Ph.D. Thesis

Integration of wastewater treatment and microalgae production for agricultural applications

Integración del tratamiento de aguas residuales y la producción de microalgas para aplicaciones relacionadas con la agricultura

University of Almeria

Department of Chemical Engineering

PhD in Biotechnology and Industrial Bioprocesses Applied to Agrifood and the Environment

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Almeria, Spain, July 2022

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CERTIFICAN

Que el trabajo descrito en la presente memoria, titulado *Integración de tratamiento de aguas residuales y producción de microalgas para aplicaciones relacionadas con la agricultura,* ha sido realizado por Ainoa Morillas España en el Departamento de Ingeniería Química de la Universidad de Almería, bajo su dirección.

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Almería, Julio de 2022

A Juan Diego.

A mi familia, en especial a los que ya no están.

A mis amigos.

Agradecimientos

Aquí termina un capítulo de mi vida, el cual no habría tenido ningún inicio si no hubiese coincidido con un entusiasta de la biotecnología de las microalgas como es el gran jefe Francisco Gabriel Acién. Con él, un equipo MARAVILLOSO, como son Ismael (quien me enseñó mucho de lo que se), José Peña (quien es un oso amoroso en pocas ocasiones) y Cintia, mi amiga además de compañera.

Eso sí, la oportunidad de empezar esta aventura no habría sido posible si no hubiese coincidido con la persona adecuada en el momento adecuado, lo que me lleva a dar las gracias a Cynthia por su confianza en mí y mis capacidades.

A veces en la vida se cruzan personas en tu camino que te hacen el trayecto más fácil, y esta persona es Tomás. Gracias por tu paciencia y por tirar de mi en esta recta final.

Cualquier persona que se dedique a la investigación sabe que nada es posible si no es con un buen equipo, y yo tengo al mejor. Silvia, Martina, Ana, José E, Rebecca y Cristina. También a quien ha formado parte: María José, Solaima, Claudia Sepúlveda, Mariana, Marta y Bruna, Gracias. Y a los que colaboran, por supuesto. José Luis Guzmán y José Luis Casas, gracias por hacerme salir de mi zona de confort.

Aunque no lo parezca, una tesis doctoral implica a muchas personas, tanto de forma directa como indirecta. Por ese motivo le quiero agradecer a mi equipo de Puerta de Europa su cariño y apoyo.

Ser quien soy es por mi familia, porque si algo les sobra es corazón. A mis abuelas Juana y María, por ser mujeres empoderadas y trasmitirme su fortaleza. Papá, Mama, Gracias por vuestro amor. Idoia, eres mi mitad, Gracias.

Por supuesto tengo que agradecer a mis amigos, Party-Party y compañía, Aguacate y Plátano y a Marta, Gracias por ser la familia que se elige.

Y como no podía ser de otra manera, a Juan Diego, por que esta tesis es tuya. Gracias por ser mi luz.

Por último me lo agradezco a mí, por demostrarme que soy capaz.

Muchas gracias a todos, de corazón.

Ainoa

Financiación

Este trabajo se ha enmarcado en el proyecto de I+D AL4BIO RTI2018-099495-A-C22 financiado por MCIN/AEI/10.13039/501100011033/, por “FEDER Una forma de hacer Europa”.

Acceso a instalaciones experimentales

La mayor parte de la investigación realizada en esta tesis doctoral se ha llevado a cabo en las instalaciones de la Estación Experimental de Cajamar Las Palmerillas, en el Centro IFAPA La Cañada, y en las instalaciones del Departamento de Ingeniería Química de la Universidad de Almería.

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RESUMEN

El concepto de escasez hídrica es relativamente nuevo pero ya afecta a casi el 40% de la población mundial. Según datos de las Naciones Unidas, 3 de cada 10 personas carecen de acceso a servicios de agua potable seguros y aproximadamente el 70% de las aguas extraidas de ríos, lagos y acuíferos naturales se utilizan para riego. A esto se suma que aproximadamente el 80% de las aguas residuales resultantes de la actividad humana se vierten al medioambiente sin ningún tratamiento, lo que supone un gran problema ambiental. La depuración de aguas residuales urbanas mediante métodos convencionales consiste en una serie de procesos físicos, químicos y biológicos que tienen como fin garantizar que el agua que se vierte al medioambiente cumple unos criterios de calidad mínimos. Estos son cada vez más exigentes. Se estima que en España se depuran alrededor de 4950 hm^3 de agua residual anuales; para ello, son necesarias más de 4700 estaciones depuradoras de aguas residuales y un presupuesto superior a los 1300 millones de euros. La eliminación de la materia orgánica presente en el agua residual es uno de los aspectos más optimizados en los procesos de tratamiento actuales. Sin embargo, a pesar de ser eficientes, gran parte de esta materia orgánica se transforma en CO_2 y acaba inevitablemente en la atmósfera. Otro inconveniente de los procesos de depuración actuales ocurre durante la eliminación del nitrógeno y del fósforo. Para eliminarlos correctamente se requieren grandes consumos energéticos y la instalación de equipos y procesos complejos.

Tanto el carbono, como el nitrógeno y el fósforo son esenciales para el crecimiento de las microalgas. Por este motivo, durante los últimos años, los sistemas de tratamiento de aguas residuales basados en microalgas han captado un gran interés comercial. Su principal ventaja frente a los procesos tradicionales es la posible valorización de la biomasa producida para obtener bioproductos sostenibles o energía. Además, al ser microorganismos fotosintéticos, son capaces de asimilar el CO_2 producido en los procesos tradicionales aumentando la sostenibilidad del proceso. Desde el punto de vista económico, el cultivo de microalgas utilizando aguas residuales como fuente de nutrientes también resulta especialmente interesante. De hecho, al combinar el tratamiento de aguas

residuales con el cultivo de microalgas, los costes de producción de la biomasa microalgal se pueden reducir teóricamente a menos de 1 €·kg^{-1}, muy por debajo de los 5-10 €·kg^{-1} actuales cuando se emplean otras fuentes de nutrientes. El principal uso de las microalgas es la producción de suplementos nutricionales, especialmente suplementos protéicos. La biomasa microalgal producida utilizando aguas residuales no puede emplearse directamente como alimento. Sin embargo, puede usarse para producir alimentos de forma indirecta: como materia prima para la obtención de bioestimulantes agrícolas o piensos para animales, especialmente para la acuicultura.

El potencial de esta tecnología es enorme. Uno de los mayores problemas relacionados con la depuración de aguas residuales urbanas mediante microalgas es que se procesan enormes cantidades de agua y se necesitan reactores de gran tamaño. Debido a su necesidad de luz, los fotobiorreactores utilizados para producir microalgas no pueden ser muy profundos, por lo que se necesitan grandes superficies. En concreto, para depurar el agua residual de una ciudad, se requieren fotobiorreactores de aproximadamente 5-6 m^2 por habitante. Hasta la fecha, la gran mayoría de los estudios científicos publicados se han realizado a escala de laboratorio o a escala piloto durante periodos de tiempo muy reducidos. La duración de los ensayos es muy relevante, ya que un proceso estable durante un periodo de tiempo muy corto no permite predecir su comportamiento en condiciones reales. La validación de dichos procesos a mayor escala y su optimización es esencial para captar el interés comercial y conseguir la implementación industrial de los procesos de depuración de aguas residuales basados en microalgas. Otro inconveniente viene dado por el reducido número de microalgas disponibles en los bancos de algas. A pesar de que hay cientos de miles de cepas de microalgas distintas en la naturaleza, muy pocas han sido estudiadas en detalle y solo algunas de ellas utilizando aguas residuales.

Así pues, en esta Tesis Doctoral se pretende validar procesos basados en microalgas a una escala precomercial, demostrar la viabilidad tecnológica de su uso para la depuración de aguas residuales y estudiar cómo influyen las variables de operación y las condiciones ambientales en su eficiencia y productividad. Para ello, se han estudiado distintos tipos de fotobiorreactores (*raceway* y *thin layer*) y se han llevado a cabo ensayos durante todas las estaciones del año, permitiendo

predecir valores de eficiencia y productividad de biomasa anuales. Los reactores tipo *raceway*, los más comunes a nivel mundial, son sistemas económicos y relativamente fáciles de operar, pero solo permiten alcanzar productividades relativamente bajas (20 $g \cdot m^{-2} \cdot día^{-1}$). Esto se debe, principalmente, a que la altura del cultivo es de unos 10-30 cm, lo que da lugar a un bajo aprovechamiento de la luz, ya que las microalgas se sombrean unas a otras. Además, la transferencia de materia en este tipo de reactores es reducida, lo que limita la asimilación de nutrientes y la eliminación de gases. Es por ello por lo que en esta Tesis también se ha llevado a cabo el estudio de otro tipo de sistemas: los reactores de capa fina o *thin layer*. Estos trabajan con una altura de cultivo inferior a 5 cm, lo que incrementa significativamente la disponibilidad de luz que tienen las células. Así, se consiguen alcanzar productividades de biomasa por unidad de superficie y tiempo muy superiores, en el rango 35-45 $g \cdot m^{-2} \cdot día^{-1}$. Hasta ahora, los reactores de capa fina no han sido optimizados a escala piloto y se desconoce su eficacia recuperando nutrientes del agua residual urbana.

En este trabajo se han utilizado fotobiorreactores tipo *raceway* de 80 m^2 y fotobiorreactores *thin layer* de 63 y 163 m^2, lo que supone un gran avance con respecto a los estudios previos donde los procesos se validaban a escalas de 10-20 m^2 como máximo. Se ha estudiado el potencial de estos sistemas utilizando agua de la red y fertilizantes comerciales como medio de cultivo, donde se han llegado alcanzar productividades de biomasa de 5100, 5600 y 9100 $kg \cdot año^{-1}$, respectivamente. A continuación, con el fín de conseguir un proceso más sostenible, se ha validado el uso de dichos reactores pero usando agua residual urbana como la principal fuente de nutrientes. Las productividades obtenidas fueron similares a las observadas en los cultivos con ferilizantes comerciales. Esto permite prever que, si se escala el proceso a un reactor de 10000 m^2, se podrían llegar a eliminar 10.6 toneladas de nitrógeno y 0.5 toneladas de fósforo al año, a la vez que se producirían hasta 56.5 toneladas de biomasa con un alto valor añadido. La utilización de aguas residuales no solo aumenta la sostenibilidad del proceso; un análisis económico preliminar ha demostrado que usando agua residual como medio de cultivo para el cultivo de microalgas se podría reducir el cose de producción en 0.44 $€ \cdot kg^{-1}$ a la vez que se obtendría agua regenerada que podría reutilizable para riego.

Esta Tesis también ha tenido como objetivo estudiar el uso de membranas de ultrafiltración para aumentar la eficiencia del proceso. El uso de estas membranas es común en los procesos tradicionales de depuración de aguas, pero no ha sido implementado aún en procesos basados en microalgas. Su uso viene justificado por el bajo contenido en nutrientes de algunas aguas residuales, lo que da lugar a productividades de biomasa muy reducidas por presentarse limitación de nutrientes. El uso de membranas de ultrafiltración hace posible separar el tiempo de retención hidráulica del tiempo de retención celular, lo que permite tratar un mayor volumen diario de agua residual por unidad de superficie y, además, aumentar el aporte de nutrientes al cultivo, con lo que se incrementa la productividad. Escalando los valores obtenidos en esta Tesis tras el uso de membranas de ultrafiltración a un reactor *raceway* teórico de 10000 m^2 acoplado a una membrana de utrafiltración, se podrían tratar 2.58 M m^3 anuales agua residual produciendo 79.92 tn de biomasa al año. Es decir, el uso de membranas permitiría procesar hasta un 130% más de agua residual por unidad de superficie, a la vez que obtener 30 tn anuales más de biomasa.

Finalmente, la biomasa obtenida en el tratamiento de agua residual mediante procesos microalgales, se propone como materia prima para la producción de bioestimulantes. Se ha estudiado así el potencial de distintas biomasas obtenidas como bioestimulante, obteniendo resultados prometedores. En concreto, una de las cepas seleccionadas (*Chlorella vulgaris* MACC 1) aumentó el desarrollo de raíces hasta un 493% e incrementó la expansión de cotiledones en un 60.9%. Los bioestimulantes agrícolas basados en microalgas ya son una realidad comercial. Sin embargo, hasta la fecha su producción se realiza utilizando agua limpia y fertilizantes comerciales como fuente de nutrientes. Los resultados de este trabajo demuestran que las aguas residuales, que suponen un problema ambiental, podrían ser utilizadas como fuente de nutrientes para la producción de bioestimulantes, lo que podrían no solo minimizar la necesidad de agua de riego y de fertilizantes, sino también mejorar la calidad de los alimentos que consumimos y la salud de los consumidores.

La investigación presentada en esta Tesis Doctoral forma parte del trabajo llevado a cabo por el Departamento de Ingeniería Química de la Universidad de Almería enmarcado en el proyecto de I+D “Microalgas para la producción sostenible de

bioproductos y agua regenerada (AL4BIO)" financiado por MCIN/AEI/10.13039/501100011033/, por "FEDER Una forma de hacer Europa" y en colaboración con GEMMA-UPC. El objetivo de dicho proyecto es producir bioproductos de alto valor y agua regenerada en sistemas basados en microalgas durante el tratamiento de aguas residuales.

ABSTRACT

Availability and access to water is fundamental for a sustainable development. Water scarcity affects more than 40% of the global population. According to a recent report of the United Nations, 3 in 10 people lack access to drinkable water and approximately 70% of all water abstracted from rivers, lakes, and natural aquifers is used for irrigation. This, together with the fact that approximately 80% of wastewater resulting from human activities is discharged into the environment without any pre-treatment represent a huge environmental problem. Wastewater treatment using conventional methods consists on a series of physical, chemical, and biological processes that aim to ensure that the water discharged into the environment meets an increasingly challenging quality criteria. It is estimated that, in Spain, around 4,950 hm^3 of wastewater are processed per year; For this, more than 4700 wastewater treatment plants have been built and a budget of over 1300 million euros per year is needed. The elimination of the organic matter present in the wastewater is one of the most well-studied and optimized aspects of current wastewater treatment processes. However, most of this organic matter is transformed into CO_2 that inevitably ends up in the atmosphere. Something similar occurs with the removal of nitrogen and phosphorus. Both are effectively removed from the wastewater but not recovered, ending up in the emission of greenhouse gases. Moreover, large energy requirements and complex equipment and processes are needed to comply with the current maximum nitrogen and phosphorus discharge limits.

Carbon, nitrogen, and phosphorus are essential for microalgal growth. For this reason, during the last few years, microalgae-based wastewater treatment processes have gained an increased commercial interest. Their main advantages when compared to traditional processes include the recovery (not removal) of nitrogen and phosphorus and their biotransformation into valuable biomass that could be used as a feedstock for the production of high-end compounds and renewable energy. Furthermore, as microalgae are photosynthetic microorganisms, they can transform the CO_2 that is produced naturally during the degradation of organic matter into complex molecules of high commercial interest. From an economic point of view, the production of microalgae using wastewater

as a source of nutrients has also attracted the interest of the wastewater treatment industry. In fact, by combining wastewater treatment with microalgae production, the production costs of microalgae biomass can be theoretically reduced to less than 1€·kg^{-1}, significantly lower than the current 5-10 €·kg^{-1} obtained when producing the biomass using freshwater and commercial nutrients. The microalgal biomass produced using wastewater cannot be used directly as food, the main use of microalgae today. However, it can be used indirectly for the production of food by being utilised as a raw material for the production of agricultural biostimulants or aquafeeds.

One of the biggest challenges of urban wastewater treatment using microalgae is the huge amount of wastewater that is daily generated in urban areas. This, together with the fact that microalgae are photosynthetic microorganisms and require large surfaces to maximise light availability leads to the need of using very large photobioreactors and large surface areas. Moreover, the up-scaling of microalgae-based processes is also a technological challenge. To date, the vast majority of the scientific literature has been carried out at a laboratory-scale or at a pilot-but for short periods of time. The validation of these processes on a demonstrative scale and during long periods of time is essential to capture commercial interest and investements and to achieve the industrial implementation of microalgae-based wastewater treatment processes. One last problem related with the processing of wastewater using microalgae is that although there are hundreds of thousands of different microalgal strains in the environment, only a very limited number of strains have been studied in detail and just a few using wastewater.

The overall objectives of this Doctoral Thesis were to up-scale current microalgae production processes and to demonstrate the technical viability of processing wastewater using microalgae. Both processes were validated at a pre-commercial scale and during the four seasos, which is essential to predict the potential industrial implementarion of the developed processes. Moreover, the assessment of the productivity and efficiency of the systems during a long period of time allos estimating the effect of environmental and operational conditions on a representative scale. The most common and economic photobioreactors are raceways, which are also easy to use and up-scale. However, despite allowing the

processing of large volumes of water, these systems lead to relatively low biomass productivities (20 $g \cdot m^{-2} \cdot day^{-1}$). The main reasons are that as the depth of the culture is around 10-30 cm, the light availability inside the culture is low because of the self-shading effect of microalgae. An inefficient mass transfer is also responsible for their relatively low productivity. For this reason, the present Thesis evaluated the potential utilisation of more innovative photobioreactor designs such as thin-layer cascade photobioreactors. These reactors work with a culture depth lower than 5 cm, which permits a greater availability of light. Thin-layer reactors have not yet been optimized on a pilot scale and the assessment of their effectiveness in recovering nutrients from urban wastewater has not yet been evaluated.

In the present work, different photobioreactors were used to produce biomass and process wastewater including 80 m^2 raceway reactors and 63 and 163 m^2 thin-layer cascade photobioreactors. Initially, the operation of the photobioreactors was optimised using freshwater and commercial fertilisers, achieving biomass productivities of 5100, 5600, and 9100 $kg \cdot year^{-1}$. The goal was to increase the sustainability of the process and, for this reason, the photobioreactors were then operated using wastewater as the sole nutrients source.The results revealed that if the process was upscales to a theoretical 10,000 m^2 reactor, it would be possible to recover approximately 10.6 and 0.5 tons of nitrogen and phosphorus per year, respectively while simultaneously producing up to 56.5 tons of valuable biomass. In addition, a preliminary economic analysis revealed that using wastewater as the culture medium could reduce the production costs by 0.44 $€ \cdot kg^{-1}$.

One third objective of the thesis was the assessment of the potential use of ultrafiltration membranes as a strategy to increase the biomass productivity and the amount of wastewater that can be processed per surface area. The use of ultrafiltration membranes is common in conventional wastewater treatment processes, but has not yet been studied in microalgae-based systems. One of their main attributes is that they allow separating the cellular from the hydraulic retention time, therefore they could be used to increase the areal biomass productivity when producing the biomass using secondary wastewater or to separate the biomass from the culture medium. In the present wotk, the use of membranes allowed achieving biomass productivities 40% higher and processing larger amounts of

water (130%) per surface area. Scaling up the values obtained with the use of ultrafiltration membranes to a theoretical 10,000 m^2 raceway reactor coupled to an ultrafiltration membrane would allow treating 2.58 M m^3, producing 79.92 tons of biomass per year. This means that not only the amount of water processed but also the annual biomass productivity was 30 tons higher when the ultrafiltration membrane was used.

Finally, the produced biomass was assessed as a raw material for the production of agricultural biostimulants with very promising results. One of the selected strains (*Chlorella vulgaris* MACC 1) allowed increasing root development by over 493% and a cytokinin activity 60.9% higher. The production and commercialisation of microalgae-based biostimulants is already a reality. However, these are produced using freshwater and commercial fertilisers as the nutrient sources. The present work demonstrates that the production of agricultural biostimulants using wastewater is feasible and that the process shows potential for further development. The processing of wastewater, which is an environmental challenge, could be carried out while simultaneously producing agricultural biostimulants that could not only minimise the amount of irrigation water and fertilisers required but also promote crop yields and promote the quality of the end products.

The research presented in this Doctoral Thesis is part of the work carried out by the Department of Chemical Engineering of the University of Almería framed in the R&D project "Microalgae for the sustainable production of bioproducts and reclaimed water (AL4BIO)" financed by MCIN/AEI/10.13039/501100011033/, for “FEDER A way of making Europe” and in collaboration with GEMMA-UPC. The objective of this project is to produce high-value bioproducts and reclaimed water in systems based on microalgae during wastewater treatment.

1. INTRODUCTION

1.1. Context and motivation

Freshwater is a scarce and necessary resource for the foundation of life on the planet. Its protection and conservation are essential for the balance of our ecosystems. Indeed, 71% of the Earth's surface is covered with water, but only 2% is drinkable. In addition, water pollution has increased significantly in recent years due to the uncontrolled disposal of industrial wastewater, an increased urban wastewater generation, and a poor human waste management. As a result, approximately 40% of the worlds population suffers from water shortages and this value is expected to reach 60% by 2025 [1]. The main objective of wastewater treatment processes is to allow the reusing or discharging of effluents into the environment without causing significant damage. The reuse of wastewater in irrigation is vital to achieve a sustainable food production in the future as today, approximately 70% of the water abstracted from rivers, lakes, and natural reservoirs is used for irrigation.

The 25th of September of 2015, world leaders adopted a set of global goals to eradicate poverty, protect the planet and ensure prosperity for all as part of a new sustainable development agenda. Each goal had specific targets to be achieved in the next 15 years. To achieve these goals, everyone has to play their role: governments, the private sector, civil society and every individual. There are 17 objectives, one of them directly related with the water problem: Goal 6, Clean Water and Sanitation. The goal is to ensure access to water and sanitation for all. Although progress has been made in expanding access to drinking water and sanitation, there are billions of people (mainly in rural areas) who still lack these basic services. Today, one in three people do not have access to safe drinking water, two in five people do not have a basic facility to wash their hands with soap and water, and more than 673 million people still defecate in the open free.

Conventional wastewater treatment processes have restrictions in terms of their high-energy consumption and their environmental impact (mainly gas emissions and greenhouse effect). Therefore, it is vital to develop technologies and processes that are less expensive, have better efficiencies and are environmentally friendly [2]. The search for an alternative wastewater treatment strategy led to an increased

interest in microalgae-based wastewater treatment processes. The use of microalgae is considered one of the most promising strategies to reclaim wastewater [3,4] and has recently been implemented on a commercial scale; for example, in Chiclana and Mérida in Spain (Aqualia) or Christchurch in New Zealand (NIWA) are already processing urban wastewater using microalgae. Their key advantage is their dual role: a valuable product that is microalgal biomass is produced together with the clean water suitable for use in agriculture. It is important to highlight that in these processes, the nutrients present in the wastewater are recovered while in the conventional processes they are just removed.

The present work was carried out in the framework of the R&D project entitled “Microalgae for sustainable production of bioproducts and reclaimed water (AL4BIO)”. This is a coordinated project funded by the Spanish Ministry of Science and Innovation and ERDF as a way of making Europe. It is composed of two sub-projects. The first one (coordinator) is entitled “Sustainable production of biopolymers and pigments from microalgae” and it has been led by the Environmental Engineering and Microbiology Research Group of the Universitat Politècnica de Catalunya in Spain. The second sub-project is entitled “Sustainable production of biostimulants and biopesticides from microalgae” and it has been led by the Chemical Engineering Department of the University of Almeria in Spain. The AL4BIO project deals with the application of the circular economy and bioeconomy concepts within the context of wastewater treatment plants. It aims at producing high-value bioproducts and reclaimed water in microalgae-based systems for tertiary wastewater treatment. The bioproducts include biopolymers, biological pigments, biostimulants, biopesticides and biogas, along with reclaimed water. Specifically, the work here presented is the result of the research performed in sub-project 2, so the main objective was to grow microalgae with wastewater as a nutrients source to obtain reclaimed water and microalgal bioproducts for agriculture.

The research described in this document was carried out at the Chemical Engineering Department of the University of Almeria, at “Las Palmerillas” - Cajamar Foundation and, the SABANA Demonstration Center located in the facilities of the Institute of Agricultural and Fisheries Research and Training (IFAPA) attached to the University of Almeria). The SABANA Demonstration Center allows studying

different species of microalgae to determine their optimal production conditions, as well as the production of these species using different technologies: raceway type reactors, thin layer cascade photobioreactors, closed tubular photobioreactors, etc. All of the systems are built at a pre-industrial scale. Another key characteristic of the Demonstration Plant is the possibility to harvest and process the biomass using different harvesting technologies (e.g. centrifugation and ultrafiltration), cell wall disruption methods (e.g. enzymatic hydrolysis, high-pressure homogenization, sonication), and drying technologies (e.g. freeze-drying, spray-drying). The production capacity of the reactor ranges from 1 to 100 kg per day since specific equipment is available for the different stages of the production process. Furthermore, the reactors can be fed with different types of water, including freshwater, primary or secondary wastewater, and seawater.

1.2. An overview of microalgae

1.2.1. Microalgal biomass production and applications

Microalgae are unicellular and photoautotrophic organisms, which means that they use CO_2 as the main carbon source and sunlight as the source of energy. In this document, the term microalgae will be used interchangeably to refer to prokaryotic cyanobacteria and eukaryotic microalgae. Cyanobacteria are a bacterial phylum able to perform photosynthesis and they are generally included in the term "microalgae". Although there is a great diversity of catalogued microalgal species, only 50-60 have been studied from a physiological point of view and less than 10 are commercially used. Different factors are currently limiting the industrial production of microalgae; these include the complexity of upscaling biological processes, high production costs, and a limited number of products on the market that leads to low consumer awareness about their potential uses.

An important characteristic of microalgae is their high biomass generation capacity: high growth rate and photosynthetic efficiency of up to 10%, one order of magnitude higher than most terrestrial plants [5]. Microalgae produce valuable biomolecules such as polyunsaturated fatty acids, carotenoids, chlorophylls, phycobiliproteins, and bioactive carbohydrates among others [6]. A hierarchical Bayesian analysis of data compiled in the literature reported that the median

macromolecular composition of microalgae is 32.2% protein, 17.3% lipid, 15.0% carbohydrate, 17.3% ash, and 5.7% RNA on a dry weight basis [7]. However, the biochemical composition of microalgal biomass is strain-dependent and changes in response to environmental and operational conditions. As they show great metabolic plasticity, it is possible to modify the composition of the biomass by changing the culture conditions. Some of the most used strategies to trigger the production and accumulation of some valuable products are hypersalinity, light stress, temperature modification, nitrogen deprivation, ammonium addition, or culture age control, among others.

Because of their small size, microalgae cannot be harvested from the environment and are produced in controlled industrial facilities. Microalgae production systems present a wide variety of designs, and they can be classified into two main groups: open and closed systems. However, only a few have been scaled up and validated outdoors [8]. Closed photobioreactors use containers with different structures (tubes, bubble columns, flat panels…) where the microalgal culture is kept under continuous mixing, using pumps or bubbling air, which can be enriched with CO_2, also to control pH. Cultures are exposed to sunlight, as artificial illumination on a commercial scale involves a high cost and lower sustainability. Closed systems, such as tubular photobioreactors, are mainly used to produce those strains that are not dominant and cannot be produced outdoors because of the risk of contamination (for example when the biomass will be used for food applications). Also, to produce strains that render high-value biomass, its high price offsets the use of a more expensive production strategy (for example, to obtain carotenoids or polyunsaturated fatty acids).

On the other hand, open systems have been used since the 1950s and are the most commonly used on a commercial scale. Most open photobioreactor designs lead to lower biomass productivity and efficiency than closed systems and are more susceptible to contamination because the culture is in direct contact with the atmosphere. However, the capital expenditure and the operational costs (CAPEX and OPEX) are lower for open systems, so the unitary cost per kg of microalgae is lower when using an open reactor. The most common designs reported in the literature include thin layer cascade photobioreactors and mainly raceway reactors, which are the most commonly used [9].

Raceway reactors are culture units that consist of a shallow pond generally formed by two channels separated by a central partition and a paddle wheel that rotate on a horizontal axis.In addition, it has a sump where the air is bubbled for oxygen desorption and CO_2 is injected on-demand as a carbon source to keep the culture's pH at the selected set-point [10]. The water depth of the culture ranges between 15 and 30 cm although higher depths have also been studied observing less energy consumption with a height of 20 cm[11]. Light availability is one of the most important factors influencing the growth of photosynthetic microorganisms. High depths lead to low biomass productivity because of the self-shading effect of microalgal cells.

A more innovative approach is the use of thin layer cascade photobioreactors. They consist of a slightly inclined surface (1-2%) where the culture flows towards a lower tank. The culture is then pumped from the tank to a bubble column where air and CO_2 injection takes place and goes back again to the inclined surface. The recirculation flow rate and the slope of the channel determine the depth of the culture layer, although depths between 0.5 and 5.0 cm are the most common [12]. The surface-volume ratio of thin layer cascade reactors is very large when compared to other photobioreactor designs. Due to the short optical path, the light availability higher, so greater biomass densities are obtained when using these systems. This is due to the high ratio of exposed surface to total culture volume and a highly turbulent flow, which allows higher volumetric and areal productivity compared to that of raceways [13]. The selection of a particular type of photobioreactor will depend on the water and nutrients source, the final application of the biomass, the location of the reactor, and the strain produced.

The microalgal biomass can be used for different applications, which mainly depend on their composition, such as their high protein content or the presence of valuable bioactive molecules. These include the production of biofuels [14], animal feed [15], or biofertilizers and biostimulants for agriculture [16]. For example, several animal feeds (especially aquafeeds) enriched in microalgal biomass have been developed and demonstrated antioxidant and antimicrobial capacity and disease-prevention properties [17]. The production of microalgal biomass for animal feed is not competitive with conventional feed ingredients (soy, fish oil, etc.) due to its higher production cost, which presents a challenge that must be addressed to obtain economically profitable microalgae-based feed for animals [17].

Also, microalgal biomass produced using wastewater from dairy cattle was used to fertilize pastures, achieving higher concentrations of minerals in the soil, including phosphorus, calcium, magnesium, and manganese, in addition to a higher dry matter content [18]. Biostimulants are, together with nutritional supplements, the most common and currently available products on the market, which are more sustainable when compared to their synthetic counterparts. These products are highly demanded, especially in the organic farming system [19] where their potential use to obtain higher yields or better-quality fruits has been demonstrated in several recent reports [20–24]

1.2.2.Microalgae-based wastewater treatment

In large and medium-sized urban agglomerations, the most common procedure for the treatment of liquid discharges is known as "active sludge", in its different forms, which since its first applications at the beginning of the 20th century has become the most widespread treatment worldwide. The stages of a conventional wastewater treatment plant are: i) primary treatment, where suspended solids and floating materials are removed; ii) secondary treatment, which includes conventional biological treatments for the elimination of organic matter; and iii) tertiary treatment, where the fundamental objective is to eliminate contaminants that persist after conventional biological treatment.

The recovery of nutrients from wastewater using microalgae involves the symbiotic association of aerobic and photosynthetic microorganisms. It is not possible to produce axenic (or even monoalgal) microalgal cultures on a large scale, especially when using open photobioreactors and wastewater. The composition of microalgae-bacteria consortia depends on several factors including the type of water source and the environmental and operating conditions [25]. For example, low light availability, generally associated with high culture depths and an increase in temperature promote the growth of nitrifying bacteria but, if the operating conditions are properly managed, more than 95% of the biomass produced will be microalgae [26–28].

One of the main advantages of using microalgae-bacteria consortia is their double function: they not only remove contaminants/nutrients but also produce valuable biomass that can be used for a wide variety of applications. Another important

advantage is that the use of microalgae in wastewater treatment can be economically viable and sustainable. The freshwater and fertilizers used for microalgae production can account for between 10 and 50% of the production cost of microalgal biomass [29]. This cost can be avoided if urban wastewater is used to produce microalgae. A techno-economic analysis revealed that in a scenario with optimal production conditions, the total cost of wastewater processing using microalgae could be 0.14 €·m^{-3}, which is 30% lower than the cost of conventional treatment with activated sludge. This cost reduction was calculated without considering the income obtained from the commercialization of the final agricultural bioproducts based on microalgal biomass [30].

Moreover, the co-products that remain after the extraction of the valuable products can be used to produce biogas by anaerobic digestion, promoting the circular bioeconomy of the process. This could increase the economic viability of the use of microalgae in the animal feed industry [31]. The process can also show a positive energy balance if coupled with biomethane production [32], making the process economically feasible and sustainable. In addition, it is necessary to evaluate the commercial potential of scaling up these processes using low-cost photobioreactors that allow treating large volumes of wastewater.

Large efforts are being made to further scale these processes since, although some studies have been carried out in relatively large reactors, their scale is still not representative of an industrial process [10]. Most of the published studies have been carried out on a laboratory scale and, although other studies use pilot plant scale reactors, the process needs to be scaled up to better predict the potential of microalgae in wastewater treatment.

1.3. Objectives

The overall objective of this Thesis was to demonstrate the technological viability of producing microalgae at a large-scale using different photobioreactor designs and technologies and different types of water and nutrient sources. During the process, novel strains that have biostimulant capacity and that are robust and able to grow in wastewater were identified. To achieve this general goal, five specific objectives were set as listed below:

- O1. To characterize the performance of pilot-scale thin layer photobioreactors in terms of productivity and efficiency compared to raceway reactors; which is the technology most widely studied and used today.
- O2. To identify the potential of *Scenedesmus* sp. and thin-layer photobioreactors to recover nutrients from wastewater and assess the effect of environmental conditions on biomass productivity and the performance of the system.
- O3. To optimise the operation of pilot-scale raceway photobioreactors and to validate the potential of the microalga *Scenedesmus* sp. to remove nutrients from wastewater throughout a whole year.
- O4. To evaluate the potential of the microalga *Scenedesmus* sp. as a biological system to recover nutrients, namely nitrogen and phosphorus, from urban wastewater using a raceway reactor combined with an ultrafiltration membrane.
- O5. To determine the viability of producing high-quality microalgal biomass using wastewater and to assess the biostimulant capacity of the produced biomass by studing different strains *in vitro*.

These objectives are linked to the research work carried out (Figure 1.1) and the scientific publications described in Chapter 2:

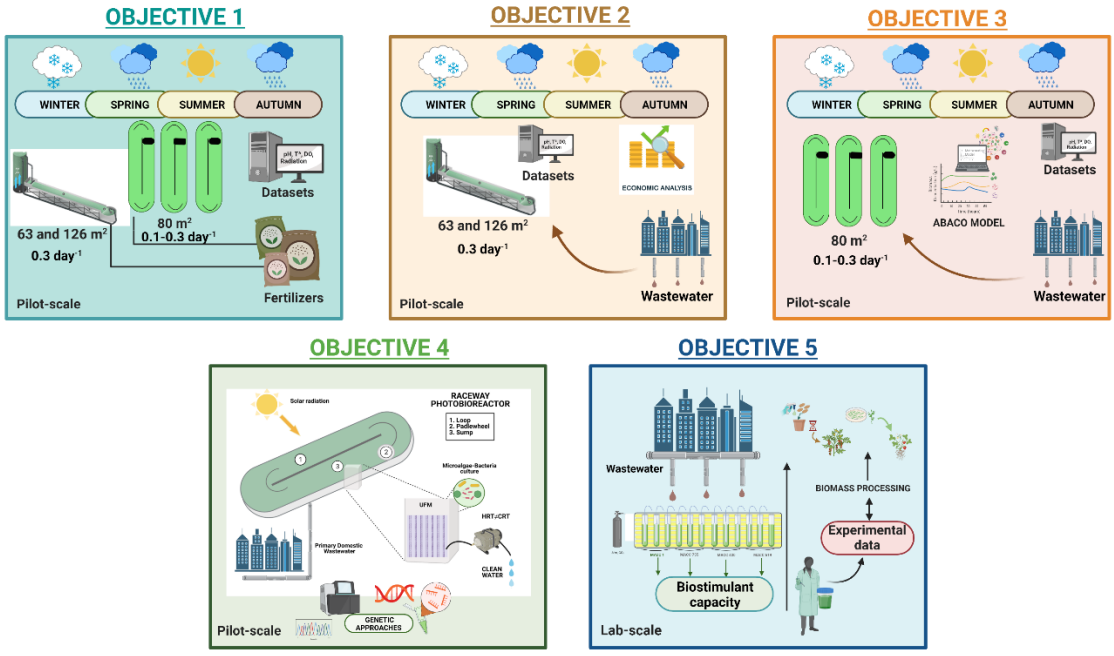


Figure 1.1. Objectives linked to the research work.

1.4. Research carried out

The present work starts with a review of the current needs and challenges of microalgae-based wastewater treatment coupled to the production of high-value agricultural products. This review was the first step of the Thesis project and serves as the basis and introduction to the different research studies and publications described in the following sections. These were divided into three categories: (i) research works related to microalgal biomass production in open photobioreactors using freshwater and fertilizers, linked to specific objectives O1 and O2; (ii) studies related with the wastewater treatment capacity of microalgae in open photobioreactors, related to specific objectives O3 and O4; and (iii) biostimulant capacity of microalgae, related to the specific objective O5. The research work carried out in each category is briefly summarized below and described in detail in Chapter 2.

1.4.1. Research on microalgae production

The biomass productivity of a given production system and a given microalgal strain depends on both the photobioreactor design and the environmental and operational conditions. Despite the reported high biomass productivities using thin layer photobioreactors, their use is still limited to laboratory or pilot scale - up to 50 m^2 [10]. The amount of information available on the performance of this type of reactor is very low when compared to that of raceways or tubular photobioreactors that have been studied for decades. Previous reports suggested that thin layer reactors have some drawbacks, such as pH and temperature gradients, as well as inadequate mass transfer capacity and oxygen accumulation in the culture [33]. More information and pilot-scale results are required before upscaling this technology to an industrial level. A major objective of this PhD Thesis was to evaluate the performance in terms of productivity and efficiency of two different pilot scale thin layer designs: a single channel photobioreactor (63 m^2) and a double channel photobioreactor (126 m^2). Both reactors were operated with a culture medium formulated by freshwater and commercial fertilizers, using nutrient concentrations comparable to those of wastewaters reported in the literature. The productivity of the system was evaluated thoughout a complete year (Figure 1.2).

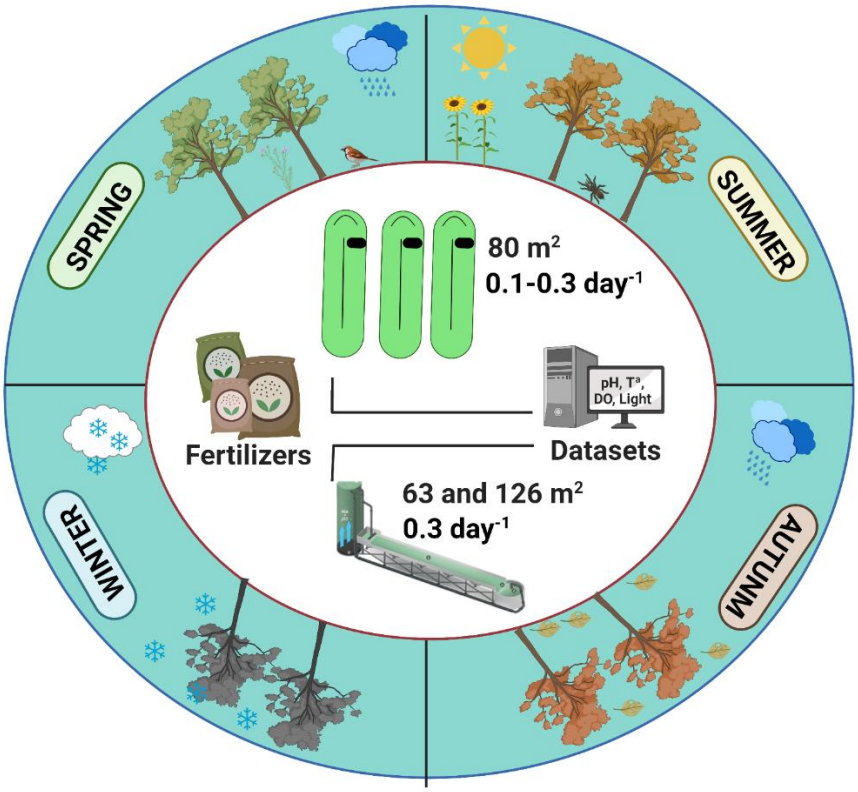


Figure 1.2 Annual production in thin layer reactors.

The results of this study, related to objective O1 and shown in Section 2.2.1, were compared to a conventional 80 m^2 raceway reactor to fully predict the commercial advantages of using novel thin layer reactors. In this initial comparison, it was observed that the maximum productivities in all the technologies are reached in the months with the highest radiation (summer), with maximum achieved values of 25 and 35 $g \cdot m^{-2} \cdot day^{-1}$ in raceway and both thin layers, respectively. A second goal related to thin layer reactors was to evaluate the potential of these systems to process wastewater using the microalga *Scenedesmus* sp. This study, described in Section 2.2.2, assessed the effect of environmental conditions on biomass productivity and the performance of the system. In this case, the productivities obtained during a year are similar to those observed with fertilizers. It is important to highlight that the concentration of the main nutrients needed for the microalgae to grow was comparable in both types of water. The results of this work demonstrated the viability of this thin layer photobioreactors for the simultaneous production of *Scenedesmus* sp. and the treatment of urban wastewater, which was the overall aim of objective O2.

1.4.2.Research on wastewater treatment

The integration of wastewater treatment and microalgae production involves the existence of microalgae-bacteria consortia. The composition of the microalgae-bacteria consortia is key to achieve high nutrient removal rates during the processing of wastewater. However, up to date data about the effect of operating and environmental conditions on the composition of the microalgae-bacteria consortia are scarce [34].

