Universitat Autònoma de Barcelona

Microalgae-based systems for micropollutants removal, resource recovery and bioenergy production towards a circular bioeconomy approach

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PhD Thesis

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Summary

Current global challenges associated with dependency on fossil fuels and limited resource availability require new approaches for the development of a cradle-to-cradle bio-based economy. Microalgae-based technologies offer a versatile and promising solution to shift the focus from wastes and wastewater treatment, toward energy and resource recovery. In these nature-based systems, microalgae remove nutrients from wastewater and produce oxygen useful for heterotrophic bacteria to biodegrade organic matter, improving water quality. This has been fully demonstrated in urban wastewater treatment, but in increasingly industrialised cities and agricultural environments, the challenge is to determine if these microalgae-based systems can degrade emerging contaminants and other organic micropollutants such as pesticides.

In addition, microalgae biomass can be further valorised for the production of biofuels and other valuable bioproducts. In this sense, anaerobic digestion is one of the most established technologies to convert organic wastes from wastewater treatment plants into renewable energy in the form of methane-rich biogas. Another opportunity is the simultaneous anaerobic co-digestion of two or more bio-wastes. The co-digestion approach contributes to overcome the drawbacks of mono-digestion and boost energy production in anaerobic digestion plants. Nonetheless, microalgae anaerobic digestion is generally hindered by the recalcitrancy of their cell walls, which lead to low methane potential.

The present PhD thesis assesses different processes included in the microalgal biorefinery concept: utilisation of algae for micropollutant degradation, energy production by algal anaerobic digestion, co-digestion with other nearby wastes, and utilisation of waste streams as fertilizers.

First, it was studied the individual degradation of three polar and three hydrophobic pesticides frequently found in surface waters by a mixed-microalgae culture. Different conditions were studied to determine the main mechanisms involved in their removal. Biodegradation plus photodegradation contributed to the removal of propanil (100%), acetamiprid (100%), oxadiazon (55%), chlorpyrifos (35%), and cypermethrin (14%) while more than 60% of chlorpyrifos and cypermethrin were removed by bio-sorption. Transformation products generated by the active microalgae were identified for chlorpyrifos, acetamiprid, and propanil. Then, it was assessed the performance of an outdoor pilot-photobioreactor operated at a HRT of 8 days in the treatment of synthetic wastewater containing a mixture of selected pesticides. During the steady-state, degradation capacity was evaluated by quantifying nutrients and pesticides removal, and transformation products were detected. $N\text{-}NO_3^-$ and $P\text{-}PO_4^{-3}$ removal efficiencies were 24 and 94%, respectively. Propanil and acetamiprid were effectively removed (99 and 71%, respectively) mainly by algal-mediated biodegradation as confirmed by the transformation products detected. Besides, the anaerobic digestion of the algal biomass was not inhibited by the retained pesticides.

To enhance the solubility and the anaerobic digestibility of algal biomass, different mild and energy-efficient pretreatments were assessed. Formerly, microalgal harvesting was tested by different cost-effective techniques that do not require large investment costs: natural sedimentation, coagulation-flocculation, and pH-induced flocculation. The pretreatments were applied before the anaerobic co-digestion of microalgae with other wastes such as activated sludge. The effect of thermal pretreatments at low temperature were evaluated for microalgae and activated sludge mixtures. In addition, the effect of

enzymatic pretreatments on microalgae cell wall solubilisation was investigated. In both cases, the effect of the pretreatment in the biogas yield was tested. Results indicate that algal biomass solubility increased and led to a higher methane yield. Nonetheless, in the co-digestion of activated sludge and algal biomass, even when biomass solubility was enhanced after the pretreatment, biogas production did not increase.

Furthermore, this thesis assesses a real case study for the integration of a microalgae-based system into the industrial wastewater treatment plant of a winery company looking for a circular approach for nutrients and bioenergy recovery from wastewater and sludge. Tertiary wastewater treatment by microalgae efficiently removed N-NH_4^+ (97%) and P-PO_4^{-3} (93%). Harvested algal biomass was co-digested in a 50 L pilot anaerobic digester with waste activated sludge obtaining a methane yield of 225.8 NL CH_4 kg VS^{-1}. The digester was operated in SBR mode showing adaptations to substrate variability over time. The valorisation of the generated bio-wastes for fertilization was assessed, indicating that mono- and co-digestion digestates and dry algal biomass improved plant biomass accumulation (growth indexes of 163, 155 and 121% relative to those of the control - organic commercial amendment-).

Resumen

Los actuales retos mundiales asociados a la dependencia de los combustibles fósiles y a la limitada disponibilidad de recursos requieren de nuevas perspectivas para el desarrollo de una bioeconomía basada en el enfoque "de la cuna a la cuna". Las tecnologías basadas en las microalgas ofrecen una solución versátil y prometedora para cambiar el foco desde el tratamiento de residuos y aguas residuales, hacia la recuperación de energía y recursos. En estos sistemas basados en la naturaleza, las microalgas eliminan los nutrientes de las aguas residuales y producen oxígeno útil para que las bacterias heterótrofas biodegraden la materia orgánica, mejorando la calidad del agua. Esto se ha demostrado plenamente en el tratamiento de aguas residuales urbanas, pero en las ciudades cada vez más industrializadas y en los entornos agrícolas, el reto consiste en determinar si estos sistemas basados en microalgas pueden degradar los contaminantes emergentes y otros micro contaminantes orgánicos, como los pesticidas.

Además, la biomasa de microalgas puede valorizarse para la producción de biocombustibles y otros valiosos bio-productos. En este sentido, la digestión anaeróbica es una de las tecnologías más consolidadas para convertir los residuos orgánicos de las plantas de tratamiento de aguas residuales en energía renovable en forma de biogás rico en metano. Otra oportunidad es la co-digestión anaeróbica simultánea de dos o más bio-residuos. La co-digestión contribuye a superar los inconvenientes de la mono-digestión y a impulsar la producción de energía en las plantas de digestión anaeróbica. Sin embargo, la digestión anaeróbica de las microalgas se ve generalmente obstaculizada por la resistencia de sus paredes celulares, que conducen a una baja producción de metano.

La presente tesis doctoral evalúa diferentes procesos incluidos en el concepto de biorrefinería de microalgas: utilización de las algas para la degradación de micro contaminantes, producción de energía mediante la digestión anaerobia de algas, la co-digestión con otros residuos cercanos, y la utilización de flujos de residuos como fertilizantes.

En primer lugar, se estudió la degradación individual de tres pesticidas polares y tres pesticidas hidrofóbicos que se encuentran frecuentemente en las aguas superficiales mediante un cultivo mixto de microalgas. Se estudiaron diferentes condiciones para determinar los principales mecanismos implicados en su eliminación. La biodegradación y la foto-degradación contribuyeron a la eliminación del propanil (100%), el acetamiprid (100%), el oxadiazon (55%), el clorpirifós (35%) y la cipermetrina (14%), mientras que más del 60% del clorpirifós y la cipermetrina se eliminaron por bio-sorción. Se identificaron los productos de transformación generados por las microalgas activas para el clorpirifós, el acetamiprid y el propanil. A continuación, se evaluó el rendimiento de un fotobiorreactor piloto de exterior operado a un TRH de 8 días en el tratamiento de aguas residuales sintéticas que contenían una mezcla de pesticidas seleccionados. Durante el estado estacionario, se evaluó la capacidad de degradación cuantificando la eliminación de nutrientes y pesticidas, y se detectaron los productos de transformación. Las eficiencias de eliminación de $N\text{-}NO_3^-$ and $P\text{-}PO_4^{-3}$ fueron del 24 y 94%, respectivamente. El propanil y el acetamiprid se eliminaron eficazmente (99 y 71%, respectivamente), principalmente por biodegradación mediada por algas, como lo confirman los productos de transformación detectados. Además, la digestión anaeróbica de la biomasa de algas no fue inhibida por los pesticidas retenidos.

Para mejorar la solubilidad y la digestibilidad anaeróbica de la biomasa algal, se evaluaron diferentes pretratamientos suaves y energéticamente eficientes. Anteriormente, se estudió la recolección de microalgas mediante diferentes técnicas rentables que no requieren grandes costes de inversión: sedimentación natural, coagulación-floculación y floculación inducida por el pH. Los pretratamientos se aplicaron antes de la co-digestión anaeróbica de las microalgas con otros residuos, como los lodos activados. Se evaluó el efecto de los pretratamientos térmicos a baja temperatura para las mezclas de microalgas y lodos activados. Además, se investigó el efecto de los pretratamientos enzimáticos en la solubilización de la pared celular de las microalgas. En ambos casos, se comprobó el efecto del pretratamiento en el rendimiento de biogás. Los resultados indican que la solubilidad de la biomasa de las algas aumentó y condujo a un mayor rendimiento de metano. Sin embargo, en la co-digestión de lodos activados y biomasa de algas, incluso cuando la solubilidad de la biomasa aumentó tras el pretratamiento, la producción de biogás no se incrementó.

Además, esta tesis evalúa un caso de estudio real para la integración de un sistema basado en microalgas en la planta de tratamiento de aguas residuales industriales de una empresa vinícola que busca aplicar un enfoque circular para la recuperación de nutrientes y bioenergía a partir de sus aguas residuales y lodos. El tratamiento terciario de las aguas residuales mediante microalgas eliminó eficazmente el $N\text{-}NH_4^+$ (97%) y el $P\text{-}PO_4^{-3}$ (93%). La biomasa de algas cosechada fue co-digerida en un digestor anaeróbico piloto de 50 L con lodos activados residuales obteniendo un rendimiento de metano de 225.8 NL CH_4 kg VS^{-1}. El digestor fue operado en modo de reactor secuencial mostrando adaptaciones a la variabilidad del sustrato con el tiempo. Se evaluó la valorización de los bio-residuos generados para su fertilización, indicando que los digestatos de mono-

digestión y co-digestión y la biomasa seca de algas mejoraron la acumulación de biomasa vegetal (índices de crecimiento del 163, 155 y 121% respecto a los del control - enmienda orgánica comercial-).

Resum

Els reptes globals actuals associats a la dependència dels combustibles fòssils i a la disponibilitat dels recursos requereixen noves aproximacions per al desenvolupament d'una economia basada en processos biològics més sostenible. Les tecnologies basades en microalgues ofereixen una solució versàtil i prometedora per canviar l'enfoc del tractament de residus i aigües residuals cap a la recuperació d'energia i recursos. En aquests sistemes basats en processos que tenen lloc en la natura (nature-based systems), les microalgues consumeixen els nutrients en les aigües residuals i produeixen oxigen, utilitzat pels bacteris heteròtrofs per biodegradar la matèria orgànica, millorant així la qualitat de l'aigua. Aquests processos s'han implementat amb èxit pel tractament de les aigües residuals urbanes, però en ciutats cada cop més industrialitzades i en entorns agrícoles, el repte actual és determinar si aquests nature-based systems poden degradar contaminants emergents i altres contaminants orgànics com els pesticides.

Altrament, la biomassa algal produïda pot valoritzar-se per a la producció de biofuels i altres subproductes valuosos. En aquest sentit, la digestió anaeròbia és una de les tecnologies millor desenvolupades i implementades per a convertir residus orgànics procedents de les plantes de tractament d'aigües residuals en energia renovable com és el biogàs. Un altra oportunitat en aquest escenari de valorització de residus i de implementació de processos sostenibles és la co-digestió simultània de dos o més residus. La co-digestió contribueix a superar alguns problemes de la mono-digestió i a augmentar la producció d'energia en les plantes de digestió anaeròbia. No obstant, en general, la digestió anaeròbia de les microalgues es veu obstaculitzada per la resistència de la paret cel·lular a la biodegradació, el que suposa un baix potencial de producció de metà.

Aquesta Tesi Doctoral avalua diferents processos que s'inclouen en el concepte de biorefineria d'algues: utilització de les algues per a la degradació de microcontaminants, producció d'energia per a la metanització de les algues, co-digestió amb altres residus orgànics propers, i utilització dels fluxos de residus generats com a fertilitzants.

Primer, s'ha estudiat la degradació individual de tres pesticides polars i tres hidrofòbics, que es troben freqüentment en aigües superficials, per un cultiu mixt de microalgues i altres microorganismes. Els estudis s'han portat a terme en diferents condicions experimentals per a determinar els principals mecanismes implicats en la seva eliminació. La biodegradació i fotodegradació contribueixen a l'eliminació del propanil (100%), de l'acetamiprid (100%), de l'oxadiazon (55%), del clorpirifos (35%) i de la cipermetrina (14%), mentre que més del 60% del clorpirifos i la cipermetrina s'eliminen per biosorpció. S'han identificat els productes de transformació del clorpirifos, l'acetamiprid i el propanil per les microalgues actives. Després s'ha avaluat el comportament d'un fotobioreactor pilot situat a la intempèrie, operat a un temps de residència hidràulic de 8 dies, en el tractament d'un aigua residual sintètica que conté una mescla de pesticides. En el període d'operació d'estat estacionari hidràulic s'ha avaluat la capacitat de degradació del sistema quantificant l'eliminació de nutrients i pesticides, i s'han detectat alguns productes de degradació. El rendiment d'eliminació de $N\text{-}NO_3^-$ i $P\text{-}PO_4^{-3}$ ha estat del 24 i 94%, respectivament. El propanil i l'acetamiprid varen ser eliminats molt eficientment (99 i 71%, respectivament) principalment per biodegradació per el sistema algal, com es confirma a partir dels productes de transformació detectats. A més a més, el procés de metanització de la biomassa algal no es veu inhibit pels pesticides adsorbits a la biomassa.

Amb l'objectiu incrementar la solubilitat i la digestibilitat de la biomassa algal, s'han avaluat diferents pretractaments suaus i de baix consum energètic. Però prèviament s'ha realitzat la collita de les microalgues per diferents tècniques senzilles que no requereixen elevats costos d'inversió per portar-les a terme: sedimentació natural, coagulació-floculació, i modificació del pH per provocar floculació. Els pretractaments s'han aplicat abans de la co-digestió anaeròbia de les microalgues amb llots procedents d'un procés de llots activats. S'ha avaluat l'efecte de pretractaments tèrmics a baixa temperatura per a la mescla, i pretractaments enzimàtics per a solubilitzar la paret de les microalgues. Per ambdós casos s'ha quantificat l'efecte del pretractament en el rendiment del biogàs i els resultats indiquen que per a la biomassa algal l'increment de solubilitat porta a un augment del rendiment. No obstant, per a la co-digestió de llots activats i biomassa algal, encara que un pretractament augmenti la solubilitat, el rendiment de producció de biogàs no augmenta.

En aquesta Tesi també s'avalua un cas estudi real per a integrar un sistema basat en microalgues en una planta de tractament d'aigües residuals d'una empresa vinícola, perseguint una proposta circular de recuperació de nutrients i energia de l'aigua residual i dels llots. El tractament terciari de l'aigua residual per les microalgues elimina eficientment $N\text{-}NH_4^+$ (97%) i $P\text{-}PO_4^{-3}$ (93%). La collita de la biomassa algal produïda es va co-digerir amb llots en un reactor pilot de 50 L obtenint un rendiment de metà de 225.8 NL CH_4 kg SV^{-1}. El digestor es va operar en mode SBR (discontinus repetits) demostrant una bona adaptació a la variabilitat estacional del substrat. Al mateix temps es va estudiar la valorització dels bioresidus generats com a fertilitzants en cultius agrícoles. Els resultats indiquen que els digestats de la mono i co-digestió, i la biomassa algal assecada

milloren el creixement dels cultius (índex de creixement de 163, 155 i 121% relatiu a un control amb fertilitzant comercial).

CONTENTS

LIST OF ACRONYMS AND ABBREVIATIONS

AcoD	Anaerobic co-digestion
AD	Anaerobic digestion
ANOVA	Analysis of variance
AOM	Algal organic matter
ARA	arachidonic acid
BBM	Bold Basal Medium
BMP	Biochemical methane potential test
CH_4	Methane
CL	Confidence level
COD	Chemical oxygen demand
DAF	Dissolved air flotation
DHA	docosahexaenoic acid
DiAF	Dispersed air flotation
DO	Dissolved oxygen concentration
EC	European Commission
EH	Enzymatic hydrolysis
EPA	eicosapentaenoic acid
EPS	Extracellular polymeric substances
GC	Gras chromatography
GHG	Greenhouse gases
GI	Growth index
H	Henry´s law constant
HESI	Heated-electrospray ionization
HPLC	High pressure liquid chromatography
HRAP	High-rate algal pond
HRT	Hydraulic retention time
IA	Intermediate alkalinity
IC	Inorganic carbon
K_H	Hydrolysis rate constant
LOD	Limit of detection
LOQ	Limit of quantification
OD	Optical density

OM	Organic micropollutant
P	Phosphorus
PA	Partial alkalinity
PBR	Photobioreactor
PE	polyethylene
polyDADMAC	Polymer of diallyl dimethyl ammonium chloride
PP	propylene
ppm	Parts per million
RDB	Ring and double bound equivalents
rpm	Revolutions per minute
SBR	Sequencing batch reactor
sCOD	Soluble chemical oxygen demand
SRT	Solid retention time
SS	Surrogate standard
STP	Standard temperature and pressure
TA	Total alkalinity
TC	Total carbon
TN	Total nitrogen
TOC	Total organic carbon
TP	Transformation products
t_R	Chromatographic retention time
TS	Total solids
TSS	Total suspended solids
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge
WRRF	Water resource recovery facility
WW	Wastewater
WWT	Wastewater treatment
WWTP	Wastewater treatment plant

SECTION 1

General aspects

CHAPTER 1.

GENERAL INTRODUCTION

1.1. Microalgae as a bioresource

An economy based on fossil resources for the industrial production of fuel, fine chemicals, pharmaceuticals, plastics, pesticides, solvents, and many other products, has generated an increase in the level of greenhouse gases (GHG) emissions, leading to current global warming and climate challenges. In addition, global population growth and an increase in energy demand led to great pressure over the consumption of water and other scarce natural resources. Limits in resource availability and current global environmental issues raise unavoidable challenges for mankind. Thus, the use of renewable fuel resources, the development of efficient wastewater treatment technologies, and circular economic approaches are becoming increasingly important. In recent years, there is a rising interest from academic and industrial researchers in microalgae-based technologies for wastewater treatment and the production of algal biomass as a source of bio-products and bioenergy (Wollmann et al., 2019; Fabris et al., 2020; Nagarajan et al., 2020b). Algae could potentially contribute to meeting the increasing demands for food, feed, energy, and materials (EC, 2020a).

Microalgae and cyanobacteria, also known as phytoplankton, live in the planktonic region of aquatic habitats and are having an important influence in the global carbon cycle and ultimately in the climate (Ramanan et al., 2016), highlighting their significance to sustain Earth's ecosystems. Microalgae and cyanobacteria include unicellular or multi-cellular autotrophic organisms that convert atmospheric CO_2 into organic matter using sunlight energy through photosynthesis. Microalgae represent the base of the food pyramid and are primary producers in the aquatic ecosystem that bio-sequester atmospheric CO_2, contributing approximately to half of the global net primary production (Field et al., 1998). It is estimated that there are more than 200.000 microalgae species on

Earth, and among 30.000 have been identified (Richmond A., 2004; Guiry, 2012). They can be broadly classified into prokaryotic (cyanobacteria) and eukaryotic microalgae such as green algae (Chlorophyta), red algae (Rhodophyta) and diatoms (Bacillariophyta) (Brennan and Owende, 2010) (the term microalgae will be used onwards for both eukaryotic microalgae and prokaryotic cyanobacteria).

Microalgae provide new dimensions in the renewable energy industry as an alternative to fossil fuels. Biofuels are renewable fuel resources such as liquid or gaseous fuels produced from diverse biomass or feedstocks obtained from photosynthesis, contributing to improve energy security and guarantee a sustainable source of energy (Suganya et al., 2016). Biomass energy is the largest renewable energy source, representing 77.4% of the global renewable energy supply (Carlos and Ba Khang, 2008). First-generation biofuels are produced directly from food crops feedstocks (starch, corn, rapeseed, sugar beet, sunflower, wheat, barley, etc.) raising conflicts and controversies due to the "food versus fuel" dilemma (Anto et al., 2020). The typically first-generation biofuels are bioethanol from corn and biodiesel from soybean. Second-generation biofuels are produced from non-food crops such as bagasse, jatropha, agricultural residues, straw, grass, etc. However, large-scale land use is required, demanding water supply, fertilizers, and pesticides (Singh et al., 2015). Limitations of first- and second-generation biofuels have led to third-generation biofuels obtained from microalgae feedstocks with larger advantages over crop plants. The interest in using microalgae as a renewable energy source was enhanced during the first energy crisis in the 1970s (Chaumont, 1993). Microalgae biomass has strengths over other substrates which are related to its abundance, widely distribution (they can grow in a diversity of habitats), and easiness of cultivation. Likewise, microalgae do not contain lignin, making it a more easily hydrolysed substrate in

comparison with crops and lignocellulosic biomasses (Singh et al., 2015), while avoiding the use of toxic solvents in biomass pretreatment (Nagarajan et al., 2020a). CO_2 conversion efficiency by microalgae and cyanobacteria is about 10-50 times higher than terrestrial plants (Li et al., 2008). They can also contribute to industrial CO_2 fixation and mitigation (1 kg of dry microalgal biomass can fix 1.83 kg of CO_2) as well as to other gases remediation (microalgae can grow using flue gases) (Judd et al., 2015; Wang and Yin, 2018). Microalgae can be grown on nonarable land and have the advantage of growing in a diversity of habitats (fresh and marine water) and diverse wastewaters containing residual nutrients such as industrial effluents (Gupta et al., 2016; Molinuevo-Salces et al., 2016; Huy et al., 2018), sewage water (Renuka et al., 2013), agricultural run-off (García-Galán et al., 2018), anaerobic digestate (Hom-Diaz et al., 2015), municipal wastewater (Arbib et al., 2014), among others, promoting wastewater valorisation and less use of the freshwater resource. Moreover, in contrast to first-generation biofuel feedstock, microalgae do not compete with food crops, do not require agricultural land nor pesticides for growing, and entails minimal land-use changes (Singh et al., 2015). Microalgae have a higher biomass yield (ca. 2-10 times higher), a short doubling time (6-12 hours), and can be cultivated all year-round (Subramanian et al., 2013). Thus, the strengths of microalgae biomass highlight their use as an innovative and low-cost organic feedstock for biofuels production. Microalgae can produce energy-rich substances such as carbohydrates and lipids, which can be used as feedstock for biohydrogen and biodiesel production, respectively (Vitova et al., 2015). Third-generation feedstock can be applied for the production of these main biofuels: (1) biomethane via anaerobic digestion, (2) bioethanol through fermentation, (3) biodiesel by transesterification, and (4) biohydrogen via direct and indirect photolysis (Bahadar and Bilal Khan, 2013; Suganya et al., 2016; Khan et al., 2018). Residual algal biomass after fuel

extraction can be applied as biofertilizer or soil amendment (Mulbry et al., 2005), used as animal feed thanks to the nutritional value of biomass (Venkata Mohan et al., 2020), and for power generation (Trivedi et al., 2015).

By the 1950s, algal biomass appears as an alternative source of protein for a high populated world (Becker, 2013). Microalgae can be potentially employed as cell factories for the synthesis of high-value bioproducts. Microalgae genera *Dunaliella*, *Botryococcus*, *Chlamydomonas*, *Chlorella* and cyanobacteria *Arthrospira* are among the most promising for commercial biotechnological applications (Suganya et al., 2016; Morais Junior et al., 2020). Currently, the most cultivated species in the European microalgae industry include *Chlorella* spp., *Nannochloropsis* spp., *Haematococcus pluvialis*, and *Spirulina* spp. (Araújo et al., 2021).

1.2. Modes of microalgae cultivation

Some microalgal species possess metabolic flexibility. They can grow under diverse metabolisms pathways and are capable of metabolic shift as a response to changes in the environmental conditions, substrate and light availability (Zhan et al., 2017). This ability highlights microalgae as promising biological systems for treating diverse sources of wastewaters. Under the photoautotrophic growth strategy, microalgae harvest light as an energy source and fix atmospheric inorganic carbon as a carbon source for biosynthesis. When microalgae are cultivated under heterotrophic conditions, organic carbons sources such as acetate, glycerol, lactate, glucose, among others, provide energy to support growth (Perez-Garcia et al., 2011; Posten and Chen, 2016). Under this condition, contamination and the cost of an organic carbon source are some challenges for application at a large scale (Chen et al., 2011). Mixotrophic metabolism combines phototrophic and heterotrophic growth modes simultaneously, achieving higher

growth rates in comparison with phototrophic cultivation (Zhan et al., 2017). Heterotrophic and/or mixotrophic growth have some advantages over autotrophic growth, such as the higher growth rates and higher biomass concentrations, but they have also disadvantages, mainly the high cost of organic substrates and the high potentiality of contamination by other heterotrophs (Velea et al., 2017). In photo-heterotrophy cultivation mode, microalgae use light and organic compounds as carbon and energy sources for growth (Hwang et al., 2014). For instance, *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Arthrospira platensis* are strains that grow under photoautotrophic, heterotrophic, as well as under mixotrophic conditions (Mata et al., 2010).

1.3. Factors affecting microalgal growth

Microalgae growth is influenced by physical, chemical, and biological factors such as nutrients availability, temperature, light, among other parameters. Microalgae can adapt to a variety of environmental conditions and have a wide tolerance against environmental stress and towards diverse environments (Jones and Mayfield, 2012; Cheah et al., 2016). During photosynthesis, microalgae use solar energy, macro-nutrients (carbon, nitrogen, phosphorus, and potassium) and essential micro-nutrients (Mg, S, Ca, Na, Cl, Fe, Zn, Cu, Mo, Mn, B and Co) to synthesize biomass and to multiply their cells (Markou et al., 2014). Among them, carbon, nitrogen, and phosphorus are the three most significant nutrients. Also, essential vitamins need to be provided (Chia et al., 2018).

The main nutrient for autotrophic microalgal growth is inorganic carbon since it is the precursor of photosynthetic reactions, which is fixed inside the algal cells and converted to an organic form through the Calvin cycle (Yao et al., 2019). Additionally, microalgae can uptake soluble carbonates as a source of CO_2

(Gonçalves et al., 2017). Microalgae species that can grow heterotrophically or mixotrophically can use organic molecules as a source of carbon and/or energy.

Nitrogen is the second most abundant element in microalgal biomass, and it constitutes essential biomass biochemical compounds, such as nucleic acids (DNA, RNA), amino acids (proteins), and pigments (chlorophylls and phycocyanin) (Markou et al., 2014). Cyanobacteria can fix atmospheric molecular nitrogen converting it into ammonia-nitrogen (NH_3-N), which can be incorporated into amino acids and proteins or excreted into the environment (Gonçalves et al., 2017). Several inorganic forms of nitrogen such as NO_3^-, NO_2^-, NO, NH_4^+, and also organic form, like urea or amino acids serve as nitrogen sources for microalgae (Glass et al., 2009). Microalgae prefer to take up the most reduced form of nitrogen, following this order of preference for nitrogen utilization: NH_4^+ > NO > NO_2^- > NO_3^- > urea (Perez-Garcia et al., 2011; Delgadillo-Mirquez et al., 2016). Ammonia is the inorganic nitrogen source most biologically accessible since less energy is required for its uptake (Delgadillo-Mirquez et al., 2016). The simultaneous presence of two or more nitrogen forms in the cultivation medium affects nitrogen uptake. Once microalgae completely remove ammonia/ammonium, they will later use the other forms (Markou et al., 2014). When ammonia is dissolved in water (water solubility about 35% (w/w) at 25 °C) it reacts with water to form a buffer system of free ammonia (NH_3)/ammonium ion (NH_4^+). The equilibrium between the forms of ammonium (ionized form) and free ammonia (unionized form) depends mainly on the pH of the cultivation medium (pK_a = 9.25 at 25 °C) and also on temperature (the pK_a value decreases as temperatures increase) (Posadas et al., 2017). When pH increases above this pK_a value, the dominant species is free ammonia, having detrimental effects on microalgae.

Phosphorus constitutes some organic molecules that are essential to microalgae metabolisms, such as nucleic acids (RNA and DNA), membrane phospholipids, and ATP (Solovchenko et al., 2016). Unlike carbon and nitrogen nutrients, phosphorus is derived from non-renewable fossil phosphate rocks (Elser, 2012). Phosphorus is mainly up taken by microalgae in the inorganic form of orthophosphate while other inorganic and organic phosphorus forms (dissolved organic phosphorus and soluble phosphorus compounds) can also be used by microalgae (Huang and Hong, 1999; Singh et al., 2018). Other inorganic forms of phosphorus have first to be converted to orthophosphate to be suitable for microalgae uptake. Cell condition and some environmental factors (light, pH, temperature, salinity of the cultivation medium) influence phosphorus uptake (Singh et al., 2018). In addition, microalgae and cyanobacteria may accumulate intracellular phosphorus reserves as polyphosphate granules when exposed to a P-sufficient environment, also known as luxury uptake (Solovchenko et al., 2016). The availability of nitrogen in the cultivation media influences the uptake of phosphorous (Beuckels et al., 2015).

Potassium is an activator for diverse enzymes involved in photosynthesis and respiration, affecting protein and carbohydrate synthesis (Markou et al., 2014). Microalgae and cyanobacteria adjust their nutrient uptake according to the nutrient availability in the surroundings. When a nutrient becomes limiting, microalgae can adjust their biomass composition accumulating carbohydrates or lipids (Chia et al., 2018). Microalgae cultivation under nutrient starvation is a strategy employed for the accumulation of long-chain fatty acids for biodiesel production (Taleb et al., 2018; Elshobary et al., 2019), and for the accumulation of carbohydrates for bioethanol production (Geada et al., 2017).

Besides, some abiotic factors such as light, temperature, pH, salinity, nutrient qualitative and quantitative profiles, dissolved oxygen concentration, and the presence of toxic compounds, influence algal growth. In addition, operational conditions of microalgae cultivation (such as hydraulic residence time, harvesting rates, gas transfer, and mixing) influence CO_2 availability, shear rates, and light exposure (Gonçalves et al., 2017).

Regarding the pH effect, the optimal pH range for many microalgal species is between 7 – 9. During the photosynthetic activity, OH^- is produced increasing the pH of the medium (Geada et al., 2017). The pH of the culture medium produces physiological changes in microalgae and it also influences nutrients removal (Gonçalves et al., 2017). For instance, an increase in the pH could be beneficial for limiting pathogenic organisms present in wastewater. However, a high pH value might affect microalgae growth and diminish nutrient utilization (Jankowska et al., 2019). The pH determines the CO_2 solubility in the culture medium, and high pH values could lead to NH_4-N stripping and PO_4-P precipitation (Gonçalves et al., 2017).

Light and temperature play an important role in nutrients uptake by microalgae. As microalgae are photosynthetic organisms, metabolic processes associated with nutrient assimilation through growth are driven by light, and photosynthesis is influenced by light intensity and day length (photoperiod) (Whitton et al., 2015). Light intensity affects the rate of photosynthesis in microalgae generating effects such as light imitation, light saturation, or light inhibition. The optimum light intensity required for efficient photosynthesis and higher biomass production is strain-dependent (Chia et al., 2018). Likewise, the light to dark cycles affects algal biomass production in autotrophic microalgae growth (Meseck et al., 2005). The optimal temperature for microalgal growth is strain-specific (Robarts and

Zohary, 1987). Generally, the optimal temperature for most species of microalgae is among 15 – 25 °C (Sutherland et al., 2015). An increase in temperature typically results in an increased metabolic activity while lower temperatures contribute to algal growth inhibition (Xin et al., 2011). However, an increase in temperature lowers the solubility of some nutrients, such as CO_2 and N-NH_4^+ (Gonçalves et al., 2017).

Relating to salinity, optimal levels differ according to microalgal strain. Salinity changes in the culture medium can have harmful effects on microalgal growth, due to osmotic stress, ion stress, and alterations in the membrane permeability to ions (Glass, 1983). In open systems, evaporative losses and rainfalls events can contribute to modify salinity in the culture medium (Gonçalves et al., 2017).

1.4. Microalgae-based systems

Microalgae-based systems are formed by a mixed consortium of algae, bacteria, protozoa, and other microorganisms that may interact with algae by diverse mechanisms (Unnithan et al., 2014). Synergetic relationships between the microorganisms with different metabolic activities, adapted to diverse environmental conditions, enhances the robustness of the biological system, the resistance to environmental fluctuations, predators, and oscillations in nutrient availability (Matamoros et al., 2015; Cuellar-Bermudez et al., 2017).

The intermediate products of algae and bacteria are crucial to their coexistence. For instance, microalgae can facilitate bacterial growth by providing organic compounds released during algal cell growth or by decomposition of algal cells (Fig. 1.1). Microalgae generates by photosynthetic aeration O_2 useful for heterotrophic bacteria to aerobically oxidize organic matter, providing simple

molecules available for microalgae assimilation. Released CO_2 from bacterial respiration can be fixed by photosynthetic microorganisms. Microalgae grow by taking up these nutrients and using sunlight and CO_2 for photosynthesis (Muñoz and Guieysse, 2006). Moreover, algae can act as a secondary habitat for bacteria, protecting them from unfavourable environmental conditions (Unnithan et al., 2014). Nonetheless, microalgae-bacteria interactions can range from symbiotic to parasitic, with microalgae benefitting or inhibiting bacteria, or vice versa (Ramanan et al., 2016). Bacteria can produce growth factors enhancing algal growth or can produce phycotoxins inhibiting algal growth. In turn, microalgae can produce exotoxins that are detrimental to bacteria (Unnithan et al., 2014).

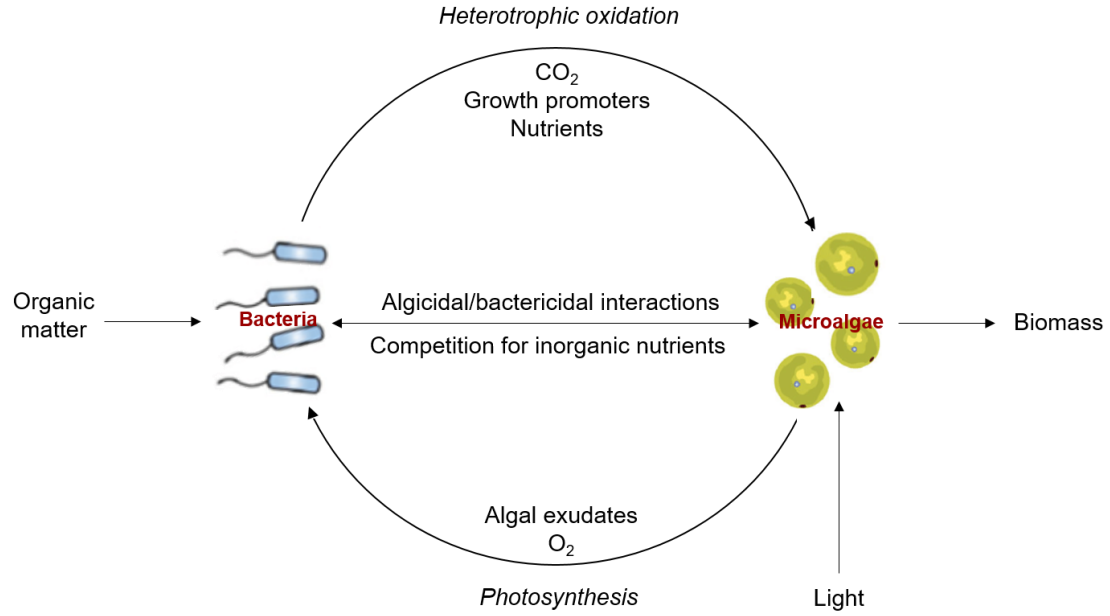

Figure 1.1 Photosynthetic oxygenation and interactions between microalgae and bacteria.

1.4.1. Microalgae cultivation systems

Two major configurations are used for microalgae production based on suspended cultures: open-culture and closed-culture cultivation systems. Another

configuration includes non-suspended cultures such as biofilm and matrix-immobilized systems. The selection of the cultivation method must be according to the target product to be obtained from the microalgae.

1.4.1.1. Suspended cultures

Open-air cultivation systems include lakes, natural ponds, circular ponds, raceways ponds and cascade systems (Geada et al., 2017). Among them, natural ponds and high-rate algal ponds (HRAPs) (Fig. 1.2a) are the most commonly used for the commercial production of microalgae (Mata et al., 2010). HRAPs consist of shallow (0.2 to 0.4 m depth) racetracks where mixing is provided by the continuous movement of paddle wheels. Length, depth, and width are important parameters in their design. For instance, a greater pond width may decrease speed affecting the mass transfer and mixing (Javed et al., 2019) while a higher water depth negatively affects light penetration and biomass concentration in these systems (Acién et al., 2017). Open pond systems are normally less expensive to build and operate, have less maintenance, have direct exposure to sunlight, self-cooling by evaporation, and low energy input requirement, being the cheapest production system for large-scale algal cultivation (Acién et al., 2017). Biomass productivity in open systems varies according to the strain, culture depth and cultivation conditions. Productivities of 13, 15 and 16.7 g m^{-2} d^{-1} were reported for *Chlorella* sp. (Hase et al., 2000), *Nannochloropsis* sp. (Chiaramonti et al., 2013), and mixed cultures (Park and Craggs, 2010), respectively. On the other hand, open systems are influenced by external conditions: diurnal temperature changes, varying sunlight, rainfall, and water loss by evaporation are not controlled in these systems and they are more susceptible to seasonal fluctuations in these parameters, especially irradiance and temperature (Mata et al., 2010; Whitton et al., 2015). Besides, the contamination

with competitors, grazers, and pathogens, lower gas transfer with liquid suspension, and limited biomass productivity are other challenges that must be addressed (Singh et al., 2015; Acién et al., 2017).

On the other side, closed cultivation systems include multiple configurations of photobioreactors (PBRs) with a flexible technical design. PBRs were mainly developed for the cultivation of a specific microalgae strain in a controlled environment required for the production of high-value products for the pharmaceutical and cosmetic industries (Geada et al., 2017). Control of culture conditions and growth parameters such as temperature, pH, mixing, CO_2 concentration, gas transfer, is possible in a PBR enhancing biomass productivity (Acién et al., 2017). Other advantages include higher cell densities, lesser water loss by evaporation, high mass transfer of gases, and better protection against culture contamination (Mata et al., 2010). PBRs are more efficient than open systems in terms of biomass productivity: 15 - 50 g m^{-2} d^{-1} were reported for tubular PBRs (Shen et al., 2009; Wiley et al., 2011; Jonker and Faaij, 2013). Richmond (2004) stated that closed systems surpass open ponds in terms of volumetric productivity (8 times higher) and cell concentration (about 16 times higher). In closed PBRs, the risk of contamination is reduced and land area requirements are lower in comparison to open configurations (Acién et al., 2017). Algal cultures are recirculated either with a mechanical pump or airlift system allowing an exchange of CO_2 and O_2 between the liquid and gas media medium while providing a mechanism for mixing (Singh et al., 2015). Even when closed systems are more efficient than open ones, they require higher energy demand. Other limitations to be addressed include cell damage by shear stress, biofouling, overheating, oxygen accumulation with subsequent growth inhibition, and difficulty in scaling-up (Mata et al., 2010; Solimeno et al., 2017). Typically, closed

systems include three main categories of PBRs: tubular (helical, manifold, and serpentine), flat-plate, and column (bubble column and airlift) PBRs (Geada et al., 2017). The most common design of closed systems is tubular PBRs (Fig. 1.2b) usually made of glass or plastic tubes in which the culture circulates using pumps or air streams. The diameter and length of the tubes and the mixing are the main factors affecting the performance of tubular PBRs (Acién et al., 2017). Tubular PBRs are suitable for outdoor cultivation and have a large illumination surface area (Mata et al., 2010). However, construction, operating and maintenance cost may also be expensive (Chen et al., 2011). Other main challenges associated with tubular PBRs include overheating, fouling (when microalgal cells attached to the walls or tubes reduce light penetration in the culture), and difficulty in scaling up (Mata et al., 2010; Singh et al., 2015). Typically, vertical column PBRs (Fig. 1.2c) are cylinders (diameter up to 0.4 m and a height up to 4 m) designed to enhance microalgal productivity (Geada et al., 2017). Column bioreactors have a constant bubbling of gas from the bottom of the reactor enabling an efficient CO_2 utilization and optimal O_2 removal while gas bubbles provoke a gentle mixing of the culture with little shear stress for the cells. However, efficient utilization of light could be a limiting growth parameter (Geada et al., 2017). Another design of closed systems is flat-plate reactors (Fig. 1.2d) that generally consists of two parallel panels made up of a transparent material with a thin layer of microalgal suspension flowing in between (Acién et al., 2017). Flat-plate have a large light exposure surface area and the panels can be placed either vertically or inclined at an optimum incident angle, achieving high volumetric biomass productivity (Richmond and Cheng-Wu, 2001). Main constraints are related to difficulty in temperature control in the culture and scalability (Geada et al., 2017).

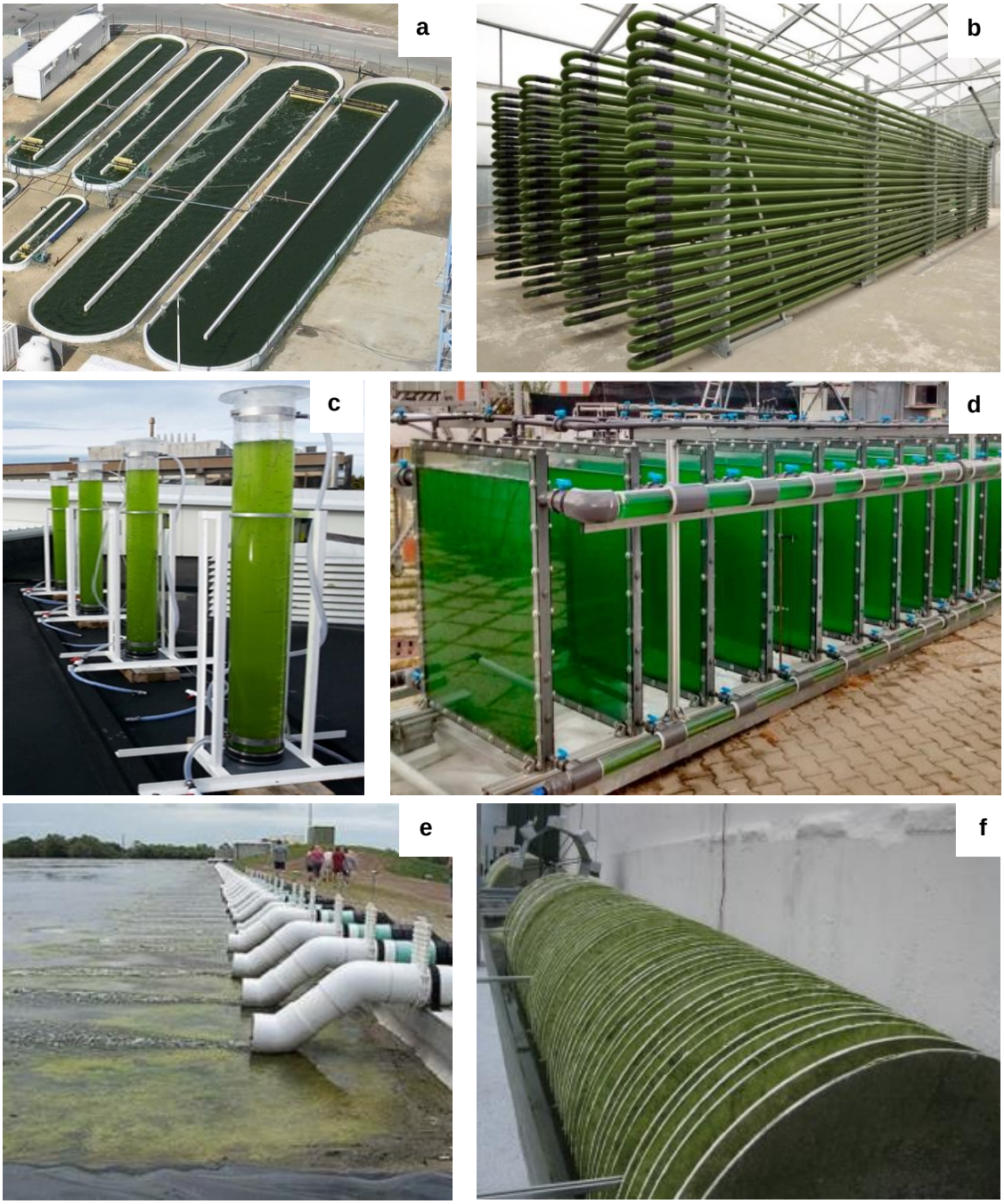

Figure 1.2 Microalgae cultivation systems: (a) HRAP (Yen et al., 2019), (b) tubular PBR, (c) column PBR (Plouviez et al., 2017), (d) flat-plate PBR (Lindblad et al., 2019), (e) algae turf scrubber (U.S. Department of Energy, 2016), and (f) rotating algal PBR (Fernández et al., 2014).

1.4.1.1. Non-suspended cultures

Non-suspended cultures include attached microalgal biofilms (open configuration) and matrix- immobilization (enclosed configuration).

Microalgal biofilm PBRs are configurations based on microalgal growth attached to artificial supporting materials such as the wall or surfaces of the PBR. Microalgae cells produce extracellular polymeric substances (EPS) consisting of polysaccharides, proteins, nucleic acids, lipids and suspended solids which contribute to creating the biofilm (Thapa et al., 2017). Some biofilm PBR includes algal turf scrubber (Fig. 1.2e) and rotating algal biofilm reactor (Fig. 1.2f), among others (Hoh et al., 2016; Li et al., 2017). Biofilm PBRs offer advantages regarding algal harvesting, more resistance to growth stresses, and high dry biomass density (Roostaei et al., 2018). However, drawbacks in light supply due to the high biomass adherence onto the tubes could reduce nutrients removal and harvesting efficiency, limiting the technical viability of this technology (Posadas et al., 2014).

In matrix-immobilization systems, photosynthetic microorganisms are entrapped or immobilized in hydrophilic synthetic (acrylamide, polyurethane, polyvinyl, resins) and natural polymer derivatives (alginate, carrageenan, agar, agarose) matrices (de-Bashan and Bashan, 2010). Immobilization enables greater biomass concentration than in suspended systems. Despite challenges associated with harvesting are reduced, cellular access to CO_2, nutrients, and photons is an issue to be considered along with the costs of the immobilisation matrix (Whitton et al., 2015; Gonçalves et al., 2017).

1.4.2. Wastewater treatment by microalgae-based systems

The potential of using algae systems for wastewater bioremediation in outdoor waste stabilization ponds was first reported in the 1960s in California cultivating autotrophic cultures (Oswald, 1988). The removal, degradation, or biotransformation of nutrients, pollutants, and xenobiotics by macroalgae, microalgae, and cyanobacteria is known as phycoremediation (Olguí, 2003). Phycoremediation comprises several applications such as nutrient removal from municipal wastewater and effluents rich in organic matter, treatment of wastewaters containing metals; CO_2 sequestration, and removal of xenobiotics (Rawat et al., 2011).

Considering that wastewater can be rich in nitrogen and phosphorus, microalgae can use wastewater as a source of nutrients and a cultivation media avoiding the use of freshwater and the supply of external nutrients. Other advantages include the nutrient recycling in algal biomass and its use for bioenergy and/or bioproducts production and the discharging of an oxygenated effluent into water bodies (Arbib et al., 2014). Algae-based technologies have been employed for bioremediation of municipal wastewater (Li et al., 2011; Arbib et al., 2014; Škufca et al., 2021), agricultural wastewater (García-Galán et al., 2018; Díez-Montero et al., 2020a), livestock wastewater (Prajapati et al., 2014; Choudhary et al., 2016), and industrial wastewater (Hernández et al., 2013; Van Den Hende et al., 2014; Mohd Udaiyappan et al., 2017). Species of the genera *Chlorella*, *Scenedesmus,* and *Chlamydomonas* are some of the most employed in wastewater treatment due to their high biomass productivity, robust environmental tolerance, and enhanced ability for nitrogen, phosphor, and COD removal (Li et al., 2011; Wang et al., 2015).

When using microalgae-based systems, algae-bacteria symbiosis enhances nutrient recovery and benefits complex degradation processes that are difficult to be accomplished using monocultures (Su et al., 2011; Ramanan et al., 2016; Gonçalves et al., 2017). Nutrients in the wastewater can be converted into biomass without an external source of organic carbon and algal biomass can be used for the production of bioenergy or bioproducts (Arbib et al., 2014). The in-situ photo-oxygenation provided by the microalgae can support microbial oxidation of recalcitrant and toxic organic contaminants and reduce energy demand for process aeration (Judd et al., 2015). Moreover, the low capital investment for PBRs installation and operation, make microalgae-based systems cheaper than the conventional activated sludge processes (Craggs et al., 2011). Several studies have dealt with microalgae-based systems for the removal of heavy metals (Suresh Kumar et al., 2015; Zeraatkar et al., 2016; Sultana et al., 2020) and organic micropollutants (Baghour, 2017; Sutherland and Ralph, 2019; Maryjoseph and Ketheesan, 2020; Nie et al., 2020; Škufca et al., 2021). Microalgae can degrade complex parent compounds to simpler molecules, highlighting their substantial biodegradation potential (Xiong et al., 2018). Thus, microalgae-based systems offer a promising alternative to shift from the wastewater treatment paradigm to energy and resources recovery.

1.5. Pesticides: occurrence, fate, and removal

Concern and scientific interest about organic micropollutants (OMs) in the environment have risen in the recent decades, boosted by the progress in the analytical technologies that allow their detection at very low levels in the different environmental compartments (Gavrilescu et al., 2015; Geissen et al., 2015). OMs include diverse compounds, such as pharmaceuticals, cosmetics, personal care products, pesticides, surfactants, flame retardants, or plasticizers (Barbosa et al.,

2016), many of which have been reported to potentially pose adverse effects on ecosystems and human health (Luo et al., 2014; Grandclément et al., 2017). The main characteristic of these compounds is their environmental persistence and bioaccumulation throughout the food chain (Bueno et al., 2012; Miniero et al., 2014). Pesticides are a wide class of chemicals used to limit, inhibit, and/or prevent the growth of harmful animals, insects, weeds, invasive plants, and fungi (Rousis et al., 2017). Pesticides are classified according to the target organisms as herbicides, fungicides, insecticides, acaricides, nematicides, rodenticide, bactericides, among others. Increase pesticide consumption mainly respond to an increase in population growth and the subsequent need for improving crop yields. Globally, more than 4 million tons of pesticide were used in 2018 (FAO, 2021). Between 2011 and 2018, around 360000 tonnes of pesticides were sold per year in the EU where Germany, Spain, France, and Italy are the main agricultural producers, and hence the main users of these products (Eurostat, 2021).

Pesticides are introduced to the environment via nonpoint and point sources (Tankiewicz et al., 2010). They are widely used not only in agriculture but also in public health (disease control such as malaria), forestry, livestock and domestic animals, maintenance of large green areas (i.e. parks, sports grounds, etc.), maintenance of water reserves, industry (i.e. paints, resins, preservation of fresh food), and for domestic uses (i.e. insect repellent) (Prieto Garcia et al., 2012). Although pesticides use and application generate many benefits in terms of improved agricultural productivity and public health, their global use has resulted in their widespread presence in soil, water, and air, representing potential risks over non-target organisms such as aquatic biota, terrestrial plants, mammals, and

soil microorganisms (Tremolada et al., 2004; Torres et al., 2008; Fenner et al., 2013a; Pietrzak et al., 2019).

