase from *Escherichia coli* and 5 suspected TPs were detected, among them, 3 were detected in this study.

Table 5.6 TPs from ERM detection in microalgal cultures.

		TP85		**TP87**		**TP88**	
Time (d)	Condition	*C. reinhardtii*	*C. sorokiniana*	*C. reinhardtii*	*C. sorokiniana*	*C. reinhardtii*	*C. sorokiniana*
0	A	×	×	✓	-	-	×
	B	×	×	✓	-	-	×
	C	×	×	✓	-	-	×
	D	×	×	✓	-	-	×
	E	×	×	✓	-	-	×
7	A	✓	✓	×	-	-	✓
	B	✓	×	×	-	-	×
	C	×	×	×	-	-	×
	D	×	✓	✓	-	-	×
	E	✓	×	✓	-	-	×
14	A	✓	✓	×	-	-	✓
	B	✓	✓	×	-	-	×
	C	✓	✓	×	-	-	×
	D	×	✓	✓	-	-	×
	E	✓	×	×	-	-	×

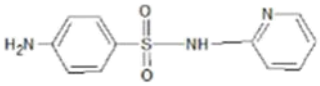
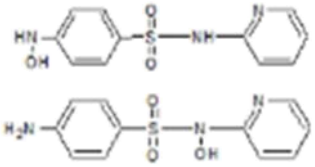
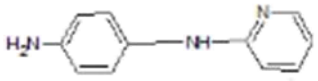
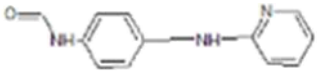
✓ indicates TP detection; × indicates no TP detection; - indicates no detection in the microalgal culture

TP85 was detected in both cultures, and no detection was found in abiotic controls 7 days after the spiking, however, at the end of the experiment it was detected. It also appears on the killed controls of *C. reinhardtii*, but not on *C. sorokiniana*. In microalgae cultures, both light and dark conditions, TP85 is identified. Llorca et al. (2015) reported TP85 as the major degradation product and the dehydration was found to be the hypothetic pathway. TP87 has been detected in *C. reinhardtii* culture at time 0 in all conditions; this may be due to easily conversion of ERM. 7 days after, it was only detected in dark abiotic and in the killed control, so photodegradation may have occurred, as well as biodegradation. At the end of the experiment it was only detected in dark abiotic control. The removal from the killed control may confirm the photodegradation of this TP, moreover, since light cannot penetrate with the same intensity in the

killed control than in the abiotic, more time is required. The proposed pathway has been the oxidation of a methyl group in the position C2 (Llorca et al., 2015). Finally, TP88 was only found in *C. sorokiniana* culture, and only appeared in live conditions, with the presence of light, that case may let us thing about *C. sorokiniana* biodegradation. Vara et al. (1989) proposed biosynthesis pathways for this compound generation. Moreover, Llorca et al. (2015) proposed that TP88 was formed due to a dehydrogenation of TP87.

The last antibiotic from which TPs were identified has been SPD, due to the high removal percentages obtained in microalgae cultures, 93% was removed in *C. reinhardtii* culture and 73% in *C. sorokiniana*. Three TPs were identified from SPD (Table 5.7); the three of them were detected in *C. reinhardtii* culture and only one in *C. sorokiniana* (Table 5.8).

Table 5.7 SPD TPs identification.

Compound	Molecular ion $[M+H]^+$	Molecular ion $[M-H]^-$	Proposed molecular structure	Reference
SPD $C_{11}H_{12}N_3O_2S$	250.06446	248.04991		(Rodríguez-Rodríguez et al., 2012)
TP51 $C_{11}H_{12}N_3O_3S$	266.05938	264.04483		(Rodríguez-Rodríguez et al., 2012)
TP64 $C_{11}H_{12}N_3$	186.10260	184.08801		(Rodríguez-Rodríguez et al., 2012)
TP66 $C_{12}H_{12}N_3O$	214.09750	212.08292		(Rodríguez-Rodríguez et al., 2012)

All the identified TPs have been detected in abiotic samples, this means that photodegradation of SPD is taking place, furthermore, it is confirmed by the absence of such compounds in dark abiotic controls. Weiss et al. (1980) mentioned that sulphonamides are relatively photostable compounds, but give complex product mixtures on prolonged irradiation. In principle the bonds can be cleaved on photolysis and SO_2 radical should be eliminated forming a simple

alkyl or aryl radical. Schwarz et al. (2010) and Rodríguez-Rodríguez et al. (2012) tested the enzymatic activity of *T. versicolor* laccase for sulphonamides transformation, including SPD, and enzymatic transformation was confirmed. Nevertheless, transformation through hydrolysis or photodegradation was not relevant in their study, contrary to the results obtained here. On the contrary, Neafsey et al. (2010) determined the TPs of 2 sulphonamides widely used after an anodic Fenton treatment, indicating the formation of similar pathways as the ones proposed here. The presence of radicals attack the molecule and SPD can be photodegraded, moreover, it was stated that the bacteriostatic properties were removed from the formed TPs. Boreen et al. (2005) also confirmed photodegradation of sulphonamides in natural water.

Table 5.8 TPs from SPD detection in microalgal cultures.

		TP51		**TP64**		**TP66**	
Time (d)	Condition	*C. reinhardtii*	*C. sorokiniana*	*C. reinhardtii*	*C. sorokiniana*	*C. reinhardtii*	*C. sorokiniana*
0	A	×	×	×	-	×	-
	B	×	×	×	-	×	-
	C	×	×	×	-	×	-
	D	×	×	×	-	×	-
	E	×	×	×	-	×	-
7	A	✓	×	✓	-	×	-
	B	×	×	✓	-	×	-
	C	✓	✓	✓	-	✓	-
	D	×	×	×	-	×	-
	E	✓	✓	✓	-	×	-
14	A	✓	×	✓	-	×	-
	B	×	×	✓	-	×	-
	C	✓	✓	✓	-	✓	-
	D	×	✓	×	-	×	-
	E	✓	✓	✓	-	×	-

✓ indicates TP detection; × indicates no TP detection; - indicates no detection in the microalgal culture

TP51 was not detected under live conditions in *C. sorokiniana* cultures, it was only present under light conditions for abiotic and killed controls, whereas, in *C. reinhardtii* live culture it was detected. This TP was previously detected in a study carried out by the fungus *T. versicolor*, and described as the hydroxylated moiety of SPD (Rodríguez-Rodríguez et al., 2012).

TP64 seems to be formed due to the presence of light and also when biomass is present, it corresponds to a desulphonated product of SPD (Rodríguez-Rodríguez et al., 2012; Schwarz et al., 2010). Previous studies have demonstrated that desulphonated products are frequently detected after the enzymatic degradation, physicochemical oxidation or photodegradation of sulphonamides (Boreen et al., 2005, 2004; Neafsey et al., 2010) which is in agreement with the detections in our study.

Finally, TP66 is a photodegradation product since it was only detected in light abiotic controls. It corresponds to the addition of a formyl group to the aforementioned desulphonated moiety (TP64) (Rodríguez-Rodríguez et al., 2012; Schwarz et al., 2010). Interestingly, in Rodríguez-Rodríguez et al. (2012) study it was not detected in the abiotic control and its formation was attributed to the presence of formic acid in the ultra-performance liquid chromatography eluents.

5.4 Conclusions

The results show that the addition of a solution containing a total concentration of 1 mg/L of 10 PhACs does not inhibit the growth of *C. reinhardtii*, *C. sorokiniana* and *D. tertiolecta*, although *P. subcapitata* seems to be affected, due to the slow growth noticed.

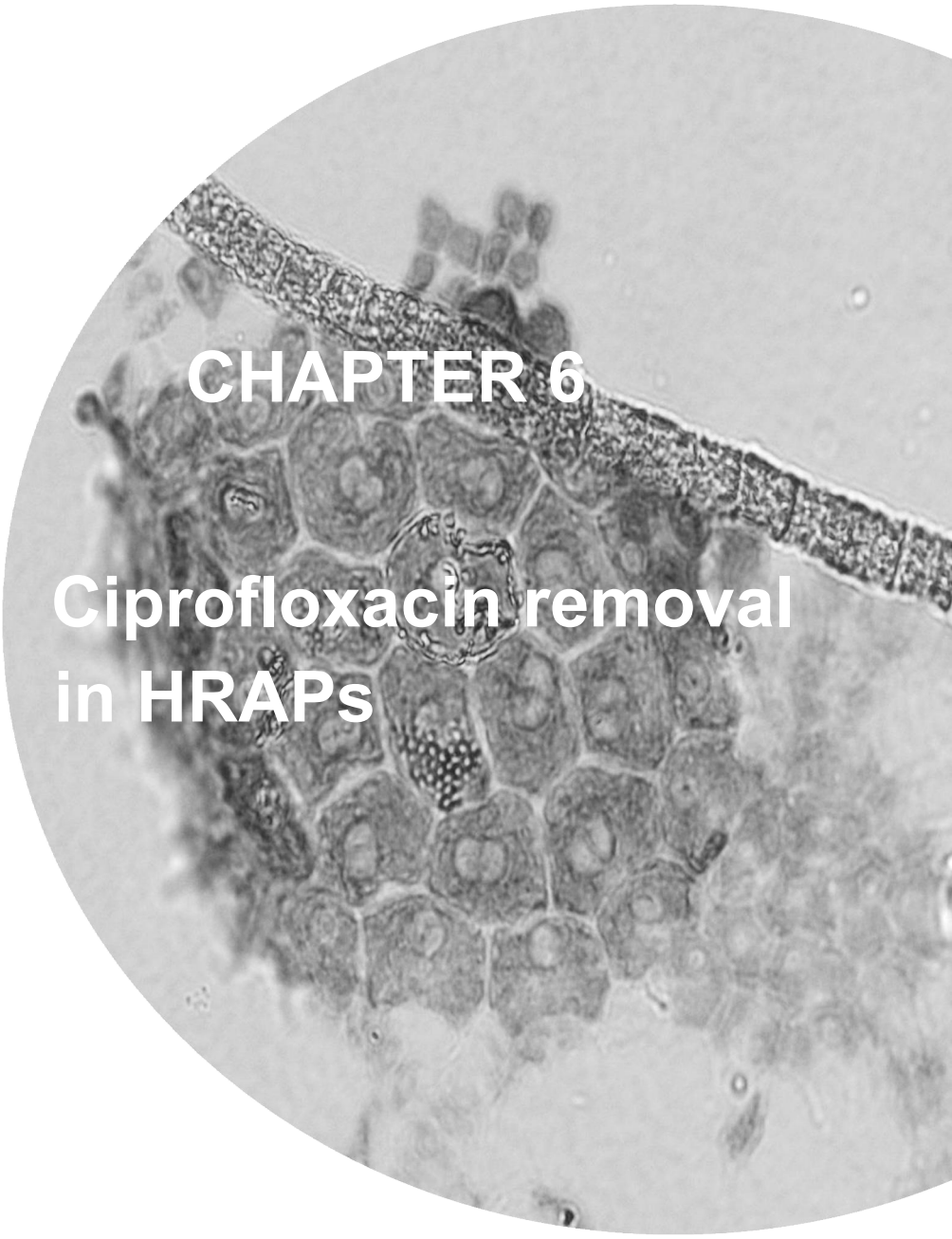
The antidepressant venlafaxine has been demonstrated to be persistent in aqueous solutions and microalgae cannot remove it. On the other hand, the combination of photodegradation, hydrolysis, sorption and biodegradation have been the responsible mechanisms for the antibiotics removal. The main removal mechanism detected for macrolides antibiotics has been photodegradation together with hydrolysis (20-33%). Azithromicin has higher removal percentages than the other macrolides. Trimethroprim presents low sorption values as well as low removal under abiotic conditions, this may let think that biodegradation can take place. Photodegradation has been detected as the main removal mechanism for fluoroquinolones (>89%), although high removal percentages under dark conditions were detected for ciprofloxacin (77%). Sulphapyridine

removal by means of biodegradation (46%) has been proposed, due to the low sorption values.

In general, photodegradation has a great impact on antibiotics removal, although the use of microalgal-based systems has the added benefit to produce biomass for further uses.

Promising results have been obtained from *C. reinhardtii* and *C. sorokiniana* cultures, due to rapid growth and good removal percentages for some of the PhACs studied.

Azithromycin, erythromycin and sulphapyridine transformation products have been identified. A TP has been proposed as a product of biodegradation as a result of enzymatic degradation. No more biodegradation TPs have been proposed since they were detected in abiotic and killed controls. TPs from sulphapyridine were due to radical attacks under light conditions.



CHAPTER 6

Ciprofloxacin removal in HRAPs

6 Ciprofloxacin removal in high rate algal ponds

Abstract

This study investigates the fluoroquinolone antibiotic ciprofloxacin (CPX) removal: hydrolysis, sorption, biodegradation, and photodegradation processes were evaluated, on different microalgal wastewater treatment systems, such as high rate algal ponds. Laboratory scale microalgae systems were fed with primary effluent wastewater containing CPX. Reactors performance was monitored measuring the main parameters of the system (pH, dissolved oxygen, temperature and nutrients concentration). Changes on light/night cycle and hydraulic retention time were evaluated during the reactors performance. 68% of CPX could be removed from laboratory scale reactors. CPX removal was also studied in an outdoor continuous pilot scale high rate algal pond and during the night high sorption onto the algae surface was detected, while photodegradation took place along the day. Laboratory scale batch studies were conducted to confirm the removal mechanisms taking place. The main CPX removal mechanisms were sorption and photodegradation, although biodegradation may occur.

6.1 Introduction

The mechanisms for pollutants removal during conventional or advanced treatments cannot be simply extrapolated to algae-based wastewater (WW) treatment as algae photosynthesis drives very specific environmental and ecological conditions to occur (Norvill et al., 2016). Hence, if high rate algal ponds (HRAPs) are to become a broadly used modern WW treatment platform, it is critical to assess (and potentially optimize) their efficiency with regards to all modern WW treatment constrains, including the removal of micropollutants.

Ciprofloxacin (CPX) is one of the most prescribed fluoroquinolone (FQ) antibiotics (Picó and Andreu, 2007) because of its high potency, broad activity spectrum, good bioavailability, high serum levels, and a potentially low incidence of side effects (Andersson and MacGowan, 2003; Kümmerer et al., 2000). However, after administration 72% of the non-metabolized form is excreted, mainly in urine and partially in faeces ending up in WW and surface water (Daughton and Ternes, 1999). He and Blaney (2015) reported that between 22 to 87% of CPX was extracted as non-metabolized, whereas Johnson et al. (2015) found out lower percentages 25-45%. CPX is one of the

most frequently encountered pharmaceuticals and it is between the 50 most frequently studied compounds. The worldwide median concentration of CPX in freshwater ecosystem has been reported to be 0.164 mg/L, and its maximum detected concentration was 6.5 mg/L (Hughes et al., 2013). Moreover, it has been detected at concentrations up to 31 mg/L in waste effluents from pharmaceutical manufacturers (Larsson et al., 2007). Through the domestic wastewater system, pharmaceutical compounds used in human medicine are conducted to wastewater treatment plants (WWTPs), where they should be removed. However, studies have demonstrated the presence of these compounds in the final effluents discharged by WWTPs and, consequently, their introduction into the aquatic environment (Gracia-Lor et al., 2012). Ineffective removal of CPX by conventional water treatment technologies has caused its continuous discharge into the environment; CPX has been detected at concentrations of 54-71 ng/L in WWTP effluents from Spain (Ripoll WWTP, Girona) (Marti et al., 2014), although in a review conducted by Speltini et al. (2010) several removal percentages in WWTP were reported. For example, in Switzerland WWTP CPX removal achieved values ranging 79 to 87% (Golet et al., 2002), lower removal percentages were attained by Seifrtová et al. (2008) in a Portuguese WWTP, 54-76% removal. Dong et al. (2016) studied the detection of several emerging pollutants, among them CPX. The influent and effluent CPX concentration in the WWTP was quantified ranging between 0.82-147 0.4-88.5 ng/L, respectively. Nevertheless, CPX has also been found in sewage sludge samples in the range of 230 mg/kg, sorption to sludge was considered as a principal removal pathway for CPX in the conventional activated sludge (CAS) process (Golet et al., 2003; Speltini et al., 2010).

In general, FQs have been reported as photosensitive and the transformation path of these compounds is resistant to hydrolysis, thermal decomposition, and biodegradation (Plósz et al., 2010; Sturini et al., 2015). Sturini et al. (2015) studied the sun-light induced CPX removal in WWTP secondary effluent, complete removal was achieved in less than in an hour. Similar values were reported by Sturini et al. (2012) in a sun-light induced study in raw river water. Various advanced oxidation processes (AOPs) have been investigated to remove CPX from surface water and wastewater, such as Fenton oxidation and

UV/H_2O_2 (Giri and Golder, 2014; Guo et al., 2013; Mahdi-Ahmed and Chiron, 2014). High operational and maintenance costs of these methods restrict their utilization at long-term applications. Adsorption may be an effective way to remove antibiotics like CPX due to high efficiency and good feasibility (Yu et al., 2016). Active carbon is a preferred adsorbent for the removal of pollutants from wastewater. However, its widespread use is restricted due to high costs. In order to decrease the costs, alternatives for sorption processes have been proposed, Sun et al. (2016) investigated the use of pomelo peel using phosphoric acid as activating agent, with maximum monolayer adsorption capacities of 400 mg/g, which was mainly attributed to its high surface area and available adsorption sites.

A recent study using the pure microalgae strain *Chlamydomonas mexicana* assessed the CPX removal capacity of the microalga, 13% was removed from an initial concentration of 2 mg/L after 11 days of cultivation in a laboratory scale study (Xiong et al., 2016). Despite the results, CPX is relatively persistent to microalgae degradation under normal growth conditions (Amorim et al., 2014; Xiong et al., 2016).

The aim of the present work is therefore to investigate the ability of HRAPs treating real wastewater to remove the antibiotic ciprofloxacin and determine the main removal mechanisms using laboratory and field experiments. CPX was selected due to its ubiquity in human sewage and animal manure and the suspected link between its environmental occurrence and the global issue of antibiotic resistance (Kümmerer, 2009a; Pruden, 2014; Rizzo et al., 2013).

6.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

6.2.1 Primary effluent wastewater

Primary effluent wastewater (PEW) collected from Palmerston North City Council WWTP (New Zealand) was used in the laboratory scale experiments for

CPX degradation and it was continuously fed to the laboratory and field pilot scale HRAPs. PEW used was characterised (Table 6.1) and stored at -20ºC until the set up of laboratory scale experiments and fed continuously in the field pilot scale HRAP.

Table 6.1 PEW characterisation prior use.

Parameter	Value
pH	8.1±1.0
TSS (mg/L)	421±88
COD (mg/L)	506±103
TN (mg/L)	30±16

6.2.2 Real wastewater treatment and CPX removal in continuous laboratory scale systems

Two double set up configurations at laboratory scale were tested for CPX removal by microalgae cultures.

The first set up configuration consisted on two 1 L beakers (PBR-A and PBR-B assays) (culture depth was 85 mm, diameter 95 mm) (Image 6.1 and Figure 6.1) initially filled with 100 mL of PEW and 600 mL of a laboratory algal/bacterial culture (from 7 L HRAPs under 7 d HRT); the dominant microorganisms were *Aphanocapsa* sp., *Gomphonema* sp., and colonial chlorophytes, the rest of species are specified in Table 6.2. The reactors were incubated at 20 ± 2ºC under continuously agitation at 150 rpm (Unimax 1010, John Morris Scientific) and intermittent artificial illumination from 09:30 am to 05:30 pm each day (8/16 light/dark cycle) using 9×30W 'full spectrum' fluorescent tubes (Viva-lite, Germany) above the beakers, providing 26-29 W/m^2 light at the culture surface (measured by actinometry tests). The reactors were weighted at the end of each dark cycle and evaporation losses were compensated with reversed osmosis (RO) water. Then, 100 mL of culture were daily withdrawn (used for analysis) and immediately replaced with 100 mL of freshly thawed PEW spiked with CPX at 14 mg/L for 9 days (Phase I) then 2 mg/L for 26 days (Phase II). The photobioreactors (PBRs) were thereby operated in a fed-batch mode at an

average hydraulic retention time (HRT) of 7 days under CPX loadings of 2.0 and 0.286 mg/($L_{reactor}$·d) during Phases I and II, respectively. During Phase III, the HRT was decreased to 3 days in one of the reactors (PBR-B assay) (thereby increasing CPX load from 0.286 mg/(L·d) to 0.667 mg/(L·d) and light period was doubled from 8 to 16 h in both reactors (09:30 am to 01:30 am, light/dark cycle of 16/8). The cultures pH, temperature, dissolved oxygen (DO) concentration, and CPX concentration (10 mL samples, from the 100 mL withdrawn) were quantified daily at the end of the dark (09:30 am) and light (05:30 pm) periods during Phases I and II, and at the end of the dark (09:30 am) and mid-light (05:30 pm) periods during Phase III. The total suspended solids (TSS) and total organic carbon (TOC) concentrations were quantified three times a week using the 100 mL of culture withdrawn at the end of each cycle.

Image 6.1 Double set up configurations. Left: laboratory scale 1 L photobioreactors (PBR-A and PBR-B); Right: laboratory scale 7 L high rate algal ponds (HRAP-A and HRAP-B).

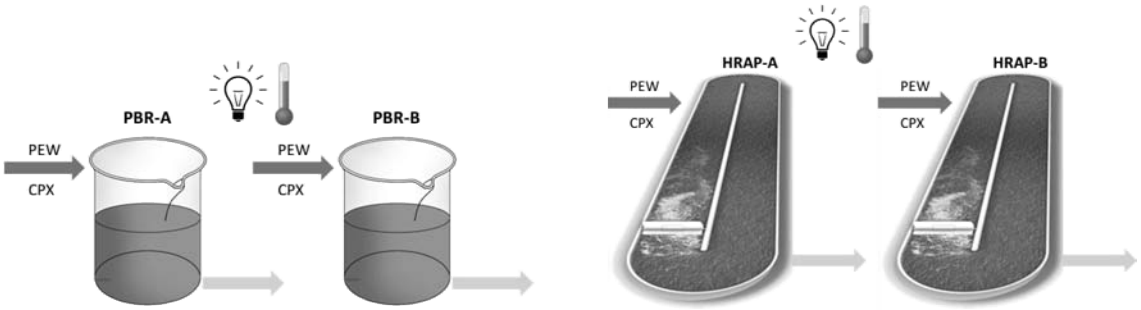


Figure 6.1 Continuous WW treatment and CPX removal at laboratory scale. Left: PBR-A and PBR-B; Right: HRAP-A and HRAP-B. Light bulb and thermometer symbols indicate controlled light and temperature, respectively.

Table 6.2 Biodiversity from a laboratory scale HRAP used as inoculum.

Species	Description	HRAP inoculation
Aphanocapsa sp. (1-2 μm)	Potentially toxic cyanobacteria	8
Pseudanabaena sp.	Potentially toxic cyanobacteria	2
Gomphonema sp.	Diatoms	6
Navicula sp.	Diatoms	1
Cryptomonas sp.	Chlorophyta	1
Monoraphidium spp.	Chlorophyta	4
Oedogonium sp.	Chlorophyta	2
Pediastrum sp.	Chlorophyta	3
Scenedesmus sp.	Chlorophyta	2
Peridinium sp.	Dinoflagellates	1
Small unicells (<5 μm)	Non-toxic species	5
Unknown colonial chlorophyte	Chlorophyta	6

1: rare; 2: rare-occasional; 3: occasional; 4: occasional-common; 5: common; 6: common-abundant; 7: abundant; 8: dominant; -: not detected

The second set up configuration consisted on additional tests in two 7 L (Image 6.1) (working volume, 0.5 m long 0.3 m wide 0.06 m deep) HRAPs continuously stirred using a paddle wheel operated at 28 rpm, which supported a liquid recirculation velocity of 0.1 m/s at the centre of the pond channel (as previously described by Alcántara et al. (2015)). The reactors were operated indoor at 20±2ºC and illuminated 16 hours each day using the light set up described above (46 W/m^2 photosynthetically active radiation (PAR) the culture surface). Initially filled with 1 L of PEW and 6 L from a field pilot scale HRAP algal/bacterial culture (where the dominant specie was *Scenedesmus* sp. and *Pseudanabaena* sp. was rated as abundant). Aliquots of reactor cultures (1 L and 2.3 L for HRAP-A and B, respectively) were daily withdrawn and replaced along the day using a pump with a freshly thawed PEW spiked with 2 mg CPX/L to maintain HRTs of 7 days (HRAP-A) and 3 days (HRAP-B). Evaporation loses were compensated with purified water. The aliquots daily withdrawn from the HRAPs were used for quantification of CPX concentration and routine wastewater analysis.

6.2.3 Real wastewater treatment in an outdoor continuous pilot scale system

A concrete HRAP (HRAP-F) was set up at Palmerston North (New Zealand) WWTP (Image 6.2). The HRAP-F was 3 m long, 1.1 m wide and 0.5 m depth with an illuminated area of 4 m^2 and a working volume of 0.9 m^3 (water depth of 0.25 m). Mixing was supplied by a stainless steel paddle wheel rotating at 11 rpm and liquid velocity was of ~0.17 m/s. The HRAP was inoculated with a mixed culture from a local WW treatment stabilization pond (Rongotea, New Zealand) and fed with PEW pumped in 1 min/20 min pulses at a flow of 0.1 m^3/d (10 d HRT).

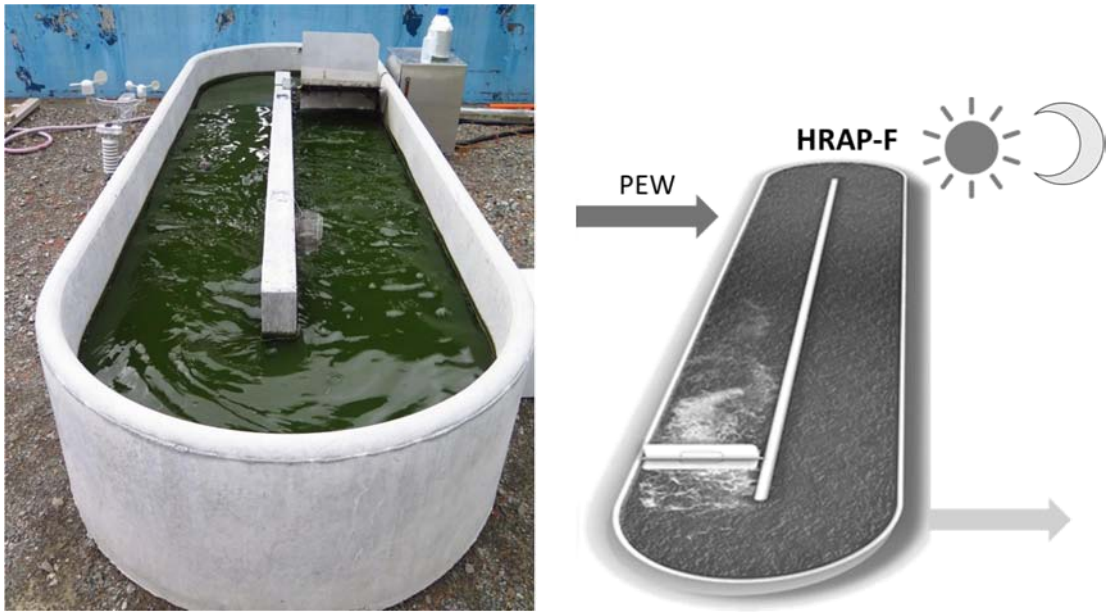


Image 6.2 WWTP pilot scale HRAP-F. Left: On-site HRAP-F. Right: Continuous WW treatment in the HRAP-F. Sun and moon symbols indicate outdoor conditions, day cycle.

6.2.4 Outdoor batch experiments for CPX removal

Spiked experiments were conducted in the pilot scale HRAP-F by stopping the influent supply during 24 h and immediately adding CPX to an initial concentration of 2 mg/L (Figure 6.2). During the spike experiment (24 h) no effluent was withdrawn. 100 mL samples were regularly withdrawn for subsequent analysis before normal continuous operation was resumed. Three batch experiments were conducted. The first one (HRAP-F1) was conducted during the day, starting after the sunrise (7:00) and ending 24 h after the spiking, although samples were only collected until 15:00 and a last sample at

24 h. The second spike was conducted through the night (HRAP-F2); it started at sunset (20:00) and ended at sunrise (6:00). Finally, the third spike (HRAP-F3) combined night and day, it was spiked after sunset (20:30) it was monitored along the night and the following day during 24 h.

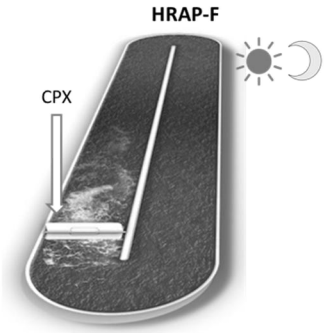


Figure 6.2 Outdoor batch experiment at pilot scale HRAP-F. Sun and moon symbols indicate outdoor conditions, day cycle.

In parallel, during the HRAP-F3 assay an additional test in 150 mL beakers using HRAP-F biomass and incubated outdoor under continuous agitation at 200 rpm (IKA® KS 260 basic) was conducted (Batch-F) (Figure 6.3). The beakers were filled with various CPX-laden solutions (e.g., PEW and HRAP-F effluent). Killed controls (3 g NaN_3/L) were also carried out. All tests were conducted in triplicates.

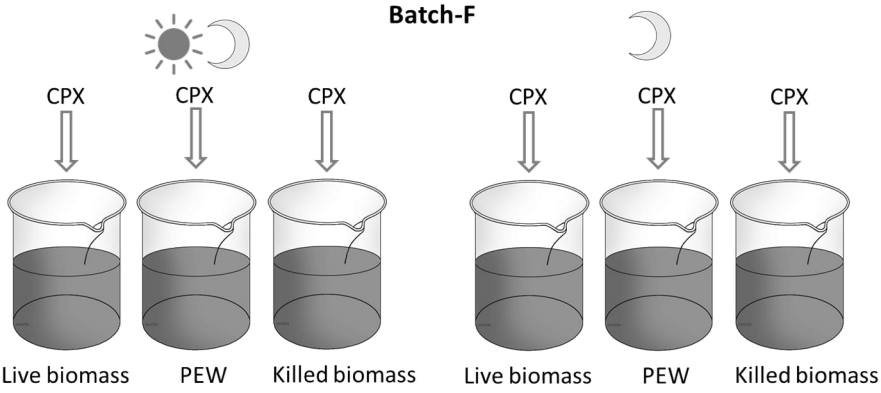


Figure 6.3 Outdoor batch experiment at laboratory scale Batch-F. Sun and moon symbols indicate outdoor conditions, day cycle. Moon symbol indicates dark conditions during the whole outdoor assay.

6.2.5 Laboratory scale batch experiment for CPX removal

Laboratory scale batch experiment was carried out in similar conditions as outdoor batch (Batch-F). 150 mL beakers incubated under continuous agitation at 200 rpm (IKA® KS 260 basic) at 25 ± 2ºC with either continuous illumination

(light intensity ranged between 5.58 and 6.37 W/m^2) using the Viva-lite® tubes, or darkness (Batch-L) (Figure 6.4) during 4 days. The beakers were filled with various solutions (PEW and HRAP-A biomass effluent). Killed controls (3 g NaN_3/L) were also carried out. All tests were conducted in triplicates.

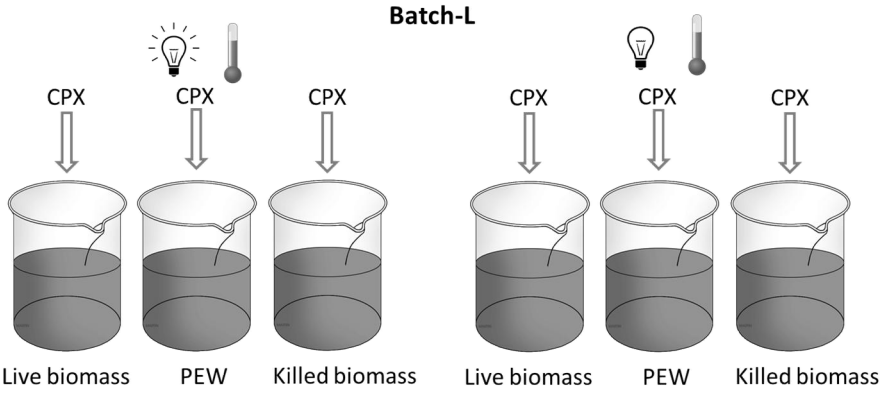


Figure 6.4 Indoor batch experiment at laboratory scale Batch-L. Light bulb and thermometer symbols indicate controlled light and temperature conditions. Off light bulb symbol indicates dark conditions during the indoor assay.

6.3 Results and discussion

6.3.1 Real wastewater treatment performance in continuous laboratory scale systems

6.3.1.1 Biomass concentration

The two 1L-photobioreactors (PBR-A and PBR-B) (Image 6.1 and Figure 6.1) operated in continuous mode showed similar wastewater treatment performance during Phases I and II (Figure 6.5). The concentration of TSS thus gradually increased during the first 3 weeks of operation, and then, stabilised around 560 ± 80 mg/L and 550 ± 30 mg/L at the end of Phase II in PBR A and B, respectively. Doubling light duration during Phase III caused TSS to increase by 30% when HRT was maintained at 7 days whereas TSS concentration decreased from 550 to 435 mg/L when HRT was reduced to 3 d (Figure 6.5).

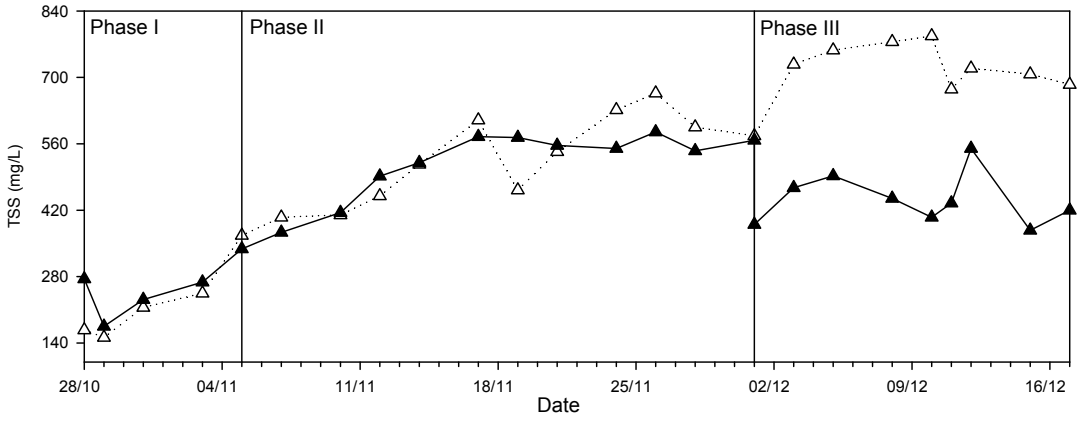


Figure 6.5 TSS concentration in laboratory scale PBRs during the three operating phases. (△) *PBR-A (HRT=7 d);* (▲) *PBR-B (HRT=7 d phase I and II, HRT=3 d Phase III).*

Modification of HRT and light cause a direct effect on biomass concentration. HRT modifies the biomass concentration in the pond by either allowing or preventing biomass accumulation on longer or shorter HRT, respectively (Sutherland et al., 2015). Whereas, light becomes the main factor affecting microalgal productivity since it is in accordance with the photosynthetic efficiency (PEF). During Phase III, despite the TSS concentration was reduced on PBR-B, productivity was enhanced (55 g/d and 102 g/L, respectively (Table 6.3)). Shading effect of microalgal biomass is less notorious, since less algae cells are in suspension, then, the microalgae from the culture may use higher amounts of the light irradiated, increasing the PEF, thus productivity.

Productivity was also increased in PBR-A as light hours and TSS were increased. The productivity during Phase II was 56 g/L, whereas during Phase III it increased to 76 g/L. Despite productivity on PBR-A was increased, higher values were attained in PBR-B when both parameters, light and HRT, were modified (Table 6.3).

In order to dissociate the effect of the HRT from the effect of the illumination time and to investigate the potential effects of feeding mode and reactor geometry, continuous laboratory scale HRAPs were tested for WW treatment and CPX removal at different HRTs of 7 and 3 days (HRAP-A and HRAP-B, respectively) but constant daily illumination time of 16 hr.

Regarding the TSS from the 7 L HRAPs (HRAP-A and HRAP-B) operated in continuous mode (Figure 6.6); variations in the concentration along the operating period were noticeable. At higher HRT (HRAP-A) the concentration

was higher than at lower HRT (HRAP-B), 409 and 244 mg/L (Table 6.3), although, the productivity was greater in HRAP-B. HRAP-A TSS content was in accordance with the values reported by Alcántara et al. (2015) using the same system operating at similar conditions (values ranged from 417±17 to 534±18 mg/L, depending on the working conditions). Whereas, in HRAP-B TSS were lower due to the wash out effect.

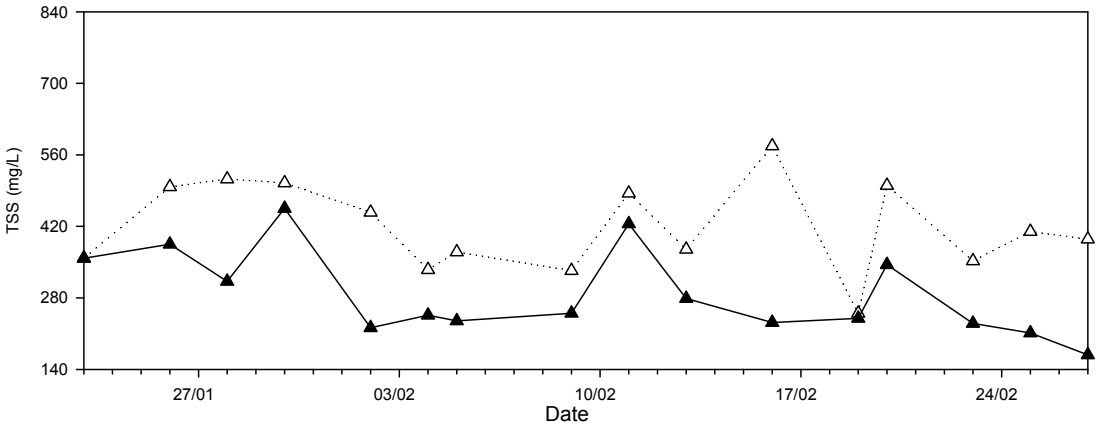


Figure 6.6 TSS concentration in laboratory scale HRAPs: (△) *HRAP-A (HRT=7d);* (▲) *HRAP-B (HRT=3d).*

The TSS concentration differences in HRAP-A and HRAP-B may be due to the light irradiance and configuration. Despite light irradiance was controlled, PBRs were made of clear glass, so more light was irradiated to the system, the degree of attenuation in the HRAPs is higher since light is only available on the culture surface. Light passing through the water column declines exponentially with depth as the microalgae absorb or scatter the light, if available light is increased within the pond water column, biomass productivity may be improved (Park et al., 2011b; Sutherland et al., 2015). Also, configuration design (i.e., pond depth and mixing) may be issues to be taken into account for TSS content. Pond depth is crucial for modifying the pond light environment, as well as governing biomass concentration. PBRs were deeper than the HRAPs but the effect of light irradiance on the overall contour was favourable for microalgal growth. Mixing determines both the amount and frequency of light exposure an individual microalgae cell experiences, as well as the nutrient uptake. To increased mixing facilitates nutrient uptake, leading to enhanced growth. Again, PBRs configuration was favourable for microalgal growth, more mixing was obtained from the shaker platform rather than with the paddle wheel from the HRAPs, enhancing light penetration and nutrient uptake.

Table 6.3 Total suspended solids concentration and productivity in continuous laboratory scale systems.

	Phase II			Phase III		
Set up configuration	**HRT (d)**	**TSS (mg/L)**	**Productivity (mg/d)**	**HRT (d)**	**TSS (mg/L)**	**Productivity (mg/d)**
PBR-A	7	560±80	56	7	729±41	73
PBR-B	7	550±30	55	3	435±38	102
HRAP-A				7	409±105	409
HRAP-B				3	244±56	569

6.3.1.2 Continuous monitoring parameters

Large daily variations in pH, DO and temperature were recorded (Figure 6.7 for laboratory PBR-A and B). Within a few days of operation both PBRs were stabilized. During Phase I and II, PBR-A and PBR-B had the same behaviour. Morning samples (after the dark cycle, when microalgal respiration takes place) maintained the pH at 8.3±0.2 and afternoon samples (after the day cycle, when microalgae are photosynthetically active) achieved higher values, 10.9±0.3. DO after the dark cycle was 8.7±0.3 for both systems, and after the light cycle it was increased to 9.7±0.4 mg O_2/L for PBR-A and 10.0±0.3 mg O_2/L for PBR-B. Temperature along both phases was the same for both reactors, after the dark cycle 19±0.7 ºC and 26±0.6 ºC after the light cycle. During phase III, when the light cycle was increased for both systems and HRT for PBR-B was decreased from 7 d to 3 d, no significant pH and DO differences can be observed, although as it has been previously observed in Figure 6.5, TSS concentration changed. PBR-A pH values after the dark cycle were slightly increased. This fact was due to the sampling time, measurements were taken few minutes after the day cycle had been started instead of at the dark cycle. pH in PBR-B was maintained since the biomass concentration decreased and less photosynthetic activity was detected on the system. Moreover, PBR-B system has more variations along the time since the system was not stable.

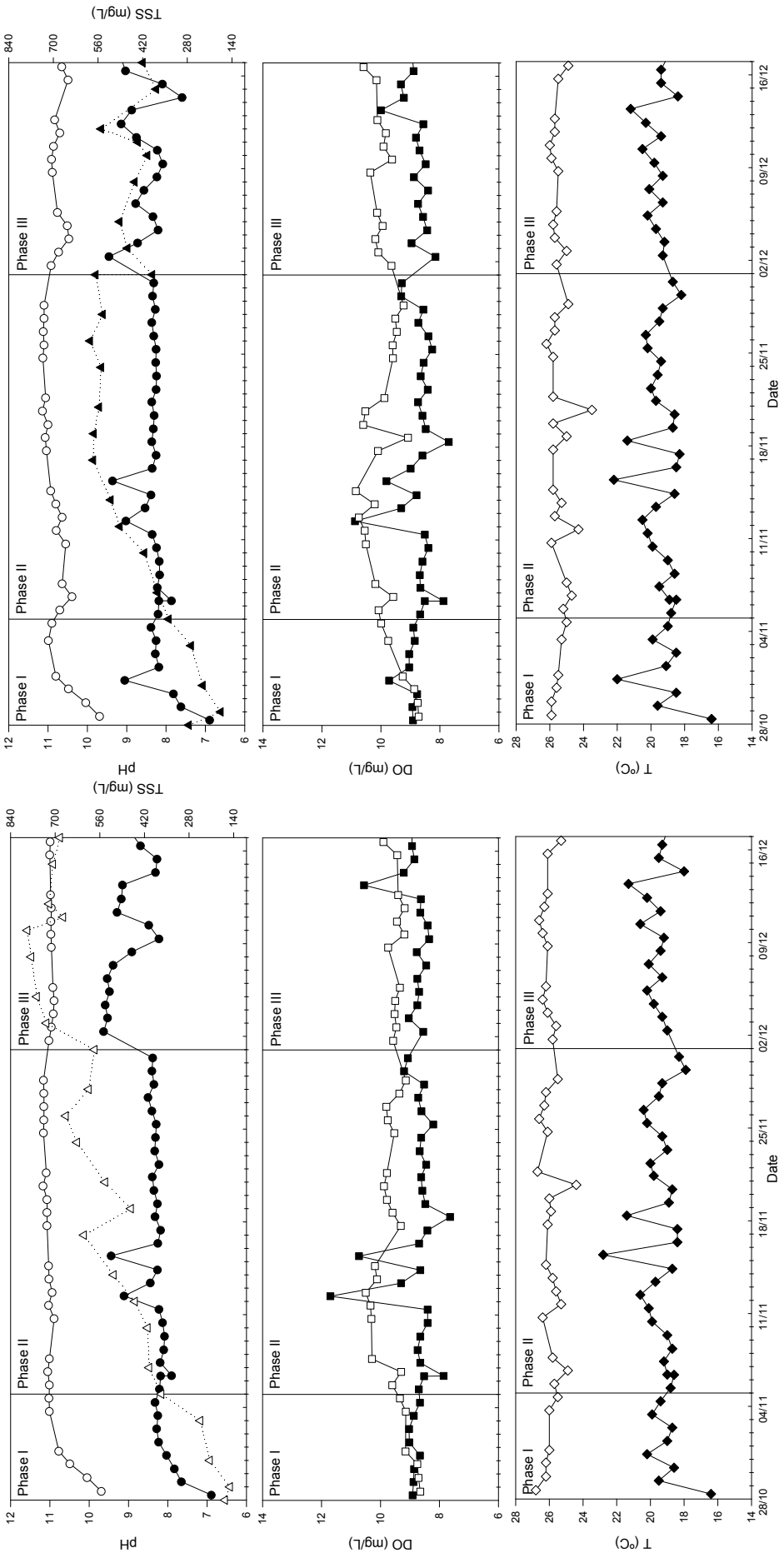


Figure 6.7 Laboratory scale PBRs performance. Left: PBR-A (HRT=7 d). Right: PBR-B (HRT=7 d Phase I & II, HRT=3 d Phase III). Top: pH and TSS monitoring: (○) *afternoon pH;* (●) *morning pH;* (△) *TSS PBR-A;* (▲) *TSS PBR-B. Middle: dissolved oxygen monitoring;* (□) *afternoon;* (■) *moming. Bottom: temperature monitoring;* (◇) *afternoon;* (◆) *morning.*

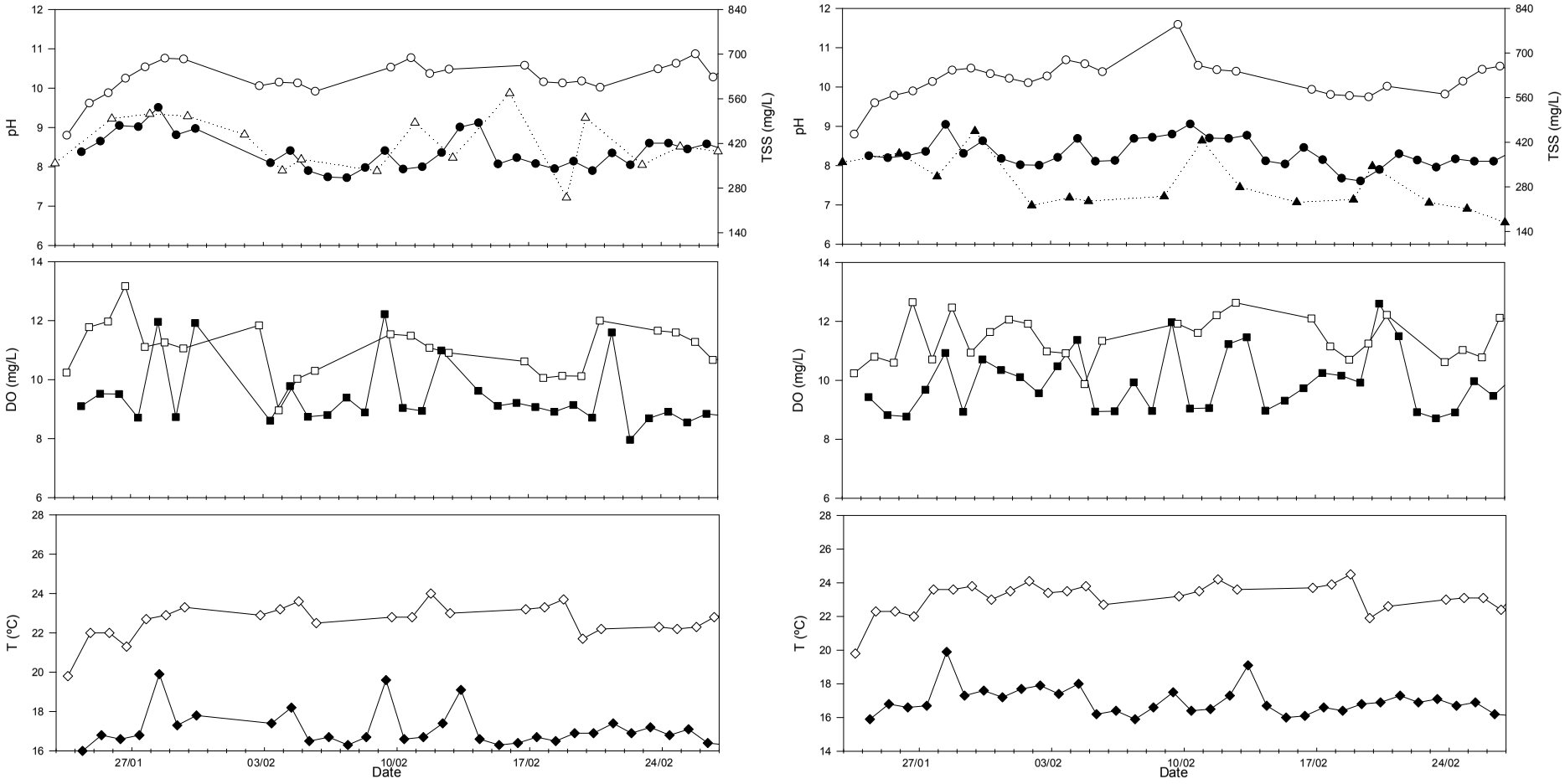


Figure 6.8 Laboratory scale HRAPs performance. Left: HRAP-A (HRT=7 d). Right: HRAP-B (HRT=3 d). Top: pH and TSS monitoring, (○) *afternoon pH;* (●) *morning pH;* (△) *TSS HRAP-A;* (▲) *TSS HRAP-B. Middle: dissolved oxygen monitoring:* (□) *afternoon;* (■) *morning. Bottom: temperature monitoring:* (◇) *afternoon;* (♦) *morning.*

Figure 6.8 shows pH, DO and T daily variations from laboratory HRAPs A and B. The behaviour of both systems was similar, pH after the dark cycle was 8.3±0.4 and after light cycle 10.3±0.4. The microalgae concentration is a parameter that directly affects the photosynthetic activity and is reflected on pH values, a lower microalgal biomass leads to lower pH values. Dissolved oxygen concentration was higher in HRAP-B (9.64±0.7 mg O_2/L after the dark cycle and 11.4±0.8 mg O_2/L after the day cycle) than in HRAP-A (9.1 ±0.6 mg O_2/L and 11.0±0.7 mg O_2/L, respectively). In comparison with the PBRs DO has been increased due to the different design of the systems, as it has been mentioned.

Temperature has been monitored along the operating periods. For both sets of systems, PBRs and HRAPs, the values recorded were in the optimum temperature range of most microalgal species between 15 to 25 ºC (Sutherland et al., 2015). TSS changes in indoor systems (laboratory PBRs and HRAPs) are not due to temperature variations.

6.3.1.3 Nutrients removal

Green microalgae have a typical biochemical composition of $C_{106}H_{181}O_{45}N_{16}P$, and require sufficient amounts of carbon, nitrogen and phosphorous to grow optimally. Nutrient load is influenced by both the water quality characteristics of the WW as the HRT (Lannan, 2011).

The following figures (Figure 6.9 and Figure 6.10) show total carbon (TC), organic carbon (TOC) and nitrogen (TN), as well as nitrite and phosphate from the inlet and effluent for the PBRs and HRAPs laboratory set up configurations.

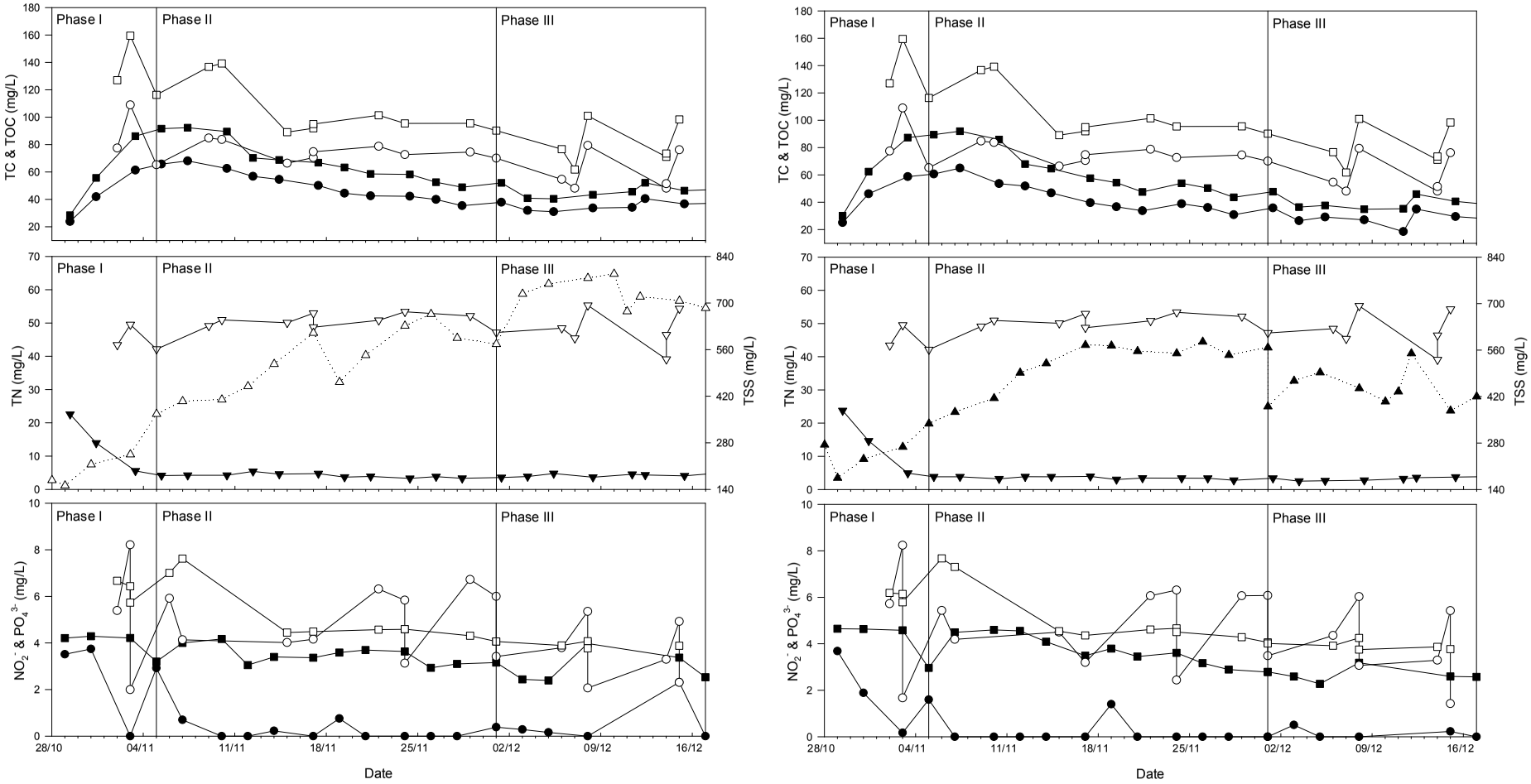


Figure 6.9 Laboratory scale PBRs nutrient load monitoring. Left: PBR-A (HRT=7 d). Right: PBR-B (HRT=7 d Phase I & II, HRT=3 d Phase III). Top: carbon monitoring: (□) *TC WW inlet;* (■) *TC PBR effluent*; (○) *TOC WW inlet;* (●) *TOC PBR effluent. Middle: total nitrogen and TSS monitoring:* (▽) *TN WW inlet;* (▼) *TN PBR effluent*; (△) *TSS PBR-A;* (▲) *TSS PBR-B. Bottom: nitrite and phosphate monitoring:* (□) NO_2^- *WW inlet;* (■) NO_2^- *PBR-A effluent;* (○) PO_4^{3-} *WW inlet;* (●) PO_4^{3-} *PBR-A effluent.*

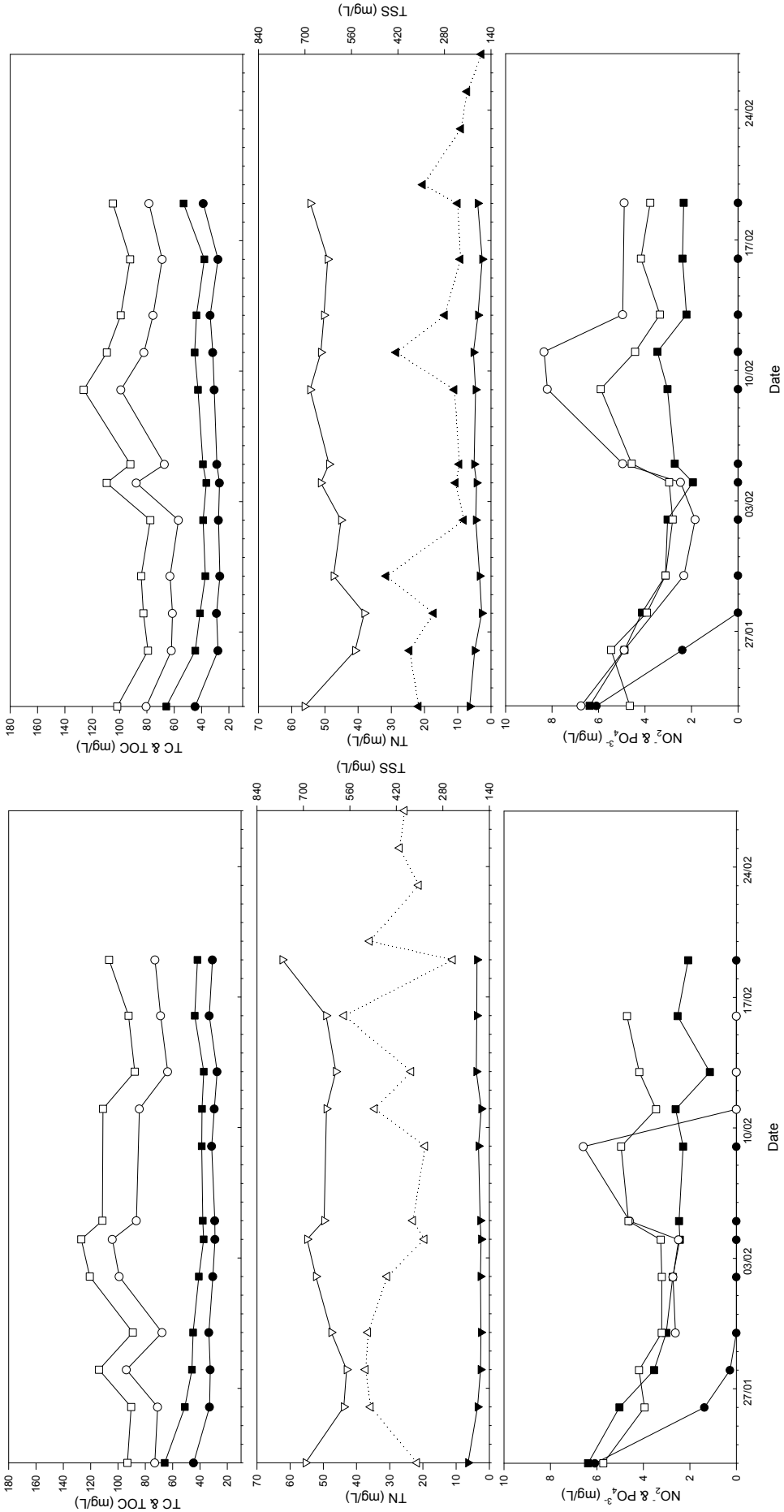


Figure 6.10 Laboratory scale HRAPs nutrient load monitoring. Left: HRAP-A (HRT=7 d). Right: HRAP-B (HRT=3 d). Top: carbon monitoring: (□) TC WW inlet; (■) TC HRAP effluent; (○) TOC WW inlet; (●) TOC HRAP effluent. Middle: total nitrogen and TSS monitoring: (▽) TN WW inlet; (▼) TN HRAP effluent; (△) TSS HRAP-A; (▲) TSS HRAP-B. Bottom: nitrite and phosphate monitoring: (□) NO_2^- WW inlet; (■) NO_2^- HRAP effluent; (○) PO_4^{3-} WW inlet; (●) PO_4^{3-} HRAP effluent.