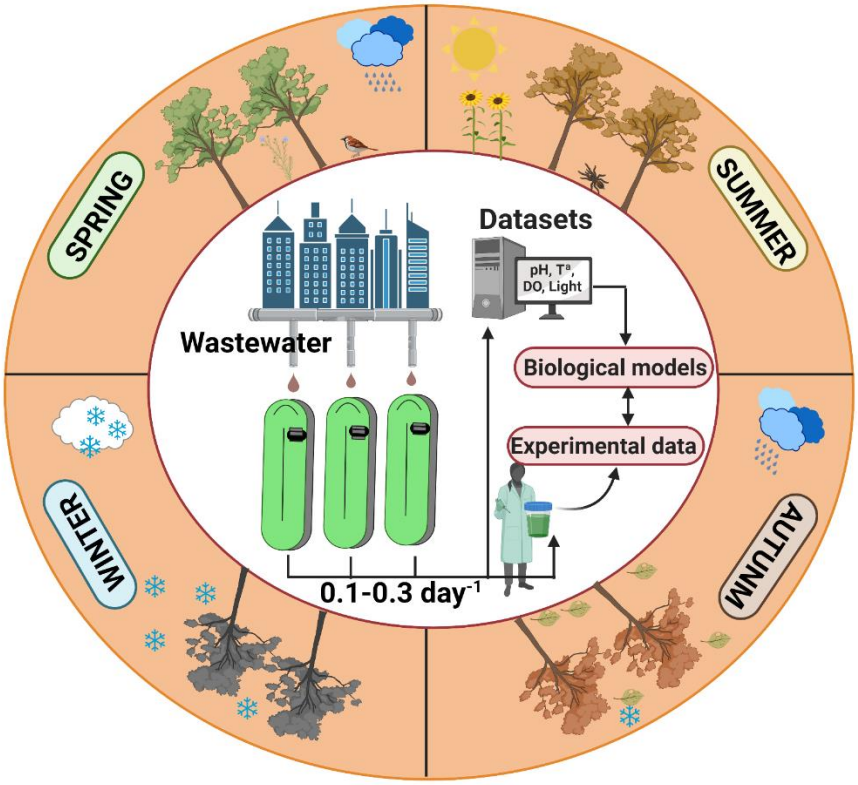


Figure 1.3. Annual production in raceway reactors.

These data are important for the correct design of the process since certain factors can promote the growth of undesirable microorganisms, such as nitrifying bacteria, which reduces the nitrogen consumption of microalgae. Despite promising results, most previous studies available in literature have been conducted under controlled conditions on laboratory scale, or outdoor but using small-sized reactors and during short time periods [35]. The need to increase microalgae productivity has been a major drawback for microalgae biotechnology; the yields of real production systems are still far from theoretical maximum values [36]. The present work is in line with the objective O3, aimed to optimise the performance of pilot scale raceway

reactors and to validate the potential of the microalga *Scenedesmus* sp. to remove nutrients from wastewater during a complete year. This work, described in Section 2.3.1, was carried out using large 80 m^2 raceway reactors operated in semi-continuous mode. The results were promising. They revealed that further upscaling the process to a theoretical 10,000 m^2 raceway reactor would allow removing 10.6 t of nitrogen and 0.5 t of phosphorous per year while simultaneously producing 56.5 t of valuable biomass.

The fourth specific objective of this Thesis (O4) was to evaluate the potential use of membrane technologies to increase the amount of wastewater processed daily and to identify the effect of operating conditions on the composition of microalgae-bacteria consortia through Illumina sequencing. This is important as one of the main challenges of microalgae-based wastewater treatment systems is the surface area needed to install microalgal photobioreactors, which nowadays is large. The results related to O4 are described in Section 2.3.2. Overall, the attachment of membranes to microalgal photobioreactors led to a 129.3% increase in the daily volume of wastewater that could be treated per square meter, and to a 48.7% increase in the biomass productivity (Figure 1.3.). These results were achieved using wastewater that had a low nutrient content; membranes allowed to increase the amount of nutrients that were introduced into the system daily.

1.4.3.Research on biostimulant effect of microalgae

Microalgae can recover (not just remove) nutrients present in wastewater while simultaneously minimising greenhouse gas emissions, saving energy, and producing valuable bioproducts [37]. The obtained biomass can be used as a feedstock to produce valuable agricultural products, such as biopesticides and biostimulants, or to produce aquafeeds that have demonstrated their effectiveness in increasing food sustainability and animal welfare [38]. There is a great interest in developing and using these natural biostimulants produced by microalgae; ongoing studies and developments in this field will certainly result in the appearance of commercial microalgae-derived biocides in the near future. It is, therefore, necessary to identify novel strains that have biostimulant capacity, that are robust, can grow in wastewater, and are highly productive. Consequently, the last specific objective of this Thesis (O5) was to determine the feasibility of producing high-

quality microalgal biomass using wastewater and to assess the biostimulant capacity of the produced biomass, as described in Section 2.4.1. In this study it was demonstrated how the biostimulant capacity of microalgal biomass is strain-dependent and that the activity of the developed extract depends as well on the concentration of biomass used. In this work, the microalga *Chlorella vulgaris* MACC 1 stands out as the most promising among those studied; the best results were obtained when using a microalgal concentration of 2 $g \cdot L^{-1}$.

2. CONTRIBUTIONS TO SCIENTIFIC JOURNALS

As a result of this thesis, five scientific articles have been already published. They are ranked in the Journal Citation Reports (JCR) as follows:

First quartile scientific articles: 2

Second quartile scientific articles: 3

Besides, the research carried out during the PhD project has resulted in another scientific article that is currently under peer review. It contains the last advances in the use of microalgae as biostimulants. This work is described at the end of Chapter 2.

Note that the papers included herein are ordered and classified as they were described at the end of Chapter 1. More precisely, Section 2.1 contains a detailed review done on microalgae, wastewater treatment, production process, and their use in agriculture. Section 2.2 includes two papers that contain the study of the production of microalgae in raceway and thin layer photobioreactors.

Specifically, Section 2.2.1 makes a comparison between both types of reactors, obtaining the optimal operating conditions for each season of the year. Section 2.2.2 includes the production of microalgae in single-channel and double-channel thin-layer reactors wastewater as a culture medium and the economic improvement in production costs that this can entail. Section 2.3 is focused on wastewater treatment using microalgae.

Namely, Section 2.3.1 includes a study on how the environmental and operating conditions influence the treatment of wastewater with microalgae in raceway reactors. Section 2.3.2 contains the comparative analysis of different hydraulic retention times by using an ultrafiltration membrane coupled to a raceway reactor. Finally, the last part corresponds to the application of microalgal biomass in agriculture. Thus, section 2.4 shows a comparison of four different microalgal strains and their effects as biostimulants in agriculture.

The reader should consider that each of the following articles contains its specific introductory sections and material and methods, since we present a doctoral thesis by compendium of articles. On the other hand, the references that the reader will find at the end of the document correspond to the bibliography of each section unified in a particular one.

Similarly, the numbering of figures and tables has been unified to facilitate their location throughout the document.

2.1. Microalgae based wastewater treatment coupled to the production of high-value agricultural products: Current needs and challenges

Title: Microalgae based wastewater treatment coupled to the production of high value agricultural products: Current needs and challenges**.**

Authors: Ainoa Morillas-España, Tomas Lafarga, Ana Sánchez-Zurano, Francisco Gabriel Acién-Fernández, and Cynthia González-López

Journal: Chesmosphere

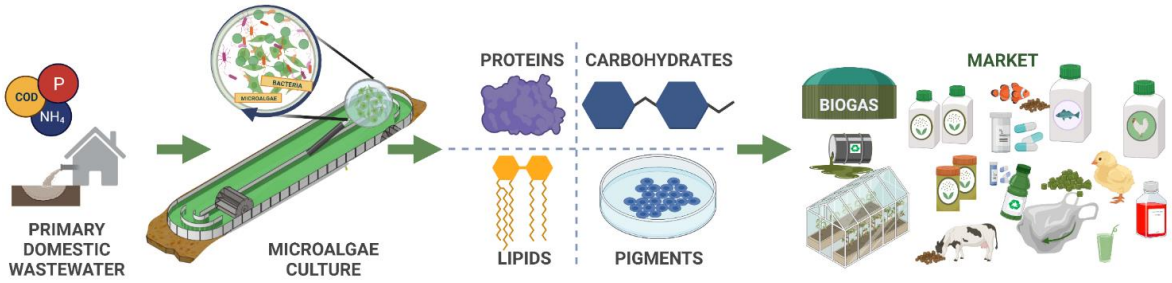
Year: 2022

Volume: 291

DOI: https://doi.org/10.1016/j.chemosphere.2021.132968

IF (JCR 2020): 7.086

Categories: ENVIRONMENTAL SCIENCES, 30/274, **Q1**



Abstract

One of the main social and economic challenges of the 21st century will be to overcome the world's water deficit expected by the end of this decade. Microalgae based wastewater treatment has been suggested as a strategy to recover nutrients from wastewater while simultaneously producing clean water. Consortia of microalgae and bacteria are responsible for recovering nutrients from wastewater. A better understanding of how environmental and operational conditions affect the

composition of the microalgae-bacteria consortia would allow for maximising nutrient recoveries and biomass productivities. Most of the studies reported to date showed promising results, although up-scaling of these processes to reactors larger than 100 m^2 is needed to better predict their industrial relevance. The main advantage of microalgae-based wastewater treatment is that valuable biomass with unlimited applications is produced as a co-product. The current paper aimed to review microalgae-based wastewater treatment processes focusing on strategies that allow for increasing both biomass productivities and nutrient recoveries. Moreover, the benefits of microalgae-based agricultural products were also discussed.

Keywords: bioremediation, cyanobacteria, nutrients, water, biostimulants, biofertilisers.

1. Introduction

You never miss the water until the well runs dry. It's an old saying that is now more relevant than ever: approximately 60% of the world population is expected to suffer a water shortage by 2025 [1]. One of the main social and economic challenges of the 21st century will be to overcome the world's water deficit expected by the end of this decade. The main causes for this deficit include increased demand for water, contamination of water resources, and lack of technologies to reclaim used water. The main goal of wastewater treatment processes is to allow the utilisation or discharge of effluents back into the environment without causing significant damage. However, conventional nutrient removal methods have restrictions concerning their high-energy requirements or environmental impact (greenhouse gas emissions) and are facing challenges to meet strict nutrient discharge standards [2].

The search for an alternative economic, sustainable, and effective strategy to process wastewater led to an increased interest in microalgae-based wastewater treatment processes. The use of microalgae is considered one of the most promising strategies to process wastewater [3,4,39,40] and is already being implemented in commercial scale (e.g. Chiclana and Mérida in Spain or Christchurch in New Zealand). In the current review, the term microalgae will be

used indistinctively to refer to prokaryotic cyanobacteria and eukaryotic microalgae. Cyanobacteria are a bacterial phylum capable of performing photosynthesis and for this reason, these microorganisms are generally included in the term “microalgae”. Recovering nutrients from wastewater using microalgae involve the symbiotic association of microalgae with aerobic and anaerobic microorganisms. It is not possible to produce 100% pure microalgal cultures at a large scale, especially when using open reactors and wastewater. The composition of the microalgae-bacteria consortia depends on several factors including the quality of the water used, environmental conditions and operational conditions [25]. For example, a low light availability (e.g. high culture depths) [41] and an increase in temperature [27] promote the growth of nitrifying bacteria. If operational conditions are managed properly, over 95% of the produced biomass will be microalgae [28].

The association of microalgae and other microorganisms is generally beneficial for microalgal growth. Yeast such as *Rhodotorula glutinis* promoted biomass and lipid productivity in the production of *Arthrospira platensis* [42]. Certain bacterial groups can synthesise micronutrients, siderophores, growth stimulants, and antibiotics that promote growth and protect algae from pathogenic microorganisms [43]. Some of the most common symbiosis factors produced by bacteria to promote microalgal growth are vitamin B_{12}, nitrogen, dimethylsulfoniopropionate, roseobacticides, virbioferrin, and acyl-homoserine lactone, among others [44,45]. Bacteria and yeast not only promote growth but also the composition of the produced biomass [44,45]. Bacteria present in microalgal cultures such as *Flavobacterium, Terrimonas,* and *Sphingobacterium* produce extracellular metabolites that facilitate harvesting by increasing the floc size and promoting the sedimentation of microalgae [46]. One of the main advantages of using microalgae-bacteria consortia is their dual role: they not only remove contaminants/nutrients but also produce valuable biomass that can be further used for a wide variety of applications. For example, microalgae produced using cattle dairy wastewater has been used for fertilising pasture and led to higher concentrations of minerals including phosphorus, calcium, magnesium, and manganese and a higher dry matter content [18]. Biostimulants are among the most common microalgae derived products currently available in the market. Biostimulants are considered environmentally friendly and cost-effective products when compared to their synthetic counterparts. These products are highly

demanded, especially in the organic cropping system [47,48]. Their potential utilisation to obtain increased yields or improved fruit quality has been demonstrated in several recent reports [20–22,24,49].

The current paper aimed to review recent findings on wastewater treatment using microalgae-bacteria consortia and to summarise and discuss the potential utilisation of microalgal biomass as feedstock for the production of biostimulants. Moreover, this review also discusses the main advantages and limitations of current microalgae production systems focusing on those processes that are coupled to wastewater treatment and production of agricultural products.

2. Producing and processing microalgal biomass

Microalgae biotechnology is a relatively new research area. As highlighted in the previous section, microalgae have potential applications in both wastewater treatment and agriculture. Microalgae are unicellular photosynthetic microorganisms with a simple reproductive and cell growth system, which allows a fast proliferation and long-term survival in harsh environments [50]. Although microalgae grow naturally in lakes, rivers, or oceans, these ecosystems allow very low biomass concentrations for large-scale harvesting. Thus, to obtain higher concentrations, microalgae need to be produced in photobioreactors that have evolved from initial open ponds to a wide variety of modern designs. The current section aims to describe and summarise the production and processing of microalgae, focusing on those methods relevant to wastewater treatment and the production of agricultural products.

2.1 Production of microalgae

Several photobioreactor designs are currently being used for microalgae production, being strains of the genus *Arthrospira, Isochrysis, Nannochloropsis, Tetraselmis, Chlorella, Haematococcus,* and *Dunaliella* the most widely produced. The selection of a certain reactor will depend on the end application of the biomass and the produced strain. For example, open ponds such as those shown in Figure 2.1.1 are the preferred option when producing microalgae using wastewater while more complex closed tubular reactors are more suitable when the biomass is used for food, cosmetic, or pharmaceutical applications. Closed systems have the advantage of providing a controlled environment that can be manipulated

according to the microalgal requirement. Higher process control allows for achieving higher volumetric productivities. For example, the productivity of the microalga *Nannochloropsis gaditana* using closed tubular photobioreactors was 0.6 $g \cdot L^{-1} \cdot day^{-1}$ [51], while the maximum productivity that could be achieved in an open raceway was 0.2 $g \cdot L^{-1} \cdot day^{-1}$ [52]. Closed photobioreactors can be controlled to satisfy specific biological and physiological demands of microalgae and allow the production of monocultures that cannot be produced in open systems. Still, contaminations represent a challenge, even for closed systems. A disadvantage of closed photobioreactors is their high-cost investment which can be around 0.6-1.2 $M€ \cdot ha^{-1}$ and leads to higher biomass production costs, in the range of 20-30 $€ \cdot kg^{-1}$. For this reason, closed systems are used for the production of high-value strains such as *Haematococcus pluvialis* [53], which is used as a source of astaxanthin, a potent antioxidant carotenoid used in the food and cosmetic industries that can reach a market price of 2,500 $€ \cdot kg^{-1}$ [54].

Figure 2.1.1 Open raceway bioreactors located at the University of Almería, Spain.

Because of their complexity and higher production costs, closed bioreactors are not recommended for wastewater treatment processes. In turn, open systems are

much simpler to operate and their fixed and operational costs are much lower – cost investment for these systems range from 0.13 to 0.37 M€·ha^{-1} [55,56]. After decades of research, biomass production costs using open systems have been reduced to 5.0-10.0 €·kg^{-1}. This value could be further reduced to under 1.0 €·kg^{-1} if the process is scaled-up sufficiently and biomass production is coupled to wastewater treatment and CO_2 capture from flue gases [57]. One of the main disadvantages of open systems is their lower biomass productivity when compared to closed photobioreactors.

The maximal biomass concentration reached in open systems is lower, around 0.5-0.6 g·L^{-1}, mainly because of the self-shading or shadow effect of microalgae. Raceway reactors can be operated at depths of 0.10-0.15 m [58]. However, higher depths of up to 1 m have also been evaluated as a strategy to avoid freezing and minimise heat loss during the cold periods [59] and lower depths (0.05 m) have been used to increase light availability [60].

Light availability is the most important factor in the growth and productivity of photosynthetic microorganisms and current raceway designs do not allow to optimise light utilisation [61]. This drawback has been improved by reducing the culture depth. Thin-layer reactors which use water depths ranging from 0.006 to 0.040 m are highly productive and allow productivities comparable to those of closed systems [62–65]. Artificial illumination is economically prohibitive for low-value applications. Therefore, wastewater treatment processes based on microalgae are carried out outdoors using the sun as the source of light. These processes are highly influenced by environmental factors namely temperature and solar radiation. For example, the areal productivity of an optimised large-scale raceway reactor used to produce *Scenedesmus almeriensis* reached 20-25 g·m^2·day^{-1} (0.14-0.17 g·L^{-1}·day^{-1}) in summer and the maximum productivities in winter and autumn were close to 15 g·m^2·day^{-1} or 0.10 g·L^{-1}·day^{-1} [65].

As open systems are exposed to the environment, other microorganisms can lead to contamination of the culture. Only a limited number of strains that are robust enough, fast-growing, and tolerant to extreme conditions can be produced in open reactors and only those that produce a valuable compound render the process economically viable. This include *Arthrospira platensis* and *Chlorella vulgaris*,

mainly used as food, and *Dunaliella salina* used as a source of β-carotene (Lafarga, 2020). The maintenance of monocultures (or cultures high a very high content of one strain) at large-scale remains challenging [39]. In this sense, several research groups are currently identifying extremophile strains that show potential for being produced outdoors and accumulate valuable biomolecules, generally carotenoids [67]. Indeed, the above-mentioned strains *A. platensis* and *D. salina* are extremophiles as they can be produced at pH values in the range 9-10 and salt concentrations up to 300 $g \cdot L^{-1}$. In this case, although the consortia contained a large number of different microorganisms including *Chlorella* strains, both *Anabaena* sp. and *Dolichospermum* sp. managed to be the predominant microorganisms [68].

Overall, the selection of the type of photobioreactor used for microalgal growth will depend on the intended use and desired quality of the biomass. When microalgae are used for wastewater treatment and as a source of bioproducts for agriculture, they are produced using open photobioreactors. The main reasons are the lower production costs and the ease of construction, operation, and scale-up of the facilities. Moreover, the surface-to-volume ratio of raceway reactors is higher than that of closed systems or thin-layer cascade designs, allowing the processing of larger volumes of water per surface unit.

2.2 Processing of microalgal biomass

Before the commercialisation of the biomass, microalgae need to be harvested. Harvesting and dewatering contribute 3-15% to algae biomass production costs [69] and refer to several processes where a diluted microalgae suspension is concentrated into a thick paste. Different methods are available for concentrating microalgal biomass and the main advantages and limitations of current harvesting strategies have been revised previously [70–73]. Ideally, the harvesting step should be effective for a large number of microalgal strains and should allow high biomass concentrations at low (moderate) costs of operation, energy, and maintenance [74]. Separating algae from water remains a major hurdle to industrial-scale processing and a universal harvesting method does not exist yet. The method used will depend on the final use of the biomass but also on fundamental properties of the microalgae such as strain, density, particle shape, or particle size [72].

For many applications, microalgal harvesting comprises two steps, pre-concentration or thickening and dewatering. However, sometimes a single step is used depending on the desired water content of the suspension (indeed, the most common current strategy at a large scale is centrifugation). Flocculation, flotation, or filtration have been used for pre-concentration, followed by centrifugation or filtration applied subsequently as the main dewatering methods to concentrate microalgal cells into pastes. Pre-concentration using flocculation results in the lowest energy requirements but because of the need for chemicals and loss of flocculants, these systems end at the same cost level as mechanical harvesting systems [69]. Bioflocculation is being evaluated as an inexpensive technology for harvesting microalgae and the main findings have been summarized in several review papers [75–77].

Bioflocculation is a flocculation process of microalgal cells assisted with microorganisms. The aggregation of bacteria and microalga creates large flocs that settle down by gravity without the need for adding chemical aids or modifying the pH of the culture. For example, the diatom *Skeletonema* was used to form flocs of *Nannochloropsis* [78] and *Citrobacter freundii* and *Mucor circinelloides* improved the flocculation of *Chlorella* [79]. In addition, the bioflocculant poly ɣ-glutamic acid produced by *Bacillus licheniformis* CGMCC 2876 was effectively used to concentrate the microalga *Desmodesmus* sp. F51 allows a flocculation efficiency higher than 99% and a harvesting efficiency of 95% [80]. This strategy can also be used as a method to improve nitrogen and phosphorus removal from wastewaters as the microalgal-bacterial flocs tend to adsorb suspended compounds [75].

Filtration techniques are currently being investigated and membrane bioreactors are widely used for municipal and industrial wastewater treatment. The use of membranes to maximise nutrient recoveries from wastewater is also common [81] One of the main limitations of membrane technologies is that microalgae lead to fouling/clogging and reduced flux and therefore to increased operational costs [71]. However, a correct design of the process and the use of anti-fouling strategies such as intermittent permeation, membrane backwashing, air backwashing, or air-induced cross flow can minimise this issue. Flocculation and membrane filtration are limited in terms of maximal concentration and are generally followed by a centrifugation step, which has potential to achieve high biomass concentrations.

However, we would like to highlight what discussed above: a universal harvesting method does not exist, and the selected method should be optimised for each process independently.

Once the biomass is concentrated, it needs to be further processed rapidly to avoid spoilage, especially in hot climates (Lafarga, 2020). When used as a source of bioactive compounds for agriculture as a biofertilizer or biostimulant, the microalgal biomass is commercialised as a liquid suspension. For this reason, drying processes will not be discussed in the current paper. Main drying strategies which include freeze-drying, rotary drying, spray drying, solar drying, and incinerator drying and have been revised previously (Chen et al., 2015; Show et al., 2015).

One of the main challenges of producing valuable bioactive compounds using microalgae is that these are (most of the time) produced and accumulated inside microalgal cells. Because microalgal cell walls are rigid and protective, a disruption step is generally required to allow the extraction of valuable biomolecules. In this sense, several strategies have been studied including enzymatic or chemical hydrolysis, bead milling, high-pressure homogenisation, sonication, microwaves, pulsed electric fields, and high-voltage electrostatic fields, and high-voltage electrical discharges (Lafarga, 2020). At a large scale, the most relevant strategies are high-pressure homogenisation, bead milling and, to a lower extent, sonication.

High-pressure homogenisation is a mechanical process during which the solution containing the biomass is forced by high pressure (50-300 MPa) through a micrometric disruption chamber. This increases the velocity and subjects the cells to intense fluid-mechanical stresses that disrupt cell walls and membranes [85]. Sonication consists of the use of ultrasonic waves, at frequencies beyond 18 kHz, to generate bubbles that collapse and generate spots of extremely high temperature and pressure that induce cell wall disruption [86]. High-energy demands are a major bottleneck for the downstream processing of microalgae. Energy consumption of high-pressure homogenisation is lower than that of sonication and is, therefore, together with bead milling, the preferred strategy for large-scale processing of microalgae. Pulsed electric fields are being industrially used in the food industry [87] and could be used to disrupt the cell wall of microalgae at the industrial level. Although this strategy has not been implemented at a large scale

(up to the best of the authors' knowledge) several laboratory-scale trials have been conducted and the results are promising [85,88].

Finally, as mentioned before, microalgae-based agricultural products are generally commercialised as a liquid suspension (rich in nutrients). This means that a thermal treatment, generally a pasteurisation step is used to increase shelf life and avoid spoilage of the product. Other technologies used to extend the shelf life of food such as high pressure processing or ohmic heating could be implemented to extend the shelf life of agricultural products, although these have not yet been evaluated (ohmic heating was assessed as a cell wall disruption step [89] but not to increase the shelf life of microalgae products).

3. Microalgae and wastewater treatment

Unfortunately, most wastewater produced globally is discharged into the environment without treatment – over 80% of sewage water is discarded untreated [90]. Wastewater treatment can include physical, chemical, and biological strategies and it allows releasing the water, once treated, into the environment. One of the main advantages of using microalgae for wastewater treatment, besides high efficiency and safety, is that the generated biomass can be further used for numerous applications. These include the production of biofuels [14], animal feed [91], or biofertilisers and biostimulants for agriculture. Another important advantage is that microalgae wastewater treatment can be economically viable and sustainable. Indeed, a techno-economic analysis revealed that (under the optimum scenario) the overall cost of processing wastewater using microalgae could be 0.15 $\$\cdot m^{-3}$ - which is a 30% lower than for activated sludge and was calculated without considering the revenues obtained from the commercialisation of the end agricultural products [92]. Moreover, the process can also show a positive energy balance if coupled with biomethane production, rendering the process economically viable and sustainable [92].

3.1. Microalgae-bacteria interactions

The development of processes to remove associated bacteria from microalgal cultures and maintain bacteria-free cultures in large-scale production has been attempted [93]. However, it is deemed impractical and unsustainable because in most cases, bacteria are introduced into microalgae cultivation systems through

algae stocks used as starter cultures, which are often not axenic [94]. Moreover, bacteria can access microalgal cultures via multiple operation processes as the culture media used during dilution or as airborne invaders in open systems. Bacteria and microalgae cannot be understood properly if they are evaluated individually. Both appear together in nature and establish many types of interactions, from mutualism or symbiotic relationships to commensalism or parasitism [45]. Microalgae-bacteria interactions are well known for a long time and were already described by Oswald in 1953 (Oswald, et al., 1953). These interactions occur in the area surrounding microalgal cells, where metabolites are exchanged between microalgae and bacteria [96] and are species-specific as the microenvironment of each microalga is different.

Currently, it is accepted that the interactions between microalgae and bacteria have the potential to improve microalgal biomass production[45]. The nutrient exchange plays a major role. Under illumination, microalgae perform photosynthesis, consuming carbon dioxide and producing oxygen. This oxygen is essential for the degradation of organic matter present in wastewater by heterotrophic bacteria. Simultaneously, during bacterial oxidation of organic matter, carbon dioxide is produced and is available for microalgae to produce photosynthesis [97]. Nitrifying bacteria or nitrifiers also are present in wastewater and also have a symbiotic relationship with microalgae. Indeed, these microorganisms transform ammonium into nitrate using the oxygen produced by microalgae [98]. Vitamins, macronutrients, and plat hormones excreted by bacteria promote microalgal growth. A survey of 326 algal species conducted in 2005 revealed that 171 require exogenous vitamin B_{12} and that the (unexpected) source of this vitamin was bacteria [99]. Since then, several studies demonstrated the exchange of vitamin B_{12} from bacteria to microalgae, for example, from *Mesorhizobium* sp. to the B_{12}-dependent microalga *Chlamydomonas nivalis* [100] or from *Sinorhizobium meliloti* to *Chlamydomonas reinhardtii* [101].

The association of microalgae and bacteria during wastewater treatment has advantages as well in terms of nutrient recoveries. For instance, a combination of *C. vulgaris* and a microalgae growth-promoting bacterium as *Azospirillum brasilense* allowed an increased removal of nutrients (nitrogen and phosphorus) than microalgae alone [102]. One of the reasons for this effect is that bacteria can

increase nutrient availability. For example, in most cases, wastewaters contain organic phosphorus that is not available (or with low availability) for uptake by microalgae. However, bacteria produce enzymes to mineralize the organic phosphorus making it bioavailable for microalgae [103]. Further studies allowing to understand better the interaction between microalgae and bacteria are needed. This might be the key to maximising productivity, nutrient recoveries and environmental and economic impact [104].

3.2. Biomass productivities and nutrient recoveries

Table 1 lists some of the most recent findings on wastewater treatment using microalgae and pilot- or large-scale photobioreactors. The selection of a robust and highly-productive strain capable to grow under a wide range of environmental conditions is of key importance. The most common genus used for wastewater treatment were *Scenedesmus* and *Chlorella* **¡Error! No se encuentra el origen de la referencia.**.These demonstrated high tolerance to adverse environmental conditions (high temperatures and solar radiation) and resistance to high $N\text{-}NH_4^+$ concentrations. $N\text{-}NH_4^+$ inhibits algal growth after a certain strain-dependent threshold (and is naturally present at high concentrations in some types of wastewater). Other microalgae such as *Arthrospira platensis* [105,106], *Tetraselmis* sp. [107,108], or *Haematococcus pluvialis* [109,110] have been studied as candidates to recover nutrients from wastewater at laboratory-scale using different photobioreactor designs.

The up-scaling of these processes using low-cost photobioreactors that allow the processing of large volumes of wastewater is necessary to assess their commercial potential. The most used reactors in the literature are raceways, which was expected because of the above-described advantages of raceways for low-value applications. However, other common designs such as bubble columns and tubular reactors or more innovative reactors have been described. Further efforts are needed to further up-scale these processes as, although some studies were conducted in relatively large reactors, their scale is not yet representative of industrial processes (reports using photobioreactors larger than 100 m^2 are not available). In terms of nitrogen and phosphorus removal capacities, all the studies demonstrated that microalgae can recover large percentages of the total nitrogen and phosphorus present in the wastewater. However, the percentage of nitrogen

or phosphorus recovered will depend largely on the initial concentration in the wastewater. It is more accurate to express the removal capacity of a process as grams of nitrogen or phosphorus that can be recovered per square meter and day. Nitrogen and phosphorus removal rates of up to 4286 and 227 $mg \cdot m^{-2} \cdot day^{-1}$ respectively have been reported in pilot-scale raceway reactors (80 m^2, 11.8 m^3) operated using primary wastewater [111]. In that study, the authors concluded that approximately 15-30% of the nitrogen removed from the wastewater was stripped into the atmosphere [111].

Little is known about the effect of environmental and operational conditions on the composition of the microalgae-bacteria consortia. In addition, the effect of the composition of the consortia on the efficiency of the process and the overall biostimulant activity of the biomass is not known. A recent study concluded that both, the dilution rate and the depth of the culture can affect significantly the microbial populations of the culture [41]. In that study, the authors observed that the abundance of nitrifiers increased with culture depth, and this could lead to the accumulation of nitrate in the system.

In a different study, a significant variation in the composition of the consortia during the year was reported, and this partially contributed (together with environmental fluctuations throughout the year) to the observed differences in the nitrogen and phosphorus removal efficiencies [116]. Similar results were observed in another report, where a strong influence of temperature, solar radiation, and nutrient content on bacterial communities was observed [25]. In that study, the authors suggested that the excretion of microalgal substances to the medium could modulate bacterial communities and therefore, the performance of the system and the overall quality of the produced biomass.

Only a limited number of studies conducted mass balances or reported phenomena that take place during wastewater treatment (nitrification, stripping, or precipitation of phosphorus, for example). This should be also considered in further studies as, for example, stripping could represent a large percentage of the total nitrogen “consumption”.

4. Microalgal agricultural products: A biorefinery approach

Microalgal biomass produced using wastewater can be used for different applications. For example, because of their high content of protein and valuable bioactive molecules, several animal feeds (especially aquafeeds) enriched using microalgal biomass have been developed and demonstrated antioxidant, antimicrobial, and disease-preventing effects [124]. High production costs of microalgal biomass, when compared to conventional feed ingredients (soybean, fish oil, etc.), have been suggested as the main challenge that needs to be addressed before fully exploiting microalgae in the animal feed industry [124]. In this sense, the use of wastewater as a source of nutrients could contribute to reducing production costs. A recent study suggested that microalgae production costs could be lower than 1 €·kg^{-1} if produced using wastewater and flue gases [125]. The utilisation of microalgal biomass or the "residues" left after the extraction of valuable microalgal products, for producing biogas by anaerobic digestion could allow increasing the economic viability of using microalgae in the feed industry [126].

4.1. Biofertilisers and biostimulants

Low production yields are mainly caused due to insufficient nutrient availability, which is a major agronomic problem in some parts of the world (while excess nutrients represent a problem in some others). Chemical fertilisers and manure have been key to agricultural intensification but they also lead to widespread nutrient pollution and the degradation of lakes, rivers, and coastal ocean while the release of nitrous oxide from fertilised fields contributes to climate change [127].

This, together with improved knowledge about the relationship between plants and soil microorganisms occurring in the rhizosphere has led to increasing development and utilisation of microbial-based fertilisers or biofertilisers worldwide [128].

Biostimulants offer a novel approach to the regulation or modification of physiological processes in plants. Not only to stimulate growth but also to mitigate stress-induced limitations and finally increase yields [129] –gure 2.1.2. Both biofertilisers and biostimulants are considered environmentally friendly and cost-effective products and are highly demanded, especially in the organic cropping system [47].

Table 2.1.1. Outdoor demonstrations of microalgae-based wastewater treatment processes.

Photobioreactor	Microalgal strain(s)	Culture medium	Operation mode	Biomass concentration/productivity	Nutrient removal	Reference
Raceway (8.3 m^2, 850 L)	*Scenedesmus* sp.	Secondary urban wastewater	Semi-continuous mode (0.1-0.3 day^{-1})	Biomass productivity ranged from 4 $g \cdot m^{-2} \cdot day^{-1}$ in winter to 17 $g \cdot m^{-2} \cdot day^{-1}$ in summer.	Average COD, TN, TP and *E. coli* removal efficiencies of 84, 79, 57, and 93%	[112]
Raceway (1.5 m^2, 470 L)	Dominated by *Stigeoclonium* sp., diatoms, *Chlorella* sp., and *Monoraphidium* during warm seasons and *Chlorella*, diatoms, and *Stigeoclonium* sp, in cold seasons	Urban wastewater	Semi-continuous mode	Biomass productivity ranged from 6-8 $g \cdot m^{-2} \cdot day^{-1}$ in winter to 13-24 $g \cdot m^{-2} \cdot day^{-1}$ in summer.	COD removal rates of 29-58 $g \cdot m^{-2} \cdot day^{-1}$. The removal of micro contaminants was season-dependent.	[113]
Tubular reactor (890 L)	*Chlorella pyrenoidosa*	Digested wheat starch processing wastewater (filtered)	Batch mode	Biomass productivity ranged from 0.11 $g \cdot L^{-1} \cdot day^{-1}$ in winter to 0.63 $g \cdot L^{-1} \cdot day^{-1}$ in summer.	Average COD, TN, and TP removal efficiencies of 66, 83, and 97% respectively	[114]
Raceway (7.2 m^2, 800 L) and tubular reactor (340 L)	*Nannochloropsis gaditana*	Centrate wastewater	Semi-continuous mode (0.2-0.3 day^{-1})	Maximum biomass productivity values around 25 and 10 $g \cdot m^{-2} \cdot day^{-1}$ for tubular and raceway reactors respectively	TN and TP removal rates of 20-30 and 1-3 $mg \cdot L^{-1} \cdot day^{-1}$ (tubular reactor) and 20-30 and 0.5-1.5 $mg \cdot L^{-1} \cdot day^{-1}$ (raceway) respectively.	[115]
Thin-layer cascade reactor (32 m^2, 1600 L)	*Scenedesmus* sp.	Primary urban wastewater	Semi continuous mode (0.3 day^{-1})	Maximum biomass productivity values varied between 28 and 47 $g \cdot m^{-2} \cdot day^{-1}$ in winter and summer respectively	$N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ removal rates varied within 15-30, 0-2, and 2-6 $mg \cdot L^{-1} \cdot day^{-1}$ respectively	[116]
Semi-open bioreactor (1500 L)	*Chlorella* sp.	Centrate wastewater	Semi-continuous mode	Biomass productivity ranged from 17.7-34.6 $g \cdot m^{-2} \cdot day^{-1}$	Average COD, TN, and TP removal efficiencies of 70, 61, and 61% respectively	[117]

Photobioreactor	Microalgal strain(s)	Culture medium	Operation mode	Biomass concentration/productivity	Nutrient removal	Reference
Raceway (1.9 m^2, 533 L) and tubular reactor (380 L)	*Scenedesmus obliquus*	Secondary urban wastewater	Semi-continuous mode (HRT 2-5 days)	Maximum biomass productivity was 8.3 and 21.7 $g \cdot m^{-2} \cdot day^{-1}$ for the raceway and tubular reactor, respectively	Average TN and TP removal efficiency of 89.6 and 86.7% (tubular reactor) and 65.1 and 58.8 (raceway) respectively.	[118]
Bubble column (33 L, 4 columns)	*Chlorella zofingiensis*	Artificial wastewater	Batch mode	Biomass productivity of 0.06 $g \cdot L^{-1} \cdot day^{-1}$	Maximum TN and TP removal efficiencies of 73.5 and 100% respectively	[119]
Rotating modified raceway (4080 L)	Dominated by *Diatoma*, *Pediastrum*, and *Chlorella*	Tertiary urban wastewater	Fed-batch mode	Biomass productivity of 31 $g \cdot m^{-2} \cdot day^{-1}$	Average TP and TN removal rates of 2.1 and 14.1 $g \cdot m^{-2} \cdot day^{-1}$ respectively.	[120]
Photo-membrane bioreactor (1300 L)	*Scenedesmus* sp.	Industrial wastewater (electric factory)	Continuous mode	Biomass concentrations ranged from 0.14 to 0.22 $g \cdot L^{-1}$	Average TP and TN removal efficiencies of 100 and 46% respectively. Phosphorus precipitation was observed.	[121]
Twin-layer bioreactor with immobilised microalgae (6 m^2, 55 L)	*Halochlorella rubescens*	Primary and secondary urban wastewater	Batch mode	Average biomass productivity of 6.3 $g \cdot m^{-2} \cdot day^{-1}$	Average TN and TP removal efficiencies in the range 70-99%	[122]
Modular offshore photobioreactor (83.6 m^2, 21000 L)	*Scenedesmus dimorphus* (s hifted to *Chlorella*, *Cryptomonas* and *Scenedesmus* after 12 months)	Urban wastewater	Semi-continuous mode	Ranged from 3.5 to 22.7 $g \cdot m^{-2} \cdot day^{-1}$	Maximum TN, TP, and BOD removal efficiencies of 75, 93, and 92% respectively.	[123]

Photobioreactor	Microalgal strain(s)	Culture medium	Operation mode	Biomass concentration/productivity	Nutrient removal	Reference
Thin-layer reactors (63 m^2, 2400 L; 126 m^2, 3600 L)	*Scenedesmus almeriensis*	Primary urban wastewater	Semi-continuous mode	Average annual productivity of 24.8 $g \cdot m^{-2} \cdot day^{-1}$ with a maximum of 32.8 $g \cdot m^{-2} \cdot day^{-1}$ in summer	TN and TP removal rates depended on the season and varied within 695-2383 and 70-118 $mg \cdot m^{-2} \cdot day^{-1}$ respectively	64
Raceway reactors (80 m^2, 11800 L)	*Scenedesmus almeriensis*	Primary urban wastewater	Semi-continuous mode	Biomass productivities range within 20-28 $g \cdot m^{-2} \cdot day^{-1}$ when operating at dilution rates of 0.3-0.5 day^{-1}	TN and TP removal rates higher than 90% and COD removal of 70% approximately	41
Raceway reactors (80 m^2, 11800 L)	*Scenedesmus almeriensis*	Primary urban wastewater	Semi-continuous mode	Maximum biomass productivity of 25.1 $g \cdot m^{-2} \cdot day^{-1}$	Maximum TN and TP removal rates of 4286 and 227 $mg \cdot m^{-2} \cdot day^{-1}$ respectively	111

Abbreviations: COD, chemical oxygen demand; TN, total nitrogen; TP, total phosphorus; HRT, hydraulic retention time; BOD, biological oxygen demand.

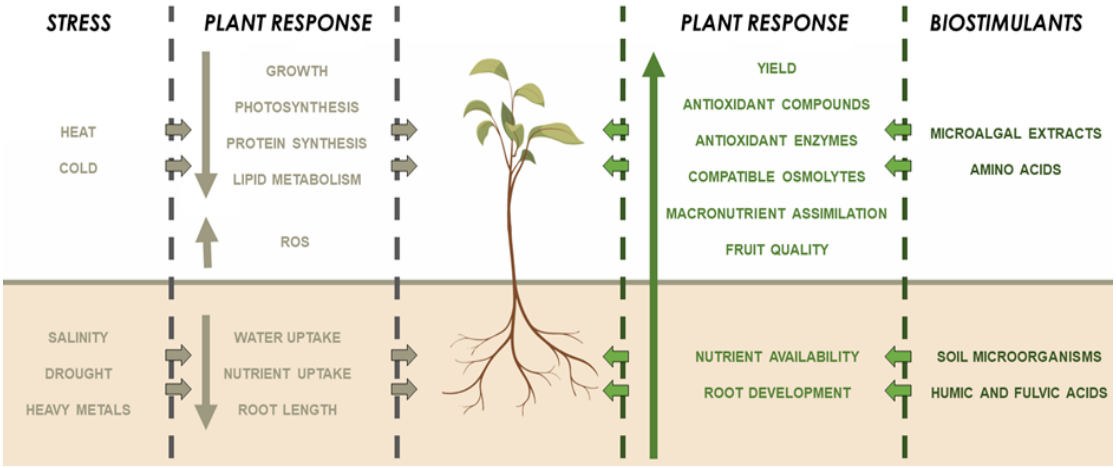
Plant biostimulants can be divided into 7 major groups: (i) humic and fulvic acids, (ii) protein hydrolysates and other N-containing compounds, (iii) algal extracts and botanicals, (iv) chitosan and other biopolymers, (vii) inorganic compounds, (vi) beneficial fungi, and (vii) beneficial bacteria. Their nature is very diverse as well as their physiological functions. To date, several physiological functions have been demonstrated and these include the protection of photosynthetic compounds against photo damage or the initiation of lateral roots – Table 2.1.. Microalgal extracts demonstrated gibberellin-like, auxin-like, and cytokinin-like effects in previous reports [49]. Gibberellins are plant hormones that promote plant growth in a variety of developmental contexts. Mutants defective in gibberellins show reduced elongation of roots, stems, and floral organs [130]. Auxins (i.e. indole-3-acetic acid, indole-3-butyric acid, phenylacetic acid) and cytokinins (*trans*-zeatin, kinetin, *N-N'*-diphenylurea) were identified in several microalgal species from *Chlorella*, *Scenedemus*, and *Acutodesmus* genus [131]. Auxins can induce growth responses in plants and have implications for most of the quantitative changes that occur during a plant's life cycle [132]. Cytokinins participate in the regulation of plant growth, physiological activities, and yield and play a key role in response to abiotic stress [133].

Microalgal biomass has the potential to prevent nutrient loss by gradually releasing nitrogen, phosphorus, or potassium into the environment. Different microalgae-based biofertilisers or biostimulants are currently commercially available and these include AlgaFert® and AlgaFert Eco® commercialised by the Spanish company Biorizon Biotech SL (Almería, Spain). Almost one century ago, Alfred Redfield concluded that plankton has an average atomic C:N:P stoichiometry of 106:16:1 [134]. In the case of freshwater microalgae, the Redfield ratio is not a rule with N:P molar ratios ranging between 8:1 and 45:1 [135].