Pesticide behaviour in the environment is strongly influenced by their physical-chemical properties, environmental factors, type of soil and/or sediment, and agricultural management practices (Navarro et al., 2007). Once pesticides are in the environment, diverse processes influence their transport such as soil surface runoff, spray drift, volatilisation, atmospheric deposition, soil leaching, and soil erosion (Schulz, 2001; Müller et al., 2002; Warren et al., 2003; Navarro et al., 2007). Moreover, their distributions in soil, water, and air are affected by transfer between phases and adsorption/desorption processes (Baghour, 2017). When pesticides reach the aquatic media, they are subject to chemical (hydrolysis and oxidation), physical (accumulation, deposition, dilution, and diffusion), photochemical (photolysis and photodegradation), and biological (biodegradation, biotransformation, and bioaccumulation) processes influencing their degradation and transformation into other compounds (Tankiewicz et al., 2010; Fenner et al., 2013a). Pesticides taken up by living organisms are susceptible to bioaccumulation (Corcellas et al., 2015), co-metabolic or partial transformation into other degradation compounds, or mineralisation (Kumar et al., 2018; Dar et al., 2019). The main mechanisms involved in the removal of pesticides by microalgae-based systems include sorption, photodegradation, bioaccumulation, and biodegradation (Norvill et al., 2016; Nie et al., 2020). Transformation products (TPs) can be formed in the environment after the degradation or conversion of the parent compounds. In some cases, these TPs may be even more hazardous than the parent compounds (Fenner et al., 2013b; Richardson and Ternes, 2014; Ccanccapa et al., 2016).

Conventional biological treatment systems such as activated sludge are not specifically designed to eliminate OMs such as pesticides and have shown limited effectiveness in their removal (Meriç et al., 2003; Köck-Schulmeyer et al., 2013; Eggen et al., 2014). It has been demonstrated that OMs concentrations in the WWTP effluents can be even higher than in the influent (Köck-Schulmeyer et al., 2013; Sadaria et al., 2016; Grandclément et al., 2017). Therefore, due to their persistence, many of these compounds can pass through wastewater biological treatment processes and WWTP effluents constitute another source of pesticides in the aquatic environment (Grandclément et al., 2017). Other technologies such as chemical precipitation, adsorption, advanced oxidation processes, chlorination and filtration, among others have been tested for the removal of pesticides from wastewater (Ormad et al., 2008; Marican and Durán-Lara, 2018; Mukherjee et al., 2020; Rempel et al., 2021). However, they are often ineffective due to pesticide low concentration in water, the formation of toxic by-products, and/or the high costs associated with their implementation and maintenance (Saleh et al., 2020). In contrast to these processes, bioremediation technology is known as one of the safest environmental restoration techniques due to its cost-efficiency, reduced risk of secondary pollutant generation, and environmentally sound alternative (Subashchandrabose et al., 2011; Helbling, 2015; Nie et al., 2020). Moreover, the use of microalgae-based systems for pollutants removal is highlighted over other bioremediation technologies due to the simultaneous resource recovery of algal biomass which can be applied as a biofertilizer or as an energy source for the production of biofuels while providing a high-quality treated effluent (Subashchandrabose et al., 2011; Craggs et al., 2012; Sutherland and Ralph, 2019).

Table 1.1 Pesticides removal from wastewater using microalgae-based systems at pilot-scale.

Pesticide	Microalgae cultivation system	Type of influent	Operational parameters	Removal efficiency (%)	Removal efficiency in WWTPs (%)	Reference
Triclosan (0-24 μg L^{-1})	HARPs (0.5 m^3)	Urban wastewater primary effluent	HRT = 4 d (warm season)	93 ± 1	71-99 [a]	Matamoros et al. (2015)
			HRT = 8 d (warm season)	95 ± 1		
			HRT = 4 d (cold season)	49 ± 5		
			HRT = 8 d (cold season)	69 ± 2		
Diazinon (0-24 μg L^{-1})			HRT = 4 d (warm season)	61 ± 4	<0 [a]	
			HRT = 8 d (warm season)	63 ± 1		
Atrazine (0-24 μg L^{-1})			HRT = 4 d (warm season)	76 ± 6	<0 -25 [a]	
			HRT = 8 d (warm season)	85 ± 3		
			HRT = 4 d (cold season)	41 ± 7		
			HRT = 8 d (cold season)	69 ± 6		
2,4-D (0-24 μg L^{-1})			HRT = 4 d (warm season)	22 ± 10	52 [b]	
			HRT = 8 d (warm season)	32 ± 26		
Triclosan (100 ng L^{-1})	Semi-closed tubular PBR (8.5 m^3)	Agricultural runoff from an irrigation channel	HRT = 16 d (winter season)	32	71-99 [a]	García-Galán et al. (2018)
Terbutryn (15 – 70 ng L^{-1})	Semi-closed tubular PBR (11.7 m^3)	Agricultural runoff from an irrigation channel	HRT = 5 d (warn season)	<0	n.a.	García-Galán et al. (2020)
Diuron (29 – 61 ng L^{-1})				<0	<0 – 72 [a-b]	
Imidacloprid (41 – 73 ng L^{-1})				<0		
MCPA (45 – 392 ng L^{-1})				89	<0 [b]	
2,4-D (9.7 – 23 ng L^{-1})				<0 - 100	52 [b]	
Diazinon (26 – 47 ng L^{-1})				<0 - 100	<0 [a-b]	
Alachor (6 ng L^{-1})				100	100 [b]	
Linuron (13 ng L^{-1})				100	100 [c]	
Cybutrine (14 ng L^{-1})				100	n.a.	
Terbuthylazine (11 ng L^{-1})				100	3 [b]	
Chlorfenvinphos (19.7 ng L^{-1})				100	n.a.	

n.a. = not available. [a] Luo et al. (2014); [b] Köck-Schulmeyer et al. (2013), [c] Rodriguez-Mozaz et al. (2015).

Table 1.1 summarises main studies on pesticides removal from water using microalgae-based systems. Overall, microalgae-based wastewater technologies support the efficient removal of pesticides with the advantages of nutrients recovery in the algal biomass and the low energy demand. Biodegradation was reported as the main removal mechanism whereas biosorption and photodegradation can also be important.

1.6. Microalgal biomass valorisation

1.6.1. Microalgae harvesting

Due to the small size of algal cells (i.e., 3-30 μm for unicellular eukaryotic microalgae and 0.2-2 μm for cyanobacteria) and their low concentration in water (~1 g L^{-1}), one of the main challenges in algal systems is the recovery of algal biomass from the culture media for the downstream processing (Postma et al., 2017; Van Haver and Nayar, 2017). Harvesting is a crucial step for algal biomass recovery and it has been estimated that contribute to 20 to 30% of the production costs depending on algal species, cell density, and culture conditions (Mennaa et al., 2015; Singh and Patidar, 2018). Several harvesting methods based on chemical, physical or biological processes have been studied to date, and there is not a single recommended technique for microalgae harvesting.

Microalgae harvesting by sedimentation is based on gravitational force. It is a low cost and low energy method, highly effective for large microalgae cells such as *Spirulina* spp. (Rawat et al., 2013). Flocculation is an enhanced process of sedimentation adding positive charge flocculants to neutralize algal negative surface charges, forming agglomeration in algal flocs, and can be used for a wide range of algal species improving biomass settling rate. Despite metal sals (aluminium sulfate, ferric chloride and ferric sulphate) are effective flocculants,

flocculation by metal salts may be unacceptable depending on the application of the biomass (Molina Grima et al., 2003). A more environmentally friendly alternative to metal salts are cationic polymers such as poly diallyl dimethyl ammonium chloride (polyDADMAC), which have demonstrated efficient flocculation of algal cells (Gerchman et al., 2017). Another flocculation technique also known as alkaline flocculation, induces algal concentration via pH adjustment of the medium without the addition of chemical flocculants (Branyikova et al., 2018). In the case of low-value products, gravity sedimentation and flocculation can provide a suitable alternative for harvesting.

Other harvesting techniques include centrifugation, flotation, filtration, and fungal co-pelletization. Centrifugation is the most rapid method for recovering suspended algae, but also more expensive due to its high energy consumption. Diverse flotation methods based on gravity separation are used for microalgal harvesting. For instance, in dissolved air flotation (DAF) small air bubbles (10-100 μm) forced the suspended microalgae to float to the liquid surface and there can be skimmed off whereas in dispersed air flotation (DiAF) bigger size bubbles (700-1500 μm) are generated (Singh and Patidar, 2018). Another technique that does not require the use of chemicals is filtration. Dead-end filtration can be used for larger algal cells harvesting (over 70 μm) (Rawat et al., 2011) whereas tangential flow filtration is more appropriate for smaller microalgae recovery (Christenson and Sims, 2011). Despite a better quality of the harvested biomass is obtained, they are energy-intensive techniques. Fungal co-pelletization of algal cells involves the self-pelletization of filamentous fungi which entrapped the microalgal biomass on the fungal pellets. This method was efficiently tested as a bio-flocculation technique (Hom-Diaz et al., 2017a; Srinuanpan et al., 2018). Other bio-flocculation techniques are based on algal–bacterial or algal–algal

interactions for microalgae harvesting (Alam et al., 2016). Overall, the selection of a specific technique depends on the microalgae species, the products to be obtained, the efficiency of the harvesting method, the energy requirements, the application of the harvested biomass, and the effects on the downstream process, among other factors (Barros et al., 2015; Singh and Patidar, 2018).

1.6.2. Anaerobic digestion

The anaerobic digestion (AD) process implies the biological transformation of the organic matter by specific microorganisms in the absence of atmospheric oxygen. AD comprises several complex processes namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis where microorganisms anaerobically degrade organic matter via cascades of biochemical conversions to biogas (Angelidaki et al., 2011). Diverse microbial communities are involved in each process stage transforming products to substrates for the next anaerobic phase (Jankowska et al., 2019). As shown in Fig. 1.3, during the hydrolysis stage, complex and high molecular-weight organic compounds (such as carbohydrates, proteins, and lipids) are broken down by extracellular enzymes from hydrolytic bacteria into simple monomers (namely monosaccharides, amino acids, and fatty acids) in a slow reaction (Lee et al., 2017). However, hydrolysis is known as the main rate-limiting step in AD of WAS and microalgae biomass (Gavala et al., 2003; Capson-Tojo et al., 2017). Later, in acidogenesis fermentation hydrolysed substances are degraded by acidogenic bacteria to organic acids, ammonia, CO_2, H_2S, and other by-products. During acetogenesis, organic acids and alcohols are converted by acetogens into acetic acid, H_2, and CO_2. Methanogenesis is the final step in the conversion of organic matter into biogas by two groups of archaea: acetoclastic methanogens (cleave acetate into methane and CO_2) and hydrogenotrophic methanogens (use H_2 as the electron donor and CO_2 as the

electron acceptor to produce methane) (Appels et al., 2008; Cavinato et al., 2017). Biogas obtained by AD is the only biofuel characterized for employing the whole organic content of the biomass for energy production (Mendez et al., 2013). It is a renewable bioenergy source used for electricity and heat cogeneration and is mainly composed of 55-75% methane, 25-45% carbon dioxide, and trace quantities of other gases (H_2, H_2S) (Harun et al., 2010).

AD is one of the most consolidated and well-known technologies to convert organic wastes into bioenergy, contributing to organic matter stabilization, nutrient recycling, and GHG emissions and odours reduction (Appels et al., 2008). It is the main technique used for WAS treatment and stabilization in WWTP facilities, providing a carbon-neutral source of energy. In the last decades, more attention was given to the AD of organic wastes from agro-industrial sources (glycerol, food wastes, lignocellulosic biomass, vinasse, animal wastes, paper mill effluents, etc.) as an alternative for their valorisation (Zahan et al., 2018; Zhang, 2018; De Farias Silva et al., 2019; Y. Li et al., 2020).

Also, AD is considered a promising process for recovering energy from algal biomass, which possesses several advantages over other biofuel feedstocks (Chia et al., 2018). Microalgal biomass composition is strain-specific, but generally algal macromolecular content varied between 6.7 – 65% of carbohydrates, 15 – 84% of proteins, and 1 – 63% of lipids based on VS basis (Xia et al., 2015). When using algal biomass for AD, all algal macromolecules are used for biogas production, and carbohydrates and lipids extraction is not required. Moreover, microalgae biomass do not contain lignin, making it a more easily hydrolysed substrate in comparison with crops and lignocellulosic biomasses (Singh et al., 2015). Methane yield from algal biomass depends on several factors, such as the microalgae species, pretreatment of the biomass, process operating parameters

(pH, temperature, nutrient availability), the presence or absence of inhibitors, among others (Mussgnug et al., 2010).

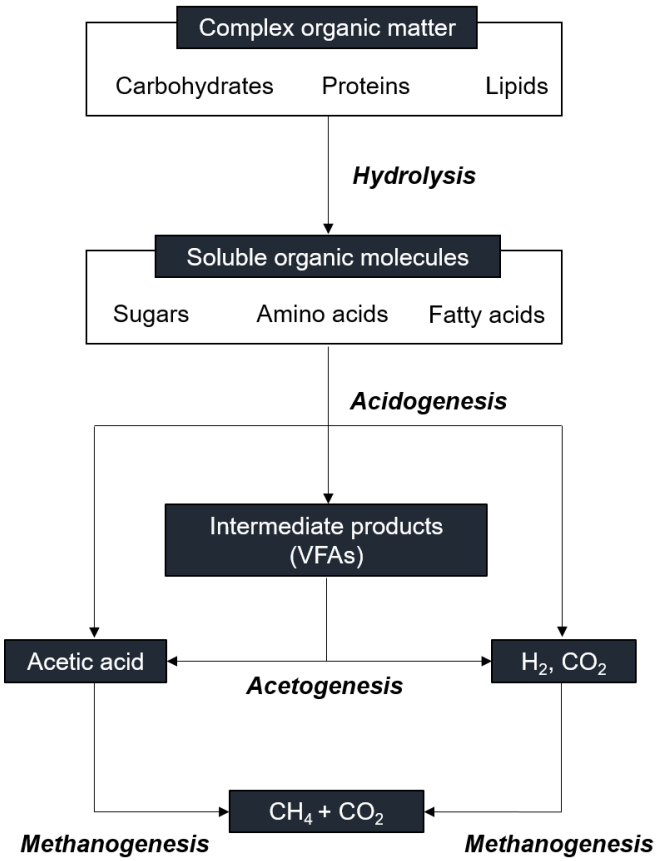

Figure 1.3 Phases involved in the anaerobic digestion process for biogas production (adapted from Appels et al., 2008).

Generally, the microalgae cell wall is constituted by a tough and resistant structure with an inner and outer layer (Yamada and Sakaguchi, 1982). For instance, green algae contain a well-defined membrane and a complex layered cell wall structure (or absence of it, depending on the microalgae species) typically formed by polymers such as cellulose, hemicellulose, pectin and starch; while in cyanobacteria glucan is the main polysaccharide (Hallenbeck and Benemann, 2002; Nagarajan et al., 2020a). Microalgae cell wall act as a protective layer that

contributes to regulating environmental interactions, provides rigidity, mechanical strength, and chemical resistance to the microalgae (Lari et al., 2019). However, the microalgae cell wall is a rigid structure resistant to hydrolysis, representing a boundary for microalgae organic matter release (Lü et al., 2013; Soto-Sierra et al., 2018). Moreover, some microalgae cell walls contain two biopolymers, namely sporopollenin and algaenan, recalcitrant macromolecules that resist acetolysis and hamper the release of organic matter from microalgae through bacterial degradation and chemical hydrolysis (de Leeuw et al., 2005; González-Fernández et al., 2012a; Ometto et al., 2014; He et al., 2016). Some algae species such as *Chlamydomonas reinhardtii*, *Dunaliella salina*, and *Asthrospira platensis* showed a higher anaerobic biodegradability and higher biogas production in comparison with *Chlor*ella *kessleri* and *Scenedesmus* sp. (Mussgnug et al., 2010). When the algal cell wall is not affected by the disruption process and intracellular content is not released, enzymes from the anaerobic consortium will have less access to algal biomass limiting biogas production (Sialve et al., 2009).

Considering the recalcitrancy of microalgae biomass due to its cell wall structure and composition, microalgae biomass pretreatment is generally a crucial step to disintegrate cell wall structure. Those pretreatments pursue cell membrane disintegration, release intracellular macromolecules followed by their solubilisation, making them bioavailable for microorganisms' degradation and further conversion into biofuels and bioproducts (González-Fernández et al., 2012b; Mendez et al., 2013). Methods for microalgae pretreatment are characterised according to the effect on the biomass and the nature of the disruption force as mechanical, chemical, thermal, and biological pretreatments. Chemical pretreatments include the use of compounds such as acids, bases, and solvents, among others, to produce chemical reactions on biomass structure.

Mechanical methods imply a reduction of biomass particle size. Chemical treatments entail a lower energy consumption in comparison with mechanical methods and are performed at room temperature. Thermal and hydrothermal pretreatments are high energy demanding methods that employ temperature for cell wall breakage (Cai et al., 2015). Biological pretreatments include the use of natural microorganisms (fungal strains, bacterial strains, microbial consortia) or enzymes for biomass hydrolysis to simpler molecules (Zabed et al., 2019). Scare literature is available on enzymatic pretreatment of algal biomass, but it has been demonstrated that the use of specific enzymes for algal cell wall disruption and biomass solubilisation increase the methane yield (Ehimen et al., 2013). Biological pretreatments are gaining interest over other methods since they require minimal energy input, they are simple, safe, with low downstream processing costs, and the absence or reduced formation of toxic compounds (Zabed et al., 2019). Many pretreatment techniques are usually combined with other methods to enhance the advantages of a single method and improve cell wall disruption and hydrolysis of the organic matter released (Yang and Wang, 2019). Generally, chemical and mechanical pretreatments are applied simultaneously; meanwhile, physical pretreatments are applied followed by biological or chemical pretreatments (Atelge et al., 2020).

Bearing in mind the microalgae differences from strain to strain, the best pretreatment method or combination of pretreatments depends on microalgal specific features and pretreatment conditions. Pretreatments to improve microalgae anaerobic digestibility should preserve its organic matter content, along with avoiding the generation of inhibitory products that could disrupt the AD process (Córdova et al., 2018). Furthermore, the pretreatment efficiency is determined by the improvement obtained in the energy or product yield and the

cost-effectiveness of the pretreatment, related to resources and energy consumption required in their application.

Another strategy that could contribute to enhancing biogas production is the simultaneous anaerobic co-digestion (AcoD) of two or more substrates containing a different macromolecular composition in a single digester. Digesters are usually oversized, and the addition of co-substrates improves the economic feasibility of AD plants due to the potential for higher biogas yield (Solé-Bundó et al., 2019). The increase in methane production from AcoD is mainly a result of an increased organic loading rate, but synergism can further enhance methane production (Ramos-Suárez and Carreras, 2014). AcoD also contributes to optimize carbon and nitrogen content, avoid, or reduce ammonia inhibition from the degradation of lipid-rich substrates. Provide alkalinity, and may supply nutrients that are missing from a single substrate (Sialve et al., 2009; Astals et al., 2014; Mata-Alvarez et al., 2014; Ajeej et al., 2015). Numerous substrates (food waste, maize straw, livestock manure, glycerol, molasses) have been tested as co-substrates to a carbon-rich waste such as WAS (Kalemba and Barbusiński, 2017; Ma et al., 2017; dos Santos Ferreira et al., 2018; Wei et al., 2019; Yang et al., 2019). In the last decades, the use of algal biomass as a co-substrate has been studied as another way to improve microalgae digestibility (Zhen et al., 2016; Thorin et al., 2018; Solé-Bundó et al., 2019; Serna-García et al., 2020). Moreover, microalgal biomass cultivated on site present some advantages over the use of other co-substrates: (i) avoid or reduce the cost associated with co-substrate transportation; (ii) minimizing the effect of the seasonality of some agro-industrial co-substrates; and (iii) provide a co-substrate in some areas where other co-substrates are not available (Astals et al., 2015; Olsson et al., 2018). Therefore,

AcoD is an opportunity to overcome the low biodegradability of microalgae mono-digestion.

1.7. Circular bioeconomy in microalgae-based systems

The transition from a fossil-based linear economy to a circular based bioeconomy is one of the fundamental requirements towards global sustainability (Venkata Mohan et al., 2020). In a circular bioeconomy scenario, resources and wastes streams circulate in a closed-looped system, pursuing their reuse, valorisation, and recycling (Venkata Mohan et al., 2016; Chojnacka et al., 2020; Nagarajan et al., 2020b). According to Stegmann et al. (2020), "the circular bioeconomy focuses on the sustainable, resource-efficient valorisation of biomass in integrated, multi-output production chains (e.g. biorefineries) while also making use of residues and wastes and optimizing the value of biomass over time via cascading".

In microalgae-based systems, single product valorisation from microalgal biomass and costs associated with large-scale facilities could hinder its economic feasibility. The cost-effective production of microalgae-based biofuels can be significantly improved in a biorefinery concept when all the components of the biomass are used to obtain bioproducts through biomass transformation (Singh and Gu, 2010; Trivedi et al., 2015). The integration of microalgae-based systems in WRRFs as a source of biomass, biofuels, bio-products, and water remediation, is a promising alternative to maximize resource recovery from algal biomass in a circular bioeconomy framework (Fig. 1.4) (Wang et al., 2013; Ajeej et al., 2015; Viruela et al., 2018; Venkata Mohan et al., 2020).

Microalgae-derived metabolites such as lipids, proteins, carbohydrates, pigments (chlorophyll, carotenoids, phycobilins), bioactive compounds (antibacterial,

antiviral, anti-fungal), and biopolyesters (polyhydroxyalkanoates) have a variety of industrial applications (Koller et al., 2014). Commercial application of algal products includes cosmetics (canthaxanthin, bixin, phycocyanin), pharmaceuticals (lutein, zeaxanthin, canthaxanthin), nutraceuticals (γ-linolenic acid, arachidonic acid -ARA, eicosapentaenoic acid -EPA-), aquaculture (astaxanthin, docosahexaenoic acid -DHA-, EPA, ARA), biofertilizers and bioplastics industries, among others (Molina Grima et al., 2003; Mobin et al., 2019).

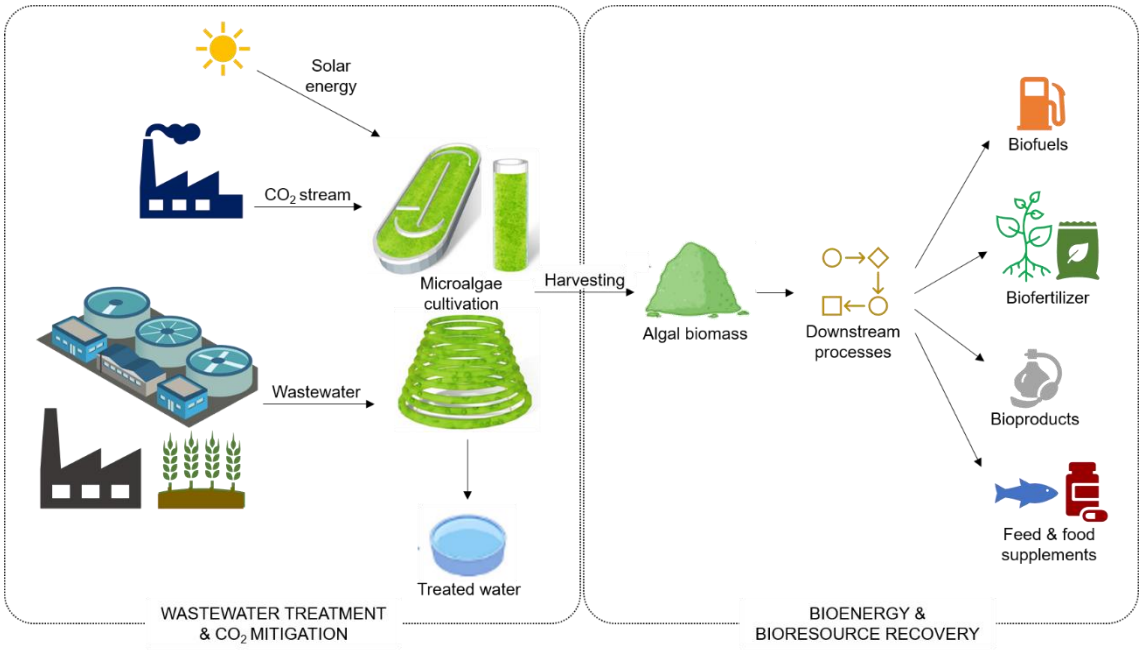

Figure 1.4 Microalgae-based systems using microalgae cultivation for wastewater treatment and the production of biofuels and bio-based products.

Also, combine biomethane production through anaerobic digestion whilst cultivating microalgae for wastewater treatment promote the transition from a finite resource economy to a sustainable resource-based economy (Barroso Soares et al., 2019). When integrating wastewater treatment with algal biofuel production, algae cultivation has a low energy requirement since the costs of algal

cultivation are covered by wastewater treatment (Vidyashankar and Ravishankar, 2016). In addition, sludge and microalgae co-digestion could optimize the management of sludge streams in WWTPs (Serna-García et al., 2020). Also, nutrients in digestate and algae can be recovered as biofertilizers and cycled on site. Digestate is a stabilized nutrient-rich by-product composed of partially degraded organic matter, microbial biomass, and inorganic compounds with potential application as a plant growth biostimulant, biofertilizer, and/or soil amendment (Sialve et al., 2009; Scaglia et al., 2017; Guilayn et al., 2019; Wang and Lee, 2021). Microalgal biomass can be used as a slow-release biofertilizer since they contain macronutrients, micronutrients, and proteins rich in essential amino acids that stimulate plant growth and increase crops yield (Tibbetts et al., 2015; Coppens et al., 2016). Apart from nutrients, microalgal biomass contains growth regulators, vitamins (A, B1, B6, B12, C, E, biotin, folic acid, among others), and phytohormones (Sruthi et al., 2016). The application of bio-based fertilizers brings environmental benefits and promotes fertilizers production in a circular economy model (EC, 2016). Hence, the circular bioeconomy approach proposes a paradigm shift and contributes to offset limitations associated with the application of microalgae-based systems at an industrial scale enhancing the economic and environmental sustainability (Gifuni et al., 2019).

CHAPTER 2.

RESEARCH MOTIVATION AND OBJECTIVES

2.1. Research motivation

The capacity of microalgae-based systems for nutrients removal in urban wastewater has been studied over the last decades and is a well-established technology applied at full scale. Recently, the use of algae-based systems for transforming or enhancing the biodegradation of diverse emerging contaminants such as pharmaceuticals from wastewater is gaining scientific interest. However, few studies to date assess the feasibility of applying algal biotechnology for the removal of other micropollutants of great concern such as pesticides from water. Hence, the study of microalgae potential for bioremediation of pesticides deserves more attention.

To address current global environmental challenges, the transition from biological conventional wastewater treatment to systems based on resource recovery from wastewater is required. New trends are focused on developing a biorefinery approach and its integration into a circular bioeconomy scenario. When coupling anaerobic digestion and microalgae cultivation in wastewater, nutrients are recycled while obtaining a profitable algal biomass that can be used as a renewable feedstock for bioenergy production. Moreover, this biomass can be co-digested with other available bio-wastes such as secondary sludge, obtaining digestate and biogas. Digestate can be further valorised by its application as a biofertilizer or soil amendment in soils. Nonetheless, the application of the presented close loop system in a real industry could present some bottlenecks and challenges that need to be overcome.

The main research motivations of this PhD thesis are:

- To study the efficiency of microalgae-based systems on pesticides removal from water, identifying processes involved in their degradation, and assessing further algal biomass valorisation through anaerobic digestion.
- To evaluate the effect of diverse algal biomass pretreatments on its anaerobic co-digestion with secondary sludge.
- To assess the coupling of a microalgae-based system with anaerobic co-digestion and bio-waste recovery in a real case study in a winery company.

2.2. Thesis outline

This PhD thesis is divided in five sections and eight chapters. Among them, Chapters 4 to 7 covers the experimental work.

Section 1 includes general aspects and comprises Chapter 1 and 2. Chapter 1 (General introduction) introduces the main topics addressed in this thesis: concern over the presence of pesticides in aquatic environments, pesticides removal using microalgae-based systems, algal-biomass harvesting and valorisation through anaerobic digestion, and circular approach in microalgae-based systems is depicted. Chapter 2 includes the main objectives of this thesis.

Section 2 comprises Chapter 3 (General materials and methods) including general materials and methods employed along the thesis. The specific materials and methods are described in each chapter.

Section 3 contains Chapter 4 (Removal of selected pesticides by microalgae) which is focused on pesticides removal by microalgae. This Chapter comprises studies at indoor batch conditions for six selected pesticides to determine their removal mechanisms and to identify their transformation products. Algal biomass with retained pesticides by sorption was valorised through anaerobic

digestion. This Chapter also includes the results obtained for the removal of polar pesticides in a semi-continuous pilot scale photobioreactor as well as the valorisation of harvested algal biomass for biogas production.

Section 4 is composed by Chapters 5, 6 and 7 addressing a real case study to evaluate the feasibility of closing waste fluxes in a winery company using a microalgae-based system to promote a circular bioeconomy approach. In this context, Chapter 5 (Low temperature thermal pretreatments) and 6 (Alkaline and enzymatic pretreatments) present different techniques for algal biomass (and secondary sludge) pretreatment prior to their anaerobic (co-) digestion. Pretreatments were selected due to their low cost and easiness of implementation. Chapter 5 describes the effects of flocculation and the low temperature thermal pretreatments of secondary sludge and microalgae mixtures on biomass solubilisation and biogas yield. Effects of alkaline and enzymatic pretreatments on algal biomass concentration and solubilisation and their further anaerobic co-digestion with secondary sludge are described in Chapter 6. Chapter 7 (Water resource recovery coupling microalgae wastewater treatment and WAS co-digestion at industrial pilot-scale) presents the co-digestion at pilot scale of microalgae and the secondary sludge. Also, the suitability of applying the obtained bio-wastes (mono- and co-digestion digestates, algal biomass, and photobioreactor effluent) as biofertilizers or for irrigation was assessed.

Section 5 comprises Chapter 8 (General conclusions) drawing the main conclusions based on the obtained results.

Later, Appendix A details specific analytical procedures from Chapter 4.

Finally, the curriculum vitae of the author including the list of publications, and the references are presented.

2.3. Objectives

In order to accomplish the research motivations, this study has the following specific objectives:

- To assess the potential of a microalgae-based system for pesticides removal from water based on their polarity, and to identify the transformation products and processes involved in pesticides removal (Chapter 4).
- To evaluate the influence of pesticides retained in algal biomass in methane production by means of anaerobic digestion and their further anaerobic degradation. To assess the removal of selected pesticides in an outdoor pilot scale photobioreactor and the valorisation of the harvested biomass by anaerobic digestion (Chapter 4).
- To evaluate the effect of low temperature thermal pretreatments, alkaline and enzymatic pretreatments on the solubility and anaerobic digestibility of algal biomass and secondary sludge-microalgae mixtures. To evaluate the efficiency of harvesting methods for microalgal concentration (Chapters 5 and 6).
- To assess the integration of a microalgae-based system for tertiary wastewater treatment and anaerobic co-digestion for bioenergy production and bio-waste recovery in a winery company (Chapter 7).

SECTION 2

General procedures

CHAPTER 3.

GENERAL MATERIALS AND METHODS

3.1. Chemicals and reagents

The suppliers of salts and reagents used in medium preparations are detailed in the medium composition tables. All chemicals used were of analytical grade.

3.1.1. Enzymes

The enzymes used in microalgal enzymatic pretreatments, their supplier, and characteristics are presented in Table 3.1.

3.1.2. Flocculants and coagulant

The flocculants used were provided by Derypol, S.A. (Barcelona, Spain). Flocculant A consist in a cationic polymer of diallyl dimethyl ammonium chloride (polyDADMAC) free of acrylamide, flocculant B is a cationic copolymer of acrylamide, while flocculant C is a cationic polysaccharide obtained from chitosan. Their properties are presented in Table 3.2. These flocculants were used in doses according to the instructions of the manufacturer. Ferric (III) chloride ($FeCl_3$) was used as a ferric salt coagulant (Merck, Germany).

Table 3.1 Description of commercial enzymes employed in microalgal enzymatic pretreatments.

Enzyme name	**Commercial name**	**Composition**	**Production**	**Supplier**
A	Celluclast® 1.5 L	Cellulase ≥700 EGU g^{-1}	Produced by a selected strain of the fungus *Trichoderma reesei.*	Novozymes, Spain
	Glucanex®	Cellulase ≥ 1000 U g^{-1} Chitinase ≥ 100 U g^{-1} Protease ≥ 10 U g^{-1}	Enzymes obtained from *Trichoderma harzianum.*	
	Shearzyme®	Cellulase 350 EGU g^{-1} Xylanase 250 FXU-S g^{-1}	Enzymes obtained from *Aspergillus oryzae* and *Trichoderma reesei.*	
B	Enovin FL®	Poligalacturonase 224.4 IU g^{-1} Pectinliase 14.7 IU g^{-1} Pectinmetilesterase 4.7 IU g^{-1}	Enzymes obtained from *Aspergillus niger* fungi.	Agrovin, Spain
C	Vinozym® Ultra FCE	Poligalacturonase 5100 PGNU g^{-1}	Enzymes obtained from *Aspergillus niger* and *Aspergillus aculeatus* fungi.	Novozymes, Spain

References: EGU: cellulase units, U: units, FXU-S: fungal xylanase units, IU: international units, PGNU: pectinase unit.

Table 3.2 Properties of the flocculants.

Flocculant	Appearance	Density (g cm^{-3})	Viscosity (cP)	pH
A	Colourless to amber transparent liquid	~ 1.1	<15000	4 - 7
B	Milky white liquid	1.2	<1000	3 - 4.2
C	Solid	0.24	-	3.7

3.2. Microalgae cultivation

3.2.1. Media for microalgae cultivation

Microalgae growth medium used for each culture is specified in the materials and methods section of each chapter. Growth media are defined in Tables 3.3 and 3.4.

Table 3.3 Modified Mann and Myers medium composition for microalgae culture (Mann and Myers, 1968).

Component	**Concentration (g L^{-1})**	**Supplier**
Macronutrients		
$NaHCO_3$	0.5	Sigma-Aldrich
KH_2PO_4	0.1	Panreac
$MgSO_4 \cdot 7H_2O$	0.6	Sigma-Aldrich
$NaNO_3$	1	Sigma-Aldrich
$CaCl_2 \cdot 2H_2O$	0.2	Panreac
NaCl	0.5	Panreac
Micronutrients		
H_3BO_3	0.006	Panreac
$FeSO_4 \cdot 7H_2O$	0.002	Sharlau
$MnCl_2$	0.0014	Sigma-Aldrich
$ZnSO_4 \cdot 7H_2O$	0.00033	Panreac
$Co(NO_3)_2 \cdot 6H_2O$	0.000007	Fisher scientific
$CuSO_4 \cdot 5H_2O$	0.00002	Panreac

Table 3.4 Bold basal medium (BBM) composition for microalgae culture (Andersen, 2005).

Component	**Concentration (g L^{-1})**	**Supplier**
Macronutrients		
K_2HPO_4	7.5	Sharlau
KH_2PO_4	17.5	Panreac
$MgSO_4 \cdot 7H_2O$	7.5	Sigma-Aldrich
$NaNO_3$	25	Sigma-Aldrich
$CaCl_2 \cdot 2H_2O$	2.5	Panreac
NaCl	2.5	Panreac
H_3BO_3	0.011	Panreac
Microelements stock solution [b]	1 [a]	-
Solution 1 [b]	1 [a]	-
Solution 2 [b]	1 [a]	-

[a] Volume concentration (mL L^{-1}). [b] See Table 3.5.

Table 3.5 Microelements stock solution, solution 1 and solution 2 composition for the preparation of the BBM.

Component	**Concentration (g L^{-1})**	**Supplier**
Microelements stock solution		
$ZnSO_4 \cdot 7H_2O$	8.82	Panreac
$MnCl_2 \cdot 4H_2O$	1.44	Panreac
MoO_3	0.71	Fluka
$CuSO_4 \cdot 5H_2O$	1.57	Fisher scientific
$Co(NO_3)_2 \cdot 6H_2O$	0.49	Fisher scientific
Solution 1		
EDTA Na_2	50	Sigma-Aldrich
KOH	31	Panreac
Solution 2		
$FeSO_4 \cdot 7H_2O$	4.98	Sharlau
H_2SO_4	1 [a]	Sharlau

[a] Volume concentration (mL L^{-1}).

3.2.2. Pilot outdoor photobioreactor

An outdoor semi-closed and tubular pilot-PBR located on the roof of the Chemical, Biological and Environmental Engineering Department at Universitat Autònoma de Barcelona (Barcelona, Spain) was used (Fig. 3.1). The PBR has a working volume of 1000 L and consists of eight tubes (length 7 m) connected at each side with two distribution chambers. The tubes are placed inside an open cuvette containing tap water to balance temperature changes between day and night. They are made of transparent low-density polyethylene (PE), they are soft and mouldable whereas the chambers and the PBR structure are made of propylene (PP) to provide robustness. The bigger chamber contains a paddle

wheel that gives movement and aeration to the microalgal culture by drawing the culture into the incoming tubes and later feeding the outgoing tubes. The paddle wheel gives a constant velocity of 0.13 m s^{-1}. Both distribution chambers have the possibility to stay covered or uncovered by placing a translucent plastic lit over it.

The PBR was exposed to rainfall events, sunlight irradiation, and ambient temperature variability.

3.3. Analytical methods

3.3.1. Biochemical methane potential tests

Anaerobic batch assays were performed according to a previously described procedure (Field et al., 1988; Martín-González et al., 2010) considering the suggestions from other authors (Angelidaki et al., 2009; Holliger et al., 2016). Biochemical methane potential (BMP) assays were carried out under mesophilic conditions 37 °C. Either 900 mL aluminium bottles (Fig. 3.2) or 120 mL glass bottles (Fig. 3.3) were used as reactors, with a working volume of 600 and 80 mL, respectively.

Figure 3.1 Semi-open tubular photobioreactor located at the roof of the Chemical, Biological and Environmental Engineering Department from Universitat Autònoma de Barcelona, Spain.

Figure 3.2 Aluminium bottles used in BMP tests.

Figure 3.3 Glass bottles used in BMP tests.

The inoculum was pre-incubated at 37 °C for 10 – 15 days to guarantee the consumption of the organic matter content and reduce background production of biogas. BMP reactors were filled with inoculum, substrate, and tap water until the working volume was reached (Fig. 3.4). Subsequently, the bottles were

flushed with pure N_2 to ensure anaerobic conditions. The reactors were closed with a gastight butyl rubber septum (in the case of the glass bottles) or using a cap with an adapted valve for manometric gas measurement (in the case of the aluminium bottles). Later, they were incubated in a temperature-controlled chamber (37 ± 1 °C). Blank reactors, containing only inoculum and water, were used to provide information about the background methane production of the inoculum, and reference reactors with crystalline cellulose were used to verify the quality of the inoculum. Reactors were shaken manually every time a gas sample was taken. Table 3.6 presents the characterization of inocula employed in BMP tests.

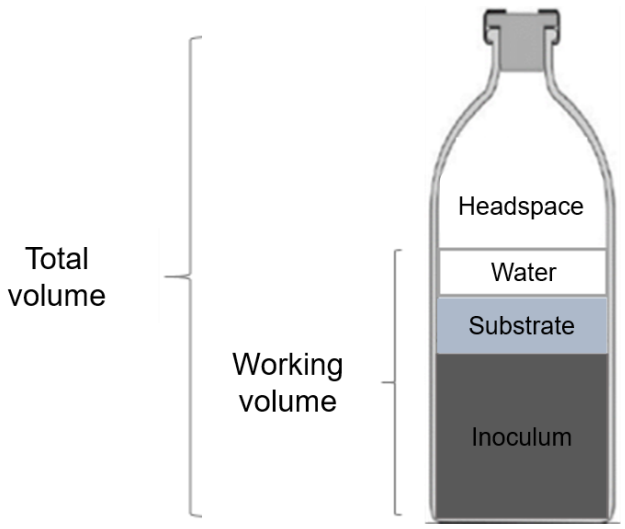

Figure 3.4 Experimental set-up for BMP tests.

Biogas production and accumulation in the headspace of the bottles were measured with a pressure switch manometer (1 bar, 5% accuracy) until biogas generation ceased. The pressure data obtained was then converted to a volumetric data according to Eq. 3.1 that expresses as the produced volume of biogas at standard conditions for temperature and pressure.

$$V_{STP} = \frac{T_{ST} \cdot P_R \cdot V_R}{P_{SP} \cdot T_R} \qquad \text{Eq. 3.1}$$

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); and T_R is the temperature inside the batch reactor (K).

Accumulated volumetric biogas or methane production was calculated considering the pressure increase in the headspace volume, expressed in standard temperature and pressure conditions (273.15 K, 1.0135 bar). The BMP value is then determined as the net cumulate biogas or methane production (calculated as the sum of the daily volumes and obtained by subtracting the biogas or methane production of the inoculum (blank) from the biogas methane production of each batch reactor containing substrate sample) divided by the mass of VS of substrate added at the initial time ($NmL_{biogas/CH4}/VS_{substrate}$). All BMP tests were performed in triplicates and results are expressed as mean ± standard deviation.

Table 3.6 Characterization of the inocula employed in BMP tests.

BMP test performed in Section	Inoculum TS (g L^{-1})	Inoculum VS (g L^{-1})	Inoculum-to-substrate ratio (ISR) based on VS	Source
4.2.4.1 Valorisation of algal biomass from indoor batch experiments	16.6 ± 0.4	10.2 ± 0.2	2	Anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona).
4.2.4.2 Valorisation of algal biomass from the pilot-PBR	23.9 ± 0.1	14.9 ± 0.1		Anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona).
5.2.3 BMP tests	29.3 ± 0.2	17.7 ± 0.2	2	Anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona).
6.2.4 BMP tests (120 mL bottles)	12.0 ± 0.1	7.5 ± 0.3	2	Anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona).
6.2.4 BMP tests (900 mL bottles)	27.5 ± 0.2	17.3 ± 0.1	2	Anaerobic digesters of the Riu Sec WWTP (Sabadell, Barcelona).

3.3.2. Biogas composition

The biogas composition (carbon dioxide and methane contents) is obtained by sampling gas from the headspace of the vessel with a syringe and the subsequent analysis of the methane content by gas chromatography (Hewlett Packard 5890, Agilent Technologies, Mississauga, Canada). The gas chromatograph was equipped with a thermal conductivity detector and a Supelco Porapack Q column (3 m x 3.2 mm) (Pennsylvania, USA). Helium was the carrier gas (338 KPa), and the oven, injector and detector temperatures were 70, 150, and 180 ºC, respectively. Injection of the samples was done manually with a 100 μL syringe

(VICI PS Syringe A-2, 0.74 mm x 0.13 mm x 50.8 mm).The run time lasted 4.5 min and result in a detection range of 0-100% (v/v).

The methane production (V_{CH4} in mL_{CH4}) is calculated as the volume of biogas multiplied by the methane percentage registered in the gas chromatograph (Eq. 3.2).

$$V_{CH4} = V_{STP} \,.\, CH_4\,(\%) \qquad \text{Eq. 3.2}$$

3.3.3. Volatile fatty acids (VFAs) determination

Before VFAs (formic, acetic, propionic, butyric, and valeric) determination, samples were centrifuged (10 min, 8000 rpm, Beckman Coulter, Avanti J20 XP, USA) and then filtered (Whatman GF/A 0.45 μm nylon syringe filter, GE Healthcare Life Sciences, Buckinghamshire, UK). The analysis was performed with a Dionex 3000 Ultimate HPLC (Thermofisher) equipped with a UV/visible detector. The VFAs content was determined by UV spectroscopy (210 nm). The chromatographic separation was achieved in an ICE-COREGEL 87H3 column (7.8 x 300 mm, Transgenomic, USA), heated at 40 ºC. The eluent was an acidic solution of 320 μL L^{-1} H_2SO_4, pump as a flow rate of 0.5 mL min^{-1}, and the analysis was isocratic. Injection of the samples was carried out with a Dionex autosampler and the injection volume was of 20 μL. Running time of the samples was 45 min.

3.3.4. Analytical procedures

3.3.4.1. Organic matter

Total solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids (VSS) were determined according to Standard Methods

(APHA, 1999). Glass fibre filters (GF/C, Whatman, GE Healthcare, USA) were used in the determination of TSS and VSS. An aliquot of biomass was dried at a constant temperature for 105 °C until constant weight. For volatile solids measurements, the sample was ignited at 550 °C (OBERSAL Mod. 12 PR1300).

3.3.4.2. Optical density

Microalgal growth was followed by measuring optical density (OD) of the algal culture at 680 nm (experiments at Chapter 4 and 6) using a spectrophotometer DR3900 (Hach Lange GmbH, Düsseldorf, Germany).

3.3.4.3. Chemical oxygen demand

Chemical oxygen demand (COD) was analysed by using Hach Lange kits (LCK314, LCK114, and LCK014) and the spectrophotometer DR 3900 (Hach Lange GmbH, Düsseldorf, Germany). Soluble COD (sCOD) was determined using a filtered supernatant (GF/A glass microfibre filters, Whatman, GE Healthcare, USA).

3.3.4.4. Ions

Nitrate ($N\text{-}NO_3^-$) and nitrite ($N\text{-}NO_2^-$) anions were analysed after filtering (45 μm syringe filter, Merck, Germany) using a Dionex ICS-2000 ionic chromatograph (Dionex Corporation, Sunnyvale, USA).

Orthophosphate ($P\text{-}PO_4^{3-}$) concentration was quantified using colorimetric test kits (LCK348 and LCK349) supplied by Hach Lange (Hach Lange GmbH, Düsseldorf, Germany) while ammonium ($N\text{-}NH_4^+$) concentration was determined using colorimetric test kits (Kit N°100683 and 114752) supplied by Merck (Germany).

3.3.4.5. Total carbon and total nitrogen

Total organic carbon (TOC), total carbon (TC), inorganic carbon (IC), and total nitrogen (TN) content were determined after filtration (0.45 μm syringe filter, Merck, Germany) by a multi-N/C 2100S analyser (Analytikjena AG, Germany).

3.3.4.6. Dissolved oxygen, pH, and temperature

Dissolved oxygen concentration (DO), pH, and temperature during PBR experiments in Chapter 4, were determined using a in situ multimeter PCE_PHD 1 (PCE Instruments, Spain). For other experiments, pH was measured by a pH meter (Crison, Spain).

3.4. Kinetic model of methane production

The modified Gompertz equation (Nielfa et al., 2015) was employed to model the biomethane production and calculate kinetic parameters for anaerobic degradation according to Eq. 3.3:

$$P_{net}(t) = P_{max}.\exp\left\{-\exp\left[\frac{R_{max}.e}{P_{max}}(\lambda - t) + 1\right]\right\} \qquad \text{Eq. 3.3}$$

where $P_{net}(t)$ is the net cumulative methane yield (NmL CH_4 g^{-1} VS) at time t, P_{max} is the methane yield potential (NmL CH_4 g^{-1} VS), R_{max} is the maximum daily methane production rate (NmL CH_4 g^{-1} VS d^{-1}), t is the digestion time (d), and λ represents the lag phase (d).

The hydrolysis rate of the anaerobic digestion was evaluated according to Eq. 3.4, adjusting the experimental data to a first-order kinetic model by the least squares method (Martín Juárez et al., 2018):

$$B(t) = B_0\left(1 - \exp^{-K_H.t}\right) \qquad \text{Eq. 3.4}$$

Where B is the cumulative methane yield at time t (mL CH_4 g VS^{-1}), B_0 is the methane yield potential (mL CH_4 g VS^{-1}), t is the digestion time (d), and K_H is the hydrolysis rate constant (d^{-1}), and t is the digestion time (d). The values of the above parameters were estimated by an algorithm developed in MATLAB R2015a (The MathWorks Inc., Natick, MA, USA).

3.5. Statistical analysis

All experiments were performed in triplicate unless otherwise stated. Results are reported as mean ± standard deviation. The experimental data was analysed statistically using the R software (R Core Team, 2020). In each chapter it is specified the statistical method employed to compare the significance of the differences among obtained data.

SECTION 3

Pesticides removal by microalgae-based systems

Section presentation

This section is focused on the removal of pesticides with different polarity by microalgae-based systems. Selected pesticides were first studied in batch at lab-scale to assess the mechanism of degradation in these systems. Moreover, transformation products (TPs) were identified. Later, the removal of selected pesticides and the detection of their TPs was performed in a tubular outdoor pilot-photobioreactor (PBR) to evaluate its performance for wastewater treatment. From a biorefinery paradigm, it is crucial the valorisation of residual algal biomass used in wastewater treatment as a resource to guarantee the economic feasibility of the process. In this sense, the conversion of microalgal biomass into methane is among the most straightforward and suitable technique for energy recovery. Therefore, aiming at algal biomass valorisation after the treatment, the concentrated biomass was anaerobically digested to assess biogas production. First, biogas production from the algal biomass used in the treatment with hydrophobic pesticides is quantified, since they are retained by sorption onto the biomass and can inhibit the process. Later, it was studied the potential of the harvested algal biomass from the pilot-PBR treating polar pesticides in water to generate biogas.

CHAPTER 4.

REMOVAL OF SELECTED PESTICIDES BY MICROALGAE

Part of this chapter was published as:

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Avila, R.; García-Vara, M.; López-García, E.; Postigo, C.; López de Alda, M.; Vicent, T.; Blánquez, P. Evaluation of an outdoor pilot-scale tubular photobioreactor for removal of selected pesticides from water. *Submitted to Science of the Total Environment.*

Abstract

Due to the detrimental effects of pesticides on non-target organisms, different technologies have been considered for their removal. This study assesses the capacity of a microalgae-based system to remove three hydrophobic (chlorpyrifos, cypermethrin, and oxadiazon) and three polar (acetamiprid, bentazone, and propanil) pesticides typically found in freshwater. Degradation of the pesticides was firstly studied individually at batch lab-scale, and an abiotic and a killed control were employed to clarify their removal pathways. After 7 days, total degradation (biodegradation plus photodegradation) contributed to the removal of 55% of oxadiazon, 35% of chlorpyrifos, and 14% of cypermethrin. More than 60% of chlorpyrifos and cypermethrin were removed by sorption onto microalgae biomass as expected due to its hydrophobic nature. Propanil and acetamiprid were totally removed whereas bentazone was not removed. One, four and two tranformation products (TPs) generated by the active microalgae were identified for chlorpyrifos, acetamiprid, and propanil, respectively. Then, the simultaneous removal of selected pesticides was studied in an outdoor pilot-photobioreactor (PBR) fed with synthetic wastewater and operated at a HRT of 8 days. During the steady-state, high removal efficiencies were observed for propanil (99%) and acetamiprid (71%). Evidences from batch experiments suggest that this removal was mainly caused by algal-mediated biodegradation. The concentration of the two detected TPs from acetamiprid raised throughout the 38 days of the study. No TPs of propanil were detected in the PBR. Phycoremediation was coupled with anaerobic degradation of the microalgae biomass containing the retained pesticides by sorption and biomass harvested from the PBR through BMP tests. Anaerobic digestion was not inhibited by the pesticides as verified by the digestion performance. The removal efficiency of the pesticides in the digestate was as follows: chlorpyrifos > cypermethrin > oxadiazon. These results highlight the potential of microalgae-based systems to couple nutrients removal, biomass production, micropollutants biodegradation, and biofuels production.

