UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

Recovery of phycobiliproteins and biogas from microalgae treating wastewater

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Terugwinning van phycobiliproteïnen en biogas uit microalgen die afvalwater behandelen

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To my family.

“Try not to become a man of success, but rather try to become a man of value.”

— Albert Einstein.

Preface

This thesis was developed within the framework of the European Joint Doctorate programme "Sustainable Product, Energy and Resource Recovery from Wastewater" (SuPER-W). This programme was established as a strong collaboration between academic and private partners, in order to train early-stage researchers to: i) optimise existing technologies and develop novel integrated technologies for product, energy and resource recovery from wastewater; ii) identify potential bottlenecks in the implementation and exploitation of these technologies; and iii) stimulate policy input formulation.

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Abstract

Current global environmental issues raise unavoidable challenges to manage natural resources towards a sustainable development. Water is undoubtedly the most essential resource of humanity. However, supplying the human population with clean water has been a major challenge for decades. Contaminants in municipal, industrial, and agricultural wastewaters endanger water bodies and the treatment of these effluents represent a high energy demand. The development of efficient wastewater treatment technologies is thus becoming increasingly important.

Due to their metabolic flexibility, microalgae represent interesting biological systems for treating a variety of wastewater. In particular, in the context of a circular and bio-based economy, microalgae biomass has shown its great potential to treat wastewater streams, while recovering sustainable bioproducts, thus implementing biorefinery concepts. However, although extensive research has been done to optimise microalgae systems in terms of operation and economic feasibility, there is still need for improvements and realistic information in order to implement these systems at large scale.

This PhD thesis aims to contribute to this quest by performing holistic studies on microalgae systems for wastewater treatment combined with different strategies for biomass valorisation. The overall content of this PhD thesis is divided in two main parts. The first part consists in experimental studies combining wastewater treatment and resources recovery using microalgae-based systems, while the second part is dedicated to the environmental analyses, through Life Cycle Assessment (LCA), of the technologies and biomass valorisation techniques investigated in this thesis.

The first part presents the investigated alternatives of microalgae-based wastewater treatment systems, testing different cultivation systems and evaluating the potential to recover bioenergy (biogas) and high-value compounds (phycobiliproteins). To start, a

well-known microalgae system, a high rate algal pond (HRAP), is studied in order to simplify its maintenance, reduce costs and the footprint. In this context, the effect of the primary treatment on the long-term performance of pilot-scale HRAPs was investigated not only in terms of wastewater treatment efficiency and biomass characteristics, but also of bioenergy recovery potential from harvested biomass. This study showed that removing the primary treatment preceding a HRAP did not significantly affect the wastewater treatment efficiency (NH_4^+-N removal of 93 and 91% and chemical oxygen demand (COD) removal of 62 and 65% in HRAP with and without primary treatment, respectively). Therefore, when water resource recovery is the main objective, this step seemed to be dispensable, but if bioenergy recovery through biogas production is considered, the co-digestion with primary sludge could significantly improve the methane yield (238–258 mL CH_4/g VS compared to 189–225 mL CH_4/g VS) and kinetics of microalgae mono-digestion.

Following, a second study investigated the cultivation of cyanobacteria-dominated biomass in lab-scale photobioreactors (PBRs) using centrate diluted in secondary effluent from the HRAPs system at different ratios. Results showed that cyanobacteria dominance was stable in a mixed culture with effective treatment efficiency (removal up to 52% of COD, 86% of NH_4^+-N and 100% of phosphorus). In addition, biomass grown in these systems could be valorised through phycobiliproteins (up to 17 mg/g dry biomass) and biogas production (from 159 to 199 mL CH_4/g VS).

Lastly, a third study investigating unialgal cultivations of *Nostoc* sp., *Arthrospira platensis* (*Spirulina*) and *Porphyridium purpureum* for further phycobiliproteins recovery was carried out. Light intensity and growth medium composition were optimised, indicating that light conditions influenced the phycobiliproteins production more than the medium composition. Conditions were then selected to cultivate these microalgae in food-industry wastewater. Efficient wastewater treatment (removal up to 98% of COD, 94% of inorganic nitrogen and 100% of phosphorus) and successful extraction of phycobiliproteins (up to 103 mg/g dry biomass) were achieved.

The second part of this thesis presents an overview of the environmental aspects associated with the microalgae systems and biomass recoveries focused in the previous

experimental studies. Two comparative LCAs were conducted in order to characterise the environmental burdens which would be caused by scaling-up the results obtained at lab and pilot-scale.

The first LCA addressed HRAPs systems for wastewater treatment in small communities comparing biogas and biofertilisers as options for resources recovery. Both microalgae systems were also compared to a conventional activated sludge plant. Moreover, an economic analysis was also carried out. This study showed that, considering the most significant impact categories, HRAPs systems implemented in warm climate region showed to be the most environmentally friendly alternative while the biofertiliser production showed to be the most economically feasible. Also, HRAPs showed lower potential environmental impacts compared to an activated sludge system.

The second LCA evaluated two microalgae systems comparing treatment of urban or industrial wastewater, with recovery of bioproducts (phycobiliproteins and digestate for reuse in agriculture) and bioenergy (biogas). Additionally, both alternatives were compared to a conventional system using standard growth media for phycobiliproteins production. The results indicate that the system treating industrial wastewater has lower environmental impacts than the system treating urban wastewater in most of the environmental indicators considered. Moreover, using wastewater appeared to be more environmentally friendly than using standard growth medium to cultivate microalgae.

In conclusion, the results obtained in this thesis suggested that microalgae systems seem to have a great potential to improve water quality and recover valuable resources from wastewater. Therefore, based on the results of this study and considering the increasing need for conceptual biorefineries, sustainable installations at full scale would be the next step towards a circular economy.

Resumen

Los problemas ambientales actuales en el mundo plantean desafíos inevitables para gestionar los recursos naturales hacia un desarrollo sostenible. El agua es sin duda el recurso más esencial de la humanidad. Sin embargo, el suministro de agua limpia a la población humana ha sido un gran desafío durante décadas. Los contaminantes en las aguas residuales municipales, industriales y agrícolas ponen en peligro las masas de agua y el tratamiento de estos efluentes representa una gran demanda de energía. El desarrollo de tecnologías eficientes de tratamiento de aguas residuales se está volviendo cada vez más importante.

Debido a su flexibilidad metabólica, las microalgas representan sistemas biológicos interesantes para tratar una variedad de aguas residuales. En particular, en el contexto de una bioeconomía circular, la biomasa de microalgas ha demostrado su gran potencial para tratar aguas residuales, al tiempo que recupera bioproductos sostenibles, implementando así conceptos de biorefinería. Sin embargo, aunque se han realizado investigaciones exhaustivas para optimizar los sistemas de microalgas en términos de operación y viabilidad económica, aún se necesitan mejoras e información realista para implementar estos sistemas a gran escala.

Esta tesis doctoral tiene como objetivo contribuir a esta búsqueda mediante la realización de estudios holísticos sobre sistemas de microalgas para el tratamiento de aguas residuales combinadas con diferentes estrategias para la valorización de la biomasa. El contenido de esta tesis doctoral se ha dividido en dos partes principales. La primera parte consistió en estudios experimentales que combinan el tratamiento de aguas residuales y la recuperación de recursos utilizando sistemas basados en microalgas, mientras que la segunda parte se ha dedicado a la evaluación ambiental, a través del Análisis del Ciclo de Vida (ACV) de las tecnologías y técnicas de valorización de biomasa investigadas.

La primera parte consistió en investigar alternativas de sistemas de tratamiento de aguas residuales a base de microalgas, probando diferentes sistemas de cultivo y evaluando el potencial para recuperar bioenergía (biogás) y compuestos de alto valor (ficobiliproteínas).

En primer lugar, se estudió un conocido sistema de microalgas, la laguna algal de alta carga (HRAP), para simplificar su mantenimiento, reducir los costes y su huella ecológica. En este contexto, se ha investigado el efecto del tratamiento primario en el rendimiento a largo plazo de las HRAPs a escala piloto, no solo en términos de eficiencia del tratamiento de aguas residuales y características de biomasa, sino también del potencial de recuperación de bioenergía de la biomasa cosechada. Este estudio mostró que la eliminación del tratamiento primario que precede la HRAP no afectaba significativamente la eficiencia del tratamiento de aguas residuales (eliminación de 93% de NH_4^+-N y 91% de demanda química de oxígeno (DQO), y 62 y 65% en HRAP con y sin tratamiento primario, respectivamente). Por lo tanto, cuando la recuperación de recursos hídricos es el objetivo principal, el tratamiento primario parece ser prescindible, pero si se considera la recuperación de bioenergía a través de la producción de biogás, la codigestión con lodo primario podría mejorar significativamente la producción de metano (238– 258 mL de CH_4/g sólidos volátiles (SV) en comparación con 189–225 mL de CH_4/g SV) y la cinética de la mono-digestión de microalgas.

A continuación, un segundo estudio investigó el cultivo de biomasa dominada por cianobacterias en fotobiorreactores (PBR) a escala de laboratorio utilizando digestado diluido en efluente secundario del sistema de HRAPs a diferentes proporciones. Los resultados mostraron que el dominio de las cianobacterias era estable en un cultivo mixto con una eficacia del tratamiento notable (eliminación de hasta 52% de DQO, 86% de NH_4^+-N y 100% de fósforo). Además, la biomasa cultivada en estos sistemas podría valorizarse a través de ficobiliproteínas (hasta 17 mg/g de biomasa seca) y la producción de biogás (de 159 a 199 mL de CH_4/g SV).

Por último, se llevó a cabo un tercer estudio que investigó los cultivos unialgales de *Nostoc* sp., *Arthrospira platensis* (*Spirulina*) y *Porphyridium purpureum* para la recuperación de ficobiliproteínas. La intensidad de luz y la composición del medio de

cultivo se optimizaron, observando que las condiciones de luz influyeron más que la composición del medio en la producción de ficobiliproteínas. A continuación, se seleccionaron las condiciones para cultivar estas microalgas en las aguas residuales de la industria alimentaria. Se logró un tratamiento eficiente de las aguas residuales (eliminación de hasta 98% de DQO, 94% de nitrógeno inorgánico y 100% de fósforo) y extracción exitosa de ficobiliproteínas (hasta 103 mg/g de biomasa seca).

La segunda parte de esta tesis presenta una visión general de los aspectos ambientales asociados con los sistemas de microalgas y la recuperación de biomasa centrados en los estudios experimentales anteriores. Se realizaron dos ACV comparativos para caracterizar las cargas ambientales que serían causadas a partir del escalado de los resultados obtenidos a escala de laboratorio y piloto.

El primer ACV abordó los sistemas de HRAPs para el tratamiento de aguas residuales en pequeñas comunidades comparando biogás y biofertilizantes como opciones para la recuperación de recursos. Ambos sistemas de microalgas también se compararon con una planta de lodo activado convencional. Además, también se realizó un análisis económico. Este estudio demostró que, considerando las categorías de impacto más significativas, el sistema HRAPs junto con la producción de biofertilizantes e implementado en la región de clima cálido demostró ser la alternativa más sostenible, mientras que la producción de biofertilizantes demostró ser la más viable económicamente. Además, los HRAPs mostraron un menor impacto ambiental potencial en comparación con un sistema de lodos activados.

El segundo ACV evaluó dos sistemas de microalgas que compararon el tratamiento de aguas residuales urbanas o industriales, con la recuperación de bioproductos (ficobiliproteínas y digestado para reúso en agricultura) y bioenergía (biogás). Además, ambas alternativas se compararon con un sistema convencional con medios de cultivo estándar para la producción de ficobiliproteínas. Los resultados indican que el sistema de tratamiento de aguas residuales industriales tiene un menor impacto ambiental que el sistema de tratamiento de aguas residuales urbanas en la mayoría de los indicadores ambientales considerados. Además, el uso de aguas residuales resultó ser más ecológico que el uso de un medio de cultivo estándar para las microalgas.

En conclusión, los resultados obtenidos en esta tesis sugieren que los sistemas de microalgas podrían tener un gran potencial para mejorar la calidad del agua y recuperar recursos valiosos de las aguas residuales. Por lo tanto, en base en los resultados de este estudio y considerando la creciente necesidad de desarrollar biorefinerías, las instalaciones sostenibles a gran escala serían el próximo paso hacia una economía circular.

Samenvatting

De huidige globale milieuproblematiek roept onvermijdelijke uitdagingen op om natuurlijke grondstoffen beter te beheren met het oog op een duurzame ontwikkeling. Water is ongetwijfeld de meest essentiële grondstof van de mens en genoeg zuiver water voorzien is reeds tientallen jaren een grote uitdaging. Verontreinigingen in huishoudelijk, industrieel en landbouw afvalwater vormen een gevaar voor de waterlichamen en de behandeling van deze effluenten vraagt veel energie. De ontwikkeling van efficiënte technologieën voor afvalwaterbehandeling wordt dus steeds belangrijker.

Vanwege hun metabolische flexibiliteit zijn microalgen interessante biologische systemen voor de behandeling van verschillende types afvalwater. Zeker in de context van een circulaire en biogebaseerde economie heeft microalgenbiomassa haar grote potentieel getoond om afvalwaterstromen te behandelen en tegelijkertijd duurzame bioproducten te produceren en dus het bioraffinageconcept te implementeren. Hoewel uitgebreid onderzoek is gedaan om microalgensystemen te optimaliseren wat betreft werking en economische haalbaarheid, is er nog steeds behoefte aan verbeteringen en realistische informatie om deze systemen op grote schaal te implementeren.

Dit doctoraatsonderzoek beoogt een bijdrage te leveren aan deze zoektocht door een holistische studie uit te voeren op microalgensystemen van microalgensystemen voor afvalwaterbehandeling in combinatie met verschillende valorisatiestrategieën voor de resulterende biomassa. De algemene inhoud van dit proefschrift is verdeeld in twee delen. Het eerste deel bestaat uit experimentele studies waarin afvalwaterzuivering wordt gecombineerd met terugwinning van grondstoffen met behulp van op microalgen gebaseerde systemen, terwijl het tweede deel is gewijd aan Life Cycle Assessment (LCA) van de technologieën en valorisatietechnieken voor biomassa die in dit proefschrift zijn onderzocht.

Het eerste deel presenteert dus de onderzochte alternatieven van afvalwaterzuiveringssystemen op basis van microalgen, het testen van verschillende cultivatiesystemen en het evalueren van het potentieel om bio-energie (biogas) en hoogwaardige verbindingen (phycobiliproteïnen) terug te winnen. Om te beginnen wordt een goed gekend microalgensysteem, een high rate algal pond (HRAP), bestudeerd om het onderhoud, de kosten en de voetafdruk te verminderen. In dit verband werd het effect van de voorbezinking op de lange termijn prestaties van HRAP's op pilootschaal onderzocht, niet alleen met betrekking tot de zuiveringsefficiëntie en biomassa-eigenschappen, maar ook met het oog op het potentieel van opwekken van bio-energie uit de geoogste biomassa. Deze studie toonde aan dat het verwijderen van de voorbezinking voorafgaand aan een HRAP geen significante invloed had op de efficiëntie van de afvalwaterbehandeling (NH_4^+-N verwijdering van respectievelijk 93 en 91% en verwijdering van chemische zuurstofbehoefte (CZV) van respectievelijk 62 en 65% in HRAP met en zonder voorbezinking). Daarom lijkt deze stap overbodig, wanneer de behandeling van huishoudelijk afvalwater het hoofddoel is. Als terugwinning van bio-energie door biogasproductie wordt overwogen, zou de co-vergisting met primair slib de methaanopbrengst (238– 258 mL CH_4/g VS vergeleken met 189-225 mL CH_4/g VS) en kinetiek van mono-vergisting van microalgen significant kunnen verbeteren.

Vervolgens werd in een tweede onderzoek de groei van door cyanobacteriën gedomineerde biomassa in lab-schaal fotobioreactoren (PBR's) onderzocht op basis van centraat dat verdund werd met secundair effluent uit het HRAP-systeem in verschillende verhoudingen. De resultaten toonden aan dat de dominantie van cyanobacteriën stabiel was in een gemengde cultuur met een effectieve behandelingsefficiëntie (verwijdering tot 52% van CZV, 86% NH_4^+-N en 100% fosfor). Bovendien zou in deze systemen gekweekte biomassa kunnen worden gevaloriseerd voor phycobiliproteïnen (tot 17 mg/g droge biomassa) en biogasproductie (van 159 tot 199 mL CH_4/g VS).

Ten slotte werd een derde onderzoek uitgevoerd naar monocultuurteelten van *Nostoc* sp., *Arthrospira platensis* (*Spirulina*) en *Porphyridium purpureum* voor verdere productie van phycobiliproteïnen. De lichtintensiteit en de samenstelling van het

groeimedium werden geoptimaliseerd, hetgeen aangeeft dat lichtomstandigheden de productie van phycobiliproteïnen meer beïnvloeden dan de samenstelling van het medium. De omstandigheden werden vervolgens gekozen om deze microalgen in het afvalwater van de voedselindustrie op te groeien. Efficiënte afvalwaterbehandeling (verwijdering tot 98% van CZV, 94% anorganische stikstof en 100% fosfor) en succesvolle extractie van phycobiliproteïnen (tot 103 mg/g droge biomassa) werden bereikt.

Het tweede deel van dit proefschrift geeft een overzicht van de duurzaamheidsaspecten die verband houden met microalgensystemen en de valorisatie van biomassa, op basis van de hierboven beschreven experimentele studies. Twee vergelijkende LCA's werden uitgevoerd om de milieubelasting te karakteriseren die zou worden veroorzaakt door het opschalen van de resultaten op laboratorium- en pilootschaal.

De eerste LCA ging in op HRAP-systemen voor afvalwaterzuivering in kleine gemeenschappen waarbij biogas en biofertilisatoren werden vergeleken als opties voor het terugwinnen van grondstoffen. Beide microalgensystemen werden ook vergeleken met een conventionele actiefslibinstallatie. Bovendien werd een economische analyse uitgevoerd. Deze studie toonde aan dat, gezien de belangrijkste impactcategorieën, het HRAP-systeem geïmplementeerd in een warm klimaatgebied het meest milieuvriendelijke alternatief bleek te zijn. Verder bleek de productie van biofertilisatoren het economisch meest haalbare te zijn. Bovendien vertoonden HRAP's lagere potentiële milieueffecten in vergelijking met een actiefslibinstallatie.

De tweede LCA evalueerde twee microalgensystemen waarin de behandeling van huishoudelijk of industrieel afvalwater werd vergeleken met oog op de terugwinning van bioproducten (phycobiliproteïnen en digestaat dat herbruikt kan worden in de landbouw) en bio-energie (biogas). Bovendien werden beide alternatieven vergeleken met een conventioneel systeem dat standaard groeimedia gebruikt voor de productie van phycobiliproteïnen. De resultaten geven aan dat het systeem dat industrieel afvalwater zuivert, lagere milieueffecten heeft dan het systeem dat huishoudelijk afvalwater

behandelt. Bovendien bleek het gebruik van afvalwater milieuvriendelijker te zijn dan het gebruik van standaard groeimedium om microalgen te kweken.

Concluderend suggereren de resultaten verkregen in dit proefschrift dat microalgensystemen een groot potentieel lijken te hebben om de waterkwaliteit te verbeteren en waardevolle grondstoffen terug te winnen uit afvalwater. Daarom zijn, op basis van de resultaten van deze studie en rekening houdend met de behoefte aan conceptuele bioraffinaderijen, duurzame installaties op volle schaal een volgende stap op weg naar een circulaire economie.

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Table of Contents

List of Figures

List of Tables

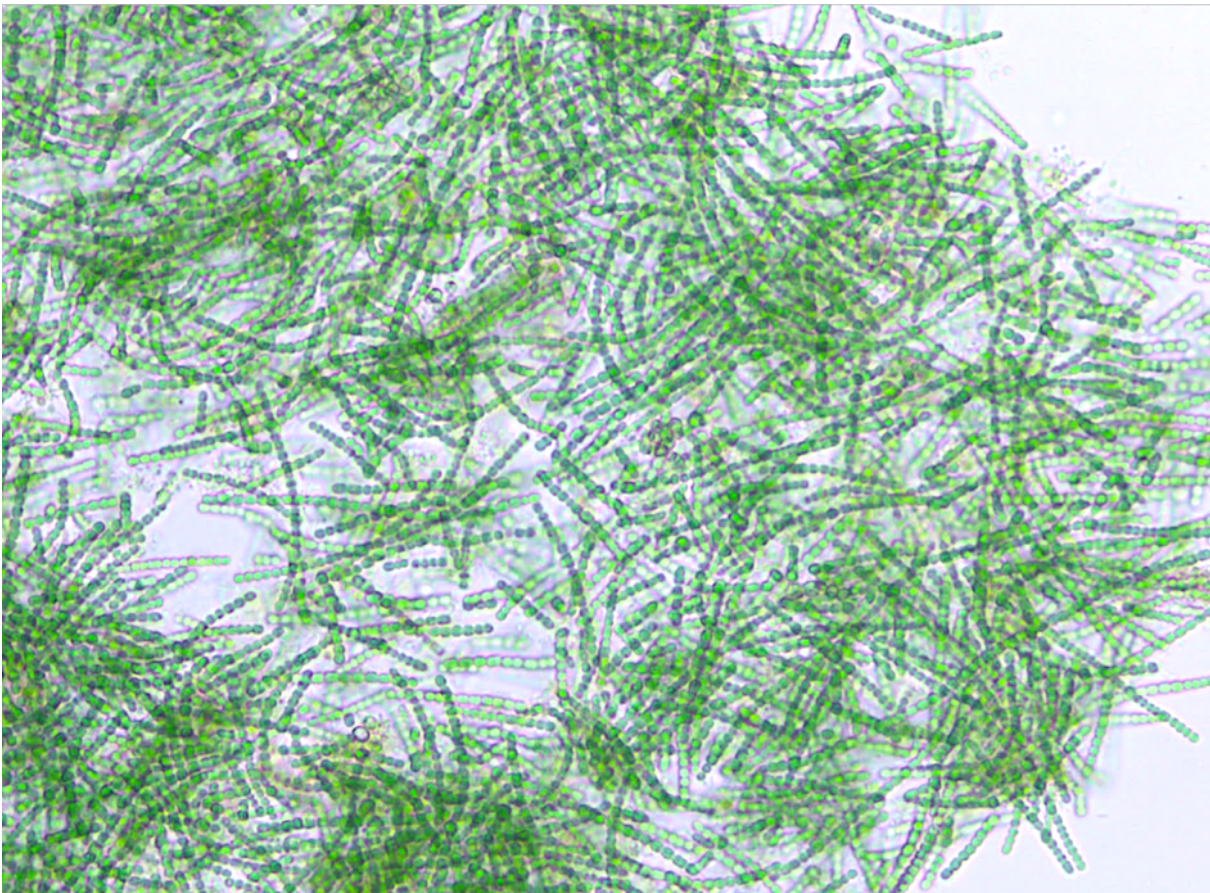
List of Acronyms and Abbreviations

APC	Allophycocyanin
ASW	Artificial sea water
BG-11	Blue-green medium (Standard cultivation medium)
BMP	Biochemical methane potential
BOD	Biochemical oxygen demand
CCA	Complementary chromatic adaptation
CHP	Combined heat and power
COD	Chemical oxygen demand
DO	Dissolved oxygen
DW	Dry weight
GHG	Greenhouse gas
HRAP	High rate algal pond
HRT	Hydraulic retention time
ISO	International standards organisation
IWW	Industrial wastewater
LCA	Life cycle assessment
LOD	Limits of detection
NER	Net energy ratio
OLR	Organic loading rate
PBP	Phycobiliprotein
PBR	Photobioreactor
PC	Phycocyanin
PE	Phycoerythrin
PS	Primary sludge
PT	Primary treatment
PVC	Polyvinyl chloride
sBOD	Soluble biochemical oxygen demand
sCOD	Soluble chemical oxygen demand

SGM	Standard growth medium
TC	Total carbon
TIC	Total inorganic carbon
TIN	Total inorganic nitrogen
TKN	Total Kjeldahl Nitrogen
TN	Total nitrogen
TOC	Total organic carbon
TON	Total organic nitrogen
TOP	Total organic phosphorus
TP	Total phosphorus
TPT	Thermal pre-treatment
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket
UWW	Urban wastewater
VS	Volatile solids
VSS	Volatile suspended solids
WRRF	Water resource recovery facility
WW	Wastewater
WWTP	Wastewater treatment plant

Chapter 1

Introduction, Objectives and Thesis Outline



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Microscopic view of *Nostoc* sp. colonies used in this research project (*Chapter 5*).

1. Introduction, Objectives and Thesis Outline

1.1 Introduction

One of the major environmental issues is wastewater generated through domestic, industrial and agricultural processes, which requires proper treatment before being discharged into water bodies. For this reason, several systems and technologies have been developed to treat contaminated effluents. The use of microalgae as an alternative for wastewater treatment has received renewed interest due to their potential capacity to treat wastewater with reduced energy consumption compared to conventional activated sludge systems (Abdel-Raouf et al., 2012). When combined with bacteria, the positive interaction is clear when microalgae take up nutrients for growth and provide oxygen necessary for aerobic bacteria to biodegrade organic pollutants. The carbon dioxide released from these bacterial processes is then consumed, in turn, by microalgae, creating the synergistic relationship for an efficient wastewater treatment (Kouzuma and Watanabe, 2015). These natural systems are appropriate solutions for wastewater treatment especially in small communities, since they reduce costs and environmental impacts associated with this process (Garfí et al., 2017).

Several systems have been developed to provide efficient wastewater treatment and, at the same time, promote algal biomass growth, which can be valorised to recover energy, nutrients and valuable compounds. The type of cultivation system and especially culture conditions, such as pH and composition of media, CO_2 and nutrients supply, and light intensity significantly affect the microalgae biomass production (Barsanti and Gualtieri, 2014). Different cultivation designs can be applied for the large production of microalgae and the most adequate configuration for each case will be a function of the overall process desired (Acién Fernández et al., 2013). In the present study, two widely practiced microalgae systems for wastewater treatment - high rate algal ponds (HRAPs) (open systems) and photobioreactors (PBRs) (closed systems) – will be addressed. In short, HRAPs represent lower costs and easier installation and maintenance, although large surface area is required, since they are shallow ponds. The PBRs, on the other hand,

represent higher biomass productivity but also higher costs and sophisticated installations (Brennan and Owende, 2010; Lam et al., 2019).

Microalgae biomass grown in these systems can be valorised for various applications, such as biofertiliser, biofuel production (e.g. biogas and biodiesel) and extraction of valuable compounds (e.g. carotenoids and pigments) (Abdel-Raouf et al., 2012; Gong et al., 2011). The high-value compounds from microalgae are usually produced within a biorefinery concept, since the composition of the microalgal cell allows for extraction of different co-products. In addition, specialty chemicals have higher revenues than bulk chemicals like algal oil for biofuels (Borowitzka, 2013). The main high-value compounds from microalgae include pigments, polysaccharides, triglycerides, fatty acids and vitamins, which are also commonly used as bulk commodities and specialty chemicals in different industrial sectors (e.g. pharmaceuticals, cosmetics, nutraceuticals, functional foods, aquaculture, biofuels) (Cuellar-Bermudez et al., 2015). In particular, phycobiliproteins are high-value pigments from microalgae which have attracted attention for their potential use in different industries, such as pharmaceutical, food, cosmetics and textile (Pagels et al., 2019). The optimisation on the extraction of these products has attracted attention of researchers, turning out as a very promising pathway for resources recovery (Chew et al., 2017; Chiong et al., 2016; Gong et al., 2011).

The anaerobic digestion of microalgae can generate energy in the form of biogas, which could minimize electricity consumption in wastewater treatment plants. Moreover, if biorefinery processes are considered in an overall system, the incorporation of anaerobic digestion could potentially enhance cost effectiveness, contributing to make the system more environmentally and economically feasible (Ward et al., 2014). Several studies have addressed the potential biogas production from algal biomass, including experiments testing the influence of various pre-treatment techniques to improve the methane yield (Passos et al., 2014) and co-digestion with other by-products (Solé-Bundó et al., 2019b). In this context, microalgae biomass valorisation techniques have to be further investigated in order to clearly understand their applicability at large scale.

Furthermore, the concern with resources scarcity and environmental damages have been increasingly discussed worldwide and effective solutions have been encouraged to face those issues. In this perspective, in order to have a concise evaluation of the diverse technologies for wastewater treatment and resources recovery, not only the technical, but also the environmental and economic aspects have to be considered. There are useful tools, such as Life cycle assessment (LCA), that could be applied to carry out a detailed evaluation of the environmental impacts caused by certain products and processes.

Therefore, the present research aims to bring forward a holistic approach of algae-based technologies for wastewater treatment, addressing not only the technology itself, but also some alternatives for the downstream processes and environmental impacts assessment. This overview will provide a more complete evaluation on the feasibility to implement these technologies within an innovative and realistic context.

1.2 Objectives

The overall goal of this research was to investigate microalgae-based technologies for wastewater treatment, addressing their feasibility and efficiency, as well as to explore alternatives for the downstream processes for resource recovery and assess environmental aspects of the systems investigated. This could provide a holistic approach on these technologies, with an attempt to overcome the main drawbacks and support their successful implementation and dissemination in the future.

The specific objectives of this research were the following:

1) To investigate the effect of removing the primary treatment (i.e. settling) on long-term performance of pilot HRAPs cultivating green microalgae, in order to demonstrate the feasibility of such simplification. Comparison of two HRAPs systems with and without primary treatment was performed in terms of: (i) wastewater treatment efficiency, (ii) biomass productivity and characteristics (microbial composition and settling capacity) and (iii) biogas production potential from green microalgae grown in both systems;

2) To investigate the treatment of centrate mixed with secondary effluent in cylindrical PBRs with mixed cultures dominated by cyanobacteria with varying influent concentrations. Comparison of three lab-scale PBRs was carried out in terms of: (i) wastewater treatment efficiency, (ii) biomass composition and productivity, (iii) content and purity of phycobiliproteins extracted from the biomass grown in each PBR, (iv) biogas production potential from biomass with and without extraction of phycobiliproteins, so the potential recovery of energy with residual biomass, aligned with a biorefinery concept, was assessed;

3) To investigate and compare the treatment of food-industry wastewater in PBRs with unialgal cultures of *Nostoc* sp., *Arthrospira platensis* (*Spirulina*) and *Porphyridium purpureum*. This study aimed to compare the biomass growth and the content and purity of phycobiliproteins extracted from the biomass grown in each PBR;

4) To compare wastewater treatment systems using HRAPs, considering different biomass valorisation techniques (biogas or biofertilisers), using the LCA methodology;

5) To compare wastewater treatment systems recovering phycobiliproteins from the biomass produced, applying different types of wastewater (urban and industrial) and different cultivation configurations, using the LCA methodology.

1.3 Thesis outline

A comprehensive literature review is presented in **Chapter 2**, embracing the multiple issues addressed in this PhD thesis. The following chapters are basically divided in two main parts: the first is composed by the experimental studies aiming at waste water treatment and resource recovery (**Chapters 3**, **4** and **5**) and the second part is dedicated to the environmental analyses (**Chapters 6** and **7**). An overview of the chapters and their interconnection is illustrated in Figure 1-1.

Chapter 3 describes a study on the effect of removing the primary settling prior to the HRAP, which would simplify the HRAPs system, reducing area needed and costs of both installation and maintenance. The study investigated the wastewater treatment efficiency and biomass characteristics regarding productivity, composition and settling

capacity. The potential bioenergy recovery through biogas production from the biomass harvested was also evaluated.

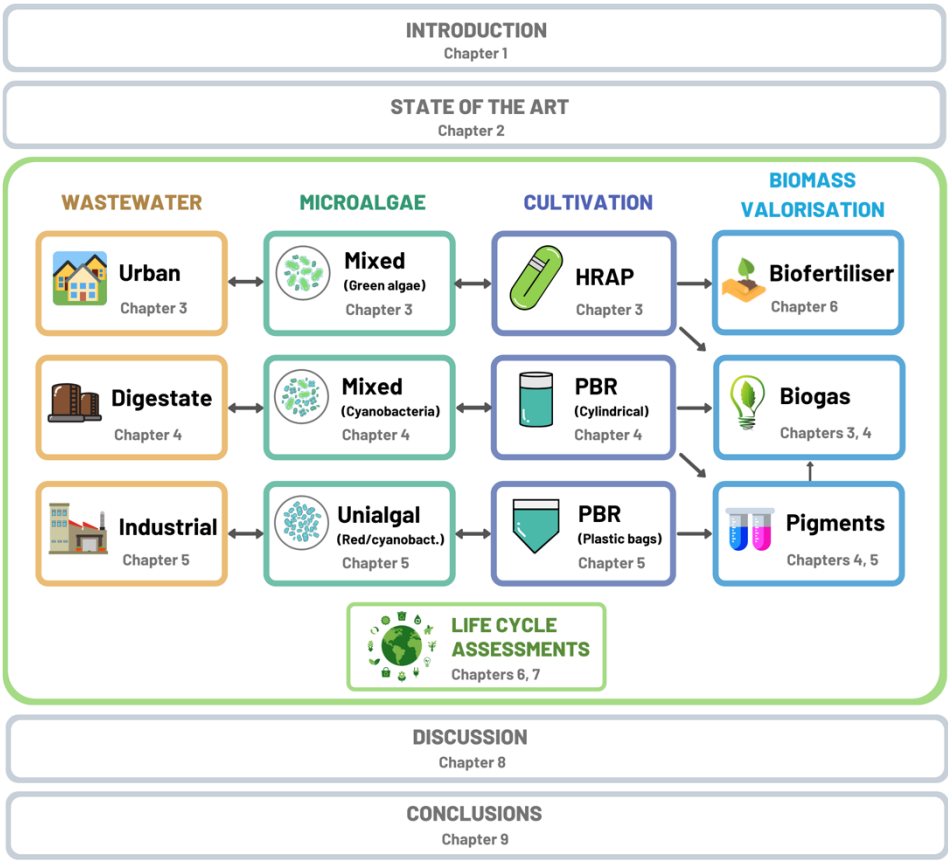


Figure 1-1. Overview of the chapters in this thesis and their interconnection.

In **Chapter 3**, the HRAPs system was shown to be effective for urban wastewater treatment, but during winter months the effluent occasionally presented nitrate concentrations higher than the discharge limits. Furthermore, biogas production was also shown to be an alternative to recover bioenergy from microalgal biomass. However, the disposal of the liquid phase of digestate (centrate) is still a challenge, unlike the solid

phase which can be used as biofertiliser. In this context, a subsequent study was carried out using centrate diluted with secondary effluent from the HRAPs system to grow cyanobacteria in PBRs. The biomass grown in these systems was valorised through high-value compounds (phycobiliproteins) and biogas production. The results of this study are presented in **Chapter 4**.

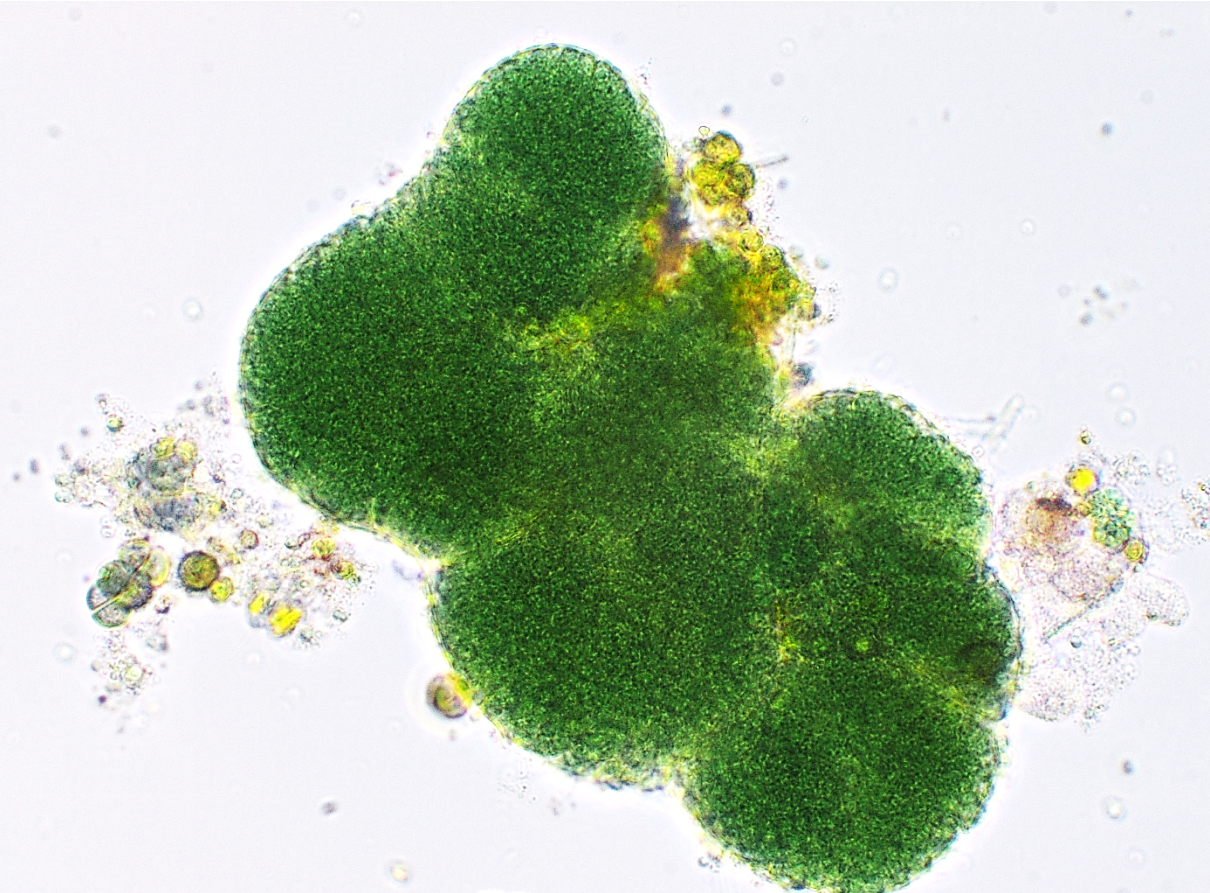
Chapter 5 addresses the use of industrial wastewater as growth medium for unialgal cultivations of three microalgae species in PBRs. The aim of this study was to promote efficient wastewater treatment and the extraction of high-value compounds (phycobiliproteins) from the biomass produced in these systems. In order to optimise the phycobiliproteins production of each species, conditions of light intensity and medium composition were tested using standard growth medium. The optimal conditions were then applied using industrial wastewater to cultivate the microalgae.

Chapter 6 shows the results of a comparative LCA of HRAPs systems using two different biomass valorisation techniques: biofertiliser and biogas. Comparison with the conventional activated sludge systems is also discussed. Similarly, **Chapter 7** describes the results of a comparative LCA of microalgae-based systems cultivating biomass for phycobiliproteins production, using different configurations and wastewater types.

Chapter 8 elucidates a general discussion that summarises and connects the main learnings generated along the research period and proposes recommendations for future research. Finally, **Chapter 9** features the main conclusions that can be extracted from this work.

Chapter 2

State of the Art



Picture on previous page:
Microscopic view of mixed culture used in experiments described in Chapter 4.

2. State of the Art

2.1 Microalgae cultivation systems

Microalgae can grow in a wide variety of cultivation systems. Currently, there are two widely practiced cultivation systems - high rate algal ponds (open systems) and photobioreactors (closed systems) - both of which are briefly discussed here.

2.1.1 High rate algae ponds (HRAPs)

HRAPs have been studied for the past decades and proved to be very effective for wastewater treatment with recovery of energy, nutrients and valuable compounds. They are economic alternatives that can be applied in locations where weather conditions are favourable for microalgae growth. HRAPs are ring-channel systems, with a typical depth of up to 0.3 m, in which the culture is typically mixed by a paddle wheel (Figure 2-1). HRAPs are characterised by low cell densities up to 0.3 g/L and are currently the cheapest cultivation system for commercial production of microalgae (Chisti, 2007; Lam et al., 2019).

Figure 2-1. High rate algae ponds for microalgae biomass production from Qualitas Health (USA) (EERE, 2020).

Recent studies demonstrated that HRAPs might help to reduce environmental impacts and costs associated with wastewater treatment compared to conventional systems, especially in small communities (Garfí et al., 2017; Maga, 2017). On the other hand, one of the main drawbacks for implementing HRAPs is the large surface area requirement, which is necessary in order to promote satisfactory effluent quality levels and biomass productivity (Kumar et al., 2015). Moreover, considering that HRAPs are open systems, there is significant evaporative water loss to the atmosphere and biomass productivity is also affected by contamination with unwanted microalgae and microorganisms that feed on microalgae. Thus, HRAPs are perceived to be less expensive than photobioreactors, due to lower building and operation costs, but they have a low biomass productivity compared with photobioreactors (Chisti, 2007).

2.1.2 Photobioreactors (PBRs)

PBRs are designed to overcome the problems associated with open pond cultivation systems. In order to design an efficient PBR, an understanding of the complex interaction between biomass production and associated environmental parameters (e.g., fluid dynamics and light transfer) within the reactor is required (Gupta et al., 2015). Based on the illuminated surface, PBRs are categorized as column, tubular, flat planel and bags (Zhang et al., 2018). Illustrations of the main types of PBRs are shown in Figure 2-2. Based on their mode of liquid flow, PBRs can be grouped as stirred type, bubble column and airlift reactor. Ideal PBRs should have high transparent surface, minimal dark zones, high mass transfer rates and prevent fouling to maximize biomass growth (Gupta et al., 2015).

PBRs have been successfully used for producing large quantities of microalgal biomass (Zhang et al., 2018) and pollutants treatment (Vo et al., 2019), as well as permitting essentially single-species culture of microalgae for prolonged durations (Xu et al., 2009). Extensive research has been carried out lately in order to reduce biomass production costs, by improving the design and shape of the PBR, controlling environmental parameters, and favouring minimal contamination risk (Khan et al., 2018). However, there are still some major drawbacks, such as limitations on light

diffusion with higher operational volumes, which results in the inefficient growth of microalgae and development of microalgal biofilm on PBR surface, thus limiting light penetration (Gupta et al., 2015). In addition, the initial investment, operational and maintenance cost of PBR is high, which eventually increases the biomass production cost (Acién Fernández et al., 2013).

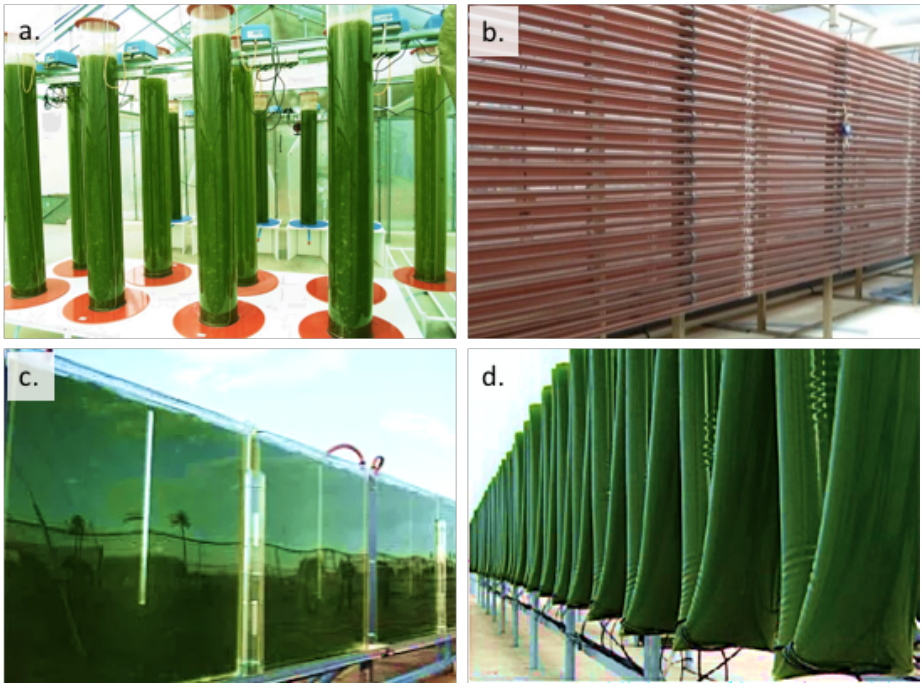


Figure 2-2. Main types of closed photobioreactor systems: a. Bubble column reactors at Sea & Sun Technology (Germany) (ABiRe, 2020); b. Tubular photobioreactor (Durmaz et al., 2017); c. Flat plate photobioreactor at Arizona State University (USA) (EERE, 2020); Plastic bags photobioreactors (Huang et al., 2017).

2.1.3 Selection of cultivation system

Several studies have indicated that the type of cultivation system and especially culture conditions, such as pH and composition of media, CO_2 and nutrients supply, and light intensity significantly affect the microalgae biomass production (Barsanti and Gualtieri, 2014). These conditions can be better controlled in a PBR and it has been

shown that the microalgae biomass productivity is increased when they are cultured in closed PBR (Xu et al., 2009). Volumetric biomass productivities in closed PBRs have been reported to be 5 to 20 times higher than in HRAPs, although the differences in areal productivities are smaller (Eriksen, 2008). This may favour using closed PBRs system for commercial production of microalgae. However, installation, operation and maintenance costs still need to be reduced considerably in order to make it economically feasible at industrial scale (Norsker et al., 2011). According to a study performed by Davis et al. (2011), the total capital cost for microalgae cultivation in a closed PBR was 153.8% higher compared to an HRAP, indicating the high investment risk in scaling-up PBRs for algal biomass production. Manufacturing costs contributed to a large portion of the total capital cost, which accounted for 52.7% or 12.7 times higher than HRAP manufacturing cost. Moreover, HRAPs required 32.7% lower operating costs compared to the closed-PBR, primarily because of the ease of operating these systems and hence, less power consumption. Some of the advantages and disadvantages for culturing in HRAPs and closed PBRs systems are outlined in Table 2-1.

Table 2-1. Main advantages and disadvantages for open and closed cultivation systems for phototrophic organisms (Brennan and Owende, 2010; Lam et al., 2019).

Cultivation system	Advantages	Disadvantages
High rate algal ponds	Construction is easy and non-expensive Easy maintenance and cleaning Low energy input	Low biomass productivity Large area of land required Water loss (high evaporation rate) Risk of contamination
Column photobioreactors	Control of growth conditions Efficient mixing → High mass transfer rates Low cost compared to other PBRs Compact and easy to operate	Small illumination area Cell sedimentation can occur if airlift system is not used
Tubular photobioreactors	Large illumination surface area High biomass productivity Potential cell damage is minimized if airlift system is used	Large area of land required Accumulation of O_2 (inhibition) in medium if tubes are too long Difficult maintenance Wall growth
Flat panel photobioreactors	Large illumination surface area High biomass productivity Low accumulation of dissolved O_2 High photosynthetic efficiency	Difficult to scale up Temperature control Wall growth

2.2 Microalgae for wastewater treatment

Microalgal-based wastewater treatments have been studied for decades, with pioneering works starting from the early 1950s (Ludwig and Oswald, 1952; Oswald et al., 1953). However, in recent years, they have received increasing attention due to the sustainability of improved systems that are moving towards smaller footprint and low energy consumption. The increasing global warming concern, the energy demand for treatment, and the high costs of sludge disposal indicate the need for a paradigm shift in the configurations of conventional wastewater treatment plants (WWTPs) to more environmentally and economically sustainable options (Foladori et al., 2018). Traditional WWTPs are increasingly regarded as water resource recovery facilities (WRRFs), reflecting the value of water, nutrients, energy and other resources, besides ensuring the required effluent quality (Solon et al., 2019). In this sense, microalgae-based systems have been recognised as particularly attractive alternatives, where symbiotic relations between microalgae and bacteria may be advantageously exploited for wastewater treatment and resources recovery (Abdel-Raouf et al., 2012). As a principal member of this symbiosis, microalgal photosynthesis provide oxygen as an electron acceptor for heterotrophic bacteria to biodegrade organic pollutants, consuming in turn the carbon dioxide released from the respiration activity, including organic wastes and aromatic pollutants (Liang et al., 2013). Moreover, both microorganisms take up nutrients for their biomass growth, which promotes satisfactory water quality indicator levels (Kouzuma and Watanabe, 2015). Bacterial growth can also enhance microalgae metabolism by excreting growth-promoting factors or by reducing dissolved oxygen in the culture broth (Fukami et al., 1997). This synergistic relationship was also reported as an efficient and economical treatment of hazardous contaminants (Muñoz and Guieysse, 2006).

Previous studies which suggested microalgae-based systems as an effective alternative for wastewater treatment are shown in Table 2-2. Park and Craggs (2011) reported that HRAPs treating anaerobically digested domestic wastewater reached removal efficiencies of up to 96.9% of NH_4^+-N and 87% of $sBOD_5$ at optimal conditions. Similar results were described by Gutiérrez et al. (2016) with HRAPs treating primary

settled urban wastewater reaching average removal of 80% of COD and 95% of NH_4^+-N. Successful studies applying this technology to treat agricultural wastes and industrial wastewater were also reported (Gupta et al., 2019; Mark Ibekwe et al., 2017; Van Den Hende et al., 2016a).

Several cyanobacteria species have also been studied for wastewater treatment. In this context, Hemlata and Fatma (2009) screened 18 cyanobacterial strains having potential for phycobiliproteins synthesis and the authors pointed that among them, phycobiliproteins yield with *Anabaena, Microchaete, Nostoc,* and *Tolypothrix* were found most promising. El-Sheekh et al. (2014) have conducted an experiment using cyanobacteria *Nostoc moscorum* to treat urban wastewater and obtained COD, NH_3 and PO_4^{3-} removal efficiencies of 50%, 91.5% and 60%, respectively. Talukder et al. (2015) also reported reduction of COD, BOD and heavy metals concentrations in textile industry effluent treated with *N. moscorum*.

Table 2-2. Summary of results obtained by previous studies using microalgae for wastewater treatment.

Wastewater type	Microalgae species	Wastewater characteristics (Removal efficiency)	Biomass production	Cultivation system	Reference
Centrate, municipal wastewater	*Chlorella* sp.	85.9 mg NH_4^+-N/L (93%) 132.3 mg TN/L (89%) 215.1 mg TP/L (80%) 2389.5 mg COD/L (90%)	920 mg/Ld	25 L coil reactor	Li et al. (2011)
Anaerobically digested domestic wastewater	*Scenedesmus* sp., *Microactinium* sp., *Pediastrum* sp., *Ankistrodesmus* sp.	56 mg NH_4^+-N/L (92 - 97%) 50.7 mg $sBOD_5$/L (84 - 87%) 7 mg PO_4^{-3}/L (70 - 73%)	10.6 - 15.3 g/m^2d	8 m^3 HRAP (outdoor), HRT: 4 d	Park and Craggs (2011)
Municipal pre-treated wastewater	*Scenedesmus obliquus*	65.12% TN (HRAP) and 89.68% TN (PBR) 58.78% (HRAP) and 86.71% (PBR)	8.26 g/m^2d (HRAP) 21.76 g/m^2d (PBR)	530 L HRAP and 380 L airlift tubular PBR	Arbib et al. (2013)
Municipal wastewater (pre- and post-treated)	*Scenedesmus acutus*	5.3 - 97.6 mg NO_3^-/L (42 - 71%) 27.7 - 207.2 mg NH_4^+/L (93%) 7.3 - 122 mg PO_4^{-3}/L (64%) 274 - 783 mg COD/L (48 -77%)	73.7 mg/Ld	20 L vertical tubular PBR (outdoor)	Sacristán de Alva et al. (2013)
Municipal pre-treated wastewater	*Chlorella* sp., *Stigeoclonium* sp. and diatoms	26 – 36 mg NH_4^+-N/L (95 %) 100 – 800 mg COD/L (80%)	3 – 26 g/m^2d	0.47 m^3 HRAP (outdoor), HRT: 8, 6 and 4 d	Gutiérrez et al. (2016)

(Table continued on the next page)

Table 2-2. *(Continued).*

Municipal wastewater	*Chlorella, Cryptomonas, Scenedesmus*	300 mg BOD/L (92%) 4.22 mg TP/L (93%) 40 mg TN/L (75)	3.5 - 22.7 g/m^2d	Large scale offshore PBR (up to 50,000 gal/d)	Novoveská et al. (2016)
Municipal wastewater	*Chlorella vulgaris, Scenedesmus obliquus*	151.9 - 475.7 mg TN/L (84 - 98%) 15.2 - 30.3 mg TP/L (92 - 100%)	100 - 900 mg/Ld	150 L PBR bubble agitation, batch mode	Gouveia et al. (2016)
Brewery wastewater	*Scenedesmus obliquus*	3635 mg COD/L (58%) 54 mg TN/L (21%)	100 mg/Ld	0.25 L Erlenmeyer	Mata et al. (2012)
Dairy wastewater	Consortia microalgae/diatoms	30.5 mg NH_4^+/L (96%) 2.6 mg PO_4^{-3}/L (99%)	21.13 - 56.2 mg/Ld	40 L rectangular aquarium tanks (outdoor), HRT: 15 d	Woertz et al. (2009)
Dairy wastewater	*Scenedesmus quadricauda* and *Tetraselmis suecica*	86 mg TN/L (45 - 86%) 8.7 mg PO_4^{-3}/L (42 - 90%)	58.75 mg/Ld	12 L Airlift PBR	Daneshvar et al. (2018)
Slaughterhouse wastewater	*Chlamydomonas subcaudata*, *Anabaena* sp., *Nitzschia* sp	- (86 - 92% COD) - (71 - 91% P)	12.7 g/m^2d	75 L HRAPs (indoor and outdoor), HRT: 10–15d	Hernández et al. (2016)
Anaerobically digested starch processing wastewater	*Chlorella pyrenoidosa*	81 - 183 mg TOC/L (11 - 62%) 56 - 275 mg TN/L (9 - 79%) 1.2 – 20.5 mg TP/L (37 - 97%)	154.7 - 342.6 mg/Ld	820 L Airlift circulation PBR, HRT: 2, 4, 10, 20 d	Chu et al. (2015)

2.3 Microalgae biomass valorisation

Microalgae have been shown to be a source of multiple bio-based products ranging from high value molecules to commodities. These microorganisms can be used for combining the treatment of wastewaters of different origins (urban, industrial, and agricultural) with the synthesis a large variety of products (Delrue et al., 2016). Among the several techniques proposed so far, the biomass valorisations covered in the present study – biogas production through anaerobic digestion and extraction of phycobiliproteins (high-value pigments) – will be briefly discussed in the following sub-sections.

2.3.1 Biogas from microalgae

During the past decades, intensive research has been developed in order to investigate the potential of microalgae to produce biofuels such as biogas and biodiesel. Several studies have reported positive results on biogas production from microalgae, which seems to contain high energy value, making anaerobic digestion of these microorganisms an attractive alternative for biofuel production (Chew et al., 2017; Jankowska et al., 2017; Montingelli et al., 2015; Uggetti et al., 2017). Indeed, anaerobic digestion is a more straightforward process to recover energy, considering that no intense concentration, drying, or complex extraction methods are required. In addition, unlike other biofuels such as bioethanol and biodiesel, anaerobic digestion uses many macromolecular components as substrate. Thus, the advantage of digesting microalgae implies less sophisticated processes while producing an energy form together with a mineralised digestate which contains valuable nutrients (González-Fernández et al., 2012a). Additionally, a microalgae biorefinery framework is considered to extract high-value compounds from the biomass, and anaerobic digestion can be applied at the end of the process to recover bioenergy from the residual biomass (Ramos-Suárez et al., 2014). In this case, a typical mix of low volume high-value products (such as pigments) and high volume low-value products (such as bioenergy) is produced (Van Den Hende et al., 2016b). The high-value products provide economic feasibility while the low-value

products can supply or minimise the energy demand of the system (Yen et al., 2013; Vulsteke et al., 2017).

Anaerobic methane production can be inhibited by the unbalanced carbon-to-nitrogen (C:N) ratios and the resistance of algal cell walls to degradation (Prajapati et al., 2013). Hence, realistic and energy-effective methods for enhanced processes could be applied to realize the full potential of anaerobic digestion systems (Bohutskyi et al., 2019). An alternative for improving microalgae digestion is to pre-treat the biomass using chemical, mechanical, or thermal processes (Bohutskyi et al., 2019). The effectiveness of these approaches depends on the pre-treatment applied and on the biomass characteristics of the processed algal species (Jankowska et al., 2017). Alternatively, production of methane can be improved through co-digestion with other types of low-cost co-substrates, such as sewage sludge, lignocellulosic residues or agriculture wastes (Lu and Zhang, 2016; Solé-Bundó et al., 2019b; Thorin et al., 2018; Yen and Brune, 2007; Zhen et al., 2016). Because pre-treatment and co-digestion can require additional energy and increased volume of the digesters, these processes could decrease the net energy output, net energy ratio (NER) and other energy balance parameters of the anaerobic digestion step. Therefore, integral assessment of final biomethane yield and their effects on the kinetics of methane production in terms of the system energy and economic balance parameters is essential for evaluating their feasibility (Bohutskyi et al., 2019). In this context, environmental and economic assessments of microalgae wastewater treatment systems with biogas production are yet to be shown.

Among the several alternatives for improving methane production from microalgae biomass, the thermal pre-treatment and co-digestion with sewage sludge were used in the present study and are briefly discussed in the next sub-sections.

2.3.1.1 Microalgae thermal pre-treatment

The thermal pre-treatments have been widely proposed to enhance biogas production of different sludge wastes (Ferrer et al., 2008; Pinnekamp, 1988) and some microalgae species (Bohutskyi et al., 2019; Passos et al., 2014; Solé-Bundó et al., 2018).

Thermal pre-treatment has been applied for sewage sludge disintegration using a range of temperatures from 50 to 270°C. However, temperatures over 180°C may lead to the production of inhibitory compounds such as some phenolic monomers (hydroquinone, resorcinol and phenol), which reduce biomass digestibility (Santos-Ballardo et al., 2016).

De Schamphelaire and Verstraete (2009) developed a pre-treatment at 80°C for 2.5 h using a mixture of *Chlorella* and *Pseudokirchneriella* but did not detect any effect in the methane production. González-Fernández et al. (2012) investigated the effect of thermal pre-treatment of *Scenedesmus* biomass, using two temperatures (70 and 90°C). The 90°C pre-treatment showed a 102% of increment in methane production compared with the raw biomass. Ehimen et al. (2013) carried out anaerobic digestion of filamentous microalgae using pre-treatments (ultrasound and enzymatic) and obtained lower methane yields, ranging from 62-97 mL CH_4/g VS. Passos et al. (2013) obtained methane yields from 105 to 170 mL CH_4/g VS testing different low-temperature pre-treatments (55, 75 and 95°C for 5, 10 and 15h) of microalgae biomass used to treat wastewater and concluded that optimum results were achieved at 75-95°C with an exposure time of 10 h.

2.3.1.2 Co-digestion of microalgae and sludge

Microalgae technologies could be incorporated in wastewater treatment plants (WWTPs) for polishing the water and, at the same time, producing biomass that can be used for several purposes, such as production of bioenergy and bioproducts. However, microalgae are likely to be a co-substrate in the biogas production step, since there will still be primary and waste activated sludge if microalgae are only partly integrated or used to treat the reject water flow (Thorin et al., 2018).

This is also related to the composition of the substrates that is needed to achieve a stable degradation process. The carbon-to-nitrogen C:N ratio is the proportion of the mass of carbon relative to the mass of nitrogen in the biomass and has a considerable effect on biodegradability by both aerobic and anaerobic processes (Milledge et al., 2019). While the C:N ratio of microalgae biomass varies from 3.1 to 14.87 (Santos-Ballardo et al., 2016), optimal ratio for anaerobic conversion of biomass to methane is

~30 to avoid lower methane yields due to nitrogen limitation (if C:N ratio is too high) or ammonia inhibition resulting from the breakdown of organic nitrogen compounds (if C:N ratio is too low) (Milledge et al., 2019; Santos-Ballardo et al., 2016; Wang et al., 2014).