The C:N:P of microalgae reflects their macromolecular composition: protein is the major reservoir of cellular nitrogen, phospholipids and nucleic acids are the major reservoirs of phosphorus, and protein, lipid, and carbohydrate content determine cellular carbon [136].

Significant phylogenetic differences in the macromolecular composition of microalgae have been identified after conducting a hierarchical Bayesian analysis

using a large number of data compiled from the literature. For example, while cyanobacteria have an average protein content of 42.2%, the protein content of other phyla such as Chlorophyta or Bacillariophyta is around 32.8 and 29.2% respectively [136]. However, the composition of microalgal biomass also depends on other factors which include culture media composition and environmental factors like temperature or solar radiation (Lafarga, 2020).



gure 2.1.2. Microalgae-based biostimulants. Biostimulants can not only modify physiological responses but also maximise crop productivities and promote root development, enhancing nutrient uptake and optimising (minimise) fertiliser consumption and use efficiency.

Thus, the chemical characteristics of microalgae-based biofertilisers or biostimulants will be highly influenced by all these factors. For example, a recent study demonstrated that the molar N:P composition of the microalgae *C. vulgaris, Stigeoclnium* sp., *S. obliquus,* and *C. sorokiniana* ranged from 7.8 to 20.3 when produced in a growth medium with an N:P ratio of 6:1 and that this difference was reduced to a ratio between 11.3 and 16.3 when the microalgae were transferred to a growth medium with an N:P ratio of 2:1 [135]. As an example, the commercial microalgal fertilisers AlgaFert® (Biorizon Biotech SL, Spain) and Spirnature (Agrinature Producciones Agrícolas SL, Spain), both based on Spirulina, have an NPK ratio of 1-7-3. As highlighted in previous sections, one of the advantages of microalgae is that this valuable resource can be produced in wastewater obtaining a dual role: (i) phycoremediation of wastewater and (ii) biomass rich in macro-and micro-nutrients essential for optimal crop growth and development – Figure 2.1.3.

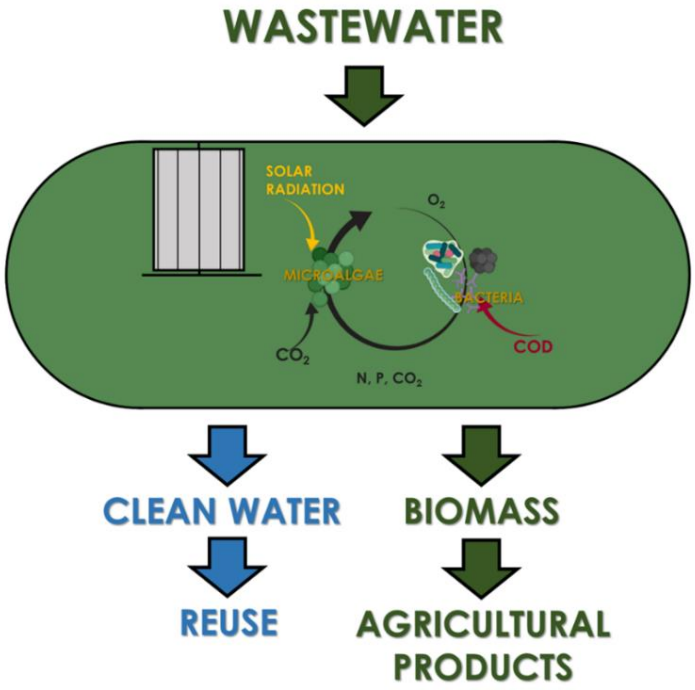


Figure 2.1.3. Dual role of microalgae-based wastewater treatment: Wastewater bioremediation and valuable biomass production.

All that glitters are not gold, and microalgae-based agricultural products have some drawbacks. For example, microalgae can accumulate heavy metals, and for this reason, an accurate chemical analysis should be performed to certify safe agricultural microalgae-derived products [47].Moreover, the effect of wastewater on the composition of the biomass, and therefore on the quality and bioactivity of the end product, has been generally overlooked. Wastewaters generally have a low content of phosphorus, and it is known that phosphorus limitation (generally) promotes lipid production and accumulation and substitution of phospholipids with glycolipids and/or betaine lipids [137]. Phosphorus limitation also led to an increased content of carotenoids, ascorbic acid, and tocopherols [138]. The exposure of microalgae to heavy metals that can be present in wastewater can also promote the synthesis of valuable compounds such as ascorbate peroxidase, catalase, superoxide dismutase, or ascorbate among other compounds [138].

Microalgae have also been suggested as biological biocides to manage pests and diseases [139]. Ongoing studies and developments in this field will certainly result in the appearance of commercial microalgae-derived biocides in the future.

Table 2.1.2. Effect of biostimulants on crop production. Table modified from (du Jardin, 2015) with permission from Elsevier.

	Humic acids	**Algal extracts**	**Protein hydrolysates**	**Glycine betaine**	**Plant growth-promoting Rhizobacteria**
Cellular mechanism (i.e. interaction with cellular components and processes)	Activate plasma membrane proton-pumping ATPases, promote cell wall loosening and cell elongation in roots	Stimulate expression of genes encoding transporters of micronutrients (i.e. Cu, Fe, Zn)	Stimulate phenylalanine ammonia-lyase enzyme and gene expression and production of flavonoids under stress	Protection of photosystem II against photo damage – likely via activation of scavengers of reactive oxygen	Release of auxins and activation of auxin-signalling pathways involved in root morphogenesis
Physiological function (i.e. action on whole plant processes)	Increase linear growth of roots and root biomass	Increased tissue concentrations and root to shoot transport of micronutrients	Protection against UV and oxidative damage	Maintenance of leaf photosynthetic activity under salt stress	Increased lateral root density and surface of root hairs
Agricultural function (i.e. output traits relevant for crop performance)	Increased root foraging capacity and enhanced nutrient use efficiency	Improved mineral composition of plant tissues	Increased crop tolerance to abiotic stress (i.e. salt)	Increased crop tolerance to abiotic stress (i.e. salt)	Increased root foraging capacity and enhanced nutrient use efficiency
Economic and environmental benefits (i.e. changes in yield, quality, ecosystem services)	Higher crop yields, saving of fertilisers and reduced losses to the environment	Enhanced nutritional value and biofortification of plant tissues	Higher crop yields	Higher crop yields	Higher crop yield, savings of fertilisers and reduced losses to the environment

4.2 Application of microalgae in soil

Several reports demonstrated the effectiveness of microalgae-based biofertilisers to improve crop yields. Indeed, rice cultivation inoculated with the microalgae *Chlorella vulgaris* or *Arthrospira platensis* led to 7-21% higher rice yields [140]. Results were consistent with those reported in a different study, where microalgae and cyanobacteria inoculation enhanced nutrient uptake and rice growth in China – microalgae inoculation also led to reduced arsenic translocation from roots to grains in arsenic-contaminated paddy soils [141]. The potential utilisation of dried biomass and extracts from *Acutodesmus dimorphus* as biofertiliser in Roma tomato plants was also investigated [22]. The authors of that study reported that *A. dimorphus*, applied 22 days before the seedling transplant, led to increased plant growth and a higher number of branches and flowers. Similar results were observed in a recent study where eighteen liquid extracts obtained from microalgae and cyanobacteria improved root and shoot length of tomato plants by 112 and 53%, respectively [142] – Figure 2.1.4. The authors of that study identified the production of a vast array of metabolites induced by microalgal extracts which led to the accumulation of palmitic acid and stearic acids in the plants as well as pyridine-3-carboxamide, an active form of vitamin B3. In a different study, the potential of *Chlorella vulgaris* or *Arthrospira platensis* alone or mixed with cow manure on growth parameters, biochemical composition, and nutritional properties of onions was evaluated [21]. Higher yields and quality were observed when manure was combined with *A. platensis* followed by manure combined with *C. vulgaris.* Both microalgae, when used alone, led to higher yields and quality than manure alone. Not only microalgal biomass but also microalgal co-products from other industrial processes can be used to improve plant yields. For example, Solé-Bundó et al., (2017) suggested that the digestate from microalgae anaerobic digestion and co-digestion with primary sludge could be a promising solution for both wastewater treatment and, because of its high organic matter and macronutrients, agriculture.

The utilisation of microalgae as a biofertiliser could promote not only yields but also quality. For example, comparable growths after cultivating tomatoes using microalgae-based and commercial organic fertilisers were reported [20]. When cultivated using microalgae-based fertilisers, fruit quality was improved by

increasing sugar and carotenoid content. In that study, microalgal biomass was produced using waste streams from aquaculture, demonstrating the previously mentioned dual wastewater treatment/biofertiliser production potential of microalgae.

Moreover, microalgae utilisation in agriculture can be beneficial not only to the plant or the fruit but also to the soil. Indeed, previous reports assessing the effect of microbial fertilisers from microalgae at four different concentrations (0.0-1.5 dose) on maize and wheat, concluded that when administered at a dose of 1.0, microalgae led to a higher amount of organic matter in soil and its water holding capacity was improved [24].

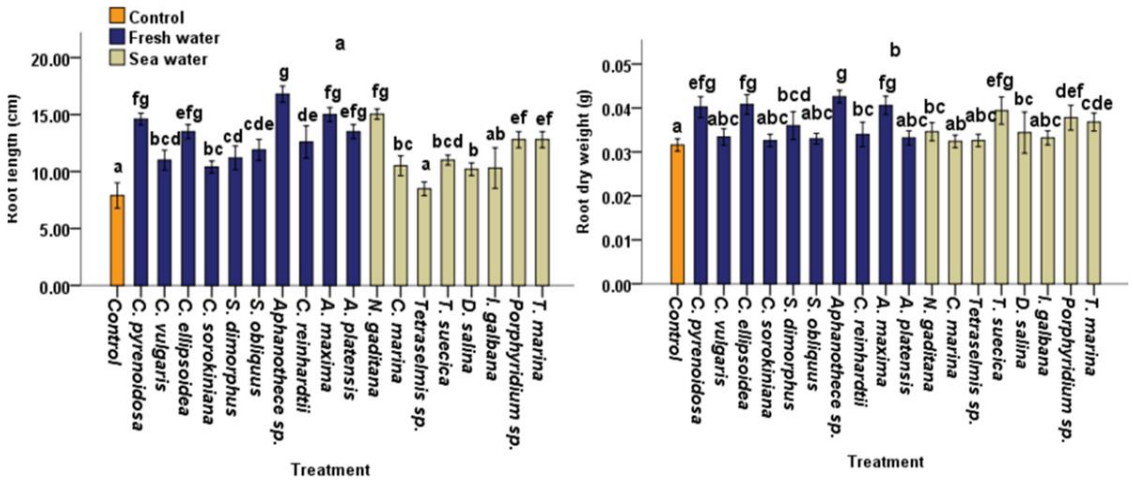


Figure 2.1.4. Effect of microalgal extracts on (A) root length and (B) root dry weight of 40 days old tomato plants. Data represent means ± standard errors of five biological replicates. Different letters indicate significant differences ($p<0.05$).

4.3 Foliar application

The number of studies evaluating the potential utilisation of microalgae and microalgae-derived compounds for foliar application is limited. This strategy is relatively novel and is one of the most innovative agricultural practices as it is environmentally safe and promotes agricultural sustainability [47]. Microalgae-derived extracts, even at low concentrations, can induce an array of physiological plant responses. Foliar application of *A. dimorphus* at a concentration of 3.75 $g \cdot mL^{-1}$ led to increased plant height and a higher number of flowers and branches per plant in tomato plants [22]. Moreover, the application of extracts of *C. vulgaris* at

different concentrations to green gram (*Vigna mungo* L.) led not only to higher yields but also improved soil physical and chemical parameters [144]. The foliar application was performed ten days before blooming after berry sitting and 21 days later. Similar results were obtained recently by the same research group on black gram (*Vigna mungo* L.) [145].

Some microalgal strains such as *A. platensis* and *A. maxima* (*Spirulina*) are naturally rich in protein [146]. These strains contain high contents of amino acids which are well-known biostimulants. Protein hydrolysates are among the active ingredients of plant biostimulants [147,148]. In this sense, the effect of foliar spraying with enzymatic hydrolysates of *Scenedesmus* sp. and *A. platensis* (10 $g \cdot L^{-1}$) on *Petunia* × *hybrida* plant development and leaf nutrient status was studied [149]. The authors reported that the application of *Scenedesmus* sp. five times (days 0, 14, 25, 35, and 42 after transplanting) at a concentration of 10 $g \cdot L^{-1}$ accelerated plant development and fastened flowering. In turn, *A. platensis* at the same studied dose and application dates led to enhanced root dry matter, water content, and the number of flowers per plant [149]. Foliar applications of algal extracts seem to be more effective if applied in the morning, when the leaf stomata are open [150], although further studies are needed to confirm this hypothesis. In a different study, foliar application of enzymatic hydrolysates of *A. platensis* promoted the growth of seedlings in organically grown lettuce – hydrolysates obtained after 4 h of hydrolysis were the most active promoting growth and increasing spermine content [151]. Results were in line with those reported in a different study, where seeds treated with *A. dimorphus* culture and with the *A. dimorphus* extracts at concentrations higher than 0.75 $g \cdot mL^{-1}$ accelerated seed germination by two days [22].

Results reported so far suggest that microalgae are excellent biofertilisers and/or biostimulants with numerous reports demonstrating their potential to improve plant growth, fruit yields, and/or the number of flowers per plant, among other benefits. The use of microalgae and microalgal extracts in agriculture is a reality and the number of novel products launched into the market is increasing every year. Overall, the most commonly utilised microalgae are *Spirulina* and *Chlorella*. However, there are thousands of microalgae strains, with varied biochemical compositions, currently available in culture collections around the world. This huge

variability suggests a bright future for microalgae-derived bioproducts in agriculture, although further research is needed to identify the most effective microalgae and extract for a given application.

Conclusions

One of the main advantages of using microalgae-bacteria consortia in wastewater treatment processes is their dual role: they not only remove nutrients and contaminants but also produce valuable biomass, which can be used for a wide variety of applications. The most common used photobioreactor designs for wastewater treatment are raceways. Not only because of their lower construction and operation costs but also because their low surface-to-volume ratio allows them to process large amounts of wastewater per surface area. So far, most of the published studies were conducted at a laboratory scale. Further studies using large (over 100 m^2) reactors are needed to better predict the potential of microalgae for the treatment of wastewater. The most common strains studied to date are *Scenedesmus* and *Chlorella*. Microalgae-derived agricultural products showed promising results and potential for being used as a sustainable and environmentally friendly strategy. Indeed, there are several commercial microalgae-based products currently available. Their production could be coupled with wastewater treatment. Moreover, biochemical fractionation of microalgal biomass and extracts derived thereof and agronomic tests of their purified compounds are needed as a useful step for in-depth study of the action mechanisms of microalgae. Overall, the benefits of a microalgal biorefinery approach to treat wastewater and produce valuable products for agriculture including biofertilisers and biostimulants go well beyond environmental health, with implications on human health, energy, and food safety, and mitigation of climate change.

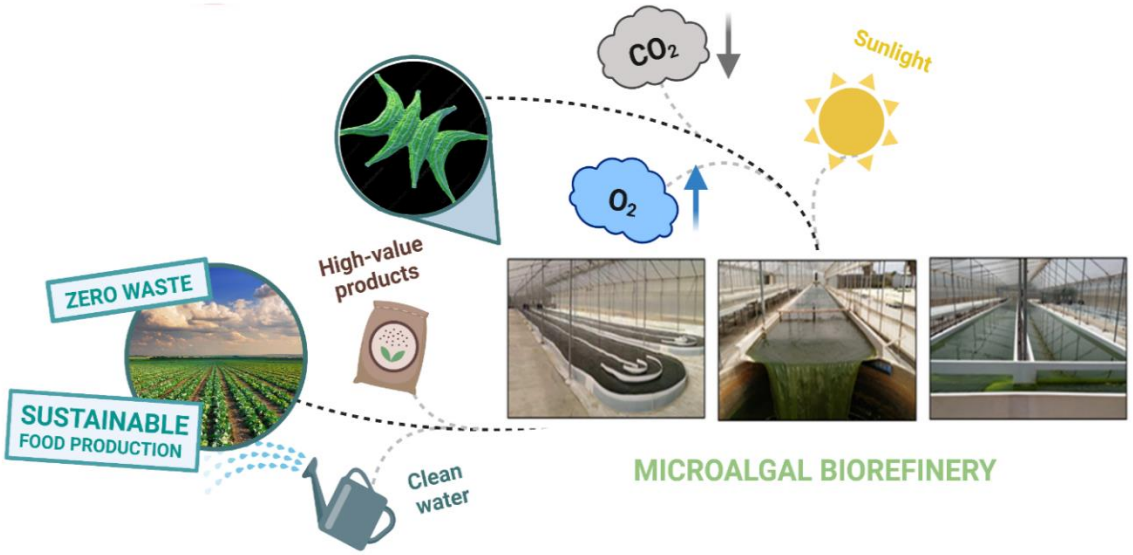
Acknowledgements

T. Lafarga thanks the Spanish Ministry of Science, Innovation, and Universities (IJC2018-035287-I). This manuscript was supported by the SABANA project of the European Unions' Horizon 2020 Research and Innovation Programme (Grant Agreement #727874) and the AL4BIO Project, funded by the Spanish Ministry of Science, Innovation, and Universities (RTI2018-099495-A-C22).

CRediT author statement

A. Morillas-España: Writing-Original Draft; **T. Lafarga:** Writing – Original Draft, Conceptualisation, Supervision; **A. Sánchez-Zurano:** Writing-Original Draft; **F.G. Acién-Fernández:** Supervision, Funding Acquisition; **C.V. González-López:** Supervision, Funding Acquisition.

2.2. Microalgal biomass productivity in open systems



2.2.1. Year-long production of *Scenedesmus almeriensis* in pilot-scale raceway and thin-layer cascade photobioreactors

Title: Year-long production of *Scenedesmus almeriensis* in pilot-scale raceway and thin-layer cascade photobioreactors

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Journal: Algal research

Year: 2020

Volume: 60

DOI: https://doi.org/10.1016/j.algal.2021.102516

IF (JCR 2020): 4.401

Categories: BIOTECHNOLOGY & APPLIED MICROBIOLOGY, 46/159, **Q2**

Abstract

Biomass of *Scenedesmus almeriensis* was produced outdoors for 12 months using three different photobioreactor designs. Optimum dilution rates to achieve the highest biomass productivities were 0.2 day^{-1} for raceways and 0.3 day^{-1} for thin-layer reactors. Biomass productivities achieved using thin-layer cascade photobioreactors during the months of higher photosynthetic activity reached 30-35 $g/m^2 \cdot day$, higher than those obtained using raceways during the same period: 20-25 $g/m^2 \cdot day$. Photosynthetic efficiency was lower in spring/summer when compared to autumn/winter, suggesting that a larger share of the solar energy that reaches the culture in spring/summer is not used for microalgal growth. During summer, culture temperature reached 40 °C in thin-layer photobioreactors, which demonstrates the importance of selecting microalgal strains able to resist these conditions. Photoinhibition was not observed at incident irradiances up to 1600 $\mu E/m^2 \cdot s$. However, dissolved oxygen values were especially high in thin-layer photobioreactors during this time of the year. They reached maximum values of 400%Sat. and showed an inhibitory effect on microalgal growth.

Keywords: microalgae, biomass, bioreactor, photosynthesis, photosynthetic efficiency.

1. Introduction

Microalgae are unicellular photosynthetic microorganisms that are naturally present in both saline and freshwater environments. These microorganisms are gaining increased importance in the context of the European bioeconomy because of their potential to produce a wide variety of valuable biomolecules with diverse applications in agriculture, aquaculture, and food production, among others [152]. Despite the thousands of microalgal strains currently available in culture collections worldwide, only a limited number of species have been studied in detail, and only a few of these have achieved commercial success [125]. These include *Arthrospira platensis* and *Chlorella vulgaris*, mainly used as food [153,154], and *Dunialiella salina* and *Haematococcus pluvialis*, which are being mass-cultured for the production of β-carotene and astaxanthin respectively [125].

Key aspects that need to be considered to achieve commercial success include (i) selection of a robust and highly productive strain, capable to grow under a wide

range of environmental conditions; (ii) selection of a photobioreactor capable of providing the optimal conditions required by the selected strain; and (iii) the possibility to adjust the production system because of the (inevitably) changing environmental outdoor conditions, both on an hourly and a seasonal basis [155]. Several species of the *Scenedesmus* genus have a certain potential for industrial use. For example, *S. almeriensis* is a fast-growing and highly productive strain that is particularly adapted to stressful conditions. The biomass of *S. almeriensis* has been suggested as a potential source of high-value biomolecules such as lutein [156] and zeaxanthin [157]. Moreover, this microalga also showed potential for being used as fishmeal in aquaculture [158], in wastewater treatment processes [159], in agriculture as a source of biofertilisers and/or biostimulants [149], and because of its high lipid productivity – 144 mg/L·day – as a promising feedstock for biodiesel production [160].

Microalgal biomass productivity of a given production system depends on both the design of the photobioreactor and environmental conditions - and how these conditions accomplish what is needed for the microalga to be cultured. Several reports assessed the biomass productivity of *S. almeriensis* using different reactor designs at different scales. At the lab-scale level, a surface response analysis predicted maximum productivity of 0.70 g/L·day, at 33 ºC and 1700 μE/m^2·s – confirmed *in vitro* as 0.73 g/L·day – when using bubble column photobioreactors with a capacity of 2 L [161]. In a different study, the maximum biomass productivity achieved, using a 2 L bubble column reactor, was 0.87 g/L·day and simulations performed *in silico* predicted that productivity of 0.95 g/L·day could be achieved under outdoor conditions [162]. At a higher-scale level, studied photobioreactors include raceways and thin-layer systems. Thin-layer reactors are characterised by their low-depth culture (0.5-5.0 cm), recirculated over a flat surface by providing an adequate slope of 0.1-2.0%. They are one of the reactors with the highest areal productivity, with values ranging between 30 and 50 g/m^2·day [155,163]. In a study conducted in Almeria (Spain), it was observed that the productivity of *Scenedesmus* sp. in a thin-layer photobioreactor could reach 42 g/m^2·day while using a raceway (both reactors were of 32 m^2) the maximum productivity was barely half this value: 24 g/m^2·day [164].

Despite the high biomass productivities reported using thin-layer reactors, their use is still limited to laboratory or pilot scales - up to 50 m^2 [165]. The amount of available information about this type of reactor is very low when compared to that of raceways or tubular photobioreactors. Previous reports suggested that thin-layer reactors present some drawbacks such as pH and temperature gradients as well as inadequate mass transfer capacity and accumulation of oxygen in the culture [155]. However, more information on the characterisation of these reactors as well as their productivity is needed before scaling up this technology to an industrial level. Thus, the current work aims to evaluate the performance in terms of productivity and efficiency of pilot-scale thin layer photobioreactors (two different designs) compared to a raceway for producing *S. almeriensis* for a year.

2. Materials and methods

2.1 *Scenedesmus almeriensis* and culture conditions

The selected strain was *S. almeriensis*, which is a fast-growing and highly productive strain that is particularly adapted to stressful conditions. It was isolated for the first time within a photobioreactor in a greenhouse exposed to high temperature (45 ºC) and irradiance (2000 μE/m^2·s) conditions [161]. *S. almeriensis* can grow well at pH, temperature, and salinity values ranging between 7-10, 26-40 ºC, and 0-5 g NaCl/l, respectively, and shows no signs of photoinhibition up to 1625 μE/m^2·s [162]. The strain was obtained from the culture collection of the Department of Chemical Engineering of the University of Almería. Inocula of *S. almeriensis* were maintained at 23 ± 2 °C, pH 8.0 ± 0.1, and 150 μE/m^2·s in batch mode until a concentration of 1 g/L and using a modified Arnon medium as described previously [164]. Once the desired concentration was achieved, the inocula were scaled-up to a final volume of 80 L using pH-controlled outdoor bubble column photobioreactors placed inside a greenhouse.

Pilot-scale production of the strain was conducted at the pilot plant facilities of the University of Almería located at IFAPA (Almería, Spain) using a culture medium that consisted of 0.90 g/L $NaNO_3$, 0.18 g/L $MgSO_4$, 0.14 g/L K_2PO_4, and 0.03 g/L of karentol® (Kenogard, Spain), which is a commercial solid mixture of micronutrients that include boron, copper, iron, manganese, molybdenum, and zinc. Chemicals used for pilot-scale production were agricultural fertilisers.

2.2 Photobioreactors and experimental conditions

Three different photobioreactor designs placed inside a greenhouse were used for the production of *S. almeriensis*: Bioreactor A, a raceway with an operating volume of 11,800 L and a land surface of 80 m^2; Bioreactor B, a single-channel thin-layer cascade photobioreactor with a total volume of 2,400 L and a land surface of 63 m^2; and Bioreactor C, a double-channel thin-layer cascade photobioreactor with a total volume 3,600 L and a land surface of 126 m^2 (Figure 2.2.1.1). Both thin layers were provided with a degasser to improve mass transfer: a 250 L bubble column with continuous injection of air (75 L/min). pH and DO probes were installed at the end of the channel (where OD values are the highest) and before the degasser. Each test was conducted in semi-continuous mode at different dilution rates during the four seasons of the year, which show different ranges of temperature and incident irradiance. The pH was controlled by the on-demand injection of CO_2. The three reactors were operated 24 h per day.

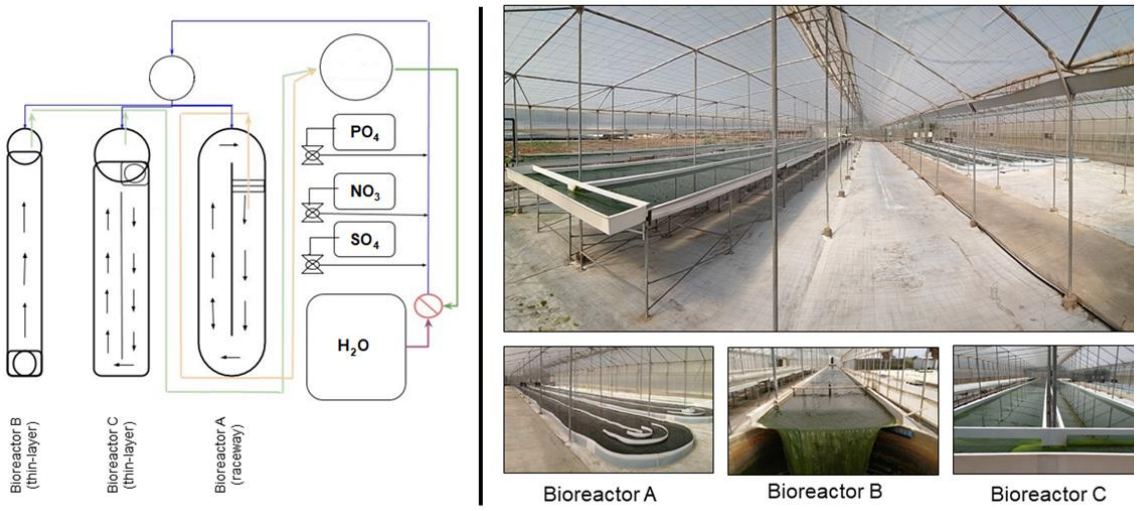

Figure 2.2.1.1.Photobioreactors design characteristics.

2.3 Culture analysis and light availability

Biomass concentration (C_b) was measured by dry weight filtering 100 mL of culture through 1 µm filters and drying at 80 °C in an oven for 24 h. Biomass productivity (P_b) was calculated as the product of biomass concentration by the dilution rate, which varied from 0.1 to 0.3 in Bioreactor A and from 0.2 to 0.4 in Bioreactors B and C. Cell status was checked daily by measuring the chlorophyll fluorescence ratio (*Fv/Fm*) with an AquaPen AP 100 fluorometer (Photon System Instruments,

Czech Republic). Absorbance at 400-700 nm was daily measured using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain) and the extinction coefficient (k_a) was calculated using the equation:

$$k_a = \frac{Abs}{C_b \cdot p}$$

where *Abs* and C_b are the above-mentioned absorbance and dry weight biomass concentration and *p* is the cuvettes' light path (1 cm).

Average irradiance inside the culture (I_{av}) was calculated as a function of the irradiance at the surface of the culture (I_0), k_a, C_b, and the light path inside the reactor (*p*) using the equation:

$$I_{av} = \frac{I_0}{k_a \cdot C_b \cdot p} \cdot (1 - e^{-k_a \cdot c_b \cdot p})$$

The percentage of photosynthetic efficiency (Ψ) was calculated as a function of *D*, k_a, and I_{av} using the equation:

$$\Psi = \frac{\Psi_b' \cdot H_b}{\lambda_{550}} \cdot 100$$

where H_b is the enthalpy of the biomass (20.6 kJ/g), λ_{550} is the energy of 1 mol of photons at 550 nm (217.5 kJ/E), and Ψ_b' is the photosynthetic efficiency (g/E) calculated as:

$$\Psi_b' = \frac{D}{k_a \cdot I_{av}}$$

where D, k_a, and I_{av} were the dilution rate, the extinction coefficient, and the average irradiance inside the culture described above.

2.4 Statistical analysis

The results shown are the mean values of three independent experiments ± standard deviation (SD). Differences between photobioreactor designs and culture conditions were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., US). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance

was in all cases $p<0.05$. To identify relationships between different variables, bivariate Pearson's' correlation analysis was carried out.

3. Results and discussion

Several aspects need to be considered to successfully up-scale the production of microalgae. These include, but are not limited to, the selection of a robust and highly productive strain and the selection of a suitable bioreactor that is capable of both, providing the optimum growth conditions to the selected microalgal strain and that can be adjusted to the changing environmental outdoor conditions [155]. As highlighted in the introduction, several species of the *Scenecesmus* genus have the potential for industrial use. In the current paper, the selected strain was *S. almeriensis*, which was isolated for the first time and is naturally present in Almería (Spain), and is therefore adapted to the unique weather conditions of the region where the bioreactors are located. Moreover, the current paper assessed the potential of using three bioreactor designs: a raceway reactor, a single-channel thin-layer photobioreactor, and a double-channel thin-layer photobioreactor.

3.1 Operating conditions: Optimum dilution rate for increased productivity

The first goal of this study was to identify the optimum dilution rate for each reactor design and season. This operating variable strongly influences biomass productivity. Studied dilution rates were 0.1-0.3 day^{-1} for Reactor A and 0.2-0.4 day^{-1} for Reactor B and Reactor C. This selection was based on previous experience of our research group. Higher dilution rates were studied for thin-layer cascade reactors because of their higher biomass productivity [163]. Overall, biomass concentration and productivity were significantly affected by reactor design ($p<0.0001$), season ($p<0.0001$), and dilution rate ($p<0.05$).

Because of the weather conditions of the region (mild winter and warm spring), the productivities observed during spring/summer and autumn/winter were comparable in all three reactor designs.

When operating using the Bioreactor A, maximum biomass concentrations and productivities ranged between 0.3-0.9 g/L and 7-20 $g/m^2 \cdot day$ depending on the season (solar irradiance and temperature) and the dilution rate used (Figure 2.2.1.2). Results were similar to those reported during the production of

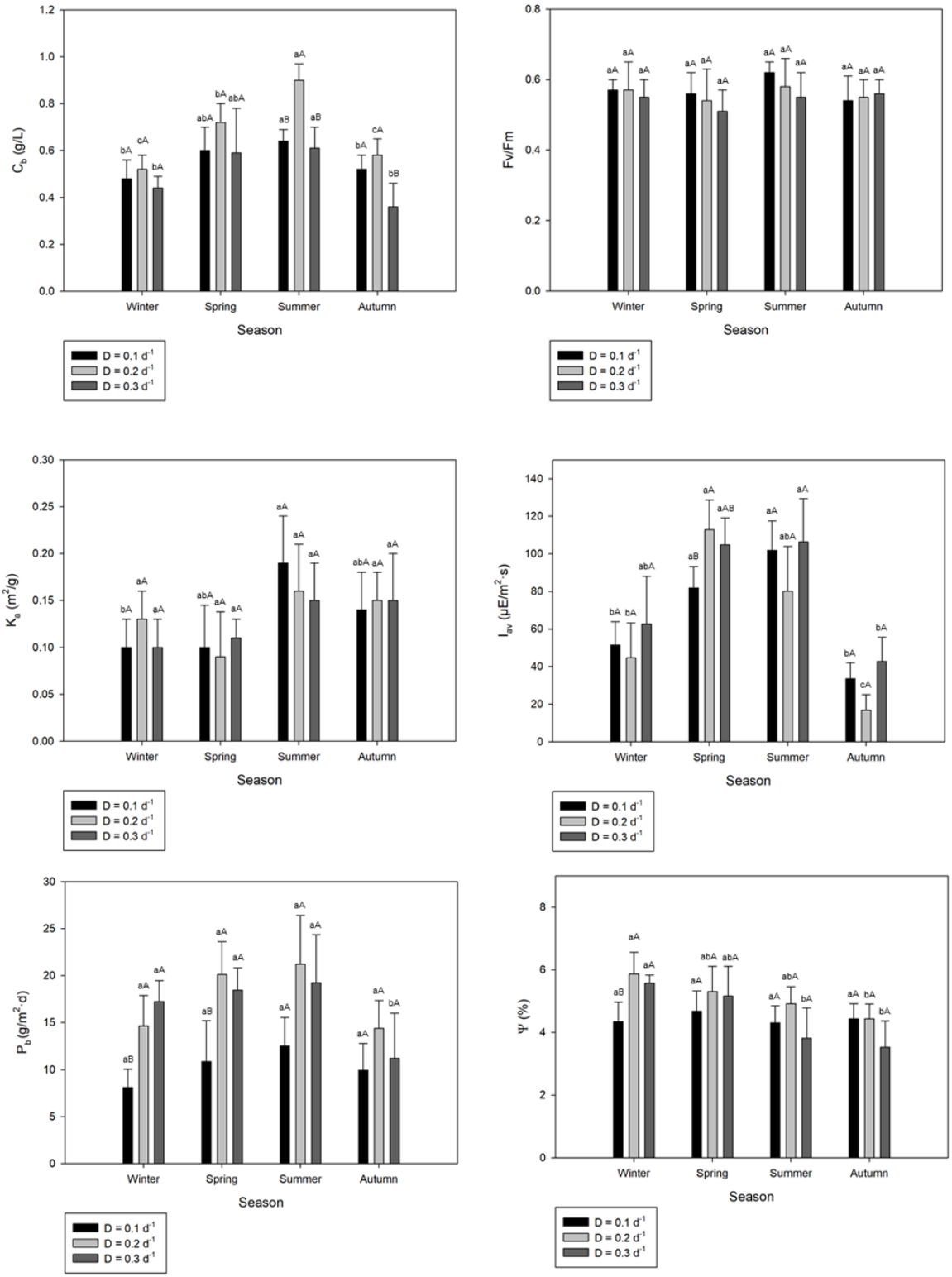

Figure 2.2.1.2. Bioreactor A (raceway): Effect of dilution rate and season on the S. almeriensis culture.

Scenedesmus spp. in raceway reactors, where productivities within the ranges of 10-15 and 20-25 g/m^2·day were achieved in winter or spring, respectively [166]. Results were slightly lower than those reported in a previous study using raceway

reactors to produce *Scenedesmus* sp. where the authors achieved biomass productivity of 24 g/m^2·day [164].

These differences can be attributed to a different reactor capacity and design, differences in the composition of culture media, and environmental factors. Overall, higher biomass productivities were obtained when operating using a dilution rate of 0.2 day^{-1}. Higher biomass concentrations were also observed under this condition, especially in summer, probably due to a higher light availability when compared to other seasons and higher nutrient availability when compared to a dilution rate of 0.1 day^{-1} (p<0.05).

Results were in line with those reported previously for raceway reactors in the south of Spain [164]. No major differences were observed in photosynthetic efficiency when operating under different dilution rates - although higher efficiencies were observed when using a dilution rate of 0.2 day^{-1}, these were not statistically significant. Photosynthetic efficiency values ranged between 4 and 6%, higher than that of previous reports with photosynthetic efficiencies of 2% in *Scenedesmus* cultures in raceway reactors [167].

Similar results were observed when producing *S. almeriensis* using Bioreactor B – Figure 2.2.1.3. Again, higher concentrations were achieved when operating at a dilution rate of 0.2 day^{-1}– the lowest studied (p<0.05). However, biomass productivity was higher in all four seasons when operating at a dilution rate of 0.3 day^{-1}. It is difficult to compare microalgae productivities reported in the literature because of differences in environmental and operating conditions, as well as algal strains used. In the current paper, all of these parameters were equivalent.

The productivity of Bioreactor B was higher when compared to that of Bioreactor A and ranged between 15 and 30 g/m^2·day depending on the season (solar irradiance and temperature) and the dilution rate used. These compare well with previous studies that demonstrated the higher productivity of thin-layer reactors when compared to the traditional raceways [164].

Recently, the biomass productivity of a thin-layer reactor using urban primary wastewater as the nutrient source ranged between 25 and 50 g/m^2·day [168]. The higher productivity of these reactors has been attributed to a higher ratio of

exposed surface to total volume and higher turbulence, allowing rapid light/dark cycles [169].

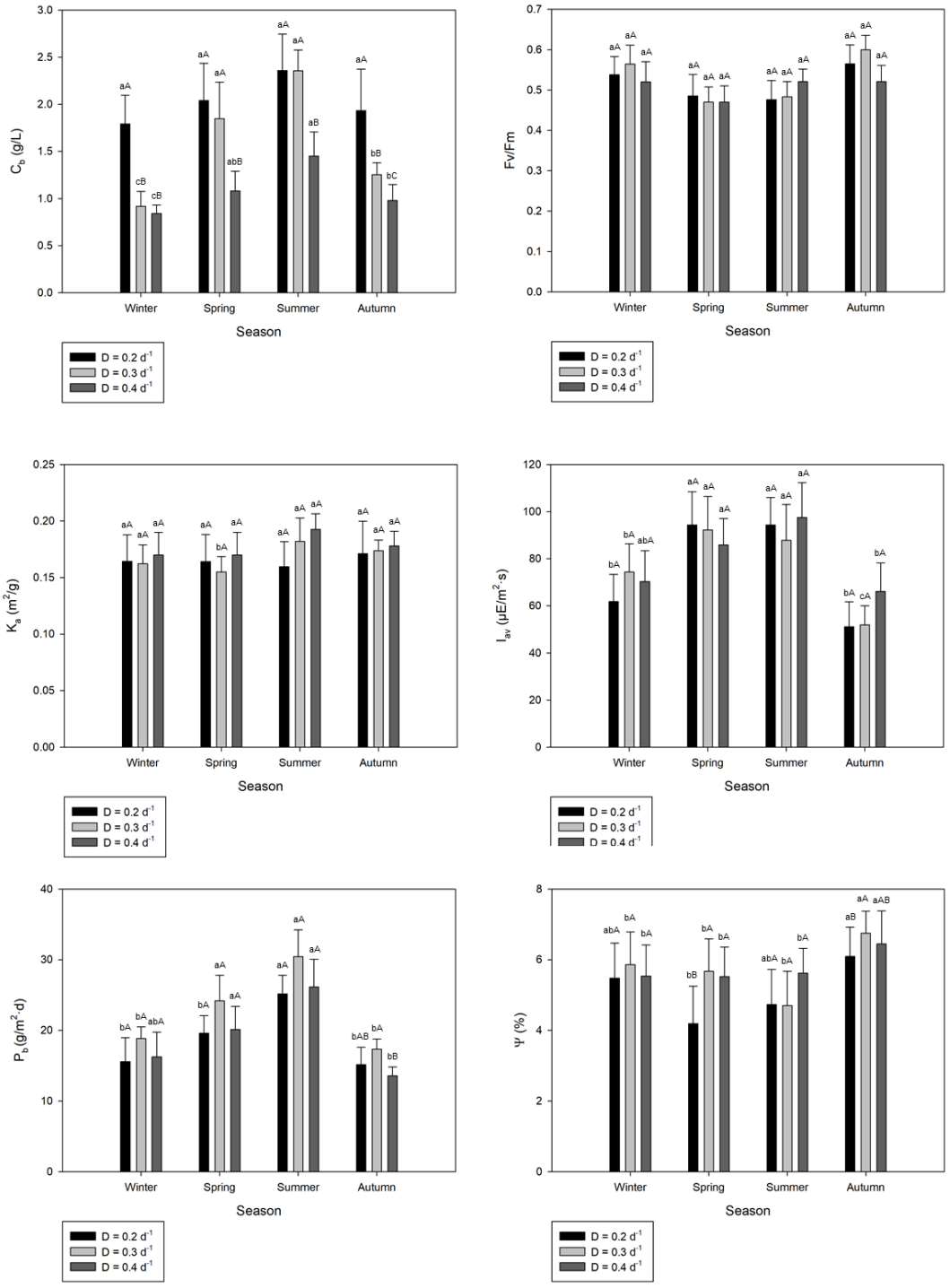

Figure 2.2.1.3. Bioreactor B (single-channel thin layer cascade): Effect of dilution rate and season on the S. almeriensis culture.

The lower culture depth allows culture temperature to decrease more rapidly in thin-layer reactors and this has also been suggested as a positive effect as it minimises biomass loss during the night due to respiration [170]. Bioreactor B also led to higher photosynthetic efficiency when compared to Bioreactor A. In this case, the photosynthetic efficiency ranged between 3 and 7% depending on the season and the dilution rate used. Results compared well with previous reports using thin-layer reactors in the Czech Republic [171].

Overall, higher efficiencies were obtained during autumn/winter when compared to spring/summer, caused by the higher biomass concentrations during the latter that darken the culture and decrease light availability and also by the extreme solar radiation during spring/summer in the region, which is not all harnessed by the microorganism.

Bioreactor C led to the higher photosynthetic efficiency, which ranged between 4 and 8% depending on the season in which the microalga was produced and the dilution rate used. Photosynthetic efficiency depends largely on solar irradiance. Although it is relevant for microalgal production and the design of photobioreactors, as the main goal is to produce large quantities of biomass, it is more valuable to optimise biomass productivity rather than photosynthetic efficiency. When operating using Bioreactor C, productivities were lower than those expected and ranged between 10 and 20 $g/m^2 \cdot day$. In this case, no differences were observed when operating at a dilution rate of 0.3 or 0.4 day^{-1}, which were higher than those obtained at 0.2 day^{-1} ($p<0.05$; Figure 2.2.1.4). Previous studies also suggested the optimum dilution rate for thin-layer reactors in the south of Spain to be 0.3 day^{-1} [164].