4.1. Introduction

Concerns about the adverse effects of agrochemicals in the environment are public and widely known. The intensive and broad use of pesticides not only in point sources but also in agriculture, leads to diffuse contamination through spray drift and runoff, which contributes to their distribution in soil, air, and water. Pesticide residues have been found in the environment at concentrations ranging from nanogram (ng) to microgram (μg) per litre (Fenner et al., 2013b). The European Drinking Water Directive 98/83/EC (EC, 1998) and its revision proposal (EC, 2018a) establishes 0.1 μg L^{-1} and 0.5 μg L^{-1} as parametric values for single and total pesticides, respectively, in water intended for human consumption, and the same values are set in the Groundwater Directive 2006/118/EC (EC, 2006).

Current advanced wastewater treatment technologies such as nanofiltration, electro-Fenton oxidation, advanced oxidation processes, and adsorption on activated carbon, among others, generate waste and do not guarantee a cost-effective elimination of pesticides (Zhang and Pagilla, 2010; Plakas and Karabelas, 2012; Zhao et al., 2012; Musbah et al., 2013). On the other side, conventional processes in WWTPs are not efficient in their degradation because they are not designed for these purposes (Köck-Schulmeyer et al., 2013; Le et al., 2017). Specially hydrophobic compounds are removed through sorption and accumulation in waste activated sludge (WAS) (Mailler et al., 2014), which could later be involved in other processes such as anaerobic digestion (AD) or composting. However, if WAS is not previously treated and xenobiotics are not degraded, they can accumulate in the media. Microalgae-based wastewater treatment systems are attractive due to their feasibility to couple the removal of nutrients and pollutants with the production of biofuels and high-added value

bio-products (Muñoz and Guieysse, 2006; Subashchandrabose et al., 2011; Parladé et al., 2018). The potential of microalgae-mediated bioremediation lies in their low cost as well as the absence of an external supply of oxygen and the addition of carbon sources or nutrients in stoichiometric balance, as required by bacteria and fungi (Xiong et al., 2018). Moreover, phycoremediation is a low cost and solar power-driven process (Katiyar et al., 2017; Sutherland and Ralph, 2019). Synergetic interactions between cyanobacteria, microalgae, bacteria, and diverse microorganisms enhance the detoxifying potential of these systems (Liu et al., 2017; Parladé et al., 2018; Xiong et al., 2018).

Recently, bioremediation of organic micropollutants (OMs) by microalgae is attracting a lot of attention, and several studies have already investigated this kind of systems for their removal (Hom-Diaz et al., 2015; Sutherland and Ralph, 2019; Tolboom et al., 2019; Vo et al., 2019; Nguyen et al., 2020). Major mechanisms driving OM removal by microalgae-bacteria based systems are biodegradation, algae-mediated photolysis, bioaccumulation, and sorption (Jonsson et al., 2001; Luo et al., 2015; Matamoros et al., 2015). Diverse studies have addressed the removal of pesticides by pure (Subramanian et al., 1994; Zhang et al., 2011; Jin et al., 2012; Kabra et al., 2014; Kurade et al., 2016) and mixed microalgae cultures (El-Bestawy et al., 2007; Smedbol et al., 2018) in batch mode at lab-scale. Also, many degradation studies were performed under sterile conditions (de Morais et al., 2014; Peng et al., 2014); however, maintaining these conditions when scaling-up could be difficult. The available literature highlights the potentiality of microalgae-bacteria systems to remove OMs from real wastewater at pilot-scale using open systems such as HRAPs (Matamoros et al., 2015; Villar-Navarro et al., 2018; García-Galán et al., 2019) and closed systems such as tubular-PBRs (Hom-Diaz et al., 2017a; García-Galán et al., 2018, 2020; Parladé et al., 2018).

Other works studied the removal of spiked OMs in HRAPs at pilot scale (De Godos et al., 2012; Hom-Diaz et al., 2017b). However, knowledge regarding pesticides removal in outdoor pilot scale tubular PBRs is limited and deserves more attention to provide useful information for their implementation at full-scale. The use of enclosed tubular PBRs for pesticides removal is interesting due to their advantages in comparison with other photobioreactors configurations, i.e. higher light utilization efficiency boosting biomass productivity, better control of operational parameters, better mixing, less evaporative loss, and lower risk of contamination (Molina Grima et al., 1999; Muñoz and Guieysse, 2006). Besides, pollutant losses by volatilisation is less likely in closed tubular PBRs (Muñoz and Guieysse, 2006).

In this study, six pesticides recently found in surface waters of an important agricultural area at levels that may pose a serious hazard for aquatic non-target organisms (maximum concentrations between 4 and 180 $\mu g\ L^{-1}$) (Barbieri et al., 2020), were selected to investigate the removal potential of microalgae systems. The selected pesticides are representative of different chemical classes, modes of action, and polarity. The pesticides under study were classified in this work in two main groups: highly to medium polar pesticides including acetamiprid, bentazone, and propanil; and hydrophobic pesticides including chlorpyrifos, cypermethrin, and oxadiazon.

Regarding polar pesticides, the neonicotinoid insecticide acetamiprid was approved by the EC until 2033 (Regulation (EU) 2018/113) (EC, 2018b) and was included in the European Watch List of substances for Union-wide monitoring (EC) 2018/840 (EC, 2018c), to decide on their consideration as priority substances. The herbicides bentazone (benzothiazinone) and propanil (anilide) are widely used in rice and cereal fields. Regarding their approval by the European

Commission (EC), bentazone was approved for use as an active substance (Regulation (EU) 2018/660) (EC, 2018d). However, even when propanil is not approved by the EU (Regulation (EC) 1107/2009 (EC, 2009a) and (EU) 2019/148 (EC, 2019)), its exceptional use in rice crops during the growing season is annually authorized by the Spanish Government (Spanish Ministry of Agriculture, 2017). These two herbicides have been also detected in dead shellfish organisms (oysters) and their presence was related with markers of tissue damage during DNA strand breakage (Ochoa et al., 2012). The environmental toxicity of propanil has been reported in various non-target animals, including amphibians, birds and fishes (Kanawi et al., 2016). Moreover, due to their low mineralisation rate and moderate sorption capacity to soil (Arena et al., 2018), both herbicides have been frequently detected in groundwater (Malaguerra et al., 2012; Lopez et al., 2015; Kanawi et al., 2016).

The chlorinated organophosphate insecticide chlorpyrifos is employed for pest control and used broadly for a great variety of crops (Cáceres et al., 2007; Aswathi et al., 2019). Despite having a moderate persistence, these pesticides have effects on non-target organisms and are highly toxic to mammals, aquatic invertebrates, freshwater fish (Kamrin, 1997; Kumar et al., 2018), and pollinators, and they have neurotoxic, immunological, and psychological effects in humans (Dar et al., 2019). In January 2020, the European Commission did not renew its approval of chlorpyrifos (Regulation (EU) 2020/18) (EC, 2020b) due to the concerns for human health, including the potential for genotoxicity and developmental neurotoxicity effects. Cypermethrin is a pyrethroid insecticide used in pest control worldwide and its use is approved by the EC (Regulation (EU) 2020/1511) (EC, 2020c). Recently, the toxic effect of cypermethrin on pollinators (Bendahou et al., 1999) and the negative effects on the fertility,

immune system, cardiovascular system, and hepatic metabolism of mammals (Wang et al., 2019) were confirmed. Oxadiazon is a selective pre-emergence oxadiazoline herbicide against annual dicotyledons, which is also used as a post-emergence measure against broad-leaved weeds (Garbi et al., 2006; Huang et al., 2017). The use of oxadiazon was no longer approved by the EC (Regulation (EC) 1107/2009) (EC, 2009a). Despite oxadiazon being toxic to non-target organisms such as aquatic microorganisms (European Food Safety Authority, 2010), it continues to be detected in bodies of water worldwide (Pietrzak et al., 2019). In humans, oxadiazon could be associated with liver cancer and harmful effects on reproductive and endocrine functions (Laville et al., 2006).

In this context, the main objectives of this work were first, to assess the individually removal of the six pesticides by a microalgae consortium at batch lab-scale in the liquid phase and the removal due to sorption in the solid phase for hydrophobic pesticides while identifying their TPs. Secondly, the performance of a semi-continuous pilot-PBR has been evaluated for treatment of synthetic wastewater containing a mixture of pesticides, quantifying the removal of nutrients, pesticides, and the detection of TPs. Finally, with the aim of the valorisation of the algal biomass its conversion to bioenergy was evaluated through AD.

4.2. Materials and methods

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

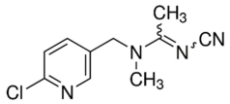
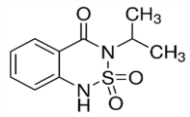
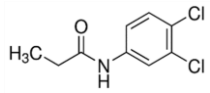
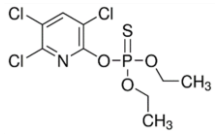
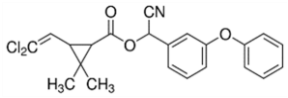
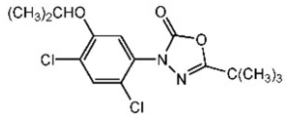
4.2.1. Chemicals and reagents

The pesticides used in the degradation studies include acetamiprid [N-[(6-chloro-3-pyridyl)methyl]-N'-cyano-N-methyl-acetamidine], bentazone [3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide], propanil [3',4'-dichloropropionanilide], chlorpyrifos [(O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate], cypermethrin [α-cyano-3-phenoxybenzyl ester of 2, 2-dimethyl-3-(2, 2-dichlorovinyl) 2-2- dimethyl cyclopropane carboxylate], and oxadiazon [5-terbutyl-3-(2,4- dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2-one]. All pesticides were purchased from Sigma-Aldrich (Steinheim, Germany). Their physical-chemical properties are presented in Table 4.1. Stock solutions of each pesticide in methanol were prepared using the analytical standards and stored in the dark at -20 °C until their use.

Isotopically labelled compounds of the polar pesticides namely acetamiprid-d_3, bentazone-d_6 and propanil-d_5 used as surrogate standards (SS) for quantitative analysis, were purchased from Sigma-Aldrich (Steinheim, Germany). Internal standards of d_{10}-chlorpyrifos and phenoxy-d_5-fenvalerate were also purchased from Sigma-Aldrich (Steinheim, Germany) while d_7-oxadiazon was purchased from LGC standards (Teddington, Middlesex, UK).

HPLC-grade acetonitrile, methanol, and formic acid (98–100%) used in the chemical analysis were supplied by Merck (Darmstadt, Germany). Chloroform was purchased from Carlo Erba (Val De Reuil, Eure, France) and methanol used to prepare stock solutions of each pesticide was purchased from Fisher Scientific (UK). The quality of all solvents was according to organic trace analysis.

Table 4.1 Physical-chemical properties of the target pesticides.

Pesticide (CAS number)	Chemical structure	Chemical family	Molecular formula	Molecular mass (g mol^{-1})	Log K_{ow}	H* (mol m^{-3}.Pa^{-1}) at 25 °C	Water solubility (mg L^{-1}) at 20 or 25 °C
Acetamiprid (160430-64-8)		Neonicotinoid	$C_{10}H_{11}ClN_4$	222.7	0.8 [a]	5.3 10^{-8} [a]	2.95 10^{3} [a]
Bentazone (25057-89-0)		Thiadiazine	$C_{10}H_{12}N_2O_3S$	240.3	-0.46 [b]	7.2 10^{-5} [b]	570 [b]
Propanil (709-98-8)		Anilide	$C_9H_9Cl_2NO$	218.1	2.29 [c]	4.4 10^{-4} [c]	95 [c]
Chlorpyrifos (2921-88-2)		Organophosphate	$C_9H_{11}Cl_3NO_3PS$	350.6	4.7 [d]	0.48 [d]	1.05 [d]
Cypermethrin (52315-07-8)		Pyrethroid	$C_{22}H_{19}Cl_2NO_3$	416.3	6.54 [e]	0.03 [e]	4 10^{-3} [e]
Oxadiazon (19666-30-9)		Oxadiazol	$C_{15}H_{18}Cl_2N_2O_3$	345.2	5.31 [f]	0.1 [g]	0.57 [h]

* H = Henry´s law constant.

[a] European Commission (2004); [b] Galhano et al. (2011); [c] Kanawi et al. (2016); [d] European Commission (2005), [e] Ware (2002), [f] Jurado et al. (2019), [g] Hoque et al. (2007), [h] Iriti et al. (2009)

4.2.2. Pesticide removal by algae at indoor batch experiments

4.2.2.1. Microalgae consortium

The microalgae samples employed at lab scale experiments were collected from a 1 m^3 outdoor tubular semi-open PBR described in previously (section 3.2.2. - Chapter 3), operating under semi-continuous mode and fed once per week using Bold Basal Medium (previously described in Tables 3.4 and 3.5 - Chapter 3) and the microalgae employed in the degradation experiments were taken from the PBR 24 h after feeding.

The consortium was composed mainly of *Chlorella* sp. and *Scenedesmus* sp., morphologically examined using an optical microscope (Zeiss, AixoCam ERc 5s) and characterized according to standard taxonomic literature (Palmer, 1962; Bourrelly, 1966; Komárek and Anagnostidis, 2005). Other microorganisms such as bacteria and protozoa were present, as reported previously for outdoor microalgae-based systems (Vidyashankar and Ravishankar, 2016; Hom-Diaz et al., 2017a; Parladé et al., 2018).

Biomass concentration evolution along experimental time was determined by optical density (OD) and was correlated to TSS concentration according to Eq. 4.1:

$$TSS\ (g\ L^{-1}) = 0.7565 \times OD_{680} - 0.0422 \quad (r^2 = 0.962) \qquad \text{Eq. 4.1}$$

4.2.2.2. Indoor batch experiments

Indoor batch experiments for individual assessment of the degradation of the six target pesticides by the microalgae system and identification of TPs were performed at lab-scale to determine their main mechanisms of removal. The

studied pesticides include three hydrophobic (log K_{ow} > 4) compounds: chlorpyrifos, cypermethrin, and oxadiazon; and three polar pesticides (log K_{ow} < 2.3): acetamiprid, bentazone, and propanil.

Experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of microalgae solution taken from the PBR described previously and the target pesticide at a concentration of 1 mg L^{-1}. The culture in the flasks was mixed and aerated by orbital shaking (100 rpm) in a constant-temperature chamber (25 ± 1 °C) and exposed to light during a 24 h photoperiod using cool white fluorescent tubes (light intensity: 35 μmol photon m^{-2} s^{-1}). Different experimental conditions were assessed in parallel to determine their influence on the removal of the target pesticides:

i. the microalgae reactor containing an active PBR microalgae culture and the target pesticide;
ii. the killed reactor containing the target pesticide and thermally inactivated biomass (PBR microalgae culture autoclaved at 121 ºC, 20 min) to determine the influence of pesticide sorption onto biomass in the overall removal;
iii. the abiotic reactor containing distilled water and the target pesticide (in absence of microalgae) to assess the influence of other losses; and
iv. a blank reactor with the microalgae consortium and without the pesticides was used to assess the effect on biomass growth.

Each condition was studied independently for each pesticide in triplicate, and experiments were run under non-sterile conditions.

Monitoring of the decayed pesticide concentration and identification of TPs was done in samples collected at time 0, and after 2 and 7 days of the start of the

experiment. Pesticide concentration was assessed in the liquid phase for all the pesticides and in the solid phase for the hydrophobic pesticides (chlorpyrifos, cypermethrin, and oxadiazon). In the case of hydrophobic pesticides, at each sampling time a triplicate of each condition was used to perform analytics, while the other reactors remained until the next sampling time.

OD was monitored in the microalgae reactors as an indicator of microalgae biomass growth. TSS were determined from the blank reactor at the beginning and the end of the experiments.

4.2.3. Pesticide removal by algae at outdoor pilot-scale

4.2.3.1. Microalgae consortium

The pilot-PBR (described in previous section 3.2.2. - Chapter 3) was inoculated with 100 L of a consortium of microorganisms from an operating outdoor hybrid PBR (located in the campus Agròpolis, Viladecans, Barcelona, Spain) treating a mixture of wastewater from an agricultural irrigation channel and domestic wastewater from a septic tank as described elsewhere (Díez-Montero et al., 2020b). The inoculum was mainly constituted by a mixed microalgae culture dominated by *Chlorella* sp. and *Scenedesmus* sp., while bacteria and protozoa were also present.

4.2.3.2. Outdoor pilot-scale experiment

The pilot-PBR was operated under semi-continuous mode and fed once a day (from Monday to Friday) with 175 L of modified Mann and Myers medium at 8 days of hydraulic residence time (HRT). The HRT was the same as the solid retention time (HRT = SRT). The employed medium is typically used to cultivate *Scenedesmus* sp. and *Chlorella* sp. (Escapa et al., 2016; Solimeno et al., 2017).

A defined volume of a methanolic solution of each pesticide was daily added to the pilot-PBR influent (125 L per day) to reach a concentration of 5 μg L^{-1} of acetamiprid and 50 μg L^{-1} of propanil. At the initial time the concentrations of acetamiprid and propanil in the PBR were 0.875 and 8.75 μg L^{-1}, respectively. These concentrations were defined based on the maximum levels found for them in a previous related study conducted in the Ebro River Delta (NE Spain) to assess the pesticides of highest concern (Barbieri et al., 2020). Experiments were run between April and June 2019 (during spring and the beginning of the summer season). The length of daylight during this period was ca. 14 h per day. Water loss due to evaporation was compensated daily by adding the influent volume.

4.2.3.3. PBR monitoring

Monitoring includes the analysis of several parameters during the experiment at outdoor pilot-scale. Biomass concentration was determined 3 to 5 times per week through TSS and VSS. Photosynthetic microorganisms were characterized according to standard taxonomic literature and using an optical microscope.

DO concentration, pH, and temperature were measured in situ. A 45 mL sample was taken daily from the PBR effluent and filtered to analyse the following parameters: nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$), total organic carbon (TOC), total carbon (TC), inorganic carbon (IC), total nitrogen TN), and orthophosphate ($P\text{-}PO_4^{3-}$). Analytical methods were previously described (section 3.3.4.4, 3.3.4.5, and 3.3.4.6 - Chapter 3). A volume of 60 mL of sample for pesticides quantification and TP detection was taken once per week before the hydraulic steady-state (n = 5) was reached, and twice per week once the steady-state was reached (n = 5). Sample collection and monitoring at the PBR were performed at the same hour (noon) every sampling day.

4.2.4. Biomass valorisation

4.2.4.1. Valorisation of algal biomass from indoor batch experiments

The potential methane production was evaluated by BMP tests (analytical method is described in previous section 3.3.1. - Chapter 3) using a concentrated microalgae suspension from the indoor batch experiments for removal of hydrophobic pesticides at the final time. A volume of 500 mL of solution from the microalgae reactors was sedimented by gravity for 12 h. Later, the supernatant was removed, and 250 mL of the settled biomass was employed as a substrate for the BMP tests. The assays were carried out using 120 mL glass bottles.

4.2.4.2. Valorisation of algal biomass from the pilot-PBR

Microalgal biomass from the pilot-PBR effluent was harvested by sedimentation, flocculation, and coagulation. Sedimentation by gravity was assessed in 1 L glass tube during 24 h. The flocculant polyDADMAC and the coagulant $FeCl_3$ were used at a dose of 250 ppm and 150 ppm, respectively, to concentrate agal biomass. Both chemicals were previously presented (section 3.1.2. - Chapter 3). Flocculation and coagulation procedures were performed according to Mir-Tutusaus et al. (2017). Harvesting efficiency was calculated considering the increase in biomass concentration at the bottom of the glass tube according to Eq. 4.2:

$$\text{Harvesting efficiency (\%)} = \frac{(TS_f - TS_i)}{TS_i} * 100 \qquad \text{Eq. 4.2}$$

where initial TS (TS_i) and final TS (TS_f) is the biomass concentration before and after the harvesting, respectively.

Biogas potential from the PBR harvested biomass was determined through BMP tests (analytical method is described in previous section 3.3.1. - Chapter 3). Reactors employed in the BMP tests were 120 mL glass bottles.

4.2.5. Analytical methods

Samples were prepared for the subsequent analytical quantification of the pesticides and identification of the TPs as followed:

i. for polar pesticides (acetamiprid, bentazone, and propanil) in the liquid phase at indoor batch experiments, 4 mL samples were taken from the flasks at each established sampling time, and 1.5 mL of the supernatant obtained after centrifugation (10000 rpm, 4 min) was added to vials containing 75 μL of the corresponding surrogate standard at a concentration of 10 μg mL^{-1}. Samples were frozen at -20 °C until their analysis.

ii. In the case of the hydrophobic pesticides (chlorpyrifos, cypermethrin, and oxadiazon) at indoor batch experiments, at each sampling time (days 0, 2, and 7) the total volume was removed from three reactors. To determine the pesticide concentration in the liquid phase, a fibre glass filter (1.6 μm, GF/A, Whatman) was used to separate biomass from the liquid phase and then 50 mL of the filtrate were spiked with internal standards (d_{10}-chlorpyrifosorpyrifos, phenoxy-d_5-fenvalerate, and d_7-oxadiazondiazon) to a final concentration of 0.1 mg L^{-1}. The liquid samples were collected in amber glass tubes, while the biomass cake retained by the filter was collected with a spatula and stored inside aluminium bags to evaluate later the concentration of pesticide in the solid or biomass phase. Additionally, to determine the pesticide sorption or retention during the filtration, the

pesticide concentration in the employed filters was determined. All samples were frozen at -20 °C until analysis.

iii. For experiments at outdoor pilot-scale, 60 mL samples withdrawn from the PBR were centrifuged (7000 rpm, 10 min). A volume of 40 mL of the supernatant was added to a vial containing the deuterated analogues of the pesticides.

Quantification of the pesticides at the liquid and solid phases and TPs identification were performed by the research group from the Institute of Environmental Assessment and Water Research (IDAEA-CSIC) involved in the project BECAS (CTM2016-75587). Detailed analytical procedures are presented in Appendix A.

In the pilot-PBR, the volumetric organic load and the volumetric load of nutrients (mg L^{-1} d^{-1}) was calculated as shown in Eq. 4.3:

$$\text{Nutrient volumetric load} = \frac{Q * N}{V} \qquad \text{Eq.4.3}$$

where Q is the flow (L d^{-1}), N is the TOC for the organic load and the nutrient concentration (N-NO_3^- and P-PO_4^{3-}) in the influent (mg L^{-1}), and V (L) is the PBR volume.

Biomass production in the pilot-PBR (g VSS L^{-1} d^{-1}) was estimated according to Eq. 4.4:

$$\text{Biomass production} = \frac{Q * VSS}{V} \qquad \text{Eq.4.4}$$

where Q is the flow (L d^{-1}), VSS is the biomass concentration in the pilot-PBR (g L^{-1}), and V (L) is the PBR volume.

4.2.6. Statistical analysis

The experimental data from batch experiments were statistically analysed using one-way ANOVA of repeated measures. A Bonferroni post-hoc test was applied when significant differences were identified ($p < 0.05$). P-values represent Bonferroni corrected significance levels. Data from BMP tests were statistically analysed employing one-way ANOVA, differences were considered significant at p values below 0.05.

4.3. Results and discussion

4.3.1. Degradation of polar pesticides and TPs identification at indoor batch experiments

Polar pesticides are characterized by a low K_{ow}, high water solubility, and low adsorption to solids (Table 4.1) and, consequently, their presence and that of their TPs was only monitored in the liquid phase. As previously reported in other studies (Hom-Diaz et al., 2015), to discriminate between potential degradation mechanisms, an abiotic control containing the pesticide in the absence of microalgae was set up to determine the possible influence of photodegradation and volatilisation among other abiotic processes on pesticide removal, and a killed control containing autoclaved (121 ºC, 20 min) dead biomass was performed to quantify the potential removal of the target pesticides by sorption.

Removal of acetamiprid, bentazone, and propanil throughout the experimental time under abiotic, killed, and microalgae conditions is presented in Fig. 4.1. The relation between the pesticide concentration in the liquid phase at a certain time with the initial pesticide concentration was used to calculate the percentage of pesticide remaining in solution. Results showed that 57.5% of acetamiprid

remained at day 2 in the microalgae batch reactors ($p < 0.01$), and the pesticide was totally removed by the microalgae consortium at day 7 ($p < 0.05$) (Fig. 4.1). Its low K_{ow} and high solubility in water (Table 4.1) make biodegradation and photodegradation feasible removal pathways. However, acetamiprid concentration was not altered by abiotic factors. The killed reactor showed slight adsorption (8.1%) on day 2, and later desorption (at day 7) of the compound as it was reported by other authors (Bai and Acharya, 2016). The higher water solubility of the pesticide implies a higher occurrence in the aqueous phase, hence a higher bioavailability for biodegradation (Blum et al., 2018).

Regarding bentazone, its concentration in the microalgae reactors remained constant, and thus, no degradation occurred throughout the 7 days (Fig. 4.1). The same behaviour was also detected in the killed reactors. However, in the absence of microalgae (abiotic control), a slight removal occurred (1.9% and 3.0% at day 2 and 7, respectively), although it was not statistically significant ($p > 0.05$). Direct photolysis of bentazone has been reported as an important removal pathway of this pesticide in water (Al Housari et al., 2011; Carena et al., 2020; Ferrando and Matamoros, 2020). The low photolysis effect on bentazone removal in the abiotic control may be due to the absence of organic matter to produce oxidant species in the presence of light (Wei et al., 2020) and the reduced light penetration in the water when the biomass is present.

Conversely, propanil was totally removed after 2 days ($p < 0.01$) in the microalgae batch reactors (Fig. 4.1). Despite propanil direct and indirect photolysis has been reported (Kanawi et al., 2016), it was not observed in the abiotic reactors. These results are consistent with studies reporting that propanil was not degraded in autoclaved water after 100 d and its main metabolite 3,4-dichloroaniline (3,4-DCA) was no detected, indicating that propanil is not susceptible to chemical

hydrolysis (Milan et al., 2012). Also, considering that propanil concentration in the killed reactors remained constant over the 7 days, the degradation could be attributed to biodegradation by the microalgae active consortium. In surface water systems, propanil is not sorbed to suspended sediment and particulate matter, nor volatilize (Kanawi et al., 2016). Propanil biodegradation has been described as a significant route of degradation in aquatic systems (Kanawi et al., 2016).

Scarce literature is available regarding the removal of these pesticides by microalgae-bacteria consortia. For instance, Zhou et al. (2014) reported 54% removal of bentazone employing a pure strain (*C. vulgaris*). John et al. (1982) observed propanil degradation by pure green algae (such as *Chlamydomonas reinhardtii, Tolypothrix tenuis,* and *UIothrix fimbriata*) and cyanobacteria (such as *Anacystis nidulans* and *Anabaena cylindrica*). More recently, Ferrando and Matamoros (2020) reported greater removal efficiencies for antibiotics (i.e., sulfacetamide, sulfamethazine and sulfamethoxazole) than for hydrophilic pesticides (i.e., bromacil, atrazine, diuron, bentazone, and mecoprop), and among them a 15% removal of bentazone when using microalgae-bacteria systems in batch (10 incubation days and 8 $\mu g\ L^{-1}$). In the present work, bentazone resulted practically unaffected while acetamiprid and propanil were completely removed, at higher concentrations (1 $mg\ L^{-1}$).

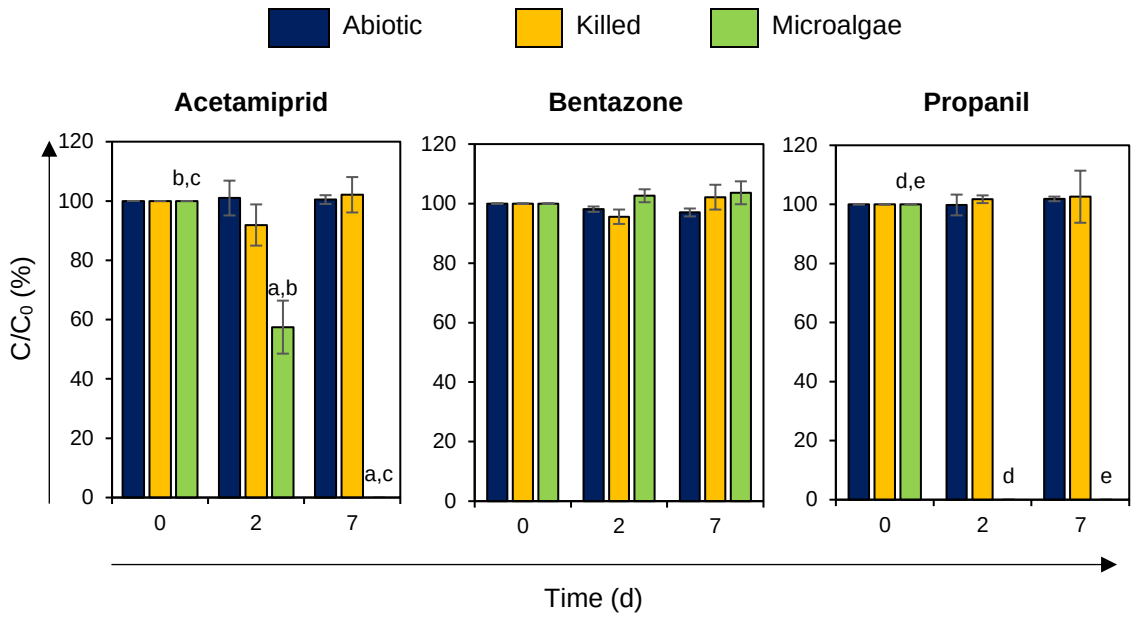


Figure 4.1 Removal of acetamiprid, bentazone, and propanil throughout the experimental time under abiotic, killed, and microalgae conditions in lab-scale batch degradation assays, expressed as the percentage of initial amount remaining in solution. Error bars indicate the relative standard deviation of the mean (n = 3, except for propanil under killed conditions where n = 2). Statistically significant differences when comparing the mean pesticide content in the reactors along time are indicated by letters as follows: a = $p < 0.05$; b, d, and e = $p < 0.01$, and c = $p < 0.001$.

Main TPs generated during the degradation of these pesticides by microalgae are shown in Table 4.2. As expected, no TPs were found in the bentazone samples, as no degradation occurred. In total 6 TPs were identified in the investigated samples. Logical tentative structures were proposed for four of them with a confidence level of 3 according to Schymanski scale (Schymanski et al., 2014), since they could not be confirmed with the analysis of pure standard solutions, and with a confidence level of 5 for the other two TPs, because MS^2 data were either missing or did not provide additional evidence of the structure.

The four TPs identified for acetamiprid were N2-carbamoyl-N1-[(6-chloro-3-pyridyl)methyl]-N1-methylacetamidine (TP240), N-((6-chloropyridin-3-

yl)methyl)-N-methylacetimidamide (TP197), N-[(6-chloropyridin-3-yl)methyl]methylamine (TP156), and 6-chloronicotinic acid (TP157). All of them remained in solution after 7 days of treatment showing an increasing trend by the end of the experiment (Fig. 4.2). TP240 is believed to be formed after addition of one water molecule to the acetamiprid structure, TP197 after the loss of the cyano group, TP156 after the loss of the N-ethylidenecynamide group, and TP157 after the loss of the group attached to the 2-chloropyridine ring and subsequent carboxylation. The Joint FAO/WHO Meeting on Pesticides Residues (JMPR) identified the previous TPs in the metabolic breakdown of acetamiprid in plants after foliar application (FAO and JMPR, 2005).

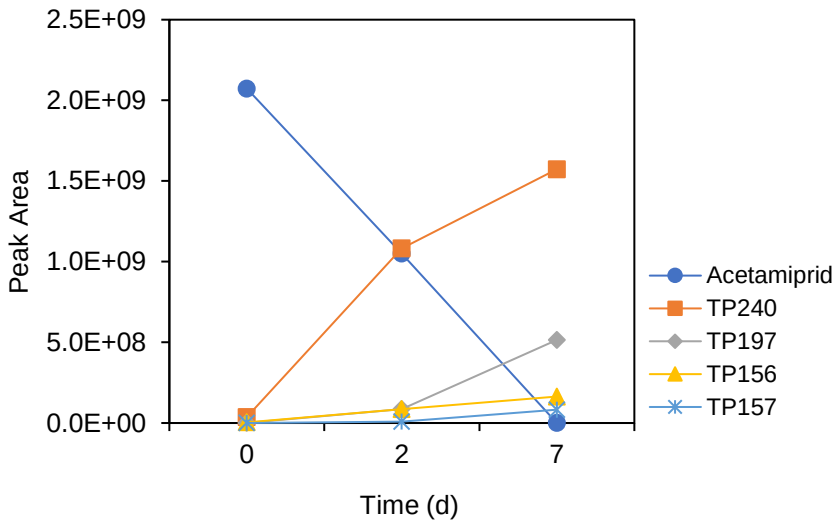
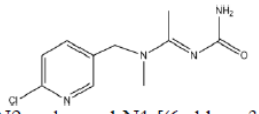
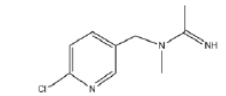
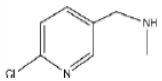
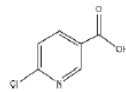
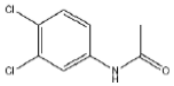
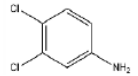


Figure 4.2 TPs identified for acetamiprid degradation by microalgae at indoor batch conditions.

Table 4.2 TPs formed during acetamiprid and propanil degradation process at indoor batch experiments.

Pesticide	TPs	t_R (min)	HESI mode	Full Scan				MS/MS				Suspect identity (Confidence level)
				m/z	Formula	RDB	Δm (ppm)	*m/z*	Formula	RDB	Δm (ppm)	
Acetamiprid	TP240	5.6	+	241.0862	$C_{10}H_{14}ON_4Cl$	5.5	4.7	224.0595	$C_{10}H_{11}ClN_3O$	6.5	5.1	 N2-carbamoyl-N1-[(6-chloro-3-pyridyl)methyl]-N1-methylacetamidine (CL 3)
								198.0804	$C_9H_{13}ClN_3$	4.5	5.8	
								181.0534	$C_9H_{10}ClN_2$	5.5	6.5	
								157.0538	$C_7H_{10}N_2Cl$	3.5	5.8	
								128.0271	C_6H_7ClN	3.5	7.1	
								126.0115	C_6H_5ClN	4.5	4.7	
	TP197	5.4	+	198.0804	$C_9H_{13}ClN_3$	4.5	5.7	198.0804	$C_9H_{13}ClN_3$	4.5	5.7	 N-((6-chloropyridin-3-yl)methyl)-N-methylacetimidamide (CL 3)
								157.0536	$C_7H_{10}ClN_2$	3.5	5.8	
								128.0270	C_6H_7ClN	3.5	6.5	
								126.0114	C_6H_5ClN	4.5	7.0	
	TP156	4.6	+	157.0536	$C_7H_{10}N_2Cl$	3.5	5.9	157.0536	$C_7H_{10}ClN_2$	3.5	5.9	 N-[(6-chloropyridin-3-yl)methyl]methylamine (CL 3)
								126.0114	C_6H_5ClN	4.5	7.2	
	TP157	7.5	+	158.0013	$C_6H_5O_2NCl$	4.5	6.3	158.0013	$C_6H_5ClN_2O$	4.5	6.3	 6-chloronicotinic acid (CL 3)
Propanil	TP203	8.7	+	203.9991	$C_8H_8ONCl_2$	4.5	6.6					 N-(3,4-dichlorophenyl)acetamide (CL 5)
	TP161	8.5	+	161.9885	$C_6H_6NCl_2$	3.5	7.9	161.9885	$C_6H_6NCl_2$	3.5	7.96	 3,4-dichloroaniline (CL 5)

t_R = chromatographic retention time; HESI = heated-electrospray ionization; Δm = mass measurement error; RDB = ring and double bound equivalents; CL = confidence level according to Schymanski scale (Schymanski et al., 2014).

In the case of propanil, two TPs, namely, N-(3,4-dichlorophenyl)acetamide (TP203) and 3,4-dichloroaniline (3,4-DCA) (TP161) were identified. Both TPs remained in solution after 7 days of treatment; however, both TPs can be considered as an intermediate by-product due to the decreasing trend observed by the end of the experiment (Fig. 4.3). It has been reported that the metabolite 3,4-DCA has a longer half-life than its parent compound (Milan et al., 2012). Thus, complete mineralisation of propanil could be expected at longer degradation times. The formation of these TPs can be explained after the loss of a methyl group (TP203), and after the loss of a propaldehyde group (TP161).

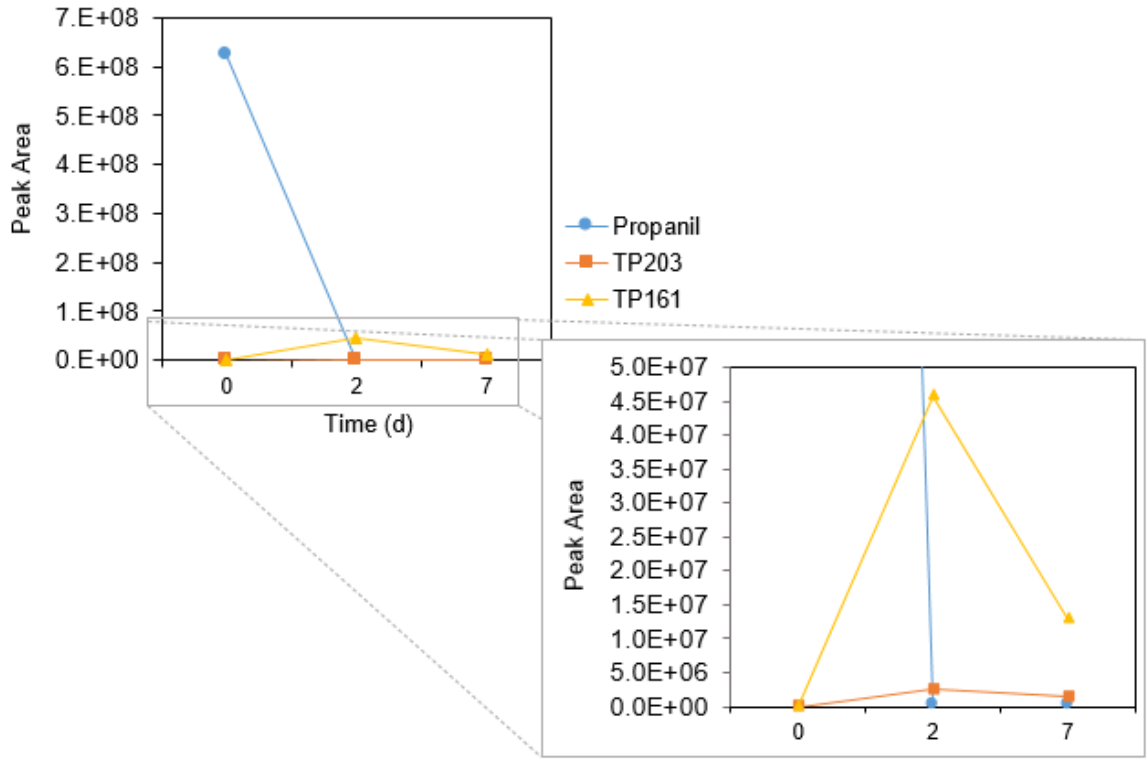


Figure 4.3 TPs identified for propanil degradation by microalgae at indoor batch conditions.

Figure 4.4 showed the evolution of biomass concentration in microalgae and blank reactors. In the case of the microalgae reactors containing bentazone, biomass concentration increased by 25%. However, biomass concentration was

reduced by 28% and 23% for reactors containing acetamiprid and propanil, respectively. However, no statistical differences were identified between the mean values of biomass in blank and microalgae reactors for each pesticide at each sampling time ($p > 0.05$, except for acetamiprid at t = 7 d). According to ecotoxicological data, chronic effects were reported for diverse algal mono-cultures after 72-h exposure to propanil being *Nostoc muscorum* the most sensitive (EC_{50} = 80 μg L^{-1}), followed by *Tolypothrix tenuis* (EC_{50} = 130 μg L^{-1}), *Scenedesmus acutus* (EC_{50} = 290 μg L^{-1}), and *Chlorella vulgaris* (EC_{50} = 5980 μg L^{-1}) (NORMAN, 2021). For bentazone, the acute 72-h EC_{50} value is 10.1 mg L^{-1} (for *Anabaena*) (University of Hertfordshire, 2020). In the case of acetamiprid, ecotoxicological data indicate acute 72-h EC_{50} values > 1.3 mg L^{-1} (for Anabaena sp.) and > 98.3 mg L^{-1} (for *Scenedesmus subs*picatus) (Bureau REACH, 2018). As previously explained (section 4.3.1.), the comparison between these ecotoxicological data and results in the present study is not viable since an algae-based consortium was employed in this work.

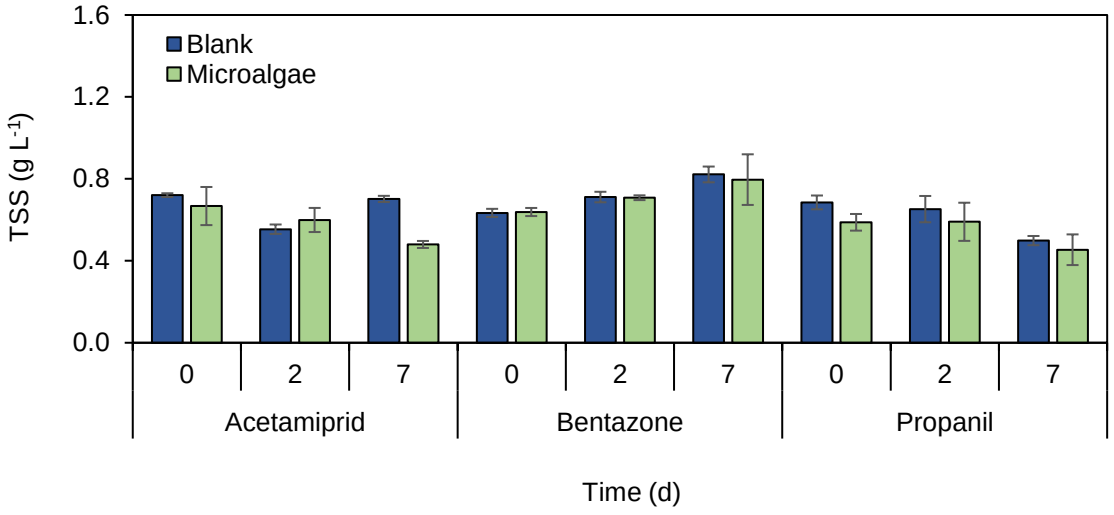


Figure 4.4 Evolution of TSS concentration in microalgae reactors with acetamiprid, bentazone, and propanil. Error bars indicate standard deviation of the mean (n = 3).

4.3.2. Degradation of hydrophobic pesticides and TPs identification at indoor batch experiments

Hydrophobic compounds have a tendency for sorption on solids and biosolids in concordance with their large log K_{ow} value and low solubility in water. They present a high solubility in lipids and an affinity for the microalgae cell wall (Rioboo et al., 2002). For that reason, they were studied in the liquid and solid phases. The biodegradation potential of the pesticides by the microalgae consortium was evaluated by studying their behaviour under the following conditions: microalgae reactor, killed control, and abiotic control.

Figure 4.5 shows the evolution of the hydrophobic pesticide distribution in the liquid and solid phases in the microalgae reactors. The remaining fraction of the target pesticide in the liquid and in the solid phases through time was calculated as the relation between the residual quantity of the pesticide in the liquid or solid phase and the total initial amount of pesticide in both phases. The solid phase included the biomass contained in the reactor and the filter used for biomass separation. Sorption of the target pesticide onto the filter was determined for the microalgae reactors (1323.3 ± 131.3 ng of chlorpyrifos, 11509.7 ± 3027.4 ng of cypermethrin, and 920.3 ± 235.3 ng of oxadiazon), and these values were also applied to killed reactors.

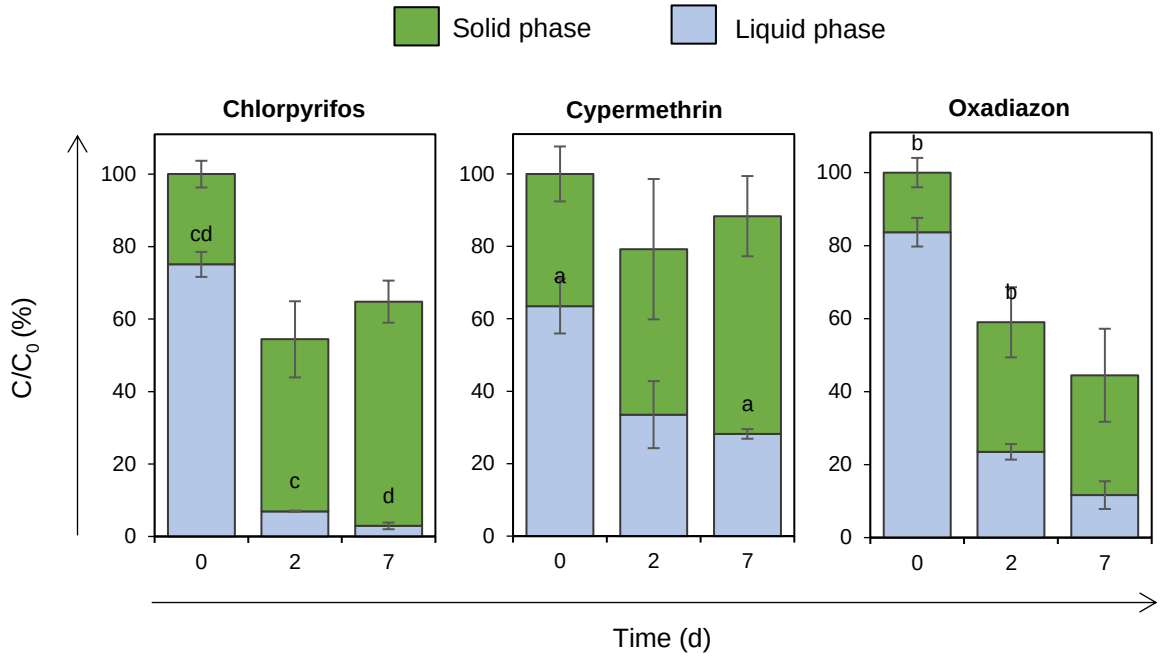


Figure 4.5 Remaining fractions of chlorpyrifos, cypermethrin and oxadiazon concentration in the solid and liquid phases during time in microalgae reactors. Remaining fractions are expressed according to the pesticide concentration at t = 0 d. Error bars indicate standard deviation of the mean (n = 3). Significant statistically differences when comparing the mean pesticide content in the liquid and solid phases respectively during time are indicated by letters as follows: a, b, c, and d ($p < 0.05$).

At the initial time, the three pesticides were mainly detected in the liquid phase, where they were added; however, the residual pesticide distribution changed gradually with time in the solid phase due to sorption onto the algae biomass. The concentration of chlorpyrifos in the liquid media was reduced by 11 times after 2 days. The oxadiazon concentration in the solid phase remained fairly constant from day 2 to day 7 (36% to 33%). Oxadiazon was gradually removed from the liquid phase due to sorption and degradation (by day 7, its concentration in the liquid phase decreased by 8-fold). At day 7, the pesticide retained by the solid phase accounted for 62%, 60%, and 33% of the chlorpyrifos, cypermethrin, and oxadiazon, respectively (Fig. 4.5), highlighting the role of sorption in

pesticide removal from the liquid phase. In algae-based systems, the microbiology community acts as a biosorbent (Gadd, 2009). Biosorption efficiency depends on the hydrophobicity and functional groups of the pollutant and sorbent (Blánquez et al., 2020). The cell wall of cyanobacteria, microalgae, and bacteria contains polysaccharides and proteins that can provide adsorption sites for the xenobiotics (Fomina and Gadd, 2014). For instance, sorption onto the solid phase was also observed for pyrimethanil (a fungicide with high log K_{ow} value) when it was in contact with microalgae (Dosnon-Olette et al., 2010).

Table 4.3 shows the distribution of the pesticides in the killed and abiotic controls after 7 days of exposure. The remaining fraction of chlorpyrifos in the solid phase of the killed control at the end of the experiment (45.9 ± 11.5%) was lower than that in the microalgae reactor (61.9 ± 5.8%). With regard to oxadiazon, it showed a higher sorption capacity of the inactivated biomass (63.8 ± 32.1%) in comparison with the active biomass (32.8 ± 12.7%). The cell wall of microalgae is constituted by an aggregation of polymers with functional groups over its surface (such as carboxyl, phosphoryl, and amine) that give it a negative charge (Xiong et al., 2018). Bearing in mind that the structure of the microalgae surface is modified after heat treatment (Schwede et al., 2013) for inactivation, sorption on the active biomass (microalgae reactor) could be different from sorption on the inactivated biomass (killed control). Furthermore, the absence of an active enzyme system after the biomass has been thermally inactivated (Luo et al., 2015) could lead to dissimilarities when both conditions are compared. Additionally, some authors have reported a major contribution of dead algal cells in the photolysis of xenobiotics (Matsumura and Esaac, 1979; Luo et al., 2015). Luo et al. (2015) proved that chlorophyll is the major active substance in dead cells and generates singlet oxygen that acts as a catalyst stimulating and accelerating the

photodegradation of xenobiotics under light irradiation, which could also explain the removal differences in both conditions. Concerning cypermethrin, sorption onto solid phase was slightly higher for killed reactors (Table 4.2) than for microalgae reactors (46% by day 2 and 60% by day 7).

Table 4.3 Distribution of chlorpyrifos, cypermethrin, and oxadiazon in abiotic (liquid phase) and killed (liquid + solid phases) controls. Mean and standard deviation (n = 3) represent the percentage of initial concentration of the pesticides along time. Significant statistically differences between mean pesticide content in the liquid and solid phases respectively in each condition along time, are indicated by letters as follows: a ($p < 0.05$); b, c ($p < 0.01$) and d ($p < 0.001$).

Pesticide	**Time (d)**	**Killed control**		**Abiotic control**
		Liquid phase (%)	**Solid phase (%)**	**Liquid phase (%)**
Chlorpyrifos	0	14.3 ± 4.6	85.7 ± 4.6 [a]	100.0 ± 0.0
	2	8.4 ± 1.4	102.5 ± 9.7 [a]	83.7 ± 4.1
	7	10.6 ± 2.4	45.9 ± 11.5	83.7 ± 4.5
Cypermethrin	0	79.6 ± 3.0 [bc]	20.4 ± 5.2 [d]	100.0 ± 0.0
	2	29.5 ± 1.9 [b]	50.0 ± 5.2 [d]	79.2 ± 12.2
	7	31.2 ± 4.2 [c]	64.2 ± 14.1	72.9 ± 12.0
Oxadiazon	0	34.7 ± 6.1	65.3 ± 6.3	100.0 ± 0.0
	2	36.2 ± 6.9	84.6 ± 46.4	122.6 ± 25.3
	7	37.0 ± 9.8	63.8 ± 32.1	104.1 ± 6.4

The removal yields at the final time for the three hydrophobic pesticides are presented in Table 4.4. Removal from the liquid phase was calculated assuming that the pesticide was in the liquid phase initially, and the removal percentage was calculated based on the remaining pesticide in the liquid phase. The total degradation is the difference between the removal from the liquid phase and the

sorption onto the solid phase. Photodegradation and other abiotic factors represent the pesticide removal percentage of the abiotic control (Table 4.3) to analyse the influence of abiotic processes on pesticide removal. Biodegradation by the microalgae consortium was defined as the difference between total pesticide degradation and photodegradation and other abiotic factors.

Table 4.4 Pesticide removal from the liquid phase, sorption onto the solid phase, total degradation, photodegradation, and biodegradation by the microalgae consortium at t = 7 d. Mean and standard deviation (n = 3) are represented by percentages based on the initial concentration of each pesticide.