Both PBRs systems (Figure 6.9) have the same tendency for nutrients removal. Both, TC and TOC were consumed as the same rate as they entered the system. At the end of Phase II the inlet content was constant and during Phase III some inlet peaks were detected, but the outlet concentration was constant along the operating period. TOC removal percentages were higher for PBR-B than PBR-A (50% and 40% respectively), although there were no differences at the end of Phase II and III in each PBR. Regarding TN concentration it was almost consumed in both systems (93%, Table 6.4), no big variations were detected from the inlet concentration. Nitrite and phosphate were also consumed, having complete removals for PO_4^{3-}. Increasing the day cycle (Phase III) does not enhance nutrient removal, similar outlet concentrations were measured in both systems. However, the biomass content in PBR-B was lower, so part of that removal may be due to wash out since the HRT was lower, although at lower HRT no nutrient limitation exists and microalgal productivity increases (Table 6.3).

In Figure 6.10 nutrient removal from HRAP-A and HRAP-B are presented. HRAPs operating mode corresponds to the PBRs operating mode during Phase III, so similar patterns are reflected in these systems for nutrient removal.

TC and TOC removal efficiency for HRAP-A was better than PBR-A, TOC removal in HRAP-A had an average value at the steady state of 59%, whereas during PBR-A operation it was around 40% (Table 6.4). HRAP-B TOC removal was also increased in this new configuration in comparison with PBR-B operation (60% and 50%, respectively). Outlet TN concentrations among systems were significantly different (one way ANOVA test, $p<0.05$). Although HRAP-A presented the lowest outlet values (3.07±0.6 mg TN/L) the same removal percentages were obtained between HRAP-A and B (92%, Table 6.4) and similar to TN removal percentages from the PBRs (91%, Table 6.4). Regarding nitrite and phosphate removals, all 4 microalgae systems had similar trends, and the discharge effluent was in the legislation limits (European commission directive 98/15/EC, 1998). Whereas phosphate was almost consumed during the operation period, nitrite was partly consumed. Phosphate removal percentages are shown in Table 6.4.

Table 6.4 TSS concentration and nutrient (TOC, TN and PO_4^{3-}) removal percentages at the steady state in continuous laboratory scale systems.

	Phase II				Phase III			
Set up configuration	**TSS (mg/L)**	**TOC (%)**	**TN (%)**	**PO_4^{3-} (%)**	**TSS (mg/L)**	**TOC (%)**	**TN (%)**	**PO_4^{3-} (%)**
PBR-A (HRT=7 d)	560±80	41±10	93±1	97±5	729±41	40±13	91±2	96±5
PBR-B (HRT=7 & 3 d)	550±30	50±6	93±1	97±8	435±38	51±12	93±1	97±6
HRAP-A (HRT=7 d)					409±105	59±6	93±1	99±2
HRAP-B (HRT=3 d)					244±56	60±7	92±2	99±3

The different laboratory microalgae systems tested, including design (1 L PBRs and 7 L HRAPs), light/dark cycle and HRT variations have demonstrated that wastewater treatment by microalgal-based technologies are feasible for nutrient removal. Increasing light cycle improves microalgae growth.

6.3.1.4 Microbial composition

WW treatment HRAPs naturally select for prolifically growing microalgae species capable for high nutrient removal and tolerant wide fluctuations in environmental variables (Sutherland et al., 2015). Dominant phototrophs were noted by microscopy and external laboratory (Cawthron Analytical Services, Nelson, New Zealand) identification. The relative dominance of algae species changed with operating conditions. Bacterial species (except some cyanobacteria) present in the systems were not identified. PBRs were inoculated using 600 mL from microalgal/bacterial suspension from operating laboratory scale HRAPs (Table 6.2). Microalgae biodiversity was analysed at the end of Phase III, results are presented in Table 6.5.

Table 6.5 Species composition from laboratory scale PBRs assay (17.12.2014).

Species	Description	PBR-A rating	PBR-B rating
Aphanocapsa sp. (<1 μm)	Potentially toxic cyanobacteria	2	-
Euglena sp.	Euglenophycota	-	1
Cymbella sp.	Diatoms	1	-
Gomphonema sp.	Diatoms	-	2
Nitschia sp.	Diatoms	1	4
Monoraphidium spp.	Chlorophyta	1	-
Pediastrum sp.	Chlorophyta	7	7
Scenedesmus sp.	Chlorophyta	8	8
Small unicells (<5 μm)	Non-toxic species	3	3
Unidentified stalked ciliate sp.	Other dominant species	5	5
Unknown colonial chlorophyte	Chlorophyta	4	5

1: rare; 2: rare-occasional; 3: occasional; 4: occasional-common; 5: common; 6: common-abundant; 7: abundant; 8: dominant; -: not detected

At the end of Phase III PBRs had a similar microbial composition, the green microalga *Scenedesmus* sp. was the dominant specie and *Pediastrum* sp. was abundant (Table 6.5, Image 6.3). This is in accordance with the frequently reported species in the literature, classified as colonial green algae (the most representative are from the genera *Pediastrum, Mucidosphaerium, Desmodesmus* or *Micractinium*) (Craggs et al., 2012; Park and Craggs, 2011; Sutherland et al., 2014).

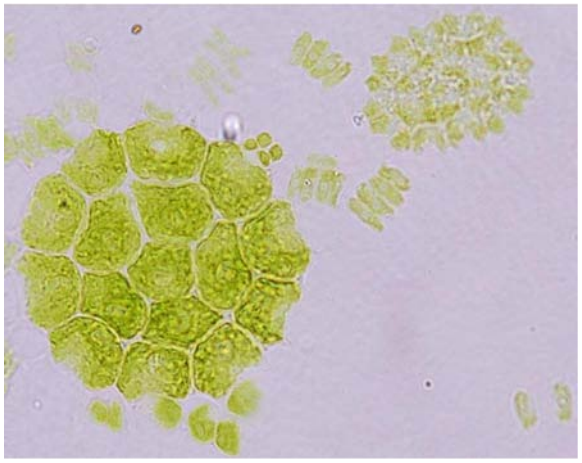


Image 6.3 Pediastrum sp. and Scenedesmus sp. from the PBRs microscopic image, 1000x magnification.

PBRs biodiversity changed from the inoculum. *Pediastrum* sp. and *Scenedesmus* sp. were occasional and rare-occasional, respectively, rated in the inoculum (Table 6.2). After approximately 2 months of operation they increased to be abundant and dominant, respectively (Table 6.5). Moreover, the potentially toxic cyanobacteria *Aphanocapsa* sp. was not detected in PBR-B (Table 6.5), whereas it was the dominant specie in the inoculum (Table 6.2). Diatoms, *Gomphonema* sp., concentration was also decreased in PBRs.

HRAPs were inoculated adding 6 L of the HRAP-F effluent. HRAP-A and HRAP-B biodiversity was evaluated a week after the inoculation. Results are shown in Table 6.6.

Table 6.6 Biodiversity of the laboratory scale HRAPs (13.01.2015).

Species	Description	HRAP-A rating	HRAP-B rating
Pseudanabaenaceae	Cyanobacteria (not toxic)	2	1
Romeria sp.	Cyanobacteria (not toxic)	1	-
Gomphonema sp.	Diatoms	-	3
Nitschia sp.	Diatoms	-	1
Reimeria sinuata	Diatoms	1	-
Synedra sp.	Diatoms	1	-
Chlamydomonas sp.	Chlorophyta	-	2
Monoraphidium spp.	Chlorophyta	-	2
Oedogonium sp.	Chlorophyta	3	6
Pediastrum sp.	Chlorophyta	4	2
Scenedesmus sp.	Chlorophyta	8	8
Stigeoclonium sp.	Chlorophyta	1	4
Peridinium sp.	Dinoflagellates	1	-
Small unicells (<5 μm)	Non-toxic species	5	4
Unknown colonial chlorophyte	Chlorophyta	7	7

1: rare; 2: rare-occasional; 3: occasional; 4: occasional-common; 5: common; 6: common-abundant; 7: abundant; 8: dominant; -: not detected

Again, *Scenedesmus* sp. was the dominant specie in both systems, however, *Pediastrum* sp. was occasional-common rated for HRAP-A and in HRAP-B was rated as rare-occasional. Unknown colonial chlorophyte were abundant for both (Image 6.4).

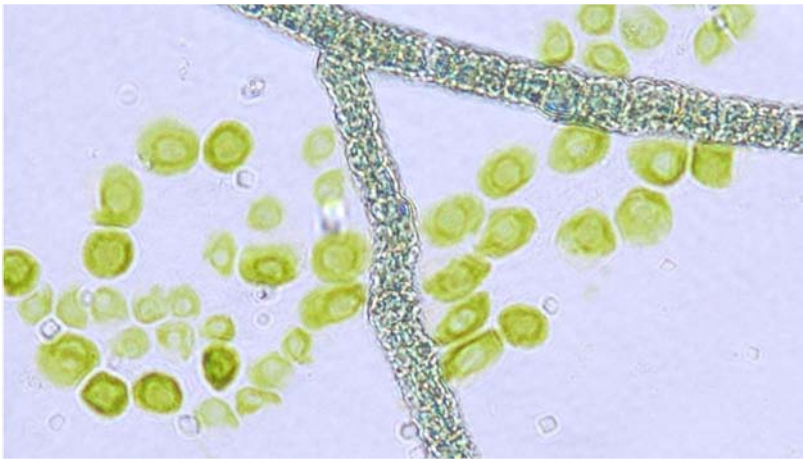


Image 6.4 Laboratory scale HRAP-A microscopic image, 1000x magnification.

6.3.2 Real wastewater treatment in an outdoor continuous pilot scale system

A pilot scale HRAP (HRAP-F) (Image 6.2) was operated during the southern hemisphere summer period (December-March). It was semi-continuously fed with PEW and located at Palmerston North (New Zealand) WWTP operating at an HRT of 10 days.

During the operating period its performance was monitored in the same way as for the two laboratory scale set up configurations (PBRs and HRAPs). Results are presented in Figure 6.11 and Figure 6.12 and in Table 6.7.

pH, DO and T were measured once a day and not at the same time, that is why lots of fluctuations appear on the graphs. Later on the day measurements present higher values.

TSS inside the pond had variations, until the 10th February the average concentration in the pond was 348±68 mg/L, whereas during the last days it was increased to 446±36 mg/L, this value is similar to the one obtained during the PBR-A Phase II operation, and also similar to the values recorded in the laboratory HRAPs (Table 6.4). Regarding the TSS content in the WW the value was maintained around 88±25 mg/L during the overall performance.

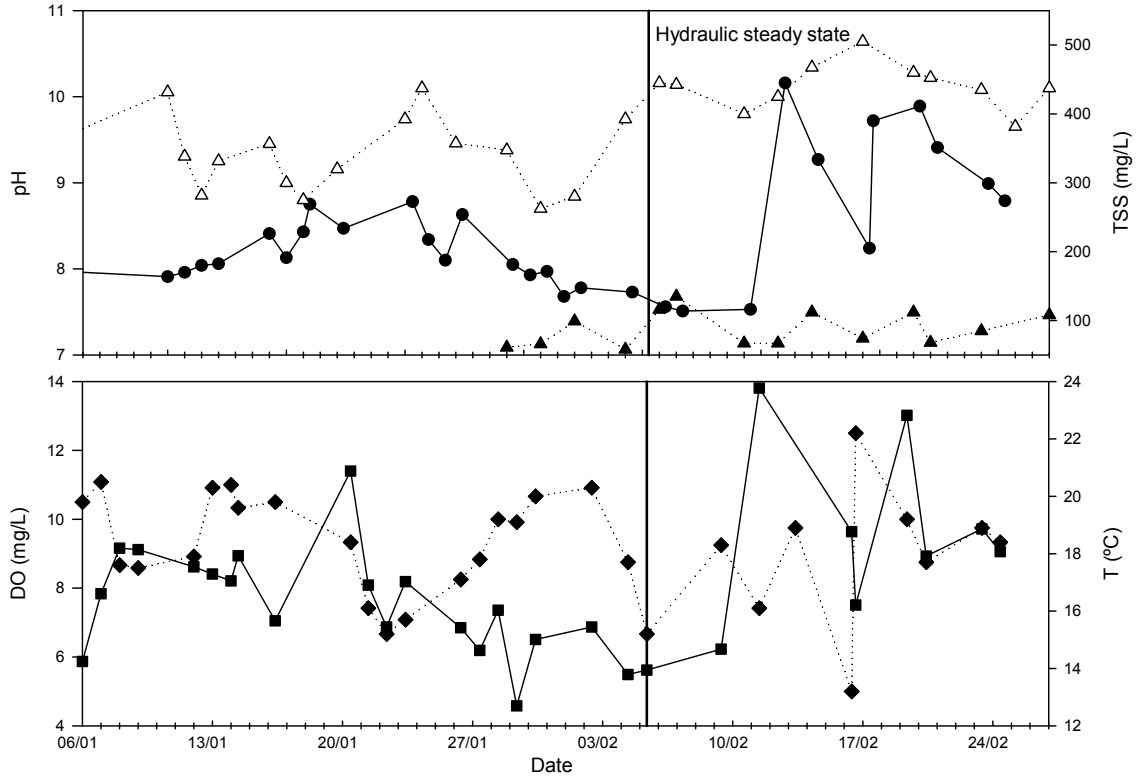


Figure 6.11 Pilot scale HRAP-F performance. Top: (●) *pH;* (△) *TSS HRAP-F;* (▲) *TSS WW inlet. Bottom:* (■) *dissolved oxygen;* (♦) *temperature. Vertical line* (|) *indicates the theoretical hydraulic steady state.*

Highly TC and TN concentrations were detected in the PEW from which the HRAP-F was fed, those load peaks were attributed to the operation of the WWTP. While in the laboratory sets the WW fed was collected the same day and frozen, in the HRAP-F WW was daily pumped into the pond. Despite these variations, the system had a similar efficiency for TC, TOC and TN removal than during the laboratory scale continuous performance. 40% TOC removal was attained at the steady state of HRAP-F operation (Table 6.7). At the end of the operating period, the system was more stable, since the theoretical steady state was reached. TC effluent concentration was 44±6 mg/L, for TOC 31±4 mg/L and for TN 20±3 mg/L. TN inlet concentration at the end of the operation dropped, achieving values below the outlet ones. Outlet concentration was constantly maintained. For that reason TN removal percentages during the steady state have not been presented in Table 6.4.

Initially, phosphorous was not removed from the system, but at the end it was completely removed (Figure 6.12 and Table 6.7). It is quite common that in HRAPs nitrification process occurs, however partial nitrification could also

occur, thus leads to nitrite accumulation in the pond as it is observed in the HRAP-F.

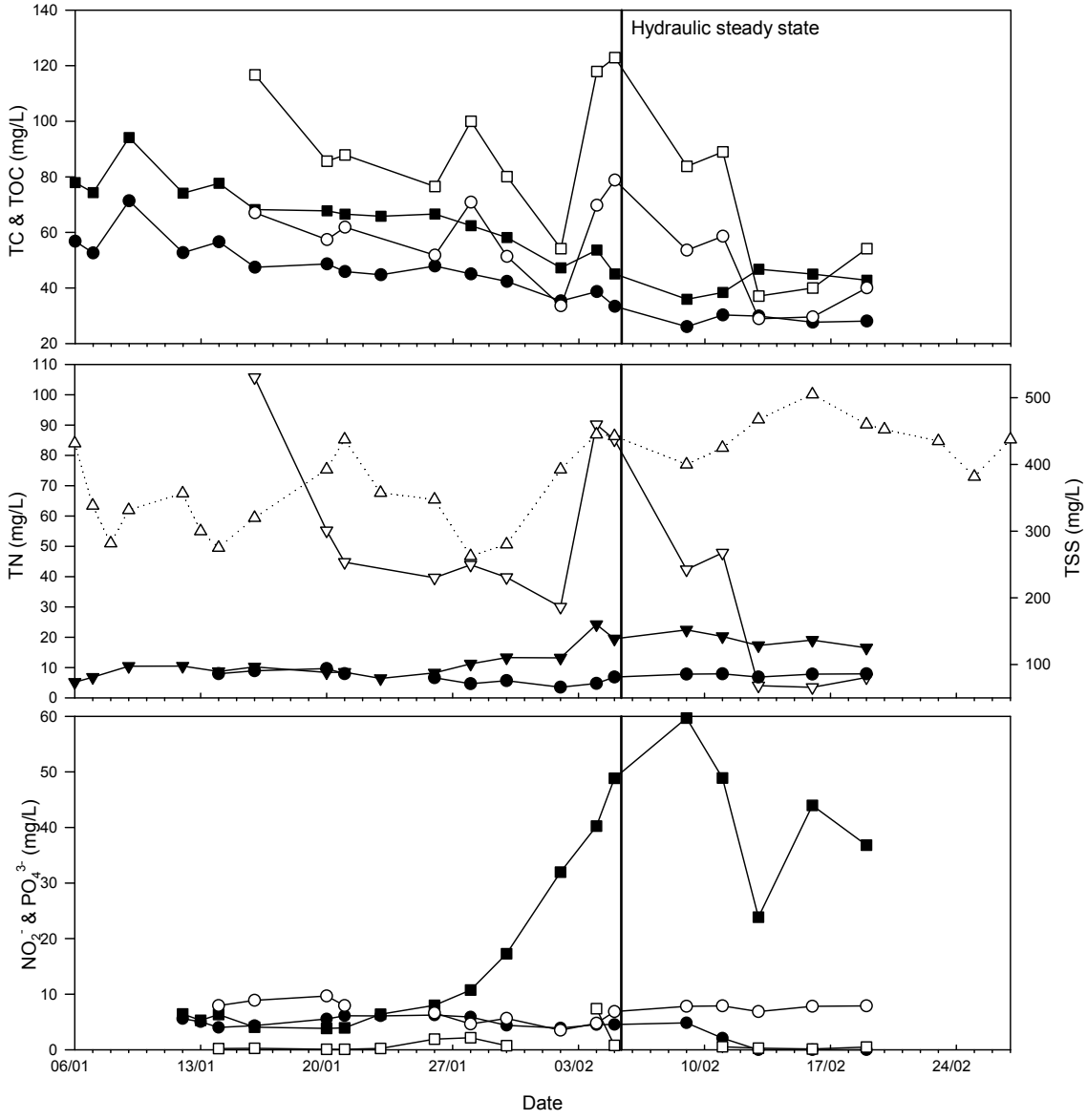


Figure 6.12 Pilot scale HRAP-F nutrients load monitoring. Top: carbon monitoring: (□) *TC WW inlet;* (■) *TC effluent;* (○) *TOC WW inlet;* (●) *TOC effluent. Middle: total nitrogen and TSS monitoring:* (▽) *TN WW inlet;* (▼) TN *effluent;* (△) *TSS. Bottom: nitrite and phosphate monitoring:* (□) NO_2^- *WW inlet;* (■) NO_2^- *effluent;* (○) PO_4^{3-} *WW inlet;* (●) PO_4^{3-} *effluent.*

Table 6.7 Theoretical hydraulic steady state nutrient (TOC, TN and PO_4^{3-}) removal percentages in the HRAP-F and TSS.

Parameter	Value
TSS (mg/L)	446±36
TOC (%)	40±19
TN (%)	-
PO_4^{3-} (%)	99±2

Microbial characterization was conducted twice during the HRAP-F operation (Table 6.8). The first was at the beginning of HRAP-F operation (17.12.2014), after the hydraulic tests were conducted and the second in the middle of its operation performance (27.01.2015).

Scenedesmus sp. was the dominant specie at the beginning and then it switched to *Pedriastrum* sp. Another chlorophyte, *Oocystis* sp., was also common-abundant during both characterizations, it has also been found in previous pilot scale HRAP systems (Passos et al., 2014b, 2013). *Pseudanabaena* sp., a potentially toxic cyanobacteria was also present at the beginning, although it was dramatically reduced during the second microbial characterization, but *Planktothrix* sp. (another potentially toxic cyanobacteria) was found to be common in the system. Several diatoms species appeared during the HRAP-F operation (Image 6.5). Diatoms are a major group of algae and are the dominant life form in phytoplankton (Demirbas and Demirbas, 2011).

Table 6.8 Biodiversity of the pilot scale HRAP-F.

Species	Description	HRAP-F rating 17.12.2014	HRAP-F rating 27.01.2015
Aphanocapsa sp. (<1 µm)	Potentially toxic cyanobacteria	3	2
Microcystis sp.	Potentially toxic cyanobacteria	-	1
Planktothrix sp.	Potentially toxic cyanobacteria	-	5
Pseudanabaena sp.	Potentially toxic cyanobacteria	7	1
Pseudanabaenaceae	Cyanobacteria (not toxic)	2	-

Species	Description	HRAP-F rating 17.12.2014	HRAP-F rating 27.01.2015
Aulacoseira sp.	Diatoms	-	1
Gomphonema sp.	Diatoms	-	3
Nitschia sp.	Diatoms	-	5
Synedra sp.	Diatoms	-	2
Actinastrum sp.	Chlorophyta	-	1
Chlamydomonas sp.	Chlorophyta	4	-
Closterium sp.	Chlorophyta	1	1
Coelastrum sp.	Chlorophyta	4	3
Crucigeniella sp.	Chlorophyta	1	-
Cryptomonas sp.	Chlorophyta	1	-
Dictyosphaerium sp.	Chlorophyta	1	2
Kirchneriella sp.	Chlorophyta	1	-
Micractinium sp.	Chlorophyta	-	1
Monoraphidium spp.	Chlorophyta	4	2
Oocystis sp.	Chlorophyta	6	6
Pediastrum sp.	Chlorophyta	2	8
Pteromonas sp.	Chlorophyta	3	-
Scenedesmus sp.	Chlorophyta	8	4
Peridinium sp.	Dinoflagellates	1	-
Small flagellates (<5 µm)	Non-toxic species	3	-
Small unicells (<5 µm)	Non-toxic species	5	7
Unknown colonial chlorophyte	Chlorophyta	1	2

1: rare; 2: rare-occasional; 3: occasional; 4: occasional-common; 5: common; 6: common-abundant; 7: abundant; 8: dominant; -: not detected

Image 6.5 Microscopic image from HRAP-F, 1000x magnification.

6.3.3 CPX removal in continuous laboratory scale systems

The fluoroquinolone antibiotic ciprofloxacin (CPX) was daily fed into the laboratory scale PBRs and HRAPs together with PEW.

During Phase I of PBR-A and B operation 14 mg/L, corresponding to 2 mg/(L·d) load, were erroneously fed to the system, then concentration was reduced to 2 mg/L. CPX load during Phase II in PBR-A and B was 0.286 mg/(L·d), whereas, during Phase III, PBR-B load was increased to 0.667 mg/(L·d), while PBR-A load was maintained at 0.286 mg/(L·d). CPX monitoring results are shown in Figure 6.13.

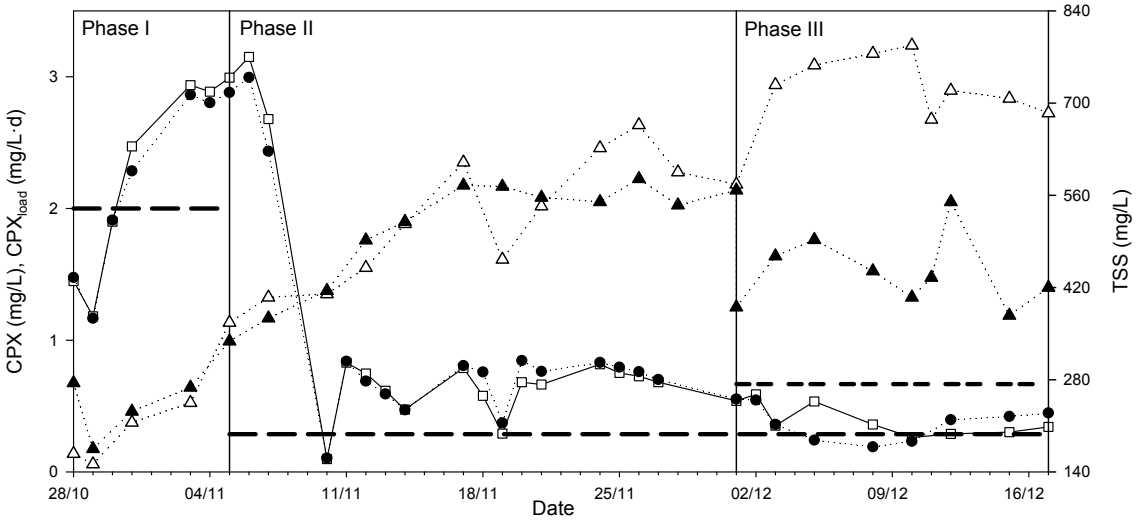


Figure 6.13 CPX monitoring in PBR-A (HRT=7 d) and PBR-B (HRT=7 d Phase I & II, HRT=3 d Phase III). (□) CPX concentration PBR-A; (●) CPX concentration PBR-B; (△) TSS PBR-A; (▲) TSS PBR-A; (— long dash) CPX load PBR-A Phase I, II and III and PBR-B Phase I and II; (- short dash) CPX load PBR-B Phase III.

CPX addition did not significantly impact algal activity during the operation period even when erroneously added at 14 mg/L during Phase I. The TSS concentration did not decrease, on the contrary it was increased, meaning that CPX did not inhibit microalgal growth inside the PBRs (Figure 6.13).

As can be seen in Figure 6.13, CPX concentration reached around 3 mg/L at the end of Phase I (78% removal efficiency, volumetric removal rate of 1.57 mg/(L·d)) then decreased and stabilized around 0.73 mg/L (64% removal efficiency, volumetric removal rate of 0.18 mg/(L·d)) during Phase II. No significant variations were observed between the two PBRs during Phase I and II. However, when PBRs operated at different HRT, thus the CPX inlet load was different (Phase III), differences among A and B appeared (Figure 6.13 and Table 6.9). Higher CPX removal percentages were obtained in PBR-A (84%) than for PBR-B (81%). But the removal rate was higher in PBR-B, due to the lower HRT, 0.54 mg/(L·d) and 0.24 mg/(L·d), respectively.

Table 6.9 TSS concentration, CPX inlet load, CPX removal percentage and CPX removal rate during Phase II and III in continuous laboratory scale PBRs and HRAPs systems.

			Phase II	
Set up configuration	**TSS (mg/L)**	**Load (mg/(L·d))**	**CPX removal (%)**	**CPX removal rate (mg/(L·d))**
PBR-A (HRT=7 d)	560±80	0.286	64±5	0.18±0.016
PBR-B (HRT=7)	550±30	0.286	64±5	0.18±50.016
			Phase III	
Set up configuration	**TSS (mg/L)**	**Load (mg/(L·d))**	**CPX removal (%)**	**CPX removal rate (mg/(L·d))**
PBR-A (HRT=7 d)	729±41	0.286	84±2	0.24±0.006
PBR-B (HRT=3 d)	435±38	0.667	81±5	0.54±0.032
HRAP-A (HRT=7 d)	409±105	0.286	67±6	0.19±0.018
HRAP-B (HRT=3 d)	244±56	0.667	67±6	0.45±0.030

It must also be noted that operational changes can impact biomass characteristics (García et al., 2006; Park et al., 2011b) and other wastewater constituents (especially pH and DO), thereby causing potential indirect effects on photodegradation (Babic et al., 2013; Salma et al., 2016) and sorption (Polesel et al., 2015; Roca Jalil et al., 2015; Vasudevan et al., 2009). There is now significant evidence that CPX can photodegrade in surface waters (Belden et al., 2007; Porras et al., 2016; Van Doorslaer et al., 2011) or sorb to biosolids during conventional biological treatment (Batt et al., 2007; Golet et al., 2003; Jiang et al., 2012; Li and Zhang, 2010). Hydrolysis and biodegradation are not considered as quantitatively significant in these environments although significant biodegradation has been reported during biological treatment (Van Doorslaer et al., 2011). Algal-based wastewater treatment systems are designed for efficient light supply, which should promote CPX photodegradation. However, light supply also supports the growth of light-shading/harvesting algae that may prevent CPX photodegradation from occurring while increasing its biosorption. As seen in Figure 6.13, doubling the daily illumination period from 8 to 16 hr at 7 d HRT (Phase II to III in PBR-A) boosted biomass concentration by 36% during operation (Table 6.9).

Therefore, it is not possible to attribute the CPX removal gain to a particular mechanism during PBR-A operation. In comparison, reducing HRT from 7 to 3 days (PBR-B) should negatively impact the quantitative significance of all removal mechanisms considered (as CPX is soluble and the reactors are well mixed). Instead, these changes were associated with a considerable increase in CPX removal percentage (CPX removal increased from 64% to 81% reducing the HRT from 7 d to 3 d (Phase II and III, respectively), Table 6.9), possibly as a consequence of the combined positive effect of increased light period and decreased TSS concentration (from 550 to 435 mg/L) on photodegradation.

CPX removal efficiency from the laboratory scale HRAPs was lower in comparison to the PBRs (Table 6.9). As can be seen in Figure 6.14, the TSS concentration in HRAP-A fluctuated around 400 mg/L at 7 d HRT operation and CPX concentration gradually increased to reach 0.65 mg/L during the last week of operation (67% removal efficiency and volumetric removal rate 0.19 mg/(L·d)). In contrast, in HRAP-B, the TSS gradually decreased from 350 to

150 mg/L at a 3 d HRT operation while CPX concentration increased up to 1.25 mg/L during the first weeks before decreasing to around 0.66 mg/L when operation ended. The reduction in the HRT, increased the volumetric removal rate in HRAP-B, 0.44 mg/(L·d) and 67% removal efficiency, at the theoretical hydraulic steady state.

Again, short HRT operation was found to increase CPX volumetric removal rate, likely as the combined effect of improved photodegradation due to reduced light shading at lower TSS concentration (244 mg/L in HRAP-B, while HRAP-A TSS concentration was 409 mg/L), and improved biosorption due to increased biomass productivity (from 409 mg/d to 569 mg/d, (Table 6.3)).

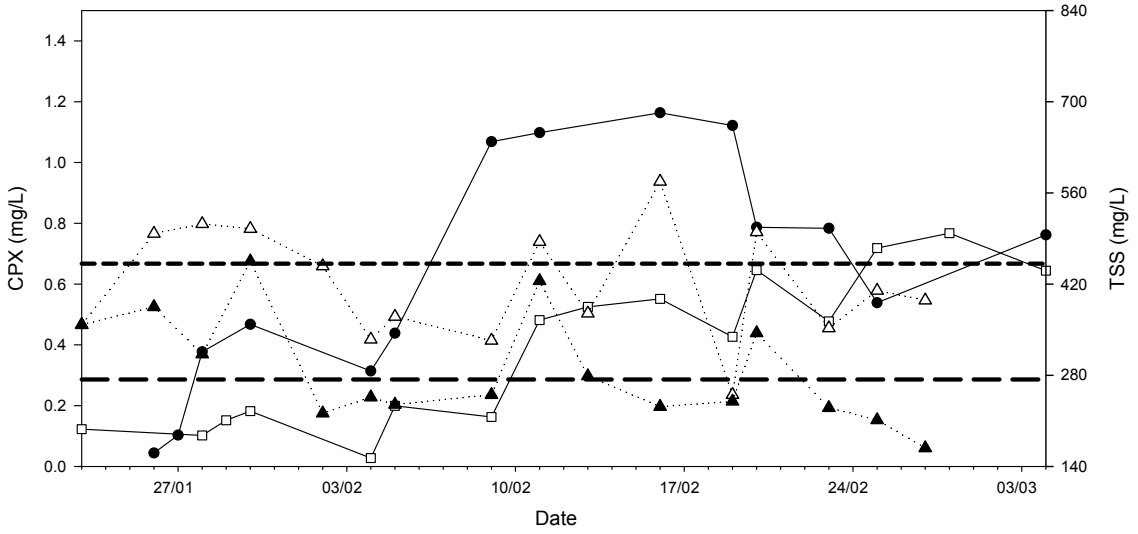


Figure 6.14 CPX monitoring in HRAP-A (HRT=7 d) and HRAP-B (HRT=3 d). (□) *CPX concentration HRAP-A;* (●) *CPX concentration HRAP-B;* (△) *TSS HRAP-A;* (▲) *TSS HRAP-B; (— long dash) CPX load HRAP-A; (- short dash) CPX load HRAP-B.*

No clear conclusion can however be made with regards to the impacts of operation mode and reactor design on CPX removal or with regards to the temporal changes, seen in Figure 6.13, given the complexity of the removal mechanisms involved and the small differences obtained during HRAPs versus PBRs treatment. Further batch tests were therefore conducted in order to widen the information on the individual removal mechanisms.

6.3.4 Outdoor batch experiments for CPX removal

CPX removal was evaluated during 24 h in outdoor batch experiments. It was conducted in the pilot scale HRAP (HRAP-F) and furtherly at laboratory scale using 150 mL flasks (Batch-F).

6.3.4.1 CPX removal from an outdoor pilot scale batch, HRAP-F

The fate of CPX was studied in the outdoor pilot scale HRAP-F for relevant information at full scale conditions. Spiked tests were conducted to further differentiate and quantify degradation during day and night periods (Figure 6.15).

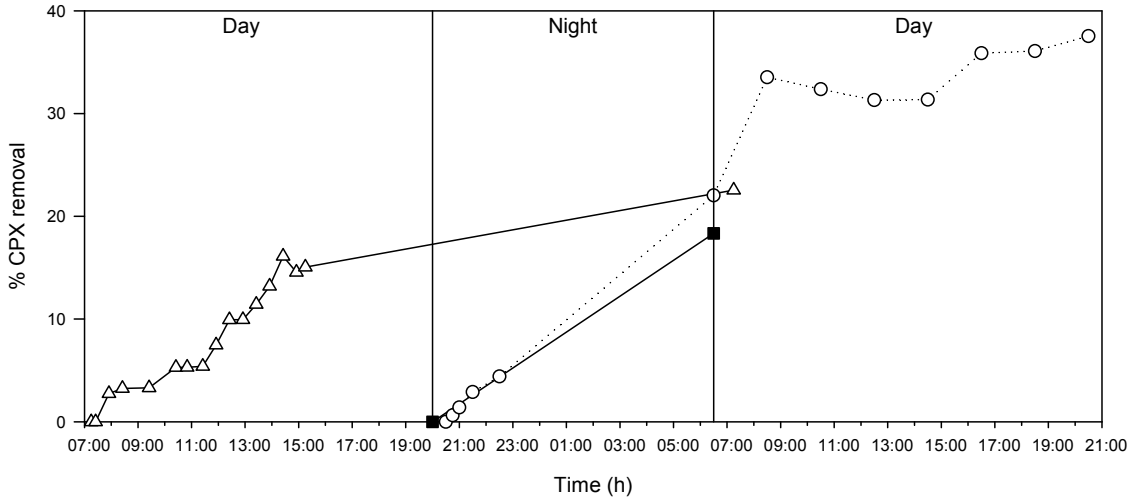


Figure 6.15 CPX removal during 24 h in outdoor pilot scale HRAP-F. (△) *HRAP-F1 assay;* (■) *HRAP-F2 assay;* (○) *HRAP-F3 assay.*

As can be seen in Figure 6.15, significant CPX removal occurred immediately after the spiking in all three assays, independently of the time of the day. Kinetic parameters could be determined and fit well into a pseudo-first order rate (Table 6.10).

Table 6.10 Biomass concentration and kinetic parameters (pseudo-first order) for CPX removal in 24 h batch assays in the pilot scale HRAP-F.

Pulse	**$TSS_{initial}$ (mg/L)**	**k (h^{-1})**	**r^2**
HRAP-F1	448 ± 40	0.020	0.95
HRAP-F2	502 ± 35	0.031	1.00
HRAP-F3	342 ± 29	0.031	1.00

Adsorption as well as photodegradation may be occurring during HRAP-F1 (Figure 6.15) (biodegradation has been considered negligible from the results obtained in the previous sections). CPX removal achieved during the day was 15% and at the end of the batch, it increased to 23%. Photodegradation also took place as seen during continuous CPX removal, moreover, it was not strongly influenced by the suspended biomass, most of the photodegradation occurred near the surface, and indirect photodegradation was more dependent on the dissolved organics than the biomass flocs. CPX removal percentages during the night, for HRAP-F2 and F3 experiments, were similar between them, 18% and 22%, respectively, attributed to sorption. HRAP-F3 was monitored along the day to evaluate photodegradation and sorption. The final CPX removal percentage was 38% in 24 h.

In HRAP-F2 and HRAP-F3 assays, the spiking was conducted after the sunset, while in HRAP-F1 assay the spiking was carried out after the sunrise. Similar k values were obtained for HRAP-F2 and F3 assays, despite initial TSS were different (Table 6.10), meaning that sorption rate during the night was the same, independently of the biomass content. k values were increased in comparison with HRAP-F1 values.

In order to confirm the results under natural sunlight, an outdoor laboratory scale batch assay (Batch-F) was conducted adjacent to the pilot scale HRAP-F fed with PEW using the biomass from the pilot reactor.

6.3.4.2 CPX removal from an outdoor laboratory scale batch, Batch-F

Batch experiments enabled more conditions to be tested and the purpose was to develop a deeper understanding of the main removal mechanisms: hydrolysis, sorption, biodegradation, direct photolysis and/or indirect photodegradation. The conditions for each sample were therefore designed to separate the effects of these mechanisms, although the differences between systems HRAP-F and Batch-F may have an influence on CPX removal. The main differences are: material used (glass versus concrete), mixing, depth, biofilms formed in the pond, temperature fluctuations or light surface.

Batch-F experiment started around 08:30 pm and was therefore submitted to darkness for approximately 10 h before sunrise (Figure 6.16). During the night

period some sorption occurred, around 10% for all conditions, whereas in HRAP—F3 night sorption achieved 20% (Figure 6.15). After the sunset, CPX removal from light samples increased. Again, it is seen that photodegradation is an important process, higher removal percentages were obtained when no microalgal biomass was present. 85% CPX removal when no biomass was present and 44% in the presence of microalgal biomass (Figure 6.16). Active biomass showed low removal percentages, the TSS increase may have inhibited part of photodegradation by scattering the light penetration due to the shading effect. However, for dark samples removal was also increased. It is possible that temperature fluctuations had an impact on CPX removal, since an increase in the temperature enhances CPX sorption.

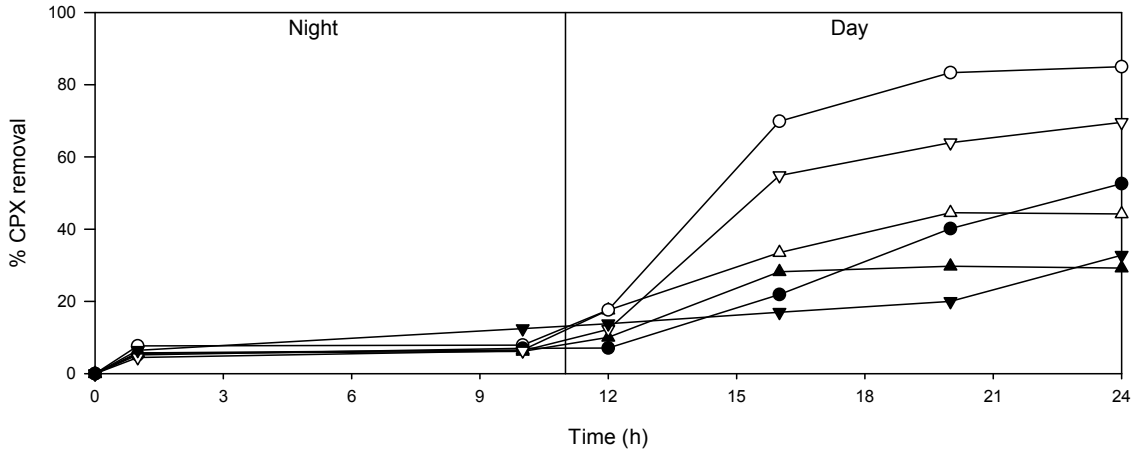


Figure 6.16 CPX removal from outdoors laboratory scale batch assay (Batch-F). (△) *biomass light;* (▲) *biomass dark;* (○) *WW light;* (●) *WW dark;* (▽) *killed control light;* (▼) *killed control dark.*

Kinetic parameters have been calculated after the sunrise and presented in Table 6.11. CPX removal has been described by a pseudo-first order rate.

Table 6.11 Kinetic parameters (pseudo-first order) for CPX removal in laboratory scale batch test conducted during 24 h outdoor, Batch-F.

	Light		**Dark**	
Condition	**k (h^{-1})**	**r^2**	**k (h^{-1})**	**r^2**
Biomass	0.0495	1.00	0.0564	1.00
WW	0.1998	0.98	0.0550	0.99
Killed control	0.1112	0.92	0.0094	1.00

Despite kinetic values are different from the pilot scale HRAP experiments (Table 6.10) previously obtained, the removal mechanisms seem to confirm what has been exposed, photodegradation and sorption are the main ones, and biodegradation could be minor.

CPX removal occurred in both, WW and biomass samples. Photosynthetic growth in the samples performed with active biomass was evidenced by pH and biomass concentration increase, pH raised from 8.42 to 10.85 and the TSS from 350 to 398 mg/L (data not shown). WW light samples also had a pH increase, from 7.52 to 8.69 (data not shown), evidencing that some bacterial growth was taking place, thus CPX removal may be enhanced. pH increase in WW dark samples was also observed, from 7.66 to 8.68 at the end of the experiment (data not shown). This pH increase may be an evidence of nitrification processes taking place in WW samples as previous works postulated. Studies have reported the ability of nitrifying conditions in activated sludge to biodegrade some pharmaceuticals (Kruglova et al., 2014; Vader et al., 2000). Lin et al. (2016) found out that in the nitrifying population increased in soils containing CPX, although, Gonzalez-Martinez et al. (2014) found out that high concentrations of CPX (350 ng/L) induced a decay of the partial-nitritation process and part of the process was damaged. Since results obtained by other authors are not in agreement, CPX removal could not be attributed to bacteria without further investigations.

6.3.5 Laboratory scale batch experiment for CPX removal

An indoor laboratory scale batch (150 mL beakers) (Batch-L, Figure 6.4) was conducted in parallel with the continuous laboratory scale HRAPs experiments discussed in the previous section 6.3.3 (Figure 6.14). This experiment (Batch-L) was similar to Batch-F experiment, the main differences were the time (4 days instead of 24 h) and it was conducted under controlled conditions of light and temperature.

As can be seen in Figure 6.17, significant CPX removal was reported during indoor assays incubated under darkness, and data was well described by pseudo-first order kinetics (Table 6.12). This ‘dark’ removal agrees with past studies on CPX fate during wastewater biological treatment (Figure 6.15 and

Batch-F results) and is therefore likely due to CPX sorption to suspended solids (Golet et al., 2002; Jia et al., 2012; Porras et al., 2016). The main removal mechanism for the killed control samples in the dark should be sorption, although hydrolysis may be occurring, and could be extrapolated to active biomass samples under the dark. Although, it should be taken into account that killed biomass could have experienced changes on biomass (denaturing proteins or disrupting cell membranes) that may have had an impact on biomass sorption (Prabhakaran et al., 2009). On the other hand, high removal percentages (43%) were also detected on WW samples under dark conditions, in that way CPX removal could be also due to sorption onto organic constituents from the WW. Previous authors reported the role of humic substances in the CPX degradation in water (Porras et al., 2016).

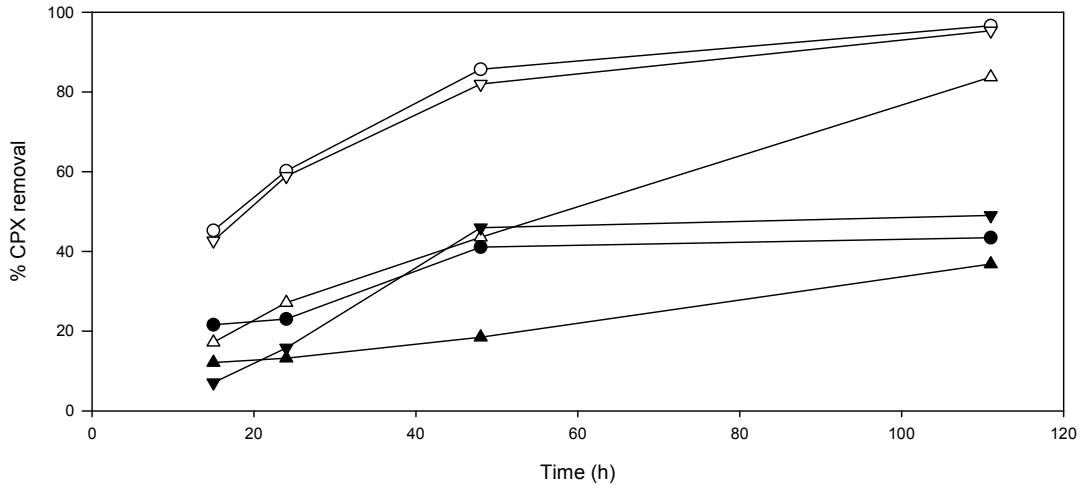


Figure 6.17 CPX removal from laboratory scale Batch-L assay. (△) *biomass light*; (▲) *biomass dark;* (○) *WW light*; (●) *WW dark;* (▽) *killed control light;* (▼) *killed control dark.*

Table 6.12 Kinetic parameters (pseudo-first order) for the laboratory scale batch test conducted indoors, Batch-L.

	Light		Dark	
Condition	**k (h^{-1})**	**r^2**	**k (h^{-1})**	**r^2**
Biomass	0.012	1.00	0.0043	0.94
WW	0.040	1.00	0.0054	0.95
Killed control	0.036	1.00	0.0071	0.79

CPX removal was significantly enhanced by light irradiation (it increased from 37% removal under dark conditions, to 84% under light conditions, Figure 6.17).

CPX removal in biomass samples was well described by a pseudo-first order kinetic rate during the firsts 48 hours of irradiation, suggesting photodegradation has the potential to cause significant CPX removal during algal-based WW treatment (Figure 6.17, Table 6.12). Photodegradation was observed: higher removal rates for the light exposed samples were obtained than for dark WW controls. Under these conditions, algal biomass concentration did not affect photodegradation, probably because the absorption of light by WW organics in this experiment was more dominant than any effect of the algal biomass.

Photosynthetic activity in illuminated assays supplied with active biomass was evidenced by biomass growth and pH increase (from 8.5 initially up to 10.5 after 24-48 h and an increase of 158 mg TSS/L at the end of the batch experiment (data not shown)). In comparison, the pH always remained around 8.5 (data not shown) in tests supplied with dead/inhibited biomass or tests supplied with active biomass and incubated under darkness. A small pH increase from 7.5 to 8.5 (data not shown) was noted in tests supplied with WW and submitted to either constant illumination or darkness.

Biodegradation must however be ruled out as significant mechanism in WW samples, since higher removal was reported in the killed samples. At the same time as CPX is photodegraded, observed from the WW samples, which follow a similar removal profile as killed controls, active biomass samples have slow sorption kinetics due to high initial concentration. The k value calculated agrees well, 0.012 h^{-1} for live biomass, 0.036 h^{-1} for the killed control and 0.040 h^{-1} for WW. Moreover, the shading effect from the higher biomass concentration could have an impact on the photodegradation rate because less light could penetrate (Mehrabadi et al., 2015; Sutherland et al., 2015).

Kinetic values can be compared with the laboratory scale batch study, Batch-F (Table 6.11) in order to compare the effect of indoor experiment (Batch-L) versus outdoor experiment (Batch-F). Better results were obtained in Batch-F, the fact of conducting the experiment outdoors, improved CPX removal, k values were higher (Table 6.11 and Table 6.12, respectively).

As it has been mentioned, sorption has a great impact on CPX removal from microalgal biomass, TSS during Batch-F assay was 443 mg/L, whereas for

Batch-L it was of 310 mg/L. Thus, apart from environmental conditions that may have enhanced CPX removal, the presence of more biomass in the reactor implies that more sorption could happen, improving the results obtained during Batch-F.

Previous authors reported faster CPX photodegradation than the values reported in the present study, this may be due to the different experimental conditions used, such as a higher initial CPX concentration, a higher working depth, and a lower light intensity. CPX (50 μg/L) was completely photodegraded 16 h after a UV degradation study (Belden et al., 2007) and 90 minutes were required for CPX (30 mg/L) removal under simulated sunlight, the addition of photocatalytic substances reduced the time required for complete CPX removal (Durán-Álvarez et al., 2016).

Based on these results, we hypothesize that conditions occurring during algal-based treatment enabled co-occurrence of CPX photodegradation and adsorption and pseudo-first order kinetics provided the best fit for the removal rates.

Although biodegradation has been shown to be negligible in these batch tests, this does not yet prove that there is no biodegradation in the continuous HRAP. Biodegradation may be discouraged in the batch test due the shock at changing conditions/increased CPX concentrations or due to the lack of established biofilms in the beakers. These results are in accordance with Rühmland et al. (2015) who reported evidences of pharmaceutical biodegradation in facultative ponds, but did not observe biodegradation during in-situ experiments in glass tubes. In other HRAP systems with different species composition or increasing solids retention time by recycling biomass may also enable biodegradation to become significant.

In order to study biodegradation mechanisms during CPX removal from biomass samples, the behaviour of the detected chromatogram peaks, together with CPX peak, along the 4 d experiment has been evaluated. The detected peaks were associated to unidentified transformation products (TP) of CPX (P0 to P7) (P3 corresponds to CPX peak). In this study, the relative peak area

obtained per each possible TP was plotted to evaluate if any of them could be attributed to photodegradation or hydrolysis (Figure 6.18).

Some of the possible TP were initially detected, meaning that the CPX stock solution already contained them. Despite it was stored under dark conditions, some light irradiation could have happened when preparation. These peaks increased along the time under light conditions, whereas under dark conditions were maintained, confirming that their formation was light dependant, also confirmed by lower relative area peaks on active biomass samples due to the shading effect, which prevents higher light irradiation.

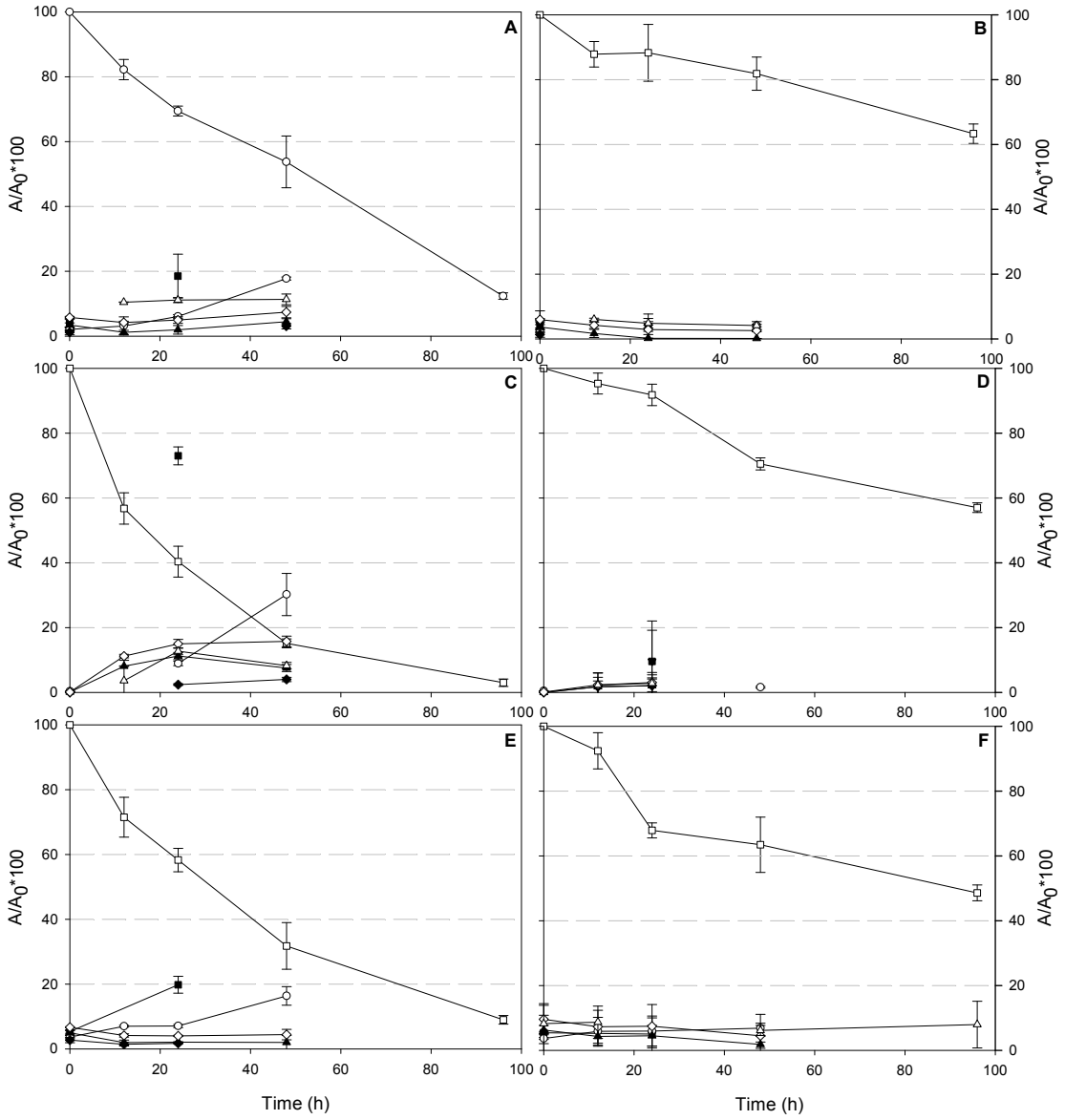


*Figure 6.18 CPX removal and transformation products formation during Batch-L assay. **A**: Active biomass light; **B**: Active biomass dark; **C**: WW light; **D**: WW dark; **E**: killed control light; **F**: killed control dark.* (□) *CPX;* (▼) *P0;* (■) *P1;* (○) *P2;* (♦) *P4;* (▲) *P5;* (◇) *P6;* (△) *P7. P indicates the transformation product peak detected on the chromatogram, they have been numbered from 0 to 7, P3 corresponds to CPX peak.*

Biodegradation could not be ruled out as a possible removal mechanism. Light is necessary for microalgal activity, thus for CPX biodegradation. Since photodegradation, sorption and biodegradation could not be separate in order to evaluate the main removal mechanism taking place, the TPs identification should be carried out and determine if any of the TPs was formed due to biodegradation.

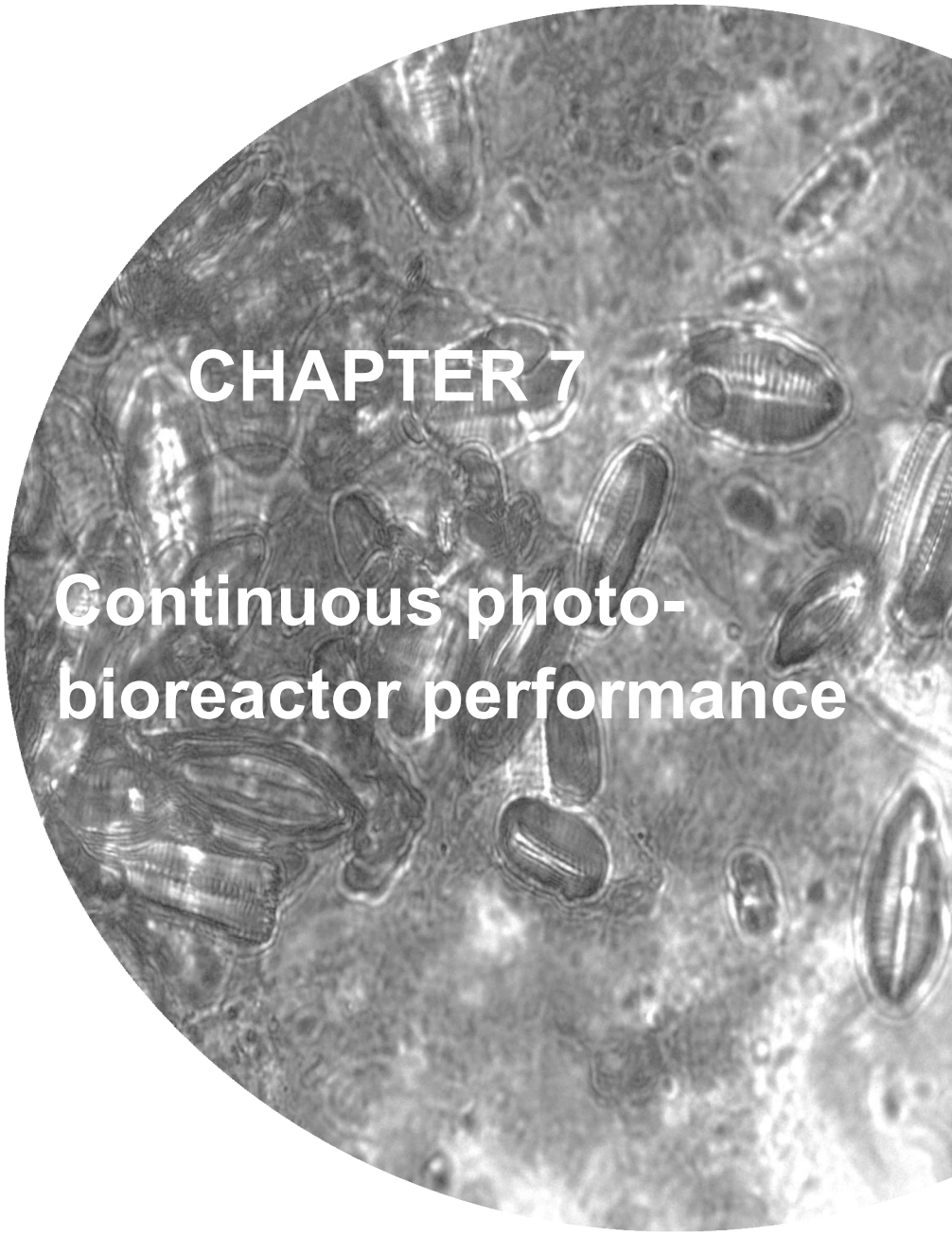
6.4 Conclusions

Laboratory scale reactors performance was in accordance with previous studies. Light and hydraulic retention time have a great impact on the reactors performance. More light irradiated into the system higher the photosynthetic activity is, increasing the TSS concentration. Photosynthetic activity was evidenced by biomass growth and pH increase. Moreover, a decrease on the HRT increases the microalgal productivity. Both systems tested, 1 L photobioreactors and 7 L high rate algal ponds, were able to successfully remove the nutrients from the primary effluent the systems were fed with. The design and operation of a pilot scale HRAP, located at a WWTP were effectively achieved. Good performance of the system was obtained, as well as confirming the results obtained during the laboratory scale experiments.