To overcome the problems with low C:N ratios, several researchers have investigated the co-digestion of microalgae with sewage sludge under different conditions. Olsson et al. (2014) observed a significant synergetic effect in co-digesting *Scenedesmus* and *Chlorella* with sewage sludge at a volatile solids (VS) percentage of 37% for microalgae, reaching 408 mL CH_4/g VS, which was 23% higher than mono-digestion of sewage sludge. Wickham et al. (2016) has achieved 139 mL CH_4/g co-substrate using dehydrated *Ulva* sp. and sewage sludge at a dry weight ratio of 6:94. Mahdy et al. (2015) observed that primary sludge supported higher anaerobic biodegradability (97%) than secondary sludge (23%), and when combined with thermally pretreated *Chlorella vulgaris*, methane yields were improved by 13–17%. Solé-Bundó et al. (2019) reported that the co-digestion of microalgae and primary sludge (25/75% on a volatile solid basis) enhanced the anaerobic digestion of microalgal biomass, since primary sludge is a more readily biodegradable substrate, increasing the methane production by 65% and reducing the risk of ammonia toxicity.

2.3.2 Phycobiliproteins

Microalgae biomass is a promising source for a diverse number of products, such as nutraceuticals, aquaculture feed, cosmetics and fine chemicals. Due to their high economic value, the microalgae biorefinery is becoming a highly attractive alternative for sustainable production of these chemicals (Chew et al., 2017).

The three multi complex light harvesting systems in microalgae are: photosystem I, photosystem II and phycobilisomes, which are used to capture light energy from the visible light spectra (400 nm to 700 nm) and convert it to chemical energy by the photosynthesis (Gao et al., 2016). Photosystem I is located at the outer surface of the thylakoid membrane, and contains chlorophyll a and b, and carotenoids. Photosystem II is located at the inner surface of the thylakoid membrane and contains chlorophyll a and

b, and xanthophylls (Yahia et al., 2019). In particular, phycobilisomes can be found in cyanobacteria and some eukaryotic algal genera such as Rhodophyta (red algae) and Cryptophyta (flagellates) (Johnson et al., 2014). Phycobilisome chromophores are linear tetrapyrroles (phycobilins), which are categorized into three types by energy: those of high energy called phycoerythrins (PE), intermediate energy phycocyanins (PC), and low energy allophycocyanins (APC) (Figure 2-3). Energy will flow from highest- to lowest-energy pigments and this is how the phycobilisomes are organised (MacColl, 1998). Thus, the energy flow of photosystem II is as follows: phycoerythrin → phycocyanin → allophycocyanin → chlorophyll (Eriksen, 2008).

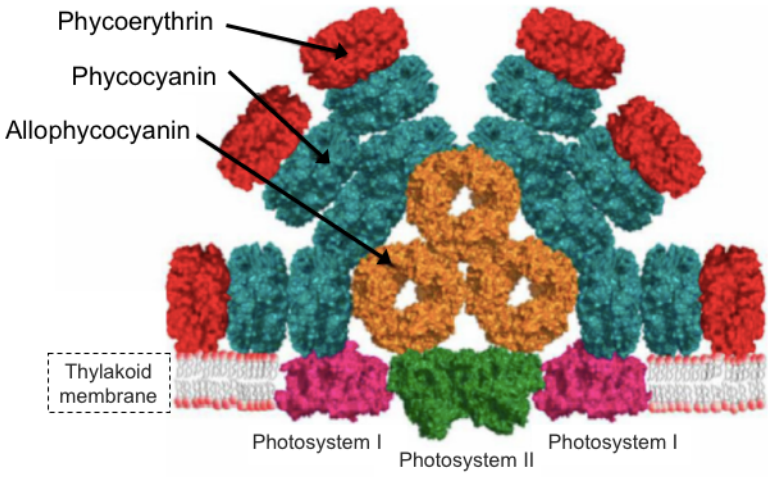


Figure 2-3. Schematic diagram of phycobilisome as part of a megacomplex situated on the thylakoid membrane. Adapted from Green (2019).

The phycobilins absorb light at different wavelengths due to small structural differences: red phycoerythrin absorbs most strongly at wavelengths around 550 nm, blue phycocyanin absorbs maximum around 615 - 620 nm and allophycocyanin absorbs around 650 nm, which is located at wavelengths in the visible light spectra where

chlorophylls have low extinction coefficients (absorbs poorly) (Figure 2-4) (Green, 2019).

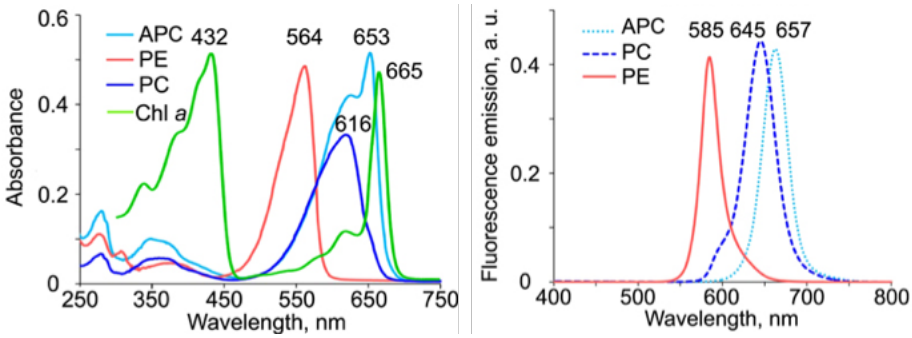


Figure 2-4. Absorption and fluorescence emission spectra of allophycocyanin (APC), phycoerythrin (PE) and phycocyanin (PC), together with the absorption spectrum of chlorophyll a (Chl a). Adapted from Bryant and Canniffe (2018).

As previously mentioned, phycobiliproteins are high-value natural products from microalgae which have attracted attention for their potential use in different industries, such as pharmaceutical, food, cosmetics and textile (Pagels et al., 2019). In light of the considerable commercial application, purity of the pigments plays a major role. Purity of phycobiliproteins defines the relationship between the presence of a certain phycobiliprotein and all other contaminating proteins. The purity, then, is usually determined as the ratio between the absorbance of respective maximum absorption to 280 nm, i.e. A_{620}/A_{280} for phycocyanin, A_{565}/A_{280} for phycoerythrin and A_{650}/A_{280} for allophycocyanin (Manirafasha et al., 2016). Purity of phycocyanin is divided into three classes: food grade (purity of 0.7), reactive grade (purity of 3.9) and analytical grade (purity greater than 4.0). Likewise, for phycoerythrin, a purity ratio greater than 4 corresponds to diagnostics and pharmaceutical grade phycoerythrin (Cuellar-Bermudez et al., 2015).

The achievement of high quality phycobiliproteins production from microalgae needs optimum production conditions, including effective microalgal biomass production and stimulation of phycobiliproteins accumulation that leads to the high

content of phycobiliproteins in the biomass (Manirafasha et al., 2016). As microalgae can be found in locations which exhibit widely dynamic chemical and physical changes like nutrient availability, light intensity and wavelength, temperature, medium conditions, synthesis of phycobiliprotein is also accordingly modulated to adapt to a particular condition. The composition and function of phycobiliproteins in microalgae have also been reported to change under stress conditions (Chakdar and Pabbi, 2016). Among various parameters affecting phycobiliprotein production, the most relevant are briefly discussed in the following sub-sections. Numerous studies in the literature have investigated how cultivation conditions can affect the phycobiliproteins biosynthesis in microalgae cultivated in standard growth medium, but information is scarce for cultivation in waste streams. Relevant research on this interaction remains to be clarified.

2.3.2.1 Parameters affecting phycobiliproteins synthesis in microalgae

2.3.2.1.1 Light

Light intensity and colour can influence phycobiliproteins synthesis. Light intensity is believed to be the most significant environmental factor influencing the light-harvesting complexes (phycobilisomes) (Chakdar and Pabbi, 2016). The influence of light intensities on phycobiliproteins content has been reported in different organisms. Hemlata and Fatma (2009) tested different experimental irradiances in *Anabaena* NCCU-9 and showed 25 µmol photons/m^2s to be the most suitable light intensity for phycobiliprotein production (124.59 mg/g of dry weight (DW)). Light irradiance of 25 µmol photons/m^2s was also reported to be optimal for *Synechocystis* sp. PCC 6701 (Hong and Lee, 2008), while light intensity of 12.5 µmol photons/m^2s was found to be optimal for *Nostoc* UAM 206 (Poza-Carrión et al., 2001). It has been suggested that cyanobacteria prefer low light intensities and stimulate phycobiliprotein synthesis because of their low specific maintenance energy rate and their pigment composition (Hemlata and Fatma, 2009). In contrast, higher light intensities were also found to be optimum for phycobiliprotein production in certain species, such as 150 µmol photons/m^2s for *Arthronema africanum* (Chaneva et al., 2007) and 90 µmol photons/m^2s for *Nostoc sphaeroides* (Ma et al., 2015). In addition, several studies have shown that

although phycobiliprotein content is increased with lower light conditions, biomass growth is significantly reduced (Castro et al., 2015; Kilimtzidi et al., 2019; Markou et al., 2012).

Regarding red microalgae, they generally prefer higher irradiance. Wang et al. (2007) investigated the effect of light in *Porphyridium cruentum* and suggested optimal light intensities of 7098 lux (~95.82 μmol photons/m^2s) for biomass growth and 7100 lux (~95.85 μmol photons/m^2s) for phycoerythrin production. Zucchi and Necchi (2001) studied the light intensity effect in seven freshwater red algae species and reported that most had the best growth performance around 65 μmol photons/m^2s, reaching phycobiliproteins up to 9.82 mg/g fresh weight. Guihéneuf and Stengel (2015) reported the highest phycobiliproteins content (~2.9% DW) reached under low light (30 μmol photons/m^2s). In contrast, Sosa-Hernández et al. (2019) have shown that increasing light intensities from 30 μmol photons/m^2s to 65 and 100 μmol photons/m^2s increased phycoerythrin production of *P. purpureum* by 43% and 30%, respectively. The authors, then, suggested that at high light intensity more phycoerythrin molecules are produced in order to harvest the maximum amount of photons reaching a limit.

Complementary chromatic adaptation (CCA) is a well-investigated phenomenon in microalgae, especially cyanobacteria, in which content of phycobiliproteins changes depending on the light colour (Bennett and Bogobad, 1973; Khatoon et al., 2018). As a result of this phenomenon, the pigment which absorbs the incident wavelengths of light most strongly becomes predominant (Wang et al., 2007). Hemlata and Fatma (2009) tested different light colours in *Anabaena* NCCU-9 and reported the order of suitable chromatic regime for the phycobiliprotein production to be white > blue > yellow > red > green, suggesting that coloured light played no stimulatory effect on phycobiliprotein production of this strain. Kilimtzidi et al. (2019) observed that shading *Arthrospira* cultures with red filters resulted in biomass with increased phycocyanin content achieving a maximum of 134 mg/g with higher purity. Stowe et al. (2011) observed that *Fremyella diplosiphon* produced a 3.3-fold increase of phycocyanin under red light cultivation and a 5.77-fold of phycoerythrin under green light. The authors also studied the CCA effect in *Gloeotrichia* UTEX 583 and observed that phycoerythrin was

synthesized under green light, but not in red light, while phycocyanin was highly abundant in both light colours. Khatoon et al. (2018) observed that white light was the most suitable light colour for maximising production of phycobiliproteins in *Pseudanabaena mucicola*. Ojit et al. (2015) also reported that fluorescent white light enhanced maximal production of phycobiliproteins in *Anabaena circinalis*, reaching concentrations of 31.53 μg/mg of phycoerythrin, 135.01 μg/mg of phycocyanin and 35.92 μg/mg of allophycocyanin after 15 days of growth.

2.3.2.1.2 Temperature

Like any living organism, all metabolic processes and biochemical composition in microalgae are also influenced by temperature. The optimal growth temperature and tolerance to the extreme values usually vary from strain to strain (Hemlata and Fatma, 2009). Likewise, phycobiliproteins synthesis is also regulated and affected by temperature (Chakdar and Pabbi, 2016). Chaneva et al. (2007) have shown that phycobiliproteins in *Arthronema africanum* was higher when temperature increased up to 36°C, but further rise resulted in lower phycobiliproteins content by 10-20%. Hemlata and Fatma (2009) described that sudden temperature changes exert stress on the organisms, especially at high temperatures, due to deficiency of oxygen, which is much less soluble in hot than in cold water. The authors suggested optimum temperature for phycobiliproteins production in *Anabaena* NCCU-9 was obtained at 30°C (127.02 mg/g DW), but yields significantly decreased at higher (by 38% at 40°C) or lower (by 23.6% at 20°C) temperatures. Regarding red algae, Zucchi and Necchi (2001) indicated that temperature had a higher effect on phycobiliproteins content than irradiance and photoperiod for seven freshwater species, with optimum temperature around 25°C. Guihéneuf and Stengel (2015) reported highest phycobiliproteins content (~2.9% DW) reached under low temperature (10°C), but maximal productivity was obtained at 20°C and under low light intensity, reaching up to 33.3 mg/L (~2% DW).

No clear correspondence between growth rates and pigment contents either for individual species or among them has been described in the literature. This highlights the challenge on determining optimal conditions for phycobiliproteins production, in which the most favourable conditions for growth are generally not coincident with those with

the highest pigment contents. Further studies to elucidate this interrelation is yet to be shown. Several hypotheses have been suggested to account for the differences in growth rates among species, such as: (i) different light harvesting properties, allowing some species to absorb available radiation more effectively than others, (ii) distinct photosynthesis/respiration ratios, yielding variable energy supply to support growth, and (iii) differences in levels of organic carbon reduction, which can explain growth rates (Zucchi and Necchi, 2001).

2.3.2.1.3 pH

Changes in pH affect solubility and bioavailability of nutrients, transport of substances across the cytoplasmic membranes, and the activity of intra-extracellular enzymes, as well as photosynthetic electron transport and osmotic potential of the cytoplasm (Chakdar and Pabbi, 2016). However, the influence of pH on the phycobiliprotein production has received little attention (Hemlata and Fatma, 2009). External pH rising from 7 to 9 significantly increased the total phycobiliprotein content in *Nostoc* sp. UAM 206, and this change was directly related to availability of inorganic carbon (Poza-Carrión et al., 2001). Similar results were described by Hong and Lee (2008), in which pH 8 was found to be optimum for *Synechocystis* sp. PCC 6701, while Deshmukh and Puranik (2012) reported optimum pH of 10 for phycobiliprotein production of *Synechocystis* sp.. Hemlata and Fatma (2009) reported that *Anabaena* NCCU-9 could grow well within the pH range 6–10, with maximum phycobiliproteins achieved at pH 8. However, in extreme pH conditions (2 and 12), the culture became white. For particular cases, it is strongly recommended that optimum pH of the medium is controlled, in order to maximize phycobiliproteins production (Chakdar and Pabbi, 2016).

2.3.2.1.4 Medium composition

The culture condition, especially nitrogen concentrations and carbon sources, also determines the production of phycobiliprotein by microalgae (Sekar and Chandramohan, 2008). In general, these microorganisms require nitrogen sources for growth, assimilating ammonium ions (NH_4^+) from external source via an active transport system,

while the unprotonated form (NH_3) is absorbed by diffusion and is trapped by protonation (Khatoon et al., 2018). Similarly, nitrate (NO_3^-) is taken up by cells via an active transport system before it is reduced to nitrite (NO_2^-) and then to NH_4^+ (Liotenberg et al., 1996). Under nitrogen limitation, microalgae use phycobiliprotein as a nitrogen source, which can contribute to a decrease in phycobiliprotein production by nblA gene expression (Eriksen, 2008). However, contradictory results were reported by Hemlata and Fatma (2009), showing that *Anabaena* NCCU-9 produced highest amount of phycobiliprotein under nitrogen free environment. Reduction in phycobiliproteins was observed with supplementation of ammonia (by 80% at 1 mM and 92% at 2 mM) and urea (by 59% at 1 mM, 75% at 2 mM and 88% at 3 mM). Similar results were reported in *Anabaena* 7120, in which phycobiliproteins exceeded in nitrogen-free media than nitrate grown cultures (Loreto et al., 2003). For red microalgae, Kathiresan et al. (2007) suggested that low concentrations of chloride, nitrate, and phosphate did not had any significant effect on the *P. purpureum* biomass production, while incrementing $NaNO_3$ above 1 g/L caused a negative impact on biomass yield as well as in phycoerythrin production. Guihéneuf and Stengel (2015) demonstrated that the highest phycobiliprotein volumetric concentration (47 mg/L) and content (1.8% DW) in *P. purpureum* were obtained in nitrogen-replete cultures (1 g $NaNO_3$ /L), while nitrogen-starvation induced (0 g $NaNO_3$/L) a strong decrease in phycobiliprotein content. In contrast, experiments showing lowest concentrations of nitrogen (0.075 g $NaNO_3$/L) leading to the best value of phycoerythrin productivity (1.08 mg/Ld) were also reported (Sosa-Hernández et al., 2019).

Salts concentrations has also been reported to affect phycobiliproteins concentration in saline species (Hemlata and Fatma, 2009; Kathiresan et al., 2007; Marrez et al., 2013). Rapid entry of sodium ions might result in detachment of phycobilisomes from the thylakoid membranes that lead to reduction in photosynthesis energy transfers from phycobiliproteins to PSII reaction centre and uptake of other mineral nutrients, such as K^+, Ca_2^+ and Mn_2^+ (Hemlata and Fatma, 2009).

Limitations of phosphorus or sulphur can also lead to partial or complete decrease of phycobiliprotein by inducing the expression of nblA gene and phycobiliprotein

degradation (Grossman et al., 1993). The availability of carbon source (such as glucose) strongly presses their synthesis by inhibiting the synthesis of other proteins thus favouring phycobiliproteins synthesis.

2.3.2.2 Phycobiliproteins production and applications

Nowadays, phycocyanin is produced commercially in autotrophic cultures of cyanobacteria *Arthrospira* spp., previously named *Spirulina*, mainly in open ponds (Spolaore et al., 2006). *A. platensis* is often chosen as a phycocyanin producing strain due to its ubiquity rather than its pigment content (Eriksen, 2008). Phycoerythrin, on the other hand, is mainly produced from the microalgae *Porphyridium* spp. (Christaki et al., 2015). However, to date, large-scale cultivation and commercial application has not achieved worldwide implementation (Li et al., 2019). Further information based on the actual phycobiliproteins production from these species will be addressed in the next sub-sections.

2.3.2.2.1 Phycocyanin production

The presence of bioactive compounds makes *Arthrospira* an alternative source for obtaining high value products such as cosmetics, nutraceuticals, and fertilisers. Several studies have demonstrated that *Arthrospira* extracts contain significant antioxidant properties with immunomodulatory and anti-inflammatory activities (Borowitzka, 2013). Besides, the demand for natural colourants in food and cosmetic industry has attracted special attention for these photosynthetic pigments (Mohsenpour et al., 2012).

More than 12,000 tons of *Spirulina* biomass are produced every year and nearly 70% is produced in China, India and Taiwan (García et al., 2017). Most of this biomass are sold as health food and animal feed, which has a low economic value (around 30 US$/kg, ~ 26.79* €/kg) relative to phycocyanin, which is considered as a high value product (Eriksen, 2008). The global phycocyanin market was estimated to be approximately US$ 120 Mn (~ € 107* Mn) in 2019, and projected to reach at least US$ 230 Mn (~ € 205* Mn) by 2029, expanding at a compound annual growth rate (CAGR) of

*Estimated based on 2019 yearly average exchange rate of US Dollar (USD) to Euro (EUR) (ECB, 2020).

around 7% during the forecast period, 2019-2029 (Transparency Market Research, 2019). The sell price varies considerably depending on the purity of the phycocyanin (Borowitzka, 2013). The commercial value of food grade phycocyanin is around 500 US$/kg (~ 446* €/kg) whilst the reactive grade and analytical grade is priced at 14 to 25 US$/mg (~ 12.5 – 22.3* €/mg) (Güroy et al., 2017).

Extensive research has been done in order to maximise the production of biomass and phycocyanin. Marrez et al. (2013) tested different cultivation media (Blue-green medium BG-11, Zarrouk's medium and synthetic human urine) to grow *A. platensis* and reached the highest biomass concentration (4.87 g/L) using Zarrouk´s medium, whereas modified BG-11 medium led to maximum content of chlorophyll (147 µg/mL) and carotenoids (140 µg/mL), phycocyanin (55.37 µg/mL) and allophycocyanin (51.73 µg/mL). Lima et al. (2018) tested strategies for using LEDs for *A. platensis* cultivation to increase its biomass productivity and high-value pigments (chlorophyll a, total carotenoids and phycocyanin) and reported that phycocyanin content using red light can be 5 to 7 times higher compared to blue light mixed with red light. Hifney et al. (2013) observed that rising salt concentration up to 0.6 M resulted in an increase of the total phycobiliprotein content in *Arthrospira* spp., from 25% to 45% of dry matter, while a further increase to 0.9 M salt affected negatively the phycobiliprotein synthesis.

2.3.2.2.2 Phycoerythrin production

The biomass productivity of *Porphyridium* sp. is high, showing strong salt resistance and abundant production of valuable products in cells, including phycoerythrin (Brody and Vatter, 1959; Fábregas et al., 1999; T. Li et al., 2019; Rebolloso Fuentes et al., 2000). However, under regular cultivation strategies, the productivity of phycoerythrin in *Porphyridium* sp. is limited, which might have prevented further commercial scale application (Golueke and Owald, 1962; Sekar and Chandramohan, 2008). Hence, to optimise the content of phycoerythrin for larger scale production process, culture methods and strategies for valuable compounds accumulation have been the primary focus in research. Fábregas et al. (1999) conducted semi-continuous cultivation of *P. purpureum* for phycoerythrin, polyunsaturated fatty acid and extracellular polysaccharide production, suggesting that the production of these

high-value products required different culture strategies. Fuentes-Grünewald et al. (2015) suggested that semi-continuous cultivation was more favourable for biomass production of *P. purpureum* as well as extracellular polysaccharide, reaching maximum values of 47 mg/Ld and 2.1 g/L, respectively. Concentrations of chlorides, nitrates and sulphates were also reported to significantly affect the synthesis of phycoerythrin by *Porphyridium* ssp. (Kathiresan et al., 2007; Velea et al., 2011; Xu et al., 2019; You and Barnett, 2004). Wang et al. (2007) also indicated that phycoerythrin biosynthesis could be maximised in *P. purpureum* under optimal cultivation conditions (pH 8; light intensity of 7100 lx; ratio of inoculum:substrate 1:20; temperature 20 °C; and light 30 μmol/m^2s).

The commercial price of phycoerythrin ranges from about €50 per mg (Anaspec, Inc., Fremont, CA, USA) (Francavilla et al., 2013) to about €650 per mg (Sigma Aldrich, Product number: P1286), depending on the purity level.

2.3.2.3 Phycobiliproteins from microalgae grown in wastewater

Phycobiliproteins seem to be thriving compounds to be extracted from cyanobacteria due to their high economic value. Several researchers have addressed the advantages and different methodologies to extract those chemicals from varies species cultivated in standard growth medium (Kuddus and Ramteke, 2012; Manirafasha et al., 2016; Ores et al., 2016; Silveira et al., 2007). However, very limited studies can be found on this valorisation strategy for microalgae grown in wastewater. Van Den Hende et al. (2016b) carried out an investigation on using microalgal-bacterial flocs to treat food industry effluent and further valorisation through high-value phycochemical extraction. The authors have reported extraction of 22.4 g phycocyanin (PC)/kg VS with a purity of 1.32 (24.5% recovery) and 9.5 g phycoerythrin (PE)/kg VS with a purity of 1.06 (20.9% recovery). Hultberg et al. (2017) cultivated *A. platensis* in anaerobic digestate effluent diluted (6%) in carbonate buffer and obtained higher phycocyanin (86.2 mg/g DW) and allophycocyanin (41.3 mg/g DW) compared to biomass grown in synthetic medium. Khatoon et al. (2018) observed phycobiliproteins production of up to 237 mg PBP/g DW (purity 1.14) using *Pseudanabaena mucicola* cultivated in wastewater from cage culture. Wood et al. (2015) demonstrated that wastewater from oil and natural gas extraction,

amended with 3 g $NaNO_3$/L and 0.5 g K_2HPO_4/L, could support growth of a cyanobacterial consortium, mainly composed by Oscillatoriales, producing phycocyanin yields up to 16.9 mg/g DW.

2.4 Life cycle assessment

Life-cycle assessment (LCA) is an accounting and management approach for assessing potential impacts to the environment. It considers all the aspects of resource use and environmental releases associated with a system, as defined by the function provided by a product, process, or activity (Curran, 2008). Crucially, an LCA is a comprehensive method for assessing all direct and indirect, upstream and downstream, environmental impacts across the full life cycle of a product system, from materials acquisition, to manufacturing, to use, and to final disposition (disposal or reuse) (Brusseau, 2019). LCA is a relative tool intended for comparison and not absolute evaluation, thereby helping decision makers compare all major environmental impacts when choosing between alternative courses of action (Curran, 2008).

According to the International Standards Organisation (ISO) 14000 series (ISO, 2006), the technical framework for LCA methodology consists of four phases (Figure 2-5):

1) Goal and scope definition: description of the purpose (product, process or activity), establishment of the context in which the assessment is to be made and identification of the boundaries (including assumptions and simplifications) and environmental effects to be reviewed for the assessment the functional unit;

2) Inventory analysis: collection of data for each unit process regarding all relevant inputs and outputs, by identifying and quantifying energy, mass flows, materials usage and environmental releases (e.g., air emissions, solid waste disposal, waste water discharges);

3) Impact assessment: evaluates the potential human and ecological effects of the examined system, product and processes identified in the inventory analysis;

4) Interpretation: evaluates the results of the inventory analysis and impact assessment to select the preferred product, process or service with a clear understanding of the uncertainty and the assumptions used to generate the results.

This feature to track and document shifts in environmental impacts can help decision makers in fully characterising the environmental trade-offs associated with product or process alternatives. The LCA provides useful information for several purposes, such as: i) determine a systematic evaluation of the environmental consequences associated with a certain product or process, ii) identify environmental trade-offs associated with one or more specific processes, iii) estimate environmental releases to air, water, and land caused by each life-cycle stage and/or major contributing process, and v) diagnose impacts to one or more specific environmental areas of concern (Curran, 2008).

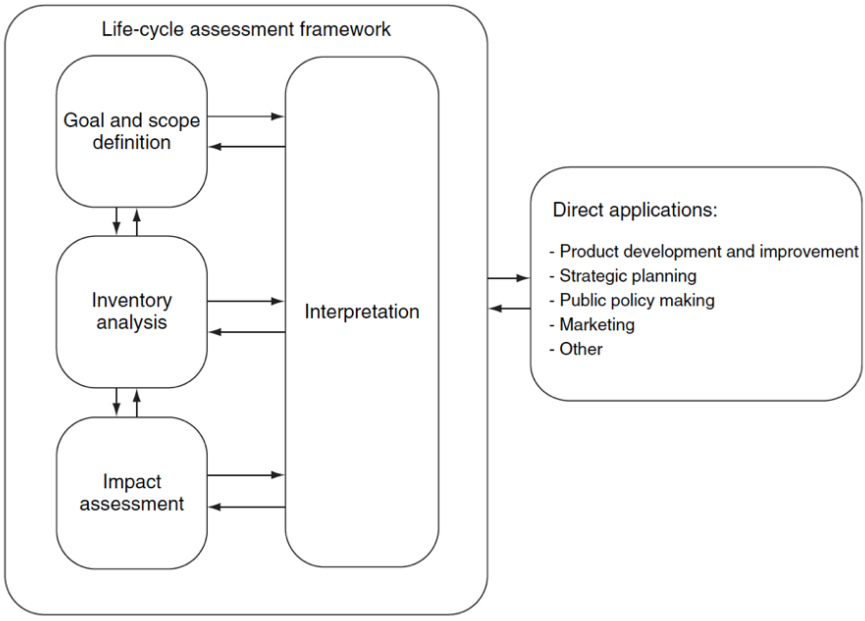


Figure 2-5. Four phases of a life cycle assessment (LCA) and example outcomes (ISO, 2006).

Several researchers have used the LCA tool to address the environmental aspects of different alternatives for wastewater treatment and resources recovery, including

microalgae systems (Collet et al., 2011; Fang et al., 2016; Garfí et al., 2017; Lam et al., 2019; Maga, 2017; Möller et al., 2018; Pan et al., 2019; Papadaki et al., 2017; Pérez-López et al., 2017; Rahman et al., 2016; Sfez et al., 2015). Corominas et al. (2013) conducted a study based on several LCAs on wastewater treatment and observed that eutrophication, toxicity and global warming impact categories are caused mainly by water discharge emissions, sludge treatment and disposal and electricity, indicating that the best alternatives seem to be the ones that provide lower nutrient emissions. Sfez et al. (2015) analysed the environmental sustainability of aquaculture wastewater treatment by microalgae, comparing the valorisation of biomass as shrimp feed and as biogas. The authors reported that valorising biomass as shrimp feed (1.9×10^{-3} kg N eq/m^3 treated water) had a lower marine eutrophication potential than as biogas (3.9×10^{-3} kg N eq/m^3 treated water), whereas the freshwater eutrophication potentials of the two options were similar (0.6 g P eq/m^3 treated water). Moreover, improvements were recommended to reduce the energy use in cultivation.

In a broader context of microalgae biomass valorisation, Vuppaladadiyam et al. (2018) also carried out a detailed evaluation of several studies of LCA and techno-economic assessments to investigate the sustainability of different microalgae biorefinery scenarios and highlighted the relevance of imminent research fields, such as integrating microalgae cultivation with wastewater or seawater, as well as with industrial wastes containing CO_2 sources, in order to reduce the water footprint and high nutrients demand. Furthermore, the concern of understanding the culturing requirements and challenges of the microalgae growth has been raised, indicating the LCA as a potential tool to investigate the impacts of different configurations and biomass valorisations (Alam and Wang, 2019).

Chapter 3

Optimisation of HRAPs systems with biogas recovery

The effect of primary treatment of wastewater in HRAPs systems: biomass and bioenergy recovery

Picture on previous page:
High rate algal ponds systems used for wastewater treatment (*Chapter 3*)
(UPC, Barcelona, Spain).

Abstract

The aim of this study was to assess the effect of primary treatment on the performance of two pilot-scale high rate algal ponds (HRAPs) treating urban wastewater, considering their treatment efficiency, biomass productivity, characteristics and biogas production potential. Results indicated that the primary treatment did not significantly affect the wastewater treatment efficiency (NH_4^+-N removal of 93 and 91% and COD removal of 62 and 65% in HRAP with and without primary treatment, respectively). The HRAP without primary treatment had higher biodiversity and productivity (18 vs. 16 g VSS/m^2d). Biomass from both systems presented good settling capacity. Results of biochemical methane potential test showed that co-digesting microalgae and primary sludge led to higher methane yields (238 - 258 mL CH_4/g VS) compared with microalgae mono-digestion (189 - 225 mL CH_4/g VS). Overall, HRAPs with and without primary treatment seem to be appropriate alternatives for combining wastewater treatment and bioenergy recovery.

This chapter has been redrafted after:

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3. The effect of primary treatment of wastewater in HRAPs systems: biomass and bioenergy recovery

3.1 Introduction

High rate algal ponds (HRAPs), as previously mentioned in Section 2.1.1, have received renewed interest due to their capacity to treat wastewater with reduced energy consumption compared to conventional activated sludge systems, while producing microalgal biomass that can be used for non-food bioproducts and biofuels production (Young et al., 2017). HRAPs consist of shallow, paddlewheel mixed, raceway ponds where microalgae assimilate nutrients and produce oxygen, which is used by bacteria to oxidise organic matter (Craggs et al., 2014; Park et al., 2011). They are low-cost technologies that can be successfully implemented in locations where weather conditions are favourable for microalgae growth (e.g. high solar radiation and temperature). These natural systems are appropriate solutions for wastewater treatment especially in small agglomerations, since they reduce costs and environmental impacts associated with wastewater treatment (Garfí et al., 2017). In this context, they were reported to treat anaerobically digested domestic wastewater reaching removal efficiencies of up to 97% of NH_4^+-N and 87% of soluble biochemical oxygen demand (sBOD) at optimal conditions (Park and Craggs, 2011). Similar results were obtained from HRAPs treating primary settled urban wastewater, reaching average removal of 80% of chemical oxygen demand (COD) and 95% of NH_4^+-N (Gutiérrez et al., 2016). Other studies applying this technology to treat agricultural wastes and industrial wastewater were also reported (de Godos et al., 2010; Ibekwe et al., 2017; Van Den Hende et al., 2016a). Moreover, HRAPs have been proven to be very effective for the recovery of bioenergy (e.g. biofuels), nutrients (e.g. biofertilisers) and valuable compounds (e.g. pigments, lipids) from wastewater (Arashiro et al., 2018; Craggs et al., 2011; Van Den Hende et al., 2016a).

The installation and maintenance of HRAPs are significantly cheaper compared to conventional activated sludge systems and closed photobioreactors (Delrue et al., 2016). Another advantage of the HRAPs is that greenhouse gas emissions are also reduced, making them an option to improve the sustainability of wastewater treatment (Acién et

al., 2016). However, one of the main drawbacks for implementing HRAPs for wastewater treatment is the large surface area requirement (up to 6 m^2/PE), which is necessary to promote satisfactory removal efficiency and biomass productivity. Indeed, a critical analysis of the latest studies on microalgae-based processes for wastewater treatment identified that the major obstacle hindering the dissemination of these technologies is the land requirement (Acién et al., 2016). In order to overcome this drawback and to simplify system operation and maintenance, the option of removing the primary treatment from the entire process could be considered. Primary treatment consists of removing settleable organic and inorganic solids from the raw wastewater by sedimentation. To date, there are several studies on optimising the HRAPs operating conditions, such as depth, hydraulic retention time (HRT) and dynamics (Amini et al., 2016; Buchanan et al., 2018; Sutherland et al., 2014). However, there are no studies in the literature which investigate, in practice, the role and effect of the primary treatment step before the HRAPs. Posadas et al. (2017) carried out a theoretical case study suggesting that primary suspended solids removal is probably unnecessary in a HRAPs system. This implication was based on the fact that the removal of biodegradable suspended solids can be efficiently reached by microalgal photosynthesis, which generates large excess in oxygenation capacity in the ponds. As suspended solids from raw wastewater may have an impact on light penetration and microalgae growth, which is directly related to biomass productivity and treatment capacity, further research is needed in order to demonstrate the feasibility of this configuration. Moreover, the possibility of incorporating a downstream process for microalgae biomass valorisation could be jeopardised in case the quality and amount of biomass was negatively affected by the absence of primary treatment.

Facing the current energy and environmental crisis, with the global economy relying on fossil fuels, extensive research has been done to valorise microalgal biomass within a biorefinery approach (Raheem et al., 2018; Šoštarič et al., 2012). Among the different biomass valorisation techniques proposed so far, biogas production seems to be the least complex option to recover bioenergy from microalgal biomass. Previous studies have reported the microalgae as a potential substrate for anaerobic digestion, especially after

undergoing pre-treatments to enhance the methane yield (González-Fernández et al., 2012a; Uggetti et al., 2017).

The aim of this research was therefore to investigate the effect of primary treatment on the long-term performance of pilot-scale HRAPs with a holistic approach, considering not only the wastewater treatment efficiency and biomass characteristics, but also the bioenergy recovery potential from harvested biomass. In particular, the present study focused on: 1) studying the performance of two parallel pilot systems: a HRAP treating raw urban wastewater and a HRAP treating primary settled urban wastewater, 2) comparing the biomass productivity, composition and settling capacity of each system, and 3) assessing the biogas production potential from microalgal biomass of each system. This is, to the best of the authors knowledge, the first study that explicitly investigated the role of the primary treatment in HRAPs systems based on pilot-scale experiments and its effect on bioenergy recovery.

3.2 Materials and Methods

3.2.1 High rate algal ponds

Experiments were carried out in a pilot plant located outdoors at the laboratory of the GEMMA Research Group (Universitat Politècnica de Catalunya, Barcelona, Spain) during 260 days (November 2016 – July 2017). The system treated real wastewater from the municipal sewer, which received a pre-treatment (screening) in the homogenisation tank (1.2 m^3) that was continuously stirred to avoid solids sedimentation. From this tank, wastewater was conveyed to two parallel treatment lines: one with a primary treatment (PT) in a cylindrical PVC settling tank (diameter: 18 cm, height: 30 cm, effective volume: 3 L, HRT: 41 min) as a control line (HRAP-PT); and another one without PT as a test line (HRAP-noPT). Subsequently, two identical HRAPs received the corresponding influents (105 L/day) with a HRT of 4.5 days. The HRAPs were made of PVC with a useful volume of 0.47 m^3, a surface area of 1.5 m^2, a water depth of 0.3 m, and with a paddle wheel constantly stirring the mixed liquor at an average velocity of 10 m/h. Both HRAPs were followed by

secondary settlers (diameter: 18 cm, height: 34 cm, effective volume: 3.3 L, HRT: 46 min) where the secondary effluent was separated from the microalgae. The biomass then was further thickened before undergoing anaerobic digestion. Details on the bioenergy recovery set-up will be described later. A schematic structure of the pilot plant is shown in Figure 3-1. The performances of both lines were compared in terms of wastewater treatment efficiency and biomass productivity, composition and settling capacity. In order to account for the seasonality, the wastewater treatment efficiency was compared in cold (November to March) and warm (April to July) periods.

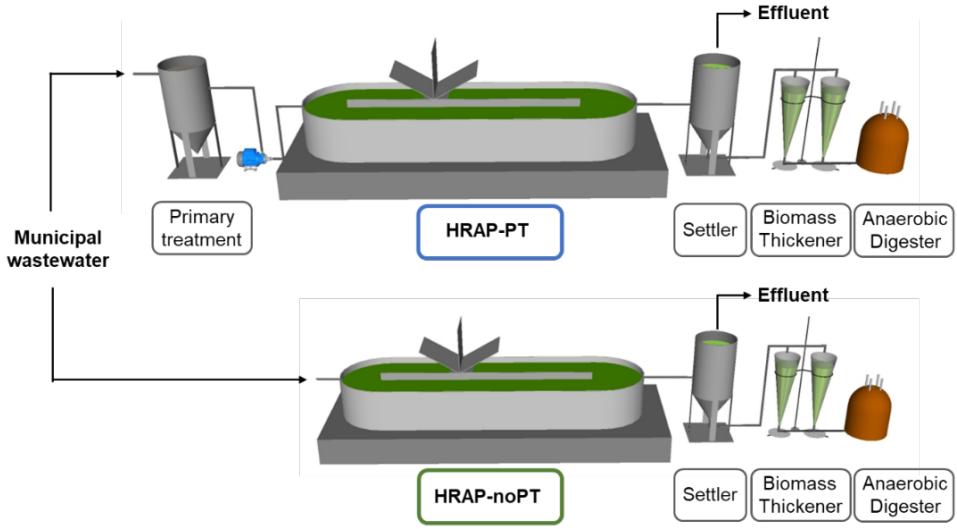


Figure 3-1. Scheme of the microalgae-based wastewater treatment pilot plant located outdoors in Barcelona (Spain). HRAP-PT is the line with primary treatment (PT) and HRAP-noPT is the line without PT.

3.2.2 Wastewater characterisation

In order to evaluate the wastewater treatment efficiency of both systems, the following parameters were monitored: dissolved oxygen (DO) and temperature

(EcoScan DO 6, ThermoFisher Scientific, USA) (daily), pH (Crison 506, Spain) and turbidity (Hanna HI 93703, USA) (three times per week), total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll-a, according to Standard Methods (APHA-AWWA-WEF, 2012), NH_4^+-N according to Solórzano method (Solórzano, 1969) and NO_2^--N, NO_3^--N and PO_4^{3-}-P through isocratic mode with carbonate-based eluents at a temperature of 30°C and a flow of 1 mL/min (ICS-1000, Dionex Corporation, USA) (limits of detection (LOD) were 0.9 mg/L of NO_2^--N, 1.12 of NO_3^--N, and 0.8 mg/L of PO_4^{3-}P) (twice a week), alkalinity, total and soluble chemical oxygen demand (COD and sCOD) according to Standard Methods (APHA-AWWA-WEF, 2012), total carbon (TC) and total nitrogen (TN) (multi N/C 2100S, Analytik Jena, Germany) (once a week). All the analyses were done in triplicate and results are given as average values.

3.2.3 Biomass composition and productivity

Samples of biomass were analysed microscopically (BA310, Motic, China) once a month, in order to observe the composition of microorganisms and measure flocs sizes during the experimental period. The identification of microalgae genera was based on conventional taxonomic books (Palmer, 1962; Streble and Krauter, 1987).

Average biomass productivity (g VSS/m²d) was calculated based on the VSS concentration in the HRAPs mixed liquor samples, using Eq. 3-1.

$$Biomass\ productivity\ = \frac{VSS\ (Q - Q_E + Q_P)}{A} \qquad \text{Eq. 3-1}$$

where VSS is the volatile suspended solids concentration of the HRAP mixed liquor (g VSS/L); Q is the wastewater flow rate (L/d); Q_E is the evaporation rate (L/d); Q_P is the precipitation rate (L/d); and A is the surface area of the HRAP (m^2). The evaporation rate was calculated using Eq. 3-2.

$$Q_E = E_p\ A \qquad \text{Eq. 3-2}$$

where A is the surface area of the HRAP (m^2) and E_p is the potential evaporation (mm/d), calculated from Turc's formula (Eq. 3-3) (Fisher and Pringle III, 2013).

$$E_p = a\,(R + 50)\,\frac{T_a}{(T_a + 15)} \qquad \text{Eq. 3-3}$$

where R is the average solar radiation in a day (cal/cm^2d); T_a is the average air temperature in a day (°C); and a is a dimensionless coefficient which varies depending on the sampling frequency (0.0133 for daily samples).

Solar radiation, air temperature and precipitation data were provided by the local automatic weather station of Barcelona – Zona Universitària (X8) (Fig. A-1, Appendix) (DAM, 2017).

3.2.4 Biomass settling capacity

Sedimentation tests were carried out monthly in order to observe the difference between the settling characteristics of the biomass produced in both HRAPs. The tests were performed in a settling column (height: 50 cm, diameter: 9 cm) with four sampling ports at different depths along the column (d_1 = 12 cm, d_2 = 20 cm, d_3 = 32 cm and d_4 = 40 cm), according to the method described by Metcalf & Eddy (2003). Mixed liquor of each HRAP was poured into the column up to 45 cm height in such a way that the distribution of particle sizes was uniform from top to bottom. At various time intervals (0, 5, 10, 20, 40, 60, 90, 120, 180 min), samples of 20 mL were withdrawn from the sampling ports and analysed for TSS concentrations. Removal efficiencies were calculated from initial and final TSS concentrations at different time intervals and column depths. Moreover, average settling velocities were estimated considering the column depth and the time needed to reach a certain biomass recovery efficiency.

3.2.5 Biochemical methane potential test

BMP tests were carried out between operational days 213 and 260 in order to compare the biogas production potential of biomass harvested from both systems. BMP

tests were performed in serum bottles of 160 mL filled up to 100 mL of liquid volume with certain amounts of inoculum and substrate, corresponding to 5 g VS substrate/L and a substrate to inoculum ratio (S/I) of 0.5 g VS substrate/g VS inoculum (Passos et al., 2013). The substrates used were primary sludge (PS) from the primary settler of the HRAP-PT and microalgal biomass from both the HRAP-PT and HRAP-noPT. PS was purged daily from the primary settler by means of a pump and microalgal biomass was harvested from the secondary settlers following the HRAPs and thickened by gravity in laboratory Imhoff cones at 4°C for 24h (Figure 3-1). The microalgae thermal pre-treatment was carried out at 75°C for 10h, according to the methodology described by Solé-Bundó et al. (2018).

Microalgal biomass was tested untreated (Microalgae-PT and Microalgae-noPT from the HRAP-PT and HRAP-noPT, respectively) and thermally pre-treated (TPT Microalgae-PT and TPT Microalgae-noPT from the HRAP-PT and HRAP-noPT, respectively). Moreover, in order to increase the C:N ratio, co-digestion (i.e. digestion of a mixture of different substrates) of Microalgae-PT and TPT Microalgae-PT with PS at two different ratios (25% Microalgae - 75% PS and 50% Microalgae - 50% PS on a VS basis) was also tested (Lu and Zhang, 2016). These ratios represent the average volume of microalgae and primary sludge obtained in warm and cold months in a pilot HRAPs system (Solé-Bundó et al., 2018). Each trial was performed in triplicate.

After being flushed with helium gas and closed with butyl rubber stoppers, the bottles were placed in a platform shaker incubator (OPAQ, Ovan, Spain) at 35°C and 100 rpm until daily methane production was less than 1% of the total accumulated methane yield in all bottles. Pressure in each bottle was periodically measured with a digital manometer (GMH 3151 Greisinger, Germany) and biogas production was calculated by subtracting the blank (only inoculum) production. The methane content in biogas was analysed by gas chromatography (Trace GC Thermo Finnigan, USA), following the procedure described by Solé-Bundó et al. (2018). The anaerobic biodegradability of each substrate was calculated based on the net methane production (mL CH_4) and the theoretical methane yield under standard conditions, which is estimated as 350 mL CH_4 for each gram of degraded COD (Chernicharo, 2007).

Microalgal biomass macromolecular composition was expressed in terms of proteins, carbohydrates and lipids over the VS content. Carbohydrates were measured by phenol-sulphuric acid method with acid hydrolysis and determined by spectrophotometry (Spectronic Genesys 8), proteins were measured from the Total Kjeldahl Nitrogen (TKN) (APHA-AWWA-WEF, 2012) and a TKN/protein conversion factor of 5.95 (González López et al., 2010) and lipids were measured with the Soxhlet extraction method, using a mixture of chloroform and methanol at the ratio of 2:1 (v/v) as extractant agents (Folch et al., 1957).

3.2.6 Energy assessment

Theoretical energy assessment of the two systems were estimated, comparing the different substrates tested. For simplification purposes, only the heating for anaerobic digestion (including pretreatment, when applicable) was considered as energy input, since it would be the main source of energy consumption in a full-scale system.

The input energy for the anaerobic digestion and co-digestion of untreated microalgae was determined by the heat required for the anaerobic digestion (T_d = 35°C) of the amount V_s (mL) of substrates (microalgae biomass alone or with primary sludge, when applicable). The initial substrates temperature was assumed to be ambient temperature (T_a = 20°C), and heat loss was considered negligible (Zupančič and Roš, 2003). The density (ρ) and specific heat (c) of digester influent (microalgae biomass alone or with primary sludge) were assumed to be the same as water (i.e. 1 g/mL and 4.18 × 10^{-3} kJ/ g °C, respectively). The input energy E_i of the anaerobic digestion step was normalised by the volatile solids (g VS) content of the substrate and was calculated by Eq. 3-4.

$$E_i = \frac{\rho\, V_s\, c\, (T_d - T_a)}{VS} \qquad \text{Eq. 3-4}$$

where E_i is the input heat energy for the anaerobic digestion (kJ/g VS); ρ is the digester influent density (g/mL); V_s is the volume of substrate (mL); c is the digester

influent specific heat (kJ/ g °C); T_d is the anaerobic digestion temperature (°C); T_a is ambient temperature (°C); and VS is the volatile solids content of the substrate (g VS).

Likewise, for the systems in which microalgal biomass was pre-treated and digested alone, the input energy was determined by the heat required for the thermal pre-treatment (T_p = 75°C) of the amount V_a (mL) of biomass. In full-scale systems, the pre-treated biomass would be cooled down before mesophilic digestion from 75 to 35°C through a heat exchanger, so that energy could be used to pre-heat the influent substrate with heat recovery efficiency (∅) of 85% (Lu et al., 2008). The input energy E_i (kJ/g VS) of the pre-treatment step was normalised by the volatile solids (g VS) content in pre-treated biomass and was calculated by Eq. 3-5.

$$E_i = \frac{\rho\, V_a\, c\, (T_p - T_a) - \rho\, V_a\, c\, (T_p - T_d)\emptyset}{VS} \qquad \text{Eq. 3-5}$$

where E_i is the input heat energy for the anaerobic digestion (kJ/g VS); ρ is the digester influent density (g/mL); V_a is the volume of microalgal biomass (mL); c is the digester influent specific heat (kJ/ g °C); T_p is the pre-treatment temperature (°C); T_d is the anaerobic digestion temperature (°C); T_a is ambient temperature (°C); ∅ is the heat recovery efficiency (85%); and VS is the volatile solids content of the substrate (g VS).

Concerning the input energy for the co-digestion of pre-treated microalgal biomass with sludge, the same approach shown in Eq. 3-5 was used for the microalgal biomass, but the heat requirement for rising up the temperature of primary sludge volume (V_{ps}) from T_a to T_d was also incorporated. The input energy E_i (kJ/g VS) for the co-digestion of pre-treated microalgal biomass and primary sludge was calculated by Eq. 3-6.

$$E_i = \frac{\rho\, V_m\, c\, (T_p - T_a) - \rho\, V_m\, c\, (T_p - T_d)\emptyset + \rho\, V_{ps}\, c\, (T_d - T_a)}{VS} \qquad \text{Eq. 3-6}$$

where E_i is the input heat energy for the anaerobic digestion (kJ/g VS); ρ is the digester influent density (g/mL); V_m is the volume of microalgal biomass (mL); V_{ps} is the volume of primary sludge (mL); c is the digester influent specific heat (kJ/ g °C); T_p is the pre-treatment temperature (°C); T_d is the anaerobic digestion temperature (°C); T_a

is ambient temperature (°C); $\emptyset$ is the heat recovery efficiency; and VS is the volatile solids content of the substrate (g VS).

In all the cases, the output energy (E_o) was determined from the methane yield, in order to assess if the methane production would at least cover the energy required for the anaerobic digestion step (i.e. $E_o - E_i = 0$). Eq. 3-7 was used to calculate E_o (kJ/g VS).

$$E_o = \Delta P_{CH_4} \xi \eta \quad \text{Eq. 3-7}$$

where ΔP_{CH_4} (m^3 CH_4/g VS) is the methane yield, ξ is the lower calorific value of methane (35800 kJ/ m^3 CH_4) and η is the heat conversion efficiency (90%) (Metcalf & Eddy, 2003).

Finally, the net energy ratio (NER) of heat were calculated as the output energy (energy produced by the system) over the input energy (energy used by the system) (Eq. 3-8). Values higher than 1 indicate net energy production.

$$\text{NER} = \frac{E_o}{E_i} \quad \text{Eq. 3-8}$$

The potential daily production of methane for each system was also estimated based on the results of methane yield obtained with the BMP tests. Microalgae biomass flow rates, harvesting efficiency and daily methane yields were estimated considering the biomass productivity and settling capacity.

3.2.7 *Statistical analyses*

Experimental data obtained from the systems HRAP-PT and HRAP-noPT regarding wastewater treatment efficiency, as well as biomass productivity and settleability, were analysed by paired two-sample t-test ($\alpha = 0.05$) using Minitab 18 (Minitab Inc., PA, USA).

For the evaluation of kinetic parameters of the BMP tests, experimental data were adjusted to a first-order kinetic model by the least square method (Schroyen et al., 2014), using the tool *Solver* from Microsoft Excel 2016 (Eq. 3-9).

$$P = P_o \cdot [1 - \exp(-k \cdot t)] \quad \text{Eq. 3-9}$$

where P_o stands for the methane production potential (mL CH_4/g VS), k is the first order kinetic rate constant (day^{-1}), P is the accumulated methane production at time t (mL CH4/g VS) and t is time (day).

The error variance (s^2) of modelled methane production from Eq. 3-9 based on the actual methane production was estimated by the following equation (Eq. 3-10):

$$s^2 = \frac{\sum_1^i (y_i - \hat{y}_i)}{N - K} \quad \text{Eq. 3-10}$$

where y_i is the experimental value, $\hat{y}_i$ is the value estimated by the model, N is the number of samples and K is the number of model parameters.

The results were statistically assessed via multi-factor analysis of variance (ANOVA) ($\alpha = 0.05$). The Fisher's Least Significant Difference (LSD) ($\alpha = 0.05$) was used as a post-hoc test using Minitab 18 (Minitab Inc., PA, USA).

3.3 Results and discussion

3.3.1 Wastewater treatment efficiency

The average values of the main parameters measured in HRAP-PT and HRAP-noPT over a period of 260 days are shown in Table 3-1 (mixed liquor) and Table 3-2 (influent and effluent). The temporal variations of water quality parameters monitored in both systems are shown in Figure 3-2. Moreover, a summary of the average removal efficiencies of the main water quality parameters is shown in Table 3-3. Additional data on average concentrations and removal efficiencies are presented in Table A-1 (Appendix).

The results obtained from the HRAPs indicated that there was no significant difference in terms of wastewater treatment efficiency between the two configurations considered. Average TSS and VSS concentration in the mixed liquor of HRAP-noPT

were 41% and 31% significantly higher than in the HRAP-PT, respectively (Table 3-1). As expected, the difference between the two systems relied more on the higher inert solids concentration discharged into the HRAP-noPT than in microorganisms' biomass (VSS). The average DO concentration in the HRAP-PT was 16% higher compared to the HRAP-noPT (Table 3-1), which is explained by its lower TSS concentration in the mixed liquor, enhancing light penetration through the pond and leading to a higher photosynthetic activity rate. However, the higher average chlorophyll-a concentration in HRAP-noPT indicates that in spite of the higher solids concentrations, microalgae growth was not hindered in this system.

Table 3-1. Summary of the average values of the main parameters monitored in the mixed liquor of both HRAPs through the entire experimental period (260 days). *P*-values for the t-test comparing values of the mixed liquor (95% confidence interval) are highlighted in bold when there is significant difference.

	HRAP-PT	**HRAP-noPT**	***P*-value**
TSS (mg/L)	261 ± 106	370 ± 131	**9.7E-15**
VSS (mg/L)	230 ± 91	301 ± 112	**1.7E-10**
pH	8.2 ± 0.5	7.9 ± 0.3	**2.1E-13**
Turbidity (NTU)	136 ± 73	160 ± 74	**4.7E-04**
TN (mg/L)	47 ± 13	52 ± 15	**1.5E-02**
TC (mg/L)	226 ± 154	240 ± 144	**2.1E-02**
DO (mg/L)	8.7 ± 2.2	7.6 ± 2.2	**7.8E-20**
Chlorophyll-a (mg/L)	1.1 ± 0.8	1.7 ± 0.8	**1.8E-06**

Regarding the wastewater quality parameters, there were no significant differences when comparing NH_4^+-N, TN, TC and COD removal efficiencies throughout the entire experimental period between the HRAP-PT and HRAP-noPT (Table 3-3). Considering the seasonal influence, there were no significant differences in removal efficiencies between the HRAP-PT and HRAP-noPT, except for NH_4^+-N and sCOD removal (Table 3-3). The NH_4^+-N removal efficiency was slightly higher in the HRAP-PT during the

warm season. This was probably because the proportion of microalgae (as mg chlorophyll-a/g VSS) increased by 61% from cold to warm season in the HRAP-PT, while in the HRAP-noPT the increase was only 6%. The higher microalgae proportion in the HRAP-PT during the warm season could have enhanced the NH_4^+-N removal in this system. Similarly, the higher sCOD removal in the HRAP-noPT during the cold season (Table 3-3) could be related to the higher biomass concentration in this system (Table A-1, Appendix).

Table 3-2. Summary of the average values of the main parameters monitored in the influent and effluent of both HRAPs through the entire experimental period (260 days).

	HRAP-PT		**HRAP-noPT**	
	Influent	Effluent	Influent	Effluent
TSS (mg/L)	201 ± 132	52 ± 37	333 ± 183	75 ± 46
VSS (mg/L)	185 ± 112	49 ± 32	280 ± 143	67 ± 38
pH	7.8 ± 0.3	8.0 ± 0.4	8.0 ± 0.2	7.7 ± 0.2
Turbidity (NTU)	135 ± 115	25 ± 22	170 ± 104	41 ± 37
TN (mg/L)	53 ± 27	28 ± 10	56 ± 28	33 ± 12
TC (mg/L)	244 ± 157	107 ± 69	258 ± 149	126 ± 88
NH_4^+-N (mg/L)	24 ± 11	1.5 ± 1.3	26 ± 11	2.2 ± 2.1
NO_3^--N (mg/L)	0.2 ± 0.4	17 ± 10	0.6 ± 1.7	16 ± 9
NO_2^--N (mg/L)	0.9 ± 1.7	1.6 ± 1.2	1.2 ± 2.3	2.3 ± 1.7
PO_4^{3-}-P (mg/L)	2.3 ± 1.8	1.5 ± 1.3	2.3 ± 1.5	1.7 ± 1.4
COD (mg/L)	353 ± 208	114 ± 65	464 ± 234	134 ± 64
sCOD (mg/L)	88 ± 48	58 ± 31	97 ± 47	61 ± 38

Despite the very high removal efficiencies of NH_4^+-N (around 90%) in both systems, the TN removal efficiencies were lower (around 45%) (Table 3-3). This was due to the fact that the influent nitrogen (mainly NH_4^+) was converted into NO_3^- (mostly) and NO_2^- (i.e. nitrification), as observed in previous studies (de Godos et al., 2016; Van Den Hende et al., 2016a). Moreover, during the warm season photosynthetic activity is enhanced, increasing pH and favouring NH_4^+ volatilisation (de Godos et al., 2016; García

et al., 2006). This explains the lower NO_3^- effluent concentrations during the warm season compared to the cold season, since a lower amount of NH_4^+ was available to be converted into NO_3^- (Figure 3-2). Average concentrations of NO_2^- in both ponds were very low (up to 2.5 mg/L). Thus, considering also that average NO_3^- concentrations in the influent and effluent of both HRAPs were similar (Figure 3-2), as well as NH_4^+ removal, it can be deduced that the nitrogen conversion pathway was similar in both systems through the experimental period. In general, NH_4^+ is the preferential form of nitrogen uptake for most microalgae species, followed by NO_3^- (Maestrini, 1982; Oliver and Ganf, 2002; Ruiz-Marin et al., 2010), which is in accordance with the results obtained in this study.

Table 3-3. Summary of the average removal efficiencies of the main water quality parameters measured in the influent and effluent of both HRAPs in cold (Nov-Mar) and warm (Apr-Jul) seasons. P-values for the t-test comparing values of the removal efficiencies (95% confidence interval) are highlighted in bold when there is significant difference.

	Cold Season			**Warm Season**			**Entire experimental period**		
	Removal (%)			Removal (%)			Removal (%)		
	HRAP-PT	HRAP-noPT	*p*-value	HRAP-PT	HRAP-noPT	*p*-value	HRAP-PT	HRAP-noPT	*p*-value
NH_4^+-N	91 ± 7	91 ± 7	0.75	95 ± 4	92 ± 9	**0.01**	93 ± 6	91 ± 8	0.05
TN	43 ± 9	46 ± 16	0.37	57 ± 21	50 ± 17	0.34	49 ± 17	48 ± 16	0.73
TC	59 ± 15	61 ± 15	0.55	54 ± 15	44 ± 14	0.15	56 ± 15	53 ± 17	0.37
PO_4^{3-}-P	12 ± 47	4 ± 55	0.66	68 ± 38	56 ± 44	0.19	37 ± 52	25 ± 52	0.22
COD	60 ± 22	63 ± 23	0.59	64 ± 23	67 ± 25	0.75	62 ± 22	65 ± 23	0.58
sCOD	44 ± 19	56 ± 22	**0.03**	33 ± 18	35 ± 16	0.77	39 ± 19	47 ± 22	0.08

On the whole, both systems presented high nutrients and organic matter removal efficiencies in spite of the seasonal changes and different operational conditions (i.e. absence of primary treatment). Average COD removal efficiencies ranged between 60 and 67% in both systems through the entire experimental period (Table 3-3). These removal efficiencies were in accordance with previous studies under similar operational conditions (Young et al., 2017; Sutherland et al., 2014). Another study which evaluated

the growth of Chlorella sp. in raw and primary treated wastewater from a conventional municipal wastewater plant (i.e. activated sludge system), also reported similar organic matter and nutrients removal efficiencies (Wang et al., 2010). Average NH_4^+-N removal efficiencies were 82.4 and 74.7%, while for COD the removal rates were 50.9 and 56.5% for microalgae cultivation in wastewater sampled before and after primary treatment, respectively (Wang et al., 2010). Although these results were obtained from batch cultures, the removal efficiencies were similar to the ones found in this work.