Pesticides	**Chlorpyrifos**	**Cypermethrin**	**Oxadiazon**
Removal from liquid phase (%)	97.3	73.9	88.4
Sorption to solid phase (%)	61.9 ± 5.8	60.1 ± 11.1	32.8 ± 12.7
Total degradation (%)	35.4 ± 7.1	13.8 ± 2.7	55.5 ± 15.6
Photodegradation and other abiotic factors (%)	16.3 ± 4.5	27.1 ± 12.0	0.0 ± 0.0
Biodegradation (%)	19.1	*	55.5

* For cypermethrin, abiotic removal was higher than total degradation, hence biodegradation could not be quantified.

According to the results shown in Fig. 4.5 and Table 4.4, oxadiazon showed the highest total degradation (photodegradation or another mechanism such as biodegradation) by day 7 (55.5 ± 15.6%) in comparison with chlorpyrifos (35.4 ± 7.1%) and cypermethrin (13.8 ± 2.7%). Oxadiazon removal increased with time from 41.0% at day 2 to 55.5% at day 7. In the case of chlorpyrifos, the total degradation after day 2 was higher (45.6 ± 10.6%) than the value at day 7, which could be attributable to pesticide adsorption on the glass flasks and its later desorption, as was reported previously by others authors considering

hydrophobic pesticide removal (Bai and Acharya, 2016). The difference in cypermethrin degradation between day 2 (20.8 ± 15.2%) and day 7 (13.8 ± 2.7%) could be associated with the same cause.

As shown in Table 4.4, removal from the liquid phase included the contribution of pesticide sorption to the solid phase plus the total pesticide degradation. In this sense, chlorpyrifos exhibited the greatest removal from the liquid media (97.3%) after 7 days of treatment with the microalgae consortium, followed by oxadiazon (88.4%) and cypermethrin (73.9%). Regarding photodegradation, chlorpyrifos removal in the absence of the microalgae consortium (abiotic control) was 16.3 ± 4.5%, suggesting a slight influence of photolysis and other abiotic factors in pollutant removal. It has been reported that chlorpyrifos has a limited potential for photodegradation by natural sunlight irradiation in water (Muhamad, 2010; Affam et al., 2014). With regard to cypermethrin, 27.1 ± 12.0% was removed from the abiotic control. For oxadiazon, no contribution of abiotic factors was detected in the removal, suggesting that the pollutant elimination was influenced by neither photodegradation nor volatilisation. Moreover, it has been reported that oxadiazon presents a medium to low volatility (European Food Safety Authority, 2010). Bearing in mind that biodegradation was assumed to be the difference between total pesticide removal and photodegradation (based on Hom-Diaz et al. (2015)), it can be suggested that oxadiazon removal can be mainly attributed to biodegradation (55.5 ± 15.6%) and sorption (32.8 ± 12.7%). Regarding cypermethrin, biodegradation was not identified, and the main removal mechanisms seemed to be sorption (60.1 ± 11.1%) and photodegradation (27.1 ± 12.0%).

The results indicate that the total pesticide degradation in the microalgae reactors was higher for oxadiazon, followed by chlorpyrifos and cypermethrin,

respectively. Biodegradation by the microalgae consortium contributed to total degradation by 19.1% for chlorpyrifos and 55.5% for oxadiazon. Moreover, algal-mediated photolysis seemed to have an effect in chlorpyrifos and cypermethrin losses (16.3% and 27.1%, respectively), because oxygen and oxidant species generated by photosynthetic microorganisms are capable of inducing indirect photolysis (Fatta-Kassinos et al., 2011). In the microalgae reactors, pesticide retention by sorption to the solid phase was similar for chlorpyrifos and cypermethrin (61.9% and 60.1%, respectively) but lower for oxadiazon (32.8%). Overall, efficient removal from the liquid phase was accomplished for the three pesticides (chlorpyrifos > oxadiazon > cypermethrin).

Given that the identification of TPs is directly associated with microalgae consortium activity, they were analysed in the microalgae reactors. As chlorpyrifos has low solubility in water, its bioavailability for microbial degradation and its use as a carbon source could be limited (Liu et al., 2001; Singh et al., 2003). However, O,O-diethyl thiophosphate (DETP) has been identified as a TP of chlorpyrifos in microalgae reactors. The corresponding retention times, measured masses, molecular formulae (calculated on the basis of their accurate mass measurements and the observed isotopic patterns), relative mass measurement errors, and degree of unsaturation, expressed as ring and double bound equivalents, for this TP are summarised in Table 4.5. Higher intensities of this metabolite were observed at day 7, suggesting that it was produced gradually as degradation proceeded. Chlorpyrifos degrading microorganisms are able to produce organophosphate hydrolysing enzymes that hydrolyse the P–O bond, leading to the corresponding dialkyl phosphate (DETP) and corresponding aryl alcohol (3,5,6-trichloro-2-pyridinol, known as TCP) (John and Shaike, 2015). This fact suggests that DETP identified in this study is a TP of chlorpyrifos by

microalgae degradation. Studies examining chlorpyrifos microbial degradation have focused mainly on bacteria and fungi (Mallick et al., 1999; Singh et al., 2004; Chen et al., 2012; Chishti et al., 2013; Abraham and Silambarasan, 2016; Kumar et al., 2018), but few studies have assessed the capability of microalgae and cyanobacteria to degrade this compound. In general, it has been reported that degrading bacteria hydrolyse chlorpyrifos, producing two main metabolites: DETP and TCP (Liu et al., 2001; Singh and Walker, 2006). In this study, TCP was not identified, suggesting further conversion into other compounds or complete mineralisation, as Barathidasan et al. (2014) found with a *Phanerochaete chrysosporium* fungal strain which could use TCP as a carbon source and completely mineralise chlorpyrifos.

Table 4.5 Identified TP with parent ion and confirmation ions' nominal mass, retention time, measured mass, mass error, molecular formula, and RDB for chlorpyrifos.

Identified TP	Nominal mass	Retention time (min)	Measured mass (m/z)	Mass error (ppm)	Molecular formula	RDB
DETP	171	4.19	171.0239	$C_4H_{12}O_3PS$	-0.222	-0.5
	115		114.9616	H_4O_3PS	2.716	-0.5
	143		142.9928	$C_2H_8O_3PS$	1.204	-0.5
	97		96.9512	H_2O_2PS	4.713	0.5
	81		80.9744	H_2O_3P	7.200	0.5

In this work, no TPs were identified in association with oxadiazon degradation, which could indicate that the molecule was mineralised. This assumption is coherent with the high biodegradation detected (55.5%). Some authors have demonstrated an ability of other microorganisms to biodegrade oxadiazon. For instance, Pseudomonas fluorescens CG5 isolated from a soil contaminated with herbicides was able to use oxadiazon as a carbon source and further catabolise

the compound, obtaining a few toxic metabolites such as indole, benzoic acid, and trimethyl benzene, including dehalogenation (Garbi et al., 2006).

Most of the published studies regarding cypermethrin biodegradation are focused on bacteria and fungi (Mir-Tutusaus et al., 2014; Pankaj et al., 2016; Gangola et al., 2018; Bhatt et al., 2020) and not on microalgae. In general, it has been reported that hydrolysis of the ester bond is the main degradation pathway of cypermethrin, producing the corresponding alcohol (2-hydroxy-2(3-phenoxyphenyl)acetonitrile, known as CPBA) and acid (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylic acid, known as DCCA) (Bhatt et al., 2020). CPBA is further transformed, leading to small molecular weight aliphatic compounds with intermediate compounds such as 3-phenoxybenzoic acid (PBA) (Bhatt et al., 2020). These aliphatic compounds include oleic acid (Pankaj et al., 2016; Bhatt et al., 2020), 2-pentadecanone (Bhatt et al., 2020), acetic acid (Gangola et al., 2018), decanoic acid (Gangola et al., 2018), 1-dodecanol (Pankaj et al., 2016), and isopropyl myristate (Pankaj et al., 2016). Likewise, photo-transformation products of cypermethrin have also been reported previously, with DCCA and PBA being the main photoproducts (Jones, 1995). In this work, no TPs related to microalgae biodegradation of cypermethrin were identified. This result is consistent with the low observed total degradation (13.8%), which indicates that the main removal mechanism of cypermethrin in the liquid phase is sorption onto the microalgae. The low degradation yields probably led to poor formation of TPs and hence no detection.

The physiological status of microalgae is in concordance with biomass growth, and cell dry weight is considered an integral parameter of cellular metabolism (Qian et al., 2018). Thus, the evolution of biomass concentration in microalgae reactors could be an indicator of the toxicity and inhibition caused by the

pesticide. As shown in Fig. 4.6, the biomass concentration in the microalgae reactors increased by 19%, 8%, and 15% for chlorpyrifos, cypermethrin, and oxadiazon after 7 days, respectively. Moreover, no statistical differences were identified between the mean values of biomass in blank and microalgae reactors for each pesticide at each sampling time ($p > 0.05$, except for oxadiazon at t = 0 d). These results could suggest that the pesticide concentration was not toxic for the microalgae consortium. On the other hand, ecotoxicological data indicate that the acute 72-h EC50 values of chlorpyrifos, cypermethrin, and oxadiazon are 0.48 mg L^{-1} (for unknown microalgae species), > 0.0667 mg L^{-1} (for *Pseudokirchneriella subcapitata*), and 0.004 mg L^{-1} (for *Scenedesmus subspicatus*), respectively (University of Hertfordshire, 2020). Bearing in mind that these toxicological studies were performed using pure microalgae species, it is not possible to relate them with the microalgae consortium employed in the present work. The advantages of working with a microalgae consortium are the synergetic interactions between microorganisms that enhance the robustness of the system and overall removal efficiency (Renuka et al., 2015; Ramanan et al., 2016; Vidyashankar and Ravishankar, 2016; Xiong et al., 2018).

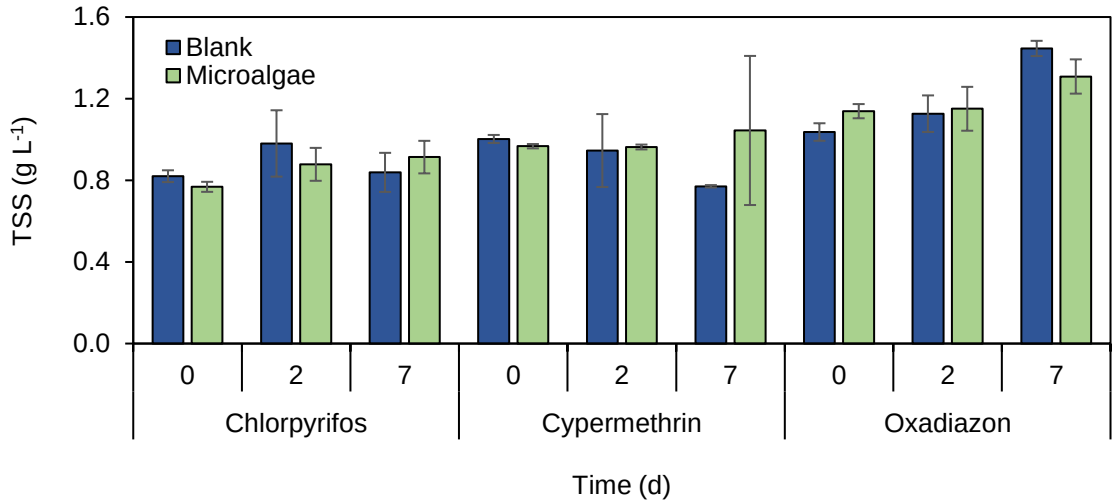


Figure 4.6 Evolution of TSS concentration in microalgae reactors with chlorpyrifos, cypermethrin, and oxadiazon. Error bars indicate standard deviation of the mean (n = 3).

4.3.3. Pesticide degradation and TPs detection in the pilot-PBR

In light of the efficient degradation of acetamiprid and propanil by the microalgae consortium in the indoor batch experiments, the removal of these two pesticides was further studied at pilot-scale in the outdoor PBR (described in section 3.2.2 - Chapter 3).

4.3.3.1. Performance of the pilot-PBR

Biomass evolution in the pilot-PBR treating simulated wastewater containing a pesticide mixture was determined by the VSS content of the PBR effluent (Fig. 4.7a). VSS represents the biomass concentration of microorganisms considering a consortium of photoautotrophs (microalgae and cyanobacteria) and heterotrophs (bacteria, protozoa and other microorganisms) as it is typically found in these systems (Posadas et al., 2014; Cuellar-Bermudez et al., 2017; Parladé et al., 2018). The VSS/TSS ratio was 80%, in agreement with values typically found in microalgae-based systems (> 70%) (García-Galán et al., 2020).

At the beginning of the operation, biomass concentration increased faster, reaching 0.7 g VSS L^{-1} after 3 days (Fig. 4.7a). From day 7 to the end of the experiment, average biomass concentration in the mixed liquor was 0.22 ± 0.12 g VSS L^{-1} and a production rate of 0.03 ± 0.01 g VSS L^{-1} d^{-1}, similar to values reported in previous experiments (Hom-Diaz et al., 2017a; Parladé et al., 2018). It is known that the optimum temperature for most microalgae species range between 15 - 30 °C (Singh and Singh, 2015; Sutherland et al., 2015). During the PBR operation, the temperature increased gradually in line with the beginning of the summer season and ranged among typical values (12.4 - 30.5 °C) (Fig. 4.7a). Temperature and sunlight irradiation have a direct effect on photosynthetic activity since they drive microalgae growth, influencing photosynthetic species composition, and PBR performance (Lee et al., 2015; Hom-Diaz et al., 2017a). As shown in Fig. 4.7a, during the first days of the PBR operation, biomass concentration increased in coherence with seasonality (rise in temperature and sunlight irradiation – average solar radiation on the horizontal surface was 4.78, 5.95, and 7.35 kWh m^{-2} d^{-1} in April, May and June, respectively (NASA, 2021). Nonetheless, from day 11 to the end of the experiment, biomass concentration decreased slowly regardless of temperature increase; meanwhile, biomass colonization of the PBR tubes, walls, and chambers was observed. Thus, reduced biomass productivity could be explained by the biofilm developing in the PBR, limiting light penetration into the mixed liquor, and negatively affecting biomass growth. Other studies using tubular PBRs reported that the biofilm is generated during PBR operation, influencing sunlight distribution throughout the entire culture (García-Galán et al., 2020). Similarly, a high microalgae growth in HRAPs produces a shading effect inside the reactor restraining higher light penetration into the system (Wang et al., 2015). Moreover, biofilm development could be correlated with microscopically observed changes in PBR populations (Table

4.6). Phototrophic biofilms are constituted by an assembly of filamentous cyanobacteria, microalgae, and heterotrophs (Sabater et al., 2002). During the period I, the PBR biomass was mainly constituted by unicellular microalgae (*Chlorella* sp. and *Scenedesmus* sp.) while the presence of filamentous microalgae (*Stigeoclonium* sp. and *Ulothrix* sp.) and cyanobacteria (*Phormidium* sp., *Oscillatoria* sp., *Nostoc* sp., and *Tolypothrix* sp.) increased towards the next periods (Table 4.6). Due to the outdoor operation, variations in the PBR populations could be associated to operational parameters, to the aforementioned dynamic changes in environmental conditions, and the predation by grazers (Deruyck et al., 2019). Moreover, the dominance of certain species could be also linked to their tolerance to pesticides in the influent. Cyanobacteria *Oscillatoria* sp. and *Phormidium* sp., as well as the green algae *Chlorella* sp., *Scenedesmus* sp., and *Stigeoclonium* sp., are considered among the most tolerant species to polluted environments (Palmer, 1969).

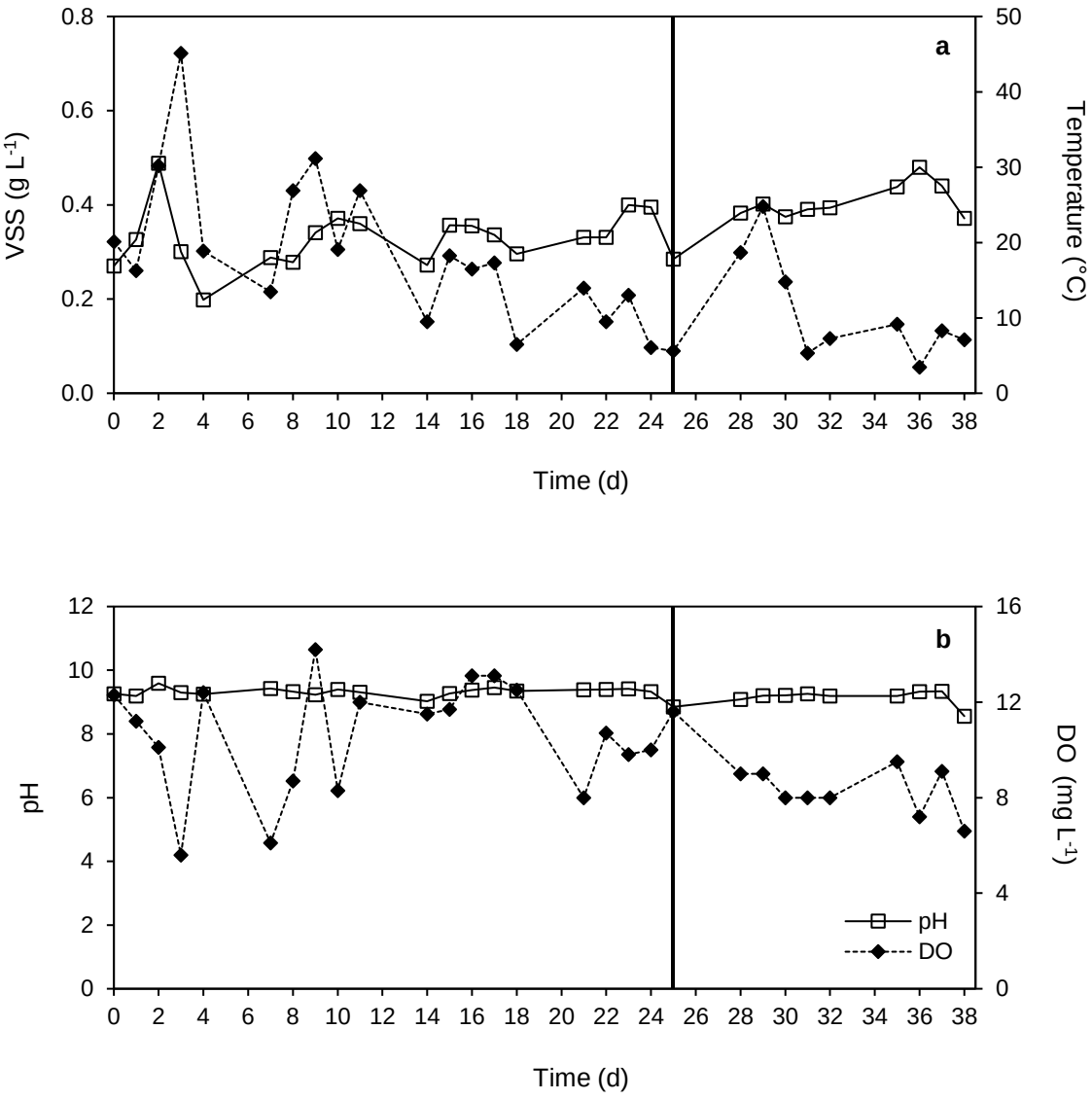


Figure 4.7 Evolution of (a) biomass concentration (VSS), temperature; (b) pH and DO in the pilot-PBR treating simulated wastewater with pesticides acetamiprid (concentration 5 μg L^{-1}) and propanil (concentration 50 μg L^{-1}). The vertical black line indicates the beginning of the steady-state.

Table 4.6 Qualitative characterization of the main microalgae and cyanobacteria genus in the pilot-PBR. References: (+++) dominant, (++) relative, (+) rare.

Species	**Period I (29th April -day 0- to 12th May -day 13-)**	**Period II (13th May -day 14- to 24th May -day 25-)**	**Period III * (25thtt May -day 26- to 6th June -day 38-)**
Algae			
Chlorella sp.	+++	+++	+++
Scenedesmus sp.	+++	+++	++
Stigeoclonium sp.	+	++	+++
Ulothrix sp.	+	+	++
Cyanobacteria			
Phormidium sp.	++	++	+
Oscillatoria sp.	+	+	++
Nostoc sp.	+	++	++
Tolypothrix sp.	+	+	++

* Period III corresponds to the steady-state.

Table 4.7 outlines the mean values of parameters analysed in the influent and the effluent of the pilot-PBR during the steady-state. The pH varied between 8.8 and 9.6 during the operation of the PBR (Fig. 4.7b), in agreement with a higher photosynthetic activity during higher irradiance periods in the spring and summer seasons (Hom-Diaz et al., 2017a). The pH in water is influenced by several factors such as microalgal growth, ammonium nitrification (release of H^+ and decrease of pH), the buffer capacity of the influent, and the excretion of acidic or basic metabolites from organic matter biodegradation (González et al., 2008). The mean DO concentration (8.6 ± 1.4 mg L^{-1}) is associated with the photosynthetic activity during the midday (Fig. 4.7b) and it is consistent with typical range values (Hom-Diaz et al., 2017a).

Table 4.7 Characterisation of some parameters from the influent and effluent of the pilot-PBR treating simulated wastewater with pesticides acetamiprid (concentration 5 μg L^{-1}) and propanil (concentration 50 μg L^{-1}) during the steady-state (mean + SD, n = 3 for the influent and n = 9 for the effluent).

Parameter	Influent	Effluent
TOC (mg L^{-1})	7.2 ± 0.7	13.1 ± 2.7
IC (mg L^{-1})	90.1 ± 6.1	80.9 ± 7.4
TC (mg L^{-1})	97.7 ± 4.7	94.0 ± 9.0
TN (mg L^{-1})	154.7 ± 30.9	128.3 ± 4.8
N-NO_3^- (mg L^{-1})	34.8 ± 2.3	26.1 ± 1.5
N-NO_2^- (mg L^{-1})	1.3 ± 0.1	5.1 ± 2.0
P-PO_4^{3-} (mg L^{-1})	6.2 ± 2.3	0.4 ± 0.1
VSS (g L^{-1})	n.a.	0.2 ± 0.1
Biomass production (g VSS L^{-1} d^{-1})	n.a.	0.02 ± 0.01
Temperature (°C)	n.a.	24.7 ± 3.3
pH	7.8 ± 0.4	9.1 ± 0.2
DO (mg L^{-1})	n.a.	8.6 ± 1.4

n.a. = not applicable.

The mean organic loading rate in the influent during the steady-state was 0.9 mg TOC L^{-1} d^{-1}. During this period, the IC was reduced by 12% and transformed to microalgal biomass, increasing the TOC in the effluent by 83% (Table 4.7), probably due to the fraction of soluble carbon released from the photosynthetically fixed carbon as reported elsewhere (García-Galán et al., 2018, 2020). The N:P ratio of the influent was 28:1 in molar basis, in line with the optimal N:P ratio reported for *Scenedesmus* sp. (Klausmeier et al., 2004). As shown in Fig. 4.8, N-NO_3^- and P-PO_4^{3-} were the sole inorganic nitrogen and phosphorous sources bioavailable to microalgae (Monfet and Unc, 2017) in the

employed culture media. $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ loading rates in the PBR influent were 4.4 and 0.8 mg L^{-1} d^{-1}, respectively. Nitrate and phosphate removal efficiencies were 24 ± 4% and 94 ± 2%, respectively. Higher values for nitrate removal were reported by other authors, which could be explained by higher microalgal biomass concentration in those studies, that were also conducted at indoor and controlled conditions. For instance, Ferrando and Matamoros (2020) evaluated the removal of 200 mg L^{-1} of $N\text{-}NO_3^-$ from a groundwater sample spiked with hydrophilic pesticides in an indoor continuous reactor with immobilized microalgae, achieving 41% attenuation of $N\text{-}NO_3^-$ at a HRT of 8 d. Likewise, Arias et al. (2018) reported 58% of $N\text{-}NO_3^-$ removal when treating secondary wastewater effluent and digestate in an indoor closed PBR with a mixed microalgae culture operating at a HRT of 8 d. As for orthophosphate, similar performances were attained when using microalgae-bacteria systems. For example, Godos et al. (2009) reported 80% PO_4^{3-} removal when treating swine slurry in a tubular biofilm PBR constituted by cyanobacteria, microalgae, and bacteria consortium. High phosphate uptake in the present study could be related to the presence of cyanobacteria with the ability to accumulate phosphate as polyphosphate granules, which can also be released to the medium with cell death (Jansson, 1988). Another mechanism influencing $P\text{-}PO_4^{3-}$ removal could be related to pH-mediated precipitation with cations (namely Ca^{2+} and Mg^{2+}) beginning at pH values between 8.9 - 9.5, which depends on the buffer capacity of the water (Craggs et al., 1996; González et al., 2008). Phosphorous could be firstly removed by pH-mediated precipitation, followed by biomass assimilation at lower concentrations (Craggs et al., 1996). Other mechanisms might be related to $P\text{-}PO_4^{3-}$ precipitation within the algal-bacterial biofilm (De Godos et al., 2009), and surface adsorption to biomass (Yao et al., 2011).

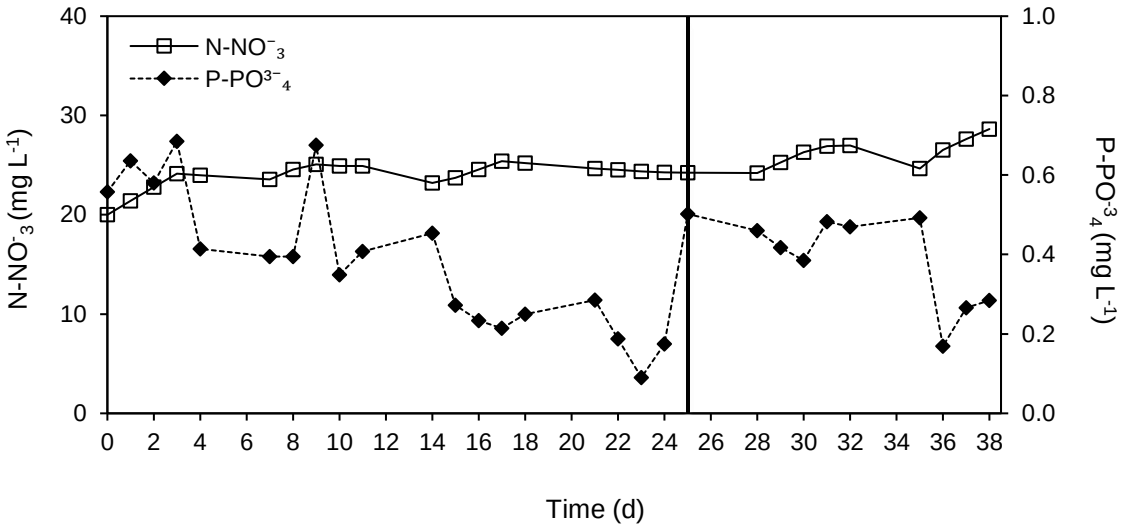


Figure 4.8 $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ evolution in the pilot-PBR effluent. The vertical black line indicates the beginning of the steady-state.

4.3.3.2. Degradation of selected pesticides and TPs detection in the pilot-PBR

The simultaneous occurrence of the targeted pesticides acetamiprid and propanil in the influent and effluent of the pilot-PBR and their removal by the microalgae-based system was analysed. Acetamiprid, propanil, and their detected TPs were monitored during 38 operational days in the PBR effluent (Fig. 4.9 and Fig. 4.10). Pesticide removal in the pilot-PBR was calculated considering the difference between the concentration of the target pesticide in the influent and the effluent at each sampling time.

Propanil was added to the pilot-PBR at a higher concentration (50 μg L^{-1}) than acetamiprid (5 μg L^{-1}) since it was degraded at a higher rate in the former indoor batch experiments. Individual concentrations of propanil and acetamiprid were lower than the threshold toxicity values reported in algal ecotoxicological studies (University of Hertfordshire, 2020; NORMAN, 2021). After 1 h of starting

pesticide addition (day 1), propanil was removed by 97% and during the following three weeks its removal efficiency was >99% (Fig. 4.9). Propanil concentration in the PBR effluent increased slightly after 25 days attaining a 97% removal. During the steady-state propanil was nearly completely removed, achieving a mean removal efficiency of 99%. These results agree with the propanil degradation rate observed at indoor lab-scale experiments (microalgae reactor) where it was completely removed within 2 d. Overall, propanil removal efficiency was enhanced over time which could be associated to biomass acclimation, as reported in previous studies (Ferrando and Matamoros, 2020).

In the present study, no propanil TPs were detected in the PBR effluent, in line with the decreasing trend of TP concentrations observed in the lab-scale batch experiments. This finding suggests that propanil and their intermediate by-products could be completely mineralised under continuous operation with a HRT of 8 d. Nevertheless, the presence of residual concentrations of the TPs in the effluent cannot be completely ruled out since TPs could be at levels below the instrumental limit of detection (which cannot be calculated in the absence of standards). For instance, Milan et al. (2012) studied the dissipation of propanil and 3,4-DCA in rice management systems, indicating a rapid conversion of propanil to 3,4-DCA due to microbial degradation, followed by a slow decrease in its concentration. Furthermore, our results at indoor batch experiments showed the absence of removal by sorption and abiotic factors, suggesting that biodegradation was the main attenuation mechanism for this pesticide.

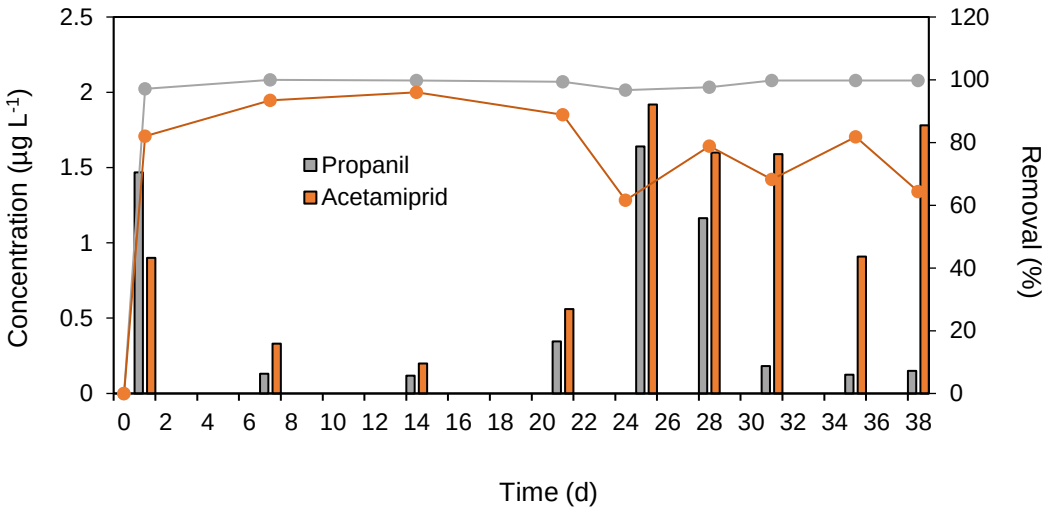


Figure 4.9 Evolution of acetamiprid and propanil concentration in the pilot-PBR effluent (bar chart) and their removal efficiency (line chart).

On the other hand, acetamiprid concentration in the effluent was reduced to 0.87 µg L^{-1} after one hour of pesticide addition (day 1) obtaining an 87% removal (Fig. 4.10). During the following two weeks, acetamiprid removal increased from 93% to 96%. By the beginning of the hydraulic steady-state (day 25), acetamiprid removal decreased to 62%, increasing its concentration in the effluent. The mean acetamiprid removal during the steady-state was 71%. According to these results, the performance of the microalgae reactor in batch and continuous operational modes indicate that acetamiprid requires a longer time than propanil for its complete degradation. At indoor batch conditions, 42% of acetamiprid removal was obtained within 2 d while a total removal was accomplished by day 7. Although acetamiprid was added to the PBR at a minor concentration than propanil, its removal was lower. Differences in removal efficiencies for acetamiprid in continuous mode could be attributed to its physical-chemical properties. Acetamiprid is stable to hydrolysis (FAO and JMPR, 2005), and its low Henry´s law constant value and log K_{ow} (Table 4.1) result in negligible volatilisation and sorption onto biomass. According to results in batch,

photodegradation did not affect acetamiprid removal, as previously reported (US EPA, 2002). In contrast, its high-water solubility contributes to its availability in aqueous phase for biodegradation. Thus, the declining biomass concentration in the pilot-PBR associated with the biomass washing effect might be a feasible explanation for the decrease in acetamiprid removal, as it has been previously reported for continuous-feeding operational mode (Ferrando and Matamoros, 2020). Moreover, different removal efficiencies observed in batch and continuous-mode reactors could be associated to the non-controlled environmental conditions in the PBR system such as light irradiance, temperature, precipitation, and presence of grazers, among others. According to our results, these factors have a greater influence over acetamiprid than propanil. Results demonstrated the good performance of semi-closed algal-based systems in nutrients and OMs removal from water under environmental conditions, as also exposed in others studies (García-Galán et al., 2018; Parladé et al., 2018). For instance, García-Galán et al. (2020) reported the removal of diverse pesticides (10 out of 16 pesticides were fully eliminated) when using a full-scale semi-closed tubular PBR for treatment of agricultural run-off.

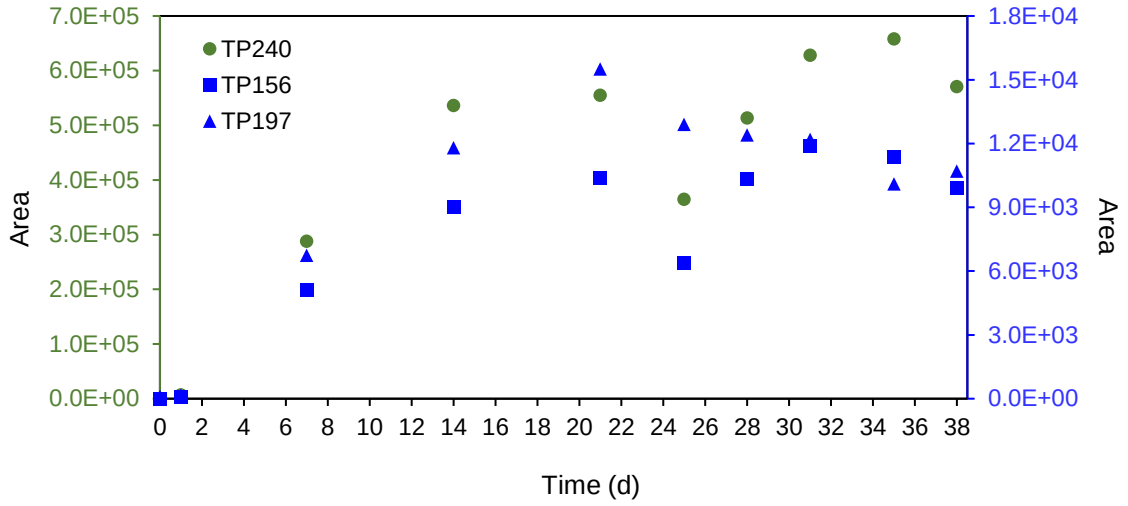


Figure 4.10 TPs from acetamiprid detected in the pilot-PBR treating simulated wastewater with pesticides acetamiprid (concentration 5 μg L^{-1}) and propanil (concentration 50 μg L^{-1}).

Only two acetamiprid TPs (TP240 and TP197) were found in PBR effluent after 7 days of operation (Fig. 4.10) and their concentration, although with fluctuations, increased throughout the studied period until day 35 and 21, respectively, when they started to decrease. MS2 spectra fitted to those obtained at the indoor batch experiments (Fig. 4.11a and Fig.4.11b). Another TP from acetamiprid, TP156, was also detected, however, no MS2 spectrum was acquired by the system (Fig. 4.11c) and, therefore, its identity could not be confirmed. Despite acetamiprid was not completely removed at a HRT of 8 d, the three TPs identified were generated right after its interaction with the microalgae-based system.

Available data on the removal of pesticides by microalgae-based systems in close or semi-close PBRs is still scarce and developed under laboratory-controlled conditions. This study verifies the capacity of these low-cost nature-based systems for the treatment of wastewater containing OMs.

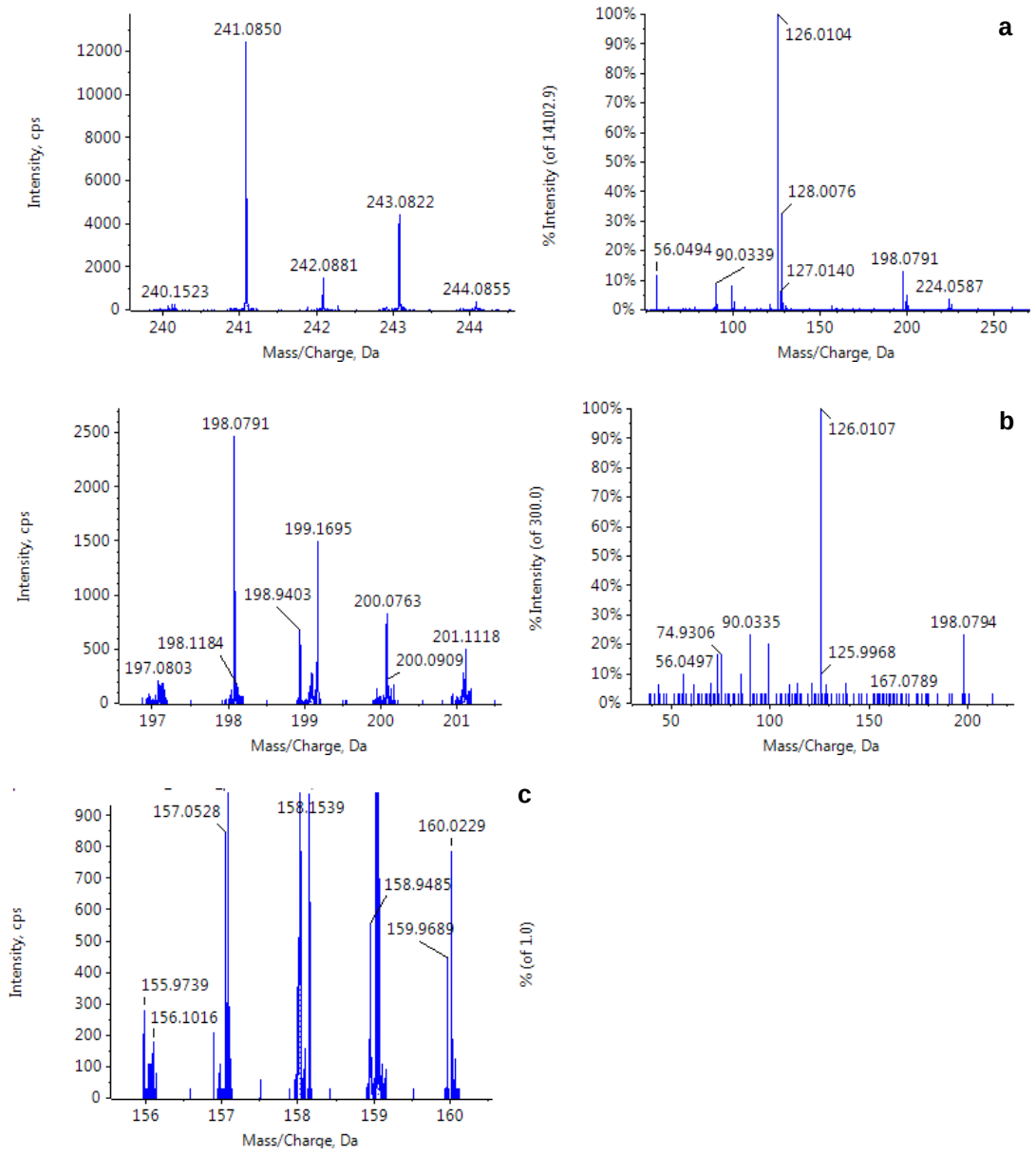


Figure 4.11 (a) TOF-MS (left) and MS/MS (right) spectra of m/z 241.0850 (TP240 from acetamiprid). (b) TOF-MS (left) and MS/MS (right) spectra of m/z 198.0791 (TP197 from acetamiprid). (c) TOF-MS (left) spectra of m/z 157.0528 (TP156 from acetamiprid).

4.3.4. Biomass valorisation

4.3.4.1. Valorisation of algal biomass from indoor batch experiments

The anaerobic biodegradation and the biogas production potential of the microalgae biomass containing the retained pesticide by sorption was assessed by BMP tests. During AD processes, pesticides and other compounds can be physically, chemically, and biologically transformed (Kupper et al., 2008) by diverse microorganisms and enzymes involved in each phase. Given the high sorption affinity of the studied hydrophobic pesticides, their concentration was determined in the biomass at the initial (t = 0 d) and final time (t = 42 d) of the BMP tests.

The net methane production (Fig. 4.12) was better for BMP reactors containing cypermethrin and chlorpyrifos, with yields of 4880 and 4558 NmL CH_4 g VS^{-1}, respectively (differences were not statistically significant). The methane yield in reactors containing oxadiazon was remarkably lower (2919 NmL CH_4 g VS^{-1}, $p < 0.05$). According to the Gompertz model, the lag phase was around 2.9 to 3.5 days (Table 4.8), after which biogas production increased sharply at a rate of 27.4, 24.3, and 21.8 mL CH_4 d^{-1}. VFAs quantified after the methanisation verified the stability of the process (Table 4.8), indicating neither inhibition from the residual pesticide nor the potential TPs.

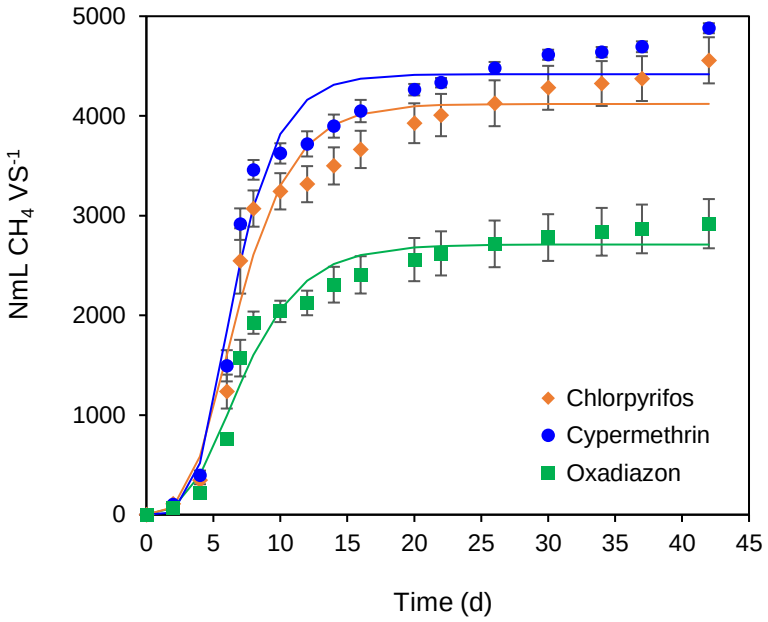


Figure 4.12 Net methane production from the microalgae biomass containing the hydrophobic pesticides retained by sorption. Dots represent experimental data and continuous lines corresponds to the fitting by the Gompertz model. Error bars represents the standard deviation of the mean (n = 3).

Analysis of the final digestate indicated that chlorpyrifos and cypermethrin were efficiently removed by 87.4 ± 0.5% and 58.7 ± 3.9%, respectively (difference of the mean pesticide content in the solid phase between the initial time and final time was statistically significant in both cases, $p < 0.01$) (Fig. 4.13). This is in agreement with the work of Lian et al. (2019), in which contaminated biomass with the insecticides parathion and malathion (organophosphorus compounds) was efficiently anaerobically transformed by reduction reactions and enzymatic hydrolysis driven by hydrolases, suggesting that hydrolytic activities provide a potential tool for biodegradation of organophosphorus compounds such as chlorpyrifos. Likewise, García-Mancha et al. (2017) reported a good removal efficiency (77%) of chlorpyrifos from wastewater under thermophilic conditions (55 °C).

Although these pesticides were retained onto algal biomass due to their hydrophobicity, biogas production was not inhibited. The results indicate that although oxadiazon was highly degraded by the microalgae consortium under aerobic conditions (55% biodegradation), its anaerobic removal was lower (18.7 ± 4.4%, $p < 0.05$) (Fig. 4.13), leading to a minor methane yield. Otherwise, chlorpyrifos and cypermethrin were successfully degraded by the anaerobic microorganisms as confirmed by their removal in the digestate. Thus, anaerobic degradation of cypermethrin was more effective than aerobic degradation by the microalgae consortium.

After the microalgae-based treatment, biomass separation is needed in order to valorise it through AD. The treated effluent could be further processed in a municipal WWTP or discharged if adequate parameters were fulfilled. As it was previously demonstrated, the solid phase containing the retained pesticides could be valorised through methanisation for biomethane production. This suggestion is in agreement with other studies (Rawat et al., 2011; Uggetti et al., 2014; Caporgno et al., 2015a) aiming at biomass valorisation. Even though AD contributed to the elimination of pesticides, the digestate obtained after the AD could be dewatered. The solid fraction could be employed for fertilization (Solé-Bundó et al., 2017b) if parameters for their agricultural reuse are fulfilled. The liquid fraction could be treated in a WWTP or properly discharged if parameters were adequate given environmental restrictions.

Table 4.8 Parameters from the BMP tests of microalgae biomass retaining the hydrophobic pesticides in lab-scale degradation experiments.

Reactor	VFAs (mg L^{-1})		Mean CH_4 content (%)	Experimental CH_4 yield (NmL CH_4 g VS^{-1})	Gompertz parameters				Hydrolysis rate	
	Acetic acid	Propionic acid			P_{max} (NmL CH_4 g VS^{-1})	R_{max} (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})	r^2
Chlorpyrifos	3.2 ± 0.0	1.1 ± 0.1	77	4557.9 ± 231.2 ^{ab}	4120.8 ± 112.8	549.9 ± 93.3	3.1 ± 0.7	0.965	0.1	0.923
Cypermethrin	7.7 ± 0.4	n.d.	77	4879.6 ± 50.0 ^{ac}	4418.1 ± 105.6	728.7 ± 122.1	3.5 ± 0.6	0.970	0.1	0.916
Oxadiazon	6.7 ± 0.1	n.d.	78	2919.1 ± 246.8 ^{bc}	2709.7 ± 70.4	323.1 ± 48.3	2.9 ± 0.6	0.970	0.1	0.929

n.d.: not detected.
Statistically differences between the mean experimental methane yield in BMP reactors at t_{42}, are shown with a superscript as indicated as follows: a ($p > 0.05$), b and c ($p < 0.001$).

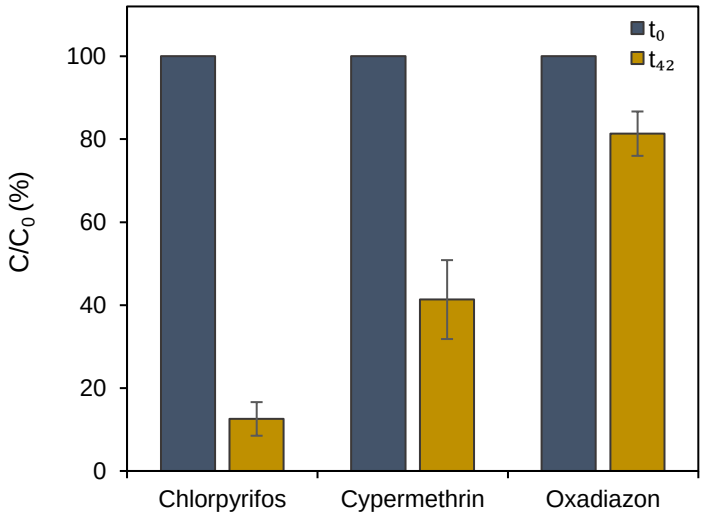


Figure 4.13 Pesticide content evolution in the solid phase before (t_0) and after (t_{42}) the AD of the microalgae biomass retaining the hydrophobic pesticides in lab-scale degradation experiments. Error bars at t_{42} represents the relative standard deviation of the mean (n = 3).

4.3.4.2. Valorisation of algal biomass from the pilot-PBR

From a biorefinery approach, coupling nutrients removal and pesticides degradation with biogas production contribute to boosting the economic feasibility of the system (Ward et al., 2014). Accordingly, AD has been explored as a technique for algal biomass valorisation after pesticides removal in the pilot-PBR. Therefore, microalgal biomass was first harvested and then valorised through AD.

Microalgae harvesting from the PBR was performed in the period of hydraulic steady-state through gravity sedimentation, flocculation with an organic flocculant and coagulation with $FeCl_3$. Coagulation and flocculation were used to increase natural sedimentation efficiency. The flocculant was employed to aggregate flocs, strengthen them while increasing their size (Edzwald and Haarhoff, 2011). The coagulant employed is widely used in wastewater treatment due to its low solubility

diminishing dissolved residual metal salts in the treated water (Edzwald and Haarhoff, 2011).

Flocculation and coagulation techniques were performed in 30 min (15 min mixing, followed by 15 min sedimentation) showing similar harvesting performances (41 – 44%) (Table 4.9). In contrast, gravity sedimentation for 24 h attained 75% biomass concentration. Settleability performance could be ascribed to the dominance of filamentous self-aggregating microalgae and cyanobacteria which contribute to immobilize microalgae cells and constitute an additional surface for bacteria colonization (Craggs et al., 1996). This mixed flocs avoid biomass from washing out, provide diverse removal pathways of nutrients by different microorganisms, enhance the robustness of the system, and overcome difficulties associated with harvesting (Liu et al., 2017).

Biogas potential production of the harvested biomass by the three different techniques was determined through BMP tests (each trial was identified with the same name of the harvesting technique). As shown in Fig. 4.14 and Table 4.9, biogas production was highest when $FeCl_3$ was employed (69.7 ± 4.8 NmL biogas g VS^{-1}), followed by flocculated biomass (54.8 ± 7.5 NmL biogas g VS^{-1}), and gravity collected biomass (50.0 ± 18.3 NmL biogas g VS^{-1}); however, statistical differences were not significant ($p > 0.05$). The greater average methane content along with the absence of VFAs in the digestate of the sedimentation trial (Table 4.9) suggest a better conversion of biomass.

Table 4.9 Harvesting efficiency and BMP tests parameters for microalgae biomass harvested from the pilot-PBR.

Trial	Harvesting		BMP							
							Gompertz parameters			
	TS after harvesting (g L^{-1})	Harvesting efficiency (%)	Experimental biogas yield (NmL biogas g VS^{-1})	CH_4 content (%)	Propionic acid (*) concentration (mg L^{-1})	VS removal (%)	P_{max} (NmL biogas g VS^{-1})	R_{max} (NmL biogas g VS^{-1} d^{-1})	λ (d)	r^2
S	5.8 ± 0.0	75	50.0 ± 18.3	73 ± 7	0.0 ± 0.0	18 ± 3	48.6 ± 2.8	3.8 ± 0.8	-1.5 ± 1.4	0.948
F	4.6 ± 0.6	41	54.8 ± 7.5	59 ± 27	49.1 ± 8.2	21 ± 7	52.2 ± 1.7	5.8 ± 1.0	-0.5 ± 0.8	0.973
C	4.8 ± 0.1	44	69.7 ± 4.8	67 ± 25	51.5 ± 12.5	30 ± 3	64.4 ± 2.3	10.7 ± 2.7	-0.1 ± 0.8	0.958

S = sedimentation. F = flocculation. C = coagulation. (*) Propionic acid was the only VFA detected.