Laboratory scale reactors were daily fed with the antibiotic ciprofloxacin, the systems achieved good removal percentages, 68%. The addition of CPX did not inhibit the reactors performance. Removal was enhanced decreasing the HRT.

The batch experiments for CPX removal conducted at pilot scale HRAP showed that CPX was sorbed onto the algae surface during the night (18-22%), whereas during the day, photodegradation occurred (15%). Laboratory scale batch assays confirmed those two mechanisms as the main CPX removal mechanisms. However, it should be confirmed at relevant concentrations.

From all the experiments conducted light is necessary for CPX removal, due to the great importance of photodegradation mechanism for its removal. Moreover, sorption has also been described as an important mechanism, whereas, biodegradation may occur but in minor proportion.



CHAPTER 7

Continuous photo-bioreactor performance

7 Continuous photobioreactor performance

Abstract

A 1000 L outdoor pilot scale microalgal photobioreactor (PBR) was used for urban wastewater (WW) treatment and pharmaceutical active compounds (PhACs) removal. During the first Period (September-October) the PBR was operated at an hydraulic retention time (HRT) of 8 days, then during the second Period (October-December) it was increased to 12 days due to low temperatures and lower light irradiance. Variations on the total suspended solids (TSS) concentration were observed along both operating periods due to seasonal changes. Nutrients from the influent WW were efficiently removed attaining the legislation limits. PhACs removal was evaluated at the theoretical hydraulic steady state for both periods. PhACs from several groups were detected (e.g., anti-inflammatories, antidepressants, diuretics, β-blockers or antibiotics) and were efficiently removed. Similar percentages to other biological treatments for urban WW were obtained during microalgal treatment (>80%). The presence of microbial populations has demonstrated the good PBR performance. The Chlorophyta (i.e., Chlorella) as well as the cyanobacterium Phormidium were detected during the whole operation. Different removal pathways have been proposed depending on the PhACs, although during real WW treatment in the PBR all mechanisms (biodegradation, photodegradation and sorption) occur at the same time, improving removal percentages.

7.1 Introduction

Photoautotrophic biological assimilation of wastewater nutrients can be less expensive, more efficient, and ecologically safer than physical/chemical removal processes (Oswald, 2003). Microalgae have been investigated in recent years for wastewater (WW) treatment because of their capacity for nutrient and organic matter removal in symbiosis with heterotrophic bacteria, with a much lower energy requirement compared to conventional activated sludge (CAS) systems which demand mechanical aeration (Ferrero et al., 2012; Park et al., 2011b). The presence of nitrogen and phosphorus from effluents could result in eutrophication if dumped into lakes and rivers and microalgae may perform nitrogen and phosphorus removal, thus reducing nutrient loads to receiving water bodies (Sturm and Lamer, 2011). Furthermore, microalgae require high amounts of nitrogen and phosphorus for protein (45–60% of microalgae dry

weight), nucleotides, nucleic acid and phospholipid synthesis for microorganism growth (Rawat et al., 2011; Riaño et al., 2011).

Wastewater treatment by microalgae removes the costs of obtaining nutrients and water for production (Hultberg et al., 2016). These systems can potentially achieve nutrient, heavy metals and pathogens removal and furnish O_2 to heterotrophic aerobic bacteria to mineralize organic pollutants, using in turn the CO_2 released from bacterial respiration in a less expensive and ecologically safer way with the added benefits of resource recovery and recycling of algal biomass for other products production (high-value products and biofuels) (Hultberg et al., 2016; Mallick, 2002; Mennaa et al., 2015; Muñoz and Guieysse, 2006; Oswald, 2003).

Microalgal-based wastewater treatment has been used in many small communities due to its low cost and efficiency (de la Noüe et al., 1992; Oswald, 1988) since it has been proved that domestic wastewater streams are readily available and cost-effective substrate for microalgal growth for biomass production and nutrient removal (Kong et al., 2010; Wang et al., 2010). Common nitrogen removal methods such as bacterial nitrification/denitrification remove the majority of the nitrogen as N_2 gas, whereas algal treatment retains useful nitrogen compounds in the biomass (Christenson and Sims, 2011).

Successful nutrient removal and sufficient microalgal biomass accumulation require careful growth chamber design. There are a variety of bioreactors that can be used for the dual purpose of removing nutrients from wastewaters with simultaneous biomass growth, each approach has relative advantages and disadvantages, and several reviews have discussed these methods in detail (Carvalho et al., 2006; Chisti, 2007; Muñoz and Guieysse, 2006; Rawat et al., 2011; Shen et al., 2009). Basically the three general types of maturation ponds employed in wastewater treatment are facultative ponds, anaerobic ponds and aerobic ponds (Rawat et al., 2011). Facultative ponds exhibit aerobic conditions on the surface due to photosynthetic oxygen production by algae and anaerobic conditions in the bottom layers and are the most common form of oxidation ponds. Anaerobic ponds are several meters deep, free of dissolved oxygen and have high biological oxygen demand (BOD) removal rates (Horan, 1998). And aerobic ponds are shallow and completely oxygenated throughout (Oswald,

1978). Aerobic algal ponds are the most common for commercial issues and can be classified in two types, open and closed system. Open systems, raceway ponds, are cheap and are also amenable to nutrient removal in domestic wastewater. High rate algal ponds (HRAPs) are the most cost-effective reactors for liquid waste management and capture of solar energy. Enclosed photobioreactors offer higher photosynthetic efficiencies and better control than open systems, unfortunately, closed systems are also more expensive to construct and difficult to operate and scale up (Muñoz and Guieysse, 2006).

Up to now, some studies have demonstrated the feasibility of cultivating microalgae in urban wastewaters to simultaneously achieve nutrient removal and biomass production using different reactors configurations (Ficara et al., 2014; Shen, 2014). Tubular photobioreactors demonstrated to be more productive than raceway reactors, but also more efficient in transforming nitrogen and phosphorous into biomass. In terms of nitrogen depuration, data from the tubular reactors show a depuration efficiency ranging from 63% to 85%, depending on the biomass productivity. Stripping occurs in both systems, but higher losses are detected in the raceway reactor due to its lower biomass productivity. High nitrogen depuration rates have been reported with freshwater microalgae cultivated in anaerobic digestion effluents, ranging from 60 to 90% (Hu et al., 2012; Huo et al., 2012; Morales-Amaral et al., 2015). Ledda et al. (2015) demonstrated the efficiency of tubular photobioreactors (340 L) and raceway reactors (800 L) for the production of *Nannochloropsis gaditana* strain using centrate from the anaerobic digestion of wastewater treatment processes as the only nutrient source. While García et al. (2006) evaluated diurnal nutrient removal variations from pilot scale HRAPs (470 L) treating urban wastewater, concluding that diurnal variations of the contaminant concentrations in HRAPs did not seriously affected their reliability in treating wastewater and good efficiencies were obtained.

Microalgal ponds have also been used for the treatment of other types of wastewater. Ammonium removals up to 80% and soluble phosphorous removal efficiency up to 91% were obtained in 75 L open-pond raceway HRAPs fed with slaughterhouse wastewater (Hernández et al., 2016). On the other hand, dilute

swine manure has been treated using HRAPs (464 L) by de Godos et al. (2009), stable and efficient carbon and nitrogen oxidation performance was reported, and biomass productivities ranging from 21 to 28 g/(m^2·d).

The presence of emerging contaminants (ECs) in the environment has attracted great interest since generalized concern arises about the possible undesirable effects of many of these pollutants in the environment and to living organisms. One of the mainsources is the discharge of effluents from wastewater treatment plants (WWTPs), where their removal is often incompleted (Kümmerer, 2008; Petrovic et al., 2003a, 2002). Some technologies have been studied for its removal including physico-chemical and biological treatments. Physico-chemical treatments include advanced oxidation processes (AOPs), activated carbon adsorption, membrane filtration, among others (Baccar et al., 2012; Fagan et al., 2016; Gimeno et al., 2016; Secondes et al., 2014).

The most popular biological treatment is the use of membrane bioreactors (MBRs) which is already implemented in WWTPs and good results for estrogenic compounds removal have been reported (efficiencies greater than 90%) as well as for antibiotics (Dorival-García et al., 2013; Nguyen et al., 2014). The use of fungi for the removal of ECs contained in WW have also been studied in the last years, obtaining good results (>66% removal) (Badia-Fabregat et al., 2012; Cruz-Morató et al., 2014, 2013a; Rodríguez-Rodríguez et al., 2012). The use of wetlands have also been studied, since they are an alternative cost-effective technology (simplicity of operation and maintenance, low environmental impact, low or no energy cost, low waste production and ability to be integrated into the landscape (Ávila et al., 2014b)). Efficient PhACs removal percentages have been obtained in pilot scale constructed wetlands (CW), PhACs removal percentages above 80% were reported by Ávila et al. (2015). PhACs removal depends on the CW configuration and the PhAC, Ávila et al. (2013) reported that horizontal flow wetlands operated under unsaturated conditions resulted in higher redox conditions and consequently enhanced removal efficiency of various ECs. Moreover, Matamoros et al. (2007b) found a high elimination of oxybenzone (>90%) operating in a vertical flow at low hydraulic loading rate (0.03-0.07 m^3/d). Microalgal-based technologies for ECs removal have not been widely studied and scarce literature is available. The

first work dealing ECs removal in microalgal ponds was published by de Godos et al. (2012) in which the antibiotic tetracycline was removed from a pilot scale HRAP treating synthetic WW and the mechanisms implied, were mainly photodegradation and biosorption. Recently, Matamoros et al. (2015) studied the hydraulic retention time (HRT) and ambient temperature/sunlight irradiation (seasonality) on the removal efficiency of 26 ECs in two HRAP pilot plants fed with real urban wastewater. The results showed that efficiencies depend on the compound, whereas some were completely removed (ibuprofen) others were hardly eliminated from the WW (carbamazepine). Moreover, it was reported that HRT had a great impact on ECs removal depending on the season the HRAPs were operated.

The aim of this study is to treat an urban WW in an outdoor pilot scale photobioreactor (PBR) and to evaluate the PhACs removal.

7.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

7.2.1 Photobioreactor design

The experimental microalgal PBR (Image 7.1) was located on the roof of the Chemical, Biological and Environmental Engineering Department (Universitat Autònoma de Barcelona, Barcelona, Spain). It consists of an enclosed 1200 L multitubular PBR. Two distribution chambers are placed at each end of the tubes, to transfer and distribute the culture evenly between the tubes. The tubes are made of low density polyethylene (PE), they are soft and mouldable, whereas the distribution chambers are made of propylene (PP), giving robustness. The tubes are placed on a PP cuvette filled with tap water in order to avoid rough temperature changes between day and night. A paddle wheel is placed in one of the distribution chambers and it is also made of PP. It gives movement and aeration to the microalgal PBR, by drawing in culture from 4 incoming tubes and raising the culture in the distribution chamber to gravity-

feed the 4 outgoing tubes. The WW inlet is placed in the same distribution chamber as the paddle wheel, together with a ball float level sensor in order to avoid a liquid overflow. The distribution chambers have the option to be covered or uncovered placing a lid over it. The lids are made of translucent plastic.

The distribution chambers have a total working volume of 0.14 m^3 with liquid level of 15 cm. The tubes have a working volume of 0.24 m^3 (230 mm internal diameter x 1 mm thickness x 7.0 m long). The paddle wheel gives a constant velocity of 0.13 m/s.

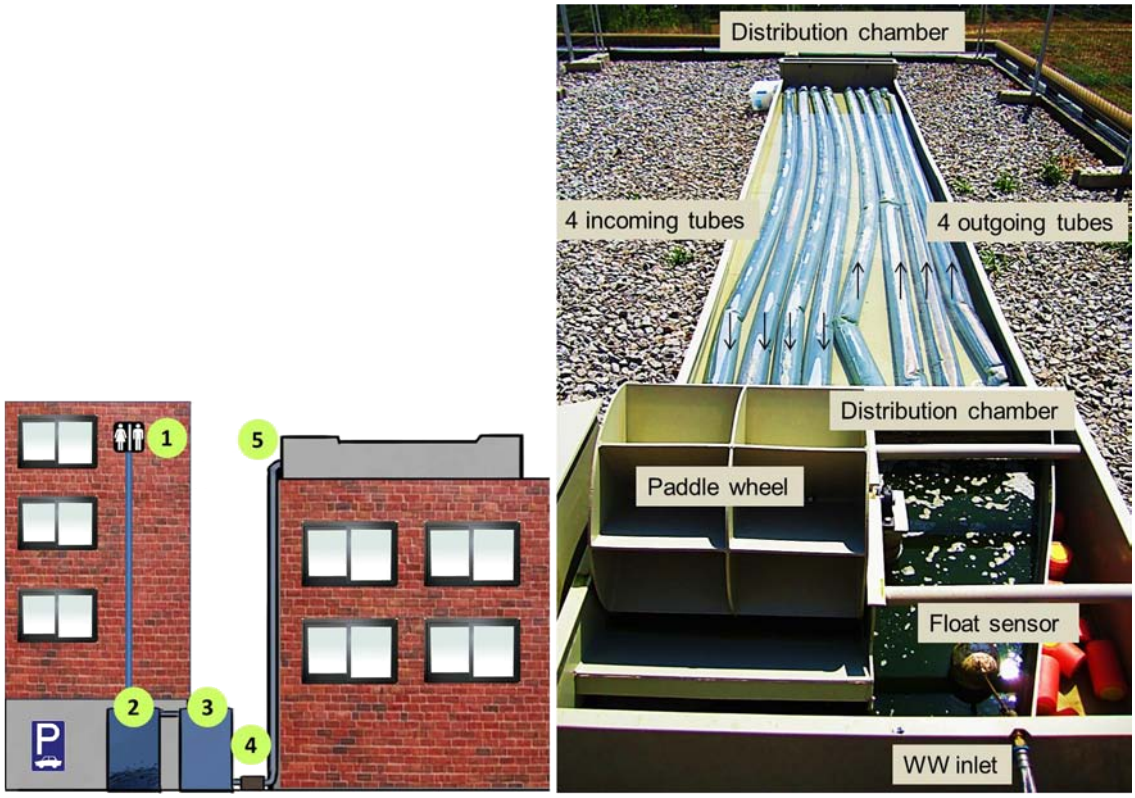


Image 7.1 Photobioreactor (PBR). Left: Schematic PBR located on the roof of the Chemical, Biological and Environmental Engineering Department from Universitat Autònoma de Barcelona, Spain: (1) toilet sewage; (2) 1st settler: (3) 2nd settler; (4) WW pump; (5) PBR. Right: PBR in operation.

7.2.2 Photobioreactor inoculation and operation

The urban WW treated in the PBR comes from the toilets drainage of the Chemical, Biological and Environmental Engineering Department (Universitat Autònoma de Barcelona, Barcelona, Spain). It goes to a first settler and the supernatant is conducted to a second settler, from which the WW is pumped by means of a peristaltic pump to the PBR entering to the distribution box

immediately after the paddle wheel (Image 7.1Left). The settlers have a total volume of 1 m^3 and are made of plastic.

The PBR was inoculated on July 10th (2015) adding 100 L of lake water from Pantà de Can Borrell (Barcelona, Spain) (Image 7.2). The rest of the PBR volume was filled with urban WW. The lake water contained microalgae as microscope observation revealed (Image 7.2).

Microalgae growth period was conducted in batch, it lasted 10 days and then continuous operation together with hydraulic tests were carried out until September 14th (2015).

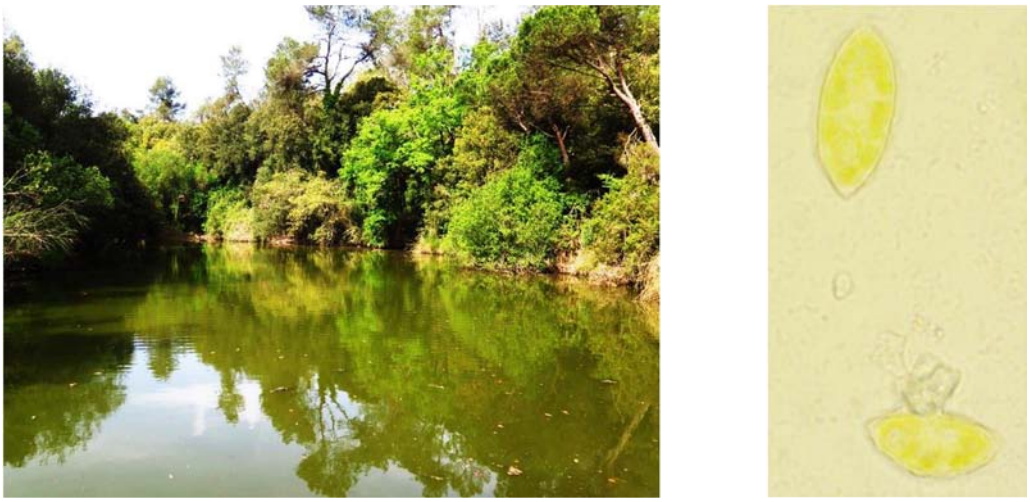


Image 7.2 Left: Pantà de Can Borrell, Barcelona, Spain. Right: Microalgae from the lake water 1000x magnification.

The continuous operation mode started on September 14th (2015) with a hydraulic retention time of 8 days and finished October 16th (2015) (Period I). During Period II the system worked at HRT of 12 days and was conducted from October 20th (2015) until December 20th (2015).

7.3 Results and discussion

7.3.1 General performance of the PBR

The PBR was monitored during the two continuous periods and between periods the PBR was used for other experiments (see Chapter 8). The most important variables (non-controlled) in PBR are: pH, dissolved oxygen (DO),

environmental temperature (Figure 7.1) and solar irradiance (Figure 7.2). Furthermore, the response of the photosynthesis rate to solar irradiance changes depends on other variables, making the microalgae culture process a complex system (Pawlowski et al., 2015).

During period I the TSS in the PBR achieved its maximum, 594 mg/L, but then dropped to the minimum value of 153 mg/L, corresponding on a decrease in the outside temperature (Figure 7.1). The low TSS concentration at the beginning of Period II was slightly increased achieving a maximum value of 513 mg/L. On November 22nd 2015 there was a temperature drop achieving values next to 0ºC which had a negative impact on biomass concentration, decreasing the content of microalgal biomass until the end of Period II, with the lowest value of 137 mg/L. Temperature changes have been previously postulated by other authors as an important factor for microalgae growth. A decrease on microalgal-based systems efficiency due to low temperatures as well as temperature gradients may have a negative effect on PBR performance (Mehlitz, 2009; Park et al., 2011b). Not only outside temperature decreased, also light hours and therefore, solar irradiation (Figure 7.2) was lower, causing more difficulties for the system to take over. Additionally, photo-deficiency is problematic for autotrophic dominated growth of microalgae (Ma et al., 2014).

Posten (2009) stated that the fundamental starting point for the optimization of photo-bioprocesses is a detailed understanding of the interaction between the bioreactor in terms of mass and light transfer as well as the microalgae physiology in terms of light and carbon uptake kinetics and dynamics. Photosynthesis increases with increasing light intensity until the maximum algae growth rate is attained at the light saturation point, however, if there is a lack of light microalgae growth could be negatively affected as happens in this study.

Temperature has as much importance as light intensity for algal productivity. A temperature increase increases algal respiration and photorespiration (Pulz, 2001; Sheehan et al., 1998). However, optimal temperature varies when nutrient or light conditions are limiting, and growth often declines when algae are subjected to a sudden temperature change. Furthermore, the optimal temperature varies between microalgae species, most commonly cultured species of microalgae tolerate temperatures between 16 and 27°C.

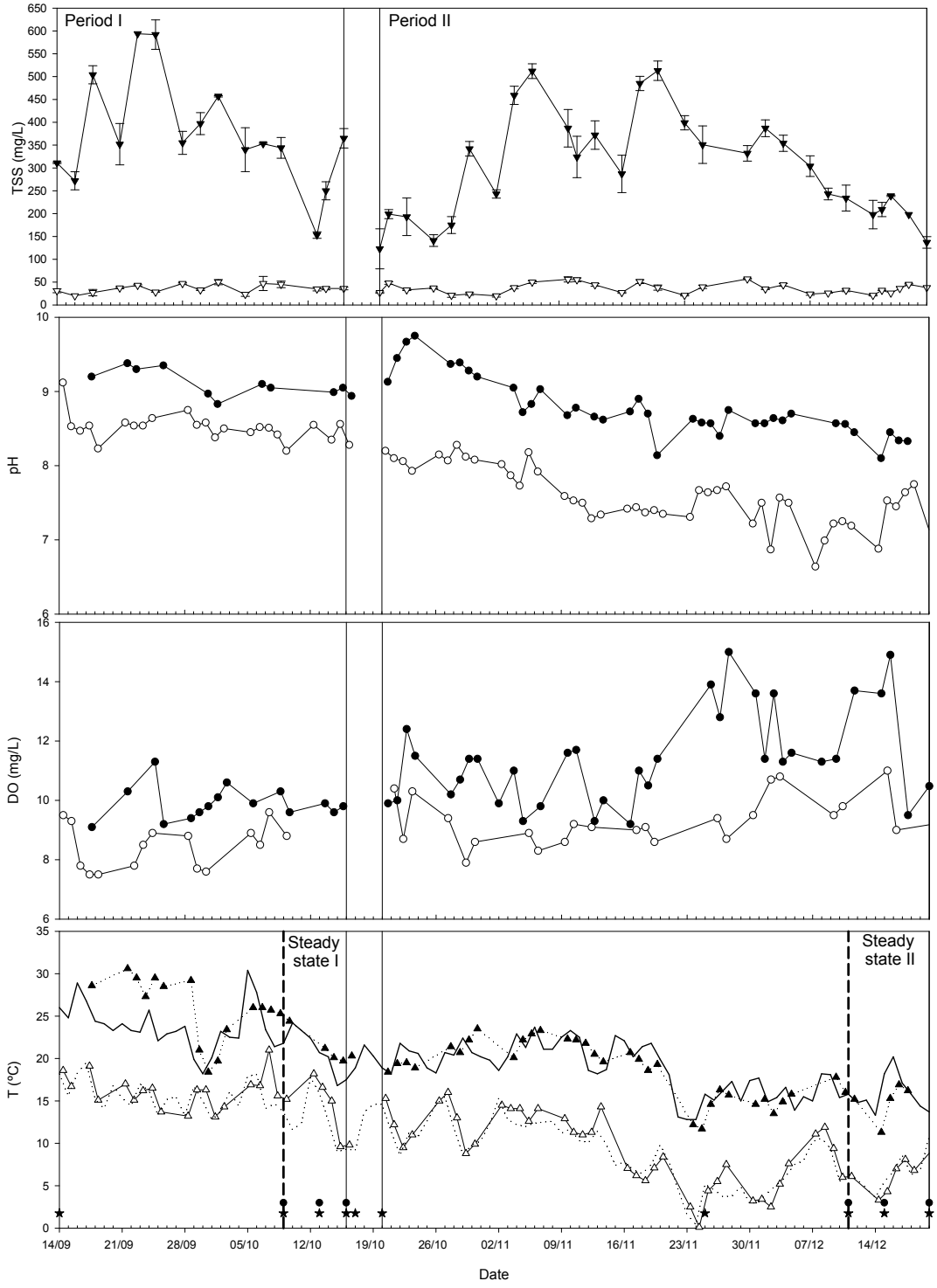


Figure 7.1 PBR performance during the two operating periods (Period I HRT=8 d; Period II HRT=12 d). Top: (▼) *PBR TSS concentration;* (▽) *WW inlet TSS concentration; Middle top:* (●) *morning pH;* (○) *afternoon pH. Middle bottom:* (●) *morning dissolved oxygen;* (○) *afternoon dissolved oxygen. Bottom:* (▲) *PBR afternoon temperature;* (△) *PBR morning temperature;* (⋯) *minimum outside temperature profiles;* (—) *maximum outside temperature profile;* (●) *PhACs analysis sample;* (★) *samples for molecular biology analysis.*

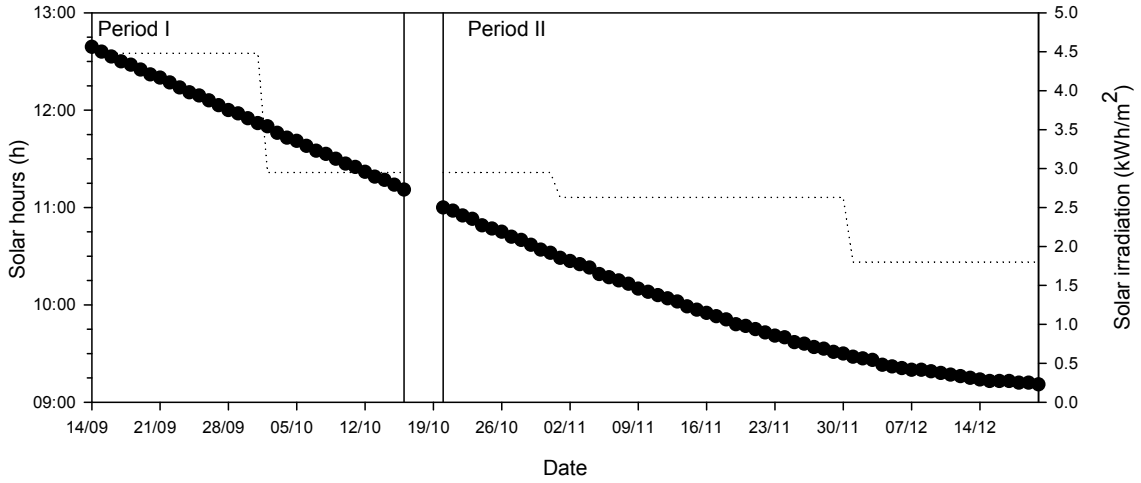


Figure 7.2 Light supply during the two PBR operating periods (Period I HRT=8 d; Period II HRT=12 d). (●) *Solar hours;* (⋯) *average monthly solar irradiation in Barcelona (adapted from AEMET, 2016).*

Temperatures lower than 16°C will slow down growth, whereas those higher than 35 °C are lethal for a number of species (FAO, 2016). Maximum temperatures in the liquor were below the low limit, that fact together with low light intensity decreases the TSS concentration at the end of Period II.

Except for solar irradiance and temperature, pH is the most important variable that influences the photosynthesis rate and affects many of the bio-chemical processes associated with algal growth and metabolism (i.e., nutrient uptake). Pond water pH is in turn a function of algal productivity, algal/bacterial respiration, alkalinity, ionic composition of the culture medium, and autotrophic and heterotrophic microbial activity (e.g., nitrification and denitrification) (García et al., 2000b; Heubeck et al., 2007; Park and Craggs, 2010). During the night the lack of algal photosynthetic activity in conjunction with the continuous respiration of microalgae and other microorganisms resulted in low DO concentration and pH measured in the morning. After sunrise, microalgae photosynthesis activity increases showing higher DO and pH (CO_2 and HCO_3^- consumption) values measured in the afternoon. Afternoon pH was higher than the optimum for most species (usually around 8 (Kong et al., 2010)).The results of the present study indicate that the DO and pH have significant variations during the day and night in the mixed liquor of the PBR (Figure 7.1). This trend has also been previously reported by García et al. (2006) on a HRAP study located outdoors and feed with urban WW, as well as other authors reported in the same type of ponds (El Ouarghi et al., 2000; Picot et al., 1993). The pH and

DO values are highly dynamic (Figure 7.1) since they depend on the photosynthesis rate and need to be kept close to their optimal values. Otherwise, overall microalgae growth will drop, thus reducing biomass productivity and, in extreme cases, lead to microorganism-damaging conditions.

7.3.2 Nutrient removal

Microalgal-based systems can significantly reduce both organic matter and nutrients present in wastewaters at minimal energy cost in simple solar-powered photobioreactors. Figure 7.3 represents the evolution of ammonium nitrogen ($N\text{-}NH_4^+$) and total phosphorus (TPh) concentration during the two PBR continuous operating periods.

Regarding ammonium nitrogen, which is taken into the algal biomass for protein synthesis (Wang et al., 2016). High removal percentages, above 80%, were achieved during periods I and II and a $N\text{-}NH_4^+$ depletion can be observed by the end of period II.

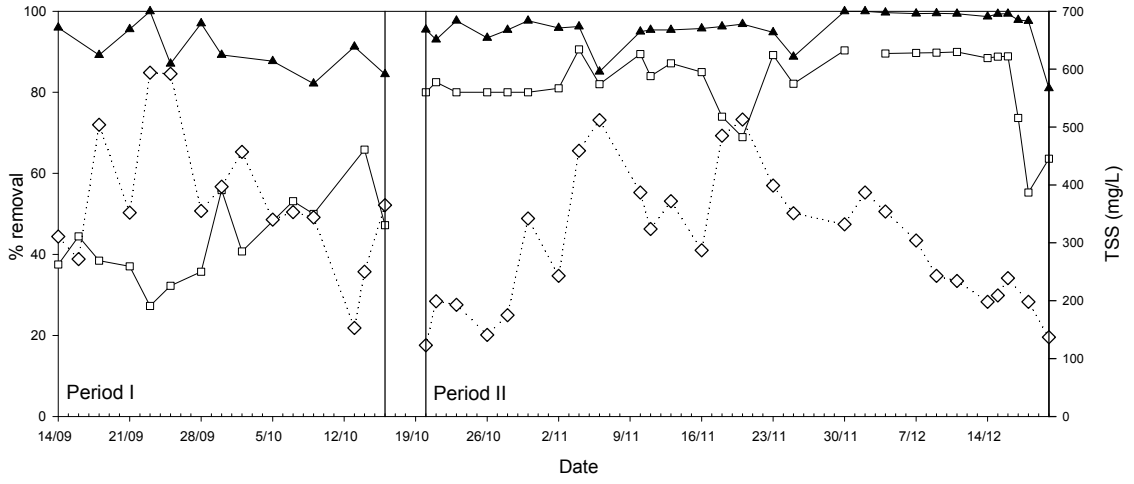


Figure 7.3 Nutrient removal and TSS during the two PBR operating periods (Period I HRT=8 d; Period II HRT=12 d). (◇) *TSS concentration;* (▲) *$N\text{-}NH_4^+$ removal;* (□) *total phosphorous removal.*

Microalgae phosphorus removal mechanisms have been described as a two-step process: may be adsorbed onto the microalgae cell wall and subsequent assimilation (Arbib et al., 2014). Although it has been reported that phosphorus in wastewaters could also be removed by struvite precipitation with other ions such as ammonium and magnesium (Huang et al., 2015). In microalgae metabolism, phosphorus, along with adenosine diphosphate (ADP) and energy

inputs, is generally converted to organic phosphates and generates adenosine triphosphate (ATP) through phosphorylation (Martinez et al., 1999). Total phosphorus removal percentages in the PBR at the beginning of period I were lower than 40%, but then the removal percentage was increased (Figure 7.3). During period II removal values remained around 80%, a sudden drop could be observed at the end of November and may be attributed to the low temperatures attained. However, the system recovered its efficiency the following days of period II, despite the TSS concentration decreased. Data for period II complies with the European commission directive 98/15/EC (80% removal for total phosphorus), however, data from period I is below the removal percentage required. In terms of limit concentration (2 mg P/L), the two periods comply with the directive, meaning that the system could be applied on WW treatment.

Microalgal phosphorus uptake has been reported to be higher than nitrogen; despite nitrogen requirements for microalgae are higher than for phosphorus (Redfield, 1958). This fact has been explained by Elrifi and Turpin (1985) who postulated phosphorus luxury consumption. Nitrogen uptake in the PBR is higher than phosphorus, which means that not all the removal is by means of biological processes. An important part could have been removed by means of abiotic processes (stripping of ammonium to ammonia gas or phosphorus precipitation) as both processes occur at pH values of above 9 (Larsdotter et al., 2007), and the pH during daytime often exceed this value (Figure 7.1).

Several studies have highlighted the potential of microalgae in removing inorganic nitrogen and phosphorus from wastewaters and evaluated the removal percentages in different microalgal systems as well as for different microalgae strains, pure cultures and microalgal/cyanobacteria consortia. Van Den Hende et al. (2014a) studied nutrient removal in a 12 m^3 high rate algal pond. $N\text{-}NH_4^+$ was detected to be removed up to 98%, while total phosphorus removal efficiency ranged between 64 and 82%. These values are similar to the ones presented in Figure 7.3, although the systems configuration and volume were different. Moreover, good nutrient removal percentages (above 99%) were reported by Liu et al. (2016) in an outdoor algal turf scrubber were microalgae where immobilized on a rotating lane and feed with horticultural wastewater.

Chemical oxygen demand (COD) as well as total carbon (TC) have also been studied to evaluate the PBR efficiency. The removal values for these two parameters, together with total organic carbon (TOC) removal are shown in Figure 7.4 and Figure 7.5. COD gives the total pollution load in the form of both organic and inorganic matter; COD inlet values were below the permissible limit (1500 mg/L according to the legislation). COD removal during period I was around 80%. The influent COD presented some variations in time, although these variations were more noticeable during period II, this led to low and strongly varying removal efficiencies. From Figure 7.4 it is observed that on November 10th COD inlet concentration started to decrease, which may be the reason of a lower TSS concentration. Outlet COD values slightly increased during Period II to a maximum of 179 mg/L on December 7th. Both effects decreased the removal efficiency of the system for COD removal, because of the low TSS (304 mg/L). as Wang et al. (2015) reported, microalgal productivity is affected by low COD inlet concentration. Similar COD removal efficiencies as at the end of Period II were reported by Van Den Hende et al. (2014a) (between 25 and 47%) in a 12 m^3 microalgal reactor treating urban WW.

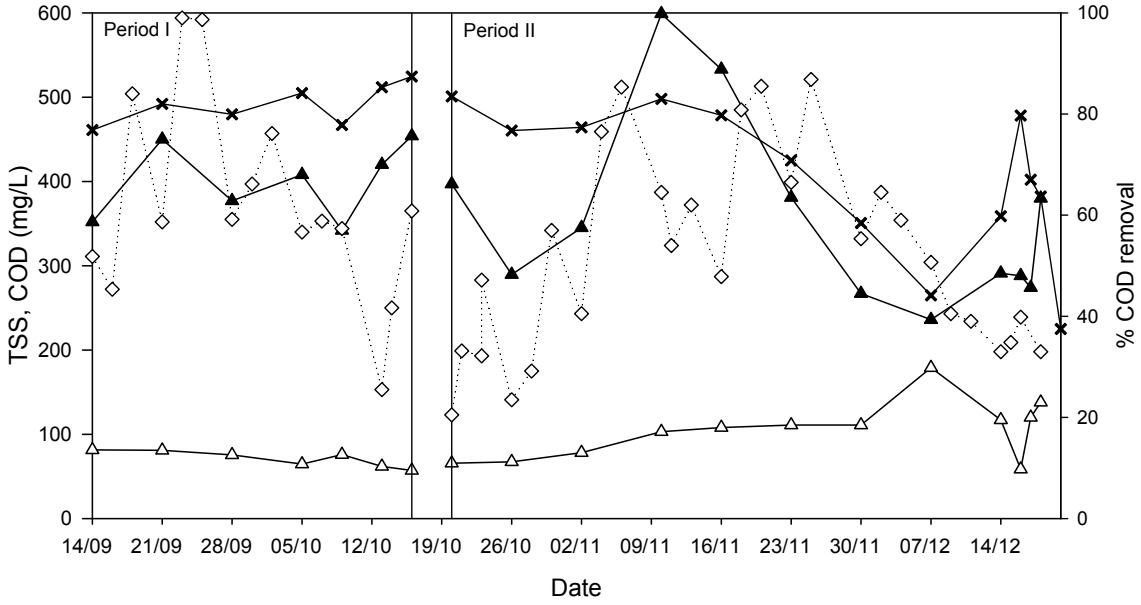


Figure 7.4 COD and TSS during the two PBR operating periods (Period I HRT=8 d; Period II HRT=12 d). (◇) *TSS concentration;* (▲) *inlet COD;* (△) *outlet COD;* (x) *COD removal.*

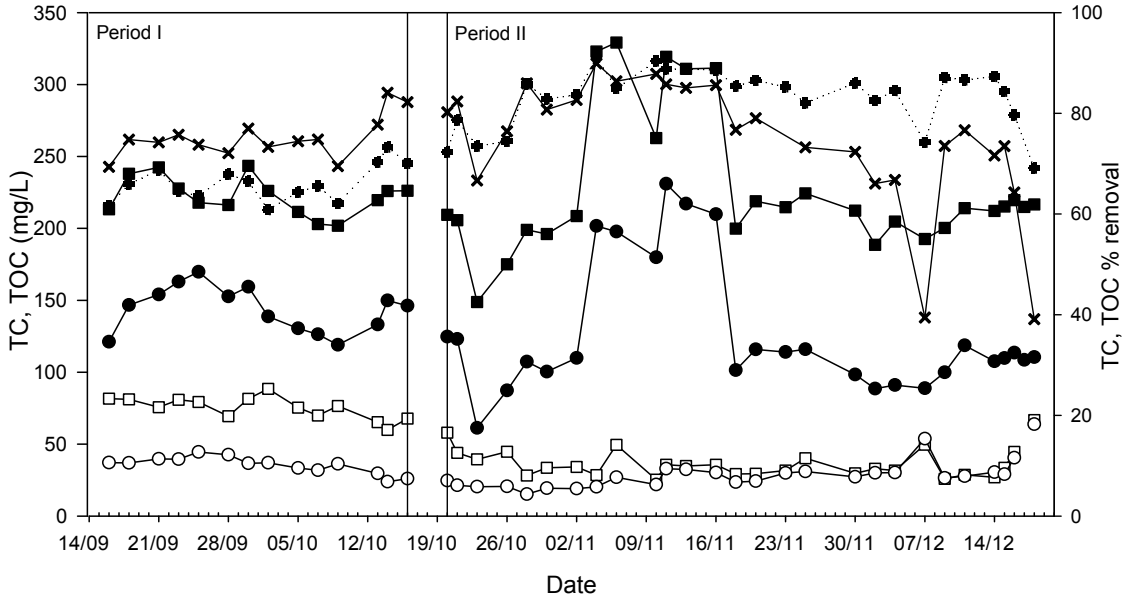


Figure 7.5 TC and TOC removal during the two PBR operating periods (Period I HRT=8 d; Period II HRT=12 d). (■) *inlet TC;* (□) *outlet TC;* (●) *inlet TOC;* (○) *outlet TOC;* (✚) *TC removal;* (x) *TOC removal.*

With regards to the carbon concentration, urban WW is an organic carbon source to support the heterotrophic (use of organic carbon) and mixotrophic (use of organic and inorganic (CO_2) carbon) growth of microalgae (Travieso et al., 2006). In the PBR heterotrophic and mixotrophic growth may occur due to the diversity of the microalgae population. During the night, microalgae could utilize organic carbon only in its heterotrophic mode, while during the day in its mixotrophic mode, obtaining extra energy for carbon assimilation from photons, this could serve as the accelerator for nutrient uptake process (Cerón García et al., 2005; Yan et al., 2012). However, some studies on combining wastewater treatment and microalgae cultivation reported that the COD or TOC concentrations increased, and this is probably due to secretion of extracellular organic matter (EOM) after the nitrogen source was significantly consumed (Farooq et al., 2013; He et al., 2013). In the PBR, TC removal percentages were not negative, indicating that the secretion of EOM was not interfered on TC removal.

Moreover, carbon concentration has a great impact on TSS concentration, greater carbon concentrations are translated into an algal biomass increase. Period I had higher TOC values with some variations in time (Figure 7.5). TOC removal was maintained around 65% and 73%. During Period II the carbon content was decreased from 220 to 200 mg TC/L and from 160 to 100 mg

TOC/L (average values for each period), whereas TC removal increased from 65% to 75%. TOC removals were kept around 85%. The difference around TC and TOC corresponds to the total inorganic carbon (TIC) (these values have not been presented). Some authors attributed that TIC removal was mainly due to carbon scavenging by $CaCO_3$ precipitation (Van Den Hende et al., 2014a).

The treatment of WW with microalgae offers many advantages over conventional treatments because it provides high amounts of inorganic nutrients necessary for algae growth. Wastewater not only contains macronutrients (N and P) but also other essential micronutrients (e.g., Fe, Cu, Mn, Zn). PBR efficiency is affected by the nutrient supply as well as by external variables, such as light and temperature that have a direct effect on the photosynthetic efficiency, thus on the microalgal growth. Furthermore, results from this work, together with other authors' studies, confirm that diversity in algae species (species richness) is positively correlated with a higher uptake rate of certain nutrients due to niche differentiation: different species have slightly different environmental optimum conditions and can complement each other in terms of total productivity (Cardinale, 2011; Ptacnik et al., 2008). So, the use of a combination of species that possess complimentary properties might be more effective than maintaining a monoculture (Nalley et al., 2014).

7.3.3 Pharmaceuticals removal

The eukaryotic algae are capable of biotransforming and biodegrading many organic pollutants present in the environment uptaking such toxic compounds from the water phase (Priya et al., 2014). Considering this statement, the study of ECs removal using microalgal PBR for WW treatment could raise interest.

93 pharmaceutical compounds have been analysed for detection in the WW influent as well as in the PBR effluent. Three samples were taken at hydraulic steady state for the two periods (Figure 7.1bottom). The detected compounds during Period I and II are presented in Table 7.1, together with removal percentages. As can be noted, the reported concentrations of PhACs in the influent reveal significant variations among the different pharmaceutical groups, which are essentially due to population necessities, metabolism (excretion rate), water consumption per person, local common diseases or environmental

persistence, among others. Anti-inflammatory and antibiotic drugs are the most representative. Anti-inflammatory compounds have also been highly detected in urban WW effluents by several authors (Cruz-Morató et al., 2013a; Jelic et al., 2011; Kasprzyk-Hordern et al., 2009; Radjenovic et al., 2009). Matamoros et al. (2015) measured the PhACs concentration from the WW treated in a pilot scale HRAP, 26 compounds were detected and biodegradation and photodegradation were stated as the main removal mechanisms occurring. Four anti-inflammatory were detected, the same as the detected in this study (except for salicylic acid). Acetaminophen and ibuprofen concentrations from this study were higher, although naproxen concentration was lower and ketoprofen concentration was similar (Table 7.1), which is also in accordance with the values reported by Jelic et al. (2011). Ibuprofen concentration was 4 times higher than the values reported by Cruz-Morató et al. (2013a) and acetaminophen 14 times higher, while ketoprofen and salicylic acid concentrations were similar. The antibiotic erythromycin (0.6 µg/L) was also detected by Cruz-Morató et al. (2013a) (0.3 µg/L) and Radjenovic et al. (2009) (0.32-2.7 µg/L). The β-blocker atenolol was detected at higher concentrations than previous studies in WW (Jelic et al., 2011; Radjenovic et al., 2009).

Several compounds detected in the WW treated in the PBR were also detected by Kasprzyk-Hordern et al. (2009), although the concentration values were not in the same range. For example, paracetamol average concentration was 54.3 µg/L (Table 7.1) while, the lowest recorded concentration by Kasprzyk-Hordern et al. (2009b) in WWTP Coslech was the double, 108.3 µg/L.

Occurrence data of PhACs in the PBR influent from Period I and II steady state is summarized in Figure 7.6. Although there are no big differences between periods and PhACs detection, climatic conditions could cause fluctuating micropollutant input, due to higher consumption of specific pharmaceuticals in certain periods (Kolpin et al., 2004).

Table 7.1 Influent (I) and effluent (E) concentrations of the detected PhACs on Period I and II from the PBR and removal percentages. [a] LOQ: limit of quantification; [b] LOD: limit of detection.

	Period I			Period II		
	I (ng/L)	**E (ng/L)**	**% removal**	**I (ng/L)**	**E (ng/L)**	**% removal**
Anti-inflammatory / Analgesic						
Naproxen	2945±368	2646±239	10.2	25043±1726	7701±623	69.2
Ibuprofen	52091±675	786±14	98.5	41450±2431	<LOQ [a]	<LOQ [a]
Acetamino-phen	54294±988	412±26	99.2	54438±4262	<LOD [b]	<LOD [b]
Salicylic acid	1349±738	<LOD [b]	<LOD [b]	368±12	245±30	33.4
Ketoprofen	472±52	301±51	36.2	6729±413	1039±75	84.6
Codeine	33±1	<LOD [b]	<LOD [b]	<LOD [b]	<LOD [b]	<LOD [b]
Antibiotic						
Azithro-mycin	385±481	43±43	88.8	-	-	-
Ciproflo-xacin	2629±142	1377±91	47.6	294±28	<LOD [b]	<LOD [b]
Ofloxacin	65±29	21±5	67.7	5662±651	1882±133	66.8
Erythro-mycin	-	-	-	661±42	100±8	84.9
Diuretics						
Hydrochlo-rothiazide	228±6	127±2	44.3	686±13	107±1	84.4
Furosemide	-	-	-	669±21	<LOD [b]	<LOD [b]
β-blocker						
Atenolol	7795±447	115±9	98.5	6906±448	997±72	85.6
Slow channel blocker						
Diltiazem	1678±189	381±23	77.3	1935±15	530±16	72.6
Antidepressant						
Lorazepam	3696±114	1581±16	57.2	2383±137	1669±64	30.0
Alprazolam	-	-		389±20	49±3	87.4
Paroxetine	-	-		1652±50	103±4	93.8

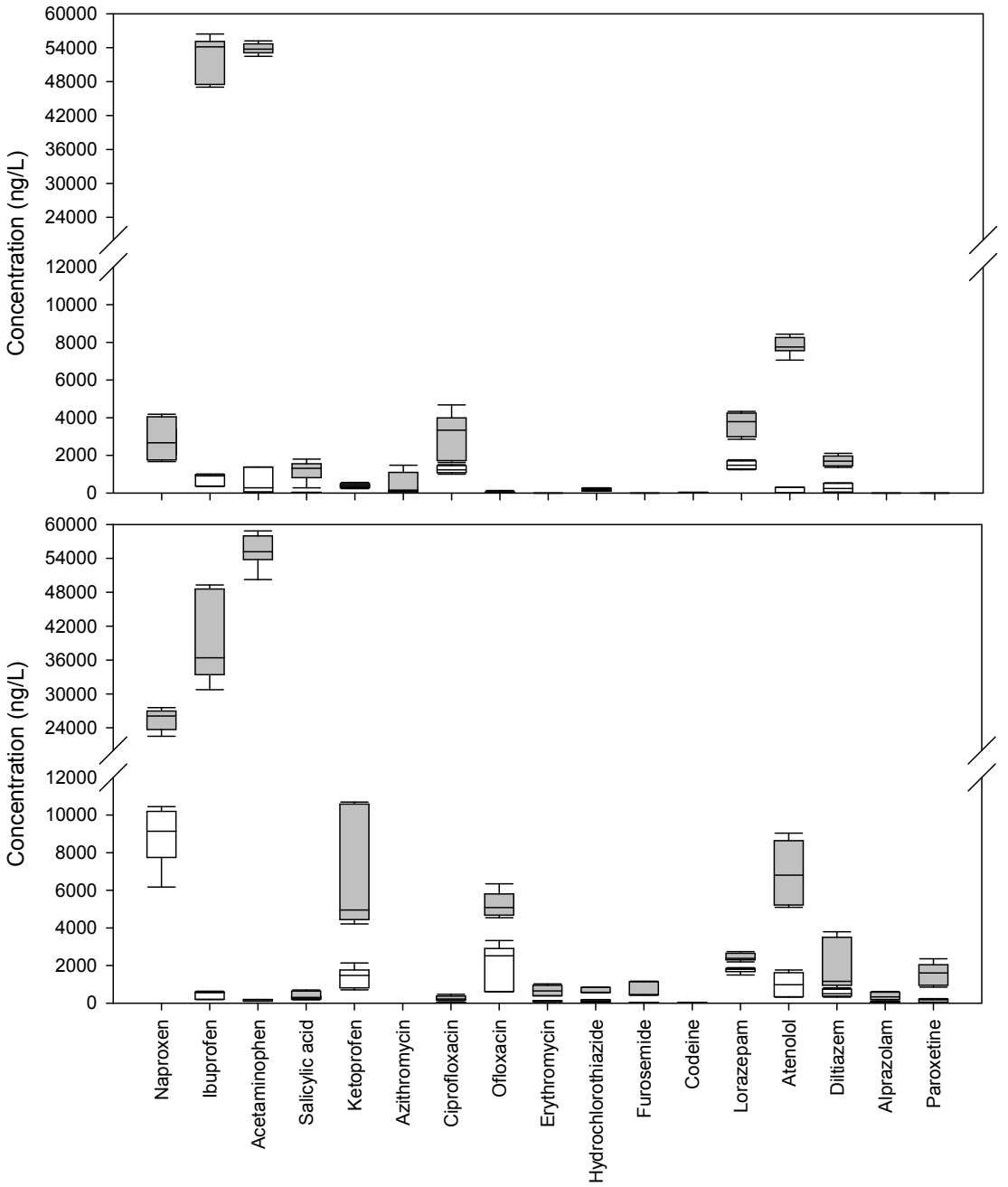


Figure 7.6 Box-plot of the occurrence of emerging contaminants in the inlet wastewater (grey boxes) and PBR effluent (white boxes) from period I (top) and period II (bottom). The box-plots indicate the median, and the 25th and 75th percentile for each compound.

Five anti-inflammatory compounds (acetaminophen, ibuprofen, naproxen, salicylic acid and ketoprofen) have been detected obtaining in the vast majority of cases good removal percentages. Removal mechanisms could not be determined, although some authors reported that the removal of these anti-inflammatory drugs via volatilization or hydrolysis is negligible (Gusseme et al., 2010; Li and Zhang, 2010; Yu et al., 2011). Despite acetaminophen (paracetamol) and ibuprofen have a low excretion rate, they are highly detected,

this is possibly because the low excretion rates are offset by the massive use of these compounds (Luo et al., 2014). Those compounds have been detected in the influent from a pilot scale HRAP, obtaining high removal percentages (>90%) (Matamoros et al., 2015), values that are in accordance with the percentages obtained in the present study (>98%, Table 7.1).

Ibuprofen has been the most abundant compound detected in the influent of four WWTPs in Spain, with concentration levels ranging from 3.73 to 603 μg/L (Santos et al., 2009). Several studies reported that the dominant ibuprofen removal mechanisms in biological systems (membrane bioreactors (Abegglen et al., 2009) and immobilized cell process (Yu et al., 2011)) is biodegradation. Besides, previous authors described that ibuprofen elimination is preferably by aerobic biodegradation during WW treatment due to the low octanol-water partition coefficient, therefore it is not expected to be sorbed onto organic matter (Ávila et al., 2010; Kasprzyk-Hordern et al., 2009). Another possible pathway for ibuprofen removal has been attributed to the presence of photosensitizers, such as dissolved organic matter (Yu-Chen Lin and Reinhard, 2005).

Direct photolysis has been described as an important mechanism for acetaminophen removal (Laurentiis et al., 2014), although other authors have described it as a readily biodegradable compound and has been characterized by significant biodegradability and bio-sorption removal percentages during WW treatment (Joss et al., 2006; Kasprzyk-Hordern et al., 2009; Radjenovic et al., 2009). According to that statement and the removal results from the PBR (Table 7.1), acetaminophen removal via biodegradation may be occurring in the microalgal system.

Ketoprofen inlet concentration has been increased by one order of magnitude between periods. Ketoprofen removal percentages are lower than the ones reported in the pilot scale HRAP by Matamoros et al. (2015), 36% and 50-95%, respectively, when similar concentrations entered the respective systems. Although, these percentages are in accordance with the percentages obtained during the activated sludge processes from WWTPs (Jiang et al., 2013).

Salicylic acid inlet concentration decreases during Period II. Salicylic acid removal from aqueous solutions has received a great deal of attention in recent

years due to its high toxicity and accumulation in the environment. Furthermore, it is a common derivate of phenol and, therefore, it is a typical pollutant in industrial wastewaters (Combarros et al., 2014), so its removal inside the system is of great importance. Escapa et al. (2015) studied the potential of *C. sorokiniana* for its removal in a semicontinuous system, obtaining high removal percentages (93%). On the other hand, Combarros et al. (2014) claimed that the biodegradation of salicylic acid by the microalgae *Pseudomonas putida* was dependent on the initial concentration; 100% efficiency was achieved 8 h after 100 mg/L were spiked, whereas 94.7% removal was obtained 24 h after 500 mg/L were added.

Naproxen exhibited the lowest removal percentage during Period I among all the anti-inflammatories detected, 10%, but increased during Period II (69%). Values from Period II are in accordance with the removal percentages reported by Matamoros et al. (2015) in a pilot scale HRAP treating urban WW (48-89%). Naproxen was described to be removed from WW mainly due to its bio-degradability, sorption was not considered due to the low octanol-water partition coefficient (Kasprzyk-Hordern et al., 2009). On the other hand, Rodarte-Morales et al. (2011) found out that naproxen was degraded inefficiently by mixed cultures of activated sludge.

Analgesics are highly prescribed and it is easily to find them in WW, surprisingly in the PBR influent only codeine was detected among the analgesics drugs, and its concentration was similar as in the urban wastewater treated by Cruz-Morató et al. (2013a) and lower to the WWTP influent reported by Kasprzyk-Hordern et al. (2009). Effluent concentration was below the limit of detection (Table 7.1).

Regarding antibiotics, variations between both periods were observed (Figure 7.6). Azithromycin was only detected in Period I, whereas erythromycin in Period II. Ciprofloxacin and ofloxacin were detected in both periods, however, their initial concentrations were different. The removal percentages obtained ranged from 48% to below the limit of detection (Table 7.1). Antibiotics removal percentages from the aqueous phase in conventional activated sludge processes ranged from 35% to 76%, although in membrane bioreactors removals were improved (25% to 95%) (Radjenovic et al., 2009). Influent concentrations were similar to the ones presented in Table 7.1.

The β-blocker atenolol has been detected at high concentration, 7.8 μg/L during both periods and removal percentages were above 80%. This high concentration is in accordance to the values reported by Kasprzyk-Hordern et al. (2009) in WWTP influents from the United Kingdom. The removal of this compound has been studied by other authors using bacteria. Escolà Casas et al. (2015b) obtained 40% removal in a continuous moving bed biofilm reactor, whereas almost complete removal was determined in a hybrid biofilm and activated sludge system (Escolà Casas et al., 2015a). Biodegradation of atenolol has been previously observed and linked to the activity of ammonia-oxidizing bacteria and heterotrophs (Sathyamoorthy et al., 2013).

A wider variety of antidepressants was detected on Period II rather than Period I and high removal efficiencies were detected. Lorazepam was detected on both Periods, obtaining removals between 30 to 57% (Table 7.1). It has been previously described that this drug could efficiently be removed by means of advanced oxidation processes (AOPs) (Sousa et al., 2012). Jelic et al. (2011) found that lorazepam was biologically degraded by 30% during WW treatment, whereas sorption onto sludge was less than 5%. Alprazolam has been described as a non-oxidizable compound due to the presence of the electron-withdrawing functional groups such as the chloro. Subedi and Kannan (2015) postulated that biodegradation and/or chemical transformation can be the dominant removal mechanism for alprazolam. A similar behavior was postulated for paroxetine removal from sludge (Radjenovic et al., 2009). High paroxetine removal efficiencies were reported in activated sludge systems and membrane bioreactors (Sipma et al., 2010).

Other pharmaceuticals were detected, including diuretics and slow channel blockers. Hydrochlorothiazide (a diuretic drug) was efficiently removed in the PBR (44% during Period I and 84% during Period II, Table 7.1), whereas on other WW treatment systems (i.e., hybrid biofilm-activated sludge process, membrane bioreactor) slow or insignificant degradation was observed (Falas et al., 2013; Kovalova et al., 2012). It has been reported that hydrochlorothiazide has a persistent behaviour (Bertelkamp et al., 2014; Radjenovic et al., 2009). Furosemide was another diuretic detected in the WW inlet and its removal was almost complete (Table 7.1). Reis et al. (2014) described that it was not

degraded by bacteria, whereas Papageorgiou et al. (2016) detected high removal efficiencies during the hole sampling campaign from a Greek WWTP. The β-blocker diltiazem was detected in the inlet concentration, its removal was maintained during both periods (1600-1900 ng/L and 73-77% removal, Table 7.1). Breitholtz et al. (2012) determined concentrations of a set of pharmaceuticals, including diltiazem, in both incoming and outgoing waters in four Swedish free water surface wetlands, obtaining removals from diltiazem varying from 30 to 88%. 13% removal was obtained in a conventional activated sludge wastewater treatment process, no significant sorption was quantified (Blair et al., 2015) and high removal percentages, ranging from 88% to 99%, were reported by Du et al. (2014) in different WWTP (i.e., municipal treatment plant, aerobic treatment plant and septic treatment system coupled with subsurface constructed wetland).

Clear differences appear on the removal efficiencies among periods (Figure 7.6). For some compounds the less influent concentration, the lower removal efficiency, which happens for naproxen, salicylic acid, ketoprofen, hydrochlorothiazide or lorazepam. This may be explained because biodegradation requires a certain compound concentration before microbial degradation can be stimulated and also because higher concentration for long time provokes microorganisms' adaptation (Spain and Van Veld, 1983). Other PhACs achieve the same removal efficiency independently to the initial concentration (i.e., codeine, ofloxacin, ibuprofen or acetaminophen). Finally, the third group in which removal efficiencies differences are observed among initial concentrations, are those that the fewer inlet concentration, the higher removal percentage, this only happens for ciprofloxacin. In general, higher removal efficiencies are obtained during period II, despite the temperature and light irradiation decreased, but it could be a consequence of an HRT increase. Some authors reported that biological wastewater treatment technologies for removing ECs are highly dependent on HRT because it enhances biodegradation, photodegradation and sorption removal processes. HRT is a key design parameter for achieving proper removal efficiency of microcontaminants from microalgal-based treatment systems. In general, the higher the HRT, the greater the EC removal efficiency (Garcia-Rodríguez et al., 2014; Víctor and

Rodríguez, 2016). Nevertheless, Hashimoto et al. (2007), pointed out that it was not observed an statistical relationship with the HRT and the removal of natural estrogens in conventional activated sludge process.

PhACs removal mechanisms from microalgal-based WW treatment systems have not been widely studied, since the complexity of the microbial variations from these systems. Microalgae produce peptides which can bind to the micropollutants, moreover on the surface of their cell walls they contain polysaccharides, proteins, or lipids which in turn contain some functional groups like amino, hydroxyl, carboxyl, and sulfate, which can act as binding sites and further used to sequester many different pollutants through adsorption or an ion-exchange process (Priya et al., 2014; Yu et al., 1999). Biodegradation of organic compounds by microalgae is the result of facultative chemoautotrophy and can be directly biodegraded (Priya et al., 2014; Semple et al., 1999).