The results of this work are in accordance with previous studies in which microalgae were cultivated at lab-scale using wastewater from different stages of municipal wastewater treatment plants, obtaining efficient treatment (Cabanelas et al., 2013; Kong et al., 2009). Furthermore, the present study corroborates with the hypothesis proposed by Posadas et al. (2017) who suggested that, based on a theoretical study, primary suspended solids removal is unlikely needed when using the HRAPs technology for treating urban wastewater.

Finally, based on the results presented in this section, the primary treatment preceding a HRAP seems to be a dispensable step when urban wastewater treatment is the main objective. Moreover, the simplification of a HRAPs system by removing the primary treatment step would also incentivise its implementation in small communities, since the wastewater treatment plant footprint and cost could be reduced.

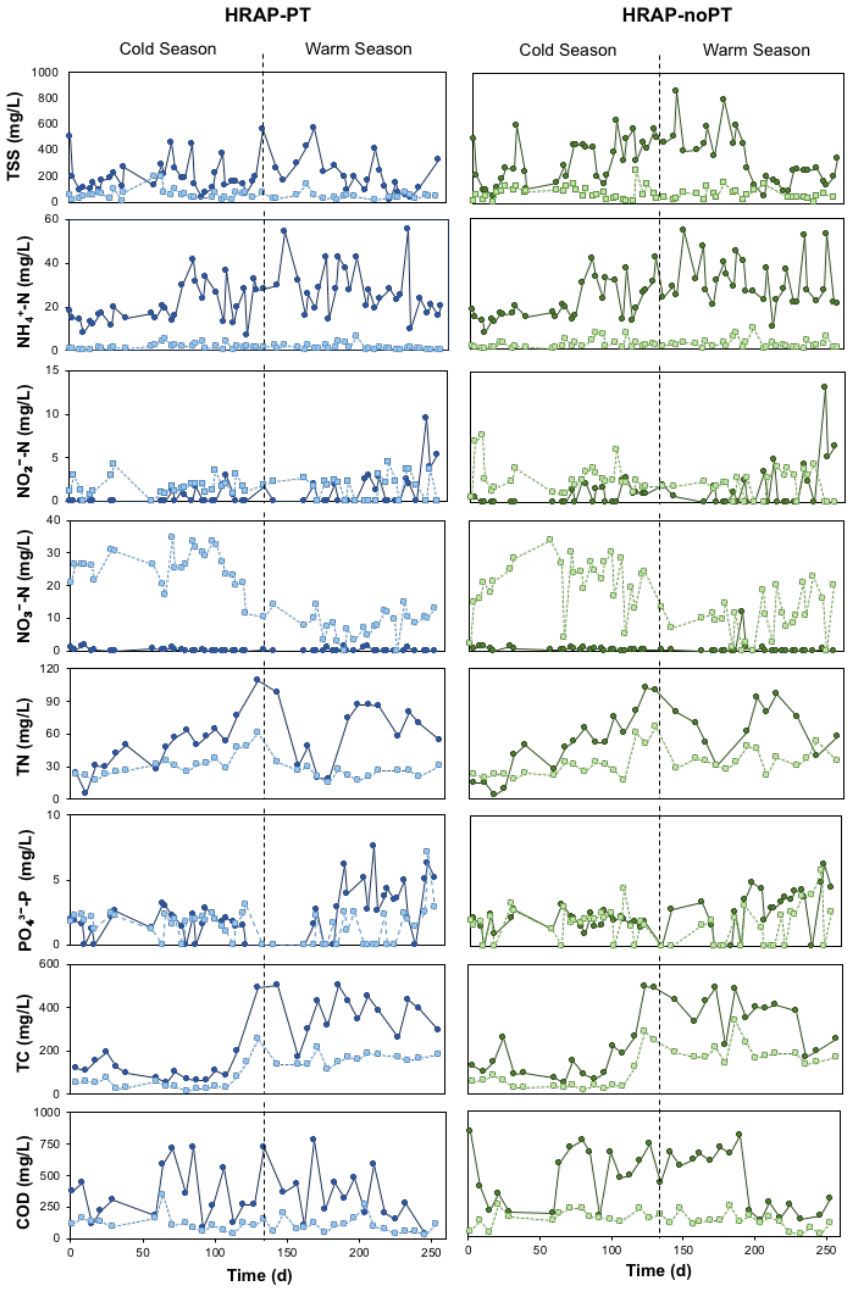


Figure 3-2. Influent (●) and effluent (■) concentrations of total suspended solids (TSS), NH_4^+-N, NO_2^--N, NO_3^--N, total nitrogen (TN), PO_4^{3-}-P, total carbon (TC) and chemical oxygen demand (COD) measured in the HRAP-PT and HRAP-noPT during the experimental period.

3.3.2 Biomass composition and productivity

Considering the entire experimental period, the HRAP-noPT had a higher biodiversity of microorganisms compared to the HRAP-PT. During the cold season, the microalgal biomass in the HRAP-PT was mainly composed of *Chlorella* sp., while in the HRAP-noPT the predominant microalgae genus was *Stigeoclonium* sp., which formed macroscopic filamentous flocs. However, during the warm season *Chlorella* sp. became the predominant genus in the HRAP-noPT system as well. Diatoms (mostly *Nitzschia* sp. and *Navicula* sp.) and grazers (ciliate and flagellate protozoans) were observed in both ponds along the entire period, but in larger quantity in the HRAP-noPT than in the HRAP-PT (Figure 3-3). The average size for the flocs observed in the HRAP-PT was 50-500 μm, while for the HRAP-noPT it ranged from 100 to 2,000 μm. The biomass diversity is a relevant parameter to be monitored, since it influences downstream processes, such as biogas and bioproducts generation. The presence of grazers, for instance, might affect the productivity of high-value compounds extracted from the biomass.

Microalgal biomass productivity of both HRAPs is shown in Figure 3-4. The overall average biomass productivity in the HRAP-noPT was 20 ± 7 g VSS/m^2d, which was significantly higher (by 30%) than in the HRAP-PT (15 ± 6 g VSS/m^2d). Park and Craggs (2010) operated a HRAP with an HRT of 4 days and reported an average biomass productivity of 20.7 g VSS/m^2d, which was slightly higher than in the present study most probably because there was CO_2 addition to control the pH and prevent carbon limitation. Similar results were described by de Godos et al. (2016), with an average biomass productivity ranging from 13.2 g VSS/m^2d (HRT of 5 days in spring) to 23.9 g VSS/m^2d (HRT of 3 days in summer) in HRAPs operated without CO_2 injection.

The higher biomass productivity observed in the HRAP-noPT might be explained by the higher influent VSS concentration (Table 3-1). Indeed, the VSS concentration in the influent was 49% higher in the HRAP-noPT than in the HRAP-PT (Table 3-1). Moreover, the VSS and chlorophyll-a concentrations in the mixed liquor were around 31% and 50% higher in HRAP-noPT than in the HRAP-PT, respectively (Table 3-1).

With this in mind, it can be assumed that part of the VSS introduced in the HRAP-noPT was consumed by the microalgal-bacterial biomass. In other words, the VSS in the influent (i.e. organic matter from the wastewater) was converted into microalgal-bacterial biomass in the HRAP-noPT system, where the microalgal proportion may have increased better than in the HRAP-PT system. As mentioned before, the difference in TSS influent concentration (Table 3-1) and, consequently, on the light availability between the two systems, did not seem to have created photo-inhibition. Indeed, previous studies, which investigated the composition of the phytoplankton community in three HRAPs submitted to different solar radiation levels, also reported that light availability was not the main influence on the growth and development of microalgal biomass. Other aspects, such as competition with other microorganisms for space and nutrients, and predation by zooplankton seemed to have a higher effect on microalgae biomass composition and productivity (Assemany et al., 2015).

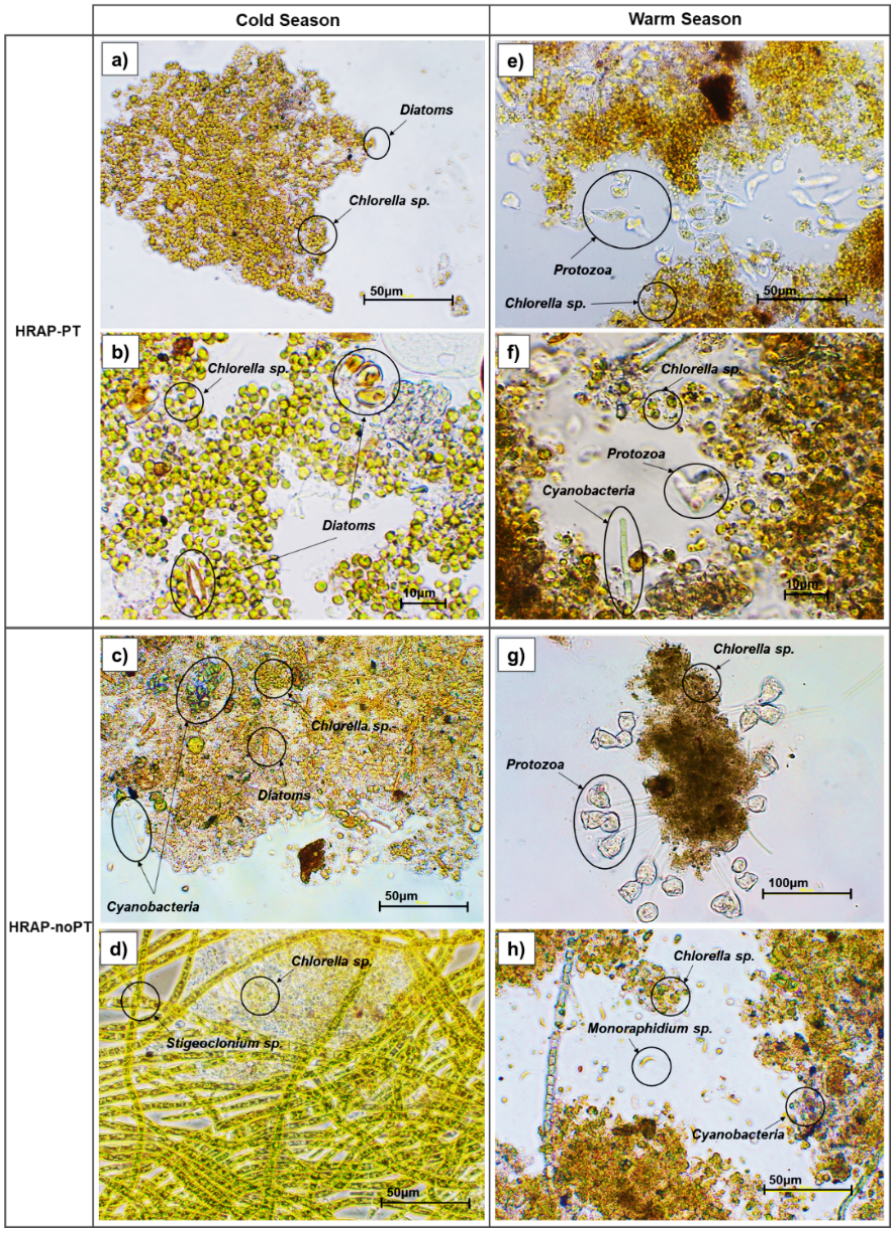


Figure 3-3. Microscopic analyses showing the biomass composition: HRAP-PT with predominance of *Chlorella* sp. in the cold season (a,b) and warm season (e,f), and HRAP-noPT with predominance of *Stigeoclonium* sp. in the cold season (c,d) and *Chlorella* sp. in the warm season (g,h). Presence of diatoms, cyanobacteria and protozoa were registered along the entire period, but in higher concentrations during the warm season.

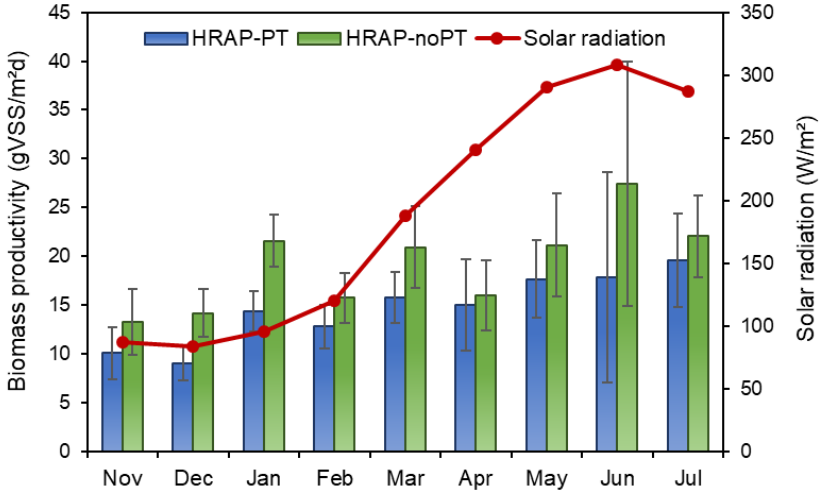


Figure 3-4. Monthly average biomass productivity in the HRAP-PT and HRAP-noPT from November 2016 to July 2017.

With regards to seasonal influence, there was a slight increase in biomass productivity in warmer months (Figure 3-4). It is worth noting that during those months, the abundance of grazers in both ponds also increased. The presence of these predators indicated that the actual biomass productivity might have been higher that the calculated values, which were based on the VSS concentrations measured in the mixed liquor of both ponds. This could possibly explain the high variation seen in June, in which the ranges of biomass productivity measured in both ponds were the largest of the entire period (HRAP-PT: 5 - 33 gVSS/m^{2}d and HRAP-noPT: 14 - 46 gVSS/m^{2}d). Biomass losses caused by these organisms have also been reported in previous studies (Mehrabadi et al., 2016; Montemezzani et al., 2016; Park et al., 2013). Finally, although the HRAP-noPT received higher organic loading, the production of microalgal biomass was not jeopardised. In addition, the higher biomass productivity would most likely lead to higher biogas production per day or other bioproducts obtained from this biomass.

3.3.3 Biomass settling capacity

The biomass sedimentation through gravity settling was assessed by monthly settling column tests. The assessment of the settling capacity helps to define further harvesting and dewatering techniques to be applied at large scale, which usually represents high energy consumption on the overall process (Fasaei et al., 2018). In this study, the initial biomass concentration in the mixed liquor varied from 0.26 – 0.39 g VSS/L for the HRAP-PT and 0.23 – 0.72 g VSS/L for the HRAP-noPT. As mentioned above, biomass recovery efficiencies were calculated from the initial and final TSS concentrations at different time intervals and column depths.

The settling tests results indicated that the biomass from both systems had good settling capacity. Figure 3-5a shows the biomass recovery over time with curves representing the four different sampling depths (12, 20, 32 and 40 cm). Based on these data, the time required to obtain certain biomass recovery efficiencies (80, 85, 90 and 95%) was calculated (Figure 3-5b). Considering average values of all settling tests, the biomass from the HRAP-noPT was faster to reach recovery efficiencies of 80, 85 and 90%, and the HRAP-PT was faster only for 95% recovery. This is in accordance with microbiology observations, that recorded higher biodiversity of microorganisms for the HRAP-noPT than the HRAP-PT during the entire period. Moreover, filamentous microalgae present in the HRAP-noPT during the cold season, which are organisms linked to flocs aggregation, also influenced the higher settling capacity of this biomass.

Biomass recovery efficiencies were lower than those found in a previous study with similar biomass composition, with about 85% recovery in less than 40 min (Gutiérrez et al., 2015). However, it is important to mention that the initial biomass concentration in that study was higher (800 mg VSS/L) than in the present one (300 - 400 mg VSS/L). In that study, the average time needed to recover 90% of biomass was 58 min, with a final effluent concentration of 80 mg VS/L. In the present study, the average times needed to reach 90% of biomass recovery was 129 min (HRAP-PT) and 114 min (HRAP-noPT), but the final effluent concentrations were much lower: 30 and 40 mg VSS/L. This highlights the importance of considering the final effluent quality when comparing results of relative removal efficiencies from different studies.

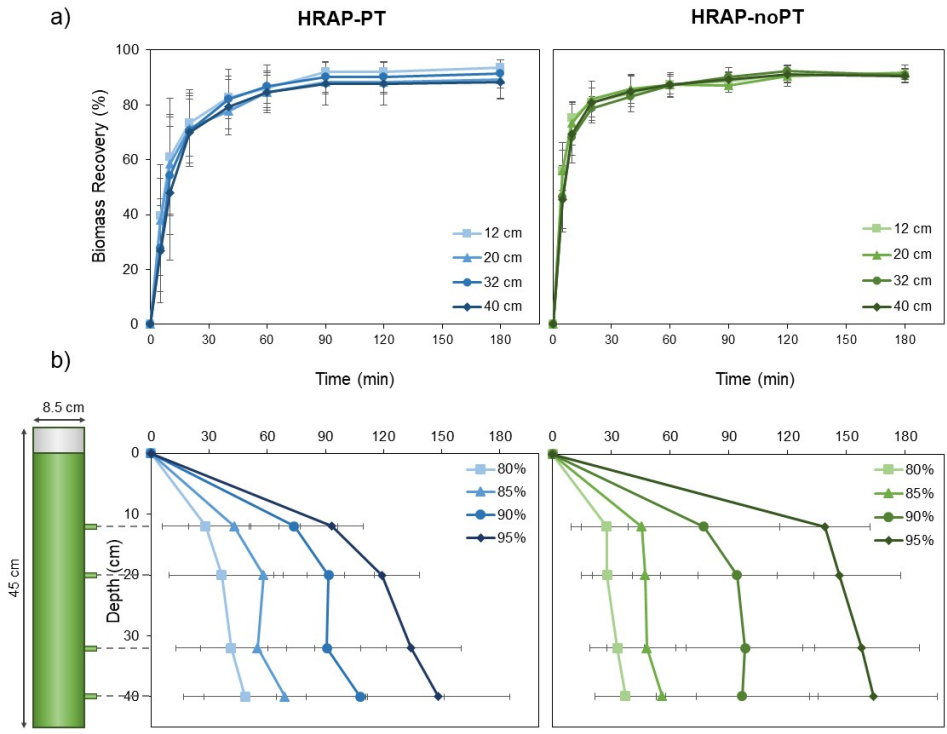


Figure 3-5. Average results of settling tests (n=8) for the HRAP-PT and HRAP-noPT: a) Removal efficiencies at depths of 12 cm (■), 20 cm (▲), 32 cm (●) and 40 cm (♦); b) Average microalgal biomass isorecovery curves of 80% (■), 85% (▲), 90% (●) and 95% (♦).

The relation between the sampling depth and settling time recorded for biomass from the HRAP-PT and HRAP-noPT is illustrated by isorecovery curves (Figure 3-5b). Each curve shows the time required to obtain a certain biomass recovery at different depths. Thus, the settling velocities were calculated by dividing the column depth (d_i) by time (t_i).

For instance, the average settling velocities for 80% recovery were 0.47 and 0.51 m/h, and for 95% recovery they were 0.13 and 0.09 m/h for the HRAP-PT and HRAP-noPT, respectively. For 80% recovery, the HRAP-noPT had a slightly higher velocity, which is explained by the larger flocs, but for 95% HRAP-PT had a higher velocity, indicating the higher amount of colloidal particles in the HRAP-noPT resulting from the

influent characteristics. The settling velocities were similar to the ones reported by Moorthy et al. (2017), which ranged from 0.03 to 0.08 m/h for *Scenedesmus abundans*, and by Peperzak et al. (2003), which fluctuated from 0.02 to 0.09 m/h for a mixture of microalgae.

Overall, the biomass from both systems presented good settling capacity with no significant differences between them. Thus, the absence of primary treatment did not affect the biomass settling capacity.

3.3.4 Biochemical methane potential test

The BMP test was performed in order to complement the comparison between the HRAP-PT and HRAP-noPT, in terms of potential bioenergy recovery from biomass harvested in each system. Biochemical analysis indicated that microalgal biomass was mainly composed of proteins (41 - 49%), followed by carbohydrates (27 - 33%) and lipids (20 - 25%) (Table 3-4), in accordance with previous studies (Dong et al., 2016; Solé-Bundó et al., 2017a).

The methane yield of each trial over an incubation period of 48 days is illustrated in Figure 3-6. The methane content in biogas was similar in all cases (around 72%).

The lowest methane yield was obtained in the mono-digestion of Microalgae-noPT, with a final yield of 188.7 mL CH_4/g VS; and the highest methane yield was from the co-digestion of 25% Microalgae-PT + 75% PS, reaching a final yield of 258.3 mL CH_4/g VS. This was 25% higher compared to the mono-digestion of the Microalgae-PT. During the initial stage of the incubation (especially the first 6 days) the kinetics and productions were better for TPT Microalgae-PT, TPT Microalgae-noPT and Microalgae-noPT (Figure 3-6a). However, after the 9th day the behaviour changed and the Microalgae-PT production slightly increased compared to Microalgae-noPT (both untreated and TPT). This performance could be explained by the fact that Microalgae-noPT contained more readily biodegradable material (which was transformed into biogas) than the Microalgae-PT, as expected, since the former was harvested from the system without primary treatment.

The final methane yield of pre-treated microalgae from the HRAP-PT, primary sludge and its co-digestion with untreated or pre-treated microalgae grown in the HRAP-PT were not statistically different from each other (Table 3-5). In addition, no significant differences were found in the final methane yield from untreated and pre-treated microalgae grown in both HRAP-noPT and HRAP-PT (Table 3-5). Nevertheless, the methane yield of untreated and pre-treated microalgae grown in HRAP-noPT were significantly lower than those obtained with the co-digestion of primary sludge and microalgae harvested in the HRAP-PT (Table 3-5).

Table 3-4. Average biochemical composition of the inoculum and substrates used for the BMP test. Microalgae-PT and Microalgae-noPT refer to microalgal biomass harvested from the HRAP-PT and HRAP-noPT, respectively; untreated or thermally pre-treated (TPT).

Parameter	Inoculum	Primary Sludge	Microalgae-PT		Microalgae-noPT	
			Untreated	TPT	Untreated	TPT
pH	7.35	6.37	6.46	6.74	6.33	6.48
TS [%(w/w)]	2.12 ± 0.01	3.13 ± 0.04	6.09 ± 0.01	6.03 ± 0.01	5.87 ± 0.02	5.80 ± 0.01
VS [%(w/w)]	1.31 ± 0.13	2.32 ± 0.40	4.65 ± 0.23	4.62 ± 0.28	3.96 ± 0.62	4.02 ± 0.11
COD (g O_2/L)	16.90 ± 0.50	15.43 ± 0.29	79.87 ± 0.88	79.70 ± 0.25	59.43 ± 1.07	59.87 ± 1.38
Carbohydrates (%VS)	-	-	29.7	26.9	29.0	32.5
Proteins (%VS)	-	-	48.8	47.4	43.6	41.2
Lipids (%VS)	-	-	20.6	25.0	22.0	19.8

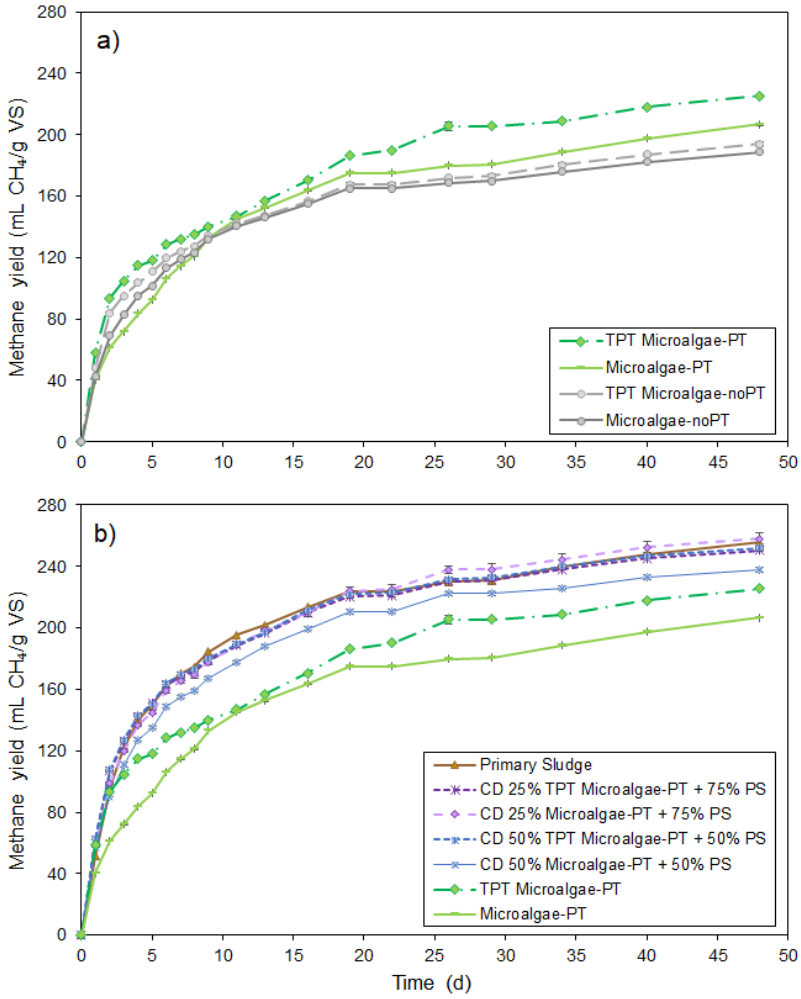


Figure 3-6. Cumulative methane yields showing the effects of: a) thermal pre-treatment (TPT), with the comparative results for microalgal biomass from the HRAP-PT and HRAP-noPT: untreated (Microalgae-PT and Microalgae-noPT) and thermally pre-treated (TPT Microalgae-PT and TPT Microalgae-noPT); and b) co-digestion (CD), with the comparative results for Primary Sludge (PS) and co-digestion of Microalgae-PT and TPT Microalgae-PT with PS at two different ratios (25% microalgae + 75% PS and 50% microalgae + 50% PS on a VS basis).

The thermal pre-treatment was applied in this study in order to increase microalgae biodegradability by breaking down their resistant cell wall, as suggested by previous studies. Several studies on microalgae pre-treatment for biogas production have been reported, including biological, chemical and physical pre-treatments (Kendir and Ugurlu, 2018). The selection of a thermal pre-treatment for this study was based on previous research comparing different pre-treatments, which showed that the thermal one would reach the highest methane yield and considerably better energy balance (Kendir and Ugurlu, 2018; Passos et al., 2015). Comparing the mono-digestions, the thermal pre-treatment improved the methane yield by 3% (HRAP-noPT) and 9% (HRAP-PT). Although no statistical difference (P-values: 0.80 for HRAP-noPT and 0.37 for HRAP-PT) was found between the methane yield of untreated and thermally pre-treated microalgae from both systems (Table 3-5), the thermal pre-treatment did improve the kinetics in all cases (by 14-22%) as compared to untreated microalgae, which is in agreement with (Solé-Bundó et al., 2018).

In contrast, the co-digestion of microalgae and sludge showed a more significant improvement, increasing the methane yield up to 25% and the kinetics up to 39% compared to microalgae mono-digestion. Moreover, the kinetics of co-digestion with thermally pre-treated microalgae at both ratios (25-75% and 50-50%) were even higher than primary sludge (Table 3-5). This highlights the synergy of co-digesting microalgae with primary sludge, as also described in previous studies on co-digestion of microalgae and other C-rich substrates (Solé-Bundó et al., 2017c; Yen and Brune, 2007). The results are also in agreement with previous studies in which the co-digestion of microalgae and sewage sludge had a synergistic effect (Olsson et al., 2014; Solé-Bundó et al., 2018).

Table 3-5. Summary of the methane yield (initial after 6 days and final after 48 days of digestion), methane content in biogas of each trial, anaerobic biodegradability (mean values ± standard deviation; n=3) and first-order kinetics constant (k) obtained from Eq. 3-9 (error variance (s^2) from Eq. 3-10 is represented in brackets).

Substrate	**Initial methane yield (mL CH_4/g VS d)**		**Final methane yield (mL CH_4/g VS)**		**Anaerobic Biodegradability (%)**		**First-order kinetics constant (day^{-1})**	
	Untreated	**TPT**	**Untreated**	**TPT**	**Untreated**	**TPT**	**Untreated**	**TPT**
Primary sludge	163.1^{a} ± 1.1		255.5^{a} ± 2.4		37.7 ± 2.4		0.202 (135)	
Microalgae-noPT	113.1^{a} ± 0.4	119.8^{a} ± 0.6	188.7^{b} ± 0.7	193.9^{b} ± 1.4	25.3 ± 0.7	25.8 ± 1.4	0.179 (78)	0.205 (150)
Microalgae-PT	106.3^{a} ± 0.2	128.5^{a} ± 0.2	206.8^{b} ± 0.7	$225.4^{a,b}$ ± 0.7	25.3 ± 0.7	26.5 ± 0.7	0.135 (63)	0.165 (326)
CD 25% Microalgae-PT + 75% PS	159.5^{a} ± 1.7	163.1^{a} ± 0.3	258.3^{a} ± 3.9	250.3^{a} ± 0.4	35.1 ± 3.9	34.5 ± 0.4	0.184 (201)	0.214 (208)
CD 50% Microalgae-PT + 50% PS	148.8^{a} ± 1.1	164.0^{a} ± 0.5	$237.6^{a,b}$ ± 1.7	251.9^{a} ± 0.5	31.1 ± 1.7	32.2 ± 0.5	0.187 (146)	0.213 (216)

^{a,b}: Letters indicate a significant difference of methane yield between trials (α = 0.05) after Fisher's LSD test.

3.3.5 Energy assessment

A simplified energy assessment of the HRAP-PT and HRAP-noPT scenarios was calculated in order to complement the comparison between these systems and to determine under which conditions the system would be energy neutral or even net energy producer (NER > 1). Table 3-6 shows the outcome of the energy assessment, as explained in Section 3.2.6.

Table 3-6. Net energy ratio (NER) of untreated and thermally pre-treated (TPT) microalgal biomass from HRAP-PT (Microalgae-TP) and HRAP-noPT (Microalgae-noPT) scenarios, and co-digestion (CD) of Microalgae-PT with primary sludge (PS).

Substrate	**Input energy (kJ/g VS)**		**Output energy (kJ/g VS)**		**Net energy ratio (NER) (kJ/g VS)**	
	Untreated	TPT	Untreated	TPT	Untreated	TPT
Primary sludge	2.71	-	8.23	-	3.04	-
Microalgae-noPT	1.59	2.18	6.08	6.25	3.83	2.86
Microalgae-PT	1.35	1.90	7.26	6.66	5.39	3.51
CD 25% Microalgae-PT + 75% PS	2.37	2.51	8.32	8.06	3.51	3.22
CD 50% Microalgae-PT + 50% PS	2.03	2.30	7.66	8.12	3.78	3.52

All the substrates combinations resulted in a positive NER, suggesting that more energy was produced than consumed in the biogas production process. The untreated Microalgae-PT led to the highest NER, while the thermally pre-treated Microalgae-noPT showed the lowest NER. This is due to the highest methane yield obtained from those substrates, as explained in Section 3.3.4. Passos et al. (2017) carried out a far more complex work to assess the energy balance of HRAPs and biogas production, considering the energy flows of the entire plant and biomass production fluctuations over the year. Likewise, the authors reported that those systems would be energy neutral or even net energy producer if thermal pretreatment of microalgal biomass and anaerobic co-digestion with primary sludge was applied.

As mentioned above, the HRAP-noPT system presented a lower methane production than the HRAP-C (especially in the case of co-digestions) (Table 3-5), which explains the less favourable energy balance of the former compared to the latter (Table 3-6). Nevertheless, it is important to note that the values shown in Table 3-5 and Table 3-6 are expressed in terms of VS (substrate). Thus, the potential methane production for each scenario was also roughly estimated considering the average volumes of biomass observed in this study (shown in Section 3.3.2) and primary sludge (as estimated by Solé-Bundó et al. (2018)) obtained per day in HRAPs systems (Table 3-7). When considering the average biomass productivity, results showed that co-digestion of microalgae and primary sludge would be the best option to maximise energy generation in this HRAPs system. Indeed, the daily methane production of the co-digestion of microalgae and primary sludge is up to 3.5-fold the values obtained from microalgae mono-digestion.

Table 3-7. Estimated daily methane production with untreated and thermally pre-treated (TPT, 75°C) microalgal biomass from HRAP-PT (Microalgae-PT) and HRAP-noPT (Microalgae-noPT) scenarios, and co-digestion (CD) of Microalgae-PT with primary sludge (PS).

Substrate	**Daily CH_4 production (L/d)**	
	Untreated	**TPT**
Primary sludge	10.6	-
Microalgae-noPT	5.3	5.5
Microalgae-PT	5.2	5.6
CD 25% Microalgae-PT + 75% PS	15.2	15.0
CD 50% Microalgae-PT + 50% PS	17.5	18.0

Nevertheless, it remains unclear whether recommending a conventional configuration with a primary treatment, which would enable co-digestion, is reasonable. In order to provide a holistic comparison, an economic analysis should be done so that the implementation and operational costs are also accounted for. In this way, a better understanding on whether the surplus energy provided by the co-digestion (HRAP-PT system) would overcome the reduction of operational demand and costs removing the

primary settler (HRAP-noPT system). This analysis should also take into account that the digestate from the anaerobic digestion of microalgae could be further utilised as biofertiliser (Garcia-Gonzalez and Sommerfeld, 2016; Huang et al., 2017; Solé-Bundó et al., 2017b). On the other hand, other biomass valorisation alternatives should be explored in order to maximise the resources recovery from HRAPs systems.

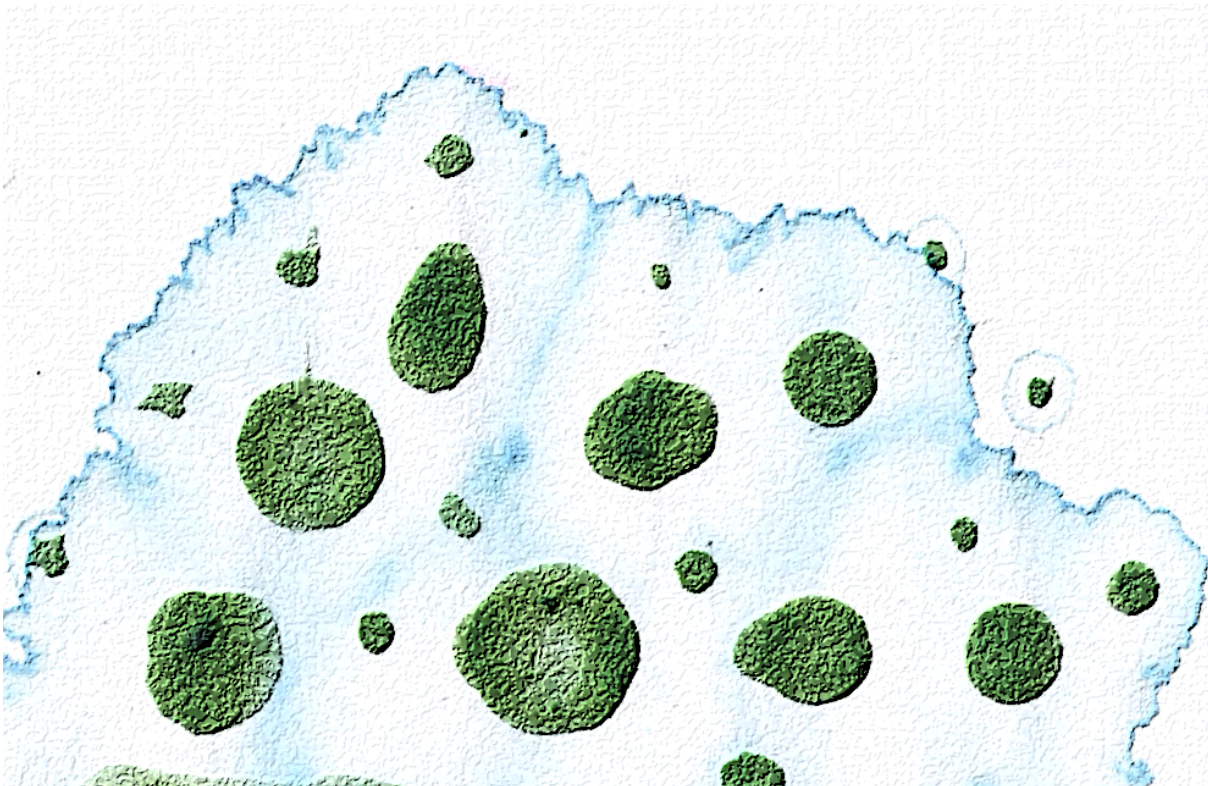
3.4 Conclusions

The removal of the primary treatment preceding a HRAP, which would simplify its maintenance, reduce costs and the footprint, did not significantly affect the wastewater treatment efficiency. Thus, it seems to be a dispensable step when urban wastewater treatment is the main objective. Although the HRAP without primary treatment received higher organic loading due to the absence of primary treatment, the production of microalgal biomass was not jeopardised. Bioenergy recovery through biogas production would be a good alternative for biomass valorisation. In particular, the co-digestion with primary sludge could improve the methane yield and kinetics of microalgae mono-digestion.

Chapter 4

Phycobiliproteins and biogas recovery from cyanobacteria

Treatment of centrate using cyanobacteria-dominated biomass and recovery of phycobiliproteins and biogas



Picture on previous page:
Phycobiliproteins extract released from cyanobacteria-dominated biomass cultivated in wastewater (*Chapter 4*).

Abstract

The aim of this study was to assess the recovery of natural pigments (phycobiliproteins) and bioenergy (biogas) from wastewater using microalgae. The biomass was grown in photobioreactors treating the secondary effluent from a wastewater treatment plant along with the anaerobic digestion centrate, with 3 different ratios (0, 15 and 25% v/v of centrate in secondary effluent). Removal efficiencies up to 52% of COD, 86% of NH_4^+-N and 100% of phosphorus were observed. The biomass composition was monitored over the experimental period in order to ensure stable cyanobacterial dominance in the mixed culture. Phycocyanin and phycoerythrin were extracted from harvested biomass, achieving maximum concentrations of 17 and 7.2 mg/g DW, respectively. The residual biomass from phycobiliproteins extraction was then used to produce biogas, with final methane yields ranging from 159 to 199 mL CH_4/g VS. The proposed process poses an example of resource recovery from wastewater and circular bioeconomy that deserves further research towards scaling-up.

This chapter has been redrafted after:

Arashiro, L.T., Ferrer, I., Pániker, C.C., Pinchetti, J.L.G., Rousseau, D.P.L., Van Hulle, S.W.H., Garfí, M., From wastewater to natural pigments and biogas: towards a circular bioeconomy. (*Submitted*)

4. Treatment of centrate using cyanobacteria-dominated biomass and recovery of phycobiliproteins and biogas

4.1 Introduction

During the last decades, the cultivation of microalgae and cyanobacteria in wastewater has been widely proposed as a sustainable alternative for biomass production and valorisation (e.g. natural pigments and biofertiliser production), while improving water quality (Acién Fernández et al., 2018; Bhattacharya et al., 2017). Among the functional components identified in cyanobacteria, natural pigments have received particular attention. The main photosynthetic pigments in cyanobacteria are chlorophylls, carotenoids and phycobilins. Phycobilins are tetrapyrrole prosthetic groups with linear discs constituted by phycobiliproteins, which act as auxiliary pigments exclusive to cyanobacteria, red algae and cryptomonads (Cuellar-Bermudez et al., 2015). Depending on their composition and content of chromophores, phycobiliproteins may be classified as phycocyanins (λ_{max} = 610-625 nm), phycoerythrins (λ_{max} = 490-570 nm), or allophycocyanins (λ_{max} = 650-660 nm) (Noreña-caro and Benton, 2018). Commercially, phycobiliproteins are high-value natural products with existing or potential biotechnological applications in nutraceuticals and pharmaceuticals, food and cosmetic industries as well as in biomedical research and clinical diagnostics (Luo et al., 2016; Manirafasha et al., 2016).

The production of phycobiliproteins generates residual biomass that can be used as biofertiliser or to recover bioenergy through biogas production (Gong and You, 2015; Ramos-Suárez et al., 2014). In this case, a typical mix of low volume high-value products (such as pigments) and high volume low-value products (such as bioenergy) is produced (Van Den Hende et al., 2016b). The high-value products provide economic feasibility while the low-value products can supply or minimize the energy demand of the system (Vulsteke et al., 2017; Yen et al., 2013). In this context, some studies have investigated the cultivation of microalgae in wastewater, in order to minimize costs of biomass production, followed by phycobiliproteins extraction from the biomass (Arashiro et al., 2020; Khatoon et al., 2018; Van Den Hende et al., 2016b; Wood et al., 2015).

Digestate from anaerobic digesters has become a major bottleneck in the development of the biogas industry, in which the solid phase is often used as agricultural biofertiliser, while the disposal of liquid phase (centrate) is still a great challenge (Xie et al., 2019). In this sense, previous researchers investigated the use of centrate diluted in synthetic medium, secondary/tertiary wastewater or seawater, in order to mitigate NH_4^+-N inhibition, lower the turbidity and enhance N/P ratio (Dulce Maria Arias et al., 2017; Ge et al., 2018; Praveen et al., 2018).

Previous studies have investigated the cultivation of cyanobacterial-dominated biomass in secondary wastewater and digestate, but to the authors' knowledge, the recovery of phycobiliproteins from this biomass, combined with a biogas production process, has not yet been reported. Thus, this study aimed to assess the recovery of pigments (phycobiliproteins) and bioenergy (biogas) while treating wastewater using cyanobacteria-dominated biomass. Following the study presented in **Chapter 3**, the secondary effluent of the HRAPs systems was used to dilute centrate (liquid part of digestate from microalgae digestion) in order to provide optimum nutrients concentrations (Ge et al., 2018; Xie et al., 2019). Moreover, this study explored the recovery of not only biogas, but also phycobiliproteins with the biomass cultivated. To this end, the following aspects were investigated: a) the potential of using wastewater (different dilution ratios of centrate in secondary effluent) to cultivate cyanobacteria-dominated biomass in photobioreactors (PBRs), b) the stability of biomass composition, monitoring the proportion of cyanobacteria, green microalgae and other microorganisms over time, and c) the potential biomass downstream processes for phycobiliproteins extraction followed by biogas production.

4.2 Materials and methods

4.2.1 Experimental set-up

The experimental set-up consisted of cylindrical photobioreactors made of polymethacrylate, with an inner diameter of 11 cm and a total volume of 3 L (working volume of 2 L). Illumination was provided by cool-white fluorescent lamps (Biolux,

Osram, Germany) with a light:dark cycle of 12:12 h, with an average intensity of 150 μmol/m^2s. A water jacket around the reactors kept the temperature at 22 ± 2 °C. The photobioreactors were continuously mixed with magnetic stirrers (AGE, Velp, Italy) at 200 rpm and aerated with 2 L air/min. pH was continuously monitored with a pH sensor (HI1001, HANNA, USA) and maintained at 7.5 with a pH controller (HI 8711, HANNA, USA) by the automated addition of 0.1M HCl or 0.1M NaOH when needed.

4.2.2 Culture conditions

Initially, one photobioreactor was inoculated with dry colonies collected from soil crusts, mostly formed by cyanobacteria (approximately 70%, with *Nostoc* sp., *Phormidium* sp. and *Geitlerinema* sp. being the most abundant species). This microbial consortium was cultivated in BG-11 medium (CCAP, UK) for 20 days, before the biomass was used to inoculate the three experimental photobioreactors.

The photobioreactors were fed with different mixtures of secondary effluent obtained from a system of high rate algal ponds (HRAP) treating urban wastewater (for details refer to **Chapter 3** (Arashiro et al. 2019), and centrate (liquid part of digestate) from a microalgae anaerobic digestion unit. The effluent from the HRAPs was filtered through 1.0 μm glass microfiber filters (GF6 Whatman, GE, Germany) to avoid any possible grazer contamination. The digestate was obtained from the anaerobic digester (working volume 400 L, HRT 20 days) of a demo scale plant using photobioreactors to treat agricultural runoff (Uggetti et al., 2018). The digestate was centrifuged (4200 rpm, 10 min) and the supernatant (centrate) was mixed with the secondary effluent from the HRAPs to feed the photobioreactors. The medium of each PBR was prepared with the following dilutions of centrate in the secondary effluent at volume proportions: PBR-0% with only secondary effluent, PBR-15% with centrate (15% volume) diluted in secondary effluent and PBR-25% with centrate (25% volume) diluted in secondary effluent. Photobioreactors were operated at a hydraulic retention time (HRT) of 10 days. The experimental set-up is shown in Figure **4-1**.

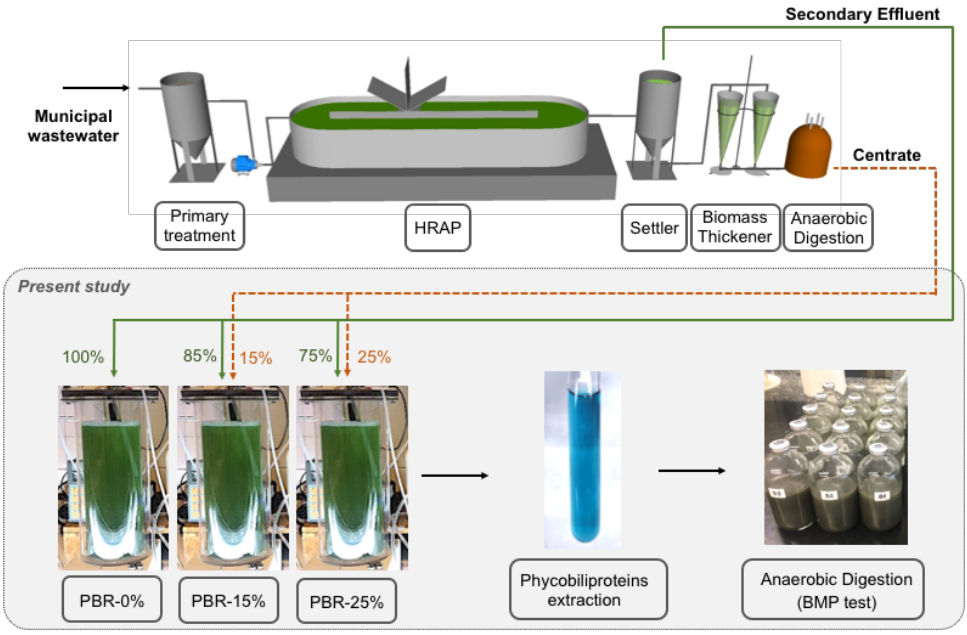


Figure 4-1. Scheme of the microalgae-based wastewater treatment pilot plant located at UPC, Barcelona, Spain (previously described in **Chapter 3**) and the experimental set-up described in this study.

4.2.3 Biomass composition

Samples from the three photobioreactors were observed under bright field (BA310, Motic, China) and fluorescent microscopy (Eclipse E200, Nikon, Japan) weekly to characterise the communities and record their relative abundance. Biomass flocs were dissociated by homogenizing the sample for 1 minute at 10x1000 rpm (Polytron PT 2500 E Homogenizer, Kinematica, USA). Cell counts were performed fortnightly, with 25 µL of homogenised sample, at 40x and alternating bright field and fluorescence microscopy with an excitation filter (510-560 nm), emission filter (590 nm) and dichroic beam splitter (575 nm) following the microscopic area counting protocol proposed by Arias et al. (2019) and Guillard and Sieracki (2005). The identification of microbial genera was based both on conventional taxonomic books (Komárek et al., 2014; Streble and Krauter,

1987), and three online databases: NCBI Taxonomy Browser, AlgaeBase, and the CyanoDB.cz.

4.2.4 Phycobiliproteins extraction

Phycobiliproteins (phycocyanin and phycoerythrin) content from the biomass of each photobioreactor was quantified. Aliquots taken daily were centrifuged (4200 rpm, 10 min) and biomass was rinsed twice with distilled water and frozen (-21°C) until further use. Microbial cells disruption was done by repeating 3 freeze-thaw cycles (-21°C to 4°C in darkness). The biomass paste was then used for determining the dry weight (DW) according to Standard Methods (APHA-AWWA-WEF, 2012) and phycobiliproteins content. Extraction of these compounds was done by adding 250 mg of the biomass paste into 15 mL covered vessels with sodium phosphate buffer at pH 7 as the solvent at a proportion 1:10 (w:w, biomass:solvent). Mixtures were then submitted to 5 ultrasonic cycles at 20KHz of 1 min each (Qsonica S-4000, USA) in ice bath to avoid overheating. The resulting slurry was centrifuged at 10,000 rpm for 15 min at 4°C (LegendMicro21, ThermoScientific, USA) to remove cell debris. The precipitate was stored for further use and the supernatant was collected and measured in a spectrophotometer at 280, 562, 615 and 652 nm, to quantify the amount of phycocyanin and phycoerythrin according to Bennett and Bogobad (1973). Purity was determined as the absorbance ratios of A_{620}/A_{280} for phycocyanin and A_{565}/A_{280} for phycoerythrin (Cuellar-Bermudez et al., 2015).

4.2.5 Biochemical methane potential test

Biochemical methane potential (BMP) tests were carried out to assess the potential to recover biogas after the phycobiliproteins extraction process. BMP tests were performed in serum bottles of 160 mL filled up to 50 mL of liquid volume with certain amounts of inoculum and substrate, corresponding to 5 g volatile solids (VS) substrate/L and a substrate to inoculum ratio (S/I) of 0.5 g VS substrate/g VS inoculum. The

substrates evaluated were the biomass grown in the three photobioreactors, before and after phycobiliproteins extraction. Each trial was performed in triplicate.

The bottles were flushed with helium gas, sealed with butyl rubber stoppers and placed in a platform shaker incubator (OPAQ, Ovan, Spain) at 35°C and 80 rpm. Pressure in each bottle was periodically measured with a digital manometer (GMH 3151 Greisinger, Germany) and biogas production was calculated by subtracting the blank (inoculum only) production. Measurements were done until the daily methane production was less than 1% of the total accumulated methane production in all bottles. Methane content in biogas was analysed by gas chromatography (Trace GC Thermo Finnigan, USA), following the procedure described by Solé-Bundó et al. (2018).

The calculation of anaerobic biodegradability of each substrate was based on the net methane production (mL CH_4) and the theoretical methane yield under standard conditions, 350 mL CH_4 for each gram of degraded COD (Chernicharo, 2007).

4.2.6 *Analytical methods*

The wastewater treatment efficiency and biomass production in the photobioreactors was evaluated by monitoring the following parameters. Total suspended solids (TSS), volatile suspended solids (VSS), chlorophyll-a, total and soluble chemical oxygen demand (COD and sCOD) were measured according to Standard Methods (APHA-AWWA-WEF, 2012). NH_4^+-N was measured following Solórzano (1969), and NO_2^--N, NO_3^--N and PO_4^{3-}-P through isocratic mode with carbonate-based eluents at a temperature of 30°C and a flow of 1 mL/min (ICS-1000, Dionex Corporation, USA) (limits of detection (LOD) were 0.9 mg/L of NO_2^--N, 1.12 of NO_3^--N, and 0.8 mg/L of PO_4^{3-}-P). Total carbon (TC) and total nitrogen (TN) were analyzed with a multi N/C 2100S, Analytik Jena, Germany. For the BMP test, total solids (TS) and volatile solids (VS) were measured according to Standard Methods (APHA-AWWA-WEF, 2012). All the analyses were done in triplicate and results are given as average values.

4.2.7 Statistical and model-based analyses

Experimental data regarding wastewater treatment efficiency, phycobiliproteins content and biochemical methane potential were statistically assessed via multi-factor analysis of variance (ANOVA) ($\alpha = 0.05$). The Tukey test ($\alpha = 0.05$) was used as a post-hoc test using Minitab 18 (Minitab Inc., PA, USA).

For the evaluation of kinetic parameters of BMP tests, experimental data were adjusted to a first-order kinetic model by the least square method (Eq. 3-9), as previously described in **Chapter 3** (Section 3.2.7). Similarly, the error variance (s^2) of modelled methane production from Eq. 3-9 based on the actual methane production was estimated by the following equation (Eq. 3-10).

4.3 Results and discussion

4.3.1 Wastewater treatment and biomass growth

Average concentrations of water quality parameters in the influent and mixed liquor of each photobioreactor are shown in Table 4-1. Variations in concentrations of influent and effluent of each photobioreactor are illustrated in Figure 4-2.

Average concentration of NH_4^+-N in the secondary effluent throughout the experimental period was very low (Table 4-1), thus PBR-0% reached a high removal efficiency of 81 ± 15%. For PBR-15% and PBR-25%, influent NH_4^+-N was much higher due to centrate addition (Table 4-1). Nevertheless, high average removal efficiencies were also reached in PBR-15% and PBR 25% (86 and 69%, respectively) upon steady state (approximately 22 days for PBR-15% and 36 days for PBR-25%) (Figure 4-2). Indeed, there was no significant difference between removal efficiencies in all photobioreactors (Table 4-2). Concentrations of NO_3^--N and NO_2^--N were also very low in the secondary effluent, so PBR-0% could reach average removal efficiency of 44% and 68%, respectively. However, for PBR-15% and PBR-25%, production of NO_2^--N during the first 30 days and accumulation of NO_3^--N concentrations were observed. Accumulation in PBR-25% was significantly higher than in PBR-0% and PBR-15%, due

to the higher NH_4^+-N concentrations in the influent, by 76-fold compared to PBR-0% and 2-fold compared to PBR-15%. These variations of NO_3^--N and NO_2^--N concentrations in all reactors suggest nitrification activity in these systems. Arias et al. (2017) also reported nitrification process in a photobioreactor treating secondary effluent and digestate.

Table 4-1. Average concentrations of the main water quality parameters measured in the influent and effluent of PBR-0% (only secondary effluent), PBR-15% (15% of centrate in secondary effluent) and PBR-25% (25% of centrate in secondary effluent).

	PBR-0%		**PBR-15%**		**PBR-25%**	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
TSS* (mg/L)	$< 10^a$	140 ± 98^b	17 ± 7^a	214 ± 108^b	25 ± 13^a	175 ± 60^b
VSS* (mg/L)	$< 10^a$	132 ± 87^b	16 ± 6^a	197 ± 95^b	23 ± 11^a	164 ± 55^b
COD* (mg/L)	--	299 ± 168	171 ± 48^a	374 ± 154^b	211 ± 49^a	313 ± 73^b
sCOD (mg/L)	59 ± 16	48 ± 19	101 ± 22^a	79 ± 34^b	148 ± 42^a	97 ± 52^b
NH_4^+-N (mg/L)	0.7 ± 0.4^a	0.11 ± 0.09^b	24.8 ± 3.0^a	3.2 ± 3.6^b	49.0 ± 5.8^a	14.4 ± 9.3^b
NO_3^--N (mg/L)	6.7 ± 1.5^a	3.5 ± 2.1^b	5.4 ± 1.5^a	12 ± 7^b	4.7 ± 1.4	14.8 ± 14.5
NO_2^-N (mg/L)	0.2 ± 0.7	0.3 ± 1.4	0.2 ± 0.8	2.9 ± 4.7	0.2 ± 0.7^a	5.7 ± 6.8^b
TP* (mg/L)	1.2 ± 0.8	2.8 ± 3.3	1.7 ± 0.8	3.8 ± 3.7	2.6 ± 1.6	3.4 ± 2.9
PO_4^{3-}-P (mg/L)	0.4 ± 1.2	0.1 ± 0.3	0.6 ± 1.5	< LOD	0.62 ± 1.49	< LOD

[a,b]: Letters indicate a significant difference (α=0.05) between influent and effluent concentrations after Tukey test.
* Effluent concentrations measured in the mixed liquor.
LOD: limit of detection.

Regarding total phosphorus, all photobioreactors showed very high removal efficiencies (Table 4-2) and its absence in the effluent suggests that this nutrient might have been a limiting factor for the growth of microorganisms in all reactors. COD removal efficiencies ranged from 30 to 52% and photobioreactors did not perform significantly different (Table 4-2). In addition, during the experimental period, COD concentrations in photobioreactors effluents were below the discharge limit of 125 mg O_2/L (Directive 98/15/EC, 1988). Other studies treating centrate have also reported high removal efficiencies of nutrients and COD (Ge et al., 2018; Xie et al., 2019).

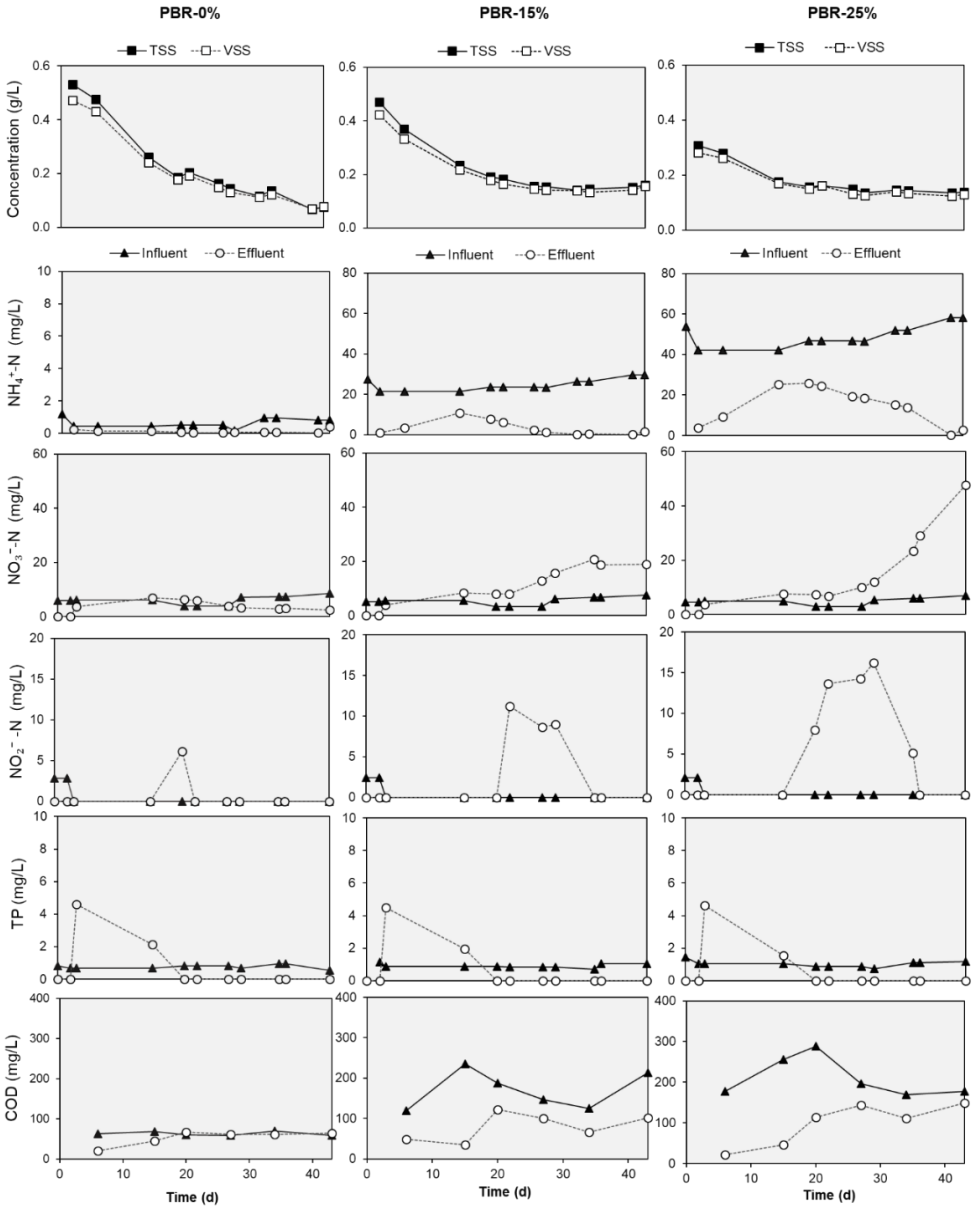


Figure 4-2. Average total suspended solids (TSS) (■) and volatile suspended solids (VSS) (□), as well as influent (▲) and effluent (○) concentrations of NH_4^+-N, NO_3^--N, NO_2^--N, total phosphorus (TP) and chemical oxygen demand (COD) measured in PBR-0% (only secondary effluent), PBR-15% (15% of centrate in secondary effluent) and PBR-25% (25% of centrate in secondary effluent).