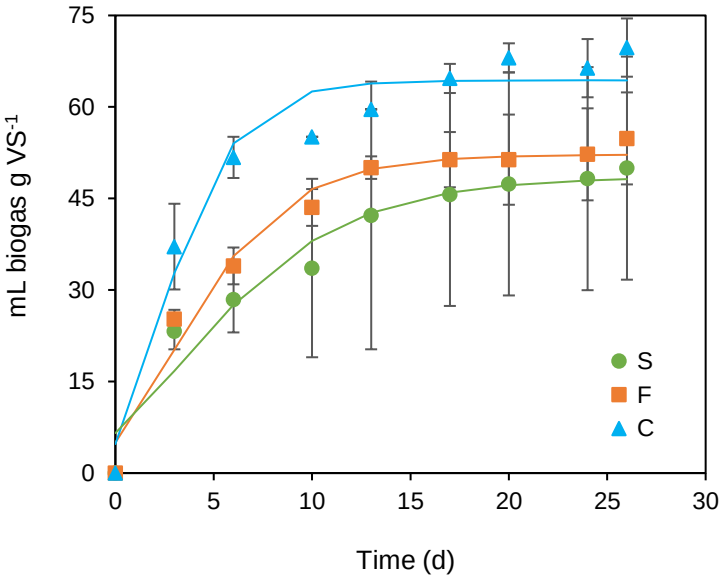


Figure 4.14 Net methane production from the harvested biomass of the pilot-PBR effluent. S = sedimentation, F = flocculation, C = coagulation. The symbols represent the experimental data while the curves are data estimated by the Gompertz model.

The use of coagulated biomass might influence the anaerobic process since Fe among other trace metals (such as Ni, Cu, Mo, and Zn) has an important role as cofactors of some enzymes or as catalytic centres at actives sites (Glass and Orphan, 2012). Indeed, Fe is one of the most required trace elements by methanogenic microorganisms for methane production (Glass and Orphan, 2012). Appropriate addition of $FeCl_3$ contributes to acetic acid use for methane production. Nonetheless, excessive doses could produce inhibition and/or toxicity altering the balance of the process (Qin et al., 2019). Some authors have also reported improvement in biogas production due to $FeCl_3$ addition (Song et al., 2001; Yu et al., 2015; Qin et al., 2019). Other authors stated that the application of pretreatment to microalgal biomass before its AD could enhance biogas yield addressing microalgae cell wall recalcitrance (Passos et al., 2014). Although results in this study did not show a rise in biogas yield after applying

the harvesting treatments, it could be suggested that neither the coagulant nor the flocculant used was toxic for the methanisation.

4.4. Conclusions

First, different conditions were studied at a batch lab-scale to determine the mechanisms of degradation by microalgae. The distribution of pesticides was evaluated in the liquid phase for polar pesticides while both liquid and solid matrices were assessed for hydrophobic pesticides. For hydrophobic pesticides, total degradation (biodegradation and photodegradation) was higher for oxadiazon (55%), followed by chlorpyrifos (35%), and cypermethrin (14%). Cypermethrin had a lower degradation concerning its low water solubility. Greater sorption to the solid phase was observed for chlorpyrifos (62%) and cypermethrin (60%). Overall, a large removal from the liquid phase (total degradation and sorption) was achieved for chlorpyrifos (97%), cypermethrin (74%), and oxadiazon (88%). In the case of polar pesticides, a complete degradation was observed for acetamiprid and propanil at t = 7 d. Neither sorption nor abiotic losses were observed in acetamiprid and propanil removal, suggesting that degradation could be attributed to biodegradation. Bentazone was not removed, confirming the recalcitrance to biodegradation of this compound as it was also described for other microorganisms. One, two and four TPs were detected for chlorpyrifos, propanil, and acetamiprid, respectively, which are formed by the degradation mechanisms taking place in microalgae-based systems (biodegradation and photodegradation).

Second, in the outdoor semi-continuous pilot-PBR treating simulated wastewater spiked with propanil and acetamiprid (concentration in the influent of 50 and 5 $\mu g\ L^{-1}$, respectively) and operating at an HRT of 8 d, degradation experiments

showed a mean removal in steady-state of 99% and 71% for propanil and acetamiprid, respectively. Three acetamiprid TPs were detected during the pilot-PBR operation while none was detected for propanil, confirming that propanil is more biodegradable than acetamiprid, as shown in the lab-scale experiments. Longer HRT could improve the removal efficiency of acetamiprid.

Third, anaerobic degradation was assessed as a process for the valorisation of the algal biomass used in the degradation experiments. AD of algal biomass with hydrophobic pesticides retained by sorption did not inhibit the process, leading to high methane generation (2919 - 4880 mL CH_4 g VS^{-1}), and further removal of the pesticides (removal efficiency was chlorpyrifos > cypermethrin > oxadiazon). This fact could suggest that pesticides are anaerobically degraded, contributing to biogas production. Regarding degradation experiments in the pilot-PBR, algal biomass was first harvested and then anaerobically digested. Similar biogas yields (50 – 69.7 NmL biogas g VS^{-1}) were obtained after the AD of the harvested biomass indicating the absence of toxicity of the employed coagulant and flocculant.

These results evidence the capacity of this nature-based treatment technology in the bioconversion of agrochemicals. Results obtained at pilot-PBR highlight that anaerobic degradation coupled to wastewater treatment make nature-based systems attractive for the simultaneous consumption of nutrients in the water, the removal and degradation of pesticides, and the generation of biomass for biofuels production.

SECTION 4

Microalgae pretreatments and co-digestion with waste activated sludge

Section presentation

This section addresses a real case study of an industrial winery WWTP ("winery WWTP" hereafter) involved in a circular bioeconomy project co-founded by the local administration (ViTech project TES/792/2017). The project aimed to evaluate the feasibility of closing bio-wastes fluxes in the winery company using a microalgae-based system promoting a circular bioeconomy approach. In a previous phase, it was evaluated the microalgal cultivation for the mitigation of the residual CO_2 from industrial fermentation processes of the winery company to reduce the carbon footprint. The scope of the ViTech project include:

1. Microalgae tertiary wastewater treatment to fullfil discharge limits.
2. Generation of profitable algal biomass to be used as:
    a. co-substrate for AcoD with WAS,
    b. a biofertilizer in the winery company arable lands.

In this sense, it was studied the integration of microalgae-based tertiary wastewater treatment into the winery WWTPs. Also, it was evaluated the valorisation of algal biomass through co-digestion with secondary sludge. Chapters 5 and 6 in this section assess different low cost pretreatments of algal and WAS mixtures to improve biomass solubility and methane yield. The last chapter of this section (Chapter 7) assesses the coupling of microalgal wastewater treatment and algal AcoD with WAS in a circular model for nutrients and bioenergy recovery from wastewater and sludge.

CHAPTER 5.

LOW TEMPERATURE THERMAL PRETREATMENTS

Part of this chapter was published as:

Avila, R., Carrero, E., Crivillés, E., Mercader, M., Vicent, T., Blánquez, P., 2020. Effects of low temperature thermal pretreatments in solubility and co-digestion of waste activated sludge and microalgae mixtures. Algal Res. 50, 101965. https://doi.org/10.1016/j.algal.2020.101965

Abstract

A low temperature thermal pretreatment was applied to three different ratios of waste activated sludge (WAS) and microalgae mixtures to increase their solubility. The performance of three organic flocculants was assessed in order to select the best flocculant for previous microalgae harvesting. The effect of the following variables in the solubilisation were evaluated: ratio of the mixture of WAS and (flocculated and non-flocculated) microalgae (50:1, 25:1 and 10:1, in v:v basis), incubation time (24, 48, and 72 h) and temperature (37 and 60 °C). A cationic polymer of diallyl dimethyl ammonium chloride free of acrylamide was selected for microalgae flocculation due to its high harvesting efficiency (95%). In pretreatments at 37 °C, effect of temperature and time in the solubilisation of the mixtures was negligible. However, results showed a high increase in solubilisation pretreatments at 60 ºC using both flocculated and non-flocculated microalgae in the mixtures. Initial soluble chemical oxygen demand in the mixtures containing WAS and flocculated microalgae was higher in comparison with that in the mixtures of the same ratio without previous flocculation of the microalgae. According to the results, the optimal pretreatment conditions were incubation of the mixtures during 24 h at 60 °C in a 25:1 (WAS:microalgae, in v:v basis) mixing ratio. Thus, these optimal mixtures using flocculated microalgae and non-flocculated microalgae were chosen to assess the effect of the pretreatment in the biogas yield. The anaerobic co-digestion (AcoD) of the selected mixtures indicated a lack of proportionality between the soluble chemical oxygen demand attained during the pretreatment and the methane yield obtained (51 and 34% lower in comparison to the control, respectively). This behaviour could be explained by the high organic matter consumption in the low temperature thermal pretreatment before the AcoD, which could have been fostered by the enzymatic activity of the sludge.

5.1. Introduction

Diverse studies reported the co-digestion of waste activated sludge (WAS) and microalgae (Olsson et al., 2014; Mahdy et al., 2015a; Wang and Park, 2015; Arias et al., 2018) and benefits when using microalgae as co-substrate to WAS. Nonetheless, due to the specific properties of both substrates, WAS and microalgae are characterized to have a low methane yield. WAS obtained from the biological wastewater treatment has a floc structure composed by aggregates of microorganisms, attached particulate organic matter and inorganic particles (Kor-Bicakci and Eskicioglu, 2019). The flocs are surrounded by polymeric network of extracellular polymeric substances (EPSs) containing polysaccharides, humic acids, lipids, proteins, etc. creating a barrier that retains compounds within this matrix (Zhen et al., 2017). Bacteria as single cells or colonies are the main microorganisms forming the floc, along with other microorganisms such as fungi, protozoa, cyanobacteria, algae, metazoan and archaeal populations (Seviour and Nielsen, 2010). In addition to the hard and resistant cell wall of these microorganisms, EPS provides protection to the floc (Zhen et al., 2017). Due to its complex composition, the organic degradation efficiency of WAS is low (ca. 30 – 50%) and usually a pretreatment is required to improve its biodegradability (Appels et al., 2008). Concerning microalgae, two main issues are needed to be addressed in valorisation of microalgae by means of anaerobic digestion (AD): their biochemical composition, as well as, their cell wall complexity, both are strain specific (Passos et al., 2014). Therefore, to avoid a limited biomethane production, a pretreatment of both substrates is recommended for organic matter freed into the soluble phase as a prior step to their anaerobic co-digestion (AcoD).

Pretreatments of WAS prior to AD contribute to disaggregate the flocs structure, disintegrate EPSs, release cell content, enhancing the kinetic and overall performance of the process (Nazari et al., 2017). Among other pretreatments, high temperature thermal pretreatment of WAS has been studied with the aim of accelerating hydrolysis and enhancing biomass biodegradability by disruption of chemical bonds in cell wall, releasing of intra- and extracellular biopolymers to the soluble phase (Climent et al., 2007; Chen et al., 2017). Thermal pretreatment at high temperature has a positive impact in biogas generation, volume reduction, dewatering, and improves the quality of bio-solid obtained after the AD (Barber, 2016; Sapkaite et al., 2017). Conversely, this pretreatment could fail to afford a positive energy balance due to energy requirements, especially when the pretreatment is implemented at high pressure (Appels et al., 2010; Prorot et al., 2011). A promising alternative to high temperature pretreatments are low temperature thermal pretreatments (< 100 °C). This pretreatment has been effectively applied to secondary sludge as a biological-enzymatic pretreatment, since thermophilic conditions promote the activity of thermophilic hydrolytic bacteria (Climent et al., 2007; Appels et al., 2010; Carvajal et al., 2013). Thus, it represents a technically and economically feasible pretreatment that accomplish a synergetic effect of temperature and hydrolytic freed enzymes in a simple operation process, without the need of catalysts (Carvajal et al., 2013).

Microalgae-based systems employed in Water resource recovery facilities (WRRFs) are predominantly constituted by microalgae along with a polyculture of microorganisms (Gonçalves et al., 2017; Parladé et al., 2018). Usually, thermal pretreatments studies applied to microalgae cultures are performed between 55 and 180 °C from some minutes to several hours (15 min - 24 h) and can be executed under pressure (Alzate et al., 2012; Lee et al., 2013; Schwede et al., 2013;

Mendez et al., 2014). In contrast, high energy consumption is demanded to reach those temperature and pressure conditions. For instance, Mendez et al. (2013) performed a thermal pretreatment of microalgae at high temperature and short time (120 ºC, 40 min) increasing methane production by 93%; nonetheless energy required in the pretreatment was 4-fold higher than the energy obtained by the AD. For that reason, pretreatments that require less energy input such as low temperature thermal pretreatment, are a sustainable alternative (Gavala et al., 2003). The main advantages reported for those pretreatments are the generation of monomers and more soluble substrates by accelerating organic matter hydrolysis rate. Besides, a positive energy balance is achieved by a reduction in energy consumption, and the technology is scalable (Appels et al., 2010; Ometto et al., 2014).

The influence of low temperature thermal pretreatments in WAS or microalgae as mono-substrates for AD has been studied by some authors (Appels et al., 2010; Carvajal et al., 2013; Mendez et al., 2015; Kinnunen and Rintala, 2016). Nonetheless, few studies analyse the effect of the same pretreatment when WAS and microalgae are mixed. For instance, Arias et al. (2018) reported the application of a thermal pretreatment (55 °C, 7.5 h) on a mixture composed by microalgae and activated sludge (50% of each substrate, on a VS basis), obtaining a solubilization ratio of 21% and a higher methane yield (2-fold) when the mixture was co-digested (in comparison with microalgae as sole substrate). Thus, incubating the mixtures of WAS and microalgae in a pre-digestion step could increase bacteria hydrolytic activity and enhance biomasses solubilisation.

The aim of this work is to assess the effectiveness of a low temperature thermal pretreatment (at 37 and 60 °C) of three different ratios of WAS and microalgae mixtures in solubility of biomasses, as well as in the methane yield of the selected

co-digested mixtures. Additionally, aiming at microalgae harvesting, algal flocculation was also assessed as a former step to the low temperature thermal pretreatment of the mixtures.

5.2. Materials and methods

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

5.2.1. Substrates

WAS and microalgae proceed from the winery company. Microscopic examination identified *Chlorella* sp. and *Scenedesmus* sp. as the main microalgal species present in the culture (Zeiss, AixoCam ERc 5 s). Microalgae were grown in batch mode in 9 L pilot PBRs located in an outdoor greenhouse at 25 ± 2 °C. PBRs were fed with grey wastewater and mixing was provided by air bubbling. Microalgae employed in the pretreatment were collected during the stationary growth phase.

WAS proceed from the industrial WWTP of the winery company with a typical activated sludge treatment. In order to define a proper ratio of the WAS:microalgae mixture to pretreat, microalgae generation in the pilot plant and its potential enlargement were considered. As a result, three different ratios of WAS:microalgae mixture were selected: 50:1, 25:1, and 10:1 in volume basis (v:v). Employed substrates are characterized in Table 5.1.

Table 5.1 Characteristics of waste activated sludge and microalgae employed in the mixtures. Results are given as mean ± standard deviation (n = 3).

Parameter	WAS	Microalgae
TS (g L^{-1})	11.9 ± 1.8	1.2 ± 0.4
VS (g L^{-1})	8.5 ± 1.7	0.8 ± 0.2
TSS (g L^{-1})	6.0 ± 2.0	0.3 ± 0.1
VSS (g L^{-1})	5.0 ± 1.6	0.3 ± 0.1
pH	7.2 ± 0.2	8.7 ± 1.2
sCOD (mg L^{-1})	161.5 ± 24.7	59.0 ± 17.1

5.2.2. Pretreatment experimental set-up

A schematic representation of the experimental set-up is presented in Fig. 5.1. WAS and microalgae biomasses mixtures were pretreated in batch mode at two low temperatures, 37 and 60 °C, during 24, 48 and 72 h under continuous orbital stirring at 100 rpm. Three different ratios of WAS and microalgae were studied: 50:1, 25:1 and 10:1 (v:v basis). As shown in Table 5.2, pretreatments for the mixtures employing non-flocculated microalgae were evaluated during 3 days at 37 °C (Mixtures A, B and C) and 60 °C (Mixtures D, E and F). Pretreatments were performed at 37 and 60 °C, since these temperatures correspond to the mesophilic and thermophilic conditions of the AD processes, respectively. According to the solubilisation achieved in these experiments, further pretreatments of the mixtures employing flocculated microalgae were assessed during 3 days at 60 °C (Mixtures G, H, and I). Triplicates of the mixtures were prepared in 1 L Erlenmeyer flasks covered with a cotton plug. A volume of 500 mL of WAS and the corresponding volume of flocculated or non-flocculated microalgae were placed inside the flasks. Pretreatments at 37 °C were performed in an orbital shaker inside a controlled temperature chamber, while pretreatments

at 60 °C were done in a controlled temperature shaker. Controls for each mixture (Mixtures J, K and L using non-flocculated microalgae; and mixtures M, N and O using flocculated microalgae) were performed in duplicates at room temperature (20 °C). All flasks were under constant agitation.

In mixtures G, H, and I, microalgae were first flocculated using three organic synthetic flocculants (characteristics of these flocculants are presented in Table 3.2 - Chapter 3). Flocculant A and B are frequently employed in WWTP to reduce sludge volume and improve its dewaterability. Flocculant C is applied for turbidity reduction in the winery industry. Flocculants were applied at two doses:

- polyDADMAC (flocculant A): at 250 and 375 mg L^{-1}),
- cationic copolymer of acrylamide (flocculant B): at 500 and 750 mg L^{-1}), and
- cationic polysaccharide obtained from chitosan (flocculant C): at 750 and 1000 mg L^{-1}).

Flocculation was carried out in triplicate in a jar-test device (Flocculator SW1 from Stuart Scientific, Staffordshire, UK) using 200 mL of microalgal suspension, according to a procedure previously described (Mir-Tutusaus et al., 2017). The optimal flocculant and its dose were selected according to the harvesting efficiency achieved, calculated as follows, in Eq. (5.1):

$$\text{Harvesting efficiency (\%)} = \frac{(TSS_i - TSS_f)}{TSS_i} * 100 \qquad \text{Eq. 5.1}$$

Where TSS_i is the initial total suspended solids and TSS_f is the final total suspended solids in the supernatant.

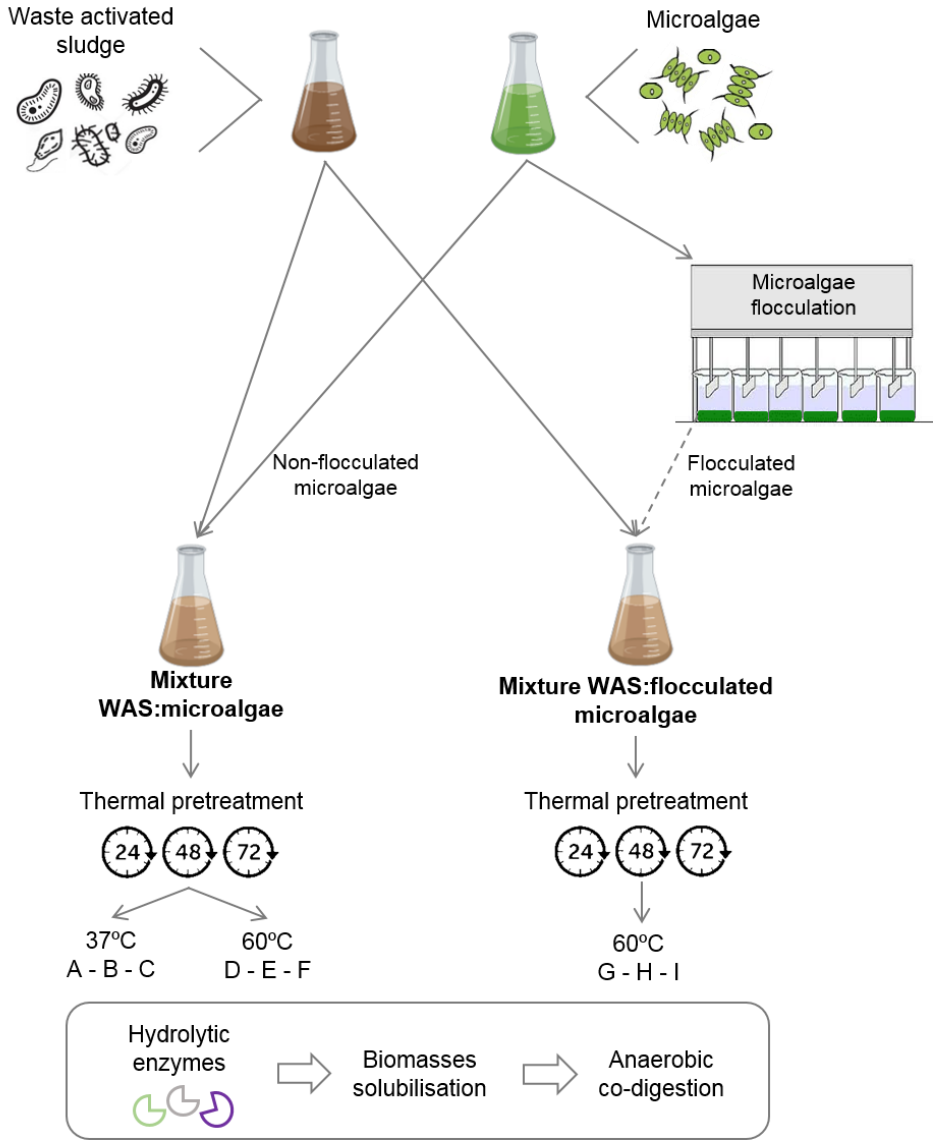


Figure 5.1 Schematic representation of the experimental set-up. A, B, C, D, E, F, G, H, and I are waste activated sludge (WAS) and microalgae mixtures identified in Table 5.2.

Table 5.2 Identification of the mixtures according to the ratio of the mixture and temperature of the pretreatment.

WAS:Microalgae ratio (v:v)	Pretreatment			Control	
	37 °C	**60 °C**	**Microalgae flocculation a 60 °C**	**20 °C**	**Microalgae flocculation a 20 °C**
50:1	A	D	G	J	M
25:1	B	E	H	K	N
10:1	C	F	I	L	O

5.2.3. BMP tests

Batch tests (previously described in section 3.3.1. - Chapter 3) were used to measure the methane production obtained from the AcoD of the selected mixtures in order to evaluate their digestibility after the thermal pretreatment. A volume of 250 mL was used as fixed inoculum volume. Characterization of the inoculum employed is presented in Table 3.6 - Chapter 3.

5.2.4. Analytical methods

The effect of hydrolytic bacteria from WAS in the solubilisation of organic matter of WAS and microalgae mixtures, was analysed by means of sCOD and VSS. Samples were filtered, and organic matter solubilisation was evaluated by means of sCOD of the filtrate using COD kits (previously described in section 3.3.4.3. - Chapter 3). TSS and VSS were calculated to assess organic matter conversion (previously described in section 3.3.4.1. - Chapter 3).

5.3. Results and discussion

5.3.1. Flocculation assays

Assuming that partial coagulation through natural processes could take place, such as biological growth and physical mixing (Adams et al., 1999), only flocculants were employed for microalgae harvesting. Polymeric flocculants agglomerate small flocs into bigger ones, creating larger, compact and denser aggregates that settle faster in comparison with the flocs obtained by coagulation, improving their removal form water (Wong et al., 2006).

Microalgae flocculation and later thermal pretreatment of the flocculated microalgae and WAS mixture was applied. As can be seen from Fig. 5.2, flocculant A reached the highest harvesting efficiency (95 ± 6%, at the lower dose), followed by flocculant B (94%, at the higher dose) and flocculant C (58 ± 9%, at the lower dose). The effectiveness and performance attained by the polyDADMAC flocculant was similar to the reported in other studies (Bolto and Gregory, 2007; Abo Markeb et al., 2019). Hence, flocculant A at 250 mg L^{-1} was selected for microalgae flocculation, before been mixed with WAS in the mixtures G, H and I; and their respective controls (mixtures M, N and O).

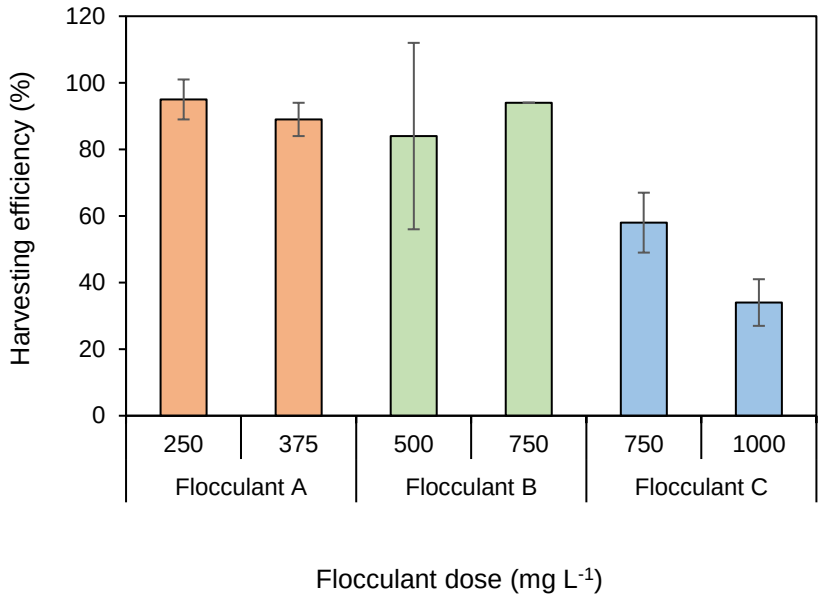


Figure 5.2 Harvesting efficiency (%) of microalgae suspension after the flocculation with flocculant A (polyDADMAC), B (cationic copolymer of acrylamide), and C (cationic polysaccharide obtained from chitosan) at their respective doses (mg L^{-1}). Results are given as mean ± relative standard deviation (n = 3).

5.3.2. Low temperature thermal pretreatment

Activated sludge was mixed with microalgae at three different ratios and incubated at 37 and 60 ºC during 24, 48 and 72 h, with the aim of assessing the combined effect of temperature and incubation time in the enzymatic activity of the WAS and the further solubilisation of the mixture.

Previous studies performing high temperature thermal pretreatment defined temperature as the main parameter determining biomass disintegration and further anaerobic biodegradability, over exposure time (Schwede et al., 2013; Sapkaite et al., 2017). In contrast to high temperature pretreatment, time seemed to have a main influence in WAS disintegration at low temperatures (Borges and Chernicharo, 2009; Appels et al., 2010; González-Fernández et al., 2012c). This

fact could be associated to the exposure time required for the hydrolysis considering the diversity of hydrolytic bacteria and microalgae structure and composition, ranging from hours to few days (Carrillo-Reyes et al., 2016). In this work, the solubilisation of the mixtures was evaluated along three days with the aim of providing enough contact time between hydrolytic bacteria from the sludge and the biomass from the mixture.

Enzymatic pretreatment to enhance biomass hydrolysis could be performed using commercial exogenous enzymes or endogenous enzymes from microorganisms. Higher sCOD is released when using enzymatic cocktail rather than single enzymes in the pretreatment (Hom-Diaz et al., 2016; Córdova et al., 2018). A promising strategy to overcome microalgae cell wall resistance is bacterial bioaugmentation employing bacterial cultures from different low-cost substrates, such as WAS, that contain a diversity and quantity of active microorganisms providing a constant source of in-situ or endogenous enzymes (Carvajal et al., 2013; Lü et al., 2013; Mahdy et al., 2015b). Particle and colloidal wastes in activated sludge are degraded by both endoenzymes and exoenzymes (esterases, aminopeptidases, lipases, glucosidases, etc.) generated by bacteria, transforming complex organic matter into soluble low-molecular weight compounds assimilated by microorganisms to be used as energy and carbon sources (Nybroe et al., 1992; Guellil et al., 2001; Gerardi, 2003). Sources of exoenzymes in sewage sludge could be the sewage effluent, the activated sludge via cell autolysis and enzymes excreted by cells (Nybroe et al., 1992). It could be assumed that hydrolytic exoenzymes could be mostly found on the EPS matrix surrounding activated sludge flocs (Frølund et al., 1995). Those enzymes could hydrolyse microalgae cell wall with the subsequent release of microalgae intracellular organic matter and further increase methane production (Sialve et

al., 2009; Wang et al., 2013; Ajeej et al., 2015). Moreover, it is assumed that once microalgae cells are damaged by a certain pretreatment, their content might be available to be degraded by hydrolytic enzymes (Schwede et al., 2013), and then the organic content could get inside the bacteria cell wall for its further digestion by endoenzymes (Gerardi, 2003). In view of that, prior hydrolysis of the substrates is desirable to let them available for subsequent bacterial and archaeal transformation into biogas (Córdova et al., 2018).

As shown in Fig. 5.3a and in Table 5.3, minor changes were detected in sCOD and VSS during the pretreatment at 37 °C. For mixture A, sCOD achieved its maximum value after 24 h pretreatment, increasing 2.2-fold, and decreased to 1.8 and 1.4-fold after 48 and 72 h pretreatment, respectively. The same parameter showed a gradually growth along time for mixture B, reaching a maximum 2.3-fold increase by 72 h pretreatment. On the other side, in mixture C sCOD increase 1.2-fold after 24 h and remains slightly constant during the treatment. For 37 °C pretreatment, time effect over the three ratios of the mixtures was similar in comparison with the same mixtures without thermal pretreatment (Mix J, K and L, Fig. 5.3c), suggesting that this temperature is not enough to increase organic matter solubilisation. This result is coherent with Prorot et al. (2011), suggesting that temperatures above 50ºC are required for solubilisation of WAS.

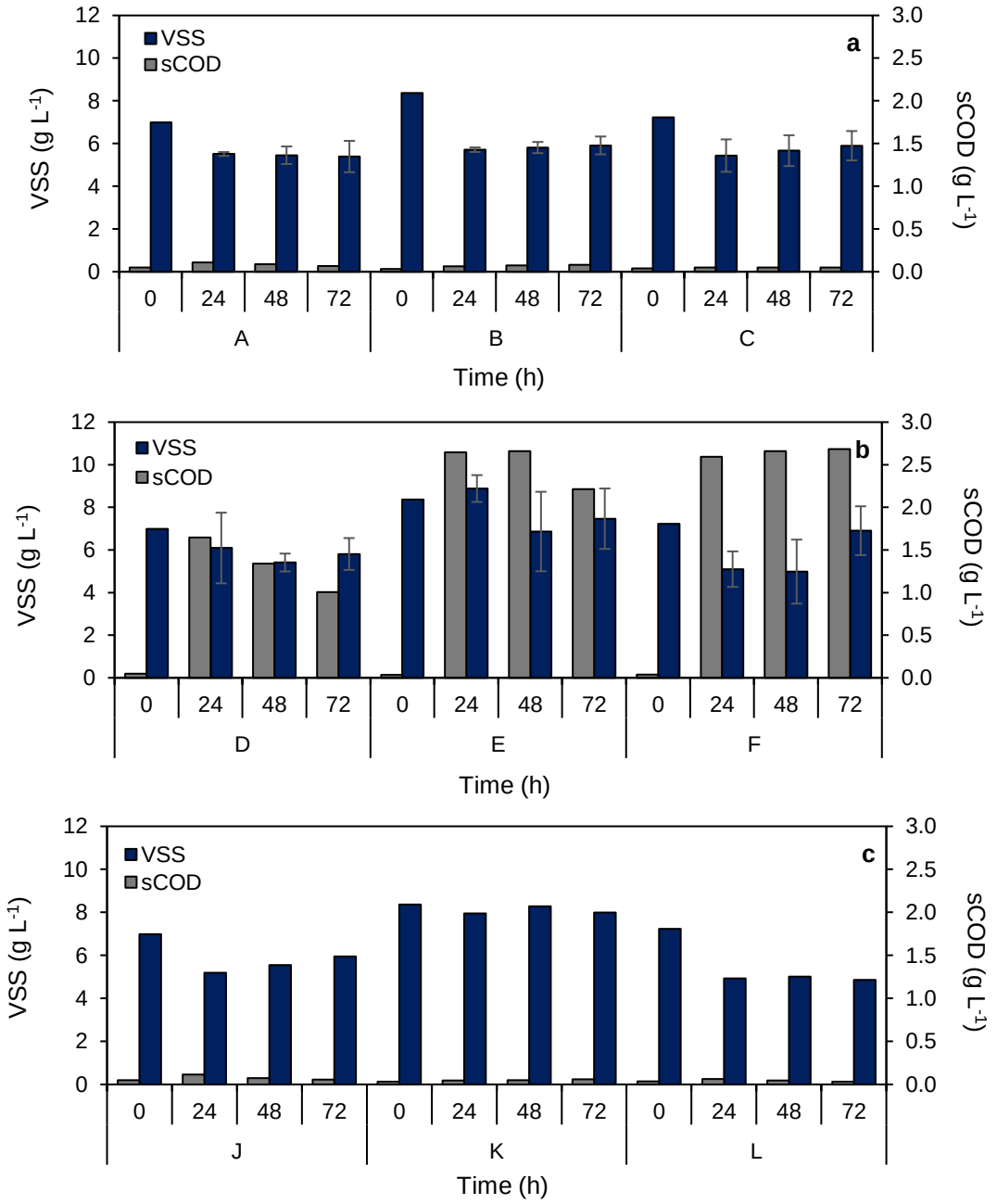


Figure 5.3 VSS and sCOD evolution during the pretreatment for mixtures containing WAS and non-flocculated microalgae(ratios are expressed in v:v basis. (a) Mixtures A (50:1), B (25:1) and C (10:1) pretreated at 37 °C; (b) Mixtures D (50:1), E (25:1) and F (10:1) pretreated at 60 °C; and (c) Mixtures J (50:1), K (25:1) and L (10:1) without thermal pretreatment (control at 20 °C). For VSS, results are given as mean ± standard deviation (n = 3).

Table 5.3 VSS and sCOD evolution for the pretreated mixtures along time. For VSS, results are expressed as mean ± standard deviation (n = 3).

Mixture Id	WAS:microalgae ratio (v:v)	Microalgae	Temperature (°C)	VSS (g L^{-1})				sCOD (g L^{-1})			
				0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
A	50:1	Non-flocculated	37	7.0	5.5 ± 0.1	5.5 ± 0.4	5.4 ± 0.7	0.05	0.11	0.09	0.07
B	25:1	Non-flocculated	37	8.4	5.7 ± 0.1	5.8 ± 0.3	5.9 ± 0.4	0.03	0.06	0.07	0.08
C	10:1	Non-flocculated	37	7.2	5.4 ± 0.8	5.7 ± 0.7	5.9 ± 0.7	0.04	0.05	0.05	0.05
D	50:1	Non-flocculated	60	7.0	6.1 ± 1.7	5.4 ± 0.4	5.8 ± 0.7	0.05	1.65	1.34	1.01
E	25:1	Non-flocculated	60	8.4	8.9 ± 0.6	6.9 ± 1.9	7.5 ± 1.4	0.03	2.65	2.66	2.22
F	10:1	Non-flocculated	60	7.2	5.1 ± 0.8	5.0 ± 1.5	6.9 ± 1.1	0.04	2.59	2.66	2.69
G	50:1	Flocculated *	60	5.2	3.8 ± 1.5	4.7 ± 0.8	5.0 ± 0.8	0.18	1.1	2.01	1.92
H	25:1	Flocculated *	60	4.8	6.8 ± 0.7	6.1 ± 0.8	5.7 ± 0.3	0.18	1.1	2.62	2.67
I	10:1	Flocculated *	60	5.4	4.6 ± 0.8	4.2 ± 0.5	4.4 ± 0.6	0.18	1.1	2.32	2.36
Control											
J	50:1	Non-flocculated	37 and 60	7.0	5.2	5.6	6.0	0.05	0.11	0.07	0.06
K	25:1	Non-flocculated	37 and 60	8.4	8.0	8.3	8.0	0.03	0.05	0.05	0.06
L	10:1	Non-flocculated	37 and 60	7.2	4.9	5.0	4.9	0.04	0.06	0.05	0.03
M	50:1	Flocculated *	60	5.2	5.7	5.8	6.1	0.18	0.18	0.29	0.24
N	25:1	Flocculated *	60	4.8	5.0	5.2	5.3	0.18	0.18	0.28	0.22
O	10:1	Flocculated *	60	5.4	6.0	6.3	6.2	0.18	0.18	0.29	0.25

* Microalgae was flocculated with polyDADMAC (flocculant A) at 250 mg L^{-1}.

Significant variations took place in mixtures pretreated at 60 °C (mixtures D, E and F) using non-flocculated microalgae (Fig. 5.3b), indicating that solubilisation increase proportionally with temperature. Afterwards 24 h treatment, the sCOD rise to 33, 74 and 65-fold for mixtures D, E and F, respectively. For mixture E, the sCOD continued relatively constant up to 48 h, while for mixture F it was maintained practically steady from 24 h up to the end of the treatment. This rise in sCOD differed remarkably from control mixtures and could be associated with the enhancing effect of thermophilic temperature (60 °C) in the activity of hydrolytic bacteria from activated sludge, fostering organic solids solubilisation (Carrère et al., 2010). During this aerobic process, a variety of aerobes and facultative anaerobes microorganisms contribute simultaneously to substrate degradation (Gerardi, 2003). Furthermore, the higher sCOD at 60 °C could be explained by the increase of ammonium concentration (released from the hydrolysis of nitrogen-containing organic molecules) leading to the accumulation of free ammonia at higher levels, which might benefit WAS and microalgae disintegration (Pijuan et al., 2012; Wang et al., 2019). Nonetheless, at 72 h pretreatment, COD solubilisation was lower (20 and 62-fold) for mixtures D and E, respectively, showing that sCOD tends to decrease at longer incubation times. It could be hypothesized that once complex molecules were degraded by hydrolysis microorganisms to soluble compounds readily bioavailable, organic matter might be further oxidized by aerobic microorganisms using molecular oxygen as final electron carrier, obtaining CO_2, water and cellular growth (Carvajal et al., 2013). Influence of time in sCOD appear to be intensive during the first 24 h, with afterwards lesser variations. In this sense, Alzate et al. (2012) verified that after 24 h pretreatment of a microalgae-bacteria consortia, aerobic degradation of the organic matter was larger. In earlier studies Scenedesmus sp. was pretreated at 70 and 90ºC during 3 h, and variations in sCOD along time

were associated to different stages in organic matter degradation with specific chemical reactions taking place at that temperature (González-Fernández et al., 2012c).

Regarding the control mixtures at 20 ºC employing non-flocculated microalgae (Fig. 5.3c), temperature have a minor effect in sCOD rise. sCOD increased by 2.3, 1.4 and 1.7-fold after 24 h pretreatment for mixtures J, K and L, respectively (Table 5.3). However, for mixtures J and L, longer incubation time tended to diminish sCOD. As a general behaviour for all control mixtures, VSS diminished at 72 h pretreatment.

The initial values of sCOD in the mixtures G, H and I containing WAS and flocculated microalgae (Fig. 5.4a) were 3.6, 5 and 4.5-times higher than the mixtures of the same ratio without previous flocculation of the microalgae (mixtures A to F, Table 5.3). The same applied to control mixtures M, N and O (Fig. 5.4b). Higher initial solubilisation might be associated with the faster biodegradation of organic matter and the organic flocculant during the previous harvesting treatment. During the first day, sCOD increase in the same proportion (around 6.1 times) for the three mixtures G, H, and I. At 48 h, solubilisation was almost doubled in the three mixtures, keeping stable until the 72 h. The control mixtures incubated at 20 ºC employing flocculated microalgae (M, N and O, Fig. 5.4b) shown constant sCOD values up to the 24 h, increasing solubilisation ca. 1.5 times after two days pretreatment. Later, solubilisation showed a slightly tendency to diminish.

Mixtures E and F (pretreated at 60ºC with non-flocculated microalgae) achieved a higher solubilisation after 24 h and remained almost stable up to 48 h. On the contrary, mixtures H and I (pretreated at 60 ºC with flocculated microalgae)

attained higher solubilisation at 48 h and it continued stable until the end of the pretreatment. Considering that sCOD along time was lesser in mixtures D and G, it could be argued that solubilisation increase proportionally with higher microalgae proportion in the mixture.

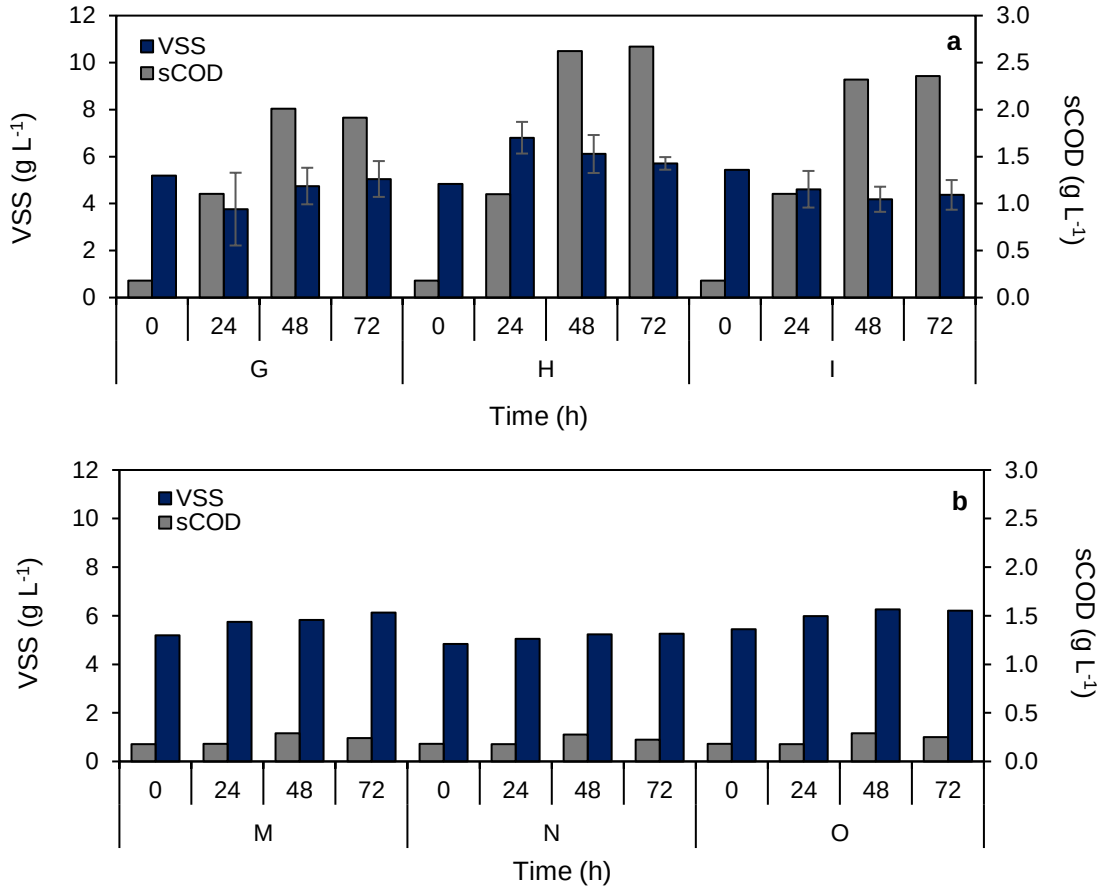


Figure 5.4 VSS and sCOD evolution during the pretreatment for mixtures containing WAS and microalgae flocculated with polyDADMAC (ratios are expressed in v:v basis). (a) Mixtures G (50:1), H (25:1) and I (10:1) pretreated at 60 °C; and (b) Mixtures M (50:1), N (25:1) and O (10:1) without thermal pretreatment (control at 20 °C). For VSS, results are given as mean ± standard deviation (n = 3).

Moreover, when comparing both pretreatments (with and without previous microalgae flocculation step) after 72 h at 60 ºC, a higher increase in sCOD was achieved when non-flocculated microalgae were used (20-fold, 62-fold and 67-

fold for mixtures D, E and F, respectively). Since initial sCOD values are higher when employing flocculated microalgae in the mixtures, sCOD rise after 72 h pretreatment is lower (11-fold, 15-fold and 13-fold for mixtures G, H, and I, respectively).

Although there are several studies that evaluate the impact of thermal pretreatments in WAS or microalgae substrates, few previous studies analyse the effect of low temperature thermal pretreatment when WAS and microalgae are mixed. Regarding microalgae, *Chlorella* sp. and *Scenedesmus* sp. biomass are mainly composed by proteins (52%), followed by carbohydrates (16%) and a minor amount of lipids (9%) (Solé-Bundó et al., 2017a). Despite of the minor fraction of lipids in those algae (González-Fernández et al., 2012c), lipids possess a higher energy content in comparison with proteins and carbohydrates, but a slow hydrolysis rate (Sialve et al., 2009). Besides, cell wall composition is strain-specific: species of genus *Chlorella* possess a hemicellulose-cellulose cell wall, while *Scenedesmus* present a complex and more resistant cell wall integrated by several layers containing sporopollenin, restraining the hydrolysis step and further methane conversion (Yen and Brune, 2007; González-Fernández et al., 2012c). According to Ometto et al. (2014) only microalgae thermal pretreatments at temperatures higher than 165 °C could achieve their complete solubilisation. However, other authors studied the auto-hydrolysis pretreatment of *Chlorella* sp. and *Scenedesmus* sp. at 50 °C during 24 and 48 h, attaining 17% and 5.7% rise in sCOD after 48 h incubation, respectively (Mahdy et al., 2014). Results from other authors verify that pretreatments at low temperature can attain important rise in solubilisation (Passos et al., 2013).

Concerning the secondary sewage sludge, Appels et al. (2010) stated that thermal pretreatment of sludge at 70 °C during 60 min just enhanced sCOD by 3 times.

Prorot et al. (2011) analysed the effect of low temperature thermal treatments (65 to 95 ºC, 20 min) on the floc structure of WAS, determining that macro-flocs were disaggregate but not complete broken. It could be presumed that a low temperature thermal pretreatment performed during short times could led to lower WAS disintegration and COD solubilisation. For this reason, in this study the incubation of the mixtures during longer time was tested.

The results obtained in soluble organic matter availability after treatments of the six mixtures at 60 °C (mixtures D to I) were significant. The optimal time and temperature conditions that increase solubility of the mixture were identified for assess the improvement in biogas yield. Pretreatment during 24 h at 60 °C for mixtures ratio of 25:1 were selected as the optimal conditions for the low temperature thermal pretreatment of WAS and microalgae mixtures. In this sense, mixture E (using non-flocculated microalgae) and H (employing previously flocculated microalgae) were selected to assess the effect of the pretreatment in the biogas yield.

5.3.3. AcoD of WAS and algal biomass

BMP tests were performed for mixture E and H obtained in previous experiments (Table 5.4). Additionally, biogas production was compared with a control mixture without thermal pretreatment employing the same ratio of WAS and non-flocculated microalgae. After 52 days, a methane yield of 153.6 ± 1.3 NmL CH_4 g VS^{-1} was obtained in control reactors, representing 34% (101.7 ± 13.8 NmL CH_4 g VS^{-1}) and 51% (75.8 ± 3.5 NmL CH_4 g VS^{-1}) more production in comparison with mixture H and E, respectively (Fig. 5.5). During the first 10 days of the digestion, the methane production by mixtures E and H was similar, later mixture H showed a slightly increment. Methane content in biogas (around

72 - 77%) was similar to the reported values for the AD of only microalgae (69-75%), pointing out an optimal biomass conversion into methane and the maintenance of the energy content of the biogas (Sialve et al., 2009; Appels et al., 2010). Additionally, the pH of the mixture after AD remains around 7 and 7.2, indicating that steady conditions were reached. The concentration of VFAs remaining in all reactors after the AcoD was low, indicating that the cease in biogas production at day 25 was not linked to VFAs accumulation.

Table 5.4 Conditions obtained from the pretreatments to assess the AcoD of the WAS:microalgae mixtures.

Mixture Id.	**Mixture ratio (WAS:microalgae)**	**Microalgae**	**Pretreatment conditions**	**sCOD increment (X-fold)**
E	25:1	Non-flocculated	60 °C, 24 h	74.1
H	25:1	Flocculated *	60 °C, 24 h	6.1
Control	25:1	Non-flocculated	20 °C, 24 h	1.4

* Microalgae flocculated with polyDADMAC (flocculant A).

The hydrolysis constant calculated from Eq. 3.4 for the studied mixtures varied among 0.07 and 0.11 d^{-1} (Table 5.5), being slightly higher when non-flocculated microalgae were used in the mixture. This values are in accordance with the K_H values determined by other authors for pure microalgae species, such as the hydrolysis rate constant of 0.1 d^{-1} found for *Chlorella vulgaris* (Mendez et al., 2013), and 0.09 d^{-1} for *Botryococcus braunii* (Neumann et al., 2015). The latter authors found similar hydrolysis constant when co-digesting a mixture of 75% of WAS and 25% of lipid-spent microalgae *Botryococcus braunii* (K_H= 0.07 d^{-1}). Lee et al. (2017) determined the hydrolysis rate in the co-digestion of several ratios of WAS and microalgae using a modified first-order kinetic model, showing that a ratio of 90% of WAS and 10% of microalgae achieved the highest hydrolysis

coefficient (K_H = 0.14 d^{-1}); while as microalgae proportion in the mixture increased more than 25%, the hydrolysis rate tended to diminish (K_H = 0.07 – 0.12 d^{-1}). This reduction could be attributed to the microalgae cell wall that hampers hydrolysis.

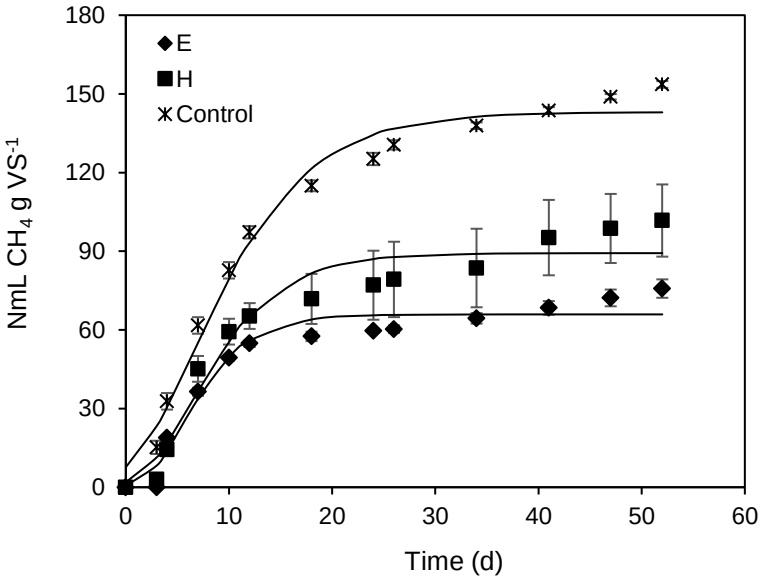


Figure 5.5 Net methane production of mixtures E (WAS:non-flocculated microalgae), H (WAS:microalgae flocculated with polyDADMAC -flocculant A-), and the control (WAS:non-flocculated microalgae without thermal pretreatment). Dots represent experimental data and curves are data estimated by Gompertz model. Results are given as mean ± standard deviation (n = 3).

The Gompertz model fits suitably the experimental data. The lag phase (λ) determined by the model was ca. 2 days for mixtures E and H, while it was 0.5 d for the control. A longer lag phase could be a sign that the substrate content was not straightforwardly hydrolysed, indicating that microorganisms from the inoculum might need more time to acclimate to the substrates obtained after the pretreatment, lengthen the beginning of the methane production. Carvajal et al. (2013) detected an inhibition effect that generates a delay of 0.6 and 2.4 d after the pretreatment of WAS at 55 °C for 12 and 24 h, respectively. However, this

delay is lower than lag phases obtained by Olsson et al. (2014) in the co-digestion of different proportions of wet and dry microalgae mixtures with sewage sludge at 37 °C, ranging from 2.1 to 13.7 d.