Although Bioreactor C is also a thin-layer cascade, productivities obtained in spring and summer were lower when compared to those obtained when using Bioreactor B ($p<0.05$; Figure 2.2.1.3). These lower productivities were caused by the high productivity of thin-layer reactors and inefficient removal of dissolved oxygen in this reactor, which will be discussed in the next section. All three bioreactors were located inside the same greenhouse and therefore had comparable environmental conditions. The average irradiance inside the culture ranged between 20 and 60

μE/m^2·s in the raceway (Bioreactor A) and between 50 and 100 μE/m^2·s in both thin-layer designs.

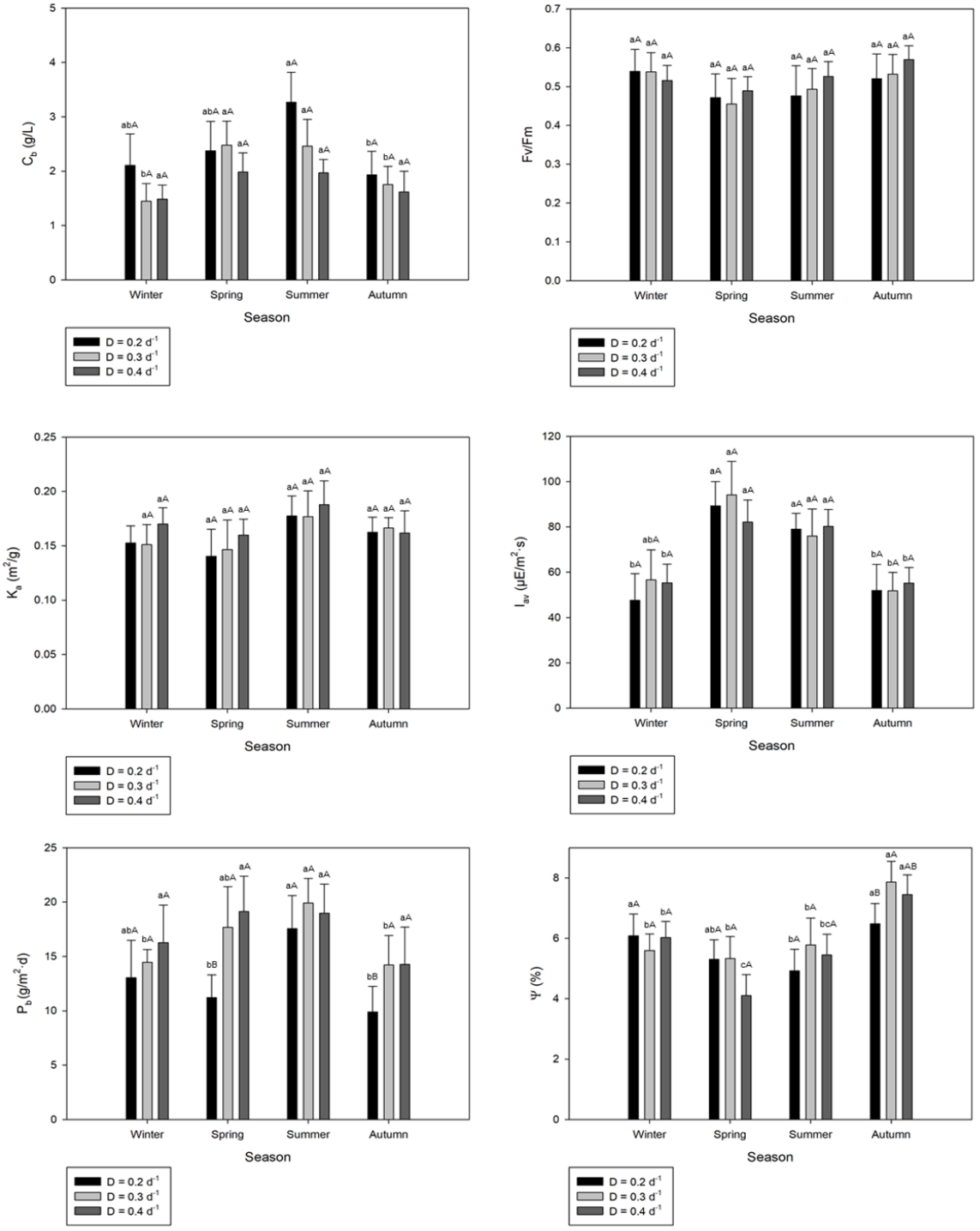

Figure 2.2.1.4. Bioreactor C (double-channel thin layer cascade): Effect of dilution rate and season on the S. almeriensis culture.

The effect of solar irradiance on productivity and efficiency will be discussed in the next section. In the current study, no differences were observed in *Fv/Fm* and k_a

values between seasons and dilution rates, suggesting the robustness of the productive process and the stability of the biological system under the studied conditions in all three bioreactors. Previous studies obtained reduced *Fv/Fm* values during summer because of photoinhibition caused by (reversible) damage of key PSII components in indoor and outdoor cultures [172].However, as discussed above, *S. almeriensis* is highly resistant to harsh environmental conditions, especially in terms of solar irradiance [162] and although a slight decrease in *Fv/Fm* values was observed during spring and summer, this was not statistically relevant and demonstrate the high tolerance of *Scenedesmus* species to seasonal temperature and radiation oscillations.

3.2 Influence of environmental and associated parameters on productivity and efficiency

Once the optimum operation conditions were selected, in this case, a dilution rate of 0.2 day^{-1} for Bioreactor A (raceway) and 0.3 day^{-1} for Bioreactor B and Bioreactor C (thin-layer), the current section will discuss the effect of different parameters on biomass productivity and photosynthetic efficiency. Figure 2.2.1.5 shows the effect of environmental and culture temperature, solar radiation, oxygen saturation, and average irradiance inside the culture on both biomass productivity and photosynthetic efficiency for Bioreactor A. Both biomass productivity and photosynthetic efficiency were influenced by the month of production (p<0.001) and therefore influenced by solar irradiance (p<0.001) and temperature (p<0.001). Spring and summer were the most productive seasons as biomass productivity was positively correlated with average irradiation (R^2=0.830; p<0.05) and the average culture media temperature (R^2=0.849; p<0.05). Although irradiance constitutes the major growth limitation, temperature also affects the biomass output of microalgae production [171].

These two parameters are linked, as higher temperatures are achieved during those months of higher solar irradiance. Indeed, in the current study, a positive correlation was also observed between maximum irradiance and average (R^2=0.853; p<0.05) and maximum (R^2=0.913; p<0.05) temperature of the culture medium. During these months, the maximum daily values of solar radiation were between 1500 and 1600 $\mu E/m^2 \cdot s$ and the maximum temperature reached in the

culture media was in the range of 30-35 °C, which was within the same range of temperature reached inside the greenhouse (no overheating of the culture was observed). These conditions are suitable for the production of *S. almeriensis*¸ which can grow under temperatures within the range of 26-40 ºC and showed no signs of photoinhibition at 1625 μE/m^2·s previously [162].

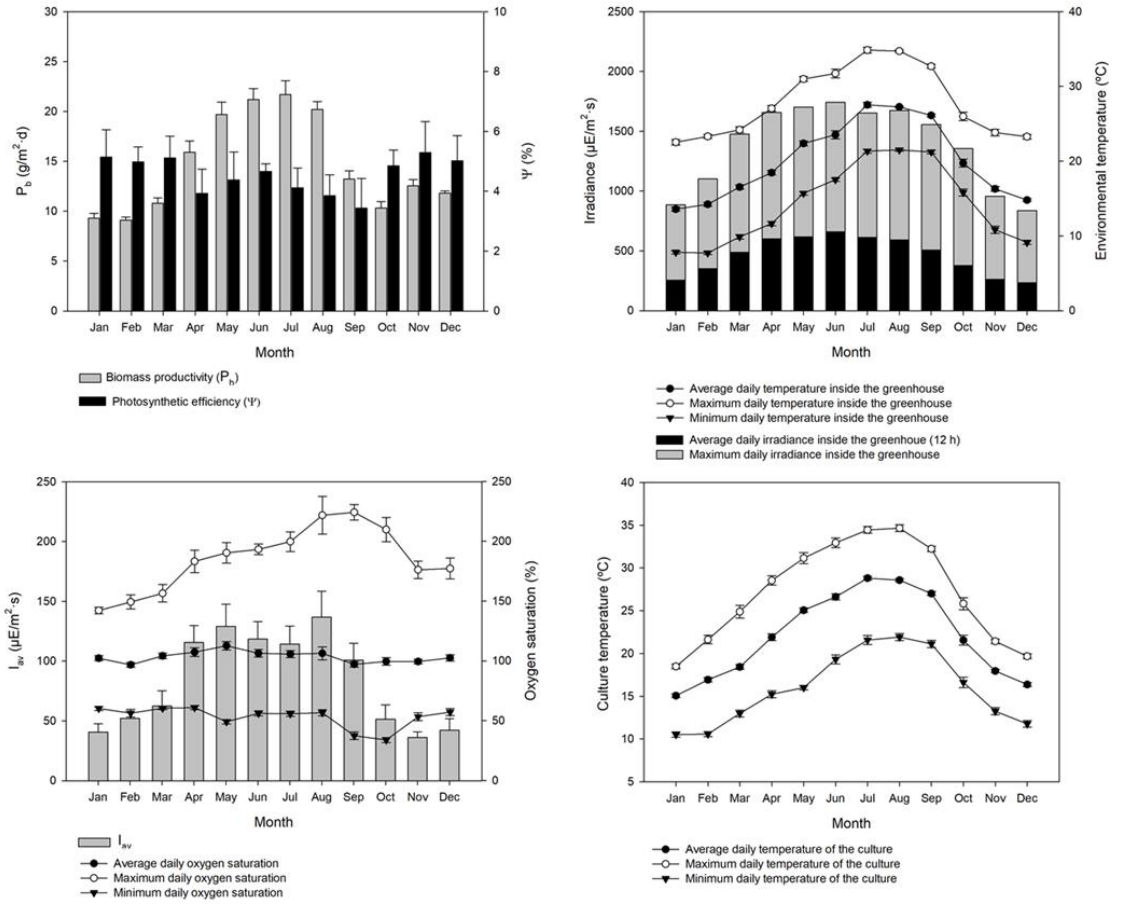

Figure 2.2.1.5. Bioreactor A (Raceway): Effect of solar irradiance, dissolved oxygen concentration, and temperature inside the greenhouse and of the culture on biomass productivity.

The average oxygen saturation values for Bioreactor A were stable throughout the whole year around 100%Sat. and showed daily peaks of nearly 200%Sat. during summer at mid-day. Moreover, photosynthetic efficiency was negatively correlated with I_{av} (R^2=0.803; p<0.05) and the average temperature of the culture (R^2=0.805; p<0.05). These results demonstrate that photosynthetic efficiency in outdoor cultures is not as important as other parameters as higher productivities were associated with lower efficiencies. The productivity and the photosynthetic efficiency of Bioreactor B were also affected by month (p<0.001), solar irradiance (p<0.001), and temperature (p<0.001) – Figure 2.2.1.6.

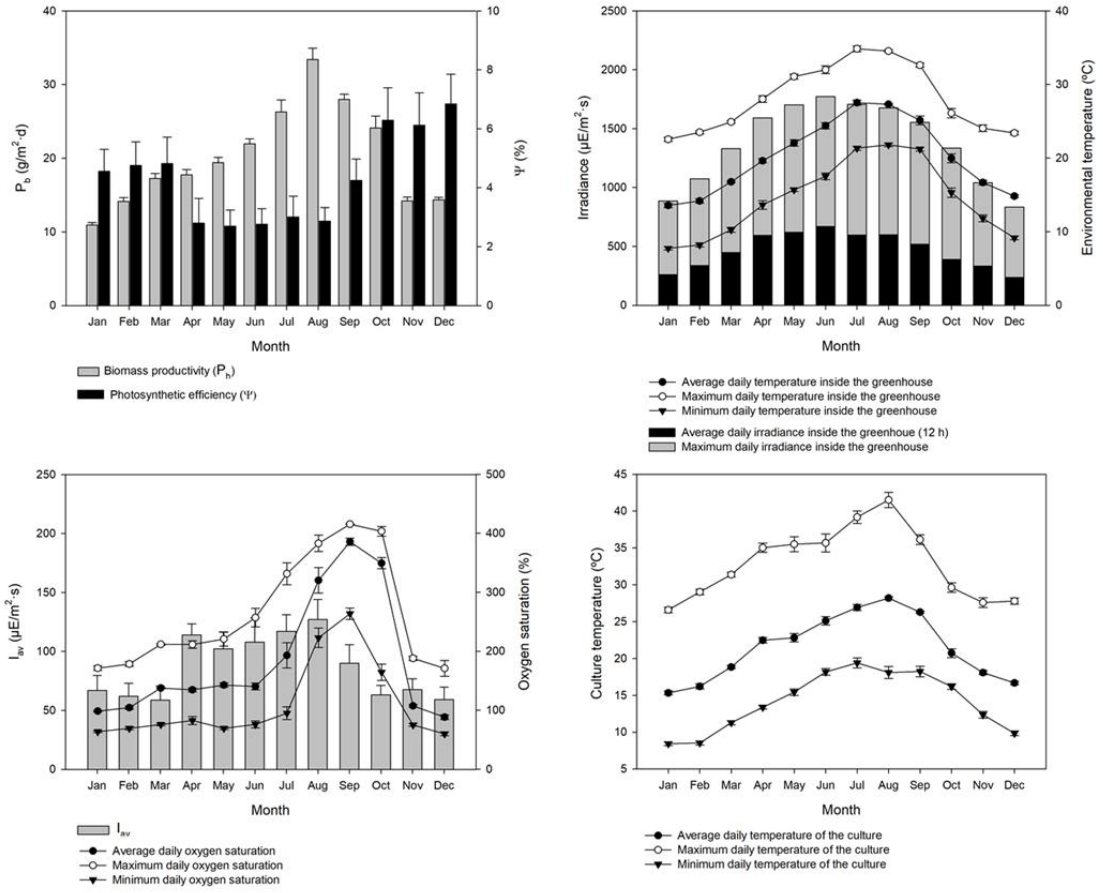

Figure 2.2.1.6. Bioreactor B (single-channel thin layer cascade): Effect of solar irradiance, dissolved oxygen concentration, and temperature inside the greenhouse and of the culture on biomass productivity.

Productivity of Bioreactor B was higher when compared to Bioreactor A in all the studied months (p<0.05), except for June, and was especially higher in summer July-September. As it happened in Bioreactor A, a positive correlation was observed between maximum irradiance and average (R^2=0.895; p<0.05) and maximum temperature (R^2=0.916; p<0.05) of the culture in Bioreactor B. However, in this case, because of the lower depth of the culture in this reactor (0.02 m against 0.13 m in Bioreactor A on average), the maximum temperature reached in the culture was extremely high during summer, surpassing peaks of 40 °C in August. Because of the high resistance of the selected strain to temperature, these peaks did not affect productivity as July, August, and September were the most productive months (p<0.05) – with an average culture temperature of 25-30 °C. Photosynthetic efficiency of Bioreactor B was negatively correlated with I_{av} (R^2=0.871; p<0.05) and the average temperature of the culture (R^2=0.867; p<0.05).

Higher efficiencies were achieved in the months where maximum and average solar irradiation were lower (Figure 2.2.1.6). In the current study, a major difference between thin-layer and raceway reactors was the extremely high oxygen saturation values measured in the former. Maximum dissolved oxygen concentration during the months of lower photosynthetic activity was within 200 and 250% saturation. However, maximum peaks of 400% were measured in summer (average daily values were over 350%), which probably limited growth in August-October (Figure 2.2.1.6). Although it is not commonly addressed in lab-scale cultures, dissolved oxygen accumulation in the culture has long been considered one of the key factors limiting the mass cultivation of microalgae, especially in highly productive and closed photobioreactors [173].

It has been suggested that high concentrations of dissolved oxygen may cause damage to the photosynthetic apparatus, membrane, and/or components of microalgal cells and therefore it has been associated with decreased cell growth rate and lower productivities [174,175]. Concentrations higher than 1.1 mM (480% saturation at 20 °C) can even become toxic to most microalgae [176]. In our pilot plant facilities, a degasser, which consists of a continuous injection of air (75 L/min) in a 250 L bubble column is used to reduce the dissolved oxygen concentration in the culture. However, it was not enough to achieve more efficient values in highly productive months. Recently, the use of substances such as perfluorocarbon nanoemulsions as oxygen scavengers has been suggested [176]. Future studies regarding Bioreactor B will include the optimisation of oxygen removal, which could probably promote biomass production, especially during the months of higher photosynthetic activity.

Photosynthetic efficiency of Bioreactor C was negatively correlated with maximum irradiance (R^2=0.730; p<0.05) and the average temperature of the culture medium (R^2=0.706; p<0.05) demonstrating that a large amount of the solar energy that reaches the culture in spring/summer was not used for microalgal growth. Photosynthetic efficiency of both thin-layer cascade reactors was higher than that of the raceway, highlighting the importance of the surface to volume ratio to achieve high photosynthetic efficiencies, as reported by other authors previously [177].

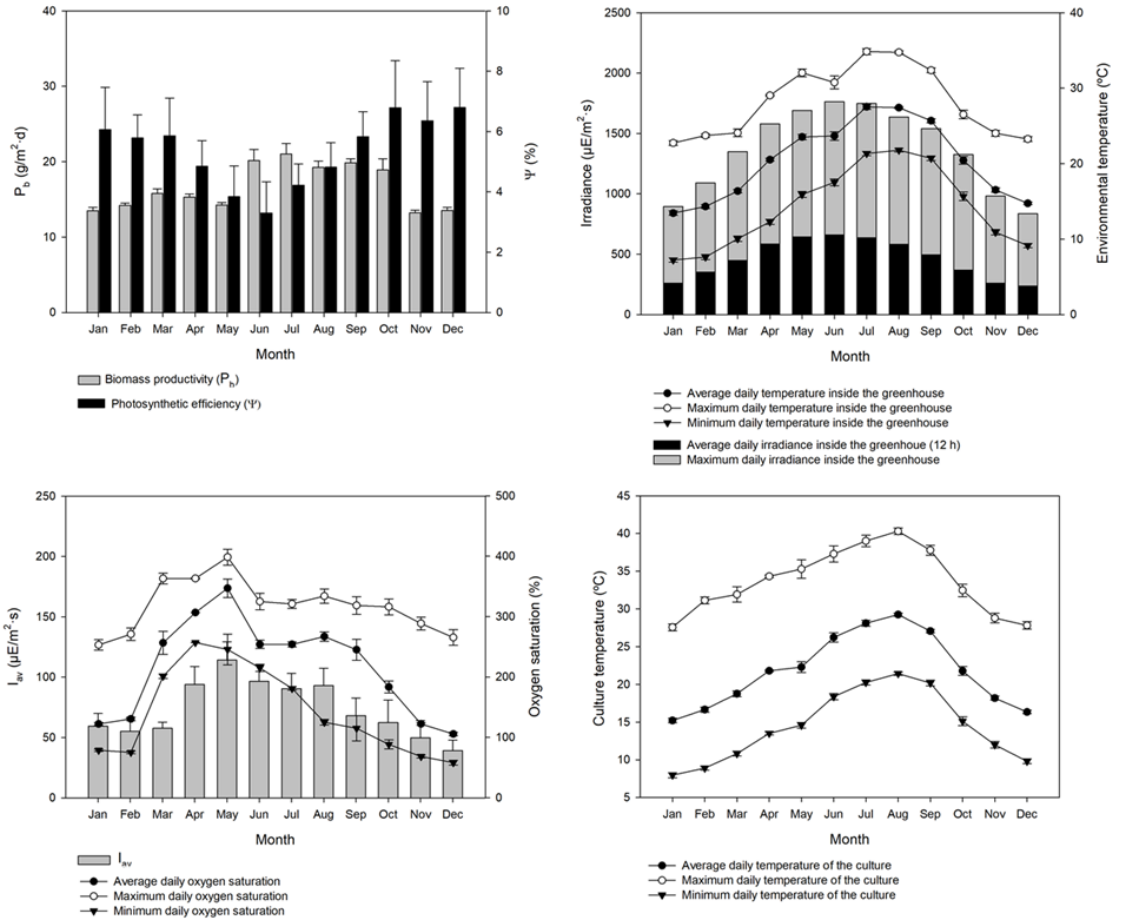

Figure 2.2.1.7. Bioreactor C (double-channel thin layer cascade): Effect of solar irradiance, dissolved oxygen concentration, and temperature inside the greenhouse and of the culture on biomass productivity.

Again, those months where the biomass productivity was higher showed a lower photosynthetic efficiency. As highlighted in the previous section, the productivity of Bioreactor C was lower than that expected, especially during spring/summer (Figure 2.2.1.7). Solar radiation and temperature both inside the greenhouse and inside the culture were similar to those of Bioreactor B. However, because of the (potentially) high productivity of the reactor, oxygen saturation in Bioreactor C was very high and reached the maximum daily values of 250-300% in autumn/winter and the range of 300-400% in spring/summer. Again, it is likely that the inefficient removal of oxygen in this reactor limited the maximum biomass productivity. Previous studies also highlighted the problem encountered while operating highly productive reactors with the accumulation of photosynthetic oxygen in the culture, with values reaching 350% in spring/summer [166].

The inhibitory effect of oxygen in Bioreactor C was higher than in Bioreactor B because both reactors share the same degasser design (and airflow injection), but

the channel surface and culture volume of Bioreactor C almost doubles that of Bioreactor B. Moreover, as the same injector is used to inject air and CO_2 (the latter used to control the pH), at highly productive seasons, especially at midday, the air injection (75 L/min) is substituted by CO_2 injection (10-12 L/min) and therefore oxygen removal is reduced. Future studies solving the low removal of oxygen from the culture are ongoing.

Conclusions

Optimum dilution rates to maximise biomass productivities were 0.2 and 0.3 day^{-1} for the raceway and thin-layer photobioreactors, respectively. Overall, these conditions led to higher biomass productivities during the 12 months of a year. As expected, the months with higher solar radiation showed higher photosynthetic activity and therefore higher biomass productivities. Once the optimum operating conditions were selected, thin-layer cascade photobioreactors led to higher biomass productivities when compared to the raceway design. The thin layer photobioreactor with a smaller surface and volume led to the highest biomass productivities. Annual productivity of the raceway (80 m^2), single-channel thin-layer cascade (63 m^2), and double-channel thin-layer cascade (126 m^2) was 5100, 5600, and 9100 kg/year. The lower areal productivity of the double-channel thin-layer reactor, when compared to the single-channel reactor, was caused by an inefficient removal of dissolved oxygen in the former. Although the optimisation of photosynthetic efficiency is relevant for the design of bioreactors, it must be considered that photosynthetic efficiency was lower in all three studied reactors in those months where solar irradiation was higher and was therefore negatively related to biomass production. Results reported in the current study, which have been collected over a year, are relevant to improving photobioreactors design and microalgal biomass productivity as they demonstrate not only the potential to produce *S. almeriensis* outdoors but also the striking effect of an inefficient oxygen removal, which can limit biomass production. Further studies will up-scale the degasser of thin-layer photobioreactors and assess the potential of these highly productive reactors to produce biomass using both, wastewater, and seawater.

Acknowledgements

This work formed part of the SABANA Project of the European Union's Horizon 2020 Research and Innovation Programme (Grant Agreement 727874) and the AL4BIO Project, funded by the Spanish Ministry of Science, Innovation, and Universities (RTI2018-099495-A-C22). Tomas Lafarga thanks the Spanish Ministry of Science, Innovation, and Universities for the *Juan de la Cierva Incorporación* postdoctoral grant (IJC2018-035287-I).

Conflict of interests

None

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable.

CRediT authorship contribution statement

A. Morillas-España: Investigation & Writing – original draft. **T. Lafarga:** Writing – original draft & Formal analysis. **C. Gómez-Serrano:** Supervision & Investigation. **F.G. Acién-Fernández:** Supervision, Funding acquisition, & Writing – review and editing. **C.V. González-López:** Funding acquisition & Writing – review and editing.

2.2.2.Annual production of microalgae in wastewater using pilot-scale thin-layer cascade photobioreactors

Title: Annual production of microalgae in wastewater using pilot-scale thin-layer cascade photobioreactors

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Journal: Journal of Applied Phycology

Year: 2021

Volume: 33

DOI: https://doi.org/10.1007/s10811-021-02565-2

IF (JCR 2020): 3.215

Categories:

MARINE & FRESHWATER BIOLOGY, 16/110, **Q1**

BIOTECHNOLOGY & APPLIED MICROBIOLOGY, 79/159, Q2

Abstract

Microalgae based wastewater treatment has been suggested as an alternative to polluting and energy-consuming conventional processes. The main advantage of this strategy is the dual role of microalgae: they recover nutrients from waste and simultaneously produce biomass with varied industrial applications. In the current study, the biomass of *Scenedesmus* sp. was produced using primary wastewater in two pilot-scale thin-layer cascade photobioreactors (63 and 126 m^2). The wastewater used for microalgal growth was not subjected to any conventional treatment process, besides the removal of solids, and contained a variable $N\text{-}NH_4^+$ content of 83.0-210.6 $mg \cdot L^{-1}$. Biomass productivity values were comparable to those obtained when operating using freshwater and commercial chemicals as nutrient sources. When operating at a dilution rate of 0.3 day^{-1}, the average annual productivity was 24.8 $g \cdot m^{-2} \cdot day^{-1}$ (82.0 $t \cdot ha^{-2} \cdot year^{1}$) with a maximum of 32.8 $g \cdot m^{-2} \cdot day^{-1}$ in summer. Inorganic nitrogen and phosphorus removal rates varied between 695.4-2383.4 and 70.4-111.8 $mg \cdot m^{-2} \cdot day^{-1}$ respectively. Production of *Scenedesmus* sp. using wastewater would allow not only to process large volumes

of water that could be reused for agricultural irrigation or safely disposed of into water streams, but also reduce production costs by 0.44 €·kg^{-1}, based on a preliminary economic analysis. Overall, results demonstrate that thin-layer cascade reactors can be used to effectively remove nutrients from wastewater while simultaneously producing valuable biomass with potential applications in agriculture or animal feed production.

Keywords: Bioremediation, microalgae, water reuse, biomass productivity, circular economy.

1. Introduction

Providing more sustainable practices for not only natural resources but also waste management is one of the key elements in the transformation towards a circular economy. Water and wastewater management is one of the biggest challenges for humans: limited access to clean water limits both production capacity and profits [178] and was ranked as the global risk of the most devastating impact by the World Economic Forum [179]. Conventional wastewater treatment plants contribute to climate change through the emission of greenhouse gases and require high levels of energy and resource consumption [2]. In the EU, water and wastewater treatment account for approximately 7-8% of the overall energy consumption [179]. Processes based on microalgae-bacteria have emerged as one of the most promising strategies to minimise the environmental impact of wastewater treatment.

The exploitation of photosynthesis to process wastewater is particularly interesting: microalgae use sunlight (an inexhaustive source of energy) and carbon dioxide (a chemical compound we want to get rid of) to transform inorganic nutrients (such as ammonia, nitrates, and phosphates) into oxygen and valuable biomass [84]. The oxygen produced by microalgae is used by heterotrophic bacteria, present in wastewater, to oxidise organic matter into inorganic nutrients and produce carbon dioxide, both needed for microalgal growth [180]. One of the main advantages of this strategy is that the biomass produced during wastewater treatment could be further used to formulate products for agriculture, such as biofertilisers or biostimulants, animal feeds and other high-value applications.

Microalgae are produced in controlled industrial facilities using photobioreactors that can be divided into two main groups: open and closed systems. The latter is highly productive and easier to control but, because of their higher cost, are used for high-end applications where monocultures are required (food, cosmetics, pharmaceutics, chemicals, etc.). Open systems are cheaper to build and operate. One of their main disadvantages is that as they are open to the environment, the risk of contamination with other microalgal strains and microorganisms is higher. However, this is not a problem in wastewater treatment processes where the interaction of microalgae with bacteria is desired. The most common open systems are open ponds or raceways. The culture depth or raceways generally ranges between 0.2 and 0.3 m, which allows the processing of large volumes of water per surface area but their (current) design does not allow to optimise light utilisation [61]. Light availability is the most important factor influencing microalgal growth. Thin-layer cascade systems are operated with a shallow water column (0.5-5.0 cm) which allows to maximise light availability and, therefore, biomass concentration and productivity [171]. For example, *Scenedesmus* sp. productivity using a pilot-scale thin-layer cascade reactor reached 30-35 $g \cdot m^{-2} \cdot day^{-1}$ while lower values of 20-25 $g \cdot m^{-2} \cdot day^{-1}$ were obtained for raceways located inside the same greenhouse during the same season [65].

Currently, microalgae-based wastewater processes are being demonstrated and validated at a large scale in several locations such as Christchurch (New Zealand) and Cadiz (Spain) [116]. However, most of the data available in the literature were obtained from indoor experiments, and the up-scaling of microalgae-based wastewater treatment processes is rare. Treatment of anaerobically digested piggery effluent was conducted outdoors using pilot-scale thin-layer reactors (0.5 cm depth, 0.35 m^3) and raceways (15 cm depth, 1.5 m^3). Thin-layer reactors allowed 1.4 times higher removal rate of N-NH4+, demonstrating the huge potential of thin-layer reactors to process waste streams and wastewaters [181]. Although thin-layer cascade reactors are highly productive and one of the top trends in microalgal biotechnology, information about their performance is limited to laboratory- or pilot-scale (up to 50 m^2) reactors. For these reasons, the goals of the current study were to assess the potential of *Scenedesmus* sp. and thin-layer cascade photobioreactors to recover nutrients from wastewater and assess the effect of

environmental conditions on biomass productivity and the performance of the system. Two different pilot-scale thin-layer cascade designs located inside a greenhouse were assessed and operated for 12 months using wastewater, with very high nitrogen and phosphorus concentration, as the sole nutrient source.

2. Materials and methods

2.1 Selected microorganism

The strain selected was *Scenedesmus* sp. (CCAP 276/24), available at the culture collection of the University of Almería, Spain. This strain was selected because it is fast-growing and highly productive and it is also particularly adapted to the environmental conditions in the south of Spain [63,65,182]. The initial inocula were prepared using 5 L photobioreactors at a laboratory scale and were further up-scaled using 80 L pH-controlled bubble columns as described previously [65]. The bubble columns were located inside a greenhouse together with the thin-layer cascade photobioreactors. The culture medium of the columns was composed of 0.90 $g \cdot L^{-1}$ $NaNO_3$, 0.18 $g \cdot L^{-1}$ $MgSO_4$, 0.14 $g \cdot L^{-1}$ K_2HPO4, and 0.03 $g \cdot L^{-1}$ of Karentol® (Kenogard, Spain), which is a commercial solid mixture of micronutrients.

2.2 Photobioreactor and operation conditions

Two different thin-layer photobioreactors were evaluated: a single-channel photobioreactor with a total volume of 2.4 m^3 and a land surface of 63 m^2, named TL1, and a double-channel photobioreactor with a total volume of 3.6 m^3 and a land surface of 126 m^2, named as TL2 (Figure 2.2.2.1). The reactor's channels were built-in glass fibre and are elevated 1.5 m from the ground. The channels had a slope of 1% and allowed an average culture velocity of 0.2 $m \cdot s^{-1}$. The surface-to-volume ratios of TL1 and TL2 were 26.3 and 35.0 $m^2 \cdot m^{-3}$ respectively. Both were operated at a culture depth was 0.02 m and were provided with a degasser (a 250 L bubble column with continuous injection of air at 75 $L \cdot min^{-1}$) and a collector to increase the volume of the system. The volume of culture in the collector and the bubble column summed up at 1.0 m^3. The pH, temperature, and dissolved oxygen concentration of the culture were measured using 5083T and 5120 probes (Crison Instruments, Spain) connected to an MM44 control-transmitter unit (Crison Instruments, Spain) and Labview data acquisition software (National Instruments, US) providing complete monitoring of the facilities. The sensors were located at

the end of the channel. The pH was controlled by on-demand injection of CO_2 and evaporation was daily compensated by the addition of freshwater.

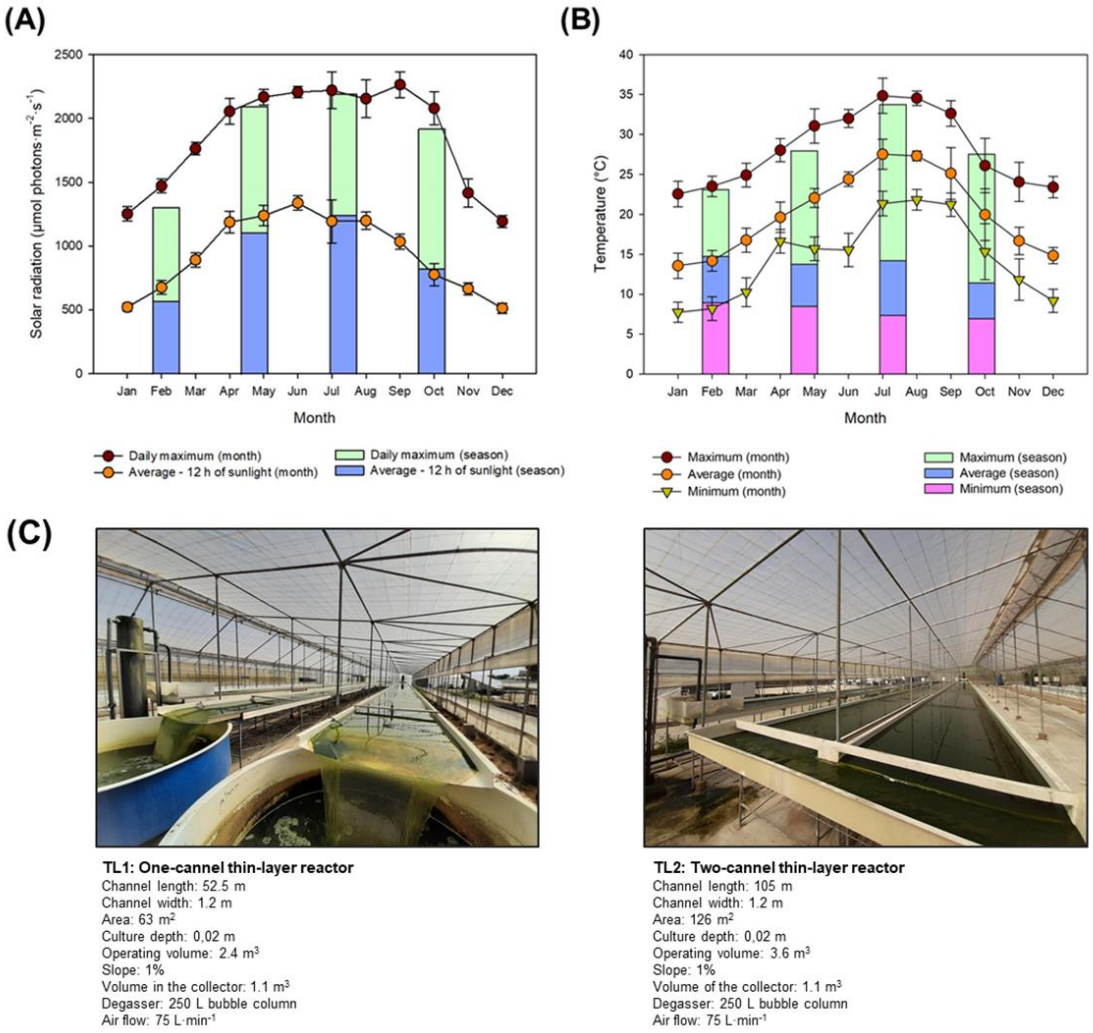


Figure 2.2.2.1. Annual variation of (A) solar radiation and (B) temperature inside the greenhouse and (C) photobioreactors used. Values represent the average of the maximum, minimum, and average daily radiation and temperature values inside the greenhouse ± SD.

Both reactors were operated on semi-continuous mode at a dilution rate of 0.3 day^{-1}, which means that every day 30% of the cultures' volume (720 and 1080 L for TL1 and TL2, respectively) was harvested and replaced with fresh culture media, in this case, primary wastewater. The reactors were operated 24 h per day and the semi-continuous mode was maintained until the total volume of the reactors was replaced at least twice (7-8 days). The production process was repeated at least three times per season, being each production run by the experimental unit. Three technical replicates were performed per natural replicate.

2.3 Wastewater composition

The domestic wastewater was collected from the sewerage of the University of Almería (Almeria, Spain). Domestic wastewater includes blackwater (wastewater from toilets) and greywater (water used for washing, bathing and kitchen. The wastewater consisted mainly of blackwater: 20,000 people with only a limited number of kitchens, showers, and washing machines. The wastewater used was not subjected to any conventional depuration treatment besides the removal of solids. For these reasons, the nutrient and bacterial load of the inlets was highly variable and, depending on the season, high when compared to conventional urban wastewater. The concentration of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ on the wastewater varied from 93.6-210.6, 1.9-3.7, and 11.3-13.0 $mg \cdot L^{-1}$, respectively.

2.4 Analytical determinations

Biomass concentration (C_b) was calculated by dry weight, filtering 100 mL of culture through 1 µm filters and drying at 80 °C in an oven for 24 h. Biomass included not only the inoculated *Scenedesmus* sp. microalga but also other microalgae and bacteria present in the microalgae-bacteria consortia. Areal biomass productivity (P_b) was calculated as the product of biomass concentration and the dilution rate (0.3 day^{-1}) using the equation:

$$P_b(g \cdot m^{-2} \cdot day^{-1}) = \frac{C_b \cdot D \cdot V}{A}$$

where D is the dilution rate imposed (0.3 day^{-1}), V is the volume of the culture expressed in L, and A is the surface of the reactor expressed in m^2.

The variable chlorophyll fluorescence ratio (F_v/F_m) was determined using an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic). Microalgal cells were dark-adapted for 5 min before the determination of F_v/F_m values [182]. The absorbance (Abs) of the culture at 400-700 nm was daily measured using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). The extinction coefficient (k_a) and the average irradiance inside the culture (I_{av}) were calculated as described previously [65] using the equations:

$$k_a = \frac{Abs}{C_b \cdot p}$$

and

$$I_{av}(\mu mol\ photon \cdot m^{-2} \cdot s^{-1}) = \frac{I_0}{k_a \cdot C_b \cdot p} \cdot (1 - e^{-k_a \cdot C_b \cdot p})$$

where I_0 is the irradiance at the surface of the culture and p the light path inside the reactor.

The concentration of ammonium, nitrates, and phosphates at the inlet and outlet of TL1 and TL2 was measured using standard official methods approved by the Spanish Ministry of Agriculture - described previously [116]. Briefly, phosphorus and nitrates were measured spectrophotometrically through the phospho-vanado-molybdate complex and by measuring the absorbance at 220-275 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). The concentration of ammonium was determined using the Nessler reactive method. Analytical determinations were conducted daily. Three technical replicates for all the studied parameters were conducted per natural replicate.

2.5 Economic analysis

An economic analysis was conducted following a previously reported methodology [183]. Briefly, the cost of equipment used was obtained from the suppliers and was used to calculate the total fixed capital by multiplying the equipment's cost and their corresponding Lang factors. Total production cost was calculated as the sum of the depreciation plus the direct production costs. Depreciation includes the amortisation of fixed capital, property taxes, insurances, and purchase taxes. Direct production costs included raw materials, utilities, labour, and others including supervision, maintenance, tax, contingency, etc. For the analysis, the present study assumed a theoretical 10,000 m^2 reactor and set: (i) the cost of commercial fertilisers and CO_2 as 0.4 and 0.1 €·kg^{-1} [63]; the cost of thin-layer cascade photobioreactors as 200 €·m^{-3} [63]; (iii) the cost of freshwater as 0.0002 €·m^{-3}; (iv) the productivity of the thin-layer cascade reactors when operated using freshwater and commercial fertilisers as 24.8 g·m^{-2}·day^{-1} [65]; and (v) 330 days of production per year, leaving 35 annual days for maintenance and cleaning operations.

2.6 Statistical analysis

Normality and homoscedasticity of the variables within each group were checked. Data were analysed using analysis of variance with JMP 13 (SAS Institute Inc., USA). A Tukey pairwise comparison of the means was carried out to identify where sample differences occurred with a criterion of $p<0.05$. A bivariate Pearson's' correlation analysis was conducted to identify relationships between different variables.

3. Results

3.1 Biomass productivity

Temperature and solar radiation values were online monitored during the 12 months of the study and the results are summarised in Figure 1. As expected, higher maximum temperatures and solar radiation values were observed in summer while no major differences were detected between spring and autumn ($p=0.002$). Environmental conditions had a striking effect on the conditions of the culture and therefore on biomass production and nutrient removal capacity. Indeed, culture temperature and solar radiation were positively correlated ($R^2=0.849$). During summer, the maximum temperature reached in the culture ranged between 35 and 40 °C (Figure 2.2.2.2). These values were maintained for a short period but still, could be lethal to certain microalgal strains and other microorganisms. Higher solar radiation on the surface led to higher average radiation inside the culture, especially higher in summer ($p=0.01$). No differences were observed between I_{av} values in TL1 and TL2 as both reactors were operated with an identical culture depth and reached similar biomass concentrations. In the current study, average I_{av} values were 52.3 ± 4.1, 94.3 ± 6.9, 108.7 ± 9.2, and 76.8 ± 8.4 μmol photons·m^{-2}·s^{-1}.