Table 4-2. Average removal efficiencies and rates of the main wastewater parameters observed in PBR-0% (only secondary effluent), PBR-15% (15% of centrate in secondary effluent) and PBR-25% (25% of centrate in secondary effluent).

	Removal efficiencies (%)			**Removal rates (mg/Ld)**		
	PBR-0%	**PBR-15%**	**PBR-25%**	**PBR-0%**	**PBR-15%**	**PBR-25%**
NH_4^+-N	81 ± 15[a]	86 ± 16[a]	69 ± 21[b]	0.62 ± 0.41	21.3 ± 5.8	34.1 ± 13.2
NO_3^--N	44 ± 48	-115 ± 112	-185 ± 204	3.31 ± 2.98	-6.17 ± 6.23	-10.0 ± 13.6
NO_2^--N	68 ± 140[a]	-189 ± 470[a,b]	-472 ± 678[b]	0.16 ± 0.66	-2.64 ± 4.92	-5.51 ± 7.01
TP[c]	97 ± 14	100 ± 0	100 ± 0	0.80 ± 1.47	0.23 ± 1.49	0.36 ± 1.48
COD[*]	30 ± 22	52 ± 20	51 ± 30	12 ± 21	92 ± 58	114 ± 76

[a,b] Letters indicate a significant difference (α=0.05) of removal efficiencies between PBRs after Tukey test.
[*] Effluent concentrations used to calculate TP and COD removal efficiencies were based on the supernatant after settling mixed liquor (soluble concentrations).

Variations of TSS and VSS in the photobioreactors showed a decrease during the first 20 days before reaching the steady state (Figure 4-2). The effluent of PBR-0%, PBR-15% and PBR-25% had very low concentrations of inorganic soluble phosphate (average of 0.7 ± 1.5, 0.64 ± 1.48 and 0.62 ± 1.49 mg/L, respectively) and inorganic carbon (average of 3.2 ± 0.8, 3.1 ± 4.0 and 2.9 ± 2.0 mg/L, respectively). Despite the constant air supply in the photobioreactors, inorganic carbon reached limiting levels, which supported the assumption of nitrification process in the systems (de Godos et al., 2014). Ge et al. (2018) carried out similar experiments treating centrate diluted in secondary wastewater reporting also a relatively low loading rates applied, which may have consequently limited the biomass productivity due to low nutrient availability.

In general, NH_4^+ is the preferential form of nitrogen uptake for most microalgae and cyanobacteria species, followed by nitrate (Ruiz-Marin et al., 2010). This is in accordance with the results obtained in this study, in which NH_4^+-N removal was very high (up to 86%), due to biomass uptake and nitrification processes. Nitrate accumulation was also observed. Based on that, it is assumed that microalgal growth was limited not only by the low availability of phosphorus and inorganic carbon, as mentioned previously, but also by competition with bacterial processes. Praveen et al. (2018) carried out a study in which a microalgal-bacterial consortium was cultivated in synthetic

wastewater mixed with anaerobic digestate, reaching 99.8% decrease in NH_4^+-N concentrations with high accumulation of NO_3^--N, also indicating presence of nitrifying bacteria. Moreover, the limitation in inorganic carbon has been related to nitrification processes and highlighted the fact that more unfavourable conditions occur in microalgae-based processes since both photosynthetic autotrophs and nitrifying bacteria compete for the same inorganic carbon sources (de Godos et al., 2014). Likewise, accumulation of nitrate and limited carbon source also indicate that denitrification did not take place in the systems, which would be achieved where dissolved oxygen concentration gradients resulted in anoxic zones within algal-bacterial biofilms (de Godos et al., 2014). Nevertheless, considering cases in which carbon sources from other waste streams (e.g. flue gas) could be combined with a mixture of centrate and secondary effluent, high nutrients removal efficiencies could be achieved and possibly implemented at full-scale (Pruvost et al., 2016).

4.3.2 Biomass composition

The microorganisms observed in each sample were grouped within three main categories: cyanobacteria, microalgae and "others". The latter included any microorganism which did not classify as either of the other two categories, such as diatoms and grazers (rotifers, amoebas, ciliates, and flagellates) (Day et al., 2017). Cyanobacteria remained the dominant clade in the three photobioreactors throughout the entire experimental period for PBR-0%, PBR-15% and PBR-25%, ranging from a minimum of 55, 65 and 55% to a maximum of 80, 72 and 73% respectively (Figure 4-3).

These results support the studies performed by Arias et al. (2017, 2019), where cyanobacterial co-cultures were used to treat secondary effluents, highlighting the relevance of cyanobacteria's dual-role of treating wastewater and producing valuable products (Fagundes et al., 2019). Average abundance of cyanobacteria in the biomass grown in PBR-0% (60 ± 6%) was significantly lower than in PBR-15% (68 ± 4%) and PBR-25% (65 ± 5%). This indicates that the addition of centrate in secondary effluent provided better conditions for cyanobacteria.

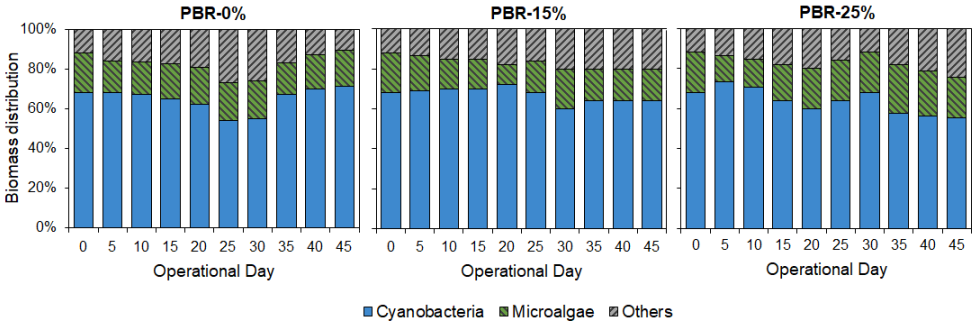


Figure 4-3. Evolution of the biomass composition in PBR-0% (only secondary effluent), PBR-15% (15% of centrate in secondary effluent) and PBR-25% (25% of centrate in secondary effluent).

Similarly to PBR-0%, the biomass from PBR-15% and PBR-25% formed flocs held together by filamentous cyanobacteria with a distinct deep blue-green colour. The taxonomical composition of the biomass showed the *Nostocales*, *Chroococales,* and *Oscillatoriales* orders as the main cyanobacterial fraction. Within these, the following 7 genera were distinguished: *Nostoc*, *Calothrix*, *Aphanocapsa, Gloeocapsa, Chroococcus, Geitlerinema,* and *Phormidium* (Figure 4-4). All genera grew and remained in equal proportions throughout the experimental period.

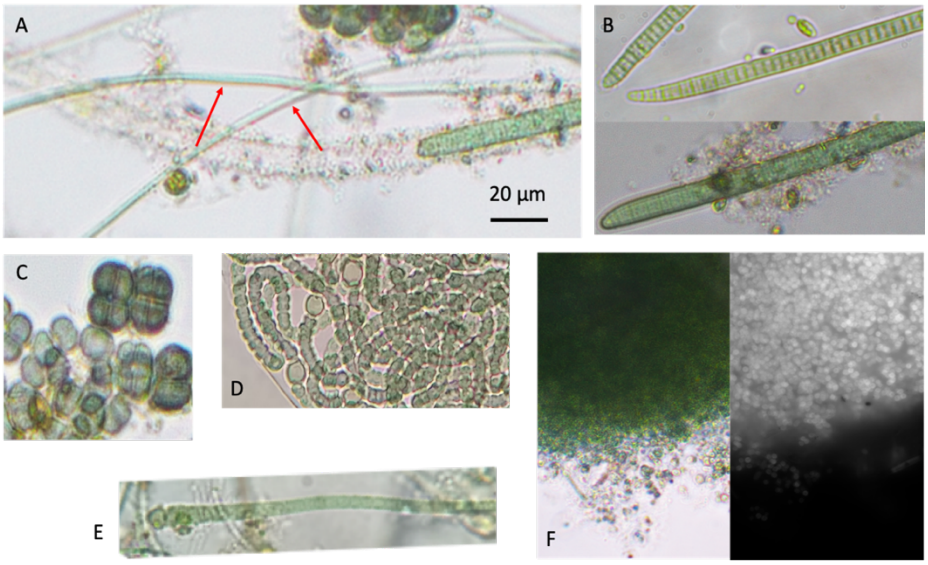


Figure 4-4. Images of biomass grown in the three photobioreactors, taken throughout the entire experimental period using a BA310 microscope (Motic, China) and an Eclipse E200 (Nikon, Japan). A: *Geitlerinema* sp., B: *Phormidium* sp., C: *Chroococcus* sp., D: *Nostoc* sp., E: *Calothrix* sp., F: *Aphanocapsa* sp. under light and fluorescence (scale applies to all images).

4.3.3 Phycobiliproteins extraction

The biomass used for the quantification of phycobiliproteins was harvested and accumulated during the experimental period. Cyanobacterial phycocyanin and phycoerythrin were detected in all photobioreactors. This was confirmed by analysing the absorbance peaks, which were observed at 618 nm and at 565 nm, which are typical for cyanobacterial phycocyanin and phycoerythrin, respectively (Chakdar and Pabbi, 2017). The average phycocyanin concentrations measured in biomass grown in PBR-0%, PBR-15% and PBR-25% were 13.5 ± 2.0, 16.4 ± 1.3 and 17.4 ± 1.2 mg phycocyanin/g DW (Figure 4-5a). Although PBR-0% had a lower content of phycocyanin than PBR-15% and PBR-25%, these concentrations were not significantly different (p-value = 0.227). The higher concentrations in PBR-15% and PBR-25% are in accordance with the abundance of cyanobacteria, which was significantly higher in these PBRs compared to PBR-0% (Section 4.3.2). Regarding phycoerythrin, the average

concentrations measured in biomass grown in PBR-0%, PBR-15% and PBR-25% were 5.5 ± 0.7, 6.7 ± 0.4 and 7.2 ± 0.8 mg phycocyanin/g DW (Figure 4-5a). Similarly to phycocyanin content, PBR-0% had the lowest concentration of phycoerythrin and PBR-25% the highest, but the concentrations were not significantly different (p-value = 0.348).

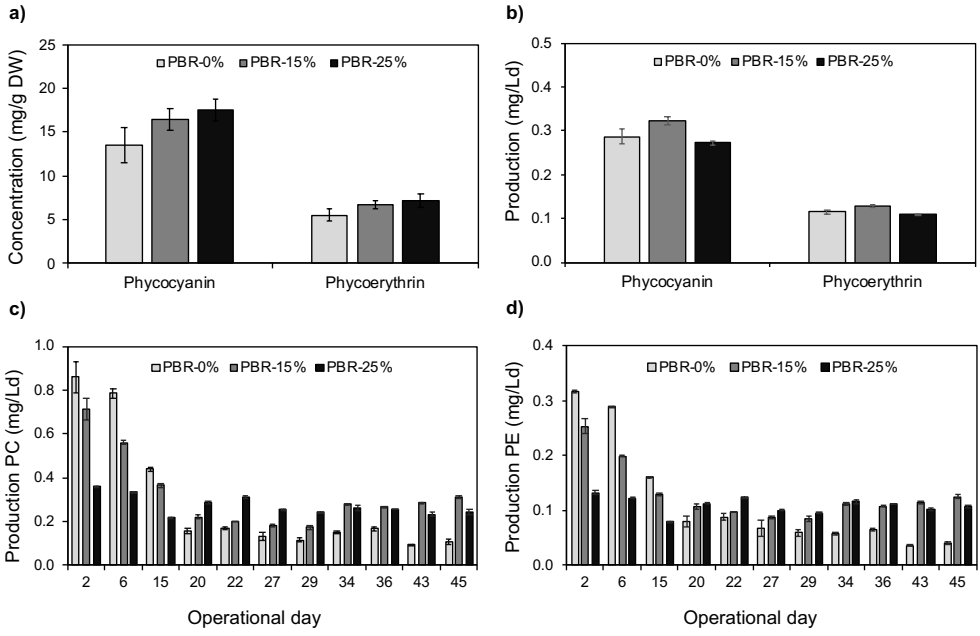


Figure 4-5. Overall average phycocyanin and phycoerythrin a) concentrations (mg/g DW) and b) production rates (mg/Ld); production rates of c) phycocyanin and d) phycoerythrin extracted from biomass grown in PBR-0% (only secondary effluent), PBR-15% (15% of centrate in secondary effluent) and PBR-25% (25% of centrate in secondary effluent).

Considering the biomass concentration in each photobioreactor, the overall average production rates of phycobiliproteins were calculated (Figure 4-5c) and the progression of the production rates of phycocyanin (Figure 4-5c) and phycoerythrin (Figure 4-5d) over time in each photobioreactor were estimated. Based on the overall average, no significant difference was found among the three photobioreactors (p-value=0.816 for phycocyanin and 0.765 for phycoerythrin). However, the biomass concentrations decreased in all photobioreactors before reaching steady state (Section 4.3.1). In this

sense, considering the steady state, the production rates of phycocyanin and phycoerythrin in PBR-0% were significantly lower than in PBR-15% and PBR-25% (p-value = $2.7x10^{-6}$ for phycocyanin and $1.5x10^{-6}$ for phycoerythrin). This is in accordance with the limiting concentrations of nutrients in all reactors, especially in PBR-0%, mentioned previously (Section 4.3.1).

Phycobiliproteins must be purified in order to meet the specific standards of diverse applications. Purity is usually determined as the absorbance ratios of A_{620}/A_{280} for phycocyanin and A_{565}/A_{280} for phycoerythrin, which define the relationship between the presence of the specific phycobiliprotein and other contaminating proteins (Cuellar-Bermudez et al., 2015). A purity ratio ≥ 0.7 refers to food grade pigment, while reagent and analytical grade correspond to ≥ 3.9 and ≥ 4.0, respectively (Borowitzka, 2013). In this study, average purity ratios of phycocyanin extracted from biomass grown in PBR-0%, PBR-15% and PBR-25% were 2.3 ± 0.2, 2.2 ± 0.1 and 2.6 ± 0.2, which were not significantly different (p-value = 0.285). Likewise, average purity ratios of phycoerythrin extracted from biomass grown in PBR-0%, PBR-15% and PBR-25% were 1.8 ± 0.1, 1.7 ± 0.1 and 1.9 ± 0.1, also not significantly different (p-value = 0.372). However, although purity ratios of phycocyanin are higher than the food grade standard, the fact that this biomass was cultivated in wastewater might hinder the application of the extracted pigment for this purpose. Therefore, the most suitable option would be to further purify the phycobiliproteins in order to reach reactive or analytical grade, increasing the market value of these bioproducts.

To date, very few studies assessing the recovery of phycobiliproteins from biomass grown in wastewaters were reported. Wood et al. (2015) demonstrated the feasibility of cultivating cyanobacteria in oil and natural gas extraction wastewater with production of phycocyanin with a maximum yield of 16.9 ± 3.4 mg/g DW and a maximum crude extract purity of 0.23 ± 0.03. The phycocyanin concentration was very similar, but the purity ratio found in the present study was much higher, most probably due to the different extraction techniques used. Khatoon et al. (2018) cultivated cyanobacteria in aquaculture wastewater and reported a much higher value of maximum phycobiliproteins (237 mg/g DW), yet with lower purity ratio (1.14) than the present study. The

discrepancies might be related to the different species or the calculation method used in that study. Van Den Hende et al. (2016) investigated the potential to cultivate cyanobacteria-dominated biomass in food-industry effluent and flue gas. They reported extraction of 61.1 mg phycocyanin/g VS with 0.43 purity ratio of crude extract, and 30.1 mg phycoerythrin/g VS with 0.36 purity ratio. In general, when comparing with other studies, the concentrations of phycobiliproteins found in the present study were lower, but purity ratios of crude extracts were higher.

4.3.4 Biochemical methane potential

The BMP test was carried out in order to investigate the potential biogas recovery from biomass harvested in each photobioreactor, with extraction (extracted) and without the extraction (unextracted) of phycobiliproteins. The methane yield of each trial over an incubation period of 43 days is shown in Figure 4-6. The methane content in biogas was similar in all cases, around 72%.

The lowest final methane yield (152.5 ± 2.1 mL CH_4/g VS) was obtained from extracted biomass of PBR-25%, and the highest final methane yield (209.1 ± 1.5 mL CH_4/g VS) was from unextracted biomass of PBR-0%. Methane production of extracted biomass was mainly observed during the initial stage of the incubation (especially the first 6 days) and remained constant after that. For unextracted biomass, methane production was rapidly increased until day 15 and very little after that. Overall, there was no significant difference between the methane yield (both initial and final) of all substrates (Table 4-3) and the average methane yields obtained were within the range reported for microalgae BMP tests (Jankowska et al., 2017).

However, the kinetics of extracted biomass were significantly faster (p-value = 0.002) than of unextracted biomass from all photobioreactors. As expected, this performance could be explained by the fact that extracted biomass contained more readily biodegradable material (which was transformed into biogas) than unextracted biomass, since extracted biomass was submitted to cell disruption. This is a matter of concern, since faster kinetics would mean lower HRT and reactor volume, hence lower costs, upon scale-up. Indeed, PBR-15% and PBR-25% Extracted showed the highest

accumulated methane yield until the 6th day, reaching 90% and 99% of the final methane yield, in a very low HRT (less than 6 days) for an anaerobic digestion process.

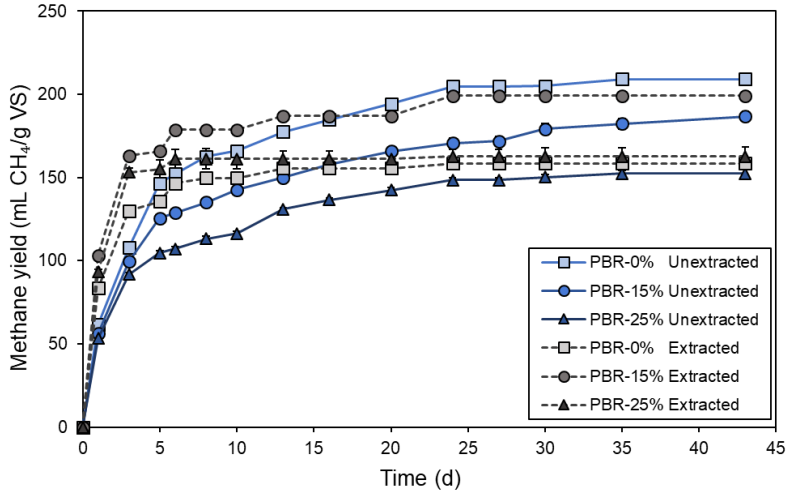


Figure 4-6. Cumulative methane yields of biomass harvested from PBR-0% (only secondary effluent), PBR-15% (15% of centrate in secondary effluent) and PBR-25% (25% of centrate in secondary effluent), unextracted and after extraction (extracted) of phycobiliproteins.

Comparing the final methane yield of extracted and unextracted biomass, for PBR-0% unextracted biomass showed a 32% higher methane yield than extracted biomass. This might be related to the abundance of cyanobacteria compared to microalgae in PBR-0%, which was lower than in PBR-15% and PBR-25% (Section 4.3.2). The ultrasonic treatment was probably more effective in biomass of PBR-15% and PBR-25% than in PBR-0%, since cyanobacterial cell walls are easier to disrupt than those of eukaryotic microalgae. Indeed, final methane yields from extracted biomass from PBR-15% and PBR-25% were 6.7% and 6.6% higher than unextracted biomass. This means that by combining the extraction of pigments and the production of biogas from residual biomass, we would not only obtain high-value compounds, but also more energy, as compared to the sole production of biogas. Economically, it has already been shown that the production of bioproducts from microalgae grown in wastewater is more profitable than the generation of biogas (Arashiro et al., 2018).

Table 4-3. Summary of the methane yield (initial after 6 days and final after 43 days of incubation), anaerobic biodegradability (mean values ± standard deviation; n=3) and first-order kinetics constant (k) obtained from Eq. 3-9 (error variance (s^2) from Eq. 3-10 is represented in brackets).

Substrate	**PBR-0%**		**PBR-15%**		**PBR-25%**	
	Unextracted	Extracted	Unextracted	Extracted	Unextracted	Extracted
Initial methane yield (mL CH_4/g VS)	152.2 ± 0.5[a]	146.6 ± 1.2[a]	128.8 ± 1.4[a]	178.8 ± 1.0[a]	107.2 ± 1.4[a]	161.0 ± 5.8[a]
Final methane yield (mL CH_4/g VS)	209.1 ± 1.5[a]	158.6 ± 3.5[a]	186.7 ± 3.0[a]	199.2 ± 0.2[a]	152.5 ± 2.1[a]	162.5 ± 5.7[a]
Methane content (%)	71.8 ± 0.1[a]	71.8 ± 0.2[a]	72.4 ± 2.7[a]	72.5 ± 0.4[a]	72.4 ± 1.6[a]	72.4 ± 1.2[a]
Anaerobic Biodegradability (%)	61.8 ± 1.6	82.1 ± 3.5	78.9 ± 3.0	95.2 ± 0.2	58.6 ± 2.1	87.1 ± 5.7
First-order kinetics constant k (day^{-1})	0.239 (102)[a]	0.661 (32)[b]	0.243 (121)[a]	0.683 (89)[b]	0.254 (109)[a]	0.877 (3)[b]
Correlation R_{model} (%)	98.8	99.2	98.1	98.5	97.6	99.9

[a,b] : Letters indicate a significant difference between trials (α = 0.05) after Tukey test.

To sum up, recovery of bioenergy as methane with residual biomass after extraction of high-value products seems to be a very promising alternative to minimize the energy demand in a microalgae cultivation system. Furthermore, the extraction of bioactive compounds prior to anaerobic fermentation can be considered as a pre-treatment of microalgal and cyanobacterial biomass in order to increase the anaerobic biodegradability (Bessette et al., 2020; Mudimu et al., 2014).

Although further improvements are needed in order to optimise processes involved (e.g. cultivation, extraction techniques and bioenergy recovery), the concept proposed in this study could potentially be applied to promote wastewater treatment while recovering high-value bioproducts from fresh biomass, and bioenergy from residual (extracted) biomass. By using wastewaters from different sources as cultivation medium for developing 'low-value-high-volume' product and 'high-value-low-volume' product, then production costs can be minimized while simultaneously remediating the wastewater (Ge et al., 2018; Vuppaladadiyam et al., 2018).

4.4 Conclusions

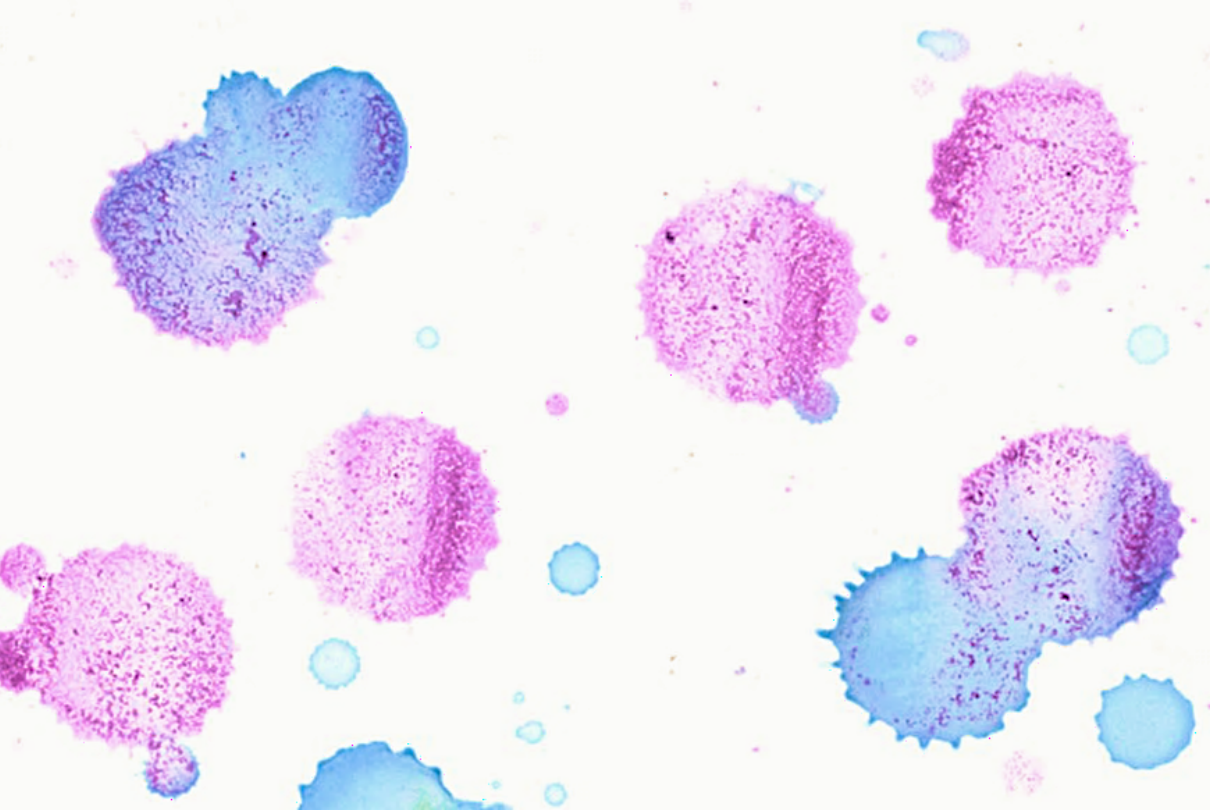
This study assessed the cultivation of microalgae and cyanobacteria in wastewater to recover high-value products and bioenergy from residual biomass. The cyanobacteria-dominated mixed culture grown in secondary wastewater and centrate achieved high NH_4^+-N, TP and COD removal efficiencies of up to 86, 100 and 52%, respectively.

Phycocyanin and phycoerythrin were extracted from harvested biomass reaching concentrations up to 17 and 7.2 mg/g DW, respectively. Biogas recovered ranged from 153 to 209 mL CH_4/g VS for unextracted biomass and from 159 to 199 mL CH_4/g VS for extracted biomass. Results of cyanobacteria-dominated biomass in this study were similar to what was reported in **Chapter 3**, in which unextracted and untreated green microalgae had an average methane yield of 207 mL CH_4/g VS. Overall, the use of wastewater with high nutrients but low solids concentrations was shown to be appropriate to produce high-value bioproducts and recover bioenergy, while reducing biomass production costs.

Chapter 5

Phycobiliproteins recovery from unialgal cultivations

Treatment of industrial wastewater using unialgal cultivations and recovery of phycobiliproteins



Picture on previous page:
Phycobiliproteins extracted from biomass cultivated in industrial wastewater (*Chapter 5*).

Abstract

The aim of this study was to investigate the cultivation of *Nostoc* sp., *Arthrospira platensis* and *Porphyridium purpureum* in industrial wastewater to produce phycobiliproteins. Initially, light intensity and growth medium composition were optimised, indicating that light conditions influenced the phycobiliproteins production more than the medium composition. Conditions were then selected, according to biomass growth, nutrients removal and phycobiliproteins production, to cultivate these microalgae in food-industry wastewater. The three species could efficiently remove up to 98%, 94% and 100% of COD, inorganic nitrogen and PO_4^{3-}-P, respectively. Phycocyanin, allophycocyanin and phycoerythrin were successfully extracted from the biomass reaching concentrations up to 103, 57 and 30 mg/g dry weight, respectively. Results highlight the potential use of microalgae for industrial wastewater treatment and related high-value phycobiliproteins recovery.

This chapter has been redrafted after:

Arashiro, L.T., Boto-Ordóñez, M., Van Hulle, S.W.H., Ferrer, I., Garfí, M., Rousseau, D.P.L., 2020. Natural pigments from microalgae grown in industrial wastewater. *Bioresource Technology* 303, 122894.

5. Treatment of industrial wastewater using unialgal cultivations and recovery of phycobiliproteins

5.1 Introduction

Microalgae are known to have a great capacity to efficiently utilize nutrients from wastewaters, since their cultivation requires high amounts of nitrogen and phosphorus. Besides enabling efficient wastewater treatment, microalgae biomass is a potential source of valuable chemicals and other products, attracting wide interest lately (Vuppaladadiyam et al., 2018). The phycobiliproteins (PBPs) are among those chemicals and are exclusive to the cyanobacteria, red algae, and the Cryptophyta and Glaucophyta (Borowitzka, 2013). These molecules are auxiliary pigments, water soluble and highly fluorescent proteins with linear prosthetic groups (bilins) that are linked to specific cysteine residues. Depending on their composition and content of chromophores, PBPs may be classified as phycocyanins (PC, λ_{max} = 610-625 nm), phycoerythrins (PE, λ_{max} = 490-570 nm), and allophycocyanins (APC, λ_{max} = 650-660 nm) (Noreña-caro and Benton, 2018).

The main commercial producers of phycobiliproteins are the cyanobacterium *Arthrospira* and the rhodophyte *Porphyridium* (Spolaore et al., 2006). Furthermore, the cyanobacterium *Nostoc* has also been recently put forward as a potential source of phycobiliproteins (Johnson et al., 2014). Besides playing an important role in the pigmentation metabolism of microalgae, phycobiliproteins also exhibit some useful biological functions, such as antioxidative, anticarcinogenic, anti-inflammatory, antiangiogenic, and neuro and hepatoprotective (Cuellar-Bermudez et al., 2015). Phycobiliproteins have high commercial value as natural colourants in the nutraceutical, cosmetic, and pharmaceutical industries, as well as applications in clinical research and molecular biology (Chiong et al., 2016) and as natural dyes in the textile industries (Okolie et al., 2019). Recent studies have shown that extracts of red pigment from the macroalgae *Gracilaria vermiculophylla* and the blue pigment from the *Arthrospira platensis* showed even distribution on the cotton and wool fabrics, with results

representing the viability and the quality of naturally dyed textiles (Ferrándiz et al., 2016; Moldovan et al., 2017).

Microalgae cultivation for chemical production using standard culture media can account for high costs. In order to improve the economic feasibility, these costs could be reduced by changing culture media concentrations, for instance through dilution (Delrue et al., 2017), or by using alternative components, including wastewater (Arashiro et al., 2019; Kumar et al., 2020; Van Den Hende et al., 2016b). Several studies have proposed alternatives to reduce costs for *Arthrospira* production, such as the use of fertilisers or seawater with and without enrichment of $NaHCO_3$ and $NaNO_3$ (Gami et al., 2011), swine wastewater (Yilmaz and Sezgin, 2014) and brine wastewater (Duangsri and Satirapipathkul, 2011; Volkmann et al., 2008). Moreover, utilizing seawater rather than freshwater for production of saline microalgae, such as *A. platensis*, would be practical and cost effective (Mahrouqi et al., 2015). The potential of the cyanobacterium *Nostoc* to promote wastewater treatment and biomass production has also been reported (El-Sheekh et al., 2014; Talukder et al., 2015).

The production of phycobiliproteins from biomass grown in wastewater has not been extensively explored in the literature. In this sense, **Chapter 4** addressed the phycobiliproteins extraction from a mixed culture dominated by cyanobacteria grown in diluted centrate. This study, diversely, investigated the performance of unialgal cultivations and phycobiliproteins production of *Nostoc* sp., *A. platensis* and *Porphyridium purpureum* grown in: i) standard growth media with varied compositions and different light intensities (in view of optimisation of the reactor conditions); and ii) food-industry wastewater under optimal light conditions. As such, this is the first study describing the possibility to recover natural pigments from these three species using effluent in order to reduce the high costs of its production (since nutrients are recovered from wastewater and therefore no chemicals would be needed for growth medium). Not only the feasibility is assessed, but also different aspects for pigments production by these microalgae under varied cultivation conditions.

5.2 Materials and methods

5.2.1 Inocula and culture conditions

A microalgae sample of *Nostoc* sp. was obtained from the Spanish Bank of Algae, University of Las Palmas de Gran Canaria (Spain) (Figure 5-1a) and samples of *A. platensis* (Figure 5-1b) and *P. purpureum* (Figure 5-1c) were obtained from the laboratory of the School of Arts (KASK), University College Ghent (Belgium). The microalgae species were cultured in Erlenmeyer flasks containing sterilized growth media in a room with constant temperature of 22°C and continuously mixed with magnetic stirrers (Hei-mix S, Heidolph, Germany) at 200 rpm. *Nostoc* sp. was cultivated in BG-11 medium (Stanier et al., 1971). *A. platensis* was cultivated in a modified Zarrouk's medium (Castro et al., 2015). *P. purpureum* was cultivated in a modified artificial sea water (ASW) (Velea et al., 2011).

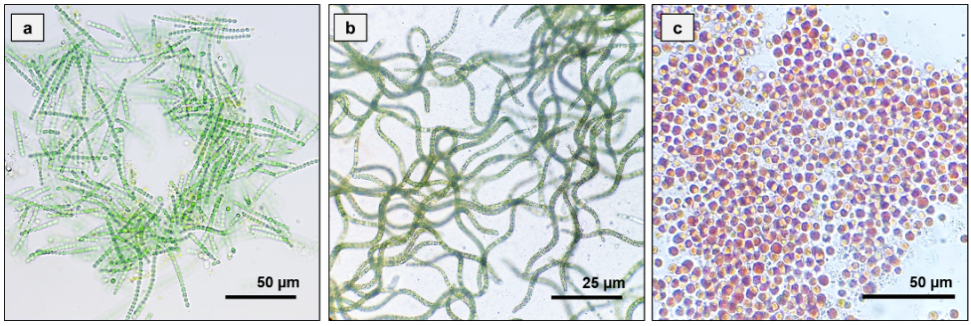


Figure 5-1. Microscopic images of the inoculum of a) *Nostoc* sp., b) *Arthrospira platensis* and c) *Porphyridium purpureum*.

5.2.2 Optimisation experiment

The optimisation experiment was carried out to find optimal conditions of light intensity and medium composition to reach high phycobiliproteins yields. The cultivations were done in 750 mL Erlenmeyer flasks filled with 600 mL culture media (Figure 5-2). The flasks were maintained in a room with constant temperature of 22°C

and aeration tubes providing approximately 3 L air/min were used to provide constant mixing and inorganic carbon. Illumination was provided by cool-white fluorescent lamps (Lumilux L 36W/840, Osram, Germany) with a light:dark cycle of 14:10 h. Two growth parameters were tested for each species: light intensity and concentration of the main ingredient of standard growth medium: $NaNO_3$ for *Nostoc* sp., $NaHCO_3$ for *A. platensis* and NaCl (as sea salt) for *P. purpureum*. Varying cultivation conditions tested as a 3^2 full factorial design (for each species) are described in Table 5-1.

Table 5-1. Varying growth conditions of light intensity and medium concentration (with respective electrical conductivity (EC)) tested for *Nostoc* sp., *A. platensis* and *P. purpureum* cultivation.

		***Nostoc* sp.**		***A. platensis***		***P. purpureum***	
Trial	**Light intensity ($\mu E/m^2s$)**	**$NaNO_3$ (g/L)**	**EC (mS/cm)**	**$NaHCO_3$ (g/L)**	**EC (mS/cm)**	**Sea salt (g/L)**	**EC (mS/cm)**
L1C1	65 ± 6	0.75	1.18 ± 0.01	8.4	13.07 ± 0.01	9.76	16.08 ± 0.02
L1C2	65 ± 6	1.5	2.22 ± 0.02	16.8	19.29 ± 0.01	19.51	29.01 ± 0.01
L1C3	65 ± 6	2.25	3.16 ± 0.02	25.2	25.13 ± 0.03	39.02	41.63 ± 0.09
L2C1	150 ± 7	0.75	1.18 ± 0.01	8.4	13.07 ± 0.01	9.76	16.08 ± 0.02
L2C2	150 ± 7	1.5	2.22 ± 0.02	16.8	19.29 ± 0.01	19.51	29.01 ± 0.01
L2C3	150 ± 7	2.25	3.16 ± 0.02	25.2	25.13 ± 0.03	39.02	41.63 ± 0.09
L3C1	230 ± 9	0.75	1.18 ± 0.01	8.4	13.07 ± 0.01	9.76	16.08 ± 0.02
L3C2	230 ± 9	1.5	2.22 ± 0.02	16.8	19.29 ± 0.01	19.51	29.01 ± 0.01
L3C3	230 ± 9	2.25	3.16 ± 0.02	25.2	25.13 ± 0.03	39.02	41.63 ± 0.09

Figure 5-2. Cultivations of *Nostoc* sp., *A. platensis* and *P. purpureum* during the optimisation experiment.

5.2.3 Wastewater treatment experiment

The set-up consisted of five photobioreactors, one for *Nostoc* sp., two for *A. platensis* and two for *P. purpureum*, made with 90 µm thick bags composed of polyamide and polyethylene (Sacs sous vide, Spain) with working volume of 8L each (Figure 5-3). The wastewater used in this experiment was an upflow anaerobic sludge blanket (UASB) effluent from a food company that markets plant-based products (Alpro, Wevelgem, Belgium). Since the wastewater has reduced organic matter concentrations (after UASB), while still containing sufficient nutrients, this effluent is promising for the production of microalgal biomass, as previously described by Van Den Hende et al. (2016).

The wastewater was filtered using 0.45 µm membrane filters to remove any suspended particles before being used in the experiment. The filtered wastewater was refrigerated at 4°C to prevent any biochemical process that could change its composition. The photobioreactors were fed with the wastewater mixed with: i) standard medium BG-11 for *Nostoc* sp., at ratio 50% WW + 50% BG-11; and ii) artificial sea water (ASW) for saline species *A. platensis* and *P. purpureum*, at two different volume ratios: 50% WW

+ 50% ASW and 75% WW + 25% ASW. The ratios of wastewater tested were defined based on the results of biomass growth and phycobiliproteins production of the optimisation experiment.

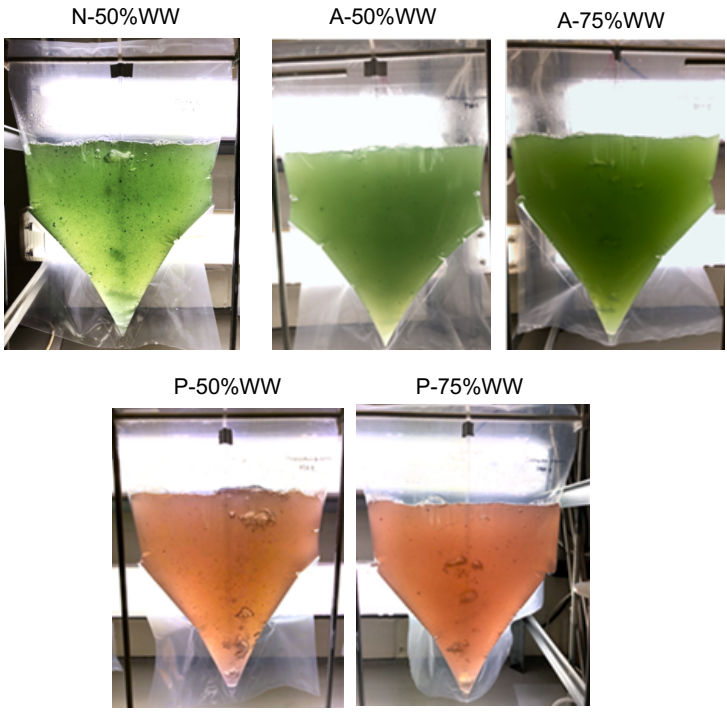


Figure 5-3. Cultivations of *Nostoc* sp., (N-50%WW) *A. platensis* (A-50%WW and A-75%WW) and *P. purpureum* (P-50%WW and P-75%WW) during the wastewater treatment experiment using food-industry effluent.

5.2.4 Biomass growth rate determination

During the optimisation experiment, biomass growth was monitored by withdrawing aliquots of 3 mL of culture media every 2 to 4 days and measuring the optical density (OD) at 680nm. Considering that working volume in this experiment was low (600 mL), biomass growth was monitored through OD to avoid withdrawing large volumes from vessels. This was done since good correlations between OD and dry

weight (DW) were observed: $R^2 = 0.989$ (*Nostoc* sp.), $R^2 = 0.999$ (*A. platensis*) and $R^2 = 0.993$ (*P. purpureum*). On the other hand, during the wastewater experiment, as the working volume was 8 L, biomass growth was monitored through volatile suspended solids (VSS) measurement by filtering 50 mL of culture media.

Maximum specific growth rate (μ_{max}) was calculated through Eq. 5-1, considering biomass concentrations during the exponential phase (Andersen, 2005).

$$\mu_{max} = \frac{\ln\left(\frac{C_f}{C_i}\right)}{(t_f - t_i)} \qquad \text{Eq. 5-1}$$

where C_i and C_f are the biomass concentrations at time initial (t_i) and final (t_f), respectively.

5.2.5 Analytical methods

Total suspended solids (TSS), volatile suspended solids (VSS) were determined according to Standard Methods (APHA-AWWA-WEF, 2012) and chemical oxygen demand (COD) was determined according to the method for high salinity proposed by Kayaalp et al. (2010). The measurement of total nitrogen (TN), total phosphorus (TP), NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P were done with spectrophotometric test kits (Hach, USA). Total inorganic nitrogen (TIN) is expressed as the sum of NH_4^+-N, NO_3^--N and NO_2^--N. pH and electrical conductivity (EC) were measured with a portable multi-parameter meter HQ30d (Hach, USA). All the analyses were done in triplicate and results are given as average values and standard deviation.

5.2.6 Phycobiliproteins extraction

Phycobiliproteins content in the biomass grown during the optimisation and the wastewater experiments was quantified. The culture medium was centrifuged at 1,160 g for 15 min (Hermle Z 300 K, Germany) and the biomass pellets were frozen (-21°C) until further use. Approximately 1 g of biomass was added into 15mL centrifuge tubes

and sodium phosphate buffer (0.1M, pH 7) was used as solvent at a proportion 1:10 (w:w, biomass:solvent). The tubes were then submitted to two freeze-thawing (-21°C to 4°C in darkness) cycles. The resulting slurry was centrifuged at 9,500 g for 15 min at 4°C (Hermle Z 300 K, Germany) to remove the cell debris. The supernatant was collected and measured in a spectrophotometer (Shimadzu UV-1280, Japan) at 280, 562, 615 and 652 nm. The amounts of phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) were calculated according to Eq. 5-2, Eq. 5-3 and Eq. 5-4, respectively (Bennett and Bogobad, 1973).

$$PC\ (mg/mL) = [A_{615} - (0.474 * A_{652})] / 5.34 \quad \text{Eq. 5-2}$$

$$APC\ (mg/mL) = [A_{652} - (0.208 * A_{615})] / 5.09 \quad \text{Eq. 5-3}$$

$$PE\ (mg/mL) = [A_{562} - (2.41*PC) - (0.849*APC)] / 9.62 \quad \text{Eq. 5-4}$$

where A_{562}, A_{615} and A_{652} are the absorbances measured at the respective wavelengths.

Purity was determined as the absorbance ratios of A_{620}/A_{280} for phycocyanin, A_{652}/A_{280} for allophycocyanin and A_{565}/A_{280} for phycoerythrin (Cuellar-Bermudez et al., 2015).

5.2.7 Statistical analyses

The average values measured during the experiments were analyzed using multi-factor analysis of variance (ANOVA) and the significant differences amongst treatments and phycobiliproteins production were determined using Fisher test at 95% confidence interval level. All statistical analyses were done using Minitab 18 (Minitab Inc., PA, USA).

5.3 Results and discussion

5.3.1 Optimisation experiment

This experiment was carried out in order to define the best conditions of light intensity and growth media composition that would suggest the optimal combinations to maximize biomass growth and phycobiliproteins production of *Nostoc* sp., *A. platensis* and *P. purpureum*.

5.3.1.1 Biomass growth and nutrients removal

Biomass growth curves of *Nostoc* sp., *A. platensis* and *P. purpureum* during this experiment are shown in Figure 5-4. Growth curves and their respective statistical significance suggest that light conditions had more influence on biomass growth than the media composition (Figure 5-4). Initial and final concentrations of nutrients as TIN and PO_4^{3-}-P are shown in Figure 5-5. The three microalgae species could grow well in all trials until the end of the batch cultivation, except the L3C1 of both *Nostoc* sp. and *P. purpureum*, which indicates that high light conditions promoted fast growth in the initial phase of cultivation, and led to nutrients starvation faster than in other trials. This can be supported by the final TIN concentrations observed in these two trials shown in Figure 5-5.

5.3.1.1.1 Nostoc sp.

The lowest biomass concentration of *Nostoc* sp. after 20 days of cultivation was measured in L1C2 with 1.1 g/L, while the highest in L3C3 with 2.2 g/L. Increasing light conditions led to higher biomass growth (except for L3C1, as previously mentioned). This is in accordance with the results shown by Spencer et al. (2011), in which growth of *Nostoc spongiaeforme* increased up to 227 $\mu E/m^2s$ (similar to L3 in this study). Initial and final TIN concentrations in the growth media of *Nostoc* sp. showed removal rates ranging from 3.8 to 7.6 mg N/Ld (28 to 94% removal efficiency). Although higher light intensities led to higher biomass growth, no significant difference was observed on TIN removal by *Nostoc* sp. when comparing different light intensities in L1, L2 and L3 (p-

value = 0.238), as well as different initial $NaNO_3$ concentrations in C1, C2 and C3 (p-value = 0.422). Likewise, PO_4^{3-}-P removal rates ranged from 0.35 to 0.39 mg P/Ld (93 to 99% removal efficiency). Likewise, no significant difference was observed on phosphorus removal by *Nostoc* sp. when comparing different initial $NaNO_3$ concentrations in C1, C2 and C3 (p-value = 0.063) and different light intensities in L1, L2 and L3 (p-value = 0.568).

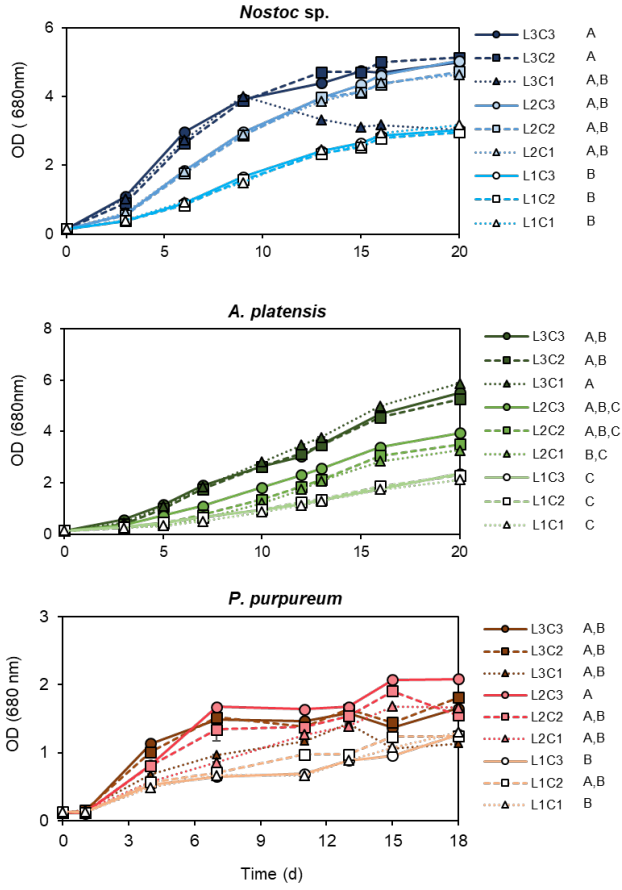


Figure 5-4. Biomass growth of *Nostoc* sp., *A. platensis* and *P. purpureum* during the optimisation experiment, measured as optical density (OD) at 680nm. Biomass growth was monitored under different conditions of low (C1), medium (C2) and high (C3) concentration of the main ingredient in standard growth medium ($NaNO_3$ for *Nostoc* sp., $NaHCO_3$ for *A. platensis* and NaCl as sea salt for *P. purpureum*) and low (L1), medium (L2) and high (L3) light intensity. Letters A, B and C indicate a significant difference (α=0.05) among trials after Fisher test.

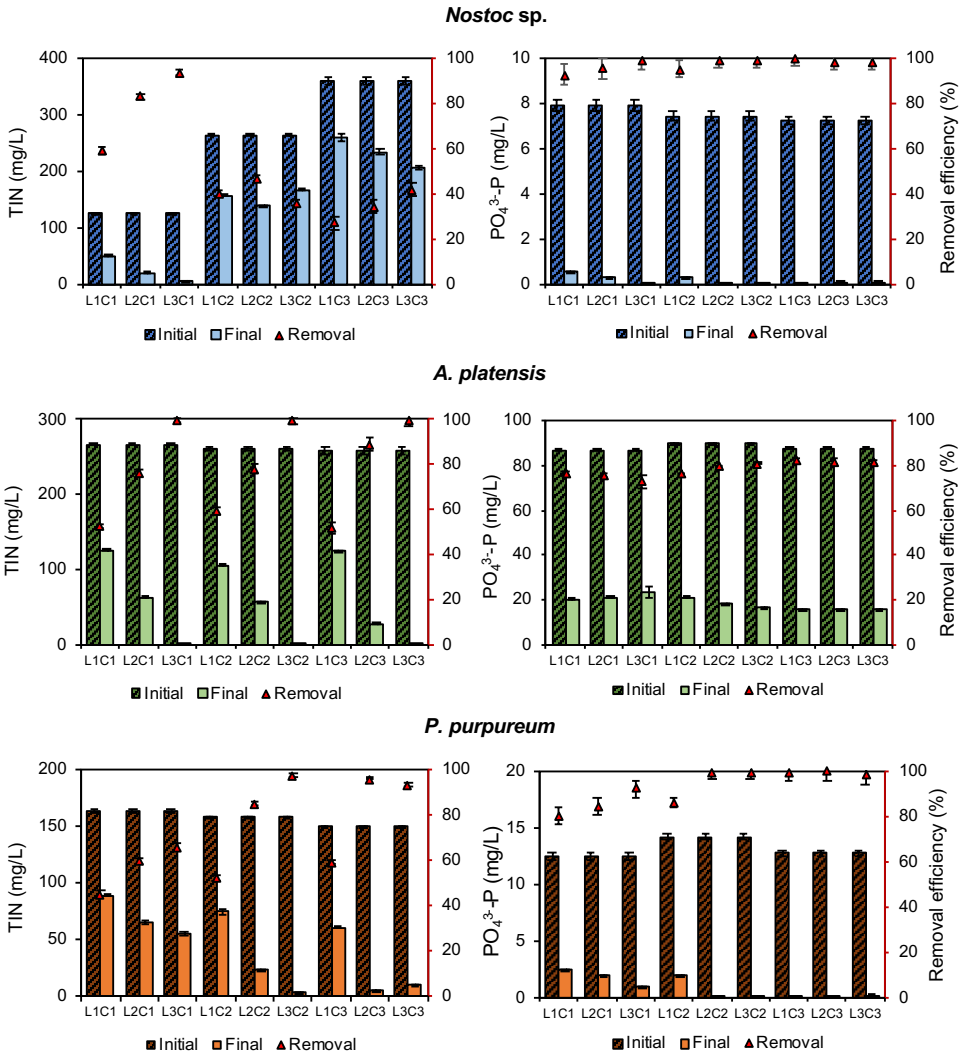


Figure 5-5. Initial and final concentrations of total inorganic nitrogen (TIN) and PO_4^{3-}-P, and respective removal efficiency (%), measured in the growth media of *Nostoc* sp., *A. platensis* and *P. purpureum* during the optimisation experiment. Variations of these parameters were monitored under different conditions of low (C1), medium (C2) and high (C3) concentration of the main ingredient in standard growth medium ($NaNO_3$ for *Nostoc* sp., $NaHCO_3$ for *A. platensis* and NaCl as sea salt for *P. purpureum*) and low (L1), medium (L2) and high (L3) light intensity.

5.3.1.1.2 *A. platensis*

The cultivations of *A. platensis* reached the lowest biomass concentration after 20 days in L1C1 with 1.0 g/L, while the highest in L3C3 with 3.0 g/L. Likewise, Markou et al. (2012) reported that increasing light intensity from 24 to 60 μE/m^2s led to 2.7-fold higher *A. platensis* biomass production in standard growth medium. Castro et al. (2015) also reported that higher light conditions and $NaHCO_3$ concentrations increased *A. platensis* biomass production. Initial and final TIN concentrations in the growth media of *A. platensis* showed removal rates ranging from 6.7 to 13.3 mg N/Ld (52 to 100% removal efficiency). Removal rates of both medium and high light intensities were significantly higher than low light intensity (p-value = 4 x 10^{-5}), while different concentrations of $NaHCO_3$ in the growth media did not have affect TIN removal (p-value = 0.996). Regarding phosphorus removal, *A. platensis* showed removal rates ranging from 3.2 to 3.6 mg P/Ld (73 to 82% removal efficiency). When comparing the influence of light conditions, L1, L2 and L3 showed similar performances of phosphorus removal (p-value = 0.983), while when varying concentrations of $NaHCO_3$, C3 showed significantly higher removal rates than C1 and C2 (p-value = 0.004).

5.3.1.1.3 *P. purpureum*

The cultivations of *P. purpureum* showed the lowest biomass after 18 days of cultivation measured in L1C1 with 0.9 g/L, while the highest in L2C3 with 2.3 g/L. The results showed faster growth under high light intensities (L3), but over time, the cultivations under medium light intensities (L2) overtook, reaching the maximum biomass concentrations at the end of the experiment. Similar results were reported by Sosa-Hernández et al. (2019) with 0.3 L cultivations of *P. purpureum* in Bold 1NV and Erdshreiber media, where light intensity of 100 μE m^{-2} s^{-1} (compared to 65 and 30 μE m^{-2} s^{-1}) resulted in higher biomass. Regarding NaCl concentrations, medium salinity level (C2) seemed to be more favourable for biomass growth, which was also reported by previous studies with the same species (Aizdaicher et al., 2014; Kathiresan et al., 2007). Similarly to *A. platensis*, removal rates by *P. purpureum* ranged from 4.1 to 8.6 mg N/Ld (45 to 98% removal efficiency), in which L2 and L3 had significantly higher TIN removal than L1 (p-value = 0.039), while concentration of NaCl in the growth media

did not have a significant influence on the TIN removal (p-value = 0.399). Regarding phosphorus removal, removal rates ranged from 0.56 to 0.79 mg P/Ld (80 to 100% removal efficiency), in which varying light conditions did not affect removal rates (p-value = 0.677), while higher concentrations of NaCl (both C2 and C3) removed better than low concentration (C1) (p-value = 0.011).

It is noteworthy, thus, to highlight that the higher light intensity did not improve the PO_4^{3-}-P removal in all trials, as observed with TIN removal (especially for *A. platensis* and *P. purpureum*). Considering the biomass stoichiometry, as PO_4^{3-}-P was not proportionally removed compared to TIN, the higher TIN removal was not related to biomass growth only, but possibly to the occurrence of nitrification-denitrification process during the cultivation period. This is in accordance with the increase in concentrations of NO_2^--N in all trials during the experiment (Table A-2, Appendix).

5.3.1.2 Phycobiliproteins

The concentrations and yield of phycobiliproteins extracted from the biomass grown during the optimisation experiment are shown in Figure 5-6. The concentrations represent the amount of phycobiliproteins per dry weight, while the yield represents the amount of phycobiliprotein per unit of volume and time in each trial, i.e. considering the content and biomass production during this period.

5.3.1.2.1 Nostoc sp.

Results of *Nostoc* sp. cultivations show that, in average, phycocyanin was the most abundant phycobiliprotein, followed by allophycocyanin and phycoerythrin. The maximum total concentration of phycobiliproteins was obtained in L1C3 (199 mg/g DW), while the minimum in L3C2 (15 mg/g DW). The concentrations observed in this study in accordance with previous results of *Nostoc* sp. cultivated in standard growth media (Khattar et al., 2015; Ma et al., 2015). Regardless of initial $NaNO_3$ concentrations, lower light intensities produced more phycobiliproteins, with higher purity observed under medium light intensity (L2) (Figure 5-6), which is in accordance with previous studies (Johnson et al., 2014; Ma et al., 2015). Regarding $NaNO_3$, varying its

concentration did not influence phycobiliproteins content as much as varying light conditions, which is in accordance with the study carried out by Rosales Loaiza et al. (2016). Under low light intensity (L1), C3 had highest concentration, while under medium (L2) and high (L3) light intensities, C1 showed highest concentrations and yields. In summary, based on the results of this experiment, the best conditions to produce phycobiliproteins with better purity levels from *Nostoc* sp. were at low and medium light intensity (L1: 65 and L2: 150 $\mu E/m^2s$) with low or high $NaNO_3$ concentrations (C1: 0.75 and C3: 2.25 g $NaNO_3$/L).

5.3.1.2.2 A. platensis

Results of *A. platensis* cultivations show that, in average, phycocyanin was the most abundant phycobiliprotein, followed by allophycocyanin and phycoerythrin. The maximum total concentration of phycobiliproteins was obtained in L1C2 (303 mg/g DW), while the minimum in L1C3 (60 mg/g DW). For low (C1) and medium (C2) $NaHCO_3$ concentrations, lower light intensities produced more phycobiliproteins. However, for high (C3) $NaHCO_3$ concentrations, higher light conditions produced more phycobiliproteins (Figure 5-6). Nevertheless, when biomass production is considered, higher light intensities led to higher phycobiliproteins yield in all cases, which was also reported in previous studies (Castro et al., 2015; Markou et al., 2012). Regarding the influence of $NaHCO_3$ in the growth medium, higher concentrations led to lower phycobiliproteins content (Sharma et al., 2014). That explains the lowest phycobiliproteins concentration and production in L1C3 (60 mg/g DW, from which 23 mg PC/g DW) and L2C3 (152 mg/g DW, from which 55 mg PC/g DW) since these trials had the less favorable condition among all others, i.e. low phycobiliproteins content due to high carbon content and low biomass production due to low light conditions. The absorbance spectra of the crude extracts (Figure A-2, Appendix) also show that L1C3 and L2C3 obtained lower absorbance for phycocyanin (λ = 615 nm). An interesting finding is that these two trials also show higher absorbance than others in the range of λ = 420-440 and 660-680 nm, which suggest the presence of chlorophyll *a*. In this sense, considering that absorbance of allophycocyanin is measured at λ = 652 nm and overlaps with chlorophyll *a*, the content of allophycocyanin might have been overestimated for

these trials, especially after observing that in these two cases only, the content of allophycocyanin is higher than phycocyanin. To sum up, based on the results of this experiment, the best conditions to produce phycobiliproteins with better purity levels from *A. platensis* were at medium and high light intensities (L2: 150 and L3: 230 µE/m^2s) combined with low and medium $NaHCO_3$ concentrations (C1: 8.4 and C2: 16.8 g/L).