From these results, it can be inferred that conditions that enhance solubility did not generate a proportional increase in methane yield. After the thermal pretreatment, solubilized organic matter might be rapidly consumed by active microorganisms from the sludge as a carbon source, lowering the organic matter content available to be anaerobically degraded for energy recovery. In coherence with this results, other authors stated that treating the sludge under thermophilic conditions and a high oxygen content, foster organic matter consumption by aerobic microorganisms from the WAS, being no longer available for methane production in the next AD process (Carvajal et al., 2013).

Table 5.5 Parameters after AD of mixtures E, H, and control. Results are given as mean ± standard deviation (n = 3).

Mix. Id.	pH	VFAs (mg L^{-1})			Mean CH_4 content (%)	Experimental net CH_4 (NmL)	Experimental net CH_4 yield (NmL CH_4 g VS^{-1})	Pmax (NmL CH_4 g VS^{-1})	Rmax (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})
		Acetic acid	Butyric acid	Valeric acid								
E	7.2 ± 0.1	n.d.	2.4	111.8	77	167.7 ± 7.8	75.8 ± 3.5	65.9 ± 2.4	6.8 ± 1.4	2.0 ± 0.9	0.96	0.11 ± 0.03
H	7.0 ± 0.2	4.0	1.9	69.1	72	225.2 ± 30.5	101.7 ± 13.8	89.3 ± 3.7	6.9 ± 1.4	1.7 ± 1.3	0.95	0.08 ± 0.02
Control	7.0 ± 0.1	n.d.	4.0	n.d.	73	340.1 ± 2.8	153.6 ± 1.3	143.1 ± 3.6	8.4 ± 0.9	0.5 ± 0.8	0.98	0.07 ± 0.01

Mix. = mixture. n.d. = not detected.

Table 5.6 shows previous reported results on sCOD and methane production increase after a low temperature thermal pretreatment of microalgae, WAS and WAS-microalgae mixtures. In the case of pure microalgae cultures, some authors achieved an increase in sCOD but not in methane production when pretreating *Chlorella vulgaris* biomass at 50 °C for 24 h (Mahdy et al., 2014). In addition, these authors observed an increase in sCOD after a thermo-alkaline pretreatment of *Chlorella* and *Scenedesmus* sp. using NaOH at 50 °C, followed by a low methane production enhancement. They attributed this behaviour to the release of soluble cell wall exopolymers rather than intracellular organic matter release after cell wall rupture. Likewise, González-Fernández et al. (2012) observed no disruption of the cell wall of Scenedesmus biomass after thermal pretreatment at 70 ºC, associating soluble organic matter rise (4 times higher) to the detachment of exopolymers from the microalgae cell wall. In this sense, the low biogas production in the mixtures E and H could be in concordance with results attained by these authors, despite the fact that microalgae consortia rather than pure cultures were employed in the present work. Results achieved by different authors regarding solubility and methane improvement after thermal pretreatment are in some way controversial. Alzate et al. (2012) pretreated a microalgae mixture as well as microalgae-bacteria consortia at 55 ºC for 12 and 24 h. When a microalgae mixture was employed, sCOD increased (11%) after 24 h, but methane productivity diminished (8%); while in the case of microalgae-bacteria consortia, both solubility and methane productivity increased (29% and 5%, respectively) which could be associated to the hydrolytic enzymes excreted by bacteria. Other authors pretreated different species of microalgae at low temperature (55, 75 and 95 ºC) during 15 h, obtaining an increase in methane production in consistency with biomass solubilisation (Passos et al., 2013). However, they determined that around 50% of the organic matter was not

anaerobically degraded. In the case of mixtures E and H, anaerobic biodegradability was 78 and 76%, respectively. This fact endorses the previous hypothesis that soluble organic matter was consumed after the thermal pretreatment, while left organic content was converted to methane.

Some authors studied the co-digestion of WAS and microalgae without previous pretreatment, reporting a synergetic effect at mesophilic conditions that boost biogas production (Olsson et al., 2014; Lee et al., 2017). Thorin et al. (2018) stated an average methane production of 317 ± 101 NmL g VS^{-1} from the co-digestion of 29 WAS and microalgae (pure cultures and polycultures) mixtures at mesophilic conditions. They outline a mean methane production of 304 ± 114 NmL g VS^{-1} for ten different activated sludges, meanwhile for thirteen different microalgae strains (pure cultures and polycultures) the generation was of 258 ± 106 NmL g VS^{-1} (high standard deviation is associated to the variety of biomass composition from the microalgae and WAS). On the other hand, Wang et al. (2013) performed the co-digestion of WAS and microalgae (*Chlorella* sp.) mixtures containing 0, 4, 11, 41 and 100% microalgae, obtaining a rather lower methane production for all the mixtures in comparison to WAS digested as a sole feed. However, they obtained a methane production of the mixtures 73 to 79% higher when comparing with AD of only microalgae. Therefore, an increase in solubilisation degree does not directly enhance methane productivity and is coherent with our results.

Table 5.6 Solubility and methane production enhancement after low temperature thermal pretreatment applied to microalgae, WAS, and WAS-microalgae mixtures.

Substrates	**Thermal pretreatment conditions**	**Increase in solubility after the pretreatment ***	**Methane production (NmL CH_4 g VS^{-1})****	**Methane yield increase after the pretreatment**	**Reference**
Microalgae					
Scenedesmus sp.	70 ºC 3 h	4-fold	85 (in COD basis)	1.1-fold	(González-Fernández et al., 2012c)
	90 ºC 3 h	4.4-fold	170 (in COD basis)	2.2-fold	(González-Fernández et al., 2012c)
Chlorella sp.	50 ºC 24 h	16.2%	-	0%	(Mahdy et al., 2014)
	50 ºC 24 h 5% w/w NaOH	18.2%	-	13%	(Mahdy et al., 2014)
Scenedesmus sp.	50 ºC 24 h	5.7%	-	0%	(Mahdy et al., 2014)
	50 ºC 24 h 5% w/w NaOH	16.8%	-	20%	(Mahdy et al., 2014)
Microalgae mixture (40% *Chlamydomonas*, 20% *Scenedesmus*, 40% unknown microalgae)	55 ºC 24 h	11%	252 ± 5	- 8%	(Alzate et al., 2012)
Microalgae (mainly *Microspora*)-bacteria consortia	55 ºC 24 h	29%	266 ± 2	5%	(Alzate et al., 2012)
Scenedesmus sp., *Coelastrum* sp.	80 ºC 3 h	13%	280 ± 9	11%	(Kinnunen and Rintala, 2016)
Microalgal (*Chlamydomonas*, *Chlorella*, *Ankistrodesmus*, *Monorraphidium*, *Scenedesmus* and diatoms *Nitzchia*) and bacterial biomass	55 ºC 15 h	400%	124.6 ± 3.3	13%	(Passos et al., 2013)
	75 ºC 15 h	1048%	160.4 ± 0.7	53%	(Passos et al., 2013)
	95 ºC 15 h	1188%	169.9 ± 3.7	60%	(Passos et al., 2013)

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(continued)

Substrates	Thermal pretreatment conditions	Increase in solubility after the pretreatment *	Methane production (NmL CH_4 g VS^{-1})**	Methane yield increase after the pretreatment	Reference
WAS					
WAS	70 ºC 1 h	2.9-fold	22.4	1.4%	(Appels et al., 2010)
	90 ºC 1 h	25.6-fold	240.4	11-fold	(Appels et al., 2010)
Polyacrylamide flocculated WAS	75 ºC pH 11 17.5 h	-	210.8	2-fold	(Liu et al., 2019)
Textile dying sludge	60 ºC 1 h	0.2-fold	82.9	2%	(Chen et al., 2017)
	70 ºC 1 h	1.7-fold	130.5	61%	
	90 ºC 1 h	4.2-fold	156.4	93%	
WAS-microalgae mixtures					
Raw sludge-*Chlorella* sp.	No pretreatment	-	116 ± 3	-	(Kim and Kang, 2015)
WAS (75%)-lipid-spent *Botryococcus braunii (25%)* (in VS basis)	No pretreatment	-	374 ± 13	-	(Neumann et al., 2015)
Undigested sewage sludge (63%)-*Scenedesmus* and *Chlorella vulgaris* (37%) (in VS basis)	No pretreatment	-	408 ± 16	-	(Olsson et al., 2014)
WAS-*Chlorella* sp. 4% (in VS basis)	No pretreatment	-	257	-	(Wang et al., 2013)
WAS-*Chlorella* sp. 11% (in VS basis)	No pretreatment	-	254	-	(Wang et al., 2013)
WAS-*Chlorella* sp. 41% (in VS basis)	No pretreatment	-	260	-	(Wang et al., 2013)
WAS (79%)-Micractinium sp. (21%) (in VS basis)	No pretreatment	-	236	-	(Wang and Park, 2015)
WAS (79%)-*Chlorella* sp. (21%) (in VS basis)	No pretreatment	-	253	-	(Wang and Park, 2015)
Mixture E	60 ºC 24 h	74-fold	75.8 ± 3.5	-51%	This study
Mixture H	60 ºC 24 h	6-fold	101.7 ± 13.8	-34%	This study

* In sCOD basis. ** Mesophilic conditions were considered.

Besides, organic matter solubilised after the thermal pretreatment could be later oxidized to complex molecules (Mahdy et al., 2014) with low and/or slow anaerobic digestibility. Sapkaite et al. (2017) applied a thermal hydrolysis pretreatment to a WAS from a municipal WWTP showing a 36% rise in solubility at 170 °C during 10 min and 140 °C during 50 min. Nevertheless, methane production decreased after 30 min pretreatment and 160 °C, indicating that solubilisation achieved did not led to higher anaerobic digestibility due to the presence of soluble but non-biodegradable substances (melanoidins) generated at high temperature pretreatment, inhibiting the AD, and reducing the methane yield. Those recalcitrant compounds are created by the Maillard reaction by non-enzymatic chemical reactions of sugars and proteins during thermal hydrolysis of sludge at temperatures higher than 140 °C (Barber, 2016) or during extended pretreatment time in low temperature thermal pretreatments (<100 °C) (Kor-Bicakci and Eskicioglu, 2019), hindering the degradation of other organic substances (Zhen et al., 2017). Likewise, minor methane yield in mixtures E and H may be attributable to the presence of low biodegradable compounds.

Regarding the influence of the flocculant in the AD, it is known the negative impact of cationic polyacrylamide accumulation in the WAS after flocculation, affecting the sludge disintegration, as well as, the overall efficiency of the AD process as a consequence of its degradation metabolites (Wang et al., 2018; Luo et al., 2020). However, Liu et al. (2019) reported an important rise in methane potential of polyacrylamide-flocculated WAS after a thermal-alkaline pretreatment (75 °C, pH 11), indicating that the pretreatment distorted the structure of the flocculant, improving its degradation by anaerobic microorganisms. In this work a flocculant was used, and the higher methane production in mixture H in comparison to mixture E could be related with the

favourable effect of the thermal pretreatment in the anaerobic degradation of the flocculant. On the other hand, the high sCOD achieved during the thermal pretreatment at 60 °C for mixture E might have generated a higher ammonium release and free ammonia accumulation, thus inhibiting methanogenesis (Zhao et al., 2018; Wang et al., 2019). This fact could explain the lowest methane yield obtained in WAS and non-flocculated microalgae mixture.

In brief, results in this study indicate that at major sCOD achieved (mixture E > mixture H > control), methane production decreases (mixture E < mixture H < control). The absence of proportionality between organic matter solubilisation after the thermal pretreatment (determined by sCOD) and organic matter digestibility (determined by methane production) could be ascribed to: 1) a faster consumption of bioavailable compounds as a result of the higher enzymatic activity of thermophilic bacteria, tending to diminish the organic content available for the anaerobic stage; 2) the release of soluble microalgae cell wall exopolymers that might increase sCOD with no influence in methane production; 3) the conversion of solubilized organic matter into complex molecules with scarce anaerobic biodegradability; and 4) the inhibition of methanogens due to toxicity of ammonia released. According to Wang et al. (2019) and bearing in mind that in our study the thermal pretreatment at 60 °C did not enhance the methane yield of the mixtures, it might be interesting to study the fermentative hydrogen production, since hydrogen consumers could have been inhibited due to the high free ammonia concentration.

5.4. Conclusions

In this chapter, low temperature thermal treatments were applied to WAS and microalgae mixtures as a previous step to AD. Microalgae harvesting showed

good clarification results when a cationic polymer of diallyl dimethyl ammonium chloride free of acrylamide was used as an organic flocculant.

Higher solubility of WAS and microalgae mixtures was achieved after the pretreatment at 60 °C in comparison with the pretreatment at 37 ºC, indicating that solubilisation increases proportionally with temperature. Initial sCOD in the mixtures containing flocculated microalgae was higher than in the mixtures of the same ratio without previous flocculation of the microalgae. Besides, the effect of longer incubation time of the mixtures (72 h) in their solubilisation was tested. When mixtures with non-flocculated microalgae were pretreated at 60 °C, influence of time in sCOD was intensive during the first 24 h, with lower later variations. Regarding to WAS:microalgae ratios, in general solubilisation increases proportionally with higher microalgae volume in the mixture.

Pretreatment at 60 °C during 24 h for mixtures ratio 25:1 using flocculated and non-flocculated microalgae were selected as the optimal pretreatment conditions to assess its effect on the methane potential. The larger methane yield obtained for the WAS and flocculated microalgae mixture could be associated with the positive effect of the low temperature thermal pretreatment in the degradation of the flocculant by anaerobic microorganisms. Nonetheless, the hydrolysis rate was higher when co-digesting the thermally pretreated WAS and non-flocculated microalgae. In comparison to the control without pretreatment, the anaerobic digestibility of the high solubilised mixtures was not enhanced after the low temperature thermal pretreatment using flocculated and non-flocculated microalgae.

CHAPTER 6.

ALKALINE AND ENZYMATIC PRETREATMENTS

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Abstract

In this study, alkaline and enzymatic pretreatments of a microalgal culture mainly constituted by *Chlorella* sp. and *Scenedesmus* sp. and cultivated in wastewater from a winery wastewater treatment plant were assessed. Microalgal enzymatic pretreatments were expected to overcome algal recalcitrancy before anaerobic digestion. The results indicated that pH-induced flocculation at pH 10 and 11 did not enhance microalgal harvesting and solubilisation, achieving a performance similar to that of natural sedimentation. Enzymatic hydrolysis of algal biomass was carried out using three commercial enzymatic cocktails (A, B, and C) at two enzymatic doses (1% and 2% (v/v)) over 3 h of exposure time at 37 °C. Since pretreatments at a 1% dose for 0.5 h and 2% dose for 2 h achieved higher solubilisation, they were selected to evaluate the influence of the pretreatment on microalgal anaerobic digestibility. Biochemical methane potential tests showed that the pretreatments increased the methane production of the raw algal biomass 3.6- to 5.3-fold. The methane yield was 9 - 27% higher at the lower enzyme dose. Hence, microalgae pretreated with enzymes B and C at a 1% dose were co-digested with waste activated sludge (WAS). The results indicated that even when the enzyme increased the methane yield of the inoculum and the WAS, the methane yield of the raw microalgae and WAS mixture was not significantly different from that obtained when algae were enzymatically pretreated. Nonetheless, co-digestion may achieve the goals of a waste recycled circular bioeconomy.

6.1. Introduction

Diverse techniques have been studied for microalgae harvesting. Among them, centrifugation, filtration, and chemical flocculation are energy-intensive and resource-demanding harvesting techniques that could render microalgal biomass production economically infeasible. Alkaline flocculation is a harvesting method that induces microalgal concentration by increasing the pH of the medium (Branyikova et al., 2018; Wan et al., 2015), thus avoiding the addition of chemical flocculants. Microalgal cells possess a negative surface charge that arises from deprotonated carboxylate, phosphate, and hydroxyl functional groups, and mutual repulsion between anionic microalgae creates stable suspensions in water (Bilal et al., 2018; Brady et al., 2014). Protonation and deprotonation of functional groups at microalgal surfaces rely on the culture conditions and microalgal growth phase (González-Fernández et al., 2013). Flocculation via pH adjustment is stimulated by H^+ when changing the H^+/OH^- ratio in the medium (Brady et al., 2014). When the pH increases to 9.5 - 11, some naturally available cations present in the medium, such as Mg^{2+} or Ca^{2+}, can precipitate and form positively charged precipitates that can interact with the negatively charged microalgal surface, allowing microalgal cells to flocculate through charge neutralisation and/or by a sweeping mechanism (Brady et al., 2014; Muylaert et al., 2015). Normally, the concentration of magnesium in water is adequate for production of microalgal flocculation when the pH of the medium is increased by the addition of a base (Vandamme et al., 2016). Afterwards alkaline flocculation, the supernatant can be recovered and reused after pH neutralization, and the quality of the harvested microalgae must be check to assess its feasibility to be employed in a defined future use (Barros et al., 2015; Li et al., 2020). Despite pH-induced flocculation is more expensive compared to gravity-based sedimentation, it is an

interesting method to pre-concentrate microalgal biomass due to its simplicity, low cost, and low energy consumption (Li et al., 2020). In this study, alkaline flocculation was assessed for microalgal concentration.

Considering that anaerobic digestion (AD) of microalgae biomass is typically restrained to the inherent nature of microalgae, a pretreatment step is recommended for biomass hydrolysis to improve the accessibility of anaerobic microorganisms and increase their digestibility for biogas production. Several pretreatment methods have been studied for microalgal cell wall disruption (Passos et al., 2014; Kendir and Ugurlu, 2018). For instance, thermal and ultrasound pretreatments break or deform microalgal cell walls without digestion (Ometto et al., 2014). Compared with thermal, mechanical, and thermochemical pretreatments, enzymatic hydrolysis is a biological treatment that digests microalgal cell walls, making them permeable and liberating intracellular compounds or improving their accessibility to microorganisms (Gerken et al., 2013). Enzymatic pretreatment is performed under mild conditions, while reduces or eliminates toxic compound formation, requires low energy consumption, and keeps downstream processing costs low (Mahdy et al., 2016; Zabed et al., 2019). Enzymatic pretreatment is more specific in cell wall hydrolysis due to enzyme specificity to a certain substrate. In this sense, the appropriate enzyme(s) could be selected according to the target microalgal species. For instance, some studies reported the use of pectinases to degrade *Scenedesmus obliquus* (Ometto et al., 2014), and proteases for degradation of proteins in *Porphyridium cruentum* (Kendir and Ugurlu, 2020) and *Chlorella vulgaris* (Mahdy et al., 2016). Meanwhile, carbohydrases are among the typically employed enzymes for microalgae enzymatic pretreatment before AD. Cellulase was used for the degradation of the cellulose inner wall layer of the marine microalgae

Nannochloropsis sp. (Maffei et al., 2018), and other carbohydrase enzymes were used for *Chlorella vulgaris* and *Scenedesmus* sp. hydrolysis (Mahdy et al., 2015b). As other authors have argued, a higher soluble chemical oxygen demand (sCOD) is obtained when using enzymatic cocktails due to a greater variety of enzymes that could interact with the microalgal cell wall (Ometto et al., 2014; Carrillo-Reyes et al., 2016), thus contributing to enhanced digestibility for biogas production. In this work, enzymatic pretreatments were tested using three different catalyst cocktails and diverse dosages.

In addition to microalgal pretreatment, biogas production can be upgraded by co-digestion of microalgae with other carbon-rich substrates, such as waste activated sludge (WAS) (Beltrán et al., 2016a; Thorin et al., 2017). Co-digestion of both substrates promotes the integration of WWTP facilities and microalgae-based systems, thus improving the economic feasibility of wastewater treatment (Solé-Bundó et al., 2019).

This chapter aims to evaluate the efficiency of alkaline and enzymatic pretreatment methods for microalgal concentration and solubilisation, respectively, as well as algal biomass valorisation through anaerobic co-digestion (AcoD) with WAS generated by the winery company. This study provides useful information since it is the first to address the effects of co-digestion of microalgae enzymatically pretreated as a co-substrate to WAS for waste streams valorisation in WWTPs.

6.2. Materials and methods

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

6.2.1. Substrates

Microalgal biomass was cultivated in 9 L column PBRs. Samples were taken from the reactors when microalgae reached the exponential growth phase. PBRs were fed with secondary effluent from a WWTP of the winery company. Inlet wastewater characterisation throughout a year is presented in Table 6.1. PBRs were located inside a greenhouse chamber in which illumination was naturally provided, and the temperature was approximately 20 ± 10 °C. Photosynthetic microalgae cultures include predominantly *Scenedesmus* sp. and *Chlorella* sp. (Image 6.1) microscopically identified as previously explained (section 5.2.1. - Chapter 5).

The WAS used in AcoD assays was obtained from the aerobic biological reactors of the company WWTP. A defined WAS and microalgal mixture (WAS:RM) composed of 93% WAS and 7% microalgae on a VS basis was used in biochemical methane potential tests (BMP)-set 2 experiments (explained below). The proportion of the mixture was established according to the daily volume production of both substrates by the company.

Table 6.1 PBR inlet wastewater characteristics.

Parameter	Value
COD (mg L^{-1})	76.8
TOC (mg L^{-1})	19.2
TS (mg L^{-1})	43.0
VS (mg L^{-1})	33.0
N-NH_4^+ (mg L^{-1})	5.7
TN (mg L^{-1})	11.9
P-PO_4^{-3} (mg L^{-1})	2.5
pH	8.3

Image 6.1 Microscopic images of the microalgae culture in the PBR.

6.2.2. Set-up for pH-induced flocculation through pH adjustment

A flux diagram of the performed experiments is presented in Fig. 6.1a. A volume of 1000 mL of microalgal biomass from the PBRs was added into a 1 L glass graduated cylinder to assess the effect of the alkaline pretreatment on microalgal concentration and solubilisation. Microalgae were flocculated by adjusting the pH of the culture medium to pH 10 (PBR-10) and pH 11 (PBR-11) by addition of 5 N sodium hydroxide (NaOH) and stirring with a magnetic stirrer until the pH was adjusted to the desired value (pH-adjusted treatments, n = 3). Additionally, controls of microalgae biomass without pH adjustment (PBR-10-C and PBR-11-C) were employed (n = 1) to compare the effect of natural sedimentation. The initial sCOD, initial and final TSS and initial and final VSS in the supernatant were determined from the pH-adjusted treatments and the controls. A 1 mL sample of the supernatant was withdrawn from the middle of the clarified zone at the initial time and at diverse exposure times during the 7 days after pH adjustment to measure the optical density (OD_{680}) and calculate the clarification efficiency (CE) according to Eq. 6.1:

$$\mathrm{CE}\ (\%) = \mathrm{OD_i} - \mathrm{OD_t} \,/\, \mathrm{OD_i} \qquad \text{Eq. 6.1}$$

where OD_i is the initial OD_{680} before adjusting the pH of the culture, and OD_t is the OD_{680} of the culture at time t after adjusting the pH to the desired value. Solids clarification in the supernatant is an indirect measurement of the concentration of solids in the thickened zone, allowing experimental measurement over time without distorting the sample.

After 7 days of flocculation, the microalgal pellet (concentrated microalgal biomass) was separated from the supernatant, and the pH of the supernatant was measured and neutralized to pH 7 by adding 2 N hydrochloric acid (HCl). Final TSS, VSS, and sCOD were determined from the neutralized supernatant.

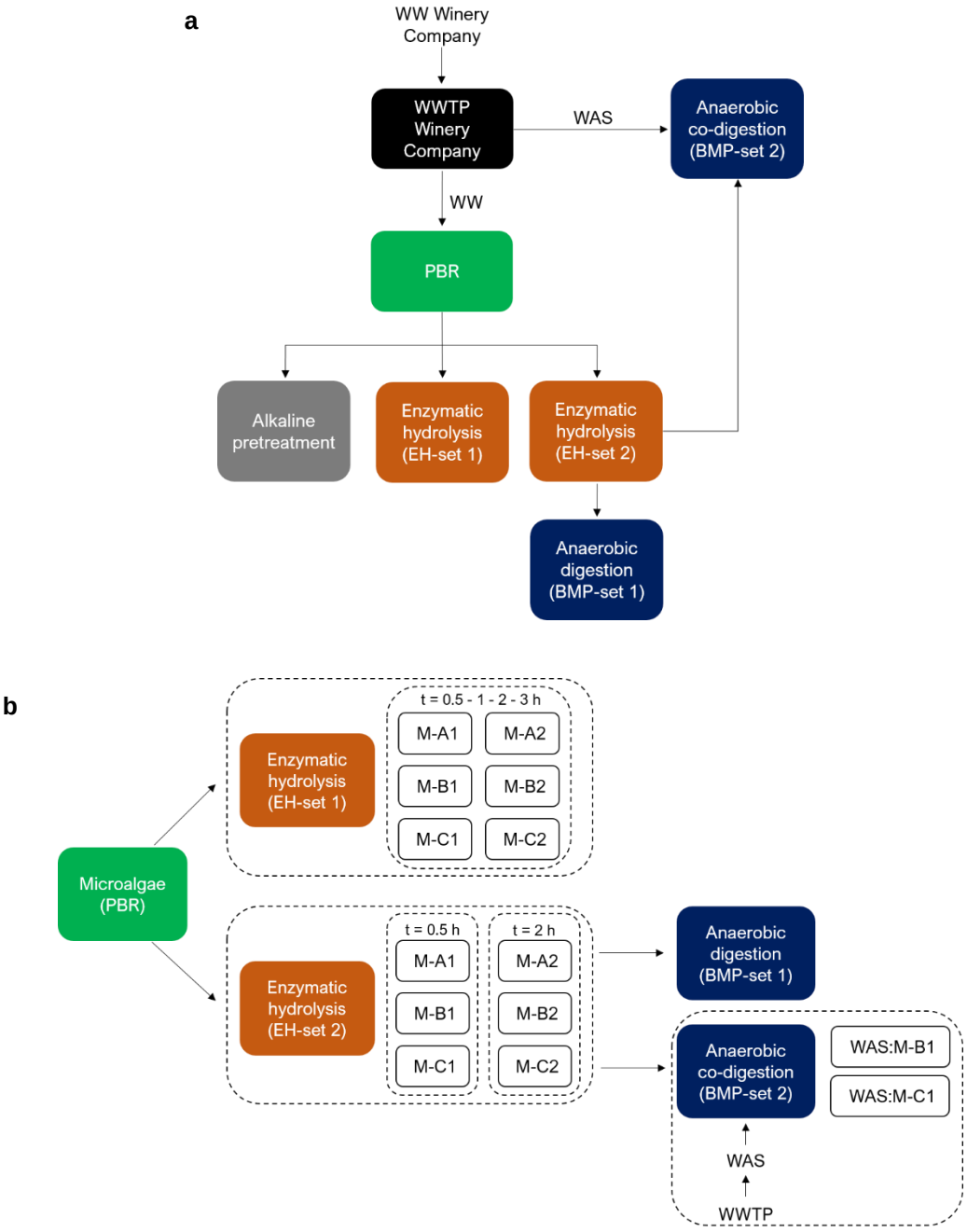

Figure 6.1 (a) Flux diagram of the performed experiments. (b) Schematic description of the experimental set-up for enzymatic pretreatments and biochemical methane potential tests, and their respective nomenclature. EH = enzymatic hydrolysis. M = microalgae. A1, B1, and C1 refer to pretreatments with the enzymes at a 1% dose while A2, B2, and C2 refer to pretreatments with the enzymes at a 2% dose.

6.2.3. Enzymatic pretreatment of algal biomass

Three enzymatic commercial preparations were applied to hydrolyse the microalgal biomass and increase microalgal digestibility: enzyme A, enzyme B, and enzyme C (a description of the enzymes is shown in Table 3.1 – Chapter 3). Enzyme A (Passos et al., 2016) and enzyme B are multi-enzymatic preparations composed of diverse enzymes. Enzymes A, B, and C were tested at two doses of 1% and 2% (v/v) to identify the following pretreatment methods (enzyme name and dose): A1, B1, C1, A2, B2, and C2. Thus, the pretreatment methods applied to the microalgal biomass (M) were identified as M-A1, M-B1, M-C1, M-A2, M-B2, and M-C2. A volume of 100 mL of microalgal solution was placed into Erlenmeyer flasks (250 mL), and the enzyme was added. Enzymatic hydrolysis was conducted at 37 °C under orbital agitation (100 rpm). The pH was not previously fixed or controlled during the pretreatment. Two sets of enzymatic hydrolysis (EH) experiments were carried out (Fig. 6.1a and 6.1b). In EH-set 1, samples were removed from all the trials to measure the total soluble organic matter released by analysing the sCOD of the filtrate at the initial time and over an exposure time of 0.5, 1, 2, and 3 h after enzyme addition. According to the results obtained, in EH-set 2, the enzymatic pretreatments were performed at the optimal exposure time in both doses. All trials were carried out in triplicate. To evaluate the effect of the pretreatment on microalgal solubilisation, a control reactor (RM) containing the raw microalgal culture without enzymatic pretreatment and exposure to 37 °C was used. The effect of the enzymatic pretreatment on the hydrolysis efficiency was determined by comparing the increase in sCOD after the pretreatment and the sCOD concentration in the control.

6.2.4. BMP tests

Two sets of BMP tests were performed: BMP-set 1 and BMP-set 2 (Fig. 6.1a and 6.1b). The aim of BMP-set 1 was to evaluate the methane yield of the selected enzymatically pretreated microalgal biomass. BMP-set 2 assessed the co-digestion efficiency of a mixture of WAS and enzymatically pretreated microalgal selected in BMP-set 1. BMP-set 1 tests were carried out using 120 mL glass reactors, and raw microalgae without pretreatment (RM) were used to compare the effect of the pretreatments on biogas production. BMP-set 2 tests were performed in 900 mL aluminium reactors, and different controls were used: WAS without pretreatment (WAS), WAS and raw microalgal mixture without pretreatment (WAS:RM), WAS with enzyme addition (WAS-enzyme-dose), and inoculum with enzyme addition (I-enzyme-dose). Characterization of the inoculum employed is presented in Table 3.6 - Chapter 3).

6.2.5. Data analysis

The experimental data were analysed statistically, and differences were considered significant at p values below 0.05. When the null hypothesis was rejected (significance level < 0.05), post hoc comparisons were performed.

6.3. Results and discussion

6.3.1. Microalgal concentration through alkaline flocculation

When the pH of the solution was adjusted to 10 (Fig. 6.2), the clarification efficiency in PBR-10 increased by 4.4% (96.8%) compared to the untreated control (PBR-10-C, 92.5%). Comparable results were obtained when adjusting the pH to 11 (Fig. 6.2). The clarification efficiency in PBR (PBR-11) increased by 5.3% compared with the control without pH adjustment (PBR-11-C) (96.7% and

91.6%, respectively). When adjusting the pH to 11, high clarification efficiencies were achieved after 2 days; however, it took at least 6 days to reach similar clarification efficiencies when adjusting the pH to 10. The evolution of the clarification during alkaline pretreatment at each pH is shown in Fig. 6.3. Overall, after 7 days of pretreatment at pH 10 and pH 11, equivalent efficiencies were attained compared with the controls under natural sedimentation, while slight differences were observed at shorter exposure times.

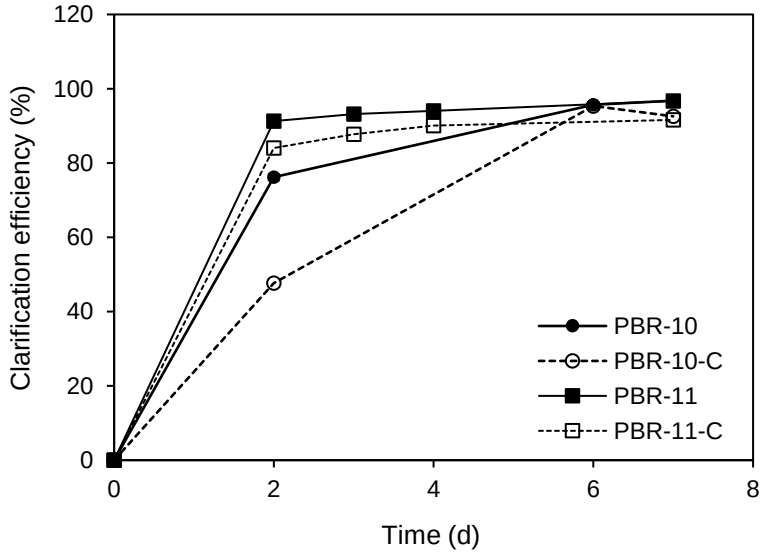

Figure 6.2 Clarification efficiency (%) after the alkaline pretreatment at pH 10 (PBR-10) and pH 11 (PBR-11), and their respective untreated controls (PBR-10-C and PBR-11-C). Error bars in PBR-10 and PBR-11 represent the standard deviation of the mean (n = 3).

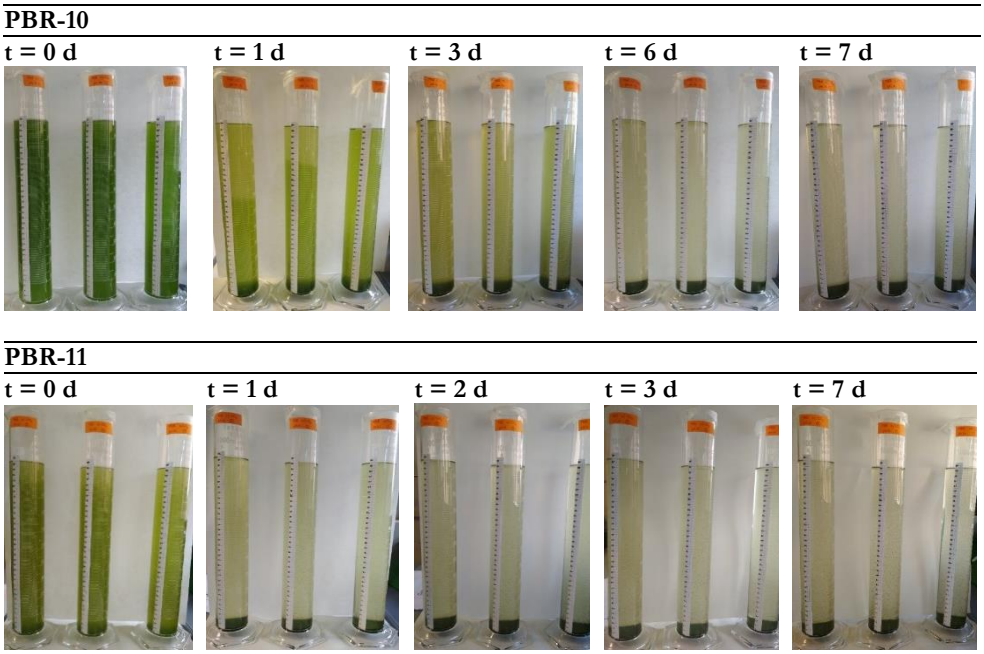

Figure 6.3 Evolution of the clarification during the alkaline pretreatment at pH 10 and 11 in the PBR effluent.

According to our results and in agreement with other authors, the absence of flocculation of *Chlorella vulgaris* biomass at up to pH 10.2-10.5 was reported (Smith and Davis, 2012; Vandamme et al., 2012), suggesting that natural sedimentation was the main mechanism involved in PBR-10 over the 7 days. Moreover, VSS reduction in the supernatant (Fig. 6.4) was higher in the untreated controls. The results under the tested conditions indicated that adjusting the pH to 10 and 11 had a slight or negligible effect on microalgal biomass flocculation compared with the controls. Contrary to our results, other authors reported >95% recovery of *Dunaliella viridis* after 24 h of adjusting the pH of the culture medium to 10 (Mixson et al., 2014) and 90% recovery of *Chlorella vulgaris* as pH increased to 10 (Branyikova et al., 2018). Ummalyma et al. (2016) obtained a 94% flocculation efficiency of the freshwater microalgae *Chlorococcum* sp. at pH 12. Diverse results could be explained by the differences in the medium composition (Mg^{2+} and Ca^{2+} content) since the amount of base needed to induce flocculation

depends on the buffering capacity of the culture and the concentrations of Ca^{2+} and/or Mg^{2+} (Vandamme et al., 2012; García-Pérez et al., 2014; Muylaert et al., 2015).

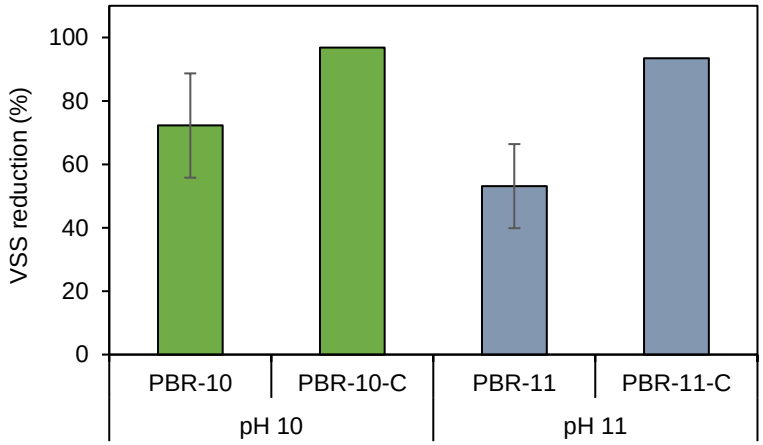

Figure 6.4 VSS reduction in the supernatant of PBR-10 and PBR-11 after pH-adjustment and neutralization, and their respective untreated controls (PBR-10-C and PBR-11-C). Error bars in PBR-10 and PBR-11 represent the standard deviation of the mean (n = 3).

Dissolved organic carbon in the supernatant increased by 1.5-fold at the end of the alkaline pretreatment for PBR-11 (Table 6.2). This fact could be associated with the presence of dissolved organic matter excreted by microalgal cells in the supernatant, also referred to as algal organic matter (AOM) (Barros et al., 2015), rather than sCOD from cell wall solubilisation. *Chlorella* sp. and *Scenedesmus* sp. are characterized by the high recalcitrance and robustness of their cell walls (González-Fernández et al., 2012), and as reported by other authors, alkaline pretreatment of *Chlorella* biomass was ineffective in biomass solubilisation (Bohutskyi et al., 2014). In addition, dissolved organic matter has a negative charge that also interacts with hydroxides, decreasing the available magnesium in the medium and requiring a higher dose of NaOH to form precipitates and a higher pH to achieve the same flocculation efficiency (Barros et al., 2015). For

instance, Vandamme et al. (2016) reported that a longer cultivation time of *Chlorella vulgaris* leads to greater excretion of AOM to the media, which mainly contains polysaccharides that interfere with and inhibit alkaline flocculation, thus increasing the dose of base addition.

Table 6.2 Initial and final sCOD in the supernatant of PBR-10 and PBR-11, and their respective controls (PBR-10-C and PBR-11-C).

pH-adjustment value	**Trial**	**Supernatant***		
		$sCOD_i$ (mg L^{-1})	**$sCOD_f$ (mg L^{-1})**	**sCOD increase (x-fold)**
pH 10	PBR-10	50.4	56.5	1.1
	PBR-10-C	50.4	75.6	1.5
pH 11	PBR-11	46.2	68.9	1.5
	PBR-11-C	46.2	54.2	1.2

* Supernatant after neutralisation at pH 7.

Thus, the lower recovery efficiencies in our study could be limited by the medium composition (the content of Mg^{2+} and Ca^{2+}) as well as the presence of AOM excreted by the microalgal biomass. Overall, the results indicate that pH adjustment of the microalgal solution to pH 10 and 11 neither enhances microalgal harvesting nor its solubility. When comparing alkaline flocculation with other harvesting techniques, such as bio-flocculation, some authors reported >98% clarification after the co-pelletization of the algal biomass with filamentous fungi (Hom-Diaz et al., 2017a) and 90% harvesting efficiency with use of a bacterial strain (Wan et al., 2013). Although these results showed higher flocculation efficiencies, additional time and costs were required for microorganism (fungal or bacterial) production.

6.3.2. Enzymatic pretreatment of algal biomass

Microalgal biomass (0.36 ± 0.07 g VS L^{-1}) from the PBR was enzymatically pretreated to evaluate the effect of the pretreatment on biomass solubility. Enzymatic pretreatments were performed at 37 °C, combining the action of temperature with the catalytic activity of the enzyme. Enzymatic hydrolysis was tested using three enzymes (A, B, and C) and two enzymatic loads (1% and 2% v/v) over exposure times of 0.5, 1, 2, and 3 h (EH-set 1, Fig. 6.1a and 1b). The selection of enzyme A was due to the effective organic matter solubilisation of microalgal biomass grown in open ponds for wastewater treatment, as reported by Passos et al. (2016). Enzymes B and C were employed due to their availability within the winery industry, as they are also applied to other industrial processes, as well as their similarity in composition to enzyme A.

Microalgal biomass without an enzymatic treatment displayed the lowest sCOD concentrations and was fairly constant over time (Table 6.3). In all cases, at higher enzyme doses, higher sCOD was released as a result of microalgal biomass solubilisation (Fig. 6.5a and 6.5b). When comparing all of the enzymatic pretreatments at the lower dose of the enzyme (Fig. 6.5a) and at the same exposure time, COD solubilisation was negligible ($p > 0.05$). However, significant differences in sCOD were found at 0.5 h when comparing pretreatments B1 and C1 ($p < 0.05$) (Fig. 6.5a). At the higher dose (Fig. 6.5b), significant variations in sCOD were identified when comparing the diverse pretreatments at each exposure time ($p < 0.05$) (Table 6.3). Furthermore, when analysing pretreatments individually, significant differences were identified in sCOD at different exposure times for pretreatments A2 and B2 ($p < 0.05$) (Table 6.3). Pretreatments A2 and B2 released greater sCOD after 2 h, and sCOD subsequently decreased. On the

other hand, the sCOD concentration in pretreatment C2 remained fairly constant from the first hour.

While pretreatments at the 1% dose exhibited faster COD solubilisation, pretreatments at the 2% dose attained greater solubilisation after 2 h of hydrolysis. According to these results, 0.5 h and 2 h were set as the optimum exposure times for the enzymatic pretreatments at 1% and 2% doses, respectively (EH-set 2). At the 1% dose and 0.5 h exposure time, sCOD increased by 138- to 159-fold, and at the 2% dose and 2 h exposure time, sCOD improved by 257- to 311-fold.

Table 6.3 sCOD of the control (raw microalgae, RM), and the enzymatically pretreated microalgae (M) with enzymes A, B, and C at a 1% dose (M-A1, M-B1, and M-C1) and a 2% dose (M-A2, M-B2, and M-C2) in EH-set 1. Significant statistically differences between the mean of the different pretreatments at the same exposure time (analysis per time), and the mean of each pretreatment at different exposure times (analysis per treatment) are indicated by letters as follows: $p < 0.05$ (a, c, d, e, j, k); $p < 0.01$ (b, i); and $p < 0.001$ (f, g, h).

Enzyme dose	Trial	Time (h)	sCOD (mg L^{-1})	sCOD increase(*) (x-fold)	*p value*	
					Analysis per time	Analysis per treatment
n.a.	RM	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	47.5 ± 9.1	1.1	n.s.	n.s.
		1	56.6 ± 8.9	1.3	n.s.	n.s.
		2	47.3 ± 6.5	1.1	n.s.	n.s.
		3	54.5 ± 11.2	1.3	n.s.	n.s.
1%	M-A1	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	6647.0 ± 114.5	152.6	n.s.	n.s.
		1	6489.3 ± 354.4	149.0	n.s.	n.s.
		2	6428.7 ± 397.3	147.6	n.s.	n.s.
		3	6398.0 ± 417.1	146.9	n.s.	n.s.
	M-B1	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	6926.3 ± 36.8	159.0	b	n.s.
		1	6635.0 ± 289.8	152.3	n.s.	n.s.
		2	6732.0 ± 392.6	154.5	n.s.	n.s.
		3	6676.3 ± 415.3	153.2	n.s.	n.s.
	M-C1	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	6048.3 ± 260.7	138.8	b	n.s.
		1	6039.7 ± 231.3	138.6	n.s.	n.s.
		2	6006.7 ± 141.0	137.9	n.s.	n.s.
		3	5978.0 ± 200.0	137.2	n.s.	n.s.
2%	M-A2	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	10367.0 ± 21.0	238.0	n.s.	f, g, h
		1	12853.3 ± 300.9	295.0	n.s.	f
		2	13221.3 ± 475.8	303.5	n.s.	g
		3	12764.7 ± 440.5	293.0	d	h
	M-B2	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	10411.0 ± 44.4	239.0	a	i, j
		1	11219.0 ± 1466.6	257.5	n.s.	k
		2	13533.3 ± 338.5	310.6	c	i, k
		3	13027.3 ± 183.1	299.0	e	j
	M-C2	0	43.6 ± 1.2	n.a.	n.s.	n.s.
		0.5	10318.7 ± 37.0	236.8	a	n.s.
		1	11190.0 ± 1339.5	256.8	n.s.	n.s.
		2	11218.0 ± 1371.1	257.5	c	n.s.
		3	11812.0 ± 397.6	271.1	d, e	n.s.

(*) sCOD increase respect to the initial time. n.a.: not applicable, n.s.: differences are not significant ($p > 0.05$).

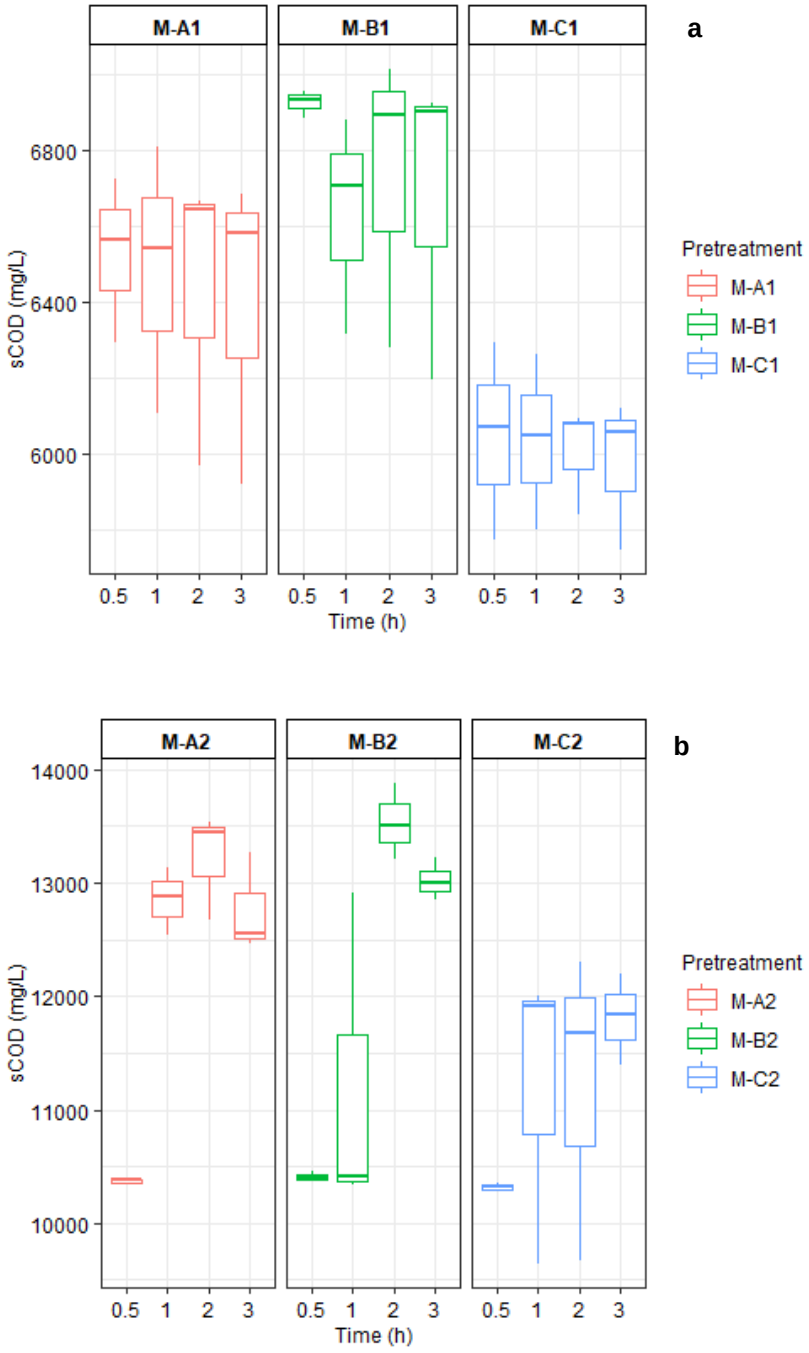

Figure 6.5 sCOD released after enzymatic pretreatments of microalgal biomass with enzymes A, B, and C, at doses of (a) 1% v/v (M-A1, M-B1, and M-C1), and (b) 2% v/v (M-A2, M-B2, and M-C2) in EH-set 1. Boxplots represent the median value, and the 25th and 75th percentile at each time.

Higher sCOD after enzymatic hydrolysis indicates effective microalgal cell wall degradation and removal of recalcitrant compounds. Comparison with other studies is not proper since the effect of the pretreatment depends on the microalgal species and the conditions applied. Enzymatic pretreatments were carried out at 37 °C in this study since mesophilic AD (37 °C) was applied after the hydrolysis treatment. Moreover, studies typically treated pure microalgal species. For instance, Mahdy et al. (2015b) stated that the differences in hydrolysis efficiency of *Chlorella vulgaris* and *Scenedesmus* sp. were due to their diversity in the cell wall and intracellular composition. Cell wall composition varies among species and growth conditions. In this work, selection of enzymes agreed with the microalgal cell wall composition. In this sense, cellulase hydrolyses cellulose, and glucohydrolase and xylanase degrade hemicellulose. Pectinliase and poligalacturonase are responsible for the degradation of pectin, and protease catalyses the breakdown of proteins. *Chlorella vulgaris* possesses a robust polymeric cell wall structure constituted by hydrolysable (xylose, mannose, galactose, glucose, fucose, arabinose, rhamnose and uronic acids) and resistant (glucosamine) compounds (Gerken et al., 2013). Pectin was also identified in *C. vulgaris* (Gerken et al., 2013) and *Scenedesmus* sp. The cell wall consists of carbohydrates composed of cellulose and hemicellulose (González-Fernández et al., 2012) in the presence of sporopollenins (Carrillo-Reyes et al., 2016). Ometto et al. (2014) tested sCOD released by three microalgal species after enzymatic pretreatment (24 h, 50 °C) using five different enzymes and doses, showing that pectinases generated higher solubilisation of *S. obliquus* biomass. Similarly, Maffei et al. (2018) reported cell damage, changes in cell morphology, and release of microalgal intracellular components after enzymatic pretreatment of *Nannochloropsis* sp. with cellulase and mannanase. Passos et al. (2016) likely obtained high solubilisation of the algal biomass when applying

enzyme A and cellulase at a 1% dose (t = 6 h, 37 °C). Due to the synergetic effect on the diverse macromolecules of the algal biomass, those researchers highlighted the use of the enzymatic cocktail (enzyme A) over the sole enzyme (cellulase), and moreover, they stated that the enzymes glucohydrolase and xylanase may have had an effect once the organic matter was hydrolysed by cellulase (Passos et al., 2016).

6.3.3. AD of enzymatically pretreated algal biomass

To further test the effect of the enzymatic pretreatment on algal biomass anaerobic digestibility, BMP tests of the enzymatically pretreated microalgal biomass were performed under the previously defined optimal conditions of 1% and 2% enzyme doses at exposure times of 0.5 h and 2 h, respectively (BMP-set 1, Fig. 6.1a and 6.1b). The microalgal biomass contained 0.44 g VS L^{-1}.