7.3.4 Microbial diversity

Efforts have been focused on the study of microalgae growth and few studies have been devoted to the identification of microalgal populations in open ponds (Cho et al., 2015; Ferrero et al., 2012; García et al., 2000b). During the PBR operation (Period I and II) several samples were taken (Figure 7.1bottom) in order to evaluate the microbial diversity and their changes when PBR operation mode was changed (from HRT 8 d to 12 d) (Table 7.2). A sample from the inoculum (Lake) was collected, then when the PBR started the operation at HRT of 8 days another sample was taken (t0), three more samples were collected during the steady state of Period I (t1 to t3). The HRT was increased to 12 d on period II and a first sample was collected (t5). During two consecutive nights in Period II temperatures dropped below 0ºC and part of the PBR was frozen (t6). When the steady state was accomplished for Period II, 3 samples were taken (t7 to t9).

Table 7.2 Samples taken for microbial diversity analysis.

	Sample	HRT (days)	Month	Operation
	Lake	-	Sept.	Inoculum
Period I	t0	8	Oct.	Start HRT 8d
	t1	8	Oct.	Steady state
	t2	8	Oct.	Steady state
	t3	8	Oct.	Steady state
Period II	t5	12	Nov.	Start HRT 12 d
	t6	12	Nov.	After freeze
	t7	12	Dec.	Steady state
	t8	12	Dec.	Steady state
	t9	12	Dec.	Steady state

In Figure 7.7 denaturing gradient gel electrophoresis (DGGE) fingerprinting cluster analysis are shown. Two first-order clusters were clearly distinguished corresponding to both periods of PBR operation, 8 days and 12 days (samples t0 to t3 and t5 to t9, respectively). These differences may be related to weather condition changes as well as a change of the HRT and also due to the effect that at the end of Period I 17β-estradiol (E2) (2 mg/L) was spiked into the system for another experiment (Chapter 8).

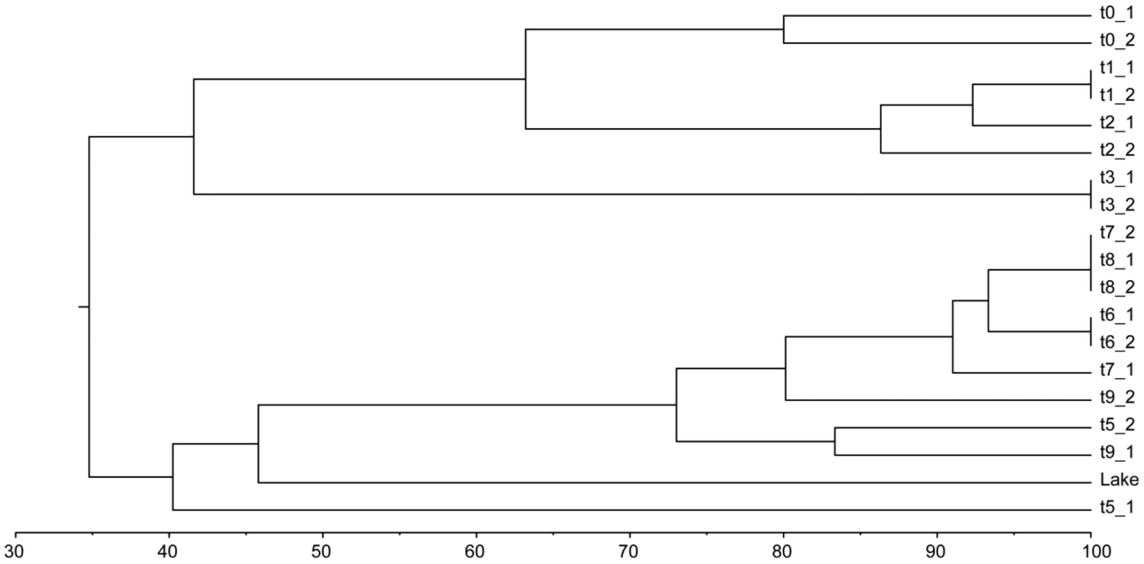


Figure 7.7 Cluster dendrogram based on UPGMA algorithm (no area sensitive) showing similarity among DGGE band patterns of 18S rRNA gene. X-axis indicates the percentage of similarity.

Overall, similarity level among samples was 35% (Figure 7.7). Period I has a similarity score of 42%, while t1 and t2 have a similarity score of 86%, the third sample of the steady state (t3) decreases the similarity of Period I. Differences among the replicates could be due to the heterogeneity of the samples, microorganisms were not equally distributed and when the polymerase chain reaction (PCR) was conducted a small change between communities was rapidly exposed since the PCR amplification is exponential. Although this bias, different duplicates are still close to each other, except for t5_1. From Figure 7.7 it is observed that some differences in the microbial diversity appear between periods (t3 and t5). One of the reasons could be attributed to the batch experiment conducted between them (Chapter 8), as well as seasonal changes.

A total of 12 representative eukaryote bands from the DGGE were excised and sequenced, obtaining 98.2 % coverage of the community when associated with the quantitative DGGE band matrix (Figure 7.8 Left). The sequences represented a total of 5 Eukaryota phyla (*Chlorophyta, Rotifera, Blastocladiomycota, Ciliophora* and *Cercozoa*) and two unidentified cultures (Table 7.3). *Chlorella* (band A)*, Scenedesmus* (band B)*, Pseudospongiococcum* (band C)*, Brachionus* (band D)*, Paraphysoderma* (band E)*, Vorticellides* (band F) and *Rhogostoma* (band G) were identified as the dominant genera in the PBR. For cyanobacteria, 5 representative bands were excised and sequenced (Figure 7.8 Right). Also, from the phylum of cyanobacteria two genus were identified, *Phormidium* (band J) and *Leptolyngbya* (band K).

Chlorophyta and cyanobacteria were detected during the whole PBR operation; while the others were not always present (Figure 7.8). Chlorophyta are photosynthetic organisms commonly known as green algae. The organisms are largely aquatic or marine. The various species can be unicellular, multicellular, cenocytic, or colonial. *Chlorella* (band A) was present during the whole PBR performance. This genus was also detected by previous authors in open algal ponds in which the microorganisms population analysis was carried out (Cho et al., 2015; Ferrero et al., 2012; Jahan et al., 2010). Many studies inoculated algal ponds with different strains of *Chlorella*, due to the advantages of this genus, as well as laboratory scale experiments for WW treatment (Aslan and Kapdan, 2006; de Godos et al., 2012). For example, *Chlorella sorokiniana* was found to

have a good growth and very good potential for wastewater treatment due to the capacity to remove nutrients efficiently under aerobic dark heterotrophic conditions (Ogbonna et al., 2000). This is in accordance with the removal results obtained in the PBR and presented in section 7.3.2.

Apart from *Chlorella* (band A), *Scenedesmus* and *Pseudospongiococcum* (band B and C, respectively) are detected during great part of the PBR operation (Figure 7.8). The green alga *Scenedesmus* is highly present in open algal ponds, including WW treatment (Park et al., 2011b; Pittman et al., 2011; Ruiz-Marin et al., 2010; Tam and Wong, 1989).

Scenedesmus (band B) was detected in the inoculum and at the end of Period I (t3), but not in t1. Band F corresponds to the Ciliophora phylum and with 100% similarity to the genus *Vorticellides*. Ciliophora is an important group of protists, common almost everywhere there is water: in lakes, ponds, oceans, rivers and soils. They are heterotrophs, feeding on smaller organisms, such as bacteria and algae (Adl et al., 2007). Thus, the presence of this microorganism may control algae and bacterial populations by predation.

During Period I, band E and I are detected just when *Vorticellides* band intensity decreased or disappeared (t3). Band E corresponds to the Blastocladiomycota phylum, from the Fungi kingdom, the closest cultured genus was *Paraphysoderma*. It has been found in some microalgal cultures as a parasite, it is a parasitic chytrid that attacks green algae. Hoffman et al. (2008) found a genus from the same phylum (Blastocladiomycota) in cultures of the green microalga *Haematococcus pluvialis.* The parasite can grow epibiotically on algae cells and cause damage to the host cultures. Moreover, the same genus (*Paraphysoderma*) was found to affect *H. pluvialis* cultures by Gutman et al. (2009), although the parasite did not affect *Chlorella* culture. Thus, the presence of this parasite fungus in the PBR has no consequences. Band I has been classified as eukaryotic, with a high percentage of similarity, despite not have been previously cultivated.

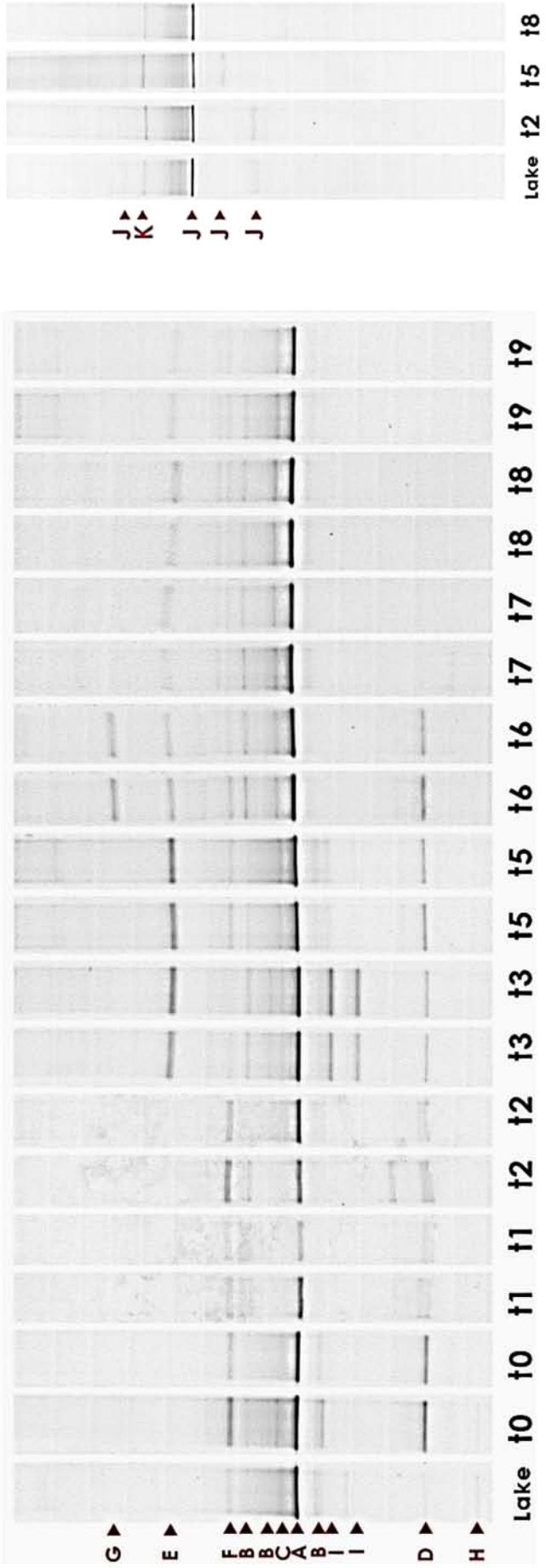


Figure 7.8 DGGE profiles of 18S rRNA fragments (left) and 16S rRNA fragments (right). DGGE profiles are from the PBR Period I (HRT=8 d, samples t0 to t3) and Period II (HRT=12 d, samples t5 to t9) operation. Bottom lane numbers refer to the samples names. The sequenced DGGE bands are indicated on the left side.

Table 7.3 Phylogenic affiliation, accession numbers of the closest relatives and sequence similarity of the DGGE bands.

Excised DGGE bands	Accession number	Closest relative (NCBI database)	Phylum	% Similarity
A	KM985375	*Chlorella* sp. QUCCCM3 18S ribosomal RNA gene, partial sequence.	Chlorophyta	100%
B	KT279469	*Scenedesmus* sp. YACCYB49 18S ribosomal RNA gene, partial sequence.	Chlorophyta	100%
C	KU057947	*Pseudospongiococcum protococcoides* strain CALU - 221 18S ribosomal RNA gene, partial sequence.	Chlorophyta	100%
D	KT729747	*Brachionus calyciflorus* isolate 82_2.1 18S ribosomal RNA gene, partial sequence.	Lophotrochozoa	100%
E	KJ563218	*Paraphysoderma sedebokerense* isolate FD61 18S ribosomal RNA gene, partial sequence.	Blastocladio-mycota	100%
F	JQ723993	*Vorticellides* sp. 2 MD-2012 small subunit ribosomal RNA gene, partial sequence.	Ciliophora	100%
G	LC032468	*Rhogostoma minus* gene for 18S ribosomal RNA, partial sequence, isolate: ZER3.	Cercozoa	99%
H	KT252432	Uncultured eukaryote clone OTU_2 18S ribosomal RNA gene, partial sequence.	Eukarya	94%
I	JF775023	Uncultured eukaryote clone RRW-I-37C-3C 18S ribosomal RNA gene, partial sequence.	Eukarya	99%
J	AB183566	*Phormidium* sp. MBIC10025 gene for 16S rRNA, partial sequence, strain: MBIC10025.	Cyanobacteria	99%
K	LN997861	*Leptolyngbya* sp. KSU-AQIQ-12 partial 16S rRNA gene.	Cyanobacteria	100%

On the other hand, the Chlorophyta *Pseudospongiococcum* which was not detected during Period I, it has been detected during the whole Period II. Between the end of Period I (t3) and the start of Period II (t5) some band changes have been observed in DGGE profile (Figure 7.8).

Regarding similarities and DGGE bands analysis for Period II operation it could be observed that only 41% of similarity (Figure 7.7) was found at the beginning of HRT 12 days operation (t5_1) and at the steady state (t7 to t9), due to the disappearance of *Brachionus calyciflorus*, from the Lophotrochozoa phylum, at the end of the Period (band D, Figure 7.8), may be due to the cold weather during that period. *Brachionus* is a genus of planktonic rotifers occurring in freshwater, alkaline and brackish water. Rico-Martinez and Dodson (1992) published that the optimal temperature for its growth was 30 ºC, far from the temperatures of that period, that were ranging between 0 and 15 ºC. Moreover, the same authors (Rico-Martinez and Dodson, 1992) confirmed that its growth was possible in the presence of *Chlorella vulgaris* cells. During the whole PBR operation *Chlorella* genus was present (Figure 7.8), thus the presence of this rotifer is observed. Besides, Groeneweg and Schlüter (1981) cultivated a rotifer of the same genus in the effluent of a HRAP which was fed with piggery manure.

DGGE band profile (Figure 7.8) shows that band G only appears in Period II steady state and sample t6, which corresponds to two consecutive freezing nights. The closest relative microorganism belongs to the Cercozoa phylum and the cultured genus is *Rhogostoma*, an amoeboid protozoa. It was detected on previous studies in which protozoan biomass distribution in a biologically productive pond was assessed (Finlay et al., 1988). In general, during Period II, the microalgal diversity distribution is maintained, without noticing major differences.

About the cyanobacteria, both genus (*Phormidium* and *Leptolyngbya*) were detected during the whole operation (Figure 7.8), as well as in the inoculum lake water. *Phormidium* genus has the advantage of the self-aggregation capacity, corresponding to the high presence of flocs during the performance. Besides, this cyanobacteria has been selected for the treatment of secondary effluents from municipal wastewater and for the treatment of fish farm effluents, moreover it has a rapid settling velocity which presents an advantage for microalgae harvesting (Duma et al., 1998; Talbot and de la Noüe, 1993). *Phormidium* has been detected in environments differing from a wide range of temperatures, which is advantageous in an open algal system exposed to

constant temperature changes, as the case in study. It has been reported to be capable to remove nutrients more efficiently than a community of green algae below 10 ºC, moreover, it was isolated from polar environments (Tang et al., 1997). On the other hand, Talbot and de la Noüe (1993) reported that *Phormidium bohneri* was a good candidate for treating wastewater at high temperatures (around 30 ºC). Furthermore, it has been found in pilot scale (Cromar and Fallowfield, 1997) and in a real eutrophic pond in Bangladesh (Jahan et al., 2010).

7.4 Conclusions

It has been observed that PBR performance is highly impacted by temperature and solar irradiation. Low temperatures and scarce light hours decrease the TSS concentration, which is directly related with productivity as well as for nutrient removal.

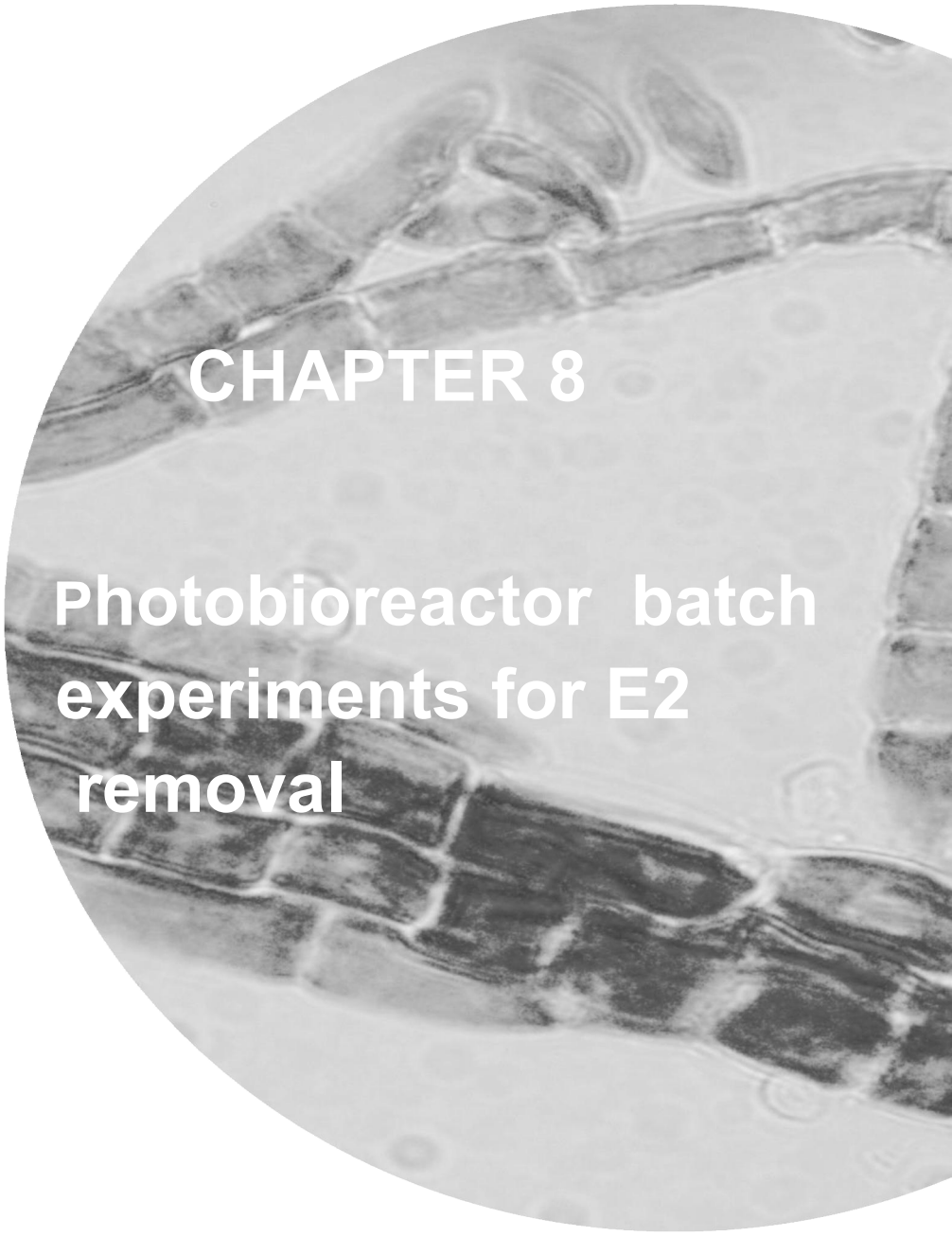
Increasing the HRT during the adverse conditions (low temperature, short light periods) for microalgal growth, maintains the PBR operation. PBR performance was successful.

High nutrient removal percentages are achieved in both periods. N-NH_4^+, total phosphorous and COD removal percentages are >80% and discharged concentrations are below the legislation limits.

From the 93 PhACs analysed for detection in the WW inlet, anti-inflammatory drugs have been the most representative, obtaining removals above 98%. Biodegradation is tentatively proposed as the main removal mechanisms for ibuprofen. In both periods, atenolol and diltiazem have been detected at the same concentration as well as their removal percentage has been similar (>86% and >73%, respectively). On the other hand, antibiotic concentration and compounds have been different between both periods, and removal were >48%. Lorazepam presented lower removal percentages (30-57%), although sorption did not take place. The persistent diuretic hydrochlorothiazide has been efficiently removed during Period II (84%). Also, almost complete removal has been detected for furosemide.

Hydraulic retention time seems to have an effect on PhACs removal, in general, the higher it is (Period II), better PhACs removals.

Chlorella (Chlorophyta) and *Phormidium* (Cyanobacteria) have been detected during the whole PBR operation, since their capacity for nutrient removal. Moreover, *Phormidium* is a self-aggregating specie which enhances microalgae harvesting.



CHAPTER 8

Photobioreactor batch experiments for E2 removal

8 Photobioreactor batch experiments for E2 removal

Abstract

The detection of the natural hormone 17β-estradiol (E2) in waters and the risk for ecosystems they possess has raised interest for its removal assessment using a pilot scale microalgal photobioreactor (PBR). E2 spiking batch experiments were conducted in a pilot scale PBR treating urban wastewater under environmental conditions, E2 concentration was monitored. In parallel, batch experiments at laboratory scale were performed under controlled conditions in order to evaluate the removal mechanisms occurring. Good removals (>70%) were obtained in both systems, PBR and laboratory scale systems. Bioaugmentation using a real microalgal concentrate effluent, mainly composed by Scenedesmus, was conducted at laboratory scale. The higher total suspended solids contained, faster E2 removal, although higher removal yields were obtained for the non-bioaugmented samples.

8.1 Introduction

Wastewater treatment plants (WWTPs) receive natural and synthetic endocrine disrupters compounds (EDCs) from urban and industrial dischargers (Snyder et al., 1999). WWTPs use a variety of treatment processes of varying efficiency such that in some cases compounds are not completely removed by the treatment processes and are ultimately discharged into surface waters, in form of complex mixture of molecules including the partially eliminated wastewater molecules but also metabolites formed during treatment processes, the amount released is sufficient to induce harmful effects on fish and other biota (Auriol et al., 2006; Cargouët et al., 2004). The principal sinks for EDCs are groundwater, river, and lakes (Auriol et al., 2006). In general, natural (estrone (E1), 17β-estradiol (E2), estriol) and synthetic (17α-ethinylestradiol (EE2), mestranol) hormones are the major contributors to the estrogenic activity observed in sewage effluents (Aerni et al., 2004; Metcalfe et al., 2013; Rodgers-Gray et al., 2001).

WWTPs have been designed for the removal of pathogens, phosphorus and nitrogen, with no particular consideration of organic micropollutants such as estrogens. The removal of natural and synthetic estrogens is incomplete in the

activated sludge process and such substances have been regularly detected in WWTP effluent globally (Bolong et al., 2009; Jarošová et al., 2014b). While various physical/chemical technologies (e.g., membrane separation, advanced oxidation, and carbon adsorption) show some promise for removing ECs during wastewater treatment, some drawbacks arose, including added costs associated with plant modification, energy/chemical consumption, and disposal of concentrated waste streams (Shreve et al., 2016). Moreover, not all physico-chemical techniques are able to remove EDCs compounds, coagulation-flocculation processes have been reported to be inefficient (Petrovic et al., 2003b; Westerhoff et al., 2005). On the other side, biological systems have been considered, due to their environmentally friendly characteristics as well as the low cost required, although these advantages, conventional biological treatments remove only a portion of the different types of EDCs, and the removed compounds are mainly polar ones (Petrovic et al., 2003a). Some advanced biological treatment techniques, such as membrane bioreactor (MBR), have been shown to successfully reduce estrogenicity of effluents (Maletz et al., 2013). According to Mastrup et al. (2001), less than 10% of natural and synthetic estrogens are removed via biodegradation process, and although a considerable amount is adsorbed to the sludge, most of the compounds remain soluble in the effluent. Biological trickling filters were found incapable to remove estrogens due to their low hydraulic retention time (HRT) properties (Servos et al., 2005; Ternes et al., 1999). The use of other biological systems have received a great deal of attention in recent years, for example the white-rot fungi due to their ability to transform a variety of recalcitrant ECs in bench scale experiments via their very powerful and non-specific extracellular oxidative enzymes (Blánquez and Guieysse, 2008; Cabana et al., 2007; Yang et al., 2013). Other studies include the use of microalgae for emerging contaminants removal (de Godos et al., 2012; Garcia-Rodríguez et al., 2014).

The natural hormones, E2 and E1, have been detected in several WWTP effluents, since they are excreted by women and consequently found in wastewater, typically, E1 is the most frequently detected estrogen in municipal wastewater effluents (Bolong et al., 2009; Cargouët et al., 2004; Liu et al., 2009; Snyder et al., 1999; Välitalo et al., 2016). In addition, E1 can be formed during

the treatment process because it is an oxidation product of E2 (Salvador et al., 2007).

E2 biodegradation is considered as a favourable and major mechanism for estrogen removal during WWTP processes. This hypothesis was strongly supported by a study showing that more than 70% of ^{14}C-labeled E2 was converted into ^{14}C-labeled CO_2 by nitrifying activated sludge in 24 h (Layton et al., 2000). Biological E2 removal from urban WW has been studied by several authors, including the use of enzymes, fungus, bacteria, microalgae as well as microorganisms consortia (Yu et al., 2013). Cardinal-Watkins and Nicell (2011) carried out an enzymatic study using immobilized fungal laccase from *Trametes versicolor* in a bench scale continuous-flow packed bed reactor. Good removal was achieved, despite T and pH were highly dependent on the efficiency. Lloret et al. (2011) also evaluated the use of laccase for E2 removal, 98% removal was obtained using a batch stirred tank reactor operated in several cycles, whereas 75% was obtained on a packed-bed reactor where laccase was immobilised in a sol–gel matrix. The use of *T. versicolor* for E2 removal in batch and continuous cultures was previously studied by Blánquez and Guieysse (2008), which was removed by more than 97% in 24 h. Previous studies showed that E2 could be cometabolically degraded by heterotrophic bacteria (Pauwels et al., 2008; Yu et al., 2007) and by ammonia-oxidizing bacteria and nitrifying activated sludge (Skotnicka-Pitak et al., 2008; Yi and Harper Jr., 2007). *Novosphin gobium* sp. strain JEM-1 is a wastewater bacterial isolate which can degrade E1, E2, and EE2 (Hashimoto et al., 2010). E2 removal in an algal treatment system fed with source separated wastewater streams was studied with the microalgae *Chlorella sorokiniana* by de Wilt et al. (2016), obtaining high percentages (60-100%) for some pharmaceutical active compounds (PhACs) (diclofenac, ibuprofen, paracetamol and metoprolol).

In previous chapters (Chapter 4) it has been proved the capacity of pure microalgae strains for E2 degradation. Transformation products (TPs) have been also identified. The objective of this work is to assess the efficiency of a pilot scale microalgal reactor containing non-selected microalgae for E2 removal spiked in real WW. Batch laboratory scale tests were performed in parallel in order to determine E2 removal mechanisms. The experiment was

performed during 3 different periods in order to evaluate the differences between seasons and operation modes. Also, bioaugmentation from a microalgae concentrate effluent has been assessed.

8.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

8.2.1 E2 removal experiments in a pilot plant PBR

Field experiments were conducted in the photobioreactor (PBR) previously described in Chapter 7. During the experiment the feed supply was stopped and immediately E2 was added to an initial concentration of 2 mg/L (no effluent withdrawal) and during 24 h its concentration was monitored.

The first E2 spiking experiment (PBR-A) was carried out when the system reached the steady state operating at an HRT of 8 days (Period I). E2 was spiked after the sunset (October 16th 2015, 21:00). The second E2 spiking experiment (PBR-B) was conducted during the steady state of the PBR at an HRT of 12 days (Period II). The spiking was started after the sunrise (December 20th 2015, 8:15). Finally, for the third E2 spiking experiment (PBR-C) the experimental conditions were the same as PBR-B (Period III), the HRT was not modified and the spiking was done after the sunrise (February 18th 2016, 7:30). Between PBR-B experiment and PBR-C the PBR performance was monitored and results will also be presented. Table 8.1 presents the initial characteristics for each PBR experiment and in Table 8.2 it is specified the time when samples were taken. Figure 8.1 shows when samples were taken for microbial diversity analysis, together with minimum and maximum temperature profile during the whole PBR operation.

Table 8.1 Field experiments characteristics.

Assay	E2 spiking	Season	T_{max} (ºC)	T_{min} (ºC)	TSS_{algae} (mg/L)	TSS_{ww} (mg/L)
PBR-A	Night	Oct. 16th	17.5	9.3	365±21.2	36±2.8
PBR-B	Day	Dec. 20th	17.1	6.2	198±11.6	38±1.4
PBR-C	Day	Feb. 18th	11.9	2.0	162±3.4	26±0.8

Table 8.2 Sample withdrawl times for E2 spike field (PBR) and laboratory (E2) experiments.

PBR-A	PBR-B	PBR-C	E2-A	E2-B	E2-C
16/10/2015 21:00	20/12/2015 08:15	18/02/2016 07:45	16/10/2015 21:45	20/12/2015 09:00	18/02/2016 08:00
16/10/2015 21:15	20/12/2015 09:15	18/02/2016 08:45	16/10/2015 22:55	20/12/2015 10:00	18/02/2016 09:00
16/10/2015 22:15	20/12/2015 10:15	18/02/2016 09:45	17/10/2015 00:40	20/12/2015 11:00	18/02/2016 10:00
17/10/2015 00:00	20/12/2015 12:15	18/02/2016 10:45	17/10/2015 07:20	20/12/2015 13:00	18/02/2016 12:00
17/10/2015 07:00	20/12/2015 14:30	18/02/2016 12:45	17/10/2015 08:15	20/12/2015 15:00	18/02/2016 14:00
17/10/2015 08:00	20/12/2015 16:30	18/02/2016 14:45	17/10/2015 10:10	20/12/2015 17:00	18/02/2016 16:00
17/10/2015 10:00	20/12/2015 18:20	18/02/2016 16:45	17/10/2015 12:10	20/12/2015 19:00	18/02/2016 18:00
17/10/2015 11:55	20/12/2015 21:00	18/02/2016 18:45	17/10/2015 15:10	20/12/2015 22:30	18/02/2016 20:00
17/10/2015 15:00	20/12/2015 22:45	18/02/2016 20:45	17/10/2015 17:00	21/12/2015 09:00	18/02/2016 22:00
17/10/2015 17:00	21/12/2015 08:00	18/02/2016 22:45	17/10/2015 20:30	-	19/02/2016 08:00
17/10/2015 20:30	-	19/02/2016 07:45	-	-	-

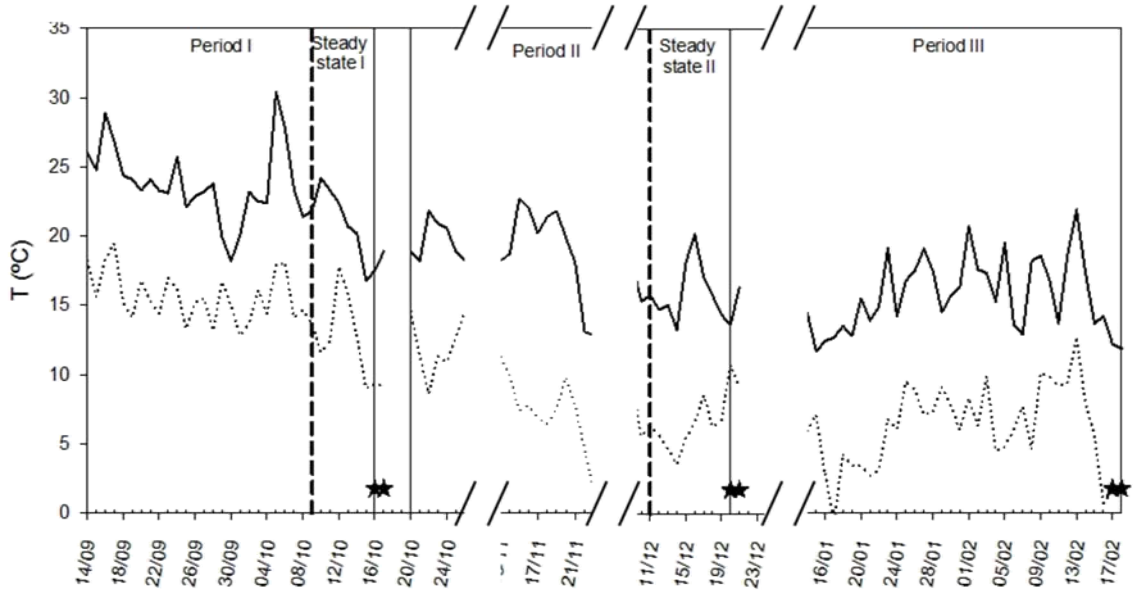


Figure 8.1 Environmental temperature profile during the PBR operation (Period I HRT=8 d, Period II & III HRT=12 d) and samples for molecular biology analysis. (⋯) *minimum outside temperature profiles;* (—) *maximum outside temperature profile (temperature data from* Meteocat (2016)*);* (★) samples for *molecular biology analysis* .

8.2.2 E2 removal experiments at laboratory scale

Batch assays (E2-A, E2-B and E2-C) were conducted in parallel using PBR biomass culture and incubated indoor. Table 8.3 shows the main biomass and WW characteristics for each experiment. Samples taken for the analysis monitoring are presented in Table 8.2.

Table 8.3 Batch experiments characteristics.

Assay	Place	Season	T_{max} (ºC)	T_{min} (ºC)	TSS_{algae} (mg/L)	TSS_{ww} (mg/L)
E2-A	Lab	Oct. 16th	27	23	365±21.2	36±2.8
E2-B	Lab	Dec. 20th	27	23	198±11.6	38±1.4
E2-C	Lab	Feb. 18th	27	23	162±3.4	26±0.8

The first batch experiment (E2-A) was carried out at once with PBR-A, the second batch experiment (E2-B) simultaneously with PBR-B and the last batch experiment (E2-C) at the same time as PBR-C. In that way, the algal biomass and influent (urban WW) were the same for outdoor and indoor experiments.

Batch experiments were carried out in 250 mL Erlenmeyer flasks incubated under continuous agitation at 130 rpm at 25±2ºC and either submitted to continuous fluorescent lamp irradiation and completely darkness (Erlenmeyer flasks were covered with foil). The Erlenmeyer flasks were filled with 100 mL of liquid medium with various compositions depending on the removal mechanism studied and were spiked with 2 mg E2/L.

Table 8.4 shows the experimental conditions tested for E2 removal determination in 24 h. Experimental conditions as well as controls were carried out under light and dark conditions. Abiotic controls contained ultrapure water and E2 at the same initial concentration as the experimental conditions (2 mg/L). These controls evaluated the E2 photodegradation (with light) as well as loses that may occur along the time (without light). WW controls were filled with the same urban WW that the PBR was fed. The presence and absence of light also gave information on photodegradation effects, moreover, if this control is compared with the abiotic, the effect of solids and bacteria contained in the WW matrix can be evaluated. The killed control, gives information about the possible sorption that may take place onto the biomass surface, although microalgae may change its cell wall properties, as well as the colour of the suspension may change. The experimental condition consisted on PBR culture spiked with E2, therefore, the results from this condition include the removal mechanisms from the previous described controls as well as they include biodegradation due to the PBR biomass.

A bioaugmentation assay (BA) was conducted (E2-D) at the same time as E2-C assay. PBR microalgal biomass was mixed with microalgal biomass coming from a microalgal industrial photobioreactor (IPBR), where the majority specie was *Scenedesmus*. Two bioaugmentation ratios (v/v) were evaluated 3:1 and 1:1 (PBR biomass:IPBR biomass), the killed controls of the respective ratios were also carried out (Table 8.5).

Table 8.4 Experimental (Exp.) conditions for E2 spiking at laboratory scale. (+ indicates the presence of light; - indicates the absence of light).

E2-A		**E2-B**		**E2-C**	
Exp. condition	**Light**	**Exp. condition**	**Light**	**Exp. condition**	**Light**
PBR Biomass	+	PBR Biomass	+	PBR Biomass	+
PBR Biomass	-	PBR Biomass	-	PBR Biomass	-
WW	+	Killed	+	Killed	+
WW	-	Killed	-	Killed	-
Abiotic	+				
Abiotic	-				
Killed	+				
Killed	-				

Table 8.5 Experimental conditions for E2-D bioaugmentation assay at laboratory scale.

Experimental condition	**Light**	**Dark**
PBR Biomass	✓	✓
3:1 PBR:IPBR Biomass	✓	✓
1:1 PBR:IPBR Biomass	✓	✓
PBR Killed	✓	✓
3:1 PBR:IPBR Killed	✓	✓
1:1 PBR:IPBR Killed	✓	✓

8.2.3 PBR biomass and wastewater initial parameters

Table 8.6 presents the main initial parameters for the WW influent and the PBR biomass prior the assays. Table 8.7 presents the initial characteristics of the IPBR biomass used for bioaugmentation assays and for the tested ratios.

Table 8.6 Initial microalgal biomass and wastewater parameters.

Parameter	PBR-A and E2-A		PBR-B and E2-B		PBR-C and E2-C	
	PBR	**WW**	**PBR**	**WW**	**PBR**	**WW**
TSS (mg/L)	365	36	198	38	162	26
$COD_{soluble}$ (mg/L)	57	454	180	288	151	245
$N\text{-}NH_4^+$ (mg/L)	19	119	25	137	21	140
P (mg/L)	11	21	3	9	5	10
pH	6.18	5.12	7.67	8.26	7.52	8.18
HRT (days)	8	-	12	-	12	-

Table 8.7 Initial microalgal biomass parameters in bioaugmentation assay (E2-D).

Parameter	IPBR	1:3	1:1
TSS (mg/L)	544	258	353
pH	8.7	7.9	8.1
$COD_{soluble}$ (mg/L)	176.1	-	-

8.3 Results and discussion

8.3.1 PBR performance monitoring

PBR performance during Period I (HRT 8 d) and Period II (HRT 12 d) has been presented in Chapter 7. In general, the total suspended solids (TSS) concentration depended on seasonal changes, although good nutrient efficiencies were obtained during the whole performance.

After PBR-B assay, PBR operation mode was resumed, Period III (HRT 12 d) was conducted in order to evaluate the effects of lower environmental temperatures (January-February) operating at the same HRT. Monitoring data was collected from January 14th (2016) to February 18th (2016), although system was operated since December.

TSS from the inlet urban WW as well as from the PBR effluent are presented in Figure 8.2 together with minimum and maximum air temperatures. During

Period III outside temperatures ranged from 6±3 ºC to 15±2 ºC, lower than during the Period II (9±3 and 18±3ºC, Figure 7.1). The TSS concentration was around 172±37 mg/L, the maximum concentration measured was 244 mg/L at the beginning of the Period III, followed by a TSS decreased and then were maintained. Temperatures next to 0 ºC were recorded at the beginning of Period III, but the subsequent effect is not observed as it happened in Period II (Figure 7.1). This fact may be compensated by the increase of solar hours (10 h 40 min at the end of Period III) as well as the solar irradiation 2.16 kWh/m^2, in comparison with Period II (Figure 7.2).

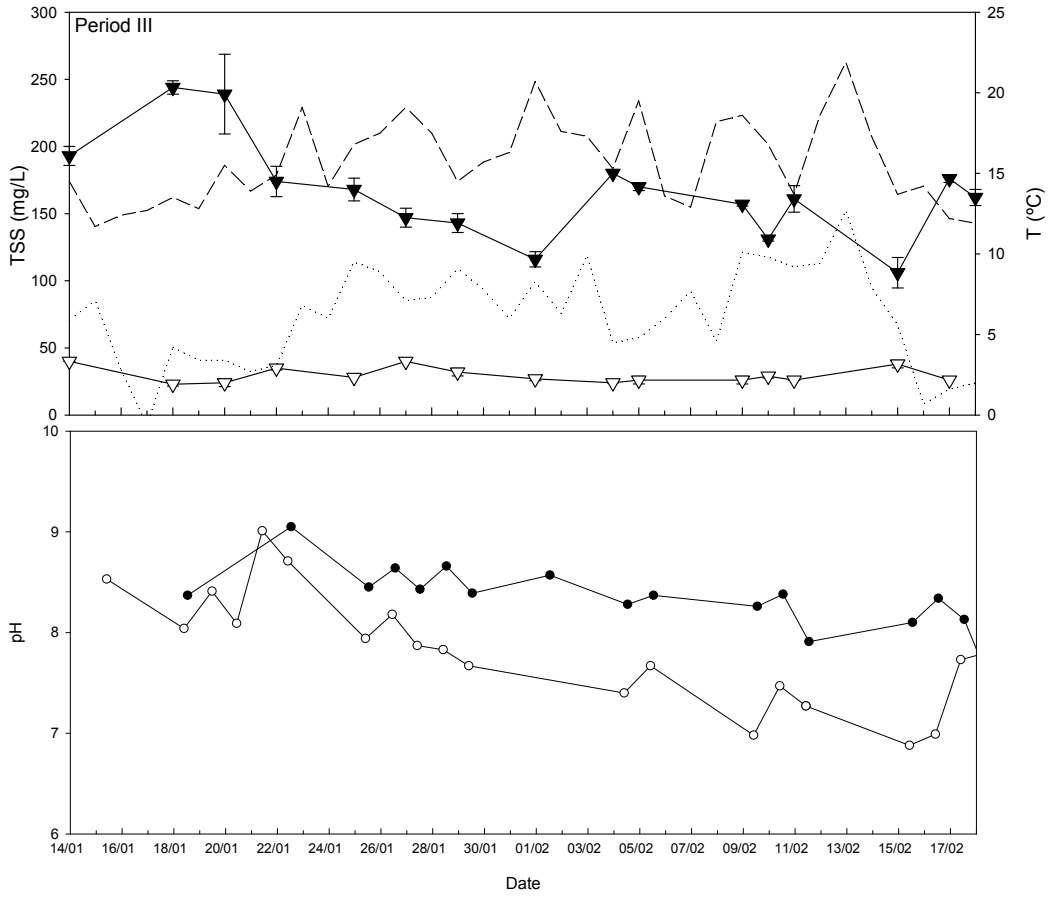


Figure 8.2 PBR performance during Period III (HRT=12 d). Top: (▼) *PBR TSS concentration;* (▽) *WW inlet TSS concentration;* (⋯) *minimum outside temperature profiles;* (---) *maximum outside temperature profile (temperature data from* Meteocat (2016)*). Bottom:* (●) *morning pH;* (○) *afternoon pH.*

pH values are presented in Figure 8.2. Higher values were reported after the day cycle (afternoon samples) than after the dark cycle (morning samples) as previously reported during Period I and II operation (Chapter 7). Morning pH values were higher than in Period II because they were measured 1 h later in comparison with Period II measurements.

Nutrients removal results are shown in Figure 8.3. During period III, ammonium nitrogen removal at the beginning of the period was 60% but at the end of the period 80% was removed. The lower removal values in comparison to Period II (>80%, Figure 7.3) can be due to lower biomass concentration in this period. Total phosphorus removal percentage was 73% and it was maintained during the whole period, attaining the legislation limits.

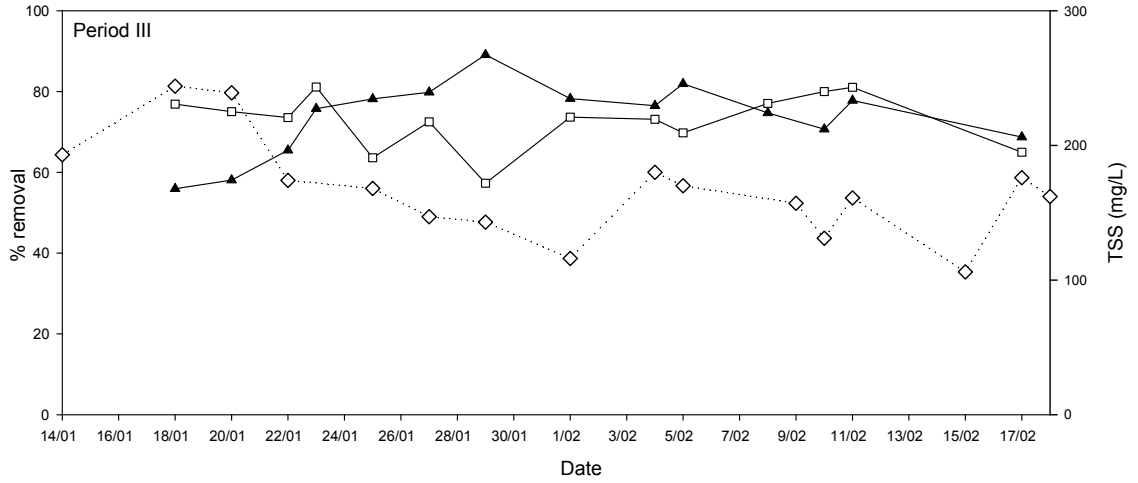


Figure 8.3 Nutrient removal during the Period III (HRT=12 d). (◇) *TSS concentration;* (▲) *N-NH4$^+$ removal;* (□) *total phosphorous removal.*

Chemical oxygen demand (COD) and carbon were monitored along Period III operation. Results are presented in Figure 8.4. COD removal percentages (40%) were in accordance with the values reported by Van Den Hende et al. (2014a) (between 25 and 47%) in a 12 m^3 microalgal reactor (high rate algal pond (HRAP) design) treating urban WW, nevertheless, were lower than Period I and II efficiencies (Figure 7.4).

Regarding the carbon concentration (Figure 8.4), inlet average values during that period were lower than during Period II, 152±30 mg/L and 227±48 mg/L, respectively. Moreover, the removal percentage was decreased due to the low biomass content, 65±15 and 75±13 %, respectively.

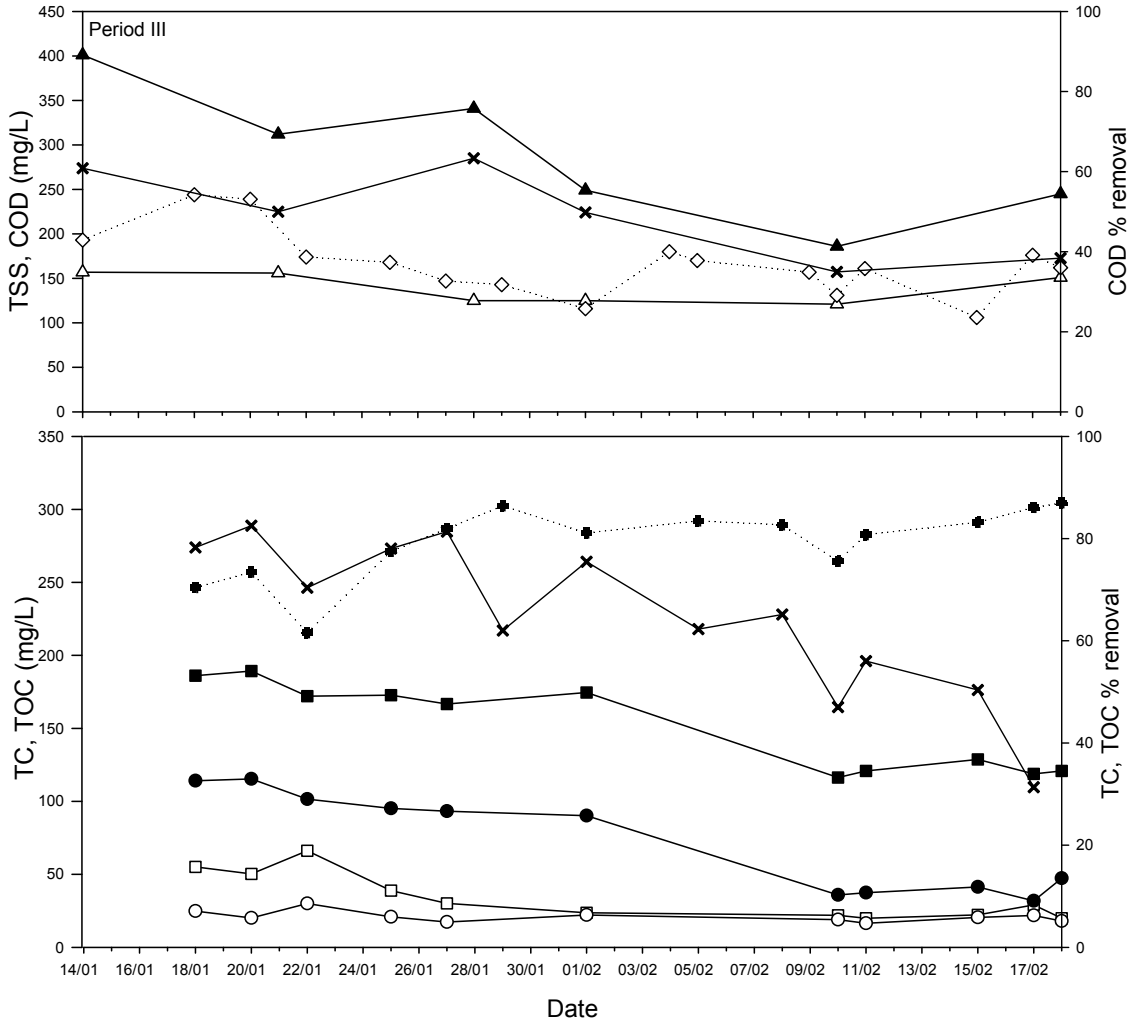


Figure 8.4 Nutrients concentration and TSS during Period III (HRT=12 d). Top: (◇) *TSS concentration;* (▲) *inlet COD;* (△) *outlet COD;* (x) *COD removal. Bottom:* (■) *inlet TC;* (□) *outlet TC;* (●) *inlet TOC;* (○) *outlet TOC;* (✚) *TC removal;* (x) *TOC removal*

Despite the low temperatures and biomass concentration during Period III, the fact that solar hours and solar irradiation were increased in comparison to Period II, nutrient removal efficiency in the PBR was maintained. The importance of light for the microalgal photosynthetic activity is also confirmed on the lower removal percentages at the beginning of Period III than at the end.

8.3.2 E2 removal batch experiments in the pilot scale PBR

17β-Estradiol (E2) removal was evaluated and estrone (E1) concentration was measured during the assay. It has been reported that under aerobic or anoxic conditions, E2 could be first oxidized to E1, further oxidized to unknown metabolites, and finally to CO_2 and water (Shi et al., 2010; Ternes et al., 1999).

Also, in Chapter 4, E1 was found in some of the experimental assays conducted.

In Figure 8.5 E2 removal along the time as well as E1 formation from the three assays conducted (PBR-A, PBR-B and PBR-C) are shown. The presence of active microalgae in the PBR was confirmed by visual observations, microscopy, and molecular biology (Figure 8.7).

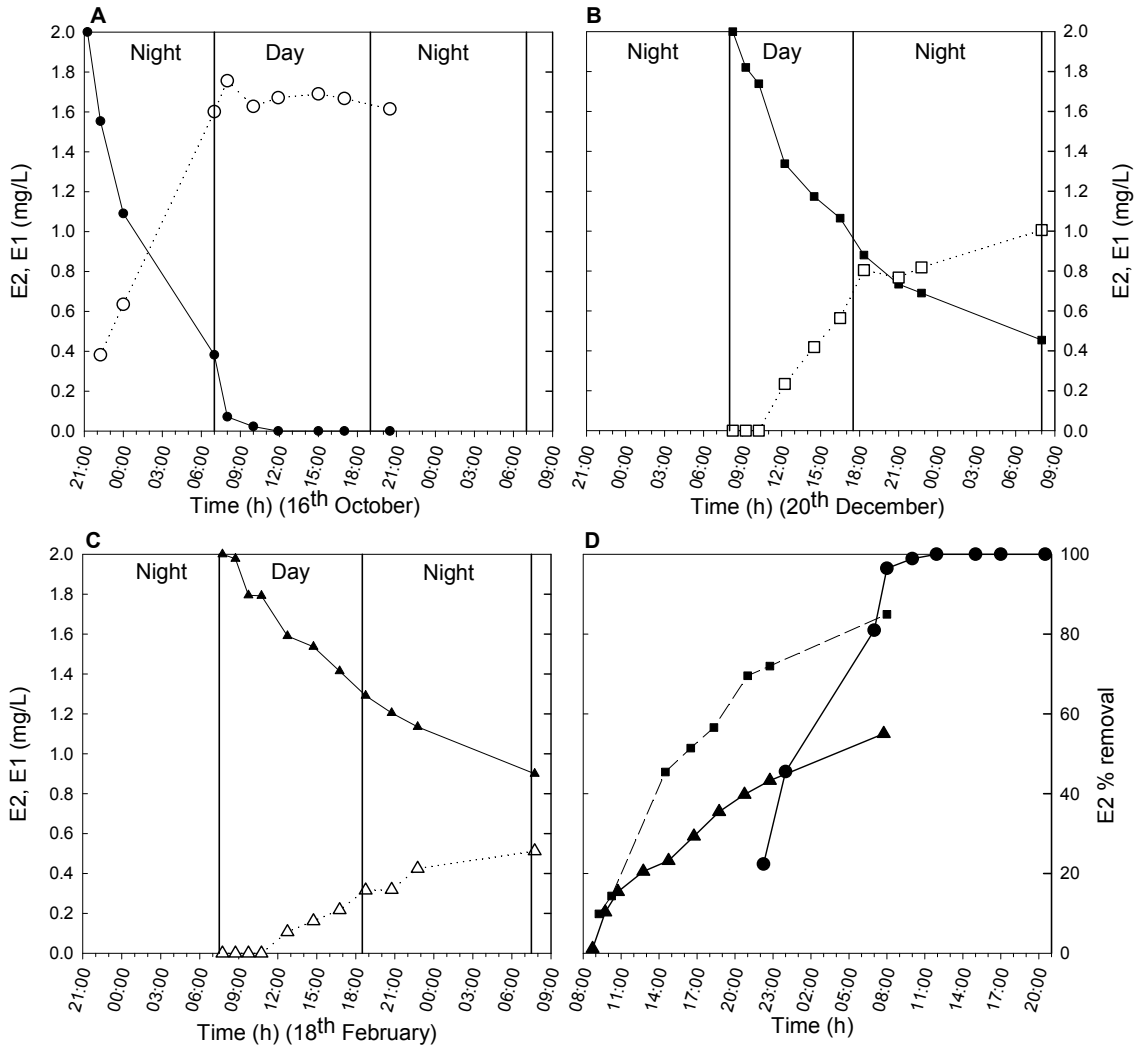


Figure 8.5 ***A:*** *PBR-A assay,* (●) *E2 and* (○) *E1 concentration;* ***B****: PBR-B assay,* (■) *E2 and* (□) *E1 concentration;* ***C****: PBR-C assay*, (▲) *E2 and* (△) *E1 concentration;* ***D****: E2 removal percentages* (●) *PBR-A assay;* (■) *PBR-B assay;* (▲) *PBR-C assay. Vertical lines* (|) *indicate the sunrise and sunset time for each PBR assay.*

The three assays (PBR-A, PBR-B and PBR-C) followed similar trends on E2 removal and E1 formation, despite the final E2 and E1 concentrations inside the PBR were different (Figure 8.5). Complete E2 removal was achieved during PBR-A assay, great part of E2 removal took place during the night, 81%. The

other two assays, PBR-B and C, had lower final removal percentages (77 and 55%, respectively). However, the behaviour for all three assays was similar, during the first 10-13 h a rapid removal took place, followed by a slower removal rate.

Shi et al. (2010) studied E2 removal in a continuous pilot scale (36 L) algal pond, inoculated with 6 pure microalgae cultures, 100 mg TSS/L, spiked with E2 and E1 (30 ng/L for each compound) at an HRT of 15 days. At the end of continuous-flow test, about 91% and 84% estrogens (E2 and E1, respectively) were removed from water phase in the algal ponds.

Due to the lack of other pilot scale microalgal systems for E2 removal in the literature, the results have been compared with other WW treatment technologies. Baronti et al. (2000) compared estrogenic compounds removal during sewage treatment, obtaining values ranging from 76 to 92% E2 removal, percentages similar than the obtained in PBR-B assay. Thus, the removal efficiency of the PBR is comparable to that of conventional activated sludge WWTPs. The apparent removal of E2 in conventional mechanical secondary and tertiary plants treatment systems was generally greater than 75% and as high as 98% (Servos et al., 2005).

Estrone (E1) has been detected in the PBR. E1 has been reported as a byproduct of biodegradation of E2 by the microalgae *Chlorella vulgaris* (Lai et al., 2002), although it can also be formed due to oxidation processes (Czajka and Londry, 2006; Servos et al., 2005; Zhao et al., 2008). The formation of estrone is likely to occur as E2 is rapidly oxidized. This would result in an apparent increase in the concentration of estrone and then will be removed from the treatment system. Jürgens et al. (2002) showed that E2 was rapidly oxidized to E1 and then was mineralized by natural bacteria in river water without the appearance of other major degradation products. PBR-A assay showed a rapid E1 formation followed by a steady state (Figure 8.5A). On that moment no E2 was detected. Similar behaviours were observed in PBR-B and C assays (Figure 8.5B and C), E1 was formed at the same rate as E2 was disappeared. At the beginning there was a rapid formation and then the rate decreased, although there was still E1 formation since E2 was not completely removed.

From the results obtained and taking into account the TSS concentration from each assay, it could be said that as more biomass there is (PBR-A, 365 mg TSS/L) more E2 removal is achieved. Therefore, it may be postulated that the decline in biomass (microalgae, heterotrophs and non-photosynthetic autotrophs organisms) decreased the biodegradability of E2. E2 concentration determination takes into account the adsorbed hormone onto the biomass surface, since an extraction is conducted. Experimental conditions give information on the possible amount biodegraded plus the adsorbed part. E2 concentration data was well described by a first order model, Table 8.8. Higher reaction rate (k) values indicate faster E2 removal (PBR-A).

Table 8.8 Kinetic rate constants for PBR batch assays.

Parameter	**PBR-A**	**PBR-B**	**PBR-C**
Final removal (%)	100	77	55
k (h^{-1})	0.338	0.063	0.034
r^2	0.99	0.98	0.99
E2 removal yield (mg E2 removed/(mg TSS·h))	$4.32 \cdot 10^{-4}$	$2.92 \cdot 10^{-4}$	$2.45 \cdot 10^{-4}$

Although, PBR-B and PBR-C had a similar TSS concentration (198 and 162 mg/L, respectively), the removal percentage was lower on PBR-C, meaning that other parameters may have had an effect on the system. Seasonality is relevant to achieve adequate EC removal efficiency in mild climates such as that of the North-Weast Mediterranean because it affects temperature, daylight duration and intensity, and biomass production, four important factors influencing biodegradation, photodegradation, volatilization and sorption on EC removal processes (Garcia-Rodríguez et al., 2014). Some season differences among the assays are shown in Table 8.1, PBR-B was conducted in December, while PBR-C in February. Figure 8.6 shows the global solar irradiance (GSI) during the PBR assays. The maximum GSI was obtained during PBR-A, 663 W/m^2, corresponding to October, autumn time. PBR-B GSI achieved a maximum value of 398 W/m^2 and then decreased. PBR-C GSI had two peaks along the day, which may have had an effect onto the biomass. The first peak achieved a higher GSI value than PBR-B maximum, 450 W/m^2, and then it dropped to 123

W/m^2. A second peak was obtained at 13:30 (solar time) and then as the day passed, the GSI decreased.