5.3.1.2.3 P. purpureum

Results of *P. purpureum* show that, in average, phycoerythrin was the most abundant phycobiliprotein, followed by phycocyanin and allophycocyanin. Similarly, reducing light conditions led to higher concentrations of phycobiliproteins (Figure 5-6), with maximum total concentration obtained for L1C3 (93 mg/g DW) and minimum for L3C2 (29 mg/g DW). However, when biomass production and purity are considered, higher productions of phycobiliproteins were obtained in medium intensity (L2). Likewise, Sosa-Hernández et al. (2019) observed highest values of phycoerythrin content under light intensities of 65 and 100 µE m^{-2} s^{-1} (3.10 and 2.71 mg/g DW, respectively), which are similar to the low (L1: 65 µE/m^2s) and medium (L2: 150 µE/m^2s) light intensities in the present study. Previous studies have also reported that high light conditions contributed to *P. purpureum* biomass accumulation, but it was adverse for biosynthesis of valuable compounds, such as phycobiliproteins, arachidonic acid and total fatty acids (Guihéneuf and Stengel, 2015; Su et al., 2016). Regarding the influence of salinity levels, high NaCl concentration (C3) led to higher phycobiliproteins concentrations, especially for low (L1) and medium (L2) light intensity. Kathiresan et al. (2007) reported similar results, in which high salinity concentration (29.62 g/L) would lead to maximum phycobiliproteins production. In summary, based on the results of this experiment, the best conditions to produce phycobiliproteins with better purity levels from *P. purpureum* were at medium light intensity (L2: 150 µE/m^2s) with medium and high salinity concentrations (C2: 19.51 and C3: 39.02 g sea salt/L).

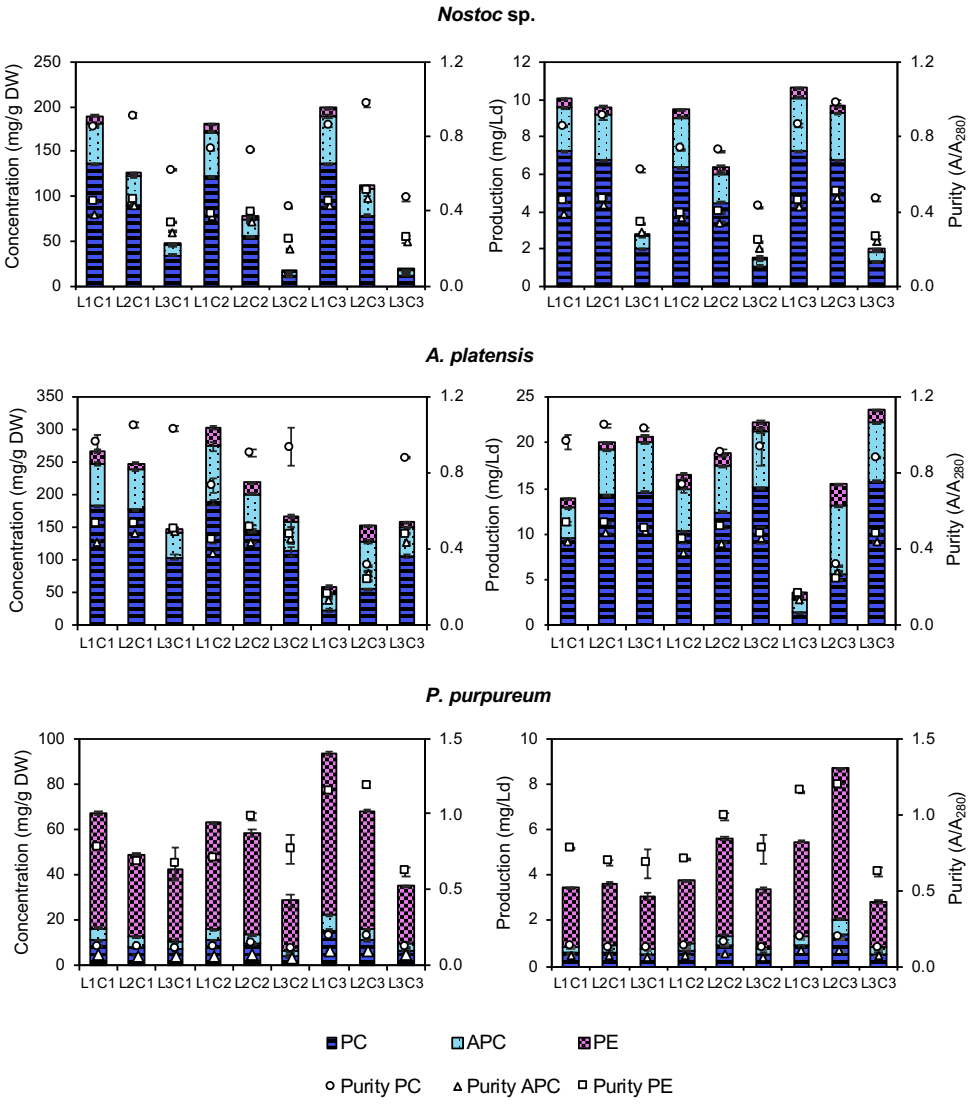


Figure 5-6. Average concentration and production of phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE), with respective purity grades, extracted from *Nostoc* sp., *A. platensis* and *P. purpureum* biomass grown during the optimisation experiment. Biomass growth was monitored under different conditions of low (C1), medium (C2) and high (C3) concentration of the main ingredient in standard growth medium ($NaNO_3$ for *Nostoc* sp., $NaHCO_3$ for *A. platensis* and NaCl as sea salt for *P. purpureum*) and low (L1), medium (L2) and high (L3) light intensity.

5.3.1.2.4 Definition of conditions for the wastewater treatment experiment

In order to select the optimal conditions for the following experiment (wastewater treatment), results were compared in terms of nutrients removal and phycobiliproteins production. For *Nostoc* sp., low (L1) and medium (L2) light conditions were optimal, but although lower light conditions (L1) reached highest phycobiliproteins yields, medium light conditions (L2) had higher purity and higher nutrients removal rates. For *A. platensis* and *P. purpurem,* medium (L2) and high (L3) light conditions were optimal. L3 led to higher TIN removal in *A. platensis* and *P. purpurem*, but L2 led to high phycobiliproteins productions with high purity while promoting similar nutrients removal compared to L3. Hence, medium light conditions (L2) was selected for the following experiment. Regarding medium composition for *Nostoc* sp., low (C1) or high (C3) concentrations of $NaNO_3$ were optimal, but removal efficiencies of C1 were much higher since C3 were limited by phosphorus. Hence, concentrations (as well as N:P ratio) similar to C1 were selected for cultivating *Nostoc* sp. in the following experiment. *A. platensis* showed higher nutrients removal in C3 and better phycobiliproteins productions in C1 and C2, so conductivities similar to C2 and C3 were selected. Finally, for *P. purpureum,* although C3 resulted in higher nutrients removal rates and phycobiliproteins, C2 and C1 were selected for the wastewater treatment experiment to avoid adding salts and avoid very low nutrients concentrations, since ASW portion would have to be much higher compared to wastewater. This way, simple and low-cost composition of growth medium using (real) wastewater mixed with seawater were evaluated, which would be more realistic in a full-scale implementation.

5.3.2 Wastewater treatment experiment

The set-up of the wastewater treatment experiment was defined based on the results of biomass growth and phycobiliproteins production of *Nostoc* sp., *A. platensis* and *P. purpureum* studied during the optimisation experiment. Five photobioreactors under medium light intensity (L2) were fed with industrial wastewater mixed with: i) standard medium BG-11 for *Nostoc* sp., at ratio 50% WW + 50% BG-11, which represented similar concentrations of C1; and ii) ASW for saline species *A. platensis* and *P.*

purpureum, at two different volume ratios: 50% WW + 50% ASW and 75% WW + 25% ASW, which represented similar conductivities of C3 and C2 for *A. platensis* and C2 and C1 for *P. purpureum*, respectively.

5.3.2.1 Biomass growth and nutrients removal

5.3.2.1.1 Overall wastewater treatment efficiency

Results showed that the three species could grow well in all photobioreactors. Cultivation period ended after 10 days, when nutrients reached low concentrations and biomass growth reached stationary phase. Biomass growth during the experiment, as well as the profiles of nitrogen species and PO_4^{3-}-P, are shown in Figure 5-7. Initial and final concentrations of the photobioreactors are summarized in Table 5-2. Removal efficiencies in the photobioreactors ranged from 45% (N-50%WW) to 84% (A-75%WW) for sCOD, 89% (P-75%WW) to 99% (A-50%WW) for TIN and 81% (A-75%WW) to 100% (N-50%WW and P-50%WW) for PO_4^{3-}-P, suggesting that the three species can potentially be applied for wastewater treatment.

Results on wastewater treatment efficiencies and specific growth rate in the present study are comparable to those previously reported on cultivation of similar microalgae in wastewaters (list of studies shown in Table 5-3). The observed nutrients removal efficiencies varied depending on the media composition and environmental conditions such as the influent concentrations, light conditions, N/P ratio, cultivation mode, and microalgae species. *Nostoc* sp. has been reported to efficiently treat municipal wastewater with removal efficiencies higher than 90% of NH_4-N and up to 60% phosphorus (El-Sheekh et al., 2014; Sharma and Khan, 2013) and to reduce COD and BOD of acidic textile effluent diluted in BG-11 medium, by 32 and 55% respectively (Talukder et al., 2015). Zhou et al. (2017) reported very similar results of nutrients removal (95% of NH_4-N and higher than 90% of PO_4^{3-}-P) by *A. platensis* grown in synthetic toilet flushing wastewater (using seawater) mixed with washing wastewater (using freshwater), in experiments with similar initial nutrients concentrations. Likewise, Chaiklahan et al. (2010) reached comparable removal of TIN by 89% and phosphorus

by 57% treating swine wastewater in a semi-continuous culture mode. Regarding the use of *P. purpureum* for wastewater treatment, no other study has been found in literature.

It is important to note that TIN removal in all photobioreactors was done not only by active biomass uptake, but also by ammonia stripping (as pH ranged from 8.62 and 9.95) and denitrification. Based on the total nitrogen balance, the amount of TIN assumed to be removed by stripping or denitrification during the cultivation period was 19.6 (N-50%WW), 13.5 (A-50%WW), 26.2 (A-75%WW), 12.1 (P-50%WW) and 20.1 (P-75%WW) mg/L. As the pH of all photobioreactors was not significantly different (p-value = 0.814), thus assuming that loss through volatilisation is similar in all photobioreactors, higher removal of nitrogen in N-50%WW, A-75%WW and P-75%WW might be related to higher nitrification-denitrification activity. The higher values of NO_3^--N and NO_2^--N in these photobioreactors, compared to A-50%WW to P-50%WW, respectively, corroborate this assumption (Table 5-2). TIN removal by ammonia volatilisation and nitrification-denitrification was also reported in other studies cultivating *A. platensis* in wastewater (Chaiklahan et al., 2010; Zhou et al., 2017).

Overall, the results showed that the three species could grow well in all photobioreactors while efficiently removing sCOD and nutrients below discharge limits for this effluent (EEA, 2019).

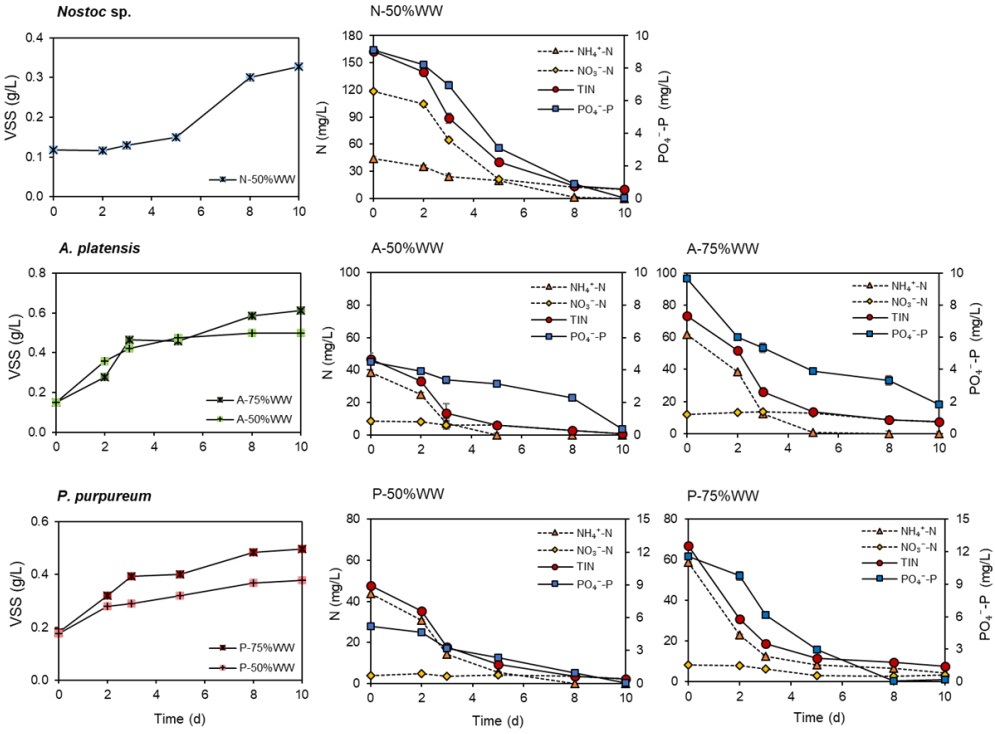


Figure 5-7. Biomass growth, measured as concentration of volatile suspended solids (VSS), and concentrations of nitrogen (N), as ammonium (NH_4^+-N), nitrate (NO_3^--N) and total inorganic nitrogen (TIN), and PO_4^{3-}-P during the cultivation in different ratios of wastewater (WW) of freshwater species *Nostoc* sp. (N-50% WW), as well as saline species *A. platensis* with 50% wastewater (A-50%WW) and 75% wastewater (A-75%WW), and *P. purpureum* with 50% wastewater (P-50%WW) and 75% wastewater (P-75%WW).

Table 5-2. Average initial and final concentrations of water quality parameters measured in the cultivations of *Nostoc* sp. with 50% wastewater (N-50%WW), *A. platensis* with 50% wastewater (A-50%WW) and 75% wastewater (A-75%WW), and cultivations of *P. purpureum* with 50% wastewater (P-50%WW) and 75% wastewater (P-75%WW).

Microalgae		***Nostoc* sp.**		***A. platensis***				***P. purpureum***			
Photobioreactor		**N-50%WW**		**A-50%WW**		**A-75%WW**		**P-50%WW**		**P-75%WW**	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
pH	-	7.61 ± 0.01^{a}	9.38 ± 0.03^{b}	8.94 ± 0.01^{a}	9.90 ± 0.01^{b}	8.62 ± 0.01^{a}	9.95 ± 0.01^{b}	8.94 ± 0.01^{a}	9.25 ± 0.01^{b}	8.62 ± 0.01^{a}	9.43 ± 0.01^{b}
EC	mS/cm	2.92 ± 0.01^{a}	2.82 ± 0.01^{b}	24.5 ± 0.06^{a}	23.60 ± 0.06^{b}	14.47 ± 0.01^{a}	14.97 ± 0.01^{b}	24.50 ± 0.06^{a}	23.90 ± 0.06^{b}	14.47 ± 0.01^{a}	14.20 ± 0.02^{b}
sCOD	mg/L	107 ± 2^{a}	59 ± 10^{b}	118 ± 9^{a}	21 ± 2^{b}	159 ± 3^{a}	26 ± 3^{b}	155 ± 4^{a}	38 ± 4^{b}	198 ± 12^{a}	41 ± 2^{b}
TN*	mg/L	205 ± 2^{a}	185.4 ± 3.4^{b}	82.5 ± 0.3	69 ± 8	98.1 ± 1.9^{a}	72 ± 1^{b}	75.5 ± 0.3	63 ± 14	112.5 ± 0.3	92 ± 8
NH_4^+-N	mg/L	44.4 ± 1.7^{a}	0.05 ± 0.01^{b}	38.4 ± 0.2^{a}	0.0 ± 0.0^{b}	61.5 ± 0.3^{a}	0.0 ± 0.0^{b}	43.6 ± 0.1^{a}	0.0 ± 0.0^{b}	58.7 ± 0.4^{a}	4.24 ± 0.01^{b}
NO_3^--N	mg/L	118 ± 2^{a}	9.9 ± 0.8^{b}	8.35 ± 0.04^{a}	0.60 ± 0.8^{b}	11.9 ± 0.1^{a}	7.52 ± 0.07^{b}	3.85 ± 0.02^{a}	2.2 ± 0.1^{b}	8.25 ± 0.02^{a}	3.4 ± 0.1^{b}
NO_2^--N	mg/L	0.10 ± 0.01^{a}	2.52 ± 0.05^{b}	0.09 ± 0.04^{a}	0.61 ± 0.01^{b}	0.14 ± 0.03^{a}	2.43 ± 0.04^{b}	0.09 ± 0.02^{a}	0.45 ± 0.02^{b}	0.14 ± 0.01^{a}	0.51 ± 0.01^{b}
TP*	mg/L	205 ± 2	14.0 ± 0.1	18.5 ± 0.1	17.4 ± 0.6	22.5 ± 0.2	19 ± 1	12.6 ± 0.7	11.1 ± 0.7	14.9 ± 0.2	12.6 ± 1.1
PO_4^{3-}-P	mg/L	9.11 ± 0.04^{a}	0.04 ± 0.01^{b}	4.5 ± 0.7^{a}	0.4 ± 0.0^{b}	9.7 ± 0.1^{a}	1.8 ± 0.2^{b}	5.24 ± 0.03^{a}	0.02 ± 0.01^{b}	11.6 ± 0.2^{a}	0.2 ± 0.1^{b}

Acronyms: EC (electrical conductivity); sCOD (soluble chemical oxygen demand); TN (total nitrogen); TP (total phosphorus).
*TN and TP were measured in the mixed liquor (influent with inoculum).
[a,b]: Letters indicate a significant difference (α=0.05) between influent and effluent concentrations after Fisher test.

5.3.2.1.2 Comparison between saline species (A. platensis and P. purpureum)

Specifically, the saline species are further compared, as they were both cultivated in the two ratios of wastewater (50% and 75% WW). First, the performance of individual species is compared in both ratios of wastewater. Later, the performances of both species are compared treating the same influent.

Biomass concentration of *A. platensis* reached 498 mg/L in A-50%WW, while in A-75%WW the concentration reached 614 mg/L, which was 23% higher (although not significantly different, p-value = 0.794). These results show that *A. platensis* could grow better in the photobioreactor with higher portion of wastewater, most probably due to the higher nutrients concentrations. The lower salinity could also have induced better growth, as previous studies suggested that higher salinity reduced *A. platensis* growth in wastewater (Duangsri and Satirapipathkul, 2011; Volkmann et al., 2008; Zhou et al., 2017).

Biomass concentration of *P. purpureum* reached 378 mg/L in P-50%WW and 496 mg/L P-75%WW at the end of the cultivation period. Similarly, the second produced 31%, although not significantly (p-value = 0.202), higher biomass concentrations at the end of the batch cultivation. These results show that *P. purpureum* could grow better in the photobioreactor with higher portion of wastewater, most probably because the influent concentrations were higher.

Although *A. platensis* produced more biomass than *P. purpureum*, there was no significant difference neither between A-50%WW and P-50%WW (p-value = 0.148) nor between A-75%WW and P-75%WW (p-value = 0.613). Likewise, in terms of treatment efficiency, despite *A. platensis* cultivations had higher removal rates than *P. purpureum* for both 50%WW and 75%WW, no significant difference was observed between TIN, PO_4^{3-}-P and COD removal rates (p-values ranging from 0.194 to 0.836). These results suggest that both species could be applied for efficient wastewater treatment.

5.3.2.2 Phycobiliproteins

The concentrations and production of phycobiliproteins extracted from the biomass grown during the wastewater treatment experiment are shown in Figure 5-8. The concentrations represent the amount of phycobiliproteins per dry weight, while the production represents the amount of phycobiliprotein per unit of volume and time in each trial, i.e. considering the content and biomass production during this period. Further discussion on the phycobiliproteins content and production from biomass grown in wastewater are provided in this section.

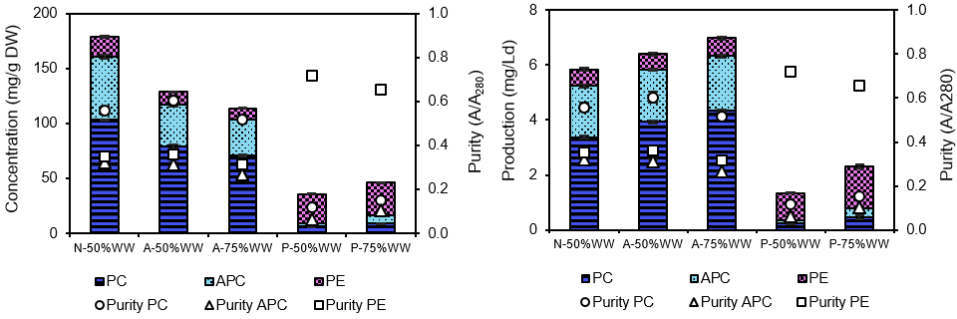


Figure 5-8. Average concentration and production of phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE), with respective purity grades, extracted from biomass grown during the cultivation in wastewater (WW) of freshwater species *Nostoc* sp. (N-50% WW), as well as saline species *A. platensis* with 50% wastewater (A-50%WW) and 75% wastewater (A-75%WW), and *P. purpureum* with 50% wastewater (P-50%WW) and 75% wastewater (P-75%WW).

Among all photobioreactors, the highest total phycobiliproteins was observed in N-50%WW with 179 mg PBP/g DW, while the lowest in P-50%WW with 36 mg PBP/g DW. However, when biomass production is considered, photobioreactors of *A. platensis* (A-50%WW and A-75%WW) had highest production of phycobiliproteins. It is important to note that the absorbance spectra of the crude extracts of *Nostoc* sp. and *A. platensis* showed absorbance in the range of λ = 420-440 and 660-680 nm, which suggest the presence of chlorophyll *a* (Figure A-3, Appendix). In this sense, as previously described in Section 5.3.1.2.2, the content of allophycocyanin might have been

overestimated for N-50%WW, A-50%WW and A-75%WW. Overall, the results suggest that the three species could produce phycobiliproteins while treating industrial wastewater. Figure 5-9 illustrates the protein solutions extracted from *A. platensis* and *P. purpureum* during this experiment.

Figure 5-9. Crude extracts of *A. platensis* (left) and *P. purpureum* (right) grown in wastewater, showing abundance of phycocyanin and phycoerythrin, respectively.

5.3.2.2.1 Comparison between saline species (A. platensis and P. purpureum)

Total phycobiliproteins concentrations in A-50%WW were 13%, although not significantly (p-value = 0.702), higher than in A-75%WW. However, when biomass production is considered, A-75%WW showed 9% higher production than A-50%WW (p-value=0.689), but with lower purity factors. Regarding *P. purpureum*, total phycobiliproteins concentrations in P-75%WW were 31% significantly higher than in P-50%WW (1.2×10^{-4}). When biomass production is considered the discrepancy is even higher, with P-75%WW showing 72% higher phycobiliproteins production than P-50%WW (p-value=2.5×10^{-6}), but with lower phycoerythrin purity factors. The results suggest that A-50%WW and P-75%WW would be the appropriate conditions for *A. platensis* and *P. purpureum* to maximize phycobiliproteins production.

Nitrogen is an important source for both the biomass and phycobiliproteins production. Zhao et al. (2017) demonstrated that a nitrate starvation led to the decline of photosynthetic performance, which is directly related to photosynthetic pigments such as chlorophyll and phycobiliproteins. Considering that the nutrients were depleted in all photobioreactors by the end of the experimental period, not only biomass but also phycobiliproteins production could possibly be improved by using higher influent nutrient concentrations or (semi) continuous culture mode.

5.3.2.2.2 Comparison of phycobiliproteins production with synthetic medium and real wastewater

The concentration of total phycobiliproteins observed in N-50%WW (179 mg PBP/g DW) was higher than in the cultivation of synthetic medium under similar conditions L2C1 (127 mg PBP/g DW). However, purity of phycocyanin, allophycocyanin and phycoerythrin were lower in N-50%WW (0.56, 0.32 and 0.35, respectively) than in L2C1 (0.91, 0.43 and 0.47). In addition, as previously mentioned, there might be an overestimation on this value after analysing the absorbance spectra of their crude extracts (Figure A-2, Appendix). Considering that TIN removal is associated with the biosynthesis of phycobiliproteins, the production from *Nostoc* sp. grown in wastewater was much lower than in standard growth medium. The productivity of total phycobiliproteins in N-50%WW was 0.44 mg PBP/mg TIN removed by biomass uptake (i.e. disregarding the TIN removed stripping or denitrification) (average production of 5.8 mg PBP/Ld), while in L2C1 the productivity was 1.8 mg PBP/mg TIN. Although initial nutrients concentrations in N-50%WW were 27% and 15% higher than in L2C1 (128 mg TIN/L and 8 mg PO_4^{3-}-P/L), respectively, total phycobiliproteins production in L2C1 was 64% higher. These results showed that the productivity of phycobiliproteins from *Nostoc* sp. biomass grown in wastewater were somewhat lower than in standard growth medium, but it still shows promise as an alternative to recover resources from wastewater. In addition, further optimisation could be explored in terms of type of wastewater and operational conditions in order to enhance the productivity.

The production from *A. platensis* biomass grown in wastewater was comparable to biomass grown in standard growth medium. The productivity of total phycobiliproteins

in A-50%WW was 2.0 mg PBP/mg TIN (average production of 6.4 mg PBP/Ld). Similar conditions were imposed during optimisation experiment L2C3, which resulted in similar productivity of 1.3 mg PBP/mg TIN. The phycobiliproteins production in this case was 2.4-fold higher, but it is important to mention that initial nutrients concentrations (259 mg TIN/L and 88 mg PO_4^{3-}-P/L) were 5.5-fold and 19.4-fold higher than in A-50%WW, respectively. Likewise, the productivity of total phycobiliproteins in A-75%WW was 1.8 mg PBP/mg TIN (production of 7.0 mg PBP/Ld). During the optimisation experiment L2C2, similar conditions were imposed and comparable productivity of 1.9 mg PBP/mg TIN was observed. In this case, phycobiliproteins production was 2.7-fold higher (19 mg PBP/Ld), also due to higher initial nutrients concentrations, which were 3.5-fold and 9.3-fold higher than in A-75%WW, respectively.

The production from *P. purpureum* biomass grown in wastewater was comparable to biomass grown in standard growth medium. The productivity of total phycobiliproteins in P-50%WW was 0.41 mg PBP/mg TIN (average production of 1.3 mg PBP/Ld). Similar conditions were imposed during optimisation experiment L2C2, which resulted in productivity of 0.75 mg PBP/mg TIN. The phycobiliproteins production in L2C2 was 4.2-fold higher (5.6 mg PBP/Ld), due to higher initial nutrients concentration, which were 3.3-fold and 2.7-fold higher than in P-50%WW, respectively. Similarly, the productivity of total phycobiliproteins in P-75%WW was 0.6 mg PBP/mg TIN (production of 2.3 mg/Ld). During the optimisation experiment L2C1, similar conditions were imposed, but productivity was 0.7 mg PBP/mg TIN. In this case, phycobiliproteins production was 1.6-fold higher (3.6 mg/Ld), also due to higher initial nutrients concentrations, which were 2.4-fold and 1.1-fold higher than in P-75%WW, respectively.

It is important to notice that several researchers have investigated the three species studied in this work, but almost all of them describe cultivations using standard growth medium. A few studies for *A. platensis* and no studies for *Nostoc* sp. and *P. purpureum* addressed optimisation of phycobiliproteins production from biomass grown in wastewater. A list of previous studies using the three species investigated in this work

(and other similar microalgae) in wastewaters is shown in Table 5-3. Results on phycobiliproteins produced in the present study are comparable to those previously reported. Hultberg et al. (2017) cultivated *A. platensis* in anaerobic digestate effluent diluted (6%) in carbonate buffer and obtained higher phycocyanin (86.2 mg/g DW) and allophycocyanin (41.3 mg/g DW) compared to biomass grown in synthetic medium. Khatoon et al. (2018) observed higher phycobiliproteins production (175 mg PBP/g DW) than this study (114 – 129 mg PBP/g DW), in spite of much lower nutrients concentrations, suggesting that *P. mucicola* might produce more phycobiliproteins than *A. platensis*. Wood et al. (2015) demonstrated that wastewater from oil and natural gas extraction, amended with 3 g $NaNO_3$/L and 0.5 g K_2HPO_4/L, could support growth of a cyanobacterial consortium, mainly composed by Oscillatoriales, but produced phycocyanin yields (16.9 mg/g DW) much lower than in this study. This might be explained by the mixed culture used (instead of unialgal cultures applied in this work) or the distinct configuration used (rotating algal biofilm reactor). Similarly, Van Den Hende et al. (2016b) carried out a study treating the UASB effluent from the same source of this work and reported lower phycobiliproteins contents and purity levels using one-step extraction, which might also be due to the mixed culture applied (containing cyanobacteria *Geminocystis* sp. and diatoms).

Although comparisons with the phycobiliproteins productions in standard growth media and wastewater have been discussed in this section, it is noteworthy to highlight that these comparisons considered only the parameters evaluated in both experiments, i.e. how light conditions and growth medium concentrations, combined with available nutrients could affect the content and production of phycobiliproteins. Hence, in the future, further optimisation can be performed in terms of other medium components and operation conditions, in order to have a holistic understanding on the performance of these three species in wastewater, especially when aiming for biomass valorisation (phycobiliproteins production and purity factor).

Table 5-3. Summary of average values of wastewater treatment efficiency and phycobiliproteins extracted in this study compared to other studies using similar microalgae species grown in wastewater.

Microalgae species	Cultivation medium	Treatment efficiency	μ_{max}	Maximum PBP concentration	Maximum Purity	Reference
		mg/Ld (removal)	**d^{-1}**	**mg/g DW**	**-**	
Nostoc sp.	50% UASB effluent (food industry) + 50% BG-11	NH_4^+-N: 4.43 (100%) NO_3^--N: 10.83 (92%) TIN: 15.25 (94%) PO_4^{3-}-P: 0.91 (100%) COD: 4.8 (45%)	0.19	Total PBP: 178.7 PC: 103.2 APC: 57.4 PE: 18.2	PC: 0.56 APC: 0.32 PE: 0.35	This study
Nostoc sp.	Urban wastewater	NH_4^+-N: 1.8 (91%) NO_3^--N: 0.05 (46%) BOD: 4.6 (92%)	ND [a]	ND [a]	ND [a]	Sharma and Khan (2013)
Nostoc muscorum	Sterilised municipal wastewater	NH_3: 1.5 (91.5%) NO_3: 0.008 (80%) PO_4^{3-}: 0.245 (60%) COD: 9.0 (50%)	ND [a]	ND [a]	ND [a]	El-Sheekh et al. (2014)
Nostoc muscorum	83.3% Textile industry effluent + 16.7% BG-11	COD: 12 mg/Ld (32%) BOD: 4 mg/Ld (55%)	ND [a]	ND [a]	ND [a]	Talukder et al. (2015)

(Table continued on the next page)

Table 5-3. *(Continued)*

A. platensis	50% UASB effluent (food industry) + 50% ASW	NH_4^+-N: 3.8 (100%) NO_3^--N: 0.7 (87%) TIN: 4.6 (98%) PO_4^{3-}-P: 0.4 (92%) COD: 9.8 (83%)	0.15	Total PBP: 128.5 PC: 78.8 APC: 38.2 PE: 11.5	PC: 0.60 APC: 0.31 PE: 0.36	This study
A. platensis	75% UASB effluent (food industry) + 25% ASW	NH_4^+-N: 6.2 (100%) NO_3^--N: 1.1 (89%) TIN: 7.2 (98%) PO_4^{3-}-P: 0.8 (81%) COD: 13.3 (84%)	0.17	Total PBP: 113.7 PC: 70.9 APC: 32.2 PE: 10.6	PC: 0.52 APC: 0.27 PE: 0.31	This study
A. platensis	Wastewater from thai rice noodle factory (supplemented with nutrients)	ND [a]	ND [a]	PC: 140	ND [a]	Vetayasuporn (2004)
A. platensis	20% UASB effluent (swine) + 25% ASW	TIN: 8 (89%) PO_4^{3-}-P: 4.3 (57%)	ND [a]	PC: 195	ND [a]	Chaiklahan et al. (2010)
A. platensis	Anaerobically treated swine wastewater (diluted and supplemented with nutrients)	NH_4^+-N: 1.0 (92%) NO_3^--N: 0.08 (49%) PO_4^{3-}-P: 0.39 (67%) COD: 0.27 (23%)	≈ 0.25	ND [a]	ND [a]	Cheunbarn and Peerapornpisal (2010)
A. platensis	Synthetic black water (seawater) + grey water (freshwater)	NH_4^+-N: 3.6 - 6.4 (50 - 95%) TP: 0.9 - 1.3 (> 90%) COD: 25 - 100 (62 - 96%)	≈ 0.22	ND [a]	ND [a]	Zhou et al. (2017)
A. platensis	Digestate diluted (6%) in carbonate buffer	ND [a]	≈ 0.43	PC: 86.2 APC: 41.3	ND [a]	Hultberg et al. (2017)

(Table continued on the next page)

Table 5-3. *(Continued)*

A. platensis	Secondary domestic effluent	NH_4^+-N: 4.1 (17%) NO_3^--N: 0.67 (24%) PO_4^{3-}-P: 0.58 (15%) COD: 14.33 (18%)	≈ 0.51	ND [a]	ND [a]	Chavan and Mutnuri (2019)
A. platensis	Palm oil mill effluent (varied concentrations)	ND [a]	0.06 - 0.30	PC: 12.96 - 22.69 (mg/L)	ND [a]	Nur et al. (2019)
Mixed cyanobacteria (mainly Oscillatoriales)	Oil and gas extraction wastewater	ND [a]	≈ 0.16	PC: 16.9	PC: 0.23	Wood et al. (2015)
Mixed culture containing *Geminocystis* sp. and diatoms)	UASB effluent (food industry)	NH_4^+-N: 2.37 (98%) NO_3^--N: -0.60 TIN: 1.28 (53%) TP: 0.14 (31%) COD: 12 (67%)	-	PC: 61.1 PE: 30.1	PC: 0.43 PE: 0.36	(Van Den Hende et al., 2016a, 2016b)
Pseudanabaena mucicola	Cage culture effluent	NH_4^+-N: 0.79 (99%) NO_2^--N: 0.62 (76%) PO_4^{3-}-P: 0.19 (28%)	≈ 0.41	Total PBP: ≈175 PC: ≈102	PC: 0.84	Khatoon et al. (2018)
P. purpureum	50% UASB effluent (food industry) + 50% ASW	NH_4^+-N: 4.4 (100%) NO_3^--N: 0.4 (100%) TIN: 4.7 (100%) PO_4^{3-}-P: 0.5 (100%) COD: 11.7 (76%)	0.09	Total PBP: 35.6 PC: 6.1 APC: 2.8 PE: 26.7	PC: 0.12 APC: 0.06 PE: 0.72	This study

(Table continued on the next page)

Table 5-3. *(Continued)*

P. purpureum	75% UASB effluent (food industry) + 25% ASW	NH_4^+-N: 5.7 (98%) NO_3^--N: 0.5 (66%) TIN: 6.3 (94%) PO_4^{3-}-P: 1.1 (98%) COD: 15.7 (79%)	0.12	Total PBP: 46.6 PC: 9.4 APC: 6.7 PE: 30.4	PC: 0.15 APC: 0.10 PE: 0.66	This study
P. purpureum	ASW*	NO_3^--N: 9.0 (60%) TIN: 8.9 (59%) TP: 1.2 (93%)	0.37	Total PBP: 93.2 PE: 70.6		This study [b]
P. purpureum	ASW*	ND [a]	ND [a]	Total PBP: 47.8 PE: 33.0	ND [a]	Kathiresan et al. (2007)
P. purpureum	F/2-RSE medium*	NO_3^--N: 2.5 (100%)	≈ 0.17	Total PBP: 14.1 PE: 12.1 PC: 1.8	ND [a]	Guihéneuf and Stengel (2015)
P. purpureum	F/2 medium*	ND [a]	0.005 – 0.08	PE: 3.10	ND [a]	Sosa-Hernández et al. (2019)

Acronyms: APC (allophycocyanin), ASW (artificial seawater), COD (chemical oxygen demand), DW (dry weight), PBP (phycobiliprotein), PC (phycocyanin), PE (Phycoerythrin), TIN (total inorganic nitrogen), TKN (total Kjeldahl nitrogen), UASB (upflow anaerobic sludge blanket).

[a] No data reported

[b] Results corresponding to L1C3

*No other study applying *P. purpureum* in wastewater has been found in the literature, so comparisons were done with cultivations in standard growth media.

The results from the present study described the potential of applying cyanobacteria, such as *Nostoc* sp. and *A. platensis,* and red microalgae, such as *P. purpureum*, for combining wastewater treatment and resources recovery. This work has focused on the phycobiliproteins production from these two species, but several researchers highlights the potential of microalgae as a factory for high-value compounds, since its composition can be modified according to operational variables to obtain protein enrichment, such as reducing residence times (Rebolloso Fuentes et al., 2000), semi continuous or continuous cultivation mode (Guihéneuf and Stengel, 2015) or mixotrophic growth (Fábregas et al., 1999).

Finally, this study showed that industrial wastewater could be applied as a medium in order to not only promote biomass growth and cleaner water, but also to reduce typical high costs to produce valuable compounds from microalgae, such as pigments. The wastewater was an effluent from a food processing company, so no potential contaminants were detected (Table A-3, Appendix). This was already expected, since the company has to comply with high standards, according to food safety regulations. However, it is important to mention that for further development of the process proposed in this study, biosafety concerns have to be considered. Depending on the desired application of the pigments, further analyses should be done to assure that the bioproducts do not present any potential risk. In this particular study, the pigments extracted will be used for a project of art and design, highlighting the applicability of such bioproducts as natural textile dyes (Ferrándiz et al., 2016; Moldovan et al., 2017) and raising no major concerns regarding potential risks by contaminants. Future research is thus encouraged in order to address the current challenges, such as cultivation systems and extraction methods. This way, once technical feasibility and economic viability of this concept is ensured, its further development as a resource recovery solution should move towards regulations analysis and decision-making processes.

5.4 Conclusions

This study suggested that, in general, light conditions had more influence on biomass growth and phycobiliproteins production than the medium composition in the

cultivations of *Nostoc* sp., *A. platensis* and *P. purpureum*. The three species showed efficient treatment of industrial wastewater reaching high COD and nutrients removal, while successfully biosynthesizing high-value compounds in their biomass.

Phycocyanin, allophycocyanin and phycoerythrin were successfully extracted from the biomass reaching concentrations up to 103, 57 and 30 mg/g DW, respectively. In general, phycobiliproteins obtained in this study were significantly higher than the results described in **Chapter 4**, in which cyanobacteria-dominated biomass reached up to 17 and 7.2 mg/g DW of phycocyanin and phycoerythrin. This shows the discrepancy most probably related to the composition of biomass (mixed vs. unialgal cultures).

This study encourages further investigations on the feasibility of this process, as well as research developments with a holistic approach to explore other synergetic opportunities associated with the nexus of water and sustainable resource recovery processes.

Chapter 6

Life Cycle Assessment – Biogas and biofertiliser recovery

LCA of HRAPs for wastewater treatment and recovery of biogas and biofertiliser

Picture on previous page:
Sustainability (Copyright free image available at *pngfuel.com*).

Abstract

The aim of this study was to assess the potential environmental impacts associated with high rate algal ponds (HRAP) systems for wastewater treatment and resource recovery in small communities. To this aim, a Life Cycle Assessment (LCA) and an economic assessment were carried out evaluating two alternatives: i) a HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production); ii) a HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production). Additionally, both alternatives were compared to a typical small-sized activated sludge system. The results showed that HRAPs system coupled with biogas production appeared to be more environmentally friendly than HRAPs system coupled with biofertiliser production in the climate change, ozone layer depletion, photochemical oxidant formation, and fossil depletion impact categories. Different climatic conditions have strongly influenced the results obtained in the eutrophication and metal depletion impact categories, with the HRAPs system located where warm temperatures and high solar radiation are predominant showing lower impact. In terms of costs, HRAPs systems seemed to be more economically feasible when combined with biofertiliser production instead of biogas.

This chapter has been redrafted after:

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6. Life Cycle Assessment of HRAPs for wastewater treatment and recovery of biogas and biofertiliser

6.1 Introduction

As previously mentioned in Section 2.1.1, HRAPs for wastewater treatment were introduced around 50 years ago and used since then not only to grow microalgae biomass but also to treat a wide variety of municipal and industrial wastewaters (Craggs et al., 2014; Oswald and Golueke, 1960). These systems are shallow, paddlewheel mixed, raceway ponds where microalgae assimilate nutrients and produce oxygen, which is used by heterotrophic bacteria to oxidise organic matter improving water quality (Craggs et al., 2014; Park et al., 2011). Since mechanical aeration is not required, energy consumption in these systems is much lower compared to a conventional wastewater treatment plant (e.g. activated sludge system) (around 0.02 kWh/m^3 of water vs. 1 kWh/m^3 of water, respectively) (Garfí et al., 2017; Passos et al., 2017). Moreover, HRAPs are less expensive and require little maintenance compared to conventional systems (Craggs et al., 2014; Garfí et al., 2017; Molinos-Senante et al., 2014). Due to their low cost and low energy consumption, HRAPs systems could have a wide range of applications in Mediterranean regions, which present suitable climatic conditions for microalgae growth (e.g. high solar radiation). However, to achieve a satisfactory performance, large land area is required compared to conventional systems (around 6 m^2/p.e. vs. 0.5 m^2/p.e. for HRAPs and activated sludge systems, respectively), making them more suitable for small communities (up to 10,000 p.e.).

Nowadays, there is an important need to shift the paradigm from wastewater treatment to resource recovery to alleviate negative effects associated with human activities, such as pollution of water bodies, greenhouse gas (GHG) emissions and scarcity of mineral resources. In this context, microalgae grown in HRAPs can be harvested and reused to produce biofuels or other non-food bioproducts. In particular, intensive research has been developed during the last years to investigate the potential of microalgae to produce biofuels such as biogas. Indeed, the biogas produced from microalgal biomass was found to contain high energy value, making microalgae

anaerobic digestion an attractive alternative for biofuel production (Chew et al., 2017; Jankowska et al., 2017; Montingelli et al., 2015; Uggetti et al., 2017). On the other hand, microalgae also offer the potential to recover nutrients from wastewater and, subsequently, to be applied as a sustainable fertiliser. During the last decade, this alternative has been described by several authors, considering the fact that microalgae contain high amounts of proteins rich in essential amino acids, as well as phytohormones that stimulate plant growth (Coppens et al., 2016; Garcia-Gonzalez and Sommerfeld, 2016; Jäger et al., 2010; Uysal et al., 2015).

Recent studies have employed the Life Cycle Assessment (LCA) methodology to assess the environmental impact of HRAPs systems for wastewater treatment. They demonstrated that HRAPs might help to reduce environmental impacts and costs associated with wastewater treatment compared to conventional systems (e.g. activated sludge system), especially in small communities (Garfí et al., 2017; Maga, 2017). These studies also highlighted that the LCA methodology is an appropriate tool to support early-stage research and development of novel technologies and processes (Fang et al., 2016; Garfí et al., 2017). Indeed, LCA methodology considers and quantifies all environmental exchanges (i.e. resources, energy, emissions, waste) occurring during all stages of the technology life cycle (Ferreira et al., 2017, 2014; ISO, 2000).

The objective of this work was to evaluate the potential environmental impacts associated with HRAPs systems for wastewater treatment considering two resource recovery strategies. To this aim a LCA was carried out comparing the following alternatives: (i) a HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production); (ii) a HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production). For the sake of comparison, both scenarios were compared to a typical small-sized activated sludge system. Additionally, an economic evaluation was addressed in order to assess the feasibility of the HRAPs alternatives based on the costs and benefits related to each of them.

This Chapter is organised as follows: Section 6.2 describes the wastewater treatment systems, as well as the methodology used for the LCA and the economic analysis; in

Section 6.3 the results of the comparative LCA and the economic analysis are described; finally, in Section 6.4 the main conclusions are highlighted.

6.2 Materials and Methods

6.2.1 Wastewater treatment systems description

The HRAPs systems were hypothetical wastewater treatment plants based on extrapolation from lab-scale and pilot-scale studies (up to 100 m^2). The systems were designed to serve a population equivalent of 10,000 p.e. and treat a flow rate of 1,950 m^3/d. The HRAPs system coupled with biogas production was considered to be implemented in Catalonia (Barcelona, Spain), where the mean temperature and global solar radiation are 15.5°C and 4.56 kWh/m^2d, respectively (AEMET, 2017). Figure 6-1 shows the flow diagrams of this scenario. For this case study, the design parameters were calculated taking into account the experimental results obtained in lab-scale and pilot systems (up to 5 m^2) located at the Universitat Politècnica de Catalunya-BarcelonaTech (UPC) (Barcelona, Spain), as described in **Chapter 3** (García et al., 2006, 2000; Gutiérrez et al., 2016; Passos and Ferrer, 2014; Solé-Bundó et al., 2019a, 2017b). This system comprises a primary settler (Hydraulic Retention Time (HRT): 2.5 h) followed by four HRAPs (Table 6-1). From these units, wastewater goes through a secondary settler (HRT: 3 h) where microalgal biomass is harvested and separated from wastewater. Treated water is then discharged into a surface water body. Part of the harvested microalgal biomass (2 and 10 % on a dry weight basis in summer and winter, respectively) is recycled in order to enhance spontaneous flocculation (bioflocculation) and increase microalgae harvesting efficiency (Gutiérrez et al., 2016). The remaining harvested biomass is thickened (HRT: 24 h), thermally pre-treated (75 °C, 10 h) and co-digested with primary sludge (35 °C, 20 days). The biogas produced is then converted in a combined heat and power (CHP) unit, while the digestate is transported and reused in agriculture. In this context, the HRT of each HRAP has to be modified over the year (8, 6 and 4 days) in accordance with the weather conditions (i.e. solar radiation and temperature) in order to accomplish wastewater treatment and meet effluent quality

requirements for discharge (García et al., 2000; Gutiérrez et al., 2016). For this reason, it was considered that during summer months (from May to July) only two HRAPs work in parallel (HRT: 4 days), whereas all of them are operated during winter months (from November to April) (HRT: 8 days). During the rest of the year (from August to October), the HRT is 6 days (3 HRAPs working in parallel).

The HRAPs system coupled with biofertiliser production was considered to be implemented in Andalucía (Almeria, Spain), where the mean temperature and global solar radiation are 19.1°C and 5.29 kWh/m^2d, respectively (AEMET, 2017). Figure 6-2 shows the flow diagrams of this scenario. For this case study, the designed parameters were determined using the results obtained in a pilot system located at the Las Palmerillas Experimental Station (Almeria, Spain) (100 m^2) (Morales-Amaral et al., 2015a). This system consists of two HRAPs operating in parallel and followed by a settler (HRT: 3 h) where microalgal biomass is separated using an organic flocculant (Table 6-2). From this unit, treated wastewater is discharged into a surface water body, while harvested microalgae biomass is dewatered on-site using a centrifuge and later sold to a local company to produce a biofertiliser (NPK = 5-1-0.75). The biofertiliser produced from the dewatered biomass is then transported and reused in agriculture. In this case, due to the more favourable climatic conditions for microalgae growth compared to Catalonia, the HRT was the same over the year (HRT: 3 days). It has to be noted that, for the same reason, the microalgal biomass production is considerably higher in the system implemented in Andalucía with respect to the one located in Catalonia (3-26 g m^2/d vs. 15-30 g m^2/d, respectively) (Gutiérrez et al., 2016; Morales-Amaral et al., 2015a).

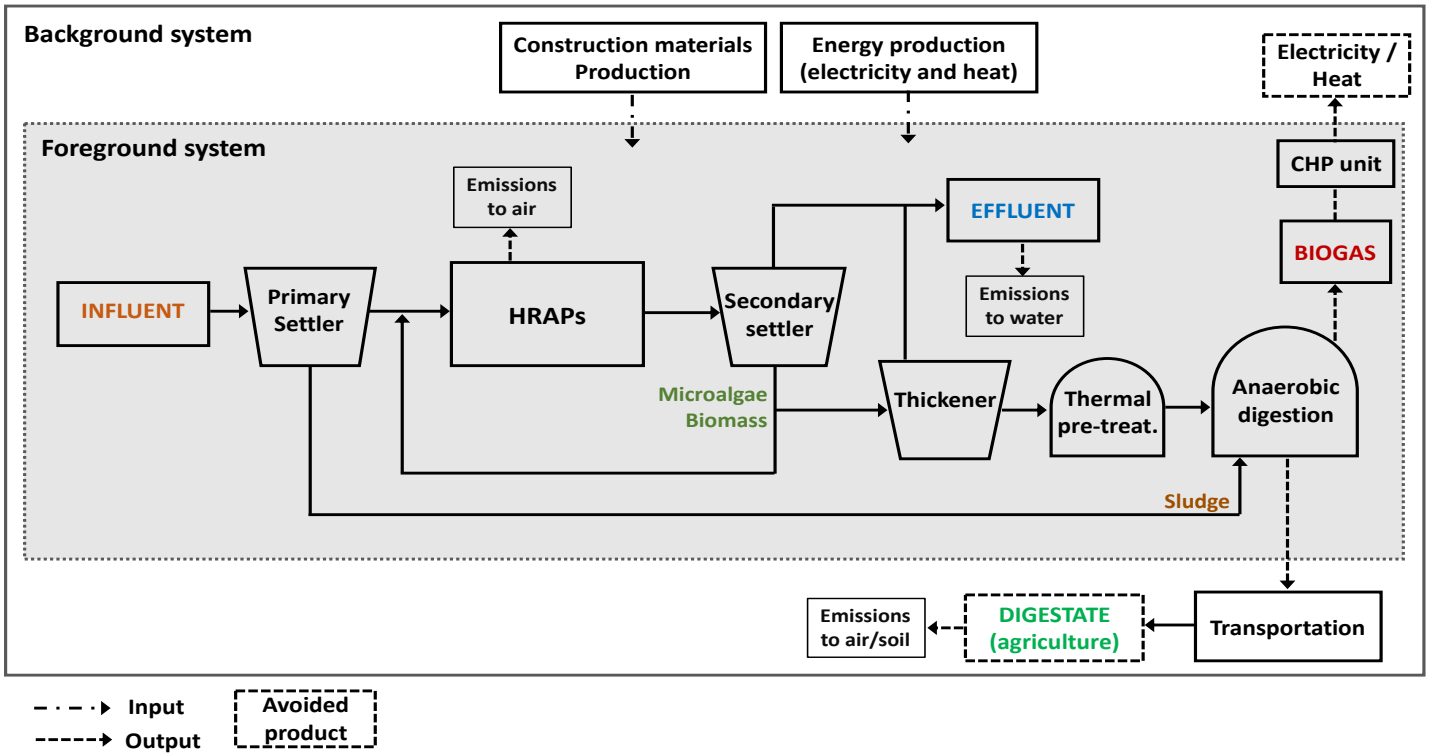

Figure 6-1. Flow diagram and system boundaries of the Scenario 1: HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production).

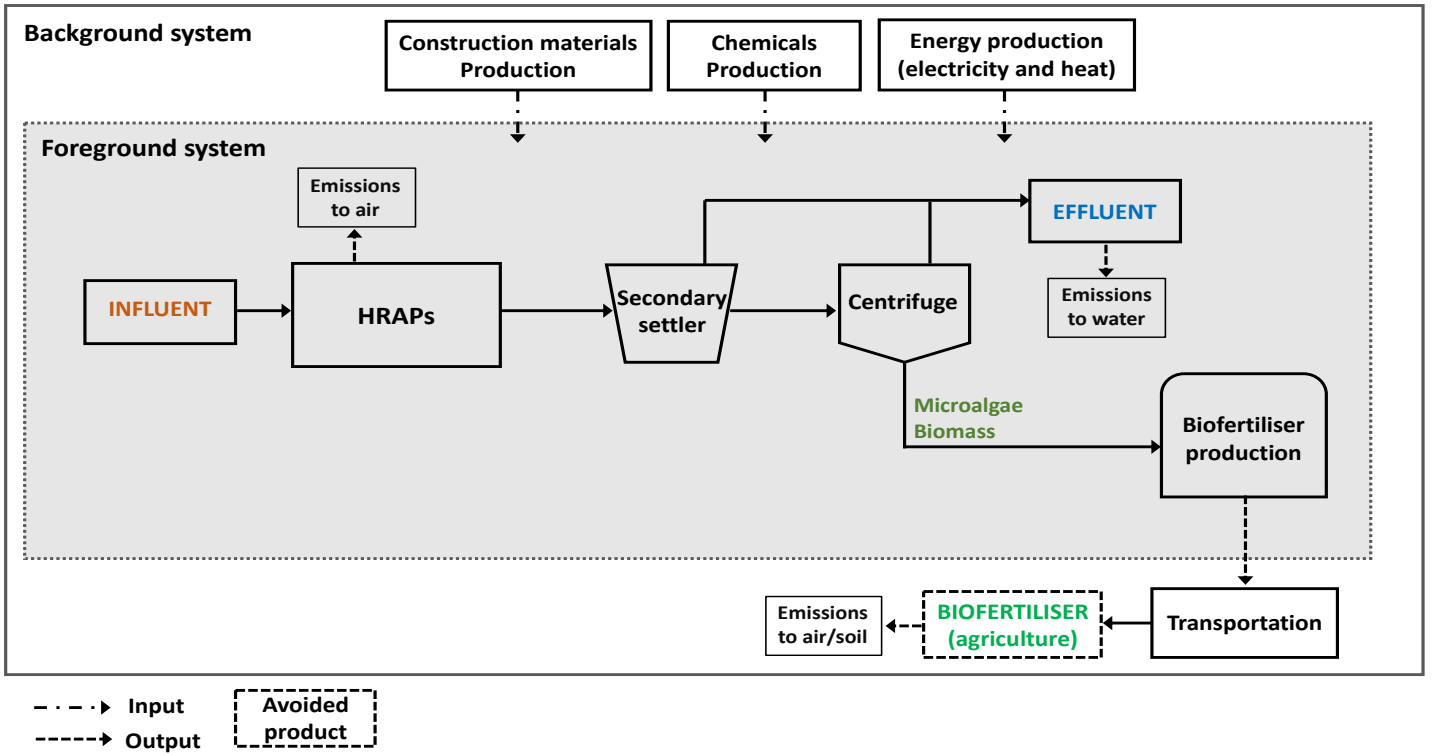

Figure 6-2. Flow diagram and system boundaries of the Scenario 2: HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production).

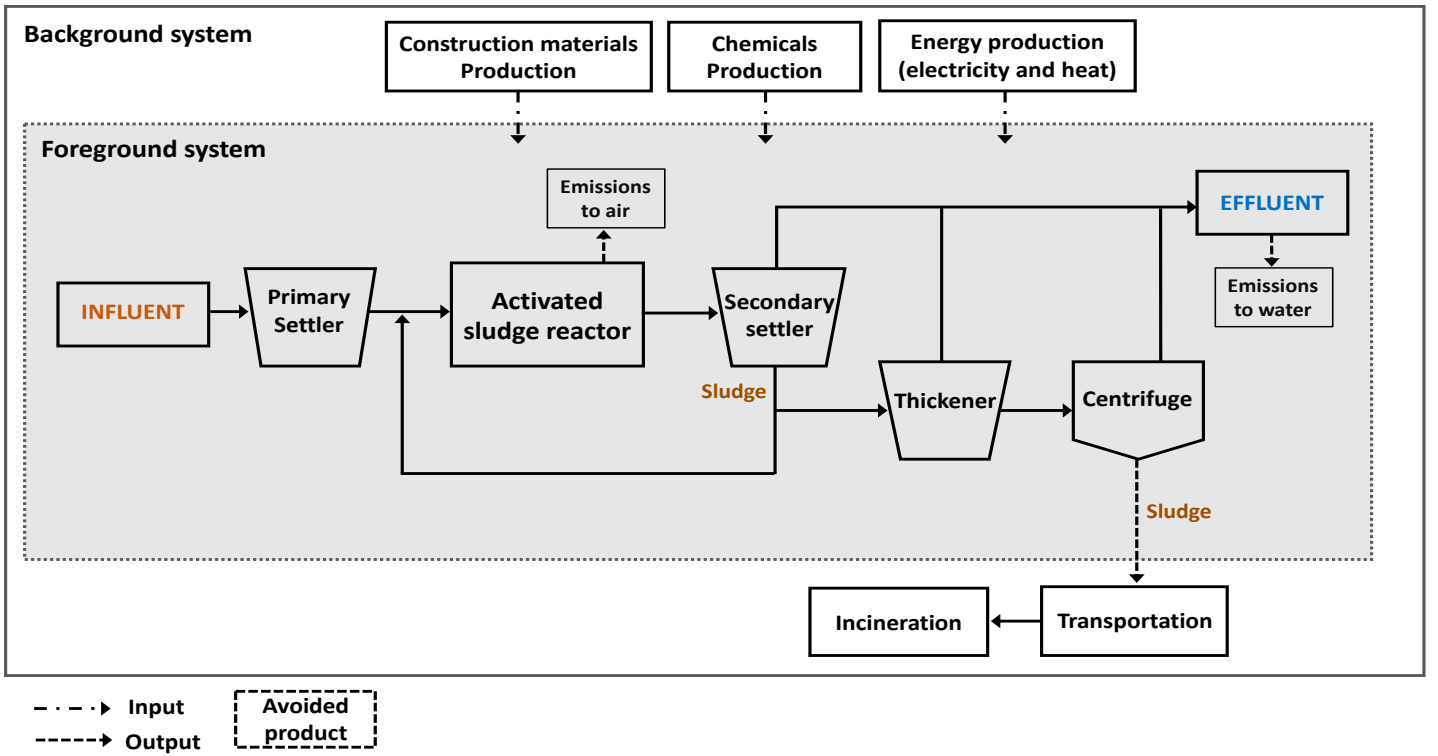

Figure 6-3. Flow diagram and system boundaries of the Scenario 3: Activated sludge system.

Table 6-1. Characteristics and design parameters of the HRAPs coupled with biogas production (Scenario 1).

System characteristics	**Unit**			
Inlet BOD_5 concentration	mg/L	300		
Outlet BOD_5 concentration	mg/L	<25		
Inlet TSS concentration	mg/L	150		
Outlet TSS concentration	mg/L	<35		
Inlet Total Nitrogen	mg/L	39		
Outlet Total Nitrogen	mg/L	9.38		
Inlet Total Phosphorous	mg/L	5		
Outlet Total Phosphorous	mg/L	3.69		
Flow rate	m^3/d	1,950		
Population equivalent	p.e.	10,000		
Total surface area	m^2	40,000		
Specific area requirement	m^2/p.e.	4		
HRAPs Design parameters	**Unit**			
Organic loading rate	g_{BOD} m^2/d	10		
Channel width	m	12		
Channel length	m	812.5		
Water depth	m	0.4		
Annual average microalgae biomass production	g m^2/d	12		
		Summer	**Winter**	**Rest of the year**
Hydraulic retention time	d	4	8	6
Number of ponds	-	2	4	3
Microalgae biomass production	g m^2/d	25.8	3.3	10.5

Acronyms: Biochemical oxygen demand (BOD); Total suspended solids (TSS).
Summer: May to July; Winter: November to April.

Table 6-2. Characteristics and design parameters of the HRAPs coupled with biofertiliser production (Scenario 2).

System characteristics	**Unit**			
Inlet BOD_5 concentration	mg/L	300		
Outlet BOD_5 concentration	mg/L	<25		
Inlet TSS concentration	mg/L	200		
Outlet TSS concentration	mg/L	<35		
Inlet Total Nitrogen	mg/L	50		
Outlet Total Nitrogen	mg/L	2		
Inlet Total Phosphorous	mg/L	10		
Outlet Total Phosphorous	mg/L	1		
Flow rate	m^3/d	1,950		
Population equivalent	p.e.	10,000		
Total surface area	m^2	30,000		
Specific area requirement	m^2/p.e.	3		
HRAPs Design parameters	**Unit**			
Organic loading rate	g_{BOD} m^2/d	20		
Channel width	m	12		
Channel length	m	1,219		
Water depth	m	0.2		
Annual average microalgae biomass production	g m^2/d	23		
Hydraulic retention time	d	3		
Number of ponds	-	2		
		Summer	**Winter**	**Rest of the year**
Microalgae biomass production	g m^2/d	30	15	25

Acronyms: Biochemical oxygen demand (BOD); Total suspended solids (TSS).
Summer: May to August; Winter: November to March.