As mentioned before, light availability is the most important factor in the production of photosynthetic organisms. Indeed, I_{av} values were positively correlated with biomass productivity in both TL1 ($R^2=0.963$; 0.05) and TL2 ($R^2=0.989$; 0.05). In the current study, oxygen concentrations are over 300%Sat. were detected in both reactors and were especially high in TL2 during the whole year (Figure 2.2.2.2). Figure 2.2.2.2 also shows the average maximum, mean, and minimum pH values measured per season. Results demonstrate an accurate pH control of the system,

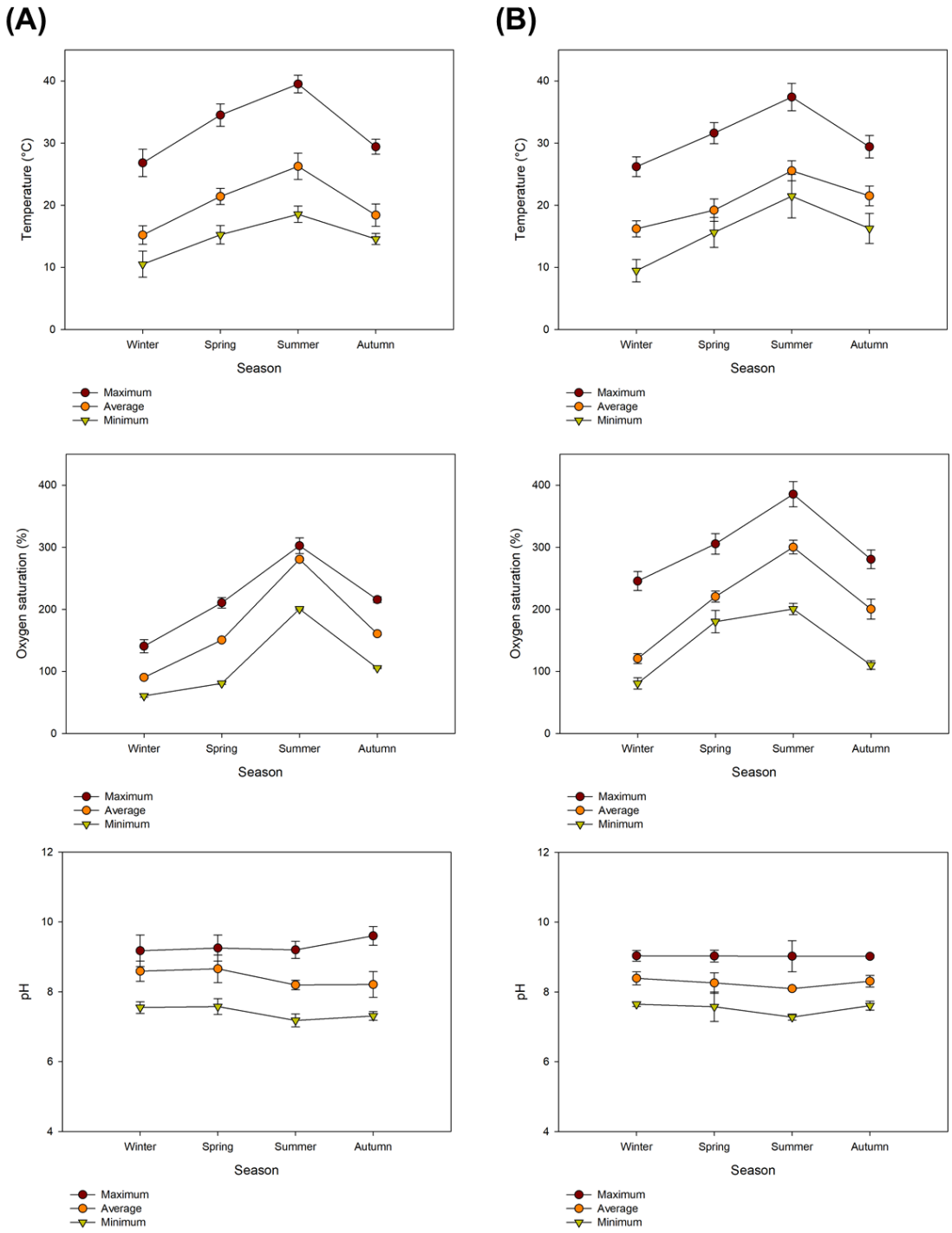


Figure 2.2.2.2 Effect of environmental conditions on culture temperature, dissolved oxygen concentration, and pH in (A) TL1 and (B) TL2. Values represent the average of the daily maximum, minimum, and average values ± SD. Temperature, dissolved oxygen concentration, and pH data of our reactors are available at http://www2.ual.es/sabana/data-center-2/.

which is important too, for example, avoid precipitation of phosphorus and maximise microalgal growth. Values were kept within the range 7.0-9.0 during the complete duration of the experiment. In addition, the F_v/F_m value measures the performance of the photochemical processes in the PSII complex, indicating if the culture is subjected to a certain stress condition. In the current study, F_v/F_m values

ranged between 0.4 and 0.5 (Figure 2.2.2.3). No major differences were observed between F_v/F_m values except for a lower value in summer for the culture produced in TL2.

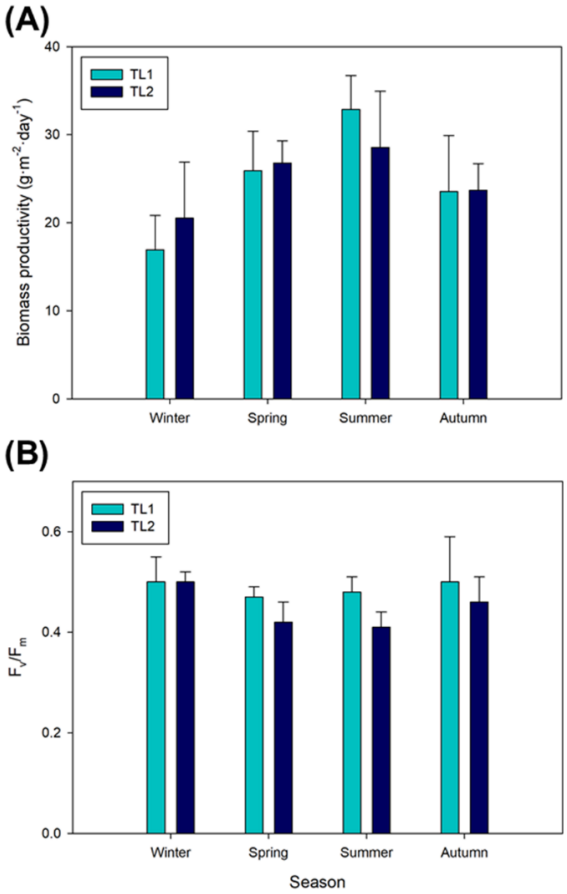


Figure 2.2.2.3. (A) Biomass productivity and (B) chlorophyll fluorescence.

3.2 Nutrient removal

The content of the main inorganic nutrients ($N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$) of the wastewater used was determined and the results are shown in Figure 2.2.2.4. The content of $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ in the inlets varied between 1.9-3.6 and 11.3-13.0 $mg \cdot L^{-1}$ and were comparable to those reported previously for urban wastewater [116]. The $N\text{-}NH_4^+$ content of the wastewaters used ranged from 93.6-210.6 $mg \cdot L^{-1}$ depending on the season. Besides inorganic nutrients, the wastewater contained organic matter including, for example, urea or proteins which contributed to the total nitrogen and phosphorus input. The content of $N\text{-}NH_4^+$ in the outlets was significantly lower during the four seasons and (almost) all the $N\text{-}NH_4^+$ present in the wastewater was either consumed by microalgae or used by nitrifying bacteria to produce $N\text{-}NO_3^-$.

Moreover, a mass balance conducted in both reactors (assuming a nitrogen content of the organic matter of 10%) demonstrated that a large percentage of N-NH_4^+ present in the media was "lost" by stripping (desorption). This was especially high for TL1 (25-55% of total N-NH_4^+). N-NH_4^+ removal capacity values ranged between 1039.0-2280.3 mg·m^{-2}·day^{-1}, being higher in those seasons with higher photosynthetic activity. Higher N-NH_4^+ removal values were observed in TL1 when compared to TL2 except during winter. N-NH_4^+ removal and biomass productivity were positively correlated in both TL1 (R^2=0.688) and TL2 (R^2=0.764).

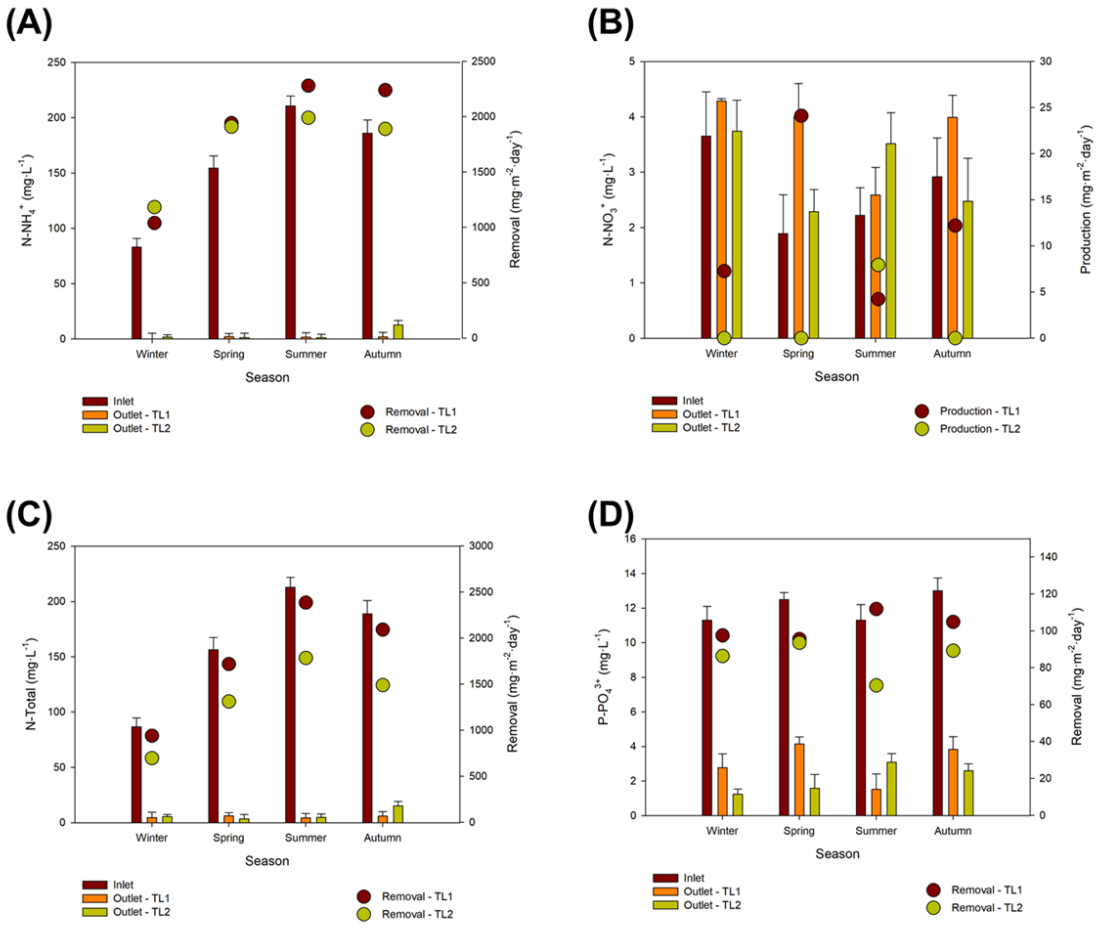


Figure 2.2.2.4. (A) N-NH_4^+, (B) N-NO_3^-, (C) total nitrogen, and (D) P-PO_4^{3-} removal capacity of the system.

Despite the low N-NO_3^- concentration in the inlet it was still present in the outlets, in some cases even at higher concentrations. Overall, the total nitrogen consumption ranged from 938.5-2383.4 mg·m^{-2}·day^{-1} in TL1 and from 695.4-1782.3 mg·m^{-2}·day^{-1} in TL2. The content of P-PO_4^{3-} in the outlets was lower than in the inlets for all four seasons and P-PO_4^{3-} removals varied between 95.5-111.8 mg·m^{-2}·day^{-1} in TL1 and between 86.3-89.2 mg·m^{-2}·day^{-1} in TL2. The P-PO_4^{3-} was

used for microalgal growth, as all the $P\text{-}PO_4^{3-}$ removed from the wastewater was either present in the outlets or the biomass (assuming a phosphorus content of 1%). Despite the lower content of $P\text{-}PO_4^{3-}$ in the outlet of TL2, higher areal recoveries were measured in TL1 because of its lower surface-to-volume ratio.

3.3 Theoretical large-scale productivity and economics

Upscaling these results to a theoretical 10,000 m^2 reactor would allow to process 21,780 m^3 and recover 5-6 t of nitrogen ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) and approximately 0.4 t of $P\text{-}PO_4^{3-}$. Using this theoretical 10,000 m^2 thin-layer cascade reactor, it would be possible to produce 80-90 $t \cdot year^{-1}$ of valuable microalgal biomass, although there is technological potential to improve this value even further. These values were calculated using the average of the seasonal productivities reported in the current study and assuming 330 days of operation per year.

A preliminary economic analysis was performed on this theoretical 10,000 m^2 reactor based on a previously reported methodology [183]. The cost of nutrients, freshwater and thin-layer cascade reactors was set as described in previous scientific publications. Briefly, the total cost of major equipment summed up to 95,835.06€ in both scenarios (freshwater and wastewater) representing 30.6% of the total fixed capital, which was 313,731.89€ - land was not considered. Construction costs (19.7%) and piping (9.2%) were the second and third most important items contributing to the total fixed capital. The direct production costs were highly influenced by the use of wastewater instead of freshwater and commercial nutrients. In the case of the photobioreactors operated using wastewater, the raw materials annual cost was 12.523.50€ and was entirely dedicated to carbon dioxide. In turn, when producing the biomass using freshwater and commercial chemicals as the source of nutrients, the cost of the raw materials was 46,649.42€ (21% nutrients and 79% carbon dioxide). The cost of water was below 1% as we assumed water recirculation. Moreover, the carbon dioxide requirements were lower in the photobioreactors operating with wastewater as in this case, microalgae can use the carbon dioxide produced by bacteria and also the organic matter present in the wastewater can be assimilated by microalgae as carbon or nitrogen sources [184].

4. Discussion

When produced outdoors, microalgal growth depends mainly on environmental conditions, namely temperature and solar radiation. Besides environmental factors, the dilution rate used directly influence biomass productivity. In the current study, the reactors were operated at a dilution rate of 0.3 day^{-1}, which was demonstrated to be the optimum independently of the season [65]. Figure 2 shows the temperature of the culture, which was equal in both reactor designs indicating that the different volume-to-surface ratios of the reactors did not influence this variable. The high temperatures achieved demonstrate the importance of selecting a robust (and productive) microalgal strain capable to grow under a wide range of environmental conditions [155]. In this case, we selected a local strain which was isolated for the first time inside a greenhouse exposed to high temperatures and solar irradiance (45 °C and 2,000 μmol photons·m^{-2}·s^{-1} respectively) (Sánchez et al., 2008) and can grow well at pH and salinity values within the ranges 7-10 and 0-5 g·L^{-1} respectively (Sánchez et al., 2008).

The oxygen produced by microalgae during photosynthesis can provide a negative effect on the performance of the system and, for this reason, the removal of the oxygen from the culture is important. Indeed, oxygen concentrations in the range 350-400%Sat. led to inhibition of microalgal growth previously when using thin-layer cascade reactors [65]. The reason for high oxygen saturation levels in thin-layer cascade reactors is their high productivity, which almost doubles oxygen production when compared to raceways. In the present study, the maximum saturation values reached suggest an ineffective oxygen removal, which will be improved in further studies. The pH of the culture was well controlled throughout the year and, as highlighted before, the strain produced during the current study can grow well under pH values of 7.0-10.0 (Sánchez et al., 2008). Overall, environmental conditions highly influenced the cultures' temperature, average irradiance inside the culture, and dissolved oxygen concentration, which play a key role in the performance of the system.

In terms of biomass productivity, the results were in line with previous reports that demonstrated that the productivity of thin-layer cascade reactors is higher than that of raceways [65]. However, maximum productivities reached herein were lower than

those obtained previously using outdoor thin-layer cascade reactors. For example, the productivity of a 32 m^2 thin-layer cascade reactor with a culture depth of 0.02 m located in the same region reached 47.3 $g \cdot m^{-2} \cdot day^{-1}$ in a previous report [116]. It is important to highlight that microalgae production using wastewater depends largely on the composition of the wastewater used. In the current study, after applying a mass balance to the system, it was observed that the microalgal growth was nitrogen-limited. Assuming a nitrogen content of the biomass of 8.0%, the mass balance indicates that microalgae utilised organic matter as a source of nitrogen and phosphorus.

Depending on the season, approximately 700-100 and 550-800 $mg \cdot m^{-2} \cdot day^{-1}$ of organic nitrogen were removed from the wastewater in TL1 and TL2 respectively. A more in-depth characterisation of the inlets and outlets of the reactors would be interesting as organic compounds and contaminants are generally overlooked in the literature. Adding nutrients to the wastewater would probably lead to increased biomass productivities, as biomass productivity values of 42 $g \cdot m^{-2} \cdot day^{-1}$ were reported for thin-layer cascade reactors operating with centrate as the nutrient source once the $N\text{-}NH_4^+$ concentration was optimised[63]. However, this would contrast with the aim of wastewater treatment processes which is removing nutrients from wastewater. Another alternative that is currently being assessed by several research groups is the utilisation of membranes that allow separating the hydraulic retention time from the cellular retention time thus allowing to process of larger volumes of water per surface area. This strategy allows for increasing the total amount of nutrients that enter the reactor without the need to supplement the wastewater with salts. Moreover, microalgal growth could have also been partially affected by the high dissolved oxygen concentrations within the range of 300-400% saturation, which led to growth inhibition previously [65].

Optimal F_v/F_m values for microalgal strains are frequently near 0.7 [186]. The F_v/F_m values obtained in the current study were lower than that and slightly lower than those reported for the same strain when produced using freshwater and commercial chemicals as the source of nutrients [65]. This indicates that the cells were subjected to some stress conditions and therefore the capacity and activity of photosynthesis were negatively affected by wastewater [187]. This was probably caused by the fact that the wastewater was not subjected to any previous

depuration process and could have had compounds that affect microalgal growth. The above-mentioned nitrogen limitation could have also affected the photosynthetic activity of microalgae. Moreover, the observed low F_v/F_m values in summer could be caused by photoinhibition caused by reversible damage of key PSII components as well as higher temperatures during this season [188]. High dissolved oxygen concentration could have partially affected the photosynthetic activity of the culture.

As highlighted before, the main goal of wastewater treatment processes based on microalgae, besides biomass production, is to recover nutrients and allow the reuse or safe disposal of large volumes of water. The $N\text{-}NH_4^+$ content in the inlet varied between 93.6 and 210.6 $mg \cdot L^{-1}$ while the $N\text{-}NO_3^-$ the content was within the range 1.9-3.7 $mg \cdot L^{-1}$. Considering that approximately 500-1000 $mg \cdot m^{-2} \cdot day^{-1}$ of organic nitrogen were removed from the wastewater in both reactors, stripping led to the challenging "loss" of 550-900 and 50-150 $mg \cdot m^{-2} \cdot day^{-1}$ of $N\text{-}NH_4^+$ in TL1 and TL2 respectively. Similar results were reported previously [63]. Further studies to understand the effect of environmental and operational conditions on this effect are needed. Moreover, $N\text{-}NO_3^-$ was still detected in the outlets of the reactors. Nitrification occurred but production of $N\text{-}NO_3^-$ was low when compared to other processes with higher culture depths. This was caused because (i) it was not consumed by microalgae and bacteria, because it is known that in the presence of both, $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$, microalgae prefer the former [189] and (ii) $N\text{-}NO_3^-$ was produced by the action of nitrifying bacteria [190]. Probably the high light availability in thin-layer cascade reactor favoured microalgal growth and limited the growth of nitrifying bacteria. The composition of the microalgae-bacteria consortia is highly influenced by environmental conditions, and this could be the cause for variability in the $N\text{-}NO_3^-$ removal efficiency shown in Figure 4.

Higher total nitrogen removal values were observed in TL1 when compared to TL2. This was caused by the lower surface-to-volume ratio of TL1, which allows the processing of a larger volume of water per surface area when compared to TL2. A different composition of the microalgae-bacteria consortia of both reactors could have also affected the nutrient removal capacity of the reactors as it is not known how different surface-to-volume ratios can affect the microalgae-bacteria consortia. It is not known how photobioreactor design parameters such as channel

length, culture depth, or the volume of the collector affect the composition of the microalgae-bacteria consortia.

The most abundant bacterial phyla present in cultures of *Scenedesmus* sp. in wastewater were Proteobacteria and Bacterioidetes, with the most dominant orders being Rhodobacterales and Sphingomonadales [116]. Moreover, a recent study suggested that the dilution rate, as well as environmental conditions, play a key role in the composition of the consortia, with heterotrophs and phototrophs mainly from the family *Rhodobacteraceae* dominating *Scenedesmus* sp. cultures [25]. Further studies on this topic are needed as the microbial community present during microalgae cultivation highly influences the performance of the system in terms of both, biomass production and nutrient recoveries [43].

Not only nitrogen but also phosphorus is a nutrient of concern in eutrophication, being both limiting factors in most growth scenarios [191]. Phosphorus and phosphorylation play a key role in the metabolism of both microalgae and bacteria [192] and previous studies demonstrated that microalgal cultures, especially when produced in thin-layer reactors, can consume large quantities of phosphorus [63]. A mass balance revealed that the phosphorus introduced into the system was either used for microalgal growth or removed with the outlet of the reactor. This demonstrates the importance of effective control of the pH as high pH values result in the precipitation of phosphates and therefore a reduction of the amount of P-PO_4^{3-} that is available for microalgal growth. As it happened with the total nitrogen, part of the phosphorus used for microalgal growth was obtained from organic matter or other inorganic phosphorus compounds not analysed in the current study. Further studies will include the in-depth analysis of the organic and inorganic compounds present in the inlets and outlets of the reactors as this information will be useful to design more efficient processes and maximise nutrient recoveries.

Overall, the reclaimed water could be reused for agricultural irrigation or even disposed of into water courses. The content of N-NH_4^+ and N-NO_3^- in the outlets was below the maximum discharge limit of Spanish regulations set at 10-15 mg·L^{-1} of nitrogen [193], and therefore based on their nitrogen content could be disposed of even into sensitive zones. However, the P-PO_4^{3-} concentration of the reactors was slightly higher than the maximum discharge limit, set at 1-2 mg·L^{-1}, which

would allow disposing of the effluents either into non-sensitive zones or into sensitive zones if the removal of phosphorus and nitrogen (in all the treatment plants of the sensitive zone) is larger than 75%, as reported herein [193]. One of the main advantages of microalgal processes, when compared to conventional wastewater treatments, is that the produced biomass can be further exploited as a source of high-value products such as animal feed or products for agriculture (biostimulants or biofertilisers). Indeed, *Scenedesmus* strains have potential for being used at commercial scale as biostimulants [194,195] and aquafeed ingredients [196,197].

The economic analysis conducted herein suggested that producing *Scenedesmus* sp. using wastewater instead of freshwater and fertilisers could lead to a reduction of reduction in the unitary production cost of 0.44 €·kg^{-1} of produced wet biomass. If used for producing agricultural products, a dehydration step is not required therefore minimising production costs. The produced biomass would consist of microalgae and bacteria. As mentioned before, the composition of the consortia depends on environmental and operational conditions but, if managed properly, over 95% of the produced biomass will be microalgae [28]. Because of the higher light availability of thin-layer cascade reactors, photoautotrophic growth would likely be favoured therefore facilitating the proliferation of photosynthetic microorganisms, although this would need to be validated in future studies. An in-depth characterisation of the biomass of *Scenedesmus* sp. produced using different types of water and photobioreactor designs is ongoing as well as a complete economic analysis comparing different reactor designs and types of water.

5. Conclusions

Scenedesmus sp. is a robust strain that can be produced outdoors using wastewater and thin-layer cascade reactors. Thin-layer cascade reactors are highly productive and allow to maximise biomass productivity per surface area. Biomass productivities were comparable to those obtained when produced using freshwater and commercial fertilisers as nutrients, demonstrating that primary wastewater is an excellent source of nutrients for microalgal production. However, because of the high productivity of thin-layer reactors, microalgal growth can be

nutrient-limited when using wastewater. The use of membranes to separate the cellular from the hydraulic retention time would allow to process of a larger amount of water per surface area and increase both, the biomass productivity, and the nutrient removal capacity of the system. The processed water can be reused for irrigation or even disposed into natural water courses. An advantage of microalgae-based wastewater treatment processes is that besides recovering nutrients from waste, the process simultaneously produces valuable microalgal biomass that can be further used for valuable applications. A preliminary economic analysis revealed that producing microalgae using wastewater could lead to a reduction in total unitary production costs of 0.44 €·kg^{-1}, which is significant when producing agricultural products.

Acknowledgements

The authors thank the Institute for Agricultural and Fisheries Research and Training (IFAPA). This work is part of the AL4BIO Project, funded by the Spanish Ministry of Science, Innovation and Universities (RTI2018-099495) and the SABANA Project funded by the H2020 Research and Innovation Framework Programme (727874). Tomás Lafarga thanks the Spanish Ministry of Science, Innovation and Universities (IJC2018-035287-I).

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

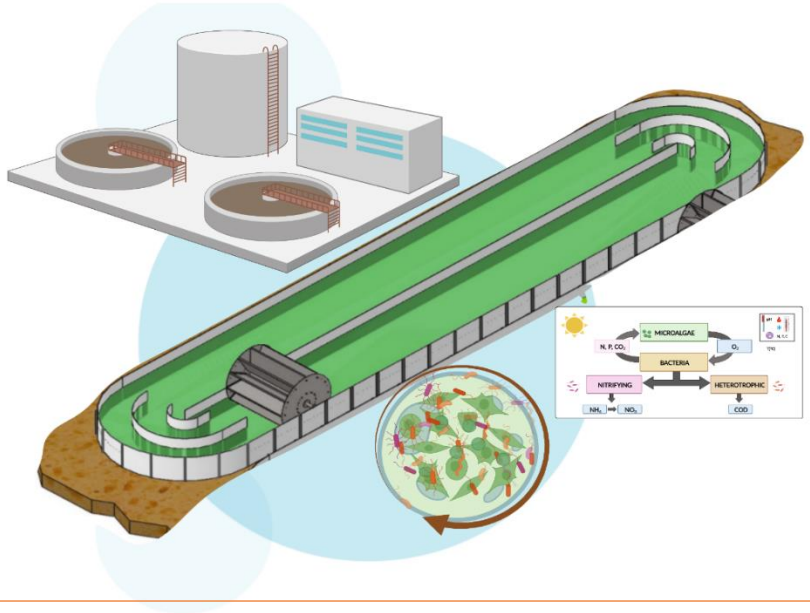
Data availability

Data from the SABANA Project are available at http://www2.ual.es/sabana/data-center-2/.

CRediT author statement

Ainoa Morillas-España: Investigation, Writing – original draft; **Tomás Lafarga:** Formal analysis, Visualization, Writing – review & editing; **Francisco Gabriel Acién-Fernández:** Supervision, Funding acquisition; **Cintia Gómez-Serrano:** Investigation, Formal analysis; **Cynthia Victoria González-López:** Supervision, Funding acquisition.

2.3. Wastewater treatment capacity of microalgae integrated systems



2.3.1.Year-long evaluation of microalgae production in wastewater using pilot-scale raceway photobioreactors: Assessment of biomass productivity and nutrient recovery capacity.

Title: Year-long evaluation of microalgae production in wastewater using pilot-scale raceway photobioreactors: Assessment of biomass productivity and nutrient recovery capacity**.**

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Journal: Algal research

Year: 2021

Volume: 60

DOI: https://doi.org/10.1016/j.algal.2021.102500

IF (JCR 2020): 4.401

Categories: BIOTECHNOLOGY & APPLIED MICROBIOLOGY, 46/159, **Q2**

Abstract

The production of *Scenedesmus* sp. using wastewater was validated with pilot-scale raceway photobioreactors during a complete annual cycle in Almería (Spain). Three different dilution rates (0.1, 0.2, or 0.3 day^{-1}) were evaluated. Biomass productivity was significantly affected by the season (temperature and solar radiation) achieving a maximum value of 25.1 $g \cdot m^{-2} \cdot day^{-1}$ when operating at a dilution rate of 0.2 day^{-1} in summer. Up to 96% of the $N\text{-}NH_4+$ present in the media was either assimilated by microalgae to produce biomass, converted to $N\text{-}NO_3^+$ by the action of nitrifying bacteria, or desorbed (stripping). Maximum nitrogen removal rates reached 4286.6 $mg \cdot m^{-2} \cdot day^{-1}$ in summer. In terms of $P\text{-}PO_4^{3+}$, up to 75% was removed, with removal rates ranging from 147.5 $mg \cdot m^{-2} \cdot day^{-1}$ in winter to 227.2 $mg \cdot m^{-2} \cdot day^{-1}$ in summer. Data reported herein was used to validate the ABACO model, which was demonstrated to be robust enough to accurately predict biomass productivity in pilot-scale outdoor open raceways throughout the year (R^2=0.929; 0.05). The current study demonstrates the potential of raceway reactors and *Scenedesmus* sp. to recover nutrients from unprocessed wastewater with an exceptionally high content of $N\text{-}NH_4^+$ at a pre-industrial scale.

Keywords: Bioremediation, microalgae, biomass, photobioreactor, water reuse, circular economy.

1. Introduction

Approximately 40% of the world's population will suffer severe water stress by 2030, caused by increased demand for water plus contamination of water resources and lack of novel technologies to reclaim used water [198]. Water is too precious to waste. Wastewater treatment and reuse are key to achieving a sustainable future. In the EU, Regulation (EU) 2020/741 known as the Water Reuse Regulation will apply from June 2023 and is expected to boost circular approaches to water reuse in agriculture. Wastewater treatment cannot be managed by a single technology mainly because of variability in scales, type and number of contaminants, and regional conditions involved. Conventional wastewater treatment facilities focus on both, the mechanical removal of suspended solids and the reduction of biological oxygen demand by activated

sludge [199]. However, the capacity of this strategy to remove nutrients and contaminants is limited and has restrictions because of their high-energy requirements and environmental impact, mainly due to the emission of greenhouse gases. Treated wastewater can still pollute water bodies if the removal capacity of the system is inefficient.

Microalgae have been proposed as an alternative to improve nutrient removal efficiencies of conventional processes. These valuable microorganisms are photosynthetic, which means that use an inexhaustive source of energy (light) and a chemical compound we need to get rid of (carbon dioxide) to produce oxygen and biomass. The microalgae-bacteria consortia that form in microalgae-based wastewater treatment processes is especially interesting. The oxygen produced by microalgae is used by bacteria, naturally present in wastewater, to remove pollutants [200]. In turn, bacteria produce carbon dioxide, micronutrients, siderophores, and growth stimulants that promote microalgal growth [116,180]. The composition of the consortia depends on environmental and operational conditions but, if managed properly, over 95% of the produced biomass will be microalgae [28]. Using microalgae in wastewater treatment processes could bring an important additional value besides sustainability and improved nutrient recovery, as the produced algal biomass can be further transformed into useful agricultural products [47,66]. For example, different *Scenedesmus* strains have shown biostimulant effects in plants [194] and can be produced using free or low-cost waste streams [201].

Several microalgal strains were suggested as potential candidates for wastewater treatment and these include *Chlorella* [202], *Arthrospira* [203], *Nitzchia* [204], and the above-mentioned *Scenedesmus* [116]. The latter has been widely studied because of its high tolerance to adverse conditions [161] and its ability to produce and accumulate valuable compounds such as lutein [205]. To date, most of the studies reported in the literature were carried out using bench-scale photobioreactors [40,201,206–208]. A limited number of studies demonstrated the robustness of *Scenedesmus* strains outdoors in pilot-scale reactors. These strains were recently produced using wastewater at a pilot scale using thin-layer cascade bioreactors [64,116]. Thin-layer cascade bioreactors are highly productive in terms of biomass production [65]. However, they have the disadvantage that because of their low

culture depth, they allow to process lower volumes of water per surface area when compared to other designs.

Because of the potential to process larger volumes of water and because of their low cost and ease of operation, raceway designs are the preferred option for wastewater treatment. Raceways are generally built on compacted soil covered by polymers and allow to achieve biomass concentrations around 0.5 $g \cdot L^{-1}$ with productivities varying from 9 to 25 $g \cdot m^{-2} \cdot day^{-1}$ depending on environmental and operational conditions [58]. For example, the maximum biomass productivity achieved using a 7.2 m^2 raceway reactor was 22.9 $g \cdot m^{-2} \cdot day^{-1}$ [209]. Raceways can be located outdoors or inside greenhouses, which is a common practice in the south of Europe. Although greenhouses represent an additional capital cost, they are necessary for microalgal production in some regions [210] and allow a higher control of the process [211].

Nutrient recovery from wastewater using microalgae-bacteria consortia has been widely studied. Despite promising results, most of the studies carried out to date were conducted under controlled conditions at a lab scale or outdoors but using "small" reactors for a short period [198]. Up-scaling microalgae production has been a major drawback for microalgae biotechnology and the yields from real production systems are still not close to theoretical values [125]. Thus, the main goal of the current study was to optimise the operation of pilot-scale raceway bioreactors and to validate the potential of the microalga *Scenedesmus* sp. to remove nutrients from wastewater for one year. Large 11.8 m^3 raceway reactors were used and operated in semi-continuous mode. Fluctuations in culture and environmental conditions can influence microbial diversity and therefore, depuration efficiency [25]. The current study will also discuss the effect of environmental conditions namely temperature and solar radiation on the performance of the system. A secondary aim of the manuscript was to validate the ABACO model, a robust tool that can be used to predict biomass productivity as a function of environmental (solar radiation, temperature, pH, and dissolved oxygen concentration) and biological parameters (microalga growth rate, nutrient saturation coefficients, and nutrient inhibition coefficients, among others) [212]. The validation of models using data generated using large reactors located outdoors is of key importance as this allows to predict the suitability of models for predicting industrial processes.

2. Materials and methods

2.1 Selected microorganism

The strain selected was *Scenedesmus* sp. because it is a fast-growing and highly productive strain that is particularly adapted to stressful and environmental conditions of the region. The microalga *Scenedesmus* sp. (CCAP 276/24) was obtained from the culture collection of the Department of Chemical Engineering of the University of Almería in Spain. The inocula were prepared using controlled 5 L photobioreactors at a laboratory scale that were further up-scaled using 80 L pH-controlled bubble columns photobioreactors located outdoors as described previously [65]. The culture medium was prepared using 0.90 $g \cdot L^{-1}$ $NaNO_3$, 0.18 $g \cdot L^{-1}$ $MgSO_4$, 0.14 $g \cdot L^{-1}$ K_2PO_4, and 0.03 $g \cdot L^{-1}$ of Karentol® (Kenogard, Spain), which is a commercial solid mixture of micronutrients.

2.2 Photobioreactor and operation conditions

The photobioreactors used for wastewater treatment and *Scenedesmus* sp. production were three raceways placed inside a greenhouse in the facilities of the Institute for Agricultural and Fisheries Research and Training (IFAPA) in Almería, Spain. All three raceway reactors were identical, which allowed for conducting the experiments in triplicate. The operating volume of the reactors was 11.8 m^3 and their surface was 80 m^2.

The reactors were inoculated with 10% of their total volume with *Scenedesmus* sp. culture and the reactors were filled up until the desired culture depth (0.135 m) using wastewater from primary treatment. Experiments were conducted in semi-continuous mode at dilution rates of 0.1, 0.2, or 0.3 day^{-1} during the four seasons of the year, which allowed for to assessment of different ranges of temperature and incident irradiance (Figure 2.3.1.1).

All the studied dilution rates were assessed at least three times per season. The reactors were operated in semi-continuous mode until the volume of the reactor was replaced at least twice. After this period, 90% of the culture was removed and the 10% remaining was used as the inoculum for the following replicate. The pH, temperature, and dissolved oxygen concentration of the culture were measured using 5083T and 5120 probes (Crison Instruments, Spain) connected to an MM44

control-transmitter unit (Crison Instruments, Spain) and Labview data acquisition software (National Instruments, US) providing complete monitoring of the facilities. The pH was controlled by on-demand injection of carbon dioxide and evaporation was compensated by the daily addition of fresh water. The culture was harvested using an industrial SSD 6-06-007 GEA separator (GEA Westfalia Separator Group, Oelde, Germany).

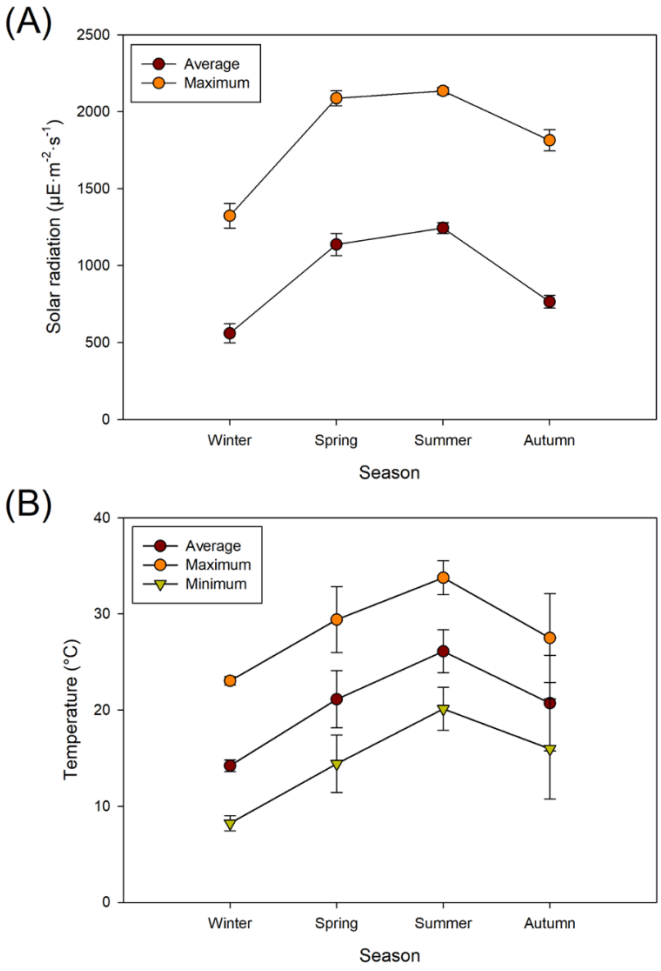


Figure 2.3.1.1. Seasonal variation of (A) solar radiation and (B) temperature inside the greenhouse. Average values are the average of all the measurements taken per day. Maximum and minimum values are the maximum and minimum values recorded every day. Results shown a are the average of all the average, maximum and minimum daily measurements taken during the assays ± SD.

2.3 Wastewater composition

The domestic wastewater collected from the University of Almería in Spain. Domestic wastewater includes blackwater (wastewater from toilets) and greywater (water used for washing, bathing and kitchen). Because of the location where the wastewater was generated, most of the wastewater consisted of blackwater:

approximately 20,000 people with only a limited number of kitchens, showers, and washing machines. The wastewater used was not subjected to any conventional depuration treatment besides the removal of solids and for these reasons, the nutrient and bacterial load of the inlets were exceptionally high when compared to conventional urban wastewater.

2.4 Analytical determinations

2.4.1 Culture parameters

Biomass concentration was calculated by dry weight by filtering 100 mL of culture through 1 µm filters followed by drying in an oven at 80 °C for 24 h. Biomass productivity was calculated as the product of biomass concentration and the dilution rate, which was either 0.1, 0.2, or 0.3 day^{-1}. The chlorophyll fluorescence ratio (F_v/F_m) was checked daily with an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic). Absorbance at 400-800 nm was daily measured using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). The extinction coefficient (k_a) and the average irradiance inside the culture (I_{av}) were calculated as described previously [65] using the equations:

$$k_a = \frac{Abs}{C_b \cdot p}$$

where Abs is the above-mentioned absorbance, C_b is the biomass concentration ($g \cdot m^{-3}$), and p is the cuvettes' light path (0.01 m), and

$$I_{av} = \frac{I_0}{k_a \cdot C_b \cdot p} \cdot (1 - e^{-k_a \cdot c_b \cdot p})$$

where I_0 is the irradiance at the surface of the culture ($\mu E \cdot m^{-2} \cdot s^{-1}$), k_a is the extinction coefficient ($m^2 \cdot g^{-1}$), C_b is the biomass concentration ($g \cdot m^{-3}$), and p is the light path inside the reactor (m).

2.4.2 Nutrient removal

The concentration of ammonium, nitrates and phosphates in the inlet and outlet of the raceways was measured using standard official methods approved by the Spanish Ministry of Agriculture as described previously [116]. Briefly, phosphorous and nitrates were measured spectrophotometrically through the phospho-vanado-

molybdate complex and by measuring the absorbance at 220-s75 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). The concentration of ammonium was determined using the Nessler reactive method.

2.5 Process modelling

Experimental results were compared to those predicted by the ABACO model [212] using MATLAB (Mathworks, MA, USA). This tool allows to simulate the dynamics of different components of the system and to predict daily biomass productivity as well as the approximate composition of the microalgae-bacteria consortia in terms of microalgae, heterotrophic bacteria, and nitrifying bacteria. The specific growth rate of the microalga (μ_{ALG}; day^{-1}) was calculated as a function of light availability inside the reactor and modified by different variables using the equation:

$$\mu_{ALG} = [\mu_{ALG}(I_{AV}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}(N) \cdot \overline{\mu_{ALG}}(P)] - m_{ALG}$$

where $\mu_{ALG}(I_{AV})$ is the specific growth rate as a function of the light availability inside the reactor (day^{-1}), and $\overline{\mu_{ALG}}(T)$, $\overline{\mu_{ALG}}(pH)$, $\overline{\mu_{ALG}}(DO)$, and $\overline{\mu_{ALG}}(CO_2)$ represent the influence of temperature, pH, dissolved oxygen concentration and carbon dioxide. The influence of nitrogen and phosphorus availability was also considered and is represented as $\overline{\mu_{ALG}}(N)$ and $\overline{\mu_{ALG}}(P)$ respectively.

Biomass productivity was calculated from the oxygen productivity that was obtained using the formula:

$$P_{O_2} = P_{O_2}ALG\left([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[CO_2]} \cdot \overline{[N]} \cdot \overline{[P]}\right) - R_{O_2}HET \cdot \left([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[N]} \cdot \overline{[P]}\right) - R_{O_2}NIT \cdot \left([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[CO_2]} \cdot \overline{[N]} \cdot \overline{[P]}\right)$$

where P_{O_2} is the oxygen productivity (g·m^{-2}·day^{-1}), $P_{O_2}ALG$ is the oxygen produced during photosynthesis by microalgae (g·m^{-2}·day^{-1}), $R_{O_2}HET$ is the oxygen consumed by heterotrophic bacteria during respiration (g·m^{-2}·day^{-1}), and $R_{O_2}NIT$ is the oxygen consumed by nitrifying bacteria during respiration (g·m^{-2}·day^{-1}). How the solar radiation, pH, dissolved oxygen concentration, the concentration of carbon dioxide, nitrogen, and phosphorus affect the specific growth rate of the culture, as well as oxygen productivity, is described in previous reports [212,213]. The

processes were modelled in triplicate and compared against experimental data obtained when operating the reactors in semi-continuous mode with a dilution rate of 0.2 day^{-1}.