The net methane yield obtained in all trials in BMP-set 1 is shown in Fig. 6.6a and 6.6b. Differences between methane yields achieved after pretreatments M-B1 and M-C1 (640.9 ± 19.7 and 652.0 ± 13.8 NmL CH_4 g VS^{-1}, respectively) were not statistically significant ($p > 0.05$) (Fig. 6.6a and Table 6.4), and biogas production amounts for both pretreatments were 5.2- and 5.3-fold higher than that obtained by the untreated biomass (RM), respectively (differences were statistically significant, $p < 0.05$). Although sCOD was reduced by 91% in M-A1 (Table 6.4), methane production was 43-46% lower (447.5 ± 40.0 NmL CH_4 g VS^{-1}) compared with M-B1 and M-C1 ($p < 0.05$). Similarly, M-B1 and M-C1 presented a greater methane production rate (4.3 mL d^{-1}) than M-A1 (3.0 mL d^{-1}). However, the bioconversion process for M-A1 (K_H = 0.194 d^{-1}) was more than 2-fold higher than that of the other pretreatments at the 1% dose (Table 6.4). For methane productivity, reactors M-A1, M-B1, and M-C1 achieved 90%

methane production after 23, 19 and 15 days, respectively. The different outputs could be associated with the assorted enzyme composition of the enzymatic cocktails and their interaction with the microalgal biomass.

Surprisingly, when the enzyme dose (2%) was increased, methane production for pretreatments M-A2, M-B2, and M-C2 decreased by 9%, 27%, and 16%, respectively (Fig. 6.6b and Table 6.4) compared with the pretreatment with the same enzyme at the lower dose (1%). Although higher solubilisation was achieved with pretreatments at the 2% dose and t = 2 h, anaerobic digestibility was lower than that with pretreatments at the lower dose (Table 6.4). Bearing in mind that the microalgal concentration was the same in all pretreatments, one hypothesis is that the excess enzyme in the pretreatments at the 2% dose might not interact with the microalgal biomass, thus inhibiting anaerobic microorganisms and reducing methane yield. In contrast to digestion of raw microalgae, the methane yield increased sharply when microalgae were pretreated with the three enzymes at the 2% dose ($p < 0.05$). Moreover, significant statistically differences were found between the M-A2 and M-C2 pretreatments.

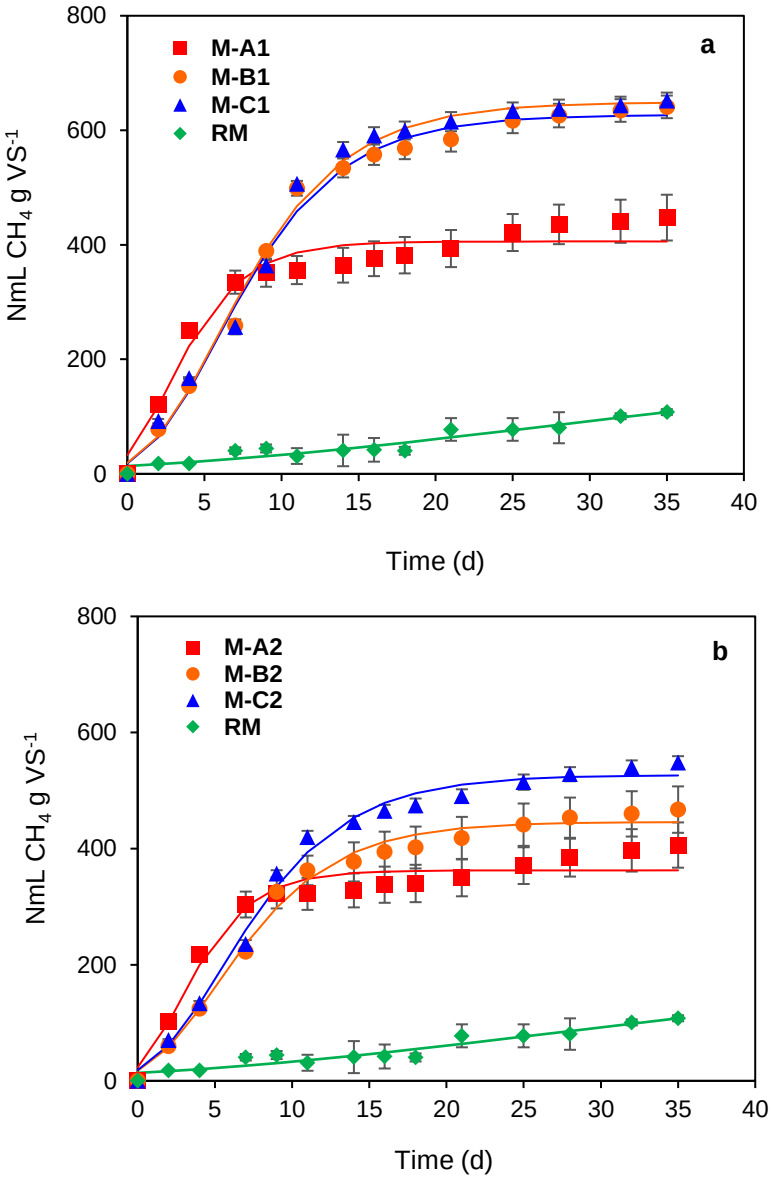

Figure 6.6 Net methane yield of microalgal biomass enzymatically pretreated at (a) a 1% dose v/v with enzyme A, B, and C (M-A1, M-B1, and M-C1) for 0.5 h; and at (b) a 2% v/v dose with enzyme A, B, and C (M-A2, M-B2, and M-C2) for 2 h, in BMP-set 1. Enzyme A = Celluclast, Glucanex, and Shearzyme; enzyme B = Enovin; enzyme C = Vinozym. RM refers to microalgal biomass without pretreatment. Dots represent the experimental data while continuous lines correspond to the fitting by the Gompertz model. Error bars represent the standard deviation of the mean (n = 3).

Methane production increased faster during the first 15 days for all pretreatments. For biogas composition, no differences were identified among the trials (Table 6.4). At the end of the BMP tests, pH values of the digestates between 7.2 and 7.7 suggest the stability of the process. Moreover, the concentration of VFAs was negligible in all cases. Fig. 6.7 shows that the relationship between methane yield and solubilisation increases after pretreatment.

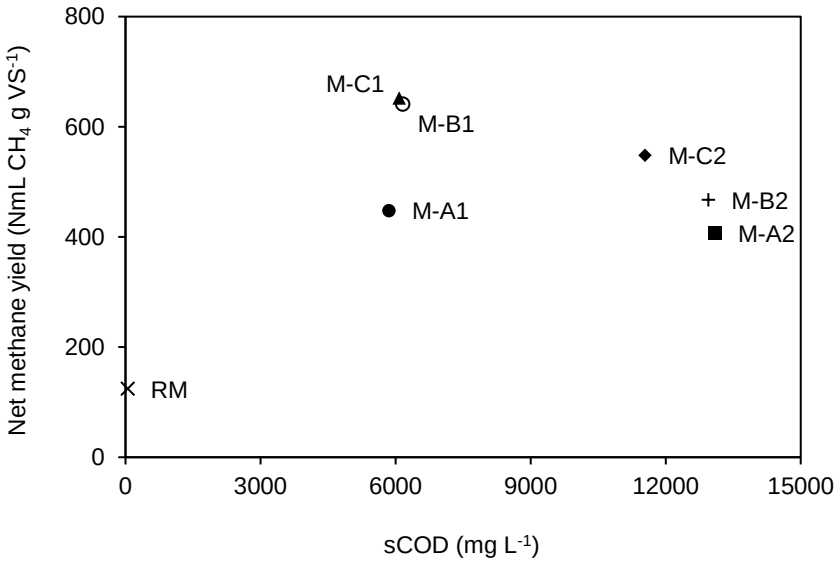

Figure 6.7 Microalgal biomass solubilisation (sCOD) vs. methane yields obtained in the enzymatic hydrolysis set 2 (EH-set 2) and the biochemical methane potential tests (BMP-set 1), respectively. Enzymatically pretreated microalgae at a 1% dose with enzymes A, B, and C = M-A1, M-B1, and M-C1. Enzymatically pretreated microalgae at a 2% dose with enzymes A, B, and C = M-A2, M-B2, and M-C2. Enzyme A = Celluclast, Glucanex, and Shearzyme; enzyme B = Enovin; enzyme C = Vinozym. RM = raw microalgae.

Table 6.4 Parameters from the enzymatic hydrolysis of microalgae (EH-set 2) and biochemical methane potential tests (BMP-set 1).

Trial	Enzymatic pretreatment (EH-set 2)	Biomethane potential test (BMP-set 1)								
					Gompertz parameters				Hydrolysis rate	
	$sCOD_f$* (mg L^{-1})	sCOD reduction (%)**	Net experimental CH_4 yield (NmL CH_4 g VS^{-1})	Methane content (%)	P_{max} (NmL CH_4 g VS^{-1})	R_{max} (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})	r^2
RM	52.8 ± 2.7	18 ± 0	124.2 ± 7.9	73 ± 4	232.5 ± 179.1	3.2 ± 0.7	1.1 ± 0.8	0.920	0.008	0.907
M-A1	5858.7 ± 1449.8	91 ± 0	447.5 ± 40.0	69 ± 1	405.8 ± 10.7	53.7 ± 9.4	-0.2 ± 0.7	0.955	0.194	0.974
M-B1	6163.3 ± 1308.0	95 ± 1	640.9 ± 19.7	69 ± 8	627.1 ± 9.9	50.9 ± 3.2	1.2 ± 0.4	0.993	0.092	0.975
M-C1	6085.3 ± 60.0	91 ± 2	652.0 ± 13.8	68 ± 10	649.1 ± 11.6	51.6 ± 3.7	1.2 ± 0.5	0.991	0.092	0.969
M-A2	13096.0 ± 1119.4	90 ± 3	406.1 ± 39.0	70 ± 1	362.6 ± 9.1	50.9 ± 8.8	0.0 ± 0.6	0.959	0.194	0.972
M-B2	12950.0 ± 500.3	90 ± 1	467.2 ± 39.0	70 ± 7	446.0 ± 8.3	38.1 ± 3.1	0.8 ± 0.5	0.989	0.103	0.982
M-C2	11546.7 ± 1012.0	92 ± 2	548.0 ± 11.2	70 ± 8	526.7 ± 9.1	43.1 ± 3.0	0.9 ± 0.4	0.991	0.096	0.980

RM = raw microalgae (without pretreatment). Enzymatically pretreated microalgae with enzymes A, B, and C at a 1% dose = M-A1, M-B1, and M-C1. Enzymatically pretreated microalgae with enzymes A, B, and C at a 2% dose = M-A2, M-B2, and M-C2. Enzyme A = Celluclast, Glucanex, and Shearzyme; enzyme B = Enovin; enzyme C = Vinozym.

* sCOD at the end of the enzymatic pretreatment (0.5 h for 1% dose, and 2 h for 2% dose).

** sCOD reduction was calculated considering sCOD values at initial and final time of the BMP tests.

The reduction in sCOD in BMP-set 1 was similar and higher than 90% for all trials. Nonetheless, the differences obtained in the methane yield for all of the trials are not consistent with their respective solubility increases after the enzymatic pretreatment. This fact suggests that the solubilized organic matter is not totally converted into methane. Consequently, it was not possible to identify a direct relationship between the reduction in sCOD and the methane yield. The compromise between a low enzymatic dose applied at a short exposure time to achieve a high methane yield represents the most favourable strategy for addressing the economic feasibility and applicability of the treatment (Fig. 6.7). Based on these outcomes, the enzymatic pretreatments of microalgae with enzyme B1 (M-B1) and C1 (M-C1) at t = 30 min were selected as the proper treatments for co-digestion studies (BMP-set 2) (Fig. 6.1a and 6.1b).

6.3.4. AcoD of WAS and enzymatically pretreated algal biomass

According to the results from the above experiments, two optimal enzymatic pretreatments (enzyme B and enzyme C at a 1% dose (v/v) at t = 0.5 h) were selected to further study the co-digestion of the pretreated microalgal biomass with WAS as a co-substrate (BMP-set 2, Fig. 6.1a and 6.1b). This set of experiments contained 4.3 ± 0.1 and 8.5 ± 0.3 g VS L^{-1} of WAS and gravity-concentrated microalgae, respectively.

As shown in Fig. 6.8 (net methane yield) and Fig. 6.9 (cumulative methane production), the methane production obtained when co-digesting enzymatically pretreated microalgae (M-B1 and M-C1) with WAS (WAS:M-B1 and WAS:M-C1) was similar to values obtained when using raw microalgae in the mixture (WAS:RM). Compared with WAS:RM, the net methane yield was 2% and 7% lower for WAS:M-B1 and WAS:M-C1 (Fig. 6.8), respectively; however, this

difference was not statistically significant ($p > 0.05$). Similarly, VS reduction was similar in the three reactors (Table 6.5). The methane yield of WAS:RM increased faster during the first days, causing a poor fit for the lag phase (λ) parameter of the Gompertz model (Table 6.5). Compared with WAS:RM, the hydrolysis rate (K_H) decreased for WAS:M-B1 (0.064 d^{-1}). However, the R_{max} values showed a higher methane production rate for this mixture (Table 6.5). This result could be explained by the effect of the previous microalgal enzymatic hydrolysis contributing to a greater bioavailability of the substrate. The major net methane yield of all the trials was obtained for WAS mono-digestion (179.3 g CH_4 g VS^{-1}) (Fig. 6.8 and Table 6.5). Although the difference was not statistically significant ($p > 0.05$), the methane yield of WAS and raw microalgae co-digested (WAS:RM) was 7% lower than that obtained by WAS. Nevertheless, the co-digestion of WAS:RM improved the K_H by 11% and the VS reduction by 27% compared with the mono-digestion of WAS. According to our results, enzymes applied to the microalgal biomass did not enhance methane production when co-digested with WAS. The values of VFAs accumulated in the reactors at the end of the BMP tests (Table 6.5) could indicate that biomasses were also converted to volatile compounds other than methane. Some studies showed an enhancement in methane yield after co-digestion of sewage sludge and raw microalgal biomass, attributing this result to higher nutrient availability, enhanced alkalinity, and a balanced C/N ratio to avoid ammonia inhibition (Olsson et al., 2014; Beltrán et al., 2016b; Solé-Bundó et al., 2020). However, Kim and Kang (2015) also observed a decline of 9% in methane accumulation when microalgae (*Chlorella* sp.) were co-digested with WAS compared with WAS mono-digestion. Caporgno et al. (2015) stated that there was no synergy between microalgae (25% *Selenastrum capricornutum*) and sludge (75% of a blend of primary and secondary sludge) co-digestion and obtained lower methane than sludge alone. In the same way, a

mixture of microalgae (*Ankistrodesmus*, *Chlorella*, *Coelastrum*, *Scenedesmus opoliensis*, *Scenedesmus quadricauda, Scenedesmus* sp., among others) and sludge (37% and 63% on a VS basis, respectively) showed low digestibility, obtaining a lower methane yield compared with the sludge alone, as reported by Olsson et al. (2018). In addition, Wang et al. (2013) observed comparable methane yields of WAS alone and WAS co-digested with raw microalgal biomass (*Chlorella* sp.). Diverse outputs after microalgae and sludge co-digestion could be related to the specific features of both substrates (microalgal composition is strain-specific) as well as to the different proportions of WAS and microalgae employed in the mixtures. The results of co-digestion assays differed slightly from those of WAS mono-digestion. Pretreated and untreated microalgal co-digestion with WAS seemed neither to have a synergetic effect nor a toxicity effect on biogas production, indicating that both substrates could be digested together, thus avoiding the costs associated with separated digestion processes (Elalami et al., 2019).

Typically, studies report sludge co-digestion with untreated co-substrates such as the organic fraction of municipal solid waste and agro-industrial and fatty wastes as co-substrates. Nonetheless, few studies have assessed the co-digestion of WAS and previously treated co-substrates such as microalgal biomass. An increase of 12% in methane production was achieved when co-digesting a mixture of 75% secondary sludge and 25% microalgae (C. *vulgaris*) when both substrates were thermally pretreated (120 °C, 40 min) (Mahdy et al., 2015a). Compared with untreated biomasses, Scarcelli et al. (2020) reported a slight increase in methane production when a thermal pretreatment (65 °C, 4 h) was applied to a WAS (60%) and microalgal (*Chlorella* sp., 40%) mixture. Similarly, Zhang et al. (2018) studied the co-digestion of microalgae (*Chlorella* sp.) pretreated with an enzymatic cocktail of cellulase, xylanase, and pectinase for lipid extraction using energy grass

(*Pennisetum* hybrid) as a co-substrate. As far as the authors are concerned, this study assesses the co-digestion of WAS and enzymatically pretreated microalgae for the first time.

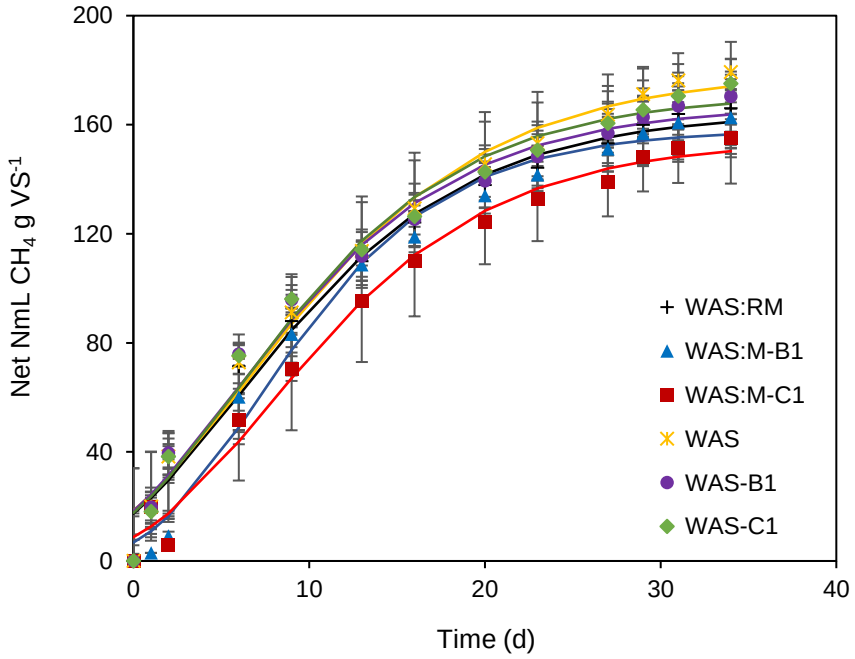

Figure 6.8 Net methane yield of the following co-digested mixtures in the BMP-set 2: WAS and raw microalgae (WAS:RM); WAS and enzymatically pretreated microalgae (WAS:M-B1 and WAS:M-C1); WAS; WAS with enzyme addition (WAS-B1 and WAS-C1); and inoculum with enzyme addition (I-B1 and I-C1). Markers represent the experimental data while the continuous lines correspond to the fitting by the Gompertz model. Error bars indicate the standard deviation of the mean (n = 3). RM = raw microalgae without pretreatment. I = inoculum. B1 = enzyme B at a 1 % dose. C1 = enzyme C at a 1% dose. M-B1 = microalgae pretreated with enzyme B at a 1% dose (t = 0.5 h). M-C1 = microalgae pretreated with enzyme C at a 1% dose (t = 0.5 h).

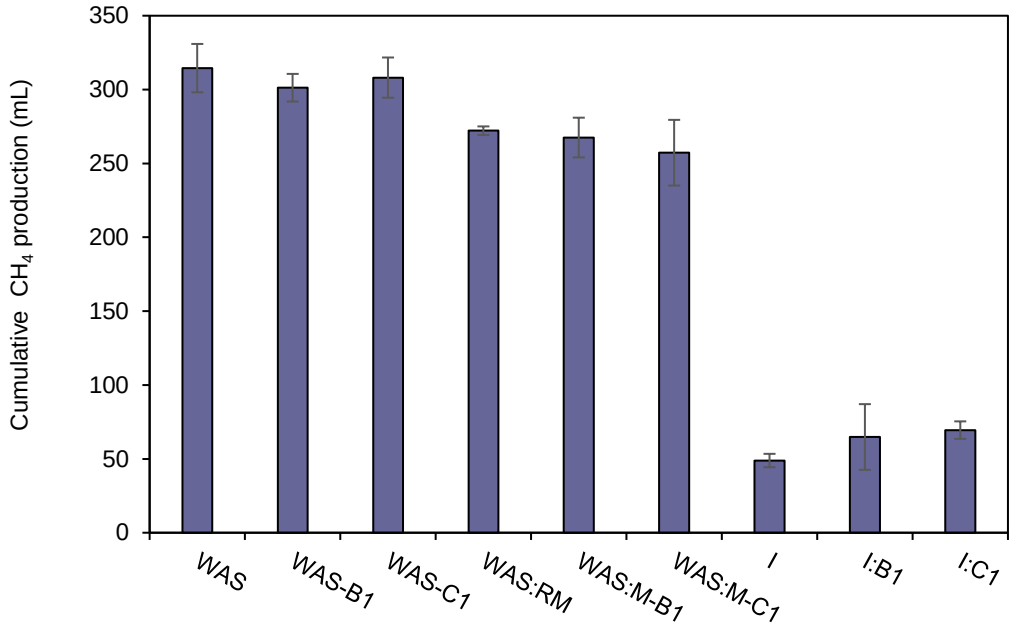

Figure 6.9 Cumulative methane production in BMP-set 2 for the following trials: WAS; WAS with enzyme addition (WAS-B1 and WAS-C1); mixture of WAS and raw microalgae (WAS:RM); mixture of WAS and enzymatically pretreated microalgae (WAS:M-B1 and WAS:M-C1); inoculum (I); and inoculum with enzyme addition (I-B1 and I-C1). Error bars indicate the standard deviation of the mean (n = 3). RA = raw microalgae without pretreatment. I = inoculum. B1 = enzyme B at a 1 % dose. C1 = enzyme C at a 1% dose. M-B1 = microalgae pretreated with enzyme B at a 1% dose (t = 0.5 h). M-C1 = microalgae pretreated with enzyme C at a 1% dose (t = 0.5 h).

Table 6.5. Experimental and calculated parameters from the biochemical methane potential tests of WAS and microalgae co-digestion (BMP-set 2).

Trial	VS reduction (%)	Net experimental CH_4 yield (NmL CH_4 g VS^{-1})	Methane content (%)	VFAs (mg L^{-1})		Gompertz parameters				Hydrolysis rate	
				Acetic acid	Propionic acid	P_{max} (NmL CH_4 g VS^{-1})	R_{max} (NmL CH_4 g VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})	r^2
WAS:RM	26 ± 2	166.0 ± 2.2	71 ± 2	34.1 ± 2.0	107.1 ± 17.9	164.7 ± 5.6	8.2 ± 0.8	-1.4 ± 1.7	0.984	0.080	0.993
WAS:M-B1	24 ± 3	162.6 ± 10.0	69 ± 10	38.5 ± 7.9	152.4 ± 4.6	158.3 ± 4.4	9.6 ± 0.9	0.9 ± 0.7	0.988	0.064	0.994
WAS:M-C1	24 ± 10	154.9 ± 16.5	70 ± 3	34.8 ± 7.2	131.5 ± 18.6	154.4 ± 5.1	7.8 ± 0.6	0.4 ± 0.7	0.989	0.055	0.992
WAS	19 ± 3	179.3 ± 11.0	70 ± 2	56.0 ± 4.6	158.3 ± 61.2	179.4 ± 6.5	8.4 ± 0.8	-1.4 ± 0.8	0.985	0.072	0.994
WAS-B1	30 ± 12	170.4 ± 6.3	70 ± 2	20.1 ± 3.7	77.1 ± 12.9	167.1 ± 6.1	8.5 ± 0.9	-1.5 ± 0.9	0.980	0.085	0.992
WAS-C1	27 ± 1	175.0 ± 9.2	68 ± 3	24.9 ± 1.7	104.7 ± 26.0	171.3 ± 6.2	8.7 ± 0.9	-1.3 ± 0.9	0.982	0.081	0.994

WAS:RM = mixture of waste activated sludge (WAS) and raw microalgae without pretreatment (RM). WAS:M-B1 and WAS:M-C1 = mixture of WAS and enzymatically pretreated microalgae with enzyme B at a 1% dose and enzyme C at a 1% dose, respectively. WAS-B1 and WAS-C1 = WAS with addition of enzyme B at a 1% dose and enzyme C at a 1% dose, respectively.

To test the effect of the enzymes on the WAS, the same dose of enzyme applied to the microalgae was directly added to the WAS in the BMP reactors WAS-B1 and WAS-C1. The results showed that enzymes B1 and C1 weakly reduced the net methane yield of WAS by 5% (WAS-B1) and 2% (WAS-C1), respectively (Fig. 6.8 and Table 6.5), but these differences were not statistically significant ($p > 0.05$). Nonetheless, a greater VS reduction took place in the WAS-B1 and WAS-C1 reactors (30% and 27%, respectively) compared with WAS (19%), suggesting that the enzymes could contribute to major solubility and further degradation of the organic matter in the WAS. Furthermore, the hydrolysis rate of the secondary sludge slightly increased in the reactors to which the enzymes were added (Table 6.5).

To verify that the enzymes did not negatively affect the inoculum, the influence of the enzymes on the inoculum in biogas production was tested. The results in Fig. 6.9 indicated that I-B1 and I-C1 exhibited 33% and 42% increases in methane production compared with the blank reactor (I), respectively. This fact suggests that enzymes B and C did not inhibit the anaerobic microorganisms present in the inoculum.

6.4. Conclusions

This chapter assessed the pretreatment of microalgal biomass for solubility enhancement and further AD of pretreated microalgae as well as its AcoD with WAS.

First, similar results were obtained when harvesting microalgae by natural sedimentation and alkaline pretreatment at pH 10 and 11. Similarly, microalgal solubility was not improved by pH adjustment. Hence, this pretreatment was rejected for algal biomass harvesting prior to valorisation.

Second, enzymatic hydrolysis pretreatments were performed to enhance the solubility of the microalgal biomass. The optimal pretreatment conditions were t = 0.5 h and t = 2 h for 1 and 2% doses, respectively. Compared with raw microalgal biomass, the enzymatic pretreatment highly enhanced the solubility and the biogas yield of the algal biomass at both doses, showing efficient solubilisation and anaerobic digestibility of the biomass. Although the organic matter solubilisation registered was higher for pretreatments at the higher dosage, the methane yield markedly increased for microalgal biomass pretreated with the lower enzyme dosage. When co-digesting microalgae with WAS, comparable methane yields were obtained for enzymatically pretreated and untreated algal biomass. Since the previous enzymatic treatment of the microalgae did not enhance the methane yield, it can be neglected to reduce costs. Overall, even though AcoD with microalgae under the studied conditions did not improve energy production, co-digestion is a promising and economically feasible alternative for diverse waste stream treatments via the integration of WWTP facilities and microalgae-based systems.

CHAPTER 7.

WATER RESOURCE RECOVERY COUPLING ALGAL WASTEWATER TREATMENT AND WAS CO-DIGESTION AT INDUSTRIAL PILOT-SCALE

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Abstract

This case study is part of a circular bioeconomy project for a winery company aiming to integrate a microalgae-based system within the existing facilities of the winery WWTP, promoting nutrient recovery and transformation into valuable products and bioenergy. Microalgae were used for wastewater treatment, efficiently removing $N\text{-}NH_4^+$ (97%) and $P\text{-}PO_4^{-3}$ (93%). A pilot anaerobic reactor was used for batch anaerobic mono-digestion of WAS and for co-digestion of WAS and algal biomass. The methane yield using WAS from two different wine production seasons was 155.4 and 132.9 NL CH_4 kg VS^{-1}. Co-digestion led to the highest methane yield (225.8 NL CH_4 kg VS^{-1}). The application of the bio-wastes for fertilization was assessed through plant growth bioassays: mono- and co-digestion digestates and dry algal biomass enhanced plant biomass accumulation (growth indexes of 163%, 155% and 121% relative to those of the control - commercial amendment, respectively), demonstrating a lack of phytotoxicity.

7.1. Introduction

Microalgae-based systems are a nature-based solution offering a breakthrough towards a new paradigm integrating wastewater treatment with resource and energy recovery (Wollmann et al., 2019). Wastewater is an abundant energy source (with an estimated energy content of 6.3 kJ L^{-1} relative to the COD) that can be recovered and reused in a circular economic approach (Nagarajan et al., 2020b). Water resource recovery facilities (WRRFs) comprise a new configuration for wastewater treatment focused on reducing energy costs and enhancing resource recovery (Seco et al., 2018). By coupling a microalgal photobioreactor (PBR) to conventional biological wastewater systems, the quality of the treated water can be improved, obtaining biomass that can be processed downstream and valorised as a bioenergy feedstock as well as an organic fertilizer (Solé-Bundó et al., 2019).

The European Commission (EC) has considered AD of organic wastes among the most energy-efficient technologies to harness the energy potential of biological wastes (EC, 2009b). Coupling microalgae production to wastewater treatment for biofuel production through anaerobic digestion (AD) or anaerobic co-digestion (AcoD) with other carbon-rich substrates is technically and economically feasible (Wang et al., 2016). Moreover, the use of microalgae for energy production increases system viability, enhances the efficiency of the process and diminishes WWTP costs (Barroso Soares et al., 2019).

The EC set a goal of a 30% reduction in non-renewable sources used in fertilizer production, with the aim of substituting recycled bio-wastes or other sources (EC, 2016). In microalgal anaerobic digestion-based biorefineries, nutrients can be recycled from wastewater, and the harvested microalgae can be used as a bio-

based fertilizer, reducing or avoiding dependency on synthetic fertilizers and their associated GHG emissions (Coppens et al., 2016). In addition, the anaerobic digestate (the solid and/or the liquid fraction) can be applied for agronomic use since it is a biologically stable organic matrix and a source of nutrients, thereby increasing nitrogen, phosphorous, and microbial biomass in the soil (Nkoa, 2014; Guilayn et al., 2019).

Currently, the disposal of the waste activated sludge (WAS) generated by the WWTP is delivered for external management and disposal, having an impact on the costs of running the winery WWTP. Efficient resource recovery is the major driving force for shifting from the current winery WWTP to a WRRF. To this end, this study evaluates integrating a microalgae-based system for tertiary wastewater treatment into the winery WWTP, bioenergy production from sludge and microalgae co-digestion, and the application of digestate and dry algal biomass as biofertilizers. This case study at the pilot scale will contribute to identifying challenges to and potentialities for establishing baselines for full-scale implementation.

7.2. Materials and methods

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

7.2.1. Algal photobioreactor

One litre of microalgal culture (TS = 0.8 g L^{-1}) obtained from a 3 L stock photobioreactor fed with modified Mann and Myers media (previously described in Table 3.3 - Chapter 3) was used as inoculum to start-up the wastewater column

PBR ("PBR" hereafter) (Fig. 7.1). Wastewater treatment was performed in a 50 L PBR (working volume of 45 L) made of transparent methacrylate and fed with 44 L of the secondary effluent from the winery WWTP. An air sparger (at a flow of 3.9 L min^{-1}) placed at the bottom of the PBR was used for microalgal stirring and aeration. The PBR was located inside a greenhouse in the facilities of the winery (Barcelona, Spain). The mean temperature during the experimental period was 19.5 °C, and the net sunlight irradiation was 26.3 MJ m^{-2} d^{-1}.

Microalgae in the PBR grew autotrophically in batch mode for 7 days until reaching the exponential growth phase and were harvested by natural sedimentation. An optical microscope and conventional taxonomic literature were used for morphological identification of algal biomass as previously explained (Chapter 6). A mixed microalgal culture of *Scenedesmus* sp. and *Chlorella* sp. as the predominant species and bacteria and protozoa at a minor abundance was identified (as previously defined in section 5.2.1. - Chapter 5 and section 6.2.1 - Chapter 6).

Figure 7.1 Column PBR

7.2.2. Pilot anaerobic reactor

A cylindrical stainless-steel digester (AISI 316 L) with a total volume of 70 L (working volume of 50 L) (Fig. 7.2) was designed and employed as the pilot anaerobic reactor. The reactor contained three outlet pipes and a safety valve (1 bar). The two upper outlet pipes of the reactor were connected to a temperature sensor (Waft, Barcelona, Spain) and a Mariotte column to measure volumetric biogas production by water displacement. The content inside the reactor was kept homogenized by continuous agitation (S.S.C. 9-2G, Agitaser, Barcelona, Spain). The reactor had a thermal jacket (Elementos calentadores, Barcelona, Spain) to maintain mesophilic conditions (35 – 37 °C).

Figure 7.2 Pilot anaerobic reactor.

7.2.3. AD process

Mono- and co-digestion (AD and AcoD) were performed in the anaerobic reactor under mesophilic conditions using WAS and WAS-microalgae mixtures, respectively. WAS was obtained from the secondary clarifier of the winery WWTP after the biological treatment, while algal biomass was collected from the PBR effluent. A mesophilic digestate from the anaerobic digester of a municipal WWTP (Vilafranca del Penedès, Barcelona, Spain) was used as inoculum. The pilot anaerobic reactor was operated in sequencing batch reactor (SBR) mode. Each cycle included the following steps: feeding, reaction, settling, and discharge. First, the reactor was fed with the inoculum and the substrate; second, the anaerobic digestion was carried out for 30 days; third, the reactor content was settled; and fourth, 1/3 of the reactor content was discharged, with the rest kept as inoculum for the next cycle. Before the beginning of a new cycle, the reactor content was left for 5 days for degasification to guarantee the depletion of the residual organic matter. Two cycles were performed for WAS mono-digestion, while two cycles were carried out for WAS-microalgae co-digestion. In the co-digestion cycles, two microalgal doses were employed: 0.2% and 1.8% (on a VS basis). These percentages were set according to the annual generation of both substrates by the winery WWTP.

AD process performance was evaluated by monitoring the following parameters: TS, VS, VFAs, pH, and alkalinity (total alkalinity (TA), partial alkalinity (PA), and intermediate alkalinity (IA)). The alkalinity index (IA/PA ratio) was employed to evaluate reactor stability, indicating to what extent the concentration of acids (estimated by the IA) exceeded the buffer capacity of the system provided by HCO_3^- (estimated by the PA) (Martín-González et al., 2010). Samples were taken from the reactor three times per week, in addition to the samples taken at the

beginning and end of each cycle. Biogas production was quantified in the Mariotte column and the produced volume of biogas was expressed at standard conditions. Periodically, samples of the generated biogas were taken in sealed gas sampling bags to analyse the CH_4 and CO_2 contents.

7.2.4. Plant growth bioassay

The reuse of liquid and solid bio-wastes from the anaerobic reactor and the PBR as a soil amendment or biofertilizer was assessed through a modified phytotoxicity assay employing plant growth (Solé-Bundó et al., 2017b). Bioassays are phytotoxicity tests used to evaluate the influence of bio-wastes on biomass accumulation in plants (Alburquerque et al., 2012) and were carried out at the end of the winter season and the beginning of the spring season (mean temperature of 23 °C and daily photoperiod of 12.6 h). They were performed in plastic pots (⌀ 8.5 cm and height 8.5 cm, with drainage holes in the bottom) placed inside holder vessels in a greenhouse located at the facilities of the winery company. The studied bio-wastes included digestate from the 1st cycle (WAS mono-digestion) (F1), digestate from the 3rd cycle (WAS-microalgae co-digestion) (F2), algal biomass harvested by sedimentation and solar dried (F3), and sedimented PBR effluent (without biomass) (F4). Moreover, an organic commercial amendment (Fervo-64, Fervosa, Barcelona, Spain) used in conventional agriculture was employed as the reference control (FC). A total of 24 pots were used to test each bio-waste (n = 120 pots). First, the pots were filled with 281.0 ± 8.9 g of compost and commercial perlite and placed inside the holder vessels. Then, the holder vessels were filled with water to reach saturation of the substrate. After 24 h, surplus water was withdrawn from the holder vessels, and after 1 h, the pots were weighed. After 6 days, the pots were weighed to quantify water evaporation and determine the amount of water to be added.

Then, lettuce seedlings (*Lactuca sativa)* were sown in each pot (day 0) and maintained under the same moisture conditions. On day 1, the bio-wastes were applied to the pots according to their mode of application as an irrigation liquid (in case of F1, F2, and F4) or by soil drench (in case of solid F3 and FC). The dose of bio-waste added to each pot was determined based on the nitrogen content in the target bio-waste, similar to the TN dose applied by other authors (Mulbry et al., 2005; Sigurnjak et al., 2017). The bioassay was performed for 40 days. On days 20, 27, and 40, a defined quantity of seedlings (on day 20, n = 7 pots; on day 27, n = 8 pots; and on day 40, n = 9 pots) were harvested from the pots, and the following parameters were measured to monitor plant growth: plant height (cm), shoot fresh weight (g), and root fresh weight (g). Plant height was measured from the point where roots started to grow to the top of the highest fully expanded leaf. At the end of the experiment, the total dry mass (TS) of the plants (shoots + roots) was determined after drying overnight at 105 °C. The growth index (GI) was used to assess the influence of the four bio-wastes and the control on plant growth and was expressed as a percentage of the total plant weight with respect to the reference control.

7.2.5. Analytical methods

TA and PA were determined by a titration method at pH 4.3 and pH 5.75, respectively, and IA was calculated as the difference between TA and PA, following procedure 2320B in Standard Methods (APHA, 2008). Biogas production was normalised and expressed as the volume of biogas or methane generated per mass of VS of added substrate (NL biogas or CH_4 kg VS^{-1}) under standard conditions (273.15 K and 1.0133 bar) (previously explained in section 3.3.2. - Chapter 3). TS, VS, and VFAs were analysed as previously defined (section 3.3.4.1. and section 3.3.3. - Chapter 3). Macro- and micronutrients and

heavy metals in the plant bioassays were analysed by an external laboratory (Eurofins Agroambiental, Lleida, Spain).

7.2.6. Data analysis

The experimental data from the bioassays were statistically analysed using ANOVA for repeated measures. Differences were considered significant when $p < 0.05$, and post hoc comparisons were performed when the null hypothesis was rejected.

7.3. Results and discussion

7.3.1. Microalgal tertiary wastewater treatment

The PBR used for tertiary wastewater treatment was fed with secondary effluent from the winery WWTP using wastewater as a nutrient source for microalgal cultivation. The average values for the parameters of the PBR influent and the settled effluent during the experimental period (from September 2018 to March 2019) are shown in Table 7.1. High removal efficiencies were achieved for $N\text{-}NH_4^+$ (97%) and $P\text{-}PO_4^{-3}$ (93%). Although the mean COD value in the effluent increased by 18%, this concentration was lower than the threshold value that was authorized for the company to discharge (160 mg O_2 L^{-1}). This surplus could be associated with the accumulation of extracellular organic matter produced during algal wastewater treatment (Wang et al., 2015; Higgins et al., 2018). Moreover, the $N\text{-}NH_4^+$ and $P\text{-}PO_4^{-3}$ concentrations in the settled effluent were in compliance with threshold values for reutilization or discharge into receiving waters (0.4 and 0.6 mg L^{-1} for $N\text{-}NH_4^+$ and $P\text{-}PO_4^{-3}$, respectively). Overall, the quality of the effluent was improved. The results obtained were similar to those

reported for alga-based tertiary wastewater treatment (Arbib et al., 2013; Arias et al., 2018).

Table 7.1 Characterization of the PBR influent and the settled effluent.

Parameter	PBR influent	PBR effluent
COD (mg L^{-1})	81.4 ± 6.5	96.0 ± 0.0
TOC (mg L^{-1})	23.1 ± 5.5	19.9 ± 0.0
TS (mg L^{-1})	62.5 ± 27.6	59.0 ± 4.2
VS (mg L^{-1})	48.0 ± 21.2	49.0 ± 1.4
N-NH_4^+ (mg L^{-1})	4.8 ± 1.3	0.1 ± 0.0
TN (mg L^{-1})	7.9 ± 5.7	3.5 ± 1.4
P-PO_4^{-3} (mg L^{-1})	2.5 ± 0.0	0.2 ± 0.0
pH	8.1 ± 0.2	8.3 ± 0.0

7.3.2. AcoD of WAS and algal biomass

This work aims for the integration of a microalgae-based system for tertiary wastewater treatment, with the ultimate goal of transitioning from a WWTP to a WRRF, as represented in Fig. 7.3. A closed-loop use of resources includes algal biomass valorisation for energy recovery through anaerobic co-digestion of WAS and algal biomass, using the biogas for tractor biofuel and the digestate as soil amendment or biofertilizer in the company vineyards. This system is also coupled to algal biomass valorisation for nutrient recovery from wastewater through the application of dry algal biomass as biofertilizer in the vineyards and the use of the PBR effluent for irrigation. The assessment of the current winery WWTP and the use of the generated biogas for tractor biofuel are beyond the scope of the present study.

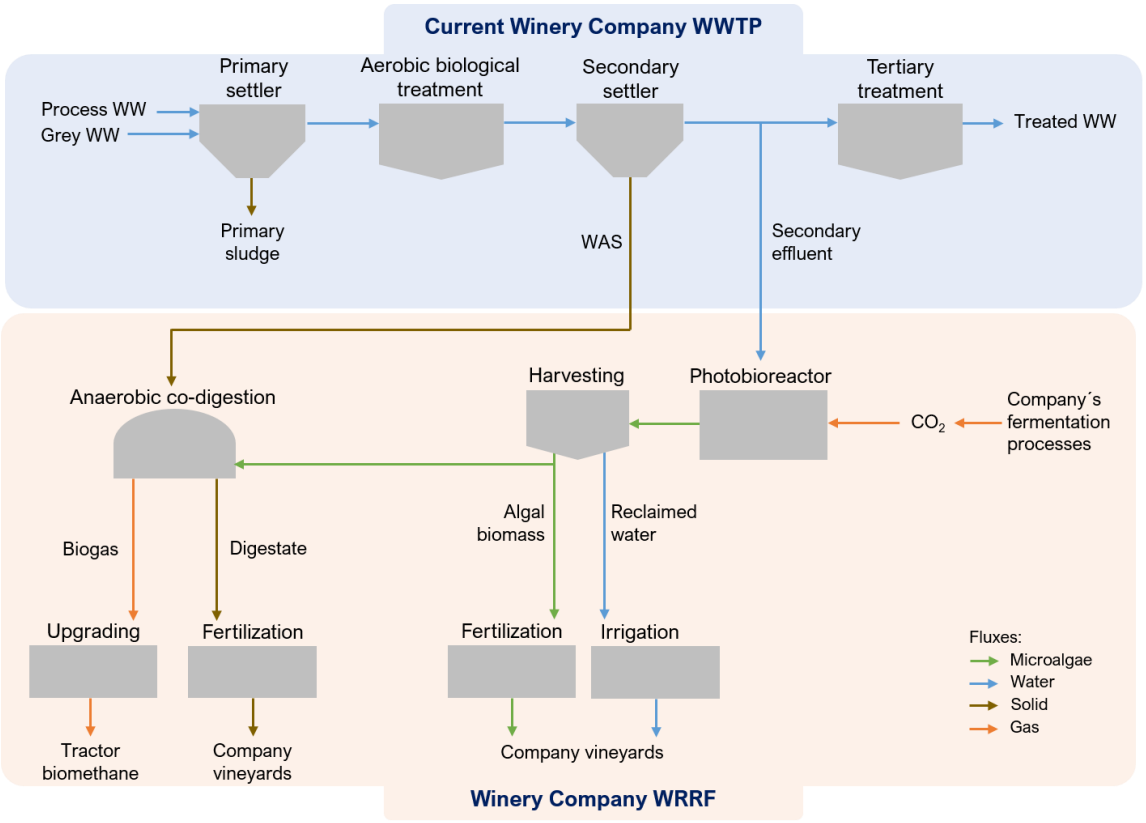

Figure 7.3 Schematic representation of the circular bioeconomy proposal for the winery company.

The performance of a pilot-scale anaerobic reactor for WAS mono-digestion (1st and 2nd cycles) and WAS and algal AcoD (3rd and 4th cycles), was tested. Table 7.2 presents the characterization of substrates and inoculum employed in cycles 1 to 4. Process stability was evaluated by monitoring the pH, alkalinity index, VFA concentration, and methane composition.

Table 7.2 Initial characterisation of SBR cycles.

SBR cycle	**1st**	**2nd**	**3rd**	**4th**
Inoculum volume (L)	33.0	31.0*	33.0*	33.0*
WAS volume (L)	15.0	15.0	14.4	16.2
Microalgae volume (L)	n.a.	n.a.	0.6	0.8
WAS and microalgae mixture volume (L)	n.a.	n.a.	15	17
VS inoculum (g L^{-1})	3.6	7.3*	5.7*	4.5*
pH inoculum	7.9	7.8*	7.7*	7.6*
VS WAS (g L^{-1})	5.0	10.6	6.9	5.0
VS microalgae (g L^{-1})	n.a.	n.a.	0.4	1.9
VS WAS and microalgae mixture (g L^{-1})	n.a.	n.a.	6.6	4.8
Relation VS_i/VS_s	1.6	1.4	1.9	1.8
Relation WAS and microalgae mixture (v:v)	n.a.	n.a.	24.0	20.0

VS_i = volatile solids in the inoculum. VS_s = volatile solids in the substrate(s). * Coming from the previous cycle. n.a. = not applicable.

When finishing the 1st cycle, a volume of 31 L of the digestate was used as inoculum for the 2nd cycle. As shown in Table 7.3, methane yields of 155.4 and 132.9 NL kg VS^{-1} were generated in SBR cycles 1 and 2, respectively, consistent with values recorded by other authors (Arias et al., 2018). The biogas production profiles of cycles 1 and 2 (Fig. 7.4a) followed a similar trend (due to operation issues, biogas production in cycle 1 was quantified from the ninth day). However, biogas production in cycle 2 decreased from day 23 onwards, suggesting that a large part of the available biodegradable organic matter had been consumed. The VS elimination in cycle 1 was 58% higher than that in cycle 2 (Table 7.3), which may be associated with the different features of the WAS employed (WAS generated during the wine grape harvesting season possessed a higher organic matter content), as previously reported (Higgins et al., 2018). Regarding VFAs, the acetic acid concentration in cycle 1 increased from day 4 of digestion,

maintaining a similar value up to day 19, and its concentration was reduced by the end of the cycle (Fig. 7.5a). The evolution of the acetic acid concentration in cycle 2 showed a similar pattern, and acetic acid was no longer detected after day 21, while the butyric acid concentration increased from day 18 to day 30, after which it tended to diminish (Fig. 7.5a and 7.5c), enhancing biogas production (Fig. 7.4a). Likewise, in both cycles, the alkalinity index (IA/PA ratio) generally remained below the threshold value of 0.4 (Martín-González et al., 2010; Astals et al., 2012), while the pH ranged between 7.7 and 8.3 (Fig. 7.6a). These values indicate stable performance of the reactor in both mono-digestions.

The third and fourth SBR cycles assessed the co-digestion of a mixture where microalgae were used as a co-substrate for WAS. In comparison to that in cycles 1 and 2, the biogas yield obtained in the 3rd cycle (137.2 NL kg VS^{-1}) was 27% and 16% lower, respectively (Fig. 7.4. Furthermore, the slight elimination of VS (4%, Table 7.3) and major propionic acid accumulation (Fig. 7.5b) in cycle 3 suggest that the biomass was partially degraded and not converted into methane, showing limited process performance. However, despite these results, no inhibition was observed. Differences between cycles 3 and 4 could be explained by the use of a different inoculum, as in cycle 3, the inoculum might not have been adapted to the microalgal substrate. Additionally, the cycles had different proportions of microalgae, and a major microalgal content in cycle 4 led to a higher biogas yield. In addition, the biogas production rate was analysed by the kinetic constant with K_H = 0.0992 d^{-1}, indicating accelerated co-digestion in comparison to that in cycle 2 (Table 7.3). Nonetheless, despite the higher kinetics, the biogas yield was lower than that observed in mono-digestion cycles 1 and 2. This fact could be explained by improved hydrolysis of algal biomass by the

hydrolytic bacteria in the WAS and the formation of non-biodegradable soluble materials, as also reported elsewhere (Scarcelli et al., 2020).

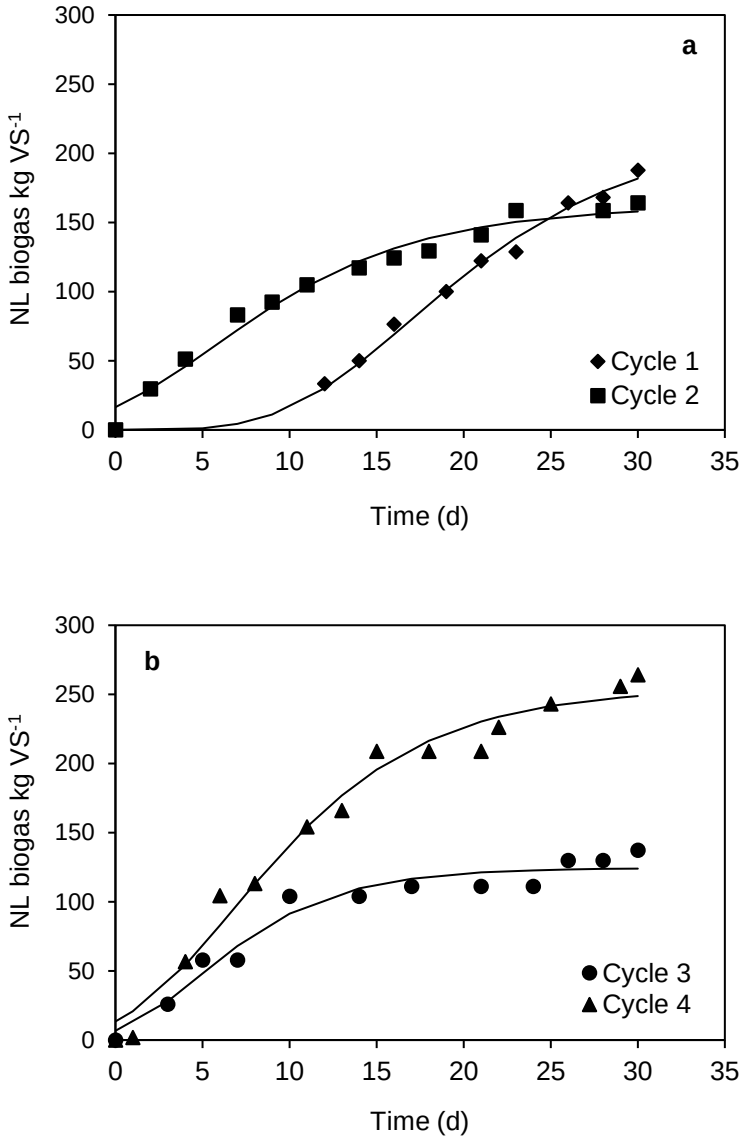

Figure 7.4 Biogas yield obtained in (a) SBR cycles 1 and 2 (WAS mono-digestion), and (b) cycles 3 and 4 (WAS and microalgae co-digestion). Dots represent experimental data and curves are data estimated by the Gompertz model.