For the sake of comparison, the potential environmental impacts of the HRAPs systems were compared to those generated by a conventional small-sized wastewater treatment plant (10,000 p.e.). For that purpose, the design of a usual small-scale activated sludge system implemented in Spain was taken into account (Gallego et al., 2008; Garfí et al., 2017; Lorenzo-Toja et al., 2015). Figure 6-3 shows the flow diagrams of this scenario. It comprises a primary settler, followed by an activated sludge reactor with extended aeration and a secondary settler (Table 6-3). Treated water is discharged into the environment and the sludge is conditioned, thickened, centrifuged on-site and then transported to an incineration facility.

Table 6-3. Characteristics and design parameters of the activated sludge system (Scenario 3).

System characteristics	**Unit**	
Inlet BOD_5 concentration	mg/L	300
Outlet BOD_5 concentration	mg/L	<25
Outlet TSS concentration	mg/L	<35
Flow rate	m^3/d	1,950
Population equivalent	p.e.	10,000
Total surface area	m^2	900
Specific area requirement	m^2/p.e.	0.6
Design parameters	**Unit**	
Primary settler HRT	h	2.5
Activated sludge reactor HRT	h	6
Secondary settler HRT	h	2

Acronyms: Biochemical oxygen demand (BOD); Total suspended solids (TSS); Hydraulic Retention Time (HRT).

6.2.2 *Life Cycle Assessment*

The LCA was conducted following the ISO standards (ISO, 2006, 2000) in order to evaluate and quantify the potential environmental impact of the investigated scenarios. It consisted of four main stages: i) goal and scope definition, ii) inventory analysis, iii) impacts assessment and iv) interpretation of the results (ISO, 2006), as previously

described in **Chapter 3**. The following sections describe the specific content of each phase.

6.2.2.1 Goal and scope definition

The goal of this study was to determine the potential environmental impact of HRAPs systems for wastewater treatment and resource recovery. In particular, two configurations were compared:

a) a HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production) (Scenario 1);

b) a HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production) (Scenario 2).

Moreover, both scenarios were compared to a typical small-sized activated sludge system implemented in Spain (Scenario 3). The functional unit (FU) for this study was set as 1 m^3 of treated water, since the main function of the technologies proposed is to treat wastewater.

The cradle-to-grave boundaries included systems construction, operation and maintenance over a 20-years period (Garfí et al., 2017; Pérez-López et al., 2017; Rahman et al., 2016) (Figure 6-1, Figure 6-2, and Figure 6-3). Input and output flows of materials (i.e. construction materials and chemicals) and energy resources (heat and electricity) were systematically studied for all scenarios. Direct GHG emissions and NH_4^+ volatilisation associated with wastewater treatment were also included in the boundaries. As treated water is discharged into the environment, direct emissions to water were also taken into account. Regarding digestate and biofertiliser reuse in agriculture in Scenarios 1 and 2, transportation (20 km) (Hospido et al., 2004) and direct emissions to soil (heavy metals), as well as direct GHG emissions, were accounted for. In the case of the activated sludge system (Scenario 3), inputs and outputs associated with sludge disposal (i.e. incineration) were also included in the boundaries. An average distance of 30 km was considered for sludge transportation to incineration facilities, based on circumstances

generally observed in our zone. The end-of-life of infrastructures and equipment were neglected, since the impact would be marginal compared to the overall impact.

Since the studied scenarios would generate by-products (i.e. biogas, biofertiliser), the system expansion method has been used following the ISO guidelines (Guinée, 2002; ISO, 2006). In this method, by-products are supposed to avoid the production of conventional products. Thus, the impact related to conventional products is withdrawn from the overall impact of the system (Collet et al., 2011; ISO, 2006; Sfez et al., 2015). In this study, the digestate and the biofertiliser produced in HRAPs systems coupled with biogas and biofertiliser production (Scenarios 1 and 2, respectively) were considered as substitutes to chemical fertiliser. Moreover, the avoided burdens of using heat and electricity produced in Scenario 1 (HRAPs systems coupled with biogas production), instead of heat from natural gas and electricity supplied through the grid, were also considered.

6.2.2.2 Inventory analysis

Inventory data for the investigated scenarios are summarized in Table 6-4, Table 6-5 and Table 6-6. In the case of HRAPs systems coupled with biogas and biofertiliser production (Scenarios 1 and 2), inventory data regarding construction materials and operation were based on the detailed engineering designs performed in the frame of this study. Treated wastewater characteristics were estimated considering the removal efficiencies and experimental results obtained in the pilot systems implemented at the Universitat Politècnica de Catalunya-BarcelonaTech (UPC) (5 m^2) (Gutiérrez et al., 2016) and at the Las Palmerillas Experimental Station (100 m^2) (Morales-Amaral et al., 2015a) for Scenarios 1 and 2, respectively. NH_4^+ volatilisation was estimated through nitrogen mass balance. NH_3 and N_2O emissions due to the application of digestate and biofertiliser on agricultural land were calculated using emissions factors from the literature (Hospido et al., 2008; IPCC, 2006; Lundin et al., 2000). In this case, CH_4 emissions were not considered since anaerobic decompositions do not occur if liquid fertiliser is used and the climate is predominantly dry (IPCC, 2000; Lundin et al., 2000). Heavy metals and nutrients (avoided Total Nitrogen (TN) and Total Phosphorous (TP))

content of the digestate and biofertiliser were gathered from experimental results obtained in the above-mentioned pilot systems (Morales-Amaral et al., 2015a; Solé-Bundó et al., 2017b). In order to estimate electricity and heat production from biogas cogeneration in Scenario 1 (HRAPs systems coupled with biogas production), biogas production obtained in lab-scale experiments was taken into account (Passos et al., 2017; Solé-Bundó et al., 2019a).

As mentioned above, data regarding the typical small-sized activated sludge system implemented in Spain (Scenario 3) were gathered from the literature (Gallego et al., 2008; Garfí et al., 2017; Lorenzo-Toja et al., 2015).

Background data (i.e. data of construction materials, chemicals, energy production, avoided fertiliser, transportation and sludge incineration process) were obtained from the *Ecoinvent 3.1* database (Moreno Ruiz et al., 2014; Weidema et al., 2013). The Spanish electricity mix was used for all electricity requirements (Red Eléctrica Española, 2016).

Table 6-4. Summary of the inventory for Scenario 1: HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production). Values are referred to the functional unit (1 m^3 of water).

Inputs	Scenario 1	Units
Construction materials		
Primary settler		
Concrete	2.55E-06	m^3/m^3
Steel	2.04E-04	kg/m^3
HRAPs		
Concrete	5.94E-04	m^3/m^3
Steel	4.76E-02	kg/m^3
Secondary settler		
Concrete	1.29E-05	m^3/m^3
Steel	1.03E-03	kg/m^3
Thickener		
Concrete	1.78E-07	m^3/m^3
Steel	1.42E-05	kg/m^3
Thermal pretreatment		
Concrete	2.77E-07	m^3/m^3
Steel	2.22E-05	kg/m^3
Digester		
Concrete	9.79E-06	m^3/m^3
Steel	7.83E-04	kg/m^3
Operation		
Energy consumption[a]		
Primary settler	4.41E-03	kWh/m^3
HRAPs	1.13E-02	kWh/m^3
Secondary settler	2.52E-03	kWh/m^3
Thermal pretreatment	1.08E-04	kWh/m^3
Digester	4.17E-02	kWh/m^3
Total energy consumption	6.00E-02	kWh/m^3

(Table continued on next page)

Table 6-4. (*Continued)*

Outputs	Scenario 1	Units
Emissions to water[a]		
Total COD	7.63E+01	g/m^3
TSS	2.40E+01	g/m^3
TN	9.38E+00	g/m^3
TP	3.69E+00	g/m^3
Emissions to air[a]		
NH_3 volatilisation in HRAPs		
NH_3	3.80E+00	g/m^3
Digestate application as fertiliser		
NH_3	6.47E+00	g/m^3
N_2O	2.59E-01	g/m^3
Emissions to soil[a]		
Digestate application as fertiliser		
Cd	3.53E-03	g/m^3
Cu	2.02E-01	g/m^3
Pb	9.08E-02	g/m^3
Zn	9.04E-01	g/m^3
Ni	4.15E-02	g/m^3
Cr	5.22E-02	g/m^3
Hg (value <)	4.52E-04	g/m^3
Avoided products[a]		
Electricity (from biogas cogeneration)	5.40E-01	kWh/m^3
Heat (from biogas cogeneration)	8.49E-01	kWh/m^3
N as Fertiliser (from digestate reuse)	2.59E+01	g/m^3
P as Fertiliser (from digestate reuse)	1.31E+00	g/m^3

[a]*Annual averages*

Table 6-5. Summary of the inventory for Scenario 2: HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production). Values are referred to the functional unit (1 m^3 of water).

Inputs	**Scenario 2**	**Units**
Construction materials		
HRAPs		
Concrete	4.32E-04	m^3/m^3
Steel	3.45E-02	kg/m^3
Secondary settler		
Concrete	1.29E-05	m^3/m^3
Steel	1.03E-03	$kg\ m^{-3}$
Centrifuge		
Steel	3.86E-05	kg/m^3
Operation		
Energy consumption[a]		
HRAPs	1.11E-02	kWh/m^3
Secondary settler	5.77E-03	kWh/m^3
Centrifuge	1.15E-02	kWh/m^3
Biofertiliser production	4.70E-02	kWh/m^3
Total energy consumption	7.54E-02	kWh/m^3
Chemicals[a]		
Organic flocculant	1.00E+01	kg/m^3
Outputs	**Scenario 2**	**Units**
Emissions to water[a]		
Total COD	1.00E+02	g/m^3
TSS	5.00E+01	g/m^3
TN	2.00E+00	g/m^3
TP	1.00E+00	g/m^3
Emissions to air[a]		
NH_3 volatilisation in HRAPs		
NH_3	5.00E+00	g/m^3
Biofertiliser		
NH_3	1.44E+00	g/m^3
N_2O	5.77E-02	g/m^3

(Table continued on next page)

Table 6-5. (*Continued)*

Outputs	Scenario 2	Units
Emissions to soil[a]		
Biofertiliser		
Cd	3.46E-04	g/m^3
Cu	4.62E-02	g/m^3
Pb	2.31E-02	g/m^3
Zn	1.15E-02	g/m^3
Ni	1.15E-02	g/m^3
Cr	3.46E-02	g/m^3
Hg (value <)	2.31E-04	g/m^3
Avoided products[a]		
N as Fertiliser (from biofertiliser)	5.77E+00	g/m^3
P as Fertiliser (from biofertiliser)	1.20E+00	g/m^3

[a]*Annual averages*

Table 6-6. Summary of the inventory for Scenario 3: typical small-sized activated sludge system implemented in Spain. Values are referred to the functional unit (1 m^3 of water).

Inputs	**Scenario 3**	**Units**
Construction materials		
Concrete	1.65E-05	m^3/m^3
Steel	1.32E-03	kg/m^3
Operation		
Energy consumption		
Electricity	8.90E-01	kWh/m^3
Chemicals		
Polyelectrolyte	1.98E+00	g/m^3
Coagulant	3.18E+00	g/m^3
Outputs	**Scenario 3**	**Units**
Emissions to water		
Total COD	1.25E+02	g/m^3
TSS	3.50E+01	g/m^3
TN	1.50E+01	g/m^3
TP	2.00E+00	g/m^3
Emissions to air		
CO_2	1.70E-01	g/m^3
N_2O	1.10E-01	g/m^3
Waste to further treatment		
Sludge (incineration)	1.24E+00	kg/m^3

6.2.2.3 Impact assessment

The LCA was performed using the software *SimaPro® 8* ("PRé Sustainability," 2014). Potential environmental impacts were calculated by the ReCiPe midpoint method (hierarchist approach) (Goedkoop et al., 2009). In this study, characterisation phase was performed considering the following impact categories: Climate Change, Ozone Depletion, Terrestrial Acidification, Photochemical Oxidant Formation, Marine Eutrophication, Freshwater Eutrophication, Terrestrial Ecotoxicity, Human Toxicity, Metal Depletion, Fossil Depletion and Particulate Matter Formation. These impact categories were selected according to the most relevant environmental issues related to wastewater treatment and used in previous LCA studies (Corominas et al., 2013; Fang et al., 2016; Gallego et al., 2008; Garfí et al., 2017; Hospido et al., 2008). Normalisation was carried out in order to compare all the environmental impacts at the same scale. This provides information on the relative significance of the indicator results, allowing a fair comparison between the impacts estimated for each scenario (ISO, 2006). In this study, the European normalisation factors have been used (Europe ReCiPe H) (Goedkoop et al., 2009).

6.2.2.4 Sensitivity analysis

In order to evaluate the influence of the most relevant assumptions have on the results, a sensitivity analysis was performed considering the following parameters: NH_3 emissions due to the application of digestate and biofertiliser on agricultural land (Scenario 1 and 2); N_2O emissions due to the application of digestate and biofertiliser on agricultural land (Scenario 1 and 2); digestate and biofertiliser transportation distance (Scenario 1 and 2). A variation of ± 10% was considered for all parameters and the sensitivity coefficient was calculated using Eq. 6-1 (Dixon et al., 2003):

$$Sensitivity\ coefficient\ (S) = \frac{(Output_{high} - Output_{low})/Output_{default}}{(Input_{high} - Input_{low})/Input_{default}} \qquad \text{Eq. 6-1}$$

where Input is the value of the input variable (e.g. NH_3 and N_2O emissions) and Output is the value of the environmental indicator (e.g. Climate Change).

6.2.2.5 Seasonality

Annual averages of potential environmental impacts from HRAPs scenarios (Scenario 1 and 2) were compared to those obtained considering the microalgal biomass production achieved in summer and winter months (highest and lowest production, respectively; Table 6-1 and Table 6-2) to assess their fluctuations over the year. In particular, the microalgal biomass production considered for Scenario 1 (HRAPs systems coupled with biogas production) was 5 and 25 g m^2/d for winter and summer months, respectively. On the other hand, for Scenario 2 (HRAPs systems coupled with biofertiliser production) a microalgal biomass production of 15 and 30 g m^2/d was considered for winter and summer months, respectively.

6.2.3 Economic assessment

The economic assessment was performed comparing the capital cost and the operation and maintenance cost of Scenarios 1 and 2 (HRAPs systems coupled with biogas and biofertiliser production, respectively). The capital cost included the cost for earthmoving and construction materials purchase. On the other hand, operation and maintenance cost comprised costs associated with energy (electricity and heat) consumption and chemicals purchase. In both scenarios, prices were provided by local companies. For Scenario 1 (HRAPs systems coupled with biogas production), the surplus electricity generated from biogas cogeneration was supposed to be sold back to the grid. Thus, the price of electricity sold to the grid was withdrawn from the overall operational and maintenance cost of the system. For Scenario 2 (HRAPs systems coupled with biofertiliser production), the dewatered microalgae biomass is sold to a local company (Biorizon Biotech S.L., Almería, Spain) to produce the biofertiliser (Romero García et al., 2012). Therefore, its price was withdrawn from the overall operational and maintenance cost of the system. Other costs (e.g. labour costs, transportation) were assumed to be similar in both scenarios and, thus, were not included in the analysis.

6.3 Results and Discussion

6.3.1 Life Cycle Assessment

6.3.1.1 Characterisation

The potential environmental impacts associated with each alternative are shown in Figure 6-4. Comparing HRAPs scenarios (Scenarios 1 and 2), the results show that Scenario 2 is the most environmentally friendly alternative in 7 out of 11 impact categories. As far as Climate Change, Ozone Depletion, Photochemical Oxidant Formation and Fossil Depletion Potentials are concerned, the potential environmental impact of Scenario 1 was lower than Scenario 2. This was mainly due to the offset energy generated from biogas cogeneration and the avoided fertiliser (Figure 6-4). In particular, the electricity generated by biogas cogeneration (avoided electricity) was around 9 times higher than that consumed for system operation in Scenario 1 (Table 6-4). It means that the surplus electricity could be sold to the grid. This is in accordance with previous studies that observed that, in a HRAPs system for wastewater treatment, the energy balance is always positive when microalgal biomass is co-digested with primary sludge and the biogas is used to cogenerate electricity and heat (Passos et al., 2017). Moreover, it has to be noticed that the contribution of the avoided fertiliser to the overall impact was higher in Scenario 1 than Scenario 2 (Figure 6-4), since TN avoided was higher in the former compared to the latter (25.9 vs. 5.77 g/m^3 of water; Table 6-4 and Table 6-5). This can be explained by the fact that, despite TN content was higher in the biofertiliser (5 g TN/kg biofertiliser) than in the digestate (1.89 g TN/kg biofertiliser), a lower amount of biofertiliser is produced in Scenario 2 (1.15 kg biofertiliser/m^3 of water) compared to Scenario 1 (13.7 kg biofertiliser/m^3 of water). Indeed, the total solids (TS) content of the microalgal biomass obtained in Scenario 1 (2% TS) is lower compared to Scenario 2 (20%TS) due to its dewatering step (i.e. centrifugation). Nevertheless, it has to be mentioned that the biofertiliser is a higher quality product compared to the digestate, since it contains high amounts of proteins rich in essential amino acids, as well as phytohormones that stimulate plant growth and improve soil quality (Coppens et al., 2016; Garcia-Gonzalez and Sommerfeld, 2016; Jäger et al., 2010; Uysal et al., 2015).

However, these benefits were not taken into account in this study. Regarding Terrestrial Acidification and Particulate Matter Formation Potentials, Scenario 2 showed lower risks to endanger the environment because this configuration causes fewer emissions to air (i.e. NH_3 emissions) derived from biofertiliser application to agricultural soil compared to digestate from Scenario 1 (Table 6-4 and Table 6-5). With regards to Freshwater and Marine Eutrophication Potentials, Scenario 1 showed higher environmental impacts compared to Scenario 2. It is explained by the quality of treated effluent (i.e. lower TN and TP removal efficiencies in Scenario 1 than in Scenario 2; Table 6-4 and Table 6-5). The reason for this difference could be primarily due to the distinct climatic conditions, since the average temperature and global solar radiation in Catalonia (Scenario 1), as previously mentioned, are lower than in Andalucía (Scenario 2). Indeed, previous studies reported that nutrient removal efficiencies are improved with higher temperature and solar radiation (Craggs et al., 2012; Mehrabadi et al., 2016). Concerning Metal Depletion Potential, Scenario 1 would impair abiotic resources more likely than Scenario 2. Since Metal Depletion Potential is mainly influenced by construction materials, the lower environmental performance of Scenario 1 is owing to the larger surface area required for its implementation compared to Scenario 2 (4 m^2/p.e. vs. 3 m^2/p.e., respectively). As mentioned above, in the system implemented in Catalonia (Scenario 1), a higher HRT is needed (especially during winter months) compared to that implemented in Andalucía (Scenario 2) in order to obtain a effluent quality suitable for discharge (García et al., 2000; Gutiérrez et al., 2016; Morales-Amaral et al., 2015a, 2015b). The influence of the geographical location on the performance of HRAPs was also addressed in previous studies, in which the use of this technology is not encouraged in northern regions, where the climatic conditions are not favourable to promote efficient wastewater treatment and biomass productivity (Grönlund and Fröling, 2014; Pérez-López et al., 2017). According to this, it is noteworthy to mention that, since in this study the two HRAPs systems (Scenarios 1 and 2) were assumed to be implemented in locations with distinct climatic conditions, it is not possible to define the best biomass valorisation strategy (i.e. biogas vs. biofertiliser production). In fact, HRAPs systems operating under similar conditions should be considered in order to enable a better comparison. In regard to Human toxicity and Terrestrial Ecotoxicity Potentials, Scenario 1 showed higher environmental impacts

compared to Scenario 2 due to the higher concentration of heavy metals in the digestate than in the biofertiliser (Table 6-4 and Table 6-5).

According to the results presented in Figure 6-4, Scenarios 1 and 2 showed lower environmental impacts in 6 out of 11 impact categories (i.e. Climate Change, Ozone Depletion, Freshwater and Marine Eutrophication, Photochemical Oxidant Formation, Fossil Depletion) compared to Scenario 3. This was primarily due to the lower energy consumption needed for system operation in HRAPs scenarios (Scenario 1 and 2) than in the activated sludge system (Scenario 3) (Table 6-4, Table 6-5 and Table 6-6). On the other hand, HRAPs scenarios (Scenario 1 and 2) showed lower environmental performance in Metal Depletion category (Figure 6-4), since a higher amount of construction materials are needed for their implementation compared to the activated sludge system (Scenario 3). Indeed, even if HRAPs systems have low raw materials requirements for their operation, a large amount of raw materials is needed for their construction. This fact could make HRAPs systems less favourable than conventional technologies (e.g. activated sludge systems) in the abiotic resources depletion impact categories. Nevertheless, this drawback can be overcome by implementing HRAPs systems in smaller agglomerations than that considered in this study (e.g. around 2,000 p.e.) (Garfí et al., 2017). As far as Terrestrial Acidification, Particulate Matter Formation, Human Toxicity and Terrestrial Ecotoxicity Potentials are concerned, the potential environmental impacts of HRAPs scenarios (Scenario 1 and 2) were higher than that caused by the activated sludge system (Scenario 3). It was mainly due to the NH_3 air emissions derived from NH_3 volatilisation in HRAPs and to the heavy metals content in the digestate/biofertiliser (emissions to soil). The results are consistent with previous studies that reported increased toxicity in a comparative LCA by integrating a side stream process into a conventional wastewater treatment facility where microalgae are cultivated, harvested and then used for fertigation (Fang et al., 2016).

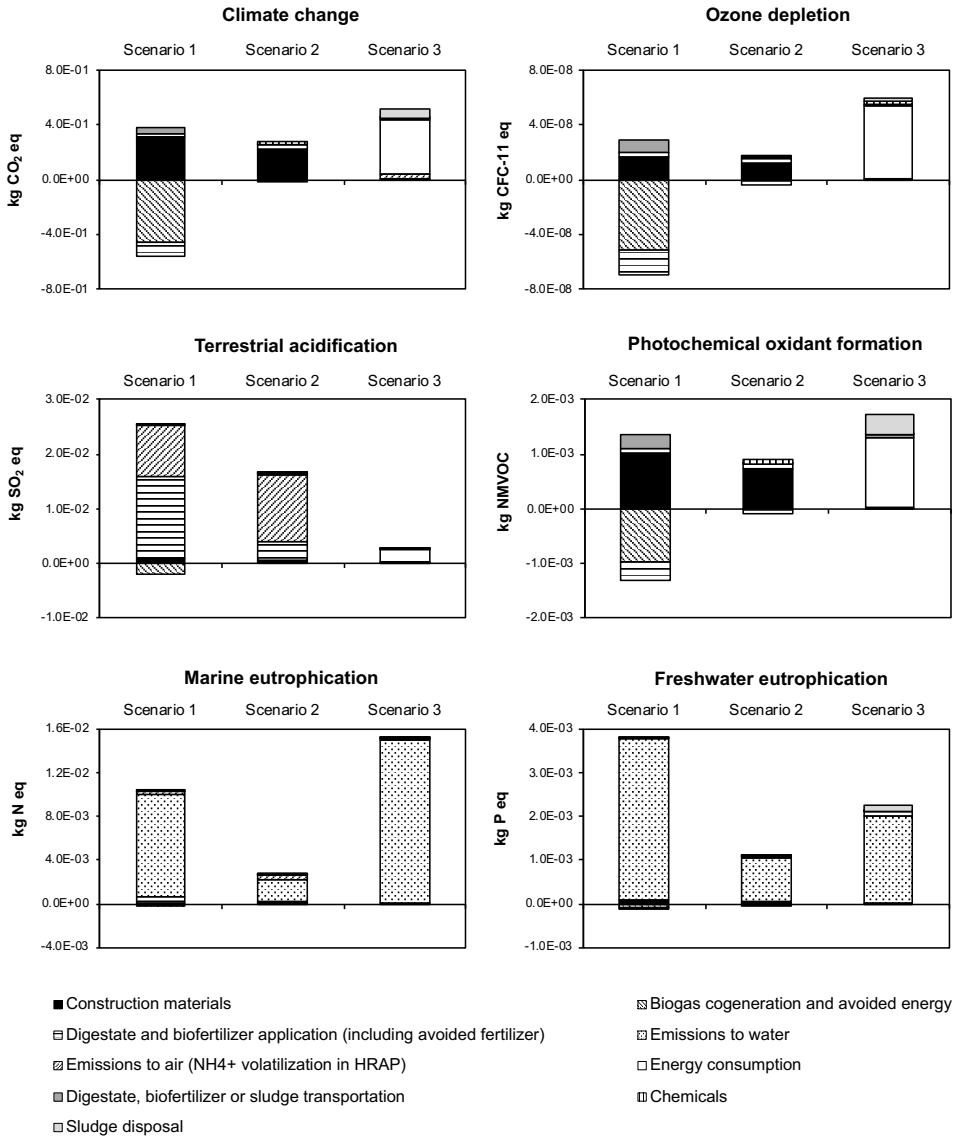

Figure 6-4. Potential environmental impacts for the three scenarios: a) HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production) (Scenario 1); b) HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production) (Scenario 2); c) activated sludge system (Scenario 3). Values are referred to the functional unit (1 m^3 of water).

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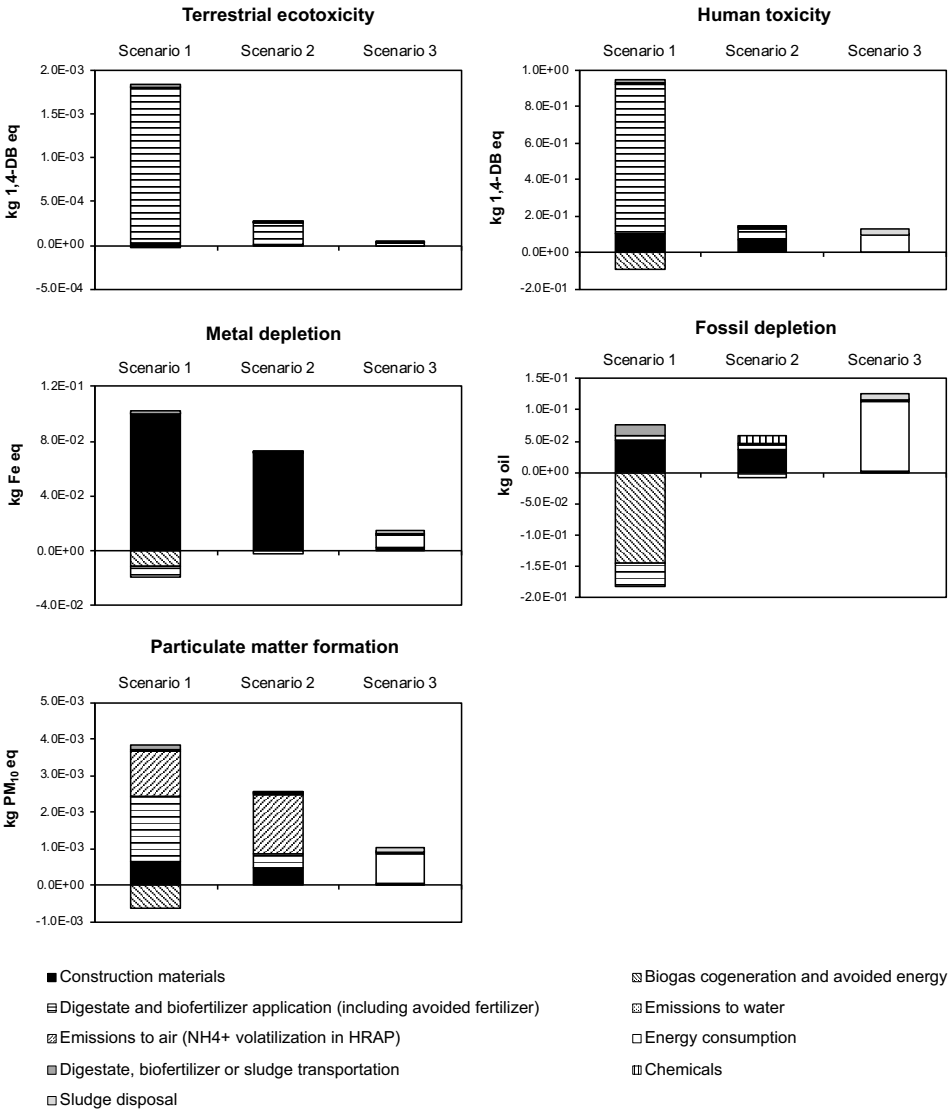

Figure 6-4. *(Continued)*.

Furthermore, it was observed that the higher impacts on terrestrial environments are unavoidable in cases where sludge and nutrients from wastewater are recycled and reused in agriculture (Tangsubkul et al., 2005). In order to address this issue, improved technologies to separate better heavy metals from recycled sludge should be encouraged

(Tangsubkul et al., 2005). In regard to Freshwater Eutrophication Potential, the activated sludge system (Scenario 3) showed higher potential environmental impact compared to Scenario 2, but lower impact than Scenario 1. This was because of the higher outlet Phosphorous concentration in Scenario 1 compared to the other scenarios, which might be related to the lower nutrients removal efficiencies caused by less favourable climatic conditions. Previous studies observed that eutrophication and toxicity impact categories were mainly affected by water discharge emissions and sludge management, indicating that the best alternatives seem to be the ones that provide lower nutrients and heavy metals emissions (Corominas et al., 2013). This corroborates with the results obtained with this study, where the configuration with higher nutrients concentration in the effluent and higher levels of heavy metals in the recycled biomass (Scenario 1) showed higher impacts in those categories.

On the whole, HRAPs systems coupled with biogas and biofertiliser production (Scenario 1 and 2) showed similar environmental performance if compared to the activated sludge system (Scenario 3). In particular, HRAPs environmental performance is better than the conventional system in the climate change, ozone layer depletion, photochemical oxidant formation, and fossil depletion impact categories. It was in accordance with previous studies, which stated that, compared to a typical medium-sized conventional wastewater treatment plant, a HRAPs system coupled with biogas production could offer clear benefits with regard to the protection of climate, protection of fossil resources and ozone depletion (Maga, 2017). In order to reduce the environmental impacts of HRAPs systems for wastewater treatment and resource recovery, the following improvements should be addressed and further assessed: i) reducing NH_3 volatilisation in HRAPs by controlling the pH through CO_2 injection; ii) ensuring higher nutrients removal efficiencies by selecting a favourable geographical location to implement the HRAPs systems; iii) studying improved technologies to separate heavy metals from recycled microalgal biomass; iv) improving HRAPs design in order to decrease the amount of construction materials used (e.g. excavation instead of concrete structure).

6.3.1.2 Normalisation

The normalised results show that Freshwater Eutrophication, Marine Eutrophication, Terrestrial Acidification and Human Toxicity Potentials are the most significant impact categories for all the scenarios considered (Figure 6-5). These results are in accordance with previous LCAs on wastewater treatment (Fang et al., 2016; Gallego et al., 2008; Hospido et al., 2004). In these impact categories, Scenario 2 showed to be the most environmentally friendly alternative.

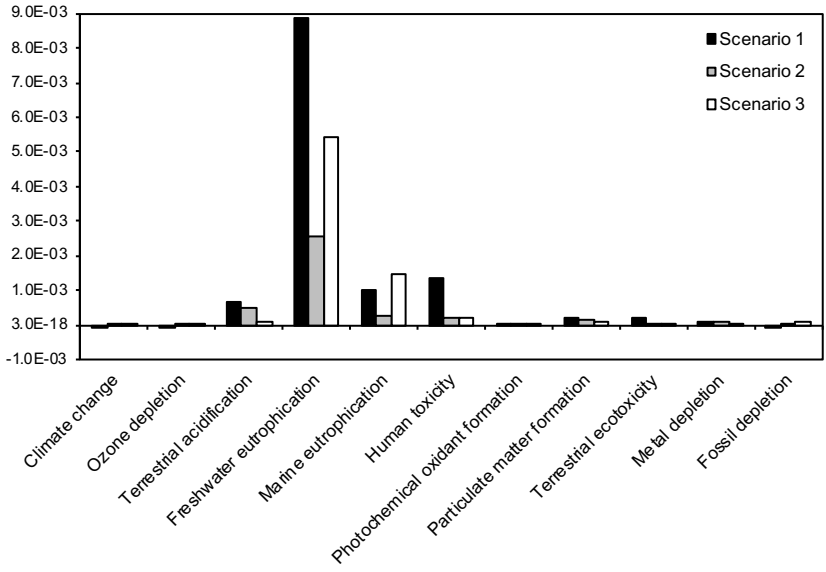

Figure 6-5. Normalised potential environmental impacts for the three scenarios: a) HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production) (Scenario 1); b) HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production) (Scenario 2); c) activated sludge system (Scenario 3).

6.3.2 Sensitivity analysis

The results of the sensitivity analysis are shown in Table 6-7, where the most sensitive inventory components are indicated by bold type. The results showed that Terrestrial Acidification and Particulate Matter Formation Potentials are somewhat sensitive to NH_3 emissions due to the application of digestate on agricultural land in Scenario 1 (sensitivity coefficient around 0.3 for both environmental indicators). Indeed, a 10% increase of this parameter would increase these indicators by around 3%.

Similarly, Climate Change Potential showed to be somewhat sensitive to N_2O emissions due to the application of digestate on agricultural land in Scenario 1 (sensitivity coefficient = 0.36). This means that a 10% increase in N_2O direct emissions would increase this environmental indicator by 3.6%.

Moreover, Photochemical Oxidant Formation Potential showed to be sensitive to digestate transportation distance in Scenario 1 (sensitivity coefficient = 2.7). Indeed, a 10% increase in digestate transportation distance would increase this environmental indicator by 27%. The transport of the sludge to agricultural applications is not a fixed parameter, as it depends on specific needs. However, the sludge is usually applied in soil relatively close to the plant location (Pasqualino et al., 2009).

In conclusion, the results were found to be sensitive to digestate transportation distance in Scenario 1. Nevertheless, since it mainly affects only one of the less significant impact categories considered (i.e. Photochemical Oxidant Formation Potential), it can be concluded that the main findings of this study are not strongly dependent on the assumptions considered.

Table 6-7. Results of the sensitivity analysis for the considered parameters: NH_3 emissions due to the application of digestate and biofertiliser on agricultural land; N_2O emissions due to the application of digestate and biofertiliser on agricultural land; digestate and biofertiliser transportation distance.

Impact categories	**Parameters**					
	Scenario 1			**Scenario 2**		
	NH_3 emissions	N_2O emissions	Digestate transport.	NH_3 emissions	N_2O emissions	Biofertiliser transport.
Climate change	±0.000	**±0.367**	±0.260	±0.000	±0.068	±0.015
Ozone Depletion	±0.000	±0.000	±0.204	±0.000	±0.000	±0.053
Terrestrial acidification	**±0.337**	±0.000	±0.008	±0.213	±0.000	±0.001
Freshwater eutrophication	±0.000	±0.000	±0.001	±0.000	±0.000	±0.000
Marine eutrophication	±0.058	±0.000	±0.001	±0.052	±0.000	±0.000
Photochemical oxidant formation	±0.000	±0.000	**±2.713**	±0.000	±0.000	±0.025
Particulate matter formation	**±0.327**	±0.000	±0.033	±0.179	±0.000	±0.003
Metal depletion	±0.000	±0.000	±0.019	±0.000	±0.000	±0.002
Fossil depletion	±0.000	±0.000	±0.153	±0.000	±0.000	±0.027
Human toxicity	±0.000	±0.000	±0.021	±0.000	±0.000	±0.011
Terrestrial ecotoxicity	±0.000	±0.000	±0.019	±0.000	±0.000	±0.011

Note: Scenario 1: HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production); Scenario 2: HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production)

6.3.3 Seasonality

The seasonal variation of the potential environmental impact for HRAPs scenarios (Scenario 1 and 2) are shown in Figure 6-6. The potential environmental impacts of Scenario 2 are fairly constant over the year. On the contrary, a strong seasonal variation was observed in Scenario 1. It was due to the fact that the microalgal biomass production range in Scenario 1 (5-25 g m^2/d) is lower than Scenario 2 (15-30 g m^2/d) and represents a high variation due to the seasonal fluctuations. It was in accordance with previous studies, which reported that meteorological conditions played a critical role in the LCA results of HRAPs for microalgal cultivation (Pérez-López et al., 2017). The authors highlighted that HRAPs are more suitable for locations where warm temperatures and high solar radiation are predominant (Pérez-López et al., 2017). Moreover, electricity and flocculants consumption, as well as water and biofertiliser characteristics, are fairly constant over the year in Scenario 2, while the biogas production and, consequently, the energy avoided, strongly depend on microalgal biomass production. These facts have a great influence on the environmental impacts' seasonality in Scenario 1. As a result, Scenario 2 remained the most environmentally friendly alternative in 7 out of 11 impact categories compared to Scenario 1 over the year. Similarly, HRAPs scenarios (Scenario 1 and 2) still showed lower potential environmental impacts in 6 out of 11 impact categories compared to activated sludge system (Scenario 3) considering seasonal fluctuations.

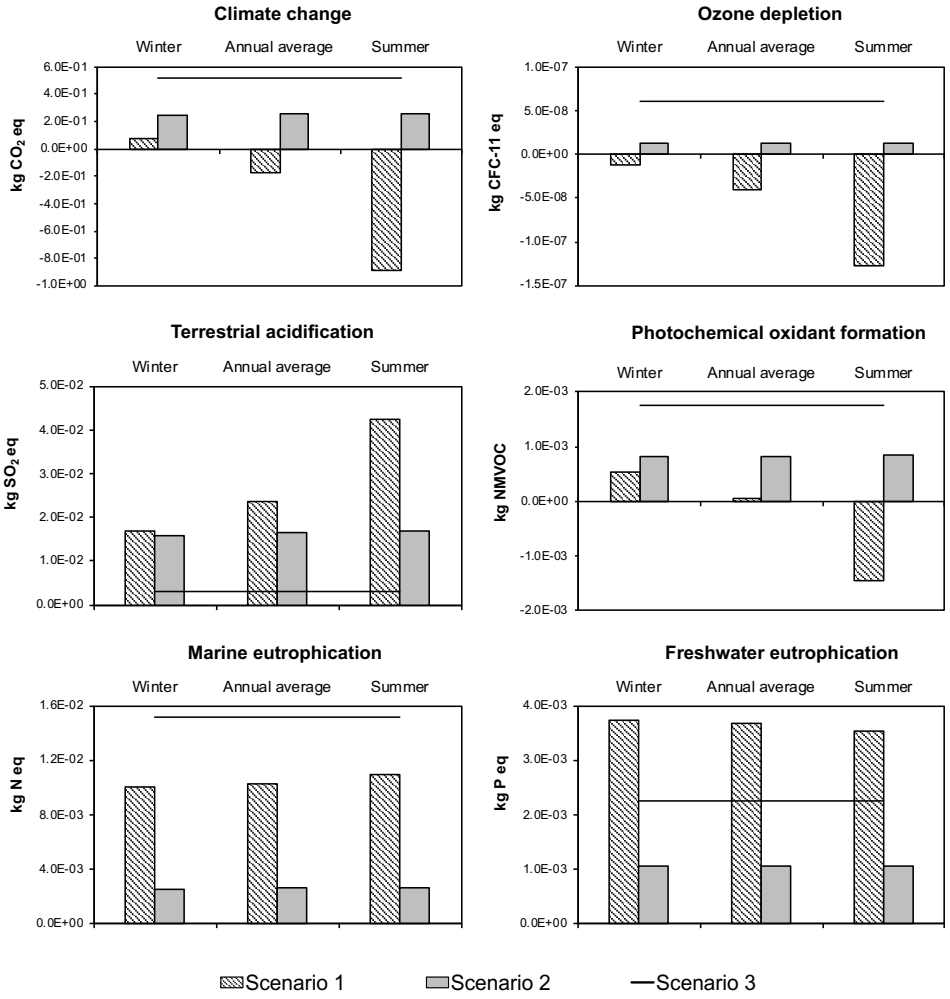

Figure 6-6. Seasonal variation of the potential environmental impacts for the three scenarios: a) HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production) (Scenario 1); b) HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production) (Scenario 2); c) activated sludge system (Scenario 3). Values are referred to the functional unit (1 m^3 of water). Potential environmental impacts were calculated considering the microalgal biomass production achieved in summer and winter months (highest and lowest production, respectively).

(Figure continued on the next page)

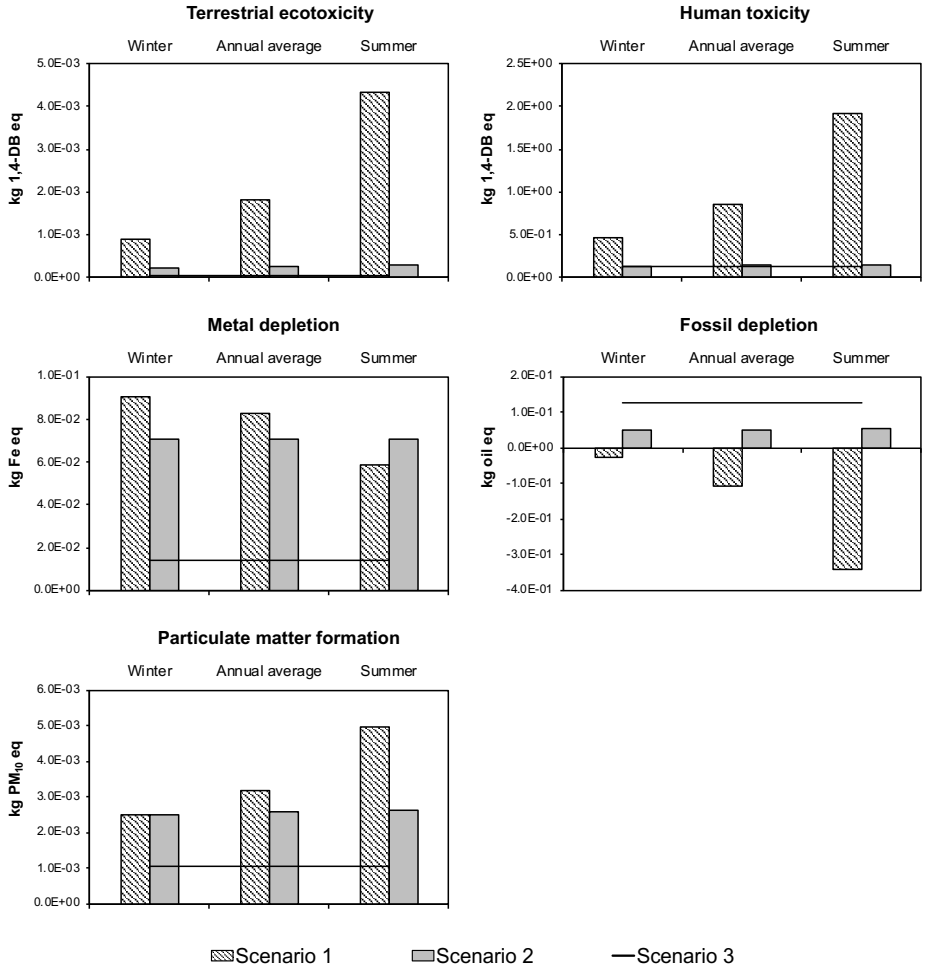

Figure 6-6. *(Continued)*.

6.3.4 Economic assessment

Results of the economic analysis are shown in Table 6-8. With respect to capital costs, Scenario 2 appeared as the less expensive alternative. It was due to its lower specific area requirement and, thus, lower amount of purchased materials, compared to Scenario 1 (3 vs. 4 m^2/p.e., respectively). Similar capital costs were found in previous studies which carried out an economic analysis of HRAPs for wastewater treatment

without any resource recovery strategies (Garfí et al., 2017; Molinos-Senante et al., 2014). In fact, in this study the capital cost for ponds implementation was around 90% of the total capital cost of the overall systems (i.e. primary settler, ponds, secondary settler, digesters).

Table 6-8. Results of the economic analysis for the HRAPs scenarios.

	Unit	**Scenario 1**	**Scenario 2**
Capital cost	€ / p.e.	192.55	139.34
Operation and maintenance cost (energy and flocculant consumption)	€ / m^3	0.007	0.02
Price of electricity sold back to the grid	€ / m^3	0.014	-
Price of microalgal biomass sold to a company to produce the biofertiliser	€ / m^3	-	8.08
Profit (calculated considering operation cost only)	€ / m^3	0.007	8.06

Note: Scenario 1: HRAPs system for wastewater treatment where microalgal biomass is valorised for energy recovery (biogas production); Scenario 2: HRAPs system for wastewater treatment where microalgal biomass is reused for nutrients recovery (biofertiliser production)

Since the highest cost is due to ponds construction, implementing downstream units for resource recovery strategies (e.g. digester) in a HRAPs system for wastewater treatment would slightly increase its capital costs. Regarding the operation costs, Scenario 2 showed to be the most expensive alternative, since this configuration requires higher expenses for energy and flocculant purchase. Nevertheless, if the price of the co-products (i.e. electricity sold back to the grid, microalgae biomass to produce the biofertiliser) that the wastewater treatment plant could sell out are considered, Scenario 2 would be the most cost-effective alternative (Table 6-8). The results of the economic assessment are consistent with previous studies, which indicated that recycling valuable compounds from microalgal biomass (such as nutrients and pigments) is likely to be more economically feasible than producing biogas from it, due to the higher added value of the final products (Ruiz Gonzalez et al., 2016; Vulsteke et al., 2017).

It is important to mention that the application of the microalgae-based products is allowed even if produced from wastewaters, as long as safety is ensured (e.g. free of

pathogens and other contaminants) (Acién Fernández et al., 2018). In addition, using the biomass on such markets would be the most efficient strategy in terms of sustainability and nutrients recovery, since microalgae are rich in proteins and thus rich in valuable amino acids, as well as carbohydrates and lipids, regardless of the species (Acién Fernández et al., 2018). The biofertiliser quality is largely dependent of the downstream processing as well as of the quality of the microalgae biomass produced. This study addressed the biofertiliser as a biostimulant, using an enzymatic hydrolysis under mild conditions, in which cells are disrupted and optimum type of enzymes are carefully dosed (Romero García et al., 2012). This type of microalgae-based products is constantly growing due to the demonstrated positive effects on plants growth and production (Acién Fernández et al., 2018).

6.4 Conclusions

In this study, the LCA methodology was a useful tool to identify the main environmental bottlenecks to scale-up high rate algal pond (HRAP) systems for wastewater treatment and resource recovery in small communities.

Results showed that HRAPs system coupled with biogas production showed to be more environmentally friendly than HRAPs system coupled with biofertiliser production in the climate change, ozone layer depletion, photochemical oxidant formation, and fossil depletion impact categories. Different climatic conditions have strongly influenced the results obtained in the eutrophication and metal depletion impact categories. In fact, the HRAPs system located where warm temperatures and high solar radiation are predominant (HRAPs system coupled with biofertiliser production) showed lower impact in those categories due to its higher nutrients removal efficiencies and lower hydraulic retention time (i.e. lower specific area requirement). The characteristics (e.g. total solids, nutrients and heavy metals concentration) of microalgal biomass recovered from wastewater appeared to be crucial when assessing the potential environmental impacts in the terrestrial acidification, particulate matter formation and toxicity impact categories.

Normalisation identified Freshwater Eutrophication, Marine Eutrophication, Terrestrial Acidification and Human Toxicity as the most significant impact categories for all the scenarios considered. In these categories, HRAPs system coupled with biofertiliser production and implemented in warm climate region showed to be the most environmentally friendly alternative.

Additionally, HRAPs systems coupled with biogas and biofertiliser production showed lower potential environmental impacts compared to an activated sludge system in the climate change, ozone layer depletion, photochemical oxidant formation, and fossil depletion impact categories.

The environmental performance of HRAPs technology for wastewater treatment and resource recovery in small communities might be improved by: i) reducing NH_3 volatilisation in HRAPs by controlling the pH through CO_2 injection; ii) ensuring higher nutrients removal efficiencies by selecting a favourable geographical location to implement the HRAPs systems; iii) studying improved technologies to separate heavy metals from recycled microalgal biomass; iv) improving HRAPs design in order to decrease the amount of construction materials used.

In terms of costs, HRAPs system coupled with biofertiliser production was the most cost-effective alternative, due to the higher added value of the biofertiliser compared to the energy obtained from biogas cogeneration.

In conclusion, HRAPs are sustainable and cost-effective technology for wastewater treatment in small communities, especially if implemented in warm climate regions and coupled with biofertiliser production. Their implementation and dissemination can help to support a shift towards resource recovery and a sustainable circular economy.

Chapter 7

Life Cycle Assessment – Phycobiliproteins recovery

LCA of microalgae systems for wastewater treatment and phycobiliproteins recovery

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Abstract

The aim of this study was to assess the potential environmental impacts associated with microalgae systems for wastewater treatment and resources recovery. In this sense, a Life Cycle Assessment was carried out evaluating two systems treating 1) municipal wastewater and 2) industrial wastewater, with recovery of bioproducts (phycobiliproteins and biofertiliser) and bioenergy (biogas). Additionally, both alternatives were compared to a conventional system using standard growth media for phycobiliproteins production. The results indicate that the system treating industrial wastewater in a UASB followed by HRAPs with unialgal cultures has lower environmental impacts than the system treating urban wastewater in HRAPs followed by PBRs with mixed cultures in the following categories: Climate Change, Ozone Depletion, Photochemical Oxidant Formation, Human Toxicity, Terrestrial Acidification, Freshwater Eutrophication, Terrestrial Ecotoxicity, Fossil Depletion and Particulate Matter Formation. Moreover, using wastewater appeared to be more environmentally friendly than using standard growth medium to cultivate microalgae.

This chapter has been redrafted after:

Arashiro, L.T., Ferrer, I., Rousseau, D.P.L., Van Hulle, S.W.H., Garfí, M., Rousseau, D.P.L. Life Cycle Assessment of microalgae systems for wastewater treatment and pigments recovery. (*In preparation*)

7. Life Cycle Assessment of microalgae systems for wastewater treatment and phycobiliproteins recovery

7.1 Introduction

Microalgae have shown a great potential for the production of several bioproducts with a wide variety of applications such as biofuels and chemicals as well as food and feed (Christaki et al., 2015; Michalak and Chojnacka, 2015; Spolaore et al., 2006). One of the greatest advantages of using microalgae is their high productivity, with the possibility to grow on marginal land in fresh or saltwater, which can avoid competition with food crops, and the option of combining biomass growth with the treatment of waste streams (Clarens et al., 2011).

Pigments from microalgae, which are particularly strong dyes even at very low concentrations, are now strongly demanded by the market as renewable natural colour enhancers for food and feed, which simultaneously provide certain health benefits (Christaki et al., 2015). Among the pigments present in microalgae cells, phycobiliproteins have important applications in the pharmaceutical, food and cosmetic industry, as well as in clinical immunodiagnostics and molecular biology, due to their fluorescence properties (Cuellar-Bermudez et al., 2015). Phycobiliproteins have been extracted and purified from several microalgae species, but commercial production is mainly from *Arthrospira* spp. (*Spirulina*) for phycocyanin, and *Porphyridium* spp. for phycoerythrin (Borowitzka, 2013; Christaki et al., 2015). In particular, *A. platensis* is widely chosen as host for phycocyanin production merely because of its availability and favourable growing conditions rather than particular qualities of its pigments (Eriksen, 2008). *A. platensis* tolerates alkaline conditions and is grown at pH values up to 10.5, being among the few photoautotrophic microorganisms able to grow in open ponds without high risks of being out-competed by contaminating organisms (Richmond and Grobbelaar, 1986).

Although the demand for natural pigments is increasing and microalgae is considered as a potential candidate for phycobiliproteins production, the requirement of

huge quantities of water and chemicals (i.e. nutrients) in large scale systems leads to high costs and further hinders the production and commercialisation of these bioproducts. Microalgae cultivation using wastewater and/or recycled water has been recently explored (Acién et al., 2016; Delrue et al., 2016; Ho et al., 2018; K. Li et al., 2019). However, little is known regarding the phycobiliproteins production from microalgae by using waste streams.

As previously mentioned in **Chapter 6**, LCA is an appropriate tool to support early-stage research and development of novel technologies and processes. LCA also has the potential to be used as a guiding tool for decision-making in process design as well as for identifying the main bottlenecks to be addressed during the scale up towards sustainable industrial facilities (Arashiro et al., 2018; Pérez-López et al., 2017).

In this context, this Chapter provides a comparative LCA of two microalgae-based systems for wastewater treatment and phycobiliproteins recovery: i) a HRAPs system treating urban wastewater followed by a PBR treating centrate (from microalgae anaerobic digestion) diluted in the secondary effluent, cultivating a mixed culture dominated by cyanobacteria (system based on **Chapter 3** and **4**); and ii) a UASB treating food-industry wastewater followed by HRAPs cultivating *A. platensis* (*Spirulina*) (system based on **Chapter 5**). The main environmental burdens and benefits of each option were evaluated, in order to compare their performances and to identify bottlenecks for up-scaling. For reference purposes, both scenarios were compared to a conventional phycocyanin production system using standard growth medium.

7.2 Materials and methods

7.2.1 Wastewater treatment systems description

The studied systems were hypothetical wastewater treatment plants based on extrapolation from lab-scale and pilot-scale studies (up to 600 m^2). The systems were designed to serve a population equivalent of 10,000 p.e. and treat a flow rate of 1,500 m^3/d. For the microalgae-based system treating urban wastewater in HRAPs followed by

PBRs with mixed cultures (hereafter referred to as Scenario UWW), the design parameters were based on experimental results obtained in lab-scale and pilot systems (up to 5 m^2) located at the Universitat Politècnica de Catalunya-BarcelonaTech (UPC) (Barcelona, Spain) (García et al., 2006, 2000; Gutiérrez et al., 2016; Passos and Ferrer, 2014; Solé-Bundó et al., 2019a, 2017b). This scenario is a combination of HRAPs for urban wastewater treatment (based on the system described in **Chapter 3**) and PBRs for cyanobacteria biomass cultivation for pigments recovery (based on the system described in **Chapter 4**). The flow diagram of this case study is shown in Figure 7-1 and characteristics and design parameters are listed in Table 7-1. Firstly, the HRAPs system comprises a primary settler (HRT: 2.5 h) followed by four HRAPs in parallel, cultivating a mixed culture of green microalgae. From these units, wastewater goes through a secondary settler (HRT: 3 h) where microalgal biomass is harvested and separated from wastewater. Part of the harvested microalgal biomass (2 and 10% on a dry weight basis in summer and winter, respectively) is recycled in order to enhance spontaneous flocculation (bioflocculation) and increase microalgae harvesting efficiency (Gutiérrez et al., 2016). The remaining harvested biomass is thickened (HRT: 24 h) and co-digested with primary sludge (35 °C, 20 days). In this context, the HRT of each HRAP has to be modified over the year (8, 6 and 4 days) according to weather conditions (i.e. solar radiation and temperature) in order to accomplish wastewater treatment and meet effluent quality requirements for discharge (García et al., 2000; Gutiérrez et al., 2016). For this reason, it was considered that during summer months (from May to July) only two HRAPs work in parallel (HRT: 4 days), whereas all of them are operated during winter months (from November to April) (HRT: 8 days). During the rest of the year (from August to October), the HRT is 6 days (3 HRAPs working in parallel). Secondly, the cultivation of cyanobacteria-dominated biomass is done in hybrid tubular PBRs, with the design based on a demo scale plant treating agricultural runoff, which is described elsewhere (García et al., 2018; Uggetti et al., 2018). For that, most of the HRAPs effluent is discharged into a surface water body, but part of it (6.5%) is used to support the cyanobacteria-dominated biomass growth. The secondary effluent is filtered (to avoid any possible grazer contamination) and used to dilute centrate (liquid part of digestate) from the microalgae anaerobic digestion unit. The portion of the secondary effluent was

estimated based on the volume of centrate available, in order to reach a similar dilution rate as the study described in **Chapter 4** (15% centrate in secondary effluent, v/v). The effluent of the PBRs goes through a tertiary settler (HRT: 3 h) where microalgal biomass is harvested and separated from wastewater that is discharged into a surface water body. The microalgae biomass is then centrifuged and the biomass paste is used for phycobiliproteins recovery, which is done through ultrasound extraction with phosphate buffer (**Chapter 4**, Section 4.2.4). The residual biomass (after extraction) is also used as a substrate for the anaerobic digester. The biogas produced is then converted in a combined heat and power (CHP) unit, while the centrate is recirculated to the PBR (as mentioned previously) and the solid part of the digestate is transported and reused in agriculture as biofertiliser.

For the microalgae-based system treating industrial wastewater in a UASB followed by HRAPs with unialgal cultures (hereafter referred to as Scenario IWW), the design parameters were based on data obtained from a company that produces plant-based food (located in Wevelgem, Belgium) and experimental results obtained in lab-scale systems at Ghent University (Kortrijk, Belgium) (**Chapter 5**) (Arashiro et al., 2020). This scenario is a combination of a UASB, to reduce the organic matter concentration of the wastewater, and HRAPs cultivating *A. platensis* (*Spirulina*) for pigments recovery (based on the system described in **Chapter 5**). The flow diagram of this case study is shown in Figure 7-2 and characteristics and design parameters are listed in Table 7-2. Firstly, industrial wastewater goes through a drum sieve (0.5 mm) to remove the large particles, which are later transported and used for compost. The wastewater is then treated in a UASB (HRT: 30 h), from which the biogas produced is converted in a CHP unit. The UASB effluent is filtered to remove suspended solids and the solids from both the UASB (digestate) and the filtration process (retained solids) are hypothetically transported and reused in agriculture. After filtration, the wastewater is mixed with sea water to ensure enough salinity to cultivate *Spirulina* biomass in the HRAPs. In this context, sea water is assumed to be directly available and the portion used was estimated in order to reach a similar concentration as the study described in **Chapter 5** (75% wastewater and 25% sea water, v/v). In this scenario, the HRT of each HRAP was also modified over the year (8, 6 and 4 days) assuming similar weather conditions than in the first scenario. The

effluent from the HRAPs goes through a secondary settler (HRT: 3 h) where microalgal biomass is harvested and separated from the treated water. The microalgae biomass is then centrifuged and the biomass paste is used for phycobiliproteins recovery, which is done through ultrasound extraction with phosphate buffer (**Chapter 4**) (Arashiro et al. (2020). The residual biomass (after extraction) is also used as a substrate for the UASB.

For reference purposes, the potential environmental impacts of the microalgae-based wastewater treatment systems were compared to those generated by a conventional phycobiliproteins production system. For that purpose, the design of a typical phycocyanin production facility from *A. platensis* (*Spirulina*) using standard growth medium (SGM) as described by Papadaki et al. (2017) was considered. The flow diagram of this case study (hereafter referred to as Scenario SGM) is shown in Figure **7-3** and characteristics and design parameters are listed in Table 7-3. It comprises HRAPs systems to cultivate the biomass, followed by a centrifuge to recover the biomass paste, which is further used for phycobiliproteins recovery. Likewise the previous scenarios, an anaerobic digester is also considered to generate biogas (later converted in a CHP unit) and the digestate is transported and reused in agriculture.