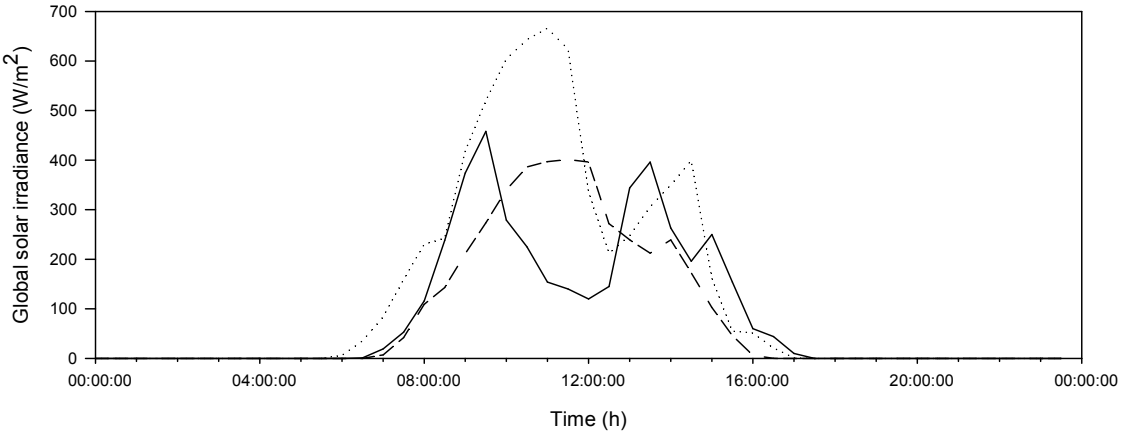


Figure 8.6 Global solar irradiance for the 3 PBR assays, solar time. (⋯⋯) *PBR-A;* (---) *PBR-B;* (—) *PBR-C. (Data supplied by* Meteocat*)*.

Vader et al. (2000) suggested that the seasonal and temperature effects on nitrification may therefore result in changes in the ability of treatment systems to remove estrogenic compounds, so this argument can also have an impact on microalgae species as well as the other microorganisms present in the PBR. Air temperatures dropped between assays (Table 8.1), as well as lower sunlight irradiation (Figure 8.6 and Chapter 7) which may have reduced the volatilization rate and may have caused a change on the biomass composition and therefore had an effect on E2 removal.

Regarding the microalgal biomass present in the PBR, visual differences were observed. Microalgal biomass for PBR-A and B was mainly composed of settling flocs before E2 addition. As it has been exposed in Chapter 7, the flocs are due to the presence of the filamentous cyanobacteria *Phormidium* sp. However, in the last assay, PBR-C, PBR culture was mainly composed of free microalgae cells and less settling flocs were present. Figure 8.7 shows the Eukaryotic denaturing gradient gel electrophoresis (DGGE) band profile, the phylogenic affiliation, accession numbers of the closest relatives and sequence similarity of the DGGE bands are found in Table 7.3. A sample before and after the spiking was taken (Figure 8.1).

From the DGGE, Figure 8.7, it is observed that microbial community does not change before and after E2 spiking. It could be mentioned that E2 does not

have an immediate effect on populations. More detailed information about the identity of the bands recovered has been detailed in Table 7.3 (Chapter 7).

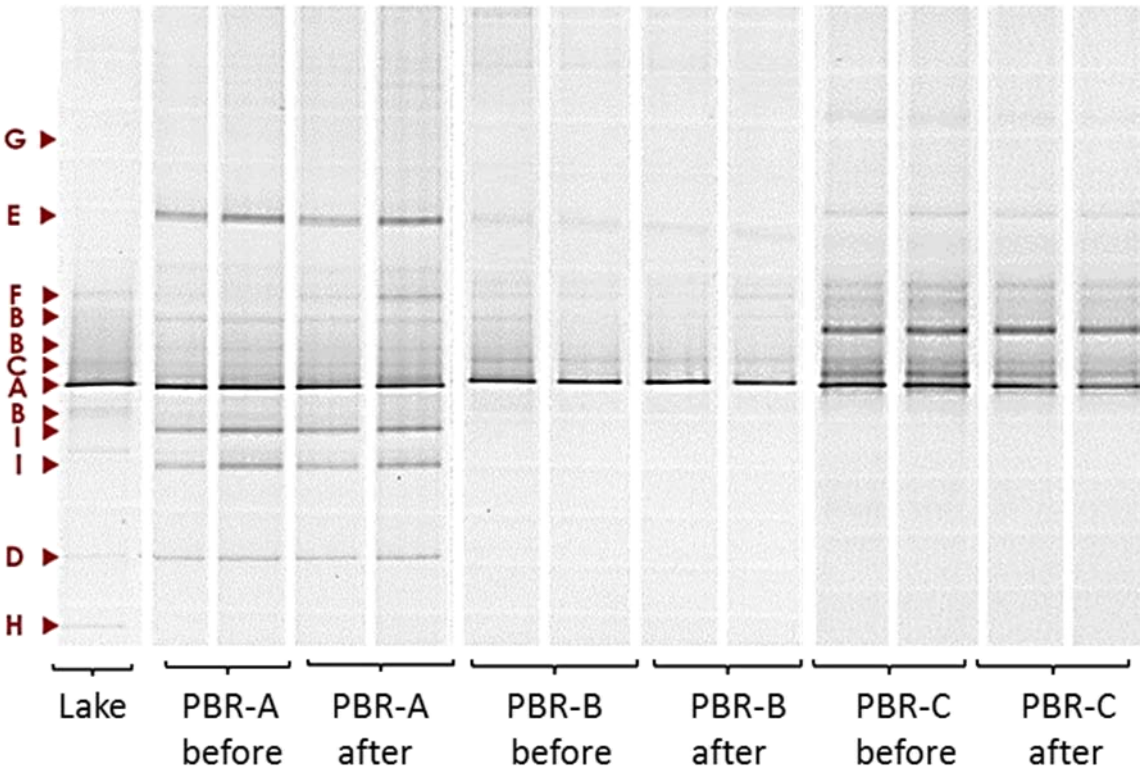


Figure 8.7 Eukaryotic DGGE band profiles from the PBR and E2-D spikings. Bottom lane numbers refer to the samples names (before and after the spiking). The sequenced DGGE bands are indicated on the left side.

Although some microalgae species, such as *Chlorella* sp. and *Pseudospongiococcum* (Band A and C, respectively) were present in all three assays, others changed along the time (e.g., *Vorticellides* sp., *Paraphysoderma sedebokerense*). Changes on the microbial community could be the responsible for the differences on E2 removal.

The efficiency of the PBR assays has been studied considering the TSS content in each assay and calculating the E2 removal yield (Table 8.8). E2 removal yield has been defined as the amount of E2 removed per mg of TSS and per hour. The results showed that the most efficient assay for E2 removal was still PBR-A, followed by PBR-B assay and finally PBR-C. These values give information about biomass composition changes that may be occurring into the system, due to season conditions (temperature and light) changing over the assays, and becoming more efficient when season conditions were more favourable for the algae growth. Differences on microalgal biomass composition

could be one of the main reasons, since not all species could be suitable for E2 removal. That behaviour has been observed previously in Chapter 4, where the 2 microalgal strains (*C. reinhardtii* and *P. subcapitata*) achieved different E2 removal percentages for experimental conditions in 24 h (89 and 47%, respectively) as well as for killed controls (27 and 2%, respectively). This behaviour not only occurs on estrogenic compounds, also, in Chapter 5, it has been shown that the percentage on each antibiotic removal depends on the microalgae strain.

Taking into account the configuration of the PBR, the most relevant removal processes that may occur in this system can be biodegradation, photodegradation, volatilization and sorption to microbial biomass. Previous experiments (data not shown) were carried out testing E2 sorption to the polyethylene (PE) tubes as well as onto the distribution chamber made of propylene (PP). No sorption was significantly detected during 48 h in either materials. As seen in Chapter 4, pure cultures may transform E2 into another product by biological mechanisms as it has been proofed by transformation products (TPs) identification. However, different microalgae species were present in the PBR consortium and the results could not be extrapolated and confirm that biodegradation of E2 was taking place in the PBR. On the other hand, the removal due to the release of microalgae exudates cannot be disregarded; neither the effect of bacteria nor other microorganisms present in the culture. It has been previously described that hydrolytic enzymatic activities of bacteria are accelerated in the presence of algal associated polymeric compounds (Martinez et al., 1996). This will therefore accelerate recycling of organic nutrients, which in turn can either favour algal growth (Daufresne et al., 2008; Grossart, 1999) or increase algae lysis and death, especially for stressed algae (Cole, 1982). In fact, it has been proved that the consortia of cyanobacteria/microalgae and bacteria can be efficient in detoxification of organic and inorganic pollutants, and removal of nutrients from wastewaters, compared to the individual microorganisms. Cyanobacterial/algal photosynthesis provides oxygen and organic exudates that serves to the pollutant-degrading heterotrophic bacteria, thus E2 removal may be affected (Subashchandrabose et al., 2011). Moreover, sorption onto the biomass can be

an important parameter to take into account for E2 removal, although hormone extraction from the biomass was conducted prior to E2 analysis. Other removal mechanisms may take place, including photodegradation or volatilization. All these mechanisms will be evaluated on the following section, where laboratory scale experiments were carried out including controls with the aim to evaluate the effect of all these mechanisms.

8.3.3 Indoor Erlenmeyer flask batch E2 removal experiments

Figure 8.8 shows E2 removal profiles for the three batch assays conducted in Erlenmeyer flasks in parallel with the PBR batch experiments. Controls and experimental conditions and kinetic constant values are shown in Table 8.9.

E2-A assay (Figure 8.8A) does not present significant differences between light and dark samples in none of the tested conditions, concluding that E2 photodegradation removal does not take place in 24 h. This fact can be confirmed with abiotic controls, there are no differences between the control light exposed and the light protected. At the end of the experiment, 23% E2 removal was achieved in both abiotic controls, this percentage was attributed to loses rather than photodegradation. From now on, abiotic controls are not going to be discussed since no differences among experiments were detected. E2 removal by means of sorption mechanisms can be considered from the killed control results, in this case, little sorption onto the microalgal biomass was occurring, 8%. Fürhacker et al. (1999) stated that when dissolved in wastewater, E2 has a low tendency for adsorption on particulate matter, so in that case seems to have the same tendency.

The highest E2 removal was achieved when microalgal/bacterial consortia biomass coming from the PBR was present (named algae experiment in Figure 8.8 and Table 8.9); however, high removal rates were achieved on WW controls. WW controls reduced completely E2 concentration in 24 h, although the removal rate was lower, 0.095 h^{-1}, whereas for the algae condition the k was 0.159 h^{-1}. WW matrix contains solids and bacteria that may have an impact on E2 removal. Although WW was used as a control to evaluate these interactions, the WW matrix and the matrix from the PBR used for the experimental conditions were not the same. Their composition was different and the results

obtained from the WW control cannot be extrapolated to the PBR. For that reason, in the next E2 spikes, WW control is not going to be shown.

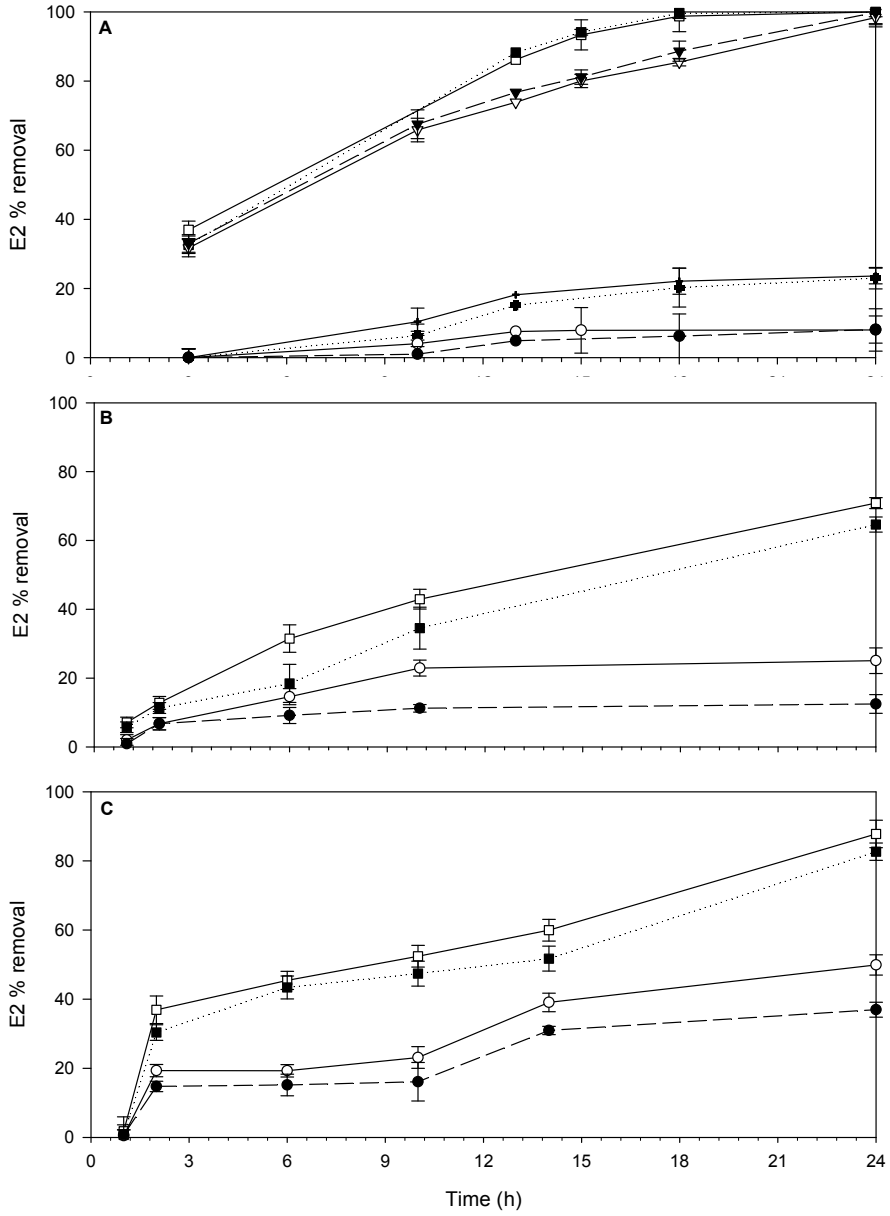


Figure 8.8 Indoor Erlenmeyer flasks E2 removal assays. ***A****: E2-A,* ***B****: E2-B,* ***C****: E2-C.* (□) *algae light;* (■) *algae dark;* (▽) *WW light;* (▼) *WW dark*; (+) *abiotic light;* (✚) *abiotic dark;* (○) *killed light;* (●) *killed dark.*

Table 8.9 Kinetic rate constants for E2 batch assays.

Condition	k (h^{-1})	r^2
	E2-A	
Algae + Light	0.159	0.971
Algae + Dark	0.171	0.973
WW + Light	0.095	0.990
WW + Dark	0.105	0.983
Abiotic + Light	0.012	0.938
Abiotic + Dark	0.012	0.936
Killed + Light	0.006	0.908
Killed + Dark	0.004	0.996
	E2-B	
Algae + Light	0.051	0.997
Algae + Dark	0.043	0.994
Killed + Light	0.011	0.976
Killed + Dark	n.a.[a]	n.a.[a]
	E2-C	
Algae + Light	0.084	0.968
Algae + Dark	0.070	0.968
Killed + Light	0.030	0.974
Killed + Dark	0.020	0.942

[a] *n.a. data not available*

Microalgal biomass experimental condition (named algae) does not show a significant difference between light and dark conditions, both profiles follow the same tendency. 88% E2 removal was achieved in 13 h, then the removal velocity was decreased achieving the complete removal in less than 24 h. This profile describes a similar trend as the behaviour occurring in the PBR (Figure 8.5). Reaction rate constants could be compared in order to evaluate the differences. Whereas E2-A has a constant rate of 0.159 h^{-1}, PBR-A has a higher value of 0.338 h^{-1}, in accordance with E2 removal, in 12 h there was 96% removal. Those results confirm that the outdoor system had a better

performance than the controlled conditions experiment; moreover, the laboratory scale study gives an idea of what is occurring in a real system. These differences could be attributed to environmental factors that may be beneficial for the PBR. Sun irradiation may be the major difference among the systems, as well as temperature changes along the 24 h. Another difference could be the presence of biofilms inside the tubes as well as on the walls from the distribution chambers. Those biofilms have a major microalgae concentration, as well as they may be composed of other microalgae species.

The next assay, E2-B, was performed in the same way as E2-A, E2 removal profiles are shown in Figure 8.8 and constant rates in Table 8.9. The removal rate for the PBR culture decreased in comparison with E2-A, k=0.051 and k=0.159 h^{-1}, respectively. 43% removal was achieved in 10 h, and 71% was the final (24 h) E2 removal obtained. Light and dark conditions were significantly different, under the light, greater E2 removal values were achieved, since microalgal photosynthesis was taking place. Microalgae can perform oxygenic photosynthesis and fix carbon dioxide through Calvin cycle; microalgae cells can trap light as energy source and assimilate CO_2 as the carbon source. Moreover, some microalgae metabolic pathways (i.e., glycolytic pathway, tricarboxylic acid cycle and mitochondrial oxidative phosphorylation) maintain high activities during illumination, indicating little effect of light on these pathways. However, when light is not available there is a decrease in nutrients removal because a portion of the CO_2 produced by the bacteria is converted back to particulate organic matter in the form of algal cells, without nutrient removal (Leslie Grady et al., 1999; Yang et al., 2000). In our particular study, different hypothesis are stated on what occurred during the dark period. On one hand, during the dark condition E2 removal by microalgae cells may be occurring, although it was higher when light was available. On the other hand, during the dark condition E2 could be adsorbed onto the microalgae surface and later removed. Finally, during the night E2 removal was due to bacteria and other microorganisms activity. No further experiments were conducted in order to determine the real E2 removal process during the dark condition, since microalgae activity seems to be taking place together with the other microorganisms activities. Several authors reported that biological diversity

enhances biological transformations of EC removal (Servos et al., 2005). Microorganisms can degrade steroidal hormones using two possible degradation mechanisms: growth-linked (metabolic) and non-growth-linked (co-metabolic) (Yu et al., 2007). For growth linked degradation, microorganisms utilize steroidal hormones as energy and/or carbon source for microbial growth. Previous studies showed that E2 could be co-metabolically degraded by heterotrophic bacteria (Pauwels et al., 2008; Yu et al., 2007) and by ammonia-oxidizing bacteria and nitrifying activated sludge (Skotnicka-Pitak et al., 2009; Yi and Harper Jr., 2007).

E2 removal yield has been calculated (Table 8.10) in order to take into account the initial microalgal biomass, E2-A had a E2 removal yield of $3.24 \cdot 10^{-4}$ mg E2/(mg TSS ·h), whereas, E2-B was lower, $2.98 \cdot 10^{-4}$ mg E2/(mg TSS ·h). The results show that despite laboratory conditions were the same, differences among the biomass and the PBR matrix may have had an effect on E2 removal achieved in both assays.

At the end of E2-B assay, between 23-25% E2 was adsorbed, conrresponding to the dark conditions results from the killed control (Figure 8.8B). Furthermore, the killed control profile between light and dark was significantly different, more E2 removal was detected under the light, than without light. This may be due to differences of the matrix from the PBR culture from the previous assay, in that case other molecules or radicals could be present and in contact with light may interfere on E2 removal, changing the molecular structure of E2 into other metabolites. The removal rate constant between the pilot scale system (PBR-B) and laboratory batch experiment (E2-B) were similar, $2.92 \cdot 10^{-4}$ and $2.98 \cdot 10^{-4}$ L/(mg TSS ·h), respectively. In that case, both systems were equally effective.

Table 8.10 Summary of kinetic rate constants for E2 batch assays and E2 removal yield.

Parameter	**E2-A**	**E2-B**	**E2-C**
Final removal (%)	100	71	88
k (h^{-1})	0.159	0.051	0.084
r^2	0.971	0.997	0.968
E2 removal yield (mg E2 removed/(mg TSS·h))	$3.24 \cdot 10^{-4}$	$2.98 \cdot 10^{-4}$	$4.52 \cdot 10^{-4}$

In Figure 8.8C it is seen that 88% E2 removal was achieved on E2-C spiking experiment at the end (24 h) for algae condition. There was an increase on E2 removal in comparison with E2-B, as well as on the rate constant, k=0.084 h^{-1}, and E2 removal yield, $4.52 \cdot 10^{-4}$ mg E2/(mg TSS·h) (Table 8.10). Experimental conditions were kept constant and the different removal rate is attributed to the microalgae community composition. As it is seen in Figure 8.7, different species were present in the PBR, being more able to degrade E2. Although the DGGE is not quantitative, band B (*Scenedesmus*) intensity is higher in PBR-C than in the other PBR assays. In that case, the initial biomass concentration was similar to E2-B, but the rate constants were different, and this was reflected on the E2 removal yield. This biomass efficiently removes E2 if the environmental conditions are favourable; laboratory assay was conducted at 25ºC whereas during the PBR-C assay air temperature ranged between 2 to 12ºC.

It is also important to point out that at the end of the experiment E2 was almost removed, the adsorption on the killed control increased considerably, 50% under the light and 37% without light (Figure 8.9C).

Few works have previously studied E2 removal using microalgae in batch assays. Shi et al. (2010) conducted a 6 days batch experiment with 6 pure microalgae strains cultured in synthetic WW containing estrogenic compounds. Around 95% of E2 was removed (100 mg TSS/L) and 60% removal was achieved in 24 h, corresponding to an E2 removal yield of $2.50 \cdot 10^{-10}$ mg E2/(mg TSS·h). This low removal yield was due to the low initial E2 spiked concentration (1 ng/L), whereas in the present work the initial concentration was in the order of mg/L. The present PBR seems to be more efficient for E2 removal. Again, PBR consortium has a great biodiversity, with well-stablished communities, while in Shi et al. (2010) study, the initial biomass composition were pure microalgae cultures, lately, changes and bacterial growth may have arisen due to the mixing with the WW.

E1 was also analysed in batch studies along the time to confirm the interconversion of E2 into E1. Results are shown in Figure 8.9.

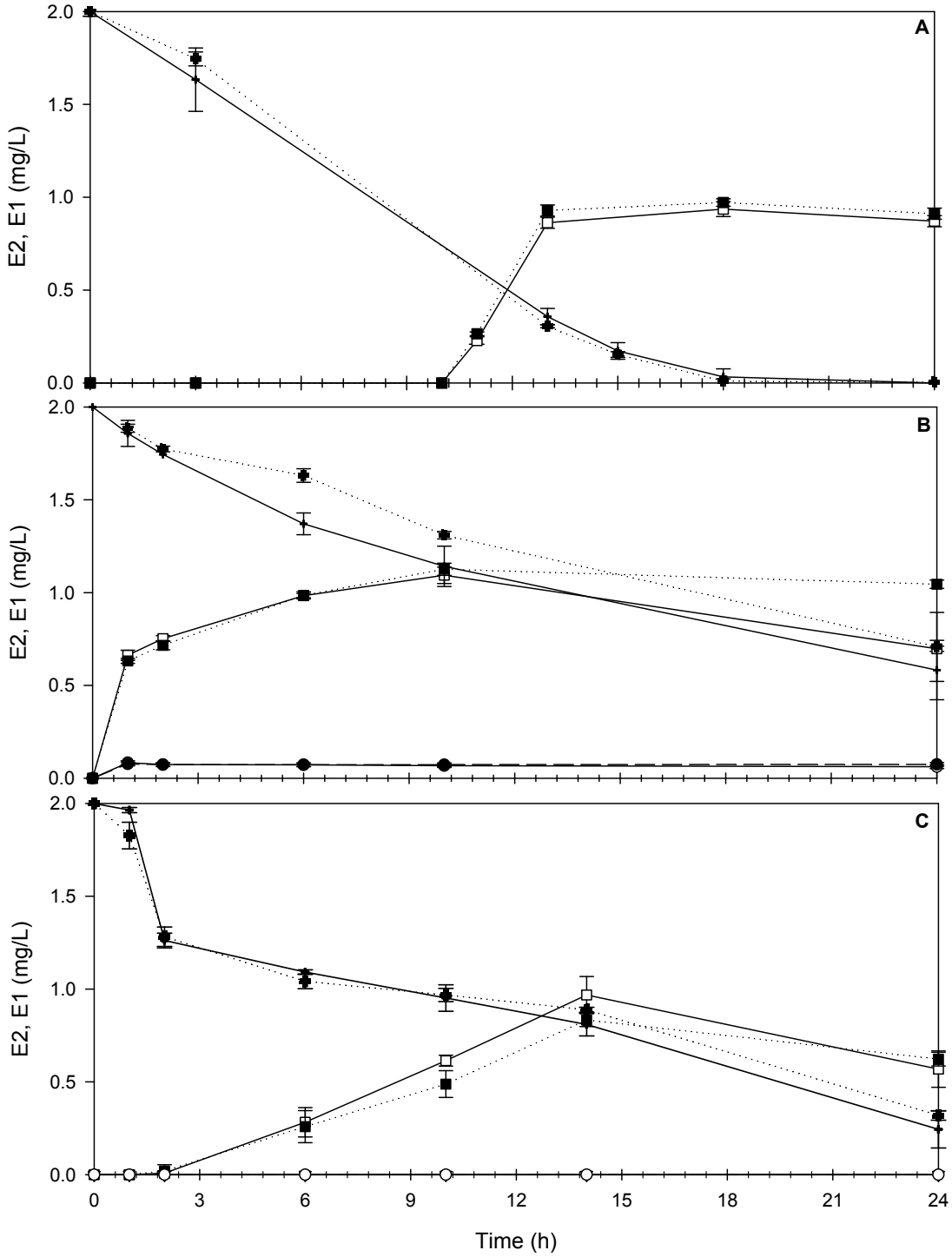


Figure 8.9 E2 removal and E1 formation on E2 assays. ***A****: E2-A assay;* ***B****: E2-B assay;* ***C****: E2-C assay.* (+) *E2 algae light;* (✚) *E2 algae dark* (□) *E1 algae light*; (■) *E1 algae dark;* (○) *E1 killed light;* (●) *E1 killed dark*.

Three different behaviours on E1 formation can be seen for algae conditions depending on the assay. In the first assay (Figure 8.9A), E1 was formed 10 h after the spiking, a rapid increase during the following 3 hours, then the concentration was maintained until the end. In that case, there was no E1

removal detected. On the other hand, in E2-B assay (Figure 8.9B), E1 was formed from the beginning of the experiment. There was a rapid increase during the firsts 3h, followed by a steady state, although light samples followed an E1 decrease, meaning that the system was also able to remove the compound. Finally, on E2-C assay (Figure 8.9C), E1 was formed 2 h after the spiking, followed by a linear increase that lasted for 10 h. In light samples E1 was detected at a lower concentration.

Overall, E1 was formed in the presence of active algal biomass and it did not depend on the light presence or absence, it was detected under both conditions. E1 was not detected in the killed controls, then it was a biodegradation product from the PBR biomass.

8.3.4 E2 bioaugmentation batch experiment

A bioaugmentation assay was carried out in order to evaluate the presence of a higher biomass density, as well as the fact of having a more specialised microalgal biomass for E2 removal, since previous authors reported better results on estrogenic compounds removal depending on the microalgae strain used. Peng et al. (2014) noted better results for *Scenedesmus obliquus* on the steroidal hormones progesterone, rather than *Chlorella pyrenoidosa* strain.

The biomass used for the bioaugmentation assay (E2-D) came from an industrial PBR (IPBR), which was mainly composed by *Scenedesmus*. During the E2-D assay, biomass from the PBR (without bioaugmentation) was used as a control, the same biomass corresponded to PBR-C and E2-C assays. Initial characteristics from IPBR and the two ratios tested are shown in Table 8.7. Figure 8.10 shows E2 removal profiles for the consortium control and the two new consortia tested (3:1 and 1:1 (v/v), PBR:IPBR) for bioaugmentation experiments.

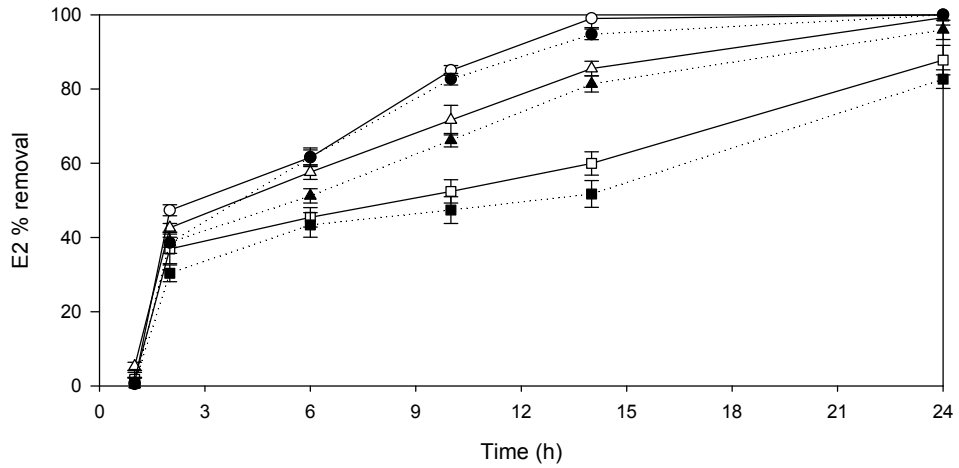


Figure 8.10 Indoor Erlenmeyer flasks E2 removal bioaugmentation assay. (□) *biomass control light;* (■) *biomass control dark;* (△) *3:1 ratio light;* (▲) *3:1 ratio dark;* (○) *1:1 ratio light;* (●) *1:1 ratio dark.*

As the TSS increased, E2 removal percentage increased, that is in accordance with the results from the PBR and Erlenmeyer flask assays. Light samples achieved better removal results, than dark samples. Complete removal was achieved in the presence of IPBR biomass, whereas the control (without bioaugmentation) only attained 88% removal at the end of the assay. Three phases can be distinguished on E2 removal, i.e., a rapid removal between 1 and 2 h, followed by a period of slower removal up to 13 h, and finally a constant removal for samples containing IPBR biomass, whereas in the control seems to increase the removal rate until the end of the experiment, since it has accomplished the maximum removal. Removal rate for each condition was determined by the k value (Table 8.11), showing that as higher IPBR biomass ratio, higher E2 removal rate was attained. E2 removal yield values have been calculated (Table 8.11) in order to take into account the initial biomass concentration, since it was different for all three conditions. The highest E2 removal yield was obtained for PBR biomass, although there was not complete removal, followed by 3:1 ratio and finally, 1:1 ratio. These results show that the PBR biomass was more efficient on E2 removal, rather than the IPBR biomass.

Table 8.11 Kinetic rate constants and E2 removal yield for bioaugmentation batch assay. E2-C control (no bioaugmentation); 3:1 and 1:1 volumetric ratio bioaugmentation experiments (E2-D assay) (PBR:IPBR).

Parameter	**E2-C**	**3:1**	**1:1**
Final removal (%)	88	99	100
k (h^{-1})	0.084	0.191	0.318
r^2	0.968	0.945	0.913
E2 removal yield (mg E2 removed/(mg TSS ·h))	$4.52 \cdot 10^{-4}$	$3.21 \cdot 10^{-4}$	$2.83 \cdot 10^{-4}$

Regarding the microbial community composition, Eukaryotic DGGE band profiles are presented in Figure 8.11, whereas the phylogenic affiliation for bands A to I is presented in Table 7.3 (Chapter 7) and for bands J to O in Table 8.12.

IPBR biomass was mainly composed by the Chlorophyta *Desmodesmus armatus* (band M) and the fungi *Gaertneriomyces semiglobifer* (band O) (Figure 8.11). *Desmodesmus armatus* is a homotypic synonym (have the same type (specimen) and the same taxonomic rank) of *Scenedesmus hystrix* var. *armatus*. It can be found in freshwater bodies all around the world, and even in the soil, their cosmopolite appearance illustrates the wide range of environmental conditions those organisms can tolerate and explains why they were among the firsts algae established in laboratory cultures (Trainor, 1998). *Gaertneriomyces semiglobifer* (band O) is a fungal member of the Phylum Chytridiomycota, is more commonly found growing in bodies of waters, primarily in freshwater environments, and in soils as well (Sime-Ngando, 2012). Some species are parasites of small marine green algae and diatoms, while in freshwater can infect a wide diversity of hosts, including both large size and small size algae (Gleason et al., 2011; Rasconi et al., 2012; Sime-Ngando, 2012).

Regarding E2-D assay, differences among the bioaugmentation consortium and the PBR are observed in Figure 8.11. PBR and IPBR suspensions were composed with different species, this is reflected on the both ratios tested during the bioaugmentation assay. For example, *Chlorella* (band A) is slightly detected on bioaugmentation ratios, since it is not detected in the IPBR biomass.

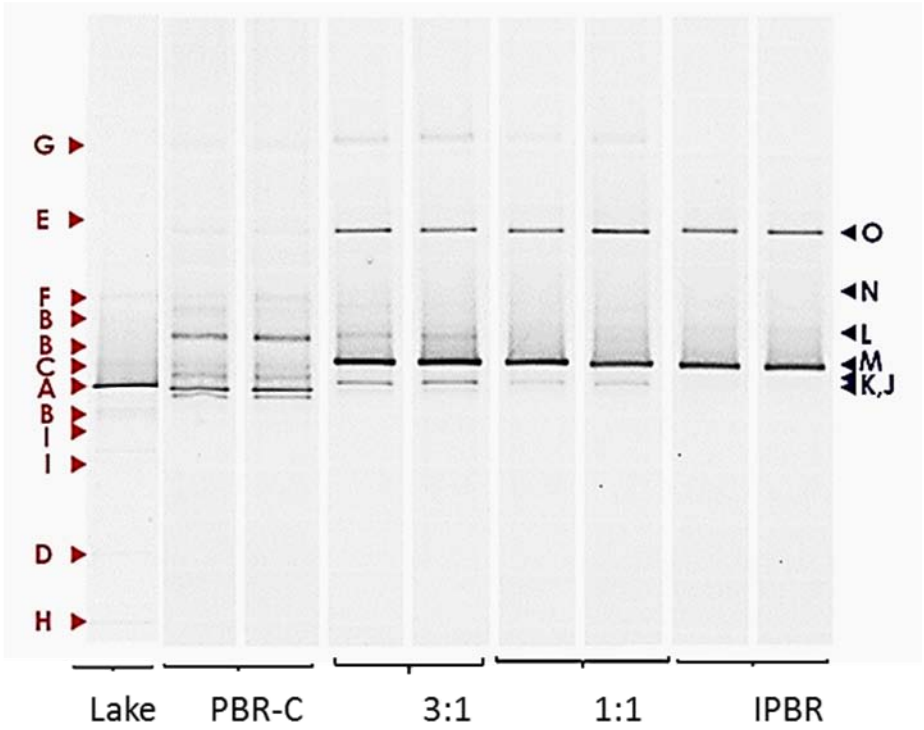


Figure 8.11 Eukaryotic DGGE band profiles from the E2-D assay. Bottom lane numbers refer to the samples names. The sequenced DGGE bands are indicated on the left and right side

Table 8.12 Phylogenic affiliation, accession numbers of the closest relatives and sequence similarity of the DGGE bands.

Excised DGGE bands	Accession number	Closest relative (NCBI database)	Phylum	% Similarity
J	AB255365	*Scenedesmus* sp. HP1-22-8	Chlorophyta	99%
K	KP726267	*Acutodesmus obliquus* strain KLL-G020	Chlorophyta	99%
L	KM020180	*Uronema minutum* strain SAG 386-1	Chlorophyta	100%
M	AB917135	*Desmodesmus armatus* GM4h	Chlorophyta	100%
N	KT279469	*Scenedesmus* sp. YACCYB49	Chlorophyta	100%
O	EF024210	*Gaertneriomyces semiglobifer* voucher DAOM BR 386	Chytridiomycetes	96%

Bioaugmentation with *Scenedesmus* did not enhance E2 removal yield (Table 8.11), although, the results from this study confirm one of the objectives tested with the bioaugmentation assay, the higher is the amount of biomass, the faster

is the E2 removal. Therefore, it may be stated that all removal mechanisms could be improved. As more biomass there is more biodegradation can occur, and also, more sorption onto the biomass surface can be adsorbed. *Scenedesmus* has not previously tested for E2, or other estrogenic compound, removal and no previous works using that microalgae strain have been described, so it could be said that the use of this microalgae strain does not improve the efficiency of the system.

Estrone has been detected and results are presented in Figure 8.12.

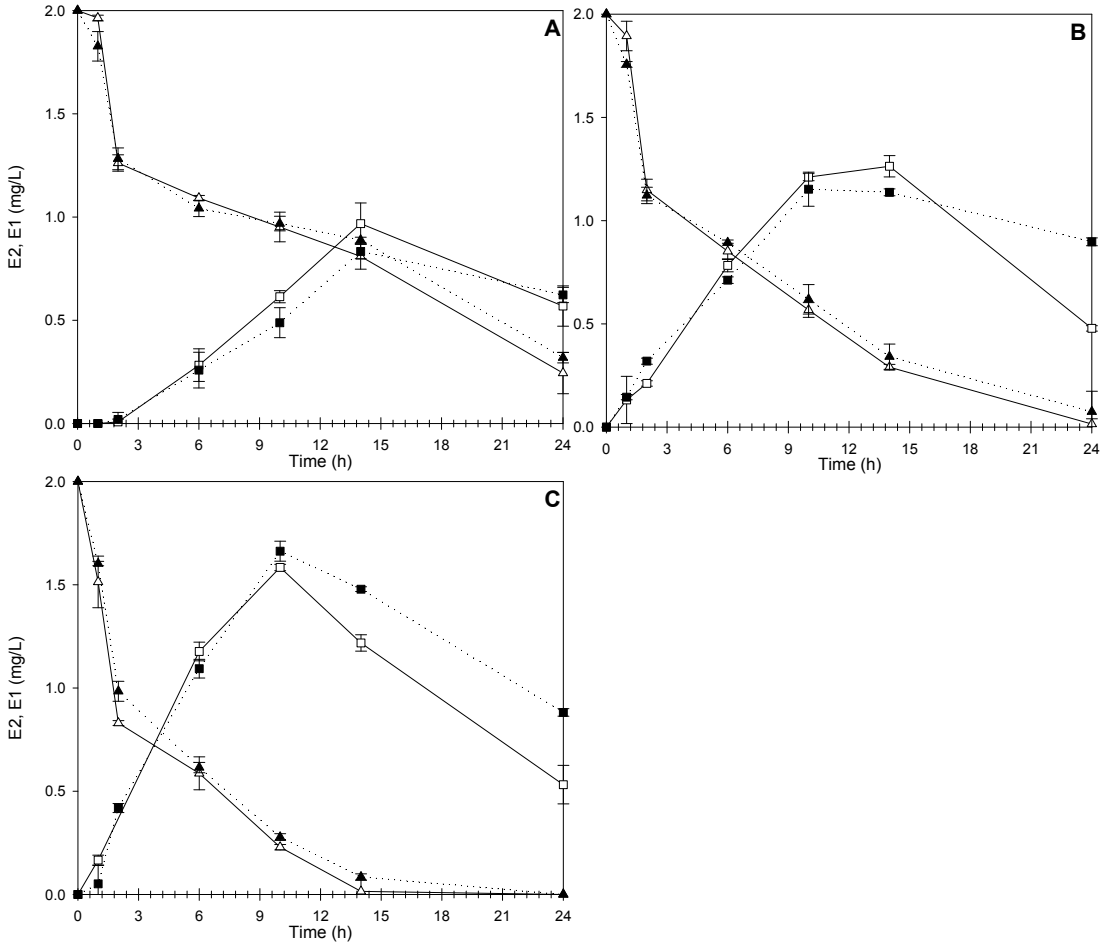


Figure 8.12 E2 removal and E1 formation on E2-D assays. ***A****: biomass control;* ***B****: 3:1 ratio;* ***C****: 1:1 ratio.* (△) *E2 biomass light;* (▲) *E2 biomass dark* (□) *E1 biomass light;* (■) *E1 biomass dark.*

E1 was formed in the consortium control (no bioaugmentation) as well as for both bioaugmentation experiments, in a similar trend. However, the maximum E1 concentration in each case was attained at different moments and at different concentrations according to E2 removal. In consortium control E1 was formed between 0 and 13 h, with a maximum detected concentration of 1 mg/L

and then it decreased to 0.6 mg/L. In experiment 3:1 ratio, E1 concentration attained the maximum (1.3 mg/L) 13 h after the spiking, then it decreased to 0.5 mg/L. Experiment 1:1 ratio had the more rapid E1 production and the maximum concentration was achieved faster (10 h) and at higher concentration, 1.6 mg/L, followed by a rapid decrease down to 0.5 mg/L at the end of the assay.

This is the first work where microalgae bioaugmentation has been conducted for emerging pollutants removal or WW treatment, although this process has been studied in projects in which the growth of microalgae with high lipids concentration was enhanced, usually for biodiesel production (Kumar et al., 2016).

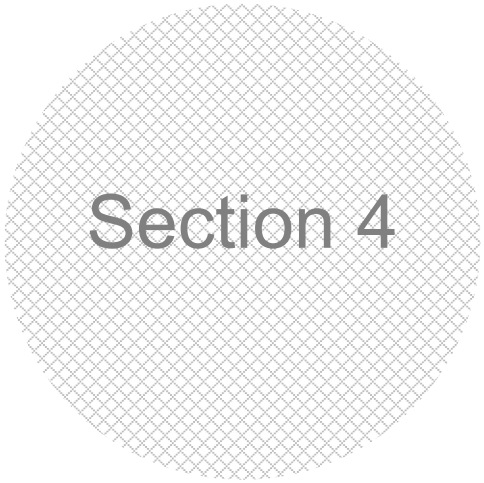
8.4 Conclusions

Seasonal changes have a great impact on TSS concentration. Despite the low temperatures during Period III, the TSS has not been affected since it has been compensated by an increase of light time and solar irradiation. Nutrient removal percentages achieved during Period III have been around 80%.

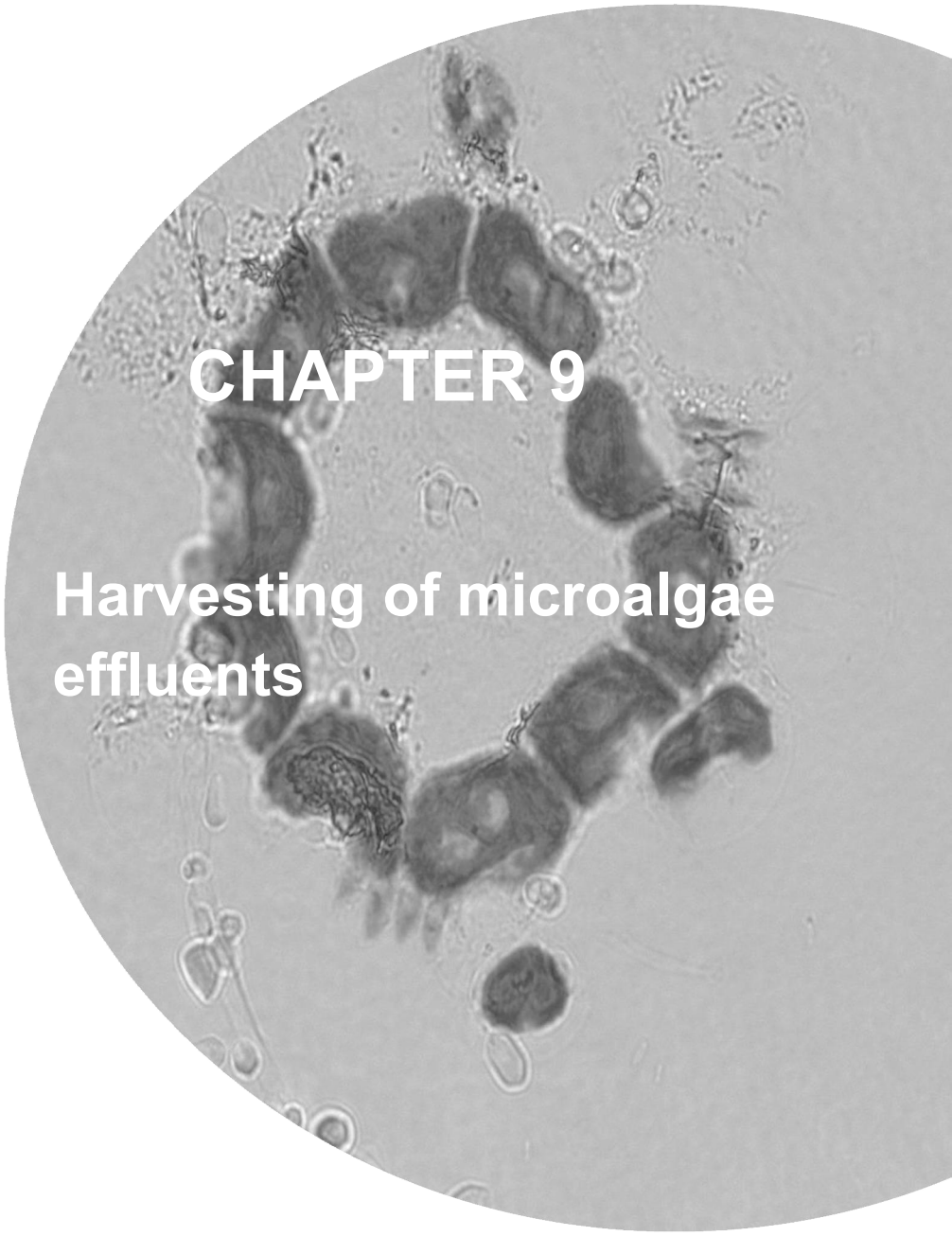
E2 removal rate depends on the biomass concentration. Increasing the TSS also increases the rate for its removal. Little sorption occurs during 24 h, then E2 removal mechanism is due to biodegradation from the PBR consortium.

Laboratory scale and pilot scale E2 removal experiments have a similar behaviour, obtaining removals higher than 70%, although, better performance is obtained in the PBR. From laboratory scale experiments it has been determined that light enhances E2 removal, then the improvement obtained outdoors could be explained by higher irradiation (sun). E2 removal also takes place during dark condition experiments, due to the high microbial diversity from the PBR consortium.

Bioaugmentation enhances E2 removal due to the higher TSS content, although, *Scenedesmus* does not enhance its removal and PBR consortium is more efficient than the use of a microalgal effluent composed mainly by one microalgae specie.



BIOMASS VALORISATION



CHAPTER 9

Harvesting of microalgae effluents

9 Harvesting of microalgae effluents

Abstract

Cost-effective biomass harvesting and thickening pose a challenge for massive microalgae production for further applications (biofuels, high value products). In this study three harvesting techniques have been applied on two real microalgae effluents and on a pure Chlamydomonas reinhardtii culture. Gravity sedimentation was conducted on a settling column. Fast and efficient results were obtained for large particles, flocs and aggregates, whereas for small cell size long time periods were required. Coagulation-flocculation was carried out in jar tests. The study tested the effectiveness of 2 coagulants and 2 flocculants. The highest efficiency was obtained for the coagulant $FeCl_3$ and the flocculant DR3000, although, the efficiency decreased as the microalgae cell size was smaller. The last technique evaluated was co-pelletization of algal biomass with ligninolytic fungi. It is a novel technology that enables the complete removal of single algae cells from the liquid medium in a sustainable way, due to the lack of chemical compounds for biomass separation, which benefits the subsequent use of both biomass and clarified effluent. Again, better results were obtained for effluents containing aggregates. C. reinhardtii culture was successfully pelletized.

9.1 Introduction

Microalgal biomass has ample post-application potential; it could be a source of high-value products for use as biofuels and bioproducts (Mallick, 2002; Mennaa et al., 2015).

One of the main challenges in microalgae biotechnology, and especially for wastewater (WW) treatment, is the efficient and reliable separation of microalgae from the effluent after treatment. It is worth mentioning that the harvesting step is one of the bottlenecks of microalgae biotechnology. The small size of the microalgae, typically in the range of 2–20 μm, the low density difference between algae and growth medium, and diluted concentrations of algal cultures, make harvesting processes a key challenge, especially at industrial scale (Li et al., 2008; Mennaa et al., 2015). Depending on the species, cell density, and culture conditions, harvesting algal biomass has been estimated to contribute 20-30% to the production costs (Christenson and Sims, 2011; Gudin and Therpenier, 1986; Molina Grima et al., 2003). The separation

of the microalgae cells from the liquid phase is particularly important for determining the cost and quality of the product. For low-value bulk products, both the investment and the operational costs must be drastically reduced to make commercial production viable (Wijffels et al., 2010).

Different technologies, including chemical flocculation, biological flocculation, filtration, centrifugation and ultrasonic aggregation have been investigated for microalgal biomass harvesting. In general, chemical and biological flocculation require lower operating costs than filtration, centrifugation, and ultrasonic flocculation, which are more costly; however, the efficiency of the process is increased (Li et al., 2008).

Consequently, the harvesting strategy has to be based on a low energy method in order to overcome the problems and make algae production economically feasible, which would in turn make the system commercially feasible (e.g., to produce biodiesel with algae technology).

The selection of separation techniques depends on the value of the target products, the species, the biomass concentration, the size of microalgae cells of interest, or the desired final product (Brennan and Owende, 2010; Li et al., 2008; Olaizola, 2003).

Gravity or natural sedimentation is a process of solid-liquid separation that separates a feed suspension into a slurry of higher concentration and an effluent of substantially clear liquid (Shelef and Sukenik, 1984). This process depends on the characteristics of the solid and liquid, because the sedimentation rate might be fast or slow depending on that (Olaizola, 2003; Shelef and Sukenik, 1984). Nevertheless, it is the most common harvesting technique for algal biomass in wastewater treatment because of the large volumes treated and the low value of the biomass generated (Nurdogan and Oswald, 1996). However, the method is only suitable for large microalgae (ca. >70 μm) (Brennan and Owende, 2010).

Conventional coagulation is applied to drinking water treatments for algae removal (Ma and Liu, 2002) and due to its effectiveness it has been widely considered as a harvesting technique combined with flocculation. Coagulants and flocculants help to concentrate the particles in suspension more easily.

Coagulation–flocculation is the coalescence of finelly divided suspended cells into larger loosely attached conglomerates, which sink to the bottom of the container. Coagulation involves pH adjustment or electrolyte addition, whereas flocculation involves addition of cationic polymers. Such approaches are quite convenient, because they allow rapid treatment of large quantities of microalgae cultures (Papazi et al., 2010). Several authors reported the importance on pH adjustment to increase the efficiency during the coagulation-flocculation process (Hu et al., 2006; Ma and Liu, 2002; Sirin et al., 2012). In general, the first stage is the aggregation of suspended cells into larger particles, resulting from the interaction of the coagulant with the negatively charged cell wall surface, destabilizing the suspended matter and changing the balance of positive and negatives charges. Then, the colloidal particles come together and aggregates are formed. A higher activity of the cations formed results in a higher efficiency of the coagulation process. Two major forces are involved: electrostatic repulsion forces dominate at large distances (negative-charged cell surfaces repel each other), while intermolecular or Van der Waals attraction forces dominate at very short distances and are stronger than electrostatic forces. Coagulant proportion is an important parameter to consider, if proportions are unbalanced, a restabilization of the colloidal matter may occur due to the over saturation of the superficial charge of the particles, thereby complicating coagulation process (Papazi et al., 2010). The second stage involves the coalescing of aggregates into large flocs that settle out of suspension (Knuckey et al., 2006; Papazi et al., 2010). The flocculant causes the formation of chemical bonds between two or more particles. Flocs formed this way are soft and porous three-dimensional structures. The specific weight of flocs differs from that of water, allowing the separation of the flocs from the liquid to occur, either by sedimentation or flotation. Flocculation depends on either, the cell and the flocculant charges. Numerous chemical flocculants have been tested in the literature (Hu et al., 2006; Ma and Liu, 2002; McGarry, 1970; Papazi et al., 2010). Metal salts (aluminium sulphate, ferric chloride, ferric sulphate, etc.) are generally preferred, because they lead to improve the harvesting efficiency. Flocculant dosage is strongly dependant on microalgae species, concentration of the culture, medium and process conditions.

Although good COD and TSS removal percentages are achieved with this methodology, some problems arise questioning its suitability. On one side, if the agent that is added to the culture is toxic, after harvesting it is essential to treat the culture before its use. It is a negative downstream effect that increases the capital cost of the process and it is also a problem from the safety hazard point of view (Talukder et al., 2014). Another drawback arises when the cell wall is damaged due to the addition of an agent, or the dosage applied is harmful to the cell. These facts may have a negative downstream effect. Another negative effect appears affecting the lipid content when microalgal biomass is recovered for biodiesel production, the addition of coagulants and flocculants produce chemical changes in the original lipids. These drawbacks can be minimized with the novel technique of co-culture microalgae with other microorganisms such as fungi (Talukder et al., 2014; Xie et al., 2013; Zhang and Hu, 2012b).

Co-pelletization using filamentous fungi is an attractive bioflocculating technique because of the self-pelletization of the fungi, which, in combination with microalgal biomass, could trap them at high efficiency. Fungal self-pelletization has been observed for numerous filamentous strains leading to development of aggregates/pellets, and several authors for biodiesel production have applied this technique (Gultom and Hu, 2013; Liu et al., 2008; Xia et al., 2014; Zhang and Hu, 2012b). Some authors reported that some filamentous fungi that naturally exist in activated sludge could immobilize or entrap the sludge solids by forming bio-flocculation/coagulation and strengthen the flocculation structure due to their unique filamentous properties (Xie et al., 2013; Zhou et al., 2013).

Numerous theories have been proposed to explain the fungal pelletization process, which can be categorized into two types of mechanisms (Liu et al., 2008; Ryoo and Choi, 1999). The first one is coagulative mechanism, where spores coagulate in the early stage of cultivation and develop into pellets through their intertwining hyphae. The second one is non-coagulative mechanism, where the spores germinate into hyphae, and then intertwine into pellets. However, the detailed mechanisms of the fungal-algal interactions are still not clear. For several authors, the ionic attraction between microalgae cells and fungal mycelium due to the difference in surface charge was the main reason for the immobilization of microalgae on fungal mycelium

(Talukder et al., 2014; Wrede et al., 2014; Zhou et al., 2013). Fungal hyphae and mycelia contain polysaccharides that were shown to be positively charged and therefore can potentially neutralize the negative charges on the algae surface. The presence of proton-active carboxylic, phosphoric, phosphodiester, hydroxyl and amine functional groups, allows the attachment of microalgae cells to the fungal cell wall (Wrede et al., 2014). Several authors are in accordance that co-pelletization efficiency depends on both, fungi and microalgae strains as well as culture conditions. Fungal assisted harvesting technology does not require the addition of chemicals or inputs of energy and has been shown to be efficient for one microalgal strain, *Chlorella vulgaris,* co-cultured with different fungi strains (Gultom and Hu, 2013). Xie et al. (2013) used the filamentous oleaginous fungus *Cunninghamella echinulate,* whereas Zhou et al. (2013) and Zhang and Hu (2012b) used the filamentous fungus *Aspergillus oryzae* and *A. niger*, respectively. The fungal/algal co-pelletization process is similar to the current flocculation process in which fungal hyphae serve as the flocculants and the attraction between fungi and algae cells can be explained through the zeta potential at each cell surface. Although the detailed co-pelletization methods have been described by different researchers, the effect of co-cultured pellets to downstream processing, including extraction of lipids, conversion to fuels, and utilization of lipid extracted algae/fungi for co-products are not substantially elucidated yet (Gultom and Hu, 2013). The choice of fungi strains will be the key issue because it determines the overall pelletization efficiency. Furthermore, it will have a direct impact on the subsequent processes. If this technology can be applied to the commercially important freshwater and seawater algae species used for biodiesel production, the procedure could offer a solution to at least one of the major problems associated with the energy-intensive and costly harvesting processes. It could be extensively applied to other microalgae processes, such as the harvesting of microalgal biomass from wastewater treatments for further methanization of the biomass (Gultom and Hu, 2013; Wrede et al., 2014).

In this chapter, three harvesting techniques have been studied in two real microalgae effluents coming from two pilot scale microalgal photobioreactors and a pure culture of *Chlamydomonas reinhardtii* in order to assess the

harvesting differences between pure and wild cultures. The techniques are natural sedimentation, coagulation-flocculation process and fungal co-pelletization.

9.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

9.2.1 Microalgae effluents

Three different microalgae effluents were tested for the harvesting tests. The first effluent tested (PBR-1 and PBR-2) came from the microalgal photobioreactor (PBR) previously described in Chapter 7, in which urban wastewater was treated. The second effluent (IPBR-1 and IPBR-2) came from an industrial photobioreactor (IPBR) from a winery industry, that was exposed to artificial light/dark cycles (14/10 h) and daily temperatures ranged from 34±3 ºC to 15±1 ºC. Agitation was achieved by addition of pressurized CO_2 and the microalgae-bacteria consortium predominantly contained *Scenedesmus* (Chapter 8). The third effluent (CR-1) consisted of *Chlamydomonas reinhardtii* suspension, previously cultured in axenic conditions. Characteristics of each effluent are presented in Table 9.1.

Table 9.1 Characteristics of the effluents used for harvesting experiments.

Parameter	PBR-1	PBR-2	IPBR-1	IPBR-2	CR-1
TS (mg/L)	1500	-	1237	2348	2620
TSS (mg/L)	454	162	548	544	240
VS (mg/L)	1020	-	-	1357	2380
VSS (mg/L)	438	150	484	958	-
OD (λ_{683nm})	-	-	0.943	2.851	0.610
$COD_{soluble}$ (mg/L)	68.6	-	176.1	-	2030
pH	8.1	7.9	8.7	8.7	8.6

9.2.2 Natural sedimentation

Natural sedimentation was conducted following the guidelines proposed by Nollet (2000). 1 L or 100 mL transparent glass measuring cylinder ([height/diameter] ratio≈5.7 and 5.8, respectively) were filled with 1 L or 100 mL microalgal suspension. It was kept vibration free and disturbance of the settled matter was avoided. Height decrease from the solid-liquid interphase and time values were recorded in order to obtain the sedimentation curve and the sedimentation velocity, using equation Eq. 9.1, which corresponds to the lineal zone of the sedimentation curve.

$$v_s = \frac{H_0 - H_f}{\Delta t} \qquad (Eq. 9.1)$$

Where,

v_s is the sedimentation velocity (m/min)

H_0 is the initial height of the suspension (m)

H_f is the final height of the suspension (m)

Δt is the time between the two measured heights (min)

After sedimentation, cell concentration of the supernatant and the settled fraction were determined by absorbance at 683 nm in a spectrophotometer. TSS determination of the two fractions was also determined.