2.6 Statistical analysis

Data shown are the mean values of three independent determinations ± standard deviation (SD). Differences between determinations were analysed using analysis of variance with JMP 13 (SAS Institute Inc., USA). A Tukey pairwise comparison of the means was carried out to identify where sample differences occurred with a criterion of $p<0.05$. A bivariate Pearson's' correlation analysis was conducted to identify relationships between different variables.

3. Results and discussion

3.1 Effect of environmental conditions on microalgal cultures

Raceways are the most widespread bioreactors used for microalgae production mainly because of their flexibility, ease of operation and scale-up, low construction costs, and low energy consumption for mixing, which is in the order of 4 $W \cdot m^{-3}$ [214]. Besides their lower costs, raceways are especially interesting for wastewater treatment because of their lower surface-to-volume ratio (when compared to other more productive designs such as thin-layer cascade photobioreactors) which allows processing of larger volumes of water per surface area [215]. When produced outdoors, microalgal growth depends largely on environmental conditions, namely temperature and solar radiation that, in turn, depend on the location of the reactor. Figure 1 shows the maximum, minimum, and average temperature and solar radiation inside the greenhouse where the raceways were located. Maximum and minimum values shown in Figure 1 and Figure 2 are the averages of all the daily maximum and minimum values measured during the experiment. Measurements were taken every 1 s and are available at the database of the H2020 SABANA project at http://sabana.ual.es/. As expected, higher temperatures and solar radiations were measured in summer ($p<0.05$), reaching an average maximum daily temperature of 33.7 °C. Minimum temperature values of 8.2 °C were achieved in winter. Average temperature throughout the year varied from 14.2 to 26.1 °C.

It is important to monitor environmental conditions as these have a striking effect on the conditions of the culture, namely temperature, dissolved oxygen concentration, and average irradiance inside the culture. In terms of culture temperature, values were in line with those measured inside the greenhouse where the reactors were located. Maximum and minimum culture temperature values were an average of 1.9 and 1.7 °C higher than the temperature inside the greenhouse (p<0.05) suggesting a slight but significant overheating of the culture.

The temperature of the culture was higher in spring and summer, reaching an average maximum value of 34.0 ± 0.9 °C (Figure 2.3.1.2). This value is relatively high and could be lethal for some strains, demonstrating the importance of selecting a robust microalga such as the *Scenedesmus* strain used in the current study. The selected strain can withstand up to 48 °C [161]. It is important to highlight that this was the maximum daily temperature reached during the experiment and was only maintained for a short period – the average culture temperature in summer was 28.0 ± 1.2 °C.

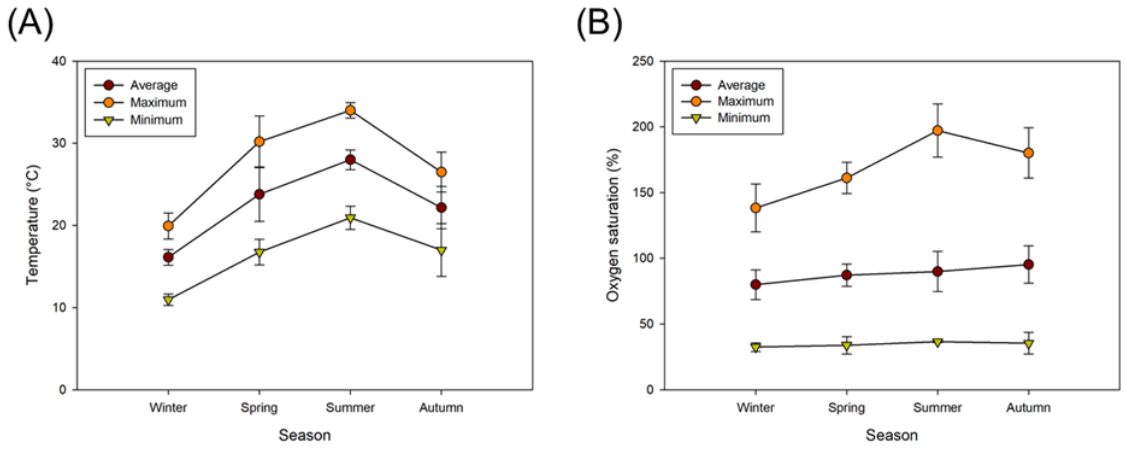


Figure 2.3.1.2. Seasonal variation of (A) temperature and (B) oxygen saturation in the culture. Average values are the average of all the measurements taken per day. Maximum and minimum values are the maximum and minimum values recorded every day. Results shown are the average of all the average, maximum and minimum daily measurements taken during the assays ± SD.

Maximum oxygen saturation values ranged between 150 and 200% and were achieved during summer and autumn (p<0.05). Higher dissolved oxygen concentrations were caused by higher biomass productivity and therefore a higher production of oxygen through photosynthesis. Controlling dissolved oxygen is of key importance as previous reports demonstrated that higher dissolved oxygen

concentrations led to limited microalgal growth, although this effect was observed in thin-layer cascade reactors that are more productive than raceways [65].

Photosynthetic efficiency depends largely on light availability inside the culture, which is the most important factor when producing any photosynthetic organism. The average irradiance inside the culture reached a maximum value of 230.3 ± 16.3 $\mu E \cdot m^{-2} \cdot s^{-1}$ in summer (p<0.05).No differences were observed in the average irradiance inside the culture in spring and autumn, while lower values were obtained in winter because of lower solar radiation (p<0.05). Overall, values were lower than those calculated when producing the same strain in the same location using freshwater [65], caused by the slight turbidity of primary wastewater.

The pH of the culture could also play an important role in affecting the performance of the system. However, in the current study, the pH of the culture was continuously online monitored and controlled at 8.0 ± 1.0 by on-demand injection of carbon dioxide. The results reported herein were comparable to those described previously by our research group when producing the same microalga in the same reactors but using freshwater instead of wastewater [65]. This means that the effect of environmental conditions on the pH, temperature, and dissolved oxygen concentration of the cultures is independent of the type of water used, demonstrating the huge potential of microalgae to process wastewater and achieve high biomass productivities. Moreover, the results demonstrate the robustness of the strain used and of the process which was not affected, in terms of biomass productivity, by the type of water used.

3.2 Biomass productivity

Besides environmental conditions, the most relevant operational conditions in open large-scale raceways are the dilution rate and culture depth. The latter was kept constant at 0.135 m, which is the optimum height of these three reactors to increase light availability and the performance of the cells while ensuring the adequate circulation of the culture through the reactor. The dilution rate represents the amount of culture that is daily harvested and substituted with fresh culture medium, in this case, wastewater. To optimise biomass productivity, it is important to operate using the dilution rate that allows for maximising light and nutrient availability. In the current study, three different dilution rates (0.1-0.3 day^{-1}) were

studied. The effect of operating at different dilution rates throughout the year is represented in Figure 2.3.1.3.

Briefly, operating at a dilution rate of 0.2 day^{-1} led to higher biomass concentration and productivity in all four seasons, especially in summer (p<0.05). Higher biomass productivity in summer can be attributed to higher light availability and more appropriate temperatures for *Scenedesmus* sp. Results were in line with those reported previously demonstrating that the optimum dilution rate for raceway reactors in the south of Spain is 0.2 day^{-1} [65]. Similar *Scenedesmus* sp. productivity values (24 $g \cdot m^{-2} \cdot day^{-1}$) were reported previously at a pilot scale using a 32 m^2 raceway reactor and centrate from anaerobic digestion as the sole nutrient source [63]. Results were also comparable to those obtained outdoors using a 7.2 m^2 raceway reactor (22.9 $g \cdot m^{-2} \cdot day^{-1}$) [209].

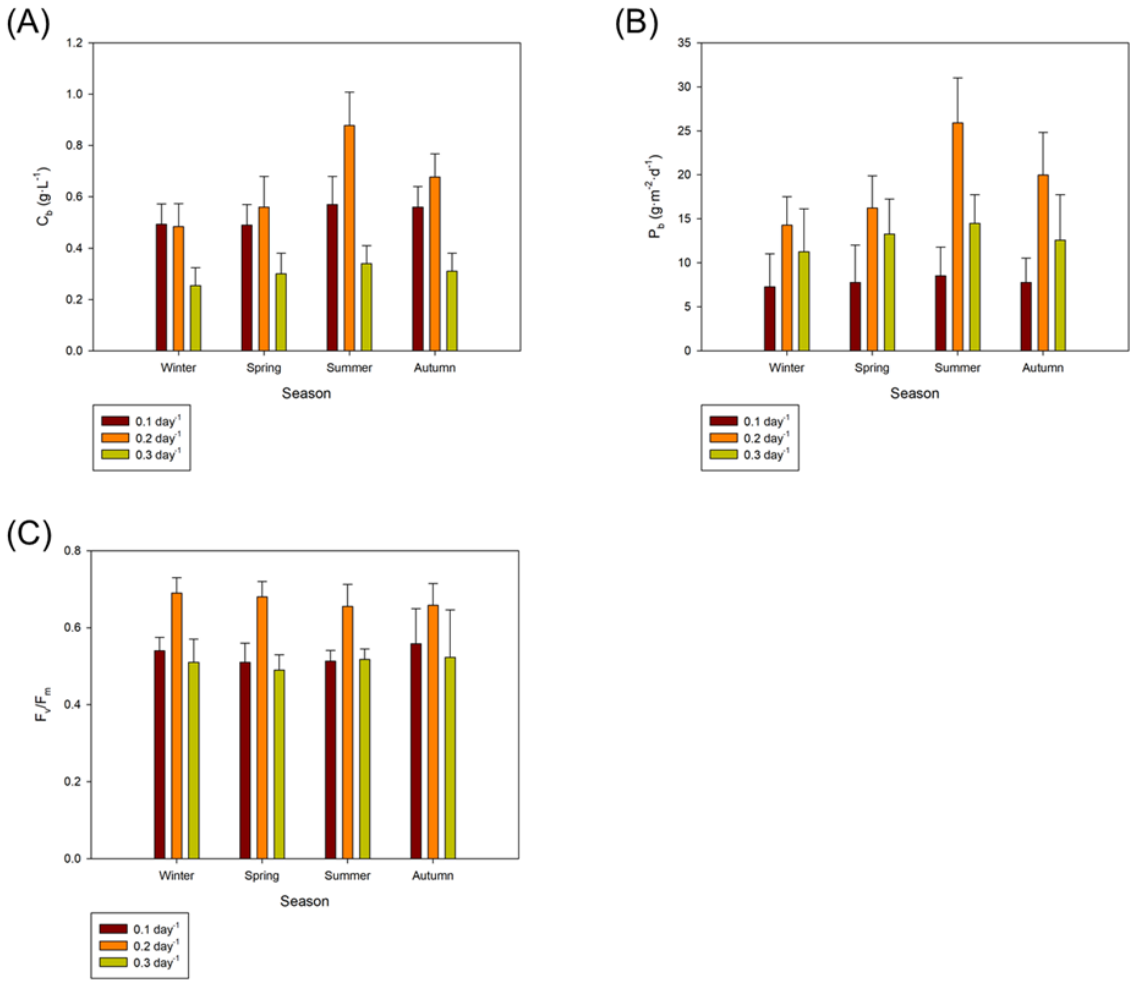


Figure 2.3.1.3. Effect of season and dilution rate on (A) biomass concentration, (B) biomass productivity and (C) chlorophyll fluorescence. The values shown are the average of three independent determinations ± SD.

Values of chlorophyll fluorescence, shown in Figure 2.3.1.3, represent a non-invasive measurement of photosystem II activity [216]. The optimal F_v/F_m value for any microalgal strain is 0.6-0.7, and lower values indicate that the culture is subjected to certain stress conditions (for example, excess of light, lack of nutrients, presence of toxins or heavy metals, or inadequate pH or temperature). In the current study, F_v/F_m values varied between 0.55 and 0.70 and were especially higher when operating at a dilution rate of 0.2 day^{-1} (p<0.05). This can be attributed to higher light and nutrient availability when operating using these conditions and correlates well with the observed higher values of biomass productivity. Because of the low oxygen saturation values of the culture, the effectiveness of the pH control, and temperatures achieved, lower F_v/F_m values could be caused by the composition of the wastewater that was especially rich in $N\text{-}NH_4^+$. Indeed, ammonia demonstrated toxic effects on *Scenedesmus* sp. previously [182].

Overall, the F_v/F_m values reported herein suggest that when the reactors were operated at 0.2 day^{-1}, microalgae were not subjected to stress conditions and that the environmental conditions of the region, as well as the temperature, pH, dissolved oxygen concentration, and light availability of the reactors, were suitable to produce *Scenedesmus* sp. at pre-industrial scale throughout the year.

3.3 Nutrient removal

The major goal of the current study, besides microalgae production, was to recover nutrients from primary wastewater and to allow its safe disposal or reutilisation. The composition of wastewaters varies with sources and has been summarised previously [40]. In the current study, the composition of the wastewater used was not constant and was especially rich in $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$, both easily assimilated by microalgae to produce biomass. Not only $N\text{-}NH_4^+$ but also $N\text{-}NO_3^+$ contribute to the total nitrogen content of wastewater. Results shown in Figure 2.3.1.4 are those obtained when operating at a dilution rate of 0.2 day^{-1}, as these conditions led to higher biomass productivity. Wastewater treatment using *Scenedesmus* sp. led to a significant decrease in the content of $N\text{-}NH_4^+$, which varied from 168.3 ± 12.5 to 210.6 ± 15.2 $mg \cdot L^{-1}$ in the inlet to 8.8 ± 1.5 to 18.6 ± 3.6 $mg \cdot L^{-1}$ in the outlet representing an 89.9-95.5% removal (Figure 2.3.1.4). Results suggest that almost

all the N-NH_4+ present in the media was assimilated by microalgae to produce biomass.

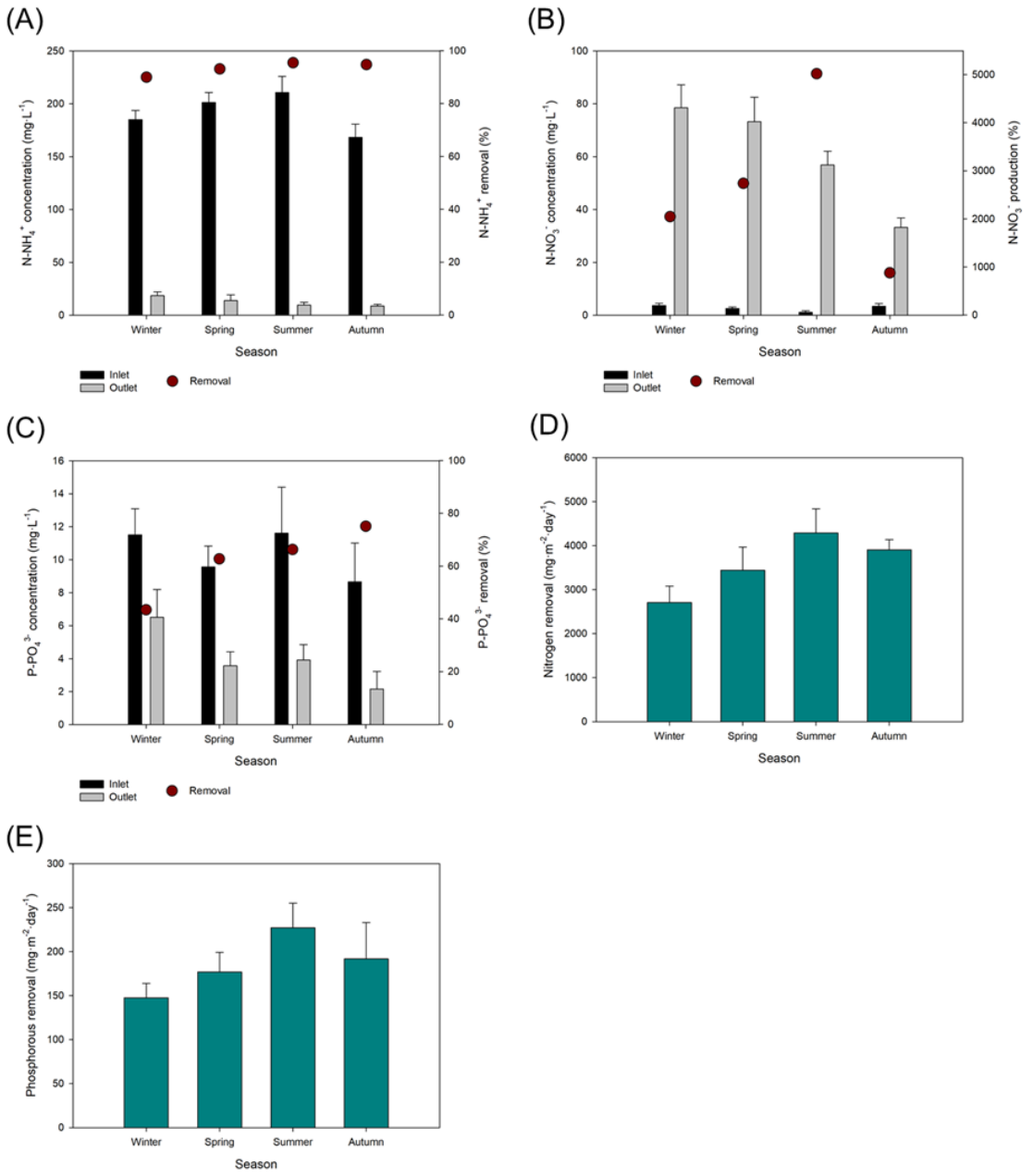


Figure 2.3.1.4. Inlet and outlet concentration of (A) ammonia, (B) nitrates, (C) phosphates and (D) nitrogen and (E) phosphorous removal. The values shown are the average of three independent determinations ± SD.

However, the content of N-NO_3+ was higher in the outlet than in the inlet (p<0.05), suggesting that part of the N-NH_4+ eliminated in the reactor was converted to N-NO_3+ by the action of nitrifying bacteria. Nitrification is the biological oxidation of ammonia to nitrite followed by the oxidation of nitrite to nitrate by ammonia-oxidising and nitrite-oxidising bacteria respectively [217]. Nitrification had an important effect on total nitrogen removal, probably caused by the low light

utilisation of current raceway designs when compared to other reactor designs [61], which facilitates the growth of non-photosynthetic microorganisms. A recent study concluded that high starting $N\text{-}NH_4^+$ loads promote the activity of ammonia oxidising bacteria and therefore, the formation of nitrites [218].

Total inorganic nitrogen removals varied between 18.3 ± 2.5 $mg \cdot L^{-1} \cdot day^{-1}$ in winter to 29.1 ± 3.7 $mg \cdot L^{-1} \cdot day^{-1}$ in summer, representing removal of approximately 50 and 80% respectively. Results were in line with those reported for centrate treatment using the marine microalga *Nannochloropsis gaditana* [219].

Removal values reported herein were higher than those obtained in a previous report where a consortium of *Chlorella* sp. and *Scenedesmus* sp. was used to process anaerobically digested piggery effluent [181]. In that study, the $N\text{-}NH_4^+$ removal rate was around 10 $mg \cdot L^{-1} \cdot day^{-1}$. Likely, the lower biomass productivity of the 1500 L raceway reactor used in that study (4.2 $g \cdot m^{-2} \cdot day^{-1}$) was the reason for the lower nitrogen removal. Indeed, thin-layer cascade reactors that are highly productive allow higher areal nitrogen removal rates – but allow to process of less wastewater per surface area [64]. Higher removal efficiencies (85-98%) were observed when processing domestic wastewater using the microalga *Chlorella variabilis* TH03 and 3.7 m^2 raceway reactors [220].

However, in that study, the concentration of nitrogen in the inlets was lower (40-70 $mg \cdot L^{-1}$) and therefore the productivity of the reactors ranged between 11 and 15 $g \cdot m^{-2} \cdot day^{-1}$. Nitrogen removal was positively correlated to biomass productivity (R^2=0.864; 0.05) and average irradiance inside the culture (R^2=0.903; 0.05) which demonstrates that part of the nitrogen removed from the water was used for the production of biomass. Indeed, approximately 20-30% of the inorganic nitrogen present in the wastewater was used for biomass production (Figure 2.3.1.5). Results are shown in Figure 2.3.1.5 also demonstrated that a large percentage of the nitrogen ($N\text{-}NH_4^+$) that entered the reactor was “lost” by stripping (desorption). These results were in line with those reported in a different study conducted in the same region using thin-layer cascade reactors [64].

In terms of phosphorous removal, values ranged from 147.5 ± 16.2 $mg \cdot m^{-2} \cdot day^{-1}$ in winter to 227.2 ± 27.7 $mg \cdot m^{-2} \cdot day^{-1}$ in summer (Figure 2.3.1.4). Approximately 50-75% of the $P\text{-}PO_4^{3-}$ that entered the reactors was effectively removed by the

microalgae-bacteria consortia. These values were lower than those reported in a previous report that demonstrated total phosphorus removal values higher than 90% [221].

Similar results were obtained in a different study with total phosphorus removal values greater than 80% [222]. In this study, the waste streams were processed using different microalgal strains (*Chlorella* sp.) and laboratory-scale photobioreactors operated using artificial and controlled illumination. When processing wastewater in outdoor conditions, the performance of the system is subjected to environmental conditions and therefore the control of the process becomes a challenge.

Recently, total phosphorus removal values close to 80% were reported when producing *Scenedesmus* sp. in 7.2 m^2 raceway reactors [219]. In that study, the microalga utilised was *Nannochloropsis gaditana* and the biomass productivity of the reactor was around 30 $g \cdot m^{-2} \cdot day^{-1}$. In the current study, $P\text{-}PO_4^{3-}$ removal was positively correlated to average irradiance (R^2=0.941; 0.05) and the average temperature of the culture (R^2=0.739; 0.05) and was therefore highly influenced by season. A positive correlation was also found between $P\text{-}PO_4^{3-}$ removal and biomass productivity (R^2=0.955; 0.05) suggesting that the $P\text{-}PO_4^{3-}$ removed from the wastewater was used for the production of biomass.

A secondary aim of the current paper was to validate the ABACO model previously described by our research group [212]. The validation of models using data generated under outdoor environmental conditions and pre-commercial scale is of key importance as data generated in the lab is not always representative of real industrial processes. The ABACO model was developed considering the main microalgal and bacterial processes that simultaneously occur in microalgae-based wastewater treatment processes. Light availability is the most important factor affecting microalgal growth [223], while the rest of the environmental parameters (temperature, pH, and dissolved oxygen) and nutritional parameters (CO_2, $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$) provide a normalizing effect in the model (0-1) and modify the productivity calculated based on the light incidence [212]. The heterotrophic growth is modelled as the product of maximum growth rate and switching functions for environmental parameters such as temperature, pH, and dissolved oxygen along

with the biodegradable soluble organic matter, ammonium nitrogen, and phosphate phosphorous.

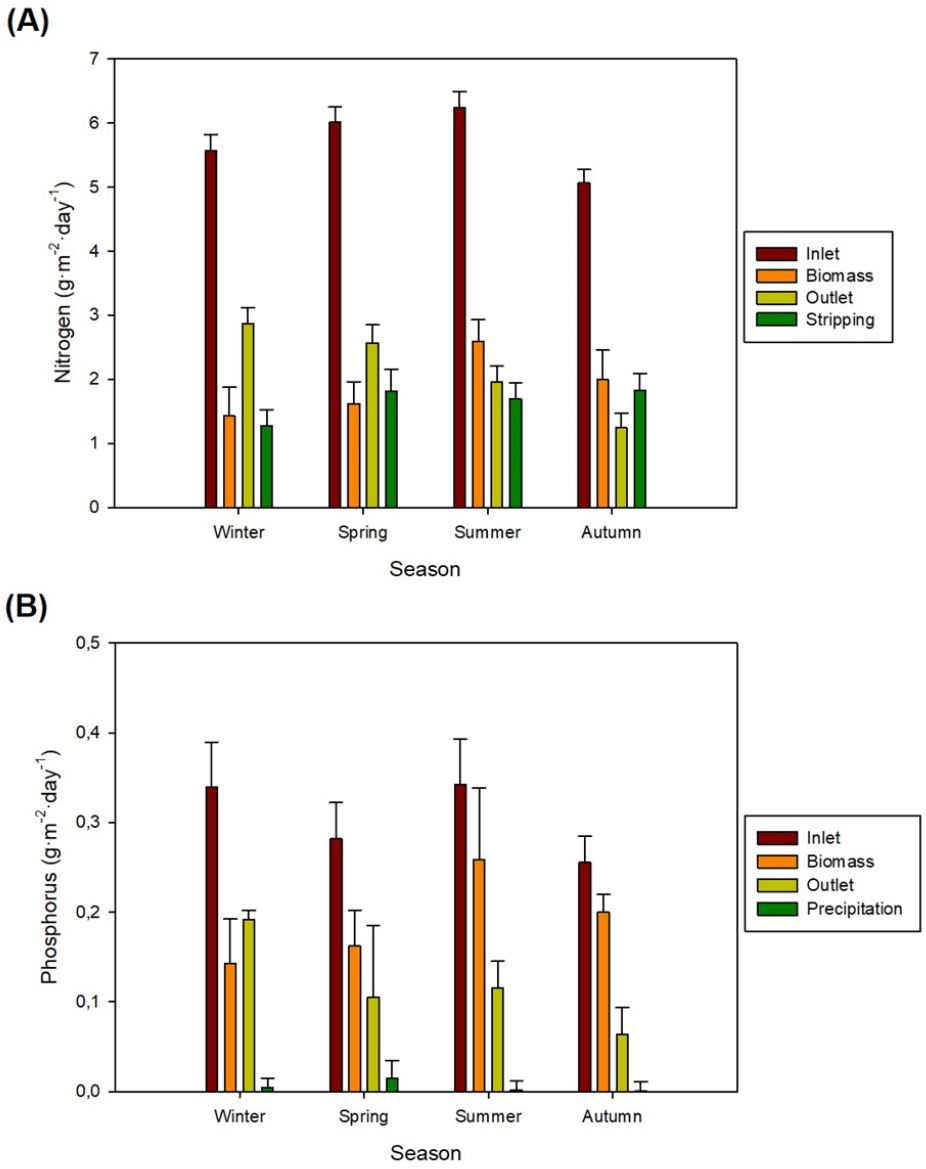


Figure 2.3.1.5. Mass balance of (A) nitrogen and (B) phosphorous when the reactors were operated with a dilution rate of 0.2 day-1. Calculations were made assuming a nitrogen and phosphorus content of the biomass of 10 and 1%, respectively. The values shown are the av erage of three independent determinations ± SD.

Indeed, a mass balance conducted in the reactor demonstrated that most of the phosphorous that entered the reactor was either used for biomass production (50-80%) or left unused (20-50%) – Figure 2.3.1.5. No phosphorous precipitation was observed, demonstrating the good pH control of the system. Removal of both nitrogen and phosphorous from wastewater is of key importance as both of them are the main nutrients of concern in eutrophication and are limiting factors in most growth scenarios [191].

3.4 Process up-scaling and simulation

The growth of nitrifying bacteria, which perform the oxidation of ammonium to nitrate, is determined by environmental parameters and the nutrients present in the culture such as CO_2, $N\text{-}NH_4^+$, and $P\text{-}PO_4^{3-}$. All these commonly measured variables (irradiance, dissolved oxygen, pH, and temperature) in the raceways were used as inputs for the model, in addition to the concentration of major components and nutrients involved into the biological process. The seasonal biomass productivity estimated using the ABACO model is shown in Figure 2.3.1.6. Although the model was developed using indoor photobioreactors, it demonstrated to be robust enough to accurately predict biomass productivity in pilot-scale outdoor open raceways. Both experimental and predicted biomass productivities were in good agreement (R^2=0.929; 0.05).

As highlighted before, domestic wastewater includes blackwater (wastewater from toilets) and greywater (water used for washing, bathing, and kitchen). Wastewater used in the current study was that generated at the University of Almeria, most of it blackwater because of a very limited number of sinks, showers, washing machines, etc. when compared to a city. The nitrogen and phosphorous content of the wastewater used in the current study doubled that of conventional primary wastewater, with nitrogen and phosphorous contents of approximately 80 and 12 $mg \cdot L^{-1}$ in Almeria [116]. For this reason, the concentration of these compounds in the outlets exceeded the maximum discharge limits of Spanish regulations set at 1-2 $mg \cdot L^{-1}$ of phosphorous and 10-15 $mg \cdot L^{-1}$ of nitrogen [193]. Up-scaling the results to a theoretical 10,000 m^2 raceway reactor, the process would allow the removal of 10.6 and 0.5 tonnes of nitrogen and phosphorous per year and produce 56.5 tonnes of valuable biomass. *Scenedesmus* strains are also interesting sources of valuable biomolecules such as lutein, which could be used for food applications [224]. However, as the biomass obtained in the current study was produced using wastewater, it cannot be used as human food. The produced biomass of *Scenedesmus* sp. could be used as a feed additive [196] or as a biostimulant in agriculture [194] – agricultural field trials are ongoing. Using a 10,000 m^2 raceway reactor would also allow to process of 81.0 m^3 of wastewater. These data were calculated considering 300 days of production and 35 days dedicated to up-scaling, cleaning, and other operations.

Overall, results suggested that *Scenedesmus* sp. could be used as a pre-treatment in the processing of wastewater with very high nitrogen and phosphorous contents. This strategy could be used as unique wastewater treatment in wastewater with lower nitrogen and phosphorous content. To improve the nutrient removal efficiency, the outlet of the reactors could be recirculated into the reactor, or membranes could be used to separate the hydraulic retention time from the cellular retention time, thus maximising nutrient recoveries. Studies on the use of membranes attached to raceway reactors and water recirculation are ongoing.

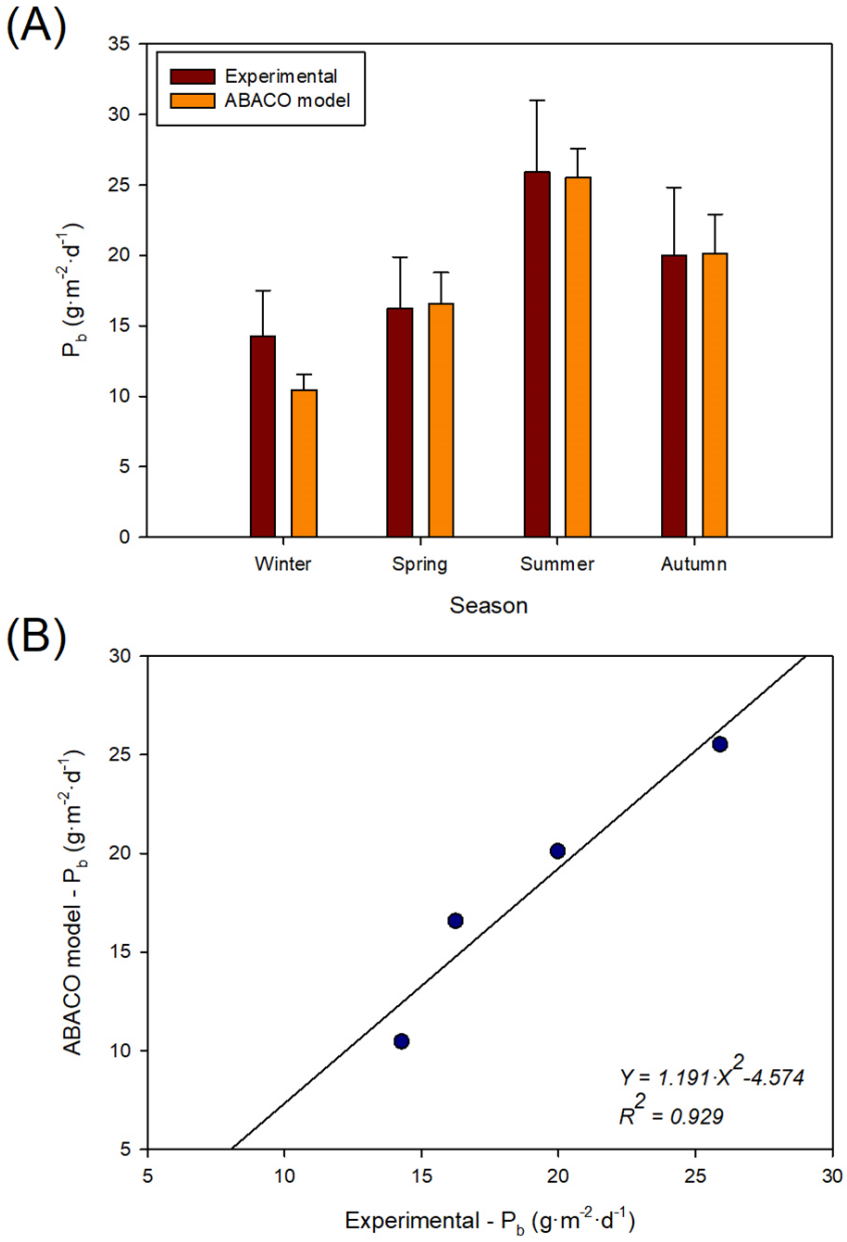


Figure 2.3.1.6. Average productivity simulated using the ABACO model. The values shown are the average of three independent determinations/simulations ± SD.

4. Conclusions

Wastewater was processed for one complete year demonstrating the robustness of microalgae-based wastewater treatment processes. Results reported herein demonstrate that the microalga *Scenedesmus* sp. can be produced at a pilot scale using primary wastewater as the sole nutrient source. Biomass productivity was comparable to that obtained when operating using freshwater and commercial fertilisers and nutrients, which would allow producing biomass at a lower price. Most of the nitrogen and phosphorous present in the wastewater were assimilated by both microalgae and bacteria to produce biomass, although some of the ammonia was used by nitrifying bacteria to produce nitrates. Moreover, the data generated in the current study was used to validate the ABACO model, which demonstrated its potential for being used to simulate biomass productivity in pilot-scale raceways. Up-scaling microalgal processes are necessary to validate the potential of these valuable microorganisms to be used on an industrial scale. The current study was carried out using 11.8 m^3 raceway reactors. Future studies will demonstrate the economic benefit of producing microalgae using wastewater and assess the potential utilisation of the produced biomass as a source for extracts with biostimulant properties in agriculture.

Acknowledgements

This work is part of the AL4BIO Project, funded by the Spanish Ministry of Science, Innovation and Universities (RTI2018-099495) and the SABANA Project funded by the H2020 Research and Innovation Framework Programme (727874). Tomás Lafarga thanks the Spanish Ministry of Science, Innovation and Universities (IJC2018-035287-I) and the BBVA Foundation for the 2020 Leonardo Grant for Researchers and Cultural Creators. Ana Sánchez-Zurano thanks the Spanish Ministry of Education (FPU16/05996). All authors thank the Institute for Agricultural and Fisheries Research and Training (IFAPA).

Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights apply to this study

CRediT author statement

Ainoa Morillas-España: Investigation, Writing – original draft; **Tomas Lafarga:** Formal analysis, supervision, Writing – review & editing; **Ana Sánchez-Zurano:** Formal analysis; **Francisco Gabriel Acién-Fernández:** Supervision, Funding acquisition; **Enrique Rodríguez-Miranda:** Formal analysis; **Cintia Gómez-Serrano:** Investigation, Formal analysis; **Cynthia Victoria González-López:** Supervision, Funding acquisition.

2.3.2.Improvement of wastewater treatment capacity using the microalga Scenedesmus sp. and membrane bioreactors

Title: Improvement of wastewater treatment capacity using the microalga Scenedesmus sp. and membrane bioreactors.

Authors: Ainoa Morillas-España, Ana Sánchez-Zurano, Tomás Lafarga, María del Mar Morales-Amaral, Cintia Gómez-Serrano, Francisco Gabriel Acién-Fernández, Cynthia Victoria González-López.

Journal: Algal research

Year: 2021

Volume: 60

DOI: https://doi.org/10.1016/j.algal.2021.102516

IF (JCR 2020): 4.401

Categories: BIOTECHNOLOGY & APPLIED MICROBIOLOGY, 46/159, **Q2**

Abstract

Primary urban wastewater was processed using the microalga *Scenedesmus* sp. in an outdoor pilot-scale raceway reactor connected to an ultrafiltration membrane. The goal was to separate cellular retention time from hydraulic retention time. This strategy led to a 129.3% increase in the daily volume of wastewater treated per square meter, and to a 48.7% increase in biomass productivity to a final value of 22.2 ± 1.9 $g \cdot m^{-2} \cdot day^{-1}$. Nutrient removal was highly influenced by permeate rate, allowing for removal up to 0.65 $mg \cdot m^{-2} \cdot day^{-1}$ of phosphates. Over 99% of ammonia was removed when the ultrafiltration membrane was used, although this was partially due to nitrate production by nitrifying bacteria: higher permeate rates led to a higher relative abundance of the nitrifying bacteria. The amplification and sequencing of the microalgae-bacteria samples led to the detection of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, such as *Bradyrhizobiaceae*, *Nitrospiraceae*, *Nitrosomonadaceae*, and *Chromatiaceae*. The most abundant families detected in the microalgae-bacteria biomass were Rhodobacteraceae and Comamonadaceae.

Keywords: *Scenedesmus* sp., bioremediation, raceway reactor, taxonomic classification, nitrification, biomass, ultrafiltration.

1. Introduction

Worldwide, one in three people does not have access to safe drinking water. A 40% deficit in freshwater resources expected during the next decade, together with an increasing global population, has our planet heading towards a global water crisis. Moreover, many of our water resources are contaminated and there is a lack of technologies to reclaim used water, enlarging, even more, the water problem. The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States, highlights this problem as Goal 6: Ensure access to water and sanitation for all.

Although nitrogen and phosphorus occur naturally, most of the nitrogen and phosphorus in our water streams come from human activities and sources (fertilisers, urban and industrial wastewater, animal waste, etc.). Despite being essential for plant growth, the overabundance of nutrients in the water, primarily nitrogen and phosphorus, lead to eutrophication: approximately 40-50% of lakes and water reservoirs are eutrophic in North America, Asia, Europe, South America, and 28% in Africa [40]. Conventional wastewater treatment processes have several limitations, which include high-energy requirements, poor nutrient removal yields, and high environmental impact, the latter mainly caused due to the emission of greenhouse gases [2,225]. The development of sustainable processes is of key importance for the future, and microalgae-based processes are called to play a key role in coming years. Microalgae are attracting increased attention in the context of bioeconomy, especially in terms of bioremediation because of their dual role: they can “clean” wastewater while producing valuable biomass with applications in agriculture and other industrial applications [66,226]. Also, the feasibility of using microalgae for wastewater treatment has been reported by a large number of research groups [4,198,199] and these valuable microorganisms are well accepted by consumers [227].

Several different photobioreactors have been proposed for microalgae production, but only a small number of designs are being used for mass production. Because of their ease of use and low costs, raceways are the most commonly used bioreactors. Raceway designs have another advantage in terms of wastewater treatment: because of their relatively high culture depth (generally between the

range 0.10-0.30 m), they allow to process of large volumes of wastewater per surface area. The use of membrane bioreactors, which is a combination of bioreactors and membranes, has been proposed as an innovative strategy to process even larger amounts of water, increasing hydraulic retention times while keeping cellular retention times constant [228].

Production of microalgae in wastewater involves the association of microalgae with aerobic and anaerobic microorganisms present in the media. Microalgae are photosynthetic microorganisms and therefore, produce oxygen. Oxygen produced by microalgae supports not only the growth of heterotrophic microorganisms, namely bacteria, but also the degradation of organic compounds [229]. The composition of the microalgae-bacteria consortia is important to achieving high nutrient removal rates. However, data on the effect of operational and environmental conditions on the composition of the microalgae-bacteria consortium are scarce [116,230]. These data are important for the correct design of the process, as certain factors can promote the growth of unwanted microorganisms such as nitrifying bacteria, which reduces microalgal consumption of nitrogen.

The main goal of the current study was to assess the potential of the microalga *Scenedesmus* sp. as a biological system to recover nutrients, namely nitrogen and phosphorus, from urban wastewater using a raceway reactor. The current study also aimed at evaluating the potential use of membrane technologies to increase the amount of water processed daily and to identify the effect of operational conditions on the composition of the microalgae-bacteria consortia by illumine sequencing.

2. Materials and methods

2.1. Microorganism and culture media

The selected strain was the freshwater *Scenedesmus* sp. This strain has been widely utilised for outdoor production because of its high tolerance to adverse conditions [185].

Briefly, *Scenedesmus* sp. was produced in 3.0 m^3 capacity tubular photobioreactors operated under continuous more. The culture medium used was

a modification of the Arnon medium [231], prepared following industrial practices and using commercial-grade fertilisers instead of pure chemicals. The inoculum was transferred to pilot-scale raceways pre-filled with primary domestic wastewater, obtained from a sewage treatment plant operated by FCC AQUALIA S.A. in Almeria (Spain). The average composition of the culture medium and the wastewater used during the experiments is listed in Table 2.3.2.1.

Table 2.3.2.1. Composition of the culture medium and primary domestic wastewater used (inlet). Concentrations are expressed as mg·L^{-1}. Values correspond to the mean values of three independent determinations ± SD (n=3).