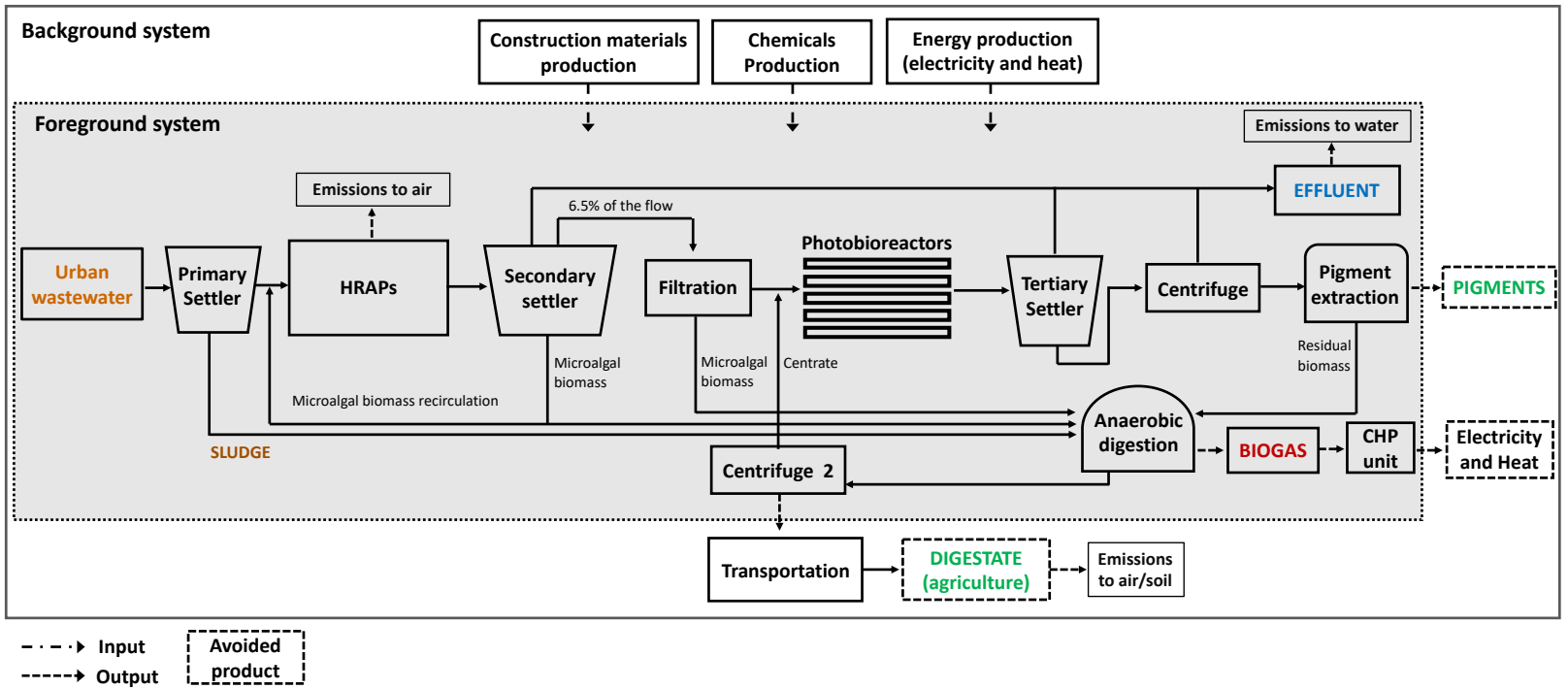

Figure 7-1. Flow diagram and system boundaries of the Scenario UWW: Urban wastewater treatment in high rate algal ponds (HRAPs), followed by photobioreactors (PBRs) cultivating cyanobacteria-dominated biomass for pigments recovery.

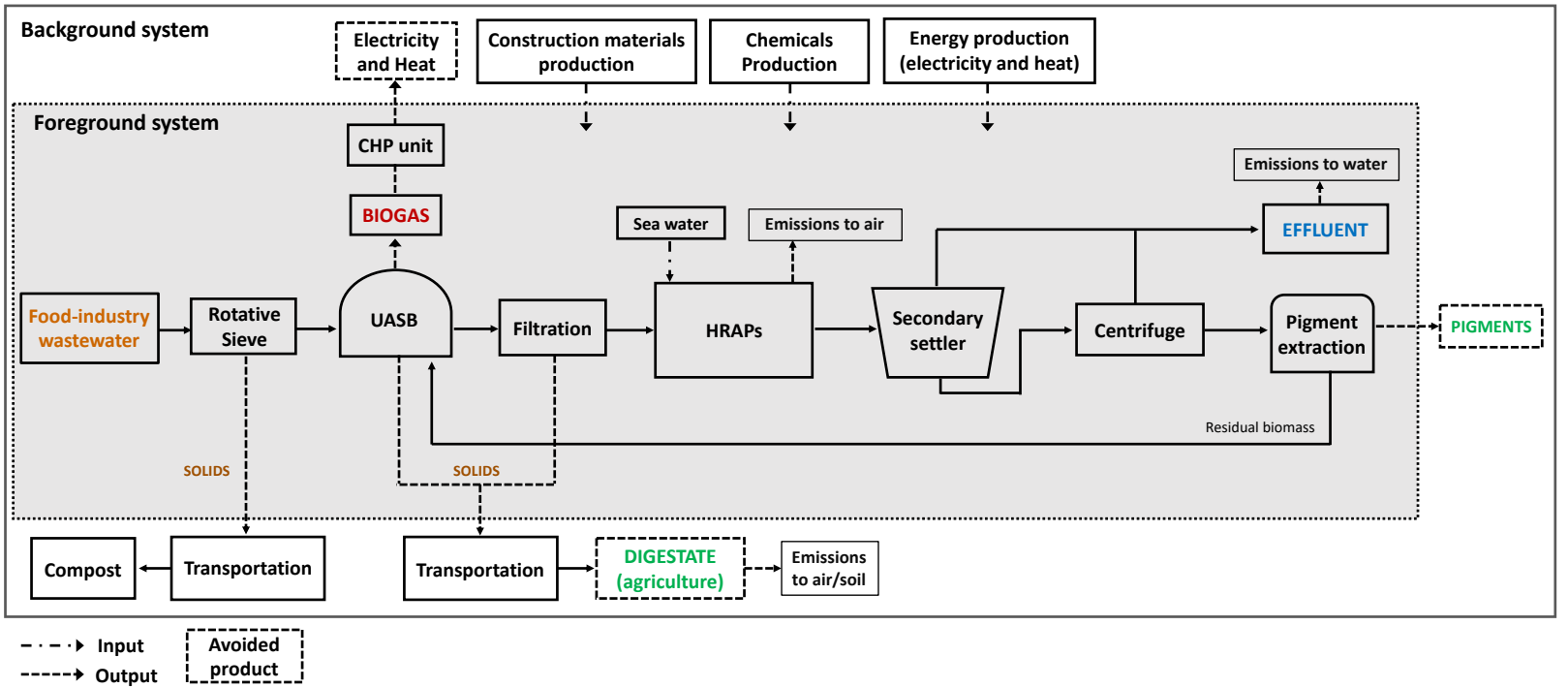

Figure 7-2. Flow diagram and system boundaries of the Scenario IWW: Industrial wastewater from a food company treated in an upflow anaerobic sludge blanket (UASB) reactor followed by high rate algal ponds (HRAPs) cultivating *A. platensis* (*Spirulina*) for pigments recovery.

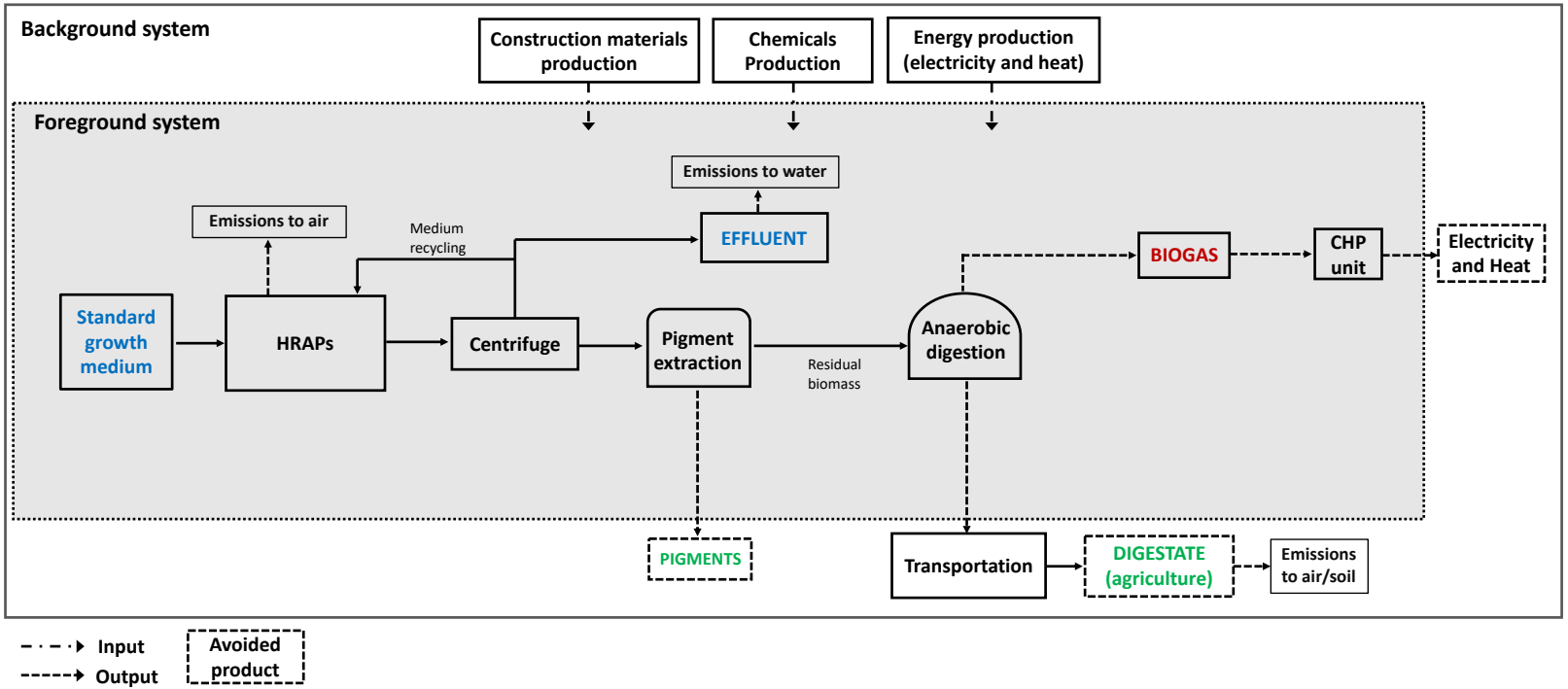

Figure 7-3. Flow diagram and system boundaries of the Scenario SGM: high rate algal ponds (HRAPs) cultivating *A. platensis* (*Spirulina*) with standard growth media (SGM) for pigments production.

Table 7-1. Characteristics and design parameters of Scenario UWW: Urban wastewater treatment in high rate algal ponds (HRAPs), followed by photobioreactors (PBRs) cultivating cyanobacteria-dominated biomass for pigments recovery.

System characteristics	**Unit**	**HRAPs**			**PBRs**
Flow rate	m^3/d	1500			96.20
Total surface area	m^2	30000			13000
Channel width	m	12			5
Channel length	m	625			50
Water depth	m	0.4			-
Influent concentrations					
BOD	mg O_2/L	300			171
TSS	mg/L	150			17
Total Nitrogen	mg/L	39			30.6
Total Phosphorus	mg/L	5			0.6
Effluent concentrations					
BOD	mg O_2/L	<25			<25
TSS	mg/L	<35			<35
Total Nitrogen	mg/L	0.94			15
Total Phosphorus	mg/L	3.69			0.6
Design parameters		**Summer**	**Winter**	**Rest of the year**	
Hydraulic retention time	d	4	8	6	5
Number of HRAP/PBR	-	2	4	3	52
Average microalgae biomass production	g TSS/m^2d (HRAP) g TSS/m^3d (PBR)	25.8	6.4	10.5	942

Acronyms: Biochemical oxygen demand (BOD); High rate algal ponds (HRAP); Photobioreactors (PBR); Total suspended solids (TSS).
Summer: May to July; Winter: November to April.

Table 7-2. Characteristics and design parameters of Scenario IWW: Industrial wastewater from a food company treated in an upflow anaerobic sludge blanket (UASB) reactor followed by high rate algal ponds (HRAPs) cultivating *A. platensis* (*Spirulina*) for pigments recovery.

System characteristics	**Unit**	**UASB**	**HRAPs**		
Flow rate	m^3/d	1500	1999[a]		
Population equivalent	p.e.	10000	10000		
Total surface area	m^2	389	40361		
Specific surface area	m^2 p.e.	0.04	4.04		
Width	m	14	12		
Length	m	27	833		
Depth	m	4.8	0.4		
Influent concentrations					
COD	mg O_2/L	3700	159		
BOD	mg O_2/L	2250	111		
TSS	mg/L	880	-		
Total Nitrogen	mg/L	182	74		
Total Phosphorus	mg/L	19	9.7		
Effluent concentrations					
COD	mg O_2/L	333	26		
BOD	mg O_2/L	202	18		
TSS	mg/L	195	35		
Total Nitrogen	mg/L	162	10		
Total Phosphorus	mg/L	17	1.8		
Design parameters			**Summer**	**Winter**	**Rest of the year**
Hydraulic retention time	h (UASB) d (HRAP)	30	4	8	6
Number of ponds	-	-	2	4	3
Average microalgae biomass production	g TSS/m^2d	-	54.43	13.44	22.15

[a]Flow of HRAPs include effluent of UASB mixed with seawater.
Acronyms: Chemical oxygen demand (COD); Biochemical oxygen demand (BOD); High rate algal ponds (HRAP); Total suspended solids (TSS); Upflow anaerobic sludge blanket (UASB).
Summer: May to July; Winter: November to April.

Table 7-3. Characteristics and design parameters of Scenario SGM: high rate algal ponds (HRAPs) cultivating *A. platensis* (*Spirulina*) with standard growth media (SGM) for pigments production.

System characteristics	**Unit**	**HRAP**		
Flow rate	m^3/d	553		
Total surface area	m^2	11057		
Channel width	m	12		
Channel length	m	111		
Water depth	m	0.4		
Average microalgae biomass production	g TSS/m^2d	30		
Design parameters		**Summer**	**Winter**	**Rest of the year**
Hydraulic retention time	d	4	8	6
Number of ponds	-	50	100	75

Acronyms: High rate algal ponds (HRAP); Total suspended solids (TSS).
Summer: May to July; Winter: November to April.

7.2.2 Life cycle assessment

The LCA was conducted following the ISO standards (ISO, 2006, 2000) in order to assess and quantify the potential environmental impacts of each scenario. The technical framework for the LCA methodology consists of four phases: 1) goal and scope definition; 2) inventory analysis; 3) impacts assessment; and 4) interpretation of the results (ISO, 2006). The following sub-sections describe the specific content of each phase.

7.2.2.1 Goal and scope definition

The goal of this study was to compare the potential environmental impacts associated with different microalgae-based systems for wastewater treatment and phycobiliproteins recovery and to identify the vulnerable aspects in which the technologies studied can potentially improve in terms of environmental performance. In particular, two configurations were compared:

a) urban wastewater treatment in HRAPs, followed by PBRs cultivating cyanobacteria-dominated biomass for phycobiliproteins recovery. (Scenario UWW);

b) industrial wastewater from a food company treated in a UASB reactor followed by HRAPs cultivating *A. platensis* (*Spirulina*) for phycobiliproteins recovery (Scenario IWW).

The functional unit (FU) for this study was set as 1 m^3 of treated water, since the main function of the technologies proposed is to treat wastewater. Additionally, both scenarios were compared to a typical facility for phycobiliproteins production using standard growth medium (Scenario SGM).

For this LCA study, cradle-to-grave boundaries comprised systems construction, operation and maintenance over a 20-years period (Garfí et al., 2017; Pérez-López et al., 2017; Rahman et al., 2016) (Figure 7-1, Figure 7-2, and Figure **7-3**). Input and output flows of materials (i.e. construction materials and chemicals) and energy resources (heat and electricity) were studied in details for all scenarios. Direct GHG emissions and NH_3 volatilisation associated with wastewater treatment were also included in the boundaries. As treated water is discharged into the environment, direct emissions to water were also taken into account. The transportation (20 km) (Hospido et al., 2004), as well as direct emissions to soil (heavy metals) and direct GHG emissions, were accounted for digestate reuse in agriculture (as biofertiliser). The end-of-life of infrastructures and equipment were neglected, since the impact would be marginal compared to the overall impact.

The investigated scenarios would generate by-products (i.e. biogas, pigments), thus considered to avoid the production of conventional products, and the system expansion method has been used following the ISO guidelines (Guinée, 2002; ISO, 2006). This way, the avoided impact related to conventional products offsets the overall impact of the system (Collet et al., 2011; ISO, 2006; Sfez et al., 2015). In this study, the digestate produced in anaerobic digesters were considered to be reused in agriculture, substituting conventional fertilisers (Coppens et al., 2016; Garcia-Gonzalez and Sommerfeld, 2016; Solé-Bundó et al., 2017b) and the pigments produced were considered as substitutes to organic chemicals. The biogas cogeneration was also considered, with avoided burdens

of using heat and electricity, instead of heat from natural gas and electricity supplied through the grid.

7.2.2.2 Inventory analysis

Inventory data for the investigated scenarios are summarized in Table 7-4, Table 7-5 and Table 7-6. The data regarding construction materials and operation for the Scenario UWW and IWW were based on the detailed engineering designs performed in this study. Treated wastewater characteristics were estimated considering the removal efficiencies and experimental results obtained in previous studies, as follows.

For Scenario UWW, biomass productivities in PBRs were estimated based on the biomass produced per nutrients removed observed by García et al. (2018) in those PBRs, but considering the influent of this study (secondary effluent and centrate, as shown in **Chapter 4**). The phycobiliproteins yields used in this scenario were also based on what was measured in the cyanobacteria-dominated biomass grown in secondary effluent and centrate described in **Chapter 4**.

For Scenario IWW, data for HRAPs were based on the lab-scale systems operated at Ghent University (Kortrijk, Belgium), as described in **Chapter 5**. For that experimental work, plastic bags were used for cultivation, but as this case study was designed for large scale, HRAPs were considered due to their simplicity and to the fact of being used for decades for the production of *Spirulina* biomass (Papadaki et al., 2017; Richmond and Grobbelaar, 1986; Ye et al., 2018) without high risks of being out-competed by contaminating organisms (Eriksen, 2008). Heavy metals and nutrients (avoided nitrogen and phosphorus) content of the digestate from the UASB were based on food digestate from the literature (Rigby and Smith, 2011). The phycobiliproteins yields used in this scenario were based on the *A. platensis* biomass grown in food-industry wastewater (A-75%WW) described in **Chapter 5**.

For Scenario SGM, as mentioned above, data regarding the conventional phycobiliproteins production were gathered from the literature (Campbell et al., 2011; Collet et al., 2011; Papadaki et al., 2017). It is noteworthy to mention that the biomass grown in standard growth medium was considered to have no heavy metals, so there

were no emissions to soil from the digestate in this scenario. Another important observation is that this scenario was included merely as reference rather than for purposes of absolute comparison. The main reason for that is due to the functional unit, which is 1 m^3 of treated water in this study. To enable a thorough comparison among all scenarios for the recovery of pigments, a separate assessment should be done with an inventory based on the production of pigments (e.g. 1 kg phycobiliproteins). However, since the main goal in this study was to treat wastewater while recovering valuable resources from it, the functional unit is maintained, as such for the Scenario SGM the inventory is based on 1 m^3 of water (standard growth medium, in this case).

The data for the pigments extraction step in all scenarios were based on the detailed study carried out by Papadaki et al. (2017), considering the extraction of the wet paste with phosphate buffer (pH 7) using ultrasound. Energy and solvent needed for the extraction were considered, but construction materials were neglected, since no substantial data was found and because the impact would be minimal compared to the overall impact of operation, considering the 20-years period of this study. NH_3 volatilisation in all scenarios was estimated through nitrogen mass balance. NH_3 and N_2O emissions due to the application of digestate on agricultural land were calculated using emissions factors from the literature (Hospido et al., 2008; IPCC, 2006; Lundin et al., 2000). In this study, CH_4 emissions were not considered since anaerobic decompositions do not occur if liquid fertiliser is used and the climate is predominantly dry (IPCC, 2000; Lundin et al., 2000). Heavy metals and nutrients (avoided nitrogen and phosphorus) content of the microalgae digestate was based on experimental results obtained in previous studies (Solé-Bundó et al., 2017b). In order to estimate electricity and heat production from biogas cogeneration in all scenarios, biogas production obtained in lab-scale experiments from previous studies were considered for mono and co-digestion (Passos et al., 2017; Solé-Bundó et al., 2019a), and results presented in **Chapter 4** (199 mL CH_4/g VS) were considered for biogas production from residual biomass.

Background data (i.e. data of construction materials, chemicals, energy production, avoided pigments, transportation and compost process) were obtained from the *Ecoinvent 3.1* database (Moreno Ruiz et al., 2014; Weidema et al., 2013).

Table 7-4. Summary of the inventory for Scenario UWW: Urban wastewater treatment in high rate algal ponds (HRAPs), followed by photobioreactors (PBRs) cultivating cyanobacteria-dominated biomass for pigments recovery. Values are referred to the functional unit (m^3).

	Scenario UWW	**Unit**
Inputs		
Construction materials		
Primary settler		
Concrete	2.788E-06	m^3/m^3
Steel	2.231E-04	kg/m^3
HRAP		
Concrete	5.945E-04	m^3/m^3
Steel	4.787E-02	kg/m^3
Secondary settler		
Concrete	2.944E-06	m^3/m^3
Steel	2.355E-04	kg/m^3
Filtration		
Polypropylene	1.30E-05	kg/m^3
Polyethylene	4.38E-06	kg/m^3
Polyurethane	1.44E-05	kg/m^3
Acrylonitrile–butadiene–styrene	4.02E-05	kg/m^3
PBR		
Polyethylene	9.971E-03	kg/m^3
Polyvinylidenchloride	2.422E-04	kg/m^3
Steel	2.512E-04	kg/m^3
Tertiary Settler		
Concrete	5.139E-07	m^3/m^3
Steel	4.111E-05	kg/m^3
Centrifuge 1		
Steel	5.023E-05	kg/m^3
Anaerobic digester		
Concrete	9.678E-06	m^3/m^3
Steel	7.742E-04	kg/m^3
Centrifuge 2		
Steel	5.023E-05	kg/m^3

(Table continued on the next page)

Table 7-4. *Continued.*

	Scenario UWW	Unit
Inputs		
Operation		
Energy consumption[a]		
Primary settler	1.997E-03	kWh/m³
HRAP	1.069E-02	kWh/m³
Secondary settler	3.970E-03	kWh/m³
Filtration	7.832E-03	kWh/m³
PBR	1.500E+00	kWh/m³
Tertiary Settler	1.002E-03	kWh/m³
Centrifuge 1	2.004E-03	kWh/m³
Pigments extraction	3.188E-01	kWh/m³
Anaerobic digester	1.955E-02	kWh/m³
Centrifuge 2	1.170E-02	kWh/m³
Total energy consumption	1.878E+00	kWh/m³
Chemicals[a]		
Filtration		
NaOH (Cleaning)	4.00E-04	kg/m³
Pigments extraction		
Sodium phosphate	3.282E-04	kg/m³
Outputs		
Emissions to water[a]		
COD	7.040E+01	g/m³
TSS	2.216E+01	g/m³
N	8.679E-01	g/m³
P	3.403E+00	g/m³
Emissions to air[a]		
NH_3 volatilisation in HRAPs		
NH_3	5.495E+00	g/m³
Digestate for agricultural reuse		
NH_3	5.532E-01	g/m³
N_2O	2.213E-02	g/m³

(Table continued on the next page)

Table 7-4. *Continued.*

	Scenario UWW	Unit
Outputs		
Emissions to soil[a]		
Digestate for agricultural reuse		
Cd	3.035E-04	g/m³
Cu	1.733E-02	g/m³
Pb	7.799E-03	g/m³
Zn	7.771E-02	g/m³
Ni	3.564E-03	g/m³
Cr	4.482E-03	g/m³
Hg	3.882E-05	g/m³
Avoided products[a]		
Biogas cogeneration		
Electricity production	2.071E-01	kWh/m³
Heat production	3.254E-01	kWh/m³
Digestate for agricultural reuse		
N as fertiliser	2.213E+00	g/m³
P as fertiliser	2.458E-01	g/m³
Pigments as organic chemical	1.001E-03	kg/m³

[a]*Annual averages*

Table 7-5. Summary of the inventory for Scenario IWW: Industrial wastewater from a food company treated in an upflow anaerobic sludge blanket (UASB) reactor followed by high rate algal ponds (HRAPs) cultivating *A. platensis* (*Spirulina*) for pigments recovery.

	Scenario IWW	Unit
Inputs		
Construction materials		
Drum sieve		
Steel	2.100E-05	kg/m³
UASB		
Concrete	1.769E-05	m³/m³
Steel	2.424E-04	kg/m³
Filtration		
Polypropylene	2.03E-04	kg/m³
Polyethylene	6.82E-05	kg/m³
Polyurethane	2.24E-04	kg/m³
Acrylonitrile–butadiene–styrene	6.26E-04	kg/m³
HRAP		
Concrete	7.918E-04	m³/m³
Steel	6.366E-02	kg/m³
Settler		
Concrete	2.943E-06	m³/m³
Steel	2.354E-04	kg/m³
Centrifuge		
Steel	5.023E-05	kg/m³
Operation		
Energy consumption[a]		
Drum sieve	5.920E-03	kWh/m³
UASB	5.000E-01	kWh/m³
Filtration	1.219E-01	kWh/m³
HRAP	1.373E-02	kWh/m³
Settler	3.860E-03	kWh/m³
Centrifuge	7.721E-03	kWh/m³

(Table continued on the next page)

Table 7-5. *Continued.*

	Scenario IWW	Unit
Inputs		
Pigment extraction	1.229E+00	kWh/m³
Total energy consumption	1.882E+00	kWh/m³
Chemicals[a]		
Filtration		
NaOH (Cleaning)	4.00E-04	kg/m³
Pigments extraction		
Sodium phosphate	1.265E-03	kg/m³
Outputs		
Emissions to water[a]		
COD	2.594E+01	g/m³
TSS	3.476E+01	g/m³
N	9.925E+00	g/m³
P	1.815E+00	g/m³
Emissions to air[a]		
NH_3 volatilisation in HRAPs		
NH_3	1.080E+00	g/m³
Digestate for agricultural reuse		
NH_3	1.273E+00	g/m³
N_2O	5.093E-02	g/m³
Emissions to soil[a]		
Digestate for agricultural reuse		
Cd	8.836E-05	g/m³
Cu	3.879E-03	g/m³
Pb	2.758E-03	g/m³
Zn	1.626E-02	g/m³
Ni	1.954E-03	g/m³
Cr	2.328E-03	g/m³
Hg	4.111E-05	g/m³

(Table continued on the next page)

Table 7-5. *Continued.*

	Scenario IWW	Unit
Outputs		
Avoided products[a]		
Biogas cogeneration		
Electricity production	6.418E-01	kWh/m³
Heat production	1.009E+00	kWh/m³
Digestate for agricultural reuse		
N as fertiliser	5.093E+00	g/m³
P as fertiliser	2.614E-01	g/m³
Pigments as organic chemical	2.513E-02	kg/m³
Waste for further treatment[a]		
Compost	5.388E-01	kg/m³

[a]*Annual averages*

Table 7-6. Summary of the inventory for Scenario SGM: high rate algal ponds (HRAPs) cultivating *A. platensis* (*Spirulina*) with standard growth media (SGM) for pigments production.

	Scenario SGM	Unit
Inputs		
Construction materials		
HRAP		
Concrete	6.531E-04	m^3/m^3
Steel	5.225E-02	kg/m^3
Centrifuge		
Steel	1.363E-04	kg/m^3
Anaerobic digester		
Concrete	1.713E-06	m^3/m^3
Steel	1.370E-04	kg/m^3
Operation		
Energy consumption[a]		
HRAP	3.726E-02	kWh/m^3
Centrifuge	1.000E+00	kWh/m^3
Pigment extraction	3.333E+00	kWh/m^3
Anaerobic digester	1.147E-03	kWh/m^3
Total energy consumption	4.372E+00	kWh/m^3
Chemicals[a]		
Medium		
Water, salt, ocean	1.00E+00	kg/m^3
Carbon dioxide	7.15E+00	kg/m^3
Nitrogen fertiliser	3.45E-02	kg/m^3
Phosphorus fertiliser	2.35E-02	kg/m^3
Iron sulphate	5.00E-04	kg/m^3
Pigments extraction		
Sodium phosphate	3.431E-03	kg/m^3
Outputs		
Emissions to water[a]		
Water	1.988E+03	kg/m^3
Salts	3.271E+00	kg/m^3

(Table continued on the next page)

Table 7-6. *Continued.*

	Scenario SGM	Unit
Outputs		
Emissions to air[a]		
Cultivation in HRAPs		
Carbon dioxide	9.650E-02	kg/m³
Nitrogen	5.000E-04	kg/m³
Digestate for agricultural reuse		
NH_3	2.501E-01	g/m³
N_2O	1.000E-02	g/m³
Avoided products[a]		
Biogas cogeneration		
Electricity production	1.143E-04	kWh/m³
Heat production	1.796E-04	kWh/m³
Digestate for agricultural reuse		
N as fertiliser	1.000E+00	g/m³
P as fertiliser	1.111E-01	g/m³
Pigments as organic chemical	6.820E-02	kg/m³

[a]*Annual averages*

7.2.2.3 Impact assessment

The environmental impacts associated with the wastewater treatment systems coupled with pigments recovery were quantified using the software *SimaPro® 8* ("PRé Sustainability," 2014). Potential environmental impacts were calculated according to the ReCiPe midpoint method (hierarchist approach) (Goedkoop et al., 2009). The selected method includes a series of impact categories, and the characterisation phase in this study was performed considering the following ones: Climate Change, Ozone Depletion, Terrestrial Acidification, Photochemical Oxidant Formation, Marine Eutrophication, Freshwater Eutrophication, Terrestrial Ecotoxicity, Human Toxicity, Metal Depletion, Fossil Depletion and Particulate Matter Formation. These impact categories were selected according to the most relevant environmental issues related to wastewater treatment and have been previously used for the evaluation of wastewater treatment and resources recovery (Corominas et al., 2013; Fang et al., 2016; Gallego et al., 2008; Garfí et al., 2017; Hospido et al., 2008). Normalisation was carried out in order to compare all the environmental impacts at the same scale. This provides information on the relative significance of the indicator results, allowing a fair comparison between the impacts estimated for each scenario (ISO, 2006). In this study, the European normalisation factors have been used (Europe ReCiPe H) (Goedkoop et al., 2009).

7.3 Results and discussion

7.3.1 Characterisation

The potential environmental impacts associated with the system treating urban wastewater in HRAPs followed by PBRs with mixed cultures (Scenario UWW) and the system treating industrial wastewater in a UASB followed by HRAPs with unialgal cultures (Scenario IWW), with the reference of the system of conventional pigments production using standard growth medium (Scenario SGM), according to the different impact categories are shown in Figure 7-4.

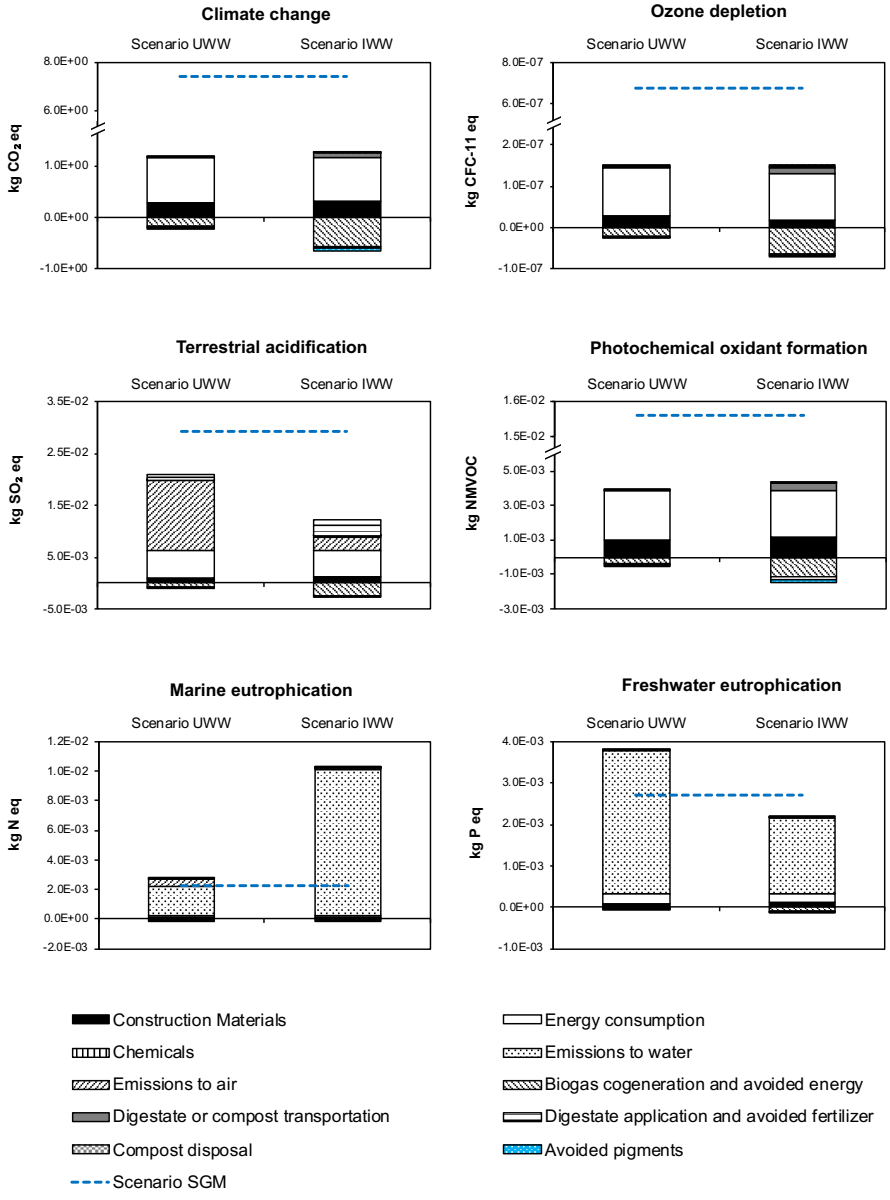

Figure 7-4. Potential environmental impacts for the three scenarios: Scenario UWW (microalgae-based system treating urban wastewater and recovering pigments), Scenario IWW (microalgae-based system treating food-industry wastewater and recovering pigments) and Scenario SGM: pigments production with standard growth media (SGM). Values are referred to the functional unit (m^3).

(Figure continued on the next page)

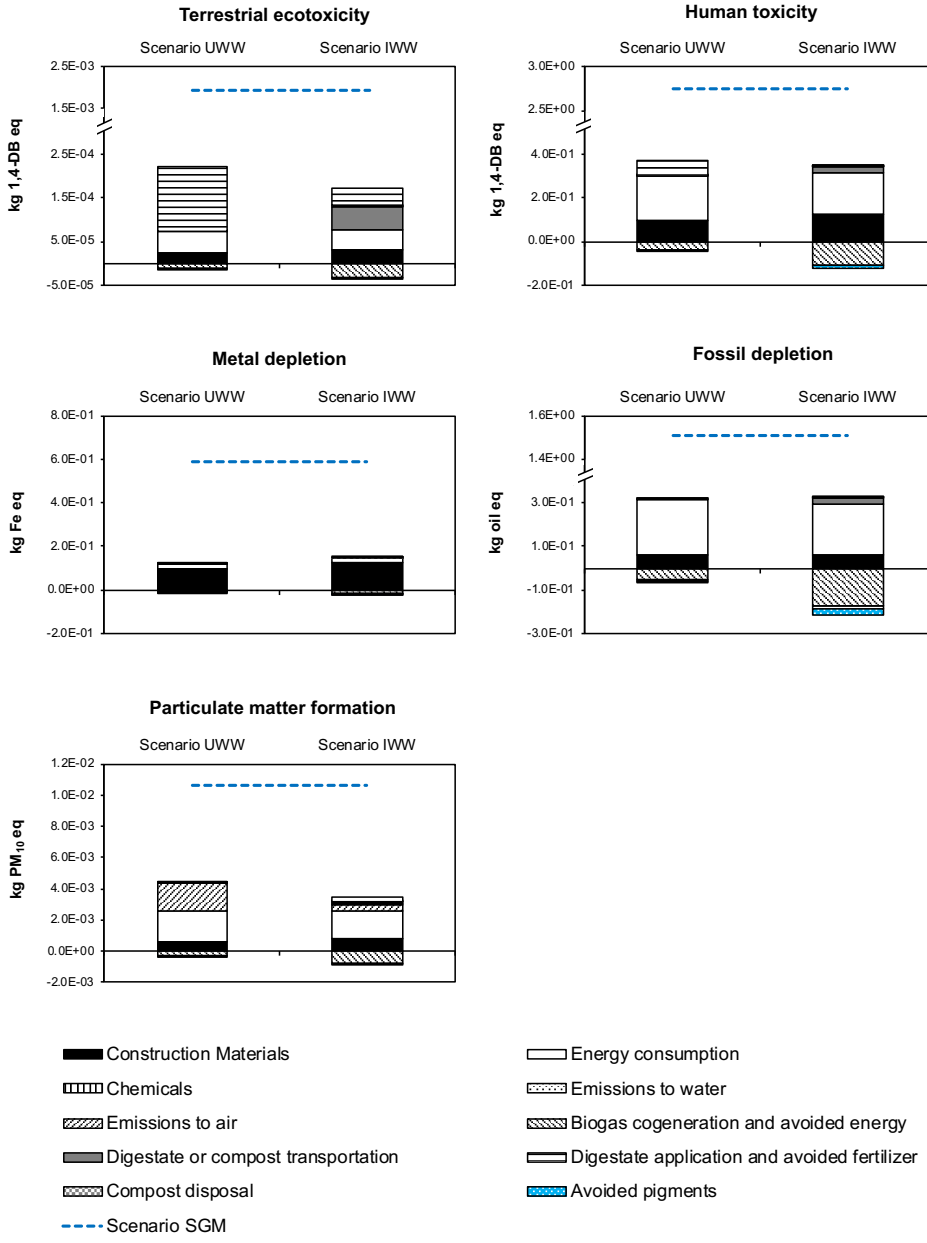

Figure 7-4. *(Continued).*

7.3.1.1 Comparison between Scenario UWW and Scenario IWW

Comparing the microalgae-based wastewater treatment systems proposed, the results indicate that Scenario UWW had higher environmental impacts (from 1.2-fold to 2.4-fold) than Scenario IWW in 9 out of 11 impact categories (i.e. Climate Change, Ozone Depletion, Terrestrial Acidification, Freshwater Eutrophication, Human Toxicity, Photochemical Oxidant Formation, Particulate Matter Formation, Terrestrial Ecotoxicity and Fossil Depletion). The main reasons for these results were the benefits generated from biogas cogeneration (Figure 7-4), which can be clearly observed especially for Climate Change, Ozone Depletion, Photochemical Oxidant Formation, Human Toxicity and Fossil Depletion potentials. Indeed, the electricity and heat generated from biogas cogeneration in Scenario IWW was more than 3 times higher than in Scenario UWW, due to the much higher BOD concentration in the industrial wastewater (2250 mg O_2/L) than in the urban wastewater (300 mg O_2/L), which would be further converted into biogas (Table 7-4 and Table 7-5). Hence, the electricity produced from biogas was equivalent to approximately 34% of the electricity consumption of the Scenario IWW, while only 11% for Scenario UWW.

Regarding Terrestrial Acidification and Particulate Matter Formation impact categories, not only the offset by the biogas cogeneration favoured Scenario IWW, but also the higher impact caused by the emissions to air (from NH_3 volatilisation) in Scenario UWW. Indeed, the average nitrogen emission from the HRAPs treating urban wastewater was higher than in Scenario IWW, with 5.495 g N/m^3 of water against 1.080 g N/m^3 of water, respectively. This was most probably related to the distinct inorganic nitrogen forms in both wastewaters. The major nitrogen form in the urban wastewater was NH_4^+ (**Chapter 3**), while in the industrial wastewater it was nitrate (**Chapter 5**). The higher concentrations of NH_4^+ caused higher NH_3 volatilisation rates, as also suggested in previous studies (Alcántara et al., 2015; Jones, 2010; Plouviez et al., 2019), leading to higher emissions to air observed in Scenario UWW (Figure 7-4).

In regard to Terrestrial Ecotoxicity potential, a major contributor for the higher impacts in Scenario UWW was the higher concentrations of heavy metals in the microalgae digestate than in the food-derived digestate (Table 7-4 and Table 7-5).

Nevertheless, the heavy metals concentrations in the microalgae digestate considered in this study were lower than the threshold established by the European sludge Directive 86/278/EEC (EEC, 1986) (Solé-Bundó et al., 2017b).

Scenario UWW showed better environmental performance than Scenario IWW in only 2 impact categories: Marine Eutrophication and Metal Depletion potentials. Regarding Marine Eutrophication, Scenario IWW showed significantly higher (by 3.74-fold) environmental impacts than Scenario UWW. This can be explained by the treated effluent quality, in which the industrial wastewater had a higher TN concentration in the effluent, with 10 mg N/L compared to 0.94 and 15 mg N/L in the effluent of HRAPs and PBRs, respectively (Table 7-1 and Table 7-2). Although the PBR effluent in Scenario UWW had higher TN concentration than the HRAPs in Scenario IWW, the volume was only 6.5% of the total flow, so the very low concentration of the major part of effluent discharged (from the HRAPs) in Scenario UWW offsets the environmental impacts. In regard to the Metal Depletion, Scenario IWW showed slightly higher impact (only by 16%) than Scenario UWW mostly due to the construction materials. Indeed, the amount of steel needed in the first case is around 29% higher than the last. This is related to the higher area of HRAPs estimated for this Scenario, since the IWW is mixed with seawater at a 75/25% (v/v%) ratio to ensure enough salinity level. This way, although the initial wastewater flow rate is the same for both cases, in Scenario UWW the flow used for pigments recovery in PBRs is only 6.5%, while in Scenario IWW the flow is increased because the industrial wastewater is mixed with seawater.

For Freshwater eutrophication potential, Scenario UWW showed higher environmental impact than Scenario IWW, also mostly as a result of emissions to water. This is explained by the higher TP concentrations in the effluent of HRAPs treating urban wastewater of 3.69 mg P/L, compared to industrial wastewater with 1.8 mg P/L (Table 7-1 and Table 7-2). The difference between the effluent quality of the two systems is related not only to its source (one being urban and the other industrial wastewater), but also to the initial nutrients concentrations when they enter the systems (industrial wastewater with concentrations about 2-fold higher than urban wastewater).

Nevertheless, in any case the effluent concentrations of phosphorus fulfil the discharge requirements.

Electricity consumption was by far the most impacting aspect in 6 impact categories (i.e. Climate Change, Ozone Depletion, Human Toxicity, Photochemical Oxidant Formation, Particulate Matter Formation and Fossil Depletion), accounting from 43 to 81% of the impacts. Following, construction materials were the major contributor for the highest impacts (83 and 85%) in Metal Depletion potential, but also as a secondary contributor in 5 impact categories (i.e. Climate Change, Ozone Depletion, Human Toxicity, Photochemical Oxidant Formation and Fossil Depletion), representing from 13 to 35% of the impacts. Afterwards, emissions to water through nutrients were the major contributor for the highest impacts in Freshwater Eutrophication potential (91% in Scenario UWW and 84% in Scenario IWW) and Marine Eutrophication potential (72% in Scenario UWW and 96% in Scenario IWW). Emissions to air of Scenario UWW through NH_3 volatilisation from HRAPs were the main contributor in Terrestrial Acidification potential (accounting for 64% of the impacts) and secondary contributor in Particulate Matter Formation and Marine Eutrophication potentials (39 and 18%, respectively). Finally, digestate reuse in agriculture was the main contributor in Scenario UWW for Terrestrial Ecotoxicity potential (accounting to 66% of the impacts, due to heavy metals concentrations) and secondary contributor in Scenario IWW for Terrestrial Acidification (accounting to 24% of the impacts, due to nitrogen volatilisation). Based on this, the major bottlenecks identified demonstrate that, in order to improve environmental performance of the microalgae-based systems studied, the following issues should still be addressed: 1) increase energy efficiency by optimising processes (e.g. pigments extraction, harvesting), maximising biogas production or integrating renewable sources to reduce impacts related to electricity consumption; 2) improve HRAPs design to reduce construction materials required (e.g. excavation instead of concrete structure); 3) improve nutrients removal efficiencies (e.g. installations in warmer regions); and 4) recover heavy metals from digestate before application in agriculture.

Overall, it is noteworthy to mention that, since in this study the two microalgae-based systems (Scenario UWW and Scenario IWW) were distinct in many aspects, such as treating different types of wastewater (urban vs. industrial), different combination of configurations implemented (HRAP, UASB and PBR) and phycobiliproteins yields (from mixed culture and unialgal culture), it is not possible to define the best system. Nonetheless, the results shown in this study suggest the use of food-industry effluent (Scenario IWW) as a more promising scenario mainly for the following reasons: 1) cultivation system: several researchers have reported that HRAPs are more energetically self-sufficient and more environmentally sustainable than PBRs, especially in cases in which the heat and power requirement of the process can be provided, totally or partially, by combusting the methane generated from the anaerobic digestion of the residual algal biomass (Stephenson et al., 2010); 2) Microalgae biomass: To be deemed suitable for producing pigments commercially, microalgae strains have to meet various criteria, such as ease of culture, lack of toxicity, high nutritional value, and presence of digestible cell walls to make the nutrients available (Christaki et al., 2015). Based on that, the most frequently used species are *Dunaliella salina*, *Haematococcus pluvialis*, *Chlorella* spp., *Muriellopsis* spp., *Scenedesmus* spp., *Arthrospira* spp. (*Spirulina*), and *Porphyridium* spp. (Borowitzka, 2013; Christaki et al., 2015; Eriksen, 2008; Ho et al., 2018; Spolaore et al., 2006). For this reason, cultivating a single species might be a better strategy than mixed cultures. This way, the cultivation parameters can be adjusted accordingly in order to maximise pigments recovery; 3) Risks of contamination and social acceptance: The application of the pigments recovered in Scenario UWW is much more limited than in Scenario IWW, since urban wastewater contains a wider variety of contaminants (e.g. pathogens, heavy metals, micropollutants) than food-industry wastewater. Although the purity of the final product could be proved to be suitable according to the application of the pigments, the cultivation in the urban wastewater could raise more concerns in terms of social acceptance and regulatory issues, which could hinder industrial scale production. For this reason, the use of food-processing waste streams could be a more appropriate alternative for providing nutrients for microalgae biomass growth while ensuring no risks of contamination. Nonetheless, sea water or streams with enough salinity (e.g. reverse osmosis reject water) would be needed in this case.

An important observation in view of the results of the present work is the contribution of the pigments produced as an offset to the impacts calculated, which might not have been incorporated as a suitable input in the software *SimaPro® 8* ("PRé Sustainability," 2014). The input parameter to be selected from the database as an avoided product (due to phycobiliproteins recovery) would be a product that would eventually be replaced by it. However, this depends on the application desired for the phycobiliproteins recovered in this process. As previously mentioned, application of pigments recovered in Scenario UWW would be more limited, such as printing dyes or creative arts sector, while in Scenario IWW the application could be extended, such as in textile, food and cosmetic industries (undoubtedly after verification of safety regulations). In this context, as most of the pigments and dyes used in the textile, cosmetic and food industries nowadays are organic synthetic pigments (Drumond Chequer et al., 2013; Kumar et al., 2017), the pigment produced in this study was introduced in the software as organic chemicals. Overall, the potential environmental impacts avoided (negative impact) indicated that the pigments produced small impact, e.g. ranging from 0.1 to 6% of the total impact for all impact categories observed for Scenario IWW, which produced significantly higher amount of pigments than Scenario UWW (0.02513 against 0.001 kg organic chemicals/m^3 water). In contrast, the environmental impacts caused by the electricity consumption for pigments extraction was the second highest for Scenario UWW and first highest in Scenario IWW (Table 7-4 and Table 7-5). Therefore, considering that electricity consumption was the major contributor for environmental impacts potential in both Scenarios, as previously discussed, the results indicate that the incorporation of pigments recovery might increase environmental impacts of the microalgae wastewater treatment systems. Furthermore, if the recovery of this high-value compound is implemented, improving the energy efficiency of the extraction step is essential. Sustainable energy alternatives (e.g. biogas) could be applied in order to tackle this issue.

7.3.1.2 *Comparison between microalgae-based wastewater treatment systems and conventional pigments production with standard growth medium*

Although the previous section indicated that incorporating pigments recovery can increase potential environmental impacts than solely microalgae-based wastewater treatment systems, when they were compared with conventional pigments production using standard growth medium (Scenario SGM), both systems investigated (Scenarios UWW and IWW) showed better environmental performance. Scenario SGM showed higher environmental impacts than Scenario UWW in 9 out of 11 impact categories (from 1.4-fold to 9.1-fold higher), while Scenario IWW in 10 out of 11 impact categories (from 1.3-fold to 14.3-fold higher). As expected, the main contributors for the higher impacts in Scenario SGM were electricity consumption and the chemicals input, which represented from 82 to 99% of the impacts in all categories evaluated. The only impact categories in which wastewater systems showed worse performance than the synthetic medium were Marine Eutrophication (Scenario UWW 23% higher and Scenario IWW 3.6-fold higher) and Freshwater Eutrophication (Scenario UWW 38% higher). Yet, it is important to note that these impacts were associated with the discharge of nutrients in the treated effluent, as previously explained. In the case of Scenario SGM there were no discharges to water bodies, since the inventory was based on systems in which all nutrients are taken up by the microalgae by recycling the medium (Papadaki et al., 2017). The benefits of using wastewater as growth medium (such as avoiding nutrients discharge in case of no treatment) were not accounted for in this study. Moreover, the impacts related to nutrients discharges could be minimised in a full-scale plant, by optimising operational conditions, which could favour even more the use of wastewater for recovering high-value compounds and bioenergy.

The results in this work are in accordance with previous research on microalgae and valuable compounds production. Ye et al. (2018) carried out a comparative LCA of an industrial scale production of *Spirulina* tablets (capsules) and found out that the most impacting stage along the entire process was the cultivation, responsible for approximately 60% of the total impacts, followed by harvesting (1-20%) and tablets production (<10%). From the cultivation stage, the growth medium was the major contributor, accounting for 80% of the impacts due to the high nutrients needed for

cultivation. In this context, extensive research has been made to identify the advantages and potential risks of either recycling growth medium or using waste streams in order to reduce costs and impacts of cultivation. However, the effects of recycling medium reported in the literature are contradictory, with some studies revealing positive aspects of recycling (Ho et al., 2018; Y. Li et al., 2019; Wang et al., 2018) while others highlighting inhibitory effects on biomass growth (Hadj-Romdhane et al., 2013; Loftus and Johnson, 2019). Therefore, the use of wastewater is a considerable option as it provides the necessary nutrients and environmental conditions required for the enhanced metabolite content of microalgae, while being a low-cost media and, thus, a better approach compared to the processing involved by using standard growth media (Alam and Wang, 2019).

7.3.2 Normalisation

The normalised results show that Freshwater Eutrophication, Marine Eutrophication, Terrestrial Acidification and Human Toxicity potentials are the most significant impact categories for all the scenarios considered (Figure 7-5), which were in accordance with previous LCAs on wastewater treatment systems (Fang et al., 2016; Gallego et al., 2008; Hospido et al., 2004). Scenario IWW showed to be the most environmentally friendly alternative in all impact categories, except for Marine Eutrophication and Metal Depletion. As Marine Eutrophication is among the most important impact categories, higher nutrients concentrations appeared to be a key factor against Scenario IWW compared to Scenario UWW. In this sense, operational conditions could be addressed in order to optimise the nitrogen removal efficiency in such system.

The conventional system using standard growth media for pigments production (Scenario SGM) showed higher impacts in all impact categories, except for the Marine and Freshwater Eutrophication, which are related to the discharge of residual nutrients from treated effluent. Significant impacts from Scenario SGM were observed in Freshwater Eutrophication and Human Toxicity, which are mostly associated with the high amount of chemicals needed for the pigments production.

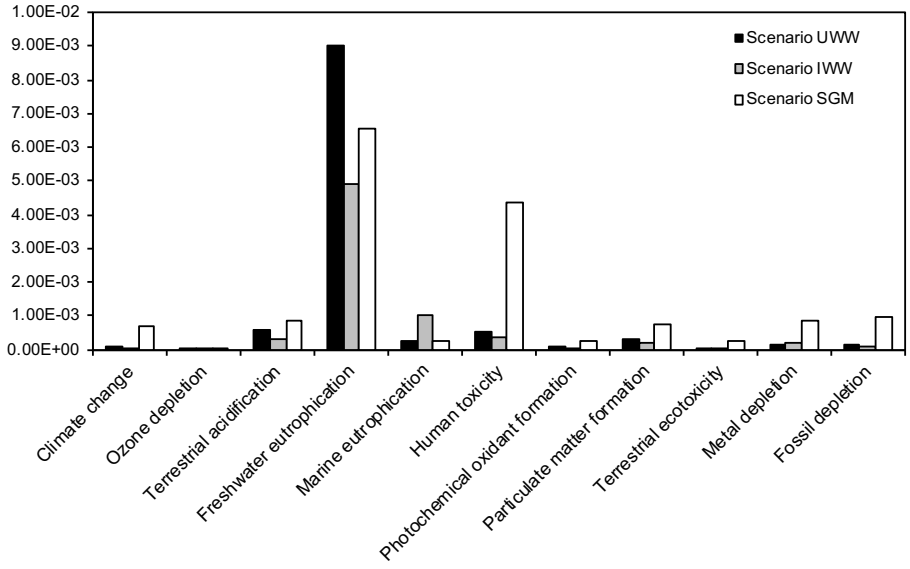

Figure 7-5. Normalised potential environmental impacts for the three scenarios: Scenario UWW (microalgae-based system treating urban wastewater and recovering pigments), Scenario IWW (microalgae-based system treating food-industry wastewater and recovering pigments) and Scenario SGM: pigments production with standard growth media (SGM).

7.4 Conclusions

The main findings from this study can be useful for identifying major bottlenecks for scaling-up microalgae-based wastewater treatment systems coupled with phycobiliproteins recovery from an environmental point of view (i.e. high electricity consumption, construction materials used, wastewater treatment efficiency, contaminants in digestate and NH_3 volatilisation). In addition, the reference impacts incorporated by the conventional system (Scenario SGM) also underlines the advantages of using wastewater as nutrients sources for producing valuable compounds within a circular bioeconomy.

The results indicate that the system treating industrial wastewater in a UASB followed by HRAPs with unialgal cultures (Scenario IWW) has lower environmental impacts than the system treating urban wastewater in HRAPs followed by PBRs with mixed cultures (Scenario UWW) in the Climate Change, Ozone Depletion,

Photochemical Oxidant Formation, Human Toxicity, Terrestrial Acidification, Freshwater Eutrophication, Terrestrial Ecotoxicity, Fossil Depletion and Particulate Matter Formation.

The conventional system using standard growth media for pigments production (Scenario SGM) showed higher impacts in all impact categories, except for the Marine and Freshwater Eutrophication, which are related to the discharge of residual nutrients from treated effluent.

Key aspects were identified when evaluating the most relevant impacts: a) Characteristics of the treated effluent (i.e. nutrients concentrations) in the Marine Eutrophication and Freshwater Eutrophication; b) Emissions of nitrogen (NH_3 from HRAPs, as well as NH_3 and N_2O from digestate reuse in agriculture) in the Terrestrial Acidification, and c) High amount of chemicals from conventional pigments production in the Human Toxicity.

Microalgae pigments such as phycobiliproteins could be a leading natural resource for innovative potential functional ingredients in nutrition. The demand for these natural pigments is significantly increasing over synthesized chemicals with numerous commercial applications. Using wastewater to cultivate microalgae biomass would be a sustainable way to reduce costs and combine waste streams treatment towards a circular economy. Nevertheless, some bottlenecks, such as low yields and operational conditions, need to be properly addressed before microalgae can be moved from niche markets to large-scale use.

Chapter 8

Discussion

Picture on previous page:
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8. Discussion

8.1 Introduction

Microalgae systems for wastewater treatment is a long-known technology, but the focus from merely removing contaminants, to also recovering resources, has significantly increased in the past years. The promising features of microalgae offer the possibility of establishing sustainable biorefineries to draw multifaceted benefits and reinforce the objectives of resource efficient bioeconomy. This thesis addressed some of the alternatives for combining wastewater treatment and resources recovery using microalgae, by characterising the benefits and challenges associated to their implementation in the future.

The Discussion sections in **Chapters 3** to **7** described specific considerations from the results obtained through the research objectives in respective chapters. This chapter, on the other hand, extends a general discussion from a macro perspective point of view. The following sub-sections will tackle the attributes, as well as challenges and research needs identified during the development of this work. In addition, a comprehensive discussion regarding the environmental aspects of the systems and recoverable resources investigated in this study is presented. To conclude the chapter, future perspectives and recommendations are delineated. This overview was mainly based on the research outcomes and particular discussions provided in the previous chapters, but also on valuable discussions with relevant experts in this field over the course of this thesis.

8.2 Main learnings, opportune attributes and challenges

Currently, there are several alternatives of microalgae systems for wastewater treatment, as well as downstream processes in microalgae technologies. Therefore, numerous combinations of such alternatives can be implemented, according to the available resources and purpose of the system. Table 8-1 recapitulates the main objectives and learnings from each chapter of this thesis, indicating the cultivation systems and biomass valorisation techniques involved.

Table 8-1. Highlights of the main objectives and findings investigated in the previous chapters of this thesis.

Chapter	Cultivation configuration		Biomass valorisation			Main Objectives	Main findings
	HRAP	*PBR*	*Biogas*	*Biofertiliser*[a]	*Pigments*		
3	X		X			- To simplify HRAPs configuration by removing primary treatment step - To recover bioenergy from biomass	- Removal of primary treatment did not affect the wastewater treatment efficiency - Co-digestion of microalgae and sludge could improve methane yield
4		X	X		X	- To maintain cyanobacteria stable while treating centrate with secondary effluent - To recover phycobiliproteins and bioenergy from biomass	- Cyanobacteria stable at certain centrate dilutions in secondary effluent - Pigments content was not affected by different centrate dilutions - Extraction of pigments improved biogas production kinetics
5		X			X	- To use unialgal cultivations treating food-industry wastewater - To recover high-value compounds from biomass	- Unialgal cultivations could efficiently treat industrial wastewater - Phycobiliproteins synthesis comparable to standard growth medium
6	X		X	X		- To carry out a comparative LCA of HRAPs systems with biomass valorisation as biogas and biofertiliser - To compare HRAPs systems to conventional activated sludge system	- Warmer climate showed lower environmental impacts and biofertiliser seems to be more profitable than biogas - HRAPs had less environmental impacts than activated sludge systems
7	X	X	X	X	X	- To carry out a comparative LCA involving configurations, wastewater types and biomass valorisations (biogas, biofertiliser and pigments) studied in previous chapters	- Food-industry wastewater could be a potential growth medium to recover pigments - Phycobiliproteins production using wastewater had less impacts than standard growth medium

[a]Biomass valorisation as biofertiliser was not carried out in this research project, but results from a collaboration project with another research group was used for the LCA (*Chapter 6*).

8.2.1 *Microalgae-based wastewater treatment systems*

In order to implement microalgae-based systems, such as the ones described in this thesis, a major challenge is merging mass production of algal biomass with minimal energy and costs input. The experimental studies presented in **Chapters 3**, **4** and **5** were carried out at lab and pilot-scale. However, when large systems are considered, the results might change considerably. For this reason, designing microalgae systems at full-scale requires a careful assessment in order to ensure the balance between the input and output energy and resources involved.

In terms of system configuration, **Chapter 3** showed considerable results using HRAPs systems for treating urban wastewater. In spite of the fact that HRAPs had been widely used for wastewater treatment purposes, there are still challenging issues hindering their implementation at full-scale, such as large area and maintenance requirement and lack of control over environmental conditions. For this reason, the study suggested an alternative to simplify their implementation by removing the primary settling step, which could reduce area and costs requirements (Section 3.3.1). Within this context, Posadas et al. (2017) had suggested that primary suspended solid removal is unlikely needed but also mentioned that this possibility had not yet been properly discussed in the literature. At first, the concept of removing the primary treatment step may appear inappropriate, since influent will have higher solids concentrations that could affect the HRAPs operation by increasing light shading effect. However, not only had this been proved to be acceptable (from results in **Chapter 3**), but also endorsed previous theoretical research that lacked experimental validation.