9.2.3 Coagulation-flocculation

The jar test is a method of simulating a full scale water treatment process, which provides to the system operators a reasonable idea of the way that a chemical treatment will behave and operate with a particular type of raw water. The method entails adjusting the amount of chemicals and the sequence in which they are added to samples of raw water held in jars or beakers (Satterfield, 2010).

Standard jar tests were conducted in a mixer six-paddle jar test apparatus (Flocculator SW1, Stuart Scientific) using 4 coagulants ($FeCl_2$, DW212, AC50

and Al_2O_3) and 4 flocculants (DR3000, GO2030, AS-77, CS209) at different doses recommended by the manufacturer. The beakers were filled with 300 mL of microalgal suspension and stirred at 200 rpm. The coagulant was added at the corresponding dose, mixed at 200 rpm for 2 minutes, and then the speed was reduced to 20 rpm and the flocculant was added at the corresponding dose. The stirring was maintained for 15 minutes. The stirrers were turned off to allow the flocs to settle to the bottom of the jar, or possibly float for 15 minutes. Supernatant samples were taken for TS, TSS and COD determination.

9.2.4 Co-pelletization

Different methodologies were applied for co-pelletization experiments (EXP-1, EXP-2 and EXP-3). The fungus used was *Trametes versicolor* in either forms of mycelium and pellets depending on the experiment. Mycelium and pellets were obtained as previously described by Font et al. (2003). All co-cultivation experiments and relative evaluations were performed in triplicate. The fungi-algae mixtures were shaken at 130 rpm and maintained at 25±1ºC.

9.2.4.1 Co-pelletization EXP-1

EXP-1 (Figure 9.1) was performed in a 1 L Erlenmeyer flask containing 50 mL of fungal defined media, 200 mL of PBR-1 effluent and 1 mL *T. versicolor* mycelia. It was cultured for 6 days.

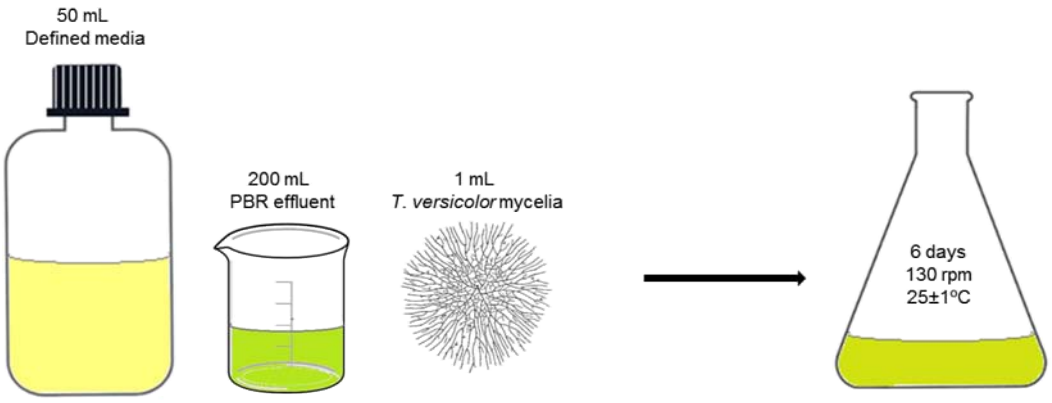


Figure 9.1 EXP-1 methodology for fungal and microalgal co-pelletization.

9.2.4.2 Co-pelletization EXP-2

EXP-2 (Figure 9.2) was performed in a 1 L Erlenmeyer flask in two steps. The first step lasted 3 days and it consisted on pellet formation by the fungus. 250 mL of *T. versicolor* defined media and 1 mL fungal mycelia were introduced into the Erlenmeyer flask for 3 days. The second step involved the immobilisation of the microalgal suspension by *T. versicolor* pellets for 3 more days. Two methodologies were carried out (EXP-2A and EXP-2B).

EXP-2A contained 200 mL PBR-1 effluent in a 1 L Erlenmeyer flask and *T. versicolor* pellets formed in step 1 were added. The pellets were filtered and washed with ultrapure water prior to microalgal contact. EXP-2B contained 200 mL of *T. versicolor* pellets suspension formed in step 1 and the centrifuged algae (6000 g^{-1}, 10 min) corresponding of PBR-1 effluent in a 1 L Erlenmeyer flask.

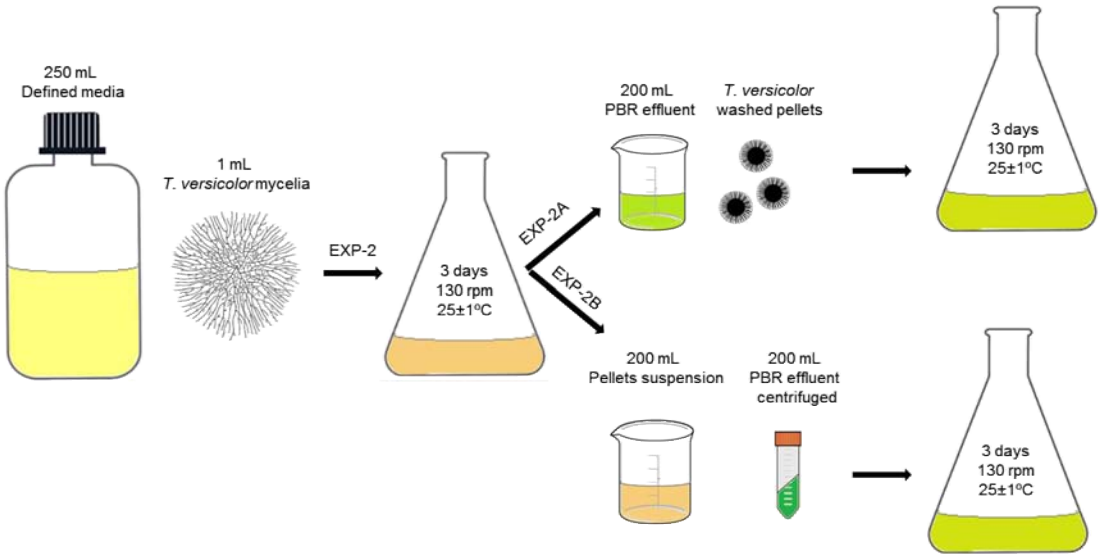


Figure 9.2 EXP-2 methodology for fungal and microalgal co-pelletization.

9.2.4.3 Co-pelletization EXP-3

EXP-3 (Figure 9.3) was performed at three different microalgae: fungal defined media volumetric ratios (1:5, 1:2, and 1:1 (v/v)) for all microalgae effluents (Table 9.1) to reach a total co-cultivation volume of 120 mL in 500 mL Erlenmeyer flasks. 0.5 mL *T. versicolor* mycelia were added to each Erlenmeyer flask. The fungus and microalgae mixtures were then cultivated: PBR-2 and IPBR-2 for 3 days, whereas CR-1 was cultured during 6 days.

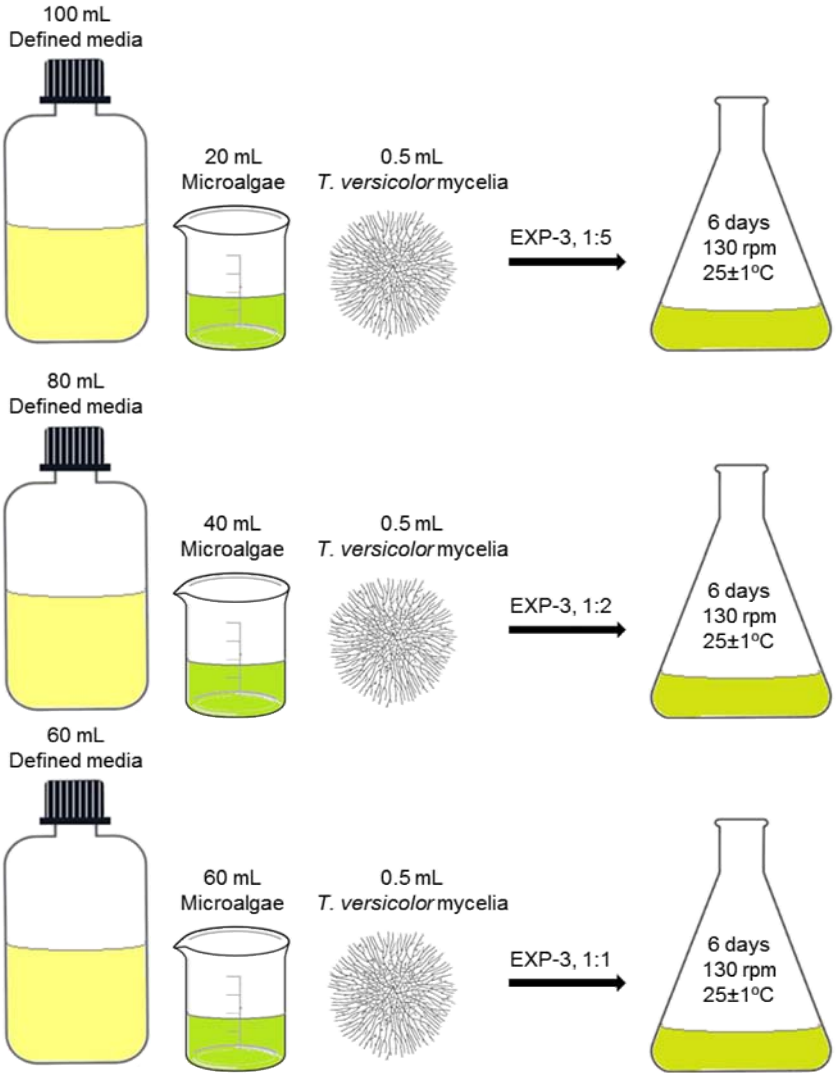


Figure 9.3 EXP-3 methodology for fungal and microalgal co-pelletization.

9.3 Results and discussion

9.3.1 Natural sedimentation

9.3.1.1 PBR sedimentation assays

PBR effluent was collected for biomass sedimentation velocity determination. The effluent was collected when the PBR was working at two different hydraulic retention time (HRT), in order to compare biomass settable differences. The HRT of the PBR was 8 days when the effluent for the first sedimentation experiment (Sed-1, PBR-1 effluent) was collected, corresponding to the autumn season (September-October). For the second sedimentation experiment (Sed-

2), the effluent was collected (PBR-2 effluent) during winter (January-February) and the HRT of the PBR was 12 days.

The gravity sedimentation profile for Sed-1 can be observed in Figure 9.4. The final sedimentation in the measuring cylinder can be observed in Image 9.1. The sedimentation profile follows a linear tendency. Initial and final parameters, together with the solid removal percentage are presented in Table 9.2. Sedimentation velocity was calculated using equation 9.1, corresponding to 0.049±0.005 m/min and 99% of TSS were removed in 7 minutes.

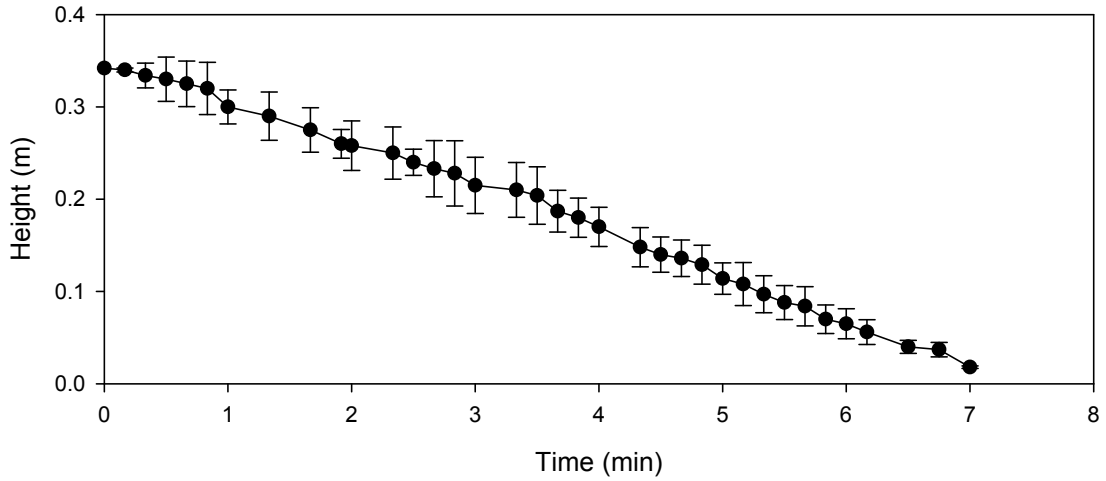


Figure 9.4 PBR-1 effluent sedimentation curve, Sed-1 assay.

Table 9.2 PBR-1 effluent parameters before and after the natural sedimentation, Sed-1 assay.

Parameter	Initial	Final, supernatant	% removal
TS (mg/L)	1500	500	63
TSS (mg/L)	454	4	99
VS (mg/L)	1020	140	86
VSS (mg/L)	438	22	95
OD (λ_{683nm})	-	0.003	-

The particles from the suspension are described as flocculating instead of discrete particles, since they change in size, shape and perhaps specific gravity; as a result, there is entrapment of water and smaller microalgae cells in interstitial spaces (Image 9.2). For that type of sedimentation Stoke's law is not applicable since as their size increases, they settle at a faster velocity.

Image 9.1 Final natural sedimentation, Sed-1 assay.

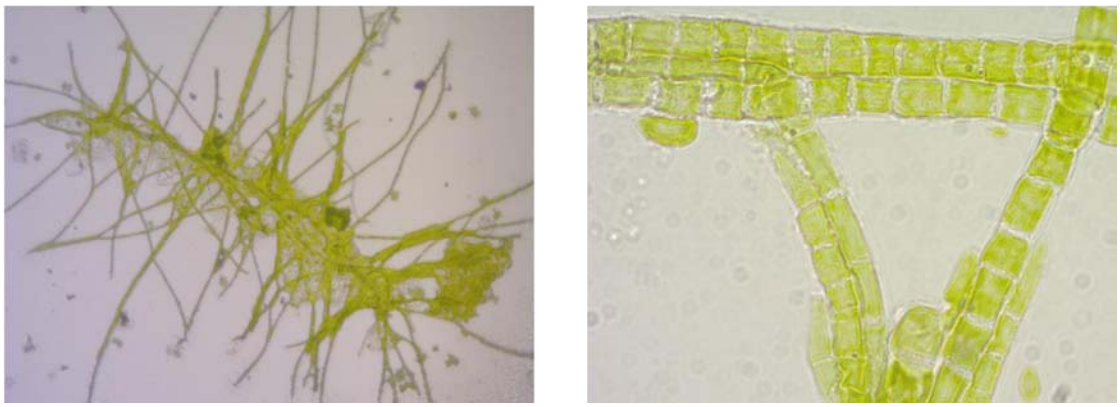


Image 9.2 PBR effluent microscopic images, Sed-1 assay. Left: 100x magnification; Right: 1000x magnification.

The filamentous microalgae species in the PBR-1 have the ability to autoflocculate and self-aggregate, immobilising the smaller microalgae cells (Image 9.2). In Chapter 7, microbial diversity of the PBR was described reporting the presence of the cyanobacteria *Phormidium*, which has the advantage of the self-aggregation capacity. Previous authors have reported lower sedimentation velocities than the values obtained for the microalgae PBR—1 effluent. Olaizola (2003) obtained a velocity of 0.01 m/min for *Haematococcus* cells since they become large and heavy during the carotenogenesis and encystment phase, coalescing into larger flocs that settle out of the growth medium quickly. This phenomenon has also been previously reported by Olguín (2003), who added *Phormidium bohneri* (a filamentous self-aggregating cyanobacteria specie) in WW treatment of farm effluents. Microalgal WW treatment based-technologies are algal-bacterial processes that

may form flocs ranging from 400 to 800 μm, which are easily removed by gravity, increasing the sedimentation velocity. Cell surface properties of the algae, extracellular polymeric substances (EPS) and other factors (e.g., content of calcium) influence the formation and stability of flocculant algal-bacterial biomass. However these mechanisms are still poorly understood and hard to induce (Garcia et al., 1998; Gutzeit et al., 2005). Moreover, autoflocculation is usually associated with carbon dioxide limitation as well as consumption of reserve sources such as lipids; factors that may cause changes in the pH and variations of the intracellular content over a period of time (Sukenik and Shelef, 1984). Gutzeit et al. (2005) studied the algal-bacterial sedimentation using a suspension of *Chlorella vulgaris* and activated sludge from a municipal wastewater treatment plant (WWTP); after 10 min of sedimentation nearly the entire biomass was settled at the bottom of the glass vessel, resulting in a supernatant with 98.2% TSS removal.

Natural sedimentation was evaluated from the same PBR, but the operation mode as well as the season was different between assays. That was reflected on the biomass composition and it was translated to the settling capacity of the system. Natural sedimentation profile for Sed-2 assay could not be obtained, due to the absence of an interphase, as can be seen in Image 9.3, for the tube settling experiment at different times. That fact is attributed to the poor settling characteristics of the microalgal biomass. During that period (January-February) the presence of filamentous species decreased and more unicellular species were observed (Image 9.4). At the initial time some aggregates were observed. After 30 minutes, the flocs were distributed at the top or bottom of the measuring cylinder depending on their density, leaving a clear green liquid in between layers. The density of the cells depends on the microalgae composition; usually the presence of lipids decreases the density of the cell allowing them to float. 7.5 h later, both layers were maintained and the liquid phase was clearer. 24 h later the liquid phase turned into an almost transparent clear liquid. Sedimentation velocity was calculated using equation Eq. 9.1, corresponding to $2.29 \cdot 10^{-4}$ m/min at 24 h, achieving a final removal of TSS in the supernatant of 88%. Initial, 24 h and final (4 days) parameters, together with the removal percentages are presented in Table 9.3.

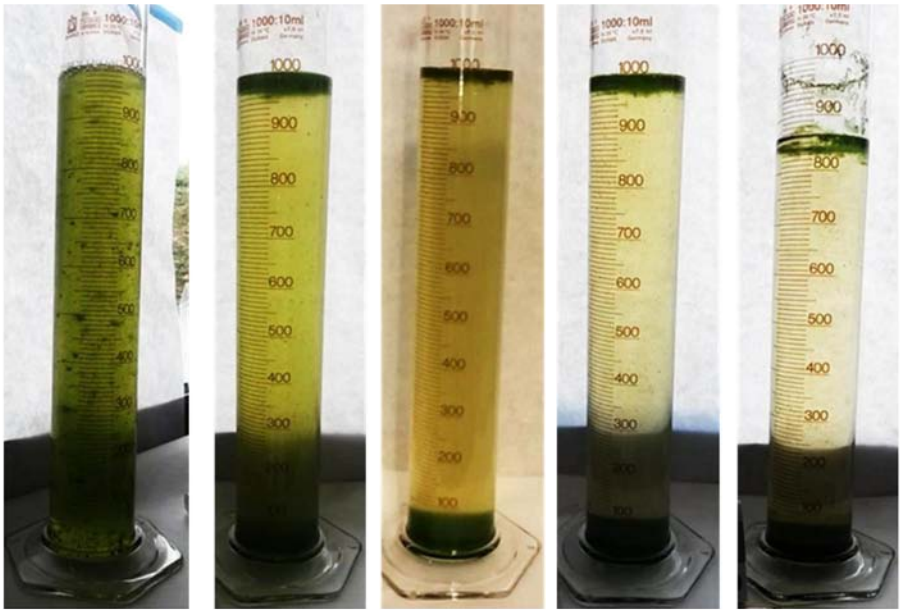


Image 9.3 Natural sedimentation, Sed-2 assay. From left to right: 0h, 0.5h, 7.5h, 24h and 4 days.

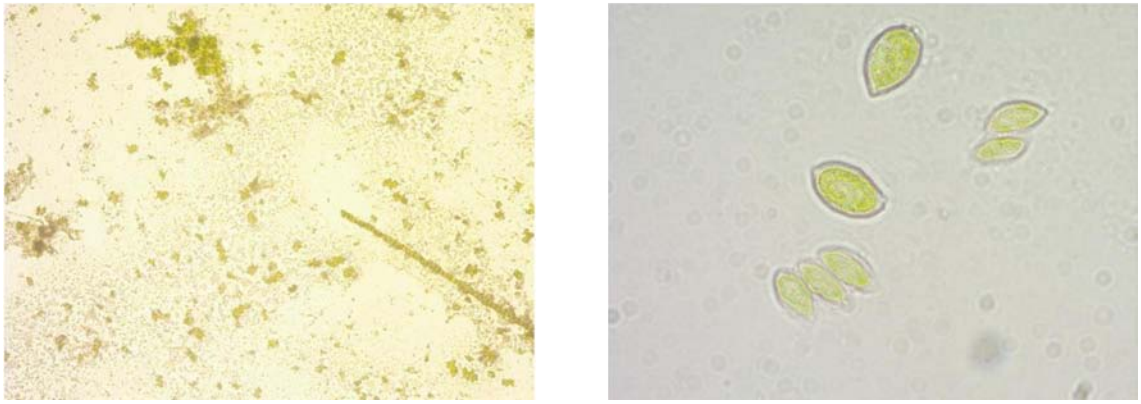


Image 9.4 PBR-2 effluent microscopic images, Sed-2 assay. Left: 100x magnification; Right: 1000x magnification.

Table 9.3 PBR-2 effluent parameters before and after the natural sedimentation, Sed-2 assay.

Parameter	**Initial**	**Final$_{24h,}$ supernatant**	**% removal$_{24h}$**	**Final$_{4d,}$ supernatant**	**% removal$_{4d}$**
TSS (mg/L)	162	20	88	16	90

From the obtained results, microalgae cells from Sed-2 assay can be classified as flocculant (the ones that have a rapid settling velocity, creating a fast layer at the bottom) and discrete particles, (the ones found in the interphase), which have more difficulties to settle down. Discrete particles does not have the tendency to flocculate and they settle as individual entities without significant interaction with neighbouring particles, moreover, their size, shape and specific gravity does not change with time.

The difference in the results obtained from both sedimentation assays using PBR effluent shows the difficulties of microalgae harvesting in outdoor systems where the environmental conditions are in constant change. Natural sedimentation gives better results for filamentous microalgae strains (PBR-1 effluent, Sed-1 assay) rather than for unicellular microalgae (PBR-2 effluent, Sed-2 assay). Park et al. (2013) stated that the harvesting efficiency was highly dependent on the dominant algae in the high rate algal pond (HRAP), and their studies were carried out on a settling unit composed of two settlers working at an HRT of 3 h (summer period) or 6 h (winter period), having variations on microalgal biomass composition. Removals between 75-85 % were achieved when *Pediastrum* sp. was dominant. Similar values are reported in this study (Table 9.2). The good *Pediastrum* sp. sedimentation rate can be attributed to its size. Despite it is not a filamentous specie; it is composed of circular colonies (made up of 8, 16 or 32 individual cells) forming high colony sizes (up to 40 μm), whereas *C. vulgaris* or *C. reinhardtii* have a diameter ranging from 2 to 10 μm.

Since previous authors reported that algae harvesting depends on the cell size, microalgae composition and other parameters are also important (Brennan and Owende, 2010; Li et al., 2008; Olaizola, 2003). Natural sedimentation of PBR effluent was compared with natural sedimentation of an effluent proceeding from an industrial microalgal bioreactor (IPBR-1) (Sed-3 assay), where *Scenedesmus* was the main specie found in the microalgae-bacteria consortium.

9.3.1.2 IPBR sedimentation assay

Image 9.5 shows the tube test (Sed-3 assay) at different times for the IPBR-1 effluent. In this case, contrary to Sed-2 the interphase can be seen. A thin layer was also located at the top, due to the low density of the cells. The settling velocity obtained was $1.48 \cdot 10^{-5}$ m/min, lower than PBR-2 effluent. In Table 9.4 the microalgal biomass removal percentages were presented. High removals were achieved but long time periods are required due to the low settling of the microalgae. Therefore, the use of natural sedimentation for the harvesting of

this effluent containing discrete microalgal particles (mainly *Scenedesmus*) (Image 9.6) is not efficient.

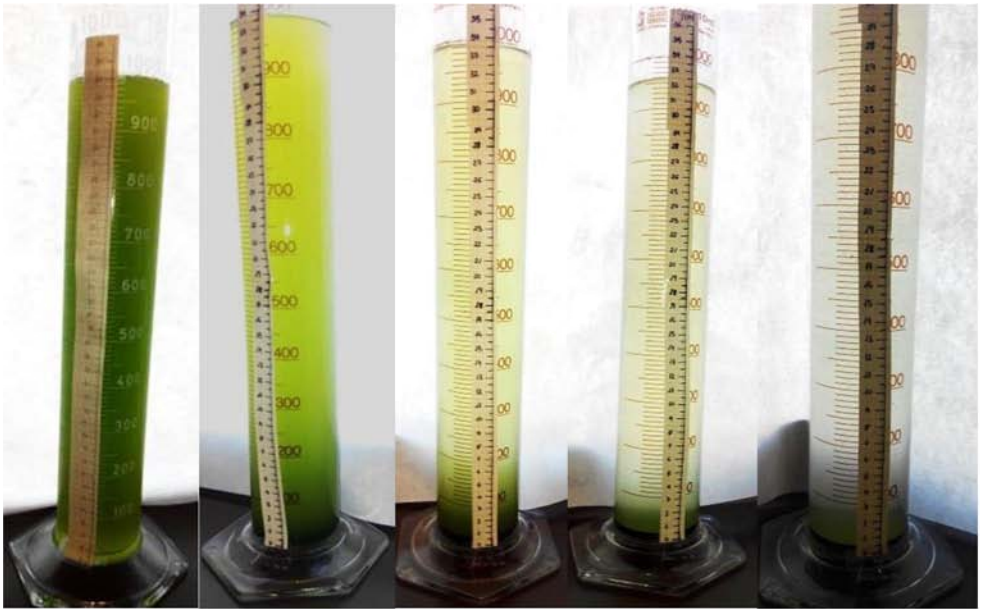


Image 9.5 Natural sedimentation, IPBR-1 effluent, Sed-3 assay. From left to right: 0h, 24h, 48h, 8 days and 14 days.

Table 9.4 IPBR-1 effluent parameters before and after the natural sedimentation, Sed-3 assay.

Parameter	**Initial**	**Final$_{24h}$, supernatant**	**% removal$_{24h}$**
TS (mg/L)	29100	1360	95
TSS (mg/L)	548	44	92
OD (λ_{683nm})	0.943	0.059	94

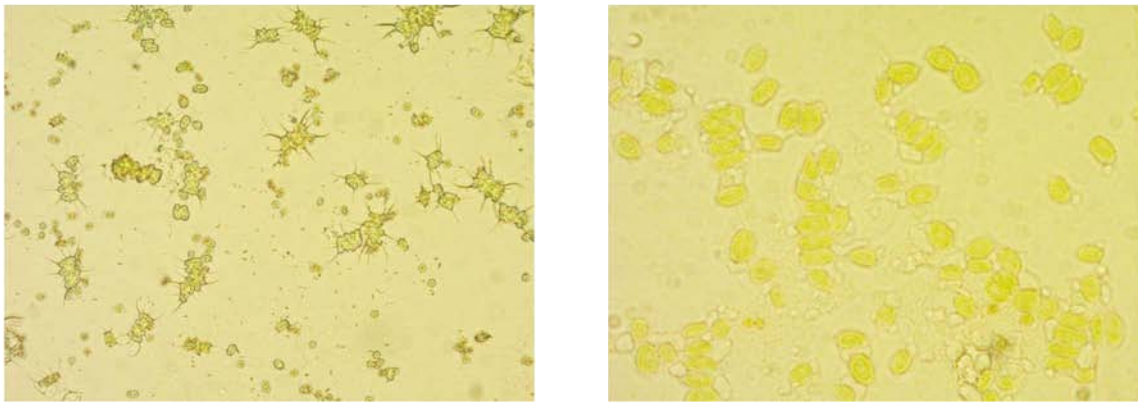


Image 9.6 IPBR-1 effluent microscopic images. Left: 400x magnification; Right: 1000x magnification.

Finally, this methodology was applied to an axenic microalgal culture (CR-1), in order to evaluate the feasibility of this harvesting technique on a pure culture (Sed-4).

9.3.1.3 CR sedimentation assay

Natural sedimentation was conducted using a pure microalgae culture of *Chlamydomonas reinhardtii* grown under axenic conditions, since it is considered a model organism for science studies. *C. reinhardtii* sedimentation velocity was very low, $2.94 \cdot 10^{-5}$ m/min (Eq. 9.1). It took 14 days to obtain a clear supernatant, due to the fact that *C. reinhardtii* cells are small and have a low density (Image 9.7).

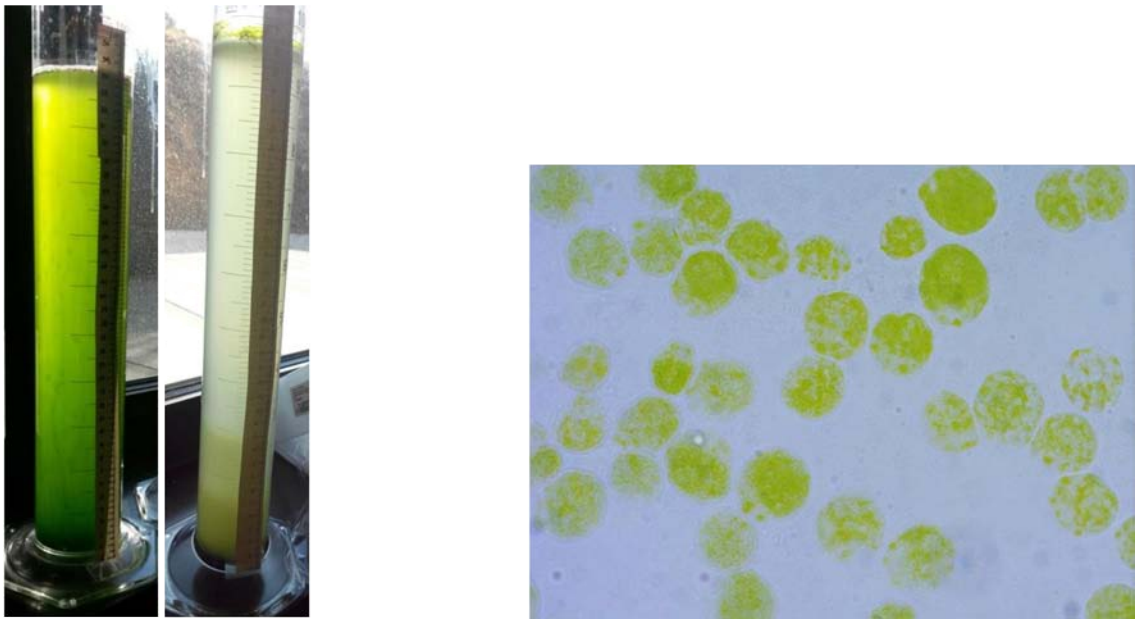


Image 9.7 Left: Chlamydomonas reinhardtii natural sedimentation at time 0 and 14 days after, Sed-4 assay; Right: Chlamydomonas reinhardtii culture microscopic image, 1000x magnification.

The initial and final (14 days) microalgal biomass parameters, together with the removal percentages are presented in Table 9.5. Removal percentages are lower in comparison with the obtained for Sed-1 assay (Table 9.2). The results confirm the importance of microalgal species behaviour in terms of settling. Gravity sedimentation is not an efficient harvesting technique for *C. reinhardtii* cultures, it requires long periods of time to attain the clarification of the suspension, and so it is not efficient and fast enough.

Table 9.5 Chlamydomonas reinhardtii parameters before and after the natural sedimentation, Sed-4 assay.

Parameter	Initial	Final supernatant	% removal
TS (mg/L)	2620	1950	26
TSS (mg/L)	240	120	50
OD (λ_{683nm})	0.610	0.229	62

Natural sedimentation is a low-cost technique for microalgae harvesting, but as it can be seen, the efficency varies depending on the microalgal biomass composition and morphology. Furthermore, real microalgal photobioreactors biomass is in constant change due to the uncontrolled conditions the system is exposed to. It can be said that this methodology is not efficient for microalgal-bacterial consortia since, depending on the majority specie, the biomass will settle down easily (Sed-1) or on the contrary, long periods of time will be required to achieve high TSS removal in the clarified phase (Sed-2 and Sed-3). For that reason, other low-cost harvesting techniques have been studied.

9.3.2 Coagulation and flocculation

There is a wide range of coagulants and flocculants in the market for wastewater treatment. The most common coagulants used include ferric sulphate, aluminium sulphate, and ferric chloride (Jiang and Lloyd, 2002). Metal salts (aluminium oxide and ferric chloride) are largely preferred in flocculation processes, because they lead to high efficiencies. Furthermore, high concentrations of colloidal organic matter probably decrease flocculant efficiency, which might explain the higher requirements of ferric salts, recorded. The need for high coagulant dosages due to the presence of organic matter has been previously described in microalgae cultures (de Godos et al., 2011; Jiang et al., 1993)

In this study two coagulants and two flocculants were tested for microalgae harvesting. $FeCl_3 \cdot 6\ H_2O$ has been chosen as a coagulant candidate, because it is widely used in an extensive range of concentrations. It has been used for the treatment of urban wastewater and also on wastewater treatment of industries that are concerned with the production of potato chips (Abdel-Shafy et al.,

1987), soap/detergent (Amoo et al., 2004) or cork processing (Peres et al., 2004). The other coagulant tested has been Al_2O_3, since aluminium (Al^{3+}) has also been used for wastewater treatment (Hu et al., 2006; Omoike and Vanloon, 1999) as well as for industrial effluents such as textile ones (Kobya et al., 2003). Commercial flocculants, DR3000 and GO2030, were used in the study according to the commercial guidelines, and because its use for wastewater treatment processes. DR3000 is effective in a wide range of pH, it can work alone or in combination with other standard products used in water treatment, its use is very common in all kind of water purification and sludge dewatering applications. The second flocculant tested is GO2030, a universal anionic dispersion flocculant, used for the elimination of suspended materials, in the chemical industries and in a wide range of industrial effluents (i.e., textiles, paintings, tanning, food, metallurgical and hydrocarbon derivatives).

Coagulation-flocculation efficiency is measured in terms of TSS removal as well as COD removal in the clarified phase or supernatant. The more TSS removal percentages, the more flocs and aggregates are formed and the better is the harvesting of the microalgal biomass in the effluent. COD gives information about the oxidative matter left in the supernatant as well as if the effluent can be released to the environment accomplishing the legislation limits.

9.3.2.1 PBR coagulation-flocculation assays

Two coagulant-flocculant combinations at different concentrations were tested in the PBR-1 effluent (PBR operating at 8 days HRT) in order to determine the efficiency of this harvesting technique.

$FeCl_3$ coagulant was applied from 50 to 300 ppm, since de Godos et al. (2011) reported maximum *Chlorella* harvesting efficiencies at a dosage of 250 ppm. DR3000 flocculant is very stable and it is not affected by hard water. Microalgae cells have negative charges on the surface of their cell walls (at natural water pH), which prevent cells from aggregating in suspension, so they should be neutralized by using cationic flocculants. Table 9.6 shows the COD and TSS removal percentages for $FeCl_3$ coagulant and DR3000 flocculant.

The highest COD and TSS removal was obtained for 250 ppm $FeCl_3$, achieving 88% and 75% removal, respectively.

The coagulant has been described as a good candidate for water with high turbidity levels and it forms dense and compact flocs. The formation of such flocs may be beneficial for the flocculation process, decreasing the concentration of suspended solids. Image 9.8 shows how the coagulant-flocculant dosage affects the aggregates formation during the jar test. $FeCl_3$ dosage has an impact during the process; de Godos et al. (2011) reported that an increase in concentrations of up to 150 and 250 mg/L $FeCl_3$ severely reduced the removals to 14±9% and 26±2%, respectively in some algae species, whereas in this study removal efficiency was maintained in the same range for all 4 doses tested (69-75%), although at the highest dose (300 ppm $FeCl_3$) the removal efficiency had the lowest value (69%).

Table 9.6 COD and TSS removal percentages after the jar test using $FeCl_3$ coagulant at different doses (ppm) and DR3000 flocculant (5 ppm).

Coagulant-flocculant dosage (ppm)	COD removal (%)	TSS removal (%)
50 $FeCl_3$ + 5 DR3000	27	72
175 $FeCl_3$ + 5 DR3000	46	72
250 $FeCl_3$ + 5 DR3000	88	75
300 $FeCl_3$ + 5 DR3000	64	69

Al_2O_3 coagulant is commonly used for drinkable and residual water treatment in the range of 10 to 50 ppm (manufacturer specifications, Aquazur, Madrid, Spain). Al_2O_3 coagulant was applied at 50 ppm using DR 3000 flocculant and also the flocculant GO2030. COD and TSS removal percentages are presented in Table 9.7.

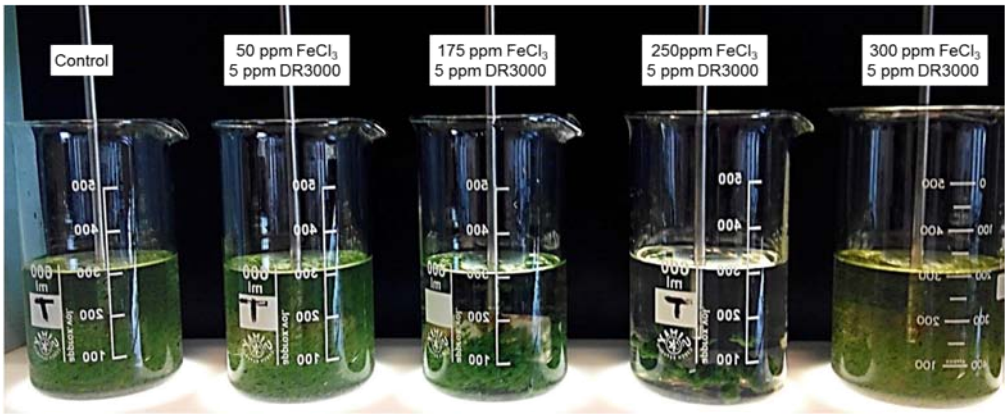


Image 9.8 Jar test for $FeCl_3$ coagulant and DR3000 flocculant at different dosage.

The best COD removal percentages, 72%, were obtained by using 10 ppm of GO2030, however, the TSS major removal was obtained by using 5 ppm DR3000, although that combination only reduced by 48% the COD from the suspension. Then, for the selection of the best flocculant combination using the Al_2O_3 coagulant, 10 ppm GO2030 was chosen, since the COD removal was higher and the TSS removal was 59% instead of 63% (obtained with 5 ppm DR3000).

Table 9.7 COD and TSS removal percentages from the supernatant after the jar test using Al_2O_3 coagulant (50 ppm) and flocculants at different doses.

Coagulant-flocculant dosage (ppm)	COD removal (%)	TSS removal (%)
50 Al_2O_3 + 5 DR3000	48	63
50 Al_2O_3 + 10 DR3000	31	57
50 Al_2O_3 + 5 GO2030	36	47
50 Al_2O_3 + 10 GO2030	72	59

An economic evaluation of the two coagulant-flocculant combinations (250 ppm $FeCl_3$ + 5 ppm DR3000 and 50 ppm Al_2O_3 + 10 ppm GO2030) is presented in Table 9.8.

The use of the coagulant $FeCl_3$ (250 ppm) and the flocculant DR3000 (5 ppm) gives the best results with regards to TSS and COD removals, combining the lowest cost. This harvesting process decreases the pH, however, the final pH is in the range stablished by the legislation (Decret Legislatiu 3/2003).

Table 9.8 TSS and COD removal percentages from the supernatant. Initial and final pH values. Coagulation-flocculation cost of the selected dosages.

Coagulant-Flocculant dosage (ppm)	COD removal (%)	TSS removal (%)	$pH_{initial}$	pH_{final}	Cost (€/$L_{treated}$)
250 $FeCl_3$ + 5 DR3000	88	75	8.1	6.3	$2.74 \cdot 10^{-6}$
50 Al_2O_3 + 10 GO2030	72	59	8.1	n.a[a]	$4.29 \cdot 10^{-6}$

[a] *n.a.: data not available*

The use of coagulation-flocculation for the PBR-1 effluent harvesting gives worse results in comparison with natural sedimentation. Moreover, coagulation-flocculation implies an additional cost and the use of chemicals damage the

environment, also the subsequent use of the biomass could be affected. No further coagulation-flocculation investigation on that effluent was conducted. However, the best coagulant-flocculant combinations were applied on IPBR-1 and CR-1 effluents in order to know the differences among them and study the settling velocity after the coagulation-flocculation process.

9.3.2.2 IPBR coagulation-flocculation assay

As previously described IPBR-1 effluent has a poor settling velocity due to the small microalgae cells. The jar test was applied to the IPBR-1 effluent followed by sedimentation in order to obtain the new settling velocity. Table 9.9 shows the COD and TSS removal results obtained for the 2 combinations of coagulant-flocculant chosen in the previous experiments, as well as the pH variation.

Table 9.9 IPBR-1 coagulation-flocculation COD and TSS removal parameters and pH values.

Coagulant-Flocculant (ppm)	COD removal (%)	TSS removal (%)	$pH_{initial}$	pH_{final}
250 $FeCl_3$ + 5 DR3000	92	93	8.72	6.17
50 Al_2O_3 + 10 GO2030	86	91	8.72	n.a[a]

[a] *n.a.: data not available*

In this case better results were also obtained for $FeCl_3$-DR3000 combination, achieving COD removal of 92% and a TSS reduction of 93%, whereas these values for Al_2O_3-GO2030 combination were lower, 86% and 91%, respectively. pH was lower at the end of the treatment but it was still within the legislative limits (Decret Legislatiu 3/2003).

Settling velocity after the coagulation-flocculation process using 250 ppm $FeCl_3$ and 5 ppm DR3000 was evaluated following the tube methodology, 1 L of the coagulation-flocculation suspension was introduced into the measuring cylinder and height was recorded along the time. Settling velocity increased, while the time required for sedimentation decreased in comparison to the results obtained from Sed-3 assay. The settling velocity increased from $1.48 \cdot 10^{-5}$ m/min (Sed-3 assay) to $4.83 \cdot 10^{-5}$ m/min after the coagulation-flocculation process, and the time required to obtain the same TSS removal (92%) was reduced by half (7 days instead of 14 days). So, the application of coagulation-flocculation

technique before the sedimentation increased the harvesting efficiency of that microalgal biomass. The presence of aggregates, which remain in suspension after the treatment (Image 9.9), does not improve the total suspended solids reduction. The harvesting of this microalgal biomass, mainly composed of *Scenedesmus,* has still settling difficulties. However, Wu et al. (2015) carried out flocculation (flocculants tested: chitosan, polyacrylamide, $Al_2(SO_4)_3$, NaOH and HNO_3) studies on *Scenedesmus* cultures, where flocculation efficiencies were higher than 90%. These differences could be related to the fact that in our study the microalgal biomass was a consortium, not pure strains and also, it was grown in WW instead of defined media (BG-11).

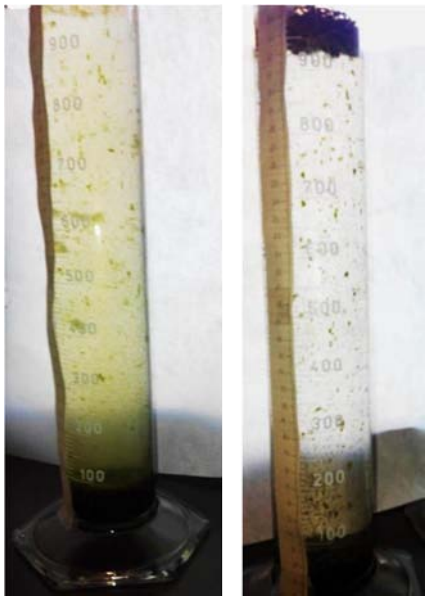


Image 9.9 Sedimentation of IPBR-1 suspension after the coagulation-flocculation process. Left: initial suspension. Right: final suspension (7 days).

As said before, IPBR-1 harvesting using coagulation-flocculation technique does not improve the TSS removal percentages, although the time required was lower.

9.3.2.3 CR coagulation-flocculation assay

Finally, the best coagulant-flocculant combination for TSS and COD removal from the PBR-1 effluent was applied on the pure strain culture of *Chlamydomonas reinhardtii* (CR-1). The COD and TSS removal percentages are presented in Table 9.10.

Table 9.10 C. reinhardtii coagulation-flocculation COD and TSS removal parameters from the supernatant and pH values.

Coagulant-Flocculant dosage (ppm)	COD removal (%)	TSS removal (%)	$pH_{initial}$	pH_{final}
250 $FeCl_3$ + 5 DR3000	14	81	8.6	7.7
50 Al_2O_3 + 10 GO2030	2	38	8.6	n.a[a]

[a] *n.a.: data not available*

Although the best removal percentages are in accordance with PBR-1 and IPBR-1 effluents, higher removal values were obtained for $FeCl_3$ and DR3000 combination, the COD and TSS removal percentages were lower than the ones obtained for the PBR-1 and IPBR-1 effluents. The matrix of the PBR-1 and IPBR-1 suspension was very different from *C. reinhardtii* suspension. The differences due to the specific nature of aqueous matrices (synthetic media and wastewater) have also been considered by other authors (Jiang et al., 1993). Moreover, the microalgal biomass in PBR, IPBR effluents and in *C. reinhardtii* suspension in terms of morphology and composition was highly different, as seen in Image 9.2, Image 9.6 and Image 9.7. Image 9.10 shows the jar test comparing the same coagulant-flocculant dosage on CR-1 and PBR-1 effluent.

Afterwards, gravity sedimentation from the resulting suspension from the coagulation-flocculation process was tested.

The sedimentation velocity obtained was $3.43 \cdot 10^{-5}$ m/min, higher than the value attained by natural sedimentation, $2.94 \cdot 10^{-5}$ m/min. This fact indicates that during the coagulation-flocculation process flocs and aggregates have been formed, increasing the weight and increasing the sedimentation ratio. TSS removal, after both techniques were applied on the same suspension, was 87%, which means an increase of 43% in comparison with natural sedimentation (Table 9.5). Image 9.11 shows the sedimentation process carried out for the *C. reinhardtii* suspension after coagulation-flocculation process. Although the efficiency of the process has been increased, the use of this harvesting methodology is not the ideal for a pure culture of *Chlamydomonas reinhardtii*, since it still requires long periods of time, 14 days, and the TSS removal is not complete.

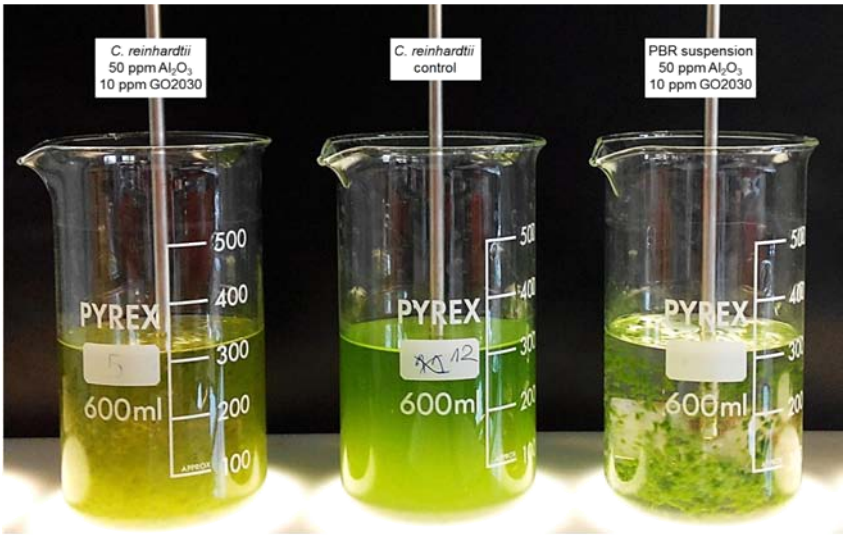


Image 9.10 C. reinhardtii culture and PBR-1 effluent jar test comparison.

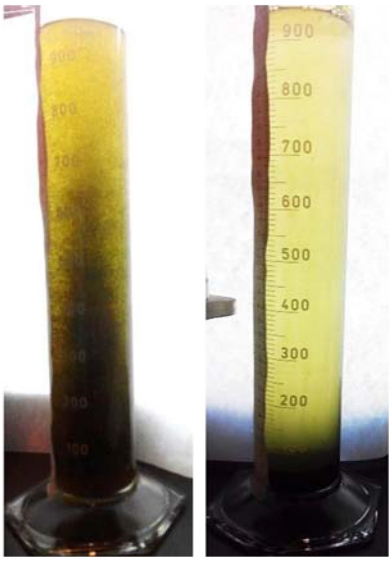


Image 9.11 Sedimentation of C. reinhardtii suspension after the coagulation-flocculation process. Left: initial suspension. Right: final suspension (14 days).

$FeCl_3$ coagulant has been widely studied by several authors for microalgae harvesting. Mennaa et al. (2015) compared the same $FeCl_3$ dosage (60 mg/L) addition to several microalgae pure strains cultured in WW with a natural algal bloom. The biomass recovery efficiency showed similar results among the natural algal bloom with several pure strains, achieving values of almost 100% biomass recovery. These results were higher than the present ones, where the TSS removal percentages have been determined with maximum values of 88% and 81% for the PBR effluent and *C. reinhardtii* culture, respectively, using a dosage of 250 mg/L in combination with DR3000 flocculant. Kim et al. (2013b) carried out a partial factorial design to screen the main factors involved, which

were the concentration of $FeCl_3$, the bioflocculant type, and the time of slow mixing for harvesting the microalga *Botryococcus braunii*. Under the optimal conditions obtained (0.79 mM $FeCl_3$, 0.58% (v/v) bioflocculant, 180 s slow mixing and 1.1 g dry cell weight/L of *B. braunii*), 90.6% clarification efficiency was obtained. De Godos et al. (2011) studied the microalgae harvesting for different microalgae cultures grown in oxygenated piggery wastewater. The main strains were *Scenedesmus obliquus, Chlorella sorokiniana, Chlorococcum* sp. and a wild type *Chlorella*, in symbiosis with a bacterial consortium. The maximum clarification efficiency reported was at 100 mg/L $FeCl_3$ (95±3%) for *S. obliquus* and in the case of *C. sorokiniana*, the maximum TSS removal from the supernatant was 66% using 250 ppm $FeCl_3$, the same dosage as in the present study. Granados et al. (2012) determined the yield of different flocculants for sedimentation experiments on *Muriellopsis* sp microalgae culture. $FeCl_3$ (dosage between 10 to 20 ppm) was the only flocculant capable to concentrate the microalgal biomass, although its clarification recovery efficiency was only 30%.

Several authors have reported coagulation-flocculation processes for microalgae cultures using other coagulants and flocculants. Papazi et al. (2010) tested 12 coagulation salts to study the differences among them for *Chlorella minutissima* harvesting. Some of the salts succeeded in coagulating totally the culture cells, while others showed no difference from the control treatment in relation to the generation of cell aggregates. In contrast to our results, Papazi et al. (2010) obtained better results with aluminium salts rather than with ferric ones. They explained it through the molecular weight and the charge of the formed cations. Aluminium ions have the higher charge density, which led to extended molecular conformation and bridging between cells, and improved charge neutralization of microalgae cell surfaces. In addition, the higher the molecular weight, the lower the solubility.

Delrue et al. (2015) tested the effectiveness of 11 coagulants and flocculants for harvesting *C. reinhardtii*. The maximum clarification efficiency achieved was higher than 95% using highly charged cationic polyacrylamides with a high molecular weight. In that sense the results are in accordance with the present

study, higher removal rates were achieved using $FeCl_3$ (with high molecular weight) and DR3000 (cationic charged).

Another study evaluating different agents at different doses on microalgae cultures is the work conducted by Wu et al. (2015). High clarification efficiencies, over 90% with the proper dosage, on *Scenedesmus* sp. and *Scenedesmus obliquus* cultures were obtained using the followings agents: chitosan, $Al_2(SO_4)_3$, NaOH and HNO_3.

Moreover, the use of biocoagulants and bioflocculants have arisen interest, since they do not affect the downstream process, reuse of the culture medium and wastewater treatment, since chemical flocculation contaminates the harvested biomass and culture medium with metal salts (Talukder et al., 2014). Zheng et al. (2012) used the microbial flocculant poly (γ-glutamic acid) to harvest oleaginous microalgae (marine *Chlorella vulgaris* and freshwater *Chlorella protothecoides*) obtaining a flocculation efficiency of 82% and a concentration factor of 15.1 for *C. vulgaris* and a flocculation efficiency of 90% and a concentration factor of 23.7 for *C. protothecoides*. Xu et al. (2013) used the natural compound, chitosan as flocculant, obtaining 99% clarification efficiency. Natural coagulants have been used in real pilot scale HRAP effluents as described by Gutiérrez et al. (2015). Jar tests showed how flocculant doses of 10 and 50 mg/L of *Ecotan* and *Tanfloc* enabled over 90% biomass recovery.

Nowadays, a new biological treatment is gaining interest amongst the microalgae harvesting. This technique is called co-pelletization (Zhang and Hu, 2012b) and it has been also tested on the three effluents in order to study the harvesting efficiency.

9.3.3 Co-pelletization

In this part of the study, fungi-assisted bio-flocculation strategy for efficient and low-cost harvesting relatively diluted algae cells in culture broth was developed and evaluated. It was hypothesized that the fungus *T. versicolor* could assist bio-flocculation of microalgae consortium from the PBR effluents by forming fungi–algae pellets. PBR co-pelletization experiments (COP-1) were conducted using PBR-1 and PBR-2 effluents following EXP-1, EXP-2 and EXP-3

methodologies. COP-2 and COP-3 assays were conducted following EXP-3 methodology using IPBR effluent and *C. reinhardtii* suspension, respectively.

9.3.3.1 PBR co-pelletization assays

The first co-pelletization study (COP-1) was conducted using the PBR-1 effluent (Table 9.1). Different methodologies were applied in order to determine the most efficient for further application on other microalgae effluents.

The first methodology tested was EXP-1 (Figure 9.1) using PBR-1 effluent. Results can be observed in Image 9.12.

Image 9.12 COP-1 assay results following EXP-1 methodology. Left: initial fungi-algae culture. Right: final (6 days) fungi-algae culture.

No pellets were formed and bacterial growth was observed. The presence of bacteria can be visually observed on a colour change of the suspension supernatant, from a colourless transparent liquid to a white-brown liquid. The main reasons attributed to the non-co-pelletization process were the presence of glucose in the fungal medium and also the presence of a wide range of microorganisms in the PBR effluent. The presence of glucose in the defined media is necessary for the fungal growth in order to form pellets, although it increases the bacterial growth as well as heterotrophic algae growth, decreasing the carbon source necessary for *T. versicolor* growth. Moreover, bacteria have a higher growth rate than microalgae and fungi, favouring bacterial growth.

In order to reduce the high competition for glucose consumption among all the microorganisms present in PBR effluent other methodologies were tested.

Afterwards, EXP-2 was conducted following the methodology from Figure 9.2, with the objective of microalgae immobilization with fungal pellets previously formed (Table 9.1). The first step was the formation of *T. versicolor* pellets within 3 more days. The second step included 2 methodologies: on EXP-2A PBR effluent was cultured with the pellets (previously sieved) for 3 days, whereas on EXP-2B PBR effluent was previously centrifuged and the concentrate was introduced into the fungal culture formed in step 1 and cultured for 3 days. EXP-2A results were not satisfactory (Image 9.13), no co-pelletization occurred and again, bacterial contamination took place in the suspension. Glucose consumption by other microorganisms instead of *T. versicolor* was observed in the pellets size, they did not increase their size after being in contact with the PBR effluent. EXP-2B results were similar to EXP-2A (Image 9.14). Despite more defined media was introduced and PBR effluent was centrifuged, bacterial contamination was observed and no pellets growth was noticeable.

Image 9.13 COP-1 assay results following EXP-2A methodology. Left: initial fungi-algae culture. Right: final (3 days) fungi-algae culture.

Image 9.14 COP-1 assay results following EXP-2B methodology. Left: initial fungi-algae culture. Right: final (3 days) fungi-algae culture.

PBR effluent biomass immobilization by *T. versicolor* mycelia (EXP-1) and pellets (EXP-2) did not succeed due to contamination problems from the different microorganisms present in the culture. The carbon source needed for growing such fungi evoked undesirable microbial contamination. This behaviour was also reported by Salim et al. (2011) who described those problems in open raceway ponds.

Finally, COP-1 assay was conducted following EXP-3 methodology (Figure 9.3) and the suspension used was PBR-2 (Table 9.1). As shown in Image 9.15, fungal pellets were grown in the presence of PBR effluent during three days for 3 different volumetric ratios PBR effluent:fungal defined media. Biomass from the PBR effluent was immobilised inside the fungal biomass, obtaining a clear transparent supernatant in the 3 ratios tested. The more algae were in the suspension the greener the fungal pellets were. After 3 days pellets were formed and the size of them allowed their simple harvesting by mesh sieve filtration.

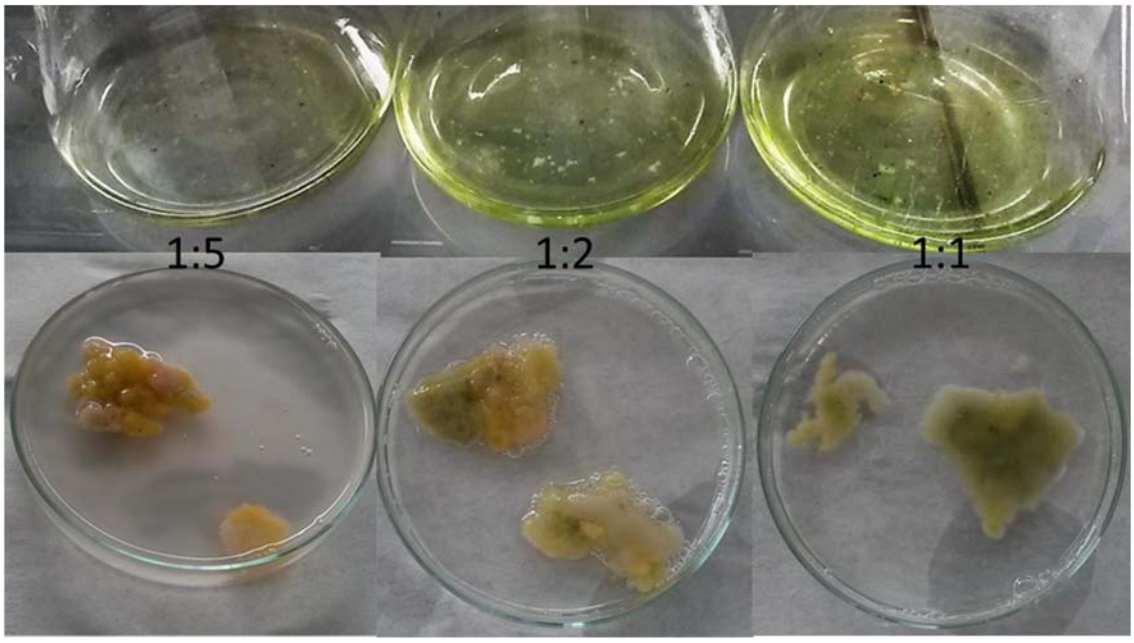


Image 9.15 COP-1 assay following EXP-3 methodology at different volumetric ratios PBR suspension:defined fungal medium. Top: initial; bottom: final, 3 days.