Parameters	Primary domestic wastewater*	Arnon medium
pH	7.9 ± 0.2 - 8.2 ± 0.1	7.5 ± 0.2
COD	296 ± 23 - 858 ± 39	16.0 ± 1.2
Sulphate	98.1 ± 0.9 - 105.3 ± 1.2	6.3 ± 0.8
Nitrogen-Nitrate	1.5 ± 0.8 - 5.6 ± 1.1	140.0 ± 4.5
Chloride	410.6 ± 3.6 - 435.4 ± 4.2	78.9 ± 2.1
Sodium	222.5 ± 5.1 - 312.1 ± 9.2	276.1 ± 7.9
Potassium	8.4 ± 0.7 - 9.8 ± 0.6	325.1 ± 6.3
Calcium	31.1 ± 0.9 - 31.9 ± 0.7	364.9 ± 5.5
Magnesium	52.1 ± 2.3 - 65.7 ± 2.2	12.2 ± 0.6
Phosphorus-Phosphate	7.9 ± 1.0 - 27.7 ± 1.2	39.3 ± 3.1
Nitrogen-Ammonium	58.2 ± 1.2 - 136.9 ± 8.0	0.0 ± 0.1
Iron	0.20 ± 0.05 - 0.22 ± 0.02	5.0 ± 0.3
Copper	0.09 ± 0.01 - 0.12 ± 0.04	0.02 ± 0.01
Manganese	0.03 ± 0.02 - 0.04 ± 0.03	0.5 ± 0.0
Zinc	0.10 ± 0.06 - 0.18 ± 0.07	0.06 ± 0.01
Boron	0.36 ± 0.11 - 0.45 ± 0.09	0.41 ± 0.08

* Minimum and maximum values

2.2. Experimental set-up

Experiments were conducted with a raceway reactor located at the pilot plant facilities of Las Palmerillas Research Centre of the Cajamar Foundation in Almería, Spain. The area of the reactor was 32 m^2 and culture depth was kept constant at 0.12 m (4.4 m^3). The pH, temperature, and dissolved oxygen concentration of the

culture were measured using 5083T and 5120 probes (Crison Instruments, Spain) connected to an MM44 control-transmitter unit (Crison Instruments, Spain). Labview data acquisition software (National Instruments, Texas, US) provided complete data monitoring and control of the installation. The gas flow rate entering the reactor was measured by a PFM 725S-F01-F mass flow meter (SMC, Tokyo, Japan). The pH of the culture was controlled at 8.0 ± 0.2 by on-demand injection of CO_2 at 10 $L \cdot min^{-1}$. Air was supplied to the reactor using a blower providing 350 mbar overpressure, through a fine bubble diffuser AFT2100 (ECOTEC, Barcelona, Spain), at 100 $L \cdot min^{-1}$ when no CO_2 is demanded and dissolved oxygen overpasses 250%Sat. The reactor was operated in continuous mode with a dilution rate of 0.2 day^{-1}, which means that 20% of the culture was daily replaced with fresh medium (in this case, wastewater). Only data from the steady-state conditions were used. Evaporation inside the reactor was compensated by the daily addition of fresh water.

As the wastewater used had a low nutrient content, a 10 m^2 Bio-Cell BC10 ultrafiltration membrane (Ecotec S.A., Spain) was used to separate cell residence time, or cellular retention time (CRT), from the hydraulic retention time (HRT) (Figure 2.3.2.1). The membrane was installed inside the sump of the reactor and was used to separate the water from the cells, keeping the latter in the reactor and allowing the release of clean water outside of the reactor.

The culture was passed through the membrane using an HP 6/11 magnetically coupled plastic centrifugal pump (Harton Anlagentechnik GmbH, Germany) operating at a flow rate of 6.2 $L \cdot min^{-1}$. A MEDO LA-120 aerator compressor (Nitto Kohki Ltd., US) was also connected to the membrane to minimise the accumulation of microalgal cells on the membrane's surface and minimise the water flux loss. The airflow of the compressor was set at 120 $L \cdot min^{-1}$ and the compressor worked continuously during the filtration process. The permeate flux varied from 0 (control) to 40% of the volume of the culture. A permeate flux of 40% means that 40% of the total volume of the culture was filtered, the permeate removed from the system, and replaced with a fresh medium. The HRT, in this case, would be 0.6 day^{-1} (dilution rate plus permeate rate) while the CRT would be 0.2 day^{-1}. Taking into account that the dilution rate was 0.2 day^{-1}, a permeate rate of 40% means that it takes 1.67 days to renew the whole culture volume.

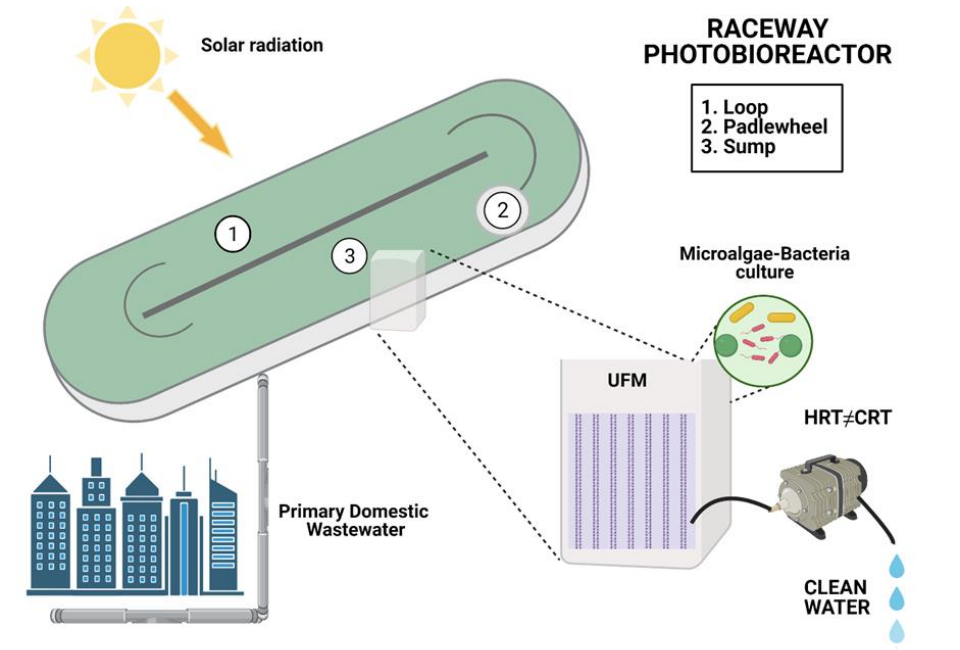


Figure 2.3.2.1 Schematic representation of the membrane photobioreactor.

2.3. Analytical determinations

Chlorophyll fluorescence (F_v/F_m) was determined with an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic). Microalgae biomass concentration was calculated by dry weight by filtering 100 mL aliquots of the culture through Macherey-Nagel glass fibre MN 85/90 and drying in an oven at 80 ºC for 24 h. Standard official methods approved by the Spanish Ministry of Agriculture were used to analyse the composition of the primary wastewater and the microalgae-bacteria samples (biomass) [63]. Phosphorus was measured by visible spectrophotometry through the phospho-vanado-molybdate complex. In addition, nitrates were quantified spectrophotometrically at 220-275 nm using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). Ammonium was measured using the Nessler reactive method and the chemical oxygen demand (COD) was determined by spectrophotometric measurement using Hach-Lange kits (LCI-400).

2.4. Genomic Analysis

Microbial communities present in the cultures were identified by genomic analyses using an Illumina MiSeq system (Illumina, San Diego, CA, US) as described

previously [116]. The QIIME pipeline was used to process the sequencing data using the “closed-reference” OTU picking strategy [232].

2.5. Statistical analysis

Differences between measurements, conducted in triplicate, were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., USA). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was in all cases $p<0.05$.

3. Results and discussion

3.1. Wastewater processing capacity and biomass productivity

Different types of wastewater and other wastes have been evaluated as potential nutrient sources for microalgae production, including municipal, agricultural, and industrial wastewaters [198]. Microalgae show huge potential for being used as a supplement for tertiary wastewater treatments. However, microalgae-based wastewater treatments have an important limitation: the processing of the extremely high amounts of wastewater produced requires very large bioreactors and the use of large surface areas.

For example, to process wastewater from a city with approximately 200,000 inhabitants, it would be necessary to use a bioreactor larger than 100 ha. When waste streams have a high nutrient concentration, they can be diluted to the optimum nutrient content for microalgal growth achieving high productivities. However, when wastewater is low in nutrients, biomass productivities are below the optimal. In this sense, it has been suggested that by incorporating membrane technologies into microalgae bioreactors, it would be possible to process larger amounts of water with low nutrient concentration by decreasing hydraulic retention times while keeping cellular retention times constant [228]. In the current study, increasing permeate rates led to lower hydraulic retention times, demonstrating the potential of this strategy to process a larger amount of water per surface area (Figure 2.3.2. 2.A).

Indeed, the volume of water that could be processed when using a permeate rate of 0.4 day^{-1} was 80.6 ± 6.2 $L \cdot m^{-2} \cdot day^{-1}$ while only 35.2 ± 4.1 $L \cdot m^{-2} \cdot day^{-1}$ could be

processed without the use of membranes (Figure 2.3.2. 2.B). Both, the hydraulic retention time and the amount of wastewater that could be processed were affected by permeate rate (p<0.001).

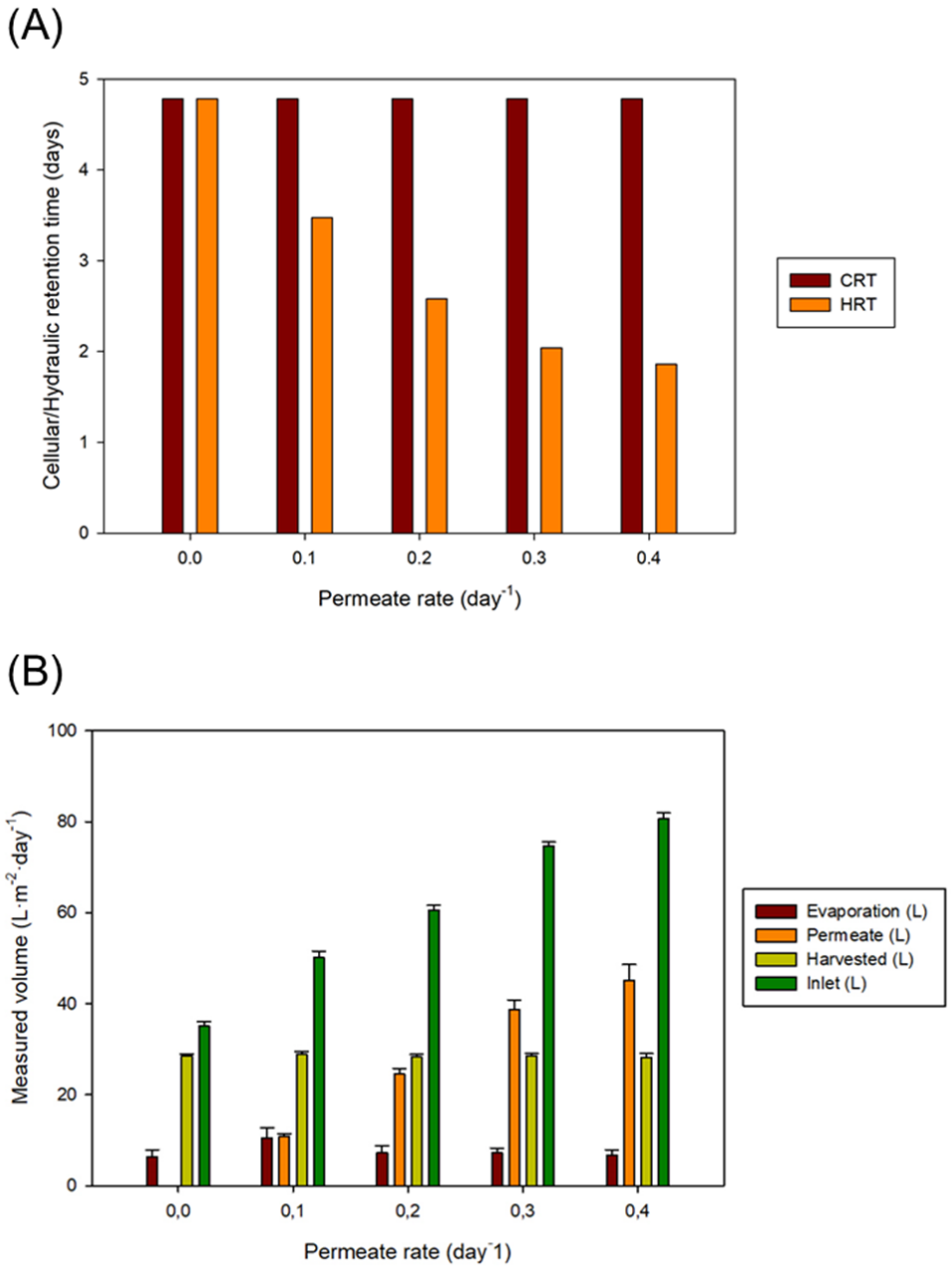


Figure 2.3.2. 2. Effect of permeate rate on (A) cellular and hydraulic retention times and (B) volume of water used to compensate for evaporation, the volume of permeate, volume of culture harvested, and total operating inlet volume per m2. Values correspond to the mean values of three independent determinations ± SD (n=3).

Increasing permeate rate not only affected the volume of water that could be processed but also biomass productivity. Indeed, biomass productivity increased from 15.2 ± 2.1 to 22.2 ± 1.9 g·m^{-2}·day^{-1} when operating at a permeate rate of 0.0

(control) or 0.3 day^{-1} respectively (p<0.05; (Figure 2.3.2.3.A). Higher permeation rates led to higher biomass productivity, attributed to higher nutrient availability. However, increasing the permeate rate over 0.2 day^{-1} did not lead to higher productivities, probably because the maximum biomass productivity of the reactor, at a dilution rate of 0.2 day^{-1}, was achieved. Indeed, although the theoretical productivity of raceway reactors can be as high as 40 $g \cdot m^{-2} \cdot day^{-1}$, experimental values are generally in the range of 20-25 $g \cdot m^{-2} \cdot day^{-1}$ because current raceway design does not allow to optimise light utilisation [61].

It is accepted that the irradiance inside the cultures is negatively affected by culture depth and biomass concentration because of the known self-shading or shadow effect of microalgae. Still, biomass productivity values were in line with those reported previously in the same region [63,65]. Different strategies have been used to increase biomass productivity in wastewater treatment processes. For example, a novel attached-growth photobioreactor equipped with cylindrical glass rods was designed to facilitate harvesting [233]. A batch experiment conducted using this reactor (3.5 L) allowed biomass concentration values of 0.25 and 0.77 $g \cdot L^{-1}$ when operated using wastewater with low and high nutrient content respectively. These were higher than the conventional suspended system that allowed reaching concentrations of 0.14 and 0.29 $g \cdot L^{-1}$ respectively [233].

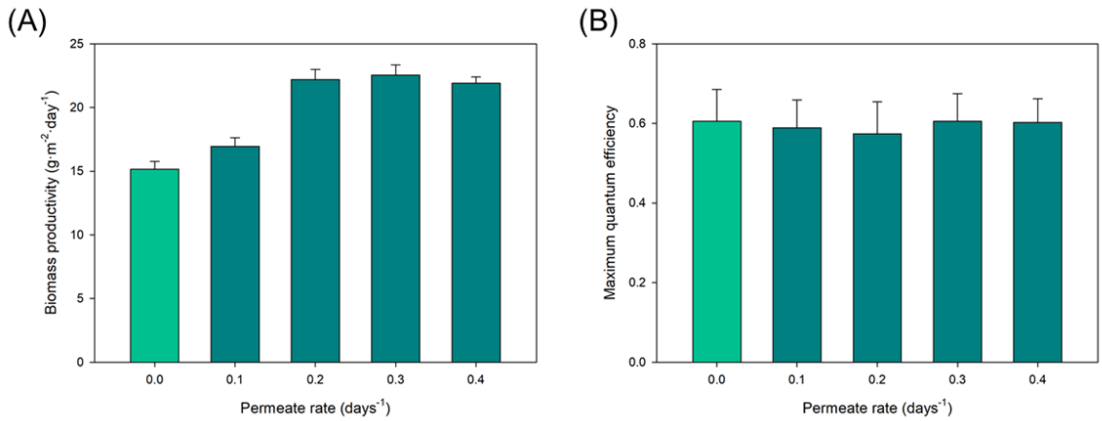


Figure 2.3.2.3. Effect of permeate rate on (A) biomass productivity and (B) maximum quantum efficacy of the PSII photochemistry – Fv/Fm. Values correspond to the mean values of three independent determinations ± SD (n=3).

This system was further validated in other studies that allowed not only higher biomass concentrations but also higher lipid productivities [234]. The use of thin-layer cascade reactors has been also studied as a potential strategy to increase biomass productivity at the pilot scale. The low culture depth of these systems permits a

high availability of light and therefore higher productivities, but the volume of water that can be processed per surface area is lower. The use of thin-layer reactors and membrane technologies has not yet been assessed, up to the best of the authors' knowledge, but this strategy shows potential for industrial implementation. Moreover, permeate rate did not affect F_v/F_m values, as shown in Figure 2.3.2.3.B. This parameter measures the performance of the photochemical processes in the PSII complex [216]. The optimal F_v/F_m value for any microalgal strain is 0.6-0.7, demonstrating that microalgae were grown without stress conditions. These values were in line with those previously reported for wastewater treatment processes [116].

Briefly, the utilisation of membranes, and increasing the permeate rate to 0.4 day^{-1}, led to a 129.3% increase in the daily wastewater volume processed per square meter. Biomass productivity increased by an average of 48.7%. Extrapolating this value to a hypothetical 1 ha facility, the use of membranes would allow to process of 2.58 M m^3 of primary wastewater while producing 79.92 Tn of valuable biomass. Membranes are already being used in conventional wastewater treatment processes because they allow improving the performance of these systems. Their industrial interest led to the development and commercialisation of reliable and low-cost membranes. Their utilisation increases wastewater treatment costs, both in terms of investment and operation (energy consumption). The current study demonstrated the potential of membranes for increasing microalgae productivity. Economic analysis must be performed based on data from larger-scale facilities.

3.2. Nutrient removal

Besides producing valuable biomass and increasing the amount of wastewater that can be processed, the current paper aimed at maximising nutrient removal per surface area. In this sense, the inlet and outlet content of ammonia, nitrates, and phosphates were measured. The composition of wastewaters varies with sources [40]. In the current study, wastewater used for microalgal growth was especially rich in ammonia and phosphates although it also had lower concentrations of nitrates – Table 2.3.2.1. Composition of the culture medium and primary domestic wastewater used (inlet). Concentrations are expressed as $mg \cdot L^{-1}$. Values correspond to the mean values of three independent determinations ± SD (n=3).. The composition of wastewater in the inlets varies because more than one week

is needed to achieve a steady state and not all the experiments were performed on the same day.

The content of ammonia in the inlet stream varied between 60 and 140 $mg \cdot L^{-1}$ for the different experiments. The ammonia content in the outlet was low for all the studied operational conditions (p<0.05). As shown in Figure 2.3.2.4 Effect of permeate rate on the removal/production of (A) NH_4^+, (B) NO_3^-, and (C) PO_4^{3-}. Values correspond to the mean values of three independent determinations ± SD (n=3)., ammonia removal was significantly affected by permeate rate, increasing from 34.2 ± 2.4 $mg \cdot L^{-1} \cdot day^{-1}$ to 76.1 ± 4.1 $mg \cdot L^{-1} \cdot day^{-1}$ when operating at permeate rates of 0.0 and 0.4 day^{-1}, respectively (representing a 122.5% increase). Results were in line with those reported previously for wastewater treatment of wastewater using *Scenedesmus* in laboratory-scale trials [235]. When operating with the membrane, the ammonia content of the outlet was below 1 $mg \cdot L^{-1}$. The observed removal increase can be attributed not to a higher removal efficiency but a larger amount of wastewater processed in the latter. When operating at a permeate rate of 0.4 day^{-1}, ammonia removal was even higher than when using thin-layer cascade reactors (15.0-30.6 $mg \cdot L^{-1} \cdot day^{-1}$), which are more productive than raceways [116]. In addition, the nitrate content in the inlet and outlet of the raceway reactor is shown in Figure 2.3.2.4.B.

The nitrate content of the wastewater (inlet) was relatively low and was around 5 $mg \cdot L^{-1}$ for all the experimental runs. The nitrate content in the outlet was higher than in the inlet for all the studied conditions (p<0.05), suggesting that ammonia was not only consumed by microalgae but also used by nitrifying bacteria to produce nitrates. The effect of processing on the content of nitrifying bacteria will be discussed in the following section.

The phosphate content in the inlet and outlet of the reactors as well as the daily removal of phosphates are shown in Figure 2.3.2.4.C. Metabolism of both microalgae and bacteria is dependent on phosphorus and phosphorylation [192]. Both phosphorus and nitrogen are the key nutrients of concern in eutrophication and are limiting factors in many growth scenarios [191]. The potential of microalgae from the *Scenedesmus* genus to remove not only nitrogen but also phosphorus

from wastewaters has been demonstrated previously (Sánchez Zurano et al., 2020; Tomás-Almenar et al., 2018).

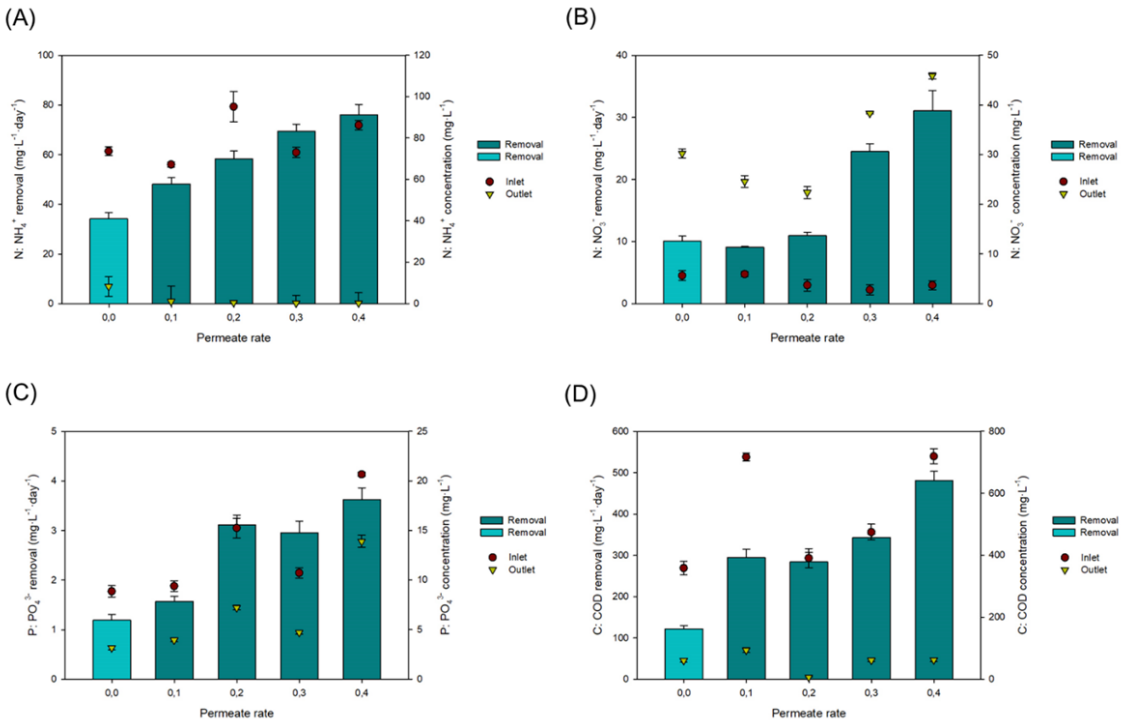


Figure 2.3.2.4 Effect of permeate rate on the removal/production of (A) NH_4^+, (B) NO_3^-, and (C) PO_4^{3-}. Values correspond to the mean values of three independent determinations ± SD (n=3).

Phosphate removal increased with increased permeate rate: when operating using a permeate rate of 0.4 day^{-1}, phosphorus removal increased by 294.6% when compared to the control without the membrane. This can be attributed to a larger volume of water processed but also to higher nitrogen content in the inlet because previous reports demonstrated that phosphorus removal improved with increased nitrogen supply – while nitrogen removal was independent of phosphorus concentration [236]. Moreover, the COD was also determined at the reactors' inlet and outlet. Results, shown in Figure 2.3.2.4.D, demonstrated large variability in the COD content of the wastewaters used. Differences between the inlets and the outlets were significant, being lower than 80 $mg \cdot L^{-1}$ in all cases (p<0.05). Similar results were observed in a recent study where *Scenedesmus rubescens* was integrated into a wastewater treatment process achieving a decrease in COD from 400 $mg \cdot L^{-1}$ in the inlet to approximately 100 $mg \cdot L^{-1}$ after 8 days [237]. Operating at higher permeate rates led to a higher COD removal, mainly caused by a larger volume of processed wastewater. Results demonstrated a huge capacity of

microalgae and bacteria to remove organic matter and depurate high COD concentrations as those evaluated in the current study (300-900 $mg \cdot L^{-1}$). COD removal values shown in Figure 3D correspond to depuration efficiencies greater than 80%, which compared well with recent reports [116,238].

Overall, the results demonstrated a high potential of the microalgae-bacteria consortium to remove nitrogen, phosphorus, and organic matter from primary wastewater. Nutrient removal was highly influenced by permeate rate, mainly caused by the larger wastewater volume processed. Results reported herein obtained operating a pilot-scale reactor located outdoors are promising, as they suggest higher nitrogen and phosphorus removal rates than in previous reports conducted indoors under controlled conditions [239]. Up-scaling the reactor could potentially lead to nitrogen (ammonia plus nitrate) and phosphorus removals of 11.8 and 2.36 $kg \cdot year^{-1}$ respectively.

3.3. Taxonomic analysis

Microalgae-bacteria interactions are determined by multiple factors: (i) microalgal and bacterial strains (as the interactions are species-specific), (ii) composition of the microalgal cell wall, (iii) nutrient availability in the culture, and (iv) cultivation conditions such as dilution rate and HRT [206]. Besides, plenty of environmental fluctuations occur during the biological process. These include variations in light intensity, mixing, or temperature and these can affect not only the interactions between them but also the community structure or composition of the consortia [97,116]. To study the effect of the permeate rate on the composition of the microalgae-bacteria consortia, the composition of the biomass was studied using Illumina sequencing. The phylogenetic diversity was determined in the cultures after filtering for quality, trimming length, and assigning taxonomies. All the samples were classified according to their phylum, class, order, and family (Figure 2.3.2.5). The microbial community consisted of 16 phyla, 21 classes, and 31 orders – only the taxa with a relative abundance higher than 1.0% were considered. On the family level, only taxa with relative abundance higher than 0.1% were considered (52 families).

Results indicate that the most abundant phyla identified in all samples were Proteobacteria (51-54%), Bacteroidetes (12-20%), Actinobacteria (5-11%) and

Verrucomicrobia (3-8%). Minoritarian phyla such as Planctomycetes (3-5%), Chloroflexi (3-5%), TM7 (1-6%), and Firmicutes (2-3%) were identified but at lower concentrations. Results agree with previous phylogenetic studies that described bacterial species associated with algal species. These species were distributed among six bacterial phyla, including Bacteroidetes, Proteobacteria, Firmicutes, Actinobacteria, Verrucomicrobia, and Planctomycetes [240]. Members of the phylum Bacteroidetes are associated with algal blooms because they are degraders of microalgal polysaccharides and are therefore key players in marine carbon cycling [241]. Ferro et al.(2020) found predominantly Proteobacteria and Bacteroidetes phyla for microalgae-based municipal wastewater treatment in open photobioreactors. Proteobacteria present a wide diversity of metabolic activities, acting in important environmental functions [242].

The classes Alpha- Beta-, Gamma- and Delta-Proteobacteria were identified in all samples, accounting for approximately more than 50% of total bacterial classes. Species of bacteria from Alpha and Gamma-Proteobacteria play an important role in the bioaccessibility of micronutrients for many species of microalgae. Specifically, some species produce siderophores, which bind Fe (III), making them available for microalgae. Consecutively, microalgae use micronutrients such as iron during photosynthesis. As a result, microalgae release dissolved oxygen and organic molecules (dissolved organic matter) that can be used for bacterial growth [45].

Within the phylum Bacteroidetes, bacteria belonging to the classes Saprospirae, Flavobacteriia, Cytophagia, and Sphingobacteria were detected in all samples. Some members of Saprospirae and Cytpophagia have been described as algicidal agents, capable to degrade algal cell wall macromolecules [243].

To take a closer look at the variability of the microbial community structure, the data was further analysed and compared at the family level. Rhodobacteraceae and Comamonadaceae, belonging to the Alpha-Proteobacteria and Beta-Proteobacteria class respectively, were the most abundant families determined. The family Rhodobacteraceae belongs to the Rhodobacterales group, which addresses a full range of metabolisms including aerobic respiration, anaerobic fermentation, sulphur oxidation, autotrophic carbon fixation, nitrogen fixation, and

hydrogen production [244]. Within these groups, heterotrophic bacteria play an important role because have been associated with phytoplankton during algal blooms acting as consumers of organic carbon [245].

The family Comamonadaceae has been identified as the dominant family present in the *Scenedesmus* phycospheres [246], the areas around microalgal cells where bacteria feed on extracellular products of microalgae. Other main bacterial families observed were Xanthomonadaceae, Saprospiraceae, Verrucomicrobiaceae, Rhodocyclaceae, and Sphingomonadaceae. The family Rhodocyclaceae includes some known organisms such as polyphosphate-accumulating organisms (PAOs), present in full-scale wastewater treatments because they facilitate the removal of large amounts of phosphorus [247]. However, the most relevant family identified was Sphingomonadaceae. Members of this group have been associated with typical conditions found in microalgae-based systems: low concentrations of dissolved organic carbon (DOC) and high radiation because they exhibit minimal DNA damage and high levels of UV-B resistance [116,248,249].

Other less abundant bacterial families but with a fundamental role in the nitrogen cycle are nitrifying bacteria. Nitrification is the biological oxidation of ammonia to nitrite by ammonia-oxidizing bacteria (AOB), followed by the oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). AOB and NOB performed this aerobic process and are known as nitrifying bacteria or nitrifiers [217]. In the samples, four families with AOB or NOB members were detected: The primary AOBs identified belonged to the families Chromatiaceae and Nitrosomonadacea. Furthermore, two families with common NOBs in wastewater treatment were detected in the microalgae-bacteria samples: Nitrospiraceae and Bradyrhizobiaceae. In the current study, nitrate production was affected by permeate rate as higher values led to larger water volumes processed and to higher nitrate production. In this sense, the amplification and sequencing of the microalgae-bacteria samples using the V3-V4 region of the 16S rRNA gene revealed a positive correlation between permeate rate and relative abundance of nitrifiers ($p<0.05$; $R^2=0.865$; Figure 4).

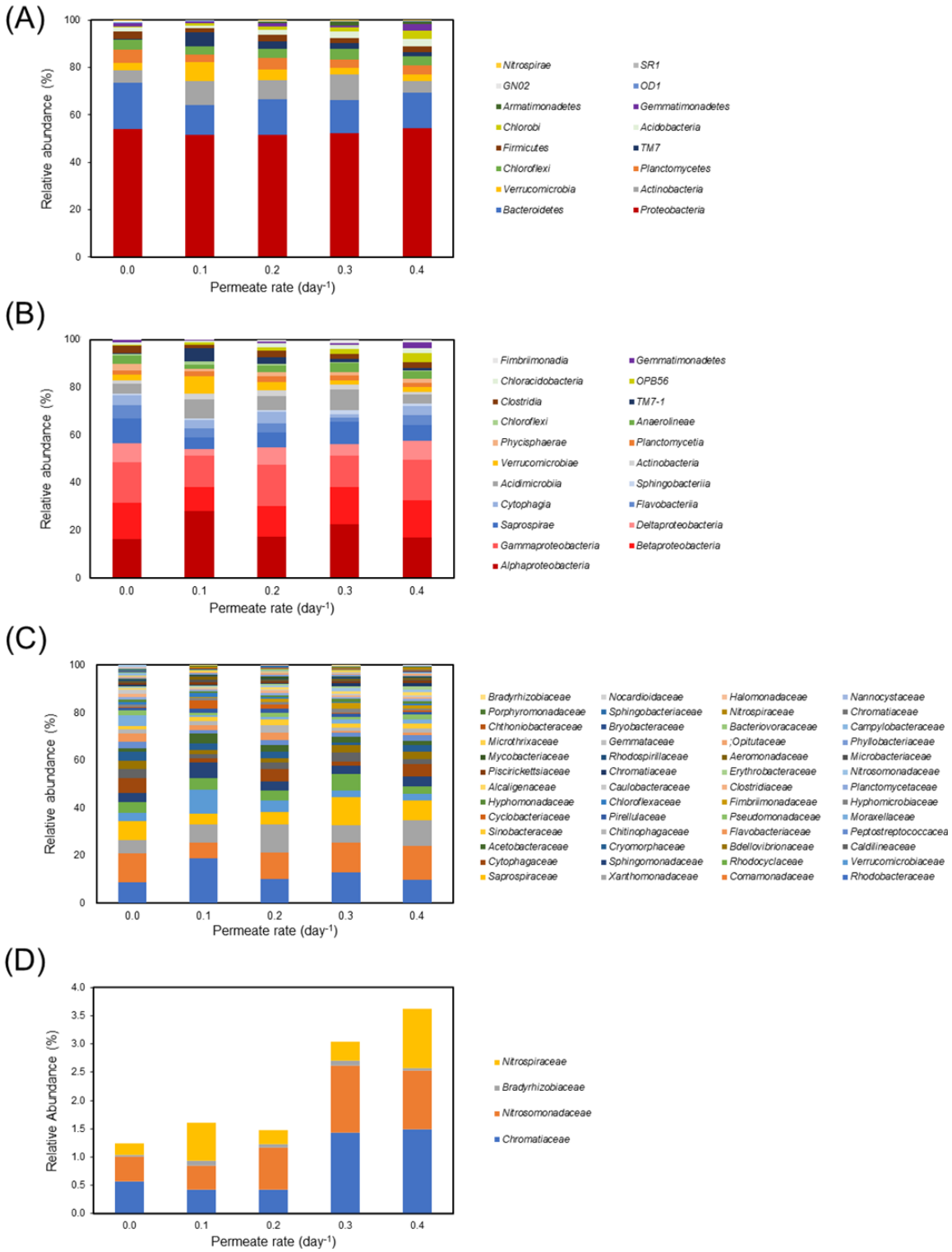


Figure 2.3.2.5. Effect of permeate rate on the relative abundance of bacteria organised by (A) phylum, (B) class, (C) family, and (D) families of nitrifying bacteria. Values correspond to the mean values of three independent determinations ± SD (n=3).

4. Conclusions

The use of ultrafiltration membranes in the large-scale microalgae-based bioremediation of wastewater shows great potential to maximise the volume of sewage that can be processed in raceway reactors. This technique could significantly improve biomass productivity. Results suggest that a raceway reactor with a total surface area of 1 ha would allow to process 2.58 M m^3 of wastewater while producing 79.92 tn of biomass. Nutrient removal was also significantly improved by increasing the permeate rate, although nitrification caused by nitrifying bacteria is a problem that still needs to be overcome. Future studies will assess the effect of operational conditions on the growth of nitrifying bacteria and optimise nutrient removal from primary wastewater.

Acknowledgements

This work forms part of the SABANA Project, funded by the European Union's Horizon 2020 Research and Innovation Programme (Grant # 727874) and the PURASOL and AL4BIO projects, funded by the Spanish Ministry of Economy and Industry (CTQ2017-84006-C3-3-R; and RTI2018-099495-A-C22 respectively). A. Sanchez-Zurano is in receipt of a FPU predoctoral grant awarded by the Spanish Ministry of Education (FPU16/05996). T. Lafarga thanks the Spanish Ministry of Science, Innovation, and Universities (IJC2018-035287-I) and the BBVA Foundation for the Leonardo 2020 Grant for Researchers and Cultural Creators.

Declaration of competing interest

The authors declare that they have no conflict of interest.

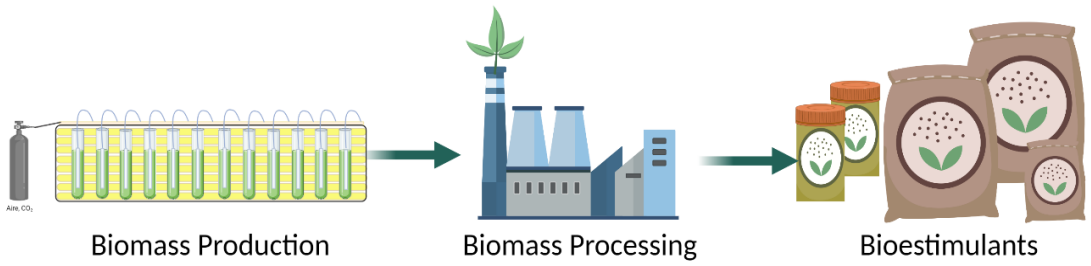
CRediT author statement

Morillas-España: Investigation, Formal Analysis, Writing – Original Draft; **A. Sánchez-Zurano**: Investigation, Formal Analysis, Writing – Original Draft; **T. Lafarga**: Writing – Original Draft, Formal Analysis, and Visualisation; **M.M. Morales-Amaral**: Investigation, Writing – Original Draft; **C. Gómez-Serrano**: Investigation and Formal Analysis; **Francisco Gabriel Acién-Fernández**: Conceptualisation, Writing – Review & Editing, Supervision, and Funding Acquisition; and **C.V. González-López**: Supervision and Funding Acquisition.

Statement of informed consent, human/animal rights

Not applicable.

2.4.Biostimulants capacity of microalgae



2.4.1.Biostimulant capacity of *Chlorella* and *Chlamydopodium* species produced using wastewater and centrate

Title: Biostimulant capacity of Chlorella and Chlamydopodium species produced using wastewater and centrate

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Journal: Biology

Year: 2022

Volume: under review

DOI:

IF (JCR 2020):

Categories:

ABSTRACT

The present study aimed to assess the potential of producing four microalgal strains using secondary treated urban wastewater supplemented with centrate and to evaluate the biostimulant effects of several microalgal extracts obtained using water and sonication. Four strains were studied: *Chlorella vulgaris* MACC-1, *Chlorella* sp. MACC-519, *Chlorella vulgaris* MACC-755, and *Chlamydopodium fusiforme* MACC-430. The highest biomass productivity was found for *C. fusiforme,* with a value of 0.38 ± 0.01 $g \cdot L^{-1} \cdot day^{-1}$. *C. vulgaris* MACC-1 achieved biomass productivity of 0.31 ± 0.03 $g \cdot L^{-1} \cdot day^{-1}$ (the highest for the *Chlorella* genus) while

the $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ removal capacities of this strain were 51.9 ± 2.4, 0.8 ± 0.1, and 5.7 ± 0.3 $mg \cdot L^{-1} \cdot day^{-1}$, respectively. *C. vulgaris* MACC-1 showed the greatest potential for use as a biostimulant - when used at a concentration of 0.1 $g \cdot L^{-1}$, it increased the germination index of watercress seeds by 3.5%. At concentrations of 0.5 and 2.0 $g \cdot L^{-1}$, the biomass from this microalga promoted adventitious root formation in soybean seeds by 220% and 493%, respectively. The cucumber expansion test suggested a cytokinin-like effect from *C. vulgaris* MACC-1; it was also the only strain that promoted the formation of chlorophylls in wheat leaves. Overall, the results of the present study suggest the potential of producing *C. vulgaris* MACC-1 using centrate and wastewater as well as the potential utilisation of its biomass to develop high-value biostimulants.

Keywords: microalgae, biostimulants, gibberellins, auxins, wastewater, biomass.

1. INTRODUCTION

The world population is expected to grow by over 2 billion people in the coming decades. Being able to feed this increasing population without further endangering the environment or depleting the world's natural resources and biodiversity is a great scientific and technological challenge [250]. In the past, the most common strategy to increase agricultural production was to expand land usage. However, today, agriculture occupies approximately 38% of the earth's terrestrial surface [251]and is responsible for the disappearance of approximately half of the world's forests [252]. For this reason, current practices now aim to increase productivity rather than land usage. Agriculture also demands huge amounts of water and energy, so it is crucial to minimise the use of these resources to ensure a sustainable future. In this way, there are high hopes for the implementation of a circular economy [253].

Plant biostimulants can promote germination, plant growth, flowering, and crop productivity as well as increase nutrient-use efficiencies and resistance to abiotic stress [254]. They are one of the top trends in agriculture and are being widely used in organic cropping systems. Microalgae are a novel and interesting source of biostimulants. Different microalgal strains from the genera *Chlorella*, *Acutodesmus*, *Arthrospira*, *Scenedesmus, Dunaliella*, and *Anabaena* have demonstrated their biostimulant capacity in scientific studies [47,48]. The biostimulant

activity of microalgae and microalgal extracts is associated with their primary metabolites (carbohydrates, proteins, and lipids), amino acids such as proline, arginine and tryptophan, vitamins, glycine betaine, and polysaccharides such as β-glucans (Ronga *et al.* 2019.). Their compositions include various hormones such as auxins, gibberellins and cytokinins, which despite being present in small quantities, provide the algal extracts with marked effects that translate into various agronomic benefits [49]. Microalgae production and microalgae-derived products have many advantages, such as reduced environmental impact and widespread consumer acceptance [255,256]. Moreover, these microorganisms can be produced on non-arable land and using different types of water and nutrient sources, including agricultural waste and urban wastewater. The main challenge is to reduce the high production cost of microalgal biomass. To achieve this, the use of wastewater and flue gases as nutrient sources has been suggested as a key strategy [257]. Using wastewater was initially proposed as an alternative to reduce production costs. However, it is now considered an alternative to using conventional wastewater treatment processes, which are polluting and require large amounts of energy. Microalgae can recover (not just remove) nutrients present in wastewater while simultaneously minimising greenhouse gas emissions, saving energy, and producing valuable bioproducts [3,199]. The produced microalgae can be used as a feedstock to produce valuable agricultural products, such as biopesticides and the above-mentioned biostimulants, or to produce aquafeeds that have demonstrated their effectiveness in increasing food sustainability and animal welfare [258].

Although there is great interest in developing and applying these natural biostimulants produced by microalgae, there is still only a limited number of well-characterized and stable products commercially available. It is, therefore, necessary to identify novel strains that have a biostimulant capacity, that is robust, can grow in wastewater, and that are highly productive. Consequently, the objective of this research is to determine the viability of producing high-quality microalgal biomass using wastewater and to assess the biostimulant capacity of the produced biomass.

2. Materials and methods

2.1. Selected microorganisms

The studied microalgal strains were supplied by the Institute of Plant Biology, Széchenyi István University (Gyor, Hungary). The selected strains were MACC-1 (*Chlorella vulgaris*), MACC-519 (*Chlorella* sp.), MACC-755 (*Chlorella vulgaris*), and MACC-430 (*Chlamydopodium fusiforme*) all of them have shown proven effectiveness as biofertilisers (Ranglová et al. 2021). The inocula were produced using 1 L controlled photobioreactors at 25 ± 1 °C and 100 µmol photons·m^{-2}·s^{-1}, and maintained using a modified Arnon medium, as described elsewhere [65].