The effluent of the HRAPs system eventually had inorganic nitrogen concentrations higher than the discharge limits. This could be addressed by increasing the HRT in colder periods, as proposed in previous studies (García et al., 2000; Gutiérrez et al., 2016). Another option would be to combine it with another (more concentrated) stream, which was the approach of the study presented in **Chapter 4**, in which the secondary effluent from the HRAPs was combined with the liquid phase (centrate) of an anaerobic digester effluent. This study was performed in PBRs, since this would allow a better control of

parameters for keeping the cyanobacteria portion in the biomass stable throughout the experimental period (Section 4.3.2). In fact, several large-scale systems have implemented PBRs for microalgae growth and the advantages rely mainly on the better control over cultivation conditions and higher biomass productivity (Acién Fernández et al., 2013). On the other hand, the energy input to operate the entire system has been reported to be approximately 350% higher than the HRAPs (Lam et al., 2019). Hence, cultivation in PBRs could easily lead to a negative energy balance in producing microalgae if no precautionary steps are taken to reduce the energy input.

Following, in **Chapter 5** another type of PBR at lab-scale was tested. This time the cultivation was done in polyethylene bags with aeration providing both inorganic carbon source and mixing. Indeed, plastic-bag PBRs have received considerable attention in the literature for their lower material cost and sterilised conditions for commercial-scale production (Huang et al., 2017; Thein et al., 2014; Zhu et al., 2018). Floating-bag PBR systems have application in ocean environments, while vertical flat-bag PBR systems are best suited for land-based operations (Zhu et al., 2018). However, non-biodegradable plastic is currently a major concern due to the pollution caused worldwide. In this sense, if this type of PBR is selected for future studied, the use of biodegradable plastic would be a more sustainable alternative.

In summary, the use of PBRs in full-scale systems should be carefully evaluated over other cultivation systems. Especially in cases of wastewater treatment as a sole purpose, closed PBRs are not the most adequate systems, due to the intense biofilm formation and high costs applied. In case high-value compounds are intended to be recovered from the biomass, as in the present work, PBRs might be a feasible option, since biomass valorisation is planned. However, a thorough economic assessment should be done in order to ensure that the economic surplus of the commercial bioproducts would counterbalance the investment and maintenance costs of PBRs.

8.2.2 Bioproducts from microalgae grown in wastewater

Regarding the bioproducts that could be recovered from microalgae cultivated in wastewater, it has been shown that several alternatives can be proposed, according to the desired purpose.

8.2.2.1 Biogas and biofertiliser

8.2.2.1.1 Opportune attributes

Firstly, **Chapter 3** showed that biomass grown in HRAPs systems could be used for bioenergy recovery through biogas production. Based on the simplified energy balance described (Section 3.3.5), the study indicates that when bioenergy is aimed for, the removal of primary treatment might not be the best option, since co-digestion of primary sludge and microalgae led to higher methane yields. On the other hand, the same study by Posadas et al. (2017) that endorsed the idea of removing primary treatment, showed that anaerobic digestion of algal-bacterial biomass generated in HRAPs systems is not economically profitable for the sole purpose of generating power, offering marginal energy savings (e.g. 10.7 €/p.e. year). The authors suggested that integrating a solar drying for the biomass (grown in HRAP without primary treatment) for further use as biofertiliser would be a more economical and energy-efficient alternative for nutrient removal and recovery (24.4 €/p.e. year). Furthermore, this is also closely linked to the results obtained in **Chapter 6**, in which the economic assessment indicated that valorising the biomass as biofertiliser (as biostimulants) would be more economic feasible than biogas. Therefore, based on the results of this thesis and on relevant studies in the literature, a configuration in which wastewater treatment is done in a HRAP without primary treatment and the biomass is solar-dried and further used as a fertiliser could be a promising alternative. Indeed, local climatic conditions and legislation for biofertiliser application should be taken into consideration. Similarly, Sfez et al. (2015) analysed the environmental sustainability of aquaculture wastewater treatment by microalgae, comparing the valorisation of biomass as shrimp feed and as biogas. The authors reported that up-scaling improves the resource footprint of the plant potential

and the valorisation as shrimp feed is overall more sustainable than as biogas. Therefore, biogas production would be recommended to maximise resources recovery by using the residual biomass (after extraction of high-value bioproducts), thus minimising the energy demand in a biorefinery, rather than a unique valorisation step (Ramos-Suárez and Carreras, 2014).

8.2.2.1.2 Drivers and obstacles

The biogas generation from microalgae has been extensively discussed and has been already applied in large scale plants. There are no direct constraints related to this type of recovery, since the biogas is just an indirect step that will be further converted in electricity and heat. However, in the case of using microalgae extracts as biofertilisers, some concerns are still to be addressed in terms of applicability and regulations. Microalgae extracts have been reported as great alternatives as both biofertiliser (by releasing their components to the plants or by improving the nitrogen and phosphorus availability of plants) and biostimulants (since they contain organic materials that, when applied in small quantities, enhance plant growth and development) (ACI, 2017). However, the advancement of their applications in agriculture is hampered by various factors. In the applied case in this thesis (**Chapter 6**), the biofertiliser/biostimulant produced from Biorizon Biotech (Almería, Spain) is a result of an enzymatic process with a 60-70% hydrolysis degree and free amino-acids concentration of 40 g/L, with a high value in the market, ranging from 5 to 20 €/L (Acién, 2016). The sales record of this bioproduct has been increasing every year, but a few technical challenges are still being addressed, such as producing biomass containing target compounds, understanding its bioactivity in real field conditions, as well as its safety and sustainability (Acién, 2016). On the whole, while there is a general consensus on the potential benefits of the interaction between microalgae and crops, there is limited scientific evidence underpinning this interaction, compared to other organic/inorganic and microbial plant biostimulants (Chiaiese et al., 2018).

Furthermore, the regulatory framework for the application of microalgae bioproducts as biofertiliser/biostimulants is still unclear worldwide. Currently, as there are different market conditions and different national regulation requirements for plant

biostimulants in different countries, the regulatory processes can lead to unfair competition between operators (Caradonia et al., 2019). This issue hampers producers to catalogue and differentiate their products from common pesticides and fertilisers. This way, presenting accurate data and information to the biostimulants industry is problematic due to the lack of an official biostimulant definition.

8.2.2.2 Phycobiliproteins

8.2.2.2.1 Opportune attributes

The possibility to not only recover high-value products from microalgae, but also generate bioenergy with the residual biomass was featured in **Chapters 4** and **5**. Among numerous high-value compounds present in microalgae cells, phycobiliproteins were explored. These coloured proteins have several applications, ranging from food pigmentation to molecular labelling. The market of pigments has significantly increased during the past years, especially protein-based colourants, which have gained attention after the discovery that synthetic colour compounds can cause detrimental effects on humans, such as mental diseases, allergies and cancer (Tang et al., 2016).

Results shown in **Chapter 4** indicated no significant differences in the overall amount of phycobiliproteins found in the three PBRs operated with varying influent concentrations. However, during the steady state, the amount in PBRs with centrate (PBR-15% and PBR-25%) had higher concentrations than the PBR with only secondary effluent (PBR-0%). This showed that limiting nutrients concentration can significantly decrease phycobiliproteins synthesis. Likewise, **Chapter 5** showed that although the content of phycobiliproteins was mostly affected by the light intensity, culture conditions could also influence. Therefore, these studies emphasize the fact that the culture media, as well as physicochemical parameters such as temperature and pH, should be optimised specifically for the cultivated microalgae. Considering that each microalgae species has specific growth requirements, these must be explored prior to its large-scale production.

Overall, public and private initiatives are actively promoting algae-derived products and their commercialisation. The results are visible both at an industry level, with the

products for nutraceutical and cosmetic sectors booming, and at the community level, with people adopting new sources for materials (e.g. bioplastics) and food.

Based on this, pigments recovered from urban wastewater (**Chapter 4**) would probably face limitations for applications, since the biomass was presumably grown in presence of contaminants, such as micropollutants and heavy metals. In fact, after several purification techniques these contaminants might be reduced in the final pigments, but further analyses should be done to ensure their quality. Nevertheless, the application of these pigments could be focused on non-food alternatives, such as paintings for textiles or arts. Relevant examples are the recent studies reporting the use of extracts of red pigment from the macroalgae *Gracilaria vermiculophylla* and blue pigment from the *Arthrospira platensis* showing even distribution on the cotton and wool fabrics, with results representing the viability and the quality of naturally dyed textiles (Ferrándiz et al., 2016; Moldovan et al., 2017). In addition, as depicted in **Chapter 5**, the phycobiliproteins from microalgae grown in food-industry wastewater offer more promising prospects, since food company process water should be free from contaminants to comply with food regulations, and therefore poses no risks for microalgae biomass cultivation.

8.2.2.2.2 Drivers and obstacles

Compared to biogas and biofertiliser, phycobiliproteins as microalgae valorisation are probably associated with more difficulties, particularly with the scale transfer and legal concerns. Most of the cited references in this thesis regarding those pigments are based on lab-scale results. It is important to emphasise, though, that extension and application of those results to industrial scale are very complex, requiring a significant capital investment. Although phycobiliproteins have already shown a great potential of applicability in the industry because of their wide range of bioactivities, part of this potential is not yet covered due to major limitations, such as efficient extraction and purification methods and pigments stability. Currently, given the lack of efficient large-scale production of phycobiliproteins, there is a need for solutions to tackle these issues. Under these circumstances, a very extensive research has been done in the past decades trying to elucidate effective methods to extract phycobiliproteins from numerous species

of microalgae. As presented in **Chapters 4** and **5**, these phycochemicals can be easily obtained with a combination of physical and chemical extraction (in this thesis, simple extraction methods tested were freeze-thawing and ultrasound in phosphate buffer, as described in Sections 4.2.4 and 5.2.6). However, in order to also increase the value of commercial phycobiliproteins, refined purification procedures are required. The most used techniques involve a first step by precipitation with different amounts of ammonium sulphate to separate the various kinds of phycobiliproteins and also exclude some other pigments. Then, specifically, a second or more purification steps by chromatographic column due to differences in the colours and polarity of the pigments, or their size (Pagels et al., 2019). Several other techniques have been proposed in the literature (Chew et al., 2019; Cruz De Jesús et al., 2016; Cuellar-Bermudez et al., 2015). In regard with phycobiliproteins stability, this can be improved by adding high concentrations of sugars and salts such as glucose and sodium chloride. However, further research is needed focussing in the chemical interaction of phycobiliproteins with stabilising agents and the food matrix. Also, more studies are needed in dealing with the bioavailability and biological potential of phycobiliproteins and their mechanisms of action (Hsieh-Lo et al., 2019).

Another limitation still to be tackled is concerning legal issues. Currently, there are no specific regulations in many countries to control the use of algae extracts as components of products, e.g. for human (e.g. nutraceuticals) or plants (e.g. biostimulants) (Michalak and Chojnacka, 2015). Particularly in Europe, lack of robust framework is mostly related to the unclear regulatory environment, related to the classification of such products (EC, 2014). This and other legislative barriers lead to more restrained and hesitant position of investors and retailers in relation to the financial and commercial support of bioproducts. In this sense, companies active in the production and commercialisation of algae products limit themselves in production volumes, which hinders their advance in the current market. Therefore, new regulations should be established, for instance as in pharmaceutical and food sectors, in order to ensure competitiveness of such businesses.

Regarding social acceptance, increased awareness of the health and environmental benefits of natural sources is undoubtedly the main driver that supports the uptake of this trend. Fortunately, the prospects for microalgae bioproducts are promising because of increased public awareness towards health care, healthy food and sustainable products. From this perspective, a robust effort in the market has been noticed, providing ways to replace synthetic chemicals and mineral fertilisers by new natural alternatives which would minimise environmental impacts.

8.2.3 Microalgae biorefinery approach

The bioproducts and bioenergy recovered in this thesis, despite being addressed at lab and pilot-scale, could potentially be considered within a microalgae biorefinery concept. In order to discuss the results obtained in the experimental part of the present work (**Chapters 3**, **4** and **5**) applied to hypothetical full-scale plants (**Chapters 6** and **7**), main input and output parameters are summarised in Table 8-2. Scenario UWW-Biogas and Scenario UWW-Biofertiliser represent Scenario 1 and Scenario 2 from **Chapter 6**, respectively, while Scenario UWW-Pigments and Scenario IWW-Pigments represent Scenario UWW and Scenario IWW from **Chapter 7**, respectively. Although the functional unit used in both LCA studies was the same (1 m^3 treated water), some characteristics are distinct, such as flow rate and type of wastewater. It is thus important to remark that results from these studies cannot be precisely compared. Nevertheless, an overall analysis can be roughly drawn to have indicators that are easy to interpret and to evaluate from a general perspective which technology or biomass valorisation technique would the suitable in terms of resources recovery.

Regarding the surface area required, all cases showed similar area needed, except for Scenario UWW-Biofertiliser. The lower area requirement in this case was due to the more favourable climatic conditions, allowing a constant low HRT throughout the entire year, as previously discussed in **Chapter 6** (Section 6.3.1.1). Furthermore, the systems could be considered to be implemented in non-arable lands, discarding then the burden of large area requirement.

Table 8-2. Comparison of the four microalgae-based systems for wastewater treatment and resources recovery investigated in this thesis: Scenario UWW-Biogas and Scenario UWW-Biofertiliser from **Chapter 6** and Scenario UWW-Pigments and Scenario IWW-Pigments from **Chapter 7**.

	Unit	Scenario UWW-Biogas	Scenario UWW-Biofertiliser	Scenario UWW-Pigments	Scenario IWW-Pigments
Specific surface area	m^2/p.e.	4	3	4.3	4.075
Electricity consumption	kWh/m^3	$6.00x10^{-2}$	$7.54x10^{-2}$	1.878	1.882
Resources recovered					
Electricity production (biogas)	kWh/m^3	$5.40x10^{-1}$	-	$2.071x10^{-1}$	$6.418x10^{-1}$
Heat production (biogas)	kWh/m^3	$8.49x10^{-1}$	-	$3.254x10^{-1}$	1.009
N as biofertiliser	g/m^3	$2.59x10^{1}$	5.77	2.213	5.093
P as biofertiliser	g/m^3	1.31	1.20	$2.458x10^{-1}$	$2.614x10^{-1}$
Pigments as organic chemical	g/m^3	-	-	$1.001x10^{-3}$	$2.513x10^{-2}$

In respect to the electricity consumption, Scenarios recovering pigments had much higher demand (by two orders of magnitude) than in Scenarios recovering biogas and biofertiliser, while producing similar (same magnitude) amount of electricity (Scenario UWW-Biofertiliser did not produce electricity). The heat production could also be used for any process in the facility, such as substrate pre-treatment, as previously discussed (Sections 3.3.4 and 4.3.4). Finally, concerning the bioproducts recovered, on the one hand Scenario UWW-Biogas showed higher nutrients recovery as avoided biofertiliser, but on the other hand Scenarios UWW-Pigments and IWW-Pigments had lower nutrients recovery, but had indeed the pigments as avoided organic chemicals. In this case, fair comparisons cannot be drawn since the bioproducts characteristics and applications are completely different. From an economic point of view, a thorough analysis would be convenient in order to demonstrate whether the overall benefits of pigments recovery could somehow compensate the higher energy demand of the entire process. Furthermore, in this study biogas has been recovered with cogeneration, but upgrading

it to biomethane is also a promising alternative to valorise this co-product, as proposed by several recent studies (Adnan et al., 2019; Rodero et al., 2019).

This general analysis comparing the four Scenarios studied in this thesis did not aim to reveal the 'best' case for microalgae-based wastewater treatment and resources recovery, but to enlighten possible ways to conduct the recovery of useful and valuable bioproducts in this context. It is also important to carefully appraise the set of technical and financial investments needed in each case, as well as the subsequent application or niche markets implied. For instance, in the case of biofertiliser produced in Scenario UWW-Biofertiliser, as discussed in Section 8.2.2.1.2, its application is very specific in terms of applicability. This is related to the targeted compounds, which depend on coinciding microalgae species and plant crops, and also to the social acceptance, since it largely depends on the interest from local farmers and producers in trying such new products. In contrast, the application of pigments could be possibly wider. On one hand, the niche market for purified phycobiliproteins is limited (cosmetics, biotechnology, pharmacology and medicine), but on the other hand, the increasing interest for natural colourants with a range variety of applications that do not require high purity, such as printing dyes, use in textile industry and creative arts sector, could expand their demand.

In summary, microalgae cultivation in wastewater is a promising biorefinery approach. The criteria to develop optimised microalgae biorefinery system includes operation cost, selection of robust microalgae strain, and process sustainability. However, many challenges are still confronted, such as contamination, low biomass yield, complex nutrients removal mechanism and impurities in the biomass after downstream processing (Javed et al., 2019). For a sustainable and economic biorefinery, future research should be dedicated to address these challenges.

8.2.4 Environmental aspects

Ever since the positive prospects of cultivating microalgae to recover nutrients from wastewater have been extensively reported in literature (Abdel-Raouf et al., 2012; Kouzuma and Watanabe, 2015; K. Li et al., 2019), recent active research and development have further propelled these systems a step closer toward scaling-up and

commercialisation. However, the issues of energy balance and economic feasibility in the entire system boundary of microalgae facilities are not clearly addressed, mainly due to limited availability of commercial cultivation plants for technical assessment.

Most of the studies based on LCA of microalgae production using standard culture medium, unfortunately revealed a negative energy balance in their assessments, especially when microalgae were cultivated in PBRs for biofuel production (Jorquera et al., 2010; Lam et al., 2019; Stephenson et al., 2010). For this reason, recent studies performing LCA of systems using wastes (e.g. wastewater and flue gas) to provide growing conditions for microalgae have significantly increased and suggested positive results (Garfí et al., 2017; Maga, 2017; Posada et al., 2016). It is important though to note that important parameters (biomass yield, lipid productivity, specific growth rate) assumed in those LCA studies were predominantly based on findings from lab-scale and might be critical to replicate the data for large-scale production. However, these results can at least create a baseline to visualise and estimate potential problems and hindrances in the microalgae-based systems. As a result, several precautionary steps could be suggested to improve further those systems before full implementation stage.

With an attempt to have an overview of the results from the two environmental assessments carried out for this thesis (**Chapters 6** and **7**), Figure 8-1 shows the normalised potential environmental impacts for the four scenarios as also assumed in Section 8.2.3 (Table 8-2).

The most relevant impact categories for both studies, as previously mentioned in Sections 6.3.1.2 and 7.3.2, affect mainly Freshwater and Marine Eutrophication, Human Toxicity and Terrestrial Acidification potentials. Based on this, one can notice that potential environmental impacts rely on the same categories when biomass valorisation is either biogas and biofertiliser (**Chapter 6**), or phycobiliproteins (**Chapter 7**). Under these circumstances, crucial improvements for better environmental performances in these systems are: 1) innovating cultivation systems designs in order to reduce materials needed for their construction; 2) ensuring the lowest nutrients concentrations possible; 3) finding better ways to decrease NH_3 and N_2O emissions from HRAPs and digestate application; 4) recovering potential heavy metals present in the digestate that would be

further applied in agriculture; and 5) improving energy efficiency (for phycobiliproteins production). Overall, the results showed that further efforts in algal-based research should be directed to improving the productivity, development of multi-product scenarios, better incorporation for valorising coproducts, integration with current industrial facilities to provide sustainable nutrient resources from waste streams, and integration of renewable technologies to minimise impacts related to electricity consumption.

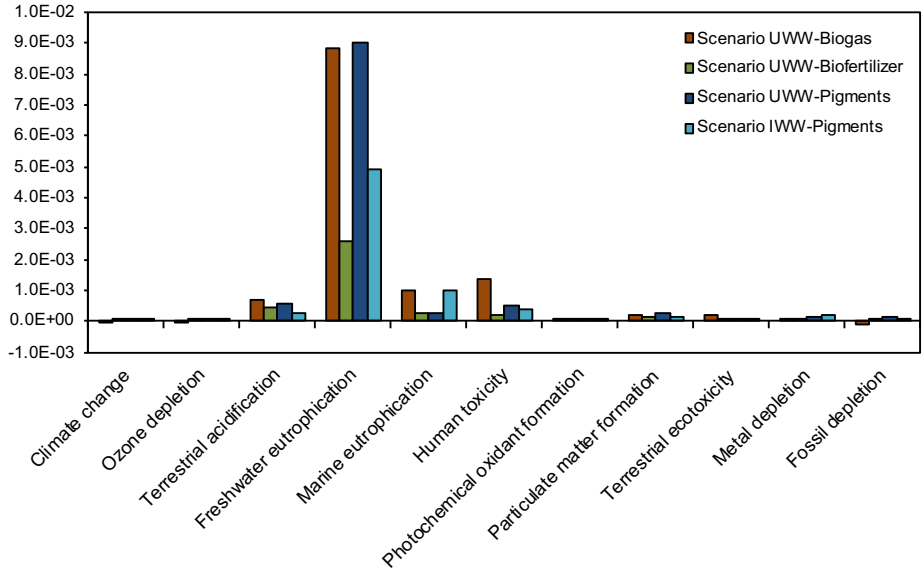


Figure 8-1. Normalised potential environmental impacts for four scenarios: Scenario UWW-Biogas (HRAPs system treating urban wastewater and recovering biogas), Scenario UWW-Biofertiliser (HRAPs system treating urban wastewater and recovering biofertiliser), Scenario UWW-Pigments (HRAPspilot and PBR systems treating urban wastewater and recovering pigments) and Scenario IWW-Pigments (HRAPs system treating food-industry wastewater and recovering pigments).

Furthermore, it is important to highlight that both LCA studies showed positive outcomes when comparing microalgae systems with conventional ones (**Chapters 6** and **7**). The first study showed that microalgae systems coupled with biogas and biofertiliser were more environmentally friendly than conventional activated sludge system, while the second LCA study demonstrated that pigments extracted from microalgae grown in

wastewater were more environmentally friendly than when they are grown in standard growth medium.

The LCA studies helped in identifying key materials and processes within the scenarios described that are likely to pose the greatest impacts, including resource demand and human health impacts. These assessments delineate the full benefits of a product or process, which can assist decision-makers to select the most effective solution in the future.

8.2.5 Economic feasibility

The assessment of the microalgae-based technologies in economic aspects is also very relevant, especially when addressing the challenges for their scaling-up. A number of studies addressed the economic analysis of cultivation of microalgae in HRAPs systems and closed PBRs in different synthetic culture media (Banerjee and Ramaswamy, 2017; Norsker et al., 2011; Resurreccion et al., 2012; Ruiz et al., 2016). A recent detailed study carried out by Javed et al. (2019) showed that several techno-economic studies on microalgae biorefinery have pointed the microalgae cultivation as a major cost as a result of high nutrients supply. For this reason, wastewater has been applied as a source for nutrients to feed microalgae. However, as microalgae require specific nutrients compositions, a subsequent challenge in using wastewater is the dilution, which would demand an additional cost for water supply (Javed et al., 2019). In this sense, the strategy used in **Chapter 4**, of diluting concentrated waste streams in secondary effluent is certainly a relevant alternative to its treatment without compromising water demand. This approach is aligned with the concept of "industrial ecology", also known as "industrial symbiosis", in which industries can share their resources and their wastes to mutual benefit, a sort of a "waste exchange" (Erkman and Ramaswamy, 2005). This way, a waste outlet of a certain process produces an intermediate product or utility (e.g. water stream or electricity) that can be used as an input to another process. This revamping increases the utilisation of existing capital, reduces energy requirements and, even more importantly, it is a sustainable way of changing a waste stream into a product stream (Jonker and Harmsen, 2012). This strategy

could certainly endorse the incorporation of microalgae-based systems into existing industrial facilities, by utilising their wastes to recover valuable compounds from microalgae biomass, as previously suggested in **Chapter 5** (Section 5.3.2).

Regarding the use of microalgae extracts as biofertiliser explored in **Chapter 6**, Coppens et al. (2016) reported that the microalgal production cost was estimated at €23 per kg of biomass produced, corresponding to €289 per kg of nitrogen. In contrast, commercial inorganic NPK fertilisers (having 14% N, 7% P, 15% K) and organic slow-release fertilisers (having 4% N, 2% P, 5% K) displayed a market value of €7.9 and €11 per kg of nitrogen, respectively (Ronga et al., 2019). These data clearly reveal that the use of microalgal biomass as biofertiliser is currently not economically competitive, compared to the commercial fertilisers. However, this economic analysis did not encompass the beneficial savings that can be obtained using microalgae systems. In particular, as discussed throughout this thesis, when wastewater is used to produce the microalgae biomass, both mutual benefits of reclaiming water and recovering nutrients, thus avoiding its release into the environment, are achieved. In addition, it is important to re-emphasize that the biofertiliser considered in this study represents more a soil amendment than a commercial fertiliser.

With the increasing interest and research of biofuels from microalgae in the past decades, several studies have reported that high production costs remain the main challenge in commercializing microalgae biofuels. From the several approaches that have been already explored, two of them have been greatly encouraged in the literature and are covered in the present work: i) The importance of choosing suitable nutrient sources (e.g. wastewater) in open pond design to enhance further the energy efficiency ratio value of algal biofuel (Javed et al., 2019; Khan et al., 2018; Lam et al., 2019; Laurens et al., 2017; Raheem et al., 2018); ii) The extraction of other high-value bioproducts from algae biomass as a necessary step to enhance the economic feasibility of algae biofuel production (Chew et al., 2017; Khoo et al., 2019; Kumar et al., 2020; Vuppaladadiyam et al., 2018; Yen et al., 2013).

Indeed, although the market volumes of secondary metabolites, such as phycobiliproteins, are very low compared to bulk chemicals and biofuels, their value is

much higher. Ruiz et al. (2016) designed a realistic cost and market analysis, in which they reported a higher profitability for high-value products (e.g. for cosmetic and food industry) with better projections for the near future. On the contrary, biofuels have a relatively low commercial value, so very large volumes or better coproduct valorisation are required to display cost and price competitiveness. These recent findings, combined with the fall of oil prices, have substantially prompted the shift in interest of microalgae-based innovative research from biofuels to high-value products (Vuppaladadiyam et al., 2018).

Under these circumstances, results obtained in **Chapters 4** and **5**, in which phycobiliproteins could be extracted and bioenergy was recovered with the residual biomass, are certainly relevant for the future of microalgae systems with a biorefinery approach. In particular, the economic feasibility from these results are also endorsed by Vulsteke et al. (2017), who evaluated the economic feasibility of HRAPs treating industrial wastewater (aquaculture and food industry) with further biomass valorisation as fertiliser, shrimp feed supplement, phycobiliproteins extraction and biogas production. The authors suggested that extraction of phycobiliproteins could generate substantial revenues if market prices stay sufficiently high and production of shrimp feed could help in considerably reducing the wastewater treatment costs, while biogas revenues are negligible.

To conclude, in order to foster economic viability, the actual costs of algal biomass production need to be minimised while significantly increasing production scale. Moreover, even when the price of biomass production is reduced, algal biomass needs to be refined into multiple products in order to increase its total value and achieve economic feasibility (Acién, 2016).

8.3 Future perspectives and recommendations

To summarise the current research trends and gaps discussed in the previous sections, Table 8-3 lists the current and validated empirical knowledge against the

challenges still to be addressed. This way, an outlook for future opportunities can be outlined, as well as recommendations for future work.

Table 8-3. Empirical knowledge and challenges for microalgae-based wastewater treatment systems with resources recovery.

Empirical knowledge	Challenges and recommendations
Microalgae-based wastewater treatment systems	
HRAPs systems can be simplified by removing primary settler, without compromising wastewater treatment and improving biomass settleability	Technical feasibility at large-scale (HRT and area needed) should be improved; Economic feasibility should be verified if anaerobic digestion is considered
HRAPs and PBRs can potentially treat wastewater while reaching high biomass productivity	Economic feasibility of cultivation and biomass valorisation and harvesting should be improved for full scale systems
Cyanobacteria-dominated biomass can efficiently treat centrate diluted with secondary effluent in photobioreactors	Photobioreactors installation and operation costs are high, so improvements are needed to enable scalability
LCA studies suggested microalgae-based wastewater treatment systems as sustainable alternatives for wastewater treatment and identified major bottlenecks for scaling-up	Further data on large-scale systems are needed in order to have more reliable interpretations of real risks and gaps in different configurations of microalgae-systems; Bottlenecks should be further addressed
Resources recovery	
Microalgae biomass can be digested (mono or co-digested) to recover bioenergy, with and without bioproducts extraction	Economic feasibility should be verified if anaerobic digestion is considered (higher methane yield by co-digesting microalgae and primary sludge, or perhaps biogas upgrading to biomethane)
Phycobiliproteins could be recovered from microalgae grown in wastewater	Technical feasibility should be improved for scalability and purity levels desired
Unialgal cultivations in food-industry wastewater could minimise costs of microalgae biomass production for further valorisation	Cultivation conditions in wastewater should be optimised (e.g. combining waste streams for favourable nutrients concentrations) to produce pigments comparable to standard media
LCA studies suggested microalgae-based wastewater treatment systems as great options to recover bioenergy, biofertiliser and	Bottlenecks should be further addressed; Legal and social aspects should be addressed and establishment of new regulations are urged in order

phycobiliproteins and identified major bottlenecks for scaling-up	to ensure biosafety of bioproducts and to facilitate these innovative solutions

Regarding microalgae system for wastewater treatment, as discussed in Section 8.2.1 and 8.2.2., a configuration in which a HRAP treats urban wastewater without primary treatment and the biomass is solar-dried and further used as a fertiliser could be a promising cost-effective alternative. This way, installation and operation costs, as well as area required could be reduced, while recovering useful resources from urban wastewater in a simple manner.

This thesis also highlighted the promising results on producing valuable compounds from urban and industrial wastewater. Facing the problematic of such a variety of industrial processes generating different types of wastewater, researchers should take advantage of the broad range of microalgae species, seeing them as potential candidates for bioremediation. In fact, increasing interest has been reported on the use of non-conventional extremophilic microalgae (thermophilic, acidophilic, and psychrophilic) to treat complex industrial effluents (Wollmann et al., 2019). Within this context, further research is encouraged to explore recovery of bioproducts from microalgae species that would potentially treat complex types of wastewaters.

Based on the results vastly discussed in this thesis, optimistic expectations have been raised concerning the recovery of phycobiliproteins (**Chapters 4**, **5** and **7**), digestate reuse in agriculture (as biofertilisers) (**Chapter 7**) and bioenergy from residual biomass (**Chapter 4)**. The results comparing these two recovered products were obtained in different systems and locations, as explained in Sections 4.2.2, 5.2.3 and 7.2.1. For this reason, future research at larger scale is encouraged, considering the entire system: wastewater as a way to produce clean water and microalgae biomass for further valorisation as pigments recovery, with residual biomass generating energy and biofertiliser.

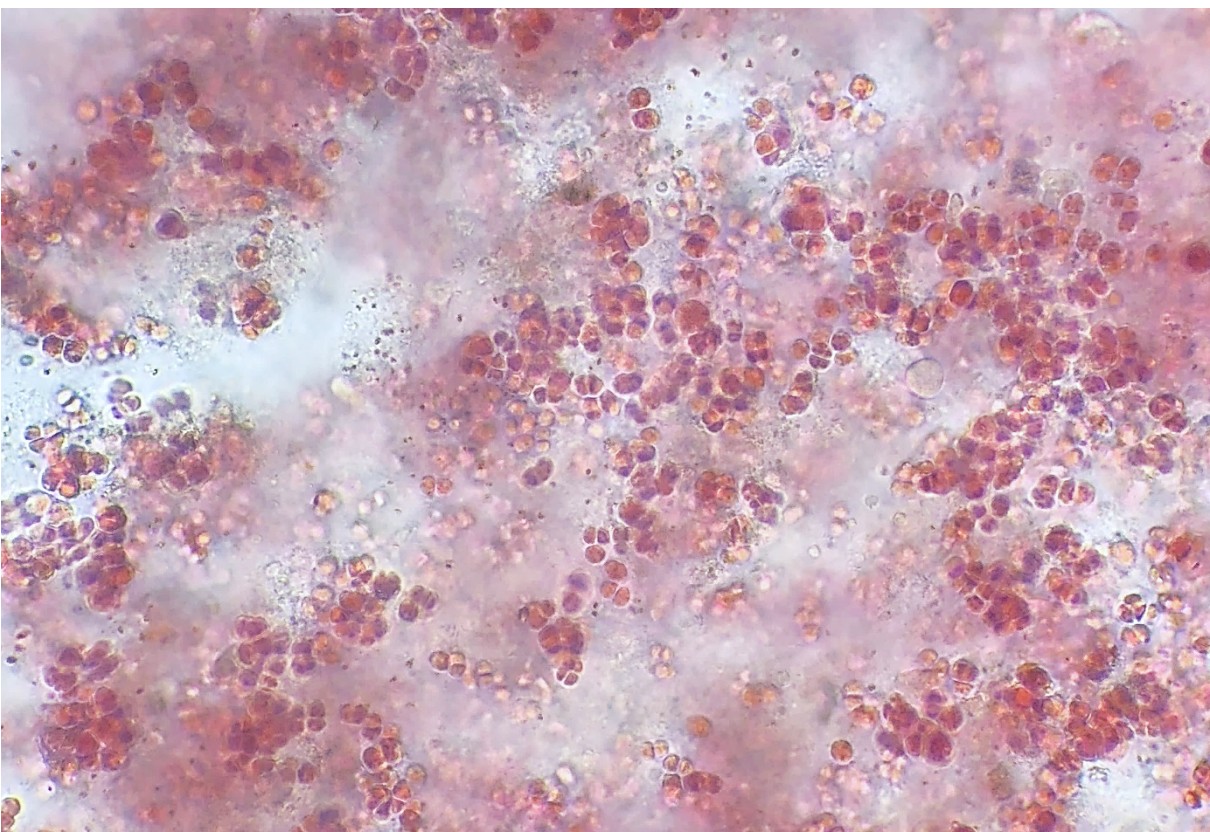
In respect to the microalgae species investigated in this study, it has been mentioned that several studies have previously shown that *A. platensis* (*Spirulina*) can be used either

for wastewater remediation solely or coupled with biomass production as a source of value-added products. This group of cyanobacteria has a great potential, since its cultivation at large scale is less vulnerable than most of other species. Furthermore, phycobiliproteins content have reported to be quite significant and the biomass can be successfully used as a substrate for biogas production. Indeed, its low lipid (< 30% cell dry weight) and high carbohydrate content make it an attractive candidate for anaerobic digestion as well (Varol and Ugurlu, 2016). However, only a limited number of studies have attempted to obtain multiple products from *Arthrospira* sp. biomass in a sequential manner by using treated wastewater as a growth medium (Chavan and Mutnuri, 2019). Therefore, as this group of microorganisms has been extensively applied from lab to industrial scale, and most regulatory advances on microalgae-derived products enlist them as major species, further exploration on their application is certainly recommended.

Finally, an in-depth understanding of the feasibility of microalgae-based systems for wastewater treatment and resources recovery in terms of technical and environmental aspects has been provided in this work. Further research is encouraged also in the economic aspects to not only maximise profits and minimise investment risks, but also to stimulate the "bigger picture" view for identifying the critical problems in scaling-up and recommending specific corrective measures. Overall, sustainable resources recovery alternatives such as the ones discussed in this work could certainly trigger a system redesign towards a circular economy.

Chapter 9

Conclusions



Picture on previous page:
Microscopic view of *Porphyridium purpureum* used in this research project (*Chapter 5*).

9. Conclusions

Cultivating microalgae as a sustainable source of biomass for recovering bioproducts and bioenergy from wastewater has been considered a new trend within the circular economy framework. The advantages and promises of microalgae are alleged to bring a revolutionary breakthrough in balancing the demand for sustainable resources while improving water quality. This thesis addressed some technical and environmental aspects within this context.

In terms of microalgae-based systems, this thesis investigated different configurations to treat distinct wastewaters. Firstly, urban wastewater was efficiently treated in simplified high rate algal ponds (HRAPs) systems, while ensuring good microalgal biomass production. The simplification could be done by removing the primary treatment, which would require less maintenance, as well as reducing costs and footprint of such systems. Furthermore, cultivation of cyanobacteria was shown to be a great approach to treat centrate diluted in secondary wastewater in photobioreactors. This study showed that addition of centrate could even be beneficial for further biomass valorisation. Lastly, food-industry wastewater was efficiently treated in unialgal cultivations of *Nostoc* sp., *A. platensis* and *P. purpureum* in plastic bag photobioreactors. This experimental work also investigated the effect of light conditions and medium composition, suggesting that the first had more influence on biomass growth and high-value compounds synthesis than the latter.

Regarding biomass valorisation, this thesis addressed the recovery of resources as bioenergy (biogas) and bioproducts (biofertiliser and phycobiliproteins). Biogas could be recovered from green microalgae grown in high rate algal ponds, demonstrating that co-digestion with primary sludge could increase methane production (238–258 mL CH_4/g VS compared to 189–225 mL CH_4/g VS) and kinetics. Phycobiliproteins were extracted from biomass harvested from mixed (up to 17 mg/g dry biomass) and unialgal cultures (up to 103 mg/g dry biomass), indicating the great potential for recovering such valuable compounds from microalgae. Further recovery of biogas from residual biomass (163 to 199 mL CH_4/g VS) was also evaluated and was higher than from biomass without phycobiliproteins extraction (153 to 187 mL CH_4/g VS). This indicates that not only

high-value compounds can be obtained, but also more energy could be recovered, as compared to the sole production of biogas.

A first life cycle assessment (LCA) study suggested that HRAPs system implemented in favourable climatic conditions has a great potential in terms of environmental aspects while HRAPs coupled with biofertiliser production is the most economically feasible solution. Additionally, HRAPs systems coupled with biogas and biofertiliser production showed lower potential environmental impacts compared to an activated sludge system. A second LCA study indicated that treatment of food-industry wastewater in a UASB followed by HRAPs with unialgal cultures has lower environmental impacts than the urban wastewater in HRAPs followed by PBRs with mixed cultures when coupled with both pigments and biogas recovery. In addition, the microalgae wastewater treatment systems showed lower environmental impacts than a conventional pigments production facility, outlining the advantages of using wastewater as nutrients sources for producing valuable compounds within a circular bioeconomy. Overall, the LCA was a useful tool to identify major bottlenecks and improvements for scaling-up microalgae-based wastewater treatment systems coupled with resources recovery in terms of environmental impacts: i) large amount of construction materials needed, which could be reduced by improving microalgae systems designs; ii) nutrients removal efficiency, which could be maximised by selecting favourable climatic conditions; iii) NH_3 volatilisation from HRAPs, which could be controlled through pH (e.g. CO_2 injection); iv) contaminants in digestate, so technologies to recover heavy metals should be explored; and v) high electricity consumption for pigments extraction, which could be minimised by improving biogas production from the anaerobic digester in the plant or using renewable sources.

In summary, the use of wastewaters for cultivating microalgae is certainly a great alternative to minimise the cost of biomass production. However, several challenges have still to be undertaken in order to benefit from the full potential of merging wastewater treatment and microalgae production. Major hindrances encourage the development of robust and efficient systems, as well as improving and innovating

cultivation and downstream processes which will allow for better growth, harvesting and bioconversion of the biomass.

To conclude, the present work has discussed promising alternatives to promote and support the transition towards a sustainable circular economy. In this context, the combination of technological innovations and improvements, economic feasibility, and regulatory progress play a critical role and are the driving forces to materialise microalgae-based systems for resources recovery from wastewater at full scale.

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Appendix

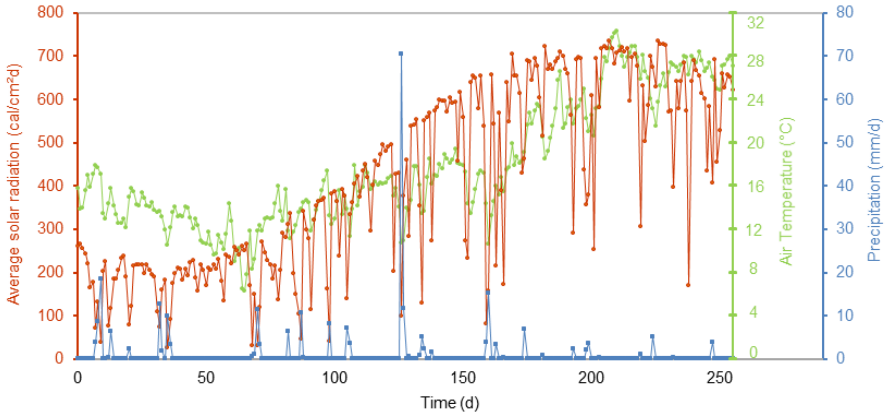

Figure A-1. Solar radiation, air temperature and precipitation data recorded by the local automatic weather station of Barcelona – Zona Universitària (X8) (DAM, 2017).

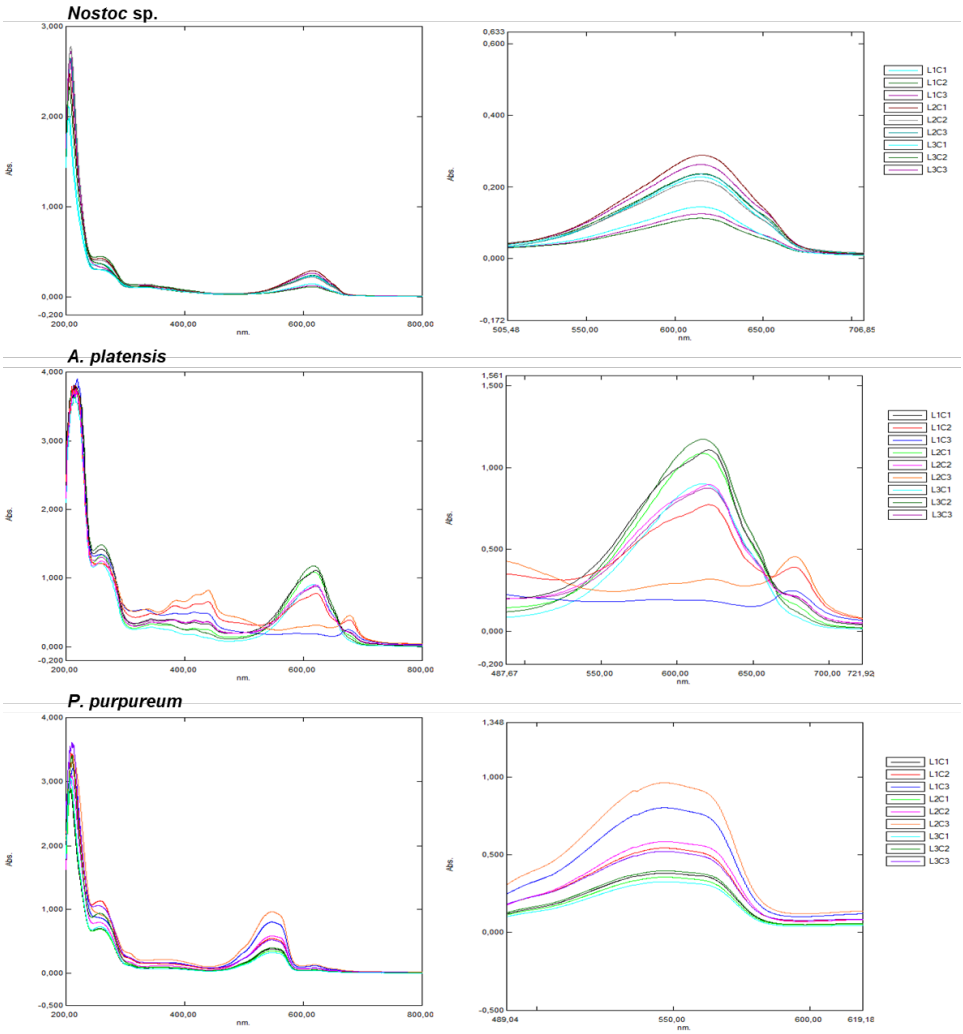

Figure A-2. Absorption spectrum of crude extracts obtained from *Nostoc* sp., *A. platensis* and *P. purpureum* grown during the optimisation experiment. Biomass growth was monitored under different conditions of low (C1), medium (C2) and high (C3) concentration of the main ingredient in standard growth medium ($NaNO_3$) and low (L1), medium (L2) and high (L3) light intensity.

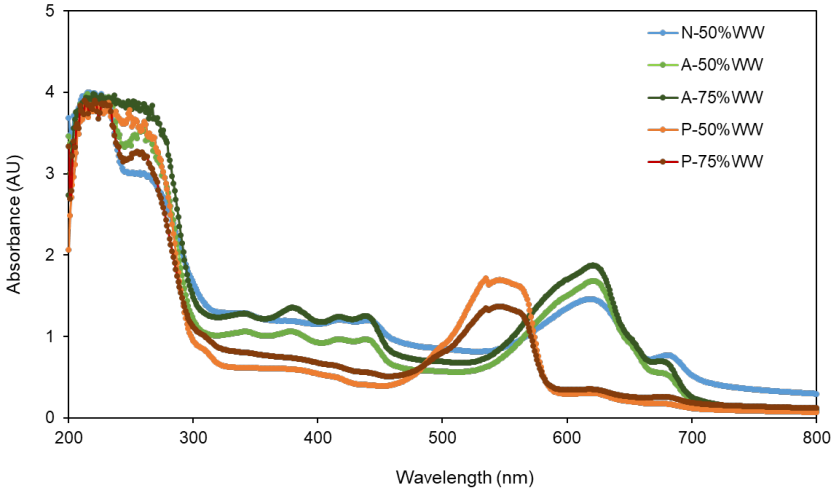

Figure A-3. Absorption spectrum of crude extracts obtained from *Nostoc* sp. (N-50% WW) and saline species *A. platensis* (A-50%WW and A-75%WW) and *P. purpureum* (P-50%WW and P-75%WW) grown during the wastewater experiment.

Table A-1. Summary of the average concentrations of the main water quality parameters measured in the influent and effluent of both HRAPs in cold (Nov-Mar) and warm (Apr-Jul) seasons, and the respective removal efficiencies. *P*-values for the t-test comparing values of the removal efficiencies (95% confidence interval) are highlighted in bold when there is significant difference.

		Cold Season							**Warm Season**						
		HRAP-C			**HRAP-T**				**HRAP-C**			**HRAP-T**			
		Influent (mg/L)	Effluent (mg/L)	Removal (%)	Influent (mg/L)	Effluent (mg/L)	Removal (%)	***P*-value**	Influent (mg/L)	Effluent (mg/L)	Removal (%)	Influent (mg/L)	Effluent (mg/L)	Removal (%)	***P*-value**
TSS	(mg/L)	201 ± 129	58 ± 42	72 ± 23	335 ± 167	80 ± 50	69 ± 22	0.49	211 ± 137	43 ± 25	83 ± 14	331 ± 205	68 ± 40	84 ± 17	0.85
VSS	(mg/L)	181 ± 106	55 ± 35	73 ± 19	284 ± 154	72 ± 43	72 ± 22	0.76	197 ± 121	40 ± 24	85 ± 15	275 ± 131	60 ± 30	85 ± 17	0.64
pH	-	7.9 ± 0.2	7.7 ± 0.2	-	7.9 ± 0.2	7.6 ± 0.2	-	-	7.8 ± 0.2	8.3 ± 0.4	-	8.1 ± 0.2	7.8 ± 0.2	-	-
Turbidity	(NTU)	130 ± 93	30 ± 24	72 ± 22	172 ± 88	47 ± 38	65 ± 32	0.22	141 ± 137	18 ± 17	80 ± 19	168 ± 124	33 ± 32	80 ± 18	0.79
NH_4^+-N	(mg/L)	20 ± 9	1.7 ± 1.3	91 ± 7	21 ± 9	2.1 ± 2.0	91 ± 7	0.75	28 ± 11	1.4 ± 1.4	95 ± 4	31 ± 11	2.4 ± 2.3	92 ± 9	**0.01**
NO_3^--N	(mg/L)	0.3 ± 0.4	25 ± 6	*	0.6 ± 0.5	21 ± 8	*	*	0.2 ± 0.4	8 ± 4	*	0.6 ± 2.4	10 ± 7	*	*
NO_2^--N	(mg/L)	0.4 ± 0.8	1.6 ± 1.0	*	0.7 ± 0.9	2.6 ± 1.7	*	*	1.5 ± 2.3	1.7 ± 1.4	*	1.8 ± 3.1	1.9 ± 1.5	*	*
TN	(mg/L)	49 ± 24	32 ± 11	43 ± 9	50 ± 30	31 ± 15	46 ± 16	0.37	58 ± 30	24 ± 5	57 ± 21	67 ± 21	36 ± 3	50 ± 17	0.34
TC	(mg/L)	132 ± 105	61 ± 59	59 ± 15	179 ± 134	75 ± 79	61 ± 15	0.55	372 ± 95	162 ± 26	54 ± 15	355 ± 105	189 ± 52	44 ± 14	0.15
PO_4^{3-}-P	(mg/L)	1.6 ± 1.0	1.7 ± 0.8	12 ± 47	1.9 ± 0.8	1.7 ± 1.1	4 ± 55	0.66	3.1 ± 2.3	1.3 ± 1.7	68 ± 38	2.8 ± 1.9	1.5 ± 1.7	56 ± 44	0.19
COD	(mg/L)	372 ± 217	125 ± 67	60 ± 22	496 ± 223	154 ± 62	63 ± 23	0.59	330 ± 203	102 ± 63	64 ± 23	430 ± 248	114 ± 61	67 ± 25	0.75
sCOD	(mg/L)	97 ± 42	68 ± 30	44 ± 19	112 ± 45	65 ± 43	56 ± 22	**0.03**	78 ± 53	48 ± 29	33 ± 18	82 ± 45	57 ± 34	35 ± 16	0.77

* There was no removal, but formation of this species due to nitrification

Table A-2. Average concentrations of the main water quality parameters measured in the initial and final growth media of Nostoc sp., A. platensis and P. purpureum during optimisation experiment. Variations of these parameters were monitored under different conditions of low (C1), medium (C2) and high (C3) concentration of the main ingredient in standard growth medium (NaNO3) and low (L1), medium (L2) and high (L3) light intensity.

		Initial	**Final**			**Initial**	**Final**			**Initial**	**Final**		
		C1	**L1C1**	**L2C1**	**L3C1**	**C2**	**L1C2**	**L2C2**	**L3C2**	**C3**	**L1C3**	**L2C3**	**L3C3**
***Nostoc* sp.**													
pH	-	7.62 ± 0.04	8.76 ± 0.03	9.05 ± 0.01	8.83 ± 0.01	7.41 ± 0.01	8.86 ± 0.02	9.01 ± 0.01	9.02 ± 0.01	7.53 ± 0.03	8.90 ± 0.01	8.95 ± 0.01	9.08 ± 0.01
EC	mS/cm	1.18 ± 0.01	1.10 ± 0.01	1.05 ± 0.00	0.93 ± 0.01	2.22 ± 0.02	2.00 ± 0.01	1.91 ± 0.01	2.26 ± 0.00	3.16 ± 0.02	2.74 ± 0.00	2.76 ± 0.01	2.58 ± 0.01
TN	mg/L	127.9 ± 0.09	133.0 ± 7.0	138.5 ± 8.5	125.3 ± 2.8	264.6 ± 2.1	248.8 ± 3.7	252.5 ± 7.5	249.8 ± 2.7	360.4 ± 7.0	342.5 ± 5.0	358.8 ± 3.8	357.5 ± 2.5
TP	mg/L	7.9 ± 0.3	7.4 ± 0.0	7.0 ± 0.0	6.6 ± 0.1	7.4 ± 0.2	7.0 ± 0.1	7.2 ± 0.2	6.8 ± 0.1	7.2 ± 0.2	7.1 ± 0.0	6.8 ± 0.0	7.2 ± 0.1
NH_4^+-N	mg/L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.10 ± 0.01	0.014 ± 0.001	0.0 ± 0.0	0.43 ± 0.01	0.17 ± 0.00	0.13 ± 0.00
NO_3^--N	mg/L	127.9 ± 0.9	50.9 ± 1.2	19.2 ± 0.8	6.0 ± 0.8	264.6 ± 2.1	156.0 ± 2.0	136.0 ± 2.0	165.0 ± 1.0	360.3 ± 7.0	259.0 ± 6.0	230.5 ± 5.5	204.5 ± 3.5
NO_2^--N	mg/L	0.014 ± 0.001	1.16 ± 0.01	2.51 ± 0.02	1.61 ± 0.04	0.016 ± 0.001	1.77 ± 0.02	3.71 ± 0.04	3.69 ± 0.03	0.020 ± 0.002	2.16 ± 0.02	4.97 ± 0.02	3.86 ± 0.01
PO_4^{3-}-P	mg/L	7.9 ± 0.3	0.58 ± 0.01	0.19 ± 0.01	0.04 ± 0.00	7.4 ± 0.2	0.37 ± 0.02	0.06 ± 0.00	0.14 ± 0.01	7.2 ± 0.2	0.06 ± 0.00	0.05 ± 0.00	0.15 ± 0.01
A. platensis													
pH	-	9.11 ± 0.01	10.11 ± 0.01	10.38 ± 0.01	10.48 ± 0.01	8.99 ± 0.00	10.21 ± 0.02	10.39 ± 0.01	10.67 ± 0.01	8.94 ± 0.00	10.31 ± 0.01	10.49 ± 0.01	10.85 ± 0.01
EC	mS/cm	13.07 ± 0.01	13.27 ± 0.07	13.70 ± 0.06	14.07 ± 0.07	19.29 ± 0.01	18.93 ± 0.08	18.80 ± 0.03	18.78 ± 0.01	25.13 ± 0.03	23.97 ± 0.03	23.90 ± 0.06	23.83 ± 0.03
TN	mg/L	267 ± 2	254.6 ± 0.6	251.3 ± 0.3	245.6 ± 0.1	261 ± 3	264.8 ± 2.7	240.8 ± 1.8	242.9 ± 0.4	259 ± 5	259.4 ± 0.1	229.2 ± 1.8	256.8 ± 0.3
TP	mg/L	86.6 ± 0.5	82.0 ± 0.1	82.7 ± 0.3	101.6 ± 6.4	89.7 ± 0.3	85.1 ± 0.5	82.6 ± 0.1	83.1 ± 1.2	87.5 ± 0.7	96.7 ± 9.4	87.2 ± 0.6	88.1 ± 0.8

(Table continued on next page)

Table A-2. *(Continued)*

		Initial	Final			Initial	Final			Initial	Final		
		C1	**L1C1**	**L2C1**	**L3C1**	**C2**	**L1C2**	**L2C2**	**L3C2**	**C3**	**L1C3**	**L2C3**	**L3C3**
A. platensis													
NH_4^+-N	mg/L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NO_3^--N	mg/L	267 ± 2	121.83 ± 1.32	57.20 ± 1.20	0.53 ± 0.12	261 ± 3	104.60 ± 0.60	51.10 ± 1.30	0.38 ± 0.05	259 ± 5	124.45 ± 0.45	27.85 ± 1.35	0.31 ± 0.01
NO_2^--N	mg/L	0.007 ± 0.001	4.50 ± 0.02	6.70 ± 0.03	0.02 ± 0.01	0.006 ± 0.001	1.79 ± 0.04	5.88 ± 0.01	0.03 ± 0.01	0.004 ± 0.001	0.42 ± 0.01	0.57 ± 0.01	0.02 ± 0.00
PO_4^{3-}-P	mg/L	86.6 ± 0.5	20.5 ± 0.6	18.0 ± 0.3	15.8 ± 0.1	89.7 ± 0.3	21.5 ± 0.2	18.6 ± 0.4	16.0 ± 0.3	87.5 ± 0.7	23.6 ± 2.5	17.2 ± 0.1	16.3 ± 0.1
P. purpureum													
pH	-	8.42 ± 0.01	8.88 ± 0.04	8.89 ± 0.01	9.15 ± 0.01	8.42 ± 0.01	8.95 ± 0.01	9.01 ± 0.01	8.77 ± 0.01	8.46 ± 0.01	9.02 ± 0.01	8.77 ± 0.01	8.95 ± 0.01
EC	mS/cm	16.08 ± 0.02	17.20 ± 0.14	17.75 ± 0.04	16.46 ± 0.01	29.01 ± 0.01	28.20 ± 0.06	28.20 ± 0.06	27.47 ± 0.09	41.63 ± 0.09	40.20 ± 0.06	38.10 ± 0.06	38.90 ± 0.01
TN	mg/L	163.9 ± 1.4	171 ± 3	150 ± 6	171 ± 3	157.9 ± 0.7	198 ± 2	146 ± 6	181 ± 7	150.1 ± 0.4	188 ± 4	190 ± 4	186 ± 6
TP	mg/L	12.5 ± 0.3	13.4 ± 0.1	10.8 ± 0.5	10.7 ± 0.6	14.2 ± 0.2	13.7 ± 0.4	11.5 ± 0.6	9.1 ± 0.1	12.8 ± 0.3	15.2 ± 0.9	11.5 ± 0.5	13.1 ± 0.2
NH_4^+-N	mg/L	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.02 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.20 ± 0.03
NO_3^--N	mg/L	163.9 ± 1.4	84.6 ± 0.8	58.5 ± 0.7	50.6 ± 1.7	157.9 ± 0.7	73.8 ± 2.4	16.1 ± 0.4	0.8 ± 0.1	150.1 ±0.4	59.7 ± 0.9	1.6 ± 0.1	5.4 ± 0.3
NO_2^--N	mg/L	0.003 ± 0.001	4.9 ± 0.2	7.3 ± 0.5	5.1 ± 0.1	0.003 ± 0.001	1.4 ± 0.2	7.4 ± 0.3	2.6 ± 0.2	0.003 ± 0.001	1.4 ± 0.01	4.3 ± 0.1	4.5 ± 0.3
PO_4^{3-}-P	mg/L	12.5 ± 0.3	2.45 ± 0.03	0.19 ± 0.01	0.11 ± 0.01	14.2 ± 0.2	1.94 ± 0.03	0.08 ± 0.01	0.02 ± 0.01	12.8 ± 0.3	0.93 ± 0.01	0.10 ± 0.01	0.22 ± 0.03

Table A-3. Wastewater metals concentrations and respective threshold limits for drinking water established by the World Health Organization (WHO) (WHO, 2017).

	Range	**WHO**
Al (mg/L)	0.1 – 1.2	(0.9)[a]
B (mg/L)	1.5 – 1.6	2.4
Ba (mg/L)	0.1 – 0.3	0.7
Ca (mg/L)	13.3 – 22.8	[b]
Cd (mg/L)	0 – 0	0.003
Cr (mg/L)	0 – 0	0.05
Cu (mg/L)	0 – 0.1	2
Fe (mg/L)	0.2 – 1.5	(2)[a]
Ga (mg/L)	0 – 0	[c]
In (mg/L)	0 – 0	[c]
K (mg/L)	46.5 – 54.6	[b]
Li (mg/L)	0 – 0	[c]
Mg (mg/L)	8.3 – 8.6	[d]
Mn (mg/L)	0 – 0.1	(0.4) [a]
Na (mg/L)	656.5 – 693.4	[e]
Ni (mg/L)	0 – 0	0.07
Pb (mg/L)	0 – 0	0.01
Sr (mg/L)	0.1 – 0.2	(4)[f]
Zn (mg/L)	0.1 – 0.2	(3)[a]
Ag (mg/L)	0 – 0.1	(0.1)[a]

[a]Guideline value not established. This is a health-based value from the Joint FAO/WHO Expert Committee on Food Additives (JECFA).
[b]Not of health concern at levels found in drinking-water.
[c]No guideline value established.
[d]Not of health concern at levels found in drinking-water. Present in natural groundwater usually at low concentrations (from negligible to about 50 mg/L).
[e]Not of health concern at levels found in drinking-water. However, concentrations in excess of 200 mg/l may give rise to unacceptable taste.
[f] Not of health concern at levels found in drinking-water. EPA lifetime health advisory limit (EPA, 2018).

Curriculum Vitae

Attention¡

Pages 274 to 281 have been removed because they contain personal data of the autor