TSS removal percentages are presented in Table 9.11. All three ratios achieve high removal percentages, indicating the feasibility of this harvesting methodology for the PBR effluent.

Table 9.11 COP-1 assay: TSS from the initial PBR effluent, TSS from the supernatant after the co-pelletization and removal percentages following EXP-3 methodology.

Ratio	$TSS_{Initial\ PBR\text{-}2\ effluent}$ (mg/L)	$TSS_{Final,\ supernatant}$ (mg/L)	Removal (%)
1:5	27	0	100
1:2	54	1	98
1:1	81	2	98

Overall, the most promising results were obtained for 1:1 ratio, since more PBR effluent could be treated and less fungal medium was added, decreasing the costs of the process.

Among the three methodologies tested for PBR effluent harvesting, the last one (EXP-3) shows encouraging results to be further studied in other microalgae cultures. The next culture studied came from an IPBR in which the majority algae genus was *Scenedesmus* (IPBR-2).

9.3.3.2 IPBR co-pelletization assay

EXP-3 methodology (Figure 9.3) was followed for the IPBR-2 effluent harvesting experiment (COP-2 assay); the characteristics of IPBR-2 effluent are shown in Table 9.1.

During the experiment, the volumetric ratio 1:1 was discarded due to high bacterial contamination observed in the Erlenmeyer flasks, inhibiting the formation of pellets and therefore the co-pelletization of the IPBR-2 effluent.

The co-cultivated IPBR-2 effluent and fungus (ratios 1:2 and 1:5) were able to form pellets of approximately 1–2 mm in size with dense biomass as shown in Image 9.16 and Image 9.17. The size of the pellets allow their simple harvest by mesh sieve filtration.

The co-cultivation was able to attain removal of microalgae cells from the liquid medium, although the presence of glucose enhanced bacterial growth. This can be observed in Image 9.16, where the microalgal suspension turned from green to yellow-brown liquid. Moreover, that fact was confirmed by the increase of the TSS (Table 9.12) and the difficulties to filter the suspension. Previous studies also experienced a biomass increase on the 3rd day of cultivation, this is the

case of the pure *C. vulgaris* culture reported by Xie et al. (2013), the un-pelletized cells were likely to grow in the culture media.

Although there was a TSS increase, the system was efficient, algal biomass was trapped by fungal pellets and COD from the supernatant was efficiently removed, values are presented in Table 9.12.

Co-pelletization of IPBR-2 suspension was feasible following the EXP-3 methodology, algal biomass was trapped inside the fungal pellets. However, bacterial growth was still a problem due to the glucose present in the media, enhancing bacterial growth.

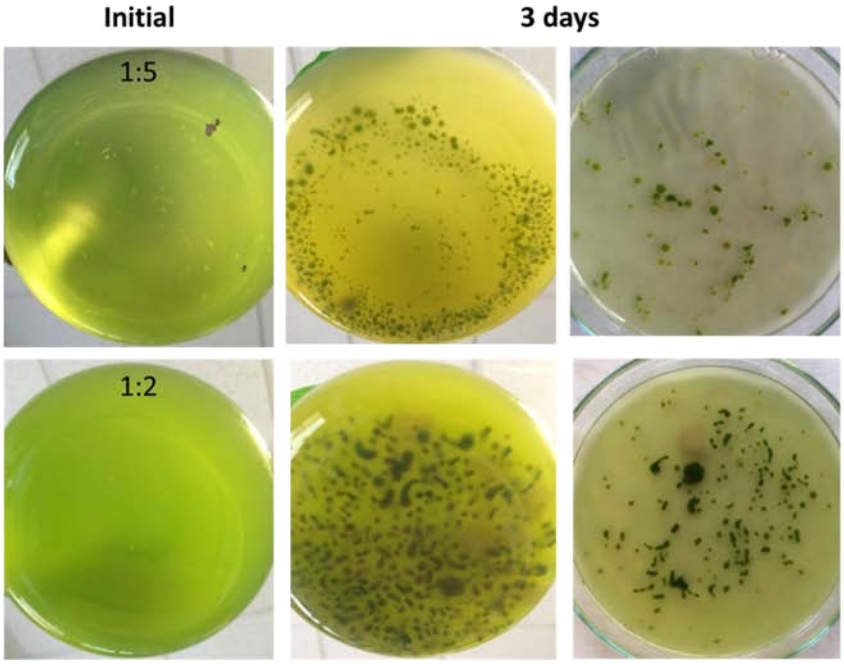


Image 9.16 COP-2 assay results following EXP-3 methodology. From left to right: initial co-pelletization suspension; final (3 days) co-pelletization suspension inside the E-flask; final (3 days) co-pelletization suspension pellets in detail. Top row: volumetric ratio microalgal suspension:defined fungal media 1:5; Bottom row: volumetric ratio 1:2.

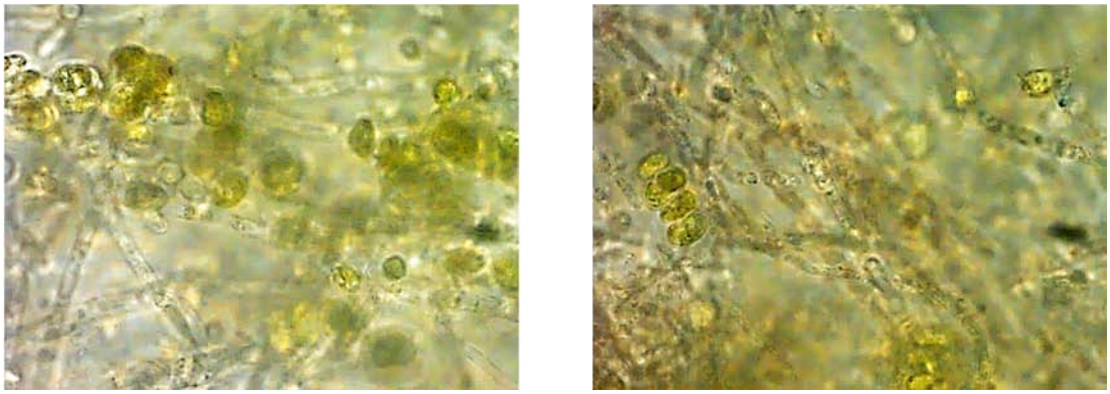


Image 9.17 IPBR biomass entrapment by T. versicolor pellets. Left: 1:2 ratio; Right: 1:5 ratio. 1000x magnification.

Table 9.12 COP-2 assay: TSS from the initial IPBR-2 effluent, TSS from the supernatant after the co-pelletization and COD removal percentages.

Ratio	$TSS_{initial\ IPBR\ suspension}$ (mg/L)	$TSS_{Final\ supernatant}$ (mg/L)	% COD removal (after co-pelletization)
1:5	178	1305	53
1:2	357	810	60

In order to separate the solid and liquid phase, gravity sedimentation of the co-pelletized suspension was conducted. The pellets sedimentation curves are shown in Figure 9.5. A rapid layer was deposited at the bottom of the column and as long as the time passed the cake compacted. Pellets from 1:5 ratio experiment achieved a lower height due to the lower biomass content. Regarding the supernatant, due to the difficulties for the interphase identification, no sedimentation curve was plotted, although sedimentation velocity was calculated (Table 9.13and Image 9.18). Sedimentation assays for both ratios lasted the same time and TSS and COD removal were evaluated (Table 9.13). Better results in terms of TSS removal and COD were obtained for 1:2 ratio (69% and 95%, respectively) than for 1:5 ratio (50% and 90%, respectively).

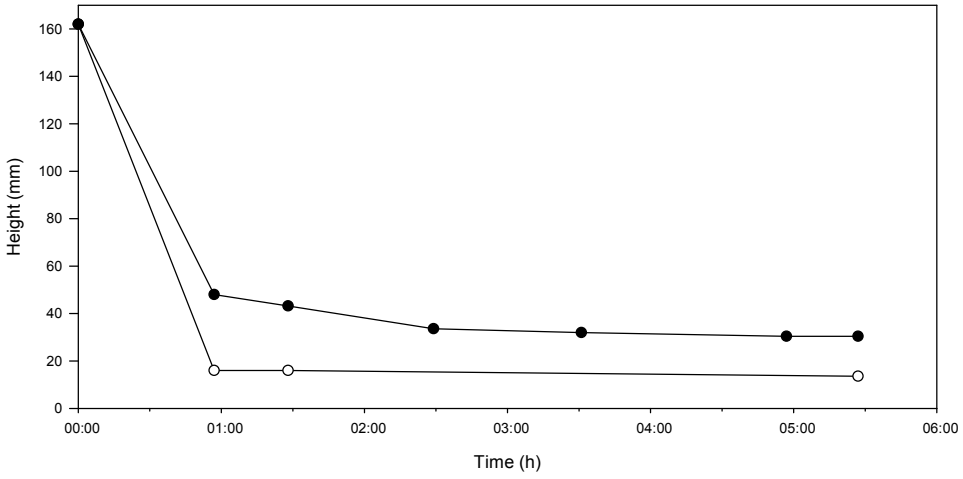


Figure 9.5 COP-2 assay: Pellets sedimentation curve after co-pelletization. (●) *1:2 ratio;* (○) *1:5 ratio.*

Table 9.13 Sedimentation velocity and TSS and COD removal after natural sedimentation of COP-2 assay.

Ratio	v_s (m/min)	% TSS removal	$\%\ COD_{total}$ removal (co-pelletization + sedimentation)
1:5	$1.51 \cdot 10^{-4}$	52	90
1:2	$1.51 \cdot 10^{-4}$	69	95

Overall, ratio 1:2 shows better results for the co-pelletization assay. Less defined medium was used and more biomass was entrapped by fungal pellets, therefore higher COD removal values were attained. Moreover, the clarification percentage (TSS removal) was also enhanced.

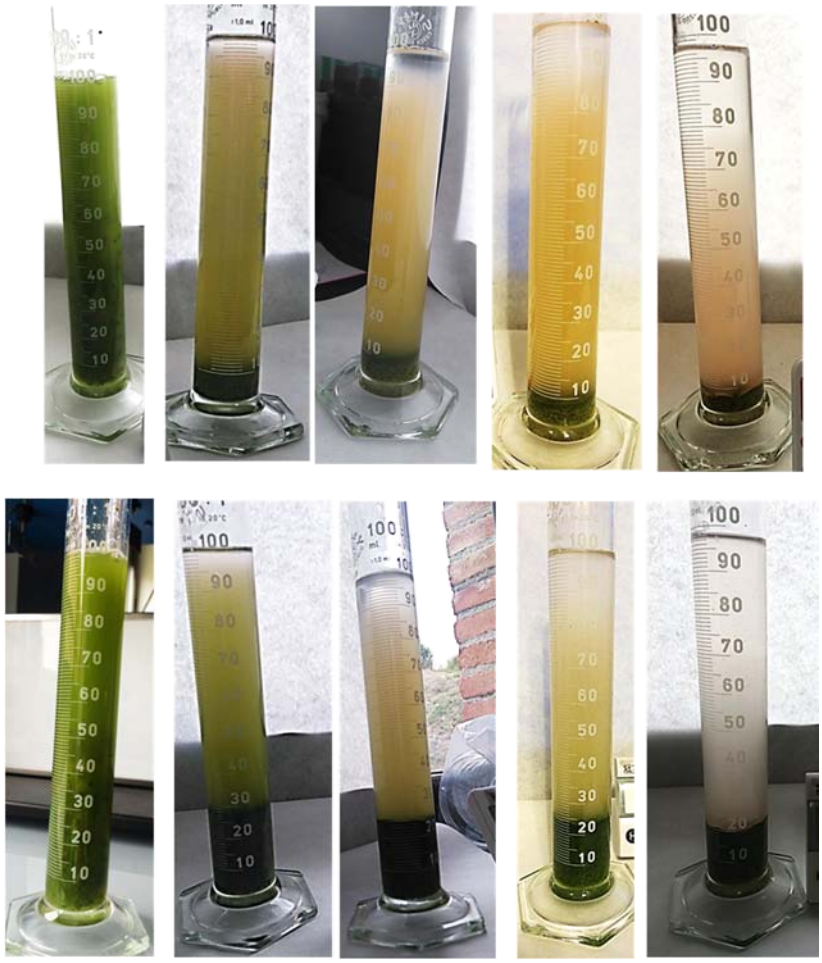


Image 9.18 Gravity sedimentation of COP-2 assay. From left to right: 0h, 1h, 3h, 6h and 18 h. Top: 1:5 ratio; Bottom: 1:2 ratio.

It is demonstrated the feasibility and performance of this strategy for the co-cultivation of IPBR-2 effluent with *T. versicolor* fungus. The technique has the potential to decrease the harvest cost of algae and increase the yield. The pelletized algae can be readily harvested by simple sedimentation in less than 5.5 h. This concentration strategy might improve the process economics and promote sustainability, because biomass could be valorised, together with other substrates for biogas production.

9.3.3.3 CR co-pelletization assay

A pure microalgal suspension was pelletized (COP-3) using *T. versicolor*. The pure microalgal suspension was the same as in previous harvesting methods, *C. reinhardtii*. Initial characteristics are presented in Table 9.1.

C. reinhardtii cells were suspended in the defined medium and inoculated with 0.5 mL of fungal mycelia, following EXP-3 methodology (Figure 9.3).

Three microalgae:defined medium volumetric ratios were analysed, in order to evaluate how the defined medium volume affected the culture. Co-pelletization results can be observed in Image 9.19.

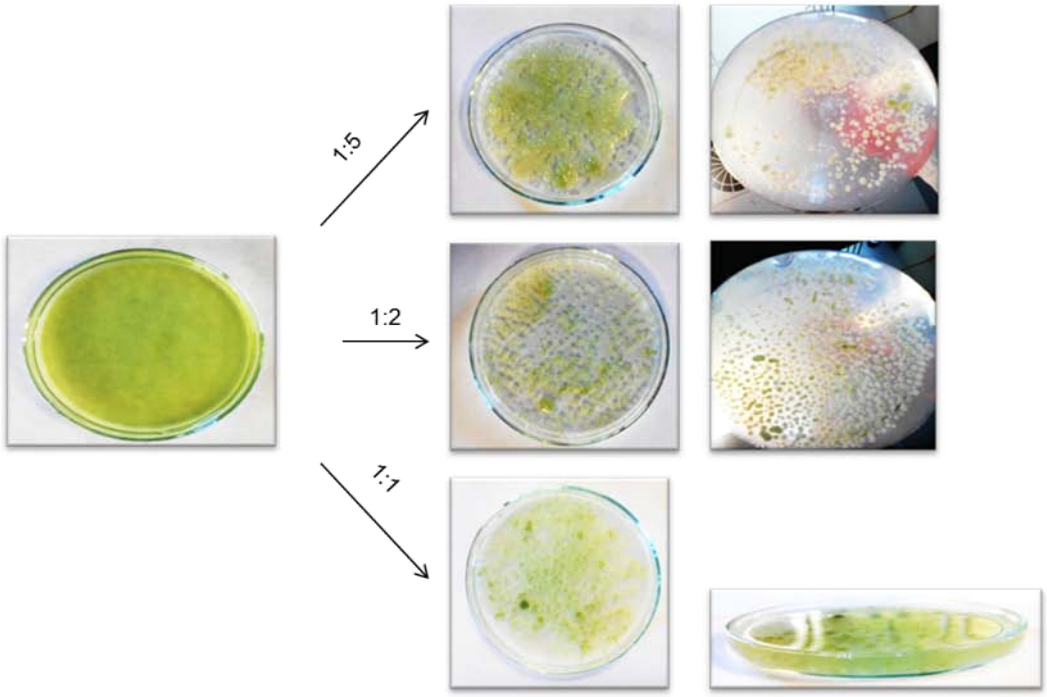


Image 9.19 COP-3 assay with C. reinhardtii and T. versicolor results following EXP-3 methodology.

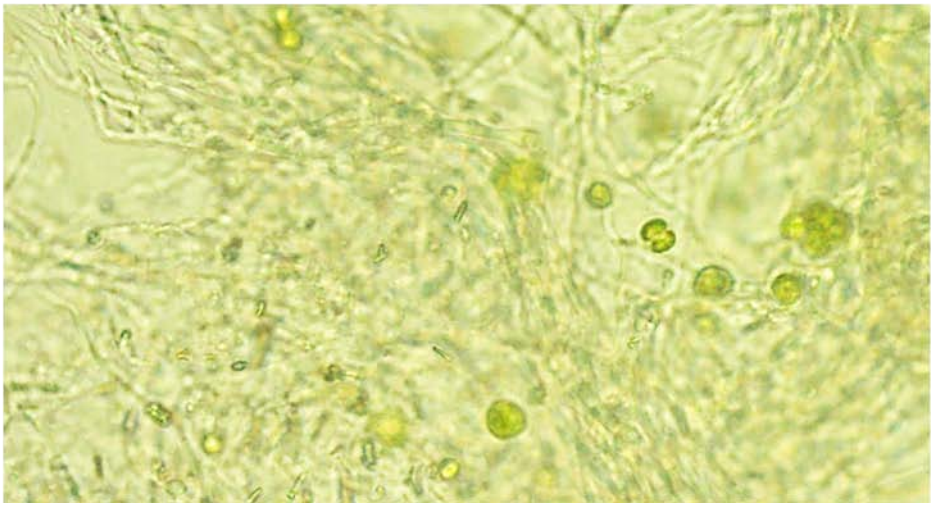


Image 9.20 C. reinhardtii cells entrapped by T. versicolor pellets (200x) during COP-3 assay, ratio 1:5.

The microalgal solution lost most of its green colour within six days of cultivation, and turned transparent, indicating that the majority of microalgae cells were co-pelletized.

Image 9.20 shows a microscope image of *C. reinhardtii* cells entrapped by *T. versicolor* pellets. The microscopic image shows that the skeleton structure of the pellets was still filamentous; while green algae cells were entrapped inside the fungal mycelia and attached on the fungal cell surface. This was also observed by Xie et al. (2013) who confirmed that the algae cells were embedded in the mesh of the fungal hyphae without significant changes in the cell morphology and lipid accumulation. Also, Zhang and Hu (2012b) observed this behaviour and suggested that cell pelletization seemed to be strain-specific and that not all the filamentous fungi strains can form pellets during their growth. Whereas *Aspergillus flavus* entrapped almost all of the individual microalgae cells, *Phanerochaete chrysosporium* and *Aspergillus oryzae* could not entrap algae cells, despite pellets were still formed. On the other side, Wrede et al. (2014) studied the pelletization of a wide range of microalgae strains including marine and motile representatives using *Aspergillus fumigatus*, obtaining that most of algae could be pelletized, but efficiencies vary among strains.

All ratios tested in this study were able to form pellets, but depending on the volumetric ratio the pellets and the supernatant appearance was different. The results obtained for each ratio are presented in Table 9.14.

Table 9.14 COP-3 assay: TSS of the CR-1 suspension and TSS of the supernatant after the co-pelletization. TSS and OD removal percentages are also presented.

Ratio	$TSS_{initial\ IPBR\ suspension}$ (mg/L)	$TSS_{Final\ supernatant}$ (mg/L)	% TSS removal	% OD removal (after co-pelletization)
1:5	51	19	64	93
1:2	102	67	34	76
1:1	153	>153	-	-

For ratio 1:5 pellets were bigger and a clearer supernatant was obtained, all the microalgae culture was immobilised by the pellets formed during the 6 days of the co-culture. 93% of the OD was reduced and 64% of TSS were removed.

Those results show higher harvesting efficiency for *C. reinhardtii* culture if compared with natural sedimentation (Table 9.5). However, Zhou et al. (2013) obtained better results, the harvesting clarification efficiency for *C. vulgaris* culture was 93%. On the other hand, Zhang and Hu (2012b) pointed out that the harvesting clarification efficiency of *C. vulgaris* was fungi strain dependant.

Focusing on ratio 1:2 of the present study, it showed smaller pellets and the supernatant was not as clear as the 1:5 ratio (Image 9.19). The TSS removal efficiency obtained was 34%. Finally, ratio 1:1 showed the smallest pellets and in a lower quantity. The supernatant was still green, most of the microalgae culture was not immobilised by the pellets, and algae growth may have occurred during the 6 days cultivation. *C. reinhardtii* is a mixotrophic microalgae, which may consume the glucose from the medium. This is in accordance with other authors, who observed glucose consumption by pure microalgae strains during co-pelletization harvesting. Xie et al. (2013) reported that co-cultivation of *C. vulgaris* by *Cunninghamella echinulata* appeared to improve co-pelletization. In this study, glucose consumption was evaluated as well as glucose consumption velocities were calculated. Results are shown in Figure 9.6 and Table 9.15.

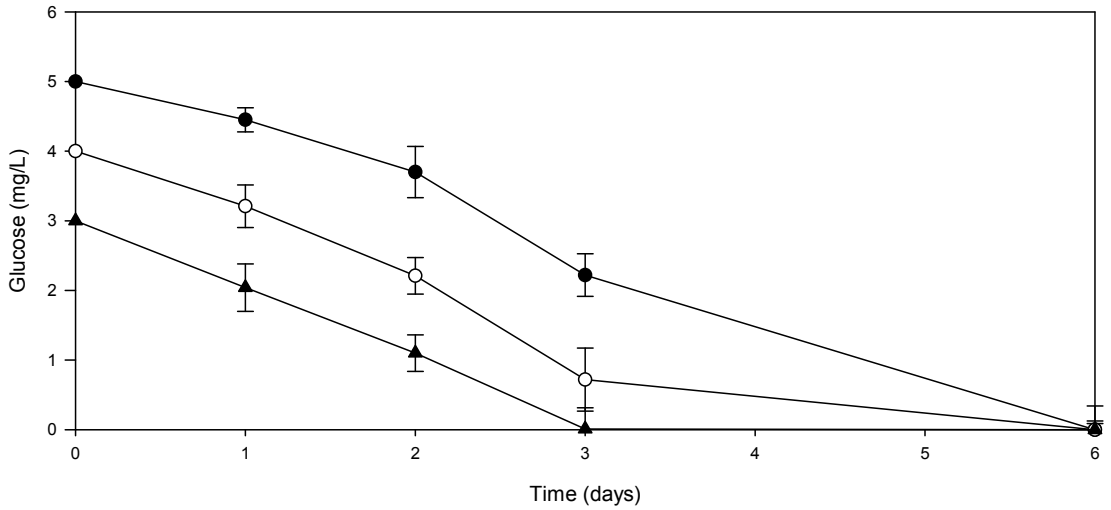


Figure 9.6 COP-3 assay: Glucose consumption. (●) *1:5 ratio;* (○) *1:2 ratio;* (▲) *1:1 ratio.*

Glucose was rapidly consumed within 3 days in ratio 1:1, although similar consumption velocities (calculated from day 0 to day 3) were obtained in all three volumetric ratios tested. Those results indicate that glucose consumption velocity does not depend on the microalgae concentration of the suspension.

However, Zhou et al. (2013) indicated that higher initial glucose concentration promoted formation of fungi–algae pellets in a short cultivation period, and Zhang and Hu (2012b) studied the growth increase when sugars were provided, reporting that the total cell biomass was significantly higher because sugars were provided and consumed both by the fungus and microalgae. However, in our study, co-pelletization of a pure microalgae culture in the presence of glucose enhanced its growth and the harvesting efficiency from the clarified phase was decreased, observing microalgal growth at the end of the experiment (ratio 1:1) (Table 9.14).

Table 9.15 COP-3 assay: Glucose concentration and consumption velocities.

Ratio	Initial glucose concentration (g/L)	Glucose concentration, 3 d (g/L)	Consumption velocity (g/(L·d))
1:5	5.0	2.22	0.93
1:2	4.0	0.72	1.09
1:1	3.0	0.0	1.00

Although the addition of sugars increases the total biomass it would be economically unfeasible if high amount of sugars are needed. Gultom and Hu (2013) proposed a life cycle assessment to evaluate the impact of this technology on the economics of co-culturing these species (i.e., nutrient requirements) as well as the subsequent use of the biomass (added cost and energy requirements/benefits for downstream processing and co-product use).

Some authors studied the factors that influence the co-pelletization mechanism. It was found that the hydrophobic proteins on the mycelial surface of some filamentous fungi might be beneficial to fungi–algae pellet formation because these hydrophobic proteins could attach to microalgae cells surface and assist the co-pelletization (Feofilova, 2010). Besides, enzymes secreted by the fungus may enhance the pellet formation. Zhang and Hu (2012b) co-cultured a fungus that produced cellulase; the enzyme was released to the culture and partially degraded the cell wall of microalgae (cellulose is reported as the main structural component of the cell wall for most microalgae species) enhancing the pellets formation. It is well documented that *T. versicolor* produces laccase enzyme

and other mediators, therefore it is possible that the release of such compounds to the suspension may have contributed to the co-pelletization process.

Fungal co-pelletization harvesting could be beneficial depending on the purpose of microalgal biomass. If the primary purpose of culturing microalgae is to generate microbial lipids, co-culturing an oleaginous fungus will increase biomass yield, as both fungus and microalgae biomass contain lipid, carbohydrate and protein, and can be processed together. Microalgae harvesting by immobilizing them on fungal mycelium may add value to the microalgae biorefinery, as a result, the total biomass of most fungal-algal pellets were found to be higher than the additive biomass of mono-cultured algae and fungi strains (Talukder et al., 2014; Wrede et al., 2014; Zhang and Hu, 2012b). The pelletized algae can be readily harvested by simple filtration and if strains are biologically safe, do not cause any secondary pollution issue while chemical flocculants may have over dosage and remain as pollutants in the water. This concentration strategy might improve the process economics and promote sustainability (Gultom and Hu, 2013; Xie et al., 2013). The co-cultivation system provides opportunities to integrate photosynthetic biorefineries with other processes, such as wastewater treatment and biomass conversion (Xie et al., 2013).

9.4 Conclusions

The study of three different microalgae harvesting techniques with three different microalgae cultures has led to the main conclusion that the source of the microalgal suspension is a key parameter that cannot be extrapolated.

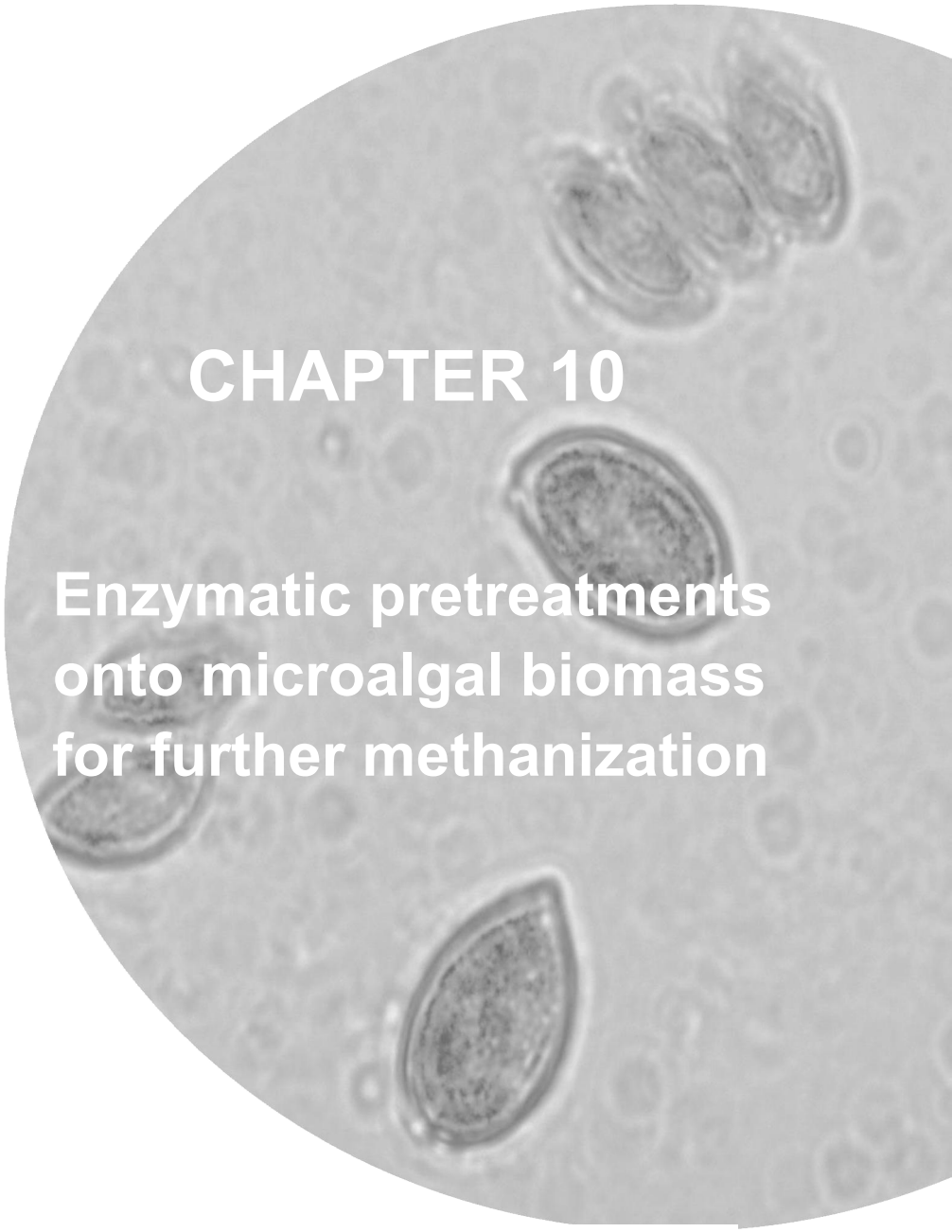
Natural sedimentation has been determined as the best harvesting method for PBR-1 effluent, since it contains microalgae species with the ability to form flocs, such as *Phormidium*. However, if there is a lack of filamentous species the settling ratio can take over 14 days as it happens using the IPBR effluent. The sedimentation process took 7 minutes with 99% TSS removal when filamentous microalgae were the majority specie. This technique is not applicable to the pure culture of *C. reinhardtii*, due to the small cell size, 14 days

were required for 50% TSS removal, which confirms the difficulties of pure microalgae cultures harvesting.

Two coagulants and two flocculants were tested in the PBR effluent at different dosages to evaluate the interactions among them. The best COD removal percentage 88%, was achieved using 250 ppm $FeCl_3$ coagulant and 5 ppm DR300 flocculant. Furthermore it was the cheapest option among the other efficient combination, resulting as the best candidate for further studies. Based on their efficiency, the same coagulant-flocculant combinations were tested on IPBR effluent as well as in *C. reinhardtii* culture. IPBR effluent achieved 90% TSS removal, whereas TSS from *C. reinhardtii* culture was 81% reduced. COD removals were lower, only 14% was reduced in *C. reinhardtii* suspension. Settling velocities were increased after the coagulation-flocculation process. The settling velocity for the IPBR was $4.83 \cdot 10^{-5}$ m/min and for *C. reinhardtii* suspension it was $3.43 \cdot 10^{-5}$ m/min. But it is still an inefficient harvesting process, since it requires long terms for pure microalgal cultures.

Finally, co-pelletization using *T. versicolor*, was an efficient harvesting technique treating microalgae effluents. 100% microalgae entrapment was achieved with PBR-2 effluent, even with the highest microalgae concentration 98% TSS removal was obtained. IPBR effluent was pelletized within 3 days, between 52 and 69% of the TSS were removed and above 90% for the COD for the major microalgae:fungal defined media ratio. *C. reinhardtii* entrapment also achieved good removal percentages (64%). Overall, co-pelletization harvesting technique for pure algae cultures attained successful results in comparison to sedimentation and coagulation-flocculation techniques, while for microalgal photobioreactors where microalgae and other microorganisms are grown, the results depend on the biomass characteristics. If flocs are easily formed good clarification results at high sedimentation velocities (low times required) are obtained using the sedimentation technique.

The developed novel harvesting technology (co-pelletization) may provide a solution to problems associated with current energy-intensive and costly algae harvesting processes. Despite the good results attained in this study further research is still needed to study the detailed pelletization conditions at large scale industrial applications.



CHAPTER 10

Enzymatic pretreatments onto microalgal biomass for further methanization

Part of this chapter has been published as:

Passos, F., Hom-Diaz, A., Blánquez, P., Vicent, T., Ferrer, I., 2016. Improving biogas production from microalgae by enzymatic pretreatment. Bioresource Technology, 199, 347-351.

Hom-Diaz, A., Passos, F., Ferrer, I., Vicent, T., Blánquez, P., Enzymatic pretreatment of microalgae using fungal broth from *Trametes versicolor* and commercial laccase for improved biogas production. *Submitted to Algal Research.*

10 Enzymatic pretreatment onto microalgal biomass for further methanization

Abstract

The effect of enzymatic hydrolysis on microalgae organic matter solubilisation and methane production was investigated in this study using microalgal biomass specific and non-specific enzymes. Among the specific enzymes, cellulase, glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase were selected based on the microalgae cell wall composition (cellulose, hemicellulose, pectin and glycoprotein). All of them increased organic matter solubilisation, obtaining high values already after 6 h of pretreatment with an enzyme dose of 1% for cellulase and the enzyme mix. Among the non-specific enzyme commercial laccase and laccase contained on a fungal broth was tested (100 U/L over an exposure time of 20 min). The fungus Trametes versicolor was cultured, and the enzymatic activity of the culture broth was analysed by measuring laccase activity. Biochemical methane potential (BMP) tests with pretreated microalgae showed a methane yield increase of 8 and 15% for cellulase and the enzyme mix, respectively and a methane yield of 203 and 217 mL CH_4/g VS, respectively. The fungal broth pretreatment increased the methane yield by 74%, while commercial laccase increased the methane production by 20% as compared to non-pretreated microalgal biomass, the methane yield values were 144 and 100 mL CH_4/g VS, respectively. T. versicolor broth further improved the results. Prospective research should evaluate enzymatic pretreatments in continuous anaerobic reactors so as to estimate the energy balance and economic cost of the process.

10.1 Introduction

Microalgal biomass may be processed for conversion into bioethanol, biodiesel, biohydrogen, and biomethane production or non-food bioproducts, biofertilizers and biomaterials.

Nevertheless, it has been shown that biogas production through anaerobic digestion (AD) is the most straightforward technology, since neither drying nor extraction techniques are needed. AD is characterised by its low complexity, minimal processing requirements and availability of a technology that has long been used for sludge treatment in wastewater treatment plant (WWTP) (Zamalloa et al., 2011). Still, pretreatment methods for biomass are crucial for enhancing the hydrolysis step and

increasing the methane yield due to the resistant and complex biomass cell structure (González-Fernández et al., 2011; Passos et al., 2014c).

Most microalgae cell walls are composed by two parts: a fibrillar part (skeleton) and an amorphous part (matrix). The fibrillar component is formed by cellulose, mannan and xylan; while the amorphous component is where the fibrillar part is submerged (Lee, 2008). Complex microalgae cell walls, such as the ones from *Chlorella* sp. and *Scenedesmus* sp., are also composed by an outer layer, which may be homogenous or have a trilaminar sheath (TLS). The TLS is resistant to the anaerobic degradation process since it is composed by sporopollenin, also called algaenan, which is a lignin-like biopolymer, formed from hydroxylated fatty acids and phenolics (Kwietniewska and Tys, 2014).

Furthermore, when dealing with microalgal biomass grown in open ponds for wastewater treatment, a mixed community of microalgae and bacteria is formed. This biomass varies in terms of population dynamics, microalgae composition and cell wall structure; generally formed of a rigid cell wall, due to the variable conditions of the system or the presence of grazers, factors that may limit the hydrolysis step (Park et al., 2011b; Passos et al., 2015b; Ward et al., 2014). Microalgae pretreatment prior to anaerobic digestion seems imperative due to its slow biodegradability. Indeed, the methane yield reached 0.05–0.15 L CH_4/g VS in continuous reactors operated at hydraulic retention time (HRT) up to 20 days (González-Fernández and Sialve, 2012). These values are low in respect to other anaerobic digestion feedstocks such as starch and sugar crops (e.g., corn 0.18–0.41 L CH_4/g VS and potatoes 0.43 L CH_4/g VS) (Frigon and Guiot, 2010), or primary sludge (0.31 L CH_4/g VS) (Kepp and Solheim, 2000), and rather similar to waste activated sludge (WAS) (0.14 L CH_4/g VS) (Bougrier et al., 2006).

Thermal, mechanical and thermochemical pretreatments are the most studied methods for improving microalgae anaerobic digestion performance (Passos et al., 2014c). Such methods are used to disrupt or weaken the cell wall structure, improving macromolecules bioavailability and biodegradability in the reactor. Nevertheless, some thermal and most mechanical methods are energetically unbalanced, i.e. the energy consumed in the pretreatment step is not compensated by the biogas gain without biomass dewatering (Passos et al., 2014c). In this

manner, research on biogas production from microalgae should focus on technologies with low energy demand, such as biological pretreatments.

Biological methods operate with mild conditions, where microalgae cell wall is degraded enzymatically rather than disrupted as in mechanical techniques (Günerken et al., 2015). Enzymes are regarded as a low-cost eco-friendly pretreatment for enhancing microalgal biomass anaerobic biodegradability (Gerken et al., 2013; Mussgnug et al., 2010). Indeed, enzymatic pretreatment consists in converting molecules from the cell wall into more usable substrates for anaerobic microorganisms. Therefore, it is necessary to know the composition of microalgae cell wall in order to select the appropriate enzymes. The hydrolysis of cellulose and hemicellulose is well studied for lignocellulosic biomass biodegradation. Celluloses are polysaccharides of glucose, more specifically they are glucose molecules linearly polymerised by β-1,4-glycosidic bonds creating cellulose chains, which are connected constituting microfibrils. Hemicelluloses are randomly branched heterogenetic polysaccharides of various mono-sugars (xylose, arabinose, galactose, mannose and rhamnose) and uronic acids (glucuronic acid, methyl glucuronic acid and galacturonic acid) (Yang et al., 2015). As a consequence of microalgae cell wall composition, the most used enzymes for microalgae pretreatment are commercial α-amylases, amyglucosidases, cellulases, xylanases, lipases or proteases (Choi et al., 2010; Ehimen et al., 2013).

Some promising results have already been shown in terms of biomass solubilisation and biogas production increase after enzymatic pretreatment of pure microalgae cultures (de C. Neves et al., 2016; Hernández et al., 2015; Ometto et al., 2014; Wieczorek et al., 2014). Nevertheless, the literature is still scarce on the effect of enzymatic pretreatment on mixed microalgal biomass grown in wastewater treatment systems. To date, results showed how the methane yield of *Chlorella vulgaris* was increased by 70% with cellulase (Onozuka®) and a hemicellulose mix (Macerozyme®) (Wieczorek et al., 2014). Similarly, the methane yield of the same microalgae species was increased by 86% with carbohydrolase and protease (Mahdy et al., 2014). For the filamentous microalgae *Rhizoclonium* sp., an enzyme mix composed by amylase, protease, lipase, xylanase and cellulase enhanced the methane yield by 30% (Ehimen et al., 2013), fact that has proved that a mixture of commercial enzymes increases the methane yield rather than using a single substrate-specific enzyme.

Furthermore, enzymes can be biologically produced. A well-known example are ligninolytic fungi, which produce non-specific intra and extracellular enzymes (such as: lipase, laccase, manganese peroxidase, among others), depending on the culture conditions (Harms et al., 2011). One of the most well-known fungus that produces laccase is the white-rot fungus *Trametes versicolor*. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are a family of glycoproteins, classified as oxidoreductases that catalyse the monoelectronic oxidation of substrates at the expense of molecular oxygen. They are used for cross-linking of monomers, degradation of polymers and ring cleavage of aromatic compounds in various environmental applications (i.e., bioremediation of soils and wastewater, decolourization of recalcitrant dyes, kraft pulp biobleaching, biorefinery processes and degradation of emerging contaminants) (Asgher et al., 2008; Blánquez et al., 2004; Marco-Urrea et al., 2009; Novotný et al., 2004; Riva, 2006). In addition, laccase can be used as a pretreatment step for cellulose hydrolysis (Qiu and Chen, 2012). Regarding the use of fungal crude enzymes, those from *Aspergillus lentullus* were effective at improving microalgae anaerobic biodegradability (Prajapati et al., 2015).

The aim of the present study is to evaluate the biogas production increase obtained by applying an enzymatic pretreatment to microalgal biomass in order to solubilise it before the biochemical methane potential (BMP) tests. The microalgal biomass comes from wastewater (WW) treatment algal reactors. Two pretreatment approaches were considered, the first one using specific microalgal cell wall enzymes; cellulase, glucohydrolase and an enzyme mix. Cellulase was tested for enhancing cellulose hydrolysis, along with glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase for enhancing hemicellulose hydrolysis. For the second enzymatic pretreatment, commercial laccase enzyme and the fungal broth from *T. versicolor* containing laccase enzyme were used.

10.2 Materials and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

10.2.1 HRAP reactor

The experimental reactor was located outdoors at the Department of Civil and Environmental Engineering of the Universitat Politècnica de Catalunya BarcelonaTech (Barcelona, Spain) (Figure 10.1). Real WW from a nearby municipal sewer was continuously pumped and treated as follows. Firstly, WW was screened and stored in a homogenisation tank (1.2 m^3). From this tank a continuous WW flow of 180 L/d was conveyed to a primary settler with a surface area of 0.0255 m^2, a useful volume of 7 L, a hydraulic surface load influent rate of 7.05 m/d and a HRT of 0.9 h. The primary effluent was continuously discharged into the high rate algal pond (HRAP) by means of a peristaltic pump with a flow rate of 60 L/d, while the excess effluent was discharged. The HRAP was built in polyvinyl chloride (PVC), it had a surface area of 1.54 m^2, a water height of 0.3 m, a useful volume of 0.47 m^3, and a HRT of 8 days. Microalgae contact with sunlight was enhanced through continuous stirring with a bladed paddle-wheel driven by an engine operated at 5 rpm, reaching an average flow velocity of 10 cm/s. Mixing also avoided biomass settling within the pond. Since the mixed liquor was under constant stirring and the HRT was 8 days, the system operated similarly to a completely mixed reactor. Microalgal biomass was harvested in a clarifier with a useful volume of 10 L, a surface area of 0.0255 m^2, a hydraulic surface load influent rate of 2.35 m/d and a HRT of 4 h. 1 L of biomass was purged from the settler (0.01 m^3 nominal volume, 4 h HRT, with an efficiency of 60%) every weekday, which had a total solid (TS) concentration of 1.0–1.5% (w/w). Subsequently, purged biomass was thickened in gravity-settling cones for 24 h to increase the TS concentration to 2.0–2.5% (w/w) before undergoing anaerobic digestion.

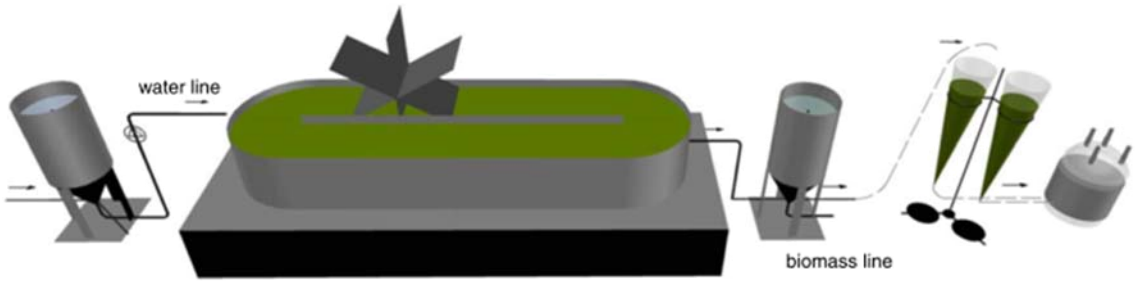


Figure 10.1 Schematic diagram of the process (Passos et al., 2015b).

10.2.2 HRAP biomass

Mixed culture of green microalgae, mainly *Oocystis* sp., diatoms, bacteria and other microorganisms such as protozoa, grown spontaneously in HRAP used for the enzymatic pretreatments for the BMP tests (BMP-E and BMP-L are referred to the pretreatment using commercial enzymes and using laccase enzyme, respectively, further information will be described) (Passos et al., 2015b). Initial characteristics of harvested biomass are summarised in Table 10.1.

Table 10.1 Main characteristics of microalgal biomass used in the BMP tests.

Parameter	Microalgal biomass BMP-E	Microalgal biomass BMP-L
pH	7.8	7.7
TS [%(w/w)]	4.87	3.28
VS [%(w/w)]	3.28	2.07
VS/TS (%)	67	63
COD (g/L)	33.5	31.3
Proteins (% VS)	58	59
Carbohydrates (% VS)	22	22
Lipids (% VS)	20	19

10.2.3 Anaerobic digestion inoculum

The inoculum was mesophilic digested sludge from an anaerobic digester of a municipal WWTP located in Gavà (Catalunya, Spain). The WWTP treats 64,300 m^3/d. It treats the domestic WW from Castelldefels, Gavà, Sant Climent de Llobregat, South-East sector of Sant Boi de Llobregat and Viladecans for around 300,000 inhabitant equivalent with a biological wastewater treatment with nutrient removal and water regeneration for agriculture purposes. The thickened sludge from primary and secondary sludge is treated in the anaerobic digester. Anaerobic digester data is presented in Table 10.2. Table 10.3 presents the initial characteristics of the inoculum used for the BMP tests after the enzymatic pretreatment.

Table 10.2 Anaerobic digester data from the WWTP Gavà-Viladecans (September 2015).

Parameter	WWTP Gavà-Viladecans
Bacteria	Mesophilic
Digester volume (m^3)	2 x 3000
HRT (d)	20-24
Methane (%)	65

Table 10.3 Initial characteristics of the inoculum used in BMP tests.

Parameter	Inoculum BMP-E	Inoculum BMP-L
pH	7.4	7.5
TS [%(w/w)]	2.71	3.63
VS [%(w/w)]	1.88	2.57
VS/TS (%)	69	71
COD (g/L)	30.1	31.2

10.2.4 Enzymatic pretreatment set up

Two enzymatic studies were applied onto microalgal biomass prior the BMP tests in order to increase biomass solubilisation and increase methane production.

The studies include the use of commercial enzymes (laccase, cellulase, glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase) and the use of a fungal broth of *Trametes versicolor* containing laccase enzyme and other isoenzymes secreted by the fungus.

The first microalgal biomass solubilisation enzymatic pretreatment was conducted using cellulase, glucohydrolase and an enzyme mix composed of cellulase, glucohydrolase and xylanase. For evaluating the best pretreatment conditions, two enzyme doses were compared (0.5 and 1%) over an exposure time of 48 h (Ehimen et al., 2013). To this end, a volume of 100 mL of microalgal biomass was placed in Erlenmeyer flasks (150 mL) where the corresponding dose of enzyme was added (0.5 and 1% w/w). Both doses were assayed in triplicate for the three studied enzymes. Trials were set in a room with controlled temperature at 37 ºC, under

continuous mixing. This temperature was set as the optimal for enzymatic activity. Samples of approximately 30 mL were removed after 6 h, 12 h, 24 h and 48 h for analysing volatile solids solubilisation after pretreatment. The best conditions were selected for the BMP test, BMP-E.

The second microalgal biomass solubilisation enzymatic pretreatment was carried out using commercial laccase and the fungal broth, afterwards, BMP test was carried out (BMP-L). A stock solution of commercial laccase was prepared and added to microalgal biomass (31 g wet weight) before BMP tests. *T. versicolor* broth was produced in 250 mL Erlenmeyer flasks containing 0.9 g cell dry weight of *T. versicolor* pellets in 100 mL of medium containing: 8 g/L of glucose, 3.3 g/L of ammonium tartrate, 1.168 g/L of 2,2-dimethylsuccinate buffer, 10 and 100 mL/L of a micro and macronutrient solution, respectively (Kirk et al., 1978); adjusted to pH 4.5 with HCl. Pellets were cultured in six Erlenmeyer flasks, 3 of them were cultured until laccase production was 100 U/L (3.5 days) and then they were sieved in order to separate the fungal pellets. The other 3 Erlenmeyer flasks were daily monitored for laccase production and glucose consumption until glucose was totally consumed.

The laccase concentration in BMP-L bottles was 100 U/L and the contact time with microalgal biomass prior to BMP tests was 20 minutes (Moilanen et al., 2011). The pretreatment was conducted at the optimal *T. versicolor* laccase production temperature, 25±1 ºC. It was kept on a 100 rpm shaker platform.

10.2.5 BMP tests set up

After the enzymatic pretreatment of microalgal biomass, BMP tests were carried out in serum bottles of 160 mL, with a useful volume of 100 mL and a headspace volume of 60 mL. The substrate to inoculum ratio was 0.5 g VS_s/g VS_i, and each bottle contained 5 g of VS, based on previous studies (Alzate et al., 2012). Afterwards, bottles were filled with distilled water up to 100 mL, flushed with helium gas, sealed with butyl rubber stoppers and incubated at 35 ºC until biogas production ceased. Biogas production was measured by the pressure increase in the headspace volume using an electronic manometer (Greisinger GMH 3151). After each measurement, biogas was purged from the reactor's headspace until atmospheric pressure; afterwards reactors were manually shaken. Samples from the gas headspace volume were taken every 2–3 days to determine biogas composition (CH_4/CO_2) by gas

chromatography (GC). A blank treatment was used to quantify the amount of methane produced by the inoculum. Results were expressed as methane yield calculated by subtracting the blank results to each trial, divided by the amount of microalgal biomass (g VS) added to each bottle.

The following trials were carried out for evaluating the enzymatic pretreatment effect under the best conditions selected in the former solubilisation assay (BMP-E): (1) cellulose (1% dose over an exposure time of 6 h before undergoing BMP tests), (2) enzyme mix (cellulase, glucohydrolase and xylanase) (1% dose over an exposure time of 6 h before undergoing BMP tests), (3) microalgal biomass control (biomass without pretreatment, exposed to 37 ºC for 6 h), (4) blank containing only inoculum, in order to quantify the methane production by endogenous respiration.

The following trials were carried out for laccase enzymatic pretreatment (BMP-L): (1) microalgal biomass pretreated with commercial laccase, (2) microalgal biomass pretreated with fungal broth, (3) non-pretreated microalgal biomass control, (4) commercial laccase control, (5) fungal broth control, and (6) blank containing only inoculum, in order to quantify the methane production by endogenous respiration. Commercial laccase control results were subtracted from microalgal biomass pretreated with commercial laccase; whereas fungal broth control results were subtracted from microalgal biomass pretreated with fungal broth.

All experimental trials, including pretreatments, controls and blank were performed in triplicate and expressed at standard temperature and pressure.

10.3 Results and discussion

10.3.1 BMP-E test

10.3.1.1 Enzymatic pretreatment

The enzymatic pretreatment was first applied for testing microalgal biomass solubilisation. To this end, the enzymes cellulase, glucohydrolase and an enzyme mix (cellulase, glucohydrolase and xylanase) were applied at doses of 0.5 and 1% over an exposure time of 48 h. The results are summarised in Figure 10.2. As can be seen, when an enzyme dose of 0.5% was applied, the maximum soluble VS

concentration was reached after 12 h and the highest value was similar in all cases (572–631 mg soluble VS/L). For the enzyme dose of 1%, cellulase and the enzyme mix exhibited a faster solubilisation of the biomass, reaching high soluble VS concentration already after 6 h of pretreatment (590 and 579 mg soluble VS/L, respectively), however the maximum solubilisation was obtained after 12 h of pretreatment for cellulase (631 mg soluble VS/L) and 24 h after for the mix (708 mg soluble VS/L). Glucohydrolase solubilisation was maintained lower along the treatment, the maximum value was obtained for a 1% concentration after 12 h (638 mg soluble VS/L).

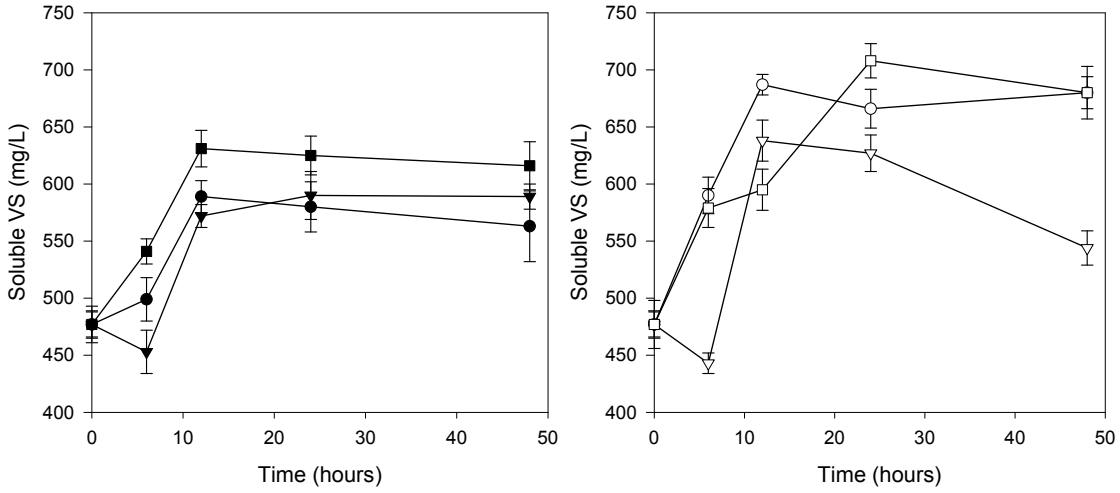


Figure 10.2 Volatile solids solubilisation after enzymatic pretreatment with cellulase [(●) and (○)], glucohydrolase [(▼) and (▽)] and the enzyme mix (cellulase, glucohydrolase and xylanase) [(■) and (□)] with a dose of 0.5% (left) and 1% (right).

The effectiveness of the enzymatic pretreatment is linked to the composition of microalgae cell wall. In our study, microalgal biomass consisted on a mixed community of microalgae and bacteria grown in HRAP. Generally, microalgae cells harvested from open ponds treating wastewater have a resistant cell wall due to the high organic content of the culture media and to the presence of grazers (e.g., protozoa and rotifers) (Park et al., 2011b; Passos et al., 2015b). In this study, microalgal biomass was composed mainly by diatoms and *Oocystis* sp. Diatoms have a resistant nanopatterned silica layer and *Oocystis* sp. is composed by multiple external layers formed by structural polysaccharides, mainly cellulose and hemicellulose.

From the enzymes investigated, cellulase is responsible for cellulose hydrolysis, while glucohydrolase and xylanase are responsible for hemicellulose hydrolysis. The

best results were reached for the enzyme mix at 0.5% dose, and cellulase and enzyme mix at 1% dose. The reason for the better performance of the enzyme mix is the synergistic effect among several macromolecules contained in the cell structure. This is to say that the enzymes glucohydrolase and xylanase may have had higher enzymatic activity after celluloses were already hydrolysed by cellulase in the enzyme mix, i.e. in this case hemicellulose would have become more available to the enzymes. Cellulase was also effective, which may be explained by the high content of cellulose in the cell wall structure of microalgae.

These hypotheses are in agreement with the results obtained by pretreating the filamentous microalgae *Rhizoclonium* sp. with an enzyme mix of amylase, protease, lipase, xylanase and cellulase, which was more effective than applying these enzymes separately (Ehimen et al., 2013). Furthermore, the same study also showed that cellulase accounted for the highest effect among the studied enzymes in 48 h. In the case of *C. vulgaris*, enzymatic pretreatment with carbohydrolase (Viscozyme L®) and protease (Alcalase 2.5 L®) increased carbohydrate and protein solubilisation by 86 and 96%, respectively in 5 h pretreatment (Mahdy et al., 2014). Besides, fungal enzymes (*Aspergillus lentulus* and *Rhizopus oryzae*) enhanced *Chrococcus* sp. cells permeability and COD solubilisation by 29% during the 48 h incubation (Prajapati et al., 2015).

10.3.1.2 Biomass solubilisation and biogas production in BMP-E test

In accordance with the previous results, the selected enzymatic pretreatment conditions for evaluating the anaerobic digestion performance in BMP tests were 1% of cellulase and enzyme mix for 6 h. Results of the pretreatment and BMP tests are shown in Table 10.4 and Figure 10.3. Biomass solubilisation was increased by 110% after enzymatic pretreatment with cellulase and by 126% with the enzyme mix (Table 10.4). These increases were calculated by comparing the results with those obtained with the temperature control at 37 ºC for 6 h. Thus, the calculated solubilisation increase was only attributed to the enzymatic effect.

The BMP-E test showed how the final methane yield was increased by the enzymatic pretreatment although there were no significant differences in terms of hydrolysis rate (Table 10.4). Indeed, the methane yield was significantly higher with the enzyme mix, 217 mL CH_4/g VS (15% increase), followed by cellulase, 203 mL CH4/g VS (8%

increase), as compared to both temperature and microalgal biomass controls (188 and 189 mL CH_4/g VS, respectively). Thus, it can be concluded that there was no thermal effect of this pretreatment, but only enzymatic, and that mixing different enzymes (cellulase, glucohydrolase and xylanase) improved the performance in respect to a single enzyme (cellulase). As can be observed, the higher the VS solubilisation, the higher the methane yield in BMP tests, i.e. pretreatment with cellulase reached 110% solubilisation increase and 8% methane yield increase, while pretreatment with enzyme mix reached 126% solubilisation increase and 15% methane yield increase.

Table 10.4 Microalgal biomass solubilisation and methane yield under enzymatic pretreatment.