2.2. Biomass production

The culture medium was prepared using secondary treated wastewater supplemented with centrate. The secondary treatment of the urban wastewater was performed using a pilot-scale thin-layer cascade photobioreactor containing a microalgae-bacteria consortium dominated by *Scenedesmus* sp. [64]. Because of the high efficiency of this initial step, the wastewater used had a low nutrient content: N-NH_4^+ 1-5 mg·L^{-1}; N-NO_3- 1-5 mg·L^{-1}, and P-PO_4^{3-} 1-5 mg·L^{-1}. For this reason, centrate was added until a total nitrogen concentration of 180 mg·L^{-1} was reached. The centrate used as the main nutrient source was kindly provided by El Bobar wastewater treatment plant (Almería).

Biomass production was performed using controlled 0.30 L bubble columns with spherical bases filled with 0.25 L of culture [259]. Each reactor was aerated at 0.2 v/v/min and the pH was controlled and kept constant at 8.0 by the on-demand injection of pure CO_2. Illumination was provided by eight 28 W Daylight T5 fluorescent tubes (Phillips, Madrid, Spain). The tubes were located horizontally 1 cm apart from each other and 4 cm away from the cultures. The illumination was carried out using a 12:12 h light/dark cycle that simulated outdoor conditions, namely, a progressive increase in light intensity from 08:00 am to 02:00 pm and a progressive decrease from 02:00 pm to 08:00 pm. The average irradiance in the centre of the columns filled with the culture medium alone was 780 µmon photons·$m^{-2}s^{-1}$. A spherical quantum sensor (SQS-100, Walz GmbH, Effeltrich, Germany) was used to determine the light intensity. Moreover, the temperature of

the cultures was maintained at 25 °C by controlling the temperature of the whole room.

The reactors were inoculated with 10% of the cultures' volume from a standard inoculum at a concentration of 1.0 $g \cdot L^{-1}$. The reactors were initially operated in batch mode until the stationary phase was reached, after which they were operated in semi-continuous mode using a dilution rate of 0.3 day^{-1} for 10-11 days. This was the period required to replace the total volume of the reactors at least twice and achieve a constant biomass concentration for a minimum of three consecutive days. The harvested microalgal biomass and the supernatant obtained after centrifuge separation were frozen at -20 °C until further analysis.

2.3. Assessment of the biomass productivity and nutrient consumption

The biomass concentration was measured on a dry-weight basis after drying the biomass in an oven at 80 ºC for 24 h. The biomass productivity (grams of biomass produced per litre of culture per day) was calculated as the product of the biomass concentration and the dilution rate (0.3 day^{-1}). The F_v/F_m ratio, which is an indicator of the maximum quantum yield of the PSII chemistry was measured daily using an AquaPen AP 100 fluorimeter (Photon System Instruments, The Czech Republic). The microalgal cells were dark-adapted for 5 min before the measurements.

The standard official methods approved by the Spanish Ministry of Agriculture were used to assess the nitrogen and phosphorus composition of the culture media and supernatant once the biomass was harvested. Briefly, $N\text{-}NO_3^-$ was quantified using a spectrophotometer to measure absorbance at 220 and 275 nm, $N\text{-}NH_4^+$ was measured by the Nessler reactive method, and $P\text{-}PO_4^{3+}$ was measured by visible spectrophotometry through the phospho-vanado-molybdate complex. The nutrient content of the media (after removing the biomass) was analysed at the beginning (day 0) before inoculation, at the end of the batch phase, and the end of the semi-continuous production. Determinations were conducted in triplicate.

2.4. Biomass processing

To obtain the biostimulant extracts, a cell wall disruption step was required. In the present study, cell disruption was carried out by sonicating the biomass using an UP 400 S ultrasonic processor (Hielsher Ultrasonics, Germany). Briefly, 0.2 L of microalgal sludge were sonicated in continuous mode at 24 kHz with a FC22K flow

cell (Hielsher Ultrasonics, Germany). The ultrasonic processor has a variable power output control; this was set at 70% during the experiments, which lasted 5 min. A magnetic stirrer was used to guarantee sample homogenization. The dry weight of the disrupted biomass was obtained by drying it in an oven at 100ºC for 48 h; this value was then used to calculate the dilutions needed to obtain microalgal extract concentrations of 0.1, 0.5, and 2.0 $g \cdot L^{-1}$.

2.5. Assessment of the biostimulant capacity

2.5.1. Germination index assessment of watercress seeds (*Lepidium sativum L.*)

The gibberellin-like effect of the extracts was determined by assessing the germination index (GI) of watercress seeds. The method used has been described elsewhere [260]. Microalgal extracts with biomass concentrations of 0.5 and 0.1 $g \cdot L^{-1}$ were prepared and compared against a control (distilled water). To assess the GI, 25 commercial watercress seeds were placed on Whatman No 5-filter paper and put inside a sterilised Petri dish. Bioassays were conducted in triplicate using three sterilised Petri dishes per treatment (75 seeds per treatment). The Petri dishes were treated with 2 mL of distilled water or microalgal extracts. The seeds were allowed to grow for 3 days at 24 °C in the dark and the GI was then determined by N (the number of germinated seeds) multiplied by L (the average length of the germinated seeds) divided by N_c (the number of germinated seeds treated with distilled water) and multiplied by L_c (the average length of the germinated seeds when treated with distilled water). The total was multiplied by a hundred to obtain the percentage.

$$GI(\%) = \frac{N \cdot L}{N_c \cdot L_c} \cdot 100$$

where:

N is the number of germinated seeds, L is the average length of the germinated seeds, N_c is the number of germinated seeds treated with distilled water, L_c is the average length of the germinated seeds when treated with distilled water.

2.5.2. Assessment of the adventitious soybean (Glycine max L.) root induction

The auxin-like effect of the extracts was assessed by determining adventitious root induction using commercial soybeans. Briefly, the soybeans were planted at a depth of 1 cm in moistened perlite. These were maintained in a growth chamber at 27 °C controlled under 12/12 h light/darkness. After 7 days of incubation, two seedlings were cut (3 cm below the cotyledon) and placed in vials containing 20 ml of the corresponding microalgal extract. They were incubated at 27 °C (12 h light/darkness) for 7 days. After this period, the adventitious roots longer than 1 mm on each hypocotyl were counted. This number increases proportionally to the auxin concentration of the sample. Four vials for each extract were placed in the chamber, so eight seedlings were counted per extract concentration. Indol-3-butyric acid (IBA, a specific auxin) was used as a positive control. The results were expressed as the percentage of variation concerning the samples treated only with distilled water (the negative control).

2.5.3. Excised cucumber (*Cucumis sativus* L.) expansion test.

The cytokinin-like effect of the extracts was determined using commercial cucumber seeds. These were placed in glass trays with 0.7% agar solidified Knop nutrient medium containing 450 mL of distilled water, 3.5 g of bacteriological agar, and 50 mL of KNOP solution (5 $g \cdot L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 1.25 $g \cdot L^{-1}$ KNO_3, 1.25 $g \cdot L^{-1}$ KH_2PO_4, 1.25 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$). Then, the trays containing the seeds were transferred to an incubator and maintained at 27 °C in darkness for 5 days. After this period, 5 uniform cotyledons were weighed using a precision balance and transferred to 60 mm Petri dishes containing a wet filter paper with 3 mL of one of the following solutions: distilled water (the negative control), 6-benzylaminopurine (BAP, the positive control), or microalgal extract (two different concentrations were studied: 0.5 and 2.0 $g \cdot L^{-1}$). Four Petri dishes were used for each extract concentration, so 20 cotyledons were weighed in total. The results are expressed as the percentage of weight variation concerning the samples treated with distilled water [261].

2.5.4. Wheat (*Triticum aestivum* L.) chlorophyll retention assay

The commercial wheat seeds were first rinsed in tap water for 4 h. They were then planted at a 1 cm-depth in moistened perlite. The trays were placed in a growth chamber (25 ºC, 65% relative humidity) that was illuminated with fluorescent lamps for 10 days (12 h light/darkness). Leaves from the seedling (approximately 10 cm long) were excised in 10 mm segments 3 cm from their apical tip. The fresh weight of ten segments was measured with an analytical balance and placed in 50 mL vials containing 10 mL of: distilled water (the negative control), 6-benzylaminopurine (BAP, the positive control), or the microalgal extracts at concentrations of 0.5 or 2.0 $g \cdot L^{-1}$. Four vials were used for each extract concentration so 40 segments were weighed in total. The vials were placed back into the controlled chamber for 4 days. Then, the leaves were blot dried and put into graduated 15 mL tubes containing 8 mL of 80% ethanol in distilled water (v/v). The test tubes were transferred to a water bath at 80ºC, and, after 10 min, the solution was chilled using an ice bath. The extracts were centrifuged, and the optical density was determined at 645 nm using a spectrophotometer. The optical density was normalized to 100 mg fresh weight and the adjusted results were compared to the control [262].

2.6. Statistical analysis

The microalgae were cultivated in three independent experimental units (photobioreactors) with three technical replicates being taken per natural replicate. The pH, temperature, and illumination were controlled and monitored online. In all cases, the fixed factor was the culture media. The normality and the homoscedasticity of the variables within each group were checked. The results were analysed using ANOVA with Statgraphics v.18 software (Statgraphics Technologies Inc., VA, US). A Duncan´s multiple range test was used to identify differences between samples.

3. Results and discussion

3.1 Biomass productivity

Only a limited number of microalgal strains achieve commercial success. Their success can be attributed to different factors, but mainly they can produce valuable

products and these can be produced using large outdoor reactors [125]. Another important attribute for a microalgal strain to achieve commercial success is that it is fast-growing and highly productive. When cultivated in wastewater, it is important to select a strain that grows rapidly thus enabling greater nutrient removal and increased wastewater treatment process efficiency. The biomass productivity of the selected strains is shown in Figure 2.4.1.1. Overall, the inoculated strain significantly affected biomass concentration and biomass productivity ($p<0.05$). The highest concentration (1.26 ± 0.03 g·L-1) and the highest biomass productivity (0.38 ± 0.01 g·L-1·day-1) were achieved when producing the microalga MACC 430 ($p<0.05$). Similar values were reported by Ruiz *et al.*, (2011) for *Chlorella vulgaris* when grown in wastewater, with the final biomass concentration ranging from 712 to 1300 mg SS·L^{-1}; differences in the final biomass concentration were related to differences in the nutrient loads. The strains MACC 1, MACC 519, and MACC 755 achieved biomass concentrations of 1.02 ± 0.10, 0.78 ± 0.08 and 0.71 ± 0.12 g·L^{-1}, respectively, and productivity values of 0.31 ± 0.03, 0.23 ± 0.02, and 0.21 ± 0.03 g·L^{-1}·day^{-1}, respectively. These results are in line with those reported for other strains when produced using wastewater or waste streams as nutrient sources [264]. Previous works conducted on the microalga MACC 430 revealed that, when produced using freshwater and pure nutrients, the biomass concentration could reach 1.9 ± 0.1 g·L^{-1} [265]. The lower values reported here could be attributable to operating the reactors in semi-continuous mode, whereas in the above-mentioned study, the experiments were carried out in batch mode. The culture medium composition might also have contributed to these results. The Redfield ratio is a commonly assumed general composition for microalgae (16), which suggests that N:P molar ratios in the culture media of 16:1 are optimal for microalgal growth [266].

In the present study, the N:P ratio in the inlet effluents was 11-12, which suggests that the culture might be phosphorus limited. The Redfield ratio is not a universal biochemical optimum; rather, it represents a suitable starting point average and can predict potential nitrogen or phosphorus limitation [267]. In our study, besides inorganic nutrients, the wastewater and centrate could have contained other compounds that might have limited microalgal growth.

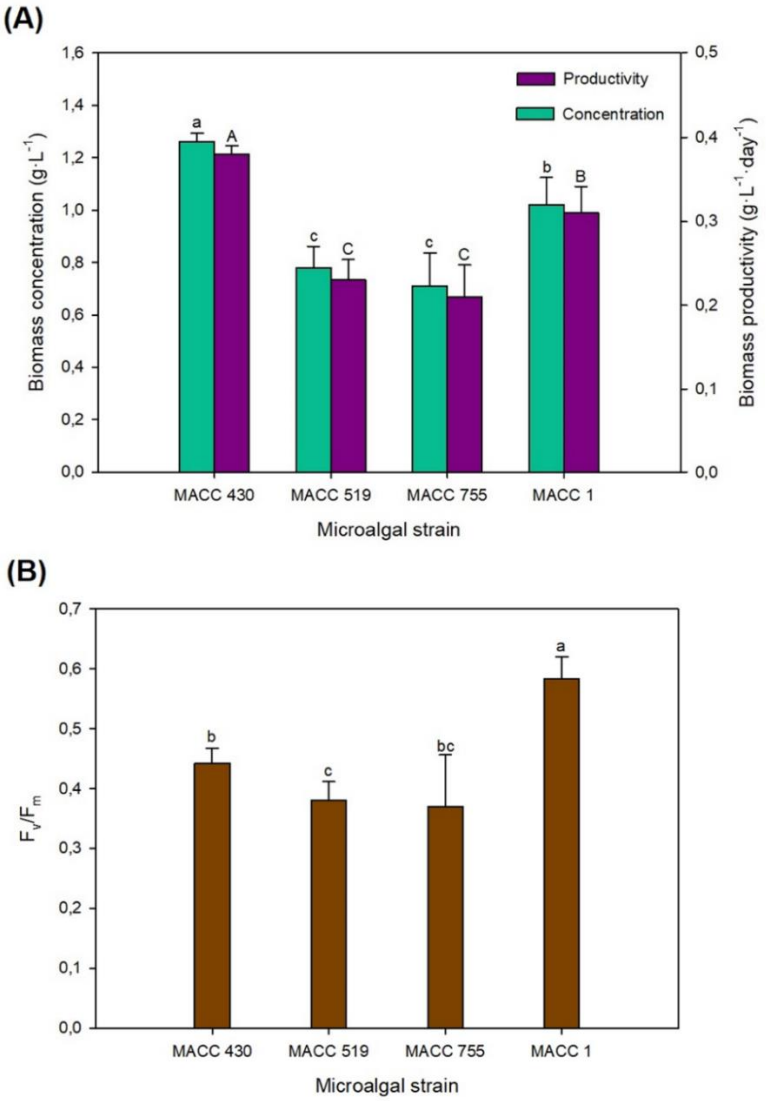

Figure 2.4.1.1 Effect of the culture media on (A) biomass productivity and (B) maximum quantum yield of PSII chemistry. Values represent the mean values ± SD. Different letters indicate significant differences ($p<0.05$).

Indeed, the F_v/F_m values (Figure 1) were lower than the theoretical optimum value (0.7) for eukaryotic microalgae [186]. The F_v/F_m values ranged from 0.37 to 0.58 and were lower in the MACC 519 and MACC 755 cultures; these were also the strains with the lowest biomass productivity. The low values indicate that the cultures were subjected to some type of stress, which might have been caused by the presence of a compound inhibiting algal growth. The nitrogen content in the centrate is usually high and so the leachate needs to be diluted because nitrogen is normally present as $N\text{-}NH_4^+$, which can inhibit algal growth [115]. The microalga MACC 1 was better able to adapt to the wastewater and centrate and thus showed greater potential for being produced in large outdoor photobioreactors.

3.2 Nutrient consumption

When producing microalgae using waste streams and wastewater, the main goal, apart from the microalgae production itself, is to recover nutrients and to allow the safe disposal of the treated water into the environment. Nitrogen and phosphorus derived from natural activities (fertilisers, wastewaters, animal wastes, etc.) are a major concern because they are harmful to health and the environment (causing eutrophication). Most of the nitrogen contained in wastewater is in the form of organic nitrogen and $N\text{-}NH_4^+$; consequently, nitrites and nitrates are produced during wastewater treatment. A secondary aim of the current study was to assess the potential of the selected strains to recover nitrogen and phosphorus from the supplemented wastewater. The results obtained for the four strains are shown in Figure 2.4.1.2. In all cases, $N\text{-}NH_4^+$ was completely removed from the media, with values higher than 50 mg $L^{-1}day^{-1}$ (Figure 2.4.1.2.A-B). This does not mean that the $N\text{-}NH_4^+$ was assimilated by the microalgae - some of it could have been transformed into $N\text{-}NO_3^-$ by the action of nitrifying bacteria and some could have been lost to the atmosphere by desorption. The $N\text{-}NO_3^-$ concentration in the outlet effluents was also lower than in the inlets, with $N\text{-}NO_3^-$ removal rates of 0.89, 0.67, 0.89 and 0.78 $mg \cdot L^{-1} \cdot day^{-1}$ for MACC 430, MACC 519, MACC 755, and MACC 1, respectively. A mass balance of the reactor, assuming a 10% nitrogen content in the biomass, revealed that not all the $N\text{-}NH_4^+$ was used by the microalgae and bacteria to produce biomass (Figure 2.4.1.2.D). Ammonia stripping or desorption was responsible for around 25-55% of the total nitrogen removal. It is important to highlight that the mass balance was conducted assuming an identical nitrogen content in the biomass; also, the organic nitrogen compounds in the wastewater were not considered.

Therefore, the results are approximate but do demonstrate that stripping occurs during wastewater treatment using microalgae even if the medium's temperature and pH are controlled. Overall, the outlet nitrogen concentration was in the 2-3 $mg \cdot L^{-1}$ range, below the maximum discharge limit (10-15 $mg \cdot L^{-1}$) set by Spanish and EU regulations [268].

Regarding phosphorus ($P\text{-}PO_4^{3-}$), the removal rates observed are shown in Figure 2.4.1.2.C. The highest $P\text{-}PO_4^{3-}$ removal was achieved using MACC 755 at a rate

of 6.8 mg·L^{-1}·day^{-1} followed by a comparable removal rate of nearly 6 mg $L^{-1}day^{-1}$ for MACC 430, MACC 519, and MACC 1. Because of the high initial P-PO_4^{3-} inlet concentration, the outlets still retained a relatively high P-PO_4^{3-} concentration and did not comply with existing regulations, which set the maximum discharge limit at 1-2 mg·L^{-1} [268].

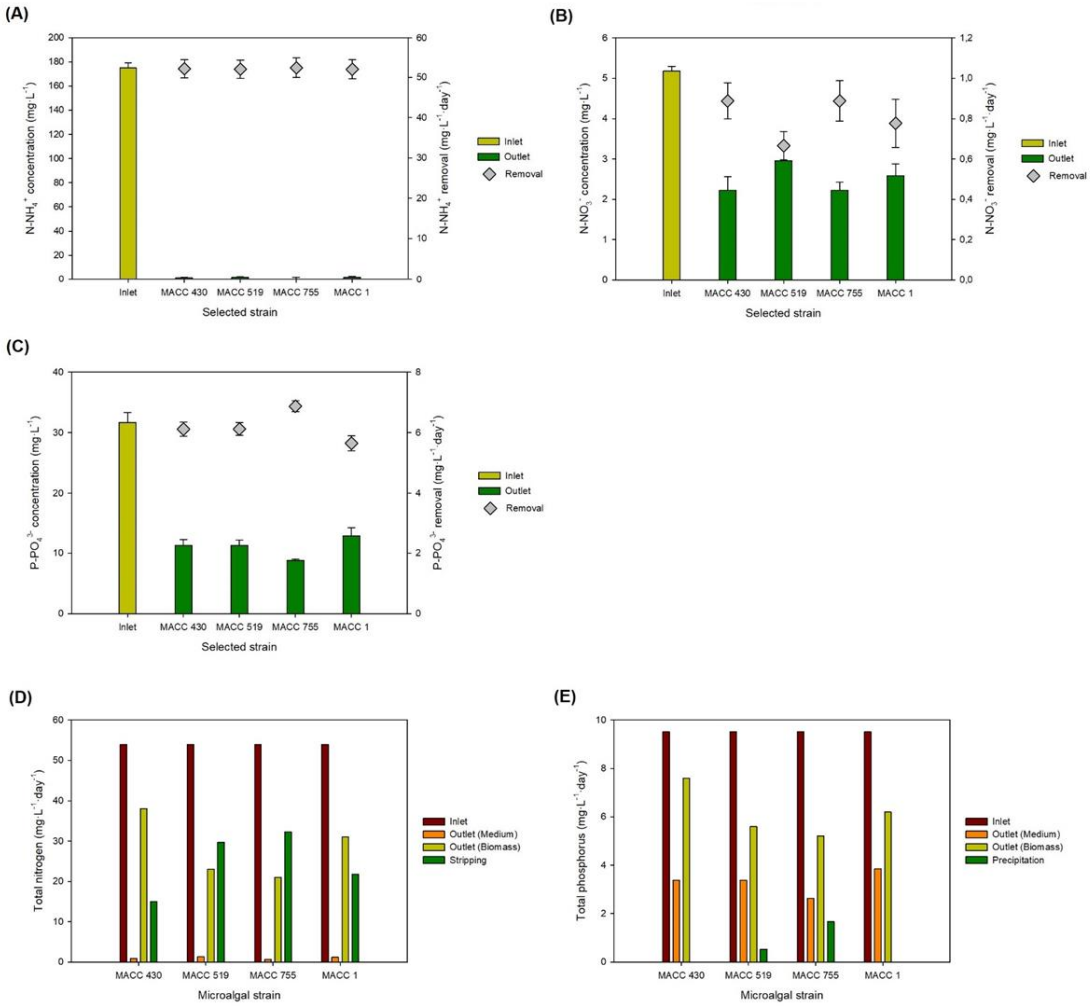

Figure 2.4.1.2. Concentration and daily removal of (A) N-NH4+, (B) N-NO3-, and (C) P-PO43- along with the mass balance of (D) nitrogen and (E) phosphorus. Values represent the mean values ± SD.

For this reason, it would be necessary either to decrease the dilution rate to increase the hydraulic retention time and thus allow greater nutrient consumption or to supplement the medium with N-NO_3^- to increase microalgal growth and therefore increase P-PO_4^{3-} uptake by the microalgal cells. It is important to note that the present study aimed to select one strain for large-scale production rather than to optimise the process.

Process optimisation must be conducted independently for each photobioreactor design and location. This is possible because laboratory-scale photobioreactors are now suitable for wastewater treatment at the commercial scale. Nevertheless, incorrect pH control must be avoided as it can cause phosphorus precipitation and reduced phosphorus availability, leading to lower biomass productivity. In our case, the pH in the reactors was monitored online and the phosphorus mass balance revealed that phosphorus precipitation was minimal. The mass balance was carried out assuming a phosphorus content in the biomass of 1.5%. Different microalgae have different compositions and, therefore, the results shown in Figure 2E are only an estimation. However, they demonstrate that the pH was correctly controlled since low phosphorus precipitation (or even no precipitation) was observed, depending on the strain.

Previous works have suggested that microalgae-based wastewater treatment processes could recover up to 90% of the total nutrients and reduce the energy consumption by half compared to conventional treatments [3]. Our results are in line with those studies, given that as a large amount of the nutrients present in the culture medium were either assimilated by the microalgae or stripped into the atmosphere. However, the results also suggest that the N/P ratio of the medium is of key importance when it comes to complying with maximum discharge limits. When the phosphorus concentration is high, the wastewater's nitrogen content will need to be adjusted using commercial nutrients to ensure safe disposal of effluent utilisation.

3.3 Biostimulant activity

One of the most interesting uses for microalgal biomass is the production of agricultural products, some of which are already available on the market. Microalgae-derived biostimulants encourage plant growth and development in a variety of ways; hence, it is necessary to assess the biostimulant effects using different bioactivity assays. Figure 2.4.1.3 shows the effects of the microalgal extracts on the germination index (GI) of watercress seeds at concentrations of 0.1 and 0.5 $mg \cdot L^{-1}$. The results are expressed as the percentage of GI variation concerning the control (distilled water, 0%). All the microalgal strains and extract

concentrations studied had a negative effect on the GI of the seeds, except for MACC 430 at a concentration of 0.1 g·L^{-1}.

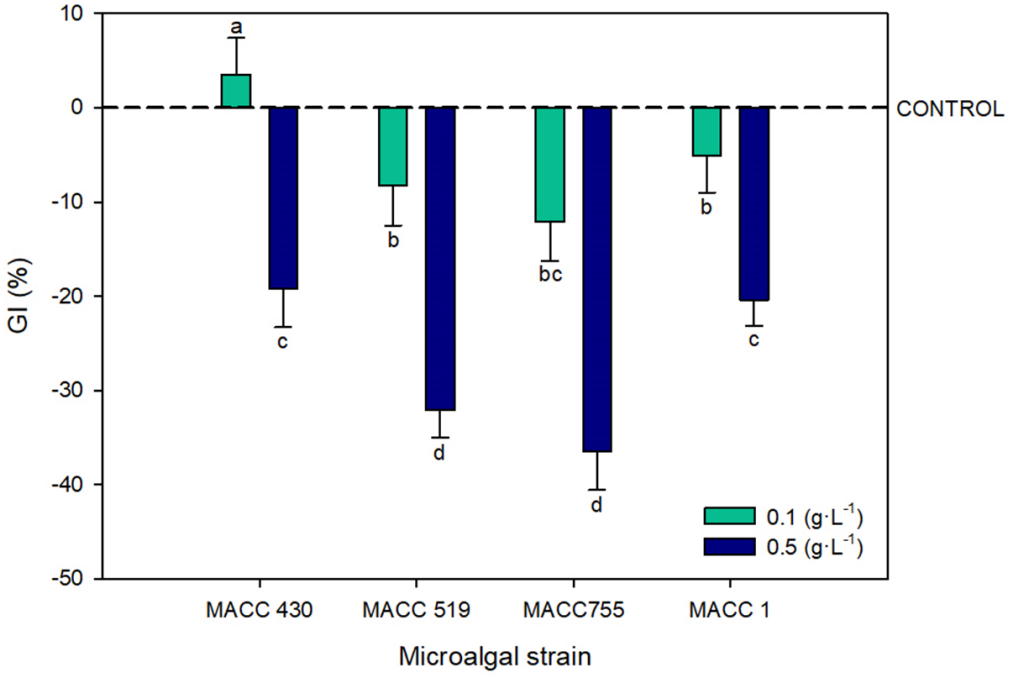

Figure 2.4.1.3. Effect of microalgal extracts on the germination index of watercress seeds. Values represent the percentage of variation concerning the control (distilled water). Values represent the mean values ± SD. Different letters indicate significant differences (p<0.05).

The results reported here suggest the presence of bioactive compounds exhibiting gibberellin-like activity. Gibberellins are a group of plant hormones that play an important role in initiating seed germination and elongating the stem [149]. The GI of the seeds was influenced both by the microalgal strain (p<0.05) and the extract concentration (p<0.01). Higher biomass concentrations led to reduced GI values, with an average decrease of 20% for MACC 430 and MACC 1, and 35% for MACC 519 and MACC 755. These results are consistent with previous reports indicating a negative correlation between watercress seed germination and the concentration of *Scenedesmus* sp. extracts [49,260]. In addition, previous work revealed that bioactive molecules, such as abscisic acid (ABA), could produce shoot growth suppression (by themselves) and form an inhibitory complex along with other biomolecules, such as lunularic acids [269].

Moreover, the biostimulant compounds are produced inside the microalgae cells so a cell disruption step is required to liberate the bioactive compounds [66].In the above-mentioned study, the authors disrupted microalgal cells using high-pressure homogenisation. They reported that a slight improvement in the GI of the seeds

could be achieved using milder disruption conditions [260]. Based on microscopic observations, we could determine that the sonication step used in the current study completely disrupted the cells. Therefore, in future studies, milder disruption conditions will be imposed on the produced biomass.

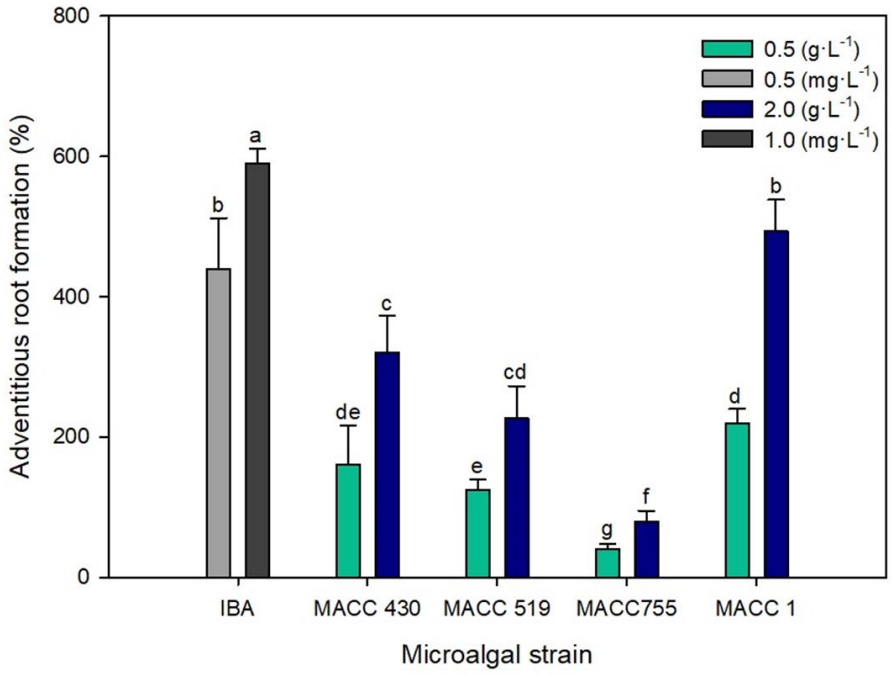

Figure 2.4.1.4. Effect of microalgal extracts on the formation of adventitious roots. Values represent the percentage of variation concerning the control (distilled water). Values represent the mean values ± SD. Different letters indicate significant differences ($p<0.05$).

Auxins play a significant role in inducing root initiation and elongation. **Figure 2.4.1.4** shows the development of mung bean roots. The results are expressed as a percentage comparison between the negative control (distilled water) and the positive control (auxin indole-3-acetic acid; IBA). All the extracts promoted growth although the best result was obtained with MACC1 at a concentration of 2.0 $g \cdot L^{-1}$; this led to root development of 493%, which was not significantly different to that of IBA at 0.5 $mg \cdot L^{-1}$ (590%). Overall, the results suggest that the produced extracts had potential biostimulant effects; for example, the MACC 430 strain at a concentration of 2.0 $g \cdot L^{-1}$ resulted in an increase of 320% compared to distilled water. These results are consistent with previous studies that demonstrated biostimulant effects of a mixed microalgal consortium containing *Chlorella* sp., *Scenedesmus* sp., *Spirulina* sp., and *Synechocystis* sp. on tomato [270]This could be because of the larger content of bioactive compounds with hormone-like activity in

the microalgal biomass, and their activity promoting root formation (or more precisely, the initiation of root formation) and elongation during the plants' early developmental stages.

Cytokinins are another group of phytohormones that control not only cell division but also bud and leaf blade development. The cytokinin-like activity of the extracts was assessed using the cucumber cotyledon expansion test. Figure 2.4.1.5 shows the cotyledon expansion when using microalgal extracts compared to the negative control (distilled water, 0%).

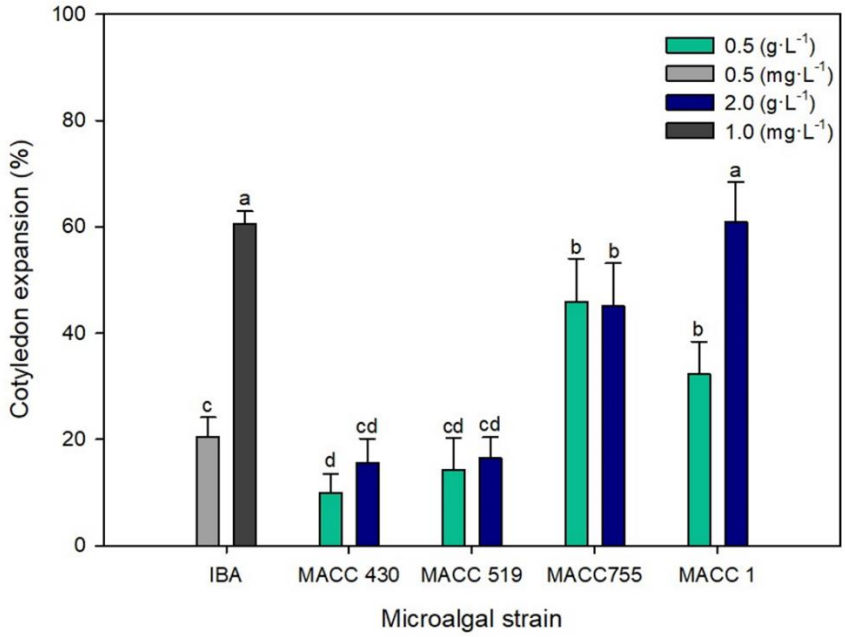

Figure 2.4.1.5. Effect of microalgal extracts on the weight of cucumber cotyledons. Values represent the percentage of variation concerning the control (distilled water). Values represent the mean values ± SD. Different letters indicate significant differences ($p<0.05$).

The obtained values are higher than the control for all four strains. In the case of MACC 1 and MACC 755, the values were even higher than those obtained with the hormone BAP at 0.5 mg·L^{-1}, being 32.3 and 45.9% higher than the negative control. The results suggest that the microalgal extracts have a biostimulant effect. For example, the MACC 1 strain at a concentration of 2.0 g·L^{-1} led to an increase of 60.9% when compared to distilled water. The results were higher than reported for other natural extracts, such as those obtained by Navarro-López *et al.*, (2020c)using a commercial biofertiliser made from algae or *Scenedesmus obliquus* biomass without any pretreatment. This could be because of a larger content of bioactive compounds having hormone-like activity, perhaps as a result of the strain produced or the culture medium, which might affect the biomass composition.

Finally, the chlorophyll retention test was carried out to determine the cytokinin-like activity of the microalgal extracts using wheat seeds. Figure 2.4.1.6 shows that only the MACC 1 strain presented positive values with respect to the control, being 7.5 and 8.0% higher at concentrations of 0.5 and 2 $g \cdot L^{-1}$, respectively. The results suggest that the MACC 1 strain has the highest content of biomolecules with biostimulant activity. However, when compared to the positive control, the results obtained for the microalgal extracts were significantly lower, even for MACC 1, despite being assessed at a much lower concentration.

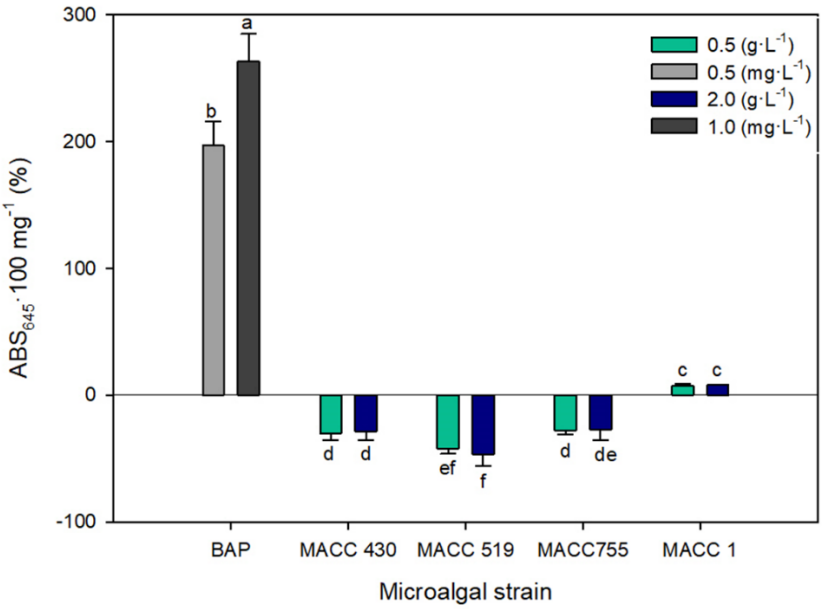

Figure 2.4.1.6. Effect of microalgal extracts on the chlorophyll content of the detached wheat leaves. Values represent the percentage of variation in the ABS645/100 mg ratio with respect to the control (distilled water). Values represent the mean values ± SD. Different letters indicate significant differences ($p<0.05$).

Conclusions

The results show that it is possible to produce the selected strains (*Chlorella vulgaris* MACC-1, *Chlorella* sp. MACC-519, *Chlorella vulgaris* MACC-755, and *Chlamydopodium fusiforme* MACC-430) using secondary wastewater supplemented with centrate. The *Chlorella vulgaris* MACC-1 strain obtained the highest biomass concentration compared to the other strains studied and is therefore a potential candidate for large-scale production. The results also revealed that this strain is a potential source of biostimulant compounds, increasing the potential for its industrial implementation in wastewater treatment processes. Furthermore, the MACC-1 microalga improved the germination index of watercress

seeds, promoted the formation of adventitious soybean roots, and increased the chlorophyll content of wheat leaves, along with other benefits to the plants. It is important to highlight that the microalgae concentration of the evaluated extracts greatly influenced the biostimulant effect; therefore, the application of the extracts must be optimised to ensure their efficacy. To confirm the results reported here, the biomass production process needs to be scaled up and the biostimulant effects verified in field trials.

Acknowledgements

This work is part of the R&D project AL4BIO RTI2018-099495-A-C22 funded by MCIN/AEI/10.13039/501100011033/, by “ERDF A way of making Europe” and the GREENFARM project, funded by the Spanish Ministry of Science, Innovation, and Universities (PDC2021-121861-C21). T. Lafarga would like to thank the Government of Andalusia (POSTDOC_21_00463) for its support.

Conflicts of interest

The authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

A. Morillas-España: Formal analysis, Research, Writing – Original Draft; **A. Ruiz-Nieto:** Formal analysis, Research; **T. Lafarga:** Supervision, Writing – Review & Editing; and **G. Acién:** Supervision, Funding acquisition; **Z. Arbib:** Resources; **C.V. González-López:** Supervision, Writing – Review & Editing, Funding acquisition.

3. CONCLUSIONS AND FUTURE WORK

3.1. Conclusions

The results shown in this PhD Thesis demonstrate and highlight the complexity of upscaling biological processes, especially when the goal is to produce photosynthetic organisms because of their strong dependence on light availability. Microalgae production yields are also highly influenced by environmental and operational conditions. However, the present work revealed that it is possible to process urban wastewater using microalgae and that the produced biomass can be used as a feedstock for the generation of valuable agricultural products besides reclaimed water for use in irrigation. Different photobioreactor designs and types of water and nutrient sources were used for producing microalgae during over 72 months. The main conclusions obtained during these period are listed below:

- The optimum dilution rates to maximize biomass productivity were found to be season-dependent (solar radiation and temperature). Overall, the dilution rates that allowed maximizing biomass productivity were 0.2 and 0.3 day^{-1} for raceway and thin layer photobioreactors, respectively. The observed difference was attributed to the higher biomass productivity of the latter.
- The average annual productivity obtained in the thin layers varied between 20 and 35 $g \cdot m^{-2} \cdot day^{-1}$ when using freshwater supplemented with fertilisers at a concentration comparable to that commonly found in urban wastewater. A similar productivity was obtained when wastewater is used as the source of nutrients.
- The high availability of light in thin layer reactors leads to high nutrient recoveries but also to an accumulation of oxygen that limits microalgal growth. In the present work, oxygen saturation values of up to 400% were achieved in summer. This indicates that the design of the degasser attached to the reactors must be improved to maximize the biomass productivity achievable in this type of system.
- The average annual productivity obtained in the raceway reactors was lower than in thin layers, ranging from 15 to 25 $g \cdot m^{-2} \cdot day^{-1}$ depending on the season.

The main reason was a lower light availability. As it happened in the thin layer photobioreactors, the production of the biomass using wastewater did not significantly affect the biomass productivity achieving comparable production yields in both cases.

- The nutrient concentration of urban wastewater is highly variable; it is directly related with the biomass productivity that can be ahieved. In some occasions, the nutrient content of the wastewater is very low. In other occasions, the concentration of nutrients in the wastewater can be too high and the system cannot meed the maximum discharge limits of current European regulations. In both cases, by coupling ultrafiltration membranes to microalgal photobioreactors could be used as an innovative strategy to maximize biomass productivity and achieve safe outlet concentrations. In the present wotk, we were able to increase the biomass productivity achieved in raceways from 15 to 22 $g \cdot m^{-2} \cdot day^{-1}$ using ultrafiltration membranes and increased the volume of wastewater that could be treated per day and per surface are by 130%.
- The microalga *Scenedesmus* sp. can be produced at a pilot scale using primary wastewater as the sole nutrient source. The nutrients present in the wastewater were assimilated by microalgae and bacteria to produce biomass; however, stripping led to the loss of nitrogen in the form of ammonia (25-55% of total N-NH_4^+) and further studies are needed to avoid this phenomenon.
- The use of wastewater instead of freshwater and commercial fertilizers would allow to produce biomass (with similar productivity) at a lower price: a reduction in the price of 1 kg of biomass of 5.7% for raceways and 5.1% for thin layers. If thin layer photobioreactors are used, the wastewater could be supplemented with commercial fertilizers to further increase the productivity of the system, or membrane systems could be implemented.
- The microalga *Chlorella vulgaris* MACC-1 showed biostimulant effects and potential for further assessment in field trials. The development of mung bean roots showed the highest development at a biomass concentration of 2.0 $g \cdot L^{-1}$. At this concentration, the root development increased by 493%. Moreover, at this same concentration an increase in the cucumber cotyledon expansion of 60.9% was observed as well as a 8.0% higher retention of chlorophylls when compared to the negative control (distilled water).

3.2. Future work

Future works will be focused on the scaling up the process from pilot to demonstrative scales and to validate the expected environmental and economic benefit of producing microalgae using wastewater. Moreover, it is important to ensure the lack of toxicity of the outlet water effluents concerning pathogens, emerging contaminants of concern, and heavy metals.

Future works will also improve the design of the thin layer photobioreactors currently available at the University of Almeria by improving mass transfer in the system. In addition, nutrient limitation has been observed in thin layer reactors when the biomass was produced using wastewater and nutrient concentrations comparable to those of wastewater. Further studies should be carried out to assess the maximal nutrient recovery capacity of these reactors and to determine the need for supplementing wastewater using commercial nutrients and/or the use of membrane systems. Preliminary trials have been carried out during this PhD Thesis.

In addition, further studies should investigate the potential use of the reclaimed water for irrigation in agriculture and the use of the microalgal biomass to obtain extracts with biostimulant properties for agriculture. The concentration and potential pre-treatment of the microalgal extracts must be optimized to ensure their safety and effectiveness and also to extend their shelf-life. Biochemical fractionation of microalgal biomass and extracts derived thereof and agronomic tests of their purified compounds are needed as a useful step for the in-depth study of the action mechanisms of microalgae.

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