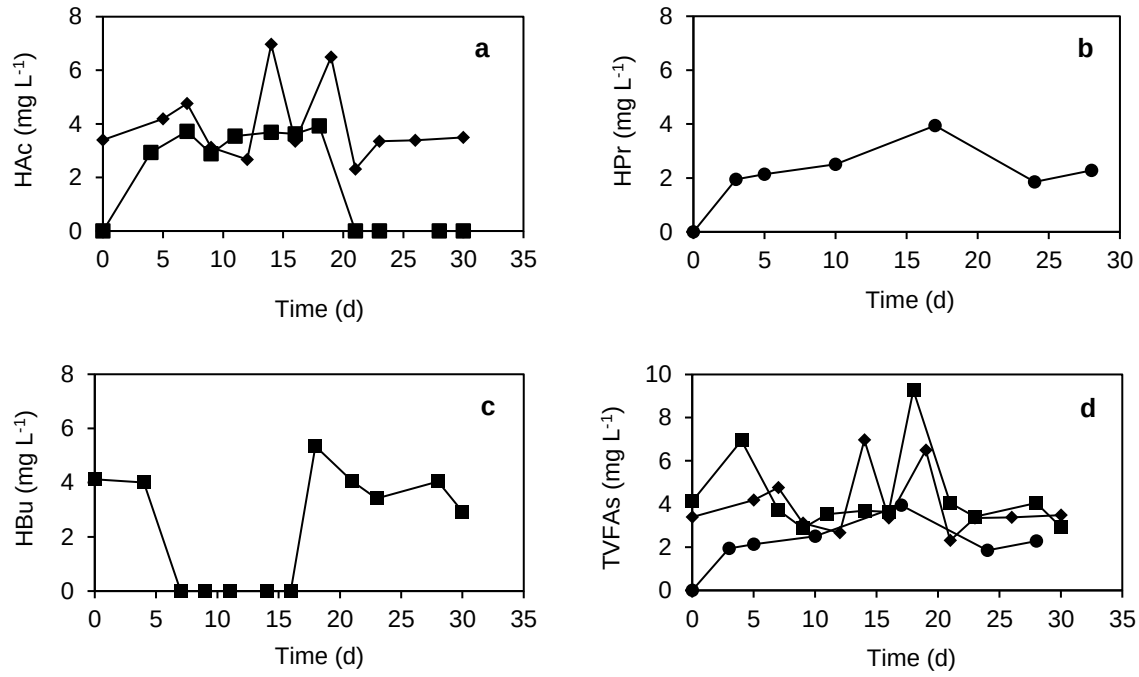

Figure 7.5 (a) Acetic (HAc), (b) propionic (HPr), (c) butyric (HBu), and (d) total VFAs (TVFAs) concentration during the SBR cycles 1 (◆), 2 (■), and 3 (●). Cycles 1 and 2 corresponds to WAS mono-digestion while cycle 3 corresponds to WAS and algal AcoD. Note: no VFAs were detected in cycle 4.

Significant improvements were attained in cycle 4, obtaining a 92% increase in biogas yield (264.1 NL biogas kg VS^{-1}) in comparison to that in cycle 3 (Fig. 7.4b) and consistent with results reported in previous WAS and microalgae co-digestion studies (Wang and Park, 2015; Olsson et al., 2018). VS was reduced by 35% (Table 7.3), attaining a better output than the other cycles. According to Wang et al. (2013), the VS reduction in WAS-microalgae co-digestion could be enhanced by algae addition. Thus, it is possible that co-digestion contributed to the degradation of some poorly degradable organic matter, as also reported by Olsson et al. (2018). Moreover, according to Fig. 7.5d and Fig. 7.6b, the system showed high stability in terms of pH (ranging from 7.5 - 8.9), VFAs (not detected), and the alkalinity index (remaining below the reference value). The co-

substate addition in cycle 4 led to an increase in biogas yield by 41% and 61% in comparison to that in cycles 1 and 2, respectively. Moreover, the digestion rate was 1.8 - 2.9-fold higher in comparison with that in WAS mono-digestion. The lag phase (λ) estimated by the Gompertz model was longer during the co-digestion cycles (Table 7.3), which could be associated with the recalcitrance of the microalgal cell wall hampering hydrolysis (Klassen et al., 2016). Despite the lower hydrolysis rate estimated for cycle 4 (K_H = 0.061 d^{-1}), the maximum biogas production rate (R_{max}) was higher than that in the other cycles (Table 7.3). Co-digestion in the 4th cycle showed an improvement in biogas yield and anaerobic digestion performance (methane content and absence of VFAs). These outcomes demonstrate that the co-digestion strategy with microalgae prevents process instabilities, leads to a more robust process, and can markedly enhance WAS mono-digestion improving the biogas yield and VS reduction. Some authors have reported similar results when co-digesting sludge and algal biomass. A 10% increase in the biogas yield was reported when WAS and *Chlorella* sp. were co-digested in comparison with WAS alone (Wang and Park, 2015). Beltrán et al. (2016) obtained a 22% higher methane yield from WAS and *Chlorella sorokiniana* (ratio 75:25) co-digestion in comparison to WAS mono-digestion. Similarly, Mahdy et al. (2015) reported a higher methane yield when co-digesting three mixtures of *Chlorella vulgaris* and WAS. Nonetheless, other authors have previously reported co-digestion of these substrates, obtaining contradictory outcomes. Wang et al. (2013) reported that co-digestion of WAS and *Chlorella* sp. yielded similar biogas production to that with WAS. The co-digestion of a 25% *Scenedesmus dimorphus* and 75% sludge mixture reduced the methane yield by 3% in comparison to that of the sludge (Peng and Colosi, 2015). Likewise, Scarcelli et al. (2020) reported that despite the co-digestion of WAS and a microalgal consortium mainly composed of *Chlorella* sp. affecting the solubilisation of the

substrate, it did not lead to a higher methane yield in comparison to that under WAS mono-digestion. Different methane yields obtained in activated sludge and microalgae co-digestion might have resulted from the diversity of algal species and their growth conditions as well as WAS features, which lead to differences in the digestibility of the substrates (Wang and Park, 2015). Overall, the operation of the anaerobic reactor in SBR mode showed adaptation to seasonal variations in WAS and microalgae production.

Additionally, the generated biogas obtained from WAS and algae co-digestion could be upgraded to high biomethane to be used as biofuel in vehicles, providing an eco-friendly alternative for transportation inside agricultural lands (Fig. 7.3). Considering the fuel consumption of tractors 100% powered by methane (model New Holland T6.180 *Methane Power*) and data from the 4th SBR cycle, for an algal production of 0.63 g L^{-1} d^{-1}, a CH_4 yield of 7.7 L d^{-1} and a production of 2.8 m^3 CH_4 $year^{-1}$ (2 kg CH_4 y^{-1}) were estimated, which is enough to fuel the tractor to run 485 km per year. Other factors influencing tractor fuel consumption should be considered, such as the type of agricultural work performed, the features of the soils, weather conditions, and tractor mechanical maintenance. A full-scale, real case study (EU FP7 All-Gas project) performed in Chiclana (Spain) has efficiently validated the use of biomethane obtained from algal biomass cultivated in wastewater as car biofuel.

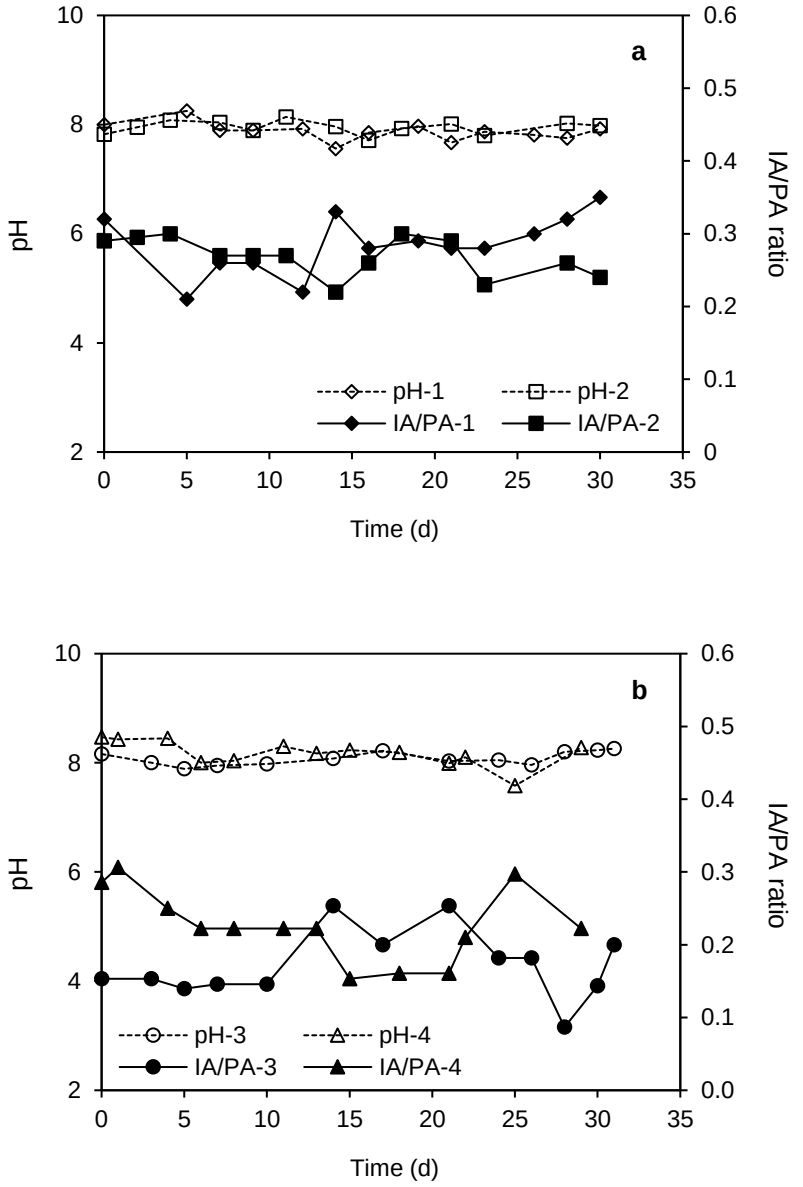

Figure 7.6 pH and alkalinity index (intermediate alkalinity/partial alkalinity (IA/PA) ratio) evolution in (a) cycles 1 (pH-1, IA/PA-1) and 2 (pH-2, IA/PA-2), and (b) cycles 3 (pH-3, IA/PA-3) and 4 (pH-4, IA/PA-4). Cycles 1 and 2 corresponds to WAS mono-digestion while cycle 3 corresponds to WAS and algal AcoD.

Table 7.3 Experimental results and estimated parameters from anaerobic SBR cycles.

Cycles	VS removal (%)	Net experimental CH_4 yield (NL CH_4 kg VS^{-1})	Methane content (%)	Gompertz model				Hydrolysis rate	
				P_{max} (NL biogas kg VS^{-1})	R_{max} (NL biogas kg VS^{-1} d^{-1})	λ (d)	r^2	K_H (d^{-1})	r^2
1	30	155.4	82.8 ± 3.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	19	132.9	81.0 ± 0.0	162.1 ± 6.9	8.9 ± 0.9	-1.2 ± 0.9	0.997	0.0815	0.9928
3	4	108.7	79.2 ± 3.1	124.4 ± 5.1	10.3 ± 2.0	0.3 ± 1.2	0.955	0.0992	0.9600
4	35	225.8	85.5 ± 2.2	254.8 ± 10.3	15.0 ± 1.6	0.5 ± 0.9	0.979	0.0610	0.9874

n.a. = not applicable (Parameters from batch test 1 could not be estimated by the models since initial biogas production was not registered due to operational issues).

7.3.3. Plant growth bioassays

The effects of the four bio-wastes on plant biomass accumulation were assessed through bioassays (Fig. 7.7). The GI was used to evaluate the phytotoxicity effect of the bio-wastes on the biomass production of lettuce. The studied bio-wastes for agricultural irrigation-fertilization included digestate from SBR cycle 1 (F1), digestate from SBR cycle 3 (F2), dry algal biomass (F3), and PBR effluent (F4). F1 and F2 can be considered biosolids since they were obtained after the anaerobic (co-)digestion of the sludge (Collivignarelli et al., 2019). A commercial organic amendment was used as a commercial reference product (FC).

Table 7.4 shows the application schedule followed in the bioassays and the volume or amount of water and bio-wastes applied to the pots considering the target dose of total nitrogen (TN) to be added and the TN content in the liquid (Table 7.5) and solid (Table 7.6) bio-wastes. In this sense, totals of 2.3 and 8.1 g of F3 and FC, respectively, were applied to the pots on day 1, and then water was added for irrigation according to the water demand of the plant. In the case of liquid bio-wastes (F1, F2, and F4), the same total volume of liquid bio-waste (350 mL) was periodically applied to the pots until day 13, and then, the pots were irrigated with water to restore solution losses by plant uptake and/or evaporation. Thus, F1 was applied at a dose 54% higher than that of F2 and F4.

The evolution of plant height and total plant (shoot and root) fresh weight on days 20, 27, and 40 is shown in Fig. 7.8a and Fig. 7.8b, respectively. Shoot plant weight and plant height were notably enhanced by both digestates (F1 and F2), achieving better outcomes than the other tested bio-wastes (Fig. 7.8b). Throughout the assay, F1 and F2 showed no significant differences ($p > 0.05$) in root and shoot weights, respectively. In contrast to the reference control, F1

significantly enhanced plant height by 66-78% ($p < 0.05$), while F2 improved it by 41-52% ($p < 0.05$) (Fig. 7.8a). Regarding root weight, both digestates had lower values throughout the assay in comparison to the reference control (reduced by 9-21% for F1 and by 6-17% for F2), showing no statistically significant differences ($p > 0.05$) (Fig. 7.8b).

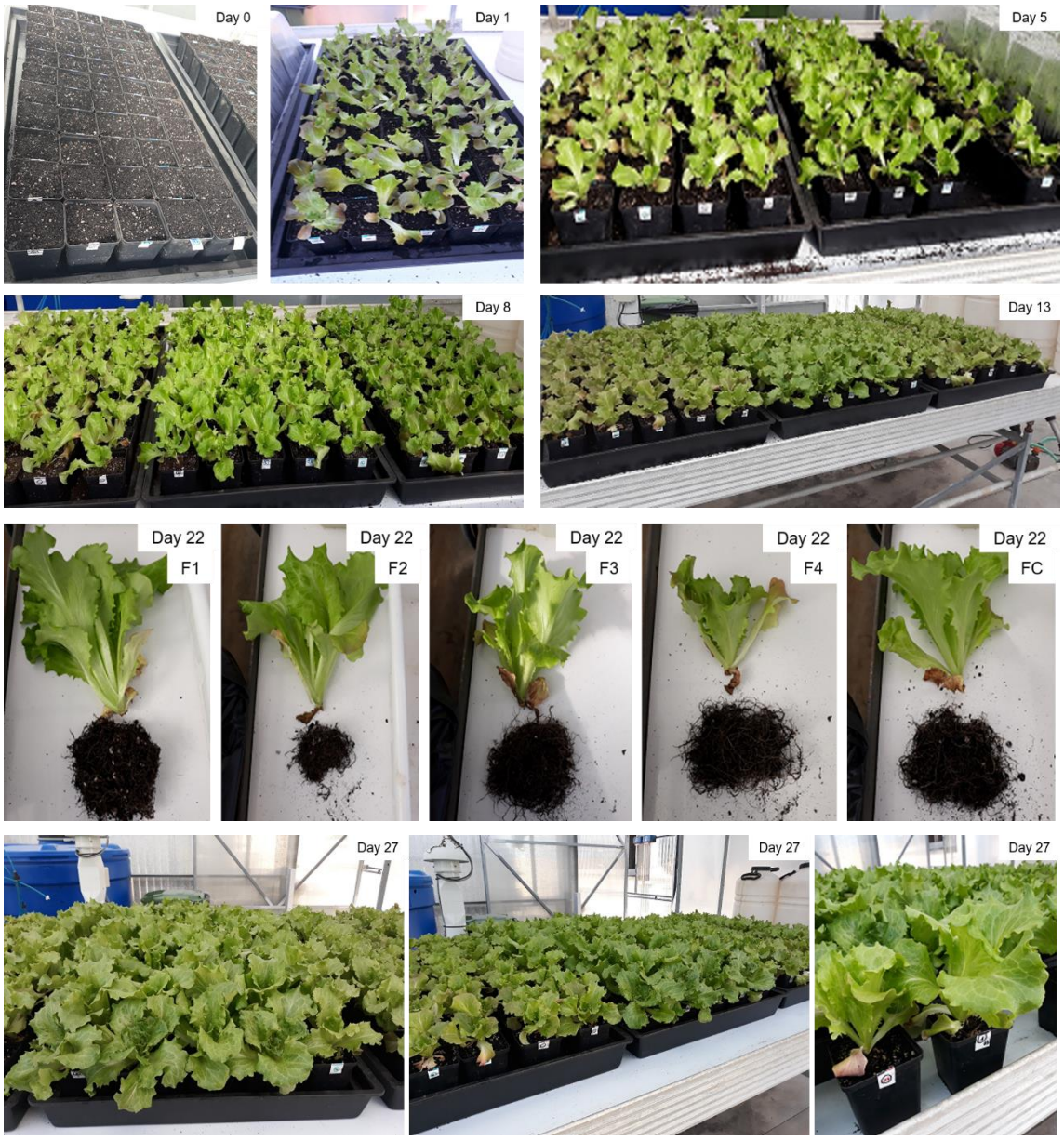

Figure 7.7 Plant growth bioassay.

Table 7.4 Dose of bio-waste and application schedule followed in the plant growth bioassay. Day 0 corresponds to seedling´s sowing.

Day of application	**0**	**1**	**5**	**8**	**13**	**16**	**20**	**16**	**29**	**30**	**33**	**35**	**36**	**37**	**40**
Liquid bio-wastes															
F1 (mL)	0	90	70	90	100	0	0	0	0	0	0	0	0	0	0
F2 (mL)	0	90	70	90	100	0	0	0	0	0	0	0	0	0	0
F4 (mL)	0	90	70	90	100	0	0	0	0	0	0	0	0	0	0
Water added (mL)	0	0	0	0	0	80	120	100	150	100	100	100	100	200	200
Solid bio-wastes															
F3 (g)	0	2.3	0	0	0	0	0	0	0	0	0	0	0	0	0
FC (g)	0	8.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Water added (mL)	0	90	70	90	100	80	120	100	150	100	100	100	100	200	200

F1 = digestate from the 1st SRB cycle (WAS mono-digestion). F2 = digestate from the 3rd SBR cycle (WAS-microalgae co-digestion). F3 = algal biomass harvested by sedimentation and solar dried. F4 = sedimented PBR effluent. FC = organic commercial amendment.

Table 7.5 Main physico-chemical parameters, macro- and micronutrients of the liquid bio-wastes F1 (digestate from the 1st SRB cycle, WAS mono-digestion), F2 (digestate from the 3rd SBR cycle, WAS-microalgae co-digestion), and F4 (sedimented PBR effluent) generated in the winery WRRF.

Parameter	**Bio-wastes**		
	F1	**F2**	**F4**
Physico-chemical			
TN (mg L^{-1})	574	374	0
TOC (mg L^{-1})	84	54	2
pH	7.76	7.63	8.27
Macronutrients			
N-NH_4^+ (mg L^{-1})	531	370	0.14
TP (P_2O_5) (%)	0.02	0.02	0.0004
TK (K_2O) (%)	0.13	0.01	0.02
Heavy metals			
Cd (mg L^{-1})	0.0003	0.0001	0
Cu (mg L^{-1})	0.091	0.052	0.011
Ni (mg L^{-1})	0.025	0.017	0.011
Pb (mg L^{-1})	0.004	0.002	<0.0016
Zn (mg L^{-1})	0.2	0.076	0.035
Hg (mg L^{-1})	0.001	0	<0.0008
Cr (mg L^{-1})	0.013	0.012	0.016
Microbiology			
Escherichia Coli CFU/100 ml	392	5	0

TN = total nitrogen. TOC = total organic carbon. TP = total phosphorus. TK = total potassium. CFU = colony formation units.

Table 7.6 Main physico-chemical parameters, macro- and micronutrients of the solid bio-waste F3 (algal biomass harvested by sedimentation and solar dried) generated in the winery WRRF and the reference control FC (organic commercial amendment).

Parameter	**Bio-wastes**	
	F3	**FC**
Physico-chemical		
Moisture (%)	11.9	35.3
TS (%)	64.7	88.1
TN (mg kg^{-1})	56	16
TOC (%)	42.4	29.9
Macronutrients		
N-NH_4^+ (%)	0.7	0.6
TP (P_2O_5) (%)	2.8	2.9
TK (K_2O) (%)	0.6	0.9
Heavy metals		
Cd (mg kg^{-1})	< 0.5	<0.5
Cu (mg kg^{-1})	166	104
Ni (mg kg^{-1})	5.9	23.8
Pb (mg kg^{-1})	< 5.0	9.9
Zn (mg kg^{-1})	356	284
Hg (mg kg^{-1})	<0.1	<0.4
Cr (mg kg^{-1})	< 10	32
Microbiology		
Escherichia Coli CFU/g	< 40	610

TS = total solids. TN = total nitrogen. TOC = total organic carbon. TP = total phosphorus. TK = total potassium. CFU = colony formation units.

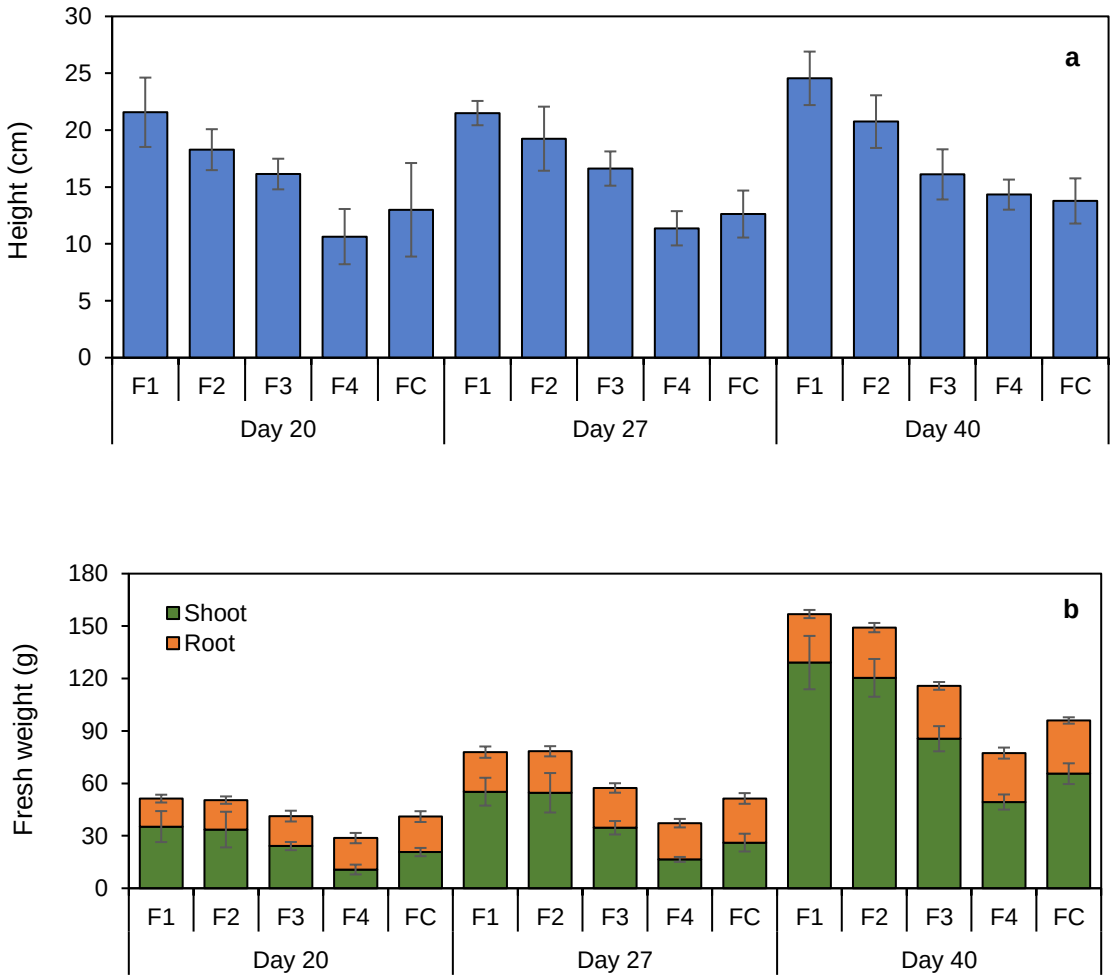

Figure 7.8 Monitoring of (a) plant height, (b) shoot fresh and root fresh weight in bioassays after application of the target organic materials: digestate from WAS mono-digestion (F1), digestate from WAS and microalgae co-digestion (F2), dry algal biomass (F3), PBR effluent (F4), and an organic commercial amendment (FC), at days 20, 27, and 40 after sowing. Error bars represent the standard deviation of the mean (n = 7 at day 20, n = 8 at day 27, and n = 9 at day 40).

When coupling microalgae tertiary wastewater treatment to the winery WWTP (Fig. 7.3), both dry algal biomass (F3) and PBR effluent (F4) are sources of nutrients with the potential to be applied in the vineyards of the company. If AD is not implemented, these bio-wastes could be valorised. When applying dry algal

biomass (F3), the plant height was constant (16-17 cm) at the three sampling times (Fig. 7.8a). By day 40, the plant heights of pots treated with F3 were statistically similar to those of the reference control ($p > 0.05$) and F4 ($p > 0.05$) (Fig. 7.8a). Although the shoot fresh weight was 31% higher than that of the control ($p < 0.05$), the root weight of plants with F3 application was reduced by 1% ($p < 0.05$). The treated water obtained from the PBR effluent (F4) slightly improved plant height during the bioassay in comparison to that in the control, with no significant differences ($p > 0.05$). F4 strongly reduced lettuce total weight compared with that in the control (shoot weight was reduced by 25-48%, and root weight was reduced by 8-18%). By day 40, the root mass of the bio-wastes was reduced compared to that of the reference control ($p < 0.05$) (Fig. 7.8b), which could be explained by the lower nutrient content in reclaimed water (Table 7.5). However, in recent years, the use of reclaimed water for agricultural irrigation has been encouraged, contributing to saving freshwater while applying the available nutrients to the soil (Delanka-Pedige et al., 2020).

The influence of the bio-wastes on the GI (expressed as a percentage of the total fresh plant mass with respect to the reference control) is shown in Fig. 7.9. After 40 days, the best performance in terms of the GI was obtained for digestates F1 and F2 (163% and 155% of the control, respectively). Meanwhile, the GI for the dry algal biomass (F3) and the PBR effluent (F4) were 121% and 81% with respect to the control, respectively. Hence, both digestates and the dry algal biomass attained a better outcome in terms of lettuce biomass accumulation in comparison to the reference material, suggesting a lack of phytotoxicity. The better outcome of F1 could be related to its application at a higher dose. The positive effects of F1 and F2 on plant biomass accumulation could be explained by the higher nitrogen available for plant assimilation in the liquid bio-wastes

(574 mg TN L^{-1} in F1 and 374 mg TN L^{-1} in F2, Table 7.5) in comparison to the other bio-wastes, with nitrogen being the main nutrient present in both digestates. Although the TP and total potassium (TK) contents in FC were higher than those in F1, F2 and F4 (Tables 7.5 and 7.6), these nutrients require water to be soluble and bioavailable. As previously reported, the GI for F2 could be related to the reduced phytotoxicity of digestates obtained from co-digestion processes, having a dilution effect of inhibitory compounds (Solé-Bundó et al., 2017b). The heavy metal concentrations in the digestates (Table 7.5) were below the threshold values defined by the sludge European Directive (CEC, 1986, 2003). Concerning hygenisation, *E. coli* was found in both digestates at lower values (Table 7.5) than the proposed value ($5 \cdot 10^5$ colony forming units per gram) by the EU Directive on spreading sludge on land (EC, 2009c). As reported by other authors, digestates could potentially be applied as biofertilizers and/or soil amendments (Guilayn et al., 2019; Wang and Lee, 2021).

Even when dry algal biomass had a similar TP and ammoniacal nitrogen content to those in the control, the nitrogen content in F3 was 3.5-fold higher, while the TK content was reduced by 33% (Table 7.6). Due to the ability of microalgae to fix nitrogen, they constitute an alternative and low-cost source of nitrogen (Dineshkumar et al., 2018). Algal biomass is considered a slow-release biofertilizer since N and P release from dry algal biomass changes over time (Mulbry et al., 2005). Thus, the application of algal biomass (F3) as a slowly released biofertilizer in agriculture could be a suitable option to offset mineral fertilizer application. Some authors have reported beneficial effects after the use of algal biomass as a biofertilizer. Coppens et al. (2016) reported the use of dry microalgal-bacterial flocs as biofertilizer for tomato cultivation, which showed an initial nitrogen availability of 7% and increased N mineralization by 11% and

25% after 21 and 95 days, respectively, ultimately yielding a plant growth rate equivalent to that when using organic fertilizers. Mulbry et al. (2005) applied dry algal biomass as a soil amendment, reporting an equivalent plant dry weight (corn and cucumber) and nutrient content to those under commercial fertilizer use. They reported that after 21 days, 30-33% of the TN and 39-75% of the TP in algal biomass was available for the plants. Diverse mineralization rates could be explained by different soil properties (type of soil, moisture content, pH, and microbial activity, among others) (Canali et al., 2011). Biofertilizer from algal biomass is a higher-quality by-product than co-digestion digestates due to its high content of proteins and phytohormones (Arashiro et al., 2018). Another advantage of its application to soils is the absence of NH_3 volatilization (Mulbry et al., 2005), and it is assumed that algal biofertilizer is not lost through runoff or leaching (Fang et al., 2016).

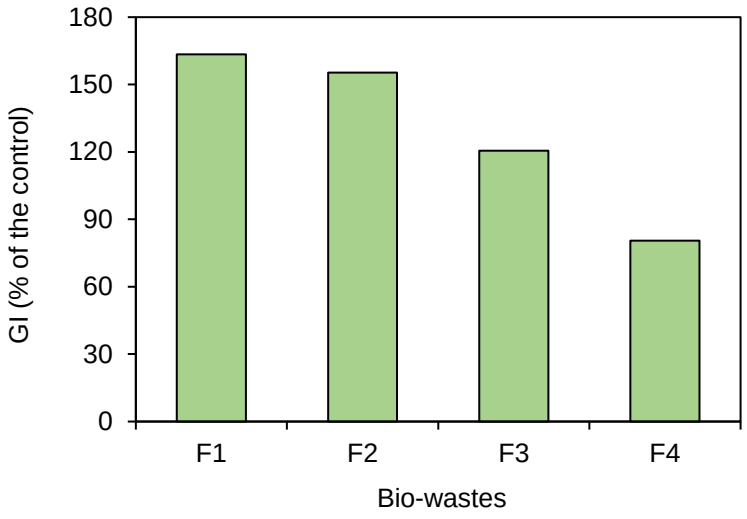

Figure 7.9 Effect of bio-wases on the GI (expressed as a percentage of the total fresh plant mass with respect to the reference control -organic commercial amendment-) of lettuce (*Lactuca sativa*) at the end of the bioassay (day 40). F1, digestate from WAS mono-digestion; F2, digestate from WAS and microalgae co-digestion; F3, dry algal biomass; and F4, PBR effluent.

Considering that the generation and application of these bio-wastes would be performed by the same company, facility, storage, transportation, and distribution costs would be negligible. In addition to the quality of the studied bio-wastes, the suitability of their application for fertilization and/or irrigation should meet the current regulations. Organic matter stability and heavy metal content in the soil should be assessed to determine the potential risks of metal accumulation and hygenisation in the soil-plant system (Alburquerque et al., 2012).

7.4. Conclusions

Microalgae efficiently removed ammonium (97%) and phosphate (93%) from the secondary effluent. The co-digestion of WAS and harvested microalgae in SBR mode (225.8 NL CH_4 kg VS^{-1}) was 45-70% higher than that for WAS mono-digestion.

Bioassays of the generated bio-wastes showed GIs of 163%, 155%, and 121% in comparison to the control for the mono-digestion digestate, co-digestion digestate, and dry algal biomass, respectively, highlighting their potential application as biofertilizers to the company´s arable lands.

This strategy contributes to moving towards the circular use of resources within the company, closing nutrient loops by anaerobic co-digestion of WAS and microalgae, generating biomass, and reusing recovered water in the vineyards.

Future research should consider substrates variability all over the year: the influence of the harvest period into the winery WWTP solid and liquid fluxes as well as the impact of seasonality in algal biomass production. Moreover, a life cycle assessment and techno-economic assessment can be useful in the

determination of the environmental impacts and the financial feasibility, providing a comprehensive perspective for the transition from a conventional winery WWTP to a sustainable WRRF.

SECTION 5

General conclusions and future perspectives

CHAPTER 8.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

8.1. General conclusions and future perspectives

In this thesis, some relevant factors regarding the potential of pesticides removal by microalgae-based systems and algal biomass valorisation have been assessed. The overall results obtained in this thesis contributed to attaining the main defined objectives and detailed conclusions of the work are presented in each chapter.

The main achievements and general conclusions that can be drawn from this thesis are summarized below:

- It has been demonstrated the feasibility of a microalgae-based system for the removal of hydrophobic and polar pesticides. The main mechanisms inherent to microalgae and involved in the removal are biodegradation plus photodegradation as shown by the transformation products identified. Besides, sorption onto algal biomass highly contributed to hydrophobic pesticides removal.

- It has been proved the capacity of microalgae-based treatment technology for the simultaneous removal of pesticides, consumption of nutrients, and the generation of biomass for biofuels production in a 1 m^3 outdoor pilot-photobioreactor.

- The transformation products resulting from the degradation and generated by the active microalgae were identified in the batch experiments and were detected during pilot-scale operation. This fact highlights the effectiveness of these nature-based systems for nutrients removal in urban wastewater treatment, but also to remove organic

micropollutants, offering new perspectives for the degradation of other groups of micropollutants.

- When using organic flocculants high harvesting efficiencies were achieved for small microalgal cells. On the other hand, filamentous species that grown in the pilot-photobioreactor contributed to their harvesting by sedimentation.

- Harvested algal biomass has been valorised by its conversion to bioenergy through of anaerobic digestion. To valorise algal biomass, synergies with another available organic substrate were sought, such as activated sludge. Pretreatments do not always increased biogas yield and depending on the substrate. For instance, enzymatic pretreatments of algal biomass enhanced algal solubility and methane production. Nevertheless, when pretreating and co-digesting activated sludge and microalgae mixtures, methane generation was not improved. Despite this fact, co-digestion of algal biomass and waste activated sludge was successful even without applying the studied pretreatments.

- An algal-based tertiary wastewater treatment coupled to the anaerobic co-digestion of algal biomass and activated sludge from the winery WWTP, contributed to applied bio-waste streams as fertilizers, closing waste fluxes into the winery company. These outcomes are useful valid for another possible scenario: the co-digestion of microalgae with sludge from a municipal WWTP taking advantage of the digesters usually oversized and obtaining more biogas.

- Results obtained from the real case study at the winery company demonstrate a useful scenario for the transition to a water resource recovery facility. Nonetheless, future research should consider substrates variability all over the year. Moreover, economic analysis and a life cycle assessment could provide useful data for future industrial-scale implementation.

APPENDIX A

Specific analytical procedures

Polar pesticides quantification and TPs identification at indoor batch experiments

Pesticide degradation and TP identification were performed after direct injection of the sample into the analytical instrument, an ultra-high performance liquid chromatography (UHPLC) system Acquity (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q Exactive (Thermo Fisher Scientific, San Jose, CA, USA), equipped with a heated-electrospray ionization source HESI. Acetamiprid was analyzed in the positive ionization mode (HESI+) while propanil and bentazone were analyzed in the negative ionization mode (HESI-). TPs formed during the degradation process were determined in both positive and negative ionization modes. Chromatographic separation was achieved with a Purospher® STAR RP-18 endcapped Hibar® HR (150 × 2.1 mm, 2 μm) column from Merck (Darmstadt, Germany) and a linear gradient of the organic constituent of the mobile phase. The mobile phase employed for HESI+ analyses consisted of (A) water and (B) methanol, both containing 0.1% of formic acid (flow rate of 0.2 mL min^{-1}), whereas in HESI- a mobile phase of (A) water and (B) acetonitrile (flow rate of 0.3 mL min^{-1}) was used. In both cases, the organic gradient employed was as follows: 5% B from the start to time (t) = 1 min, 20 % B at t = 3 min, 80% B at t = 6 min, 100% B at t = 7 min. Pure organic conditions were maintained until t = 9 min. Finally, initial conditions (5% B) were again achieved at t = 9.5 min and held for 4.5 min for column re-equilibration. The injection volume was 10 μL.

The specific conditions used in the HESI interface were: ion spray voltage, 3.0 kV in HESI+ and -2.5 kV in HESI-; sheath gas flow rate, 40 arbitrary units; auxiliary gas, 10 arbitrary units; capillary temperature, 350 ºC; and vaporizer temperature, 400 ºC. Nitrogen (>99.98%) was employed as sheath, auxiliary and

sweep gas. Accurate mass detection was conducted in data-dependent acquisition (DDA) mode. First, a full scan was acquired over the m/z range 70-1,000 at a full width at half maximum (FWHM) resolution of 70,000 (at m/z 200). Then, data-dependent MS/MS scan events (FWHM resolution of 17,500 at m/z 200) were recorded for the five most intense ions (>$10e^5$) detected in each scan, with a normalized collision energy of 40%. Data acquisition was controlled by Xcalibur 2.2 software (Thermo Fisher Scientific).

The software Compound Discover 3.1 from Thermo Fisher Scientific was used to process the HRMS data generated with the LC-Orbitrap MS to identify the TPs formed during batch degradation experiments. Briefly, experimental samples (i.e., those collected at time 2 and 7 days) were compared with control samples (i.e., samples collected at t = 0 days). The software was used for peak alignment and deconvolution using 2 min as maximum retention time shift and 5 ppm of mass tolerance. Then, the different peaks detected were grouped and their elemental composition predicted. In parallel, a search by formula or mass was performed in various MS libraries and compound databases (mzCloud, mzVault, ChemSpider) for assignment of potential compound identity. The list of potential candidates was subsequently revised to identify TPs that were only present in experimental samples and absent in control samples (killed and abiotic reactors). Once identified, the molecular structures proposed by the software were evaluated according to the elemental composition of the molecular and fragment ions, fragment rationalization (assisted by fragment ion search scoring), and isotopic patterns. Quantification of the pesticide removal was done using the corresponding deuterated analogue as internal standard. Limit of detection (LOD) and limit of quantification (LOQ) for propanil, acetamiprid and

bentazone were 0.20 and 0.66 μg L^{-1}, 0.22 and 0.74 μg L^{-1} and 2.9 and 9.6 μg L^{-1}, respectively.

Hydrophobic pesticides quantification and TPs identification at indoor batch experiments

A 30 mL volume of liquid sample was ultrasonically extracted for 5 min with chloroform. Then, the sample was centrifuged (3.500 rpm, 5 min) and the organic phase was recovered. The liquid phase was extracted once more, and the organic phases were evaporated until dry under a nitrogen stream. The residue was reconstituted with 50 μL of ethyl acetate and then subjected to gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) analysis.

Freeze-dried biomass samples and filters were extracted by pressurised liquid extraction using a 350 ASE system (Dionex, USA). Prior to extraction, biomass samples were spiked with 15 ng of internal standard and left overnight at 4 °C. Then, samples and 2 g of Florisil were loaded into an ASE extraction cell (22 mL) previously filled with 6 g of Florisil. Hexane and dichloromethane (1:1, v/v) were used as the extraction solvent. Temperature and pressure were set at 100 °C and 1650 psi, respectively. Extracts were evaporated to dryness under a nitrogen stream, and the residue was reconstituted with 50 μL of ethyl acetate prior to GC-MS/MS analysis.

Pesticide concentrations were determined through GC-MS/MS on a 7890B GC coupled to a 7000C triple quadrupole (Agilent technologies, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm, 0.25 μm). The operating conditions were 80 °C for 2 min, raised at 25 °C min^{-1} to 180 °C for 6 min, at 5 °C min^{-1} to 240 °C for 5 min, at 10 °C min^{-1} to 280 °C for 5 min, and at 30 °C min^{-1} to 325 for 2 min. The temperatures corresponding to the transfer line and

the ionisation source were 300 and 280 °C, respectively. The collision energy was 70 eV. Two different transitions were monitored for each pesticide.

No analytes of interest were observed in the method blank samples. Recoveries ranged from 50 to 58%. LOD were 1.7, 2.8, and 0.9 ng L^{-1} for chlorpyrifos, cypermethrin, and oxadiazon, respectively. LOQ were 5.6, 9.2, and 2.1 ng L^{-1} for chlorpyrifos, cypermethrin, and oxadiazon, respectively.

Identification of TPs was carried out on a Waters Acquity UHPLC system (Waters, Milford, MA, USA) coupled to a hybrid quadrupole-Orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific; San Jose, CA, USA) equipped with a HESI II heated-electrospray ionisation source. Chromatographic separation was performed on a Purospher STAR RP-18 end-capped (2 μm) Hibar HR 150-2.1 UHPL column (Merck). The mass spectrometer performed a Fourier transform mass spectrometry scan event of 50-700 m/z at a resolution of 70,000 and a subsequent MS/MS scan event was acquired at a resolution of 35,000. To identify all potential TPs, the total ion current chromatograms acquired at 2 and 7 days were compared with those obtained at initial time using Compound Discoverer (Thermo Fisher Scientific). This software allows differential analysis of selected sets of samples by simultaneously comparing thousands of MS spectra to find significant differences between the control and samples. The accurate masses of the potential TPs were then extracted to confirm their presence. Identification of the potential TPs was based on their accurate mass, mass error, molecular formula, and degree of unsaturation of the parent ion and product ions.

Pesticide quantification and TPs detection at outdoor pilot-scale

Pesticide removal and TPs formation were monitored after large-volume direct injection of the sample into an analytical system consisting of a SCIEX Exion LC™ AD chromatograph, that incorporates a Shimadzu FCV-11AL Reservoir Selection Valve and a 0.5 mL injection loop, coupled to a SCIEX X500R QTOF detector, equipped with a Turbo V™ source (Sciex, Framingham, MA). Chromatographic separation was achieved with a Purospher® STAR RP-18 endcapped Hibar® (125 × 4 mm, 5 μm) column from Merck (Darmstadt, Germany) and a mobile phase consisting of (A) water and (B) acetonitrile, with the following organic gradient: 5% B from the start to time (t) = 1 min, 80% B at t = 15 min, 100% B at t = 17 min. Pure organic conditions were maintained for 3 min and then, in 2 min, initial conditions (5% B) were again achieved and held for 4 min for column re-equilibration. The injection volume was 400 μL and the flow rate 1 mL min^{-1}.

The specific conditions used in the ion source were a curtain gas of 35 arbitrary units, ion source gas 1 and 2 of 60 and 45 psi, respectively, temperature 700 ºC, with an ion spray voltage of -4500 V (negative mode) and 5500 V (positive mode). Accurate mass detection was achieved through a full TOF-MS scan over the m/z range 70-650, using an accumulation time of 0.125 s, a declustering potential of 70 V with a spread of 20 V, and a collision energy of 10 V, followed by an Information Dependent Acquisition (IDA) TOF-MS/MS analysis was performed with a workflow for small molecules, i.e., a maximum of 6 candidate ions, an intensity threshold of 100 counts/s, with dynamic background subtraction and dynamic accumulation, a mass tolerance of 20 mDa and an inclusion list containing the m/z of the target pesticides and the TPs identified in the batch experiments. Data acquisition was controlled by SCIEX OS v.1.5

Pesticide removal and TP formation during pilot-scale PBR experiments were monitored using the software Sciex OS v.1.5. (Sciex, Framingham, MA). To detect degradation by-products, a suspect list was set up including potential TP candidates generated using the EAWAG BBD Pathway Prediction System and those found in the present study in the lab-scale batch reactors. Therefore, a suspect screening and a comprehensive analysis of MS^2 spectra was performed for the structural elucidation of the possible TPs. Quantification of the pesticide removal was done with the internal standard method, using the corresponding deuterated analogue as internal standard. LOD and LOQ obtained for both compounds were 0.017 and 0.05 μg L^{-1}, respectively. Then, a semi-quantitation was performed for the TPs formed.

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Después de varios años de haber terminado mi etapa universitaria, sabía que volver al ámbito académico iba a ser un todo un desafío. De hecho, fue una travesía interesante y con sorpresas. Lo planificado sólo fue un mapa, pero el territorio me mostró que planificar es sólo una guía. Y como suele suceder, sobre la marcha las cosas siempre fluyen y se adaptan momento a momento. Y hoy mirándolo en perspectiva, fue una etapa con mucho crecimiento y aprendizaje que también me permitió conocer lindas personas que hicieron posible esta etapa.

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Curriculum vitae of the author

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Academic background

2017-Present Spain	**PhD candidate in the Environmental Science and Technology Doctoral Program.** Thesis tittle: "Microalgae-based systems for micropollutants removal, resource recovery and bioenergy production towards a circular bioeconomy approach". Universitat Autònoma de Barcelona.
2015-2017 Spain	**Master in Biological and Environmental Engineer** Universitat Autònoma de Barcelona.
2010-2011 Germany	**Master in Natural Resource Management** Cologne University of applied Sciences.
2009-2010 Mexico	**Master in Environmental Sciences** Universidad Autónoma de San Luis Potosí.
2004-2008 Argentina	**Bachelor in Environmental Sciences** Universidad Blas Pascal.

Former scientific activities

Jan2018 -Dec 2020	**Teaching assistant** Teaching assistant in the Department of Chemical, Biological and Environmental Engineering at Universitat Autònoma de Barcelona Chemical engineering, and biotechnology lab-experimentation. (Barcelona, Spain).
May 2019	**Statistical course** Advanced statistical computing using R. ICTA-UAB (Barcelona, Spain).
May 2018-Dec 2018	**Tutor in Argó Programme** Tutor of students at the UAB laboratory. Topic: Bioremediation using microalgae cultivated in a photobioreactor**.**
Jul 2018	**Circular economy course** Generation and development of model business from a circular economy approach. Parc de Recerca UAB (Barcelona, Spain).

Publications in journals related with this thesis

Avila*, R.;* Justo Á.; Carrero, E.; Crivillés, E.; Mercader, M.; Vicent, T.; Blánquez, P. Water resource recovery coupling microalgae wastewater treatment and sludge co-digestion at industrial pilot-scale. *Submitted to Bioresource Technology.*

Avila, R.; García-Vara, M.; López-García, E.; Postigo, C.; López de Alda, M., Vicent, T.; Blánquez, P. Evaluation of an outdoor pilot-scale tubular photobioreactor for removal of selected pesticides from water. *Submitted to Science of the Total Environment.*

Avila, R., Carrero, E., Vicent, T., Blánquez, P., 2021. Integration of enzymatic pretreatment and sludge co-digestion in biogas production from microalgae. Waste Manag. 124, 254 263. https://doi.org/10.1016/j.wasman.2021.01.042

Avila, R., Peris, A., Eljarrat, E., Vicent, T., Blánquez, P., 2021. Biodegradation of hydrophobic pesticides by microalgae: Transformation products and impact on algae biochemical methane potential. Sci. Total Environ. 754, 142114. https://doi.org/10.1016/j.scitotenv.2020.142114

Avila, R., Carrero, E., Crivillés, E., Mercader, M., Vicent, T., Blánquez, P., 2020. Effects of low temperature thermal pretreatments in solubility and co-digestion of waste activated sludge and microalgae mixtures. Algal Res. 50, 101965. https://doi.org/10.1016/j.algal.2020.101965

Publications in preparation

Pla, M., Avila, R., Vicent, T., Blánquez, P., Martínez-Alonso, M., Gaju, N. Effect of organic toxic compounds on the evolution of microbial populations in a continuous outdoor pilot- photobioreactor. *In preparation*.

Other publications

Abad, V., Avila, R., Vicent, T., Font, X., 2019. Promoting circular economy in the surroundings of an organic fraction of municipal solid waste anaerobic digestion treatment plant: Biogas production impact and economic factors. Bioresource Technology, *283*, 10-17. https://doi.org/10.1016/j.biortech.2019.03.064

Avila, R., 2011. Remoción de azul de metileno en agua empleando bagaso de *Agave salmiana* como biosorbente (MSc thesis). Available from Academia repository:https://www.academia.edu/24194289/CINTIA_ROMINA_AVILA

Avila, R, Abril, G, 2008. Impacto de la actividad cinegética sobre los niveles ambientales de plomo en el norte de la provincia de Córdoba: evaluación y estrategias de gestión (Final tesis). Available from UBP repository: https://bib.ubp.edu.ar/cgi-bin/koha/opac-detail.pl?biblionumber=36014

Conferences

Avila R. Circular bioeconomy project for wastes streams management and valorisation in a winery company. Workshop CYRUS project: "Trends in Circular Bioeconomy". October 2020. Barcelona, Spain. Oral presentation.

Avila, R., Vicent, T., Blánquez, P. Low-temperature thermal pre-treatment with and without flocculation to enhance microalgae solubility and digestibility. IWA Conference on Algal Technologies and Stabilization Ponds for Wastewater Treatment and Resource Recovery, 2019. Valladolid, Spain. Oral presentation.

López-García, E., Postigo, C., Avila, R., Blánquez, P., Vicent, T., López de Alda, M. High resolution mass spectrometry to investigate the fate of pesticides during microalgae-based bioremediation treatment of water. SETAC Europe 29th Annual meeting, 2019. Helsinki, Finland.

López-García E., Postigo C., Avila R., Blánquez P., Vicent T., López de Alda, M. Evaluation of a new water treatment technique based on microalgae for bioremediation of pesticides using high resolution mass spectrometry. 4th International mass spectrometry school, 2019. Sitges, Spain. Poster presentation.

Avila, R., Peris, A., Eljarrat, E., Vicent, T., Blánquez, P. Biodegradation of non-polar pesticides by microalgae-mased systems: impact on biochemical methane potential and transformation products generation. 28th European Biomass Conference & Exhibition. Transition to a Bioeconomy. EUBCE 2020. Marseille, France. Accepted. Oral presentation. Postponed for 2022.

Stay in internationally recognized research centers

Laboratoire de Biotechnologie de l'Environnement (LBE), French National Institute for Agricultural Research (INRA), Narbonne, France. Less than 1 month (Interrupted by COVID-19 measures). Research topic: Enzymatic microalgae pretreatments for hydrogen production by dark fermentation. The research at the laboratory was re-defined and a review paper is in preparation: "Opportunities and challenges of coupling dark fermentation and microalgae cultivation for biomass valorisation".

Participation in R + D projects

ViTech – Waste fluxes closure for a circular viniculture (TES/792/2017), financed by the Generalitat de Catalunya. Funded company: Miguel Torres S.A.

BECAS - Development of bioremediation processes for the elimination of agricultural (pesticides) and industrial (halogenated solvents) pollutants in water and soil (CTM2016-75587-C2-1-R).

CYRUS - Creating Virtual Reality e-resources for European Sustainable Education in circular bioeconomy with bio-based products. Erasmus + project KA203 Strategic Partnerships for higher education (2019-1-RO01-KA203-063773).

Professional experience

Apr 2014-Aug 2015 Buenos Aires City Government	**Environmental manager** Restoration of the Matanza-Riachuelo river basin. Implementation and monitoring of environmental KPIs. Environmental industrial control.
Jan 2012-Jun 2013 Odebrecht	**Environmental coordinator** Management of environmental permits during construction phase. Environmental supervision of subcontracted companies. Compliance with environmental legislation.
May 2008-Apr 2011 MetroQuímica	**Freelance Teacher** Development and teaching of the Environmental Impact Assessment course.
Sep 2007-Apr 2008 Environmental consultancy Molina & Sbarato	**Environmental Analyst** Environmental audits at industries. Verification of compliance of environmental legislation. Development of environmental management plans.

Languages

Spanish	Native proficiency.
English	Advanced proficiency (C1). TOEFL iBT (101/120).
German	Intermediate proficiency (B1).
Catalan	Elementary proficiency (A2).

Digital tools

Numerical simulation	RStudio - Matlab - Python - FlexPDE
GIS	Global Mapper - QGIS
Life cyle assessment	SimaPro - GaBi

Volunteer experience

Sep 2020 – Present	**Surfrider Foundation Europe** Organization of ocean clean-up events.
Jun 2017 – Jan 2019	**United Nations** Crowdmaping rural Tanzania to fight FGM.

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