Trial	Soluble VS (mg/L)	Hydrolysis rate (d^{-1})	Methane yield (mL CH_4/g VS)
Microalgal biomass control	33.2 (0.6)[a]	0.21 (0.004)[a]	188.6 (3.2)[a]
Temperature control	50.4 (3.7)[a]	0.20 (0.002)[a]	188.3 (0.8)[a]
Cellulase	105.9 (9.1)[b]	0.18 (0.002)[a]	203.0 (0.4)[b]
Enzyme mix (glucohydrolase, cellulase, xylanase)	114.0 (7.4)[b]	0.20 (0.001)[a]	217.3 (7.2)[c]

[a,b,c] *Stand for significantly different values within columns (ρ=0.05).*

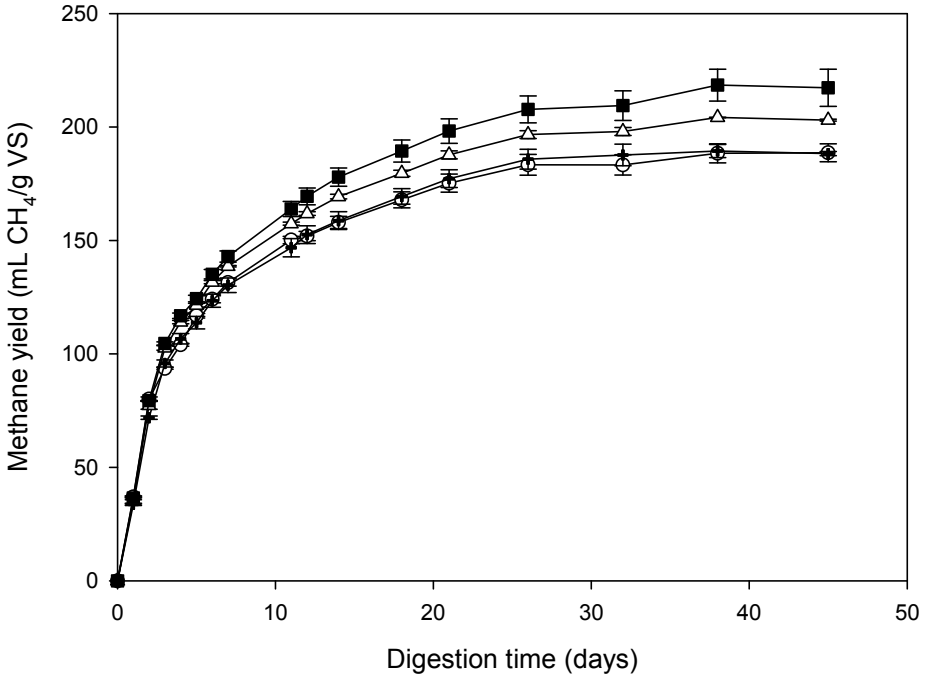


Figure 10.3 Accumulated methane yield in biochemical methane potential (BMP) tests under enzymatic pretreatment with cellulase and the enzyme mix (cellulose, glucohydrolase and xylanase). (○) *microalgal biomass control;* (+) *temperature control*; (△) *cellulase;* (■) *enzyme mix.*

Results obtained in our study are in accordance with the literature. For instance, the anaerobic digestion of the filamentous microalgae *Rhizoclonium* sp. reached the highest methane yield after pretreatment with an enzyme mix composed by amylase, protease, lipase, xylanase and cellulase (31% increase), followed by the pretreatment with only cellulase (20% increase) (Ehimen et al., 2013). Besides, the enzymatic pretreatment of *C. vulgaris* and *Chlamydomonas reinhardtii* with carbohydrolase (Viscozyme L®) and protease (Alcalase 2.5 L®) enhanced the methane yield of *C. vulgaris* by 14%, while the methane yield of *C. reinhardtii* did not increase due to the lack of cellulose on its cell wall (Mahdy et al., 2014). Gruber-Brunhumer et al. (2015) prior the BMP test conducted an enzymatic pretreatment consisting of cellulase, xylanase, betaglucanase and protease mix, at 37 ºC for 24 h. Methane production was 14% increased, which is in accordance with the present study.

The results from the present study show lower methane yields than thermal and mechanical pretreatments described in the bibliography. Literature results showed how the methane yield increased by 15–220% for thermal pretreatment at 70–170ºC; up to 90% for ultrasound pretreatment and up to 78% for microwave pretreatment compared to non-pretreated biomass (Passos et al., 2014c). Thermal pretreatment at low temperatures (<100 ºC) seems to be the most promising physical method so far. In fact, when comparing the effect of thermal, hydrothermal, microwave and ultrasound pretreatments on the same biomass harvested from microalgae-based wastewater treatment systems, the highest methane yield increase was achieved after thermal pretreatment at 95 ºC (72%), in comparison with the other methods (8–28%) (Passos et al., 2015a). Even if the enzymatic pretreatment achieved a lower methane yield increase (8–15%, results from the present study), biological pretreatments have lower energy requirements compared to physical methods and, therefore, they are more likely to be compensated by the energy gain from biogas production. On the other hand, Ometto et al. (2014) reported that enzymatic hydrolysis showed the greatest biogas yield increments (>270%) followed by thermal hydrolysis (60-100%) and ultrasounds (30-60%).

Specific enzymes increase the microalgal biomass solubilisation, however, the use of an enzyme mix, gives better results due to the synergistic effects. This increase on

the methane production was compared using a non-specific enzyme, such as laccase, that has been used in a wide range of environmental applications.

10.3.2 BMP-L test

10.3.2.1 Fungal broth production

Trametes versicolor cultured in Kirk's nutrient medium produces laccase enzyme and is appropriated for studying the ligninolytic activity of fungal cultures (Casas et al., 2013). Laccase production and glucose consumption from *Trametes versicolor* culture are shown in Figure 10.4.

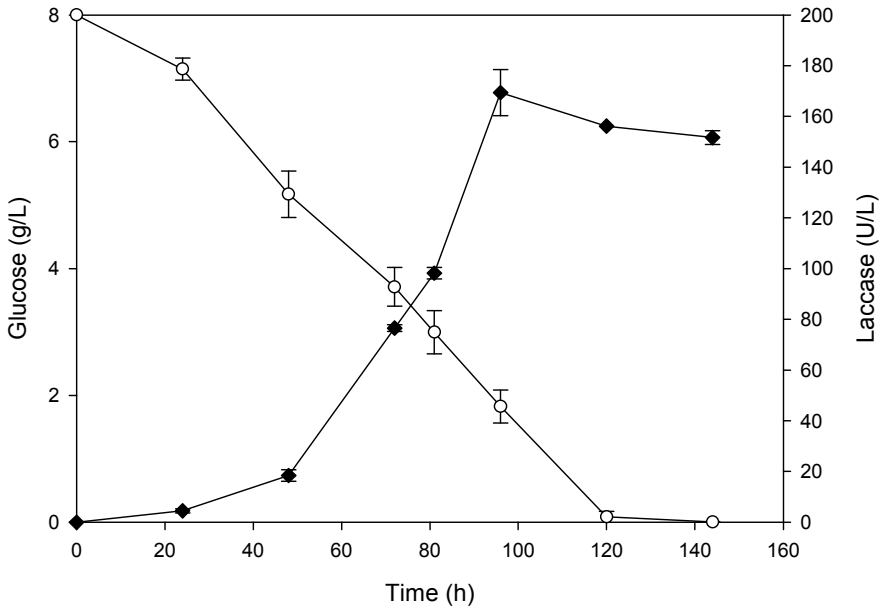


Figure 10.4 Glucose consumption (○) *and laccase production* (♦) *by Trametes versicolor.*

Laccase enzyme is excreted by *T. versicolor* to the broth, which is associated to both growth and glucose consumption. Enzyme production increased over the first 4 days and, after reaching a maximum activity level (170 U/L, 4 days), it started to decrease, since the carbon source (glucose) was consumed. The same laccase activity behaviour was observed by other authors (Blánquez et al., 2004; Borchert and Libra, 2001).

The fungal broth obtained from *T. versicolor* culture in Kirk's medium is mostly rich in laccase enzyme, among other enzymes or mediators and unconsumed glucose. After 3 days of cultivation, other enzymes could be secreted by *T. versicolor,* such as

cellulases and hemicellulases (Singh et al., 2013), possibly important for microalgae cell wall degradation.

10.3.2.2 Biogas production in BMP-L test

The fungal broth and commercial laccase were applied at a dose of 100 U/L of laccase enzyme and were used as a pretreatment for microalgal biomass solubilisation in order to evaluate the increase of the anaerobic biodegradability in BMP-L tests. The experiment lasted 32 days, until accumulated biogas production reached an asymptote (Figure 10.5). As can be seen from the results, both pretreated trials increased the biogas production compared to non-pretreated microalgae. Moreover, the fungal broth pretreatment attained the highest value. The methane content was measured along the experiment obtaining an average concentration of 68±4.5% CH_4. Control trials from both laccases (commercial and fungal broth) were subtracted from the corresponding pretreatment, along with the production of the inoculum, to obtain the net biogas and methane production along with the net methane yield (Table 10.5).

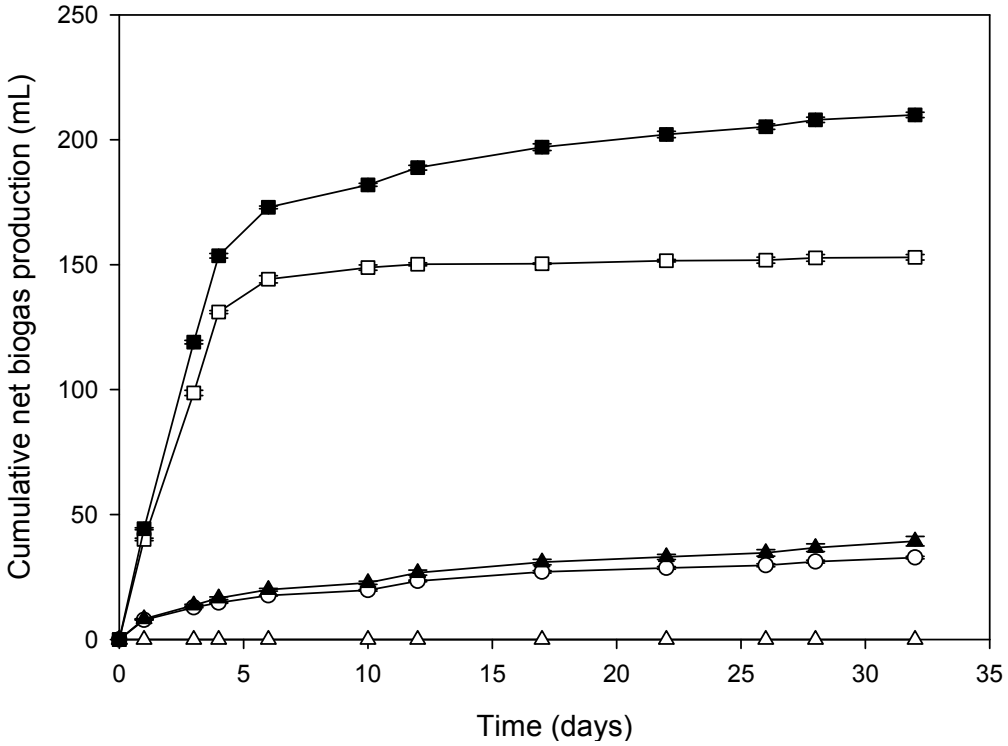


Figure 10.5 Cumulative net biogas production for the anaerobic digestion of microalgal biomass using two enzymatic pretreatments and their respective controls. Commercial laccase control (△)*; Microalgal biomass control* (○); *Commercial laccase pretreatment* (▲)*; Fungal broth control* (□)*; Fungal broth pretreatment* (■)*.*

Table 10.5 Net methane production and yield for the different trials of the BMP-L test.

Trial	Biogas production (mL)	Methane production (mL CH_4)	Methane yield (mL CH_4/g VS)
Microalgal biomass control	33±0.5	22±0.5	83±1
Commercial laccase control	0.0	0.0	-
Fungal broth control	153±1.1	104±1.1	-
Commercial laccase pretreatment	40±1.3	27±1.3	100±7
Fungal broth pretreatment	210±0.3	143±0.3	144±2

Regarding the control trials, commercial laccase control did not produce any biogas. Microalgal biomass control produced little methane (22 mL CH_4), whereas the fungal broth control produced 104 mL CH_4. Indeed, reactors containing fungal broth produced more biogas than the rest, since they contained part of the nutrients (mainly glucose) present in the media for laccase production, which were not completely consumed by *T. versicolor.* This can be seen from Figure 10.4: when 100 U/L of laccase were obtained, the concentration of glucose was 3 g/L. The amount of biogas produced from glucose remaining in the culture broth was theoretically calculated and compared with experimental results using Buswell equation (Symons and Buswell, 1933). 108 mL CH_4 were theoretically produced, due to the remaining glucose in the media. This theoretical value is in accordance with the experimental one (104 mL CH_4).

With regards to the pretreatment trials, commercial laccase pretreatment increased the methane yield by 20%, whereas fungal broth pretreatment increased the methane yield by 74% in respect to non-pretreated biomass. The results suggest that laccase may solubilise part of the microalgal biomass substrate, enhancing its bioavailability and/or biodegradability by anaerobic microorganisms. However, better results were achieved using the fungal broth. This is probably due to the presence of other enzymes, radicals and other mediators produced by *T. versicolor* during its culture, which may also contribute to microalgal biomass solubilisation (Riva, 2006). It is worth pointing out that even though laccase is not specific on glycoproteins and polysaccharides degradation (the main components of microalgal cell wall), the pretreatment was effective. Therefore, results confirm that laccase played a role on

microalgae enzymatic pretreatment, although a mixture of different enzymes would be preferred. This is common for complex cultures, such as the one of the present study, composed by several microalgae species, bacteria and other microorganisms with different cell wall compositions.

Results obtained are in accordance with previous studies, where microalgae methane yield was increased when non-specific enzymes were added confirming the synergistic effect (Ehimen et al., 2013; Prajapati et al., 2015). Nevertheless, a previous study using filamentous microalgae reported higher values than those obtained in our study. Ehimen et al. (2013) obtained 115-145 mL CH_4/g TS after the pretreatment carried out over 2 days, whereas the values obtained in the present study based on the same units are 63 and 91 mL CH_4/g TS for commercial laccase and fungal broth pretreatment, respectively, for a 20 minutes pretreatment. From those results, contact time seems to be an important parameter that should be investigated.

On the other hand, the methane yield of *Chlorella vulgaris* was increased by 14% after pretreatment with the hydrolytic enzyme carbohydrolase and by 51% after pretreatment with protease after an exposure time of 5 h (Mahdy et al., 2014). Moreover, the same study with *Chlamydomonas reinhardtii* showed no increase after pretreatment with carbohydrolase and only 8% increase after pretreatment with protease. This increase was lower than the ones obtained in our study (20 and 74% increase) and highlights that pretreatment effectiveness is species-specific and depends on the biomass complexity and composition.

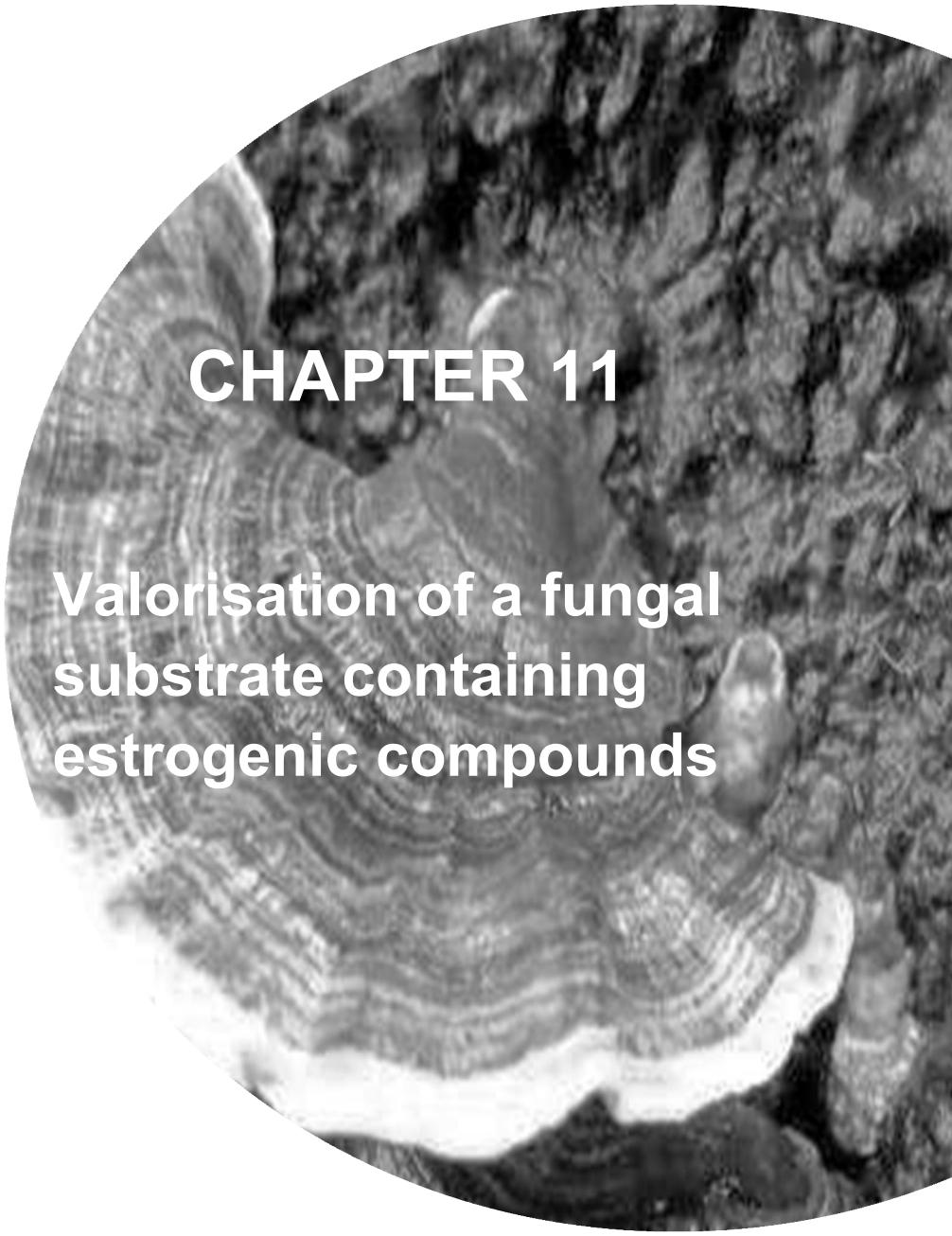
The results obtained in this study demonstrates that enzymatic pretreatment may be applied prior to microalgae anaerobic digestion, with better results for crude fungal enzymes probably due to the presence of other enzymes and other molecules produced by the fungus. This may be more cost-effective compared to commercial enzymes. Nevertheless, these results should be evaluated in continuous anaerobic reactors in order to estimate the energy balance and economic cost of the process, which is yet to be determined.

10.4 Conclusions

The use of the enzymatic pretreatment methods was observed to lead to greater CH_4 conversions than the raw microalgal biomass. The application of an enzyme mix composed by microalgal biomass specific enzymes composed by cellulase, glucohydrolase and xylanase gave better solubilisation results than the use of cellulase or glucohydrolase alone. Methane production was 15% increased using the enzyme mix pretreatment for 12 h, whereas only 8% was increased with cellulase. The use of a non-specific microalgal biomass enzyme, such as laccase, has been tested for the first time for microalgal biomass solubilisation obtaining good results. Better results were observed for the fungal broth rather than commercial laccase, methane production was 74% and 20% increased, respectively, when compared to non-pretreated biomass. This fact may be due to the synergistic effect of laccase and other radicals or mediators produced by *T. versicolor*. Moreover, the use of a fungal broth for an algal pretreatment reduces enzyme purifying costs from the process.

Although the use of fungal broth has a major methane production increase in terms of methane yield, better results were achieved using the enzyme mix, 144 and 217 mL CH_4/g VS, respectively. Then, the use of specific enzymes is preferred; however, for both pretreatments the synergistic effect between enzymes and/or mediators has a great impact on the methane yield. Enzymatic pretreatment time should be further investigated because it seems to play an important role in the biomass solubilisation process.

Enzymatic pretreatments are promising due its low energy requirement, and therefore they should be further investigated in continuous reactors to estimate the energy balance, economic costs of the process and for evaluating full scale viability.



CHAPTER 11

Valorisation of a fungal substrate containing estrogenic compounds

Part of this chapter has been published as:

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11 Valorisation of a fungal substrate containing estrogenic compounds

Abstract

Biochemical methane potential tests of exhausted fungal biomass were assessed in batch assays at mesophilic temperature to study the feasibility of an anaerobic digestion treatment to valorise this substrate. The inocula used were collected from two different sources: the anaerobic digester of a wastewater treatment plant (WWTP) and an anaerobic reactor treating the organic fraction of municipal solid waste (OFMSW). The results showed higher methane yields when using the WWTP inocula (595 mL CH_4/g VS_{feed}) compared with the inocula obtained from the digester treating OFMSW (281 mL CH_4/g VS_{feed}). Further experiments evaluated biogas production of fungal biomass when 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) were spiked onto the substrate at a concentration of 2 mg/L respectively. No differences in methane yield were detected at the end of the experiments for spiked and non-spiked biomass (367 and 307 mL CH_4/g VS_{feed}, respectively), confirming no toxic effect of the hormones at the concentration tested. This study confirms the feasibility of anaerobic digestion treatment for the valorisation of fungal substrate containing these emerging pollutants.

11.1 Introduction

The biogas produced during anaerobic digestion (AD) processes has evolved from a waste treatment by-product into a desirable energy source that can be used for internal heating and electricity production. Sustainable AD treatments are required to optimize the biogas production of existing waste streams and to expand the range of feedstocks with high energy content and the potential to increase biogas yields (Asam et al., 2011).

Sludges from wastewater treatment plants (WWTPs) and wastes from the agricultural and farming sectors were typically used as substrates in earlier anaerobic digesters, followed by a wide range of industrial organic wastes, municipal solid wastes, energy crops and algal biomass (Khalid et al., 2011; Lorenz et al., 2013; Nges et al., 2012). Some authors highlight an increasing interest in the valorization of industrial wastes by anaerobic digestion instead of promoting the use of crop wastes generated in biofuels production that compete with the use of agricultural land (Tufvesson et al., 2013). Depending on the

industrial processes and the nature and amounts of the waste streams, centralized biogas plants can be a more feasible and economic option rather than an “on-site anaerobic digester” (Mahanty et al., 2014). Alternatively, co-digestion of such wastes in anaerobic reactors from WWTPs, which usually operate below their full capacities, would improve the energy efficiency of the whole process (Schwarzenbeck et al., 2008).

In the last several decades, industries involving fermentation processes have used fungi to obtain metabolites (enzymes, flavorings, industrial chemicals and antibiotics). Such fungal biomass is not reused and poorly valorized (Fourest and Roux, 1992; Svecova et al., 2006). Therefore, relatively large quantities of fungal biomass waste could represent a disposal problem. That fact gives the opportunity to valorize this biomass in different ways, such as composting (Gabarrell et al., 2012; Wood, 1979), using fungi as a tool for algae harvesting (Prajapati et al., 2014b, 2013) or anaerobic digestion.

White-rot fungi have been studied as an alternative to bacteria for the biodegradation of xenobiotics such as dyes, pharmaceuticals, pesticides and industrial chemicals. These fungi are attractive candidates for use in bioremediation when compared with bacteria (i.e., they are extremely versatile as they are able to degrade a wide range of contaminants individually or in mixtures) (Asgher et al., 2008; Blánquez and Guieysse, 2008; Cruz-Morató et al., 2013b; Harms et al., 2011; Pointing, 2001). Some studies have tested the application of continuous laboratory scale reactors that require fungal purges (Blánquez et al., 2008, 2006). Gabarrell et al. (2012) carried out a life cycle assessment to evaluate the use of fungal biomass for the removal of a metal-complex dye. In that study 87 g of residual fungal biomass (1.73 kg wet weight) were obtained per 1 m^3 of simulated effluent with the dye for the removal of 90%. That fungal waste was not valorised, but composting was thought as an alternative. Blánquez and Guieysse (2008) used *Trametes versicolor* pellets for the removal of the hormones 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in a fungal bioreactor. The study showed that between 30 and 40% of the initial hormone concentrations were adsorbed onto the fungal biomass and the rest were degraded. The post-treatment of such biomass, which must be replaced periodically, has only been evaluated in a theoretical study where composting

was considered (Gabarrell et al., 2012). Alternatively, anaerobic digestion is potentially a feasible option for the valorization of such waste for biogas production, despite containing micropollutants.

Micropollutants, like pharmaceutical active compounds (PhACs), are detected in the environment, gaining interest on the study of their fate and degradation. Non-assimilated PhACs enter to WWTPs through urban wastewaters (WW), but most of them are non-degraded and are released into the stream effluent or sorbed onto the sludge, depending on their hydrophobic properties (Vicent et al., 2013). The results achieved from previous works including anaerobic digestion of sludge containing estrogens (Esperanza et al., 2007; Ternes et al., 1999) concluded that this kind of emerging pollutants are not toxic and that good removals percentages are reached. Some authors (Carballa et al., 2006) noticed that EE2 contained in the sludge requires an adaptation period for its anaerobic degradation.

The aim of the present study is to assess the suitability of fungal biomass as a feedstock for biogas production and to evaluate the effect of adsorbed hormones onto exhausted fungal biomass. This evaluation was conducted while considering the future potential of fungal biomass co-digestion in existing anaerobic plants.

11.2 Material and methods

The main relevant characteristics from the experiments are presented as follows. More detailed information is presented in Chapter 3 (Materials and methods).

11.2.1 Sabadell wastewater effluent

Inoculum (Inoc-1 and Inoc-3) used for the biochemical potential tests (BMP) was collected from the anaerobic digester of Riu Sec WWTP, which is located in Sabadell (Spain). The WWTP treats 50,000 m^3/d. The watershed is the 'Conques internes de Catalunya' and it treats the domestic wastewater of Sant Quirze del Vallès and Sabadell for around 200,000 inhabitant equivalent with a physico-chemical and biological treatment. The thickened sludge from

primary and secondary sludge is then sent to the anaerobic digester (anaerobic digester data is presented in Table 11.1). The plant is directed by the Ajuntament de Sabadell.

Inoc-1 and Inoc-3 were pre-incubated at 37 °C for 7 days to ensure its degasification and depletion of the residual biodegradable organic material. The characteristics of Inoc-1 and Inoc-3 are presented in Table 11.2.

Table 11.1 Anaerobic digester data from the WWTP Sabadell (May 2013).

Parameter	**WWTP Sabadell**
Bacteria	Mesophilic
Digester volume (m^3)	2 x 3,500
HRT (d)	26
Methane (%)	60
VFA (g/L)	0.21

Table 11.2 Initial characteristics of the inoculums (Inoc-1 and Inoc-3).

Parameter	**Inoc-1**	**Inoc-3**
TS (g/g) (wet basis)	0.023 ± 0.0003	0.008 ± 0.0004
VS (g/g) (wet basis)	0.016 ± 0.0002	0.005 ± 0.0003
COD (g/L)	24.0 ± 1.4	N.D.[a]
VFA (g/L)[b]	0.55 ± 0.05	<0.50
pH	7.8 ± 0.2	7.4 ± 0.0

[a]N.D.: no data available; [b]acetate was the only VFA detected

11.2.2 Ecoparc II municipal solid waste

Inoculum (Inoc-2) used for the BMP tests was collected from the anaerobic reactor of a mechanical and biological treatment (MBT) plant, Ecoparc II, treating the 13% of the organic fraction of municipal solid waste (OFMSW) of Barcelona metropolitan area, which is located in Montcada i Reixac (Spain). Its treatment capacity is 287,500 t/y. The OFMSW is then sent to the anaerobic digester (Table 11.3). The plant is directed by Ecoparc Besòs SA.

Because of its significant solids content, Inoc-2 was passed through an 8 × 8 mm mesh sieve. It was pre-incubated at 37 °C for 7 days to ensure its degasification and depletion of the residual biodegradable organic material. The characteristics of the Inoc-2 are presented in Table 11.4.

Table 11.3 Anaerobic digester data from the MBT Sabadell (January 2013).

Parameter	MBT Ecoparc II
Bacteria	Mesophilic
Digester volume (m^3)	4,500
HRT (d)	28
Methane (%)	55
VFA (g/L)	1.05
Biogas production (Nm^3/kg)	0.55
Biogas production (Nm^3/y)	11,592,512
Engine power (MW)	4.156

Table 11.4 Initial characteristics of the inoculum (Inoc-2).

Parameter	Inoc-2
TS (g/g) (wet basis)	0.130 ± 0.001
VS (g/g) (wet basis)	0.067 ± 0.001
COD (g/L)	117.4 ± 42
VFA (g/L)[b]	0.62 ± 0.16
pH	7.9 ± 0.1

[b] *acetate was the only VFA detected*

11.2.3 Experimental set up

In order to evaluate the biogas production potential from fungal biomass BMP test was carried out.

The first set of batch assays (BMP-1) was conducted to assess the methane potential of the fungal pellets and to evaluate the effects of the inoculum source in terms of methane production. The second set of batch assays assessed the

effects of the adsorbed estrogenic compounds on the *T. versicolor* pellets (BMP-2) and the feasibility to completely remove them without previous adaptation of the inocula to the micropollutant. The total solids (TS) and volatile solids (VS) content of the pellets for each BMP assay are presented in Table 11.5. The pellets were spiked with 2 mg/L of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), respectively, in the second set of BMP tests (BMP-2) to simulate the fungal bioreactor purge after the biodegradation treatment of these micropollutants (Blánquez and Guieysse, 2008).

Table 11.5 Substrate characteristics.

Assay	TS (g TS/g)[a]	VS (g VS/g)[a]
BMP-1, Inoc-1	0.032 ± 0.001	0.026 ± 0.002
BMP-1, Inoc-2	0.068 ± 0.004	0.062 ± 0.004
BMP-2, Inoc-3	0.068 ± 0.004	0.062 ± 0.004

[a] *wet basis (g)*

Commercial aluminum bottles (1 L) were used as batch reactors. The caps of each bottle included a manual valve that was used to measure biogas production (Figure 11.1) (Ferrer et al., 2008). The methodology was adapted from Field et al. (1988) and Martín-González et al. (2010). The initial inoculum concentration was 5 g VS/L, and two inoculum:substrate ratios ($r_{I/S}$) were tested; 2:1 and 5:1 (VS/VS). $NaHCO_3$ was added to all tests to maintain a neutral pH. Reactors were filled with tap water to a final volume of 600 mL. All experimental reactors, including control reactors without any substrate, were prepared in triplicate and incubated at 37 °C until methane production ceased. Table 11.6 shows the initial compositions of the batch reactors for BMP-1 and Table 11.7 for BMP-2. The methane production of control reactors was subtracted to present the net methane production relative to fungal biomass degradation without the inoculum contribution. Before starting the experiments, all bottles were flushed with nitrogen for 30 s to remove ambient air.

The biogas production and methane composition were analyzed twice per week. The biogas produced was measured using a pressure switch manometer (1 bar, 5% accuracy; SMC, Vitoria, Spain). After each measurement, the biogas

was purged from the reactor headspace by opening the manual valve, then the reactors were closed and manually shaken.

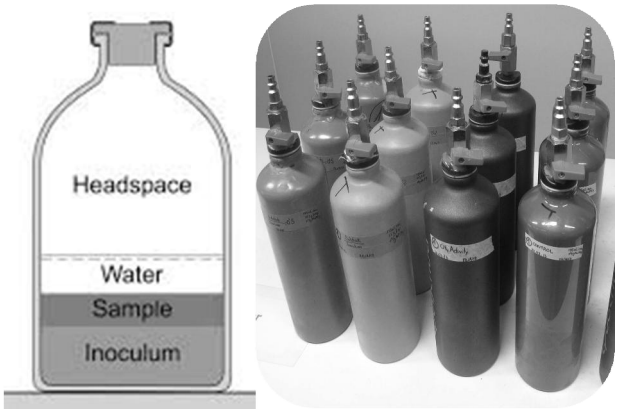

Figure 11.1 Left: Experimental set up for BMP tests. Right: Commercial aluminium bottles and manual valve cap used for BMP tests.

Table 11.6 Initial content of batch reactors for BMP-1 test.

	Inoc-1		**Inoc-2**	
	$r_{I/S}$ = 2/1	**$r_{I/S}$ = 5/1**	**$r_{I/S}$ = 2/1**	**$r_{I/S}$ = 5/1**
Inoculum (mL)	185	185	48	48
Substrate (g_{wet})	56.2	22.5	24	9.6
Water (mL)	415	415	555	555
$NaHCO_3$ (g)	1.5	0.6	1.5	0.6
Volume (mL)	660	620	620	610

Table 11.7 Initial content of batch reactors for BMP-2 test.

	Inoc-3, non-Spiked		**Inoc-3, Spiked**	
	$r_{I/S}$ = 2/1	**$r_{I/S}$ = 5/1**	**$r_{I/S}$ = 2/1**	**$r_{I/S}$ = 5/1**
Inoculum (mL)	555	555	555	555
Substrate (g_{wet})	24	9.6	24	9.6
Water (mL)	45	45	45	45
$NaHCO_3$ (g)	1.5	0.6	1.5	0.6
E2-EE2 (mg/L)	-	-	2	2
Volume (mL)	600	590	600	590

11.2.4 Anaerobic biodegradability assay

Prior to the BMP tests, inoculum methanogenic activity assays were performed according to Field et al. (1988) in order to assure the suitability of each inoculum for use in further experiments. This test can be used to select an adapted sludge as inoculum, to follow changes in sludge activities due to a possible build-up of inert materials, to estimate maximum applicable loading rate to certain sludge or to evaluate batch kinetic parameters. The activity of the inoculum is also important for prediction of methanization characteristics of specific waste that could be introduced in a specific reactor. Higher methanogenic activities contribute to prevent the acidification during anaerobic batch biodegradation of waste, since the inoculum is able to process a higher flow of metabolites such as hydrogen, acetate and other volatile fatty acids (VFAs), preventing their accumulation. The activity assay should be carried out under the same BMP test temperature condition. The assay should be done in triplicate placing a sample of inoculum into 100-2000 mL serum bottles which headspace is flushed with nitrogen gas. Substrate (acetic, propionic and butyric acids), macro- and micro-nutrients are then added in the reactor (Angelidaki et al., 2009; Field et al., 1988).

The initial rate of methane accumulation is the optimal estimate of methanogenic activity of the biomass. Bacterial growth and adaptation changes the biomass characteristics. Likewise, pH and concentration of substrate and nutrients in the vials are changing during process. Therefore, only the initial linear rate of the methane accumulation curve should be considered to indicate that adaptation or no significant growth of biomass has taken place during the test period. Therefore, the test period of the assay could be limited to the linear part of the methane production period which is usually depending on the activity and is in the range of 0.5-10 days (Angelidaki et al., 2009; Field et al., 1988).

The experimental conditions are adapted from Field et al. (1988) and are compiled in Table 11.8 for the specific BMP assays. An anaerobic basic medium containing nutrient solution, trace metals solution, yeast extract and sodium sulfide solution was prepared for the methanogenic activity assays (Table 3.16) (Kortekaas et al., 1995). The substrate solution contained a

mixture of acetic:propionic:butyric acid (73:21:4) (v/v) (Table 3.17) and the initial concentration of this mixture in each batch reactor was 1.5 g/L.

Table 11.8 Reactors composition for methanogenic activity tests.

Parameter	Inoc-1 (mL)	Inoc-2 (mL)	Inoc-3 (mL)
Inoculum	56	15	165
Water	517	556	407
Substrate solution	24	24	24
Macronutrients solution	1.2	1.2	1.2
Trace elements solution	1.2	1.2	1.2
Sulphur solution	1.2	1.2	1.2
Yeast extract	0.12 [a]	0.12 [a]	0.12 [a]

[a] *weight (g)*

Field et al. (1988) established a methanogenic activity range of 0.02-0.2 g COD-CH_4/g VSS while Angelidaki et al. (2009) showed the minimum of the methanogenic activity on acetate to be 0.1 g COD-CH_4/(g VSS·d) for sludge inoculum and 0.3 g COD-CH_4/(g VSS·d) for granular sludge inoculum.

Calculations for the methanogenic activity are presented in Chapter 3.

11.3 Results and discussion

11.3.1 Methanogenic activity tests

Cumulated methane production of each inocula (Inoc-1, Inoc-2 and Inoc-3) used for BMP-1 and BMP-2 are shown in Figure 11.2.

The methanogenic activity results of Inoc-1 and Inoc-3, both collected from a WWTP, were 0.102±0.05 and 0.127±0.06 g CH_4-COD/(g VS·d), respectively, while Inoc-2, collected from a digester used to treat OFMSW, had a methanogenic activity of 0.110±0.05 g CH_4-COD/(g VS·d). The values obtained agreed with those recommended by Angelidaki et al. (2009) and Field et al.

(1988). Therefore, the inocula used in this study were suitable for performing the BMP tests.

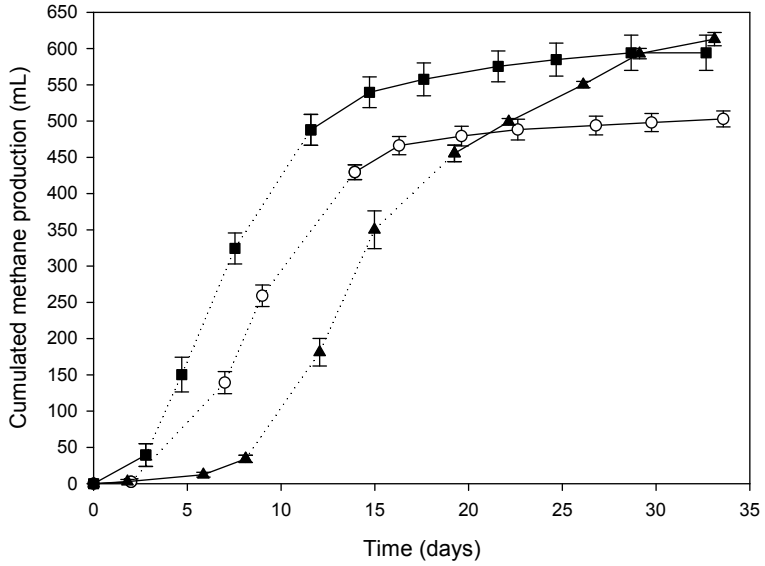

Figure 11.2 Cumulated methane production of the inocula for methanogenic activity determination. (○) *Inoc-1, BMP-1;* (▲) *Inoc-2, BMP-1;* (■) *Inoc-3, BMP-2; Dotted lines indicated the maximum slope of methane production.*

11.3.2 BMP tests using fungal biomass

The mean cumulative net methane production values obtained in BMP-1 for the two tested inoculum:substrate ratios are presented in Figure 11.3. This experiment was conducted until the biogas production between two consecutive measurements no longer exceeded a difference of 10% which took 33 days.

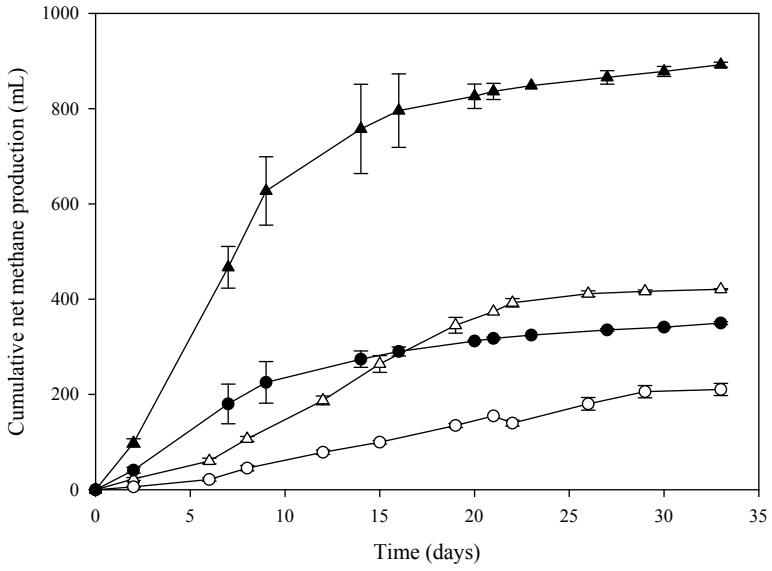

Figure 11.3 Cumulative net methane production for the anaerobic digestion of fungal biomass using two different inocula. (▲)*Inoc-1, r I/S=2;* (△) *Inoc-2, r I/S=2;* (●) *Inoc-1, r I/S=5;* (○) *Inoc-2, r I/S=5.*

The methane production in batch reactors containing Inoc-1 was statistically different from reactors containing Inoc-2 ($p<0.001$) for both inoculum:substrate ratios (see Figure 11.3). These results confirm the influence of the inoculum source on the methane potential values, as previously reported by Elbeshbishy et al. (2012). Although both inocula were collected from anaerobic digester, their compositions were considerably different. Inoc-1 was obtained from a digester that was used to treat sludge wastes from a WWTP, and Inoc-2 was obtained from a reactor treating OFMSW. The net cumulative methane production profiles indicate that the Inoc-2 reactors have a slower methane production rate.

The results of BMP-1 are shown in Table 11.9. The pH remained constant during the assay because of the addition of sodium bicarbonate as a buffer. The mean methane content in the biogas present values ranged between 65% and 69%, commune data for a healthy anaerobic process and in accordance with other authors (Bolzonella et al., 2003; Davidsson et al., 2007).

The VS reduction values, varied from 16 to 40% and were similar to those obtained in previous studies that used similar inocula and other biodegradable substrates, such as OFMSW (Cabbai et al., 2013). A higher methane yield was achieved in the reactors containing Inoc-1 for $r_{I/S}=2$ and $r_{I/S}=5$ (595 mL CH_4/g VS_{feed} and 583 mL CH_4/g VS_{feed}, respectively) compared with the reactors containing Inoc-2 (281 mL CH_4/g VS_{feed} and 329 mL CH_4/g VS_{feed}, respectively). As we stated above, these differences could be related to the different origins of inocula and their adaptation to the substrate, microbial communities differ between inocula leading to different methane yields (Symsaris et al., 2015).

Methane yield values for Inoc-1 were not affected by the inoculum:substrate ratio used, final values were not statistically different ($p>0.001$) and the VS reduction was the same. However, Inoc-2 had statistically differences ($p<0.001$), which indicates the importance of the ratio used for methane production.

Inoc-2 results using the high inoculum:substrate ratio presented unexpected values; although the methane yield was higher the volatile solids reduction was lower. Because of this, the data obtained for this inoculum:substrate ratio using

Inoc-2 has been considered an experimental error and has been ignored for the following discussion.

Table 11.9 Final results of BMP-1 test.

	$r_{I/S} = 2$		$r_{I/S} = 5$	
	Inoc-1	**Inoc-2**	**Inoc-1**	**Inoc-2**
Final net methane (mL)	893 ± 5	421 ± 2	350 ± 3	210 ± 13
% of methane in the biogas	65 ± 1	65 ± 2	69 ± 4	67 ± 1
Methane yield (mL CH_4/g VS_{fed})	595 ± 18	281 ± 25	583 ± 16	329 ± 22
pH	7.04 ± 0.1	7.28 ± 0.1	7.06 ± 0.1	7.26 ± 0.01
VS reduction (%)	40 ± 1	38 ± 8	40 ± 1	16 ± 3

Experimental results can be contrasted with the theoretical methane production results. Using equation 3.4 the theoretical methane potential (TMP) obtained is 421 mL CH_4/gVS, lower than the obtained using Inoc-1 and higher than Inoc-2. Theoretical molecular formulas for proteins, lipids and carbohydrates, as well as stoichiometric methane potential (SMP) were used to have general predictions, however, they do not take into account specific conditions that the experimental assay does.

Because of the lack of previous studies on the valorization of fungal biomass through anaerobic digestion, our results can only be compared with previous studies that used other organic substrates. Among the studies that used inocula from anaerobic digesters of WWTP, Cabbai et al. (2013) tested different types of municipal solid wastes from canteens, supermarkets, markets, households, restaurants and bakeries as substrates. The authors reported methane yields between 200 and 700 mL CH_4/g VS_{feed}. Higher values, between 940 and 1400 mL CH_4/g VS_{feed}, were obtained by Elbeshbishy et al. (2012) when food waste was digested by an inocula obtained from a WWTP anaerobic reactor. However, when the substrate was primary sludge, the methane yield decreased to 220–273 mL CH_4/g $VS_{feed.}$ Our study showed a methane yield of 595 mL

CH_4/g VS_{feed} when using Inoc-1, this value is within the range of values reported by Cabbai et al. (2013).

Among the studies that have used inocula obtained from OFMSW digesters, Martín-González et al. (2010) used a mixture of OFMSW and oil and grease from sewage treatment plants as a substrates. The authors tested different inoculum:substrate ratios and observed methane yields of 277 mL CH_4/g VS_{feed} when an inoculum:substrate ratio of 2:1 was used (the same ratio as used in this study). Elbeshbishy et al. (2012) obtained methane yields that varied from 440 to 790 mL CH_4/g VS_{feed} when food waste was anaerobically degraded by an inocula obtained from a digester treating source separated organics. A methane yield of 329 mL CH_4/g VS_{feed} was obtained in the present study when using Inoc-2, proceeding from an OFMSW digester.

Other biomass-type substrates, such as algae, have been studied in BMP tests. The cell walls of algae and fungi have some macromolecular similarities. Algae are composed of cellulose, hemicelluloses, pectin and glycoproteins (Wang and Evangelou, 1995). In contrast, fungi contain chitin (glucosamine polymer), glucan (glucose polymer) and mannoproteins (mannose-containing glycoproteins) (Webster and Weber, 2007). The absence of cellulose in the cell walls of fungi leads to higher methane yields than in studies using algae as a substrate. For instance, Costa et al. (2012) used digested anaerobic sludge from a WWTP as an inoculum and macroalgae collected from aquaculture tanks as a substrate and observed methane yields lower than 200 mL CH_4/g VS_{feed}. Low yields (150 mL CH_4/g VS_{feed}) were also obtained by Yen and Brune (2007), who anaerobically co-digested microalgae and waste paper for methane production. Another example of anaerobic digestion with algae is the study carried out by Prajapati et al. (2014a) who tested exhausted algal biomass as a cosubstrate together with cattle dung. The obtained values were higher than those previously mentioned, 292 mL CH_4/g VS_{feed}.

Our results show that fungal biomass could be considered as a potential methane production source. The obtained methane yields were higher than those obtained from algae and within the range of values achieved using common organic wastes.

11.3.3 BMP tests using fungal biomass spiked with estrogens

The main objective of this set of BMP tests was to assess the valorization of the fungal biomass that was used in a previous biodegradation treatment, where non-degraded micropollutants were potentially adsorbed onto the biomass (Blánquez and Guieysse, 2008). According to the results obtained in BMP-1 tests, in order to maximize methane production, Inoc-3 (anaerobic sludge from a WWTP) was used as the inoculum in BMP-2 that used *T. versicolor* biomass spiked with hormones, both inoculum:substrate ratios were tested. The amount spiked onto the fungal biomass was based on a previous work (Blánquez and Guieysse, 2008), where the same fungal biomass was used on a biodegradation study. Also, Shi et al. (2013) determined that 1.5 mg/L of estrone (E2 precursor) was adsorbed on activated sludge. The cumulative net methane production profiles over 33 days are shown in Figure 11.4. No statistically significant differences were observed ($p>0.05$) between the reactors that only contained fungal biomass and the reactors that contained fungal biomass spiked with E2 and EE2. Therefore, it seems that E2 and EE2 did not have inhibitory or toxic effects on the methanogenic population contained in Inoc-3.

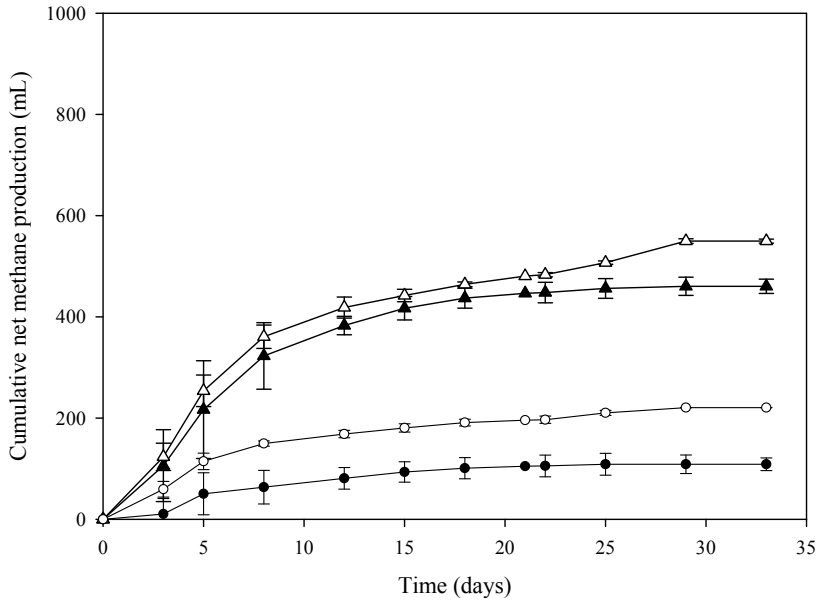

Figure 11.4 Cumulative net methane production. (▲) *non-spiked biomass,* $r_{I/S}$*=2;* (△) *E2 and EE2 spiked biomass,* $r_{I/S}$*=2;* (●) *non-spiked biomass,* $r_{I/S}$*=5;* (○) *E2 and EE2 spiked biomass,* $r_{I/S}$*=5.*

The experimental values and results that were obtained at the end of BMP-2 are presented in Table 11.10. The net methane production was slightly higher when the substrates were spiked, which confirmed that no inhibition or toxic effects resulted from presence of the hormones. The net methane production that was obtained when no estrogens were spiked in BMP-2 using Inoc-3 was lower than the production recorded in the BMP-1 test when using Inoc-1 (Table 11.9). This difference was related to the VS content of each inoculum (see Table 11.2 and Table 11.4). Although both inocula were obtained from the same anaerobic WWTP digester, they were collected at different times. Inoc-3 was more diluted than Inoc-1, which resulted in a lower methane production (data not shown). This was because not all of the VS content of Inoc-3 corresponded to biomass, as the VS method quantifies both the filterable (dissolved organic matter) and non-filterable portions.

Table 11.10 Final results of BMP-2 test, non-spiked and spiked fungal biomass.

	$r_{I/S}$ = 2		**$r_{I/S}$ = 5**	
	Non-spiked	**Spiked**	**Non-spiked**	**Spiked**
Final net methane (mL)	460 ± 14	550 ± 4	109 ± 12	221 ± 1
% of methane in the biogas	65 ± 1	64 ± 2	65 ± 1	64 ± 2
Methane yield (mL CH_4/g VS_{fed})	307 ± 18	367 ± 20	181 ± 12	368 ± 21
pH	7.34 ± 0.1	7.31 ± 0.1	7.36 ± 0.1	7.35 ± 0.1
VFA (g/L)[a]	0.337 ± 0.07	0.327 ± 0.08	0.337 ± 0.07	0.327 ± 0.08
VS reduction (%)	46 ± 1	46 ± 1	37 ± 2	46 ± 1
E2 removal (%)	-	92 ± 1	-	89 ± 1
EE2 removal (%)	-	0	-	0

[a] *Acetate was the only VFA detected*

Differences between inoculum:substrate ratios were observed on the methane yield and the volatile solids reduction of non-spiked substrate, whereas the spiked substrate had the same behaviour for both ratios (Table 11.10). The

values obtained using the high inoculum:substrate ratio for the non-spiked biomass have been considered an experimental error, and have been ignored for the following discussion.

The spiked substrate results confirm the values obtained for BMP-1 test, where the ratio inoculum:substrate does not have an influence on the methane yield and the VS reduction.

The results from the BMP-2 test revealed similar ranges of methane in the biogas (60–70%) relative to those obtained in BMP-1 (Inoc-1) (Table 11.9). The pH remained close to neutral because of the effects of the $NaHCO_3$ buffer. Thus, acidification or alkalinization did not occur during the process. The VS reduction values (46%) were not different between the spiked biomass and the non-spiked biomass. In addition, the final methane yields were similar for the low I/S ratio (307 and 367 mL CH_4/g VS_{feed}, respectively) and also for the spiked substrate using the high ratio (368 mL CH_4/g VS_{feed}) which corresponded with the reduced VS contents.

In BMP-2, the E2 and EE2 concentrations were quantified at the beginning and end of the experiment to evaluate degradation of these pollutants during the anaerobic digestion process. The degradation percentages of both hormones are presented in Table 11.10 and indicate that E2 was almost completely removed for low and high inoculum:substrate ratio (92% and 89%, respectively), but EE2 was not removed (0%). No other peaks were detected on the chromatogram after 33 days of experiment, reducing the possibility of E2 conversion to estrone (E1). As others authors reported (des Mes et al., 2008) E2 and E1 are biologically interconvertible under anaerobic conditions, possibly catalyzed by other biological conversions. Des Mes et al. (2008) did not detect a substantial decline in the sum of E1 and E2 in long-term anaerobic experiment, contrary to the results of this work. However, E2 was partially removed in the experiments carried out by Czajka and Londry (2006) converting E2 to E1 because of the presence of lingering oxygen. That conversion may be as an alternative electron acceptor to regenerate cofactors. Moreover, Czajka and Londry (2006) detected other transformation products (e.g., 17α-estradiol) stating that multiple enzymes may be involved in E2 removal.

On the other hand, EE2 is less biodegradable because of its persistence and recalcitrance, as previously described in the literature (Ifelebuegu, 2011; Ternes et al., 1999). Muller et al. (2010) conducted an experiment to determine the removal of some estrogenic compounds (E2 and EE2, among others) in urban sewage sludge during a plant scale sludge anaerobic digestion process. In this case, a maximum removal of 30–40% was achieved in all endocrine compounds, which indicated persistence of the estrogenic compounds throughout the digestion process. However, Carballa et al. (2006) conducted a laboratory scale study and achieved 85% removal efficiency for E2 and EE2 in a BMP test. These EE2 removal results contrast with the results obtained in this study, where no EE2 removal was achieved. Carballa et al. (2006) indicated that EE2 removal began after an adaptation period of the sludge, which was not performed in this study. However, the present results for EE2 removal are in accordance with des Mes et al. (2008) since they did not detect EE2 removal under anaerobic conditions in the presence of sludge. Moreover, Czajka and Londry (2006) confirmed that EE2 was not degraded under anaerobic conditions since EE2 blocks the potential formation of a ketone and sterically hinders access to an hydroxyl group.

Finally, Hamid and Eskicioglu (2013) observed that microwave hydrolysis (MWH) pretreatment of mixed sludge containing steroidal hormones enhanced their removal during anaerobic digestion from 17% without MWH pretreatment to 72% following MWH pretreatment. From the results of this study and several others, it can be confirmed that anaerobic digestion is suitable for the biodegradation of E2, but pretreatment might be necessary for the biodegradation of EE2.

11.4 Conclusions

Biochemical methane potential tests of exhausted *Trametes versicolor* biomass using two inocula from different sources indicated that higher methane yields were obtained when the anaerobic sludge from a WWTP digester was used rather than the digestate from an anaerobic reactor used to treat OFMSW.

Two different inoculum:substrate ratios were tested, showing no significant differences using the anaerobic sludge from the WWTP. However, no conclusive results could be said about different inoculum:substrate ratios using the anaerobic digested inoculum from an OFMSW due to the incongruent data obtained. Thus, the fungal biomass could be used as a substrate in anaerobic digestion processes. *Trametes versicolor* biomass spiked with E2 and EE2 was successfully used as substrate for methanization. No significant differences were observed in the methane yield between the spiked and non-spiked biomass at the concentrations tested showing the absence of a toxic effect related to E2 and EE2. Different inoculum:substrate ratios showed the same behavior when E2 and EE2 were present on the substrate. E2 was 89-92% removed, whereas no changes were detected on EE2 concentration after the 33 days experiment.

Section 5

GENERAL CONCLUSIONS

12 General conclusions

In the present thesis, some relevant factors about emerging contaminants degradation by microalgae have been assessed. The overall results obtained in this thesis contributed to attain the main objectives presented and detailed conclusions of the works conducted are presented in each chapter.

The main achievements and conclusions that can be drawn from this thesis are next summarized:

- It has been proved the feasibility of microalgae for emerging contaminants removal. Several removal mechanisms occur at the same time related to microalgal cultures. The main removal mechanisms taking place are: photodegradation, biodegradation and sorption.

- Transformation products of estrogenic compounds from microalgal degradation have been detected. These compounds have not been previously described as photodegradation transformation products. This fact confirms the biodegradation as a removal mechanism in microalgae cultures.

- Microalgal capacity for pollutants removal has been evidenced in effluents with different characteristics such as urban wastewater or digestate.

- Microalgal-based systems for urban wastewater treatment are efficient for nutrients as well as emerging contaminants removal. Temperature and light supply are the parameters with a major impact on the photosynthetic activity. Therefore, microalgal photobioreactors could be an alternative to other biological systems for low load effluents treatment.

- Microalgal harvesting needs further investigation, although depending on the microalgal culture some techniques are preferred. Sedimentation is a low cost technique but single small cells are not well harvested. On the other hand, filamentous species could be simply harvested by sedimentation; this is the case of the microalgal biomass from

photobioreactors, where microalgal/bacteria consortia could enhance the separation by aggregates formation.

Another harvesting technique which has the benefit to clarify the effluent as well as further biomass valorisation has been proposed, the co-pelletization using the fungus *Trametes versicolor*.

- Valorisation of exhausted biomass (fungal and microalgal) by means of anaerobic digestion improves the economy of the process. In order to increase the amount of biogas produced from microalgal biomass prior pretreatments should be considered. Enzymatic pretreatments are an economic and environmentally friendly alternative. Moreover, methane yield is increased when an enzyme mixture or a culture broth where enzymes have been released, are used.

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Education

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Oct 2014 – Apr 2015	Research stay at School of Engineering and Advances Technology (SEAT), Massey University (Palmerston North, New Zealand).
2010 - 2011	Master in Biotechnology, Advanced Biotechnology, Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain). Final mark: 3.0/4.0 Title:"*Algal growth and treatment of centrate wastewater: A preliminary screening*."
2005 - 2010	Chemical Engineering (University degree), Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain). Final mark: 2.05/4.0

Former scientific activities

2013 - 2016	Phd grant (FI- AGAUR, Generalitat de Catalunya).
2010 - 2014	Teaching assistant in the Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain). AGAUR grant. Chemical engineering and biotechnology lab-experimentation, chemical engineering elementary operations and Matlab simulation.
November 2014	Statistical course: Deducer, Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain).
June 2012	Wastewater treatment course: Innovative technologies for urban wastewater treatment plants summerschool, Novedar Consolider, Escola Técnica Superior de Enxeñería, Universidad de Santiago de Compostela (USC) (Santiago, Spain).

August 2011	Arctic Technology course: Implementation of an hydraulic turbine for energy supply, Technical University of Denmark (DTU) in collaboration with Building and Construction School (Sanaartormik Ilinniarfik) (Sisimiut, Greenland).
August 2009	Laboratory in chemical/biochemical engineering course, Technical University of Denmark (DTU) (Copenhagen, Denmark).

Language and computer skills

Catalan: Native speaking and writing
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English: Advanced speaking and writing (C.A.E 2014)
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Sigmaplot (advanced level), Windows and Office (advanced level), MATLAB-Simulink (medium level), AutoCAD (medium level), process simulator HYSYS (low level), Deducer (medium level).

Publications in journals

Hom-Diaz, A., Passos, F., Ferrer, I., Vicent, T., Blánquez, P., Enzymatic pretreatment of microalgal biomass using fungal broth from *Trametes versicolor* and commercial laccase for biogas production improvement. *Submitted* (Algal Research).

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Conferences

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Jaén-Gil, A., Hom-Diaz, A., Rodríguez-Mozaz, S., Vicent, T., Blánquez, P., Barceló, D., Suspect screening of antibiotic transformation products during microalgae water treatment by on-line turbulent flow liquid-chromatography coupled to high resolution mass spectrometry LTQ-Orbitrap. Non target 2016, Ascona, Switzerland. Poster presentation.

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Norvill, Z., Shilton, A., Hom-Diaz, A., Guieysse, B., Antibiotic fate in algal wastewater ponds: Tetracycline. 25th SETAC Europe Annual Meeting 2015. Barcelona, Spain. Poster presentation.

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